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Anti-bacterial evaluation and phytochemical analysis of some Iranian medicinal plants against plant pathogenic *Xanthomonas* pathovars

S. Babu, S. Satish, D.C. Mohana, M.P. Raghavendra, and K.A. Raveesha*

Agricultural Microbiology Laboratory, Department of Studies in Botany, University of Mysore, Manasagangotri, Mysore-570006. India

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The aqueous, different solvent extracts and isolated constituents of seven higher medicinal plants viz., *Althea officinalis* L. (Malvaceae), *Origanum vulgare* Oregano (Lamiaceae), *Plantago lanceolata* L. (Plantaginaceae), *Polygonum bistorta* L. (Polygonaceae), *Satureja hortensis* L. (Lamiaceae), *Solanum dulcamara* L. (Solanaceae), and *Quercus robur* L. (Fagaceae), were screened *in vitro* for anti-bacterial activity by cup diffusion method against important phytopathogenic *Xanthomonas* pathovars viz., *Xanthomonas axonopodis* pv. *malvacearum* (*X. a*. pv. *m*.), *Xanthomonas axonopodis* pv. *phaseoli* (*X. a*. pv. *p*.) and *Xanthomonas campestris* pv. *vesicatoria* (*X. c*. pv. *v*.) associated with angular leaf spot of cotton, common blight of beans and bacterial spot of tomato. All the plants recorded antibacterial activity against *Xanthomonas* pathovars, whereas, among the seven plants tested methanol extract of *Origanum vulgare* and *Althea officialis* recorded highly significant antibacterial activity against all pathovars. The antibacterial activity is more significant in solvent extracts as compared to aqueous extract in all the plants indicating that the active principle responsible for antibacterial activity is more soluble in organic solvents. Comparison of the inhibitory activity of the extracts with the antibiotics bacterimycin 2000 and streptocycline revealed that methanol extracts of *Origanum vulgare* and *Althea officialis* were significantly higher than that of the antibiotics tested. Phytochemical analysis of methanol extract of *Origanum vulgare* and *Althea officialis* revealed that antibacterial activity is due to the presence of phenolic and acidic fractions respectively. The results suggest that *Origanum vulgare* and *Althea officialis* are potential candidate plants for the management of phytopathogenic *Xanthomonas* which are known to cause diseases on many crop plants.

Key words: antibacterial activity, Iranian medicinal plants, phytochemical analysis, *Xanthomonas* pathovars

* Corresponding author: Dr. Raveesha, K. A.; e-mail: karaveesha@gmail.com

Introduction

Pesticides have made great contribution for quick and effective management of plant diseases and microbial contaminations in several agricultural commodities. In-spite of use of all available means of plant protection, about 1/3 of the yearly harvest of the world is destroyed by pests and loss due to this is expected to be nearly $300 billion per year (Chandler, 2005). Incessant and extensive use of these synthetic pesticides are posing serious problem to the life supporting systems due to their residual toxicity (Campos *et al*., 2005). It is estimated that hardly 0.1% of the agro-chemicals used in crop protection reaches the target pest, leaving the remaining 99.9% to the environment to cause hazards to non target organisms including humans. In recent years a large number of synthetic pesticides have been banned in the Western world because of their undesirable attribute such as high and acute toxicity, long degradation periods, accumulation in the food chain and extension of their power to destroy both useful and harmful pests (Ortelli *et al*., 2005). Many pathogenic microorganisms have acquired resistance to synthetic pesticides (White *et al*., 2002). Pathovars of *Xanthomonas* are known to cause diseases on several vegetable and cash crops and are reported to have developed resistance to kanamycin, ampicillin, penicillin and streptomycin (Mandavia *et al*., 1999). This seriously hinders the management of diseases of crops and agriculture products. Considering the deleterious effects of synthetic pesticides on life supporting system, there is an urgent need for alternative agents for the management of pathogenic microorganisms (Mahajan and Das, 2003).

A green plant represents a reservoir of effective chemotherapeutants and can provide valuable sources of natural pesticides (Cowan, 1999; Newman *et al*., 2000; Gibbons, 2005). Reports are available on the use of active agents from higher plants, in place of chemical fungicides, that are non-phytotoxic, more systemic and easily biodegradable (Gottlieb *et al*., 2002). This led the authors to screen *in vitro*, a large number of plants for antibacterial activity against important seed borne phytopathogenic *Xanthomonas* pathovars, with the ultimate aim of developing plant based formulations for plant disease management (Kiran and Raveesha, 2006; Raghavendra *et al*., 2006; Kiran and Raveesha, 2007).

Materials and methods

Collection of plant materials

Fresh leaves of seven different plant species free from diseases were collected from Iran localities, washed thoroughly 2-3 times with running tap water and once with sterile water, shade-dried, powdered and used for extraction. A voucher specimen of the plant is deposited in the herbarium of Department of Studies in Botany, University of Mysore, Mysore.

Preparation of extracts

Aqueous extract

Samples (50g) of dried leaves, root and seeds of *Althea officinalis, Origanum vulgare, Plantago lanceolata, Polygonum bistorta, Satureja hortensis, Solanum dulcamara, and Quercus robur* were macerated with 100 ml sterile distilled water in waring blender (Waring International, new Hart ford, 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 Whatman No.1 filter paper and sterilized at 120°C for 30 min. The extract was preserved aseptically in a brown bottle at 5ºC until further use. The extract was subjected to antibacterial activity assay.

Solvent extracts

Fifty gm of shade dried, powder of leaf of seven medicinal plants collected from Iran locality were filled separately in the thimble and extracted successively with 200 ml each of Petroleum ether, Benzene, Chloroform, Methanol and Ethanol using a Soxhlet extractor for 48 hrs. All the extracts were concentrated using rotary flash evaporator. After complete solvent evaporation, each of these solvent extract was weighed and preserved at 5°C in airtight bottles until further use. One gm of each solvent residue was dissolved in 5 ml of methanol, which served as the test extracts for antibacterial activity assay.

Phytochemical analysis

Methanol extracts of *Origanum vulgare* and *Althea officinalis,* which recorded the highest antibacterial activities were subjected to phytochemical

analysis (Anon. 1985; Harborne, 1998). Fraction I (Phenolic compounds), Fraction II (Neutral compounds), Fraction III (Bases) and Fraction IV (Weaker acids) were obtained by sequential extraction of methanol extract of both the plants following the procedures of Roberts *et al*., (1981). All the fractions were subjected to antibacterial activity assay at 50µl concentration.

Plant pathogenic bacterial cultures

Authentic pure cultures of phytopathogenic *Xanthomonas axonopodis* pv *malvacearum* (*X. a*. pv. *m*.) isolated from cotton (*Gossypium herbaceum* L.), *Xanthomonas axonopodis* pv *phaseoli* (*X. a*. pv. *p*.) isolated from french bean (*Phaseolus vulgaris* L.) and *Xanthomonas campestris* pv *vesicatoria* (*X. c*. pv. *v*.) isolated from tomato (*Lycopersicon esculentum* mill.) were obtained from DANIDA lab, University of Mysore, India.

Anti-bacterial 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 were made in nutrient agar plate using sterile 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 50 µl each of all aqueous, solvent extracts and isolated constituents were placed in the cups made in inoculated plates. The treatments also included 50 µl of sterilized distilled water and methanol separately which served as control. Antibiotics as bacterimycin 2000 (Nitro propane hexadiol) (3µg/ml) (Source: T. Stanes and Company Ltd., 23, Race-cource Road, Coimbatore-641018, India) and streptocycline (Streptomycin sulphate I.P. and 90% Tetracycline Hydrochloride I.P. 10%) (1µg/ml)(Source: Hindustan Antibiotics Ltd., PIMPRI, Pune-411018, India) at their respective recommended dosage were also tested for comparative efficacy. The plates were incubated for 24 h. at 37°C and zone of inhibition if any around the wells were measured in mm (millimeter). For each treatment six replicates were maintained. The data was subjected to statistical analysis using SPSS for windows software.

Results

Antibacterial activity assay

Aqueous extract

Antibacterial activity of aqueous extracts of all the seven plants was presented in Table 1. Highly significant antibacterial activity was observed in *Origanum vulgare* followed by *Althea officinalis* and *Quercus robur*, respectively against all pathovars of *Xanthomonas*. Among the phytopathogenic *Xanthomonas* pathovars, *X. a.* pv. *m.* was highly susceptible followed by *X. a.*pv. *p.* and *X. c.* pv. *v.*, respectively.

Table 1. Zone of Inhibitory activity (in millimeter) of different extracts of some Iranian medicinal plants and antibiotics against some plant pathogenic pathovars of *Xanthomonas* at 50µl concentration.

Extracts		*X. a.* pv. *m*	*X. a.* pv. *p*	*X. c.* pv. *v*
Control aqueous	C	0.00±0.00	0.00±0.00	0.00±0.00
Control methanol	C	0.00±0.00	0.00±0.00	0.00±0.00
Althea officialis	A	11.2±0.25	10±0.49	9.8±0.32
	M	22.0±0.26	18.3±0.50	17.8±0.24
Origanum vulgare	A	14.4±0.33	13±0.21	12.8±0.29
	M	29.2±0.35	19.6±0.25	18.6±0.32
Plantago lanceolata	A	0.00±0.00	0.00±0.00	0.00±0.00
	M	16.8±0.26	15.4±0.33	14.2±0.36
Polygonum bistorta	A	0.00±0.00	0.00±0.00	0.00±0.00
	M	17.1±0.33	15.3±0.24	14.9±0.25
Satureja hortensis	A	0.00±0.00	0.00±0.00	0.00±0.00
	M	15.4±0.25	14.6±0.28	14.3±0.33
Solanum dulcamara	A	8.2±0.22	7.9±0.36	6.6±0.22
	M	16.3±0.31	15.2±0.34	14.9±0.25
Quercus robur	A	9.9±0.33	9.0±0.33	8.5±0.26
	M	18.5±0.32	17.3±0.31	16.9±0.24
Streptocycline	A	19.9±0.25	16.0±0.026	14.63±0.26
Bacterimycin 2000	A	10.00±0.43	11.38±0.026	11.25±0.25

Data given are mean of six replicates ± standard error, $p < 0.001$
A- Aqueous extract, M- Methanol extract, C- Control, A-Antibiotic.
X. a. pv. *m* - *Xanthomonas axonopodis* pv. *malvacearum*
X. a. pv. *p* - *Xanthomonas axonopodis* pv. *phaseoli*
X. c. pv. *v* - *Xanthomonas campestris* pv. *vesicatoria*

Solvent extracts

The ANOVA analysis of the data revealed that among the five solvents tested in each plants, methanol extract of all the plants showed highly significant activity against all the test pathogens (Table 1). Tukey HSD analysis of the data revealed that *X. a.* pv. *m.* was highly susceptible among the *Xanthomonas* pathovars, where as *X. c.* pv. *v.* showed the least susceptibity. Anti-bacterial activity of the methanol and aqueous extract of *Origanum vulgare* and *Althea officinalis* were highly significant when compared to Streptocycline and Bacterimycin 2000.

Phytochemical analysis of methanol extract Origanum vulgare and Althea officinalis

The phytochemical analysis of methanol extracts of *Origanum vulgare* and *Althea officinalis* revealed the presence of carbohydrates and glycosides, protein and amino acid, phenolic compounds, saponin, tannin, flavonoids, oils, gum and mucilage (Table 2). Further phytochemical analysis (Roberts *et al*., 1981) revealed that the antibacterial activity of methanol extract is due to the presence of phenolic and acidic compounds (Table 3).

Table 2. Zone of Inhibitory activity (in millimeter) of different fraction of methanol extract of of different extracts of *Origanum vulgare* and *Althea officinalis* against *Xanthomonas* at 50µl concentration.

Plants	**Fractions**	***X. a.* pv. *m.***	***X. a.* pv. *p.***	***X. c.* pv. *v.***
Origanum vulgare	Acidic fraction	15.4±0.35	15.0±0.25	14.3±0.45
	Basic fraction	0.00±0.00	0.00±0.00	0.00±0.00
	Neutral fraction	0.00±0.00	0.00±0.00	0.00±0.00
	Phenolic fraction	18.0±0.55	16.0±0.35	15.8±0.45
Althea officialis	Acidic fraction	12.4±0.35	12.2±0.65	11.6±0.75
	Basic fraction	0.00±0.00	0.00±0.00	0.00±0.00
	Neutral fraction	0.00±0.00	0.00±0.00	0.00±0.00
	Phenolic fraction	11.0±0.35	10.4±0.55	9.4±0.55

Data given are mean of six replicates ± standard error, $p < 0.001$

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

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

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

Discussion

Field existences of antibiotic resistant phytopathogenic bacteria are increasing in recent years (Mandavia *et al*., 1999). WHO banned many agriculturally important pesticides due to wide range of toxicity against non-target organisms including humans which are known to cause pollution problem (Barnard *et al*., 1997). Some of the developing countries are still using these pesticides despite their harmful effects. Exploitation of naturally available chemicals from plants, which retards the reproduction of undesirable microorganism, would be a more realistic and ecologically sound method for plant protection and will have a prominent role in the development of future commercial pesticides (Gottlieb *et al*., 2002). Many reports of antibacterial activity of plants extracts against human pathogens and their pharmaceutical application are available (Bylka *et al*., 2004; Kilani, 2006), but not much has been reported on the antibacterial activity of plants extract against plant pathogens (Satish *et al*., 1999).

Table 3. Preliminary phytochemical analysis of methanol extract of *Origanum vulgare* and *Althea officinalis.*

Phytochemical tests	***Origanum vulgare***	***Althea officinalis***
Alkaloids	--	--
Carbohydrates/Glycosides	+ +	++
Flavonoids	--	--
Gums and mucilage	+ +	++
Oils	++	++
Phenolic compounds	+ +	++
Phytosterols	--	++
Proteins/Aminoacids	+ +	++
Saponin	++	--
Tannin	+ +	++
Terpenoids	--	++

++: Present and --: Absent

Xanthomonas is an important phytopathogenic bacteria which involved in important diseases on many crop plants including cotton, pepper, tomato, beans and paddy rice etc. (Mohana *et al*., 2006) Seed sanitation to overcome plant diseases caused by seed borne 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 (Raghavendra *et al*., 2006). Even though these methods are effective, copper compounds and chlorine derivatives cannot be used in seeds

for human consumption (Campos *et al*., 2005). Hence, alternatives from biological sources would 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 active fractions of *Origanum vulgare* and *Althea officinalis* against the seed borne *Xanthomonas* pathovars *in vitro*. These results indicate the 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 present investigation as attempted to evaluate seven plants belonging to different families of the plant kingdom to show the fact the plants are still a reservoir of many pharmaceuticals which can be isolated and used in plant disease management. Several species of genus *Origanum*, which was used in the present study, was native to the Mediterranean, all of which are traded as a spice and *Althea officinalis* leaf is used for the lungs and urinary system, it may also be used in cases of urethritis and urinary gravel (Chevallier, 1996). The leaves of *Althea officinalis* make an excellent poultice for leg ulcers (Uphof, 1959). Since these pants are already known to the world for its medicinal value, the present study is successful in identifying a potential candidate plant which can be exploited for management of diseases caused by *Xanthomonas* on crop system as good seed protectant with minimal or no side effect on the nontarget organisms including man.

The anti-bacterial activities of aqueous, solvent extracts, fractions were compared with standard Streptocycline and Bacterimycin 2000 and the results are reported in Table 1 and 2. The results showed that the methanol extract of all test plants had more inhibitory effect than the other extracts. This tends to express that the active ingredients of the plant parts may be better extracted with methanol than other solvents. The phytochemical analysis of methanol extract revealed that the antibacterial activity of *Origanum vulgare* and *Althea officinalis* is due to the presence of phenolic and acidic compounds. Phenolic compounds are widely distributed in plant kingdom and main purpose is found to be defensive (Raskin *et al*., 2002.). The results of the present investigation is successful in identifying the nature of the bioactive principle and its solubility, which will help in further isolation and characterization of the active principle responsible for the activity.

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 *Origanum vulgare* and *Althea officinalis* has been demonstrated for the first time against phytopathogenic bacteria.

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