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Research Article

EVALUATION OF FOUR EXTRACTS FROM *DILLENIA OVATA* STEM BARK AND LEAVES FOR ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY

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ABSTRACT

Background: *Dillenia* species are widely used by the indigenous people of Southeast Asia traditionally as it has many medicinal values. *Dillenia* sp. has been shown to possess antimicrobial and antioxidant properties but no studies have yet been carried out for *D. ovata*.

Methods: In this study, the antimicrobial activity of n-hexane, ethyl acetate, methanol and water extracts of *Dillenia ovata* stem bark and leaf against twenty species of bacteria, fungi and yeasts was evaluated. The antimicrobial properties of *D. ovata* were evaluated through the disk diffusion method and the modified broth microdilution test using the resazurin assay.

Results: In the disk diffusion assay, the most potent extract of *Dillenia* for both the stem bark and leaf was the n-hexane extract. A low concentration of the n-hexane extract was observed to be sufficient for the inhibition of bacterial growth compared to the methanol and ethyl acetate extracts. No antimicrobial activity was detected in the water extract for all strains tested and no inhibitory effects were observed for the fungal and yeast strains tested for all extracts.

Conclusion: The compounds in the n-hexane extract will need to be elucidated further in order to investigate the efficacy of *D. ovata* as an antibacterial agent.

Keywords: *Dilleniaovata*, Resazurin, Antimicrobial activity.

INTRODUCTION

The prevalence of increasing bacterial resistance against synthetic antibiotics in the past several decades has spurred the search of substitutes for ineffective conventional antibiotics. Plant derived antimicrobials have many advantages over synthetic antibiotics in terms of reproducibility, fewer side effects and lower cost of production making the discovery of novel antimicrobial activity from plants advantageous in helping to produce better antibiotics. In recent years, the use of medicinal plants in primary health care has also increased tremendously.

The genus *Dillenia* (family *Dilleniaceae*) is a tropical plant native to Southeast Asia and approximately ten species can be found in Malaysia [1]. *Dillenia* species are widely used by the indigenous people as it has many medicinal values. Other species of *Dillenia* such as *Dilleniapentagyna* and *Dilleniaindica* have been shown to contain antimicrobial properties [2, 3].To date, pharmacological and phytochemical properties of other *Dillenia* species such as *Dilleniaindica, Dilleniapentagyna* and *Dilleniasuffruticosa* have been extensively studied. The leaf, bark, and fruits of *Dilleniaindica* have been traditionally used for treatment of diarrhoea, cancer, fever and cough[4]. It is established that the methanol and ethyl acetate extracts of the fruit of *Dilleniaindica* possesses anti-leukemic effect and that the active compound which was responsible for the activity was betulinic acid [5]. Methanol, ethyl acetate and water extracts of fruit of *Dilleniaindica* also displayed antioxidant activity attributed to high total phenolic content [6]. To date, no studies have yet been carried out for *Dilleniaovata*. Therefore, the objective of this study was to evaluate the antimicrobial activity of different extracts of *Dillenia ovata* stem bark and leaf against bacteria, fungi and yeast.

MATERIALS AND METHODS

Preparation of the extracts

The leaves of *Dillenia ovata* were collected from the Chebar Besar Forest Reserve, Kedah, Malaysia in April 2011. The plant material was identified by a taxonomist and the voucher specimen HTP3183 were deposited in the Herbarium Taman Botani, Putrajaya, Malaysia. The powdered plant material was sequentially extracted until the solution became colourless in the siphon of the extractor in a Soxhlet apparatus with n-hexane, ethyl acetate, methanol and water in an increasing order of polarity. The extracts were filtered before evaporation under reduced pressure in a rotary evaporator and concentrated by drying on water bath. Finally, they were stored in the desiccator over silica gel until use. The water extract, however, was lyophilized using a freeze dryer to obtain dry solid mass and maintained in a 4^{o}C refrigerator until further use. The yields of each extract are reported in Table 1.

Table 1: Yields of n-hexane, ethyl acetate, methanol and water extracts by percentage from starting material

Extract	Stem bark (%)	Leaf (%)
n-hexane	0.42	1.26
Ethyl acetate	1.13	2.28
Methanol	5.21	4.80
Water	5.45	5.63

Test microorganisms

Bacillus cereus, Bacillus subtilis, Staphylococcus epidermidis, Staphylococcus aureus, Staphylococcus saprophyticus ATCC 15305, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiellapneumoniae*, *Proteus vulgaris* and *Enterobacteriaeaerogenes* NCIMB 10102 were the bacterial strains used in this study. *Staphylococcus aureus*ATCC 25923 and *Escherichia coli* ATCC 25922, were used as reference strains and with the antibiotic gentamycin. *Candida guilliermondii* ATCC 6260, *Candida parapsilosis* ATCC 22019, *Candida lusitaniae* ATCC 34449, *Candida tropicalis* ATCC 201380,*Saccharomyces cerevisiae*, *Penicillium chrysogenum*, *Aspergillusniger* ATCC 16404 and *Aspergillusfumigatus* were the yeast and fungal strains used. *Candida albicans* ATCC 60193 was used as the reference strain for fungi and yeasts with the antibiotic amphotericin B. All microorganisms were obtained from either American Type Culture Collection (ATCC, USA) or the Institute for Medical Research (IMR, Malaysia). The bacterial strains were subcultured on tryptone soy agar (Merck, Germany) whereas yeasts and fungi were maintained on malt extract agar (Merck, Germany). The working media used for

susceptibility testing were Mueller-Hinton agar (Oxoid, UK) and Mueller-Hinton broth (MH broth) (Oxoid, UK) [7].

Preparation of inoculum

All inoculum were freshly prepared prior to testing and were used within 30 minutes of preparation [8]. Turbidity was visually assessed and adjusted to meet 0.5 McFarland (prepared as described) [9] which was equivalent to approximately 10^7-10^8 cfu/mL of bacterial cells. The suspension was also verified by measuring its absorbance spectrophotometrically to ensure its optical density was within 0.08-0.13 at 625nm [9]. The antifungal disk diffusion susceptibility test was carried out according to the National Committee for Clinical Laboratory Standards (NCCLS) guidelines [10]. The inocula were prepared using direct colony suspension. Inoculum suspensions with inoculum size ranging from 1×10^6 to 5×10^6 cells/mL were prepared for all fungal strains.

Disk diffusion test

The extract stock solution was sterilized by filtration through a 0.45µm pore filter (Millipore, Ireland). Sterile filter paper disc (Oxoid, England), 6mm in diameter was impregnated with 20µL of filtered plant extracts at concentrations of 50, 25, 15, 10, 5 and 3mg/ml, then left to dry at room temperature before applying on the inoculated Muller-Hinton agar (Oxoid, England) surface. For fungi, the Mueller-Hinton agar was supplemented with 2% glucose and 0.5µg/mL methylene blue dye for better visibility. The plates were then inverted and placed into the incubator for 18 hours at 37°C for bacteria and 48 hours at 35°C for fungi and yeasts. Triplicates were performed. Zones of inhibition for each disk were measured after the incubation period. Subsequently, only extracts that exhibited promising antimicrobial effects were proceeded to the broth microdilution test with resazurin indicator dye for determination of the minimum inhibitory concentration (MIC).

Modified Broth microdilution

Inoculum suspensions were prepared according to NCCLS and CLSI guidelines [11, 12]. The plant extracts (50µL) were serially diluted two-fold with broth to a final concentration ranging from 0.125 to 64mg/ml. Bacterial suspensions were compared to the 0.5McFarland turbidity standard to obtain 10^7 - 10^8 cfu/ml and was diluted accordingly to achieve 10^6 cfu/ml. For yeasts and moulds, 50µL of inoculum suspension was added to give a final concentration of 5 x 10^2 to 2.5 x 10^3cells/ml and 0.4 x 10^4 to 5 x 10^4 cells/ml respectively. Sterility and growth controls were included in the plates. Cell suspensions were added into all wells except for sterility control row. Resazurin of 0.02% (w/v) was prepared by dissolving resazurin sodium salt (Sigma-Aldrich, USA) in distilled water. The final concentration of resazurin was 0.002% (w/v). The plates were then incubated at 37°C for 16-20 hours for bacteria and 35°C for 48 hours for fungi and yeasts. After incubation, the colour changes of resazurin dye were observed visually to determine the minimum inhibitory concentration (MIC).

RESULTS

In the disk diffusion assay, inhibition of growth was obtained for all Gram positive bacteria tested against the stem bark n-hexane and methanol extracts. However, the n-hexane extract from leaves only inhibited *S. saprophyticus, S. aureus* and *S. epidermidis* and inhibitory effects against *B. cereus* was only effective at 50mg/ml, which was the highest concentration used (Table 2a).

For the methanol extract from leaves, the inhibitory effects exerted was less effective and inhibition was observed only at higher concentrations compared to the same extract obtained from the stem bark inhibiting *S. epidermidis* only at 10mg/ml, *B. cereus* at 15mg/ml and *B. subtilis* 25mg/ml. The methanol extract from stem bark also inhibited the growth of *P. vulgaris* at 50mg/ml, the only effect against all the Gram negative bacteria tested.

The ethyl acetate extract from stem bark was only effective at the highest concentration of 50mg/ml for all Gram positive bacteria tested. Interestingly, the same extract from leaves only inhibited the growth of the *Bacillus* sp.

Water extracts from stem bark only exerted inhibitory effects at 50mg/ml against *S. aureus* ATCC 25923 (7.25 ± 0.354 mm) and *S. epidermidis*(6.5 ± 0 mm) whereas water extract from leaves showed inhibition against *S. epidermidis* at the same concentration (Table 2b).

Table 2a: Antimicrobial activity of different extracts of *Dillenia ovata* stem bark against bacteria using disk diffusion test

Microorganisms	Concentration (mg/mL)	Zone of inhibition (mm) (n=3)				
		n-hexane	Ethyl acetate	Methanol	Water	[a]Gentamicin (10 µg)
Staphylococcus saprophyticus ATCC 15305	3	6.0 ± 0	6.0 ± 0	6.0 ± 0	6.0 ± 0	30.5 ± 0.67
	5	7.67 ± 0.58	6.0 ± 0	6.0 ± 0	6.0 ± 0	
	10	8.67 ± 0.58	6.0 ± 0	7.0 ± 0	6.0 ± 0	
	15	9.33 ± 1.15	6.0 ± 0	8.0 ± 0	6.0 ± 0	
	25	10.67 ± 1.15	6.0 ± 0	9.0 ± 0	6.0 ± 0	
	50	11.67 ± 1.15	8.0 ± 0	10.0 ± 0	6.0 ± 0	
Staphylococcus aureus ATCC 25923	3	6.0 ± 0	6.0 ± 0	6.67 ± 0.577	6.0 ± 0	23.7 ± 1.15
	5	6.83 ± 0.289	6.0 ± 0	6.83 ± 0.289	6.0 ± 0	
	10	7.0 ± 0.707	6.0 ± 0	7.5 ± 0.5	6.0 ± 0	
	15	7.33 ± 0.577	6.0 ± 0	8.67 ± 0.289	6.0 ± 0	
	25	7.33 ± 0.289	6.0 ± 0	8.67 ± 0.289	6.0 ± 0	
	50	10.7±0.58	7.25 ± 0.354	9 ± 1	7.25 ± 0.354	
Staphylococcus epidermidis	3	7 ± 0	6.0 ± 0	8 ± 0	6.0 ± 0	27.0 ± 0
	5	7 ± 0	6.0 ± 0	8 ± 0	6.0 ± 0	
	10	7 ± 0	6.0 ± 0	8.67 ± 0.577	6.0 ± 0	
	15	7 ± 0	6.0 ± 0	8.67 ± 0.577	6.0 ± 0	
	25	7.25 ± 0.354	6.0 ± 0	8.83 ± 0.289	6.0 ± 0	
	50	7.67 ± 0.577	6.5 ± 0	9.33 ± 0.577	6.5 ± 0	
Bacillus cereus	3	6.0 ± 0	6.0 ± 0	6.83 ± 0.289	6 ± 0	23.7 ± 0.58
	5	6.0 ± 0	6.0 ± 0	6.83 ± 0.289	6 ± 0	
	10	6.0 ± 0	6.0 ± 0	8 ± 0	6 ± 0	
	15	7 ± 0	6.0 ± 0	8.667 ± 0.577	6 ± 0	
	25	7.17 ± 0.289	6.0 ± 0	8.667 ± 0.577	6 ± 0	
	50	7.83 ± 0.289	6.83 ± 0.29	9 ± 1.0	6 ± 0	
Bacillus subtilis	3	6.0 ± 0	6.0 ± 0	7.0 ± 0	6.0 ± 0	24.3 ± 0.58
	5	6.0 ± 0	6.0 ± 0	7.33 ± 0.577	6.0 ± 0	
	10	6.75 ± 0.354	6.0 ± 0	7.67 ± 0.577	6.0 ± 0	
	15	6.75 ± 0.354	6.0 ± 0	7.83 ± 0.289	6.0 ± 0	
	25	7 ± 0	6.0 ± 0	7.83 ± 0.289	6.0 ± 0	
	50	8.67 ± 0.577	6.50 ± 0	7.83 ± 0.764	6.0 ± 0	

Proteus vulgaris	3	6.0 ± 0	6.0 ± 0	6.0 ± 0	6.0 ± 0	19.89 ± 0.96
	5	6.0 ± 0	6.0 ± 0	6.0 ± 0	6.0 ± 0	
	10	6.0 ± 0	6.0 ± 0	6.0 ± 0	6.0 ± 0	
	15	6.0 ± 0	6.0 ± 0	6.0 ± 0	6.0 ± 0	
	25	6.0 ± 0	6.0 ± 0	6.0 ± 0	6.0 ± 0	
	50	6.0 ± 0	6.0 ± 0	6.667 ± 0	6.0 ± 0	

*6mm is the diameter of disk,indicates no antimicrobial activity.

[a]Gentamicin 10 µg as positive control.

Disk diffusion test were performed in triplicates and diameter of zone of inhibitions were expressed as mean**±** standard deviation in mm.

Table 2b: Antimicrobial activity of different extracts of *Dillenia ovata* leaves against bacteria using disk diffusion test

Microorganisms	**Concentration (mg/mL)**	**Zone of inhibition (mm)(n=3)**				
		n-hexane	**Ethyl acetate**	**Methanol**	**Water**	**[a]Gentamicin (10 µg)**
	3	8 ± 0	6 ± 0	6 ± 0	6 ± 0	
Staphylococcus saprophyticus	5	9.33 ± 058	6 ± 0	6 ± 0	6 ± 0	32.58 ± 0.67
	10	10.67 ± 0.58	6 ± 0	6 ± 0	6 ± 0	
	15	12 ± 0	6 ± 0	6 ± 0	6 ± 0	
	25	13.33 ± 0.58	6 ± 0	6 ± 0	6 ± 0	
	50	14.33 ± 0.58	6 ± 0	6 ± 0	6 ± 0	
Staphylococcus aureus ATCC 25923	3	6 ± 0	6 ± 0	6 ± 0	6 ± 0	
	5	6 ± 0	6 ± 0	6 ± 0	6 ± 0	24 ± 0
	10	6 ± 0	6 ± 0	6 ± 0	6 ± 0	
	15	7± 0	6 ± 0	6 ± 0	6 ± 0	
	25	8.5 ± 0.71	6 ± 0	6 ± 0	6 ± 0	
	50	17.33 ± 1.15	6 ± 0	6 ± 0	6 ± 0	
Staphylococcus epidermidis	3	6.5 ± 0	6 ± 0	6 ± 0	6 ± 0	
	5	7.3 ± 0.75	6 ± 0	6 ± 0	6 ± 0	28 ± 0
	10	7.5 ± 0.5	6 ± 0	6.7 ± 0.71	6 ± 0	
	15	8 ± 0	6 ± 0	7.5 ±0.47	6 ± 0	
	25	8 ± 0	6 ± 0	7.9 ± 0.47	6 ± 0	
	50	8 ± 0	6 ± 0	8± 0	15.7 ± 0.94	
Bacillus cereus	3	6 ± 0	8 ± 0	6 ± 0	6 ± 0	
	5	6 ± 0	8.3 ± 0.24	6 ± 0	6 ± 0	27.3 ± 0.47
	10	6 ± 0	9.8 ± 0.24	6 ± 0	6 ± 0	
	15	6 ± 0	10 ± 0	7 ± 0	6 ± 0	
	25	6 ± 0	11 ± 0	7.5 ± 0.71	6 ± 0	
	50	7.7 ± 0.24	11 ± 0	8 ± 0	6 ± 0	
Bacillus subtilis	3	6 ± 0	7.5 ± 0.71	6 ± 0	6 ± 0	
	5	6 ± 0	8 ± 0.41	6 ± 0	6 ± 0	27 ± 0
	10	6 ± 0	9 ± 0.82	6 ± 0	6 ± 0	
	15	6 ± 0	9.3 ± 0.47	6 ± 0	6 ± 0	
	25	6 ± 0	9.7 ± 0.47	7.5 ± 0.41	6 ± 0	
	50	6 ± 0	10 ± 0.82	7.7 ± 0.47	6 ± 0	

*6mm is the diameter of disk,indicates no antimicrobial activity.

[a]Gentamicin 10 µg as positive control.

Disk diffusion test were performed in triplicates and diameter of zone of inhibitions were expressed as mean**±** standard deviation in mm.

There was no antimicrobial activity observed for all extracts of the stem bark and leaf against the Gram negative bacteria, fungi and yeasts tested in the disk diffusion test (results not shown). Therefore, the broth microdilution test was only proceeded with for the Gram positive bacteria and *Proteus vulgaris*.

In the resazurin broth microdilution method, the extract which was efficacious at the lowest concentration among the extracts tested was the methanol extract from stem bark which gave the lowest MIC values (Table 3a). This is in agreement with the results from the disk diffusion method wherein the methanol disks at the lowest concentration of 3mg/ml exhibited clearance zones for most Gram positive bacteria tested. For *B. cereus*, all extracts tested from stem bark and leaves had an MIC of <0.06 mg/ml. The levels of MIC obtainedfrom the broth microdilution method mirrored the pattern of degree of inhibition obtained from the disk diffusion method (Tables 3a and 3b). For example, the methanol extract from stem bark which showed larger clearance zones at low concentrations of 3mg/ml compared to the n-hexane extract in the disk diffusion test possessed a lower MIC when compared to the MIC obtained from the n-hexane extract in the broth microdilution test.

Table 3a: Antimicrobial activity of different extracts of *Dillenia ovata* stem bark against bacteria using broth microdilution test

Microorganisms	**Minimum Inhibitory Concentration (mg/mL) (n=3)**				
	n-hexane	**Ethyl acetate**	**Methanol**	**Water**	**Gentamicin***
Staphylococcus saprophyticus ATCC 15305	1.0 ± 0	2.0 ± 0	0.25 ± 0	-	0.03 ± 0
Staphylococcus aureus ATCC 25923	0.21±0.072	1.33±0.58	0.21±0.072	0.50±0	0.67±0.29
Staphylococcus epidermidis	0.50±0	1.00±0	0.25±0	0.50±0	0.25±0
Bacillus cereus	<0.06	<0.06	<0.06	NA	<0.06
Bacillus subtilis	<0.06	0.67±0.29	0.25±0	NA	1.67±0.58
Proteus vulgaris	-	-	1.0 ± 0	-	0.5 ± 0.0

NA indicates not applicable.

*Gentamicin as positive control

Broth microdilution test was performed in triplicates and minimum inhibitory concentrations were expressed as mean ± standard deviation in mg/mL.

Table 3b: Antimicrobial activity of different extracts of *Dillenia ovata* leaves against bacteria using broth microdilution test

Microorganisms	Minimum Inhibitory Concentration (mg/ml) (n=3)				
	n-hexane	Ethyl acetate	Methanol	Water	Gentamicin*
Staphylococcus saprophyticus	0.42 ± 0.14	NA	NA	NA	0.003 ± 0
Staphylococcus aureus ATCC 25923	0.33 ± 0.12	NA	NA	NA	1.00 ± 0
Staphylococcus epidermidis	0.67 ± 0.24	NA	0.38 ± 0.13	3.00 ± 1.00	0.50 ± 0
Bacillus cereus	<0.06	<0.06	<0.06	<0.06	1.00 ± 0
Bacillus subtilis	NA	0.25 ± 0.00	0.50 ± 0.00	2.00 ± 0.00	2.00 ± 0

NA indicates not applicable.

*Gentamicin as positive control

Broth microdilution test was performed in triplicates and minimum inhibitory concentrations were expressed as mean ± standard deviation in mg/mL.

DISCUSSION

Emergence of microbial resistant strains, increased occurrences of microbial infection and severe side effects of current antimicrobial agents bring about an imperative need to discover more antimicrobial agents from natural sources with prominent pharmacological activity. In the present study, n-hexane, ethyl acetate, methanol and water extracts from the stem bark and leaves from *D. ovata* were tested for antimicrobial activity against twenty microbial pathogens. For the classification of the antibacterial activity as Gram-positive or Gram-negative, it is generally expected that a much greater number would be active against Gram-positive than Gram-negative bacteria [13]. This could be attributed to the difference in cell wall composition and the presence of a lipopolysaccharide in the Gram negative bacteria.

Overall, *D. ovata* methanol extract from stem bark is the most effective in inhibiting the growth of Gram positive bacteria followed by the n-hexane extract based on the size of the clearance zones obtained. The n-hexane and dichloromethane fractions of *Dillenia indica Linn.* Bark has been found to show remarkable activities against all the tested bacteria [14]. The ethyl acetate extract from leaves was only effective against the *Bacillus* sp. and the same extract from stem bark was found to be effective only from 50mg/ml. This contradicts with the finding that the ethyl acetate extract from *Dilleniapentagyna*stem bark showed promising sensitivity against all the gram positive strains and most of the Gram negative bacteria [15].

No antimicrobial activity was detected in the water extract for all strains tested except for the highest concentration tested at 50mg/ml against *S. aureus* ATCC 25923 (7.25± 0.354 mm) and *S. epidermidis*(7.0± 0 mm). This is in partial agreement with the findings for *Dilleniaindica* leaves which showed moderate antibacterial and antifungal activity compared to kanamycin for all fractions tested with the exception of the aqueous fraction [2]. The n-hexane and methanol extracts from stem bark would need to be elucidated further in order to identify the specific compounds inhibiting bacterial growth towards the development of new antibiotics. The results indicate that the antibacterial and antifungal activity may be variable with the species of the plants.This study is a preliminary evaluation of antimicrobial activity of *D. ovata*. The crude extracts demonstrating antimicrobial activity could result in the discovery of new antibacterial agents for the maintenance of animal or human health in our ongoing warfare with emerging antimicrobial resistance.

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