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Research Article

Genotyping and Phytochemical Analysis of Kayu Pule Plant as Local Bali Medicinal Plant

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ABSTRACT
The bark of Kayu Pule plants in Bali is empirically known as a traditional medicinal ingredient and has been developed as a cosmetic and other health ingredient; however, scientific research has yet to be conducted on the profiles of the plant. This study aimed to determine the plant species, examine the scientific function of the compounds, and the antioxidant activity of the plant's ethanolic extract. This study performed a DNA analysis of the plant using matK primer, and the amplified DNA sequences were used to determine the phylogenetic tree. Based on the molecular analysis, the Kayu Pule plant bark from Bali, which was used as medicine, was *Alstonia scholaris*. The main compounds in Kayu Pule bark, such as ergost-5-en-3-ol and 12-oleanen -3-yl acetate, had anti-inflammatory, antioxidant, and antimicrobial properties. The antioxidant strength of the Kayu Pule plant was measured with IC50 of 3.7 µg mL^{-1} with a very strong category. This research showed the potential of Kayu Pule for developing medicinal and cosmetic products.

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INTRODUCTION

The greater public attention to the dangers of chemical products, the more organic products in food and herbal medicines are increasingly in demand by the public (Rahayu et al. 2020). Various types of medicinal plants have been developed and used traditionally in various regions of Indonesia. The medicinal properties of plants in Bali are listed in Lontar *Taru Pramana*, so their efficacy is believed to be hereditary. Some medicinal plants in the lontar include *Moringa oleifera* (Vergara-Jimenez et al. 2017; Tenri & Rivai 2020; Qadir et al. 2022). Several plants, including *Syzygium polyanthum*, *Andrographis paniculata*, *Clitoria ternatea*, tangi wood (Kayu Tangi), and pule wood (Kayu Pule) (Andila et al. 2023), are used to cure various diseases. One of the plants the community has widely used is the Kayu Pule plant. The Kayu Pule plant traditionally used its bark (*babakan)* for traditional scrubs (*boreh*), tea, and relatively modern a simple flour (simplisia) preparations used as scrubs or cosmetics without scientific proof. Therefore, we conducted this research by focusing on genotyping, and analysing phytochemical content (bioactive compounds) and antioxidant power.

Plants used in the community for generations generally lack scientific proof. Therefore, scientific studies are essential to support the development of traditional herbal medicine, mainly to correctly identify species information of plants, in particular for species with similar morphological features. Identification can be done by molecular analysis through DNA sequences and comparing them with the genetics present in Genbank. This study aims to genetically analyse to determine plant species based on MatK primers, determine the content and function of chemical compounds, and the strength of plant antioxidants. Therefore, the urgency of this research is very strategic for identifying which Balinese plant materials that can be used for health purposes, particularly as anti-inflammatory, antioxidant, and antimicrobial compounds.

MATERIALS AND METHODS

Genotyping

This study was conducted by isolating total DNA from the leaves of Kayu Pule plants with Quick DNA Plant/Seieid Miniprep Kit (Zymo Research), following the kit procedure. The isolated DNA was followed by polymerase chain reaction (PCR) using matK primer with Biorad RtPCR thermal cycler. The amplified DNA fragments were sequenced at Genetics Science Indonesia Jakarta using Next Generation Sequencing. The DNA fragment sequence of matK was compared (homology analysis) with DNA sequence data in the GenBank using the Blast method. Phylogenetic tree analysis was carried out to determine the molecular position of species or variety of Kayu Pule plants (Wirawan et al. 2020; Wirawan et al. 2022; Ariati et al. 2022).

Plant extraction

The extraction method refers to (Sarada et al. 2006; Azwanida 2015; Molino et al. 2018). The dried sample powder was extracted using maceration in 96 % ethanol for three days. The filtrate was then vacuumed to obtain crude extract.

Active compound identification

The ethanol extract of the Kayu Pule bark was analysed by the GC-MS method (HP-5MS Ultra Inert column, 30 cm in length, a diameter of 0.25 mm, and a column thickness of 0.25 µm). The sample of 1 µL was injected at a temperature of 50 °C for the first 5 minutes; then, for 2 minutes, the temperature was held until it reached 100 °C. The temperature was raised by 7 °C every minute to 300 °C, and in the last 3 minutes, the column was heated to 325 °C,

which was the final temperature. Phytochemical compounds were identified using Willey database version 7.0 by comparing the mass spectrum and fragmentation patterns of reference compounds stored in Willey's library.

Antioxidant activity assay using DPPH (2,2-diphenyl-1-picrylhydrazyl) method.

The DPPH technique was applied to perform an antioxidant activity assessment. A DPPH solution was prepared by dissolving 6 mg of DPPH in 50 mL of methanol. A combination of extracts at varying concentrations (0; 0.114; 0.170; 0.227; 0.284 µg mL^{-1}) was combined with 2.5 mL of DPPH solution and then stored in the dark at ambient temperature for 30 minutes. The absorbance was measured with a UV-Vis spectrophotometer at a wavelength of 517 nm. The percentage of radical inhibition was calculated using the subsequent formula:

Inhibition percentage = (A control - A sample) / A control x 100 %

The control absorbance (A control) denotes the absorbance of the DPPH solution without any extract, while sample absorbance (A sample) indicates the absorbance measurement of the sample. A linear regression model was constructed for each extract. The IC_{50}, or half-maximal inhibitory concentration, denotes the antioxidants required to diminish the initial DPPH concentration by 50 %. Each sample was analysed in triplicate.

RESULTS AND DISCUSSION

Genotyping of Kayu Pule Plant

Genotyping was used to molecularly identify the species of the sample. This research was conducted using matK primer. DNA amplification (Figure 1) showed a clear amplification at 500 kb. The size of the amplified PCR corresponds to the expected target size. Clear bands of the expected size in this study indicate that the PCR successfully reproduced the target DNA fragment well and was specific to the desired target, without producing non-specific amplification. The DNA obtained was then sequenced (Figure 1) and used for phylogenetic tree construction (Figure 2).

Alignment using BLAST (https://blast.ncbi.nlm.nih.gov/) showed the proximity of the sample to *Alstonia scholaris* MK9825941. Pairwise distance analysis was used to show the proximity of the Kayu Pule samples compared to the sequences generated from the blast analysis. Pairwise distance is a measure of the difference between two molecular sequences. Pairwise distance is used to estimate how far two organisms or species have diverged from each other from a common ancestor. Pairwise distance between species is shown in Figure 2. The value of 0.00 resulting from the comparison of nu-

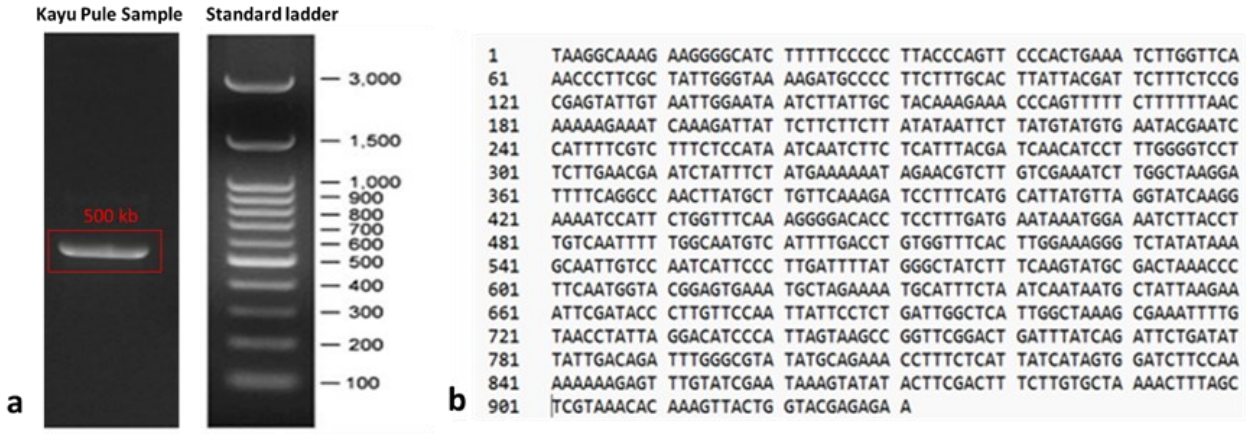

Figure 1. Visualisation of PCR products of Kayu Pule by electrophoresis 1 % TBE agarose (a) and DNA sequence (b) of Kayu Pule plant.

Pairwise distance	Pule	MK9825941_Alstonia scholaris	DQ6605151_Dyera costulata	KT9553461_Ochrosia kilneri	KT9553311_Catharanthus longifolius	DQ6605131_Craspidospermu verticillatuml	DQ6605251_Melodinus cochinchinensis	EF4563721_Carissa spinarum	DQ6605051_Carissa macrocarpa	KT9553501_Ochrosia poweri	DQ6605181_Gonioma kamassi	HQ3845531_Acokanthera oblongifolia	MN3701951_Hunteria simii	MT5941381_Mitragyna speciosa
Pule		0.00	0.01	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.10
MK9825941_Alstonia scholaris	0.00		0.01	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.09
DQ6605151_Dyera costulata	0.01	0.01		0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.09
KT9553461_Ochrosia kilneri	0.03	0.03	0.03		0.01	0.02	0.02	0.03	0.02	0.00	0.03	0.03	0.02	0.09
KT9553311_Catharanthus longifolius	0.03	0.03	0.03	0.01		0.03	0.03	0.03	0.03	0.01	0.03	0.03	0.03	0.10
DQ6605131_Craspidospermu verticillatuml	0.03	0.03	0.03	0.02	0.03		0.01	0.02	0.02	0.02	0.02	0.02	0.02	0.09
DQ6605251_Melodinus cochinchinensis	0.03	0.03	0.03	0.02	0.03	0.01		0.02	0.02	0.02	0.02	0.02	0.02	0.10
EF4563721_Carissa spinarum	0.03	0.03	0.03	0.03	0.03	0.02	0.02		0.01	0.03	0.02	0.01	0.02	0.09
DQ6605051_Carissa macrocarpa	0.03	0.03	0.03	0.02	0.03	0.02	0.02	0.01		0.02	0.02	0.01	0.02	0.09
KT9553501_Ochrosia poweri	0.03	0.03	0.03	0.00	0.01	0.02	0.02	0.03	0.02		0.03	0.03	0.02	0.09
DQ6605181_Gonioma kamassi	0.03	0.03	0.03	0.03	0.03	0.02	0.02	0.02	0.02	0.03		0.02	0.01	0.09
HQ3845531_Acokanthera oblongifolia	0.03	0.03	0.03	0.03	0.03	0.02	0.02	0.01	0.01	0.03	0.02		0.02	0.10
MN3701951_Hunteria simii	0.03	0.03	0.03	0.02	0.03	0.02	0.02	0.02	0.02	0.02	0.01	0.02		0.10
MT5941381_Mitragyna speciosa	0.10	0.09	0.09	0.09	0.10	0.09	0.10	0.09	0.09	0.09	0.09	0.10	0.10	

Figure 2. Pairwise distance between Kayu Pule sample and other species sequences collected from blast analysis.

cleotides in Kayu Pule and *Alstonia scholaris* samples indicates that both sequences are identical. Alignment of sample sequence compared to *Alstonia scholaris* MK9825941 is shown in Figure 3.

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Query  1    GAAATCTTGGTTCAAACCCTTCGCTATTGGGTAAAAGATGCCCCTTCTTTGCACTTATTA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    GAAATCTTGGTTCAAACCCTTCGCTATTGGGTAAAAGATGCCCCTTCTTTGCACTTATTA  60

Query  61   CGATTCTTTCTCCGCGAGTATTGTAATTGGAATAATCTTATTGCTACAAAGAAACCCAGt  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   CGATTCTTTCTCCGCGAGTATTGTAATTGGAATAATCTTATTGCTACAAAGAAACCCAGT  120

Query  121  ttttctttttttAACAAAAAGAAATCAAAGATTATTCTTCTTCTTATATAATTCTTATGTA  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  TTTTCTTTTTTAACAAAAAGAAATCAAAGATTATTCTTCTTCTTATATAATTCTTATGTA  180

Query  181  TGTGAATACGAATCCATTTTCGTCTTTCTCCATAATCAATCTTCTCATTTACGATCAACA  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  TGTGAATACGAATCCATTTTCGTCTTTCTCCATAATCAATCTTCTCATTTACGATCAACA  240

Query  241  TCCTTTGGGGTCCTTCTTGAACGAATCTATTTCTATGAAAAAATAGAACGTCTTGTCGAA  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  TCCTTTGGGGTCCTTCTTGAACGAATCTATTTCTATGAAAAAATAGAACGTCTTGTCGAA  300

Query  301  ATCTTGGCTAAGGATTTTCAGGCCAACTTATGCTTGTTCAAAGATCCTTTCATGCATTAT  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  ATCTTGGCTAAGGATTTTCAGGCCAACTTATGCTTGTTCAAAGATCCTTTCATGCATTAT  360

Query  361  GTTAGGTATCAAGGAAAATCCATTCTGGTTTCAAAGGGGACACCTCCTTTGATGAATAAA  420
            |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  361  GTTAGGTATCAAGGAAAATCCATTCTGGTTTCAAAGGGGACACCTCTTTGATGAATAAA  420

Query  421  TGGAAATCTTACCTTGTCAATTTTTGGCAATGTCATTTTGACCTGTGGTTTCACTTGGAA  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  TGGAAATCTTACCTTGTCAATTTTTGGCAATGTCATTTTGACCTGTGGTTTCACTTGGAA  480

Query  481  AGGGTCTATATAAAGCAATTGTCCAATCATTCCCTTGATTTTATGGGCTATCTTTCAAGT  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  AGGGTCTATATAAAGCAATTGTCCAATCATTCCCTTGATTTTATGGGCTATCTTTCAAGT  540

Query  541  ATGCGACTAAACCCTTCAATGGTACGGAGTGAAATGCTAGAAAATGCATTTCTAATCAAT  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541  ATGCGACTAAACCCTTCAATGGTACGGAGTGAAATGCTAGAAAATGCATTTCTAATCAAT  600

Query  601  AATGCTATTAAGAAATTCGATACCCTTGTTCCAATTATTCCTCTGATTGGCTCATTGGCT  660
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  601  AATGCTATTAAGAAATTCGATACCCTTGTTCCAATTATTCCTCTGATTGGCTCATTGGCT  660

Query  661  AAAGCGAAATTTTGTAACCTATTAGGACATCCCATTAGTAAGCCGGTTCGGACTGATTTA  720
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  661  AAAGCGAAATTTTGTAACCTATTAGGACATCCCATTAGTAAGCCGGTTCGGACTGATTTA  720

Query  721  TCAGATTCTGATATTATTGACAGATTTGGGCGTATATGCAGAAACCTTTCTCATTATCAT  780
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  721  TCAGATTCTGATATTATTGACAGATTTGGGCGTATATGCAGAAACCTTTCTCATTATCAT  780

Query  781  AGTGGATCTTCCaaaaaaaaGAGTTTGTATCGAATAAAGTATATACTTCGACTTTCTTGT  840
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  781  AGTGGATCTTCCAAAAAAAAGAGTTTGTATCGAATAAAGTATATACTTCGACTTTCTTGT  840

Query  841  GCTAAAACTTTAGCTCGTAAACACAAA  867
            |||||||||||||||||||||||||||
Sbjct  841  GCTAAAACTTTAGCTCGTAAACACAAA  867
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Figure 3. Nucleotide base alignment of Kayu Pule sample (sbjct) with *Alstonia scholaris* MK9825941 (query).

This result is supported by phylogenetic analysis (Figure 4), which shows the kinship between Kayu Pule samples and *A. scholaris* in the same cluster. The phylogenetic tree shows that Kayu Pule and *Alstonia scholaris* are in the same clade, indicating that both species originated from a common ancestor. This is also confirmed because both species have the same nodes. The

bootstrap number shows a high value (99 %) which indicates that the evolutionary relationship between the two species has a high level of confidence in its accuracy and truth.

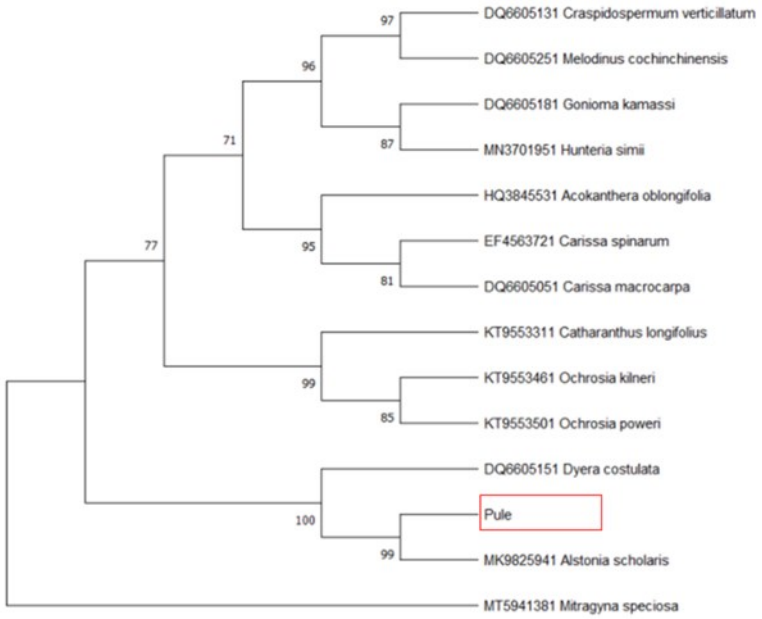

Figure 4. Phylogenetic tree of Kayu Pule plant based on matK primer.

The matK gene has emerged as a significant marker in molecular species identification, particularly in the context of DNA barcoding. Its utility stems from its high variability and substitution rates, enhancing its ability to distinguish between closely related species. MatK has shown remarkable discrimination power in specific taxonomic groups, such as the Orchidaceae, where it can identify over 90 % of species (Parveen et al. 2011; Saddhe et al. 2016). A study on conifer species in Vietnam reported a species identification rate of 98 % when combining matK with rbcL (Pham et al. 2021). Similarly, in identifying medicinal plants, the combination of matK and other loci, such as Internal Transcribed Spacer (ITS), has proven effective, highlighting matK's role in enhancing the accuracy of species identification (Thanh Pham et al. 2021). However, the effectiveness of matK is not uniform across all plant families. The matK gene is a valuable tool for identifying molecular species, mainly when used with other genetic markers. Its high variability and substitution rates contribute to its effectiveness, although its performance can vary significantly across different plant families.

Analysis of Antioxidant

Antioxidant activity assay showed the Inhibitory Consentration 50 % (IC_{50}) value of 3.7 µg mL^{-1} (regression equation y=14,91x-5,79) which showed that 3.7 µg mL^{-1} of Kayu Pule ethanol extract could act as an antioxidant to inhibit DPPH (free radicals) by 50 %. According to Molyneux (2004) and Kusumawati et al. (2021), this IC_{50} value belongs to the very strong category. The inhibitory power categories are IC_{50} of >250 µg mL^{-1} classified as inactive antioxidant compounds, IC_{50} of 100-250 µg mL^{-1} as weak, IC_{50} of 50-100 µg mL^{-1} as medium, IC_{50} of 10-50 µg mL^{-1} as strong antioxidant compounds, and IC_{50} of <10 µg mL^{-1} as very strong antioxidant compounds.

The antioxidant activity of Kayu Pule has been the subject of various studies, highlighting its potential as a source of natural antioxidants. The presence of bioactive compounds such as flavonoids, alkaloids, and phenolic acids in Kayu Pule contributes significantly to its antioxidant properties (Kanase & Mane 2018; Islamc 2020). As a comparison, a study reported that

the methanolic extract of *A. scholaris* leaves exhibited strong antioxidant activity with an IC_{50} value of approximately 69.50 µg mL^{-1} (Pratiwi 2023). Another study focusing on the chloroform extracts of *A. scholaris* found the IC_{50} values ranging from 45.77 to 62.03 µg mL^{-1} (Khanum 2014). The aqueous and ethanolic extracts of the bark of *A. scholaris* were evaluated for their cytotoxic and antioxidant properties, yielding IC_{50} values of 13.38 and 14.21 µg mL^{-1}, respectively (Jayashree et al. 2020). These values highlight the potential of *A. scholaris* extracts as an effective natural antioxidant, particularly in preventing oxidative stress-related diseases.

Phytochemical identification using GC-MS

Based on the results of gas chromatographic analysis of crude Kayu Pule extract, 103 compounds were obtained, as shown in chromatogram peaks in Figure 5 indicated the total chemical compounds detected in extract using GC-MS containing of secondary and primary metabolites especially fatty acid. Compounds were detected based on peaks in the chromatogram corresponding to each compound's retention time (RT). Compounds with a quality of >90 percent were selected 68 compounds (Table 1). The percentage of quality refers to the degree of match or similarity between the mass spectra of the detected compounds and the mass spectra of reference compounds in the GC-MS library database. The higher this quality number (>90 %) shows the greater confidence of the identified compound to the database.

Based on several publications, the compounds found in Kayu Pule ethanol extract showed various pharmacological activities, including anti-inflammatory (21 compounds, total AUC 17.85 %), antioxidant (23 compounds, total AUC 16.21 %), antimicrobial (16 compounds, total AUC 9.77 %), antibacterial (9 compounds, total AUC 8.97 %), antifungal (5 compounds, total AUC 3.07 %), and anticancer (7 compounds, total AUC 6.64 %). The primary function of extracts based on percent abundance was anti-inflammatory and antioxidant, with a total AUC of 17.85 and 16.21 %, respectively, supported by 21 and 23 compounds.

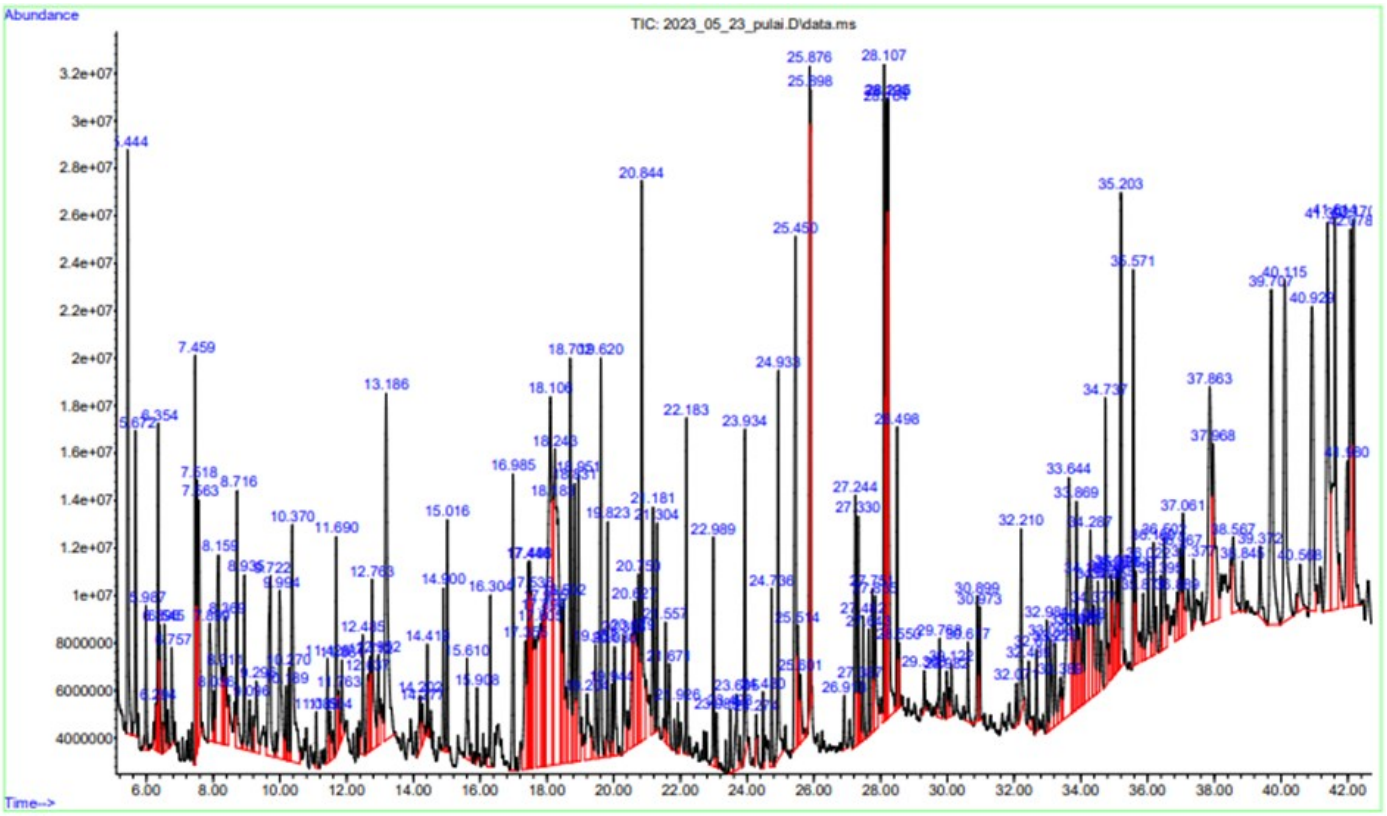

Figure 5. GC-MS chromatogram of ethanol extract of Kayu Pule.

Table 1. Compounds detected in ethanol extracts of Kayu Pule plants on GCMS machine.

No.	Compound name	Chemical formulas	RT	AUC (%)	Potential biological activity
1.	2-Furanmethanol (alcohol)	$C_5H_6O_2$	5.444	1.56	Antibacterial (Franko et al. 2017)
2.	3-Ethylpyridine (alkaloids groups)	C_7H_9N	7.459	0.79	Anticancer (Ji et al. 2002)
3.	5-Methylfurfural (5-methylfuran -2-carbaldehyde) (aldehydes)	$C_6H_6O_2$	7.563	0.83	Anticancer (Wang et al. 2023)
4.	Phenol (basic constituents of polyphenol groups)	C_6H_6O	7.899	0.53	Antioxidants, antimicrobial, and antiseptics (Diniyah et al. 2020)
5.	3-Methylcyclopentane-1,2-dione (diketone)	$C_6H_8O_2$	8.935	0.40	Flavoring agents (PubChem 2023a)
6.	4-Hydroxy-2,5-dimethyl-3(2H)-furanone (=strawberry furanone)	$C_6H_8O_3$	9.722	0.97	As a food and beverage additive (Xiao et al. 2021)
7.	Guaiacol (2-Methoxyphenol) (constituent groups of polyphenols)	$C_7H_8O_2$	10.270	0.21	Antioxidants and antimicrobial (Orlo et al. 2021)
8.	2,3-Dihydro-3,5-dihydroxy-6-methylpyran (organic compounds)	$C_6H_{10}O_3$	11.428	0.29	Antioxidants (Yu et al. 2013)
9.	Benzenemethanol (alcohol)	$C_8H_{10}O$	12.485	0.30	There has been no supporting research related to the function of these compounds
10.	1,2-Benzenediol catechol (constituent groups of polyphenols)	$C_6H_6O_2$	12.637	0.30	Precursors such as parfume and pharmaceuitical (Fiege et al. 2000)
11.	Furfuraldazine (2-furancarboxaldehyde, (2-furanyl methylene) hydrazone)	$C_{10}H_8N_2O_2$	13.186	1.85	Antimicrobial (Kapusterynska et al. 2023)
12.	Isobornyl acetate (1,7,7-trimethyl-2-bicyclo [2.2.1] heptanyl] acetate)	$C_{12}H_{20}O_2$	14.419	0.16	Anti-inflammatory (Wang et al. 2022)
13.	2-Methoxy-4-vinylphenol (constituent groups of polyphenols)	$C_9H_{10}O_2$	14.900	0.37	Antimicrobial (Rubab et al. 2020) and anticancer (Kim et al. 2019)
14.	Benzeneethanol (alcohol)	$C_8H_{10}O$	15.016	0.45	There has been no supporting research related to these compounds
15.	Phenol, 2,6-dimethoxy	$C_8H_{10}O_3$	15.610	0.35	Antimicrobial and fragrance (Dionisio et al. 2018)
16.	Benzoic acid, 4-formyl-, methyl ester	$C_9H_8O_3$	15.908	0.15	Antibacterial and antifungal (Farooq & Ngaini 2018)
17.	1-Tetradecene (alkenas)	$C_{14}H_{28}$	16.304	0.40	There has been no supporting research on the specific function of this compound
18.	Tricyclo[5.2.1.0 (2,6)] dec-4-en-8-yl acetate (acetic groups)	$C_{12}H_{16}O_2$	16.985	0.80	There has been no supporting research on the specific function of this compound
19.	4-Cyclopropyl-2-methoxyphenol (constituent groups of polyphenols)	$C_{10}H_{12}O_2$	17.446	0.56	Antibacterial (Rathinavel et al. 2023)

Table 1. Contd.

No.	Compound name	Chemical formulas	RT	AUC (%)	Potential biological activity
20.	1,2-Benzenedicarboxylic acid (phthalic acid)	$C_8H_6O_4$	17.536	0.50	Larvicide and antibacterial (Pachaiyappan et al. 2021)
21.	Phenol, 2,4-bis(1,1-dimethylethyl) (constituent groups of polyphenols)	$C_{14}H_{22}O$	18.502	0.50	Antifungal defense compound (Teresa et al. 2014)
22.	Lily aldehyde (lilial)	$C_{14}H_{20}O$	18.831	1.02	Lotions and cosmetics (Scherer et al. 2017)
23.	Benzoic acid (carboxylic acid group)	$C_7H_6O_2$	18.951	0.98	Antioxidant (Velika & Kron 2012), antibacterial, antifungal, and antioxidants (Liu et al. 2020)
24.	2,6-Dimethyl-3-methoxymethyl-p-benzoquinone	$C_{10}H_{12}O_3$	19.458	0.38	Detected but its use is unknown
25.	Benzoic acid (carboxylic acid group)	$C_7H_6O_2$	19.620	1.16	Antioxidant (Velika & Kron 2012), antibacterial, antifungal, and antioxidants (Liu et al. 2020)
26.	Hexadecene (alkenas)	$C_{16}H_{32}$	19.823	0.48	Antimicrobial and antioxidants (Mou et al. 2013)
27.	Hexadecene (alkenas)	$C_{16}H_{32}$	19.944	0.18	Antimicrobial and antioxidants (Mou et al. 2013)
28.	2-Pentyl-3-phenyl-2-propenal (cinnamaldehyde groups)	$C_{14}H_{18}O$	20.844	1.82	Flavouring agents (PubChem 2024a)
29.	1-(4-Isopropylphenyl)-2-methylpropyl acetate	$C_{15}H_{22}O_2$	21.181	0.68	There has been no supporting research related to these compounds
30.	1-Hexyl salicylate (benzoat ester)	$C_{13}H_{18}O_3$	21.304	0.48	Active ingredients cosmetics compound (PubChem 2024b)
31.	Heptadecane (alkanas)	$C_{17}H_{36}$	21. 557	0.28	Antiinflammatory (Kim et al. 2013), antibacterial, anticancer (Popović-Djordjević et al. 2016), antifungal, antioxidants, and antiseptic (Chehregani et al. 2010)
32.	E-15-heptadecenal (fats with aldehyde functional groups)	$C_{17}H_{32}O$	22.989	0.51	Anti-inflammatory and antioxidants (Chansiw et al. 2018)
33.	Octadecane (alkanas)	$C_{18}H_{38}$	23.089	0.13	Antidepressant (Guo et al. 2021), and antitumor (Tang et al. 2020)
34.	Neophytadiene (terpenoid)	$C_{20}H_{38}$	23.685	0.33	Antidepressant (Gonzalez-Rivera et al. 2023) and Anti-inflammatory (Bhardwaj et al. 2020)
35.	1,3,7-Trimethylpurine-2,6-dione (caffeine, alkaloids groups)	$C_8H_{10}N_4O_2$	23.934	0.90	Anti-inflammatory and antioxidants (Herman & Herman 2012)

Table 1. Contd.

No.	Compound name	Chemical formulas	RT	AUC (%)	Potential biological activity
36.	Hexadecene (alkenas)	$C_{16}H_{32}$	24.274	0.04	Antimicrobial and antioxidants (Mou et al. 2013)
37.	Pentadecanoic acid (fatty acid group)	$C_{15}H_{30}O_2$	24.480	0.15	Protecting cardiometabolic, immune, and liver health (Venn-Watson & Schork 2023)
38.	5,8-Dimethoxy-2,2-dimethyl-2h-chromene (basic constituents of flavonoids)	$C_{13}H_{16}O_3$	24.736	0.38	Formation of drugs (PubChem 2023b)
39.	Palmitic acid (fatty acid group)	$C_{16}H_{32}O_2$	24.933	1.03	Anti-inflammatory, antioxidant, and antimicrobial (Carta et al. 2017)
40.	Hexadecanoic acid or palmitic acid (fatty acid group)	$C_{16}H_{32}O_2$	25.450	1.66	Anti-inflammatory, antioxidant, and antimicrobial (Carta et al. 2017)
41.	Thiosulfuric acid (strong acids)	$H_2S_2O_3$	25.601	0.26	There has been no supporting research related to these compounds
42.	Hexadecanoic acid or palmitic acid (fatty acid group)	$C_{16}H_{32}O_2$	25.876	1.28	Anti-inflammatory, antioxidant, and antimicrobial (Carta et al. 2017)
43.	Hexadecanoic acid or palmitic acid (fatty acid group)	$C_{16}H_{32}O_2$	25.898	0.79	Anti-inflammatory, antioxidant, and antimicrobial (Carta et al. 2017)
44.	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	$C_{19}H_{34}O_2$	27.244	0.21	Anti-inflammatory and antioxidants (El-Ashmawy et al. 2024)
45.	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	$C_{19}H_{34}O_2$	27.330	0.60	Anti-inflammatory and antioxidants (El-Ashmawy et al. 2024)
46.	Hexadecene (alkanas)	$C_{16}H_{32}$	27.482	0.29	Antimicrobial and antioxidants (Mou et al. 2013)
47.	Octadecadienoic acid (alkenes, unsaturated fatty acids)	$C_{19}H_{34}O_2$	27.643	0.21	Anti-inflammatory and antioxidants (El-Ashmawy et al. 2024)
48.	9,12-Octadecadienoic acid (Z,Z)-, methyl ester (unsaturated fatty acids)	$C_{19}H_{34}O_2$	27.751	0.42	Anti-inflammatory and antioxidants (El-Ashmawy et al. 2024)
49.	9,12-Octadecadienoic acid (Z,Z)-, methyl ester (unsaturated fatty acids)	$C_{19}H_{34}O_2$	27.834	0.56	Anti-inflammatory and antioxidants (El-Ashmawy et al. 2024)
50.	Linoleic acid ethyl ester (unsaturated fatty acids, omega-6)	$C_{20}H_{36}O_2$	28.107	1.74	Anti-inflammatory (PubChem 2023b)
51.	Linoleic acid ethyl ester (unsaturated fatty acids, omega-6)	$C_{20}H_{36}O_2$	28.164	0.78	Anti-inflammatory (PubChem 2023b)
52.	Ethyl oleic acid (fatty acid group)	$C_{20}H_{38}O_2$	28.190	1.19	The role of nonoxidative alcohol metabolism in liver disease (Song et al. 2015)
53.	Ethyl oleic acid (fatty acid group)	$C_{20}H_{38}O_2$	28.235	1.88	The role of nonoxidative alcohol metabolism in liver disease (Song et al. 2015)

Table 1. Contd.

No.	Compound name	Chemical formulas	RT	AUC (%)	Potential biological activity
54.	Methyl 17-methyloctadecanoate (fatty acid group)	$C_{20}H_{40}O_2$	28.498	0.69	Anticancer, antitumor, and anti-inflammatory (Tang et al. 2020)
55.	Decosane (hydrocarbons)	$C_{22}H_{46}$	28.550	0.13	Antimicrobial (Lammers et al. 2021)
56.	Tricosane (hydrocarbons)	$C_{23}H_{48}$	29.786	0.22	Components of female sex pheromone of the bee (Francke & Schulz 2010)
57.	Acetic acid (13-tetradecenyl) ester (organic ester compounds)	$C_{16}H_{30}O_2$	30.144	0.14	There has been no supporting research related to these compounds
58.	2,5-Furandione,3-(dodecenyl) dihydro- (terpenoids)	$C_{16}H_{26}O_3$	30.617	0.23	It has a role as a plant metabolite (PubChem 2024c)
59.	Adipic acid (dicarboxilic aid) (organic acid)	$C_6H_{10}O_4$	30.973	0.30	Anti-inflammatory, anticancer, and antimicrobial (Liao et al. 2020)
60.	n-Eicosane (saturated organic compounds)	$C_{20}H_{42}$	32.071	0.13	There has been no supporting research related to these compounds
61.	2,6,10,14,1,22-Tetracosahexaene (terpenoids)	$C_{24}H_{38}$	35.571	1.02	There has been no supporting research related to these compounds
62.	17,24-Dihydroxy-3-oxopregn-4-en-21-al (steroid)	$C_{21}H_{30}O_4$	37.061	0.45	There has been no supporting research related to these compounds
63.	Ergost-5-en-3-ol (steroid)	$C_{28}H_{48}O$	39.707	2.09	Antidiabetic, antirheumatic, anthelmintic, antipsoriatic, antioxidants, antiepileptic, and antigonorrhea (Qadir et al. 2022)
64.	Stigmasterol (steroid)	$C_{29}H_{48}O$	40.115	1.86	Antioxidants and anticancer (Wang et al. 2022)
65.	Stigmast-5-en-3-ol (steroid)	$C_{47}H_{84}O_2$	40.929	1.86	Antioxidants and anticancer (Wang et al. 2022)
66.	12-Oleanen-3-yl acetate (steroid)	$C_{32}H_{52}O_2$	41.392	2.24	Antibacterial, antiprotozoal, and anti-inflammatory (Pérez-González, A. et al. 2017)
67.	Olean-12-ene, 3-methoxy- (terpenoid)	$C_{30}H_{50}$	42.087	1.54	Antibacterial (Muhammad et al. 2000)
68.	Amyrin (terpenoid)	$C_{30}H_{50}$	42.170	1.31	Anti-inflammatory (Okoye et al. 2014)

RT, retention time; AUC, area under curve

Compounds that act as anti-inflammatory in Kayu Pule extracts include unsaturated fatty acids such as *Octadecadienoic acid* (*linoleic acid ethyl ester*) and flavonoid group compounds. *Octadecadienoic acid* or *linoleic acid* is an essential fatty acid that is anti-inflammatory. Cytokine compounds as proinflammatory compounds, such as interleukin-1 beta and tumor necrosis factor-alpha, can be inhibited by linoleic acid. In addition, the linoleic acid present in Kayu Pule plants can reduce the expression of the cyclooxygenase-2 (COX-2) enzyme,

which synthesizes prostaglandin hormones that spur inflammation (Kusumawati 2002).

The anti-inflammatory properties of Kayu Pule have been the focus of several studies, revealing its potential therapeutic benefits in managing inflammation-related conditions. One significant study by Subraya et al. (2012) demonstrated that the stem bark extract of *A. scholaris* exhibited substantial anti-inflammatory activity comparable to that of indomethacin, a well-known anti-inflammatory drug. This study utilised models such as carrageenan-induced paw edema, dextran-induced edema, and cotton pellet-induced granuloma to assess the anti-inflammatory effects. The results indicated that *A. scholaris* effectively reduced edema, suggesting its potential as a natural anti-inflammatory agent. In another investigation, the bark extract exhibited significant inhibition of inflammatory mediators in a rat model. The study highlighted the extract's ability to restore antioxidant enzyme levels and prevent the rise of inflammatory mediators, indicating a dual action of antioxidant and anti-inflammatory effects (Zehra & Sanaye 2021). This aligns with the traditional use of the plant in folk medicine for treating inflammatory conditions. Moreover, Shang et al. (2010) conducted pharmacological evaluations that confirmed the anti-inflammatory and analgesic properties of *A. scholaris*. The study noted that the extract could inhibit the production of pro-inflammatory mediators, which are crucial in the inflammatory response (Shang et al. 2010). The inhibition of cyclooxygenase (COX) and lipoxygenase (LOX) pathways was also discussed, suggesting that the extract could modulate these pathways to exert anti-inflammatory effects. Additionally, the latex of *A. scholaris* has been evaluated for its anti-inflammatory properties. A recent study by Banik and Das (2023) highlighted the latex's effectiveness in various in vitro assays, demonstrating its potential in managing inflammatory diseases. The study focused on protein denaturation and membrane stabilization mechanisms, which are critical in the inflammatory process. Several studies showed that *Alstonia scholaris* exhibits significant anti-inflammatory activity through various mechanisms, including the modulation of inflammatory mediators and pathways.

The grouping of phytochemical compounds in the 68 compounds identified in the GC-MS analysis was also carried out mainly on alkaloids, phenolic compounds (tannins and flavonoids), steroids, terpenoids, and fatty acids, as shown in Table 2. These compounds are secondary metabolites that function to defend themselves from biotic and abiotic stress and are not directly involved in plant growth. Plants produce different metabolites, even a compound produced by only one plant species (Verpoorte & Alfermann 2000). Secondary metabolites such as salicylic acid generally have pharmacological solid effects, so they can be used as drugs or as new drug models. Phenolics (tannins and flavonoids) have antioxidants to prevent cancer-preventing free radicals. Flavonoid compounds have essential properties in the body, namely as anti-inflammatory, antioxidants, and anticancer, and belong to the largest class of natural phenol compounds and are easily obtained in various types of plants, including Kayu Pule plants (Panche et al. 2016; Ademiluyi et al. 2018; Tungmunnithum et al. 2018).

Steroids are among the dominant compounds in Kayu Pule extracts with a total AUC of 8.5 % (Table 2) consisting of 5 compounds such as 17.24-dihydroxy-3-oxopregn-4-en-21-al (AUC 0.45 %), ergost-5-en-3-ol (AUC 2.09 %), stigmasterol (1.86 %), stigmast-5-en-3-ol (1.86 %), and 12-oleanen-3-yl acetate (AUC 2.24 %) (Table 1). Steroids are anti-inflammatory, regulate body metabolism, as well as affect body growth and development (Patadiya 2020).

The compound 12-oleanen-3-yl acetate (Figure 6) was the most abundant compound with an AUC of 2.24 % and has properties as antibacterial,

Table 2. Group of compounds contained in Kayu Pule extract based on AUC value and number of function support compounds.

No.	Group of compounds	AUC value of all compounds (%)	No. of supporting compounds
1	Alkaloids	1.69	2
2	Phenolics (tannins and flavanoids)	3.2	8
3	Steroids	8.5	5
4	Terpenoids	4.10	4
5	Fatty acids	11.97	14
6	There have been no reports/studies related to the compound	3.46	8

Compound function based on Willey data base version 7, AUC=Area Under Curve in percent, the number of supporting compounds were compounds with the same group.

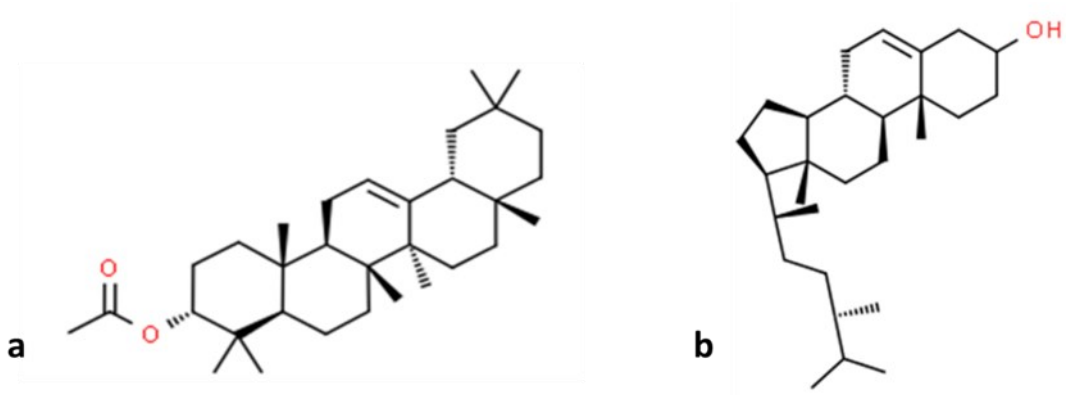

Figure 6. Chemical structure of 12-oleanen-3-yl acetate (a) and ergost-5-en-3-ol (b).

antiprotozoal, and anti-inflammatory (Pérez-González, M.Z. et al. 2017) and is cytotoxic with the molecular formula $C_{32}H_{52}O_2$ which appeared in RT 41,392, average mass 468,754 Da, and ChemSpider ID8727640.

Ergost-5-en-3-ol (Figure 6) is the second most abundant steroid compound (AUC 2.09 %) appearing at RT 39.707 with the molecular formula $C_{28}H_{48}O$, having a mass of 400.370514 Da, and ChemSpider ID18902059. Ergost-5-en-3-ol is antidiabetic, antirheumatic, anthelmintic, anti-psoriatic, antioxidants, antiepileptic, and anti-gonorrhea (Qadir et al. 2022).

Groups of steroid compounds can be cyclic or acyclic and often have aldehyde groups, carboxylic acids, or alcohols. Steroids have significant bioactivity, such as hormone formation, cell membrane parts, and vitamin D formation. Steroids can also act as an attractor or insect repellent and antimicrobial. Based on the results of GCMS, the bark extract of the Kayu Pule plant contains many compounds that have the potential for various pharmacological activities, including relieving convulsions, thinning phlegm, lowering blood sugar, curing malaria, and lowering blood pressure (Candrasari et al. 2018). Kayu Pule bark extract is proven to contain alkaloids, tannins, saponins, and steroids. However, terpenoids and flavonoids are found only in the leaves.

The presence of steroid groups in *Alstonia scholaris* has been documented in various studies focusing on the phytochemical composition of the plant. The plant contains various bioactive compounds, including alkaloids, flavonoids, saponins, steroids, and triterpenoids. One significant study by Islam et al. (2020) reported that the ethanolic extracts of *A. scholaris* revealed the presence of steroids among other phytochemicals such as tannins, glycosides, and alkaloids. This finding underscores the importance of *A. scholaris* as a source of steroid compounds known for their various biological activities, including anti-inflammatory and antioxidant effects. Additionally, a review by Verma et

al. (2015) highlighted that *A. scholaris* is rich in flavonoidal glycosides, indole alkaloids, and steroids, indicating a diverse phytochemical profile contributing to its medicinal properties. The presence of these compounds suggests potential therapeutic applications, particularly in traditional medicine systems where steroids are often utilized for their anti-inflammatory and immunomodulatory effects. Moreover, the study by Raju et al. (2022) identified several phytochemical constituents in the bark extract of *A. scholaris*, including steroids, saponins, flavonoids, and triterpenoids. Furthermore, the isolation of specific triterpenoids such as lupeol and betulin from *A. scholaris* has been reported, reinforcing the notion that steroidal compounds are indeed present in this plant (Zehra & Sanaye 2021). *Alstonia scholaris* contains various phytochemicals, including steroid groups, specifically in the form of triterpenoids and other steroidal compounds. These phytochemicals contribute to the plant's pharmacological activities, including anti-inflammatory effects, making it a valuable resource in traditional medicine and potential therapeutic applications.

CONCLUSIONS

Molecular identification with the matK marker showed that Kayu Pule is close to the species *Alstonia scholaris* (MK9825941). The compounds contained in the Kayu Pule plant have several pharmacological activities including anti-inflammatory, antioxidant, and antimicrobial properties. The antioxidant strength of Kayu Pule was measured with IC_{50} of 3.7 µg mL^{-1}, including the very strong category. Due to the solvent used in this research being polar (ethanol), further extraction using a non-polar solvent is necessary to identify the whole chemical in Kayu Pule.

AUTHOR CONTRIBUTION

I.K.S. organised the research, wrote the manuscript's draft; I.G.P.W. contributed substantially to the writing of the paper, I.N.W. data analysis and created the tables and the figures, validated the final version of the paper; A.A.S.I.G. data analysis and created the tables and the figures; G.A.P.T.A.H. provided a number of references; M.M.V.S. produced the final version of this manuscript; P.K.K. did a summary of relevant literature; I.M.O.P. provided a number of references. All authors approved the final version of the manuscript.

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CONFLICT OF INTEREST

We have no conflict of interest.

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