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ANTIMICROBIAL EVALUATION AND PHYTOCHEMICAL ANALYSIS OF A KNOWN MEDICINAL PLANT *SAMANEA SAMAN* (JACQ.) MERR. AGAINST SOME HUMAN AND PLANT PATHOGENIC BACTERIA AND FUNGI

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ABSTRACT

The present investigations evaluate the antimicrobial efficacy of six different solvent extracts and isolated constituents of *Samanea saman* (Jacq.) Merr. against 21 microorganisms. Our result revealed that, the methanol extract showed a highest antibacterial activity with zone of inhibition ranging from 11.0mm to 30.5mm at 1mg/ml concentration. The MIC value of the methanol extract for the tested bacteria ranged between 15µg/ml to 500µg/ml. The most susceptible organism in the present investigation was *Streptococcus faecalis* (MIC 15µg/ml) followed by *Staphylococcus aureus* (MIC 62µg/ml), while the most resistant was *Proteus vulgaris* (MIC 500µg/ml). The highly significant antifungal activity against all the fungi was observed in methanol extract with percentage of inhibition ranging from 20.4% to 81.6% depending upon fungal species at 1mg/ml concentration. The IC_{50} value of the methanol extract ranged from 0.3mg/ml to 5mg/ml. Among the tested fungi, *Fusarium moniliforme* (IC_{50} 0.3mg/ml) was highly sensitive and *Aspergillus tamari* (IC_{50} 5mg/ml) was least sensitive.

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KEY WORDS

Samanea saman, antimicrobial activity, human pathogenic bacteria, seed-borne fungi, active fraction.

INTRODUCTION

Infectious diseases are serious health problem worldwide. Commercial antimicrobial drugs used haphazardly in the treatment of many infectious diseases have inevitably led multiple drug resistance (MDR) in both human and plant pathogenic microorganisms[1]. Eventhough pharmacological industries have produced a number of new antibiotics in the last three decades, resistance to these drugs by microorganisms has increased[2]. As a result, society is facing one of the most serious public health dilemmas over the emergence of infectious bacteria displaying resistance to many and in some case all, effective antibiotics. Much like the situation in human medicine, substantial use of chemical pesticides and antibiotics in agriculture has also accelerated the development of antibiotic resistant strains of bacteria and fungi[3]. Furthermore residues of chemical pesticides in agricultural products cause damage to the health of animals and humans, and also affect the export of agricultural products[4]. Considering these there is an urgent need to search for alternative method for the management of antibiotic resistant bacteria and fungi without any toxicity to the consumer, which are eco-friendly and effective.

In the last few years, a number of studies have been conducted in different countries to prove antimicrobial efficacy of botanicals[5,6]. The potential of higher plants as a source for new drug and botanical pesticides is still largely unexplored. This is also true in India and only a small percentage of plants of this region have been evaluated for antibacterial activity[7]. This led the authors to screen *in vitro*, a large number of plants for antimicrobial activity. During regular screening *Samanea saman* (Jacq.) Merr. recorded significant antibacterial and antifungal activity. *S. saman* is belongs to the family Fabaceae and it is globally distributed throughout the tropical regions. It is folk remedy for curing various diseases *viz.,* stomach cancer, cold, diarrhoea, headache, intestinal ailments, sore throat and stomach ache[8]. The antibacterial activity of *S. saman* was reported against some human pathogenic bacteria[9]. A scientific and systematic investigation with regard to the antifungal activity of this plant is lacking. Thus considering vast potentiality of plants as a source of new chemotherapeutic and fungicidal agents, detailed investigations was conducted to test the efficacy of *S. saman* against some plant and human pathogenic bacteria and fungi.

MATERIALS AND METHODS

(i) ***Plant materials :***
Fresh disease free leaves of *S. saman* were collected from Southern part of Karnataka, washed thoroughly 2-3 times with tap water and once with sterile distilled water, shade dried, powdered and used for extraction. An authenticated voucher specimen of the plant is deposited in the herbarium of Department of studies in Microbiology and Biotechnology, Bangalore University, Bangalore.

(ii) ***Preparation of Aqueous extract :***
Fifty grams of shade dried, powdered leaves of *S. saman* were macerated with 100ml sterile distilled water in a waring blender for 10 min. The macerate was first filtered through double-layered muslin cloth and then centrifuged at 4000*g* for 30 minutes. The supernatant was filtered

through Whatman No. 1 filter paper and heat sterilised at 121^0 C for 30 min and subjected to antimicrobial assay[10].

(iii) ***Preparation of Solvent extract :***

Fifty grams of shade dried powder of *S. saman* was filled in the thimble and extracted successively with 200 ml of petroleum ether, toluene, chloroform, methanol and ethanol using a soxhlet extractor until colourless extract obtained on the top of the extractor. Each of the solvent extract was concentrated separately under reduced pressure using rotary flash evaporator. After complete evaporation of the solvent each of these solvent extract was weighed, dissolved in dimethyl sulfonoxide (DMSO) and subjected to antimicrobial activity assay. Only methanol extract, which recorded highest antimicrobial activity, was used for determination of the minimal inhibitory concentration (MIC) [10].

(iv) ***Phytochemical analysis and separation of the active fraction from methanol extract of S. saman :***

Phytochemical analysis of different extracts of *S. saman* was conducted following procedure of Rojas *et al.,* (2003)[11]. The methanol extract which is showed highest antimicrobial activity was further fractionated in to four different fractions viz., hexane (Fraction 1), chloroform (Fraction 2), butanol (Fraction 3) and water (Fraction 4)[12]. All the four fractions were dried under reduced pressure, dissolved in DMSO and subjected to antibacterial activity assay. The fraction which showed highest antibacterial activity was selected for further isolation of the active principle.

(v) ***Isolation of the active compound from butanol fraction of S. Saman by TLC system :***

The butanol fraction which showed significant antibacterial activity was subjected to compound separation by TLC with methanol:ammonium hydroxide (1:1(v/v)) as an eluting solvent. The separated bands were identified under iodine vapour and retardation factor (R_f) value of the spots were determined[3]. The respective bands were scraped out separately along with silica and dissolved in methanol and filtered through Whatman No. 1 filter paper and the filtrate was collected in glass vials and allowed to dry. After complete evaporation of methanol, all the bands were dissolved in DMSO and subjected to antibacterial activity assay.

(vi) ***Organisms used for antimicrobial assay :***

Seven human pathogenic bacteria *viz., Escherichia coli* (NCIM 2065), *Klebsiella pneumoniae* (NCIM 2957), *Proteus vulgaris* (NCIM 2027), *Pseudomonas aeruginosa* (NCIM 5031), *Salmonella typhi* (NCIM 2501), *Staphylococcus aureus* (NCIM 2079), *Streptococcus faecalis* (NCIM 5025) and plant pathogenic *Xanthomonas campestris* (NCIM 2954), *Fusarium moniliforme* (NCIM 1099) and *Fusarium oxysporum* (NCIM 1043) were obtained from National centre of industrial microorganisms, National chemical laboratory, Pune, India.

A total of remaining eleven seed-borne phytopathogenic fungi *viz., Alternaria brasicola*, *Alternaria geophila, Aspergillus flavus, Aspergillus fumigatus, Aspergillus tamari, Curvularia tetramera, Fusarium equiseti, Fusarium lateratum, Fusarium udum, Penicillium chrysogenum* and *Penicillium citrinum* which were isolated from seeds of sorghum, maize and paddy using standard procedure[13]. These bacteria and fungi were sub-cultured and served as test pathogens for the antimicrobial assay.

(vii) ***Antibacterial activity assay :***

Antibacterial activity of the aqueous, solvent extracts and isolated constituents of *S. saman* were determined by disc diffusion method on the Muller-Hinton agar (MHA) medium[5]. In this method, 6 mm sterilized filter paper discs (Whatman No.1) are saturated with sterilized extracts and

isolated constituents of desired different concentrations. The impregnated discs are then placed on to the surface of MHA medium. The MHA media has pre-inoculated with test bacteria (inoculum size 1 X 10^8 CFU/ml). For each treatment six replicates were maintained. The disc devoid of extract and presence of DMSO served as control. The plates were kept at 4 ^{0}C for 1 hour for diffusion of extract, thereafter the plates were incubated at 37 ^{0}C for 24 hours. After incubation, zone of inhibition if any around the disc was measured in mm (millimetre). Augmentin (30mcg/disc), Bacitracin (10U/disc), Erythromycin (10mcg/disc) and Penicillin-G (10U/disc) were used as positive reference to determine the sensitivity of each bacterial species tested. The last concentration of methanol extract showing a clear zone of inhibition was taken as the MIC[14].

(viii) ***Antifungal activity assay :***

Aqueous, solvent extracts and isolated constituents of *S. saman* were subjected to antifungal activity assay by poisoned food technique[15]. The desired different concentrations of each extracts and isolated constituents were separately added to the Czapeck-Dox-Agar (CDA) medium, autoclaved and poured into petridishes (20 ml each) and allowed to cool. After complete solidification of the medium, 5 mm disc of 7 day old culture of the test fungi were inoculated. Four replicates were maintained for each extracts. The petridish containing media devoid of extract and presence of DMSO served as control. The plates were incubated at 26±1^{0}C for seven days. The fungi toxicity of the extract in terms of Percentage Inhibition (%) of mycelial growth was calculated by using the formula,

Percentage Inhibition = dc-dt X 100/dc

where,

dc - Average increase in mycelial growth in control.

dt - Average increase in mycelial growth in treatment

The synthetic fungicides, viz., Blitox and Dithane M-45 which are commonly used for seed treatment were obtained from Bangalore agrochemical market. They were tested at their recommended dosage (2mg/ml) for antifungal activity by poisoned food technique for comparison.

RESULTS AND DISCUSSION

1. ***Antibacterial activity assay :***

The inhibitory activity of the six different extracts and isolated constituents of leaves of *S. saman* against some human pathogenic bacteria and a phytopathogenic *X. campestris* is presented in Table 1. Among the six different extracts tested, methanol extract recorded highest antibacterial activity followed by ethanol and water extracts, whereas no significant antibacterial activity was observed in petroleum ether and toluene extracts. The control DMSO did not inhibit any of the bacteria tested. These results clearly indicate that the active principle responsible for antimicrobial activity is soluble in more polar solvents. The methanol extract exhibited antibacterial activity with zone of inhibition ranging from 11.0mm to 30.5mm at 1mg/ml concentration depending upon bacterial species. All the test bacteria were inhibited by methanol extract of *S. Saman* demonstrating broad spectrum of activity. The most susceptible organism in the present investigation was *Strep. faecalis* followed by *Staph. aureus* and *Pr. vulgaris* was found to be most resistant bacteria against all the extracts tested. In the present study clearly indicates Gram-positive bacteria were more susceptible than Gram-negative bacteria. The antibacterial activity of synthetic antibiotics viz., augmentin, bacitracin, erythromycin and penicillin-G reveal that all the pathogenic bacteria were highly susceptible to erythromycin with zone of inhibition ranging from14mm to 38mm. Bacitracin was not active against all the test pathogens except *Staph. aureus* and *Strep.*

faecalis. Augmentin and penicillin-G was not effective on *Kl. pneumonia, Pr. vulgaris and X. campestris.* The results of the present investigation demonstrate that *Kl. pneumonia, Pr. vulgaris and X. campestris* were resistant to augmentin, bacitracin and penicillin-G. However these bacteria were effectively inhibited by methanol and ethanol extracts of *S. saman*.

MIC value of methanol extract and antimicrobial activity of different fractions of methanol extract of *S. saman* was evaluated and presented in Table-2. The range of MIC of the extract was 15µg/ml to 500µg/ml depending upon bacterial species. In the present investigation lowest MIC value 15µg/ml was observed in *Strep. faecalis*, whereas highest MIC value 500µg/ml was observed in *Pr. vulgaris.* When methanol extract was sub-fractionated further by solvent partition using hexane, chloroform, butanol and water, the highest antibacterial activity was retained in the butanol sub-fraction with zone of inhibition ranging from10.2mm to 18.6mm at 500µg/ml concentration. Further separation of butanol fraction on TLC showed three bands (R_f value 0.47, 0.70 and 0.92). Activity guided antibacterial assay revealed that band 2 (R_f value 0.70) recorded significant antibacterial activity.

Table 1
Antibacterial activity of aqueous, different solvent extracts of S. saman and synthetic antibiotics against some human and plant pathogenic bacteria

Organisms	A	Solvent extracts (1mg/ml)					Synthetic antibiotics			
		P	T	C	M	E	Aug (30mcg)	Bac (10U)	Ery (10mcg)	Pen (10U)
E. coli	7.3±0.3	0±0.0	0±0.0	8.5±0.2	12.87±0.5	12±0.7	8.0±0.4	0±0.0	14.0±0.2	0±0.0
Kl. pneumoniae	9.0±0.3	0±0.0	0±0.0	0±0.0	13.0±0.4	11.0±0.4	0±0.0	0±0.0	28.0±0.5	0±0.0
Pr. vulgaris	0±0.0	0±0.0	0±0.0	0±0.0	12.2±0.4	8.5±0.2	0±0.0	0±0.0	15.0±0.7	0±0.0
Ps. aeroginosa	0±0.0	0±0.0	0±0.0	0±0.0	15.7±0.4	10±0.5	28.0±0.6	0±0.0	26.0±0.4	16.0±0.6
Salm. typhimurium	7.3±0.3	0±0.0	0±0.0	8.2±0.3	11.0±1.1	9.7±1.7	16.0±0.5	0±0.0	15.0±0.4	12.0±0.3
Staph. aureus	12.6±0.3	0±0.0	7.0±0.3	11.2±0.2	14.7±0.5	13.5±0.5	20.0±0.5	28.0±0.4	22.0±0.5	32.0±0.4
Strep. faecalis	25.6±0.3	0±0.0	7.3±0.3	18.7±0.4	30.5±0.5	26.5±0.2	23.0±0.7	28.0±0.4	38.0±0.3	18.0±0.5
X. campestris	9.1±0.1	0±0.0	0±0.0	0±0.0	15±0.4	11.0±0.4	0±0.0	0±0.0	28.0±0.3	0±0.0

Data given are the mean of six replicates ± standard error, p > 0.001
Note : A = Aqueous extract., P = Petroleum Ether, T = Toluene., C = Chloroform., M = Methanol., E = Ethanol., Aug = Augmentin ., Bac = Bacitracin., Ery = Erythromycin., Pen = Penicillin.

Table 2
MIC value and antibacterial assay of different fractions of methanol extract of *S. saman* against some human and plant pathogenic bacteria

Organisms	MIC value of methanol extract (µg/ml)	Different fractions of methanol extract(0.5mg/ml)			
		Hexane	Chloroform	Butanol	Water
E. coli	62	0±0.0	7.7±0.4	10.2±0.6	8.2±0.2
Kl. pneumoniae	62	0±0.0	0±0.0	11.4±0.5	9.0±0.3
Pr. vulgaris	500	0±0.0	0±0.0	10.5±0.6	9.6±0.4
Ps. aeroginosa	250	0±0.0	0±0.0	13.4±0.6	9.7±0.5
Salm. typhimurium	125	0±0.0	11.7±0.4	17.0±0.8	14.0±0.4
Staph. aureus	62	0±0.0	11.0±1.2	15.5±0.5	14.2±0.4
Strep.faecalis	15	0±0.0	0±0.0	18.6±0.5	15.6±0.4
X. campestris	250	0±0.0	0±0.0	13.0±0.3	10.6±0.7

Data given are the mean of four replicates ± standard error, p > 0.001

2. *Antifungal activity assay :*

The antifungal activity of six different extracts and isolated constituents of *S. saman* is presented in the Table-3. In aqueous extract antifungal activity was observed against all the test fungi and the extent of percent of mycelial growth inhibition was varied from 40.2% to 91.6% at 50% concentration. Among the thirteen test fungi, *F. moniliforme* (PI 91.6%) is highly susceptible followed by *Al. geophila* (PI 91.0%) and *F. udum* (PI 90.5%). Among different solvent extracts tested, methanol and ethanol extracts recorded highest antifungal activity against tested fungi with percent of mycelial growth inhibition ranging from 20.4% to 81.6% and 10.4% to 66.9% respectively at 1mg/ml concentration. In the present investigation clearly confirm that the highest antifungal activity observed in field fungi viz., species of *Alternaria, curvularia* and *Fusarium* than storage fungi viz., species of *Aspergillus* and *Penicillium*. The range of IC_{50} value of the methanol extract was 0.3mg/ml to 5mg/ml depending upon fungal species. The percent mycelial inhibition of two synthetic fungicides viz., Blitox and Dithane M-45 revealed that, among the 13 fungi tested against Blitox, *F. moniliforme* (PI 96.2%) was highly susceptible and *Penicillium citrinum* (73.9%) was least activity. In case of Dithane M-45, *Al. brassicola* was highly sensitive (79.1%) while *A. tamari* (24.4%) was least sensitive. The antifungal activity of aqueous, methanol and ethanol extracts were almost equivalent to that of synthetic fungicides tested. The present investigation clearly demonstrated the first time antifungal property of *S. saman*.

Table 3

Antifungal activity of aqueous, different solvent extracts of leaves of S. saman and synthetic fungicides against some phytopathogenic seed-borne fungi

Test organisms	Aqueous extract		Solvent extracts (1 mg/ml)					IC_{50} value of methanol extract (µg/ml)	Synthetic fungicide (2000 µg/ml)	
	10%	50%	Petroleum ether	Toluene	Chloroform	Methanol	Ethanol		Blitox	Dithane M-45
Al. brassicola	50.0±0.3	72.2±0.6	15.8±2.3	15.5±0.8	23.1±0.9	74.7±0.2	45.1±0.4	0.5	83.4±0.5	79.1±0.2
Al. geophila	5.2±1.2	91.0±0.0	45.7±0.5	45.7±0.6	42.8±0.5	64.2±0.4	50.0±0.5	1.0	90.9±0.3	68.9±0.3
Aspergillus flavus	6.6±1.5	42.1±5.5	21.9±0.6	30.8±0.4	12.1±0.4	21.3±0.5	22.5±0.6	3.0	92.3±0.3	47.6±0.3
A. fumigatus	22.1±0.8	51.6±0.57	40.4±0.3	41.0±0.3	35.5±0.6	36.8±0.5	24.3±0.5	3.0	96.0±0.3	63.1±0.1
A. tamari	15.5±1.1	62.9±0.6	20.2±0.6	22.5±0.5	21.6±0.5	20.4±0.4	10.4±0.5	5.0	95.4±0.2	24.4±0.3
C. tetramera	53.1±0.3	77.1±0.8	33.2±0.8	32.2±0.5	42.7±1.8	73.0±0.6	53.2±0.7	0.5	83.5±0.3	78.9±0.3
Fusarium equiseti	61.8±0.3	89.0±0.3	11.4±0.8	49.6±0.4	55.8±0.4	66.9±02	39.1±0.4	0.5	94.5±0.3	74.0±0.6
F. lateratium	46.4±0.0	87.2±0.3	11.9±0.4	50.5±4.2	58.8±0.7	71.9±0.2	66.9±3.1	0.5	83.8±0.2	58.9±0.3
F. moniliforme	63.5±0.6	91.6±0.0	10.4±0.8	16.4±1.7	33.7±0.8	81.6±0.4	43.8±0.6	0.3	97.2±0.4	69.8±0.8
F. oxysporum	8.6±0.2	40.2±0.4	2.1±1.0	20.8±1.9	28.1±0.6	43.4±0.2	28.4±1.2	0.5	89.6±0.6	69.1±0.1
F. udum	51.3±1.2	90.5±0.0	14.1±1.1	47.2±0.2	52.5±2.6	70.2±0.8	58.8±0.9	0.5	87.8±0.2	68.3±0.3
Penicillium chrysogenum	20.4±0.3	52.6±0.3	16.0±0.4	23±0.4	14±0.8	23.1±0.6	14±0.4	5.0	89.0±0.0	72.4±0.3
P. citrinum	27.9±0.3	77.9±0.0	19.7±0.5	20±0.4	15.5±0.6	21.3±0.5	10.8±0.5	4.0	93.9±0.3	63.9±0.3

Data given are the mean of four replicates ± standard error, p > 0.001

3. *Phytochemical analysis :*

Investigation on phytochemical analysis of different extracts viz., aqueous, petroleum ether, toluene, chloroform, methanol and ethanol extracts of *S. saman* is presented in the table-4. Methanol extract which recorded significant highest antibacterial and antifungal activity revealed the presence of saponins, glycosides, quinines, cardiac glycosides, steroids, alkaloids, flavonoids, phenolic compounds.

The spread of multi drug resistant (MDR) strains of bacteria and fungi necessitates the discovery of new class of antibiotics and fungicide that inhibits antibiotic resistant pathogens[10]. Association of variety of fungi causing significant loss in seed quality and nutritional quality of sorghum, maize and paddy has been reported[16]. Chemical fungicides such as copper carbonate, sulphur, organic acid, inorganic mercurial compounds, carboxin, benomyl, captan, thiram, carboxin etc. are generally applied for the management of seed borne fungal diseases and fungal bio-deterioration[17]. Even though effective and efficient control of seed borne fungi of seeds can be achieved by the use synthetic chemical

fungicides, the same cannot be applied to grains for reasons of pesticide toxicity[7]. In view of these, the result of the present investigations clearly demonstrates *S. saman* is one of the potent plant for exploitation in medical and agricultural microbiology. Further work going on to identification and characterisation of active principle based on Nuclear Magnetic Resonance (NMR), Mass Spectral analysis (MS) and Infra Red (IR) spectral analysis.

Table 4
Phytochemical analysis of aqueous and different solvent extracts of leaves of S. saman

Extracts Tests	Aqueous extract	Petroleum ether	Toluene	Chloroform	Methanol	Ethanol
Saponins	+	-	-	-	+	-
Tannins	+	-	-	-	+	-
Alkaloids	+	-	-	+	+	+
Cardiac Glycosides	+	+	-	-	+	+
Cardbohydrates	+	+	-	-	+	+
Flavanoids	-	-	-	-	+	+
Phlobatannins	+	-	-	-	-	-
Steroids	+	+	+	+	+	-
Terpenoids	-	-	-	-	-	+
Glycosides	+	+	-	-	+	+
Amino acids	-	-	-	-	-	-
Phenolic Compounds	-	-	-	-	+	-
Quinones	+	-	-	-	-	+
Anthraquinones	-	-	-	-	-	-

+ Present -Absent

CONCLUSION

The present investigation it is an important step in developing plant based pesticides and drugs which are eco-friendly for the management of the pathogenic bacteria and fungi and development of commercial formulation of botanicals. Further investigations are necessary for developing commercial formulation based on field, animal trails and toxicological experiment.

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