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Inhibition of growth of antibiotic-resistant *Staphylococcus* sp. and *Proteus* sp. by mangrove plant extracts

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Abstract

Mangrove plants *Avicennia marina, Bruguiera sexangula, Derris trifoliata, Excoecaria agallocha, Lumnitzera racemosa* and *Rhizophora apiculata* were used to obtain plant extracts by grinding and sequential Soxhlet extraction method. Plant extracts were obtained from mature leaves, immature leaves, shoot and bark of above selected mangrove plants. In sequential Soxhlet extraction method, plant extracts were obtained in petroleum ether, chloroform, ethyl acetate, ethanol and water. The antibacterial activity of these mangrove plant extracts was tested against antibiotic-resistant bacterial species of *Staphylococcus* sp. and *Proteus* sp. The degree of antibacterial activity was assayed by measuring the length of inhibition zone in millimeters. Ethanol extracts were found to be much more effective on both bacterial strains than aqueous extracts in grinding procedure. In Soxhlet extraction method, almost all plant extracts could inhibit growth of *Staphylococcus* sp more than *Proteus* sp. Among the plant extracts, the most clear antibacterial activity was observed extracts of *L. racemosa* against both bacterial strains. Soxhlet extracts of fresh plant materials and charcoal treated Soxhlet plant extracts of *L. racemosa* were able to inhibit both bacterial strains more than those of dried Soxhlet extracts of plant materials and untreated Soxhlet plant extracts. Antibacterial activity of plant extracts of both fresh and dried plant materials had been reduced for both bacterial strains with the time after extraction. The biological active compounds in plant extracts will be isolated, purified and characterized using chromatographic methods and known spectroscopic techniques.

Introduction

Mangroves are of great importance to many people specially, who live along the tropical shorelines. Mangroves are widely used by mangrove dwellers for bush medicine e.g. *Lumnitzera racemosa* is used for skin disorders (Bandaranayake, 1998). A number of mangroves and mangrove associates contain biological activite compounds such as antifungal, antibacterial and poisonous (Bandaranayake, 1998). Mangrove plants are also a rich source of steroids, triterpenes, saponins, flavonoids, alkaloids and tannins (Bandaranayake, 1995). Several reported biological activities such as antibacterial, antitherpetic, anthelmintic are mainly tanins (Bandaranayake, 1998).

It is important to note that 'new' diseases are continuing to arise. In order to combat the hospital-acquired antibiotic resistant strains and new infectious diseases are appearing. Some hospital-acquired infections are known to be resistant to antibiotics. The discovery of new antibiotics in the early twentieth century provided an increasingly important weapon against bacterial diseases. Therefore, we have to find more and more new antimicrobial compounds against them. Since several mangroves have been reported to contain biological activities, screening of antibacterial compounds of mangrove plant extracts for infectious diseases is very important in order to investigate new antimicrobial compounds.

Materials and methods

A. Plant materials and sample preparation

Avicennia marina, Bruguiera sexangula, Derris trifoliate, Excoecaria agallocha, Lumnitzera racemosa and *Rhizophora apiculata* were used to obtain plant extracts by grinding and sequential Soxhlet extraction method (Table 1). In grinding method, 0.3 g of plant materials (mature leaves, immature leaves, shoot and bark) of each selected mangrove plants were extracted separately using 1 ml of water and 1 ml of 95% ethanol using a mortar and pestle.

Table 1. Tested plant species and bacterial species for antibacterial activity screening

Plant species	Bacterial species*
Avicennia marina	*Staphylococcus* sp
Brugueira sexangula	*Proteus* sp.
Derris trifoliate	
Excoecaria agallocha	
Lumnitzera racemosa	
Rhizophora apiculata	

**obtained from the General hospital, Matara*

In sequential Soxhlet extraction, plant extracts were obtained from crushed materials of 50 g of freshmature leaves and bark of *Avicennia marina, Bruguiera sexangula, Excoecaria agallocha, Lumnitzera racemosa* and *Rhizophora apiculata* and bark of *L. racemosa* in a Soxhlet extractor using 300 ml of petroleum ether, chloroform, ethyl acetate, ethanol and water as solvents respectively. The extraction time was three hours for each solvent. The resulting extracts were evaporated to about 10 ml using rotary evaporator at 40 °C and were stored at 4 °C. Soxhlet plant extracts obtained from fresh plant materials were treated with small amount of activated charcoal and incubated at 40 °C for 10 minutes and filtered through a fluted filter paper. Soxhlet plant extracts of dried plant materials of *L. racemosa* that were dried in an oven for over night at 40 °C were

prepared in order to determine the effect of dryness of plant materials on the growth of tested bacterial strains.

B. Bacterial strains and antibacterial activity

Antibiotic resistant bacterial species of *Staphylococcus* sp. and *Proteus* sp. (Table 1) isolated from wounds were used as tested bacterial species. Both *Staphylococcus* sp. and *Proteus* sp. are identified as antibiotic-resistant bacteria. *Staphylococcus* sp. is resistance to Ceftazidime, Cephalexin, Cotrimoxazole, Mecillinam, Cloxacillin, Ticarcillin/Clavulanic acid, Gentamicin, and Kenamycin whereas *Proteus* sp. is resistant to Gentamicin and Kanamycin. Above-mentioned plant extracts were tested for antibacterial activity against above bacterial strains by agar diffusion technique (de Castillo *et al.* 1998). The degree of antibacterial activity was assessed by measuring the length of inhibition zone in millimeters from the edge of the well to the edge of the growing bacterial colony. Since *L. racemosa* showed the best inhibition for the both bacterial strains, it was decided to use for further investigations. Changes of antibacterial activity of Sohlet extracts of *L. racemosa* were tested once a month. For control, petroleum ether, chloroform, ethyl acetate, ethanol and water were used instead of plant extracts.

C. Phytochemical screening

To identify chemical constituents that are present in plant crude extracts, phytochemical screening was carried out to test for alkaloids, coumarins, flavonoids, sterols, saponins and triterpenes. Plant extracts for phytochemical screening were prepared using powdered bark and mature leaves (100 g) of *L. racemosa*. They were extracted with 300 ml of 95 % ethanol using Soxhlet extractor. After about one hour of refluxing on a steam bath with occasional swirling, extract was cooled to room temperature. The mixture was filtered and the plant material was washed with 50 ml of fresh 95 % ethanol. The total volume of the extract was measured and portions were used in the screening tests.

Mayer's test and Wagner's test were carried out to test alkaloids. Picric acid and silicotungstic acid were used to determine presence of alkaloids. Using Dragendorff's reagent the probable number of alkaloids present in plant extracts were estimated. Quaternary alkaloids were also tested using Mayer's reagent. Froth test was carried out for saponins. Salkowski test and Liebermann- Burchardt test were used for steroids and triterpenoids (Harbone, 1984, NARESA, SLASS and UNESCO (SCAMAP), 1986).

Results

Out of forty-one aqueous and ethanolic extracts (Table 1), almost all ethanolic extracts showed more inhibition than aqueous extracts against both bacterial strains (Fig. 1 and Table 2). The highest antibacterial activity was shown for both bacterial strains by *L. racemosa* extracts. Neither *A. marina* nor *D. trifoliata* showed antibacterial activity against *Proteus* sp. (Fig. 2). Moreover, none of the plant extracts from tender leaves of *D. trifoliata* and shoot from *E. agalocha* was able to inhibit both bacterial strains.

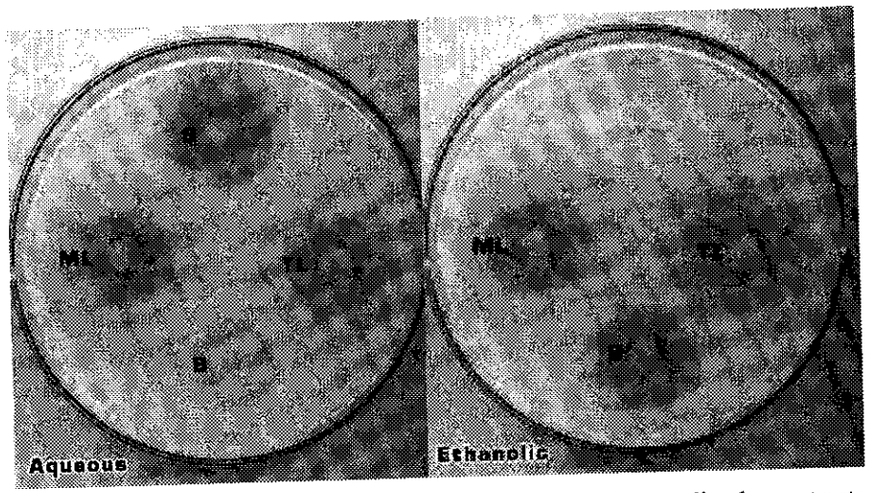

Figure. 1 Inhibition of *Staphylococcus* sp. by aqueous and ethanolic plant extracts of mature leaves (ML), tender leaves (TL) shoots (S) and bark (B) of *L. racemosa* obtained by grinding method

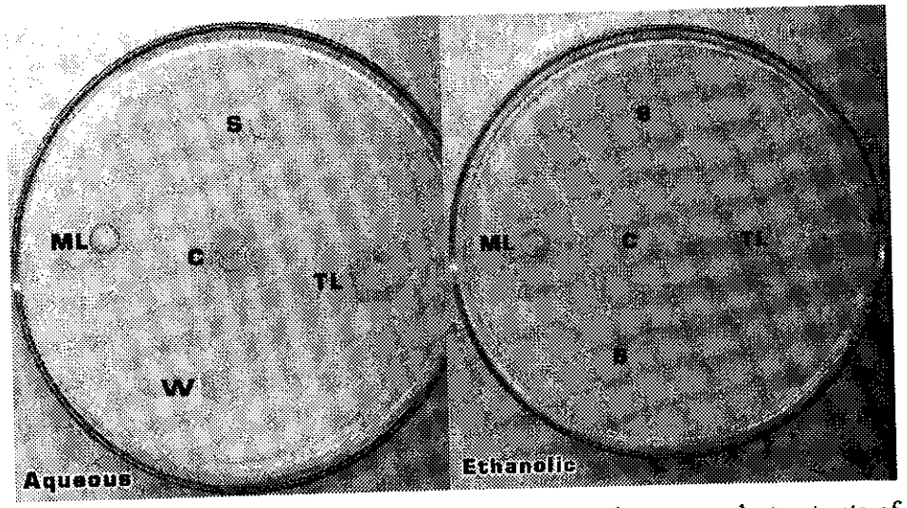

Figure. 2. No inhibition of *Proteus* sp. by ethanolic and aqueous plant extracts of mature leaves (ML), tender leaves (TL) shoots (S) and bark (B) of *D. trifoliata* obtained by grinding method.

Table 2. Degree of growth inhibition of bacterial species measured in mm, from the edge of the well to the edge of the inhibition zone by plant extracts of *A. marina, B. sexangula, D. trifoliata, E. agallocha, L. racemosa* and *R. apiculata*; ML- mature leaves, TL- tender leaves, B-bark, S-shoot, W-water, ET-ethanol, *~ Represents the non availability of plant material; - ~ mark represents no inhibition

Plant material		Bacterial strain			
		Staphylococcus sp.		*Proteus* sp.	
		W	ET	W	ET
A. marina	ML	2	3	-	-
	B	-	2	-	-
	TL	4	5	-	-
B. sexangula	ML	2	6	3	3
	B	5	5	5	8
	TL	3	4	3	3
D. trifoliata	ML	-	3	-	-
	B	3	3	-	-
	TL	-	-	-	-
E. agallocha	ML	6	8	8	7
	B	6	5	3	6
	TL	7	8	7	9
	S	-	-	-	-
L. racemosa	ML	7	9	6	7
	B	6	11	6	8
	TL	9	11	6	8
	S	11	*	6	*
R. apiculata	ML	-	4	2	5
	B	2	5	2	3
	TL	3	4	3	3
	S	-	3	-	3

Ethanolic extracts of bark and tender leaves and aqueous extract of shoot of *L. racemosa* gave the highest inhibitory activity for *Staphylococcus* sp. It is clear that antibacterial activity of these plant extracts was not similar for both bacterial strains. For *Proteus* sp. the highest antibacterial activity was exhibited by ethanolic extract of tender leaves of *E. agallocha*. Ethanolic extract of bark of *B. sexangula,* aqueous extract of mature leaves of *E. agallocha*, ethanolic extracts of bark and tender leave of *L. racemosa* were also found to be effective against *Proteus* sp. However, comparatively the extracts of *L. racemosa* showed the most effective antibacterial activity among tested mangrove plants in grinding method. Extracts of *E. agallocha* exhibited the second most efficient antibacterial activity against both bacterial strains.

Almost all fifty-five extracts obtained by Soxhlet extraction method, exhibited more inhibition for *Staphylococccus* sp. than *Proteus* sp. Out of fifty-five Soxhlet extracts, the highest antibacterial activity against *Staphylococcus* sp. was exhibited by the extracts of

mature leaves of *E. agallocha* in ethyl acetate. The second most efficient antibacterial activity was shown by ethanolic extracts of mature leaves of *E. agallocha* and ethanolic extracts of bark of *L. racemosa*. The highest antibacterial activity was exhibited by extract of bark of *A. marina* against *Proteus* sp.

None of the extracts of mature leaves of *A. marina, E. agallocha* and *R. apiculata* and bark extracts of *R. apiculata* showed any inhibitory effect on *Proteus* sp. However, these extracts could suppress the growth of *Staphylococcus* sp. Also, none of the Soxhlet extracts of petroleum ether and water showed antibacterial activity against *Proteus* sp. However, in Soxhlet extraction method, the highest antibacterial activity for both bacterial strains was shown by extracts of *L. racemosa* among tested mangrove plants (Table 3). No inhibition was given for control in both grinding and Soxhlet extraction method.

Table 3. Degree of growth inhibition of bacterial species measured in mm, from the edge of the well to the edge of the inhibition zone by plant extracts of mature leaves (ML), and bark (B) of *A.marina, B. sexangula, E. agallocha, L. acemosa,* and *R. apiculata* and bark (B) of *L. racemosa* in petroleum ether (PE), chloroform (C), ethyl acetate (EA), ethanol (ET), and water (W). (- Represents no inhibition).

Plant material		*Staphylococcus* sp.					*Proteus* sp.				
		PE	C	EA	ET	W	PE	C	EA	ET	W
A. marina	ML	2	10	6	4	-	-	-	-	-	-
	B	6	11	5	6	-	-	-	6	8	-
B. sexangula	ML	4	4	5	6	-	-	4	-	-	-
	B	-	9	9	7	2	-	4	-	-	-
E. agallocha	ML	-	5	15	12	-	-	-	-	-	-
	B	-	6	6	-	-	-	-	3	2	-
L. racemosa	ML	5	5	9	11	8	-	-	5	3	-
	B	4	6	8	12	6	-	1	5	3	-
	B	-	1.5	2.5	8	6	-	1.5	1.5	-	-
R. apiculata	ML	-	4	6	3	-	-	-	-	-	-
	B	-	-	6	-	2	-	-	-	-	-

Extracts of fresh plant materials could suppress the growth of both bacterial strains more than those of extracts of dried plant materials (Table 4). Charcoal treated extracts could inhibit the growth of both bacterial strains more than those of untreated extracts. The degree of growth inhibition showed by all extracts (fresh and dried plant materials) decreased with the time after extraction for both bacterial strains.

Table 4. Comparison of degree of growth inhibition measured in mm from the edge of the well to the edge of the inhibition zone of tested bacterial species by fresh and dried plant extracts of mature leaves (ML) and branch bark (B) of *L. racemosa* in petroleum ether (PE), chloroform (C), ethyl acetate (EA), ethanol (ET) and water (W). – mark represents no inhibition.

Plant material			50 µl				40 µl			
			Staphylococcus sp		Proteus sp		Staphylococcus sp		Proteus sp	
			Fresh	Dry	Fresh	Dry	Fresh	Dry	Fresh	Dry
L. racemosa	ML	PE	-	-	-	-	-	-	-	-
		C	2	1	-	-	1	-	-	-
		EA	11	2	5	0.5	10	2	4	-
		ET	9	3	4	-	8	2	3	-
		W	7	2	-	-	6	2	-	-
	B	PE	-	-	-	-	-	-	-	-
		C	6	2	2	1	5	1.5	-	-
		EA	10	3	5	1	9	2	4	-
		ET	11	9	3	-	8	8	2	-
		W	7	6.5	-	-	5	5	-	-

Discussion

A. marina, B. sexangula, Derris trifoliate, E.agallocha, L. racemosa and *R. apiculata* were selected as test plants as it has been reported that the people living in coastal areas have already used them against microbial infections (Bandaranayake 1998). Since mangrove plant extracts showed antibacterial activity against antibiotic-resistant bacteria, these extracts should contain antibacterial compounds. According to preliminary studies, it has been recorded that the mangrove plant extracts have antibacterial activity against clinical isolates from different sources (Abeysinghe *et al.* 2002).

Therefore, it is possible to speculate that these compounds are responsible for inhibition of bacterial growth in the plate assay. However, some plant extracts could not inhibit the bacterial growth. It can be due to the presence of some kind of resistant mechanisms e.g. enzymatic inactivation, target sites modification and decreased intracellular drug accumulation (Schwarz and Noble 1999) and the presence of low concentration of the active components in plant extracts. Since crude plant extracts were used in plate assay, there is a possibility that the compounds as a mixture are responsible for the inhibition instead of a single compound. This could be clarified by separating the molecules in the active fractions by conducting further experiments. Petroleum ether, chloroform, ethyl acetate and ethanol are volatile they may get evaporated with the time of incubation. Therefore, no inhibition was given for control.

According to the results, the highest antibacterial activity was exhibited by *Staphylococcus* sp. by ethyl acetate extract of bark of *L. racemosa*. For almost all extracts showed lower or no inhibitory activity against *Proteus* sp. Therefore, these results indicated that *Proteus* sp. was much more resistant to all types of plant extracts. Since *L. racemosa* gave the best inhibition, it was used for further investigations. In this research mature leaves, immature leaves, bark and shoot were used as plant materials. Furthermore, roots, flowers and fruits can also be used as plant materials in addition to the above plant materials.

Since charcoal treated plant extracts (Perrin *et al.* 1985) showed more inhibition on bacterial growth than those of charcoal untreated extracts, it is possible to assume that plant pigments may contribute to increase of bacterial growth. The degree of antibacterial activity of extracts of dried plant material was lower than those of fresh plant materials for both bacterial strains. This difference could be due to several possibilities. Some of the antibacterial compounds that are volatile might get evaporated during drying. On the other hand, there is a chance of converting antibacterial compounds to non-antibacterial compounds on drying and some of compounds can be destroyed due to heat.

The results of preliminary phytochemical screenings of crude extracts of plants provide some clues that alkaloids, steroids and flavonoids were present in mature leaves and bark extracts of *L. racemosa*. These results comparable with the results reported earlier (Bandaranayake 1995). Further research in this regard would reveal the nature (classes) of compounds, which could be responsible for the biological activity. It is believed that the secondary plant metabolites such as alkaloids, steroids, flavonoids show antimicrobial activities (Bandaranayake 1995). Since tested antibiotic resistant-bacterial strains are sensitive to some of the plant extracts, the discovery of novel antibacterial compounds from these plants will be very important and interesting.

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