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BOTANICAL FORMULATIONS AGAINST CITRUS CANKER DISEASE (*XANTHOMONAS AXONOPODIS* PV. *CITRI*)

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ABSTRACT
Antibacterial activity of plant extracts were tested against *Xanthomonas axonopodis* pv. *citri* (XAC), causal organism of citrus canker. Under *in-vitro* study, out of 40 plant extracts screened, only nine organic solvent extracts *viz., Bixa orellana, Jatropha curcas, Lantana camara, Polyalthia longifolia, Polygonum hydropiper, Terminalia arjuna, Terminalia chebula, Zingiber officinale* and *Saraca asoca;* and three aqueous extracts *viz., Allium sativum, Bixa orellana* and *Terminalia chebula* were found to be effective against the pathogen. The probit analysis on plant extracts against the XAC with respect to 100 ppm streptomycin sulfate reveals that, *P. longifolia* (MeOH) was found most effective in lower concentration, with the lowest ED50 of 0.392% followed by ED50 of *T. chebula* (MeOH) (0.399%) and *Zingiber officinale* (CHCl3) (0.449%). The spraying of formulated plant extract 20 EC @ 10ml liters^{-1} positively reduced the disease symptom as compared to control.

INTRODUCTION

Citrus canker is highly contagious disease caused by the bacterium, *Xanthomonas axonopodis* pv. *citri*. An infestation can destroy entire orchard, but the disease poses no health risk to humans or animals. It can be a serious disease where rainfall and warm temperatures are prevalent during periods of shoot emergence and early fruit development. Citrus canker is mostly a leaf spotting and fruit rind blemishing disease, but when conditions are highly favorable infections cause defoliation reducing fruit quality and quantity, shoot dieback, and fruit drop. Citrus canker seriously limits citrus production in Asia and South America.The disease causes heavy losses when the infection occurs at early stages of plant growth (Gupta and Sharma 2008). Copper-based fungicides are a standard control measure worldwide for controlling citrus canker (Leite and Mohan, 1990). Long-term use of copper bactericides has several disadvantages including induction of copper-resistance in Xanthomonas populations (Canteros 2000) and accumulation of copper in citrus soils with potential phytotoxic and adverse environmental effects (Jinghua *et al*., 2011). The antibiotic streptomycin-resistant strain of XAC has also been reported by several researchers (Pruvost *et al*., 1999, Islam, *et al*., 2014). The use of botanicals and antimicrobial is a time honored practice for control of plant diseases and pests Ambarish, *et al*. (2011). Development of non toxic, safe and biodegradable alternative to synthetic pesticides has become the necessity. The humid tropics, especially the rainforest ecological zones, are endowed with abundant flora of families of plants and herbs with untapped pesticide potentials (Amadioha, 2003). Organic and integrated cropping systems have been proposed as possible solutions for reducing pesticide use, but the effect of reducing pesticide use on crop yield remains unclear (Hossard *et al.,* 2014). So, in an attempt to explore the plant base pesticides, an investigation was carried out to study the antibacterial activity of some potential plant extract formulations in laboratory as well as field condition against citrus canker which is a common disease of citrus in west Bengal

MATERIALS AND METHODS

Isolation of the bacteria

The bacteria infected samples were collected from the University Research Farm, Mohanpur, Bidhan Chandra Krishi Viswavidyalaya. The XAC was isolated from the disease sample using potato sucrose proton agar and was incubated at 29 ± 1°C in BOD. The bacterial culture thus obtained was preserved at the cold deep fridge and was used for further assessments Yumlembam *et al*.(2016).

Collection and identification of the plant samples for extraction

40 species of widely growing plants (Table 1) were collected from University campus at Mohanpur, Nadia. The plant samples (leaves or aerial parts and well ground seed), duly identified by the expertise, were well dried at 50°C, packed and used for extraction.

Methods of extraction

The plant samples were well dried under 50°C. The soxhlet apparatus was used for hot extraction following sequential extraction of the plant extracts from the solvents *viz.,* hexane, chloroform and methanol. The crude extract obtain from the soxhlet apparatus was collected and concentrated in a Buchi Rotary evaporator at 45°C then transferred in a pre-weighed

conical flask. The conical flask was allowed to evaporated the excess solvent under the water bath, after which only crude will remain. The crude plant extract was kept under cool dry place for further analysis.

Preparation of primary emulsifiable concentrates of the extracts

The crude plant extracts of known weight was mixed along with a light solvent naptha (LSN) and Tween 80 (the surfactant) to formulate 20 EC (Emulsifiable concentrate) of the plant extract. Formulation of the plant extracts were stored under cool dry and dark place until further assessment.

Testing of Bio efficacy of the plant extract

Disk diffusion technique using a paper disc was used for the testing of Bio efficacy of the plant extract. A bacterial lawn was prepared using spread plate technique, 0.1 ml of the bacterial suspension which is of 0.1 OD was spread upon the PSPA agar plates and allow to dry for 15 minutes aseptically under the laminar airflow. A filter paper disc impregnated with plant extracts is placed on bacterial lawn and were incubated at 29 ± 1 °C in BOD incubator for 24 hrs. Zone of Inhibition, *i.e.*, the area of no growth around the disc was then measured and taken as observation. The relative percentage inhibition of the test extract with respect to Streptomycin Sulfate 100 ppm was calculated by using the formula: (Ajay *et al.*, 2003; Gaurav *et al.*, 2010).

$$\text{Relative percentage inhibition by the test extract} = \frac{(x - y)}{(z - y)} X100$$

Where,

X: total area of inhibition of the test plant extract

Y: total area of inhibition of the solvent

Z: total area of inhibition of the antibiotic

Dose response studies

Dose response studies for the effective extracts against the test pathogen were studied *in vitro* following paper disc method at four different concentrations *i.e.*, 0, 0.1, 0.2, 0.3 and 0.5% and the effective dose (ED50) for inhibition of 50 % growth of the test pathogen and its Minimum inhibitory concentration were calculated.

Field evaluation of the extracts against the citrus canker

A healthy citrus plant was chosen from the research plot. A branch having three to four almost fully expanded leaves, each of Branches of the citrus plant was sprayed with 0.2% of 20 EC of the treatments and tagged. Twenty-four hours after spraying, four leaves per plant as well as four areas per leaf were selected for puncturing. In each area, 20 wounds were made and the upper leaf surfaces immediately sprayed with mixture of XAC strains. Inoculated seedlings were immediately covered with plastic bags for 48h. Inoculated leaves were immediately covered with plastic bags to maintain the relative humidity of 80-90%. After 14 days, numbers of lesions on treated leaves were counted using a X10 magnifier (Graham and Leite, 2004). The treatments include leaves inoculated with bacterial suspension (0.1 OD) as positive control, leaves sprayed only with sterile distilled water as negative control and other treatments *viz.*, *P. longifolia* in MeOH (Methanol) (20 EC) @ 0.2%, *T. chebula* in MeOH (20 EC) @ 0.2%, Salicylic acid @ 2g.L^{-1} (0.2%), Streptomycin 100 ppm (0.01%), and Solvent (LSN) @ 0.2% as shown in Table no. 2. The experiment was carried out in completely randomized design with 7 treatments and 3 replicatess. The mean number of lesions observed in each treatment were analyzed statistically. Infection rate was measured by dividing mean lesion number in each treatment by number of wounds on leaves produced by needle puncturing (40 wounds) as below: (Samavi *et al.*, 2009)

RI (%) = LN/NW× 100

Where: RI = Infection Rate,

LN = Mean number of lesions in each treatment,

NW = Number of wounds on leaves (40 wounds).

Another index was infection inhibition which was estimated through the following function:

II (%) = (RIC %-RII %)

Where: II = Infection inhibition,

RIC = Infection rate in positive control treatment (C+),

RII = Infection rate in expected treatment.

RESULTS AND DISCUSSION

Screening of effective plant extract by paper disc method

The plant pathogenic bacteria *X. axonopodis* pv. *citri*, causal organism of citrus canker was tested with solvent extracts *viz,* methanol, chloroform and hexane extract. The results of the study is presented in Table 3. Methanol extract of *T. chebula* gave the highest percentage ZOI of 50.20% which was statistically at par with *P. longifolia* (45.42%). Minimum ZOI was produced by *S. asoca* (35.19) and it was statistically at par with *T. arjuna* (39.12%) and *B. orellana* (40.46%). Chloroform extract of *T. chebula* gives the highest ZOI with a relative percentage of 44.39% which is statistically at par with *Z. officinale* (42.89%), *P. longifolia* (41.99%), *B. orellana* (40.44%), *P. hydropiper* (41.75%), *L. camara* (37.21%), and

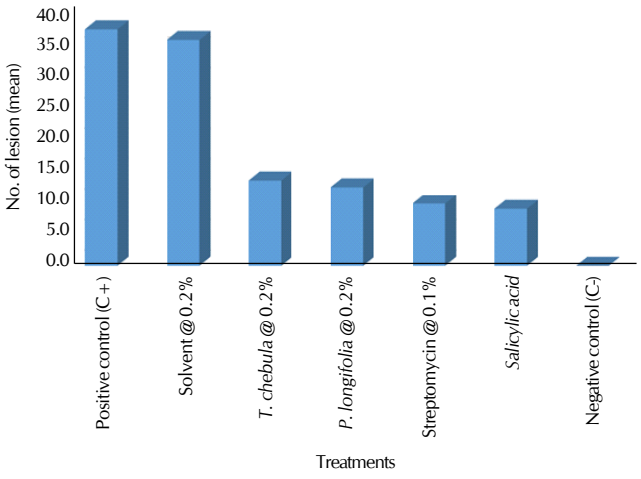

Figure 1: Effect of treatments on means of lesion numbers in field experiment

Table 1 : List of plants selected and its parts used for extraction

Sl.no	Common name	Scientific name	Family	Parts used
1.	Goat weed	*Ageratum conyzoides* C. Morren.	Berberidaceae	Tender leaves
2.	Chatim	*Alistonia scholaris* R. Br	(Apocynaceae	Leaves
3.	Onion	*Allium cepa* L.	Alliaceae	Fresh and dried bulbs
4.	Garlic	*Allium sativum* L.	Alliaceae	Fresh and dried bulbs
5.	Dil	*Anethum graveolens* L.	Umbelliferae	Seeds
6.	Custard apple	*Annona squamosa L.*	Annonaceae	Seeds
7.	Neem	*Azadirachta indica* A. Juss.	Meliaceae	Seeds
8.	Brahmi	*Bacopa monnieri* L.Penn.	Scrophulariaceae	Tender shoot
9.	Annatto	*Bixa orellana L.*	Bixaceae	Leaves
10.	Calotropis	*Calotropis procera* Aiton.	Apocynaceae	Leaves
11.	Cassia	*Cassia fistula* L.	Fabaceae	Leaves
12.	Clerodendron	*Clerodendrum inerme* L.	Verbenaceae	Leaves
13.	Fern	*Cryptogramma sp.*	Pteridaceae	Leaves
14.	Amanda	*Curcuma amada* Roxb.	Zingiberaceae	Fresh and dried rhizome
15.	Palmarosa	*Cymbopogon martini* Roxb.	Poaceae	Leaves
16.	Bamboo	*Dendrocalamus strictus*	Poaceae	Leaves
17.	Wild fig	*Ficus hispida* L.	Moraceae	Leaves
18.	Phalsa	Grewia asiatica L.	Tiliaceae	Leaves
19.	Khuleykhara	*Hygrophila spinosa* T.Anderson.	Acanthaceae	Tender shoot
20.	Ixora	*Ixora singaporensis* L.	Rubiaceae	Leaves
21.	Jatropha	*Jatropha curcas* L.	Euphorbiaceae	Mature leaves
22.	Lantana	*Lantana camara* L.	Verbenaceae	Tender leaves
23.	Curry Leaf	Murraya koenigii L. Sprengel	Rutaceae	Leaves
24.	Nicotiana	*Nicotiana tabacum* L	Solanaceae	Leaves
25.	Basil	*Ocimum basilicum* L.	Lamiaceae	Tender shoot
26.	Pachyrhizus	*Pachyrhizus erosus* L.	Fabaceae	Seeds
27.	Parthenium	*Parthenium hysterophorus* L.	Asteraceae	Young and mature plant
28.	Devdar	*Polyalthia longifolia* Sonn.	Annonaceae	Leaves
29.	Polygonum	*Polygonum hydropiper* L.	Polygonaceae	Leaves
30.	Pongamia	*Pongamia pinnata*. Linn.	Leguminosae	Seeds
31.	Bangikat	*Populus ciliata*	Salicaceae	
32.	Sarpagandha	*Rauvolfia serpentina* L.Benth.	Apocynaceae	Leaves
33.	Ruei	*Ruta graveolens* L.	Rutaceae	Leaves
34.	Wild brinjal	*Solanum indicum L.*	Solanaceae	Leaves
35.	Teak	*Tectona grandis* Linn.	Verbenaceae	Fresh and Fallen dried leaves
36.	Arjun	*Terminalia arjuna* Roxb.	Combretaceae	Mature leaves and fruits
37.	Haritaki	*Terminalia chebula*	Combretaceae	Mature leaves and fruits
38.	Nishinda	*Vitex negundo* Linn.	Verbenaceae	Tender shoot
39.	Ashwagandha	*Withania somnifera* Dunal	Solanaceae	Leaves
40.	Ginger	*Zingiber officinale* Roscoe	Zingeberaceae	Fresh and dried rhizome

Table 2 : Treatment details for controlling citrus canker disease of citrus.

Sl.No	Treatment number	Treatment details	Conc^{n}
1.	T_1	Salicylic acid	0.20%
2.	T_2	*P. longifolia* MeOH (20 EC)	0.20%
3.	T_3	*T. chebula* MeOH (20 EC)	0.20%
4.	T_4	Streptomycin sulphate	0.01%
5.	T_5	Solvent	0.20%
6.	T_6	Positive Control (Inoculated)	-
7.	T_7	Negative Control (un- Inoculated)	-

Solvent : Light solvent naptha (LSN) + Surfactant (Tween 80) (Solvent system used to prepare 20 EC of formulations.

T. arjuna (37.21%). Minimum ZOI was produced by *J. curcas* (21.06%) which was statistically at par with *L. camara* (27.27%) and *S. asoca* (34.12%). The hexane extract *L. camara* and *P. longifolia* gave ZOI of 20.61% and 27.27% respectively which was statistically at par. Overall maximum ZOI was given by *T. chebula* methanol (50.20 %) and *P. longifolia* methanol (45.42%).

Dose response studies

Dose response studies for the effective extracts against the pathogens were studied *in-vitro* following paper disc method at four different concentrations *i.e.*, 0, 0.1, 0.2, 0.3 and 0.5% and the Minimum inhibitory concentration was determined. The probit analysis on *Xac* (Table 4) reveals that, out of the three plant extracts tested, *P. longifolia* (MeOH) has the lowest ED_{50} of 0.392%. The ED_{50} of *T. chebula* (MeOH) and *Zingiber officinale* ($CHCl_3$) (chloroform) were achieved at 0.399% and 0.449% respectively.

Against citrus canker caused by *Xanthomonas axonopodis* pv. *citri*

The leaves were inoculated with bacterial suspension by pin-

Table 3 : Relative zone of inhibition produced by 0.2% of the plant extracts against *XAC* by using paper disc diffusion method.

Sl. No.	Scientific name	% of relative zone of inhibition		
		Methanol	Chloroform	Hexane
1.	*Bixa orellana* L.	40.46^{bc}	40.44^{a}	0.00^{b}
2.	*Jatropha curcas* L.	ND	27.27^{bcd}	0.00^{b}
3.	*Lantana camara* L.	ND	37.21^{ab}	20.61^{a}
4.	*Polyalthia longifolia* Sonn.	45.42^{ab}	41.99a	27.27^{a}
5.	*Polygonum hydropiper* L.	ND	41.75^{a}	0.00^{b}
6.	*Terminalia arjuna* Roxb.	39.12^{bc}	37.21^{abc}	0.00^{b}
7.	*Terminalia chebula*	50.20^{a}	44.39^{a}	0.00^{b}
8.	*Zingiber officinale* Roscoe	ND	42.83^{a}	0.00^{b}
9.	*Saraca asoca*	35.19^{c}	34.12^{abcd}	0.00^{b}
10.	Solvent (LSN @ 0.2%)	0.00^{d}	0.00^{e}	0.00^{b}
11.	Control (water)	0.00^{d}	0.00^{e}	0.00^{b}
S Em±		2.9	3.2	3.1
CD(0.05)		8.8	9.4	9

ND = NOT DONE (Figures followed by the same letter are not significant at $P < 0.05$)

Table 4 : Response of *X. axonopodis* pv. *citri* against different doses of plant extracts on paper disc method

Sl.No.	Extract	Solvent	% inhibition of *Xac* at different doses					Regression equation	R^2	ED_{50}
			Control	0.1	0.2	0.3	0.5			
1	*P. longifolia*	MeOH	0	0	40	47	53	y = 0.023 x - 0.717	0.982	0.392
2.	*T. chebula*	MeOH	0	0	40	42	56	y = 0.018 x - 484	0.98	0.399
3	*Z. officinale*	$CHCl_3$	0	0	38	40	53	y = 0.018 x - 444	0.98	0.449

Table 5 : Mean of lesion numbers and effect of plant extracts on citrus bacterial canker under field conditions

Sl. No.	Treatments	Conc ^{n}	Mean of lesions	Infection rate	Infection inhibition %
1.	*T. chebula* MeOH	0.2%	14.00c	35.00	62.12
2.	*P. longifolia* MeOH	0.2%	12.88d	32.19	64.94
3.	Salicylic acid	0.2%	9.37f	23.43	73.69
4.	Streptomycin	0.01%	10.25e	25.63	71.50
5.	Solvent	0.2%	37.13b	92.82	4.31
6.	Positive control (C+)	-	38.85a	97.13	0.00
7.	Negative control (C-)	-	0.00g	0.00	
	SEm±		0.144		
	CD 5%		0.438		

Figures followed by the same letter are not significant at $P < 0.05$

prick method on the lower surface of each leaf. The other treatment includes leaves inoculated with bacterial suspension as positive control, leaves sprayed only with sterile distilled water as negative control and other treatments as mentioned in the materials and method. Results showed a significant difference among all treatments at $P < 0.05$ on the number of lesions observed in each leaf. According to the Duncan multiple range test, treatments fell into seven groups ("a" to "d" in Table 5 and Fig. 1). The lowest number of lesion was observed in the negative control (C-) followed by Salicylic acid (9.38) and Streptomycin (10.25). These two latter treatments were in the same statistical group (c). On the other hand, as expected, the highest lesion numbers were observed in the positive control (C+) (38.88) and Solvent (37.13). These two latter treatments were in the same statistical group (a). The mean lesion numbers in the following treatments were not significantly different ($P < 0.05$): *P. longifolia* (12.88) and *T. chebula* (14.00). The results also showed that the highest inhibition of infection was afforded by Salicylic acid (73.75) and Streptomycin Sulfate (71.56). The inhibition of infection due to the plant extract of *T. chebula* MeOH and *P. longifolia* MeOH were 62.19 and 65.00, suggesting that these compounds could be effective in the control of citrus canker.

According to Rangaswami *and Lakshaman* (1959), 500-1000 ppm Streptomycin Sulfate was effective when sprayed with 1% glycerine on lime. Six sprays of 1000 ppm Streptomycin Sulfate along with two prunings reduced bacterial canker in acid lime (Balaraman and Purushotman, 1981). Other effective antibiotics were Agrimycin (Sawant *et al.,* 1985), Streptocycline (Mathur *et al.,* 1973) and Streptocycline in combination with Bordeaux mixture (Krishna and Nema, 1983). Kale *et al*. (1994) also suggested that for better control of canker, spraying of Streptocycline + copper oxychloride (0.1%) should preferably be done at 7th or 15th day intervals. Because copper-based bactericides are a standard control measure for citrus canker world-wide (Koizumi, 1985; Leite and Mohan,1990) and after long-term use resistance to copper in Xanthomonas populations was claimed (Rinaldi and Leite, 2000). Integrated pruning of infected twigs and application of 0.3% copper oxychloride, 100ppm Streptocycline and neem cake suspension was found very effective in controlling citrus canker (Das and Singh, 2000). The efficacy of *Terminalia chebula* and *Galla chinensis* to control late blight on potato plants in the field was low compared with a copper-based fungicide. In potato cultivar "Nicola" treated with *T. chebula* and *G. chinensis*, the percentages of inhibition were around 30%

and 10%, respectively. There was no control of late blight in potato cv. 'Agria' treated with *G. chinensis*. However, *T. chebula* did control late blight (40%) in potato cultivar 'Agria'. Poor rain fastness of plant extracts was proposed as the main factor for the limited late blight control in the field (Cao *et al*., 2004). Protection of field-grown potato and tomato plants may depend on the association of effective plant extracts with adhesive adjuvants. On the other hand, use of extracts without adhesives might be suitable for greenhouse-grown tomatoes (Eduardo *et al*., 2007).

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