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Screening of Petroleum ether, Chloroform, Ethyl acetate, Ethanol and Water Extracts of Medicinal Plant, Avicennia *marina* for Antibacterial Activity against Antibiotic Resistant Bacteria Species, *Staphylococcus* and *Proteus*

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Abstract:

The secondary metabolites of the young leaves of *Avicennia marina* were obtained by sequential Soxhlet extracts with petroleum ether, chloroform, ethyl acetate, ethanol and water as solvents. Antibacterial activity against antibiotic resistance and pathogenic bacterial species of *Staphylococcus* and *Proteus* was screened by agar diffusion technique and paper disk method. The extracts exhibited different degree of growth inhibition against tested bacterial strains. Ethyl acetate extract exhibited the highest antibacterial activity while water extract did not show any growth inhibition. The extracts of *A. marina* when purified with activated charcoal showed more inhibition than the untreated extracts. Components of young leaf extracts were separated by Thin Layer Chromatography (TLC). The results of TLC analysis of petroleum ether, chloroform, and ethyl acetate and ethanol Soxhlet extracts of *A. marina* may be a mixture of 4, 8, 4 and 4 respectively. The results of the two dimensional TLC analysis for the chloroform extract isolated from the Soxhlet plant extracts of *A. marina* may be a mixture of twenty components. Chloroform, ethyl acetate and ethanol extracts were fractionated using column chromatography and obtained fourteen, seven and seven fractions respectively. Phytochemical screening revealed that young leaf of *A. marina* contained alkaloids, steroids. Phytochemical screening of Soxhlet extracts of chloroform contained alkaloids and steroids/triterpenoids. Ethyl acetate and ethanol extract contained steroids/ triterpenoids and alkaloids respectively. Chemical tests revealed that fractions obtained from column chromatography of chloroform, ethyl acetate, ethanol extracts contained carbonyl groups and chloroform extract contained phenolic groups.

Key Words: Antibacterial activity, *A. marina*, extracts, growth inhibition metabolites, TLC

Introduction:

Microbes and viruses have a vast multitude of ploys for gaining entry into humans by countering body defenses [1]. Bacteria, fungi, viruses and other microorganisms are potentially pathogenic and cause many diseases in humans, animals and plants, even deadly diseases. Bacterial infections are one of the major issues and serious problems in health care systems all over the world. Antibiotic resistant bacteria outbreaks have been reported in hospitals throughout the world [2]. Some 'hospital-acquired' infections may be resistant to a number of clinically efficacious antibiotics and become less effective even to the high dosage of antibiotics. Therefore, the emergence of bacteria resistance to antibiotics is a severe problem, and cost of treating these infections has become a burden to National Health Service [3,4]. It is therefore essential to discover or develop new compounds probably with novel mode of action to overcome this problem [5, 6].

Traditional medicine plays a significant role in the health care system of developing countries, especially in Asia including Sri Lanka. Medicinal plants have been used for centuries as remedies for human diseases because they contain components of therapeutic value [7]. Tropical forests are the hot spots for the plant diversity in the world. Plants are a gift of nature providing food, forage, timber, cloths, cosmetics, firewood, charcoal, building materials, tannin, flavors and etc. Also they supply limitless drugs and medicines to cure infectious diseases including chronic diseases [8, 9]. In Sri Lanka, there is a rich mangrove ecosystem. The total cover of mangroves in Sri Lanka has been estimated as 4000 ha [10]. Mangroves are widely used by mangrove dwellers for bush medicine. Extracts from different mangrove plants are reported to possess diverse medicinal properties [11]. Many species of mangroves are being used to treat diseases such as rheumatism, small pox, ulcers, hepatitis, leprosy, asthma, snake bites, toothache, etc and purgative and so on. Since mangrove plants offer a wide array of molecules with therapeutic value [11]. For example, *Acanthus ilicifolius, Avicennia marina* and *Exoecaria agallocha* showed significant analgesic activity [12]. *Avicennia marina* is used for rheumatism, small pox and ulcers [11]. Compare to the other selected mangrove species studied [13]. *A. marina*, was found to be highly active towards some selected bacterial strains; *Escherichia coli* (from blood), *Pseudomonas* sp. (from a wound) and *Shigella* sp. (from a wound). The aim of the study was assess the antibacterial activity of different Soxhlet extracts of *A. marina* against antibiotic resistant and human pathogenic two bacterial species of *Staphylococcus* and *Proteus*.

Materials and Methods:

Plant Materials and Sample Preparations:

Extracts of young leaves of *A. marina* were obtained by sequential Soxhlet extraction with different solvents of increasing polarity. Two hundred grams of crushed fresh

young leaves were sequentially extracted in a Soxhlet extractor using 1500 ml of petroleum ether, chloroform, ethyl acetate, ethanol and water. The extraction time was four hours for each solvent. The resulting extracts were evaporated using rotary evaporator and 2 ml of each were stored at 4^0 C. Portions of these extracts were treated with small amount of activated charcoal and incubated at 40^0 C for 10 minutes, filtered through a fluted filter paper and concentrated by rotary evaporator.

Bacterial Strains and Antibacterial Activity:

Antibiotic resistant bacteria species of *Staphylococcus* and *Proteus* were used as test bacterial species. Plant extracts were tested for antibacterial activity against these two bacterial strains by agar diffusion technique and paper disk method [4]. The degree of antibacterial activity was assessed by measuring the length of inhibition zone in millimeters from the edge of the well or paper disk to the edge of the growing colony. Each possible combination of plant extracts was selected. i.e. petroleum ether and chloroform, petroleum ether and ethyl acetate, petroleum ether and ethanol, etc. Each plant extracts were mixed according to above manner and assessed the antibacterial activity. Antibacterial activity of the extracts was carried out during first, second, and third month after extraction. Pure solvents of petroleum ether, chloroform, ethyl acetate, ethanol and water were used as controls.

Thin Layer Chromatographic (TLC) Analysis of the Soxhlet Plant Extracts:

Silica coated plates were air dried at room temperature one hour and dried at 80^0 C in an oven 30 minutes. The extracts were separated by thin layer chromatography (TLC) and chloroform extracts were further separated by preparative TLC. Compounds were visualized using UV, I_2, phosphomolybdic acid (PMA) and anisaldehyde spray.

Separation of the Components Present in Soxhlet Plant Extracts by Column Chromatography:

Plant extracts (1.5 ml) obtained by sequential Soxhlet extraction of *A. marina* were separated by flash chromatography. Fractions were collected and analyzed by TLC. Screening process was repeated for fractions obtained from column chromatography which gave positive result for bioassay.

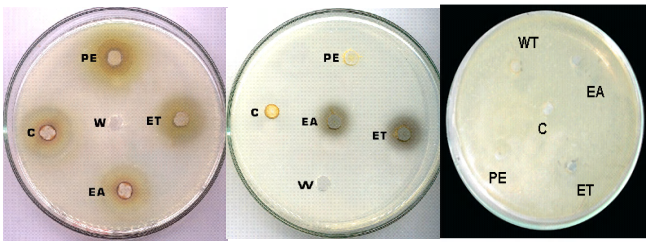
Phytochemical and Chemical tests for Fractions Taken from Column Chromatography:

Phytochemical screening for alkaloids, saponins and steroids/triterpenoids was done according to Harbone, (1984). Brady's, Fehling's, Sodium nitropraside and Diazotization tests were carried out for the partially purified mixture of metabolites isolated from the column chromatography of *A. marina* to test presence of carbonyl groups, amines, phenolic groups respectively [14].

Results:

Antibacterial assays for Soxhlet plant extracts of *A. marina* and the controls were also carried out. Five different extracts; ethyl acetate, petroleum ether, chloroform, ethanol and water exhibited different degree of growth inhibition against bacterial strains ***(Figure 1)***.

Figure 1: **Inhibition of plant extracts of young leaves of *A. marina*, extracted in petroleum ether (PE), chloroform (C), ethyl acetate (EA) ethanol (ET), and water (W) against *Staphylococcus* and *Proteus sp.***



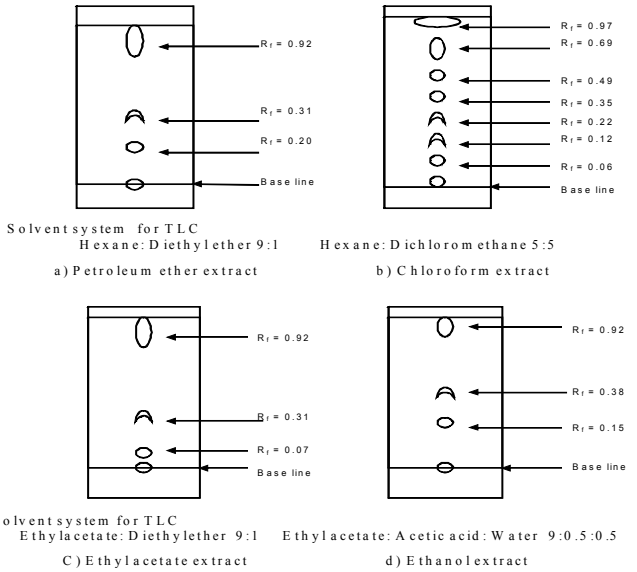
Plant extract of ethyl acetate showed the highest inhibition compared to the extracts obtained with petroleum ether, chloroform, ethanol and water.

No inhibition was given for controls. Charcoal treated extracts of showed more inhibition of the growth of tested bacterial strains than untreated extracts. Any possible combinations of leaf extracts taken from mixing by all of Soxhlet extracts unable to exhibit synergistic activity against tested bacterial species, *Staphylococcus* and *Proteus.* All combinations exhibited antagonistic effects.

The degree of growth inhibition of ethyl acetate and ethanol extract of young leaves of *A. marina* for *Proteus sp*. reduced slowly during three months after plant extraction. But petroleum ether and chloroform extracts of young leaf of *A. marina* for *Proteus* sp. did not reduce growth inhibition during three months after plant extraction.

The results of TLC analysis of petroleum ether, chloroform, ethyl acetate and ethanol extracts of *A. marina* may be a mixture of 4, 8, 4 and 4 compounds **(Fig.2a, Fig.2b, Fig.2c and Fig.2d)** Two dimentional analyzes of preperative TLC of Chloroform extracts of *A. marina* may be a mixture of twenty or more components.

Figure 2: The diagrammatic representations of the TLC of Soxhlet extracts after visualization under UV light and I_2 spray. Solvent systems and solvent used for the extraction are given.



Column chromatography of the chloroform, ethyl acetate and ethanol extracts obtained fourteen, seven and seven fractions respectively. According to phytochemical screening of the chloroform, ethyl acetate and ethanol

extracts, it contained two important secondary metabolites belonging to two classes of natural products viz alkaloids and terpinoids. ***(Table 1).***

Table 1: **Phytochemical screening results for petroleum ether (PE), chloroform (C), ethyl acetate (EA), ethanol (E) and water (W) extracts**

Test	Alkaloids				Saponins	Steroids/ Triterpenoids		Cardiac glycosides	Flavonoids
Extraction	Mayer's	Wagner's	Quaternary	Dragondroff's	Froth test	Salkowski test	Liebermann-Burchard	Killer-Kilani	
PE	-	+	-	-	-	Cherry red	Bluish green	-	-
C	-	+	-	-	-	Cherry red	Bluish green	-	√
EA	-	+ +	-	-	-	Cherry red	Bluish green	-	√
ET	+	+ +	+	-	-	Cherry red	Bluish green	-	√
WT	+	+	-	-	-	-	-	-	√

Discussion:

Inhabitants in coastal areas have already used plant extracts of *A. marina* against microbial infections [11]. According to preliminary studies, it has been recorded that mangrove plant extracts in general had antibacterial activity against clinical isolates from different sources [13]. Extracts of mature leaf of *A. marina* and tender leaf of *A. officinalis* exhibited considerable antibacterial activity against tested bacterial strains. In addition to above tested mangrove plant species, other mangrove plants can be used to identify and isolate antibacterial components against pathogenic bacteria species. Evidence proved that, *Exoecaria agallocha*, *Lumnitzera racemosa* and *Rhizophora apiculata* had antibacterial activity against *Staphylococcus* sp. and *Proteus* sp [4,15].

The ability of bacteria to develop resistant to antimicrobial agents has become a significant problem in the treatment and control of bacterial infectious diseases. Probably these two species of bacteria may have evolved a number of different mechanisms to resist antimicrobial agents. However, our results of this experiment also proved that different plant extracts of *A. marina* in different solvents showed the antibacterial activity against the tested antibiotic resistant bacteria species causing wounds and urine infections. This revealed that these extracts contained compounds which were able to inhibit the growth of bacteria. Mangroves have a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids and flavonoids which have antibacterial properties [15-17]. According to the phytochemical screening of active compounds of Soxhlet extracts, it was clear that it contained alkaloids and steroids/ triterpinoids and contained carbonyl and phenolic groups. These secondary metabolites and active groups may have activity against the growth of bacterial species and may have some kind of resistance mechanisms e.g. enzymatic inactivation, target sites modifications and decrease intracellular drug accumulation [18]. Secondary compounds present in the plants may play a role in plants' defense through cytotoxicity towards pathogenic microorganisms [19] and this could prove the usefulness of these as antimicrobial medicines for humans [16].

The crude extracts of plant materials contain different type of pigments, such as chlorophyll, caratinoids, etc. They may act as a masking agent for antibacterial compounds. To test whether there is an influence of pigments in plant extracts on bacterial growth, charcoal treated and untreated plant extracts were used. Activated charcoal was used to remove pigments in plant extracts [15]. Charcoal treated plant

extracts showed more inhibition of all tested bacterial strains than untreated plant extracts showing that plant pigments may involve the growth of bacteria. It can assume that plant pigments may contribute to the increase of bacterial growth and may increase the survival ability of bacteria [13]. *Staphylococcus* sp. and *Proteus* sp. gave considerable inhibition with ethyl acetate extracts, but did not give any inhibition with water extracts. Water extract may contain low concentration of antibacterial compounds or may not extract antibacterial compounds or all antibacterial compounds may have extracted by other solvents; petroleum ether, chloroform, ethyl acetate and ethanol. Fractions obtained from column chromatography was able to exhibit antibacterial activity against bacteria species. It may be due to sufficient concentration of active compounds in separated fractions. Interestingly, fractions taken from ethyl acetate and ethanol extracts gave more inhibition than the original Soxhlet extracts.

Conclusion:

A. marina plant extracts have an antibacterial activity against *Staphylococcus sp.* and *Proteus sp*. These results suggest that extracts of young leaves of *A. marina* could be used as a potential application to combat pathogenic bacteria as well as antibiotic resistance bacteria like *Staphylococcus sp*. and *Proteus* sp. According to results of phytochemical screening of Soxhlet extracts, it is clear that they contained alkaloids, steroids/triterpinoids and contained carbonyl and phenolic groups.

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