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ANTIBACTERIAL ACTIVITY OF *JUNIPERUS COMMUNIS* L. AND *VITEX NEGUNDO* AGAINST *XANTHOMONAS AXONOPODIS* PV *PUNICAE IN VITRO*

Digvijay*[1], Satyavrat Bhardwaj[1], Pankaj Kumar[2]

[1]Department of Biotechnology, Dr Y S Parmar University of Horticulture and Forestry, Nauni, Solan (HP) 173230.

[2]Dept of biotechnology, Dr Y S Parmar University of Horticulture and Forestry Nauni Solan India.

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***Correspondence for Author**
Dr. Digvijay Singh
Department of Biotechnology, Dr Y S Parmar University of horticulture and Forestry, Nauni, Solan (HP).

ABSTRACT

Leaf solvent extracts of *Juniperus communis* L. and *Vitex negundo* were prepared in different solvents (methanolic, ethanolic, petroleum ether and water) and were evaluated for antibacterial activity against *Xanthomonas axonopodis* pv *punicae in vitro*. Different working concentrations for each extract were prepared separately viz. 20, 40, 60, 80, 100, 200, 300 ppm and evaluated for their antibacterial activity at 24, 48 and 72 hours respectively. Among these plants extracts concentrations methanolic and ethanolic extracts at 300ppm were more effective than that of other extracts concentrations for both plants. The maximum zone of inhibition was found to be exhibited by ethanolic extract of *Juniperus communis* L. (21 mm) and methanolic extract of *Vitex negundo* (26 mm) at 300 ppm concentration after 48 hour and 72 hours respectively. All the plant extracts show inhibitory effect on linear growth of *Xanthomonas axonopodis* pv. *punicae*.

KEYWORDS: Medicinal plants, Antibacterial activity, zone of inhibition, *Xanthomonas axonopodis* pv. *punicae*.

INTRODUCTION

Bacterial blight of pomegranate caused by *Xanthomonas axonopodis* pv. *punicae*, has become a major constraint in important pomegranate producing states of Maharashtra, Karnataka and Andhra Pradesh of India. In addition to this, *X. axonopodis* pv. *punicae* has

also become a serious threat to pomegranate cultivation and also to the tremendous export potential such cultivation represents because of the effect of its disease on the quality and quantity of the fruit. As India is an area where *X. axonopodis* pv. *punicae* infections are presently endemic, the species is also a potential threat to pomegranate-growing areas of the world where such infections are not endemic. In view of enormous losses which may extend up to 60-80% in unmanaged orchards under epidemic conditions. [1]

Control of these pathogens is a major challenge in agriculture. Uses of plant-derived products as disease control agents have been studied, since they tend to have low mammalian toxicity, less environmental concerns and wide public acceptance. [2] In agriculture, however crop loss due to plant pathogens has become a major concern. Plant based natural constituents can be derived from any part of the plant like bark, leaves, flowers, roots, fruits, seeds etc. [3] The beneficial effects of plant materials typically results from the combination of secondary products present in the plant. [4]

North-West Himalayas are well known as a treasure house of medicinal plants. Himachal Pradesh, a North-West Himalayan state having a geographical area of 55,673 Km^2 aptly showcases its medicinal plant richness and diversity of the zone that is spread over its different agro-climatic zones. Medicinal plant diversity in the state can also be appreciated from the fact that its medicinal plants are spread across more than 100 plant families. Besides the trade of medicinal plants harvested from the wild, some species are also cultivated for sale. [5]

At present, quick and effective management of plant diseases and microbial contamination in several agricultural commodities is generally achieved by the use of synthetic pesticides. [6] However, the indiscriminate application of these chemical pesticides has caused health hazards in animals and humans due to their residual toxicity. [7] This seriously hinders the control of crops diseases and agricultural product. Considering the deleterious effects of synthetic pesticides on life supporting systems, there is an urgent need for alternative agents for the management of pathogenic micro-organisms. [8, 9] Biological control is an alternative to chemicals in the control of plant pathogens, or in order to reduce environmental pollution. It has been described as a non-hazardous strategy to recently, evaluation of plant extracts against many *Xanthomonas* species is becoming an important area. Thus, a focus should also be on indigenous practices of the farmer to look for their effectiveness.

In the light of these above enumerated facts, the present study was carried out on biological control of *Xanthomonas axonopodis* pv. *punicae* by using solvent extracts of *Juniperus communis* L. and *Vitex negundo*(*in vitro*).

MATERIAL AND METHODS

Collection of plant material

The leaves of *J. communis* and *Vitex negundo* were collected in the month of March- April and Feb- March from Kinnaur and Mandi districts of Himachal Pradesh, India respectively in their active growth period.

Preparation of plant extract

Leaves of the plants were thoroughly washed and dried under shade at the room temperature (20 ± 2°C). The dried leaves were then ground to a fine powder in an electric grinder. Stock solutions of the extract were prepared by adding ground leaf powder to 200 ml of each solvent (w/v, 50 g/ 200 ml). Various solvents which were used for extraction were petroleum ether, methanol, ethanol, and water i.e. extraction according to polarity. Prepared extracts were then shaken for at least 6 h for homogenous mixing of ground leaf powder in the solvent. After that each extract was passed through Whatmann filter paper no.1. Final filtrate was then concentrated to 25% crude extract on a rotary evaporator under vacuum at 20°C and was utilized for the experiments.

Isolation and maintenance of the pathogen

The bacterial strain was isolated from the infected leaves, small twigs and fruits of affected *Punica granatum* trees from fields of Department of Fruit Science, Dr Y.S. Parmar University of Horticulture and Forestry, Nauni, Solan, H.P. These tissues were washed, air dried, cut into small sections with sterilized razor blades and then disinfected with 0.1% $HgCl_2$ for about 1-1½ minute and washed thrice with sterile distilled water to remove traces of $HgCl_2$.They were macerated with sterilized blade in an autoclaved petriplate containing few drops of sterile distilled water in order to allow the bacteria to diffuse out. A loopful of suspension was then transferred with the help of bacteriological needle to autoclaved petriplates filled with nutrient agar medium with sucrose and incubated at 28±2°C for 24-72 hr. Incubated petriplates were observed for the presence of typical pale yellow, glistening colonies after 48 to 72 hr. and then transferred to the nutrient agar medium with sucrose slants. The cultures were maintained on yeast extract glucose agar with charcoal slant for further studies i.e. *in vitro* antibacterial activity.

Identification and characterization of pathogen

Identification and characterization of *Xanthomonas axonopodis* pv. *punicae* was performed by evaluating bacterial isolates through Gram staining, potassium hydroxide (KOH) solubility test, Kovac's oxidase test. [10] starch hydrolysis, Lipase activity and Arginine dehydrogenase test. [11] gelatin hydrolysis, and catalase tests.

Screening of antibacterial activity

Antibacterial tests of selected microorganism were carried out using disc-diffusion method.[12] Nutrient agar plates (90 mm size) were prepared and cooled down at room temperature (20 ± 2°C). A small sterile cotton swab was dipped into the 24 hour old culture of bacteria and was inoculated by streaking the swab over the entire agar surface. This process was repeated by streaking the swab two or more times rotating the plates approximately 60° each time to ensure even distribution of inoculum. After inoculation the plates were allowed to dry at room temperature (20 ±2°C) for 15 minutes in laminar chamber for settle down of inoculum. The filter paper discs (5 mm) loaded with 40 µl of extract were placed on the surface of the bacteria seeded agar plates and it was allowed to diffuse for 5 min then these plates were incubated at 37 ± 1°C for 24 hour.

Statistical analysis

Statistical analysis of collected data was conducted using Statistical Package for the Social Sciences (SPSS) 16.0 software package. The least significant difference at 5 per cent level was used for testing the significant differences among treatments. The data for both the years of investigation were pooled after performing homogeneity test. The heterogeneous data were pooled by the weighed means method. [13]

RESULTS

Effect of essential oils of *Juniperus communis* L. on growth of *Xanthomonas axonopodis* pv. *punicae*

In the present studies, bacterial strain was found to be sensitive when incubated with four different plant solvent extracts containing essential oils. The maximum zone of inhibition in case of *Juniperus communis* was observed to be 21 mm exhibited by ethanolic extract at 300 ppm after 48 hr. of incubation (Table 1and Figure 3). Among all the four plant solvent extracts, ethanolic extract exhibited maximum antibacterial activity followed by methanolic, petroleum ether and aqueous extract respectively.

The interactions of different factors i.e. Incubation Time (D), concentration (C) and type of solvent extract (E) were studied using statistical three way CRD Factorial analysis. All the mean values generated from this analysis revealed that maximum zone of inhibition was observed at 300 ppm of ethanolic extract at 48hr. of incubation, completely supporting the results given in Table 1 and graphical presentation shown in Figure 1 of the present studies.

Effect of essential oils of *Vitex negundo* on growth of *Xanthomonas axonopodis* pv. *Punicae*

The methanolic extract of *Vitex negundo* exhibited maximum zone of inhibition against *Xanthomonas axonopodis* pv. *Punicae* at a concentration of 300 ppm after 72 hr. of incubation. All the four plant solvent extracts of *Vitex negundo* were found effective against the pathogen and the order of their antibacterial activity was methanolic extract followed by ethanolic, petroleum ether and aqueous extracts (Table 2 and Figure 3).

When the means of interactions between factors like Incubation Time (D), concentration (C) and type of solvent extract (E) were compared using statistical three way CRD Factorial analysis, revealed and supported the results shown by Table 2 i.e. the maximum zone of inhibition was exhibited by 300 ppm methanolic extract at 72 hrs. These *in vitro* zones of inhibition are also shown in graphical presentation (Figure 2).

DISCUSSION

Polarity of the extracting solvent greatly influences the antimicrobial property. The activity of plant extracts against both gram positive and gram negative bacteria may be an indicative of the presence of broad spectrum antibiotic compounds or simply general metabolic toxins in the plant. As evident from the available literature, *J. communis* L. is well documented for its use for remedies of various ailments. Due to stressful climatic and geophysical conditions, Western Himalayan region plants offer greater possibilities of having novel molecules and even larger quantities of active compounds. [14, 15] Relying upon the results obtained in the present investigation, it is clear that almost all extracts of leaves of *J. communis* were effective against the pathogenic bacteria except aqueous extract. The ethanol fraction showed more activity followed by methanol, petroleum ether extract and aqueous extract. This might be due to the various substances that show activity against bacteria are more soluble in organic solvents than aqueous medium and therefore, not present in aqueous extract. [16] Using crude extracts of *J. communis* (ethanol, methanol, chloroform, petroleum ether and aqueous) against some animal pathogenic bacteria using agar-well method showed significant

inhibition of bacterial strains. [17] It is also found that chloroform extract of *J. communis* leaves was most effective but in the present study the methanol extract showed highest activity against *Xanthomonas axonopodis* pv. *punicae*. This variation in the results may be as a result of different techniques followed (Disc diffusion method). Other researcher also conducted antimicrobial activity of isolated compounds of *J. communis* against animal pathogenic bacteria and their result is in agreement with this our study. [18, 19] On the basis of available literature it was observed that there is no previous record on the sensitivity of these plant pathogenic bacterial strains *E. chrysanthemi*, *A. tumefaciens* and *X. phaseoli* which are responsible for various plant diseases like crown gall, leaf blight, leaf spot and rot disease. It concludes that *J. communis* leaves extracts possess a broad spectrum activity against a panel of bacteria responsible for the most common human and plant bacterial diseases. [20]

In contrast to our results for plant extracts of *Vietx negundo,* no antibacterial activity was reported against bacterial strains like *B. subtilis, S. aureus, S. epidermidis, E. coli, and P. aeruginosa* when pathogenic bacterial strains were incubated with dichloromethane: methanol (1:1 v/v) extracts. [21] The antibacterial activity of petroleum ether, chloroform, methanol and aqueous extracts of *V. negundo* bark and leaf against *B. subtilis, S. aureus, S. epidermidis, S. typhimurium, P. aeruginosa, V. cholerae,* and *V. alginolyteus* had shown little activity. [22] Dichloromethane, ethyl acetate, ethanol, methanol and aqueous extracts of leaf, flower and fruit of *Vitex negundo* and bulb of *Allium sativum* were subjected for the antibacterial activity against two phytopathogenic bacteria *Pseudomonas solanacearum* and *Xanthomonas axonopodis* and were found to be effective against these bacterial strains. [23, 24] The data clearly revealed the antibacterial activity of ethyl acetate extract of flower and fruit of *Vitex negundo* and bulb of *Allium sativum* and ethanol extract of flower of *Vitex negundo* against all the test bacteria. It was also observed that the effect was higher in ethyl acetate extract and less in other four extracts. [25, 26] It has been already reported that the ethyl acetate extract of *Vitex agnus-castus* and *Vitex negundo* showed higher inhibitory effect on some other bacterial pathogens. [27, 28, 29] However, our results have shown comparatively better inhibitory effects with plant solvent extracts of *Juniperus communis* L. and *Vitex negundo*.

Table 1: Zone of inhibition of *Juniperus communis* L. against *Xanthomonas axonopodis* pv. *Punicae.*

Extracts	24 Hours				48 Hours				72 Hours				C×E			
	Concentration (ppm)				Concentration (ppm)				Concentration (ppm)				Concentration (ppm)			
	100	200	300	Mean	100	200	300	Mean	100	200	300	Mean	100	200	300	Mean
Methanol	13.00	15.00	16.00	14.67	16.00	16.00	18.00	16.67	14.00	16.00	18.00	16.00	14.33	15.67	17.33	15.78
Ethanol	10.00	17.00	12.00	13.00	18.00	18.00	21.00	19.00	15.00	18.00	20.00	17.67	14.33	17.67	17.67	16.56
Petroleum Ether	7.00	8.00	5.60	6.87	14.00	11.00	15.00	13.33	8.00	8.80	10.50	9.10	9.67	9.27	10.37	9.77
Water	7.00	12.00	13.00	10.67	8.00	10.00	12.00	10.00	9.00	11.20	13.50	11.23	8.00	11.07	12.83	10.63
Mean	9.25	13.00	11.65	11.30	14.00	13.75	16.50	14.75	11.50	13.50	15.50	13.50	11.58	13.42	14.55	

$CD_{0.05}$ D 1.13 **C** 1.13 **E** 1.30

D x C 1.95 **D x E** 2.26 **C x E** NS

D x C x E NS

Table 2: Zone of inhibition of *Vitex negundo* against *Xanthomonas axonopodis* pv. *Punicae.*

Extracts	24 Hours				48 Hours				72 Hours				C×E			
	Concentration (ppm)				Concentration (ppm)				Concentration (ppm)				Concentration (ppm)			
	100	200	300	Mean	100	200	300	Mean	100	200	300	Mean	100	200	300	Mean
Methanol	21.00	21.00	22.00	21.33	26.00	18.00	20.00	21.33	20.00	21.00	26.00	22.33	22.33	20.00	22.67	21.67
Ethanol	18.00	19.00	15.00	17.33	15.00	16.00	18.00	16.33	15.00	16.00	18.00	16.33	16.00	17.00	17.00	16.67
Petroleum Ether	13.00	19.00	10.00	14.00	10.00	15.00	19.00	14.67	5.00	10.00	10.20	8.40	9.33	14.67	13.07	12.36
Water	5.00	7.00	7.20	6.40	7.00	8.00	10.00	8.33	8.20	10.20	13.60	10.67	6.73	8.40	10.27	8.47
Mean	14.25	16.50	13.55	14.77	14.50	14.25	16.75	14.77	12.05	14.30	16.95	14.43	13.60	15.02	15.75	

CD0.05 D 0.47 **C** 0.47 **E** 0.54

D x C 0.81 **D x E** 0.94 **C x E** 0.94

D x C x E 1.63

*****D- means incubation time, C- means concentration of plant extract in ppm, E- means type of plant extract used.

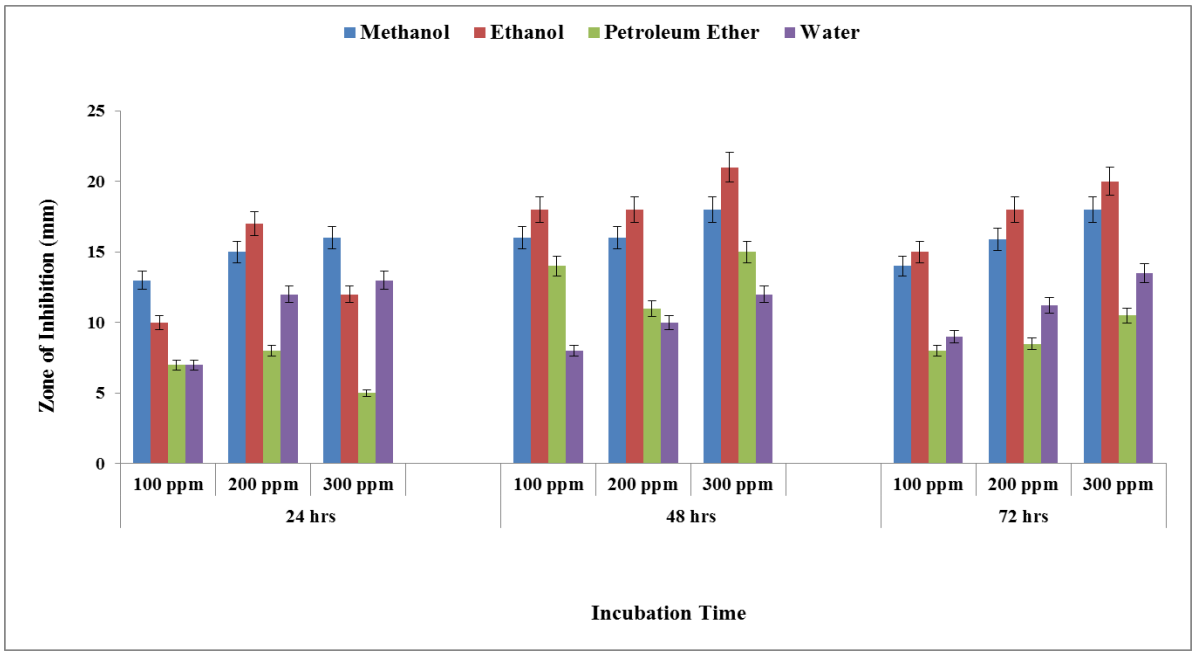

Figure1: Graph showing zone of inhibition of *Juniperus communis* L. against *Xanthomonas axonopodis* pv. *punicae*.

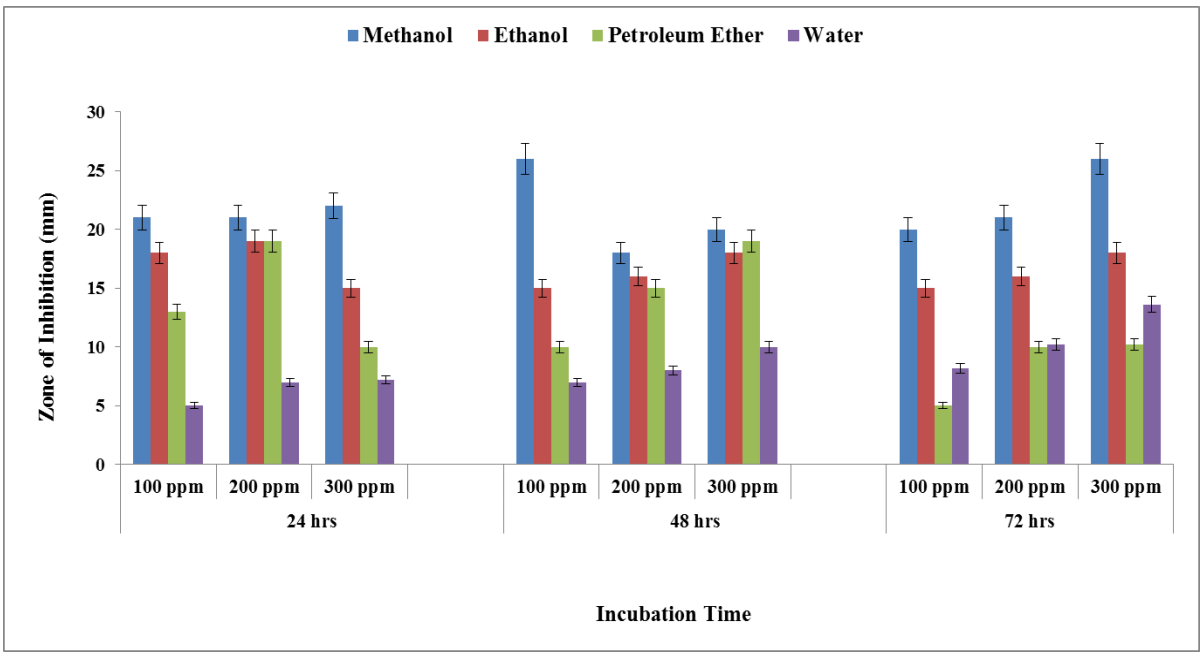

Figure 2: Graph showing zone of inhibition of *Vitex negundo* against *Xanthomonas axonopodis* pv. *punicae.*

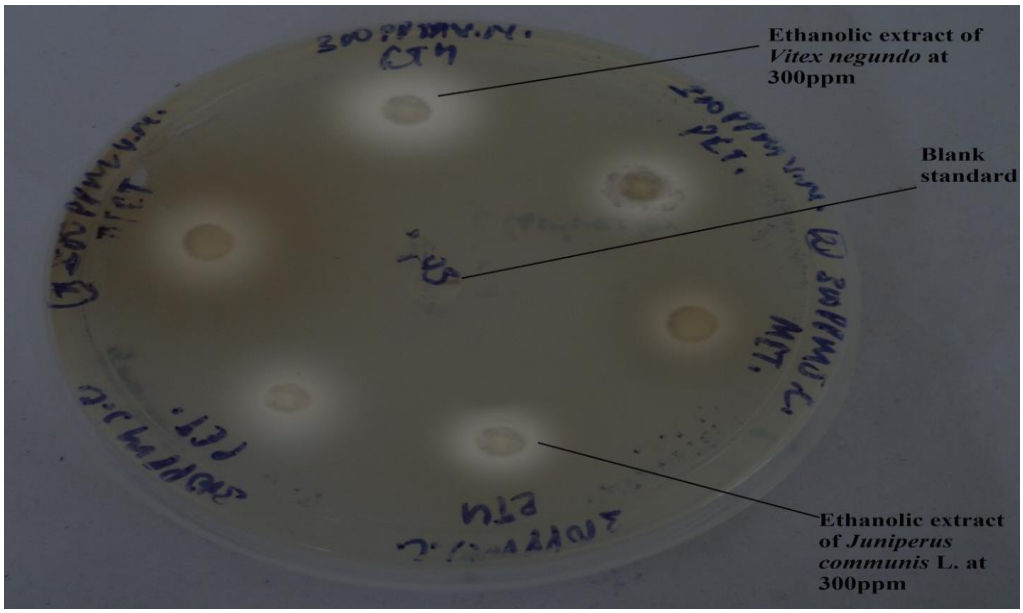

Figure 3: Pertiplate showing *in vitro* zone of inhibition of *Juniperus communis* L. And *Vitex negundo* against *Xanthomonas axonopodis* pv. *Punicae.*

CONCLUSION

Several chemicals and bactericides in the market, efforts aim to treat diseases caused by *Xanthomonas axonopodis* pv *punicae*. However, a search for more efficient agent has resulted in the screening of several medicinal plants for possible activity. It is easy to perceive the potential in these plants as attractive targets for future studies, and possible to uncover new alternatives to the existing control agents for diseases caused by this pathogen. Furthermore, in vivo activity of the active compounds needs to be determined in plants grown in fields, so as to determine their efficacy in a metabolic environment. Such future studies necessary to expand the existing limited uses for majority of such diseases.

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