In vitro evaluation of anti-bacterial spectrum and phytochemical analysis of *Acacia nilotica*

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The antibacterial activity of aqueous extract, different solvent extracts and isolated constituents of leaves of *Acacia nilotica* (Family: *Fabaceae*) were evaluated by the cup diffusion method against three phytopathogenic *Xanthomonas* pathovars viz., *Xanthomonas axonopodis* pv. *malvacearum*, *X. a*. pv. *phaseoli* and *X. campestris* pv. *vesicatoria* associated with angular leaf spot of cotton, common blight of bean and bacterial spot of tomato respectively and 14 human pathogenic bacteria. Aqueous, methanol and ethanol extracts showed significant antibacterial activity against all the pathovars of *Xanthomonas* and 14 human pathogenic bacteria tested. 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 5, 6 and 7 µg/ml for *Xanthomonas* pathovars and 6-12 µg/ml for human pathogenic bacteria) compared with synthetic antibiotics like Bact-805 and K-cycline for phytopathogenic bacteria and Gentamicin and Streptomycin for human pathogenic bacteria. The results also revealed that further separation of active fraction resulted in the loss of antibacterial activity, indicating synergistic effect of the isolated active fraction.

Key words: *A. nilotica*, acidic fraction, antibacterial activity, *Xanthomonas pathovars*

Introduction

Management of plant and human disease is generally achieved by the use of synthetic pesticides and antibiotics (Mathur and Tannan, 1999), however the incessant and indiscriminate use of these synthetic antibiotics has caused health hazards in animals and humans due to their residual toxicity and many a times management of pathogenic microbes becomes difficult due to the resistance of pathogens.

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Pathovars of *Xanthomonas*, known to cause diseases on several vegetable and plantation crops have developed resistance to Ampicillin, Copper compounds, Kanamycin, Penicillin and Streptomycin (Verma *et al*., 1989; Cooksey, 1987; Rodriguez *et al*., 1997). There are also several reports of antibiotic resistance of human pathogens to available antibiotics (Mitsuyama *et al*., 1987; Gutmann *et al*., 1988; Gandhi and Banker, 1999; Garg *et al*., 2000; Ganguly *et al*., 2001; Martino *et al*., 2002).

Bioactive principles isolated from plants appear to be one of the alternatives for the control of these antibiotic resistant plant and human pathogens. Though the literature is replete with information on the antimicrobial effect on human pathogenic bacteria, a similar investigation on plant pathogens has not been given an adequate attention. Considering these, higher plants are routinely screened for antibacterial properties against important phytopathogenic and human pathogenic bacteria in our laboratory. *Acacia nilotica* (*Fabaceae*) showed significant antibacterial activity, hence further investigations were undertaken to identify the bioactive principle responsible for the antibacterial activity. *Acacia nilotica* is found in the warmer and drier parts of the world. Indian acacias yield three major products: tannin, gum and timber (Kirtikar and Basu 1975).

Materials and Methods

Collection of plant material

Healthy disease free, mature leaves of *Acacia nilotica* (L.) Willd. exDelile ssp. Indica (Benth.) Brenan collected from Mysore, Mysore district, Karnataka (India) was used for 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, *X. a.* pv. *phaseoli* (*X.a.p*.) causal agent of common blight of bean and *X. campestris* pv. *vesicatoria* (*X.c.v*.) causal organism of bacterial spot of tomato were obtained from DANIDA laboratory, Department of Studies in Applied Botany, University of Mysore, Mysore.

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

Preparation of the aqueous extract

Samples (50g) of thoroughly washed fresh leaves of *A. nilotica* were macerated with 100ml sterile distilled water in a Waring blender (Waring International, new Hartford, CT, USA) for 10min. 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 120^0C for 30 min. These extracts were allowed to cool to room temperature and their pH was determined just before subjecting it to antibacterial activity assay.

Preparation of 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 48h. All the extracts were concentrated using rotary flash evaporator and preserved at 5^0C 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 (Anon, 1996). 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, solvents 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 Gentamicin and Streptomycin (5 mm in diameter) for human pathogens and recommended dosage of K-cycline and Bact-805 for plant pathogens were also used as positive controls. All the plates were incubated for 24h. at 37^0C 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 (1985) and Harborne (1998). Methanol extract was separated in to different fractions as Fraction I (Stronger acids), Fraction II (Neutral compounds), Fraction III (Bases) and Fraction IV (Weaker acids) following the procedures of Roberts *et al*., (1981). The active fraction was resolved by TLC and column chromatography using silica gel G and H (Merck) respectively with mobile phases Acetic acid: chloroform (1:9 v/v), benzene: chloroform (1:1 v/v) and chloroform: methanol: acetic acid: water (170:25:25:04 v/v). All the corresponding spots were again subjected to antibacterial activity.

Determination of Minimal Inhibitory Concentration (MIC)

MIC was determined by both agar and broth dilution methods. For broth dilution tests, 0.1ml 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) and incubated for 24h. at 37^0C for human pathogens and at room temperature for plant pathogens. In agar plating method dilutions having 0-20 µg/ml 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.

The data were subjected to statistical analysis using SPSS for windows software.

Results

Antibacterial activity

Aqueous extract

Highly significant antibacterial activity of the aqueous extract at 50 µl was observed against all pathovars of *Xanthomonas* and human pathogenic bacteria except *Klebsiella* sp., *Pseudomonas* sp. and *Salmonella typhimurium*. Among the phytopathogenic *Xanthomonas* pathovars, *Xanthomonas*

axonopodis pv. *phaseoli* was highly susceptible followed by *Xanthomonas axonopodis* pv. *malvacearum* and *Xanthomonas campestris* pv. *vesicatoria* at 50 µl concentration. Among the eleven susceptible bacteria tested *Staphylococcus aureus* was highly susceptible to the aqueous extract at 50 µl concentration (Table 1).

Solvent extracts

The results revealed that among the five solvents tested, methanol extract recorded highly significant antibacterial activity against all the *Xanthomonas* pathovars and all the 14 human pathogenic bacteria following the ethanol extract. Chloroform extract showed significant antibacterial activity against *Xanthomonas axonopodis* pv. *phaseoli* and *Xanthomonas campestris* pv. *vesicatoria* but no activity against *Xanthomonas axonopodis* pv. *malvacearum* whereas, petroleum ether and benzene extracts did not show antibacterial activity against all the test pathogens. *Xanthomonas campestris* pv. *vesicatoria* was highly susceptible among the *Xanthomonas* pathovars (Table 2) and among the fourteen bacteria *Streptococcus faecalis* was highly susceptible (Table 3) to methanol extract.

Table 2. Antibacterial activity of different solvent extracts of *Acacia nilotica* on phytopathogenic bacteria (Zone of inhibition measured in mm)

Extracts	*X. a. m.*	*X. a. p.*	*X. c. v.*
Petroleum ether	0.00 [a]	0.00 [a]	0.00 [a]
Benzene	0.00 [a]	0.00 [a]	0.00 [a]
Chloroform	0.00 [a]	11.75±0.25 [b]	13.50±0.81 [b]
Ethanol	19.75±0.04 [b]	19.82±0.11 [c]	19.37±0.12 [c]
Methanol	22.17±0.11 [c]	27.47±0.14 [d]	27.90±0.10 [d]

Average of four trials ± standard error
X. a. m.: *Xanthomonas axonopodis* pv. *malvacearum*
X. a. p.: *Xanthomonas axonopodis* pv. *phaseoli*
X. c. v.: *Xanthomonas campestris* pv. *vesicatoria*
Figures followed by different letters in rows differ significantly when subjected to TUKEY ($P< 0.001$)

Phytochemical analysis

Phytochemical analysis of all the solvent extracts revealed the presence of carbohydrates and glycosides, phytosterols, phenolic compounds, saponins, flavonoids, proteins and amino acids, gums and mucilages in both methanol and ethanol extract (Table 4). Further phytochemical analysis (Roberts *et al*., 1981) revealed that the antibacterial activity of methanol and ethanol extract is due to the presence of acidic compounds. Antibacterial activity was not observed in isolated compounds indicating the loss of antibacterial activity on further separation of the active fraction.

Table 4. Phytochemical analysis of *Acacia nilotica*

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 mucilage	--	--	--	++	++
Volatile oils	--	--	--	--	--

--: Absent
++: Present

Determination of Minimal Inhibitory Concentration

Minimal Inhibitory Concentration of the acidic fraction varied for different test pathogens. The Minimal Inhibitory Concentration required for each test pathogens is presented in Table 5 and 6.

Comparative antibacterial activity assay of the acidic fraction with that of the synthetic antibiotic gentamicin revealed highly significant activity against all the human pathogenic bacteria except *Staph*. *aureus*, *Salmonella paratyphi* B and *Shigella sonnei*, whereas similar evaluation with streptomycin revealed highly significant antibacterial activity against all the tested human pathogenic bacteria except *Staph*. *aureus* (Table 5).

Table 5. Comparative evaluation of MIC of acidic fraction with the synthetic antibiotics against human pathogenic bacteria (Zone of inhibition measured in mm)

Organisms	MIC (µg/ml)	Acidic fraction	Gentamicin	Streptomycin
Proteus mirabilis	12	15.50±0.04[a]	9.25±0.15 [a]	0.00 [a]
Citrobacter sp.	10	22.62±0.06 [g]	15.75±0.15 [d]	13.62±0.10 [g]
Klebsiella sp.	12	17.75±0.15 [c]	15.62±0.10 [d]	11.75±0.15 [e]
E. coli	12	15.72±0.17 [a]	14.62±0.10 [c]	10.62±0.10 [d]
S. aureus	10	18.60±0.00 [d]	22.37±0.10 [h]	22.75±0.15 [j]
Streptococcus faecalis	08	23.62±0.11 [h]	15.75±0.15 [d]	0.00 [a]
Pseudomonas aeruginosa	12	17.92±0.09 [c]	14.62±0.10 [c]	7.5±0.00 [b]
Salmonella paratyphi A	06	29.92±0.02 [i]	18.37±0.10 [f]	16.37±0.10 [h]
Salmonella paratyphi B	10	20.65±0.02 [f]	21.62±0.10 [i]	18.75±0.15 [i]
Salmonella typhi	10	20.65±0.07 [f]	20.62±0.10 [h]	0.00 [a]
Salmonella typhimurium	10	20.65±0.07 [f]	16.62±0.10 [e]	12.00±0.17 [f]
Shigella boydii	10	20.65±0.10 [f]	21.75±0.10 [i]	0.00 [a]
Shigella flexneri	10	19.70±0.12 [e]	14.40±0.10 [b]	9.25±0.15 [c]
Shigella sonnei	12	16.80±0.12 [b]	18.75±0.15 [g]	0.00 [a]

Average of four trials ± standard error

Figures followed by different letters in columns differ significantly when subjected to TUKEY ($P< 0.001$)

Among the phytopathogenic bacteria acidic fraction recorded highly significant antibacterial activity against all the *Xanthomonas* pathovars compared with synthetic antibiotics K-cycline and Bact-805 (Table 6).

Table 6. Comparative evaluation of MIC of acidic fraction with the synthetic antibiotics against phytopathogenic bacteria (Zone of inhibition measured in mm)

	X.a.m.	X.a.p.	X.c.v.
Fraction I	23.25±0.18	28.20±0.18	28.27±0.50
MIC	4 µg/ml	3 µg/ml	2 µg/ml
K-cycline	20.00±0.40	18.66±0.40	15.66±0.40
Bact-805	21.66±0.40	16.00±0.00	14.66±0.40

Average of four trials ± standard error

X. a. m.: *Xanthomonas axonopodis* pv. *malvacearum*

X. a. p.: *Xanthomonas axonopodis* pv. *phaseoli*

X. c. v.: *Xanthomonas campestris* pv. *vesicatoria*

Discussion

Further separation of the active fraction on TLC showed that the antibacterial activity was not observed in the isolated compounds, suggesting that, there may be synergism between the compounds in the fraction, hence antibacterial activity was not observed in the isolated pure compounds. It has been observed from the literature survey that there is a possibility of synergism between the compounds in a crude decoction than in isolated constituents (Daniel, 1999).

Among the plant diseases caused by bacteria, blackarm of cotton is severe. It was first described in 1891 by Atkinson from the United States, but the bacterial nature of the disease was not established until 1901 by E.F. Smith from the same country. 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 it has been studied in great detail by many workers in various parts of India and also in other countries. It is a disease of major concern in Maharashtra, Karnataka, Andhra Pradesh, Tamil Nadu and Madhya Pradesh (Rangasamy and Mahadevan, 1999).

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 acidic fraction of *A. nilotica* 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 (Aiyelagabe *et al*., 2000, Prashanth *et al*., 2001, Mouniswamy *et al*., 2002, Woldemichael *et al*., 2003, Truiti *et al*., 2003, Kubo *et al*., 2004, Chacha, 2005). In recent years development of multidrug resistance in the pathogenic bacteria and parasites has created major clinical problems in the treatment of infectious diseases (Davies, 1994). This and other problems such as toxicity of certain antimicrobial drugs on the host tissue (Idose *et al*., 1968, Maddux and Barrere,

1980) 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 *A. nilotica* has been demonstrated for the first time against phytopathogenic bacteria and human pathogenic bacteria suggesting the possibility of this plant in the management of plant pathogenic bacteria in an ecofriendly way, since compounds of biological origin are known to posses minimal residual effect.

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Table 1. Antibacterial activity of aqueous extract of *A. nilotica* against different pathogenic bacteria (Zone of inhibition measured in mm)

Organisms	10µl	20µl	30µl	40µl	50µl
Proteus mirabilis	9.05±0.02 [a]	12.05±0.02 [b]	13.10±0.05 [c]	14.95±0.05 [d]	15.90±0.04 [e]
Citrobacter sp.	8.66±0.09 [a]	10.40±0.05 [b]	11.92±0.04 [c]	12.95±0.05 [d]	13.66±0.12 [e]
Klebsiella sp.	0.00 [a]	0.00 [a]	0.00 [a]	0.00 [a]	8.92±0.02 [b]
E. coli	8.47±0.02 [a]	9.70±0.12 [b]	11.90±0.05 [c]	13.60±0.05 [d]	14.92±0.02 [e]
S. aureus	12.62±0.12 [a]	15.05±0.02 [b]	18.27±0.10 [c]	20.27±0.10 [d]	25.12±0.12 [e]
Streptococcus faecalis	7.07±0.02 [a]	7.57±0.07 [b]	8.40±0.05 [c]	9.40±0.05 [d]	11.99±0.02 [e]
Pseudomonas aeruginosa	0.00 [a]	0.00 [a]	0.00 [a]	7.25±0.14 [b]	9.05±0.02 [e]
Salmonella paratyphi A	7.17±0.03 [a]	8.65±0.09 [b]	9.60±0.05 [c]	10.45±0.05 [d]	11.40±0.05 [e]
Salmonella paratyphi B	7.27±0.10 [a]	8.45±0.02 [b]	9.57±0.07 [c]	10.85±0.05 [d]	11.60±0.05 [e]
Salmonella typhi	8.25±0.08 [a]	9.12±0.04 [b]	10.20±0.07 [c]	11.90±0.05 [d]	12.15±0.02 [e]
Salmonella typhimurium	0.00 [a]	0.00 [a]	0.00 [a]	7.57±0.07 b	8.82±0.11 c
Shigella boydii	9.27±0.10 [a]	10.85±0.06 [b]	11.82±0.11 [c]	13.52±0.10 [d]	14.65±0.08 [e]
Shigella flexneri	9.77±0.06 [a]	10.52±0.10 [b]	11.85±0.08 [c]	13.62±0.12 [d]	14.32±0.11 [e]
Shigella sonnei	8.67±0.11 [a]	9.82±0.06 [b]	10.85±0.05 [c]	11.75±0.09 [d]	12.70±0.12 [e]
X.a.m.	9.57±0.02 [a]	12.95±0.02 [b]	14.85±0.08 [c]	15.82±0.11 [d]	17.00±0.07 [e]
X.a.p.	10.75±0.12 [a]	12.60±0.05 [b]	14.25±0.04 [c]	15.65±0.08 [d]	18.40±0.05 [e]
X.c.v.	10.42±0.14 [a]	12.25±0.14 [b]	13.30±0.11 [c]	14.80±0.05 [d]	16.67±0.16 [e]

Average of four trials ± standard error
X. a. m.: *Xanthomonas axonopodis* pv. *malvacearum*
X. a. p.: Xanthomonas axonopodis pv. *phaseoli*
X. c. v.: *Xanthomonascampestris* pv. *vesicatoria*
Figures followed by different letters in columns differ significantly when subjected to TUKEY ($P < 0.001$)

Table 3. Antibacterial efficacy of different solvent extracts of *Acacia nilotica* against human pathogenic bacteria (Zone of inhibition measured in mm)

Organisms	Petroleum ether	Benzene	Chloroform	Methanol	Ethanol
Proteus mirabilis	0.00 [a]	0.00 [a]	0.00 [a]	14.37± 0.07 [c]	10.55± 0.10 [b]
Citrobacter sp.	0.00 [a]	0.00 [a]	0.00 [a]	13.92±0.02 [c]	12.33±0.06 [b]
Klebsiella sp.	0.00 [a]	0.00 [a]	0.00 [a]	12.82±0.11 [c]	12.87±0.04 [b]
E. coli	0.00 [a]	0.00 [a]	0.00 [a]	11.97±0.04 [c]	11.35±0.09 [b]
S. aureus	0.00 [a]	0.00 [a]	0.00 [a]	16.72±0.13 [c]	16.40±0.05 [b]
Streptococcus faecalis	0.00 [a]	0.00 [a]	0.00 [a]	19.82±0.11 [c]	14.87±0.04 [b]
Pseudomonas aeruginosa	0.00 [a]	0.00 [a]	0.00 [a]	13.20±0.12 [c]	11.25±0.14 [b]
Salmonella paratyphi A	5.75±0.14 [a]	5.75±0.14 [a]	5.62±0.12 [a]	14.37±0.12 [c]	13.12±0.12 [b]
Salmonella paratyphi B	5.75±0.14 [a]	5.75±0.14 [a]	5.75±0.14 [a]	15.15±0.06 [c]	12.90±0.06 [b]
Salmonella typhi	5.75±0.14 [a]	5.75±0.14 [a]	5.75±0.14 [a]	13.90±0.04 [c]	11.37±0.07 [b]
Salmonella typhimurium	5.75±0.14 [a]	5.87±0.12 [a]	5.75±0.14 [a]	14.77±0.09 [c]	12.02±0.04 [b]
Shigella boydii	5.75±0.14 [a]	5.87±0.12 [a]	5.75±0.14 [a]	14.85±0.11 [c]	11.35±0.08 [b]
Shigella flexneri	5.75±0.14 [a]	5.75±0.14 [a]	5.75±0.14 [a]	18.00±0.04 [c]	15.25±0.14 [b]
Shigella sonnei	5.75±0.14 [a]	5.87±0.12 [a]	6.00±0.00 [a]	18.65±0.15 [c]	15.75±0.08 [b]

Average of four trials ± standard error

Figures followed by different letters in columns differ significantly when subjected to TUKEY ($P< 0.001$)