Chapter II

Studies on the bioactivity and chemistry of some selected mangrove plants
References:

3. Everett RR, Kanofsky JR and Butler A. Biol. Chem. 1990, 265: 4903


BIOACTIVE SUBSTANCES FROM THE MANGROVES- A REVIEW.

The mangrove ecosystem is one of the important ecosystems in the tropics and has great economic and ecological value. The mangrove forests provide habitats for species, which are adapted to a saline tidal environment and a large number of diverse migratory waterfowl and terrestrial animals and are very important as habitat of aquatic organisms. The term mangrove is also used to designate halophytic and salt resistant marine tidal forests comprising of trees, shrubs, palms, epiphytes, ground ferns and grasses, which are associated in stands or groves. Mangroves are usually found only in tropical climates, as they need consistently warm conditions for development and survival.

Mangrove flora can be classified into true mangroves and mangrove associates. True mangrove species consist of plants which are absolutely confined to salt or brackish water, while mangrove associates are plants which belong to the littoral zone and/inland vegetation but can frequently been found with back mangrove. The mangal associates are salinity tolerant plant species, which are not found exclusively in the proximity of mangroves and may occur only in transitional vegetation, landwards and seawards. However, they do interact with true mangroves. Mangroves (mangroves, mangrove minors and mangal associates) are highly productive ecosystem with various important economic and environmental functions. The uses of mangroves fall in two major categories: First is its indirect use in control of coastal erosion and protection of coastal land, stabilization of sediment, natural purification of coastal water from pollution. Second use of economic importance is associated with prawn fisheries and many other species including crabs, shrimp, oysters, lobsters and fish. Traditionally, besides their use in folklore medicine, the mangroves have been exploited for firewood and charcoal and their uses also include construction of dwellings, furniture, boats and fishing gear and production of tannins for dying and leather production. The mangrove leaves are useful contributors to the nutrient system of the mangrove environment. It is known that mangrove leaves contain sufficient amounts of minerals, vitamins and amino acids, which are essential for the
growth, and nourishment of marine organisms and livestock. Mangrove foliage plays an important role in the formation of detritus, which is utilized by several estuarine and marine detritivorous organisms, and mangrove leaves make a superior fodder due to their high salt and iodine content.  

Two basic factors justify the study of the chemical constituents of mangrove plants. First is their ability to thrive in a very peculiar environment under stressful conditions such as violent environments, high concentration of moisture, high and low tides of water, and abundant living microorganisms and insects and serve as a bridging ecosystem between freshwater and marine systems. These have imposed several modifications in these plants and due to these reasons one expects them to contain chemical compounds, which protect them from these destructive elements. The second and the most important reason is that numerous mangrove plants are being used in folklore medicine, and recently, extracts from mangroves and mangrove-dependent species have proven activity against human ailments and animal and plant pathogens. These plants can be used as alternative medicine, but scientific proof of their effectiveness and research into possible side effects are still needed before they can be widely recommended. Only limited investigations have been carried out to identify the metabolites responsible for their bioactivities. Metabolites, some with novel chemical structures, and belonging to a diversity of ‘chemical classes’, have been characterized from mangroves and mangal associates. But, here we will restrict only to molecules with unusual structures and products of secondary metabolism having toxicological, pharmacological and ecological importance identified from selected mangroves and mangal associates.

Medicinal uses, bioactivity of extracts, and metabolites characterized from selected mangroves and mangal associates:

*Acanthus illicifolius*, is an evergreen spiny herb used traditionally in the treatment of paralysis, asthma, rheumatic pains and possessing analgesic, anti-inflammatory and, leishmanicidal activities. Chinese use this plant to cure skin itches and abscesses. It is a rich source of long chain alcohols, triterpenes, steroids and triterpenoidal saponins. Stigmasterol (1), a common plant steroid, abundantly present in *A. illicifolius*, has been shown to have hypercholesterolemic effects.
Benzoxazoline (2), used extensively as a central nervous system depressant, also exhibiting antipyretic, hypnotic, and muscle relaxant activity was first identified as anti *Fusarium* factor from this plant. Its precursors, benzoxazinoids, have also been identified as anti-inflammatory agents. Furthermore, the ribose derivatives of this compound are active as anticancer and anti-viral agents. Jongsuvat (1981) found that the extracts of the plant were non-toxic to experimental mice but displayed significant anti-leukemic activity. Minocha and Tiwari report besides triterpenoidal saponins a novel alkaloid, acanthicifolin, from the root of this spiny herb. Benzoquinones have been identified from *Aegiceras corniculatum* and *Kandelia candel*. Decoctions made from the rhizome of the reed grass *Arundo donax* has been used as emollients and diuretics and are said to stimulate menstrual discharge and diminish secretion of milk. Triterpenes, sterols, alkaloids, and the novel compound, N-(4'-bromophenyl)-2,2-diphenylacetanilide (3), hitherto known only as a synthetic compound, has been isolated from different parts of this plant. Significant anti-feedant activity was exhibited by the isolates tricontanol (4), flavonoid tricin (5), and tetramethyl-N, N-bis (2,6-dimethylphenyl) cyclobutane-1,3-diimine (6). Known triterpenes, steroids, and a novel triterpenoid esters have been isolated from *Acrostichum aureum* and *Rhizophora apiculata*, a mangrove fern and tree respectively. The extracts of these plants are being used in folklore medicine. Rocaglamide (7), a substituted benzofuran, along with its congeners, has been identified as the active insecticidal constituent of the Chinese rice flower bush *Aglaia odorata*. Clopentabenzoferan, and aglaiastatin, two protein synthesis inhibitors have also been identified. Anti-plasmodial and cytotoxic activities of *Alstonia macrophylla* are due to an array of alkaloids present in the extracts. *Avicennia alba* is a rich source of naphthoquinones. An associate of *A.marina* fungus belonging to the genus *Phytophthora*, produced three chemically related phytoalexins (8). Naphthoquinone derivatives occurring in the *Diospyros* species have potent anti-tumor promoting activity. Tannin from species of *Diospyros* has anti-hemorrhages (snakebite) effects. Plants of the family *Rhizophoraceae* may be generally divided into mangrove species and inland species. Either group of the plants contains organic sulfur containing compounds. The alkaloids brugine (9) and gerradine
1,2-dithiolane (sulfur containing) compounds isolated from *Bruguiera sexangula* (mangrove species) and *Cassipourea gerrardii* (mangal associate) respectively. Extracts of *B. sexangula* bark were active against, Sarcoma 180 and Lewis Lung Carcinoma cell lines. The activity was partly associated with tannins and partly with tannin-free aqueous residue containing the alkaloid brugine (9) as well as tropine and its acetic acid ester. The alkaloids were found to be toxic. Kato and coworkers identified 1,2-dithiolane compounds, brugierol (11), isobrugierol, and 4-hydroxy-1, 2-dithiolane-1-oxide, from the mangrove species *B. conjugata* with antibacterial and insecticidal activities. The bark of *Gymnotroches axillaris* has yielded hygroline (12).

Metabolites belonging to different 'chemical classes' have been identified as antifungal agents and in chemical narcosing of fish. Antifungal metabolites include alkaloids, flavonoids and related compounds, modified fatty acids, oxygen heterocyclics, proanthocyanidins, quinones, stilbenes, terpenoids, triterpenoids and saponins. The bark of the mollucidal and piscicidal plant *Balanites aegyptiaca*, besides being used for the treatment of abdominal pains, as a purgative, and as an anthelmintic, is also employed as a detergent, fish poison, and also as a remedy for malaria and syphilis. The leaf is edible and has been once regarded as an effective medicine for sleeping sickness. The piscicidal effect of *B. aegyptiaca* to the Nile Tilapia and the molluscicidal activity is due the metabolites balanitin, 1,2, and 3. The saponins are the main constituents responsible for the piscicidal activity of *Aegiceras majus*, *Derris trifoliata*, *D. elliptica* and *D. urucu*. Rotenone (13), a well-known fish poison and a natural insecticide, is found among tropical plant species such as, *Derris, Lonchocarpus*, and *Tephrosia*. The sesquiterpenes heritianin, heritol, heritonin, vallapin (14) and vallapianin are the ichthyotoxins isolated from the mangrove plant *Heritiera littoralis*. Vallapin and vallapianin also showed activity against Boll Weevils. A triterpene ester isolated from *H. littoralis* showed significant anti-fungal and Boll Weevil anti-feedant activities. The piscicidal activity of the extracts of *Aegiceras corniculatum* is due the benzoquinones embelin (15) and 5-O-methyl embelin (16). 5-O-Methyl embelin also inhibited the growth of the fungi *Pythium ultimum*. Fagaronine (17), an antileukemic alkaloid, has been isolated from *Fagara*
The alkaloid was found to be bactericidal but not mutagenic. *Caesalpinia bonducuella*, extensively used in Jamaican folk medicine is a rich source of furanoditerpenes collectively referred to as caesalpins (18). The oleoresin from the bark of *Calophyllum inophyllum* (Guttiferae) is used as a cicatrisant, whereas an infusion or decoction of the leaves has been traditionally used for the treatment of eye diseases and as an ingredient in aromatic powders and liniments. Anti-bacterial, anti-inflammatory, and phagocytosis stimulant activities have been reported for this plant. Guttiferaceous species are a rich source of xanthones, biflavonoids, benzophenones, neo-flavonoids, and coumarin derivatives. Recently, various bioactivities such as cytotoxic, and antitumour, anti-inflammatory, antifungal, enhancement of choline acetyltransferase activity, and inhibition of lipid peroxidase due to xanthones have been revealed. Two new xanthones, calaxanthones A and B have been isolated from the root bark of *C. inophyllum*. The giant African snail, *Achatina fulica*, feeds on the leaves of *C. inophyllum* and ingest inophyllums and calophyllolides from the plant. A xanthone derivative, subelliptenone, and related compounds showed strong inhibitory effect against topoisomerases 1 and 11, in *in vitro* experiments. These xanthones are claimed to be prospective lead compounds for anticancer drugs and inophyllums as active against HIV-1 in cell culture. Earlier phytochemical studies had revealed that *C. inophyllum* to be a rich source of benzopyrans, coumarins, steroids, triterpenes, and xanthones. Plants of the genus *Clerodendron* are well known for their pesticidal properties. They are used as armyworm antifeedants and to arrest bleeding from cuts, wounds, as well as post-partum hemorrhage. *Clerodendron inerme*, a mangal associate, is recognized medicinal plant exhibiting antipyretic, larvicidal, antiviral and uterine stimulant activities. Extracts of *C. inerme* were effective as surface protectants for cowpea seeds against pulse beetle infestation. The anti-viral resistance-inducing protein isolated from the plant is a polynucleotide. A number of flavonoids, a neolignan, and novel complex iridoids, phenyl propanoids, sterols and known terpenes, a new diterpene acid cleroinermin, have been characterized from the plant. *Cyprus rotundus* was used in traditional control of insect pests, the effectiveness being due to the presence of novel sesquiterpene alkaloids. Triterpenes,
steroids, long chain aliphatic carboxylic acids are responsible for the antifeedant activity of *Eleocharis dulcis* \(^{41}\). The sap of *Hippomane mancinella* is known to cause a reaction characteristic of a burn and contact with eye, produce severe conjunctivitis, which, if complicated by secondary infection, could result in loss of sight\(^{42}\). Surprisingly, the poisonous latex, a source of various metabolites has been used as an ingredient in many native preparations. 2-Hydroxy-2, 6-dimethoxyacetophenone, mono, di and trimethyl ethers of ellagic acid (23), and a novel alkaloid have been isolated from various parts of the plant. The toxic principle of the extracts of leaves and twigs was identified as tannins, hippomanin A and B. The irritant factor was assigned to esters of deoxyphorbol (24), resiniferonol, and 13-hexadeca-2, 4, 6-trienoic acid\(^{43}\). Apart from its folk medicinal utilization, *Excoecaria agallocha* contains toxic principles injurious particularly to the skin. The latex though mildly active against certain fungi and inactive against bacteria and yeast it is well known for its biocidal effects on marine organisms and phytoplankton. It causes metabolic depression of the rice field crab, *Oziotelphusa senex* and is used as an uterotonic, fish poison, dart poison, and contains novel chalcones and piperidine alkaloids. Soil bacteria and yeast actively degrade the latex, which probably helps in the detoxification of the latex in nature. The infusion of leaves posses antioxidant and anti-tumor promoting properties. Bioassay guided isolation led to the characterization of excoecarin, an irritant and a tumor promoter and excoecariatoxin (25), piscicidal agent with activity comparable to natural rotenone. A novel phorbol ester was isolated as the anti-HIV principle of the leaves and stems\(^{44-49}\). Sesquiterpenoid quinones, the hibiscones (26), hibiscoquinones and benzoquinones (27) are the major constituents of *Hibiscus tileaceous*. *Ipomoea pes-caprae* is a traditional medicinal plant used in the treatment of headache and various types of inflammation including jellyfish sting dermatitis. The extracts from the leaves exhibits anti-inflammatory activity, reduce prostaglandin synthesis *in vitro*, and inhibit smooth muscle contraction. 2-Hydroxy-4, 4, 7-trimethyl-1 (4H)-naphthalenone, mellein, eugenol, and 4-vinylguaicol, were the inhibitors of prostaglandin synthesis. The antispasmodic and anti-nociceptive activities\(^{50,52}\) was exhibited by the presence of the isoprenoids \(\beta\)-damascenone (28) and E-phytol
Melaleuca leucadendron exists in three chemotypes, the volatile leaf oil of two of which is characterized by very high content of phenylpropanoids. In addition to small amounts of known mono-, di-, sesqui-, and tri- terpenes, stilbene glycosides, novel triterpenoid esters, and hydrolysable tannins, have been isolated from the plant, whose extracts possess antifungal properties. Terpenoids along with stilbenes (30), inhibited histamine release from rat mast cells and were active against Bacillus and Staphylococcus. Known glycosides, fatty acid esters, and a novel trisaccharide have been characterized from ripe fruits of Morinda citrifolia, which is toxic to nematodes and Drosophila. Octanoic acid, toxic to many insect species, along with hexanoic and other carboxylic acids, are the main toxic compounds isolated from the extracts. Hirazumi and Furusawa reported the presence of a ‘polysaccharide-rich substance’ with antitumor activity in the fruit juice of M. citrifolia, which also showed anticancer and analgesic activity. A polysaccharide extracted from the leaves of B. cylindrica, E. agallocha, R. apiculata, R. mucronata, Salicornia brachiata, Sesuvium portulacastrum, Sueda maritima and S. monica showed positive activity against human immunodeficiency viruses.

Antioxidant activity of extractives of Pandanus odoratissimus has been demonstrated as due to phenolics, lignans, and a benzofuran derivative. A number of Pluchea species are noted for their ethnomedical properties, of which the reputed viper venom neutralization activities of P. odorata and P. indica are probably the best known. Neuropharmacological actions (including viper venom neutralization) of the shrub P indica have been investigated. The leaves and roots of the shrub have been reported to possess anti-inflammatory and anti-ulcer, especially gangrenous ulcers, astringent and antipyretic properties and are used as a diaphoretic in fevers. The crushed leaves and young shoots mixed with alcohol, are used to relieve lumbago and rheumatic pains and in baths to treat scabies. A number of known compounds and a new eudesmane derivative have been identified from the leaves.

(Z)-5-Tetradecenyl acetate and tetradecyl acetate were identified as sex pheromone components of an unnamed Planotortrix leaf roller moth species found in Avicennia resinifera. All parts of the plant Pongamia pinnata, used as a
crude drug for the treatment of tumors, piles, skin diseases, wounds, ulcers, is a rich source of flavonoid and related compounds\textsuperscript{63-64}. Extracts of the plant showed positive activity against human and simian immunodeficiency viruses\textsuperscript{65-67}. The indole derivative, rhizophorine (32) is a major constituent of the leaves of \textit{Rhizophora mucronata} and a novel type of water soluble polymer has been isolated from the leaves of \textit{R. stylosa}\textsuperscript{11}. Triterpenoids from \textit{R. mangle} possess insecticidal properties and has clinical use in the control of diabetes. Warm aqueous extract of the bark of \textit{R. apiculata} is used as an astringent for diarrhoea, nausea, and vomiting, and as an antiseptic. The extract is also used to stop bleeding in fresh wounds and for the treatment of chronic typhoid fever. A nitrogen containing phorbol ester, sapintoxin A (33), a piscicidal agent, has been isolated from the poisonous plant \textit{Sapium indicum}. Sapinine, a diterpene ester (a phorbol derivative), and a non-biologically active metabolite was isolated using traditional purification techniques\textsuperscript{68}. Skin irritant and tumor promoting diterpene ester, 12-deoxyphorbol, has been identified from \textit{Sapium sebiferum}\textsuperscript{69}. \textit{Sesuvium portulacastrum}, a salt marsh halophyte, is a rich source of an array of amino acids\textsuperscript{70}. An unusual secondary metabolite, 2-nitro-4- (2'- nitroethenyl) phenol (34) has been isolated from the fruits of \textit{Sonneratia acida}. The fruits are used as poultice in swelling and sprains. Fermented juice of the fruit is useful for arresting haemorrhage. The wood has yielded three anthraquinones and the leaves contain plant growth regulators, the diterpenoid gibberellins (35)\textsuperscript{71}. \textit{Bruguiera gymnorrhiza}, \textit{Rhizophora mucranata} and \textit{Sonneratia apetala} were also found to contain gibberellins\textsuperscript{72-73}.

\textit{Terminalia catappa} is used in folk medicine for preventing hepatoma and treating hepatitis and is a rich source of tannins. Antioxidant and hepatoprotective activity, anti-sickling potential, and the effects of the major tannin components, punicalagin and punicalin of \textit{T. catappa} on carrageenan-induced inflammation in rats, bleomycin-induced genotoxicity in rabbits, have been evaluated\textsuperscript{47,74,75}. The triterpene lupeol is the antibacterial principle of the leaves of \textit{Thespesia populnea}, and gossypol was the active ingredient in the flowers, which accounted for its antifertility activity\textsuperscript{76}. Naturally occurring quinones, the mansonones, extracted from the heartwood of \textit{T. populnea} showed cytotoxicity, antibacterial and anti-
steroidogenic activities. The limonoid ester, xylocensins (36), the esters of alcohols, isobutyrate and alpha-methylbutyrates, methyl angolensate, gedunin, phragmalin were the novel constituents of X. moluccensis. Alvi et al. characterized two new liminoids, xylocensin 1 and 2, devoid of any biological activity, from the Fijian species of X. granatum and X. moluccensis. The fruit of X. molluscensis is used in folk medicine in East Africa as aphrodisiacs. The bitter principle of young fruits, an unusual monoterpenoid having a nonglycosidic hemiacetal function xylomollin (37), tested positive as an antifeedant and strongly inhibited the respiratory reactions of mitochondria from rat liver. Insect antifeedant bioassays employing African armyworms and Mexican bean beetle has led to the isolation and characterization of N-methylflindersine (38) and several benz [C] phenanthridone alkaloids from the extracts of X. granatum. The former metabolite has been identified as the principle responsible for insect antifeedant activity.

Drugs of 'natural' origin, either the 'original' natural product, products derived 'Semi-synthetically' from natural products or synthetic products based on natural products models, play an invaluable role in the drug discovery process. Marine organisms and plants produce novel metabolites unique to the environment. Mangroves and mangal associates living in yet another different environment to that of marine and terrestrial plants, produce metabolites, which are unique to these plants and are of interest to the 'curious' chemist. Although the chemistry of the natural products of mangrove plants is little known, there have been some examples in recent years to support the need to study the chemistry of the mangroves. This belief is well supported by the illustrated examples. The chemistry of mangrove plants tends to establish that they are not only source of novel compounds but also provide a new source for many already known biologically active compounds including toxic compounds. Rotenoids, alkaloids, terpenoids are among the classes of natural products, which provide numerous toxins. Toxin in plants often has the role of feeding repellents. A remarkable number of insecticidal plants seem to have been recognized first as fish poisons. Knowledge of the toxins in higher plants has led to a variety of useful drugs. Metabolites though toxic, are still used clinically for the treatment of diseases.
Typical examples are those of the toxic drugs, sodium stibogluconate and pentamidine, used in the treatment of *Leishmania donovani* infection\(^7\). Though numerous mangroves and mangal associates are recommended in traditional medicine as active against various diseases, very little attempts have been made to investigate the veracity of these assertions in controlled experiments. Few workers have investigated the reputation of such plants by performing *in vitro* and *in vivo* experiments in order to demonstrate whether there are any protective effects, using drugs or mixtures of drugs prepared using traditional formulae.

In our ongoing program on 'Bioactive substances from Indian Ocean' we have directed our efforts towards detecting pharmacological activities of the extracts of marine organisms including mangrove plants and towards isolating and characterizing those active substances which show promise as potential therapeutic agents. During these investigations we had an opportunity to study the bioactivity and chemistry of mangrove plants, *Lumnitzera racemosa*, *Aegiceras corniculatum* and *Sesuvium portulacastrum*. Accordingly this chapter has been divided into three sections:

**SECTION-I-** Antibacterial flavonoids identified from *Lumnitzera racemosa* are described. This section has been divided into two parts:

**Part-1:** Antimicrobial activity of the tonga mangrove, *Lumnitzera racemosa*.

**Part-2:** Chemical investigation of the active n-butanol fraction by Tandem mass spectrometry.

**SECTION-II-** Deals with the antimicrobial and CNS depressant properties of *Aegiceras corniculatum*.

**SECTION-III-** Chemical investigation on *Sesuvium portulacastrum* exhibiting oxytocic activity has been discussed.
References:


Introduction:

Infectious diseases are still a major scourge of human life, and recent emergence of multidrug resistance to antibiotics by bacteria due to genetic mutation or gene acquisition has left physicians with little recourse for the treatment of what were once routine infections. Current threats posed by *Staphylococcus aureus* are disturbing, with deaths associated with infection by methicillin-resistant *S. aureus* and the increased prevalence of drug-resistant *S. pneumoniae* in community-acquired pneumonia. While attention commonly focuses on resistance to antibacterial agents, resistance to antifungal, antiparasitic and antiviral drugs is also on the rise. This has created an urgent need for the rapid and continued development of new antimicrobial agents to replace the current regimens.

During the general screening of the extracts for biological activity the crude methanolic extract of *Lumnitzera racemosa* Willd exhibited considerable activity against antibacterial infection. Fractionation of the crude extract, by partitioning into solvents of increasing polarity i.e. successively with petroleum-ether, chloroform, n-butanol and the residue is considered as water-soluble. Testing of these fractions located the activity in n-butanol soluble fraction.

This section has been further divided into two subsections:

The first one relates to the antibacterial activity observed in methanolic extracts of this mangrove, and the bioassay guided identification of the antibacterial principle from the n-butanol fraction of the methanolic extract of the plant *Lumnitzera racemosa* Willd.

The second subsection deals with the flavonoids identified from the active n-butanol fraction by ESI-MS/MS.
2.1.1 Antimicrobial activity of the tonga mangrove, *Lumnitzera racemosa* (Willd):

The plants of *Lumnitzera racemosa* Willd, a mangrove belonging to family *Combretaceae* are large glabrous shrubs, growing on the salt marshes along with mangroves on the coast of India and on the Andaman and Nicobar Islands. The wood of *L.racemosa* is used as a fuel for its calorific value and the leaves of the plant are eaten in South Pacific Island during periods of scarcity. The reddish brown bark contains 15-19% tannin while the leaves and wood contain smaller quantities. A fluid obtained from incisions made in the stem was reported to be employed as an external application for the treatment of herpes and itches. According to the folk medicine, the fruits of this plant are curative in skin disorders. Antihypertensive activity has been reported for the aqueous acetone extract of the plant. The blood pressure lowering activity was due to the presence of hydrolysable tannins. Chemical examination of this plant occurring in various parts of the world was reported to give a large number of compounds, long chain rubber like polyisoprenoid alcohols in leaves, flavonoids and long chain fatty acids and low molecular weight carbohydrates. Chemical examination of Indian species was reported to give friedlin, β-amyrin, taraxerol, betulin, betulinic acid and tricontanol besides a new aromatic ester and an enzyme cinnamyl alcohol-NADPH-dehydrogenase. The presence of trace elements is also reported.

\[ G = \text{galloyl} \quad \text{HHDP} = \text{hexahydroxydiphenoyl} \quad \text{CHEB} = \text{chebuloyl} \quad \text{GALA} = \text{gallayl} \quad \text{NECH} = \text{neochebuloyl} \]

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Results:

Crude methanolic extract of the tonga mangrove (*Lumnitzera racemosa*) was screened *in vitro* against fungi (*Candida albicans, Aspergillus fumigatus, Mucor sp.*), virus (*Hepatitis B*) and pathogenic gram-positive (*Staphylococcus aureus*) as well as gram-negative bacteria (*Salmonella typhi, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Shigella flexineri, Proteus mirabilis, Vibrio cholerae*). The extract and the fractions were ineffective against fungi and the *Hepatitis B* virus tested even at 500 µg/ml concentration, but the crude methanolic extract and the n-butanol fraction were bactericidal with an inhibition zone greater than 10mm at 50µg/ml against all the pathogenic bacteria tested (Table-1). The active n-butanol fraction on bioassay-guided fractionation and chromatography over sephadex LH20 with methanol yielded two yellow compounds identified as flavonoids, quercetin (1), M+ 302 and myricetin (2), M+ 318, on the basis of spectral data (Table-2) and these data compared well with the literature values15.

Compound 1 was obtained as yellow amorphous powder with an elemental composition of C15H10O7 as evidenced by its electron impact mass spectra (EI-MS, Fig-2.1.1), which showed molecular weight of 302. The infrared spectrum (Fig-2.1.2) indicated the presence of hydroxyl group (3294.2 cm⁻¹) and α,β unsaturated carbonyl groups (1668.3cm⁻¹). Aromaticity was evident from the absorption bands at 1614.3, 1517.9 cm⁻¹. Its nuclear magnetic resonance (NMR) data was characteristic of flavonoids with ¹H NMR (Fig-2.1.3) (Table-2) showing two meta coupled protons, as doublets at δ 6.183 (J=2.1 Hz) and 6.38 (J=2.1Hz) ascribed to H-6 and H-8 and are indicative of 5,7 disubstitution in ring A. The aromatic protons on the ring B of 1 showed an ortho coupling doublet δ 6.88 (J=8.7 Hz), a meta coupling doublet at δ 7.73 (J=2.1Hz) and a double doublet at δ 7.64dd (J= 2.1, 8.4 Hz) assigned to H-5', H-2' and H-6' respectively.
**Table 1. Antibacterial activity of the methanolic extract, fractions, and flavonoids.**

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<th>Concentration (μg/ml)</th>
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<th>P. mirabilis</th>
<th>P. aeruginosa</th>
<th>S. typhi</th>
<th>S. fie.</th>
<th>S. aureus</th>
<th>V. cholerae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract</td>
<td>500</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Petroleum ether</td>
<td>50</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Chloroform</td>
<td>50</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>n-butanol</td>
<td>50</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Quercetin</td>
<td>30</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++++</td>
<td>-</td>
<td>+</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>Myricetin</td>
<td>30</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>(Quercetin+ Myricetin) 1:1</td>
<td>30</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

- No zone of inhibition (inactive); (++) 2-3 mm zone of inhibition (moderately active); (+++) 3-5 mm zone of inhibition (strongly active); (++++) 5-7 mm zone of inhibition (significantly active).

Bioassays that were carried out on the extract against the fungi and virus below showed no inhibitory activity.

- *Aspergillus fumigatus.*  
- *Mucor sp.*  
- *Candida albicans.*  
- *Hepatitis B virus*
The compound also exhibited singlets at δ 13.4, 13.6, 14.6 and 14.9 due to D₂O exchangeable phenolic hydroxyl protons. This ¹H NMR data coupled with its ¹³C NMR values (Fig-2.1.4)(Table-2) identified compound 1 as quercetin, the data being well in agreement with the literature reports¹⁵.

![Compound 1](image1)

Compound 2 with molecular formula of C₁₅H₁₀O₈, as evidenced by ESI-MS (M⁺ 318) (Fig-2.1.5a), MS/MS (Fig-2.1.5b) had spectral characteristics similar to that of compound 1. The only difference observed was that the protons in tetrasubstituted ring B appeared as a two-proton singlet at δ 7.3 indicating their equivalence. This value was assigned to H-2' and H-6' of ring B. On the basis of spectral data observed and its comparison with the literature values the compound was identified as myricetin¹⁵. The structure of compound 2 was confirmed by its IR (Fig-2.1.6), ¹H NMR(Fig-2.1.7) and ¹³C (Fig-2.1.8) (Table-2). The fragmentation pattern observed in its MS/MS spectrum is well in agreement with the structure of myricetin (2).

![Compound 2](image2)

It is evident from bioassay-guided fractionation of the active n-butanol fraction and identification of the active constituents that the observed activity was due to the presence of flavonoids identified as quercetin (1) and myricetin (2). Quercetin was effective only against three bacterial strains (P. aeruginosa, S. flexineri and...
Section I

Antibacterial flavonoids from Lumnitzera racemosa
Fig 1.2.10: $^{13}$CNMR of Compound (2).

Fig 1.2.11: IR of Compound (2).
Fig 1.2.12: $^1$HNMR of Compound (2).

Fig 1.2.13: HMQC of Compound (2).
Fig 1.2.14: HMBC of Compound (2).

Fig 1.2.15: TOCSY of Compound (2).
Fig 1.2.16: $^1$H-$^1$H COSY of Compound (2).

Fig 1.2.17: NOESY of Compound (2).
Fig 1.2.18: ESI-MS of Compound (2).
S. aureus) among eight tested at 30 µg/ml (MICs, 6µg/ml; Table 3). At the same concentration, myricetin was effective in inhibiting all of the gram-positive as well as gram-negative bacteria (MIC - 6µg/ml), with the strongest activity being observed against P. aeruginosa (MIC of 1.5µg/ml). Salmonella typhi and V. cholerae were not susceptible to myricetin.

Table 2: NMR spectral data (300 MHz; DMSO) of Flavonoids.

<table>
<thead>
<tr>
<th>Carbon No.</th>
<th>¹²C</th>
<th>¹'H</th>
<th>¹²C</th>
<th>¹'H</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-2</td>
<td>148.0</td>
<td>-</td>
<td>156.5</td>
<td>-</td>
</tr>
<tr>
<td>C-3</td>
<td>137.2</td>
<td>-</td>
<td>135.9</td>
<td>-</td>
</tr>
<tr>
<td>C-4</td>
<td>177.3</td>
<td>-</td>
<td>175.6</td>
<td>-</td>
</tr>
<tr>
<td>C-5</td>
<td>162.5</td>
<td>-</td>
<td>160.9</td>
<td>-</td>
</tr>
<tr>
<td>C-6</td>
<td>99.2</td>
<td>6.18(d, J=2.1Hz)</td>
<td>98.5</td>
<td>6.17(d, J = 2.1Hz)</td>
</tr>
<tr>
<td>C-7</td>
<td>165.5</td>
<td>-</td>
<td>164.0</td>
<td>-</td>
</tr>
<tr>
<td>C-8</td>
<td>94.4</td>
<td>6.38(d, J=2.1Hz)</td>
<td>93.6</td>
<td>6.37(d, J = 2.1Hz)</td>
</tr>
<tr>
<td>C-9</td>
<td>158.2</td>
<td>-</td>
<td>156.5</td>
<td>-</td>
</tr>
<tr>
<td>C-10</td>
<td>104.5</td>
<td>-</td>
<td>103.2</td>
<td>-</td>
</tr>
<tr>
<td>C-1'</td>
<td>121.6</td>
<td>-</td>
<td>121.7</td>
<td>-</td>
</tr>
<tr>
<td>C-2'</td>
<td>116.0</td>
<td>7.73(d, J=2.1Hz)</td>
<td>107.6</td>
<td>7.34(s)</td>
</tr>
<tr>
<td>C-3'</td>
<td>146.2</td>
<td>-</td>
<td>145.4</td>
<td>-</td>
</tr>
<tr>
<td>C-4'</td>
<td>148.7</td>
<td>-</td>
<td>135.3</td>
<td>-</td>
</tr>
<tr>
<td>C-5'</td>
<td>116.2</td>
<td>6.88(d, J=8.7Hz)</td>
<td>145.4</td>
<td>-</td>
</tr>
<tr>
<td>C-6'</td>
<td>124.1</td>
<td>7.52 (dd,J=8.4,2.1Hz)</td>
<td>107.6</td>
<td>7.31(s)</td>
</tr>
</tbody>
</table>

Table 3. MIC (µg/ml) of flavonoids against one strain each of eight medically important bacteria.

<table>
<thead>
<tr>
<th>Compound</th>
<th>E. coli</th>
<th>K. pneumoniae</th>
<th>P. mirabilis</th>
<th>P. aeruginosa</th>
<th>S. typhi</th>
<th>S. flexineri</th>
<th>S. aureus</th>
<th>V. cholerae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quercetin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>6</td>
<td>-</td>
<td>6</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>Myricetin</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>1.5</td>
<td>-</td>
<td>6</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>Q + M.</td>
<td>-</td>
<td>-</td>
<td>6</td>
<td>-</td>
<td>-</td>
<td>6</td>
<td>6</td>
<td>-</td>
</tr>
</tbody>
</table>

Q (Quercetin) + M (Myricetin)
A strong inhibitory activity of myricetin against the most resistant organism, *P. aeruginosa* and methicillin-resistant organism, *S. aureus* have significant clinical implications. Based on the current findings, it can be concluded that this plant has antibacterial activity, which is being reported here for the first time.

**Discussion**

In this study, strong antibacterial activity by quercetin from *Lumnitzera racemosa* was observed only against the bacteria *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella typhi* and *Shigella flexineri*, with MICs at 6 µg/ml. Quercetin is reported by Aziz et al. to inhibit microorganisms such as *Escherichia coli*, *Klebsiella pneumoniae*, *Bacillus cereus*, *Aspergillus parasiticus*, *Aspergillus flavus* at a concentration of 100-200 µg/ml. Sensitivity of *Staphylococcus aureus*, *Staphylococcus epidermis*, *Bacillus subtilis*, *Mucor luteus* and *Escherichia coli* to quercetin is also reported at higher concentrations (500 µg/ml). Surprisingly, no significant activity against *Pseudomonas aeruginosa* ATCC 9027 and *Escherichia coli* ATCC 8739 was noted by Rauha et al. even at 500 µg/ml, perhaps because the strain used (*P. aeruginosa* ATCC9027) was a genetically mutated, resistant strain in comparison to the strain used in the present investigation. Gatto et al. reported that quercetin and its 3-O-acyl derivatives did not exhibit significant activity against gram-positive strains of bacteria (*S. aureus*, *B. subtilis*, *Listeria ivanovi*, *Listeria monocytogenes*, *Listeria serligeri*) as well as gram-negative strains (*E. coli*, *S. flexneri*, *Shigella sonnei*, *Salmonella enteritidis*, *Salmonella typhimurium*) and yeasts (*Candida albicans* and *Candida glabrata*) tested, and attributed the inactivity to the lower concentrations used for screening (100 µg/ml). In the present study, myricetin was found to be more inhibitory than quercetin, and was effective against six of eight bacterial species (one strain each) tested. *Salmonella typhi* and *Vibrio cholerae* were the two strains that showed resistance to myricetin. These data are supported by Puupponen et al., who found that myricetin inhibited growth of all lactic acid bacteria derived from gastrointestinal tract flora, but did not affect a *Salmonella*
enterica sv. typhimurium E-981151 strain. The flavonoid, myricetin is also known to inhibit other medically important, multidrug resistant bacteria in addition to methicillin-resistant Staphylococcus aureus, by inhibiting their ability to synthesize essential proteins.

To check whether mixing the two flavonoids could enhance the antibacterial activity, the mixture in 1:1 proportion was tried. It was observed that quercetin and myricetin, in 1:1 combination had no effect on the MIC value (6 µg/ml) when Shigella flexineri and Staphylococcus aureus were used as the test bacteria. Quercetin alone did not show any activity against Proteus mirabilis, and its addition had no effect on MIC for myricetin. Although both flavonoids were strongly inhibitory to Pseudomonas aeruginosa (MIC for myricetin, 1.5 µg/ml), mixing rendered them inactive. In contrast, Arima et al reported enhancement of antibacterial activities of flavonoids by combining or mixing them, especially when quercitrin was mixed either with quercetin, morin or rutin. Better potency of myricetin as compared to quercetin seems to be associated with the additional phenolic hydroxyl in ring B of myricetin.

Conclusion

In the present investigation, extract from the tonga mangrove, Lumnitzera racemosa, as well as a n-butanol fraction inhibited eight species (one strain each) of medically important gram-positive and gram-negative bacteria tested. Two flavonoids were partly responsible for the observed activity. L. racemosa, a plant belonging to Combretaceae, has been reported to contain large amount of tannins castalgin, punicalin and punicalagin. These tannins, and/or synergistic action of the constituents of flavonoids from the active fraction and other tannins present in the plant could be responsible for the activity against Salmonella typhi and Vibrio cholerae, which were resistant to the two flavonoids isolated and tested in this study. Tannins have antimicrobial activity although, paradoxically, some organisms (e.g. the fungus, Candida sp.) are capable of using tannins as a carbohydrate source.
Tea extract in combination with β-lactam antibiotic is reported to have a synergistic effect with methicillin against methicillin-resistant *Staphylococcus aureus* (MRSA), due to the presence of catechin compounds and polyphenols including quercetin and myricetin. A pharmaceutical composition containing 0.412% quercetin derived from St John's wort was shown to be suitable for clinical or veterinary use. Commercially the above-mentioned composition is known as Novoimamine, and is effective against *S. aureus* infection. Quercetin is a better bactericidal agent against *S. aureus* as compared to conventionally used sulfanilamide. In this study, the extract and n-butanol fraction from *Lumnitzera racemosa* showed broad-spectrum antibacterial activity against all the pathogenic bacterial strains tested, suggesting that this mangrove species has similar pharmacological value.

2.1.2: Chemical investigation of the active n-butanol fraction by Tandem mass spectrometry:

1. Characterization of flavonol glycosides from *L. racemosa*:

The active n-butanol fraction from the methanol extract of leaves of *L. racemosa* was analyzed by ESI-MS and Tandem mass spectrometry. It showed mass spectral characteristics of flavonoids, flavonoid glycosides, and biflavonoids. (Table 4) lists their molecular masses and fragmentation observed.

First order ESI-MS(Fig.2.1.9a,b) spectrum of the flavonoid rich fraction indicated that quercitrin (quercetin-3-O-α-L-rhamnopyranoside) (3) is a major constituent of the fraction followed by quercetin 3-O-hexoside (4).

![Compound 3: R = H; R' = Rha.](image)

123
(Fig 2.1.10) represent the MS/MS spectrum of a protonated flavonol glycoside [M+H]^+ at m/z 471. It was identified as quercitrin (3). As evident from the spectrum compound (3) loosens its terminal rhamnose unit to yield sodiated fragment at m/z 325 and also to produce a product ion at m/z 301 followed by Retro Diels-Alder (RDA) fragmentation 1,3 A to give ion at m/z 151.

RDA reaction of flavonoids is an important fragmentation reaction, which may occur in six-membered cyclic structures containing a double bond and involves the relocation of three pairs of electrons in the cyclic ring. The net result of these rearrangements is the cleavage of two-bonds and the formation of two-bonds, for example, cyclohexene will fragment into butadiene and ethylene. Two (complementary) fragments, 1,3 A^+ and 1,3 B^+, are formed and charge retention can occur on either side of the cleavages, as depicted in Fig. A.

The MS/MS spectrum (Fig 2.1.11) [M + Na]^+ peak at m/z 487 / [M+H]^+ at m/z 465 was attributed to the flavonoid, Quercetin-3-O-hexoside (glucoside/galactoside) (4). The fragmentation observed could be explained as shown below. The loss of hexose moiety from the molecular ion yields a fragment at m/z 301, which corresponds to quercetin aglycone suggesting that this compound is either quercetin glucoside or galactoside.
Compound 4

The aglycones kaempferol 4'-methylether (5) M⁺ 301.1345 (Fig.2.1.12), kaempferol 3,4'-dimethylether (6) M⁺ 315.148 (Fig.2.1.13), have also been identified in the fraction solely on the basis of fragmentation pattern observed in their tandem mass spectra (Table-4) and comparison with the literature values

Compound 5

Compound 6
Table 4: Tandem mass of Flavonoids

<table>
<thead>
<tr>
<th>Compound</th>
<th>[M+Na]/[M+H]+</th>
<th>ESI-MS/MS (% base peak)- Major ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quercitrin (Quercetin-3-O-rhamnoside) [3]</td>
<td>471</td>
<td>325(100), 301(9.6), 119(40), 197(24), 165(17.6), 105(12), 151(8), 129(6.4)</td>
</tr>
<tr>
<td>Quercetin-3-O-hexoside. (glucoside/galactoside) [4]</td>
<td>487</td>
<td>119(100), 87(28), 105(20), 151(28), 137(5), 245(7), 301(7), 343(6), 119(100), 87(28), 151(28), 137(5), 455(15), 473(2), 301(7).</td>
</tr>
<tr>
<td>Kaempferol-4’methylether [5]</td>
<td>301</td>
<td>55(100), 286(15), 153(11), 141(25), 109(0.5), 73(15), 87(50).</td>
</tr>
<tr>
<td>Bi-isorhamnetin [7]</td>
<td>615</td>
<td>87(100), 301(50), 209(20), 187(10), 105(20).</td>
</tr>
<tr>
<td>Myricetin-7-O-methyl ether (3→8'”)quercetin-3-O-rhamnoside [8]</td>
<td>763</td>
<td>119(100), 87(50), 105(44), 151(16), 195(22), 467(20), 617(12).</td>
</tr>
</tbody>
</table>

The product ion mass spectrum (Fig 2.1.12) of isosakurametin (5) (kaempferol 4’ methyl ether) contained ions at m/z 286 corresponding to the fragments [M + H – CH₃]. The RDA fragmentation products wherein bonds 1 and 3 undergo scission leading to the formation of the 1'3 A ion at m/z 153 and the sodiated ion 1'3 B at m/z 141 with simultaneous elimination of –OCH₃ group were also evident.

Kaempferol 3,4’ dimethyl ether (6) exhibited fragmentation (Fig 2.1.13) with the RDA fragmentation producing ions at 1'3 A m/z 155 and 1'3 B sodiated ion at m/z 187 which produces ion at m/z 173 with the loss of –CH₂ group. Cleavage 1'2 B led to the formation of ion at m/z 119. These product ion mass spectra were similar to those observed in other mass spectrometry studies of flavonoids and provided unequivocal identification of the relevant flavonols.
Quercetin besides being antibacterial is an effective antioxidant. Its glycosidic form should also be equally effective since in biological system glycosides undergo enzymatic hydrolysis to the corresponding aglycone. Quercetin is known to get metabolized to 3-O-methyl quercetin in intact rat lens. Interestingly, both the aglycone and its methyl ether are known to inhibit hydrogen peroxide induced sodium and calcium influx and lens opacification and thus play a role in the prevention of cataract formation.

2. Characterization of biflavonoids:
The majority of naturally occurring biflavonoids contain C-C linked monomers with ring A usually being involved in the inter flavonoid linkage. The combination so far found in nature are C-6→C8; C-3’→C6”; C8→C8”; C3’→C8” and C-3→C8”. In the present investigation the tandem mass spectra of the flavonoid constituents of the active n-butanol fraction was also indicative of the presence of biflavonoids, bi-isorhamnethin (7) (MS/MS spectrum (Fig 2.1.14)) 5,7,4’-trihydroxy-3’-methoxy (3’→8”)-3”,5”,7”,4’’-tetrahydroxy-3’’-methoxy flavone and myricetin-7-O-methyl ether (3→8”) quercetin-3-O-rhamnoside (8) (MS/MS spectrum (Fig 2.1.15)) 5,3’,4’,5’-tetrahydroxy-7-methoxy flavone (3→8”)-5”,3’”,4’’-tri hydroxy-3”-O-rhamnoside in the active fraction, the fragmentation pattern observed being well in agreement with the structure assigned to these biflavonoids.

![Compound 7](image)
Biflavonoids are a series of naturally occurring compounds that include flavone-flavone, flavanone-flavone and flavanone-flavanone subunit linkages. More than 100 biflavonoids have been identified from plants since the isolation of gingetin in 1929\textsuperscript{40}. A variety of biological activities for biflavonoids have been published including anti-inflammatory, antimicrobial, anti-oxidants and others\textsuperscript{41,42}. Biflavonoids \textit{Rheedia garderiana} volkensiflavone, fukugetin, fukugiside are antibacterial against \textit{E.coli}, \textit{P.aeruginosa}, \textit{S aureus} and \textit{B. cerrus} with MIC ranging from 0.15-1.0 mg/ml concentrations\textsuperscript{43}. Antimicrobial biflavonoids from the aerial parts of \textit{Ouratea sulcata} are reported active against \textit{S aureus}, \textit{B subtillis}, \textit{V anguillarium} and \textit{E coli} at MIC ranging from 0.85-12.5 µg/ml it being almost as effective as the standard streptomycin used. They were inactive against \textit{E.coli}.

\textit{L. racemosa}, a plant belonging to \textit{Combretaceae}, has been reported to contain large amount of tannins castalgin, punicalin and punicalagin besides corilagin\textsuperscript{22}. In the present investigation, we have identified flavonoids and biflavonoids from the active n-butanol fraction and the antimicrobial activity observed in this fraction could partly be due to the presence of the tannins. Corilagin is known to cause marked potentiation of β-lactams against methicillin-resistant \textit{Staphylococcus aureus} \textsuperscript{44}.
EXPERIMENTAL:

Plant material:

The tonga mangrove, *L. racemosa* as authenticated by Dr T. G. Jagtap, National Institute of Oceanography, Goa was collected from Ratnagiri, Maharashtra, along the West coast of India. A voucher specimen is deposited at National Institute of Oceanography Herbarium, Dona Paula, Goa, India, bearing the number NIO/DOD/DIO-1466. For this study, leaves and stems of 2 small plants (2.5Kg wet weight) were cut into small pieces. Extracts were prepared by percolation with 90% aqueous methanol that yielded 11.2gms of the crude extract on removal of aqueous methanol. Fractionation of the crude methanolic extract was done by partitioning successively with crude extract:petroleum ether (1:2, v/v, thrice), chloroform (1:2, v/v, thrice), n-butanol (1:1, v/v, thrice), and the insoluble aqueous residue and thus the petroleum ether, chloroform, n-butanol and aqueous fractions were obtained.

Microorganisms:

The microorganisms used in this study were one strain each of two moulds (*Aspergillus fumigatus* and *Mucor* sp.), a yeast (*Candida albicans*), a virus (*Hepatitis B*), and eight bacterial species: one gram-positive bacterial strain (*Staphylococcus aureus*), and seven gram-negative strains (*Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Proteus mirabilis, Staphylococcus typhi, Shigella flexineri and Vibrio cholerae*). All microorganisms were clinical isolates obtained from the stock cultures maintained in the microbiology laboratory of Goa Medical College, Goa, India.

Antibacterial and antifungal assays:

The active crude extract and fractions were subjected to bioassay-guided fractionation and the flavonoids to quantitative bioassays for the determination of the minimum inhibition concentration (MIC), defined as the lowest concentration
of the compounds showing no visible microbial growth after incubation time\textsuperscript{28}. Initially, antimicrobial spectrum of the extract was determined by the Agar well diffusion method\textsuperscript{28}. One loop full of a 24-hr-old culture containing $10^4$-$10^6$ cells\textsuperscript{29} was spread on the surface of the Mueller-Hinton agar plates. Wells 5-7mm in diameter were dug in the medium using a sterile borer, and were filled with a 500 µg/ml concentration of the \textit{L. racemosa} crude extract. Zones of inhibition were measured after overnight incubation, and expressed as the diameter of zone in millimeters. Crude extracts with an inhibition zone greater than 10mm were selected for further investigations. Bioassays of the fractions and active constituents with concentrations are given in Tables 1 and 2. Ampicillin and nystatin were used as antibacterial and antifungal controls.

The MIC was determined by tube dilution method\textsuperscript{30}, using Mueller-Hinton broth. Tubes that had been inoculated with bacterial cultures ($10^4$-$10^6$ cells/tube) which were found to be sensitive to the testing material were incubated overnight at a temperature of 37°C. Graded dilution of the testing material, which can inhibit growth of these organisms, was assessed.

\textbf{Antiviral assay:}

Since the \textit{Hepatitis B} virus cannot be grown on tissue cultures\textsuperscript{31} human plasma positive for hepatitis B surface antigen (HbsAg) was used as the virus source and the extract at 500µg/ml concentration was mixed with \textit{hepatitis B} surface antigen (HbsAg) positive plasma. This mixture was retested for \textit{hepatitis B} surface antigen (HbsAg) by enzyme-linked immunosorbent assays (ELISAs)\textsuperscript{32} after regular intervals of incubation (2hrs) with suitable controls (antigen-enzyme II from Abbott Laboratories). Negative antigen activity indicates promising anti HBV property.

\textbf{Isolation and purification of active constituents:}

The active n-butanol fraction (3.5g/300ml of butanol) was resuspended in methanol (100%) and loaded on to a column of Sephadex LH20, obtained from
Amersham Pharmacia Biotech Asia Pacific Ltd., Hong Kong, which was equilibrated with methanol. Bioassay-guided fractionation led to the isolation of two yellow solids, identified as quercetin (38mg/2.5Kg wet wt. of plant material) and myricetin (23mg/2.5Kg wet wt. of plant material) on the basis of spectral data (Table 3). The retardation factor [(Rf) - defined as the distance travelled by the compound divided by the distance travelled by the solvent] values on thin layer chromatography (TLC) silica gel F254 plates obtained from E. Merck (Germany) developed in methanol:chloroform solvent (1:4 ratio) were 0.76 and 0.58, respectively, and in agreement with the standards obtained from Sigma-Aldrich Company.

**ESI-QSTARXL MS/MS spectrometry:**

Full scan positive ion ESI mass spectra were obtained for each of the flavonols by direct infusion of diluted methanol mixture. In addition to full scan mass spectra, collision induced dissociation was undertaken in the MS/MS mode to yield diagnostic product ion mass spectra, which were characteristic of the structural moieties present in the analyte. While infusing the mixture, the collision energy was varied from 20V-50V so as to obtain optimum product ion mass spectra.
Fig 2.1.1: ESI-MS spectrum of compound 1

Fig 2.1.2: IR spectrum of compound 1
Fig 2.1.3: $^1$H NMR of compound 1

Fig 2.1.4: $^{13}$C NMR of compound 1
Fig 2.1.5a: ESI-MS spectrum of compound 2

Fig 2.1.5b: MS/MS spectrum of compound 2
Fig 2.1.6: IR spectrum of compound 2

Fig 2.1.7: $^1$H NMR of compound 2
**Fig 2.1.8:** $^{13}$C NMR of compound 2

**Fig 2.1.9a:** ESI-MS spectrum of the flavonoid rich fraction
Fig 2.1.9b: ESI-MS spectrum of the flavonoid rich fraction

Fig 2.1.10: MS/MS spectrum of compound 3
Fig 2.1.11: MS/MS spectrum of compound 4

Fig 2.1.12: MS/MS spectrum of compound 5

Fig 2.1.13: MS/MS spectrum of compound 6
**Fig 2.1.14:** MS/MS spectrum of compound 7

**Fig 2.1.15:** MS/MS spectrum of compound 8
References:


5. Griffiths, PD. Int. J. Infectious Disease. 6 (Supplement 1) 2002: S32-S37.


