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Original Article

Quantification of Tannins, Antioxidant Capacity and Antibacterial Activities of Stem extracts of Avicennia marina (L)

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ABSTRACT

Majority of regular synthetic antibiotics are in the process of being labeled out-dated owing to multi drug resistance among pathogens. Consequently researchers are devoting interest towards identification and isolation of potent antibacterial compounds from available herbal sources. Aim: The present study was aimed at evaluation of tannin levels, antioxidant potential and antibacterial activity of *Avicennia marina L* (AM) extracts on selected Gram positive and Gram negative bacterial species. Methods: Stem extracts prepared in different solvents in the order of increasing polarity (ethyl acetate, acetone, ethanol and methanol) were subjected to – quantification of tannins by Folin-Denis method, antioxidant capacity by ABTS and Blue Cro5 method and antibacterial activity by agar well diffusion method against selected bacterial species. The zones of inhibition obtained were measured and the results were statistically analysed. Results: Tannin level was found to be significantly high in methanol extract followed by that in ethanol, acetone and ethyl acetate. Extract in all the solvents showed antioxidant activity. However, extracts in methanol and ethanol exerted higher potentials. Like wise, extracts in all the solvents showed various degrees of antibacterial activity against the test cultures used. The methanol extract on *Bacillus subtilis, Staphylococcus aureus* and *Streptococcus pyogens* exhibited higher activity than that of Gentamicin. Conclusions: The present experimental data reveals that the stem of AM is rich in tannins and exhibits potential antioxidant and antibacterial activities. A comparative analysis is also provided with respect to the solvent used for the study of bioactivities.

1. Introduction

Plants are the potential and inexhaustible sources of secondary metabolites such as alkaloids, flavonoids, glycosides, saponins and Tannins [1]. Crude extracts of plant parts in polar and non-polar solvents are endowed with antimicrobial, antioxidant, antidiuretic and antidiabetic and other therapeutic properties. Population worldwide is known to use herbal medicines from times immemorial in the treatment of many disorders, as plants are considered as diverse and rich sources of potential secondary metabolites. The advantages of use of plants and plant based products are their easy availability, less cytotoxicity, and economic for self-medicating groups [2]. Medicinal plants used in the traditional systems of medicine (TSM) serve as important sources for the discovery of potent phytomedicines. According to Word Health Organization more than 80% of the world population depends on traditional medicine for their primary health care [3,4]. Tropical and sub-tropical areas of the world are bestowed with abundant flora and herbs that have properties yet to be exploited.

Tannin and tannin like substances are widely distributed in many species of plants. Tannins play an important role in protection from predation and contribute to plant growth regulation. They are found in leaves, seeds, roots and stems. Tannins are a group of high molecular weight poly-phenolic compounds that are divided into two major groups hydrolysable tannins and condensed tannins [5]. Tannins exhibit diverse biological activities [6].

Oxidative stress arises when the antioxidant defense system of the human body is not entirely efficient. Antioxidant defense of the body increases in response to mild oxidative stress. However, severe oxidative stress causes a rise in free radical levels that can lead to cellular injury and death. Scientific evidences suggest that antioxidants reduce the risk of stress induced diseases including cancer and myocardial infarctions. Most of the antioxidant compounds in a typical diet are derived from plant sources. They

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belong to various classes such as alkaloids flavonoids and tannins. The potentiality of an antioxidant depends mainly on its ability to neutralize free radicals. Antioxidant compounds scavenge free radicals such as peroxides, hydroperoxides, lipid peroxides and therefore inhibit the oxidative mechanisms that lead to degenerative diseases.

Pathogenic microorganisms are continuously developing resistance to the existing antimicrobial compounds [7]. Hence there is a need to research and design alternative drugs from natural products to combat microbial infections. Nature poses a rich resource of potential compounds that are structurally novel and biologically active metabolites. Mangroves are a diversified group of plants that grow in estuarine environment. Mangrove plants have been used in folklore medicine to treat various diseases over the centuries [8]. The chemical and pharmacological complexity of the plant ascribes its diverse medicinal properties [9].

Extracts from mangrove species are known to exhibit inhibitory activity against human, animal and plant pathogens [10-12]. However, the precise antibacterial activity of mangrove plants has not been studied as extensively as that of other plant species. Avicennia marina L is a tropical plant widely distributed in Indian mangroves. It is particularly prominent in mangroves of Kakinada, locally called as Tella mada. It belongs to the family Avicenniaceae. The present study was taken up to determine the tannin content, antioxidant and antibacterial activities of stem extracts of Avicennia marina L in various solvents.

MATERIALS AND METHODS

Collection of Plant Sample: Stems of Avicennia marina L. (AM) were collected from Corangi Reserved Forest, Kakinada, East Godavari, Andhra Pradesh, India. Geographic location - between 16o 39' N longitude – 17o N longitude and 82o 14' E latitude - 82o 23'E latitude. Collected plant material was transported to the laboratory in new polythene bags. All the stems were surface sterilized with 1% mercuric chloride solution and thoroughly washed with filter sterilized distilled water. The washed stems were chopped to small pieces and shade dried.

Extraction: Different solvents in the increasing order of polarity were employed to prepare the stem extracts. Ethyl acetate, acetone, ethanol and methanol were used to prepare the crude extracts of AM. The chopped material (100gm) was first soaked for 12h in 500ml of the respective solvent. The material was subjected to extraction by refluxing for 6 - 8h below the boiling point of the respective solvent. The extract was concentrated by evaporating at reduced pressure using rotary evaporator. The concentrated extract was further dried at 37oC for 3-4 days in order to facilitate complete evaporation of the solvent. The dried plant extract of 100mg each was dissolved in 10ml of 1:10 diluted DMSO in sterile distilled water so as to obtain the final concentration of 10mg /1ml [13].

Determination of tannin content: Tannin content in the extracts was estimated by Folin-Denis method [14] with tannic acid as standard solution at a concentration of 100µg/ml. One ml (10mg/1ml) of the individual extract was mixed with 0.5ml of Folin-Denis reagent and 1ml of saturated carbonate solution. The contents were vortexed and allowed to stand for 30min and the intensity of the colour was read at 760nm against reagent blank. All the values were determined in triplicates and the mean was calculated.

Determination of antioxidant activity by ABTS method: Total antioxidant capacity of each extract was measured using 2, 2´-azinobis [3-ethylbenzthiazoline]-6-sulfonic acid (ABTS) assay [15]. ABTS and potassium persulfate were separately dissolved in deionized distilled water to a final concentration of 7mM and 2.45mM respectively. The two solutions were mixed and allowed to stand in dark at room temperature for 16h before use in order to produce ABTS radical (ABTS+). The resultant intensely-coloured ABTS+ radical cation was diluted with 0.01M PBS (phosphate buffered saline), pH 7.4, to give an absorbance value of ~0.70 at 734 nm. The test compound was diluted 100X with the ABTS solution to a total volume of 1ml. Absorbance was measured spectrophotometrically at time intervals of 3min after addition of each extract. The assay was performed at least in triplicate. Controls were run using PBS in place of the extract. The assay relies on the antioxidant capability of the samples to inhibit the oxidation of ABTS to ABTS+ radical cation. Percent inhibition was calculated using the following formula.

% inhibition of oxidation of ABTS to ABTS

$$+ = \frac{\text{Initial absorbance - Final absorbance}}{\text{Initial absorbance}} \times 100$$

Determination of antioxidant activity by the blue CrO_5 method: Chromium peroxide (CrO_5) is a strong pro-oxidant produced in an acidic environment by ammonium dichromate in the presence of H_2O_2. It is a deep blue potent oxidant compound, miscible and relatively stable in polar organic solvents that can be easily measured by spectrometry [16].

Reagent preparation: Solution1: 1a, 1b and 1c these three solutions are mixed in a 1:1:3 ratios (v/v/v). 1a) 10 mL of sulphuric acid (25 mM), 1b) 10 mL of 20 mM ammonium dichromate solution, 1c) 30 mL of 99.5 % DMSO (v/v). Solution2: 1.6 M H_2O_2

Procedure: 400µl of solution1 was mixed with 4µl (10mg /ml) of AM extract and incubated for 192s. The first absorbance was measured at 416nm (A416). Subsequently, 40µL of solution2 was added and the solution was reincubated for another 192s. Absorbance was measured at 608nm (A608). The net absorbance was determined as $A = A_{608} - A_{416}$.

Determination of antibacterial activity: Six test cultures three each of Gram negative and Gram positive bacterial strains were selected for this study viz., Escherichia coli (MTCC 7410), Enterobacter aerogenes (MTCC 7324), Pseudomonas aeruginosa (MTCC 7083), Bacillus subtilis (MTCC 736), Staphylococcus aureus (MTCC 737) and Streptococcus pyogens (MTCC 1925). The antibacterial activity of each extract (100µl) was tested by agar well

diffusion method [17]. The zones of inhibition were measured. Each experiment was performed in triplicate and the mean zone size was calculated. Gentamicin (1mg/100µl) was taken as positive control [18].

RESULTS AND DISCUSSION

Tannins are bitter high molecular weight poly-phenolic compounds. They are associated with organic molecules including proteins, celluloses, hemicelluloses, pectins, chlorophylls and certain inorganic substances like divalent metals [19]. Tannins have diverse effects on biological system, as they are associated with potential activities such as metal ion chelation and biological antioxidantion. Tannins being complex molecules there is no ideal solvent to isolate them from natural sources. Hence there is a preliminary need to select the best solvent for their extraction and estimation. In our study we have used four different solvents viz., Ethyl acetate, Acetone, Methanol and Ethanol to extract the tannins. Tannin content in the extracts was estimated by Folin-Denis method. Table-1 depicts the amount of Tannin content in the stem extracts of AM in Ethyl acetate, Acetone, Methanol and Ethanol. The results showed that methanol is most suitable for the extraction of tannins. Our results are in concurrence with the study of ShuDong et.al [20].

Non-enzymatic antioxidant activities of natural sources are endorsed to their total secondary metabolite content [21]. Antioxidant compounds in food play an important role as a health protecting factor [22]. The primary sources of naturally occurring antioxidants are whole grains, fruits and vegetables. Plant derived antioxidants like vitamin C, vitamin E, carotenes, poly phenols, tannins, phytate and phytoestrogens have been recognized as having the potential to reduce risk of contacting chronic diseases [23]. Antioxidant activity of AM was determined by ABTS and CrO5 method. The results of ABTS are shown in Fig-1. All the fractions were found to be positive for antioxidant activity. However, methanol and ethanol extracts showed significantly high antioxidant activity when compared to that of the rest of the solvents. These results co-relate with the tannin content of the methanol extract. Our results are best supported by those of Sahreen et.al [24]. The strong antioxidant potency of tannins can be attributed to the presence of phenolic hydroxyl groups and formation of a stable reaction product. Polyphenols act as scavengers of Reactive Oxygen Species (ROS), peroxide decomposers, quenchers of singlet oxygen and inhibitors of lipoxygenases [25]. The antioxidant activity by CrO5 method is depicted in Fig-2. The maximum antioxidant activity was seen in ethanol extract followed by methanol, acetone and ethyl acetate extracts. This result however, does not correlate with the tannin content. This could be due to the effect of secondary metabolites other than tannins that may have krept in.

Numerous mangrove plants have been used in folk medicine and recently extracts from mangroves and mangrove dependent species have proven to associate with potent activity against human, animal and plant pathogens. Only limited investigations have been carried out to identify the potent plant part for efficient isolation of the metabolites responsible for their bioactivity. The mangroves are promising sources of bioactive compounds such as alkaloids, flavonoids, glycosides, tannins, lignans, steroids and triterpenoids. These compounds are known to posses many medicinal values [26,27]. Abeysinghe and Wanigatunge [28] reported the antibacterial activity of Avicennia marina leaf extracts in petroleum ether, chloroform, ethyl acetate and ethanol against Escherichia coli, Pseudomonas species, Proteus species, Shigella species and Staphylococcus species. In the present study, we have evaluated the antibacterial activity of AM stem extracts in ethyl acetate, acetone, methanol and ethanol on the selected Gram positive and Gram negative bacteria. The result showing inhibitory effects of these extracts is presented in Fig 3. Acetone, ethanol and methanol extracts were active against all the selected test cultures. The zones of inhibition produced by the methanol extracts against Bacillus subtilis, Staphylococcus aureus and Streptococcus pyogens are higher than that of the standard antibiotic Gentamicin. This further connects with the relatively higher tannin content in the methanol extract. Our results are in agreement with the findings of Suraya sulaiman et.al [29], Hisanori et.al [30].

However, ethyl acetate extracts did not have any inhibitory effect against the Gram negative cultures used in our study. The resistance shown by Gram negative test cultures could due to mechanisms such as enzymatic inactivation, target site modification or decreased intracellular drug accumulation. Farag et.al and Marino et.al have reported that plant extracts generally have more inhibitory effect against Gram positive bacteria than Gram negative bacteria [31,32]. The outer membrane in Gram negative bacterial cell wall is rich with lipopolysaccharides that restrict diffusion of the bioactive principles present in the extract. Among the test cultures used Bacillus subtilis showed maximum susceptibility to all the extracts of AM stem. The results of the present study support the traditional use of AM in folk medicine [8].

Table1. Tannin content of Avicennia marina in different solvent systems

Solvent	Concentration µg/ 10 mg of the crude extract
Ethyl acetate	15.33
Acetone	22.66
Methanol	86.66
Ethanol	47.33

Fig 1- Antioxidant activity of the Stem Extracts of Avicennia marina by ABTS method

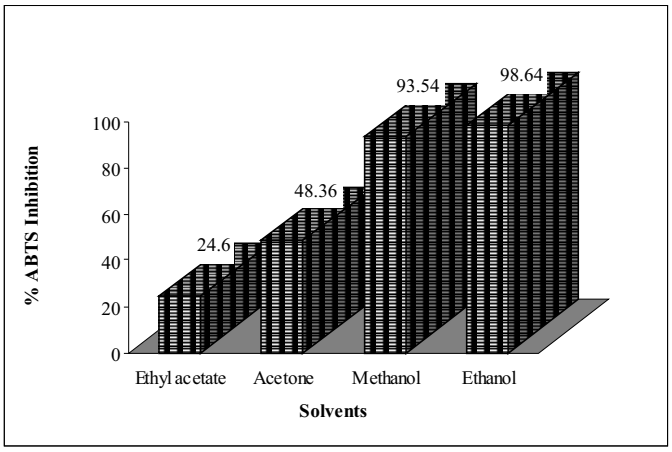


Fig 2 - Antioxidant activity of the Stem Extracts of *Avicennia marina* by CrO_5 method

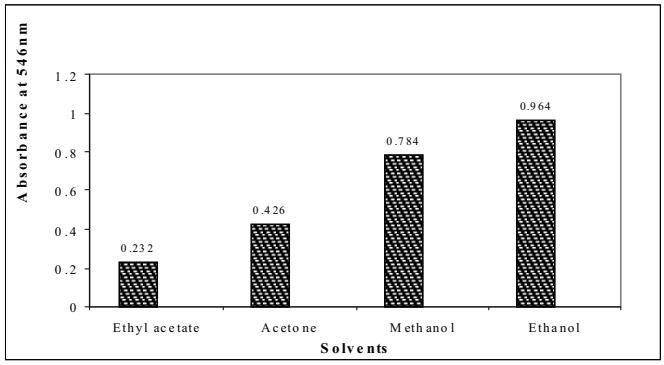
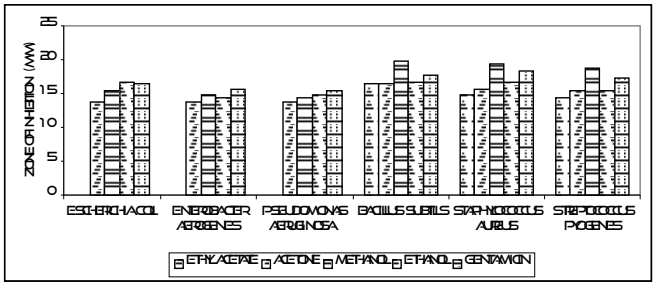


Fig. 3- Antibacterial activity of the Stem extracts of *Avicennia marina* on selected bacteria



CONCLUSION

Stem extracts of *Avicennia marina L* in ethyl acetate, acetone, methanol and ethanol were tested for tannin content, antioxidant activity and antibacterial activity. This study revealed that *Avicennia marina* is rich with tannins and it has considerable antioxidant as well as antibacterial activity. Therefore, *Avicennia marina* is strongly recommended as a valuable source of alternate medicine. Detailed study may be proceeded to reveal the nature of the active . phytochemical constituents in order to get novel bioactive compounds for combating chronic diseases. Finally, there is a need to explore this area further to understand the potentiality of the mangrove plants towards the development of a new era of medicines

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