SCREENING OF "LAB-LAB", SELECTED MANGROVE PLANT AND SEAWEED FOR ANTIMICROBIAL COMPOUNDS

By

K. PAU BIAK LUN, B.F.Sc.

THESIS

Submitted in partial fulfillment of the requirement for the degree of

MASTER OF FISHERIES SCIENCE

Faculty of Fisheries

Kerala Agricultural University

2008

DEPARTMENT OF AQUACULTURE
COLLEGE OF FISHERIES
PANANGAD, COCHIN

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Dedicated
To
My family
&
Dr.Devika Madam

DECLARATION

I hereby declare that this thesis entitled **"SCREENING OF "LAB-LAB", SELECTED MANGROVE PLANT AND SEAWEED FOR ANTIMICROBIAL COMPOUNDS**" is an authentic record of the work done by me and that no part thereof has been presented for the award of any degree, diploma, associateship, fellowship or any other similar title.

Place: Panangad — K.PAU BIAKLUN

Date: 16-08-2008 — 2006-14-108

CERTIFICATE

Certified that this thesis entitled "**SCREENING OF "LAB-LAB", SELECTED MANGROVE PLANT AND SEAWEED FOR ANTIMICROBIAL COMPOUNDS**" is a record of research work done independently by Mr. K. Pau Biaklun under my guidance and supervision and that it has not previously formed the basis for award of any degree, fellowship or associateship to him.

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EXTERNAL EXAMINER

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INTRODUCTION

1. INTRODUCTION

Frequent occurrence of disease is causing heavy mortality of cultured aquatic organisms. In recent years, several disease outbreaks have caused major problems and brought devastation in farming. Disease problems are tackled either by preventing disease outbreaks or by treating the disease with drugs or chemicals. The regular use of commercial antibiotics adversely affects not only human health but also the environment. Decreased efficacy and resistance of pathogens to antibiotics has necessitated development of new alternatives (Smith *et al.*, 1994)

In the search for effective antimicrobial products, natural products are being increasingly looked at as an alternative for chemical drugs and antibiotics. Exploration of the marine environment as an exciting and rich resource to isolate new compounds began during the 1960s. So far, more than 3700 new natural products have been separated from these groups (Ninawe, 2006).

Mangrove plants and seaweeds widely found in the inter-tidal zones and in clear tropical waters contribute an important source of natural antimicrobial activity. The fatty acids in the litter fall of mangroves were suggested to have a positive role on the growth of fishes and shrimps (Changyi *et al*., 1997). Mangroves were also found to have good antiviral activity. Surprisingly, however, there are not many studies on the antibacterial activity of mangroves.

Many bioactive and pharmacologically active substances have been isolated from micro and macroalgae. Seaweed contains compounds like acrylic acids, halimedatrial and alkaloids and flavanoids as their natural bioactive compounds. There are several reports of studies on the crude extracts of seaweeds and other microalgae in different solvents, that were tested against gram positive and gram negative bacteria (Rao and Parekh

1981a; Pesando and Caram 1984). Studies have shown that fatty acids in the microalgae were responsible for the antibacterial activity (Borowitzka and Borowitzka 1989).

Seaweeds have been traditionally used as food by the people of the far east, mainly, the Japanese, Chinese and Koreans. The green tiger shrimp *Penaeus semisulcatus* and the Kuruma shrimp *Marsupenaeus japonicus* are known to hide or take shelter within sea grasses. It is also known that *Penaeus monodon* cultured along with the sea weed *Gracilaria* sp. grows faster. It is likely that apart from shelter that the sea weeds provide, they also help in the better growth and survival of shrimps. This prompted us to study the antibacterial properties of *Gracilaria*.

Cyanobacteria have been identified as one of the most promising group of organisms from which novel and biochemical active natural products are isolated. They represent a rich opportunity for discovery since they are largely unexplored. “Lab-lab” is a plant animal complex that consists of a benthic community of cyanobacteria, diatoms, and associated invertebrates. The major algae in lab-lab consisted of the blue green algae *(Cyanophyta*), the green algae (*Chlorophyta*) and the diatoms (*Bacillariophyta)*. The cyanobacteria, particularly, *Lyngbea* and *Oscillatoria* predominate in ‘lab-lab’. It grows as a mat on the pond bottom and is the most desired natural food for milkfish. The presence of associated organisms gives “lab-lab” its high food value and may contribute to its antimicrobial properties. This may be the reason for the lower incidences of disease occurrence in milkfish. The diatoms were dominant during the dry season while the cyanophyta dominated during the wet season (Fortes and Pinosa 2007)

In the present study the antibacterial properties of the mangrove *Avicennia officinalis*, the red algae *Gracilaria corticata* and “lab-lab” against gram positive and negative bacteria were studied. An attempt was also made to identify the active ingredient responsible for the antibacterial activity.

REVIEW OF LITERATURE

2. Review of literature

Bacterial diseases are responsible for heavy mortality in aquaculture. As a result, 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. Repeated use as a prophylactic and treatment measure has led to decreased efficacy of many of the chemicals/drugs. In addition, development of resistance of pathogens to antibiotics is a serious concern that needs to be addressed. This has the necessitated development of new alternatives (Smith *et al*., 1994).

The aquatic environment is a reservoir of bioactive natural products. Use of natural compounds for disease treatment started in 1970s and great progress have been made over the past three decades (Yan 2004). Many antimicrobial compounds and other pharmacologically active substances have been isolated from marine microbes, blue green algae, seaweeds, sponges, corals etc. Some of the relevant information on antimicrobial activity of algae, mangroves and cyanobacteria has been reviewed here.

2.1. Plant antibacterial activity

Nowadays, the plant natural products are seem to be of great importance than the synthetic chemical compounds used for the resistances in the development of disease in world. The plant natural products are better and non-toxic to the natural environment. Plants are considered as house of medicines and played an important role in nearly every culture on earth including Asia, Africa, Europe and the Americas. A study was conducted to analyze the leaves and seeds of *Foeniculum vulgare* for different bioactive compounds against methanol, n-propanol, ethanol and diethyl ether. The compound includes saponins, total proteins, amino acids, fat and flavonoids with one and two dimensional thin layer and column chromatography followed by spectrophotometric analysis. Results indicated that leaves

contained higher concentration of flavonoids and fat. Where as level of Saponins, proteins, amino acids, total minerals and other organic compounds was high in seeds (Zaidi, 1998).

Ajith and Janardhanan (2001) reported the antimicrobial activity of a wood inhibiting polypore macrofungus, *Phellinus rimosus* (berk) in which ethyl acetate, methanol and water extracts showed activity against *E.coli, P.aeruginosa*, *S. aureus*, *S. typhi, P. vulgaris, Agrobacterium tumifaciens*, *Klebsiella pneumoniae* and *Shigella spp.* at a concentration of 800 microgram per well. However, aqueous extract solution did not show antibacterial activity upto 1 microgram per well.

Moreover, the studies on bark of a plant called the Marula bark which is widely used for bacteria-related diseases by indigenous cultures in Africa. Bark and leaves were extracted with acetone and MIC values were determined using a microplate serial dilution technique with *Staphylococcus aureus, P.aeruginosa, E.coli* and *Enterococcus faecalis* as test organisms. Based on minimum inhibitory concentration values, inner bark extracts tended to be the most potent followed by outer bark and leaf extracts. Using bark may be detrimental to the plant, but leaf material can also be used for antibacterial application (Eloff, 2001).

2.1.1. Algae antibacterial activity

Many bioactive and pharmacologically active substances have been isolated from algae. The antimicrobial activities of five compounds extracted from marine algae were tested against *Staphylococcus aureus, S. choleraesuis and E. coli* (James *et al*., 1974). Three of the compounds cycloendesmol, laurinterol and debromolaurinterol exhibited activity at concentration approaching that of streptomycin. There are similar reports of studies on the antibacterial activity of extracts of different marine algae (Sachithananthan and Sivapalan, 1975, Mahasneh *et al*., 1995; Siddhanta *et al.,* 1997). Analyses of the antibacterial activities of microalgae showed that in most cases fatty

acids in the algae contributed to the antibacterial activity (Cooper *et al*., 1983; Findlay and Patil, 1984; Viso *et al*., 1987; Kellam *et al.*, 1988).

Just as macroalgae, the microalgae also are significant resources for bioactive metabolites. Moore *et al.* (1988) has shown that the marine microalgae such as the blooms of *Phaeocystis pouchetii* produce chemicals such as acrylic acid, which constitutes about 7.0% of the dry weight of the algae and shows antibacterial activity.

The studies on microalgae strains isolated from different freshwater reservoirs situated in various topographies in Turkey were tested by agar-well diffusion method for their antimicrobial agent production on various organisms (*B.subtilis*, *B. thuringiensis*, *B. cereus*, *B. megaterium*, *Yersinia enterocolitica*, *E. coli*, *S. aureus*, *Micrococcus luteus*, *Micrococcus flavus*, *P. aeruginosa*, *Saccharomyces cerevisiae*, *Candida albicans* and *Candida tropicalis*). The study revealed that acetone and ether extracts were found to possess antimicrobial activity against gram negative bacteria, the methanol extracts on gram positive bacteria and ethanol extracts on both gram positive and gram negative organisms. Chloroform extracts, on the other hand, did not show any antimicrobial activity (Hikmet *et al*., 2006).

Rajeev *et al*. (2006) studied the marine microalgal extracts for their antibacterial activity against multi-drugs resistant human pathogens. Among the microalgae which were cultured and extracts screened against human pathogens, *Isochrysis galbana* extract showed highest percentage of antibacterial activity. Among the solvents used for the extraction of antimicrobials, n-butanol showed maximum extraction of antimicrobials.

Goud *et al*. (2007) reported that microalgae are of significant attraction as natural source of bioactive molecules. Different fresh water algal species were screened by them for their antibacterial activity and biomolecules. Maximum antibacterial activity was observed in methanol extracts and least in aqueous extracts. Maximum activity was observed in the extracts of *Nostoc, Lyngbya, Mougeotia and Pithophora* sp. Gram-positive bacteria were more

susceptible than gram-negative bacteria. The species contain tannins, phenols, steroids, flavonoids and saponins. The authors suggested that the pharmacological activities and bioactive molecules from these microalgae can be highly exploited.

2.1.1.1. Cyanobacteria

The fact that cyanobacteria in general and marine forms in particular are one of the richest sources of novel bioactive compounds including toxins with wide pharmaceutical applications is unquestionable. Cyanobacteria (blue-green algae) and other eukaryotic algae occur in freshwater, marine and terrestrial (soil) habitats. A number of cyanobacteria and eukaryotic algae, produce various biologically active compounds. These include antibiotics, which in laboratory tests inhibited bacteria and fungi that incite diseases to humans.

Many cyanobacteria and algae are known to produce a large number of antibacterial and antifungal materials. Among the five divisions of microalgae, studies of biomedical natural products have been concentrated on only two divisions, i.e., Cyanophyta (blue-green algae) and Pyrrophyta (Dinoflagellates). Although several metabolites have been isolated from cyanophytes, most of them are isolated from fresh water species, which are cultured easily in comparison to marine organisms.

The organic solvent extracts of cyanobacteria and eukaryotic freshwater algae, were screened by Cannell *et al*. (1988) for antibacterial activity against *B.subtilis, E.coli* and *S.aureus*. Activity was detected in these algae in the methanol extracts. In the preliminary screening, activity was found only against the gram-positive bacteria and not against *E.coli*.

The *in vitro* antimicrobial activity of the methanol extract of the blue-green alga, *Microchaete tenera* against *Proteus vulgaris, Bacillus cereus, Escherichia coli, Pseudomonas aeruginosa, Aspergillus niger Aspergillus flavus* and

Rhizopus nigricans was studied using agar cup-plate method (Koehn *et al*., 1992). They observed significant antibacterial activity against *Pseudomonas aeruginosa*, good antimicrobial activity against *Proteus vulgaris* and *Aspergillus niger.*

Namikosh and Rinehart (1996) reported that cyanobacteria produce a large number of compounds with varying bioactivities and antibacterial activity. In general, cyclic peptides and depsipeptides are the most common structural types, but a wide variety of other types were also found such as linear peptides, guanidines, phosphonates, purines and macrolides. The authors opined that the close similarity or identity observed in structures between cyanobacterial products and compounds isolated from sponges, tunicates and other marine invertebrates suggest that the latter compounds may be derived from dietary or symbiotic blue-green algae.

Prasantkumar *et al*. (2006) have worked on the antimicrobial activity of the methanol extract of a blue-green alga and two green algae against *Proteus vulgaris, Bacillus cereus, Escherichia coli, Pseudomonas aeruginosa, Aspergillus niger, Aspergillus flavus* and *Rhizopus nigricans* using agar cup-plate method. Blue-green alga (*Microchaete tenera*), green algae (*Nitella tenuissima*) and *Sphaeroplea annulina* showed significant antibacterial activity against *Pseudomonas aeruginosa*. *Microchaete tenera* showed good antimicrobial activity against *Proteus vulgaris* and *Aspergillus niger*.

2.1.1.2. Seaweed antibacterial activity

More than ten thousand species of marine algae (seaweed) have been reported from all over the world, some of which have very important economic value for their nutritional and healing properties. Seaweeds are rich in iodine and used in the preparation of food products as well as agar. There are a number of studies on the antibacterial activity of seaweeds.

Lipid extracts of 13 algae from Eastern Sicily were tested for antimicrobial activity against *Bacillus subtilis* and *Phoma tracheiphila*, and for antiviral activity against tobacco mosaic virus. *Zanardinia prototypus* and *Cystoseira balearica* exhibited the best antimicrobial and antiviral activity among the species tested, while *Lophocladia lallemandii* was inactive against the bacterium it had high inhibitory effect against the virus (Caccamese *et al.,* 1981). Enrichment of the antibacterial activity of *Laurencia obtusa* was possible during fractionation process.

Enteromorpha intestinalis and *G. corticata* collected from Gujarat coast of India were tested for antibacterial activity (Rao and Parekh 1981b). The crude extracts of these seaweeds were active throughout the year with a peak during the winter season. They were active only against gram-positive bacteria. The authors also tested the acetone and ethanol extracts of marine algae *Cladophora fascicularis, Caulerpa taxifolia, Chaetomorpha antennina, Ulva lactuca* and *G. corticata* collected from south-west coast of India in three seasons and found good inhibitory activity against *Bacillus subtilis*.

Different species of British marine microalgae have been screened for the production of antibacterial properties by Hornsey and Hide (1993). Of these, *Asparagopsis armata, Bonnemaisonia asparagoides, Bonnemaisonia hamifera*, *Chondrus crispus* and most members of the family Rhodomelaceae appear to possess outstanding antibacterial properties. In the case of two closely related and morphologically similar species, *Chondrus crispus* and *Gigartina stellata,* the former possessed considerable degrees of antimicrobial activity whilst the latter exhibited no such activity.

Extracts from seaweeds (macroalgae) sprayed on plants have been reported to reduce the incidence of *Botrytis cinerea* (gray mold) on strawberries, *Erysiphe polygoni* (powdery mildew) on turnips, and damping-off of tomato seedlings (Kulik, 1995).

Vlachos *et al*. (1996) has studied the effect of post collection storage time and season of selected Southern African marine macro algae, of *Sargassum incisifolium*, *Zonaria subarticulata* on the antibacterial activity. Antibacterial activity was determined by pipetting extracts into wells in overlay agar seeded with test bacteria. The antibacterial activity of *Zonaria subarticulata* increased post collection storage time, while that of *Sargassum incisifolium* decreased.

Ethanol extracts from 56 Southern African seaweeds from the divisions Chlorophyta (green algae), Phaeophyta (brown algae) and Rhodophyta (red algae) was tested against *Aeromonas* and *Psuedomonas* for antibacterial activity and the highest antibacterial activity was reported for Phaeophyta for both the bacteria (Vlachos *et al*., 1997). Similar results were reported earlier by Caccamese and Azzolina (1979) and Pesando and Caram (1984) for screening studies on seaweeds of Mediterranean and Eastern Sicily coast.

In a study on the effect of *Ulva* meal supplementation on fish, the seaweed was found to provide disease resistance to red sea bream in Japan and high growth rate in black sea bream (Nakagawa *et al.*, 1987).

Febles *et al*. (2000) carried out studies on a number of brown (Phaeophyta) and green (Chlorophyta) seaweeds that were collected from the littoral of Tenerife (Canary Islands). Extracts from them showed the potential of antibacterial and antifungal activity. Three different solvents n-hexane, ethyl acetate and methanol have been used to obtain extracts from the Phaeophyta: *Sargassum desfontainesii* (Turner) *C. Agardh*, *Halopteris scoparia* (Linnaeus) *Sauvageau* and *Stypopodium zonale* (Lamouroux) Papenfuss; and the Chlorophyta: *Codium intertextum* and *Ulva rigida* (Clemente) *C. Agardh.* The activities of the extracts were tested using gram-positive and gram-negative bacteria. The methanol extract showed highest antibacterial activity. The extracts were mainly active against gram positive bacteria.

Some of the macro-algal crude extracts indicated their potential therapeutic nature when challenged with potential pathogens among fish and shellfish. Among the crude extract of the red algae, halogenated lipids have been isolated, particularly from the *Laurencia sp*. The rare chemical prostaglandin was also reported to occur in *Gracilaria pichenoids*. (Lipton, 2001).

The green alga *Codium iyengarii* from the Karachi coast of the Arabian Sea has been found as the source of a steroid, iyengadione and two new steroidal glycosides, iyengarosides A and B. Iyengaroside-A displayed moderate activity against a range of bacteria (Ali *et al.,* 2002).

Bhosalel *et al*. (2002) studied the antifouling potential of some marine organisms from India against species of *Bacillus* and *Pseudomonas*. The crude methanolic extracts of brown algae were screened for antibacterial properties against strains of bacteria from *B. pumilus* and *P. vesicularis.* The *Padina tetrastromatica* (brown algae) extract exhibited significant activity against *B. pumilus* and *P. vesicularis*.

Certain studies showed a variation in the antibacterial activity of different parts of the same algae. For instance, Southern African seaweeds were analyzed to assess the distribution of antibacterial activity in extracts from selected portions of their thalli. Seaweeds were separated into meristem and thallus, extracted and tested for antibacterial activity against gram-positive and gram-negative food-associated bacteria by agar diffusion. Extracts of the meristem of *Ecklonia radiata* showed more antibacterial activity than corresponding blade and stipe extracts. The antibacterial activity of meristem extracts was less or equal to that of thallus extracts. (Vlachos, 1999). Similarly, Pelegrin and Morales (2004) reported that evaluation of the antimicrobial activities associated with extracts from different thallus regions (apical, basal and stolon) of selected *Caulerpa spp.* (*C. ashmeadii, C. paspaloides and C. prolifera*) showed the highest antibacterial activity in stolon of *Caulerpa*.

Felix and Rajeev (2004) tested the extracts of two seaweeds viz., *Gracilaria verrucosa* and *Ulva lactuca* against shrimp bacterial pathogens viz., *Vibrio alginolyticus* and *V. parahaemolyticus*. Among the seaweed samples, *G.verrucosa* showed better inhibitory activity against both *V. alginolyticus* and *V. parahaemolyticus*. Butanol extracts of *G. verrucosa* recorded the maximum inhibitory activity against the shrimp pathogens *V. parahaemolyticus* and *V. alginolyticus*.

Pelegrin and Morales (2004) studied ethanolic and lipid-soluble extract from marine algal species of the coast of Yucatan, Mexico and evaluated them for antibacterial activity against pathogenic gram-positive and gram-negative microbes. All species of Chlorophyta, Phaeophyta and Rhodophyta were active against the gram-positive bacteria (*Bacillus subtilis, Streptococcus faecalis* and *Micrococcus luteus*) and most of the algal species exhibited activity against *B. subtilis*.

The methanol, dichloromethane, hexane, chloroform and volatile oil extracts of the red alga *Jania rubens* were tested *in vitro* by Yavasoglu *et al.* (2004) for their antimicrobial activity against gram-positive and gram-negative bacteria and fungi *Candida albicans*. Analysis of the volatile components of *J. rubens* showed that *n*-docosane (6.35%), *n*-eicosane (5.77%) and *n*-tetratriacontane (5.58%) were the major components. The methanol and chloroform extracts (4 mg/disc) showed more potent antimicrobial activity than the hexane and dichloromethane extracts.

Surface extracts of *Bonnemaisonia hamifera* tested on bacteria by a standard disc-diffusion assay showed that metabolites are naturally present at sufficiently high concentrations in order to inhibit bacterial growth on the surface of the seaweed (Nylund *et al.,* 2005). *In situ* quantification of bacteria on *B. hamifera* also showed that this alga had significantly fewer bacteria on its surface compared to a co-existing alga.

In vitro screening of organic solvent extracts of three marine algae viz., *Gracilaria corticata*, *Ulva fasciata* and *Enteromorpha compressa* and

five mangroves viz., *Aegiceras corniculatum*, *Aegialitis rotundifolia*, *Aglaia cucullata*, *Cynometra iripa* and *Xylocarpus granatum* showed species specific activity in inhibiting the growth of six virulent strains of bacteria pathogenic to fish viz., *Edwardsiella tarda*, *Vibrio alginolyticus, Pseudomonas fluorescens*, *Pseudomonas aeruginosa* and *Aeromonas hydrophila* (2 strains). Three methanol extracts of *C. iripa* were active against all the six pathogens, whereas *A. corniculatum* and *A. cucullata* were active against four of the pathogens. The chromatographic fractionation of active extracts of *A. corniculatum, C. iripa* and *G. corticata* resulted in enriched fractions with wide spectrum activity and lowered values of minimum inhibitory concentration (Choudhury *et al*., 2005).

The screening of seaweed species from the coast of Urla for antimicrobial activity by using methanol, acetone, diethyl ether and ethanol extracts were carried out *in vitro* against *Candida spp., Enterococcus faecalis, Staphylococcus aureus, Streptococcus epidermidis, Pseudomonas aeruginosa,* and *Escherichia coli* by the disc diffusion method (Ney *et al*., 2006). Diethyl ether was found to be the best solution for extracting the effective antimicrobial materials from the algae species used in this experiment. Diethyl ether extracts of fresh *Cystoseira mediterranea, Enteromorpha linza, Ulva rigida, Gracilaria gracilis,* and *Ectocarpus siliculosus* were effective against all tested organisms. In addition, a comparison of dried and fresh extract on antimicrobial activity showed that all tested organisms were more sensitive to fresh extracts of the algae. Although fresh extracts of *G. gracilis, D.linearis,* and *E. siliculosus* inhibited the tested bacteria, their dried extracts had no inhibition activity on either gram negative or gram-positive bacteria.

2.1.2. Mangrove antibacterial activity

The mangroves found abundantly in the coastal swampy areas are a favoured habitat for different kinds of aquatic organisms. Studies have shown that mangroves have potent antimicrobial properties. Several bioactive compounds have been identified in them (Kokpal *et al*., 1990). Some

mangroves showed insecticidal activity (Ishibashi *et al*., 1993; Miki *et al*., 1994). The study of Premnathan *et al.* (1992, 1996) revealed that the mangroves were found highly effective for antiviral activity as compared to seaweeds and sea grasses.

Changyi *et al*. (1997) opined that the fatty acids (PUFA) in litter fall of mangroves might have positive role on the growth of fishes and shrimps. However, literature on the antibacterial activity of mangroves is scanty.

Two subspecies of *Physostegia virginiana* from seven different origins were investigated for iridoid glycosides. The plant material included the authentic sources of 'deoxyloganic acid'. This substance has now been identified as a mixture of 7-deoxy-8-epiloganic acid and 10-deoxygeniposidic acid. The isolated compounds were of iridoids *viz* stegioside-I from *P. virginiana* (origin Freiburg) and stegiosides-II and-III from *P. virginiana* (origin Chicago) (Nass and Rimpler, 1998).

In vitro assessment of the antibacteriophage, antibacterial and anticandidal activities as well as cytotoxicity were evaluated for both aqueous and ethanol extracts prepared from roots, cotyledons, leaves and stems of *Avicennia marina* (Khafagi *et al.,* 2003). Aqueous extracts of both shoots and roots of the seedlings demonstrated anti-bacteriophage activity using coliphage against *Escherichia coli* which indicated antiviral activity. Aqueous extracts also exhibited moderate cytotoxicity against the larvae of the brine shrimp *Artemia salina*, suggesting antiplasmodial and antimalarial activities. However, both aqueous and ethanol extracts of various parts of the seedlings lacked antimicrobial activities against eight microbial test strains. Phytochemical analysis of ethanol extract of the shoot system indicated the presence of some biologically active metabolites including tannins, flavonoids, sterols, iridoid glycosides and organic acids.

Methanol extracts of leaf, trunk, and bark of six species of mangrove trees were examined for antimicrobial activity. The screening of the

antimicrobial activity by using methanol extract of *Rhizophora mucronata* bark showed it to be active on *Staphylococcus aureus* (Kazuhiko *et al.,* 2003).

Agoramoorthy *et al.* (2007) have reported that fatty acids are widely occurring in blind-your-eye mangrove and they are known to have antibacterial and antifungal properties. They reported that the antibacterial and antifungal properties of the blind-your-eye mangrove (*Excoecaria agallocha)* was composed of palmitic acid and lauric acid, which were tested on *Bacillus subtilis, Staphylococcus aureus, Bacillus pumilus, Micrococcus luteus, Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Escherichia coli* and fungi *Candida albicans, C. krusei and C. parapsilosis and Candida tropicalis* against the solvents of methanol, benzene and sulphuric acid (200: 100: 10 v/v). The highest zone of inhibition was produced by extract of *Excoecaria agallocha* against *B. subtilis* and *S. aureus*.

2.2. "Lab-Lab"

"Lab-lab", a plant-animal complex is found commonly in fertilized brackishwater ponds. It is comprised of blue green algae (Cyanobacteria), and the green algae (Chlorophyta), diatoms (Bacilliarophyta) and dinoflagellates as the major plant components. The diatoms were dominant during the dry season while the cyanobacteria dominated during the wet season (Fortes and Pinosa 2007). Twenty eight genera were observed during the dry season and 25 genera were noted in the wet season. Surprisingly, however, there is no report on the antibacterial activity of this complex.

MATERIALS AND METHODS

3. Materials and methods

The leaves and bark of the mangrove *Avicennia officinalis*, the seaweed *Gracilaria corticata* and "lab-lab" were screened for antibacterial activity. Leaves and bark of *Avicennia* sp. were collected from the brackishwater areas of the college itself while *G.corticata* was collected from the rocky shores in Kollam region in Kerala during low tide. Lab-lab was collected from the brackishwater ponds of Kerala Agricultural University Fisheries Research Station, Puduveypu, Kochi during September-October. While seaweeds occur throughout the seas of the world, mangroves are common in the coastal regions of the tropics. "Lab-Lab" is naturally formed in milkfish ponds after manuring and is their preferred food.

3.1. Preparation of Extracts

The collected materials were brought to the laboratory in sealed polyethylene bags. They were then thoroughly cleaned with fresh water and used for extraction immediately. Whenever it was not possible to carry out the extraction immediately, the materials were stored in a refrigerator at 4^0C and extracted later.

Leaves and bark of *Avicennia officinnalis* (Plate 1) were cut into small pieces and ground with glass powder to ensure proper grinding and better extraction. The extractions were carried out using different solvents varying in polarity, with 1:1 ratio of sample:solvent (w/v), i.e., 5 g of sample was extracted with 5 ml of each of the different solvents. The solvents used were butanol, methanol, chloroform and water.

Gracilaria corticata (Plate 2) was also extracted in each of the solvents as described above.

Plate 1. *Avicennia officinnalis*

Plate 2. *Gracilaria corticata*

"Lab-lab", collected was covered with a clean plastic wrapper and stored immediately in cold condition in a thermocole box. Extraction was done immediately since they are more sensitive to temperature change. The ratio of sample:solvent was maintained as described earlier. Since "lab-lab" did not dissolve in any of the solvents, viz., chloroform, methanol, butanol or water, extraction in cold acetone solution was tried.

The above samples mixed with the respective solvents were ground well and centrifuged @ 12,000 rpm at 4^0C for 15 min. The crude extracts were then filtered.

3.2. Preparation of discs

Whatman filter paper 6mm size blank discs were used for study of antibacterial activity by disc diffusion test. The different extracts were absorbed onto the disc till saturation and then allowed to dry. A disc with solvent alone was similarly prepared as negative control for each of the polar and non- polar solvents. A broad-spectrum antibiotic disc of tetracyclin l was used as the positive control.

3.3. Sterilization of glassware and culture media

Glassware and culture media were sterilized in an autoclave at 121^0C, 15 lbs pressure for 15 min and 10 min, respectively. Nutrient agar and nutrient broth (Himedia, India) were used for preparation of plates and culture medium, respectively.

3.4. Preparation of bacterial cultures

The bacterial cultures were obtained from the stock cultures maintained in the Pathology Laboratory of College of Fisheries, Panangad, Cochin. Both gram negative and gram positive bacteria were used for the test. *Pseudomonas spp., Aeromonas hydrophila, Salmonella sp and Escherichia*

coli were the gram negative strains used while *Bacillus megaterium* was the gram positive strain used.

Pure culture of bacteria were subcultured in 100 ml of nutrient broth each for 16-18 h at 37^0C to obtain young and growing cultures for the disc diffusion assay.

3.5. Disc diffusion test

The procedure as described by Casida (1986) was followed. For the test**,** each of the bacterial cultures was streaked as a lawn on nutrient agar plates using sterile cotton swab. The discs were carefully placed on the plate using sterile forceps. The plates were incubated overnight at 37^0C.

The zone of inhibition of bacteria around the disc was measured and marked such that –ve mark denoted no inhibition, +ve for inhibition and ++ve for the strong inhibition above 5mm to 10mm.

3.6. Thin Layer Chromatography

3.6.1. Silica gel G - Thin-layer Preparation

Glass plates (20 x 20 cm) were coated with 0.25 mm of silica-gel (G). The glass plates were cleaned with soap solution and washed with distilled water to remove all stains, dirt and oil marks. 40g of silica gel-G was mixed with 90ml of distilled water. The slurry was then spread quickly and evenly with glass spreader on the glass plates. It was allowed to set for 30 min and then activated at 105^0 C for 1hr.

3.6.2. Phyto-chemical analysis

The crude aqueous extract of *Avicennia* sp., which gave the maximum zone of inhibition were fractionated by thin layer chromatography on silica

gel (G) to analyze the compound responsible for the antibacterial activity. The phytochemical analysis was carried out as described by Ajith and Janardhanan (2001). The preliminary analysis of the extract was done by applying 50 µl of aqueous extract of *Avicennia* leaf as a concentrated spot on the TLC plate about 2cm from the base of the plate at an angle of 45º to the base. The mobile phase used was a mixture of n-butanol:aceic acid: water in the ratio of 4:1:5 (v/v/v). This was taken in a glass chamber/tank in which the glass plates were placed vertically. The tank was closed and the solvent allowed to ascend until it reached the desired height. The plates were then removed and the solvent present was measured. The plates were then sprayed with the following reagents and observed for development of spots.

The reagents used for spraying were 1% alcoholic $FeCl_3$ in the ratio of 0.5g in 50ml alcohol to detect polyphenols/flavonoids, 1%vanillin-H_2SO_4(0.5g vanillin in 50ml sulphuric acid) for terpenoids, 10% alcoholic KOH (1g in 10ml alcohol) for quinones and Ninhydrin in the ration of 0.2 gm in 100ml acetone for peptides. For detection of fats, some quantity of iodine was kept inside the glass tank and covered with a lid for development of colour spot, if any.

The distances moved by the solvent from and the spot from the origin were measured. The R_f value was calculated using following equation.

$$\text{The } R_f\text{- value} = \frac{\text{Distance moved by the spot}}{\text{Distance moved by the solvent front}}$$

RESULTS

4. RESULTS

A large number of micro algal extracts and extra cellular products have been found to have antibacterial activity. However, pH of the medium, incubation period and temperature of incubation were very important for the biosynthesis of antimicrobial agent products as secondary metabolites. The seaweed *Gracilaria corticata*, "lab-lab", the tender leaves and bark of the mangrove *Avicennia officinalis* were screened for their antibacterial activities. The details of the observations made during the study are presented below. Antibacterial activity of crude extracts of mangrove leaf and bark, the seaweed *Gracilaria corticata* and "lab-lab" are shown in Table 1. Positive control (Chloramphenicol) against *Aeromonas, Salmonella, E.coli, Pseudomonas* and *Bacillus* are shown in Plate 1 to 5 respectively.

4.1. Mangrove antibacterial activity

Extracts of *Avicennia sp.* leaves and bark were prepared in butanol, methanol, chloroform and water and screened for antibacterial activity against four gram negative and one gram positive bacteria. It was observed that among all the extracts tested, maximum zone of inhibition of 27 mm was obtained with the aqueous extract against the gram positive bacteria, *Bacillus megaterium* (Plate 10). The zone of inhibition obtained was higher than that obtained for positive control with chloramphenicol, in which case a zone of inhibition of 24 mm was recorded (Plate 5). The antibacterial activity in terms of the zone of inhibition was very high even in case of all the gram negative bacteria tested. Maximum activity was observed against *Salmonella* (24 mm) (Plate 7) followed by that against *Aeromonas hydrophila* (19 mm) (Plate 6). There was no zone of inhibition with *E.coli* (Plate 8) and *Pseudomonas* (Plate 4). In the butanol extract, antibacterial activity was observed only against *E. coli* (14 mm) (Plate 8). In chloroform, methanol and acetone extracts, there appeared to be no antibacterial activity as no zone of inhibition was found against any of the bacteria tested. The respective negative controls with solvent alone did not show any zone of inhibition.

The graphical presentation of the antibacterial activity of mangrove leaf and bark (*Avicennia officinalis*) extract in different solvents against pathogenic bacteria shown in Fig.1 and Fig.2.

For the bark of *Avicennia* sp., antibacterial activity was observed in the butanol extract against gram negative *Salmonella* sp. (10 mm) (Plate 7) and gram positive *B. megaterium* (8 mm) (Plate 10). In the aqueous extract, however, zone of inhibition was observed only against *Salmonella* sp. (10 mm) (Plate 7). Chloroform, methanol and acetone extracts of *Avicennia* bark did not show any activity.

4.2. Antibacterial activity of Seaweed

In the case of seaweed *Gracilaria corticata*, maximum antibacterial activity was obtained in the butanol extract. Zone of inhibition recorded was highest against *Aeromonas hydrophila* (16 mm) (Plate 11) followed by that against *Salmonella* (10 mm) (Plate 12) and *E. coli* (10mm) (Plate 13). In the chloroform extract, good activity was observed against *Salmonella* (12 mm) (Plate 12) and *E. coli* (12mm) (Plate 13). In acetone extract, activity was observed against *Aeromonas hydrophila* (10 mm) (Plate 11) but not against any of the other bacteria tested.

The graphical presentation of the antibacterial activity of seaweed (*Gracilaria corticata*) extract in different solvents against pathogenic bacteria shown in Fig.3.

4.3. "Lab-Lab" antibacterial activity

For 'lab-lab', extracts were made in all the five solvents mentioned above, viz., water, butanol, methanol, chloroform and acetone. However, the complex could not be dissolved in any of the solvents and hence no activity was observed. Therefore, acetone was chilled well and extraction was repeated

TABLE 1: Antibacterial activity of crude extract of mangrove leaf and bark, seaweed and "Lab-Lab"

Name of Material/ commercial antibiotics	Solvent of Extraction	Activity against Pathogen				
		Aeromonas	***Salmone-lla***	***E.coli***	***Pseudo-monas***	***Bacillus***
Avicennia (Leaf)	Methanol	-ve	-ve	-ve	-ve	-ve
	Butanol	-ve	-ve	14 mm	-ve	-ve
	Chloroform	-ve	-ve	-ve	-ve	-ve
	Distilled water	19 mm	24 mm	-ve	-ve	27 mm
	Acetone	-ve	-ve	-ve	-ve	-ve
Avicennia (Bark)	Methanol	-ve	-ve	-ve	-ve	-ve
	Butanol	-ve	10 mm	-ve	-ve	8 mm
	Chloroform	-ve	-ve	-ve	-ve	-ve
	Acetone	-ve	-ve	-ve	-ve	-ve
	Distilled water	-ve	10 mm	-ve	-ve	-ve
Gracilaria Seaweed	Methanol	-ve	-ve	-ve	-ve	-
	Butanol	16mm	10 mm	10 mm	-ve	-
	Chloroform	-ve	12 mm	12 mm	-ve	-
	Acetone	10 mm	-ve	-ve	-ve	-
	Distilled water	-ve	-ve	-ve	-ve	-
"Lab-Lab"	Methanol	-ve	-ve	-ve	-ve	-ve
	Butanol	-ve	-ve	-ve	-ve	-ve
	Chloroform	-ve	-ve	-ve	-ve	-ve
	Acetone (cold form)	-ve	-ve	-ve	-ve	-ve
	Distilled water	-ve	-ve	-ve	-ve	-ve
Commercial antibiotic Chloramphe-nicol		-ve	23mm	26mm	21mm	24mm

* All the negative control is negative

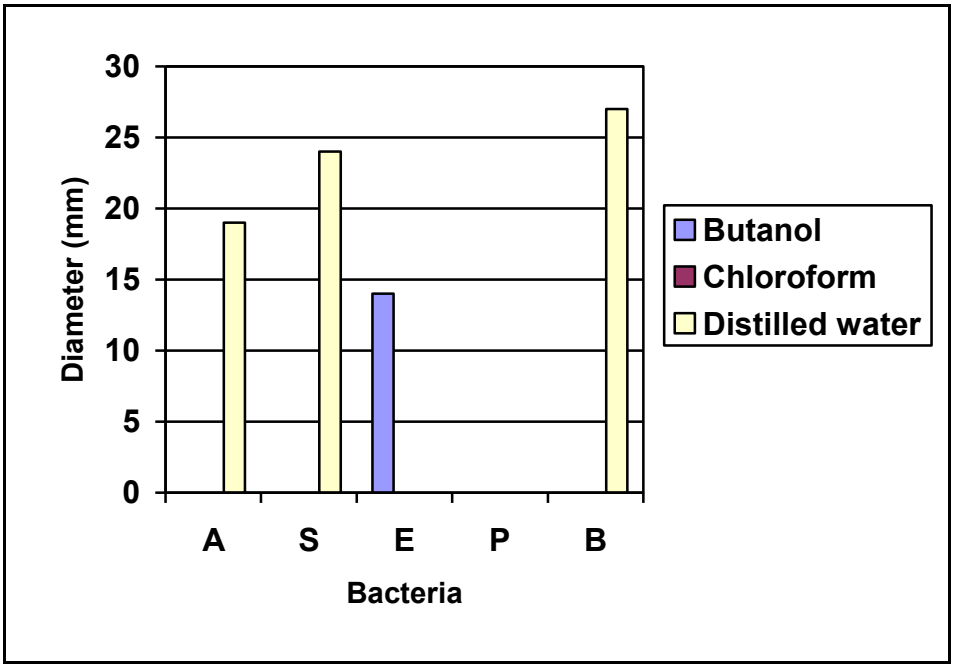

Fig.1.The antibacterial activity of mangrove leaf extract in different solvents against pathogenic bacteria

Where, B - *Bacillus*,
S - *Salmonella*,
E - *E.coli*
A - *Aeromonas*
P - *Psuedomonas*

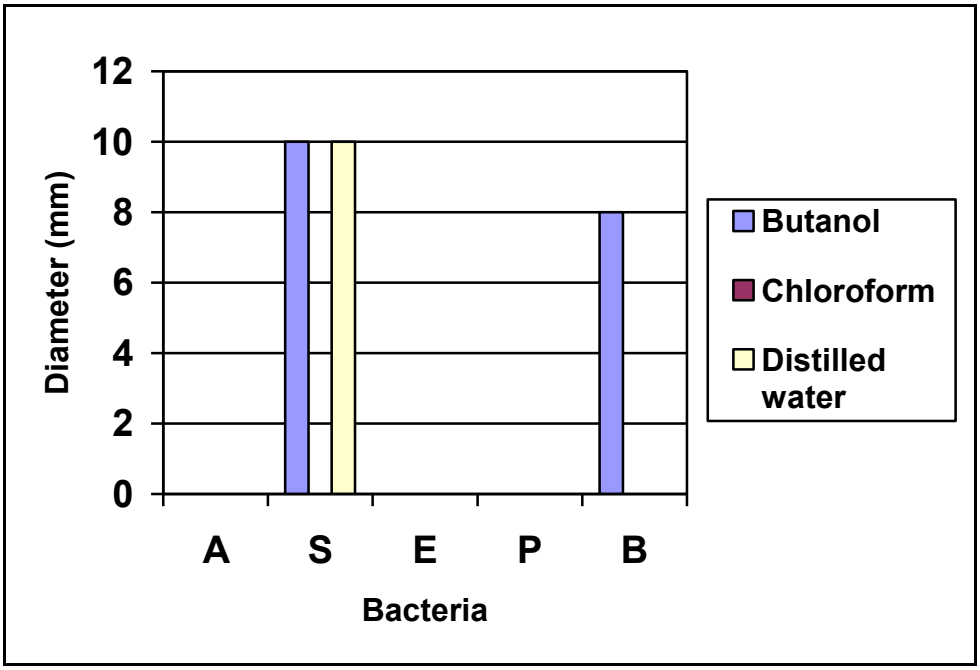

Fig.2.The antibacterial activity of mangrove bark extract in different solvents against pathogenic bacteria

Where, B - *Bacillus*,
S - *Salmonella*,
E - *E.coli*
A - *Aeromonas*
P - *Psuedomonas*

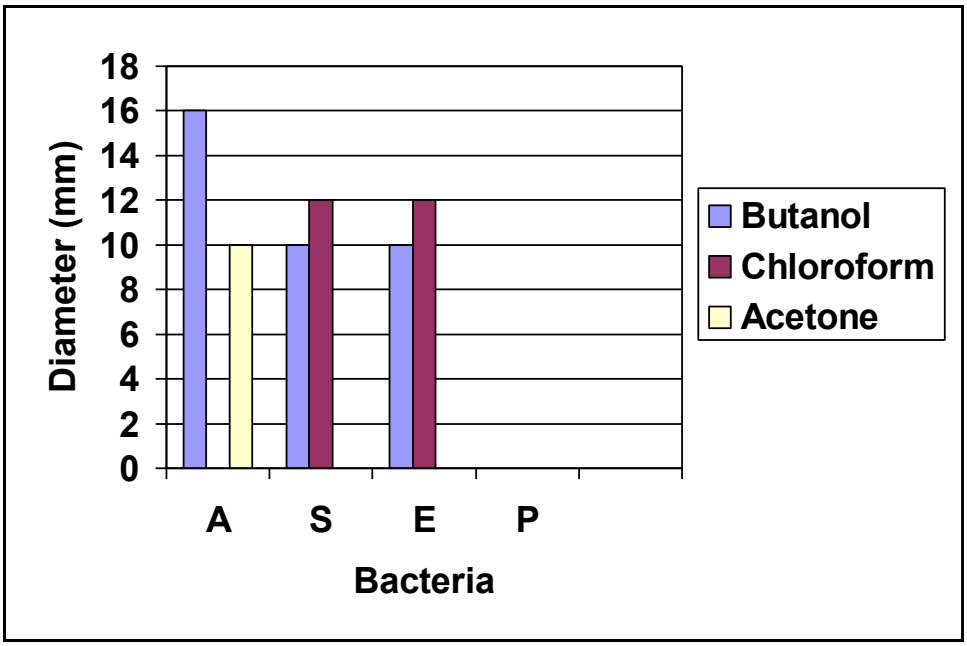

Fig.3.The antibacterial activity of seaweed (*Gracilaria corticata*) extract in different solvents against pathogenic bacteria

Where, B - *Bacillus*,
S - *Salmonella*,
E - *E.coli*
A - *Aeromonas*
P - *Psuedomonas*

in cold acetone. However, no activity was observed even in cold acetone.

The zone of inhibitions obtained against different bacteria using the broad spectrum antibiotic chloramphenicol as positive control are shown in Table 1 and Plates 15 to 19.

4.4. Phyto-chemical analysis

In the disc diffusion assay, very strong antibacterial activity (as observed by the maximum zones of inhibition) was obtained with the aqueous extract of *Avicennia* leaf. To determine the compound (s) responsible for the antibacterial activity, phytochemical analysis of the aqueous extracts of mangrove leaf was done by thin layer chromatography. Five plates, each coated with silica gel-G and spotted separately with the aqueous leaf extract of *Avicennia* and run in Butanol: acetic acid
:water solvent system were developed individually, using alcoholic $FeCl_3$ (1%) to detect the presence of polyphenols/flavonoids, if any, vanillin sulphuric acid (0.5 g vanillin in 50 ml H_2SO_4) for the presence of terpenoids, alcoholic KOH (10%) for quinones, iodine for the presence of fats and ninhydrin (0.2% in acetone) for peptides, respectively.

On spraying the plates with alcoholic $FeCl_3$ (1%), a distinct brownish colour developed indicating the presence of polyphenols/flavonoids (Plate 22). The R_f value was found to be 0.88 (Table 2). With vanillin-sulphuric acid, a dark red colour spot with nearly the same R_f value (0.86) showing the presence of terpinoids was observed (Plate 24), while on spraying alcoholic KOH (10%), a reddish coloured spot developed (Rf value- 0.89) for quinone (Plate 21). However, with ninhydrin, colour development was not obvious (Plate 23). On keeping iodine in the glass chamber with activated plate, a clear darkish brown colour development was noted (R_f value- 0.85) (Plate 20) showing the presence of an unsaturated lipids components.

The R_{f-} values, calculated from the ratio of the distance moved by the spot to the respective distance moved by the solvent front, are shown in table-2 for each of the components used.

Table-2. Comparison of distances movement and Rf -value of mangrove leaf extract

1	Distance moved by the solvent front	13 cm
	Distance moved by the spot	11cm
	R_f -value of the solute revealed by I_2	0.85
2	Distance moved by the solvent front	14.6 cm
	Distance moved by the spot.	13 cm
	R_f -value of the solute revealed by alcoholic KOH	0.89
3	Distance moved by the solvent front	12.5 cm
	Distance moved by the spot	11 cm
	R_f-value of the solute revealed by alcoholic $FeCl_3$	0.88
4	Distance moved by the solvent front	13.5 cm
	Distance moved by the spot.	12 cm
	R_f -value of the solute revealed by Ninhydrin	0.89
5	Distance moved by the solvent front	13.1 cm
	Distance moved by the spot	11.3 cm
	R_f -value of the solute revealed by Vanillin	0.86

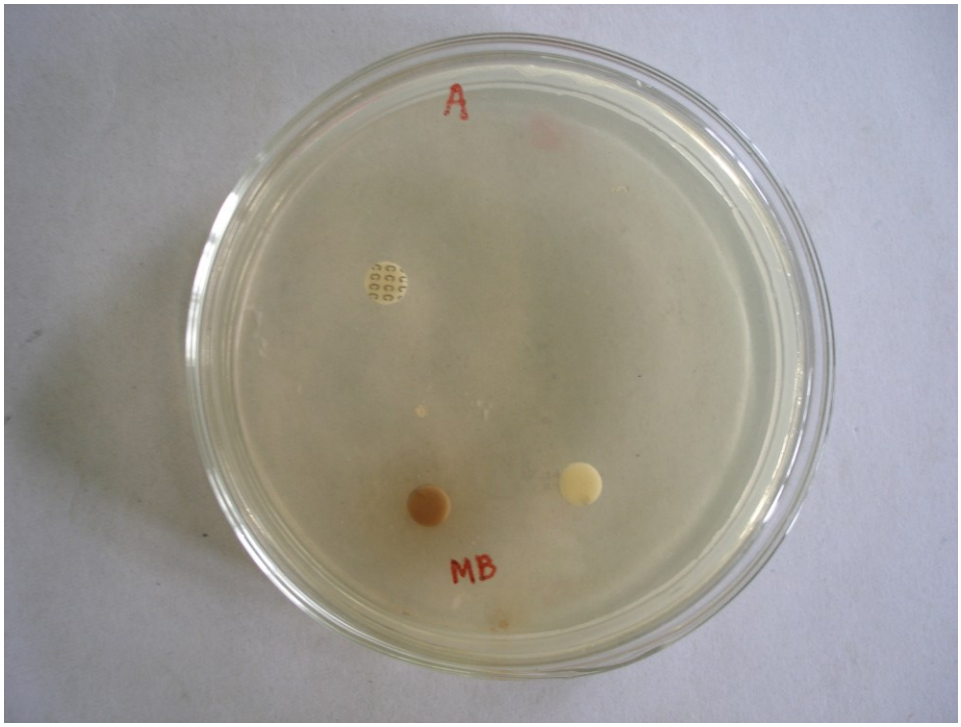

Plate-1. Positive control (Chloramphenicol) against *Aeromonas*

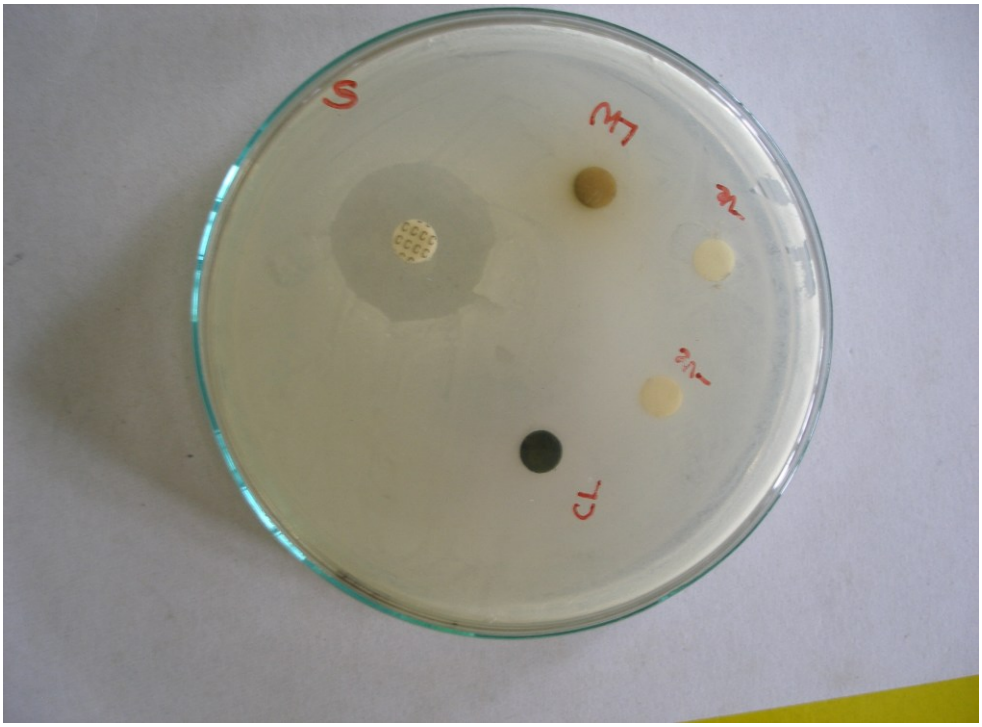

Plate-2. Positive control (Chloramphenicol) against *Salmonella*

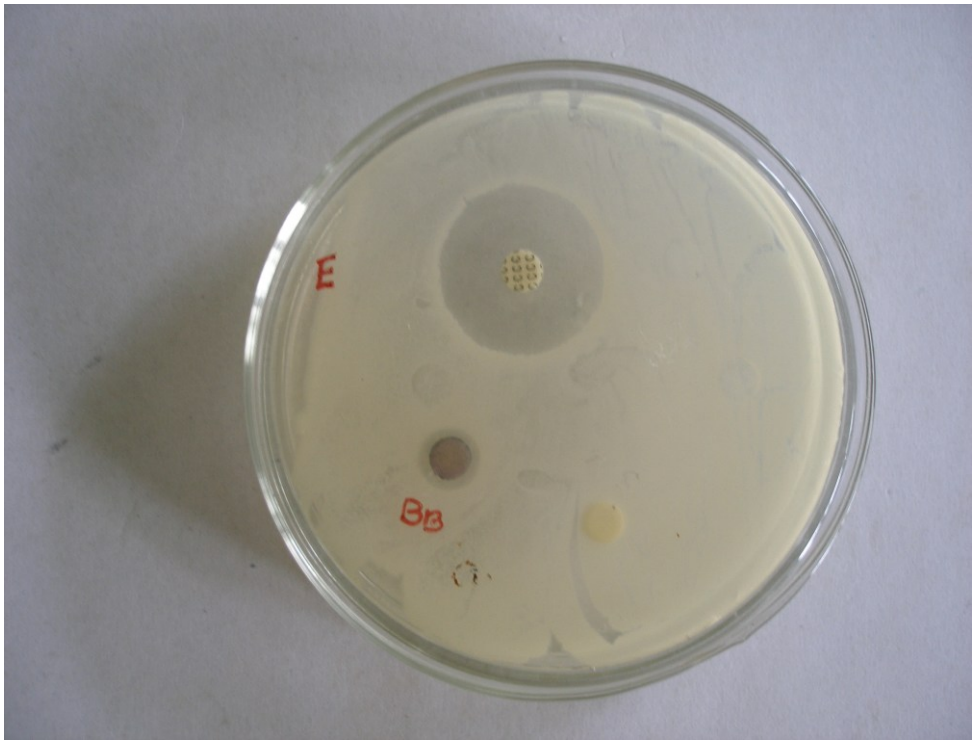

Plate-3. Positive control (Chloramphenicol) against *E.coli*

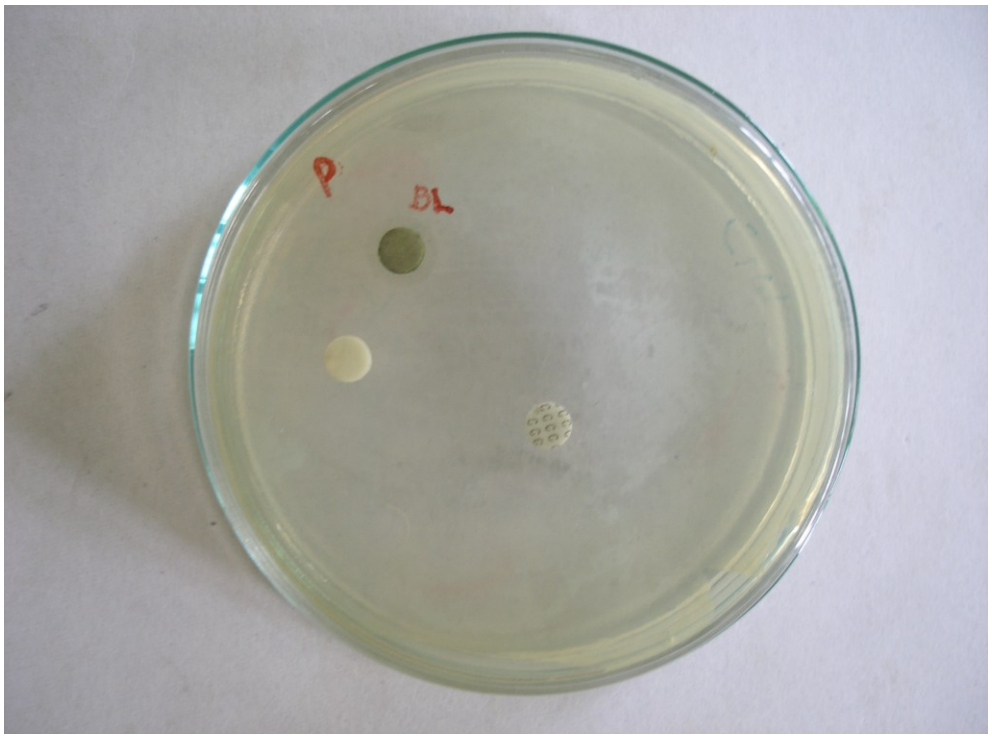

Plate-4. Positive control (Chloramphenicol) against *Pseudomonas*

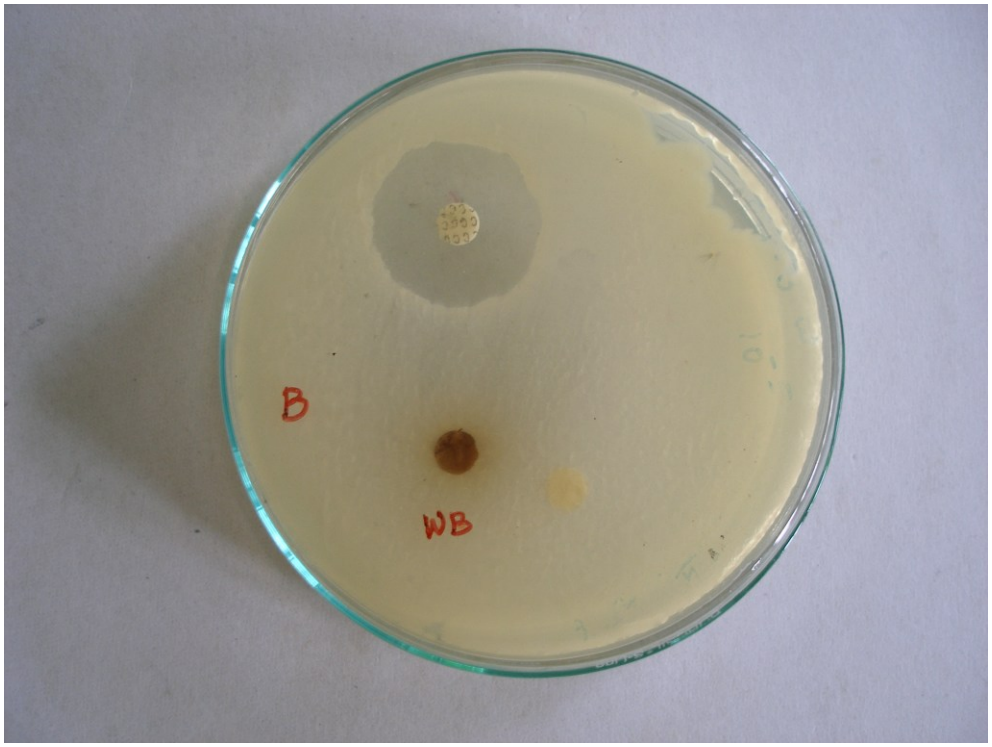

Plate-5. Positive control (Chloramphenicol) against *Bacillus*

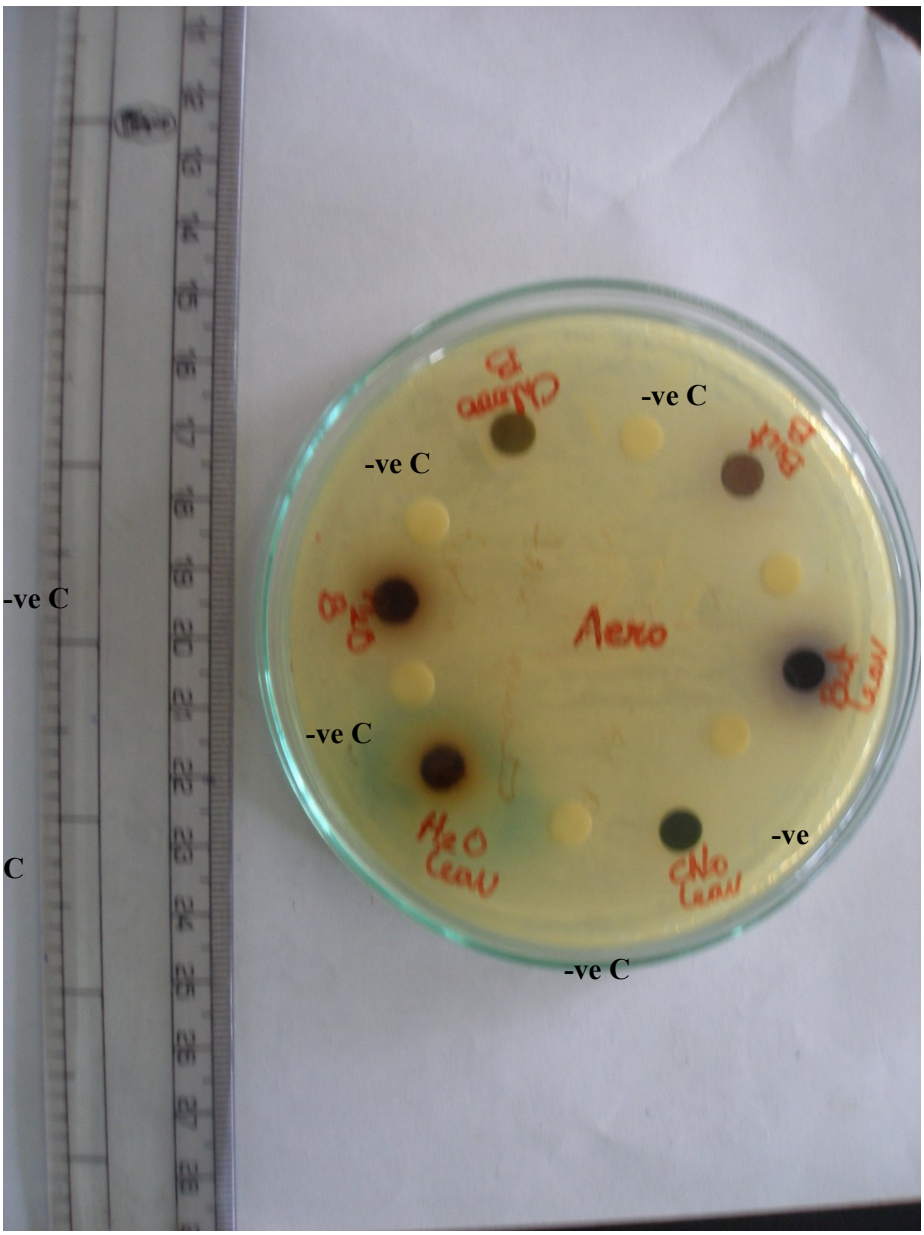

Plate-6. Antibacterial activity of mangrove leaf and bark extracts against *Aeromonas*.

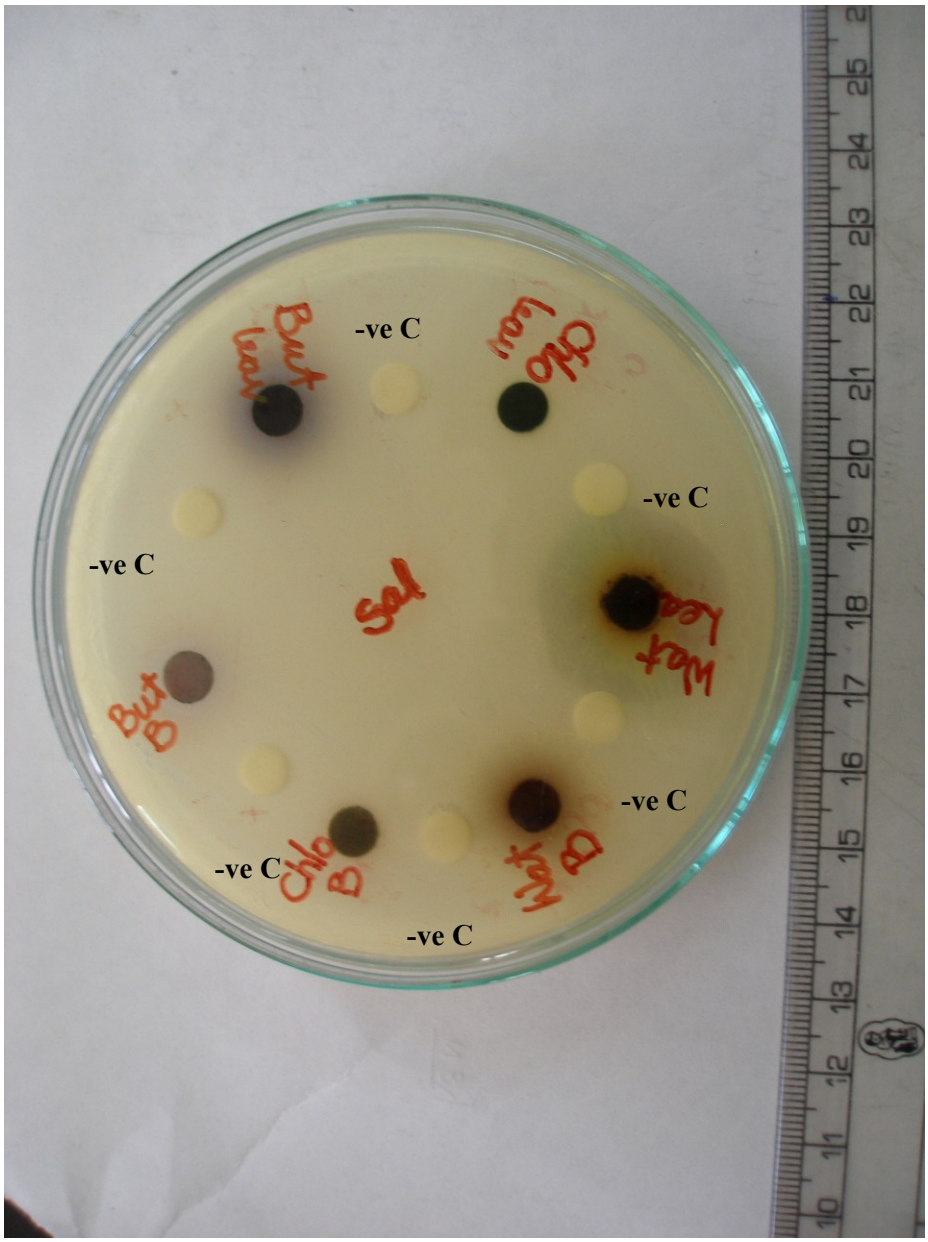

Plate-7. Antibacterial activity of mangrove leaf and bark extracts against *Salmonella*

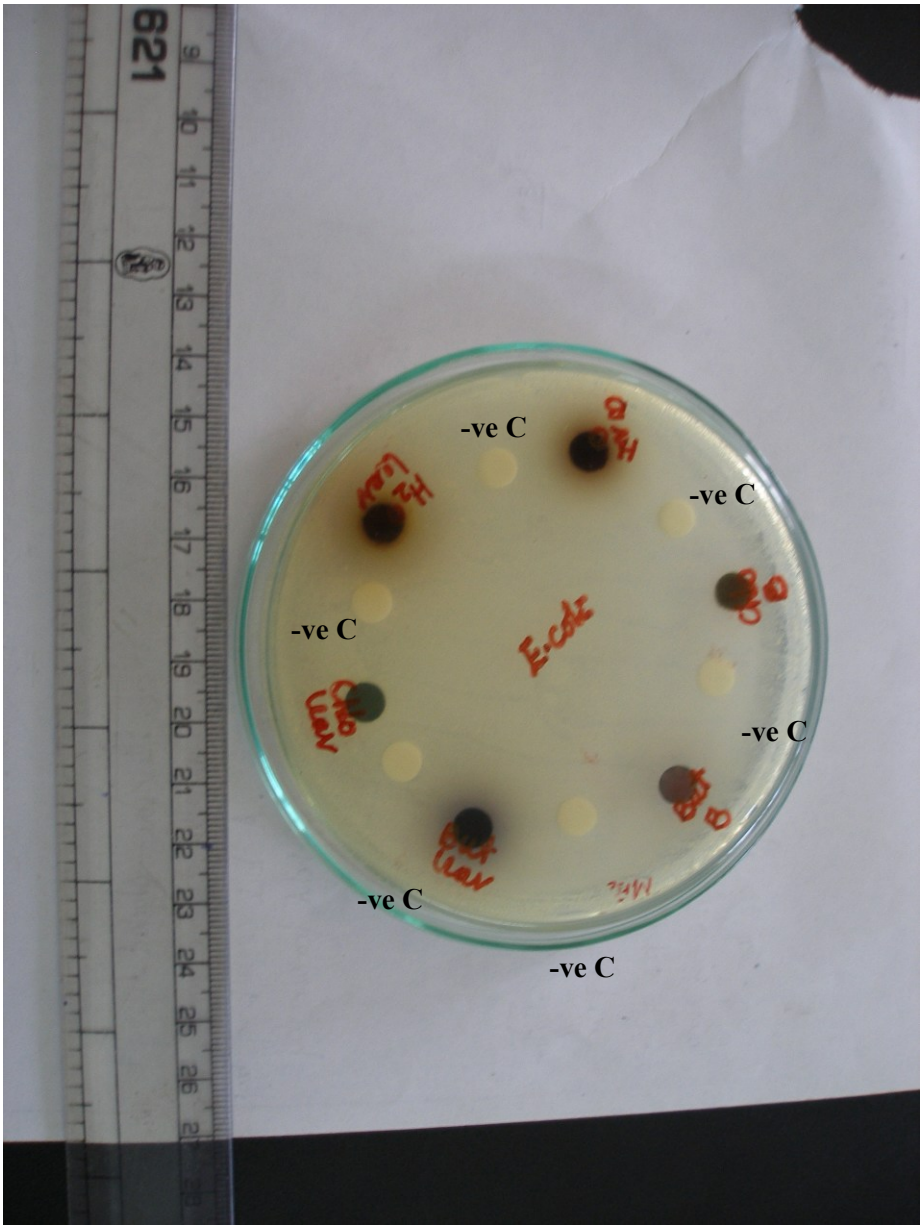

Plate-8. Antibacterial activity of mangrove leaf and bark extracts against
E. coli.

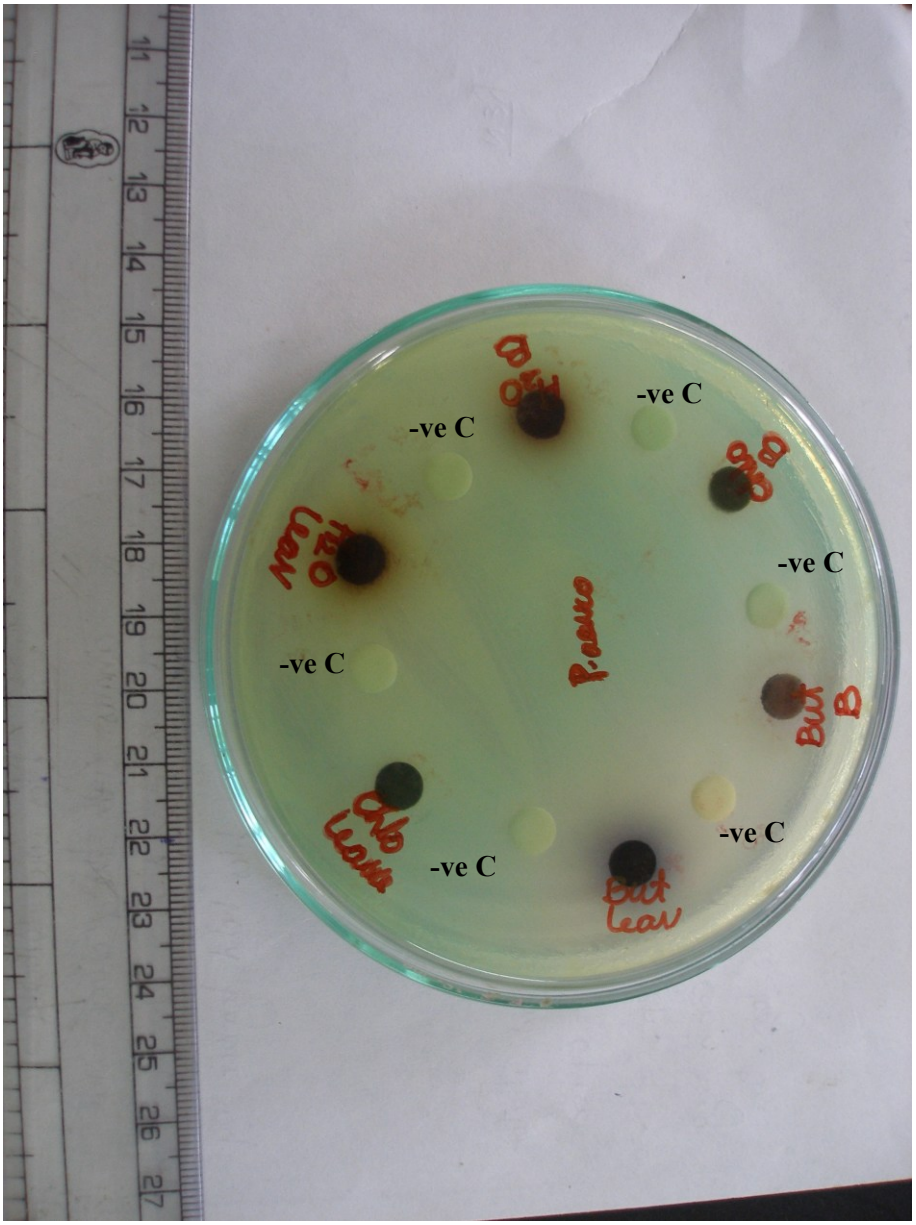

Plate-9. Antibacterial activity of mangrove leaf and bark extracts against *Psuedomonas*.

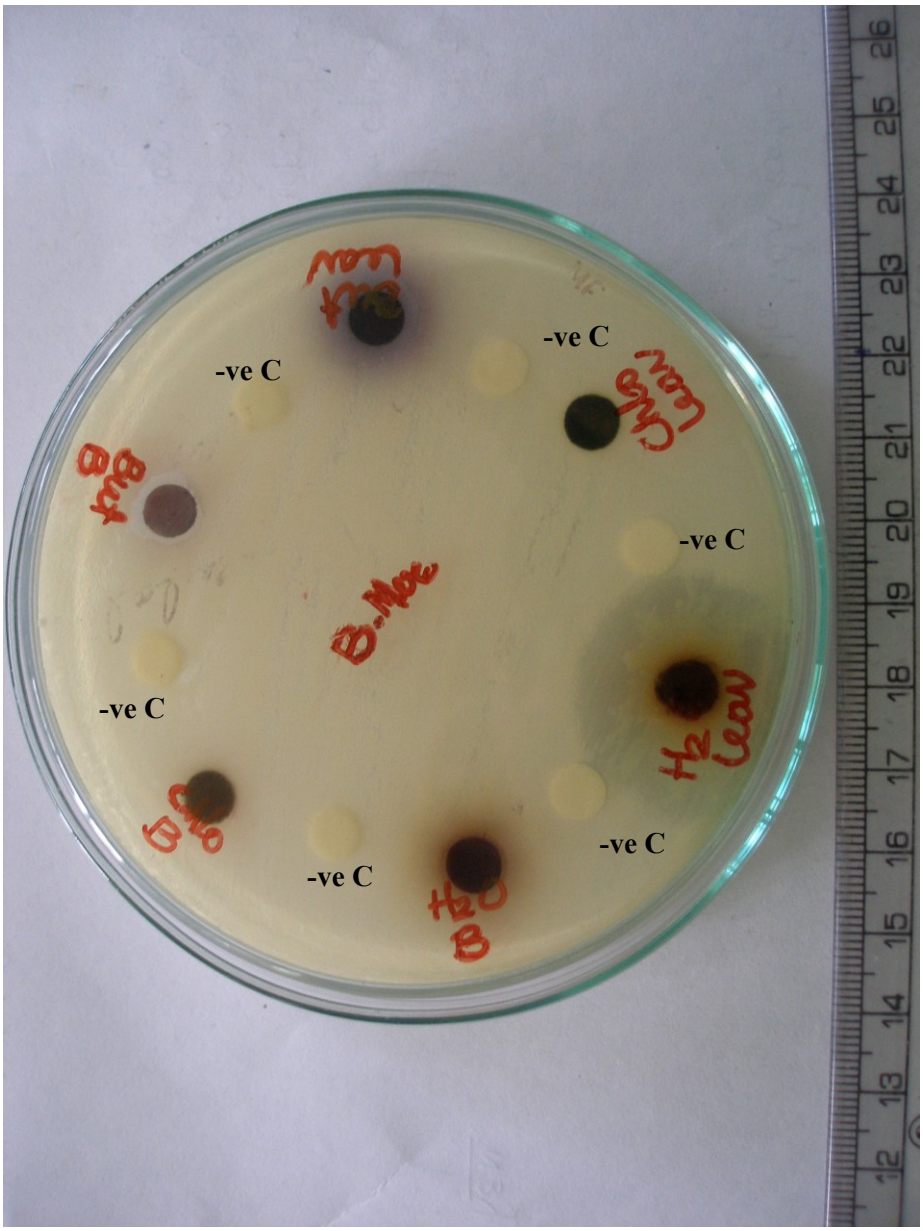

Plate-10. Antibacterial activity of mangrove leaf and bark extracts against *Bacillus*.

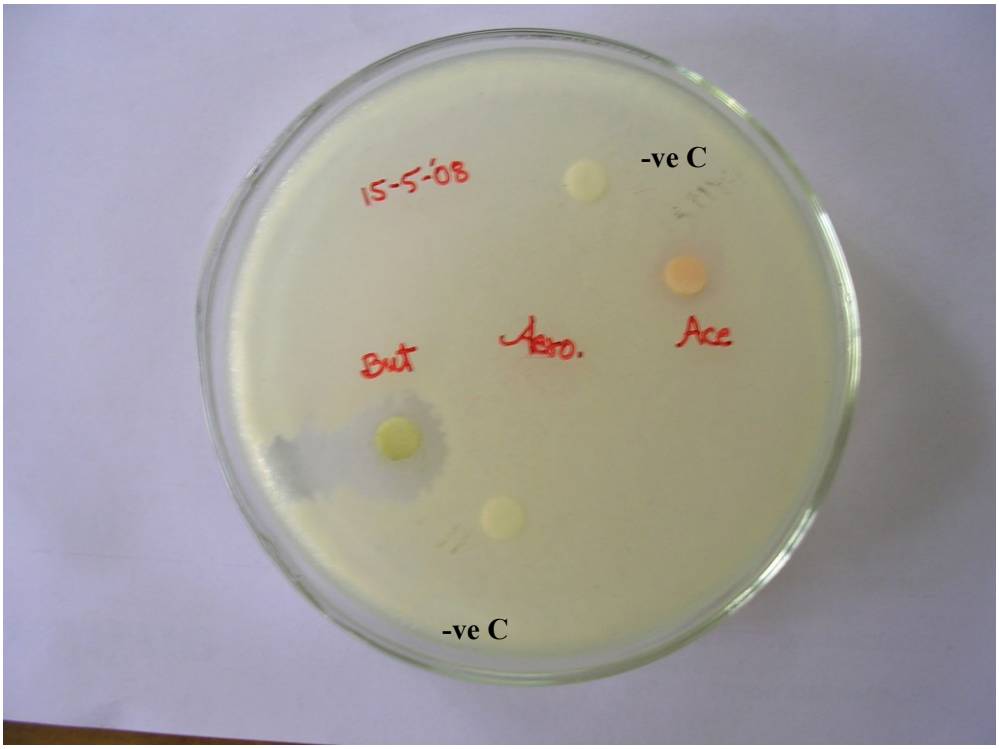

Plate-11. Antibacterial activity of seaweed (*Gracillaria*) extracts against *Aeromonas.*

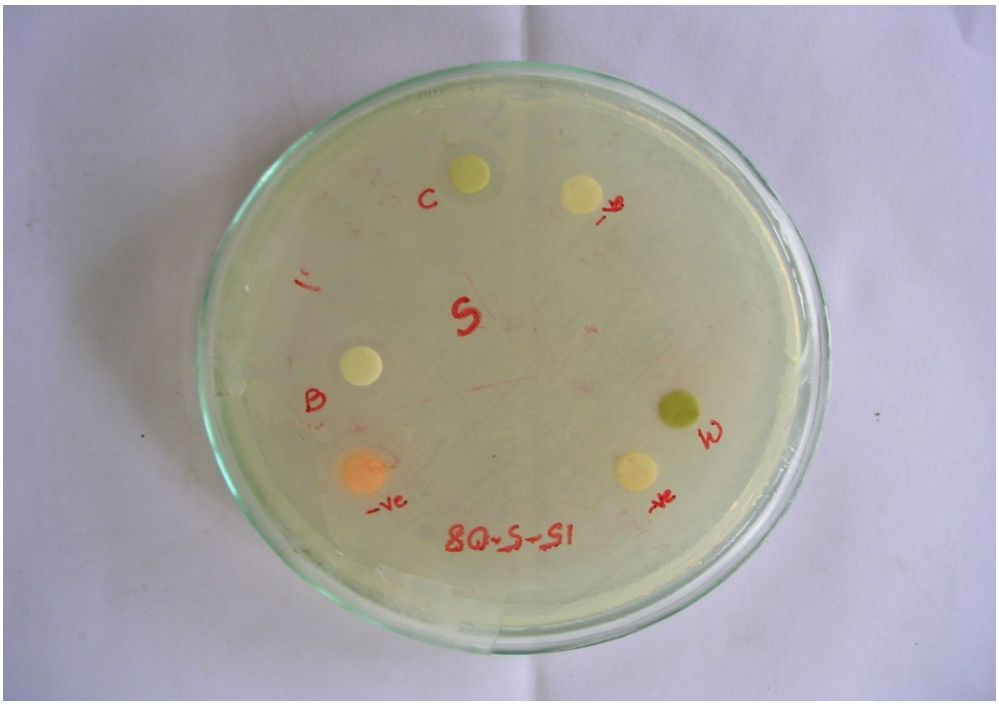

Plate-12. Antibacterial activity of seaweed (*Gracillaria*) extracts against *Salmonella.*

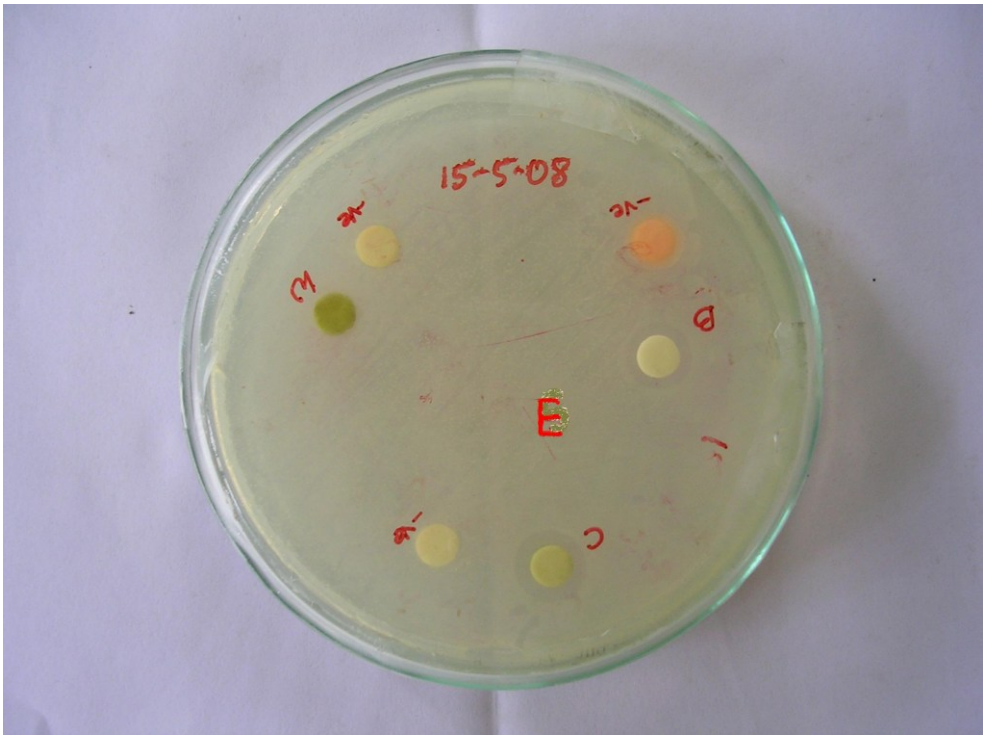

Plate-13. Antibacterial activity of seaweed (*Gracillaria*) extracts against *E. coli.*

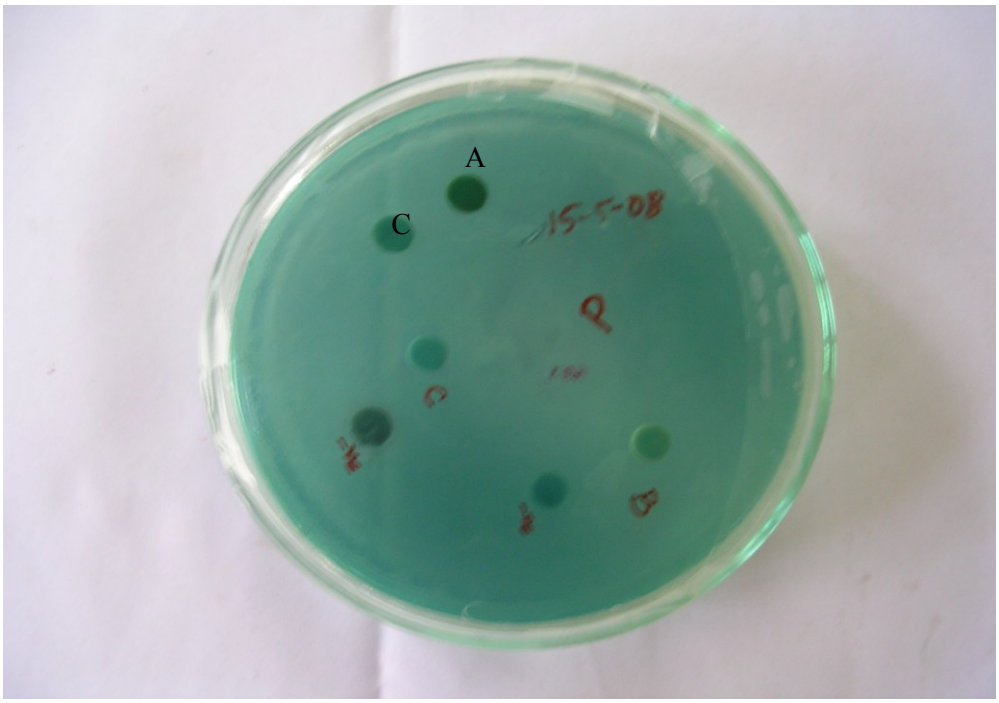

Plate-14. Antibacterial activity of seaweed (*Gracillaria*) extracts against *Psuedomonas.*

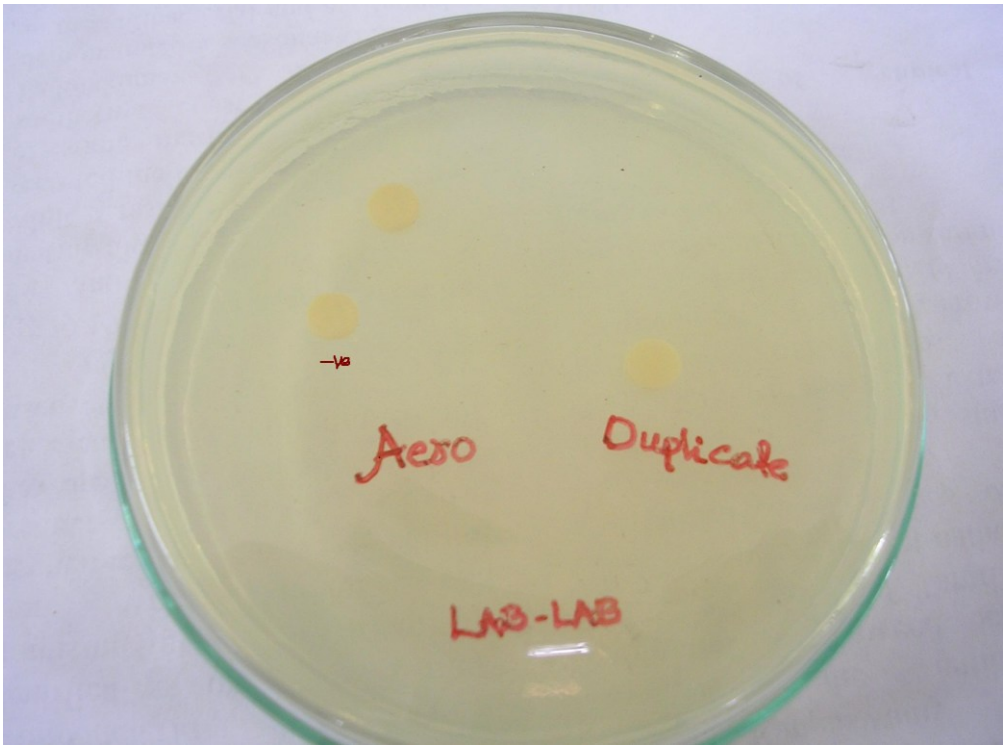

Plate-15. Antibacterial activity of “Lab-Lab” extracts against *Aeromonas*.

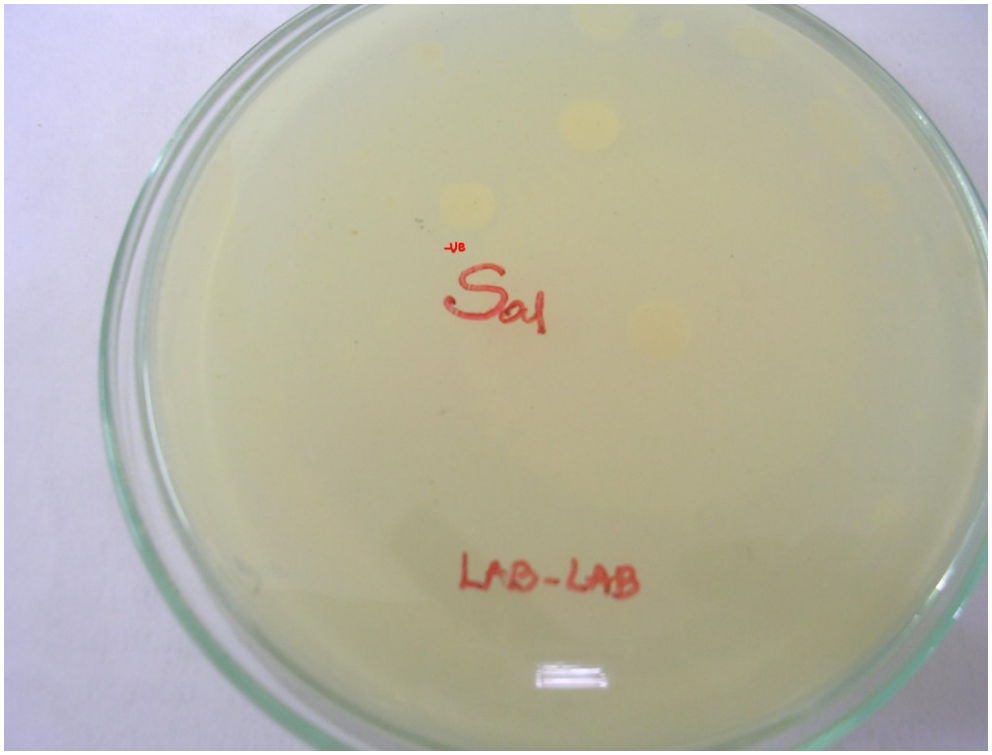

Plate-16. Antibacterial activity of “Lab-Lab” extracts against *Salmonella*

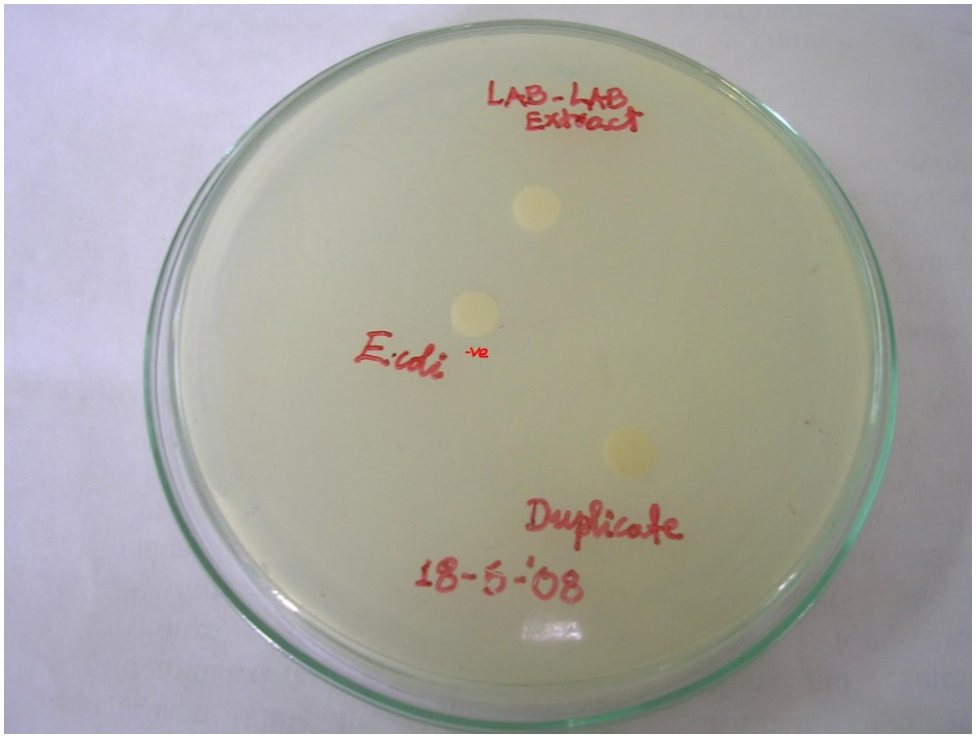

Plate-17. Antibacterial activity of "Lab-Lab" extracts against *E.coli*

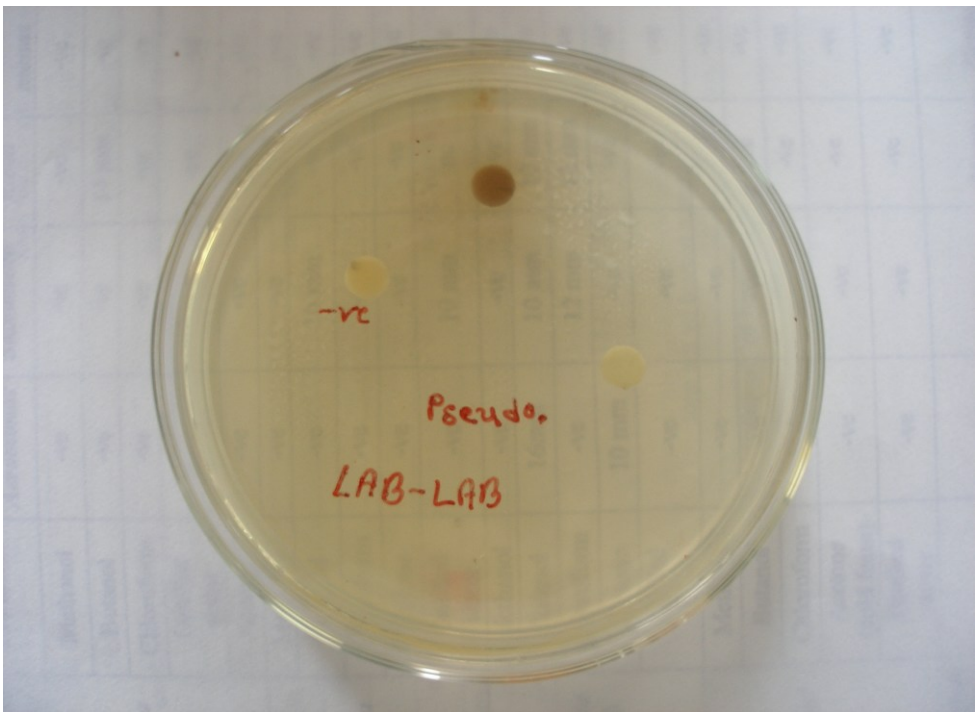

Plate-18. Antibacterial activity of "Lab-Lab" extracts against *Pseudomonas*

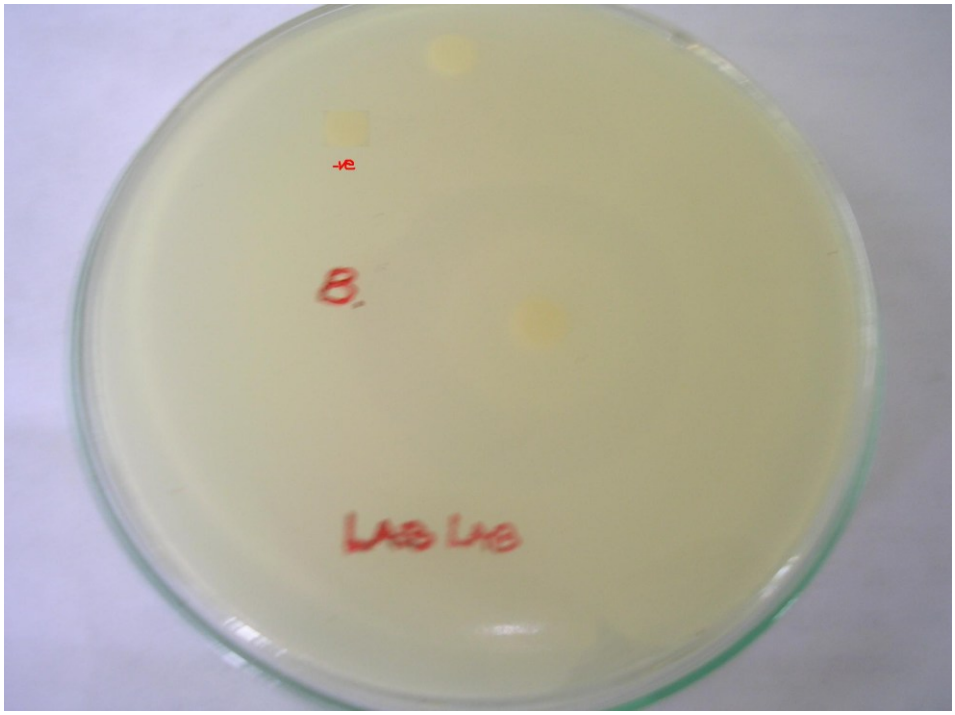

Plate-19. Antibacterial activity of “Lab-Lab” extracts against *Bacillus*

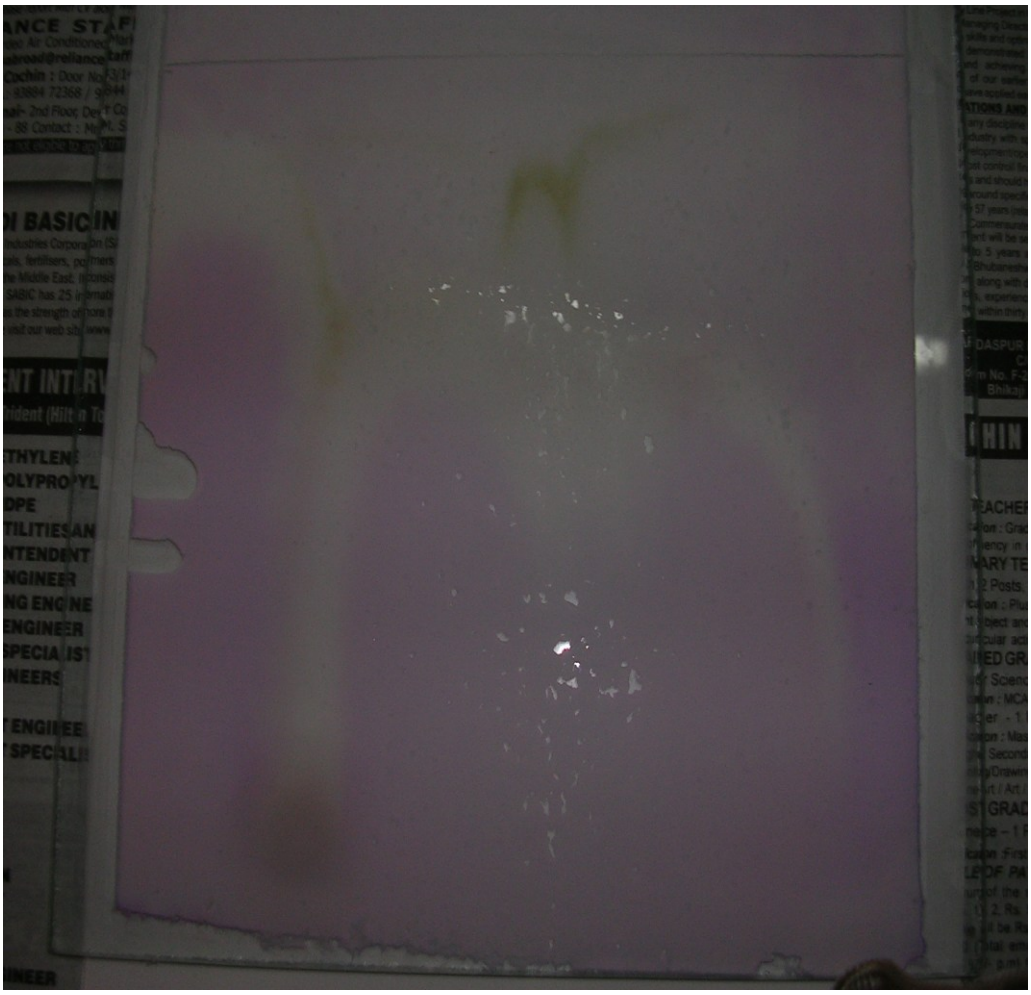

Plate-20. Iodine showing the presence of fats or lipid compound in aqueous mangrove leaf extract.

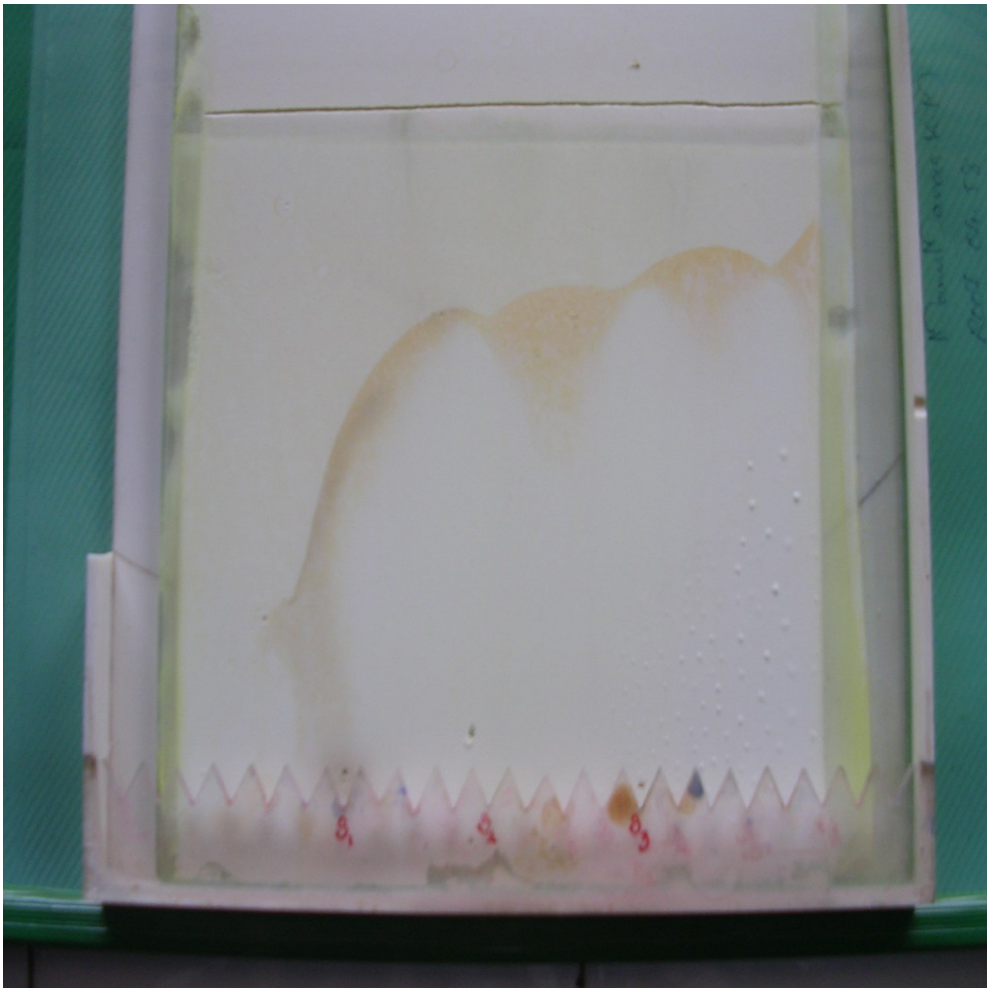

Plate-21. Alcoholic KOH showing the presence of quinone compound in aqueous mangrove leaf extract.

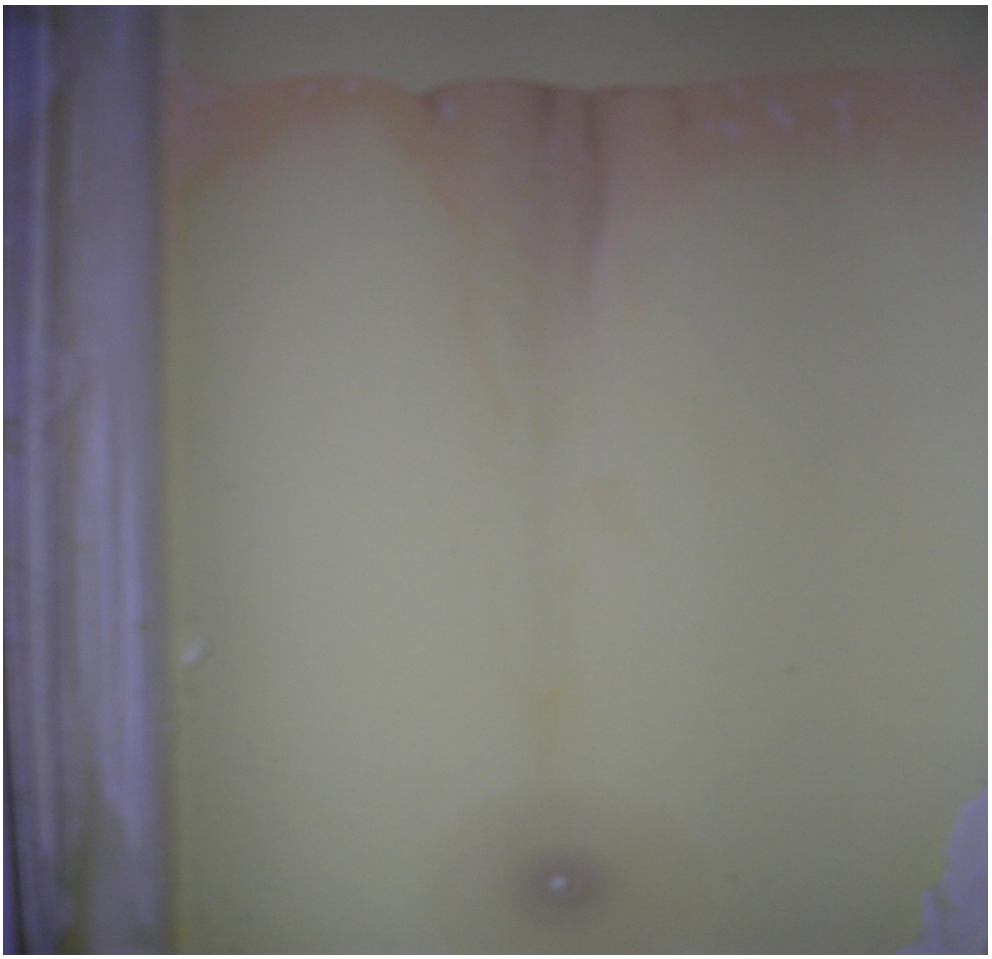

Plate-22. Alcoholic ferric chloride ($Fecl_3$) showing the presence of polyphenol compound in aqueous mangrove leaf extract.

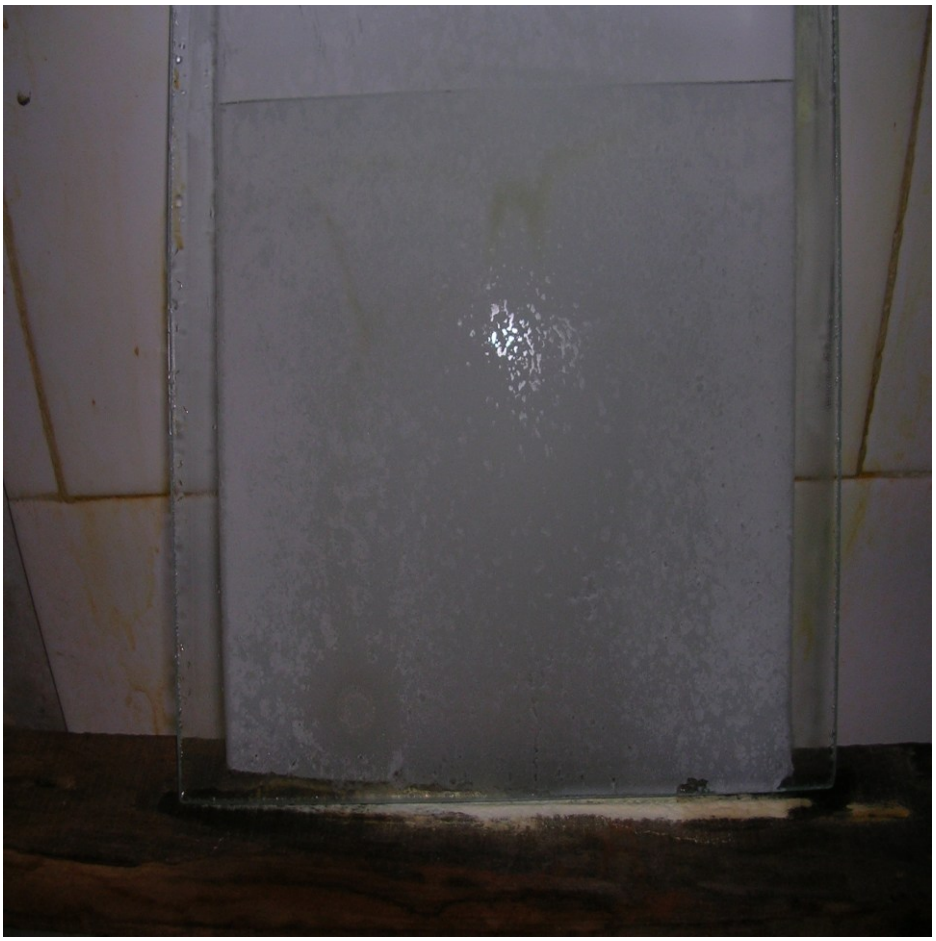

Plate-23. Ninhydrin showing the presence of peptides compound in aqueous mangrove leaf extract.

Plate-24. Vanillin sulphuric acid showing the presence of terpenoid compound in aqueous mangrove leaf extract.

DISCUSSION

5. DISCUSSION

The antibacterial activity in the extracts of seaweed *Gracilaria corticata*, mangrove *Avicennia officinalis* and the plant-animal complex 'lab-lab' were screened and the results obtained are discussed below.

5.1. Antibacterial activity

5.1.1 Seaweed

Gracilaria corticata, a species belonging to Rhodophyceae, exhibited considerable antibacterial activity, in the present study, in the butanol, chloroform and acetone extracts. Maximum zone of inhibition was obtained in the butanol extract. However, no activity was observed in the methanol extract.

Hodgson (1944) opined that the use of organic solvents is always better for extraction compared with aqueous extraction in algae. This finding is corroborated by the studies of Padmakumar and Ayyakkannu (1986), who reported that toluene-methanol (1:3) extracts of species belonging to Rhodophyceae exhibited broad-spectrum activity. These algae were more active than the species belonging to Chlorophyceae and Phaeophyceae. Similarly, Vidyavathi and Sridhar (1991) reported that chloroform-methanol extract of fully grown *G. corticata* showed maximum activity against *S. aureus and Pseudomonas* compared to medium and young stages of growth. In the present study, butanol extracts of *Gracilaria corticata* showed activity against Aeromonas, *Salmonella* and *E.coli* while the chloroform extract showed moderate activity against the latter two bacteria. Acetone extract showed activity only against *Aeromonas* and no activity against any of the other bacteria tested. No activity was observed in the aqueous extract. This agrees with the finding of Hodgson (1944).

Vijaya (2004) suggested that methanol is a better solvent for algal extraction and separation of a variety of phytochemicals that produce maximum inhibitory effect on both gram positive and gram negative bacteria. Febles *et al.* (2000) also observed the best activity in methanol extract of the green and brown seaweeds they studied. However, in the present study, antibacterial activity could not be detected in the methanol extract against either gram positive or gram negative bacteria.

Rao and Parekh (1981) analysed *Enteromorpha intestinalis* and *G. corticata* collected from Gujarat coast of India for antibacterial activity. The algae were active throughout the year with a peak during the winter season. This is in contrast to the results obtained earlier by Hornsey and Hide (1974) who tested 151 species of British marine algae and found that, although antibacterial activity was more evident in some taxonomic groups, it also varied seasonally. They did not find any activity in *Gracilaria* sp., *Enteromorpha* sp. and *Cladophora dalmatica*. Crasta *et al*. (1997) also recorded significantly different inhibitory activity from season to season. In the present study, although antibacterial activity was observed in the *Gracilaria* extract, seasonal changes in the activity was not studied.

5.1.2 Lab-Lab

Cannel *et al*. (1988) screened more than 100 cyanobacterial cultures and obtained positive results in less than 10%. Zornitza *et al.* (2000), however, showed that a broad spectrum antimicrobial is produced by *Nostoc sp* which inhibits the growth of bacteria, notably multiresistant *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The same authors tested the methanol extract of *Spirogyra* against a wide range of bacteria and reported that *Spirogyra varians* showed inhibitory activity against gram positive and gram negative bacteria. In the present study, the antibacterial activity of 'lab-lab' was studied. Cyanobacteria, particularly, *Lyngbea* and *Oscillatoria*, predominate in lab-lab. However, no antibacterial activity was observed in 'lab-lab' in any of the solvent extracts. This may be because the extraction procedure might not have been efficient enough to break the mucilaginous covering and extract

the antimicrobial compound from it. It is surprising that no activity was shown in any of the extracts since 'lab-lab' contains a complex of both plant and animal matter.

Vlachos *et al.* (1997) found that fractionation of crude extracts of marine algae enhanced their activity against both gram negative as well as the resistant gram positive pathogens. Enrichment of the antibacterial activity of lipid extracts of marine algae *Laurencia obtusa* was achieved during fractionation process by thin layer chromatography (Caccamese *et al* 1981).

5.1.3 Mangrove

The leaves of *Avicennia officinalis* extracted in water showed excellent antibacterial activity against both gram positive and gram negative bacteria, in the present study. The zone of inhibition obtained was greater than that obtained with the broad spectrum antibiotic chloramphenicol. Although the bark of this mangrove also showed antibacterial activity against gram negative *Salmonella* and gram positive *Bacillus* in the butanol and aqueous extracts, the zones of inhibition were much smaller compared to that obtained for leaf. The extracts of bark did not show activity against *Aeromonas, Psuedomonas* or *E. coli*.

The methanol extract of the mangrove *Rhizophora mucronata* showed moderate activity against *Staphylococcus aureus* (Kazuhiko *et al*., 2003). However, in the present study, no activity was recorded in the methanol extract of leaf or bark. Khafagi *et al.* (2003) evaluated the antibacterial activity in addition to antibacteriophage and anticandidal activities in the aqueous and ethanol extracts of roots, cotyledons, leaves and stems of *Avicennia marina* and demonstrated only antibacteriophage activity. On screening the organic solvent extracts of five mangroves, viz., *Aegiceras corniculatum, A. rotundifolia, Aglaia cucullata, Cynometra iripa and Xylocarpus granatum*, Choudhury *et al.* (2005) reported that methanol extract of *C. iripa* were effective in inhibiting the growth of all the six virulent strains of bacteria pathogenic to fish that were tested, whereas *A. corniculatum* and

A.cucullata were active against four of the pathogens. The result of the present study, where maximum antibacterial activity was obtained in the aqueous extract of mangrove leaf is in contrast to the results of the above studies.

Antibacterial activity of mature leaves, tender leaves and bark extracts of *Avicennia marina*, *Avicennia officinalis* and *Bruguiera sexangula* were evaluated using Soxhelt extraction method (Abeysinghe *et al*., 2006). Petroleum ether, chloroform, ethyl acetate and ethanol were used as solvents. Extracts of *A. marina*, *A. officinalis* and *B. sexangula* exhibited different degree of growth inhibition against tested bacterial strains. Mature leaf extracts of *A. marina* and tender leaf extracts of *A. officinalis* in ethyl acetate exhibited promising antibacterial activity than other plant extracts. The authors observed that all plant extracts in ethyl acetate showed strong inhibition compared to other extracts on all tested bacterial strains.

5.2 Phyto-chemical analysis

On thin layer chromatography, coloured spots that appeared in the individual plates had nearly the same Rf value, indicating the presence of a single compound. Differently coloured spots developed with alcoholic ferric chloride indicating the presence of polyphenol, alcoholic KOH showing the presence of a quinone, iodine vapours clearly showing the presence of an unsaturated lipid component and finally, vanillin sulphuric acid indicating the presence of terpenoid or isoprenoids. Thus, the compound responsible for the antibacterial activity in the aqueous extract of mangrove leaves is a polyphenolic lipid quinone with isoprenoid side chains. In a study of bioactive compounds in the mangrove *Avicennia officinalis* collected from the coast of Ongole in Andhra Pradesh, India, Subrahmanyam *et al.* (2006) reported the presence of diterpenes. The hexane extract of the root of *A. officinalis* yielded rhizophorin B1, which showed antibacterial activity against *Bacillus subtilis*. This finding agrees well with the result obtained in the present study, where maximum antibacterial activity was observed against *Bacillus* sp. and a terpenoid component has been identified in the thin layer chromatographic analysis. Changyi *et al.* (1997) suggested that the fatty acids (PUFA) in litter

fall of mangroves might have a positive role on the growth of fishes and shrimps. Similarly, Agoramoorthy *et al.* (2007) reported that the fatty acids, mainly palmitic acid and lauric acid, were responsible for the antibacterial and antifungal activity in blind-your-eye mangrove *Excoecaria agallocha*.

Phytochemical investigation of the stem of the marine mangrove *Bruguiera gymnorrhiza* yielded five new aromatic compounds. Among them, 3 showed moderate activity against gram-positive and gram-negative bacteria including mycobacteria and resistant strains (Han *et al.*, 2005).

The extract of *Avicennia marina* fractionated by Preparative Thin Layer Chromatography (PTLC) exhibited moderate antibacterial activity against *Pseudomonas* sp., *Shigella* sp. and *E. coli*. The components did not show any antibacterial activity against *Proteus* sp. and *Staphylococcus* sp. Phytochemical screening revealed that mature leaf of *A. marina* contained alkaloids, steroids, triterpenoids and flavonoids (Abeysinghe *et al*., 2006).

The fact that maximum antibacterial activity was obtained in the aqueous extract of mangrove leaf is particularly advantageous from a practical point of view. Application of the extract on fishes/shellfishes to treat bacterial infection becomes much easier since the antibacterial compound gets extracted in water. At a time when the use of many antibiotics, especially chloramphenicol, in aquaculture is banned, the result obtained in the present study assumes significance. With further *in vivo* studies and standardization of dose, leaf extract of *Avicennia officinalis* can be recommended for use in aquaculture as an effective alternative to antibiotics.

SUMMARY

6. SUMMARY

1. The objective of the present study was to screen for the antibacterial activity in leaves of the mangrove *Avicennia officinalis,* its bark, the seaweed *Gracilaria corticata* and the plant-animal complex "lab-lab" and to evaluate the nature of the antibacterial compound.

2. Five gram of each sample were ground in mortar and pestle with the addition of glass powder in order to ensure complete grinding and subsequent extracting of the bioactive compound. The different solvents used were butanol, chloroform, methanol, acetone and water.

3. The antibacterial activity was tested by disc diffusion assay, with a disc of broad spectrum antibiotic chloramphenicol as the positive control and disc absorbed with solvent alone as the negative control.

4. Maximum zones of inhibition were observed in the aqueous extract of *Avicennia* leaf. In the butanol extract, activity was detected only against *E. coli*. The butanol, chloroform and aqueous extracts of bark showed less activity compared to that of leaf. *Gracilaria* extract in butanol also showed good antibacterial activity against *Aeromonas, Salmonella* and *E. coli*. In chloroform too, zone of inhibition was observed against *Salmonella* and *E. coli.* "Lab-lab" did not show any antibacterial activity in any of the above-mentioned solvents.

5. Phytochemical analysis to determine the active compound for the antibacterial activity in aqueous extract of *Avicennia* leaf was conducted by thin layer chromatography.

6. Samples of *Avicennia* leaf extract in water were applied on five numbers of activated glass plates coated with silica gel-G. The solvent used for running the sample was a mixture of butanol, acetic acid and water in the ratio of 4:1:5 (v/v/v).

7. Each of the plates were sprayed with one of the following reagents, 1% alcoholic $FeCl_3$ for detection of polyphenol/flavonoids, 1% vanillin-H_2SO_4 for terpenoids, 10% alcoholic KOH for quinones, iodine for fats and 0.2% ninhydrin for peptides. The presence of the compound was detected by the development of colour.

8. Thin layer chromatographic analysis suggests that the antibacteriall compound in *Avicennia* leaf is a polyphenolic lipid quinone with isoprenoid side chain.

9. The strong antibacterial activity seen in extracts of mangrove leaves and seaweed indicates that they have the potential to be used as effective alternatives to antibiotics in aquaculture. However, *in vivo* studies need to be carried out with these extracts to treat bacterial infections in fish/shellfish. Also, further studies are required to determine whether these extracts have antifungal or antiviral activities.

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SCREENING OF "LAB-LAB", SELECTED MANGROVE PLANT AND SEAWEED FOR ANTIMICROBIAL COMPOUNDS

By

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ABSTRACT OF THESIS

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ABSTRACT

The present study aimed to find out the feasibility of using mangrove leaf, "lab-lab" and seaweed as a bioactive compound source and to evaluate the antibacterial activity of each *in vitro* against pathogenic bacteria. *In vitro* screening of the extracts of the seaweed viz., *Gracilaria corticata*, and the mangrove *Avicennia officinalis* by disc diffusion test showed species specific activity in inhibiting the growth of bacteria pathogenic to fish viz., *Pseudomonas* spp*., Bacillus megaterium, Aeromonas hydrophila, Salmonella* spp. and also *E.coli*. The solvents used were butanol, methanol, chloroform, acetone and water. The aqueous extracts of *A. officinalis* leaves showed very high activity against *Bacillus*, *Salmonella* and *Aeromonas,* the zones of inhibition being greater than or comparable to that obtained with the broad spectrum antibiotic chloramphenicol used in the study as a positive control. The butanol extract of mangrove leaf was active against *E. coli.* The butanol and aqueous extract of the bark of *A. officinalis* showed good activity against *Salmonella* and *Bacillus,* whereas in case of *Gracilaria corticata*, butanol, chloroform and acetone extracts showed considerable activity against *Aeromonas, Salmonella* and *E. coli,* respectively. However, extracts of "lab-lab" in the different solvents did not show any activity against the bacteria tested. Water was the best solvent to extract the antimicrobial compounds from the mangrove leaves while butanol was the best solvent medium for extracting the effective antimicrobial compounds from the marine algae *Gracilaria*. Phyto-chemical analysis of the aqueous extract of *Avicennia* leaves by thin layer chromatography to analyze the compound responsible for the antibacterial activity indicated the presence of a polyphenolic lipid quinone with isoprenoid side chains. Thus, present study shows the potential of using mangrove and seaweed extracts for development of antibacterial agents for use in aquaculture as an alternative to antibiotics.