

## ***In vitro* Analysis of Antimicrobial Susceptibility of *Xanthomonas axonopodis* pv. *punicae* and Comparative Study of Protein Profiling under Biotic Stress**

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### **Authors' contributions**

*This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.*

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### **ABSTRACT**

Bacterial blight of pomegranate caused by *X. axonopodis* pv. *Punicae* (*XAP*) assumed epidemic form and resulted in economic burden on farmers. In the current study the pathogen infected samples were collected and the isolated *XAP* was identified and confirmed through the morphological, biochemical characterization and Pathogenicity test. Bacterium was reisolated from infected plant to prove Koch's postulates. Efficacy of different chemicals and oils were tested by disc diffusion assay and turbidometrically. Bronopol 3000 ppm (25.6±1.6 mm) and Clove oil (18.0±0.7 mm) formed highest zone of inhibition Turbidometri showed the highest O.D. (0.908 nm) by Copper oxy chloride and Neem oil showed maximum inhibition of growth with O.D. (0.842 nm). Biotic stress (pathogen) induced protein response was studied by using SDS-PAGE method after protein extraction from *XAP*, healthy *P. granatum* L. and infected *P. granatum* L. The protein band pattern showed the unique band no. 2 (Mol.Wt.66000 Da) in infected *P. granatum* L. as compared

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to the banding pattern of XAP and healthy *P. granatum L.* The over expressed protein due to biotic stress could be useful as a marker for detection of the disease at the early stage and for control of the diseases after knowing the biochemical significance of the protein.

**Keywords:** Pomegranate; bacterial blight; *Xanthomonas axonopodis pv. punicae*; *in vitro* efficacy; antimicrobial activity; SDS-PAGE.

## 1. INTRODUCTION

Pomegranate (*Punica granatum L.*) belongs to the family Lythraceae, is an ancient, medical fruit and cash crop of Maharashtra. It is also grown in commercially in other state of india viz. Karnataka, Andhra Pradesh, Rajasthan, Gujarat, and Tamil Nadu [1]. India is one of the major producers of pomegranate in the world with average total production of 8 lakh tons per annum [2].

Many diseases occurred on the pomegranate plant out of them bacterial disease caused by the *Xanthomonas axonopodis pv. punicae* (Xap) strongly damaged the pomegranate production, including large economic losses to the Indian growers [3]. Bacterial disease cause substantial loss to the productivity of major crop plants. Unlike fungal disease, bacterial disease not control effectively through chemical methods.

The symptoms of the bacterial blight were observed on the leaves stems and fruits as small (2 to 5 mm) in size, irregular, prominent water soaked spots, which later become black often leads to the breaking of branches and cracking of fruits, reducing to the market value of the fruit [4,5].

It is gram negative, aerobic rods belonging to the family *Pseudomonadaceae*. The pathogen causes disease actively at high temperature (up to 32°C) and high rainfall and humidity and pH at 7.0. The pathogen causes destruction of seedlings and adult plants and thus results in yield reduction. The aim of the study was to screen *in vitro* a range of antimicrobial agents and their efficacy against Xap and protein profiling; in laboratory conditions.

## 2. MATERIALS AND METHODS

### 2.1 Collection and Isolation of Disease Sample

The samples were collected from two different districts of Maharashtra viz. Ahmednagar and pune as per typical symptoms showed by plant externally. The fresh infected leaves samples

were used for isolation employing tissue isolation method by using Nutrient Agar medium [6].

### 2.2 Purification and Maintenance of Bacterial Culture

The respective bacterial cultures were maintained on NA medium at room temperature by adopting subsequent subculturing at periodical, regular intervals. Three days old cultures were used for further studies [7].

### 2.3 Identification of the Pathogen

The identification of the pathogen involved in causing of oily spot in pomegranate was determined by conducting studies on its morphological [8,9] and biochemical [10,11] features of the pathogen as per standard microbiological procedures.

### 2.4 Pathogenicity Test

Veinlets of some leaves were injected with 48 h old bacterial suspension with the help of sterile Dispo Van syringe. Some plant leaves were inoculated by injuring the leaves with sharp needle and by cotton swabbing the bacterial suspension on injured leaves. The plant was kept for incubation. After 10 – 12 days observations made for typical symptoms of bacterial blight on leaves, the organism was reisolated from artificially inoculated leaves of Pomegranate plants showing typical symptoms of disease. The re-isolation was carried out on NAS medium and the growth of organism was observed for next 2-3 days [12,13].

### 2.5 *In vitro* Efficacy of Different Chemicals and Plant Derived Oils against XAP by Using Paper Disc and Turbidimetric Method

*In vitro* efficacy of different chemicals and oils was checked against *Xanthomonas axonopodis pv. Punicae* by using paper disc and turbidimetric method (Copper oxy chloride: 2500 ppm, 1000 ppm, Bronopol: 3000 ppm, 1000 ppm, Bordeaux mixture: 3000 ppm, 1000 ppm, Bactomycin: 1200 ppm, 1000 ppm, Clove

oil: 100%, Nilgiri oil: 100%, Neem oil: 100%) [14,15].

## 2.6 Isolation of Protein from Healthy and Infected Pomegranate Leaves

Kumar et al. [16] standardized the protein isolation protocol from plant leaves.

## 2.7 Isolation of Protein from *Xanthomonas axonopodis* pv. *Punicae*

Bioera's teaching kit [17] described the protein extraction from bacteria and clear result was observed.

### 1. Day I:

- i) The LB plates were inoculated with loopful stock culture of *Xanthomonas axonopodis*.
- ii) The plates were incubated overnight at 37°C.

### 2. Day II:

- iii) A single colony was picked from the LB plate and inoculated into 5 ml LB Broth.
- iv) The LB broth was incubated at 37°C on a shaker overnight.

### 3. Day III:

- v) The overnight incubated 1 ml of culture was inoculated into 50 ml of LB Broth (in a 500 ml conical flask).
- vi) The culture flask was incubated at 37°C on the shaker, until cells reach mid log phase of growth that is  $A_{600}$  is 0.5 (approximately 4-5 hours).
- vii) 5 ml of the culture from step 6 was transferred into test tube.
- viii) The 3 ml (1.5 ml at a time) of culture was centrifuged in 1.5 ml vials at 8000 rpm for 10 minutes. The supernatant was discarded at each time.
- i) The cell pellets were resuspended in 100  $\mu$ l of cell lysis buffer.
- ii) 25  $\mu$ l of protein loading dye was added to suspension and mixed gently.
- iii) The samples were boiled in water bath for 20 minutes.
- iv) The samples were centrifuged at 6000 to 8000 rpm for 10 minutes.
- v) The 50  $\mu$ l protein marker and 35  $\mu$ l of sample supernatant was loaded on to SDS-PAGE.

- vi) The SDS-PAGE was run at 50 volts until bromophenol blue reaches near the bottom of separation gel.
- vii) Stained the gel with protein staining solution.

## 3. RESULTS AND DISCUSSION

### 3.1 Collection and Isolation of Diseased Sample

Bacterial blight infected samples were collected from Ahmednagar and Pune districts of Maharashtra, associated bacterium *Xanthomonas axonopodis* pv. *punicae* was isolated. Purified culture was maintained on NSA slants and coded as Xap I, Xap II respectively. [18].

### 3.2 Identification of the Pathogen

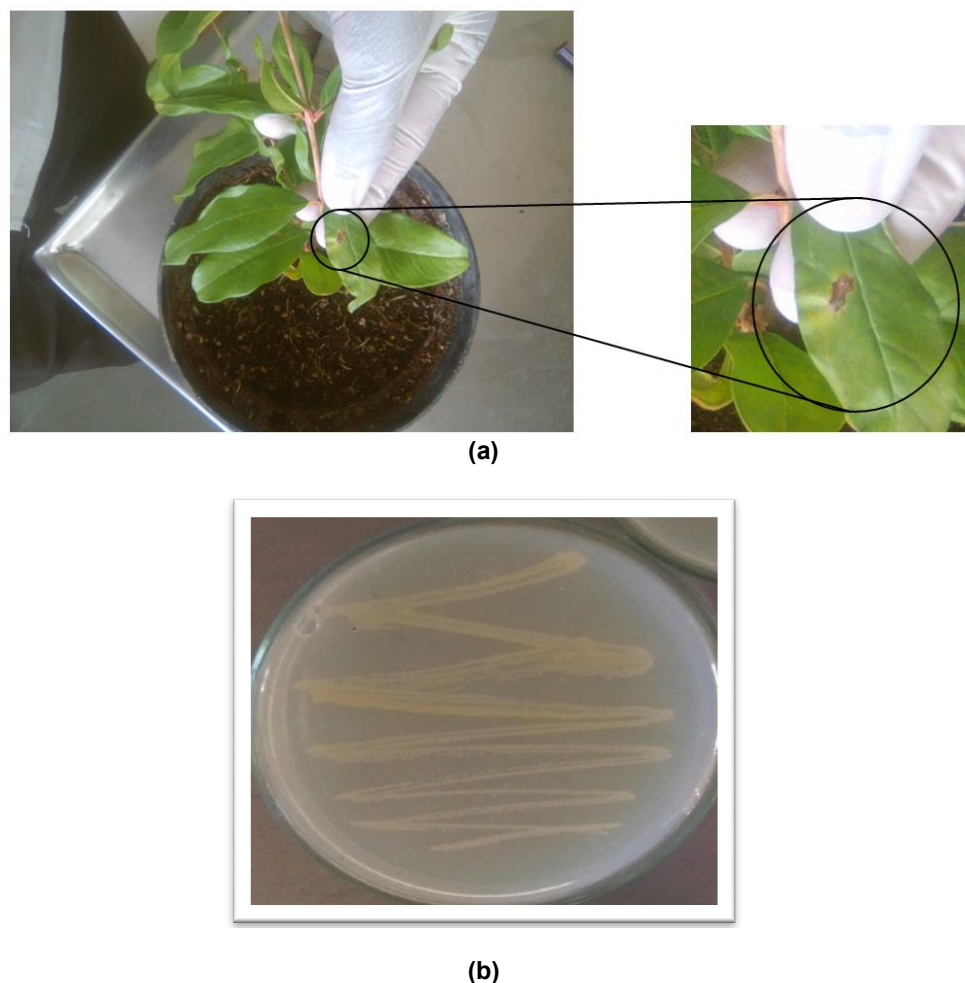
Morphological and biochemical characteristics viz., shape, colony colour, Gram reaction, starch hydrolysis, gelatin liquefaction, indole production, acid and gas production, KOH, catalase test were performed and confirmed to identify the bacterium as *Xanthomonas axonopodis* pv. *punicae* and following Table 1 showed the morphological and biochemical test. [19,20].

### 3.2 Pathogenicity Test

Pathogenicity test of isolates was confirmed by injecting bacterial solution to disease free pomegranate plant. Occurrence symptoms of bacterial blight symptoms on plant leaf confirmed pathogenicity test of isolates (Fig. 1). Bacterium was reisolated from infected plant to prove Koch's postulates [21].

### 3.3 *In vitro* Efficacy of Different Chemicals and Plant Derived Oils against XAP by Using Paper Disc Method

In order to assess the efficacy of chemicals and oils against two isolates of *Xanthomonas axonopodis* pv. *Punicae* an experiment was conducted and the evaluation was made by paper disc method. The data presented in (Table 2) revealed the significant differences among the different treatments. In the chemical treatment Bronopol was significantly superior in inhibiting the growth of bacteria (18.6 $\pm$ 0.7 mm, 25.6 $\pm$ 1.6 mm) against Xap. Among the oils Clove oil showed maximum zone of inhibition 18.3 $\pm$ 0.7 mm (Fig. 2) [22].



**Fig. 1. (a) Symptoms of bacterial blight observed after 10-12 days of inoculation (b) Reisolated *Xanthomonas axonopodis* pv. *punicae* after pathogenicity test**

### **3.4 *In vitro* Efficacy of Different Chemicals and Plant Derived Oils against XAP by Using Turbidimetric Method**

Efficacy different chemicals and oils was tested turbidometrically. After 24 hours of incubation list O.D. 0.713 nm was exhibited by Bronopol(3000 ppm), followed by Copper oxy chloride (3000 ppm) with O.D. 0.908 nm and Bordeaux (3000 ppm) 0.834 nm respectively whereas Bronopol (1000 ppm) with O.D 0.219 nm showed minimum inhibition of growth and Among the oils tested Neem oil showed maximum inhibition of growth with O.D. 0.842 nm, followed by Clove oil with O.D. 0.810 nm, Nilgiri oil showed minimum inhibition of growth among oils showed in following Table 3.

### **3.5 Protein Isolation from Healthy Pomegranate Leaves, Infected Pomegranate Leaves and XAP I, XAP II Bacteria**

#### **3.5.1 Data analysis of healthy pomegranate leaves, infected pomegranate leaves and bacteria**

Following data analysis Table shows the actual position of bands on the gel as compared to the standard marker on the basis of molecular weights provided by BioEra's Teaching Kit.

The protein banding pattern showed the unique band no. 2 (Mol.Wt.66000 Da) in infected *P. granatum L.* as compared to the banding pattern of *X. axonopodis* pv. *punicae* and healthy *P. granatum L.* (Fig. 3) [23].

**Table 1. Morphological and biochemical characteristics of *Xanthomonas axonopodis* pv. *punicae* isolates**

Sr. No.	Morphological and biochemical test	XAP I	XAP II
1.	Colony color	Yellow	Yellow
2.	Gram's reaction	-ve	-ve
3.	Starch hydrolysis	+++	++
4.	Indole production	++	+
5.	Catalase test	+++	+++
6.	KOH test	++	+
7.	Gelatin liquefaction	++	++
8.	Acid production	++	+

- Negative, + Positive, Varying degree of + ve reaction (+ poor, ++ moderate, +++ strong)

**Table 2. Revealed the significant differences among the different treatment by paper disc method**

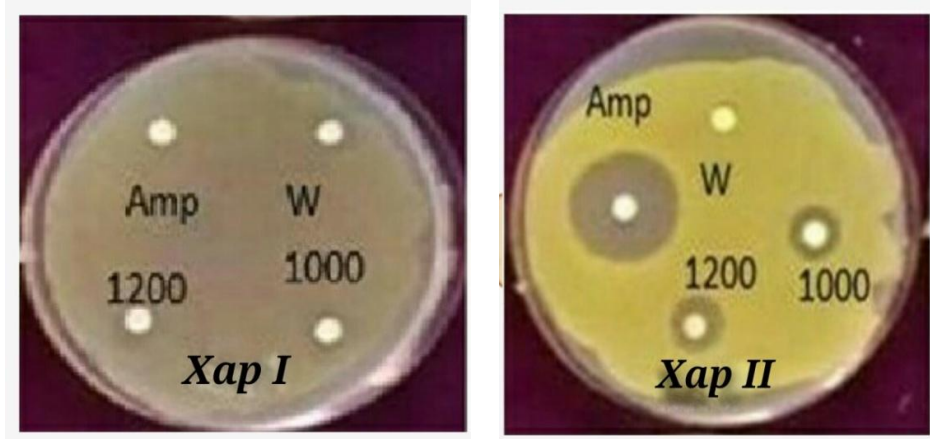
Sr. No.	Treatment	Concentration	Zone of inhibition produced by XAP I	Zone of inhibition produced by XAP II
T <sub>0</sub>	Control(+ve) water	10 µl	00	00
T <sub>1</sub>	Control(-ve) Ampicillin	15 µl	17.9±0.6	27.7±0.7
T <sub>2</sub>	Bronopol	1000 ppm	10.8±0.4	18.6±0.7
T <sub>3</sub>	Bordeaux	1000 ppm	10.2±0.2	9.3±0.1
T <sub>4</sub>	COC	1000 ppm	12.1±0.3	11.9±1.5
T <sub>5</sub>	Bactomycin	1000 ppm	10.1±0.3	13.2±0.9
T <sub>6</sub>	Bronopol	3000 ppm	11.6±0.4	25.6±1.6
T <sub>7</sub>	Bordeaux	3000 ppm	9.7±0.7	8.1±.02
T <sub>8</sub>	COC	2500 ppm	7.1±1.0	9.2±0.4
T <sub>9</sub>	Bactomycin	1200 ppm	11.2±0.4	15.3±0.2
T <sub>10</sub>	Clove oil	100%	15.9±0.9	18.3±0.7
T <sub>11</sub>	Neem oil	100%	13.4±1.6	7.3±1.1
T <sub>12</sub>	Nilgiri oil	100%	8.3±0.9	10.6±0.4

**Table 3. Efficacy of chemicals and oils against *Xanthomonas axonopodis* pv *punicae* by turbidometric method**

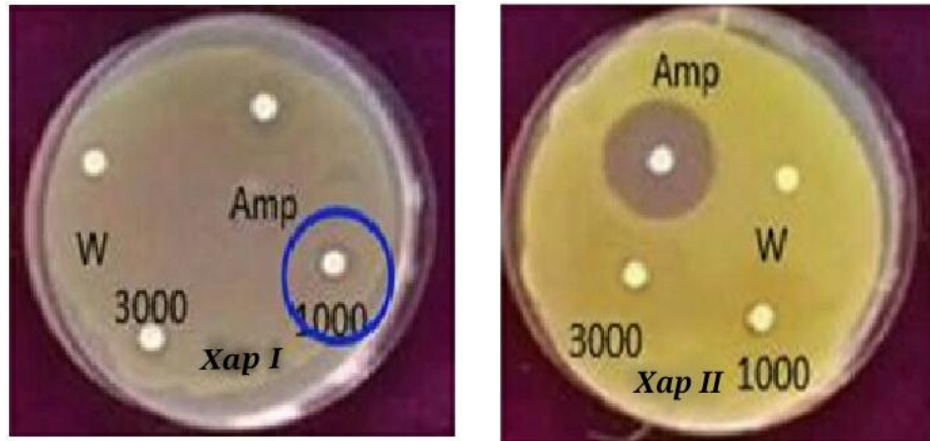
Sr. No.	Treatment	Concentration	O.D. of culture	O.D. after 24 hrs at 620 nm
T <sub>0</sub>	Control(+ve)nutrient broth	1 ml		00
T <sub>1</sub>	Control (-ve) antibiotic	500 µl		0.324
T <sub>2</sub>	Bronopol	1000 ppm		0.219
T <sub>3</sub>	Bordeaux	1000 ppm	0.998	0.337
T <sub>4</sub>	COC	1000 ppm		0.410
T <sub>5</sub>	Bronopol	3000 ppm		0.713
T <sub>6</sub>	Bordeaux	3000 ppm		0.834
T <sub>7</sub>	COC	3000 ppm		0.908
T <sub>8</sub>	Clove oil	100%		0.810
T <sub>9</sub>	Neem oil	100%		0.842
T <sub>10</sub>	Nilgiri oil	100%		0.392

**Table 4. Data analysis of healthy pomegranate leaves, infected pomegranate leaves and bacteria**

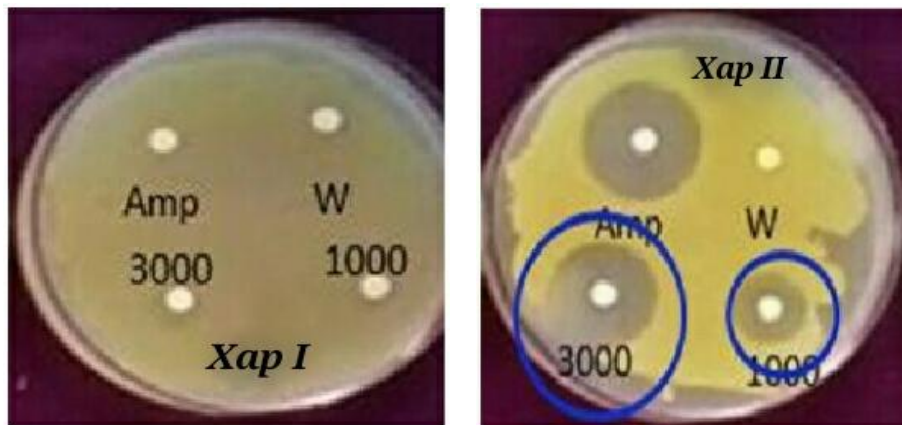
Sr. No.	Standard marker (Band no)	Molecular weight	Healthy plant (Band no)	Infected plant (Band no)	XAP I proteins (Band no)	XAP II proteins (Band no)
1.	1	97,400	Absent	Absent	Absent	Absent
2.	2	66,000	Absent	Present	Absent	Absent
3.	3	43,000	Present	Present	Absent	Absent
4.	4	29,000	Present	Present	Absent	Absent
5.	5	20,100	Present	Present	Present	Present
6.	6	14,300	Present	Present	Present	Present



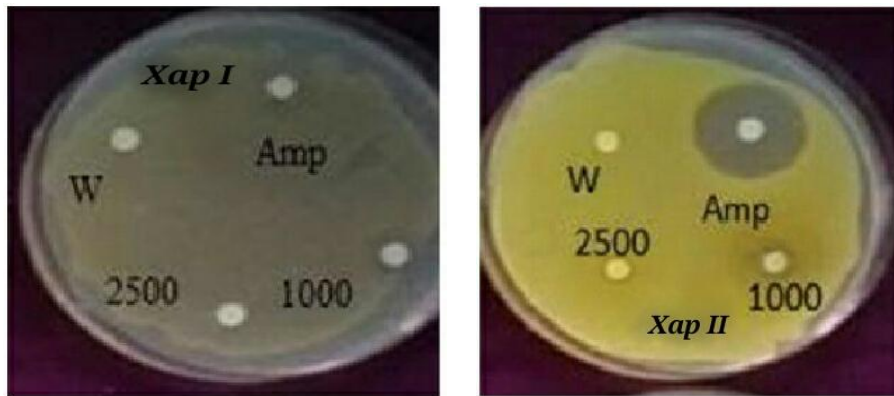
(a) Bactomycin



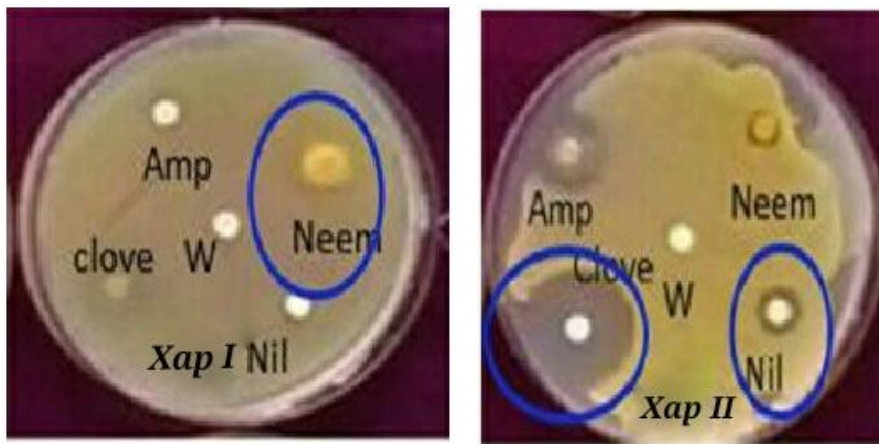
(b) Bordeaux



(c) Bronopol



(d) Copper oxychloride



(e) Oils

Fig. 2. Efficacy of different chemicals and oils against *Xanthomonas axonopodis pv. Punicae* by Paper disc method

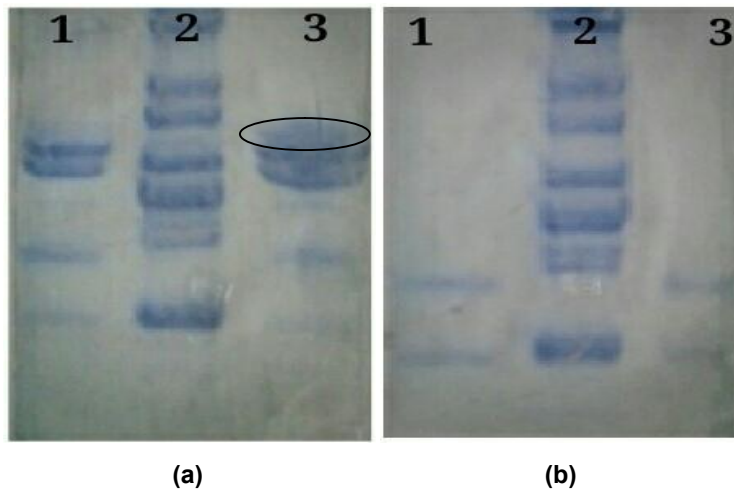


Fig. 3. (a) Isolated proteins of 1) Healthy, 2) Marker, 3) Infected leaves (b) 1) *Xap I*, 2) Marker and 3) *Xap II* Bacteria (*Xanthomonas axonopodis pv. punicae*)

#### 4. CONCLUSION

In the current study the pathogen infected samples identified as infected with XAP was through morphological, biochemical characterization and Pathogenicity test. Efficacy of different chemicals and oils were tested by disc diffusion assay and turbidometrically. Bronopol 3000 ppm (25.6±1.6 mm) formed highest zone of inhibition and Clove oil formed highest zone of inhibition (18.0±0.7 mm). Turbidometri showed the highest O.D. (0.908 nm) by Copper oxy chloride and Neem oil showed maximum inhibition of growth with O.D. (0.842 nm).

Biotic stress (pathogen) induced protein response was studied by using SDS-PAGE method after protein extraction from XAP, healthy *P. granatum L.* and infected *P. granatum L.* The protein banding pattern showed the unique band no. 2 (Mol.Wt.66000 Da) in infected *P. granatum L.* as compared to the banding pattern of *X. axonopodis pv. punicae* and healthy *P. granatum L.* The over expressed protein due to biotic stress could be useful as a marker for detection of the disease at the early stage and for control of the diseases after knowing the biochemical significance of the protein.

#### COMPETING INTERESTS

Authors have declared that no competing interests exist.

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