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Antimicrobial activities of stembark and wood extracts from *Nauclea subdita* against pathogenic microorganisms

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ABSTRACT

Aims: The aim of the study was to investigate the antimicrobial activity of *Nauclea subdita* (Korth) Steud against six pathogenic microorganisms.
Methodology and results: Young and matured trees of *N. subdita* were cut and separated into bark and wood parts, respectively, prior to extraction process. Phytochemical screening tests, antimicrobial activity, minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values were determined. Preliminary screening for phytochemical components showed that both young and matured tree had similar constituents. Extracts from matured tree showed more potency in terms of the zones of inhibition sizes than the young tree. Extract of *N. subdita* was more potent to both marine bacteria, *Vibrio parahaemoliticus* and *V. alginolyticus*, while *Candida albican* and *Aspergillus niger* were resistant to it. The sensitivity test showed that 500 µg/mL is the optimum concentration for extract of bottom sapwood of mature tree to act as bactericidal.
Conclusion, significance and impact study: The results from this study suggest that *N. subdita* bark and wood extracts may serve as potential source of antimicrobial agents for future development in medicine applications.

Keywords: Nauclea subdita, antimicrobial activity, minimal inhibitory concentration, minimal bactericidal concentration

INTRODUCTION

In 2012, infectious disease caused around 6.1 million (10.9%) death globally (WHO, 2014). There are several bacteria as well as fungi that responsible for a variety of infections in humans. Take for example the organisms that used in this study, *Bacillus subtilis* is rod-shaped Gram-positive bacterium which generally regarded as safe and only causes disease in severely immunocompromised patients (Barbe *et al*., 2009). Both *Vibrio parahaemolyticus* and *V. alginolyticus* are pathogenic Gram-negative marine bacteria that can be detected in seafood. These bacteria are believed to cause gastroenteritis in human through undercooked seafood (Rojas *et al*., 2011). Another Gram-negative bacterium *Burkholderia cepacia* was named after onion in Latin by Burkholder (1950) as they was identified as the organism that caused soft rot of onions. *B. cepacia* was said to be a potential pathogen in immunocompromised patients, particularly individuals with cystic fibrosis, a serious disease of the glands which usually affects children and can cause breathing difficulty (Govan *et al*., 1996). On the other hand, *Candida albicans* is a dimorphic fungus that localized in mucosal surfaces of the oral cavities and the digestive tract and causes candidiasis in humans (Molero *et al*., 1998). Differed from *C. albicans*, *Aspergillus niger* is generally regarded as a safe fungus which had been exploited in industry to produce citric acid (Singh Dhillon *et al*., 2011).

The information on medicinal plants as novel compounds to fight the infectious diseases is therefore useful for further research and development. Many medicinal materials has been destructively obtained from plants such as roots, bark, sapwood, heartwood and other plant parts in the forest (Kaviarasan *et al*., 2007). Antibacterial and antifungal properties of the extractives from plant parts have been investigated by several researchers. El-Mahmood *et al*. (2008) studied the antibacterial properties of the extracts of leaves, barks and roots of *Nauclea latifolia* and *Daniella oliveri* and found that it successfully inhibited the growth of tested pathogenic organisms. Salem *et al*. (2014) found that the bark extract of *Delonix regia* effectively restricted the growth of pathogenic fungus. In the recent study by Fatin

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et al. (2012), crude extracts from heartwood of *N. subdita* showed promising free radical scavenging effect. *N. subdita*, or locally known as Kedamba, is one of the two *Nauclea* species that existed in Malaysia apart from *N. officinalis* (Liew *et al*., 2014). It is a timber species that generally grows in lowland to hill forests, also in swampy areas and habitually along streams and rivers and normally planted to stabilize slopes and river banks (Lim *et al*., 2004). Young leaves of *N. subdita* are edible and used by traditional practitioners, particularly the ethnic in Semporna, Sabah, Malaysia as remedies for stomach ache, blood pressure, diabetes and skin problems.

However, there is little or no information on the antimicrobial properties of the extractives from other parts of *N. subdita*. Therefore, the objective of this research was to evaluate the potential of stem bark and wood extracts of *N. subdita* as antimicrobial agents against some pathogenic microorganism strains.

MATERIALS AND METHODS

Sample preparation

Fresh *N. subdita* plant materials from young and matured trees, respectively, were collected from forest sources in Pitas, Sabah in March 2010. The plants were identified by expert at the Forest Research Institute Malaysia (FRIM), Kepong. Barks were then separated from the woods of the young and matured trees of *N. subdita*, respectively. Separately, both of the barks and woods were chopped into smaller pieces, ground into particles or powder form and then dried in the room temperature for two weeks. The dry powdered wood materials were weighed and blended using a laboratory blender (Waring®, Z272213 Aldrich, Sigma-Aldrich, U.S.) prior to extraction process in accordance to the procedures reported by Fatin *et al*. (2012). Table 1 shows the samples used in this study.

Table 1: Samples classification according to maturity groups and different parts of tree.

Samples	Maturity	Parts
P1b	Young	Bark
P1T	Young	Top
P1B1	Young	Bottom sapwood
P1B2	Young	Bottom heartwood
P2b	Matured	Bark
P2T	Matured	Top
P2B1	Matured	Bottom sapwood
P2B2	Matured	Bottom heartwood

Plant material and extraction

One hundred gram of ground samples in the form of dried fine powder were placed in a cellulose thimble and extracted in a soxhlet extractors (Z556203 Aldrich, Sigma-Aldrich, U.S.) using polar solvent (methanol) in accordance to the method suggested by Sengul *et al*. (2009). The air-dried woods and barks in powder form was macerated in 300 mL of methanol for 72 h and filtered. The filtered extracts were concentrated under vacuum at a temperature of 60 °C using a rotary evaporator (Rotavapor® R-100, Buchi Labortechnik AG, Switzerland). The solid residues obtained after rotary evaporation were dried, kept in glass vials and stored in a refrigerator. The yield of the obtained powder were recorded and stored at 4 °C until further use. Portions were taken from the refrigerator to be used for each of the experiment below. Phytochemical screening was performed by using standard procedures. A qualitative phytochemical test to detect the presence of phenolic compounds (ferric chloride and gelatin tests), flavanoids, tannins, phytosterols (Libermann-Burchard's test), terpenoids (Salkowski test), steroids, alkaloids, cardiac glycoside (Keller-Killiani test) and saponins were carried out according to the standard procedures described by Kumar *et al*. (2007) and Abu Bakar *et al*. (2009).

Antimicrobial assay

Nauclea subdita extracts, woods and barks, were evaluated as antimicrobial agents against the growth of Gram-positive bacteria *B. subtilis* (ATCC 6633), Gram-negative bacteria *V. parahaemoliticus* (ATCC 33844), *B. cepacia* (ATCC 53795) and *V. alginolyticus* (ATCC 17749) as well as fungus *C. albican* (ATCC 90028) and *A. niger* (ATCC 16404). These isolates were obtained from Microbiology Laboratory, Kulliyyah of Science, International Islamic University Malaysia. Streptomycin was used as positive control for bacteria and Nystatin was used as positive control for fungi. Methanol was used as negative control for both types of microorganisms. The bacteria were maintained on nutrient broth (NB) at 37 °C and fungus was maintained on Sabouraud broth at 28 °C. Bacteria were incubated for 24 h and fungi for 48 h in their respective broths.

Media preparation

Mueller-Hinton agar was used as agar media for bacteria while Sabouraud agar was used as media for fungi (Reich *et al*., 2013). Thirty eight gram of Mueller-Hinton powder and 65 g of Sabouraud powder were added into 1 L of distilled water, respectively. The above ingredients were stirred and autoclaved at 121 °C, 20 psi for 30 min. The media were let to cool to about 55 °C, then poured into sterile plastic Petri dishes and allowed to solidify overnight. The Petri dishes were stored upside down in the refrigerator at 4 °C.

Antibiotic stock

One gram of Streptomycin powder was mixed with 50 mL of 70% ethanol and poured into a bottle and vortex. The solution was filtered using 0.22 µm Milipore membrane filter (Merck, German). The solution was stored at 20 °C until further use. Nystatin dehydrate stock solution with concentration of 20 mg/mL was prepared by dissolving 20 mg of Nystatin powder into 1 mL of 100% dimethyl

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sulfoxide (DMSO). The solution was filtered and stored at –20 °C until further use.

Disc diffusion method

Two hundred milligram of each crude extract were dissolved in 0.5 mL of methanol and then 20 µL of extract were applied to each disc to give concentration of 10 mg/disc. The discs were dried in the laminar flow for 30 min. Each microorganism with volume of 100 µL were spread on Mueller-Hinton and Sabouraud agar and allowed to stand for several minutes until the inoculates were completely absorbed by the medium before the discs were placed on it (Valgas *et al.*, 2007). Both Streptomycin and Nystatin were used as positive control while methanol was used as negative control. The test was conducted in duplicate. Bacterial plate was incubated for 24 h at 37 °C while fungi plate was incubated for 48 h under room temperature. The inhibition zones were measured with ruler after 24 h of incubation.

Determination of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

The crude extracts that showed 12 mm of inhibition zones were proceed for broth microdilution method in 96 wells microtiter plate. Prior to the MIC test, each crude extract was diluted in methanol and the MICs were then determined using two folds serial broth microdilution at concentrations ranging from 1 µg/mL to 2000 µg/mL. Twenty microlitre of tested extracts were added to 180 µL of sterile nutrient broth into microtiter plates before 100 µL of the bacterial suspension prepared were added. One hundred microlitres of the mixture from the first well were pipetted into the second well and so on. Each extract was assayed in duplicate. The bacterial suspensions were used as positive control and extracts in broth were used as negative control. The MIC values were taken as the lowest concentration of the extracts in the wells of the microtiter plate that showed no turbidity after 24 h of incubation at 37 °C. The turbidity of the wells in the microtiter plate was interpreted as visible growth of the microorganisms. The minimum bactericidal concentration (MBC) was determined by subculture of the well showing no apparent growth in a sterile agar plate. The least concentration showing no visible growth on agar subculture was taken as MBC value (Yilmaz, 2012).

RESULTS AND DISCUSSION

Phytochemical screening and extraction yield analysis

The results of phytochemical screening indicated the presence of natural compounds in the extracts. Screening tests of the methanolic crude extracts of *N. subdita* revealed the presence of phenolic compound, flavanoids, terpenoids, alkaloids, phytosterols and saponins in the bark and wood of both young and matured tree. However, only phytosterols were detected in hexane extracts. Therefore, based on results of the screening tests, only the methanolic crude extracts of *N. subdita* were used for the further study of antimicrobial properties.

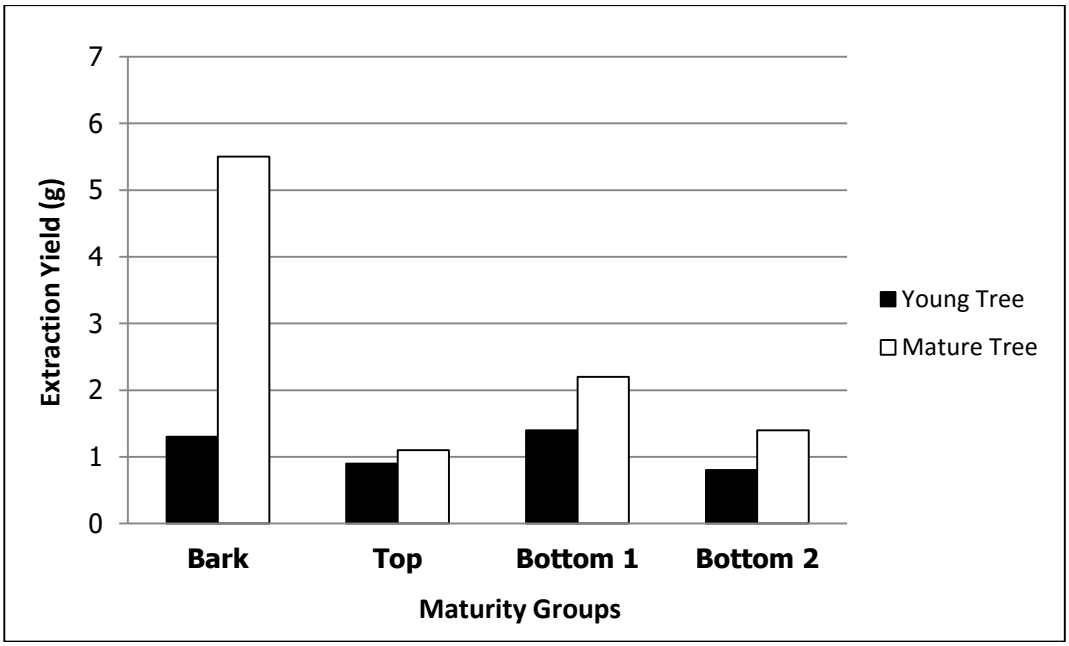

Figure 1: The value of extraction yield from different portions of both maturities tree groups. bottom 1, bottom sapwood; bottom 2, bottom heartwood.

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Figure 1 shows the extraction yields in gram from different portions of both young and matured tree. Based on the results exhibited in Figure 1, bark from matured tree obtained the highest extraction yield, which was 5.5 g, compared to the other extracted parts of the tree, while the extraction yield from heartwood of young tree showed the lowest value. All the other samples shows that the extraction yields ranged from 0.8 g to 2.2 g. Hexane extraction has yielded negligible weight of residue which indicated that the low non-polar content of the sample, and the relatively high presence of polar compounds. Further study was continued using methanol extracts only and hexane extracts were eliminated. The percentage yields from methanol extractions from different parts of both young and matured *N. subdita* by using a soxhlet extractor showed that matured tree generally had three times higher content of crude extracts compared to young tree. For both young and matured trees, the bark contained higher crude extracts than the sapwood and heartwood. Overall, the quantity of crude extracts also increased from top to bottom and from sapwood to heartwood. Kawamura *et al.* (2010) reported that although the concentration of secondary metabolites in part of trees is not uniform, methanol extracts from bark or heartwood of many of the wood species had higher yields than those from sapwood.

Antimicrobial activity

All of the sample extracts were further tested in antimicrobial study using disc diffusion method. Tables 2 and 3 show the results of antimicrobial properties of the samples by determining the diameters of the inhibition zones of the tested microbes. All of the negative controls showed no inhibition zones. From Tables 2 and 3, one can see that *V. parahaemoliticus* showed larger zone of inhibition compared to the other tested strains with *V. alginolyticus* showed second larger inhibition zones. This can be proved by the diameter of the inhibition zones which were more than 12 mm. It can be concluded that *V. parahaemoliticus* showed higher activity and highly susceptible to the extracts. Diameters of the inhibition zones for the other samples ranged from 7 mm to 12 mm. It can be categorized as intermediate susceptibility. Differed from the other samples, the growth of *C. albican* and *A. niger* was inhibited by the extracts with almost no inhibition zones was detected. This can be concluded that they are resistant toward the extracts. Figure 2 exhibits the action of *C. albican* in the extracts of both young [Figure 2(A)] and matured trees [Figure 2(B)]. All the tested samples showed no inhibition zones were existed. The similar results were found against *A. niger* as shown in Figure 2(C) and Figure 2(D).

Table 2: Inhibition zones (mm) of extracted parts from young trees against tested microorganisms.

Microbes	Samples	Replicate 1 (mm)	Replicate 2 (mm)	Average (mm)	Positive control (mm)	Negative control (mm)
B. subtilis	P1b	9	10	9.5	23	NA
	P1T	9	10	9.5	23	NA
	P1B1	9	10	9.5	23	NA
	P1B2	8	8	8	23	NA
V. parahaemoliticus	P1b	18	8	13	11	NA
	P1T	14	18	16	11	NA
	P1B1	14	12	13	11	NA
	P1B2	15	15	15	11	NA
V. alginolyticus	P1b	9	9	9	7	NA
	P1T	9	9	9	7	NA
	P1B1	15	15	15	7	NA
	P1B2	NA	15	7.5	7	NA
B. cepacia	P1b	12	9	10.5	11	NA
	P1T	9	9	9	11	NA
	P1B1	9	9	9	11	NA
	P1B2	12	11	11.5	11	NA
C. albican	P1b	7	NA	7	14	NA
	P1T	NA	NA	NA	14	NA
	P1B1	NA	NA	NA	14	NA
	P1B2	7	NA	7	14	NA
A. niger	P1b	NA	NA	NA	7	NA
	P1T	NA	NA	NA	7	NA
	P1B1	NA	NA	NA	7	NA
	P1B2	NA	NA	NA	7	NA

P1, Young Tree; P2, Mature Tree; b, Bark; T, Top; B1, Bottom sapwood; B2, Bottom heartwood

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Table 3: Inhibition zones (mm) of extracted parts from matured trees against tested microorganisms.

Microbes	Samples (Mature Trees)	Replicate 1 (mm)	Replicate 2 (mm)	Average (mm)	Positive control (mm)	Negative control (mm)
B. subtilis	P2b	7	7	7	23	NA
	P2T	7	7	7	23	NA
	P2B1	9	9	9	23	NA
	P2B2	10	11	10.5	23	NA
V. parahaemoliticus	P2b	10	11	10.5	11	NA
	P2T	19	18	18.5	11	NA
	P2B1	19	18	18.5	11	NA
	P2B2	16	7	11.5	11	NA
V. alginolyticus	P2b	7	7	7	7	NA
	P2T	17	7	12	7	NA
	P2B1	12	7	9.5	7	NA
	P2B2	15	15	15	7	NA
B. cepacia	P2b	NA	11	11	11	NA
	P2T	11	11	11	11	NA
	P2B1	NA	NA	NA	11	NA
	P2B2	11	7	9	11	NA
C. albican	P2b	NA	NA	NA	14	NA
	P2T	NA	NA	NA	14	NA
	P2B1	NA	NA	NA	14	NA
	P2B2	NA	NA	NA	14	NA
A. niger	P2b	NA	NA	NA	7	NA
	P2T	NA	NA	NA	7	NA
	P2B1	NA	NA	NA	7	NA
	P2B2	NA	NA	NA	7	NA

P1, Young Tree; P2, Mature Tree; b, Bark; T, Top; B1, Bottom sapwood; B2, Bottom heartwood

In this study, the extracts from matured tree showed more potency in terms of the zones of inhibition sizes than the young tree. The antimicrobial potency may be due to the presence of some active compounds like alkaloids, phenols, cardiac glycosides and saponins. Saponin also had been reported by Abdallah (2011) as a phytochemical compound of antimicrobial activity. Higher extraction yields and the presence of higher amount of natural compounds from matured tree might contribute to better antimicrobial ability against pathogens. Liew *et al*. (2012) reported that genus *Nauclea* are rich in alkaloid compound that may potent in antimicrobial activities. The results of Table 3 demonstrate that the both *V. parahaemoliticus* and *V. alginolyticus* showed higher activity in methanolic extract of *N. subdita*. According to results shown in Table 3, methanolic extract of *N. subdita* is more potent to marine microorganism while *C. albican* and *A. niger* were resistant to it.

Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

Minimum inhibitory concentration (MIC) values were measured using modified microdilution broth method described by Sengul *et al*. (2009). The MIC was defined as the minimum concentration of test sample that inhibits the visible growth of microorganisms after overnight incubation. MBC was defined as the lowest concentration that allows no visible growth on the medium. The experiments were performed in triplicate. All extracted samples against *V. parahaemoliticus* and P1B1, P2T and P2B2 extracts against *V. alginolyticus* were further tested by MIC method because they showed greater inhibition zones by these bacteria than the other tested strains. Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC) were done directly after the MIC was observed. Swabs from the observed MIC wells were cultured on their respective agar, incubated for 24 h for bacteria and 48 h for fungi. The cultured plates that exhibited no growth were considered as bactericidal or fungicidal.

Table 4 shows the MIC value of *V. parahaemoliticus* and *V. alginolyticus*. P2B1 showed the lowest MIC value against *V. parahaemoliticus*, which is 250 µg/mL while the MIC values for extract P1B1 and P2B2 against *V. alginolyticus* was 1000 µg/mL. The MIC values of all the other extracts against both *V. parahaemoliticus* and *V. alginolyticus* were 500 µg/mL. Table 5 showed that at the concentration of 500 µg/mL of P2B1, no visible growth of *V. parahaemoliticus* after 24 h of incubation was observed. Meanwhile, growth of *V. parahaemoliticus* and *V. alginolyticus* was detected for other tested samples. It can be concluded that 500 µg/mL is the optimum concentration for P2B1 as a bactericidal.

The MIC and MBC tests showed that the extracts were more potent than the standard antibiotics Streptomycin used as positive control in the study against *V. parahaemoliticus* and *V. alginolyticus*. These extracts

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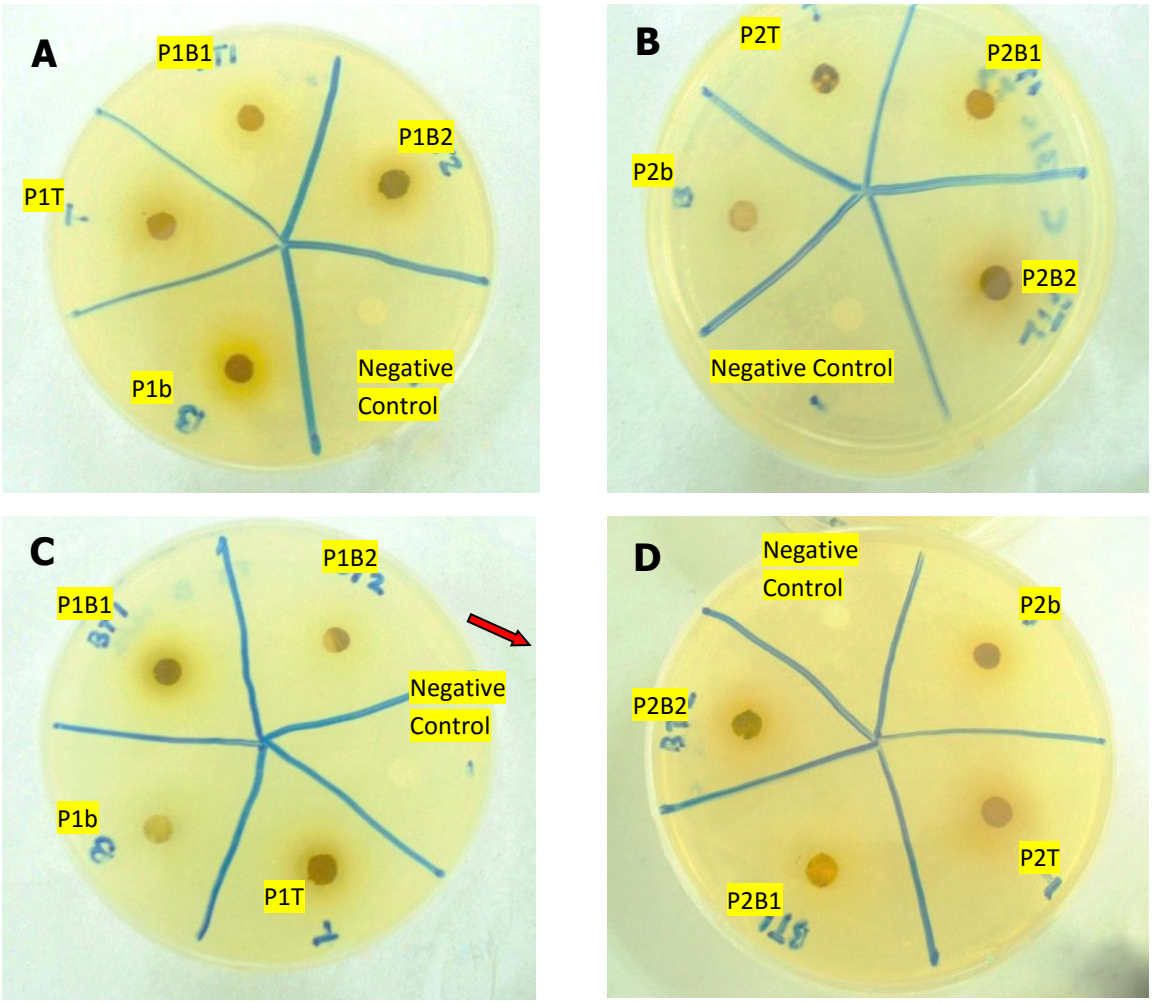

Figure 2: Photographs of agar plates showing inhibition zones in antifungal test for methanol extracts. A, young trees; B, matured trees against *C. albicans*; C, young tree; D, matured tree against *A. niger*.

Table 4: MIC value of extracted parts against *V. parahaemoliticus* and *V. alginolyticus*.

Samples / Microbes	Young Trees (µg/mL)				Mature Trees (µg/mL)			
	P1b	P1T	P1B1	P1B2	P2b	P2T	P2B1	P2B2
V. parahaemoliticus	500	500	500	500	500	500	250	500
V. alginolyticus	-	-	1000	-	-	500	-	1000

P1, Young Tree; P2, Mature Tree; b, Bark; T, Top; B1, Bottom sapwood; B2, Bottom heartwood

Table 5: MBC value of extracted parts against *V. parahaemoliticus* and *V. alginolyticus*.

Sample extracts	*V. parahaemoliticus*	*V. alginolyticus*
P1b	Grew in all concentration tested	Grew in all concentration tested
P1T	Grew in all concentration tested	Grew in all concentration tested
P1B1	Grew in all concentration tested	Grew in all concentration tested
P1B2	Grew in all concentration tested	Grew in all concentration tested
P2b	Grew in all concentration tested	Grew in all concentration tested
P2T	Grew in all concentration tested	Grew in all concentration tested
P2B1	Bactericidal. Killed at 500 µg/mL	Grew in all concentration tested
P2B2	Grew in all concentration tested	Grew in all concentration tested

P1, Young Tree; P2, Mature Tree; b, Bark; T, Top; B1, Bottom sapwood; B2, Bottom heartwood

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showed high activities without any fractionation or purification. As been reported by previous study, heartwood showed higher durability than sapwood because of the higher amount of extracts (Kawamura *et al.*, 2010). However, the results in this study showed exceptionally high activities in sapwood. The difference of antimicrobial activity of the extracts against tested strains might influence by the difference of species and parts used and thus their reactions toward the extracts are expected to be different from each species.

CONCLUSION

From the studied antimicrobial activity, disc diffusion assays have revealed that the crude extracts from top and bottom sapwood, bottom heartwood and bark of young and matured trees have shown considerable inhibition zones against the two bacteria strains *V. parahaemoliticus* and *V. alginolyticus,* which were even greater than the inhibition zones shown by the positive standard (Streptomycin). However, the MIC and MBC results showed that 250 µg/mL is considered as the MIC for the bottom sapwood of matured tree extract against *V. parahaemoliticus*. The sensitivity test results showed that the extracts were more potent than the standard antibiotics Streptomycin used as positive control in the study against *V. parahaemoliticus* and *V. alginolyticus*. These plant extracts could be used as antimicrobial to treat water borne disease cause by *V. parahaemoliticus* which might cause gastroenteritis in human. Generally, the reduced efficacy of the extracts, relative to the standard antibiotics, used in the study may be due to the fact that they are still crude and require further purification. The extracts from young and matured trees were resisted by two fungi strains *C. albican* and *A. niger* in this study. The current studies revealed that *N. subdita* tree is an important plant in term of providing extracts of phytochemical compounds that have antioxidant and antimicrobial ability. Although the tree is low in market value for the timber industry, but it might have high value in the nutraceutical and pharmaceutical industries. In order to encourage the use of *N. subdita* in those industries, proper plantations and commercialization of the tree byproduct should be set up with special incentives for development.

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