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Phytochemical contents, antimicrobial and antioxidative activities of *Solanum sisymbriifolium*

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ABSTRACT

The present study was aimed at screening the phytochemical contents, antimicrobial and antioxidative activities from the aerial parts of *Solanum sisymbriifolium*. *S. sisymbriifolium* is known to be traditionally used in various kinds of ailments including pain and inflammation, some of which may be due to infection by various microbes found in our environment. Phytochemical analyses revealed the presence of phenolics, flavonoids, tannins, alkaloids and saponins in this plant. Screening for the antimicrobial activity of tissue extracts by disc diffusion assay against test microorganisms revealed the presence of antimicrobial activity, maximum activity being found in the methanolic extracts. The activity was stable against drastic thermal and pH treatments. The antioxidative activity of its aerial tissue extracts was determined using DPPH-radical scavenging method. Antioxidant activities have been implicated as agents for fighting many other diseases such as cancer that result from oxidative damages arising out of synthesis of compounds via natural metabolic routes. The study suggests the use of *Solanum sisymbriifolium* species for further isolation and characterization of its bioactive compounds leading to the development of new antimicrobial drugs.

INTRODUCTION

The use of herbs and medicinal plants as relief from various ailments is a universal phenomenon. Living organisms on earth have relied on the vast variety of natural chemistry found in plants for their therapeutic properties (Serrentino, 1991). A large number of therapeutic agents in use today have been isolated or derived from plant sources (Cowan, 1999). The emergence of multidrug resistant microbial strains due to the indiscriminate use of antibiotics has posed a serious increasing threat to the world population. This has necessitated the need for novel antimicrobials which are not based on the existing antibiotics for eliminating multidrug resistant pathogens and plants have proven to be a potential source for the purpose (Chopra *et al.*, 1997; Shah, 2005). In today's world, growing number of reports are available on diseases like cancer, liver cirrhosis, atherosclerosis, cardiovascular diseases, cataracts etc. in which free radical pathogenicity leading to "oxidative stress" pose a major role (Aruoma, 1998).

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Biological systems protect themselves against this free radical or oxidative damage by enzymes (superoxide dismutase, glutathione peroxidase and catalase) and compounds (α-tocopherol, β-carotene, ascorbic acid, glutathione etc.) (Niki *et al.*, 1994). Deficiency or disruption of these protective mechanisms due to clinical reasons, require antioxidant supplements to combat oxidative damage (Hazra *et al.*, 2008). Plant constitutes a diversified group of phytochemicals and compounds, many of which possess antimicrobial and/or antioxidant activity. Moreover there is a growing concern in using synthetic antioxidants and food preservatives such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propylgallate (PG) etc. because of their potential health hazards and toxicity (Tawaha *et al.*, 2007). So plants can be a potent source for isolation of natural antimicrobials and antioxidative agents, thus helping in the process of newer drug discovery, food preservatives and natural supplements (Cos *et al.*, 2006; Lee *et al.*, 2003). *Solanum sisymbriifolium* (Solanaceae) is a densely prickly perennial shrub 0.8-1.5 m in height. Ethno-medicinally the plant has been reported to be used to treat pain, inflammation (Uddin *et al.*, 2008) and CNS disorder (Chauhan *et al.*, 2011). A number of compounds from the plant have already been reported to possess hypotensive activity (Ibarrola *et al.*, 1996;

Ibarrola *et al.,* 2000), cardiovascular effect (Ibarrola *et al.,* 2006) and anticonvulsant activity (Chauhan *et al.,* 2011). The plant has also been reported to possess molluscicidal activity (Silva *et al.,* 2005). Schmeda-Hirschmann *et al.* (2005) reported the free radical scavenging activity of the fruits from the species. The present study is aimed at determining the phytochemical contents, antimicrobial activity and antioxidant activity from the aerial parts of *S. sisymbriifolium*. It is important to note that while earlier studies on this species from Argentina and Bangladesh failed to demonstrate the presence of any antimicrobial activities from this plant (Anesini and Perez, 1993; Uddin *et al.,* 2008), our findings suggest presence of antimicrobial and antioxidant activities in the aerial parts of this plant. A very recent study by Maheshwari *et al.* (2013) has shown the antibacterial activity of the species at a preliminary level. Our study on this promising activity extends the knowledge further.

MATERIALS AND METHODS

Plant materials and extraction processes

Aerial parts of *S. sisymbriifolium* were collected randomly from Santiniketan, West Bengal. The sample was identified and the dried specimen of this plant (Voucher no. VB/CBT/AR/007) was deposited in the Herbarium of Botany department, Visva-Bharati University, India.

The plant materials 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 and the powdered tissues were extracted three times with each of the selected organic solvents – hexane, benzene, chloroform and methanol at room temperature. An aqueous extract of the aerial parts of the plant was 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 was evaporated at room temperature. The aqueous extract was lyophilized using a lyophilizer. The dried crude extracts were stored at -20 °C until use.

Quantitative phytochemical analysis of *S. sisymbriifolium*

The powdered tissues of aerial part of *S. sisymbriifolium* 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 aluminium 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.

Antimicrobial activity assays

Microorganisms used

A total of four microorganisms including two Gram-positive, one Gram-negative bacteria and one fungus were used for this study: *Bacillus subtilis* (MTCC 121), *Bacillus coagulans*, *Escherichia coli* (MTCC 484) 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 were stored at 4 °C.

Inoculum preparation and antimicrobial assays

The inoculums of the bacteria and fungus (10^8 cfu/ml and 10^6 cfu/ml respectively) were prepared and the antimicrobial activities of the dry extracts dissolved in dimethylsulphoxide (DMSO) at 250 mg/ml were tested against the microorganisms as described previously (Gupta *et al.,* 2010). As positive controls, 3 µl of antibiotics such as ampicillin (at a concentration of 125 and 500 µg/ml) and fluconazole (at a concentration of 10 mg/ml) were used. DMSO was used as a negative control.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The MIC of the plant extracts were determined on LB-agar medium following the method described by Rios *et al.* (1988) and Collins *et al.* (1995). Paper discs of 6.0 mm diameter were placed on the agar plates spread with *B. subtilis* suspension and spotted with 3 µl of the methanolic extract in decreasing order of concentration (250, 125, 62.5, 31.3, 15.6, 7.8, 3.9, 1.95, 0.98, 0.49 mg/ml). The plates were then incubated at 30 °C for 24 h. The MIC value was considered as the least concentration of plant extract that completely inhibited growth of the test organisms. The method of Greenwood (1989) was used to determine the MBC of the extracts. Crude extracts dissolved in LB-broth at final concentrations of 12.5, 6.25, 2.5 and 1.25 mg/ml were prepared in test tubes. Then overnight grown test organism (10 µl) was pipetted into these test tubes containing various concentrations of the crude extracts as mentioned above and incubated at 30 °C for 24 h at 180 rpm. At the end of this incubation period, the contents of the tubes were plated on LB-agar plates and grown for 24 h at 30 ^{o}C to determine the bactericidal activities in extracts. The concentration of crude extract in which no microbial growth occurred (as evidenced from absence of subsequent microbial growth in the LB-agar plates) was recorded as the MBC of that crude extract.

Thermal and pH stability of extracts

Heat treatment

The methanolic extract was dissolved in DMSO at a concentration of 250 mg/ml and incubated in a water-bath for 30 min at 40 ℃, 60 ℃, 80 ℃, 100 ℃ and autoclaved at 121 ℃. It was then cooled to room temperature and stored at -20 ℃ until use. The residual antibacterial was determined against the target organism with the help of disc diffusion method described above. Untreated extracts were used as controls.

pH treatment

Aliquots of DMSO were adjusted to pH 3.0, 6.0 and 9.0 with 1 N HCl and 1 N NaOH as appropriate and autoclaved. The methanolic extract was then dissolved in pH-adjusted DMSO at a concentration of 250 mg/ml and stored at -20 ℃ until use. The residual antibacterial activity was determined against the target organism with the help of disc diffusion method described above. pH-adjusted DMSO was used as acid or alkali control solution to determine whether the changes in inhibition, if any, were because of acidic or alkaline pH or because of extracts. pH-untreated extracts were used as controls.

Antioxidant activity by DPPH assay

The free radical scavenging activity of methanolic extract of *S. sisymbriifolium* aerial part was determined according to Liu *et al.* (2008). Briefly, the sample stock solutions (1 mg/ml) were diluted with methanol to final concentrations of 300, 200, 100, 80, 60 and 40 µg/ml. 2 ml of each of this sample solution at different concentrations was mixed with 1ml of 0.1 mM DPPH solution in methanol and allowed to react at room temperature in dark. After 30 min, the absorbance was measured at 517 nm. 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 (%) = [(A_{517} of control – A_{517} of sample) / A_{517} of control] × 100. All the analysis were done in triplicate and averaged. Lower IC_{50} value indicated higher free radical scavenging activity.

Statistical analysis

All the analysis were carried out in triplicate and expressed as mean ± SD. The IC_{50} value calculation and all other statistical analysis were carried out using Microsoft Office Excel 2007.

RESULTS AND DISCUSSION

Quantitative phytochemical analysis

Plants live under various kinds of hostile biotic and abiotic stresses. To thrive in such hostile conditions they often synthesize various kinds of secondary metabolites that act as countermeasures to such situations (Edreva *et al.,* 2008; Shulaev *et al.,* 2008; Reyes and Zevallos, 2003). Many of these secondary metabolites possess significant biological and other medicinal properties which play an invaluable role in the drug discovery process (Cowan, 1999; Tsuchiya *et al.,* 1996; Merschjohann *et al.,* 2001; Zhu *et al.,* 1998; Cheeke, 2000). The different phytochemicals of this plant has been investigated quantitatively to study the secondary metabolite profile of this plant (Table 1). Alkaloids have been found to be present in significant amount (67.77 ± 1.24 mg/g) in the aerial parts of *S. sisymbriifolium*. The presence of phenolics (07.13 ± 0.64 mg GAE/g) and flavonoids (30.00 ± 0.50 mg QE/g) have been found to be in moderate quantity along with the tannin content (23.83 ± 0.28 mg TAE/g). Saponins (2.50 ± 0.70 mg/g) have also been found to be present in the plant.

Table. 1: Quantitative estimation of phytochemicals from aerial parts of *S. sisymbriifolium.*

Sl. No.	Phytochemicals	Amount
1.	Total phenolics	07.13 ± 0.64 mg GAE/g dry weight
2.	Total flavonoids	30.00 ± 0.50 mg QE/g dry weight
3.	Total tannin	23.83 ± 0.28 mg TAE/g dry weight
4.	Total alkaloid	67.77 ± 1.24 mg/g dry weight
5.	Total saponin	02.50 ± 0.70 mg/g dry weight

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

Antimicrobial activities and MIC/MBC studies

The screening for antibacterial and antifungal activities have been performed using hexane, benzene, chloroform, methanol and aqueous extracts of the aerial part of *S. sisymbriifolium* against four microbial strains including Gram-positive bacteria (*Bacillus subtilis*, *Bacillus coagulans*), Gram-negative bacteria (*Escherichia coli*) and fungus (*Saccharomyces cerevisiae*). The screening result is represented in Table 2. All of the extracts except the aqueous one have exhibited mild to moderate activity against *B. subtilis*, of which, the methanolic extract (9.16 ± 0.28 mm) is the most potent one. But in case of *B. coagulans* only benzene (6.16 ± 0.14 mm) and methanolic (6.15 ± 0.13 mm) extracts have demonstrated a mild activity. None of the extracts at the concentration used has been found to exhibit any activity against *E. coli* and *S. cerevisiae*. DMSO has served as the negative control for all the antimicrobial assays conducted in this study and has shown no inhibitory activity against the strains tested, thus validating the antibacterial property of the extracts of *S. sisymbriifolium*. The antibiotics ampicillin (125 µg/ml and 500 µg/ml) and fluconazole (10 mg/ml) have served as positive controls in these assays.

The screening of antimicrobial activity using different organic solvents extracts of *S. sisymbriifolium* aerial part has revealed that the methanolic extract is exhibiting the maximum antibacterial activity against *B. subtilis* showing an inhibition zone of 9.16 ± 0.28 mm. The MIC and MBC values of the extract against *B. subtilis* are demonstrated in Table 3. The extract has exhibited an MIC value of 1.95 mg/ml i.e. 5.85 µg/disc when used against *B. subtilis*. This shows the potent inhibitory activity of the methanolic extract of the plant part against *B. subtilis* despite its use in such a minute amount. The MBC value of the extract has been found to be 6.25 mg/ml of the culture. The presence of phenolics, flavonoids, tannins, alkaloids and saponins in various quantities, as revealed in our experiments described above, may be

linked with the observed antimicrobial activity of the extracts in *S. sisymbriifolium*. It has already been reported earlier about the difference in sensitivity to antibiotics, external agents and detergents between Gram-positive and Gram-negative bacteria 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.

Effect of thermal and pH treatment on the antibacterial activity

The methanolic extract of *S. sisymbriifolium* aerial part when subjected to thermal treatment (40 °C, 60 °C, 80 °C, 100 °C and autoclaved), no significant loss in the activity has been observed demonstrating the stability of the antimicrobial compound(s) in the extract responsible for the activity (Table 4). A very slight drop in the activity has been noticed when the extract is been treated at 100 °C and 121 °C (autoclaved). Also no significant alteration in the activity has been noticed when the extract is been treated at different pH (3.0, 6.0 and 9.0) (Table 4). pH adjusted DMSO solutions (pH 3.0, 6.0 and 9.0) and normal DMSO has shown no inhibitory activity against the strains tested (data not shown).

The methanolic extract of aerial part of *S. sisymbriifolium* has been found to be stable at drastic conditions and exhibited its activity despite the tough physico-chemical treatments. Therefore the plant part proves to be a valuable source of natural preservatives to be used in the food processing applications. pH tolerance is one of the main challenges in the field of drug discovery (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. Loss of activity at certain pH values can limit oral exposure of the compound. The loss of activity may be due to change in molecular structure or charge of the compound responsible for the activity.

DPPH radical scavenging activity

DPPH is nitrogen centered free radical, which is stable at room temperature. When dissolved in methanol it produces violet color, which changes to yellow upon reduction in the presence of antioxidants or free radical scavengers (Liu *et al.*, 2008). DPPH exhibits a strong absorption at 517 nm. Easy and rapid determination of the antioxidative potential can be performed using DPPH.

The percentage of DPPH radical scavenging activity at various concentrations of methanolic extract of *S. sisymbriifolium* aerial parts along with the Quercetin as standard reference has been represented in Table 5. Lower IC_{50} value corresponds to higher antioxidant activity. Thus the methanolic extracts of *S. sisymbriifolium* aerial parts demonstrated a low DPPH radical scavenging activity (IC_{50}= 211.4 µg/ml) compared to the standard antioxidant Quercetin (IC_{50}= 1.85 µg/ml).

The result obtained in the present study reveals that the aerial parts of *S. sisymbriifolium* possess a moderate quantity of phytoconstituents which may be linked with the antibacterial and antioxidant activities exhibited by this plants tissue extracts. The methanolic extract has retained its antimicrobial activity despite extreme pH and thermal treatment. The methanolic extract is also found to possess moderate scavenging activity against DPPH radical. Therefore, in view of the thermo-stable and pH stable antibacterial activity, further characterization of these bioactive compounds from *S. sisymbriifolium* should be undertaken, which may be a potential source of antimicrobial compound(s) for use as natural preservatives in food processing industries and in the newer drug discovery process. We have mentioned earlier that previous Argentine and Bangladeshi efforts to identify antimicrobial activities in this species have not yielded any detectable activities (Anesini and Perez, 1993; Uddin *et al.*, 2008). On the other hand our studies and the study by Maheshwari *et al.* (2013) have detected antimicrobial activities in this species. This may be due to differential gene expression of this plant under changed environmental conditions in which they grew and were collected for experiments under different circumstances. This species should be investigated further with regard to its antimicrobial properties.

Table. 2: Antimicrobial activities of *S. sisymbriifolium* aerial parts extracts by disc diffusion method.

Sl. No.	Extract	Inhibition zone diameter (mm)[a]			
		Microorganisms			
		B. subtilis	***B. coagulans***	***E. coli***	***S. cerevisiae***
1.	Hexane	6.15 ± 0.12	-	-	-
2.	Benzene	6.16 ± 0.05	6.16 ± 0.14	-	-
3.	Chloroform	6.16 ± 0.02	-	-	-
4.	Methanol	9.16 ± 0.28	6.15 ± 0.13	-	-
5.	Aqueous	-	-	-	-
	Controls				
6.	Ampicillin[b]	13.33 ± 0.28	22.5 ± 0.50	10.33 ± 0.57	-
7.	Fluconazole[c]	-	-	-	15.16 ± 0.28
8.	DMSO	-	-	-	-

Each value is the mean ± standard deviation from three replicates. All the extracts used were 750 µg/disc. [a] Inhibition zone diameter including disc diameter of 6.0 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] Fluconazole used was 3 µL/disc of conc. 10 mg/mL against *S. cerevisiae*.

Table. 3: MIC and MBC value of methanolic extract of *S. sisymbriifolium* aerial parts against *B. subtilis.*

Extract	Test organism: *B. subtilis*	
	MIC (mg/mL)	MBC (mg/mL)
Methanol	1.95[a]	6.25

[a] 3 µL of the methanolic extract of conc. 1.95 mg/mL dissolved in DMSO i.e. 5.85 µg of the extract on the disc.

Table. 4: Effect of thermal and pH treatment of methanolic extract[a] of *S. sisymbriifolium* aerial parts on inhibitory zone[b] (mm) against *B. subtilis.*

Temperature	Zone of inhibition (mm) *B. subtilis*	pH	Zone of inhibition (mm) *B. subtilis*
Untreated	9.12 ± 0.53	Untreated	9.12 ± 0.53
40 °C	9.00 ± 0.00	3.0	9.08 ± 0.14
60 °C	9.00 ± 0.00	6.0	9.12 ± 0.12
80 °C	9.10 ± 0.08	9.0	8.00 ± 0.25
100 °C	8.50 ± 0.25		
Autoclaved	8.25 ± 0.00		

[a] Extract used was 750 µg/disc. [b] Inhibition zone diameter including disc diameter of 6.0 mm and is the mean ± standard deviation from three replicates.

Table. 5: In vitro free radical scavenging activity of *S. sisymbriifolium* aerial parts by DPPH method.

	DPPH radical scavenging activity (%)[a]						
Conc.	40 µg/mL	60 µg/mL	80 µg/mL	100 µg/mL	200 µg/mL	300 µg/mL	IC_{50} (µg/mL)
Methanol extract	12.42±0.78	16.88±0.21	22.59±0.60	26.60±0.90	42.18±0.72	55.28±0.78	211.4
Conc.	0.5 µg/mL	1 µg/mL	2 µg/mL	3 µg/mL	4 µg/mL		
Quercetin	11.84±0.94	24.82±0.26	56.61±0.64	81.19±0.75	85.16±0.25		1.85

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

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