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RESEARCH PAPER

Gravity column chromatography of n-hexane fraction of *Rhizophora mucronata* leaves from West Nusa Tenggara and their antioxidant activity

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Abstract: In the last few decades, many diseases have been caused by Radical Oxidative Stress (ROS) or free radicals. Compounds that act as natural antioxidants have been widely developed to counteract these free radicals. Rhizpora mucronata was a mangrove species reported to possess strong antioxidant activity. However, the research is limited to extracts; fractions with more specific compound components have not been explored. The *n*-hexane fraction of R. mucronata leaves was reported to have high total flavonoid content (TFC). The TFC has a positive correlation with antioxidant activity. Therefore, this study aimed to simplify the compound components in the *n*-hexane fraction of R. mucronata leaves using gravity column chromatography (GCG) and determine their antioxidant activity. The n-hexane fraction was fractionated using GCG with increasing solvent polarity (n-hexane, chloroform, ethyl acetate, and methanol). The fractions were tested for antioxidant activity using 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) methods. The fractionation using GCG resulted in 80 fractions. These fractions were grouped based on their thin-layer chromatography (TLC) profiles, resulting in 12 groups of fractions (A-L). Fractions D, F, G, H, J, K, and L, the fractions with enough yield, determined their antioxidant activity using the DPPH and ABTS methods. Fractions D and J tested by the DPPH method had IC50 values of 28.68 ± 1.58 ppm and 9.59 ± 0.39 ppm, respectively. Meanwhile, fractions D and J tested by the ABTS method had IC50 values of 15.10 ± 1.00 ppm and 3.16 ± 0.55 ppm, respectively. Meanwhile, fractions F, G, H, K, and L exhibit antioxidant activity with IC50 values greater than 100 ppm. Fractions D and J have potent antioxidant activity, and both were tested using DPPH and ABTS methods. Therefore, fractions D and J can be further developed as natural antioxidants.

.**Keywords:** Rhizpora mucronata leaves fraction, antioxidant activity, gravity column chromatography, DPPH, ABTS.

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INTRODUCTION

Free radicals are atoms or molecules that have unpaired electrons. This condition causes free radicals to be unstable and reactive. To stabilize themselves, free radicals bond to take electrons from other compounds, such as DNA or nucleic acids, causing these compounds to become free radicals and initiating a chain radical reaction that ultimately damages the body's cells. Therefore, free radicals significantly influence various physiological conditions of the human body, which can cause various diseases [1]. Several diseases caused by free radicals were cardiovascular disease, metabolic disease, Alzheimer's, and cancer [2]. Excess free radicals in the body can be overcome with antioxidants.

Antioxidants neutralize free radicals by donating electrons to the free radicals before damage to certain molecules increases. Antioxidants are naturally produced by the body, for example, Superoxide dismutase (SOD), Glutathione peroxidase (GPx), Catalase (CAT), Glutathione reductase (GRx), and non-enzymatic antioxidants such as lipoic acid, glutathione, L-arginine, uric acid, and bilirubin [3]. However, if the number of free radicals is too high, antioxidants from outside the body are needed. The exogenous antioxidants were vitamin C, E, carotenoids, polyphenols, proteins, and micronutrients [4]. Polyphenols were reported to act as antioxidants through an electron transfer mechanism [5].

Polyphenols are secondary metabolites that contain hydroxyl groups and aromatic rings [6]. Polyphenolic compounds can be obtained from plants. *Rhizpora mucronata* was a mangrove species reported to have high total phenolic content [7]. In addition, *R. mucronata* leaf extract obtained from North Sulawesi has very strong antioxidant activity with an IC_{50} value of 20.99 ± 0.33 µg/mL [8]. The compound content in the extract is still large and varied. The phytochemical analysis of *R. mucronata* leaf extract revealed the presence of saponin, steroid, triterpenoid, phenolic, and flavonoid groups [9]. *R. mucronata* leaves extract detected using liquid chromatography-high resolution mass spectrometry (LC-HRMS) was reported to contain some flavonoid compounds such as rutin, quercetin trifolin, hispidulin, and kaempferol [10]. In addition, the n-hexane fraction of *R. mucronata* leaves, as detected using gas chromatography-mass spectrometry, contained squalene as the major compound [11]. The ethyl acetate fraction, n-hexane fractions, and water fraction of *R. mucronata* leaves, when fractionated using liquid-liquid chromatography (LLE), revealed significantly different antioxidant activities. The n-hexane fraction of *R. mucronata* leaves has been reported to exhibit strong antioxidant activity [9]. The LLE method can separate compounds only within a limited range of polarities. The fractionation using gravity column chromatography (GCC) employed a gradient polarity solvent to obtain compounds of varied polarity [12]. So, the compounds obtained in each fraction were more specific. So, a fraction with more potent antioxidant activity was expected to be obtained. Fractionation of the n-hexane fraction of *R. mucronata* leaves from Lombok Island using the GCC method has not been performed. Variations in geographical location influence the composition of compounds and the associated biological activities of *R. mucronata* leaves. However, this study aimed to fractionate *R. mucronata* leaves using gravity column chromatography. In addition, each fraction would be tested for its antioxidant activity using the DPPH and ABTS methods. The fraction that has the highest antioxidant activity can be a candidate for development as a natural antioxidant.

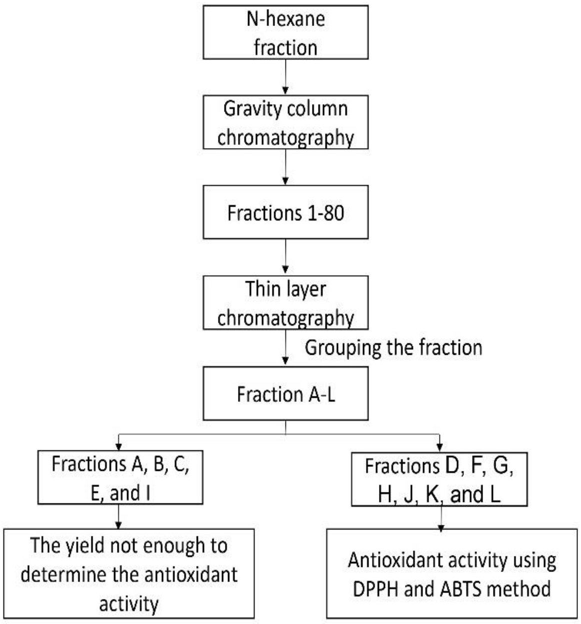

Figure 1. Scheme of Fractionation of *R. mucronata* Leaves

MATERIALS AND METHODS

Materials

Reagents with pro-analysis grade (Sigma-Aldrich), such as 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), 2,2-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS), and quercetin. Silica gel 60 F_{254} Merck 1.09385.1000 (230-400 Mesh) for column chromatography. F_{254} silica plate. Solvent with pro analytical grade (E. Merck) consisting of *n*-hexane, methanol, ethyl acetate, chloroform, and dichloromethane. *R. mucronata* leaves were obtained from Buwun Mas Village, Sekotong District, West Lombok Regency, West Nusa Tenggara, Indonesia.

Method

Extraction and Fractionation

R. mucronata leaves were extracted using the sonication method by Sammanta et al. (2024) [7]. The *n*-hexane fraction was obtained through a liquid-liquid fractionation process by Sammanta et al. (2024) [7]. The *n*-hexane fraction (5 grams) was fractionated using the gravity column chromatography (45 x 3 cm). The stationary phase used is Silica gel 60 F_{254} Merck 1.09385.1000 (230-400 Mesh) for column chromatography. The solvents used are *n*-hexane, chloroform, ethyl acetate, and methanol with a gradient composition. The eluent composition and gradient can be seen in Table 1. The fractionation process was monitored based on the colour of the separated compounds on the chromatography column or the collection of a 20 mL fraction in a vial. The metabolite profile of each vial was identified using thin-layer chromatography (TLC). Fractions that have similar TLC profiles were combined. The fractionation procedure is available in Figure 1.

Thin Layer Chromatography (TLC)

The compound profile of fractions 1-80 was examined using TLC. The stationary phase used was silica F_{254}. Fractions 1-56 were eluted using the mobile phase *n*-hexane: chloroform (1:4). Fractions 56-62 were eluted using the mobile phase ethyl acetate: methanol (1:4). Fractions 62-75 were eluted using ethyl acetate: methanol (1:9). Fractions 75-80 were eluted using a 100% methanol mobile phase. Meanwhile, fraction A-L was eluted using a mobile phase of *n*-hexane: chloroform (1:4).

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

The fractions D, F, G, H, J, K, and L were made into a concentration series of 0.156, 0.3125, 0.625, 1.25, 2.5, 5, 10, 25, 50, and 100 µg/mL. Each 100 µL fraction and 100 µL 0.25 mM DPPH were put into a 96-well plate. The solution mixture was homogenized using a shaker machine for 30 seconds. The mixture was incubated in the dark for 30 minutes [13]. A mixture of 100 µL methanol and 100 µL DPPH was used as a control. The absorbance of each sample was measured at a wavelength of 517 nm using a multiscan spectrometer (Multiscan SkyHigh, Thermo Scientific, Finland). The DPPH free radical scavenging was calculated using the following formula:

$$DPPH\ Radical\ Scavenging\ activity\ (\%) = \frac{(Abs\ Control - Abs\ Sample)}{Abs\ Control}\ x\ 100\%$$

The concentration series and the sample's DPPH free radical scavenging activity were used to create a calibration curve, yielding the Y=bx-a equation. The IC_{50} of the sample can be calculated using the equation.

Table 1. The solvent for fractionation of the *n*-hexane fraction of *R. mucronata* leaves using GCC

Solvent composition	n-hexane (mL)	Chloroform (mL)	Ethyl acetate (mL)	Methanol (mL)	Number of fractions
1	100	-	-	-	1-5
2	75	25	-	-	6-10
3	50	50	-	-	11-15
4	25	75	-	-	16-20
5	-	100	-	-	21-25
6	-	80	20	-	26-30
7	-	60	40	-	31-35
8	-	40	60	-	36-40
9	-	20	80	-	41-45
10	-	-	100	-	46-50
11	-	-	80	20	51-55
12	-	-	60	40	56-60
13	-	-	40	60	61-65
14	-	-	20	80	66-70
15	-	-	-	200	71-80

Antioxidant activity assay using 2,2´ -azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) method

Five millilitres of 7 mM ABTS were combined with 88 µL of 140 mM potassium persulfate. The mixture was left for 12-16 hours in the dark at room temperature to obtain an ABTS positive radical solution (ABTS·+). The ABTS·$^{+}$ solution was diluted using methanol until its absorbance reached 0.70 ± 0.02 at 734 nm. One hundred µL of each fraction (0.156; 0.3125; 0.625; 1.25; 2.5; 5; 10; 25; 50; and 100 µg/mL) was put into a 96-well plate, then added to 100 µL ABTS·+ solution. The mixture was allowed to stand for 6 minutes, after which the absorbance was read at a wavelength of 734 nm using a multiscanning spectrometer (Multiscan SkyHigh, Thermo Scientific, Finland). The control solution used was a mixture of 100 µL methanol and 100 µL ABTS·+ solution. The ABTS free radical scavenging activity can be calculated with the following equation:

$$ABTS\ radical\ scavenging\ activity\ (\%) = \frac{(Abs\ Control - Abs\ sample)}{Abs\ control}\ x\ 100\%$$

Statistical analysis

Data analysis was carried out using SPSS software, version 16. The % DPPH and ABTS radical scavenging activity of the tested fractions (100 ppm) was analyzed using the One-Way ANOVA. The level of confidence used is 95%.

RESULTS AND DISCUSSION

Yield of Extract and Fractions

R. mucronata leaves were extracted using ethanol (96%) by the sonication method. The sonication method utilizes sound wave energy that disturbs the particles in the sample. The vibration of sonication enhanced the contact of the sample with the solvent. Therefore, the separation process of the compounds becomes quicker and more efficient [14]. The ethanol (96%) was chosen because it is selective, non-toxic, has good absorption, and can filter non-polar, semipolar, and polar compounds [15]. The ethanol extract of *R. mucronata* leaves obtained was 26.56 g, corresponding to a yield of 10.624%. The extract has yielded above 10%, indicating the maximum extraction process.

R. mucronata ethanol extract was extracted using the liquid-liquid extraction method. The method used two solvents that do not mix (water and n-hexane). Liquid-liquid extraction was a fractionation method that allowed for the easy separation of compounds based on their polarity [16]. The *n*-hexane fraction obtained was 14.9 g, corresponding to a yield of 74.5%. For further separation of compounds, fractionation was performed using the gravity column chromatography (GCC) method. This method can separate more specific components based on their polarity and affinity with the stationary phase on the chromatography column [17].

Thin-Layer Chromatography (TLC) of Fractions

Table 2. Weight of fractions of *R. mucronata* leaves

Fraction	Fraction from vial number	Weight (mg)
A	1-18	1
B	19-24	17.3
C	25-32	7.8
D	33-35	496.9
E	36	5.9
F	37-38	180
G	39-52	53.3
H	53-59	18.2
I	60-61	18.6
J	62-65	154
K	66-70	1538.9
L	71-80	6302.7

Figure 2. TLC profile of Fractions *R. mucronata* leaves. Eluent: *n*-hexane: chloroform (1:4), stationary phase: F254 silica plate, visualization under UV 366.

The fractionation using GCC produced 80 fractions. These fractions were analyzed for their compound profiles using TLC. Based on the TLC profile, 12 fractions (fractions A-L) were obtained. Each fraction has a different weight, as seen in Table 2. The fraction with the highest number was fraction L, which corresponded to fractions 71-80, eluted using a methanol solvent (Table 1). So, it was concluded that the dominant compound in *R.*

mucronata leaves was polar. The TLC profile also showed that the L fraction was polar, as evidenced by the L fraction spot not eluting when eluted with a semipolar eluent (Figure 2).

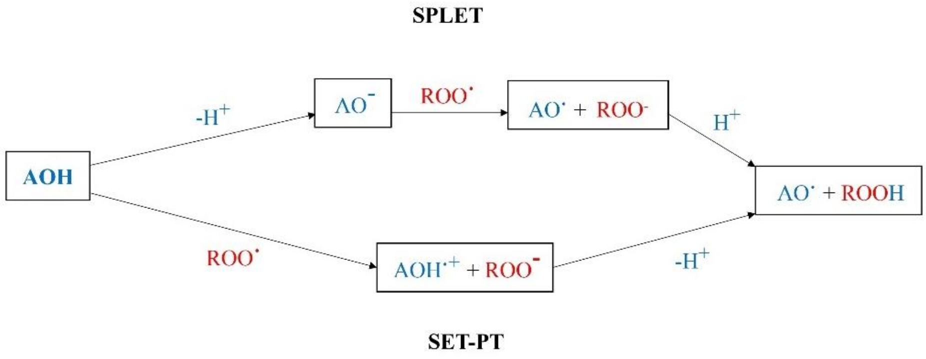

Figure 3. Antioxidant Mechanism Scheme of Single Electron Transfer (SET). Electron Transfer followed by a Proton Transfer (SET-PT), Sequential Proton Loss Electron Transfer (SPLET), Antioxidant (AOH), Free Radical ($ROO^{\cdot}$), Anion (ROO^{-}), Positif Proton (H^{+}).

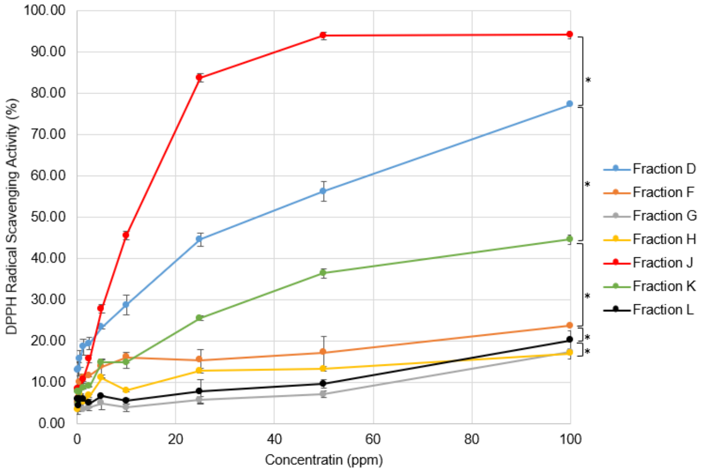

Figure 4. Antioxidant Activity of *Rhizpora mucronata* Leaves Using the DPPH Method. The % inhibition of fractions at 100 ppm was significantly different (*) with $p<0.05$.

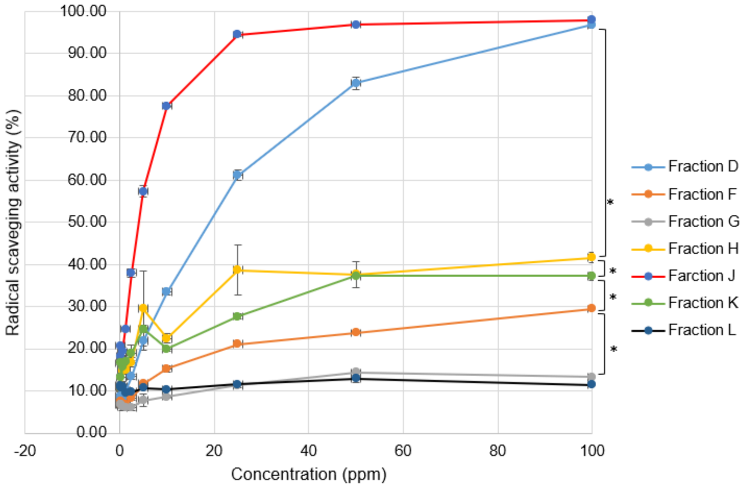

Figure 5. Antioxidant Activity of *Rhizpora mucronata* Leaves Using the ABTS Method. The % inhibition of fractions at 100 ppm was significantly different (*) with $p<0.05$.

Antioxidant activity of fractions

Only fractions D, F, G, H, J, K, and L were tested for antioxidant activity. Meanwhile, the other fractions were not tested because the numbers were insufficient. ABTS and DPPH methods have the same principle: transferring a single electron to a free radical, often called Single Electron Transfer (SET). The SET mechanism is divided into 2, namely electron transfer followed by a proton transfer (SET-PT) and sequential proton loss electron transfer (SPLET). Antioxidants that are more easily ionised and deprotonated will be more reactive in stabilising radicals through the SET-PT mechanism (Figure 3).

Meanwhile, the SPLET mechanism is the release of protons from antioxidant compounds followed by the transfer of anions to radical compounds (Figure 3). In the SET-PT method, antioxidants directly react with free radicals (ROO), then donate electrons to anions (ROO-). The DPPH method mainly reacts via the SPLET mechanism when conducted in organic solvents such as ethanol and methanol. Meanwhile, in distilled water, the ABTS method primarily proceeds through the SPLET mechanism [18]. Therefore, if the sample was dissolved in aquadest, the ABTS method was more appropriate.

Fractions D and J of *R. mucronata* leaves, tested using the DPPH method at a concentration of 50 ppm, showed % DPPH radical inhibition with values of 56.20 ± 2.40% and 93.84 ± 0.34%, respectively (Figure 4). These two fractions have IC_{50} of 28.68 ± 1.58 ppm and 9.59 ± 0.39 ppm, respectively. The % inhibition at the D and J concentrations of 100 ppm was significantly different. Meanwhile, based on the ABTS antioxidant test method, fractions D and J at a concentration of 50 ppm showed % inhibition of ABTS radicals, respectively, of 82.96 ± 1.50 % and 96.86 ± 0.02% (Figure 5). The IC_{50} value of fraction D and J was 15.1 ± 1.00 ppm and 3.16 ± 0.55 ppm, respectively. The antioxidant activity of fractions D and J was classified as very strong [19]. The differences in antioxidant activity of the tested fractions, as determined by various methods, were attributed to variations in the reactivity of the compounds towards DPPH radicals or ABTS radicals. Compounds with a high number of hydroxyl groups correlated with their antioxidant activity, as tested using the DPPH method.

Meanwhile, the number of hydroxyl groups in the compound did not correlate with its antioxidant activity, as determined using the ABTS method. For example, the compounds naringenin and naringin have few hydroxyls. Their antioxidant activity, as measured by the DPPH method, was low, but high when using the ABTS method. In addition, naringenin has lower antioxidant activity than naringenin because naringenin has a sugar residue at position 7 in ring A. Therefore, the steric hindrance of naringenin was high, making it difficult to react with DPPH (18). Therefore, fraction D tested with DPPH had lower antioxidant activity than the ABTS method. So, most of the compounds in fraction D contain compounds with a low number of hydroxyl groups.

Meanwhile, fraction J was estimated to have many hydroxyl groups. So, its antioxidant activity was high when tested using the DPPH method. One example of a compound that has high activity when tested using the DPPH and ABTS methods is proanthocyanidins. This compound has a high number of hydroxyls [20].

Fraction D was a combination of fractions 33-35. This fraction was eluted using a solvent mixture of chloroform and ethyl acetate (3:2), indicating that the compound was predicted to be semipolar. Some examples of semipolar compounds include phenolics, terpenoids, alkaloids, and aglycones. The phenolic compounds in fraction D probably have long carbon chains, so they are attracted to semipolar solvents [21]. Phenolic compounds have a hydroxyl group attached to an aromatic group, which reduces

Captured free radicals through a hydrogen atom donor mechanism [22]. Fraction J was a combination of fractions 62-65, eluted with the solvent ethyl acetate: methanol (2:3). Therefore, the compounds contained in fraction J were polar. Some examples of polar compounds include coumaric acid, caffeic acid, quercetin, chlorogenic acid, and hesperidin. These compounds were classified as flavonoid compounds. These compounds were classified as flavonoid compounds. Quercetin was the major flavonoid detected in the polar extract of *R. mucronata* leaves, as determined using LC-HRMS (10). There were also polar phenolic compounds, such as gallic acid [23]. Flavonoid compounds neutralize free radical compounds through hydrogen donor transfer [24].

CONCLUSION

R. mucronata leaves have relatively strong antioxidant activity. The fractionation process using Gravity Column Chromatography makes separating compounds in a sample easier based on their polarity. Different antioxidant activity testing methods will produce different antioxidant activities as well. Each type of compound has a different reactivity in the antioxidant test method. Most fractions, such as D, F, H, and J, had high antioxidant activity when tested using the ABTS method. Fractions G, K, and L had high antioxidant activity when tested using the DPPH method. The J fraction, as tested using the DPPH and ABTS methods, exhibited the highest activity, with IC_{50} values of 9.59 ± 0.39 ppm and 3.16 ± 0.55 ppm, respectively. The antioxidant activity of fraction J was relatively strong. Therefore, fraction J can be a promising natural antioxidant candidate that warrants further development.

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