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RESEARCH ARTICLE

ANTIOXIDANT AND ANTIBACTERIAL ACTIVITY OF MANGROVE *Brugueira gymnorrhiza* STEM EXTRACTS AGAINST PATHOGENIC BACTERIA *Vibrio cholerae*

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ABSTRACT

Background : The treatment of some diseases caused by free radicals and pathogenic bacteria usually by using antioxidants and antibiotics. Due to excessive use of antibiotics and other environmental cues, some bacteria are now resistant to certain antibiotics or even to multiple antibiotics. Some *Vibrio cholerae* bacterial strains are multiresistant to many antibiotics.

Objective : The antioxidant and antibacterial activities of *Brugueira gymnorrhiza* stem extracts against pathogenic bacteria *V. cholerae*.

Method : The *B. gymnorrhiza* stem was extracted by gradient maceration method. The DPPH method was used to determine the antioxidant activity and the disc diffusion method was used to determine the antibacterial activities. The column chromatography method was used to fractionate the selective extract with the best activity. The LC-MS/MS method was used to identify the compound obtained from the fraction with the best antioxidant and antibacterial activity.

Result : Ethyl acetate extract of *B. gymnorrhiz*a stem had the best antibacterial activity with MIC and MBC values of 62.50 mg/L. Ethyl acetate extract also showed the best value of antioxidant activity as indicated by an IC50 value of 255.03 mg/L. The results of fractions test showed that fraction 3 had the best antibacterial and the best antioxidant activities with both the MIC and MBC values of 7.90 mg/L and IC50 value of 348.91 mg/L, respectively.

Conclusion : Ethyl acetate extract of *B. gymnorrhiza* stem has good potential as antioxidant and antibacterial. The compound which is thought as antioxidant and antibacterial from Ethyl acetate extract is 2-Ethyl-4-methyl-1H-imidazole.

Keywords : Antibacterial, Antibiotics resistant, *B. gymnorrhiza*, Stem, *Vibrio cholerae*

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INTRODUCTION

There are many diseases caused by free radicals such as cancer, premature aging and heart disease, while others are caused by pathogenic bacteria such as cholera, dysentery and tuberculosis. Cholera is an acute diarrheal disease caused by *Vibrio cholerae* (*V. cholerae)* intestinal infection. Patients with this illness will experience dehydration due to quick loss of body fluids resulting in death. Patients with cholera are usually treated with antibiotics. However, continues use of the same antibiotics can lead to bacterial resistance. There are *V. cholerae* bacteria that are multiresistant or resistant to many antibiotics.[1]

Therefore, it is necessary to search for new antibacterial, especially against *V. cholerae*. Research related to *Bruguiera gymnorrhiza* (*B. gymnorrhiza)* as a source of bioactive compounds has been explored utilizing different parts of the plant which include leaf, bark, fruit and roots.[2] In previous studies, research related to the discovery of antioxidant and antibacterial agents from *B. gymnorrhiza* had been performed. Ethanol extract of the leaf of this plant had shown as potential antioxidant.[3] Ethanol extracts of bark and roots had the potential to reduce cholesterol levels.[4] The people of the Solomon Islands had traditionally used bark extract of the plant to treat burns and medicine for diarrhea and malaria.[5] Antibacterial-related research on *B. gymnorrhiza* had also been carried out using leaf extract of the plant against *S. aureus* and *E. coli.*[6] Ethanol extract of the leaf had been evaluated against *E. coli* and *S. aureus.*[7] Leaf and bark extracts were tested against *S. aureus*, *Bacillus cereus*, *E. coli* and *Pseudomonas aeruginosa*.[8] Fruit extracts of the plant had been tested against *B. cereus* and *S. aureus.*[9]

However, the use of stem of *B. gymnorrhiza* as potential antioxidant and or antibacterial has not been previously conducted. Therefore, to the best of authors' knowledge, this is the first study to use the stem of *B. gymnorrhiza* as a source of antioxidants and or antibacterial.

MATERIAL AND METHODS

This research had been conducted according to ethical regulation in which all biological assays were performed in an accredited Biosafety Laboratory Level 2 at the Eijkman Institute for Molecular Biology, Jakarta, Indonesia. This is because *V. cholerae* is categorized as Risk Group 2 pathogen. However, this research did not require animal or clinical ethical permission because the research did not use animal or human object.

Sample Preparation, Extraction and Fractionation

The stem of *B. gymnorrhiza* was collected from Karya Tani Village, Labuhan Maringgai District, East Lampung, Lampung Province, Indonesia in June 2018. The *B. gymnorrhiza* stems were freshly obtained from the trees then dried and stored for further use. Simplisia of the stem sample was prepared by grinding method using blender. Water content of the simplisia was determined using standard method of the Association of Official Analytical Chemistry.[10]

The samples were extracted using gradient maceration method. The maceration process was carried out for 24 hours with a ratio of simplisia to solvents was 1:5 (Kg/L) using four types of solvent with different polarity, namely n-hexane,

ethyl acetate, ethanol and water. Maceration mixtures were filtered and concentrated using rotary evaporator. All of the extracts were stored until used for qualitative phytochemical, antioxidant and antibacterial assays. The fractionation process was carried out on the extract that has the best antioxidant and antibacterial activity by using column chromatography.

Qualitative Phytochemical Assays

Qualitative phytochemical assays were carried out based on the standard method.[11] Phytochemical tests were performed on simplisia and extracts. Phytochemical tests included flavonoids; steroids and triterpenoids; alkaloids; saponins and tannins using specific reagents such as the Lieberman-Burchard reagent for the triterpenoid-steroid, Mayer, Wagner and Dragendrof reagents for alkaloid testing, $FeCl_3$ for the hydroquinone phenolic reagents, saponin test by heating the extract in distilled water and tannin testing carried out by heating the extract in distilled water then the mixture is filtered. The filtrate was reacted with $FeCl_3$ 1% (w / v).

DPPH Assays

All extracts obtained previously were used for the antioxidant activity test using DPPH method.[12] The mixture of DPPH solution and extract were incubated and the absorbance measured at a wavelength of 517 nm. Vitamin C was used as positive control and the blank was made by mixing ethanol with DPPH solution.

Antioxidant measurements were also carried out to find the IC_{50} value (free radical inhibitory concentration of 50%). The smaller IC_{50} value of a material or sample is the better antioxidant activity. The same procedures were used to test antioxidant activity of the fractions.

Antibacterial Assays

Antibacterial activity assays were carried out using disc diffusion method.[8] Bacteria (*V. cholerae*) were cultured on Nutrient Agar media and then incubated. The cultured bacteria were grown in Nutrient Broth media with overnight incubation time. After incubation, 20 µL NB containing test bacteria was added to 20 mL of Mueller Hinton Agar (MHA), then homogenized and poured onto a petri dish and allowed to stand until solidified. Paper discs that had been treated by extracts (1000 mg/L) were placed on top of the MHA and then incubated. The zone of inhibition (ZOI) formed by the bacteria was then measured. Tetracycline (30 mg/L) was used as positive control, whereas 10% DMSO was used as negative control.

Antibacterial activity tests were also carried out by determining the minimum inhibitory concentration (MIC) and the minimum bactericide concentration (MBC). The MIC and MBC values are the smallest concentration values of a material or sample that can be used to inhibit or kill bacteria. The determination of the MIC and MBC was performed by liquid dilution method. All extracts and fractions 1-5 of the ethyl acetate extract with several concentrations were used to determine the MIC and MBC values. All samples with different concentrations were transferred into a test tube and added with bacterial suspension that had been adjusted to Mc Farland's solution. The MIC value was obtained at the minimum concentration in which the turbidity was absent. The MBC value was obtained by conducting further tests by streaking solid media method. MBC value was indicated by the absence of bacterial growth at the lowest concentration of the samples.

LC-MS/MS Analysis of Antioxidant and Antibacterial Candidate Compounds

Analysis of antioxidant and antibacterial candidate compounds were carried out on the fraction with the best antioxidant and antibacterial activity. The fraction was dissolved into the mixture of methanol: water with 90:10 ratio (v/v). The mixture was injected into the LC column at a flow rate of 0.20 mL/min at 40ºC. The system of LC-MS/MS was LC System Ultra Performance Liquid Chromatography (UPLC) tandem Quadrupole Time of Flight (QTOF) mass spectrometer, which equipped with electrospray ionization (ESI) (Qmicro QAA842). The ionization mode on the MS system used was positive ionization. Scan mode used was full scan from 100-500 m/z. UPLC column used was acquity UPLC HSS C18 1.8 µm (2.1 × 150 mm). The eluent used was H_2O and acetonitrile. The results of the analysis were displayed using mass chromatogram.

Statistical Analysis

The data of antioxidant and antibacterial activity were analyzed using statistical analysis in the form of one-way analysis (ANOVA) to show the effect of extracts and fractions addition towards the antioxidant and antibacterial activity. The ANOVA test generates results in the form of a p-value, with $p < 0.05$ showing a significant effect on a confidence level of 95 % (α=0.05).

RESULTS

Bruguiera gymnorrhiza Stem Yield of Extracts and Phytochemical Content

The content of water obtained was 7.67%. The yield of the extracts were 1.21 % n-hexane extract, 2.97% ethyl acetate extract, 4.46 % ethanol extract and 3.50 % water extract (Table 1).

Antioxidant and Antibacterial activity of *B. gymnorrhiza* Stem Extracts

The results of antioxidant and antibacteria test of *B. gymnorrhiza* stem extracts showed that the potential of each extract as antioxidant and antibacterial varied as shown in IC_{50} values and MIC and MBC values (Table 2). Ethyl acetate extract produced the highest antioxidant and antibacterial activity as indicated by IC_{50} values of 255.03 mg/L and MIC and MBC values of 62.50 mg/L.

Fractionation

The best eluent for fractionation of ethyl acetate extract was obtained from the TLC experiment results, namely Dichloromethane: Ethyl acetate with 9:1 ratio (v/v). Five fractions (1-5) were obtained from separation results using column chromatography method. The yield of fractions 1 to 5 were 5.35 %, 13.67%, 23.02 %, 6.55% and 15.43%, respectively (Table 3).

Antioxidant and Antibacterial Activity of Factions 1-5

Antioxidant and antibacterial activity of fractions 1-5 were analyzed using the same method of the extract activity. The results showed that fraction 1 had the best antioxidant activity indicated by the lowest IC_{50} value of 251.48 mg/L. Meanwhile, fraction 3 showed the best antibacterial activity indicated by the lowest MIC and MBC values of 7.90 mg/L with the IC_{50} value of 348.91 mg/L (Table 3).

Table 1. Yield and phytochemical content of simplisia and stem extracts of *B. gymnorrhiza*

Extract	Yield (%)	Test	Samples				
			Simplisia	n-hexane extract	Ethyl acetate Extract	Ethanol Extract	Water Extract
n-hexane	1.21	Flavonoid	+	+	+	+	+
Ethyl acetate	2.97	Steroid	-	-	-	-	-
Ethanol	4.46	Triterpenoid	+	+	+	+	+
Water	3.50	Saponin	+	-	+	+	+
		Tanin	+	-	-	+	-
		Alkaloid	+	-	+	-	-

Table 2. MIC, MBC and IC_{50} value of *B. gymnorrhiza* stem extracts

Sample	MIC (mg/L)	MBC (mg/L)	IC_{50} (mg/L)
n-hexane	1000.0	1000.0	712.08 ± 6.68^{c}
Ethyl acetate	62.5	62.5	225.03 ± 2.25^{b}
Ethanol	1000.0	1000.0	$>1000.00 \pm 0.00^{d}$
Water	1000.0	1000.0	638.17 ± 14.81^{c}
Tetracycline	3.9	3.9	-
Vit C	-	-	3.10 ± 0.02^{a}

The IC_{50} value is presented as mean ± SD. IC_{50} presented with different alphabetic letters are significantly different ($p<0.05$)

Table 3. Yield, MIC, MBC and IC_{50} values of fractions 1-5

Sample	Yield (%)	MIC (mg/L)	MBC (mg/L)	IC_{50} (mg/L)
Fraction 1	5.4	250.0	250.0	251.48 ± 6.12^{b}
Fraction 2	13.7	250.0	250.0	$>1000.00 \pm 0.00^{g}$
Fraction 3	23.0	7.9	7.9	348.91 ± 2.24^{b}
Fraction 4	6.6	62.5	125.0	$>1000.00 \pm 0.00^{e}$
Fraction 5	15.4	62.5	125.0	$>1000.00 \pm 0.00^{f}$
Tetracycline	-	3.9	3.9	-
Vit C	-	-	-	3.13 ± 0.02^{a}

The IC_{50} value is presented as mean ± SD. IC_{50} presented with different alphabetic letters are significantly different ($p<0.05$)

Table 4. Antibacteria and antioxidant compounds of fraction three ethyl acetate extracts of *B. gymnorrhiza* stem

Retention Time (Minute)	*M/Z*	IUPAC Name	Peak Area (%)
1.33	111.093	2-Ethyl-4-methyl-1H-imidazole	8.18
2.15	309.086	3,6-dihydro-2H-pyran-3,5,6-triol; triacetate	3.91
13.13	209.154	4-(Heptyloxy)Phenol	8.18
13.89	279.164	2-Hexanoyl-4,4,6,6-tetramethyl-3,5-dioxo-1-cyclohexane-1-olate	7.52
16.17	409.201	N, N, N', N'-Tetrabutyl-1,3-tri sulfane dicarboxamide	15.26
17.11	776.228	Unidentified	12.83
17.76	391.284	7-Deoxycholate	8.97
18.39	338.343	(13Z)-13-Docoseamide	30.57

LC-MS/MS Analysis of Antioxidant and Antibacterial Compounds of Fraction 3

The identification of bioactive compounds was carried out on fraction 3. The results showed that there were eight major compounds in fraction 3 (Table 4).

DISCUSSION

Phytochemical tests were carried out to qualitatively observe secondary metabolites contained in simplisia and extracts. Secondary metabolites such as alkaloids, flavonoids, saponins and tannins have important roles in biological activity of a plant.[13] The results showed that simplisia and stem extracts of *B. gymnorrhiza* contain different secondary metabolites (Table 1). The difference in secondary metabolites content in simplisia and extract due to the difference in solvent polarity. Different secondary metabolites resulted in different antioxidant and antibacterial activity of the extracts and fractions used.

The highest antioxidant and antibacterial activity from ethyl acetate extract were due to bioactive compounds contained in the sample. According to phytochemical test, the difference in phytochemical content of ethyl acetate extract and other extracts lies in the group of alkaloid compounds (Tabel 1). Ethyl acetate extract contains alkaloid compounds that were known to have biological activities such as antioxidants, antibacterial, anticancer and some other biological activities. The activity of alkaloid compounds as antibacterial was supported by previous research in which the alkaloid was effective against multiresistant E. coli.[14] The results of this study strongly indicated that ethyl acetate extract had good activity as antioxidant and antibacterial due to its alkaloids content. However, the IC_{50} value of ethyl acetate extract was much higher than the IC_{50} value of vitamin C, which is 3.13 mg/L and the MIC and MBC values were higher than the MIC and tetracycline values, which is 3.90 mg/L. The good activity of vitamin C and tetracycline are because vitamin C and tetracycline are pure compounds, while ethyl acetate extract consisted of many chemical constituents. This condition may result in following consequences such as lowering actual concentration of the active compound or the activity of the compound affected by other compounds. Several compounds will increase their biological activity if given simultaneously while some others will be increased their biological activity as a single compound.[15]

The difference in the yield among fractions indicated that different chemical constituents interacted differently with the stationary phase or mobile phase according to their polarity, charge of other factors.[16] The separation principle using column chromatography depends on interaction of compounds with stationary phase or mobile phase. The stationary phase used was silica which has high polarity. This condition can cause the high polarity fractions have stronger bonding than low polarity fractions. So, the separation happened because the low polarity fractions were carried out firstly then high polarity fractions.

The results of antioxidant and antibacterial test of fraction 1-5 showed that there is no significant difference against antioxidant activity of ethyl acetate extract which is showed by IC_{50} value. On the other hand, the MIC and MBC values indicate an increase in antibacterial

activity which is shown by the significant difference with the antibacterial activity of ethyl acetate extract.

The MIC and MBC results can be used to show whether antibacterial compounds in ethyl acetate extract and fraction 3 are bacteriostatic or bactericidal. At high concentrations, a compound can be bactericidal, whereas at low concentrations it will be bacteriostatic. According to the results, the similarity of MIC and MBC values explain that antibacterial compounds contained are bactericidal.[17]

Antibacterial activity tests were also carried out on the solvents used for extracting. It aimed to performed that antibacterial activity only comes from compounds contained in the extract. Test results showed n-hexane, ethyl acetate, ethanol 70%, water and DMSO were used to dissolve extracts and fractions did not form inhibitory zones.

The results showed that there were 8 compounds in the selected fraction. The abundance of compounds contained in the selected fraction is indicated by the value of % peak area (Table 4). The compounds thought to act as antioxidants and antibacterials was an alkaloid compound, namely 2-Ethyl-4-methyl-1H-imidazole. This compound is a derivative of imidazole which is proven to have biological activities such as antioxidants, anticancer, antibacterial and anti-inflammatory.[18] The antibacterial mechanism of alkaloids compounds is known to act as DNA intercalator and inhibits the topoisomerase enzyme.[19] Another mechanism of alkaloids as antibacterial is interfering the constituent components of peptidoglycan which causes bacterial cell death due to a nonintact of cell walls formation.

The reaction mechanism between 2-Ethyl-4-methyl-1H-imidazole with DPPH was shown in Figure 1. Another compound was thought to have antioxidant and antibacterial activity shown in Table 4 was 2-Hexanoyl-4,4,6,6-tetramethyl-3,5-dioxo-1-cyclohexane-1-olate. This compound is one of 3000 compounds which are potential as antitubercular agents.[20]

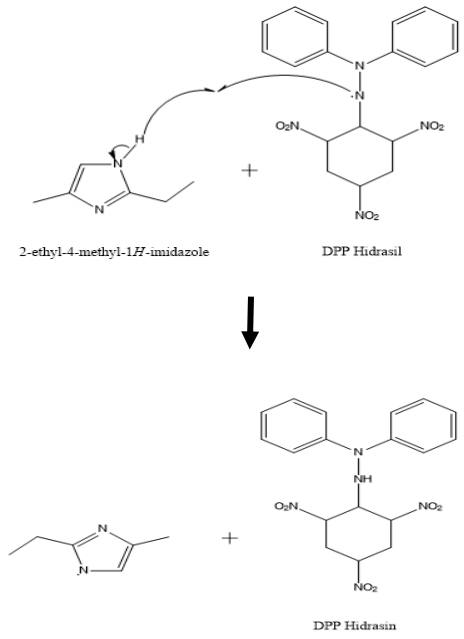

Figure 1. Reaction of 2-Ethyl-4-methyl-1H-imidazole with DPPH

The (13Z)-13-Docosenamide compound which was thought to be neuroactive is suspected as antioxidant and antibacterial agent.[21] The result also showed several other compounds such as 7-Deoxycholate; 3,6-Dihydro-2H-pyran-3,5,6-triol; triacetate; 4-(Heptyloxy) Phenol and N, N, N ', N'-Tetrabutyl-1,3-tri sulfane dicarboxamide which has unknown biological activity. The diversity of compounds contained in fraction 3 influenced antioxidant and antibacterial activity. The determination of antioxidant and antibacterial compounds in this study is only limited to compound estimation,

thus it needs further purification to determine the role of suspected compound.

CONCLUSION

According to the results of this study, these data suggested that ethyl acetate extract of *B. gymnorrhiza* stem has good potential as antioxidant and antibacterial. Ethyl acetate extract and its fraction 3 showed the best antioxidant and antibacterial activity. The increasing antibacterial activity of the fraction compares to ethyl acetate extract showed that compounds contained in *B. gymnorrhiza* stem extract would have better biological activity as a pure/single compound. The compound which is thought as antioxidant and antibacterial is 2-Ethyl-4-methyl-1H-imidazole.

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