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Phytochemical screening, antimicrobial and cytotoxic activity of different fractions of *Sesbania sesban* bark

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Abstract – In Bangladesh, the tree *Sesbania sesban* (L) Merr. is used as the traditional medicine for the treatment of a number of ailments. The present study has been undertaken for antimicrobial activity of the ethanol, ether (diethyl ether) and chloroform extracts of *S. sesban* bark. Antimicrobial activity has been investigated against five Gram-positive bacteria, nine Gram-negative bacteria and seven fungi by disc diffusion and broth macro-dilution assay. The zone of inhibition has been observed with almost all bacteria and fungi with some exceptions. Minimum inhibitory concentrations (MIC) of these extracts were found to be significant. In brine shrimp lethality bioassay test, the LC_{50} values of ethanol, ether and chloroform extracts of bark of *S. sesban* were found to be 1280, 640 and 320 µg/ml, respectively. Findings of the study justify the use of the plant in traditional medicine and suggests for further investigation.

Keywords – *Sesbania sesban*, Phytochemical screening, Antimicrobial activity, MIC, Brine shrimp lethality

1. Introduction

Sesbania sesban (Family- Papilionaceae) is a small perennial tree with woody stems, yellow flowers which is commonly known as jayant (Bengali); sesbania (English); jainti, jait, rawasan (Hindi); champai, chithagathi, karunchembai (Tamil); sesaban (Arabic). It is found widely in tropical Asia especially in the tropical region of India and throughout in Bangladesh [1]. *S. sesban* has been found to contain triterpenoids, carbohydrates, vitamins, amino acids, proteins, tannins, saponins glycosides, sterol, kampferol, fat and steroids [2]. Flowers contain cyanidin and delphinidin glucosides. Pollen and pollen tubes contain alpha-ketoglutaric, oxaloacetic and pyruvic acids. Reports suggest that, previous phytochemical investigations of the plant led to the isolation of oleanolic acid, stigmasta-5, 24(28)-diene-3-ol-3-0-β-D-galactopyranoside, fatty acids and amino acids. Various types of lignins composed of guaiacyl, syringyl and P-hydroxyphenylpropane building units and also antitumor principal kaempferol disaccharide [3-4]. Leaves are useful in diabetes, colic, skin diseases [5] inflammatory rheumatic swelling [6-7] and found to have clinical application in Vicharchika (a skin disease like Eczema) [8]. Seeds are stimulant, emmenagogue, astringent and also used in diarrhea [5] excessive menstrual flow, to reduce enlargement of spleen and in skin disease [6-7]. Barks are useful in ulcers, leucorrhoea, vitiated conditions of pitta, anemia, bronchitis, tumors, dysentery, inflammations, cirrhosis of the liver and hypertension [9].

Since no literature is currently available to substantiate antimicrobial activities from 95% ethanol, ether and chloroform extract of *S. sesban* bark, therefore the present study is a part of our on-going antimicrobial and chemical screening of selected *S. sesban* bark and designed to provide scientific evidence for its use as a traditional folk remedy by investigating the antimicrobial activities.

2. Materials and Methods

2.1. Plant material

The plant *S. sesban* was collected from the road side area of Mothbaria Thana, Pirojpur, Bangladesh. The plant was collected at the 10 February, 2010 on the day time. The plant was identified by the experts of Bangladesh National Herbarium, Mirpur, Dhaka (Accession no.: DACB - 35001) and a voucher specimen was also deposited there. The necessary plant parts were carefully cleaned and separated from other parts of the plant as well as from undesirable materials. After cutting into small pieces, these were dried under shade with ample aeration. After complete drying, the plant material was grinded into a coarse powder with the help of a suitable grinder (Capacitor start motor, Wuhu motor factory, China). The powdered plant material was weighed using an electric balance, kept in a suitable airtight container and then stored in a dark, cool and dry place for further use.

2.2. Extraction

The powdered plant material (crushed barks) was macerated in 95% ethanol, hexane and chloroform respectively for three days with occasional shaking. It was then filtered through a piece of clean, white cloth and then through a cotton plug to remove the plant debris. The filtrate was evaporated using a rotary vacuum evaporator at a temperature of 50 $^{\circ}$C to yield the crude extract.

2.3. Phytochemical screening

The freshly prepared crude extract was qualitatively tested for the presence of chemical constituents, by using the following reagents and chemicals, for example, alkaloids were identified by the Dragendorff's reagent and Mayer's reagent, flavonoids with the use of Mg and HCl, tannins with ferric chloride and potassium dichromate solutions, steroids with Libermann-Burchard reagent and sulphuric acid, gums with molish reagent and sulphuric acid and Reducing sugars with Fehling's solutions A and B and Benedict's reagent [10-12].

2.4. Test microorganism

Five Gram-positive bacteria, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Bacillus subtilis*, nine Gram-negative bacteria, *Shigella boydii*, *Shigella flexneri*, *Shigella sonnei*, *Shigella dysenteriae*, *Escherichia coli*, *Proteus vulgaris*, *Erwinia amylovora*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, and seven fungi, *Trichophyton rubrum*, *Microsporum fulvum*, *Candida albicans*, *Curvularia lunata*, *Aspergillus fumigatus*, *Fusarium oxysporum* and *Sacharomyces cerevacae* were taken for the test. The bacterial strains used for this investigation were obtained from the bacterial stocks preserved in animal cell culture laboratory of Bangladesh Council of Scientific and Industrial Research, Dr. Qudrat-E-Khuda Road, Dhaka-1205, Bangladesh.

2.5. Antimicrobial assay

The antimicrobial activity was investigated using two methods: disc diffusion and broth macro-dilution assay [13-15]. Reference microorganisms from the stock were streaked onto nutrient agar plates and the inoculated plates were incubated overnight at 37°C. Using a sterile loop, small portion of the subculture was transferred into test tube containing nutrient broth and incubated (2-4 h) at 37°C until the growth reached log phase. Nutrient agar media seeded with standard inoculums suspension was poured in Petri-dishes and allowed to solidify. Discs (BBL, Cocksville, USA) impregnated with ethanol, ether and chloroform extract (250µg/disc), ethanol, ether and chloroform extract (500µg/disc), standard antibiotic disc (Kanamycin 30 µg/disc, Oxoid Ltd, UK) and blank (solvent ethanol, ether and chloroform) discs were placed on the Petri-dishes with sterile forceps and gently pressed to ensure contact with the inoculated agar surface. Finally the inoculated plates were incubated at 37° C for 18 h and the zone of inhibition was measured in millimeters.

The broth macro-dilution assay was carried out to determine the minimum inhibitory concentration (MIC). Stock suspension of the extract was prepared in nutrient broth with tween-80 concentration not exceeding 5%. Serial dilution of the stock was carried out to obtain six different concentrations (8, 4, 2, 1, 0.5 and 0.25 mg/ml) in six vials containing 1 ml each. The same procedure was followed for the standard antibiotic solution of ceftriaxone to obtain six different concentrations (8, 4, 2, 1, 0.5 and 0.25 µg/ml) in six vials containing 1 mL each. Then 1 ml of freshly grown inoculum was added to each vial and incubated at 37° C for 12 h. After incubation period, the vials were checked for turbidity and the lowest concentrations of the extract/standard showing no

turbidity were regarded as the MIC of the test substance.

2.6. Brine shrimp lethality bioassay

In this assay, the eggs of *Artemia salina* were hatched for 24 h at room temperature (25-30^{o}C) in artificial sea water (20 g NaCl and 18 g table salt in 1L of distilled water) to obtain nauplii (shrimp larvae). Test samples (ethanol, ether and chloroform extracts of bark of *S. sesban*) dissolved in DMSO was added in test tubes in such a way that the each contains 4 ml of sea water with sample concentrations of 5, 10, 20, 40, 80, 160, 320, 640, 1280 and 2560 µg/ml where the concentration of solvent should be not more than 5%. Same procedure was followed for the standard drug chloramphenicol. The final volume for each test tube was adjusted to 10 ml with artificial sea water with 10 living nauplii in each. The process also includes control test tubes containing 10 living nauplii in 10 mL of artificial sea water. The test tubes were observed and the number of survived nauplii in each test tube counted and the results were noted after a period of 24 h. The percentage of dead nauplii in the test and standard group was established by comparing with that of control group [16-17].

3. Results

Phytochemical screening of the ethanol, ether and chloroform extract of bark of *S. sesban* indicates the presence of carbohydrates, flavonoids, steroids, alkaloids, tannins and saponins (Table 1).

In the antimicrobial assay, the chloroform extracts (both 250 µg/ml and 500 µg/ml) and 500 µg/ml of the ethanol extract of *S. sesban* bark inhibited all bacteria. In disk diffusion assay, 250 µg/ml of the ethanol extract of bark inhibited all the microorganisms except P. vulgaris and E. faecalis and the ether extracts (both 250 µg/ml and 500 µg/ml) of *S. sesban* bark inhibited all the microorganisms except P. vulgaris (Table 2). The highest zone of inhibition was 14.2 mm against F. oxysporum. Zone of inhibition for the standard Kanamycin discs ranged between 25.80 to 44.30 mm (Table 2).

The data obtained from broth macro dilution assay for determining MIC is presented in Table 3. Minimum inhibitory concentration (MIC) of the ethanol extract of bark was 8000 µg/ml for *S. boydii*, *S. flexneri*, *S. sonnei*, *P. vulgaris*, *P. aeruginosa* and *E. faecalis*; 4000 µg/ml for *S. dysenteriae*; 2000 µg/ml for *E. coli*, *S. aureus*, *T. rubrum* and *S.* cerevacae; 1000 µg/ml for *E. amylovora*, *K. pneumonia*, *S. epidermidis*, *M. fulvum*, *C. albicans* and *A. fumigatus*; 500 µg/ml for *S. pyogenes* and *C. lunata*; 250 µg/ml for *B. subtilis* and *F. oxysporum*. MIC of the ether extract of bark was 8000 µg/ml for *S. boydii*, *S. flexneri*, *S. sonnei*, *P. vulgaris* and *E. faecalis*; 2000 µg/ml for *P. aeruginosa* and *T. rubrum*; 1000 µg/ml for *S. dysenteriae*, *E. coli*, *S. epidermidis*, *S. aureus*, *C. albicans* and *S. cerevacae*; 500 µg/ml for *E. amylovora*, *K. pneumonia* and *M. fulvum*; 250 µg/ml for *S. pyogenes*, *B. subtilis*, *C. lunata* and *F. oxysporum*. MIC of the chloroform extract of bark was 8000 µg/ml for *S. boydii*, *S. flexneri*, *P. vulgaris* and *E. faecalis*; 4000 µg/ml for *S. sonnei*; 1000 µg/ml for *S. aureus* and *T. rubrum*; 500 µg/ml for *S. dysenteriae*, *E. coli*, *E. amylovora*, *P. aeruginosa*, *S. epidermidis*, *C. albicans* and *S. cerevacae*; 250 µg/ml for *K. pneumonia*, *S. pyogenes*, *B. subtilis*, *M. fulvum*, *C. lunata*, *A. fumigatus* and *F. oxysporum*.

In the brine shrimp lethality bioassay, the percent mortality the nauplii caused by the test extracts, as well as chloramphenicol is represented in Table 4 and Fig. 1. Probit analysis software LdP (LdP Line software, USA) was used to calculate the LD_{50} and was found to be 1280 µg/ml for the ethanol extract of bark of *S. sesban*, 640 µg/ml for the ether extract of bark of *S. sesban* and 320 µg/ml for the chloroform extract of bark of *S. sesban* whereas 20 µg/ml for chloramphenicol.

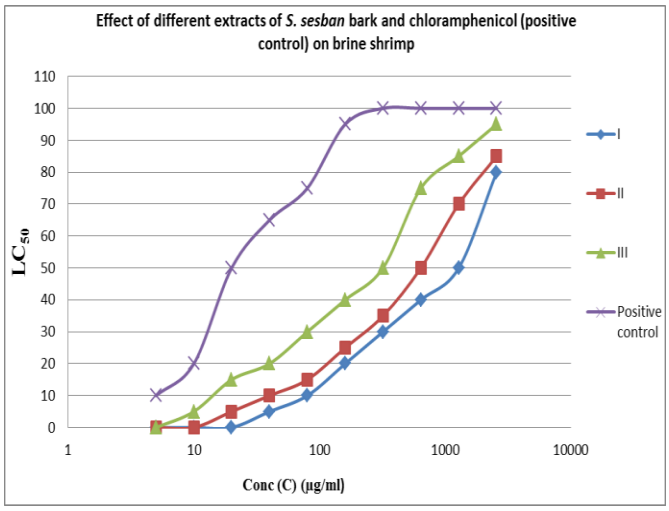

Fig.1. Mortality of nauplii by positive control and *S. sesban* bark extracts (I=ethanol extract of bark of *S. sesban*, II=ether extract of bark of *S. sesban*, III=chloroform extract of bark of *S. sesban*) in brine shrimp lethality bioassay

Table 1. Phytochemical constituents of extracts of *S. sesban* bark

Extracts	Steroids	Alkaloids	Reducing Sugars	Tannins	Gums	Flavonoids	Saponins
Ethanol extract of S. sesban bark	+	+	+	+	-	+	+
Ether extract of S. sesban bark	+	+	+	+	-	+	+
Chloroform extract of S. sesban bark	+	+	+	+	-	+	+

+ = Presence of constituents; - = Absence of constituents

Table 2. Results of the disc diffusion assay of *S. sesban* bark

	Diameter of zone of inhibition (mm)						
	I	II	III	IV	V	VI	Kanamycin (30 µg/disc)
Gram negative bacteria							
Shigella boydii	4	4.4	4.9	5.1	5.1	5.4	32.4
Shigella flexneri	3.1	3	3.4	3.6	4.3	4.5	33.5
Shigella sonnei	4.6	4.2	3.6	3.9	5.6	5.7	31.2
Shigella dysenteriae	5.1	5.2	7.3	7.8	8.4	8.9	35.4
Escherichia coli	5.2	5.9	6.3	6.7	7.1	6.4	35.7
Proteus vulgaris	0	2	0	0	3.2	3	33.1
Erwinia amylovora	5.9	6.7	6.5	7.4	7.8	8.7	33.4
Klebsiella pneumonia	6	6.8	7.6	8	8.5	9.5	37.8
Pseudomonas aeruginosa	4	4.8	6.3	6.9	6.4	7.9	33.1
Gram positive bacteria							
Staphylococcus epidermidis	5.3	6	6.9	4.5	6.4	6.5	25.8
Streptococcus pyogenes	8.2	10	11.5	11.9	10.5	13.2	36.7
Staphylococcus aureus	6.1	7.2	6.9	6.4	6.2	6.7	28.5
Enterococcus faecalis	0	1.6	2.9	3.7	3	4.4	30.8
Bacillus subtilis	9.2	12.2	10.5	12.4	13.6	13.8	30.2
Fungi							
Trichophyton rubrum	6	6.7	6.6	5.9	6.1	7.3	41
Microsporum fulvum	8.1	7.9	8.4	8.8	9.1	7.2	43.5
Candida albicans	7.4	7.6	7.8	8.3	8.6	8.4	43.2
Curvularia lunata	10.2	10.5	11.1	12.3	12	11.8	44.1
Aspergillus fumigatus	8.1	8.3	8.7	8.4	8.6	9.2	44.3
Fusarium oxysporum	9.7	10.3	10.9	13.4	12.3	14.2	40.1
Sacharomyces cerevacae	6.9	7.2	8.1	8.5	8	8.3	43.5

I=ethanol extract of bark of *S. sesban* (250µg/ml), II= ethanol extract of bark of *S. sesban* (500µg/ml), III=ether extract of bark of *S. sesban* (250µg/ml), IV=ether extract of bark of *S. sesban* (500µg/ml), V=chloroform extract of bark of *S. sesban* (250µg/ml), VI=chloroform extract of bark of *S. sesban* (500µg/ml)

Table 3. MICs of *S. sesban* bark extracts

	Minimum Inhibitory Concentration (MIC)			
	I	**II**	**III**	**Ceftriaxone (µg/ml)**
Gram negative bacteria				
Shigella boydii	8000	8000	8000	2
Shigella flexneri	8000	8000	8000	2
Shigella sonnei	8000	8000	4000	1
Shigella dysenteriae	4000	1000	500	0.5
Escherichia coli	2000	1000	500	0.5
Proteus vulgaris	8000	8000	8000	2
Erwinia amylovora	1000	500	500	0.25
Klebsiella pneumonia	1000	500	250	0.25
Pseudomonas aeruginosa	8000	2000	500	0.5
Gram positive bacteria				
Staphylococcus epidermidis	1000	1000	500	1
Streptococcus pyogenes	500	250	250	0.25
Staphylococcus aureus	2000	1000	1000	0.25
Enterococcus faecalis	8000	8000	8000	2
Bacillus subtilis	250	250	250	0.25
Fungi				
Trichophyton rubrum	2000	2000	1000	1
Microsporum fulvum	1000	500	250	0.25
Candida albicans	1000	1000	500	0.5
Curvularia lunata	500	250	250	0.25
Aspergillus fumigatus	1000	500	250	0.25
Fusarium oxysporum	250	250	250	0.25
Sacharomyces cerevacae	2000	1000	500	1

I=ethanol extract of bark of *S. sesban* (µg/ml), II=ether extract of bark of *S. sesban* (µg/ml), III=chloroform extract of bark of *S. sesban* (µg/ml)

Table 4. Effect of different extracts of *S. sesban* bark and chloramphenicol (positive control) on brine shrimp

Conc (C) (µg/ml)	**% Mortality**				**LC_{50} (µg/ml)**			
	I	**II**	**III**	**Positive control**	**I**	**II**	**III**	**Positive control**
2560	80	85	95	100				
1280	50	70	85	100				
640	40	50	75	100				
320	30	35	50	100				
160	20	25	40	95	1280	640	320	20
80	10	15	30	75				
40	5	10	20	65				
20	0	5	15	50				
10	0	0	5	20				
5	0	0	0	10				

I=ethanol extract of bark of *S. sesban*, II=ether extract of bark of *S. sesban*, III=chloroform extract of bark of *S. sesban*, Positive control= chloramphenicol.

4. Discussions

Highly significant degree of activity was observed against the test bacteria *B. subtilis* with 13.60 mm in diameter followed by *E. coli* with 7.1 mm in diameter at 250 µg/ml of the chloroform extract. The carbon tetrachloride partitionate of the methanol leaf extract of *S. sesban* showed the strongest inhibitory activity against *E. coli* having the zone size 12 mm [18]. A fluctuating trend of inhibition zone was found against some pathogens in the analysis. Similar fluctuation trend of inhibition zone was reported by Kunjal Bhatt *et al.*, (2003) [19] and Uma and Sasikumar (2005) [20]. This may be due to the fact that at higher concentrations, the rate of diffusion may perhaps be varied and hence, it might not be available to react with the microorganisms. In most of the bacteria examined, a better zone of inhibition was obtained at 500 µg/ml of the extract. The ethanol extracts of bark showed moderate antimicrobial activity whereas ether and chloroform extracts of bark showed relatively higher antimicrobial activity in both the assays. Although 250µg/ml of the ethanol extract of bark did not show antimicrobial activity against *E. faecalis* and *P. vulgaris* and the ether extract (both 250µg/ml and 500µg/ml) of ether did not show antimicrobial activity against *P. vulgaris* but inhibited the same microorganism in broth marco dilution assay. However, the MIC was obtained at a higher concentration (8000 µg/mL) than the extract content in the disc (250 µg/ml and 500µg/ml). Therefore, concentration may play a role for the observed activity in latter experiment. Antimicrobial activity offered by nonpolar compound(s) may also be a reason as it may fail to diffuse in agar media to exhibit antimicrobial activity in disc diffusion assay [17]. A difference in inoculums size used for the assay can lead to variable results for a given sample. In the present study we adjusted to keep the inoculums size as close to the recommended standard of 5×10^5 CFU/ml [15].

The plant is also reported to contain saponins, so there is growing interest in natural saponins caused as much by the scientific aspects extraction and structural analysis of these compounds, as by the fact of their wide spectrum of pharmacological activities; for instance, bactericidal, antiviral, cytotoxic, analgesic, anti-inflammatory, anti-cancer and anti-allergic [21]. Phytochemical constituents such as tannins, flavonoids, alkaloids and several other aromatic compounds of plant that serve as defense mechanisms against predation by many microorganisms, insects and herbivores. The antimicrobial activity of flavonoids is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell

walls [22-24]. Several reports are available in support of antimicrobial activity of saponins against bacterial and fungal pathogens [25]. The alkaloids are known to have antimicrobial and antiparasitic properties. Verpoorte have reported about 300 alkaloids showing such activity [26]. Similar results on antimicrobial activity were reported on related species of the genus Mahonia [27-29].

The brine shrimp lethality bioassay is a rapid, simple and easily mastered technique for identifying biologically active compound present in a crude extract since it does not require aseptic techniques, inexpensive and very small amount of test material is needed [30]. Result of the present study indicates that the bark extracts of *S. sesban* might have compounds with biological activity with actions like enzyme inhibition, ion channel interference, antimicrobial, pesticidal and/or cytotoxic activity [31-33]. Both the test extracts and chloramphenicol showed a gradual increase in percent mortality of the shrimp nauplii with the increase in concentration. The LD_{50} obtained for the extracts was relatively low than that of chloramphenicol. However, it is still high as a crude extract and infers that there may be one or more compounds present in the extract having biological activity.

Plants are important source of potentially useful structures for the development of new chemotherapeutic agents. The first step towards this goal is the in vitro antimicrobial activity assay and in the recent years several reports available on the antimicrobial activity of plant extracts on human pathogenic bacteria [34]. The beneficial effects of treatment can be obtained with the bark extract of *S. sesban* for various bacterial infectious diseases like pneumonia, diarrohea, urinary tract infection and even some skin disease. The broad antimicrobial and antifungal activities could be as a result of the plant secondary metabolites like alkaloids, flavonoids, tannins, steroids etc., present in the extracts. Usman and Osuji (2007) [35] reported that tannins had been widely used topically to sprains, bruises and superficial wounds as such, it could be probable that tannins and other plant phenols from this extract were responsible for these broad activities.

5. Conclusion

The present study provides a rationale for the use of *S. sesban* bark in traditional medicine in Bangladesh. Further, it reflects a possibility for the development of more novel chemotherapeutic agents or templates from the plant so that the plant may serve for the production of improved therapeutic plant based drugs in future. But *in vivo* studies on the medicinal plant are necessary and should seek to determine toxicity of active constituents, their side effects, serum-attainable levels, pharmacokinetic properties and diffusion in different body sites. The antimicrobial activity could be enhanced if active components are purified and adequate dosage is determined for proper administration. It goes a long way in curbing administration of inappropriate concentration, a common practice among many traditional practitioners. This represents a preliminary report on the antimicrobial activity of the medicinal plant *S. sesban* in Bangladesh and for rational use of the traditional plant it requires further scientific study as necessary on it.

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