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Pomegranate bacterial blight: *Abutilon indicum, Prosopis juliflora* and *Acacia arabica* as Antibacterial Agents for *Xanthomonas axonopodis PV. Punicae*

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Abstract
Xanthomonas axonopodis pv. punicae causes Bacterial blight disease in Pomegranate. Complete range of symptoms of bacterial blight caused by *Xanthomonas axonopodis pv. punicae appear* on various pomegranate plant parts expect roots. The present investigation was initiated to find a suitable alternative to synthetic antibiotics for the management of plant diseases caused by bacteria. The study was aimed to use wild plant species viz., *Abutilon indicum, Prosopis juliflora and Acacia arabica* as Antibacterial agent against *Xanthomonas axonopodis pv. Punicae*. Aqueous extracts of *Abutilon indicum, Prosopis juliflora and Acacia arabica* plants has Antibacterial activity against *Xanthomonas axonopodis pv. Punicae.* The antibacterial activity was tested by well diffusion assay, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The maximum activity recorded in *P. juliflora* (MIC = 1.03 mg ml^{-1} and MBC = 0.15 mg ml^{-1}) and *A. arabica* (MIC = 1.00372 mg ml^{-1} and MBC = 2.58 mg ml-1) against *X. axonopodis pv. Punicae*, while the lowest activity was recorded by *A.indicum* (MIC = 0.619 mg ml^{-1} and MBC = 0.923 mg ml^{-1}). Highest ZOI was shown by *P.juliflora* while lowest ZOI was shown by *A.indicum* the results infer that the extracts of *Prosopis juliflora* and *Acacia arabica* are highly sensitive against the *Xanthomonas axonopodis pv. punicae*. Plant extracts exhibited antibacterial activity with a potential to be used in the management of many plant diseases as an alternative to chemical antibiotics. Further phytochemical analysis is required to identify the bioactive compounds responsible for antibacterial activity.

Keywords: *Prosodies juliflora,* minimum bactericidal concentration, *acacia arabica,* synthetic antibiotics

1. Introduction
Pomegranate (*Punica granatum* L.) is an ancient fruit, belonging to the smallest botanical family punicaceae. Pomegranate is a native of Iran, where it was first cultivated in about 2000 BC, but spread to the Mediterranean countries at an early date. It is extensively cultivated in Spain, Morocco and other countries around the Mediterranean, Egypt, Iran, Afghanistan, Arabia and Baluchistan. Pomegranate is a good source of carbohydrates and minerals such as calcium, iron and sulphur. It is rich in vitamin-C and citric acid is the most predominant organic acid in pomegranate (Malhotra *et al*., 1983) [11]. Apart from the fleshy portion of the fruit, the crop residues are also finding place in industries. The rind of the fruit is a good source of dye, which gives yellowish brown to khaki shades and is being used for dying wool and silk. The flower and buds yield light red dye, which is used for dying of cloths in India. The bark of the stem and root contains a number of alkaloids belonging to pyridine group. The bark is used as a tanning material especially in Mediterranean countries in the East (Bose, 1985). Pomegranate is regarded as the "Fruit of Paradise". It is one of the most adaptable subtropical minor fruit crops and its cultivation is increasing very rapidly. In India, it is regarded as a "vital cash crop", grown in an area of 1, 16,000 ha with a production of 89,000 MT with an average productivity of 7.3 MT (Srivastsva and Umesh, 2008) [21]. Successful cultivation of pomegranate in recent years has met with different traumas such as pest and diseases. Among diseases bacterial blight caused by *Xanthomonas axonopodis pv. Punicae* is a major threat. Since 2002, the disease has reached the alarming stage and hampering the Indian economy vis-à-vis export of quality fruits. The disease accounted up to 70-100 per cent during 2006 in Karnataka and Maharashtra resulting in wipe out of pomegranate. During the year 2007, the total output of pomegranate production in India was down by 60 per cent (Raghavan, 2007) [15].
The causal organism of blight is *Xanthomonas axonopodis pv. punicae* (Hingorani and Singh) Vauterin, Hoste, Kersters and Swings (Hingorani and Mehta, 1952; Vauterin *et al*., 1995) [8, 22].

The plant is susceptible to blight during all stages of growth and results in huge economic loss.

Bacterial blight primarily affects the above ground plant parts, especially leaves, twigs and fruits. While the leaves show early water soaked lesions to late necrotic blighting, the fruits show isolated or coalesced water soaked lesions followed by necrosis with small cracks and splitting of the entire fruit (Petersen *et al*., 2010) [14]. Stems show lesions around nodes or injuries, forming cankers in later stages. Suspected symptoms on floral parts have also been reported (Chand and Kishun, 1991; Rani *et al*., 2001) [6, 17]. It is also presumed that the stem canker could be an outcome of systemic spread of bacterium from leaf (Chand and Kishun, 1993) [10]. Although reported by Chand and Kishun (1991) [6] and Rani *et al*. (2001) [17] attempts to reproduce the field symptoms of blight on detached leaves, twigs and fruits were unsuccessful in the artificial inoculations.

Management of bacterial blight of pomegranate is a major concern. This disease could not be effectively managed with conventional antibiotics like streptocycline in field conditions. Thus this invasination is carried out on management of disease by aqueous extracts of *Abutilon indicum*, *Prosopis juliflora* and *Acacia Arabica* plants. Continual and indiscriminate use of synthetic antibiotics to control bacterial disease of crop plants has caused health hazard in animals and humans due to their residual toxicity (Raghavendra *et al*. 2006) [16]. A bioactive principle isolated from plant appears to be one of an alternative for control of plant and human pathogens developed resistant to antibiotics. Plant originated-antibacterial compounds can be one approach to plant disease management because of their eco-friendly nature (Bolkan and Reinert 1994) [5].

2. Materials and Methods

2.1 Plant Material

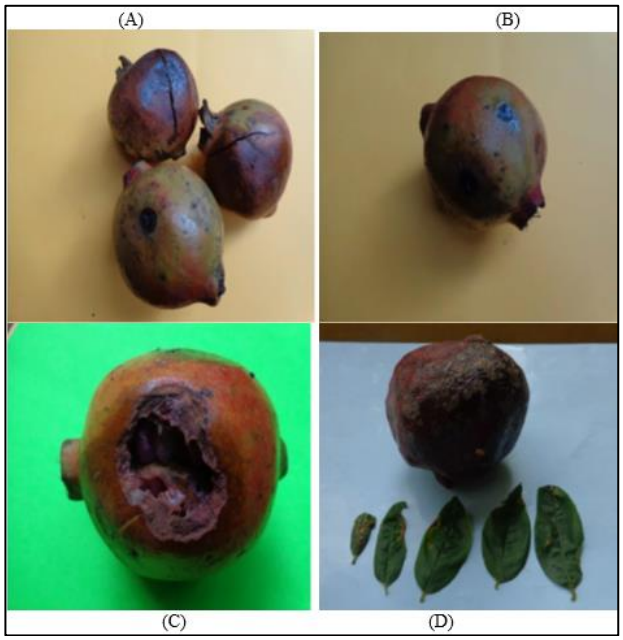
Commonly available weed plants were collected in the surrounding areas of Latur district, Maharashtra. The plants used for this study are *Abutilon indicum, Prosopis juliflora and Acacia arabica*.

2.2 Preparation of plant extracts

Plant leaves were washed with tap water followed by sterile distilled water and then air dried at room temperature. Dried leaves were powdered; 50 gram of powdered material was percolated with 250 ml water for 72 hours. The percolate was mixed thoroughly for every 12 hours. Percolate was filtered through double-layered muslin cloth followed by Whatman no.1 filter paper. The filtrate was concentrated at 35°C and stored at 4°C until further use.

2.3 Field visit and collection of bacterial samples

Field visit was undertaken in major pomegranate growing regions of Latur, Maharashtra, India i.e., Murud and Harangul during the month of June to August. 2018. During the field survey, the randomly selected plant parts were inspected at the fields of for the incidence of bacterial blight. Distribution of bacterial blight of pomegranate was recorded in these areas. Plants were diagnosed as infected based on typical symptoms of bacterial blight, viz., yellow water soaked lesions at early stages and corky, dark oily spots at later stages of infection. The suspected plant leaves and fruits were collected and transferred into sterilized plastic bags and brought to Research laboratory of Microbiology Department of Dayanand Science College, Latur for the further studies.



A. Bacterial blight symptoms on pomegranate fruit
B. Bacterial blight symptoms on fruit
C. Bacterial blight symptoms on pomegranate fruit
D. Bacterial blight symptoms on fruit and leaves

Fig 1: Bacterial blight on pomegranate fruits and leaves

2.4 Storage of sample

Samples were surface sterilized and stored in laboratory condition.

2.5 Isolation of the bacteria

The bacteria were isolated from the infected leaves, and fruits of pomegranate collected from regions of Latur, Maharashtra, India i.e., Murud and Harangul during the month of June to August. 2018. These samples were washed, air dried, and then disinfected with 0.1% $HgCl_2$ for about 30-60 seconds and washed thrice with sterile water to remove traces of $HgCl_2$. They were macerated with sterilized blade in a sterile Petri dish containing few drops of sterile distilled water in order to allow the bacteria to diffuse out. A loop full of suspension was then transferred with the help of sterilized bacteriological needle to sterilized Petri plates filled with nutrient agar medium with (NA) and incubated at 28°C for 24-72 hr. After 2- 3 days, incubated plates were observed for the presence of typical pale yellow, glistening colonies (Figure 2) which were transferred to the NA slants and maintained in laboratory condition for further studies.

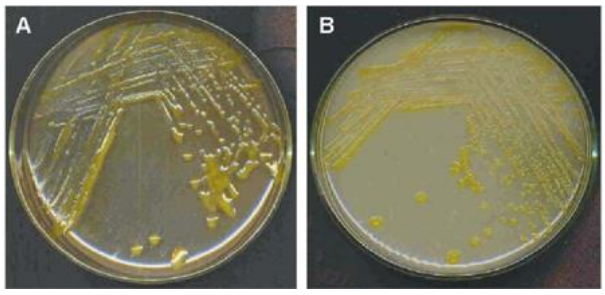


Fig 2: Pale yellow, glistening colonies of Xanthomonas on NA medium

2.6 Identification and Conformation of isolate

Identification and Conformation of *Xanthomonas axonopodis pv. Punicae was done by using following methods.*

2.6.1 Morphological characters

The morphological characters such as shape, gram reaction and pigmentation characters were studied as described by Society of American Bacteriologists, Bradbury (1970) [4] and Schaad and Stall (1992) [20].

Table 1: Cultural and morphological characteristics of *Xanthomonas axonopodis pv. Punicae* isolate on nutrient agar media

Colony characters	*Xanthomonas axonopodis pv. punicae*
Colour	Yellowish
Size of colony	Medium to large
Shape of colony	Small Circular colonies
Cell shape	Single rod
Appearance	Slightly raised, glistening
Elevation	Convex
Margin	Entire margin
Texture	Highly mucoid

2.6.2 Growth rate at 28^0C and 37^0C

The effect of varied temperature levels on the growth of *Xanthomonas axonopodis pv. punicae* was studied and data so obtained is presented in Table 2. *Xanthomonas axonopodis pv. punicae* isolate were tested at temperature 28 ^{0}C and 37 ^{0}C on NA agar medium. The data clearly indicated that the temperature of 28^0C was found optimum for the growth of the pathogen as significantly maximum number of colonies was observed at this temperature. Isolate grew well at 28 ^{0}C but no growth was observed at temperature of 37 ^{0}C. Growth of *Xanthomonas axonopodis pv. punicae* started 72 hrs after incubation. Maximum growth was observed after 120 hrs of incubation at 28^0C but no growth was observed after 48 hrs of incubation. At 370C no growth was observed upto 120 hrs. Similar work on temperature requirement was carried out by Hingorani and Mehta (1952) [8]. They found that the pomegranate bacterium grows well at a cordial temperature of 300C and can tolerate a minimum and maximum temperature of 5 and 400C, respectively. Gour *et al.*, (2000) [7] also got the similar results while working with *X. axonopodis pv. Vincula*, the causal agent of leaf blight of cowpea. They have recorded the maximum growth of the pathogen at a temperature level of 300C, whereas, Manjula (2002) [12] recorded the highest number of colonies of *Xap* at a temperature of 27^0C.

Table 2: Growth rate at 28^0 C and 37^0 C

S. No.	Isolate	Growth rate							
		28^0 C				37^0 C			
		48 hrs	72 hrs	96 hrs	120 hrs	48 hrs	72 hrs	96 hrs	120 hrs
1	*Xanthomonas axonopodis pv. punicae*	+	++	+++	++++	-	-	-	-

(Colony growth: '-': no growth, '+': less growth, '++': moderate growth, '+++': maximum growth)

2.6.3 Biochemical variability

Methodology followed for the following experiments on biochemical variability is according to Schaad (1992) [19].

2.6.3.1 Lactose utilization

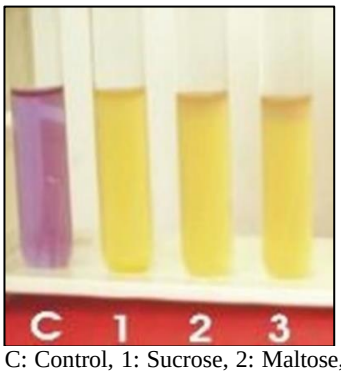
Carbon source (lactose) was filter sterilized and mixed with autoclaved, cooled Dye's medium along with 1.2 per cent purified agar. The pH was adjusted to 7.2. Bacterial isolate were spot inoculated with replica plating method and incubated at 300C for 3, 7 and 14 days. Growth was compared with control, where carbon source was not supplemented (Schaad, 1992) [19].

2.6.3.2 Starch hydrolysis

Medium used for Starch hydrolysis (see Appendix) was sterilized by autoclaving and poured into sterilized Petri plates. These plates were inoculated and incubated at 300C for 7 days. The plates were flooded with Lugol's iodine and allowed to act for few min. The presence of starch hydrolysis was indicated by the presence of clear zones and *vice-versa*. The zone hydrolysed was measured for each isolate.

2.6.3.3 Acid production from Sucrose, Maltose, Dextrose

The acid production by the isolate of *Xanthomonas axonopodis pv. punicae* was tested by using Peptone water medium of Dye. Ten ml of medium was dispensed in each test tube. This medium was sterilized in an autoclave for 15 min. To these tubes, filter sterilized carbohydrates *viz*., Sucrose, Maltose and Dextrose were added at 0.14 per cent concentration. The tubes were inoculated with 0.1 ml of 24 hrs old bacterial culture and incubated at room temperature for three days. Change in the colour of the medium confirmed the acid production.



C: Control, 1: Sucrose, 2: Maltose, 3: Dextrose

Fig 3: Acid production from sucrose, maltose, dextrose

2.7 Testing Antibacterial Activity

After conformation of *Xanthomonas axonopodis pv. Punicae*, the antibacterial activity of plant extract was preliminarily screened by well diffusion assay. LB agar plates were spread plated with 20 µL of bacterial strain (1x108 cfu/ml). The wells of 6 mm diameter were made in the agar plates. Each plant extract was tested for antibacterial activity by adding 40 µL of extracts in different concentrations *viz*., 50, 100 and 200 mg ml-1. The experiment was repeated thrice. The plates were incubated at 37oC for 24 hour. Subsequently, the plates were examined for zone of inhibition (ZOI) and diameter was measured in mm after subtracting well diameter (Ahmad *et al*. 1998). To determine Minimum inhibitory concentration (MIC), required quantity of extracts were added in to the LB broth of 4 ml to bring initial concentration of 20 mg ml-1. In each test tube 0.1 ml of standardized inoculum (1x108 cfu/ml) was added. Two control tubes were maintained for each test batch namely extract control (tube containing plant extract

and LB medium without inoculum) and organism control (tube containing LB medium and inoculum). The test tubes were incubated at 37^0 C for 24 hour. The lowest concentration (highest dilution) of plant extract that produced no visible growth (no turbidity) recorded as minimum inhibitory concentration. Minimum bactericidal concentration (MBC) was assed by sub-culturing test dilutions on to a drug free solid medium. The plates were incubated for 24 hours at 37^0C. The lowest concentration of the antimicrobial at which no single colony observed after sub-culturing is regarded as Minimum bactericidal concentration. (Akinyemi *et al*. 2005) [1].

3. Results and Discussion

3.1 Well diffusion assay

The preliminary screening of selected three plant extracts against the *Xanthomonas axonopodis pv. punicae* was done using well diffusion method. The zone of inhibition greater than 5 mm diameter is found to be having significant activity against particular bacteria (Palombo and Semble 2001). The aqueous extracts were sensitive against *Xanthomonas axonopodis pv. punicae* tested at different concentrations (Table 3)

The extract of *Acacia Arabica* was sensitive against *Xanthomonas axonopodis pv. punicae* tested at different concentrations. The same extract was much effective at low concentration of 50 mg ml^{-1} i.e 11mm ZOI. *P. juliflora* extract arrested the growth of *Xanthomonas axonopodis pv. punicae* (24 mm) at 200 mg ml-1. The *P. juliflora* extract shows a zone of inhibition about 21 mm and 19 mm at 100 and 50 mg ml-1 respectively. Based on ZOI, Satish *et al*. (1999) [16] reported significant antibacterial activity *of P. juliflora* and *A. arabica* against *X. campestris* pathovars. The extracts of *A. indicum was* exhibited the antibacterial activity of (11 mm) ZOI at 200 mg ml^{-1}.

Table 3: Antibacterial activity of plant extracts showing zone of inhibition

Plant species	Conc. mg ml^{-1}	Zone of inhibition (mm)*
Acacia arabica	50	11
	100	15
	200	16
Prosopis juliflora	50	19
	100	21
	200	24
Abutilon indicum	50	6
	100	9
	200	11

3.2 Minimum inhibitory and bactericidal concentration

The antibacterial activity of *A. arabica* and *P. juliflora* extracts are found to be high against all the bacteria. The maximum activity recorded in *P. juliflora* (MIC = 1.03 mg ml-1 and MBC = 0.15 mg ml-1) and *A. arabica* (MIC = 1.00372 mg ml-1 and MBC = 2.58 mg ml-1) against *X. axonopodis pv. Punicae*. Raghavendra *et al*. (2006) [16] reported significant antibacterial activity of *Acacia nilotica* extracts against *Xanthomonas* pathovars and human pathogenic bacteria tested, while the lowest activity was recorded by A.ndicum (MIC = 0.619 mg ml^{-1} and MBC = 0.923 mg ml^{-1}). These findings indicate that the extracts of *Prosopis juliflora* and *Acacia arabica* are potential to use in the management of plant diseases. Further phytochemical analysis is required to identify the active components of plant extracts showing antimicrobial activity.

Table 4: MIC and MBC of Plant extracts against *Xanthomonas axonopodis pv. Punicae*

Plant species	MIC (mg ml-1)	MBC(mg ml-1)
Acacia arabica	1.00372	2.58
Prosopis juliflora	1.03	0.15
Abutilon indicum	0.619	0.923

4. Conclusion

Many synthetic antibiotics are used to control several phytopathogens. The increased awareness of environmental problems with these chemical antibiotics has led to the search for non-conventional chemicals of biological origin for the management of these diseases. Bactericides of plant origin can be one approach to disease management because of their eco-friendly nature (Bolkan and Reinert 1994) [5]. The products of plant origin are of greater advantage to user, the public and radical environmentalist. Laboratory screening of plant extracts has given encouraging results, indicating their potential use in the management of disease caused by *Xanthomonas* species. Plant extracts resulted in antibacterial activity is potential to use in the management of plant diseases as an alternative to chemical antibiotics. Further phytochemical analysis is required to identify the bioactive compounds responsible for antibacterial activity.

5. Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

6. Acknowledgement

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