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IN VITRO INVESTIGATIONS OF SOME ANTIMICROBIAL AND ANTIPARASITIC POTENCIES OF METHANOLIC EXTRACT OF MANGROVE PLANT, AVICENNIA MARINA

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ABSTRACT
This study was designed to assess the antimicrobial and anthelmintic activities of methanolic extract of mangrove leaves (*Avicennia marina*). Various concentration of the extract (50-300 µg/ml) were subjected to different methods for identifying and screening the antimicrobial and anthelmintic activities. The obtained results established that the mangrove plant has broad-spectrum antimicrobial and anthelmintic properties and can be considered as a potential alternative source for treating many infectious diseases. However, further research work is needed for phytochemical screening, identifying, isolation, and fractionation of the active principle(s) responsible for antimicrobial activity.

KEYWORDS: *Avicennia marina, antibacterial, anthelminthic.*

1. INTRODUCTION
Ethnobotany is a science that studies the interaction between plants and humans. The association of plants and human cultures is not limited to the use of plants as a source of food, manufacturing of clothes and refuge but also includes their use in the field of health (Sofowora *et al*., 2013). The term Pharmacognosy means studying of drugs that derived from natural products, now it is considered as one of the main interests of scientists to discover new active principles from plant origin which can be used in the treatment of many infectious and non-infectious diseases as they possess new mechanisms of actions (Anand *et al*., 2019). The presence of many plant components as secondary metabolites like quinines, phenol, terpenoids, flavones, tannins, essential oils, alkaloids and others, render the plant having many therapeutic benefits especially antimicrobial activities against many new or reemerging infectious diseases (Ravikumar *et al*., 2010). Mangroves are group of plants that constitute an important component for water purification and protection of the western coast from wind and waves of the coastal ecosystem of Saudi Arabia. They have wide applications in folk medicine due to their high biological productivity and have been recently considered as a precious source for chemical components with potential medical values (Eswaraiah *et al*., 2020). Although Mangrove extracts have been studied by many authors as a source of anti-helminthic, anti-inflammatory, anti-bacterial, anti-fungal, as well as antioxidant products (Chi *et al*., 2019) but still up till now have not been completely explored. Further investigations will result so far in the discovery of several new compounds with potential medicinal value for detection of new chemotherapeutic agents. One of mangrove tree species is *Avicennia marina* that extremely accommodates due to its flexible growth model is widely distributed all over the western coast of Saudi Arabia. Although the information about chemical compounds extracted from *Avicennia marina* is very rare, many important classes of constituents like alkaloids, benzofurans, terpenoids, quinines and essential oils have been recorded to show many biological activities (Nabeelah Bibi *et al*., 2019). Therefore, the objective of the current study was to screen and evaluate the different biological activities of methanol *Avicennia marina* leaf extract in vitro experimental models.

2. MATERIALS AND METHOD
2.1. Drugs and Chemicals
All drugs, chemicals and reagents employed in this work were of analytical grade.

2.2. Experimental animals
2.3. Ethical statement
2.4. Collection and Processing of plant material
The leaves of *Avicennia marina* were collected from the southern coast of Saudi Arabia on the red sea. The plant

was identified in the Botany department, faculty of Science and Art, King Abdul-Aziz University (KAU).

2.5. Preparation of methanol extract of the plant

Freshly collected *Avicennia marina* leaves were subjected to extraction by using methanol as an organic solvent. At first, 100 g of leaves were washed thoroughly by distilled water to get rid of any salts or contaminants to which one liter of methanol was added for 24 hours at room temperature followed by filtration. The filtrate was evaporated to remove the solvent at 40 ^{o}C, reduced pressure and rotation of 20 rpm in vacuum rotatory evaporator. The obtained extract was scraped off and transferred to an air-tight container and kept for storage at -20 ^{o}C until subsequent use. The extraction process has been described in detail previously (Shoibe *et al*., 2017).

2.6. Phytochemicals analysis of methanol extract of *A. marina* leaves

The qualitative phytochemical analysis was accomplished to detect the various phytochemical components like phenols, flavonoids alkaloids, saponins, and terpenoids by employing standard procedures explained previously (Tiwari *et al*., 2011). The extract was subjected to chemical analysis by using gas chromatography-mass spectroscopic (GC-MS); Clarus 500 Perkin-Elmer (Auto System XL) which equipped and coupled with a mass detector turbo mass gold – Perkin Elmer Turbomas 5.2 spectrometer and an Elite-1 (95% dimethyl poly siloxane/5% diphenyl). A capillary column: 30 mm × 0.25 mm × 0.25 µm was used with helium as carrier gas at rate of 1ml/min. The device oven was conserved at 110°C for 2 minutes, then raised up to 280°C and maintained constant for 10 min at 5°C/min. The split ratio was adjusted in a mode as 10:1 where 2µl of the samples were injected. MS scan range was set at *m/z* 45-450 Da. The Peaks of mass spectra were identified by comparing them with those stored in the spectrometer database of National Institute of Standards and Technology MS database (Dinesh *et al*., 2016).

2.7. In vitro antibacterial activity of the extract

2.7.1. Test organisms

Three standards Gram-negative strains; *Escherichia coli* (ATCC® 25922™), *pseudomonas aeruginosa* (ATCC® 27853™) and *Klebsiella pneumoniae* (ATCC® 10031™), and two Gram-positive strains; *Staphylococcus aureus* **(**ATCC® 25923™) and *Streptococcus faecalis* (ATCC® 29212™) in addition to yeast; *Candida albicans* (ATCC® 90025™), were subjected to assess the antibacterial effect of *A. marina* leave extract. All tested microorganisms were sub-cultured and maintained on nutrient agar slants (Oxoid, UK) while *C. albicans* was cultured and preserved on Sabouraud dextrose agar slants (Oxoid, UK) at 4 ^{o}C till further use.

2.7.2. Disc diffusion assay

The disc diffusion assay was established to assess the antimicrobial activity of the extract from which, 100 mg was dissolved in one ml ethanol (85%) then 20 µl equivalent to 2 mg dried extract were impregnated on dry sterile filter paper discs with 6 mm in diameter (Whatman No.1). The prepared discs were kept at 4 ^{o}C after the evaporation of the ethanol. The prepared discs were placed on Mueller Hinton agar (MHA) medium (Oxoid, UK) plates consistently seeded with the test pathogen (10^6 cfu/ml) and incubated at 37 ^{o}C for 24 hours and the diameters of inhibition were measured in millimeter. The efficiency of the leaf extract as an antibacterial agent was compared with streptomycin (200 µg/ml, Oxoid, UK) and the solvent ethanol. The assay was performed in triplicate and the result was recorded as average. The antifungal activity was assessed (Madhurima & Punarbasu. 2014) on Sabouraud`s dextrose agar (SDA) against *Candida albicans* and nystatin (200 µg/ml) was employed as a positive control. The result of inhibition in millimeters was recorded after 72 hours incubation at 30 ^{o}C.

2.7.3. Determination of MIC.

The minimum inhibitory concentration (MIC) of the extract was determined according to the micro-dilution method in 96 multi-well plates following the recommendations of CLSI (2017). The stock concentration of the crude plant extract was 10 mg/ml. Two-fold dilution was prepared using tryptic soy broth (Oxoid, UK) as diluent. An equal volume (5 µl) of pathogen suspension containing 10^8 cfu/ml (adjusted to 0.5 McFarland standards turbidity) was inoculated in each well and incubated overnight at 37 ^{o}C. To assess the inhibitory activity of the ethanol, a negative control was encompassed for all pathogens via preparing the solvent with broth instead of plant extract. Streptomycin and nystatin were used as positive controls for bacteria and yeast, respectively. The microbial growth was detected by the addition of 40 µl of P-iodonitrotetrazolium (0.2 mg/ml of water) into each well and the plates were incubated for 30 min at 37 ^{o}C. The test was repeated three times. The lowest concentration of the extract that inhibited the pathogens' growth was reported as the MIC.

2.7.4. Determination of MBC and MFC

The minimum bactericidal concentration and minimum fungicidal concentration of the plant extract were estimated by sub-culturing the content of each well showing no visible growth onto MHA and SDA plates for bacteria and yeast, respectively. The inoculated plates were incubated at 37 ^{o}C until growth was detected in the control plates. The consequent concentrations required for killing 99.9% of the microbes were considered MBC for bacteria and MFC for yeast (Scorzoni *et al*., 2007).

2.8. In vitro anti-helminthic activity of the extract

Adult earthworms (*Pheretima posthuma*) of about 3.5-5.0 cm in length and 0.2-0.5 cm in width were subjected to the laboratory assessment of anti-helminthic activity of the ethanol extract of *Avicennia marina* leaves according to the method described by (Panda *et al*.,

2011). Earthworms were collected from moist soil and washed with physiological saline to remove all contaminant matters. The collected worms were divided into seven groups containing six worms each. A stock solution was made, and different concentrations were prepared (25, 50 and 75 mg/ml). Distilled water (DW) was used as a negative control while standard drug Piperazine citrate (25, 50 and 75 mg/ml in DW) used as a positive control. A volume of 25 mL of DW and each concentration of the methanol extract and standard drug were poured in seven Petri dishes. Six earthworms were placed in each Petri dishes and the time taken for paralysis and death was recorded per minute. Paralysis time was calculated based on the lack of movement of the earthworm in the normal saline medium, even if it was severely shaken. Immotile earthworms with faded body color were considered dead when dropped in warm water (50-60 °C).

2.9. Statistical analysis

All obtained data were subjected to single-factor analysis of variance (ANOVA) and Duncan's test. Significant differences between the means of antimicrobial and anthelminthic effects of mangrove leaf extract were determined in the confidence level of 95% ($P \leq 0.05$) using Statistical Package for the Social Sciences (SPSS) ver. 24.0 program for windows.

3. RESULTS

The components of *Avicennia marina* leaves were extracted by using methanol as a solvent. The phytochemical analysis of the obtained extract is illustrated in table (1).

Table 1: Phytochemical screening of methanolic extract of *avicennia marina* leaves.

Components			
Alkaloids	Flavonoids	Carbohydrates and glycosides	Saponins
+	+++	++	++
Coumarins	Tannins	Sterol and Terpenes	Anthraquinones
+	++	+	-

The crude methanolic extract of *Avicennia marina* leaves was analyzed by GC-MS to evaluate the chemical compounds responsible for antimicrobial activity. The spectral data revealed the presence of a mixture of seven compounds with retention times as illustrated in figure (1) and table (2). The major ingredients were benzeneethanol,4-hydroxy-, benzaldehyde,3-methyle-, and cyclobuta (1,2:3,4) dicyclooctene, hexadec at retention time (RT)12.195, 7.321, and 11.842, respectively.

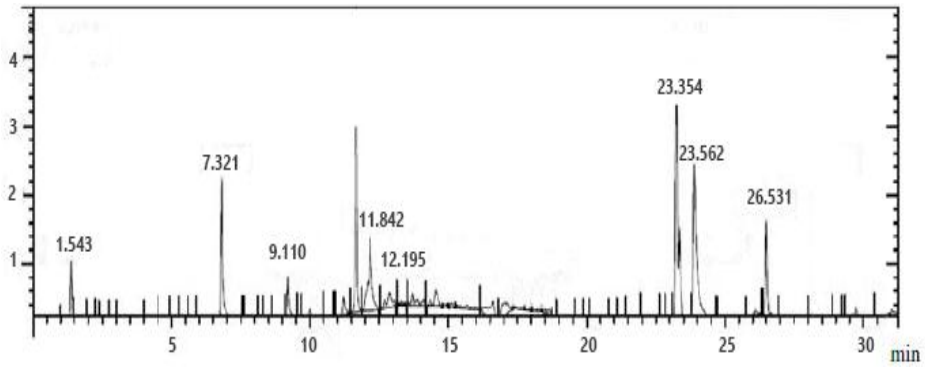

Figure 1: Gas chromatography-mass spectrometry chromatogram of methanolic extract of *Avicennia marina* leaves.

Table 2: The main phytochemicals identified from methanolic extract of *A. marina* by using GC-MS.

Peak No.	RT	Name	Hight
1	7.321	Benzaldehyde,3-methyle-	945366
2	9.110	2-Methoxy-4-vinylphenol-	177364
3	11.842	Cyclobuta(1,2:3,4)dicyclooctene,hexadec	835421
4	12.195	Benzeneethanol,4-hydroxy-	5933457
5	23.354	2-Dicarboxy-3-(4, chlorophenyl) 2,3(1H)	550618
6	23.562	Phenol,2-(1,1-dimethylethyl)-4-(1-methy	204142
7	26.531	Methyl p-(2-phenyl-1-benzimidazolyl) benzoate	233982

In vitro antimicrobial activity of the methanol extract of *Avicennia marina* leaves have estimated by the disc diffusion assay is presented in table (3). The different concentrations of the extract (from 50 up to 300 µg/ml)

showed different zones of inhibition against Gram-positive bacteria, Gram negative bacteria and yeast. The better activity was exerted at the highest extract concentration against all tested microorganisms. The maximum inhibition zone was obtained against *Staphylococcus aureus* (21±0.29). However, the methanolic extract exhibited better antibacterial property against Gram-positive than negative ones. In the same context, the methanolic leave extract exhibited maximum percentage of relative inhibition was reported against *Staphylococcus aureus* (95.5%), while the minimum percentage was against *Pseudomonas aeruginosa* (47.6%), as shown in table (3). The organic solvent, methanol, did not show inhibition for any tested microorganism and regarded as negative control.

Table 3: Potential antimicrobial activity of methanolic extract of *avicennia marina* leaves.

Antibacterial substances		***Tested bacteria* and zone of inhibition in mm**					
		Gram negative strains			**Gram positive strains**		**Yeast**
Item	**Conc. (µg/ml)**	***E. coli***	***P. aeruginosa***	***K. pneumoniae***	***Staph. aureus***	***S. faecalis***	***Candida albicans***
Plant extract (test)	50	2±0.6	0.00	1±0.32	1± 0.33	0.00	2±0.22
	100	5±0.66	2±0.16	3±0.65	3±0.75	4.00	5±0.31
	150	8±0.65	5±0.07	5±0.57	8±0.42	9.00	7±0.4
	200	10±0.33	9±0.44	8±0.66	12±0.13	11±0.33	10±0.3
	250	11±0.53	13±0.25	10±0.16	18±0.43	14±0.09	12±0.2
	300	12±0.14	10±0.54	11±0.33	21±0.29	18±0.11	17±0.15
Streptomycin (+ve control)	200 (µg/disc)	23±1.03	21±1.70	19±0.45	22±0.43	21±0.77	ND
Fluconazole (+ve control)	100 (unit/disc)	NA	NA	NA	NA	NA	28.0±0.2
Methanol (-ve control)	Absolute	0.00	0.00	0.00	0.00	0.00	0.00
Relative inhibition percentage		52.2%	47.6%	57.9%	95.5%	85.7%	60.7%

The sensitivity of the tested microorganisms was evaluated quantitively via using 96 wells plate assay. The methanolic extract revealed a conceivably good antimicrobial activity. The MIC values for tested microorganisms were ranged between 78.1 and 1250 mg/ml. The methanolic extract showed the ratio MBC/MIC ≥4 in case of all tested bacteria whereas for yeast the ratio was <4, as presented in table (4).

Table 4: MIC and MBC activity of *Avicennia marina* leaves extract (300 ug/ml).

	Tested microorganisms					
	Gram negative strains			**Gram positive strains**		**Yeast**
Item	***E. coli***	***P. aeruginosa***	***K. pneumoniae***	***Staph. aureus***	***S. faecalis***	***C. albicans***
MIC	625	312.5	625	78.1	156.25	1250
MBC	2500	2500	2500	312.5	625	2500
MBC/MIC	4	8	4	4	4	2

It was found that the highest concentration of the extract was faster in its paralytic effect and shorter in its time to death for all tested earthworms. The methanolic leaves extract with concentration of 25, 50 and 75 mg/ml produced paralysis depending on the dose where the paralysis and death time at concentration of 75 mg/ml were (14.73±0.85, 35.62±0.83) (P<0.01) respectively, as compared to the positive control. The result of anthelmintic activity is depicted in **table (5)** and figure (2).

Table 5: Anthelminthic activity of methanolic extract of *avicennia marina* leaves.

Group	**Concentration (mg/ml)**	**Time taken for paralysis (min)**	**Time taken for death (min)**
Methanolic leaf extract	25	37.36±0.26**	61.32±1.2*
	50	22.43±0.55**	45.33±1.02*
	75	14.73±0.85**	35.62±0.83**
Piperazine citrate (Positive control)	25	33.77±0.48	62.27±0.77
	50	25.87±0.88	31.63±0.87
	75	19.54±0.66	17.67±0.93
Phosphate Buffer Saline (PBS) (Negative control)		-	-

Comparisons between standard and treated group were carried out via statistical analysis. n=6 was taken in each group. Asterix represents statistical significance: *P<0.05, **P<0.01.

Fig. 2: Methanolic extract of earth Worm and Its effect in paralysis anddeath.

4. DISCUSSION

Recently, Medical plants have received the attention of microbiologists and pharmacists because they contain biologically active compounds, which are the main source of many active principles known as secondary metabolites such as steroids, phenols, alkalis, flavonoids and terpenoids, which are used to treat many diseases and eliminate many pathogenic microorganisms (Ali *et al*., 2018). Previous studies have shown that antimicrobial activity of crude *A. marina* leaves extract against tested microbes varies according to the type of solvent used in the extraction process. Owing to the discovery of the inhibitory activity in the methanolic extract, it can be concluded that the antimicrobial components present in *A. marina* are more soluble in methanol than others. Accordingly, methanol was selected as the solvent for extraction. (Manilal *et al*., 2016 and Kamilla *et al*., 2017). In the present study, the antimicrobial activity of *Avicennia marina* leaves extract was carried out against five clinically important pathogenic bacterial and yeast strains. The high inhibitory effect of the methanolic extract may be attributed to the higher concentration of the antimicrobial constituents. In our study, the mangrove extract showed activity against all tested microbes with various degrees and the maximal antimicrobial activity was shown against the Gram-positive bacterial pathogens whereas the lower activity was recorded against the yeast and the minimal range of activity was noted against Gram-negative bacteria. Since the 1980s, the inhibitory effect of the mangrove extract has been demonstrated against multidrug resistant clinical pathogens (Manilal *et al*., 2010). Mostly, Gram-negative bacteria are more resistant than Gram-positive ones. The difference in the susceptibility may be attributed to the presence of additional protection afforded by the outer membrane which formed mainly of lipophilic impermeable lipopolysaccharide layer. The inhibition zones of the highest concentration of the extract (300 µg/ml) ranged from 21±0.29 mm to 10±0.54 mm, the highest inhibition zone was reported against *S. aureus* while the lowest zone was recorded against *Pseudomonas aeruginosa* as presented in table (3). Based on the MBC/MIC ratio, the substance that has antimicrobial activity is considered bactericidal if the ratio >4 and bacteriostatic when the ratio <4 (Hossan *et al*., 2018). In our study, the leaf extract showed the ratio ≥ 4 for all tested bacterial strains, suggesting that the extract could be classified as a bactericidal agent while may be classified as fungistatic in case of *C. albicans* where the ratio was 2 (<4), as represented in table (4). In concordance with our study**,** Manilal *et al*., (2010) reported that *Staph. aureus* was the most susceptible strain and considered as one of the most important microorganisms causing nosocomial infection. Up till now, a potent antibiotic that can overcome the multidrug-resistant *S. aureus* has not been discovered. So, our results suggest that the *A. marina* extract ingredients are promising anti-staphylococcal drugs in the future. Species of genus *Candida* are considered the most common opportunistic pathogen causing many serious infectious diseases in human especially those with immunodeficiency due to infection with AIDS virus (Köhler *et al*., 2014). In agreement with previous work (Manilal *et al*., 2016**)**, *A. marina* extract displayed a minimal degree of activity against the *C. albicans* when compared with bacterial strains. This resistance may be attributed to the concentration of extract used and consequently, much higher extract concentration is essential to achieve a stronger inhibition. Accordingly, *A. marina* can be deemed as an innovative source to produce a novel antimycotic drug. Conversely, the results obtained in previous studies (Köhler *et al*., 2014) did not show such activity against *C. albicans*. This discrepancy may be due to the low concentration of the ingredients responsible for the inhibition of *C. albicans* or the geographical source of the plant. On considering disc diffusion, MIC, MBC, and relative inhibition index values, it can be concluded that methanolic *A. marina* leaves extract shows potent antibacterial and considerable antifungal activities against some clinically important microbes.

Helminthic infections can cause chronic diseases and even death of human and animal (Akter *et al*., 2014). In this regard, some medicinal herbs are an important source of effective agents, especially in developing

countries. Many previous studies have confirmed that medicinal plants possess many active principles that can be used as helminthicide. Besides, these natural substances have mostly no side effects or less than the drugs currently used, and they are compatible with human physiological processes (Jamkhande & Barde, 2014). In this study, the potential of the extract was assessed for paralysis and death of the worms, while piperazine citrate was used as a reference drug. Earthworm (*Pheretima posthuma*) was selected for evaluation of the anthelmintic activity due to its anatomical and physiological similarity with the intestinal helminth, *Ascaris lumbricoids* of human beings beside its easy availability (Sreejith *et al*., 2013). Many synthetic anthelmintic drugs have been developed but using such drugs result in serious side effects and emergence of resistance leading to more severe infections. Therefore, attention has been paid to the production of safer medicines from natural sources to be mimic the effects of synthetic drugs for the treatment of parasitic worms. The results obtained in the current study showed promising potential for anti-helminthic activity in a dose-dependent fashion. Results indicate that time taken for piperazine citrate causing paralysis and death is near to that of the methanolic extract, as demonstrated in table (5). This may be described by the result obtained via qualitative phytochemical screening of the *A. marina* extract where several bioactive constituents which have significant anthelmintic potential due to a single compound or combined effect of these phytochemicals such as alkaloids, tannins and flavonoids, and phenolics (Jayaramu & Prathiba, 2016). It was reported earlier that alkaloids (Jamkhande & Barde, 2014) act on CNS of the worm causing paralysis while phenolics and tannins kill the worm via either binding to gastrointestinal tract free proteins or interfering the energy generation in helminth (Raju *et al*., 2013). This plant could be useful in controlling gastrointestinal nematode infections. Further studies need to be applied using in vivo models to establish the in vivo pharmacological efficacy for the use of *A. vacinna* as anthelmintic drugs. In conclusion, medicines of plant origin are increasingly being accepted as they serve as promising sources of novel antimicrobial and anthelmintic agents. The results obtained in the current study clearly indicate that the methanolic extract of *A. marina* leaf possesses bioactive compounds that have considerable antimicrobial and anthelmintic activities. These potential activities established that folk medicine can be as effective as modern medicine to combat pathogenic microorganisms and helminth infestations. Moreover, they represent a more economical and safer alternative. However, further research work is needed for phytochemical screening, identifying, isolation, and fractionation of the active principle(s) responsible for antimicrobiall activity.

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