



Research Journal of **Microbiology**

ISSN 1816-4935



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Bio-Control of *Vibrio fluvialis* in Aquaculture by Mangrove (*Avicennia marina*) Seeds Extracts

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Abstract: The microbial community associated with mangrove plant (*Avicennia marina*) in Safaga (Red Sea) was studied, the heterotrophs (TVC), *Vibrio*, *Aeromonas* and *Staphylococcus* counts in sea water were 56000, 200, 300 and 160 cfu mL⁻¹, respectively. The mangrove stems harboured lower values and the roots harboured higher values. The dominant heterotrophs isolated from the roots and stems were: *Bacillus*, *Vibrio*, *Aeromonas* and *Pseudomonas*. Different extracts of the different parts of the plant (seeds, leaves, stems and roots) were applied on different bacterial pathogens such as: *P. aeruginosa*, *V. fluvialis*, *V. vulnificus*, *S. faecalis*, *E. coli*, *S. aureus* and *B. subtilis*. The chloroform extracts showed considerable activities against the different pathogens, while the activity of the ethanol extracts showed lower values. The chloroform seeds extracts inhibited the growth of all pathogens efficiently and recorded the highest activity unit (AU = 25.0) against the fish pathogen *V. fluvialis*. Chemical composition of the extract contained carbohydrates, proteins and lipids (2.58, 0.74 and 0.074 mg), respectively, in addition to flavonoids, triterpenoids, lignin and tannin (8.6, 3, 11 and 8%), respectively. The study extended to apply these extracts on *Nile tilapia* sp. (*Oreochromis niloticus*) aquaculture, 2.5 and 5 ml L⁻¹ of the chloroform seeds extracts were applied, 5 ml L⁻¹ showed satisfied results while the efficiency ranged from 64.1% in the second day to 79.4% in the six day.

Key words: Biocontrol, mangrove, *Vibrio fluvialis*, aquaculture, extracts, activity unit, triterpenoids

INTRODUCTION

The intensive use of antibiotics to prevent and control bacterial diseases in aquaculture has led to an increase in antibiotic resistant bacterial population (Alderman and Hastings, 1998; Teuber, 2001). Therefore, several alternative strategies to the use of antimicrobials have been proposed, such as the use of probiotics as biological control agents (El-Sersy *et al.*, 2006; Abdel-Tawwab *et al.*, 2008) or the use of the natural products (Henkel *et al.*, 1999). Marine macro and micro-organisms produce a dizzying array of active compounds including terpenes, steroids, peptides and alkaloids, which have potent activities as anti-microbial agents (Wanger-Dobler *et al.*, 2002).

Among the marine ecosystems, mangroves constitute the second most important ecosystem in productivity and sustained tertiary yield after coral reefs. Several mangrove species were used for antiviral activity against Newcastle disease, vaccinia, Semliki forest, encephalomyocarditis and hepatitis viruses. Bark extract of *Rhizophora mucronata* and leaf extract of *Bruigiera cylindrica* were highly effective against all viruses tested (Premanathan *et al.*, 1996).

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The chemistry of floral scent of a number of mangrove species has been investigated by Azuma *et al.* (2002). A total of 61 compounds were found and these were fatty acid and carotenoid derivatives or terpenoids. The chemical profiles of individual species appear to be unique ranging from only 2 compounds in *Kandelia candel* to 25 in *Nypah fruticans*. Synergistic action needs also to be taken into consideration, e.g., berberine isolated from rhizomes of *Berberis aristata* exhibits antimicrobial and antifungal activity and when used in conjunction with santonin, its activity increases (Singh *et al.*, 2001).

Nile tilapia, *Oreochromis niloticus* (L.) is an important species for fresh water aquaculture. Improving fish performance and disease resistance of cultured organisms are major challenges facing fish culturists. Moreover, bacterial diseases are one of the limiting factors for fish culture including Nile tilapia. In particular, *Vibrio fluvialis* causes mass mortalities in several species and is the aetiological agent of several diseases (Li *et al.*, 2006).

Therefore, this study was firstly designed to evaluate and identify the bacterial flora associated with the mangrove environment. Moreover, controlling the fish pathogen *V. fluvialis* in fish aquacultures by using mangrove extracts.

MATERIALS AND METHODS

Sample Collection

Mangrove plant (*Avicennia marina*) were collected from Safaga region (in the Red Sea Fig. 1a, b). This type of mangrove plant was the most dominant species in Red Sea which were collected during a project sampling process (Ecological monitoring of mangrove in the Red sea: Distribution, microbial community and bio-active extracts.) National Institute of Oceanography and Fisheries (NIOF).



Fig. 1: (a) Mangrove plant and (b) Mangrove forest on Red Sea shores (Safag)

Bacterial Indicators

Bacterial indicators used for detecting the antagonistic properties of mangrove extracts were *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 8739, *Bacillus subtilis*, *Vibrio fluvialis*, *Vibrio vulnificus*, *Streptococcus faecalis* and *Escherichia coli*. These strains were kindly provided by Dr. Gehan Abou El-ela, Dr. Nermeen El-Sersy and Dr. Hassan Ebrahim (NIOF).

Bacterial Cultures

All bacterial pathogenic strains were maintained on nutrient agar slants incubated at 30°C. Bacterial cultures were prepared by inoculating 100 mL nutrient broth medium (Oxoid LTD., Basing stoke, Hampshire, England), then shaken at (250 rpm) at 30°C for 24 h. The optical density of the used cultures at $A_{550} = 1$.

All media used were of pure grade and purchased from Sigma chemicals, USA.

Bacterial Identification

Twenty-five strains of heterotrophic bacteria were isolated from the roots and stems of the mangrove plants, purified and identified using Bergy's Manual of Systematic Bacteriology (Holt and Williams, 1994) and API 20A Kit (Biomereieux Comp).

Extraction Procedures of Antimicrobial Agent from Mangrove Parts

The mangrove plant were washed and shade-dried and then each part of the plant (stem, root, seed and leaf) was powdered separately. Ten grams of each part were macerated with 30 mL of 70% aqueous ethanol. The lipid-soluble extracts were prepared by adding 30 mL of chloroform-methanol (2:1 v/v) to the different powders. After soaking for a month, the extracts were filtered through Whatman 542 filter paper. Both ethanolic and lipid soluble extracts were then concentrated until complete dryness and finally resuspended in 4 mL of the appropriate solvent (Ballantine, 1987).

Antagonistic Action Against Pathogenic Bacteria

The antagonistic activity of mangrove extracts was detected using well cut-diffusion technique in which cut (5 mm) was punched upon the surface of nutrient agar plates inoculated with indicator strains mentioned before.

The radius of clear zone around each well (Y) and the radius of the well (X) were linearly measured in mm to calculate the activity unit (AU), which was calculated according the following equation:

$$AU = Y^2/X^2$$

where, Y is the radius of clear zone around each well and X is the radius of the well itself. This equation was applied according to El-Masry *et al.* (2002) to evaluate the activity unit. This test was done in duplicates and repeated twice.

Determination of the Different Components of Mangrove Seed Extract

Determination of the different components of mangrove seed extract was performed with the aid of Ghorban Association Private Laboratory, Alexandria Egypt.

Fish Culture and Feeding Regime

Tilapia sp. (*Oreochromis niloticus*) (10 cm) were obtained from El max fish farm, Egypt. Healthy fish were kept in an indoor glass tank for 2 weeks for acclimation to the laboratory conditions. Settled fish wastes were siphoned daily along with three quarters of the aquarium's water, which was replaced

by aerated water from tap water. 0.1 g of sodium thiosulphate was added for dechlorination purposes. Water temperature range was 26–27°C. Fish (40 g) were randomly distributed at three 12 L aquarium (12 fish per aquarium). Each aquarium was supplied with compressed air via air-stones using aquarium air pumps. The diets were fed at 10% of body weight during the period of study. Each diet was fed to aquaria once daily. The net calculated bacterial count from blank aquarium was done for the consideration of any possible bacterial contamination (the net viable bacterial count which shown in Table 3 = total viable count of *Vibrio* –blank count), where blank was the count of *Vibrio* in aquarium free from both *Vibrio fluvialis* and the mangrove extract.

Infection Study

At a late logarithmic phase of growth ($A_{550} = 1$), *V. fluvialis* (10^4 cfu mL⁻¹) was inoculated to each aquarium. The mangrove seeds extract were added to aquariums 1 and 2 at the concentrations of 2.5 and 5 ml L⁻¹, respectively. Aquarium 3 was used as control without the extract. Any dead fish was daily recorded and removed.

Bacterial Count

Vibrio sp. associated with fish water was monitored during the period of the experiment. Counting was applied by spreading 100 µL of fish culture water over a TCBS (Thiosulphate- Citrate-Bile-Sucrose) agar plates and incubating at 37°C for 24 h (Kobayashi *et al.*, 1963).

RESULTS

Bacterial Flora Associated with the Mangrove Environment

The data in Table 1, showed that the heterotrophs count in sea water was 56000 cfu mL⁻¹ while *Vibrio* group, *Aeromonas* group and *Staphylococcus* group were 200, 300 and 160 cfu mL⁻¹, respectively. In addition, no fecal pollution was detected; *Salmonella* group was also absent. In respect to the plant parts of the mangroove, the roots exhibited the higher count. Total viable counts (TVC) were 67233 and 3484 cfu g⁻¹ on both the root and the stem, respectively. While the count of the different groups on root was approximately double that on the stem.

The total viable count was estimated by following pour plate method on sea water agar. For detection of all other pathogenic strains spreading of 100 µL sample over their corresponding selective medium was applied in duplicates.

Identification of Heterotrophic Bacteria Associated with the Mangroove

Twenty five strains of heterotrophic bacteria were isolated from the roots and stems of the mangrove plants. The dominant species were *Bacillus* sp. (ten strains), *Vibrio parahaemolyticus* (four strains), *Pseudomonas aeruginosa* (three strains), *Aeromonas hydrophila* (three strains). While five strains were not identified.

The *Pseudomonas aeruginosa* was not counted by using selective medium. While during the identification of the 25 isolates from heterotrophic viable count (TVC), three isolates were identified as *Pseudomonas aeruginosa*.

Table 1: Viable count of different bacterial groups in sea water (cfu mL⁻¹) and mangrove plant (stem and root) (cfu g⁻¹)

Sample	TVC	<i>Vibrio</i> sp.	<i>Aeromonas</i> sp.	<i>Salmonella</i> sp.	<i>Staphylococcus</i> sp.	<i>S. faecalis</i>	<i>E. coli</i>
Sea water	56000	200	300	0	160	0	0
Stem	3484	166	130	0	170	0	0
Root	67233	250	300	0	320	0	0

The Antimicrobial Activities of the Mangroves Extracts

Different extracts from different plant parts were examined to determine their ability to produce antimicrobial agents against different pathogens. As shown in Fig. 2a and b, production of antimicrobial agents was found in the different parts of mangrove plant (roots, stems, leaves and seeds) which was extracted with chloroform. Moreover, it was also detected in mangrove seeds extracted with ethanol. No activity was found for the other different parts with ethanolic extraction. The highest productivity of antimicrobial agents was detected in chloroform extract of the seeds. This extract inhibited the growth of all pathogens efficiently. The detected activity units were 27.04 AU against *E. coli* followed by 25 AU against *V. fluvialis* and *P. aeruginosa*.

The root extracts also showed high activity (18,16,16 and 14 AU) for *V. fluvialis*, *V. vulnificus*, *S. aureus* and *E. coli*, respectively.

The leaf extract gave its highest activity against *V. fluvialis* (12 AU), *P. aeruginosa* and *S. aureus* (10.24 AU). The lowest activity estimated was detected in the stem extract ranging from 8.0 against *V. fluvialis* to 4.84 against *E. coli*. On the other hand, only ethanolic extract of seeds showed considerable efficiency against the different pathogens. The highest activity units were detected against *P. aeruginosa*, *S. faecalis* and *B. subtilis* (16 AU) (Fig. 2b).

We can conclude that the effect of chloroform extract on *Pseudomonas aeruginosa* exceeds that effects resulted from leafs, steams, seeds and roots extracts by approximately 2.5, 4 and 3 fold increase respectively.

The activity against *V. fluvialis* exceeded that produced from the other parts by 2, 3 and 1.5 fold, respectively. Similar results were observed for *E. coli* and *Streptococcus* sp. It is amazing that the effect of roots extracts on *S. aureus* exceeded that of the seeds extracts.

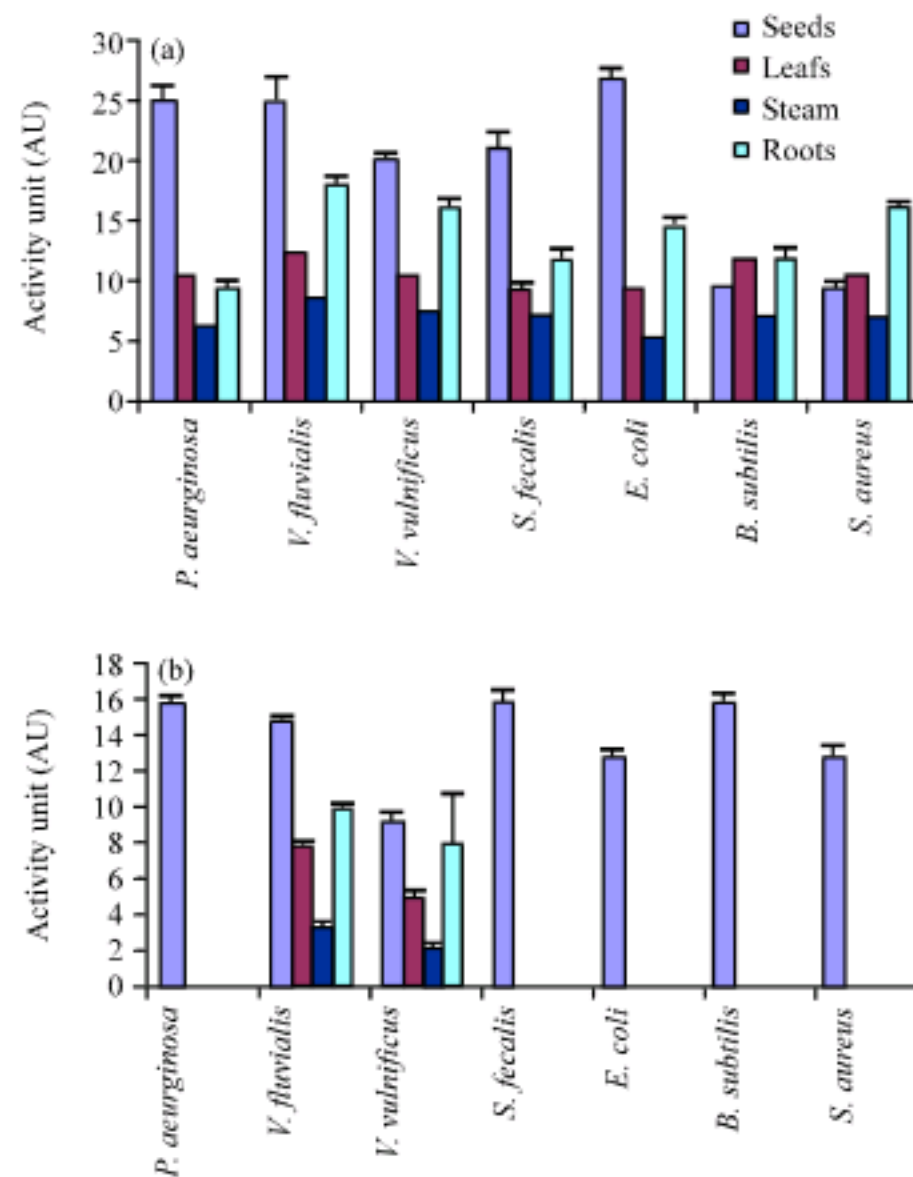


Fig. 2: The activity units produced by (a) chloroform and (b) ethanol extract from different mangrove plant parts against the different pathogens

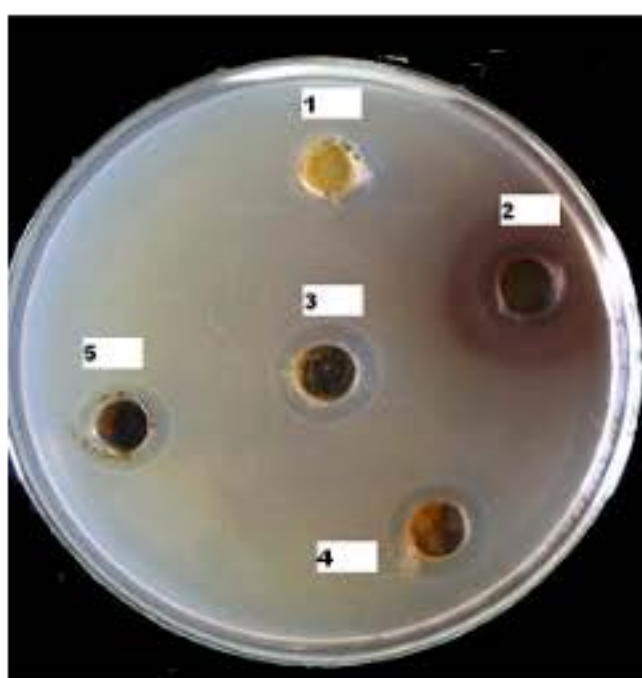


Fig. 3: Micrograph showing highest antagonistic activity of chloroform extract of different parts of mangrove plant (1; control, 2; seed, 3; root, 4; leaf, 5; stem) against *V. fluvialis*

Table 2: The chemical composition of chloroform seeds extracts

Compounds	Amount
Carbohydrates	2.58 mg mL ⁻¹
Proteins	0.74 mg mL ⁻¹
Lipids	0.074 mg mL ⁻¹
Amino-acids	833 μmol g ⁻¹
Flavonoids	8.6 %
Triterpenoids	3 %
N-alkanes	1.5 mmol g ⁻¹
Lignin	11%
Tannins	8 %

This part of study was done with the aid of Ghorban Association Private Laboratory previously mentioned at material and method section

The estimation of minimal inhibitory concentration (MIC) of each part of the extract on the different bacterial pathogens, was not the aim, but we tried to investigate the efficiency of the crude extract of the most efficient part of mangrove plant body in aqua-culture infected with *V. fluvialis*.

Each extract was resuspended in the same solvent which has no effect against the tested pathogens when tested separately as a control. This can be shown in Fig. 3 at spot no. 1.

This test was applied by testing 200 μL of different chloroform extract of different mangrove plant parts (from spot no. 2-5) against fish/pathogen *V. fluvialis* (spot no. 1 is control with chloroform only).

The Chemical Composition of Chloroform Seeds Extract

The extract contained carbohydrates (2.58 mg mL⁻¹), proteins (0.74 mg mL⁻¹), lipids (0.074 mg mL⁻¹), amino-acids (833 μmol g⁻¹). In addition, the extract also contained anti-microbial agents such as flavonoids (8.6%), triterpenoids (3%) and N-alkanes (1.5 mmol g⁻¹) (Table 2).

Determination of the Efficiency of the Chloroform Seeds Extract in Aqua-Culture Infected with *V. fluvialis*

The efficiency of the chloroform seeds extract was determined daily for seven successive days. Treatment 1 represented the lower concentration of the extract (2.5 mL) and treatment 2 was the higher concentration (5 mL). Treatment 2 was more effective than treatment 1 and its efficiency ranged from

Table 3: Application of chloroform seed extract on *Vibrio* counts in fish infected aquaculture

Day	<i>Vibrio</i> sp. count cfu mL ⁻¹				
	*Control	** Trial 1	Efficiency (%)	*** Trial 2	Efficiency (%)
1	700	250	64.3	200	71.5
2	1340	700	47.8	480	64.1
3	2400	1050	56.3	560	76.7
4	2500	1200	52.0	600	76.0
5	3200	1510	52.8	710	77.8
6	3500	1700	51.4	720	79.4
7	8000	3250	59.4	2000	75.0

*Control: Pathogen only; **Trial 1: Pathogen+2.5 ml L⁻¹ of antimicrobial agent; ***Trial 2: Pathogen+5 ml L⁻¹ of antimicrobial agent

64.1% in the second day to 79.4% in the six day. On the other hand, the efficiency of treatment 1 did not exceed 64.3% in the first day then, decreased to 47.8% in the 2nd day and ranged from 56.3 to 51.4% from 3rd to 6th day at the 7th day, it was 59.4% (Table 3).

We used 12 fishes per aquarium, at the third day, three fishes were lost from each aquarium i.e., 25%. At the fourth day, one fish from each aquarium was dead (one fish from the remaining nine fishes) i.e., approximately 11%.

Generally this type of research always done in an open system to be mimic to the actual fish aquaculture tanks. So the aim is to investigate the effect of present extract on water quality as well as fish. So, no need to be sure that the counted pathogens are identical with the inoculated one.

The extract had no lethal effect on fishes, the rate of mortality (25% at the third day) and (11% at the fourth day) equals that recorded for the blank where the blank was free from both the pathogen and the extract.

DISCUSSION

Bacterial diseases are responsible for heavy mortality in wild and cultured fish. The problems in farms usually tackled by preventing disease outbreaks or by treating the actual disease with drugs or chemicals. The use of antimicrobial agents has increased significantly in aquaculture practices (Alderman and Michel, 1992). Antibiotics used in both human as well as veterinary medicines have been tried experimentally to treat bacterial infections of fish. Problems including solubility, palatability, delivery and governmental restrictions have limited the available antibiotics to a select few, especially in food fish culture. Decreased efficacy and resistance of pathogens to antibiotics has necessitated development of new alternatives (Smith *et al.*, 1994).

Mangroves have enormous ecological value. They protect and stabilize coastlines, enrich coastal waters, yield commercial forest products and support coastal fisheries. Mangrove forests are among the world's most productive ecosystems, producing organic carbon well in excess of the ecosystem requirements and contributing significantly to the global carbon cycle. Extracts from mangroves and mangrove-dependent species have proven active against human, animal and plant pathogens. Mangroves may be further developed as sources of high-value commercial products and fishery resources and as sites for a burgeoning ecotourism industry. Their unique features also make them ideal sites for experimental studies of biodiversity and ecosystem function (Kathiresan and Bingham, 2001).

Two basic factors justify the study of the chemical constituents of mangrove plants. Firstly, they possess modifications to establish water and salt economy. There are modifications or alterations in other physiological processes such as carbohydrate metabolism or polyphenol synthesis and due to these reasons, they may have chemical compounds, which protect them from these destructive elements. The second reason is that numerous mangrove plants have been used in folklore medicine, and recently, extracts from mangroves and mangrove-dependent species have proven active against

human, animal and plant pathogens, but only limited investigations have been carried out to identify the metabolites responsible for their activities (Bandaranayake, 2002).

Mangroves are highly productive marine ecosystems where bacteria (culturable and nonculturable) actively participate in biomineralization of organic matter and bioremediation of minerals. *Vibrio* sp., *Aeromonas* sp., *Serratia* sp., *Pseudomonas* sp. and *alcaligenes* sp. were detected in mangrove environment (Acosta *et al.*, 2006) while in this environment *Vibrio* sp., *Aeromonas* sp. and *Pseudomonas* sp. were detected.

These flora found in our environment and other environments associated with the mangrove habitats i.e., these flora is specific to the mangrove habitats and their presence not only affected by the water pollution or the environmental factors but mainly due to presence of mangrove plants.

Choudhury *et al.* (2005) reported that screening of organic solvent extracts of five mangroves (*Aegiceras corniculatum*, *Aegialitis rotundifolia*, *Aglaia cucullata*, *Cynometra iripa* and *Xylocarpus granatum*) showed species specific activity in inhibiting the growth of six virulent strains of bacterial pathogens to fish viz., *Edwardsiella tarda*, *Vibrio alginolyticus*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa* and *Aeromonas hydrophila* (2 strains).

The studies of Premnathan *et al.* (1992, 1996, 1999) revealed that the mangroves were found highly effective for antiviral activity as compared to seaweeds and seagrasses. Kokpal *et al.* (1990) has also reported the bioactive compounds from mangrove plants. Some mangroves had shown insecticidal activity (Miki *et al.*, 1994; Ishibashi *et al.*, 1993). Wu *et al.* (1997) reported the cytotoxic and antiplatelet aggregation activity of methanol extract of *Aglaia elliptifolia*.

Abraham *et al.* (2002) reported that antibacterial and antifungal activities of alcoholic extracts of *Holothurian* species such as *Actinopyga echinites*, *A. miliaris*, *Holothuria atra* and *H. scabra* of Tamil Nadu coast were studied. Bacteria such as *Aeromonas hydrophila*, *Escherichia coli*, *Enterococcus* sp., *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Staphylococcus aureus* and *Vibrio harveyi* and fish born mold, *Aspergillus* sp. were inhibited at varying levels by the extracts of *A. milaris*, *H. atra* and *H. scabra*. *Bacillus* sp. was not affected by *Holothurian* sp. extracts. The results of this study revealed the presence of antimicrobial substances possibly steroidal sapogenins with medical importance in *Holothuria* sp. Moreover, Padmakumar and Ayyakkannu (1997) found that *S. aureus* was the most susceptible bacterial pathogen to mangrove extracts followed by *Vibrio* sp. where as *P. aeruginosa* was most resistant but in disagreement with this results, *E. coli*, followed by *P. aeruginosa* and *V. fluvialis* were the most susceptible bacterial pathogens while *B. subtilis* and *S. aureus* were the most resistant.

Opsahl and Benner (1999) reported that in mangrove wood, the total carbohydrate yields can represent up to 65.5% of organic carbon. In this study, carbohydrates concentration was 2.58 mg mL⁻¹ in seed extract.

Amino acids can represent up to 9% of the mangrove leaves biomass (Hernes *et al.*, 2001). But very few studies have described their composition in mangrove tissues. Zieman *et al.* (1984) reported that concentrations of total amino acids in *Rhizophora* leaves of 833 Mmol g⁻¹ with glutamic acid, leucine and glycine representing each more than 10%. *Avicennia* leaves, on the other hand, contain mostly glycine, glutamic acid and aspartic acid (Tremblay and Benner, 2006). The concentration of amino acids was 833 mmol g⁻¹ in present seed extract.

Tannins in vascular plants occur as two types, condensed and hydrolysable. They are more abundant in plant leaves than in woody tissues and contribute to the color and astringency of the bulk organic matter (Hernes *et al.*, 2001). Tannins concentrations are 8% in the present study.

Lignin is a nitrogen free copolymer of various phenylpropenyl alcohols that is present in vascular plants. Mangrove species exhibit a typical vascular-plant lignin signature, with great variations between leaves and wood, the latter being richer in lignin oxidation product. Marchand *et al.* (2005) reported that total carbohydrates ranged from 3.8 to 5.1% in leaves and wood material, respectively, but in present results it reached up to 11%.

Alencar *et al.* (2007) showed that, propolis has been used as a medicinal agent to treat infections. Its antimicrobial activity against *S. aureus* ATCC25923 and *S. mutans* UA159 was evaluated and the chloroform fraction was the most active and the hexane fraction having the highest concentration of total flavonoids showed the best sequestering activity for the free radical. In this research, flavonoids concentration was measured as 8.6% in the seed extract.

Moreover, the triterpenoids represented 3%, in the same extract. Dodd *et al.* (1998) reported 11 triterpenoids in epicuticular waxes accounting for up to 3.5% of *Rhizophora mangle* leaves from West Africa.

Oku *et al.* (2003) suggested that triterpenoids may have a special function in the degradation of mangrove to salt stress, which may explain their richness.

Chen *et al.* (2008) isolated a new triterpenoid from the rhizome of *Vladimiria muliensis*. Compounds 2 and 4 exhibited modest antimicrobial activity against *Escherichia coli*, *Candida albicans*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Bacillus cereus* and *Staphylococcus aureus*.

N-alkanes comprises 1.5 mmol g⁻¹. Long chain n- alkanes (between 25-35 carbons) are characteristic components of epicuticular waxes of mangrove leaf surfaces and can also be used as tracers of higher plant remains (Versteegh *et al.*, 2004). Dodd *et al.* (1999) suggested that the n-alkane composition of mangroves can be linked to environmental conditions and attributed the dominance of longer chained C31 and C33 n-alkanes of *A. marina* in the United Arab of Emirates to its evolution under arid conditions.

Overall, the results indicate that extracts prepared from Red Sea mangrove parts exhibit significant antibacterial activity. The chloroform extract, in particular, had considerable activity against most pathogens especially *Vibrio fluvialis*, so these extracts can safely be applied in aquaculture for recovery and treatment purposes. In addition, these compounds have medical importance with low cost.

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