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RESEARCH ARTICLE

IN VITRO INHIBITORY ACTIVITY OF CRUDE EXTRACTS OF PLANTS AGAINST CITRUS CANKER CAUSED BY *XANTHOMONAS AXONOPODIS* PV. *CITRI*

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ABSTRACT

Citrus canker is the most serious bacterial disease caused by bacterium *Xanthomonas axonopodis* pv. *citri*. In the present investigation the effect of crude methanolic, ethanolic and aqueous extracts of ginger rhizomes, curry leaves, neem, tulsi and parthenium leaves on *in vitro* growth of plant pathogen *Xac* causing citrus canker were tested. Different concentrations (25%, 50%, 75% and 100%) of these extracts were prepared. Among them all extracts of neem were found effective in inhibition of the growth of the test pathogen and its effect gradually increased with concentration. The methanolic extract of neem at 100% conc. given highest inhibition zone i.e. 14.0 mm followed by that of parthenium (10.6 mm) and tulsi (9.6 mm) extracts.

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INTRODUCTION

Lime or Acid lime (*Citrus aurantifolia)* is a shrub like tree native to Asia, belongs to *Rutaceae* family. In India, Andhra Pradesh, Gujarat, Bihar, Karnataka, Maharashtra, Assam & Chattisgarh are major lemon producing states. According to the data published by National Horticultural Board, India produced around 2835.02 metric tons of Lime in 2013-14 as the total area under cultivation of Lime was 286.41 thousand ha. Maharashtra contributes about 11% (306.0MT) of total Lime production in India. Citrus canker is the most serious bacterial disease caused by bacterium *Xanthomonas axonopodis* pv. *citri*. Citrus canker was first reported from Punjab (Luthra and Sattar, 1942).

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Once this disease becomes endemic in an area, it is very difficult to manage with commercially acceptable methods under favorable conditions for disease development (Das, 2003). The disease symptoms appear on leaves, branches and fruit stalks. Canker lesions appear as yellowish spots, which gradually enlarge and appear as raised, rough brownish pustules. These pustules are surrounded by a characteristic yellow hallow. Canker lesions on the fruits are confined to the rind only and do not penetrate into the flesh of the fruit. Appearance of canker lesions on the surface of fruits makes fresh fruits unacceptable for market (Goto, 1992). Lesion size depends on cultivar and the age of host tissue at the time of infection (Gottwald *et al.* 2002). The disease affected leaves drops very early, they may not serve as the main source of inoculums (Nirvan, 1960), but Rao and Hingorani (1963) found that the bacterium survives up to 6 months in the infected leaves. The disease is carried from season to season mainly in the cankers on twigs and branches. The most

effective management of canker is possible by supplementing the use of resistant cultivars with integrated systems of compatible cultural practices and phytosanitary measures. The basic strategies of the specific methods are to avoid, exclude, or eradicate the pathogen, to reduce the amount of inoculum available for infection, to minimize dissemination of the pathogen, and to protect susceptible tissue from infection (Civerolo, 1984) with integrated application of pruning of infected twigs and use of Copper oxychloride (0.3%), streptocycline (100 ppm) (Das and Singh, 2001) proved the control of citrus canker spray of neem cake solution (1kg /20 liter water) was found effective. The extracts from *Tamarindus indica* (Tamarind) was the most effective control of citrus canker which disease incidence was 48% by one spray after leaf puncture inoculation whereas the control was 100 % (Leksomboon *et al.,* 2001). Thus the aim of present study is to investigate the novel and more effecive solution against *X. axonopodis* pv. *citri* through herbal extracts using different concentrations.

MATERIALS AND METHODS

Isolation of *Xanthomonas axonopodis* pv. *citri*

Cirtrus canker affected leaves samples of Acid lime were collected from disease affected field. The collected samples were washed thoroughly with tap water and allowed to dry under shade. Then surface sterilized with 1% Sodium hypochlorite for 1 min and later wiped using sterile tissue paper. Again plant material washed with sterile water and placed on sterilized slide. Selected lesions were excised using sterile scalpel blade. Carefully used sterilized scalpel blade to halve and then quarter the excised lesion. A drop of sterilized water was added and kept for 2 to 5 minutes for maximum efflux of bacteria into saline. Small volume of exudate was collected using sterile wire loop and inoculated by streaking on nutrient agar plates aseptically. Further plates were incubated at room temperature and growth was observed after 2-3 days and as required thereafter. Bacteria streaked onto the plates containing nutrient agar medium were incubated at 30^{o}C for 72 hours and then desirable colonies were picked up and sub cultured for pure culture. Further cultures were stored in the refrigerator at 4^{o}C, which served as a stock culture for further studies.

Pathogenecity test by detached leaf method

Pathogenecity of the isolate of *Xac* was proved by detached leaf method. Disease free and fresh leaves of Acid lime were collected in sterile plastic bags just before the inoculation. Leaves were washed under sterile distilled water and then soaked in a fresh solution of 1% sodium hypochlorite for 2 mins. Then again rinsed with sterile water. Later it was air dried and placed in a petri plate containing moist filter paper in aseptic condition. For the inoculation of control, 10ml of sterilized distilled water was aseptically poured onto a healthy leaf in a petridish. A sterilized needle was used gently to prick the surface of leaf at the location of the droplets of sterile water 2-3 times each. For test, small amount of isolated *Xac* culture was resuspended in 10ml sterile distilled water. This resuspended culture was then poured onto another healthy leaf.The sterilised needle was used gently to prick the surface of each leaf at the location of the droplate of bacterial suspension. Then the both petridishes were wrapped and incubated at 25 to 30 ^{0}C for 8 to 10 days in a growth cabinate or on incubator equipped with white light. Observations were made for symptoms expression of canker at regular intervals.

Morphological and biochemical characteristics of *Xac*

The identification of the pathogen causing of Citrus canker on acid lime was determined by conducting studies on its morphological and biochemical features. Biochemical tests *viz*. Gram's reaction, Starch hydrolysis, Indole Production, Catalase test, KOH test, Gelatin liquefaction, Acid production etc. were carried out for biochemical confirmation of *Xanthomonas axonopodis* pv. *citri* as per standard microbiological procedures.

Preparation of plant extracts

The details of plants and their parts used in extract are given as follows,

Table 1. Name of the plats and their parts used in the work

S.No.	Common Name	Botanical Name	Plant part used
1.	Ginger	*Zingiber officinale*	Rhizome
2.	Curry tree	*Murraya koenigii*	Leaf
3.	Neem	*Azadirachta indica*	Leaf
4.	Tulsi	*Ocimum tenuiflorum*	Leaf
5.	Parthenium	*Parthenium hysterophorus*	Leaf

The inhibitory activity of selected plants as mentioned in Table no. were tested against *Xac*. Their extraction was carried out by using different solvents i.e. Methanol, Ethanol and Water. For the extraction fresh, disease free plant materials were collected, washed with tap water and then shade dried. After drying plant materials were powdered using grinder and stored in air tight bottles.10 grams of grinded powder of each plant was added into 100 ml of solvent (Methanol, Ethanol and Sterile water), mixed well, plugged with cotton plug and shaken periodically. After 24 hrs each supernatant was collected and directly used as crude extracts against *Xac*. The collected concentrated supernatants were further diluted as 75%, 50% and 25% with their respective solvents.

In vitro efficacy of selected plants against *Xac* by Paper disc method

Effect of selected plant extracts against *Xac* was tested by paper disc inhibition assay method. The culture of bacterium *X. axonopodis* pv. *citri* was multiplied by inoculating the loopful culture in 250 ml conical flask containing 100 ml of nutrient broth medium. The inoculated flasks were incubated at 28^{0}C for 72 hours. The 20 ml bacterial suspension was added to autoclaved molten cooled 1000 ml nutrient agar medium at room temperature. The seeded medium was thoroughly mixed and poured into the sterilized petriplates and media was allowed to solidify. Plant extracts were prepared at different concentrations i.e. 25%, 50%, 75%, 100%.The filter paper discs (Whatman No. 42) measuring 5 mm in diameter were

soaked in the respective extract solution for 5 minutes and transferred onto the surface of the seeded medium in petriplates. The plates were incubated at 25 – 30 °C temperature for 72 hours and observed for the inhibition zone around the filter paper discs and obtained results were analysed and noted down. Paper discs soaked in respective solvents were served as control.

RESULTS AND DISCUSSION

Isolation of *Xac* from diseased leaves sample

A bacterium was isolated from infected leaves collected from local area of Loni.

Bacterial Citrus canker symptoms were detected in collected citrus samples. Nutrient Agar plates showed the bacterial colonies after 48 hours of incubation at 30ºC temperature. The Pale yellow to yellow pigmented bacterial colonies identical to *Xanthomonas* were selected for further study [Plate 1.1 (a)]. The isolate was then purified by streak plate method and maintained on NA slants to preserve for longer period.

Pathogenicity test by detached leaf method (*in vitro*)

Plates showing well separated, typical, yellow colonies of *Xanthomonas* bacterium were used to check pathogenicity on detached leaves of Pomegranate to confirm the isolate.

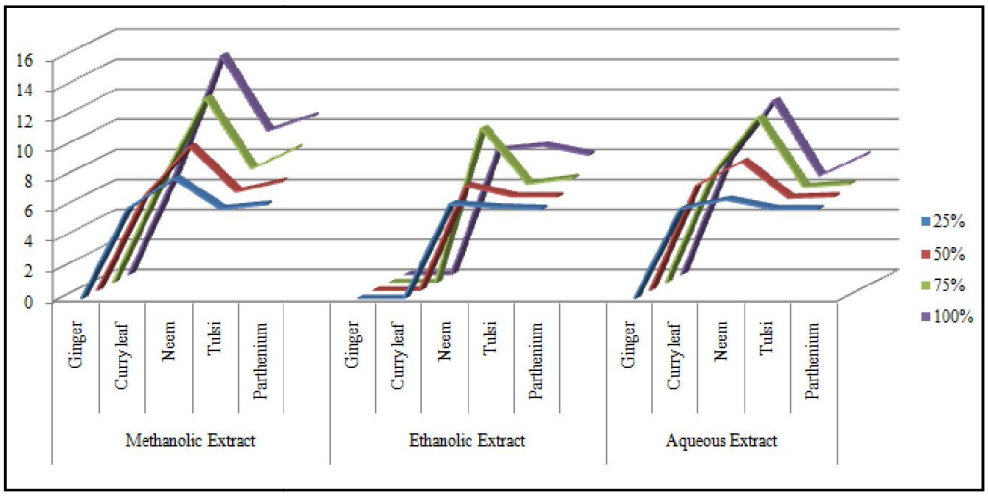
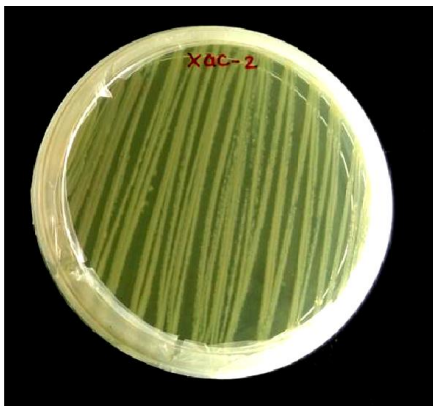


Fig.1. Inhibitory effect of different solvent extracts on *X. axonopodis* pv *citri* with its respective zone of inhibition



1.1 (a)

1.1 (b)

Plate 1.1 (a). Isolated colonies of Xac on NA plate (b) Symptoms of citrus cankert observed on leaves after 10-12 days of inoculation in pathogenicity test

1.2 (a)

1.2(b)

Plate 1.2 (a). Control plate (b) Plate showing maximum inhibitory effect of Neem methanolic extract at 100 % concentration

The characteristic symptoms of the disease appeared after 10 to 12 days of inoculation on leaves [Plate 1.1 (b)]. Reisolation carried out from artificially inoculated plants yielded the bacterial colonies similar to the previous one. Present findings corroborates with the findings of Jabeen *et al.* (2011) who reported that three methods of inoculation, clipping, pin prick and paint brush were tested both on detached leaves and on attached leaves *In -vitro* and *In- vivo* experiments successfully. The results are also confined with the findings of Al-Saleh *et al*. (2014) who were reported pathogenicity of *Xac* strains on detached grapefruit leaves.

Table 2. Presented in gallery proof does not differentiate the extract type of solvent with plant used and their respective zone of inhibition

Extract Type	Plant used	Concentration (%)			
		25%	50%	75%	100%
		Zone of inhibition (mm)			
Methanolic	Ginger	0	0	0	0
	Curry leaf	6	6.2	6.5	6.7
	Neem	8	9.6	12.3	14.6
	Tulsi	6	6.6	7.6	9.6
	Parthenium	6.3	7.3	9	10.6
Ethanolic	Ginger	0	0	0	0
	Curry leaf	0	0	0	0
	Neem	6.3	7	10.3	8.3
	Tulsi	6.1	6.3	6.6	8.6
	Parthenium	6	6.3	7	8
Aqueous	Ginger	0	0	0	0
	Curry leaf	6	7	7.2	7.6
	Neem	6.6	8.6	11	11.6
	Tulsi	6	6.2	6.4	6.6
	Parthenium	6	6.3	6.6	8

Morphological and biochemical characteristics of *Xac*

The isolated bacterium was shown gram negative reaction for gram staining. The bacterium was positive for Starch hydrolysis, Indole Production, Catalase test, KOH test, Gelatin liquefaction, Acid production test. Results are in agreement with Vernière *et al*. (1998), Gottwald *et al*., (2002), and Mubeen *et al.* (2015) who reported the *Xanthomonas axonopodis* is a rod shaped Gram negative bacterium with the same biochemical characteristics.

In vitro* efficacy of selected plants against *Xac

In order to assess the efficacy of selected plants against isolates of *Xac* an experiment was conducted and the evaluation was made by paper disc method (Plate 1.2(a) and 1.2(b)). The antibacterial activity of methanolic, ethanolic and aqueous extracts of selected plants at different concentrations were tested. The data presented in Table 1.2 revealed the significant differences in zones of inhibition (in mm) among them. The methanolic, ethanolic and aqueous extracts of Neem were found significantly superior in controlling the growth of *X. axonopodis* pv. *citri* producing maximum zones of inhibition (14.6 mm), (10.3 mm) and (11.6 mm) respectively at its 100% concentration. Apart from this, methanolic extract of Parthenium at 100% concentration shown 10.6 mm zone of inhibition. Tulsi extracts showed average zone of inhibition in all solvants. Among the all extracts of Ginger, zone of inhibition was not found. While curry leaf extracts were also not much effective against *X. axonopodis* pv. *citri. (Fig. 1)*. The present findings are in agreement with findings of Abhang (2015), who reported that, neem extract (5%) was effective in inhibiting the growth of *Xanthomonas axonopodis* pv. *citri* as assessed *in vitro* by paper disc method. Das and Singh (2000) reported that neem cake suspension was found very effective in controlling the *Xanthomonas axonopodis* pv. *citri.* Patel *et.al* (2015) found the ethanol extracts of *Azadirachta indica* (Neem) effective against *X. axonopodis* pv. *punicae.*

Conclusion

On the basis of the present *in vitro* study against *Xac*, the maximum zones of inhibition were recorded in methanolic, ethanolic and aqueous extracts of Neem. It could be concluded that, Neem extract in different solvents were found significantly superior in inhibiting the growth of *X. axonopodis* pv. *citri* than other tested extracts.

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