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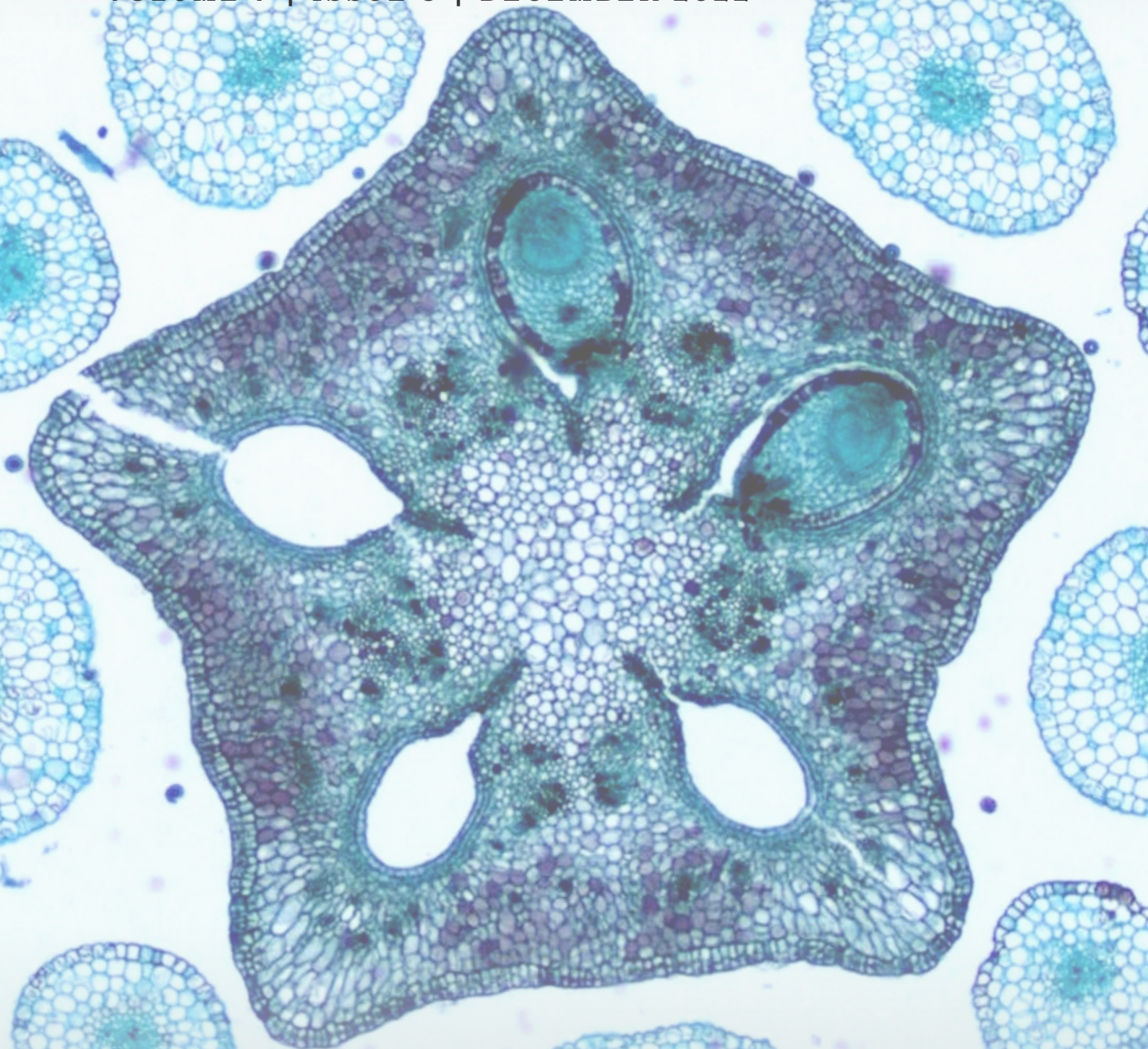

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Short Communication

Molecular Bird Sexing of Small Yellow-crested Cockatoo (*Cacatua sulphurea,* Gmelin 1788) Using Polymerase Chain Reaction Method

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ABSTRACT
The yellow-crested cockatoo (*Cacatua sulphurea,* Gmelin 1788) is an endemic bird in eastern part of Indonesia with monomorphic characteristics and included in the list of endangered birds. A method of sex determine in monomorphic birds is by molecular sexing which is based on the PCR amplification of the *chromodomain helicase DNA-binding 1* (CHD-1) gene of the bird sex chromosome. This study was aimed to sex determine of the *C. sulphurea* by amplifying the CHD-1 gene on the Z and W chromosomes and comparing the PCR amplification results from peripheral blood and plucked feathers samples. The samples used were four birds of *C. sulphurea* from the Wildlife Rescue Centre (WRC), Yogyakarta. The feathers obtained from the ventral wings of each bird were plucked. Through the cutting of the birds' nails, the peripheral blood samples were collected into microhematocrit tubes which contained Heparin. The amplification of the CHD-1 gene used the PCR method with specific primers, such as NP, P2, and MP. Moreover, the PCR results were visualized on 1.5% agarose gel using UV-Transilluminator, at a wavelength of 280 nm. The PCR products (amplicons) were in 297 bp and 392 bp DNA bands, depending on the sex of the bird being tested. It was also observed that the male *C. sulphurea* produced single 392 bp DNA fragment of the Z chromosome. However, the female birds produced two DNA fragments of the Z and W chromosomes, with a length of 297 bp and 392 bp. The results showed that samples obtained from peripheral blood produced clearer DNA bands compared to plucked feather. It concludes that the extracted DNA from peripheral blood samples have a better quality compared to samples from plucked feathers. Amplification of the CHD-1 gene in male *C. sulphurea* generated only a single DNA fragment in size of 392 bp, so the four *C. sulphurea* were male birds.

More than 35,000 wild animals in the world have been categorized as endangered species (CITES 2015), including a variety of birds. The main causes of these extinctions are the destruction of natural habitats and illegal hunting. Moreover, wildlife trade has been observed to be a serious threat to the survival of animals in nature. According to reports, about 95% of traded species originated from natural hunting with only 5% from captivity (Zein et al. 2017). One of the birds experiencing such population decline is the small

yellow-crested Cockatoo (*Cacatua sulphurea*). This species is one of the endemic birds in the eastern part of Indonesia, with other habitats ranging from Nusa Penida island to the Wallacea bioregion area. The economic value of this bird has led to increased hunting, due to the great demand from collectors or bird hobbyists (Widhiantara et al. 2016).

The number of illegally captured cockatoos continued to increase, as it is being reported to be a threat to the survival of these birds in nature. Therefore, the Ministry of Environment and Forestry of the Republic of Indonesia is campaigning for the cockatoo rescue program, where people are urged to voluntarily release the illegally captured birds to the government. This rescue service is likely to be accompanied by a programwhich enables the release of these birds into their natural habitat. The process of releasing the cockatoos that have lived with humans for a long time requires several stages, in order for them to possess the abilities to live naturally in the wild environment. These stages involved are as follow: (1) Identification of each individual bird. (2) Preparation of the bird's condition for proper release. (3) Release of birds into their natural habitats by the proper distribution process. (4) Monitoring the birds after being released to the wild environment. Since these cockatoos are to be returned to the distribution area of their original habitat; phenotypic and genotypic studies are needed to determine the species; lineage and sex of these birds (Zein et al. 2017).

The use of molecular technology for sex determination is also reported to be beneficial for conservational efforts and the matchmaking process of birds, e.g., the small yellow crested cockatoo (*C. sulphurea*). These cockatoos are classified as monomorphic birds, with males and females having similar physical characteristics, making them difficult to be phenotypically differentiated. Several methods that have been used in determining the sex of birds include the vent and steroid sexing, laparoscopy, and karyotyping. Even though these methods have been widely used to determine the sex of monomorphic birds, they still possess several weaknesses, such as being expensive and invasive, as well as consuming a large amount of time (Morinha et al. 2012). Moreover, molecular bird sexing has been applied to various species, especially in the Psittacidae family (curve-billed birds). This method has been reported to have the advantage of being non-invasive, as it less threatens the safety and depends on the age of the birds, while it is also providing faster and more accurate results (Kurniawan & Arifianto 2017).

Furthermore, molecular bird sexing is known to be the simplest and most widely used method of sex determination, based on DNA amplification. A wide variety of specific nucleotide primers pairs to amplify intron segments of the Chromodomain Helicase DNA-binding I (CHD-1) gene, has also been reported to be used. This intron segment amplification is also used as a marker for sex determination, due to having a significant difference in the size of the amplified DNA, for both male and female birds (Morinha et al. 2012). Also, the analysis of this sex determination was based on size differences of the CHD-1 gene's amplification results on the Z and

W chromosomes, via the use of the PCR method. This amplification also has the ability to multiply the target genes in most monomorphic birds, by producing single and double fragments of DNA amplicon in male (Z chromosome) and female (Z and W chromosomes) species, respectively (Fridolfsson & Ellegren 1999; Nugroho & Moch 2015). Moreover, this molecular sexing by amplification of CHD-gene on the Z and W chromosomes have also been successfully performed by researchers on other monomorphic and Kutilang birds, such as Peach-faced Lovebird (*Agapornis roseicollis*) (Nugraheni et al. 2019), with Sooty-headed and Black-crested bulbuls (*Pycnonotus aurigaster & Pycnonotus melanicterus*) (Pamulang & Haryanto 2021). The aim of this study was to determine the sex of the yellow crested cockatoo (*C. sulphurea*) on the Chromodomain Helicase DNA-binding I (CHD-1) target gene in the Z and W chromosomes, via the use of the PCR method. It also aimed to compare the quality of isolated and produced DNA from the PCR amplification derivatives of two different samples, namely peripheral blood and plucked feathers.

The samples in this study were collected from peripheral blood and plucked feathers of 4 small yellow-crested cockatoos (*C. sulphurea*), which were obtained from the Wildlife Rescue Centre (WRC) collection, in Kulon Progo, Yogyakarta. Preparations of these samples were carried out at the Biochemistry Laboratory, the Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta. The peripheral blood samples were coded SD1, SD2, SD3 and SD4, with that of the plucked feathers being SB1, SB2, SB3, and SB4.

This research met the ethical requirements of both the Ethical Clearance Commission of the Veterinary Medicine Faculty, Universitas Gadjah Mada, Yogyakarta (Approval no. 0013/EC-FKH/Int./2020), and local laws regulations.

According to the standard procedures of the Geneaid GSYNC™ DNA Extraction Kit Quick Protocol, DNA isolation from the samples was performed. This genetical isolation from the peripheral samples was carried out by collecting blood in a microhematocrit tube and plucked feather samples was performed by removing two or three primary feathers from *C. sulphurea*. The results of the DNA isolation from both peripheral blood and calamus feathers of *C. sulphurea* were then used as genetic templates in the amplification process, via the use of the PCR method. These DNA templates were then amplified by targeting the CHD-1 encoding gene on the Z and W chromosomes, by the use of the specific nucleotide primer pairs P2, NP, and MP which have been designed by Griffiths et al. (1998) and Ito et al. (2003). The nucleotide sequence, number of bases in each primer, with the annealing temperature (Tan) and melting temperatures (Tm) based on Thammakarn et al. (2007), they were presented in Table 1.

The compositions of the PCR reagent mixture in one reaction of a 25 µL total volume (consisted MyTaq™ DNA Polymerase), P2, NP, and MP

primers, and the DNA template isolated from both peripheral blood and feather calamus samples, were presented in Table 2.

Table 1. Nucleotide sequence, annealing temperature (Ta) and melting temperature (Tm) of P2, NP, and MP primers for amplification of the CHD-1 gene.

Primer	Nucleotide Sequence	∑ N Base	Tan (°C)	Tm (°C)
NP-F	5'-GAGAAACTGTGCAAAACAG-3'	19	46	49.5
P2-R	5'-TCTGCATCGCTAAATCCTTT-3'	20	46	41.9
MP-R	5'-AGTCACTATCAGATCCGGAA-3'	20	46	52.3

Furthermore, the mixture was placed into a thermocycler, with the conditions for the temperature and duration of the PCR reaction as follows, (1) Pre-denaturation at 94°C for 2 mins, (2) Denaturing at 94°C for 20 secs, (3) Annealing at 46°C for 30 secs, (4) Elongation at 72°C for 40 secs, (5) Post -elongation at 72°C for 10 mins. Moreover, repetition of the denaturation, annealing, and elongation stages were still carried out in 40 cycles. This PCR condition was performed according to Savitri et al. (2021), who has performed a molecular bird sexing by PCR on sulphur-crested cockatoo (*C. galerita*).

Furthermore, the extracted DNA from the samples (peripheral blood & plucked feathers) and PCR products were also electrophoresed in agarose gel with a concentration 1.5% in a 1x Tris-Buffered-EDTA (TBE) supplemented with 2 µL SYBR Safe DNA staining.

Afterwards, eight DNA samples (4 peripheral blood and 4 plucked feather samples) were ready to be loaded in the 1.5% agarose gel. In each of these samples, 8 µL of DNA was collected and added with 2 µL loading dye, as 4 µL of Hyperladder 100 bp DNA. The voltage was set to 100 volts with an electric current at 80 mA, for 45 mins running duration. The agarose gel electrophoresis of extracted DNA and PCR products was then observed on the UV-Transilluminator, with a wavelength of 280 nm. This DNA electrophoresis method was performed according to Argarini et al. (2020), who have a molecular bird sexing on fischeri lovebird (*Agapornis fischeri*) by using PCR amplification.

Table 2. Compositions of PCR reagents mixtures for yellow-crested Cockatoo (*C. sulphurea*) DNA in one reaction for the CHD-1 gene.

Sample Code	MyTaq™ DNA Polymerase (µl)	Forward Primer NP (20 pmol/µl) (µl)	Reverse Primer P2 (20 pmol/µl) (µl)	Reverse Primer MP (20 pmol/ µl) (µl)	DNA Template (100 ng/µl) (µl)	Total Volume (µl)
SD1	12.5	1	1	1	9.5	25
SD2	12.5	1	1	1	9.5	25
SD3	12.5	1	1	1	9.5	25
SD4	12.5	1	1	1	9.5	25
SB1	12.5	1	1	1	9.5	25
SB2	12.5	1	1	1	9.5	25
SB3	12.5	1	1	1	9.5	25
SB4	12.5	1	1	1	9.5	25

The DNA was successfully extracted from the four samples of the peripheral blood and plucked feathers of *C. sulphurea* birds. The extracted DNA was then used as a template for PCR amplification, by targeting the Chromodomain Helicase DNA-binding-1 (CHD-1) gene on the Z and W chromosomes, via the use of specific nucleotide primers pairs, namely NP, P2, and MP. The results of the electrophoresis extracted DNA from the peripheral blood and plucked feathers of *C. sulphurea,* were presented in Figure 1.

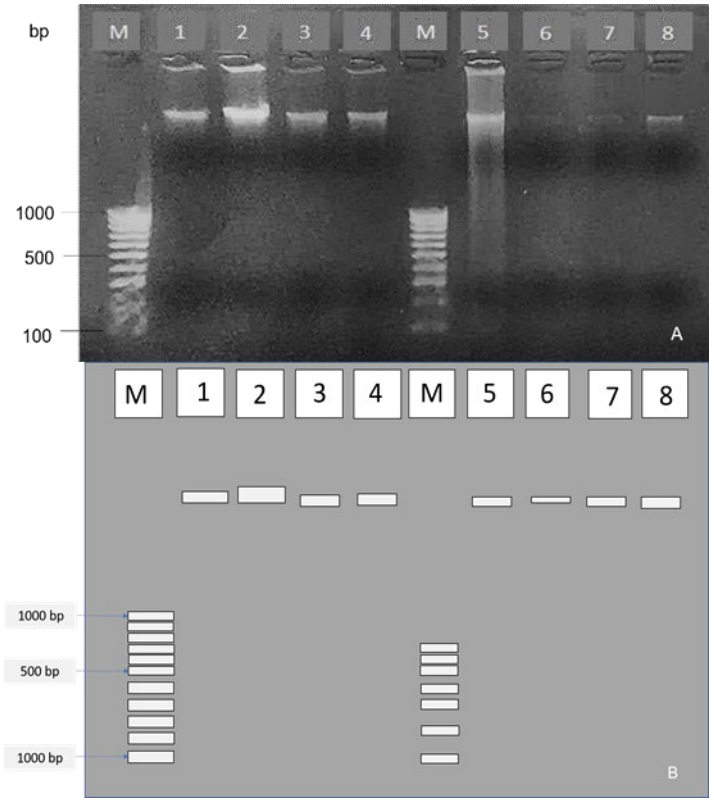

Figure 1. A. Electrophoresis of total extracted DNA. Note: M = DNA marker (hyperladder 100 bp), 1-4 = blood samples of *C. sulphurea*, 5-8 = plucked feather samples of *C. sulphurea*. B. Electrophoregram of Figure 1A.

Comparisons of the amplified CHD-1 gene electrophoresis with the 100 bp Hyperladder DNA Marker, which were observed on the UV-Transilluminator, resulted in products (PCR) in the form of DNA bands, with a length of 297 bp and 392 bp, respectively. The results of this PCR products electrophoresis on 1.5% agarose gel, were presented in Figure 2.

Sample number 1, 2, 3, 4 and 5 produced a fairly clear DNA bands, with values 6, 7 and 8 exhibiting a very thin display. Due to this result, the visualization of the PCR amplification of the CHD-1 gene in these samples was difficult to observe. Generally, the results of DNA electrophoresis showed that samples from peripheral blood produced a clearer image of DNA bands, compared to the thin display obtained from the plucked feathers. Furthermore, the electrophoresis of PCR amplified CHD-1 gene (from blood samples of *C. sulphurea* birds) compared with control samples obtained from the male and female *Cockatoo sp.,* was presented in Figure 3.

Moreover, the visualization results of the amplified CHD-1 gene in samples 1, 2, 3, and 4, showed only single DNA band, which was in size of 392 bp. The electrophoresis of PCR products in Figure 2 and 3 showed the results that were in line with Bosnjak et al. (2013), who have studied the feasibility of non-invasive molecular methods for sexing of Parrots.

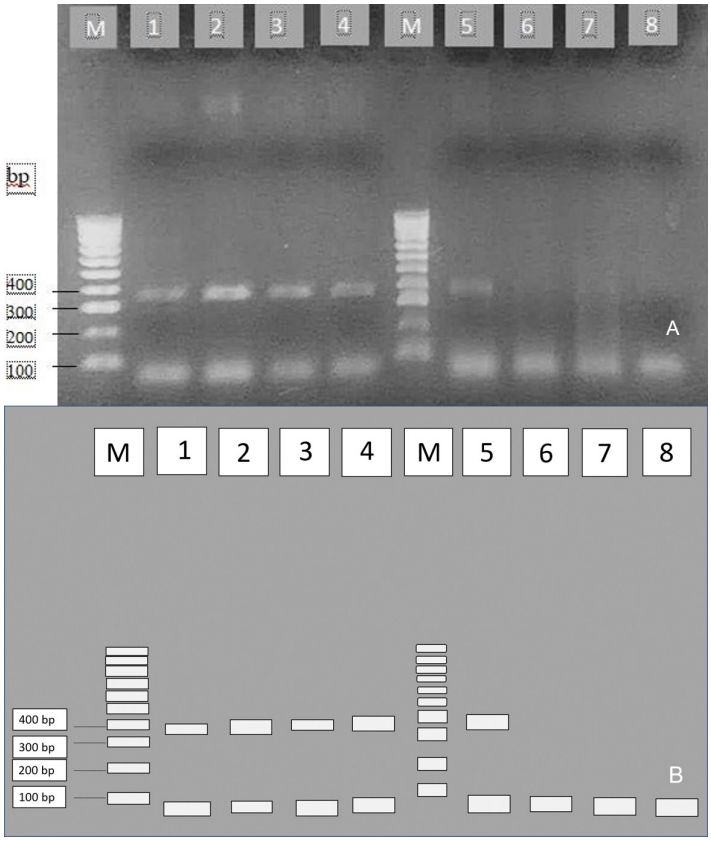

Figure 2. A. Electrophoresis of the amplified CHD-1 gene from samples taken from *C. sulphurea* bird. M= hyperladder 100 bp DNA marker, lane no. 1-4 = peripheral blood sample of *C. sulphurea*, lane no. 5-8 = plucked calamus samples of *C. sulphurea*. B. Electrophoregram of Figure 2A.

It was well known that extracted DNA from peripheral blood has better quality than extracted DNA from lucked feather. However to sex determine of endangered birds, a non-invasive method is required, because the invasive sample collection method has a high risk for species of endangered birds, therefore, the extracting DNA method from plucked feathers taken still needs to be improved as have been successfully performed in Avian by Khaerunnisa et al. (2013).

Table 3. Comparison between the quality of extracted DNA and PCR amplified DNA from peripheral blood samples and plucked feather samples from *C.sulphurea*.

No	Sample Code	Extraction		PCR Amplification	
		Blood	Plucked Feather	Blood	Plucked Feather
1.	***C. sulphurea* 1**	+++	++	+++	++
2.	***C. sulphurea* 2**	+++	+	+++	+
3.	***C. sulphurea* 3**	+++	+	+++	+
4.	***C. sulphurea* 4**	+++	+	+++	+

Note: +++: clear, ++: clear enough, +: less clear.

Based on Table 3, the comparison of the quality between extracted and PCR amplified DNAs indicated that the genetic origination from peripheral blood samples was better, compared to those obtained from the plucked feathers. Also, it indicated that blood sample was a better source of DNA in molecular bird sexing. Remedios et al. (2010) also stated that blood sample was an effective source of genetic template for molecular bird sexing, due to the fact that erythrocytes in birds had a nucleus which made them rich sources of DNA. The source of DNA in plucked feathers was only obtained from the calamus, which contained inhibitory protein (keratin) that made extraction process more difficult, with less quantity of DNA being obtained (Hickman et al. 2004).

The results of the DNA extraction also showed that the bands from the blood samples were visualized clearly, compared to those from the feathers. In Figure 1, samples with clearly visible DNA bands indicated that the quantity of extraction was numerous, as visualization was significant. However, samples with thin DNA bands indicated that the quantity of extraction was very little, as visualization was not clear enough.

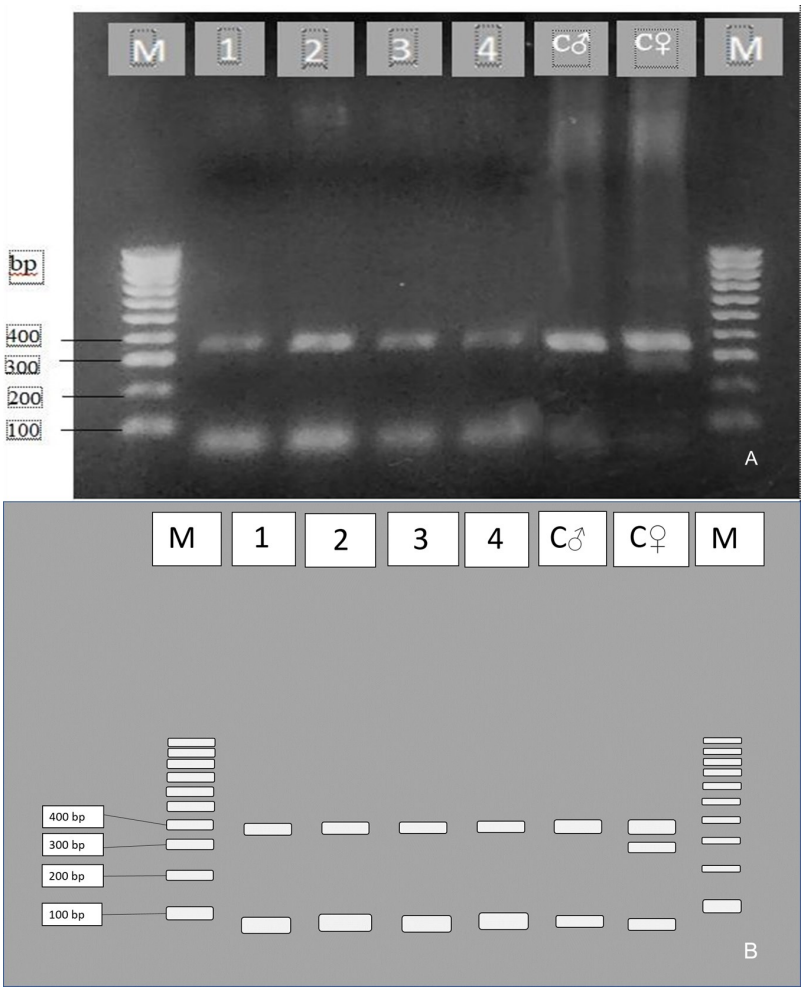

Figure 3. A. Electrophoresis of CHD-1 gen amplification result of *C. sulphurea* peripheral blood samples compared to control male and female cockatoos. M = Hyperladder 100 bp DNA marker, Lane 1-4 = peripheral blood sample of *C. sulphurea*; C♂ = control male *Cacatua sp*; C♀ = control female *Cacatua sp*. B. Electrophoregram of Figure 3A.

In this study, DNA amplification was observed to have used the PCR method, which targeted the CHD-1 gene on the W and Z chromosomes in *C. sulphurea*. This PCR was an *in vitro* reaction, which was used to multiply the number of DNA molecules on a particular gene. Moreover, this reaction was carried out by using a specific nucleotide primer pair, which complemented the target DNA that was to be amplified. However, this reaction occurred with the help of the DNA polymerase enzyme. The specific primers used in this study were the NP, P2, and MP, which were developed by Ito et al. (2003). These primers were used as a substitute for the those often used, namely P2 and P8, which were developed by Griffiths et al. (1998), due to the fact that they were unable to detect differences in the introns of the CHD gene on the Z and W chromosomes. The results of a study conducted by Lee et al. (2008), showed that out of the 29 species that tested, NP, P2, and MP, the primers only succeeded in determining the sex of 25, which belonged to seven different orders, namely Ciconiformes, Falconiformes, Gruiformes, Columbiformes, Strigiformes, Caprimulgiformes, and Passeriformes. A research by Thammakarn et al. (2007) also observed that these primers were used to determine the sex of several bird species, such as conures, macaws, and parrots, which belonged to the order Psittaciformes. Use of three primers (NP, P2, MP) for sex determination of *C. sulphurea*, was that these primer pairs have been successfully performed to sex determine by other researchers with other types of Cacatua birds, as reported in previous study by Savitri et al. (2021) to sex determine of *C. galerita* as well as by Hidayat et al. (2021) for *C. goffiniana.*

Also, the amplification of the CHD-1 gene was carried out *in vitro* with a thermocycler, at the optimized annealing temperature of 46°C for 30 secs, in 40 cycles. Sex determination with NP, P2, and MP primers was also carried out by looking at the visualization results on the UV-transilluminator, with a wavelength of 280 nm. PCR amplification in male *C. sulphurea* birds was also observed to produce one DNA band of 392 bp. However, female *C. sulphurea* produced two DNA bands of 297 bp and 392 bp, respectively. This was due to the fact that birds have different sex chromosomes, compared to mammals. The heterogametic and homogametic characters of sex chromosomes in birds were discovered in females and males, as ZW and ZZ genes, respectively (Ellegren 1996). The CHD-1 gene also showed differences in Z and W alleles in female birds, due to the linkage between their positions (Griffiths & Korn 1997).

According to Garofalo et al. (2016), NP were forward primers that attached to both the CHD-1W and CHD-1Z on the W and Z sex chromosomes, respectively. There were also two reverse primers, namely P2 and MP, which anneal to CHD-1Z and CHD-1W genes on the Z and W sex chromosomes, respectively. Therefore, in female birds, two DNA bands (Z and W) of 297 and 392 bp were observed to have occurred, compared to the males (Z) only in size of 392 bp.

Furthermore, the visualization under UV-transilluminator compared to Hyperladder 100 bp DNA Marker showed PCR products with length around 297 bp and 392 bp. The electrophoresis of the amplified CHD-1 gene was also presented in Figure 2. Samples coded SD1, SD2, SD3, SD4, and SB1 were observed to have produced clear DNA bands, compared to the very thin display of SB2, SB3 and SB4. These thin observations made visualization very difficult to conduct. Also, the 1.5% agarose gel electrophoresis showed that the blood samples produced a clear DNA band, compared to the thin display of the plucked feathers. According to Harvey et al. (2006), the amount of DNA that were isolated from a feather sample was not as much as those from the blood. However, the DNA produced from plucked feather samples was easier to degrade.

The electrophoresis of the amplified CHD-1 gene (*C. sulphurea* blood samples) compared with control male and female *Cacatua sp.,* were also presented in Figure 3. This comparison showed the PCR amplification results of the CHD-1 gene on SD1, SD2, SD3, SD4, with the production of one DNA band at 392 bp. This was in line with the results from a research on curve-billed birds by Purwaningrum et al. (2019), which discovered that male birds produced one DNA band of 392 bp, as a result of CHD-1 gene amplification on the Z sex chromosome, with the females producing two bands of 392 bp from the process. Lee et al. (2008) also stated that amplification of the CHD-1 gene segment in male birds only produced one fragment of DNA amplicon from the Z chromosome, with two fragments displayed in females from the Z and W alleles.

Based on the results of electrophoresis in Figure 3, sex determination using the PCR method on the small yellow-crested cockatoo (*C. sulphurea*), was interpreted as shown in Table 4. This interpretation indicated that the four *C. sulphurea* birds tested were male, since only single DNA band of 392 bp was produced in all samples. This interpretation was also in line with the previous studies conducted by Lee et al. (2008) and Purwaningrum et al. (2019) on the curved-billed birds, as well as by Savitri et al. (2021) that had successfully conducted the molecular bird sexing on Sulphur-crested Cockatoo (*Cacatua galerita*) and Hidayat et al. (2021) on Tanimbar Cockatoo (*Cacatua goffiniana*) by PCR amplification.

Table 4. Interpretation of the results of sex determination of *C. sulphurea* birds by PCR method.

No	Sample Code	Electrophoresis Results	Interpretation
1.	**SD1**	single DNA band	Male
2.	**SD2**	single DNA band	Male
3.	**SD3**	single DNA band	Male
4.	**SD4**	single DNA band	Male
5.	**SB1**	Single DNA band	Male
6.	**SB2**	Unclear	Unidentified
7.	**SB3**	Unclear	Unidentified
8.	**SB4**	Unclear	Unidentified

Based on the DNA quality for molecular bird sexing in *C. sulphurea,* samples derived from peripheral blood have a better quality than those from plucked feathers, which were used as a source of DNA. In male *C. sulphurea*, amplification of the CHD-1 gene generated a single DNA fragment in size of 392 bp for the Z chromosome, through the use of NP and P2 primers. However, female birds generated double DNA fragments, in sizes of 297 bp and 392 bp in the W and Z chromosomes, respectively.

In Molecular bird sexing of *C. sulphurea*, the extracted DNA from peripheral blood samples have a better quality when compared to samples from plucked feathers. Amplification of the CHD-1 gene in male *C. sulphurea* generated only a single DNA fragment in size of 392 bp, so the four of tested *C. sulphurea* in this study were male birds. Although extracted DNA template from peripheral blood samples have a better quality, we suggested that to sex determine of *C. sulphurea* which is an endangered bird to use an extracted DNA from non-invasive samples as plucked feather, because it was easier to perform, low risk, more convenient and has a low stress level for birds (Ratri 2020).

AUTHORS CONTRIBUTION

INDR carried out all of laboratory works, data analysed and wrote the draft manuscript, IP took care of the research permit and collected the field samples, WPN collected and handled the field samples, AH designed the research, supervised all of the research process and finished the draft manuscript.

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CONFLICT OF INTEREST

The authors declare that they have no competing interest

REFERENCES

Argarini, A.D. et al., 2020. Molecular bird sexing on Fischeri Lovebird (Agapornis fischeri) by using Polymerase Chain Reaction Method. *BIO Web of Conference*, 20, 04003. doi: 10.1051/bioconf/20202004003.

Bosnjak, J. et al., 2013. Feasibility of non-invasive molecular method for sexing of Parrots. *Pakistan Journal of Zoology*, 45(3), pp.715-720.

CITES (Convention on International Trade in Endagered Species of Wild Fauna and Flora), 2015. '*Cacatua sulphurea'*, in *CITES*, viewed 18 January 2020, from https://cites.org/eng/taxonomy/term/2843

Ellegren, H., 1996. First gene on the avian W chromosome (CHD) provides a tag for universal sexing of non-ratite birds. *Proceedings of the Royal Society B: Biological Sciences*, 263(1377), pp.1635–1641. doi: https://doi.org/10.29007/g21c.

Fridolfsson, A.-K. & Ellegren, H., 1999. Nordic Society Oikos A Simple and Universal Method for Molecular Sexing of Non-Ratite Birds Published by: Wiley on behalf of Nordic Society Oikos Stable. *Journal of Avian Biology*, 30(1), pp.116–121. doi: https://doi.org/10.2307/3677252.

Garofalo, L. et al., 2016. Comparison between Two molecular protocols for sex determination in birds, with implications for the management and conservation of the eurasian Griffon vulture Gyps fulvus. *Avocetta*, 40 (1), pp.17–22. doi: https://doi.org/10.30456/AVO.2016103.

Griffiths, R. & Korn, R.M., 1997. A CHD1 gene is Z chromosome linked in the chicken Gallus domesticus. *Gene*, 197(1–2), pp.225–229. doi: 10.1016/s0378-1119(97)00266-7..

Griffiths, R. et al., 1998. Short Communication A DNA test to sex most birds. *Molecular Ecology*, 7, pp.1071–1075. doi: 10.1046/j.1365-294x.1998.00389.x.

Harvey, M.G. et al., 2006. A comparison of plucked feathers versus blood samples as DNA sources for molecular sexing. *Journal of Field Ornithology*, 77(2), pp.136–140. doi: https://doi.org/10.1111/j.1557-9263.2006.00033.x.

Hickman, C.P. et al., 2004. *Integrated principles of zoology* 11st ed., Singapore: McGraw-Hill.

Hidayat, R.F.K. et al., 2021. Molecular bird sexing of tanimbar cockatoos (*Cacatua goffiniana*) by using polymerase chain reaction method. *Journal of Tropical Biodiversity and Biotechnology*, 6(2). doi: 10.22146/jtbb.59997.

Ito, H. et al., 2003. Sex identification by alternative polymerase chain reaction methods in Falconiformes. *Zoological Science*, 20(3), pp.339–344. doi: https://doi.org/10.218/zsj.20.339.

Khaerunnisa, I. et al., 2013. Avian sex determination based on chromo helicase DNA-binding (CHD) genes using polymerase chain reaction (PCR). *Media Peternakan*, 36(2), pp.85–90. doi: 10.5398/medpet.2013.36.2.85.

Kurniawan, N. & Arifianto, A., 2017. *Ornitologi: sejarah, biologi, dan konservasi*, Malang: UB Press.

Lee, M.Y. et al., 2008. Application of two complementary molecular sexing methods for east Asian bird species. *Genes and Genomics*, 30(4), pp.365–372.

Morinha, F. et al., 2012. Molecular sexing of birds: A comparative review of polymerase chain reaction (PCR)-based methods. *Theriogenology*, 78(4), pp.703–714. doi: 10.1016/j.theriogenology.2012.04.015.

Nugraheni, P. et al., 2019. Sex determination of peach-faced lovebird (Agapornis roseicollis) using polymerase chain reaction (PCR) techniques. *IOP Conference Series: Earth and Environmental Science*, 355(1). doi: 10.1088/1755-1315/355/1/012111

Nugroho, A.H. & Moch, S.A.Z., 2015. Evaluation of sexing methods on black caped lory (Lorius lory , Linnaeus 1758). *Zoo Indonesia*, 24(2), pp.83–93.

Olsena, M.E. et al. 2012. DNA from keratinous tissue Part II: Feather. *Annals of Anatomy*. 194(1): 31-35. doi: https://doi.org/10.1016/ j.aanat.2011.03.003.

Pamulang, Y.V. & Haryanto, A., 2021. Short communication: Molecular bird sexing on kutilang (pycnonotus sp.) based on amplification of chd-z and chd-w genes by using polymerase chain reaction method. *Biodiversitas*, 22(1), pp.449–452. doi: 10.13057/biodiv/d220155.

Purwaningrum, M. et al., 2019. Molecular techniques for sex identification of captive birds. *Veterinary World*, 12(9), pp.1506–1513. doi: 10.14202/ vetworld. 2019.1506-1513.

Ratri, I.N.D., 2020. *Penentuan jenis kelamin secara molekuler pada Kakatua kecil jambul kuning (Cacatua sulphurea) dengan metode Polymerase Chain Reaction*. Fakultas Kedokteran Hewan, Universitas Gadjah Mada.

Remedios, D.N. et al., 2010. Molecular sex-typing in shorebirds: a review of an essential method for research in evolution, ecology and conservation. *Wader Study Group Bulletin*, 117 (June 2016), pp.109–116.

Savitri, D. et al., 2021. Molecular bird sexing of sulphur-crested cockatoo (*Cacatua galerita*) by polymerase chain reaction method. *Indonesian Journal of Biotechnology*, 26(1), pp.1–6. doi: 10.22146/ijbiotech.54611.

Thammakarn, C. et al., 2007. Sex identification of some Psittacine birds by polymerase chain reaction. *Journal of Mahanakorn Veterinary Medicine*, 2 (2), pp.30–34. doi: http://www.vet.mut.ac.th/journal_jmvm...

Widhiantara, I.G. et al., 2016. Burung Kakatua kecil jambul kuning. *Jurnal Virgin*, II(I), pp.15–21.

Zein, M.S.A. et al., 2017. Aplikasi kajian DNA molekuler dan fenotipik pada program pelepasliaran burung Kakatua. *Jurnal Biologi Indonesia*, 13(1), pp.157–169. doi: 10.14203/jbi.v13i1.3105.

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Research Article

The Effectiveness of Stingless Bees on Pollination of Bitter Melon Plants *Momordica charantia* L. (Cucurbitaceae)

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ABSTRACT

This study aimed to measure the effectiveness of stingless bee *Tetragonula* cf. *biroi* pollination on the fruit formation of bitter melon *Momordica charantia* plants. We used hoods on the observed bitter melon plants. In the first hood, stingless bees are inserted to help pollinate 100 bitter melon plants, while in the other hoods, stingless bees are not inserted so that there is no assistance in pollinating the other 100 bitter melon plants. The method used is the focal sampling method for 25 days of observation. Based on the results of the study, stingless bee pollination assistance increased the percentage of the number of flowers that became fruit by 390%, the weight of seeds/fruit by 64%, number of seeds/fruit by 260%, fruit weight by 163%, fruit diameter by 91%, and fruit length by 86%. In addition to the size of the fruit, the shape of the bitter melon pollinated by bees is standard (long and straight). In contrast, the bitter melon that does not get pollination assistance grows with a bent shape resembling the letter "C." Bitter melon is an agricultural commodity that needs pollinating agents such as stingless bees because of its monoecy.

INTRODUCTION

Pollination is an essential factor in forming seeds and fruit of Angiosperms plants through the mechanism of pollen transfer from the anther to the stigma on the flower, which is the initial stage of the reproductive process. Some bees, such as *Amegilla*, *Apis*, stingless bees, and *Xylocopa*, are the primary pollinators of most crops (Delaplane & Mayer 2000). The decline in the population of pollinating bees around agricultural land will decrease the frequency of visits to flowers. Pollination by bees contributed significantly to fruit production, namely 41% on cranberries, 7% on blueberries, 26% on tomatoes, 45% on strawberries, and 22-24% on cotton (Delaplane & Mayer 2000), with estimated economic value amounted to US$ 14,564,000 (FAO 2006). Most horticultural crops require pollinating agents for the successful fertilization

process. Bitter melon *Momordica charantia* is one of the crops that require a pollinating agent. Bitter melon is a monoecious plant and has sticky pollen so the presence of pollinating insects can play an essential role in the process of pollen transfer to the stigma. Several studies have reported the effectiveness of insect pollinators on bitter melon plants such as Apidae, Halictidae, Syrpidae, Megachilidae, and Formicidae (Deyto & Cervancia 2009; Subhakar et al. 2011; Balina et al. 2012). Bitter melon is one of vegetables with high economic value in Indonesia. Bitter melon fruit has high nutritional value especially, high ascorbic acid, and iron and as well as medicinal properties against various diseases, including boosting the immune system in HIV-AIDS patients (Behera 2004; Njoroge & van Luijk 2004). The increasing demand for bitter melon is an important reason for optimizing the yield through improved agronomic practices and intensive pollination.

Pollinating bees that intensively visit flowers can speed up the process of pollination and fertilization (Husby et al. 2015). The mutualism symbiosis between pollinating bees and flowering plants provides very good results for the ecosystem, including increasing agricultural crop yields. Diversity and intensive visiting behavior of pollinating insects are positively correlated with fruit yield (Atmowidi et al. 2007). The use of stingless bees in helping pollinate cucurbitaceae plants has been widely reported. The number of seeds produced is better and the size of the fruit is larger (Bisui et al. 2020; Wayo et al. 2020). In this study, we used a stingless bee *T.* cf. *biroi* as a pollinating agent in bitter melon. This study aims to measure the effectiveness of the stingless bee on the success of pollinating bitter melon plants.

MATERIALS AND METHODS

Materials

We conducted this research in March-August 2021 in residential areas outside the agricultural location, Biringere Village, Kec. North Sinjai, Kab. Sinjai, South Sulawesi, Indonesia. In this study, we used one colony of *T.* cf. *biroi* with healthy and active colony group as a pollinating agent against bitter melon plants covered with insect nets.

Methods

Plant setup

A total of 200 bitter melon seeds were sown in polybags that had been given liquid organic fertilizer. After the shoots of bitter melon begin to appear or are about 15-20 days old, the seeds of bitter melon are transferred to land with a spacing of 25 cm x 25 cm. The seeds of bitter melon that have been transferred are watered every day and fertilized once a week using liquid organic fertilizer until the plants flower. The bitter melon plant is covered with insect net if the bitter melon plant has grown and has begun to propagate. Pest control is done manually without using pesticides.

Utilization of *T.* cf. *biroi* as pollinators of bitter melon plants

A total of 100 bitter melon plants that began to flower were covered with insect net mesh 50, size 3 x 4 x 3 m. After the bitter melon begins to blossom, as many as one colony of stingless bees is put in a hood. We also covered 100 other bitter melon plants, but We did not put stingless bees in the lid to prevent bee pollination from assisting.

Observation of foraging behavior of *T.* cf. *biroi*

The activity of bee visits on bitter melon was observed visually for 25 days using the focal sampling method (Martin & Bateson 1986). The visit activities observed were the number of flowers visited per minute (foraging rate), length of visits per flower (flower handling time), and length of visits to bitter melon plantations (Dafni 1992).

Environmental factor measurement

We measured environmental parameters during the bee diversity observation, including humidity and air temperature, wind speed, and light intensity. Environmental data measurements were carried out at 07.00, 09.00, 11.00, 13.00, and 14.00 WIB. The correlation between physical factors and foraging behavior was analyzed using Principal component analysis (PCA) in PAST software.

Measuring the effectiveness of pollinating bees

The effectiveness of bee pollinators was measured on 100 bitter melon plants caged with gauze and contained stingless bee colonies. Another hundred bitter melon plants were confined with gauze, without stingless bee pollination. The effectiveness of bees in pollinating bitter melon was measured by the percentage of the number of fruits formed, fruit size, number of seeds, and seed weight per fruit.

RESULTS AND DISCUSSION

Results

Foraging behavior of *T.* cf. *biroi* on bitter melon

Observation results show that the foraging *T.* cf. *biroi* activity in the cage occurs when the sun rises at 05.30 hrs until the sun sets at 18.00 hrs. The highest number of flowers visited per minute was in the third week, namely 39.8±0.91 flowers, and the least in the fifth week was 30.23±1.03 flowers (Figure 1a). The highest total number of plants visited per trip was 13.33±4.62 plants (first week), and the least in the third week was 10.39±0.62 plants (Figure 1c).

Correlation between environmental parameters and stingless bee's foraging activity

During the 25-day observation, the air temperature increased at 11.00 and decreased in the afternoon, starting at 16.00. The humidity is highest in the

morning and gets lower as the air temperature rises. The highest light intensity is at 11.00, and the lowest is at 16.00 (Table 1). The wind speed is relatively low because this research was carried out in a hood, so the wind does not affect the pollination of bitter melon plants (Table 2).

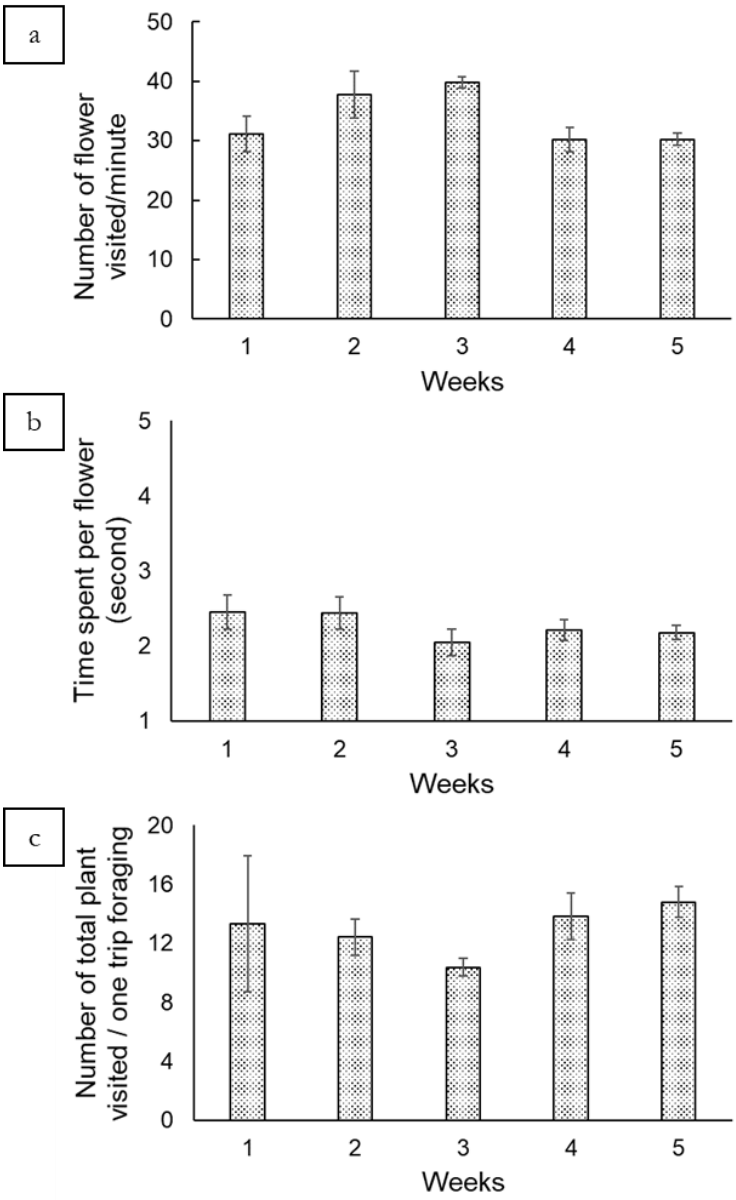

Figure 1. The behavior of *T.* cf. *biroi* visits on caged bitter melon. a) foraging rate, b) flower handling time, c) plant handling time. The first to third week is in May 2021, and the fourth to fifth week is the beginning of June 2021. Bars = standard deviation.

Table 1. Conditions of abiotic factors in the hood for 25 days of observation.

Times	Abiotic factors											
	Temperature			Humidity			Light intensity			Wind speed		
	Max	Min	Avrg	Max	Min	Avrg	Max	Min	Avrg	Max	Min	Avrg
07.00	32	17	21,98	73	23	59	1548	176	662,09	1,5	0	0,13
09.00	37	22	27,56	67	23	46,65	1304	99	793,68	10,6	0	0,70
11.00	36	24	29,28	64	19	41,28	1861	210	886,40	20,1	0	1,33
13.00	38	22	27,15	63	27	40,34	1502	113	743	10,5	0	0,79
16.00	30	19	24,8	72	30	36,09	897	53	540,51	0,2	0	0,72

Max = maximum; Min = Minimum; Avrg = Average

Table 2. Pearson correlation between the number of individuals and environmental parameters.

	Number of individuals	Temperature	Humidity	Light intensity	Wind speed
Number of individuals	1				
Temperature (°C)	0,092724996	1			
Humidity (%)	0,32651301	-0,69726	1		
Light intensity (Lux)	0,1070385	0,54137	-0,50304	1	
Wind speed (m/s)	-0,089986299	0,228328	-0,3444	0,159456	1

The effectiveness of *T.* cf. *biroi* on pollinating bitter melon plants

Significant differences were seen in bee-pollinated plants with non-pollinated plants (Table 3). In plants pollinated by *T.* cf. *biroi*, bitter melon fruit is longer and wider in diameter than plants without the help of bee pollination. The number of seeds per fruit in bee-pollinated plants is also more, and the weight of the seeds is heavier than plants that are not pollinated. The percentage of flowers that become fruit on plants pollinated by bees is 98%, while plants that are not pollinated by bees are only 20%, meaning that most (80%) flowers fail to become fruit. The shape of the bee-pollinated bitter melon also looks better, with a straight shape, long and 30.18±1.56 cm larger in size. Meanwhile, plants that were not pollinated by bees had an abnormal shape, bent, with a shorter size of 16.14±2.03 cm (Figure 2).

Figure 2. Bitter melon fruit pollinated by bees (top), and unpollinated bitter melon fruit (bottom).

Table 3. Comparison of fruit pollinated by a stingless bee with fruit without pollination assistance.

Component	n	Plantation		Enhancement (%)
		Pollinated by bees	Bees do not pollinate them	
Fruit length (cm)	25	$30,18^{b} \pm 1,56$	$16,14^{a} \pm 2,03$	86
Fruit diameter (cm)	25	$7,72^{b} \pm 0,51$	$4,04^{a} \pm 0,92$	91
Fruit weight (g)	25	$415,46^{b} \pm 35,37$	$157,69^{a} \pm 26,5$	163
Number of seeds/fruit	25	$31,75^{b} \pm 4,94$	$8,8^{a} \pm 3,1$	260
Seed weight/fruit (g)	25	$2,34^{b} \pm 0,36$	$1,42^{a} \pm 0,32$	64
Flower to fruit (%)	25	98	20	390

*Different letters in the same row indicate a significant difference with the 95% level T-test.

Discussion

Visiting activities can be a measure of the effectiveness of pollinating insects. A species has the potential as a pollinator if its visitation activity is intensive on one type of plant and is supported by distinctive morphological characters, such as the number of hairs on its body, and has a pollen basket as a pollen storage place (Dafni 1992).

The foraging activity of bees is influenced by the availability of feed and environmental conditions (Sadeh et al. 2007). The flying activity of bees is reduced at low temperatures and high humidity because the bees require a large amount of energy to heat the thoracic temperature to 35 °C (Yao et al. 2006). Visiting activities and the suitability of pollinating bees' body characters with flowers affect the success of pollination. Pollinating bees that visit flowers intensively can speed up the process of pollination and fertilization (Husby et al. 2015). The length of the bee's visit to the flower is influenced by the behavior of taking pollen (Mensah & Kudom 2011; Depra et al. 2014). Bitter melon plants are monoecious, where on one tree, male and female flowers are separated, with the ratio of male and female flowers being 15:1 (Kishan et al. 2017). In general, the Cucurbitaceae have large, sticky pollen. The character of the flower causes the bitter melon plant to need a pollinating agent to transfer pollen to the stigma. Dorjay et al. (2017) reported that the percentage of bitter melon fertilization increased by 87.14%, while in plants that carried out self-pollination, they formed only 2.17% of the fruit. *Cucumis melo* pollinated by a stingless bee produces more fruits and seeds, larger fruit size, and sweeter taste (Azmi et al. 2018). Increases in fruit length and weight and the number of fruits per plant also increased in cucumbers pollinated by *Tetragonula irridipennis* in India (Kishan et al. 2017). In this study, bitter melon fruit from stingless bee pollination had larger fruit, straight fruit shape, and more seeds.

Meanwhile, bitter melon plants that are not pollinated have smaller fruit, fewer seeds, and a curved fruit shape resembling the letter "C". In plants that are not pollinated by bees, there is a decrease in pollen viability after a few hours or a few days (Kahriman et al. 2015). This condition can cause fertilization failure, or fruit malformation occurs when fertilization occurs when pollen viability has decreased.

The number of individuals and the highest number of pollinating bee species occurred in the morning and the least in the afternoon. It was also reported by and Rianti (2010) that the highest abundance of pollinating insects occurred in the morning. This can be affected by the time the bitter melon bloom in the morning. Perfectly blooming flowers with yellow inflorescences are more attractive to pollinating bees (Faheem et al. 2004; Atmowidi et al. 2007).

Environmental factors influence the local distribution of bees in foraging, reproducing and molting (Gottlieb et al. 2005). Light intensity was positively correlated with the number of individuals and species of pollinating bees. Bee fly activity is reduced at low temperatures and high humidity, be-

cause it requires a large amount of energy to heat the chest temperature to 35°C (Yao et al. 2006). At low temperatures, the number of individual bees looking for food is also reduced, because the calories needed are also higher (Gerling et al. 1989). Klein et al. (2002) also reported an increase in the number of solitary bees in coffee plantations, along with an increase in light intensity. However, the number of species and the number of individuals were negatively correlated with wind speed.

CONCLUSION

The stingless bee, *T.* cf. *biroi* is a potential pollinator for monoecious bitter melon plants. Pollination by stingless bees can increase the quality and quantity of bitter melon fruit set. The match between the morphology of the bitter melon flower and the body size of the stingless bee causes pollination to occur optimally.

AUTHORS CONTRIBUTION

In this research, A.G.M. was tasked sampling and observing the behavior of bees in the field. RC.H.S. supervised all data analysis. R.E.P. designed the research and supervised all the process. R.R. controlled the sampling activity of bee behavior data and its effectiveness. H.P. supervised the results of data analysis and the manuscript. A.A. supervised data analysis and manuscript writing. S.K. controlled sampling activities in the field, and manuscript writing.

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CONFLICT OF INTEREST

There is no conflict of interest in this research.

REFERENCES

Atmowidi, T. et al., 2007. Diversity of pollinator insects in relation to seed set of mustard (*Brassica rapa* L: Cruciferae). *Hayati Journal of Biosciences*, 14(4), pp.155-161. doi: 10.4308/hjb.14.4.155.

Azmi, W.A. et al., 2018. Effects of pollination by Indo-Malaya stingless bee (Hymenoptera: Apidae) on the quality of greenhouse-produced rockmelon. *Journal of Economic Entomology*, 112(1), pp.20-24. doi: 10.1093/jee/toy290.

Balina, P.K. et al., 2012. Diversity, abundance, and pollination efficiency of native bee pollinators of bitter melon (*Momordica charantia* L.) in India. *Journal of Apicultural Research*, 51(3), pp.227-231. doi: 10.3896/IBRA.1.51.3.02

Behera, T.K. 2004. Heterosis in bitter gourd. *Journal of New Seeds,* 6, pp.217-221.

Bisui, S. et al., 2020. Utilization of Indian Dammar Bee (Tetragonula iridipennis Smith) as a Pollinator of Bitter Gourd. *Acta Agrobotanica*, 73 (1), pp.1-7. doi: 10.5586/aa.7316

Dafni, A., 1992. *Pollination Ecology a Practical Approach*, New York, US: Oxford Univ Press.

Delaplane, K.S. & Mayer, D.F., 2000. *Crop Pollination by Bees*, New York, US: CABI Publishing.

Depra, M.S. et al., 2014. Pollination Deficit in Open-Field Tomato Crops (*Solanum lycopersicon* L., Solanaceae). *Journal of Pollination Ecology*, 12(1), pp.1-8. doi: 10.26786/1920-7603(2014)7.

Deyto, R.C. & Cervancia, C.R., 2009. Floral biology and pollination of ampalaya (*Momordica charantia* L). *The Philippine Agricultural Scientist,* 92(1), pp.8 -18.

Dorjay et al., 2017. Insect visitor on cucumber and bittergourd flowers and impact on quantity of crop production by different pollination treatment. *Journal of Apiculture*, 32(2), pp.77-88. doi: 10.17519/ apiculture.2017.06.32.2.77.

Faheem, M. et al., 2004. Pollination ecology with special reference to insects a review. *Journal of Scientific Research*, 4, pp.395-409.

Food and Agricultural Organization of The United Nations, 2006. *Economic Valuation of Pollination Services*, Food and Agriculture Organization of the United Nations Agriculture Department, Seed and Plant Genetic Resources Division (AGPS).

Gerling, D. et al., 1989. Bionomics of the large carpenter bee *Xylocopa* pubescens and its implications for the evolution of sociality. *Annual Review of Entomol*ogy, 2, pp.123–128. doi: 10.1146/ annurev.en.34.010189.001115

Gottlieb, D. et al., 2005. Possible foraging benefits of bimodal daily activity in *ProXylocopa olivieri* (Lepeletier) (Hymenoptera:Anthophoridae). *Environmental Entomology*, 34, pp.417-424. doi: 10.1603/0046-225X-34.2.417

Husby, J.F. et al., 2015. Pollinators May Not Limit Native Seed Set at Puget Lowland Prairie Restoration Nurseries. *Journal of Pollination Ecology,* 15 (5), pp.30-37. doi: 10.26786/1920-7603(2015)4

Kahriman, F. et al., 2015, 'The Role of Artificial Pollination and Pollen Effect on Ear Development and Kernel Structure of Different Maize Genotypes', *Journal of Pollination Ecology,*15(2), pp.6-14. doi: 10.26786/1920-7603(2015)1

Kishan, T.M. et al., 2017. Stingless bee *Tetragonula irridipennis* Smith for pollination of greenhouse cucumber. *Journal of Entomology and Zoology Studies*, 5(4), pp.1729-1733.

Klein, A.M. et. al., 2002. Effects of landuse intensity in tropical agroforestry systems on coffe flower-visiting and trapnesting bees and wasps. *Conservation biol*ogy, 16, pp.1003-1014.

Martin, P., & Bateson, P., 1986. *Measuring behaviour: An introductory guide*, Cambridge University Press.

Martin, P. & Bateson, P., 1993. *Measuring Behavior*, Cambridge, GB: Cambridge Univ Press.

Mensah, B.A. & Kudom, A.A., 2011. Foraging Dynamics and Pollination Efficiency of *Apis mellifera* and *Xylocopa olivacea* on *Luffa aegyptiaca* Mill (Cucurbitaceae) in Southern Ghana. *Journal of Pollination Ecology,* 4(5), pp.34-38. doi: 10.26786/1920-7603(2011)6

Njoroge, G.N, & van Luijk, M.N., 2004. Momordica charantia L. In: Grubben, GJH. And Denton, OA. (eds.). PROTA 2: Vegetables/Légumes. PROTA, Wageningen, Netherlands.

Rianti, P. et al., 2010, Diversity and Efectiveness of Insect Pollinators of *Jatropha curcas* L. (Euphorbiaceae). *Hayati Journal of Bioscience*, 17(1), pp.38-42. doi: 10.4308/hjb.17.1.38

Sadeh, A. et al., 2007. The Carpenter Bee *Xylocopa pubescens* as an Agricultural Pollinator in Greenhouse. *Apidologie*, 38, pp.508-517. doi: 10.1051/apido:2007036

Subhakar, G. et al., 2011. Pollinator diversity and abundance in bitter melon *Momordica charantia* Linn. *Pest Management in Horticultural Ecosystems*, 17 (1), pp.23-27.

Wayo, K. et al., 2020. Local and Landscape Compositions Influence Stingless Bee Communities and Pollination Networks in Tropical Mixed Fruit Orchards, Thailand. *Diversity*, 12(482), pp.1-17. doi: 10.3390/d12120482

Yao, Y.F. et al., 2006. Nectar and pollen sources for honeybee (*Apis cerana cerana* Fabr.) in Qinglan mangrove area, Hainan Island, China. *Journal integrative plant Biology,* 48(11), pp.1266-1273. doi: 10.1111/j.1744-7909.2006.00353.x

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Research Article

Reef Fish Diversity in Jayapura City, Indonesia: A Preliminary Study

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ABSTRACT
As one of the marine areas included in the world's Coral Triangle region, Yos Sudarso Bay have a potential reef fish diversity that needs to be studied. However, there is very little information about reef fish diversity in these waters to date. This study aims to determine the species diversity of reef fish in Yos Sudarso Bay, Jayapura City, Indonesia. The study was conducted in April 2020 at seven sites; six of them located inside the Yos Sudarso Bay and one more located outside the bay. Sampling was carried out using the Underwater Visual Census method (25 m long and 5 m wide). Relative abundance by species, and diversity (H´), evenness (E), and dominance (C) indices were calculated. A total of 1,075 individual reef fish was recorded in seven study sites, representing 122 species and 26 families. Locations showed differences in reef fish abundance (86 to 215 individuals/125 m^2), diversity (H' = 2.462 to 3.358), evenness (E = 0.770 to 0.887), and dominance (C = 0.047 to 0.155). This study has provided preliminary information on species diversity, fish abundance, and the ecological index of reef fish in Yos Sudarso Bay, Jayapura City.

INTRODUCTION
Coral reefs are a complex and productive ecosystem in coastal areas (Marshell & Mumby 2015). This ecosystem provides many important habitats that have high biodiversity and provide benefits to people in many tropical regions (Veron 2002; Madduppa et al. 2012). Several important ecological functions of coral reef ecosystems including a habitat for protection from predators, spawning ground, nursery ground, and feeding ground for various species of reef fish (Cole et al. 2008; Madduppa et al. 2012).

Indonesian waters have the highest number of reef fish species in the world. There are 2,139 species of reef fish in Indonesia reported in the FishBase (Froese & Pauly 2020) and about 197 species are endemic (Allen & Erdmann 2012). The reef fish species are spread from the Western to the Eastern part of Indonesia with a variety of different habitat types, causing differences in the structure of the community. In particular, Papua's waters in the world's Coral Triangle region have a high level of reef fish diversity. Ac-

cording to Allen and Erdmann (2009), 1,511 species have been identified in 451 genera and 111 families of reef fish around the Bird's Head peninsula and surrounding waters in West Papua Province. Live coral cover and diversity of coral lifeforms are important indicators of reef fish species abundance and diversity (Garpe & Öhman 2003; Komyakova et al. 2018; Paulangan et al. 2019a), including variations in coral species and coral reef habitat zones (Bell & Galzin 1984; Paulangan et al. 2019a). The diversity of reef fish species correlates with the condition of coral reefs, where the loss of more than 20% corals can reduce the richness of reef fish species (Wilson et al. 2006). Therefore, the diversity of coral reef habitats is one of the key factors and can explain a large number of reef fish species and individuals in the ecosystem (Roberts & Ormond 1987).

As one of the marine areas included in the world's Coral Triangle region, Yos Sudarso Bay also has a potential reef fish diversity need to be studied. However, there is very little information about reef fish diversity in these waters to date (Tebaiy et al. 2014; Hamuna et al. 2019) who evaluates only reef indicator species (Chaetodontidae) and species present in seagrasses respectively. Data and information on reef fish diversity are very important because they are one of the components for the management and development of the fisheries and tourism sector in coral reef areas. Based on the results of research by Hamuna et al. (2019), the condition of coral reefs in Jayapura City in several locations is still in moderate to good condition with the percentage of live coral cover ranging from 32 to 60%. This shows that the coral reef ecosystem in Jayapura City has the potential to become a habitat for various species of reef fish. This study aims to determine the number of reef fish species, and to analyze the abundance and ecological index of reef fish in the coral reef ecosystem of Yos Sudarso Bay, Jayapura City, Papua Province, Indonesia.

MATERIALS AND METHODS

Study Site

Yos Sudarso Bay is one of the bays in Jayapura City, Papua Province, Indonesia. Geographically, Yos Sudarso Bay is directly adjacent to the Pacific Ocean. Generally, as one of the coastal areas in tropical waters, the coastal waters of Jayapura City, including Yos Sudarso Bay, are an area that is quite rich in natural resources, including the three main ecosystems in the coastal area, namely mangrove and seagrass ecosystems in Youtefa Bay (Rumahorbo et al. 2019; Rumahorbo et al. 2020) and coral reef ecosystems located in the Yos Sudarso Bay (Hamuna et al. 2019; Rumahorbo et al. 2020), as well as high potential fishery resources (Tebaiy et al. 2014; Hamuna et al. 2020; Pujiyati et al. 2021). In particular, the condition of coral reefs in Yos Sudarso Bay is in a moderate to good category with live coral cover ranging from 32 to 60% (Hamuna et al. 2019). However, at certain sites, the condition of the coral reefs has been damaged due to destructive fishing as indicated by the amount of rubble.

The observation of reef fish was conducted in April 2020 in seven sites (Table 1; Figure 1). Six of them were inside the Yos Sudarso Bay (S2-S6) and one more was outside the Yos Sudarso Bay (S1).

Table 1. Location of study sites and coordinates in Yos Sudarso Bay, Indonesia.

Site code	Site name	Coordinates	
		S	E
S1	Base-G coastal	2° 31' 15.062"	140° 44' 36.441"
S2	Southern of the Argapura coastal	2° 33' 24.036"	140° 43' 15.442"
S3	Northern of the Argapura coastal	2° 32' 55.921"	140° 43' 9.863"
S4	Eastern of the Kosong Island	2° 32' 51.458"	140° 43' 37.533"
S5	Western of Kosong Island	2° 32' 49.226"	140° 43' 22,136
S6	Kayupulo Island	2° 32' 41.64"	140° 43' 19.458"
S7	Lampu Merah reefs	2° 32' 31.598"	140° 43' 5.847"

Data Collection

Before conducting reef fish observations, a field survey was conducted to determine the reef fish observation points. Observations of reef fish only during the day using the Underwater Visual Census (UVC) method refers to English et al. (1997). UVC is a method that has been widely used for monitoring or evaluating reef fish resources. At each study site, observation of reef fish was only carried out on one transect at a depth of 3 to 5 m depending on the condition of the coral reef. The 25 m transect line was placed parallel to the coastline and reef fish observations were carried out 15 to 20

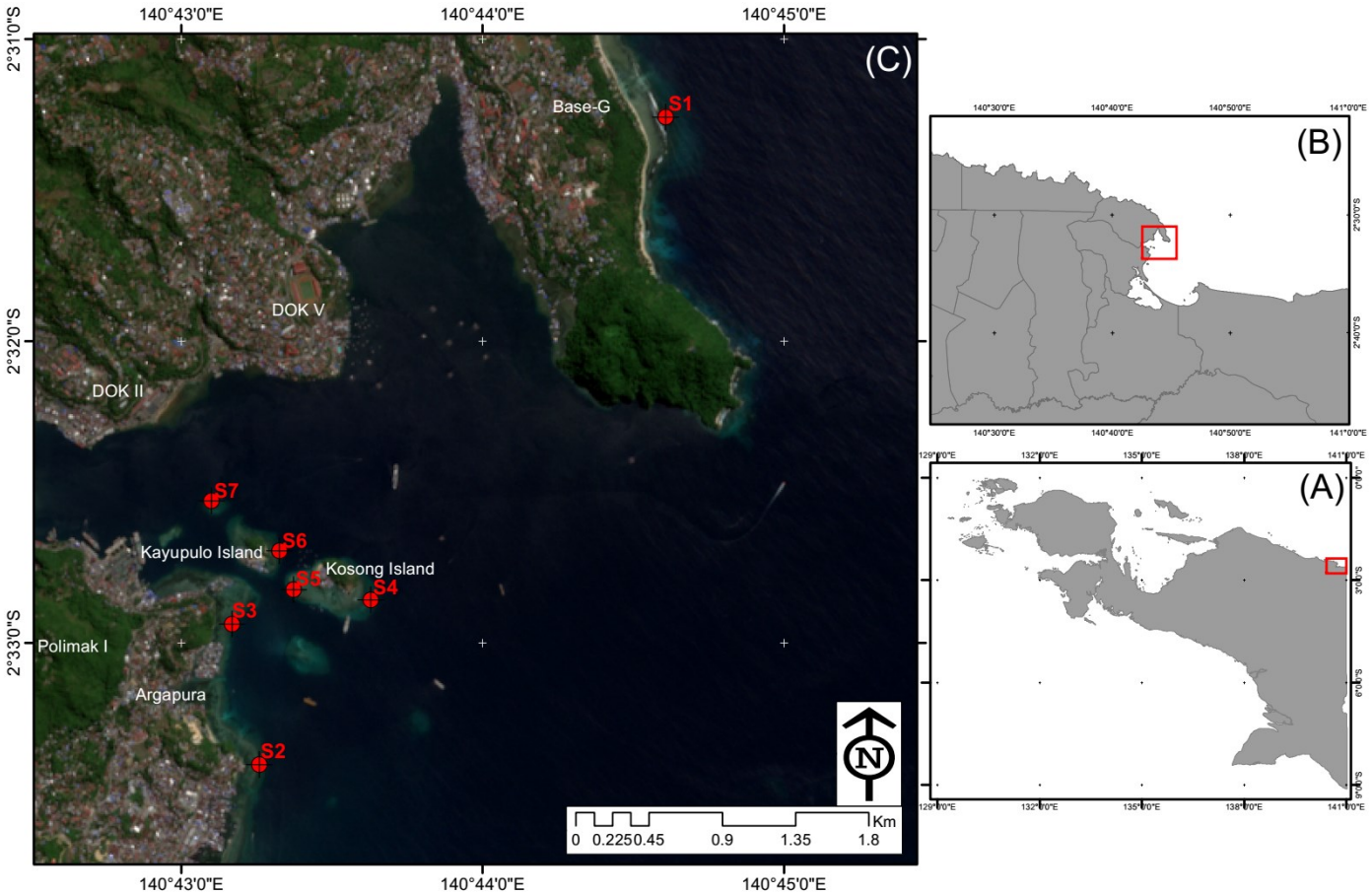

Figure 1. Map of study sites; (A) Papua Island (Papua and West Papua Provinces), (B) Jayapura City, and (C) seven sites (S1-S7) for reef fish observation in Yos Sudarso Bay, Papua Province, Indonesia.

minutes after that. The number of individuals of each reef fish species found was counted to the observation distance limit of 2.5 m on both sides of the transect line (transect area = 125 m^2). In addition to direct field observations, underwater photos and videos were also taken to re-identify certain species and families of reef fish that were not recognized during field observations, where re-identification refers to Allen et al. (2000).

Data Analysis

Analysis of reef fish diversity was carried out at the species and family level. Furthermore, reef fish are grouped based on their role, namely major fish, target fish, and indicator fish according to English et al. (1997) and Madduppa et al. (2012). The major fish group includes colored small ornamental fish (generally 5-25 cm in size) which tends to be territorial, such as the families Pomacentridae, Apogonidae, Balistidae, Labridae, Gobiidae, and Blenniidae. Target fish are represented by economically important species for human consumption, such as the families Acanthuridae, Lutjanidae, Serranidae, Holocentridae, Nemipteridae, and Haemulidae. Target fish groups were confirmed based on direct interviews with several local fishermen. Indicator fish are reef fish species that typically inhabit coral reef ecosystems and as indicators of the fertility of these ecosystems, such as reef fish from the Chaetodontidae family.

Data on the number of individuals from each type of reef fish were analysed to determine the abundance of reef fish in each location. Abundance of reef fish was determined based on the ratio between the numbers of individual reef fish with the area of observation (English et al. 1997). Ecological indices of reef fish, including the diversity index (Shannon-Wiener), evenness index (Pielou's), and dominance index (Simpson) were determined referring to the following equations (Magurran 1991):

$$H' = -\sum_{i=1}^{s} [p_i \ln p_i] \; ; \; p_i = \frac{n_i}{N}$$

$$E = \frac{H'}{\ln S}$$

$$C = \sum_{i=1}^{s} p_i^{\,2}$$

where H', E, and C are the diversity index, the evenness index, and the dominance index, respectively. Whereas S is the number of reef fish species, n_i is the number of individuals of each reef fish species, and N is the total individuals of all reef fish species.

Multivariate analysis is used to determine the level of similarity between sites. The data used to determine the similarity index is the relative abundance of each species of reef fish. Non-metric Multidimensional Scaling (nMDS) based on Bray-Curtis Similarity is used to visualize the level of similarity between sites (Clarke 1993) using PRIMER 7 software. The quality of MDS 2-dimensional plots can be determined based on stress value, namely

stress value <0.2 (poor representation), stress value <0.1 (good representation), and stress value <0.05 (excellent representation) (Kruskal 1964; Field et al. 1982).

RESULTS AND DISCUSSION

Species Diversity and Abundance

The results of reef fish observations showed that the number of families, species, number of individuals, and abundance of reef fish found varied in the seven study sites. There were 1,073 individuals from 122 reef fish species representing 26 families during the observation in seven study sites (Table 2). The reef fish species that was most frequently found during the observation at the seven study sites were *Ctenochaetus cyanocheilus, Zanclus cornutus, Zebrasoma scopas, Ctenochaetus binotatus, Ctenochaetus striatus, Nectamia savayensis, Pomacentrus moluccensis, Neopomacentrus filamentosus, Pomacentrus taeniometopon*, and *Chromis ternatensis*. However, only *C. cyanocheilus* and *C. binotatus* were found at all study sites. Pomacentridae and Acanthuridae were the most common reef fish families, as many as 348 individuals (35 species) and 308 individuals (11 species), respectively. Both families reached 61.02% of the total reef fish individuals surveyed.

Based on the composition of the roles of each species, the reef fish found were dominated by major fish groups of 68 species and 581 individual fish. The number of species and individual groups of target fish and indicator fish was 42 species (376 individuals) and 12 species (118 individuals), respectively. Although English et al. (1997) and Madduppa et al. (2012) classify Labridae species as major fish, several species were included in the target fish group due to their importance to Papua fishermen., such as *Bodianus anthioides*, *Bodianus mesothorax*, *Cheilinus chlororus*, *Cheilinus fasciatus*, *Cheilinus tribolatus*, *Halichoeres argus*, and *Hemigymnus melapterus*.

Abundance and Ecological Index of Reef Fish Communities

The abundance of reef fish at the study sites ranged from 112 to 215 individuals/125 m^2 (Table 3). Ecological indices values ranged between 2.468 to 3.358, 0.770 to 0.887, and 0.047 to 0.155 for diversity index, evenness index, and dominance index, respectively. The higher diversity and evenness index values are inversely proportional to the lower dominance index. However, there are certain reef fish species that have a high dominance index compared to other reef fish species, such as *N. filamentosus* (C = 0.031) at S1 site, *Z. cornutus* (C = 0.025) at S2 site, *N. savayensis* (C = 0.079) at the S3 site, *C. ternatensis* (C = 0.122) at the S4 site, *C. cyanocheilus* (C = 0.012) at the S6 site, and *P. moluccensis* (C = 0.018) at the S7 site.

Analysis of Similarity Index

The results of the analysis of the similarity index between sites using nMDS showed a stress value of 0.06 so that the resulting nMDS plot had a good representation (Figure 2). Based on the number of individuals for each reef

Table 2. Richness of reef fish species in each site surveyed in Yos Sudarso Bay, Jayapura City, Indonesia (M = major fish; T = target fish; and I = indicator fish).

Family and Species	S1	S2	S3	S4	S5	S6	S7	Total	Fish Category
Acanthuridae									
Ctenochaetus cyanocheilus	14	15	11	4	13	23	15	95	T
Ctenochaetus binotatus	4	4	7	7	10	9	12	53	T
Zebrasoma scopas	-	18	13	1	10	9	2	53	T
Ctenochaetus striatus	4	8	-	-	9	20	10	51	T
Acanthurus nigrofuscus	3	-	-	-	11	4	11	29	T
Acanthurus lineatus	2	1	-	-	-	12	-	15	T
Acanthurus maculiceps	-	4	-	-	-	-	-	4	T
Acanthurus pyroferus	-	-	-	2	-	1	-	3	T
Acanthurus thompsoni	3	-	-	-	-	-	-	3	T
Acanthurus leucosternon	1	-	-	-	-	-	-	1	T
Naso lituratus	-	-	-	-	-	-	1	1	T
Apogonidae									
Nectamia savayensis	-	-	45	-	-	-	-	45	M
Taeniamia zosterophora	-	-	28	-	-	-	-	28	M
Cheilodipterus artus	-	-	2	-	-	-	-	2	M
Ostorhinchus multilineatus	-	-	-	-	1	-	-	1	M
Balistidae									
Sufflamen chrysopterum	-	1	-	-	-	1	1	3	M
Balistapus undulates	-	-	-	-	-	1	-	1	M
Blenniidae									
Meiacanthus grammistes	-	-	-	-	-	-	2	2	M
Centriscidae									
Centriscus scutatus	-	13	-	-	-	-	-	13	M
Chaetodontidae									
Chaetodon trifasciatus	-	7	7	3	6	1	3	27	I
Chaetodon vagabundus	-	9	-	9	5	4	-	27	I
Chaetodon triangulum	-	6	1	-	4	4	3	18	I
Heniochus chrysostomus	-	8	1	2	2	-	2	13	I
Chaetodon citrinellus	-	2	-	-	-	9	1	12	I
Chaetodon kleinii	-	-	-	-	4	1	2	7	I
Chaetodon rafflesi	-	-	1	2	-	-	2	5	I
Chaetodon ornatissimus	-	-	3	-	-	-	-	3	I
Chaetodon meyeri	-	-	-	-	1	-	-	1	I
Chaetodon oxycephalus	-	1	-	-	-	-	-	1	I
Forcipiger longirostris	-	-	-	-	-	-	1	1	I
Heniochus varius	-	-	-	-	-	1	-	1	I
Cirrhitidae									
Paracirrhites forsteri	1	-	-	-	-	2	-	3	M
Amblycirrhitus bimacula	-	-	-	-	1	-	-	1	M
Cirrhitichthys falco	-	-	-	-	1	-	-	1	M
Diodontidae									
Lophodiodon calori	-	-	-	-	1	-	-	1	M
Gobiidae									
Cryptocentrus cinctus	-	-	-	-	1	-	-	1	M
Eviota punctulata	-	-	-	-	-	1	-	1	M
Haemulidae									
Plectorhinchus lineatus	-	2	-	-	-	-	-	2	T
Plectorhinchus polytaenia	-	1	-	-	-	-	-	1	T
Holocentridae									
Myripristis kuntee	-	-	3	-	-	-	2	5	T
Neoniphon sammara	-	-	4	-	-	-	-	4	T
Myripristis amaena	-	-	-	-	-	-	2	2	T
Sargocentron diadema	-	-	-	-	-	1	1	2	T

Table 2. Contd.

Family and Species	S1	S2	S3	S4	S5	S6	S7	Total	Fish Category
Myripristis berndti	-	-	1	-	-	-	-	1	T
Labridae									
Thalassoma hardwicke	2	8	1	-	2	3	1	17	M
Thalassoma lunare	-	-	-	-	1	2	5	8	M
Cheilinus chlorourus	-	1	-	2	1	1	1	6	T
Labroides dimidiatus	-	1	4	1	-	-	-	6	M
Halichoeres argus	-	3	-	-	-	-	-	3	T
Hemigymnus melapterus	-	-	3	-	-	-	-	3	T
Labrichthys unilineatus	-	-	3	-	-	-	-	3	M
Stethojulis trilineata	-	2	-	-	1	-	-	3	M
Anampses elegans	-	1	-	-	1	-	-	2	M
Bodianus anthioides	-	-	-	-	-	2	-	2	T
Bodianus macrourus	-	-	-	-	2	-	-	2	T
Bodianus mesothorax	-	-	-	-	1	1	-	2	T
Halichoeres biocellatus	-	-	-	-	2	-	-	2	T
Stethojulis interrupta	-	-	-	-	2	-	-	2	M
Cheilinus fasciatus	-	1	-	-	-	-	-	1	T
Cheilinus trilobatus	-	-	-	-	1	-	-	1	T
Halichoeres hortulanus	-	-	-	-	1	-	-	1	T
Monacanthidae									
Cantherhines pardalis	-	-	-	-	2	-	-	2	M
Cantherhines dumerilii	-	-	-	-	-	1	-	1	M
Mullidae									
Parupeneus multifasciatus	-	2	-	-	2	3	-	7	M
Parupeneus bifasciatus	-	1	-	-	-	1	-	2	M
Nemipteridae									
Scolopsis bilineata	-	-	-	1	1	-	-	2	T
Scolopsis lineata	-	-	-	-	1	-	-	1	T
Scolopsis taenioptera	-	-	-	-	1	-	-	1	T
Ostraciidae									
Ostracion cubicum	-	1	-	-	-	1	-	2	M
Ostracion meleagris	-	-	-	-	-	-	1	1	M
Pempheridae									
Pempheris adusta	-	6	-	-	1	4	-	11	M
Pinguipedidae									
Parapercis hexophtalma	-	-	-	-	-	2	-	2	M
Parapercis millepunctata	-	1	-	-	-	-	-	1	M
Pomacanthidae									
Centropyge bicolor	-	3	-	-	-	-	2	5	M
Pomacentridae									
Pomacentrus moluccensis	-	-	11	1	-	9	22	43	M
Neopomacentrus filamentosus	19	20	-	-	-	-	-	39	M
Pomacentrus taeniometopon	-	-	-	-	10	13	10	33	M
Chromis ternatensis	-	-	-	30	-	-	-	30	M
Plectroglyphidodon dickii	5	-	-	-	8	9	3	25	M
Chromis margaritifer	2	-	4	1	-	9	5	21	M
Chrysiptera cyanea	-	-	-	-	-	9	10	19	M
Plectroglyphidodon lacrymatus	14	-	-	-	-	-	-	14	M
Chrysiptera unimaculata	-	-	-	-	-	13	-	13	M
Chromis caudalis	8	-	-	-	-	-	3	11	M
Pomacentrus reidi	-	-	-	-	11	-	-	11	M
Abudefduf vaigiensis	10	-	-	-	-	-	-	10	M
Chromis xanthura	7	-	-	-	2	-	-	9	M
Pomacentrus armillatus	2	-	-	-	-	-	6	8	M
Neoglyphidodon nigroris	-	7	-	-	-	-	-	7	M
Amblyglyphidodon aureus	-	-	-	6	-	-	-	6	M

Table 2. Contd.

Family and Species	S1	S2	S3	S4	S5	S6	S7	Total	Fish Category
Pomacentrus lepidogenys	-	-	-	-	-	-	6	6	M
Amblyglyphidodon leucogaster	-	-	-	-	4	-	-	4	M
Pomacentrus brachialis	2	-	-	-	-	-	2	4	M
Pomacentrus emarginatus	4	-	-	-	-	-	-	4	M
Amphiprion frenatus	-	-	-	-	-	-	3	3	M
Amphiprion sebae	-	-	-	3	-	-	-	3	M
Chromis lepidolepis	-	-	-	-	3	-	-	3	M
Chrysiptera brownriggii	-	-	-	-	-	3	-	3	M
Pomacentrus littoralis	-	-	-	3	-	-	-	3	M
Amphiprion clarkii	-	-	-	-	-	-	2	2	M
Amphiprion polymnus	-	-	-	2	-	-	-	2	M
Chromis atripectoralis	-	-	1	-	-	1	-	2	M
Chromis retrofasciata	2	-	-	-	-	-	-	2	M
Pomacentrus bankanensis	-	-	-	-	-	1	1	2	M
Pomacentrus nigromarginatus	-	-	-	-	-	-	2	2	M
Abudefduf septemfasciatus	-	-	-	1	-	-	-	1	M
Abudefduf sexfasciatus	-	-	-	1	-	-	-	1	M
Chromis opercularis	-	-	-	-	-	1	-	1	M
Dascyllus reticulatus	-	-	-	-	1	-	-	1	M
Scaridae									
Calotomus spinidens	-	4	1	-	-	1	-	6	T
Chlorurus bleekeri	-	4	-	-	1	1	-	6	T
Scarus oviceps	-	-	1	-	1	-	-	2	T
Scarus globiceps	-	-	-	-	1	-	-	1	T
Scarus hypselopterus	-	-	1	-	-	-	-	1	T
Scorpaenidae									
Pterois volitans	-	-	-	-	-	1	-	1	M
Serranidae									
Cephalopholis argus	-	-	-	-	-	1	-	1	T
Siganidae									
Siganus canaliculatus	-	-	-	2	-	-	-	2	T
Tetraodontidae									
Arothron caeruleopunctatus	-	-	-	-	-	2	-	2	T
Arothron nigropunctatus	-	-	-	-	1	-	-	1	T
Canthigaster papua	-	-	-	-	-	1	-	1	T
Canthigaster valentini	-	-	1	-	-	-	-	1	T
Zanclidae									
Zanclus cornutus	-	31	2	2	2	15	2	54	M
Number of species	20	34	26	22	45	46	37		

fish species, three groups were obtained that had similarities based on the similarity index analysis. The three groups are group A consisting of one sub-district (S1), group B consisting of four sub-districts (S2, S5, S6, and S7), and group C consisting of two sub-districts (S3 and S4). The average similarity of groups B and C was 42.12% and 30.33%, respectively. Five fish species that contributed to group B were *C. cyanocheilus* (11.90%), *C. striatus* (9.35%), *C. binotatus* (8.02%), *Z. scopas* (7.05%), and *C. triangulum* (5.93%). Meanwhile, five fish species that contributed to group C were *C. binotatus* (19.18%), *C. cyanocheilus* (14.50%), *C. trifasciatus* (12.56%), *Z. cornutus* (10.25%), and *Z. scopas* (7.25%).

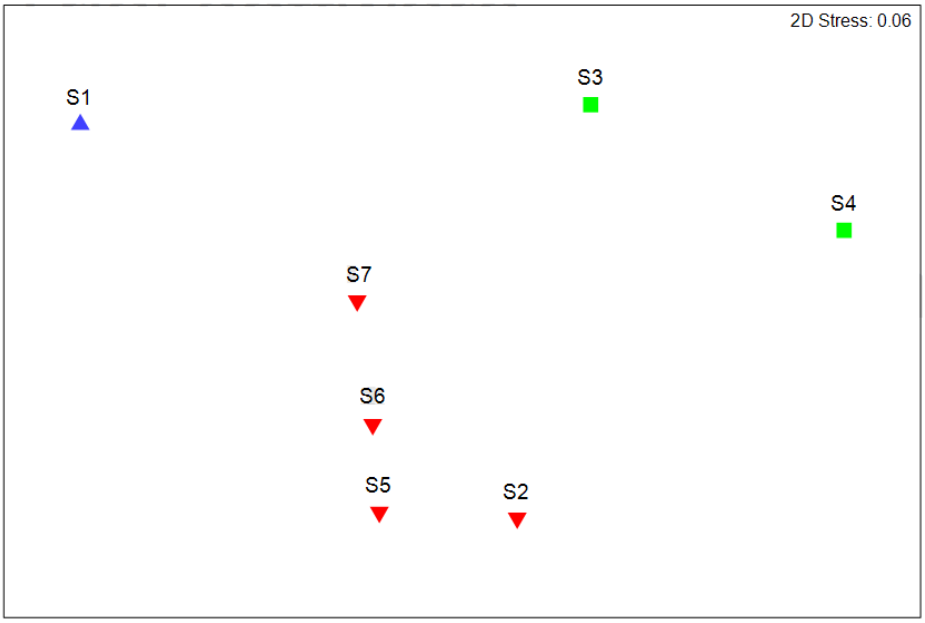

Figure 2. nMDS plot based on Bray-Curtis similarity showing three group associations of 123 species of reef fish at seven sites.

Discussion

The results of this study provide an overview of the diversity of reef fish species in the Yos Sudarso Bay, Jayapura City. The number of reef fish species and families found in this study is relatively the same as the reef fish diversity in Depapre Bay (Tanah Merah Bay) Jayapura Regency. The results of the study by Paulangan et al. (2019a), found 130 species of reef fish from 26 families in seven observation sites in Depapre Bay, Jayapura Regency. However, several species and families of reef fish found in the Depapre Bay were not found at this study site, such as reef fish species which belongs to the target fish group of the Caesionidae, Lutjanidae, and Lethrinidae families, as well as major fish groups from the Pseudochromidae family. Besides, the number of individual reef fish found was greater than the results of this study. Generally, apart from their relatively close location, the characteristics of the coral reef ecosystem in the two locations are also relatively similar, namely fringing coral reefs with live coral cover in the moderate to good category (32.00-60.00% in Yos Sudarso Bay and 46.33-68.00% in Depapre Bay) which is dominated by Coral Massive, Acropora Branching, Acropora Tabulate, and Coral Branching (Paulangan et al. 2019b; Hamuna et al. 2019).

In this study, the number of individuals from the target fish group of the Acanthuridae family, such as *C. binotatus*, *C. cyanocheilus*, *C. striatus*, and *Z. scopas* (only *C. binotatus* and *C. cyanocheilus* found at all study sites) were found to be higher compared to reef fish species from the Pomacentridae family (major fish group). The high abundance of target fish from the Acanthuridae family can indicate that all study sites are potential reef fishing grounds. This result was shown from interviews with several fishermen that medium to large size reef fish of the Acanthuridae family were their catch targets, in addition to target fish from the Labridae, Scaridae, Siganidae, and Holocentridae families. Generally, diversity, presence, and absence of target fish species

can be used as a guide in monitoring the condition of coral reef ecosystems and the status of coral reef capture fisheries (Gisawa & Lokani 2001), can also be used as a guide to the level of disturbance anthropogenic (Obura & Grimsdith 2009), especially reef fish species of the Serranidae, Lutjanidae, Lethrinidae, and Haemulidae families (Suharti et al. 2014).

The ecological index is an indicator that indicates the health status of the community, where higher ecological parameter values will indicate the community is stable, and vice versa (Magurran 1991). According to Galib et al. (2013), the diversity index provides more information to determine the condition of species in a community than mere information on the number and abundance of species. In this study, the results of diversity index analysis showed that the reef fish community in S5, S6, S7, and S2 sites were classified as high and more diverse, and the reef fish community was stated to be more stable than S1, S3, and S4 sites (reef fish diversity is classified as moderate). The higher diversity of fish species results in a more stable fish community (Albaret & Lae 2003). The evenness index in each study site is high, on the contrary, the dominance index shows a low value. The values of these two indicate that the abundance and distribution of reef fish species in each study site are almost the same (high level of evenness) and no specific reef fish species dominate (there is no concentration of individual fish in one particular species in each study site). However, the nMDS plot shows that the six sites inside Yos Sudarso Bay form a separate group from those outside Yos Sudarso Bay. This shows that the reef fish community structure (number of species and abundance) between the two types of sites is different.

Overall, the results of this study have provided preliminary information on the species diversity and ecological indices of reef fish in Yos Sudarso Bay, Jayapura City. We realize that there are still many limitations to this study so that some reef fish species that have been reported in previous studies were not recorded in this study, such as several types of reef fish from the Chaetodontidae family found by Hamuna et al. (2019) and several reef fish species found in seagrass ecosystems reported by Tebaiy et al. (2014). Therefore, to improve the database of reef fish species diversity in Jayapura City, further studies are needed by considering the addition of the number of sites and observation transects, placement of transects at different water depths, and different seasons. This is very important because it will have an impact on the diversity and abundance of reef fish found. To preserve the coral reef ecosystem as a habitat for reef fish, it is necessary to have regulations from the local government. This is due to the practice of destructive fishing on coral reefs using explosives (although with a low incidence) carried out by local communities which is still common today. This regulation is expected to suppress the destruction of coral reef ecosystems which have a direct impact on increasing the diversity and abundance of reef fish.

CONCLUSION

In this study, preliminary information was obtained about the diversity, abun-

dance, and ecological index of reef fish in Yos Sudarso Bay, Jayapura City. The diversity of reef fish species and families is quite high. The results also showed that the reef fish diversity index was moderate to high with an even distribution of reef fish species in each study site. Further research is needed to determine the exact diversity of reef fish by considering the addition of the number of locations and observation transects, placing transects at water depths and in various profiles of coral reef habitats, as well as conducting observations in different seasons. This is very important because it will have an impact on the diversity and abundance of reef fish found.

AUTHORS CONTRIBUTION

Research design: BH, LD; field data collection: BH, LD; data analysis: BH, LD, AA; financial gain: BH, LD; original manuscript writing: BH, AA; manuscript revision: BH, AA.

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CONFLICT OF INTEREST

The authors declare no competing interests regarding the research or the research funding.

REFERENCES

Albaret, J.J. & Laë, R., 2003. Impact of fishing on fish assemblages in tropical lagoons, the example of the Ebrie Lagoon, West Africa. *Aquatic Living Resources,* 16(1), pp.1–9. doi*:* 10.1016/S0990-7440(03)00002-0

Allen, G., Swainston, R. & Ruse, J., 2000. *Marine fishes of South East Asia: A field guide for anglers and divers*. Singapore: Periplus Edition Ltd.

Allen, G.R. & Erdmann, M.V., 2009. Reef fishes of the bird's head peninsula, West Papua, Indonesia. *Check List,* 5(3), pp.587–628. doi: 10.15560/5.3.587

Allen, G.R. & Erdmann, M.V., 2012. *Reef fishes of the East Indies, volume I-III.* Perth: Tropical Reef Research.

Bell, J.D. & Galzin, R., 1984. Influence of live coral cover on coral-reef fish communities. *Marine Ecology Progress Series,* 15, pp.265–274. doi: 10.3354/meps015265

Clarke, K.R., 1993. Nonparametric Multivariate Analyses of Changes in Community Structure. *Australian Journal of Ecology*, 18, pp.117–143. doi: 10.1111/j.1442-9993.1993.tb00438.x

Cole, A.J., Pratchett, M.S. & Jones, G.P., 2008. Diversity and functional importance of coral-feeding fishes on tropical coral reefs. *Fish and Fisheries*, 9(3), pp.286–307. doi: 10.1111/j.1467-2979.2008.00290.x

English, S., Wilkinson, C. & Baker, V., 1997. *Survey manual for tropical marine resources,* 2nd edition. Townsville: Australia Institute of Marine Science.

Field, J.G., Clarke, K.R. & Warwick, R.M., 1982. A practical strategy for analysing multispecies distribution patterns. *Marine Ecology Progress Series,* 8, pp.37–52. doi: 10.3354/meps008037

Froese, R. & Pauly, D., 2020. *FishBase*, World Wide Web electronic publication. www.fishbase.org, version (12/2020).

Galib et al., 2013. Fish diversity of the River Choto Jamuna, Bangladesh: Present status and conservation need. *International Journal of Biodiversity and Conservation,* 5(6), pp.389–395. doi: 10.5897/IJBC2013.0552

Garpe, K.C. & Öhman, M.C., 2003. Coral and fish distribution patterns in Mafia Island Marine Park, Tanzania: fish-habitat interactions. *Hydrobiologia*, 498, pp.191–211. doi: 10.1023/A:1026217201408

Gisawa, L. & Lokani, P., 2001. Trial community fishing and management of live reef food fisheries in Papua New Guinea. *SPC Live Reef Fish Information Bulletin*, 8, pp.3–5.

Hamuna, B., Kalor, J.D. & Rachmadani, A.I., 2019. Assessing the condition of coral reefs and the indicator fish (family: Chaetodontidae) in coastal waters of Jayapura City, Papua Province, Indonesia. *European Journal of Ecology,* 5(2), pp.126–132. doi: 10.2478/eje-2019-0020

Hamuna et al., 2020. Distribution and density of demersal fishes in Youtefa Bay, Papua, Indonesia: A study using hydroacoustic technology. *Indian Journal of Fisheries,* 67(1), pp.30–35. doi: 10.21077/ijf.2019.67.1.88578-05

Komyakova, V., Jones, G.P. & Munday, P.L., 2018. Strong effects of coral species on the diversity and structure of reef fish communities: A multi -scale analysis. *PLOS ONE*, 13(8), e0202206. doi: 10.1371/journal.pone.0202206

Kruskal, J.B., 1964. Multidimensional scaling by optimizing goodness of fit to a nonmetric hypothesis. *Psychometrika,* 29, pp.1–27. doi: 10.1007/BF02289565

Madduppa et al., 2012. Fish biodiversity in coral reefs and lagoon at the Maratua Island, East Kalimantan. *Biodiversitas*, 13(3), pp.145–150. doi: 10.13057/biodiv/d130308

Magurran, A.E., 1991. *Ecological diversity and its measurement, 2nd edition*. London: Chapman and Hall.

Marshell, A. & Mumby, P.J., 2015. The role of surgeonfish (Acanthuridae) in maintaining algal turf biomass on coral reef. *Journal of Experimental Marine Biology and Ecology,* 473, pp.152–160. doi: 10.1016/j.jembe.2015.09.002

Obura, D.O. & Grimsdith, G., 2009. *Resilience assessment of coral reefs: assessment protocol for coral reefs, focusing on coral bleaching and thermal stress.* Gland: International Union for Conservation of Nature.

Paulangan et al., 2019a. Keanekaragaman dan kemiripan bentuk profil terumbu berdasarkan ikan karang dan lifeform karang di Teluk Depapre Jayapura, Provinsi Papua, Indonesia. *Jurnal Ilmu dan Teknologi Kelautan Tropis,* 11(2), pp.249–262. doi: 10.29244/jitkt.v11i2.24140

Paulangan et al., 2019b. Distribution and condition of coral reef ecosystem in Tanah Merah Bay, Jayapura, Papua, Indonesia. *AACL Bioflux*, 12(2), pp.502–512.

Pujiyati et al., 2021. Distributions of environmental parameters and fish at Humbold Bay, Jayapura. *IOP Conf. Series: Earth and Environmental Science*, 944, 012003. doi:10.1088/1755-1315/944/1/012003

Roberts, C.M. & Ormond, R.F.G., 1987. Habitat complexity and coral reef diversity and abundance on Red Sea fringing reefs. *Marine Ecology Progress Series*, 41, pp.1–8. doi:10.3354/meps041001.

Rumahorbo, B.T., Keiluhu, H.J. & Hamuna, B., 2019. The economic valuation of mangrove ecosystem in Youtefa bay, Jayapura, Indonesia. *Ecological Questions,* 30(1), pp.47–54. doi: 10.12775/EQ.2019.003

Rumahorbo, B.T., Hamuna, B. & Keiluhu, H.J., 2020. An assessment of the coastal ecosystem services of Jayapura City, Papua Province, Indonesia. *Environmental & Socio-economic Studies*, 8(2), pp.45–53. doi: 10.2478/environ-2020-0011

Suharti et al., 2014. Monitoring ikan karang. In: S. Suharsono, O.K. Sumadhiharga (eds), Panduan Monitoring Kesehatan Terumbu Karang: Terumbu Karang, Ikan Karang, Megabenthos dan Penulisan Laporan, pp. 32–59. Jakarta: COREMAP-CTI LIPI.

Tebaiy et al., 2014. Struktur komunitas ikan pada habitat lamun di Teluk Youtefa Jayapura Papua. *Jurnal Iktiologi Indonesia,* 14(1), pp.49–65. doi: 10.32491/jii.v14i1.95

Veron, J.E.N., 2002. Reef corals of the Raja Ampat Islands, Papua Province, Indonesia, in S.A. McKenna, G.R. Allen, S. Suryadi (eds), A Marine Rapid Assessment of the Raja Ampat Islands, Papua Province, Indonesia, pp. 26–36. Washington DC: Conservation International.

Wilson et al., 2006. Multiple disturbances and the global degradation of coral reefs: are reef fishes at risk or resilient? *Global Change Biology*, 12(11), pp.2220–2234. doi: 10.1111/j.1365-2486.2006.01252.x

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Research Article

Management Strategies of Mangrove Biodiversity and the Role of Sustainable Ecotourism in Achieving Development Goals

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ABSTRACT

Mangrove forest is a unique and vulnerable ecosystem. This ecosystem serves both ecological and economic purposes. The Siak government has begun to develop the Sungai Apit District mangrove area, which has potential. The goal of this research was to develop a sustainable mangrove ecotourism strategy through five research goals: (1) identification of mangrove species diversity; (2) identification of ecotourism supply; (3) identification of ecotourism demand, (4) development strategy of mangrove ecotourism, and (5) development of the potential for mangrove ecotourism to increase the SDGs value. This research was conducted from January to April 2020. The supply and demand of natural tourism objects and attractions were assessed using ADO-ODTWA criteria analysis. The IFAS/EFAS and SWOT analysis was used to develop a mangrove ecotourism development strategy based on the valuation of ADO-ODTWA aspects. The contribution of mangrove ecotourism to UNESCO's SDG indicators for sustainable development. According to research, there are 35 species of mangroves on the Sungai Apit coast. The outcomes demonstrated that the feasibility level of tourism attractions (204 points) and supporting elements (472 points) met high-level criteria. It indicated that the area had a high potential for development as a mangrove ecotourism area. Based on IFAS/EFAS, SWOT analysis and the grand strategy selection matrix, the position of mangrove ecotourism strategy was in Quadrant I (Strength-Opportunity). The strategy that could be developed included (1) developing special interest mangrove ecotourism product; (2) increasing facilities; (3) improving the quality of human resources; (4) developing a network on the website and (5) increasing coordination with the Government. By implementing ecotourism strategy, the SDGs can be achieved, including: no poverty (goal 1); decent work and economic growth (goal 8); climate action (goal 13); life below water (goal 14); life on land (goal 15); and partnerships to achieve goals (goal 17).

INTRODUCTION

Mangrove areas serve a variety of ecological and socioeconomic functions in addition to being physically functional. One of mangroves' ecological functions is to maintain and stabilize shorelines and riverbanks, as well as to pro-

tect them from crashing waves and currents. The biological function of mangroves includes various types of fish, birds, monitor lizards and other types of primates, while the economic function of mangroves is one of the natural tourism areas, the results of which can be developed in the form of tourism industrial products as foreign exchange earners (Abdullah et al. 2014; Goh 2015; Hakim et al. 2017).

The permanent development of mangroves areas into ecotourism areas is a very rational alternative use in coastal areas because it can provide economic benefits and environmental services without exploitation of mangroves. The use of environmental services, such as ecotourism, will encourage the preservation of the mangrove ecosystem as a buffer zone for conservation areas (Kathiresan 2012; Elliott 2012; Cheia 2013; Malik et al. 2015; Kenny 2017; Susilo et al. 2017; Tracey et al. 2017).

The mangrove area on the coast of Siak Regency's Sungai Apit District has the potential to be developed as an ecotourism area and become one of the natural tourism destinations for the people of Siak Regency and its surroundings. On the other hand, the mangrove area on the coast of Sungai Apit District has the potential to be developed into an object of ecotourism attraction, but on the other hand, this mangrove area does not yet have an ecotourism development strategy. This is due to the fact that potential supply and demand for ecotourism in the coastal mangrove area of Sungai Apit District have not been identified as a basis for management planning and development strategies for ecotourism in mangrove areas.

The mangrove area on the coast of Sungai Apit District is one of the areas for regional development in the context of spatial use for regional tourism activities, according to Siak Regency Government policies. Regional development aspects, tourism product development, supporting transportation development, tourist market development, marketing and promotion development, human and institutional development, investment development and other supporting infrastructure development are all part of Siak regency tourism (Bappeda Siak 2019).

Ecotourism in the coastal mangrove area of Sungai Apit District can be an alternative development to mangrove ecotourism. Based on the potential of the mangrove area on the coast of Sungai Apit District, it is necessary to identify internal and external factors in the development of ecotourism, namely the strengths, weaknesses, opportunities and threats to formulate a coastal mangrove ecotourism development strategy. Sungai Apit District coast in a sustainable manner in this regard, the purpose of this research was to develop a strategy for developing ecotourism in the coastal mangrove areas of Sungai Apit District, as well as to explore the potential for mangrove ecotourism to increase the value of SDGs (Bappeda Siak 2019).

MATERIALS AND METHODS

Materials

The study was conducted in the coastal area of Sungai Apit Subdistrict,

which covers three villages (Kampung), Kampung Mengkapan, Kampung Sungai Rawa and Kampung Rawa Mekar Jaya (Figure 1) The study lasted four months, from January to April 2020. The equipment used is a 1: 50,000 scale work map, camera, binoculars, Global Position System (GPS) as well as the Dharmawan et al. (2020) and Melana et al. (2000). The Mangrove Lovers Group in Kampung Mengkapan, Kampung Sungai Rawa, and Kampung Rawa Mekar Jaya, as well as government leaders, community informal leaders, and the community in the three villages, participated in the research.

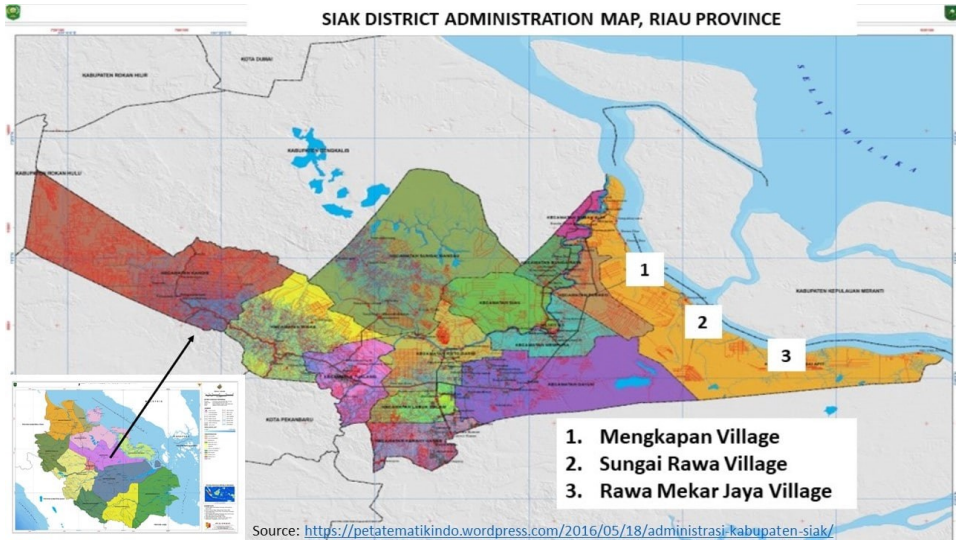


Figure 1. Map of Study area.

Methods

The survey method was used for the research, which included field observations and interviews. The potential for an area to be developed into an ecotourism attraction necessitates an evaluation of several elements required as a potential supply. The analysis of potential ecotourism offers employs a scoring and weighting system based on the Analysis of Operational Areas for Natural Tourism Objects and Attractions (ADO-ODTWA) guidelines (PHKA 2003) with the ecotourism planning criteria modified. These elements include object attractiveness, infrastructure, facilities and services, market potential, security, community socioeconomic conditions, institutional elements, environmental quality and accommodation. The potential supply of ecotourism in the development of mangrove ecotourism on the coast of Sungai Apit District, Siak Regency is classified as high, medium, or low. Meanwhile, the indicators along with the SDGs targets developed by the United Nations, are used as a tool in this study to determine the SDGs values that will be achieved if this ecotourism development strategy is implemented.

The descriptive analysis of ecotourism demand is based on tabulated data that has been processed, compiled and presented into important information based on visitor characteristics, visiting patterns, motivation, perceptions, preferences and expectations. In addition, an ADO-ODTWA analysis was conducted to support the IFAS/EFAS and SWOT analysis of all internal and external factors in developing ecotourism in the coastal mangrove area

of Sungai Apit District, Siak Regency.

IFAS/EFAS and SWOT analysis were used to develop strategies in the coastal mangrove area of Sungai Apit District, Siak Regency. IFAS/EFAS and SWOT analyses are used to identify various factors in order to develop a mangrove ecotourism development strategy. The stages of IFAS/EFAS and SWOT analysis are as follows:

a. identification and weighting of internal and external factors
b. analysis of internal and external factors

Weights and ratings can be assigned to each of the predetermined parameters based on the internal and external matrices that have been created to obtain a weighted value. This value will then be used to provide guidance on the prospects for ecotourism development in mangrove areas along the coastline of Sungai Apit District, Siak Regency.

These elements are then linked together in the form of a matrix to generate a number of strategic options. This matrix will produce four potential strategies for developing mangrove ecotourism along the coast of Siak Regency's Sungai Apit District. The creation of a grand strategies matrix to determine the strategy for developing mangrove ecotourism on the coast of Sungai Apit District, Siak Regency, followed the formulation of alternative strategies for developing mangrove ecotourism.

The outcomes of the IFAS/EFAS and SWOT analysis are then incorporated into the development plan formula's synthesis. The quantitative synthesis findings will guide the development of ecotourism in the coastal mangrove area of Sungai Apit District, Siak Regency based on the potential and conditions of the ecotourism object.

RESULTS AND DISCUSSION

Mangrove Diversity on the Coastline of Sungai Apit District, Siak Regency

According to the findings of surveys and interviews, 35 species of Mangrove plants can be found in various coastal areas of the Sungai Apit. The various types of mangroves are depicted in Table 1.

Ecotourism Attraction

Based on the information in Table 2, the mangrove area on the coast of Sungai Apit Subdistrict, Siak Regency, has a high classification of attractiveness which includes natural beauty, uniqueness of mangroves, cleanliness and comfort of the area, and variations in High-value tourism activities.

The mangrove area on the coast of Sungai Apit District, Siak Regency has the potential for limited (exclusive) ecotourism development. Limited ecotourism development in mangrove forest areas is a method of using mangrove areas as ecotourism objects without disturbing or degrading the quality of mangrove forests.

The analysis of the elements of attraction and support for ecotourism activities and support for ecotourism activities is used to determine the po-

Table 1. Mangrove diversity on the coastline of Sungai Apit.

No	Species	Local Name	Location
1	*Acanthus ilicifolius* Linn	Jeruju/Bakau Kurap	2
2	*Acrostichum aureum* Linn	Paku Laut/ Piai Raya	1,2,3
3	*Acrostichum speciosum* Wild	Piai Lasa	1,2
4	*Aegiceras corniculatum* Linn	Gedangan	2
5	*Avicennia alba* Blume	Api-api	1,2,3
6	*Avicennia marina* Vierch	Api-api Putih	2,3
7	*Bruguiera cylindrical* Blume	Tumu Putih	2
8	*Bruguiera gymnorhiza* Linn	Tumu Mera	2,3
9	*Bruguiera parviflora* Rosch	Lenggadai	3
10	*Bruguiera sexangular* Lour	Temusing/ Lindur	2,3
11	*Calophyllum inophyllum* Linn	Mentagur	2
12	*Cerbera mangas* Linn	Bintaro	1,2
13	*Ceriops decandra* Graff	Tengau	2
14	*Ceriops tagal* Pers	Tengar	2,3
15	*Derris trifolia* Lour	Ambung	2
16	*Excoecaria agallocha* Linn	Buta-buta	1,2,3
17	*Heritiera littoralis* Aiton	Dungun	2
18	*Hibiscus tiliaceus* Linn	Waru Laut	1
19	*Heritiera globose* Aiton	Dungun	3
20	*Knadelia candel* Steud	Berus-berus	3
21	*Lumnitzera littorea* Wild	Sesop	1,2
22	*Melastoma candidum* Blume	Senduduk	2
23	*Nypa fruticans* Wumb	Nipah	2,3
24	*Osbornia octodonta* F.v. Muell	Baru-baru	2
25	*Pandanus odoratissima* Forssk	Pandan Hutan	1,2
26	*Rhizophora apiculata* Blume	Bakau	1,2,3
27	*Rhizophora mucronate* Lam	Belukap/ Bangka Hitam/ Merah	1,2,3
28	*Rhizophora stylosa* Griff	Bakau Merah	1,2,3
29	*Scyphiphora hydrophyllacea* C.F. Gaegtn	Cingam	2,3
30	*Sonneratia alba* Sm	Pedada	1,2,3
31	*Sonnertia caseolaris* Lam	Perepat	1,2,3
32	*Sonneratia ovate* L.F	Kedabu	1,2
33	*Terminalia catappa* Linn	Ketapang	2
34	*Xylocarpus granatum* K.D. Koening	Nyirih	2
35	*Xylocarpus moluccensis* Lam	Nyireh	2

Description:

1: Mengkapan Mangrove area

2: Sungai Rawa Mangrove area

3: Rawa Mekar Jaya Mangrove area

tential supply of ecotourism in the coastal area of Sungai Apit District, Siak Regency. Furthermore, Figure 2 also demonstrates that the value of the element of ecotourism offering, which includes the value of the element of ecotourism attraction, is 220, while the value of the supporting elements is 470, indicating that the area has the potential to be developed as a mangrove ecotourism area according to the ODTWA analysis classification. Visitors to the coastal mangrove area of Sungai Apit District, Siak Regency can be seen based on their characteristics, visit patterns, motivation, preferences, perceptions and expectations to the development of ecotourism in the coastal mangrove area of Sungai Apit District, Siak Regency.

IFAS/EFAS and SWOT analysis were used to develop a limited ecotourism development strategy in the coastal mangrove area of Sungai Apit

Table 2. Assessment of elements attractiveness of ecotourism at coastline of Sungai Apit District, Siak Regency.

No	Attractiveness element	Value	Category
1	Tourist attraction	220	high
2	Market potential	230	high
3	Access to locations	350	moderate
4	Conditions around the area	125	moderate
5	Safety of the site	425	moderate
6	Management and services	320	moderate
7	Climate	200	moderate
8	Accommodation	330	moderate
9	Supporting facilities and infrastructure	350	moderate
10	Availability of clean water	310	moderate
11	The relationship between tourist objects around	395	moderate
12	Carrying capacity of the area	435	high
13	Visitor settings	330	moderate
14	Marketing	360	moderate
15	Market share	380	moderate

District, Siak Regency. The management of the area, namely the Mangrove Lovers Group, Kampung Mengkapan, Kampung Sungai Rawa, and Kampung Rawa Mekar Jaya, serves as the unit of analysis. The management of the area manager and the condition of the mangrove forest area located on the coast of Sungai Apit District, Siak Regency, which includes positive aspects (strengths) and negative aspects (weaknesses) are seen as internal factors while factors outside the management Mangrove areas that are threats (negative) and opportunities (positive) are considered external factors.

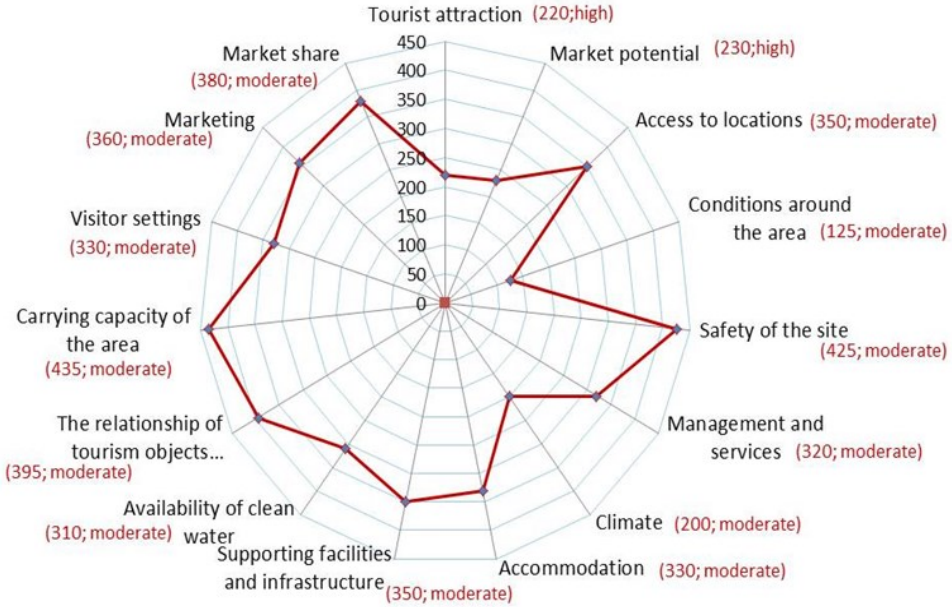


Figure 2. Assessment of elements attractiveness of ecotourism at coastline of Sungai Apit District, Siak Regency.

Identification of Internal and External Factors

Internal and external strategic factors for ecotourism development in Sungai Apit District, Siak Regency are determined using the ADO-ODTWA development criteria method (PHKA 2003) which is modified based on local ecotourism planning criteria.

Analysis of Internal and External Factors

Internal and external factor analysis includes IFAS-EFAS matrix and internal -external matrix analysis. The internal-external matrix positioning strategy is based on the number of weighted values of internal and external factors.

IFAS and EFAS Matrix

The IFAS Matrix was created based on the results of internal factors identification (Table 3), as shown in Table 4. The EFAS Matrix was also created based on the results of external factor identification (Table 4), as shown in Table 5.

The strengths and weaknesses of the Mangrove Lovers Group in Kampung Mengkapan, Kampung Rawa Mekar Jaya, Kampung Sungai Rawa as

Table 3. Matrix identification and weighting of internal factors.

No	Internal factor	Value of ADO-ODTWA	Weighting
I. Strengths			
1	Cleanliness of mangrove areas; Free of industrial and household waste, noise, sting odor and dust	25	0.07
2	Characteristic and uniqueness of mangrove vegetation and wild life	55	0.15
3	The natural beauty and physical shape of the beach area	46	0.12
4	Existing planning and management of tourist zone	20	0.05
5	Safety of the region	24	0.06
6	The existence of tourist facilities (Restaurant, worship facilities, toilets, clinic parking lot; fishing rig, interpretation boards, shelters, canoe /boat)	50	0.13
7	Availability of infrastructure (Roads, clean water and telecommunications networks and the Internet)	145	0.36
	Total I	361	
II. Weaknesses			
1	Human resource to manage mangrove tourism is not available, no tour guide /no interpreter	5	0.01
2	Interpretation facilities is minimal	12	0.03
3	No detail concept of mangrove ecotourism	5	0.01
	Total II	22	
	Total number (I+II)	383	1.00

Remarks : Weighted value based on the results of ADO-ODTWA

Table 4. Matrix identification and weighting of external factors.

No	External factor	Value of ADO-ODTWA	Weighting
I. Opportunities			
1	Support for ecotourism development policy	23	0.21
2	The support of the local government of Siak Regency	23	0.21
3	Position close to the downtown area of the district	23	0.21
4	The site is in priority of tourist destination development in Siak Regency	20	0.17
	Total I	89	
II. Threats			
1	Degradation of environmental quality	7	0.05
2	Site security impaired	7	0.05
3	Changes in site status	7	0.05
	Total II	21	
	Total number (I+II)	110	1.00

Remarks : Weighted value based on the results of ADO-ODTWA

Table 5. Ecotourism development of mangrove IFAS matrix coastline of Sungai Apit District, Siak Regency.

Code	Internal factor	Weighting	Rating	Weighted point
Strength factor				
S1	Cleanliness of mangrove areas; Free of industrial and household waste, noise, sting odor and dust	0,07	3	0,21
S2	Characteristic and uniqueness of mangrove vegetation and wild life	0,14	4	0,60
S3	The natural beauty and physical shape of the beach area	0,12	4	0,48
S4	Existing planning and management of tourist zone	0,05	4	0,20
S5	Safety of the region	0,06	3	0,18
S6	The existence tourist facilities) (Restaurant, worship facilities, toilets, clinic parking lot; fishing rig, interpret ation boards, shelters, canoe /boat	0,13	3	0,39
S7	Availability of infrastructure) (Roads, clean water and telecommunications networks and the Internet	0,36	3	1,08
	Total A			**3,14**
Weaknesses factor				
W1	Human resource to manage mangrove tourism is not available) (no tour guide /no interpreter	0,01	4	0,04
W2	Interpretation facilities is minimal	0,03	3	0,09
W3	No detail concept of mangrove ecotourism	0,01	4	0,04
	Total B			**0,17**
	Total number (A + B)	1,00		3,31

Table 6. Ecotourism Development of Mangrove EFAS matrix coastline of Sungai Apit District, Siak Regency.

Code	Internal factor	Weighting	Rating	Weighted point
Opportunities				
O1	Support for ecotourism development policy	0,21	4	0,84
O2	The support of the local government of Siak Regency	0,21	3	0,63
O3	Position close to the downtown area of the district	0,21	3	0,63
O4	The site is in priority I of tourist destination development in Siak Regency	0,17	4	0,68
	Total A			2,61
Threats				
T1	Degradation of environmental quality	0,05	4	0,20
T2	Site security impaired	0,05	4	0,20
T3	Changes in site status	0,05	4	0,20
	Total B			0,60
	Total number (A + B)	1.00		3,21

area managers are internal factors in the development of ecotourism in the coastal mangrove area of Sungai Apit Subdistrict, Siak Regency. Internal factors in the strength aspect have a weighted value of 3.14 whereas external factors in the weakness aspect have a weighted value of 0.17.

Table 6 displays that the external factors are external factors that contribute to the development of ecotourism in the coastal mangrove area of Sungai Apit District, Siak Regency. External factors that are opportunities have a weighted value of 2.64 whereas threats that may arise from ecotourism activities have a weighted value of 0.60.

Internal External Matrix

The ecotourism development strategy in the coastal mangrove area of Sungai

Apit District, Siak Regency is positioned in cell 1, namely the growth strategy with the expansion or diversification of ecotourism activities. This cell is intended to achieve growth in terms of product, marketing and assets. This condition can be achieved by creating new ecotourism products, improving human resources quality of services, and facilities.

SWOT analysis

By examining the combination of internal and external strategic factors, a SWOT analysis was used to determine the ecotourism development strategy in the coastal mangrove area of Sungai Apit District, Siak Regency (Table 7).

Table 7. Mangrove ecotourism development strategy formulation at coastline of Sungai Apit District, Siak Regency.

Internal	**Strengths/S**	**Weaknesses/W**
External	1. Cleanliness of mangrove areas; Free of industrial and household waste, noise, sting odor and dust 2. Characteristics and uniqueness of mangrove vegetation and wildlife 3. The natural beauty and physical shape of the beach area 4. Existing planning and management of tourist zone 5. Safety of the region 6. The existence of tourist facilities; Restaurant, worship facilities, toilets, clinic parking lot; fishing rig, interpretation boards, shelters, canoe /boat 7. Availability of infrastructure; Roads, clean water and telecommunications networks and the Internet	1. Human resource to manage mangrove tourism is not available; no tour guide /no interpreter 2. Interpretation facilities is minimal 3. No detail concept of mangrove ecotourism
Opportunities/O	**Strategy SO**	**Strategy WO**
1. Support for ecotourism development policy 2. The support of the local government of Siak Regency 3. Position close to the downtown area of the district 4. The site is in priority of tourist destination development in Siak Regency	1. Developing ecotourism products of special interest mangrove 2. Adding facilities and mangrove ecotourism activities 3. Improve the quality of competent human resources in the mangrove ecotourism activities 4. Make networking through special interest mangrove ecotourism website 5. Coordination with the Government of Siak regarding preservation and cleanliness of mangrove areas	1. Develop technical design and mangrove eco-tourism package as part of the management of mangrove areas in Siak Regency 2. Building mangrove areas as ODTWA in local government development program 3. Training as a travel interpreters 4. Improve the means of supporting eco-tourism in the region and outside the region structuring region)
Threats/T	**Strategy ST**	**Strategy WT**
1. Degradation of environmental quality 2. Site security impaired 3. Changes in site status	1. Conducting socialization of ecotourism mangrove 2. Raise awareness of the importance of environmental education 3. Prioritize the tourist zone with border village 4. Monitoring and evaluation of the impact of ecotourism activities 5. Improving the ecological benefits of mangrove areas and environmental services	1. Improve forest security with relevant agencies 2. Enhance training/education as a force travel interpreters 3. Increase supervision and services to the visitors 4. Adding interpretation boards

Position of Ecotourism Development Strategy in the coastal area of Sungai Apit District, Siak Regency

Based on the space matrix analysis and the grand strategy selection matrix, the position of the ecotourism development strategy in the coastal mangrove area of Sungai Apit District, Siak regency was determined. The space matrix is used to fine-tune the strategy analysis based on ecotourism's internal and external factors.

The ordinate position of the grand strategy for development of ecotourism in the coastal mangrove area of Sungai Apit District, Siak Regency is at [3,36; 1,92], which is in quadrant I, according to the space matrix analysis. The vector line in Figure 3 is positive for both internal and external factors. This means that the strategy can be developed to support an aggressive growth policy (growth-oriented strategy) by leveraging existing strengths to capitalize on existing opportunities. Quadrant I suggests an aggressive strategy, which includes leveraging internal strength to capitalize on external opportunities, overcoming internal weaknesses, and avoiding external threats (Majiol et al. 2016; Masud et al. 2017).

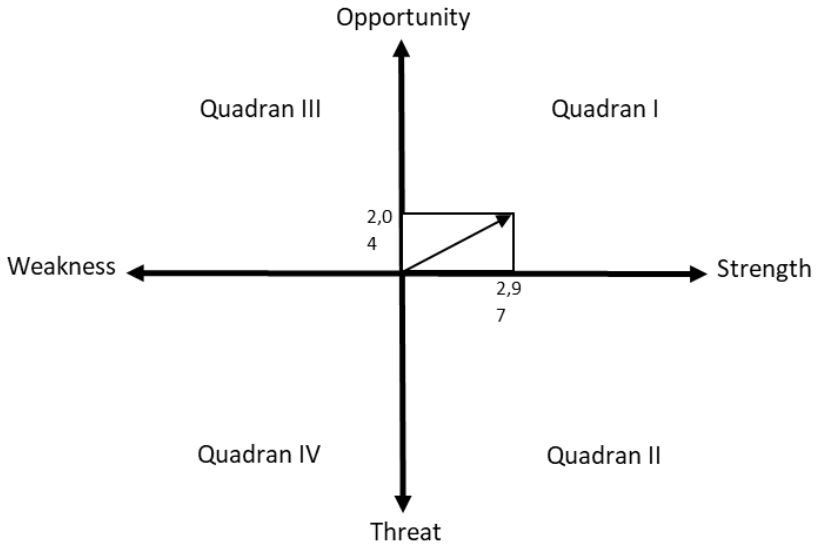


Figure 3. Grand strategy selection matriks of Mangrove ecotourism at coastline of Sungai Apit District, Siak Regency.

The quadrant strategy I have a better strategic position, and it is recommended that I pursue an alternative strategy that includes product diversification. A SWOT analysis was used to study the potential of the mangrove ecosystem on the coast of Sungai Apit District, Siak Regency. The highest strength of this ecosystem is the factor of environmental attractiveness around the mangroves, and its weakness is that no effort has been made to procure ecotourism products (Liu et al. 2014; Mojiol et al. 2016; Kenny 2017; Masud et al. 2017). Ecosystem potential in mangrove areas is expected to be developed through the implementation of strategies, specifically optimizing potential and minimizing existing obstacles to achieve the goals of mangrove ecosystem conservation. Strategic recommendations for the development of

ecotourism in the coastal mangrove areas of Sungai Apit District, Siak Regency are as follows:

Developing Mangrove Special Interest Ecotourism Products

The development of ecotourism products in the Sungai Apit District, Siak Regency coastal mangrove area is based on the cleanliness of the mangrove area, the uniqueness of the vegetation and animals of the mangrove ecosystem, facilities, infrastructure, area security, area status as an effort to support sustainable mangrove preservation in Siak Regency. Ecotourism products offered in the mangrove area must be safe and comfortable, in accordance with the potential of attractive, beautiful, and natural resources, facilities and road conditions to tourist objects that are easy to reach, and can fulfill and provide the desired satisfaction and experiences that are difficult to measure by visitors (Hussin et al. 2014; Hakim et al. 2017).

The development of ecotourism products in the coastal mangrove areas of Sungai Apit District, Siak Regency, is aimed at special interest ecotourism that includes elements of mangrove conservation efforts as well as tourism destinations. Bird and other animal watching, enjoying the beauty of mangrove vegetation via a wooden platform (boardwalk), fishing, boating among the mangrove vegetation (canoeing), and photography with the beauty and uniqueness of the vegetation and mangrove animals as an interesting object are some of the special interest ecotourism programs that can be developed in the coastal mangrove area of Sungai Apit District, Siak Regency. Mangrove education tours and tracking, bird watching, fishing, mangrove tree plantation or adoption, canoeing and boating are some of the ecotourism programs with a special interest in mangroves that have been developed in various locations based on the potential of mangrove areas as tourist attractions as well as efforts to rehabilitate and conserve mangrove areas. The mangrove education tour and tracking program is the most popular among visitors. Bird watching programs are only popular with a select group of people because they necessitate a specific amount of time and equipment (Jaafar 2012; Kathiresan 2012; Liu et al. 2014; Jaafar et al. 2015; Mojiol et al. 2016; Kenny 2017; Masud et al. 2017).

Development of distinctive tourism products in accordance with the potential of tourist objects and activities

The attraction is its natural beauty and local wisdom, as well as customs and local community culture developed in the coastal areas of Sungai Apit District, Siak Regency, but there is no special packaging in the form of programs. A fairly traditional community culture can be added attraction. The potential that can be developed are as follows:

a) Beauty of Nature

The view of the mangrove forest is one of the natural attractions or beauty that we can enjoy when visiting these tourist objects in Sungai Apit District, particularly those in Kampung Sungai Rawa and Kampung Rawa Mekar Jaya,

which are located at the estuary and along the banks of the Sungai Rawa. Green water, river water, and cool sea breezes lead directly to the Danau Zamrud National Park which consists of 2 (two) adjacent lakes. The two lakes are side by side with each other, namely the *Tasik Besar* and the *Tasik Bawah*.

Table 8 depicts the various types of Flora and Fauna in Zamrud Lake National park, which contains 38 species of aves or birds, 12 of which are protected species, including the White stork (*Ciconia ciconia*), Enggang dua warna, Enggang palung, Enggang benguk, dan Enggang ekor hitam. Spesies aves lain yaitu Finches (*Pycnonotus aurigaster*), Celepuk (*Otus spp.*), Bubut (*Cuculus spp.*), Murai batu (*Copsychus malabarius*), Layang-layang (*Delichon dasypus*), Rangkong gading (*Buceros virgil*), Rangkong papan (*Buceros bocrnis*), Punai (*Treron spp.*), Srigunting (*Dicrurus macrocercus*), Serindit (*Loriculus galgulus*), and Tekukur (*Geopelia striata*). Meanwhile, the types of fish that inhabit the lakes and rivers in this area are known to consist of 14 species with 8 species that have high economic value. Several types of fish that can be found in this area are Arwana (*Scleropages forosus*) which is an iconic ornamental fish, catfish (*Pangasius hypothalmus*), Gabus (*Channa striata*), and Limbat (*Clarias batrachus*).

Table 8. Various types of Flora and fauna in Zamrud Lake National Park.

No.	Family	Species	local name
	Flora		
1	Anacardiaceae	*Gluta wallichii* (Hook.f.) Ding Hou	Rengas burung
2		*Gluta aptera* (King) Ding Hou	Rengas paya
3		*Gluta renghas* L.	Rengas
4	Anisophylleaceae	*Combretocarpus rotundatus* (Miq.) Danser	Perepat
5	Apocynaceae	*Alstonia spatulata* Blume	Pulai
6	Aquifoliaceae	*Ilex cymosa* Blume	Kelat
7		*Ilex wallichii* Hook.f.	Mengkulat
8	Burceraceae	*Santiria laevigata* Blume	Balam
9	Calophyllaceae	*Calophyllum pisiferum* Planchon & Triana	Bintangur
10		*Calophyllum sclerophyllum* Vesque	Bintangur
11		*Calophyllum canum* Hook.f. ex T.Anderson	Bintangur
12	Chrysobalanaceae	*Parastemon urophyllus* (Wall. ex A.DC.) A.DC	Kelat
13	Clusiaceae	*Garcinia rostrata* Hassk. ex Hook.f.	Manggis hutan
14	Dipterocarpaceae	*Anisoptera marginata* Korth.	Mersawa
15		*Dipterocarpus coriaceus* Slooten	Keruing
16		*Shorea balangeran* Burck	Belangiran
17		*Shorea hemsleyana* King ex Foxw.	Meranti rawang
18		*Shorea macrantha* Brandis	Meranti kunyit
19		*Shorea platycarpa* F.Heim	Meranti kait
20		*Shorea teysmanniana* Dyer ex Brandis	Meranti lilin
21		*Shorea uliginosa* Foxw.	Meranti paya
22	Ebenaceae	*Diospyros maingayi* (Hiern) Bakh.	Asam kelat
23	Elaeocarpaceae	*Elaeocarpus griffithii* (Wight) A.Gray	Merawa

Table 8. Contd.

No.	Family	Species	local name
	Flora		
24	Fabaceae	*Dialium indum* L.	Keranji
25		Koompassia malaccensis Benth.	Mengeris
26	Hypericaceae	*Cratoxylum arborescens* (Vahl) Blume	Geronggang
27	Lauraceae	*Litsea grandis* (Nees) Hook.f.	Medang
28		*Litsea machilifolia* Gamble	Medang
29	Lecythidaceae	*Barringtonia reticulata* (Blume) Miq	Putat
30	Sapotaceae	*Madhuca motleyana* (de Vriese) J.F.Macbr	Nyatoh
31		*Palaquium burckii* H.J.Lam	Nyatoh
32		*Palaquium ridleyi* King & Gamble	Nyatoh
33	Thymelaeaceae	*Gonystylus bancanus* (Miq.) Kurz	Ramin
	Fauna		
1		*Panthera tigris sumatreansis*	Harimau sumatera
2		*Neofelis nebulosa*	Harimau dahan
3		*Helarctos malayanu*	Beruang madu
4		*Tragulus napu*	Napu
5		*Tragulus javanicus*	Kancil
6		*Macaca fascicularis*	Monyet ekor panjang
7		*Macaca nemestrina*	Beruk
8		*Presbytis melalophos*	Kokah
9		*Presbytis thomasi*	Ungko
10		*Tapirus indicus*	Tapir
12		*Muntinacus muntjak*	Kijang
13		*Felis spp*	Kucing hutan
14		*Hylobates syndactylus*	Siamang
15		*Manis javanica*	Trenggiling

b) Mangrove Forest

According to the findings of surveys and interviews, mangroves are abundant in Sungai Apit District, Siak Regency, and can be found on nearly every riverbank. Mangroves are owned in the following varieties: *pedada, bebetak, serokan, cingarn, piyoe (paku laut), selada, parepat, kedabu senoh, segamit, bulukap, pandan, jeruju, nipah*. There are several fruits from the mangrove tree that can be consumed such as nipah fruit (kolang-kaling), buah lindur fruit (*Bruguiera gymnorrhiza*), api-api fruit (*Avicennia alba*), pedada fruit (*Sonneratia alba*).

c) Mangrove Planting

Efforts to protect and preserve the mangrove forest area have included nurseries or the planting of mangrove seedlings, all planting and facility development activities are carried out independently in groups with the members of the community in the surrounding area. Other activities besides planting mangrove seeds include learning mangrove crab cultivation, *kelulut* bee cultivation, and fishing for fish and giant prawns.

d) Indigenous *Anak Rawa*

The Anak Rawa Indigenous People are inland communities who live in the Kampung Sungai Rawa area and a portion of Kampung Rawa Mekar Jaya. The state of a very traditional society that lives as a coastal community,

reliant on the waters of rivers, estuaries, lakes and sea. The Anak Rawa Tribe has generally accepted various religions, including Christianity, Hinduism-Buddhism, Konghuchu and Islam. Despite the fact that several religions have spread among the people, belief in ancestral spirits and persists to this day.

The Indigenous *Anak Rawa* tribe is still living in a time when tribal customs are prevalent. The *Anak Rawa* Tribe's origin, which first inhabited the Sungai Lancur Darah (Kampung Sungai Rawa) today, is due to a large number of *Anak Rawa* Tribe newcomers moving to Kampung Penyengat, the majority of whom now inhabit Mata Rimba and Sungai Mungkal. Because of its trustworthiness and honesty, the *Anak Rawa* Tribe once held a special place in the Siak Kingdom.

e) Traditional Dance and Music

This Gong dance performance has existed since the ancestors of the indigenous swamp tribe; however, the Gong dance, particularly in Sungai Apit sub-district, is not well known by the larger community and has received little attention needed in order to preserve this culture. So, in this case, it is possible that this dance will disappear because those who can dance it are elderly, with a few young people learn it, and there is a general lack of interest in the Gong dance.

Tradition is the nation's cultural heritage that, from time to time, requires attention in the direction of cultural development and is passed down from generation to generation. The gong dance resurfaced and was introduced by the Siak Regency Government in 2010 through a traditional festival art event of the Rawa Tribe. The Gong has a mystical meaning based on ancient story that if the Gong is struck, his voice will touch the hearts of the indigenous people. Indigenous people who are currently employed can resign. This is still going on in the area where the swamp children live today. Gong has its own set of meanings. Following the story of the Gendong dance, the Orang Rawa Indigenous people invented the Gong dance. The Gendong dance is based on legend held by the Anak Rawa Tribe.

Improvement of Mangrove Ecotourism Facilities

The development of ecotourism in Sungai Apit District, Siak Regency, cannot be separated from the provision of facilities for ecotourism activities with special interest in mangroves. Improved facilities for mangrove ecotourism activities must be based on conservation, spatial, safety, and comfort aspects, and must be tailored to the ecotourism activities offered in order to achieve a high level of visitor satisfaction (Ramli et al. 2018; Aye et al. 2019; Musa et al. 2020). The mangrove area is said to be optimal as an ecotourism object if the location and type of activity can be determined, the order and harmony of facilities and infrastructure are adjusted to the condition of the object, and visitors comfort and safety are guaranteed (Yeo et al. 2013; Susilo et al. 2017; Tracey et al. 2017; Situmorang 2018; Sofian et al. 2019).

Additional facilities are required to support ecotourism activities in the Sungai Apit District, Siak Regency, including the construction of wooden

platforms (board walks), viewing towers (for bird and animal watching activities), shelters, information huts, interpretation boards, nursery areas and wooden boats. Facilities must reflect the nature of the mangrove ecosystem while remain comfortable, unique and adapted to the ecotourism activities of special interest in mangroves that are being developed (Liu et al. 2014; Aye et al. 2019).

The layout of the facilities still takes into account the needs and aesthetics of the area. Visitors are not only interested in the quality of natural attractions, but also in the quality of facilities, beginning with the moment they depart from the visitor's origin to the destination of their destination, and throughout their tour, visitors get satisfaction and convenience.

Improving the Quality of Competent Human Resources in Mangrove Ecotourism Activities

Ecotourism products must be offered in conjunction with a certain level of competence from the manager and the availability of skilled personnel. Area managers must own competency standards in the development of ecotourism activities, which include knowledge, skills and attitudes in carrying out an activity (Ramli et al. 2018; Situmorang 2018; Aye et al. 2019). Training and education for officers involved in ecotourism activities in the coastal mangrove areas of Sungai Apit District, Siak Regency can be organized in collaboration with government agencies, universities or competent institutions by providing ecotourism management training and education. Training and education in support of ecotourism development activities, with a focus on mangroves, including interpretation planning techniques, ecotourism activity implementation, visitor management, ecotourism guides, and evaluation of the prevention and control of ecotourism impacts.

Ecotourism education and training not only demonstrate that the products offered have no negative impact on the environment, but also provide added value to visitor satisfaction, managers' ability to compete in the global ecotourism market, and all forms of ecotourism activities in accordance with utilization norms. In order to conserve the area, sustainable forest environmental services must be provided (Kathiresan 2012; Liu et al. 2014; Kenny 2017; Aye et al. 2019).

Mangrove Special Interest Ecotourism Website

In accordance with the development of exclusive ecotourism, the development of coastal mangrove ecotourism, Sungai Apit District, Regency by creating a network through ecotourism websites has piqued the interest of visitors, particularly middle to upper class visitors. This website offers interesting information about the natural beauty, comfort of the area, the uniqueness of mangrove plants and endemic animals, as well as ecotourism activities with a focus on mangroves. The success of tourism development in Bangladesh's Sundarbans mangrove area is supported via a more widely accessible website, particularly for visitors who are interested in visiting with exclusive infor-

mation about facilities (Liu et al. 2014; Kenny 2017; Tracey et al. 2017).

Coordination with the Siak Regency Government regarding Mangrove Areas

Cooperation and coordination between the area management and the Siak Regency government, as well as other related parties, is required in the development of ecotourism in the coastal mangrove area of Sungai Apit District, Siak Regency, in order to realize the development of mangrove ecotourism and the conservation of sustainable mangrove areas. Based on current environmental, political, and economic policies, this is the primary foundation for supporting the development of conducive and sustainable mangrove ecotourism (Liu et al. 2014; Kenny 2017; Tracey et al. 2017; Basyuni et al. 2018).

Coordination between the local government and management to support the preservation of mangrove areas and maintain the area's quality and cleanliness in developing ecotourism in coastal mangrove areas, Sungai Apit District, Siak Regency is as follows:

1) Equal perceptions of mangrove ecosystems environmental protection in order to maintain the attractiveness of ecotourism objects, including regulations on the use of environmental resources
2) Integration of land use related to the development plan of ecotourism objects in the mangrove area of Siak Regency
3) Improved coordination and outreach to stakeholders in tourism institutions strengthening by facilitating and expanding the network of tourism groups and organizations
4) Increase research and development of biodiversity in the coastal mangrove area of Sungai Apit District, Siak Regency in supporting efforts to conserve endemic vegetation and animals in the mangrove area
5) Assessing and monitoring the impact of mangrove ecotourism activities.

An ecotourism area is said to be good and successful if it can achieve three goals: 1) environmental preservation 2) ensuring visitor satisfaction and 3) increasing the integration of community development around the area and its development zone (Arriagada et al. 2012; Arkwright 2018; Adegboyega et al. 2019).

Based on several strategies proposed in the development of Mangrove ecotourism Sungai Apit, Siak Regency, it is expected to establish a system for utilizing environmental services that at the very least contributes to the regional economy, particularly local communities. Sustainable ecotourism, as discussed in Fennell's book (2015), is a nature-based tourism activity that contributes to the improvement and capacity building of local communities, provides knowledge and experience, and can be morally and socially aggravated. This ecotourism development could be one of the activities that contribute to the achievement of sustainable development goals.

Results should be clear and concise. The discussion should explore the significance of the results of the work. Avoid extensive citations and discussion of published literature. If appropriate, Results can be written in a sepa-

rate section from Discussion. This is especially if the Discussion is extensive and includes all the Results of the study.

SDGs Value

Mangrove ecotourism developed in Sungai Apit, Siak Regency, can help to achieve several Sustainable Development Goals (SDGs), as shown in Table 9.

According to the study's findings, the development of sustainable ecotourism can aid in the achievement of several priority SDGs, namely:

Goal 1 (No Poverty)

Essentially, all living things on this planet have values that can be used for humans, in addition to the resources' own survival. Ecotourism, according to Boo (1992), has the potential to benefit the economy. They can develop various types of businesses particularly for local communities, such as developing mangroves products as souvenirs, providing transportation services, lodging, and so on. Based on the results of the study, mangroves have been tentatively estimated at an average of US$ 4185 per hectare per year (Brander et al. 2012). Nobi et al. (2021) through a study in Bangladesh found that the estimated annual economic contribution of tourism in the Sundarban mangroves to Bangladesh economy is USD 53 million. Ecotourism provides several economic benefits, including 1) funding for protected areas; and 2) job creation for people living near the area. Furthermore, Boo (1990) and Lindberg (1991) state that ecotourism is viewed as a means of generating income and employment in areas that have not been impacted by development. So this activity is able to reduce the number of poor people.

Goal 8 (Decent Work and Economy Growth)

One of SDG 8's targets is to create jobs and promote local culture through sustainable tourism by 2030. Based on the potential discovered in the Sungai Apit Mangrove area, there are the *Anak Rawa* Native tribes, whose distinct culture and customs have been preserved to this day. Tourists, particularly foreign tourists, will be drawn to this. According to Wirakusuma (2014) and Muktaf and Zulfiana (2018), the uniqueness of traditional culture (such as traditional rituals, the uniqueness of the local community, culinary or traditional food) is what attracts foreign tourists to Indonesia., in addition to natural beauty. According to Sedarmayanti (2005), ecotourism activities that attract a large number of tourists have helped the country's foreign exchange while also creating job opportunities in the surrounding community. Communities not only gain jobs and income, but also create new jobs to support tourism activities. Furthermore, Purnamasari et al. (2015) and Fistiningrum and Harini (2021), argue that mangrove ecotourism can be an alternative to optimize the potential by constantly emphasizing the ecosystem sustainably and creating an economically valuable area.

Goal 13 (Climate Action)

Mangrove ecosystems have enormous carbon storage potential, allowing them to play a role in climate change mitigation. The term Blue Carbon refers to the

Table 9 Achievable Goals of Sustainable Development.

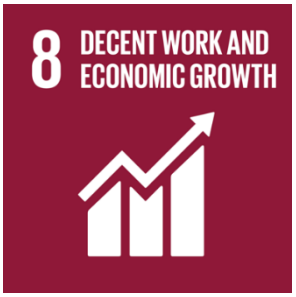
Goals		Target
	1.4	Ensure that all men and women, in particular the poor and the vulnerable, have equal rights to economic resources, as well as access to basic services, ownership and control over land and other forms of property, inheritance, natural resources, appropriate new technology and financial services, including microfinance
	1.5	Build the resilience of the poor and those in vulnerable situations and reduce their exposure and vulnerability to climate-related extreme events and other economic, social and environmental shocks and disasters
	1.a	Ensure significant mobilization of resources from a variety of sources, including through enhanced development cooperation, in order to provide adequate and predictable means for developing countries, in particular least developed countries, to implement programmes and policies to end poverty in all its dimensions
	1.b	Create sound policy frameworks at the national, regional and international levels, based on pro-poor and gender-sensitive development strategies, to support accelerated investment in poverty eradication actions
	8.2	Achieve higher levels of economic productivity through diversification, technological upgrading and innovation, including through a focus on high-value added and labour-intensive sectors
	8.3	Promote development-oriented policies that support productive activities, decent job creation, entrepreneurship, creativity and innovation, and encourage the formalization and growth of micro-, small- and medium-sized enterprises, including through access to financial services
	8.6	Substantially reduce the proportion of youth not in employment, education or training
	8.9	By 2030, devise and implement policies to promote sustainable tourism that creates jobs and promotes local culture and products
	8.10	Strengthen the capacity of domestic financial institutions to encourage and expand access to banking, insurance and financial services for all
	13.3	Improve education, awareness-raising and human and institutional capacity on climate change mitigation, adaptation, impact reduction and early warning
	14.7	Increase the economic benefits to small island developing States and least developed countries from the sustainable use of marine resources, including through sustainable management of fisheries, aquaculture and tourism
	15.1	Ensure the conservation, restoration and sustainable use of terrestrial and inland freshwater ecosystems and their services, in particular forests, wetlands, mountains and drylands, in line with obligations under international agreements
	15.2	Promote the implementation of sustainable management of all types of forests, halt deforestation, restore degraded forests and substantially increase afforestation and reforestation globally
	15.b	Mobilize significant resources from all sources and at all levels to finance sustainable forest management and provide adequate incentives to developing countries to advance such management, including for conservation and reforestation

Table 9 Contd.

Goals		Target
	17.14	Enhance policy coherence for sustainable development
	17.16	Enhance the Global Partnership for Sustainable Development, complemented by multistakeholder partnerships that mobilize and share knowledge, expertise, technology and financial resources, to support the achievement of the Sustainable Development Goals in all countries, in particular developing countries
	17.17	Encourage and promote effective public, public-private and civil society partnerships, building on the experience and resourcing strategies of partnerships

use of environmental services provided by the mangrove ecosystem as a form of mitigation in the face of climate change. Blue Carbon was introduced as a metaphor to highlight coastal ecosystems, which have a greater impact on organic carbon (C) than terrestrial forests (green carbon) (Manafe et al. 2016). About 22% of mangrove forests in Indonesia are preserved in conservation areas and provide 0.82–1.09 PgC hectare^{-1} of carbon storage (Sidik et al. 2018). As a result, mangrove conservation efforts are required to ensure the long-term viability of the area's mangrove ecosystem. Conservation efforts can be carried out by improving the quality of coastal waters and creating alternative sources of income, such as ecotourism and non-wood mangrove products (Sidik et al. 2018).

Goal 14 (Life Below Water)

Goal 14 requires the ability to use and conserve oceans and marine resources in a sustainable manner in order to achieve sustainable development. Mangroves play an important ecologically and physically role. Physically, mangroves have the role of protecting the coast from the intrusion of seawater into freshwater sources and tidal waves, preventing abrasion also. Meanwhile, mangroves play an ecological role as a source of organic matter in food chain. Furthermore, mangroves play an ecological and economic role in shrimp and fish spawning and rearing, so the presence of mangroves boosts the productivity of coastal and offshore fisheries. This is where the mangrove forest area is developed and managed to ensure the sustainability of the existing resources. According to Tuwo (2011), coastal and marine ecotourism almost never exploits natural resources, but instead relying on natural and community services to meet physical and psychological needs, as well as tourist knowledge. Furthermore, conservation is one of the goals of ecotourism, specifically through the sustainable use of mangrove ecosystem services to help provide effective funding or economic incentives to conserve, increase biodiversity, and protect the natural heritage that exists on this planet (Fennel 2015).

Goal 15 (Life on Land)

One of the goals of ecotourism development is to reduce pressure on forests as resources (Flamin & Asnaryati 2013), so the possibility of disturbance from visitors must be considered. To minimize environmental damage, the number of visitors must be limited in accordance with the carrying capacity of the ar-

ea. It should be noted, however, that this will create a conflict between conservation and economic interest. As a result, an economic strategy, such as the use of a price mechanism as a solution to economic and conservation problems, is required. When the number of visitors does not exceed the carrying capacity, the price can be set to the standard rate. However, if the number of visitors exceeds the carrying capacity, the policy adopted is to maintain a normal number of visitors while increasing the price proportionally.

Goal 17 (Partnership for the Goals)

According to Yoeti (2008), one of the positive effects of ecotourism, from a macroeconomic standpoint, is that it can encourage increased investment from the tourism industry sector and other economic sectors. Ecotourism will attract foreign tourists, resulting in financial resource partnerships for developing countries like Indonesia. In this case, however, it is necessary to anticipate the occurrence of leaks in tourism development. Import leakage typically occurs when there is a demand for international standard equipment used in the tourism industry, as well as imported food and beverage ingredients that cannot be supplied by local or domestic communities. The amount of revenue generated by the tourism industry is accompanied by the high costs that must be incurred in order to import products that meet international standards. Meanwhile, export leakage is common in the development of tourist destinations, especially in poor or developing countries which tend to require large capital and investment to build infrastructure and other tourist facilities. Conditions like these will entice foreign investors with deep pockets to build resorts or hotels as well as tourism facilities and infrastructure. In exchange, their business and investment profits will flow freely back into their home country (Istiqomah et al 2021; Tampubolon & Wulandari 2021). To prepare for this, the government will need to regulate investment policies and partnership patterns that support sustainable ecotourism.

CONCLUSION

According to the research findings, the Sungai Apit area has a relatively high diversity of mangrove species and has the potential to be developed as an ecotourism area. This area can be developed as an ecotourism destination by creating traditional mangrove products, improving facilities and human resources, and establishing networks with both the government and the private sector. As a result, it can help to achieve a number of sustainable development goals (SDGs).

AUTHORS CONTRIBUTION

P.W.T. designed the research, supervised all the process and wrote the manuscript, E. analyzed the data and wrote the manuscript, I.C. collected the data and wrote the manuscript, N.J.H.N., and R.S.W. collected the data.

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CONFLICT OF INTEREST

We have no conflicts of interest to disclose.

REFERENCES

Abdullah, M. et al., 2013. Local community participatory process and intervention procedure in mangrove ecotourism of Marudu Bay, Sabah. *Pertanika Journal Tropical Agriculture Science*, 36, pp.173-180.

Adegboyega et al., 2019. Evaluation of unsustainable land use/land cover change on ecosystem services in coastal area of Lagos State, *Nigeria. Applied Geomatics*, 11(1), pp.97-110.

Arkwright, D. & Kaomaneng, I.S., 2018. Mangrove ecotourism development on Kakaralamo Island North Halmahera: Community perception, participation and development strategies. *IOP Conference Series: Earth and Environmental Science*, 175(1), pp.22-32. doi: 10.1088/1755-1315/1/012232.

Arriagada, R.A. et al., 2012. Dopayments for environmental services affect forest cover? a farm-level evaluation from Costa Rica. *Land Econ*., 88(2), pp.382–399.

Aye, N.W. et al., 2019. Contribution of mangrove forest to the livelihood of local communities in Ayeyarwaddy region, Myanmar. *Forest*, 10(5), 414. doi: 10.3390/f10050414.

Bappeda Siak, 2019. *Peta Jalan Siak Menuju Kabupaten Hijau,* Sedagho Siak & Pemerintah Kabupaten Siak.

Basyuni, M. et al., 2018. Developing community-based mangrove management through eco-tourism in North Sumatra, Indonesia. *IOP Conference Series: Earth and Environmental Science*, 126, pp.12-19. doi: 10.1088/1755-1315/126/1/012109.

Boo, E., 1990. *Ecotourism: The Potentials and Pitfalls*, Washington, DC: World Wildlife Fund.

Boo, E., 1992. *The Ecotourism Boom: Planning for Development and Management*, Washington, DC: Wildlands and Human Needs, World Wildlife Fund.

Brander, M.L. et al., 2012. Ecosystem service values for mangroves in Southeast Asia: A meta-analysis and value transfer application. *Ecosistem Services*, 1(1), pp.62-69.

Cheia, G., 2013. Ecotourism: Definition and Concepts. *Journal of Tourism*,15, pp.46-59.

Dharmawan, I.W.K. et al., 2020. *Panduan Monitoring Struktur Komunitas Mangrove di Indonesia*, PT Media Sains Nasional.

Elliott, J., 2012. An Introduction to sustainable development. 4thed. Abingdon, Oxon: Routledge

Flamin, A. & Asnaryati, 2013. Ecotourism potential and strategy development of Tahura Nipa-Nipa, Kendari City, Southeast Sulawesi. *Jurnal Penelitian Kehutanan Wallacea*, 2(2), pp. 154-168.

Fennell, D.A., 2015. *Ecotourism-fourt edition,* New York.

Fistiningrum, W. & Harini, R., 2021. The impacts of mangrove ecotourism management on the socio-economic conditions of people in kulonprogo regency. *IOP Conf. Series: Earth and Environmental Science*, 683, 012116. doi:10.1088/1755-1315/683/1/012116

Goh, H.C., 2015. Nature and community-based tourism (CBT) for poverty alleviation: A case study of Lower Kinabatangan, East Malaysia. *Geografia-Malaysian Journal of Society and Space*, 11(3), pp.42-52.

Hakim, L., Siswanto, D. & Makagoshi, N., 2017. Mangrove conservation in East Java: The ecotourism development perspective. *Journal of Tropical Life Science*, 7(3), pp.277-285.

Hussin, R., Mohd. Yassir, S. & Kunjuraman, V., 2014. Potential of homestay tourism based on seaweed cultivation from the views of seaweed cultivators in district of Semporna Sabah, East Malaysia. *SHS Web of Conferences*, 12(01005), pp.1-11.

Istiqomah et al., 2021. Does ecotourism contribute to sustainable development? A case at in Banyuwangi. *E3S Web of Conference 232*, 04009. doi: 10.1051/e3sconf/202123204009

Jaafar, M. & Maideen, S.A., 2012. Ecotourism-related products and activities, and the economic sustainability of small and medium island chalets. *Tourism Management*, 33(3), pp.683-691.

Jaafar, M. et al., 2015. Development of ecotourism products in Kilim Geopark based on tourist perceptions. *Journal of Sustainability Science and Management*, 10(1), pp.1-18.

Kathiresan, K., 2012. Importance of Mangrove Ecosystem. *International Journal of Marine Science*, 2(10), pp.70-89.

Kenny, L., 2017. The Road Less Travelled: A Case Study on Ecotourism and Sustainable Developmentinthe Sundarbans, Bangladesh. Thesis Department of Sociology. Lund University. http://lup.lub.lu.se/luur/downloadfunc=downloadFile&recordOId=8912611&fileOId=8912628

Lindberg, K., 1991. *Policies for Maximising Nature Tourism's Ecological and Economic Benefits*, Washington, DC : World Resources Institute.

Liu, J. et al., 2014. The role of social capital in encouraging residents' pro-environmental behaviours in community-based ecotourism. *Tourism Management*, 41, pp.190-201.

Malik, A., Fensholt, R. & Mertz, O., 2015. Mangrove exploitation effects on biodiversity and ecosystem services. Biodiversity and Conservation, 24 (14), pp.3543-3557.

Manafe G., Kaho, M.R. & Risamasu, F. 2016. Estimasi Biomassa Permukaan dan Stok Karbon pada Tegakan Pohon Avicennia marina dan Rhizophora mucronata di Perairan Pesisir Oebelo Kabupaten Kupang. *Jurnal Bumi Lestari*, 16(2), pp.163-17

Masud, M.M. et al., 2017. Community-based ecotourism management for sustainable development of marine protected areas in Malaysia. *Ocean & Coastal Management*, 136, pp.104-112.

Melana, D.M. et al., 2000. *Mangrove Management Handbook*, Department of Environment and Natural Resources, Manila, Philippines through the Coastal Resource Management Project, Cebu City, Philippines

Mojiol, A.R. et al., 2016. Contribution of mangrove forest and socio-economic development of local communities in Kudat district, Sabah Malaysia. *International Journal of Agriculture, Forestry and Plantation*, 2, 122-129.

Muktaf, Z.M. & Zulfiana, E.R., 2018. Persepsi Wisatawan Asing terhadap Wisata Indonesia. *Cakrawala Jurnal Penelitian Sosial*, 7(1), pp.83-106.

Musa, F., Fozi, N.M. & Hamdan, D.D.M., 2020. Coastal communities' willingness to pay for mangrove ecotourism in Marudu Bay, Sabah, Malaysia. *Journal of Sustainability Science and Management*, (15)4, pp.130-140.

Nobi, M.N. et al., 2021. Economic valuation of tourism of the Sundarban Mangroves, Bangladesh. *Journal of Ecology and Natural Environment,* 13(4), pp.100-109.

Perlindungan Hutan danKonservasiAlam (PHKA), 2003. Pedoman Analisis Daerah Operasi Obyek dan Daya Tarik Wisata Alam (ADO-ODTWA). Direktorat Jenderal Perlindungan Hutan dan Konservasi Alam. Bogor.

Purnamasari, R, Suprapto, D. & Purwanti, F., 2015. Pengembangan Ekowisata Mangrove Desa Karangsong, Kabupaten Indramayu. *Diponogoro Journal of Maquares,* 4(4), pp.146-154.

Ramli, F., 2018. Factors affecting user's perception towards conservation of biodiversity in Matang Mangrove Forest Reserve (MMFR), Perak, Malaysia. *International Journal of Business and Society*, 19(SI), pp.26-36.

Sedarmayanti, 2005. *Membangun Kebudayaan dan Pariwisata (Bunga Rampai Tulisan Pariwisata)*, Bandung (ID): Penerbit Mandar Maju.

Sidik et al., 2018. Mangrove conservation for climate change mitigation in Indonesia. *Wiley Interdisciplinary Reviews: Climate Change*, 9(5).

Situmorang, R.O., 2018. Social capital in managing mangrove ecotourism by the Muara Bambai community. *Indonesian Journal of Forestry Research*, 5(1), pp.21-34.

Sofian, A. et al., 2019. Ecosystem services-based mangrove management strategies in Indonesia: a review. *Aquaculture, Aquarium, Conservation & Legislation*, 12(1), pp.151-166.

Susilo, H., Takahashi, Y. & Yabe, M., 2017. The opportunity cost of labour for valuing mangrove restoration in Mahakam Delta, Indonesia. *Sustainability*, 9(12), 2169. doi, 10.3390/su9122169.

Tampubolon, N. & Wulandari, C., 2021. The importance of community-based ecotourism sustainability and its supports to the SDGs: goals number 8 and 17. Journal of Tourism & Sports Management (JTSM), 4(1), pp.928 -933.

Tracey, L.C. et al., 2017. Achieving the national development agenda and the Sustainable Development Goals (SDGs) through investment in ecological infrastructure: A case study of South Africa. *Ecosystem Services,* 27, pp.253–260.

Tuwo, A., 2011. *Pengelolan Ekowisata Pesisir dan Laut*. Surabaya: Brilan Internasional.

Wirakusuma, R.M., 2014. Analisis Karakter Wisatawan Mancanegara terhadap Fasilitas Wisata Kawasan Prawirotaman. *Jurnal Pariwisata,* 1(1), pp.42-52.

Yeo, S.C., Noor, A.G.A. & Lee, P.C., 2013. The estimation of economic benefits of urban trees using contingent valuation method in Tasik Perdana, Kuala Lumpur. *Pertanika Journal of Tropical Agricultural Science*, 36(1), pp.99-114.

Yoeti, O.A., 2008. *Ekonomi Pariwisata: Introduksi, Informasi, dan Implementasi*, Jakarta (ID): Kompas.

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Research Article

Effect of Carbon Source Variations on Growth, Physiological Stress, and Saponin Levels of *Talinum paniculatum* Gaertn. Adventitious Roots

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ABSTRACT

Monosaccharide and disaccharide as carbon sources can affect the production of secondary metabolites. The study aims to determine the effect of variations in carbon sources on growth, physiological stress, and saponin levels of the adventitious roots of *Talinum paniculatum* Gaerthn. Adventitious roots are subculture in liquid MS medium treated with various sugars: 3% sucrose, 3% glucose, 3% fructose, 3% lactose, 3% maltose, 3% dextrose, sucrose + fructose (1.5% + 1.5%), sucrose + glucose (1.5% + 1.5%), glucose + fructose (1.5% + 1.5%), sucrose + dextrose (1.5% + 1.5%) for 6 weeks. The results of this study show that the 3% fructose treatment produces the highest fresh and dry biomass, which are 1.30 g and 0.23 g compared to the control. The morphology of adventitious roots in the treatment of carbon source variation is not different from the control treatment. The highest MDA (malondialdehyde) levels are found in the sucrose + fructose treatment (1.5% + 1.5%). Meanwhile, the highest proline levels are fo und in the 3% maltose treatment. Saponin levels analyzed using thin layer chromatography show the data in the form of color intensity and stain area based on ImageJ software analysis. The 3% fructose treatment shows the highest color intensity and stain area compared to the control. Variations in carbon sources affect physiological stress, biomass, and saponin levels of adventitious roots of *T. paniculatum*, but do not effect on root morphology.

INTRODUCTION

Talinum paniculatum Gaertn. (java ginseng) is a family of Portulacaceae. *T. paniculatum* is widely used as a substitute for Korean ginseng which is still imported because it is relatively cheap, easy to obtain, and easy to cultivate (Widiyani 2006). The roots of *T. paniculatum* were known to have androgenic potential (Winarni 2009) and sperm viability potential (Rahmi & Widyasari 2011). Saponins can be used as anti-inflammatory drugs, have androgenic effects, as well as can induce cells through cell receptors (Zuo et al. 2009) and increase the body's resistance to disease (Hu et al. 2003). In its natural habitat, java ginseng root grows slowly. Getting the root in the form of bulbs

takes approximately two to three years, so efforts are needed to obtain bioactive compounds of java ginseng root through in vitro culture techniques. Enhancement of saponin accumulation in adventitious roots culture of java ginseng have been done in various treatment, for instance by elicitation (Faizal et al. 2019), sucrose and potassium nitrate treatment (Manuhara et al. 2015), but the production of saponin compounds that influenced by the carbon source (sugar) not done yet. However, plant cells and culture media do not have autotrophic capabilities. Thus, it requires an external carbon source for metabolism.

Sugar has been known as a compound that can be utilized by plants as a carbon source that can affect metabolism, development, growth, and gene expression. It is either monosaccharide or disaccharide sugar that can act as the carbon source (Wang & Weathers 2007). The culture of plant cells, tissues, or organs usually requires the introduction of a carbon source into the culture medium (George 1993). Among the many available carbon sources, sucrose is the main one (Fuentes et al. 2005). However, it can lead to hypoxia and cell ethanol accumulation due to its rapid metabolism (Scott 1995). In some cases, sucrose is completely or partially replaced by another carbon source (George 1993). Many studies report the use of other carbon sources such as maltose (Percival & Fraser 2005); glucose (Sami et al. 2016); fructose (Uozumi et al. 1991); glucose + fructose (1:1), sucrose + fructose (1:1), sucrose + glucose (1:1) (Praveen & Murthy 2012).

Based on the ideas that have been described, this study is conducted to provide a variety of carbon sources, including sucrose, lactose, maltose, glucose, fructose, dextrose, sucrose + fructose, sucrose + glucose, sucrose + dextrose, and glucose + fructose to determine the effect on morphology, physiological stress, biomass production, and levels of saponin compounds in adventitious roots of *T. paniculatum*. The changes in malondialdehyde (MDA) and proline levels as markers for cellular oxidative stress were also evaluated. The results of this study can be used as a basis for the development of java ginseng adventitious root culture to increase biomass and saponin compounds on a larger scale.

MATERIALS AND METHODS

Material Preparation

The *T. paniculatum* plant used in this study is obtained from the Bratang flower market, Surabaya, and has been identified by Indonesian Institute of Sciences, Purwodadi Botanical Garden, Pasuruan, East Java, Indonesia. The materials used in this study include materials for making media Murashige and Skoog, *Indole Butyric Acid* (IBA) 2 mg/L, sucrose (Gulaku), glucose (Glucolin), lactose (Grdane Lactose 200), maltose (Merck kGaA), fructose (Krystar 300), dextrose (R&W), agar compaction, 0.5 mL *anisaldehyde*, 10 mL glacial acetic acid, 5 mL concentrated sulfuric acid, 2-propanol, distilled water, 4.5 mL H_2SO_4 1%, aqueous solution *Trichloro Acetic Acid* (TCA) 0.5 mL, *Trichloro Acetic Acid* (TCA) + *Thiobarbizuric Acid* (TBA) 0.5 mL,

Sulfosalycilyc Acid (SAS) 4.5 mL, Ninhydrin 1 mL, Toluene 1.5 mL, Folin-Ciocalteu reagent, and Na_2CO_3.

Adventitious roots were induced in MS solid medium supplemented with IBA 2 mg/L, sucrose 3% and agar 7 g/L. After four weeks cultured adventitious roots were sub cultured in MS liquid medium supplemented with IBA 2 mg/L and various carbon source; there are 3% sucrose (control) (Murashige & Skoog 1962), 3% glucose, 3% fructose, 3% lactose, 3% maltose, 3% dextrose, sucrose + fructose (1.5% + 1.5%), sucrose + glucose (1.5% + 1.5%), glucose + fructose (1.5% + 1.5%), sucrose + dextrose (1.5% + 1.5%); and were cultured for 6 weeks. Treatments were replicated three times respectively based on Federer formula.

Morphological Observations

Adventitious roots that are six weeks old in liquid culture that have been mentioned above were harvested. Their morphology was observed using a stereomicroscope at 30x magnification. The length of the root branch and its diameter was measured using an inverted microscope. The results of morphological observations are presented in images. Meanwhile, the measurements are presented in the form of an average, which is then analysed quantitatively.

Malondialdehyde (MDA) and Proline Tests

The MDA test is conducted based on the method by Peixoto et al. (1999) with a few modifications. In the MDA test, a fresh sample of 0.5 g is required, which is mixed with 4.5 mL of 1% sulphuric acid solution. Afterward, it is centrifuged, and the supernatant is taken as much as 0.5 mL. The supernatant is mixed with 0.5 mL TCA solution and centrifuged again. The supernatant is mixed with 2 mL of TCA + TBA solution and heated in a 100°C water bath for 60 minutes. The next one is cooled in ice cubes for 30 minutes, then centrifuged again and taken about 200 µL on a microplate for spectrophotometry at a wavelength of 532 nm. The proline test is conducted based on the method by (Bates et al. 1973) with slight modifications. In the proline test, a fresh sample of 0.5 g is mixed with 4.5 mL of SAS solution and centrifuged. In addition, take 0.5 mL of the supernatant and mix with 1 mL of ninhydrin, and then heat it in a 100°C water bath for 60 minutes. Then, cooled in ice cubes for 30 minutes, added with 1.5 mL toluene, and vortexed for 1 minute. Finally, about 200 µL is taken on a microplate for spectrophotometry at a wavelength of 520 nm.

Biomass Measurement

Adventitious roots that are six weeks old in liquid culture in each treatment are harvested and weighed using an analytical balance to determine their fresh weight, followed by heating in the oven at a temperature of around 60° C for ± five days. Then it was weighed using an analytical balance to determine the dry weight. The measurement results are presented in the form of

an average and then analysed quantitatively. The number of samples used in this study was three replicates. The growth index was calculated using the following formula:

$$Growth\ index = \frac{(final\ fresh\ weight) - (initial\ fresh\ weight)}{initial\ fresh\ weight}$$

Saponin Extraction

To extract the saponins, the adventitious root of *T. paniculatum* is weighed 0.02 g dry, grounded using a mortar to become a powder, given 5 mL of 96% ethanol, and left for 24 hours. That was followed by heating for 45 minutes in a water bath at 80°C. The sample is filtered using filter paper to separate the precipitate and liquid. The obtained liquid is tested using thin-layer chromatography (three replicates).

Thin Layer Chromatography Test

T. paniculatum adventitious root extract samples are spotted on 20 µL *silica gel* GF_{254} TLC plates of each treatment. To compare the presence of saponins in the sample, a standard saponin solution is made, which is also spotted on the TLC plate. Afterward, elution is carried out using a vessel containing a solution of 2-propanol: water (14: 3) (Yachya 2012). Moreover, it is sprayed on the surface of the TLC plate using the *anisaldehyde-Sulfuric Acid* solution and then dried in an oven at a temperature of 100-110°C for 7-10 minutes (Stahl 1985). Finally, the researchers observed the appearance of saponin stains was observed on the TLC plate. The saponin was determined by the green color. When spraying with a stain viewer, if a dark green (Manuhara et al. 2015), purple or red-purple color appears (Marliana et al. 2005) then the extract contains saponins.

RESULTS

The Effect of Carbon Source Variations on Adventitious Root Biomass

Adventitious roots are obtained from the induction of leaf explants of *T. paniculatum* on solid *Murashige and Skoog* (MS) media plus 2 mg/L IBA for four weeks, then sub cultured in a 250 mL Erlenmeyer for six weeks culture with the addition of various carbon sources, result shown in Figure 1.

The yield of adventitious root culture (Figure 1) is weighed to determine the fresh and dry biomass. The averages of the fresh and dry biomass are summarized and presented in Table 1. Plant propagation by in vitro culture is also shown by the growth index obtained from fresh biomass using the growth index formula (IP), which is the difference between fresh biomass at harvest and fresh biomass inoculated. Afterward, it is divided by fresh biomass inoculated. Among all treatments, the highest IP is obtained in adventitious roots with 3% fructose treatment, which is 0.30. The IP value of sucrose + dextrose (1.5% + 1.5%) treatment is lower, i.e. 0.01 compared to sucrose alone. The mean biomass and growth index of *T. paniculatum* adventitious roots are shown in Table 1.

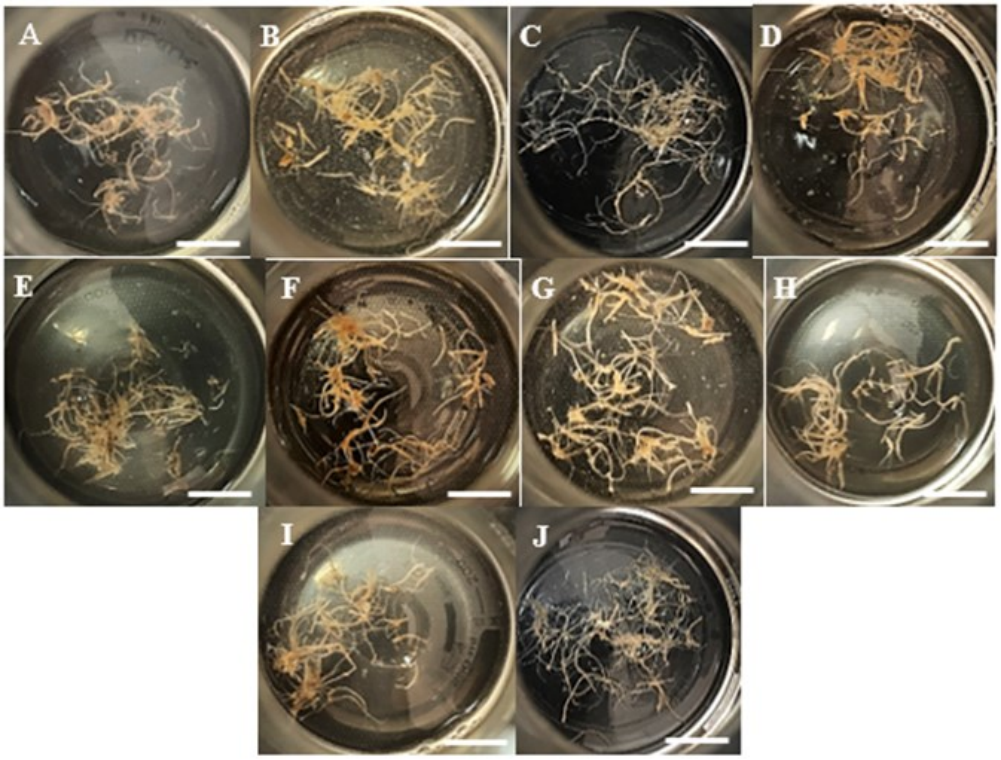

Figure 1. Adventitious roots of *T. paniculatum* cultured for 6 weeks in liquid MS medium with various carbon sources treatment. **A.** 3% sucrose; **B.** Lactose 3%; **C.** Maltose 3%; **D.** 3% glucose; **E.** Dextrose 3%; **F.** Fructose 3%; G. Sucrose+Fructose (1.5%+1.5%); **H.** Sucrose+Glucose (1.5%+1.5%); **I.** Sucrose+Dextrose 1.5%+1.5%; **J.** Glucose+Fructose (1.5%+1.5%). Bars = 1 cm.

Table 1. Average biomass and growth index of *T. paniculatum* adventitious roots at 6 weeks of culture age in various treatments of carbon sources (n=3).

Treatment	Initial inoculum fresh biomass (g)	Fresh biomass (g)	Dry biomass (g)	Growth Index (IP)
Sucrose 3% (control)	1	1.06 ± 0,02	0.07 ± 0.00	0.06 ± 0,02
Lactose 3%	1	1.21 ± 0,10	0.14 ± 0.07	0.21 ± 0.03
Maltose 3%	1	1.24 ± 0,16	016 ± 0.19	0.24 ± 0.16
Glucose 3%	1	1.05 ± 0,00	0.05 ± 0.03	0.05 ± 0.00
Dextrose 3%	1	1.14 ± 0,01	0.06 ± 0.01	0.14 ± 0.01
Fructose 3%	1	1.30 ± 0,07*	0.23 ± 0.12*	0.30 ± 0.07*
Sucrose+Fructose (1.5% + 1.5%)	1.	1.02 ± 0,01	0.05 ± 0.00	0.02 ± 0.01
Sucrose+Glucose (1.5% + 1.5%)	1	1.03 ± 0,02	0.03 ± 0.01	0.03 ± 0.02
Sucrose+Dextrose (1.5% + 1.5%)	1	1,01 ± 0,00	0.02 ± 0.00	0.01 ± 0.00
Glucose+Fructose (1.5% + 1.5%)	1	1,25 ± 0,09	0.20 ± 0.16	0.25 ± 0.09

*highest yield of fresh biomass and dry biomass

The Effect of Carbon Source Variations on Adventitious Root Morphology

The results of the observation of the morphology of the adventitious roots of T. *paniculatum* using a stereo microscope with a magnification of 30x are shown in Figure 2. The adventitious roots cultured in the treatment media of various carbon sources show the growth of root branches and root hairs as in control. In addition, this study also measures the root branch length and

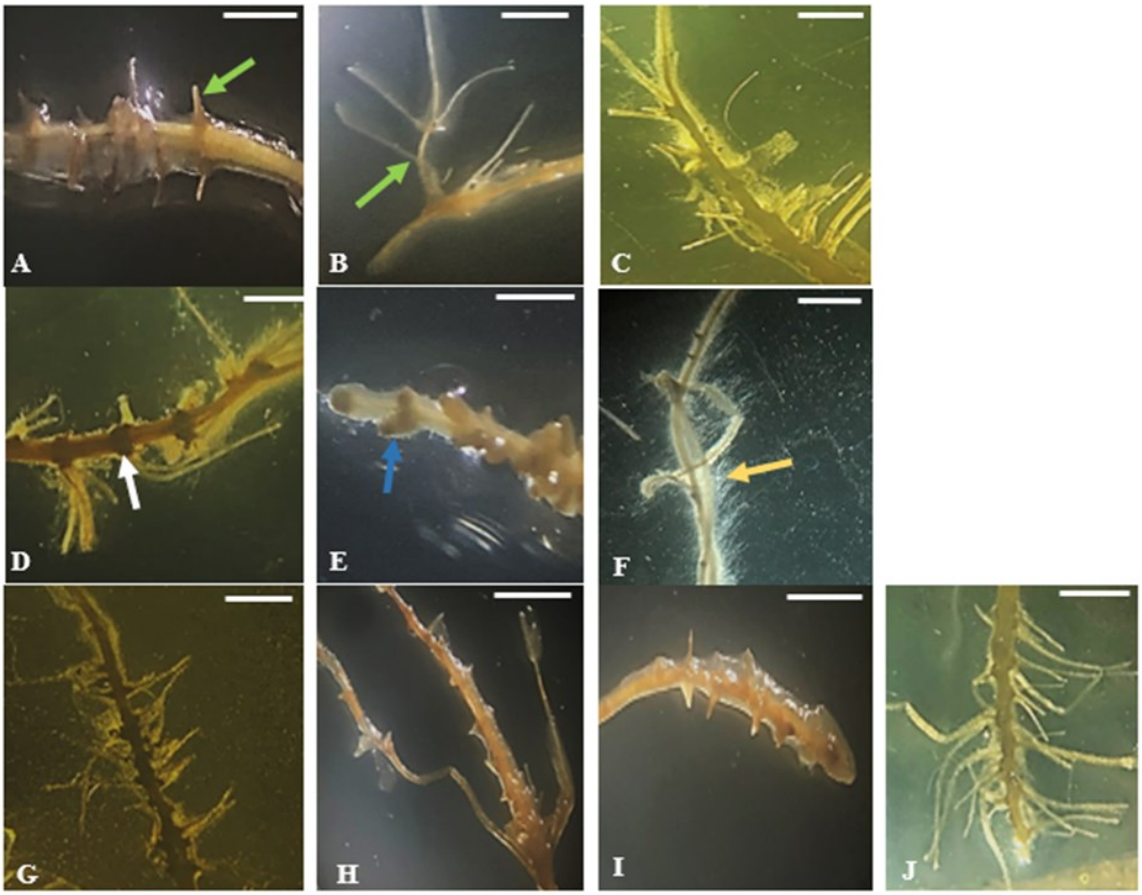

Figure 2. Morphology of adventitious roots of *T. paniculatum* cultured for six weeks in liquid MS medium with various carbon sources treatment. **A.** 3% sucrose; **B.** Lactose 3%; **C.** Maltose 3%; **D.** 3% glucose; **E.** Dextrose 3%; **F.** Fructose 3%; G. Sucrose+Fructose (1.5%+1.5%); **H.** Sucrose+Glucose (1.5%+1.5%); **I.** Sucrose+Dextrose (1.5%+1.5%); **J.** Glucose+Fructose (1.5%+1.5%). Observed with a 30x magnification stereo microscope. Bars = 1mm. Green arrow = root branching; white arrow = root initiation; blue arrow = root initiation starts to grow; yellow arrow = root hair.

root diameter using an inverted microscope. Measurements are conducted with 3 repetitions so that the average root branch length and root diameter average are obtained, as presented in Table 2.

Table 2. Average branch length and diameter of adventitious roots of *T. paniculatum* cultured for 6 weeks in liquid MS medium with various carbon source treatments (n=3).

Treatment	Root Branch Length (mm)	Root Diameter (mm)
Sucrose 3% (control	0.89 ± 0.34	0.76 ± 0.06
Lactose 3%	2.63 ± 0.29	0.70 ± 0.13
Maltose 3%	1.39 ± 0.61	0.72 ± 0.05
Glucose 3%	1.80 ± 0.16	1.53 ± 0.05*
Dextrose 3%	1.14 ± 0.25	0.68 ± 0.28
Fructose 3%	2.94 ± 0.17	0.56 ± 0.10
Sucrose+Fructose (1.5% + 1.5%)	1.97 ± 0.08	0.18 ± 0.06
Sucrose+Glucose (1.5% + 1.5%)	1.69 ± 1.15	0.73 ± 0.04
Sucrose+Dextrose (1.5% + 1.5%)	0.81 ± 0.18	1.28 ± 0.13
Glucosa+Fructose (1.5% + 1.5%)	3.86 ± 0.09*	0,41 ± 0.02

The Effect of Carbon Source Variations on MDA and Proline Levels of Adventitious Roots

MDA and proline test were performed to know whether the adventitious roots of *T. paniculatum* could do adaptation during cultured. The damage of cells membrane due to lipid peroxidation can be predicted through the accumulation of MDA (Baque et al. 2014). Since oxidative stress occurred in cells, they could activate defence mechanisms through increased activity of antioxidants (Esfandiari et al. 2007; Baque et al. 2013). The highest MDA was found in the treatment of sucrose and fructose combination with 1.5% concentration, respectively, whereas the highest proline was found in the treatment of maltose at 3%. The measurement results are presented in Table 3. It's indicated that adventitious roots have stress in medium supplemented with sucrose and fructose 1.5%. The combination of sucrose and fructose in the media will produce a high concentration of sugar in the media because some of the sucrose undergoes hydrolysis during autoclave sterilization into glucose and fructose molecules. High proline levels determine the physiological stress of roots on drought stress due to sugar stress. The sugar in the culture media causes hypertonic conditions in the root cells, so the cells shrink due to lack of water.

Table 3. MDA and proline levels of adventitious roots of *T. paniculatum* cultured for 6 weeks in a variety of carbon source treatment media were measured using a spectrophotometer.

Treatment	MDA level (nmol/0.5 g FW)	Proline level (µmol/0.5 g FW)
Sucrose 3% (control)	169.321	223.772
Lactose 3%	49.476	1033.621
Maltose 3%	48.672	1894.666*
Glucose 3%	60.737	512.339
Dextrose 3%	41.433	261.007
Fructose 3%	52.693	256.352
Sucrose + Fructose (1.5% + 1.5%)	183.799*	591.462
Sucrose + Glucose (1.5% + 1.5%)	42.237	247.044
Sucrose + Dextrose (1.5% + 1.5%)	41.433	1112.744
Glucose + Fructose (1.5% + 1.5%)	45.454	195.847

*the results of the highest MDA and proline levels

The Effect of Carbon Source Variations on Levels of Adventitious Root Saponin Compounds

Based on TLC test results (Figure 3), highest saponin levels were obtained at 3% dextrose treatment. This treatment produces saponin stains with large colour intensity and area. Afterward, it is analysed using ImageJ software to obtain results in the form of colour intensity and area of saponin stains as

shown in Figure 4. Based on ImageJ software, the highest saponin content was also found at 3% dextrose, which was shown by the largest area and colour intensity of *T. paniculatum* adventitious roots (Figure 4A and 4B).

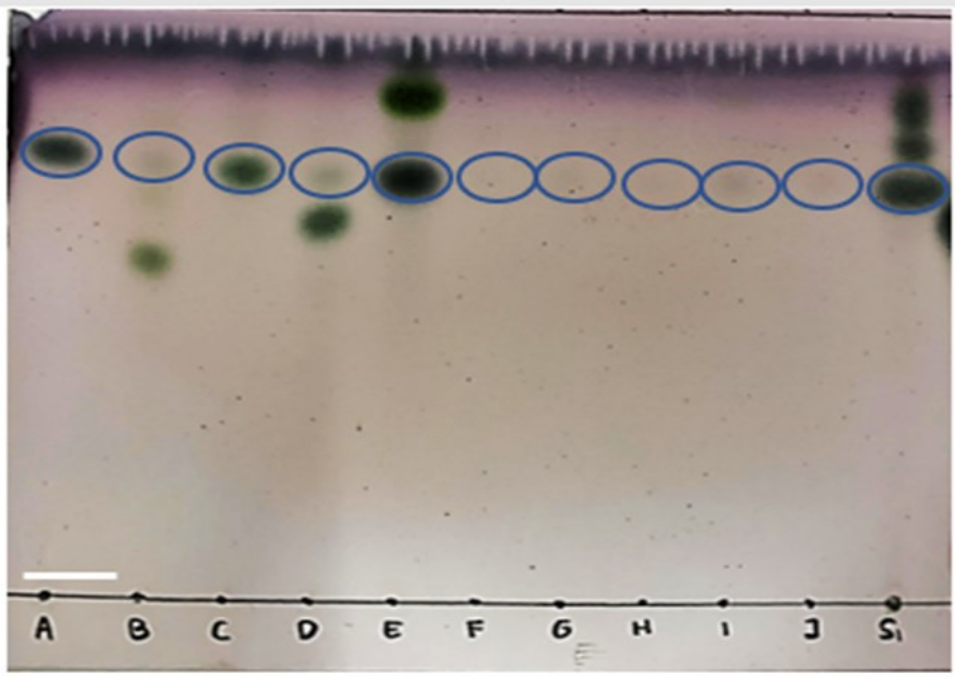

Figure 3. Saponin stains of adventitious roots of *T. paniculatum* on Thin Layer Chromatography of silica gel GF254 using 2-propanol:water (14:3) as eluent. **A.** 3% sucrose; **B.** Lactose 3%; **C.** Maltose 3%; **D.** 3% glucose; **E.** Dextrose 3%; **F.** Fructose 3%; G. Sucrose+Fructose (1.5%+1.5%); **H.** Sucrose+Glucose (1.5%+1.5%); **I.** Sucrose+Dextrose (1.5%+1.5%); **J.** Glucose+Fructose (1.5%+1.5%); S1 = standard saponins. Bars = 1 cm.

DISCUSSION

The data in Table 1 shows that there is an increase in the fresh weight of the roots compared to the fresh weight of the initial inoculum. The increase in fresh biomass that occurred indicates adventitious root growth activity and is a result of the increase in root cell mass compared to the initial inoculum. The increase in biomass occurs due to the presence of sufficient energy sources in the liquid medium to support the need for adventitious roots to grow. The energy is in the form of sugar, which can be utilized by plants as a carbon source for metabolism, development, growth, and gene expression. It is either monosaccharide or disaccharide sugar that can act as a carbon source (Wang & Weathers 2007).

The results show that the highest fresh biomass is produced by 3% fructose treatment, which is 1.30 g compared to sucrose as a control. This is similar to the research of Uozumi et al. (1991), which stated that there is an increase in root hair biomass infected by *Agrobacterium rhizogenes* A4 in carrot culture with the administration of a fructose concentration of 30 g/L on day 48 of the culture period. For fed-batch culture, the average root hair growth rate increased to 0.8 g-dry biomass/L/day and 0.25 g-dry biomass/L/day for batch culture. According to Uozumi et al. (1991), if the plant cell is ready to

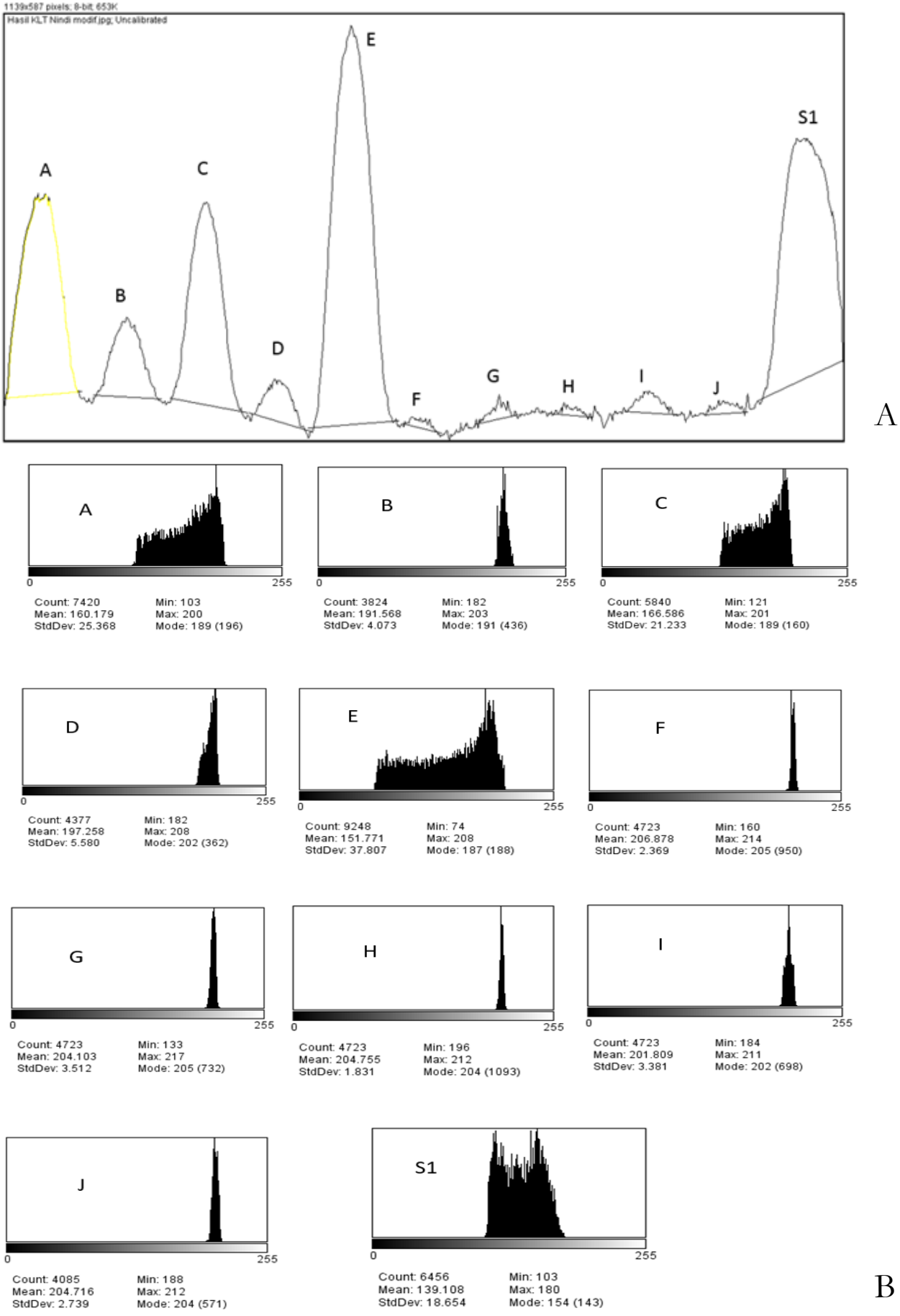

Figure 4. The results of the analysis of saponin levels using Image J software. (a) The size of the area; (b) color intensity of adventitious root saponins of *T. paniculatum* on Thin Layer Chromatography plate. **A.** 3% sucrose; **B.** Lactose 3%; **C.** Maltose 3%; **D.** 3% glucose; **E.** Dextrose 3%; **F.** Fructose 3%; G. Sucrose+Fructose (1.5%+1.5%); H. Sucrose+Glucose (1.5%+1.5%); **I.** Sucrose+Dextrose (1.5%+1.5%); **J.** Glucose+Fructose (1.5%+1.5%); (S1) standard saponins.

consume carbohydrate, as in the case of bacteria, the medium containing the monosaccharide fructose can achieve a higher cell mass. However, the researchers are unable to identify the factors that caused the increase in cell density completely. In this study, supplemented sucrose 3% as a control was not showed the maximum fresh weight biomass, even though in another research showed the maximum yield was achieved at 3% sucrose (Cui et al. 2014; Thanh et al. 2014). Effect of carbohydrate source on biomass accumulation also showed that treatment of sucrose 3% has the highest yield in cell suspension culture, adventitious roots and hairy roots of *Whitania somnifera* (Nagella & Murthy 2014). The yield of *T. paniculatum* adventitious root biomass in this study is low. This happens because the inoculum that is immersed continuously in the medium is prone to hyperhydricity. Hyperhydricity is the occurrence of tissue damage and improper shape changes in explants, such as symptoms of excess water in plant cells or tissues (Solim 2017).

The combination treatment of glucose and fructose (1.5% + 1.5%) resulted in the longest average root branch length of 3.86 mm compared to the sucrose treatment. This can be explained by the fact that glucose is the raw material for respiration and can be used as energy along with light in metabolism for the production of ATP and NADH so that growth can be accelerated (Yang et al. 2007). Administration of fructose increased the growth of *M. griffithii* significantly about 1-4 times after 25 days of culture compared to control. According to Yee (2015), if the two carbon sources were combined, it will result in rapid root branch growth. The largest average diameter produced by the addition of glucose is 1.53 mm (double the root diameter in the control treatment). This is because glucose molecules are active as raw materials for respiration and used as energy in primary plant metabolisms, such as cell enlargement and elongation to produce large root diameters. The results of this study are in accordance with the research by Yusuf et al. (2021), that the supplementation of 20 mM glucose can produce a larger leaf area per plant than the control. In the other study showed that was added of 5% sucrose increase biomass of adventitious root of *G. procumbens* (Noviyanti et al. 2017; Lestari et al. 2017).

MDA levels in this study indicate physiological stress of root cells, not acclimatization or a form of root adaptation to culture conditions. This research is in line with Ikhtimami (2012) research, which shown root hair achieves stationary growth (no increase root hair length) in the 4th week, as indicated with a horizontal line at weeks four to ten. Although the subculture treatments are different, the three treatments in this study show the same results in terms of achieving stationary growth. Different things are shown in the 2-week subculture treatment. In this study, adventitious roots of *T. paniculatum* are subcultured in a liquid medium for six weeks (Figure 2), which was in line with Ihktimami's research.

The highest MDA levels are obtained in the sucrose + fructose treatment: 183.799 nmol/0.5 g fresh weight (Table 3). If the MDA level is high,

describe that the cell is experiencing oxidative stress, which affects the decrease in biomass. The results of this study can be explained that the combination of sucrose and fructose in the media will produce a high concentration of sugar in the media because some of the sucrose undergoes hydrolysis during autoclave sterilization into glucose and fructose molecules. In another study (adventitious roots culture of *Hypericum perforatum* L.) showed an increase of sucrose levels followed by an increase of MDA levels (Cui et al. 2014). Aeration was used in liquid culture by shaking, consequently, shear stresses occurred inside the culture caused cells damage. The damage of cells membrane due to lipid peroxidation can be predicted through the accumulation of MDA (Baque et al 2014). Since oxidative stress occurred in cells, they could activate defences mechanisms through increased activity of antioxidants (Esfandiari et al. 2007; Baque et al. 2013).

This study also measures proline levels to determine the physiological stress of roots on drought stress due to sugar stress. The sugar contains in the culture media causes hypertonic conditions in the root cells so that the cells shrink due to lack of water. The root cells produce proline so that the fluid inside and outside the cell is the same (isotonic). The highest proline content is obtained in the 3% maltose treatment. This is similar to the research of (Ibrahim & Abdellatif 2016), maltose and trehalose treatment significantly increase the concentration of soluble protein and proline compared to the control. According to Luo et al. (2010), externally applied maltose and trehalose accumulate rapidly, are transported by leaf and root tissues, as well as play an important role as osmoprotectants. Maltose has a hydrophilic group, and replacing water molecules with maltose can reduce damage caused by drought (Lerbret et al. 2005).

Based on the results of the TLC test, the highest levels of saponins were obtained in the 3% fructose treatment. This is similar to the results of Wang & Weathers (2007) that the production of artemisinin in a medium with fructose resulted in 2 times the levels of artemisinin compared to sucrose. However, when these sugars are combined, such as sucrose + glucose or fructose in seed culture, there is a decrease in artemisinin levels compared to sucrose alone. In this study, the TLC results were then analysed using ImageJ software, which showed the area of the stain and the intensity of the colour (Figure 4). The results of both show that the 3% fructose treatment produced the highest levels of saponins.

CONCLUSION

Based on the results of the research above, it can be concluded that the variation of carbon sources has an effect on biomass, MDA levels, proline levels, and saponin levels of adventitious roots of *T. paniculatum*. The adventitious root biomass obtained in the treatment of various carbon sources increases compared to the initial inoculum biomass. The levels of MDA, proline levels, and levels of adventitious root saponins produced in the treatment of various carbon sources are higher than the control. However, the morphology of ad-

ventitious roots in the treatment of variations in carbon sources does not differ from the morphology of roots in control, so that the provision of variations in carbon sources has no effect on the morphology of adventitious roots. The highest yield of root biomass and content of saponin compounds is produced by 3% fructose treatment. Hence, 3% fructose can be used as a substitute for sucrose for the liquid culture of adventitious roots of *T. paniculatum*.

AUTHORS CONTRIBUTION

Y.S.W.M designed the research and supervised all the process, A.Y and A.N.K collected and analyzed the data, D.S designed the research and supervised the data, and N.N.E analyzed the data and wrote the manuscript.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

Bates, L.S., Waldren, R.P. & Teare, I.D., 1973. Rapid determination of free proline for water stress studies. *Plant and Soil*, 39(1), pp.205-207. doi:10.1007/BF00018060

Baque, M.A. et al., 2013. Production of Biomass and Bioactive Compounds by Adventitious Root Suspension Cultures of *Morinda citrifolia* (L.) In A Liquid-Phase Airlift Balloon-Type Bioreactor. *In Vitro Cell. Dev. Biol.-Plant,* 49(6). doi: 10.1007/s11627-013-9555-3

Baque, M.A., Murthy, H.N. & Paek, K.Y., 2014. Adventitious Root Culture of *Morinda citrifolia* in Bioreactors for Production of Bioactive Compounds. In *Production of Biomass and Bioactive Compounds Using Bioreactor Technology*, 1st edition. Heidelberg: Springer Science and Business Media Dordrecht. pp.186-223. doi: 10.1007/978-94-017-9223-3

Cui, X-H, Murthy, H.N. & Paek, K-Y., 2014. Production of Adventitious root Biomass and Bioactive Compouns from *Hypericum perforatum* L. Trough Large Scale Bioreactor Cultures. In *Production of Biomass and Bioactive Compounds Using Bioreactor Technology*. Springer Dordrecht. pp. 167-184. doi: 10.1007/978-94-017-9223-3

Esfandiari, E., Shekari, F. & Esfandiari, M., 2007. The Effect of salt stress on antioxidant enzymes, activity and lipid peroxidation on the wheat seedling. *Not. Bot. Hort. Agrobot. Cluj*., 35. doi: 10.15835/nbha351251

Faizal, A. & Sari, A.V., 2019. Enhancement of saponin accumulation in adventitious root culture of Javanese ginseng (*Talinum paniculatum* Gaertn.) through methyl jasmonate and salicylic acid elicitation**.** *African Journal of Biotechnology*, 18(6), pp.130-135. doi: 10.5897/AJB2018.16736

Fuentes, G. et al., 2005. Exogenous sucrose can decrease in vitro photosynthesis but improve field survival and growth of coconut (*Cocos nucifera* L.) in vitro plantlets. *In Vitro Cellular and Developmental Biology - Plant*, 41 (1), pp.69-76. doi: 10.1079/IVP2004597

George, E.F., 1993. *Plant propagation by tissue culture. Part 1: The technology* 2nd ed., Exegetics limited.

Hu, X.Y. et al., 2003. Activation of plasma membrane NADPH oxidase and generation of H2O2 mediate the induction of PAL activity and saponin synthesis by endogenous elicitor in suspension-cultured cells of *Panax ginseng*. *Acta Botanica Sinica*, 45(12), pp.1434-1441.

Ibrahim, H.A. & Abdellatif, Y.M.R., 2016. Effect of maltose and trehalose on growth, yield and some biochemical components of wheat plant under water stress. *Annals of Agricultural Sciences*, 61(2), pp.267-274. doi: 10.1016/j.aoas.2016.05.002

Ikhtimami, A., 2012. *Pengaruh periode subkultur terhadap kadar saponin akar rambut tanaman ginseng jawa (Talinum paniculatum Gaertn.)*. Universitas Airlangga.

Lerbret, A. et al., 2005. Influence of homologous disaccharides on the hydrogen-bond network of water: Complementary Raman scattering experiments and molecular dynamics simulations. *Carbohydrate Research*, 340 (5), pp.881-7 doi: 10.1016/j.carres.2005.01.036

Lestari, S.R. et al., 2017. Biomass and flavonoid production of *Gynura procumbens* (Lour.) Merr. axillary shoots culture induced by sucrose and erythrose 4-phosphate. *Scholars Academic Journal of Biosciences*, 5(4).

Luo, Y. et al., 2010. Exogenously-supplied trehalose protects thylakoid membranes of winter wheat from heat-induced damage. *Biologia Plantarum*, 54(3), pp.495-501 doi: 10.1007/s10535-010-0087-y

Manuhara, Y.S.W., Kristanti, A.N. & Utami, E.S.W., 2015. Optimization of culture conditions of *Talinum paniculatum* Gaertn.adventitious roots in balloon type bubble bioreactor using aeration rate and initial inoculum density. *Asian Journal of Biological Sciences*, 8(2), pp.83-92 doi: 10.3923/ajbs.2015.83.92

Marliana, S. D., Suryanti, V. & Suyono, 2005. Skrining Fitokimia dan Analisis Kromatografi Lapis Tipis Komponen Kimia Buah Labu Siam (Sechium edule Jacq. Swartz) dalam Ekstrak Etanol. *Biofarmasi*, 3(1), p.29.

Murashige, T. & Skoog, F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum* 15, pp.473-497.

Nagella, P. & Murthy, H.N., 2014. Production of whitanolides from cell and organ culture of *Withania somnifera* (L.) Dunal, In*Production of Biomass and Bioactive Compounds Using Bioreactor Technology*. Springer Dordrecht. pp.167-184. doi**:** 10.1007/978-94-017-9223-3

Noviyanti, R. et al., 2017. Biomass and flavonoid production of *Gynura procumbens*adventitious roots induced by sucrose, phenylalanine and tyrosine. *Bioscience Research* 14(4), pp. 934-941.

Peixoto, P.H.P. et al., 1999. Aluminum effects on lipid peroxidation and on the activities of enzymes of oxidative metabolism in sorghum. *Revista Brasileira de Fisiologoia Vegetal*, 11(3), pp.137-143

Percival, G.C. & Fraser, G.A., 2005. Use of sugars to improve root growth and increase transplant success of birch (*Betula pendula* Roth.). *Journal of Arboriculture*, 31(2), pp.66-72. doi: 10.48044/jauf.2005.008

Praveen, N. & Murthy, H.N., 2012. Synthesis of withanolide A depends on carbon source and medium pH in hairy root cultures of Withania somnifera. *Industrial Crops and Products*, 35(1), pp.241-243. doi: 10.1016/j.indcrop.2011.07.009

Rahmi, E. & Widyasari, K., 2011. Potency of Java Ginseng (*Talinum Paniculatum* Gertn.) Roots Extract on Quality and Viability of Mice Sperm. , 11, pp.7-10.

Sami, F. et al., 2016. Role of sugars under abiotic stress. *Plant Physiology and Biochemistry*, 109, pp.54-61. doi: 10.1016/j.plaphy.2016.09.005

Scott, J.D., 1995. *Sugar Transport in Strawberry Fruit: Uptake Competition, and Developmental Changes in Invertase Levels*. Oregon State University.

Solim, M.H., 2017. *Peningkatan Produksi Biomassa dan Saponin Akar Adventif Talinum paniculatum Gaertn. Dalam Bioreaktor Bergelembung Tipe Balon Sistem Batch dan Continous*. Universitas Airlangga.

Stahl, E., 1985. *Analisis Obat Secara Kromatografi dan Mikroskopi*, Bandung: Institut Teknologi Bandung.

Thanh, N.T., Murthy, H.N. & Paek, K.Y., 2014. Ginseng cell culture for production of ginsenoside. In *Production of Biomass and Bioactive Compounds Using Bioreactor Technology*. Springer Dordrecht. pp.167-184. doi: 10.1007/978-94-017-9223-3

Uozumi, N. et al., 1991. Fed-batch culture of hairy root using fructose as a carbon source. *Journal of Fermentation and Bioengineering*, 72(6), pp.457-460. doi: 10.1016/0922-338X(91)90054-K

Wang, Y. & Weathers, P.J., 2007. Sugars proportionately affect artemisinin production. *Plant Cell Reports*, 26(7), pp.1073-1081. doi: 10.1007/s00299-006-0295-2

Widiyani, T., 2006. Efek antifertilitas ekstrak akar som Jawa (*Talinum paniculatum* Gaertn.) pada mencit (*Mus musculus* L.) jantan. *Bul. Penel. Kesehatan*. 34(3).

Winarni, D., 2009. *Potensi androgenik akar ginseng Jawa (Talinum paniculatum Gaertn.) pada kondisi testosterone rendah*. Universitas Airlangga.

Yachya, A., 2012. *Pengaruh Laju Aerasi Dan Kerapatan Inokulum Terhadap Biomassa Dan Kandungan Saponin Kultur Akar Rambut Ginseng Jawa Dalam Bioreaktor Tipe Balon*. Universitas Airlangga.

Yang, S.H., Wang, L.J. & Li, S.H., 2007. Ultraviolet-B irradiation-induced freezing tolerance in relation to antioxidant system in winter wheat (*Triticum aestivum* L.) leaves. *Environmental and Experimental Botany*, 60(3), pp.300-307. doi: 10.1016/j.envexpbot.2006.12.003

Yee, W., 2015. Feasibility of various carbon sources and plant materials in enhancing the growth and biomass productivity of the freshwater microalgae *Monoraphidium griffithii* NS16. *Bioresource Technology*, 196, pp.1-8. doi: 10.1016/j.biortech.2015.07.033

Yusuf, M. et al., 2021. Glucose modulates copper induced changes in photosynthesis, ion uptake, antioxidants and proline in *Cucumis sativus* plants. *Carbohydrate Research*, 501, pp.108271. doi: 10.1016/j.carres.2021.108271

Zuo, G. et al., 2009. Total saponins of *Panax ginseng* induces K562 cell differentiation by promoting internalization of the erythropoietin receptor. *American Journal of Chinese Medicine*, 37(4), pp.747-57. doi: 10.1142/S0192415X09007211

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Research Article

Evaluation of Bioactive Secondary Metabolites from Ponyfish Associated Bacteria (*Photobacterium leiognathi)*

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ABSTRACT

The marine environment continues to surprise us by producing novel bioactive substances with a wide range of benefits for humans. Materials and Methods: Marine bioluminescent bacteria *Photobacterium leiognathi* was isolated from pony fish, *Secutorruconius* which was confirmed with microscopic and molecular characterization. The secondary metabolite of the isolated bacteria was extracted with dichloromethane. The chemical fingerprinting of the isolated metabolite was analyzed through TLC, FT-IR, and HPLC. The nature of the compound present in the metabolite was identified in the gas chromatography-mass spectrometry analysis (GC - MS). The isolated extract was investigated for its antibacterial property against 10 human pathogenic bacteria and also its antioxidant activity using different assays such as 1, 1-Diphenyl-2-picrylhydrazyl, Phosphomolybdenum, Metal chelating, Hydroxyl radical scavenging and hydrogen peroxide scavenging activity. Results: The Presence of functional groups including phenols, sugars, and amino acids in the extracts were identified by TLC. Totally, nine peaks were obtained for the crude extract through the FTIR spectrum range of 400 to 4000 cm^{-1} for the active sample. The DCM extract showed a broad spectrum of antibacterial activity against the six human bacterial pathogens. Secondary metabolites from the bioluminescent bacteria, *P. leiognathi,* have strong antioxidant properties. These results will be instrumental in developing novel products with biosensors and bio-imaging applications using *P. leiognathi.*

INTRODUCTION

Being one of the least investigated ecosystems with only one percent of the oceans being explored, the marine environment still contains many novel compounds with various applications including cosmetics, nutraceuticals, pharmaceuticals, feed formulation, food supplements, textiles, etc. One of the most understudied ecosystems on the planet is the marine environment. Many studies have identified marine natural products for a variety of purposes, including nutraceuticals, pharmaceuticals, feed formulation, textiles, and so on, although they represent only 1% of ocean exploration and abundance. On the other hand, still the marine environment, continues to surprise us by producing novel bioactive substances with a wide range of benefits for humans. Bioluminescence is one such characteristic that provides spectacular

advantages to the populace while also adding to the beauty of the ocean. Bioluminescence is produced by the extracellular biochemical compounds with chemiluminescence properties (Morin-Crini et al. 2019). Bioluminescence producing bacteria is attached to surface of several marine flora and fauna (Ramesh & Mohanraju 2017). The need for the production of bioluminescence molecules differs among species. Some species make biochemical substances to attract the opposite gender and to light up the deeper waters for food-seeking purposes.

Chemists have identified that the bioluminescence organisms could emit light at a visible range which is produced by naturally occurring enzymatic reactions. The photoprotein from some organisms emit light when combined with luciferin or luciferase enzyme, but not all photoproteins require them to emit light (Haddock et al. 2010). The majority of luminescent bacteria inhabits the ocean. Mainly, two genera of marine bacteria, *Vibrio* and *Photobacterium*, are the most abundant bioluminescent bacteria. They are found in seawater, intestinal tract, and on the surfaces of marine animals. However, the only terrestrial luminescent bacterial genus known so far is *Photorhabdus* (Engebrecht et al. 1983). *Photobacterium leiognathi* is a marine bacterium that can naturally emit light by secreting the photoprotein and reacting with that atmospheric oxygen. The bioluminescence proteins of the bacteria were highly sensitive to a wide variety of toxic substances including heavy metals such as Hg, Al, Zn and Cr (Kannahi & Sivasankari 2014).

The applications of bioluminescence are used for sensing and controlling hygienicity for many industries such as fish and milk industries. Moreover, bioluminescence-based assays can be applied for pollution mapping in ecosystems and sensing of pH, metal ions, transmembrane potential, drug molecules and other metabolites, gene assays and observation of protein–protein interactions (Kim et al. 2018; Sharifian et al. 2018). Bioluminescence research is also being conducted for use in the medical field. The bioluminescent bacterium has been used as an imaging component in the medical field (Nunes-Halldorson & Duran 2003; Menz et al. 2013). *P. leiognathi* was isolated from the marine ponyfish *Secutorruconius* and its secondary metabolite was extracted. Furthermore, the antibacterial and antioxidant properties of the secondary metabolite were evaluated using standard methods.

MATERIALS AND METHODS

Sample Collection and Bacterial Isolation

Live fish of *Secutorruconius* (ponyfish of the Leiognathidae family) were collected from Mudasalodai, Tamilnadu, India (Lat11°30'501" N and long 079° 49'669" E) and stored in an ice chest. The fishes were washed with sterile sea water then rinsed by double distilled water. The fish body surface was swabbed with sterile cotton swab for the isolation of luminescent bacteria. The upper layer of the skin of the fish was peeled off with the help of a sterile blade without touching the light organ and the tissue was homogenized with 1 ml of sterile seawater. The homogenate was serially diluted up to 10^5

and used for the isolation of luminescent bacteria. The swab and homogenate dilution (100µl) were spread on freshly prepared luminescent agar and Zobell marine agar plates. The plates were incubated at 22 °C for 24 hrs and monitored for the growth of luminescent bacteria. High-intensity light emitting luminescent bacterial colonies were sub-cultured for future works, the isolated pure colonies were subjected to morphological and molecular identification. The pure culture was also used for secondary metabolite production and purification. Further, the glycerol stock was also prepared and stored for future studies (Firudoz et al. 2020).

Morphological and Biochemical Characterization of Bacteria

The isolated pure luminescent bacterial colonies were subjected to morphological characterization by microscopy and colony characteristics. Initially, the cultures were subjected to the Gram staining technique to classify the bacteria according to the composition of the cell wall. Later, the cultures were grown on luminescent agar and are kept in dark to identify the luminescence of the bacteria, and the cultures were also grown in thiosulphate-citrate -bile salts-sucrose (TCBS) agar, a selective medium for identifying marine bacteria, especially *Vibrio* sp. The cultures were then subjected to a catalase test in which a loopful of liquid culture was added to a clean slide with 3% hydrogen peroxide and observed foran immediate effervescence. Another biochemical test is the starch hydrolysis test, in which cultures were inoculated in nutrient agar supplemented with starch at a final concentration of 2% and after incubation, the plates were flooded with iodine solution and clear zones around the culture as a result of starch hydrolysis (Sarkar et al. 2019).

Molecular Characterization of Bacteria

The use of molecular characterization helps in the specific identification of the isolate and thus was carried out accordingly. The genomic DNA of the isolate was extracted using a Bacterial Genomic DNA extraction kit according to the manufacturer's protocol (QIAGEN, QIAamp DNA Mini Kit) with some modifications. The isolated DNA was then amplified using the following PCR mix: 1 µl of bacterial universal 16S rRNA primers forward E9F (5-GAGTTTGATCCTGGCTCAG-3) (Farrelly et al. 1995) and 1µl of reverse primer U1510R (5-GGTTACCTTGTTACGACTT-3) (Reysenbach & Pac 1995), 2 µl of genomic DNA and 6µl of PCR grade water were added and the PCR amplification was done. Amplified sequence threads were submitted to the NCBI database and NCBI BLAST (http://www.ncbi.nlm.nih.gov/Blast) was carried out to distinguish the nearest neighbors of the isolates and then a phylogenetic tree was constructed using MEGA X software.

Extraction of Secondary Metabolites from Luminous Bacteria

A loop full of bacterial culture was inoculated in 250 ml conical flasks containing 150 ml Zobell marine broth and incubated at 28°C for 72 hours un-

der constant agitation of 220 RPM. Post-incubation, the cultures were taken and centrifuged at 6000 RPM for 20 minutes to collect the supernatant. To the collected supernatant, an equal volume of dichloromethane (DCM) was added and continuously agitated for 1 hour to obtain a homogeneous mixture. The organic phases were collected using a separating funnel and the solvent was evaporated using a rotary evaporator. The sample was lyophilized and stored at 4 ℃ for further analysis (Klöppel et al. 2008).

Characterization of Secondary Metabolites

Gas Chromatography – Mass Spectroscopy

The crude extract was analyzed using GCMS (Gas Chromatography Mass Spectrometry) to identify and to confirm the presence of a various compound in them. Helium gas is used as a carrier gas for GCMS, the injection volume was 1 µl with a flow rate of 1.0 µl/min. NIST database spectrum is used for standard. The crude extract spectrum was compared with the NIST database spectrum. The high-intensity chromatogram peak was adjusted with ESI Compass Data Analysis Version 4.0. Moreover, the non-polar column is performed (Bakaraki et al. 2016).

Fourier Transform Infrared Spectroscopy

The lyophilized sample was dissolved in 1 mg/ml of HPLC grade water and subjected to FTIR spectral evaluation. All spectra were recorded within an infrared range from 400 to 4000 cm^{-1} using an FTIR spectrometer (Shimadzu, Japan).

Thin Layer Chromatography

Thin Layer Chromatography (TLC) is a simple yet effective technique for the identification of different groups of secondary metabolites. The crude sample along with the DCM fraction was prepared at a 1 mg/ml concentration. The study was conducted using a silica gel plate for the stationary phase. The crude sample was spotted on silica plate and was transferred to a twin chamber which contains the developing solvent system with water: glacial acetic acid: n-butanol: ethyl acetic acid in the ratio of 1:1:1:1. *P-Anisaldehyde* – sulphuric acid was sprayed and the plate was allowed to dry and then heated to visualize the bands (Al-Massarani et al. 2017).

HPLC Analysis

HPLC was used for the separation and identification of compounds from the crude extract. The lyophilized DCM extract was dissolved in HPLC grade water (1 mg/ml). The sample was analysed with a reverse phase HPLC C18 column and the purified fraction was collected. Initially, the column was washed for 5 min. The crude sample was loaded on the column and the fraction was eluted using methanol and HPLC grade water for 30 min (These et al. 2009).

Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis

The protein content in the crude sample was determined using SDS-PAGE according to the method of Laemmeli (1970). The crude sample (20 µl) was mixed with Laemmeli loading dye (10 µl) and the mixture was loaded in 10% SDS-PAGE. After 4 hours, the gel was removed from the plates and stained with a staining solution containing 0.1% Coomassie brilliant blue and Methanol: Glacial acetic acid and de-stained with glacial acetic acid: methanol: water (10:30:60). The molecular weight was estimated by comparing it with protein marker and BSA with a molecular weight of 66.5 kDa (He 2011).

Biological Activity

Antibacterial Assay

The antibacterial activity for the crude and DCM fractions of secondary metabolites was determined by Bauer et al. (1966). In this test, human bacterial pathogen cultures namely *Bacillus subtilis* (ATCC 23857), *Pseudomonas, Shigella, Staphylococcus aureus* (ATCC 23235), *Salmonella paratyphi-A* (ATCC 9150) and *Salmonella paratyphi-B* (ATCC BAA-1250/SPB7), *Proteus vulgaris* (ATCC 8427), *Escherichia coli* (ATCC 25922D-5), *Klebsiellapneumoniae* (ATCC BAA-1705D-5) and *Proteus* mirabilis (ATCC 12453) were used in this test. The spread plate technique is employed and followed as per (Bauer 1966). The plates were incubated at 37 °C for 24 hours and observed for clear zones of inhibition formed around the discs, and the diameter of the zones is measured in millimeters (mm). Amoxicillin (10 µl /disc) was used as a positive control.

Antioxidant Assay

Antioxidant activity of the secondary metabolite was evaluated using different assay and the percentage of inhibition was calculated. DPPH radical Scavenging Activity was performed as per Yen and Chan (1995) method (Yen & Chen 1995).The total antioxidant capacity of the crude DCM extract was carried out using the phosphomolybdenum method to determine the total quantity of fat and water-soluble antioxidants presence (Saeed et al. 2012; Singh & Chahal 2018). The ability of secondary metabolite extract to scavenge hydroxyl scavenging was carried out according to the protocol of Halliwell and Gutteridge (Gutteridge & Halliwell 1988).The ability of the crude extract to scavenge hydrogen peroxide is determined as per the method mentioned by Ruch et al. (Ruch et al. 1989).The metal chelating activity of the extract was determined as described by Soler-Rivas (Soler-Rivas et al. 2000). Finally, the percentage of inhibition and the total amount of antioxidants present in the sample were calculated.

Statistical Analysis

Results were expressed as mean SD. The one-way ANOVA followed by Tukey's multiple tests was used to analyze data.

RESULTS AND DISCUSSION

Bacterial Characterization

The bacterial culture isolated from pony fish was gram-negative rod-shaped bacteria when observed microscopically, as shown in Figure 1. In TCBS agar, luminescent green colored colonies were observed macroscopically in the dark, as shown in Figure 2 and green colonies were observed in TCBS agar when provided with a light source as shown in Figure 3. Figures 4 and 5 indicate positive results for catalase and starch in biochemical assays. Gram staining and biochemical analysis revealed that the isolates tested were gram-negative rods, taxonomically identified as *Photobacterium leiognathi*. In the current study, secondary metabolites were extracted from *Photobacterium leiognathi* which was obtained from skin of *S. ruconius*. For *photobacterium*, the tests for catalase and cytochrome oxidase are both positive. Although D-glucose produces acids, it does not produce gas. Nitrate is broken down into nitrite (Nogi et al. 1998).

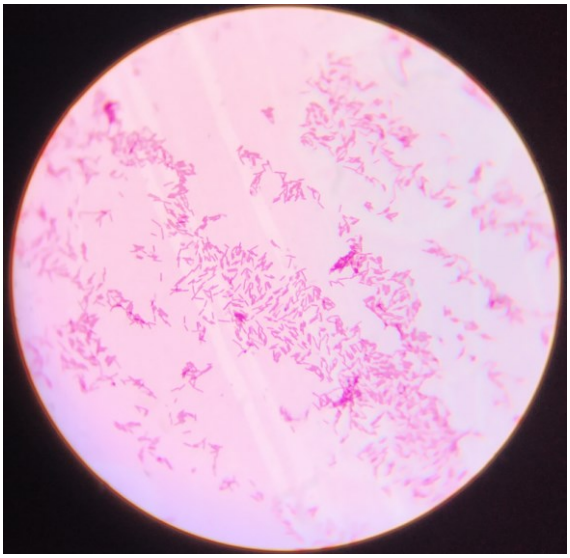

Figure 1. Gram staining – Gram Negative Rods.

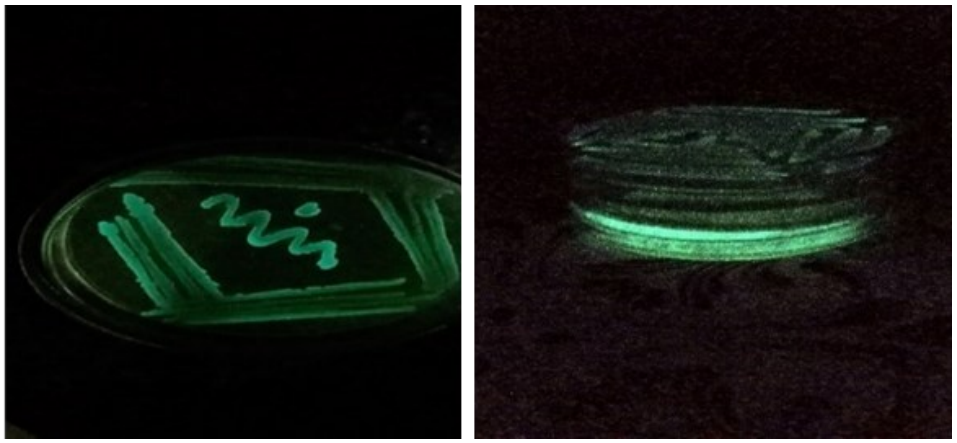

Figure 2. Bioluminescence from *P. leiognathi* in dark.

Gel electrophoresis for PCR product of the sample and a positive control for 16S rRNA gene. The sample and the positive control were amplified by 2% gel electrophoresis as shown in Figure 4.

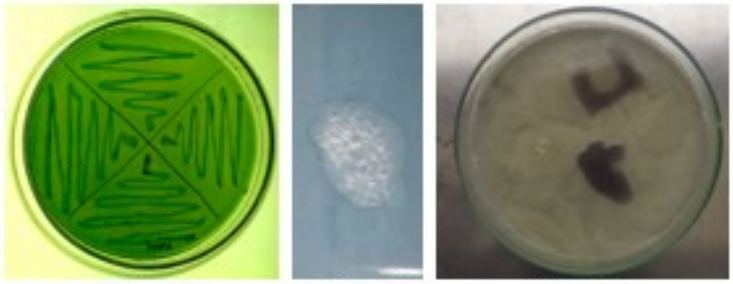

Figure 3. a) Green colour colonies in light (TCBS Agar); b) Catalase test – Positive; c) Starch hydrolysis test - Positive.

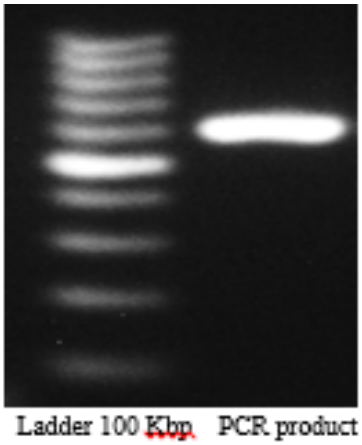

Figure 4. Agarose gel electrophoresis – Left: DNA Ladder 100Kbp, Right: Bacterial DNA.

After sequencing, amplified 16s rRNA of the isolated sample was subjected to BLAST analysis and a phylogenetic tree was constructed with the neighbourhood joining method. The sample of pony fish was found to be similar to that of *P. leiognathi.* The sequence of *P. leiognathi* (ATCC25521) was the closest of the operational taxonomic unit. The species *P. damselae* (ATCC33539) and *P. piscicola* (NCCB100098) have the closest sequence similarity of 99%. The molecular characterization by 16S rRNA sequencing confirmed that the isolated bacterium was a bioluminescent strain of *P. leiognathi* as shown in Figure 5 and was found to be in correlation with similar studies showing the isolation of *P. leiognathi* from pony fish (Molina et al. 2016).

Characterization of Secondary Metabolites

Gas Chromatography – Mass Spectroscopy

GC-MS spectrum of the crude secondary metabolite from the bacterial isolate shows a numerous presence of various components with different retention times as illustrated in Figure 6 and Table 1. Results depicted some of the most common secondary metabolite which were not yet reported from *P. leiognathi* and they are as follow: Cyclopropane, 1-Butyl-2-(2-Methyl Propyl), Oleic acid, Emylcamate, 2-Propenoicn acid, Oxybis (Methyl-2,1-Ethanediyl) ester, 17-(1,5-Dimethyihexyl)-10,13-Dimethyl-1,7,8,9,10,11,12,13,14,15,16,17 -Dodeca,2-Chloropropionic acid, 2,2-Dimethyl Propyl ester, 2,6-Lutidine 3,5-Dichloro-4-Dodecylthio-, 3-Butoxy-1,1,1,5,5,5-Hexamethyl-3-

(Trimethylsicoxy) Trisicoxane. In certain marine bacteria, specific GC-MS compounds such as phenol, dibutyl phthalate, butyl octylester, and indole were observed (Gromek et al. 2016).

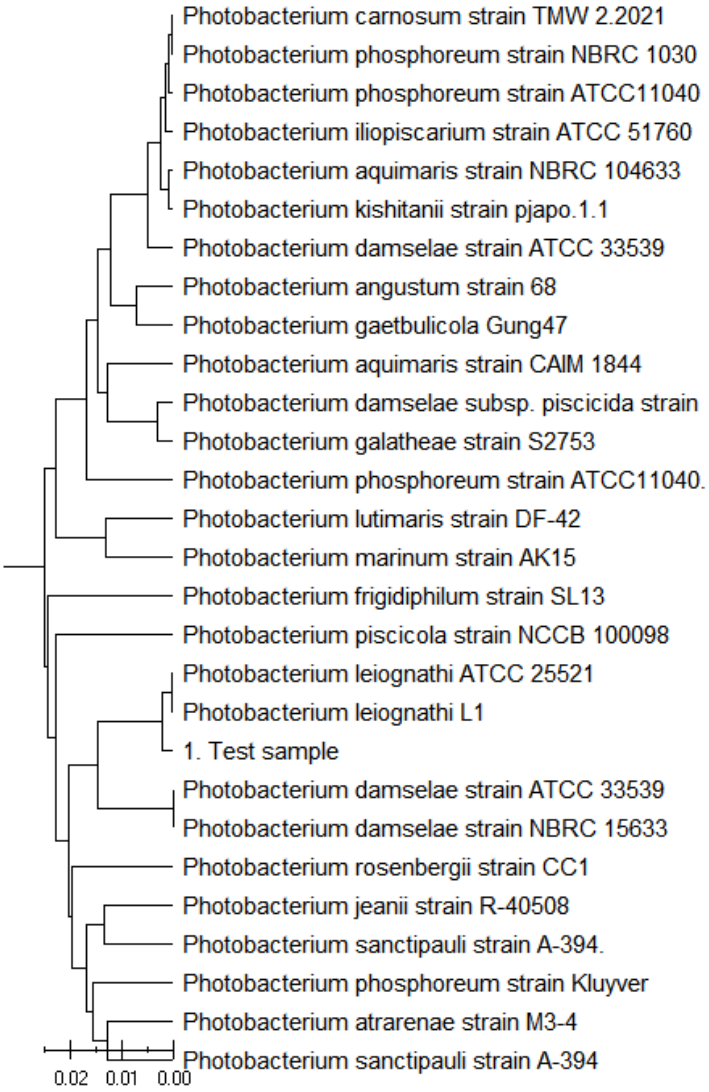

Figure 5. Phylogenetic tree of *P. leiognathi*.

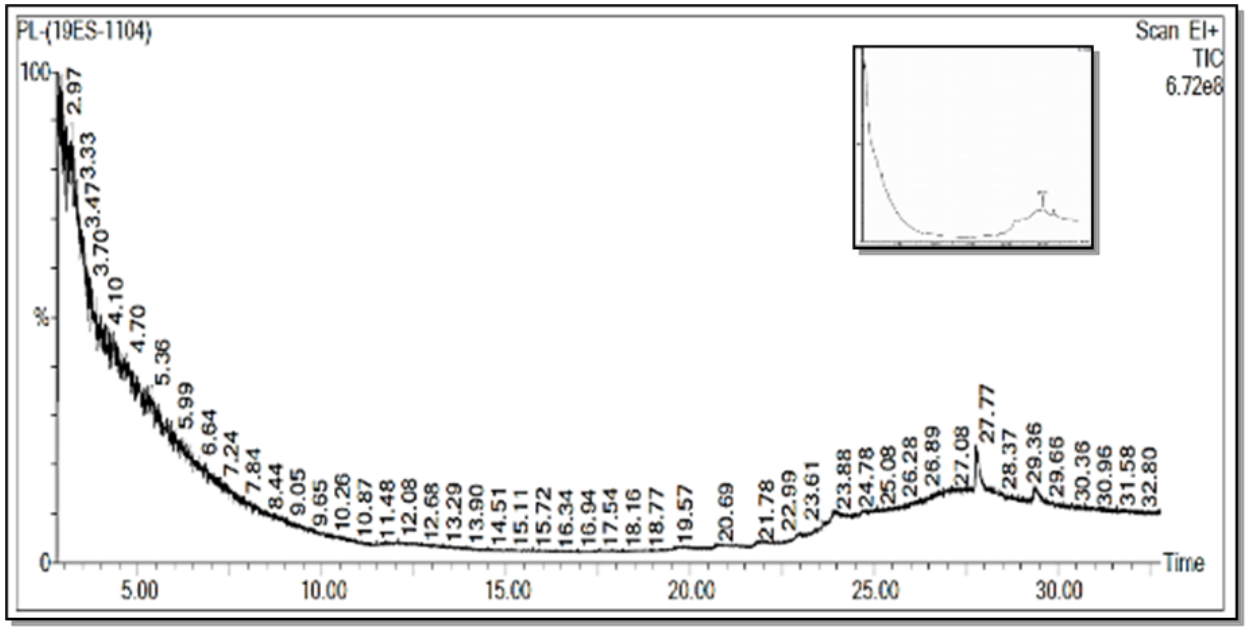

Figure 6. GCMS analysis of secondary metabolites from DCM extract.

Table 1. GCMS analysis of secondary metabolites from DCM extract.

S No.	Retention time	Molecular mass (m/z)	Compound name	Molecular formula
1.	3.083	84	Methylene Chloride	CH_2Cl_2
2.	23.967	154	Cyclopropane,1-Butyl-2-(2-Methyl Propyl)	$C_{11}H_{22}$
3.	24.782	282	Oleic Acid	$C_{18}H_{34}O_2$
4.	25.277	282	Oleic Acid	$C_{18}H_{34}O_2$
5.	26.698	145	Emylcamate	$C_7H_{15}O_2N$
6.	27.138	240	2-Propenoicn Acid, Oxybis(Methyl-2,1-Ethanediyl) Ester	$C_{15}H_{28}O_2$
7.	27.453	382	17-(1,5-Dimethyihexyl)-10,13-Dimethyl-1,7,8,9,10,11,12,13,14,15,16,17-Dodeca	$C_{27}H_{42}O$
8.	27.793	178	2chloropropionic Acid, 2,2-Dimethyl Propyl Ester	$C_8H_{15}0_2Cl$
9.	28.249	375	2,6-Lutidine 3,5-Dichloro-4-Dodecylthio-	$C_{19}H_{31}NC_{12}S$
10.	29.359	368	3-Butoxy-1,1,1,5,5,5-Hexamethyl-3-(Trimethylsicoxy)Trisicoxane	$C_{13}H_{36}O_4S_{14}$

Fourier Transform Infrared Spectroscopy

The lyophilized crude sample was analyzed an FTIR spectrometer and its result is shown the Figure 7. The FTIR spectrum obtained the spectral range of 400 to 4000 cm^{-1} for the active crude sample. Totally, 9 peaks were obtained for crude extract. Apart from 4 peaks, range peaks were also recorded. Infrared spectroscopy is a useful analytical technique for the detection of functional groups of the compound. IR spectrum corresponds to the presence of P-H phosphine at wavelength of 2362.80 cm^{-1}, while the c=c stretching frequency around 1667.78 cm^{-1} conform the alkenes groups and amides group are present in 1548.84 cm^{-1} wave number. A collection of bands in the region of 1408.04 are due to the S=O sulfate group, while the absorption band in the region 1093.64 cm^{-1} are C-F structuring functional group of Ether and alkyl halides functional group present wavelength of 557.43 cm^{-1} structure of C-Br- group. FTIR spectra of the crude extract of the marine bacterium *Pseudomonas aeruginosa* revealed major bands at 3396.01 cm^{-1}, 2928.88 cm^{-1}, 1726.24 cm^{-1}, 1510.88 cm^{-1}, 1726 cm^{-1}, and 1046.43 cm^{-1} (Nair et al. 2021).

Thin Layer Chromatography

The developed TLC plate sprayed with the derivatization agent is shown in

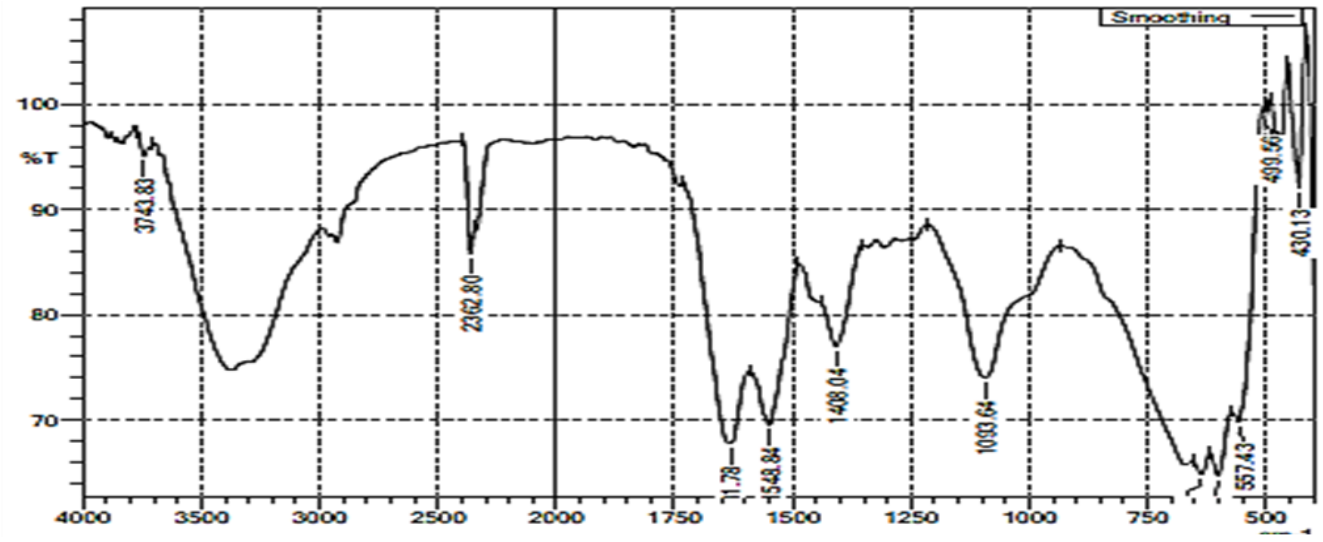

Figure 7. FTIR analysis of DCM extract of bioluminescent bacteria.

figure 8. Crude extract on the right side showed multiple bands with different RF whereas the fraction showed 3 prominent bands in the TLC plate under white light, 254 nm and 366 nm suggesting the presence of individual compounds. Further the colour developed with derivatization agent confirms the presence of functional groups including phenols, sugars, and amino acids in the fraction. TLC bioautography overlay assays were used to detect antimicrobial activity in all fractions using Staphylococcus aureus as the test microorganism, and fraction numbers 13–18 revealed a robust antimicrobial inhibition zone with an Rf value of 0.42 (Zheng et al. 2005).

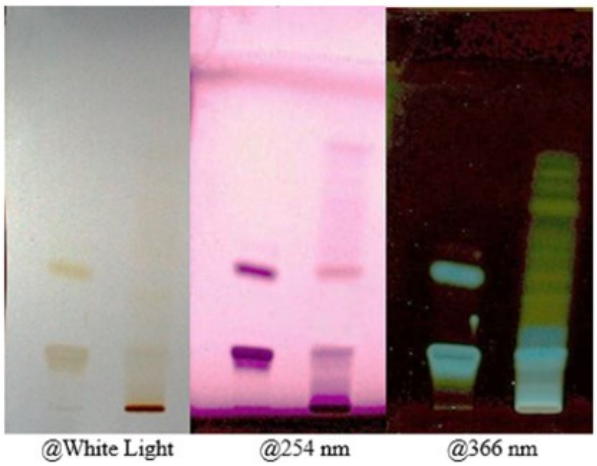

Figure 8. TLC plate analysis of secondary metabolites: A Fraction; B - Crude extract.

High Performance Liquid Chromatography

The DCM extracts were analysed through HPLC. These results showed nine peaks at different retention time and are represented in Figure 9. Among these, the maximum intensity was recorded in the second peak at 7.93 RT. The obtained peak was further purified through preparative HPLC and collected for further analysis. HPLC is generally used for the analytical estimation of various compounds. In the present study, the analysis of DCM extract was performed using reverse phase HPLC column (C-18) with the retention time up to 17.5 min at 258 nm. As a result, nine peaks were observed

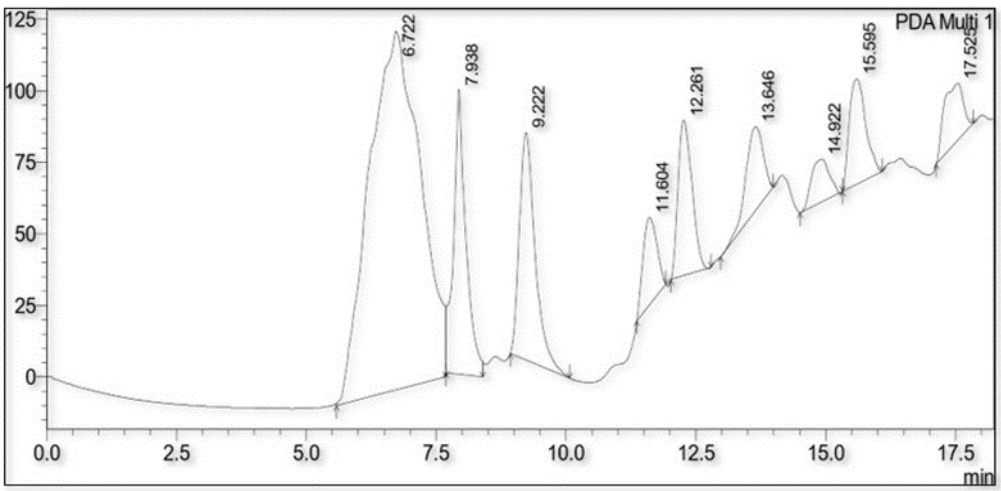

Figure 9. HPLC analysis of secondary metabolites from DCM extract.

with the retention time of 6.72, 7.93, 9.22, 11.60, 12.26, 13.64, 14.92, 15.59 and 17.52. The highest intensity (6.72RT) was observed and further bioactivity evaluations were performed similarly. In the HPLC-PDA, three peaks with maximum absorption wavelengths of 246.5, 281.9, and 337.7 nm were identified in marine bacteria (Zheng et al. 2005).

Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis

The crude extract of secondary metabolites of *P. leiognathi* was analysed in 10% SDS –PAGE. After electrophoresis, the banding pattern was observed as shown in Figure 10. As a result, nine prominent bands were observed and the same was compared to protein marker with a molecular range between 10 to 203 kDa. Several prominent bands are observed in the molecular range of the crude extract between 10 to 110 kDa. Bacteriocins are proteins (>10 kDa) or short peptides (>10 kDa) that differ greatly from antibiotics (secondary metabolites) in their mode of action and chemical structure (Dobson et al. 2012). Crude toxins in the aqueous extract of *Halichondria panicea* produced nine bands on SDS-PAGE on a 12 percent gel, ranging from 14.3 to 116 kDa, with three well-defined bands at 19.5, 39.0, and 66.2 kDa (Purushottama et al. 2009). To our knowledge, the current study holds the novelty of the first ever report regarding the protein band pattern of DCM extract produced by bioluminescent bacteria, *P. leiognathi*.

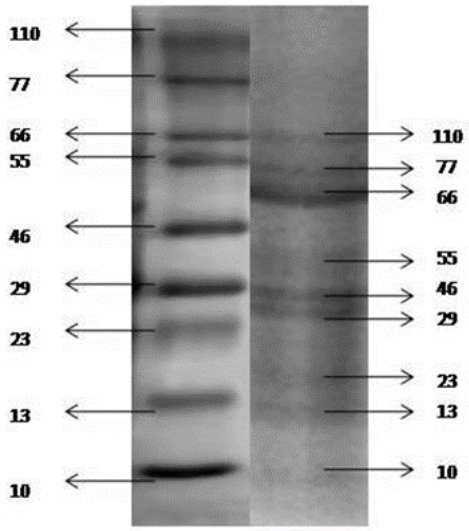

Figure 10. SDS - PAGE analysis of metabolites – 1: Protein Marker, 2: Isolated Proteins

Biological Activity

Antibacterial Assay

The antibacterial activity showed that the crude extract and the fraction exhibited significant antibacterial activity against all ten pathogenic bacteria, as shown in Figure 11 and Table 2. The antibacterial activity was noted for the sample in comparison with the standard antibacterial drug Amoxicillin. The inhibition zone was observed against all pathogenic bacteria in the range of 12 mm to 20 mm for both the crude sample and fraction at a concentration

of 30 µl. However, the maximum inhibition zone of 20±0.32 mm was observed against *Shigella* sp. by crude and 15±0.22 mm by a fraction. Secondary metabolite of the bacterium *P. leiognathi* was extracted using DCM to study its bioactive potential against human pathogens and its antioxidant capacity. The DCM extract showed a broad spectrum of antibacterial activity against the six human bacterial pathogens. The sample at a concentration of 30 µg/ml presented a maximum zone of inhibition against *Shigella* sp. When compared to the standard drug amoxicillin and minimum inhibition towards *P. mirabilis*., Yalla et al. (2018) reported that the ethyl acetate extract (50 mg/ml) of *V. furnissi* showed wide spectrum activity against *S. sonnei* (10.6 mm). The micronutrient-rich medium for the growth of bacteria determines the nature of metabolite produced by them, and thus, the bioactivity varies accordingly (Armstrong et al. 2001; Kelecom 2002). Different antimicrobial compounds produced by *Photobacterium* sp. have been reported. Compounds such as Unarmicins A and C, Holomycin, Ngercheumicin A, B, C, D and E are produced by *Photobacterium* and showed antibacterial and antifungal activities. Antibacterial compounds such as phenol, 2,4-bis (1,1-dimethylethyl) -, Indolizine and 1,2-benzenedicarboxylic acid, butyl octyl ester are produced by *Photobacterium* (Ramesh & Mohanraju 2017). However, these compounds have not been detected in the current study; antibacterial activity was still significant against clinical pathogens.

Table 2. Antibacterial activity of bioluminescent bacteria.

Bacterial pathogen	Inhibition zone in mm		
	Crude (30 µl)	Fraction (30 µl)	Standard (30 µl))
Bacillus subtilis	12±0.32	12±0.36	17±0.18
Salmonella paratyphi-A	12±0.18	12±0.49	14±0.22
Salmonella paratyphi-B	12±0.12	12±0.22	15±0.29
Proteus mirabilis	11±0.49	11±0.31	12±0.11
Proteus vulgaris	12±0.38	13±0.29	13±0.31
Klebsiella pneumoniae	15±0.15	12±0.13	25±0.25
Shigella	20±0.32	15±0.22	30±0.19
Pseudomonas	7±0.37	7±0.24	7±0.21
Staphylococcus aureus	12±0.23	12±0.49	10±0.16
Escherichia coli	17±0.31	10±0.22	20±0.19

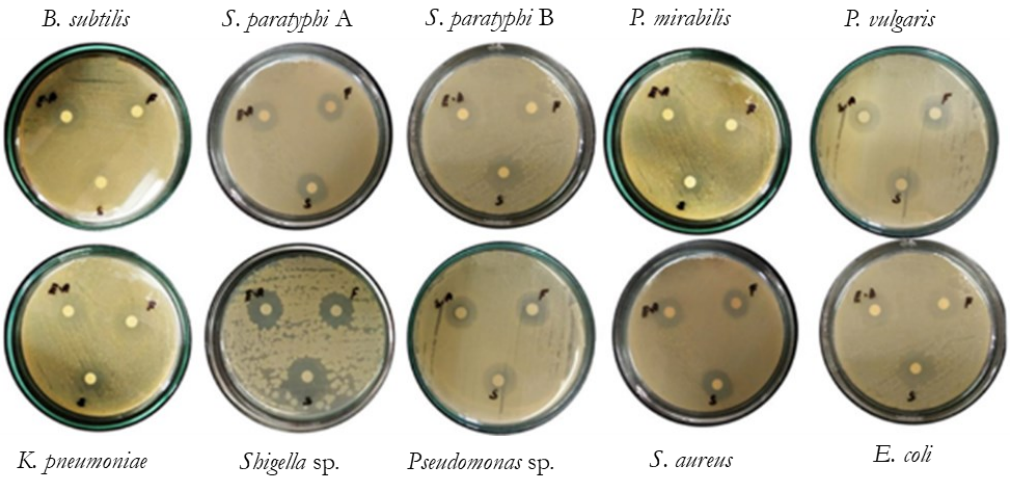

Figure 11. Antimicrobial susceptibility assay for extracts of *P. leiognathi*

Antioxidant Assay

The antioxidant potential of the crude sample was examined using DPPH, HRSA, H2O2, and Metal chelating activity (Figure 12). The results of the antioxidant activity are reported in Table 3. Total antioxidant capacity using the phosphomolybdenum technique is shown in Table 4 Ascorbic acid was used as the standard. The DPPH scavenging activity results showed 98.74±0.18% inhibition for 100 µl of standard and the crude extract showed 86.21±0.29% at 300 µl of concentration. The difference between standard extract and the crude extract is 12.53% which reveals significant DPPH scavenging activity. The hydroxyl radical scavenging activity results showed 98.26±0.21% inhibition for 100 µl of standard and the crude extract showed 83.11±0.31% at 300 µl of concentration. The difference between the standard and the crude extract is 15.55% which reveals significant hydroxyl radical scavenging activity. The results of the hydrogen peroxide scavenging activity showed 89.91±0.19% inhibition for 100 µl of the standard at a concentration of 100 µl and the crude extract showed 81.68±0.34% at 300 µl of concentration. The difference between the standard and the crude extract is 8.23%, which reveals significant hydrogen peroxide scavenging activity. Metal chelating activity results showed 99.12±0.15% inhibition for 100 µl of standard and the crude extract showed 98.45±0.35% at 300 µl of concentration. The difference between the standard and the crude extract is 0.67%, which reveals significant metal chelating activity. The total antioxidant capacity was carried out using the phosphomolybdenum method where a standard ascorbic acid graph was plotted. When 100 µg/ml of the crude extract was evaluated for phosphomolybdenum activity, the optical density when compared to the

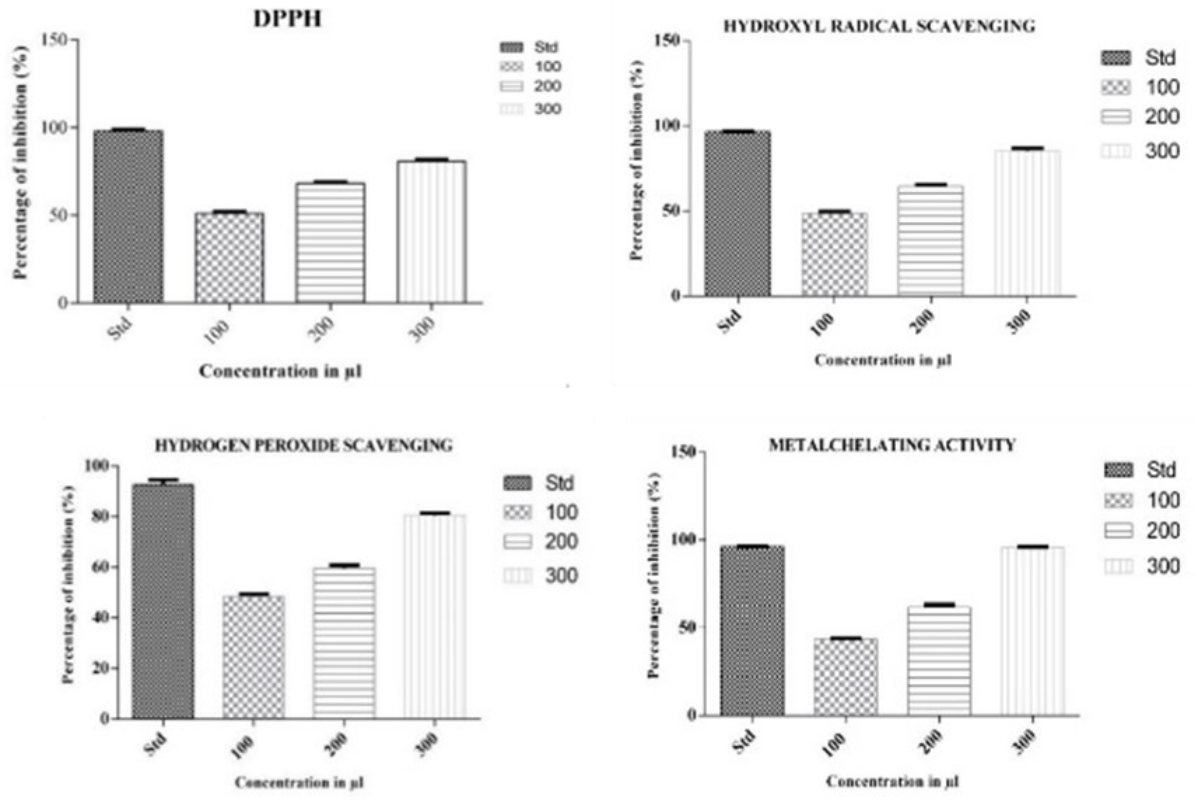

Figure 12. Antioxidant activity of Secondary Metabolites of DCM extracts of bioluminescent bacteria *Photobacterium leiognathi.*

Table 3. Percentage Inhibition of the Crude Extract.

Concentration	Percentage of Inhibition			
	DPPH Scavenging Activity	Hydroxyl Radical Scavenging Activity	Hydrogen Peroxide Scavenging Activity	Metal Chelating Activity
Ascorbic Acid (Standard) – 100µl	98.74±0.18%	98.26±0.21%	89.91±0.19%	99.12±0.15%
Extract – 100µl	40.71±0.34%	39.54±0.48%	49.27±0.28%	47.84±0.22%
Extract – 200µl	68.38±0.41%	62.17±0.32%	59.28±0.22%	53.47±0.18%
Extract – 300µl	86.21±0.29%	83.11±0.31%	81.68±0.34%	98.45±0.35%

standard graph shows that the total antioxidant capacity of the secondary DCM crude extract of the bacterial metabolite to be 50.11 µg/ml. Therefore, out of 100 µg/ml crude extract, 50.11 µg/ml concentration consists of antioxidants revealing that 50% of the extract has antioxidants. The oxidation reaction can produce free radicals which can set off chain reactions that damage cells. Antioxidants stop these chain events by neutralizing or stabilising free radicals. The number of active groups has a favourable relationship with antioxidant activity (OH or NH2) (Chandra et al. 2020). The study has clearly proved that the secondary metabolites identified from the bioluminescent bacteria *P. leiognathi* by GCMS and FTIR have excellent antioxidant properties. The results of the DPPH scavenging activity were supported by (Kumagai et al. 2018). In this activity, crude sample reacts with DPPH and reduces the free radicals of the hydroxyl group (Matthäus 2002). As the activity of DPPH showed a minimum of 40.71±0.34% activity and a maximum of 86.21±0.29% when compared to standard ascorbic acid, the ability to reduce Fe^{3+} ions may be due to the active compound present in the solvent extract (Kekuda et al. 2010). Various disorders related to oxidative stress can be prevented by determining hydrogen peroxide activity (Poongodi et al. 2012). ROS like hydroxyl free radical is produced when hydrogenperoxide reacts with metal ions (Fe2C and/or Cu2C which leads to a toxic effect; in addition, hydrogen peroxide can cross the cell membrane easily (Floyd & Lewis 1983). The reducing capacity of a compound may help to improve cell signaling. The sample serves as a potential antioxidant activity because it has increased reducing power. Although the standard ascorbic acid has a high reducing power compared to that of the extract, the antioxidative property presence is significant for a crude extract without any purification, thus, the extract has the potential to provide electron donors for radical chain reactions.

Table 4. Phosphomolybdenum activity.

Total Antioxidant Capacity - Phosphomolybdenum Method	
Concentration	**Optical Density at 680nm**
Ascorbic Acid – 10 µg/ml	0.067
Ascorbic Acid – 50 µg/ml	0.101
Ascorbic Acid – 100 µg/ml	0.116
Ascorbic Acid – 250 µg/ml	0.195
Ascorbic Acid – 500 µg/ml	0.385
Crude Extract – 100 µg/ml	0.092
Total Antioxidant Capacity – 50.11 µg/ml	

CONCLUSION

Bioluminescence is an area of surging research especially due to its ties with the ocean or the marine environment as many bacteria and other organisms either in their free-state or symbiotic relationship express bioluminescence which can be used for the human welfare development. To apply bioluminescence for industrial usage, isolation of bioluminescence bacteria must be carried out from different sources based on the stable light intensities. The current study is the first successful attempt to isolate *P. leiognathi* from *S. ruconius* and their morphological, biochemical, and molecular identification was documented. Then, crude secondary metabolites were extracted using dichloromethane, and their compositional and functional group analysis were done using GC-MS and FTIR. Cyclopropane, 1-Butyl-2-(2-Methyl Propyl), Oleic acid, Emylcamate, 2-Propenoicn acid, Oxybis(Methyl-2,1-Ethanediyl) ester etc., were the functional group identified by GCMS. The FTIR spectrum obtained the spectral range of 400 to 4000 cm^{-1} for the active crude sample. Totally, 9 peaks were obtained for crude extract. The extracts were also characterized using chromatographic techniques, including TLC and HPLC, finally, protein analysis was reported using SDS-PAGE. TLC confirms the presence of functional groups including phenols, sugars, and amino acids in the fraction. In HPLC, nine peaks were observed with a retention time of 6.72, 7.93, 9.22, 11.60, 12.26, 13.64, 14.92, 15.59 and 17.52. The metabolites were then profiled for their antibacterial activity against ten different clinical bacterial pathogens and showed excellent activity. Another important bioactivity that was extensively carried out was the antioxidant activity and total antioxidant capacity of the secondary metabolites. One of the most important findings is that the secondary metabolites showed excellent antioxidant activity when studied using different assays and the analysis of the total antioxidant capacity revealed that 50% of the extract contains rich antioxidants. These results will be instrumental in developing novel products with biosensor and bio imaging applications using *P. leiognathi*.

AUTHORS CONTRIBUTION

S.T. performed the experiments, wrote, analyzed and interpreted the data. U.S. and S.R. analyzed the data, K.S. discussed the article. A.M. designed, supervised physicochemical experiments, and discussed the article.

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CONFLICT OF INTEREST

The authors report that there is no conflict of interest.

REFERENCES

Al-Massarani, S.M. et al., 2017. Isolation, biological evaluation, and validated HPTLC quantification of the marker constituent of the edible Saudi plant Sisymbrium irio L. *Saudi Pharmaceutical Journal*, 25(5), pp.750–759. doi: 10.1016/j.jsps.2016.10.012

Armstrong, E. et al., 2001. The symbiotic role of marine microbes on living surfaces. *Hydrobiologia*, 461(1), pp.37–40. doi: 10.1023/A:1012756913566

Bakaraki, N. et al., 2016. Development of a sensitive liquid--liquid extraction method for the determination of N-butyryl-l-homoserine lactone produced in a submerged membrane bioreactor by gas chromatography mass spectrometry and deuterated anthracene as the internal standard. *Analytical Methods*, 8(12), pp.2660–2665. doi: 10.1039/C6AY00317F

Bauer, A.W., 1966. Antibiotic susceptibility testing using a standardized single disc method. *Am J clin pathol*, 45, pp.149–158. doi: 10.1128/am.13.2.279-280.1965.

Chandra, P., Sharma, R.K. & Arora, D.S., 2020. Antioxidant compounds from microbial sources: A review. *Food Research International*, 129, p.108849. doi: 10.1016/j.foodres.2019.108849

Dobson, A. et al., 2012. Bacteriocin production: a probiotic trait? *Applied and environmental microbiology*, 78(1), pp.1–6. doi: 10.1128/AEM.05576-11

Engebrecht, J., Nealson, K. & Silverman, M., 1983. Bacterial bioluminescence: isolation and genetic analysis of functions from Vibrio fischeri. *Cell*, 32(3), pp.773–781. doi: 10.1016/0092-8674(83)90063-6

Farrelly, V., Rainey, F.A. & Stackebrandt, E., 1995. Effect of genome size and rrn gene copy number on PCR amplification of 16S rRNA genes from a mixture of bacterial species. *Applied and environmental microbiology*, 61(7), pp.2798–2801. doi: 10.1128/aem.61.7.2798-2801.1995

Firudoz, B. et al., 2020. Molecular variation and phylogenetic status of ponyfish (Perciformes: Leiognathidae) in Karaikal, South India. *Notulae Scientia Biologicae*, 12(2), pp.251–257. doi: 10.15835/nsb12210661

Floyd, R.A. & Lewis, C.A., 1983. Hydroxyl free radical formation from hydrogen peroxide by ferrous iron-nucleotide complexes. *Biochemistry*, 22 (11), pp.2645–2649. doi: 10.1021/bi00280a008

Gromek, S.M. et al., 2016. Leisingera sp. JC1, a bacterial isolate from Hawaiian bobtail squid eggs, produces indigoidine and differentially inhibits vibrios. *Frontiers in microbiology*, 7, p.1342. doi: 10.3389/fmicb.2016.0134

Gutteridge, J.M. & Halliwell, B., 1988. The deoxyribose assay: an assay both for'free'hydroxyl radical and for site-specific hydroxyl radical production. *Biochemical Journal*, 253(3), p.932. doi: 10.1042/bj2530932

Haddock, S.H.D., Moline, M.A. & Case, J.F., 2010. Bioluminescence in the sea. *Annual review of marine science*, 2, pp.443–493. doi: https://doi.org/10.1146/annurev-marine-120308-081028

He, F., 2011. Laemmli-sds-page. *Bio-protocol*, e80. doi: 10.21769/BioProtoc.80

Kannahi, M. & Sivasankari, S., 2014. Isolation and identification of bioluminescent bacteria from marine water at Nagapattinam sea shore area. *Int. J. Pharm. Sci. Rev. Res*, 26(2), pp.346–351.

Kekuda, P.T.R. et al., 2010. Studies on antioxidant and anthelmintic activity of two Streptomyces species isolated from Western Ghat soils of Agumbe, Karnataka. *Journal of Pharmacy Research*, 3(1), pp.26–29.

Kelecom, A., 2002. Secondary metabolites from marine microorganisms. *Anais da Academia Brasileira de Ciências*, 74(1), pp.151–170. doi: 10.1590/S0001-37652002000100012.

Kim, S.U. et al., 2018. Adenosine triphosphate bioluminescence-based bacteria detection using targeted photothermal lysis by gold nanorods. *Analytical chemistry*, 90(17), pp.10171–10178. doi: 10.1021/acs.analchem.8b00254. Epub 2018 Aug 20.

Klöppel, A. et al., 2008. HPTLC coupled with bioluminescence and mass spectrometry for bioactivity-based analysis of secondary metabolites in marine sponges. *JPC-Journal of Planar Chromatography-Modern TLC*, 21(6), pp.431–436. doi: 10.1556/jpc.21.2008.6.7

Kumagai, M. et al., 2018. Antioxidants from the brown alga Dictyopteris undulata. *Molecules*, 23(5), p.1214. doi: 10.3390/molecules23051214

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227, pp.680-685. https://doi.org/10.1038/227680a0

Matthäus, B., 2002. Antioxidant activity of extracts obtained from residues of different oilseeds. *Journal of Agricultural and Food Chemistry*, 50(12), pp.3444–3452. doi: 10.1021/jf011440s

Menz, J., Schneider, M. & Kümmerer, K., 2013. Toxicity testing with luminescent bacteria--characterization of an automated method for the combined assessment of acute and chronic effects. *Chemosphere*, 93(6), pp.990–996. doi: 10.1016/j.chemosphere.2013.05.067

Molina, A.J., Abisado, R.G. & Calugay, R.J., 2016. Bioluminescent Vibrio spp. with antibacterial activity against the nosocomial pathogens Staphylococcus aureus and Klebsiella pneumoniae. *AACL Bioflux*, 9(2), pp.185-194.

Morin-Crini, N. et al., 2019. Applications of chitosan in food, pharmaceuticals, medicine, cosmetics, agriculture, textiles, pulp and paper, biotechnology, and environmental chemistry. *Environmental Chemistry Letters*, 17 (4), pp.1667–1692. doi: 10.1007/s10311-019-00904-x

Nair, A. V, Vijayan, K.K. & others, 2021. Antibacterial assay guided isolation of a novel hydroxy-substituted pentacyclo ketonic compound from Pseudomonas aeruginosa MBTDCMFRI Ps04. *Brazilian Journal of Microbiology*, 52(1), pp.335–347. doi: 10.1007/s11033-021-06146-x

Nogi, Y., Masui, N. & Kato, C., 1998. Photobacterium profundum sp. nov., a new, moderately barophilic bacterial species isolated from a deep-sea sediment. *Extremophiles*, 2(1), pp.1–8. doi: 10.1007/s007920050036

Nunes-Halldorson, V. da S. & Duran, N.L., 2003. Bioluminescent bacteria: lux genes as environmental biosensors. *Brazilian journal of Microbiology*, 34, pp.91–96. doi: 10.1590/S1517-83822003000200001

Poongodi, S. et al., 2012. Marine actinobacteria of the coral reef environment of the gulf of mannar biosphere reserve, India: a search for antioxidant property. *Int. J. Pharm. Pharm. Sci*, 4, pp.316–321.

Purushottama, G.B. et al., 2009. Bioactivities of extracts from the marine sponge Halichondria panicea. *Journal of Venomous Animals and Toxins including Tropical Diseases*, 15(3), pp.444–459. doi: 10.1590/S1678-91992009000300007

Ramesh, C.H. & Mohanraju, R., 2017. Antibacterial activity of marine bioluminescent bacteria. Indian Journal of Geo Marine Sciences. Vol. 46 (10), pp. 2063-2074

Reysenbach, A.-L. & Pace, N.R., 1995. Reliable amplification of hyperthermophilic archaeal 16S rRNA genes by the polymerase chain reaction. *Archaea: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY*, pp.101–107. doi: 10.1023/A:1015122926687

Ruch, R.J., Cheng, S. & Klaunig, J.E., 1989. Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from Chinese green tea. *Carcinogenesis*, 10(6), pp.1003–1008. doi: 10.1093/carcin/10.6.1003

Saeed, N., Khan, M.R. & Shabbir, M., 2012. Antioxidant activity, total phenolic and total flavonoid contents of whole plant extracts Torilis leptophylla L. *BMC Complementary and alternative medicine*, 12(1), pp.1–12. doi: 10.1186/1472-6882-12-221

Sarkar, M.K.D., Ahmmed, T. & others, 2019. Antibiotic resistance analysis of Vibrio spp. isolated from different types of water sources of Bangladesh and their characterization. *Eur J Med Health Sci*, 1, pp.19–29. doi: 10.34104/ejmhs.01929

Sharifian, S. et al., 2018. The emerging use of bioluminescence in medical research. *Biomedicine \& Pharmacotherapy*, 101, pp.74–86. doi: 10.1016/j.biopha.2018.02.065

Singh, R. & Chahal, K.K., 2018. Phytochemical analysis and in vitro antioxidant capacity of different solvent extracts of Saussurea lappa L. roots. *J Pharma Phytochem*, 7(3), pp.427–432. doi: 10.1016/j.sjbs.2022.01.040

Soler-Rivas, C., Esp\'\in, J.C. & Wichers, H.J., 2000. Oleuropein and related compounds. *Journal of the Science of Food and Agriculture*, 80(7), pp.1013–1023. doi: 10.1002/(SICI)1097-0010(20000515)80:7<1013::AID-JSFA571>3.0.CO;2-C

These, A., Scholz, J. & Preiss-Weigert, A., 2009. Sensitive method for the determination of lipophilic marine biotoxins in extracts of mussels and processed shellfish by high-performance liquid chromatography--tandem mass spectrometry based on enrichment by solid-phase extraction. *Journal of Chromatography A*, 1216(21), pp.4529–4538. doi: 10.1016/j.chroma.2009.03.062. Epub 2009 Mar 27.

Yalla, S.K., Cherian, T. & Mohanraju, R., 2018. Antimicrobial potential of secondary metabolites extracted from Vibrio furnissii, a luminescent bacterium associated with squid, Uroteuthis duvauceli. *Int. J. of Pharm. and Biol. Sci*, 8(1), pp.530–534.

Yen, G.-C. & Chen, H.-Y., 1995. Antioxidant activity of various tea extracts in relation to their antimutagenicity. *Journal of agricultural and food chemistry*, 43(1), pp.27–32. doi: 10.1021/jf00049a007

Zheng, L. et al., 2005. Antimicrobial screening and active compound isolation from marine bacterium NJ6-3-1 associated with the sponge Hymeniacidon perleve. *World Journal of Microbiology and Biotechnology*, 21(2), pp.201–206. doi: 10.1007/s11274-004-3318-6

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Research Article

In Vitro Culture of *Phalaenopsis amabilis* (L.) Blume Orchid for Seedling Production with Banana Extract Supplementation and Light Treatment for *Ex Situ* Conservation

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ABSTRACT
In vitro culture is one of the effective cultivation methods for seedling production of orchids that can be used as a powerful tool in the conversation of orchids. The aims of the present study were to (i) investigate the effects of the addition of banana extract (0 g/l, 100 g/l, and 150 g/l) on media (NP media), in two different light regimes (light and dark conditions) on the growth of plantlets of an epiphytic orchid *Phalaenopsis amabilis* in *in vitro* culture. Methods used included (i) subculturing orchid seedlings in treatments media, (ii) measuring leaves and roots chlorophyll content and growth parameters, (iii) anatomical preparation of leaves and roots of the seedlings. The results showed that the best condition for getting greater seedlings of *P. amabilis* plantlets is in media with an addition of 100 g/L banana extract in light condition. The highest amount of chlorophyll in the *P. amabilis* leaves was found in medium with the addition of 100 g/L banana extract medium in light conditions. The thickness of mesophyll and the largest root diameter of *P. amabilis* seedlings were also found in media with the addition of 100 g/L banana extract medium in light condition. In conclusion, the addition of 100gr/L banana extract into basic culture medium will be beneficial for seedlings production of *P. amabilis* with great appearance, for *ex situ* orchid conservation programs.

INTRODUCTION
One of the orchid genera that has high commercial value and is widely traded is the *Phalaenopsis* genus. *Phalaenopsis amabilis* orchid is a popular plant (Theng & Korpenwar 2014) and is also used as a mother plant to generate superior hybrids of *Phalaenopsis* orchids (Semiarti et al. 2007). Overcollection of these orchids from their natural habitat in the forest for cultivation at home or nurseries and for commercialisation (Setiari et al. 2018) causes the population to decrease and even become endangered (Rukmana 2000). Therefore, propagation of orchids to generate many seedlings is required to fulfil marker demand on the *Phalaenopsis* and to reduce overcollection from their natural habitats. *In vitro* is one of cultivation methods for facilitating mass propagation of orchids to produce numerous seedlings as well as one of the programs of *ex-*

situ conservations of orchids (Semiarti et al. 2010).

The nutrient composition in medium is one of the crucial and essential factors in the *in vitro* growth period of orchid seedlings. Several studies reported that the addition of some organic substances in medium culture, such as fruit extract can enhance plantlet growth. The fruit extracts in culture medium contain many nutrients providing hormones and plant growth regulator useful to increase plant growth (Souza et al. 2013). The addition of banana extract (50 g/L) in culture medium has been shown to promote protocorm-like bodies (PLBs) viability (Yulianti et al. 2016)

Musa acuminata x *Musa balbisiana* is one of the Indonesian banana cultivars called "Pisang Raja" that Indonesian people widely consume. Djajanegara (2010) showed that the insertion of 100 g/L banana extract of "Pisang Raja" into medium culture affects the percentage of shoots number, plantlet height, leaves number, and roots number on *P. amabilis* orchids.

Other studies also reported that the addition of 100 g/L banana extract into medium culture affects the percentage of shoots number, plantlet height, leaves number, and roots number on *P. amabilis* orchids (Djajanegara 2010). Furthermore, the addition of 150 g/L banana extract into culture medium can increase the number of roots and growth of *Dendrobium lasianthera* (Utami et. al. 2016). Banana is known to contain high nutrients including sugar, phosphorus, thiamin which can accelerate cell division in root meristems (Pazil 2009; Sallolo et al. 2012; Hapsari & Lestari 2016).

There are some factors affecting plantlet growth in *in vitro* culture including culture media (Qomariyah & Dewanti 2019) and light conditions. Currently, there are no studies of plantlet growth of *P. amabilis* in relation to the addition of banana extract into culture media and light conditions. The present study aimed to investigate the effects of (i) the addition of banana extracts into culture media (ii) light conditions on the growth of *P. amabilis* plantlets. Furthermore, morphological, physiological, and anatomical characteristics of the plantlets were also investigated. This study is a part of programs to support *ex situ* conservation of orchids.

MATERIALS AND METHODS

Plant Materials, Medium Preparation and Culture Conditions

Plant materials used in this study were 18 months-old *P. amabilis* plantlets with ≥ 2 leaves without root generated from seed germination in *in vitro* culture. The plantlets were cultivated in basic medium, New Phalaenopsis (NP) medium with the addition of banana extracts (0 g/l: 100 g/l and 150 g/l) (Arditti 2008), under two light regimes (light and dark conditions) at 25° C. The plantlets were planted in culture bottle with 5 plantlets in each bottle as replication. Every treatment has 2 bottle culture with 10 plantlets in total as replication. Plantlets were sub-cultured every two weeks. The measurements of plantlet growth were carried out by photographing the plantlets using a Canon 800D DSLR *camera* and processed using an Image Raster 3.0.

The Banana extract was made by weighing the peeled banana fruit us-

ing a digital scale, 100 g and 150 g, respectively. The bananas were mashed using a blender with 100 ml of water for 100 g peeled banana and 150 ml of water for 150 g peeled banana. The homogenate obtained was filtered twice, then added to the NP basic medium and homogenized.

Measurement of Chlorophyll Content in Leaves and Roots of *P. amabilis*

The methods used for measuring chlorophyll content are based on Harborne (1998) with modification. Leaves sample was weighed at 0.03 g and then put into a microtube and crushed with a micro-pestle. The crushed leaves were dissolved in 1.5 ml of 80% acetone. After that, the sample was vortexed for a few moments for homogenization. The homogeneous sample was centrifuged at 8000 rpm for 15 minutes. The supernatant was taken to measure the content of chlorophyll a and b and it was performed by determining the absorbance value using spectrophotometer with wavelength of 646 nm and 663 nm.

Anatomical Preparation

The anatomical preparation were prepared by free-hand sectioning method based on Berlyn and Miksche (1976). The samples of *P. amabilis* leaves and roots were taken after eight weeks of treatment with banana extract and light the samples were sliced transversely as thin as possible using a razor blade. The incision of the sample is placed into the water for a while. Subsequently, the sample is placed in an object glass and dripped with water and then covered with cover glass to be observed under a microscope (Nikon, Japan) and optilab (Miconos, Indonesia).

Data Analysis

Quantitative data for plant morphological and physiological analysis were carried out by measuring plant height, leaves length and number, root length and number; and chlorophyll contents. The quantitative data for anatomy were collected by measuring root diameter and mesophyll thickness. The quantitative data were analyzed using SPSS (Statistical Package for the Social Sciences) v.23 which includes analysis of variance (ANOVA). Significant variance between treatments were subsequently tested with Duncan's test or Duncan's Multiple Range Test (DMRT).

RESULTS AND DISCUSSION

The Growth Response of Plantlets

Results showed that treatments of lighting condition and the addition of banana extract in basic medium affect the growth of *P. amabilis* plantlets after eight weeks of subculturing. Leaf length, root length and plant height of *P. amabilis* plantlets in the treatment of the addition of 100 g/L banana extract in NP medium in the dark showed the highest values compared to other treatments (Table 1). Futhermore, leaf number was not significantly different

Table 1. The growth of *P. amabilis* plantlets under treatment of medium and light conditions for 8 weeks culture.

Treatments / Parameters		Average of Leaves Number	Length of Leaves (µm)	Average of Roots Number	Length of Roots (µm)	Plant Height (µm)
Light Treatments (1224 lux)	NP 0	3.9±1.101[a]	6.46±1.821[b]	1.2±0.422[a]	3.24±1.321[a]	16.02±5.096[bc]
	NP 100	3.5±0.849$_a$	6.74±1.296[b]	1.3±0.483[a]	2.93±1.165[a]	16.49±2.963[bc]
	NP 150	3.3±0.823[a]	4.08±0.618[a]	1.3±0.483[a]	2.87±0.769[a]	10.93±1.498[ab]
Dark Treatments (3 lux)	NP 0	3.5±0.971[a]	6.73±1.484[b]	2.1±1.449[b]	6.55±3.892[b]	16.97±2.691[c]
	NP100	3.5±0.849[a]	8.53±2.955[c]	1.2±0.422[a]	6.64±1.830[b]	18.45±4.456[c]
	NP150	3.6±0.966[a]	4.46±1.007[a]	1.0±0.000[a]	3.87±1.948[a]	13.43±4.153[ab]

Note: Data in the same column followed by the same letters are not significantly different by Duncan's test at $p \leq 0.05$. Details: NP 0: control medium; NP 100: NP+100 g/l banana extract; NP 150: NP+150 g/l banana extract

between *P. amabilis seedlings in all treatments*. This might be influenced by internal factors such as genotype and plantlet physiological conditions (Haris & Mercuriani 2018).

Leaf length and plant height of *P. amabilis* plantlet were highest in the treatment of the addition of 100 g/l banana extract (Table 1). This might be due to the auxins and cytokinin contained in banana extract that commonly has effects to promote plant growth, where auxin plays a key role in cell elongation while cytokinin regulates plant cell proliferation and differentiation (Armarego-Marriott et al. 2020). Hasanah et al (2014) reported that banana extract contains 0.00035% IAA (auxin) and 0.00020% cytokinin. Furthermore, the action of auxin and cytokinin was influenced by light, where both hormones were degraded when exposed to light (Manzur et al. 2014). This might explain the higher values of plant height and leaf length in medium NP+100 g/l banana extract in the dark than in light conditions.

Root length of seedlings in medium NP+100 g/l banana extract was also higher in the dark than in the light condition (Table 1), as in light conditions auxin activity decreased as the auxin is degraded in light conditions. However, dark conditions can cause etiolation, and the etiolation process causes the plantlets to have pale green colour leaves leading to skotomorphogenic phenotype (Armarego-Marriott et al. 2020).

Apart from auxin and cytokinin, banana extract also contains thiamine which can stimulate cell division in root meristem to grow faster which affects the length of the roots (Sallolo et al. 2012). Furthermore, potassium (K) plays an important role for root growth by affecting N distribution and controlling the photosynthesis rate by carbohydrate translocation (Xu et al. 2020). The combination between auxin, thiamine, and potassium contained in banana extract that have promoting effects can lead to the increase of plant growth, especially in the dark. In the present study, leaves length, plant height and roots length were the highest in medium NP+100 g/L banana extract in dark conditions. Results of the present study were similar to the results of study by Djajanegara (2010).

The present study also showed that the best medium for root growth is NP 0 basic medium (without the addition of banana extract) (Table 1). The largest number of roots on medium without the addition of banana extract

indicates that the amount of endogenous auxin that is essential for the formation adventitious roots and elongation of root cells of the seedlings, is sufficient. Therefore, the addition of some growth regulator such as auxin contained in banana extract did not have significant effects (Arimarsetiowati & Ardiyani 2012). This is also relevant with the theory stated by Moore (1989) that the use of plant hormones generally must be in accordance with their needs. Adding exogenous hormones that exceed the critical level will interfere with plant metabolism or have no significant effect (Moore 1989).

The present study also showed that the addition of 150 g/l banana extract into medium culture did not significantly affect the growth of *P. amabilis* plantlets, both in dark or light conditions. This might be due to the PGR contained in 150 g/l banana extract exceeded the optimal levels for plantlets growth of *P. amabilis*. In contrast, Utami et al. (2016) reported the addition 0f 150 g/L banana extract of same cultivars improve the number of roots in other orchid species, *Dendrobium lasianthera*. This indicates that the concentration of banana extract gives a different response on different species of orchids that might be related to the amount of endogenous auxin of each species. Each plant requires different levels of PGR and gives a different effect related to the amount of endogenous auxin of each species (Moore 1989).

Although, the growth of the plantlets showed the highest value in the dark conditions, as seen in Figure 1, this condition was not recommended for continuous treatment. The dark condition caused plantlets to have pale green leaves compared to the light treatment, as seen in Figure 1. It proves that the leaves contain lack of chlorophyll and are in line with Figure 2A. Dark conditions are usually applied to stimulate plantlet's root growth (Monteuuis & Bon 2000). When the root starts to appear, culture is placed in normal condition (Monteuuis & Bon 2000) with sufficient light intensity for multiplication phase of *in vitro* culture around 1000-10.000 lux (Yuniardi 2019).

Chlorophyll Content in Leaves and Roots of *P. amabilis*

Results of the present study demonstrated that the highest leaf chlorophyll content was found on light treatment in NP+100 g/L banana extract medium (Figure 2A). This might be related to the essential compounds contained in banana including vitamins, iron, and magnesium which is important for the formation of chlorophyll and avoid chlorosis (McCauley et al. 2009). In addition, potassium (K) levels which are found in banana extract at appropriate concentration can increase leaf chlorophyll levels and facilitate the integrity of the chloroplast ultrastructure (Tränkner et al. 2018). Perceiving light signal in plant affects the uptake and utilize multiple nutrients including macronutrients and micronutrients. Availability of light also activates some cryptochrome that regulates plant development and also influences chlorophyll content in plant. Light also triggers expression of gene that controlling nutrient utilization and plant hormone (Xu et al. 2021).

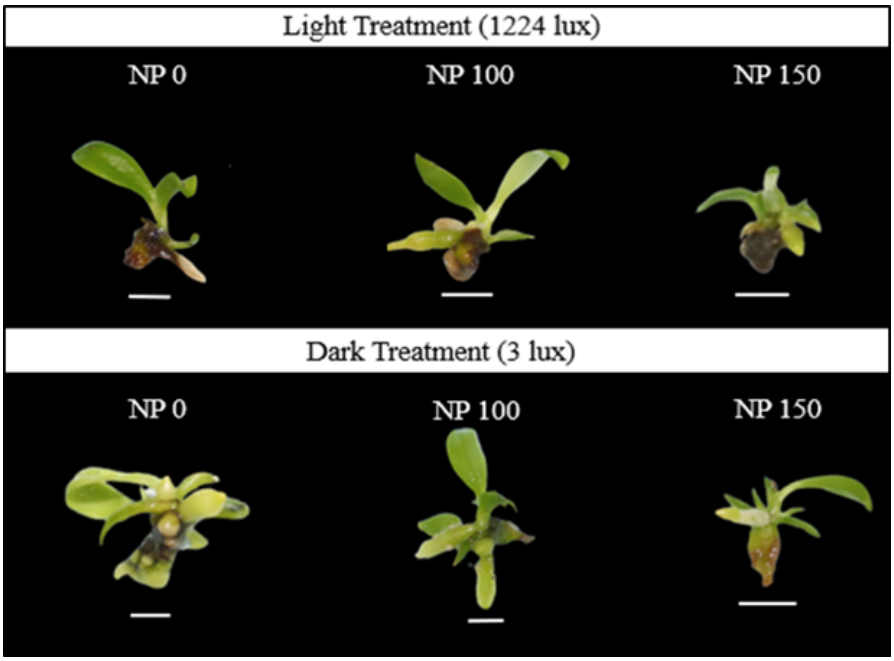

Figure 1. Growth comparison of *Phalaenopsis amabilis* plantlets on NP medium and dark-light treatment after 8 weeks of culture. Details: NP0: control medium; NP 100: NP+100 g/L banana extract; NP 150: NP+150 g/L banana extract. Bars: 0.5 cm

Furthermore, the highest chlorophyll content in the *P. amabilis* roots was found in NP0 basic medium under light treatment (Figure 2B). The nutrients contained in the NP0 medium might be sufficient for the synthesis of chlorophyll in *P. amabilis* roots.

Almost all the light treatment conditions have higher chlorophyll content than the dark treatments. This condition showed that the formation of chlorophyll is strongly influenced by light. Light plays an important role in converting proplastids into normally functioning chloroplasts, while dark conditions cause proplastids to develop into etioplast (Cortleven & Schumulling 2015).

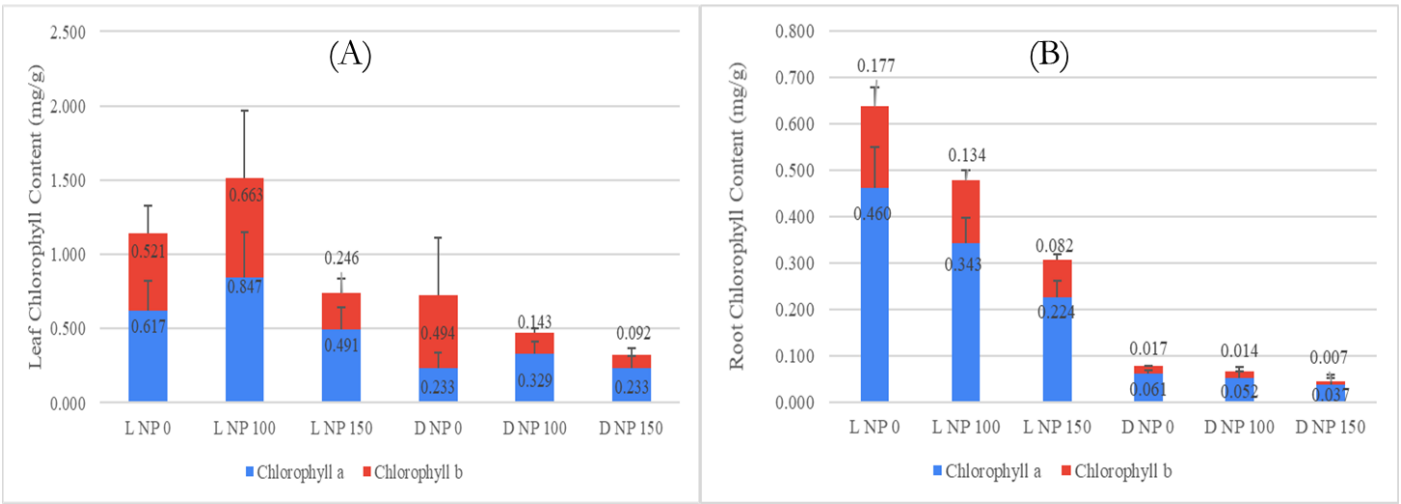

Figure 2. Chlorophyll content in leaves and roots of *Phalaenopsis amabilis*. (A) leaves, (B) roots with various medium treatment and dark-light conditions. Details: D: dark 3 lux; L:light 1224 lux; NP0: control medium; NP 100: NP+100 g/L banana extract; NP 150: NP+150 g/L banana extract.

Anatomical Features of Leaves and Roots of *P. amabilis*

The present study also showed anatomical features of *P. amabilis* leaves and roots. Anatomical features of *P. amabilis* leaves consisted of upper and lower epidermis, stomata, mesophyll, and vascular bundle (xylem and phloem) (Figure 3).

The outermost layer of the leaf is the epidermis which acts as a protective tissue, prevents water loss and gas exchange through epidermis derivatives. The tissue under the epidermis is the mesophyll which is homogeneous with parenchyma cells that are polygonal in shape and contain chloroplasts, in which photosynthesis occurs (Bercu et al. 2011). *P. amabilis* leaves have closed collateral type of vascular bundle, where the xylem located opposite to phloem and fascicular cambium is not present between phloem and xylem (Rindyastuti et al. 2018; Pradhan & Bajracharya 2020). In the *P. amabilis* leaves, the stomata are found on abaxial and adaxial epidermis surface with parallel position (Bercu et al. 2011).

Table 2 shows that the thickest of the mesophyll in *P. amabilis* was found in light treatment (1224 lux) on NP+100 g/L banana extract medium. The type of nutrient availability influenced the quantitative variation and differentiation of leaf tissue in orchid (Silva Junior et al. 2013). Thus, nutrient contained in NP+100 g/L banana extract, especially macronutrients such as N, P, and K helps differentiation of leaf tissue and affect the photosynthesis process. This is also in accordance with previous study by Silva Junior et al. (2013) where nitrogen application to *Laelia purpurata* orchid in appropriate levels will affect significantly on leaf mesophyll thickness. However, higher concentration of N application can cause toxicity, resulting in a reduction in thickness of mesophyll (Silva Junior et al. 2013).

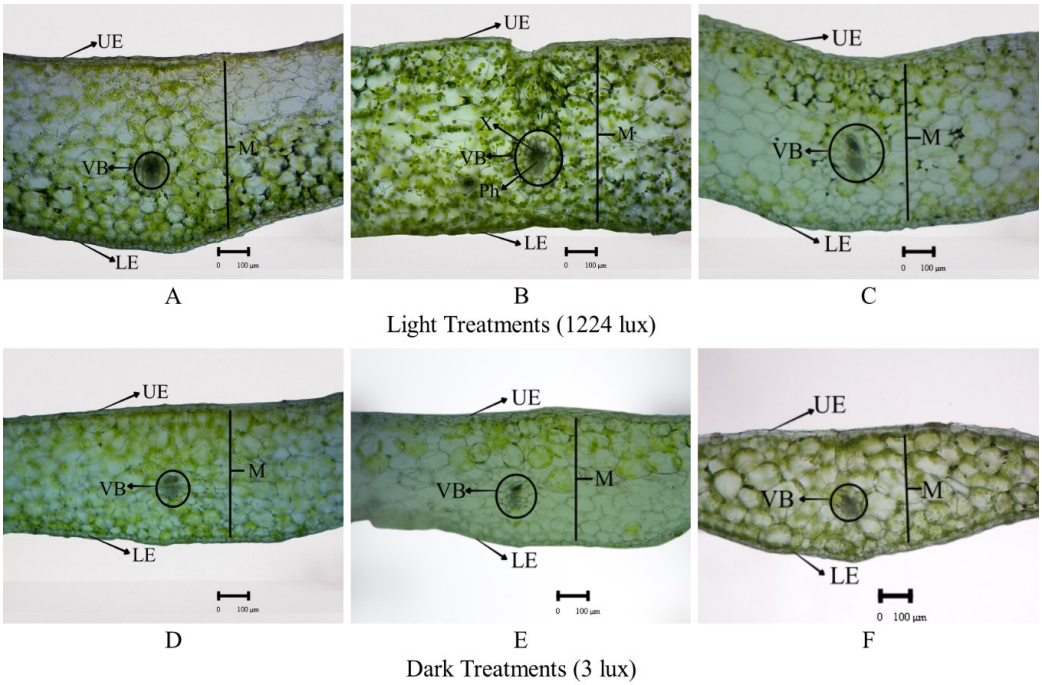

Figure 3. Cross section of leaves of *Phalaenopsis amabilis* orchids. (A) NP0; (B) NP100; (C) NP150; (D) NP0; (E) NP100; (F) NP150. Details: M: Mesophyll; LE: Lower Epidermis; Ph: Phloem; UE: Upper Epidermis; VB: Vascular Bundles; X: Xylem. Bars: 100µm

Table 2. Comparison of roots diameter and mesophyll thickness in *P. amabilis* under different medium and lighting condition.

Treatments / Parameters		Roots Diameter (mm)	Mesophyll Leaves Thickness (µm)
Light (1224 lux)	NP 0	1.64±0.031101^{bc}	471. 25±66.420^{c}
	NP 100	1.795±0.079^{c}	550.65±1.486^{d}
	NP 150	1.65±0.004b^{c}	412.09±24.637^{bc}
Dark (3 lux)	NP 0	1.54±0.019^{bc}	377.45±9.963^{b}
	NP 100	1.37±0.021^{ab}	350. 33±12.684^{ab}
	NP 150	1.19±0.006^{a}	286.97±27.381^{a}

Note: Data in the same column followed by the same letters are not significantly different by Duncan's test at $p \leq 0.05$. Details: NP 0: control medium; NP 100: NP+100 g/L banana extract; NP 150: NP+150 g/L banana extract.

Moreover, light changes the anatomical structure of leaves by affecting the differentiation of parenchymal cell arrangement of mesophyll layer (Zheng & Van Labeke 2017). Leaves with thick mesophyll can effectively reduce transpiration that is beneficial for the growth of *in vitro* culture plantlets after acclimatization (Zheng & Van Labeke 2017). Plants that get sufficient light tend to have a thicker mesophyll layer indicating the high effectiveness of photosynthesis, while plants that get low intensity have a thinner mesophyll layer due to barriers to the differentiation process of mesophyll cells (Paiva et al. 2003).

The present study also demonstrated anatomical features of orchid roots which consisted of epidermis, velamen, exodermis, cortex, endodermis, and vascular bundle. Epidermis as the outermost layer of root anatomy orchids forms a derivative layered, known as velamen which is composed of cellulose with suberin and lignified cells in assorted size (Nurfadilah et al. 2016). Velamen has essential functions in defence, assimilation of water and nutrients, and defence against water loss caused by evaporation (Simpsons 2019). The thickness of the velamen layer depends on light intensity and humidity fluctuations (Moreira et al. 2013).

The outer layer of cortex is exodermis which functions as a control of water and nutrients pathway to the roots, protection against water evaporation, and controlling the entry of mycorrhizae (Beck 2010; Nurfadilah et al. 2016). The cortex is composed by parenchyma cells which have thin wall in various proportions and includes the chloroplast inside (Nurfadilah et al. 2016). The cortex serves as a pathway for water and nutrients entry from external environment to inside cell of the stele and as food reserve (Metusala et al. 2017). The innermost layer of the cortex is the endodermis which is arranged by one layer of cells and the cell wall can be thickened by lignin and suberin substance to prevent backflow in the cortex and prevent water loss (Muthukumar & Shenbagam 2018). Root anatomy of *P. amabilis* has radial vascular bundle type where xylem located alternate with phloem position and forming circular radii or *arch* (Beck 2010).

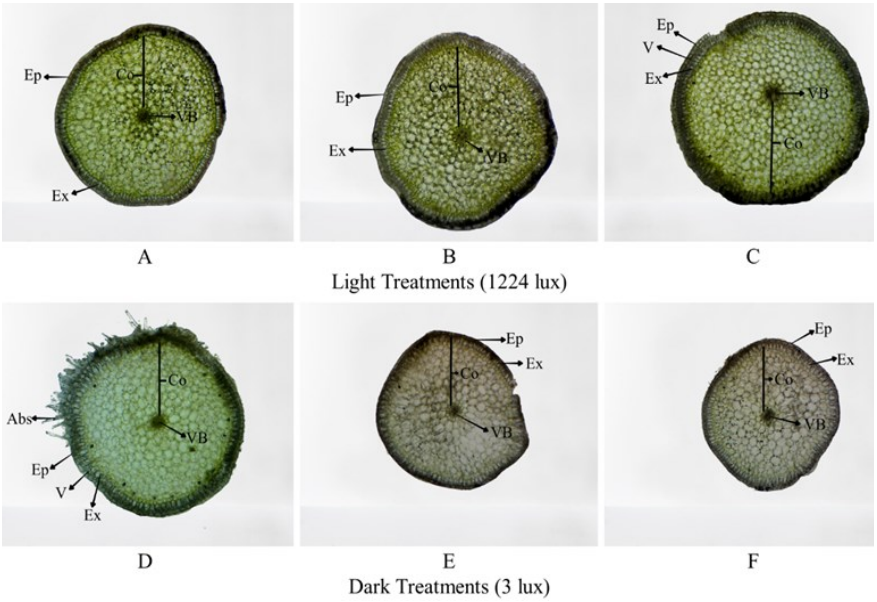

Figure 4. Cross section of roots *Phalaenopsis amabilis* orchids. (A) NP0; (B) NP100; (C) NP150; (D) NP0; (E) NP100; (F) NP150. Details: Abs: Absorbing hair; Ep: Epidermis; V: Velamen; Ex: Exodermis; Co: Cortex; VB: Vascular bundles; Ed: Endodermis; P: Pith; X: Xylem; Ph: Phloem. Bars: 100µm

The present study also showed that the largest root diameter in *P. amabilis* was found in NP+100 g/L banana extract medium in light conditions (Table 2 and Figure 4). It can be concluded that medium type NP+100 g/L banana extract and in light conditions produced better root anatomical characteristics compared to other treatments. Nutrients obtained in 100 g/L banana extract are at sufficient levels and the presence of light is needed for the development of root cells of *P. amabilis*. Light signaling interacts with the root environment, including nutrient acquisition (Joca et al. 2017). Root of dark-grown still develop but have a far thinner diameter than the ones of light-grown seedlings (van Gelderen et al. 2018). That previous research was consistent with the results, where plantlets roots diameter grown in dark conditions had thinner diameter than the light ones.

The largest diameter of roots in NP+100 g/L banana extract might be related to the phosphorous (P) content that has essential functions to support greater root growth (Heydari et al. 2018). According to Heydari et al. (2018) the root diameter decreases significantly under the lack of P. Besides P, potassium (K) also affect root diameter (Filho et al. 2017). The roots diameter increases linearly with the increase of K rate (Filho et al. 2017).

Data of morphological, physiological, and anatomical features showed that *P. amabilis* plantlets grow better in NP+100 g/L of banana extract. Therefore, medium NP+100 g/L banana extract can be recommended for seedling production of *P. amabilis* to produce many seedlings with great performance to fulfill market demand. This work could contribute to reduce exploitation of *P. amabilis* in natural habitats.

CONCLUSION

It can be concluded that NP+100 g/L of banana extract is the best culture

medium for *P. amabilis* plantlets as providing seedlings with high quality. Light treatment at 1224 lux becomes the best condition for *P. amabilis* plantlets growth. The combination of light treatment at 1224 lux and medium NP+100 g/L banana extract is suitable for increasing seedling production of *P. amabilis* by affecting morphology growth, chlorophyll content and anatomy structure. This work could contribute to provide a mean to produce many seedlings for *ex situ* orchid conservation and reduce exploitation of *P. amabilis* in natural habitats.

AUTHORS CONTRIBUTION

DAPA carried out the analysis of growth plantlets, anatomical studies, and chlorophyll content in *P. amabilis*, also drafted the manuscript. ES was responsible for coordinating the implementation of the research and discussion of the research results. All authors read and approved this final manuscript.

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CONFLICT OF INTEREST

The authors declare there is no competing interest.

REFERENCES

Arditti, J., 2008. *Micropopagation of Orchid Second Edition*, Oxford: Blackwell Publishing.

Arimarsetiowati, R., & Ardiyani, F., 2012. The effects of shooting and rooting of arabica coffee propagation through Embryogenesis Somatic auxin uses). *Pelita Perkebunan (a Coffee and Cocoa Research Journal)*, 28(2), pp.82-90.

Armarego-Marriott, T., Sandoval-Ibañez, O. & Kowalewska, Ł., 2020. Beyond the darkness: recent lessons from etiolation and de-etiolation studies. *Journal of experimental botany*, 71(4), pp.1215-1225. doi: 10.1093/jxb/erz496

Beck, C.B., 2010. An Introduction to Plant Structure and Development. New York: Cambridge University Press

Bercu, R., Bavaru, A. & Broască, L., 2011. Anatomical Aspects of *P. amabilis* (L.) Blume, *Annals of RSCB,* pp. 103-109.

Berlyn, G.P. & Miksche, J.P., 1976. *Botanical microtechnique and cytochemistry,* Ames: Lowa State University Press.

Cortleven, A. & Schmülling, T., 2015. Regulation of chloroplast development and function by cytokinin. *Journal of experimental botany*, 66(16), pp.4999-5013. doi: 10.1093/jxb/erv132

Djajanegara, I., 2010. Pemanfaatan limbah buah pisang dan air kelapa sebagai bahan media kultur jaringan anggrek bulan (*Phalaenopsis amabilis*) tipe 229. *Jurnal Teknologi Lingkungan*, 11(3), pp.373-380. doi: 10.29122/jtl.v11i3.1182

Filho, A.C.A.C. et al., 2017. Influence of Potassium Levels on Root Growth and Nutrient Uptake of Upland Rice Cultivars. *Revista Caatinga*, 30, pp.32-44. doi: 10.1590/1983-21252017v30n104rc

Hapsari, L. & Lestari, D. A., 2016. Fruit characteristic and nutrient values of four Indonesian banana cultivars (*Musa* spp.) at different genomic groups. *AGRIVITA: Journal of Agricultural Science*, 38(3), pp.303-311. doi: 10.17503/agrivita.v38i3.696

Harborne, J.B., 1998. *Phytochemical Methods, a Guide to Modern Techniques of Plant Analysis*. London: Chapman and Hall.

Haris, A. & Mercuriani, I.S., 2018. Pertumbuhan Anggrek *Rhynchostylis retusa* pada Medium Kultur *In Vitro* dengan Penambahan Jus Buah Pisang. *Biologi-S1*, 7(6), pp.367-381.

Hasanah, U. & Suwarsi, E., 2014. Pemanfaatan Pupuk Daun, Air Kelapa dan Bubur Pisang sebagai Komponen Medium Pertumbuhan Plantlet Anggrek Dendrobium Kelemense. *Biosaintifika: Journal of Biology & Biology Education*, 6(2), pp.137-144. doi: 10.15294/biosaintifika.v6i2.3784

Heydari, M.M., Brook, R.M. & Jones, D. L., 2019. The role of phosphorus sources on root diameter, root length and root dry matter of barley *(Hordeum vulgare* L.). *Journal of plant nutrition*, 42(1), pp.1-15. doi: 10.1080/01904167.2018.1509996

Joca, T.A.C. et al., 2017. The velamen of epiphytic orchids: variation in structure and correlations with nutrient absorption. *Flora,* 230, pp.66-74. doi: 10.1016/j.flora.2017.03.009.

Manzur, J.P., Calvache-Asensio, M.D.L.N. & Rodriguez-Burruezo, A., 2014. Growth regulators and darkness increase efficiency in in vitro culture of immature embryos from peppers. *Scientia Agricola*, 71, pp.488-493. doi: 10.1590/0103-9016-2013-0230

McCauley, A., Jones, C. & Jacobsen, J., 2009. *Plant Nutrition Function and Deficiency and toxicity Symptoms*. Bozeman: Montana State University

Metusala, D. et al., 2017. Comparative leaf and root anatomy of two *Dendrobium* species (Orchidaceae) from different habitat in relation to their potential adaptation to drought. *AIP Conference Proceedings*, 1862. doi: 10.1063/1.4991222

Monteuuis, O. & Bon, M. C., 2000. Influence of auxins and darkness on in vitro rooting of micropropagated shoots from mature and juvenile *Acacia mangium*. *Plant cell, tissue and organ culture*, 63(3), pp.173-177. doi: 10.1023/A:1010611126950

Moore, T.C., 1989. *Biochemistry and Physiology of Plant Hormone Ed-2*. New York: Springer-Verhag.

Moreira, F.P.A.S. et al., 2013. Structural adaptations of two sympatric epiphytic orchids (Orchidaceae) to a cloudy forest environment in rocky outcrops of Southeast Brazil. *Revista de Biología Tropical*, 61(3), pp. 1053-1065.

Muthukumar, T., & Shenbagam, M., 2018. Vegetative anatomy of the orchid *Bulbophyllum* sterile (Orchidaceae: Epidendroideae). *Lankesteriana,* 18(1), pp.13-22. doi: 10.15517/lank.v18i1.32701

Nurfadilah, S., Yulia, N.D., & Ariyanti, E.E., 2016. Morphology, anatomy, and mycorrhizal fungi colonization in roots of epiphytic orchids of Sempu Island, East Java, Indonesia. *Biodiversitas Journal of Biological Diversity*, 17(2). doi: 10.13057/biodiv/d170229

Paiva, É.A.S., et al. 2003. The influence of light intensity on anatomical structure and pigment contents of *Tradescantia pallida* (Rose) Hunt. cv. purpurea Boom (Commelinaceae) leaves. *Brazilian Archives of Biology and Technology*, 46, pp.617-624. doi:10.1590/S1516-89132003000400017

Pazil, S.N., 2009. Perbandingan Aktivitas Antioksidan Ekstrak Daging Pisang Raja (*Musa* AAB 'Pisang Raja') Dengan Vitamin A, Vitamin C, dan Katekin Melalui Penghitungan Bilangan Peroksida, *Srkipsi*, Fakultas Kedokteran, Universitas Indonesia

Pradhan, B.M. & Bajracharya, D.M., 2020. Anatomical Study of *Dendrobium* (Orchidaceae) of Nepal. *Annals of Plant Sciences,* 9(7), pp.3916-3948. doi:10.21746/aps.2020.9.7.1

Qomariyah, F.L., & Dewanti, P., 2019. Pertumbuhan Planlet Anggrek *Dendrobium* sp. pada Media Tahap III secara *In-Vitro*. *Jurnal Ilmiah Inovasi*, 19 (1), pp.13-16. doi: 10.25047/jii.v19i1.853

Rindyastuti, R. et al., 2018. Leaf anatomical characters of four epiphytic orchids of Sempu Island, East Java, Indonesia: The importance in identification and ecological adaptation. *Biodiversitas Journal of Biological Diversity*, 19(5), pp.1906-1918. doi: 10.13057/biodiv/d190543

Rukmana, R., 2000. *Anggrek Bulan,* Yogyakarta: Penerbit Kanisius.

Sallolo, S.T., Sadimantara, I.G.R. & Wijayanto, T., 2012. Pertumbuhan anggrek *Dendrobium Candy Stripe Lasianthera* pada media sapih Vacin dan Went secara *in vitro* dengan penambahan ekstrak pisang raja dan fish emulsion. *J. Penelitian Agronomi*, 1(1), pp.57-62.

Semiarti, E. et al., 2007. Agrobacterium-mediated transformation of the wild orchid species *Phalaenopsis amabilis*. *Plant Biotechnology*, 24(3), pp.265-272. doi: 10.5511/plantbiotechnology.24.265

Semiarti, E. et al., 2010. High-frequency genetic transformation of *Phalaenopsis amabilis* orchid using tomato extract-enriched medium for the preculture of protocorms. *The Journal of Horticultural Science and Biotechnology*, 85(30), pp.205-210. doi: 10.1080/14620316.2010.11512655

Setiari, N. et al., 2018. Micropropagation of *Dendrobium phalaenopsis* orchid through overexpression of embryo gene *AtRKD4*. *AGRIVITA, Journal of Agricultural Science*, 40(2), pp.284-294. doi: 10.17503/ agrivita.v40i2.1690

Silva Júnior, J.M.D. et al., 2013. Changes in anatomy and chlorophyll synthesis in orchids propagated in vitro in the presence of urea. *Acta Scientiarum: Agronomy*, 35(1), pp.65-72. doi:10.4025/actasciagron.v35i1.15356

Simpsons, M.G., 2019. *Plant Anatomy and Physiology*. In: M.G. Simpson, (eds) Plant Systematic Third Edition. New York : Academic Press.

Souza, G.R.B. et al., 2013. Pulp fruit added to culture medium for in vitro orchid development. *Semina: Ciências Agrárias*, 34(3), pp.1141-1146.

Theng, P.A. & Korpenwar, A.N., 2014. Phytochemical, Phermacognostic and Physiocochemical evaluation of endangered terrestrial orchid *Geodorum densiflorum* (Lam.) Schltr. *IJSR*, 3(9), pp.1250-1253.

Tränkner, M., Tavakol, E. & Jákli, B., 2018. Functioning of potassium and magnesium in photosynthesis, photosynthate translocation and photoprotection. *Physiologia plantarum*, 163(3), pp. 414-431. doi: 10.1111/ppl.12747

Utami, E.S.W., Harianto, S. & Manuhara, Y.S.W., 2016. Pengaruh Pemberian Ekstrak Pisang pada Media VW terhadap Induksi Akar dan Pertumbuhan Tunas *Dendrobium lasianthera* JJ Sm. *Jurnal Agrotop*, 6(1), 35-42.

van Gelderen, K., Kang, C. & Pierik, R., 2018. Light signaling, root development, and plasticity. *Plant physiology*, 176(2), pp. 1049-1060. doi: 10.1104/pp.17.01079

Xu, X. et al., 2020. Effects of potassium levels on plant growth, accumulation and distribution of carbon, and nitrate metabolism in apple dwarf rootstock seedlings. *Frontiers in Plant Science*, 11, pp. 904. doi: 10.3389/fpls.2020.00904

Xu, J. et al., 2021. Light regulation of horticultural crop nutrient and utilization. *Horticultural Plant Journal*, 7(5), pp.367-379. doi: 10.1016/j.hpj.2021.01.005

Yulianti, Y., Aisyah, S. I. & Sukma, D., 2016. Pengaruh Bahan Organik Nabati dan Hewani terhadap pertumbuhan protocorm like bodies *Phalaenopsis amabilis* (L.) blume. *Jurnal Hortikultura Indonesia*, 7(3), pp. 176-186. doi: 10.29244/jhi.7.3.176-186

Yuniardi, F., 2019. Aplikasi Dimmer Switch pada Rak Kultur Sebagai Pengatur Kebutuhan Intesitas Cahaya Optimum Bagi Tanaman In Vitro. *Indonesian Journal of Laboratory*, 1(4), pp. 8-13.

Zheng, L. & Van Labeke, M. C., 2017. Long-term effects of red-and blue-light emitting diodes on leaf anatomy and photosynthetic efficiency of three ornamental pot plants. *Frontiers in plant science*, 8, pp. 917. doi: 10.3389/fpls.2017.00917

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Research Article

Population structure and habitat preference of cave crickets (*Rhaphidophora* sp. (Orthoptera: Rhaphidophoridae)) in Sanghyang Kenit cave, Citatah karst area, West Java

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ABSTRACT

Cave crickets are considered as a keystone species that can be used as a cave ecosystem bioindicator. Developing caves as tourism has considerable potential to disturb cave cricket populations. This study aimed to investigate cave cricket population structure and their habitat preference in Sanghyang Kenit cave one year after it developed into a show cave. Data were collected through standardized visual searching in three cave zones: entrance, twilight, and dark. Besides cave crickets, other macroarthropods discovered in each zone were also recorded. Abiotic parameters of habitat comprised air and soil temperatures, RH, soil moisture, soil pH, and light intensity were measured. Data were analyzed to show cave crickets abundance, density, sex ratio, and age structure. Statistical analysis comprising Kruskal Wallis, non-metric multidimensional scaling, and correlation tests were performed. The cave cricket population in Sanghyang Kenit belonged to a single species, *Rhaphidophora* sp. The population was around 78-108 individuals and distributed in all cave zones. The abundance and density in twilight and dark zones were significantly higher than in the entrance. The number of males outperformed females with a 2.16 ratio. Besides, the population was dominated by the sub-adult class. Environmental parameters of twilight and dark zones tended to be similar to one another compared to the entrance. Cave crickets preferred habitats with dark, humid, and acidic soil pH. *Heteropoda* sp. and *Catagaeus* sp. were considered potential predators. This study implies the importance of protecting cave crickets in Sanghyang Kenit.

INTRODUCTION

Caves represent several extreme environments that generate unique ecosystem characters. The typical cave environments include total darkness, high humidity, and a stable microclimate (Prous et al. 2015; Kurniawan & Rahmadi 2019). Besides, caves are well-known as one of the most challenging environments for organisms due to the absence of sunlight, which limits visual organs from working properly. The darkness also inhibits photosynthetic organisms, resulting in low food availability and/or variation (Ravn et al. 2020). Caves biodiversity is generally lower than surface ecosystems because

those extreme conditions act as limiting factors for a diverse group of living creatures (Prakarsa et al. 2021). Caves only can be inhabited by adaptive taxa that can cope with such a harsh environment. Therefore, caves and their biodiversity are unique and extremely sensitive (Mammola 2019).

All inhabited cave animals can be referred to as cavernicoles. These animals are classified into three groups according to their degree of cave adaptations and cave usages as their habitat: troglophile, trogloxene (subtroglophile), and troglobite/troglobiont (Howarth & Moldovan 2018a). Troglophiles are permanent resident of cave habitats, which also can inhabit surface habitats. Trogloxenes are partial cave dwellers that must leave caves periodically for biological functions, such as feeding. Meanwhile, troglobites are obligate cave animals that only can live exclusively in the caves realm (Culver & Pipan 2009; Prakarsa et al. 2021).

One of the essential cave-dwelling animals is cave crickets. Taxonomically, they belong to order Orthoptera, suborder Ensifera, and family Rhaphidophoridae. This family consists of more than 550 described species distributed into 80 genera (Ingrisch & Rentz 2009). All the family members have a humpbacked morphological appearance, relatively larger body size than the common cricket (subfamily Gryllinae), and are wingless. Moreover, they also can be recognized from their long antennae and strong enlarged hind legs (Di Russo & Rampini 2017; Song et al. 2020). All rhaphidophorids are nocturnal (Allegrucci et al. 2010). They occur in various humid habitats such as under logs, rocks, damp humus, tree holes, and many species restricted to caves (Epps et al. 2014; Hu et al. 2014). The cave dwellers are called cave crickets or cave weta, while surface inhabitants are known as camel crickets or spider crickets. Cave crickets are categorized as troglophiles that can complete their whole life cycle in caves. However, they can also be classified as trogloxene since several species periodically leave caves at night, mainly for foraging (Deharveng & Bedos 2012; Chandoo et al. 2013).

The existence of cave crickets population is vital for the continuity of caves ecosystem because it provides food for many cavernicoles. In Indonesian caves, cave crickets are the main prey for many predatory species, including whip spiders (order Amblypygi), huntsman spiders (family Sparassidae), and centipedes (order Scutigeromorpha) (Kurniawan & Rahmadi 2019; Prakarsa et al. 2021). In temperate caves where bats' guano supply is limited, their droppings and carcasses are the primary food sources for other cave-dwellers. Besides, their eggs are also consumed by smaller-sized arthropod groups like carabid beetles (Lavoie et al. 2007; Culver & Pipan 2009). Thus, cave crickets are often considered as keystone species in many cave ecosystems (Yoder et al. 2010; Epps et al. 2014).

Caves ecosystem in Indonesia has been experiencing escalated threats due to overexploitation by humans. Besides the rising numbers of extractive industries such as limestone quarries (Mulyani 2011; Subekti 2016), the increase of human visits to caves, mainly for tourism purposes, is a significant challenge for caves ecosystem continuity (Kurniawan & Rahmadi 2019;

Kurniawan et al. 2020). Caves have exciting attributes, such as beautiful speleothems or cave ornaments and cultural, historical, and religious values, that can attract people. Thus, many caves are developed as show caves, i.e., natural caves opened to public as tourist attractions. Several previous studies revealed that intense human visits to caves could alter cave microclimate conditions, including increased air temperature and CO_2 concentration, and aerobiological content (Fernandez-Cortes et al. 2011; Pacheco et al. 2020). Those environmental changes can bring serious menace for cave-dwelling animals resulting in population decrease and biodiversity loss.

Nowadays, the cave tourism industry in Indonesia is on a positive trend because of the excellent response from the community. This trend leads the opening of many new show caves, including Sanghyang Kenit, a show cave situated in West Java Province. This cave has been operated as a tourist place since 2019 and is managed by the local community. According to the preliminary observation of this study, the cave was inhabited by cave crickets. This study aimed to investigate cave cricket population structure and habitat preference in Sanghyang Kenit. Investigation related to the cave cricket population is essential to provide a baseline condition of a keystone species that can be used to detect ecological changes caused by tourism activities.

MATERIALS AND METHODS

Study Site

This study was conducted in Sanghyang Kenit cave, located in the Citatah karst area. This cave is administratively situated in the Cipatat sub-district, Bandung Barat district, West Java province, at the geographical coordinate 6° 51'35" S 107°20'51" E (Figure 1). The cave entrance is moderate in size and located in the Citarum riverside. This cave has a horizontal passage approximately 400 m in length, divided into three zones: entrance, twilight, and dark. The cave passage is a part of the Citarum drainage basin which is naturally passed by a high level of water current. Still, the Saguling hydroelectric power plant decreases the water flow, making it possible for humans to visit the cave. Sanghyang Kenit has both terrestrial and aquatic types of habitats. The terrestrial habitat consists of rocks, pebbles, soils, and sand substrates. The cave ceiling was inhabited by insectivorous bat populations that generated guano piles on the cave floor.

Field Data Collection

Data collection was carried out through a visual searching method by hand collecting and direct counting (Wynne et al. 2019). All visible cave cricket individuals during cave explorations were captured directly by hand with the help of gloves, small hand nets, and tweezers. The collection was conducted during daylight (1.00-4.30 pm) with three repetitions for each cave zone starting from the entrance, twilight, and dark zones. The method was standardized by a similar duration of sampling efforts (30 minutes per zone)

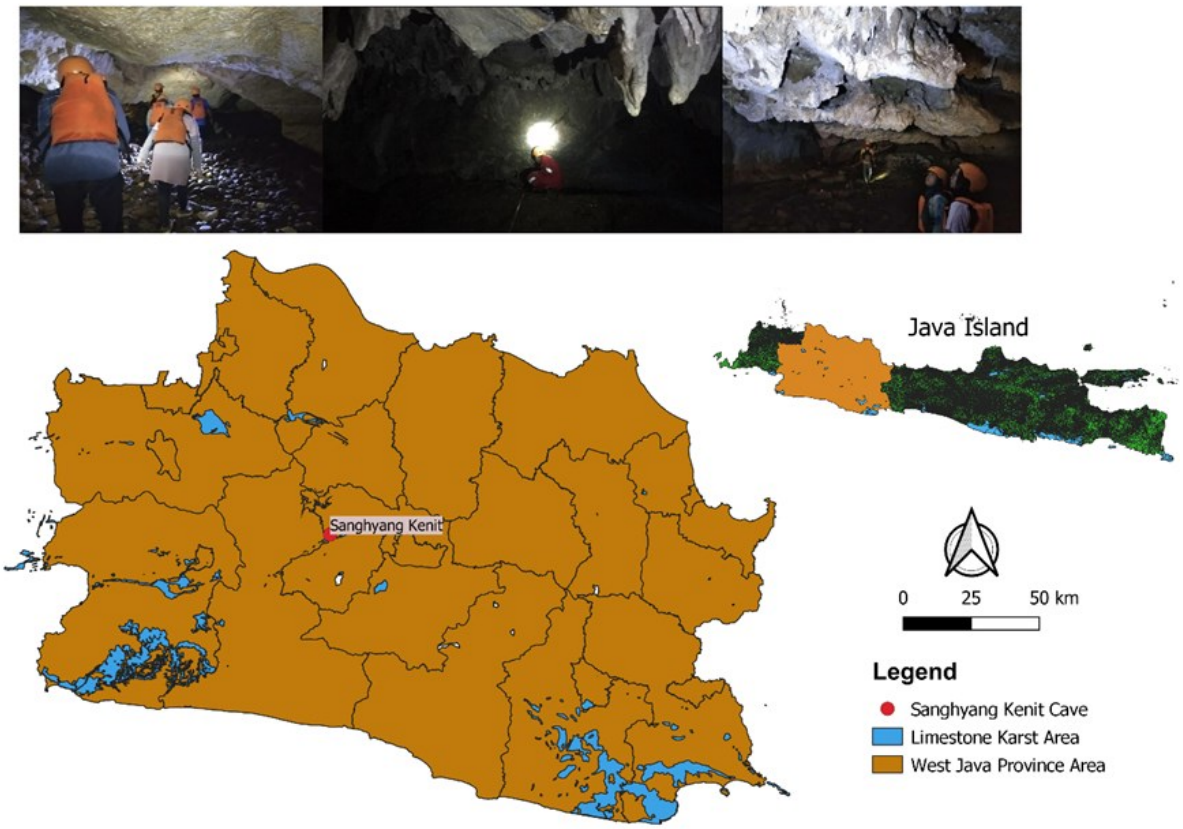

Figure 1. The location of Sanghyang Kenit cave and several images of its passage conditions.

and performed by three collectors, with good experience collecting cave-dwelling arthropods. Every collector was assigned to collect data in the different sampling areas, namely the right, left, and middle parts of the cave passage.

All collected cave crickets from each zone were placed in a container box to be counted for their number of individuals and categorized into sex and age class. The number of individuals was calculated by hand tally counter. Sex categorization (male/female) was distinguished based on the presence and absence of an ovipositor. At the same time, age structure was estimated based on three different body size categories (Carchini et al. 1994), namely small (< 3 cm), medium (3-5 cm), and large (> 5 cm). The classification of cave crickets based on their body size aimed to reveal the age proportion among juvenile (small), sub-adult (moderate), and adult (large) classes within the population. Cave crickets were released into their habitat immediately after the measurements were completed. Several individuals from each sex and body size category were carried off and preserved using 70% alcohol for morphological and morphometrical studies in the laboratory.

Along with the cave cricket collection, the richness and the number of individuals of the other cave-dwelling arthropods were also recorded. The data were used to detect potential predators and competitors of cave crickets. All collected arthropods were identified based on morphological characters up to the lowest possible taxon category. Besides, they were also classified into their role in the cave ecosystem.

Abiotic Parameters Measurement

Several associated abiotic parameters were measured during the study. Abiotic parameters were utilized to describe the habitat characteristic and its relation to cave cricket population. Soil and air temperatures, relative humidity (RH), soil moisture, light intensity, and soil pH were among the abiotic parameters that were assessed. Soil moisture and pH were measured using soil tester (Takemura DM-5), light intensity using light meter (Lutron LX-100), RH and air temperature using thermo-hygrometer (HTC-1), while soil temperature using soil thermometer (71200.080-vr). The measurements were conducted after the animal collection was completed. Each parameter was measured five times for each zone with three repetitions in the different sampling efforts. In addition, the total area of each zone was also estimated by measuring the length and width of the cave passage. The total area was used to calculate population density.

Data Analysis

The population structure was indicated by calculating the cave cricket abundance, density, sex ratio, and age structure. The formulas for each aspect are provided as follow:

Abundance:

$$N = \sum_{i=1}^{S} \mathrm{Ni}$$

where

N : abundance index

Ni : the number of individuals of targeted species,

Density:

$$Di = \frac{ni}{A}$$

where

Di : density of targeted species

ni : the number of individuals of targeted species

A : the total of the studied area (m^2)

Sex ratio:

$$X = \frac{J}{B}$$

where

X : sex ratio of targeted species

J : the number of male individuals

B : the number of female individuals

Meanwhile, the age structure analysis was carried out by comparing the relative abundance of each size category. The formula of relative abundance is as follow:

$$Relative\ Abundance\ (\%) = \frac{The\ number\ of\ Individual\ a\ size\ category}{Total\ Number\ of\ Individual\ all\ size\ category} x\ 100\%$$

Several statistical analyses were performed for different purposes. The Kruskal Wallis test was used to determine the abundance and density differences among cave zones. The test was processed using SPSS version 25. To highlight similarity and dissimilarity among cave zones according to their measured abiotic parameters and their relationship with cave cricket abundance, non-metric multidimensional scaling (NMDS) was performed. On the other hand, a correlation test was conducted to show the relatedness of cave cricket population with the other arthropod species discovered in the cave. NMDS and correlation tests were processed using R Studio under Vegan (function: metaMDS and combined with envfit) (Oksanen et al. 2020) and Corrplot packages (function: corrplot(cor) (Wei & Simko 2021), respectively. The results of the analyses were presented as table, plot, and graph to make better visualization and interpretation.

RESULTS AND DISCUSSION

Cave crickets occurring in Sanghyang Kenit consisted of only a single species. This species belonged to subfamily Rhaphidophorinae and genus *Rhaphidophora*. The typical characteristics of this subfamily include wingless, undeveloped sound organs, and membranous genitalia without complex structures. There are two genera from this subfamily that bear a great degree of similarity in Indonesia area: *Rhaphidophora* and *Stonycophora*. The Sanghyang Kenit population is classified into *Rhaphidophora* due to the absence of large copulatory processes on the male abdomen, which differs from *Stonycophora* (Di Russo & Rampini 2017). Moreover, *Stonycophora* was never reported inhabiting caves previously. Males can be easily distinguished from females by the absence of ovipositors (Figure 2b).

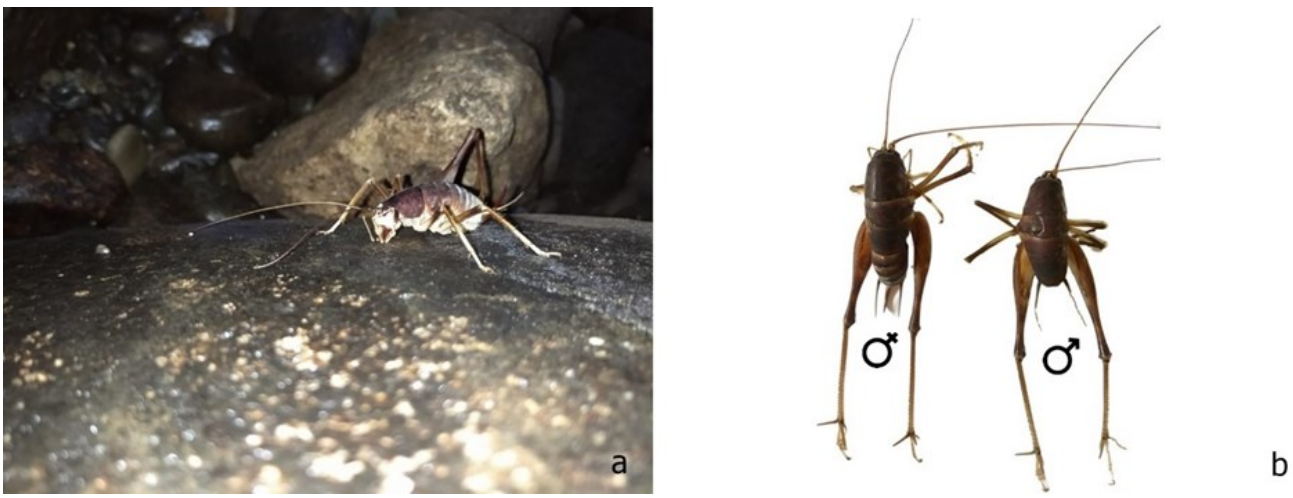

Figure 2. a) Sanghyang Kenit's cave crickets habitus, b). Comparison of female and male morphology.

There are two known genera of cave crickets living in Java: *Diestrammena* and *Rhaphidophora* (Rahmadi 2008). Up to recently, the distribution of these two genera is still unclear and seems to overlap. But many previous cave-dwelling arthropods studies had revealed that *Rhaphidophora* occurred in many karst area in Java including Tuban, Gunungsewu, Cilacap, Tasikmalaya, and Ciampea (Rahmadi 2002; Rahmadi & Suhardjono 2007; Prakarsa & Ahmadin 2017; Kurniawan et al. 2018a; Hasibuan & Lidiawati 2020; Hidayaturrohmah et al. 2021). Overall, at least 12 species belonging to genus *Rhaphidophora* have been described from Java, but only two species are reported to occupy cave realm. These species are *R. dammermani* and *R. dehaani* (Prakarsa et al. 2021). It is challenging to distinguish these two species based on morphological character because it needs specific taxonomical expertise. Moreover, the available references for these two species are limited. Thus, most previous studies only justified cave crickets until genera level and commonly used a uniform name, *Rhaphidophora* sp. Only a few studies had named cave crickets until species level, but, likely, the justifications were not made based on morphological characters but biogeographical evidence instead. Considering this approach, the species of Sanghyang Kenit's population can be suspected as *R. dammermani*. It is because the geographical location of the Citatah karst area is not far from Ciampea in Bogor, where the first specimens of this species were collected (Rahmadi 2011). A further sophisticated taxonomical study is needed to prove this hypothesis.

Abundance and Density

Cave crickets in Sanghyang Kenit were distributed in all cave zones. However, the number of individuals that occurred in each cave zone was highly different (Figure 3). The difference is confirmed by the result of Kruskal Wallis test, which shows a lower significant value than α ($\alpha < 0.05$). Most cave crickets were discovered in dark zone with 60-79 individuals, followed by twilight zone (16-26 individuals). The smallest abundance was in entrance zone, where only 2-4 individuals were recorded. Overall, around 78-109 individuals of cave crickets inhabited Sanghyang Kenit.

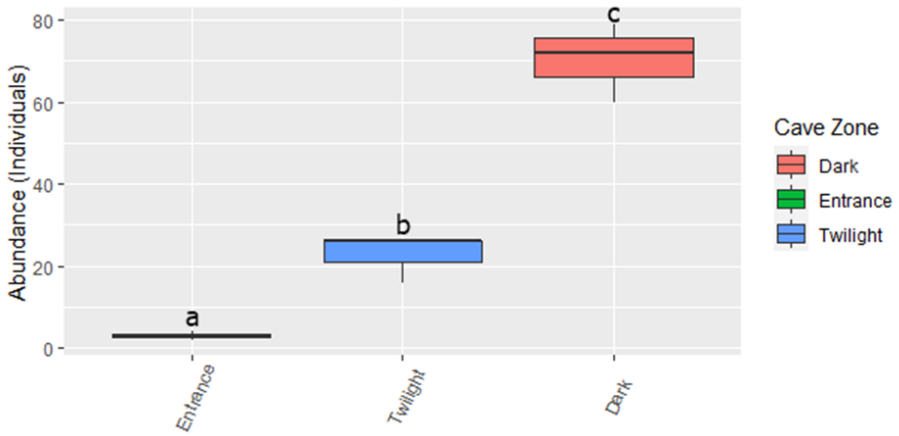

Figure 3. Comparison of cave cricket abundance across different cave zones. The different letter indicates different abundance among cave zones.

Even the cave has been operated as a tourist attraction, the population of cave crickets was still in great abundance. This abundance is relatively higher than previous cave cricket populations monitoring in the other show caves in Java. The study conducted by Kurniawan et al. (2017), performed by a similar sampling technique, demonstrated that cave cricket populations in the frequently visited show caves in the Gunungsewu karst area were only around 5-24 individuals. Furthermore, the study also revealed that wild caves were inhabited by more abundant cave crickets, with about 56-180 recorded individuals. As mentioned previously, Sanghyang Kenit is a new show cave where human visit is less intense. Minor disturbance allows the population to thrive prosperously.

In line with abundance, the cave cricket density among cave zones was also significantly different ($\alpha < 0.05$). However, the density in twilight and dark zones was relatively similar (Table 1). The entrance hosts the most significant area (3000 m^2), but the abundance of cave crickets in the zone was fewer. This result indicates that space is not the main factor deciding cave cricket distributions in caves. The difference in environmental parameters among cave zones is considered as the most important driving factor.

Table 1. The density of cave crickets across different cave zonation.

Cave Zone	Mean of Abundance (Individuals)	Zone Area (m^2)	Density (Ind/m^2)*
Entrance	3	3000	0.001a
Twilight	22.67	690	0.032b
Dark	70.33	1800	0.039b

*Kruskal Wallis test is significant at $\alpha = 0.05$. The different letter illustrates different density among cave zones.

Only few previous studies contained information regarding cave cricket density. Carchini et al. (1994) demonstrated that during three years of continuous monitoring of a cave cricket population (*Dolichopoda geniculata*), the density fluctuated but was always lower than one individual/m^2. However, the study was conducted in the temperate region, where caves condition is significantly different from the tropical region. Unfortunately, there is no published data about cave cricket density from Indonesian species for comparison.

The comparison of cave cricket density among cave zones (Table 1) shows that the value increases from the entrance to the dark zone. This result shows a similar pattern with the previous study conducted by Carchini et al. (1994), which revealed that density in the deeper area of a cave was essentially higher than near entrance area. However, the density between twilight and dark zones in this study was not statistically different. Most animals belonging to troglophiles are commonly abundant in twilight and dark zones due to preferable microclimate conditions suitable for this group (Howarth & Moldovan 2018b).

Sex Ratio

The result of the sex ratio analysis illustrates that males predominated in all cave zones. The sex ratio varied from 2.10 to 3.50. Overall, the number of males was twice higher than females (Table 2). Several previous studies had examined the sex ratio of different cave cricket species, and the results were highly varied. Lavoie et al. (2007) reported that a cave cricket species from the genus *Hadenoecus* had significantly more females than males, while Bernardini & Di Russo (2004) said a fair sex ratio (1:1) in the genus *Dolichopoda*. Up to the present, there is no specific information about the sex ratio of the genus *Rhaphidophora*.

Table 2. The sex ratio of cave cricket across different cave zonation.

Cave Zone	Mean of Abundance (Individuals)		Sex Ratio (Male/ Female)
	Male	Female	
Entrance	2.33	0.67	3.50
Twilight	15.67	7	2.23
Dark	47.67	22.67	2.10
Total	65.67	30.33	2.16

Most rhaphidophorids appear to have a polyandry mating system where females mate with more than one male (Fea & Holwell 2018). One essential benefit of this mating system is a sufficient sperm supply that improves fertilization probability (Slatyer et al. 2012). However, even though females can copulate with multiple males, rhaphidophorids and other ensiferans commonly have various mechanisms to control fertilization success more toward one male than other mates. There are compelling evidences of cryptic female choice mechanisms in which females manage fertilization in their favor. The mechanisms include manipulation of spermatophore attachment duration, sperm uptake regulation, re-copulation with the same male, modification in the rate of oviposition and/or the differential allocation of resources to eggs, and persistence during mating (Vahed 2015). Conversely, intraspecific competition among males to fight over females is evident within raphidophorids (Stritih & Čokl 2012). Sometimes, the agonistic behavior of males involves physical conflict (male-male combat). Thus, males have a particular structure on their hind legs used as armament. At the same time, it is also utilized to capture and grasp females during copulation (Conroy & Gray 2015).

Age Structure

The relative abundance (RA) comparison among size groups showed a homogenous pattern across cave zonation. The greatest RA came from the moderate class, followed by the large class, and the least was the small class (Figure 4). Thus, the accumulative RA from all zones also indicated a similar pattern. This result is in accordance with Lavoie et al. (2007), which reported the predominance of adult to juvenile. However, the comparison between sub-adult and adult was inconsistent with Carchini et al. (1994), that showed

the domination of adult to sub-adult. In contrast, this study revealed the opposite result.

Carchini et al. (1991) revealed that age structure within cave crickets populations was diverse and highly dependent on the season and the natural condition of inhabited caves. According to the study, the population of cave crickets in artificial caves is extremely seasonal since food resources and climatic conditions mainly rely on surface environments. Nevertheless, in natural caves, where colonization is older and climatic condition less fluctuates, age structure tends to be more stable. However, further investigation and continuous monitoring, which cover both rainy and dry seasons, are needed to confirm whether the age structure of the Sanghyang Kenit's population is seasonal or year-round.

The domination of the sub-adult class is a good indicator for the sustainability of cave cricket population in Sanghyang Kenit. This domination indicates that the population is on a positive trend since many individuals are achieving sexual maturity. According to this, the reproduction rate probability is projected to increase, generating new individuals. As mentioned previously, cave crickets are a keystone species that provide food for a diverse group of cave-dwellers, both when they are in the form of eggs, nymphs, and adults (Culver & White 2012; Kurniawan & Rahmadi 2019). Therefore, the successful reproduction of cave crickets will guarantee their existence and the other species that rely on them (Benoit et al. 2004).

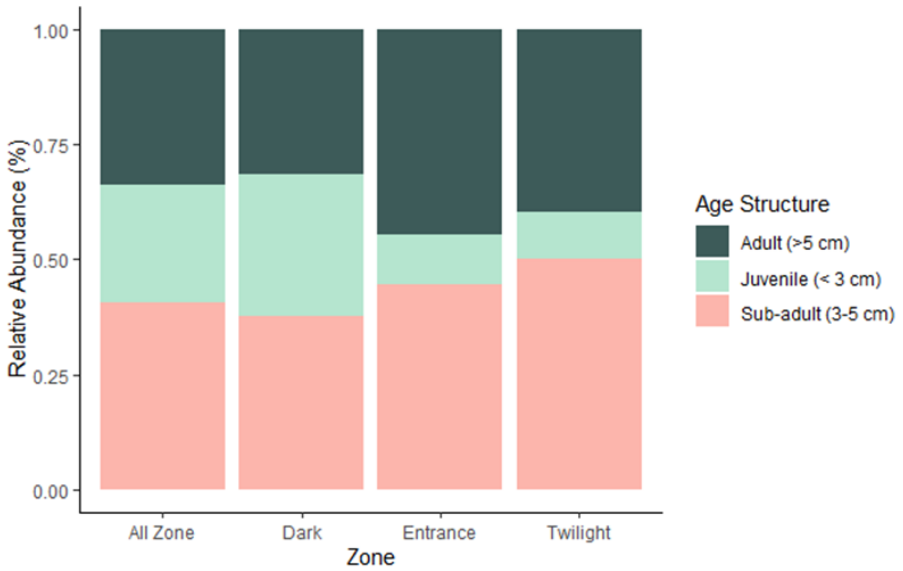

Figure 4. Comparison of relative abundance among size groups across cave zonation.

Habitat Preference based on Environmental Parameters

The results of abiotic environmental parameters measurement were varied across cave zonation. Nevertheless, twilight and dark zones embraced a remarkable similarity to one another than the entrance. This result is in line with previous studies that also indicated the same (Kurniawan et al. 2018b). NMDS output presented in Figure 5 illustrates that the similarity between

those two zones occurred in almost all measured environmental parameters. In contrast, the entrance zone had a higher light intensity, air and soil temperatures, and soil pH, while relative humidity and soil moisture were significantly lower than in twilight and dark zones.

According to the cave cricket abundance and density that have been already discussed, it can be inferred that cave crickets in Sanghyang Kenit preferred twilight and dark zones as their habitat. Figure 5 also showed that abundance (blue arrow) steers from the area of twilight zone towards dark zone. This preference matched the environmental condition where both zones tended to be similar in most measured parameters. Hu et al. (2014) and Epps et al. (2014) mentioned that most cave crickets prefer dark and humid places since they are nocturnal animals. Low light intensity and high humidity were the characters of twilight and dark zones. In this regard, cave crickets were abundant in those two zones.

Cave crickets possess several morphological adaptations that support them to thrive in dark and humid environments. Even cave crickets have eyes, limited sunlight or total darkness in caves obstruct their visual organs from working properly. But, cave crickets have elongated appendages, particularly antennae, which they use as a sensory organ for orientation (Culver & Pipan 2009). In addition, they also have elongated legs that may be used for walking on irregular surfaces in the darkness (Lavoie et al. 2007). High humidity is stressful for most surface animals, but it brings crucial benefits to cave cricket's life. Compared to their surface relatives, the outer cuticle of cave crickets is significantly thinner, making them very sensitive to moisture loss caused by sunlight exposure, high temperature, and low humidity. Staying in humid caves prevents them from experiencing such dreadful evaporation (Lavoie et al. 2007; Howarth & Moldovan 2018a).

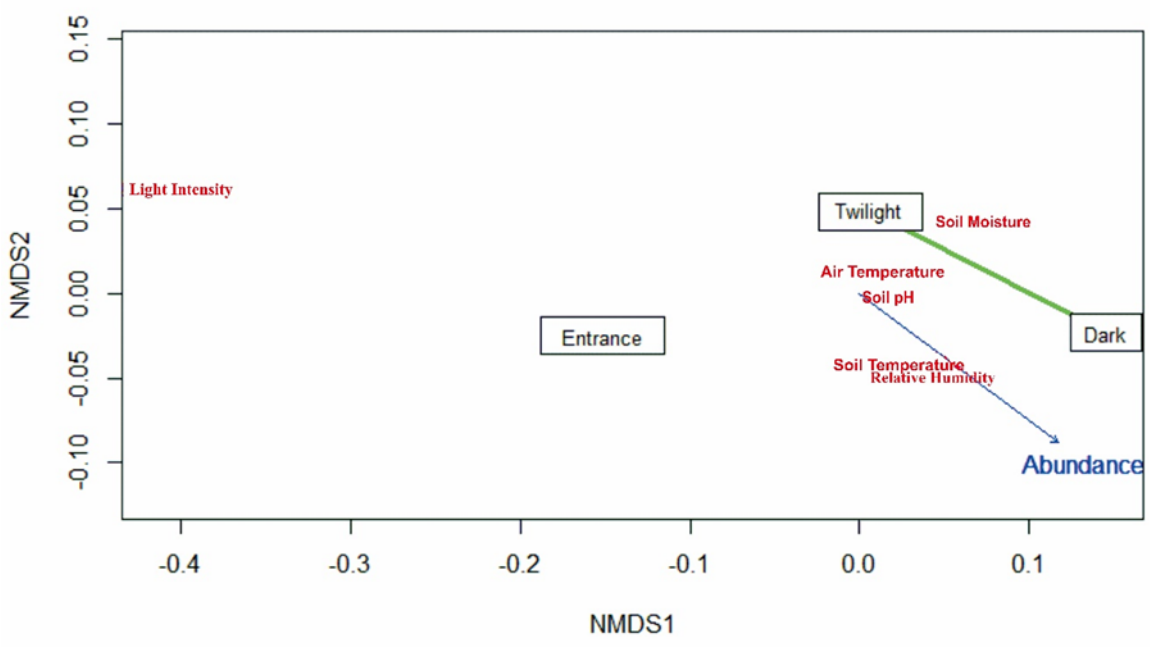

Figure 5. Relatedness among cave zones based on environmental parameters and its relation with cave cricket abundance.

Another essential factor promoting cave cricket preference over twilight and dark zones is food availability, particularly bats guano. This kind of organic matter is the most critical food source in tropical caves (Kovac 2018). A considerable proportion of cave-dwelling animals are guano-dependent, including cave crickets (Moulds 2004; Ferreira 2019). Bats population in Sanghyang Kenit was distributed from twilight to dark zones, which generated guano piles on the cave floor. Commensurate with cave crickets, bats prefer humid and dark places for their roosting sites (Furey & Racey 2016; Lizarro et al. 2020; Newman et al. 2021). The measurement of soil pH showed that twilight and dark zones have more acidic pH than the entrance. This is caused by guano deposits spread in those zones since guano has acidic pH (Mazebedi & Hesselberg 2020). Multiple studies have examined that guano was one of the main deciding factors for distribution, composition, and abundance of various cave-dwelling animals belonging to troglophiles and troglobiont (Tobin et al. 2013; Iskali & Zhang 2015). Therefore, conservation of cave crickets and other troglophiles in Sanghyang Kenit can be carried out by maintaining the bats population inside the cave.

Potential Predator and Competitor

Six other macroarthropods species were successfully recorded during the study. Those species are distributed into two classes (Arachnida and Insecta) and four orders (Aranea, Amblypygi, Blattodea, and Hymenoptera). These groups are well-known to have cavernicolous representatives (Romero 2009; Prakarsa et al. 2021). According to their roles in the ecosystem, most species are true predators, and only a single species acts as a detritivore. In addition, they occupied different cave zones. There were three species exclusively inhabiting entrance, two species in dark zone, and one species that occurred both in entrance and twilight zones (Table 3).

According to the correlation test result presented in correlogram (Figure 6), it can be seen that there are two species of macroarthropods having a strong positive correlation with cave cricket abundance, namely *Heteropoda* sp. and *Catagaeus* sp. This is indicated by the almost perfect circle form with dark blue in the correlogram between *Rhaphidophora* sp and the two mentioned species. This relation happened because *Heteropoda* sp. and *Catagaeus* sp. individuals were concentrated only in dark zone where cave crickets were abundant. Several studies have documented predatory activities of those

Table 3. The list of other macroarthropods in Sanghyang Kenit with their role and distribution within cave passage.

Class	Order	Morphospecies	Ecological Role	Distribution
Arachnida	Araneae	*Heteropoda* sp.	Predator	Dark
		Araneus sp.	Predator	Entrance
		Pholcus sp.	Predator	Entrance
	Amblypygi	*Catagaeus* sp.	Predator	Dark
Insecta	Blattodea	*Periplaneta americana*	Detritivore	Entrance, Twilight
	Hymenoptera	*Polistes* sp.	Predator	Entrance

two species to cave crickets in many Indonesian caves (Kurniawan & Rahmadi 2019; Prakarsa et al. 2021). Thus, the species were considered as potential predators for cave crickets.

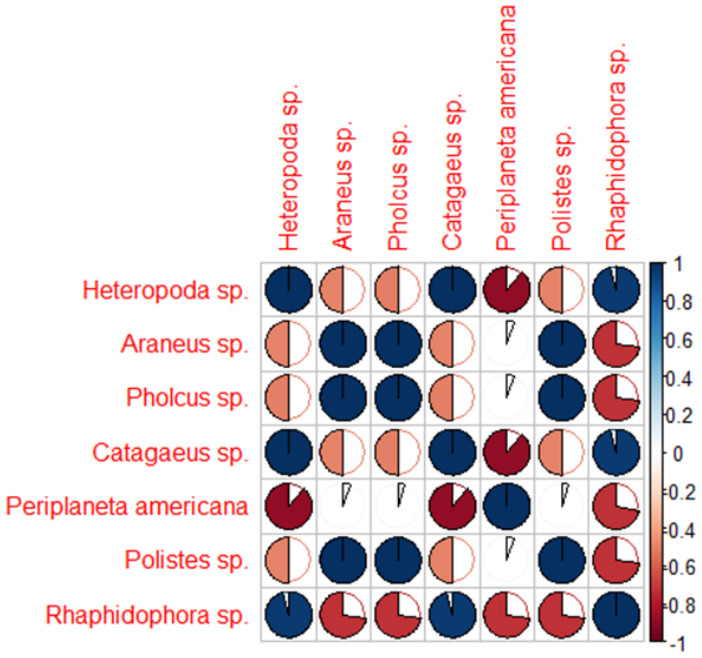

Figure 6. Correlation between cave crickets and the other recorded macroarthropods. A correlation of 1 (solid blue) indicates a perfect positive correlation, while -1 (solid red) depicts a perfect negative correlation.

The existence of predators indicates that cave crickets are an essential component of the cave's food web. Their population can influence the fate of upper trophic level species. In other words, disturbance of cave cricket population can indirectly affect those two predators' survival. This result implies an essential suggestion to the Sanghyang Kenit' show cave managers to ensure they manage the cave wisely. As previously discussed, intensely visited show caves appear to have a lower abundance of cave crickets than wild caves (Kurniawan et al. 2017). This strongly indicates that uncontrolled tourism activities can disturb cave crickets, resulting in population decline.

Among recorded arthropods, only one species can potentially be competitor for cave crickets, namely cockroaches (*Periplaneta americana*). This species was the only detritivore recorded during the sampling efforts. Like cave crickets, many cave-dwelling cockroaches are also guano consumers (Rahmadi & Suhardjono 2007; Ferreira 2019). They are common in a cave with rich guano deposits, and many are restricted to living in guano (guanobites) (Lucañas & Lit 2016). However, the competition likelihood for food between cave crickets and cockroaches in Sanghyang Kenit is considered low. According to their distribution within the cave, both species prefer different habitats. Cockroaches were abundant in entrance and twilight zones, while most cave crickets were concentrated in twilight and dark zones. There was a prospective competition in twilight zone where many individuals from both species gathered. But considering enough guano availability and

their ability to leave the cave for foraging at night (since twilight zone is close to entrance), they are unlikely to compete for food in the cave. In this regard, they could coexist appropriately in the same area.

CONCLUSION

The cave cricket population that occurred in Sanghyang Kenit belongs to *Rhaphidophora* sp. This species was distributed in all cave zones with a total population of around 78-108 individuals. However, the abundance and density of cave crickets in twilight and dark zones were greater than entrance zone. Males predominated females with a sex ratio of 2.16. Moreover, the population was in a positive trend since the sub-adult class was dominating. Cave crickets preferred specific habitats with low light intensity, high humidity, and acidic pH. Two species of arthropods were considered potential predators, namely *Heteropoda* sp. and *Catagaeus* sp. This study implies the importance of protecting cave crickets in Sanghyang Kenit since the population is still growing and highly relies on the natural condition of the cave environment. Besides, they also play a vital role in the cave food web. Therefore, tourism activity in the cave should be managed wisely to prevent disturbances in the cave cricket population.

AUTHORS CONTRIBUTION

I.D.K designed the research framework, gathered references, analyzed data, and wrote the original manuscript, M.K.W and B.S collected and organized raw data, R.T.M.A and R.A.U revised and finalized the manuscript.

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CONFLICT OF INTEREST

The authors declare there is no conflict of interest in any part of this research.

REFERENCES

Allegrucci, G. et al., 2010. Cave crickets and cave weta (Orthoptera, Rhaphidophoridae) from the Southern End of the World: A molecular phylogeny test of biogeographical hypotheses. *Journal of Orthoptera Research*, 19(1), pp.121–130. doi: 10.1665/034.019.0118.

Benoit, J.B. et al., 2004. Mycoflora of a trogloxenic cave cricket, Hadenoecus cumberlandicus (Orthoptera: Rhaphidophoridae), from two small caves in Northeastern Kentucky. *Annals of the Entomological Society of America*, 97(5), pp.989–993. doi: 10.1603/0013-8746(2004)097 [0989:MOATCC]2.0.CO;2.

Bernardini, C. & Di Russo, C., 2004. A general model for the life cycle of Dolichopoda cave crickets (Orthoptera: Rhaphidophoridae). *European Journal of Entomology*, 101(1), pp.69–73. doi: 10.14411/eje.2004.015.

Carchini, G., Russo, C.D. & Sbordoni, V., 1991. Contrasting age structures in cave cricket populations: patterns and significance. *Ecological Entomology*, 16, pp.305–314. doi: 10.1111/j.1365-2311.1991.tb00221.x.

Carchini, G., Rampini, M. & Sbordoni, V., 1994. Life cycle and population ecology of the cave cricket Dolichopoda geniculata (Costa) from Valmarino cave (Central Italy). *International Journal of Speleology*, 23(3/4), pp.203–218. doi: 10.5038/1827-806x.23.3.6.

Chandoo, M. et al., 2013. Frequency of missing legs in the cave cricket, Hadenoecus subterraneus. *Scientia Discipulorum*, 6, pp.56–61.

Conroy, L.P. & Gray, D.A., 2015. Male armaments and reproductive behavior in "Nutcracker" camel crickets (Rhaphidophoridae, Pristoceuthophilus). *Insects*, 6(1), pp.85–99. doi: 10.3390/ insects6010085.

Culver, D.C. & Pipan, T., 2009. *The Biology of Caves and Other Subterranean Habitats*, New York: Oxford University Press.

Culver, D.C. & White, W.B., 2012. *Encyclopedia of Caves Second Edition* 2nd ed., USA: Elsevier Academic Press.

Deharveng, L. & Bedos, A., 2012. Diversity Patterns in The Tropics. In W. B. White & D. C. Culver, eds. *Encyclopedia of Caves*. China: Academic Press, pp. 238–250.

Epps, M.J. et al., 2014. Too big to be noticed: Cryptic invasion of Asian camel crickets in North American houses. *PeerJ*, 2014(1), pp.1–15. doi: 10.7717/peerj.523.

Fea, M. & Holwell, G., 2018. Combat in a cave-dwelling wētā (Orthoptera: Rhaphidophoridae) with exaggerated weaponry. *Animal Behaviour*, 138, pp.85–92. doi: 10.1016/j.anbehav.2018.02.009.

Fernandez-Cortes, A. et al., 2011. Detection of human-induced environmental disturbances in a show cave. *Environmental Science and Pollution Research*, 18(6), pp.1037–1045. doi: 10.1007/s11356-011-0513-5.

Ferreira, R.L., 2019. Guano communities. In W. B. White, D. C. Culver, & T. Pipan, eds. *Encyclopedia of Caves (Third Edition)*. Academic Press, pp. 474 –484. doi: 10.1016/b978-0-12-814124-3.00057-1.

Furey, N.M. & Racey, P.A., 2016. Conservation Ecology of Cave Bats. In C. V. Christian & T. Kingston, eds. *Bats in the anthropocene: Conservation of bats in a changing world*. Switzerland: Springer, pp. 463–500. doi: 10.1007/978-3-319-25220-9.

Hasibuan, R.S. & Lidiawati, I., 2020. Potential Study of Sibiuk Cave as a directive for special interest tourism in Ciampea District , Bogor. , (2012), pp.623–635.

Hidayaturrohmah, N., Hernawati, D. & Chaidir, D.M., 2021. Keanekaragaman Arthropoda Berdasarkan 3 Zona Pencahayaan Di Gua Sarongge Tasikmalaya. *BIOTIK: Jurnal Ilmiah Biologi Teknologi dan Kependidikan*, 8(2), pp.245–258. doi: 10.22373/biotik.v8i2.7778.

Howarth, F.G. & Moldovan, O.T., 2018a. The Ecological Classification of Cave Animals and Their Adaptations. In Oana T. Moldovan, L. Kovac, & S. Halse, eds. *Caves Ecology*. Switzerland: Springer, pp. 41–67.

Howarth, F.G. & Moldovan, O.T., 2018b. Where Cave Animals Live. In Oana Teodora Moldovan, L. Kovac, & S. Halse, eds. *Caves Ecology*. Switzerland: Springer, pp. 23–37.

Hu, C., Yang, J. & Tu, W., 2014. Antennal epicuticular structure of camel crickets (Orthoptera: Rhaphidophoridae) for identifying the prey of Mustella sibrica Pallas. *Advances in Entomology*, 02(01), pp.1–7. doi: 10.4236/ae.2014.21001.

Ingrisch, S. & Rentz, D.C.F., 2009. Orthroptera. In V. H. Resh & R. T. Carde, eds. *Encyclopedia of Insect*. USA: Academic Press, pp. 732–743.

Iskali, G. & Zhang, Y., 2015. Guano subsidy and the invertebrate community in bracken cave: The world's largest colony of bats. *Journal of Cave and Karst Studies*, 77(1), pp.28–36. doi: 10.4311/2013LSC0128.

Kovac, L., 2018. Caves as Oligotrophic Ecosystem. In O. T. Moldovan, L. Kovac, & S. Halse, eds. *Caves Ecology*. Switzerland: Springer, pp. 297–307.

Kurniawan, I.D. et al., 2017. The Detection of Human Activities' Impact on Show Caves Environment in Pacitan, Indonesia. In S. Moore, Kevin; White, ed. *Proceedings of the 17th International Congress of Spelology*. Sydney, pp. 175–178.

Kurniawan, I.D. et al., 2018a. The difference on Arthropod communities ' structure within show caves and wild caves in Gunungsewu Karst area , Indonesia. *Ecology, Environment and Conservation*, 24(1), pp.72–81.

Kurniawan, I.D. et al., 2018b. Cave-dwelling Arthropod community of Semedi Show Cave in Gunungsewu Karst Area , Pacitan , East Java , Indonesia. *Biodiversitas*, 19(3), pp.857–866. doi: 10.13057/biodiv/d190314.

Kurniawan, I.D. & Rahmadi, C., 2019. *Ekologi Gua Wisata: Dampak Aktivitas Wisata terhadap Lingkungan dan Biota Gua serta Upaya Konservasinya*, Yogyakarta: Graha Ilmu.

Kurniawan, I.D. et al., 2020. Correspondence between bats population and terrestrial cave-dwelling arthropods community in Tasikmalaya karst area. *Communications in Mathematical Biology and Neuroscience*, 2020(59), pp.1–21. doi: https://doi.org/10.28919/cmbn/4830.

Lavoie, K.H., Helf, K.L. & Poulson, T.L., 2007. The biology and ecology of North American cave crickets. *Journal of Cave and Karst Studies*, 69(1), pp.114–134.

Lizarro, D. et al., 2020. Characterization of caves as bat roosts in the brazilian-paranense biogeographic region of bolivia. *Therya*, 11(3), pp.390–397. doi: 10.12933/therya-20-1008.

Lucañas, C.C. & Lit, I.L., 2016. Cockroaches (Insecta, Blattodea) from caves of Polillo Island (Philippines), with description of a new species. *Subterranean Biology*, 19(1), pp.51–64. doi: 10.3897/subtbiol.19.9804.

Mammola, S., 2019. Finding answers in the dark: caves as models in ecology fifty years after Poulson and White. *Ecography*, 42(July), pp.1331–1351. doi: 10.1111/ecog.03905.

Mazebedi, R. & Hesselberg, T., 2020. A preliminary survey of the abundance, diversity and distribution of terrestrial macroinvertebrates of Gcwihaba cave, northwest Botswana. *Subterranean Biology*, 35, pp.49–63. doi: 10.3897/subtbiol.35.51445.

Moulds, T., 2004. Review of Australian cave guano ecosystems with a checklist of guano invertebrates. *Proceedings of the Linnean Society of New South Wales*, 125(January), pp.1–42.

Mulyani, E., 2011. Produksi, Konsumsi Semen Dan Bahan Bakunyadi Indonesia Periode 1997 – 2009 Dan Prospeknya 2010 – 2015. *Jurnal Teknologi Mineral dan Batubara*, 7(2), pp.82–89.

Newman, B.A., Loeb, S.C. & Jachowski, D.S., 2021. Winter roosting ecology of tricolored bats (Perimyotis subflavus) in trees and bridges. *Journal of Mammalogy*, 102(5), pp.1331–1341. doi: 10.1093/jmammal/gyab080.

Oksanen, J. et al., 2020. Package 'vegan.' *Community Ecology Package*, pp.1–298. Available at: https://cran.r-project.org/web/packages/vegan/vegan.pdf [Accessed August 31, 2021].

Pacheco, G.S.M. et al., 2020. Tourism effects on the subterranean fauna in a Central American cave. *Insect Conservation and Diversity*, 2020 (September), pp.1–13. doi: 10.1111/icad.12451.

Prakarsa, T.B.P. & Ahmadin, K., 2017. Diversitas Arthropoda Gua di kawasan Karst Gunung Sewu, Studi gua-gua di Kabupaten Wonogiri. *BIOTROPIC The Journal of Tropical Biology*, 1(2), pp.31–36. doi: 10.29080/biotropic.2017.1.2.31-36.

Prakarsa, T.B.P., Kurniawan, I.D. & Putro, S.T.J., 2021. *Biospeleologi: Biodiversitas Gua, Potensi, dan Permasalahannya*, Yogyakarta: Bintang Pustaka Madani.

Prous, X., Ferreira, R.L. & Jacobi, C.M., 2015. The entrance as a complex ecotone in a Neotropical cave. *International Journal of Speleology*, 44(2), pp.177–189. doi: 10.5038/1827-806X.44.2.7.

Rahmadi, C., 2002. Keanekaragaman Arthropoda di Gua Ngerong, Tuban, Jawa Timur. *Zoo Indonesia*, 29, pp.19–27.

Rahmadi, C. & Suhardjono, Y.R., 2007. Arthropoda Gua di Nusakambangan Cilacap, Jawa Tengah. *Zoo Indonesia*, 16(1), pp.21–29.

Rahmadi, C., 2008. *Cave Fauna of Java*, Bogor. Available at: https://www.rufford.org/files/40.11.06 Detailed Final Report.pdf.

Rahmadi, C., 2011. Biospeleologi of java caves, Indonesia: a Review. In E. Haryono, T. N. Adjie, & Suratman, eds. *Asian Trans-Disciplinary Karst Conference 2011*. Yogyakarta, pp. 241–250.

Ravn, N.R., Michelsen, A. & Reboleira, A.S.P.S., 2020. Decomposition of Organic Matter in Caves. *Frontiers in Ecology and Evolution*, 8(October), pp.1–12. doi: 10.3389/fevo.2020.554651.

Romero, A., 2009. *Cave Biology Life in Darkness*, New York: Cambridge University Press.

Di Russo, C. & Rampini, M., 2017. A new species of the genus Rhaphidophora from Seram island (Moluccas, Indonesia) with notes on the geographic distribution of the subfamily Rhaphidophorinae (Orthoptera, Rhaphidphoridae) Claudio. *International Journal of Entomology Research*, 2(2), pp.55–58.

Slatyer, R.A. et al., 2012. Estimating genetic benefits of polyandry from experimental studies: A meta-analysis. *Biological Reviews*, 87(1), pp.1–33. doi: 10.1111/j.1469-185X.2011.00182.x.

Song, H. et al., 2020. Phylogenomic analysis sheds light on the evolutionary pathways towards acoustic communication in Orthoptera. *Nature Communications*, 11(1), pp.1–16. doi: 10.1038/s41467-020-18739-4.

Stritih, N. & Čokl, A., 2012. Mating Behaviour and Vibratory Signalling in Non-Hearing Cave Crickets Reflect Primitive Communication of Ensifera. *PLoS ONE*, 7(10), pp.1–10. doi: 10.1371/journal.pone.0047646.

Subekti, T., 2016. Konflik Samin vs PT. Semen Indonesia. *Jurnal Transformative*, 2(2), pp.189–202. Available at: https://transformative.ub.ac.id/index.php/jtr/article/view/136.

Tobin, B.W., Hutchins, B.T. & Schwartz, B.F., 2013. Spatial and temporal changes in invertebrate assemblage structure from the entrance to deep -cave zone of a temperate marble cave. *International Journal of Speleology*, 42(3), pp.203–214. doi: 10.5038/1827-806X.42.3.4.

Vahed, K., 2015. Cryptic Female Choice in Crickets and Relatives (Orthoptera: Ensifera). In A. V. Peretti & A. Ainseberg, eds. *Cryptic Female Choice in Arthropods*. Springer International Publishing, pp. 285–324. doi: 10.1007/978-3-319-17894-3_11.

Wei, T. & Simko, V., 2021. An Introduction to corrplot Package. *Package 'corrplot'*, pp.1–26. Available at: https://cran.r-project.org/web/packages/corrplot/corrplot.pdf [Accessed July 13, 2022].

Wynne, J.J. et al., 2019. Fifty years of cave arthropod sampling: Techniques and best practices. *International Journal of Speleology*, 48(1), pp.33–48. doi: 10.5038/1827-806X.48.1.2231.

Yoder, J.A. et al., 2010. The pheromone of the cave cricket, Hadenoecus cumberlandicus, causes cricket aggregation but does not attract the co-distributed predatory spider, Meta ovalis. *Journal of Insect Science*, 10(1), pp.1–10. doi: 10.1673/031.010.4701.

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Research Article

Antifungal Activities of *Neobalanocarpus heimii* (Cengal) Heartwood Extracts on *Trametes versicolor* and *Coniophora puteana*

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ABSTRACT

Neobalanocarpus heimii (Cengal) is from the family Dipterocarpaceae. It is a long-lasting wood that is also one of the most robust timbers in the world. This species is native to Peninsular Malaysia and southern Thailand**.** In this study, the Cengal heartwood was studied concerning the amount of water extractive content with antifungal properties from the *Neobalanocarpus heimii*. The dilution method was used to test the antifungal properties. Wood meals samples were subjected to the sequential extractive beginning with hexane followed by dichloromethane, methanol and water. The extracts were collected and underwent evaporation by using rotary evaporator to obtain pure crude extract. The antifungal activities were determined using agar dilution method. Two selected fungi *Trametes versicolor* (*T. versicolor*) and *Coniophora puteana* (*C. puteana*) were used. The antifungal index (%) which compares the diameter of the growth zone for the experimental plate and control plate was calculated. The total percentage of yield from *Neobalanocarpus heimii* was 0.28%. The highest antifungal index obtained for *Trametes versicolor* (*T. versicolor)* was 81.22%, while *C. puteana* was 43.24%. The crude extracts from *Neobalanocarpus heimii* were effective in inhibiting the growth of *Trametes versicolor* and *Coniophora puteana.*

INTRODUCTION

The traditional toxic wood preservatives that create environmental hazards can be replaced by secondary metabolites of timber with antifungal properties and can be used as natural biodegradable fungicides. Antifungal refers to a chemical compound produced biosynthetically or synthetically that could destroy or usefully suppress the metabolism of various harmful microscopic organisms (Kawamura et al. 2010).

An abundance of naturally durable wood species demonstrates improved efficiency and durability in outdoor exposure without preservatives. When assessing and forecasting the efficiency of a naturally durable wood species, wood extractives are widely considered to be a contributing factor (Kirker et al. 2013). Wood extractives are the non-structural components of wood. It is often produced by the standing tree as defensive compounds to

environmental stresses and is typically concentrated in the heartwood (Taylor et al. 2002). In terms of wood extractives, extractive content varies greatly not only from tree to tree but also within an individual tree, and by employing various extraction methods, extractives from wood species with high natural resistance can be used to improve the durability of non-durable wood against insects, fungi, mould, and termites (Kadir & Hale 2019).

According to (Bernhoft 2010), plants generate bioactive compounds as secondary metabolites, a form of compound other than primary metabolites that are thought to aid plants in increasing their overall ability to thrive and resolve local challenges by enabling them to interact with their environment. The occurrence of wood-decay fungi species may affect factors such as the degree of decay, the amount of bark left on the log and the previous fungal population (Renvall 1995; Niemelä et al. 1995; Kruys et al. 1999). The community of decomposers can be affected in many ways by the internal wood characteristics partly related to the tree growth rates. A higher concentration of total nitrogen and amino acids was found in the fast-growing wood from *Pinus sylvestris* than slow-growing wood shown by (Sundberg et al. 1993). N and P concentrations are essential determinants of fungal growth rates (Edman et al. 2006), and this is also probably true for amino acids.

Neobalanocarpus heimii (*N. heimii* = Cengal) is well known throughout the Peninsular. According to (Symington et al. 2004), the most botanical difference of this species is in flower, having oblong anthers and short appendages quite unlike the oval anthers and filiform appendages of balanocarpus bed., 7 which is now considered a synonym within the type of hopea sections. *Neobalanocarpus heimii* comes from the family of dipterocarpaceae, one of Malaysia's heavy hardwoods' products. Furthermore, many dipterocarp plants have been found to contain a variety of terpenoids and sesquiterpenes, which is thought to act as a defence compound against fungi and insects (Kadir & Hale 2019). Yamamoto (1988) stated that Cengal has a durable wood with air -dry density ranging from over 915 to 980 kg/m^3 even under adverse conditions. According to Symington et al. (2004), Cengal is among the most robust timbers globally that is 50% stronger than teak and resistant to termite and fungi. In addition, high extractive contents in the heartwood of *N. heimii* give a high degree of decay resistance (Kim et al. 2006). It also offers high economic value due to its strength and durability. Thus, it produces a strong and naturally durable wood used for heavy construction, boats, buildings, bridges, to which strength is essential (Tnah et al. 2012). Furthermore, it can improve the cooling effect for Malaysia's green building which is designed to save energy consumption, to minimize the impact on climate change, and to reduce the rate at which we consume natural resources. Cengal has good thermal conductivity and resistivity and can circulate heat more effectively than Meranti (Mohamed et al. 2015).

MATERIALS AND METHODS

Sample collection

A sample of the heartwood of *Neobalanocarpus heimii* was obtained from Bentong, Pahang. 8.0 kg of fresh sample was collected and air-dried for two months from September to November 2017. The heartwood sample of Cengal was milled to a very fine homogenous composition and grounded into a fine powdery mixture (Hosseinihashemi et al. 2013).

Solvent extraction

Extraction of wood was carried out using solvent extraction method according to the procedure described by (Chang et al. 1999) with slight modification. The wood meal of *Neobalanocarpus heimii* was placed in the 3000 mL separator funnel and immersed in hexane solvent (non-polar) for two weeks to remove the hexane-soluble compound from the samples. The wood meals in the separator funnel were evenly stirred with a glass rod twice a day every day for two consecutive weeks. The crude extract was drained and the solvent was subsequently replaced with dichloromethane (semi-polar), methanol and water (polar). The solvent for the extraction was replaced by a polar solvent from a non-polar one to get the pure crude of water extract from *Neobalanocarpus heimii* wood meals. The extract was evaporated into dryness using a vacuum rotary evaporator at 100˚C. Pure crude extracts were dried in an oven at 60˚C and weighed.

The collected crude extracts were tested to determine their antifungal properties. The percentage yield of the crude extracts was obtained from the weight of the sample before and after the extraction procedure. The percentage was calculated as follows (Chang et al. 1999):

$$Y\,(\%) = \frac{S_2}{S_1} \times 100\% \qquad (1)$$

Where:

Y = Yield percentage of pure crude

S_1 = Weight of wood sample before extraction

S_2 = Weight of crude extract

Dilution method

The method that was used for the antifungal properties is the dilution method. In this method, 10.0 g of crude extract was diluted with distilled water to prepare the stock solution. This dilution was done in order to obtain final concentration of 50 mg/ml, 25mg/ml and 10 mg/ml. For each concentration, seven replications were prepared. Diluted water crude mixed with agar was used as treatment while agar without crude extract acted as a control. The diameter of growth of the mycelium on agar was observed and recorded after 6 days.

Determination of Antifungal

Preparation of fungi pure culture

The method used for fungi culture was described by (Sibero et al. 2016) with slight modification. 7.0 g of Malt Extract Agar (MEA) powder was weighed and dissolved in 200 mL of distilled water. MEA solution was stirred with a stirrer on the hotplate machine before autoclaving at 121˚C for two hours. MEA solution was poured onto the sterile disposable petri dishes and left to cool and solidify. Two selected fungi (*Trametes versicolor* and *Coniophora puteana*) were taken from stock culture and then transferred to Malt Extract Agar (MEA) and incubated at room temperature for 7 days. It was done aseptically in the lamina flow hood to prevent contamination. Fungi growth was checked frequently to make sure there was no contamination. The growing mycelia with a 0.5 cm diameter were then harvested and tested with different concentrations of crude heartwood extract from *N. heimii*.

Antifungal analysis

The antifungal activity assay against of *T. versicolor* and *C. puteana* growth was carried out according to Özgenç et al. (2017). Each petri dish was filled with 15 mL of Malt Extract Agar (MEA) medium which contained the extracted sample at concentrations of 50, 25, and 10 mg/ml. The petri dish's centre was filled with a 1 cm disc of a tested fungi culture that had been growing for seven days. The colony's diameter was measured in centimetres after incubation. The following formula was used to calculate the antifungal index which was expressed as a percentage of inhibition (Chang et al. 1999),

$$\text{Antifungal index} = \left(1 - \frac{Da}{Db}\right) \times 100\% \quad (2)$$

Where Da is the mean diameter of growth zone in the experimental plate (cm) and Db is the mean diameter of growth zone in control plate (cm).

RESULTS AND DISCUSSION

Crude extract of *Neobalanocarpus heimii* was obtained using successive extraction of different solvent polarity and the total percentage of water crude extract obtained was 0.28% with 19 g mass crude (Table 1).

The amount of extractive content found by percentage yield of water crude extractive obtained from *N. heimii* heartwood meal was 0.28%. The percentage obtained was too low. Low percentage yield might be caused by the temperature of the solvent extraction itself. According to Ahmad (2013), on previous study, removing extractives from sound wood in cold water was a prolonged process with an initial rate of 0.012% hour^{-1} compared to hot water with an equivalent rate of 0.197% hour^{-1}. The duration of extraction does not play an essential role as a more extended extraction period resulted in a higher percentage of crude extract due to the longer time between the solvent and the solute. However, the solvent and sample would be stopped with extractive content after some duration, so excessive extraction was unnecessary. This is based on Fick's law of diffusion (Baldosano 2015).

Table 1. Percentage yield of crude extracts from N. heimii heartwood after extracted with hexane, dichloromethane, methanol, and distilled water.

Wood meal sample (g)	Crude extract (%)
Neobalanocarpus heimii	0.28

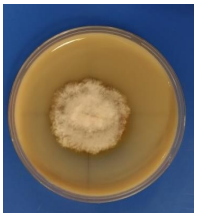
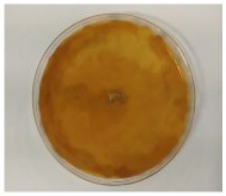
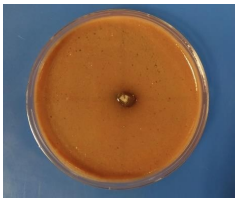
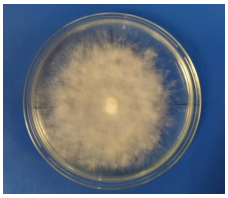
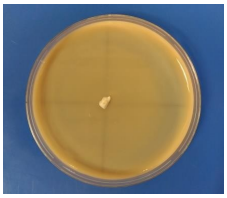
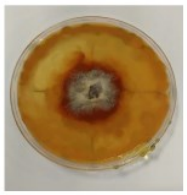
Among the three concentrations of crude extract used as seen in Table 2, the maximum antifungal index was 100% at the highest concentration 50 mg/ml for both fungi, *T. versicolor* and *C. puteana*. At a 25 mg/ml concentration, the antifungal index was 100% and 43.24% for *T. versicolor* and *C. puteana,* respectively. The record also shows that the concentration of crude extract at 25 mg/ml inhibits the growth of T. versicolor but is susceptible to *C. puteana*. When *T. versicolor* was tested using 10 mg/ml concentration, the antifungal index showed 81.22%. Low concentration allows the growth of fungi and the diameter of fungi growth would be increased due to the high susceptible enzyme of *T. versicolor* with the crude extract. This indicates that the antifungal activities depend on the concentration of extracts, the higher concentrations completely inhibited mycelial growth (Bopenga et al. 2020). In fact, decay activity of the timbers that caused by the fungi was reduced by increasing the concentrations of wood extracts due to compound in the extracts that slowed fungal attack and decreased weight loss in a susceptible wood species (Kadir & Hale 2019).

Table 2. Antifungal index of T. versicolor and C. puteana in different concentration of water crude extract from *N. heimii.*

Concentration (mg/ml)	Antifungal index (%)	
Control	***Trametes versicolor***	***Coniophora puteana***
	0	0
10	81.22	0
25	100	43.24
50	100	100

The effectiveness of extractives against wood-rotting fungus *T. versicolor* and *C. puteana* has been evaluated in bioassay for antifungal activity. The fungal growth on wood extract on agar medium was tabulated in Table 3. The result showed that a high concentration of heartwood extracts inhibits fungus growth for both types, *C. puteana* and *T. versicolor* compared to the control sample. The results of antifungal assays of *T. versicolor* and *C. puteana* were tested with different concentrations of water crude extracts from *N. heimii*. Saidan et al. (2020) reported that Cengal wood showed antifungal activities against *C. albicans* and *A. brasiliensis*. Furthermore, some researchers also found the extract of Cengal heartwood have effective termiticidal and fungicidal properties against subterranean termites (*C. curvignathus* and *C. gestroi*) and fungi (*T. versicolor*, *C. puteana*, and *L. sajor-caju*) (Kadir & Hale 2019). The fungi *T. versicolor* was able to grow at a concentration of 10 mg/ml (81.22%) because the concentration of the extract was not effective in inhibiting the growth of fungi.

Table 3. Antifungal activity of *T. versicolor* and *C. puteana* in three different concentrations of sample.

Types of fungi	Concentration of sample			
	Control	10 mg/ml	25 mg/ml	50 mg/ml
Trametes versicolor				
Coniophora puteana				

According to Teeri (1997), White-rot fungi secrete one or more of three extracellular enzymes important for lignin degradation, such as lignin peroxidase, manganese-dependent peroxidase, and laccase. As a result, they can completely mineralize all cell wall polymers in hardwood decay. In addition, plant diversity affects extractive material variation. Alkaloids, terpenoids, condensed tannins, and a variety of other chemicals can be found in extractives, and they are responsible for natural durability (Taylor et al. 2002).

There is no growth of *C. puteana* recorded in the concentration of 10 mg/ml. This might be due to some error when handling the laboratory session. At a concentration of 25 mg/ml and 50 mg/ml, *T. versicolor* did not show a positive growth rate as the increase in concentration lead to the effective growth inhibition of fungi. The antifungal index of *C. puteana* showed 43.24% at a concentration of 25 mg/ml. The fungi could still grow because the enzyme secreted by this species of brown-rot fungi is still active to degrade the extractive contained in the MEA solution. Antifungal properties start to inhibit the growth of *C. puteana* at a 50 mg/ml concentration whereby the extractives had produced a toxic effect on the continued growth of fungi. Scheffer et al. (1966) stated that some researchers attempted to correlate the natural resistance of wood to fungal decay with several factors such as toxic wood extractives, structural features of wood, lignin and cellulose content of cell walls, depletion of reserve food materials, moisture content and nitrogen content of the wood. In spite of this they are vital in determining wood properties such as durability. The ability of fungi to degrade wood is determined by its chemical properties and structural features (Kadir & Hale 2019).

CONCLUSION

This study was demonstrated to determine the amount of water extracts from *Neobalanocarpus heimii* and to show how crude extracts affected the growth decay of two fungi, *Trametes versicolor* and *Coniophora puteana*. Crude

extracts obtained from *Neobalanocarpus heimii* were 0.28%. Antifungal properties of *N. heimii* were shown through the inhibition of growth of both fungi by crude extracts according to its level of concentration.

The antifungal activities determined by the antifungal index revealed that at a concentration of 25 mg/ml, *Trametes versicolor* was inhibited. Crude extracts have the higher inhibitory ability on *Trametes versicolor* which starts to inhibit growth at a concentration of 25 mg/ml while *Coniophora puteana* starts to inhibit growth at a concentration of 50 mg/ml. This study has shown that water crude extracts from *N. heimii* contain a compound that inhibit the growth of *Trametes versicolor* (white rot) and *Coniophora puteana* (brown rot).

AUTHORS CONTRIBUTION

N.A.M. carried out the research, wrote, and revised the article. I.J. conceptualised the central research idea and provided the theoretical framework. F.P. supervised the writing progress.

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CONFLICT OF INTEREST

The authors agree that this research was conducted in the absence of any self-benefits, commercial or financial conflicts and declare the absence of conflicting interests with the funders.

REFERENCES

Ahmad et al., 2013. Changes in extractive content on wood surfaces of Chengal (Neobalanocarpus heimii) and effects on performance. *Journal of Tropical Forest Science*, pp.278-288.

Bernhoft, A., 2010. A brief review on bioactive compounds in plants. *Bioactive compounds in plants-benefits and risks for man and animals*, 50, pp.11-17.

Baldosano et al., 2015. Effect of particle size, solvent and extraction time on tannin extract from Spondias purpurea bark through soxhlet extraction. *Proceedings of the DLSU Research Congress*, 3, pp.1-6.

Bopenga et al., 2020. Relationships between chemical composition and decay durability of Coula edulis Baill as an alternative wood species in Gabon. *Wood science and technology*, 54(2), pp.329-348. doi: 10.1007/s00226-020-01158-5.

Chang et al., 1999. Antifungal compounds in the ethyl acetate soluble fraction of the extractives of Taiwania (Taiwania cryptomerioides Hayata) heartwood. *Holzforschung*, 53(5), pp.487-490. doi: 10.1515/HF.1999.080.

Edman, M., Möller, R. & Ericson, L., 2006. Effects of enhanced tree growth rate on the decay capacities of three saprothrophic wood-fungi. *Forest Ecology and Management*, 232(1-3), pp.12-18. doi: 10.1016/j.foreco.2006.05.001.

Hosseinihashemi, S.K., Safdari, V. & Kanani, S., 2013. Comparative Chemical Composition of n-Hexane and Ethanol Extractives from The Heartwood of Black Locust. *Asian Journal of Chemistry*, 25(2). pp.929-933.

Kadir, R. & Hale, M., 2019. Biocidal potential of the extractives of four Malaysian timbers against subterranean termites and wood decay fungi. *European Journal of Wood and Wood Products*, 77(1), pp.147-155. doi: 10.1007/s00107-018-1361-5.

Kim et al., 2006. Micromorphological and chemical characteristics of cengal (Neobalanocarpus heimii) heartwood decayed by soft rot fungi. *Journal of the Korean Wood Science and Technology*, 34(2), pp.68-77.

Kawamura et al., 2010. Antifungal activities of extracts from heartwood, sapwood and bark of 11 malaysian timbers against *Gloeophyllum trabeum* and *Pycnoporus sanguineus*. *Journal of Tropical Forest Science*, pp.170-174.

Kruys et al., 1999. Wood-inhabiting cryptogams on dead Norway spruce (*Picea abies*) trees in managed Swedish boreal forests. *Canadian Journal of Forest Research*, 29(2), pp.178-186.

Kirker et al., 2013. The role of extractives in naturally durable woods species. *International Biodeterioration & Biodegradation*, 82, pp.53-58. doi: 10.1016/j.ibiod.2013.03.007

Mohamed et al., 2015. CFD simulation using wood (cengal and meranti) to improve cooling effect for Malaysia green building. *ARPN Journal of Engineering and Applied Sciences*, 10(20), pp.9462-9467.

Niemelä, T., Renvall, P. & Penttilä, R., 1995. Interactions of fungi at late stages of wood decomposition. *Annales Botanici Fennici*, 32(3), pp. 141-152.

Özgenç, Ö. et al., 2017. A comparison between some wood bark extracts: Antifungal activity. *Kastamonu University Journal of Forestry Faculty*, 17(3), pp.502-508. doi: org/10.17475/kastorman.282637.

Renvall, P., 1995. Community structure and dynamics of wood-rotting Basidiomycetes on decomposing conifer trunks in northern Finland. *Karstenia*, 35, pp.1-51.

Saidan et al., 2020, August. Effect of Chengal Wood (*Neobalanocarpus heimii*) on Physicochemical and Antimicrobial Properties of Coconut Sap (Cocos nucifera). *IOP Conference Series: Earth and Environmental Science*, 549(1), 012073. doi: 10.1088/1755-1315/549/1/012073.

Sibero et al., 2016. Evaluation of antimicrobial activity and identification of yellow pigmented marine sponge-associated fungi from Teluk Awur, Jepara, Central Java. *J. Biotechnol*, 21, pp.1-11.

Sundberg et al., 1993. The relationship between crown size and ring width in *Pinus sylvestris* L. stems: dependence on indole-3-acetic acid, carbohydrates and nitrogen in the cambial region. *Tree Physiology*, 12(4), pp.347-362. doi: 10.1093/treephys/12.4.347.

Symington, C. F. et al., 2004. *Foresters' manual of dipterocarps. Second edition*. Malayan Forest Records No. 16. Forest Research Institute Malaysia, Kepong.

Taylor, A.M., Gartner, B.L. & Morrell, J.J., 2002. Heartwood formation and natural durability-a review. *Wood and Fiber Science*, 34(4), pp.587-611.

Teeri, T.T., 1997. Crystalline cellulose degradation: new insight into the function of cellobiohydrolases. *Trends in biotechnology*, 15(5), pp.160-167. doi: 10.1016/S0167-7799(97)01032-9

Tnah et al., 2012. DNA extraction from dry wood of *Neobalanocarpus heimii* (Dipterocarpaceae) for forensic DNA profiling and timber tracking. *Wood science and technology*, 46(5), pp.813-825. doi: 10.1007/s00226-011-0447-6.

Yamamoto, K. & Hong, L.T., 1988. Decay resistance of extractives from chengal (Neobalanocarpus heimii). *Journal of Tropical Forest Science*, 1 (1), pp.51-55.

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Research Article

Morphometric Analysis of Sumatran, Kalimantan, and Javan *Cyrtodactylus*, which were Labelled as *Cyrtodactylus marmoratus*, Revealed Undescribed Species

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ABSTRACT
Cyrtodactylus marmoratus is originally described based on the specimen from Java. Due to the marbled body colour pattern and the similarity in morphology, many specimens from outside of Java have been identified as *C. marmoratus*. The ongoing research, both molecular and morphological studies, showed that *C. marmoratus* is restricted to Java. The taxonomic status of specimens labelled as *C. marmoratus* from outside Java remains unresolved. Here, we study the morphometric and meristic of Javan, Sumatran, and Kalimantan *Cyrtodactylus* which were labelled as *C. marmoratus* to reveal their taxonomic status. We examined 11 morphometric and 19 meristic characters in 51 specimens at Museum Zoologicum Bogoriense (MZB) originally from, Java, Sumatra, and Kalimantan labelled as *C. marmoratus* as well as other recognized species. Principal Component Analysis (PCA) results show that *C. marmoratus* from Java differs from previous specimens which labelled as *C. marmoratus* from Sumatra and Kalimantan. The PCA results also show that *C. marmoratus* from Martabe differs from *C. marmoratus* Java which is supported by statistical analysis on interorbital, HeadW, HeadD, tubercle on the ventrolateral fold, dorsal tubercle and ventral scales. We hypothesized that *Cyrtodactylus* from Martabe is a suspected undescribed species-await formal description, and overall molecular analyses are needed for future study.

INTRODUCTION
The genus *Cyrtodactylus* (Gray 1827) is the most diverse genera among gekkonids with 305 validated species (Uetz et al. 2021), and it is widely distributed in India, Indochina, Sundaland, Wallacea and Papua (Wood et al. 2012; Grismer et al. 2020). Despite the diversity of *Cyrtodactylus* is relatively high, several species are difficult to recognize because of their morphological similarity to other known species (Davis et al. 2021). The diversity of *Cyrtodactylus* in Sumatra, Java and Borneo islands is still underestimated and much remains undescribed species (O'connell et al. 2019; Davis et al. 2021). Currently, Sumatra consists of seven recognized species (*C. agamensis, C. lateralis,*

C. quadrivirgatus, C. semicinctus, C. psarops, C. consobrinus and *C. marmoratus* (Harvey et al. 2015), whereas Borneo consists of 14 species (*C. consobrinus, C. malayanus, C. baluensis, C. pubisulcus, C. cavernicolus, C. ingeri, C. matsuii, C. hantu, C. limajalur, C. miriensis, C. muluensis, C. quadrivirgatus, C. philippinicus* and *C. yoshii*) (Hikida 1990; Davis et al. 2019; Uetz et al. 2021) and Java consists of three species (*C. marmoratus*, *C. semiadii, C. petani*) (Uetz et al. 2021; Riyanto et al. 2020).

The Java island has a diversity of *Cyrtodactylus* that need further research to reveal hidden species. O'Connel et al (2019) provide the phylogenetics tree of *Cyrtodactylus* on Sumatra and West Java, which West Java has one putative new species in *C. marmoratus* group. However, the dispersal pattern of *C. marmoratus* group in West Java remained unresolved. In this case, *C. marmoratus* is a widely distributed species that can be found in Sumatra, Java, Borneo, Malay Peninsula, and West Papua. Meanwhile, Dring (1979) transferred all specimens of *C. marmoratus* from the Malay Peninsula and Singapore to *C. quadrivirgatus*. The type locality of *C. marmoratus* was mentioned from Java. Mecke et al. (2016b) were done redescription the type series of *C. marmoratus*, which is then based on the compatibilities of morphological characters. Subsequently, Riyanto et al. (2020) determined the specimens from Cibodas as the "true *marmoratus*. Both of Mecke et al (2016b) and Riyanto et al (2022) agree that *C. marmoratus* is restricted to Java.

The taxonomic status of *C. marmoratus* outside Java is remain unresolved. Mecke et al. (2016a) redescription of *C. marmoratus* from Sulawesi resulted that the Sulawesi population belong to *C. fumosus*. These results indicated that the specimens were identified as *C. marmoratus* from Sumatra and Borneo suspected to be different species. In this paper, we examined museum specimens which were labelled as *C. marmoratus* to the revealed taxonomic status of *C. marmoratus* in Sumatra and Kalimantan.

MATERIALS AND METHODS

Materials

We follow Riyanto et al. (2020) to identify specimens from Cibodas (West Java) as the true *C. marmoratus* due to the similarity in its morphology to the recent type series description by Mecke et al. (2016b). A total of 51 specimens collected from Kalimantan and Sumatra which have labelled and have similar pattern to *C. marmoratus* were examined. We included *C. consobrinus, C. malayanus,* and *C. lateralis* as the comparisons. All specimens examined here are stored in Museum Zoologicum Bogoriense (MZB) (Table 1), localities information presented in Figure 1. We applied a methylene blue in 70% ethanol to stain the skin of specimens to get clear visualization of some minute structures, such as sub-digital scales and pores following Harvey et al. (2015).

Methods

Morphological data

Measurements were taken with Mitutoyo® dial callipers to the nearest 0.1

Table 1. Locations, sample-sizes and grouping into primary Operational Taxonomic Units (OTUs).

Localities	Sample size	Species ID	Primary OTU
Bukit Lawang, Sumatra	2	*C. consobrinus*	*C. consobrinus*
Mt. Bondang Murung, Central Kalimantan	1	*C. consobrinus*	*C. consobrinus*
Mt. Meratus, East Kalimantan	2	*C. consobrinus*	*C. consobrinus*
Seulawah Agam, Aceh	6	*C. lateralis*	*C. lateralis*
Ketambe, Aceh, Sumatra	1	*C. lateralis*	*C. lateralis*
Bukit Lawang, Sumatra	1	*C. lateralis*	*C. lateralis*
Bentung Kerihun, West Kalimantan	1	*C. malayanus*	*C. malayanus*
Maruwai, Central Kalimantan	3	*C. malayanus*	*C. malayanus*
Meratus, Central Kalimantan	2	*C. malayanus*	*C. malayanus*
Kutai, East Kalimantan	2	*C. malayanus*	*C. malayanus*
Kerinci National Park, Jambi, Sumatra	3	*C. marmoratus*	*C. marmoratus* (Kerinci)
Cibodas, Bogor, West Java	11	*C. marmoratus*	*C. marmoratus* (Java)
Nunukan, East Kalimantan	3	*C. marmoratus*	*C.marmoratus* (Nunukan)
Martabe, Sumatra	8	*C. marmoratus*	*C.marmoratus* (Martabe)
Kubu Perahu, Lampung, Sumatra	2	*C. marmoratus*	*C.marmoratus* (Lampung)
Mandau, Siak, Riau, Sumatra	3	*C. marmoratus*	*C.marmoratus* (Riau)
Kutai Kartanegara, East Kalimantan	1	*C. marmoratus*	*C.marmoratus* (Kutai)
Sebangau, Palangkaraya, Central Kalimantam	1	*C. marmoratus*	*C.marmoratus* (Kutai)

mm, we followed Grismer et al. (2012) by measuring 11 external body characters: snout-vent length (SVL), measured from the tip of snout to the cloaca opening; tail length (TailL), measured from the cloaca opening to the tip of the original tail; axilla to groin length (AGL), measured from the arm to groin, specimen in supine position; head length (HeadL), measured from the posterior margin of the retroarticular process of the lower jaw to the snout; head width (HeadW), maximum wide measured the angle of the jaws; head

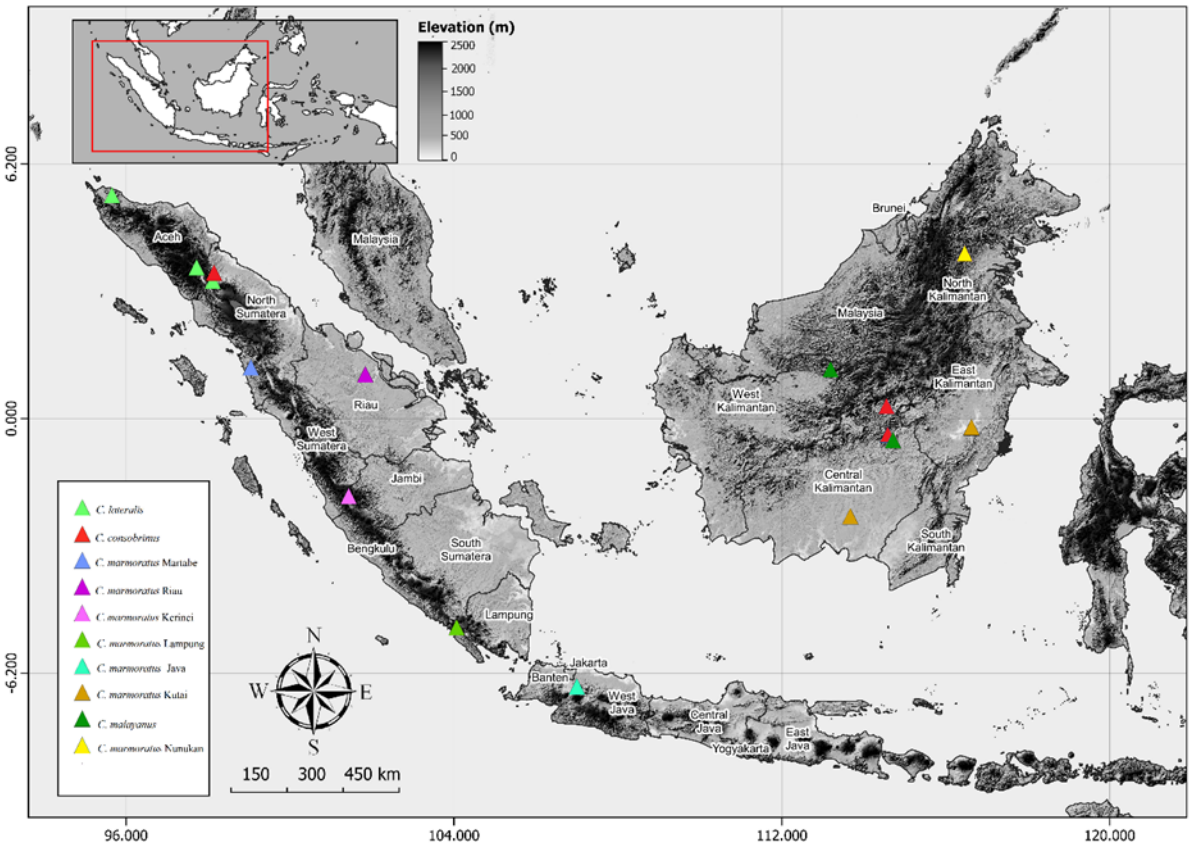

Figure 1. Distribution on the Sunda land (Sumatra, Java, and Kalimantan) of the 10 OTUs specimens used in this study. Map modified by QGIS 3.16 Hannover following this website: https://www.qgis.org/en/site/forusers/download.html.

depth (HeadD), the maximum height of head measured from the top-to-bottom side; eye diameter (EyeD), the greatest horizontal diameter of the eyeball; eye to ear distance (EyeEar), measured from the anterior edge of the ear opening to the posterior edge of the eyeball; eye to snout distance (EyeS), measured from anterior margin of the eye ball to the snout; eye to nostril distance (SnEye), measured between the anterior margin of the eyeball to the posterior margin of the nares; interorbital distance (InteroD), measured between the anterior edges of the orbit; ear length (EarL), the greatest length of the ear opening; and internarial distance (IN), measured between the nares.

We counted the number of supralabials (SuL), number of infralabials (InL), number of dorsal tubercles (DT), number of paravertebral tubercles (PVT), number of ventral scales between lateral body folds at midbody (VS), number of pores at femoral (FP), number of pores at precloacal (PP), number of precloacal and femoral pores which continuous arranged (PFP), and number of subdigital lamellae under 4th toe were counted following Bauer et al. (2010) under a stereoscope (AmScope, magnification 10x to 60x). All measurements and counts were taken on the right side. The illustration for measurement and counted meristic characters is presented in Figure 2. We followed Riyanto et al. (2020) for checked the presence of tubercle on upper (TubBrachium) and lower arms (TubAntebrachium), presence of tubercle on ventrolateral fold, presence of enlarged post cloacal scales and number of tubercle post cloaca. We followed Mecke et al. (2016b) and checked the presence of enlarged femoral scales (EFS), enlarged precloacal scales (EPS), enlarged median subcaudal (SubC) and the condition of precloacal depressions. A groove is always longitudinal and relatively narrow, with some or all of the precloacal scales (usually pore-bearing) on the left and right sides in contact with each other or narrowly separated. The groove may have an entire length slit shape, or a "ʌ" shape with the depression broadening posteriorly. Meanwhile, pit is a triangular depression, with most or all the posterior enlarged precloacal scale series widely separated from each other.

Data analysis

Specimens were divided into a priori Operational Taxonomic Units (OTUs) as a representing putative species due to the upon locations and obvious differences in morphology, and localities. As the consequence, all specimens labelled as *C. marmoratus* both from Kalimantan and Sumatra were relabelled to primary OTUs with added to their respective localities (Table 1).

All morphometric characters before their inclusion in the statistical analyses were adjusted by divided to SVL. We follow Zar (2010) for the small sample size, in performing separate Kruskal-Wallis one-way analysis of variance tests for each of nine morphometric ratios and 15 meristic characters to detect statistical differences between OTUs. The characters which have significant statistical differences will be performed to Dunn test. We performed statistical analysis only specimens which were labelled as *C. marmoratus*. Homogeneity of the priori-OTUs examined by Principal Com-

ponent Analysis (PCA) followed by ordination of specimens along their first two Principal Components (PCs) and the resulting pattern was inspected visually. A biplot of the principal component scores were used to examine the morphometric differentiation between OTU. All statistical analyses were performed in R. (version 4.1.2) (R Core Team 2021).

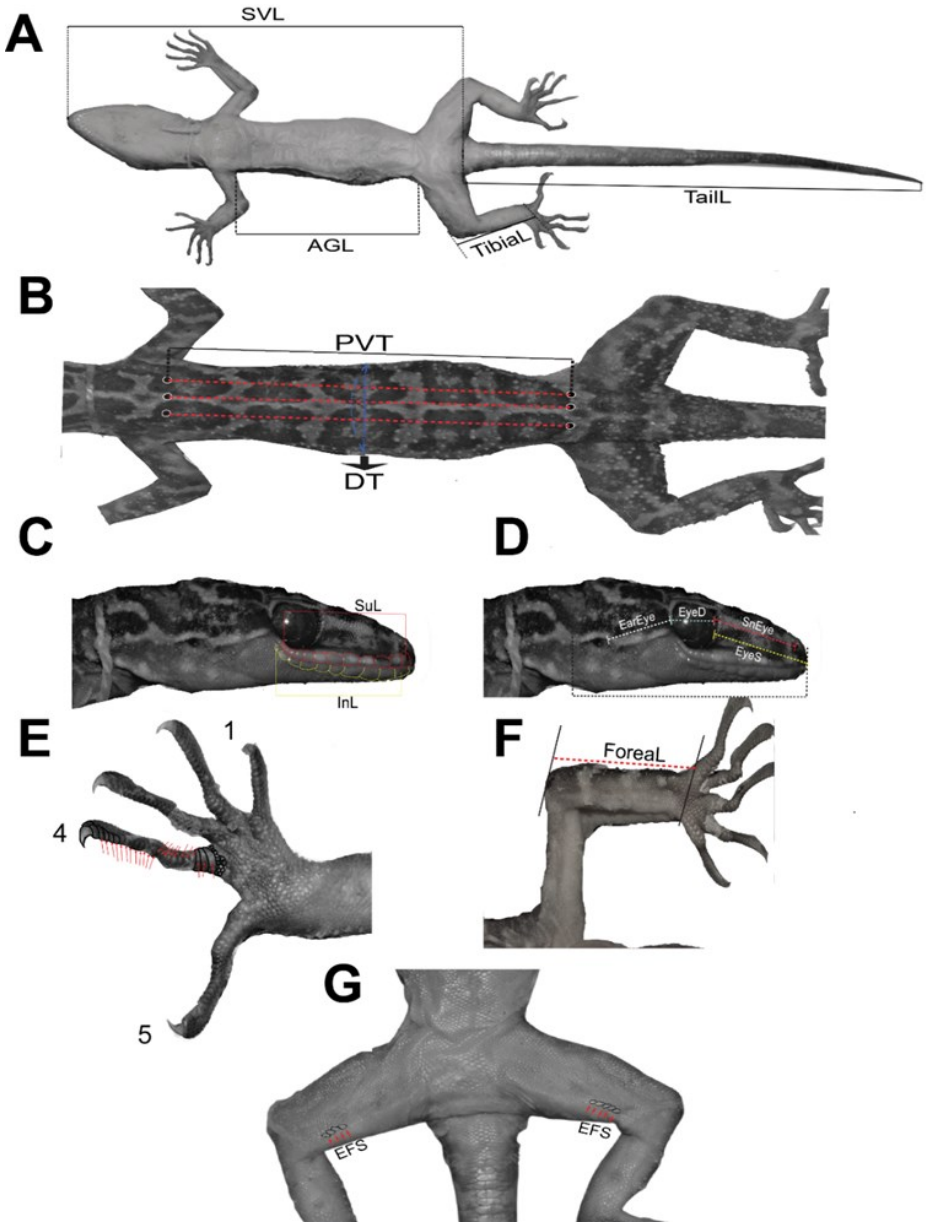

Figure 2. Illustration of measured characters in *Cyrtodactylus*. **A**. Ventral view of the body, **B.** Dorsal view of the body, **C & D.** Lateral view of the head, **E.** View of the subdigitall lamellae under 4th toe, **F.** Ventral view of the forelimb, **G.** View of enlarged femoral scales.

RESULTS AND DISCUSSION

Results

Principal component analysis showed that five components reach 58,5 % for explaining the variation and the total eigenvalue reach 14.0438 (Table 2). In the first PC, morphometric characters have the highest positive correlation including HeadW, HeadD, SnEye, EyeS, and IO, meanwhile, in meristic characters only VS has the highest positive correlation. In contrast, spines

Table 2. Summary analysis of principal component analysis score of Sumatran, Kalimantan and Javan *Cyrtodactylus*

Character	PC1	PC2	PC3	PC4	PC5
AGL/SVL	0.156979329	-0.1047731520	-0.017577464	0.161303205	-0.07102509
HeadL/SVL	0.247976590	0.1548045588	0.152607599	0.154439302	-0.12785407
HeadW/SVL	0.387401006	-0.0007062378	-0.152955681	-0.093274456	0.01332827
HeadD/SVL	0.321194914	-0.1247859036	-0.146126871	0.042263714	-0.03890982
EyeS/SVL	0.339506083	0.0374904712	0.113425315	0.013576318	-0.11152219
EyeD/SVL	-0.048223921	0.2631359450	0.039981276	0.325266169	0.24191758
SnEye/SVL	0.328166997	0.1025988828	0.117406218	-0.006155799	0.10230859
EarEye/SVL	0.159169926	0.1081510718	0.047441474	0.020439091	-0.08187862
IO/SVL	0.257882384	-0.0991033079	-0.401818209	0.153104059	0.19897514
TubLA	-0.005029344	-0.1951094491	0.386515065	-0.119819618	0.38423815
SubCa	0.105099365	-0.4090890411	0.211396239	-0.131384872	-0.15510277
Spines	-0.304670099	-0.0819113423	-0.299208775	0.255526172	0.05072730
SuL	-0.124357557	0.3223170942	0.163640764	-0.241888743	0.13300855
InL	0.075308957	0.0722428672	-0.037937543	-0.293111049	0.33601431
TubBrachium	-0.138683850	0.2024149568	-0.143979780	0.049260554	-0.23023212
TubAntebrachium	0.040920158	0.2024149568	-0.134921092	-0.232552812	-0.03316241
PVT	-0.184273135	-0.1186416582	0.091083435	0.359726487	-0.30461448
DT	-0.251274883	-0.0516968099	0.120578545	-0.289238110	-0.05011188
VS	0.281242210	0.1980686805	0.006832648	-0.194381401	-0.22696191
Toe4th	-0.030271656	0.2419296242	-0.278781743	-0.155091016	-0.05536596
EFS	0.014774424	-0.3248167963	0.039410353	-0.195999762	-0.32730647
EPS	0.130307246	0.0208115941	0.127516061	0.370515709	0.31658363
Enlarge Post Cloaca Scale	0.002137374	0.2472410278	0.501559727	0.094961333	-0.03050341
Tubercle Post Cloaca	0.056978099	0.1950744514	0.137696274	0.228704799	-0.36339726
Standard deviation	2.1260	1.8790	1.5649	1.41876	1.23757
Proportion of variance	0.1883	0.1471	0.1020	0.08387	0.06382
Cumulative proportion	0.1883	0.3354	0.4375	0.52134	0.58516
Eigenvalue	4.51973073	3.53071478	2.44891599	2.01287319	1.53156920

and DT have the highest negative correlation. In the second PC, EyeD, SuL, and Toe4th showed the highest positive correlation on the morphometric and meristic characters whereas SubCa and EFS showed the highest negative correlation. The highest positive correlation on the third PC are enlarged post cloaca scales and TubLa while IO and spines showed the highest negative correlation. In the fourth and fifth PC, EyeD and EPS showed similar value of positive correlation, while EPS character has the highest positive correlation in the fourth PC and TubLa in the fifth PC, respectively.

Principal component analysis based on the nine morphometric characters and 15 meristic characters (PC1–PC2, PC1–PC3, and PC2–PC3 (Figure 3) showed that there are five distinct OTUs, i.e. *C. malayanus, C. consobrinus, C. lateralis, C. marmoratus* from Martabe and *C. marmoratus* from Java. It clear that *C. marmoratus* (specimens originally from Cibodas, West Java) is differs from specimens previously labelled as *"marmoratus"* that originally from Martabe. We hypothesized that *C. marmoratus* frrom Martabe is suspected undescribed species. It is considered by statistical difference on interobital, HeadW, HeadD, tubercle on ventrolateral fold, dorsal tubercle and ventral scales. The comparison of morphometric and meristic among *C. marmoratus* group are presented in table 3 and 4.

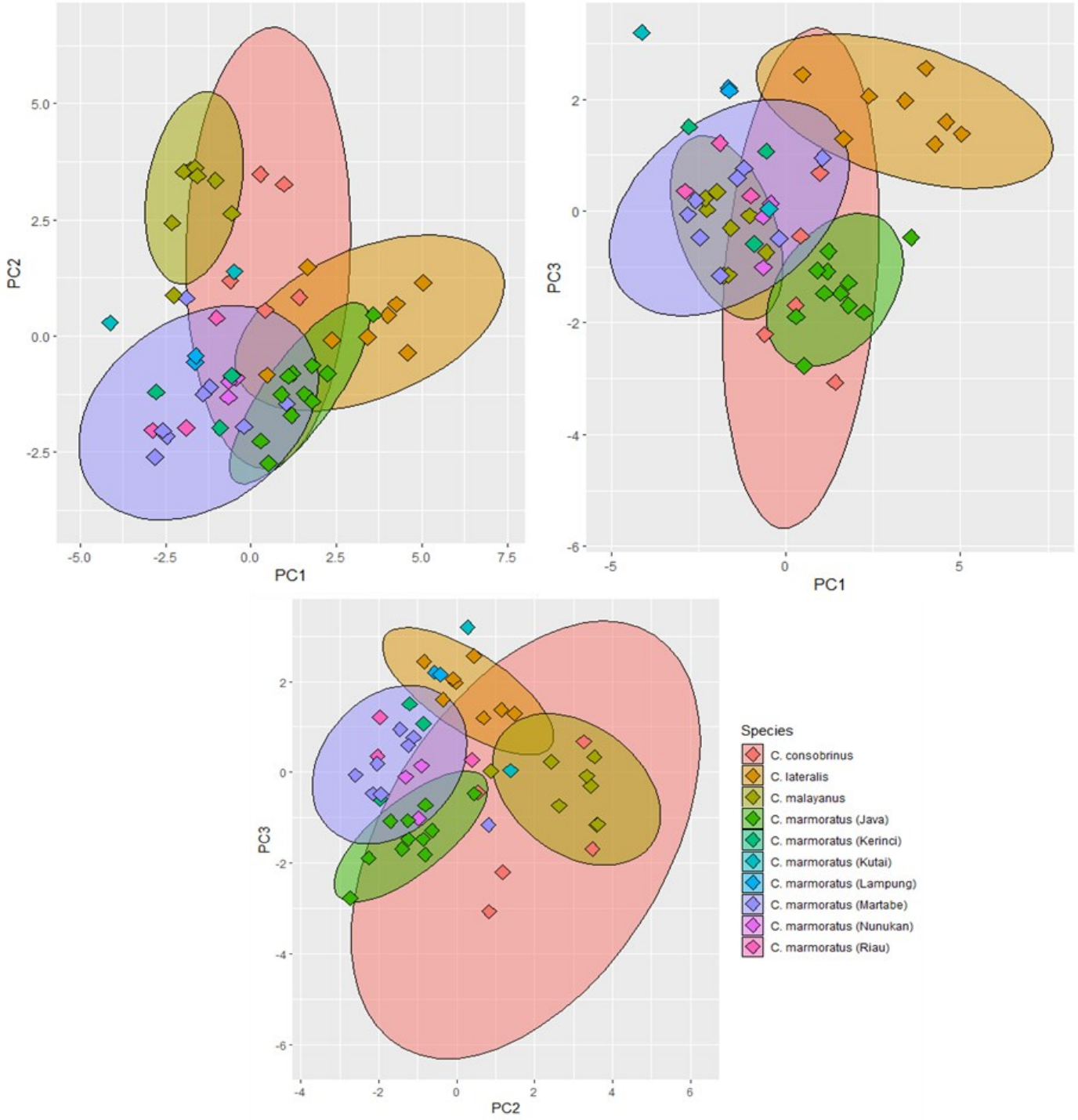

Figure 3. Principal component analysis biplot of morphometric variation in primary OTUs, each point represents an individual specimen.

Referring to the biplots on three PC (Figure 3), show that the *C. marmoratus* from Java is clearly different from other specimens which labelled as *C. marmoratus* as well as other recognized species. It means similarity on dorsal color pattern is not supported as a diagnostic character to distinguish *C. marmoratus*. *Cyrtodactylus marmoratus* is easily distinguished from the other OTUs in this study by the following combination of characters such as the absence of tubercles on the upper arms, subcaudal not enlarged, presence of enlarged precloacofemoral, vary in arrangement of precloacal and femoral pores vary continuous and not, and presence of groove precloacal depression in male (Table 5).

Table 3. Summary statistics of Kruskal-Wallis and Dunn test of the morphometric characters on the specimens which were labelled as *C. marmoratus*.

Head Length	**Z**	**P.adj**	**Head Depth**	**Z**	**P.adj**
Java – Kerinci	2.01355599	0.2312949	Java – Kerinci	2.35080182	0.098348294
Java - Kutai	0.85096053	0.7536924	Java - Kutai	2.61591570	0.093437952
Java – Lampung	1.68301082	0.2771190	Java – Lampung	1.99187798	0.162345567
Java – Martabe	-0.34151660	0.9051182	Java – Martabe	3.49598294	0.009918697
Java – Riau	2.24023648	0.2020011	Java – Riau	2.35080182	0.131131058
Java – Nunukan	1.52256573	0.3356519	Java – Nunukan	2.24169287	0.104921161
Kerinci – Kutai	-0.72011152	0.8250486	Kerinci – Kutai	0.52548679	1.000000000
Kerinci – Lampung	-0.01946247	0.9844722	Kerinci – Lampung	0.00000000	1.000000000
Kerinci – Martabe	-2.17162431	0.2091881	Kerinci – Martabe	0.13777677	1.000000000
Kerinci – Riau	0.26111648	0.8775819	Kerinci – Riau	0.00000000	1.000000000
Kerinci – Nunukan	-0.39167473	0.9734180	Kerinci – Nunukan	-0.08703883	1.000000000
Kutai – Lampung	0.63960215	0.7836469	Kutai – Lampung	-0.47970161	1.000000000
Kutai – Martabe	-1.02815479	0.7090464	Kutai – Martabe	-0.48879490	1.000000000
Kutai – Riau	0.95366121	0.7145358	Kutai – Riau	-0.52548679	1.000000000
Kutai – Nunukan	0.36978700	0.9338978	Kutai – Nunukan	-0.60333668	1.000000000
Lampung – Martabe	-1.83719463	0.2779609	Lampung – Martabe	0.11798498	1.000000000
Lampung – Riau	0.25301216	0.8716474	Lampung – Riau	0.00000000	1.000000000
Lampung - Nunukan	-0.33086205	0.8642068	Lampung - Nunukan	-0.07784989	1.000000000
Martabe - Riau	2.39975617	0.2460899	Martabe - Riau	-0.13777677	1.000000000
Martabe – Nunukan	1.69924682	0.3124544	Martabe – Nunukan	-0.24274955	1.000000000
Nunukan – Riau	0.65279121	0.8301314	Nunukan – Riau	0.08703883	1.000000000
Head Width	**Z**	**P.adj**	**Eye Diameter**	**Z**	**P.adj**
Java – Kerinci	1.7955026	0.254007779	Java – Kerinci	1.4481733	0.23838004
Java - Kutai	2.1685722	0.210806224	Java - Kutai	1.8973268	0.13483124
Java – Lampung	1.8218528	0.287604735	Java – Lampung	-1.9162370	0.14525419
Java – Martabe	4.0894495	0.000908036	Java – Martabe	-1.4208134	0.23305654
Java – Riau	3.0503705	0.023998717	Java – Riau	0.6298562	0.61692013
Java – Nunukan	1.9319013	0.280201349	Java – Nunukan	-1.6614317	0.18446931
Kerinci – Kutai	0.5449992	0.946218101	Kerinci – Kutai	0.5644117	0.60109765
Kerinci – Lampung	0.2530353	0.933614382	Kerinci – Lampung	-2.6468964	0.04264797
Kerinci – Martabe	1.0793503	0.841294828	Kerinci – Martabe	-2.3684483	0.06252007
Kerinci – Riau	1.0010383	0.831621814	Kerinci – Riau	-0.6527912	0.63480640
Kerinci – Nunukan	0.1088085	0.913354376	Kerinci – Nunukan	-2.4806066	0.05508680
Kutai – Lampung	-0.2665253	0.975678110	Kutai – Lampung	-2.9315098	0.07083692
Kutai – Martabe	0.2949895	1.000000000	Kutai – Martabe	-2.6799445	0.05154406
Kutai – Riau	0.3503566	1.000000000	Kutai – Riau	-1.1482859	0.35119076
Kutai – Nunukan	-0.4476779	0.981578488	Kutai – Nunukan	-2.7831337	0.05652844
Lampung – Martabe	0.6321203	0.922789472	Lampung – Martabe	1.0281548	0.39883859
Lampung – Riau	0.6423205	0.993997027	Lampung – Riau	2.0630222	0.11733156
Lampung - Nunukan	-0.1557141	0.968496142	Lampung - Nunukan	0.4281744	0.66852414
Martabe - Riau	0.1279473	0.943100215	Martabe - Riau	1.5811524	0.19922558
Martabe – Nunukan	-0.9481223	0.800490153	Martabe – Nunukan	-0.6232759	0.58921939
Nunukan – Riau	0.8922298	0.781766571	Nunukan – Riau	1.8278154	0.14191225

Table 3. Contd.

Interorbital	Z	P.adj
Java – Kerinci	1.66143166	0.4058324877
Java - Kutai	2.40159971	0.0856986879
Java – Lampung	1.22286180	0.5811274881
Java – Martabe	4.17380216	0.0006290716
Java – Riau	2.75252112	0.0413968328
Java – Nunukan	2.80707559	0.0524931847
Kerinci – Kutai	0.83688636	0.6039846680
Kerinci – Lampung	-0.15569979	1.0000000000
Kerinci – Martabe	1.26623411	0.7190024721
Kerinci – Riau	0.87038828	0.6204502492
Kerinci – Nunukan	0.91390769	0.6887338511
Kutai – Lampung	-0.90610304	0.6385422996
Kutai – Martabe	0.11798498	1.0000000000
Kutai – Riau	-0.05838742	1.0000000000
Kutai – Nunukan	-0.01946247	0.9844721731
Lampung – Martabe	1.26412474	0.6185557719
Lampung – Riau	0.93419873	0.7354229173
Lampung - Nunukan	0.97312368	0.7711476177
Martabe - Riau	-0.21650635	1.0000000000
Martabe – Nunukan	-0.16401996	1.0000000000
Nunukan – Riau	-0.04351941	1.0000000000

Table 4. Summary statistics of Kruskal-Wallis and Dunn test of the meristic characters of specimens which were labelled as *C. marmoratus*.

Tubercle on Ventrolateral Fold	Z	P.adj
Java – Kerinci	-2.7692096	0.03933473
Java - Kutai	0.2346403	0.95023595
Java – Lampung	-2.3464027	0.09951698
Java – Martabe	-3.3480083	0.01709286
Java – Riau	-1.7538328	0.18540472
Java – Nunukan	-2.7692096	0.05900209
Kerinci – Kutai	2.1734327	0.08924331
Kerinci – Lampung	0.0000000	1.00000000
Kerinci – Martabe	-3.3480083	0.93727903
Kerinci – Riau	0.8099905	0.73140483
Kerinci – Nunukan	0.0000000	1.00000000
Kutai – Lampung	-1.9840635	0.12402800
Kutai – Martabe	-2.1959559	0.11799943
Kutai – Riau	-1.4489551	0.30943525
Kutai – Nunukan	-2.1734327	0.10411719
Lampung – Martabe	0.3137080	0.93109409
Lampung – Riau	0.7244776	0.75724805
Lampung - Nunukan	0.0000000	1.00000000
Martabe – Riau	0.6105533	0.81224299
Martabe – Nunukan	-0.3663320	0.99976430
Nunukan – Riau	0.8099905	0.79789618

Dorsal Tubercle	Z	P.adj
Java – Kerinci	-1.2612422	0.36263779
Java - Kutai	-1.8120993	0.18367351
Java – Lampung	-3.0666295	0.04546227
Java – Martabe	-2.6231004	0.04574514
Java – Riau	-2.6321577	0.05939114
Java – Nunukan	0.4386929	0.73045078
Kerinci – Kutai	-0.6260202	0.65631389
Kerinci – Lampung	-1.6824292	0.21579985
Kerinci – Martabe	-0.5869309	0.65012510
Kerinci – Riau	-1.0936122	0.44281746
Kerinci – Nunukan	1.3560791	0.33423216
Kutai – Lampung	-0.9643651	0.50229439
Kutai – Martabe	0.2202482	0.82567788
Kutai – Riau	-0.3521363	0.76097282
Kutai – Nunukan	1.8389342	0.19777459
Lampung – Martabe	1.4400842	0.31467150
Lampung – Riau	0.7042727	0.63165766
Lampung - Nunukan	2.8953432	0.03976814
Martabe - Riau	-0.7320150	0.64982318
Martabe – Nunukan	2.2224239	0.09189135
Nunukan – Riau	-2.4496913	0.06005106

Table 4. Contd.

Paravertebral Tubercles	Z	P.adj
Java – Kerinci	-0.1546255	0.87711658
Java – Kutai	1.1474557	0.43958832
Java – Lampung	1.8448045	0.22773095
Java – Martabe	-2.7163266	0.04620753
Java – Riau	-1.1971005	0.44151043
Java – Nunukan	1.0524509	0.43888909
Kerinci – Kutai	1.0765704	0.45500899
Kerinci – Lampung	1.6637906	0.20192399
Kerinci – Martabe	-1.7155831	0.20122287
Kerinci – Riau	-0.8316074	0.53239014
Kerinci – Nunukan	0.9629138	0.46982707
Kutai – Lampung	0.5360563	0.65422700
Kutai – Martabe	-2.7122540	0.03508436
Kutai – Riau	-1.8203827	0.20610224
Kutai – Nunukan	-0.2153141	0.87099858
Lampung – Martabe	-3.3903175	0.01466046
Lampung – Riau	-2.4076029	0.06744207
Lampung - Nunukan	-0.8025343	0.52159547
Martabe - Riau	0.7126268	0.55542283
Martabe – Nunukan	2.8769009	0.04216818
Nunukan – Riau	-1.7945212	0.19091628

Ventral Scales	Z	P.adj
Java – Kerinci	1.7871873	0.17245017
Java - Kutai	-1.3160638	0.35920050
Java – Lampung	2.6511092	0.04211964
Java – Martabe	2.9177854	0.02467688
Java – Riau	0.1991295	0.84216144
Java – Nunukan	1.1300600	0.38767645
Kerinci – Kutai	-2.3833939	0.07204609
Kerinci – Lampung	0.9572648	0.47380710
Kerinci – Martabe	0.2831803	0.81589052
Kerinci – Riau	-1.2668319	0.35912696
Kerinci – Nunukan	-0.5242063	0.66330717
Kutai – Lampung	3.0495901	0.04812231
Kutai – Martabe	2.9946073	0.02885383
Kutai – Riau	1.2503050	0.34115010
Kutai – Nunukan	1.9145295	0.16665760
Lampung – Martabe	-0.8628530	0.47956380
Lampung – Riau	-2.0903536	0.12805116
Lampung - Nunukan	-1.4261291	0.32304526
Martabe - Riau	-1.8110371	0.18410467
Martabe – Nunukan	-0.9153969	0.47247807
Nunukan – Riau	-0.7426256	0.53399312

Supralabial	Z	P.adj
Java – Kerinci	-1.83487298	0.23283579
Java - Kutai	-2.29250012	0.15313761
Java – Lampung	-3.12703613	0.03708143
Java – Martabe	-2.03680405	0.21876583
Java – Riau	-2.46293416	0.14469548
Java – Nunukan	-1.34955297	0.46504348
Kerinci – Kutai	-0.62126485	0.70143330
Kerinci – Lampung	-1.32400707	0.43283496
Kerinci – Martabe	0.36735791	0.78844176
Kerinci – Riau	0.50101951	0.76138267
Kerinci – Nunukan	0.38715144	0.81508478
Kutai – Lampung	-0.64151294	0.72966527
Kutai – Martabe	1.03196138	0.63438944
Kutai – Riau	0.17313939	0.90566896
Kutai – Nunukan	0.96754363	0.58322660
Lampung – Martabe	1.84341819	0.27412550
Lampung – Riau	0.87588160	0.57164161
Lampung - Nunukan	1.67028584	0.28458847
Martabe – Riau	-0.97161017	0.63237591
Martabe – Nunukan	0.09956429	0.92069024
Nunukan – Riau	-0.88817094	0.60487884

Enlarged Femoral Scales	Z	P.adj
Java – Kerinci	0.000000	1.0000000000
Java - Kutai	1.935782	0.1851303342
Java – Lampung	0.000000	1.0000000000
Java – Martabe	0.000000	1.0000000000
Java – Riau	0.000000	1.0000000000
Java – Nunukan	4.569196	0.0001028158
Kerinci – Kutai	1.630074	0.2164800825
Kerinci – Lampung	0.000000	1.0000000000
Kerinci – Martabe	0.000000	1.0000000000
Kerinci – Riau	0.000000	1.0000000000
Kerinci – Nunukan	3.644957	0.0014040396
Kutai – Lampung	-1.488048	0.2610459144
Kutai – Martabe	-1.882248	0.1794070958
Kutai – Riau	-1.630074	0.2706001031
Kutai – Nunukan	1.630074	0.2405334250
Lampung – Martabe	0.000000	1.0000000000
Lampung – Riau	0.000000	1.0000000000
Lampung - Nunukan	3.260149	0.0046768555
Martabe - Riau	0.000000	1.0000000000
Martabe – Nunukan	4.395984	0.0001157857
Nunukan – Riau	-3.644957	0.0018720528

Table 4. Contd.

Infralabial	Z	P.adj
Java – Kerinci	-2.3910922	0.11758834
Java – Kutai	-0.8818600	0.61037718
Java – Lampung	-2.3742384	0.09232227
Java – Martabe	0.2539041	0.79956964
Java – Riau	-0.3068924	0.83881214
Java – Nunukan	-1.6278641	0.27182848
Kerinci – Kutai	0.9634662	0.58679881
Kerinci – Lampung	-0.2932289	0.80781459
Kerinci – Martabe	2.4747159	0.28001861
Kerinci – Riau	1.6626162	0.28916783
Kerinci – Nunukan	0.6088454	0.75967770
Kutai – Lampung	-1.1472004	0.58636387
Kutai – Martabe	1.0067051	0.59960059
Kutai – Riau	0.5236230	0.78820982
Kutai – Nunukan	-0.4188984	0.78783882
Lampung – Martabe	2.4578116	0.14677580
Lampung – Riau	1.7803180	0.31510050
Lampung - Nunukan	0.8377967	0.60321732
Martabe - Riau	-0.4695253	0.78897522
Martabe – Nunukan	-1.7404208	0.28624808
Nunukan – Riau	1.0537709	0.61317446

Tubercle post cloaca	Z	P.adj
Java – Kerinci	2.5173879	0.04965601
Java - Kutai	2.1330294	0.11522809
Java – Lampung	0.8295114	0.65716279
Java – Martabe	-0.7841682	0.56823565
Java – Riau	2.5173879	0.06207001
Java – Nunukan	1.4917854	0.35635795
Kerinci – Kutai	0.0000000	1.00000000
Kerinci – Lampung	-1.0976604	0.51994629
Kerinci – Martabe	-2.9601699	0.03228429
Kerinci – Riau	0.0000000	1.00000000
Kerinci – Nunukan	-0.8181477	0.57858197
Kutai – Lampung	-1.0020222	0.55358248
Kutai – Martabe	-2.5349381	0.07872705
Kutai – Riau	0.0000000	1.00000000
Kutai – Nunukan	-0.7317736	0.57355543
Lampung – Martabe	-1.2674690	0.47830447
Lampung – Riau	1.0976604	0.57194092
Lampung - Nunukan	0.3658868	0.83352448
Martabe - Riau	2.9601699	0.06456858
Martabe – Nunukan	1.9734466	0.14533411
Nunukan – Riau	0.8181477	0.61990925

Enlarged median subcaudal	Z	P.adj
Java – Kerinci	0.000000	1.000000000
Java - Kutai	3.679465	0.004908202
Java – Lampung	0.000000	1.000000000
Java – Martabe	0.000000	1.000000000
Java – Riau	0.000000	1.000000000
Java – Nunukan	0.000000	1.000000000
Kerinci – Kutai	3.098387	0.008172250
Kerinci – Lampung	0.000000	1.000000000
Kerinci – Martabe	0.000000	1.000000000
Kerinci – Riau	0.000000	1.000000000
Kerinci – Nunukan	0.000000	1.000000000
Kutai – Lampung	-2.828427	0.016372072
Kutai – Martabe	-3.577709	0.003639503
Kutai – Riau	-3.098387	0.013620416
Kutai – Nunukan	-3.098387	0.010215312
Lampung – Martabe	0.000000	1.000000000
Lampung – Riau	0.000000	1.000000000
Lampung - Nunukan	0.000000	1.000000000
Martabe - Riau	0.000000	1.000000000
Martabe – Nunukan	0.000000	1.000000000
Nunukan – Riau	0.000000	1.000000000

Enlarged post cloacal scales	Z	P.adj
Java – Kerinci	0.000000	1.0000000000
Java - Kutai	0.000000	1.0000000000
Java – Lampung	0.000000	1.0000000000
Java – Martabe	-4.459790	0.0001722842
Java – Riau	0.000000	1.0000000000
Java – Nunukan	-3.181580	0.0153797960
Kerinci – Kutai	0.000000	1.0000000000
Kerinci – Lampung	0.000000	1.0000000000
Kerinci – Martabe	-3.060970	0.0115825990
Kerinci – Riau	0.000000	1.0000000000
Kerinci – Nunukan	-2.538023	0.0292637106
Kutai – Lampung	0.000000	1.0000000000
Kutai – Martabe	-2.621258	0.0306620401
Kutai – Riau	0.000000	1.0000000000
Kutai – Nunukan	-2.270076	0.0487261938
Lampung – Martabe	-2.621258	0.0367944481
Lampung – Riau	0.000000	1.0000000000
Lampung - Nunukan	-2.270076	0.0541402153
Martabe - Riau	3.060970	0.0154434653
Martabe – Nunukan	0.000000	1.0000000000
Nunukan – Riau	2.538023	0.0334442407

Table 5. Morphometric (in mm) and meristic characters of OTUs examined in this study. The number of precloacal pores not given for specimen with precloacal and femoral pores in a continuous series.

Character	OTU									
	C. marmoratus (n=11)	*C. lateralis* (n=8)	*C. malayanus* (n=8)	*C. marmoratus* Nunukan (n=3)	*C. consobrinus* (n=5)	*C. marmoratus* Kerinci (n=3)	*C. marmoratus* Riau (n=3)	*C.marmoratus* Martabe (n=8)	*C. marmoratus* Lampung (n=2)	*C. marmoratus* Kutai (n=2)
SVL	73.7±7.5 (59.6-85.2)	74.7±11.2 (57.6-88.9)	85.9±8.4 (68.6-95.1)	58.1±6.61 (50.5-62.3)	80.1±30.2 (42.5-118.6)	69.4±4.8 (65.2-74.8)	60.9±6.5 (53.6-66.2)	57.4±5.0 (50.5-62.9)	53.6±2.7 (51.7-55.6)	68.7±1.5 (67.6-69.8)
TL/SVL	0.822±0.214 (0.450-1.003)	0.970±0.207 (0.502-1.173)	1.008±0.368 (0.278-1.331)	1.064±0.102 (0.952-1.154)	1.038±0.233 (0.781-1.352)	1.026±0.116 (0.894-1.115)	1.214±0.083 (1.118-1.274)	0.900±0.419 (0.126-1.351)	0.747±0.077 (0.692-0.802)	1.219±0.203 (1.075-1.363)
AGL/SVL	0.473±0.062 (0.385-0.630)	0.457±0.045 (0.402-0.544)	0.428±0.023 (0.383-0.453)	0.449±0.036 (0.407-0.477)	0.413±0.017 (0.390-0.435)	0.427±0.033 (0.406-0.466)	0.433±0.030 (0.401-0.462)	0.423±0.024 (0.377-0.449)	0.423±0.003 (0.421-0.426)	0.396±0.022 (0.380-0.412)
HeadL/SVL	0.277±0.016 (0.260-0.316)	0.288±0.016 (0.273-0.320)	0.278±0.016 (0.266-0.318)	0.261±0.009 (0.251-0.269)	0.273±0.021 (0.238-0.289)	0.259±0.003 (0.255-0.262)	0.254±0.008 (0.248-0.264)	0.279±0.015 (0.263-0.308)	0.256±0.017 (0.244-0.268)	0.264±0.020 (0.250-0.273)
HeadW/SVL	0.187±0.011 (0.170-0.215)	0.191±0.029 (0.151-0.250)	0.162±0.011 (0.139-0.174)	0.163±0.005 (0.157-0.168)	0.174±0.007 (0.166-0.183)	0.166±0.016 (0.150-0.183)	0.151±0.016 (0.132-0.161)	0.156±0.007 (0.148-0.168)	0.161±0.007 (0.156-0.167)	0.160±0.008 (0.153-0.166)
HeadD/SVL	0.119±0.005 (0.110-0.130)	0.112±0.013 (0.094-0.134)	0.096±0.009 (0.081-0.109)	0.104±0.005 (0.100-0.110)	0.112±0.010 (0.103-0.128)	0.100±0.014 (0.086-0.114)	0.102±0.010 (0.091-0.111)	0.102±0.006 (0.094-0.114)	0.105±0.006 (0.100-0.109)	0.098±0.004 (0.094-0.101)
EyeS/SVL	0.118±0.007 (0.107-0.129)	0.123±0.004 (0.116-0.128)	0.111±0.003 (0.106-0.116)	0.107±0.001 (0.105-0.109)	0.112±0.009 (0.096-0.120)	0.107±0.009 (0.099-0.117)	0.112±0.003 (0.108-0.115)	0.113±±0.010 (0.103-0.135)	0.110±0.002 (0.108-0.112)	0.105±0.010 (0.097-0.113)
EyeD/SVL	0.055±0.004 (0.051-0.065)	0.057±0.007 (0.045-0.067)	0.070±0.011 (0.059-0.089)	0.066±0.010 (0.057-0.077)	0.068±0.007 (0.059-0.075)	0.049±0.002 (0.048-0.052)	0.053±0.004 (0.483-0.057)	0.065±0.019 (0.049-0.111)	0.067±0.003 (0.065-0.070)	0.039±0.007 (0.034-0.044)
SnEye/SVL	0.081±0.005 (0.074-0.090)	0.091±0.009 (0.079-0.106)	0.078±0.006 (0.069-0.090)	0.083±0.002 (0.081-0.085)	0.082±0.009 (0.070-0.092)	0.069±0.002 (0.068-0.072)	0.076±0.016 (0.057-0.088)	0.079±0.009 (0.069-0.100)	0.080±0.001 (0.079-0.081)	0.071±0.008 (0.065-0.077)
EarEye/SVL	0.088±0.006 (0.076-0.100)	0.094±0.007 (0.086-0.110)	0.088±0.022 (0.065-0.138)	0.093±0.004 (0.089-0.099)	0.087±0.002 (0.827-0.090)	0.091±0.011 (0.083-0.104)	0.083±0.008 (0.074-0.090)	0.083±0.007 (0.071-0.095)	0.077±0.002 (0.075-0.079)	0.086±0.009 (0.079-0.093)
IO/SVL	0.051±0.003 (0.046-0.056)	0.041±0.008 (0.024-0.050)	0.034±0.001 (0.032-0.037)	(0.033±0.002 (0.030-0.035)	0.054±0.017 (0.036-0.080)	0.041±0.006 (0.036-0.049)	0.034±0.004 (0.029-0.038-)	0.032±0.004 (0.027-0.040)	0.040±0.001 (0.039-0.041)	0.034±0.008 (0.028-0.040)
Suprlabial	8–11	10–12	12–13	10–11	8-12	10–13	11–12	10–12	12–13	11–12
Infralabial	9–11	9–11	9–11	10–11	8–14	10–12	9–10	8–10	11	10
Tubercle on brachium	absent	absent	absent	absent	absent	absent	Absent	absent	absent	absent
Tubercle on antebrachium	present	present	present	absent	present	present	Absent	present	present	present
Tubercle on hindlimbs	present	present	present	present	present	present	present	present	present	present
Tubercle on ventrolateral fold	absent	present	present	present	absent	absent	absent	present	absent	present
DT	14–23	17–22	17–23	16–18	14–20	18–25	22–25	19–24	18–19	20–24
PVT	24–33	22–30	24–32	26–30	26–32	27–33	31–33	31–36	28	27

Table 5. Contd.

Character	OTU									
	C. marmoratus (n=11)	*C. lateralis* (n=8)	*C. malayanus* (n=8)	*C. marmoratus* Nunukan (n=3)	*C. consobrinus* (n=5)	*C. marmoratus* Kerinci (n=3)	*C. marmoratus* Riau (n=3)	*C.marmoratus* Martabe (n=8)	*C. marmoratus* Lampung (n=2)	*C. marmoratus* Kutai (n=2)
VS	39–47	51–60	34–53	35–42	44–48	28–46	40–43	30–40	30	48–49
EPS	present	present	absent	present	present	present	present	present	absent	present
EFS	present	present	absent	absent	present	present	present	present	present	absent
PP	-	7–10	6–10	5–6	8-10	-	-	-	0	7
EPFS	yes	no	no	no	No	yes	yes	yes		no
PFP	45-52	0	0	0	0	35–45	46–49	39–47	0	0
Precloacal depression	groove	groove	pit	pit	pit	pit	pit	Pit	N/A	no
Tubercle post cloacal	1–2	2–3	1–3	1–2	0–3	1	1	2	1–2	0
Enlaraged median sub-caudal	absent	absent	present	absent	present	absent	absent	absent	N/A	present (in male)
Enlarged post cloacal scales	absent	present	present	present	present	absent	absent	present	N/A	absent
Subdigital lamella 4th toe	19–24	21–22	18–26	17–19	23–27	20–24	22–23	19–22	21–23	20–23

Cyrtodactylus marmoratus from Martabe differs from the other OTUs by its unique combination of characters such as the absence of tubercle on the upper arm, presence of tubercles along ventrolateral folds, and presence of EPFS. As the result of our statistical analysis, *C. marmoratus* from Martabe (Sumatra) is differ with *C. marmoratus* from Java. The *C. marmoratus* from Martabe can be distinguished from Java specimens by combination characters: more supralabial (10-12 vs 8-11), presence of tubercle on ventrolateral fold (vs absent), more number of paravertebral tubercle (31-36 vs 24-33), precloacal depression in groove form (vs pit), and presence of enlarged post cloacal scales (vs absent) (Figure 4).

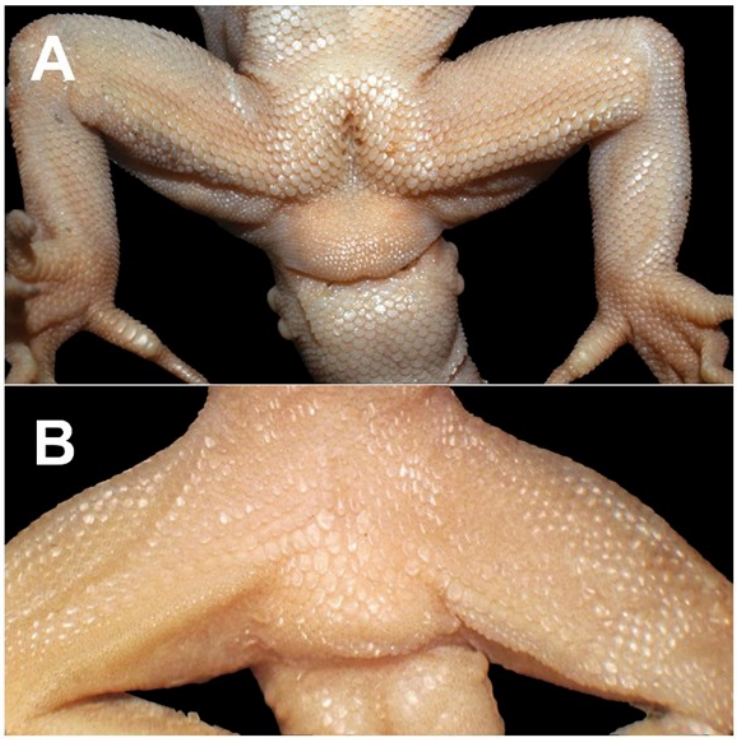

Figure 4. Comparison of precloacal region. **A** *C. marmoratus* from Java, **B** *C. marmoratus* from Martabe

Discussion

We assigned that specimens *C. marmortaus* from Kerinci belong to *C. semicinctus* described by Harvey et al. (2015). It is based on similarity in its morphology including fewer supralabial (10-13 vs 10-15), presence of tubercles on antebrachium, absence of tubercle on brachium, matching the number of paravertebral tubercles (27-33 vs 24-27), number of ventral scales (28-46 vs 33-44), number of precloacofemoral pores (35-45 vs 36-38), number of the lamella under the fourth toe (20-24 vs 19-22). This assignment is also supported by the similarity of the collected specimens with the type locality of *C. semicinctus*.

Cyrtodactylus semicinctus, C. marmoratus from Nunukan and *C. marmoratus* from Riau are grouped on the *C. marmoratus* from Martabe in all PC. The overlaping of *C. marmoratus* from Lampung, *C. marmoratus* from Riau, *C. marmoratus* from Nunukan and *C. marmoratus* from Kutai may be due to small

samples size. According to Rogell et al. (2020), this phenomenon can lead to an erroneous taxonomic conclusion. The position of these OTUs can be evident when the sample sizes for each OTU in sufficiently large, as suggested by Chan & Grismer (2021). Although *C. marmoratus* from Nunukan are overlapping within Martabe group, these species are distinct species according to Riyanto et al. 2021. *Cyrtodactylus marmoratus* from Nunukan is described as a new species namely *C. hamidyi*. These species are closely related to morphological characters within *C. matsuii*.

In this paper, we reported that *C. marmoratus* from Martabe has morphological variations compared to *C. marmoratus* from Java. In concordant, the comparison between *C. marmoratus* from Martabe and *C. agamensis* resulted that it has still unique morphological variations. *Cyrtodactylus marmoratus* from Martabe is easily distinguished from *C. agamensis* by fewer supralabial (10-12 vs 10-13), fewer infralabial (8-10 vs 9-12), widely dorsal tubercles (19-24 vs 17-21), fewer ventral scales (30-40 vs 50-67), a lower subdigital lamella on 4th toe (19-22 vs 21-26) (Milto & Bezman-Moseyko 2021).

Cyrtodactylus marmoratus group is a unique species regarding phylogenetic relationship and a gap-wide distribution (Riyanto et al. 2022). This group consists of four species, including *C. marmoratus, C. semiadii, C. papuensis* and *C. papeda* (Riyanto et al. 2022). *Cyrtodactylus marmoratus* group has potentially several undescribed species from Sumatra and Java (O'Conell et al 2019; Grismer et al. 2021). According to Riyanto et al. (2022) which stated that *C. marmoratus* is resticted to Java. It is indicated that specimens of *C. marmoratus* outside of Java island potential as a distinct species. Integrative taxonomic studies are needed to reveal hidden diversity on *C. marmoratus* and explain of phylogenetic relationship of this group.

CONCLUSION

The morphometric and meristic study of the *C. marmoratus* from Sumatra and Kalimantan revealed one suspected undescribed species, *C. marmoratus* from Martabe. Further study on molecular analysis is an ongoing issue to better understand phylogeny and reveal the taxonomic status of *Cyrtodactylus* in Sumatra and Kalimantan. The diagnostic charateristics to determine specimens to be *C. marmoratus* are the absence of tubercle on the upper arms, absence of enlarged median subcaudal, absence of tubercle on ventrolateral fold, vary in arrangement of precloaco femoral pores, absence of enlarged post cloacal scales, and presence of groove precloacal depression in male.

AUTHORS CONTRIBUTION

M.A.F. designed and conducted the research, analysed and interpretation the data, and wrote the draft of manuscript. A.R. designed the research, analysed and interpretation the data, reviewed the draft of manuscript, and supervised all the process. N.K. designed the research, reviewed the draft of manuscript, and supervised all the process.

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CONFLICT OF INTEREST

The authors declare there is no competing interest.

REFERENCES

Bauer, A.M. et al., 2010. *Cyrtodactylus dumnuii* (Squamata: Gekkonidae), a new cave-dwelling gecko from Chiang Mai Province, Thailand. *Zootaxa*, 2570, pp. 41– 50. doi: 10.11646/zootaxa.2570.1.2.

Chan, K.O. & Grismer, L.L., 2021. Correcting for Body Size Variation in Morphometric Analysis. *bioRxiv*. doi: 10.1101/2021.05.17.444580.

Davis, H.R., 2019. Uncovering karst endemism within Borneo: two new *Cyrtodactylus* species from Sarawak, Malaysia. *Zootaxa,* 4614(2), pp. 331-352. doi: 10.11646/zootaxa.4614.2.4.

Davis, H.R., 2021. Genetically diverse yet morphologically conserved: Hidden diverity revealed among Bornean geckos (Gekkonidae:*Cyrtodactylus*). *Journal of Zoological Systematic and Evolutionary Research,* 00, pp.1-23. doi: 10.1111/jzs.12470.

Dring, J.C.M., 1979. Amphibians and reptiles from Northern Trengganu, Malaysia, with descriptions of two new geckos: *Cnemaspsis* and *Cyrtodactylus*. *Bulletin of the British Museum* (Natural History) *Zoology,* 34(5), pp. 181-241

Gray, J.E., 1827. A synopsis of the genera of saurian reptiles in which some new genera are indicated, and the others reviewed by actual examination. *Philosophical Magazine*. 2(2), pp. 54–58.

Grismer, L.L., 2012. *Cyrtodactylus majulah,* A new species of bent-toed gecko (Reptilia: Squamata: Gekkonidae) from Singapore and the Riau archipelago. *The Raffles Bulletin of Zoology,* 60(2), pp. 487-499.

Grismer, L.L. et al., 2020. Two new species of *Cyrtodactylus* Gray, 1827 (Squamata: Gekkonidae) from a karstic archipelago in the Salween Basin of southern Myanmar (Burma). *Zootaxa*, 4718, pp. 151–183. doi: 10.11646/zootaxa.4718.2.1.

Grismer, L.L. et al., 2021. Phylogenetic partioning of the third-largest vertebrate genus in the world, *Cyrtodactylus* Gray, 1827 (Reptilia; Squamata; Gekkonidae) and its revelance to taxonomy and conservation. *Vertebrate Zoology,* 71, pp 101-154. doi: 10.3897/vz.71.e59307.

Harvey, M.B. et al., 2015. Two new species of *Cyrtodactylus* from the Southern Bukit Barisan Range of Sumatra and an estimation of their phylogeny. *Zootaxa*, 4020(3), pp. 495–516. doi: 10.11646/zootaxa.4020.3.5.

Hikida, T., 1990. Bornean gekkonid lizards of the genus *Cyrtodactylus* (Lacertidae: Gekkonidae) with descriptions of three new species. *Japanese Journal of Herpetology*, 13(3), pp. 91–207.

Mecke, S. et al., 2016a. Redescription of *Cyrtodactylus fumosus* (Muller, 1895) (Reptilia: Squamata: Gekkonida), with a revised identification key to the bent-toed geckos of Sulawesi. *Acta Herpetologica,* 11(2), pp. 151-160. doi: 10.13128/Acta_Herpetol-17874.

Mecke, S. et al., 2016b. Historical considerations and comments on the type series of *Cyrtodactylus marmoratus* Gray, 1831, with an updated comparative table for the bent-toed geckos of the Sunda Island and Sulawesi. *Zootaxa*, 4175(4), pp. 353–365. doi: 10.11646/ zootaxa.4175.4.5.

Milto, K.D.& Bezman-Moseyko, O.S., 2021. Herpetofauna of the Maninjau caldera, West Sumatra, Indonesia, with special account to geckos. *Proceedings of the Zoological Institure RAS*, 325(4), pp. 430-446. doi: 10.31610/trudyzin/2021.325.4.430.

O'Connell, K.A. et al., 2019. Diversification of bent-toed geckos (*Cyrtodactylus*) on Sumatra and west Java. *Molecular Phylogenetics and Evolution*, 134, pp. 1–11. doi: 10.1016/j.ympev.2019.01.021.

R Core Team, 2021. R: A language and environment for statistical computing. R Foundation for statistical computing. Vienna. Austria. https:// www.R-project.org/.

Riyanto, A. et al., 2020. Taxonomic evaluation of two similar bent-toed geckos (Squamata: Gekkonidae: *Cyrtodactylus* Gray, 1827) from East Java, Indonesia, *Zootaxa*, 4830(1), pp.186–196. doi: 10.11646/ zootaxa.4830.1.8.

Riyanto, A. et al., 2021. Another new-bent toed gecko, genus *Cyrtodactylus* Gray 1837 (Squamata: Gekkonidae), from Borneo. *Zootaxa,* 5026(2), pp. 286-300.

Riyanto, A. et al., 2022. A new bent-toed gecko of the *Cyrtodactylus marmoratus* group (Reptilia: Gekkonidae) from Obi island, Indonesia. *Herpetologica,* 78(1), pp. 30-39. doi: 10.11646/zootaxa.5026.2.8.

Rogell, B. et al., 2020. Controlling for body size leads to inferential biases in the biological sciences, *Evolution Letters*, 4(1), pp.73–82. doi: 10.1002/ evl3.151.

Uetz, P. et al., 2021. 'Diversity of the genus *Cyrtodactylus'*, in *Reptile Database*, viewed 13 June 2021 from http:// www.reptile-database.org

Wood, P.L. et al., 2012. Phylogeny of bent toed geckos (*Cyrtodactylus*) reveals a wes to east pattern of diversification. *Molecular Phylogenetic and Evolution*, 65, pp. 992-1003. doi: 10.1016/y.ympev.2012.08.025.

Zar, J.H., 2010. *Biostatistical Analysis, 5th edition*. Prentice Hall Inc., USA.

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Research Article

The Effect of Nanoparticles of *Piper crocatum* Leaves Ethanolic Extract on Liver Insulin Receptor Expression of Diabetic Rat's Induced by Streptozotocin

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ABSTRACT

Diabetes mellitus is a disease related to hyperglycemia and insulin resistance that can lead to the outcome of chronic liver diseases such as nonalcoholic fatty liver disease (NAFLD). Red betel leaves are known as traditional plants that have anti-hyperglycemic potential. This study aimed to investigate the effect of ethanolic extract of red betel leaves nanoparticle (RbL-Nps) on the liver and hepatic insulin receptor's (INSR) expression in diabetic rats. Thirty rats were included in this study and further divided into five groups containing six rats each. Group I (GI) comprised of the normal rats; while group II (GII), III (GIII), IV (GIV) and V (GV) comprised of diabetic rats induced by streptozotocin (STZ) at dose of 45 mg/kg bw and nicotinamide (NA) at dose of 110 mg/kg bw, intraperitoneally. Group I and II were treated with 0,5% Na-CMC orally. Group III, IV and V were given the oral administration of RbL-Nps at the doses 30, 60, and 90 mg/kg bw diluted in 0,5% Na-CMC, respectively. All groups were treated once daily and subsequently euthanized after 28 days. Liver tissues were collected for immunohistochemistry method to see the INSR expression and haematoxylin-eosin (HE) staining. Result in this study revealed that INSR expression on the GI, GIII and GIV were significantly higher compared to that on the GII ($p < 0.05$). On the other hand, there were no significant differences on the INSR expression between GV and GII ($p > 0.05$). Histologically, liver tissues retrieved from GII showed severe vacuolic and necrotic hepatocytes with dilatated sinusoid. Mild vacuolic and necrotic hepatocytes were observed from GV. There were no pathological changes observed in the liver tissues retrieved from GI, GII, and GIV. We concluded that RbL-Nps improved the liver condition of diabetic rats at doses of 30 and 60 mg/kg bw, but not at doses of 90 mg/kg bw.

INTRODUCTION

Red betel (*Piper crocatum* Ruiz & Pav.) is a plant which belongs to the *Piperaceae* family that can be found in various countries. According to The Plant List (2013), red betel was originally identified in Peru and later found in many countries, including Indonesia. Red betel has been well described to possess anti-inflammatory, anti-microbial, antifungal, antihyperglycemic, and antipro-

liferative potentials (Parfati & Windono 2016). It also has been known to contain active compounds such as flavonoids, polyphenols, and tannins (Dewandari et al. 2013). Recently, there has been growing interest in the study of red betel as a potential source of plant-based antioxidant and also anti-diabetic, anti-inflammatory, and anti-glycative therapeutic agents for some diseases, especially diabetes mellitus (DM).

DM is one of metabolic diseases that affects all of the systems in the body, including liver function. One of the most common features of DM is hyperglycemia which leads to insulin resistance. Insulin resistance, which is exacerbated by oxidative stress and exaggerated inflammatory signals, has been recognized as a dominant contributor to liver injury (Mohamed et al. 2016). Several substances that were released or expressed by the adipose tissues contributed to the development of proinflammatory markers that can be localized in the liver or widely expressed throughout the body. The increase of free fatty acid flow from adipose tissue to non adipose organs as a result of aberrant lipid metabolism, leads to hepatic triglyceride build up and contributes to poor glucose metabolism and insulin sensitivity in muscle and liver as target organs (Bugianesi et al. 2005). In addition, insulin resistance may progress to the chronic and life-threatening diseases such as cirrhosis and end-stage of liver failure (Mohamed et al. 2016).

Previous studies showed that traditional plants such as red betel leaves contain antioxidant and antidiabetic properties such as flavonoids, tannins and polyphenols. Flavonoids have a limited bioavailability but a major health potential that could be explored using various value-added drug delivery technologies. The main limiting factors for flavonoids to bypass the biological barrier and absorbed systematically upon oral administration are water solubility and gastrointestinal stability. Due to these conditions, many promising in vitro bioactivities showed only little or no in vivo activity. On the other hand, flavonoids have substantially improved stability and absorption qualities when administered via nano-sized delivery systems. As an outcome, the activity becomes more intense, noticeable, and prolonged (Bilia et al. 2014).

Thus, this study was conducted to investigate the potential role of red betel leaves nanoparticles (RbL-Nps) as antioxidant and antidiabetic agent targeting the liver and insulin receptor in diabetic rats induced by streptozotocin-nicotinamide (STZ-NA).

MATERIALS AND METHODS

Materials

Red betel leaves were obtained from local farmers in Sleman and Bogor areas, chitosan, sodium tripolyphosphate (Na-TPP), STZ (Nacalai Tesque), NA, Mouse/Rabbit Probe HRP Labeling Kit (BIOTNA, Cat. No. TAHC03D-15), insulin receptor rabbit polyclonal antibody (ABCLONAL, Cat. No. A0287), and sodium carboxymethyl cellulose (Na-CMC).

Methods

Plant determination

Plant sample had been determined as *Piper crocatum* Ruiz & Pav. species by Laboratory of Plant Systematic, Faculty of Biology, Universitas Gadjah Mada (number: 0149494/S.Tb./I/2021).

Plant materials and extractions

The fresh red betel leaves were determined by Laboratory of Plant Systematic, Faculty of Biology, Universitas Gadjah Mada (UGM). For the extraction, fresh leaves were washed, cleaned, and dried using oven at the temperature of 60-70 °C. Dried leaves were ground into powder then processed to maceration extraction using ethanol 96% at room temperature for 48 hours. The filtrate was then evaporated by evaporator (Abubakar & Haque 2020).

Preparation of nanoparticle synthesis

Ionic gelation method between tripolyphosphate (TPP) and chitosan was used to prepare the red betel leaf extract-loaded chitosan nanoparticles (Sundari et al. 2014). One percent of chitosan (w/v) suspension was added dropwise into 5% red betel leaf extract solutions under constant stirring until dissolved. Subsequently, under stirred condition, 1% natrium tripolyphosphate (Na-TPP) solutions were added dropwise to this mixed solution until a homogeneous solution was obtained. Thereafter, the homogenous mixed solution was filtered then dried by evaporator.

Particle size

Particle size distributions of RbL-Nps were performed using Malvern Zetasizer nano instrument. The measurements were performed in triplicates. The data were recorded by computer system. In this study the nanoparticles defined as particles with size range from 10-1000 nm.

Animal preparation

Treatment for the animal model was approved by Institutional Animal Care and Use Committee (IACUC) Faculty of Veterinary Medicine, Universitas Gadjah Mada (Number: 0005/EC-FKH/Int/2021). In this study, 30 adults male Wistar rats weighing 180 ± 20 g, age 3-4 months were used as the animal model. The rats were housed in standard single caged at room temperature with a natural dark-light cycle, provided with commercial pellet diet (Japfa Comfeed, AD II pellets) and free access to water. Prior to experiment, the rats were quarantined for a week.

Experimental design of animal treatment

Rats were randomly separated into 5 groups (GI, GII, GIII, GIV, and GV), each group consists of 6 rats and placed in individual caged. Group I (GI) was the normal control group; while group II (GII) was the diabetic control group. Group II (GII), III (GIII), IV (GIV) and V (GV) were groups in-

duced by streptozotocin (STZ) and nicotinamide (NA). Group I (GI) and GII were then treated with 0,5% Na-CMC orally as placebo. Meanwhile Group III, IV and V were treated by oral administration of RbL-Nps.

Prior to diabetes induction for the GII, GIII, GIV and GV, rats were fasted overnight. The rats of GII, GIII, GIV and GV were then injected intraperitoneally with nicotinamide (NA) at the dose of 110 mg/kg body weight (bw) which was diluted in normal saline. After 15 minutes, the rats were injected with STZ at the dose of 45 mg/kg bw which was diluted in buffer citrate (pH 4.5). After 72 hours, animals with fasting blood glucoses level more than 150 mg/dL were considered diabetic (Ghasemi et al. 2014).

Diabetic rats of GIII, GIV, and GV were treated orally with RbL-Nps at the dose of 30, 60, and 90 mg/kg bw, respectively. The RbL-Nps were diluted in Na-CMA 0,5% before given to the animals. The treatments were conducted once daily using intragastric tube every morning, for 28 days.

On the 29th day, all of the rats were anesthetized using ketamine at the dose of 90 mg/kg body weight after overnight fasting. After the rats were deeply anesthetized, the animals were perfused and subsequently proceeded to the fixation process using 10% neutral buffered formalin. The rat's livers, the left lateral lobe (Garcia-Moreno et al. 1994), were collected and immersed in 10% neutral buffered formalin. The fixed livers were trimmed into 4 mm and were processed into paraffin embedding. Livers were cut into 5 µm thickness and mounted on the object glass and coated slides.

Livers sections of the left lateral lobe were mounted on uncoated slides, deparaffinized, rehydrated and stained following the hematoxylin-eosin protocols. Histological changes in organs were examined under a light microscope and the microscopic images were captured with Optilab. Histopathological findings of the liver hematoxylin-eosin staining were analyzed descriptively.

Immunohistochemistry

Immunohistochemistry procedure for antibody insulin receptor (INSR) was in accordance with Mouse/Rabbit Probe HRP Labeling Kit (BIOTNA, TAHC03D) protocol. Insulin receptors expression was evaluated semi-quantitatively using the modified immunoreactive score (IRS) of Remmele method (Nowak et al. 2007). The method considers both the percentage of positive cells (immunoractive cells) and the intensity of the response colour, and the final result is a product of the parameters, ranging from 0 to 12 points. No reaction = 0 point (-); poor reaction = 1-2 point (+); moderate reaction = 3-4 point (++); severe reaction = 6-12 point (+++). The positive cells (immunoractive cells) are marked by the brown colour on the hepatosite as the result of the use of DAB chromatogen. The images were captured using Optilab and cell counting was processed using imageJ. Immunoreactivity score were then analyzed with Kruskall-Wallis test and Mann-Whitney test using SPSS program ver. 25.

RESULTS AND DISCUSSION

Results

Size distributions of the RbL-Nps

The data presented in the Figure 1a were sorted from the smallest to the largest size of the nanoparticles, then accumulated to the point of 100% and determined the d80 (Figure 1b). Based on the curve the size of samples had d80 = 712.4 nm. This means that 80% of the diameter of the RbL-Nps has a size of 712,4 nm or smaller. However, this size confirmed that the RbL-Nps synthesized for this research were determined as nanoparticles.

The size distributions of RbL-Nps samples were obtained from particle size distribution curve as presented in Figure 1.

Histological feature of liver with hematoxylin-eosin staining

Histological features of rat's liver on group I (GI) showed normal structure of the hepatocytes and the sinusoids. Meanwhile, the group II (GII) showed severe pathological changes of the hepatocytes. Lipid accumulation was seen with expanded vacuoles pushing the nucleus into the periphery of the hepatocyte. Necrosis was also observed in some cells with enlarged sinusoids.

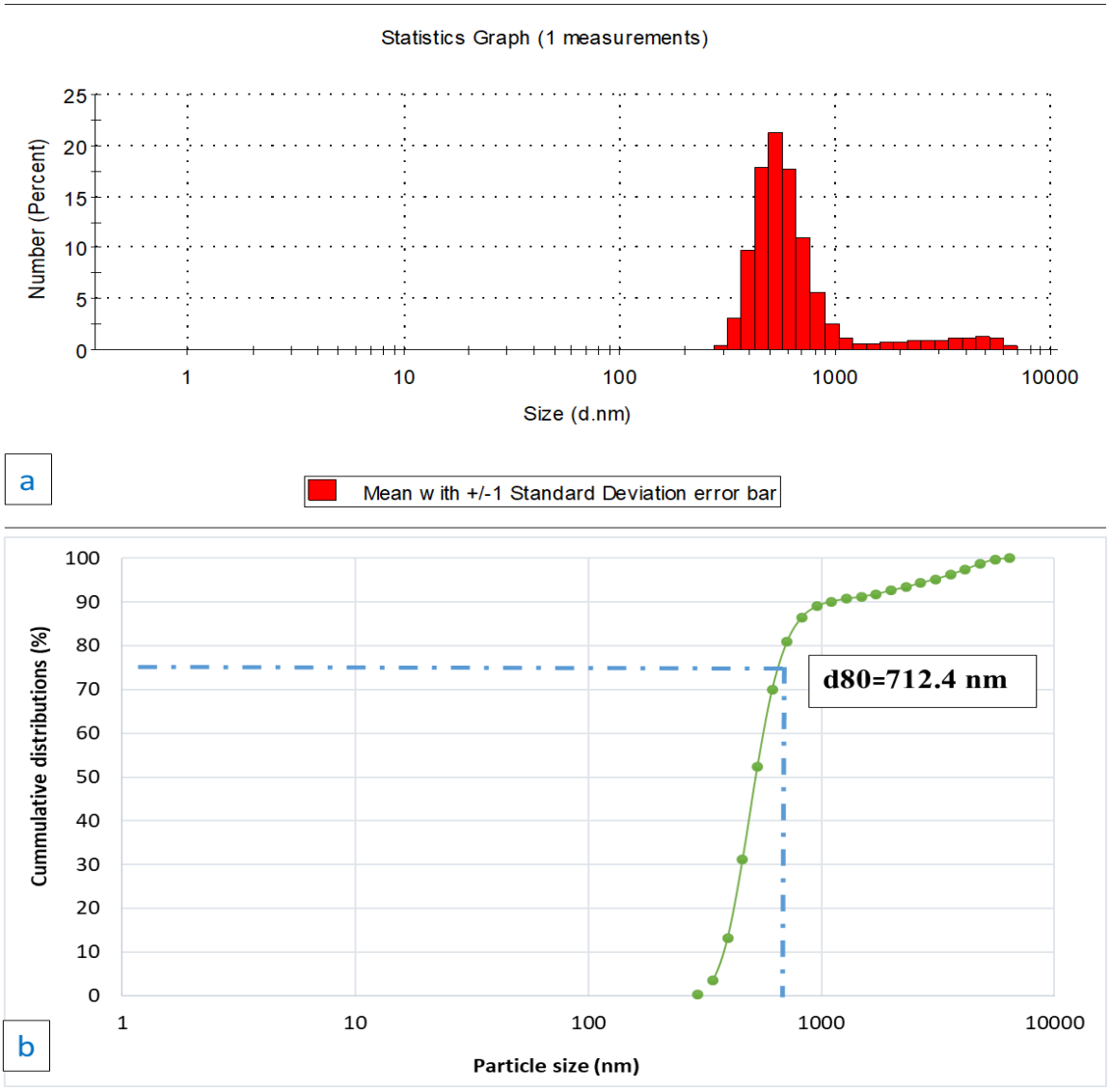

Figure 1. Particle size distribution by number (a) and cumulative distribution curves (b) of RbL-Nps.

All groups that were administered with RbL-Nps showed various histological changes depending on the given doses. Group III (GIII) that were administered with RbL-Nps at the dose of 30 mg/kg bw showed few histological changes of the hepatocytes and the sinusoids. Hepatocytes appeared normal and few of them appeared to have lipid accumulation. The nucleus appeared normal in the center of the hepatocyte and with normal features of sinusoids. Groups that were administered with RbL-Nps at the dose of 60 mg/kg bw (GIV) also showed a similar histological change with GIII.

Compared to GIII and GIV, changes were found on the group V (GV) that were administered with RbL-Nps at the dose of 90 mg/kg bw. Lipid accumulation with small to large vacuoles appeared evenly throughout the hepatocytes and pushed the nuclei to the periphery of the hepatocytes. Widening of the sinusoid were also found in this group. Necrotic area was observed in some hepatocytes with sinusoids filled with red blood cells. Histological changes of the rat's liver were presented on Figure 2.

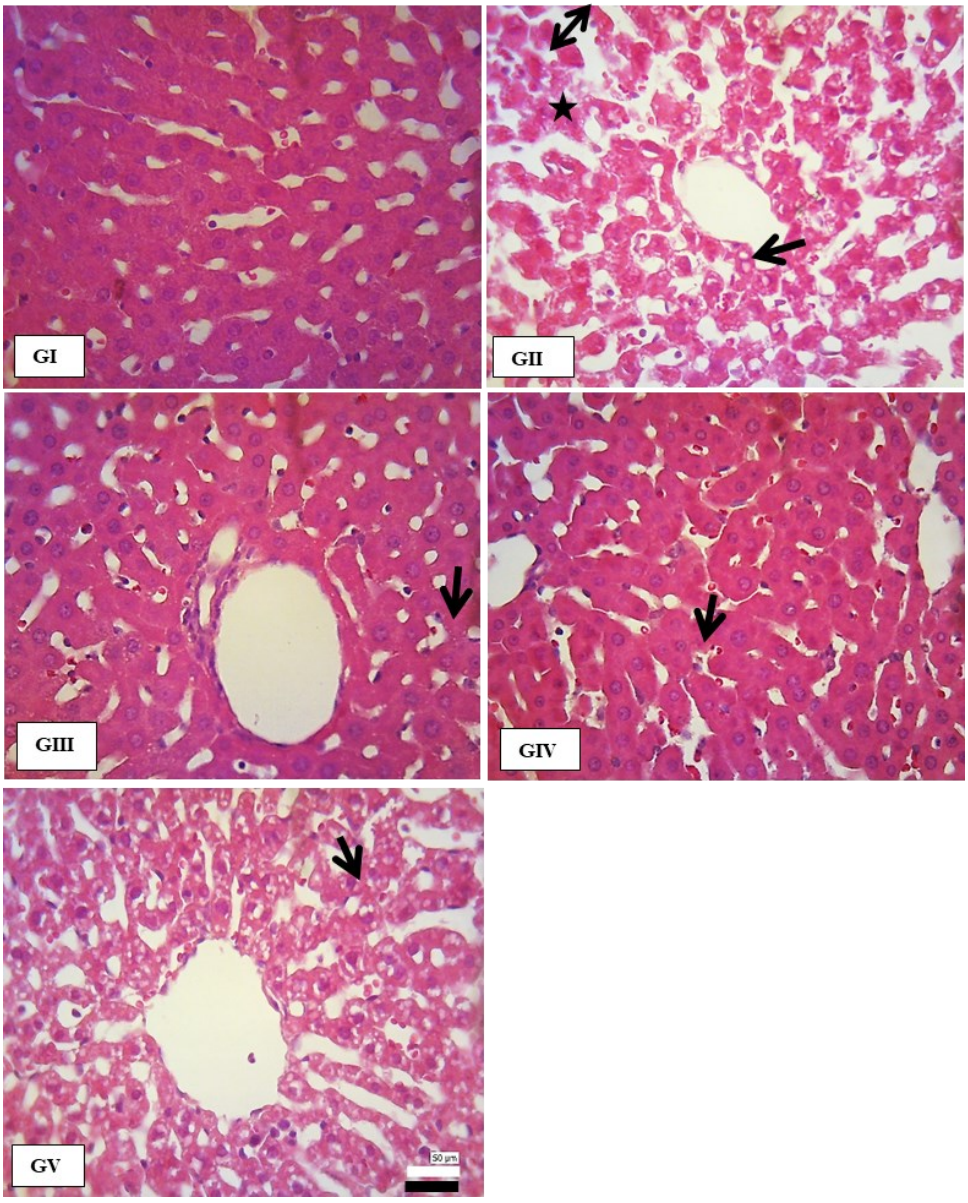

Figure 2. Histological features of the rat's liver, H&E staining. (GI) normal control group; (GII) diabetes control group; (GIII) diabetic control group treated with RbL-

Nps at the dose of 30 mg/kg bw; (GIV) diabetic group treated with RbL-Nps at the dose of 60 mg/kg bw; (GV) diabetic group treated with RbL-Nps at the dose of 90 mg/kg bw. Double headed arrows; star icons; and black arrows represented the widening of sinusoid; necrotic area of the hepatocytes; and fatty degeneration of the hepatocytes, respectively. 50 μm scale bar shown on GV was applied for all images.

Immunohistochemistry Features of Insulin Receptor (INSR) Expression on the Liver

In addition to the histopathological changes, immunohistochemistry staining method was done to determine the expression of INSR on the liver. Insulin receptor can be found on the perivenous area, especially at the cell membrane, cytoplasm, and nucleus of the hepatocyte. Statistical analysis of the insulin receptor's expression on the liver was shown on Table 1 bellow.

Table 1. Average of immunoreactivity score of INSR on the liver.

Treatment's Group	Immunoreactivity Score of INSR (Mean rank ± SD)
GI (normal control)	83.92 ± 0.563^{a}
GII (diabetes control)	60.30 ± 0.450^{b}
GIII (diabetes + RbL-Nps at the dose of 30 mg/kg bw)	83.20 ± 0.498^{ac}
GIV (diabetes + RbL-Nps at the dose of 60 mg/kg bw)	79.05 ± 0.547^{ad}
GV (diabetes + RbL-Nps at the dose of 90 mg/kg bw)	71.03 ± 0.430^{ab}

Note: Different superscript (^{a,b,c,d}) on the different group means significant difference ($p < 0.05$).

Based on the data analysis of the INSR immunoreactivity score shown on the Table 1 there was significant difference ($p < 0.05$) on the INSR immunoreactivity score between GI and GII ($p = 0.013$), GII and GIII ($p = 0.011$) as well as GII and GIV ($p = 0.040$). On the other hand, immunoreactivity score of the INSR among GI, GIII, GIV showed significant differences compared to GII. There was also no significant difference between GII and GV.

According to Table 1, administration of RbL-Nps at doses of 30 and 60 mg/Kg bw to GIII and GIV showed a positive result marked by the increase of insulin receptor expression in hepatocytes compared to the diabetic control group (GII) which were not administered RbL-Nps. There was no statistically significant difference or remained similar mean rank in insulin receptor expression among GI, GIII and GIV.

Immunoreactivity to INSR expression on the liver sections were shown on Figure 3. According to the Figure 3, we found strong expression of INSR on the GI as the nondiabetic control group. Well expressed INSR were shown on the GIII, GIV and GV. The lowest expression of INSR showed on the GII as the diabetic control group.

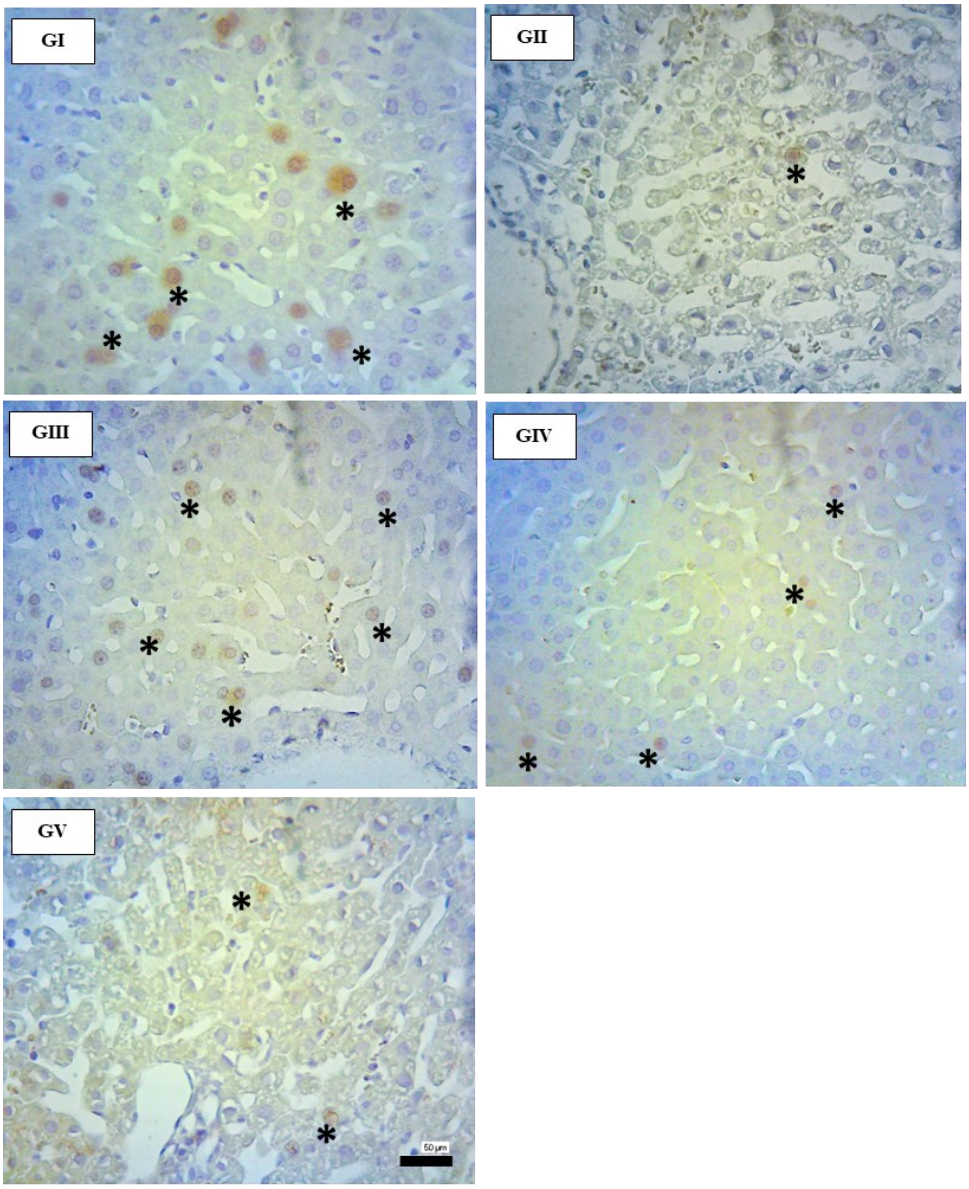

Figure 3. Photomicrograph of insulin receptors immunoreactivity in the rat's liver tissues. (GI) normal control group; (GII) diabetes control group; (GIII) diabetic control group treated with RbL-Nps at the dose of 30 mg/kg bw; (GIV) diabetic group treated with RbL-Nps at the dose of 60 mg/kg bw; (GV) diabetic group treated with RbL-Nps at the dose of 90 mg/kg bw. Immunohistochemistry staining with antibody specific to insulin receptor. 50 µm scale bar shown on figure GV was applied for GI, G II, G III and G IV. Brown colour (*) represented the immunoreactive cells.

Discussion

In this study, red betel leaves or "daun sirih merah" that were obtained from Sleman and Bogor area were used and confirmed as *Piper crocatum* species. Morphological features of the leaves included the dark green color in upper side with silvery color around the leaf bone and purple color on the bottom (Parfati & Windono 2016). Based on previous study, the young and adult leaves had cordate shapes, and when it went into flowering season, the plant formed hanging branches and the leaves underwent shape changes into elliptical (Astuti et al. 2011).

The RbL-Nps particle size distribution in this study had d80 = 712.4

nm, which means that 80% of the samples RbL-Nps were smaller than 712.4 nm. According to the theory, nanoparticles were defined as particles with a size between 1-1000 nm (Duncan & Gaspar 2011). The particles sizes of RbL -Nps in current study were larger than previous study (Dewandari et al. 2013) probably due to the difference of NaTPP and chitosan concentration. High concentrations of chitosan (1%) and NaTPP (1%) were used in this study. It was demonstrated before that the high concentrations of TPP and chitosan could increase the particle sizes and when lowering both concentrations decreased particle sizes (Mudhakir et al. 2014). Crosslinking between NaTPP and chitosan resulted in the increase of viscosity and homogenization capabilities which lead to aggregation forming larger nanoparticles (Rodrigues et al. 2012).

Nanoparticles have been described to be able to increase bioavailability of drugs or herbal. Particles in nano size have a larger surface area so that the distribution of particles expand and their solubility increase. Therefore, the use of nano-sized particles would increase the contact of the particles with the surrounding materials (Abdullah et al. 2008; Dizaj et al. 2015), which in this case, increased the contact of RbL-Nps with gastric and intestinal mucosa. Chitosan as a nanocarrier has the ability to open the tight junction reversibly, thereby increasing paracellular permeability, aside from that it also has mucoadhesive property (Yeh et al. 2011). Those mucoadhesive and tight junctions opening property may slow and sustain the release of bioactive compound from nanoparticles into the circulation system via paracellular pathway (Sung et al. 2012). It has been reported that the release of total phenol from red betel leaf-chitosan nanoparticles was slowed and sustained in gastric condition (Dewandari et al. 2013).

Observation of the diabetes rat's liver induced by streptozotocin on GII showed the damaged of the liver characterized by the presence of fatty accumulation on the hepatocytes and the occurrence of tissue necrosis. Diabetes mellitus (DM) condition due to insulin reduction could cause hyperglycemia and the upregulation of the hormone-sensitive lipase on the adipose tissue resulting in lipid breakdown into free fatty acid. Free fatty acid in the blood circulation then accumulated in the liver causing fatty liver. Liver damage could be exacerbated by the release of tumor necrosis factor α and leptin which resulting in the mitochondrial oxidative stress on the hepatocytes (Mohamed et al. 2016).

The improvement of the liver damage due to DM condition could be seen on the groups that were administered with RbL-Nps. Observation on the histological changes of the liver tissue showed an improvement in liver structure indicated by the reduction of the fat vacuoles in the group administered with RbL-Nps at the doses of 30 (GIII) and 60 mg/kg bw (GIV) compared with diabetes control group (GII). Group administered with RbL-Nps at the dose of 90 mg/kg bw (GV) also showed an improvement on the liver histological structure but not as good as GIII and GIV. Findings in this study demonstrated the potential role of RbL-Nps in repairing liver damage caused

by free fatty acid accumulation and oxidative stress on the liver.

Red betel leaf extract has been widely known to contain active compounds in the form of polyphenols, tannins, and flavonoids (Dewandari et al. 2013). Study conducted by Lister et al. (2019) had shown the main compounds of the red betel leaf ethanolic extract were eugenol and hydroxychavichol which demonstrated an antioxidant activity indicated by the result of 1,1-diphenyl--2-picrylhydrazyl (DPPH) scavenging test, scavenging of H_2O_2, 2,2'-Azinobus-(3-ethylbenzo thiazoline-6sulfonic acid) (ABTS) reduction and the ferric reducing antioxidant power (FRAP) assay.

Lister et al. (2020) also showed that antioxidant properties of red betel leaves extract at low concentration could diminish TNF-α level, reduce the necrotic cell percentage and reactive oxygen species level; as well as gaining the glutathione peroxidase (GPX) gene expression in H_2O_2-induced liver injury model. This was also explained by Dinda et al. (2019) that flavonoids could affect the genes related to inflammation such as TNF-α, IL-6, IL-1β, MCP-1, TLR-2/4, TGF-β, INOS, ICAM-1, and NOX by decreased their activation.

Flavonoids could also act as an antidiabetic property due its function to improve the insulin sensitivity on the target organ. Flavonoids have major therapeutic targets on various genes that are metabolically related to target tissues. Flavonoids could increase adiponectin secretion that leads to activation of p-AMPK and SIRT1 that could diminish FoxO1 activation. This condition could lead to the decrease of gluconeogenesis activity, G6Pase and PEPCK on the liver. Increased p-AMPK could also affect the increase of fatty acid oxidation and the increase of p-PPARKγ which caused the decrease of the lipogenesis activity (Dinda et al. 2019).

In our research, the rat model for DM was treated by nicotinamide injection and then followed by injection of streptozotocin. Nicotinamide plays a role to protect the pancreas from severe damages by streptozotocin that make a chronic response of DM. The chronic of DM caused DM type 2 characterized by the disturbance of INSR. Samuel and Shulman (2016) reported that INSR activation plays an important role in initiating a complex of insulin signaling pathways. According to Wang et al. (2019) malfunction of INS thought to be the cause of type 2 diabetes mellitus which is characterized by insulin resistance. Our current study showed that RbL-Nps at the doses of 30 and 60 mg/kg bw increased insulin receptor expression on the group of diabetic rats. Also, in line with our previous study, diabetic rats given RbL-Nps at same doses had significantly increased plasma insulin levels (Nuraniyati 2021) and increased cardiac INSR expressions (Pramesti 2021) than the diabetes control group. The flavonoids compounds contained in RbL-Nps could increase the insulin action on the liver. The increased insulin action on the liver caused the increased of p-IRS1/2 activations which is a substrate formed due to the binding between insulin and insulin receptor (Dinda et al. 2019). Group V that was administered with RbL-Nps at the dose of 90 mg/kg bw showed slightly increased of insulin receptor expres-

sion compared to the diabetes control group, although statistically non significantly different.

In insulin resistance condition, insulin's ability to suppress glycogenolysis or gluconeogenesis is diminished, but it continues to promote hepatic lipogenesis, which contributes to the development of hepatic steatosis (Perry et al. 2014). The latter is characterized by hepatic lipid accumulation (steatosis) in accordance with overt inflammation, which results in hepatocyte death, fibrosis, and impaired hepatic function (Michelotti et al. 2013). This was proven in our study by the accumulation of vacuolic hepatocytes on the control diabetic group. Vacuolic hepatocytes also were found on group administered with RbL-Nps at the dose of 90 mg/kg BB. We assume that at highest dose of RbL-Nps can exacerbate the liver function. It has been known that herbal supplement can induce liver injury (Lin et al. 2019).

Based on our findings, administration of RbL-Nps to the diabetic rats could repair the liver damage due to fatty acid accumulation and oxidative stress. Thus, administration of RbL-Nps at the doses of 30, 60 and 90 mg/kg bw can repair liver damage due to fat accumulation and oxidative stress and has the potential to increase insulin sensitivity by increasing insulin receptor expression on the diabetic rat's liver. However, the outcomes at the dose of 90 mg/kg bw were not as favorable as in the two lower dose groups. RbL-Nps at the dose of 90 mg/kg bw showed induce disturbance in the livers of rats.

Futher research is needed to study the effect of administration of RbL-Nps at the dose of 30 and 60 mg/kg bw of normal control group on liver, for safety reason to healthy people, since it is possible people will consume nanoparticle of red betel leaves extract in normal situations. The expression levels of genes related to inflammation and antioxidant genes in liver tissues also need to study further which could give a better understanding of the molecular mechanisms involved.

CONCLUSION

Administration of RbL-Nps at the doses of 30, 60 and 90 mg/kg bw could improve the repairment of the liver damage and improve the expression of the insulin receptor (INSR) on the diabetic rats induced by streptozotocin. RbL-Nps administration at the doses of 30 and 60 mg/kg bw resulting the best effect at repairing the liver damage and improve the INSR expression better than RbL-Nps at the dose of 90 mg/kg bw. These findings indicated that RbL-Nps at the dose of 30 and 60 mg/kg bw improved diabetic rats' liver condition, however at the dose of 90 mg/kg bw, it did not ameliorate liver damaged of diabetic rats.

AUTHORS CONTRIBUTION

Tri Wahyu Pangestiningsih designed the research and supervised all the process, Citra Ayu Pramesti collected, prepared, and stained the sample, Nu-

saibah Nuraniyati analyzed the data, Bambang Sutrisno examined microscopic finding and Agus Purnomo maintained and cared animal treatment.

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CONFLICT OF INTEREST

The authors declare that they have no known competing personal interest and relationship that could have appeared to influence the work reported in this paper.

REFERENCES

Abdullah, M. et al., 2008. Review: Sintesis Nanomaterial. *J. Nanoscience & Nanotechnology*, 1(2), pp.33-57.

Abubakar, A.R. & Haque, M., 2020. Preparation of Medicinal Plants: Basic Extraction and Fractionation Procedures for Experimental Purposes. *Journal of Pharmacy and Bioallied*, 12(1), pp.1-10. doi: 10.4103/jpbs.JPBS_175_19.

Astuti, I.P. et al., 2011. Heteroblastic Development in Six Species of Wild Piper: Piper baccatum Blume, Piper firmum Blume, Piper majusculum C.DC, Piper miniatum Blume, Piper crocatum Ruiz & Pav. and Piper retrofractum Vahl.1. *Berita Biologi,* 10(5), pp.621–625. doi: 10.14203/beritabiologi.v10i5.1920.

Bilia, A.R. et al., 2014. Flavonoids Loaded with Nanocarriers: An Opportunity to Increase Oral Bioavailability and Bioefficacy. *Food and Nutrition Science,* 5(3), 1212-1227.

Bugianesi, E. et al., 2005. Insulin Resistance: A Metabolic Pathway to Chronic Liver Disease. *Journal of Hepatology,* 42, pp. 987-1000. doi: 10.1002/hep.20920.

Dewandari, K.T. et al., 2013. Ekstarksi dan Karakterisasi Nanopartikel Ekstrak Sirih Merah (Piper crocatum). *Jurnal Pascapanen*, 10(2), pp.58–65. doi: 10.21082/jpasca.v10n2.2013.58-65.

Dinda, B. et al., 2019. Dietary Plant Flavonoids in Prevention of Obesity and Diabetes. *Journal of Advances in Protein Chemistry and Structural Biology*, 120, pp. 159-235. doi: 10.1016/bs.apcsb.2019.08.006.

Dizaj, S.M. et al., 2015. Nanosizing of drugs: Effect on dissolution rate. *Research in Pharmaceutical Sciences*, 10(2), pp.95-108.

Duncan, R. & Gaspar, R., 2011. Nanomedicine(s) under the microscope. *Molecular Pharmaceutics*, 8(6), pp.2101–2141. doi: 10.1021/mp200394t.

Garcia-Moreno et al., 1994. Behaviour of the nucleolar organizer regions on the different Wistar rat liver lobes. *Lab Anim*; 28, pp.50–54.

Ghasemi, A. et al., 2014. Streptozotocin-nicotinamide-induced rat model of type 2 diabetes. *Acta Physiologica Hungarica*, 101(4), pp.408–420. doi: 10.1556/APhysiol.101.2014.4.2.

Lin, N.H. et al. 2019. Herb Induced Liver Injury After Using Herbal Medicine: A systemic review and case-control study. *Medicine*, 98(3), 1-8.

Lister, I.N.E. et al., 2019. Antioxidant Properties of Red Betel (*Piper crocatum*) Leaf Extract and its Compounds. *Journal of Natural Remedies,* 19(4), pp. 2330-3358. doi: 10.18311/jnr/2019/23633.

Lister, I.N.E. et al., 2020. Hepatoprotective Properties of Red Betel (*Piper crocatum* Ruiz and Pav) Leaves Extract Towards H_2O_2-induced HepG2 Cells Via Anti-inflammatory, Antinecrotic, Antioxidant Potency. *Saudi Pharmaceutical Journal*, 28, pp. 1182-1189. doi: 10.1016/j.jsps.2020.08.007.

Michelotti, G.A. et al., 2013. NAFLD, NASH and liver cancer. *Nature Reviews Gastroenterology & Hepatology*, 10(11), pp. 656–665. doi:10.1038/nrgastro.2013.183.

Mudhakir, D. et al., 2014. Encapsulation of Risperidone into Chitosan-based Nanocarrier via Ionic Binding Interaction. *Procedia Chemistry*, 13, pp.92–100. doi: 10.1016/j.proche.2014.12.011.

Mohamed, J. et al., 2016. Mechanisms of Diabetes-Induced Liver Damage: The Role of Oxidative Stress and Inflammation. *Sultan Qaboob University Med J,* 16, pp. 132-141. doi: 10.18295/squmj.2016.16.02.002.

Nowak, M. et al., 2007. Intensity of Cox2 Expression In Cells of Soft Tissue Fibrosarcomas in Dogs as Related to Grade of Tumour Malignancy. *Bulletin of Veterinar Institute in Pulawy,* 51(2), 275-279.

Nuraniyati, N., 2021. *Effect of Nanoparticles Red Betel Leaf Extract on the level of Plasma Insulin, Plasma Malondialdehyde, and Insulin Expression in Pancreatic Islet Langerhans of Diabetic Type 2 Rats*. Gadjah Mada University.

Parfati, N. & Windono, T., 2016. Sirih Merah (*Piper crocatum* Ruiz & Pav.) Kajian Pustaka Aspek Botani, Kandungan Kimia, dan Aktivitas Farmakologi. *Media Pharmaceutica Indonesiana*, 1(2), pp.106–115. doi: 10.24123/mpi.v1i2.193.

Perry, R.J. et al., 2014. The role of hepatic lipids in hepatic insulin resistance and type 2 diabetes. *Nature*, 510(7503), pp.84–91. doi: 10.1038/nature13478.

Pramesti, C.A., 2021. *Effectiveness of Ethanolic Extract of Red Betel Leaves Nanoparticle on Blood Glucose Levels, Expression of Insulin Receptor, GLUT4 and Intracellular Hsp70 on Type 2 Diabetes Mellitus Rats*. Gadjah Mada University.

Rodrigues, S. et al., 2012. Chitosan/Carrageenan Nanoparticles: Effect of Cross-linking with Tripolyphosphate and Charge Ratios. *Carbohydrate Polymers*, 89, pp.282-289. doi: 10.1016/j.carbpol.2012.03.010.

Samuel, V.T. & Shulman, G.I., 2016. The pathogenesis of insulin resistance: integrating signaling pathways and substrate flux. *The Journal of clinical investigation*, 126(1), pp.12–22. doi: 10.1172/JCI77812.

Sundari, et al., 2014. Effect of Nanocapsule Level on Broiler Performance and Fat Deposition. *International Journal of Poultry*, 13(1), pp.31-35. doi: 10.3923/ijps.2014.31.35.

Sung, H.W. et al., 2012. pH-Responsive Nanoparticles Shelled with Chitosan for Oral Delivery of Insulin: From Mechanism to *Therapeutic Applications. Accounts of Chemical Research*, 45(4), pp.619-629. doi: 10.1021/ar200234q.

The Plant List, 2013. *Piper crocatum* Ruis & Pav., Fl. Peruv. In *International Plant Names Index,* viewed 16 October 2021, from https://www.ipni.org/n/681064-1.

Wang, Y., et al. 2019. Restoration of insulin receptor improves diabetic phenotype in T2DM mice. *JCI Insight*, 4(15), e124945. doi: 10.1172/jci.insight.124945.

Yeh, T.H. et al., 2011. Mechanism and consequence of chitosan-mediated reversible epithelial tight junction opening. Biomaterials, 32(26), pp.6164-6173.

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Research Article

Molecular Identification and Phylogenetic Tree Reconstruction of Marine Fish Species from the Fishing Port of Kutaradja, Banda Aceh

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ABSTRACT
The enormous potential of marine resources possessed by Banda Aceh Province is expected to be utilised optimally. Accuracy in marine fish resource identification is a critical requirement to support their utilisation and preservation in Banda Aceh Province. In this study, a molecular identification approach was carried out in addition to conducting a morphological identification, commonly used by several scientists. The results were 47 COI sequences generated representing 33 genera, 19 families, and five orders. From the resulting COI partial sequences, there is one potential haplotype from the Scombridae family (*Auxis thazard*), two potential haplotypes from the Carangidae family (*Elagatis bipinnulata* and *Decapterus macarellus*), and two potential haplotypes from the Serranidae family (*Variola albimarginata* and *Cephalopholis sonnerati*). This study is essential for fisheries biological studies and other fisheries studies to support the sustainable utilisation of marine fisheries potential in Banda Aceh.

INTRODUCTION
Aceh is the westernmost province of the Indo-Malaya Archipelago (IMA), an area known as a hot spot of tropical marine biodiversity (Briggs 2005; Hoeksema 2007; Bellwood & Meyer 2009; Veron et al. 2009; Gaither et al. 2011). This province has a high fisheries potential, with a water area reaching 295,370 km^2 and a coastline length of 2,666.3 km (Fikri 2013). One of the centres of fishing activity and the most significant fish landing site in Aceh is the Fishing Port of Kutaradja. Marine fisheries production at this fishing port increased from 8,922 tons in 2013 to 12,305 tons in 2017 (Yusuf 2003; Yeni & Naufal 2017; Mardhatillah et al. 2019) The fish landing site suffered massive damage due to the tsunami that struck Aceh Province and rebuilt in 2004 (Zulmaidah et al. 2015). The rebuilding of the Kutaradja fishing port has revived the economy and fisheries activities in the Banda Aceh region.

Regarding the fishing grounds for fishers at this fishing port, all the fishing zones include the Indian Ocean, Andaman Sea, and Malacca Strait. Two of the three fishing zones are included within two out of the 11 Indone-

sian Fisheries Management Areas (FMA), namely FMA 571 and 572. Since 2009, Indonesia has determined the management of territorial waters into several areas according to Law no. 31 of 2004 in conjunction with Law No. 41 of 2009 (Suman et al. 2017), which called Indonesian Fisheries Management Areas (FMA). The management area in western Sumatra includes FMA 571 in the Malacca Strait (Damanik et al. 2016) and FMA 572 in the Indian Ocean waters west of Sumatra. In the framework of fisheries management policies in Indonesia, the 11 FMAs stretch from the Malacca Strait in the west of Indonesia to the Arafura Sea in the east of Indonesia (Damanik et al. 2016). The level of utilization of pelagic and demersal fish resources in the two FMAs is categorized in the overexploited category (Suman et al. 2017; Salmarika & Wisudo 2019).

Previous research on the types of fish landed by the many traditional fishers of the Kutaradja Fishing Port were conducted based on fish morphology and anatomy. From the inventory carried out at the Kutaradja Fishing Port, 11 species were identified (Munawwarah et al. 2016). However, another report on the types of marine fish species in Simeuleu Island identified around 77 marine fish species which are members of 54 genera, 26 families, and seven orders (Batubara et al. 2017). The reef-associated fish inventory at Ulee Lheue, Banda Aceh, also mentioned that there were 87 species of reef fishes from 28 families in this location (Fadli et al. 2019). In different areas (i.e. Lhoknga and Lhok Mata Ie Beaches) 25 fish species which are members of eight orders, 11 families, and 19 genera were recorded from 51 fish samples (Nur et al. 2019); 71 species were identified in Pusong Bay, Lhokseumawe belonging to 54 genus, 37 families and 15 orders (Damora et al. 2020); 50 species were identified in the Weh Island, Sabang belonging to 24 families and eight orders (Zulfahmi et al. 2022). The morphological approach is the most widely used method in many regions in Indonesia, including in Banda Aceh.

This research identified marine fish at the molecular level in the Cytochrome C Oxidase subunit I (COI) region of the mitochondrial gene to complete the morphological identification that was also carried out. This COI Region is the region that some gene markers have agreed on globally for molecular identification. Research on barcoding of several aquatic biota has been carried out such as for marine fish in Australia (Ward et al. 2005), marine fish in India (Lakra et al. 2011), marine fish in Turkey (KeskİN & Atar 2013), marine fish in China (Wang et al. 2012; Zhang & Hanner 2012), and marine fish in Taiwan (Chang et al. 2017; Bingpeng et al. 2018). Whereas research on fish molecular identification in Aceh has been carried out on some species such as groupers (Kamal et al. 2019), and *Scomber* spp. (Edwarsyah et al. 2019). This research is the first study to carry out molecular identification on the marine fish landed at the Kutaradja fishing port.

The purpose of this research is to identify marine fish to species level by using a molecular approach. This approach has higher accuracy of identification until species level. In addition, the research aims to identify Aceh's

potential haplotype for the Scombridae, Serranidae, and Carangidae groups, which are pelagic fish resources with significant economic important. DNA Barcoding will strengthen genetic information availability and it can be used for other studies such as breeding, fishery management, as well as conservation (Afriyie et al. 2019). One of the studies which is essential is haplotype analysis. Haplotype analysis can only be conducted based on genetic information, especially the DNA sequences from the number of unique species in a particular region.

MATERIALS AND METHODS

Sampling site

A total of 47 fish samples were collected from the Kutaradja Fishing Port on 19 July 2019 (5°35'09"N -95°19'06"E) (Nasution et al. 2019). Morphological identification and species confirmation have been carried out together with the molecular identification carried out in this study. Morphological identification by guideline of FAO Species Identification Guide for Fishery Purposes (Carpenter and Niem 2001). No specific permit was required for this study, and a digital camera was used to take individual photographs. All samples collected from the fish market were already dead upon purchasing. All specimens have been deposited to the Fisheries Laboratory, Faculty of Fisheries and Marine, Universitas Airlangga. All specimens keep in ethanol 90% with samples code AC no 01-47.

DNA extraction and PCR condition

Each specimen wascollected based on the morphological characters and following collection were directly preserved in 90% ethanol for further experimental purposes. Genomic DNA were extracted using an Accuprep® Genomic DNA Extraction Kit (Bioneer) following the manufacturer's guidelines. Around 1 cm tissue was dissected from the anal fin and mixed with 6X lysis buffer, which was further homogenized using the TissueLyser II (Qiagen). Quantification of purified genomic DNA was performed using NanoDrop (Thermofisher Scientific D1000), aliquoted and stored at the -70°C for further analysis.

One set of universal fish primer targeting cytochrome c oxidase I (COI) region, BCL-BCH (Baldwin et al. 2009, Handy et al. 2011), was used to obtain the partial sequences of each gene. The PCR mixture (20µL) included 11.2 µL ultra-pure water, 1 µL primer forward and reverse (0.5 µM), 0.2 µL Ex Taq DNA polymerase (TaKaRa, Japan), 2 µL 10X ExTag Buffer, 2 µL dNTPs (1 µM, TaKaRa, Japan), and 2 µL genomic DNA as template. The PCR condition was carried out under the following setting: 95°C for 5 min in initial denaturation, followed by denaturation at 95°C for 30 s in 40 cycles, 50°C for 30 s in annealing, and 72°C for 45 s in extension step, and a final extension at 72°C for 5 min. The PCR products were purified with the AccuPrep®Gel purification kit (Bioneer, Korea). The experiment was conducted at Molecular Physiology Laboratory, Department of Marine Biology,

School of Fisheries Science, Pukyong National University, Busan Korea. All PCR products were sent to Macrogen (Seoul, Korea) for sequencing.

Sequence alignment and data analyses

All sequences were aligned and submitted to GenBank (Table 1). All raw files after sequencing were trimmed and the sequences quality were checked using Chromash® (downloaded from http://technelysium.com.au/wp/chromas/) to read the ab1 file format. Then, the reverse sequence was aligned with Clustal-omega using online system through https://www.ebi.ac.uk/Tools/msa/clustalo/, but reverse complement (https://www.bioinformatics.org/sms/rev_comp.html) was also performed on reverse sequences to make them have the same direction with the forward sequences. The BLASTN which is provided on NCBI system was applied for sequences identification (https://blast.ncbi.nlm.nih.gov/Blast.cgi). After all sequences have been identified (species name) using BLASTN, the phylogenetic tree was then constructed. The pairwise evolutionary distance among the families was determined by the Kimura 2-Parameter method. The Neighbour-joining (NJ) tree constructed, and 1000 bootstrap analysis was carried out by MEGA7 (Kumar et al. 2016). Besides, nucleotides composition and genetic distance were also generated by MEGA7, including sequences alignment and transition/transversion bias after phylogenetic trees reconstruction was conducted.

RESULTS AND DISCUSSION

Results

Species Identification

In this study, a pair of universal COI primers BCL-BCH succeeded in obtaining DNA target sequences of more than 600 bp (Baldwin et al. 2009, Handy et al. 2011), it is effectiveness and efficiency to be used as a standard for molecular identification at species level. This strengthens previous research which has also succeeded in using these primers in molecular identification down to the species level (Pringgenis & Susilowati 2016; Serdiati et al. 2020). Here, we report the identification of marine fish from the Lampulo fish market, Aceh which is one of the center for fisheries in the province. A total of 47 COI sequences were generated representing 37 species, 33 genera, 19 families, and five orders with % identity ranging between 99-100% when compared to the GenBank dataset on BLASTN online system. Common names, taxonomic designation, habitat, IUCN list, as well as the GenBank accession number for all specimens are listed in Table 1. The sequencing of the COI gene produced more than 600 nucleotide base pairs per taxon. The un-ambiguity and simplicity were observed among all the sequences and no stop codons, deletions, and insertions were observed in all the sequences. Here, we cluster them into two groups in phylogenetic reconstruction, namely “Perciformes” and “other order”.

Table 1. The marine fish species list was identified by COI region from Lampulo fish market, Banda Aceh, Indonesia

No.	ID (AC).	Species Name	Family	GenBank Acc No.	Order	Common name	Habitat	IUCN list
1	16	*Myripristis berndti*	Holocentridae	MN257521	Beryciformes	Blotcheye soldierfish	Indo-Pacific and Eastern Pacific	LC
2	17	*Myripristis berndti*	Holocentridae	MN257522	Beryciformes	Blotcheye soldierfish	Indo-Pacific and Eastern Pacific	LC
3	36	*Sardinella jussieu*	Clupeidae	MN257539	Clupeiformes	Mauritian sardinella	Western Indian Ocean	DD
4	37	*Sardinella jussieu*	Clupeidae	MN257540	Clupeiformes	Mauritian sardinella	Western Indian Ocean	DD
5	38	*Stolephorus commersonnii*	Engraulidae	MN257541	Clupeiformes	Commerson's anchovy	Indo-West Pacific	LC
6	39	*Stolephorus commersonnii*	Engraulidae	MN257542	Clupeiformes	Commerson's anchovy	Indo-West Pacific	LC
7	40	*Thryssa baelama*	Engraulidae	MN257543	Clupeiformes	Baelama anchovy	Indo-Pacific	LC
8	41	*Thryssa baelama*	Engraulidae	MN257544	Clupeiformes	Baelama anchovy	Indo-Pacific	LC
9	1	*Scolopsis xenochroa*	Nemipteridae	MN257509	Perciformes	Oblique-barred monocle bream	Indo-West Pacific	NE
10	4	*Lutjanus bengalensis*	Lutjanidae	MN257511	Perciformes	Bengal snapper	Indo-West Pacific:	NE
11	5	*Upeneus sulphureus*	Mullidae	MN257512	Perciformes	Sulphur goatfish	Indo-West Pacific	LC
12	7	*Pristipomoides filamentosus*	Lutjanidae	MN257513	Perciformes	Crimson jobfish	Indo-Pacific	LC
13	8	*Parascolopsis eriomma*	Nemipteridae	MN257514	Perciformes	Rosy dwarf monocle bream	Indo-West Pacific	NE
14	9	*Epinephelus areolatus*	Serranidae	MN257515	Perciformes	Areolate grouper	Indo-Pacific	LC
15	10	*Variola albimarginata*	Serranidae	MN257516	Perciformes	White-edged lyretail	Indo-Pacific	LC
16	11	*Cephalopholis sonnerati*	Serranidae	MN257517	Perciformes	Tomato hind	Indo-Pacific	LC
17	12	*Parastromateus niger*	Carangidae	MN257518	Perciformes	Black pomfret	Indo-West Pacific	LC
18	14	*Parupeneus macronemus*	Mullidae	MN257519	Perciformes	Long-barbel goatfish	Indo-West Pacific	LC
19	15	*Parupeneus macronemus*	Mullidae	MN257520	Perciformes	Long-barbel goatfish	Indo-West Pacific	LC
20	18	*Priacanthus tayenus*	Priacanthidae	MN257523	Perciformes	Purple-spotted bigeye	Indo-West Pacific	LC
21	19	*Lethrinus rubrioperculatus*	Lethrinidae	MN257524	Perciformes	Spotcheek emperor	Indo-Pacific	LC
22	25	*Megalaspis cordyla*	Carangidae	MN257528	Perciformes	Torpedo scad	Indo-West Pacific	LC
23	26	*Pomadasys argyreus*	Haemulidae	MN257529	Perciformes	Bluecheek silver grunt	Indo-West Pacific	NE
24	27	*Terapon jarbua*	Terapontidae	MN257530	Perciformes	Jarbua terapon	Indo-Pacific	LC
25	28	*Equulites leuciscus*	Leiognathidae	MN257531	Perciformes	Whipfin ponyfish	Indo-West Pacific	LC
26	29	*Gazza minuta*	Leiognathidae	MN257532	Perciformes	Toothpony	Indo-Pacific	LC
27	30	*Leiognathus striatus*	Leiognathidae	MN257533	Perciformes	Toothpony	Western Indian Ocean	NE
28	31	*Photopectoralis bindus*	Leiognathidae	MN257534	Perciformes	Orangefin ponyfish	Indo-West Pacific	NE
29	32	*Gerres filamentosus*	Gerreidae	MN257535	Perciformes	Whipfin silver-biddy	Indo-Pacific	LC
30	33	*Equulites leuciscus*	Leiognathidae	MN257536	Perciformes	Whipfin ponyfish	Indo-West Pacific	LC
31	34	*Gazza minuta*	Leiognathidae	MN257537	Perciformes	Toothpony	Indo-Pacific	LC
32	35	*Megalaspis cordyla*	Carangidae	MN257538	Perciformes	Torpedo scad	Indo-West Pacific	LC
33	43	*Lutjanus lutjanus*	Lutjanidae	MN257545	Perciformes	Bigeye snapper	Indo-West Pacific	LC
34	46	*Caranx sexfasciatus*	Carangidae	MN257546	Perciformes	Bigeye trevally	Indo-Pacific	LC
35	47	*Siganus sutor*	Siganidae	MN257547	Perciformes	Shoemaker spinefoot	Indian Ocean	LC
36	48	*Siganus sutor*	Siganidae	MN257548	Perciformes	Shoemaker spinefoot	Indian Ocean	LC

Table 1. Contd.

No.	ID (AC).	Species Name	Family	GenBank Acc No.	Order	Common name	Habitat	IUCN list
37	49	*Nemipterus bipunctatus*	Nemipteridae	MN257549	Perciformes	Delagoa threadfin bream	Indian Ocean	NE
38	50	*Terapon jarbua*	Terapontidae	MN257550	Perciformes	Jarbua terapon	Indo-Pacific	LC
39	51	*Terapon jarbua*	Terapontidae	MN257551	Perciformes	Jarbua terapon	Indo-Pacific	LC
40	52	*Coryphaena hippurus*	Coryphaenidae	MN257552	Perciformes	Common dolphinfish	Atlantic, Indian and Pacific	LC
41	53	*Auxis thazard*	Scombridae	MN257553	Perciformes	Frigate tuna	Atlantic, Indian and Pacific (Western Central)	LC
42	54	*Auxis thazard*	Scombridae	MN257554	Perciformes	Frigate tuna	Atlantic, Indian and Pacific (Western Central)	LC
43	55	*Thunnus albacares*	Scombridae	MN257555	Perciformes	Yellowfin tuna	Worldwide in tropical and subtropical seas	NT
44	56	*Decapterus macarellus*	Carangidae	MN257556	Perciformes	Mackerel scad	Circumglobal	LC
45	57	*Katsuwonus pelamis*	Scombridae	MN257557	Perciformes	Skipjack tuna	Cosmopolitan in tropical and warm-temperate waters	LC
46	24	*Psettodes erumei*	Psettodidae	MN257527	Pleuronectiformes	Indian halibut	Indo-West Pacific	NE
47	20	*Platycephalus sp.*	Platycephalidae	MN257525	Scorpaeniformes	Bartail flathead	Indo-West Pacific	DD

Least Concern (LC); Not Evaluated (NE); Data deficient (DD); Near Threatened (NT)

Perciformes

From the total of 37 samples, we successfully identified 31 species from 14 families under Perciformes (30 genera). The nucleotide frequencies of COI sequences are 29.65% (T/U), 23.95% (A), 28.80% (C), and 17.6% (G). The average of transitional pair (si=5.07) was lower than the average of transvertional pair (sv=14.86) with an overall transition/transvertion ratio bias of 1.57. The phylogenetic tree was constructed from the COI sequences for the Perciformes and shows that the average K2P distance within taxonomic levels measured for COI sequences is 0.226 (Figure 1).

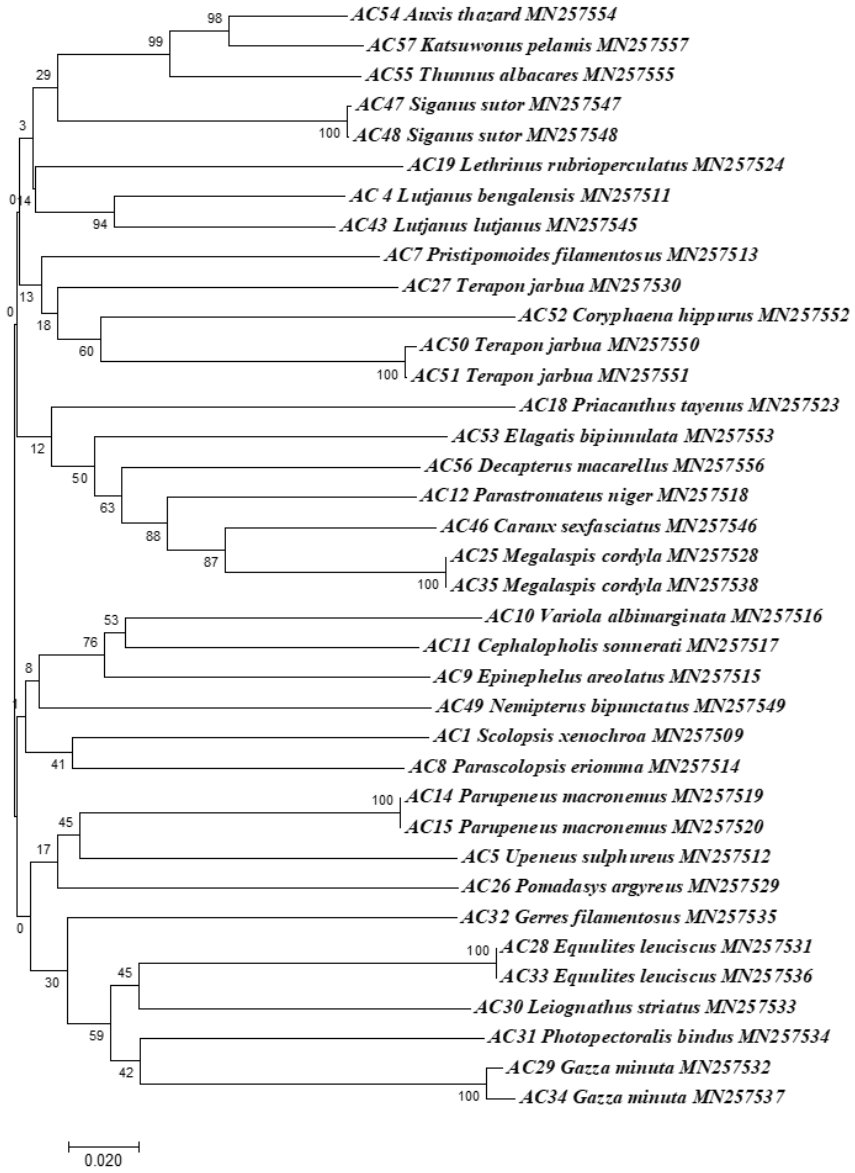

Figure 1. Phylogenetic tree of several Perciformes order by Neighbor-Joining tree algorithm using Mega7

Clupeiformes and Others

In addition to Perciformes, Clupeiformes (3 genera) were also identified from 6 samples which were distributed in three species and three families. For the rest of the samples, one species was from Scorpaeniformes (*Platycephalus* sp.), one species from Pleuronectiformes (*Psettodes erumei*), and one species from

Beryciformes (*Myripristis berndti*). The nucleotide frequencies of the COI sequences were 28.17% (T/U), 23.04% (A), 30.11% (C), and 18.68% (G). The average of transitional pair (si=1.43) was lower than the average of transvertional pair (sv=22.13) with an average transition/transvertion bias of 8.71. The phylogenetic tree was constructed using the COI sequences for the small number order, including the Clupeiformes, Beryciformes, Pleuronectiformes, and Scorpaeniformes (Figure 2). The average K2P distance within taxonomic levels measured for COI sequences is 0.214.

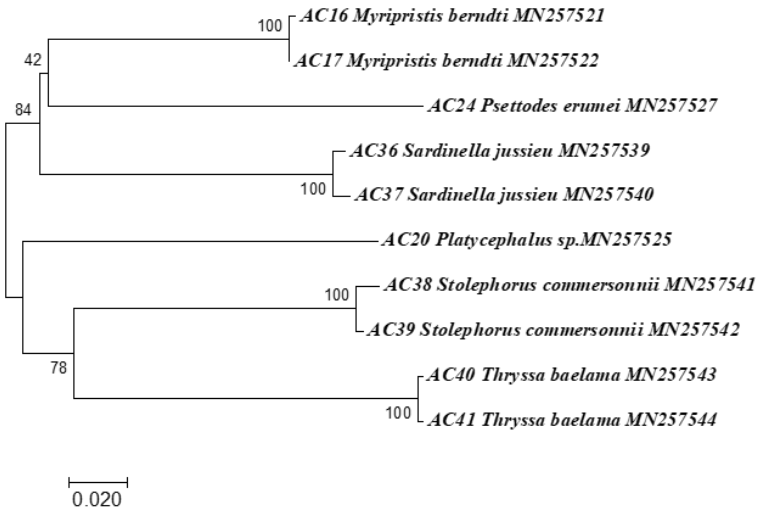

Figure 2. Phylogenetic tree of Clupeiformes including Beryciformes, Pleuronectiformes, and Scorpaeniformes by Neighbor-Joining tree algorithm using MEGA7.

The haplotype of Scombridae, Serranidae, and Carangidae from Aceh

In this study, the sample from Aceh had several unique potential haplotypes when compared to the same species from the GenBank database. By aligning the sequence generated with the reference sequence, some different nucleotides produced genetic variations (Table 2). The phylogenetics tree reconstruction of those sequences show that several potential haplotypes were found in this study (Figure 3). The identified haplotype in the Carangid group was found in the *Decapterus macarellus* species (MN257556) which had similarities with sequences from China and Malaysia, having a genetic distance with an Indian sequence of 0.002. Also, *Elagatis bipinnulata* (MN257553) is closer to the similarity of the sequence owned by the same type of fish (KF461174) from Alabama, USA. While the genetic distance of *Elagatis bipinnulata* with the same species is 0.003 (Philippines) and 0.02 (India and China). In the Carangid group, *Caranx sexfasciatus* (MN257546) and *Megalaspis cordyla* (MN257528 and MN257538) species were not found to be polymorphic in the sequences obtained.

In the Scombridae family group, potential haplotypes were found in *Auxis thazard* fish (MN257554) which differed from Chinese, Indian, and Spanish haplotypes with a genetic distance of 0.002. While in the Serranidae family, haplotypes were found in *Variola albimarginata* fish (MN257516) and *Cephalopholis sonnerati* (MN257517). This *Variola albimarginata* species

(MN257516) has similarities with sequences from India but is different from Chinese haplotypes with a genetic distance of 0.007. While species of *Cephalopholis sonnerati* (MN257517) differ only from Chinese haplotypes, this species merged in one clade with samples from the Philippines, Australia, and Indonesia with genetic distance 0.00-0.002. In *Epinephelus arelatus* species, there are no potential haplotypes and sequences obtained from samples originating from China and Saudi Arabia.

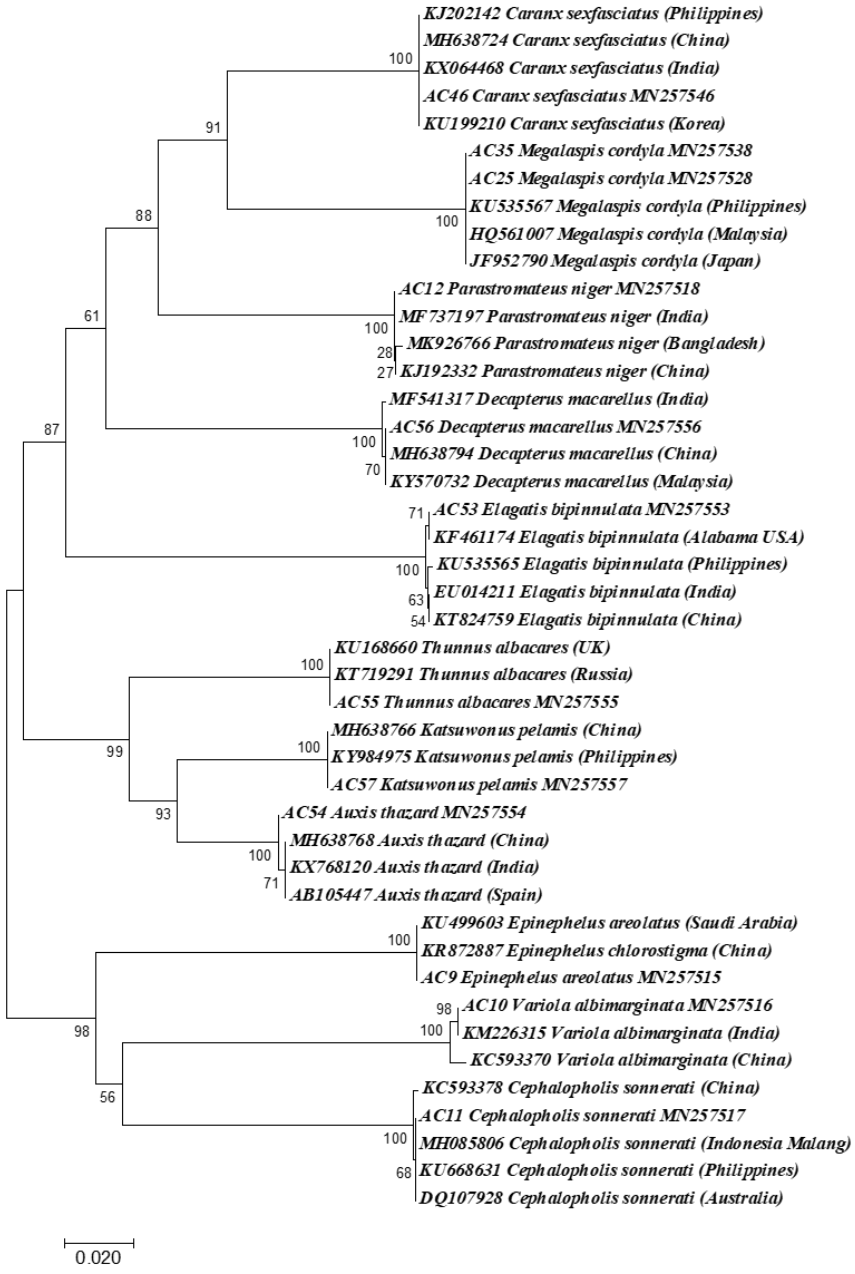

Figure 3. Phylogenetic tree reconstruction of three families (Carangidae, Scombridae, and Serranidae) by Neighbor-Joining algorithm using MEGA7. All sequence on this figure have code with AC, then another sequence has been downloaded from GenBank databased as the reference.

Discussion

Research on molecular identification is now extensive in the field of fisheries and marine sciences. In this study, molecular identification is used to com-

Table 2. Alignment result of several marine fish species from Aceh showing nucleotides different from the references (GenBank database) based on Clustal Omega online system.

No.	Species name	GenBank Acc Number	Origin	Sequence number							
				123	171	213	249	258	328	408	471
1	*Elagatis bipinnulata*	MN257553	Aceh 53	-	-	A	-	-	T	-	-
		KU535565	Philippines	-	-	G	-	-	C	-	-
		KF461174	USA	-	-	A	-	-	T	-	-
		EU014211	India	-	-	A	-	-	C	-	-
		KT824759	China	-	-	A	-	-	C	-	-
2	*Decapterus macarellus*	MN257556	Aceh 6	-	C	-	-	-	-	-	-
		MH638794	China	-	C	-	-	-	-	-	-
		KY570732	Malaysia	-	C	-	-	-	-	-	-
		MF541317	India	-	T	-	-	-	-	-	-
3	*Auxis thazard*	MN257554	Aceh 54	-	-	-	-	-	-	-	-
		MH638768	China	-	-	-	-	-	-	-	-
		KX768120	India	-	-	-	-	-	-	-	-
		AB105447	Spain	-	-	-	-	-	-	-	-
4	*Variola albimarginata*	MN257516	Aceh 10	C	-	-	G	-	-	G	C
		KM226315	India	C	-	-	G	-	-	G	C
		KC593370	China	T	-	-	A	-	-	A	T
5	*Cephalopholis sonnerati*	MN257517	Aceh 11	-	-	-	-	A	-	-	-
		MH085806	Indonesia	-	-	-	-	A	-	-	-
		KU668631	Philippines	-	-	-	-	A	-	-	-
		DQ107928	Australia	-	-	-	-	A	-	-	-
		KC593378	China	-	-	-	-	G	-	-	-

plete the morphological identification and, at the same time, determine the position of the species identified in the phylogenetic tree created. Conventional identification that has been done at this time still faces obstacles with the difficulty of getting taxonomists in the process of determining species, in addition to the long time period required for the identification process, errors in identification also still occur in some cases. By doing a combination identification approach, the results are expected to be more valid in identifying the fish species obtained.

In this study, several marine fish that were landed at the Kutaradja Fishing Port are part of the essential fishery commodity in Banda Aceh. After the 2004 tsunami disaster in this province, several activities that are able to mobilize economic activities continue to be carried out, including capture fisheries activities in the Kutaradja Fishing Port (Zulmaidah et al. 2015). Previous studies have also reported the identification of marine fish species from Kutaradja Fishing Port at Lampulo. There are still inaccurate information regarding marine fish identification in some reports. Some identifications were also only done based on morphological-based characteristics and were not done by taxonomists, the results of which may be incorrect for species justification. In an earlier report, the species *Sardinella sirin* (Serranidae) was reported to exist in the Kutaradja Fishing Port (Munawwarah et al. 2016). Still, an inaccurate determination of taxonomy made the identification results unreliable. The genus *Sardinella* spp. is a group of fish in the family Clupeidae, order Clupeiformes (www.fishbase.org), and is not included in Serranidae.

In this report, the family Perciformes is identified as a group that dominates the fish composition caught by fishermen in Banda Aceh, who landed their catch at the Kutaradja Fishing Port. These are fish used for human consumption that are essential export commodities with high economic value

such as skipjack tuna (57%) followed by yellowfin tuna (23%) (Lubis et al. 2016). Based on the identification results, the Scombridae family is a group of pelagic fish that is quite commonly found. The types identified in this report include *Thunnus albacares, Auxis thazard*, and *Katsuwonus pelamis*. In addition, three species from the genus Lutjanidae (snapper) were also found, namely *Lutjanus bengalensis, Lutjanus lutjanus*, and *Lethrinus rubrioperculatus*. Other groups that are targeted by fishermen are reef fish that have significant economic value, such as groupers and carangids. The groupers identified in this study include *Epinephelus areolatus, Variola albimarginata*, and *Cephalopholis sonnerati*, whereas the carangids group includes *Parastromateus niger, Megalaspis cordyla, Caranx sexfasciatus,* and *Decapterus macarellus* (Table 1).

In another group from the Clupeiformes order, two families were found in Lampulo fish market, namely Clupeidae (*Sardinella jussieu*) and Engraulidae (*Stelephorus commersonnii* and *Thryssa baelama*). In connection with the types of fish caught by fishermen, it is shown that captured fisheries in Banda Aceh use purse seine, which collects a group of pelagic fish in large quantities. Previous studies have explained that the fishermen in Banda Aceh mostly use purse seine (Wiryawan et al. 2016; Hariati 2017). The purse-seine is also a fishing gear generally used to catch *Thunnus tonggol, Euthynnus affinis*, *Auxis thazard*, and *Auxis rochei* (Salmarika & Wisudo 2019).

The small number of fish collected in this study are fish that are associated with coral reefs such as grouper fish groups that use coral reef areas as their nursery ground, feeding ground, and spawning ground. The diversity of reef fish around Banda Aceh experiences a natural gradient, which shows an increase in the area far from the mainland of the island of Sumatra. Variety in the region of small islands around Banda Aceh still shows good conditions when compared to the status of coral reefs on the shores of mainland Sumatra (Edrus et al. 2016). The species of *Epinephelus areolatus*, *Variola albimarginata*, and *Cephalopholis sonnerati* are groups of fish that utilize coral reefs as their habitat. However, several pelagic fish found around the shallow seas of Banda Aceh are still the primary target. The Indian mackerel *Rastrelliger kanagurta* (Hariati et al. 2015; Hariati & Fauzi 2017), yellowfin tuna *Thunnus albacares* (Neliyana et al. 2014), mackerel scad *Decapterus macrosoma*, and the anchovy *Stolephorus* spp. (Kurnia et al. 2016) were also obtained in this study.

In this report, sequences from several Acehnese fish also have similarities with those collected in some previous studies, and some are unique to other sequences. Species *Auxis thazard* that was identified from the Kutaradja Fishing Port at Lampulo, may have been caught from the area around the seas of Western Banda Aceh Province, indicating a catch distance of about 50-190 nautical miles (Salmarika & Wisudo 2019). Although it is still in the Indian Ocean region, there may be specialization in this species so that the Aceh haplotype separated from the same species in the resulting phylogenetic tree analysis.

In this study, a phylogenetic tree analysis of three prominent marine fish families, namely Scombridae, Serranidae, and Carangidae, was carried

out. The results of the investigation found that the Scombridae *Auxis thazard* (Aceh) became separated from the same clade species even though it only has a genetic distance of only 0.002. This haplotype appeared likely to occur due to differences compared to species populations analyzed from India, China (Xu et al. 2019), and Spain (Catanese et al. 2008). While for other haplotypes found from the reef fish *Variola albimarginata* and *Cephalopholis sonnerati*, the *Variola albimarginata* from Aceh might be from a population previously described from the results of a study conducted in India that allows the sharing of habitats in the Indian Ocean in the Western part of Sumatra Island. Previous studies on molecular identification of *Variola albimarginata* species have been carried out in the Andaman Islands and Nicobar Island (Basheer et al. 2017). This area is part of Indian sea territory, which may potentially have reef fish that are of almost the same as the species in Aceh. While *Cepalopholis sonnerati* fish species also have similarities with populations from Australia and the Philippines, however they are slightly different to populations from China (Zhuang et al. 2013). The study of *Cepalopholis sonnerati* shows the possibility of differences in the structure of coral fish populations in the South China Sea with the Indian Ocean, especially in Aceh waters. Although integrated with Indian Ocean waters, no similarity with Indian populations was found in the *Cepalopholis sonnerati* sample species, similarities were only found in previous studies conducted in the Philippines (Alcantara and Yambot 2016), and Australia (Ward et al. 2005). The speciation process that occurs in coral reef ecosystems occurs with an allopathic pattern that makes geographic isolation becomes the leading cause for the emergence of different species. However, the presence of pelagic larvae in reef fish species also becomes a big question even though it is believed that the allopatric pattern is the main speciation pattern occurring in coral reefs (Rocha and Bowen 2008).

Referring to the IUCN data, almost all marine fish in this study are included in the LC category (Table 1). In addition, there are also fish species that are categorized as Not Evaluated (NE), Data deficient (DD) and even Near Threatened (NT). This shows that studies on marine fish species in Indonesia need to be improved so that the conservation status of marine fish is in a well-monitored condition. The type of fish *Thunnus albacares* is getting a lot of attention because it is one of the world's important fishery commodities. Research on biological characteristics (Pecoraro et al. 2017; Mullins et al. 2018), migration (Wang et al. 2018) and various aspects has been carried out. Moreover, this fish also has a fairly high price in the world fish market (Krčmář et al. 2019; Primyastanto et al. 2021).

CONCLUSION

From this study, the identification of marine fish landed at the Kutaradja fishing port in Aceh confirmed 47 specimens (33 genera) of marine fish. Almost all fish species were considered important as fishery commodities and became the main target of the Province of Banda Aceh's exports, including

the yellowfin tuna (*Thunnus albacares*) and the skipjack tuna (*Katsuwonus pelamis*). Beside Perciformes, Serranidae, Lethrinidae and Lutjanidae was identified as fisheries resources of Banda Aceh. More in-depth research on haplotype analysis using suitable application (bioinformatic software) is very much needed to maintain a record of the genetic biodiversity presence in the waters of Banda Aceh, Indonesia.

AUTHORS CONTRIBUTION

SA. designed the research and supervised all the process including laboratory analysis and wrote the manuscript, AD. collected and analysed the data and wrote the draft manuscript, HWK. designed research and manuscript finalization

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CONFLICT OF INTEREST

The authors state that they do not have any conflict of interest. The authors are solely responsible for the article's content and writing.

REFERENCES

Afriyie, G. et al., 2019. DNA barcoding of Ghanaian fish species: Status and prospects. *African Journal of Biotechnology*, 18, pp.659-663. doi: 10.5897/AJB2019.16792

Alcantara, S.G. & Yambot, A.V., 2016. DNA barcoding of commercially important grouper species (Perciformes, Serranidae) in the Philippines. *Mitochondrial DNA Part A*, 27, pp.3837-3845. doi: 10.3109/19401736.2014.958672

Baldwin, C.C. et al., 2009. Genetic identification and color descriptions of early life-history stages of Belizean Phaeoptyx and Astrapogon (Teleostei: Apogonidae) with comments on identification of adult Phaeoptyx. *Zootaxa*, 2008, pp.1-22. doi: 10.11646/zootaxa.2008.1.1

Basheer, V. et al., 2017. Mitochondrial signatures for identification of grouper species from Indian waters. *Mitochondrial DNA Part A*, 28, pp.451-457. doi: 10.3109/19401736.2015.1137899

Batubara, A.S. et al., 2017. Check list of marine fishes from Simeulue Island waters, Aceh Province, Indonesia. *Aceh Journal of Animal Science*, 2, pp.77-84. doi: 10.13170/ajas.2.2.9584.

Bellwood, D.R. & Meyer, C.P. 2009. Searching for heat in a marine biodiversity hotspot. Journal of Biogeography, 36, 569–576. doi: 10.1111/j.1365-2699.2008.02029.x

Bingpeng, X. et al., 2018. DNA barcoding for identification of fish species in the Taiwan Strait. *PloS one*, 13, e0198109. doi: 10.1371/journal.pone.0198109

Briggs, J.C., 2005. The marine East Indies : diversity and speciation. *Biogeography*, 32, pp.1517–1522. doi: 10.1111/j.1365-2699.2005.01266.x

Carpenter, K.E. & Niem, V.H., 2001. FAO species identification guide for fishery purposes. The living marine resources of the Western Central Pacific. Volume 6. Bony fishes part 4 (Labridae to Latimeriidae), estuarine crocodiles, sea turtles, sea snakes and marine mammals. FAO Library.

Catanese, G. et al., 2008. Complete mitochondrial DNA sequences of the frigate tuna Auxis thazard and the bullet tuna *Auxis rochei*: Full Length Research Paper. *DNA Sequence*, 19, pp.159-166. doi: 10.1080/10425170701207117

Chang, C.H. et al., 2017. DNA barcodes of the native ray-finned fishes in Taiwan. *Molecular ecology resources*, 17, pp.796-805. doi: 10.1111/1755-0998.12601

Damanik, M.R.S. et al., 2016. Kajian pendekatan ekosistem dalam pengelolaan perikanan di wilayah pengelolaan perikanan (WPP) 571 Selat Malaka Provinsi Sumatera Utara. *Jurnal Geografi*, 8, pp.165-176. doi:10.24114/jg.v8i2.5780

Damora, A. et al., 2020. Diversity of marine fish and their conservation status in Pusong Bay, Lhokseumawe City, Aceh Province, Indonesia. *European Journal of Environmental Sciences*, 10, pp.115-123. doi:10.14712/23361964.2020.13

Edrus, I.N. et al., 2016. Struktur komunitas ikan karang di perairan Pulau Raya, Pulau Rusa, Pulau Rondo dan taman laut Rinoi dan Rubiah, Nanggroe Aceh Darussalam. *Jurnal Penelitian Perikanan Indonesia*, 19, pp.175-186. Doi: 10.15578/jppi.19.4.2013.175-186

Edwarsyah, E. et al., 2019. The DNA Barcoding of Several Commercial Fish from Simeulue Islands Coast of Aceh Using Cytochrome Oxidase Sub Unit I (COI) Gene Marker. *Budapest International Research in Exact Sciences (BirEx) Journal*, 1, pp.62-70. doi:10.33258/birex.v1i1.140

Fadli, N. et al., 2019. The composition of coral reefs in Ulee Lheue breakwater, Banda Aceh, Aceh, Indonesia. *IOP Conference Series: Earth and Environmental Science*, 012077. doi:10.1088/1755-1315/348/1/012077

Fikri, F., 2013. Pelaksanaan Perlindungan Terhadap Sumberdaya Perikanan dalam Laut Teritorial dan Zona Ekonomi Ekslusif Indonesia di Provinsi Aceh. *Kanun Jurnal Ilmu Hukum*, 15(3), 415-435. http://www.jurnal.unsyiah.ac.id/kanun/article/view/6181

Gaither, M. et al., 2011. Phylogeography of the reef fish Cephalopholis argus (Epinephelidae) indicates Pleistocene isolation across the indo-pacific barrier with contemporary overlap in the coral triangle. *BMC Evolutionary Biology*, 11, 189. doi:10.1186/1471-2148-11-189

Handy, S.M. et al., 2011. A single-laboratory validated method for the generation of DNA barcodes for the identification of fish for regulatory compliance. *Journal of AOAC International*, 94, pp.201-210. doi: 10.1093/jaoac/94.1.201

Hariati, T. et al., 2015. Umur, Pertumbuhan Dan Laju Pemanfaatan Ikan Banyar (*Rastrelliger kanagurta* Cuvier, 1816), di Selat Malaka (Wilayah Pengelolaan Perikanan-571). *Jurnal Penelitian Perikanan Indonesia*, 21, pp.1-8. doi: 10.15578/jppi.21.1.2015.1-8

Hariati, T., 2017. Status dan perkembangan perikanan pukat cincin di Banda Aceh. *Jurnal Penelitian Perikanan Indonesia*, 17, pp.157-167. doi: 10.15578/jppi.17.3.2011.157-167

Hariati, T. & Fauzi, M., 2017. Aspek reproduksi ikan banyar, *Rastrelliger kanagurta* (Cuv. 1817) di perairan utara Aceh [Reproductive aspect of indian mackerel *Rastrelliger kanagurta* (Cuv. 1817) of northern Aceh waters]. *Jurnal Iktiologi Indonesia*, 11, pp.47-53. doi: 10.32491/jii.v11i1.155

Hoeksema, B.W., 2007. *Delineation of the Indo-Malayan centre of maximum marine biodiversity: the Coral Triangle*. In Biogeography, time, and place: distributions, barriers, and islands (pp. 117-178). Springer, Dordrecht. doi: 10.1007/978-1-4020-6374-9_5

Kamal, M.M. et al., 2019. Autentikasi spesies ikan kerapu berdasarkan marka gen MT-COI dari perairan Peukan Bada, Aceh. *Jurnal Biologi Tropis*, 19, pp.116-123. doi: 10.29303/jbt.v19i2.1245

KeskİN, E. & Atar, H.H., 2013. DNA barcoding commercially important fish species of Turkey. *Molecular ecology resources*, 13, pp.788-797. doi: 10.1111/1755-0998.12120

Krčmář, P. et al., 2019. Identification of tuna species Thunnus albacares and Katsuwonus pelamis in canned products by real-time PCR method. *Acta Veterinaria Brno*, 88, pp.323-328. doi:10.2754/avb201988030323

Kumar, S. at al., 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular biology and evolution*, 33, pp.1870-1874. doi:10.1093/molbev/msw054

Kurnia, K. et al., 2016. Pemetaan daerah penangkapan ikan pelagis kecil di Perairan Utara Aceh. *Jurnal Ilmiah Mahasiswa Kelautan Perikanan Unsyiah*, 1.

Lakra, W. et al., 2011. DNA barcoding Indian marine fishes. *Molecular Ecology Resources*, 11, pp.60-71. 10.1111/j.1755-0998.2010.02894.x

Lubis, A.M. et al., 2016. The Contribution of the Lampulo Fishing Port, for Fishery Sector in Banda Aceh City, Nanggroe Aceh Darussalam Province. *Jurnal Online Mahasiswa Fakultas Perikanan dan Ilmu Kelautan Universitas Riau*, 3, pp.1-13.

Mardhatillah, I. et al., 2019. Application of Schaefer model on mackerels fishery in Aceh waters, Indonesia. *IOP Conference Series: Earth and Environmental Science*, 012114. doi: 10.1088/1755-1315/348/1/012114

Mullins, R.B. et al., 2018. Genomic analysis reveals multiple mismatches between biological and management units in yellowfin tuna (*Thunnus albacares*). *ICES Journal of Marine Science*, 75, pp.2145-2152. doi: 10.1093/icesjms/fsy102

Munawwarah, A. et al., 2016. Identifikasi Jenis-Jenis Ikan Yang Terdapat Di Tempat Pelelangan Ikan (TPI) Di Gampong Lampulo Kecamatan Kuta Alam Banda Aceh. *Serambi Saintia: Jurnal Sains dan Aplikasi*, 4. doi: 10.32672/jss.v4i1.115

Nasution, M.H. et al., 2019. Forecasting The Amount of Tuna/Madidihang (Yellowfin tuna) Landed in PPS Kutaraja Banda Aceh City With The Triple Exponential Smoothing Method. *Samakia: Jurnal Ilmu Perikanan*, 10, pp.08-14. doi: 10.35316/jsapi.v10i1.231

Neliyana, W.B. et al., 2014. Analisis Kelayakan Usaha Perikanan Pukat Cincin di Pelabuhan Perikanan Pantai (PPP) Lampulo Banda Aceh Propinsi Aceh. *Jurnal Marine Fisheries*, 5, pp.163-169.

Nur, F.M. et al., 2019. Checklist of coral fishes in Lhoknga and Lhok Mata Ie Beaches, Aceh Besar, Indonesia. *IOP Conference Series: Earth and Environmental Science*, 012104. doi: 10.1088/1755-1315/348/1/012104

Pecoraro, C. et al., 2017. Putting all the pieces together: integrating current knowledge of the biology, ecology, fisheries status, stock structure and management of yellowfin tuna (*Thunnus albacares*). *Reviews in Fish Biology and Fisheries*, 27, pp.811-841. doi: 10.1007/s11160-016-9460-z

Primyastanto, M. et al., 2021. Sustainable operational analysis of the cultivation of Indonesian *Thunnus albacares* by bioeconomic approach. *Croatian Journal of Fisheries: Ribarstvo*, 79, pp.61-70. doi: 10.2478cjf-2021-0007

Pringgenis, D. & Susilowati, R., 2016. Highly commercial fisheries tawar fish: molecular analysis DNA mitochondrial COI gene sequence and proximate analysis from malacca Strait, Riau. *Jurnal Teknologi*, 78. doi: 10.11113/jt.v78.8149

Rocha, L. & Bowen, B. 2008. Speciation in coral-reef fishes. *Journal of Fish Biology*, 72, pp.1101-1121. doi: 10.1111/j.1095-8649.2007.01770.x

Salmarika, S. & Wisudo, S.H., 2019. Status Pengelolaan Sumber Daya Ikan Tongkol di Perairan Samudera Hindia Berbasis Pendaratan Pukat Cincin di Pelabuhan Perikanan Samudera Lampulo, Aceh: Suatu Pendekatan Ekosistem. *Jurnal Penelitian Perikanan Indonesia*, 24, pp.263-272.

Serdiati, N. et al., 2020. Morphological variations and phylogenetic analysis of Oryzias nigrimas Kottelat, 1990 (rice fish) from Lake Poso, Central Sulawesi, Indonesia. *Biodiversitas Journal of Biological Diversity*, 21. doi: 10.13057/biodiv/d210305

Suman, A. et al., 2017. Potensi dan tingkat pemanfaatan sumber daya ikan di wilayah pengelolaan perikanan Negara Republik Indonesia (WPP NRI) Tahun 2015 serta Opsi Pengelolaannya. *Jurnal Kebijakan Perikanan Indonesia*, 8, pp.97-100. doi: 10.15578/jkpi.8.2.2016.97-100

Veron, J.E.N. et al., 2009. Delineating the Coral Triangle. Galaxea. *Journal of Cournal Reef Studies*, 11, pp.91-100. doi:10.3755/galaxea.11.91

Wang, X. et al., 2018. Relationship between water migration and quality changes of yellowfin tuna (*Thunnus albacares*) during storage at 0 C and 4 C by LF-NMR. *Journal of aquatic food product technology*, 27, pp.35-47. doi: 10.1080/10498850.2017.1400630

Wang, Z-D. et al., 2012. DNA barcoding South China Sea fishes. *Mitochondrial DNA*, 23, pp.405-410. doi: 10.3109/19401736.2012.710204

Ward, R.D. et al., 2005. DNA barcoding Australia's fish species. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 360, pp.1847-1857. doi: 10.1098/rstb.2005.1716

Wiryawan, B. et al., 2016. Analisis Kelayakan Usaha Perikanan Pukat Cincin Di Pelabuhan Perikanan Pantai (Ppp) Lampulo Banda Aceh Propinsi Aceh (Analysis Financial Fisheries of Purse Seine in Lampulo Fishing Port Banda Aceh Provinsi Aceh). *Marine Fisheries: Journal of Marine Fisheries Technology and Management*, 5, pp.163-169. doi: 10.29244/jmf.5.2.163 -169

Xu, L. et al., 2019. A molecular approach to the identification of marine fish of the Dongsha Islands (South China Sea). *Fisheries Research*, 213, pp.105-112. doi: 10.1016/j.fishres.2019.01.011

Yeni, E. & Naufal, A., 2017. Identifikasi Aktivitas Pengembangan Fasilitas Pelabuhan Perikanan Pantai Lampulo Banda Aceh. *Prosiding SEMDI-UNAYA (Seminar Nasional Multi Disiplin Ilmu UNAYA)*, pp.355-363.

Yusuf, Q., 2003. Empowerment of Panglima Laot in Aceh. *International workshop on Marine Science and Resource*, pp.11-13.

Zhang, J. & Hanner, R., 2012. Molecular approach to the identification of fish in the South China Sea. *PLoS One*, 7, e30621. doi: 10.1371/ journal.pone.0030621

Zhuang, X. et al., 2013. A comprehensive description and evolutionary analysis of 22 grouper (Perciformes, Epinephelidae) mitochondrial genomes with emphasis on two novel genome organizations. *PLoS One*, 8, e73561. doi: 10.1371/journal.pone.0073561

Zulfahmi, I. et al., 2022. Commercial marine fish species from Weh Island, Indonesia: Checklist, distribution pattern and conservation status. *Biodiversitas Journal of Biological Diversity*, 23. doi: 10.13057/biodiv/d230432

Zulmaidah Z, Zain J, Hutauruk RM. 2015. Facilities Utilization in Lampulo Fisheries Port, District of Kuta Alam, Banda Aceh City, Province Nanggroe Aceh Darussalam. *Jurnal Online Mahasiswa*, 2, pp.1-13.

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Research Article

Autecology of *Castanopsis argentea* (Blume) A.DC. in Telaga Warna Nature Reserve Area, Bogor Regency

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ABSTRACT
The red list of the International Union for the Conservation of Nature and Natural Resources (IUCN) reported *Castanopsis argentea* as an endangered species. Studies about autecology on its natural habitat become important to perform species conservation. This study aimed to analyze the population structure, distribution pattern, and environmental factors that influence the presence of *C. argentea* in the Telaga Warna Nature Reserve. Data was collected in September 2020 by making 21 plots with a single plot. The purposive sampling method was used based on *C. argentea* representatives to determine plot location. Measurement of environmental factors, including soil sampling was carried out on each plot. Population structure was analyzed based on plant density, and Morisita index determined the distribution pattern. Environmental data were analyzed using PCA with Minitab 19 programs. Our field observation showed that *C. argentea* seedling has the highest density (1071 ind/ha) and decreased in the mature phase. *C. argentea* was found to have a clumped distribution pattern with an Id value of 1.03. PCA analysis showed differences in environmental factors that were thought to influence the presence of *C. argentea* individuals in four growth phases. The highest population structure of *C. argentea* was found in the growth phase of seedlings and saplings at an altitude of 1400 m asl. The spread population distribution of *C. argentea* was clumped. The influences of environmental variables on the existence of *C. argentea* were Mg, Ca, CEC, pH, and soil moisture.

INTRODUCTION
Castanopsis argentea (saninten) is a woody plant from the Fagaceae family. This plant is distributed naturally in Assam, India, Myanmar, Indonesia, and Thailand (POWO 2022) and is a native plant from Indonesia that grows in Borneo, Sumatra, and Java (Soepadmo 1968; POWO 2022). On Java Island, *C. argentea* is found in primary forest at an altitude of 150-1750 m asl (Zuhri et al. 2018). Ecologically, this tree species is a place for wildlife, especially birds and mammals, used for foraging, resting, and nesting. *C. argentea* seeds are food for wild animals such as wild boars and other primates (Heriyanto et al. 2007). This species can be used to reforest rocky mountainous land (Wibowo 2006) and become one of the plant species that have the potential for

revegetation activities of ex-mining land as well as to repair damaged ecosystems (Ahmad et al. 2013). In other fields, the wood of this plant can be used as a building material, the fruit can be used as a stomach ache medicine, and the peel of the fruit can be used as a dye (Setyawati 2010).

Since 2018, *C. argentea* has been listed as an endangered species and added to the red list according to the International Union for the Conservation of Nature and Natural Resources (IUCN) (Barstow & Kartawinata 2018). As the case in Sumatra, *C. argentea* in nature is disrupted due to land conversion, such as forest clearing into plantations of palm oil. This species habitat loss has led to a population decline of approximately 50% over the last three decades (Handayani & Hidayati 2020). The most significant impact of *C. argentea* declinine in the population is the existence of anthropogenic activities. The anthropogenic activity threat to this species is the reduced ability of natural regeneration caused by fruit over-harvesting and the wood being cut down for timber (Barstow & Kartawinata 2018).

The Telaga Warna Nature Reserve Area is one of the remaining natural habitats of *C. argentea* in West Java. Based on an initial scoping study, *C. argentea* trees are commonly found growing in the Telaga Warna Nature Reserve Area. That makes the Telaga Warna Nature Reserve considered effective for obtaining data related to the interaction of *C. argentea* species on environmental factors and other plant species in the area. Therefore, a study is needed on the autecology of the *C. argentea* species in its natural habitat. The information obtained can be used as a reference for conservation and added to the biodiversity database in the Telaga Warna Nature Reserve Area. The purposes of this study were to analyze the population structure, distribution pattern, and environmental factors that influence the presence of *C. argentea* in the Telaga Warna Nature Reserve Area.

MATERIALS AND METHODS

Materials

The study was conducted in September 2020 in the Telaga Warna Nature Reserve, Bogor Regency (Figure 1). Biotic and abiotic data were collected at three different altitudes (1400, 1500, and 1600 m asl). The separation of elevation range 100 meters impacted the growth of *C. argentea* (Dewi et al. 2019). The materials used in this study were field-collected plant specimens and soil samples from three different altitudes.

Methods

The population structure of *C. argentea* and community structure at the study site was carried out by collecting biotic data on four growth phases (seedling, sapling, pole, and tree) with a total sampling area of 0.84 hectares. Seedlings are young trees from sprouts to < 1.5 m high; saplings are young trees that have 1.5 m high and < 10 cm of diameter breast height (dbh); poles are young trees with dbh range from 10 cm to < 20 cm, while trees are adult individuals with a > 20 cm dbh (Wahyudi et al. 2014). The purposive

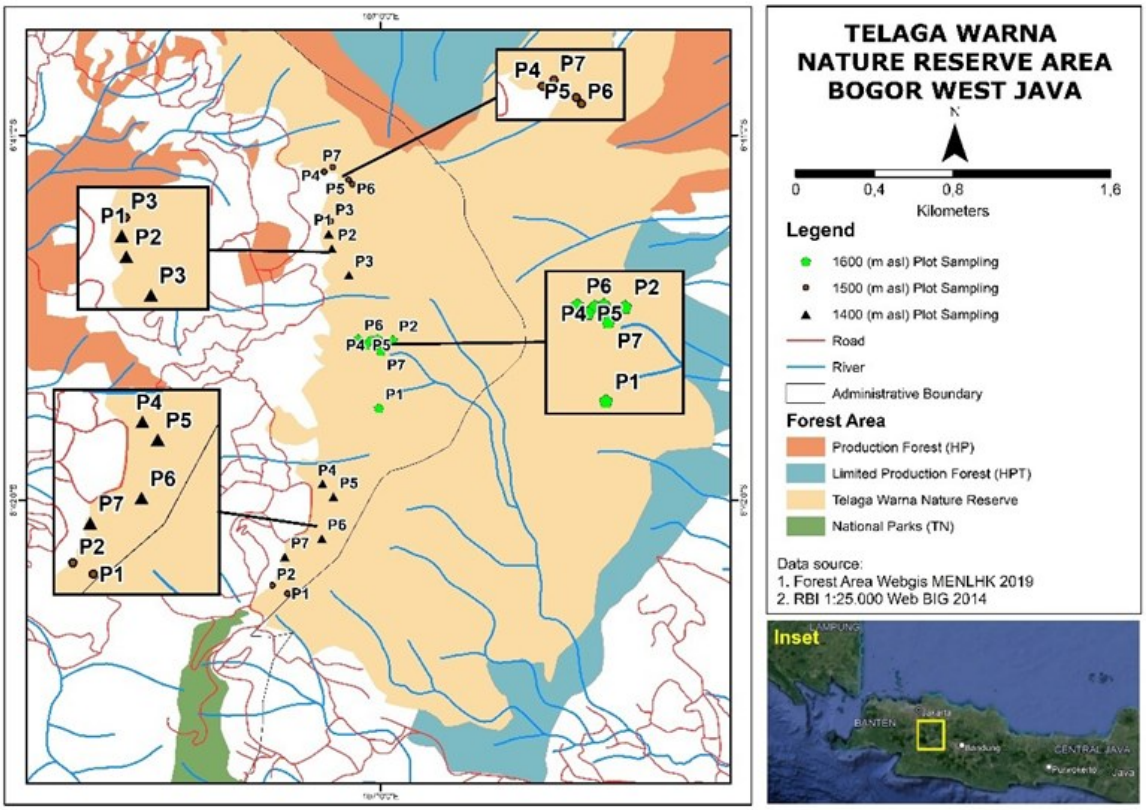

Figure 1. A map of the research location and plot sampling in the Telaga Warna Nature Reserve Area, Bogor Regency.

sampling method was used based on *C. argentea* representatives to determine plot location. The total number of plots is 21, divided by three based on each elevation (1400, 1500, and 1600 m asl). A Single quadrat plot of 20 m x 20 m was used for vegetation analysis, modified from Metananda et al. (2015). The sample plot size was used as 20 m x 20 m for the tree phase sampling. In plot 20 m x 20 m, sub-plots were made for other phases of sampling; 2 m x 2 m is made for the seedling phase, 5 m x 5 m for the sapling phase, and 10 m x 10 m for the pole phase.

Plant data inventory was obtained by recording each individual plant contained in each plot, then calculating the number of individuals for each species. Population structure was measured by dbh (diameter at breast height). For this method, the dbh level (1.3 m above the ground) was measured for each species present within the quadrat (Pradhan et al. 2018). The identification method was done by taking photography and herbarium preparation. A sample of leaves, flowers, and fruit for herbarium preparation was brought to the Laboratory of Ecology and Plant Resources to be identified and verified through various websites such as Plants of Southeast Asia, GBIF, and Plants of the World.

Environmental factors measured by using a 4-in-1 environmental meter (Lutron LMB8000A) were air humidity, air temperature, light intensity, and wind speed. The topographic factors measured were altitude by using GPS and altimeter, whereas the slope of the place was measured with a clinometer. In contrast, edaphic factors such as pH and soil moisture were measured using a soil tester. Determination of soil sampling following the

location of vegetation plot created to test chemical properties. Soil samples were taken with a sample ring of 2 inches in diameter and stored in sealed plastic. For each altitude, seven sampling points were taken based on the presence of a study plot, and then the soil was composited (Risna 2009).

Data on plant abundance at each growth phase in each sample plot was obtained by calculating the Important Value Index (IVI). In contrast, population structure data was obtained by calculating the density value (D). The Important Value Index (IVI) for the seedling and sapling phases was obtained based on the sum of relative density (RD) and relative frequency (RF). In contrast, the IVI for the pole and tree phases was obtained based on the sum of relative density (RD), relative frequency (RF), and relative dominance (RDo) (Gonçalves et al. 2018). Data on the basal area (BA) is needed to determine the dominance of a species. Basal area (BA) is calculated using a formula $\frac{1}{4}\ \pi\ (dbh)^2$ (Yahya et al. 2019). Density (D), relative density (RD), frequency (F), relative frequency (RF), Dominance (Do), and relative dominance (RDo), were calculated as follows:

$$D = \frac{\text{Number of individuals of each species}}{\text{Sample plot area (ha)}}$$

$$RD = \frac{\text{Density of each species}}{\text{Total density of all species}} \times 100\ \%$$

$$F = \frac{\text{Number of study plots found for each species}}{\text{Total number of study plots created}}$$

$$RF = \frac{\text{Frequency of each species}}{\text{Total frequency of all species}} \times 100\ \%$$

$$Do = \frac{\text{Basal area of each species}}{\text{Sample plot area (ha)}}$$

$$RDo = \frac{\text{Dominance of each species}}{\text{Total dominance of all species}} \times 100\ \%$$

The Morisita index was used to determine the distribution pattern of *C. argentea* in the study area (Krebs 1989). The Morisita index was calculated as follows:

$$I_\delta = n\left[\frac{\sum x^2 - \sum x}{(\sum x)^2 - \sum x}\right] ; M_u = \frac{\chi^2_{0.975};df^1 - n + \sum xi}{(\sum x^2) - 1} ; M_c = \frac{\chi^2_{0.025};df^1 - n + \sum xi}{(\sum xi) - 1}$$

where:

I_δ = Morisita index
M_u = uniform distribution pattern index
M_c = aggregate distribution pattern index
n = number of plot
Σx = total number of plot
Σx^2 = sum of the squares of the number of plot
Σxi = number of each individual plant in a plot i (i = 1,…n)

$\chi^2_{0.975}$ = Chi-squared in db (n-1), 97.5%

$\chi^2_{0.025}$ = Chi-square in db (n-1), 2.5%

The observed plant distribution pattern was determined using the following criteria:

a) If the value of $I_\delta \geq M_c \geq 1.0$, then $I_p = 0.5 + 0.5\left(\frac{I\delta - Mc}{n - Mc}\right)$

b) If the value of $M_c > I_\delta \geq 1.0$, then $I_p = 0.5\left(\frac{I\delta - 1}{Mc - 1}\right)$

c) If the value is $1.0 > I_\delta > M_u$, then $I_p = 0.5\left(\frac{I\delta - 1}{Mu - 1}\right)$

d) If the value is $1.0 > M_u > I_\delta$, then $I_p = 0.5 + 0.5\left(\frac{I\delta - Mu}{Mu}\right)$

The distribution pattern of the species is random when $I_p = 0$; clumped when $I_p > 0$; and uniform when $I_p < 0$ (Krebs 1972).

Environmental and soil chemical data from each research plot were analyzed using Principal Component Analysis (PCA) with the Minitab 19 program. Principal Component Analysis (PCA) was used to determine the effect of environmental factors on the presence of *C. argentea*.

RESULTS AND DISCUSSION

Overview of Research Sites and Population Structure of *Castanopsis argentea*

The Telaga Warna Nature Reserve Area study site has dense vegetation conditions (Figure 2). Some tree species with a diameter > 50 cm were found growing in this location, such as *Castanopsis argentea*, *Castanopsis tunggurut*, *Castanopsis acuminatissima*, and *Lithocarpus indutus* from the Fagaceae family, *Cinchona succirubra* (Rubiaceae), *Schefflera polybotrya* (Araliaceae), *Turpinia sphaerocarpa* (Staphyleaceae), *Ficus elastica* (Moraceae), *Sloanea sigun* (Elaeocarpaceae), *Manglietia glauca* (Magnoliaceae), *Villebrunea rubescens* (Urticaceae), *Schima wallichii* (Theaceae), and *Neolitsea javanica* (Lauraceae).

The results of the vegetation analysis showed that the *C. argentea* tree dominated the study area. Trees of this species had the highest IVI of 59.95 with values of relative density, relative frequency, and relative dominance, 12.40, 12.74 , and 34.81% respectively (Table 1).

The number of individuals per hectare was used as a parameter to study the population structure of *C. argentea* at the study site. The results showed that each study site in the Telaga Warna Nature Reserve had a

Table 1. Important Value Index of *Castanopsis argentea* in all study sites in the Telaga Warna Nature Reserve.

Growth phases	D (ind/ha)	RD (%)	F	RF (%)	Do	RDo (%)	IVI
Seedling	1071.43	1.01	0.19	3.25	-	-	4.27
Sapling	323.81	6.23	0.52	7.97	-	-	14.20
Pole	38.10	5.44	0.38	7.41	0.66	5.55	18.40
Tree	36.90	12.40	0.94	12.74	21.49	34.81	59.95

Notes: ind = individuals; ha = hectare; D = density; RD= relative density; F = frequency; RF = relative frequency; Do = dominance; RDo = relative dominance; IVI = Important Value Index.

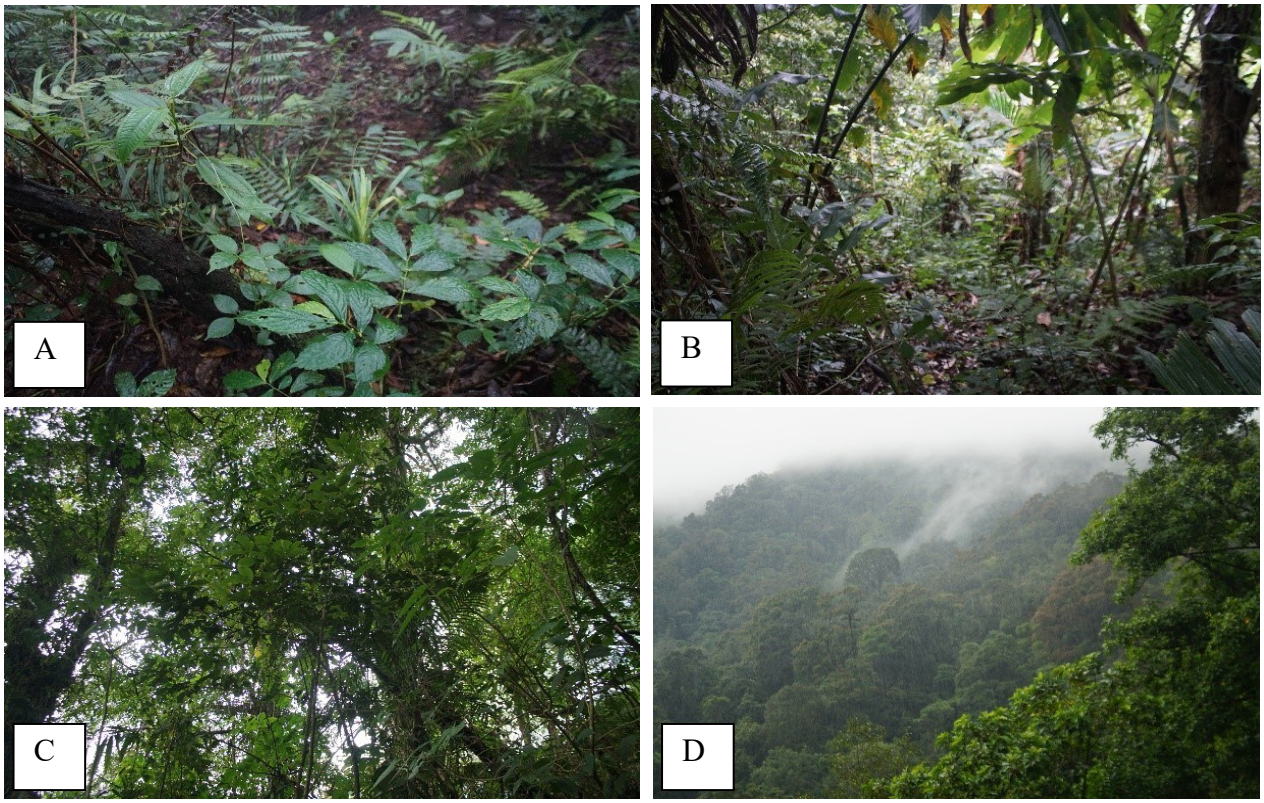

Figure 2. Vegetation conditions in the Telaga Warna Nature Reserve. A-B: forest floor condition; C-D: tree vegetation cover.

different population structure of *C. argentea* (Figure 3). The rejuvenation of *C. argentea* in seedling and sapling phases was more commonly found at an altitude of 1400 m asl, which indicates the environmental conditions of 1400 m asl were suitable for the growth of rejuvenation *C. argentea*. Seedlings of *C. argentea* grow very well at an altitude of 1400 m asl, indicated by the high value of seedling density (Heriyanto et al. 2007).

Meanwhile, at an altitude of 1600 m asl, no individual seedlings were found in the study plots. The individual density of the seedling level tends to decrease with increasing altitude (Hilwan 2012). Although the density of the tree phase at this location is greater than in the other two locations, the abundance of trees is not always a parameter in terms of increasing the success of seeds in growing into seedlings. Most seeds cannot survive due to unfavorable growing environment conditions (Yang et al. 2015). A growing environment with enough high light intensity can increase the seed density and viability of seedling life compared to a growing environment with low light intensity (Zhao et al. 2021). Given that *Castanopsis* is a tree species with a wide canopy cover, the number of trees in this location can withstand light from entering the forest floor (low light intensity value is 242 lux). That can affect seed germination to grow into seedlings and develop into sapling phase. *C. argentea* saplings at this location had a lower population density than the other two locations (Figure 3). Danková & Saniga (2013) found that 6 out of 8 species of saplings had low specific densities in locations with large canopy cover so that only a small amount of light entered the forest floor through the canopy gap.

The existence of competition also affects the development of the *C. argentea* seedling phase. As time passes, individuals in the seedling phase require much energy to compete among plants for sunlight and nutrients used for growth and development (Waskitaningtyas et al. 2018). Slow seedling growth can also be caused by competition between individuals of the same or different species in terms of struggle nutrients, water, growth space, and sunlight, causing some *C. argentea* seedling individuals to be defeated.

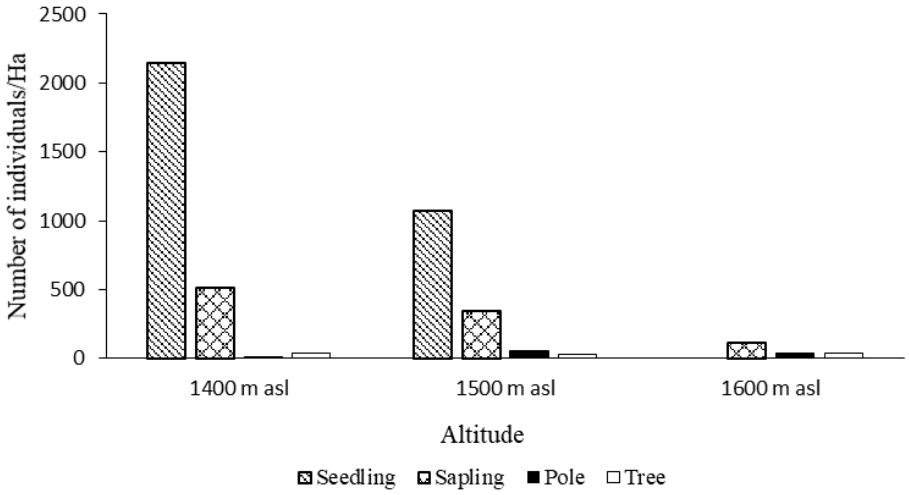

Figure 3. Population structure of *C. argentea* at three altitudes in the Telaga Warna Nature Reserve.

Several dominant plant species in the seedling and understorey phases were found around *C. argentea* (Table 2). *Elastostema strigosum* was the dominant understorey species at three altitudes. *E. strigosum* could grow well and even dominate previously disturbed forest park areas (affected by landslides) (Raihandhany et al. 2019). In addition, this alleged competition showed that the state of seedlings at an altitude of 1500 m asl decreased due to other dominant plant species besides *E. strigosum*, namely *Cliemia hirta*. At that altitude, *C. hirta* has the second-highest IVI. *C. hirta* is an invasive plant that can threaten and harm the ecosystem. In addition, this species also can spread fast because it has a high germination ability and is shade tolerant (Nursanti & Adriadi 2018). *C. hirta* also has allelopathic compounds that can inhibit seed germination, roots, and stems from other plant seedlings (Ismiani 2015). The dominance of this species is feared to change the condition of the forest floor and even inhibit the growth of other plant species such as *C. argentea* seedlings which are native plants in the area.

Distribution Pattern of *Castanopsis argentea* at the Study Site

The calculation of the Morisita dispersion index (Table 3) showed that the population of *C. argentea* in the Telaga Warna Nature Reserve has a clumped distribution pattern ($I_p > 0$). Hilwan & Irfani (2018) research in the Gede

Table 2. Dominant plant species in the seedling and understorey phases found around *Castanopsis argentea* at three altitudes.

No.	Species Name*)	Altitude		
		1400	1500	1600
1	*Elatostema strigosum* Hassk.*	√	√	√
2	*Cyrtandra grandis* Blume*	√	-	-
3	*Clidemia hirta* (L.) D.Don*	-	√	-
4	*Strobilanthes filiformis* Blume*	√	-	√
5	*Elatostema acuminatum* (Poir.) Brongn.*	√	-	-
6	*Costus* sp.*	√	-	-
7	*Cyathea contaminans* (Hook.) Copel.	-	-	√
8	*Psychotria montana* Blume	-	-	√

Notes: *) Plant species with INP ≥ 10; * understorey; √ = found; - = not found.

Pangrango National Park area showed similar results. Random plant distribution patterns rarely occur in nature, and between plants usually have a clumped pattern (Rayburn et al. 2011).

Differences in the distribution pattern of a plant species are influenced by various biological factors and environmental conditions available in an ecosystem, such as the presence of wind, water flow, light intensity, animals, characteristics possessed by a plant species (Duman et al. 2016), environmental heterogeneity (Perry et al. 2009), seed dispersal (Schurr et al. 2004), and the presence of certain disturbances (Rayburn & Monaco 2011). The clumped distribution pattern in fruit plants, including *C. argentea* is influenced by reproductive factors (Sumihadi et al. 2019). Naturally, the fruit of *C. argentea* will not fall too far from the mature plant. However, because trees of this species are found growing on relatively steep land, some individual trees are found growing in steep places, causing the regeneration of *C. argentea* to be found quite far from the mature plant. This situation allows *C. argentea*, who are relatively the same age, to live in groups. Therefore, it was rare to find seedlings growing together in the same plot with mature individuals during the research.

The different characteristics of *C. argentea* at various growth phases are related to this species distribution pattern in nature. Judging from the seed or fruit distribution mechanism, *C. argentea* can grow well if the seeds or fruit of *C. argentea* are carried to a particular place in a suitable environment to support its growth process. The process of growing from seed dispersal is influenced by high light intensity and the steep slope affects the success of tree phase growth. Different habitat conditions, such as elevation and slope, cause tree species to have different distribution patterns (Mirmanto 2014).

Table 3. The value of the standardized Morisita index and the distribution pattern of *C. argentea* at the study site.

Morisita dispersion index (I_δ)	Uniform index (M_u)	Clumped index (M_c)	Standardized Morisita index (I_p)	Distribution pattern
1.03	0.84	1.22	0.07	Clumped

Habitat Characteristics Affecting the Presence of *Castanopsis argentea*

Based on the analysis of environmental factors (Table 4 and Table 5), two components can describe the habitat characteristics of *C. argentea* in the Telaga Warna Nature Reserve Area, namely edaphic factors and climatic factors. PCA analysis of environmental data obtained from three different study sites showed that altitude affected the presence of *C. argentea* individuals (Figure 4A). At an altitude of 1400 m asl, there were 26 individuals of *C. argentea* starting from the seedling phase to the tree phase, an altitude of 1500 m asl as many as 22 individuals, and an altitude of 1600 m asl as many as 17 individuals. That means the number of *C. argentea* individuals in the four growing phases decreased with increasing altitude.

Altitude has an essential role in the process of plant growth and development. The difference in altitude has affected the amount of sunlight received, water absorption, and the availability of soil nutrients (Zhu et al. 2019). These varying factors can cause an increase or decrease in the presence of a plant species along an altitude gradient (Körner 2007). Therefore, some plants can grow well in high lands while others can only grow in low to medium lands. *C. argentea* found at the study site could grow well at an altitude of 1400 m asl. Because at that altitude, there were environmental factors that were favorable for the existence of this species, namely the high content of Mg, Ca, Cation Exchange Capacity (CEC), pH, and soil moisture (Table 4).

Table 4. Analysis of soil chemical properties at the study site.

Parameters	Altitude (m asl)					
	1400		1500		1600	
	Value	Criteria*	Value	Criteria*	Value	Criteria*
pH H_2O	5.41	Acidic	4.97	Acidic	4.84	Acidic
C (%)	8.88	Very high	10.25	Very high	4.84	High
N (%)	1.01	Very high	1.16	Very high	2.08	Very high
C/N ratio	8.79	Low	8.84	Low	13.69	Medium
P_2O_5 (ppm)	14.85	Very high	16.40	Very high	44.06	Very high
Ca ($cmol_c$/kg)	17.97	High	7.96	Medium	1.23	Very low
Mg ($cmol_c$/kg)	4.14	High	2.63	High	0.67	Low
K ($cmol_c$/kg)	1.26	Very high	8.28	Very high	2.62	Very high
Na ($cmol_c$/kg)	0.30	Low	0.32	Low	0.32	Low
CEC ($cmol_c$/kg)	63.33	Very high	50.94	Very high	54.21	Very high
BS (%)	37.39	Medium	37.67	Medium	8.91	Very low

Notes: *soil fertility criteria follow Eviati & Sulaeman (2009).

Table 5. Average microclimate at the study site.

Parameters	Altitude (m asl)		
	1400	1500	1600
Soil moisture (%)	58	57	56
Air humidity (%)*	62	70	73
Air temperature (°C)	24.4	24.4	21.8
Light intensity (lux)	968	904	242
Wind speed (m/s)	0.04	1.70	0.30

Notes: *air humidity data is an absolute value.

C. argentea in the four growth phases had different tendencies in PCA biplots. All seedlings and saplings of *C. argentea* (Figure 4B) collected at location 1 (1400 m asl) were influenced by the high availability of macronutrients (Ca and Mg), cation exchange capacity, base saturation, pH, and microclimates such as air temperature, the intensity of light and soil moisture. The seeds must be in favorable environmental conditions to grow and develop into a more mature phase to increase the chances of survival. The rejuvenation of *C. argentea*, especially in the seedling phase at an altitude of 1400 m asl was growing in a slightly shaded environment. It can be said that the seedling of this species is a light-demanding plant. The light intensity at this location is also higher than in the other two locations. This study's results align with the research by Handayani et al. (2019), which showed that *C. argentea* seedlings planted in the open area experienced a rapid increase in height.

In the pole phase, the presence of individual *C. argentea* at location 2 (altitude 1500 m asl) was more influenced by the availability of macronutrients K and Na as well as microclimates such as wind speed and humidity. In contrast to its regeneration, *C. argentea* in the pole phase required higher macronutrients K and Na concentrations than the other two macronutrients (Ca and Mg). This condition was supported by the high value of CEC (> 40) at the study site. CEC plays an essential role in retaining soil nutrients (Luo et al. 2015) and controlling the supply of exchangeable cations, namely Ca^{2+}, Mg^{2+}, K^{+}, Na^{+}, Al^{3+}, and Fe^{3+} (Mueller et al. 2012), so the soil with a high CEC able to absorb and provide sufficient nutrients for plant growth (Siburian et al. 2020).

C. argentea trees were dominant at an altitude of 1600 m asl (location 3). That can explain the growing *C. argentea* trees were heavily influenced by soil macronutrients such as N, C-organic, C/N ratio, and phosphate. The high C/N ratio (13.69) indicated that the mature phase of *C. argentea* required fewer nutrients than the younger phase. Meanwhile, high nitrogen availability can help maximize nutrient absorption and play an essential role in mature plants' flowering and fruit formation process (Pescie et al. 2018). That can also answer why the number of trees in this location was abundant.

Different altitudes affect soil formation, which can determine soil texture and vegetation composition. The Telaga Warna Nature Reserve Area has an andosol soil type with a dusty to clay texture (Effendi et al. 2019). The finer the texture of the soil, the greater its ability to hold water used for plant growth (Fitriani et al. 2018). The research by Rukhmi et al. (2017) at three altitudes shows that soil with a clay texture has a larger surface area than sandy loam so that, it stores more nutrients, providing sufficient water content for air circulation in the soil. The soil condition could be another supporting factor for discovering *C. argentea* in the Telaga Warna Nature Reserve Area.

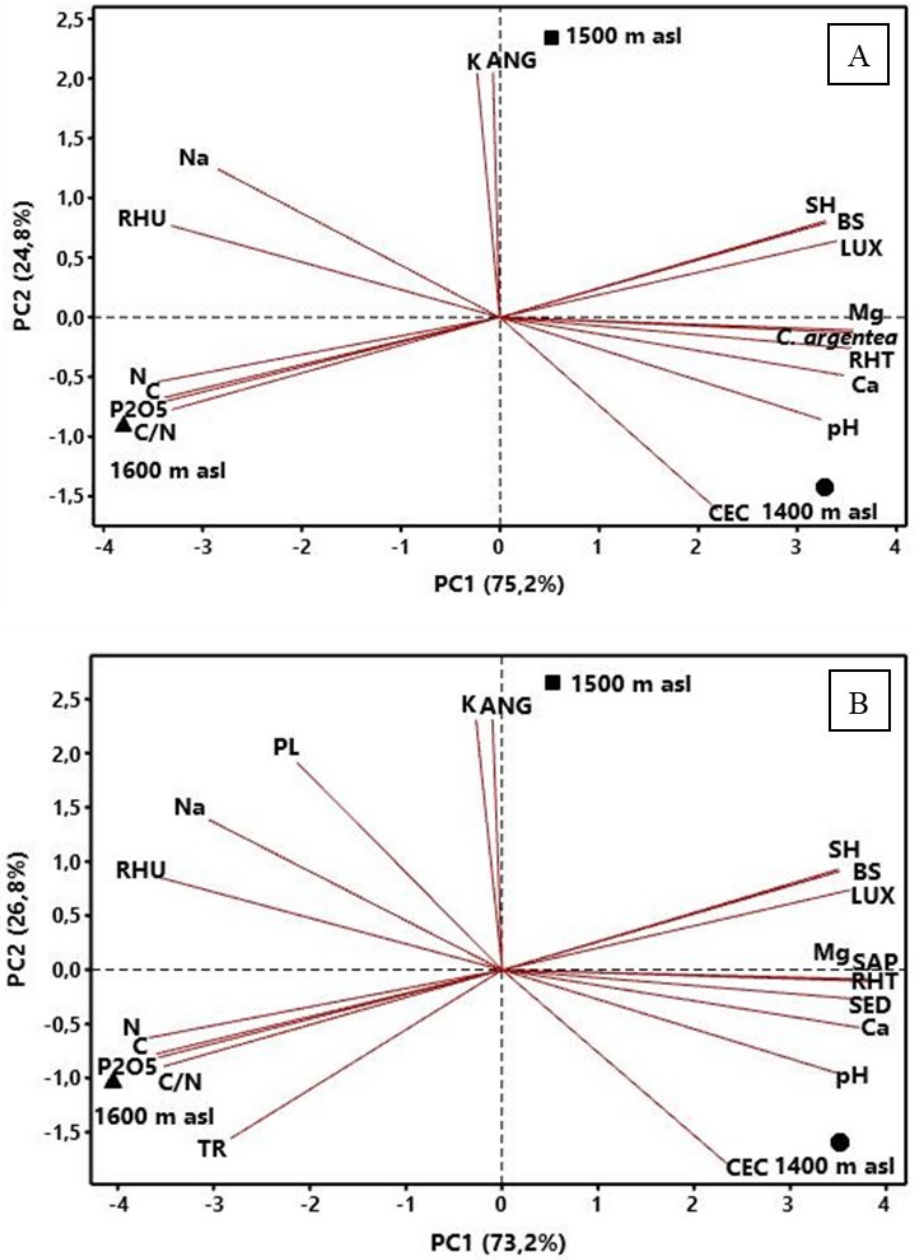

Figure 4. Results of PCA analysis of environmental factors determining the presence of *C. argentea* in the Telaga Warna Nature Reserve. (A) All phases; (B) four growth phase. SED: seedling; SAP: sapling, PL: pole; TR: tree; ANG: wind speed; SH: air temperature; LUX: light intensity; RHT: soil moisture; RHU: air humidity, BS: base saturation; CEC: cation exchange capacity.

CONCLUSION

The highest population structure of *C. argentea* was found in the growth phase of seedlings and saplings at an altitude of 1400 m asl, decreasing population with ever-increasing elevations. The population of *C. argentea* spreads clumped in the Telaga Warna Nature Reserve. Meanwhile, PCA analysis showed differences in environmental factors, namely edaphic and climatic factors, that were thought to influence the presence of *C. argentea* individuals in four growth phases. *C. argentea* could grow well at an altitude of 1400 m asl because environmental factors were favorable for the existence of this species, namely the content of Mg, Ca, CEC, pH, and soil moisture.

AUTHORS CONTRIBUTION

D.M.P. contributed to design the research concept, data collection, analyzed the data, and authored the manuscript. S. and N.R.D. contributed equally to this manuscript, developed the research concept, examined the data, authored, reviewed, and approved the final manuscript.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

REFERENCES

Ahmad, T.L.S. et al., 2013. Kajian pemilihan jenis tumbuhan untuk restorasi hutan berdasarkan beberapa parameter fotosintesis. *Jurnal Biologi Indonesia*, 9(2), pp.233-243. doi: 10.20886/jphka.2015.12.1.89-104.

Barstow, M. & Kartawinata, K., 2018. *Castanopsis argentea*. The IUCN Red List of Threatened Species 2018: e.T62004506A62004510 (10 February 2020). Accessible at https://www.iucnredlist.org/species/62004506/62004510.

Danková, L. & Saniaga, M., 2013. Canopy gaps and tree regeneration patterns in multi-species unmanaged natural forest Sitno (preliminary results). *Beskydy*, 6(1), pp.17-26. doi: 10.11118/beskyd201306010017.

Dewi, S.P. et al., 2019. Dataset on the reproductive period of three local species in a tropical sub-mountainous forest. *Data in Brief*, 25, pp.1-4. doi: 10.1016/j.dib.2019.104238.

Duman, T. et al., 2016. Dissipation intermittency increases long-distance dispersal of heavy particles in the canopy sublayer. *Boundary-Layer Meteorol*, 159 (1), pp.41–68. doi: 10.1007/s10546-015-0112-y.

Effendi, H. et al., 2019. *Telaga Warna Puncak: Kekayaan Alam yang Terpendam*. Bogor: IPB Press.

Eviati & Sulaeman, 2009. *Analisis Kimia Tanah, Tanaman, Air, dan Pupuk*. 2nd Ed. Prasetyo BH, Santoso D, Widowati LR, editor. Bogor: Balai Penelitian Tanah.

Fitriani, D.A. et al., 2018. Beberapa sifat fisika dan kimia tanah pada areal revegetasi tanaman sengon di *waste dump* tambang batubara di Kalimantan Selatan. *Jurnal Tanah dan Air*, 15(2), pp.55-60. doi: 10.31315/jta.v15i2.4110.

Gonçalves, F.M.P. et al., 2018. Species diversity, population structure and regeneration of woody species in fallows and mature stands of tropical woodlands of southeast Angola. *Journal of Forestry Research*, 29, pp.1569-1579. doi: 10.1007/s11676-018-0593-x.

Handayani, A. et al., 2019. Evaluasi kesintasan dan pertumbuhan beberapa jenis pohon lokal di area restorasi Cagar Biosfer Cibodas. *Jurnal Pengelolaan Sumberdaya Alam dan Lingkungan*, 9(3), pp.541-548. doi: 10.2924 4/jpsl.9.3.541-548.

Handayani, A. & Hidayati, S., 2020. Ethnobotany of Mountain Regions, *Castanopsis argentea* (Blume) A. DC. Fagaceae, in Franco F (eds.), pp:1-6, Springer, Cam.

Heriyanto, N.M., 2007. Kajian ekologi permudaan saninten (*Castanopsis argentea* (Bl.) A.DC.) di Taman Nasional Gunung Gede Pangrango, Jawa Barat. *Buletin Plasma Nutfah*, 13(1), pp.34-42. doi: 10.21082/ blpn.v13n1.2007.p34-42.

Hilwan, I., 2012. Komposisi jenis dan struktur tegakan pada areal bekas tebangan di PT Salaki Summa Sejahtera, Provinsi Sumatera Barat. *Jurnal Silvikultur Tropika*. 03(03), pp.155-16. doi: 10.29244/j-siltrop.3.3.%25p.

Hilwan, I. & Irfani, E., 2018. Pola penyebaran dan regenerasi jenis saninten (*Castanopsis argentea* Blume) di Resort Selabintana, Taman Nasional Gunung Gede Pangrango. *Jurnal Silvikultur Tropika*, 9(1), pp.53–59. doi: 10.29244/j-siltrop.9.1.53-59.

Ismiani, L., 2015. Pengaruh alelopati tumbuhan invasif (*Clidemia hirta*) terhadap germinasi biji tumbuhan asli (*Impatiens platypetala*). *Prosiding Seminar Nasional Masyarakat Biodiversitas Indonesia*, 1(4), pp.834-837. doi: 10.12057/psnmbi/m010429.

Körner, C., 2007. The use of altitude in ecological research. *Trends in Ecology & Evolution*, 22(11), pp.569-574. doi: 10.1016/j.tree.2007.09.006.

Krebs, C.J., 1972. *Ecology: The experimental analysis of distribution and abundance*. New York: The University of British Columbia.

Krebs, C.J., 1989. *Ecological Methodology*. New York: Harper & Row.

Luo, W.T., et al., 2015. Contrasting pH buffering patterns in neutral-alkaline soils along a 3600 km transect in northern China. *Biogeosciences*, 12(23), pp.7047–7056. doi: 10.5194/bg-12-7047-2015.

Metananda, A.A., et al., 2015. Populasi, sebaran, dan asosiasi kepuh (*Stercularia foetida* L.) di Kabupaten Sumba, Nusa Tenggara Barat. *Media Konservasi*, 20(3), pp.277-287. doi: 10.29244/medkon.20.3.%25p.

Mirmanto, E., 2014. Fitososiologi hutan pegunungan di Lereng Tenggara Gunung Salak. *Jurnal Biologi Indonesia*, 10(1), pp.27-38. doi: 10.14203/ jbi.v10i1.326.

Mueller, K.E., et al., 2012. Tree species effects on coupled cycles of carbon, nitrogen, and acidity in mineral soils at a common garden experiment. *Biogeochemistry*, 111(1-3), pp.601–614. doi: 10.1007/s10533-011-9695-7.

Nursanti & Adriadi, A., 2018. Keanekaragaman tumbuhan invasif di Kawasan Taman Hutan Raya Sultan Thaha Saifuddin, Jambi. *Media Konservasi*, 23(1), pp.85-91. doi: 10.29244/medkon.23.1.85-91.

Perry, G.L.W. et al., 2009. Nearest-neighbor interactions in species-rich shrublands: the roles of abundance, spatial patterns and resources. *Oikos*, 118(2), pp.161-174. doi: 10.1111/j.1600-0706.2008.16947.x.

Pescie, M.A. et al., 2018. Absorption, distribution and accumulation of nitrogen applied at different phenological stages in southern highbush blueberry (*Vaccinium corymbosum* interspecific hybrid). *Scientia Horticulturae*, 230, pp.11-17. doi: 10.1016/j.scienta.2017.11.005.

Plant of the World Online (POWO), 2022. Plant of the World Online, viewed 28 August 2022, from https://powo.science.kew.org.

Pradhan, A. et al., 2018. Diversity, population structure, and regeneration potential of tree species in five sacred forests of western Odisha, India. *Écoscience*, 26(1), pp.1-13. doi: 10.1080/11956860.2018.1522148.

Raihandhany, R. et al., 2019. Perbandingan struktur komunitas tumbuhan pada area longsor dan nonlongsor di Taman Hutan Raya Ir. H. Djuanda, Bandung. *Prosiding Seminar Nasional*, pp.220-230.

Rayburn, A.P. et al., 2011. Use of precise spatial data for describing spatial patterns and plant interactions in a diverse Great Basin shrub community. *Plant Ecology*, 212(4), pp.585-594. doi: 10.1007/s11258-010-9848-0.

Rayburn, A.P. & Monaco, T.A., 2011. Linking plant spatial patterns and ecological processes in Grazed Great Basin plant communities . *Rangeland Ecology & Management*, 64(3), pp.276-282. doi: 10.2111/rem-d-10-00130.1.

Risna, R.A., 2009. *Autekologi dan Studi Populasi Myristica teijsmannii Miq. (Myristiceceae) di Cagar Alam Pulau Sempu, Jawa Timur*. Bogor: Institut Pertanian Bogor.

Rukhmi et al., 2017. Sifat fisik dan kimia tanah pada berbagai ketinggian tempat di habitat eboni (*Diospyros celebica* Bakh.) DAS Sausu Sulawesi Tengah. *Warta Rimba,* 5(1), pp.28-36.

Schurr, F.M. et al., 2004. Spatial pattern formation in semi-arid shrubland: a priori predicted versus observed pattern characteristics. *Plant Ecology*, 173(2), pp.271-282. doi: 10.1023/b:vege.0000029335.131948.87.

Setyawati, T., 2010. Pemanfaatan pohon berkhasiat obat di Cagar Alam Gunung Picis dan Gunung Sigogor, Kabupaten Ponorogo, Jawa Timur. *Jurnal Penelitian Hutan dan Konservasi Alam*, 8(2), pp.177–192. doi: 10.20886/jphka. 2010.7.2.177-192.

Siburian, R.H.S. et al., 2020. Growing site characteristics of *Agathis labillardieri* Warb in the Natural Forests of Siwi Momiwaren, West Papua. *Jurnal Sylva Lestari*, 8(3), pp.297-307. doi: 10.23960/jsl38297-307.

Soepadmo, E., 1968. Florae Malesianae Praecursores XLVII. Census of Malesian *Castanopsis* (Fagaceae), *Reinwardita*, 7(4), pp.383-410.

Sumihadi et al., 2019. Kepadatan dan pola penyebaran *Ficus* spp. di stasiun penelitian Cabang Panti Taman Nasional Gunung Palung Kalimantan Barat. *Protobiont*, 8(3), pp.115-121. doi: 10.26418/ protobiont.v8i3.36877.

Wahyudi, A. et al., 2014. Keanekaragaman jenis pohon di hutan pendidikan konservasi terpadu Tahura Wan Abdul Rachman. *Jurnal Sylva Lestari*, 2 (3), pp.1-10. doi: 10.23960/jsl321-10.

Waskitaningtyas, E. et al., 2018. Distribusi dan regenerasi jamuju (*Dacrycarpus imbricatus* (Blume) de Laub) di Cagar Alam Gebugan Kabupaten Semarang. *Jurnal Akademika Biologi*, 7(3), pp.21-26.

Wibowo, C., 2006. *Hubungan Antara Keberadaan Saninten (Castanopsis argentea BLUME) Dengan Beberapa Sifat Tanah: Kasus di Taman Nasional Gunung Gede Pangrango, Jawa Barat*. Bogor: Institut Pertanian Bogor.

Yahya, N. et al., 2019. Species diversity, population structure and regeneration status of woody species on Yerer Mountain Forest, Central Highlands of Ethiopia. *Tropical Plant Research*, 6(2), pp.206-213. doi: 10.22271/tpr.2019.v6.i2.030.

Yang, Y. et al., 2015. Completing the life history of *Castanopsis fargesii*: Changes in the seed dispersal, seedling and sapling recruitment patterns. *European Journal of Forest Research*, 134(6), pp.1143–1154. doi:10.1007/s10342-015-0916-9.

Zhao, Z. et al., 2021. Influence of slope direction on the soil seed bank and seedling regeneration of *Castanopsis hystrix* seed rain. *Forests*, 12(500), pp.1-13. doi: 10. 3390/f12040500.

Zhu, Z.X. et al., 2019. Tree abundance, richness, and phylogenetic diversity along an elevation gradient in the tropical forest of Diaoluo Mountain in Hainan, China. *Acta Oecologica*, 101, pp.103481. doi: 10.1016/ j.actao.2019.103481.

Zuhri, M. et al., 2018. The detection of wood decay of trees collection *Agathis borneensis* and *Castanopsis argentea* at the public area in Cibodas Botanical Garden. *IOP Conference Series: Earth and Environmental Science*, 203(1), pp.1-9. doi: 10.1088/1755-1315/203/1/012034.

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Research Article

The Hypolipidemic Effect of Mountain Papaya and Bitter Melon Fruit Ethanolic Extract in Diabetic Rats

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ABSTRACT

Traditional medicine has been developed rapidly throughout the world to treat hyperlipidemia. However, the use of a single compound in hyperlipidemia treatment usually have low efficacy. Therefore, a combination of ingredients is bound to have more synergistic impact in therapy. This research aimed to examine the hypolipidemic potential of mountain papaya (MPE) and bitter melon fruit ethanolic extract (BME) in alloxan-induced rats. Forty rats divided into eight groups were used in this study. Groups are divided into normal control, negative control, positive control, as well as MPE and BME groups which divided into single doses and three combination doses. Induction of 150 mg/kg alloxan intraperitoneally were performed to generate a model of diabetes and hyperlipidemia. The treatment was carried out for four weeks of the experiment. The single and combination doses of both extracts sufficiently exhibited hypolipidemic activity ($p < 0.05$). The levels of lipid profiles total such as cholesterol, triacylglycerides, low-density lipoprotein, high-density lipoprotein, and very high-density lipoprotein were decreased after MPE and BME administration ($p < 0.05$). The combination of MPE and BME also has hypolipidemic action equivalent to simvastatin. The single and combined doses of mountain papaya, as well as bitter melon fruit ethanolic extracts, have the potential to improve the biochemical (lipid profile) modifications of alloxan-induced.

INTRODUCTION

Diabetes mellitus is a metabolic disorder identified by hyperglycemia because of deficiencies in insulin secretion and/or insulin activity (Laela et al. 2021). In 2017, the International Diabetes Federation (IDF) assessed the number of diabetes cases reached 425 million and predicted that this value would be rise to 629 million by 2045 (Cho et al. 2018). According to numerous studies, diabetes mellitus tends to increase lipid levels, blood pressure, and leads to other complications (Alwardat et al. 2018; Susanti et al. 2018). Dyslipidemia is identified by elevated total blood cholesterol levels, low-density lipoprotein cholesterol, and decreased levels of high-density lipoprotein cholesterol. The condition of hyperlipidemia is the primary cause of cardiovascular disorders

(Duraipandiyan et al. 2016). Numerous studies on diabetic therapy showed that controlling lipid levels help to prevent cardiovascular system complications (Donate-Correa et al. 2020).

In hyperlipidemia therapy, several synthetic drugs, including statin groups and fibrates, have been used. However, these drugs have been discovered to have several side effects like myopathy, hepatic dysfuction, rhabdomyolysis, and peripheral neuropathy (Ramkumar et al. 2016). Therefore, there is a challenge to discover non-detrimental alternatives for the theraphy. Indonesia is abundant of prospective plants especially medicinal plants (Susanti et al. 2018). These plants are used as sources of active secondary metabolites use for complementary or alternative treatments (Kamel et al. 2017). Several advancements in oral hyperlipidemia therapy from natural metabolites have been made. However, the use of a single compound in hyperlipidemia treatment is usually has low efficacy. Therefore, a combination of ingredients is bound to have a more synergistic impact in therapy (Sasongko et al. 2020).

Plants with flavonoid have hypolipidemic activity related to inhibition of HMG-CoA reductase and hence mevalonate production (Ziaee et al. 2009; Ling et al. 2020). Mountain papaya (*Vasconcellea pubescens*) and bitter melon (*Momordica charantia*) are two Indonesian plants known to exhibit this activity (Elangovan et al. 2019; Gao et al. 2019).

According to previous study, ten chemicals were detected in the fruits and active fractions of mountain papaya, tentatively identified as hydroxycinnamic acid glycosides and nine as quercetin glycoside derivatives (Simirgiotis et al. 2009). Furthermore, the fruit's extract contains additional metabolites, including tannins, triterpenoids, as well as polyphenols, and these compounds exhibit anti-diabetic properties (Sasongko et al. 2020; Sasongko et al. 2021). Meanwhile, bitter melon is known to contain phytochemicals including proteins, polysaccharides, flavonoids, triterpenes, saponins, ascorbic acid, and steroids (Jia et al. 2017). Several studies shown this fruit has hypoglycemic and hypolipidemic properties. Moreover, the potential mechanism of this action is possibly through induction of insulin release from the remaining pancreatic beta cells (Mahwish et al. 2017; Raish 2017). This study, therefore, purposed to investigate the hypolipidemic efficacy of a mixture of mountain papaya and bitter melon fruit ethanolic extracts.

MATERIALS AND METHODS

Materials

Mountain papaya and bitter melon were collected from Dieng, Wonosobo, and traditional market at Surakarta, Central Java, Indonesia respectively. Wistar rats (*Rattus norvegicus*) were obtained from Faculty of Medicine, Universitas Sebelas Maret, Surakarta, Indonesia. The fruits were subjected to taxonomic identification at the Faculty of Mathematics and Sciences, Universitas Sebelas Maret, Indonesia (No. 207/UN27.9.6.4/Lab/2019).

Fruit Extract Preparation

The fresh fruit samples were washed under running water, sliced into small pieces, and oven-dried separately at 50 °C. This was followed by pulverization using a mechanical grinder and extraction using the maceration method in 70% ethanol (1:5) for 3 x 24 hours (Sugiyarto et al. 2018). The extract's yield was then evaporated by a rotary evaporator at a temperature below 60 °C until thick extracts were obtained from the mountain papaya (MPE) and bitter melon (BME).

Phytochemical Screening

Flavonoid

One gram of the extract was added to each separate reaction tube. Then, 3 drops of HCl 2N, a few milligrams of magnesium powder, and 1 milliliter of amyl alcohol were added to each test tube, and they were agitated until homogenous. The reaction is positive if a yellow to crimson solution is produced (Santoso et al. 2018).

Saponin

One gram of extract was added to each separate reaction tube. Then added aquadest in each tube and shaken strongly. The presence of saponins is shown by the foam formation (Weli et al. 2018).

Alkaloid

One gram of extract was added to each separate reaction tube. This test was carried out using four reaction tubes, as well as five drops of chloroform to each tube. Tube 1 served as a control, while a drop or two Mayer's reagent, Dragendorff's reagent, and Wagner's reagent were added to tubes 2, 3, and 4, respectively. The formation of white, orange, and brown precipitates indicated a positive result for tubes 2, 3, and 4, respectively (Santoso et al. 2018).

Tannin

This test was performed by filling 2 separate reaction tubes with 1 gram of MPE and BME, then added 2 to 3 drops of 1% $FeCl_3$, as well as 1% gelatin solution to each tube. The formation of a white precipitate shows the presence of tannins (Santoso et al. 2018).

Phenolic compounds

This test was performed by filling 2 separate reaction tubes with 1 gram of MPE and BME, then adding 2 to 3 drops of 1% $FeCl_3$ to each tube. The formation of a black precipitate shows the presence of phenolic compounds (Santoso et al. 2018).

Animal Preparation

This study received approval for all animal handling protocols from the Ethics Committee of Moewardi Hospital, Surakarta, Indonesia (No: 1046/III/

HREC/2019). Male Wistar rats (aged 10–12 weeks) weighing 150 to 180 grams were obtained from the Integrated Laboratory, Universitas Sebelas Maret Surakarta, Indonesia, and were fed with corn seed, as well as water ad libitum, and allowed to be acclimatized to the laboratory conditions for a week before the experiment.

In Vivo Experimental

Hyperglicemia induction in the rats was done using 150 mg/kg intraperitoneally alloxan monohydrate (Mourya et al. 2017). Alloxan is a toxic glucose analog that can be used for rapid induction of diabetes with hyperlipidemia as the side effect (Erejuwa et al. 2016). Forty rats were divided into eight groups: three control groups and five test groups. The normal control group (I) was not given any treatment, the positive control group (II) was orally administered 0.9 mg/kg of simvastatin suspension, and the negative control group III was orally administered a suspension of 0.25% sodium carboxymethyl cellulose (CMC-Na). Meanwhile, the test groups were orally administered a combination of MPE-BME at the ratios of 50% : 50% (IV), 25% : 75% (V), and 75% : 25% (VI), as well as 100% MPE (VII), and 100% BME (VIII) at the dosage of 173.900 mg/kg, and 378.170 mg/kg, respectively. The extract dosage was based on the total flavonoid activity (quercetin equivalent), 121.334 ± 3.404 mg/100 g ethanol extract, and 55.795 ± 1.601 mg /100 g ethanol extract, for MPE and BME, respectively (Sasongko et al. 2020). Furthermore, the lipid profile measurements were performed 28 days after the treatment, using the Biochemistry Semi Analyzer by Biosystem (BTS350) at a wavelength of 500 nm.

Determination of Lipid Profile

The rat was anesthetized with ketamine hydrochloride (50 mg/kg BW) intraperitoneally. The 2 mL of the blood was collected with cardiac puncture method. The blood was centrifuged for 10 minutes at 4000 rpm and 4 °C to separate and collect the serum for biochemical study. Subsequently, the lipid profile, comprising the total cholesterol (TC), triacylglycerides (TG), low-density lipoprotein (LDL), high-density lipoprotein (HDL), very high-density lipoprotein (VLDL), atherogenic index (AI), atherogenic coefficient (AC), and cardiovascular risk index (CRI), was determined using the Biosystems instrument commercial kits (Biosystems, Spain). Meanwhile, the atherogenic index, atherogenic coefficient, and cardiovascular risk index were calculated using the following formulae, respectively (Kinosian et al. 1994; Azizian et al. 2018):

Atherogenic Index (AI) = (Total Cholesterol – HDL Cholesterol) / HDL Cholesterol

Atherogenic Coefficient (AC) = LDL Cholesterol / HDL Cholesterol

Cardiovascular Risk Index (CRI) = Total Cholesterol / HDL Cholesterol

Statistical Analysis

The data were subjected to a one-way analysis of variance (ANOVA), followed by a Tukey HSD post hoc test to analyze the significant differences between groups at $p < 0.05$. Subsequently, all data were proved as mean ± standard error of the mean (SEM).

RESULTS AND DISCUSSION

Results

Phytochemical qualitative analysis

The results of this study showed a slightly lower percentage yield of 21.1% for the BME, compared to the MPE of 26.43% (Table 1). This was calculated using a 100% weight comparison between extract and dried fruit. Meanwhile, the qualitative phytochemical analysis of MPE-BME indicated the presence of alkaloids, flavonoids, saponin, polyphenols, and tannins. There have been numerous previous studies that demonstrated qualitatively (Sugiyarto et al. 2018).

Table 1. The percentage yields of MPE and BME extraction.

Extract sample	Dried fruit (gram)	Extract (gram)	Yield (%)
MPE	454	119.97	26.43
BME	479	100.62	21.01

Note: MPE = mountain papaya extract, BME = bitter melon extract.

The effect of MPE and BME treatment on body weight

Figure 1 shows the administrative effect of MPE and BME on body weight profile. Based on the statistical analysis at the 28th day, all the dose of MPE and BME treatments significantly influenced the body weight of alloxan-induced diabetic rats besides MPE: BME (75% : 25%) compared with negative control. Statistically, intraperitoneal administration of a single alloxan dose led to a significant ($p < 0.05$) reduction of the rats' body weight, com-

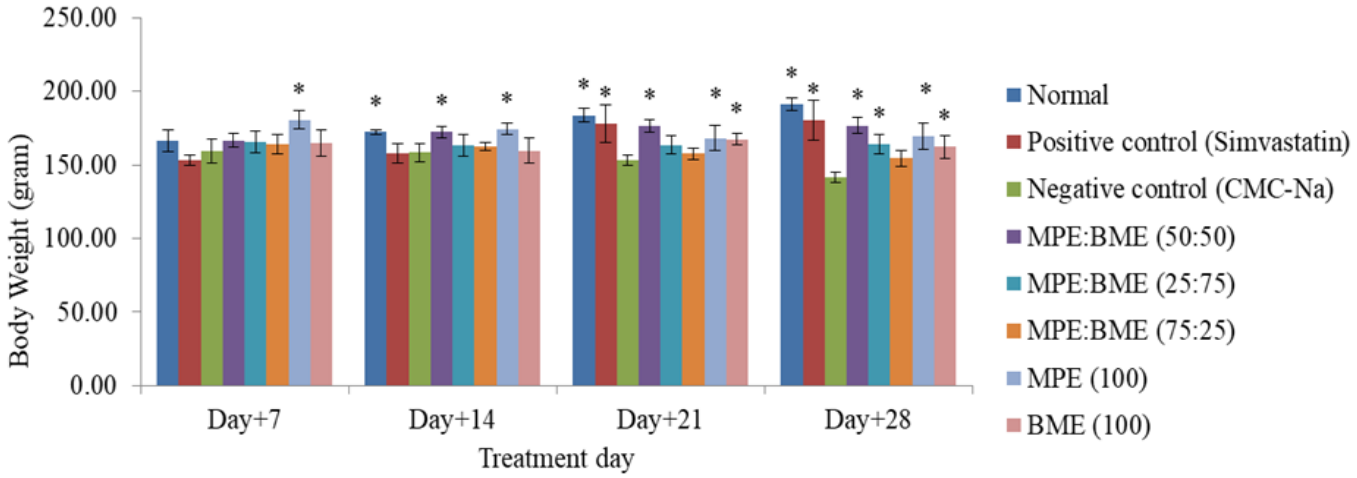

Figure 1. The effect administration of MPE and BME on body weight profile. The data are represented as means ± SEM (n = 5). *$p < 0.05$ compared with the negative control group. MPE = mountain papaya and BME = bitter melon extract.

Table 2. The ability of MPE and BME to improve lipid profiles in diabetic rats after 28 days of testing.

Groups	Lipid profiles							
	TC ± SEM (mg/dL)	TG ± SEM (mg/dL)	HDL ± SEM (mg/dL)	LDL ± SEM (mg/dL)	VLDL ± SEM (mg/dL)	AI ± SEM	AC ± SEM	CRI ± SEM
Normal	90.137 ± 5.17*	89.93 ± 0.9*	30.5 ± 0.2*	28.57 ± 2.3*	31.067 ± 1.86*	1.95 ± 0.12*	0.936 ± 0.21*	2.955 ± 0.18*
Negative control (CMC-Na)	255.467 ± 3.37	223.51 ± 3.8	23.64 ± 0.3	210.2 ± 3.9	21.627 ± 1.3	9.81 ± 0.59	8.892 ± 0.53	10.806 ± 0.65
Positive control (Simvastatin)	98. 379 ± 3.72*	108.13 ± 6.2*	30.25 ± 0.3*	50.14 ± 3.8*	17.989 ± 1.08*	2.25 ± 0.14*	1.657 ± 0.12*	3.252 ± 0.2*
MPE (100)	99.746 ± 4.93*	99.28 ± 3.3*	30.17 ± 0.55*	53.73 ± 2.1*	15.846 ± 0.95*	2.31 ± 0.14*	1.781 ± 0.18*	3.306 ± 0.18*
BME (100)	98.69 ± 3.04*	94.14 ± 4.6*	30.36 ± 0.51*	49.5 ± 2.4*	18.83 ± 1.13*	2.25 ± 0.15*	1.63 ± 0.14*	3.250 ± 0.21*
MPE:BME (50:50)	100.703 ± 1.88*	106.91 ± 5.7*	31.31 ± 0.31*	48.01 ±0.8* #	21.383 ± 1.28	2.216 ± 0.13*	1.533 ± 0.13*	3.216 ± 0.19*
MPE:BME (25:75)	99.043 ± 1.44*	106.25 ± 6.1*	30.85 ± 0.36*	46.94 ± 1.9* #	21.253 ± 1.28	2.21 ± 0.13*	1.522 ± 0.14*	3.210 ± 0.22*
MPE:BME (75:25)	102.042 ± 4.8*	101.72 ± 9.5*	30.11 ± 0.21*	51.59 ± 3.9*	20.342 ± 1.22	2.38 ± 0.14*	1.713 ± 0.11*	3.388 ± 0.25*

Note: The data are represented as means ± SEM (n=5). *$p < 0.05$ compared with the negative control group; # $p < 0.05$ compared with single extract group . MPE = mountain papaya extract; BME = bitter melon extract; TC = total cholesterol; TG = triglycerides; HDL = high - density lipoprotein; LDL = low - density lipoprotein; VLDL = very high - density lipoprotein; AI = atherogenic index; AC = atherogenic coefficient; CRI = cardiovascular risk index.

pared to the normal control. Meanwhile, in each week of observations, it was shown that there were several groups that decreased and increased in terms of body weight, especially in the normal group and simvastatin positive control group.

The effect of MPE and BME treatments on lipid profile of diabetic rats

Table 2 showed the rats' lipid profiles after oral administration of MPE and BME. The hyperlipidemia group (negative control) has a significantly higher ($p < 0.05$) difference in serum lipids after 28 days, compared to the normal group. Furthermore, the treatment with 0.9 mg/kg simvastatin caused a significant decrease in the hyperlipidemic profile of the diabetic rats ($p < 0.05$). According to Table 2, all the treatment groups exhibited hypolipidemia ($p < 0.05$). The lipid profile evaluation on the 28th day showed that the single and combined extracts did not differ significantly from the positive control ($p > 0.05$). This showed that the administration of single or combined extracts have similar effect as simvastatin. Therefore, the single and combination doses of MPE and BME have the similar capacity to improve the lipid profiles ($p > 0.05$).

Discussion

In this study, mountain papaya and bitter melon were extracted to evaluate their hypolipidemic effect. These extracts were shown that they contained saponins, flavonoids, alkaloids, and tannins in this research. According to another study, bitter melon contained anthraquinones, glucosinolates (Joseph & Jini 2013), carbohydrates, glycosides, proteins, and amino acids (Shukla & Kashaw 2018; Li et al. 2020). In addition, the phytochemical analysis of mountain papaya ethanolic extract showed the presence of flavonoids, tannins, and phenolic compounds (Simirgiotis et al. 2009; Laily et al. 2012; Sasongko et al. 2018). Figure 1 showed that alloxan induced weight loss in the diabetic rat model. Alloxan caused diabetes in rats by damaging the insulin-secreting cells of the pancreas, leading to hyperglycemia and hyperlipidemia (Shatynska et al. 2020; Atanu et al. 2021). According to the American Diabetes Association (American Diabetes Association 2007), polyphagia and polydipsia, as well as loss in body weight, were significant signs of diabetes mellitus, and these symptoms were exhibited by the diabetic groups in this study. This is possibly associated with structural protein atrophy and muscle wasting (Frier et al. 2008). The diabetic rats group showed a reduction of the body weight each week. However, the hyperlipidemic rats given MPE and BME kept their body weight the same, while the rats given simvastatin gained weight.

Alloxan injection causes diabetes mellitus, which is often found in unstable lipid profile conditions (TG, TC, HDL, LDL, and VLDL). Similar to one previous study, uncontrolled hyperglycemia leaded to hyperlipidemia (Rahimi-Madiseh et al. 2017). Alloxan contributes to insulin secretion loss, resulting in elevated plasma glucose levels, by killing Langerhans islets β-cells

(Roghani & Aghaie 2007). A study by Akbari et al (2013) showed the inappropriate activity of lipolytic hormones on adipose tissues was possibly attributed to alloxan-induced hyperlipidemia. In vivo, fatty acid mobilization from adipose tissue's triglyceride stores is regulated by hormone-sensitive lipase. According to (Cignarelli et al. 2019), insulin regulates the mobilization of lipids in the body from adipose tissue. Therefore, in the absence of insulin, hormone-sensitive lipase is activated, leading to a rise in serum lipid levels (Okazaki et al. 2002).

Generally, lipid disorders are related to diabetes and this leads to cardiovascular conditions, including elevated levels of TC, TG, LDL, and reduced HDL (Elangovan et al. 2019). Similar to the report by Sasongko et al. (2020), alloxan-induced diabetes triggered a considerable rise in serum MDA and a significant decreased in antioxidant enzymes in this study. As a distinctive characteristic of oxidative stress, lipid peroxidation (LPO) plays significant role in the development of diabetes mellitus, disrupting components of the cell membrane, necrosis, inflammation, and serves as a buffer against oxidative stress. Increased TC, TG, LDL, VLDL, and HDL levels, as well as decreasing HDL levels, are typically associated with diabetes, which lead to cardiovascular disease (Elangovan et al. 2019). This explains why untreated diabetic rats (negative control) have higher amounts of TC, TG, LDL, and VLDL but lower levels of HDL. For 28 days, MPE and BME extracts were given and significantly lowered TC, TG, LDL, and VLDL levels while increasing HDL levels. In the MPE and BME combination-treated group of rats, similar effect was seen. Bitter melon, a health-promoting vegetable, is traditionally used for medical nutrition therapy to cure diabetes, but to reap maximum health claims, vigilant control of its substances in the diet is crucial as part of curative action for effective diabetes management. On the other hand, the lipid profile in the negative control group was found to be higher. This might be related to insulin insufficiency in a hyperglycemic condition, which could lead to hormone-sensitive lipase-mediated free fatty acid release from adipose tissue (Goldberg 2001).

CONCLUSION

This study concluded that single and combined doses of mountain papaya, as well as bitter melon fruit ethanolic extracts, have the potential to improve the biochemical (lipid profile) modifications of alloxan-induced. In addition, the MPE and BME combination provided similar effective activity as simvastatin against hypolipidemia. The combination of both extracts had no synergistic effects on most lipid profiles, with the exception of LDL levels, which improved when compared to single extracts. Therefore, the ameliorative role of both extracts towards alloxan-induced hyperlipidemia in the rats' livers is possibly linked to the phenolic compounds present.

AUTHORS CONTRIBUTION

The authors contribution: H.S designed the research, wrote the manuscript

and supervised all the process; R.G.L collected and analyzed data especially for lipid profile; R.D.I conducted the extraction process and phytochemical screening; R.D.W conducted phytochemical screening and analyzed the data; S.M conducted the extraction process, analyzed the data and wrote the manuscript.

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CONFLICT OF INTEREST

All authors declare no conflict of interest.

REFERENCES

Akbari, F. et al., 2013. Effect of Turnip on Glucose and Lipid Profiles of Alloxan-Induced Diabetic Rats. *Iranian Journal of Endocrinology and Metabolism*, 14(5), pp.492–497.

Alwardat, N. et al., 2018. Association between hypertension and metabolic disorders among elderly patients in North Jordan. *Diabetes & Metabolic Syndrome: Clinical Research and Reviews*, 12(5), pp.661–666. doi: 10.1016/j.dsx.2018.04.011

American Diabetes Association, 2007. Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care*, 30(1), S42–S47. doi: 10.2337/dc07-S042

Atanu, F.O. et al., 2021. Anti-hyperglycemic and Anti-hyperlipidemic effects of Hydroethanol Leaf and Stem Extracts of Vitex doniana in Diabetic Rats. *Asian Journal of Research in Biochemistry*, 8(3), pp.53–59. doi: 10.9734/ajrb/2021/v8i330184

Azizian, H. et al., 2018. Cardioprotective and anti-inflammatory effects of G-protein coupled receptor 30 (GPR30) on postmenopausal type 2 diabetic rats. *Biomedicine and Pharmacotherapy*, 108, pp.153–164. doi: 10.1016/j.biopha.2018.09.028

Cho, N.H. et al., 2018. IDF Diabetes Atlas: Global estimates of diabetes prevalence for 2017 and projections for 2045. *Diabetes Research and Clinical Practice*, 138, pp.271–281. doi: 10.1016/j.diabres.2018.02.023

Cignarelli, A. et al., 2019. Insulin and Insulin Receptors in Adipose Tissue Development. *International journal of molecular sciences*, 20(3), 759. doi: 10.3390/ijms20030759

Donate-Correa, J. et al., 2020. Inflammatory Targets in Diabetic Nephropathy. *Journal of Clinical Medicine*, 9(2), 458. doi: 10.3390/jcm9020458

Duraipandiyan, V. et al., 2016. Hypolipidemic activity of friedelin isolated from Azima tetracantha in hyperlipidemic rats. *Revista Brasileira de Farmacognosia*, 26, pp.89–93. doi: 10.1016/j.bjp.2015.07.025

Elangovan, A. et al., 2019. Antidiabetic and hypolipidemic efficacy of skin and seed extracts of Momordica cymbalaria on alloxan induced diabetic model in rats. *Journal of ethnopharmacology*, 241, 111989. doi: 10.1016/j.jep.2019.111989

Erejuwa, O.O. et al., 2016. Nigerian Honey Ameliorates Hyperglycemia and Dyslipidemia in Alloxan-Induced Diabetic Rats. *Nutrients*, 8(3), 95. doi: 10.3390/nu8030095

Frier, B.C., Noble, E.G. & Locke, M., 2008. Diabetes-induced atrophy is associated with a muscle-specific alteration in NF-κB activation and expression. *Cell Stress and Chaperones*, 13(3), pp.287–296. doi: 10.1007/s12192-008-0062-0

Gao, H. et al., 2019. Fermented Momordica charantia L. juice modulates hyperglycemia, lipid profile, and gut microbiota in type 2 diabetic rats. *Food Research International*, 121, pp.367–378. doi: 10.1016/j.foodres.2019.03.055

Goldberg, I.J., 2001. Diabetic dyslipidemia: causes and consequences. *The Journal of Clinical Endocrinology and Metabolism*, 86(3), pp.965–971. doi: 10.1210/jcem.86.3.7304

Jia, S. et al., 2017. Recent Advances in Momordica charantia: Functional Components and Biological Activities. *International Journal of Molecular Sciences*, 18(12), 2555. doi: 10.3390/ijms18122555

Joseph, B. & Jini, D., 2013. Antidiabetic effects of Momordica charantia (bitter melon) and its medicinal potency. *Asian Pacific Journal of Tropical Disease*, 3(2), pp.93–102. doi: 10.1016/S2222-1808(13)60052-3

Kamel, F.O. et al., 2017. Knowledge, attitude, and beliefs toward traditional herbal medicine use among diabetics in Jeddah Saudi Arabia. *Complementary Therapies in Clinical Practice*, 29, pp.207–212. doi: 10.1016/j.ctcp.2017.10.007

Kinosian, B., Glick, H. & Garland, G., 1994. Cholesterol and coronary heart disease: predicting risks by levels and ratios. *Annals of internal medicine*, 121(9), pp.641–647. doi: 10.7326/0003-4819-121-9-199411010-00002

Laela, N., Legowo, A.M. & Fulyani, F., 2021. The effect of kefir-spirulina on glycemic status and antioxidant activity in hyperglycemia rats. *Potravinarstvo Slovak Journal of Food Sciences*, 15, pp.101–110. doi: 10.5219/1445

Laily, A.N., Suranto, S. & Sugiyarto, S., 2012. Characterization of Carica pubescens in Dieng Plateau, Central Java based on morphological characters, antioxidant capacity, and protein banding pattern. *Nusantara Bioscience*, 4(1), pp.16-21. doi: 10.13057/nusbiosci/n040104

Li, Z. et al., 2020. The pharmacological properties and therapeutic use of bitter melon (Momordica charantia L.). *Current Pharmacology Reports*, 6(3), pp.103-109. doi: org/10.1007/s40495-020-00219-4

Ling, Y. et al., 2020. Hypolipidemic effect of pure total flavonoids from peel of Citrus (PTFC) on hamsters of hyperlipidemia and its potential mechanism. *Experimental Gerontology*, 130, 110786. doi:10.1016/j.exger.2019.110786

Mahwish, S.F. et al., 2017. Hypoglycemic and hypolipidemic effects of different parts and formulations of bitter gourd (Momordica Charantia). *Lipids Health Dis*, 16, pp.1–11. doi: 10.1186/s12944-017-0602-7

Mourya, P. et al., 2017. Hypoglycemic and hypolipidemic effects of ethanolic and aqueous extracts from Ziziphus oenoplia (L) Mill on alloxan-induced diabetic rats. *Beni-Suef University Journal of Basic and Applied Sciences*, 6(1), pp.1–9. doi: 10.1016/j.bjbas.2016.12.002

Okazaki, H. et al., 2002. Lipolysis in the Absence of Hormone-Sensitive Lipase: Evidence for a Common Mechanism Regulating Distinct Lipases. *Diabetes*, 51, pp.3368–3375. doi: 10.2337/diabetes.51.12.3368

Rahimi-Madiseh, M. et al., 2017. Effect of hydroalcoholic Allium ampeloprasum extract on oxidative stress, diabetes mellitus and dyslipidemia in alloxan-induced diabetic rats. *Biomedicine and Pharmacotherapy*, 86, pp.363–367. doi: 10.1016/j.biopha.2016.12.028

Raish, M., 2017. Momordica charantia polysaccharides ameliorate oxidative stress, hyperlipidemia, inflammation, and apoptosis during myocardial infarction by inhibiting the NF-κB signaling pathway. I*nternational Journal of Biological Macromolecules*, 97, pp.544–551. doi: 10.1016/j.ijbiomac.2017.01.074

Ramkumar, S., Raghunath, A. & Raghunath, S., 2016. Statin therapy: review of safety and potential side effects. *Acta Cardiologica Sinica*, 32(6), pp.631-639. doi: 10.6515/ACS20160611A

Roghani, M. & Aghaie, M., 2007. The effect Allium ampeloprasum feeding on serum level of glucose, triglyceride, and total cholesterol of diabetic rats. *Koomesh*, 8(2), pp.73–78.

Santoso, B.S.A. et al., 2018. Hypoglycemic Activity and Pancreas Protection of Combination Juice of Mengkudu (*Morinda citrifolia* Linn.) Juice and Temulawak (C*urcuma xanthorrhiza* Roxb.) Juice on Streptozotocin-Induced Diabetic Rats. *Indonesian Journal of Pharmacy*, 29(1). doi: 10.14499/indonesianjpharm29iss2pp%p

Sasongko, H., Efendi, N.R. & Sugiyarto, 2018. The ethanolic extract of mountain papaya (*Vasconcellea pubescens* A.DC.) fruit against lipid peroxidation of rat liver tissues. *AIP Conference Proceedings*, 2019, 050001. doi: 10.1063/1.5061894

Sasongko, H. et al., 2020. Antidiabetic and Antioxidant Effect Combination Vasconcellea pubescens A.DC. and Momordica charantia L. Extract in Alloxan- Induced Diabetic Rats. *Pharmacognosy Journal*, 12(2), pp.311–315. doi: 10.5530/pj.2020.12.49

Sasongko, H., Shofa, A.N. & Advaita, N. 2021. The influence of mountain papaya and bitter melon extract supplementation on aspartate transaminase enzyme in diabetic rats models. *Journal of Physics: Conference Series*, 1912(1), 012043). doi:10.1088/1742-6596/1912/1/012043.

Shatynska, O. et al., 2020. Dietary supplementation with magnesium citrate may improve pancreatic metabolic indices in an alloxan-induced diabetes rat model. *Potravinarstvo Slovak Journal of Food Sciences*, 14, pp.836–846. doi: 10.5219/1375

Shukla, R. & Kashaw, V., 2018. Extraction of Momordica charantia, Pongamia glabra and Piper nigrum: *Qualitative and Quantitative assessment*, 8(6-s), 155–165. doi: 10.22270/jddt.v8i6-s.2105

Simirgiotis, M.J., Caligari, P.D.S. & Schmeda-Hirschmann, G., 2009. Identification of phenolic compounds from the fruits of the mountain papaya Vasconcellea pubescens A. DC. grown in Chile by liquid chromatography–UV detection–mass spectrometry. *Food Chemistry*, 115(2), pp.775–784. doi: 10.1016/j.foodchem.2008.12.071

Sugiyarto, S. et al., 2018. Antibacterial activity of ethyl acetate and n-hexane fractions of Carica pubescens rind and seeds. *AIP Conference Proceedings*, 2019, 050005. doi: 10.1063/1.5061898

Susanti, H. et al., 2018. Antihypercholesterol activity of costus speciosus water extract. *Thai Journal of Pharmaceutical Sciences (TJPS)*, 42(2), pp.66-68.

Weli, A.M. et al., 2018. Biological and phytochemical studies of different leaves extracts of Pteropyrum scoparium. *Beni-Suef University Journal of Basic and Applied Sciences*, 7, pp.481–486. doi: 10.1016/j.bjbas.2018.05.001

Ziaee, A. et al., 2009. Effects of Rutin on Lipid Profile in Hypercholesterolaemic Rats. *Basic and Clinical Pharmacology and Toxicology*, 104, pp.253–258. doi: 10.1111/j.1742-7843.2008.00368.x

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Research Article

Flower Structures of *Averrhoa dolichocarpa* Rugayah & Sunarti

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ABSTRACT

Hermaphrodites are believed to be the ancestral characters of flowering plants. However, plants have developed spatially and functionally in arrangements to reduce the chances of self-fertilization. One well-known spatial arrangement is heterostyly. This arrangement is found in almost all Oxalidaceae species, including *Averrhoa* spp. The question that arises with the discovery of two new species of *Averrhoa* is how the spatial flower arrangement of the new species is. This study observed flowers of *A. dolichocarpa* to prove heterostyly of the species. We also compared morphological and anatomical characteristics among flower morphs of *A. dolichocarpa*. Three flower morphs, S-morph, M-morph, and L-morph, were observed, proving that *A. dolichocarpa* is tristyly. Morphologically and anatomically, there was no significant difference between the three flower morphs. Differences in morphometry were found in three flower morphs. In addition to the notable differences in style length in heterostyly, differences in ovary height between flower morphs were observed. The flower morphology and anatomy of *A. dolichocarpa* are similar to that of *A. carambola* and *A. bilimbi* and follow the general pattern of Oxalidaceae.

INTRODUCTION

The reproductive structures of flowering plants are spectacularly diverse. This diversification results from co-evolution and adaptation to available pollinators in most flowering plants. Pollinators have played an essential role in flower form and function diversification, attributed to unique mating strategies and sexual systems variations. The seed-plant reproductive organs shape mating outcomes through their influence in the pollination and progamic phase (Barrett & Harder 2017).

Hermaphrodite flowers are believed to be the ancestral characters of Angiospermae (Sauquet et al. 2017) and are found in most flowering plants (Barrett 2002). Hermaphroditism increases the chances of successful fertilization in the self-compatible plant through self-pollination. However, self-fertilization increases the risk of inbreeding depression (Charlesworth & Willis 2009). Plants have developed several mechanisms to avoid that risk. The structure of the hermaphrodite flower can be separated spatially within the

flower (herkogamy) or function at different times (dichogamy). Those phenomena thereby reduce the chances of self-pollination. Heterostyly, enantiostyly, and flexistyly are forms of sexual polymorphism in male and female reproductive organ spatial arrangement (Barrett 2002).

Heterostyly is a phenomenon that Darwin first described in his book "Different Forms of Flowers on Plants of the Same Species". In heterostylous species, populations are composed of two (distyly) or three (tristyly) floral morphs, which are distinguished by a reciprocal arrangement of stigma and anther heights (Darwin 1877; Barrett 1992; Lloyd & Webb 1992). Heterostyly was found in 28 families (Barrett 2002), with tristyly only reported in six families (Barrett 1993; Thompson et al. 1996). Oxalidaceae is one of 28 flowering plant families that undergoes heterostyly. Oxalidaceae consists of six genera and homostyly was reported in *Biophytum* and certain species *Oxalis* (Cocucci 2004; Veldkamp 1971). Distyly was found in *Sarcotheca, Dapania pentandra, Averrhoa carambola*, several species of *Oxalis*, and several species of *Biophytum* (Veldkamp 1967, 1971; Cocucci 2004). Meanwhile, tristyly was found in *Oxalis*, *Biophytum*, and *A. bilimbi* (Veldkamp 1967, 1971; Cocucci 2004).

The distribution of style polymorphism in Oxalidaceae can be used to study the evolution of heterostyly. It is also essential to obtain detailed information on flower structure to understand the underlying evolutionary pathways of heterostyly in Oxalidaceae. In this study, we will observe the heterostyly in *Averrhoa dolichocarpa*. *Averrhoa* is a genus of the Oxalidaceae family previously known to consist of two species, one tristylous species and one distylous species (Cocucci 2004; Veldkamp 1971). Later, two wild species of *Averrhoa* were described, *A. dolichocarpa* and *A. leucopetala* (Rugayah & Sunarti 2008). Distyly was reported on *A. dolicocarpha* based on observations of two flower morphologies called short-styled morph (S-morph) and long-styled morph (L-morph) (Kapsah et al. 2016). However, the images on Kapsah et al. (2016) showed L-morph and mid-styled morph (M-morph) flowers. We suspected the species is tristyly. Therefore, we further studied the flowers to gather information on the heterostyly of the species. We also performed a comparative study of floral morphology and anatomy to obtain detailed information on the flower structure of *A. dolichocarpa.*

MATERIALS AND METHODS

Materials

Flowers were collected from four *A. dolichocarpa* individuals in Bogor Botanical Gardens and the Cibinong Science Center (Table 1). Flowers were collected at the anthesis stage and stored in 70% alcohol. *A. dolichocarpa,* as other *Averrhoa,* has two whorls of anthers. The plant is distinguished by the reciprocal arrangement of stigma and anther heights on its flowers. The flower with two anther whorls higher than the flower style is identified as S-morph. Meanwhile, flowers with a style whose height is between two anthers whorls categorized as M-morph. L-morph is determined by a style higher than two anther whorls (Figure 1).

Table 1. Locality and flower morphology of plant materials.

Taxon	Locality	Flower Morphology
A. dolichocarpa	Vak VII.D.96, Bogor Botanical Garden	L-morph
A. dolichocarpa	Kandang Badak Nursery, Bogor Botanical Garden	S-morph
A. dolichocarpa	Gedung IX Nursery, Bogor Botanical Garden	M-morph
A. dolichocarpa	Botany Building Park, Cibinong Science Center	M-morph

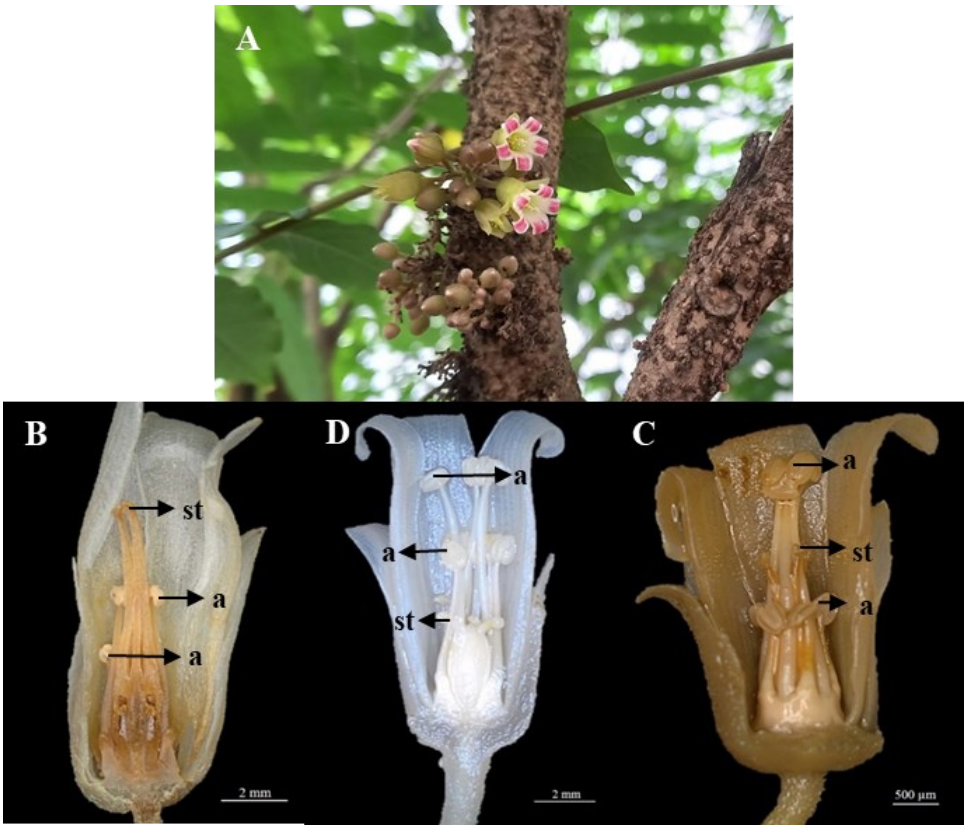


Figure 1. Flower morphology of *A. dolichocarpa*. (A) inflorescence, (B) L-morph flower, (C) M-morph flower, (D) S-morph flower. a: anther, st: stigma.

Methods

Forty flowers per morph were measured. Two sepals and petals are stripped for measurement, and the ovary base is the basis for all sizes except style-stigma (Figure 2). The measurement was conducted under a stereomicroscope (Olympus) with an LC-micro program for the image analyzer. Measured characters are (1) stamen height, (2) anther length, (3) style-stigma height, (4) ovary height, (5) petal length, and (6) sepal length. The ovary height was compared between morphs using one-way ANOVA (α=0.005).

Flower anatomy was observed by making anatomical slides following Sass (1951) method. The examined flowers were in the same stage which was fully developed. Flowers were dehydrated with a multi-grade solution of a combination of aquadest:tert-butanol:ethanol, then infiltrated using paraffin. The samples were sectioned longitudinally and transversally with a rotary microtome (15 - 18 µm). Staining was carried out using safranine and fast-green solution. Slides observation was conducted using Nikon Eclipse 80i and measurements using the Beta View program. For the full pictures, the slides were scanned using a slide scanner.

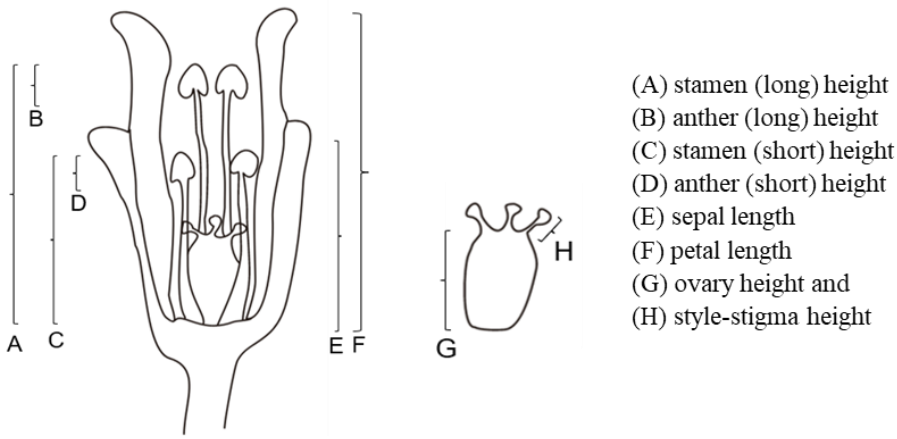


Figure 2. Flower measurement (S-morph flower).

RESULTS AND DISCUSSION

Averrhoa dolichocarpa has cluster-type inflorescence (Figure 1A) with actinomorphic, bisexual, 5-merous (isomerous), and obdiplostemonous flowers. Tristyly can be confirmed in *A. dolichocarpa* with three flower morphs, S-morph, M-morph, and L-morph, observed (Figure 1B-1D). The morphology and anatomy of the three flower morphs of *A. dolichocarpa* have the relatively same structure. However, there are differences in the morphometry of the flower parts as follows.

Calyx: *A. dolichocarpa* calyx has five separate sepals (aposepalous and pentasepalous). Our observation obtained that sepal has the same shape as the previous report by Rugayah & Sunarti (2008), namely lanceolate with a slightly recurved apex. The sepal lengths range of L-morph, M-morph, and S-morph are 6.53-8.72 mm, 5.08-8.05 mm, and 5.25-9.09 mm, respectively (Table 2).

Anatomically, the sepal consists of an epidermis layer with an irregular shape (Figure 3A). Irregular and undifferentiated parenchyma cells are located below the epidermis. There are 2-3 layers of parenchyma cells with small and dense sizes (Figure 3A). The inner part consists of larger parenchyma cells and several simple vascular bundles (Figure 3A). Simple filiform non-glandular trichomes with thick walls were observed in the outer epidermis of all samples (Figure 3A). The result agreed with Rugayah & Sunarti (2008), who stated that the sepal surface of *A. dolichocarpa* is glabrous inside and hairy outside. The presence of trichomes in the L-morph flowers of *A. dolichocarpa* was less in number than the other flower morphs. The presence of trichomes on the sepals with a simple, unicellular, lignified, frequent tanniferous form is a characteristic and representative of all the Oxalidales families (Matthews & Endress 2002).

Corolla: Corolla consists of five petals (pentapetalous) in all observed flowers. As previously described, the petal shape of *A. dolichocarpa* is oblong-ovate (Rugayah & Sunarti 2008). Pentapetalous is also common in other *Averrhoa* species, even though modifications to 4-6 petals in *A. carambola* and *A. bilimbi* were reported (Soumya & Nair 2013). The petals of *A. dolichocarpa* follow the petal-type of Oxalidaceae that are postgenitally united into a basal

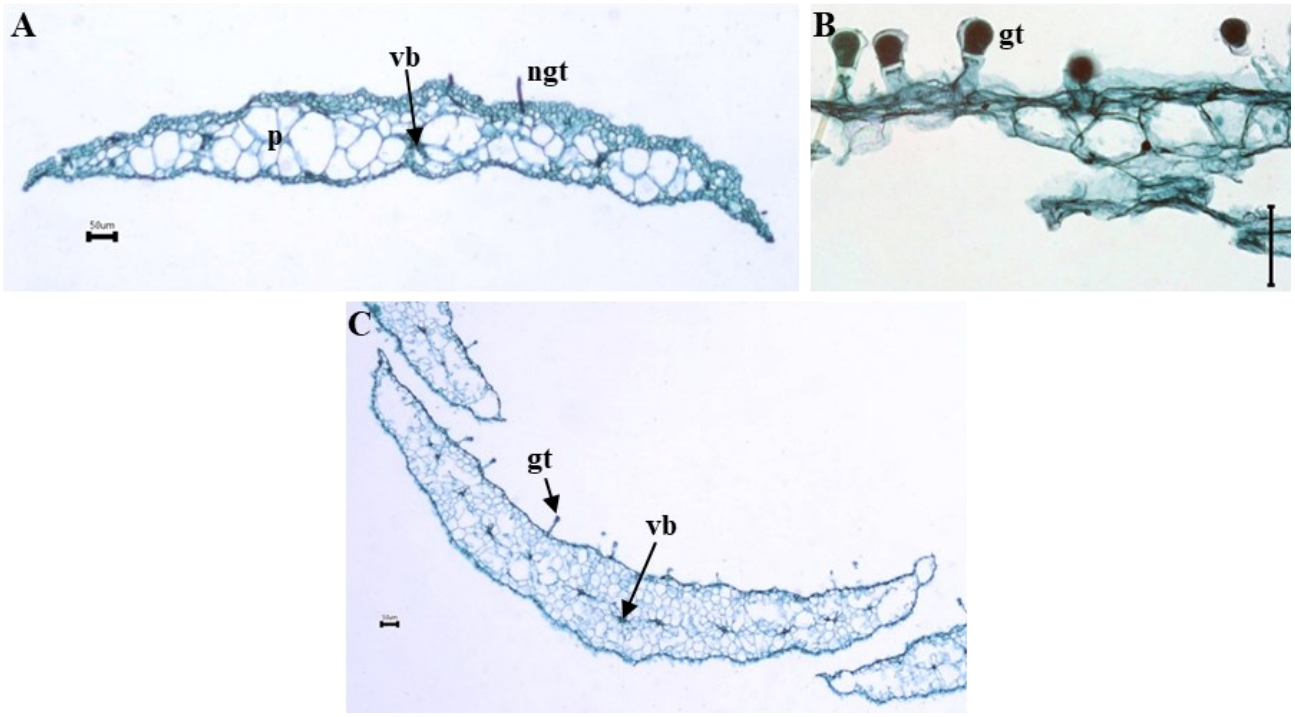


Figure 3. (A) Cross-section of sepal with simple filiform trichome (S-morph), (B) Glandular trichome of M-morph, (C) Cross-section of the petal (S-morph). gt: glandular trichome, ngt: non-glandular trichome, p: parenchyma, vb: vascular bundle. Scale bar: 50 mm.

tube but free at the insertion zone (Matthews & Endress 2002). The petal lengths of L-morph, M-morph, and S-morph are 10.22-13.41 mm, 8.52-12.35 mm, and 9.58-12.44 mm, respectively (Table 2).

The anatomical structure of the corolla consists of a layer of epidermis with a more orderly arrangement than the epidermis of the calyx (Figure 3C). Parenchyma cells of fairly uniform size and several simple vascular bundles are located inside the epidermic cells. Glandular trichomes are capitate forms with rounded heads (Figure 3B). Similar trichomes were also reported in abundance on the petals of *A. carambola* and in small numbers to absent on *A. bilimbi* (Soumya & Nair 2013). These glandular hairs are also common characters on the petal of Oxalidaceae and were found with uni- or multicellular head (Matthews & Endress 2002).

Androecium: Androecium consists of two whorls of stamen, with the outer whorl opposite the petals and the inner whorl opposite the sepals (obdiplostemonous). An alternating arrangement of stamens between five short stamens and five long stamens was found in all observed flowers of *A. dolichocarpa*. The pattern in *A. dolichocarpa* contrasts with *A. bilimbi,* which has an inconsistent pattern and varies 10-12 stamens. The arrangement of the stamens in *A. bilimbi* was modified to 6+6, 6+4, 7+3, 6+5, or 4+6 in different flowers (Soumya & Nair 2013).

The stamens are excurved, curving outward from the axis (Figure 1B-D). The androecium as a whole is not attached to the floral envelope. Based on its attachment, the anther type is dorsifixed with an elongated anther shape. The same anther type was observed in *A. carambola* (Matthews & Endress 2002).

In obdiplostemonous flowers, the epipetalous stamens are generally shorter than the episepalous ones (Matthews & Endress 2002). This size reduction can vary in the filament's length, width, and thickness. An extreme example of the reduction was observed in the absence of anthers in the epipetalous stamen of *A. carambola* (Matthews & Endress 2002; Soumya & Nair 2013). All stamens of *A. dolichocarpa* are fertile. There is a difference in long and short stamen length among flower morphs. The long stamens length for L-morph, M-morph, and S-morph range from 5.49-6.35 mm, 6.13-8.83 mm, and 7.46-8.90 mm, respectively (Table 2). Meanwhile, the short stamen length ranges from 3.38-4.85 mm, 3.30-4.30 mm, and 5.3-6.27 mm for L-morph, M-morph, and S-morph (Table 2). The shortest average of short stamen was observed in M-morph and the longest in S-morph (Table 2).

The longitudinal section showed that the filaments consist of a single layer of the epidermis and rectangular parenchyma cells with a length of 2-3 times the epidermal cells (Figure 4A). The cells are smaller and denser at the ends of the filaments that contact the anthers (Figure 4A). The tip of the filament is connected to the center of the anther. Based on cross-section, the filaments are spherical, with the outermost part being a single-layer epidermis with a square cell shape (Figure 4B, left). Parenchyma cells are composed of polyhedral to rounded cell shapes. The parenchyma cells get smaller towards the middle, and a simple vascular bundle was found in the center (Figure 4B, left).

The cross-section of the anther showed that the anther type is tetrasporangiate (Figure 4C). Observations were made on mature flowers, so the anthers had matured and released their spores. The remaining cells in the anther wall of the mature anther are the epidermis and the endothecium. The epidermis is rounded, while the endothecium is rectangular-trapezoidal with a uniform size (Figure 4C). However, smaller endothecium cells are found in the stomium and facilitate the release of spores as they mature (Figure 4C). Connectivum consists of parenchyma cells, some of which are tanniferous. In the center of the connectivum, vascular bundles originating from the ends of the filaments were found (Figure 4C).

Gynoecium: Gynoecium consists of five attached carpels (syncarp). Variations in the number of carpels with 4-7 carpels were reported in *A. carambola* and *A. bilimbi* (Soumya & Nair, 2013). Meanwhile, there was no varia-

Table 2. Floral dimension of L-morph, M-morph, and S-morph flowers (mm).

Character length (mm)	L-morph			M-morph			S-morph		
	Average	Max	Min	Average	Max	Min	Average	Max	Min
Short stamen	4.11	4.85	3.38	3.69	4.30	3.30	5.86	6.27	5.3
Long stamen	5.89	6.35	5.49	7.64	8.83	6.13	8.33	8.90	7.46
Short anther	0.71	0.82	0.43	0.64	0.88	0.35	0.80	0.96	0.65
Long anther	0.74	0.86	0.56	0.71	0.87	0.54	0.80	0.96	0.69
Style-stigma	5.11	5.82	4.30	2.51	3.17	1.94	0.95	1.14	0.57
Petal	11.72	13.41	10.22	10.29	12.35	8.52	11.31	12.44	9.58
Sepal	7.69	8.72	6.53	6.37	8.05	5.08	7.15	9.09	5.25
Ovary	3.40	4.06	2.72	2.88	3.55	2.27	2.48	3.12	1.84

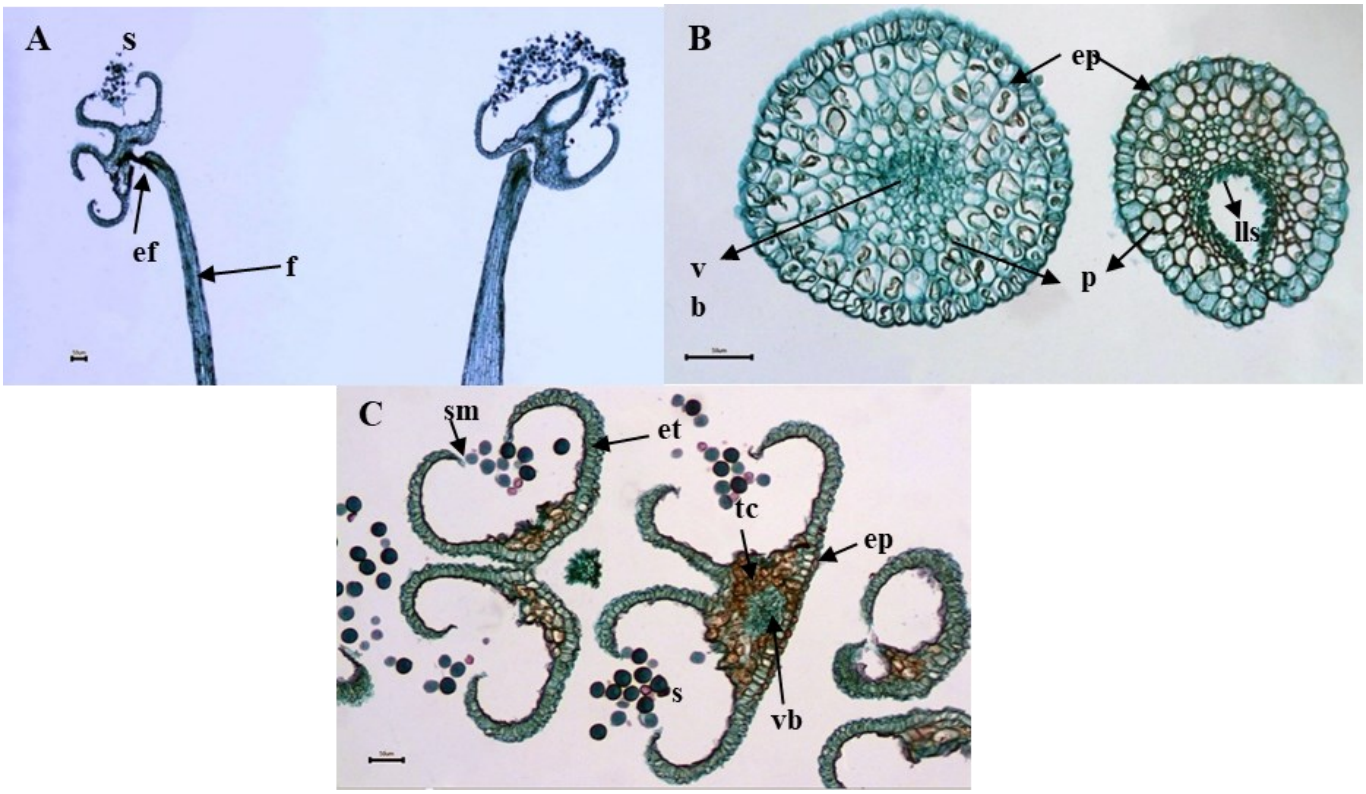


Figure 4. (A) Stamen of L-morph, (B) Filament (left) and stylus (right) of M-morph, (C) Tanniferous cells at connectivum area (M-morph). ep: epidermis, ef: end of filaments, et: endothecium, f: filament, lls: lobe-like structures, p: parenchyma, s: spores, sm: stomium, tc: tanniferous cells, vb: vascular bundle. Scale bar: 50 μm.

tion in the number of carpels of *A. dolichocarpa*. The ovary position is superior, and simple trichomes were found on the ovary, similar to the calyx trichomes (Figure 6A). Trichomes were observed in small numbers in all flower morphs. The difference in style length has long been known as a characteristic of heterostyly plants. It turns out that the height of the ovary in the three *A. dolichocarpa* morphs is also different. The ovary length range from 1.84-3.12 mm in S-morph, 2.27-3.55 mm in M-morph, and 2.72- 4.06 mm in L-morph flowers. One-way ANOVA showed that the ovary height differed significantly (Figure 5).

The type of *A. dolichocarpa* ovule is inverted and straight, with the microphyle situated next to the funiculus (anatropous) (Figure 6A-C). Two ovules in each loculus were found in S-morph flowers (Figure 6A), while four to six ovules per loculus were observed in L-morph and M-morph flowers (Figure 6B-C). The number of ovules in *A. dolichocarpa* corresponds to the number of ovules in each carpel in Oxalidales, which are generally two or slightly more (Matthew & Endress 2002). Three to six ovules per loculus were also reported in *A. bilimbi* (Soumya & Nair 2013). The wall of the ovary consists of a layer of rectangular epidermal cells and several layers of polyhedral to round parenchyma cells and the cell size towards the center increases (Figure 6E). Many tannin cells were found near the epidermis. The vascular bundle is scattered in parenchyma cells. Mature ovules with square outer and rectangular inner integuments can be observed at the loculus. There are three layers of parenchyma cells between the integuments.

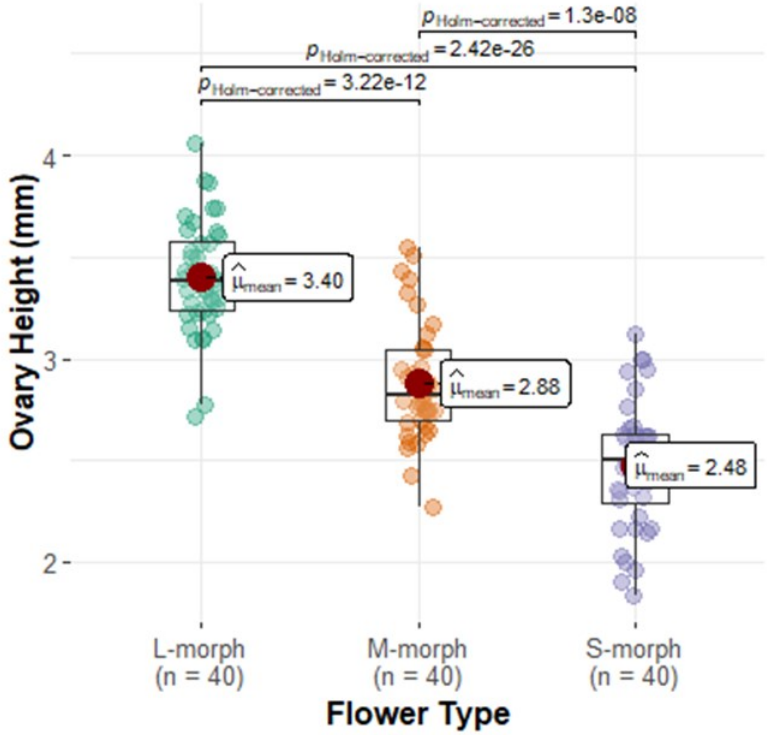


Figure 5. One way ANOVA boxplot comparing ovary height of *A. dolichocarpa*.

Averrhoa dolichocarpa has five branches of stylus ending with five capitate stigmas as reported in most Oxalidales (Matthew & Endress 2002; Rosenfeldt & Galati 2009). The number of stigma in all flower morphs equals the number of carpels. The style area close to the stigma has a hollow texture with a lobe-like structure (Figure 4B, right) that allows entry of the stamens during fertilization. The stylus base (near the ovary) has a solid structure. The center of the stylus develops into the transmission tissue (Figure 6F). The transverse section shows five styli with the same structure (Figure 6G). The epidermis consists of a layer of square-shaped cells. Inside the epidermis, rounded parenchyma cells with a size that is getting smaller towards the center were observed. There are two lateral vascular bundles observed (Figure 6G). The median vascular bundle was discovered on the stylus close to the ovary (Figure 6G).

Floral Vascularization: The vascularization observed in all flower morphs of *A. dolichocarpa* was similar to that in *A. carambola* (Estelita-Teixeira 1980; Matthews & Endress 2002). There is a slight difference with *A. carambola* in the anther tissue unification at the base of the flower. There are 5-6 simple vascular bundles and some small ones in sepal (Figure A1, B1, C1). This result supports Matthews & Endress (2002), who found sepals have at least three main vascular bundles and traces in some species of Oxalidaceae (Matthews & Endress). The vascular bundle in the sepals maintains its shape and presence until several parts of the sepals unite at the base of the flower. The number of vascular bundles in sepals is higher than in petals. Closer to the base of the flower, the bundles that are close together will form more elongated vascular (Figure 7B3-B5) and eventually become a single bundle (Figure 7A4-A5).

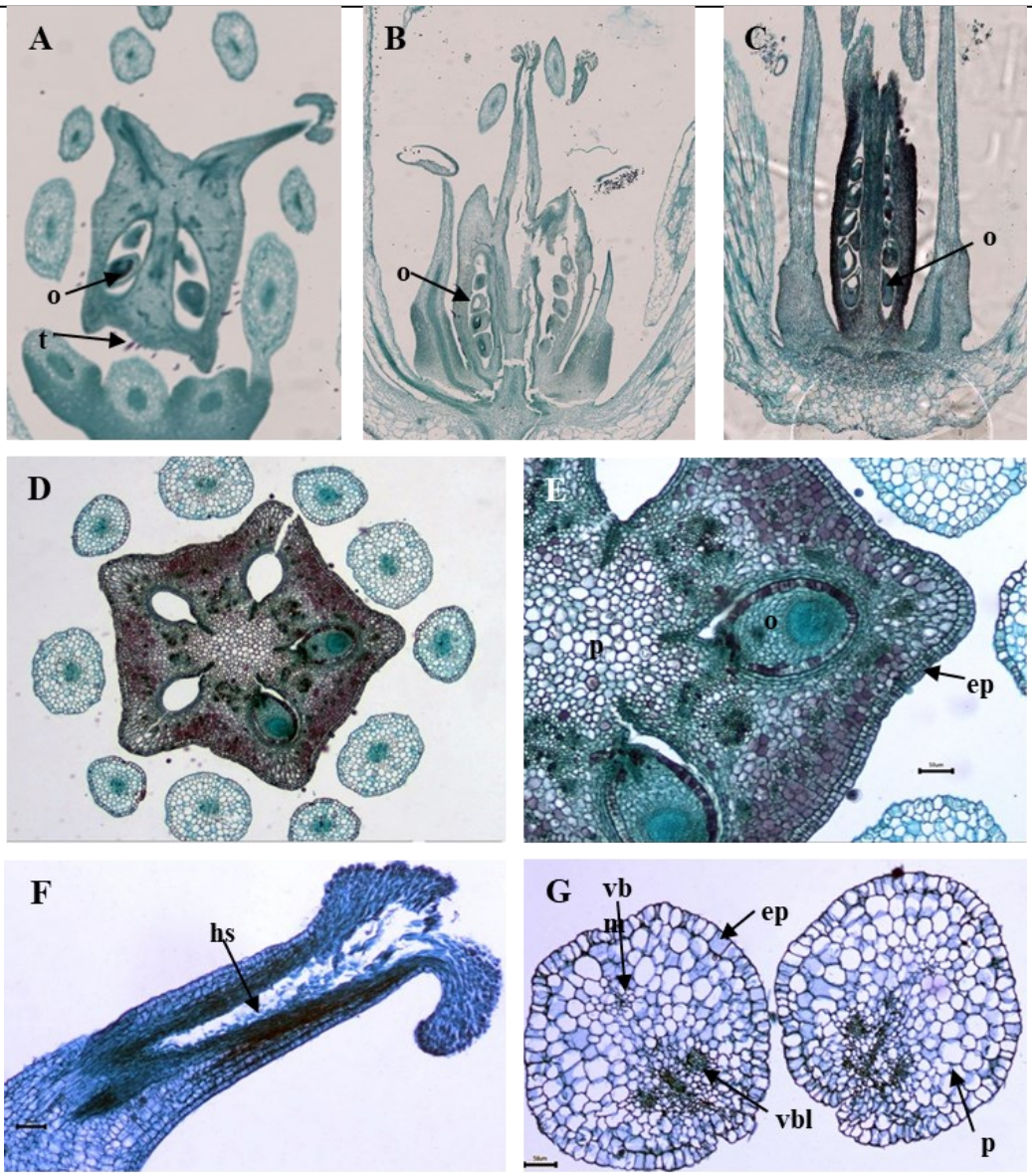
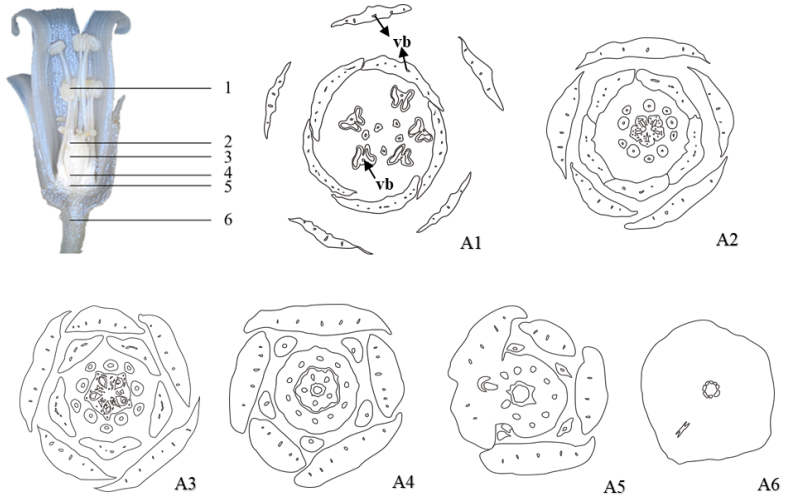


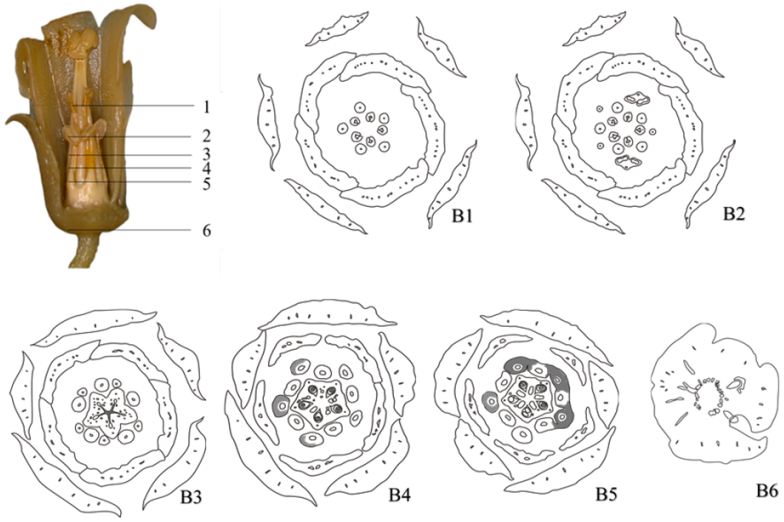
Figure 6. Gynoecium longitudinal section of (A) S-morph, (B) M-morph, and (C) L-morph, (D&E) Cross-section of ovary M-morph, (F) Longitudinal section of stigma S-morph, (G) The stylus of L-morph with two lateral vascular bundles and median vascular bundle (weak). ep: epidermis, hs: hollow structure, o: ovule, p: parenchyma, vbl: lateral vascular bundle, vbm: median vascular bundle. Scale bar: 50 mm.

There is one vascular bundle in the anther. All the anthers will fuse and form ten traces at the base of the flower. Two collateral vascular bundles in the right and left lobes of the stylus were observed. The median vascular bundle is visible on the stylus with a solid center close to the ovary (Figure 7B2). Additional vascular bundles will be formed between the lateral and median bundles and then unite into a larger lateral bundle (Figure 7B5, 7C4). After a larger lateral bundle is formed, the median vascular bundle could still be found at the corner of the ovary and some small bundles could also be observed. Toward the basal of the flower, the sizeable lateral vascular bundles unite in the center of the ovary and form a star-shaped central vascular bundle (Figure 7C5). The median bundle will also merge with the central bundle. Toward the base of the flower, the star-shaped bundle will turn into a circular shape (Figure 7A4-A6) and the transport bundle in the pedicellus will end in a siphonostele form (Figure 7C6).

A. S-morph



B. M-morph



C. L-morp

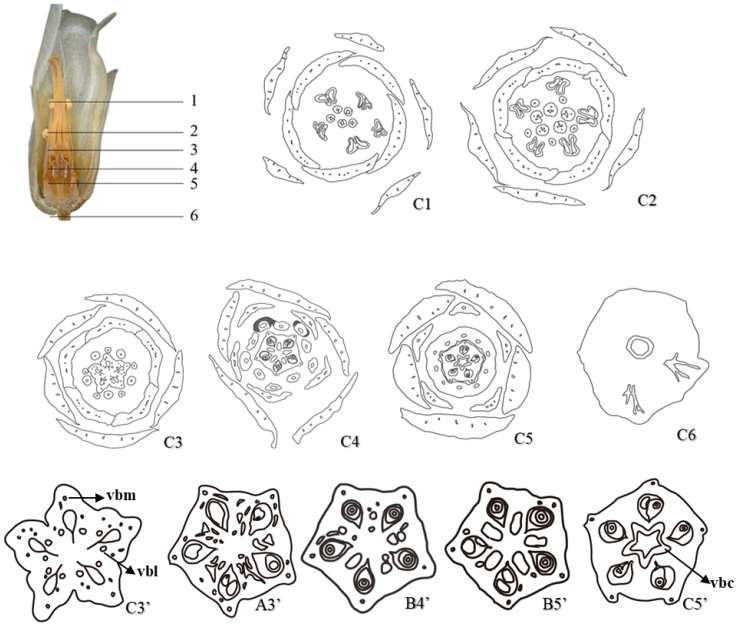


Figure 7. Floral vascularization of *A. dolichocarpa*. (A) S-morph, (B) M-morph, and (C) L-morph. (A1) Slide on short anther dan filament level, (B2, C2) Slides on short anther, filament, and stylus level, (A2, B3, C3) slides on the top of the ovary level, (A3, B4, C4) slides on the middle ovary level, (A4, B5, C5) slides on the low part of the ovary level, (A5) slide on flower receptacle level, (A6, B6, C6) slides on flower pedicel level. (C3'- C5': vascularization in ovary). vb: vascular bundle, vbm: median vascular bundle, vbl: lateral vascular bundle, vbc: central vascular bundle.

CONCLUSION

Averrhoa dolichocarpa was proven tristyly by finding three types of flowers with different arrangements between stigma and anther heights. The flower structure of the three different flower morphs is generally similar. The flower structure of *A. dolichocarpa* follows the general structure of *Averrhoa* and Oxalidaceae. The results of this study provide new information on the anatomy of *A. dolichocarpa* that has not been studied before.

AUTHORS CONTRIBUTION

The description of the author's contributions is listed: T.Y.I.W contributes to the research design, morphometry and anatomical study, and manuscript writing. S.K.S contributes to research concepts, morphometry study, data analysis, and manuscript writing. I.P.A contributes to sample collections and review of the manuscript. All of the authors are responsible for the content writing in this manuscript.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

REFERENCES

Barrett, S.C.H., 1992. Heterostylous genetic polymorphisms: model systems for evolutionary analysis. In Barett, S.C.H. (Ed.), *Evolution and Function of Heterostyly*. New York, USA: Springer-Verlag Berlin Heidelberg. pp.1-24.

Barrett, S.C.H., 1993. The evolutionary biology of tristyly. In: Futuyma, D.J., Antonovics, J. (Eds), *Oxford surveys in evolutionary biology*, Vol. 9. Oxford, UK): Oxford University Press, pp.283–326.

Barrett, S.C.H., 2002. The evolution of plant sexual diversity. *Nature Reviews Genetics*, *3*(4), pp.274–284. doi: 10.1038/nrg776

Barrett, S.C.H. & Harder, L.D., 2017. The ecology of mating and its evolutionary consequences in seed plants. *Annual Review of Ecology, Evolution, and Systematics*, *48*(1), pp.135–157. doi: 10.1146/annurev-ecolsys-110316-023021

Charlesworth, D. & Willis, J.H., 2009. The genetics of inbreeding depression. *Nature Review Genetics*, 10, pp.783–796. doi: 10.1038/nrg2664.

Cocucci, A.A., 2004. Oxalidaceae. In Kubitzki, K. (Ed). *The Families Genera Of Vascular Plants Volume VI*. New York, USA: Springer-Verlag Berlin Heidelberg. pp.285-290.

Darwin, C., 1877. *Different Forms of Flowers on Plants of The Same Species*. London, UK: John Mueray, Albemarle Street. doi: 10.1017/CBO9781107415324.004

Estelita-Teixeira, M.E., 1980. Floral vascularization in Oxalidaceae. *Boletim de Botanica da Universidade de Sao Paulo*, 8, pp.75-85.

Kapsah, Dorly, & Astuti, I.P., 2016. Morfologi dan viabilitas polen pada dua spesies belimbing hutan (*Averrhoa dolichocarpa* dan *A. leucopetala*). *Buletin Kebun Raya*, 19(2), pp.79–90.

Lloyd, D.G. & Webb, C.J., 1992. *The evolution of heterostyly*. In Barrett S.C.H., (Ed.), *Evolution and Function of Heterostyly*. Heidelberg, Berlin: Springer-Verlag, pp. 151–178.

Matthews, M.L. & Endress, P., 2002. Comparative floral structure and systematics in Oxalidales (Oxalidaceae, Connaraceae, Brunelliaceae, Cephalotaceae, Cunoniaceae, Elaeocarpaceae, Tremandraceae). *Botanical Journal of the Linnean Society*, *140*(4), pp.321–381. doi: 10.1046/j.1095-8339.2002.00105.x

Rugayah & Sunarti, S., 2008. Two new wild species of *Averrhoa* (Oxalidaceae) from Indonesia. *Reinwardtia*, 12(4), pp.325–331.

Rosenfeldt, S & Galati, B.G., 2009. The structure of the stigma and the style of *Oxalis* spp. (Oxalidaceae). *Journal of the Torrey Botanical Society,* 136 (1), pp. 33–45.

Sass, J.E., 1951. *Botanical Microtechnique 2nd edition*. Iowa, USA: The IOWA State College Press.

Sauquet, H. et al., 2017. The ancestral flower of angiosperms and its early diversification. *Nature Communications* 8:16047. doi: 10.1038/ncomms16047

Soumya, S.L. & Nair, B.R., 2013. Floral morphological features and variability in two species of *Averrhoa* L. (Oxalidaceae). *The International Journal of Plant Reproductive Biology*, 5(2), pp. 200–209.

Thompson, J.D. et al., 1996. Tristyly in the endangered Mascarene Island endemic *Hugonia serrata* (Linaceae). *American Journal of Botany*, 83, pp. 1160–1167. doi: 10.2307/2446199.

Veldkamp, J.F., 1967. A revision of *Sarcotheca* BL. and *Dapania* Korth. (Oxalidaceae). *Blumea,* 20 (2), pp. 519–543.

Veldkamp, J.F., 1971. Oxalidaceae. In van Steenis, C.G.G.J. (Ed), *Flora Malesiana series I Vol 7*. Jakarta, ID: Noordhoff-Kolff. pp. 151–178.

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Research Article

The Gastrointestinal Parasites in Habituated Group of Sulawesi Black-crested Macaque (*Macaca nigra*) in Tangkoko, North Sulawesi

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ABSTRACT
The Sulawesi black-crested macaque (*Macaca nigra*) is an endemic primate of North Sulawesi that is categorized as critically endangered (IUCN 2015). Endoparasite contributes to the decline of *M.nigra*. Therefore, this study aims to determine the prevalence of endoparasites in the Sulawesi black-crested macaque (*M. nigra*). We collected 80 fresh fecal samples representing all sex from the two habituated groups. We analyzed them using the direct examination technique (0.9% NaCl, iodine, methylene blue) and flotation technique with the modified McMaster test. A total of 15 endoparasite taxa were recorded and 78 of 80 samples were infected with at least one or several endoparasite taxa. Around 93.75% (75/80) samples were positive for protozoa (*Balantidium* sp., *Entamoeba* sp., *Giardia* sp., and *Isospora* sp.) and 88.75% (71/80) samples were positive for helminths (*Ancylostoma* sp., *Strongyloides* sp., *Haemonchus* sp., *Trichuris* sp., *Trichostrongylus* sp., *Ascarid* sp., *Diphyllobothrium* sp., *Echinococcus* sp., *Hymenolepis* sp., *Schistosoma japonicum* and *Schistosoma mekongi*). The abundance of protozoa was higher than helminth, although the number of helminth taxon (11) was higher. The average temperature and monthly rainfall did not affect the number of endoparasites (EPG). The prevalence was higher in females than males due to different social styles; female crested macaques are more tolerant than males. The group with a larger number of individuals had a higher prevalence of endoparasites. These results confirm the presence and high diversity of gastrointestinal endoparasites in *M. nigra*, which can help to understand transmission dynamics and zoonotic potential, as well as to consider conservation policies.

INTRODUCTION
The Sulawesi black-crested macaque (*Macaca nigra*) is an endemic primate of North Sulawesi that is categorized as critically endangered (IUCN 2015). The highest population is in Tangkoko with a density of 15 individuals/km^2 (Maneasa et al. 2021). Due to anthropogenic pressures such as hunting and habitat fragmentation, their population has decreased due to the loss of their natural habitat (O'Brien & Kinnaird 1997; Melfi 2010). Humans also can reduce the primate population density by threatening the primate's health

through direct or indirect pathogen transmission due to their close phylogenetic proximity (Wolfe et al. 2007).

Humans contact *M. nigra* when they keep them as a pet or when humans enter their habitat to engage in illegal activities (Palacios et al. 2012). Protozoa and nematodes have been found in eight *Macaca* species in Sulawesi that are kept as pets by humans (Jones-Engel et al. 2004). Parasite transmission is also influenced by several factors such as climate, habitat type, and sex of the host (Benavides et al. 2012; Chapman et al. 2006). Female and male primates have behavioral differences (Duboscq et al. 2013) that may influence the probability of parasite encounter as the previous study showed that climate and home range also affect the presence of endoparasites (Wenz-Mücke et al. 2013; Boundenga et al. 2018).

As an endangered species, they become vulnerable to many threats including zoonotic disease. However, there have been no studies that investigate the presence of endoparasites in habituated *M. nigra* by biological and environmental factors. The information obtained can help to understand the parasite-host dynamic to avoid the pathogen transmission that can lead to the *M. nigra* population decline. Therefore, this study aims to analyze the prevalence of gastrointestinal parasites from different genders and groups through different seasons to fill the knowledge gap.

MATERIALS AND METHODS

Materials

Fecal samples were collected from April 2018 to March 2019 in the Tangkoko, Bitung, North Sulawesi, Indonesia (1N 32'39", 125E 12'42"). A total of 80 fecal samples were collected every month during wet and dry seasons from two habituated groups (R1, n= 120; PB1B, n = 79). The samples were collected immediately after defecation and sprayed with 10% formalin (Gillespie 2006). Both groups are habituated and individually identifiable. The collection of environmental data in the form of temperature and rainfall was carried out every day at the same time using a thermometer and ombrometer.

Methods

Endoparasite Examination and analysis

The examination and identification of parasites were carried out from February to November 2021 at the Laboratory of Biosystematics and Animal Ecology, Department of Biology, Bogor Agricultural University. Fecal samples were examined using two methods (direct examination and flotation with the modified McMaster test) to maximize the detection of all possible endoparasites.

Direct examination: A small amount or about two milligrams of fecal for each sample was placed in three different object glasses. Each object glass was given 1-2 drops of a different solution (0.9% NaCl, iodine, and methylene blue) then mixed evenly before being covered with a coverslip. The

slide was observed under a microscope with 10x and 40x magnification (Gillespie 2006).

Flotation method: Two grams of fecal sample were mixed with 28 ml of saturated salt solution. The precipitate was then pipetted into the *McMaster* glass until the two chambers are filled. The sample was then allowed to stand for five minutes before examining the number of eggs using a microscope with 10x and 40x magnifications. A saturated salt solution was obtained by adding 400 g of NaCl into 1.000 mL to get a saturated salt solution with a concentration of 40% and SG 1.18, which was measured using a hydrometer (WHO 2019).

The eggs/endoparasite cysts found were then identified through photos taken with 10x and 40x magnifications using a camera-embedded microscope Olympus CX31LEDRFS1. The identification was based on morphological characteristics of eggs/cysts such as size, shape, color, stage of development, and other unique characteristics of each species according to Chitwood et al. (1950), Cuomo et al. (2009), WHO (2019), and Zajac and Conboy (2012).

Calculation of Endoparasites Prevalence and Egg per gram (EPG)

Parasite prevalence was calculated by dividing the number of each sample with one or more endoparasites by the total number of samples examined and written in percentage (McKenna & Dohoo 2006), using the following formula:

$$\text{Prevalence} = \frac{\text{The number of each sample with one or several parasites}}{\text{The number of all samples examined}} \chi\ 100$$

The quantitative examination of gastrointestinal parasites was performed by flotation with a modified *McMaster* test. Following Zajac and Conboy (2012), the eggs/cysts found in both chambers within the grid of the *McMaster* glass were counted and multiplied by 50 to obtain the number of each parasite per gram of feces (EPG) and estimate the infection rate in each host (Thienpont et al. 2003).

Data Analysis

The significance of all parasitic taxa prevalence was determined using Kruskal-Wallis and the prevalence of endoparasites between sexes and different groups were compared using the Chi-square test (χ^2). We computed the parasite abundance of each month through a year with monthly rainfall and temperature using the Pearson and Spearman correlation. The whole analysis was carried out using the R program (R Core team 2018) with a confidence level of 0.05.

RESULTS AND DISCUSSION

Diversity and Prevalence of Endoparasites

A total of 15 endoparasite taxa were recovered from the stool samples. Almost all of the samples (78/80) were infected at least with one

endoparasite taxon, which are nematodes (A1-F1), cestodes (A2-C2), trematodes (A3-B3), and protozoa (A4-D4) (Figure 1). Compared to data from the previous studies (Jonas & Melfi, in Hilser et al. 2013; Jones-Engel et al. 2004), to our knowledge, this is the first report of *Diphyllobothrium* sp., *Echinococcus* sp., *Schistosoma mekongi*, *Giardia* sp., and *Isospora* sp. found in *Macaca nigra*.

Primates are generally contaminated with gastrointestinal endoparasites through direct transmission from the soil, water, and zoonotic links in their natural habitat (Klaus et al. 2017), Invasion then occurs when the host ingests something that has been contaminated with endoparasite eggs (Cuomo et al. 2009). The Sulawesi black-crested macaque spends 60% of their daily activity on the ground looking for food than on trees, making them a semiterrestrial primate (O'Brien & Kinnaird 1997). The prevalence of nematodes in terrestrial primates was higher than in arboreal primates (Huffman et al. 2013).

Statistical analysis showed a significant difference between the total prevalence of all endoparasite species found (X^2 = 422.14, df = 14, p-value <2.2e-16). The protozoa group had the highest prevalence of 93.75% (75/80), followed by nematodes 86.25% (69/80), cestodes 26.25% (21/80), and trematodes 26.25% (21/80). This result shows that protozoan parasites had higher prevalence and abundance, in which *Balantidium* sp. and *Entamoeba* sp. were responsible for most of the infection (Table 1). In contrast, *Trichuris* sp. and *Diphyllobothrium* sp. from the worm group was not found in the McMaster test. We can assume that the abundance of these parasites was low.

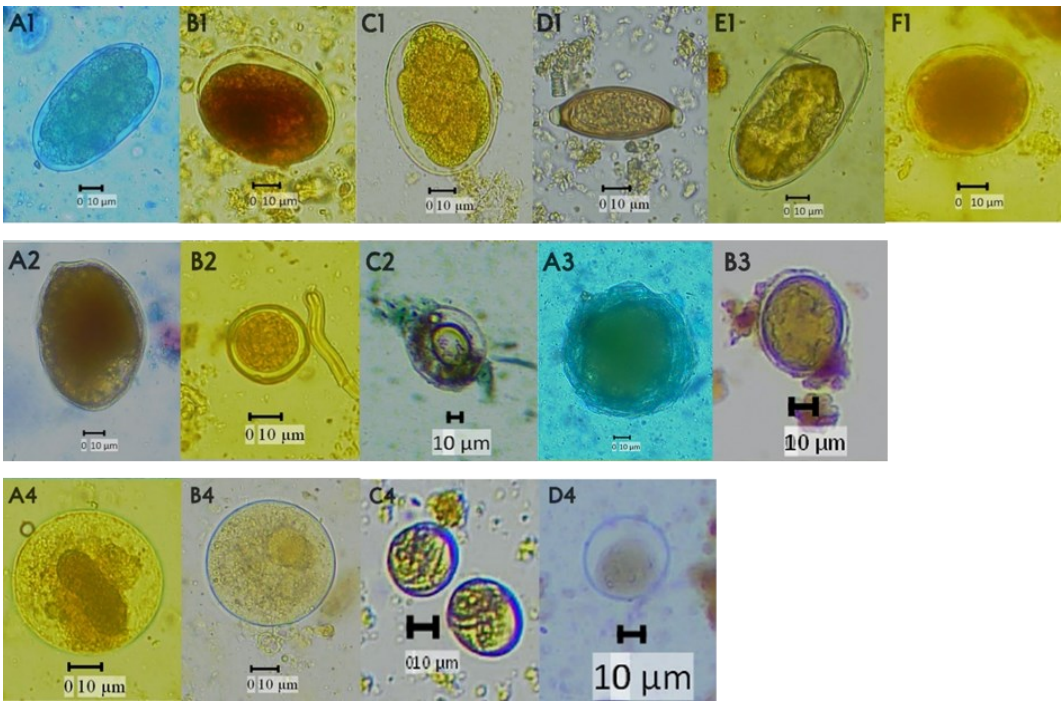

Figure 1. Endoparasites found in the fecal of *M. nigra* using saline, iodine, methylene blue and flotation methods. A1. *Ancylostoma* sp., B1. *Strongyloides* sp., C1. *Haemonchus* sp., D1. *Trichuris* sp., E1. *Trichostrongylus* sp., F1. *Ascarid* sp., A2. *Diphyllobothrium* sp., B2. *Echinococcus* sp., C2. *Hymenolepis* sp., A3. *Schistosoma japonicum*, B3. *Schistosoma mekongi*, A4. *Balantidium* sp., B4. *Entamoeba* sp., C4. *Giardia* sp. and D4. *Isospora* sp.

Table 1. The endoparasites prevalence and infection state of Sulawesi black-crested macaque.

No.	Endoparasite taxon	Number of positive samples	Prevalence (%)	*Mean EPG±SE	Infection
	Nematodes				
1	*Ancylostoma* sp.	44	55	62.50±20.71	light
2	*Strongyloides* sp.	38	47.5	71.43±12.05	light
3	*Haemonchus* sp.	31	38.8	62.50±8.33	light
4	*Trichostrongylus* sp.	20	25	50.00±6.52	light
5	*Ascarid* sp.	43	53.8	40.00±23.02	light
6	*Trichuris* sp.	3	3.75	0.00±0.00	-
	Cestodes				
1	*Diphyllobothrium* sp.	7	8.75	0.00±0.00	-
2	*Hymenolepis* sp.	6	7.5	50.00±5.61	light
3	*Echinococcus* sp.	8	10	50.00±5.61	light
	Trematodes				
1	*Schistosoma japonicum*	11	13.8	50.00±6.52	light
2	*Schistosoma mekongi*	6	7.5	50.00±4.16	light
	Protozoa				
1	*Entamoeba* sp.	64	80	497.0±596.7	light
2	*Balantidium* sp.	66	82.5	1101±1411.9	moderate
3	*Giardia* sp.	8	10	75.00±17.54	light
4	*Isospora* sp.	4	5	50.00±11.23	light

*mean EPG is calculated using data from modified *McMaster* test only. Standard errors were shown in the table.

A previous study of eight macaque species in Sulawesi also showed that protozoan has a higher prevalence than nematodes (Jones-Engel. et al 2004). The high prevalence is probably because these amoebas have a small size, large feeding strategy, and are able to make cysts (Schuster & Ramirez-avila 2008). The high prevalence of the protozoan group may indicate that the water *Macaca nigra* drunk is contaminated since protozoa are water-based parasites (Finlay et al. 2013).

Endoparasite Prevalence Between Different Gender

The prevalence between male and female *M. nigra* was not significant ($p = 0.33$). For both sexes, *Balantidium* sp. and *Entamoeba* sp. have a high prevalence (Table 2). For other taxa, females had a slightly higher prevalence than males, especially for helminths, including *Strongyloides* sp. (50 vs 42,5), *Trichostrongylus* sp. (32.5 vs 20), *Ascarid* sp. (62.5 vs 42,5), *Trichuris* sp. (5 vs 2.5), *Diphyllobothrium* sp. (12.5 vs 7.5), *Hymenolepis* sp. (10 vs 5), *Schistosoma japonicum* (15 vs 12.5), and *Giardia* sp. (12.5 vs 7.5).

This result may be due to the different behavior between female and male *Macaca nigra* including social style and diet. The females are engaged in physical contact such as grooming and playing with others more often than males (Sueur et al. 2011; Duboscq et al. 2013). Furthermore, the matrilineal system that *M. nigra* adopt makes females accompanied by an infant for most of their daily activity. The degree of second-hand contact then increases via their offspring (Mul et al. 2007). Meanwhile, males *M. nigra* rarely make physical contact because they prefer to rest more (O'Brien & Kinnaird 1997).

Regarding dietary behavior, *M. nigra* females significantly eat more often than the males measured by percentage of time feeding (3.9 vs 2.9) (O'Brien & Kinnaird 1997). Females primates also forage more than the males due to their duty as mothers that have to feed their offspring (Bicca-Marques 2003). Therefore, females *M. nigra* have a higher susceptibility to parasite infection. This aligns with previous research reporting that females are more likely to be infected with a higher parasite prevalence, more severe symptoms, and slower immune development than males (Escobedo et al. 2010; Mul et al. 2007).

Table 2. Endoparasite prevalence (helminth and protozoa) between gender.

Parasites taxa	Sex				Comparison test	
	Male		Female		X	P-Value
	N/40	P(%)	N/40	P(%)		
Helminth						
Ancylostoma sp.	22	55	22	55	0.81	0.36
Strongyloides sp.	17	42.5	21	50	0.20	0.65
Haemonchus sp.	18	45	13	35	0.46	0.49
Trichostrongylus sp.	8	20	12	32.5	1.03	0.30
Ascarid sp.	17	42.5	26	65	2.45	0.11
Trichuris sp.	1	2.5	2	5	0.00	1.00
Diphyllobothrium sp.	3	7.5	4	10	0.00	1.00
Hymenolepis sp.	2	5	4	10	0.18	0.67
Echinococcus sp.	5	12.5	3	7.5	0.01	0.70
S.japonicum	5	12.5	6	15	0.00	1.00
S.mekongi	4	10	2	5	0.18	0.67
Protozoa						
Giardia sp.	3	7.5	5	12.5	0.13	0.70
Entamoeba sp.	32	80	32	80	0.00	1.00
Balantidium sp.	34	85	32	80	0.08	0.76
Isospora sp.	3	7.5	1	2.5	0.26	0.60

*Notes: N = Number of samples; P = Prevalence.

Although females are more susceptible to infection, there are several types of parasites that are higher in males *M. nigra*. This is probably because males still have possible transmissions through food contamination and physical contact although they rarely occur, such as fighting and grooming (Engelhardt et al. 2017). Thus, transmission from others possibly happens during these contact. However, male and female primates generally still show the same response when they were infected with parasites, which is reducing activity and increasing duration of rest (Ghai et al. 2015).

Endoparasite Prevalence Between Group

The results show that *Balantidium* sp. has a higher prevalence among other taxa for both groups (87.5% and 77.5%). Meanwhile, for the other ten taxa,

the R1 group has a higher prevalence (Table 3). However, the significant differences in prevalence occur only for *Strongyloides* sp. (X2 = 12.80, df=1 p<0.0003), which is higher in the R1 group than in PB1B. The incidence in the R1 population may be related to the high contact rate with humans as intermediate hosts for some endoparasites as the home range of R1 is in the recreation areas of Tangkoko. It was reported by Saroyo (2010) that 53.8% of visitors come to the recreation area to see *Macaca nigra*.

The presence of humans in the primate's habitat can cause behavioral changes; they spend more time on the ground and eat more food from humans, this change is correlated with parasite infection (Wenz-Mücke et al. 2013). Human and nonhuman primates are known to share a wide range of gastrointestinal parasites because their phylogenetic proximity will increase the chance of pathogen's exchange (Wolfe et al. 2007). According to Jones-Engel et al. (2004) and Hasegawa et al. (1992), pet macaques have been infected with parasites commonly found in human populations in Sulawesi and the endoparasites burden were higher in pets than wild *M. nigra*. Thus, the endoparasites may be transmitted by other species or humans which live sympatrically with *Macaca nigra*.

Table 3. Endoparasite prevalence between different groups.

Endoparasites	Group				Comparison test	
	R1		PB1B		X2	P-value
	N/40	P(%)	N/40	P(%)		
Helminth						
Ancylostoma sp.	22	52.5	22	55	0.00	1.00
Strongyloides sp.	28	67.5	10	25	12.80	0.0003
Haemonchus sp.	12	32.5	19	47.5	1.30	0.25
Trichostrongylus sp	13	35	7	17.5	2.32	0.12
Ascarid sp.	24	60	19	47.5	0.45	0.50
Trichuris sp..	2	5	1	2.5	0.00	1.00
Diphyllobothrium sp.	5	12.5	2	5	0.62	0.42
Hymenolepis sp.	5	12.5	1	2.5	1.62	0.20
Echinococcus sp.	6	15	2	5	1.25	0.26
S.japonicum	6	15	5	12.5	0.00	1.00
S.mekongi	3	7.5	3	7.5	0.00	1.00
Protozoa						
Giardia sp.	2	5	6	15	1.25	0.26
Entamoeba sp.	34	85	30	75	0.70	0.40
Balantidium sp.	35	87.5	31	77.5	0.77	0.37
Isospora sp.	2	5	2	5	0.00	1.00

*Notes: N = Number of samples; P = Prevalence.

Moreover, group size also plays an important role in parasite exchange due to the mobility of the host and the transmission route of the parasite (Patterson & Ruckstuhl 2013). The R1 group has a higher population density than the other groups, making them have a wider home range (Rismayanti

2020). The largest group will travel further but it includes more fragmented areas with human density and spends longer time to forage than the smaller group does (O'brien & Kinnaird 1997). Thus, the chance of the R1 group encountering more parasites from the inter-intraspecies transmission is greater than the PB1B group which lives in the primary forest. Previous studies have reported that the diversity and prevalence of endoparasites appear to be higher in fragmented locations with a higher density of human appearance (Gillespie & Chapman 2008; Boundenga et al. 2018; Joesoef et al. 2018).

There was no significant difference in the endoparasites prevalence between both groups except *Strongyloides* sp. It is most likely because they still live in the same habitat and geographic area. In addition, groups R1 and PB1B were the groups that most often passed each other and shared the same sleeping tree more than other groups (Rismayanti 2020). Therefore, the diversity of endoparasites found in these two groups does not differ much. As Jones-Engel et al. (2004) reported, there are no significant differences in the endoparasite prevalence of Macaca sub-groups in the Sulawesi area. The biased parasitism is most likely found in primates exposed to different environmental conditions or different host-specific (Boundenga et al. 2018).

Climates Impact on Endoparasites Abundance

Pearson's correlation showed that rainfall was positively correlated with parasite abundance that invaded each host (r = 0.18), but it was not significant (t = 0.59868, df = 10, p-value = 0.562). Conversely, the monthly temperature negatively correlated (r = 0.46) with the abundance of endoparasites, but it was also not significant (S = 417.69, p-value = 0.132). The correlation value of 0.18 is very weak, while 0.46 is quite strong (Dancey et al. 2004). Although this is not significant, we can provide evidence on the correlation of average climate with endoparasite abundance; endoparasite abundance tends to increase when rainfall also increases (Figure 2) but tends to be low when temperatures are high (Figure 3).

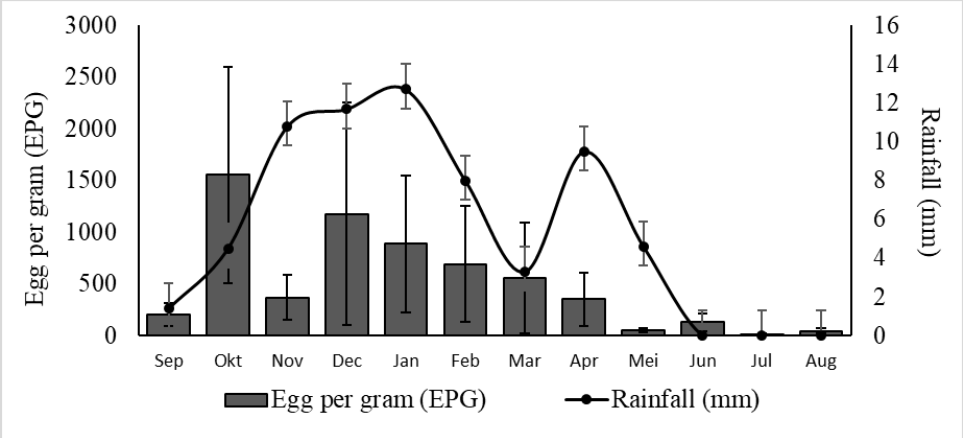

Figure 2. Average rainfall and EPG on each month. Error bars indicate standard errors.

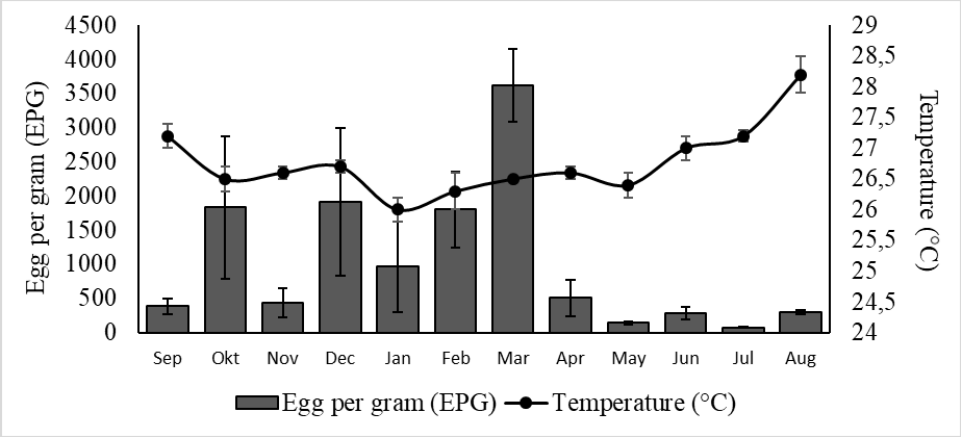

Figure 3. Average temperature and EPG on each month. Error bars indicate standard errors.

The highest parasite abundance occurred in October-March with the highest rainfall (12.2 mm) and the lowest temperature (26 °C). On the contrary, the lowest parasite abundance was in May-August with a lower rainfall range (4.6 - 0 mm) and a higher temperature (26.4 - 28.6 °C). Our finding is similar to the findings of previous studies which also showed a positive correlation between rainfall and parasite richness in primate populations (Benavides et al. 2012; Joesoef et al. 2018). Climate factors such as temperature and rainfall can temporally influence the presence of endoparasites. Some species of endoparasites have favorable environmental conditions to survive and to complete their life cycle (Cuomo et al 2009). A wet environmental condition during the rainy season supports the development of worm eggs and makes the rate of parasitic infection during this season increases (Joesoef et al. 2018).

As for the primates, the climate change in every season influences the availability and distribution of foods that will change the primate's daily ranges and activities (Hurtado et al. 2017). These changes include food composition, feeding intensity, and range of foraging movement. They eat fruit 4.6 times in the rainy season while in the dry season this number decreases to 3.7 times (O'Brien & Kinnaird 1997). Changes related to seasons will indirectly affect the possibility of *Macaca nigra* being infected by endoparasites. Even though the number of parasite's eggs in the rainy season is lower than in other seasons (Thienpont et al. 2003) because the feeding activity of Sulawesi black-crested monkeys increases, the possibility of being exposed to parasites through eating is still high.

Meanwhile, because of the high temperatures in the dry season, they will rest more and reduce their social activities (O'Brien & Kinnaird 1997). Even so, food scarcity in dry seasons will lead to host stress that causes immunosuppression and makes them vulnerable during this period; thus, the parasite load will increase (Chapman et al. 2006). Therefore, *M. nigra* still face the risk of being infected by endoparasites at every season because there is no clear pattern of the climate-gastrointestinal link mechanism.

CONCLUSION

This study reveals that there is an increase in the number of taxa and the prevalence of endoparasites found in *Macaca nigra* from Tangkoko, Bitung, North Sulawesi compared to previous studies. All of the taxa in this study have been found in other primate species including humans. This indicates that there is a potential zoonotic transfer between the two sympatric living species. There is no gender bias but the females have a slightly higher prevalence than the males. This, may be due to behavioral differences between the females and the males such as social style and diet, where the females eat and engage in social activities more often than males. The R1 group with more individuals had a higher prevalence of parasites than PBIB. This is probably because R1 travel more and have a wider home range, including fragmentation areas with human disturbance. The environmental factors show that rainfall is positively correlated, while temperature is negatively correlated to EPG.

AUTHORS CONTRIBUTION

S.A.M.W. contributed to designing the research concept, data examination, analyzed the data, and authored the manuscript. D.P.F and E.S contributed equally to this manuscript, developed the research concept, authored, reviewed, and approved the final manuscript.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

REFERENCES

Benavides, J.A. et al., 2012. From parasite encounter to infection: Multiple-scale drivers of parasite richness in a wild social primate population. *Am J Phys*, 147(1), pp.52–63. doi:10.1002/ajpa.21627.

Bicca-Marques, J.C., 2003. Sexual selection and the evolution of foraging behavior in male and female tamarins and marmosets. In *Sexual Selection and Reproductive Competition in Primates: New Perspectives and Directions*. Norman, OK: American Society of Primatologists, pp.455-475.

Boundenga, L. et al., 2018. Diversity and prevalence of gastrointestinal parasites in two wild Galago species in Gabon. *Infect Genet Evol*, 63(4), pp.249–256. doi:10.1016/j.meegid.2018.04.035.

Chapman, C.A. et al., 2006. Do food availability, parasitism, and stress have synergistic effects on red colobus populations living in forest fragments. *American journal of Physical Anthropology*, 131, pp.525-534. doi: 10.1002/ajpa.20477.

Chitwood, B.G., et al. 1950. *An introduction to nematology*. Baltimore, Md: Monumental Printing Company.

Cuomo, M., Noel L.B. & White, D.B., 2009. *Diagnosing Medical Parasites: A Public Health Officers Guide to Assisting Laboratory and Medical Officers*. Defense technical information center.

Dancey, C., Reidy, J. & Using S., 2004. *Statistics Without Maths for Psychology for Windows*. 4th ed. England: Pearson Education Limited.

Duboscq, J. et al., 2013. Social Tolerance in Wild Female Crested Macaques (*Macaca nigra*) in Tangkoko-Batuangus Nature Reserve, Sulawesi, Indonesia. *Am J Primatol,* 75(4), pp.361–375. doi: 10.1002/ajp.22114.

Engelhardt, A. et al., 2017. Highly polymorphic microsatellite markers for the assessment of male reproductive skew and genetic variation in critically endangered crested macaques (*Macaca nigra*). *Int J Primatol*, 38(4), pp.672 -691. doi: 10.1007/s10764-017-9973-x

Escobedo, G., Leon-Nava, M.D. & Morales-Montor, J., 2010. Sex Differences in Parasitic Infections : Beyond the Dogma of Female-Biased Resistance. *Sex Horm Immun to Infect*, pp.187–204. doi:10.1007/978-3-642-02155-8.

Finlay, B.J., Mary, Q. & Esteban, G., 2013. Freshwater protozoa : Biodiversity and ecological function Freshwater protozoa: biodiversity and ecological function. *Biodivers Conserv*, 7(9), pp.1163–1186. doi:10.1023/A.

Ghai, R.R. et al., 2015. Sickness behaviour associated with non-lethal infections in wild primates. *Proc R Soc B Biol Sci*, 282(1814) pp.1-8. doi:10.1098/rspb.2015.1436.

Gillespie, T.R., 2006. Noninvasive Assessment of Gastrointestinal Parasite Infections in Free-Ranging Primates. *Int J Primatology*, 27(4), pp.1129-1141. doi: 10.1007/s10764-006-9064-x.

Gillespie, T.R & Chapman, C.A., 2008. Forest fragmentation, the decline of an endangered primate, and changes in host-parasite interactions relative to an unfragmented forest. *Am J Primatol,* 70(3), pp.222–230. doi:10.1002/ajp.20475.

Hasegawa, H. et al., 1992. Intestinal parasitic infections in Likupang, North Sulawesi, Indonesia. *Southeast Asian J Trop Med Public Health*, 23(2), pp.219–227.

Hilser, H. et al., 2013. *Sulawesi Crested Black Macaque Macaca nigra Species Action Plan: Draft 1. Selamatkan Yaki*. Pacific Institute, Manado, Indonesia.

Huffman, M.A., 2013. Preliminary survey of the distribution of four potentially zoonotic parasite species among primates in Sri Lanka. *J Natl Sci Found Sri Lanka*, 41(4), pp.319–326. doi:10.4038/ jnsfsr.v41i4.6246.

Hurtado, R.R. et al., 2017. Primates adjust movement strategies due to changing food availability. *Behavioral ecology*, 29(2), pp.369-376. doi: 10.1093/beheco/arx176.

IUCN, 2015. IUCN Red List of Threatened Species. Version 2011, viewed 23 October 2020, from http://www.iucnredlist.org

Joesoef, J.A. et al., 2018. Keragaman Endoparasit pada *Macaca fascicularis* dan Potensi Zoonotiknya dengan Cuaca Berbeda di Kota Kupang. *J Vet*, 19 (36), pp.451–459. doi: 10.19087/jveteriner.2018.19.4.451.

Jones-Engel, L. et al., 2004. Prevalence of enteric parasites in pet macaques in Sulawesi, Indonesia. *Am J Primatol*, 62(2), pp.1–82. doi: 10.1002/ ajp.20008.

Klaus, A. et al., 2017. International Journal for Parasitology: Parasites and Wildlife Co-infection patterns of intestinal parasites in arboreal primates proboscis monkeys (*Nasalis larvatus*) in Borneo. *International Journal for Parasitology*: *Parasites and Wildlife*, 6(3), pp.320-329. doi: 10.1016/j.ijppaw.2017.09.005.

Maneasa, E., Simandjuntak, S. & Semuel M.Y., 2021. Density of the crested black macaque (*Macaca nigra*) and habitat quality in Tangkoko, Bitung, North Sulawesi. *Jurnal Biologi Tropis*, 21(2), pp.587-592. doi: 10.29303/ jbt.v21i2.2789

McKenna, S.L. & Dohoo, I.R., 2006. Using and interpreting diagnostic tests. *Vet Clin North Am Food Anim Pract*, 22(1), pp.195-205. doi: 10.1016/ j.cvfa.2005.12.006

Melfi, V., 2010. Selamatkan Yaki! Conservation of Sulawesi Crested Black Macaques Macaca nigra. In *Gursky S., Supriatna J. (eds) Indonesian Primates. Developments in Primatology: Progress and Prospects*. Springer, New York, NY.

Mul, I.F. et al., 2007. Intestinal parasites of free-ranging, semi-captive, and captive *Pongo abelii* in Sumatra, Indonesia. *Int J Primatol,* 28(2), pp.407–420.doi:10.1007/s10764-007-9119-7.

O'Brien, T.G. & Kinnaird, M.F., 1997. Behavior, diet, and movements of the Sulawesi crested black macaque (*Macaca nigra*). *Int J Primatol*, 18(3), pp.321–351. doi:10.1023/A:1026330332061.

Palacios, J.F.G. et al., 2012. Status of, and conservation recommendations for, the critically endangered crested black macaque *Macaca nigra* in Tangkoko, Indonesia. *Oryx*. 46(2), pp.290–297. doi: 10.1017/ S0030605311000160.

Patterson, J.E.H. & Ruckstuhl, K.E., 2013. Parasite infection and host group size: A meta-analytical review. *Parasitology*, 140(7), pp.803–813. doi:10.1017/S0031182012002259.

R Core Team, 2018. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.

Rismayanti, 2020. Intergroup Competition For Sleeping Sites And Their Function In Home Range And Group Defense In Crested Black Macaques (*Macaca nigra*). Institut Pertanian Bogor.

Saroyo, K.R., 2010. Pemetaan Distribusi dan Densitas Monyet Hitam Sulawesi (M*acaca nigra*) di Sulawesi Utara. *Biosfera*, 27(3), pp.133-139. doi: 10.20884/1.mib.2010.27.3.203.

Schuster, F.L. & Ramirez-avila, L., 2008. Current World Status of *Balantidium coli*. *Clin Microbiol Rev*, 21(4), pp.626–638. doi: 10.1128/CMR.00021-08.

Sueur, C. et al., 2011. A comparative network analysis of social style in macaques. *Anim Behav*, 82(4), pp.845–852. doi:10.1016/ j.anbehav.2011.07.020.

Thienpont, D., Rochette, F. & Vanparijs, O.F.J., 2003. *Diagnosing Helminthiasis by Coprological Examination*. *3rd ed*. Beerse, Belgium: Janssen Animal Health.

Wenz-Mücke, A. et al., 2013. Human contact influences the foraging behaviour and parasite community in long-tailed macaques. *Parasitology*, 140(6), pp.709–718. doi: 10.1017/S003118201200203X.

WHO, 2019. *Bench Aids For the Diagnosis of Intestinal. 2nd ed*. Geneva: World Health Organization.

Wolfe, N.D., Dunavan, C.P. & Diamond, J., 2007. Origins of major human infectious diseases. *Nature*, 447(7142), pp.279–283. doi: 10.1038/ nature05775.

Zajac, A. & Conboy, G., 2012. *Veterinary Clinical Parasitology*. 8th ed. USA: Wiley-blackwell.

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Research Article

Molecular Mechanism of Inhibition of Cell Proliferation: An In Silico Study of the Active Compounds in *Curcuma longa* as an Anticancer

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ABSTRACT
Cancer is one of the death causes in the world. Many plants act as anticancer, one of them is *Curcuma longa*. The purpose of this study was to analyze the molecular mechanism of compounds in *Curcuma longa* as an anticancer using in silico. These research methods included exploration of the active compounds of *Curcuma longa* plants, prediction of their activity, human intestinal absorption test, test of Lipinski's rule of five, molecular docking, and interactions of receptor with compounds as well as signaling pathways. The results showed that *Curcuma longa* had 20 compounds that have the potential as an anticancer. As many as 5 of the 20 active compounds, namely α-curcumene, curcumenol, curcumin, curcumin II, and curcumin III had a value of Pa > 0.3 and HIA above 80%. The results of molecular docking of α-curcumene, curcumenol, curcumin, curcumin II, and curcumin III compounds with protein receptors of VEGFR-2, EGFR, and FGFR-1 showed ΔG_{bind} values of -5.0 to -7.5 kcal/mol. The compound in *Curcuma longa* that had the most effective activity as an anticancer was curcumin with a ΔG_{bind} value of -7.5 kcal/mol at the FGFR-1 receptor. Curcumin molecular mechanism as antiproliferative was revealed computationally through inhibition of the PI3K/AKT/mTOR pathway.

INTRODUCTION
Cancer is one of the leading causes of human death in the world, characterized by the presence of some cells that grow uncontrollably and spread to other parts of the body (American Cancer Society 2016). Understanding the molecular changes in cancer development is one of the key factors to prevent and treat cancer and underlies the development of anticancer drugs (Hamzehzadeh et al. 2018; Tomeh et al. 2019). Various studies were conducted to find new drugs that have the potential as anticancer, one of them is turmeric (*Curcuma longa* Linn.).

Curcuma longa Linn. belongs to the Zingiberaceae family, and is a native plant of Asia, especially in India, Indonesia, and China (Abdurrahman 2019). Turmeric phytochemical studies show that turmeric contains curcuminoids

and essential oils as its main components. Curcumin and its derivatives have great attention in the last decade because of their anticancer activity (Nagahama et al. 2016; Chao et al. 2018). Curcumin is safe to use in animals and humans because curcumin can still be accepted by the body though in very high doses. However, curcumin has low bioavailability and low water solubility (Anisa et al. 2020). The properties of curcumin and its derivatives in cancer treatment led to the tyrosine kinase signaling pathway because curcumin inhibits receptor tyrosine kinases (RTK). The changes in genetic and gene expression of tyrosine kinase are responsible for the loss of cell growth control and oncogenic properties that appear in cancer (Golonko et al. 2019).

The first member of the receptor tyrosine kinases (RTK) superfamily is the epidermal growth factor receptor (EGFR). Other RTK proteins in humans, namely vascular endothelial growth factor receptor (VEGFR), fibroblast growth factor receptor (FGFR) are key to the activity of the PI3K/AKT/mTOR signaling pathway (Rahimi 2017; Rawluk & Waller 2018; Golonko et al. 2019; Astolfi et al. 2020), which regulates cell proliferation, survival, and differentiation (Papadimitrakopoulou 2012; Hamzehzadeh et al. 2018). This study aimed to analyze the molecular mechanism of compounds in *Curcuma longa* as an anticancer using in silico.

MATERIALS AND METHODS

Materials

The materials used in this study were ligands (compounds in *Curcuma longa*) obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/), and receptors obtained from the PDB database (Protein Data Bank) (https: //www.rcsb.org/).

Methods

This study was an exploratory descriptive study which included exploration of the active compound of *Curcuma longa*, its activity prediction, human intestinal absorption test, Lipinski's rule of five test, molecular docking, and interaction of receptor with compounds and signaling pathways.

Collection of active compounds in *Curcuma longa*

The active compound in *Curcuma longa* was collected from Dr. Duke's Phytochemical and Ethnobotanical Databases by Dr. Jim Duke from the Agricultural Research Service/USDA (Ezealisiji & Awucha 2020), which can be accessed via (https://Phytochem.nal.usda.gov/) using keywords of *Curcuma longa*. SMILES compounds collection was carried out using PubChem via (https://pubchem. ncbi.nlm.nih.gov/). SMILES is used to predict the bioactivity of a compound.

Prediction of compound activity in *Curcuma longa* using test of PASS (prediction of activity spectra for substances)

The PASS Online test in this study was used to predict the possibility of

compound activity in preventing, inhibiting, and killing the growth and spread of cancer cells based on activities of pharmacological, biological, and ADME (absorption, distribution, metabolism, and excretion). PASS Online is a computer-based program to predict the biological activity of a compound (Jamkhande et al. 2014). The PASS test was carried out online via http://www.pharmaexpert.ru/passonline/. The results of the PASS test for each compound showed Pa (Potential Activity) and Pi (Potential Inhibitory) values based on the activity similarity of a compound structure with the drug compound. The PASS test results were analyzed and grouped based on the Pa value.

Test of HIA (human intestinal absorption) and toxicity hazard

The HIA test was carried out to predict the absorption ability of *Curcuma longa* compounds on intestinal wall. This test was carried out online using admetSAR (Moon et al. 2017) via http://lmmd.ecust.edu.cn/admetsar2/ by entering the SMILES compounds in the search column and running until the data was generated. The results of the HIA test were analyzed based on the percent value (%), with values of high (70-100%), medium (20-70%), and low (0-20%). Toxicity hazard (when administrated orally) was predicted using Toxtree. It was categorized into three classes, that are low (class I) which indicates efficient mode of metabolism, intermediate (class II) which possess less innocuous than class I, and high (class III) which indicates significant toxicity or had reactive functional group so the dose was crucial for oral use.

Test of Lipinski's rule of five

Test of Lipinski's rule of five is to determine the ability of compounds to penetrate cell membranes and reach target receptors (Jadhav et al. 2015). According to this law, a drug compound must comply with two or more absolute values of Lipinski's rules consisting of (1) a molecular weight is less than 500 g/mol, (2) a log P-value is less than 5, (3) a value of Hydrogen Bond Donors (HBD) is not more than 5, (4) the value of the Hydrogen Bond Acceptor (HBA) is not more than 10, and (5) the value of the Molar refractivity should be between 40-130 (Syahputra et al. 2014). Test Lipinski's rule of five was carried out through admetSAR on the http://lmmd.ecust.edu.cn/admetsar2/. The results of Lipinski's rule of five were analyzed by grouping the test ligand compounds that fulfilled 4 or 5 criteria of the rule of five.

Molecular docking and visualization

The structure of *Curcuma longa* compound and anticancer drugs downloaded through PubChem (https://pubchem.ncbi.nlm.nih.gov/) in 3D, saved in SDF format (*sdf) (Dallakyan & Olson 2014). The use of anticancer drugs of sunitinib, lapatinib, and gefitinib were selected based on the ability of the drug mechanism to act on a protein receptor through the drug bank (https://go.drugbank.com/). The receptor structure was downloaded through the protein data bank (https://www.rcsb.org/). The downloaded

receptor structure data was prepared using the discovery studio visualizer application. Furthermore, the removal of water molecules and ligands on protein macromolecules that were not needed was carried out. The structure formed was saved in .pdb format, which then can be used in the docking process.

Molecular docking between ligands (*sdf) and protein macromolecules (*pdb) was performed using Pyrx 0.8 software and analyzed by AutoDock Vina which functions as an anchor. Vina AutoDock is found on Pyrx which is included in the Vina Wizard section (Dallakyan & Olson 2014). In addition to the active compounds and target proteins, molecular docking was also carried out on the drug control compounds to determine the similarity of interactions between the test compounds and the control compounds. The molecular docking results were binding affinity scores, or bond energy values (ΔG_{bind}) and the interaction results were visualized into 2D and 3D structures using the Discovery Studio Visualizer. The results of molecular docking were analyzed by grouping the ligands based on the value of the bond energy (ΔG_{bind}), the type of bond formed, and the analysis of signaling mechanism.

Receptor interaction with compounds and signaling pathways

The interaction of three receptors (VEGFR-2, EGFR, and FGFR-1) with *Curcuma longa* compounds was evaluated to know the relationship between receptors and compounds in analyzing signaling pathways or biological pathways in cancer by their proteins. This interaction can be analyzed using STRING on page https://string-db.org/ (Franceschini et al. 2013). The next step was to analyze the cancer inhibition signaling pathway using the KEGG pathway.

RESULTS AND DISCUSSION

Predictions of active compounds from *Curcuma longa* based on Dr. Duke's phytochemical and ethnobotanical databases obtained 267 compounds with 658 activities. From 267 compounds, 20 compounds that have activities of anticarcinogenic, anticancer, and anti-tumor were found (Table 1).

This database (Dr. Duke's phytochemical and ethnobotanical databases) is often used to predict the activity of a test compound. Maduabuchi and Awucha (2020) used this database to predict the activity of *Pterocarpus mildbraedii* compounds as medicine for malaria and digestive disorders. Anand and Gokulakhrisna (2014) used Dr. Duke's databases in a study related to *Hybanthus enneaspermus* plant activity, especially the characteristics of bioactive compounds in ethanol extracts.

A total of 20 phytochemical compounds of *Curcuma longa* were obtained, based on the results of PASS Online screening, 5 compounds with anticarcinogenic activity were obtained, with a Pa value more than 0.3, namely curcumene, curcumenol, curcumin, curcumin II, and curcumin III (Table 2). According to Filimonov et al. (2014), the PASS test performs an analysis based on the relationship of the compound structure and its biological activi-

ty or SAR (Structure Activity Relationship).

Table 1. Collection of *Curcuma longa* compounds as anticancer.

No	Compound	Molecular Formula
1	Alpha-curcumene (Curcumene)	$C_{15}H_{22}$
2	Alpha-terpineol	$C_{10}H_{18}O$
3	Alpha-tocopherol	$C_{29}H_{50}O_2$
4	Ar- tumerone	$C_{15}H_{20}O$
5	Ascorbic acid	$C_6H_8O_6$
6	Beta carotene	$C_{40}H_{56}$
7	Beta sitosterol	$C_{29}H_{50}O$
8	Beta turmerone	$C_{15}H_{22}O$
9	Bis-desthoxycurcumin (Curcumin III)	$C_{19}H_{16}O_4$
10	Caryophyllene	$C_{15}H_{24}$
11	Curcumenol	$C_{15}H_{22}O_2$
12	Curcumin	$C_{21}H_{20}O_6$
13	Curcuminoid	$C_{21}H_{18}N_2O_6$
14	Curcumenol	$C_{15}H_{22}O_2$
15	Curcumol	$C_{15}H_{24}O_2$
16	Curdione	$C_{15}H_{24}O_2$
17	Demethoxycurcumin (Curcumin II)	$C_{20}H_{18}O_5$
18	Limonene	$C_{10}H_{16}$
19	Quercetin	$C_{15}H_{10}O_7$
20	Tetrahydrocurcumin	$C_{21}H_{24}O_6$

Curcumin, curcumin II, and curcumin III had anticancer activity (anticarcinogenic) in moderate criteria ($0.5 < Pa < 0.7$). Pa value indicated that the compound has probability to be active and in mid-range score is a good candidate for drug discovery. While curcumene and curcumenol compounds had low anticancer activity ($Pa < 0.5$) prediction. The greater the Pa value, the more possibility of the compound to block receptors in laboratory experiments. Nevertheless, compounds with low Pa value are not certain to have low activity, because there have not been many studies on these compounds (Pramely & Raj 2012; Filimonov et al. 2014; Ivanov et al. 2018).

Table 2. PASS online prediction results.

Compound	Value	
	Pa	Pi
α-Curcumene	0.357	0.039
Curcumenol	0.454	0.024
Curcumin	0.611	0.012
Curcumin II	0.645	0.011
Curcumin III	0.555	0.015

Comparative analysis of the Pa and Pi values of the five compounds in *Curcuma longa* showed that all of them had Pa value above 0.3 (Table 2). Only compounds with Pa value > 0.3 were categorized as good activity (Pramely & Raj 2012; Druzhilovskiy et al. 2016).

Prediction of compound absorption with parameters of HIA

Based on the results of the HIA (Human Intestinal Absorption) prediction,

the HIA values for α-curcumene, curcumenol, curcumin, curcumin II, curcumin III, sunitinib, gefitinib, and lapanitib were more than 84% (Table 3). The value obtained showed that anticancer drugs and these compounds can be well absorbed in the intestines because they had a value of more than 70%. According to Nerkar et al. (2012), if the predicted absorption of a compound is more than 70%, it can be stated that the intestines have a high ability to absorb these compounds and can reach their target receptors. The result of toxicity hazard also suggests that the compound has reactive functional group when orally administrated. For medical application the effective dose is crucial to be considered. All active compounds from *Curcuma longa* could be used as a treatment by considering all pharmacokinetics properties. Compounds in *Curcuma longa* are predicted to have the same speed as anticancer drugs to reach the target of cancer cell receptors in the body, thus blocking the receptors from binding and inhibiting the growth of cancer cells (Lazzeroni et al. 2012).

Table 3. Result of HIA prediction and toxicity hazard for oral use.

		Toxic Hazard		
Compounds	HIA (%)	Low (Class I)	Intermediate (Class I)	High (Class I)
α-Curcumene	98.87	○	-	-
Curcumenol	84.59	-	-	○
Curcumin	97.70	-	-	○
Curcumin II	97.70	-	-	○
Curcumin III	97.37	-	-	○
Sunitinib	97.60	-	-	○
Lapatinib	98.14	-	-	○
Gefitinib	99.37	-	-	○

Note: ○= categorized; - = Not categorized.

Prediction of compound potential using parameters of Lipinski's rule of five

When a compound is able to be absorbed in intestinal wall and enters the blood circulation properly, it will be distributed throughout the body and penetrate the cell membrane to reach its target receptor. Therefore, it is important to pay attention to factors related to pharmacology, because drug interactions will not occur if the compound can't reach its target (Jadhav et al. 2015).

Lipinski's prediction results showed that 5 compounds of *Curcuma longa* and 2 compounds of anticancer drugs (sunitinib and gefitinib) met all the criteria of the rule of five. Lapatinib did not meet the 3 criteria, namely a molecular mass of more than 500 Da, a LogP of more than 5, and a molar refractivity of more than 130 (Table 4). However, lapatinib still complied with the Lipinski's rule of five, because it still had 2 criteria (Jadhav et al. 2015).

Table 4. Results of Lipinski's rule of five test for ligand compounds.

Ligand	Molecular Mass (Da)	H- Donor	H- Acceptor	LogP	Molar Refractivity
α-Curcumene	202	0	0	4.84	69.55
Curcumenol	234	1	2	3.18	69.25
Curcumin	368	2	6	3.37	102.80
Curcumin II	338	2	5	3.14	96.31
Curcumin III	308	2	4	3.35	89.82
Sunitinib	398	3	3	3.33	116.31
Lapatinib	581 *	2	8	6.14 *	153.88 *
Gefitinib	446	1	7	4.28	121.66

*Value does not meet requirements of the rule of five.

According to Syahputra et al. (2014), compounds with molecular mass of more than 500 will be difficult to penetrate cell membrane, because they do not diffuse into cell. Conversely, compounds with small molecular mass can enter the cell through the diffusion process. Almost all of the tested compounds also had values of H-donor and H-acceptor less than 5, so these compounds can penetrate cell membranes. Too large value of H-donor and H-acceptor will slow down the compound to reach the target. Likewise, with the logP value, if the value is too large, the compound will be difficult to pass through the cell membrane and tend to have a high level of toxicity. The greater the logP value, the more hydrophobic the molecule and more easily retained in the lipid bilayer of the cell membrane. It will cause the compound to be widely distributed, thereby reducing bond selectivity of compounds to the target (Kilo et al. 2019; Weni et al. 2020).

Molecular docking of active compound from *Curcuma longa*

The result of the molecule binding to the target is binding affinity (ΔG_{bind}) and the interaction of ligand with protein receptor. The results of the ligand-receptor interaction are based on ΔG_{bind} value because each binding of the ligand to the protein macromolecule (receptor) produces a ligand conformational based on ΔG_{bind} rank. The smaller the ΔG_{bind} value, the more stable the ligand binding to the receptor. ΔG_{bind} is energy required by ligand when it interacts or binds to the receptor binding site.

Table 5. Value of ΔG_{bind} (kcal/mol) docking of 7 compounds with 3 receptors.

Compound (Ligand)	Receptor		
	VEGFR-2	EGFR	FGFR-1
α-Curcumene	-5.8	-6.0	-6.8
Curcumenol	-6.9	-6.7	-7.2
Curcumin	-7.3	-7.1	-7.5
Curcumin II	-7.2	-7.3	-7.0
Curcumin III	-7.0	-7.2	-6.8
Sunitinib	-6.9	-7.6	-7.0
Lapatinib	-7.2	-7.8	-8.5
Gefitinib	-7.2	-7.4	-6.9

The value of ΔG_{bind} in Table 5 showed that curcumin could form a complex more efficiently than other compounds because it had the most

negative ΔG_{bind} (binding affinity) value. While in anticancer drugs, interaction of the FGFR-1 receptor with lapatinib had the highest ΔG_{bind} value of -8.5 kcal/mol. Interaction between ligands and macromolecular residues on receptor can be seen based on visualization using discovery studio visualizer software. The interactions that occur can be in the form of hydrogen, hydrophobic, and electrostatic bonds (Arwansyah et al. 2014). Affinity determination based on the negative or lowest ΔG_{bind} value is preferred, because it relates to the amount of hydrogen or various types of residues that interact with ligand. The difference in ΔG_{bind} value in each compound is influenced by the bonds formed. Role of hydrogen bond determines the value of resulting ΔG_{bind} rather than hydrophobic bond. However, hydrophobic bond plays an important role in determining the stability of ligand to receptor (Arwansyah et al. 2014).

Based on Table 6, it can be seen that the compound of curcumin-FGFR-1 had fewer hydrogen bonds (3) than curcumin II (8), but the ΔG_{bind} value of curcumin-FGFR-1 (-7.5) was higher than curcumin II-FGFR-1 (-7.0). Likewise, the lapatinib-FGFR-1 interaction had fewer hydrogen bonds (1) than gefitinib (12) and sunitinib (3), but the ΔG_{bind} of lapatinib-FGFR-1 was higher (-8.5) than gefitinib (-6.9) and sunitinib (-7.0). The similarity of the amino acid residues that interacted between ligand compounds and control drug showed that the ligand had potential as a substitute for the control drug. The molecular docking results for *Curcuma longa* compounds which have same amino acid residues with control cancer drugs were summarized in Table 7.

Interaction similarity of amino acid residues of ligand with control drug indicates that ligand is able to inhibit activity of target protein and has the potential to substitute control drug (Arwansyah et al. 2014; Chamata et al. 2020). Active compounds that have strong binding to target protein receptors are compounds that have same hydrogen bonds and amino acid residues as control drugs (Bintari 2018). From the docking results, it was found that not all ligand compounds had same amino acid residue as control drug. Curcumin compounds had same amino acid residues as the three drugs, both in the interaction with VEGFR-2, EGFR, and FGFR-1. It showed that curcumin bound well to FGFR1 in the binding pocket and can be a substitute for

Table 6. Number of hydrogen and hydrophobic bonds in the interaction of *Curcuma longa* compounds (ligands) with receptors.

Compound (Ligand)	VEGFR-2		EGFR		FGFR-1	
	Hydrogen Bond	Hydrophobic Bond	Hydrogen Bond	Hydrophobic Bond	Hydrogen Bond	Hydrophobic Bond
α-Curcumene	-	12	-	10	-	14
Curcumenol	-	12	-	11	-	15
Curcumin	4	7	3	14	3	12
Curcumin II	3	11	2	15	8	12
Curcumin III	1	12	1	15	5	11
Sunitiib	1	8	1	15	3	12
Lapatinnib	2	15	4	12	1	17
Gefitiib	6	11	5	13	12	12

cancer drugs, according to the results of in vivo studies (Figure 1) (Puteri 2020). According to Devassy et al. (2015), curcumin had been shown to have many positive effects on suppressing transformation, angiogenesis, metastasis, and suppressing cancer cell growth through cell proliferation. It is stated that curcumin has effect of preventing various types of cancer, such as prostate, pancreatic, lung, colon, and breast cancer. The three anticancer drugs are cancer inhibitors (data from the DrugBank website). Sunitinib inhibits cellular signaling by targeting multiple RTK, lapatinib inhibits RTK, and gefitinib inhibits the intracellular phosphorylation of many tyrosine kinases associated with cell transmembrane surface receptors.

Table 7. Amount of similarity of amino acid residues between *Curcuma longa* compounds and anticancer drugs.

Ligand	Receptor		
	VEGFR-2	EGFR	FGFR-1
α-Curcumene - Sunitinib	0	0	0
Curcumenol - Sunitinib	0	14	0
Curcumin - Suntinib	0	10	3
Curcumin II - Sunitinib	5	12	1
Curcumin III - Sunitinib	0	10	1
α-Curcumene - Lapatinib	2	0	0
Curcumenol - Lapatinib	0	8	1
Curcumin - Lapatinib	1	9	2
Curcumin II - Lapatinib	6	9	3
Curcumin III - Lapatinib	0	7	8
α-Curcumene - Gefitinib	12	0	0
Curcummenol - Gefitinib	0	7	1
Curcumin - Gefitinib	9	9	4
Curcumin II - Gefitinib	2	13	1
Curcumin III - Gefitinib	0	10	3

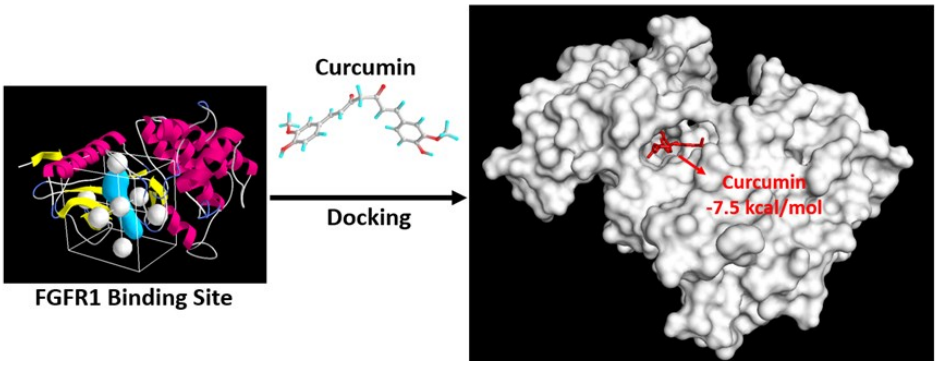

Figure 1. Binding pocket detection and the molecular interaction between curcumin and FGFR1 with binding energy -7.5 kcal/mol.

The number of similarities of amino acid residues possessed by each ligand compound does not affect the ΔG_{bind} value. There were many similarities in amino acid residues in the three control drugs to α-curcumenol, curcumin, curcumin II, and curcumin III, but curcumin had the highest ΔG_{bind} value (-7.5 kcal/mol). Study results by Arfi et al. (2020) showed that cyclocurcumin ligand had a stronger potential to bind to its target protein than

mebendazole ligand, although cyclocurcumin ligand had fewer amino acid residues than mebendazole ligand.

PI3K/AKT signaling pathway analysis

The signaling pathway mechanism was analyzed using STRING to determine the signaling interactions of proteins to form interaction networks. Proteins that affect signaling were marked in red with various line colors. Blue lines show interactions based on an accurate database, purple lines show interactions based on experimental research, green lines show interactions based on gene-environment predictions, red lines show cell fusion, dark blue lines show gene-occurrence interactions, yellow lines show text mining, black lines show co-expression, and gray lines show protein homologs (Franceschini et al. 2013). Pathway analysis used KEGG pathway contained in STRING by entering the three receptors in the column presented and selecting PI3K/AKT pathway on the KEGG list. STRING results were shown in Figure 2.

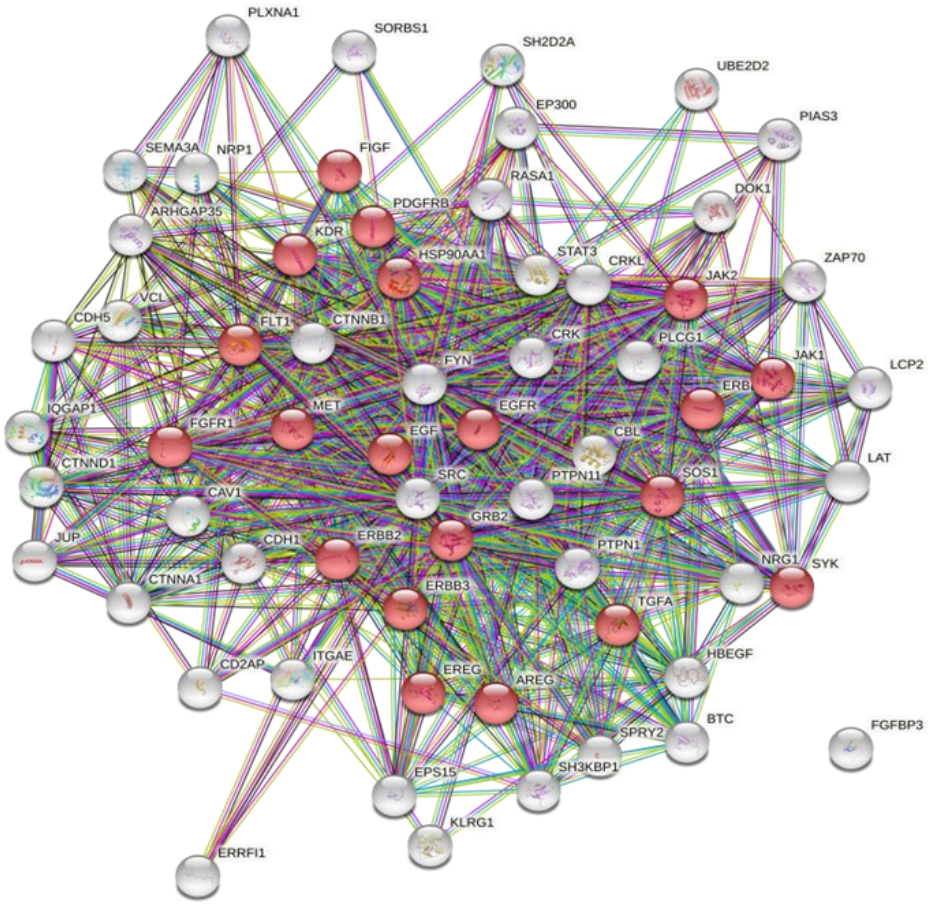

Figure 2. Results of STRING analysis of on KEGG pathway.

The results of the protein interaction network showed that many proteins contribute to the same function. Several proteins that contribute to the PI3K/AKT pathway include KDR (VEGFR-2), EGFR, and FGFR-1. Curcumin compounds in *Curcuma longa* can inhibit angiogenesis, induce apoptosis, and suppress cancer cell proliferation. Inhibition mechanism of tumorigenesis and anticarcinogenic in curcumin can occur through various levels of

signaling pathways, such as NF-Kb, PI3K/AKT, mTOR, Ras/Raf/Erk, STAT, etc. (Abdurrahman 2019; Cas & Ghidoni 2019). Mechanism of curcumin action occurs both intrinsically and extrinsically (Abdurrahman 2019).

Curcumin can inhibit cell proliferation, differentiation, migration, and transcription, and also increase apoptotic death by inhibiting its signaling pathways. Apoptosis induction, growth inhibition, and differentiation enhancement by curcumin occur through PI3K/AKT signaling pathway (Figure 3). In PI3K/AKT signaling pathway, curcumin reduces malignant potential of cancer cells by inhibiting cell proliferation (Hamzehzadeh et al. 2018).

Phosphatidylinositol-3-kinase (PI3K) signaling is a pathway that plays an important role in aspects of cell growth, cell cycle, apoptosis, and cell proliferation in cancer development. Therefore, this signaling pathway is closely related to tumor development and can be an important anticancer target (Shi et al. 2019).

RTK (receptor tyrosine kinase) is a cell surface receptor for many growth factors, hormones, and cytokinins (Mele & Johnson 2019). RTK has phosphotyrosine kinase activity which is attached to plasma membrane but in inactive condition (Haddadi et al. 2018). RTK activation occurs due to the binding of ligands (extracellular molecules) nor growth factors and receptor-associated proteins (Insulin Receptor Substrate-1; IRS1). When ligand binds to RTK, it activates an intracellular tyrosine kinase domain that results in phosphorylation of tyrosine receptors on cell surface and attracts PI3K (phosphatidylinositol 3-kinase) to plasma membrane. The p85 regulatory subunit of PI3K binds directly to the tyrosine receptor. The binding of p58 results in activation of the p110 catalytic subunit in plasma membrane, then p110 catalyzes the phosphorylation of PIP2 (phosphatidylinositol-4,5-bisphosphate) to PIP3. PIP3 regulates many cellular processes such as cell proliferation, angiogenesis, growth, survival, and motility (Papadimitrakopoulou 2012).

RTK activation further activates the PI3K/Akt/mTOR pathway, a central pathway for cancer growth, survival, and motility, making this pathway a target for cancer research and therapy (Cidado & Park 2012). PIP3 contributes to the recruitment and activation of various downstream signaling targets, including Akt (protein kinase B). PIP3 localizes Akt to plasma membrane and it is activated through phosphorylation of phosphoinositide-dependent kinase (PDK1). After phosphorylated, Akt escapes from plasma membrane and activates targets that promote the increase of cell growth, metabolism, survival, and cell proliferation in nucleus and cytoplasm. Akt which is phosphorylated by PIP3 second messenger will activate mTOR (mammal target of rapamycin), mTOR2 will phosphorylate Akt for the second time after PIP3 is fully activated. mTOR acts as a regulator of cell proliferation and growth located downstream and upstream of Akt (Papadimitrakopoulou 2012).

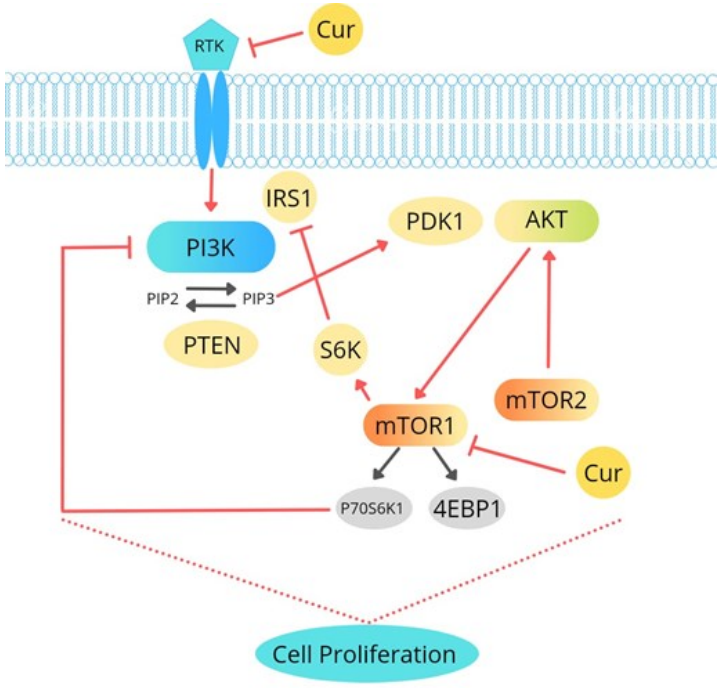

Figure 3. Targeting anti-cancer signals by curcumin.

Curcumin inhibits Akt phosphorylation in cancer cells thereby inhibiting mTOR signaling. Akt Inhibition is due to dephosphorylation of PIP3 to PIP2 which is inactive by PTEN or other inhibition by mTOR. Curcumin reduces cancer cell proliferation by reducing the expression of Raptor and Rictor components. mTORC1 has a significant role in controlling cell proliferation and the downstream pathway is 70 kDa ribosomal protein S6 kinase 1 (p70S6K1) and eIF4E 1 (4EBP1) binding protein which can provide negative feedback on Akt pathway. mTORC1 signaling is regulated by Akt through TSC1/TSC2 complex, which can induce GTPase and Rheb activity. Curcumin directly suppressed Akt and mTORC1 so the inhibition of downstream pathway like phosphorylation of p70S6K1 and 4EBP1 can be achieved. These downstream targets are involved in cell proliferation and growth (Tamaddoni et al. 2020).

CONCLUSION

Curcuma longa is well-known as anticancer and curcumin as one of metabolites could be concluded that had the most promising anticancer activity by interaction with FGFR-1 receptor protein. Curcumin molecular mechanism as antiproliferative was revealed computationally through by inhibiting the PI3K/AKT/mTOR pathway. The anti-cancer activity of *Curcuma longa* compounds needs to be tested further by in vivo and in vitro to find out more about the inhibitory activity of *Curcuma longa* compounds as anticancer.

AUTHORS CONTRIBUTION

This research was conducted with the cooperation of all the authors, with the following details: conceptual and design research by RS and DHU; data collection by SMWK; data validation and accuracy by RS; data visualization by

DHU; writing original draft manuscript by SMWK; review of draft manuscripts by DHU and RS; supervision of all activities by RS.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

REFERENCES

Abdurrahman, N., 2019. Kurkumin pada Curcuma longa sebagai Tatalaksana Alternatif Kanker (Curcumin in *Curcuma longa* as an Alternative Cancer Treatment). *Journal Agromedicine*, 6(2), pp.410-415.

American Cancer Society, 2016. Cancer Fact and Figure. Atlanta: American Cancer Society Inc.

Anand, T. & Gokulakrishnan, K., 2014. GC-MS Analysis and Anti-Microbal Activity of Bioactive Components of *Hybanthus enneaspermus*. *International Journal of Pharmacy and Pharmateucal Science*, 2(3), pp.646-650.

Anisa, D.N., Anwar, C. & Afriyani, H., 2020. Sintesis Senyawa Analog Kurkumin Berbahan Dasar Veratraldehida Dengan Metode Ultrasound. *Analit: Analytical and Environmental Chemistry*, 5(01), pp.74-81. doi: 10.23960%2Faec.v5i1.2020.p74-81

Arfi, A.S., Lestari, R.D. & Damayanti, D.S., 2020. Studi In Silico Senyawa Aktif Rimpang Kunyit (*Curcuma domestica*) terhadap Penghambatan Acetylcholinesterase, Microtubulin (Beta tubulin), dan Aktivasi Calcium channel sebagai Terapi Antelmintik. (In Silico Study: Active Compound of Turmeric Rhizome (Curcuma domestica) Towards Acetylcholinesterase and Microtubulin (Beta Tubulin) Inhibition, and Calcium Channel Activation as an Anthelmintic Therapy). *Jurnal Kedokteran Komunitas*, 8(2), pp.36-47.

Arwansyah, A., Ambarsari, L. & Sumaryada, T.I., 2014. Simulasi Docking Senyawa Kurkumin dan Analognya Sebagai Inhibitor Reseptor Androgen pada Kanker Prostat. *Current Biochemistry*, 1(1), pp.11-19. doi: 10.29244/cb.1.1.11-19

Astolfi, A. et al., 2020. The Emerging Role of the FGF/FGFR Pathway in Gastrointestinal Stromal Tumor. *International Journal of Molecular Science*, 27(3313), pp.1-14. doi: 10.3390/ijms21093313

Bintari, Y.R., 2018. Studi In Silico Potensi Ekstrak Lipida *Tetraselmis chuii* sebagai Antioksidan. *Jurnal Ketahanan Pangan*, 2(1), pp.76-81.

Cas, M.D. & Ghidoni, R. 2019. Dietary Curcumin: Correlation Between Bioavailability and Health Potential. *Nutrient*s, 11(2147), pp.1-14. doi: 10.3390/nu11092147

Chamata, Y., Watson, K.A. & Jauregi, P., 2020. Whey-Derived Peptides Interactions with Ace by Molecular Docking as A Potential Predictive Tool of Natural Ace Inhibitors. *International Journal of Molecular Sciences*, 21(3), pp.1-13. doi: 10.3390/ijms21030864

Chao, I.C. et al., 2018. Simultaneous Quantification of Three Curcuminoids and Three Volatile Components of *Curcuma longa* Using Pressurized Liquid Extraction and High-Performance Liquid Chromatography. *Molecules*, 23(7), pp.1568. doi: 10.3390/molecules23071568

Cidado, J. & Park, B.H., 2012. Targeting the PI3K/Akt/mTOR Pathway for Breast Cancer Therapy. *Journal Mammary Gland Biology and Neoplasia*, 17 (3-4), pp.205-216. doi: 10.1007/s10911-012-9264-2

Dallakyan, S. & Olson, A.J., 2014. Small-Molecule Library Screening by Docking with PyRx. *Methods in Molecular Biology*, 1263, pp.243-250. doi: 10.1007/978-1-4939-2269-7_19

Devassy, J., Nwachukwu, I. & Jones, P., 2015. Curcumin and Cancer: Barriers to Obtaining a Health Claim. *Nutrition Reviews*, 73(3), pp.155-165. doi: 10.1093/nutrit/nuu064

Druzhilovskiy, D. S. et al., 2016. Online Resources for the Prediction of Biological Activity of Organic Compounds. *Russian Chemical Bulletin*, 65(2), pp.384-393. doi: 10.1007/s11172-016-1310-6

Ezealisiji, K.M. & Awucha, N.E., 2020. Phytochemistry and GC-MS analysis of methanolic leaf extract of *Newbouldia leavis* (Bignonacea). *Journal of Pharmacognosy and Phytochemistry*, 9(2), pp.1998-2004.

Filimonov, D.A. et al., 2014. Prediction of the Biological Activity Spectra of Organic Compounds Using the PASS Online Web Resource. *Chemistry of Heterocyclic Compounds*, 50(3), pp.444-457. doi: 10.1007/s10593-014-1496-1

Franceschini, A. et al., 2013. STRING v9.1: Protein-Protein Interaction Networks, With Increased Coverage and Integration. *Nucleic Acids Research*, 41(1), pp.808-815. doi: 10.1093/nar/gks1094

Golonko, A. et al., 2019. Curcumin as Tyrosine Kinase Inhibitor in Cancer Treatment. *European Journal of Medicinal Chemistry*, 181(111512). pp.1-25. doi: 10.1016/j.ejmech.2019.07.015

Haddadi, N. et al., 2018. PTEN/PTENP1: 'Regulating the Regulator of RTK -Dependent PI3K/Akt Signalling', New Targets for Cancer Therapy. *Molecular Cancer*, 17(37), pp.1-14. doi: 10.1186/s12943-018-0803-3

Hamzehzadeh, L. et al., 2018. The Versatile Role of Curcumin in Cancer Prevention and Treatment: A Focus on PI3K/AKT Pathway. *Journal of Cellular Physiology*, 233(10), pp.6530-6537. doi: 10.1002/jcp.26620.

Ivanov, S.M. et al., 2018. ADVERPred-Web Service for Prediction of Adverse Effects of Drugs. *Journal of Chemical Information and Modeling*, 58(1), pp.8-11. doi: 10.1021/acs.jcim.7b00568.

Jadhav, P.B., Yadav, A.R. & Gore, M.G., 2015. Concept of Drug Likeness in Pharmaceutical Research. *International Journal of Pharma and Bio Science*, 6 (4), pp.142-154.

Jamkhande, P. G. et al., 2014. Antioxidant, Antimicrobial Activity and In Silico PASS Prediction of *Annona reticulata* Linn. Root Extract. *Beni-Suef University Journal of Basic and Applied Sciences*, 3(2), pp.140-148. doi: 10.1016/j.bjbas.2014.05.008

Kilo, A.L. et al., 2019. Studi Potensi Pirazolin Tersubstitusi 1-N dari Tiosemikarbazon sebagai Agen Antiamuba Melalui Uji in Silico (Study of Potential of 1-N-Substituted Pyrazoline Analogues of Thiosemicarbazones as Antiamoebic Agent using In Silico Screening). *Indonesia Journal of Chemical Research*, 7(1), pp.9-16. doi: 10.30598//ijcr.2019.7-akr

Lazzeroni, M. et al., 2012. Oral low dose and topical tamoxifen for breast cancer prevention: modern approaches for an old drug. *Breast Cancer Research*, 14(5), 1–11. doi: 10.1186/bcr3233

Maduabuchi, E.K. & Awucha, N.E., 2020. Phytochemistry and GC-MS Analysis of Methanolic Leaf Extract of *Newbouldia laevis* (Bignonacea). *Journal of Pharmacognosy and Phytochemistry*, 9(2), pp. 1998-2004.

Mele, S. & Johnson, T.K., 2019. Receptor Tyrosine Kinases in Development: Insights from Drosophila. *International Journal of Molecular Sciences*, 21 (188), pp.1-21. doi: 10.3390/ijms21010188

Moon, A. et al., 2017. Insilico Prediction of Toxicity of Ligands Utilizing Admetsar. *International Journal of Pharma and Bio Sciences*, 8(3), pp.674-677. doi: 10.22376/ijpbs.2017.8.3.b674-677

Nagahama, K. et al., 2016. Discovery of A New Function of Curcumin Which Enhances Its Anticancer Therapeutic Potency. *Scientific Reports*, 6(30962), pp.1-14. doi: 10.1038/srep30962

Nerkar, A.G. et al., 2012. In Silico Screening, Synthesis and Pharmacological Evaluation of Novel Quinazolinones as NMDA Receptor Inhibitors for Anticonvulsant Activity. *International Journal of Pharmacy and Pharmaceutical Sciences*, 4(3), pp.449-453.

Papadimitrakopoulou, V., 2012. Development of PI3K/AKT/mTOR Pathway Inhibitors and Their Application in Personalized Therapy for Non -Small-Cell Lung Cancer. *Journal of Thorac Oncology*, 7(8), pp.1315-1326. doi: 10.1097/JTO.0b013e31825493eb

Pramely, R. & Raj, T.L.S., 2012. Prediction of Biological Activity Spectra of A Few Phytoconstituents of *Azadirachta indicia* A. Juss. *Journal of Biochemical Technology*, 3(4), pp.375-379.

Puteri, F.D., 2020. Efek Kurkumin Pada Kunyit (*Curcuma longa*) Sebagai Pengobatan Kanker Lambung (Effect of Curcumin on Turmeric (*Curcuma longa*) as a Tretment for Stomach Cancer). *Jurnal Ilmiah Kesehatan Sandi Husada*, 9(2), pp.860-864. doi: 10.35816/jiskh.v10i2.426

Rahimi, N., 2017. Defenders and Challengers of Endothelial Barrier Function. *Frontiers in Immunology*, 8(1847), pp.1-10. doi: 10.3389/ fimmu.2017.01847

Rawluk, J. & Waller, C.F., 2018. Gefitinib. *Recent Results Cancer Research*, 211, pp.235-246. doi: 10.1007/978-3-319-91442-8_16

Shi, X. et al., 2019. Research Progress on the PI3K/AKT Signaling Pathway in Gynecological Cancer (Review). *Molecular Medicine Reports*, 19(6), pp.4529-4535. doi: 10.3892/mmr.2019.10121

Syahputra, G, Ambarsari, L. & Sumaryada, T., 2014. Simulasi docking kurkumin enol, bisdemetoksikurkumin dan analognya sebagai inhibitor enzim12-lipoksigenase. *Biofisika*, 10(1), pp.55-67.

Tamaddoni, A. et al., 2020. The Anticancer Effects of Curcumin via Targeting the Mammalian Target of Rapamycin Complex 1 (mTORC1) Signaling Pathway. *Pharmacological Research*, 156(104798), pp.1-11. doi: 10.1016/j.phrs.2020.104798

Tomeh, M.A., Hadianamrei, R. & Zhao, X., 2019. A Review of Curcumin and Its Derivatives as Anticancer Agents. *International Journal of Molecular Sciences*, 20(1033), pp.1-26. doi: 10.3390/ijms20051033

Weni, M., Safithri, M. & Seno, D.S.H., 2020. Molecular Docking of Active Compounds Piper crocatum on The Alpha-Glucosidase Enzyme as Anticiabetic. *Indonesian Journal of Pharmaceutical Science and Thechnology*, 7 (2), pp.64-72. doi: 10.24198/ijpst.v7i2.21120

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Review Article

An Extensive Review on Production, Purification, and Bioactive Application of Different Classes of Bacteriocin

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ABSTRACT
Lactic Acid Bacteria (LAB) synthesize various metabolites during their growth phase and are Generally Recognized as-- Safe (GRAS) and Qualified Presumption of Safety (QPS). Ribosomally synthesized Antimicrobial Peptides (AMP) or Bacteriocins from the genera of Lactic Acid Bacteria and other prokaryotic genera are cationic, heat-stable, amphiphilic and the membrane permeabilizing peptides built with an excess amount of lysyl and arginyl residues. Antimicrobial compounds produced by LAB depend on the physical and biological conditions of microbial culture. Different classes of bacteriocin are produced by both Gram-positive and Gram-negative bacteria. The production of bacteriocin is influenced by various environmental factors. Bacteriocin has a wide variety of applications in various fields. The application spectrum of bacteriocins can be expanded in various domains such as food processing, biomedical, and personal care due to the increase in the number of newly discovered bacteriocins. Bacteriocins acquire a wide spectrum of antimicrobial activity with minimal level of cytotoxicity. In addition, bacteriocins were studied for their anticancer activity against different cancer cell lines. Selective binding of bacteriocins (cationic) towards cancer cells (anionic) increases the cytotoxicity of cancer cells. Bacteriocin peptides initiate necrosis by communicating with the cell surface which selectively targets and kills the cells with tumor formation and does not cause any damage to the normal healthy cells. In this review, the bacteriocins synthesized from lactic acid bacteria along with their interaction with cancer cell lines and other applications are discussed along with a few examples of other bioactive compounds produced by LAB.

INTRODUCTION
Lactic Acid Bacteria (LAB) are gram-positive, non-sporulating, facultative aerobic, cocci or rods which produce lactic acid during fermentation. LAB are most recognized for their employment as starter cultures in the production of acidophilus milk, yogurt, buttermilk, cottage cheese, hard cheeses, and soft cheeses. In addition, they are also used in the processing of meats, alcoholic beverages, and vegetables such as sausage, cured hams, wines, beer, fortified spirits, pickles, sauerkraut, etc. LAB is generally recognized as safe (GRAS) which had sought the attention of industrialists to develop new products. LAB also produces various metabolites such as bacteriocin or bactericidal proteins, organic acids, and vitamins during the fermentation process. Lactic Acid Bacteria consist of diverse genera which are grouped into

homo fermenters and hetero fermenters based on their end products formed during fermentation. Homo fermenters produce lactic acid as the major end product of fermentation from glucose whereas hetero fermenters synthesize several products from the fermentation of glucose besides lactic acid which includes carbon dioxide, acetic acid, and ethanol. Homo fermenters possess the enzyme aldolase which leads to the direct conversion of glucose into lactic acid. Hetero fermenters use an alternate pentose monophosphate pathway and convert six-carbon sugars (hexoses) to five-carbon sugars (pentoses) by the enzyme phosphoketolase, producing in the process both aldehyde and diacetyl-highly desirable aromatic and flavor-enhancing substances which are often used in industries. The genera of homo fermenters include Lactococcus*, Streptococcus, Pediococcous,* and *Lactobacillus*. On the other hand, the genera of hetero fermenters include *Betabacteria* (*Lactobacillus*) and *Leuconostoc* (Carr et al. 2002).

Bacteriocin produced by Gram-positive bacteria was classified into four groups based on the presence of monosulfide and disulfide bond (lanthionine) bonds. Bacteriocins were divided into four categories: (1) antibiotics containing unusual post-translationally modified amino acids such as dehydroalanine, dehydrobutyrine, lanthionine, or -methyl-lanthionine (lantibiotics); (2) antibiotics containing at least one disulfide bridge essential for their activity (cystibiotics); (3) antibiotics to be in active form, compounds with a single -SH residue should be in a reduced form (thiolbiotics); and (4) antibiotics without cysteine residues (Jack et al. 1995). Bacteriocins generated by gram-negative microorganisms were of particular interest.

Optimization of fermentation conditions is necessary for the commercial production of bacteriocin. The strain, medium composition (carbohydrate and nitrogen sources, cations, etc.), fermentation conditions (pH, temperature, agitation, and aeration), as well as the mode of fermentation, influence the yield per unit biomass (batch, fed-batch, and continuous fermentations) (Parente & Ricciardi 1999). Influencing variables may vary with different types of bacteriocin and strains producing bacteriocin. The effects of two key parameters for bacteriocin production include medium compositions and cultivation circumstances (Leroy et al. 2003). Bacteriocin-producing bacteria require complex nutritional requirements to develop, which not only raise manufacturing costs but also complicate bacteriocin purification (Li et al. 2002). An ideal bacteriocin production technique would be one that could be applied to large-scale purification and result in bacteriocin yields of more than 50% and purity of over 90% (Schöbitz et al. 2006). The bacteriocin produced by lactic acid bacteria can be used for various bioactive applications. Cancer is the predominant cause of death worldwide. Cancer-causing risk factors, lifestyle changes, aging, and the building of population are the common cause of cancer incidence and mortality which affect socioeconomic development (Bray et al. 2021). World Health Organization (WHO) reported that cancer is the leading cause of death in 2019 in 112 countries for the people below 70 years. The cancer incidence rate (2006-

2015) was steady in women and decreased by about 2% each year in males, although the cancer death rate (2007-2016) decreased by 1.4 percent and 1.8 percent, respectively, over the last decade of dated from 1991 to 2016, the global cancer death rate fell by a total of 27%, resulting in about 2,629,200 fewer cancer deaths than would have been expected if death rates had maintained at their high. Even though the racial difference in cancer mortality is shrinking, socioeconomic inequalities are rising with the most noteworthy discrepancies occurring in malignancies that are the most preventable. For example, between 2012 and 2016, mortality rates in the poorest regions were 2-fold higher for cervical cancer and 40% higher for male lung and liver cancers when compared to the wealthiest regions (Siegel et al. 2019). In 1940s the generation of Chemotherapy began with the initial usage of nitrogen mustard seeds and antifolate medicines. Since then, cancer medication development has evolved from a low-budget, government-supported research project to a high-stakes, multibillion-dollar enterprise. Although the targeted-therapy revolution has arrived, the concepts and limitations of chemotherapy revealed by the pioneers remain valid. Chemotherapy is widely followed treatment to inhibit the growth of tumor cells. In case of metastasis, the continuation of chemotherapy remains unclear (Chabner & Roberts 2005). Cancer cell resistance and normal cell destruction are other major upcoming problems in cancer treatment (Porta et al. 2015). There is a need for the development of new cancer therapeutics in the future. Nowadays, antimicrobial peptides (AMP) have reported anticancer activity without disturbing the normal cells (Lao et al. 2014). In addition to their broad-spectrum activity and distinct methods of action to traditional antibiotics, antimicrobial peptides (AMPs) have been investigated as potential therapeutic sources of future antibiotics. Although AMPs offer a lot of potential as new generation antibiotics, they still have several drawbacks in terms of clinical and commercial development, such as possible toxicity, protease susceptibility, and expensive peptide synthesis costs. Extensive efforts have been made to overcome those obstacles. To avoid proteolytic breakdown, for example, unique amino acids or peptide-mimetics are used, and the construction of short peptides with antibacterial activity is suggested as a cost-effective approach (Seo et al. 2012). Antimicrobial peptides (AMPs) are a critical component of innate immunity that evolved over 2.6 billion years in most living species to combat microbial assault. These tiny cationic peptides have exhibited direct antibacterial activity against a variety of bacteria, viruses, fungi, and parasites, and are multifunctional as innate immune effectors on the skin and mucosal surfaces (Gordon et al. 2005). Such AMP synthesized by Lactic Acid Bacteria is completely recognized as safe. Bacteriocins are mostly employed in the food processing industry. The application spectrum of bacteriocins can be expanded in various domains such as horticulture, biomedical, and personal care due to the increase in the number of newly discovered bacteriocins.

The review article mainly focuses on the different types of bacteriocins produced by lactic acid bacteria during fermentation, and their application to

anticancer agents that have been previously reported by the original authors. It also focuses on the various application of classes of bacteriocins. In addition, the article also discusses the other bioactive compound produced by lactic acid bacteria.

BACTERIOCIN

Bacteriocins are low molecular weight (10 KDa) antimicrobial compounds biologically synthesized from the ribosomes of Lactic Acid Bacteria during the primary phase of their growth. Lactic acid bacteria create a wide range of antagonistic factors, including metabolic end products, antibiotic-like compounds, and bacteriocins (bactericidal proteins). Bacteriocins from lactic acid bacteria have a large range of inhibitory action, inhibiting a diverse spectrum of Gram-positive microorganisms, or a narrow range of inhibitory activity, inhibiting only those strains that are closely related to the producer organism. Bacteriocins are bactericidal proteins or protein complexes that target bacteria that are usually closely related to the producer bacterium. Genetic determinants and transfer mechanisms for bacteriocin production and immunity are expected to play an important role in the development and the use of genetic technologies for lactic acid bacteria in this area (Lao et al. 2014). In comparison to peptide antimicrobials produced by eukaryotic cells which typically have 10^2-10^3-fold lower activities, most bacteriocins are extraordinarily active, demonstrating antimicrobial activity at nanomolar concentrations. Surprisingly, the producer cells are impervious to the bacteriocins that they make. The bacteriocin is produced in multiple microbial environments which are used to eliminate other unwanted microbes. Initially, the antimicrobial activity was restricted particularly to the strains of the same species but later they exhibited a broad range spectrum (both gram-positive and gram-negative bacterium). The degradation of bacteriocins by the enzyme proteases makes it safe for human utilization. Bacteriocins are cationic, heat-stable, amphiphilic, and membrane permeabilizing peptides that contain an excess amount of lysyl and arginyl residues (Chu et al. 2015). They act on the targeted cells by interacting with specific surface receptors. They do not affect the normal commensal of the environment. Bacteriocins and other products from LAB are recognized as safe and Qualified Presumption of Safety (QPS) (Carr et al. 2002).

DIFFERENT CLASSES OF BACTERIOCINS

Bacteriocins of gram-positive bacteria can be divided into four main classes which include classes: 1, 2, 3 & 4 shown in Figure 1. The classifications are made according to their differences in size or molecular weight, primary structure, genetic characters, post-translational modifications, and physicochemical characteristics (Rodr et al. 2000). All the classes show good antimicrobial activity even at low concentrations.

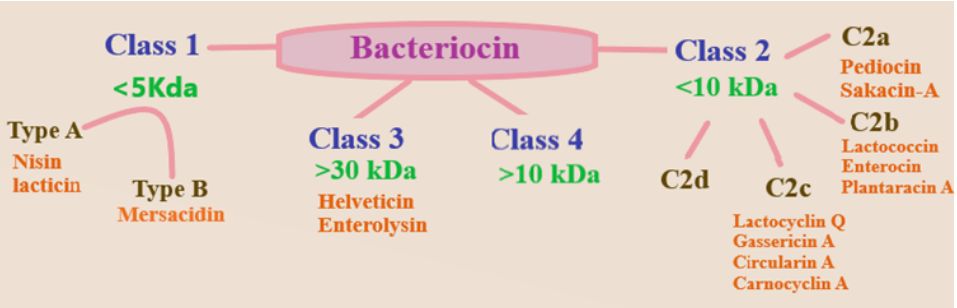

Figure 1. Represents the classification of Bacteriocins.

Class 1

The class 1 bacteriocins are a lantibiotic class of small, heat-stable peptides (<5 kDa) build with methyllanthionine, dehydroalanine, and 2 amino isobutyric acids (unsaturated amino acids). Based on the structural similarities and net charge, lantibiotics are further divided into two types. Type A is amphipathic and positively charged screw-shaped lantibiotics with a molecular mass of 2 to 4 kDa which acts through membrane depolarization and pore formation in the cytoplasmic membrane. The depolarization occurs due to the binding of N- terminal domain bacteriocin with the precursor of peptidoglycan called lipid II. C terminal region also plays an important role in pore formation and membrane destruction. Nisin and lacticin 3147 are the major representatives of this type (Rodríguez et al. 2003; Zacharof & Lovitt 2012). Type B lantibiotics are globular tertiary structures with a molecular mass of 2 to 3 kDa with no net charge or a net negative charge which interferes with cellular enzymatic reactions (Alvarez-Sieiro et al. 2016). The type B bacteriocin also inhibits peptidoglycan synthesis. Example: Mersacidin

Class 2

Non-lantibiotic class 2 bacteriocins (<10 kDa) are built with heat-stable membrane-active peptides (Shaw 2021). The transporter is required for their maturation rather than other enzymes. The non-lantibiotics do not undergo post-translational modification at the peptide chain. So, they can be determined as lanthionine or β-lanthionine (Cotter et al. 2005). They possess a helical structure which is very helpful for depolarization and cell death. Cell death or depolarization occurs by the insertion of a helical structure into the membrane of the target cell (Cleveland et al. 2001). The class 2 bacteriocins are classified into four subclasses C2a, C2b, C2c, and C2d. Subclass C2a is a monomer constructed with a consensus N-terminal sequence. They have antimicrobial activity at a higher rate (Example: pediocin and sakacin-A) (Patton & Donk 2003). Subclass C2b has two independent heterodimeric bacteriocin peptide codings for short-chain amino acids which work in collaboration to exhibit antimicrobial activity. They exhibit antimicrobial activity by forming cation or anion-specific pores (Example: Lactacin, lactococcin, Enterocin, and Plantaracin A) (Deegan et al. 2006). Subclass C2c is circular. The structure is formed by a covalent bond linking the C and N terminal regions of the peptide (Cintas et al. 2001). They have a very stable structure

with one or two cysteine residues at the leader peptide sequence. They contain two transmembrane channels that induce antimicrobial activity by forming pores in the target cells. (Example: Lactocyclin Q, gassericin A, circularin A, and carnocyclin A) (Ovchinnikov et al. 2016). The subclass C2d contains various bacteriocins that contain a single linear peptide but are not similar to pediocin. With pediocin-like Listeria active peptides such as pediocin PA1 and leucocin A as examples, Class 2a is attracting attention in food preservation. Fermented meat, fermented vegetables, dairy products, smoked salmon, and the human gastrointestinal tract have all yielded over 50 different types of class 2a bacteriocins. Plantaricin A and enterocin X are examples of Class IIb bacteriocins that require the synergistic activity of two complementary peptides to exhibit antibacterial activity. Although some of these peptides have antibacterial activity by themselves, the presence of the corresponding peptide dramatically increases this activity. The complementary peptide pair is active at nanomolar to picomolar concentrations. Class 2b bacteriocins are predominantly cationic and comprise amphiphilic and hydrophobic areas. The genes that code for the two peptides are genetically related and encoded in the same operon (Mokoena 2017).

Class 3

Bacteriocins with large-molecular weight (greater than 30 kDa) synthesize antimicrobial compounds composed of different domains with bacteriolytic activity. The enzymatic activity presented in them is linked with the antibiotic which induces lysis in the target cell. The synthesized bacteriocins can be destroyed when exposed to high temperatures (heat-labile) (Example: helveticin and enterolysin) (Ross et al. 2002). This group of bacteriocin is not well characterized.

Class 4

Bacteriocins of this class contain higher molecular weight peptides. They usually combine with carbohydrates or lipids. The antimicrobial activity is exhibited by disturbing the cell membrane of the targeted microorganism (Jeevaratnam et al. 2005).

PRODUCTION OF BACTERIOCIN FROM NOVEL SOURCES

Initiation and biosynthesis of LAB antimicrobial compounds depend on the physical and biological condition of microbial culture. The operon clusters containing plasmids are responsible for the bacteriocin-producing and immunity-building genes. They can be found on mobile genetic components including chromosomes and transposons, as well as plasmids. Initially, inactive bacteriocin peptides with N-terminal sequence are synthesized ribosomal where the modification is done into active peptides before they are exported out of the cell by a macromolecular protein compound. Serine and threonine residues dehydrate to add cysteines on unsaturated amino acids by thioether cross-links called lanthionine.

Biosynthesis of lantibiotics which belongs to class-I shown in Figure 2 starts with the formation of prebacteriocin which is then qualified by the enzymes (LanB and LanC), where2, 3 –di dehydro alanine along with 2, 3-di dehydro butyrine are yielded as the result of removal of hydroxyl amino acids, serine and threonine, catalyzed by the enzyme LanB and LanC . The mature peptide holds some desiccated amino acids without cysteine residues; whereas the intramolecular Michael addition reaction leads to the synthesis of thioether bridges by the initiation of double bond interaction between thiol groups of neighboring cysteine residues and didehydroamino acids. Serine protease (LanP) modifies the synthesized prebacteriocin and ABC-transporter (LanT) translocates the prebacteriocin which ends with the release of matured bacteriocin (Sugrue et al. 2019). The released matured bacteriocin is sighted by Histidine protein kinase (HPK) which transfers phosphoryl group (p) to response regulator (RR) through the process called autophosphorylation. Phosphorylated regulators will initiate transcription along with LanI, LanFEG, and ABC-transport proteins (immunity proteins), to protect their cells against the synthesized bacteriocin. LanI protein plays an important role in protecting the cell membranes from forming pores and also by retarding back the synthesized bacteriocin molecules (Example: nisin, epidermin, subtilin, and Pep5). Lantibiotics of class II use LanM enzyme for modification and the process is mediated by LanT (P) transporter (Example: cytolysin, lacticin 481, and mersacidin).

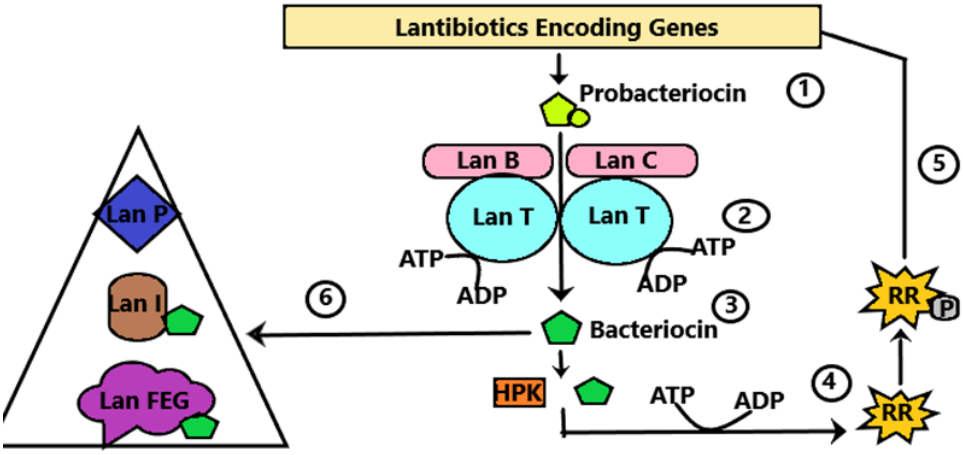

Figure 2. Schematic representation for the biosynthesis of lantibiotics (Class I Bacteriocin). (1) prebacteriocin production (2) LanB, LanC, LanT (ABC- transporter), and LanT (Processor) modifies the produced bacteriocin to release mature bacteriocin (3) Histidine protein kinase (HPK) is autophosphorylated (4) Transfer and addition of phosphoryl group (P) to subsequently response regulator (RR) (5) The transcripted (RR) response regulator activates regulated genes (6) immunity proteins are synthesized..

Unlike lantibiotics, picturized in Figure 3 the modification process is not initiated in bacteriocins of class II. The elimination of leader peptide along with synthesized prebacteriocin export is done by a transporter (ABC)

along with supplementary protein (Van Kraaij et al. 1999; Dufour et al. 2000; Rodali et al. 2013).

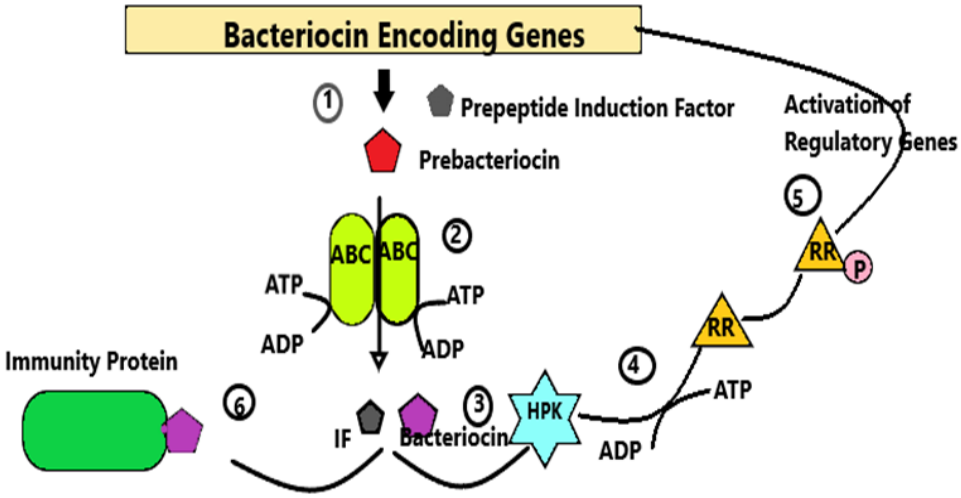

Figure 3. Diagrammatic representation for class II bacteriocins biosynthesis (1) prebacteriocin and induction factor (IF) is formed (2) prebacteriocin translocation and release of mature bacteriocin by a transporter called ABC along with processor (IF) (3) Histidine protein kinase (HPK) autophosphorylate (4) Addition of phosphoryl group (P) to RR (5) regulated genes are activated by the transcription of RR (6) Regulation of immunity producing proteins.

To attain a good quantity of bacteriocin the physical and chemical composition of the culture medium is essential for *in-vitro* production. The highest bacteriocin activity was achieved in De Man Rogosa and Sharpe Agar, LAPTg Agar, and M17 Agar (lactic *streptococci*) improved by the addition of inconsistent carbohydrates. The optimization of physiological conditions is the principal part of bacteriocin production. This review aims at experiments as examples that were conducted by the original author. A good amount of antimicrobial compound synthesized from *Lactobacillus plantarum* was obtained under different physiochemical conditions (temperature-22 to 27°C, NaCl - 2.3 to 2.5%, inoculum size- 107.3 to 107.4 CFU/ml, glucose concentration - 2%) under anaerobic incubation (Leal-Sánchez et al. 2002). Bacteriocin synthesized from *Lactobacillus acidophilus* at pH of 6.0, under the temperature of 34 °C, incorporated with 4% phenyl acetamide (Mahrous et al. 2013). (Onwuakor et al. 2014) reported that *Lactococcus lactis, L. fermentum, L. casei*, and *L. plantarum* exhibited good bacteriocin activity after adding 2 percent of sodium chloride at pH ranging from 5 to 6 when incubated at 35° C for 72 hours. The addition of cysteine and glycine into the production medium induced bacteriocin production after 20 hours of incubation at 37° C. Adding 1 % of glycerol and pyruvic acid in the production medium increased the concentration of bacteriocin. Sometimes, bacteriocins on their initiative the process of biological synthesis after the addition of bacteriocin compound in to the production medium (Yi et al. 2013).

FACTORS INFLUENCING THE PRODUCTION OF BACTERIOCINS

Production of Bacteriocins in Distinct Broths

The production of four different types of bacteriocin (AU ml-l) was determined by growing a strain from an individual genus in Tryptone Glucose Extract (TGE) broth. In another study, the impact of the addiction to broth with the increased concentration of supplements such as tryptone (1.5%), glucose (2%) and yeast extract (1·5%) or pantothenic acid, niacinamide, and biotin (each 0·5 μg ml-l) was analyzed on the production of bacteriocins. The strains used for the production of pediocin AcH (chimeric protein) were *P. acidilacti* B42-923, for nisin *L. lactis* sp. ATCC 11454, *L. carnosum* for leuconocin Lcml and *L. sake* B 706 for sakacin.A 1% level overnight culture was inoculated in both 1L GE and TGE buffer broths. The degree of pediocin AcH production differed little across *P. acidi lactici* strains, the level of nisin and leuconocin Lcm1 production varied significantly. The study results specified that for a large scale and economical production an increased producer strain must be selected.

Significant Effect of Media Composition on Bacteriocin Production

Bacteriocin Pediocin AcH was produced in high quantity by strain *P.acidilactici* B42-923 in TGE broth than in TGE buffer broth. It has been found that a buffered medium produces less pediocin AcH and that high pediocin production requires a pH of 3.6 to 3.7. It was suggested that pediocin AcH was initially produced as a secondary metabolite. Prepediocin to active pediocin AcH post-translation processing was found to be efficient at pH levels below 5.0 in recent investigations. The production of bacteriocins such as nisin, leuconocin LCM1, and sakacin A was reduced in TGE broth when compared to TGE buffer broth. This was due to the demand for an increase in pH for the production of the three bacteriocins. Different researchers have found increased nisin synthesis by *L. lactis* strains by increasing glucose or sucrose concentrations and adding acetate, citrate, phosphate, and pantothenic acid to various complex broths.

Effect of Final pH on the Production of Bacteriocin

At an initial pH of 6.8, the strain was grown in broth and the final pH of broth was varied. The production varied accordingly to the pH of different broths. The growth and the production of bacteriocin varied greatly concerning their pH. The maximum pH for the production of pediocin AcH was 3.7, and the maximum production of leuconocin Lcm1 was at pH 5, followed by nisin at pH 5.8 and pH 4.5 for sakacin A production. Because the final pH for optimum bacteriocin production varies, a producer strain should be cultivated in a broth at the pH where bacteriocin production is highest for economical production (Yang & Ray 1994).

PURIFICATION OF BACTERIOCIN

Bacteriocin biosynthesis occurs inside the fermentation medium, where crude extract contains media components. A few purification steps carried out by different authors are reviewed. Initially, the purification is carried out by the ammonium sulfate precipitation method to precipitate the proteins secreted into the culture medium (Carolissen-Mackay et al. 1997). Since this method does not allow a greater level of purification multiple chromatographic techniques such as ion exchange hydrophobic interaction method (Beaulieu et al. 2006), gel filtration technique (Martínez et al. 1998), and reversed-phase high-pressure liquid chromatography (RP-HPLC) are adopted (Abriouel et al. 2003). In addition, a simple protocol has been developed for purification which includes extraction using chloroform or methanol followed by extraction or precipitation. Finally, the RP-HPLC technique is adopted for purification (Callewaert et al. 1999). In addition, crude bacteriocins are purified by expanded bed adsorption chromatography and hydrophobic gel interaction chromatography by adjusting the pH of the crude fermentation medium by maximizing the bioavailability of bacteriocin through titer analysis (Callewaert & De Vuyst 1999). Pediocin PA-1 yielded 110% by Cation-exchange chromatography (81% yield) followed by Reverse-phase HPLC (RP-HPLC).

ANTICANCER ACTIVITY OF BACTERIOCIN

The quantity of positively charged amino acids in bacteriocins, hydrophobicity, and the ability to form amphipathic structures and oligomers may all influence their cytotoxicity and potential to harm cancer cells. Bacteriocins are thought to primarily target the cytoplasmic membrane of eukaryotic cells. Bacteriocins have been shown to kill organisms that are close to the bacterium that produces them. Because they are active against bacteria of the same or phylogenetically related species which include cancer cells, bacteriocins have a relatively narrow spectrum of activity. Induction of apoptosis and/or depolarization of the cell membrane leads to alterations in permeability, which are examples of cytotoxicity mechanisms. Some of them can produce necrosis and apoptosis in the same cell.

Studies of membrane potential show that a sensitive eukaryotic cell's surface is depolarized and its permeability increases within seconds of interacting with cytotoxic bacteriocin, resulting in cell death. Cytotoxic bacteriocin's rapid degradation may imply a non-receptor mechanism of action. Bacteriocins are advantageous as therapeutic agents since they are short peptides that are not immunogenic. Second, they are simple amino acids that are quickly hydrolyzed. Bacteriocins are advantageous as therapeutic agents since they are short peptides that are not immunogenic (Ankaiah et al. 2017).

Second, they are simple amino acids that are quickly hydrolyzed. The loss of stability in the intestines or human tissues is one of the major drawbacks of employing bacteriocins as medications. Chemically synthesizing peptides using D-amino acids which are less sensitive to proteolytic cleavage

in the stomach, has been attempted. Lactococcin G analogs were made by replacing the N- and C-terminal residues with D-amino acids which have a lower sensitivity to exopeptidases but did not affect activity. Changes in salivaricin P trypsin recognition sites resulted in a stable molecule with a minor alteration in activity. Such research is justified to improve the stability and efficiency of anticancer bacteriocins. Furthermore, functional carriers for the targeted and regulated distribution of bacteriocins can improve their in vivo stability (Soltani et al. 2021).

Different Types of Bacteriocin against Cancer Cell

The surface variations provide selective action among the cancer cells and normal cells. The inner and outer surface of normal mammalian cells is distributed with phospholipids as a bilayered phospholipid membrane. Normal cells contain sphingomyelin and phosphatidylcholine as neutral choline-containing zwitter ionic phospholipids on their outer surface whereas the interior layer contains amino phospholipids such as phosphatidyl serine and phosphatidyl ethanol amine. In the case of cancer cells, the phospholipids are loosely bound with the asymmetric structure where the cell membrane carries anionic phosphatidyl serine along with O-glycosylated mucins, gangliosides, and heparin sulfates at a greater quantity than imparts predominantly negative charge on cancer cells (Schweizer 2009; Riedl et al. 2011). Whereas the bacteriocin is structured with a cationic cell surface which gets easily attracted to anionic cell surfaces rather than neutral cell surfaces (normal cells) (Dobrzyńska et al. 2005; Hoskin & Ramamoorthy 2008). The fluidity nature of cancer cell membranes is quite high rather than normal cells for this reason the facilitation becomes easy for bacteriocins to provoke a destabilization of membranes (Sok et al. 1999). In addition, cancer cells are structured with a high number of microvilli which paves the way for easy attachment of bacteriocins rather than normal cell structures with less amount of microvilli on their cell surface (Chan et al. 1998). For the above-mentioned reasons, bacteriocin peptides start to communicate with the cell surface which selectively targets and kills the cells with tumor formation and does not cause any damage to the normal healthy cells. Interestingly, such factors may facilitate the bacteriocins that initiate the pathway of cell lysis (apoptosis) by disrupting the quality of mitochondria, particularly on their surfaces, and releasing cytochrome (Smolarczyk et al. 2010). In addition, the results also found that necrosis occurs through cell membrane damage (Maher & McClean 2006; Vaucher et al. 2010).

The mechanism behind the suppression of cancer cells such as HNSCC, SW480, LS180, HT29, Caco2, SW1088, A375, and IMR-32 by bacteriocins starts with apoptosis (Schenkel & Bakovic 2014). Apoptosis index Bax/BCL-2 is initiated after the treatment of cancer cells with bacteria. After the initiation of the apoptosis index, the cell cycle becomes eventually arrested finally it inhibits the proliferation and migration of cancer cells by bringing down the expression genes such as *cea, ceam6, and mmp2f* (Kim et al. 2006).

Bacteriocin also disturbs the cell membrane and releases LDH (Lactate Dehydrogenase) and leads to the rapid accumulation of ROS (Reactive Oxygen Species) as shown in Figure 4. As a result of such reactions cancer cells completely loses their energy by inhibiting mitochondrial responsibilities (Maher & McClean 2006; Ahmadi et al. 2017). I hereby discussed the anticancer activity of some bacteriocins synthesized from lactic acid bacterium against different types of cancer cell lines that have been previously reported by the original author which gives a perspective view of the development of bacteriocins as a cancer drug. Anticancer activity of different classes of bacteriocin is given in Table 1.

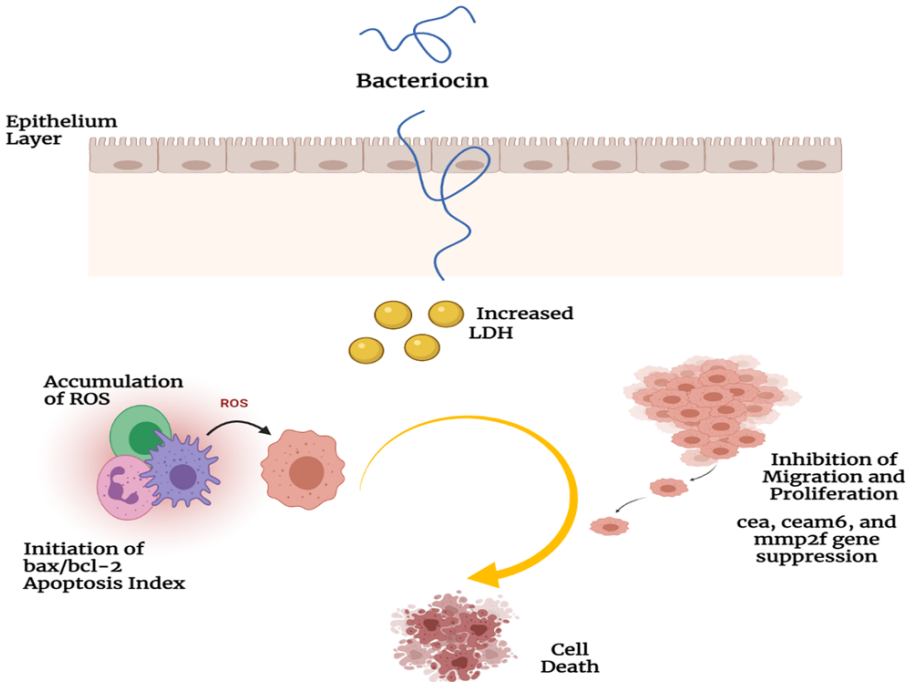

Figure 4. Pictorial representation of cell death initiation by bacteriocin**.** Bacteriocin exhibits anticancer activity by increasing the LDH, Accumulating the ROS, initiating the Apoptosis index followed by inhibiting cell proliferation and migration. The picture is created in biorender, https://biorender.com/

Nisin

Nisin is a 3.49 kDa lantibiotics bacteriocin synthesized by *Lactococcus lactis*, composed of 34 amino acids that exhibit a broad spectrum of antimicrobial activity by inhibiting gram-positive and negative bacteria. Nisin A and Nisin

Table 1. Anticancer activity of bacteriocin

S.no	Bacteriocin	Organism	size (kda)	Cancer cell line	Reference
1	Pediocin K2a2-3	*P.acidilacti* PAC1.0	3.5	DLD-1, A549	(Beaulieu et al. 2005)
2	Microcin E492	*K.pnuemoniea*	7.9	RJ2.25, HeLa, Jurkat,	(Hetz et al. 2002)
3.	Colicin E3	*E.coli*	9.8	P388, HS913T HeLa,	(Fuska et al. 1979)
4	Pediocin K2a2-3	*P.acidilacti* K2a2-3	4.6	HeLa HT2a,	(Villarante et al. 2011)
5	Nisin	*L. lactis*	3.5	HepG2, MCF7	(Paiva et al. 2011)
6	Pyosin S2	*Pseudomonas aeruginosa* 42A	74	mKS-A ,HepG2, HeLa, AS-II, TU-7	(Abdi-Ali et al. 2004)
7	Plantaricin A	*L.plantarum* C11	2.4	PC12, Jurkat, GH4, Reh,	(Zhao et al. 2006)

Z are the two variant natural polypeptides. They differ from each other in the 27th position by single amino acid. Nisin A contains histidine in the 27th position whereas Nisin Z contains asparagine in the 27th position (Norouzi et al. 2018). Nisin was accepted by FDA and WHO as a food preservative in 1988, with promising therapeutic applications (Zainodini et al. 2018). The potential anti-tumor activity of nisin Z was analyzed using different HNSCC cell lines, where oral keratinocytes served as a control. The cytotoxic activity occurred by apoptosis and cell growth arrest at the G2 phase during the cell cycle through a significant increase in calcium influx. In addition, an Affymetrix gene microarray was used to analyze the HNSCC cells treated with nisin Z on 39,000 genes which resulted in four-fold upregulation of apoptosis, regulation of cation transporter, and CHAC1. The *in vivo* study reported that injection of nisin for three weeks at the dosage of 200 mg per kilogram resulted in a significant reduction of a tumor with no evidence of fibrosis, necrosis, or inflammation. The long-term ingestion of nisin Z increased the rate of survival (Lewies et al. 2018). Nisin A and Z were analyzed for cytotoxic effects on HNSCC cells *in vitro* and *in vivo* method showed decreased proliferation in cells at different doses ranging from 100 to800 μg/ml (Kamarajan et al. 2015). Also, oral gavage of Nisin A and Z were tested for their properties along with their body weight for 3 weeks at the dosage of 800 mg/kg. The result signified a 3- and 17-fold reduction of the tumor. (Preet et al. 2015) tested the effect of nisin in combination with doxorubicin in a mice model. The individual compound analysis of nisin and doxorubicin showed mean tumor reduction after 4 weeks of treatment with volumes ranging from 14 to 51.3%. whereas 66.82 % reduction in tumor volume was reported by using nisin combined with doxorubicin due to chromatin condensation and marginalization of nuclear material when compared to the untreated group which may be due to apoptosis in tumor tissues. Nisin also exhibited a cytotoxic effect in HepG2 along with MCF-7. The inhibitory concentration of half of the cells (IC50) values for MCF-7 and HepG2 cell lines resulted at 105.46 and 112.25 μM, at the high concentration of 140 μM, both the cell lines' viabilities were less than 20%. Microscopic observations showed shrinkage in a cell, cytoplasm vacuolization along with condensation, lateralization, and cell detachment at concentrations above IC50 (Paiva et al. 2012). In another study, IC50 cytotoxicity test results were seen to be 225μM in Jurkat cell lines treated with nisin where no apoptosis was observed in the cancer cell line (Begde et al. 2011). Adenocarcinomas of the colon (HT29) and colorectum (Caco-2) human cell lines showed significant cytotoxic activity with IC50 values of 89.9μM and115μM when administered with nisin (Maher & McClean 2006). Also, evaluated the cytotoxic activity of nisin against SW480 (colon cancer cells) which resulted in anti-proliferative impact and increased apoptotic index. In addition, (Ahmadi et al. 2017) reported the intrinsic apoptotic pathway which is responsible for the cytotoxicity of nisin. A recent study also reported that nisin has an antitumor and antiproliferative effect with decreased cyclin D1 in colon cancer cell lines SW480 (Kamarajan et al. 2015).

The other recent work has been done by combining Thioridazine and Nisin as an antitumor drug against hepatocellular carcinoma cell lines (HepG2). The result indicated PI3K/AKT proliferation pathway inhibition, ROS induction, and angiogenic inhibition against hepatocellular carcinoma cell lines (HepG2). From the existing reports, Nisin has an anti-cancer activity by inducing apoptosis and arresting the cell cycle. However, the mechanism of antitumor activity remains unclear. Furthermore, potential analysis is required for the clinical application of nisin as cancer therapeutics.

Plantaricin

Plantaricin A (plnA) with 2.4kDa is a class II antimicrobial bacteriocin synthesized by *Lactobacillus plantarum* which can act as a hormone. They exhibit 3 variants that contain Plantaricin gene derived from the residues of 48 precursor genes. Plantaricin exhibited antimicrobial activity at the pH ranging 4.0–6.5 against lactic acid bacteria of closely related species by forming a hole in the cell membrane of target bacteria due to the amphiphilic nature of plnA that results in its oligomerization. In addition, different glycosylation patterns intake activities against different cell types including cancerous cells (Sand et al. 2010; Andersland et al. 2010; Sand et al. 2013). The Cytotoxicity of plantaricin at a rate of 25 μM at 20°C was determined against the human T cell leukemia (Jurkat) under in vitro conditions. It caused a 75% loss in the cell viability, whereas at 37◦C it decreased to 55%. The reason behind the loss of cell viability is apoptosis along with necrosis by fragmentation of cancer cells in cell nuclei and plasma membrane which was induced by Plantaricin with the increasing impact on intracellular concentration of caspase-3 in cancerous cells. In addition, plnA binds with negatively charged phospholipids (phosphatidylserine) by the formation of the amyloid-like fibrils which effectively concentrate on the targeted cell membrane (Zhao et al. 2006). Similar reports on plnA exposure with phosphatidylserine on the various cancer cell surface. The permeability study of Plantaricin A towards normal cell and GH4 cancerous cell lines was analyzed by whole-cell patch-clamp recordings and microfluorimetry. Interestingly, plnA was able to concentrate on all cancerous cells within a few seconds at a rate of 1 mM concentration, on the other hand, plnA was not able to permeabilize into normal cells even at the concentration of 1 Mm (Sand et al. 2007). Highlighted that membrane permeabilizing activity of plnA towards eukaryotic cell membranes is significantly due to the negative surface charge which is imparted by glycosylated membrane proteins. Plantaricin C belongs to a class of lantibiotics with 3.5 Kilo Dalton synthesized by *Lactobacillus plantarum*. The cytotoxic effects completely changed when a high concentration of plnC was used (Turner et al. 1999). In addition, plnC did not show any cytotoxic activity against HT29 and HeLa cell lines at variable concentrations (Martín et al. 2015).

Pediocin

Pediocin (class IIa bacteriocins) are small (>5 kDa), thermostable, cationic

encoded antimicrobial peptides built from 44 amino acids synthesized by the genera of *Pediococcus* (Papagianni 2003). The pediocins are assembled with the N terminal region by a conserved motif known as a "pediocin box" which forms a β-sheet structure built with conserved cysteines connected by Disulfide Bridge. The β-sheet region at a cationic site with N-terminal initiates binding whereas the hairpin-like C-terminal region initiates to enter inside the target cell surface in the hydrophobic sector leading to membrane leakage (Fimland et al. 2005). Cell lines including lung carcinoma and colorectal adenocarcinoma reported inhibition by recombinant pediocin PA-1 pideocin (*Pediococcus acidilactici* PAC1). Pediocin synthesized by *Pediococcus acidilactici* showed a cytotoxic reaction in different cell lines such as human liver carcinoma cell lines, cervical cancer cell lines, and mammalian gland adenocarcinoma cell lines.

Enterocin

Enterocin (65 kDa) is a heat-stable antimicrobial peptide synthesized by "*Enterococcus* sp." which shows activity at a wide range of pH and temperatures. Enterocin exhibits a broad spectrum of antimicrobial activity against gram-positive and gram-negative bacteria. Antimicrobial peptides act on the cytoplasmic membrane by creating pores and destroying the transmembrane channel which leads to cell damage. Antitumor activity of some enterocins has been reported by (Fimland et al. 2005) observed anticancer activity of enterocin-A by late apoptosis mechanism, cell cycle arrest at sub-G, G1 phase against HeLa, HT-29, Caco-2 cancer cell lines without showing any disturbance towards normal intestinal cell lines (INT-407). In addition (Drider et al. 2006) reported *in-vitro* anticancer activity of enterocin LNS18 against HepG2 cells at low concentrations with induction of ROS and cell cycle arrest at the G0 phase. According to the data provided, enterocin poses antitumor activity. Furthermore, therapeutic analysis can provide a solution for the findings.

Fusion Protein

The cloning of different bacteriocin peptides results in the formation of fusion proteins. The fusion protein has been developed to identify new cancer drugs. (Villarante et al. 2011), reported that the fusion of three different bacteriocins such as Enterocin A- R type Pyocin- Lactocin induced apoptosis in gastric cancer cell lines (AGS) at a concentration of 80 µg/ml. the recombinant fusion protein activates apoptosis which leads to death internally. Similar way different bacteriocins can be fused to develop promising cancer therapeutics.

OTHER APPLICATIONS OF BACTERIOCIN

Bacteriocin-a possible substitute for antibiotics

Emerging antibiotic-resistant bacteria, as well as the fact the application of broad-spectrum antibiotics would reduce the normal commensal of human

microbiota (Laxminarayan et al. 2013). Bacteriocins could be a viable alternative to antibiotics. Peptides, which are produced by various bacteria, can be bioengineered and have high potency and low toxicity. They can also be created in situ by probiotics. Bacteriocins can be broad-spectrum or narrow-spectrum. Bacteriocins work through a variety of processes that are often not the same as those used by antibiotics. Bacteriocins are divided into two types: those that target the cell membrane and those that target DNA, RNA, and protein metabolism within the cell (Lopetuso et al. 2019). Many bacteriocins have characteristics that suggest they could be useful in clinical situations. To date, however, the primary focus of their use has been on animal health rather than human health (Hanchi et al. 2018).

To fight against intestinal infections, bacteriocins can be produced in situ by the probiotic bacteria that are present in the gut. The bacteriocins namely lantibiotics and thiopeptides the class I bacteriocins act against gram-positive pathogens (Osbelt 2020). Nisin type of lantibiotics, planosporicin, Pep5, epidermin, gallidermin, mutacin B-Ny266, lacticin 3147, and actagardine (and their bioengineered derivatives) have a significant *in vitro* activity opposing pathogen that is clinically essential which include *Streptococcus pneumoniae, staphylococci* (including methicillin-resistant Staphylococcus aureus (MRSA)), *vancomycin-resistant enterococci* (VRE), various mycobacteria, *Propionibacterium acnes* and *Clostridium difficile. C. difficile*-associated diarrhea (CDAD) is an excellent illustration of this situation because the disease is frequently caused by and treated with drugs that can modify the resident gut microbiota (Van Staden 2015).

Application of bacteriocin in meat

LAB are found most commonly in meat, bacteria that produce bacteriocin are isolated and studied. Many bacteriocins that are isolated from food-related LAB are not the most effective in all food systems. But under appropriate conditions, many bacteriocins have an effective role in food applications (Deegan et al. 2006). One of the best examples and well-studied is the usage of nisin in meat systems**.** To prevent clostridial growth in meat, nitrates are used mostly. Based on safety the food industries are looking out for an alternative preservation method. Nisin or its compound along with the smaller number of nitrates can be used as a preservative to prevent the growth *of Clostridium.* Sausage is a commonly studied subject because its deterioration is often caused by lactic acid bacteria that can be found in the environment where the bacteriocins are the inhibitors (Vandenbergh 1993).

In other research, nisin is used in combination with Latic acid showed an increased effect on the growth of gram-negative bacteria. When used in a cold meat-binding system, nisin is also effective at inhibiting *Brochothrix thermosphacta.* In another investigation, pediocin AcH A-1 successfully inhibited the growth of *L. monocytogenes* in raw chicken. After 28 days of storage at 58^0C, the chickens treated with 2,400 AU/g pediocin had 2.8 log cfu/g *L.*

monocytogene but the control chickens had up to 8.1 log cfu/g (Branen & Davidson 2004).

Application in the food system

The use of bacteriocins in food systems, particularly nisin, has been examined. The bacteriocin's activity can be influenced significantly by the chemical composition and physical circumstances of the diet (Ghanbari & Jami 2013). At pH 2, for example, nisin is 228 times more soluble than at pH 8. Lactic acid bacteria are widely utilized as starter cultures in food fermentations, and researchers looked at using bacteriocin makers (Gillor et al. 2008). Inoculating Manchego cheese with a bacteriocin-producing *Ent. faecalis* strain reduced the organism's survival by 6 logs in 7 days, whereas the organism's survival in cheese prepared with a commercial starting culture was unaffected (Silva et al. 2018). Similarly, when a naturally contaminated salami sausage was inoculated with the bacteriocin producer *L. plantarum* MCS1, the amount of *L. monocytogenes* that survived was reduced (Ndlovu et al. 2013). Although the majority of commercial beginning cultures do not create bacteriocins, a few bacteriocin-producing meat starter cultures are currently available. A commercial *L. lactis* starter culture for Gouda cheese was developed using transposon-encoding nisin production and immunity (Roberts & Zottola 1993). Because *Pediococcus* sp. isn't used as cheese starting cultures, the plasmid-encoded pediocin was expressed in *Lac. lactis* to aid in the preservation of cheddar cheese and to ensure the microbiological quality of the fermentation process. According to the findings, the control cheese prepared from milk spiked with 106 CFU /ml was superior to the cheese created from milk spiked with 106 cfu /ml. After 2 weeks of ripening, *L. monocytogenes* had 107 cfu/g, but cheese prepared with the pediocin-producing strain had only 102 cfu/g. *Streptococcus thermophilus*, a key bacterium in dairy fermentations, has also been found to express pediocin PA-1 (Bagenda & Yamazaki 2007).

Another study found that *Lac. lactis* co-expressed pediocin PA-1 and nisin, two separate families of bacteriocins that have both been proved to be safe and effective (Reddy 2008). Even though the transformed cells produced only 11.8 percent of the pediocin produced by the control pediocin producer, the co-production of bacteriocins could have significant implications for food safety and reducing the chance of resistant organisms (Ahmad et al. 2017) Pediocin PA-1 has also been found to be expressed in the yeast *Saccharomyces cerevisiae* which aids in the preservation of wine, bread, and other yeast-containing foods, and the United States, it is approved as a food additive for this purpose (Van Reenen et al. 2003). Various applications of bacteriocin in food are given in Table 2.

OTHER BIOACTIVE COMPOUNDS PRODUCED BY LACTIC ACID BACTERIA

Antimicrobial compounds such as organic acid, hydrogen peroxide, CO_2, ethanol, fatty acid, Diacetyl, ethanol, reuterin, and bacteriocin are commonly

Table 2. Application of bacteriocin in food.

No	Bacteriocin	Organism	Target Pathogens	FoodProcessing	Reduction (Log Cfu G^{-1})	Reference
1	AcH Pediocin	*Lactobacillus plantarum*	*L. monocytogenes*	Cheese	1.0-2.0	(Loessner et al. 2003)
2	Enterocin	*Enterococcus faecium* *Enterococcus faecalis*	*L. monocytogenes* *S. aureus*	Milk Sausage	2.0 5.3	(Elotmani et al. 2002) (Ananou et al. 2005)
3	Nisin	*Lactococcus lactis*	*Brochothrix thermosphacta* *L. monocytogenes*	Pork Fermented milk	3.5 6.0	(Nattress et al. 2001)
4	Nisin Z	*Lactococcus lactis*	*S. aureus*	Afuega'l pitu cheese	2.0	(Rilla et al. 2004)
5	Aureocin A70	*Staphylococcus aureus A70*	*L. monocytogenes*	Skim milk	5.5	(Fagundes et al. 2016)

produced by Lactic Acid Bacteria (Ross et al. 2002). They tend to inhibit the growths of microflora as well as bacteria and pathogens causing spoilage (Jeevaratnam et al. 2005).

Organic Acid

Fermentation decreases usable carbohydrates and produces a variety of tiny molecular mass organic compounds with antibacterial activity, the most prevalent of which are lactic, acetic, and propionic acid (Pereira Da Costa & Conte-Junior 2015). The amount and kind of organic acids produced during fermentation are determined by the organisms' species, culture composition, and growth circumstances (Güzel-Seydim et al. 2000). The major metabolite of LAB is lactic acid. Many bacteria, fungi, and yeasts are poisonous to the undissociated form of lactic acid, which is present at low pH. Other organic acids generated by LAB through heterofermentative routes include acetic and propionic acids (Caplice & Fitzgerald 1999). Due to the high pKa values (lactic acid 3.08, acetic acid 4.75, and propionic acid 4.87), acetic acid and propionic acid have an effective antimicrobial activity.

Carbon dioxide

Carbon dioxide is mostly produced during hexoses heterofermentative LAB. Its antibacterial action's precise mechanism is still unknown. However, CO_2 generation creates an anaerobic environment, which inhibits enzymatic decarboxylation, and CO_2 accumulation in the membrane lipid bilayer which may result in permeability dysfunction. Many food spoilage germs, particularly Gram-negative psychotropic bacteria, can be efficiently inhibited by carbon dioxide whereas, in LAB, some yeasts exhibited significant tolerance. Various microorganisms have different levels of carbon dioxide resistance. Some food spoilage-causing bacteria are inhibited by carbon dioxide concentrations greater than 50% the inhibition of microorganisms rises linearly with increasing carbon dioxide concentration, depending on the food and microflora (Caplice & Fitzgerald 1999).

Hydrogen Peroxide (H_2O_2)

In the presence of oxygen, LAB produces hydrogen peroxide by the activity of flavoprotein oxidases or nicotinamide adenine hydroxy dinucleotide (NADH) peroxidase. The antibacterial activity of hydrogen peroxide is due to the oxidation of sulfhydryl groups which causes denaturation of several enzymes, as well as the peroxidation of membrane lipids which results in enhanced membrane permeability. It is used as a precursor for bactericidal free radical production which includes superoxide and hydroxyl radicals that damages the DNA. A study found that *Lactobacillus* and *Lactococcus* strains produced H_2O_2 which inhibited *Staphylococcus aureus*, *Pseudomonas* sp., and other psychotropic microbes in food (Caplice & Fitzgerald 1999).

Diacetyl

Some species and strains of the genera *Streptococcus, Leuconostoc, Lactobacillus*, and *Pediococcus*, as well as other microbes, generate diacetyl. Since the 1930s, diacetyl has been known to have antibacterial properties. It inhibits Gram-negative bacteria growth by interfering with arginine utilization by reacting with the arginine-binding protein. Gram-negative bacteria, yeasts, and mold are more sensitive to diacetyl than Gram-positive bacteria, and its mode of action is thought to be related to interference with arginine use by reacting with Gram-negative bacteria's arginine-binding proteins. It has a limited application as a food preservative (Caplice & Fitzgerald 1999).

Reuteri

Lb. reuteri, a heterofermentative species that live in the gastrointestinal tracts of humans and animals produce reuterin. It is formed during the *Lb. reuteri's* anaerobic growth on a mixture of glucose and glycerol or glyceraldehydes. 3-hydroxypropanal (β-hydroxypropionaldehyde), a highly soluble pH-neutral molecule in equilibrium with its hydrated monomeric and cyclic dimeric forms, has been chemically discovered.

(Olaoye & Ntuen 2011) have indicated that when natural preservative techniques are used, probiotics food may be acquired while food-borne pathogens and spoilage pollutants can be reduced. They also described how bacteriocins' inability to permeate the outer membrane of Gram-negative bacteria prevented them from acting on the bacteria. The principal dangers associated with spoilage and pathogenic bacteria found in fresh and processed aquatic foods subjected to short-term storage, as well as the biological techniques that can be employed to reduce their proliferation, were also underlined. (Parada et al. 2007) emphasized that bacteriocin has been proposed as a bio preservative agent capable of reducing the growth of some contaminating bacteria in meat and meat products. However, commercial availability is restricted and expensive. They also looked into selecting *Lactobacillus* sp. isolates with the ability to produce bacteriocins to inhibit the growth of *E. coli, Salmonella typhimurium, and Listeria monocytogenes*, as well as optimizing the bacteriocin production process. (Onwuakor et al. 2014) has demonstrated the

separation, identification, and investigation of physical and cultural aspects of the bacteriocins produced by LAB from traditional Indian fermented foods. Bacteriocin-producing *Lactobacillus lactis* strains derived from maritime environments were investigated and found to have a wide spectrum of antibacterial activity against some of the most common food-borne infections. Their research found that bacteriocin can be used as a food preservative and the *L. lactis* strain can be used as a probiotic.

Bacteriocin-like inhibitory compounds (Bt-BLIS) produced by Mexican *Bacillus thuringiensis* strains had a mild to a wide range of antibacterial activity, being harmful to clinically significant Gram-positive and Gram-negative bacteria, such as common causative agents of human diseases such as strep throat and scarlet fever, septicemia, pneumonia, urinary tract infection, and staph infections. The discovery of a bacteriocin-like inhibitory substance (BLIS) produced by a soil isolate of *Lactobacillus* animalis with a broad inhibitory spectrum against Gram-positive bacteria was the first. An antimicrobial peptide produced by a bacterium isolated from a cattle abattoir's effluent pond was extracted and characterized (Rodali et al. 2013). *Geobacillus stearothermophilus* strains isolated from oil wells in Lithuania developed four novel heat-stable bacteriocin-like compounds. The secretion of the examined bacteriocins began during initial logarithmic growth and abruptly decreased once the culture reached its stationary phase. After pretreatment with proteolytic enzymes, the bacteriocins' antibacterial activity on sensitive indicator cells vanished, demonstrating that they are proteinaceous. The biotechnology sector is interested in bacteriocins with the interaction between various spectra, especially antipathogenic activity because they could be employed as antimicrobial agents in medicine, agriculture, and food item.

CONCLUSION

LAB produce biologically active byproducts as a result of fermentation. The bacteriocins produced by LABs have gained attention due to its non-toxicity. In addition, to antimicrobial property bacteriocins also posses' anticancer activity. Nisin, plantaricin, pediocin, enterocin and fusion proteins had shown sufficient anticancer property against different cell lines. The production, purification and the factors affecting the production of bacteriocins should be considered for pharmaceutical aspects. During the fermentation process LABs also synthesize other chemical compounds such as organic acid, carbon dioxide, hydrogen peroxide and diacetyl which also contributes for the benefits of human. In this regard, the utilization of chemical compounds produced by LAB will pave the way to meet the need for natural requirements. However, much research is needed for the industrial application of bacteriocin and other compounds.

AUTHORS CONTRIBUTION

M.V. collected and analyzed the data and wrote the manuscript, T.S. designed and supervised.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding this manuscript.

REFERENCES

Abdi-Ali, A. et al., 2004. Cytotoxic effects of pyocin S2 produced by Pseudomonas aeruginosa on the growth of three human cell lines. *Canadian journal of microbiology*, 50(5), pp.375–381. doi: 10.1139/w04-019

Abriouel, H. et al., 2003. A simple method for semi-preparative-scale production and recovery of enterocin AS-48 derived from Enterococcus faecalis subsp. liquefaciens A-48-32. *Journal of microbiological methods*, 55(3), pp.599–605. doi: 10.1016/s0167-7012(03)00202-1

Ahmad, V. et al., 2017. Antimicrobial potential of bacteriocins: in therapy, agriculture and food preservation. *International journal of antimicrobial agents*, 49(1), pp.1–11. doi: 10.1016/j.ijantimicag.2016.08.016

Ahmadi, S., 2017. The apoptotic impact of nisin as a potent bacteriocin on the colon cancer cells. *Microbial pathogenesis*, 111, pp.193–197. doi: 10.1016/j.micpath.2017.08.037

Alvarez-Sieiro, P et al., 2016. Bacteriocins of lactic acid bacteria: extending the family. *Applied microbiology and biotechnology*, 100(7), pp.2939–2951. doi: 10.1007/s00253-016-7343-9

Ananou, S. et al., 2005. Control of Staphylococcus aureus in sausages by enterocin AS-48. *Meat science*, 71(3), pp.549–556. doi: 10.1016/j.meatsci.2005.04.039

Andersland, K. et al., 2010. Peptide pheromone plantaricin a produced by Lactobacillus plantarum permeabilizes liver and kidney cells. *The Journal of membrane biology*, 235(2), pp.121–129. doi: 10.1007/s00232-010-9263-4

Ankaiah, D. et al., 2017. Probiotic characterization of Enterococcus faecium por1: cloning, over expression of Enterocin-A and evaluation of antibacterial, anti-cancer properties. *Journal of Functional Foods*, 38, pp. 280–292.

Bagenda, D.K. & Yamazaki, K., 2007. Application of bacteriocins in food preservation and safety, *Food*, 1, pp. 137–148.

Beaulieu, L., 2005. Production, purification et caracterisation de la pediocine PA-1 naturelle et de ses formes recombinantes: Contribution a la mise en evidence d'une nouvelle activite biologique (French and English text).

Beaulieu, L. et al., 2006. An improved and simplified method for the large-scale purification of pediocin PA-1 produced by Pediococcus acidilactici. *Biotechnology and applied biochemistry*, 43(Pt 2), pp.77–84. doi: 10.1042/BA20050041

Begde, D. et al., 2011. Immunomodulatory efficacy of nisin--a bacterial lantibiotic peptide. *Journal of peptide science: an official publication of the European Peptide Society*, 17(6), pp.438–444. doi: 10.1002/psc.1341

Branen, J.K. & Davidson, P.M., 2004. Enhancement of nisin, lysozyme, and monolaurin antimicrobial activities by ethylenediaminetetraacetic acid and lactoferrin. *International journal of food microbiology*, 90(1), pp.63–74. doi: 10.1016/s0168-1605(03)00172-7

Bray, F. et al., 2021. The ever-increasing importance of cancer as a leading cause of premature death worldwide. *Cancer*, 127(16), pp.3029–3030. doi: 10.1002/cncr.3358

Callewaert, R. & De Vuyst, L., 1999. Expanded bed adsorption as a unique unit operation for the isolation of bacteriocins from fermentation media. *Bioseparation*, 8(1-5), pp.159–168.

Callewaert, R. et al., 1999. Characterization and production of amylovorin L471, a bacteriocin purified from Lactobacillus amylovorus DCE 471 by a novel three-step method. *Microbiology (Reading, England)*, 145 (Pt 9), pp.2559–2568. doi: 10.1099/00221287-145-9-2559

Caplice, E. & Fitzgerald, G.F., 1999. Food fermentations: role of microorganisms in food production and preservation. *International journal of food microbiology*, 50(1-2), pp.131–149. doi: 10.1016/s0168-1605(99)00082-3

Carolissen-Mackay, V., Arendse, G. & Hastings, J.W., 1997. Purification of bacteriocins of lactic acid bacteria: problems and pointers. *International journal of food microbiology*, 34(1), pp.1–16. doi: 10.1016/s0168-1605(96)01167-1

Carr, F.J., Chill, D. & Maida, N., 2002. The lactic acid bacteria: a literature survey. *Critical reviews in microbiology*, 28(4), pp.281–370. doi: 10.1080/1040-840291046759

Chabner, B.A. & Roberts, T.G.Jr., 2005. Timeline: Chemotherapy and the war on cancer. *Nature Reviews Cancer*, 5(1), pp.65–72. doi: 10.1038/nrc1529

Chan, S.C. et al., 1998. Microscopic observations of the different morphological changes caused by anti-bacterial peptides on Klebsiella pneumoniae and HL-60 leukemia cells. *Journal of peptide science: an official publication of the European Peptide Society*, 4(7), pp.413–425. doi: 10.1002/(SICI)1099-1387(199811)4:7%3C413::AID-PSC160%3E3.0.CO;2-W

Chu, H.L. et al., 2015. Novel antimicrobial peptides with high anticancer activity and selectivity. *PloS one*, 10(5), e0126390. doi: 10.1371/journal.pone.0126390

Cintas, L.M. et al., 2001. Review: Bacteriocins of Lactic Acid Bacteria. *Food Science and Technology International*, 7(4), pp.281–305. doi: 10.1106/R8DE-P6HU-CLXP-5RYT.

Cleveland, J. et al., 2001. Bacteriocins: safe, natural antimicrobials for food preservation. *International journal of food microbiology*, 71(1), pp.1–20. doi: 10.1016/s0168-1605(01)00560-8

Cotter, P.D., Hill, C. & Ross, R.P. 2005. Bacteriocins: developing innate immunity for food. *Nature reviews Microbiology*, 3(10), pp.777–788. doi: 10.1038/nrmicro1273

Deegan, L.H. et al., 2006. Bacteriocins : Biological tools for bio-preservation and shelf-life extension, 16(9), pp. 1058–1071. doi: 10.1016/j.idairyj.2005.10.026

Dobrzyńska, I. et al., 2005. Changes in electric charge and phospholipids composition in human colorectal cancer cells. *Molecular and cellular biochemistry*, 276(1-2), pp.113–119. doi: 10.1007/s11010-005-3557-3

Drider, D. et al., 2006. The continuing story of class IIa bacteriocins. *Microbiology and molecular biology reviews: MMBR*, 70(2), pp.564–582. doi: 10.1128/MMBR.00016-05

Dufour, A. et al., 2000. IS1675, a novel lactococcal insertion element, forms a transposon-like structure including the lacticin 481 lantibiotic operon. *Journal of bacteriology*, 182(19), pp.5600–5605. doi: 10.1128/JB.182.19.5600-5605.2000

Elotmani, F. et al., 2002. Characterization of anti-Listeria monocytogenes bacteriocins from Enterococcus faecalis, Enterococcus faecium, and Lactococcus lactis strains isolated from Raïb, a Moroccan traditional fermented milk. *Current microbiology*, 44(1), pp.10–17. doi: 10.1007/s00284-001-0067-8

Fagundes, P.C. et al., 2016. The four-component aureocin A70 as a promising agent for food biopreservation. *International journal of food microbiology*, 237, pp.39–46. doi: 10.1016/j.ijfoodmicro.2016.08.017

Fimland, G. et al., 2005. Pediocin-like antimicrobial peptides (class IIa bacteriocins) and their immunity proteins: biosynthesis, structure, and mode of action. *Journal of peptide science: an official publication of the European Peptide Society*, 11(11), pp.688–696. doi: 10.1002/psc.699

Fuska, J. et al., 1979. Effect of colicin E3 on leukemia cells P388 in vitro. *Experientia*, 35(3), pp.406–407. doi: 10.1007/BF01964380

Ghanbari, M. & Jami, M., 2013. Lactic Acid Bacteria and Their Bacteriocins: A Promising Approach to Seafood Biopreservation, in M. Kongo (ed.), Lactic Acid Bacteria - R & D for Food, Health and Livestock Purposes, IntechOpen, London. doi:10.5772/50705.

Gillor, O., Etzion, A. & Riley, M. A., 2008. The dual role of bacteriocins as anti- and probiotics. *Applied microbiology and biotechnology*, 81(4), pp.591–606. doi: 10.1007/s00253-008-1726-5

Gordon, Y.J., Romanowski, E.G. & McDermott, A.M., 2005. A review of antimicrobial peptides and their therapeutic potential as anti-infective drugs. *Current eye research*, 30(7), pp.505–515. doi: 10.1080/02713680590968637

Güzel-Seydim, Z.B. et al., 2000. Determination of organic acids and volatile flavor substances in kefir during fermentation. *Journal of Food composition and Analysis*, 13(1), pp.35–43. doi: 10.1006/jfca.1999.0842

Hanchi, H. et al., 2018. The Genus *Enterococcus*: Between Probiotic Potential and Safety Concerns-An Update. *Frontiers in microbiology*, 9, pp.1791. doi: 10.3389/fmicb.2018.01791

Hetz, C. et al., 2002. Microcin E492, a channel-forming bacteriocin from Klebsiella pneumoniae, induces apoptosis in some human cell lines. *Proceedings of the National Academy of Sciences of the United States of America*, 99(5), pp.2696–2701. doi: 10.1073/pnas.052709699

Hoskin, D.W. & Ramamoorthy, A., 2008. Studies on anticancer activities of antimicrobial peptides. *Biochimica et biophysica acta*, 1778(2), pp.357–375. doi: 10.1016/j.bbamem.2007.11.008

Jack, R.W., Tagg, J.R. & Ray, B., 1995. Bacteriocins of gram-positive bacteria. *Microbiological reviews*, 59(2), pp.171–200. doi: 10.1128/mr.59.2.171-200.1995

Jeevaratnam et al., 2005a. Biological preservation of foods–Bacteriocins of lactic acid bacteria. *Indian J. Biotechnol*,4.

Kamarajan, P. et al., 2015. Nisin ZP, a Bacteriocin and Food Preservative, Inhibits Head and Neck Cancer Tumorigenesis and Prolongs Survival. *PloS one*, 10(7). doi: 10.1371/journal.pone.0131008

Kim, R., Emi, M. & Tanabe, K. 2006. Role of mitochondria as the gardens of cell death. *Cancer chemotherapy and pharmacology*, 57(5), pp.545–553. doi: 10.1007/s00280-005-0111-7

Lao, Y. et al., 2014. Application of proteomics to determine the mechanism of action of traditional Chinese medicine remedies. *Journal of ethnopharmacology*, 155(1), pp.1–8. doi: 10.1016/j.jep.2014.05.022

Laxminarayan, R. et al., 2013. Antibiotic resistance-the need for global solutions. *The Lancet. Infectious diseases*, 13(12), pp.1057–1098. doi: 10.1016/S1473-3099(13)70318-9

Leal-Sánchez, et al., 2002. Optimization of bacteriocin production by batch fermentation of Lactobacillus plantarum LPCO10. *Applied and environmental microbiology*, 68(9), pp.4465–4471. doi: 10.1128/AEM.68.9.4465-4471.2002

Leroy, F. et al., 2003. The stimulating effect of a harsh environment on the bacteriocin activity by Enterococcus faecium RZS C5 and dependency on the environmental stress factor used. *International journal of food microbiology*, 83(1), pp.27–38. doi: 10.1016/s0168-1605(02)00316-1

Lewies, A. et al., 2018. The antimicrobial peptide nisin Z induces selective toxicity and apoptotic cell death in cultured melanoma cells. *Biochimie*, 144, pp.28–40. doi: 10.1016/j.biochi.2017.10.009

Li, C. et al., 2002. Optimization of a cultural medium for bacteriocin production by Lactococcus lactis using response surface methodology. *Journal of biotechnology*, 93(1), pp.27–34. doi: 10.1016/s0168-1656(01)00377-7

Loessner, M. et al., 2003. A pediocin-producing Lactobacillus plantarum strain inhibits Listeria monocytogenes in a multispecies cheese surface microbial ripening consortium. *Applied and environmental microbiology*, 69 (3), pp.1854–1857. doi: 10.1128/AEM.69.3.1854-1857.2003

Lopetuso, L.R. et al., 2019. Bacteriocins and Bacteriophages: Therapeutic Weapons for Gastrointestinal Diseases? *International journal of molecular sciences*, 20(1), pp.183. doi: 10.3390/ijms20010183

Maher, S. & McClean, S., 2006. Investigation of the cytotoxicity of eukaryotic and prokaryotic antimicrobial peptides in intestinal epithelial cells in vitro. *Biochemical pharmacology*, 71(9), pp.1289–1298. doi: 10.1016/ j.bcp.2006.01.012

Mahrous, A.H. et al., 2013. Study Bacteriocin Production and Optimization Using New Isolates of *Lactobacillus* spp. Isolated from Some Dairy Products under Different Culture Conditions. *Food and Nutrition Sciences*, 4(3), pp.342-356. doi: 10.4236/fns.2013.43045.

Martín, R. et al., 2015. Surface glycosaminoglycans protect eukaryotic cells against membrane-driven peptide bacteriocins. *Antimicrobial agents and chemotherapy*, *59*(1), pp.677–681. doi: 10.1128/AAC.04427-14

Martínez, J.M. et al., 1998. Generation of polyclonal antibodies of predetermined specificity against pediocin PA-1. *Applied and environmental microbiology*, 64(11), pp.4536–4545. doi: 10.1128/AEM.64.11.4536-4545.1998

Mokoena, M.P., 2017. Lactic Acid Bacteria and Their Bacteriocins: Classification, Biosynthesis and Applications against Uropathogens: A Mini-Review. *Molecules (Basel, Switzerland)*, 22(8), pp.1255. doi: 10.3390/ molecules22081255

Nattress, F.M., Yost, C.K. & Baker, L.P., 2001. Evaluation of the ability of lysozyme and nisin to control meat spoilage bacteria. *International journal of food microbiology*, 70(1-2), pp.111–119. doi: 10.1016/s0168-1605(01) 00531-1

Ndlovu, B., et al., 2015. Screening, identification and characterization of bacteriocins produced by wine-isolated LAB strains. *Journal of applied microbiology*, 118(4), pp.1007–1022. doi: 10.1111/jam.12752

Norouzi, Z. et al., 2018. Nisin, a potent bacteriocin and anti-bacterial peptide, attenuates expression of metastatic genes in colorectal cancer cell lines. *Microbial pathogenesis*, 123, pp.183–189. doi: 10.1016/ j.micpath.2018.07.006

Olaoye, O.A. & Ntuen, I.G., 2011. Spoilage and preservation of meat: a general appraisal and potential of lactic acid bacteria as biological preservatives. *International Research Journal of Biotechnology*, 2(1), pp.33–46.

Onwuakor, C.E. et al., 2014. Effect of Varied Culture Conditions on Crude Supernatant (Bacteriocin) Production from Four *Lactobacillus* Species Isolated from Locally Fermented Maize (Ogi). *American Journal of Microbiological Research*, 2(5), pp.125-130.

Osbelt, L., 2020. *Influence of the intestinal microbiota composition on the individual susceptibility towards enteric infections in healthy individuals and hematological patients*. Otto-von-Guericke Universität Magdeburg. doi: 10.25673/35695.

Ovchinnikov, K.V. et al., 2016. Novel Group of Leaderless Multipeptide Bacteriocins from Gram-Positive Bacteria. *Applied and environmental microbiology*, 82(17), pp.5216–5224. doi: 10.1128/AEM.01094-16

Paiva, A.D. et al., 2012. Toxicity of bovicin HC5 against mammalian cell lines and the role of cholesterol in bacteriocin activity. *Microbiology (Reading, England)*, 158(Pt 11), pp.2851–2858. doi: 10.1099/mic.0.062190-0

Paiva, A.D., Breukink, E. & Mantovani, H.C., 2011. Role of lipid II and membrane thickness in the mechanism of action of the lantibiotic bovicin HC5. *Antimicrobial agents and chemotherapy*, 55(11), pp.5284–5293. doi: 10.1128/AAC.00638-11

Papagianni, M., 2003. Ribosomally synthesized peptides with antimicrobial properties: biosynthesis, structure, function, and applications. *Biotechnology advances*, 21(6), pp.465–499. doi: 10.1016/s0734-9750(03)00077-6

Parada, J.L. et al., 2007. Bacteriocins from lactic acid bacteria: Purification, properties and use as biopreservatives. *Brazilian Archives of Biology and Technology*, 50(3), pp.521–542. doi: 10.1590/S1516-89132007000300018

Parente, E. & Ricciardi, A., 1999. Production, recovery and purification of bacteriocins from lactic acid bacteria. *Applied microbiology and biotechnology*, 52(5), pp.628–638. doi: 10.1007/s002530051570

Patton, G.C. & van der Donk, W.A., 2005. New developments in lantibiotic biosynthesis and mode of action. *Current opinion in microbiology*, 8(5), pp.543–551. doi: 10.1016/j.mib.2005.08.008

Pereira Da Costa, M. & Conte-Junior, C.A., 2015. Chromatographic methods for the determination of carbohydrates and organic acids in foods of animal origin, *Comprehensive Reviews in Food Science and Food Safety*, 14(5), pp.586–600. doi: 10.1111/1541-4337.12148

Porta, C. et al., 2015. Renal effects of targeted anticancer therapies. *Nature reviews. Nephrology*, 11(6), pp.354–370. doi: 10.1038/nrneph.2015.15

Preet, S. et al., 2015. Effect of nisin and doxorubicin on DMBA-induced skin carcinogenesis--a possible adjunct therapy. *Tumour biology: the journal of the International Society for Oncodevelopmental Biology and Medicine*, 36(11), pp.8301–8308. doi: 10.1007/s13277-015-3571-3

Reddy, J.R. 2008. *The Influence of The Leader Sequence on Antimicrobial Activity of Leucocin A, An Antilisterial Bacteriocin Produced by Leuconostoc Gelidum UAL 187-22*. University of KwazuluNatal.

Riedl, S. et al., 2011. In search of a novel target - phosphatidylserine exposed by non-apoptotic tumor cells and metastases of malignancies with poor treatment efficacy. *Biochimica et biophysica acta*, 1808(11), pp.2638–2645. doi: 10.1016/j.bbamem.2011.07.026

Rilla, N., Martínez, B. & Rodríguez, A., 2004. Inhibition of a methicillin-resistant Staphylococcus aureus strain in Afuega'l Pitu cheese by the nisin Z-producing strain Lactococcus lactis subsp. lactis IPLA 729. *Journal of food protection*, 67(5), pp.928–933. doi: 10.4315/0362-028x-67.5.928

Roberts, R.F. & Zottola, E.A., 1993. Shelf-life of pasteurized process cheese spreads made from cheddar cheese manufactured with a nisin-producing starter culture. *Journal of dairy science*, 76(7), pp.1829–1836. doi: 10.3168/jds.S0022-0302(93)77515-3

Rodali, V.P. et al., 2013. Biosynthesis and potential applications of bacteriocins', *Journal of Pure and Applied Microbiology*, 7(4), pp. 2933–2945.

Rodr, E. et al., 2000. Diversity of bacteriocins produced by lactic acid bacteria isolated from raw milk', 10. doi: 10.1016/S0958-6946(00)00017-0

Rodríguez, J. M. et al., 2003. Heterologous production of bacteriocins by lactic acid bacteria. *International journal of food microbiology*, 80(2), pp.101–116. doi: 10.1016/s0168-1605(02)00153-8

Ross, R.P., Morgan, S. & Hill, C., 2002. Preservation and fermentation: past, present and future. *International journal of food microbiology*, 79(1-2), pp.3–16. doi: 10.1016/s0168-1605(02)00174-5

Sand, S.L. et al., 2007. The bacterial peptide pheromone plantaricin A permeabilizes cancerous, but not normal, rat pituitary cells and differentiates between the outer and inner membrane leaflet. *The Journal of membrane biology*, 216(2-3), pp.61–71. doi: 10.1007/s00232-007-9030-3

Sand, S.L. et al., 2010. Plantaricin A, a peptide pheromone produced by Lactobacillus plantarum, permeabilizes the cell membrane of both normal and cancerous lymphocytes and neuronal cells. *Peptides*, 31(7), pp.1237–1244. doi: 10.1016/j.peptides.2010.04.010

Sand, S.L. et al., 2013. Plantaricin A, a cationic peptide produced by Lactobacillus plantarum, permeabilizes eukaryotic cell membranes by a mechanism dependent on negative surface charge linked to glycosylated membrane proteins. *Biochimica et biophysica acta*, 1828(2), pp.249–259. doi: 10.1016/j.bbamem.2012.11.001

Schenkel, L. C. & Bakovic, M. 2014. Formation and regulation of mitochondrial membranes. *International journal of cell biology*,709828. doi: 10.1155/2014/709828

Schöbitz et al., 2006. Bacteriocin like substance production by Carnobacterium piscicola in a continuous system with three culture broths. Study of antagonism against Listeria monocytogenes on vacuum packaged salmon. *Brazilian Journal of Microbiology - Braz J Microbiol.* 37. doi:10.1590/S1517-83822006000100010.

Schweizer, F., 2009. Cationic amphiphilic peptides with cancer-selective toxicity. *European journal of pharmacology*, 625(1-3), pp.190–194. doi : 10.1016/j.ejphar.2009.08.043

Seo, M. D. et al., 2012. Antimicrobial peptides for therapeutic applications: a review. *Molecules (Basel, Switzerland)*, 17(10), pp.12276–12286. doi: 10.3390/molecules171012276

Shaw, A., 2021. Are Bacteriocins an alternative to Antibiotics? Food *and Agriculture Spectrum Journal*, 2(03), pp.273–276.

Siegel, R.L., Miller, K.D. & Jemal, A., 2019. Cancer statistics, 2019. *CA: a cancer journal for clinicians*, 69(1), pp.7–34. doi: 10.3322/caac.21551

Silva, C., Silva, S. & Ribeiro, S.C., 2018. Application of Bacteriocins and Protective Cultures in Dairy Food Preservation. *Frontiers in microbiology*, 9, pp.594. doi: 10.3389/fmicb.2018.00594

Smolarczyk, R. et al., 2010. Anticancer effects of CAMEL peptide. *Laboratory investigation; a journal of technical methods and pathology*, 90(6), pp.940–952. doi: 10.1038/labinvest.2010.58

Sok, M., Sentjurc, M., & Schara, M. 1999. Membrane fluidity characteristics of human lung cancer. *Cancer letters*, 139(2), pp.215–220. doi: 10.1016/s0304-3835(99)00044-0

Soltani, S. et al., 2021. Bacteriocins as a new generation of antimicrobials: toxicity aspects and regulations. *FEMS microbiology reviews*, 45(1). doi: 10.1093/femsre/fuaa039

Sugrue, I. et al., 2020. *Actinomyces* Produces Defensin-Like Bacteriocins (Actifensins) with a Highly Degenerate Structure and Broad Antimicrobial Activity. *Journal of bacteriology*, 202(4). doi: 10.1128/JB.00529-19

Turner, D.L. et al., 1999. Solution structure of plantaricin C, a novel lantibiotic. *European journal of biochemistry*, 264(3), pp.833–839. doi: 10.1046/j.1432-1327.1999.00674.x

Van Kraaij, C. et al., 1999. Lantibiotics: biosynthesis, mode of action and applications. *Natural product reports*, 16(5), pp.575–587. doi: 10.1039/a804531c

Van Reenen et al., 2003. Characterization and heterologous expression of a class IIa bacteriocin, plantaricin 423 from Lactobacillus plantarum 423, in Saccharomyces cerevisiae. *International journal of food microbiology*, 81(1), pp.29–40. doi: 10.1016/s0168-1605(02)00164-2

Van Staden, A.D.P., 2015. In vitro and in vivo characterization of amyloliquecidin, a novel two-component lantibiotic produced by Bacillus amyloliquefaciens. *Stellenbosch: Stellenbosch University.*

Vaucher, R.A., Teixeira, M.L. & Brandelli, A., 2010. Investigation of the cytotoxicity of antimicrobial peptide P40 on eukaryotic cells. *Current Microbiology*, 60(1), pp.1-5 doi: 10.1007/s11274-013-1575-y

Vandenbergh, P.A., 1993. Lactic acid bacteria, their metabolic products and interference with microbial growth. *FEMS Microbiology Reviews*, 12(1-3), pp.221-237. doi: 10.1016/0168-6445(93)90065-H

Villarante, K.I. et al., 2011. Purification, characterization and in vitro cytotoxicity of the bacteriocin from Pediococcus acidilactici K2a2-3 against human colon adenocarcinoma (HT29) and human cervical carcinoma (HeLa) cells. *World Journal of Microbiology and Biotechnology*, 27(4), pp. 975–980. doi: 10.1007/s11274-010-0541-1

Yang, R. & Ray, B., 1994. Factors influencing production of bacteriocins by lactic acid bacteria. *Food Microbiology*, 11(4), pp. 281–291. doi: 10.1006/fmic.1994.1032

Yi, H. et al., 2013. Effect of exogenous factors on bacteriocin production from Lactobacillus paracasei J23 by using a resting cell system. *International journal of molecular sciences*, 14(12), pp.24355–24365. doi: 10.3390/ijms141224355

Zacharof, M.P. & Lovitt, R.W., 2012. Bacteriocins Produced by Lactic Acid Bacteria a Review Article. *APCBEE Procedia*, 2, pp.50–56. doi: 10.1016/j.apcbee.2012.06.010

Zainodini, N. et al., 2018. Nisin Induces Cytotoxicity and Apoptosis in Human Asterocytoma Cell Line (SW1088). *Asian Pacific journal of cancer prevention: APJCP*, 19(8), pp.2217–2222. doi: 10.22034/APJCP.2018.19.8.2217

Zhao, H. et al., 2006. Interaction of the antimicrobial peptide pheromone Plantaricin A with model membranes: implications for a novel mechanism of action. *Biochimica et biophysica acta*, 1758(9), pp.1461–1474. doi: 10.1016/j.bbamem.2006.03.037

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Review Article

Immunonutrition and Hepatoprotectant Aspects of *Moringa Oleifera* Leaf Nanoemulsion Syrup as an Antituberculosis Adjuvant for Children with Tuberculosis

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ABSTRACT

Tuberculosis in children is a global health problem that decreases the quality of life of children. Based on data from the Indonesian Ministry of Health in 2016, nearly 69.000 children had tuberculosis and the case keeps increasing every year. *Moringa oleifera* leaf nanoemulsion syrup has immunonutrition and hepatoprotectant effects in children with tuberculosis. *Moringa oleifera* leaf nanoemulsion syrup contains proteins, micronutrients, and minerals which have a biological role as an immunity agent and prevent toxic effects of tuberculosis drugs. Until now, the use of *Moringa oleifera* leaf nanoemulsion syrup has been carried out for the immunomodulatory and hepatoprotective aspects. Immunomodulatory and hepatoprotective aspects will be discussed further in this literature review. The sources of articles in this literature review are pubmed.com, ncbi.com, plosone.com, sciencedirect.com, and googleschoolar.com from 2010-2020, except when there is no new research against the article. The authors searched for the keywords: "immunonutrition", "tuberculosis in children", "hepatoprotectant", and "*Moringa oleifera*". As an immunomodulator, *Moringa oleifera* leaf nanoemulsion syrup stimulate activation of polimorphonuclear (PMN) cells. As a hepatoprotectant, *Moringa oleifera* leaf nanoemulsion syrup work by reducing the side effects of conventional tuberculosis drugs such as rifampicin by suppressing the action of cytochrome p450 (CYP1A2 and CYP2B), thus decreases the production of toxic hydrazine which causes liver toxicity in tuberculosis patient. Seeing the various interests in the immunomodulatory and hepatoprotective aspects, *Moringa oleifera* leaf nanoemulsion syrup can be used as an adjuvant therapy in overcoming tuberculosis in children by stimulating the activation of immunity cell such as PMN, increasing nutrient absorption, and suppressing the action of cytochrome p450 (CYP1A2 and CYP2B).

INTRODUCTION

Tuberculosis is a tropical infectious disease caused by *Mycobacterium tuberculosis* (Zarb et al. 2012). Until now, tuberculosis has become one of the highest infectious diseases in the world with increasing mortality rate. Indonesia is the third rank on the highest number of tuberculosis cases and it is estimated that the mortality rate in 2020 reach around 1.4 million people (Buonsenso et

al. 2021). Tuberculosis cases not only attacked adult population but also children. According to the data from the Indonesian Ministry of Health in 2016, nearly 69.000 children aged 0-15 years old had tuberculosis and nearly 95% of patients come from the middle to lower socio-economic groups (Kemenkes RI 2016b).

Children in their early development phase (golden period) are susceptible to tuberculosis infection due to the lack of cellular immunity (vanden Driessche et al. 2013). Currently, the main solution in preventing tuberculosis is using the Bacille Calmette – Guérin (BCG) vaccination which is a mandatory vaccine from an early age (Davenne & McShane 2016). Immunity provided by BCG vaccination is a nonspecific immunity of up to 80% so that some children are still able to become infected with tuberculosis in the future (WHO 2017). If children are left infected from childhood and do not get proper care, it will decrease the quality of the nation's future human resources due to high morbidity (Aggarwal 2019).

Current treatment for pediatric tuberculosis is carried out according to standards guidelines with a long duration of drug administration around 2-3 months in the form of crushed tablets. It makes the children less obedient to take the antituberculosis drugs (Munawarah et al. 2019). In addition, the consumption of antituberculosis drugs is known to cause hepatotoxicity (Sanchez-Codez et al. 2020). A safer method of treatment is needed for children with tuberculosis. One potential therapeutic method is using herbal medicine, such as *Moringa oleifera* leaf (Abd elhameed et al. 2018).

Moringa oleifera is a plant that has a cultural-medical-magical aspect and is easily found in Indonesia, and rarely used as an economic commodity (Kasolo et al. 2010). This plant is often called "nutritional dynamite" in Europe and America since it contains phytochemicals that have many functions as antioxidants, painkillers (analgesics), and immunostimulants (Stohs & Hartman 2015). Research conducted *in vitro* in Nigeria stated that the use of *Moringa oleifera* leaf showed antibacterial potential because unsaturated fatty acids have an inhibitory power to prevent bacterial growth by directly causing lysis of bacterial cells and preventing electron transport in oxidative phosphorylation (Kasolo et al. 2010). Beside the potency as antibacterial, antioxidant, immunostimulant, and analgesic, *Moringa oleifera* as a traditional potential medicine also has potency as antidiabetic, anti infertility, antiinflammatory, anticancer, and antiarthritis (Sahoo et al. 2015).

Adjuvant administration in the form of *Moringa oleifera* leaf nanoemulsion syrup can be given in line with the medicines given by the doctor if the child has been confirmed as an active tuberculosis patient (Harausz et al. 2018). The pathophysiological process of tuberculosis in children has a latent phase and it is diagnosed before the primary or gohn complex is formed (Delgado & Bajaj 2019). When the complex is formed, it will only cause clinical manifestations in the form of redness or induration of the skin which is called a positive tuberculin test (Aggerbeck et al. 2018). The tuberculin test began to mark its clinical course on the third day when it became infected,

this event indicated that the cellular immunity process had formed specific antibodies against *Mycobacterium tuberculosis*. In addition, based on the progressive pathophysiological process, tuberculosis in children will lead to impaired nutritional status so that they are prone to triggering malnutrition (Hoyt et al. 2019). These impaired nutritional status in children continue to increase, along with the prevalence of tuberculosis in children that also continues to increase (Kemenkes RI 2016a). According to WHO, there are 50.000 cases that are not reported every year. This shows that there are many undetected tuberculosis cases in children that need to be treated (WHO 2014).

Starting from the condition of the positive tuberculin test results, the adjuvant supplementation of *Moringa oleifera* leaf nanoemulsion syrup which has been added with glucose or sucrose type (which also functions as a natural preservative) can be given to children along with the administration of antituberculosis drugs which tend to be bitter (Pal et al. 2011). The administration of nanoemulsion syrup from *Moringa oleifera* leaf also contains protein and micronutrients which help to improve drug function because children with tuberculosis tend to become weak and experience weight loss (Leone et al. 2015).

The preparation of nanoemulsion from *Moringa oleifera* leaf is extracted using 96% ethanol by maceration technique (Puspitasari & Proyogo 2017). By adding maltodextrin with the *Moringa oliefera* leaf extract, it will increase its solubility and can be used as a carrier to protect secondary metabolites in *Moringa oleifera* (Jusnita & Tridharma 2019). The nanoemulsion of *Moringa oleifera* will increase absorption in the digestive mucosa (Nkya et al. 2014). This nanoemulsion will increase the antioxidant effect in *Moringa oleifera* leaf (Wright et al. 2017). The use of nanoemulsion is to improve the *Moringa oleifera* potency as an antituberculosis adjuvant in children because it increases the absorption capability of the patient's intestinal mucosa (Jusnita & Tridharma 2019).

Therefore, the use of *Moringa oleifera* leaf is a better choice because it is a tropical plant that is relatively easy to cultivate (Leone et al. 2015). The abundant number of ingredients that are rarely known to the public and their various mechanisms make *Moringa oleifera* leaf in the form of nanoemulsion syrup such a promising modality in the co-management of tuberculosis in children (Famewo et al. 2017).

MATERIALS AND METHODS

The source of articles in this literature review is collected using search engines: pubmed.com, ncbi.com, plosone.com, sciencedirect.com, and googleschoolar.com.The authors searched for the keywords "immunonutrition","tuberculosis in children", "hepatoprotectant", and "*Moringa oleifera*". We selected the proceeding or journal in Indonesian or English. Furthermore, it is carried out by reading and understanding the abstract and article content. The inclusion criteria were all research articles concerning the

relationship of nutrition in *Moringa oleifera* with tuberculosis in children. Articles from 2010-2020 are included, except when there is no new research against the article. Of the 130 articles that were reviewed, only 63 articles were suitable as references.

RESULTS AND DISCUSSION

Immunopathology Concept of Tuberculosis in Children

Transmission of tuberculosis in children is often as a result of staying at home. At home, children tend to have close contact with an active tuberculosis family member (Aristoff et al. 2010). Tuberculosis in children is generally primary tuberculosis which is a tuberculosis infection characterized by a positive tuberculin skin test (Hunter 2016; Ter Beek et al. 2019; Starke 2020).

Mycobacterium tuberculosis is transmitted when its 1-5 µm droplet nuclei are aerosolized from patients with pulmonary or laryngeal tuberculosis and are inhaled into the alveoli. The first process begins with innate immunity by alveolar macrophages and dendritic cells that activate phagocytosis process of *Mycobacterium tuberculosis*. Furthermore, it will trigger the activation of the complement pathway, stimulate the production of proinflammatory cytokines in the form of interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α). It also increases the opsonization as the first introduction of germs into the body and phagocytosis to control the infection that occurs. This opsonization causes the body to generate a second body defense response called adaptive immune response. The adaptive immune response induces T cells, such as T helper (Th)-1 CD4+ T cells, CD8+ cytotoxic T cells, and gamma delta (γδ) T cells which then trigger the secretion of cytokines that control the growth of *Mycobacterium tuberculosis* (Thomas 2019).

In children, their immunity has not yet matured, so it will be difficult to control the growth of *Mycobacterium tuberculosis*, thus triggering severe inflammation that will invade the lung parenchyma (Thomas 2019). Infants and neonates are at higher risk due to the absence of production and function of toll-like receptors (TLRs), dendritic cells, and macrophages, as well as deficiency of CD4+ function to express Th1. This weak immune system makes children, especially babies and neonates, are highly susceptible to tuberculosis infection. Tuberculosis tends to put children in a state of progressive weight loss. This surely leads to chronic inflammation and malnutrition in children (Koethe & von Reyn 2016; Ter Beek et al. 2019) (Figure 1).

Tuberculosis patients often experience heavy weight loss. The compound that plays a role in body weight regulation is leptin. The inflammation that occurs in tuberculosis which causes the increase of TNF-α will reduce leptin levels. Leptin is important because it is related to Cell-Mediated Immunity (CMI). If the levels of leptin are low, it contributes to the decline in T cell function (Van Crevel et al. 2002). The decreasing of T cell function reduces the production of type 1 cytokines. The examples of type 1 cytokines

are anti-inflammatory interleukin-2 (IL-2) and IFN-γ which are the main mediators of immunity and increase disease progression. Tuberculosis will also increase energy expenditure which leads to conditions of weight loss or wasting that lead to cachexia (Van Crevel et al. 2002; Chandrasekaran et al. 2017).

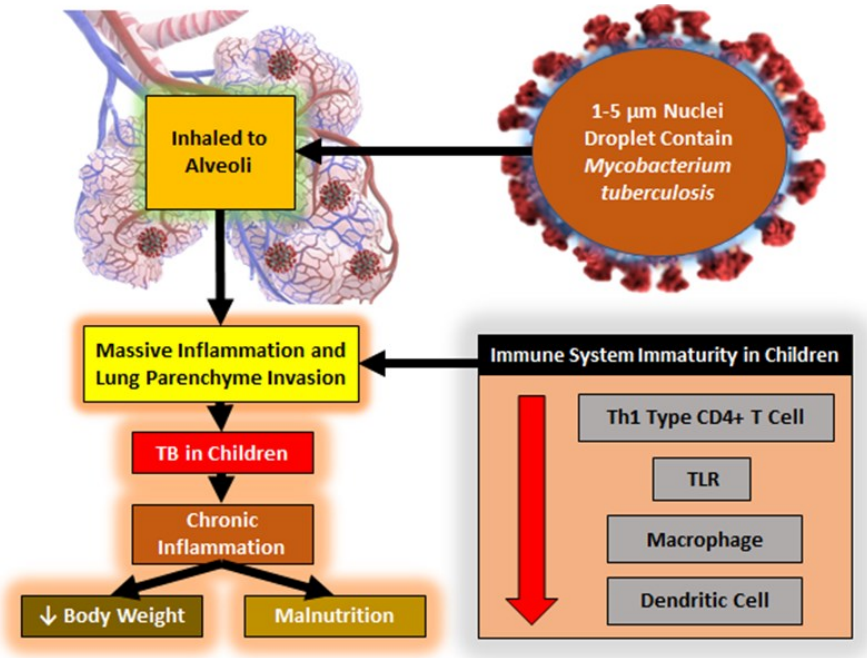

Figure 1. Pathogenesis of Tuberculosis in Children (Pal et al. 2011; Leone et al. 2015). (The red down arrow in "Immune System Immaturity in Children" box indicates 'decreasing'; TB = tuberculosis, TLR = toll-like receptor).

Decreased appetite resulted in protein energy malnutrition (PEM) has a direct effect on the work of T-cells. Severe PEM triggers atrophy of the thymus and peripheral lymphoid organs, then it triggers leukopenia, a decrease in the CD4/CD8 ratio, and increases immature T cells in peripheral blood. Protein deficiency also reduces the expression of CD25 and CD27, the molecules for the activation and proliferation of T cells. Under malnutrition conditions, there can be interference with complement factor C3 opsonization and the ability of phagocytes to kill pathogens. In addition, various antigen-presenting cells (APCs), such as B lymphocytes, macrophages, dendritic cells, and Kupffer cells will decrease in number if they are malnourished (Chandrasekaran et al. 2017) (Figure 2).

The Role of *Moringa oleifera* Leaf Nanoemulsion Syrup in Imunonutrition

Moringa oleifera leaf nanoemulsion syrup has a number of macronutrients such as vegetable protein also micronutrients such as vitamins, minerals, as well as trace elements which are associated with immunomodulatory effects against intracellular pathogens such as *Mycobacterium tuberculosis* (Chandrasekaran et al., 2017). Nfambi et al. (2015) tested the administration of *Moringa oleifera* methanol extract against hematological parameters in an *in vivo* study and found an increase in several parameters, especially leukocytes that play an important role in immunity compared to immunocompromised mice without

Moringa oleifera administration (Kant et al. 2015; Nfambi et al. 2015).

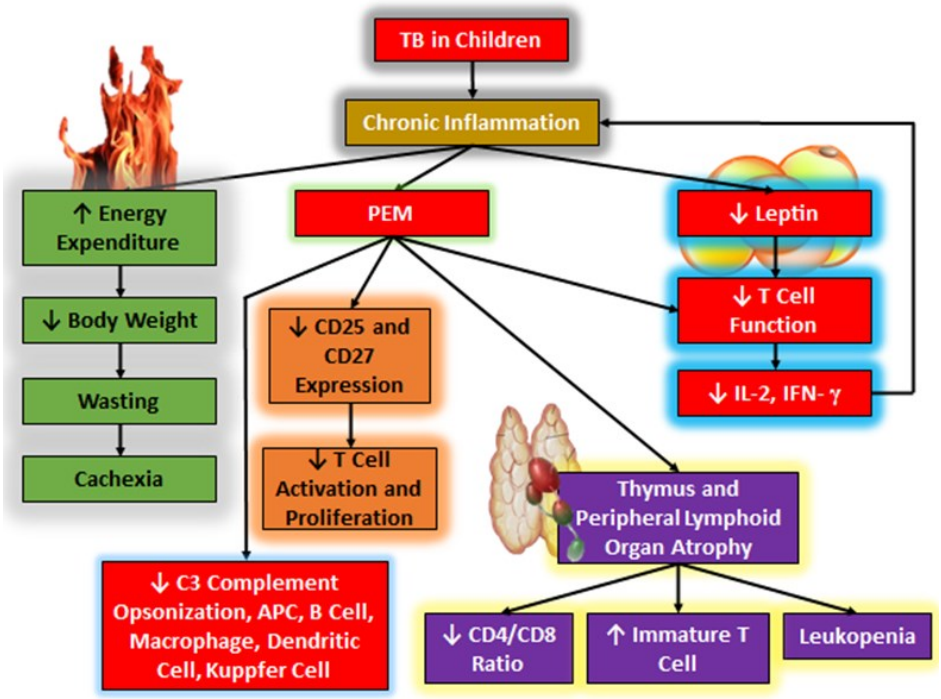

Figure 2. Pathogenesis of Tuberculosis Complication in Children (Pari & Kumar 2002; Tostmann et al. 2008; Wright et al. 2017). (↑ = increasing; ↓ = decreasing; TB = tuberculosis; PEM = protein energy malnutrition; CD = cluster of differentiation; APC = antigen presenting cell; IL = interleukin; IFN = interferon).

Moringa oleifera has potency as an immunomodulator because it contains prebiotics (Alabi et al. 2017). Prebiotics are dietary products rich in fiber that provide a food source for the normal bacterial flora present in the intestines (La Fata et al. 2017). These prebiotics will be fermented by the normal intestinal flora bacteria to produce short-chain fatty acids (SCFAs) as a by-product (Goyal et al. 2020). These SCFAs are very easily absorbed by the body (Feng et al. 2018). While absorbing, this prebiotic triggers the colonization of good bacteria in the intestine such as *Lactobacillus* sp. and *Bifidobacterium* sp. which suppress the presence of bad bacteria such as *Salmonella* sp. and *Campylobacter* sp. (Hemalatha et al. 2017; Feng et al. 2018; Kim et al. 2019). Prebiotics also affect the lungs and can be explained by the theory of the gut-lung axis, in which a healthy intestinal condition will impact the health of other organs, like lung. This is because prebiotics will be assimilated by gut microbiote and it produces SCFAs metabolite and stimulates the migration of immune cell to distal organ such as lung (Zhang et al. 2020). SCFAs as a result of prebiotic metabolism by normal intestinal flora bacteria contained in *Moringa oleifera* can modify the intestinal environment by increasing the good bacteria and thereby increasing nutrient absorption (Al-Sheraji et al. 2013). The increase of nutrient absorption will increase the children weight. In addition, SCFAs have an antidiarrheal effect and do not trigger lactose intolerance, making them safe for children (Goyal et al. 2020) (Figure 3).

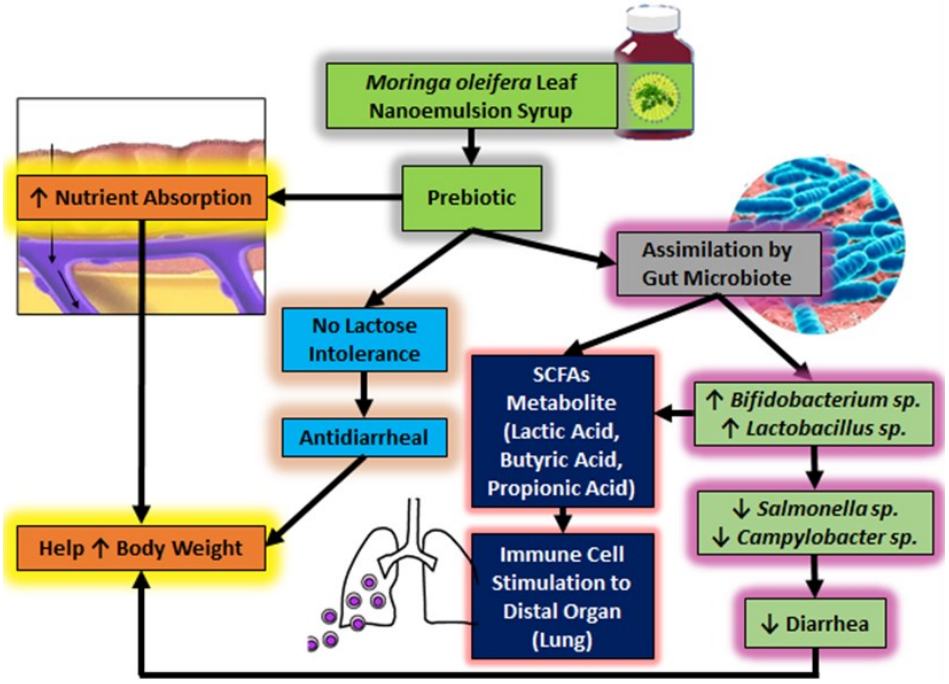

Figure 3. Mechanism of Action of Prebiotic from *Moringa oleifera* Leaf Nanoemulsion Syrup in Immunonutrition Aspect (Al-Sheraji et al. 2013; Alabi et al. 2017; la Fata et al. 2017; Hemalatha et al. 2017; Feng et al. 2018; Kim et al. 2019; Goyal et al. 2020; Zhang et al. 2020).

Low leptin in children with tuberculosis is due to chronic inflammation. Therefore, leptin administration could have a positive impact on tuberculosis patients because it would increase CMI. However, this is not feasible if it is done in Indonesia because there are no leptin drugs available in Indonesia (van Crevel et al. 2002). Because there are no leptin drugs available in Indonesia, thus a traditional potent herbal medicine application, such as *Moringa oleifera* leaf nanoemulsion syrup had an appetite-enhancing or orexigenic effect from zinc and vitamin E may result in statistically significant weight gain in animal studies (van Crevel et al. 2002; Uwaifo 2020).

Vegetable protein contained in *Moringa oleifera* leaf nanoemulsion syrup has the effect of increasing the CD4/CD8 ratio, IL-2, IFN-γ, and expression of CD25 as well as CD27. This increases the patient immunity by increasing the body natural response of microbial infection by differentiating between foreign body such as bacteria and "self" cell in the body (Chandrasekaran et al. 2017).

Various trace elements contained in *Moringa oleifera* leaf nanoemulsion syrup, such as zinc, copper, and iron have benefits in the treatment of tuberculosis. Zinc and copper can strengthen the innate immune response which acts on the phagolysosome. The more its concentration in the phagolysosome, the better its bactericidal effect (Djoko et al. 2015). Iron may increase antimicrobial function by increasing the production of IL-4, IL-10, IL-5, and Forkhead Box P3 (FOXP3). Activation of FOXP3 increases the function of T cells (Chandrasekaran et al. 2017).

Various minerals are also present in the nanoemulsion syrup from *Moringa oleifera* leaf, one of which is calcium. Calcium is needed by the macro-

phages because it requires concentration ten times higher to fight tuberculosis than without tuberculosis (Chandrasekaran et al. 2017).

Various vitamins are also contained in *Moringa oleifera* leaf nanoemulsion syrup, such as vitamins A, B6, C, and E. Vitamin A increases cellular and lymphoproliferative immune response. Meanwhile, vitamin B6 increases the activity of natural killer (NK) cells and lymphocytes. Vitamin C reduces ROS and thus reducing tissue damage caused by tuberculosis. Vitamin E reduces oxidative stress and improve T cell function (Chandrasekaran et al. 2017) (Figure 4).

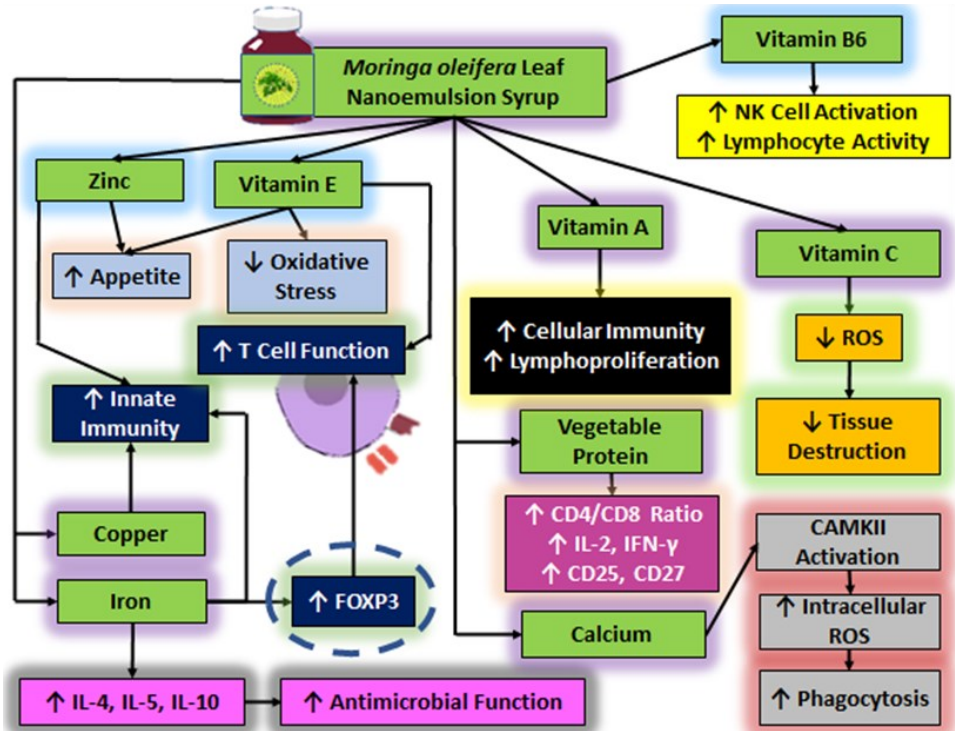

Figure 4. Mechanism of Action of Macronutrient and Micronutrient from *Moringa oleifera* Leaf Nanoemulsion Syrup in Immunonutrition (van Crevel et al. 2002; Djoko et al. 2015; Nfambi et al. 2015; Chandrasekaran et al. 2017; Uwaifo 2020). (ROS = reactive oxygen species; CAMKII = Ca^{2+}/CAM-dependent protein kinase II; IL = interleukin; IFN = interferon; CD = cluster of differentiation; FOXP3 = Forkhead Box P3; NK = Natural Killer).

Pathophysiology of Antituberculosis Drug-Induced Hepatotoxicity

Antituberculosis drugs have been reported to have toxicity to the liver via cytolytic mechanisms that damage membranes and organelles through degradation of phospholipids (Pari & Kumar 2002; Tostmann et al. 2008; Abd el-hameed et al. 2018; Mangwani et al. 2020). In general, antituberculosis drugs have side effects on the liver because they contain toxic metabolites, reactive oxygen species (ROS), and free radicals (Jia et al. 2019). In addition, antituberculosis drugs also activate CYP2E1 thereby increasing fatty acid accumulation and LDL uptake. All of these will contribute to liver damage (Mangwani et al. 2020).

Antituberculosis drugs that contribute to liver toxicity include: isoniazid (INH), RIF, and pyrazinamide (PZA) (Mangwani et al. 2020). INH

causes liver toxicity by increasing the production of toxic compound called acetyl hydrazine (Chan et al. 2017; Mangwani et al. 2020). RIF can increase INH metabolism and produce toxic intermediates such as hydrazine through the INH hydrolase enzyme (Pari & Kumar 2002; Wang et al. 2016). In addition, RIF can also impair membrane permeability, thereby impairing glucose-6-phosphate activity. This leads to high lipid peroxidation in the liver which has a negative effect on liver enzymes (Shehu et al. 2017). PZA causes liver toxicity by increasing the production of 5-hydroxypyrazinoic acid which has a toxic effect on the liver (Mangwani et al. 2020).

Moringa oleifera Leaf Nanoemulsion Syrup as Hepatoprotectant

Some compounds from *Moringa oleifera* leaf such as beta carotene, alkaloids, flavonoids, glycosides, saponins, tannins, and terpenoids can repair damage of the liver cells and improve the function of the SGOT, SGPT, and ALP enzymes (Pari and Kumar 2002; Mangwani et al. 2020). These compounds also have a hepatoprotective effect by reducing levels of lipid peroxidation markers such as thiobarbituric acid reactive substances (TBARS) and hydroperoxides through increased glutathione peroxidase (GPx) by kaempferol. This *Moringa oleifera* leaf nanoemulsion syrup can improve hepatic cell structure by increasing phospholipid levels (Pari & Kumar 2002; Abd elhameed et al. 2018). Decreased malondialdehyde (MDA) and tumor necrosis factor-alpha (TNF-α) as well as increased adiponectin were also identified in *in vivo* studies. This adiponectin later activates AMP-activated kinase and peroxisome proliferator-activated receptor alpha (PPAR-α) which suppress TNF-α and trigger IL-10. In addition, adiponectin also suppresses the proliferation and migration of hepatic stellate cells by binding to adipore-ceptor-2 thus suppressing hepatic fibrosis (Abd elhameed et al. 2018). These compounds also reduce serum bilirubin so that the symptoms of jaundice can be minimized. Phytochemicals compound such as glucosinolates contained in the *Moringa oleifera* leaf nanoemulsion syrup also suppresses the action of cytochrome p450 (CYP1A2 and CYP2B) (Mishra et al. 2011; Mangwani et al. 2020). This causes RIF, which is an inducer of the p450 pathway as previously described, inhibits INH metabolism, thus decreases the production of toxic hydrazine (Mangwani et al. 2020).

SCFAs are one of the products of prebiotic metabolism contained in the *Moringa oleifera* leaf nanoemulsion syrup. These SCFAs can improve cir-rhosis of the liver (Goyal et al. 2020) (Figure 5).

Moringa oleifera leaf at the dose of 1000 mg/kgBW was more effective than silymarin and showed significant differences in the groups that were given only RIF and INH to test its different potency for treating liver disease. Compared with *Moringa oleifera,* silymarin is a well-known plant that has been clinically used since 2000 years ago as a plant for treating liver disease. *Moringa oleifera* also showed a decrease in MDA and TNF-α (as a proinflammatory marker), and also a dose-dependent increase in GPx and adiponectin (as an antioxidant and antiinflammatory marker). In addition,

Moringa oleifera is known to increase RIF concentration in plasma, allowing reduction of the dose and duration of antituberculosis drugs (Abd elhameed et al. 2018; Chandra Dinda 2017).

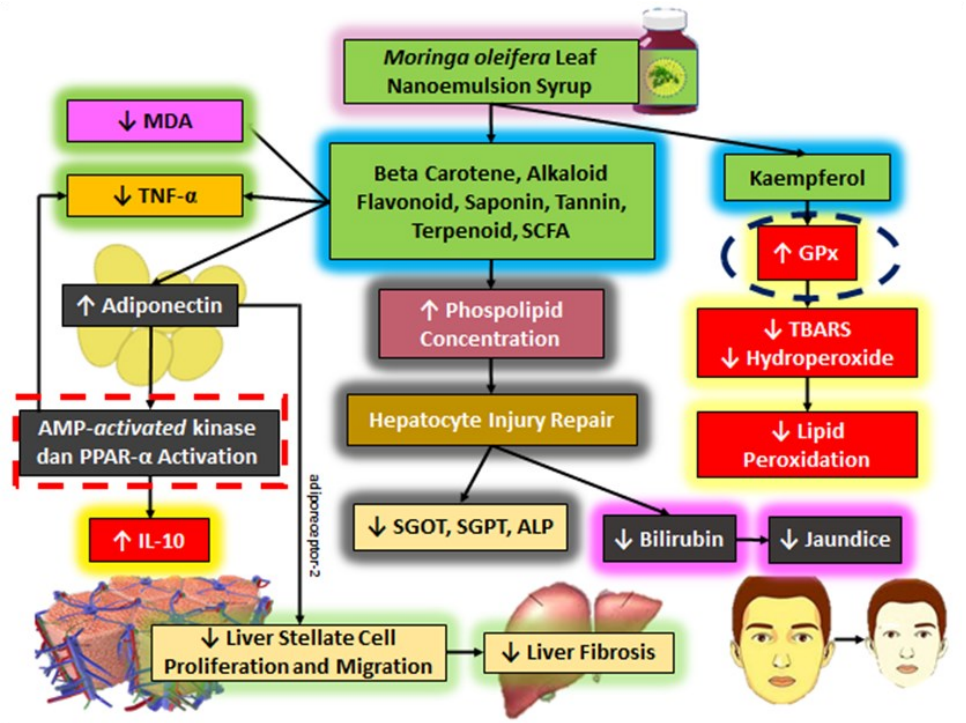

Figure 5. Mechanism of Action of Secondary Metabolites and SCFA from *Moringa oleifera* Leaf Nanoemulsion Syrup as Hepatoprotectant) (Pari & Kumar 2002; Mishra et al. 2011; Abd elhameed et al. 2018; Goyal et al. 2020; Mangwani et al. 2020) (MDA = malondialdehyde; TNF = tumor necrosis factor; AMP = adenosine monophospate; PPAR = peroxisome proliferator-activated receptors; IL = interleukin; SCFA = short-chain fatty acids; SGOT = serum glutamic-oxaloacetic transaminase; SGPT = serum glutamic-pyruvic transaminase; ALP = alkaline phosphatase; GPx = glutathione peroxidase; TBARS = thiobarbituric acid reactive substances).

The Advantages of *Moringa oleifera* Leaf Nanoemulsion Syrup as an Adjuvant Agent in Children with Tuberculosis

The advantages of *Moringa oleifera* leaf nanoemulsion syrup can be seen from several aspects, such as the form of nanoemulsion syrup, various efficacies of the *Moringa oleifera* leaf and the economic potential of this modality.

The packaging of the drug in the form of nanoemulsion syrup aims to increase the compliance of the patient. This is because *Moringa oleifera* that is packaged in other forms like powder may induce a bitter taste and thereby can reduce the patient compliance. In addition, the form of nanoemulsion syrup also increases the absorption of the *Moringa oleifera* (Tayeb & Sainsbury 2018; Harwansh et al. 2019; Nakano et al. 2019).

Moringa oleifera leaf is known to be beneficial against tuberculosis because it is rich in nutrients, a hepatoprotectant agent, and may enhance the actions of rifampicin (RIF bio-enhancer). *Moringa oleifera* contains four times of calcium in milk, seven times of vitamin C in oranges, three times of potas-

sium in banana, three times of iron in spinach, four times of vitamin A in carrot, and two times of protein in milk (Moyo et al. 2011). Children nutritional status must be taken as consideration in the management of tuberculosis in children, therefore giving *Moringa oleifera* as an adjuvant is very compatible because it is rich in nutrients and may increase the children appetite (Uwaifo 2020).

Previous studies found that *Moringa oleifera* can induce hepatoprotective effect significantly (Buraimoh et al. 2011; Pari & Kumar 2002; Saalu et al. 2011). The administration of *Moringa oleifera* was able to reduce the level of serum AST and ALT which was previously high due to the administration of anti-tuberculosis drugs (INH and RIF). This mechanism is caused by the activity of anti-lipid peroxidase induced by *Moringa oleifera*, resulting in the improvements of the plasma membrane and liver structural tissue (Abd elhameed et al. 2018). In addition, *Moringa oleifera* also plays a role as a bio-enhancer for RIF. *Moringa oleifera* is able to inhibit cytochrome P450 which acts in the process of RIF metabolism. This inhibition leads to the increase of RIF concentration in the plasma (Pal et al. 2011). This allows reduction of the dose and timing of therapy and may reduce the risk of drug toxicity. To date, there are no studies that found the side effects of *Moringa oleifera* both in humans and experimental animals. *Moringa oleifera* has been consumed traditionally by people especially in Indonesia, therefore making it is such a safe drug for consumption (Stohs & Hartman 2015).

Previous study found that the content of *Moringa oleifera* is different in that plant domestically compare to that live in the wild (Chodur et al. 2018). *Moringa oleifera* leaf nanoemulsion syrup can also be consumed directly, cooked, or stored for months without using refrigerator and still provide the same nutritional value (Mary 2015). When compared with other conditions, giving *Moringa oleifera* is also very beneficial because *Moringa oleifera* continue to grow during the dry season when other food sources are usually scarce.

The Limitations of *Moringa oleifera* Leaf Nanoemulsion Syrup as an Adjuvant Agent in Children with Tuberculosis

The main limitation is *Moringa oleifera* leaf nanoemulsion syrup only can be the adjuvant for the conventional antituberculosis drugs for some aspects of tuberculosis patophysiology. Another limitation is the lack of comprehensive and detailed mechanism of action from compound in *Moringa oleifera* such as why kaempferol increase GPx. This encourages the need for an integrated research on *Moringa oleifera* against tuberculosis in children.

CONCLUSION

Based on the review that has been done, *Moringa oleifera* leaf nanoemulsion syrup is a potential adjuvant agent in the management of children with tuberculosis. The immunonutrition and hepatoprotectant mechanism induced by *Moringa oleifera* as well as the form of nanoemulsion syrup is a suitable form of drug for children by stimulating the activation of immunity cell such as

PMN, increasing nutrient absorption, and suppressing the action of cytochrome p450 (CYP1A2 and CYP2B). Further research on *Moringa oleifera* at preclinical and clinical levels are needed to fully understand and explore the role of *Moringa oleifera* in the management of tuberculosis in children.

AUTHORS CONTRIBUTION

N.B.W.S. designed the research, colleted and analyzed the data, and wrote the manuscript. A.I.Y.D.P. collected and analyzed the data. M.I.D.S. wrote the manuscript. A.W.I. designed the research and wrote the manuscript. I.A.I.W. provided language help and writing assistance.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

REFERENCES

Abd elhameed, M.F., 2018. Protective Effects of *Moringa oleifera* extract on Isoniazid and Rifampicin Induced Hepatotoxicity in Rats: Involvment of Adiponectin and Tumor Necrosis Factor-α. *Egyptian Journal of Veterinary Sciences*, 1(1), pp.25-34. doi: 10.21608/ejvs.2018.2349.1025

Aggarwal, A.N., 2019. Quality of life with tuberculosis. *Journal of Clinical Tuberculosis and Other Mycobacterial Diseases*, 17, 100121. doi: 10.1016/j.jctube.2019.100121

Aggerbeck, H. et al., 2018. C-Tb skin test to diagnose Mycobacterium tuberculosis infection in children and HIV-infected adults: A phase 3 trial. *PLoS ONE*, 13(9), e0204554. doi: 10.1371/journal.pone.0204554

Alabi, O.J. et al., 2017. Effect of aqueous *Moringa oleifera* (Lam) leaf extracts on growth performance and carcass characteristics of hubbard broiler chicken. *Revista Brasileira de Ciencia Avicola*, 19(2), pp.273–280. doi: 10.1590/1806-9061-2016-0373

Al-Sheraji, S.H. et al., 2013. Prebiotics as functional foods: A review. *Journal of Functional Foods*, 5(4), pp.1542–1553. doi: 10.1016/j.jff.2013.08.009

Aristoff, P.A. et al., 2010. Rifamycins - Obstacles and opportunities. *Tuberculosis* 90(2), pp.94–118. doi: 10.1016/j.tube.2010.02.001

Buonsenso, D. et al., 2021. COVID-19 effects on tuberculosis care in Sierra Leone. *Pulmonology*, 27(1), pp.67–69. doi: 10.1016/j.pulmoe.2020.05.013

Buraimoh, A.A., Bako, I.G., & Ibrahim, F.B., 2011. Hepatoprotective Effect of Ethanolic Leave Extract of *Moringa oleifera* on the Histology of Paracetamol Induced Liver Damage in Wistar Rats. *International Journal of*

Animal and Veterinary Advances, 3(1), pp.10–13.

Chan, S.L. et al., 2017. Association and clinical utility of NAT2 in the prediction of isoniazid-induced liver injury in Singaporean patients. *PLoS ONE*, 12(10), e0186200. doi: 10.1371/journal.pone.0186200

Chandra Dinda, S., 2017. Exploring the Use of Lipid Based Nano-Formulations for the Management of Tuberculosis. *Journal of Nanosciences: Current*, 2(2), 1000112.

Chandrasekaran, P. et al., 2017. Malnutrition: Modulator of immune responses in tuberculosis. *Frontiers in Immunology*, 8, 1316. https://doi.org/10.3389/fimmu.2017.01316

Chodur, G.M. et al., 2018. Wild and domesticated *Moringa oleifera* differ in taste, glucosinolate composition, and antioxidant potential, but not myrosinase activity or protein content. *Scientific Reports*, 8(1), 7995. doi: 10.1038/s41598-018-26059-3

Davenne, T. & McShane, H., 2016. Why don't we have an effective tuberculosis vaccine yet? *Expert Review of Vaccines*, 15(8), pp.1009–1013. doi: 10.1586/14760584.2016.1170599

Delgado, B.J., & Bajaj, T., 2019. *Ghon Complex* (3rd ed., Vol. 2018). StatPearl Publishing.

Djoko, K.Y. et al., 2015. The role of copper and zinc toxicity in innate immune defense against bacterial pathogens. *Journal of Biological Chemistry*, 290(31), pp.1854–1861. doi: 10.1074/jbc.R115.647099

Famewo, E.B., Clarke, A.M. & Afolayan, A.J., 2017. The effect of polyherbal medicines used for the treatment of tuberculosis on other opportunistic organisms of humans infected with tuberculosis. *Pharmacognosy Magazine*, *13*(51), S539–S543. doi: 10.4103/pm.pm_468_16

Feng, W., Ao, H. & Peng, C., 2018. Gut microbiota, short-chain fatty acids, and herbal medicines. *Frontiers in Pharmacology*, 9, 1354. doi: 10.3389/fphar.2018.01354

Goyal, N., Yadav, V. & Rastogi, M., 2020. *Food and Agriculture Spectrum Journal Probiotics, prebiotics & synbiotics: A new Era*. www.gsrl.orghttps://fasj.org/index.php/fasj/article/view/3

Harausz, E.P. et al., 2018. Treatment and outcomes in children with multidrug-resistant tuberculosis: A systematic review and individual patient data meta-analysis. *PLoS Medicine*, 15(7), e1002591. doi: 10.1371/journal.pmed.1002591

Harwansh, R.K., Deshmukh, R. & Rahman, M.A., 2019. Nanoemulsion: Promising nanocarrier system for delivery of herbal bioactives. *Journal of Drug Delivery Science and Technology*, 51, pp.224–233. doi: 10.1016/j.jddst.2019.03.006

Hemalatha, R. et al., 2017. Effect of probiotic supplementation on total lactobacilli, bifidobacteria and short chain fatty acids in 2–5-year-old children. *Microbial Ecology in Health and Disease*, 28(1), 1298340. doi:

10.1080/16512235.2017.1298340

Hoyt, K.J. et al., 2019. Effect of malnutrition on radiographic findings and mycobacterial burden in pulmonary tuberculosis. *PLoS ONE*, 14(3), e0214011. doi: 10.1371/journal.pone.0214011

Hunter, R.L., 2016. Tuberculosis as a three-act play: A new paradigm for the pathogenesis of pulmonary tuberculosis. *Tuberculosis*, 97, pp.8–17. doi: 10.1016/j.tube.2015.11.010

Jia, Z.L. et al., 2019. Mechanism of isoniazid-induced hepatotoxicity in zebrafish larvae: Activation of ROS-mediated ERS, apoptosis and the Nrf2 pathway. *Chemosphere*, 227, pp.541–550. doi: 10.1016/j.chemosphere.2019.04.026

Jusnita, N. & Tridharma, W.S., 2019. Karakterisasi nanoemulsi ekstrak daun kelor (*Moringa oleifera* Lamk.). *Jurnal Sains Farmasi & Klinis*, 6(1), pp.16–24.

Kant, S., Gupta, H. & Ahluwalia, S., 2015. Significance of Nutrition in Pulmonary Tuberculosis. *Critical Reviews in Food Science and Nutrition*, 55(7), pp.955–963. doi: 10.1080/10408398.2012.679500

Kasolo, J.N. et al., 2010. Phytochemicals and uses of *Moringa oleifera* leaves in Ugandan rural communities. *Journal of Medicinal Plants Research*, 4(9), pp.753–757. doi: 10.5897/JMPR10.492

Kemenkes RI., 2016a. *Infodatin: Tuberkulosis Temukan Obari Sampai Sembuh*. Kemenkes RI.

Kemenkes RI., 2016b. *Regulation of the Minister of Health No. 67 on Tuberculosis Control Jakarta (Peraturan Menteri Kesehatan Nomor 67 Tentang Penanggulangan Tuberkulosis Jakarta)*. Kemenkes RI.

Kim, S.A. et al., 2019. Potential for prebiotics as feed additives to limit foodborne Campylobacter establishment in the poultry gastrointestinal tract. *Frontiers in Microbiology*, 10(91). doi: 10.3389/fmicb.2019.00091

Koethe, J.R. & von Reyn, C.F., 2016. Protein-calorie malnutrition, macronutrient supplements, and tuberculosis. *International Journal of Tuberculosis and Lung Disease*, 20(7), pp.857–863. doi: 10.5588/ijtld.15.0936

La Fata, G. et al., 2017. Recent development of prebiotic research—statement from an expert workshop. *Nutrients*, 9(12), 1376. doi: 10.3390/nu9121376

Leone, A. et al., 2015. Cultivation, genetic, ethnopharmacology, phytochemistry and pharmacology of *Moringa oleifera* leaves: An overview. *International Journal of Molecular Sciences*, 16(6), pp.12791–12835. doi: 10.3390/ijms160612791

Mangwani, N., Singh, P.K. & Kumar, V., 2020. Medicinal plants: Adjunct treatment to tuberculosis chemotherapy to prevent hepatic damage. *Journal of Ayurveda and Integrative Medicine*, 11(4), pp.522–528. doi: 10.1016/j.jaim.2019.02.004

Mary, G.A., 2015. *Moringa Oleifera Leaf Supplementation on Vitamin A Status of*

Children in Ada-East District of Ghana, University of Ghana.

Mishra, G. et al., 2011. Traditional uses, phytochemistry and pharmacological properties of *Moringa oleifera* plant: An overview. *Der Pharmacia Lettre*, 3 (2), pp.141-164.

Moyo, B. et al., 2011. Nutritional characterization of Moringa (*Moringa oleifera* Lam.) leaves. *African Journal of Biotechnology*, 10(60), pp.12925–12933. doi: 10.5897/ajb10.1599

Munawarah, M. et al., 2019. Pengaruh penggunaan sediaan fixed dose combination (fdc) dibandingkan dengan tablet lepas obat anti-tuberkulosis terhadap peningkatan nilai sgpt dan sgot pada pasien tuberkulosis di balai besar kesehatan paru masyarakat makassar. *Majalah Farmasi Dan Farmakologi*, 23(1), pp.32–34.

Nakano, Y. et al., 2019. Development of a Novel Nano-emulsion Formulation to Improve Intestinal Absorption of Cannabidiol. *Medical Cannabis and Cannabinoids*, 2(1), pp.35–42. doi: 10.1159/000497361

Nfambi, J. et al., 2015. Immunomodulatory activity of methanolic leaf extract of *Moringa oleifera* in Wistar albino rats. *Journal of Basic and Clinical Physiology and Pharmacology*, 26(6), pp.603–611. doi: 10.1515/jbcpp-2014-0104

Nkya, J.W., Erasto, P., & Chacha, M. (2014). *Antimycobacterial and cytotoxicity activities of Moringa oleifera Lam extracts* (Vol. 2, Issue 9). www.usa-journals.com

Pal, A. et al., 2011. Influence of Moringa oleifera on pharmacokinetic disposition of rifampicin using HPLC-PDA method: A pre-clinical study. *Biomedical Chromatography*, *25*(6), pp.641–645. doi: 10.1002/bmc.1494

Pari, L. & Kumar, N.A., 2002. Hepatoprotective Activity of Moringa oleifera on Antitubercular Drug-Induced Liver Damage in Rats. *JOURNAL OF MEDICINAL FOOD*, 5(3).

Puspitasari, A.D. & Proyogo, L.S., 2017. Perbandingan Metode Ekstraksi Maserasi dan Sokletasi Terhadap Kadar Fenolik Total Ekstrak Etanol Daun Kersen (Mungingia calabura). *Cendekia Eksakta*, 2(1), pp.1–8.

Saalu, L.C. et al., 2011. The hepato-protective potentials of Moringa oleifera Leaf Extract On Alcohol-Induced Hepato-toxicity in Wistar Rat. *American Journal Biotechnology and Molecular Sciences*. doi: 10.13140/RG.2.2.33221.76002

Sahoo, D. et al., 2015. Review on Nutritional and Pharmacological Potencies of Moringa Oleifera. *European Journal of Pharmaceutical and Medical Research*, 3(1), pp.150-155.

Sanchez-Codez, M. et al., 2020. Hepatitis in children with tuberculosis: A case report and review of the literature. *BMC Pulmonary Medicine*, 20(1). doi: 10.1186/s12890-020-01215-6

Shehu, A.I., Ma, X. & Venkataramanan, R., 2017. Mechanisms of Drug-Induced Hepatotoxicity. In *Clinics in Liver Disease*, 21(1), pp.35–54. doi: 10.1016/j.cld.2016.08.002

Starke, J.R., 2020. Tuberculin skin test versus the interferon-γ Release Assays: Out with the old, in with the new. *Pediatrics*, 145(1). doi: 10.1542/

PEDS.2019-3021

Stohs, S.J. & Hartman, M.J., 2015. Review of the safety and efficacy of Moringa oleifera. *Phytotherapy Research*, 29(6), pp.796–804. doi: 10.1002/ptr.5325

Tayeb, H.H. & Sainsbury, F., 2018. Nanoemulsions in drug delivery: Formulation to medical application. *Nanomedicine*, 13(19), pp.2507–2525. doi: 10.2217/nnm-2018-0088

Ter Beek, L. et al., 2019. Tuberculosis-Related Malnutrition: Public Health Implications. *The Journal of infectious diseases*, 220(2), pp.340–341. doi: 10.1093/infdis/jiz091

Thomas, T.A., 2019. Tuberculosis in Children. *Thoracic surgery clinics*, 29(1), pp. 109–121. doi: 10.1016/j.thorsurg.2018.09.009

Tostmann, A. et al., 2008. Antituberculosis drug-induced hepatotoxicity: Concise up-to-date review. *Journal of Gastroenterology and Hepatology (Australia)*, 23(2), pp.192–202. doi: 10.1111/j.1440-1746.2007.05207.x

Uwaifo, 2020. Evaluation of weight and appetite of adult wistar rats supplemented with ethanolic leaf extract of Moringa oleifera. *Biomedical and Biotechnology Research Journal*, 4(2), pp.137–140. doi: 10.4103/bbrj.bbrj_17_20

Van Crevel, R. et al., 2002. Decreased Plasma Leptin Concentrations in Tuberculosis Patients are Associated with Wasting and Inflammation. *The Journal of Clinical Endocrinology & Metabolism*, 87(2), pp.758-763. doi: 10.1210/jcem.87.2.8228

Vanden Driessche, K. et al., 2013. Immune vulnerability of infants to tuberculosis. *Clinical and Developmental Immunology*, 2013, 781320. doi: 10.1155/2013/781320

Wang, P. et al., 2016. Isoniazid metabolism and hepatotoxicity. *Acta Pharmaceutica Sinica B*, 6(5), pp.384–392. doi: 10.1016/j.apsb.2016.07.014

WHO, 2014. *Guidance for national tuberculosis programmes on the management of tuberculosis in children*. WHO.

WHO, 2017. *Global tuberculosis report 2018*. WHO.

Wright, R.J. et al., 2017. An investigation of the antioxidant capacity in extracts from Moringa oleifera plants grown in Jamaica. *Plants*, 6(4). doi: 10.3390/plants6040048

Zarb, P. et al., 2012. The European Centre for Disease Prevention and Control (ECDC) pilot point prevalence survey of healthcare-associated infections and antimicrobial use. *Euro Surveill*, 17(46), 20316. doi: 10.2807/ese.17.46.20316-en.

Zhang, D. et al., 2020. The Cross-Talk Between Gut Microbiota and Lungs in Common Lung Diseases. *Frontiers in Microbiology*, 11, 301. doi: 10.3389/fmicb.2020.00301

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Review Article

Review: Current Checklist of Local Names and Utilization Information of Indonesian Wild Mushrooms

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ABSTRACT
Mushrooms have been considered an important part of human life due to their various benefits and potential. In Indonesia, many indigenous people get used to foraging and using wild mushrooms as part of their daily lives. To date, there was no update following prior local name checklist of wild mushroom and their uses in Indonesia. Thus, this review aims to provide the latest work on that information known so far in the country. A literature review was focusing on available publications containing the local names and the use of wild mushrooms in Indonesia. 107 mushrooms in total are known to have 170 local names with 36 of them having more than 1 indigenous name. Some of them: *Coprinus* spp., *Polyporus* spp., *Schizophyllum commune*, *Scleroderma* spp., *Termitomyces* spp., and *Trametes* spp. are known to have 5 local names for each region and ethnicity that uses them. 50 species of mushrooms in total are used as food and traditional medicine. The information was derived from 8 provinces and 8 tribes, of which West Kalimantan Province and Javanese ethnicity contributed to the highest number of it. The number of local names is expected to increase as more investigations are conducted in the near future.

INTRODUCTION
The mushroom is a cosmopolitan-heterotrophic-organism due to its distribution in natural ecosystems to man-made sites in Indonesia (Putra et al. 2017; 2018; 2019). Based on the existing definitions, the mushroom is categorized as a group of fungi with macroscopic fruiting bodies, visible without a microscope, and can be picked up by hand (Arora 1986; Hawksworth 2001; Anon 2002). Taxonomically, most mushrooms belong to the phylum of Basidiomycota and a few of Ascomycota. Moreover, the mushroom is one of the living creatures with a large number of species in the world (Hawksworth 2001). Hence, it is increasing the chance of interaction with other organisms, including humans.

Wild mushroom is one of the important natural resources that have been utilized by various local people around the world (Boa 2004; Pala et al. 2013; Osarenkhoe et al. 2014; Semwal et al. 2014; Lazo et al. 2015; Álvarez-Farias et al. 2016; Ao et al. 2016; Yilmaz & Zencirci 2016; Merida Ponce et

al. 2019), including Indonesia (Putra & Hafazallah 2020). Mushroom contains high nutrients and bioactive compounds which are good for health (Lima et al. 2012; Wang et al. 2014). Mushrooms can grow on a broad range of habitats, different seasons, used as functional food, and some are known to have the potential for poisoning in Indonesia (Putra 2020a;b). However, the indigenous people are used to foraging and using the wild mushroom in their daily life without experiencing mushroom poisoning (Putra & Hafazallah 2020). This habit has been passed down through generations and became their local wisdom and identity. Yet, the documentation of indigenous knowledge regarding wild mushrooms in Indonesia is still partially done (Putra & Hafazallah 2020). Thus, a better collaboration between researchers and local people is needed in order to organize the local name and utilization of Indonesian wild mushrooms. Moreover, wild mushroom has the potential to increase the mushroom supply in the market which can contribute to the Indonesian economy. It is stated that mushroom is the largest commodity from horticulture (nonfruit commodities) which reach 33.000 tons y-1 (Statistics Indonesia 2021).

Hitherto, Indonesia does not have a checklist of Indonesian mushrooms diversity in contrast to Malaysia (Lee et al. 2008) and Vietnam (Kiet 2008). In addition, the checklist of Indonesian mushroom's local names has not been updated since Bisema (1968) and Heyne (1987). The participation of indigenous people in reporting ethnomycological information will contribute to Indonesian mushroom biodiversity and its potential utilization. The indigenous people are considered to have the empirical experience in utilizing wild mushrooms (Reyes-Lopez et al. 2020). The information regarding local name and uses of the wild mushrooms should be summarized, validated, and passed on to the next generation. Therefore, this review is aimed to providing the current checklist of Indonesian wild mushroom to optimize its potential uses in the future. Then, it might contribute to the Indonesian government's program, especially the ministry of agriculture, in developing mushroom cultivation from wild mushrooms and its production to support the food self-sufficiency program of world food barn 2045.

MATERIALS AND METHODS

We investigated the available published articles and records from books on the ethnomycology studies in Indonesia. The information was collected using related keywords such as local names of Indonesian mushrooms, local people knowledge about wild mushrooms, the use of wild mushrooms by Indonesian tribes. The information (including some photographs) was also obtained through periodically online communication with indigenous peoples, member of the Indonesian Mushroom Hunter Community (KPJI) from 2020 to 2021. Some of the wild edible mushroom specimens used by them were deposited to Herbarium Bogoriense and investigated for future taxonomical studies. Furthermore, the obtained taxonomical position was validated based

on Index Fungorum (2021) (http://www.indexfungorum.org/names/Names.asp). The data then presented in checklist (tables) and graphs.

RESULTS AND DISCUSSION

The total of 107 wild mushrooms (Basidiomycota 103 species, Ascomycota 4 species) are known to have long been used by local people from various provinces (West Java, West Kalimantan, South Sumatra, Lampung, Bangka Belitung, Jambi, Central Sulawesi, Riau) in Indonesia. Most of them have been identified up to the species level (Table 1), with 170 local names recorded. This data will contribute to the record of Indonesian fungal biodiversity. Until 2017, there are only 2273 species of fungi in Indonesia (micro-and macroscopic fungi), or only approximately 0.15% of the total species in the world and did not include the information on their use (Indonesian Institute of Sciences 2019). Blackwell (2011) suggested that 70,000 fungal species have been described, of an estimated total of 1,500,000 species exist on earth. To date, the estimated number of fungal species in the world is increasing between 2.2–3.8 million (Cannon et al. 2018). Furthermore, Hawksworth (2001) argued that fungi in the tropics should be more diverse than the temperate areas, but the documentation has not been done well, and Indonesia is no exception. Currently, Indonesia does not have a checklist of mushroom species and the updated information of index of local mushroom names. Thirty-six species of wild mushrooms in this report are known to have more than one local name, either from the same or different local ethnicity knowledge (Table 1, Figure 4). They have between 2-5 names for each species with a total of 99 local names, while the other 71 mushrooms have only one local name (Table 1). Some of wild mushrooms include *Coprinus* spp., *Polyporus* spp., *Schizophyllum commune*, *Scleroderma* spp., *Termitomyces* spp., and *Trametes* spp. have five different local names from each province and the ethnicity that uses them. The result shows that these mushrooms are popular in various tribes in Indonesia.

The mushroom information is obtained from 8 provinces in Indonesia (Figure 2), with West Kalimantan (40.2%), West Java (36.2%), and South Sumatra (18.9%) having the highest data, respectively. This result indicates that these areas have the records of wild mushrooms by indigenous peoples provided in appropriate scientific publications. The information related to ethnomycology in Indonesia should be higher than the presented data, considering the vast territory of Indonesia with the extravagant of ethnic groups. According to The Consensus of the Central Statistics Agency of Indonesia (2010), there are 1340 ethnic groups in Indonesia. It is implied that the possibility to obtain more information on the use of wild mushrooms in Indonesia. Meanwhile, information regarding the use of wild mushrooms was obtained from 8 tribes (Figure 3), with Javanese (28.3%), Dayak (26.7%), Baduy (20.4%), and Malay (15.7%) having the highest data, respectively. Until recently, these tribes contributed the most information related to wild mushroom uses in Indonesia (Putra & Hafazallah 2020). A total of 50 species of mushrooms (Table 2) are used by various tribes primarily as food and traditional medicines (Figures 1; 5). The wild mushrooms are generally

consumed by themselves, but some communities sell them in conventional or modern markets (Putra 2020a; Putra & Hafazallah 2020). Some of the mushrooms sold by local people include 'pelawan' mushroom/*Heimioporus* sp. (Bangka Belitung Province), 'melinjo' mushroom/*Scleroderma* spp. (South Sumatra Province), and *Favolaschia manipularis* (Central and East Java Provinces). Many wild mushrooms have been reported as a low-calorie food source and rich in vegetable protein, minerals, and vitamins (Chang & Miles 2004; Wang et al. 2014). In addition, mushrooms contain bioactive ingredients that function as anticancer, antibacterial, antifungal, antioxidant, anti-inflammatory, and other derivative active ingredients (Adhikari 2020). The mushrooms used by the community in this review were generally collected from soil, wood, straw, and oil palm bunches (Figure 6). Due to their cosmopolitan lifestyle, mushrooms are known to grow in various environmental conditions in Indonesia (Putra et al. 2018).

Figure 1. Some of wild edible mushrooms used as food by local people in Indonesia. A-B. *Termitomyces eurizhus*. C-D. *Schizophyllum commune*. E-F. *Scleroderma* sp. G-H. *Cookeina speciosa*. (With permission: A-D, G-H Putra & Hafazallah (2020), E-F Indonesian Mushroom Hunter Community).

Table 1. List of local names of Indonesian wild mushrooms.

Local Name	Scientific Name	Location	Ethnicity Group	Literatures
Supa baseuh	*Agaricus* sp.	West Java	Baduy	Khastini et al. 2019
Kulat ipoh	*Amanita vaginata*	West Kalimantan	Dayak	Syafrizal et al. 2014
Supa kamanden	*Amauroderma* sp.	West Java	Baduy	Khastini et al. 2018, 2019
Kulat gadong	*Amauroderma rugosum*	West Kalimantan	Dayak	Syafrizal et al. 2014; Yunida et al. 2014
Supa jambu	*Armillaria* spp.	West Java	Sunda	Putra & Hafazallah 2020
Supa ceuli, Supa lember lutung, Supa lamber sungu	*Auricularia* spp.	West Java	Baduy , Sunda	Al Ulya et al. 2017; Khastini et al. 2019; Putra & Hafazallah 2020
Kulat kuping	*Auricularia auricula-judae*	West Kalimantan	Dayak	Syafrizal et al. 2014
Kulat kerup	*Auricularia delicata*	West Kalimantan	Dayak	Syafrizal et al. 2014
Supa kebo	*Boletus* sp.	West Java	Sunda	Al Ulya et al. 2017
Supa koneng	*Calocera* sp.	West Java	Baduy, Sunda	Al Ulya et al. 2017; Khastini et al. 2019
Kulat mata sapi, Kulat mate	*Calostoma* sp.	West Kalimantan	Dayak	Syafrizal et al. 2014; Yunida et al. 2014
Jamur impes	*Calvatia excipuliformis*	South Sumatra	Malay	Riastuti et al. 2018
Kulat nangka, Supa brui	*Cantharellus* sp.	Lampung, West Java	Malay, Sunda	Putra & Hafazallah 2020
Supa padali bodas	*Cellulariella* sp.	West Java	Baduy	Khastini et al. 2019
Supa koja	*Clavaria* sp.	West Java	Baduy	Khastini et al. 2019
Supa jangkar	*Clavariadelphus* sp.	West Java	Baduy	Khastini et al. 2019
Jamur gagang	*Clitocybe desembris*	South Sumatra	Malay	Riastuti et al. 2018
Jamur payung rayun	*Clitocybe infundibuliformis*	South Sumatra	Malay	Riastuti et al. 2018
Kulat nyiur	*Collybia* sp.	West Kalimantan	Dayak	Yunida et al. 2014
Jamur ati	*Coltricia perennis*	South Sumatra	Malay	Riastuti et al. 2018
Supa taneh lojor, Supa jarum	*Conocybe* sp.	West Java	Baduy, Sunda	Al Ulya et al. 2017; Khastini et al. 2019
Kulat mangkok	*Cookeina speciosa*	West Kalimantan	Dayak	Syafrizal et al. 2014; Putra & Hafazallah 2020
Kulat mangkok	*Cookeina sulcipes*	West Kalimantan	Dayak	Yunida et al. 2014
Kulat mangkok, Kulat terap	*Cookeina tricholoma*	West Kalimantan, South Sumatra	Dayak, Malay	Syafrizal et al. 2014; Yunida et al. 2014; Riastuti et al. 2018; Putra & Hafazallah 2020
Kulat beras, Supa taneh leutik, Supa amis, Supa jarami, Supa taneh bodas	*Coprinus* spp.	West Kalimantan, West Java	Dayak, Baduy	Yunida et al. 2014; Khastini et al. 2019
Jamur krikik	*Coriolus pubescens*	South Sumatra	Malay	Riastuti et al. 2018
Jamur racun	*Coriolus versicolor*	South Sumatra	Malay	Riastuti et al. 2018
Supa kasungka	*Cortinarius* sp.	West Java	Baduy	Khastini et al. 2019
Kulat tepus	*Crepidotus applanatus*	West Kalimantan	Dayak	Syafrizal et al. 2014
Supa batok	*Cyathus* sp.	West Java	Baduy	Khastini et al. 2019
Kulat gadong	*Cymatoderma elegans*	West Kalimantan	Dayak	Syafrizal et al. 2014

Table 1. Contd.

Local Name	Scientific Name	Location	Ethnicity Group	Literatures
Supa kayas bodas, Supa kayang	*Daedalea* spp.	West Java	Baduy	Khastini et al. 2019
Jamur racun	*Daedalea elegans*	South Sumatra	Malay	Riastuti et al. 2018
Supa padali coklat	*Daedaleopsis* sp.	West Java	Baduy	Khastini et al. 2019
Jamur krikik	*Daedaleopsis confraggosa*	South Sumatra	Malay	Riastuti et al. 2018
Kulat bulat	*Daldinia* sp.	West Kalimantan	Dayak	Yunida et al. 2014
Jamur batu	*Daldinia concentrica*	South Sumatra	Malay	Riastuti et al. 2018
Kulat gadong putih	*Filoboletus manipularis*	West Kalimantan	Dayak	Syafrizal et al. 2014
Supa letah	*Fistulina hepatica*	West Java	Sunda	Putra & Hafazallah 2020
Kulat gadong, supa bereum	*Fomes fomentarius*	West Kalimantan, West Java	Dayak, Baduy	Yunida et al. 2014; Khastini et al. 2018
Supa tutung, supa awi	*Fomitopsis* spp.	West Java	Baduy	Khastini et al. 2019
Jamur mata kerbau	*Galiella* sp.	Riau	Malay	Putra & Hafazallah 2020
Supa tutung bodas, Supa tutung hideng	*Ganoderma* spp.	West Java	Baduy	Khastini et al. 2019
Kulat gadong, Kulat gadong hitam, Supa coklat kayas	*Ganoderma applanatum*	West Kalimantan, West Java	Dayak, Baduy	Syafrizal et al. 2014; Yunida et al. 2014; Khastini et al. 2018
Kulat gadong amas	*Ganoderma australe*	West Kalimantan	Dayak	Yunida et al. 2014
Kulat gadong, Supa tutung bodas	*Ganoderma lucidum*	West Kalimantan, West Java	Dayak, Baduy	Syafrizal et al. 2014; Khastini et al. 2018
Kulat gadong	*Gloeophyllum sepiarium*	West Kalimantan	Dayak	Syafrizal et al. 2014
Jamur dipa	*Grifolla frondosa*	South Sumatra	Malay	Riastuti et al. 2018
Supa catang, Supa Wood, Supa awi	*Gymnopus* sp.	West Java	Baduy	Khastini et al. 2019
Kulat minyak	*Gymnopus dryophilus*	West Kalimantan	Dayak	Syafrizal et al. 2014
Kulat pelawan	*Heimioporus* sp.	Bangka Belitung	Malay	Putra & Hafazallah 2020
Kulat sisik	*Hexagonia papyracea*	West Kalimantan	Dayak	Syafrizal et al. 2014
Supa kasongket	*Hygrocybe* sp.	West Java	Baduy	Khastini et al. 2019
Kulat tiong, kulat siung	*Hygrocybe conica*	Bangka Belitung, West Kalimantan	Malay, Dayak	Putra & Hafazallah 2020
Kulat gadong, Kulat kalimbauan	*Hymenochaete rubiginosa*	West Kalimantan	Dayak	Syafrizal et al. 2014; Yunida et al. 2014
Supa kincir coklat	*Hymenopellis* sp.	West Java	Baduy	Khastini et al. 2019
Supa akar hideng	*Laccaria* sp.	West Java	Baduy	Khastini et al. 2019
Supa nyiruan	*Laetiporu*s sp.	West Java	Baduy	Khastini et al. 2019
Kulat kuning	*Laetiporus sulphureus*	West Kalimantan	Dayak	Yunida et al. 2014
Kulat tawak, Supa cau, Supa kayas bereum	*Lentinus* sp.	West Kalimantan, West Java	Dayak, Baduy	Yunida et al. 2014; Khastini et al. 2019
Jamur tui, kulat ngkasehan, jamur lot	*Lentinus sajor-caju*	South Sumatra, West Kalimantan	Malay, Dayak	Yunida et al. 2014; Riastuti et al. 2018; Putra & Hafazallah 2020
Supa tai kotok, supa wereu	*Lepiota* sp.	West Java	Baduy, Sunda	Al Ulya et al. 2017; Khastini et al. 2019
Jamur barat	*Lepiota cristata*	South Sumatra	Malay	Riastuti et al. 2018
Jamur telor	*Lycoperdon pyriforme*	South Sumatra	Malay	Riastuti et al. 2018

Table 1. Contd.

Local Name	Scientific Name	Location	Ethnicity Group	Literatures
Supa amis, Suapa glenter	*Marasmiellus* spp.	West Java	Sunda	Al Ulya et al. 2017
Kulat kerang	*Marasmiellus affixus*	West Kalimantan	Dayak	Syafrizal et al. 2014
Kulit putih	*Marasmius* spp.	West Kalimantan	Dayak	Yunida et al. 2014
Kulat papan, Supa arey	*Microporus* spp.	West Kalimantan, West Java	Dayak, Baduy	Yunida et al. 2014; Khastini et al. 2019
Kulat gadong	*Microporus xanthopus*	West Kalimantan	Dayak	Syafrizal et al. 2014
Kulat terang, Supa lumar catang, Supa payung, suum caum oranye	*Mycena* spp.	West Kalimantan, West Java	Dayak, Baduy	Yunida et al. 2014; Khastini et al. 2019
Kulat gadong putih	*Mycena chlorophos*	West Kalimantan	Dayak	Syafrizal et al. 2014
Jamur kelapa	*Mycena haematopus*	South Sumatra	Malay	Riastuti et al. 2018
Kulat pangku anak	*Mycena leaiana*	West Kalimantan	Dayak	Syafrizal et al. 2014
Supa tikukur, kulat tawe	*Oudemansiella* sp.	West Java, West Kalimantan	Baduy, Dayak	Khastini et al. 2019; Putra & Hafazallah 2020
Kulat tangar	*Mycena subcana*	West Kalimantan	Dayak	Yunida et al. 2014
Jamur wulu	*Panus rudis*	South Sumatra	Malay	Riastuti et al. 2018
Jamur iwak	*Phaeolus schweinitzii*	South Sumatra	Malay	Riastuti et al. 2018
Jamur pengantin	*Phallus* spp.	West Java, West Kalimantan	Sunda, Dayak	Putra & Hafazallah 2020
Supa bereum	*Phellinus linteus*	West Java	Baduy	Khastini et al. 2018
Supa akar, Supa akar Wood	*Pleurotus* spp.	West Java	Baduy	Khastini et al. 2019
Kulat pangku anak, Kulat beras	*Pleurotus ostreatus*	West Kalimantan	Dayak	Syafrizal et al. 2014; Yunida et al. 2014
Jamur gromo	*Pleurotus pulmonarius*	South Sumatra	Malay	Riastuti et al. 2018
Kulat labang, Cendawan elang, supa kincir, supa liat, supa lipit	*Polyporus* spp.	West Kalimantan, Jambi, West Java	Dayak, Kerinci, Sunda	Syafrizal et al. 2014; Saputra et al. 2018; Putra & Hafazallah 2020
Kulat gelang	*Polyporus durus*	West Kalimantan	Dayak	Syafrizal et al. 2014
Supa cau hideng, Supa kiray	*Psathyrella* spp.	West Java	Baduy	Khastini et al. 2019
Supa bereum	*Pycnoporus* sp.	West Java	Baduy	Khastini et al. 2019
Jamur merah, Kulat areh	*Pycnoporus cinnabarius*	South Sumatra, West Kalimantan	Malay, Dayak	Yunida et al. 2014; Riastuti et al. 2018
Kulat gadong, kulat areh	*Pycnoporus sanguineus*	West Kalimantan	Dayak	Syafrizal et al. 2014; Yunida et al. 2014
Jamur karang	*Ramaria kunzei*	South Sumatra	Malay	Riastuti et al. 2018
Supa kayas hideng, Supa lumar catang	*Rigidoporus* spp.	West Java	Baduy	Khastini et al. 2019
Kulat gadong, kulat krikik	*Rigidoporus microporus*	West Kalimantan, South Sumatra	Dayak, Malay	Yunida et al. 2014; Riastuti et al. 2018
Supa kayas hideung	*Rigidoporus stereum*	West Java	Baduy	Khastini et al. 2018
Kulat kawi	*Russula vesca*	West Kalimantan	Dayak	Syafrizal et al. 2014
Supa kincir bereum	*Sarcoscypha* sp.	West Java	Baduy	Khastini et al. 2019
Kulat manis	*Stereum ostrea*	West Kalimantan	Dayak	Syafrizal et al. 2014
Jamur grigit, kulat karang, Supa mireg, tanggidi, taggojo	*Schizophyllum commune*	South Sumatra, West Kalimantan, West Java, Central Sulawesi	Malay, Dayak, Baduy, Kaili	Yunida et al. 2014; Riastuti et al. 2018; Khastini et al. 2019; Putra & Hafazallah 2020
Jamur so, jamur melinjo, jamur kodok, jamur tangkil, supa buled	*Scleroderma* spp.	Sumatra, Java, Kalimantan	Malay, Betawi, Sunda, Java	Al Ulya et al. 2017; Putra & Hafazallah 2020
Suung tunggal, Jamur rayap, jamur barat, jamur bulan, jamur sempagi	*Termitomyces* spp.	Java, Kalimantan, Sumatra	Baduy, Sunda, Malay, Java	Al Ulya et al. 2017; Putra & Hafazallah 2020
Jamur barat, supa bulan, suung rampak, kulat elong	*Termitomyces eurhizus*	West Java, East Java, West Kalimantan	Sunda, Java, Dayak	Putra & Hafazallah 2020

Table 1. Contd.

Local Name	Scientific Name	Location	Ethnicity Group	Literatures
Kulat papan, Supa kayas putih, Supa cau leutik, Supa Wood putih, Supa cau leutik	*Trametes* spp.	West Kalimantan, West Java	Dayak, Baduy	Yunida et al. 2014; Khastini et al. 2019
Kulat gadong	*Trametes hirsuta*	West Kalimantan	Dayak	Syafrizal et al. 2014
Kulat gadong	*Trametes pubescens*	West Kalimantan	Dayak	Syafrizal et al. 2014
Jamur mata kerbau	*Trichaleurina javanica*	West Kalimantan	Dayak	Putra & Hafazallah 2020
Kulat hati bekut	*Tricholoma sulphureum*	West Kalimantan	Dayak	Yunida et al. 2014
Suum cau hiding, jamur lengkuas	*Volvariella* sp.	West Java, Central Java, West Kalimantan	Baduy, Java, Dayak	Khastini et al. 2019; Putra & Hafazallah 2020
Jamur sawit	*Volvariella volvacea*	South Sumatra	Malay	Riastuti et al. 2018
Jamur rambut sawit	*Xylaria* sp.	South Sumatra	Malay	Riastuti et al. 2018

Table 2. Information on the utilization of wild mushrooms by local people in Indonesia.

Species	Habitat	Uses	References
Agaricus spp.	Soil	Food	Khastini et al. 2019; Putra & Hafazallah 2020
Agaricus campestris	Soil	Food	Putra & Hafazallah 2020
Amauroderma sp.	Soil	Fever medicine	Khastini et al. 2018
Amauroderma rugosum	Soil	Fever medicine	Putra & Hafazallah 2020
Armillaria spp.	Wood	Food	Putra & Hafazallah 2020
Auricularia spp.	Wood	Food, Fever medicine	Al Ulya et al. 2017; Khastini et al. 2019; Putra & Hafazallah 2020
Auricularia cornea	Wood	Food	Putra & Hafazallah 2020
Auricularia delicata	Wood	Food	Putra & Hafazallah 2020
Auricularia mesenterica	Wood	Food	Putra & Hafazallah 2020
Auricularia nigricans	Wood	Food	Putra & Hafazallah 2020
Boletus spp.	Soil	Food	Putra & Hafazallah 2020
Calvatia spp.	Soil	Food, Bump medicine	Putra & Hafazallah 2020
Calvatia excipuliformi	Soil	Food (young basidiomata), Wound medicine	Riastuti et al. 2018
Clitocybe decembris	Soil	Food	Riastuti et al. 2018
Coltricia perennis	Wood	Food	Riastuti et al. 2018
Cookeina speciosa	Wood	Food	Riastuti et al. 2018
Cookeina tricholoma	Wood	Food	Riastuti et al. 2018
Coprinellus sp.	Soil	Food	Al Ulya et al. 2017
Coprinus spp. (young basidiomata)	Soil, Straw	Food	Khastini et al. 2019; Putra & Hafazallah 2020
Coprinus comatus	Soil	Food	Putra & Hafazallah 2020
Daldinia concentrica	Wood	Itchy medicine	Riastuti et al. 2018
Favolaschia manipularis	Wood	Food	Putra & Hafazallah 2020
Favolus spp.	Wood	Food (flavour seasoning)	Putra & Hafazallah 2020
Fomes fomentarius	Wood	Fever medicine	Khastini et al. 2018
Galiella sp.	Wood	Wound medicine	Putra & Hafazallah 2020
Ganoderma lucidum	Wood	Cancer medicine	Khastini et al. 2018
Grifolla Frondosa	Wood	Food	Riastuti et al. 2018
Heimioporus sp.	Soil	Food	Putra & Hafazallah 2020
Hygrocybe conica	Soil	Food	Putra & Hafazallah 2020
Laetiporus sulphuratus	Wood	Food	Khastini et al. 2019; Putra & Hafazallah 2020
Lentinus sajor-caju	Wood	Food	Khastini et al. 2019; Putra & Hafazallah 2020
Lycoperdon pyriforme	Soil	Food	Riastuti et al. 2018
Marasmiellus sp.	Wood	Food	Al Ulya et al. 2017
Mycena sp.	Wood	Food	Khastini et al. 2019
Mycena haematopus	Wood	Food	Riastuti et al. 2018

Table 2. Contd.

Species	Habitat	Uses	References
Oudemansiella sp.	Wood	Food	Khastini et al. 2019; Putra & Hafazallah 2020
Phaeolus schweinitzii	Wood	Food	Riastuti et al. 2018
Phellinus linteus	Wood	Blood circulation disorder medicine	Khastini et al. 2018
Pleurotus sp.	Wood	Food	Al Ulya et al. 2017
Pleurotus pulmonarius	Wood	Food	Riastuti et al. 2018
Polyporus spp.	Wood	Food	Putra & Hafazallah 2020
Rigidoporus stereum	Wood	Immune deficiency medicine	Khastini et al. 2018
Russula spp.	Soil	Food	Putra & Hafazallah 2020
Schizophyllum commune	Wood	Food	Khastini et al. 2019; Putra & Hafazallah 2020
Scleroderma spp. (young basidiomata)	Soil	Food	Putra & Hafazallah 2020
Tremella sp.	Wood	Food	Putra & Hafazallah 2020
Trichaleurina javanica	Wood	Eye medicine	Putra & Hafazallah 2020
Volvariella spp.	Wood, Palm bunch, Straw	Food	Khastini et al. 2019; Putra & Hafazallah 2020
Volvariella bombycina	Wood	Food	Putra & Hafazallah 2020
Volvariella volvaceae	Palm bunch	Food	Riastuti et al. 2018

One of the mushrooms reported in this review has the highest economic value in Indonesia, namely the 'pelawan' mushroom, commonly consumed and traded by the local people in Bangka Belitung Province (Putra 2020a; Putra & Hafazallah 2020). This macrofungus forms the the ectomycorrhizae symbiosis with 'pelawan' plant (*Tristaniopsis* spp.) and has not been successfully cultivated. To date, the Malay indigenous people in Bangka Belitung still depend on the production of this mushroom in nature. This mushroom has a high price, both in fresh conditions (ranging between 200-500 thousand of Indonesian rupiah/kg) or dry condition (> 1 million Indonesian rupiah/kg, depending on the season and availability). This fact has become the concern of the local government to preserve the 'pelawan' forest, the habitat of pelawan' mushroom.

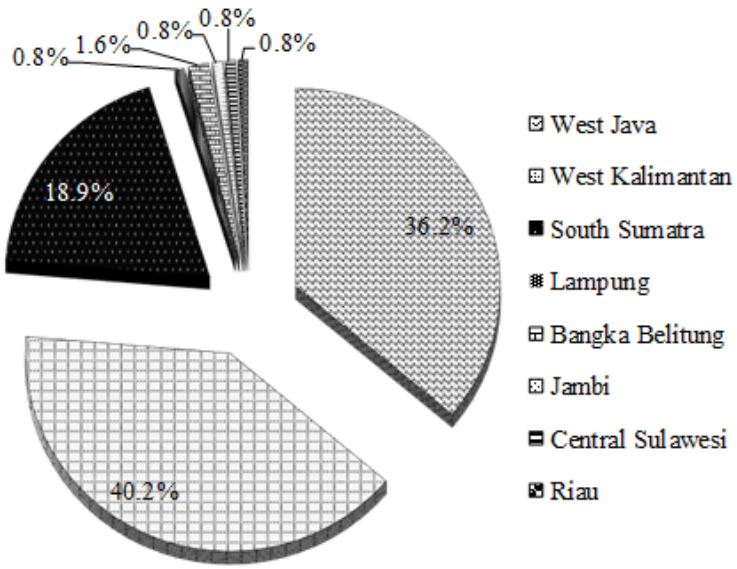

Figure 2. Percentage of local knowledge distribution of mushrooms utilization in the provinces of Indonesia.

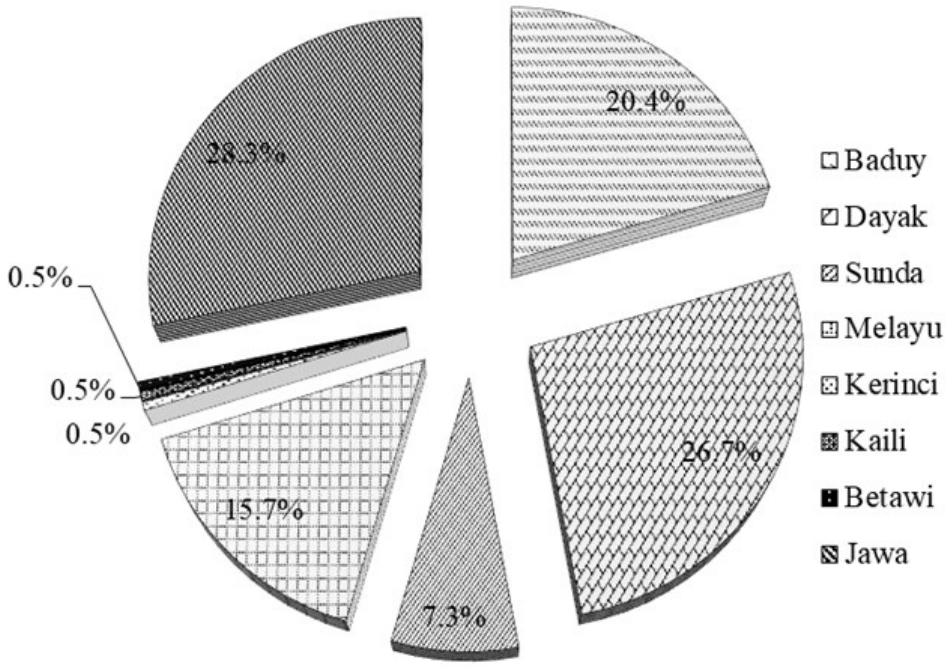

Figure 3. Percentage of local knowledge distribution of mushrooms utilization by ethnic groups in Indonesia.

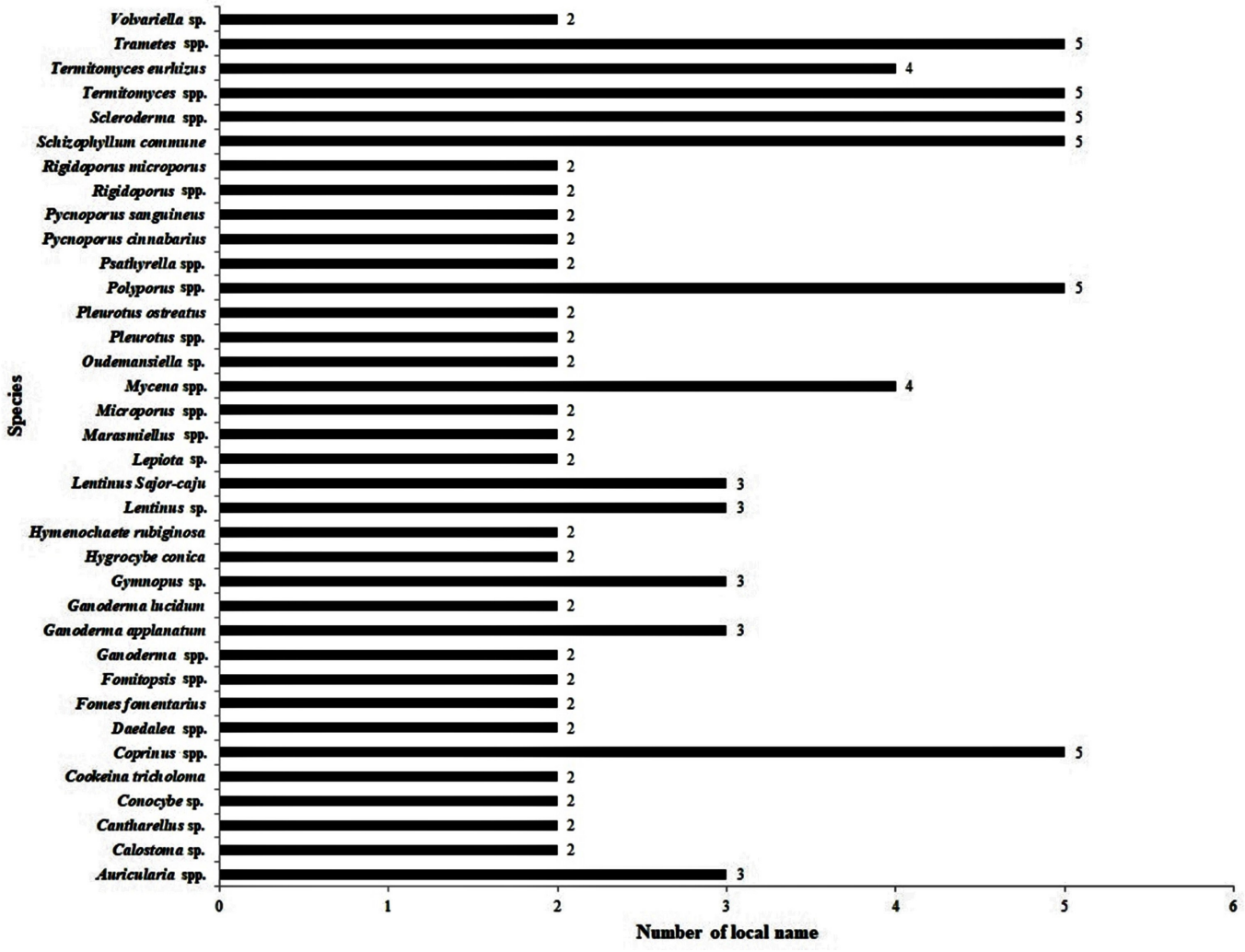

Figure 4. Indonesian wild mushrooms with more than one local name.

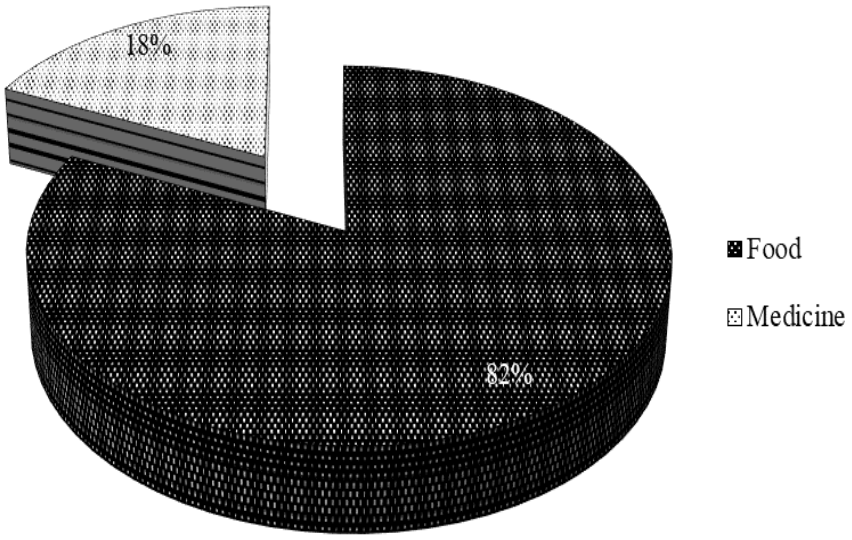

Figure 5. Indonesian wild mushrooms utilization by local people in Indonesia.

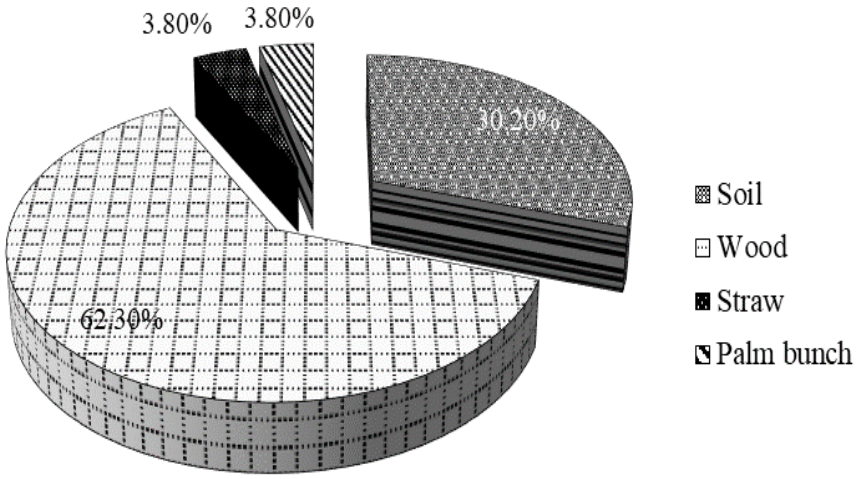

Figure 6. The habitat of Indonesian wild mushrooms used by local people in Indonesia.

Ethnomycology focuses on the interaction between local communities and fungi (Reyes-Lopez et al. 2020). However, this topic is less popular in Indonesia. We consider that this discipline is helpful to collect the data on mushroom germplasm in Indonesia, especially from the indigenous knowledge. We suggest that the collection of information in this review needs a special attention regarding the biodiversity records in Indonesia. Our data revealed that the distribution of mushroom taxonomy information is not centered only on Java Island. Indonesian Institute of Sciences (2019) stated that until 2017 most of the reports on fungal biodiversity in Indonesia were obtained mainly from Java Island. Hence, the validation taxonomy of wild mushrooms at the species level should be more comprehensive, especially from West Kalimantan with higher data contributions than provinces in Java Island. Further research on ethnomycology in Indonesia needs to be carried out thoroughly on the comprehensive aspects by considering biology (identification), conservation, cultivation, production, marketing, and the preservation of the local knowledge to optimize its benefits in the future.

CONCLUSION

In conclusion, we present the current checklist of local names of Indonesian wild mushrooms and their uses. Total of 107 wild mushrooms have 170 local names, with 36 of them having more than one name. Considering the vast territory of Indonesia and the existing tribes, we suspect that there is still more information which has not been studied and published in the scientific publications. We expect the data should be periodically updated, and the information will be in flux.

AUTHORS CONTRIBUTION

I.P.P. and N.N. contributed to the study conception and design. R.H. and M.P.A. obtained the data. I.P.P. and N.N. analyzed the data. All authors wrote the manuscript. All authors read, critically revised, and approved the final manuscript.

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CONFLICT OF INTEREST

The authors declare no competing interests.

REFERENCES

Adhikari, M.K., 2020. Some antiviral mushrooms of Nepal. *Nepal Journal of Science and Technology*, 19(1), pp.54–59. doi: 10.3126/njst.v19i1.29767

Al Ulya et al., 2017. Biodiversitas dan potensi jamur basidomycota di Kawasan Kasepuhan Cisungsang, Kabupaten Lebak, Banten. *Al-Kauniyah: Jurnal Biologi*, 10(1), pp.9–16. doi: 10.15408/kauniyah.v10i1.4513.

Álvarez-Farias et al., 2016. Ethnomycological knowledge of wild edible mushrooms in Tlayacapan, Morelos. *Mycosphere,* 7(10), pp.1491–1499. doi: 10.5943/mycosphere/si/3b/1.

Anon., 2002. *Termitomyces* mushrooms: a tropical delicacy. *Mycologist,* 16(1), pp.1–9. doi: 10.1016/s0269-915x(07)60014-7

Ao et al., 2016. Wild edible mushrooms of Nagaland, India: a potential food resource. *Journal of Experimental Biology and Agricultural Sciences*, 4(1), pp.59–65. doi: 10.18006/2015.4(1).59.65.

Arora, D., 1986. *Mushrooms demystified*, Teen Speed Press.

Bisema, J., M., 1968. *Djamur: jang dapat dimakan, jang beratjun dan pengusahaan djamur merang di Indonesia*, PT Kinta.

Blackwell, M., 2011. The fungi: 1, 2, 3 … 5.1 million species? *American Journal of Botany*, 98(3), pp.426–438. doi: 10.3732/ajb.1000298.

Boa, E., 2004. *Wild edible fungi: a global overview of their use and importance to people*, Food and Agriculture Organization of The United Nations.

Cannon et al., 2018. Definition and diversity. In *state of the world's fungi 2018*. Royal Botanic Gardens Kew, pp.6–12.

Chang, S.T., Miles, P., 2004. *Cultivation techniques. In mushroom, cultivation, nutritional value and medicinal effect and environmental impact*, CRS Press.

Hawksworth, D.L., 2001. The magnitude of fungal diversity: the 1.5 million species estimate revisited. *Mycological Research,* 105(12), pp.1422–1432. doi: 10.1017/s0953756201004725

Heyne, K, 1987. *Tumbuhan berguna Indonesia. Jilid II*, Badan Penelitian dan Pengembangan Departemen Kehutanan .

Index Fungorum., 2021. Index Fungorum. http://www.indexfungorum.org/names/Names.asp

Indonesian Institute of Sciences., 2019. *Status keanekaragaman hayati Indonesia: kekayaan jenis tumbuhan dan jamur Indonesia,* A. Retnowati, Rugayah, J. S. Rahajoe, D. Arifiani (eds.), LIPI Press.

Khastini et al., 2018. Ethnomycology of bracket fungi in baduy tribe Indonesia. *Biosaintifika: Journal of Biology & Biology Education,* 10(2), pp.424–432. doi: 10.15294/biosaintifika.v10i2.14082

Khastini et al., 2019. Inventory and utilization of macrofungi species for food in Cikartawana inner Baduy Banten. *Biodidaktika, Jurnal Biologi Dan Pembelajarannya*, 14(1), pp.7–13. doi: 10.30870/biodidaktika.v14i1.4838

Kiet, T.T., 2008. Preliminary checklist of macrofungi of Vietnam. *Feddes Repertorium*, 109(3-4), pp.257–277. doi: 10.1002/fedr.19981090309.

Lazo et al., 2015. Ethnomycological survey of cacrofungi utilized by gaddang communities in Nueva Vizcaya, Philippines. *Current Research in Environmental & Applied Mycology,* 5(3), pp.256–262. doi: 10.5943/cream/5/3/8

Lee et al., 2008. C*hecklist of Literature on Malaysian macrofungi*, Forest Research Institute Malaysia (FRIM).

Lima et al., 2012. Poisonous mushrooms; a review of the most common intoxications. *Nutricion Hospitalaria,* 27(2), pp.402–408. doi: 10.1590/s0212 16112012000200009

Merida Ponce et al., 2019. Ethnomycological knowledge among Kaqchikel, indigenous maya people of Guatemalan Highlands. *Journal of Ethnobiology and Ethnomedicine*, 15(36), doi: 10.1186/s13002-019-0310-7

Osarenkhoe et al., 2014. Ethnomycological conspectus of West African Mushrooms: An awareness document. *Advances in Microbiology*, 4(1), pp.39–54. doi: 10.4236/aim.2014.41008

Pala et al., 2013. Ethnomycological studies of some wild medicinal and edible mushrooms in the Kashmir Himalayas (India). *International Journal of Medicinal Mushrooms*, 15(2), pp.211–220. doi: 10.1615/intjmedmushr.v15.i2.100

Putra et al., 2017. Ragam jamur asal serasah dan tanah di Taman Nasional Ujung Kulon Indonesia. *Jurnal Sumberdaya HAYATI*, 3(1), pp.1–7. doi: 10.29244/jsdh.3.1.%25p

Putra et al., 2018. Ragam dan potensi jamur makro asal Taman Wisata Mekarsari Jawa Barat. *Al-Kauniyah*, 11(2), pp.133–150. doi: 10.15408/kauniyah.v11i2.6729

Putra et al., 2019. Catatan beberapa jamur makro di Hutan Kampus Institut Pertanian Bogor. *Biota*, 12(2), pp.57–71. doi: 10.20414/jb.v12i2.192

Putra, I.P., 2020a. Potensi beberapa jamur pangan liar yang bernilai ekonomi di Pulau Belitong, Provinsi Kepulauan Bangka Belitung. *Jurnal Wasian*, 7 (2), pp.121–135. doi: 10.20886/jwas.v7i2.6109

Putra, I.P., 2020b. *Scleroderma* spp. in Indonesia : poisoning case and potential utilization. *Jurnal Sains Dan Teknologi*, 3(2), pp.37–45. doi: 10.31764/justek.vXiY.3517

Putra, I.P., Hafazallah, K., 2020. *Catatan komunitas pemburu jamur Indonesia : kolaborasi lintas profesi dan generasi mengenai etnomikologi jamur-jamur Indonesia*, Haura.

Reyes-Lopez et al., 2020. Folk classification of wild mushrooms from San Isidro Buensuceso, Tlaxcala, Central Mexico. *Journal of Ethnobiology Ethnomedicine*, 16(53), doi: 10.1186/s13002-020-00408-x

Riastuti et al., 2018. Eksplorasi jamur makroskopis di perkebunan kelapa sawit. *BIOEDUSAINS: Jurnal Pendidikan Biologi Dan Sains*, 1(2), pp.126–135. doi: 10.31539/bioedusains.v1i2.454

Saputra et al., 2018. Study of wild edible *Polyporus* (cendawan elang) from West Wood Aro Subdistrict, Kerinci Regency, Jambi. *Metamorfosa: Journal Of Biological Sciences,* 5(1), pp.112–116. doi: 10.24843/metamorfosa.2018.v05.i01.p17

Semwal et al., 2014. Edible mushrooms of the Northwestern Himalaya, India: a study of indigenous knowledge, distribution, and diversity. *Mycosphere*, 5(3), pp.440–461. doi: 10.5943/mycosphere/5/3/7

Statistics Indonesia., 2021. *Statistical Yearbook of Indonesia 2021*, Badan Pusat Statistik.

Syafrizal et al., 2014. Inventarisasi jamur makroskopis di Hutan Adat Kantuk dan implementasinya dalam pembuatan flipbook. J*urnal Pendidikan Dan Pembelajaran Untan*, 3(9), pp.1–15. doi: 10.26418/jppk.v3i9.6985

The Consensus of the Central Statistics Agency of Indonesia., 2010. Suku Bangsa. https://indonesia.go.id/profil/suku-bangsa#:~:text=Indonesia memiliki lebih dari 300,mencapai 41%25 dari total populasi

Wang et al., 2014. A mini-review of chemical composition and nutritional value of edible wild-grown mushroom from China. *Food Chemistry*, 151, pp.279–285. doi: 10.1016/j.foodchem.2013.11.062

Yilmaz, H. & Zencirci, N., 2016. Ethnomycology of macrofungi in the Western Black Sea Region of Turkey: Identification to Marketing. *Economic Botany*, 70(3), pp.270–283. doi: 10.1007/s12231-016-9353-z

Yunida et al., 2014. Inventarisasi jamur di Gunung Senujuh Kabupaten Sambas dan implementasinya dalam pembuatan flash card. *Jurnal Pendidikan Dan Pembelajaran Khatulistiw*a, 3(10), doi: 10.26418/jppk.v3i10.7502