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"*In vitro* Antibacterial Potential of Alkaloids of *Samanea saman* (Jacq.) Merr. Against *Xanthomonas* and Human Pathogenic Bacteria"

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Abstract: Aqueous extract, different solvent extracts and isolated constituents (alkaloids) of leaves of *Samanea saman* (Jacq.) Merr. (*Fabaceae*) were assayed for antibacterial activity by cup diffusion method against three phytopathogenic *Xanthomonas* pathovars viz., *Xanthomonas axonopodis* pv. *malvacearum*, *X. a.* pv. *phaseoli* and *X. campestris* pv. *vesicatoria* and 14 human pathogenic bacteria. The pathovars are associated with angular leaf spot of cotton, common blight of bean and bacterial spot of tomato respectively. Aqueous and methanol extracts showed significant antibacterial activity against all pathovars of *Xanthomonas* and the activity varied among 14 human pathogenic bacteria. Methanol extract was subsequently fractioned and monitored by bioassay leading to the isolation of active fraction by further phytochemical analysis. This active fraction recorded highly significant antibacterial activity *in vitro* (MIC 6, 6 and 4 μg ml^{-1} for *Xanthomonas* pathovars and 3-11 μg ml^{-1} for human pathogenic bacteria) compared with synthetic antibiotics such as Bacterimycin 2000 and Sreptocycline tested at recommended dosage for phytopathogenic bacteria and Gentamycin and Streptomycin discs for human pathogenic bacteria. The active fraction was further confirmed as alkaloids.

Key words: Alkaloids · *Samanea saman* · Antibacterial activity

INTRODUCTION

Worldwide, plant diseases with the potential to wipe out crops are exploding. There are several reasons for this explosion, many plant pathogens and the insects that often spread them have overcome the pesticides, agricultural practices and biocontrols that once held them in check. At the same time, some effective chemicals, such as methyl bromide, are being banned because of environmental concerns [1, 2].

The increasing interest in the possible application of the secondary metabolites to pest management has directed investigation towards search for new sources of biologically active natural products. Considering the recent need for biologically active natural products with low mammalian toxicity, lack of neurotoxic mode of action, low persistence in the environment and biodegradability, which may also avoid the development of resistance in pests, higher plants are routinely screened for antibacterial activity against *Xathomonas* pathovars in particular and human pathogenic bacteria in general in our laboratory. During the screening *Samanea saman* has recorded highly significant antibacterial activity; hence in the present study further investigation was undertaken by antibacterial activity guided fractionation to isolate the active fraction and also to confirm the nature of the active compound by phytochemical analysis. The main aim is to develop herbal remedies for plant disease management.

Samanea saman (Jacq.) Merr. (Fabaceae) is distributed in the tropics and generally called as a rain tree. It is cultivated as an ornamental shade tree, its pods and leaves are valued as cattle fodder [3]. Rain tree is a folk remedy for cold, diarrhea, headache, intestinal ailments and stomachache [4]. The antibacterial activity of alkaloid fraction of leaves was reported against *Mycobacterium tuberculosis* [5] and the bark is known to contain two alkaloids – $C_8H_{17}ON$ and $C_{17}H_{36}ON_3$ (Pithecolobine) and a saponin (samarin) [6]. Rain tree is also known to have anticancer property; the root decoction is used in hot baths for stomach cancer in Venezuela [7]. The leaf infusion is used as laxative [8]. All reports are dates back to several years and recent reports on phytochemical analysis and biological activity of this medicinal plant is scanty, hence the present study.

MATERIALS AND METHODS

Collection of plant material: Healthy disease free, mature leaves of *Samanea saman* (Jacq.) Merr. collected from Mysore, Mysore district, Karnataka (India) was used for

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the preparation of aqueous and different solvent extracts. A voucher specimen of the plant is deposited in the herbarium of Department of Studies in Botany, University of Mysore, Mysore.

Test pathogens: Three pathovars of *Xanthomonas* viz., *Xanthomonas axonopodis* pv. *malvacearum* (X.a.m.) known to cause angular leaf spot of cotton (*Gossypium herbaceum* L.), *X. a.* pv. *phaseoli* (X.a.p.) causal agent of common blight of bean (*Phaseolus vulgaris* L.) and *X. campestris* pv. *vesicatoria* (X.c.v.) causal organism of bacterial spot of tomato (*Lycopersicon esculentum* mill.) were obtained from DANIDA laboratory, Department of Studies in Applied Botany, University of Mysore, Mysore.

Cultures of fourteen human pathogenic bacteria were obtained from the Government Medical College, Mysore, Karnataka, which served as test pathogens.

Preparation of extracts: *Aqueous extract*: Samples (50 g) of thoroughly washed fresh leaves of *S. saman* were macerated with 100 ml sterile distilled water in a Waring blender (Waring International, new Hartford, CT, USA) for 10 min. The macerate was first filtered through double-layered muslin cloth and then centrifuged at 4000 *g* for 30 min. The supernatant was filtered through Whatmann No.1 filter paper and sterilized at 120°C for 30 min. These extracts were cooled to room temperature and their pH was determined just before subjecting it to antibacterial activity assay.

Solvent extracts: Thoroughly washed mature leaves were shade dried and then powdered with the help of waring blender. Twenty-five grams of the powder was filled in the thimble and extracted successively with petroleum ether, benzene, chloroform, ethanol and methanol using a Soxhlet extractor for 48 h depending on increase in polarity. All the extracts were concentrated using rotary flash evaporator and preserved at 5°C in airtight bottle until further use. All the extracts were subjected to antibacterial activity assay and phytochemical analysis.

Antibacterial activity assay: Antibacterial activity of aqueous extract, solvent extracts and isolated constituents was determined by cup diffusion method on nutrient agar medium [9]. Cups are made in nutrient agar plate using cork borer (5 mm) and inoculum containing 10^6 CFU/ml of bacteria were spread on the solid plates with a sterile swab moistened with the bacterial suspension. Then aqueous extract, solvent extracts and isolated constituents (Fractions I to IV) were placed in the cups made in inoculated plates, similarly each plate carried a blank cup with solvent only in the center to serve as a control and the antibiotic discs of Gentamycin and Streptomycin (5 mm in diameter) for human pathogens and recommended dosage of bacterimycin 2000 (Nitro propane hexadiol) (3 μg ml^{-1}) (Source: T. Stanes and Company Ltd., 23, Race-cource Road, Coimbatore-641018, India) and streptocycline (Streptomycin sulphate I.P. 90% Tetracycline Hydrochloride I.P. 10%) (1 μg ml^{-1}) (Source: Hindustan Antibiotics Ltd., PIMPRI, Pune-411018, India) for plant pathogens were also used as positive controls. All the plates were incubated for 24 h at 37°C for human pathogens and at room temperature for plant pathogens and zone of inhibition if any around the wells was measured in mm (millimeter).

Phytochemical analysis: Phytochemical analysis of all the evaporated solvent extracts was conducted following the procedures of Anon [3] and Harborne [10]. Methanol extract was separated in to different fractions as Fraction I (Stronger acids), Fraction II (Phenolic compounds), Fraction III (Alkaloids) and Fraction IV (Weaker acids) following the procedures of Roberts *et al.* [11].

Determination of Minimal Inhibitory Concentration (MIC): MIC was determined by both agar and broth dilution methods. For broth dilution tests, 0.1 ml of standardized suspension of bacteria (10^6 CFU/ml) was added to each tube containing different concentrations of the active fraction (0-20 μg ml^{-1}) and incubated for 24 h at 37°C for human pathogens and at room temperature for plant pathogens. In agar plating method dilutions having 0-20 μg ml^{-1} of active fraction was placed in the cups on the inoculated plate and incubated as mentioned above. The lowest concentration of the tube or plate that did not show any visible growth by macroscopic evaluation was considered as the MIC. Each assay was performed in quadruplet.

Stastical analysis: Treatment effect was determined by one-way analysis of variance (ANOVA) using SPSS for windows software. The significance ($p<0.05$) of difference between treatments was determined. The values given are mean of four replicates ± Standard Error.

RESULTS AND DISCUSSION

Aqueous extract: The inhibitory activity of aqueous extract was not observed up to 20 μl against all the *Xanthomonas* pathovars, where as it is highly significant at 50 μl concentration. X.a.m. recorded high susceptibility to the aqueous extract followed by X.a.p. and X.c.v. at

Table 1: Antibacterial activity of aqueous extract of *Samanea saman* on phytopathogenic *Xanthomonas* pathovars at different concentrations (Zone of inhibition measured in mm)

Phytopathogenic bacteria	Concentrations (µl)				
	10	20	30	40	50
X.a.m.	0.00[a]	0.00[a]	8.70±0.12[b]	11.00±0.07[c]	16.20±0.12[d]
X.a.p.	0.00[a]	0.00[a]	0.00[a]	8.80±0.04[b]	15.80±0.12 [c]
X.c.v.	0.00[a]	0.00[a]	6.75±0.14[b]	9.27±0.10[c]	12.32±0.11[d]

Values are the means of four replicates ± standard error

Figures followed by different letters in columns differ significantly when subjected to ANOVA ($p<0.05$)

X.a.m.: *Xanthomonas axonopodis* pv. *malvacearum*, X.a.p.: *X. a.* pv. *phaseoli*, X.c.v.: *X. campestris* pv. *vesicatoria*

Table 2: Antibacterial activity of aqueous extract of *Samanea saman* on human pathogenic bacteria at different concentrations (Zone of inhibition measured in mm)

Human pathogenic bacteria	Concentrations (µl)				
	10	20	30	40	50
Proteus mirabilis	0.00	0.00	0.00	0.00	0.00
Citrobacter sp.	8.85±0.08[a]	10.90±0.05[b]	11.85±0.08[c]	13.75±0.14[d]	14.25±0.14[e]
Klebsiella sp.	11.12±0.08[a]	11.92±0.05[b]	12.90±0.08[c]	14.90±0.07[d]	15.45±0.08[e]
E. coli	0.00[a]	11.32±0.11[b]	12.65±0.11[c]	13.77±0.10[d]	15.27±0.12[e]
Staphylococcus aureus	14.75±0.14[a]	16.07±0.07[b]	17.92±0.04[c]	18.55±0.05[d]	20.25±0.14[e]
Streptococcus faecalis	0.00	0.00	0.00	0.00	0.00
Pseudomonas aeruginosa	0.00	0.00	0.00	0.00	0.00
Salmonella paratyphi A	0.00	0.00	0.00	0.00	0.00
Salm. paratyphi B	0.00	0.00	0.00	0.00	0.00
Salm. typhi	0.00	0.00	0.00	0.00	0.00
Salm. typhimurium	0.00	0.00	0.00	0.00	0.00
Shigella boydii	0.00	0.00	0.00	0.00	0.00
Sh. flexneri	0.00	0.00	0.00	0.00	0.00
Sh. sonnei	0.00	0.00	0.00	0.00	0.00

Average of four replicates ± standard error

Figures followed by different letters in columns differ significantly when subjected to TUKEY ($p< 0.05$)

Table 3: Antibacterial activity of different solvent extracts *S. saman* against important pathovars of phytopathogenic *Xanthomonas* (Zone of inhibition measured in mm)

Bacteria	Solvents				
	Petroleum ether	Benzene	Chloroform	Ethanol	Methanol
X.a.m.	10.40±0.10[d]	6.45±0.05[b]	5.75±0.14[a]	9.25±0.14[c]	18.25±0.14[e]
X.a.p.	10.12±0.12[d]	5.42±0.07[a]	8.10±0.13[c]	6.55±0.05[b]	16.85±0.15[e]
X.c.v.	6.45±0.05[b]	5.67±0.12[a]	6.50±0.12[b]	5.87±0.12[a]	17.32±0.11[c]

Values are the means of four replicates ± standard error

Figures followed by different letters in columns differ significantly when subjected to TUKEY ($p< 0.05$)

X.a.m.: *Xanthomonas axonopodis* pv. *malvacearum*, X.a.p.: *X. a.* pv. *phaseoli*, X.c.v.: *X. campestris* pv. *vesicatoria*

50 µl concentration (Table 1). The activity was increased with increased concentration of the aqueous extract over 20 µl concentration.

Citrobacter spp., *Klebsiella* spp., *E. coli* and *Staph. aureus* were found highly susceptible to aqueous extract at 50 µl concentration, where as other test pathogens were found not affected even at 50 µl concentration. The susceptibility was more in *Staph. aureus* compared to other human pathogenic bacteria (Table 2).

Solvent extracts: Among the different solvent extracts, methanol recorded significant antibacterial activity both against plant and human pathogenic bacteria (Table 3).

Table 4: Antibacterial activity of different solvent extracts of *Samanea saman* against important human pathogenic bacteria (Zone of inhibition measured in mm)

	Solvent extracts				
Bacteria	Petroleum ether	Benzene	Chloroform	Ethanol	Methanol
Proteus mirabilis	0.00[a]	0.00[a]	0.00[a]	0.00	0.00
Citrobacter sp.	0.00[a]	0.00[a]	0.00[a]	6.25±0.14[c]	10.12±0.12[b]
Klebsiella sp.	0.00[a]	0.00[a]	0.00[a]	6.12±0.12[b]	6.00±0.00[b]
E. coli	0.00[a]	0.00[a]	0.00[a]	6.12±0.12[b]	8.87±0.12[c]
Staphylococcus aureus	0.00[a]	0.00[a]	12.75±0.14[a]	11.05±0.25[b]	18.37±0.12[d]
Streptococcus faecalis	0.00[a]	0.00[a]	0.00[a]	8.75±0.14[b]	9.75±0.14[b]
Pseudomonas aeruginosa	0.00[a]	0.00[a]	0.00[a]	0.00[a]	10.18±0.11[b]
Salmonella paratyphi A	0.00[a]	0.00[a]	0.00[a]	6.00±0.00[b]	8.37±0.12[c]
Salm. paratyphi B	0.00[a]	0.00[a]	7.87±0.12[c]	6.25±0.14[b]	13.75±0.14[d]
Salm. typhi	0.00[a]	0.00[a]	6.45±0.05[c]	5.87±0.12[b]	7.87±0.12[d]
Salm. typhimurium	0.00[a]	0.00[a]	7.87±0.12[b]	7.87±0.12[b]	9.75±0.14[a]
Shigella boydii	0.00[a]	0.00[a]	0.00[a]	7.87±0.12[b]	10.00±0.00[c]
Sh. flexneri	0.00[a]	0.00[a]	0.00[a]	6.00±0.00[b]	10.75±0.14[c]
Sh. sonnei	0.00[a]	0.00[a]	0.00[a]	6.75±0.14[b]	13.75±0.14[c]

Values are the means of four replicates ± standard error

Figures followed by different letters in columns differ significantly when subjected to ANOVA ($p < 0.05$)

Table 5: Phytochemical analysis of *Samanea saman*

	Solvent extracts				
Test for	Petroleum ether	Benzene	Chloroform	Methanol	Ethanol
Alkaloids	--	--	++	++	++
Carbohydrates and Glycosides	--	--	--	++	++
Phytosterols	++	--	--	++	++
Fixed oils and fats	--	--	--	--	--
Phenolic compounds/tannins	--	--	--	++	++
Saponins	--	--	--	++	++
Flavonoids	--	--	++	++	++
Proteins and Aminoacids	--	--	--	++	++
Gums and mucilages	--	--	--	++	++
Volatile oils	--	--	--	--	--

-- : Absent ++ : Present

Table 6: Comparative efficacy of antibacterial activity of alkaloid fraction of *S. saman* with antibiotics against phytopathogenic bacteria (Zone of inhibition measured in mm)

	X.a.m.	X.a.p.	X.c.v.
Fraction I	--	--	--
Fraction II	--	--	--
Fraction III	19.32±0.18	17.40±0.18	18.25±0.50
Fraction IV	--	--	--
MIC ($\mu g\ ml^{-1}$)	6	6	4
Streptocycline	19.9±0.25	16.0±0.026	14.63±0.26
Bacterimycin 2000	10.00±0.43	11.38±0.026	11.25±0.25

Values are the means of four replicates ± standard error

X.a.m.: *Xanthomonas axonopodis* pv. *malvacearum*, X.a.p.: *X. a.* pv. *phaseoli*, X.c.v.: *X. campestris* pv. *vesicatoria*

The methanol extract recorded highly significant antibacterial activity was recorded against X.a.m. followed by X.c.v. and X.a.p.

For human pathogenic bacteria the inhibitory activity was highly significant against *Staph. aureus* followed by both *Salm. paratyphi* B and *Sh.sonnei* in the methanol extract, where as no activity was observed against *Pr. mirabilis*. Petroleum ether and benzene extracts was not effective against all the test human pathogenic bacteria (Table 4).

Phytochemical analysis: The results revealed the presence of alkaloids, carbohydrates and glycosides, phytosterols, phenolic compounds/tannins, saponins and flavonoids, proteins and aminoacids and gums and mucilages, whereas fixed oils and fats and volatile oils were absent. Chloroform extract was found to contain alkaloids and flavonoids and petroleum ether extract was found to contain phytosterols (Table 5). Among four fractions, only fraction III (Alkaloids) recorded

Table 7: Comparative efficacy of antibacterial activity of alkaloid fraction of *S. saman* with synthetic antibiotics against human pathogenic bacteria (Zone of inhibition measured in mm)

		Zone of inhibition		
Organisms	MIC (μg ml^{-1})	Alkaloid fraction	Gentamycin	Streptomycin
Proteus mirabilis	20	0.0^{a}	9.25±0.15^{a}	0.00^{a}
Citrobacter sp.	08	13.12±0.06^{f}	15.75±0.15^{d}	13.62±0.10^{g}
Klebsiella sp.	11	9.22±0.15^{b}	15.62±0.10^{d}	11.75±0.15^{e}
E. coli	09	10.75±0.17^{d}	14.62±0.10^{c}	10.62±0.10^{d}
Staph. aureus	03	22.10±0.02^{h}	22.37±0.10^{h}	22.75±0.15^{j}
Strep. faecalis	09	11.10±0.11^{d}	15.75±0.15^{d}	0.00^{a}
Pseudomonasaeruginosa	09	12.25±0.09^{e}	14.62±0.10^{c}	7.5±0.00^{b}
Salmonella paratyphi A	09	10.20±0.02^{c}	18.37±0.10^{f}	16.37±0.10^{h}
Salm. paratyphi B	07	15.00±0.015^{g}	21.62±0.10^{i}	18.75±0.15^{i}
Salm. typhi	11	9.90±0.07^{c}	20.62±0.10^{h}	0.00^{a}
Salm. typhimurium	09	11.00±0.07^{d}	16.62±0.10^{e}	12.00±0.17^{f}
Shigella boydii	09	12.10±0.10^{e}	21.75±0.10^{i}	0.00^{a}
Sh. flexneri	09	12.50±0.12^{e}	14.40±0.10^{b}	9.25±0.15^{c}
Sh. sonnei	07	15.10±0.12^{g}	18.75±0.15^{g}	0.00^{a}

Values are the means of four replicates ± standard error

Figures followed by different letters in rows differ significantly ($p< 0.05$)

antibacterial activity. This fraction was further tested for the MIC.

Determination of MIC: The MIC of alkaloid fraction, which recorded highly significant antibacterial activity against all the pathovars of *Xanthomonas*, was 6, 6 and 4 μg ml^{-1} for X.a.m. X.a.p. and X.c.v. respectively.

The zone of inhibition of alkaloid fraction was highly significant compared to bacterimycin 2000 and streptocycline tested at the recommended dosage, even though zone of inhibition observed is more, the MIC values are high compared to antibiotics tested, suggesting that at high concentration the alkaloid fraction is highly significant compared to antibiotics (Table 6).

Alkaloid fraction recorded almost equal antibacterial activity against *Staph. aureus*, compared to the tested antibiotics but antibacterial activity against other human pathogenic bacteria were not found significant compared to gentamycin whereas compared to streptomycin it was found significant against *Strep. faecalis*, *Ps. aeruginosa*, *Sh. boydii*, *Sh. flexneri* and *Sh. sonnei* out of fourteen human pathogenic bacteria (Table 7).

DISCUSSION

The antibacterial activity of aqueous extract of *S. saman* against *Xathomonas* pathovars was reported from our laboratory [12]. The present study also describes similar result, which is a part of antibacterial-guided fractionation, which always begins with aqueous extract. The phytochemical analysis of different solvent extracts of leaves of *S. saman* is first to report from the present investigation.

The observation indicates the solubility of the active principle, which is more polar, as it is evident from the study that, water and methanol extracts recorded antibacterial activity, where as no activity was observed in other nonpolar solvents used in the present study.

Phytochemical analysis revealed the presence of alkaloids and even among the different fractions obtained from methanol extract, alkaloid fraction recorded highly significant antibacterial activity. Further investigations need to be carried out whether the alkaloids previously reported from this plant [6] are responsible for the activity or any novel alkaloid involved in it.

Xanthomonas pathovars were selected as test organisms for the present study due to its varied response to the available antibiotics. Among the plant diseases caused by bacteria, blackarm of cotton is severe. Atkinson from the United States first described it in 1891, but E.F. Smith from the same country established the bacterial nature of the disease in 1901. Since then the disease has been reported from African countries, Australia, China, Egypt, India, Pakistan, South America, Srilanka, Sudan and U.S.S.R. The disease was first observed in India in Tamil Nadu in 1918, since then many workers in various parts of India and also in other countries has studied it in great detail. It is a disease of major concern in Maharashtra, Karnataka andhra Pradesh, Tamil Nadu and Madhya Pradesh [13].

Seed sanitation from phytopathogenic bacteria including *Xanthomonas* pathovars was achieved so far with acid compounds (e.g., HCl, acetic acid), copper compounds or chlorine derivatives and heat treatments with a certain efficacy. Even though these methods are effective, copper compounds and chlorine derivatives cannot be used in seeds for human consumption. Hence alternatives from biological sources will be highly useful in the management of these pathogens in an ecofriendly way both in field and in the storage. The present investigation clearly demonstrates the significant antibacterial activity of various extracts and the alkaloid fraction of *S. saman* against the seed borne *Xanthomonas* pathovars *in vitro*. These indicate a potential use of this plant in management of seed borne bacterial diseases caused by *Xanthomonas* pathovars, since the genus *Xanthomonas* is an important phytopathogenic bacteria causing a large number of diseases in many important crop plants.

The importance of medicinal plants in providing health care against various ailments including infectious diseases is well documented [14-17]. In recent years development of multidrug resistance in the pathogenic bacteria and parasites has created major clinical problems in the treatment of infectious diseases [18]. This and other problems such as toxicity of certain antimicrobial drugs on the host tissue [19, 20] triggered interest in search of new antimicrobial substances/drugs of plant origin.

Considering the rich diversity of plants, it is expected that screening and scientific evaluation of plant extracts for their antimicrobial activity may provide new antimicrobial substances; hence in the present investigation the antibacterial activity of *S. saman* and nature of the active principle has been demonstrated for the first time against phytopathogenic bacteria and human pathogenic bacteria. The results suggest the possible exploitation of this plant in the management of plant pathogenic bacteria in an ecofriendly way and also human Staphylococcal disease, since compounds of biological origin are known to posses minimal residual effect.

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