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Evaluation of antibacterial activity of different mangrove plant extracts

P.D. Abeysinghe, R.P. Wanigatunge
Department of Botany, University of Ruhuna, Matara, Sri Lanka , pushpa@bot.ruh.ac.lk,

R.N. Pathirana
Department of Chemistry University of Ruhuna, Matara, Sri Lanka , ranjithnp@chem.ruh.ac.lk

Antibacterial activity of mature leaves, tender leaves and bark extracts of *Avicennia marina*, *Avicennia officinalis* and *Bruguiera sexangula* were evaluated using Soxhelt extraction method. Petroleum ether, chloroform, ethyl acetate and ethanol were used as solvents in order to get the plant extracts. The antibacterial activity was screened by using agar diffusion technique against pathogenic bacteria species of *Staphylococcus* sp. (from urine), *Proteus* sp. (from a wound), *Escherichia coli* (from infected blood), *Shigella* sp. (from a wound) and *Pseudomonas* sp. (from a wound). The length of inhibition zone was measured in millimeters from the edge of the well to the edge of the inhibition zone. Twelve different plant extracts in *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. All plant extracts in ethyl acetate showed strong inhibition compared to other extracts on all tested bacterial strains. Among all bacterial strains, *Pseudomonas* sp. and *E. coli* showed considerable growth inhibition against almost all plant extracts. Mature leaf extract of only *A. marina* was used for further investigations since a large amount of tender leaves of *A. officinalis* was not readily available. Charcoal treated mature leaf extracts of *A. marina* showed more inhibition for all tested bacterial strains than untreated plant extracts. Combinations of mature leaf extracts of *A. marina* in different solvents failed to exhibit synergistic activity against tested bacterial strains. Most combinations showed antagonistic effect against *Proteus* sp., *E. coli* and *Shigella* sp. Antibacterial activity of plant extracts of *A. marina* gradually declined during 4 months after extraction, for all tested bacterial strains. Components of mature leaf extracts of *A. marina* in chloroform, ethyl acetate and ethanol were separated by Analytical Thin Layer Chromatography (ATLC) when the eluents were hexane and diethyl ether. Isolated components in Preparative Thin Layer Chromatography (PTLC) exhibited moderate antibacterial activity against *Pseudomonas* sp., *Shigella* sp. and *E. coli*. Separated 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.

Key words: *A. marina*, antibacterial activity, plant extracts, growth inhibition

1. Introduction

Microorganisms have potential to cause human diseases. Most of the time viruses, bacteria and fungi act as major pathogenic organisms. The discovery of antibiotics in the early twentieth centaury provided an increasingly important tool to combat bacterial diseases. As antibiotics are increasingly used and misused, the bacterial

strains become resistant to antibiotics rapidly. Therefore, screening of antibacterial activity of medicinal plants is very important since vast number of medicinal plants have been used for centuries as remedies for human diseases. Among them extracts from different parts of mangroves and mangrove associates are widely used throughout the world. For instance, stem of *Avicennia marina* is used for ulcers and bark of *Bruguiera sexangula* is used for antitumors (Bandaranayake 1998). Mangrove and mangrove associates contain biologically active antiviral, antibacterial and antifungal compounds (Bandaranayake 1998). They provide a rich source of steroids, triterpenes, saponins, flavonoids, alkaloids and tannins (Bandaranayake 1995). Therefore, it is worth to screen mangrove plants for the presence of new antibacterial compounds to combat the normal pathogenic bacterial strains and hospital acquired antibiotic resistant bacterial strains.

The main objectives of this study were to screen antibacterial activity of some selected mangrove plant species against pathogenic and antibiotic resistant bacterial strains and also to isolate and characterization of chemical components which are responsible for antibacterial activity.

2. Materials and Methods

2.1. Plant materials and preparation of plant extracts

Mature leaves, tender leaves and bark of *Avicennia marina*, *Avicennia officinalis* and *Bruguiera sexangula* were used as plant materials. Sequential Soxhlet extraction method was carried out to obtain plant extracts. Fifty grams of mature leaves of each selected mangrove species were ground and extracted separately with petroleum ether (300 ml) followed by chloroform (300 ml), ethyl acetate (300 ml) and finally with ethanol (300 ml). The extraction time period was three hours for each solvent. The same procedure was followed for tender leaves and bark of tested plants. Extracts were concentrated to 3 ml portions using a rotary evaporator at 50°C and stored at 4°C .

Four mature leaf extracts of *A. marina* obtained from Soxhlet extraction method were treated with appropriate amount of activated charcoal and incubated at 40°C for 10 minutes in a water bath. Charcoal treated extracts were centrifuged at 10,000 rpm for 2 minutes and the organic layer was obtained. Mature leaf extracts of *A. marina* were mixed with the following combinations of solvents: petroleum ether and chloroform, petroleum ether and ethyl acetate, petroleum ether and ethanol were used to test synergistic activity of plant extracts.

Plant extracts for phytochemical screening were prepared using 100g of ground fresh mature leaves of *A. marina*. They were extracted using 300ml of 95% (v/v) ethanol in Soxhlet apparatus. The mixture was refluxed on a steam bath about one hour, then cooled to room temperature and plant extract was washed with 50 ml of fresh 95 % (v/v) ethanol. The final volume of the extract was measured and portions of this extracts were used in phytochemical screening for alkaloids, flavonoids, saponins, terpenoids, steroids and cardiac glycosides.

Table 1 Tested bacterial strains for antibacterial activity.

Bacterial strain	Source
Escherichia coli (from blood)	General hospital, (Matara)
Proteus sp.* (from a wound)	General hospital, (Matara)
Pseudomonas sp. (from a wound)	General hospital, (Matara)
Shigella sp. (from a wound)	Faculty of medicine, (University of Ruhuna)
Staphylococcus sp.* (from urine)	General hospital, (Matara)

*antibiotic resistant bacterial strains.

2.2. Tested bacterial strains

Staphylococcus sp. is resistant to many common antibiotics in use such as Ceftazidime, Cephalexin, Cotrimoxazole, Cloxacillin, Gentamycin, Kenamycin and Ticarcillin/Clavulanic acid although *Proteus* sp. is resistant only to Gentamycin and Kenamycin as indicated in the Microbiology report of the General Hospital, Matara 2003.

2.3. Determination of antibacterial activity

Antibacterial activity of tested bacterial strains was assayed using all plant extracts by agar diffusion technique (de Castillo et al. 1998). Antibacterial activity was observed as inhibition zone on Petri plates containing nutrient agar. Size of the inhibition zone was measured in millimeters using a metric ruler from the edge of the well to the edge of the inhibition zone. Petroleum ether, chloroform, ethyl acetate and ethanol were used as controls instead of plant extracts. Stability of antibacterial activity of plant extracts was tested during a period of 4 months and results were obtained once a month using agar diffusion technique. Antibacterial activity against tested bacterial strains (Table 1) was tested for components separated by PTLC.

2.4. Separation of active components

Mature leaf extract obtained from Soxhlet extraction was separated using Thin Layer Chromatography (TLC). Pure solvents of hexane, diethyl ether, petroleum ether, ethyl acetate and water, a mixture of hexane : diethyl ether; petroleum ether: ethyl acetate; ethyl acetate: water; hexane: water were used as mobile phase with different ratios as 9 : 1 , 8 : 2 , 7 : 3 , 6 : 4 , 5 : 5 , 4 : 6 , 3 : 7 , 2 : 8 and 1 : 9. Plates were observed under UV light and developed using I_2 vapour.

2.5. Phytochemical screening

Phytochemical screening was carried out for crude extract of mature leaves of *A. marina* for alkaloids, flavonoids, saponins, terpenoids, steroids and cardiac glycosides. Mayer's reagent and Wagner's reagent were used to detect alkaloids. Dragendorff's reagent was used to estimate the probable number of alkaloids present. Mayer's reagent was also used to determine quaternary alkaloids. Saponins were screened using froth test. Liebermann - Burchardt test and Salkoweski test were carried out to determine steroids and triterpenoids. Keller- killiani test was performed for screening cardiac glycosides. Flavonoids were tested using octanol (Harbone 1984).

Figure 1 Inhibition of *E. coli* by plant extracts of mature leaf of *A. marina*, *A. officinalis* and *B. sexangula* extracted in petroleum ether (PE), chloroform (C), ethyl acetate (EA), and ethanol (ET) using Soxhlet extraction method.

3. Results

Twelve different extracts from *A. marina*, *A. officinalis* and *B. sexangula* exhibited different degree of growth inhibition against tested bacterial strains (Table 2). According to Table 2, mature leaves of *A. marina* and tender leaves of *A. officinalis* exhibited considerable antibacterial activity against tested bacterial strains. Further tests were carried out using only mature leaves of *A. marina* because tender leaves of *A. officinalis* are not easily obtainable.

Plant extracts of mature leaf of *A. marina* showed more inhibition than mature leaf extracts of *A. officinalis* and *B. sexangula* against *E. coli*, *Staphylococcus* sp., and *Shigella* sp. (Fig. 1 and Table 2). Tender leaf extracts of *A. officinalis* exhibited more pronounced inhibition against *Staphylococcus* sp. and *Pseudomonas* sp. (Fig. 2 and Table 2). Bark extracts of *A. marina* showed strong inhibition against *Shigella* sp. and *E. coli* (Fig. 3 and Table 2) compared to the extracts of *B. sexangula* and *A. officinalis*.

Mature leaf extract of *A. officinalis* was unable to inhibit the growth of *Shigella* sp. and *Staphylococcus* sp. while tender leaf extract did not exhibit antibacterial activity against *Proteus* sp. Moreover, none of the tender leaf extracts of *B. sexangula* were able to inhibit *Proteus* sp., *Pseudomonas* sp. and *Shigella* sp. Also mature

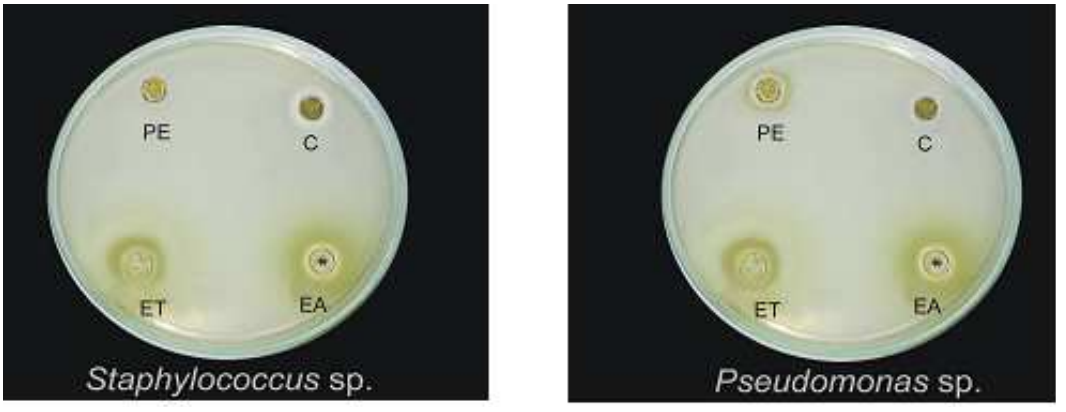

Figure 2 Inhibition of *Staphylococcus* sp. and *Pseudomonas* sp. by plant extracts of tender leaf of *A. officinalis* extracted in petroleum ether (PE), chloroform (C), ethyl acetate (EA) and ethanol (ET) using Soxhlet extraction method.

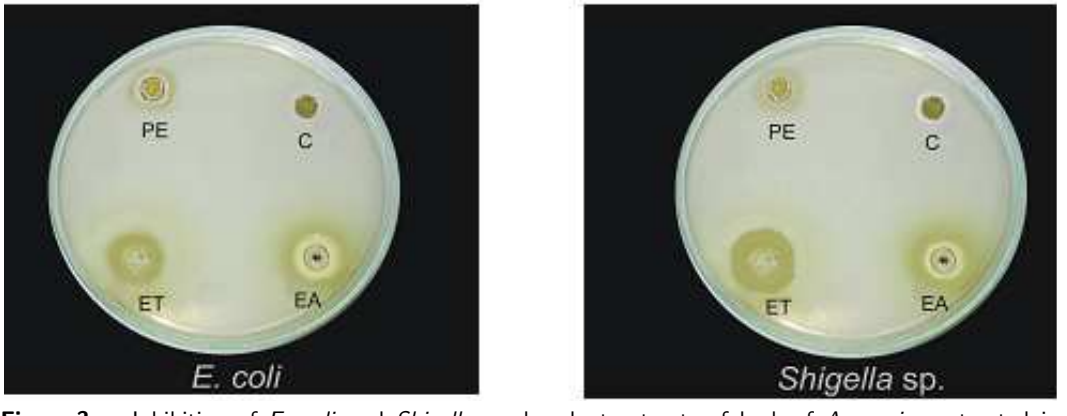

Figure 3 Inhibition of *E. coli* and *Shigella* sp. by plant extracts of bark of *A .marina* extracted in petroleum ether (PE), chloroform (C), ethyl acetate (EA) and ethanol (ET) using Soxhlet extraction method.

leaf extract of *B. sexangula* did not exhibit antibacterial activity against *Pseudomonas* sp. and bark extracts were unable to inhibit *Staphylococcus* sp (Table 2).

No inhibition of the growth of *Proteus* sp., *Staphylococcus* sp. and *Shigella* sp. was observed in extracts in petroleum ether except bark extract of *A. marina*. Ethyl acetate extracts of bark of *A. officinalis*, mature leaf and tender leaf extract of *A. marina* and chloroform extract of mature leaf of *A. marina* were able to inhibit the growth of all tested bacterial strains. Ethanolic extract of bark of *A. officinalis* greatly inhibited, the growth of *Pseudomonas* sp, *E. coli* and *Shigella* sp. Controls did not exhibit inhibitory effect against any of the tested bacterial strains. Charcoal treated mature leaf extracts of *A. marina* were able to inhibit the growth of all tested bacterial strains more than untreated extracts (Table 3). None of the tested possible combinations of mature leaf extracts were able to exhibit synergistic activity

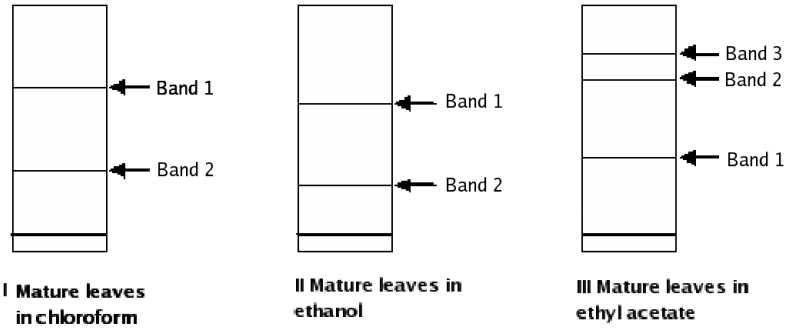

Figure 4 A diagrammatic representation of PTLC plates showing separated bands in plant extracts of mature leaves in chloroform, ethanol and ethyl acetate when the eluents were hexane: diethyl ether 4: 6.

Table 2 A comparison of the degree of growth inhibition of bacterial species in millimeters (mm) from the edge of the well to the edge of the inhibition zone by charcoal treated (T) and untreated (UT) plant extracts of mature leaves of *A. marina* in petroleum ether (PE), chloroform (C), ethyl acetate (EA) and ethanol (ET).

Plant material		*Proteus* sp.		*Staphylococcs* sp.		*Pseudomonas* sp.		E. coli		*Shigella* sp.	
		UT	T	UT	T	UT	T	UT	T	UT	T
Mature leaves	PE	-	-	-	-	2	3	1	2	-	-
	C	1	3	1	1	1	3	2	2	2	3
	EA	4	5	4	7	4	6	6	8	7	9
	ET	2	4	-	-	-	-	5	7	5	8

(- mark indicates no inhibition)

against tested bacterial strains. Most combinations exhibited antagonistic effect against *Proteus* sp., *E. coli* and *Shigella* sp. (Table 4). The degree of antibacterial activity of mature leaf extract of *A. marina* decreased with time for all tested bacterial strains. When TLC was carried out, chloroform and ethanol extracts of mature leaves separated into two bands and ethyl acetate extract of mature leaves separated into three bands (Fig. 4). Separated components of TLC could inhibit the growth of tested bacterial species except *Proteus* sp. and *Staphylococcus* sp. (Table 5). According to phytochemical screening, mature leaves of *A. marina* contained alkaloids, steroids and flavonoids.

4. Discussion

A. marina, *A. officinalis* and *B. sexangula* were used as test plants due to the presence of much evidence that prove their therapeutic value against microbial infections (Bandaranayake 1998). Also preliminary studies have been demonstrated that the mangrove plant extracts have antibacterial activity against pathogenic bacterial strains; *Staphylococcus* sp. and *E. coli* and *Pseudomonas* sp. (Abeysinghe *et al.* 2002) and antibiotic resistant bacterial strains; *Staphylococcus* sp and *Proteus* sp. (Abeysinghe *et al.* 2003). The results of the present study clearly showed that mangrove plant extracts showed antibacterial activity against tested pathogenic bacterial strains including antibiotic resistant strains. The effectiveness of the active

Table 3 Degree of growth inhibition of bacterial species measured in millimeters (mm) from the edge of the well to the edge of the inhibition zone by possible combinations of mature leaf extracts of *A. marina* in petroleum ether (PE), chloroform, ethyl acetate (EA) and ethanol (ET)

Bacterial strains	Combination of plant extracts														
	PE	C	EA	ET	PE + C	PE+EA	PE+ET	C+EA	C+ET	EA+ET	PE+C+EA	PE+C+ET	ET+C+EA	EA+PE+ET	PE+C+ET+EA
Proteus sp.	-	1	3	2	1	2	1	2	1	3	2	2	4	-	-
Pseudomonas sp.	1	1	7	-	-	-	-	4	-	-	-	-	-	-	-
Staphylococcus sp.	-	1	5	-	1	-	-	-	-	-	-	-	-	-	-
Shigella sp.	-	2	8	4	1	2	4	9	4	6	4	2	4	5	7
E. coli	1	1	8	6	2	5	-	7	-	6	3	1	4	3	4

(- indicates no inhibition).

Table 4 Level of inhibition caused by the components of mature leaf extracts of *A. marina* in chloroform (MC), in ethanol ((ME) and in ethyl acetate (MEA) separated by PTLC measured in millimeters (mm) from the edge of well to the edge of inhibition zone against *Proteus* sp. (Pr), *Staphylococcus* sp. (St), *Pseudomonas* sp. (Ps), *E. coli* and *Shigella* sp. (Sh) by TLC method,B1- band one, B2- band two, B3-band three)

Bacterial strain	Isolated bands						
	MCB 1	MCB 2	MEB 1	MEB 2	MEAB 1	MEAB 2	MEAB 3
Pr	-	-	-	-	-	-	-
Ps	-	2	1	1	-	-	-
St	-	-	-	-	-	-	-
E.coli	2	1	1	-	1	2	-
Sh	1	1	2	-	-	-	-

(-mark indicates no inhibition).

compounds present in plant extracts cause the production of growth inhibition zones that appear as clear areas surrounding the wells. Antibacterial activity may be due to active components which are present in plant extracts. However, some plant extracts were unable to exhibit antibacterial activity against tested bacterial strains. These bacterial strains may have some kind of resistance mechanisms e.g. enzymatic inactivation, target sites modification and decrease intracellular drug accumulation (Schwarz and Noble 1999) or the concentration of the compound used may not be sufficient. No inhibition was observed with controls, which proves that solvents could not act as antibacterial agents. Further investigations were done using extracts of mature leaves of *A. marina* only, since they showed considerable antibacterial activity. In almost all tests, crude ethyl acetate extracts showed better inhibition against all tested bacterial strains, indicating that active ingredients in plant materials could be extracted into ethyl acetate. However, highest antibacterial activity was observed against *E. coli*.

Charcoal treated plant extracts showed more inhibition against all tested bacterial strains than untreated plant extracts. It may be assumed that plant pigments may increase the survival ability of bacteria. The degree of antibacterial activity of plant extracts decreased during the period of four months after extraction. It may be due to degradation or volatility of antibacterial compounds or they may have converted into non-antibacterial compounds. A combination of plant extracts in different solvents unable to exhibit synergistic activity against tested bacterial strains. Mixing of plant extracts in different solvents seems to have diluted down the toxic effect against tested bacterial stains or might have increased the survival ability of pathogenic bacteria. TLC was carried out using different solvent systems. But only hexane and diethyl ether solvent system gave best separation. Separated components showed positive results against *Pseudomonas* sp., *E. coli* and *Shigella* sp. whereas antibacterial activity was not observed against *Staphylococcus* sp and *Proteus* sp. According to preliminary studies, it has been reported that active components from mature leaves of *A. marina* have been separated using TLC (Withanawasm 2002).

Phytochemical screening of mature leaf extracts of *A. marina* revealed the presence of biologically active substances such as alkaloids, steroids, triterpenoids and flavonoids. The results obtained from preliminary phytochemical screening are comparable with the results reported earlier (Bandaranayake, 1995). Preliminary phytochemical screening of *Luminetzera racemosa* reveled the presence of secondary metabolites such as alkaloids, flavonoids and steroids (Abeysinghe *et al.*, 2003). These secondary metabolites may exert antibacterial activity against tested bacterial strains. In addition to above tested phytochemical groups tannins, anthocyanins, polyphenols, coumarins and essential oils should be subjected to phytochemical screening. It is promising that the tested mangrove plant species could be used to synthesis novel antibiotics for bacterial infections, especially for antibiotic resistant bacterial infections. Further research is necessary for successful separation, purification and characterization of biologically active compounds using chromatographic methods and spectroscopic techniques. Further studies are being carried out in order to separate the individual components that are present in plant extracts of *A. marina* using column chromatography.

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