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Research Article

THE CONTROL OF BACTERIAL BLIGHT OF POMEGRANATE USING THE METHANOLIC EXTRACTS OF INDIAN BAEL AND *LEUCAS ASPERA*

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ABSTRACT

Objectives: Blight disease occurring due to *Xanthomonas axonopodis* (pv. Punicae) has been creating a problem in pomegranate cultivation whose symptoms appear on leaves as well as on fruits and stem and thereby causing a detrimental effect on yield by reducing it to nearly 70–75%. In recent years, the disease has been controlled by nanoparticles (silver and copper). We are looking forward to an organic and more economic method for the control of blight disease of pomegranate which is farmer and consumer friendly.

Methods: In the present work, the causative agent of disease was isolated from the infected pomegranate fruit which was collected from Bellary, Karnataka on nutrient agar media using streak plating method. Secondary metabolites were extracted from *Leucas aspera* and Indian bael using methanol as solvent from maceration and soxhlet extraction techniques for about six hours. These methods are considered as the traditional methods for extracting phenolic compounds along with the ultrasound assisted solvent extraction (UASE) method. Since UASE degrades the phenols and certain metabolites which act as nutrients we did not go for this technique. The Phytochemical screening of extracts was conducted to check the presence of secondary metabolites having antimicrobial activity. The methanolic extracts were characterised using thin layer chromatography (TLC) and Fourier transform infra-red (FTIR) spectroscopy. The antimicrobial activity for extracts of *L. aspera* and Indian bael were tested by agar disc diffusion method.

Results: Clear Zone of Inhibition (ZOI) were observed for methanolic extracts of *L. aspera* (40 mg/ml and 100mg/ml) and Indian bael (160 mg/ml) which shows that the blight disease could be controlled.

Conclusion: Indian bael methanolic extract (160 mg/ml) shows better control against growth of *Xanthomonas* species with higher ZOI values than *L. aspera* extracts (100 mg/ml and 40 mg/ml).

Keywords: Bacterial blight, *Xanthomonas axonopodis* (pv. Punicae), Secondary metabolites, *Leucas aspera*, Indian bael (*Aegle marmelos)*, Phytochemical screening, Agar disk diffusion, Zone of inhibition, Thin-layer chromatography, Fourier transform infrared spectroscopy.

INTRODUCTION

Punica granatum (pomegranate) is fruit crop which is considered important since it serves as rich source of carbohydrates, vitamins, and minerals. It helps in the prevention of various health disorders in human beings. It is major crop that can be grown in dry regions of Maharashtra located to the West and Karnataka, Andhra Pradesh located to the South of India. Pomegranate cultivation in Karnataka accounts for the majority of state income. Decrease in its yield is major loss of income of state. Even though pomegranate has antibacterial and antiviral activity due to secondary metabolites present, still it gets affected by bacteria. The main reason of decline in its yield is the disease due to *Xanthomonas axonopodis* pv. Punicae which shows symptoms first on leaves than fruits and finally stem. Blight disease symptoms were first observed on leaves as black-greenish spots or oily spots on further investigation of causative agent it was found to be because of *X. axonopodis* and these symptoms controlled using biocontrol potential of certain organisms or biologically active compounds [1]. The cultural and morphological characteristics of the causative agent of disease of infected pomegranate fruits were compared with standard causative agents of bacterial blight disease of pomegranate [2]. The disease due to *X. axonopodis* pv. Punicae has been controlled using actinomycetes [3] and copper nanoparticles [4]. Hence, to reduce the effect of *Xanthomonas* species on yield of pomegranate, the methanolic extracts of *Leucas aspera* and Indian bael are used because of its ability to inhibit the growth of broader range of bacteria including Gram positive and Gram negative. Existence of phytocompounds in the methanolic extracts of *L. aspera* and Indian bael was checked through phytochemical analysis [5,6]. There is a certain concentration of extracts that is enough to inhibit the growth of micro-organisms referred to as minimum inhibitory concentration. The methanol in the extract can be evaporated and the residue can be sprayed directly on the pomegranate plants. The soil contains certain beneficial organisms which contribute to maximum production, if we spray methanolic extracts directly into the soil might hinder the activity of these organisms. In recent research articles, the new blight disease of pomegranate was discovered due to *Pseudomonas* species cultivated in Maharashtra [7] so we took it as a standard causative agent of blight disease.

From the infected pomegranate fruits obtained from different farms in Bellary, Karnataka, isolated colonies of the causative agents of bacterial blight disease appeared as yellow colonies due to yellow pigment produced by the *Xanthomonas* genus termed as xanthomonadin. The methanolic extracts of *L. aspera* and Indian bael were prepared through the Soxhlet and maceration extraction method considered the traditional method for the extraction of phytocompounds from plants [8]. The bioactive compounds present in the methanolic extracts can be characterized by thin-layer chromatography (TLC) considered as a basic chromatographic technique, and Fourier transform infrared (FTIR) spectroscopy [9,10] considered as a powerful analytic tool for characterizing the secondary metabolites present in extracts that contribute to its antimicrobial properties. For antimicrobial testing, we took streptomycin as a positive control (PC) because through already discovered research, it was found that streptocycline, a combination of streptomycin sulfate and tetracycline hydrochloride was effective against the bacterial leaf blight of pomegranate [11].

METHODS

Collection of infected pomegranate fruit and isolation of causative agent of blight disease

Pomegranates are the largest production of fruits that are being imported from Karnataka. The infected pomegranate fruits were collected from a farm located in Bellary. The pomegranate plant had weak stems and was lacking in nutrition. It has been infected by an organism which causes the disease to the fruit. Due to this disease, there has been a decrease in the yield of pomegranates plant and different stages of blight disease are shown in Fig. 1.

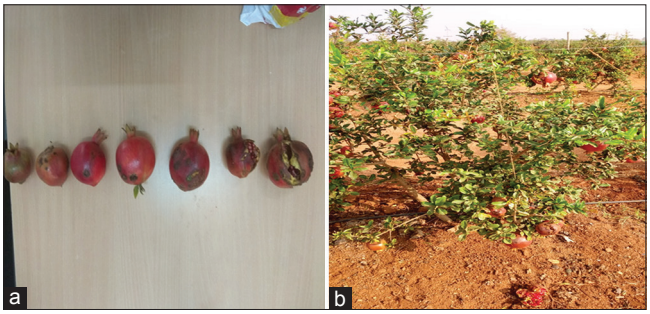

Fig. 1: (a and b) Infected pomegranates showing different stages of disease symptoms from greenish patches to break opening of fruits obtained from Bellary, Karnataka

Then to isolate the causative organism from the diseased fruit, we prepared nutrient agar media, a culture media. The bacterium was isolated using sterile water and spread on the Petri plates using the spread plate technique at aseptic conditions. This was incubated at 37°C for 2 days, as shown in Fig. 2. The mother culture was then subcultured twice to obtain the pure colonies; the colonies obtained were inoculated into the broth, as shown in Fig. 3. This was subjected to preliminary biochemical tests.

Collection of medicinal plants –*L. aspera* and Indian bael

L. aspera, commonly known as Thumbe gida in Kannada, is shown in Fig. 4. It is found in the majority of regions in India. It is widely acknowledged for its tremendous therapeutic qualities such as anti-incentive, antifungal, antimicrobial, and properties. *Aegle marmelos,* also known as Indian bael (Bilva patra in Kannada), is well known medicinal plant, as shown in Fig. 5, which is very known for its healing properties. It is mostly found in India and Bangladesh. Both of these medicinal plants were obtained from Bengaluru, Karnataka, as shown in Figs. 4 and 5.

The stems, leaves, buds, flowers, and roots of both the plants were separated and sun dried at a temperature of 34°C for 2 days. Once they

Fig. 2: Isolated pure yellow colonies of the causative agent of disease obtained from infected pomegranate fruits

Fig. 4: *Leucas aspera* plant before sun drying obtained from RV College of Engineering, Bengaluru, Karnataka

Fig. 3: Broth culture of the causative agent of disease of pomegranate after 2 days of incubation at 37°C

Fig. 5: Indian bael plant before sun drying obtained from Bengaluru, Karnataka

are completely dried, it was powdered and stored in a zip lock cover for further use, as shown in Fig. 6.

Preliminary microbial testing of causative agent

Gram staining

The cells were air-dried and heat-fixed by smearing it on the glass slide. Then, it was flooded with crystal violet. The slide was then rinsed with tap water, drop by drop 1 ml of gram's iodine was placed on the glass slide and kept aside for a minute. Then, it was cleaned again using water falling dropwise. The same slide was then flushed with 95% ethanol – a decolorizing agent and then waited for 10 to 15 s. The glass slide was flushed with safranin (counterstain) and kept aside for duration of 45 s to 1 min, it was then blotted dry and then viewed under microscope.

Hydrolysis of starch

In the starch agar media for hydrolysis, the bacteria may secrete an enzyme called alpha-amylase. This extracellular enzyme cleaves the glucosidic linkage of starch thereby releasing individual glucose molecules. This test was done in a sterile technique, a single streak inoculation of organism which was made into the center of the labeled plate. The plates containing the bacteria were inoculated for 48 h at 37°C. Then following the incubation, with the use of a dropper the surface of the plates was flooded with iodine solution for 30 s. The excess iodine was then washed off. Ultimately, it was then checked for the clear zone of inhibition (ZOI) around the line of bacterial growth.

Test to check the utilization of citrate by bacteria

This test is to check whether the bacteria utilizes citrate for its metabolism from the source. The bacterium (incubated for a period of 18–24 h) was inoculated on Simmons citrate agar slant media using the sharp point of the inoculating loop. Azure blue color was seen, this denotes that bacteria have used the citrate and highlights the process of alkalization.

Indole production test

In this test, the suffusion of L-tryptophan broth was done with the emulsified isolated colony of the test organism. It was then kept for incubation (37°C) for 24–48 h. A chemical agent Kovac's solution (0.6 ml) was put to the L-tryptophan broth culture. If red colored rings were noted, then this shows if the bacteria produce indole from L-tryptophan by splitting up of amino acids.

Methyl red (MR) test

This test is to check whether the bacteria utilizes the glucose from the media and turns it into acid. Two of the test tubes having MR Voges–Proskauer (VP) broth was suffused by including the broth culture of the test bacteria. The incubation time for these test tubes was 4 days. About 0.8 ml of pH indicator (MR) solution was put to both tubes, a positive result is noted if the color of the medium turns red.

VP test

The organism was taken from a culture of 18–24 h of the lightly inoculated medium. It was then incubated aerobically at 37°C for 24 h. Following the 24-h incubation, aliquot 2 ml of the broth had been put in the test tube. The aliquot was re-incubated for an individual for an additional 24 h. 0.8 ml of 5% alpha-naphthol was combined well. The test tube was then observed for pink coloration at the surface of the top layer for 30 min after vigorous shaking of the test tube.

Extraction of secondary metabolites from *L. aspera* and Indian bael by Soxhlet extraction and maceration method

Maceration method

3 g in 30 ml of methanol of the powdered form of *L. aspera* and 4 g in 25 ml of methanol (160 mg/ml) *A. marmelos* were taken in a beaker. It was stirred with a magnetic stirrer for 6 h at high speed, as shown in Fig. 7. Then, the extract was filtered using Whatman filter paper (42 mm) into two separate conical flasks consecutively

Fig. 6: Ground powder of *Leucas aspera* and Indian bael

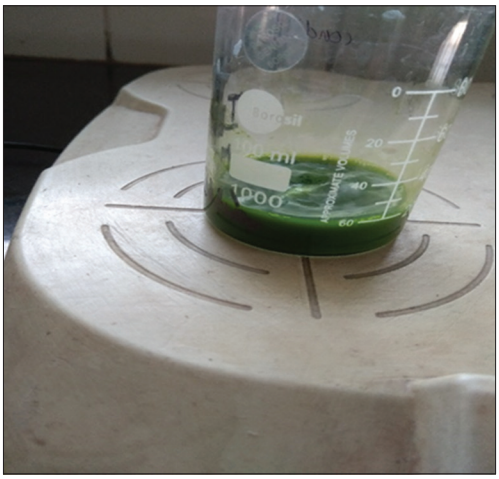

Fig. 7: Extraction of secondary metabolites from Indian bael of concentration 160 mg/ml and *Leucas aspera* of concentration 100 mg/ml by maceration extraction method

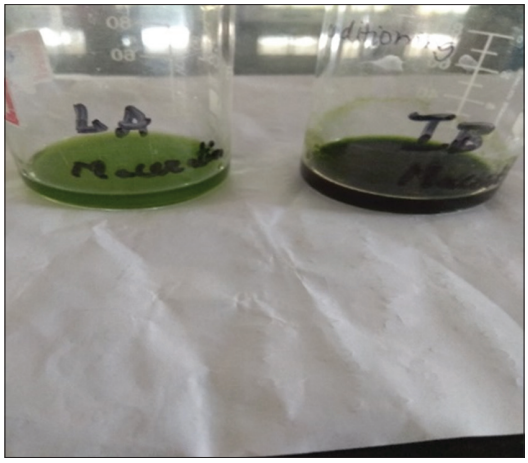

Fig. 8: The methanolic extracts of *Leucas aspera* and Indian bael after maceration method completion

as shown in Fig. 8. This was then wrapped in aluminum foil and stored in the refrigerator at −4° to be used for agar disk diffusion method in vials of 2 ml for further analysis by FTIR spectroscopy and TLC and for checking for its antimicrobial activity by agar disk diffusion method.

Procedure for Soxhlet extraction

Soxhlet extractor

It is an apparatus used for extracting the particular type of solids. This is used to extract partially soluble components from *L. aspera* in methanol.

Principle

This is a common method for continuous extraction of metabolites from plant materials (solid) that are partially soluble in methanol (liquid). Cellulose paper (thimble) containing the plant material, through which methanol is allowed to run continuously.

Concentration of the extract

10 g of dried *L. aspera* was weighed. This was then diluted with methanol (250 ml).

Procedure

Thimble is made out of a thick cellulosic material whose pore size is such that it allows only particular compounds to pass through. The plant material containing the desired compound kept within the thimble. This is kept in the main column of Soxhlet extractor. This entire apparatus was stacked on the round bottom flask containing methanol then the air-line condenser was fitted onto it. Methanol (solvent) was boiled at a temperature of 65°C. Vapors of the solvent were allowed to escape up the distillation limb, and floods into the main column containing the plant material. The compartment having the solid material gets accumulated by warm vapors of the methanolic solvent. Few of the desired components was diffused in warm vapors. As soon as the compartment fills up it is naturally drained through siphon side limb, with the solvent flowing to the round bottom flask. The continuous travelling of this solvent from the round bottom flask and back to it is referred as one cycle. Similarly, four separate cycles done to completion. Every cycle, some quantity of the partially soluble compound was dissolved in the methanol. After four cycles, the desired compound was dissolved in the solvent. Soon, after four cycles are finished that the metabolites were concentrated in the round bottom flask, as shown in Figs. 9-11.

Fig. 9: Extraction of secondary metabolites from *Leucas aspera* of concentration 40 mg/ml by Soxhlet extraction method (during 1st cycle)

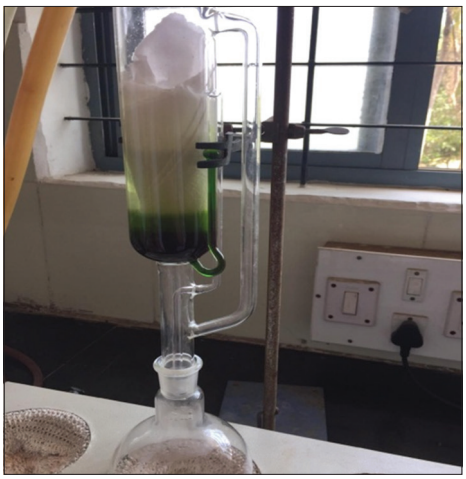

Fig. 10: Soxhlet extraction of phytocompounds from *Leucas aspera* in methanol after 1st cycle

Phytochemical screening of *L. aspera* and Indian Bael

Preparation of reagents used in the screening of polyphenolic compounds.

1 percent ammonia: 0.5 ml of ammonia solution suffused in 49.5 ml of H_2O (distilled).

Mayer's reagent

Preparing the reagent, for examining the presence of alkaloids:

i. 0.68 g of $HgCl_2$ suffused in 30 ml of H_2O (distilled)
ii. 2.5 g of KI suffused in 10 ml of H_2O (distilled).

Solutions (i) and (ii) are diffused thoroughly and made up to the volume (50 ml) using H_2O (distilled).

Wagner's reagent

This reagent is usually used for the detection of alkaloids. 0.635 g of iodine, 1 g of KI was suffused to a volume of 2.5 ml of H_2O, this is made up to 50 ml using H_2O (distilled).

Test for tannins

Ferric chloride test

1 ml of extract was taken and boiled with 5 ml of ethanol (45%) left for 5 min that of *L. aspera* and Indian bael.

Alcoholic solution of the extract was filtered using filter paper. A small amount of this percolate was mixed with 0.6 ml of ferric chloride solution (5% in distilled water). The transitory change from Sacramento green color to black color. Tannins existence is proved by this test.

Fig. 11: Methanolic extract (light greenish) of *Leucas aspera* (40 mg/ml) after completion of four cycles

Checking for the presence of terpenoids

Evaluation Salkowski (test)

Extract (methanolic) taken and diffused with 1 ml of chloroform and conc. H_2SO_4 was affixed to the test tubes to form a division. Showing up of auburn color at interface confirmed the terpenoid's presence.

Examination of alkaloids

Mayer's chemical reagent and Wagner's chemical reagent were utilized in this test.

1 ml of methanolic extract had been prepared and kept aside in warm condition with 2% sulfuric acid for 2 min. Then, it was filtered and a few drops of reagents were added.

a. Test of Mayer's – A milky green appearance in extract after the addition of Mayer's reagent showed alkaloid's presence
b. Test of Wagner's – Appearance of the reddish-brown precipitate. This happened to confirm the existence of alkaloids.

Test for flavonoids

1 ml of methanolic extract was boiled with ethyl acetate (10 ml) in water bath, whose temperature was 77°C, for a duration of 5 min. This mixture was filtered. The filtrate from this mixture was used to do these following tests:

Ammonium test

1 ml (1%) of dilute ammonia was taken and added to 1 ml of filtrate, which was collected earlier. Separation of two layers occurred. The development of yellow coloration was seen in the ammonia layer, which confirmed its presence.

Examination of saponins in methanolic extracts

Foam test

A diluted form of the extract with 10 ml H_2O (distilled.), this was agitated in the test tube for 15 min continuously. The occurrence of the layer of foam will have indicated the existence of saponin.

Test for sterols

Liebermann–Burchard test

Methanolic extract of 2 ml in volume was blended with chloroform (1 ml). 1 ml of ethanoic anhydride was taken and 0.8 ml of conc. H_2SO_4 and run down along the sides, then mixed thoroughly by shaking it for 1 min. A notable color change from red to blue and finally green color will have indicated the presence of sterols.

TLC of *L. aspera* and Indian bael

The mobile phase petroleum ether: n-Hexane: ethyl acetate: acetone:methanol in the ratio is in the ratio 30:8:5:5:2. This was kept at equilibrium for half an hour. The samples were then spotted on the TLC sheet. These samples were allowed to run till the solvent reached 3/4th distance of the TLC sheet, as shown in Fig. 12.

FTIR spectroscopy analysis of a methanolic extract of *L. aspera*

Merieux NutriSciences is a well-known company for testing biological samples. It is located in Rajaji Nagar, Bengaluru Karnataka. As part of the project, the samples were given to this test house to conduct the analysis of Fourier series infrared spectroscopy. They used PerkinElmer spectrum version 10.03.07, instrument model number-spectrum two, instrument serial number 89230.

Sample specification:

1. *L. aspera* – 40 mg/ml (obtained by Maceration method)
2. *L. aspera* – 100 mg/ml (obtained by Soxhlet extraction method)
3. *A. marmelos* – 160 mg/ml (obtained by Maceration method).

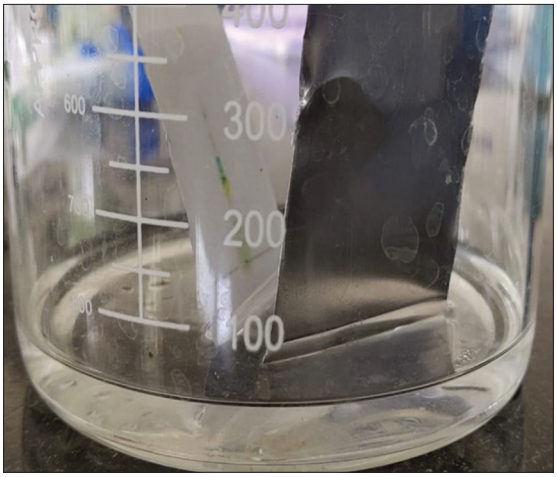

Fig. 12: The running of the sample of Indian bael (160 mg/ml) and solvent (mobile phase) to 3/4th distance of the thin-layer chromatography sheet

Fig. 13: Isolated pure culture of the causative agents of bacterial blight after 2 days of incubation at 37°C

Table 1: Comparison of the test bacteria, causative agent of disease in collected pomegranates with the most common causative agent of blight disease of pomegranate *Xanthomonas axonopodis* and *Pseudomonas* species

Characteristics	Test bacteria	*Xanthomonas axonopodis*	*Pseudomonas* Species
Shape	Rods	Rods	Rods
Gram staining	–	–	–
Starch hydrolysis	–	–	+
Indole production	–	–	–
Methyl-red	–	–	–
Voges–Proskauer	–	–	–
Citrate utilization	+	+	+

Antimicrobial activity testing of the methanolic extracts of *L. aspera* and Indian bael for control of blight disease by agar disk diffusion method

Luria Bertani agar (LBA)-plate 1

LBA media were prepared and poured onto the Petri plate. This was then kept aside for 20 min to solidify. *Xanthomonas* species was poured on

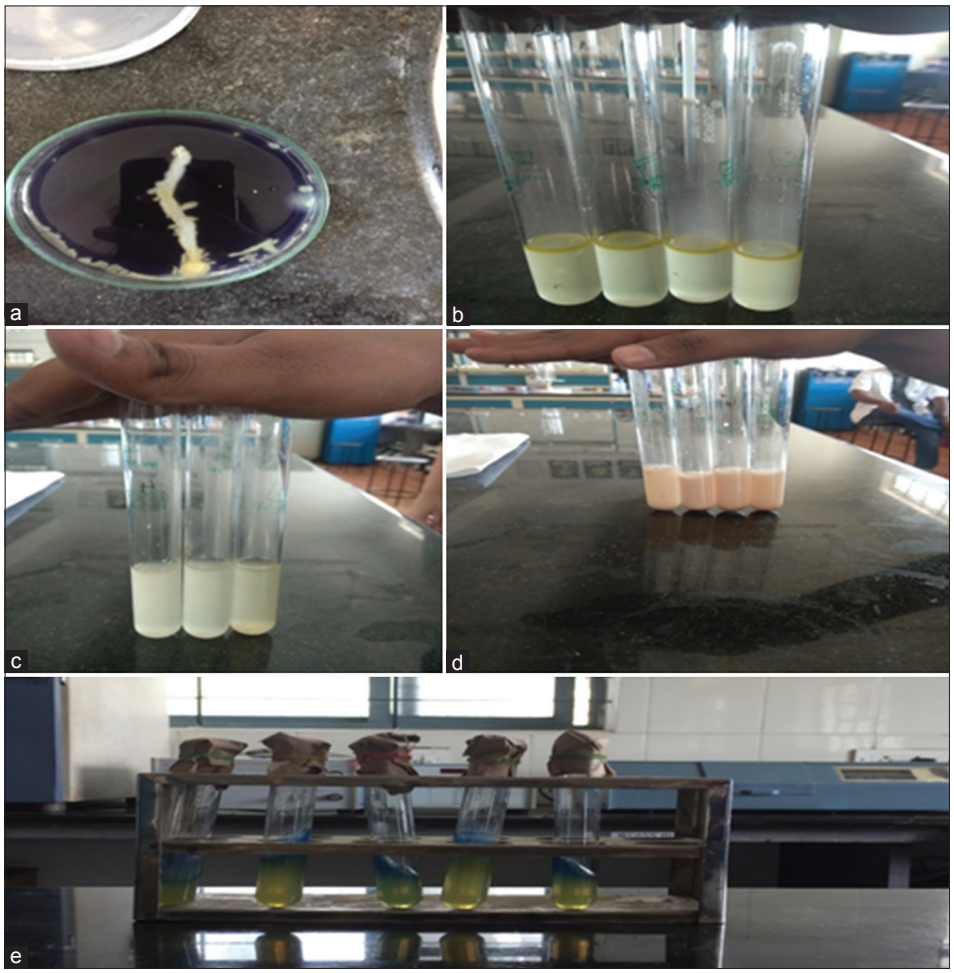

Fig. 14: (a) Starch hydrolysis test there was no clear zone observed hence negative. (b) Indole red ring was not observed hence indole test showed negative results. (c) Methyl-red test showed negative results since culture medium had not turn red on addition of methyl red, pH-sensitive reagent. (d) Voges–Proskauer test showed negative results as the medium did not turn red in the presence of alpha-naphthol. (e) Citrate utilization test showed positive results since growth was visible on slant surface and media turned Prussian blue in color

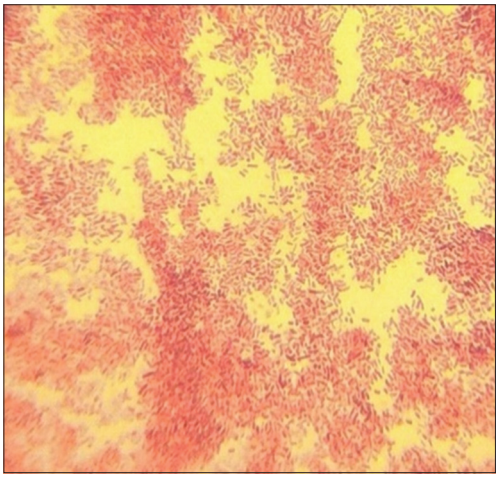

Fig. 15: Gram staining image of the causative agent appeared as Gram-negative rods

the media using a micropipette and spread uniformly by incorporating the spread plate technique, all by maintaining a sterile condition in the laminar airflow. Streptomycin disk (10 mcg) was taken as the PC and negative control (NC) taken was 1 ml of methanol put on disk. 1 ml of

Table 2: Phytochemical screening results of the phytocompounds having antimicrobial activity in our liquid extracts where "+" indicates present and "–" indicates absent

Phyto compounds	Liquid extract *Leucas aspera* (stem)	Liquid extract Indian bael (entire plant excluding fruit)
Tannins	+	+
Terpenoids	+	+
Flavonoids	+	+
Alkaloids	+	+
Sterols	+	+
Saponins	–	–

L. aspera (40 mg/ml) was put on the sterile disk of 5 mm diameter. After this, 1 ml of Indian bael (160 mg/ml) was put on another sterile disk of the same diameter was placed on LBA media.

LBA- plate 2

LBA media were prepared and poured on the Petri plate. This was kept aside to solidify for 20 min. *Xanthomonas* species was poured on the media using a micropipette. This was spread uniformly on the plate by incorporating the spread plate technique, all this is done by maintaining a sterile environment in the Laminar airflow. Streptomycin

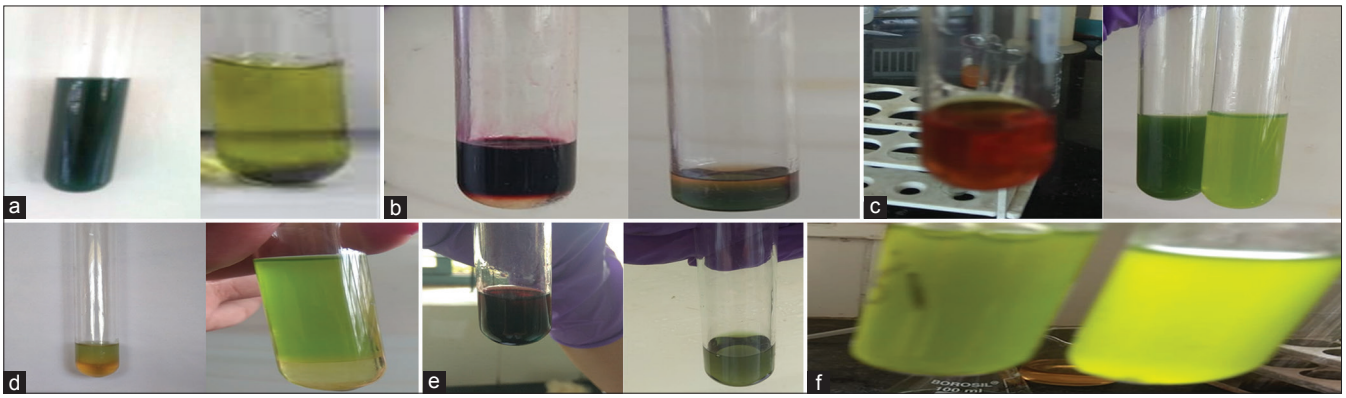

Fig. 16: The results that appeared for phytochemical tests have been summarized. (a) Ferric chloride test for tannins (i) ferric chloride test for tannins showed positive results for *Leucas aspera* extract by appearance of transient greenish to black (ii) $FeCl_3$ examination for tannins showed positive result for Indian bael extract by appearance of black color at the bottom. (b) Salkowski test for terpenoids (triterpenoids) (i) salkowski test for terpenoids showed positive result for *L. aspera* extract by appearance of red color top layer (ii) salkowski test for terpenoids showed positive result for Indian bael extract but present in low amounts by appearance light red color top layer. (c) Test for alkaloids (i) Wagner's test for alkaloids showed positive result for *L. aspera* extract be appearance of reddish-brown precipitate (ii) Mayer's test for alkaloids showed positive results for Indian bael extract by appearance of creamy green color from dark green, actual color of extract. (d) Ammonium test for flavonoids (i) ammonium test for flavonoids showed positive result for *L. aspera* extract since yellow color was observed in ammonia layer (bottom layer) (ii) ammonium test for flavonoids showed positive results for Indian bael extract since yellow color was observed in ammonia layer (bottom layer). (e) Liebermann–Burchard test for Sterols (i) Liebermann–Burchard test for sterols showed positive results for *L. aspera* extract by appearance of first red color and finally green color (ii) Liebermann–Burchard test for sterols showed positive result for Indian bael extract by appearance of first blue to green color. (f) Foam test for saponins (i) foam test for saponins gave negative results for both *L. aspera* and Indian bael extracts since no appearance of foam

Fig. 17: Thin-layer chromatography sheet image of methanolic extract of Indian bael 160 mg/ml, distance moved by solvent system was 8 cm and the spots were numbered from start to top of sheet (the direction in which solvent moved)

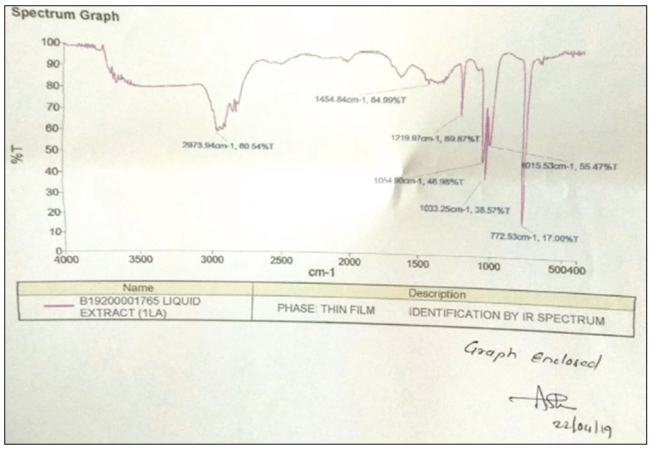

Fig. 18: Fourier transform infrared spectrum of methanolic extract of *Leucas aspera* by Soxhlet extraction method

Table 3: The Rf values of the spots of Indian bael have been summarized

Sample spot	Color	Distance moved by sample spot in cm	Rf
1	Light greenish	1.1	0.1375
2	Yellowish	3.9	0.4875
3	Parrot greenish brighter than other spots	4.3	0.5375
4	Greenish	4.7	0.5875

disk (10 mcg) was taken as PC and NC was 1 ml of methanol put on disk. 1 ml of *L. aspera* (100 mg/ml) was put on the sterile disk of 5 mm diameter. After this, 1 ml of Indian bael (160 mg/ml) was put on another sterile disk of the same diameter was placed on LBA media.

Mueller-Hinton (MH) agar-plate 3

MH agar media were prepared and poured onto the Petri plates. This was then kept aside for 20 min to solidify. *Xanthomonas* species was poured on the media using a micropipette and spread uniformly by incorporating the spread plate technique, all by maintaining a sterile condition in the laminar airflow. Streptomycin disk (10 mcg) was taken as the PC and the NC taken was 1 ml of methanol put on sterile disk. 1 ml of *L. aspera* (40 mg/ml) was put on the sterile disk of 5 mm diameter. After this, 1 ml of Indian bael (160 mg/ml) was put on another sterile disk of the same diameter was placed on MH agar media.

Nutrient agar plates

For obtaining more clearer zones, we prepared nutrient agar media and poured on the two Petri plates (a and b) and kept aside for 20 min to solidify. *Xanthomonas* species was poured on the plate and spread uniformly using the spread plate technique maintaining sterile conditions under laminar airflow. Streptomycin disk (25 mcg) was taken as PC and NC was 2 ml of methanol put on disks and placed in both plates. 2 ml of Indian bael (160 mg/ml)

was put on disks and 2 ml of 100 mg/ml put on disks and placed on both plates.

RESULTS AND DISCUSSION

Isolation of causative agent of bacterial blight of pomegranate

Colonies of causative agent of blight disease were observed on nutrient agar media after incubation period of two days as shown in Fig. 13.

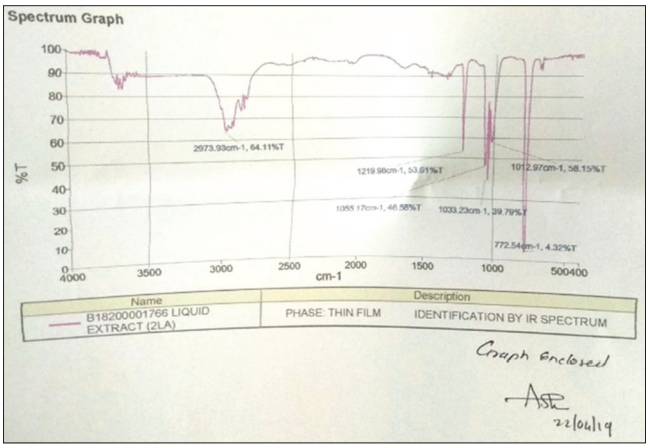

Fig. 19: Fourier transform infrared spectrum of the methanolic extract of *Leucas aspera* by maceration extraction method

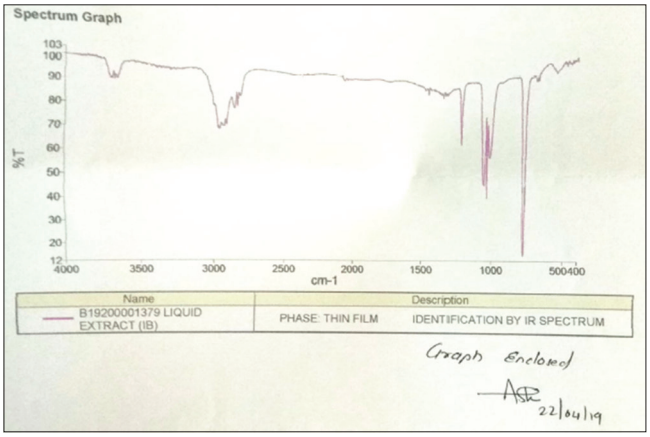

Fig. 20: Fourier transform infrared spectrum of the methanolic extract of Indian bael by maceration extraction method

Preliminary microbial testing of the causative agent

The biochemical tests (starch hydrolysis, indole formulation, MR, VP, and citrate usage tests) results shown in Fig. 14 as well as Gram staining shown in Fig. 15, the basic microbial test for the test bacteria culture is summarized in Table 1.

From the preliminary tests, we conducted for the test bacteria and through comparative analysis with the major causative agents of blight disease of pomegranate that is *X. axonopodis* and *Pseudomonas* species, we found that the causative agent of the disease of infected pomegranate fruit we obtained from Bellary was *X. axonopodis*. In recent studies, it has been found that new blight disease of pomegranate is caused by *Pseudomonas* species, which has affected pomegranate cultivation in Maharashtra. Hence, we are comparing that whether the test bacteria, the causative agent of the disease is *Xanthomonas* or *Pseudomonas* species, which is inferred in Table 1.

Screening for polyphenolic compounds of the methanolic extracts of *L. aspera* and Indian bael

The screening of polyphenolic compounds revealed the existence of secondary metabolites which were extracted using methanol as solvent from medicinal plants *L. aspera* (stem) and Indian bael (entire plant excluding fruits), of which tannins, terpenoids, flavonoids, alkaloids, sterols, and saponins have antibacterial and antifungal activity and results. Using this property of these compounds present in the extract, we inhibited the growth of *Xanthomonas* species.

The results for all the above tests for detection of presence of some of secondary metabolites, which we are focussing on because of its antimicrobial activity are summarized in Table 2 and Fig. 16.

TLC of methanolic extract of *L. aspera* (40 mg/ml) and Indian bael (160 mg/ml)

The solvent system used was petroleum ether: n-Hexane: ethyl acetate: acetone:methanol in the ratio 30:8:5:5:2. For 40 mg/ml of *L. aspera* methanolic extract distance moved by solvent system was 6 cm, but we got a very light green spot and its Rf value corresponded to that flavonoids, terpenoids, and alkaloids and for Indian bael we got four spots which could be observed in visible light as shown in Fig. 17. The Rf value is calculated using an equation.

Rf = Distance moved by solvent system/distance moved by sample spot.

The Rf values summarized in Table 3 show the presence of flavonoids, alkaloids, terpenoids, and steroids in Indian bael.

FTIR analysis of extracts of *L. aspera* and Indian bael

The FTIR analysis of the methanolic extract of stem of the *L. aspera* obtained through maceration extraction method of concentration 100 mg/

Table 4: ZOI values measured for 1 ml of each extract and negative control for petri plates in Fig. 21

Sample specifications	Luria Bertani agar (1) ZOI (in cm)	Luria Bertani agar (2) ZOI (in cm)	Mueller-Hinton agar (3) ZOI (in cm)
PC	1.0	1.2	2.0
NC	0.0	0.0	0.0
40 mg/ml of *L. aspera*	0.7	-	0.6
100 mg/ml of *L. aspera*	-	0.9	-
160 mg/ml of Indian bael	1.2	1.0	0.6

ZOI: Zone of inhibition, *L. aspera*: *Leucas aspera*, PC: Positive control, NC: Negative control

Table 5: ZOI in cm of methanolic represented in the form of average±standard deviation

Species	ZOI in cm for 40 mg/ml of *L. aspera*	ZOI in cm for 100 mg/ml of *L. aspera*	ZOI in cm for 160 mg/ml of Indian bael
Xanthomonas species	0.65±0.05	0.9±0.0	0.93±0.25

ZOI: Zone of inhibition, *L. aspera*: *Leucas aspera*

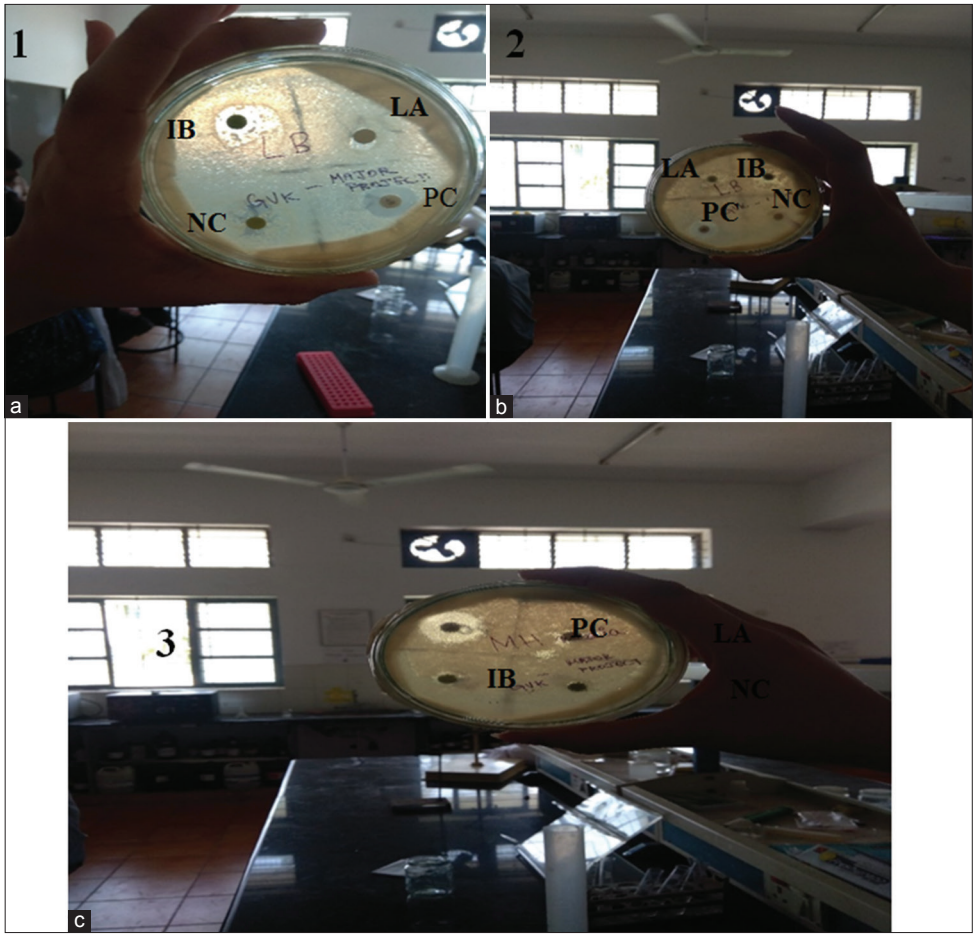

Fig. 21: (a and b) Zone of inhibition for *Xanthomonas* species due to the antimicrobial activity of extracts on Luria Bertani agar media, positive control used was 10 mcg (millionth of gram) of streptomycin it is not present in our extract just for reference of clear zone and negative control used was methanol since we used it as solvent for extraction. (c) Zone of inhibition for *Xanthomonas* species due to antimicrobial activity of extracts on Mueller-Hinton agar media, positive control was 10 mcg (millionth of gram) of streptomycin and negative control was methanol since used as solvent for extraction

ml (green color liquid) is depicted in Fig. 18, spectrum obtained using PerkinElmer spectrum software version 10.03.07 with resolution of 4 cm^{-1} and number of scans was 1 and checked for the existence of functional groups present in the stem extract. The major peaks were observed at 2973.94, 1454.84, 1219.97, 1054.90, 1033.25, 1015.53, and 772.53 cm^{-1}. The peak at 2973.94 cm^{-1} is due to C-H bond stretch in alkanes, 1454.84 cm^{-1} corresponds to C-H bond bend in alkanes, 1219.97 cm^{-1} corresponds to C-O stretch in phenols, and 1033.25 cm^{-1} corresponds to O-H group in flavonoids, terpenoids, and non-flavonoids. 772.54 cm^{-1} C-H bond out of plane bend in aromatic compounds or C-H bond bend in cis-alkene.

FTIR analysis of the methanolic extract of *L. aspera* stem obtained through Soxhlet extraction method of concentration 40 mg/ml (light green color liquid) to reveal the functional groups that is present in the extract is depicted in Fig. 19, spectrum obtained using PerkinElmer spectrum software version 10.03.07 with resolution of 4 cm^{-1} and number of scans was 1. FTIR spectrum of the extract showed major peaks at 2973.93, 1219.98, 1055.17, 1033.23, 1012.97, and 772.54 cm^{-1}. The peak at 2973.93 cm^{-1} corresponds to C-O stretch in phenols, 1219.98 cm^{-1} corresponds to C-O stretch in phenols, 1055.17 cm^{-1} corresponds to C-O stretch in alkyl substituted ethers or primary alcohol, and 1033 cm^{-1} and 1012.97 cm^{-1} show presence of O-H group that is present in flavonoids, terpenoids, and non-flavonoids and 772.54 cm^{-1} corresponds to C-H out of plane bend in aromatic compounds(ortho), C-H bend in cis-alkene.

FTIR analysis of plant extract of Indian bael using methanol as solvent obtained by maceration extraction method of concentration 160 mg/ml

Table 6: The values of ZOI in cm including the disk diameter for methanolic extracts of 100 mg/ml *L. aspera* and 160 mg/ml of Indian bael have been summarized for Petri plates (a and b) shown in Fig. 22

Sample	Nutrient agar (a) ZOI (in cm)	Nutrient agar (b) ZOI (in cm)
Positive control	2.0	1.9
Negative control	2.0	1.9
100 mg/ml of *L. aspera*	1.8	1.9
160 mg/ml of Indian bael	1.8	2.5

ZOI: Zone of inhibition

Table 7: ZOI in cm has been measured as mean±standard deviation for *L. aspera* and Indian bael for *Xanthomonas* species

Species	ZOI in cm for 100 mg/ml of *Leucas aspera*	ZOI in cm for 160 mg/ml of Indian bael
Xanthomonas species	1.85±0.05	2.15±0.35

ZOI: Zone of inhibition

(green color liquid) is depicted in Fig. 20, spectrum obtained using PerkinElmer spectrum software version 10.03.07 with resolution 4 cm^{-1} and number of scans was 1. The spectrum showed major peaks between 3500–3400, 3000–2970, 1300–1210, 1060–1050, 1035–

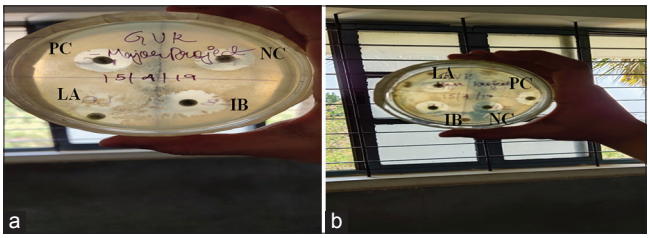

Fig. 22: (a and b) Clearer zone of inhibition for *Xanthomonas* species using methanolic extracts of *Leucas aspera* and Indian bael increasing the volume on nutrient agar media. Positive control used was 20 mcg (millionth of gram) of streptomycin and negative control was methanol but volume used was 2 ml and even the volumes of methanolic extracts of *Leucas aspera* and Indian bael used were 2 ml

1030, 1020–1010, and 780–770 cm^{-1}. Broad peak between 3500 and 3400 cm^{-1} is responsible for O-H stretching in polyphenolic compounds such as flavonoids, non-flavonoids, and triterpenoids. 3000–2970 cm^{-1} corresponds to C-H stretching in alkanes, 1300–1210 cm^{-1} C-O stretching in phenols, and 1060–1050 cm^{-1} are due to presence of alkyl substituted ethers or primary alcohol, 1035–1010 cm^{-1} corresponds to O-H group in flavonoids, terpenoids, and non-flavonoids. 780–770 cm^{-1} is due to the presence of C-H out of plane bend in aromatic compounds, C-H bend in cis-alkenes.

The presence of alkyl, methyl groups, alcohols, ethers, esters, carboxylic acid, and anhydrides indicated the appearance of C=O, C-H, C=C, C-O, and C-C bonding structures. The FTIR analysis confirms the presence of secondary metabolites such as alkaloids, tannins, flavonoids, steroids, and terpenoids and phenols, these compounds contribute to medicinal property of Indian bael (*A. marmelos*) and *L. aspera*. Proteins and carbohydrates are also present but we are interested in secondary metabolites having antimicrobial activity. We know phenols are used as antioxidants.

Antimicrobial activity testing of methanolic extracts by agar disk diffusion method to control blight induced due to *Xanthomonas* species

ZOI of negative control, methanol was 0.0 cm in Table 4 since no clear zone was observed even though the disc diameter (0.5 cm).

To obtain clearer ZOI, we increase volume of the extract but did not use 40 mg/ml of *L. aspera* methanolic extract obtained through Soxhlet extraction method, but used 100 mg/ml of *L. aspera* methanolic extract obtained through maceration extraction method and 160 mg/ml of Indian bael methanolic extract by maceration extraction method since we did not get larger zones in case of 40 mg/ml of *L. aspera* in the previous case for *Xanthomonas* species. By increasing the volume, we found the ZOI got higher.

Larger zones of inhibition can be correlated to minimum inhibitory concentrations of extracts for *Xanthomonas* species. Therefore, the minimum inhibitory concentrations of Indian bael extract required for *Xanthomonas* species (*X. axonopodis*) were found to be 160 mg/ml and of *L. aspera* extract was 100 mg/ml.

CONCLUSION

From the above study, we concluded that infection of pomegranate plant was caused due to *Xanthomonas* species which we inferred from preliminary microbial tests of test bacteria which we had isolated from infected pomegranate. By the use of secondary metabolites in methanolic extracts of Indian bael and *L. aspera,* we were able to control the blight disease which was caused by *Xanthomonas s*pecies. The presence of secondary metabolites in extracts was confirmed using TLC and FTIR spectroscopy. This could replace the use of nano copper and silver nanoparticles for the control of bacterial blight disease in pomegranate that is being used at present. Silver nanoparticles and copper nanoparticles are being used because of their antimicrobial activity against most of the bacteria including *Xanthomonas* species. Since we evaluated the antimicrobial activity of the methanolic extracts of *L. aspera* and Indian bael by agar disk diffusion method by the appearance of ZOI, we found it has significant effect controlling *X. axonopodis* (pv. Punicae) growth with minimum inhibitory concentration required 100 mg/ml of *L. aspera* and 160 mg/ml of Indian bael. Indian bael showed more significant effect against growth of *Xanthomonas* species when compared to *L. aspera* which could be inferred from Table 5-7. As shown in Table 5, NC methanol showed similar result as PC streptomycin but we cannot use methanol for the control of blight disease of pomegranate since it degrades some phenols and fatty acids which act as nutrients to the infected leaves and stems, because we know the stem fall apart from plant after being infected with blight due to lack of nutrients.

FUTURE SCOPE

Using the antimicrobial activity of the secondary metabolites that are present in *L. aspera* and Indian bael methanolic extracts, we can control many diseases caused by bacteria like *Xanthomonas* species. These extracts are even effective against many fungal diseases like *Candida albicans*. *Xanthomonas* species are major threat to cultivation of food crops such as rice, citrus fruits, and pomegranate fruit. We cannot use the methanolic extracts directly as methanol is highly viscous as it causes degradation of many fatty acids, proteins, and many other necessary biomolecules. Hence, we need to evaporate the methanol used for extraction of secondary metabolites in methanolic extracts and spray the residue that remains with water as solvent. This residue will provide the necessary nutrients as well as control the disease caused by *Xanthomonas* species. It has been reported recently that bacterial disease of pomegranate is being caused by *Pseudomonas* species in Maharashtra, the secondary metabolites that have been extracted are also effective against *Pseudomonas* species growth. It is also economical preparing plant extract when compared to preparation of silver nanoparticles and copper nanoparticles which have relative antimicrobial activity against *Xanthomonas* and other bacterial and fungal organisms. Hence, these plant secondary metabolites could be used for cultivation of pomegranate, because silver and copper nanoparticles have certain side effects on consumer health, since cannot be directly consumed.

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AUTHORS' CONTRIBUTIONS

Alisha Susana Rodrigues and Ananya B have conducted the research work looking for possible approaches for execution, G Vijaya Kumar and Trilok Chandran guided us in this project.

REFERENCES

1. Hingorani S. Biocontrol of Bacterial Blight of Pomegranate Caused by *Xanthomonas axonopodis* pv. *Punicae*, Department of Plant Pathology. Bengaluru: University of Agricultural Sciences GKVK; 2015.
2. Patil AG, Ambadkar CV, Kanase KM, Kashid VS. Cultural and morphological charateristics of different *Xanthomonas axonopodias* pv. *punicae* isolates on nutrient agar media. Int J Curr Microbiol Appl Sci 2017;6:1678-83.
3. Chavan NP, Pandey R, Nawani N, Nanda RK, Tandon GD, Khetmalas MB. Biocontol potential of actinomycetes against *Xanthomonas axonopodis* pv. *punicae*, a causative agent for oily spot disease of pomegranate. Biocontrol Sci Tech 2015;26:351-72.
4. Mondal KK, Mani C. Investigation of the antibacterial properties of nanocopper against *Xanthomonas axonopodis* pv. *punicae*: The incitant

of pomegranate bacterial blight. Ann Microbiol 2013;62:889-93.
5. Sheel NJ. Preliminary phytochemical screening of methanolic extract of clerodendron infortunatum. IOSR J Appl Chem 2014;7:10-3.
6. Roopalatha UC, Nair V. Phytochemical analysis of successive reextracts of the leaves of *Moringa oleifera* Lam. Int J Pharm Pharm Sci 2013;5:629-34.
7. Smita BJ, Sonawane MS, Kapadnis BP. A new bacterial blight of pomegranate caused by *Pseudomonas* sp. in Maharashtra. India Aust Plant Dis Notes 2018;13:27.
8. Vasudha K, Archana D, Mutyalamma B, Kishori B. Phytochemical screening, antimicrobial and antioxidant activities of root and leaf extract of *Leucas aspera*. Asian J Pharm Clin Res 2019;12:141-7.
9. Hasana H, Desalegn E. Characterisation and quantification of phenolic compounds from leaf of *Agarista salicifolia*. J Herb Med 2017;3:1.
10. Renatha Nunes Oliveira BQTP. FTIR analysis and Quantification of Phenols and Flavonoids of five commercially available plants extracts used in wound healing. Matéria (Rio J) 2016;21:767-79.
11. Kumar MR, Wali SY, Benagi VI, Patil HB, Patil SS. Management of bacterial blight of pomegranate through chemicals/antibiotics. Acta Hortic 2011;890:481-4.