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Bioactivities of Plant Extracts Collected In Halmahera Island, Indonesia: A Bioprospection Study of Underexplored Plant Species

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ABSTRACT

The discovery of new antibiotics to overcome the growing resistance problem, as well as the discovery of new natural and safe antioxidants to combat oxidative stress, are still urgently needed. Medicinal plants are known to produce potential therapeutic substances which are more biologically selective than synthetic compounds. Therefore, we explored the bioactivities of 35 ethanolic extracts from 24 underexplored plant species collected in Halmahera island, Indonesia, to find potential sources for antibacterial and antioxidant agents. Dried plant parts were extracted using ethanol 96%. Thin layer chromatography-direct-bioautography (TLC-DB) and minimum inhibitory concentration (MIC) determination were used to evaluate the antibacterial effect. Antioxidant activity was determined against 2,2-Diphenyl-1-picrylhydrazyl (DPPH) using TLC-DB and microdilution assay. Total phenolic content (TPC) was determined using Folin-Ciocalteu's method. The ethanolic plant extracts from Halmahera island exhibited moderate to weak antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*. However, 14 extracts from the leaf, stem, or stem bark of 10 underexplored plant species from Halmahera Island, Indonesia, displayed strong to very strong antioxidant activities against DPPH with antioxidant activity index (AAI) values between 1.12 to 13.42. A strong correlation between TPC and antioxidant activity with $r=0.8712$, $p<0.0001$ was observed. This strong correlation between TPC and antioxidant activity exhibited a prominent role of phenolic compounds in the plants' antioxidant properties. These findings indicate that collected plants from Halmahera are potential to be studied and developed further as potential sources for novel antioxidants.

Keywords: antibacterial, antioxidant, underexplored plants, phenolic content, Halmahera island.

INTRODUCTION

Natural products are essential in drug discovery and development. Many FDA-approved new molecular entities (NME) are originally from plants (Patridge *et al*., 2016). The plant kingdom consists of a wide variety of plant species that generate various bioactive compounds with distinct chemical scaffolds (Atanasov *et al*., 2015). These natural compounds usually have a better pharmacokinetic profile, better binding affinity, and possess more complex stereochemical configurations. These properties are in contrast to the more planar and less stereochemically complex structures of synthetic compounds (Rodrigues *et al*., 2016).

Secondary metabolites with potential pharmacological effects are widely distributed in plants. However, from approximately 500,000 species of plants, only a small fraction has been screened for their pharmacological effects (Stanković *et al*., 2016), leaving a wide chance for discoveries. Indonesia is one of the world's richest nations in terms of biodiversity (Paoli *et al*., 2010). Local communities in Indonesia, including Halmahera Island, have traditionally used these diversities to meet their basic necessities (Asteria *et al*., 2021; Tamalene, 2017). Plants from several genera that have been reported to have pharmacological properties such as from genera *Elaeocarpus, Alpinia, Gmelina, Morinda, Smilax,*

Aquilaria, Ziziphus, Timonius, Micromelum, Colubrina, Cinnamomum, and *Garcinia* (Hashim *et al*., 2016; Packer *et al*., 2015; Sangsopha *et al*., 2018; Singh *et al*., 2015; Srithi *et al*., 2019; Vasconcelos *et al*., 2018) are found and used locally in Halmahera island. However, many species from these genera are still underexplored in regard to their bioactivities and active metabolites (phytochemistry).

In this present study, we collected 24 underexplored plant species from 16 genera in Halmahera island and explored their biological activity. Antibacterial and antioxidant activities were examined because antibiotic resistance and the detrimental effect of long-term use of synthetic antioxidants are still serious problems today (Barbieri *et al*., 2017; Jiang & Xiong, 2016). In this study, the antibacterial and antioxidant activities of some collected plant species were firstly reported.

MATERIALS AND METHODS

Material collection and identification

Plant materials were collected from Weda, Central Halmahera, North Maluku, Indonesia (Figure 1). Each plant was identified, and the voucher specimens were deposited at the Herbarium Bogoriense, Research Center for Biology, Indonesian Institute of Sciences.

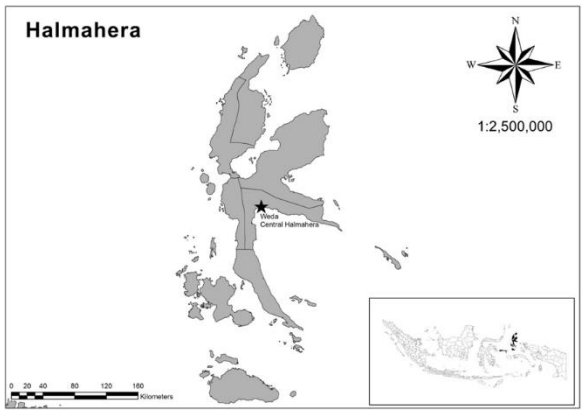

Figure 1. Research sampling location at Weda, Central Halmahera District, North Maluku Province, Indonesia

Extraction

The plant parts were dried under sunlight, ground into powder, and macerated with 96% ethanol. The filtrate was dried using a rotary evaporator and further dried using nitrogen before use.

Thin-layer chromatography (TLC) profiling

Onto TLC plate (silica gel GF254, Merck, Germany), 10 µl of each extract (10 mg/ml) was transferred and developed in CH_2Cl_2:MeOH (10:1). The TLC chromatogram was visualized under ultraviolet light (254 nm and 366 nm) and with spray reagents (1% Ce(SO4), $_2$/10% sulphuric acid, and 1% vanillin-sulphuric acid).

Antibacterial activity detection using thin layer chromatography-direct-bioautography (TLC-DB) method

Detection of antibacterial activity using the TLC-DB method was performed as described before (Jesionek *et al*., 2013) with modifications. Briefly, for dot blot TLC-DB, 10 µl of ethanolic extract (10 mg/ml) was transferred onto silica gel GF_{254} - TLC glass plate (Merck, Germany). The plate was immersed into the suspension of *S. aureus* InaCC B5 (InaCC, Indonesia) or *E. coli* Ina CC B4 (InaCC, Indonesia) and incubated for 18h at 37°C under a humid atmosphere. After incubation, the TLC plate was sprayed with 4mg/mL Iodonitrotetrazolium p-violet (INT) (Sigma-Aldrich, Germany). The inhibition zone was observed as a clear zone against a purple background. Further analysis for the active extracts was done by developing the extract on a TLC plate (developed TLC-DB) using CH_2Cl_2:MeOH (10:1) as the mobile phase. Subsequently, the developed extract was run through the same procedure as dot blot TLC-DB to detect antibacterial activity.

Minimum inhibitory concentration (MIC) determination

The MIC of active extracts (extracts that showed a positive result in the antibacterial TLC-DB examination) were determined by serial microdilution assay as described before (Praptiwi *et al*., 2018). Each test was performed in triplicate. Final concentrations of the extract were in the range of 4 – 512 µg/ml. Chloramphenicol (Sigma-Aldrich, Germany) was used as the positive control. The MIC was determined as the lowest concentration where the clear wells were observed.

Antioxidant activity detection using the TLC-DB method

Detection of antioxidant activity using the TLC-DB method was performed as described before (Gu *et al*., 2009) with modifications. Briefly, for dot blot, TLC-DB, 10µL ethanolic extract

(10mg/mL) was loaded onto the TLC plate. The TLC plate was sprayed with 0.02% 2,2-Diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, Germany) in methanol and incubated for 15min in a dark environment. The inhibition zone was observed as a clear zone against a purple background. Further analysis for the active extracts was done by developing the extract on a TLC plate using CH_2Cl_2:MeOH (10:1) as the mobile phase. The developed extract was run through the same procedure to detect antioxidant activity in a similar way to the undeveloped extract (dot blot). (+)-Catechin (Sigma Chemical, USA) was used as a positive control.

IC_{50} and antioxidant activity index (AAI) determination of active extract

The IC_{50} determination of active extracts against DPPH with microdilution assay in 96-well microtiter plate (Thermo Scientific, China) and the AAI calculation were determined as described before (Praptiwi *et al*., 2018; Scherer & Godoy, 2009). Each test was performed in triplicate with the final concentrations of the extract from 5.86 – 750µg/mL. Catechin was used as the positive control and methanol (Merck, Germany) as the negative control.

Total phenolic content (TPC) determination

TPC was measured using Folin-Ciocalteu's photometric assay. Into 200µL extract (1mg/mL), 200µL of 50% Folin-Ciocalteu's solution, and 4mL of 2% Na_2CO_3 were added and incubated for 30min in a dark environment. The absorbance was read using a UV-Vis spectrophotometer (Shimadzu, Japan) at 750nm. The calculation of the TPC value was based on the standard curve of gallic acid. The TPC value was expressed as mg gallic acid equivalents (GAE) per gram of dry extract (DE).

Correlation analysis between TPC and antioxidant activity of the plant extracts

Pearson's correlation analysis was performed to analyze the correlation between TPC and IC_{50} of the plant extracts against DPPH. Statistical data analysis was performed using Graph Pad Prism 7 (La Jolla, USA). A probability (p) value ≤0.05 indicates statistical significance.

RESULTS AND DISCUSSION

Antibacterial activity of the plant extracts

In this study, we analyzed 35 extracts from 24 underexplored plant species collected from Halmahera Island, Indonesia (Table I). The underexplored plants studied in this research are the plants that are less studied in terms of their bioactivities and their active metabolites. The TLC analysis of the underexplored plants indicated different chemical compounds within each extract, which appeared as spots or stains with different retention factors (R_f) (Figure 2). The extracts' initial antibacterial and antioxidant activity from various plant species was performed qualitatively by observing the presence or absence of an inhibition zone (white to yellow area against the purple background) on the TLC plate using the dot-blot technique. In this assay, from the 35 extracts that we observed (Figure 3), ±85.71% extracts (30 extracts) had antibacterial activity against *S. aureus* (gram-positive), and ±91.42% (32 extracts) had the antibacterial activity against *E. coli* (gram-negative), and ±82.86% (29 extracts) had the antibacterial activity against both of the pathogenic bacteria.

To further evaluate the antibacterial effect, developed TLC-DB was performed to separate the phytochemicals within the plant extracts. From this step, we observed that all of the developed extracts exhibited clear areas or clear bands against a purple background (Figure 3). However, 31 extracts contained more potential compounds against *S. aureus*, and 32 extracts contained more potential compounds against *E. coli*. This developed TLC-DB result also can be used for further investigation, for instance, as guidance for bioactive compound isolation from the extract.

MIC as a quantitative indicator of the antimicrobial effect was assessed, and the classification of the antibacterial activity described before (Pessini *et al*., 2003) was followed. The extract from the rhizome of *R. lanata* and the stembark of *O. glomerata* exhibited moderate antibacterial activity against *S. aureus*. The extract from the stem bark of *D. esculentoides* displayed moderate antibacterial activity against *E. coli*, while other extracts showed weak antibacterial activity against *S. aureus* and *E. coli* (Table I).

A previous study reported that the methanol extract of root and the stembark of *O. glomerata* had an antiplasmodial activity with IC_{50} ≤11µg/mL against chloroquine-sensitive or chloroquine-resistant clones of *Plasmodium falciparum* (Horgen *et al*., 2001). The bark of this species contains several alkaloids such as tetraphylline pseudoindoxyl and tetraphylline oxindole A, while the leaf contains tetraphylline oxindole B (Buckingham *et al*., 2010).

Tabel I. Antibacterial and antioxidant activities of Halmahera island plant extracts

No.	Plant Species	Plant parts	Antibacterial activity		Antioxidant activity		
			MIC against *S. aureus* (µg/mL)	MIC against *E. coli* (µg/ml)	C_{50} against DPPH (µg/mL)	AAI against DPPH	Criteria of AAI value
1	*E. dolichostylus* Schltr.	Leaf	>512	>512	10.203	9.80	**very strong**
2	*E.dolichostylus* Schltr.	Stem	>512	>512	15.934	6.27	**very strong**
3	*Alpinia gigantea* Blume	Rhizome	>512	>512	249.471	0.40	weak
4	*E. multiflorus* (Turcz) Fern. – Vill.	Leaf	>512	>512	21.722	4.60	**very strong**
5	*Gmelina lepidota* Scheff.	Leaf	>512	>512	65.621	1.52	**strong**
6	*T. morotaiense* Kosterm	Leaf	>512	>512	864.528	0.11	weak
7	*M. umbellata* L.	Leaf	NT	NT	NT	NT	-
8	*M. umbellata* L.	Fruit	NT	>512	794.752	0.12	weak
9	*Morinda umbellata* L.	Stem	>512	>512	551.237	0.18	weak
10	*Smilax australis* R. Br.	Leaf	>512	>512	56.335	1.77	**strong**
11	*Smilax australis* R. Br.	Stem	>512	>512	69.318	1.44	**strong**
12	*S. ovalifolia* Roxb. Ex. D. Don	Leaf	>512	NT	NT	NT	-
13	*M. glomerata* (Gaudich.) T. Koyama	Leaf	>512	>512	55.768	1.79	**strong**
14	*R. lanata* (Scheff.) K. Schum. ex Valeton	Rhizome	128	>512	309.429	0.32	weak
15	*Aquilaria filaria* (Oken) Merr.	Leaf	>512	>512	168.646	0.59	moderate
16	*Z. angustifolius* (Miq.)	Leaf	>512	>512	450.427	0.22	weak
17	*Z. angustifolius* (Miq.)	Stem bark	>512	>512	102.905	0.97	moderate
18	*Iodes cirrhosa* Turcz	Leaf	>512	>512	NT	NT	-
19	*Timonius rufescens* (Miq.) Boerl.	Leaf	>512	>512	807.672	0.12	weak
20	*Timonius rufescens* (Miq.) Boerl.	Stem bark	>512	>512	59.033	1.69	**strong**
21	*Micromelum minutum* Wight. & Arn.	Leaf	>512	>512	1300.766	0.07	weak
22	*C. asiatica* (L.) Brongn.	Leaf	>512	>512	88.512	1.12	**strong**
23	*C. asiatica* (L.) Brongn.	Stem bark	>512	>512	72.863	1.37	**strong**
24	*C. sintoc* Blume	Leaf	>512	>512	75.732	1.32	**strong**
25	*C. sintoc* Blume	Stem bark	>512	>512	7.449	13.42	**very strong**
26	*Garcinia latissima* Miq.	Leaf	>512	>512	372.701	0.26	weak
27	*Garcinia latissima* Miq.	Stem bark	>512	>512	12.483	8.01	**very strong**
28	*D. esculentoides* M. Kato	Leaf	>512	>512	NT	NT	-
29	*D.esculentoides* M. Kato	Stem bark	512	256	181.393	0.55	moderate
30	*O. glomerata* (Blume) F. Muell.	Leaf	>512	>512	879.541	0.11	weak
31	*O. glomerata* (Blume) F. Muell.	Stem bark	128	>512	849.029	0.11	weak
32	*Psychotria celebica* Miq.	Leaf	>512	>512	21.645	4.62	**very strong**
33	*G.papuanum* (S Moore) L.A.S Johnson	Leaf	>512	>512	111.413	0.89	moderate
34	*Erythroxylum ecarinatum* Hochr	Leaf	NT	NT	NT	NT	-
35	*Polyscias schulzei* Harms.	Leaf	NT	NT	NT	NT	-

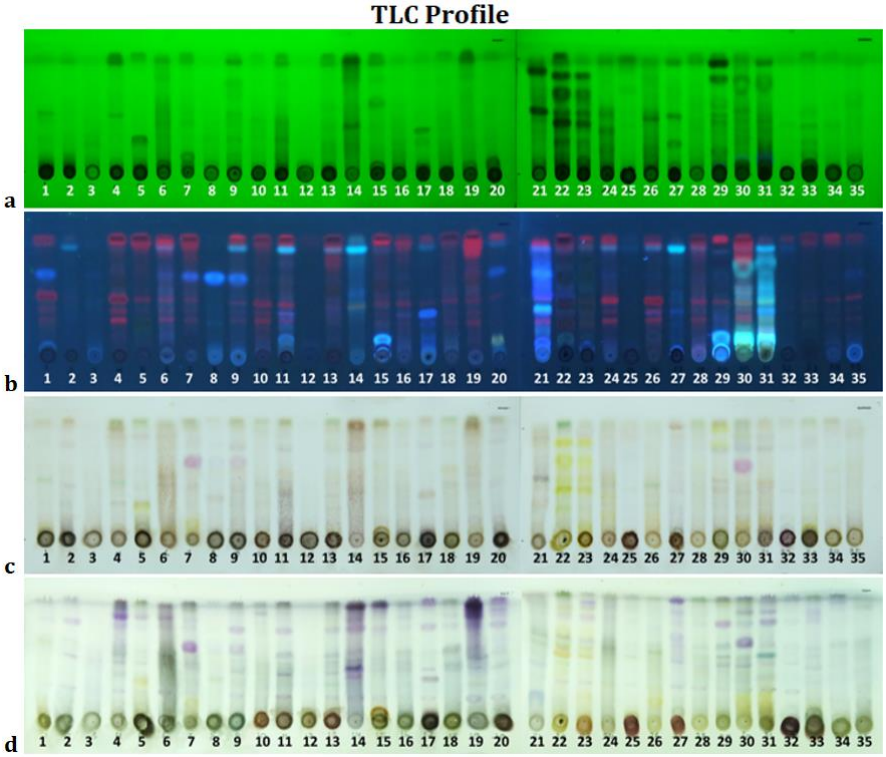

Figure 2. The TLC profile of Halmahera plant extracts. The TLC spots were visualized (a) under UV 254nm, (b) UV 366nm, (c) with cerium, and (d) vanillin. The list of Halmahera plant extracts (number 1-35) (Table I)

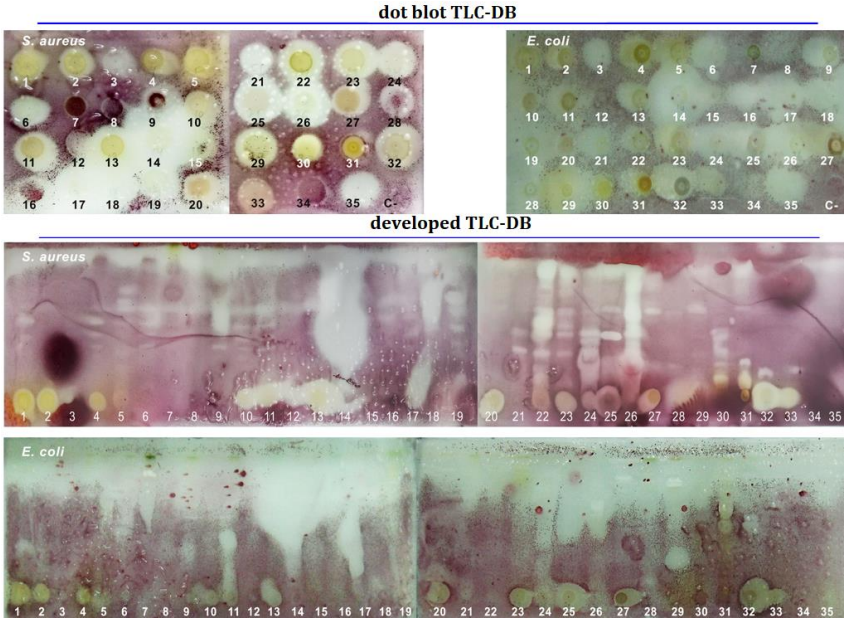

Figure 3. Qualitative antibacterial activity evaluation of Halmahera plant extracts using TLC direct bioautography (TLC-DB) against *S. aureus* and *E. coli*. Dichloromethane-methanol with a ratio of 10:1 was used as the mobile phase for developed TLC-DB. The list of Halmahera plant extracts (number 1-35) (Table I).

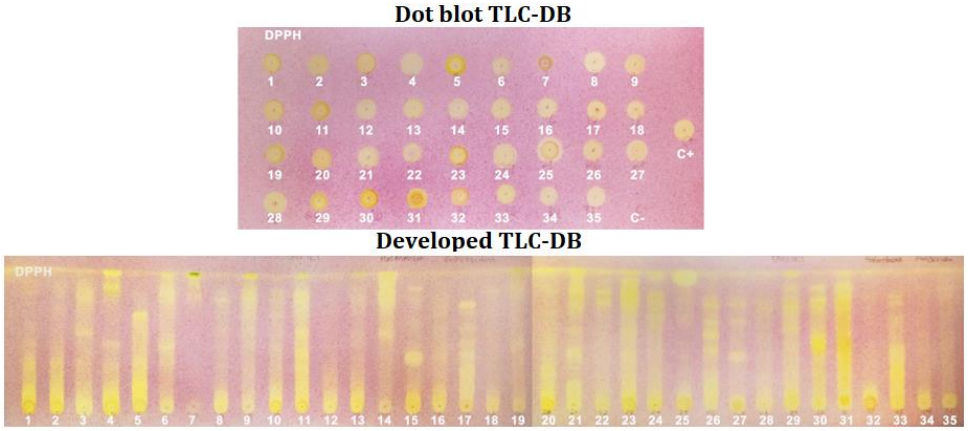

Figure 4. Qualitative antioxidant activity evaluation of Halmahera plant extracts using TLC direct bioautography method (TLC-DB) against DPPH. Dichloromethane-methanol with a ratio of 10:1 was used as the mobile phase for developed TLC-DB. The list of Halmahera plant extracts (number 1-35) (Table I).

Alkaloids from genus *Ochrocia* and family Apocynaceae, in general, have been reported to have a wide range of pharmacological effects (Dey *et al*., 2017) and may contribute to the antibacterial and antiplasmodial effect of *O. glomerata*. However, further study is needed to confirm this statement.

To our knowledge, this is also the first report on the antibacterial activity of *R. lanata* (Scheff.) and *D. esculentoides*. The information regarding *R. lanata* and *D. esculentoides* is very limited. In Indonesia, these two species may only grow in the east part of this country since the specimen records showed only Maluku, Halmahera, and Papua as the collection places in the GBIF database. *R. lanata* belongs to the Zingiberaceae family in which several species from this family also have antibacterial activity against *S. aureus,* for instance, *Curcuma aeruginosa* Roxb., *Curcuma glans* K. Larsen & J. Moodand *Curcuma* cf. *Xanthorrhiza* Roxb. (Akarchariya *et al*., 2017). However, unlike the *Curcuma* genus from the same family, the biological activities of the *Riedelia* genus have not been heavily studied. Our preliminary data from this study showed that one of the plant species from this genus has potency as a resource for antibacterial agents, especially for gram-positive bacteria. Further biological activity studies of plants from this genus could be promising for drug discovery and development research.

The stem bark extract of *D. esculentoides* was the only extract that showed moderate activity against *E. coli*. Finding a potent antibiotic for gram-negative bacteria has been a challenge because gram-negative bacteria have an outer membrane that makes them less susceptible to antibiotics. As only a little information could be found regarding *D. esculentoides*, future studies to explore its antibacterial mechanism and the phytochemical constituents of this plant are needed.

Antioxidant activity of the plant extracts

In this study, we also evaluated the antioxidant activity of the plant extracts. The qualitative screening using dot-blot TLC indicated that almost all extracts had antioxidant activity against DPPH, which was recorded as yellow spots (Figure 4). However, in the developed TLC-DB which exhibited the substances within the extracts that produced antioxidant effects, the result indicated that 29 out of 35 extracts contained more potent antioxidant compounds inside the extracts (Figure 4). These active spots can also be isolated in future studies to identify the compounds responsible for the antioxidant activity.

For further analysis, the IC_{50} determination against DDPH to calculate each extract's antioxidant activity index (AAI) was done. The AAI value is advantageous to compare the antioxidant strength of the plant extracts (Scherer & Godoy, 2009). Based on the classification of AAI described by Scherer and Godoy (2009), eight extracts had strong antioxidant activity and six extracts had very strong antioxidant activity (Table I).

Until today, the demand for natural antioxidants in the food and health industry is still high because of the health risk concern of synthetic antioxidant consumption (Jiang and Xiong, 2016).

Table II. Total phenolic compounds of selected plant extracts

Plant species	Plant parts	TPC (mg GAE/ g DE)
Elaeocarpus dolichostylus Schltr.	Leaf	307.86±4.65
Elaeocarpus dolichostylus Schltr.	Stem bark	271.61±7.10
Elaeocarpus multiflorus (Turcz) Fern. – Vill.	Leaf	264.92±2.31
Gmelina lepidota Scheff.	Leaf	130.48±1.96
Smilax australis R. Br.	Leaf	127.43±3.74
Smilax australis R. Br.	Stem	177.55±4.32
Machaerina glomerata (Gaudich.) T. Koyama	Leaf	146.51±3.89
Timonius rufescens (Miq.) Boerl.	Leaf	196.50±8.86
Colubrina asiatica (L.) Brongn.	Leaf	128.03±2.41
Colubrina asiatica (L.) Brongn.	Stem bark	193.16±7.23
Cinnamomum sintoc Blume	Leaf	73.49±4.63
Cinnamomum sintoc Blume	Stem bark	411.17±8.48
Garcinia latissima Miq.	Stem bark	268.85±8.75
Psychotria celebica Miq.	Leaf	271.01±6.90

Antioxidants are usually used in the food industry to protect the food from oxidative degradation, while in the medical area, antioxidants are used to reduce oxidative stress that can induce the development of various diseases like cancer, inflammatory diseases, and heart-related diseases (Yashin *et al*., 2017). Thus, 14 extracts from the ten underexplored plant species reported in this study can be used as potential antioxidant sources. From these ten underexplored plant species with strong and very strong antioxidant activity, only *C. sintoc, P. celebica,* and *G. latissima* have been reported before for their antioxidant activity (Ambarwati *et al*., 2018; Praptiwi *et al*., 2021; Yashin *et al*., 2017). To the best of our knowledge, the antioxidant activity of the other seven plant species was first reported in this present study. Hence, the ten underexplored plant species reported in this study can be studied further and used as potential antioxidant sources.

Total phenolic content of the plant extracts

In this study, we also analyzed the TPC of 14 extracts with strong and very strong antioxidant activity (Table II). Almost all of the tested extracts have a high TPC value, except the leaf extract of *C. sintoc*. We also evaluated the correlation between TPC and IC_{50} of plant extract against DPPH using Pearson correlation analysis with r=-0.8712, p<0.0001. This high correlation indicates that the total phenolic content of selected plant extracts may be responsible for their antioxidant properties. This correlation is in accordance with previous findings which also found linear correlations between TPC and antioxidant activity of other plant species (Butsat & Siriamornpun, 2016; Złotek *et al*., 2016). Furthermore, studies also reported that phenolic compounds, for instance, phenolic acids, phenylpropanoids, and flavonoids are accountable for the plants' antioxidant properties (Shahidi & Ambigaipalan, 2015). Hence, this study presents valuable data displaying plants with rich phenolic compounds as the sources for novel antioxidant agents.

CONCLUSION

The antibacterial evaluation in this study revealed that ethanolic extracts of two plant species collected from Halmahera had moderate activities against *S. aureus* and one plant species had moderate activity against *E. coli.* However, this present study displayed ten underexplored plant species with strong and very strong antioxidant activities. The correlation analysis displayed a high correlation between TPC and antioxidant activity, indicating a strong role of phenolic compounds in the selected plants' antioxidant properties. Further studies are needed to reveal compounds that are responsible for these antioxidant activities. These findings indicate that collected plants from Halmahera are potential to be developed as the sources for novel antioxidants.

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