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GREENER METHODS OF CONTROL FOR MICROBIAL DISEASES OF POMEGRANATE USING COPPER OXIDE NANOPARTICLES AND BETEL LEAF EXTRACT

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ABSTRACT

Pomegranate (*Punica granatum*) is an important exportable fruit crop of India that faces major losses when infected by fungal wilt and bacterial blight. The present investigation is aimed to isolate and identify the causative organism of the plant disease infecting the stem of pomegranate. The identification based on microscopic, morphological characterization and 18SrRNA sequencing confirms as *Mariannaea elegans*. The crude extracts of various leaves of plants viz *Psidium guajava*, *Picrorhiza kurroa* and *Piper betel* were used to evaluate antifungal activity using agar well diffusion method. Results confirm that ethanolic extract of *Piper betel* extract (0.5mg/ml) show zone of inhibition against *Mariannea* elegans. The research demonstrates that commonly used fungicides viz., Manocozeb/ Matalxy and Carbendazim showed very little inhibition as compared to the *Piper betel* ethanolic extracts and hence the latter proves to be a better and eco-friendlier control alternative.

The present study was also aimed at finding remedies for bacterial blight. The blight pathogen was isolated from the fruit pericarp and identified using morphological and biochemical characters to be *Xanthomonas axonopodis pv. punicae* (Xap). Nanoparticles synthesized using green method, Copper oxide nanoparticles (1mg/ml) synthesized using *Azadirachta indica* leaf extract showed excellent inhibitory effect against the blight pathogen as compared to Bordeaux mixture, commonly used as an antimicrobial spray by farmers.

Keywords: Pomegranate, *Piper betel*, nanoparticles, blight, wilt

INTRODUCTION

Pomegranate (*Punica granatum*) is one of India's most significant fruit crops cultivated in the tropical and sub-tropical regions in states of Andhra Pradesh, Gujarat, Karnataka, Maharashtra, Rajasthan and Tamil Nadu (**Sharma *et al.*, 2009**). Known for its commercial, medicinal and nutritional value, pomegranate is also exported worldwide (**Saeed *et al.*, 2018; Kumar *et al.*, 2016**). Bacterial blight caused by *Xanthomonas axonopodis pv. punicae* (Xap) has been a disease of grave concern causing major losses to pomegranate production in India as the bacterium suppresses the immunity of the crop and affects all the plant parts including the fruit (**Soni & Mondal, 2017; Mondal *et al.*, 2012**). Another emerging infection causing harm to the crop is fungal wilt. Different fungi have been reported as the causal organisms of pomegranate wilt from different places, *Ceratocystis fimbriata* in India and China, *Colletotrichum gloeosporiodes* in Greece and *Pilidiella granati* in Mediterranean region (**Yang *et al.*, 2016; Xu *et al.*, 2011; Huang *et al.*, 2003; Somasekhara, 1999**). Newer techniques need to be developed to combat these infections so as to maintain good yield of pomegranate crops in the country. Various metal nanoparticles have been used as nanopesticides for control of pathogens that cause damage to plants, out of which Copper nanoparticles have known to exhibit better efficiency as antifungal and antibacterial agents (**Achari & Kowshik, 2018**). The objectives of our study was to isolate and identify the causal organisms of pomegranate blight and wilt in India and further test the inhibitory effect of various nanoparticles on the same. The crude extracts of leaves of plants, *Psidium guajava*, *Picrorhiza Kurroa* and *Piper betel* were also used to evaluate antifungal activity. The research provides interesting perspectives in demonstrating that commonly used fungicides viz., Manocozeb/ Matalxy and Carbendazim showed very little inhibition as compared to the *Piper betel* ethanolic extracts. We also propose that copper, silver and iron nanoparticles synthesized using green methods also provide for eco-friendly options to effective control of bacterial blight as they showed excellent inhibitory effects in-vitro.

MATERIALS AND METHODS

Study of Fungal Wilt of Pomegranate

Isolation and Identification of fungi

The stem of the pomegranate plant infected with fungal disease was selected from the agricultural fields of Indapur, Pune, India. The plant part was surface sterilized using 70 % alcohol and 0.1% $HgCl_2$ solution for few minutes and explants of approximately 5 mm were cut using sterile blade and forceps and dried (**Rajput et al., 2006**). Aseptically loopful of sample was taken and placed on DCPA selective medium (Peptone 3.5g%, K_2HPO_4 0.1g%, $MgSO_4.7H_20$. 0.05g%, Dichloran 0.004g% Chloramphenicol 0.02g% agar-1.5g%) (**Petrini, 1986**). Isolated fungi were subcultured aseptically on sterile PDA plates (potato-20g%, dextrose-2g%, Yeast Extract 0.01g%, agar-1.5g%) and incubated at 28ºC for 3 days. The plates after inoculation were checked every day for 30 days for any contamination. The isolates were morphologically identified after staining with lactophenol cotton blue, slide culture technique (**Kali *et al.*, 2014**), examining colonies for asexual or sexual reproductive structures using optical microscopy (40X) and taxonomic keys (**Barnett and Hunter, 1972**; **Petrini 1986**). The fungus was also identified based on 18S ribosomal RNA gene sequencing using NCBI Database.

Media Used

All solutions and reagents were prepared with distilled and deionized water and analytical grade chemicals were used without further purification (Sigma-Aldrich Co., USA).

PDA and MEA agar (NA) was used for recovery and maintenance of the fungal strain. The fungal suspension made in sterile saline was inoculated on the MEA slants. These were then incubated for 48 hrs to get sufficient growth. The fresh culture was used for further studies.

Preparation of plant extract

Psidium guajava, *Picrorhiza Kurroa*, *Vitex negundo* and *Piper betel* leaves were used for testing antifungal activity. Wet and dry weight for leaves was taken and grinded into fine powder (**Pawar *et al.*, 2017**). Plant material was dissolved in absolute ethanol. Mixtures were kept in the dark for 3 days at 30ºC $\pm$ 2ºC in beakers wrapped with aluminum foil to avoid evaporation and exposure to sunlight was avoided. After 3 days, mixtures were filtered through Whatman no.1 filter paper and kept in incubator at 30ºC $\pm$ 2ºC till all the solvent had completely evaporated. Mixtures were re-suspended in DMSO (Dimethyl sulfoxide) for bioactivity. (**Suprat and Khalini, 2012; Pandey and Shweta, 2011).**

Isolation of Organic components

The Piper betel leaves were purchased from local market of Pune, India. The isolated components were purified on Silica Gel (100-200 mesh) Column Chromatography using n-Hexane and ethyl acetate solvent system. Further

purification and isolation was carried out on preparative TLC technique and all the steps were monitored by thin layer Chromatography (TLC) technique using Silica gel 60 F254, preloaded Silica gel on Alumina sheets. The compound with high yield was characterized by 1H NMR spectroscopy and compared with the previously reported compounds. ^{1}H NMR spectra were recorded on Bruker 200MHz at room temperature using d-$CDCl_3$.

Antifungal Activity

The antifungal activity of ethanolic plant extracts (0.5mg/ml) was evaluated against the isolated fungal strain by using agar well diffusion method. 100 µl of freshly prepared fungal culture (10^8 spores per ml) was spread on Potato dextrose agar medium plates. 5 mm wells were bored using a sterile borer and 20 µl of plant extracts was added in it. For control, DMSO and ethanol solvent were also put separately in the wells. Plates were incubated at 37ºC $\pm$ 2ºC for 24 - 48 hrs and zones of inhibition observed. (**CLSI 2004, 2010**). Also, as a comparative measure, two fungicides Manocozeb/ Matalxy and carbendazim (0.1 mg/ml) which were already in use at the agricultural fields were taken and the antibacterial sensitivity was measured as the zone of inhibition (in mm).

Study of Bacterial Blight of Pomegranate

Isolation and Identification of Bacteria

The fruit pericarp of the pomegranate plant showing typical symptoms of bacterial blight (yellow water soaked lesions at early stages and corky, dark oily spots at later stages of infection) was selected from the agricultural fields of Indapur, Pune, India. The lesions with some part of healthy portion on diseased fruits were cut into 2-3 mm pieces and surface sterilized with 70% alcohol for 2-3 min, washed three times with sterile distilled water and then macerated. These infected pieces of pomegranate fruit were placed in a sterile petri plate containing few drops of sterile distilled water in order to diffuse out the bacteria. A loopful of the suspension was taken and inoculated on Nutrient Agar (NA) and Yeast Dextrose Calcium Carbonate Agar media (YDCA) pertiplates and incubated for 72h at 30ºC. After the incubation period, observations were made for the development of well-separated typical, bright yellow, mucoid colonies (**Chowdappa *et al*., 2018**). The discrete colonies were sub cultured on nutrient agar slants for further studies and the slants were stored in the refrigerator at 5ºC which served as a stock culture for further studies (**Raghuwanshi *et al*., 2013**).
The causal pathogen of pomegranate blight was identified based on morphological and biochemical characters of the pathogen as per standard microbiological techniques. The morphological characteristics of the pathogen such as cell shape, cell measurement, pigmentation, gram reaction and spore forming characters were studied as per the standard procedures described by **Anon (1957), Salle (1961), Bradbury (1970) and Schaad (1992)**. The bacterial isolates were subjected to various biochemical tests such as citrate utilization, motility indole urease test, starch hydrolysis, gelatin liquefaction, catalase production, and hydrogen sulphide production by following the standard laboratory protocols for identification of plant pathogens as described by **Schaad and Stall in 1988** and **Schaad in 1992.**

Green synthesis of Nanoparticles and their characterization

Preparation of plant extracts: Medicinally important plant powder of *Azadirachta Indica* was purchased from Ayurved Rasashala centre in Pune. The extract was prepared by mixing 10g of the powder in 100 ml of distilled water, heated for 10 min after boiling started and the filtered using filter paper. The filtrate was used as the extract while the residue was discarded (**Shete *et al*., 2014**).
Synthesis of nanoparticles: For synthesis of silver, copper, cadmium, iron and zinc nanoparticles, 250ml of 1mM solutions of silver nitrate, copper nitrate/copper sulphate, cadmium chlorite, ferric nitrate and zinc acetate were prepared respectively in conical flasks and mixed with 5ml of the plant extracts, sonicated for 30min and incubated overnight. Color change was noted and optical measurements were carried out by using a UV-Visible spectrophotometer and scanning the spectra between 200-1100 nm at the resolution of 1nm (**Chandran *et al.*, 2006**). The combination of the plant extract and metal solution which yielded the best nanoparticles according to the UV spectra and also gave better antibacterial activity were characterized and stored for further experimentation.
Characterization of nanoparticles: The formation of nanoparticles was confirmed and their characterization carried out by performing UV Visible spectra and X-ray Diffraction (XRD) analysis techniques (**Mourdikoudis *et al*., 2018**).

Antibacterial activity

The solid nanoparticles obtained after centrifugation of the solution of nanoparticles were used to test its antibacterial potential against 24h old sub cultured cultures of *Xanthomonas axanopodis pv punicae* by method of well diffusion assay according to National Committee for Clinical Laboratory Standards (NCCLS). The culture was spread on nutrient agar plates with a sterile swab moistened with the bacterial suspension. Subsequently, wells of 8 mm diameter were punched into the agar medium and filled with 20µl, 50µl, 100µl of nanoparticle solutions (1mg/ml concentrated) in DMSO. Wells containing the same volume of DMSO (10%) served as negative controls while discs dipped in Bordeaux mixture (mixture of copper sulphate, $CuSO_4$ and slaked lime $Ca(OH)_2$) which is used as an antibacterial and fungicide by the farmers was used as a positive control (**Ranjard *et al.*, 2006; Somerville, 1986**). Bordeaux mixture spray is used as antimicrobial was also confirmed by the farmers of Indapur from where the infected fruits used in this study were collected. After incubation for 72h at 30ºC, the diameters of the zones of inhibition for different concentrations of nanoparticles were measured in mm and recorded (**Dahiya and Purkayastha, 2012**). Three replicates were carried out for each of the nanoparticles.

RESULTS

Study of Fungal Wilt of Pomegranate

Isolation and Identification of fungi

The fungi identified based on Internal transcribed spacer (ITS) was processed and the report was generated using NCBI Database. The confidence in identification was limited by both the availability and the extent of homology shown by the ~550 bp sequence of the samples with its closest neighbor in the database. Isolate was reported with the first five hits observed in the said database for phylogenetic analysis. Parameters used for Database Search were Standard Nucleotide BLAST, Data Set: both type and non-type strains, both environmental (uncultured) sequences and isolates: near-partial length sequences (>=700 bases): good quality sequences. The results showed highly similar sequences (megablast) (**Altschul *et al*., 1997**). Closest Neighbour (Accession #) E Value Similarity-Isolate was *Mariannaea elegans* var. elegans strain DUCC400 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence as ahown in Figure1.

Antifungal Activity

Psidium guajava, *Picrorhiza Kurroa and Vitex negundo* ethanolic extracts did not show any significant antifungal activity. However, *Piper betel* extract (0.5mg/ml) showed 20 mm zone of inhibition when dissolved in ethanol against *Mariannea elegans* (Fig 2). Fungicides Manocozeb/ Matalxy (1 mg/ml) and Carbendazim (1mg/ml) show zone of inhibition of 5mm respectively against *Mariannea elegans* (Fig 3). The differences in the inhibitory effect of the plant extract may be attributable to quantitative difference in the antifungal principles/ compounds made available when dissolved in varied solvents. It is presumed that if the antifungal compounds were found to be in higher degree so the extract of the above plant may be utilized as phytofungicide to control the pathogenic fungi on this economically important crop plant.
Figure 4 explains HPLC data with three prominent peaks. Isolation of bioactive compound: The ^{1}H NMR of Spectrum in Fig.5 predicted that the above observations for isolated compound. A peak at δ value doublet at 3.28 indicates the presence of $–OCH_2$ - group which correlates with observed up field shift of protons (-CH) of all benzene ring at δ 6.0 to 6.82 ppm. A multiplet at δ 5.10 ppm suggestive of olefin protons (=CH) whereas singlet at δ 1.4 ppm showed the presence of $–CH_3$ group. Thus the ^{1}H NMR spectrum predicted that the compound is having phenolic Eugenol with aliphatic side chain.

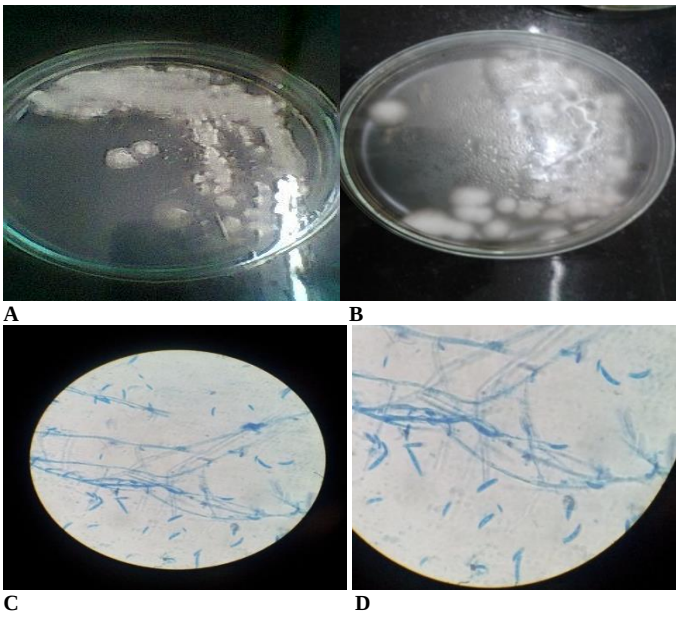


Figure 1 Isolation of fungi from stem on DCPA medium (a) , white coloured, and on Potato Dextrose Agar medium (b) appeared cottony white, its microscopic view (c) , close view of macroconidia (d). They are found hooked ta the apical region whereas basal region is blunt. Chlamydospores not seen.

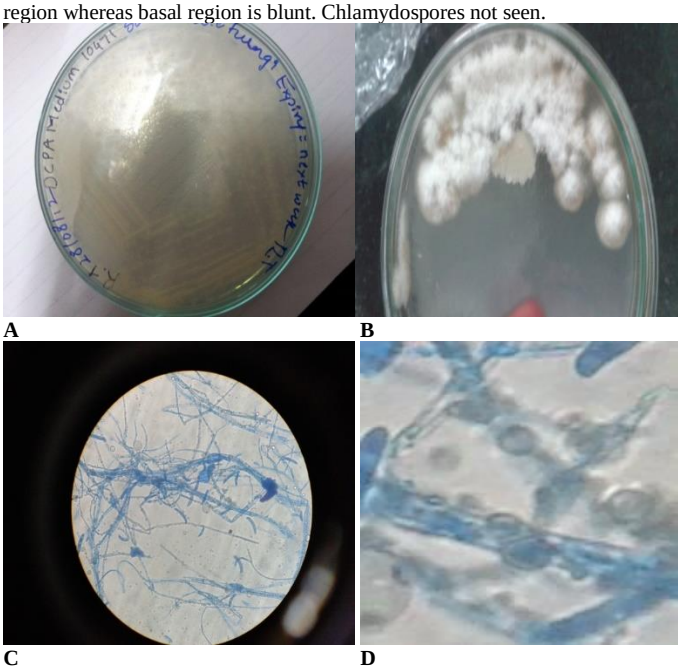


Figure 2 Isolation of fungi from root on DCPA medium (a) , white coloured , and on Potato Dextrose Agar medium (b) appeared cottony white, its microscopic view (c) , Chlamydospores seen in close view (d). They are found hooked ta the apical region whereas basal region is notched.

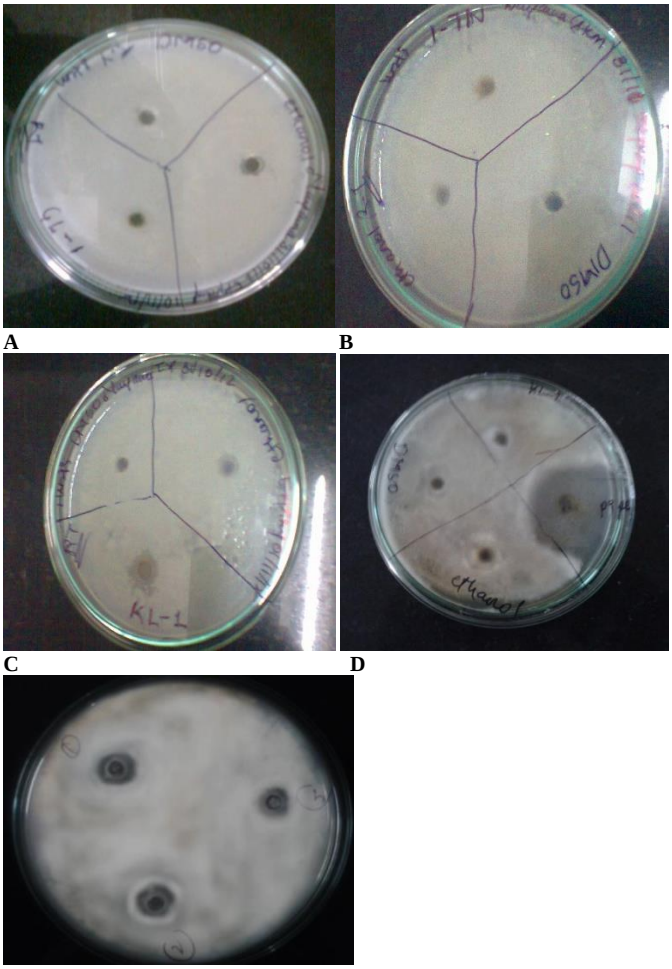
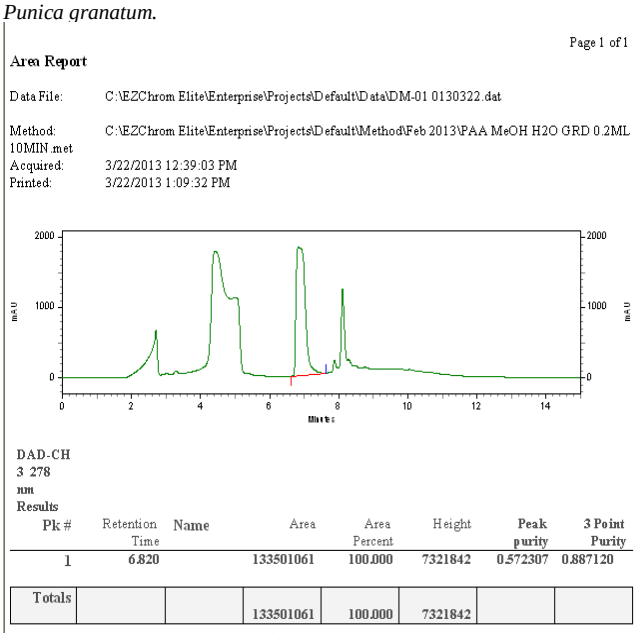


Figure 3 Isolate1 showed no zone of inhibition against ethanol extracts of Gvava leaves (a) and Nirgundi leaves (b), 5 mm zone for Kutki leaves (c) and 20 mm zone for piper betel leaves (d) Anti-fungal activity of 1. Manocozeb/ Matalxy (5 mm), 2. Carbendazim (5 mm) 3. Demildex (no zone) used in agricultural farms of *Punica granatum.*



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Area Report

Data File: C:\EZChrom Elite\Enterprise\Projects\Default\Data\DM-01 0130322.dat

Method: C:\EZChrom Elite\Enterprise\Projects\Default\Method\Feb 2013\PAA MeOH H2O GRD 0.2ML 10MIN.met

Acquired: 3/22/2013 12:39:03 PM

Printed: 3/22/2013 1:09:32 PM

DAD-CH 3 278 nm

Results

Pk #	Retention Time	Name	Area	Area Percent	Height	Peak purity	3 Point Purity
1	6.820		133501061	100.000	7321842	0.572307	0.887120
Totals			133501061	100.000	7321842		

Figure 4 HPLC of isolated phenolic derivative of cavicol/Eujenol/allylpyrocatechol from alcoholic extract of Piper betel.

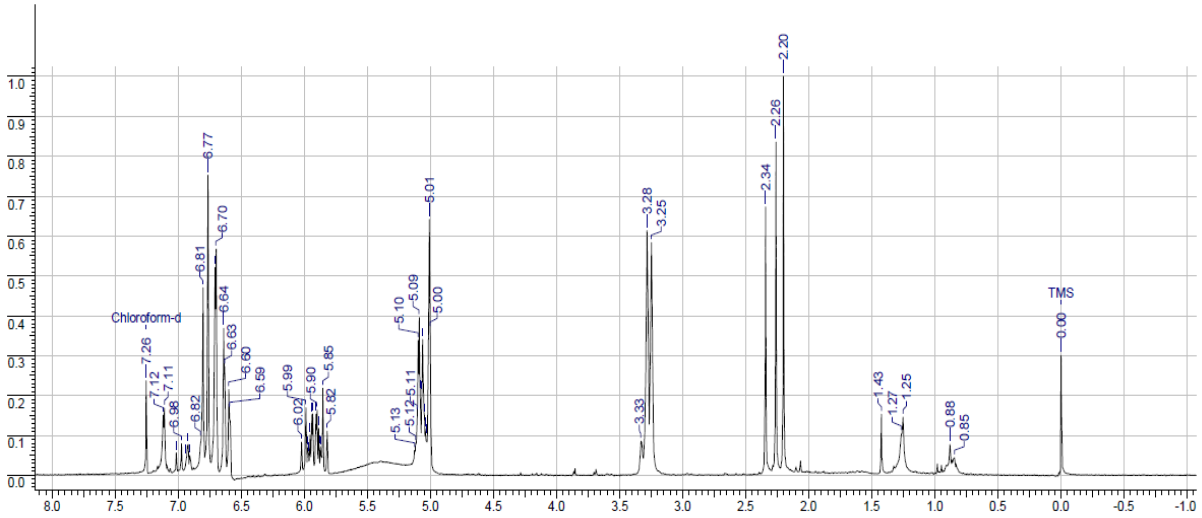


Figure 5 ^{1}H NMR spectrum of isolated compound from alcoholic extract of Piper betel , 1D ^{1}H-NMR spectrum of the active ingredient of Piper betle extract is suggestive of molecule from the phenyl propanoid- eugenol family molecule.

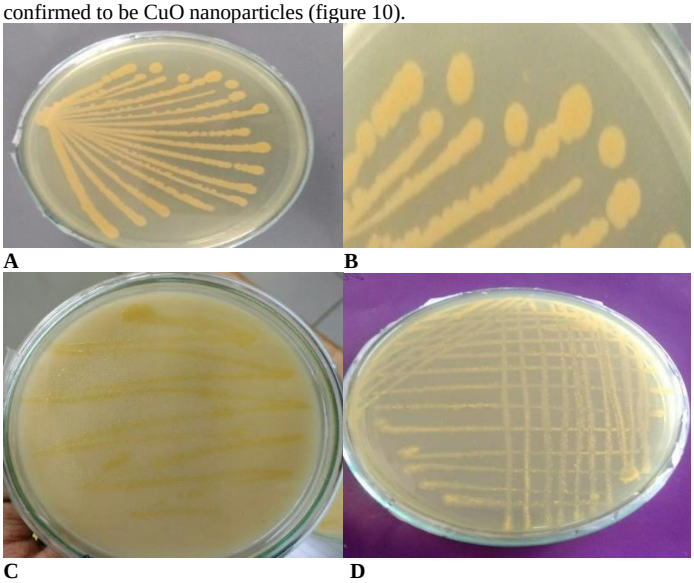
Study of Bacterial Blight of Pomegranate

Isolation and identification of the bacterium

The causal pathogen of the bacterial blight was isolated, identified and confirmed as *Xanthomonas axonopodis pv. Punicae* based on its morphological and biochemical characteristics (figure 6).

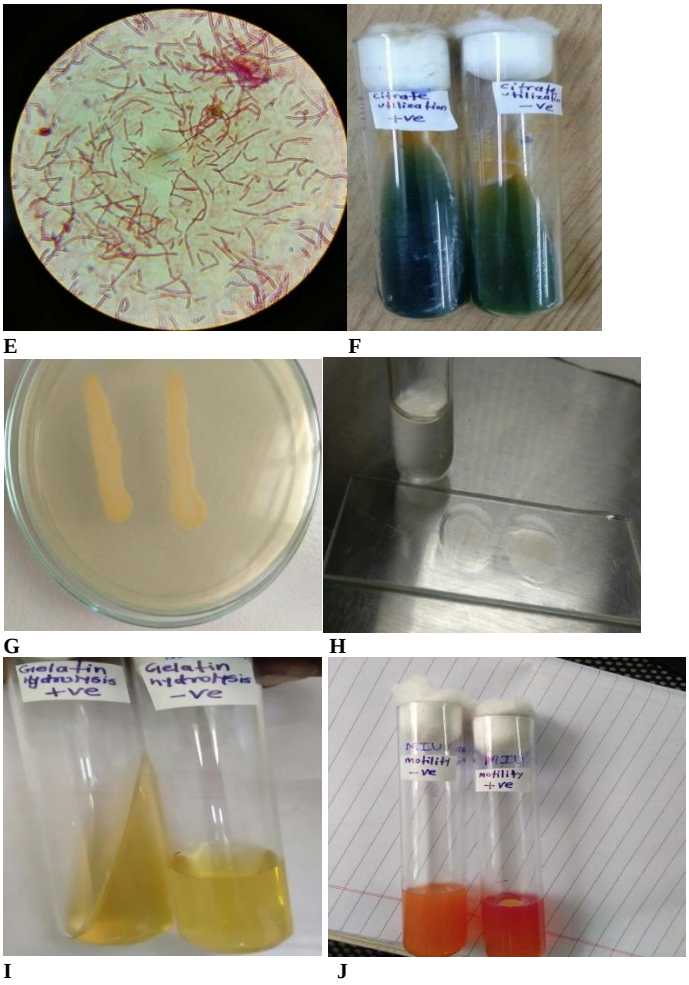
Antibacterial activity

The copper and copper oxide nanoparticles synthesized using Neem plant (*Azadirachta Indica*) showed the best antibacterial activity against *Xanthomonas axonopodis pv. Punicae* compared to the rest of the nanoparticles. The sizes of the zones of inhibition for different concentrations of all the nanoparticles are given table 1. The antibacterial assay plates showing zones of inhibition formed by CuO and CdO nanoparticles are shown in figure 7. The inhibition zones formed by the Bordeaux mixture used as the positive control are shown in the figure 8. One of the most interesting observations made were that although the Bordeaux mixture showed good inhibitory activity initially, the size of the growth clearance zones decreased with subsequent incubation. This could be as a result of the copper in the mixture getting completely utilized by the bacteria for their growth but this requires further detailed investigation. The UV spectra of the Fe, Ag, Cu, Zn nanoparticles are shown in figure 9. The synthesized Cu oxide nanoparticles when subjected to characterization using UV and XRD were confirmed to be CuO nanoparticles (figure 10).



A **B**

C **D**



E **F**

G **H**

I **J**

Figure 6 Isolated pure culture of *Xanthomonas axanopodis pv punicae* from infected pomegranate fruit (a) pure culture of *Xanthomonas* on Nutrient Agar medium plate (b) Close view of *Xanthomonas* colony isolated, shining yellowish colored (c) Pure culture of *Xanthomonas* on YDC medium plate (d) Streak plate of *Xanthomonas* isolate on NA plate. Morphological and biochemical tests (e) Gram staining of isolate observed under 100X. Isolate tested positive for citrate utilization (f), negative for starch

hydrolysis and catalase test (g and h), positive for gelatin hydrolysis (i) and was motile as checked by the motility indole urease test (j).

Table 1 The results of antibacterial assay against *Xanthomonas axanopodis pv punicae* using well diffusion technique, sizes of zones of inhibition for different concentrations of all the nanoparticles

Nanoparticles of	Antimicrobial activity against *Xanthomonas*	Volume of nanoparticles with DMSO (in µL) Concentration of nanoparticles 1mg/ml	Diameter of zone of inhibition (in cm)
Silver (Ag)	+	20	0.5
		50	0.7
		100	1
Copper (Cu)	+	20	1.3
		50	1.4
		100	1.5
Iron (Fe)	+	20	0.4
		50	0.4
		100	0.4
Copper Oxide (CuO)	+	20	1
		50	1.2
		100	1.5
Cadmium Oxide (CdO)	+	20	0.9
		50	1
		100	1.1
Zinc (ZnO)	+	20	0.3
		50	0.5
		100	0.8
Negative Control (DMSO)	-		
Positive Control (Bordeaux mixture)	+	20	0.7
		50	0.85
		100	1

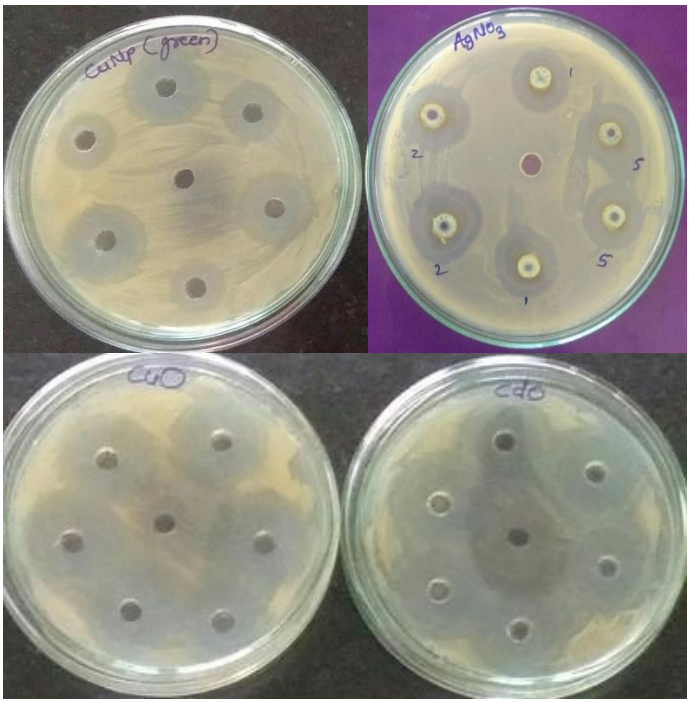


Figure 7 Antibacterial assay plates, (a) Cu nanoparticles (0.1mg/ml) forms zones of inhibition against the test organism (b) Ag nanoparticles (1mg/ml) showing zones of inhibition against test organism (c) Zones of inhibition caused by CuO and CdO nanoparticles (1mg/ml).

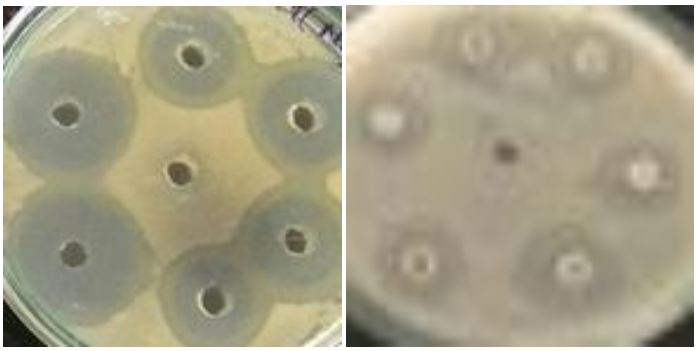


Figure 8 (a) The zones of inhibition formed by Bordeaux mixture (positive control) after 72h of incubation, (b) the decrease in the size of zone of inhibition after one more day of incubation.

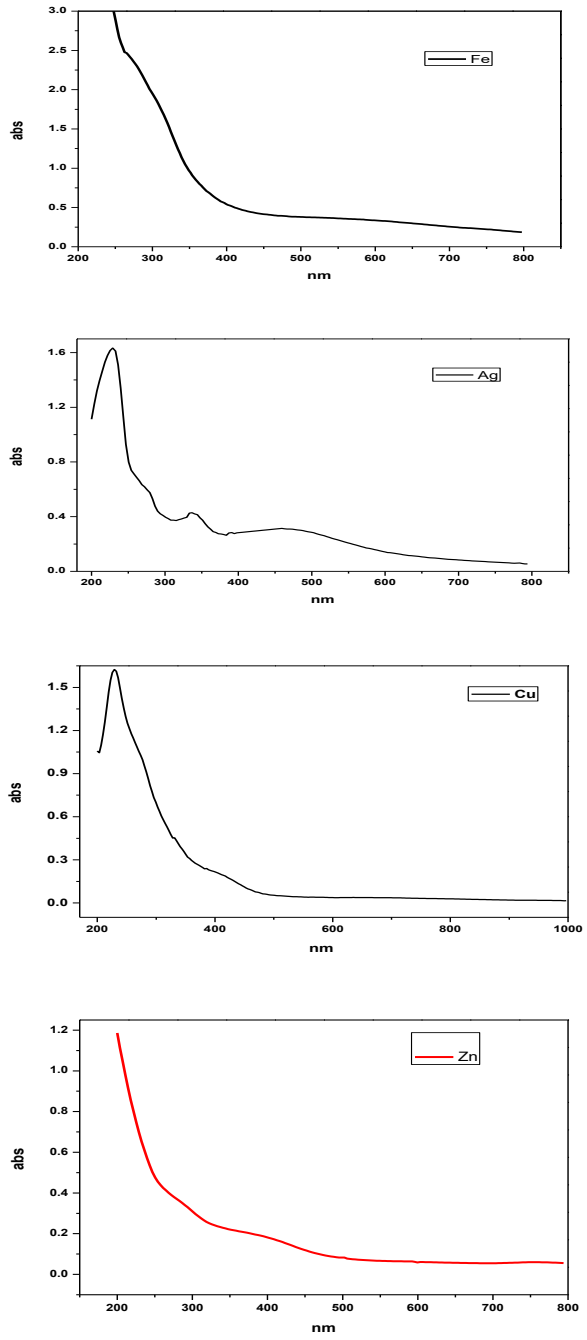


Figure 9 UV spectra of (a) Fe Nanoparticles (characteristic UV peak at ~550nm), (b) Ag Nanoparticles (characteristic UV peak at around 478nm), (c) Cu Nanoparticles (characteristic UV peak at around 400nm) and (d) Zn Nanoparticles (characteristic UV peak at around 350nm)

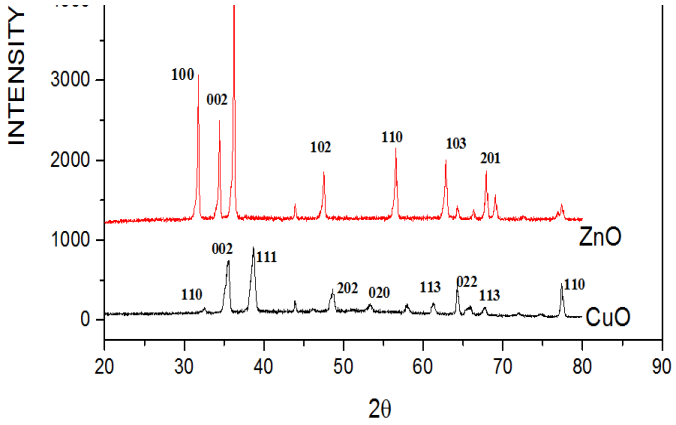


Figure 10 X-ray diffraction spectra of CuO and ZnO

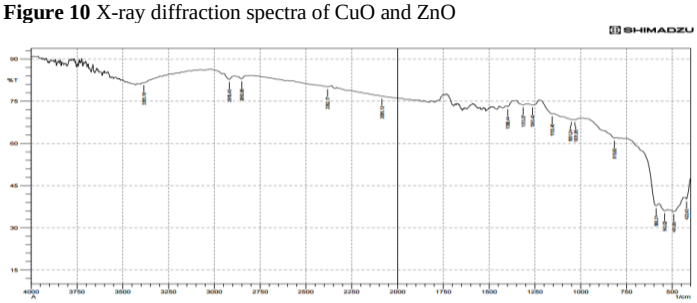


Figure 10 IR spectra of CuO nanoparticles.

DISCUSSION

The antioxidant and antimicrobial potential of *Piper betle* leaves has been evaluated by **Sarma *et al.* in 2018** with its antibacterial activity attributed to the presence of phytosterols in the leaves. Traditionally betel leaf has not only been known to harbour antimicrobial potential but also anticariogenic and anti-inflammatory activity, anti-protozoal, anti-larval, anti-allergic and antiulcer effects, antidiabetic, hepatoprotective and cardio-protective properties (**Fazal *el al.*, 2014**). The immunogenic activity of the leaves is majorly attributed to the presence of active ingredient Eugenol. *P. betle* dried leaf extract and its oil has also shown antitoxin potential against *Aspergillus flavus* without affecting fungus mycelia growth (**Yazdani *et al.*, 2013**) and fungicidal activity against human pathogens *Candida albicans*, *Candida tropicalis*, *Candida glabrata*, and *Candida parapsilosis* respectively (**Rath and Mohapatra, 2015**). Our study also reports the antifungal activity of betel leaf extract against the pomegranate wilt pathogen *Mariannea elegans.* The research further demonstrated the interesting insights that commonly used fungicides viz., Manocozeb/ Matalxy and Carbendazim showed very little inhibition as compared to the *Piper betel* ethanolic extracts. The purification and characterization of the bioactive molecules using Chromatographic techniques illustrates the presence of compounds viz. derivatives of Eujenol. These compounds can be further purified, identified, formulated and thus harnessed as control agents as they exhibit a wide range of medicinal properties. Hence, pomegranates fungal wilt being an emerging disease yet less studied can be put in check with the help of the natural extracts of *P. Betel*. Cavicol, eugenol and allylpyrocatechol are three main ingredients as shown in HPLC plot in Figure 4 from three prominent peaks of HPLC analysis. Figure 5 explains 1D , ^{1}H NMR spectrum of isolated compound from alcoholic extract of Piper betel and shows the active ingredients of Piper betel extract is suggestive of molecule from the phenyl propanoid- eugenol family molecule.

The bacterial blight on the other hand is a much widely studied pomegranate disease and hence many control measures are already explored and also followed. Integrated disease management schedule has been designed for easy implementation which includes practices like orchard sanitation and prudent sprays of antibiotic Streptocycline in combination with fungicides like carbendazim/mancozeb/copper oxychloride or antibiotic Bactronol (2-Bromo, 2-Nitropropane, 1-3 diol) that can achieve up to 80% of disease control and also increase crop yield (**Sharma *et al.*, 2009**). The in-vitro inhibitory effect of methanol extracts of medicinal plants *Acalypha indica*, *Aerva lanata* and *Phyllanthus amarus* has been evaluated against other strain of Xanthomonas, *Xanthomonas campestris* (**Britto *et al.*, 2011**) while in another study the

effectivity of Streptomycin sulphate against *Xanthomonas axonopodis* pv. *Malvacearum* has been checked (**Sarker *et al.*, 2017**). Chemical treatment regimens of copper oxychloride and copper hydroxide in combination with a few known antimicrobials have shown to completely control the *X. axonopodis* pv. *punicae* blight in vitro (**Raghuwanshi *et al.*, 2013**). However, most chemicals used as pesticides have been reported to often cause biomagnifications in the ecosystem and hence nanoparticles are being used as the newer, safer and cheaper tools of bio-control in modern agriculture (**Baker *et al.*, 2017; Mukhopadhyay, 2014**). Our study therefore tests the antibacterial activity of various nanoparticles synthesized using entirely green approaches, thus making them eco-friendly options for control of bacterial blight caused by *Xanthomonas axonopodis pv. Punicae*. The study shows that copper oxide nanoparticles synthesized using *Azadirachta indica* leaf extract effectively inhibit the causal pathogen of pomegranate blight in vitro even at concentrations as low as 0.1mg/ml. Further, our study shows that Bordeaux mixture which is sprayed as an antimicrobial on pomegranate crops to protect them from blight is only effective in control in earlier phase of infection while it promotes growth of the pathogen in the later phase of infection. Apart from the one found in our study, there are other disadvantages of using Bordeaux mixture as well, such as its wash-off leads to losses during rainfall season, its application results in increase in total copper content of the crop and also sometimes affects soil biology depending on the soil type as studied by **Rodriguez *et al.* (2013, 2016), Wang *et al.* (2016) and Mathew *et al.* (2015)**. Copper nanoparticles hence can prove to be a good alternative as they show better inhibition of the pathogen than Bordeaux mixture (as seen in table 1 and figures 7, 8) and being nano-sized also can solve the problem of copper accumulation in crops.

The Powder XRD explains the pattern of different planes in XRD spectra given in Figure 10 is well matching with JCPDS file No 80-0076 for CuO and JCPDS file No 80-0074 for ZnO. IR spectra explains only –OH stretching around 3000cm^{-1} with M-O stretch in Figure 11 which is around 600cm^{-1}, which confirms formation of pure CuO nanoparticles.The particle size of CuO nanoparticles is around 100nm scale from XRD calculation. UV spectra of Fe Nanoparticles (characteristic UV peak at ~550nm), (b) Ag Nanoparticles (characteristic UV peak at around 478nm), (c) Cu Nanoparticles (characteristic UV peak at around 400nm) and (d) Zn Nanoparticles (characteristic UV peak at around 350nm) which is shown in Figure 9.

The control methods elaborated in this study can be implemented in vivo conditions (actual pomegranate fields that are affected by wilt/blight) in the near future with a further large scale evaluation as they are not only environment friendly but also highly effective.

CONCLUSION

Green methods of control for pomegranate fungal wilt and bacterial blight were explored and elaborated in this study. Use of Copper oxide nanoparticles in place of Bordeaux mixture which includes both $CuSO_4$ and $Ca(OH)_2$ is a cost-effective and also a beneficial alternative owing to the latter's disadvantages. Use of Betel leaf extract to control fungal plant diseases opens new gates of research in the field.

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