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ANTIBACTERIAL ACTIVITY OF CHLOROFORM EXTRACT AND RHIZOPHORA SPP LEAVES METHANOL EXTRACT ON MOUTH BACTERIA

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Abstract

The purpose of this study was to obtain the phytochemical profile of the methanol and chloroform extracts of the leaves of *Rhizophora apiculata* and to find the antibacterial activity of the methanol and chloroform extracts against human oral bacteria. This research was carried out in 3 stages, namely *Rhizopora spp*. Leaf Sampling, Extraction and Analysis of Secondary Metabolite Content and Antibacterial Activity Test using the agar diffusion method. Based on the results of the phytochemical screening examination of the leaf extract of *Rhizopora spp*., the results showed that it was rich in chemical compounds of alkaloids, flavonoids, tannins, and saponins in both methanol extract and chloroform extract. The results of the antibacterial activity test of methanol extract obtained that the smallest concentration in bacterial isolate bacteria 2 was 30 mg/ml, while the smallest concentration in bacterial isolate 1 was 50 mg/ml. The results of the antibacterial activity test of the chloroform extract obtained that the smallest concentration in the bacterial isolate bacteria 2 was 20 mg/ml while the smallest concentration in the bacterial isolate 1 was 30 mg/ml. Both methanol extract and chloroform extract had better activity on gram-positive bacteria.

Keywords: Antibacterial, Chloroform Extract, Methanol Extract, *Rhizophora Spp*

INTRODUCTION

Mangrove forest is a typical forest type found along the coast of river mouths and is influenced by tides. This forest is also known as coastal forest, tidal forest, brackish forest or mangrove forest. Mangroves grow on sheltered beaches or flat beaches. Usually in places where there are no river mouths, mangrove forests are quite thin, but in places with large river mouths and deltas where the river flows contain a lot of mud and sand, mangroves usually grow well and expand. Until now, the utilization of mangrove forests has only been limited to direct use, namely as fuel, building materials, fishing gear, food, beverages, household appliances, agriculture (fertilizer), paper products and as a fishing ground for marine organisms. The specialty of mangroves is that they can live in high salinity, have muddy, soft soil and contain little humus. This of course can be related to many problems which include biological, physical and economic aspects of waters. However, here, a study will be conducted on the role of mangroves as

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a source of pathogenic antibacterial bioactive ingredients.

The Mangrove Indonesia Center reported that in 2006, Indonesia is one of the countries that has the largest mangrove forest in the world. The area of mangrove forests in Indonesia reaches 25% of the total 18 million hectares of mangroves in the world. This year, the area of mangrove forests in Indonesia has shrunk to 1.9 million hectares. However, the diversity of plant species in Indonesia's mangrove forests is quite high, around 89 species, consisting of 35 tree species, 5 herb species, 9 shrub species, 9 liana species, 29 epiphytic species and 2 parasitic species.

Indonesia's mangrove forests with the diversity of mangrove species in it are an advantage as well as a challenge in relation to the use of mangrove forests as a source of marine natural products. It can be an advantage because the high biodiversity of mangrove forests will provide a high diversity of secondary metabolites (biochemical diversity). This is because each type of mangrove plant has a different biosynthetic process so that it produces different compounds both in terms of physical and chemical properties. This is a challenge due to the lack of information on the results of exploration activities of Indonesian mangroves as a source of marine natural products. Whereas traditionally, people have been using some extracts of mangrove plants for medicine. In general, the existence of mangrove forests is underestimated because it is not considered to provide a significant economic value for the income of an area. Economic considerations take precedence over ecological considerations of mangrove forests so that mangrove forests are always converted into places that are considered more economically productive. For this reason, in order to maintain the existence of mangrove forests, reasons must also be considered regarding other economic aspects that can be obtained from mangrove forests.

Utilization of mangrove forests as a source of marine natural products is a strategy to add value to the existence of mangrove forests so that they are no longer underestimated. The output in the form of lead compounds that can be used in the field of medicine with high commercial value is a strong supporting reason for maintaining the existence of mangrove forests. Mangrove forests are no longer considered only ecologically, but also economically profitable.

Mangrove plants contain compounds such as alkaloids, flavonoids, phenols, terpenoids, steroids and saponins. This group of compounds is an ingredient in modern medicines. Several mangrove plants of the genus Rhizophoraceae have been tested for their toxicity to insect larvae, including *Brugueira cylindrica, Ceriops decandra, Rhizophora apiculata, Rhizophora lamarckii,* and *Rhizophora mucronata*. Several studies on the use of *Rhizpora spp*, namely petroleum ether extract from the *Rhizophora apiculata* plant, were the most effective against *Culex quinquefasciatus* mosquito larvae with an LC50 value of 25.7 mg/L. Research has also been carried out on plants of the species *Rhizophora apiculata* on the potential of various plants that produce toxins for insecticides, including 5 long chain aliphatic alcohols, 11 long chain aliphatic saturated carboxylic acids, and 3 steroids, namely 2,6-dimethoxy-p-benzoquinone, syringaldehyde, and sitosteryl 3-glucoside from the wood center of *Rhizophora apiculata*. The purpose of this study was to obtain the phytochemical profile of the methanol and chloroform extracts of the leaves of *Rhizopora spp* and to find the antibacterial activity of the methanol and chloroform extracts against

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human oral bacteria. The expected result of this research is the discovery of bioactive substances obtained from mangrove plants of the genus *Rhizopora spp* which can be used as antimicrobial agents, especially for oral bacteria which is the main cause of oral disease. From the results of the literature search until now there has been no patented bioactive substance derived from mangroves for both pharmaceuticals and food, so it is an opportunity to patent bioactive substances derived from mangroves as bioactive for antibiotics or antimicrobials

MATERIALS AND METHODS

Place and time of research

This research was conducted at the Biology Laboratory of the Faculty of Mathematics and Natural Sciences, UNIMA, sub-laboratory of Biochemistry and Microbiology. The research was conducted for 5 (five) months.

Tools and materials

The tools used in this study include glassware, blender (Philips), electric oven (Mammert), rough balance (O'Haus), electric balance (Vibra AJ), desiccator, mortar, stamper, porcelain dish, Rotary evaporator (Buchi 461), incubator (Mammert), oven (Gallenkam), autoclave (Fison), capillary, loop needle, sprite lamp, refrigerator (Karl Kolb), tweezers, rubber pump, hot plate, spatula, water bath, micro pipette, caliper, filter paper, aluminum foil, metal printer, spatula, microscope (Olympus), object glass, cover glass, drying cabinet, test tube rack, Erlenmeyer 50 mL, test tube, shaker, Laminar Air Flow (LAF), incubator, petri dish, bunsen burner, loop needle, 100 L-1000 L micropipette and 20 L-200 L micropipette. Puncher, caliper, petri dish, 100 L-1000 L micropipette and 20 L-200 L micropipette.

The plant materials used are Rhizophora spp. leaves, all materials used are of pro-analytical quality unless otherwise stated; distilled water, methanol 96% (distilled), methanol pa, dilute hydrochloric acid chloroform, concentrated hydrochloric acid, chloroform, iron (III) chloride, lead (II) acetate, anhydrous acetic acid, sodium chloride, barium chloride, potassium iodide , Mercury (II) chloride, iodine, -naphthol, nitric acid, bismuth nitrate, isopropanol, magnesium powder, mmethanol, n-hexane, concentrated sulfuric acid, amyl alcohol, chloral hydrate, bacterial culture

Research procedure

1 Leaf Sampling of *Rhizopora spp*

The sample that will be used as an extract comes from the mangrove plant of the genus *Rhizopora spp* which was obtained from the Kombi Coast of Minahasa Regency. Plant samples in the form of leaves were brought to the laboratory for further treatment.

2. Extraction and Analysis of Secondary Metabolites

a. Extraction

Extraction of mangrove leaves was carried out with 2 types of solvents. Samples of Mangrove Plant Leaves were washed with running water and washed with detergent then rinsed until clean and drained. Then the extract was made into wet extract simplicia and dry extract simplicia using methanol

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and n-hexane. For wet simplicia mashed using a mortar, and for dry simplicia dried and mashed using a blender.

In the extraction process, a ratio of 1: 5 (simplicia: solvent) was used, i.e. 50 grams of simplicia was extracted each with 250 milliliters of methanol and chloroform. Extraction is done by maceration method. Maceration was carried out in a closed vessel for 2x24 hours and occasionally shaken. Then it is filtered using filter paper to produce filtrate, which is then evaporated in a rotary evaporator at a temperature of 35-37oC (48-50 rpm). So that a crude extract will be produced from the leaves of the Mangrove Plant which will be used to be tested in the next stage.

b. Phytochemical Analysis (Harbourne method, 1996)

Alkaloid test. A total of 0.1 grams of extract was added 3 mL of chloroform and 3 drops of ammonia. The chloroform fraction was separated and acidified with 10 drops of 2 M H2SO4. The acid fraction was taken, then Meyer and Wagner's reagent was added. The presence of alkaloids was indicated by the formation of a white precipitate by the Meyer reaction and a brown precipitate by the Wegner reaction. As a comparison, use the blood footprint.

Saponin and Flavonoid Test. A total of 1 gram of extract was put in a beaker then added 100 ml of hot water and boiled for 5 minutes, after that it was filtered and the filtrate was used for testing. The saponin test was carried out by shaking 10 ml of the filtrate in a closed test tube for 10 seconds and then left for 10 minutes. The presence of saponins is indicated by the formation of stable foam. Another 10 ml of the filtrate was added with 0.5 grams of magnesium powder, 2 ml of carbohydrate alcohol (a mixture of 37% HCL and 95% methanol in a ratio of 1:1) and 20 ml of amyl alcohol and then shaken vigorously. The formation of red, yellow and orange colors on the amyl alcohol layer indicates the presence of flavonoids.

Tannin Test. A total of 0.1 grams of extract was added to 2 mL of water and then boiled for several minutes. Then filtered and the filtrate was added with 1 drop of 1% FeCl3 (w/v). Dark blue or greenish black color indicates the presence of tannins.

Triterpenoid and Steroid Test. A total of 0.1 grams of extract added 2 mL of 30% methanol and then heated and filtered. The filtrate was evaporated and then 1:1 ether was added. The ether layer was added with Lieberman Burchard's reaction (3 drops of acetic anhydride and 1 drop of concentrated H2SO4). Red and green colors indicate the presence of triterpenoids and green colors indicate the presence of steroids.

3. Antibacterial activity test

a. Tool Sterilization

The tools used in this anti-bacterial activity test are sterilized before use. Glass utensils were sterilized in the oven at 170oC for 2 hours. The media was sterilized in autocave at 121oC for 15 minutes. Ose needle and tweezers with Bunsen lamp (Lay, 1994)

b. Preparation of NA media and test bacteria inoculation

NA media is made by dissolving 20 grams of NA media powder in aquadest, to a volume of 1 liter. The media solution was heated until the NA medium powder was completely dissolved, and put in 5 mL

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test tubes each. Then sterilized using an autoclave for 15 minutes at a pressure of 1 atm, a temperature of 121°C. The test tube is then tilted so that the NA medium in it freezes in an oblique shape. The source of bacterial isolates from the human mouth grown on NA media with the scratch technique.

The bacteria in NA slanted media were then incubated for 12-18 hours in an incubator at 37ºC. Colonies formed, indicating bacterial growth. The stock of bacteria can be used immediately for testing or when not in use, can be stored in the refrigerator. Bacteria are regenerated every two weeks in the same way.

c. Activity test against oral pathogenic bacteria

This test is carried out using the agar diffusion method which depends on the diffusion of the antibiotic compound into the agar. The antibiotic compound was impregnated on a paper disc with a diameter of 6 mm. This disc paper is placed on the surface of the media that has been inoculated with pathogenic bacteria to be tested. After being incubated for 24 hours at a temperature of 35-37 ^{0}C, the inhibition area around the paper disc was observed. The inhibition area formed is a clear area around the disc paper, which indicates that the pathogenic bacteria or microorganisms tested have been inhibited by antimicrobial compounds that diffuse into the agar from the disc paper (Naerobi et. al. 2005).

This extraction was taken at a concentration of 10% w/v, for soaking paper discs with a diameter of 6 mm. The positive activity response is indicated by the presence of a clear zone around the medium that has been inoculated with bacteria, where this clear area is an inhibition zone formed by the extract and the chemical compounds contained in the extract. The effectiveness of antibiotics will be seen with the distance of the highest inhibition zone at small concentrations.

Research data analysis

The research data were analyzed descriptively qualitatively.

RESULTS AND DISCUSSION

a. Extraction

Leaf samples of *Rhizopora spp* were obtained from the combi coast and brought to the laboratory in a fresh state. Selected leaves that are not deformed (torn or there are symptoms of fungal/bacterial infection). The leaves of Rhizopora were then washed with detergent in running water repeatedly until they were free of detergent. The leaves of *Rhizopora spp* were finely chopped and then blended until smooth for extraction by maceration method. In the extraction process, a ratio of 1: 5 (simplicia: solvent) was used, ie each 200 grams of simplicia was extracted with 1000 milliliters of mmethanol and chloroform. Each solvent was made in 3 replications so that the total weight of the blended leaf was 1200 gr. Maceration was carried out in a 1000 ml Erlenmeyer covered with aluminum foil for 48 hours and occasionally shaken to mix the solvent and solute. Then filtered using Whatman filter paper no. 1 to produce the filtrate. The filtrate is evaporated with a rotary evaporator at a temperature of 35-37ºC (48-50 rpm), resulting in a crude extract that will be used for testing in the next stage. The average weight of the crude extract of the largest *Rhizopora spp*. extract obtained from the chloroform extract was 3.67 g, while

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the mmethanol extract the average weight of the yield obtained was 3.18 g (Table 1).

Table 1 *Rhizopora spp* . leaf extract yield

No	Extract Type	Yield weight (gr)
1	Methanol Extract$_1$	2,53
	Methanol Extract$_2$	3,48
	Methanol Extract$_3$	3,53
2	Cloroform2 Extract$_1$	3,63
	Cloroform2 Extract$_2$	3,43
	Cloroform2 Extract$_3$	3,98

Secondary Metabolite Content Analysis

Based on the results of the phytochemical screening examination of the leaf extract of *Rhizopora spp*., the results showed that it was rich in chemical compounds of alkaloids, flavonoids, tannins, and saponins. Tannins and flavonoids are a class of polyphenolic compounds that have been known to have antibacterial properties

Table 2. Contents of Groups of Secondary Metabolic Compounds Rhizopora Leaf Crude Extract

Type of Crude Extract (Crude Extract)	Phytochemical Group					
	Alkaloids	Flavonoids	Saponins	Tannins	Steroids	Triterpenoids
Methanol extract	+++	+++	+++	+++	-	-
Extract chlorforom	+++	+++	++	++	+	+

The +++ symbol indicates a very positive compound content, ++ indicates a positive compound content, + indicates a positive compound content but tends to be negative while, - indicates a negative compound content in the tested crude extract. Tests using the parareax according to the Harborne Method (1996) where indicators of the presence of each compound are shown qualitatively through color intensity, presence of precipitate, presence of stable foam, etc.

Table 3 the results of the antibacterial activity of the methanol extract of leaves of *Rhisopora spp*

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No	Concentration Crude extract solution (mg/ml)	Diameter of bacterial growth inhibition (mm)	
		Isolate 1	Isolate 2
1	500	20,10	21,5
2	400	18,2	18,2
3	300	17,25	17,3
4	200	15	17
5	100	12	15.5
6	90	12	14
7	80	10	13,2
8	70	9,5	12,2
9	60	8.4	10,0
10	50	8.2	9,5
11	40	-	9,5
12	30	-	7,1
13	20	-	-
14	10	-	-
15	5	-	-
16	Blanko	-	-

Information :

* measurement is carried out 3 times; - no clear zone formed

Table 3 results of the antibacterial activity of *Rhisopora spp* . leaf chlorforom extract

No	Concentration Crude extract solution (mg/ml)	Diameter of bacterial growth inhibition (mm)	
		Isolat 1	Isolate 2
1	500	22,10	23,50
2	400	20,30	20,20
3	300	20,25	20,20
4	200	20,00	19.20
5	100	20,00	18.20
6	90	18,25	18.00
7	80	18,20	16,20
8	70	15,20	14,20
9	60	15,20	12,00
10	50	10,20	12,00
11	40	-	10,50
12	30	-	8,10
13	20	-	7.20
14	10	-	-
15	5	-	-
16	Blanko	-	-

Information :

* measurement is carried out 3 times; - no clear zone formed

Many factors affect the yield weight obtained from the filtrate extracted by evaporation using Rotapavor. However, the nature of the solvent (including boiling point), pressure, the ratio of solvent and solute are the main factors. The average weight of the highest extract yield was obtained in chlorforom solvent because this solvent has a lower boiling point than methanol. The low boiling point causes the solvent evaporation process using a rotapavor to be better. Evaporation by rotapavor is strongly influenced by temperature and pressure. The maximum temperature that can be used to obtain good secondary

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metabolites does not exceed 55^{0}C. Thus the boiling point of a solvent greatly affects the evaporation process. Chlorforom is able to produce a larger yield which is also influenced by the nature of this solvent, which is semi-polar. This property causes polar and non-polar compounds to be attracted compared to polar methanol so that they are only able to attract polar compounds as well.

The group of phytochemical compounds obtained where alkaloids, flavonoids, saponins and tannins were very positive in line with the results of research from Eryanti et. al. (1999) who also found the content of alkaloids, flavonoids, phenols, saponins, terpenoids and steroids in Avicenia and Rhizopora. Only in this study the presence of terpenoids was still negative in methanol solvents except for chloroform solvents. Several factors that influence this result include the simplicia used fresh leaves (basic simplicia) which is different from other studies using dried leaf simplicia as well as stem bark and roots. However, many groups of flavonoid, saponin and tannin compounds have been reported to have strong antibacterial activity.

The method used in this research is to determine the diameter of the inhibition zone, the diameter of the inhibition zone which increases with increasing concentration. This proves that an increase in the concentration of Rhizopora leaf extract has a positive correlation with an increase in the diameter of the growth inhibition zone of the 2 isolates of oral bacteria used.

From the data above, it shows that the chlorforom leaf extract shows the ability to inhibit the growth of bacterial isolates which is stronger than the methanol extract. While the blank did not show antibacterial activity against the 2 bacterial isolates used.

The results of the antibacterial activity test of the mmethanol extract obtained the smallest concentration in the bacterial isolate of bacteria 2 of 30 mg/ml while the smallest concentration on the isolate of bacteria 1 was 50 mg/ml. Thus the mmethanol extract of Rhizopora leaves was stronger in inhibiting the growth of bacterial isolate 1 compared to bacterial isolate 2. Bacteria isolate 1 was a gram negative bacterium while isolate 2 was a gram positive bacterium. From the results of the study, it was seen that the chloroform extract of mangrove leaves gave greater inhibitory power to gram-positive bacteria than gram-negative bacteria.

The results of the antibacterial activity test of the chloroform extract obtained that the smallest concentration in the bacterial isolate bacteria 2 was 20 mg/ml while the smallest concentration in the bacterial isolate 1 was 30 mg/ml. Thus, the chloroform extract of Rhizopora leaves was stronger in inhibiting the growth of bacterial isolate 1 compared to bacterial isolate 2. Bacteria isolate 1 was gram-negative, while bacterial isolate 2 was gram-positive. From the results of the study, it was seen that the chloroform extract of mangrove leaves gave greater inhibitory power to gram-positive bacteria than gram-negative bacteria.

Both methanol extract and chloroform extract had better activity on gram-positive bacteria. This is due to differences in the composition and structure of the cell walls. The cell walls of gram-negative bacteria contain less amount of peptidoglycan than the cell walls of gram-positive bacteria. It is the cell wall that causes these two groups of bacteria to respond differently to treatments such as staining

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(Pelczar, 1986). The limit of the inhibition area is considered effective if it has an inhibitory diameter of approximately 14 mm to 16 mm (Depkes RI, 1995).

Antibacterial activity can be caused by the presence of chemical compounds, namely alkaloids, flavonoids, saponins and tannins obtained from phytochemical screening results with a very positive intensity. The results of the antibacterial activity test showed that the leaf extract of *Rhizopora spp*. had an inhibitory effect on 2 isolates of oral bacteria.

CONCLUSION

1. Based on the results of the phytochemical screening examination of the leaf extract of *Rhizopora spp*., the results showed that it was rich in chemical compounds of alkaloids, flavonoids, tannins, and saponins in both methanol and chloroform extracts.
2. The results of the antibacterial activity test of the mmetanol extract obtained the smallest concentration in the bacterial isolate bacteria 2 of 30 mg/ml while the smallest concentration on the bacterial isolate 1 was 50 mg/ml.
3. The results of the antibacterial activity test of the chloroform extract obtained the smallest concentration in the bacterial isolate bacteria 2 of 20 mg/ml while the smallest concentration on the bacterial isolate 1 was 30 mg/ml.
4. Both methanol extract and chlorforom extract had better activity on gram-positive bacteria.

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