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Antibacterial Activity of Alkaloid Extracts from *Ochrosia oppositifolia* (Aktiviti Antibakteria Ekstrak Alkaloid daripada *Ochrosia oppositifolia*)

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ABSTRACT

Ochrosia oppositifolia can be found along the coastline and is locally known as ‘Mempelam pasih’ or ‘Mangga laut’. In this study, the alkaloids from the leaves, stem-bark and roots were extracted and tested for their antibacterial activity. So far, no previous studies have been carried out to determine the antibacterial potential of the extracts. Each extract was tested using disc-diffusion and minimum inhibitory concentration (MIC) methods. The highest inhibitory diameters shown by 10 mg/mL extracts of the leaves and stem-bark against *Staphylococcus aureus* and the roots against MRSA were 10.0 ± 2.8 and 10.5 ± 2.1 and 14.0 ± 2.8 mm. On the other hand, the MIC values exhibited by the leaf and stem-bark extracts against *Bacillus subtilis*, *Salmonella thyphimurium* and *Serratia marcescens* and the root extract against *Vibrio fluvialis* were 3.75 and 0.94 and 0.12 mg/mL. This study broadens the bioactivity potential of the plant and the information obtained can be utilized for pharmacological purposes.

Keywords: Alkaloid extract; antibacterial activity; disc diffusion; minimum inhibitory concentration; *Ochrosia oppositifolia*

ABSTRAK

Ochrosia oppositifolia adalah tumbuhan pesisir pantai dan dikenali orang tempatan sebagai ‘Mempelam pasih’ atau ‘Mangga laut’. Dalam kajian ini, alkaloid daripada daun, kulit dan akar diekstrak dan diuji aktiviti antibakteria mereka. Tiada kajian terdahulu yang dijalankan setakat ini bagi menentukan potensi aktiviti ekstrak tersebut. Setiap ekstrak diuji menggunakan kaedah resapan-cakera dan kepekatan perencatan minimum (MIC). Diameter perencatan tertinggi ditunjukkan oleh ekstrak 10 mg/mL bagi daun dan kulit terhadap *Staphylococcus aureus* dan akar terhadap SARM adalah 10.0 ± 2.8 dan 10.5 ± 2.1 dan 14.0 ± 2.8 mm. Selain itu, nilai MIC yang diperlihatkan oleh ekstrak daun dan kulit terhadap *Bacillus subtilis*, *Salmonella thyphimurium* dan *Serratia marcescens* serta ekstrak akar terhadap *Vibrio fluvialis* adalah 3.75 dan 0.94 dan 0.12 mg/mL. Kajian ini memperluaskan potensi bioaktiviti tumbuhan tersebut dan maklumat yang diperoleh dapat digunakan bagi tujuan farmakologi.

Kata kunci: Aktiviti antibakteria; ekstrak alkaloid; kepekatan perencatan minimum; *Ochrosia oppositifolia*; resapan-cakera

INTRODUCTION

Ochrosia oppositifolia or also known as *Calpicarpum oppositifolium* is of the family Apocynaceae. It is a small tree with stout branches, leaves fleshy with whorls of 7.5–10 cm and obovate in shape 10–25 cm long. Their flowers are white and the fruits or drupes are ovoid in shape 5–7 cm long (Ridley 1894). The timber is moderately hard and grained (Burkill 1966). It was first found in areas ranging from the Mascarene Island to Polynesia. Observed along the coastline in Singapore a century ago, it still occurs in the group of islands in Langkawi, Malaysia (Gamble 1922).

The roots were taken as an antidote for eating poisonous fishes and crustaceans (Gamble 1922). In Vietnam, the local people credited the bark, wood and leaf with febrifugal and stomachic properties (Van Valkenberg & Bunyapraphatsara 2001). Meanwhile in the Seychelles, a bitter bark decoction is taken to purify the blood, as an appetizer, purgative and carminative and in high doses as an abortifacient. A leaf decoction is also used to

wash the abdomen of women after childbirth (Gurib & Brendler 2004). Furthermore, local people in Acheh used the decoction of the stem-bark to treat malaria (Susiarti 2006). *Ochrosia oppositifolia* also has similar uses in Southeast Asian countries (Van & Hendrian 2001).

In previous study, two compounds of neisosposinine and reserpine were isolated from the bark and leaves of *Ochrosia oppositifolia*. While, another indole alkaloids of isoreserpiline and alkaloid D were also isolated from the bark and leaves of *O. oppositifolia*, respectively. Two ferulic acid esters were another compounds found in the bark of *O. oppositifolia* (Nasab 2013; 2012). All extracts and pure compounds were tested for antiplasmodial activity in which the hexane bark extract showed highest activity with IC_{50} 0.0505 μ g/mL and the alkaloid D was the most potent compound with IC_{50} 0.0123 μ mol/L (Nasab 2012). Other related study reported that screening of the appropriate extracts of *Ochrosia maculata* elicited both oncolytic and neurosedative activities. The former was found to be associated with 9-methoxyellipticine

while the latter with reserpine. 9-Methoxyellipticine possesses a moderate degree of potency as an antitumor agent expressed by its activity against several of the solid mouse neoplasm (Svobodo et al. 1968).

The above findings showed that the local had used parts of the tree for treatments of various health conditions. The above studies also showed that the tree of *Ochrosia oppositifolia* and the related genus contains profound alkaloids with various promising potency of bioactivity such as antiplasmodial and antitumor. However, there is still many other bioactivity potential of *O. oppositifolia* that not yet discovered. Therefore, in this study, the antibacterial potential of the alkaloid extracts of *O. oppositifolia* against four Gram-positive and five Gram-negative bacteria were evaluated as bacterial resistance against existing drugs nowadays are steadily increasing. Thus, there are needs to overcome this antibiotic resistance of pathogenic species. Furthermore, as reported earlier, *O. oppositifolia* plant extract chemically consists of indole alkaloids which are nitrogen bearing structures known to be pharmacologically active.

MATERIALS AND METHODS

PLANT MATERIALS COLLECTION AND IDENTIFICATION

The studied plant parts of *Ochrosia oppositifolia* were leaves, stem-bark and roots. They were collected from Pulau Pangkor, Perak, Malaysia on July 2014. The plant species were identified by Mr. Sani Miran, a botanist at Universiti Kebangsaan Malaysia. For further reference, a plant voucher specimen of WYA517 was deposited at the Universiti Kebangsaan Malaysia Herbarium (UKMB).

SCREENING OF ALKALOIDS

The alkaloids for different parts of the plant were detected by using Mayer precipitating reagent (K_2HgI_4) described by Culvenor and Fitzgerald (1963) and Dragendoff's reagent.

EXTRACTION OF ALKALOIDS

The leaves, stem-bark and roots of *Ochrosia oppositifolia* were air dried, the latter was cut into small pieces and each of the three plant materials was grounded into powder by using a grinder. In an empty amber solvent glass bottle, 300 g of each powder was soaked in 1.5 L methanol for three days with regular shaking. The methanol solution was filtered using cotton wool and the filtrate evaporated under reduced pressure using Buchi rotary evaporator at less than 40°C until about one tenth of its original volume remained (Neli et al. 2011). The concentrated solutions were used for the extraction of alkaloids following Hadi and Bremner (2001) with minor modification to yield 0.20 (0.07), 0.70 (0.23) and 0.20 g (0.07%) of the leaf, stem-bark and root alkaloid extracts. They were stored below 4°C prior to use.

PREPARATION OF EXTRACTS FOR ANTIBACTERIAL ASSAY

Stock solutions of the test extracts were prepared from 10 mg/mL for disc-diffusion method (in methanol) and 30 mg/mL for minimum inhibitory concentration method (in DMSO). For disc-diffusion method, blank 6 mm diameter discs were prepared from the cut out of filter paper Whatman No.2 and sterilized using autoclave machine at 120-124°C for 1-2 h before being dipped into 10 mg/mL of each of the test extract solutions and allowed to air-dry in a plastic Petri-dish at room temperature. The air-dried discs were prepared before use.

BACTERIAL STRAINS

Bacterial strains of four Gram-positive of *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 11774, *B. thuringiensis* ATCC 10792, methicillin resistant *S. aureus* (MRSA) and bacterial strains of five Gram-negative of *Escherichia coli* ATCC 10536, *Salmonella thyphimurium* ATCC 51812, *Serratia marcescens* ATCC 13880, *Vibrio cholera* and *V. fluvialis* were obtained from the Microbiology Laboratory culture collection, School of Biosciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia.

PREPARATION OF CULTURE MEDIA FOR ANTIBACTERIAL ASSAY USING MUELLER-HINTON AGAR (MHA) AND MUELLER-HINTON BROTH (MHB)

To prepare MHA, 800 mL distilled water was added to a Schott bottle (1 L) containing 19 g of Mueller-Hinton agar powder. Meanwhile, to prepare MHB, 200 mL distilled water was added to a Schott bottle (500 mL) containing 4.4 g of Mueller-Hinton broth powder. As for minimum inhibitory concentration method, 200 mL of Tween 80 solution (0.5% v/v) was used instead of distilled water and added with 2.6 g of Mueller-Hinton broth powder to prepare the MHB solution. The mixtures were then shaken thoroughly to dissolve the powder. Then, 10 mL of each of the two MHB solutions were poured into 9 universal bottles (20 mL) and 5 mL of the same solutions were then poured into 9 universal bottles (10 mL). After that, the MHA and 36 bottles of MHB solutions were sterilized for 1-2 h at 120-124°C using the autoclave machine. The sterilized MHB solutions were kept at 4°C until use and the MHA solution was used to prepare agar plates.

PREPARATION OF AGAR PLATES

The laminar hood was first sterilized with 70% ethanol. Then all the empty disposable commercial plastic Petri-dishes (diameter 9 cm) were assembled in the laminar hood and sterilized using UV light for 15-30 min. Later, the hot sterilized MHA solution was poured into each of the dishes enough to cover their surfaces; they were cooled under UV light for 15-30 min to form agar. Finally, the plates were concealed with their covers and stored in the cool room to prevent contamination until use.

PREPARATION OF BACTERIAL INOCULUMS

The sterilized 9 universal bottles with 5 mL of each of the two MHB solutions were seeded with bacteria from the pure culture stocks using a sterilized wire-loop and incubated at 37°C for 24 h to obtain the cultures. After that, 300 µL of each culture were taken and put into the 10 mL sterilized MHB solution prepared earlier in the 20 mL universal bottle. The turbidity of culture was measured and standardized against the McFarland 0.5 (optical density of 0.13 at 625 nm).

DISC-DIFFUSION ASSAY

The disc-diffusion assay according to CLSI (2009) and Scorzoni et al. (2007) was carried out to determine antibacterial activity of the alkaloid extracts by measuring the inhibition diameters. The bacteria suspensions in MHB solution (distilled water) were loaded on a sterile cotton swabs and streaked over the dried surface of MHA plates for inoculation. The sterile filter paper discs previously impregnated with each of the extracts (10 mg/mL) were then placed on the inoculated agar. The plates were incubated at 37°C for 24 h. The antibacterial activity was determined by measuring the diameter of inhibition zone in millimeter including the disc diameter against the bacteria. All tests were carried out in triplicate. Streptomycin (10 µg), vancomycin (30 µg), penicillin (30 µg), kanamycin (30 µg) and tetracycline (30 µg) were used as positive controls. A disc which was impregnated with methanol and air-dried was used as negative control.

MINIMUM INHIBITORY CONCENTRATION

MIC was used to determine the lowest concentration of the assayed antibacterial agent that, under defined test conditions, inhibits the visible growth of the bacteria (Irith et al. 2008). MIC was evaluated on bacterial strain that showed sensitivity to the extracts in the disc-diffusion assay according to Irith et al. (2008) and CLSI (2009). MIC was carried out using broth dilution assay with two-fold serial micro-dilution in 96-well microplate. A 100 µL of 30 mg/mL DMSO solution of each of the test alkaloid extracts was loaded into the first well and serially two-fold diluted to obtain the extract concentrations down to 0.12 mg/mL. A 50 µL of the MHB solution (Tween 80) of bacterial inoculums was then dispensed using micropipette into each of the above loaded well. Each microplate was only used for one bacterium. All tests were carried out in triplicate. For positive controls, chloramphenicol of two-fold dilution from 128 down to 0.25 µg/mL and vancomycin of two-fold dilution from 32 down to 0.06 µg/mL were used. The preparation of these antibiotic stock solutions and their ranges for MIC determination was done according to suggestions from literature (Jennifer 2006). Meanwhile, the last three wells of the line were used as negative control by filling 50 µL of the above bacteria inoculums and 50 µL DMSO. As for the growth control, the well was filled with 100 µL of the blank MHB solution and 50 µL of 30 mg/

mL DMSO extract solution. The microplates were covered with aluminum foil and incubated at 37°C for 16-24 h in an incubator.

DETERMINING BACTERIAL GROWTH USING MTT ASSAY

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a tetrazolium dye (greenish yellow) which can be reduced by the live bacteria into an insoluble purple formazan. This reagent therefore can be used to differentiate between the live and the dead bacteria (Stevens & Olsen 1993). MTT was prepared by dissolving in a phosphate-buffered saline (PBS) solution at 0.2 mg/mL. In this study, the MTT assay was incorporated into the MIC method to visually differentiate between the live (the colour of mixture turns purple) and the dead bacteria (the colour of mixture remains). After 16-24 h incubation in the MIC method, 50 µL of the MTT reagent were added into each well. The colour development was observed and recorded after 2 h incubation (Figures 1-4).

RESULTS AND DISCUSSION

It is well known that alkaloids have many pharmacological properties such as antitumor, cytotoxic, antiprotozoa, antimicrobial and antiparasitic properties (Neli et al. 2011). Some other studies also found that *Neisosperma oppositifolia* (Kartini et al. 2010), *Ochrosia maculata* (Svobodo et al. 1968) and *Ochrosia sandwicensis* (Jordan & Werner 1965) have profound alkaloids content. All these studies showed that the alkaloids presence give promising bioactivity such as antitumor and vasorelaxant effect.

In this study, the alkaloid extracts of the leaves, stem-bark and root of *Ochrosia oppositifolia* showed significant results of the disc-diffusion assay against Gram-positive bacteria. For instance, the leaf, stem-bark and root extracts gave the highest inhibition diameter zones against Gram-positive bacteria of *Staphylococcus aureus* for both leaf and stem-bark extracts (10.0±2.8, 10.5±2.1 mm) and MRSA for root extract (14.0±1.8 mm). Otherwise, there was no inhibition observed against Gram-negative bacteria of *Vibrio cholera* for root and stem-bark alkaloid extracts. Besides that, there were also no inhibition observed against one Gram-positive bacterium of *Bacillus thuringiensis* and one Gram-negative bacterium of *Vibrio fluvialis* for both leaf and stem-bark extracts (Table 1). These results were in agreement with the studies conducted by Damintoti et al. (2005), Jonathan et al. (2000), Masikaa and Afolayan (2002) and Tonia and Johannes (1997) which indicated that plant extracts inhibited mostly the Gram-positive bacteria compared to the Gram-negative bacteria.

It was reported that Gram-positive bacteria have a thick peptidoglycan layer with the absence of impenetrable cell wall compared to Gram-negative bacteria which have a thin peptidoglycan layer with the presence of impenetrable cell wall. Thus, Gram-positive bacteria do not have an effective barrier against the lipophilic solutes whereas the Gram-negative bacteria have an outer phospholipidic

TABLE 1. Inhibition diameters of antibacterial activity for *Ochrosia oppositifolia* leaf, stem-bark and root alkaloid extracts at 10 mg/mL

Bacteria	Inhibition diameter (mm)			Positive control*
	Leaf	Stem-Bark	Root	
MRSA	7.5±2.1	7.0±1.4	14.0±1.8	16.7±0.1
<i>Staphylococcus aureus</i>	10.0±2.8	10.5±2.1	11.5±1.2	14.3±1.2
<i>Bacillus subtilis</i>	8.5±3.5	9.0±0.8	9.5±2.1	12.7±3.2
<i>Bacillus thuringiensis</i>	NI	NI	7.0±0.6	16.7±6.7
<i>Staphylococcus typhimurium</i>	8.5±4.0	8.5±2.1	7.5±0.7	19.0±1.7
<i>Serratia marcescens</i>	7.5±0.7	8.5±0.7	10.5±0.7	22.7±0.6
<i>Escherichia coli</i>	7.5±2.1	8.0±1.4	7.0±1.4	23.7±0.6
<i>Vibrio fluvialis</i>	NI	NI	11.0±0.5	25.0±0
<i>Vibrio cholera</i>	7.0±1.4	NI	NI	10.0±0

NI - No inhibition; *Antibiotic streptomycin (10 µg) for all bacteria except *Bacillus subtilis* (penicillin, 30 µg) and *Vibrio cholera* (kanamycin, 30 µg)

membrane that enables the cell wall to be impermeable to lipophilic solutes, while the porines constitute a selective barrier against hydrophilic solutes with an exclusion limit of about 600 Daltons (Nikaido & Vaara 1985; Scherrer & Gerhandt 1971). Many observations have confirmed these findings, but some findings have also showed that both Gram-positive and Gram-negative bacteria can be inhibited by the plant extracts (Suhanya et al. 2009). The study showed that methanolic and alkaloid extracts of *Mitragyra speciosa* leaves inhibit the growth of the Gram-positive (*Bacillus subtilis*) and Gram-negative bacteria (*Staphylococcus typhimurium*). This finding is also in agreement with the study conducted by Doughari (2006).

The MIC results in this study also agree with the above findings. The lowest MIC values for the alkaloid extracts were observed against Gram-positive bacterium of *Bacillus subtilis* and Gram-negative bacteria of *Serratia marcescens* and *Staphylococcus thphymurium* for both extracts of the leaves (3.75 mg/mL) and stem-bark (0.94 mg/mL) and Gram-negative bacterium of *Vibrio fluvialis* for root extract (0.12 mg/mL) (Table 2). The MIC values at ≤ 0.064, 0.128-0.512, 1.023-0.513 and ≥ 1.024 mg/mL were considered high, moderate, low and no activity (Kakuko et al. 2005). Therefore, from the MIC data obtained, the root alkaloid extract showed moderate antibacterial activity while the stem-bark alkaloid extract showed low

antibacterial activity and the leaf alkaloid extract showed no antibacterial activity. Moreover, the root alkaloid extract showed the lowest MIC value against all bacteria tested compared to the leaf and stem-bark alkaloid extracts.

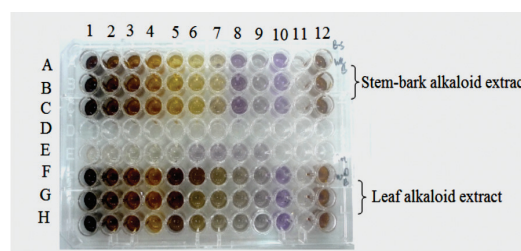


FIGURE 1. MIC determination for stem-bark and leaf alkaloid extracts of *Ochrosia oppositifolia* against *Bacillus subtilis*

Microtiter plate wells A1-A9, B1-B9, C1-C9 for triplicate of stem-bark alkaloid extract concentrations that range from 30 to 0.12 mg/mL; F1-F9, G1-G9, H1-H9 for triplicate of leaf alkaloid extract concentrations that ranges from 30 to 0.12 mg/mL; A10, B10, C10, F10, G10, H10 for negative control; A11, B11, C11, A12, B12, C12, F11, G11, H11, F12, G12, H12 for growth control; E1-E9 for positive control.

TABLE 2. Minimum inhibitory concentration for *Ochrosia oppositifolia* leaf, stem-bark, root alkaloid extracts and positive control

Bacteria	MIC (mg/mL)			
	Leaf	Stem-Bark	Root	Positive control (µg/mL)
MRSA	15	7.50	1.88	0.06
<i>Staphylococcus aureus</i>	7.50	1.88	0.94	0.25
<i>Bacillus subtilis</i>	3.75	0.94	0.94	8.00
<i>Bacillus thuringiensis</i>	NI	NI	3.75	0.25
<i>Staphylococcus typhimurium</i>	3.75	0.94	0.46	0.25
<i>Serratia marcescens</i>	3.75	0.94	0.46	0.25
<i>Escherichia coli</i>	7.50	7.50	0.94	0.25
<i>Vibrio fluvialis</i>	NI	NI	0.12	0.50
<i>Vibrio cholera</i>	7.50	NI	NI	0.50

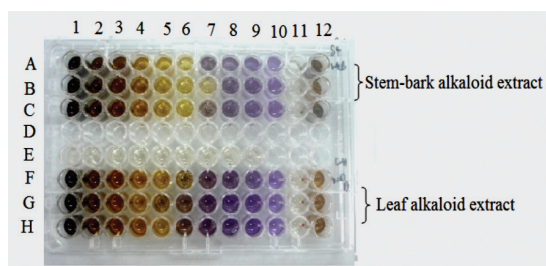


FIGURE 2. MIC determination for stem-bark and leaf alkaloid extracts of *Ochrosia oppositifolia* against *Staphylococcus typhimurium*

Microtiter plate wells A1-A9, B1-B9, C1-C9 for triplicate of stem-bark alkaloid extract concentrations that range from 30 to 0.12 mg/mL; F1-F9, G1-G9, H1-H9 for triplicate of leaf alkaloid extract concentrations that ranges from 30 to 0.12 mg/mL; A10, B10, C10, F10, G10, H10 for negative control; A11, B11, C11, A12, B12, C12, F11, G11, H11, F12, G12, H12 for growth control; E1-E9 for positive control.

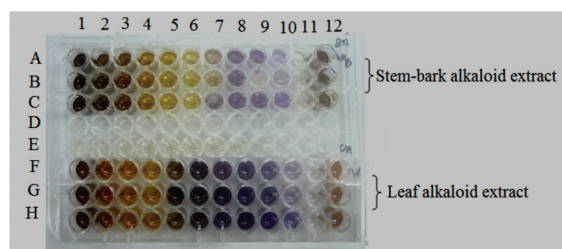


FIGURE 3. MIC determination for stem-bark and leaf alkaloid extract of *Ochrosia oppositifolia* against *Serratia marcescens*

Microtiter plate wells A1-A9, B1-B9, C1-C9 for triplicate of stem-bark alkaloid extract concentrations that range from 30 to 0.12 mg/mL; F1-F9, G1-G9, H1-H9 for triplicate of leaf alkaloid extract concentrations that ranges from 30 to 0.12 mg/mL; A10, B10, C10, F10, G10, H10 for negative control; A11, B11, C11, A12, B12, C12, F11, G11, H11, F12, G12, H12 for growth control; E1-E9 for positive control.

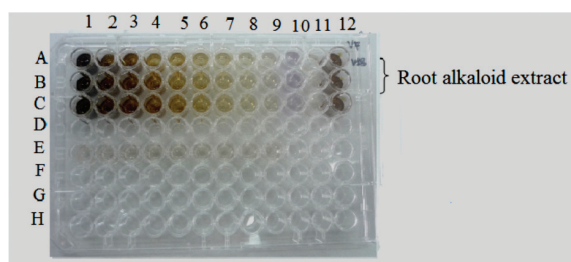


FIGURE 4. MIC determination for root alkaloid extract of *Ochrosia oppositifolia* against *Vibrio fluvialis*

Microtiter plate wells A1-A9, B1-B9, C1-C9 for triplicate of root alkaloid extract concentrations that range from 30 to 0.12 mg/mL; A10, B10, C10, F10, G10, H10 for negative control; A11, B11, C11, A12, B12, C12 for growth control; E1-E9 for positive control.

CONCLUSION

In conclusion, the leaf, stem-bark and root alkaloid extracts showed sensitivity against both Gram-positive and Gram-negative bacteria. The root alkaloid extracts showed more potent antibacterial activity in contrast to leaf and stem-bark alkaloid extracts. Furthermore, the leaf and stem-bark alkaloid extracts demonstrated similar activities against the same bacteria with slightly different values of inhibition zones. This similarity might be due to the presence of neisosposinine and reserpinine alkaloids in both extracts which was shown in the previous study (Nasab 2013, 2012). Further isolation of the active chemical components of the root alkaloid extract is now under progress and it is predicted that there will be the presence of indole alkaloids but with different chemical structures as compared to the indole alkaloids in stem-bark and leaves extracts.

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