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Full Length Research Paper

Analysis of and correlation between phytochemical and antimicrobial constituents of *Ceriops decandra,* a medicinal mangrove plant, from Indian Sundarban estuary

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The present study was aimed at screening the phytochemical contents and antimicrobial activities of different solvent extracts as well as partial identification of the bioactive constituents from leaf, root, wood and bark of *Ceriops decandra,* a mangrove plant from Sundarban estuary, India. Phytochemical analysis revealed the presence of high phenolics (GAE 42.33 - 163.44 mg/g), flavonoids (QE 39.16 - 582.22 mg/g), tannins (TAE 16.71 - 134.77 mg/g), alkaloids (34.04 - 150.50 mg/g) and saponins (16 - 38 mg/g). Antimicrobial activities of different tissue extracts were screened by disc diffusion method using two Gram-positive (*Bacillus subtilis* and *Bacillus coagulans*), two Gram-negative (*Escherichia coli* and *Proteus vulgaris*) bacteria and two fungi (*Saccharomyces cerevisiae* and *Aspergillus niger*). The extracts exhibited significant antimicrobial activities against all the microorganisms tested except *A. niger*. Selected plant extracts, when subjected to pH (3.0, 6.0 and 9.0) and heat (40, 60, 80 and 100°C) treatments, retained their antimicrobial activities in these extreme conditions thus demonstrating the stability of these compounds. The phytochemical components in each of the selected tissue extracts were also separated using thin layer chromatography (TLC); the antimicrobial constituents' positions on TLC-plates were determined by TLC-bioautography, followed by partial identification of the nature of active phytochemical constituents exhibiting these antimicrobial activities. This study suggests the plant as a potential source of stable antimicrobial compounds for use as phytotherapeutic agents and in food-processing industries.

Key words: *Ceriops decandra,* phytochemicals, antimicrobials, Sundarban, mangroves, TLC-bioautography, TLC-fingerprinting.

INTRODUCTION

The uses of medicinal botanicals as sources of remedy for various ailments have been known for centuries in different parts of the world and can be traced back to 60,000 years ago when Neanderthals used plants such as hollyhock, which are still used for ethnomedicinal purpose around the world (Cowan, 1999). A large number of therapeutic agents in use today have been isolated or derived from plant sources (Cowan, 1999). In recent years, the emergence of multidrug resistant microbial strains due to the indiscriminate uses of antibiotics has led to a growing need for novel anti-microbials (Chopra et al., 1997). The use of new antimicrobials not based on the existing synthetic anti-biotics may check the further growth and control the problem (Shah, 2005). The bioactive compounds possessing antimicrobial activities from plants are good sources for this purpose. Because the natural products

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either as pure compounds or as crude plant extracts are highly chemically diversified, they have huge applications in the newer drug discovery processes (Cos et al., 2006). In this respect, mangrove plants offer a very rich source of valuable medicines and merits serious considerations for the discovery of novel drugs since these plants as a group has been studied scantily from this perspective in spite of their extensive use in ethnomedicine/folk medicine.

Mangroves are woody, specialized types of trees of the tropics that can live on the edge where rainforests meet oceans. Found on sheltered coastlines and river deltas, they grow in brackish wetlands between land and sea where other plants cannot grow. The Sundarban (latitude 21° 31' - 22° 30' North and longitude 88° 10' - 89° 51' East) is the largest single block of tidal halophytic mangrove forest in the world (Ghosh et al., 2002). The total area under Indian Sundarban mangrove forest covers 4,266.66 km^2 (Naskar, 2004). Highly stressful habitat involving daily changes in pH of soil and water, humidity, salinity, temperature and tidal cycles may be possible reasons for many of these plants to synthesize a large number of different bioactive phytochemicals, many of which have been found to have extensive use in industry and human health care. Due to their medicinal values, different parts of these plants have been used for ages by the local people as folk medicine for curing many diseases (Bandaranayake, 1998) in the Sundarbans areas.

Ceriops decandra (Griff.) Ding Hou of the Rhizophoraceae family is a shrubby mangrove species and it has been traditionally used as remedial measures for wounds, boils, hepatitis, ulcers, angina, diabetes, diarrhea and dysentery (Watt and Breyer-Brandwijk, 1962; Duke and Wain, 1981; Kathiresan and Ramanathan, 1997; Bandaranayake, 1998). The species is distributed across East Africa and Madagascar, throughout tropical Asia and Queensland to Melanesia and Micronesia (Tomilson, 1986). A few studies prior to this present one have reported some pharmacologically important biological activities of this plant species. *C. decandra* have been reported for its antioxidant (Banerjee et al., 2008), antinociceptive (Uddin et al., 2005) and antidiabetic (Nabeel et al., 2010) properties. Though a few earlier studies have reported very preliminary antimicrobial activities (Sakagami et al., 1998; Vadlapudi and Naidu, 2009; Chandrasekaran et al., 2009; Ravikumar et al., 2010) in this species, an in-depth and coherent study is required for identification of nature of the constituents responsible for these activities (as antiseptics in wounds and boils) in order to realize their full potential in the health care industry.

Therefore, besides screening of the antimicrobial activities of leaf, root, wood and bark of *C. decandra* using different organic extraction systems for fine tuning the extraction of these antimicrobials, the present study also describes pH and thermal stability of these antimicrobials and total estimation of phytochemicals in the different tissues of this plant. In order to identify and correlate the antimicrobial constituents with these phytochemicals for future research involving characterizations of active principals, techniques such as TLC-bioautography and TLC-fingerprinting etc. have been used. Chromatographic fingerprinting has been used for identification and evaluation of stability of the chemical constituents in case of herbal drugs (Sharma et al., 2009) in other systems earlier.

MATERIALS AND METHODS

Collection and identification of plant materials

C. decandra (Griff.) Ding Hou was collected from the Indian Sundarban forest, West Bengal. The dried specimen of this plant after proper identification was deposited in the Herbarium of Botany department, Visva-Bharati University, India. Samples collected in different batches were always checked for appropriate activities before studies were carried out using these materials.

Preparation of plant extracts

The plant materials separated into leaf, wood, bark and root were washed and cleaned thoroughly with tap water and then with distilled water and air-dried in shade for several weeks. They were then finely ground to powder using a grinder. Dried and powdered plant tissues were extracted three times with each of the selected organic solvents– hexane, benzene, chloroform and methanol at room temperature. Aqueous extracts of *C. decandra* leaf, wood, bark and root were also prepared by soaking powdered tissues in sterile distilled water with intermittent shaking for 24 h at room temperature. The extracts were then filtered through Whatman No. 1 filter paper. The organic solvent extracts of the plant were evaporated at room temperature while the aqueous extracts were lyophilized using a lyophilizer. The dried extracts were stored at -20°C until use.

Quantitative phytochemical analysis of *C. decandra*

The powdered tissues of *C. decandra* were analyzed directly for quantitative estimation of phytochemical constituents such as phenolics, flavonoids, tannins, alkaloids and saponins. Total phenolics content was determined by the Folin-Ciocalteu colorimetric method (Singleton and Rossi, 1965) using gallic acid as a standard phenolic compound. The total phenolic content was expressed as gallic acid equivalents (GAE) in milligrams per gram of dry material. Total flavonoids content was measured by the aluminum chloride colorimetric assay (Zhishen et al., 1999) using quercetin as a standard flavonoid. The total flavonoid content was expressed as quercetin equivalents (QE) in milligrams per gram of dry material. Tannin determination was carried out according to the method described by Van-Buren and Robinson (1969) with slight modifications as described by Kaur and Arora (2009) using tannic acid as standard. The tannin content was expressed as tannic acid equivalents (TAE) in milligrams per gram of dry material. Alkaloid estimation was performed according to the method of Harborne (2005). The residue was dried, weighed and expressed as alkaloids in milligrams per gram of dry material. Quantitative estimation of saponin was carried out according to the method described by Obadoni and Ochuko (2001). The residue was dried, weighed and

expressed as saponins in milligrams per gram of dry material.

Antibacterial and antifungal activities

Test organisms

A total of six microorganisms including two Gram-positive, two Gram-negative bacteria and two fungi were used for this study; they were *Bacillus subtilis* (MTCC 121), *Escherichia coli* (MTCC 484), *Proteus vulgaris* (MTCC 426), *Bacillus coagulans, Aspergillus niger and Saccharomyces cerevisiae.* All the bacterial strains were maintained on LB (Luria-Bertani)-agar, *S. cerevisiae* on YPD (Yeast extract powder-Mycological peptone-Dextrose)-agar and *A. niger* on PD (potato dextrose)-agar and were stored at 4°C.

Microbiological methods

Growth and handling of the microorganisms were carried out following routine microbiological methods. Bacterial strains were grown in LB-broth; the fungi *S. cerevisiae* and *A. niger* were grown in YPD and PD broth, respectively. The compositions of the media were as described by Sambrook and Russel (2001). The incubation temperature for *B. subtilis*, *B. coagulans*, *P. vulgaris*, *S. cerevisiae* and *A. niger* were 30°C, while for *E. coli* it was 37°C. Inoculums were prepared by diluting actively growing overnight culture of bacterial and fungal suspensions with sterile 0.9% saline solution and adjusting the turbidity to a suitable final concentration for various experiments.

Antimicrobial activity assay of C. decandra extracts

The antibacterial and antifungal activity was determined by disc diffusion method (Bauer et al., 1966) using LB-agar, YPD-agar and PD-agar plates. The assay plates were prepared by pouring 30 ml of media into sterile 90 mm Petri dish and allowed to solidify. The prepared inoculum as described above was then spread uniformly on agar plates using a sterile cotton swab dipped into the experimental microbial suspension. Then paper discs (made from Whatman No. 1 filter paper) of 5.5 mm diameter were placed on the agar surface and 8 µL extract of concentration 250 mg/ml dissolved in dimethylsulphoxide (DMSO) was spotted on each disc (2 mg/disc). The plates were then incubated at respective ambient temperatures for 18 h (for bacteria) and up to 36 h (for fungus) and then the diameter of the inhibition zone around each disc was measured in mm. 3 µL of antibiotics such as ampicillin (125 and 500 µg/ml), chloramphenicol (10 mg/ml) and commercial fluconazole, an antifungal (10 mg/ml) were used as standards, while DMSO was used as a negative control to determine the sensitivity of the microorganisms tested.

Determination of minimum inhibitory concentration (MIC) / minimum bactericidal concentration (MBC) / minimum fungicidal concentration (MFC)

The MIC of the plant extracts were determined on LB-agar and YPD-agar medium following the method described by Sharma et al. (2009). Paper discs of 5.5 mm diameter were placed on the agar spread with respective test microorganism and spotted with 8 µL of different concentration of each plant extract. The concentration range 0.48 - 250 mg/ml (0.48, 0.97, 1.95, 3.9, 7.81, 15.62, 31.25, 62.5, 125, 250 mg/ml) of a plant tissue extract was prepared by serial dilution of a stock solution in DMSO. The plates were then incubated at respective incubation temperature for 18 h (for bacteria) to 36 h (for fungus). The MIC value was recorded as the least concentration of plant extract that completely inhibited the growth of the test organisms.

The MBC/MFC was determined according to the method described by Greenwood (1989), with some modifications. The tissue extracts (stock solution 250 mg/ml in DMSO) were added to 1 ml of LB or YPD broth containing 10 µL of overnight grown test organism and incubated at appropriate temperature for 24 h at 180 rpm. Final concentration of extracts in the growth media was in the range of 1 - 20 mg/ml. After 24 h, the contents of the culture tubes were plated on to fresh agar plates and grown for another 18 h (for bacteria) or 36 h (for fungus). The least concentration of the extract at which the corresponding sub-cultured plate did not show any growth was recorded as the MBC/MFC value of a particular extract.

pH and thermal stability of antimicrobial activities

pH treatment

Aliquots of DMSO were adjusted to pH 3.0, 6.0 and 9.0 with 1 N hydrogen chloride (HCl) or 1 N sodium hydroxide (NaOH) as appropriate and autoclaved. The extracts from each of root, wood and bark showing maximum antimicrobial activity were then dissolved in the above-mentioned pH-adjusted DMSO at a concentration of 250 mg/ml. Residual antibacterial or antifungal activities of these various pH-treated samples were determined against the target organisms using disc diffusion method described above with suitable controls.

Heat treatment

The extracts from each of root, wood and bark showing maximum antimicrobial activity were dissolved directly in DMSO at a concentration of 250 mg/ml and incubated in a water-bath for 30 min at 40, 60, 80 and 100°C. The samples were then cooled to room temperature and stored at -20°C until use. The residual antibacterial or antifungal activities were determined against the target organisms with the help of disc diffusion method described above. Untreated extracts dissolved in DMSO were used as positive controls.

Thin layer chromatography (TLC) of plant extracts

TLC Silica gel 60 F_{254} plates (Merck) of 0.2 mm thickness were used for TLC. Extracts from each of root, wood and bark showing maximum antimicrobial activities were dissolved in solvents at a concentration of 50 mg/ml and were applied on replicate TLC plates. The chromatograms of root-hexane extract and wood-chloroform extract dissolved in hexane and chloroform respectively were developed using benzene: ethanol: ammonia (18:2:0.2) as mobile phase. The chromatograms of bark-water extract dissolved in methanol were developed using upper layer of butanol: acetic acid: water (20:5.5:25) as mobile phase. At the end of the run, the spots and bands on TLC plates were visualized under visible and ultraviolet (254 and 366 nm) light and their R_f values were determined (distance travelled by phytochemical component/ distance travelled by the mobile phase).

TLC-bioautography

The bioautography technique was carried out according to the method described by Gupta et al. (2010) for the detection of antimicrobial constituents present in the extracts. After separating these extract constituents by TLC and drying the plates thoroughly so that no residue of any solvent was left on the plate, inoculums of

the test microorganisms containing 10^8 CFU/ml (for bacteria) and 10^6 CFU/ml (for fungus) in molten LB-agar and YPD-agar respectively were poured on these TLC plates. After solidification of the agar medium on the TLC plates, they were incubated at respective temperature for 24-36 h. The TLC-bioautograms were stained by spraying with 1% aqueous solution of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT). The staining method is based on the reduction of MTT by mitochondrial dehydrogenase of viable cells resulting in a blue formazan product zone after incubation. Where there are no viable cells, the region appears as a clear zone without any colour. The incubation was for 1 - 4 h at respective temperature. Growth Inhibition zones failed to take the bluish stain and indicated the presence of active antimicrobial constituents at those sites of the TLC plates.

Partial identification of nature of the active constituents

Partial identification of nature of the various extract constituent(s) was carried out by using different spraying reagents giving colour reactions specific for phenols, flavonoids, tannins, alkaloids etc. as described (Krebs et al., 1969; Harborne, 2005). For phenols, the TLC plates were sprayed with 1 N Folin-Ciocalteu reagent and the plates were heated at 80°C for 10 min. The colour and R_f values of the spots were recorded under visible light. Blue coloured spots demonstrated positive for phenols. For flavonoids, the TLC plates were sprayed with 1% ethanolic solution of aluminium chloride ($AlCl_3$). Spots with yellow fluorescence under ultraviolet light at 366 nm were tested positive for flavonoids. Screening the spots for tannin on TLC plates was carried out by spraying the chromatograms with alcoholic $FeCl_3$ (5% w/v). The colour and R_f values of the spots were recorded under visible light. For alkaloids, the TLC plates were sprayed with Dragendorff's reagent and observed under both visible and ultraviolet light at 254 nm. Orange coloured spots demonstrated positive for alkaloids. Subsequently, the colour reaction patterns obtained from various compounds separated on TLC plates were compared with their TLC-bioautogram counterparts. This allowed us to determine the nature of phytochemical constituents at the growth inhibition zones of microorganisms found on TLC-bioautograms.

RESULTS AND DISCUSSION

Quantitative phytochemical analysis

Mangroves like *C. decandra* thrive in extremely stressful and hostile environment. To sustain in such hostile condition, alternations in the level and nature of their secondary metabolites have occurred which are thought to protect them from the destructive elements of nature (Edreva et al., 2008; Shulaev et al., 2008; Reyes and Zevallos, 2003). Many of these compounds or molecules have significant biological and other medicinal properties which play invaluable role in the drug discovery process. For examples, several classes of phenols and polyphenols such as phenolics, flavonoids and tannins contribute to plant defence mechanism in resisting pathogenic microorganisms (Cowan, 1999). According to Tsuchiya et al. (1996), flavonoids exhibit antimicrobial activity by forming complexes with extracellular and soluble proteins and with the cell wall of the micro-organisms. Merschjohann et al. (2001) and Zhu et al. (1998) have described the ability of berberine, a well-known alkaloid to intercalate with DNA and inhibit protein synthesis, thus resulting in antimicrobial activity. Saponins are natural surfactants and form complex with cholesterols in protozoal cell membranes, causing cell lysis (Cheeke, 2000). Hence, quantification of these major phytochemicals is necessary for obtaining a preliminary idea regarding the biological potential of this experimental plant species.

The quantitative picture of the various phyto-constituents recorded in *C. decandra* is represented in Table 1. The data clearly outline the richest phenolic contents in the bark (163.44 ± 3.39 mg GAE/g dry weight) and root (93.00 ± 1.80 mg GAE/g dry weight). The leaf of the plant used as an alternative source of tea (Bandaranayake, 1998) has been found to be a rich source of natural phenolics (67.66 ± 1.41 mg GAE/g dry weight). The flavonoid content has been found to be the highest in the bark (582.22 ± 43.88 mg QE/g dry weight), followed by root (458.61 ± 39.62 mg QE/g dry weight). The bark of the plant has been found to be a rich source of tannin (134.77 ± 8.48 mg TAE/g dry weight) and used by local fishermen for tanning their fishing-nets to prevent damage to cotton threads resulting due to saline water soaking, thus increasing the durability of the net (Raju et al., 2008). The bark has also been found to contain highest alkaloid (150.50 ± 0.53 mg/g dry weight) and saponin (38.00 ± 4.24 mg/g dry weight) compared to the other parts of the plant examined. Thus, the quantitative analysis has showed the presence of significant amounts of phytochemical contents in *C. decandra*.

Antimicrobial activities

Antibacterial and antifungal activities

The determination of antibacterial and antifungal activities have been carried out using hexane, benzene, chloroform, methanol and aqueous extracts of leaf, root, wood and bark of *C. decandra* against six microbial strains, including Gram-positive bacteria (*B. subtilis* and *B. coagulans*), Gram-negative bacteria (*E. coli* and *P. vulgaris*) and fungi (*S. cerevisiae, A. niger*). The findings are represented in Table 2. All of the extracts have exhibited some activities in the disc diffusion assays against the microorganisms tested except *A. niger*. Among the extracts tested, the aqueous extract of bark has exhibited maximum broad-spectrum activity against both Gram-positive and Gram-negative bacteria. Methanol extract of bark, aqueous extract of leaf, methanol and aqueous extracts of root also have shown inhibitory activities against all the bacteria tested. Aqueous extract of bark has been found to exhibit the highest mean activity against *B. subtilis* (12 mm), *E. coli* (10.66 ± 0.28 mm) and *P. vulgaris* (10.16 ± 1.04 mm), while, the chloroform extract of wood has been found to exhibit its highest activity against *B. coagulans* (15.16 ±

Table 1. Quantitative determination of phytochemicals from leaf, root, wood and bark of *C. decandra.*

Parts examined	Total phenolics (mg GAE/g dry weight)	Total flavonoids (mg QE/g dry weight)	Total tannin (mg TAE/g dry weight)	Total alkaloid (mg/g dry weight)	Total saponin (mg/g dry weight)
Leaf	67.66 ± 1.41	39.16 ± 10.53	30.11 ± 4.28	91.42 ± 7.09	16.00 ± 1.41
Root	93.00 ± 1.80	458.61 ± 39.62	52.11 ± 4.34	77.98 ± 1.38	16.00 ± 0.00
Wood	42.33 ± 1.80	154.72 ± 17.82	16.71 ± 3.74	34.04 ± 1.07	21.50 ± 2.12
Bark	163.44 ± 3.39	582.22 ± 43.88	134.77 ± 8.48	150.50 ± 0.53	38.00 ± 4.24

Each value is the mean ± standard deviation from three replicates.

Table 2. Antimicrobial activity of *C. decandra* tissue extracts against Gram-positive, Gram-negative bacteria and fungi by disc diffusion method.

Parts examined	Extract	Inhibition zone diameter (mm)[a]					
		Microorganisms					
		B. subtilis	***B. coagulans***	***E. coli***	***P. vulgaris***	***S. cerevisiae***	***A. niger***
Leaf	Hexane	07.50 ± 0.00	07.33 ± 0.28	-	-	-	-
	Benzene	06.41 ± 0.14	-	-	-	-	-
	Chloroform	06.66 ± 0.28	-	-	-	-	-
	Methanol	07.58 ± 0.28	-	06.50 ± 0.00	-	-	-
	Aqueous	08.50 ± 0.50	07.16 ± 0.28	06.83 ± 0.57	06.66 ± 0.28	-	-
Root	Hexane	08.75 ± 0.25	07.33 ± 0.28	-	-	13.33 ± 0.76	-
	Benzene	07.41 ± 0.14	07.33 ± 0.28	-	-	08.58 ± 0.52	-
	Chloroform	06.91 ± 0.14	07.16 ± 0.28	-	-	07.83 ± 0.28	-
	Methanol	09.83 ± 0.28	07.25 ± 0.43	08.00 ± 0.00	06.66 ± 0.28	-	-
	Aqueous	09.00 ± 0.00	06.83 ± 0.28	09.08 ± 0.38	07.66 ± 0.57	-	-
Wood	Hexane	08.50 ± 0.86	06.91 ± 0.52	-	-	-	-
	Benzene	08.91 ± 0.94	09.00 ± 0.50	-	-	-	-
	Chloroform	09.00 ± 2.00	15.16 ± 0.28	-	-	-	-
	Methanol	08.66 ± 0.57	-	-	-	-	-
	Aqueous	08.00 ± 0.00	-	06.66 ± 0.28	06.91 ± 0.38	-	-
Bark	Hexane	09.50 ± 0.50	09.00 ± 0.50	-	-	08.08 ± 0.94	-
	Benzene	08.50 ± 0.50	07.83 ± 0.28	-	-	07.16 ± 0.28	-
	Chloroform	07.83 ± 0.38	07.33 ± 0.57	-	-	-	-
	Methanol	10.25 ± 0.43	08.50 ± 0.00	09.16 ± 0.38	07.83 ± 0.76	-	-
	Aqueous	12.00 ± 0.00	09.50 ± 0.50	10.66 ± 0.28	10.16 ± 1.04	-	-

Table 2. Contd.

Control	Ampicillin[b]	11.16 ± 0.83	23.16 ± 0.25	08.41 ± 0.40	-	-	-
	Chloram.[c]	-	-	-	15.62 ± 0.88	-	-
	Fulconazole[d]	-	-	-	-	18.83 ± 0.81	12.50 ± 0.35
	DMSO	-	-	-	-	-	-

Each value is the mean ± standard deviation from three replicates. All the extracts have been used at 2 mg/disc. [a] Inhibition zone diameter, including disc diameter of 5.5 mm. [b] Ampicillin used at 3 µL/disc (conc. 125 µg/ml) against Gram-positive bacteria and 3 µL/disc (conc. 500 µg/ml) against *E. coli* . [c] Chloram. = Chloramphenicol at 3 µl/disc (conc. 10 mg/ml) against *P. vulgaris.*[d] Fulconazole used was 3 µL/disc (conc. 10 mg/ml) against fungus.

0.28 mm). The sensitivity of *E. coli* to aqueous extract of leaf of this plant is in line with an earlier study by Sakagami et al. (1998). Hexane, benzene, chloroform extracts of root and hexane, benzene extracts of bark have been found to possess antibacterial activities against Gram-positive bacteria and antifungal activities against *S. cerevisiae*. Hexane extract of root has exhibited the highest activity against *S. cerevisiae* (13.33 ± 0.76 mm). Therefore, the Gram-positive bacteria, Gram-negative bacteria and fungi are sensitive to different tissue extracts of this plant, demonstrating the broad-spectrum antimicrobial capability of *C. decandra*. DMSO has served as the negative control for all the antimicrobial assays conducted in this study and has not exhibited any inhibitory activity against the strains tested, thus validating the antimicrobial properties of the extracts of *C. decandra*. The antibiotics ampicillin (125 and 500 µg/ml), chloramphenicol (10 mg/ml) and fluconazole (10 mg/ml) used as positive controls have been found to be effective against the respective strains tested.

Thus, the screening and quantitative analysis of phytoconstituents of leaf, root, wood and bark of *C. decandra* revealed the presence of phenolics, flavonoids, tannins, alkaloids and saponins in significant amounts, which may be linked with the observed antimicrobial activities of the extracts. The differences in susceptibility to antibiotics, external agents and detergents between Gram-positive and Gram-negative bacteria have already been reported earlier where Gram-negatives are reported to exhibit more resistance to these external agents. This is due to the presence of lipopolysaccharides in their outer membranes, which make these cells impermeable (Nikaido and Vaara, 1985), whereas the Gram-positive bacteria possess an outer peptidoglycan layer, which is an inefficient permeability barrier (Scherrer and Gerhardt, 1971), thus making the Gram-positives much more sensitive to external agents compared to Gram-negatives.

MIC and MBC/MFC of C. decandra extracts

The extracts from each of leaf, root, wood and bark of *C. decandra* exhibiting maximum activity against each of the microbial strains tested with a minimum inhibitory zone of 8.5 mm diameter in the antimicrobial assay, have been selected further to determine their MIC and MBC/MFC. The results obtained for the MIC and MBC/MFC of the selected extracts of *C. decandra* leaf, root, wood and bark are represented in Table 3. The MIC and MBC/MFC values have been found to be extract and strain dependent. The chloroform extract of wood has significant MIC value of 1.95 mg/ml against *B. subtilis* and *B. coagulans* and MBC value of 2 and 4 mg/ml, respectively. The aqueous extract of bark possesses significant MIC and MBC values of 1.95 and 3 mg/ml, respectively, against *B. subtilis* and MIC values of 15.62, 125, 31.25 mg/ml and MBC values of >20 mg/ml for *B. coagulans*, *P. vulgaris* and *E. coli*, respectively. The hexane extract of root appears to have MIC value of 3.9 mg/ml and MFC value of 9 mg/ml against *S. cerevisiae*. The methanol extract of root and aqueous extract of leaf have exhibited MIC values of 1.95 and 31.25 mg/ml and MBC values of 3 and >20 mg/ml respectively against *B. subtilis.* The aqueous extract of root has been found to possess MIC of 125 mg/ml and MBC value of >20 mg/ml against *E. coli*.

The aforementioned results, therefore, clearly demonstrate the bactericidal effects of methanol extract of root, chloroform extract of wood and aqueous extract of bark against *B. subtilis*, chloroform extract of wood against *B. coagulans* and fungicidal effect of hexane extract of root against *S. cerevisiae*. The aqueous extract of bark has been found to exhibit growth inhibitory effect against *B. coagulans*, *P. vulgaris* and *E. coli* at the maximum extract concentration (20 mg/ml) used. The aqueous extracts of leaf and root have also been found to show inhibitory activity against *B. subtilis* and *E. coli*, respectively, at the highest concentration (20 mg/ml) of crude extract used in this study. The results also seem to indicate

Table 3. MIC and MBC/MFC value of *C. decandra* leaf, root, wood and bark extracts[a] against bacteria and fungi.

Organisms	MIC (MBC/MFC) value in mg/ml					
	Plant part-extract					
	Leaf- aqueous	Root- hexane	Root- methanol	Root- aqueous	Wood-chloroform	Bark- aqueous
B. subtilis	31.25 (>20)	-	1.95 (3)	-	1.95 (2)	1.95 (3)
B. coagulans	-	-	-	-	1.95 (4)	15.62 (>20)
P. vulgaris	-	-	-	-	-	125 (>20)
E. coli	-	-	-	125 (>20)	-	31.25 (>20)
S. cerevisiae	-	3.9 (9)	-	-	-	-

[a] Extracts from each of leaf, root, wood and bark of *C. decandra* exhibiting maximum activity against each microbial strain with a minimum inhibitory zone of 8.5 mm diameter in disc diffusion test (Table 1) have been considered. Also, 8 µL of each of the stated concentration have been used for MIC. MBC/MFC values are shown in brackets.

presence of different antimicrobial components (as bacteriostatic or bactericidal) in varying amounts in various tissue extracts. The presence of a broad spectrum of various kinds of growth inhibitory/bactericidal properties is consistent with the usage of the plant tissue extracts for healing different skin lesions practiced by the local population.

Effect of pH and thermal treatments on antimicrobial activities

The *C. decandra* extracts exhibiting maximum activity against respective microbial strains have been considered further to determine the effect of pH and temperature on their antimicrobial activities and the results obtained are represented in Table 4. The hexane extract of root has shown a significant decrease in activity at pH 9.0 compared to the control against *S. cerevisiae.* The chloroform extract of wood has also exhibited a slight drop in the activity at pH 9.0 compared to the control against *B. coagulans*. All the other pH and temperature treated extracts, except the two examples mentioned earlier, have largely retained their activities, thus suggesting that these activities are pH and temperature tolerant. Slight variations in the inhibitory zone diameter of positive control values (Table 4) from that of the diameter obtained during antimicrobial activity assays (Table 2) of the selected extracts observed might be due to the slight errors involved in conducting same experiments in separate occasions. pH adjusted DMSO solutions (pH 3.0, 6.0 and 9.0) and normal DMSO showed no inhibitory activity against the strains tested (negative controls; data not shown).

Thus, the *C. decandra* extracts tested for pH and thermal stability have exhibited significant stability and have retained their antimicrobial activities under extreme conditions. These results prove this plant's worth as a valuable source for extraction of antimicrobial compounds useful as: i) natural preservatives in food-processing applications to check microbial growth and ii) drugs that can be administered orally. pH tolerance is one of the main challenges in the field of drug discovery (Di and Kerns, 2009) since the pH value of gastrointestinal tract varies from low pH in the stomach (pH 1.2) to high pH (pH 8.0) in the small and large intestine. Loss of activity at certain pH values due to change in molecular structure or charge of the compound can limit oral exposure of the intended drugs.

TLC and bioautography of *C. decandra* extracts

Using the TLC technique, organic compounds can be separated based on their molecular weight and polarity. Haugland and Johnson (1999) described the technique as an ideal method for the separation of natural substances and have huge applications in analyzing biological and chemical samples for identification and determination of their composition. TLC of hexane extract of root and chloroform extract of wood of *C. decandra* have been carried out using benzene: ethanol: ammonia (18:2:0.2) as mobile phase and has been able to separate different compounds/phytoconstituents present in the respective extracts using this method. TLC of aqueous extract of *C. decandra* bark has been performed using upper layer of butanol: acetic acid: water (20:5.5:25) as mobile phase.

The chromatograms of respective extracts have been visualized under UV light at 366 nm (Figure 1: Lanes a, g and l) and 254 nm (Figure 1: Lanes b, h and m), and shown to contain distinct and fused bands of different R_f values viz. root-hexane extract (Figure 1: Lane a: R_f 0.092, 0.796, 0.907; Lane b: R_f 0.370, 0.453, 0.527, 0.750, 0.842, 0.879), wood-chloroform extract (Figure 1: Lane g: R_f 0.216, 0.726, 0.839; Lane h: R_f 0.216, 0.255, 0.547, 0.811, 0.867, 0.896) and bark-aqueous extract (Figure 1: Lane l: R_f 0.523; Lane m: R_f 0.180, 0.523). Many of these fractions observed might be the active substances responsible for the observed antimicrobial activities. The TLC-bioautography method as described by Hostettmann and Wolfender (1997), can be used to resolve these bioactivities using microbial cultures and other biological agents. TLC-bioautography of *C.*

Table 4. Effect of pH and thermal treatment of *C. decandra* extracts[a] on antimicrobial activities[b] (mm) against bacteria and fungi.

Organisms	Plant part-extract	Positive control	pH			Temperature (°C)			
			3.0	6.0	9.0	40	60	80	100
S. cerevisiae	Root-hexane	12.93 ± 0.37	12.12 ± 0.17	12.12 ± 0.17	07.87 ± 0.17	12.87 ± 0.53	12.62 ± 0.17	12.37 ± 0.17	12.12 ± 0.17
B. coagulans	Wood-Chloroform	14.87 ± 0.25	14.50 ± 0.00	13.00 ± 0.00	12.25 ± 0.35	15.00 ± 0.00	15.00 ± 0.00	14.75 ± 0.35	14.75 ± 0.35
B. subtilis	Bark-aqueous	11.37 ± 0.14	11.62 ± 0.17	11.37 ± 0.17	11.25 ± 0.00	11.50 ± 0.35	11.37 ± 0.17	11.50 ± 0.00	11.37 ± 0.17
P. vulgaris	Bark -aqueous	09.62 ± 0.25	09.37 ± 0.17	09.12 ± 0.17	09.50 ± 0.35	09.75 ± 0.35	10.00 ± 0.00	09.75 ± 0.35	09.75 ± 0.35
E. coli	Bark -aqueous	11.18 ± 0.23	10.37 ± 0.17	10.62 ± 0.17	11.12 ± 0.17	11.25 ± 0.35	11.00 ± 0.00	11.25 ± 0.35	11.25 ± 0.35

[a] Extracts exhibiting maximum activity against respective microbial strain have been considered; All the extracts used at 2 mg/disc. [b] Inhibition zone diameter includes disc diameter of 5.5 mm, and is the mean ± standard deviation from two replicates except control; Positive control value is the mean ± standard deviation from four replicates. Dried extracts dissolved in DMSO served as positive controls in both pH and thermal stability experiments.

decandra root-hexane extract chromatograms have been performed using *B. subtilis*, *B. coagulans* and *S. cerevisiae*. Clear zones of inhibitions obtained on the TLC plates suggest the presence of active substances at those regions, which inhibited the growth of *B. subtilis* (Figure 1: Lane c: R_f 0.092, 0.370, 0.453, 0.527, 0.750, 0.842, 0.879 and 0.907) and *B. coagulans* (Figure 1: Lane d) at identical positions but no activity has been detected against *S. cerevisiae* on the TLC plate (data not shown).

Bioautography of *C. decandra* wood-chloroform extract chromatograms have been carried out using *B. subtilis* (Figure 1: Lane i) and *B. coagulans* (Figure 1: Lane j). The active constituent at R_f 0.547 (Figure 1: Lanes i and j) exhibits the maximum activity against both the bacteria at identical positions. A few other active constituents have shown minor activities against *B. subtilis* (Figure 1: Lane i: R_f 0.028, 0.141, 0.188, 0.216, 0.811, 0.839, 0.867 and 0.896) and *B. coagulans* (Figure 1: Lane j: R_f 0.028, 0.147 and 0.867). Bioautography of aqueous extract of bark have been done using the replicate chromatogramswith *B. subtilis*, *B. coagulans*, *E. coli* and *P. vulgaris*. The active constituent at R_f 0.180, 0.523 and the stretch onwards R_f 0.523 has shown inhibitory zone against *B. subtilis* (Figure 1: Lane n), but no detectable activity on the TLC-bioautography plate of bark-aqueous extract has been obtained against *B. coagulans*, *E. coli* and *P. vulgaris* (data not shown). The clear inhibitory zones suggest the presence of antimicrobial constituents at those regions.

It is interesting to note that while in the TLC-bioautography studies, no antimicrobial activity has been recorded for root-hexane extract against *S. cerevisiae* and bark-aqueous extract against *B. coagulans*, *E. coli* and *P. vulgaris*, these same extracts have shown activity against the respective organisms during antimicrobial activity assessment using disc diffusion method (Table 2). This might be due to the synergistic activity of more than one substance or compounds present in the extract. After the separation of these extract components on TLC plates, the compounds responsible for the activity at synergistic level might have separated from each other and lost their antimicrobial activities as an individual substance or compound. The other possibility might be the small amount of extract spotted (3 µL of 50 mg/ml) on the TLC plates, which is insufficient for exhibiting any activity against the organisms.

Partial identification of the nature of the active constituents exhibiting antimicrobial activity by TLC-fingerprinting

The TLC replicates of *C. decandra* root-hexane, wood-chloroform and bark-aqueous extracts have been sprayed with different colour reagents specific for phenols, flavonoids, tannins, alkaloids etc., to detect the nature of the constituents at the regions of growth inhibitory zones on the TLC-bioautography plates. The TLC replicate of root-hexane extract sprayed with phenol-specific reagent developed few bluish spots, suggesting the presence of phenols at those regions, of which the one at R_f 0.750 (Figure 1: Lane e shown with an arrow) is at the identical position showing one of the inhibitory zones on TLC-bioautography plates (Figure 1: Lanes c and d). Similarly, the flavonoid specific reagent has demonstrated positive for flavonoid at R_f 0.842 (Figure 1: Lane f shown with an arrow) and is identical to the region showing antimicrobial activity on TLC-bioautography plates (Figure 1: Lanes c and d). No other spots have been found to be positive for tannins and alkaloids on the TLC replicates of root-hexane extract (data not shown). Therefore, the analysis for root-hexane extract suggests

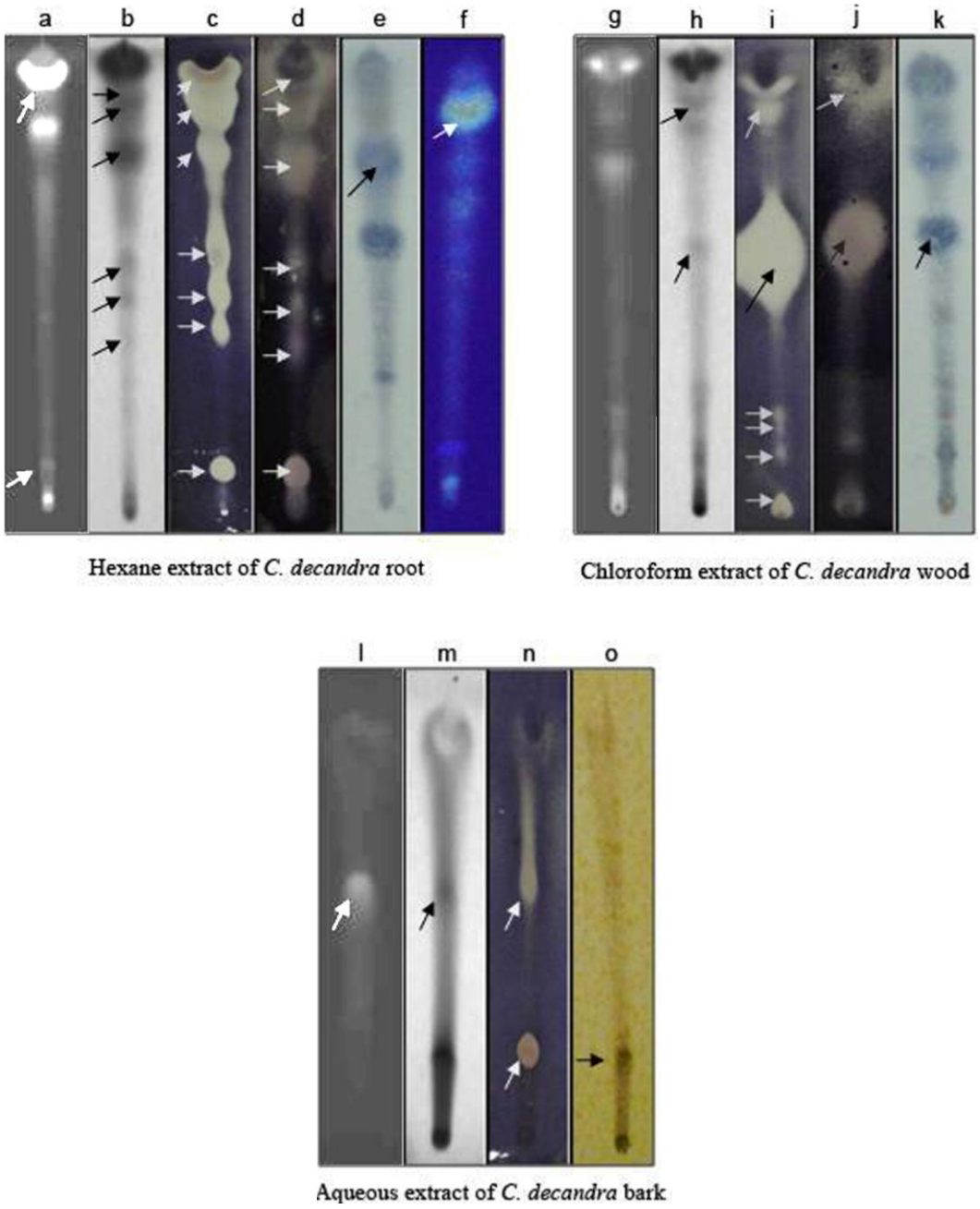

Figure 1. TLC, TLC-bioautography and TLC-bioactive constituent nature detection plates of *C. decandra* root, wood and bark extracts. Lanes a, g and l were visualized under long wave UV light (366 nm); lanes b, h and m under short wave UV light (254 nm). Arrows indicate active bands. Lanes c, i and n are bioautography plates with *B. subtilis*. Lanes d and j are bioautography plates with *B. coagulans.* Arrows indicate antibacterial activity. Lanes e and k were sprayed with F.C. reagent and visualized under visible light for detection of phenol spots. Lane f was sprayed with ethanolic $AlCl_3$ and visualized under UV light at 366 nm for flavonoid spot detection. Lane o was sprayed with alcoholic $FeCl_3$ and viewed under visible light for tannin detection. Arrows indicate active spot.

that the constituents present at two of the regions showing inhibitory zones against *B. subtilis* and *B. coagulans* (Figure 1: Lanes c and d, respectively shown with arrows), are of phenolic (R_f 0.750) and flavonoid (R_f 0.842) nature. However, the nature of other growth inhibitory constituents exhibited in these two lanes (Figure 1: Lanes c and d) cannot be ascertained at present. The TLC replicate of *C. decandra* wood-chloroform extract exhibits few phenol specific bluish spots, of which the distinct spot at R_f 0.547 (Figure 1:

Lane k shown with an arrow) is at identical position, showing maximum inhibitory activity at R_f 0.547 on TLC-bioautography plates against *B. subtilis* and *B. coagulans* (Figure 1: Lanes i and j shown with arrows). None of the spots on the TLC replicates of wood-chloroform extract has demonstrated positive for flavonoids, tannins and alkaloids specific reagents (data not shown). Therefore, microbial growth inhibitory activity of wood-chloroform extract at R_f 0.547 (Figure 1: Lanes i and j) might be due to the presence of phenolic constituent(s). Moreover, the TLC replicate of *C. decandra* bark-aqueous extract when sprayed with tannin specific alcoholic $FeCl_3$ reagent, has developed a dark brown spot at R_f 0.180 (Figure 1: Lane o) distinctly on the brown stretch demonstrating the presence of tannin at the region. The spot is at an identical position of R_f 0.180 in the TLC-bioautography result showing inhibitory activity (Figure 1: Lane n shown with lower arrow) against *B. subtilis*, suggesting the presence of tannin-like substances in this region. The nature of the growth inhibitory constituent(s) at R_f 0.523 region (Figure 1: Lane m shown with upper arrow) has not been detected. Also, none of the TLC replicates of bark-aqueous extract has demonstrated positive for phenol, flavonoids and alkaloids (data not shown).

From the TLC-fingerprinting experiments, it is apparent that: i) there are several growth inhibitory substances present in different tissues of the plant (Figure 1: Lanes c, d, i, j and n), ii) these inhibitory components are different from each other and extract from the plant tissues differently with various organic solvents. Of these growth inhibitory components resolved by the TLC/ bioautography experiments, it is not possible to differentiate at this point, which ones are having bactericidal properties and which ones are acting as microbial growth inhibitors. However, in the light of earlier MIC/MBC studies, it is clear that both classes of components are present in the different tissue extracts of *C. decandra*.

In conclusion, the results obtained in the present study show the presence of significant amounts of phytochemicals, especially phenolics in the leaf, root, wood and bark of the mangrove plant *C. decandra*. Considerable antimicrobial activities against Gram-positive, Gram-negative bacteria and fungus have also been exhibited by extracts of different tissues from this mangrove species and the observed activities have been correlated with some of the phytochemicals found to be present in the species. These findings should be useful in further studies for the isolation and characterization of active principals from this plant directed at the wound healing process reported by others. The microorganisms used in this study are the representative species and the extracts showing activity against these microorganisms are expected to exhibit similar antimicrobial activity against microbes found at the wound sites. Selected extracts of this species exhibiting maximum antimicrobial activities have also been found to be tolerant to high pH and thermal treatments, confirming the stable nature of the antimicrobial substances. Thus, the findings from this study justify the use of *C. decandra* as a potent source of antimicrobials and its use as antiseptic in wounds and boils in traditional medicine.

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