Isolation, identification of alkaloid from *Rhizophora mucronata* and the activity of its methanol extract against barnacles

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Abstract. A study was conducted to isolate, identify alkaloid content of *Rhizophora mucronata* and to determine its effectiveness of methanol extract against barnacles. The use of tributyltin (TBT) was effective to reduce biofouling. However, TBT has been prohibited because they are detrimental to non-target organisms and for surrounding environment. *Rhizophora mucronata* is useful as antifouling compounds because of the secondary metabolites. Its active compound will replace TBT as it is safe against the biota and the marine environment. The root's bark, trunk's bark and leaves powder were macerated using methanol. The methanol extract was used for total alkaloid isolation. The separation of alkaloids was done by preparative TLC and analyzed with FTIR and LC-MS. The results showed that the alkaloid metabolite identified was benzamide ($C_{17}H_{14}N_2O_2$). The methanol extract had antifouling activity against barnacles. The highest yield was found in *Rhizophora mucrophora* leaf extracts at about 24.8%. Antifouling activity test against barnacles showed that a mixture of wood paint and *Rhizophora mucronata* leaf, root and bark extracts were not significantly different.

1. Introduction

Submarine objects, especially in coastal waters, are attached by biofouling organisms. Barnacle is a macrofouling that forms a fouling community causing damage to beach buildings and increase fuel consumption, damage to pipes, pumps and vessels can be reversed in bad weather conditions [1,2]. Biofouling or biological fouling is the accumulation of microorganisms, plants, algae or animals on wetted surfaces [3].

Antifouling agent is a substance that prevents or retards fouling or marine underwater growth on plants, rocks or ships' bottoms [3]. Currently fouling prevention is done using antifoulant paint, which active compound is tributyltin (TBT). This compound is harmful to organisms. The copper content in TBT and continuous accumulation of TBT will poison the organisms in the sea, causing impaired immune systems and shells to form changes such as malformation [4,5].

The greatest interest in the use of natural products as antifoulants is based on their potential to function as nontoxic antifoulants. The new diterpene methoxy-ent-8 (14)-primarenely-15-one (1) and three metabolites: ent-8(14)-primarene-15R, 16-diol (2), stigmasterol (3) and β-sitosterol (4) were isolated from the roots of mangrove plant *Ceriops tagal* [6-8]. The objective of this research was to

isolate and identify alkaloids in *Rhizophora mucronata* extract derived from leaves, stems and roots, as well as to analyze the activity of the methanol extract as antifouling agent.

2. Methods

2.1. Extraction and fractionation

Isolation and fractionation of alkaloid metabolites was done from the roots, stems and leaves powder of mangrove. Extraction was carried out by maceration with methanol then concentrated using a rotary evaporator. The methanol extract was added with HCl 1 N, then extracted with ethyl acetate. The acid layer was added with ammonium hydroxide then re-extracted with ethyl acetate. The ethyl acetate layer was concentrated with a rotary evaporator to obtain a total alkaloid extract [9,10].

2.2. Separation, purification, and identification

The total alkaloid was purified by thin layer chromatography (TLC) and eluted with methanol, ethyl acetate, and hexane using silica gel plate 60 GF254. The spots were observed under UV lamp at 366 nm. Further separation was done using preparative TLC to obtain the pure alkaloid. The alkaloid isolate was analyzed and identified by FTIR and LC-MS [11,12].

2.3. Testing of alkaloid metabolite

The alkaloid of roots, barks and leaves of *Rhizophora mucronata* was determined with Dragendorff's test. Two mg of methanol extract was acidified with 2 M HCl and added by 1 mL of Dragendorff's reagent. Formation of orange or orange red precipitate indicated the presence of alkaloids. Wagner's test was done by preparing 2 mg of methanol extract, acidified with 1.5 % v/v of HCl, and added with several drops of Wagner's reagent. A yellow or brown color indicated the presence of alkaloids. Furthermore, the extract was added with HCl and NaOH followed by extraction with ethyl acetate.

2.4. Antifouling activity determination

The antifouling activity test was conducted in the sea waters of Teluk Awur Beach, Jepara, Central Java Indonesia. Temperature, pH, salinity, and brightness were recorded during the experiments for 16 days. Antifouling activity was observed by counting the number of macrofouling barnacle which attached on wooden board media. A 7 x 14 cm^2 area of wooden board was painted using a commercial paint mixed with extract of roots, stems or leaves of mangrove at 500 or 1000 ppm concentration. As a positive control used was wooden board of the same size painted with commercial antifouling, whereas the negative control used was the same commercial-painted wooden board without mixing with neither mangrove extract nor plain wood planks. The wooden boards were weighted and tied to a pivot on a dock and were spaced 50 cm from the sea level at the lowest tide; the distance between the boards and the beach was about 12 m [13-15].

2.5. Data analysis

The data was analyzed descriptively by standard deviation (STDEV) based on the number of attachment of macrofouling biota on wood board media. The standard deviation is a measure of how widely values are dispersed from the average value (the mean). The experiment was repeated three times. The standard deviation was calculated using the "n-1" method with the following formula:

$$\frac{\sqrt{\Sigma(x-\bar{x})2}}{(n-1)}$$

3. Results and discussion

3.1. Extraction and fractionation

Extraction by methanol submergence method was intended to bind the polar compounds. Alkaloids are polar compounds that have to be extracted with polar reagent. Methanol is the most polar reagent after water. The rendement of mangrove extract is shown in Table 1.

Table 1. Rendement of root's bark, stem's bark and leaves extracts of *Rhizophora mucronata*.

Sample	Sample weight (gr)	Extract weight (gr)	Rendement (%)
Root's bark	401.6	69.7	17.4
Stem's bark	500.1	89.2	17.9
Leaves	387.2	96.0	24.8

Rendement is a comparison between the weights of the ingredients used with the total weight of the material. The value of rendement is used to determine the effectiveness of bioactive metabolite material. The higher the yield value indicated the higher the value of the methanol extract produced. The highest rendement value was obtained from the leaves extract of *Rhizophora mucronata* because the leaves had smoother texture compared with stem's and root's barks. The tiny size of the leaves extract is easy to withdraw the compound. It correlates with the highest activity against macrobiota [7,8,11,16,17].

3.2. Alkaloid test of mangrove extract

The methanol extracts of roots, stems and leaves of *Rhizophora mucronata* showed positive alkaloid reactions proven by white deposits when Mayer and Dragendorff reagents were added (Table 2).

Table 2. Testing of Alkaloid metabolite of *Rhizophora mucronata.*

Alkaloid metabolite	Root's bark		Stem's bark		Leaves	
	Simplicia	Methanol extract	Simplicia	Methanol extract	Simplicia	Methanol extract
Sample + (Mayer + Dragendorff) reagent	+	+	+	+	+	+

+ = contains metabolite alkaloids

3.3. Separation and purification

The separation showed three spots of TLC. The ratio of eluent was 21 methanol: 6.5 ethyl acetate: 5.5 n-hexane. The results showed that the topmost spot of a reddish-purple shine indicated an alkaloid compound. The Rf spot was 0.9 (Figure 1).

Figure 1. TLC of alkaloid isolate.

Separation of alkaloid compounds with preparative TLC was performed two yellow and one dark green bands. On the yellow bands are scraped subjected for purity by TLC again using the same developer.

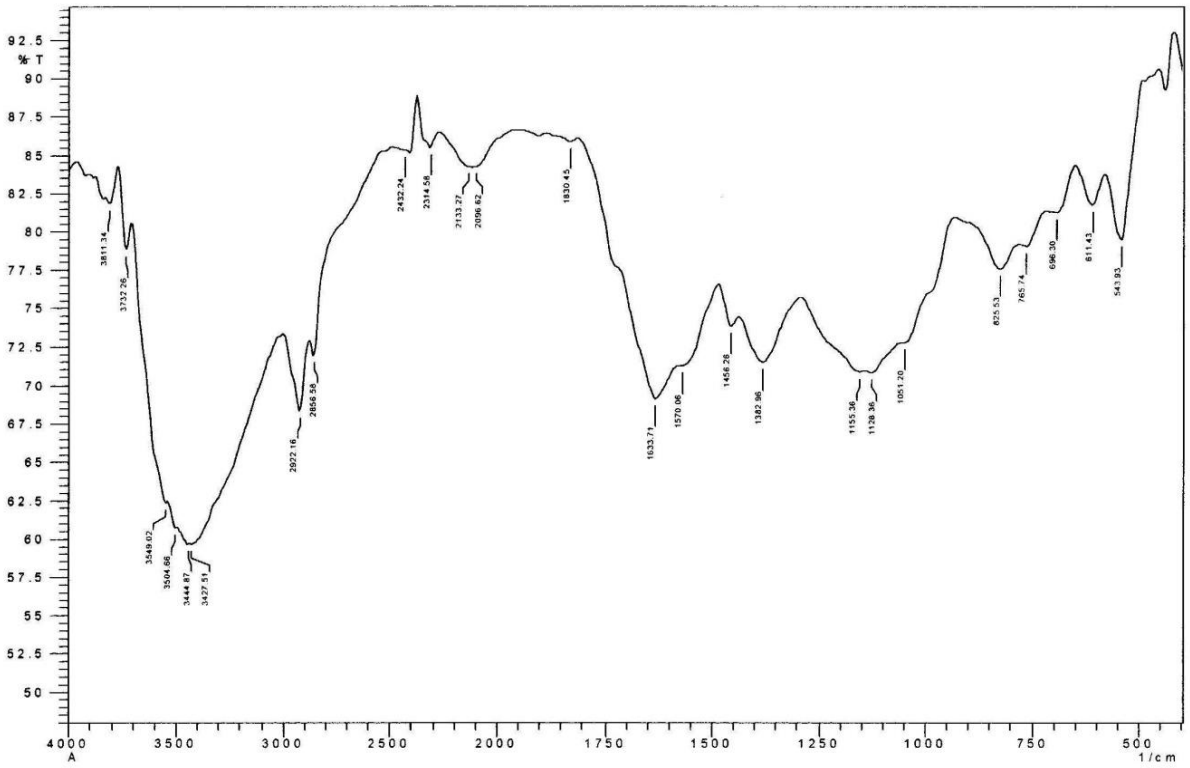

Figure 2. FTIR spectogram of alkaloids of mangrove leaves extract.

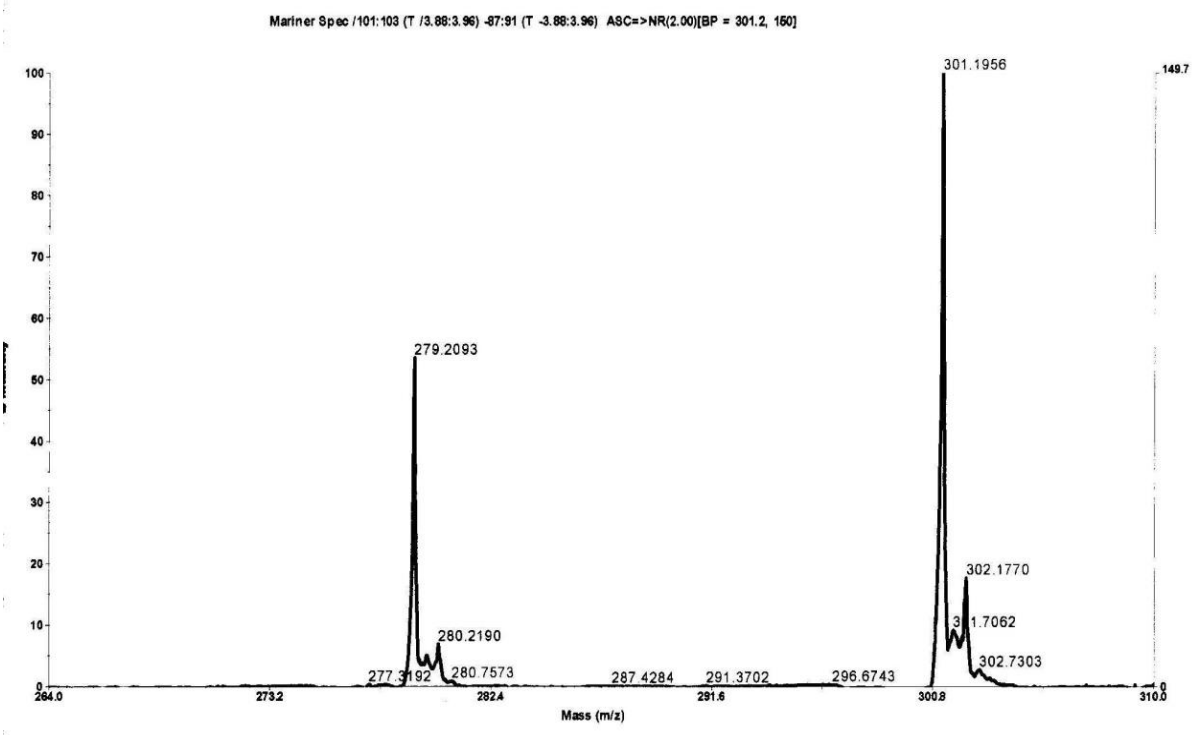

Figure 3. Spectogram of LC-MS.

3.4. Identification of compounds

Based on FTIR spectroscopy analysis, the alleged alkaloid compounds contained in the mangrove leaves were N-H, NH, C-O, C-N, CH and C-C groups. FTIR spectogram of alkaloids isolated from the leaves extract of *Rhizophora mucronata* is presented in Figure 2.

The results of FTIR spectroscopy showed the functional groups of the isolate samples. Figure 2 shows the vibration peaks with an absorption at the wavelength of 3444.87 cm^{-1}, which was the absorption of the vibration stretching of the NH group. It was also supported by the absorption at the wavelength of 1570.06 cm^{-1}, which was the vibration buckling of the NH group. The wavelength of 1051.20 cm^{-1} was a buckling vibration of C-O. The presence of vibration in the wave number 1382.96 cm^{-1} was the absorption of C-N, while at 2432.24 cm^{-1} wavelength was the bending vibration of CH and at 1633.71 cm^{-1} was the vibration of C-C group.

The isolate was analyzed using Liquid Chromatography-Mass Spectroscopy (LC-MS) to determine the molecular weight of the alkaloids. Spectogram of the LC-MS is shown in Figure 3.

The molecular weight of the isolated compound indicated by the LC-MS spectrometer was 278 g/mol. It is shown in Figure 3 in the presence of m/z 279.2093 [M + H]+ and m/z 301.1956 [M+Na]+. Based on these results, the isolate of alkaloids from mangrove leaf contained a benzamide compound, *N*-1-isoquinolinyl-3methoxy- with the chemical formula $C_{17}H_{14}N_2O_2$. The chemical structure of the benzamide alkaloid ($C_{17}H_{14}N_2O_2$) is shown in Figure 4.

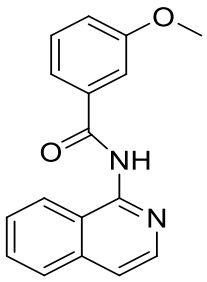

Figure 4. Alkaloid benzamide.

Six new 4-phenyl-3,4-dihydroquinolone derivatives along with the related of inquinolone 4 were isolated and identified from the cultures of an endophytic fungus obtained from the fresh leaves of the marine mangrove plant *Rhizophora stylose* [18]. Many bioactive metabolites including anthracenediones, xyloketals, sesquiter penads, chromones, lactones, coumarin and isocoumarin derivatives, xanthones and peroxides have been isolated from various mangrove-derived fungi in the South China Sea [19]. The methanol extract of *Rhizophora mucronata* contained alkaloid, flavonoid, and tannin [16], seven compounds were identified in the fraction of ethyl acetate of *Rhizophora mucronata* stem bark namely quinone, steroid, alkaloid and aromatic group with the highest secondary metabolite content was alkaloid group (74.8%) [20]. 2-isocyanato-2-hexanamine that was successfully isolated from mangrove plant was active against bacterial disease in shrimp [21]. The compounds contained in the skin of the mangrove stem *Sonneratia alba* is a phenolic group with lactone rings [22], the main secondary metabolites in mangrove plant in the form of alkaloid class of polar compounds. The white crystalline powder form characteristics had a melting point of 172°C with molecular weight of 232, and the molecular formula was $C_{12}H_{12}N_2O_3$ [23].

3.5. Antifouling activity

The data obtained was in the form of the number of attachment of macrofouling biota on wood board media. The data was analyzed descriptively with three replicates based on related sources in the form of journals, books, and thesis and presented in tabular form. The antifouling activity of *Rhizophora mucronata* extract at 500 and 1000 ppm was not significantly different with the amount of attachment between 247-315 barnacles (Table 3).

Table 3. Barnacles attachment.

Sample	Concentration of extract (500 ppm)			Concentration of extract (1000 ppm)		
Wood+paint+ extract (*Rhizophora mucronata*)	Root skin	Bark stem	Leaf	Root skin	Bark stem	Leaf
Barnacles attachment (individual)	280±35.68 a	315±15.95 a	253±42.74 a	282±23.67 a	286±47.51 a	247±66.16 a

Table 4. Barnacles attachment (control).

Control	Standard	Barnacles attachment (individual)
Positive (+)	Wood + commercial antifouling paint	4 ± 3.0a
Negative (-)	Wood + paint without antifouling	395 ± 60.68c
	Wood without painting	190 ± 98.38b

The results indicated that concentration of the extracts did not affect barnacles attachment. All values were not statistically different (Table 3).

3.6. The effectiveness of antifouling extract of Rhizophora mucronata

The bacteria initiate the attachment and form a primary thin film on the surface of submerged objects. The formed thin layer allows benthic diatoms, algae spores and larvae of various types of aquatic animals to attach and grow on the surface of the object. At the end, cauced bryozoa, hydrozoa, ciripedia, tunikata, algae and other biota to be a group of biota that developed later after forming layer film by bacteria. *Rhizophora mucronata* extract inhibited the growth of biofilm bacteria that reduced macrofouling attachment [17].

The number of barnacles attached to the wood media was due to the wood media surface was uneven and had many gaps so that the barnacles were attached to the cracks on the wooden surface. Selection of wood species for the test board was based on the type of wood commonly used in the manufacture of boats e.g. teak, meranti, and camphor. Meranti wood was selected because the price was more economical and easily obtained [24].

In addition to media and habitats, the attachment rate of barnacles was influenced by brightness, temperature, and current velocity [25]. Barnacles grow well at depth and brightness between 20-130 cm [26]. Water temperature during observation was 27°C on average; the temperature was quite good for the growth of barnacles because barnacles like waters with temperature of 15-35°C [26]. Soluble salinity or salinity in the study area was 36 ppt, the salinity was particularly suitable for growing barnacles because barnacles are able to live in estuary waters and have a salinity tolerance between 15-41 ppt [26]. The degree of acidity or pH of the waters at the time of observation was very good that reached 8, barnacles can live optimally at a pH between 6-9 [27].

Based on the number of wooden barnacles attached to wood with wood paint mixture, *Rhizophora mucronata* 1000 ppm leaf extract was a more effective anti fouling than root bark extract. However, the mixture of wood paint and *Rhizophora mucronata* 1000 ppm leaf extract would be much less effective when compared with commercial antifouling paint because the concentration of the active ingredients or secondary metabolites in commercial paints was very high compared with the active ingredients in mangrove leaf extract, due to the copper content.

4. Conclusion

Mangrove leaf extract (*Rhizophora mucronata*) contained betanidine alkaloid compounds ($C_{17}H_{14}N_2O_2$) and showed an antifouling activity against barnacles.

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