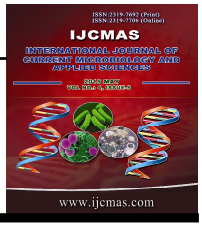
Int.J.Curr.Microbiol.App.Sci (2015) 4(5): 824-833

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 5 (2015) pp. 824-833**
http://www.ijcmas.com



Original Research Article

In vitro evaluation of antibacterial potential of *Pongamia pinnata* L. against *Xanthomonas axonopodis* punicae, phytopathovar of Bacterial blight of Pomegranate (*Punica granatum*)

V. A. Gargade[1] and D.G. Kadam[2]*

[1]Walchand Centre for Biotechnology, Walchand College of Arts and Science Solapur-413006, Maharashtra, India
[2]D.B.F. Dayanand College of Arts and Science, Solapur-413002, Maharashtra, India

**Corresponding author*

A B S T R A C T

The present investigation was undertaken to assess the potential of *Pongamia pinnata* L. as antibacterial agent against *Xanthomonas axonopodis punicae*, phytopathovar of Bacterial blight of Pomegranate (*Punica granatum*).The aqueous extracts and solvent extracts of leaves and seeds of *Pongamia pinnata* L.were evaluated by agar cup diffusion method. The leaves and seed extracts were prepared in six solvents viz. water, ethanol, methanol, diethyl ether, benzene, chloroform and tested for its antibacterial activity against *Xanthomonas axonopodis punicae.* Aqueous, ethanol, methanol extracts of leaves and seeds of *Pongamia pinnata* showed significant antibacterial activity against *Xanthomonas axonopodis punicae*. Minimum inhibitory concentration of these extracts ranged between 400-500µg/ml. Among the selected solvent extracts, methanol extract of leaves and aqueous extract of seeds showed maximum antibacterial activity against the pathogen. GCMS analysis of these extracts further revealed presence of phytoconstituents that may have potential to inhibit the growth of *Xanthomonas axonopodis punicae* indicating their possible use in controlling bacterial blight of Pomegranate in field.

Keywords

Pongamia pinnata, *Xanthomonas axonopodis punicae*, antibacterial activity, Minimum Inhibitory Concentration, GCMS analysis

Introduction

The Pomegranate (*Punica granatum* L.) is an ancient fruit crop of India. Pomegranate has been associated with high nutritional value, many health benefits and its entire plant has great economic and therapeutic value (Julie Jurenka,2008).India is one of the major producers of Pomegranate in the world with average total production of 8 lakh tonnes per annum(Pawar *et al.*,2013).

Recently yield and quality of Pomegranate is affected by bacterial blight disease caused by *Xanthomonas axonopodis punicae.* The pathogen can infect in any stage of growth of the plant.The damage is observed on fruits which develop black oily spots later become completely black splits and dries off. In advanced stage of infection tissue necrosis occurs on leaves and twigs.

The primary disease management includes spraying bleaching powder, Bordeaux mixture, streptocycline, urea, farmyard manure, bronip, bactinash, bactrinashak, plantomycin to control bacterial blight of pomegranate (Yenjerappa *et al.*,2014).Use of chemicals in agriculture causes several adverse and environmental hazards (Shanthi,2011).

Regular use of chemicals in agriculture land causes killing of flora and fauna of the soil, increase in development of resistance in plant pathogen against chemicals and residual toxicity remains in plant and animals. To overcome this problem, there is growing interest worldwide in the ultilisation of ecofriendly material for pathogen control (Madhiazhagan *et al.*,2002).

Our study is focussed on evaluation of antibacterial potential of *Pongamia pinnata* against *Xanthomonas axonopodis punicae*, phytopathovar of Bacterial blight of Pomegranate (*Punica granatum*).*Pongamia pinnata* is an ancient plant of great medicinal value. It is medium sized glabrous tree belonging to family Fabaceae (Papilionaceae) popularly known as Karanj.It is widely distributed in various countries like India, Bangladesh, China, Florida, Hawai, Malaysia.

Pongamia is versatile medicinal plant,is source of various type of chemical compounds(Khatri Pankaj *et al.*,2012,Dubey Swati,2014).Shanti *et al,*.(2011), Murgan *et al*.,(2012)showed antibacterial activity of *Pongamia pinnata* plant extracts against *Xanthomonas oryzae pv.oryzae* causative agent of bacterial leaf blight of paddy. The present study was undertaken to explore the potential of aqueous and different solvent extracts of *Pongamia pinnata* against *Xanthomonas axonopodis punicae*.

Materials and Method

Collection of diseased plant parts of Pomegranate plant

Diseased Pomegranate fruits were collected from field located at village Kurul, district Solapur, Maharashtra, India .

Isolation and Identification of pathogen from lesions on diseased fruit

The infected portion of fruits were surface sterilized with 0.1% mercuric chloride solution for one minute and washed three times with sterile distilled water and squeezed gently with sterile scalpel in sterile saline. The presence bacteria in fruit lesion was confirmed by performing ooze test. The suspension was serially diluted and plated on sterile petriplate with Nutrient Glucose Agar medium with composition Beef extract-0.3%,peptone-0.5%,glucose-0.25%, agar-2%, pH-6.8 (Mondal *et al*., 2009).The inoculated plates were incubated at 30ºC for72hours.After incubation typical mucoid, yellow coloured, well isolated colony was selected and screened for morphological and biochemical characteristics. The culture was further identified on the basis of pathogenicity test.

Pathogenicity test

Identification of the pathogen was done by performing pathogenicity test (Schaad,1992). Healthy and vigorously growing, six months old (Bhagava variety) plants were used for pathogenicity test.The cells from NG slant grown in nutrient glucose broth were taken.Cell density is adjusted to10^6 -10^7cfu/ml on the basis when culture reaches optical units at 600nm with spectrophotometer. For leaf spotting, pathogen was streaked on the upper part of the stems and young petiole of young leaves

was punctured with sharpe sterile needle with syringe in several locations. Bacterial isolate was inoculated in punctured leaves holding finger beneath the leaf. Control plants were similarly inoculated with sterile distilled water. The plant surfaces were kept wet. The plants were covered with polythene bags and kept under greenhouse conditions for development of symptoms. The isolate that showed symptoms of bacterial blight was subjected for molecular identification on the basis of 16s rRNA sequencing.

Collection of *Pongamia pinnata* leaves and seeds

Fresh healthy leaves and seeds of *Pongamia pinnata* were collected and identified from Department of Botany, Walchand College of Arts and Science, Solapur, Maharashtra, India. The plant parts were washed thoroughly with running tap water and once with sterile distilled water. The plant material was dried with blotting paper under shade.

Preparation of aqueous extract

The dried leaves and seeds of *Pongamia pinnata* were macerated with sterile distilled water in waring blender. The macerate was kept for 24hr at room temperature (Nidaullah *et al.*,2005).The macerate was filtered through double layered musclin cloth and was centrifuged at 4000rpm to separate supernatant .The supernatant was then evaporated to dryness at room temperature and stored at 4ºC.

Preparation of solvent extracts:

Twenty five grams of power of dried plant material was filled in the thimble prepared with musclin cloth and extraction was carried out in soxhlet apparatus by using different solvents and it was then concentrated by rotary flash evaporator and preserved at 4ºCfor further use (Raghavendra *et al.,* 2006)). All the extracts were subjected to antibacterial activity assay.

Antibacterial activity assay

The antibacterial activity of 100µl of aqueous and solvent extracts against the isolate was done by cup diffusion method on Nutrient Glucose Agar medium.

Determination of minimum inhibitory concentration

MIC of plant extracts was determined by broth dilution method. For broth dilution tests, 0.1ml of standardised suspension of bacterial isolate (10^6cfu/ml) was added in each tube containing different concentrations of plant extracts ranging from 100µg/ml to 1000ug/ml.

Phytochemical analysis

The plant extracts showing maximum antibacterial activity were subjected to qualitative phytochemical analysis (Raaman,2006,Deshpande and Kadam,2013).

All the experiments were confirmed by repeating three times for satisfied confirmations.

Gas chromatography mass spectroscopy analysis

GCMS analysis of methanol extract of leaves and aqueous extract of seeds of *Pongamia pinnata* showed maximum antibacterial activity was carried out by using Helwett Packard GCD1800A model with electron ionisation detector operator through a data system.1µl of extract was

used to inject in injection port of GC column and scanning was done. While the instrument was run, the computer generated graph from the signal called chromatogram is obtained .Each of the peak in the chromatogram represented the signal created by compound when eluted from gas chromatography column into the detector. As individual compounds eluted from the Gas chromatographic column, they entered the electron ionization (mass spectroscopy) detector where they were bombarded with a stream of electrons causing them to break apart into fragments. The fragments obtained were actually charged ions with a certain mass .The M/Z (Mass /Charge) ratio obtained was calibrated from the graph obtained which was called as the Mass spectrum graph which is the fingerprint of a molecule (Sagwan, 2012).

Interpretation and Identification of individual components were done by using National Institute of Standards and Technology (NIST) Library.

Results and Discussion

Isolation and identification of pathogen

The isolated organism was identified on the basis of morphological, cultural, biochemical characteristics and 16SrRNA.In pathogenicity test, the isolate showed presence of oily black spots on the leaves after incubation, indicates causative agent of bacterial blight of Pomegranate.

Molecular Identification of isolate

Phylogenetic placement of Xavp1 (Accession Number KP168824) was done based on 16S rRNA analysis. Phylogenic tree based on 16S rRNA gene sequences showed relationships among strain Xavp1 and the most close type strain species of *Xanthomonas axonopodis pv. punicae* AP-5. The optimal tree *w*ith the sum of branch length = 0.16279831 is shown. Numbers at nodes indicate percentages of bootstrap support based on a Neighbor-joining analysis of 1,000 resampled datasets. Bar 0.005 substitutions per nucleotide position.

Antibacterial activity

The aqueous, methanol, ethanol extracts of *Pongamia pinnata* leaves and seeds showed significant antibacterial activity against *Xanthomonas axonopodis punicae* (Table 2) (Fig.2).

Minimum inhibitory concentration of plant extracts

The MIC of selected plant extract required for inhibition of growth of *Xanthomonas axonopodis punicae* is presented in Table 3.

Phytochemical analysis

Results of qualitative phytochemical analysis of aqueous extracts of *Pongamia pinnata* leaves revealed the presence of saponin and terpenoids. While methanol and ethanol extracts showed alkaloid, flavonoids, terpenoids, flavonoids. Phytochemical analysis of aqueous extract of *Pongamia pinnata* seeds showed presence of alkaloids, fixed oils and fats, tannins, terpenoids, flavonoids (Raaman, 2006).

Gas chromtography mass spectroscopy analysis

Chromatogram obtained after GCMS analysis of *Pongamia pinnata* leaves shown in figure 4.GCMS analysis of methanol extract of *Pongamia pinnata* leaves (Table 4) and aqueous extract of *Pongamia pinnata* seeds are depicted in Table 5 with name of the compound identified, their

Retation time, Molecular formula and Molecular weight.

The compounds detected in methanol extract of *Pongamia pinnata* leaves were Myo-Inositol (aromatic compound),4-(-methy, Hexadecanoic acid methyl ester,2-Methyl amino-3- (methyl-N-phenylamino) -1,4 naphtoquinone,2H-1-benzopyran-6-ol,3,4-dihydro-2,8-dimetyl-2-[4,8,12-trimethyl-tridecyl]- (Table 4).

The compounds detected in aqueous extract of *Pongamia pinnata* seeds were 1-(5-ethyl-@ methyl piperidinol)-3-(o-nitrobenzyoloxy) propane, Naphthol(1,2-b)furan-6,9-dione,4,5,7,8-tetrahydroxy-, n-Hexadecanoic acid, Lupeol,2-5(-(-methyl-benzoonazol-7-yl-1H-pyrazol-3-yl)-phenol. (Table 5).

The compound n-hexadecanoic acid also known as palmitic acid is a 16-carbon saturated fatty acid generally present in natural oils and fats. It is also found in all plant parts of *Pongamia pinnata*. The presence of fatty acids in *Pongamia pinnata* shows pharmacological properties. Karanj (*Pongamia pinnata*) seed oil as it contains n-hexadecanoic acid ,inhibits fourteen strains of pathogenic bacteria. Another compound Lupeol (Triterpene in nature) was observed in *Pongamia pinnata* leaves displaying antimicrobial property (Sagwan *et al*., 2012).This compound was also reported in other plants,viz. *Stevia rebaudiana* (Korobko, 2008) and *Mimosa pudica* (Sriram, 2011).

It was reported that presence of 14 bioactive compounds in flower extract of *Cassia javanica* L. showed antibacterial activity against Gram negative bacteria *E.coli., S. typhi* and Gram positive bacteria *Bacillus subtilis, S.pyogenes*. GCMS analysis of flower extract of *Cassia javanica* L. showed presence of myo-inisitol, n-hexadecanoic acid which are also found in Leaves *of Pongamia pinnata* (Rameshbabu,2014).

Vivek *et al.*,(2009) and Satish Kumar(2011) also reported that the chloroform, ethyl acetate, methanol extract of *Pongamia pinnata* leaves showed antibacterial potential against *Bacillus subtilise* ATCC6633, *Staphylococcus aureus* ATCC6538, *Listeria monocytogenes* ATCC19118, *Listeria monocytogenes* ATCC19166, *Pseudomonas aeruginosa* ATCC6432 and *Salmonella typhimurium* ATCC2512 suggest that *Pongamia pinnata* leaves have significant antibacterial potential.

Shanthi *et al*. (2011) showed antixanthomonas activity of alcoholic fraction of *Pongamia pinnata* leaves and cowurine extract against bacterial leaf blight of Paddy.

Recently bacterial blight of pomegranate is controlled by using bactericides,Copper oxychlorides, micronutrients, antibiotic streptocycline.More ever growing public concern about the use of pesticides and its hazardous effect on plant and human health, has promoted the search for alternative approach. Our study is focussed on use of *Pongamia pinnata* for in vitro control of *Xanthomonas axonopodis punicae.*

Use of plants as a source of medicine has been inherited and is important componant of heathcare system. The secondary plant metabolites which are divided into different categories based on their mechanism function like chemotherapeutic, bacteriostatic, bacteriocidal, and antimicrobial (Shariff, *et al.*, 2006). The property of antibacterial activity of secondary metabolites can be exploited for controlling bacterial blight on Pomegranate.

Table.1 Morphological, cultural and biochemical characters of bacterial isolate

Morphological/biochemical character	
Size	3mm
Shape	Circular
Colour	Yellow
Margin	Irregular
Opacity	Opaque
consistency	Mucoid
Elevation	Elevated
Gram nature	Gram negative short rods
Motility	Motile
Starch hydrolysis	+
Hydrogen sulphide production	+
Gelatin liquefaction	-
Catalase production	+
Oxidase production	+
Growth at 35ºC	+
Glucose	+
Fructose	+
Mannose	+
Rhamnose	-
Indol	-
Methyl Red	+
Voges Prauskeur	_
Citrate	+

+positive test -negative test

Table.2 Diameter of Zone of inhibition of *Pongamia pinnata* against *Xanthomonas axonopodis* punicae

Sr, No	Plant part used	Aqueous extract	Ethanol extract	Methanol extract	Petroleum Ether extract	Benzene extract	Chloroform extract
1	Leaves	26±1.53	24±0.58	27±1.00	--	--	--
2	seeds	30±1.00	16±1.53	26±2.51	--	--	--
3	Solvent	0.00±0.00	7±0.42	5±1.00	--	--	--

Zone of Inhibition in mm (± SD)

Table.3 The minimum inhibitory concentration of the *Pongamia pinnata* against *Xanthomonas axonopodis* punicae

Sr.No.	Plant material used	MIC in µg/ml		
		Aqueous	Ethanol	Methanol
1	Seeds	400	800	400
2	leaves	600	500	500

Table.4 Phytocomponents identified in the methanol extract of *Pongamia pinnata* leaves using GCMS analysis

Sr.No	R.T.	Name of the Compound	Molecular Formula	Molecular Weight
1	15.16	Myo-Inositol,4-C-methyl-	$C_7H_{14}O_6$	194
2	16.69	Hexadecanoic acid methyl ester,	$C_{17}H_{34}O_2$	270
3	28.74	2-Methylamino-3[-N-phenylamino]-1,4naphtoquinone	$C_{18}H_{16}N_2O_2$	292
4	33.98	2H-1-benzopyran-6-ol,3,4-dihydro-2,8-dimetyl-2-[4,8,12-trimethyltridecyl]-	$C_{27}H_{46}O_2$	402

Table.5 Phytocomponents identified in the aqueous extract of Pongamia pinnata seeds by using GCMS analysis

Sr.No	R.T.(Min.)	Name of the Compound	Molecular Formula	Molecular Weight
1	2.85	1-(5-ethyl-2-methylpiperidino)-3-(o-nitrobenzoyloxy)propane	$C_{18}H_{26}N_2O_4$	334
2	28.41	Naphthol(1,2-b)furan-6,9-dione,4,5,7,8-tetrahydroxy-	$C_{12}H_6O_7$	262
3	17.35	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256
4	28.15	Lupeol	$C_{30}H_{50}O$	426
5	28.76	2-[5(-methyl-benzooxazol-7-yl)-1H-pyrazol-3-yl]-phenol	$C_{17}H_{13}N_3O_2$	291

Figure.1 Phylogenetic placement of Isolate

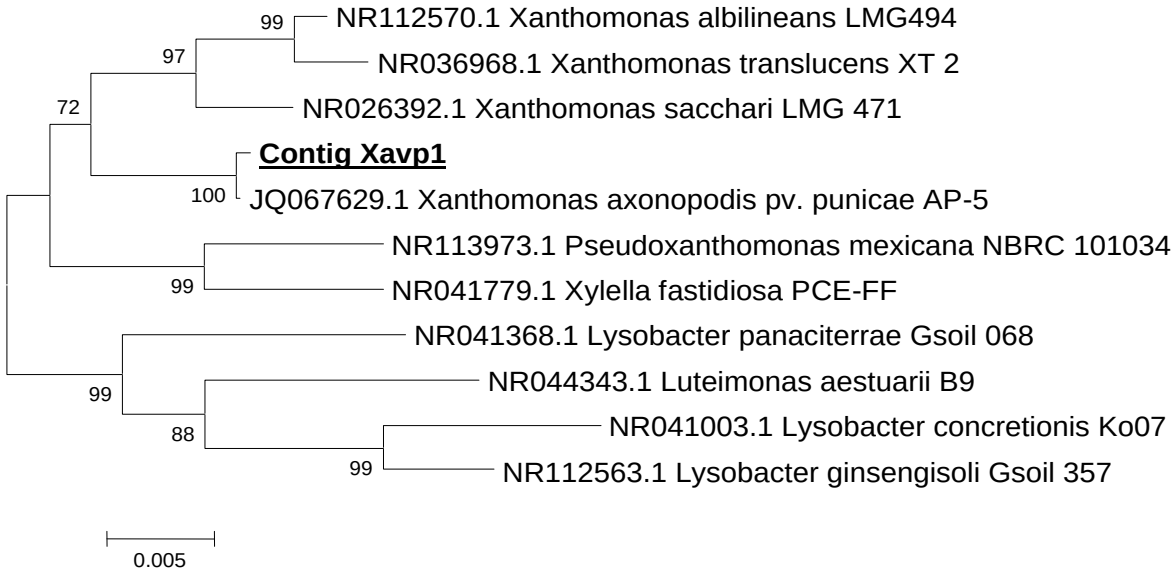


Fig.2 Antibacterial activity of aqueous extract *Pongamia pinnata* against Xavp1

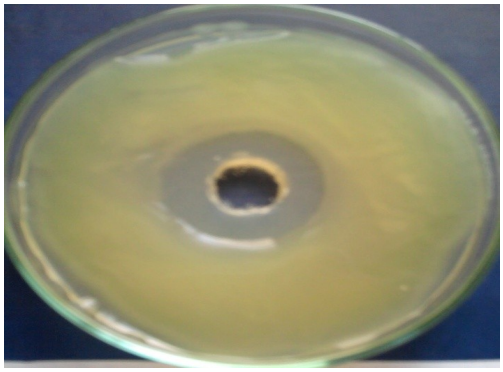


Fig.3 Chromatogram obtained after GCMS analysis of methanol extract of *Pongamia pinnata* leaves

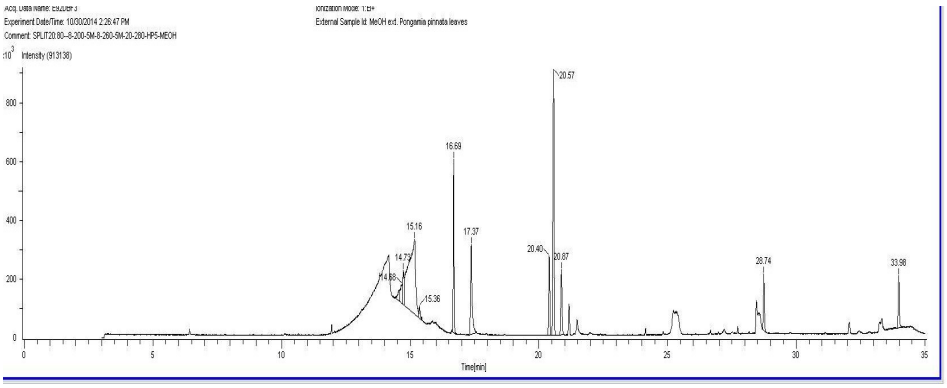
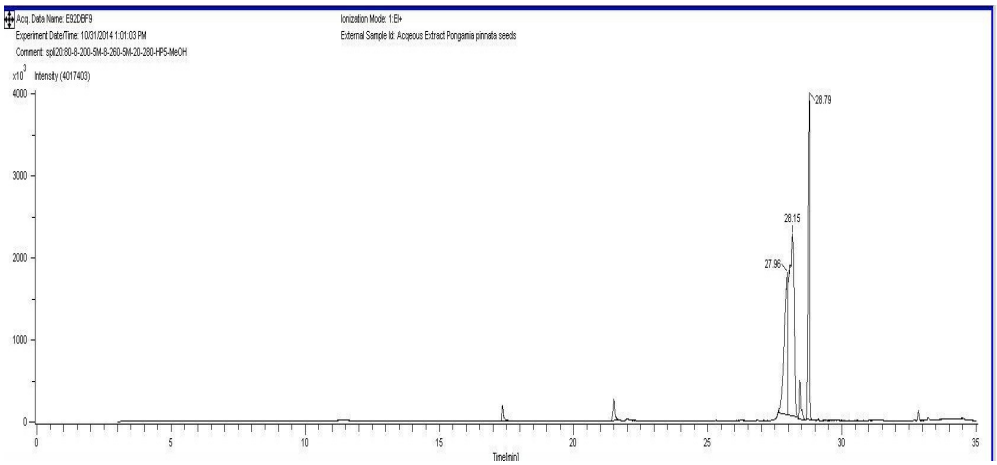


Fig.4 Chromatogram obtained after GCMS analysis of aqueous extract of *Pongamia pinnata* seeds



GCMS analysis of *Pongamia pinnata* leaves and seed extracts showed presence of bioactive compounds previously reported for their antibacterial activity. The use of *Pongamia pinnata* plant extracts showed significant antibacterial activity against

Xanthomonas axonopodis punicae indicates it can be used to control bacterial blight on Pomegranate. Results of this work will help to identify the compounds, which may be used for controlling bacterial blight of Pomegranate. Furthermore, the identification of plant metabolites is the first step to explain the benefits of traditionally used medicinal plants to control plant pathogen in future.

Acknowledgement

Authors are thankful to Dr. Shirishkumar, Rajiv Gandhi Centre for Biotechnology Thycaud P.O., Thiruvananthapuram, Kerala, India for providing facility for DNA barcoding We extend our thanks to SAIF, Indian Instistute of Technology, Powai, Mumbai for GCMS analysis of plant extracts.

References

Deshpande S.N., Kadam D.G.,2013. Phytochemical Analysis and Antibacterial activity of Acacia nilotica against *Streptococcus mutans*, International Journal of pharmacy and pharmaceutical Sciences. 5(1), pp.236-238.

Dubey Swati,2014.Phytochemical analysis of seeds of certain medicinal plants, International Research Journal of Pharmacy.5(2),pp.102-105

Julie Jurenka MT 2008.Therapeutic applications of pomegranate (*Punica granatum* L.):A review. Alternative Medicine Review.13,pp.123-144.

Khatri Pankaj, Patel Rakesh,2012.An phytochemical overview of various parts of *Pongamia pinnata*,World Journal of Pharmaeutical Research.2(1),pp.146-165.

Korobko N.V.,Turko Ya A.,Shokun V.V.,E. N. Chernyak L.M., Pokrovskii O.N. Smetankina B.F., Kerimzhanova, U.A.Baltaev.2008.GC-MS Investigations. II. Lipid composition of *Stevia rebaudiana*,Chemistry of Natural Compounds,44(3),pp.359-360.

Mahesh Pawar, Manoj Kadam and Bipinraj Kunchiraman Nirichan.2014.In vitro control of bacterial blight causing organism(*Xanthomonas axonopodis* pv. *punicae*) of pomegranate. Journal of Environmental Research and Development.8(3a), 568-573

Mondal,K.,Rajenran,T.,Phaneendra,C.,Mani, C.,Sharma ,Jyotsana,Shukla Rucha,Pooja,Verma Geeta,Kumar,R.,Singh, D.,Kumar,A.,Saxena,A.K.,Jain,R.,2012.The reliable and rapid polymerase chain reaction(PCR)diagnosis for *Xanthomonas axonopodis pv.punicae* in pomegranate,African Journal of Microbiology Research.6(30), pp.5950-5956.

Murugan, A.M., S.Shanti, C.Arunachalam, N.Sivakumar, S.Elamathy and K. Rajapandian 2012. Study on cowurine and *Pongamia pinnata* Linn seed in farmyard: A natural, cost effective, ecofriendly remedy to bacterial leaf blight (BLB) of paddy. African Journal *of Biotechnology*. 11(40),pp 9591-9598.

N.W.Schaad 1992.Laboratory Guide For Identification of plant Pathogenic Bacteria, International Book Distributing Co. Lucknow.pp81-93.

Nidaullah H.Durrani F.R.,Ahmad S.,John I.U.,and Gul S.,2005.Aqueous extract from different medicinal plants as coccidial growth promotive and immunostimulants in broilers,

Asian Research Publishing Network Journal of Agricultural Biological Science,5(1),pp.53-59.

Raaman N.,2006, Phytochemical Techniques, New India Publishing Agency, New Delhi, Chapter 5,pp.19-24.

Madhiazhagan K, Ramadoss N, Anuradha R.2002. Antibacterial effect of plant on common pathogen: Journal of Mycology plant pathology. 32(1), pp.68-69

Raghavendra M.P., Satish, S. and Raveesha, K.A. 2006.In vitro evalution of anti-bacterial spectrum and phytochemical analysis of *Acacia nilotica*., Journal of Agricultural Technology.2(1),pp.77-88.

Rameshbabu Bhuvaneshwari Rajgopal Gopalkrishnan, 2014 Antimicrobial potential and structural elucidation of bioactive compounds from flower extract of Cassia javanica L, Indian Journal of natural products and Resources. 5(1),pp.34-39.

S.T. Yenjerappa, V.B. Nargund, M.R. Ravikumar and A.S. Byadagi 2014:Efficacy of bactericides and antibacterial chemicals against bacterial blight of Pomegranate, International Journal of Plant Protection.7(1),pp. 201-208

Sagwan S., Rao D.V., Sharma R.A.2012. Studies on gc/ms spectroscopic analysis of some different *invivo* plant extracts of (karanj) *pongamia pinnata* (l.).Pierre Journal of Global Pharma Technology. 8(4):pp.1-10

Satish Kumar B.N.2011.Phytochemistry and pharmacological study of *Pongamia pinnata* (Linn.) Pierre,Journal of Pharmaceutical Sciences Review and Research.9(2),pp.12-19.

Shanthi S,Elamathy S, Panneerselvam.A, Radha N.2011.Antixanthomonas activity of *Pongamia pinnata* linn leaves – cow urine extract- a natural cost effective ecofriendly remedy to bacterial leaf blight of paddy (BLB). Journal of Pharmacy Research. 4(3),pp.650-652

Shariff, N., Sudarshan, M., Umesha,S., Hariprasad, P. 2006. Antimicrobial activity of *Rauvolfia tetraphylla* and *Physalis minima* leaf and callus extracts, African Journal of Biotechnology. 5(10),pp.946-950.

Sriram Sridharan, Meenaa Vaidyanathan, Kavitha Venkatesh and Agnel Arul John Nayagam, 2011:GC-MS study and phytochemical profiling of *Mimosa pudica* Linn.Journal of Global Pharmacy Research. 4(3),pp. 741-742.

Vivek K Bajpai, Atiqur Rahman, Savita Shukla, Archana Mehta, Shruti Shukla, Yassir Arafat SM, Mizanur Rahman M, Zennat Ferdousi.2009, Antibacterial activity of leaf extracts of *Pongamia pinnata* from India, Pharm Biol. 47(12),pp.1162-1167.