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Original Article

Assay of antibacterial agents against drug resistant and drug sensitive bacteria and identification of biologically active principles from *Ceriops tagal* stem extracts

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ABSTRACT

Plants are being used as traditional medicine due to their active natural ingredients. Screening of plants for biologically active compounds against human pathogens mostly drug resistant strains is as renewed interest in research field. The main objective of this study is to evaluate the antibacterial potentialities of *Ceriops tagal* stem extracts in different solvents against drug resistant strains-*Staphylococcus aureus* (MTCC87), *Bacillus subtilis*(MTCC 441),*Bacillus cereus* (MTCC430), *Escherichia coli* (MTCC40), *Klebsiella pneumonia* (MTCC39) and drug sensitive strains-*Bacillus subtilis* (MTCC121), *Enterobacter aerogenes* (MTCC111) and *Pseudomonas aeruginosa* (MTCC424) by agar-well diffusion method and qualitative screening of bioactive principles. The crude stem extracts demonstrated broad spectrum activity against all bacteria tested with inhibition zones in the range of ≤1to≥14mm at about 20mg/ml concentration. The most active solvent extracts methanol, acetone, and alcohol and water range from 9 to ≥14mm on drug - resistant Gram-positive *Staphylococcus aureus*. Of the eight extracts studied only four extracts showed good antimicrobial activity against the tested bacteria whereas remaining extracts were showed less activity. However the methanol extracts of *Ceriops tagal* stem was found to be most active towards drug resistant Gram-positive *Staphylococcus aureus*. Qualitative screening of stem extracts shows that, this plant had potent source of alkaloids, flavonoids, saponins, steroids and terpenoids.

***Keywords*:** *Ceriops tagal*; Secondary metabolites; Antibacterial activity

INTRODUCTION

Multidrug-resistant bacteria species are considered as the potential risk sources of numerous clinical problems worldwide. The development and increase of resistance among pathogens causing Nosocomial and community–acquired infections are known to be associated with the abuse of antibiotics [1-3].Infectious diseases caused by resistant microorganisms are responsible for increase health costs as well as high morbidity and mortality, particularly in developing countries [4].Literature reveals that plants have been used as an alternate, nontoxic and safe medicine to mankind. Therapeutic efficiency of plants is associated with the quality and quantity of secondary metabolites. Hence it is necessitated to search plant parts for biologically active compounds which are beneficial to human beings [5-7]. Mangroves are usually found only in tropical climates, as they need consistently warm conditions for development and survival. Mangrove plant extracts have been used for centuries as a popular method for treating several health disorders. Plant – derived substances have recently become of great interest owing to their versatile applications. Mangroves are biochemically unique, producing a wide array of novel natural products such as alkaloids, flavonoids, triterpenes, saponins and tannins. Mangrove and Mangrove associates contain biologically active antiviral, antibacterial, antifungal, antioxidant, anticancer, antihyper-cholestermic compounds [8, 9]. In this connection, the present study is an attempt to explore the mangrove plant *Ceriop*s *tagal* (*C.tagal*) from Estuarine Corangi Reserve Forest, Kakinada and East Godavari. The main objective of the authors is to exploit stem extracts of *C. tagal* for antibacterial activity. The taxonomic authenticity of *C.tagal* was identified by M.S. Swaminathan Research Institute, Kakinada; East Godavari. *C.tagal* is a true mangrove plant belonging to the family

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Rhizophoraceae and has been extensively studied for its bioactive potential. Use of this plant as a folk remedy is reported from different parts of the world[10,11].In India the decoction of the bark of *C. tagal* is being used to treat hemorrhages and malignant ulcers [12], while in China it is used against sores [13].Use of this plant in the treatment of malaria is also reported and its roots are used as a substitute for quinine [14, 15].This plant is a rich source of tannins and tri terpenoids [16].So far, twenty three di terpenes and twenty nine triterpenes have been reported from the stems, twigs, roots, leaves, hypocotyls and fruits of *C. tagal.*

MATERIALS AND METHODS

Plant Material: *C.tagal* stems were collected from Corangi Reserve Forest, Kakinada, East Godavari, Andhra Pradesh, India. The stems were surface sterilized with 1% mercuric chloride and thoroughly washed with plenty of distilled water. The plant material was dried under shade with occasional shifting. Later, the stems were chopped into small piece and stored in an airtight container.

Preparation of the Plant Extract: The chopped stem material *C. tagal* (100g) was extracted separately into different solvents in the increasing order of polarity viz hexane, benzene, ethyl acetate, chloroform, acetone, absolute alcohol, methanol and distilled water [17].The chopped material was extracted sequentially into 500 ml of the respective solvent by initial soaking for 12 hours followed by refluxing for about 10 hours below the boiling point of the respective solvent. Resulting extracts in different solvents were evaporated and concentrated using rotary evaporator. Concentrated extracts were dissolved in 1-2 ml of dimethyl sulfoxide (DMSO) and the concentration was adjusted to 100mg/ml with water and stored at 4°C.

Bacterial Strains: Pure cultures of *Staphylococcus aureus*(MTCC87), *Bacillus subtilis* (MTCC441), *Bacillus cereus* (MTCC430), *Escherichia coli* (MTCC40), *Klebsiella pneumonia* (MTCC39)(drug resistant strains) (these strains are resistant to the following drugs *S.aureus* - erythromycin, chloramphenicol and hydrophilic fluoroquinolone; *B.subtilis*-cephalexin and pencilin;*Bacillus cereus*-penicillin;*E.coli*-cephalexin and ampicillin; *K.pneumonia*- ciprofloxacin, penicillin, ampicillin and imipenem); *Bacillus subtilis* (MTCC121), *Enterobacter aerogenes* (MTCC 111) and *Pseudomonas aeruginosa* (MTCC 424) (drug sensitive strains) were procured from Microbial Type Culture Collection (MTCC) Chandigarh to determine the antibacterial activity. The active cultures were generated by inoculating a loopful of culture in separate 100mL nutrient broth and incubated at 37°C overnight. The cells were harvested by centrifuging at 4000 rpm for 15min, washed with normal saline, spun at 4000 rpm for 15 min again and diluted in normal saline to obtain 1-2×10 CFU/ml [18].The amount of bacteria needed to undertake the study was determined using UV/Vis spectrophotometer (ELICO, India) at 625 nm so that the absorbance of the suspension was held at 0.01nm.

Determination of antibacterial activity: The antibacterial activity of *C. tagal* stem extracts was performed by agar well diffusion method [19]. About 20 ml of molten Nutrient agar was mixed with 0.5 ml of bacterial suspension homogeneously and allowed to solidify in petri dishes (143 mm diameter). Wells with 10mm diameter were prepared using a sterile cork borer on the solidified medium. These wells were filled with 200µl of the crude extract containing 20mg.All the tests were performed in triplicates. Tetracycline, Gentamycin and Rifampicin was used as standard reference antibiotics and the diameters of the inhibition zones were measured and their means were calculated. DMSO in water was taken as control.

Identification of secondary metabolites: The crude stem extracts of hexane, benzene, ethyl acetate, chloroform, acetone, ethanol, methanol and distilled water were qualitatively analysed for the identification of secondary metabolites viz, alkaloids, flavonoids, saponins and tannins by using standard protocols [20-22].

Test for Alkaloids: 5 ml of the crude extracts was stirred with 10 ml of 1% aqueous HCl in water bath and then filtered. To 2ml filtrate 4-6 drops of Dragendroff's reagent was added. Formation of orange – red precipitate was considered as positive to alkaloids. To 2 ml filtrate few drops of Mayer's reagent was added and appearance of buff-colored precipitate was taken as existence of alkaloids.

Test for Anthraquinones: Borntrager's test - About 0.2gm of each extract to be tested was shaken with 10ml of benzene and then filtered. The filtrate was shook well after the addition of 5ml of the 10% ammonia solution. Appearance of pink, red or violet colour in the ammonical (lower) phase indicates the presence of free anthraquinones.

Test for Flavonoids: About 5ml of the test solution was boiled with 10 ml of distilled water and then filtered. Later, 2 ml of lead acetate solution was added to 2 ml of the filtrate. Appearance of buff colored precipitate considered as positive to flavonoids. To 2 ml of the filtrate, 5 ml of dilute ammonia solution was added followed by 4–6 drops of concentrated sulphuric acid. Appearance of yellow color indicates the presence of flavonoids.

Test for glycosides: To 1cm^3 of the extract in the test tube, 10cm^3 of 50%H_2SO_4 was added. The mixture was heated in boiling water for 15minutes. 10cm^3 of Fehling's solution was added, a brick red precipitate indicates the presence of glycosides.

Test for phenols: 2ml of extracts treated with3-4 drops of ferric chloride solution. Formation of bluish-black colour indicates the presence of phenols.

Ellagic acid test- To 2ml of extract, add few drops of 5%glacial acetic acid and 5%sodium nitrite. Muddy yellow or chocolate brown color appears, indicates the presence of phenols.

Test for Saponins: About 5 ml of crude extract was shaken with 5 ml of water in a test tube and it was warmed in a water bath. The persistent froth indicates the presence of saponins.

Test for steroids: Salkowskii test - About 0.2gm of each extract was dissolved in 2ml of chloroform, followed by the addition of conc.H_2SO_4 to form reddish brown colour at interphase indicates the presence of steroids.

Keller-Killiani test - To 0.5ml of test solution, 2ml of 3.5% $FeCl_3$, small amount of glacial acetic acid and 2ml of conc. H_2SO_4 were added carefully. Appearance of reddish brown ring at interphase. Indicates the presence of steroids.

Liebermann-Burchard test - To 0.2gm of each extract, 2ml of acetic acid was added and the solution was cooled well in ice followed by the addition of conc. H_2SO_4 carefully. Colour development from violet to blue or bluish-green indicates the presence of a steroidal ring.

Test for Terpenoids: Small quantity of crude extract was dissolved in ethanol.To this, 1ml of acetic acid, followed by the addition of few drops of conc.H_2SO_4. A change in colour from pink to violet confirms the presence of terpenoids.

Test for tannins: About 5ml of each extract was stirred with about 10ml of distilled water and then filtered. Few drops of 1% ferric chloride solution was added to 2ml of the filtrate. Occurrence of blue-black, green or blue-green precipitate. Indicates the presence of tannins.

About 5 ml of each extract was added with 1 ml of 1% HCl solution. Formation of red precipitate indicates the presence of tannins.

Phlobatannins test: About 0.2gm of each extract was added with 1% HCl solution. Formation of red precipitate indicates the presence of tannins.

RESULTS

Invitro determination of crude stem extracts of *C.tagal* possess broad spectrum activity against all tested bacteria with inhibition zones in the range of 1-14mm as depicted in Table: 1a&1b. Maximum activity was shown with acetone, alcohol, methanol and aqueous extracts (9.3mm, 10.1mm, 14.5mm &10.5mm) on drug- resistant Gram-positive *S.aureus*. Of these, methanol exerted highest activity on drug-resistant Gram-positive *S.aureus* and least activity was found with hexane extract about 1mm on drug-sensitive Gram-negative *E.aerogenes*. Tetracycline (30mcg), Gentamycin (30mcg) and Rifampicin (15mcg) were used as standard reference antibiotics and the diameters of the inhibition zones were measured and their means were shown in Table: 2a and 2b. DMSO in water was taken as control. An assessment of phytochemical studies of *C. tagal* stem crude extracts showed the presence of bioactive principles as shown in Table: 3

DISCUSSION

Our study shows that Gram-positive bacteria observed to be more susceptible than Gram-negative bacteria. These observations are more likely to be the fact that an outer membrane in Gram- negative bacteria, which acts as a barrier to many environmental substances including antibiotics [23, 24]. Among all the solvent extracts methanol exhibited highest activity (14mm). This may be due to the active ingredients of the plant parts are better extracted with methanol than other solvents and also depends on polarity of solvents. The active ingredients are mostly flavonoids, glycosides, steroids, tannins and terpenoids. Results obtained from this study indicate the potential usefulness of *C. tagal* in the treatment of various pathogenic diseases as mentioned in previous literature [25, 26]. So in future, our aim is to investigate, isolate and characterization of pure bioactive compounds of this plant that would be helpful for further applications of pharmaceutics.

CONCLUSION

Our study concluded that, this work may be suspected the first report on *C.tagal species* was made the comparison of drug resistant and drug sensitive bacteria. This study showed that species had promising antimicrobial activity against eight different clinical pathogens. Hence *C.tagal* is strongly recommended for considering as a valuable source for isolation, identification and characterization of bioactive principles responsible for antibacterial activity. However, further work in this direction could lead to the discovery of powerful bioactive principle from the *C.tagal*.

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Table 1a: Antibacterial activity of *C. tagal* stem extracts on drug resistant bacterial strains

S.N	Drug resistant strains	H	B	EA	C	A	E	M	W
1	*Escherichia coli*(MTCC 40)	2.03±0.35	3.1±0.08	5±0.99	3±1.73	5.1±0.13	6±0.108	5.1±0.135	5±0.1057
2	*Klebsiella pneumonia*(MTCC 39	4±0.629	3±1.732	4±0.865	3±1.047	6±0.236	7±0.887	7±0.981	6.6±0.00
3	*Bacillus cereus* (MTCC 430)	2±0.051	1.8±0.129	2.9±0.0814	1.8±0.108	8.2±0.2188	5.2±0.3672	7.1±0.17032	7.2±0.162
4	*Bacillus subtilis* (MTCC 441)	6.9±0.998	8.1±0.089	3.1±0.1085	1.1±0.1356	7±0.602	5±0.1085	7±0.1085	5.3±0.2448
5	*Staphylococcus aureus* (MTCC 87)	6±0.872	5.9±0.1723	6.9±0.9948	6.1±0.4711	9.3±0.232	10.1±0.1366	14.5±0.1342	10.5±0.713

H-Hexane; B-Benzene; EA-Ethyl Acetate; C-Chloroform; A-Acetone-E-Ethanol; M- Methanol;W- Water

Table1b: Antibacterial activity of *C. tagal* stem extracts on drug sensitive bacterial strains

S.N	Drug sensitive strains	H	B	EA	C	A	E	M	W
1	*Bacillus subtili*s (MTCC121)	2.0±0.332	2±0.8602	4.1±0.1085	2±0.1085	7±0.8579	5.1±0.0814	6±0.18999	5.1±0.371
2	*Enterobacter aerogenes*(MTCC 111)	1±0.000	3.1±0.1090	7±0.2367	5.2±0.1628	6±0.1085	7.1±0.1085	6.4±0.1628	6.2±0.2205
3	*Pseudomonas aeruginosa*(MTCC 424)	2.1±0.1356	3±0.0857	4.1±0.1356	3.1±0.1356	5.1±0.1091	4.1±0.1091	5.4±0.7297	4.3±0.1362

Experimental data statistically measured in mean ± SEM (standard error mean), where n=3usingexcelsoftware, windows8.1version

Table 2a: Antibacterial activity for standard antibiotics on drug resistant strains

S.N	Drug resistant strains	Gentamycin(30 mcg)	Rifampicin(15 mcg)	Tetracycline(30mcg)
1	*Escherichia coli*(MTCCC40)	13.6±0.360555	10.166±0.2886	19.433±0.4041
2	*Klebsiella pneumonia* (MTCC 39)	15.3±0.264575	8.833±0.76376	11.266±0.2516
3	*Bacillus cereus*(MTCC 430)	11.2±0.264575	7.633±0.55075	10.433±0.4509
4	*Bacillus subtilis* (MTCC 441)	16.133±0.2309	14.3±0.264575	16.466±0.4163
5	*Staphylococcus aureus* (MTCC 87)	10.666±0.2886	11.466±0.4163	12.433±0.3785

Table 2b: Antibacterial activity for standard antibiotics on drug sensitive strains

S.N	Drug sensitive strains	Gentamycin (30mcg)	Rifampicin (15mcg)	Tetracycline (30mcg)
1	*Bacillus subtilis* (MTCC 121)	15.033±0.152	12.466±0.450	14.333±0.2886
2	*Enterobacter aerogenes*(MTCC 111)	15.433±0.404	11.3±0.26457	16.133±0.1527
3	*Pseudomonas aeruginosa*(MTCC 424)	13.266±0.251	10.266±0.305	14.233±0.2516

The standard antibiotics values measured in mean ± SEM (standard error mean)

Table: 3 phytochemical analysis of *C. tagal* stem extracts in different solvents

S. N	Phytochemical	H	B	Ea	C	A	E	M	W
1	Alkaloids	-	-	+	-	-	-	+	-
2	Flavonoids	+	+	+	+	+	-	+	+
3	Glycosides	+	+	+	+	+	+	+	+
4	Phenols	-	-	-	-	+	-	+	+
5	Saponins	+	+	-	+	-	-	+	+
6	Steroids	+	+	+	+	+	+	+	+
7	Terpenoids	+	+	+	+	+	+	+	+
8	Tannins	+	+	+	+	+	-	+	-
9	Anthraquinones	-	-	-	+	+	+	+	+

H-Hexane; B-Benzene; EA-Ethyl Acetate; C-Chloroform; A-Acetone; E-Ethanol; M-Methanol; W-Water

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