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Original Research Article

A Study of a Unique "Herbal Elixir" as a Potential Green Pesticide for Integrated Pest Management of Pomegranate

Vaidehi Dande*, Anupama Sonawane, Shruti Goradiya, Ashwini Ghule, Manali Mahajan and Sonali Renake

Dept. of Microbiology, P. E. Society's Modern College of Arts, Science & Commerce, Ganeshkhind, Pune- 16, India

**Corresponding author*

A B S T R A C T

Traditional antimicrobials having an immense potential can be exploited to develop alternative methods that are economical and suited for adoption even by the small-scale pomegranate cultivators. In this study, cow urine was mixed with different botanicals such as neem leaves, neem seed powder, pomegranate rind and garlic bulb. The mixture was fermented in an earthen pot which was buried in soil for 21 days. The fermented broth was tested against the wilt isolate Fusarium and blight isolate *Xanthomonas* by well diffusion assay. *Fusarium* and *Xanthomonas* were isolated from infected pomegranate plants from an orchard located in the village Natepute, Dist. Solapur. Fermented cow urine and fermented cow urine- with botanicals, exhibited the potential to inhibit the fungal as well as the bacterial isolate.

Keywords

Pomegranate, Fungal wilt, Bacterial blight, Cow urine, Botanicals

Introduction

Pomegranate (*Punica granatum* L.) is one of the delicious fruits consumed worldwide. Being the most adaptable subtropical fruit crop, its cultivation has increased rapidly creating its image as an important cash crop in global market. It is also ruling Indian agro economy in a short span of time by exporting the fresh fruits to Middle Eastern and European countries (Report of GOI-UNCTAD DFID project 2007). According to National Horticulture Board of India, India is the largest pomegranate producer (7.43 lakh tones in 2010-2011) in the world sharing about 36 per cent of the world's production and about 30 per cent of the international pomegranate trade by exporting 30,158 tonnes of this fruit in 2011- 12 (Raghuwanshi et al 2013). Maharashtra State (area 82.0 thousand ha) is the largest producer of pomegranate in India contributing about 66.2 per cent (4.92 lakh tonnes) of pomegranate production followed by Karnataka, Andhra Pradesh, Gujarat, Rajastan and Tamilnadu.

However, in recent years the crop yield is greatly affected by many insect and microbial pests. The most prevalent and

dreadful ones observed during the surveys are bacterial blight by *Xanthomonas axonopodis pv punicae* (up to 100.0% severity in some orchards) and pomegranate wilt complex by *Fusarium spp* (70.0% severity). Bacterial blight caused by *X. axonopodis pv. punicae*, first reported by Hingorani and Mehta in 1952, was of minor importance until 1991, when it appeared in epidemic proportion at IIHR Experimental plot in Bangalore, resulting in 60-80 per cent yield losses (Chand and Kishun, 1991). The disease was observed throughout the year on pomegranate trees in Western Maharashtra particularly from Solapur district (Dhandar *et al.*, 2004). All the commercial grown cultivars are susceptible to this disease. The disease affects all the above ground plant parts but it is more destructive when fruits are infected. The symptoms on fruits are seen when immature, which turn to deep brown or black spots which coalesce together and form irregular lesions, as a result of which, skin of the fruit become rough which split opens with 'L'/'Y' shaped cracks at final stages.

Pomegranate wilt is a destructive disease caused by the soil – borne fungi *Fusarium oxysporum* or *Ceratocystis fimbriata*. Affected plants show yellowing of leaves in some branches, followed by drooping and drying of leaves leading to sudden wilting of the plant. There is formation of nodules on roots and brown discoloration in the stem. The entire tree dies in few months or a year (Gaigole *et al.*, 2011).

The current pest management of blight includes the use of antibiotic Streptocycline (100ppm) and of wilt disease with copper oxychloride (0.3%) which alone have proved to be ineffective in field. Hence multiple applications of chemicals like Bordeaux mixture (1%), captan (0.25%), Copper oxychloride (0.3%), Copper hydroxide (0.3%), bromopol (500ppm) and antibiotic Streptocycline (250ppm) alone or in combinations have been suggested (Raghuwanshi *et al.*, 2013).

But multiple applications and inappropriate doses of these pesticides would be responsible for the development of resistance among all the cultivars being used. Being toxic, chemical pesticides also pose a threat to the environment. Also because of high Minimum Residual Levels (MRLs) the fruits are graded as low quality by regulatory authorities and farmers suffer heavy losses (Report of GOI-UNCTAD DFID project, 2007). Hence Indian Agriculture Research Institute has recommended Integrated Pest Management (IPM) which emphasizes the growth of a healthy crop with the least possible disruption to agro-ecosystems and encourages natural pest control mechanisms, which also ensures low MRLS. This has led to the emergence of botanicals and bio-pesticides as viable alternatives endowed with potential bactericidal, fungicidal and viridical properties. Similarly, traditional antimicrobials like dung and urine of cattle restrict microbial pests and insects. Cow urine distillates of botanicals were found more inhibitory to pathogens than cow urine distillate alone. In rural areas the small scale farmers soak bitter tasted and obnoxiously odoured neem leaves in cow urine in an earthen pot and spray to control plant pathogen, pests and insects (Murugan *et al.*, 2012).

Pomegranate rind is known to have antimicrobial potential. The present study was carried out to scientifically validate the use of fermented cow urine with various botanicals including pomegranate rind for the management of pomegranate microbial pests.

Materials and Methods

Collection of samples

Infected samples of pomegranate plants i.e. fruits with small, oily, black spots, roots with infective nodules and brown discoloured, infected stems were collected from the orchard in village Natepute, Dist. Solapur of Maharashtra, India. All the samples were transported to laboratory within 18h.

Isolation and identification of oily spot disease causing bacterial pathogen

Younger portions of spots were cut using sterile razor blade and were surface sterilized by dipping in 0.1% mercuric chloride. The cut portions were immediately rinsed in sterile distilled water repeatedly and minced finely in sterile water in a petridish. This suspension was streaked on sterile malt extract glucose yeast extract peptone (MGYP) medium and incubated for 48 h at 30˚C.Characteristic yellow, mucoid colonies were further streaked on selective SX agar medium. Purple centered colonies surrounded by clear zone of starch hydrolysis on SX agar were selected. After studying Gram character and motility, the isolates were subjected to Xanthomonadin extraction. (The Laboratory Guide for the Identification of Phytopathogenic Bacteria, 3rd edition.) The extracted residue was subjected to TLC and spectroscopic analysis by the method of Starr and Stephens (1964). The isolated bacterial pathogen was identified as *Xanthomonas* spp.

Isolation and identification of pomegranate wilt pathogen

Infected root and stem samples were cut into small pieces and inoculated in sterile potato dextrose agar medium (PDA, Himedia). All the plates were incubated at 30°C and observed everyday for fungal growth. Inoculated brown discoloured bits from infected stem showed white, fluffy, cottony growth which was purified and identified by using slide culture technique. The isolated fungal pathogen was identified as *Fusarium* spp.

Selection of botanicals

Allium sativum (Garlic) bulbs, *Punica granatum* (pomegranate) fruits, *Azadirachta indica* (Neem) dried seeds were obtained from local market. *Azadirachta indica* (Neem) leaves were collected from Pashan in Pune.

Dried *A. indica* seeds, fresh *A. indica* leaves and peeled, *A. sativum* bulbs were crushed using surface sterilized mortar and pastel. Rind of fresh *P. granatum* (pomegranate) fruits was minced finely using sharp razor blade.

Fermentation of botanicals in Cow urine

Different botanicals i.e. crushed fresh neem leaves 100g, crushed garlic cloves 200g, minced pomegranate rind 200g, and neem seed powder 146g were separately mixed with 600 ml Cow urine collected from a single cow and was mixed in earthen pots. Only cow urine 600ml was also fermented in a separate earthen pot. All the earthen pots were buried in soil for 21 days. After every 7 days intervals, 15 ml broth was collected for 21 days for phytochemical analysis. After 21 days, fermented broths were filtered through sterile muslin cloth and were tested for their antimicrobial activity by well diffusion method.

Condensation of fermented Cow urine

After 21 days, all the fermented broths of cow urine with different botanicals and only fermented cow urine were condensed at

40ºC in hot air oven till the one tenth volume was obtained. All the condensed fermented broths were tested for their antimicrobial activity by well diffusion method.

Antimicrobial assay

Antimicrobial activity of fermented broths of cow urine with different botanicals, and condensed cow urine extracts was assayed using the well diffusion method. 0.1 ml of 18 to 24 h old culture of *Xanthomonas* having O.D. of 0.15 at 600nm was spread on sterile nutrient agar medium with sterile swab. 0.1 ml *Fusarium* spore suspension prepared in saline with tween 80 was spread on sterile potato dextrose agar by using sterile swab. 30 µl of fermented broths were added in the well and incubated at 4°C for 15 min and then at 30ºC for 48 h. Streptocycline (100ppm) and Tin 20(100ppm) were used as positive controls for *Xanthomonas* and *Fusarium* respectively. Antimicrobial activity was assayed by measuring the diameter of the inhibition zone formed around the well using Hi-media standard scale.

Phytochemical analysis

Preliminary phytochemical analysis of all the fermented broths was performed as per the standard method (Harborne 1984).

Separation of active compounds

Fermented broth of cow urine with pomegranate rind which demonstrated maximum antimicrobial activity was subjected to standard separation procedure using silica gel column. 0.5g of dry crude extract was packed in the column made up of silica gel (Silica Gel 60-120 Mesh, Lobachemie). Hexane, chloroform, ethyl acetate, methanol, ethanol and water were used in different proportion in the order of increasing polarity. All solvents were evaporated in water bath; residue was dissolved in 1ml dimethyl sulfoxide and the antimicrobial activity of the fraction was checked by the standard method. The methanol fraction exhibited maximum antimicrobial activity. Hence this fraction was subjected to FT-IR spectral analysis.

Results and Discussion

The bacterial pathogen isolated from infected pomegranate fruits was identified as *Xanthomonas* based on standard morphological, biochemical screening and by characterization of unique xanthomonadin pigment. The fungal pathogen isolated from the discolored brown stem of pomegranate was identified as *Fusarium* on the basis of morphological features observed in slide culture and characteristic pink pigment production on potato dextrose agar.

All the fermented broths exhibited antimicrobial potential on 15th and 21st day. On 15th day of fermentation, fermented cow urine with neem leaves (FCU+NL), fermented cow urine with neem seeds (FCU+NS), fermented cow urine with pomegranate rind (FCU+PR) and streptocycline inhibited *Xanthomonas* equally (13 mm) followed by fermented cow urine with garlic cloves (FCU+GC) (12mm) and fermented cow urine alone (FCU)(11mm). On 21st day there was slight reduction in the inhibition potential of FCU+PR, FCU+GC and FCU (10mm, 10mm and 9mm respectively) whereas FCU+NL and FCU+NS proved to be more effective (17mm and 14mm). Upon condensation the anti *Xanthomonas* potential was enhanced in FCU+PR and FCU+NS (14mm and 15mm) but retained as that of 15th day by FCU and reduced substantially

in FCU+NL and FCU+GC (7mm and 10mm). (Figure 1)

FCU+PR had the highest anti *Fusarium* potential (30mm) on 15th day as well as after condensation amongst all other fermented broths and Tin 20. The lowest anti *Fusarium* activity was demonstrated by FCU+GC (10mm) which diminished upon condensation. FCU alone had consistent activity throughout the process and after condensation (15mm) (Figure2).

Preliminary phytochemical analysis was performed for FCU+NL, FCU+NS, FCU+GC and FCU+PR on 0, 7th, 14th & 21st day. Secondary metabolites like flavanoids and proteins were present in all the broths throughout the fermentation process. FCU+PR showed presence of phenols and tannins, FCU+NL, FCU+NS, FCU+GC contained alkaloids and glycosides (Table 1).

All the solvent fractions of FCU+PR and FCU i.e. hexane, chloroform, ethyl acetate, methanol, ethanol and water collected by silica gel separation were subjected to check their antimicrobial potential. Methanol extract of FCU+PR exhibited the highest anti *Xanthomonas* as well as anti *Fusarium* activity with zones of inhibition of 10mm & 30mm respectively. Hexane and water extract could inhibit *Fusarium* equivalent to methanol extract whereas no activity was found against *Xanthomonas*. Chloroform, ethyl acetate and methanol fractions of only FCU could inhibit *Xanthomonas* alone. (Figure 3)

In the FTIR spectrum of methanol fraction of FCU+PR, a broad absorption at 3338 cm -1 is due to N-H and O-H stretching frequency. At 2943 and 2832 cm^{-1} the stretching vibrations were due to methoxy group. The frequencies at 1721 cm^{-1} can be assigned to C=O vibrational stretching indicating aliphatic acids, while at 1597 cm^{-1}, strong stretching vibrations were observed for the C=C and N-H group indicating presence of aromatic compounds and primary amines. The presence of a small stretching vibration at 1452 cm^{-1} may be due to C=C phenyl groups and at 1257 cm^{-1} maybe due to C-N symmetric and C-O-C asymmetric stretching and O-H out of plane bending. At 1118 cm^{-1} strong streching of C-N is observed followed by a very sharp peak at 1020 cm^{-1} indicating stretching of carbonyl group. Out of plane bending of N-H was observed at 849 cm^{-1} indicating primary amine whereas broad stretch of acetylenes and esters are observed at 633 and 608 cm^{-1} (Figure 4).

In the FTIR spectrum of methanol fraction of FCU, the stretching vibration at 3420cm^{-1} can be assigned to N-H vibration and intra-molecularly H-bonded O- H group. The small peak at 2999 cm^{-1} is due to stretching of C=O of acids, 2916 cm^{-1} indicates the asymmetrical stretching in methyl group, at 1659 cm^{-1} stretching is due to presence of C=N group C=C group. Secondary amines are indicated at 1572 cm^{-1} due to N-H starching. A sharp and intense band at 1430 and 1408 cm^{-1} is due to N-H stretching indicating ammonium salts. At 1313 cm^{-1} streching in C-N corresponds to primary amine. At 1019 cm^{-1} a sharp, intense peak due to stretching of carbonyl group indicating aromatic benzene with a shoulder peak of aromatic methane at 950 cm^{-1}. A sharp peak at 699 cm^{-1} is due to C-H out of plane bending of aromatic methane. The small peak at 664 cm^{-1} is due to bending in O-N=O of nitrite ester. A broad peak at 561 cm^{-1} is due to polychloro compounds. (Figure 5) The FCU+PR broth is a concentrated mixture of nondegraded products from cow urine and pomegranate rind i.e. urea, creatinine, uric acid and

flavonoids, tannins, phenols respectively and the degraded products like alcohols, benzoic acids, phenols, aliphatic acids and esters formed due to fermentation. The highest anti *Xanthomonas* and anti *Fusarium* activity of FCU+PR hence can be attributed to these fermented products.

Bacterial blight and wilt of pomegranate are the dreadful diseases which need the serious attention so as to reduce the exorbitant losses suffered by farmers. The IPM has recommended use of sustainable and environment friendly agronomic practices like use of green pesticides and scientific traditional methods of farming. One of these traditional practices is use of cow products like buttermilk, dung and urine as insect repellents and antifungals. The scientific validation of these methods has been reported in recent studies. Kumar *et al.* (2013) found all the animal products to be effective for management of fungal blight of urdbean through botanicals and animal products, with highest reduction in disease severity by cow urine. The Pharmaceutical composition of cow urine states antimicrobial principles in cow urine are urea, uric acid and creatinine. Another traditional practice in rural areas to control plant pathogens is spraying broth of botanicals in soaked in cow urine. Harender and Sharma (2013) have reported the bio efficacy of cow urine based bio formulations to control fungal pathogens. In this traditional method, the incubation of botanicals with cow urine is carried out by keeping the earthen pot in the pit dug in soil which provides the ambient temperature for the fermentation enhancing the extraction of the bioactive principles (Murugan *et al.*, 2012). In the present investigation, the fermentation of cow urine with neem leaves, neem seeds garlic cloves and pomegranate rind was carried out by the said traditional method. All the fermented broths, i.e. FCU+NL, FCU+NS, FCU+GC, FCU+PR and only FCU exhibited antimicrobial potential against bacterial blight pathogen, *Xanthomonas* as well as wilt causing fungus *Fusarium* isolated from the infected plant samples obtained from the same orchard in Dist. Solapur.

Polyphenols, flavonoids, condensed and hydrolysable tannins extracted from fruits, vegetables, herbs and spices have been explored as potential agents for treating or preventing a wide range of microbial infections. *Azadirachta indica* having active principles like Azadirachtin, nimbin, nimbinin, nimbidin is proven traditional botanical used in rural India to manage plant pathogens. The results in this study are parallel confirming neem as the most common antimicrobial botanical since FCU+NL and FCU+NS demonstrated the moderate antimicrobial potential. Garlic (*Allium sativum*) is one of the oldest vegetables used as a medicine having allicin as its antibacterial principle which has also inhibited the growth of *Xanthomonas* and *Fusarium* upon condensation,

FCU+GC activity had reduced which may be due to unstable nature of allicin and other sulfur compounds formed in fermentation.

Pomegranate (*Punica granatum*) is an ancient fruit known for its several medicinal properties and considered as ‘healing food’ in various countries. The pomegranate peels are characterized by substantial amounts of phenolic compounds, including flavonoids (anthocyanins, catechins, quercetin, kaempferol and other complex flavonoids) and hydrolyzable tannins (punicalin, pedunculagin, punicalagin, gallic and ellagic acid).

Figure.1 Antimicrobial activity of fermented cow urine broths on 15th, 21st day and after condensation on *Xanthomonas*

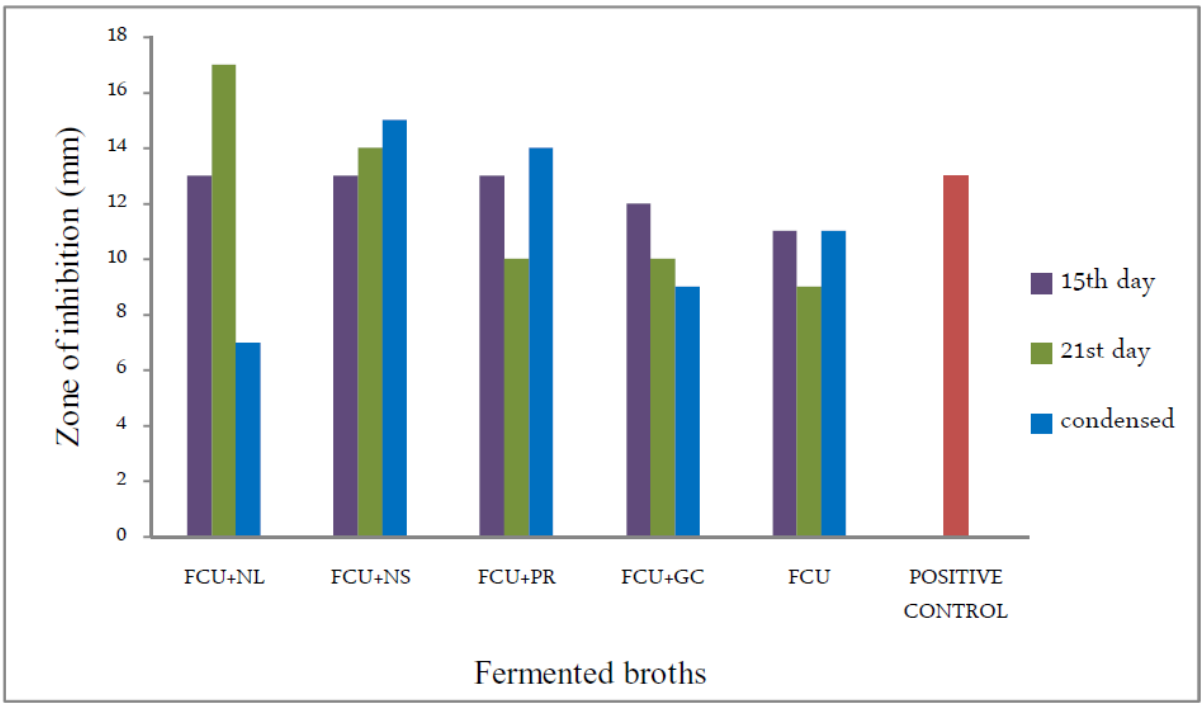


Figure.2 Antimicrobial activity of fermented cow urine broths on 15th, 21st day and after condensation on *Fusarium*

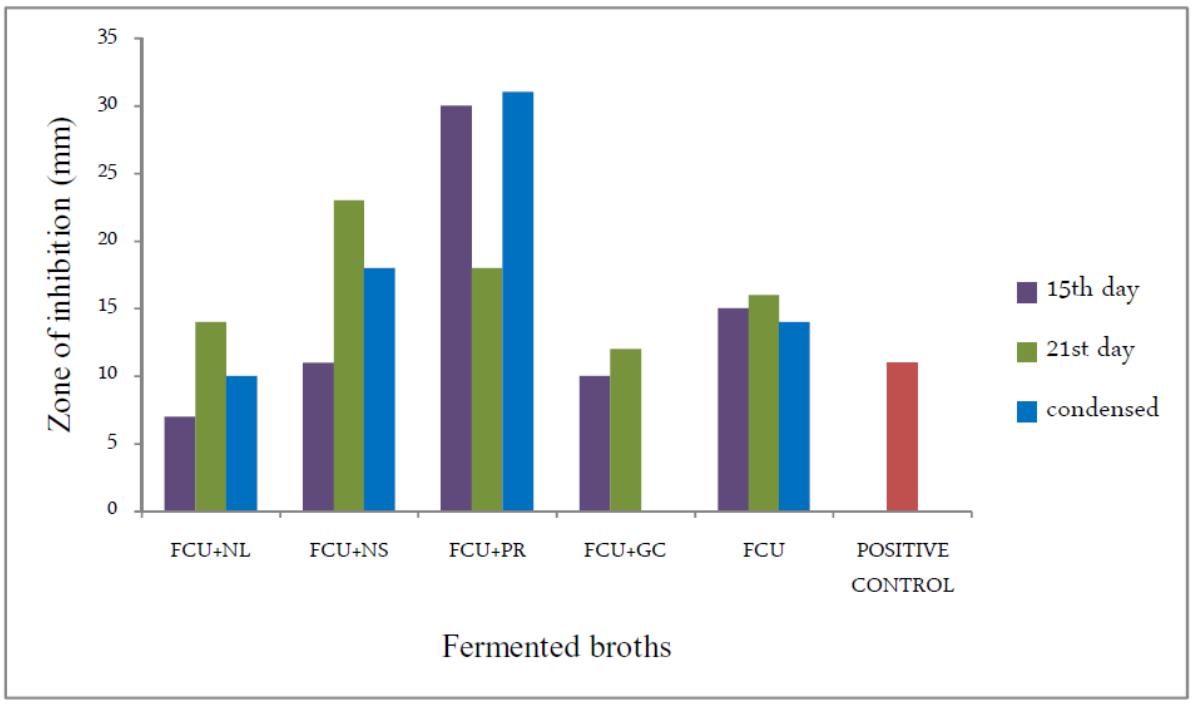
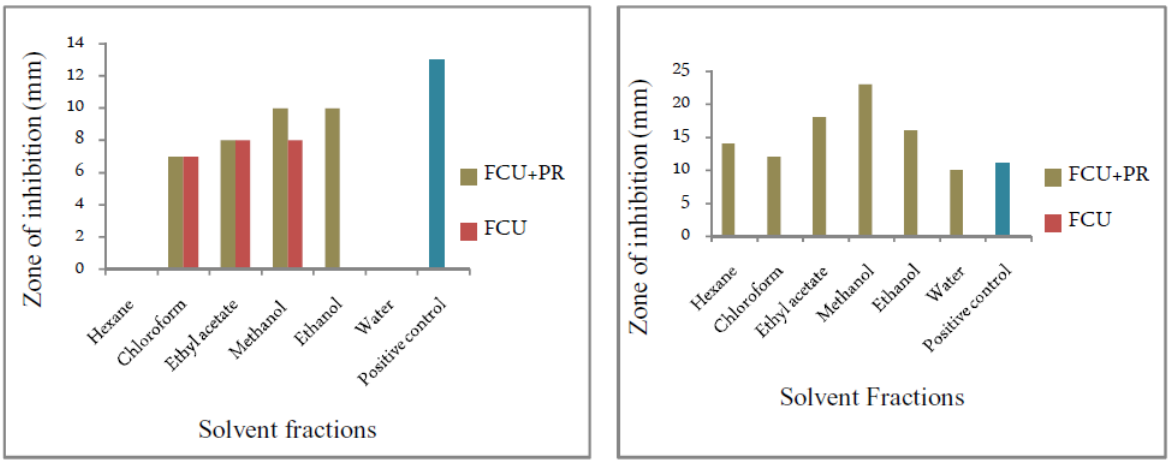
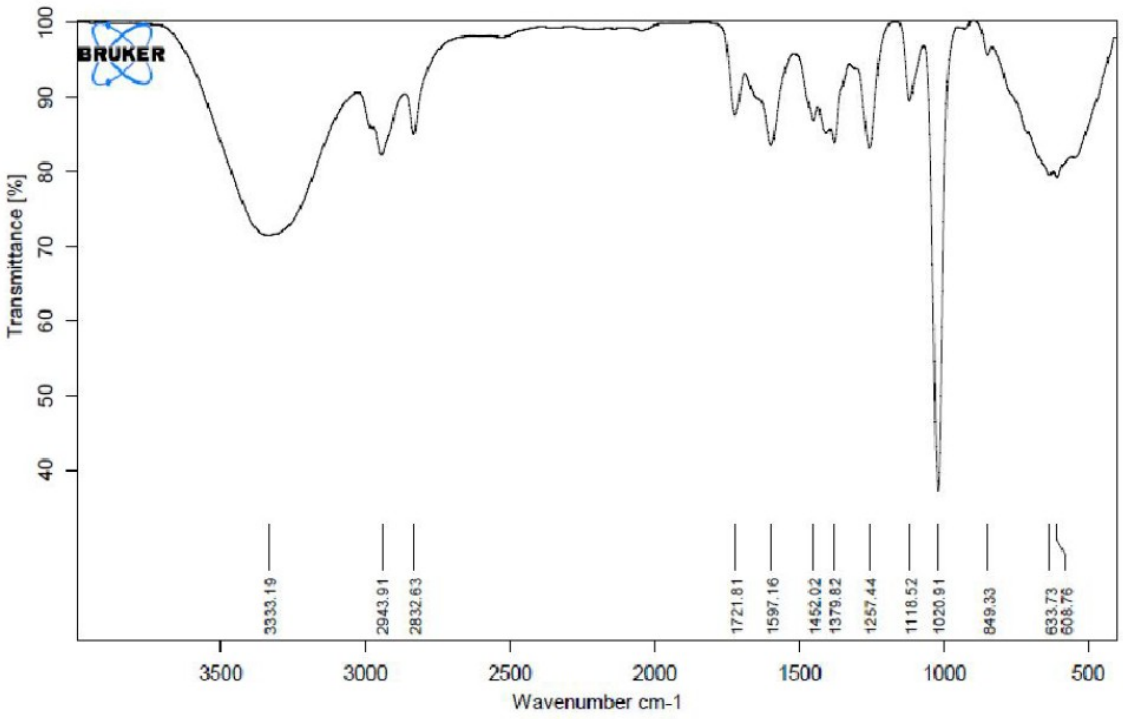


Figure.3 Antimicrobial activity of different solvent fractions of FCU+PR and FCU



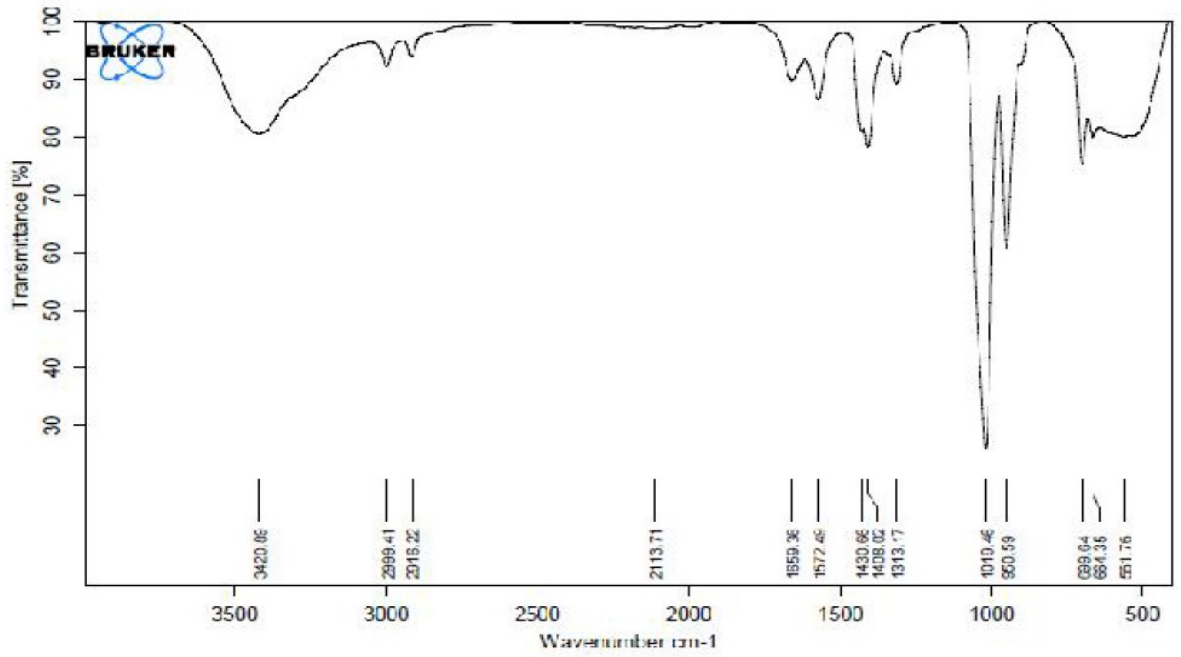
Xanthomonas *Fusarium*

Figure.4 FT-IR spectrum of methanol: ethanol (96:4) fraction of FCU+PR



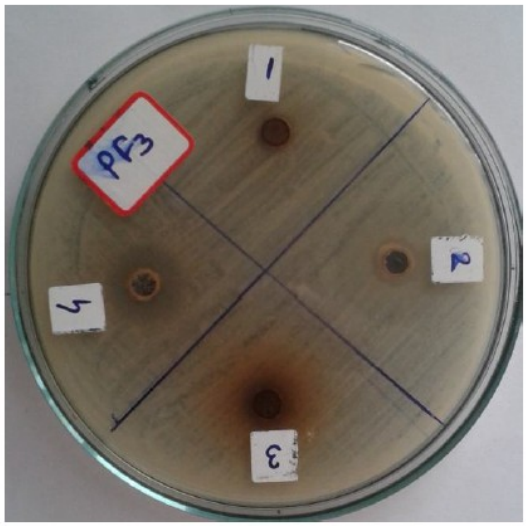
3333.19 71.86 2943.91 81.86 2832.63 85.58 1721.81 87.44 1597.16 80.00 1452.02 83.72 1379.82 83.10 1257.44 83.00 1118.52 90.00 1020.91 47.52 849.33 95.58 633.73 74.96 608.76 74.34

Figure.5 FT-IR spectrum of methanol: ethanol (96:4) fraction of FUC

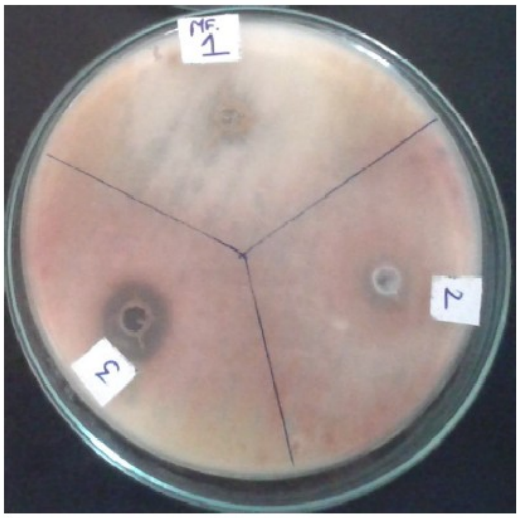


3420.89 80.71 2999.41 91.42 2916.22 94.26 2113.71 98.58 1659.36 90.72 1572.49 86.39 1430.66 80.71 1408.02 77.10 1313.17 90.00 1019.46 26.45 950.59 61.42 699.64 74.97 664.35 81.42 561.76 80.00

Figure.6 Antimicrobial activity of fermented cow urine broths. Well 3= FCU+PR



Xanthomonas



Fusarium

Table.1 Preliminary phytochemical analysis of cow urine fermented with various botanicals

Phytochemical Tests ↓	FCU+NL				FCU+NS				FCU+GC				FCU+PR			
Days →	0	7th	14th	21st	0	7th	14th	21st	0	7th	14th	21st	0	7th	14th	21st
Protein	+	+	+	+	–	–	–	–	+	+	+	+	+	+	+	+
Carbohydrates	–	–	–	–	–	–	–	–	+	+	–	+	–	–	+	–
Tannin/Phenol	+	+	+	+	–	–	–	–	+	+	–	–	+	+	+	+
Flavanoid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Saponin	+	+	–	–	–	–	–	–	+	+	+	+	–	+	+	–
Glycosides	+	+	+	–	+	+	+	–	+	+	+	+	–	–	–	–
Steroids	+	+	+	–	–	–	–	–	+	+	+	+	–	–	+	+
Terpenoids	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Alkaloids	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–

Pomegranate rind rich in these flavonoids and tannins as natural antimicrobial agents, have been widely exploited against Staphylococcus aureus and hemorrhagic Escherichia coli for their ability to precipitate membrane proteins and inhibit enzymes such as glycosyltransferases, leading to cell lysis. Satish et al 2009 have reported the inhibitory activity of *Punica granatum* extracts against *Fusarium* spp. as well. The toxicity profile of pomegranate peel extract indicates its safe use even for oral consumption up to 70mg/kg body wt. Among all the broths used the highest anti *Xanthomonas* and anti *Fusarium* potential was found to be present in FCU+PR. Hence the same was subjected to Standard separation procedure. Only the methanolic fraction of FCU+PR was effective against *Xanthomonas* and *Fusarium*. This indicates the polar nature of the bioactive principle. The complex compounds like flavonoids and tannins would be getting converted into simpler forms during fermentation process in earthen pot which is evident in FTIR analysis of FCU+PR which clearly reveals the significant presence of aliphatic acids, benzoic acids, phenols and alcohols. The concentration of phenols and benzoic acids appear to be less in FTIR of FCU alone. This is the first report establishing the in vitro anti *Xanthomonas* as well as anti *Fusarium* potential of fermented cow urine with pomegranate rind. This study strongly supports the traditional practice of using fermented cow urine with the recommendation of the most effective, safe and easily available pomegranate rind for the management of bacterial blight and wilt of pomegranate.

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