EXCLI Journal 2014;13:997-1010 – ISSN 1611-2156
Received: June 20, 2014, accepted: July 21, 2014, published: August 29, 2014

Original article:

ANTIMICROBIAL AND ANTIOXIDATIVE ACTIVITIES IN THE BARK EXTRACTS OF *SONNERATIA CASEOLARIS*, A MANGROVE PLANT

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ABSTRACT

The present study deals with the phytochemical contents, antimicrobial and antioxidative activities of bark tissue of *Sonneratia caseolaris,* a mangrove plant from Sundarban estuary, India. Phytochemical analyses revealed the presence of high amounts of phenolics, flavonoids, tannins, alkaloids and saponins. Antimicrobial efficacies of various extracts of *S. caseolaris* were assessed by disc diffusion method against two Gram-positive (*Bacillus subtilis* and *Bacillus coagulans*), two Gram-negative (*Escherichia coli* and *Proteus vulgaris*) bacteria and one fungus (*Saccharomyces cerevisiae*). The methanolic extract among others showed significant minimum inhibitory concentration (MIC) values. The antioxidant activity as indicated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity of the bark tissue extract from the species was found to be quite appreciable. The extracts were found to retain their antimicrobial activities despite pH and thermal treatments, thus indicating the stability of their activity even at extreme conditions. The antioxidant activity was also found to be considerably stable after thermal treatments. The components of the tissue extracts were subjected to separation using thin layer chromatography (TLC). The constituents with antimicrobial and antioxidative properties were identified using TLC-bioautography by agar-overlay and DPPH spraying methods respectively. A number of bioactive constituents with antimicrobial and radical scavenging properties were observed on the developed bioautography plate. The fractions with antimicrobial properties were isolated from the reference TLC plates and subjected to gas chromatography-mass spectrometry (GC-MS) analysis for partial characterization and identification of the metabolites that might be responsible for the activities. The study suggests *Sonneratia caseolaris* bark as a potential source of bioactive compounds with stable antimicrobial and antioxidative properties and can be used as natural antimicrobial/antioxidative agents in clinical, pharmaceutical and food processing industries.

Keywords: *Sonneratia caseolaris*, phytochemical, antimicrobial, antioxidant, mangrove plants, TLC bioautography

INTRODUCTION

Infectious diseases are one of the major causes of death in developing countries. Microbes with multi drug resistance character are sources of growing concern. Emergence of multi drug resistance results due to indiscriminate uses of antibiotics for controlling such diseases (Westh et al., 2004). Tradition of using plants as means for curing diseases exists in many parts of the globe since time immemorial. Nearly 30 % of the modern drugs in use today are derived from plants

and their extracts. These medicinal plants have major role in homeopathic or ayurvedic medicines (Hassan et al., 2013). A number of plants reported to be rich in phytochemicals possessing strong activity towards human pathogens have been found to be important both clinically and pharmacologically (Ray and Majumdar, 1976; Cox, 1994).

Oxidative stress resulting due to imbalance of oxidizing agents and natural antioxidants in the body induces the severity of a number of diseases like atherosclerosis, cancer, cardiovascular ailments, neurodegenerative disorders and diabetes (Aruoma, 1998). As defensive measure against such oxidative damages, biological systems have evolved an array of enzymatic machineries and scavengers. These include dietary antioxidants (α-tocopherol, β-carotene, ascorbic acid, glutathione, uric acid), hormones (estrogen, angiotensin), enzyme systems (superoxide dismutase, glutathione peroxidase, catalase) etc. (Halliwell and Gutteridge, 1990; Martinez-Cayuela 1995). A number of antioxidative agents, both natural (e.g. α-tocopherol) and synthetic (e.g. butylated hydroxyanisole, butylated hydroxytoluene, *tert*-butyl hydroquinone and propyl gallate) are widely used in the food industry to prolong shelf life as they inhibit lipid oxidation (Tawaha et al., 2007; Bhatt and Negi, 2012). However, the use of these synthetic antioxidants is increasingly getting restricted due to their toxicity and health risks (Tawaha et al., 2007). Therefore, discovery of novel antioxidative and antimicrobial agents of natural origin is the pressing need of the hour and plants can be a good source for the purpose.

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). Various parts of these mangrove plants have been extensively used in folk medicines by traditional healers and local people for a long time (Bandaranayake, 1998). Due to the hostile nature of their habitat involving high heat, humidity, daily tidal cycles, changing salinity etc. the mangrove plants have undergone considerable physiological and morphological adaptations in order to thrive in such hostile environment (Silva et al., 2011) and are known to produce, as defensive stress responses, secondary metabolites of various kinds such as steroids, triterpenoids, saponins, flavonoids, tannins, alkaloids etc.; these secondary metabolites have been shown to be basically responsible for the biological activities such as antioxidant, antibacterial, antilarval, antiviral, antifungal, antiinsectidal etc. along with other beneficial activities found to be associated with these plants (Kokpol et al., 1990; Bandaranayake, 2002; Simlai and Roy, 2013; Gupta et al., 2014).

Sonneratia caseolaris (L.) Engl. (Sonneratiaceae) is such a mangrove plant found in the Indian Sundarban delta. It is an evergreen, medium to tall tree normally attending up to a height of 10 m (Naskar, 2004). Fruits from the species are used traditionally to treat bleeding, hemorrhages, piles, sprain poultices (Bandaranayake, 1998). A number of studies reported on the pharmacologically important biological activities (Avenido and Serrano Jr., 2012; Kaewpiboon et al., 2012) and chemical constituents (Wu et al., 2009; Tiwari et al., 2010; Sadhu et al., 2006) of this species, among which antimicrobial properties (Yompakdee et al., 2012; Shamsuddin et al., 2013) and antioxidative activities (Wetwitayaklung et al., 2013; Howlader et al., 2012) of the species had been described. An in-depth study by Minqing et al. (2009) reported the isolation of twenty four compounds from stem and twigs of *S. caseolaris* of Chinese origin, but none of the compounds exhibited significant antibacterial activities against *Candida albicans* and *Staphylococcus aureus*. Sadhu et al. (2006) reported to have isolated two flavonoids, luteolin and luteolin 7-*O*-*β*-glucoside both of which have been reported to possess antioxidant activities. In the present study, we report on the major phytochemical contents, antimicrobial activities, antioxidant efficacies and other relevant properties etc. found in the bark extracts of a mangrove plant *Sonneratia caseolaris*. We have also identified components in the bark extracts

that appear to be responsible for the antimicrobial activities using TLC linked bioassays and partially characterized those using GC-MS studies in an effort to identify the compounds responsible for the bioactivities.

MATERIALS AND METHODS

Plant materials

Sonneratia caseolaris plant samples were collected from the Indian Sundarban forest, West Bengal. Bark of this species was used for preparation of the solvent extracts.

Preparation of plant extracts

The bark from the plant was washed thoroughly under tap water and then with distilled water and air-dried in shade for several weeks. They were then ground into fine powder using an electric grinder.

For antimicrobial activity assay

The dried powder (40 g) was extracted with hexane, benzene, chloroform, methanol and water by sequential extraction each for three days at room temperature. The extracts obtained were filtered with Whatman No. 1 filter paper and dried using a rotary evaporator. However, the aqueous extract was freeze-dried using a lyophilizer. The dried extracts were stored until use.

For antioxidant activity assay

The dried powder (10 g) was extracted two times with absolute methanol at room temperature. The extract was then filtered using Whatman No. 1 filter paper and dried using a rotary evaporator. The dried extract was stored until use for antioxidant activity determination.

Quantitative phytochemical analysis

The powdered bark of *S. caseolaris* was tested for quantitative estimation of phytochemical contents such as phenolics, flavonoids, tannins, alkaloids and saponins accordingly as described previously (Simlai and Roy, 2012).

Test microorganisms, their growth and maintenance

Four bacterial strains viz., *Bacillus subtilis* (MTCC 121), *Bacillus coagulans*, *Escherichia coli* (MTCC 484), *Proteus vulgaris* (MTCC 426) and one fungal strain i.e. *Saccharomyces cerevisiae* were used as test microorganisms to evaluate the antimicrobial activity. The growth and maintenance of these microbial strains and preparation of the inoculums were performed as described earlier (Simlai and Roy, 2012).

Antimicrobial activity assay

Antimicrobial assay of the extracts was carried out by disc diffusion technique (Bauer et al., 1966) as described previously (Simlai and Roy, 2012). Each of the paper discs was loaded with 6 µL of extract [250 mg/mL conc. in dimethylsulphoxide (DMSO) i.e. 1.5 mg/disc] in the present study. Minimum inhibitory concentration (MIC) of the bark extracts was determined as described previously (Simlai and Roy, 2012) by spotting each disc with 6 µL extract of different concentrations.

Antioxidant activity determination by DPPH assay

The free radical scavenging activity of methanolic extract of *S. caseolaris* bark was performed using a modified version of the method described previously (Simlai et al., 2014). In the present study, the assay procedure was adapted to ‘micro test plate’ based method. Briefly, the serial concentrations (6.25, 12.5, 25, 50 µg/mL) of bark methanolic extracts were made in methanol. 200 µL of each of this sample solution of different concentrations was mixed with 100 µL of 0.5 mM DPPH solution (also in methanol) and left in the dark at room temperature. After 30 min, the optical density was measured at 517 nm using a Molecular Devices SpectraMax M3 plate reader. For obtaining the control value, methanol was used in place of the sample. Quercetin was used as the standard antioxidant. The percentage of scavenging activity was calculated using the formula, DPPH radical scavenging activity (%) =

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Received: June 20, 2014, accepted: July 21, 2014, published: August 29, 2014

[(A_{517} of control – A_{517} of sample) / A_{517} of control] × 100. Lower IC_{50} value indicated higher free radical scavenging activity.

pH and thermal stability of bioactivities

The pH and heat treatments of the bark extracts and evaluation of residual antimicrobial activities of these treated samples were carried out as described earlier (Simlai and Roy, 2012).

To determine the thermal stability of the antioxidant activities observed in the bark extract of the plant, the bark methanolic extract was heated in water-bath at 80 °C and 100 °C for 30 min and the residual antioxidative activity was evaluated as described previously.

Thin layer chromatography (TLC) and TLC-bioautography

TLC of the bark methanolic extract was performed as described previously (Simlai and Roy, 2012) using toluene: ethyl acetate: formic acid (60:40:1) (TEaF) as mobile phase. TLC-bioautography technique was carried out accordingly as described previously (Gupta et al., 2010) for the detection of the antimicrobial constituents present in the extract. Potential antimicrobial fractions identified using bioautography was scraped from reference TLC plates, dissolved in methanol, vortexed and centrifuged at 10,000 rpm for 10 min. The supernatant obtained was collected carefully in a conical flask. The procedure was repeated four times. The collected supernatant was then dried using a rotary evaporator; the residue obtained was weighed and stored for further analysis.

The TLC-bioautography for the purpose of identification of antioxidative constituents in the bark extract was performed as stated earlier (Simlai et al., 2014) using the mobile phase TEaF as before.

GC-MS analysis

The antimicrobial fractions purified from the TLC plates were subjected to GC-MS analysis using a Gas Chromatograph (Model: Trace GC Ultra; Make: Thermo Scientific) coupled with Mass Spectrometer (Model: Polaris Q; Make: Thermo Scientific). The diluted samples (0.6 µL each) were injected into a Thermo TR-1MS column (30 m length × 0.25 mm i.d. × 0.25 µm film thickness). Helium gas (99.999 %) was employed as the carrier gas at a flow rate of 1 mL/min. The GC injector and MS transfer line temperatures were set at 250 and 290 ºC, respectively. The oven temperature was initially 50 ºC for 2 min and gradually increased upto 300 ºC (at 10 ºC/min) with a hold time of 15 min at the final temperature. For MS detection, the ion source temperature was maintained at 230 ºC, electron ionization (EI) was performed with an ionization energy of 70 eV and mass range at m/z 40 – 600.

Statistical analysis

The computation of mean, standard deviation (SD), IC_{50} values and analysis of variance (ANOVA) were done using Microsoft Office Excel 2007. ANOVA was carried out to study the differences at 5 % level of significance among the results of the antimicrobial activities, antioxidant activities and residual bioactivities after the pH and thermal treatments.

RESULTS AND DISCUSSION

Quantitative phytochemical analysis

A wide array of secondary metabolites including phenolics, flavonoids, tannins, alkaloids, saponins etc. are synthesized by the plants which are potentially toxic to herbivores and pathogens, and thus contribute in the plants' defensive mechanism (Wittstock and Gershenzon, 2002). Few phenolic compounds of botanical origin have been reported to destabilize the outer membrane found in the Gram-negative bacteria, releasing lipopolysaccharide and increasing the permeability of the cytoplasmic adenosine triphosphate (Nohynek et al., 2006). Antioxidant properties of the phenolics as reducing agents, hydrogen donors, heavy metal chelators, singlet oxygen and hydroxyl radical quenchers are attributed to their redox potential (Kaur and Kapoor, 2002). The conjugated ring structures and hydroxyl groups of phenolics have

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a significant role in the antioxidative activity as superoxide anion, singlet oxygen and lipid peroxy radical scavenger and thereby stabilizing free radicals involved in oxidative process either by hydrogenation or complexing with oxidizing agents (Liu et al., 2008). Flavonoids comprising maximum polyphenolic compounds including anthocyanins, proanthocyanins, flavonols and catechins exhibit their antioxidant activities by chelating or scavenging (Mervat and Hanan, 2009; Cook and Sammam, 1996). Cushnie and Lamb (2011), in their review, have discussed in detail the antimicrobial efficacy of the flavonoids and their mode of action. The study has delivered a wide perspective regarding the antibacterial activity of flavonoids which proposes multiple mechanism of action. These include interference with various bacterial virulence factors comprising enzymes, toxins and signal receptors apart from the existing reports like β-lactamase inhibition, efflux pump inactivation, cytoplasmic membrane destabilization and topoisomerase inhibition. Tannins are reported to possess reducing power and impart hepato-protective activity by inhibiting lipid peroxides formation (Okuda et al., 1983). Considering a number of previous reports on the antimicrobial efficacy of tannins, Chung et al. (1998) have cited few mechanisms by which tannins impart their toxicity effect on microbial population. These include their action on the microbial membranes, inhibition of electron transport system and deficiency of essential metal ions due to their complex formation with tannins. Berberine, a well-known isoquinoline alkaloid has been reported to inhibit microbial protein biosynthesis by intercalating with DNA, resulting in antimicrobial activity (Cernakova and Kostalova, 2002). Saponins are natural surfactants and impart their toxic effect by causing cell lysis, as a result of their complex formation with the cholesterols present in the protozoal cell membranes (Cheeke, 2000). Due to their presence in extremely stressful and hostile conditions, mangrove plants have evolved to be a rich source of many of such secondary metabolites. For this reason we wanted to investigate the secondary metabolite profile of this plant tissue. The result of phytochemical analysis of bark of *S. caseolaris* is represented in Table 1. The data clearly indicates the bark of the species to be a rich source of flavonoids (90.04 ± 3.57 mg QE/g dry weight). The bark has been found to be a good source of phenolics (50.70 ± 0.74 mg GAE/g dry weight), alkaloids (56.96 ± 2.66 mg/g dry weight), tannin (48.04 ± 0.91 mg TAE/g dry weight) and saponins (8.00 ± 1.41 mg/g dry weight). Presence of these phytochemicals indicated that *S. caseolaris* tissue extracts may have significant biological activities of beneficial nature.

Table 1: Quantitative estimation of phytochemicals from *S. caseolaris* bark extracts

Sl. No.	Phytochemicals	Amount
1.	*Total phenolics*	50.70 ± 0.74 mg GAE/g dry weight
2.	*Total flavonoids*	90.04 ± 3.57 mg QE/g dry weight
3.	*Total tannin*	48.04 ± 0.91 mg TAE/g dry weight
4.	*Total alkaloid*	56.96 ± 2.66 mg/g dry weight
5.	*Total saponin*	08.00 ± 1.41 mg/g dry weight

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

Antimicrobial activities

In view of the reported antimicrobial activities of various phytochemicals in other systems, *in vitro* antimicrobial activities of *S. caseolaris* bark extracts have been studied. For this purpose, the ground bark tissue of the plant has been extracted with hexane, benzene, chloroform, methanol and water and tested against five microbial strains including Gram-positive bacteria (*B. subtilis, B. coagulans*), Gram-negative bacteria (*E. coli, P. vulgaris*) and fungus (*S. cerevisiae*). Different organic solvent extractions have been carried out to facilitate extraction of phytochemicals with unknown properties. The findings are represented in Table 2. All the extracts have shown activity against *B.*

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subtilis, among which the methanolic (18.33 ± 0.76 mm) and the aqueous (15.83 ± 0.29 mm) extracts have been found to be most effective. Against *B. coagulans*, all the extracts except the aqueous one have exhibited activity, of which the methanolic extract is the most potent one (19.50 ± 0.50 mm). In case of *P. vulgaris,* a Gram-negative bacterium, both the methanolic and aqueous extracts have exhibited activity. Therefore, the methanolic extract of bark of *S. caseolaris* has been found to exhibit the highest activity against *B. subtilis* (18.33 ± 0.76 mm), *B. coagulans* (19.50 ± 0.50 mm) and *P. vulgaris* (12.67 ± 0.58 mm)(Figure 1). None of the extracts have shown activity against *E. coli* and *S. cerevisiae* at the concentration used. Significant differences in the antimicrobial activities among *S. caseolaris* bark extracts have been observed when the results were subjected to statistical testing using ANOVA (P = 0.000). Ampicillin, chloramphenicol and fluconazole were used as positive controls while, DMSO, the bark tissue extract solubiliser was used as negative control. The quantitative analysis of certain phytochemicals from the bark of *S. caseolaris* has revealed the species to be a moderately good source of phenolics, flavonoids, tannin, alkaloid and saponin which can be linked to the observed antimicrobial activities of the extracts.

MIC of S. caseolaris extracts

MIC of an antimicrobial agent is defined as the minimum concentration of the product that will still inhibit the growth of test microorganisms. The extracts from the bark of *S. caseolaris* showing maximum activities against the tested microbial strains (Table 2) have been selected further to determine their MIC values. The results obtained for the MIC of the selected extracts of *S. caseolaris* bark are represented in Table 3. The MIC values of the extracts have been found to vary with the strains used. The methanol extract has significant MIC value of 3.90 mg/mL against *B. subtilis* and 7.81 mg/mL against *B. coagulans* whereas in case of *P. vulgaris* it has been found to be 62.5 mg/mL. The aqueous extract has revealed MIC value of 15.62 mg/mL against *B. subtilis,* and 125 mg/mL against *P. vulgaris.* The chloroform extract possesses MIC value of 7.81 mg/mL against *B. coagulans.* So, the

Table 2: Antimicrobial activities of *S. caseolaris* bark extracts by disc diffusion method

Sl. No.	Extract	Inhibition zone diameter (mm)[a]				
		Microorganisms				
		B. subtilis	***B. coagulans***	***E. coli***	***P. vulgaris***	***S. cerevisiae***
1.	*Hexane*	09.33 ± 0.58	09.17 ± 0.29	-	-	-
2.	*Benzene*	08.33 ± 0.58	09.50 ± 0.50	-	-	-
3.	*Chloroform*	07.17 ± 0.29	10.17 ± 0.29	-	-	-
4.	*Methanol*	18.33 ± 0.76	19.50 ± 0.50	-	12.67 ± 0.58	-
5.	*Aqueous*	15.83 ± 0.29	-	-	08.67 ± 0.58	-
	Controls					
6.	*Ampicillin*[b]	20.83 ± 0.76	22.50 ± 0.50	13.00 ± 1.04	-	-
7.	*Chloram*[c]	-	-	-	17.17 ± 1.26	
8.	*Fluconazole*[d]	-	-	-	-	20 ± 1.73
9.	*DMSO*	-	-	-	-	-

Each value is the mean ± standard deviation from three replicates. All the extracts used were 1.5 mg/disc. [a] Inhibition zone diameter including disc diameter of 5.5 mm. [b] Ampicillin used was 3 µL/disc of conc. 125 µg/mL against Gram-positive bacteria and 3 µL/disc of conc. 500 µg/mL against *E. coli*. [c] Chloramphenicol used was 3 µL/disc of conc. 10 mg/mL against *P. vulgaris.* [d] Fluconazole used was 3 µL/disc of conc. 10 mg/mL against *S. cerevisiae*. DMSO served as negative control.

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Received: June 20, 2014, accepted: July 21, 2014, published: August 29, 2014

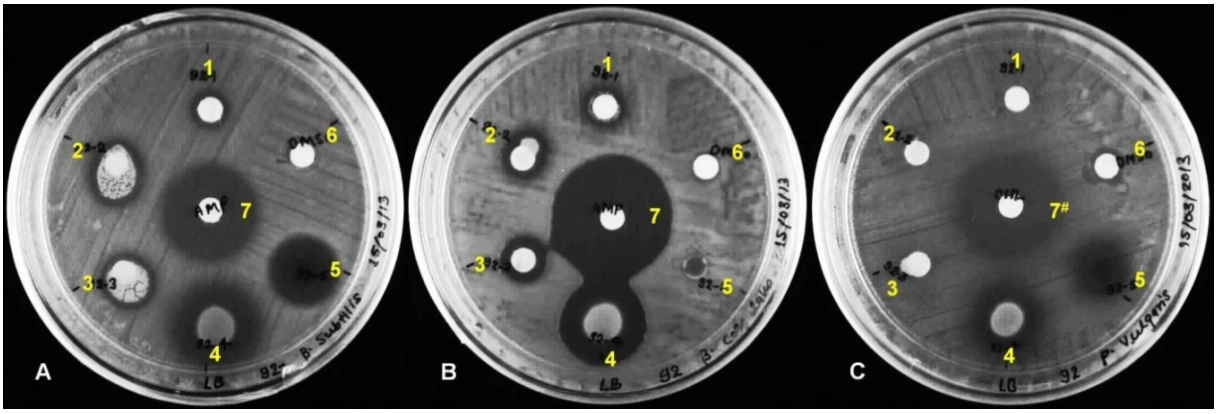

Figure 1: Disc diffusion assay of hexane (1), benzene (2), chloroform (3), methanol (4) and aqueous (5) extracts of *S. caseolaris* bark with *B. subtilis* (A), *B. coagulans* (B) and *P. vulgaris* (C) using DMSO (6) and Ampicillin (7) / Chloramphenicol ($7^{\#}$) as negative and positive control respectively.

result clearly indicates the broad spectrum nature of the methanolic extract of *S. caseolaris* bark showing inhibitory activity at even considerably low concentrations. It also needs to be pointed out that the observed MIC values are against crude bark extracts.

Table 3: MIC values of *S. caseolaris* bark extracts against test microorganisms.

Extracts	MIC values in mg/mL		
	Test Microorganisms		
	B. subtilis	***B. coagulans***	***P. vulgaris***
Chloroform	-	7.81	-
Methanol	3.90	7.81	62.50
Aqueous	15.62	-	125

For the determination of MIC, 6 µL of the extracts of concentration stated above has been used.

DPPH radical scavenging activity

In the biological systems, both endogenous (metabolic byproducts by small cytoplasmic molecules and proteins, peroxisomes, mitochondrial electron transport chain, membrane enzymes and microsomic electron transport systems) and exogenous (ionizing radiation, pesticides, pollutants, tobacco smoke etc.) factors ensure constant supply of free radicals in the system (Martinez-Cayuela, 1995). These free radicals, responsible for lipid peroxidation process, possess a major role behind a number of life threatening chronic disorders (Roy and Urooj, 2013). The antioxidative potential as indicated by free radical scavenging activity of the methanolic extract of *S. caseolaris* bark has been determined by DPPH radical scavenging assay. DPPH is a stable radical which when dissolved in methanol produces violet colour due to the unpaired nitrogen electron at the center and gets reduced by either hydrogen- or electron-donation forming yellow coloured DPPH-H in presence of antioxidant compound (Liu et al., 2008; Edziri et al., 2012). Therefore, the antioxidant compounds are also known to be radical scavengers. As shown in Table 4, the untreated sample of the methanolic extract of *S. caseolaris* bark has exhibited appreciable free radical scavenging activity and the percentage of scavenging activity has been found to be > 90 %. The IC_{50} value of the sample is 21.74 µg/mL whereas for a standard antioxidant compound like Quercetin, the IC_{50} value is 10.14 µg/mL. The antioxidant activity of Quercetin has been found to be better than the methanolic extract of *S. caseolaris* bark. Further isolation and purification of antioxidative compounds from the crude extract of the species might show better activity compared to the crude one.

Effect of pH and thermal treatments on bioactivities

The *S. caseolaris* bark extracts showing maximum antimicrobial activities have been considered further to determine the effect of pH and temperature on the activities of these extracts. The results obtained are represented

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in Table 5. The changes in antibacterial activity imparted by the extracts before and after the pH and thermal treatment has been found to be more or less significant in case of methanolic ($P = 0.035$) and aqueous ($P = 0.006$) against *B. subtilis*; chloroform ($P = 0.000$) against *B. coagulans*; methanolic ($P = 0.028$) and aqueous ($P = 0.012$) against *P. vulgaris*. The minor changes in the antibacterial activity as observed with the methanolic extract against *B. coagulans* has not been found to be significant ($P = 0.166$). It has been observed that despite drastic pH treatment of the selected extracts, they have retained their activities against the respective microbial strains. Therefore, the study suggests the activities of the extracts are tolerant to pH change. The heat treated extracts have also shown quite good retention in their activity except the chloroform extract which has revealed a gradual decrease in its activity with the temperature change against *B. coagulans*. 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).

The methanolic extract of *S. caseolaris* bark, when subjected to heat treatment at 80 °C and 100 °C for 30 min, a very minor deviation with regard to antioxidative activity has been noticed compared to the untreated one (Table 4). This minor difference in the result has not been found to be significant when analyzed using ANOVA ($P = 0.122$). The IC_{50} value of the 80 °C treated sample (21.19 µg/mL) has been found

Table 4: In vitro free radical scavenging activity by DPPH method

Sample	DPPH radical scavenging activity (%)[a]				
	6.25 µg/mL	12.5 µg/mL	25 µg/mL	50 µg/mL	IC_{50} (µg/mL)
Untreated	19.43 ± 5.95	39.06 ± 3.27	74.38 ± 0.95	90.30 ± 0.68	21.74
80 °C	21.59 ± 0.61	41.48 ± 0.21	74.46 ± 0.19	90.78 ± 0.05	21.19
100 °C	20.16 ± 0.64	35.93 ± 0.55	69.47 ± 0.45	90.52 ± 0.21	22.55
	5 µg/mL	10 µg/mL	15 µg/mL	20 µg/mL	
Quercetin	25.17 ± 2.60	50.89 ± 1.37	80.05 ± 1.37	90.56 ± 0.15	10.14

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

Table 5: Effect of pH and thermal treatment of *S. caseolaris* extracts[a] on antimicrobial activities[b] (mm)

Organisms	Tissue extract	Positive Control	pH				Temperature (°C)			
			3.0	6.0	9.0		40	60	80	100
B. subtilis	Methanol	17.25 ± 0.65	16.50 ± 0.00	16.50 ± 0.71	16.25 ± 0.35		18.00 ± 0.00	17.25 ± 0.35	16.75 ± 0.35	16.25 ± 0.35
	Aqueous	14.88 ± 0.85	15.25 ± 0.35	14.50 ± 0.00	15.00 ± 0.71		14.00 ± 0.00	12.75 ± 0.35	13.75 ± 0.35	13.25 ± 0.35
B. coagulans	Chloroform	10.50 ± 1.22	10.75 ± 0.35	10.50 ± 0.00	11.50 ± 0.71		09.75 ± 0.35	07.75 ± 0.35	06.75 ± 0.35	07.50 ± 0.71
	Methanol	19.63 ± 0.25	18.00 ± 0.71	19.25 ± 1.06	19.75 ± 0.35		19.50 ± 0.00	19.25 ± 0.35	19.75 ± 0.35	19.50 ± 0.71
P. vulgaris	Methanol	13.50 ± 1.22	13.63 ± 0.18	13.75 ± 0.35	14.25 ± 0.35		12.25 ± 0.35	12.00 ± 0.71	12.75 ± 0.35	11.75 ± 0.35
	Aqueous	08.81 ± 0.24	07.75 ± 0.35	07.50 ± 0.00	07.88 ± 0.18		08.25 ± 0.35	08.75 ± 0.35	08.25 ± 0.35	08.00 ± 0.00

[a] Extracts exhibiting maximum activity against respective microbial strain have been considered; All the extracts used at 1.5 mg/disc. [b] Inhibition zone diameter including 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.

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to be slightly less whereas for the 100 °C treated sample, IC_{50} value (22.55 µg/ mL) has been found to be little higher than the untreated one. A study by Kaur and Kapoor (2001) suggests that heat treatment can trigger the development of certain compounds with antioxidative potential like Maillard reaction products or enhance the activity of natural antioxidants, augmenting the total antioxidant activity in turn. The study also reveals that, thermal treatment results in augmenting the bioavailability of β-carotene which might be responsible for increasing the antioxidative activity.

Therefore, the *S. caseolaris* bark extracts screened for the pH and heat stability of their antimicrobial/antioxidative activities has been found to be quite stable despite treatment under harsh conditions. The stability of the antimicrobial/antioxidative activities of these extracts suggests the possibility of the use of active principles extracted from bark tissue of *S. caseolaris* as natural preservatives in food processing applications. pH tolerance is one of the major challenges in the drug discovery domain (Di and Kerns, 2009) as 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. Alteration in molecular structure or charge of the compound at certain pH values can lead to loss of activity and can limit oral exposure of the intended drugs.

TLC and bioautography

TLC is a widely used technique for separation of natural substances and possesses applications in analyzing biological and chemical samples for identification and determination of their composition (Haugland and Johnson, 1999). In our study, methanolic extract of bark of *S. caseolaris* has been found to exhibit maximum antibacterial activities by disc diffusion method and therefore, has been considered for further detailed studies. TLC of this methanolic extract has been carried out using toluene: ethyl acetate: formic acid (60:40:1) as mobile phase. The developed chromatograms, when visualized under UV light at 254 nm (Figure 2: Lane A) and 366 nm (Figure 2: Lane B), have exhibited a number of bands or spots at different R_f values representing the separated phytochemicals. The chromatogram under 254 nm has been found to contain bands at R_f 0.13, 0.37, 0.44, 0.58 and under 366 nm at R_f 0.19,

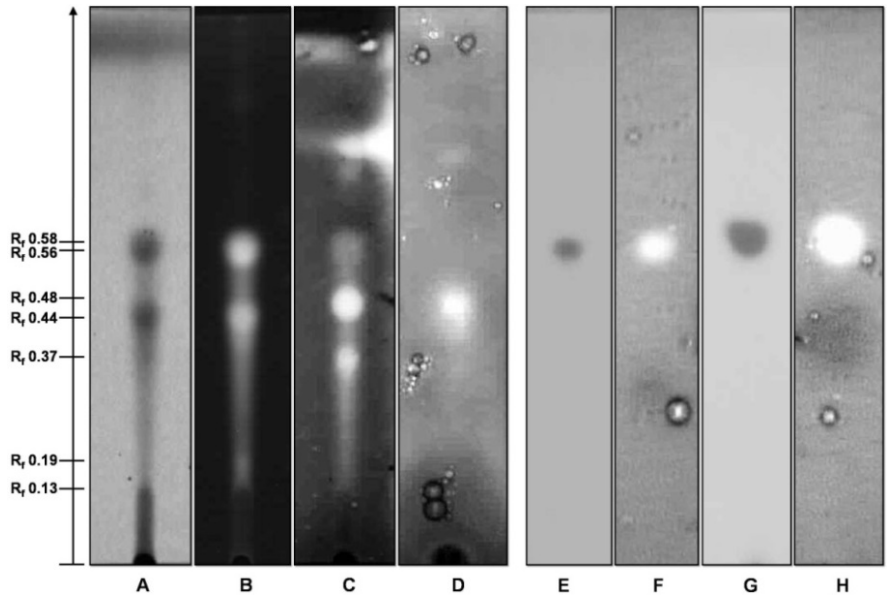

Figure 2: TLC/TLC-bioautography of the methanolic extract (lanes A-D) obtained by sequential extraction process and purified antimicrobial fractions (lanes E-H) of *S. caseolaris* bark. Lanes A, E and G as visualized under short wave UV light (254 nm); lane B as visualized under long wave UV light (366 nm). Lanes C, F and H are bioautography plates with *B. coagulans* and lane D with *B. subtilis*.

EXCLI Journal 2014;13:997-1010 – ISSN 1611-2156
Received: June 20, 2014, accepted: July 21, 2014, published: August 29, 2014

0.44, 0.58. These separated phytochemicals on the TLC plate have been analysed in the next step by using TLC bioautography using *B. coagulans, B. subtilis* and *P. vulgaris* as test microorganisms. TLC-bioautography is a well practiced method used for identification of the active fractions on the TLC plate possessing biological activities (Hostettmann and Wolfender, 1997). Annegowda et al. (2013) described TLC-bioautography as a method not only useful for identification and separation of bioactive substances, but also as an efficient technique in judging the contribution of the phyto-constituents within the extract for the observed pharmacological activity. In our data (Figure 2) clear zones of inhibitions obtained on the TLC plates implied the presence of active antimicrobial substances at these locations inhibiting the growth of *B. coagulans* (Figure 2: Lane C: R_f 0.37, 0.48 and 0.58) and *B. subtilis* (Figure 2: Lane D: R_f 0.48). No activity except a weak inhibition zone at the point of application on the TLC plate has been detected against *P. vulgaris* (data not shown). The bioactive fractions as observed above at R_f 0.48 and R_f 0.58 (Figure 2: Lane C) in case of *B. coagulans* have been scraped off of a reference TLC plate and subjected to further tests by TLC and TLC-bioautography (Figure 2: Lanes E, F, G and H) with regard to their antibacterial activities. The results have indicated that both the bands retained their activities against *B. coagulans* (Figure 2: Lanes F and H). It is to be noted that the R_f 0.48 component from the crude bark extract ran with a higher R_f value of 0.56 when separated from the primary TLC plate. The reason for this mobility shift is unclear. The R_f 0.48 component active against *B. subtilis* has not been studied further.

The antioxidative potential of the methanolic extract, obtained directly from the powdered *S. caseolaris* bark, has been studied by TLC using toluene: ethyl acetate: formic acid (60:40:1) as mobile phase. The chromatogram under 254 nm (Figure 3: Lane A) has been found to contain spots at R_f 0.2, 0.43, 0.53, 0.65 and under 366 nm (Figure 3: Lane B) at R_f 0.53, 0.65. When sprayed with 0.02 % (w/v) DPPH solution in methanol to identify the bioactive fractions having radical scavenging properties, the chromatogram exhibited convincing radical scavenging activities at R_f 0.53 and as a smear from just above the point of application to R_f 0.2.

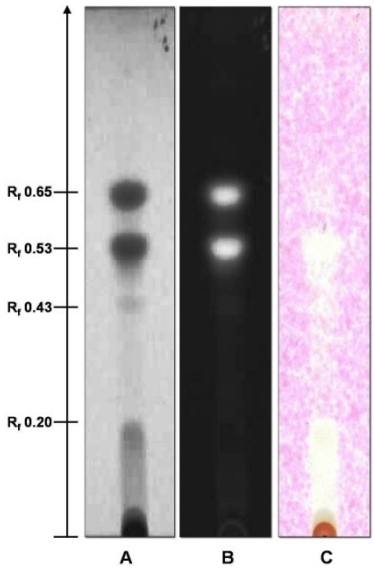

Figure 3: TLC/TLC-bioautography of the methanolic extract (lanes A-C) for antioxidative activity of *S. caseolaris* bark. Lane A and B as visualized under short (254 nm) and long (366 nm) wave UV light respectively. Lane C is the bioautography plate after DPPH spray.

GC-MS studies

The R_f 0.48 and 0.58 bands possessing antimicrobial activities against *B. coagulans* (Figure 2: Lane C) have been subjected to GC-MS analysis. Hexane and ethyl acetate have been used to prepare the R_f 0.48 and R_f 0.58 components. From the overall nature of the GC-MS spectrum (Figure 4), it is evident that a number of phytochemicals may be present in both the components.

In the hexane fraction of R_f 0.48 (Figure 4A) the components present may be β-sitosterol, cholest-5ene-diol and pentacyclic triterpene like oleanolic or ursolic acid and betulin or lupeol. Spectrum analysis of R_f 0.48 in ethyl acetate (Figure 4B) has revealed the possible presence of glycoside of kaempferol, pentacyclic triterpene like oleanolic or ursolic acid and betulin or lupeol,

EXCLI Journal 2014;13:997-1010 – ISSN 1611-2156
Received: June 20, 2014, accepted: July 21, 2014, published: August 29, 2014

ellagic acid derivative. In the case of ethyl acetate fraction of R_f 0.58 (Figure 4C), only a few peaks have been found to be present indicating the presence of triterpene constituents in the fraction. Interestingly, the compounds present, as indicated by the GC-MS spectrum analysis in the current study, have been found to be in line with the earlier study by Minqing et al. (2009). Except cholest-5ene-diol, all of the aforementioned compounds, from other plant origin, have been reported to possess antimicrobial activities (Kiprono et al., 2000; Wolska et al., 2010; Prachayasittikul et al., 2010; Gallo and Sarachine, 2009; Mary and Merina, 2014; Rahman et al., 2001).

In the present studies, the bark of *Sonneratia caseolaris* has been found to contain fair amounts of phenolics, flavonoids, tannin, saponin and alkaloids. These phytochemicals are known to be responsible for various kinds of bioactivities known to be present in some plant species, some examples of which have been discussed earlier. The quantification of these phytochemicals gives an indication on the bioactive potential of the plant tissue. In contrast to the previous report of Minqing et al. (2009) on the antimicrobial activities of *S. caseolaris* tissue extracts on *S. aureus*, a Gram-positive bacterium and our preliminary tests on the bark extracts of *S. caseolaris* have exhibited strong antimicro-

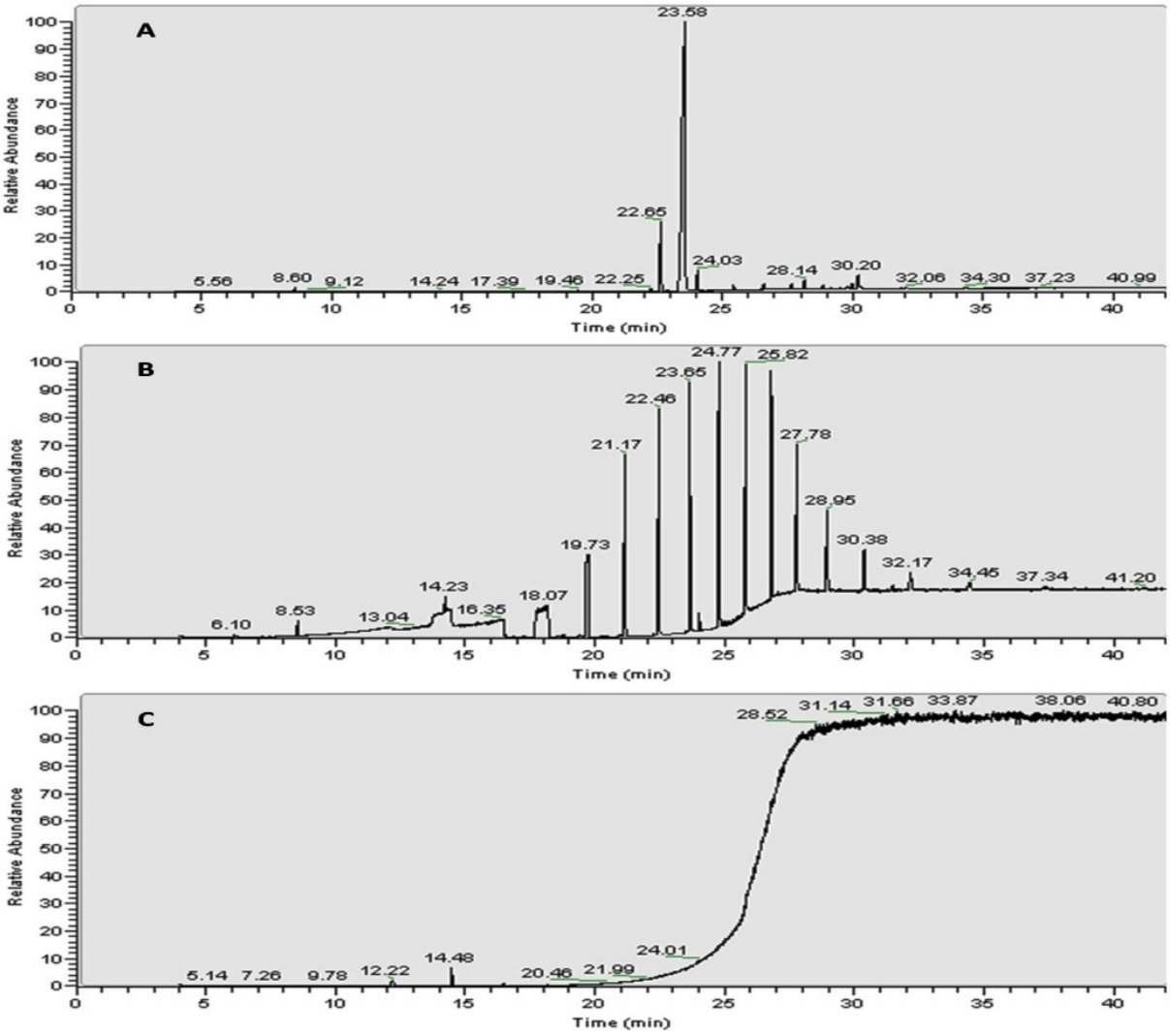

Figure 4: GC-MS analysis of **A.** Hexane fraction of R_f 0.48, **B.** Ethyl acetate fraction of R_f 0.48, **C.** Ethyl acetate fraction of R_f 0.58 from *S. caseolaris* bark.

bial activities against other test organisms of both Gram-positive and Gram-negative types. This prompted us to carry out more detailed biological profiling of the bark extract of this plant with respect to its antimicrobial properties. The methanolic extract of bark of *S. caseolaris* has been found to possess the highest activity against *B. subtilis*, *B. coagulans* and *P. vulgaris*. Though, the microbial strains used in the current study are non-pathogenic in nature, they have been used as representative organisms and the extracts are expected to retain their activity against some other harmful microbes, too. The methanolic extract has also been found to have good antioxidative property as indicated by radical scavenging activity. The broad spectrum activity of the methanolic extract against both Gram-positive and Gram-negative bacteria, stability of its antimicrobial and antioxidative properties despite drastic treatments widens up the possibility of exploitation of the species in both pharmaceutical and food processing industries. The GC-MS studies of the two antimicrobial fractions isolated from the methanolic extract have indicated possible presence of the compounds β-sitosterol, cholest-5ene-diol, glycoside of kaempferol, ellagic acid derivative, pentacyclic and other triterpenes. All of these compounds except cholest-5ene-diol have been reported to exhibit activities against various microorganisms in other systems. Minqing et al. (2009) have studied these individual components and came to the conclusion that "none of these compounds showed strong antibacterial activities against tested bacterial strains….". On the other hand the strong activities that we have observed in the bark extracts of this plant may actually be due to the synergistic effects of several phytochemicals, as detected by GC-MS studies.

Acknowledgements

This work has been supported in part by a grant from UGC [F. No. 40-176/2011 (SR)] to AR and other departmental funds from UGC and DBT. Authors wish to profusely thank the Director, Bose Institute, India and Prof. Parimal Chandra Sen for the use of institutional GC-MS facilities and Smriti Ranjan Maji for assistance with the GC-MS experiments.

Conflict of interest

The authors declare that there are no conflicts of interest.

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