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Antibacterial phenolics from the mangrove *Lumnitzera racemosa*

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Antimicrobial activity of the methanolic extract and fractions of *Lumnitzera racemosa* was evaluated against fungi, virus and pathogenic bacteria. Crude methanolic extract and n-butanol fraction were ineffective against fungi and virus tested but exhibited significant bactericidal activity on all the bacteria tested. Flavonoids, quercetin and myricetin are the main active constituents with quercitrin, quercetin-3O-hexoside, kaempferol 4′-methyl ether, kaempferol-3,4′dimethyl ether, and bi-isorhamnetin and myricetin-7O-methylether(3→8″)quercetin –3O-rhamnoside, which are also constituents of the active fraction being partly responsible for the observed activity. Myricetin showed the most potent activity (MIC – 1.5 µg/ml) against *Pseudomonas aeruginosa* being markedly active against others (MIC – 6 µg/ml). Based on the current findings it can be concluded that this plant has antibacterial activity.

[**Keywords:** *Lumnitzera racemosa*, Antibacterial, Flavonoids, Quercetin, Myricetin]

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 created an urgent need for the rapid and continued development of new antimicrobial agents to replace the current regimens.

L. racemosa Willd, (Combretaceae), a mangrove species found on the coast of India and in Andaman and Nicobar islands is known for its great economic importance. A fluid obtained from incisions made in the stem is useful in the treatment of herpes and itches. Its aqueous acetone extract is known to be antihypertensive. Phytochemical investigations of this plant have resulted in the identification of rubber-like polyisoprenoid alcohols, flavonoids and long-chain fatty acids, low-molecular-weight carbohydrates, triterpenes and an aromatic ester 3-(4-hydroxyphenyl)-propyl-3′-(3,4-dihydroxyphenyl)-propionate[1].

This communication relates to the identification of antibacterial principle in *L. racemosa* which is associated with polyphenolic constituents. The efficacy of this mangrove has been studied in terms of their ability to inhibit the growth of the commonly occurring clinical pathogens under *in vitro* conditions. In addition, some of the flavonoids present in the active n-butanol fraction have also been identified using electrospray ionisation tandem mass spectrometry.

The twigs of *L. racemosa* were collected in November 2006 from Ratnagiri, Maharashtra, West coast of India. Voucher specimen bearing reference no. NIO/DOD/DIO-1466 is deposited at the National Taxonomy Centre of National Institute of Oceanography.

Fresh twigs (2.5 kg) were extracted thrice with 90% aqueous methanol at room temperature. Removal of the solvent yielded 11.2 g of crude methanolic extract which was partitioned successively with petroleum ether, chloroform and n-butanol, to yield four fractions of different polarities including the most polar, insoluble aqueous residue. Bioassay-guided fractionation of the active n-butanol fraction (3.5 g) by repeated column chromatography over sephadex LH_{20} coupled with thin layer chromatography (silica gel $60F_{254}$, E. Merck) yielded two yellow solids, identified as quercetin (38 mg) and myricetin (23 mg) on the basis of spectral data.

Full scan positive ion electrospray ionisation mass spectra were obtained for each of the flavonols by direct infusion of diluted methanol mixture. Collision induced dissociation spectra were undertaken in the MS/MS mode to yield diagnostic product ion mass spectra, which were characteristic of the structural moieties present in the analyte. The collision energy was varied from 20V-50V so as to obtain optimum product ion mass spectra.

The clinical pathogens listed in Tables1 and 2 were used for antibiotic studies. Antibacterial and antifungal assays were performed using Agar well diffusion method[2, 3] and MIC was determined by tube dilution method using Himedia Mueller-Hinton broth[4]. Streptomycin and nystatin were used as antibacterial and antifungal positive controls while solvent methanol was used as negative control. For anti-viral bioassays human plasma positive for hepatitis B surface antigen (HbsAg) was used as the virus source. The extract (500 µg/ml) was mixed with HbsAg positive plasma. This mixture was retested for hepatitis B surface antigen (HbsAg) by enzyme-linked immunosorbent assays (ELISAs) as described by Shanmugam *et al.*[5] after regular intervals of incubation (2h) with suitable control, antigen-enzyme II from Abbott Laboratories. Negative antigen activity indicates promising anti HBV property.

The crude methanolic extract and n-butanol fraction demonstrated good antibacterial activity against all the strains tested at 500 µg/ml and 50 µg/ml concentration respectively with the different degree of inhibition (Table 1). However, no fungistatic or fungicidal and antiviral activities were detected at the same concentration. The polyphenolics were found to be the main constituents responsible for the observed bactericidal activity with quercetin **1** and myricetin **2**, being identified as the major contributors to the observed activity. Their structure was established using NMR and Mass spectra and confirmed by direct comparison with published information and with standards (Sigma Aldrich). Antibacterial screening results and the MICs of these flavonoids are as shown in Tables 1 and 2. It is to be noted that at the same concentration i.e. 30 µg/ml reference antibiotic streptomycin showed moderate activity only against *Escherichia coli.*

The effect, on the observed antibacterial activity, of the mixture in equal proportion (15 µg/ml) of the two active flavonoids quercetin and myricetin, was also checked. As evident (Table 2) 1:1 combination had no effect on the MIC value (6 µg/ml) of *Shigella flexineri* and *Staphylococcus aureus* (Table 2). Quercetin alone was ineffective against *Proteus mirabilis* and its addition to myricetin had no effect on the MIC values (6 µg/ml) of the latter. Although,

Table 1—Antibacterial activity of the methanolic extract, fractions, and flavonoids

	Concentration (µg/ml)	*Escherichia coli*	*Klebsiella pneumoniae*	*Proteus mirabilis*	*Pseudomonas aeruginosa*	*Salmonella typhi*	*Shigella flexineri*	*Staphylococcus aureus*	*Vibrio cholerae*
Extract	500	+++	+++	+++	+++	+++	+++	+++	+++
Petroleum ether	50	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Chloroform	50	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
n-butanol	50	+++	+++	++	+++	+++	++	++	+++
Quercetin	30	Nil	Nil	Nil	++++	Nil	++++	+++	Nil
Myricetin	30	+++	++++	++++	++++	Nil	++++	+++	Nil
(Quercetin+ Myricetin) 1:1	30	Nil	Nil	+++	Nil	Nil	+++	+++	Nil
Streptomycin/ Nystatin	30	++	Nil	Nil	Nil				

(Nil) No zone of inhibition (inactive); (++) 2-3 mm zone of inhibition (moderately active); (+++) 3-5 mm zone of inhibition (significantly active); (++++) 5-7 mm zone of inhibition (strongly active).
Bioassays that were carried out on the extract against the fungi [*Aspergillus fumigatus, Mucor* sp., *Candida albicans* and *Hepatitis B virus]* showed no inhibitory activity.

Table 2—MIC (µg/ml) of flavonoids against one strain each of eight medically important bacteria

Compound	*Escherichia coli*	*Klebsiella pneumoniae*	*Proteus mirabilis*	*Pseudomonas aeruginosa*	*Salmonella typhi*	*Shigella flexineri*	*Staphylococcus aureus*	*Vibrio cholerae*
Quercetin	Nil	Nil	Nil	6	Nil	6	6	Nil
Myricetin	6	6	6	1.5	Nil	6	6	Nil
Q + M	Nil	Nil	6	Nil	Nil	6	6	Nil

Q (Quercetin) + M (Myricetin)

both flavonoids were strongly inhibitory to *Pseudomonas aeruginosa* mixing the two flavonoids rendered them inactive. Similarly, *Escherichia coli* and *Klebsiella pneumoniae* that were susceptible to myricetin but not quercetin, were resistant towards the mixture. This is contrary to the observations made by Arima *et al.*[6] who reported the enhancement of antibacterial activity on mixing of quercetin with quercitrin, morin or rutin.

There are conflicting reports on the antibacterial activity of quercetin probably owing to inter and intra-assay variation in susceptibility testing or due to the difference in genetic variation of the strain. Quercetin is reported by Aziz *et al.*[7] to inhibit *Escherichia coli,* and *Klebsiella pneumoniae, Bacillus cereus, Aspergillus parasiticus and Aspergillus flavus* at concentration between 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.*[8] even at 500 μg/ml, perhaps because *Pseudomonas aeruginosa* ATCC9027 used was a genetically mutated, resistant strain in comparison to the strain used in the present investigation. Gatto *et al.*[9] reported that quercetin and its 3-O-acyl derivatives did not exhibit significant activity against gram-positive as well as gram-negative strains and yeasts 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 strains tested. *Salmonella typhi* and *Vibrio cholerae* were the two strains that showed resistance to myricetin. These data are supported by Puupponen *et al.*[10], 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 strains. 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[11]. Various researchers have sought to elucidate the antibacterial mechanisms of action of selected flavonoids. The activity of quercetin, for example, has been at least partially attributed to inhibition of DNA gyrase[12].

Better potency of myricetin as compared to quercetin is attributable to the extra phenolic hydroxyl, since this is the only contrasting feature between the two structures. The overall antibacterial activity observed in the crude methanolic extract, may also be partly attributed to the presence of emodin as well as the tannins castalgin, punicalin, punicalagin and corilagin. These are known to be constituents of *L. racemosa*[13,14]. Emodin is reported to be antifungal[15] as well as antibacterial[16] and the polyphenolic corilagin is not only antimicrobial but also known to markedly decrease the MIC of oxacillin and other β-lactam antibiotics against methicillin resistant *Staphylococcus aureus*[17]. Corilagin by itself shows weak anti MRSA activity (MIC,128 μg/ml) against the two MRSA strains tested[17]. Corilagin could be hydrolysed to glucose, ellagic acid and gallic acid however, none of the hydrolysable products reduced the MICs of β-lactam antibiotics[17].

According to Miller and Jeremy[18] tannins present in tea extract in combination with β-lactam antibiotic are reported to have a synergistic effect with methicillin against MRSA, due to the presence of catechin compounds and polyphenols including quercetin and myricetin. In addition, Kwaja and Fiedman[19] report suitability of a pharmaceutical composition containing 0.412% quercetin, commercially known as novoimamine (derived from St John's wort, *Hypericum perforatum*), for clinical or veterinary use effective against *Staphylococcus aureus* infection. These examples provide enough evidence that the tannins or synergistic action of the flavonoids and tannins present in the plant could also be responsible for the initial activity of the crude extract from *L. racemosa*.

Chemical investigation of the active n-butanol fraction by ESI-MS/MS led to the identification of polyphenolic compounds **3-8** on the basis of its molecular masses (Table 3) and fragmentation observed in the MS/MS spectrum of the flavonoids selected from the first order ESI-MS spectrum of the flavonoid rich fraction. The product ion mass spectra were similar to those observed in other mass spectrometry studies of flavonoids,[20,21] and provided unequivocal identification of the relevant flavonols. Except quercetin, quercitrin and isoquercitrin this is the first report of polyphenolics, quercetin glycosides [quercitrin **3**, quercetin-3O-hexoside (glucoside/ galactoside) **4]**, aglycones [kaempferol 4′-methyl ether **5**, Kaempferol-3,4′ dimethyl ether **6**] and

Table 3—Fragmentation in tandem mass spectra of flavonoids

Compound	$[M+Na]^+/[M+H]^+$	ESI-MS/MS (% base peak)- Major ions
Quercitrin (Quercetin-3O-rhamnoside)[**3**].	471	325(100), 301(9.6), 119(40), 197(24), 165(17.6), 105(12), 151(8), 129(6.4)
Quercetin-3O-hexoside. (glucoside/galactoside) [**4**]	487 465	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).
Kaempferol-4′methyl ether[**5**]	301	55(100), 286(15), 153(11), 141(25), 109(0.5), 73(15), 87(50)
Kaempferol-3,4′dimethyl ether [**6**]	315	87(100), 55(38), 73(25), 155(32), 119(5), 187(12.5), 173(6).
Bi-isorhamnetin[**7**]	615	87(100), 301(50), 209(20), 187(10), 105(20).
Myricetin-7O-methyl ether(3→8″) quercetin-3O-rhamnoside[**8**]	763	119(100), 87(50), 105(44), 151(16), 195(22), 467(20), 617(12)

biflavonoids [bi-isorhamnetin **7** and myricetin-7O-methyl ether (3→8″)quercetin –3O-rhamnoside **8**] from the mangrove plant, *L. racemosa*[22].

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[23] and thus contribute to some extent to the observed antibacterial activity.

A variety of biological activities for biflavonoids have been published including anti inflammatory, antimicrobial, anti-oxidants and others[24,25]. Biflavonoids from *Rheedia garderiana*[26] are antibacterial against *Escherichia coli, Pseudomonas aeruginosa*, *Staphylococcus aureus, Bacillus cerrus* (MIC 0.15-1.0mg/ml). Antimicrobial biflavonoids from the aerial parts of *Ouratea sulcata* are reported to be active against *Staphylococcus aureus, Bacillus subtillis, Vibrio anguillarium and Escherichia coli* (MIC 0.85-12.5 µg/ml), it being almost as effective as the standard streptomycin used. They were inactive against *Escherichia coli.*

The present study provides an important basis for the use of extracts from *L. racemosa* for the treatment of the infections associated with the studied microorganisms particularly against the MDR *Staphylococcus aureus.* The activity is due to the presence of flavonoids quercetin, myricetin and quercetin glycosides, anthraquinone derivative emodin and the polyphenolic tannins particularly corilagin in the extract. It has been suggested that because flavonoids are widely distributed in edible plants and beverages and have previously been used in traditional medicine, they are likely to have minimal toxicity. However, this family of compounds has a diverse range of activities in mammalian cells and in vivo confirmation of their side effects would be necessary for a full evaluation of their practical usefulness in the field of modern medicine.

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