IN VITRO EVALUATION OF ANTIFUNGAL POTENTIAL OF SOME MEDICINAL PLANTS AGAINST *Ceratocystis fimbriata* VMB12

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ABSTRACT

Pomegranate (Punica granatum L.) is one of the most significant arid zone fruit crop which was affected by soil born pathogen like Ceratocystis fimbriata, Fusarium oxysporum Schl., and nematode which are responsible to cause wilt disease in pomegranate. Pomegranate wilt disease is considered to be one of the very devastating disease and causes losses about more than 5%-10% and that's why the study was undertaken on this disease with the objectives like isolation of causal organism and antifungal potential of some medicinal plants against the wilt causing Ceratocystis fimbriata VMB12.The antifungal activity of several medicinal and locally available plants extracts Viz. leaves, bark, stem and root which are frequently found in the surrounding fields on which some fungi were tested in the lab conditions. Three different plants Viz. Azadirachta indica,Ricinus communis and Pongamia pinnata were selected for testing.All these plants showed antifungal activity against the Ceratocystis fimbriata VMB12. Of which Azadirachtaindicacrude extracts of leaves, bark, stem and root showed best inhibition activity against the Ceratocystis fimbriata VMB12 and suppressed the mycelial growth of above mentioned pathogens.It is investigated in present study that Azadirachta indica,Ricinus communis and Pongamia pinnata can be utilized against the management of fungal diseases like wilt complex disease of Pomegranate (Punica granatum L.) caused particularly by Ceratocystis fimbriata VMB12.

Keywords : *Pomegranate,Plants extract,Antifungal activity, Wilt Complex Disease, %Inhibition.*

Introduction

Pomegranate (Punica granatum L.) is an ancient fruit which belongs to the family Punicaceae. This is one of the central cash crops of our country and it is commonly called as '**Fruit of Paradise**'. The successful cultivation of pomegranate is frequently suffers from different diseases. Amongst these wilt of pomegranate caused by Ceratocystis fimbriata Ellis and Halst. is a major threat for it's the cultivation and is second most important and destructive disease of pomegranate cultivation which causes extensive yield loss. More than a few agents such as Ceratocystis, Rhizoctonia and Fusariumare known to cause wilt in Pomegranate, but Ceratocystis fimbriata is the major cause of Pomegranate found which is associated in 77% of wilt samples collected from diverse locations of Maharashtra and Karnataka (Sharma,et al., 2010; Sharma, 2009). A survey of 44 locations in Maharashtra in India from 1995 to 1998 showed 7.5% crop losses amounting to Rs.30 lakhs (Somasekhara, 1999). Somasekhara and Wali (2000) reported 12.29% mean incidence ranging from 1.62 to 63.50% in Maharashtra and Karnataka; monetary losses mounted to Rs. 67.45 lakhs during 1996-97 and Rs. 26.9 lakhs during 1999-2000. Different reports suggest that 45% of crop is severely affected by wilt pathogen Ceratocystis fimbriata and day by day its severity is increasing many folds.

Plants having possible antimicrobial activity should be checked against some microbes to confirm the activity (Shinwariet al., 2009). The activity of these plant extracts on bacteria and fungi has been studied by a very large number of researchers in various parts of the world (Vuuren and Naido, 2010; Walter et al., 2011; Bhengraj et al., 2008).The medicinal plants represent a dominant source of antimicrobial agents (Adnan et al., 2010; Mahesh and Satish, 2008).Many of the plant materials used in traditional medicineare quickly available in rural areas at relatively cheaper than modern medicine (Gilani et al.,2010; Mann et al., 2008; Hussainet al., 2012). The Plant metabolites and plant based pesticides seemto be one of the better alternatives as they are known to have negligible environmental impact and danger to the consumers in contrast to the synthetic pesticides (Hussain et al., 2014; Varma and Dubey, 1999).

In the present study, three medicinal plants Viz. Azadirachta indica, Ricinus communis

and Pongamia pinnata were studied to determine its antifungal effects on mycelial growth of Ceratocystis fimbriata VMB12.

Materials and Methods

1. Collection of Diseased Plant Parts of Pomegranate Tree

Pomegranate plants were observed for presence of symptoms of wilt complex disease. The yellowing of single branch from top to bottom at early stages and later progression of disease resulting in complete wilting of plants were collected from pomegranate field located at Chikurda, Latur District, Maharashtra, India. Samples of infected stem and roots were collected from wilt affected pomegranate plants.The sterile scissor used to cut the branches. Then the infected stem and roots were put in polythene bag and closed it securely. It was then brought to the laboratory and kept it in shade, cool place and preserved for further study (Divya, 2013; Nelson and Bushe, 2006).

2. Collection of Medicinal Plant parts for Antifungal Activity

The different medicinal plants used in the present study Viz. Azadirachta indica, Ricinus communis and Pongamia pinnata were collected from different parts of Latur city. The collected plant parts were identified from the Department of Botany, Lal Bahadur Shastri Mahavidyalaya,Dharmabad, Nanded, Maharashtra. The medicinal plants used in this study are generally found in this geographical area and their medicinal importance is emphasized in the literature of utilization of medicinal plants in plant disease control. These plant parts were then further used to perform In vitro antifungal activity against the isolated representative pathogen Viz. Ceratocystis fimbriata VMB12 of wilt complex of pomegranate(Fatima et al., 2012; Govindappa et al., 2011; Dhanya and Mary, 2006).

3. Isolation and identification of the pathogen from affected parts of Pomegranate plant

Ceratocystis fimbriata VMB12, associated with wilt was isolated from the infected stems and roots of pomegranate plant which were collected from Chikurda village. The slices of the collected stem portions having characteristic symptoms of vascular staining were further surface sterilized with 1% sodium hypochlorite ($NaHCO_3$) for about 2 minutes and washed in 70% alcohol and twice with sterile distilled water (D/W) to remove traces of $NaHCO_3$. Pathogen isolation was made using carrot bait technique (Moller et al., 1968) in which, stems were kept in between the carrot disks and placed in a humid chamber and incubated at 25 ± 2^0C for 12 hour photoperiod (Moller et al., 1968). Following perithecium formation, a portion of the fungus was transferred to the freshly prepared potato dextrose agar (PDA) to allow the full development of fungus. In order to confirm the identity of the fungus, the ascospores, endoconidia, aleroconidia and perithecia were observed under the high power (40x) objective of the microscope. The identification of pathogen has done as explained by Sharma et al., (2010) and 18s rRNA analysis.

4. Molecular identification of the bacterial pathogen

Molecular identification of the promising bacterial isolate was carried out by 18S rRNAwere sequenced (Mondal et al., 2012) at National Center for Cell Sciences, University of Pune Campus, Pune and Maharashtra, India.

5. Phylogenetic analysis:

The generated sequences were analyzed at the National Center for Biotechnology Information Bethesda, MD. www.nbi.nlm.nih.gov/BLAST for closed homology using BLASTn algorithm. The related sequences for the isolates were downloaded from the NCBI database were aligned by using CLUSTAL X2 multiple sequence alignment tool, the Phylogenetic evolutionary history was inferred using the Neighbor Joining Method analysis (Tamura et al., 2004). Phylogenetic analyses were conducted in MEGA 4.0. Phylogenetic tree building was performed using MEGA 4.0 (Tamura et al., 2007).

6. Collection of Different Medicinal Plant Parts

Different medicinal plant Viz. Azadirachta indica, Ricinus communis and Pongamia

pinnata were collected from various sources. Healthy, fresh, disease free plant parts Viz. root, stem, leaves and bark were cut with the help of sterile scissor and knife. It was kept in polythene bag and closed it securely. Afterwards, all these plant parts were brought to the laboratory and thoroughly washed with the distilled water. These parts of the plants were shade dried and then powdered with the help of warring blender. These powdered forms of plants were used for the preparation of solvent extracts.

7. Preparation of Medicinal Plant Extracts

7.1. Preparation of Aqueous Extract of Selected Medicinal Plant Parts

Twenty gram of thoroughly washed fresh plant parts Viz. root, stem, leaves and bark were macerated with 100ml sterile distilled water taken separately in a warring blender for 10 minutes. The crush of each plant was then filtered through muslin cloth having double layer and then centrifuged (Remi Centrifuge model R-8C DX+R-81A) at 4000 rpm for 30 minutes. The supernatant was then filtered just before subjecting it to antifungal activity assay (Raghavendra et al., 2006).

7.2. Preparation of Solvent Extracts of Selected Medicinal Plants

Twenty gram of dried powder of each plant was filled in the thimble and extracted consecutively with the solvents Viz. aqueous, ethanol and methanol by using a Soxhlet extractor (Soxhlet Complete Borosil, Code 3840) for 48 hours. All these extracts were separately prepared in Soxhlet extractor. Further, all these extracts were concentrated using rotary flash evaporator (Superfit Rotary Vacuum Flash Unit PBU-6D) and preserved at 4^0C in airtight bottle for further use. Afterwards, all these extracts were subjected to antifungal activity assay (Raghavendra et al., 2006).

8. Antifungal activity of plant extract:

The different medicinal plant extracts were assessed In Vitro by Poison food technique (Nene and Thapliyal, 2000). The supernatant was considered as a standard plant extract solution (100%). Furthermore, the plant extract was diluted by adding distilled water to get different per cent concentrations. Theplant extracts were further subjected to boiling temperature of 50^0C in a water bath for to avoid contamination and after that incorporated into Potato dextrose agar (PDA) media by transferring 2ml of each kind of plant extract in to a sterile petri plate containing 20ml melted warm Potato dextrose agar (PDA) medium and gentle shaking for thorough mixing of the extract. The sterile Potato dextrose agar (PDA) plates having the plant extracts were inoculated aseptically with given pathogens by transferring 6mm diameter agar disc of 07 days old culture of the pathogen Ceratocystis fimbriata VMB12 to the centre of Potato dextrose agar (PDA) medium in Petridish. All the experiments done in triplicates. The basal medium (PDA) without any phytoextract served ascontrol. All the inoculated petri plates were incubated at 25±1°C.The radial growth of the test fungus in the treated plates was measured in all treatments when the pathogen growth touched the periphery in the control Petridishes. The per cent inhibition of fungal growth was determined by using the formula given by Vincent (1927).

$$I = \frac{C - T}{C} \times 100$$

Where, I = per cent inhibition.

C = Colony diameter in control.

T = Colony diameter in treatment.

Results

Three medicinal plants Viz. Azadirachta indica, Ricinus communis and Pongamia pinnata were tested against Ceratocystis fimbriata VMB 12 bypoisoned food technique. In vitro evaluation of different medicinal plants extract Viz. aqueous, ethanolic and methanolic was taken for to test the antifungal evaluation.

Table 1: In Vitro evaluation of Antifungal Potential of Azadirachta indica against Ceratocystis fimbriata VMB12

Aqueous Extract													
Sr. No.	Plant used	Leaves			Bark			Stem			Root		
		Control	Treated	% Inhibition	Control	Treated	% Inhibition	Control	Treated	% Inhibition	Control	Treated	% Inhibition
1.	Azadirach-taindica	15.5	1.8	88.38	15.5	2.2	85.80	15.5	3.0	80.64	15.5	4.7	69.67
Ethanolic Extract													
1.	Azadirach-taindica	15.5	2.0	87.09	15.5	2.9	81.29	15.5	3.9	74.83	15.5	4.3	72.25
Methanolic Extract													
1.	Azadirach-taindica	15.5	2.4	84.51	15.5	2.4	84.51	15.5	3.4	78.06	15.5	3.8	50.96

Table 2: In Vitro Evaluation of Antifungal Potential of Ricinus communis against Ceratocystis fimbriata VMB12

Aqueous Extract													
Sr. No.	Plant used	Leaves			Bark			Stem			Root		
		Control	Treated	% Inhibition	Control	Treated	% Inhibition	Control	Treated	% Inhibition	Control	Treated	% Inhibition
1.	Ricinus-communis	15.5	2.2	85.80	15.5	3.4	78.06	15.5	3.5	77.41	15.5	5.2	66.45
Ethanolic Extract													
1.	Ricinu-scommunis	15.5	2.5	83.87	15.5	3.2	79.35	15.5	3.7	76.12	15.5	5.0	67.74
Methanolic Extract													
1.	Ricinus-communis	15.5	1.8	88.38	15.5	2.5	83.87	15.5	3.4	78.06	15.5	3.7	76.12

Table 3: In Vitro Evaluation of Antifungal Potential of Pongamia pinnata against Ceratocystis fimbriata VMB12

Aqueous Extract													
Sr. No.	Plant used	Leaves			Bark			Stem			Root		
		Control	Treated	% Inhibition	Control	Treated	% Inhibition	Control	Treated	% Inhibition	Control	Treated	% Inhibition
1.	Pongamia-pinnata	15.5	2.1	86.45	15.5	3.0	80.64	15.5	3.5	77.41	15.5	4.6	70.32
Ethanolic Extract													
1.	Pongamia-pinnata	15.5	2.5	83.87	15.5	3.0	80.64	15.5	3.1	80.00	15.5	3.7	76.12
Methanolic Extract													
1.	Pongamia-pinnata	15.5	2.2	85.80	15.5	3.0	80.64	15.5	3.5	77.41	15.5	3.8	75.48

Phylogenic Analysis of VMB12:

The phylogenetic tree was constructed by using Neighbour joining method by Kimura – 2 parameter with 1000 replicates in MEGA 4.0. According to the sequencing similarities an multiple alignments, the present isolate was identified. The sequence obtained of the present isolate have been deposited in DNA Databank of Japan (DDBJ) and accession number obtained (**Figure I)**.

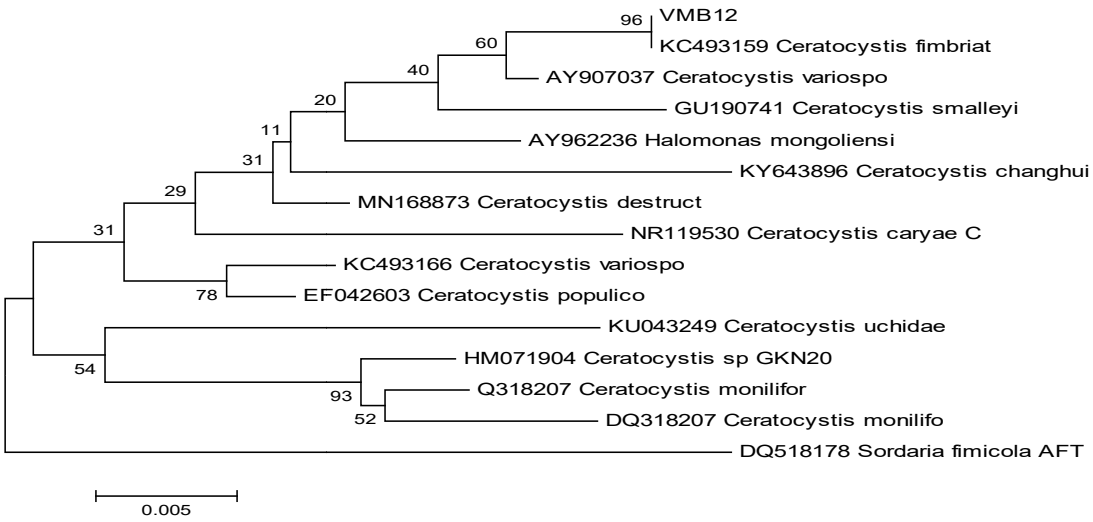

Figure I: Phylogenetic placement of VMB12 (Accession Number LC532385).

The gene sequences showing relationships among strain VMB12 and the closest type strain species of Ceratocystis. Numbers at nodes indicate percentage of bootstrap support based on a Neighbor-joining analysis of 1,000 resampled datasets. Bar 0.005 substitutions per nucleotide position.

In Vitro evaluation of Antifungal Potential of Some Medicinal Plants against Ceratocystis fimbriata VMB12

All the aqueous plant extract of three medicinal plants Viz.Azadirachta indica, Ricinus communis and Pongamia pinnata showed antifungal activities against the test fungal pathogen Ceratocystis fimbriata VMB12 with some showing a better antifungal activities than others. Whereas the control (only distilled water means without medical plant extract) of all the extracts were also taken for antifungal test evaluation against Ceratocystis fimbriata VMB12.

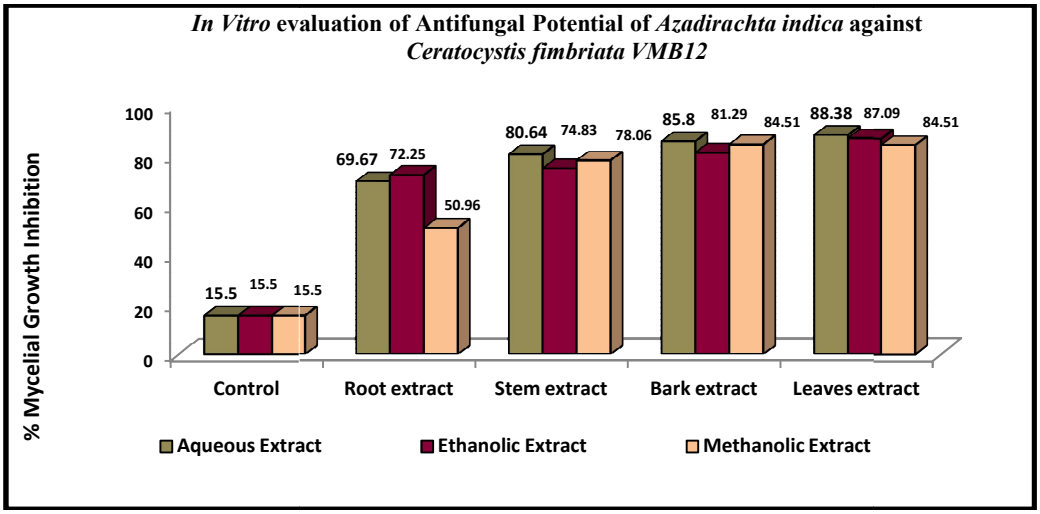

Figure II: In Vitro evaluation of Antifungal Potential of Azadirachta indica against Ceratocystis fimbriata VMB12

It is found from the **Figure II** that the promising fungal isolate VMB12 shows the largest percent inhibition of mycelial growth i.e. 88.38% to aqueous leaves extract of Azadirachta indica followed by bark extract, stem extract and root extract which is 85.80%,

80.64% and 69.67% respectively.Similarly, the promising fungal isolate VMB12 shows the largest percent inhibition of mycelial growth i.e. 87.09% to ethanolic leaves extract of Azadirachta indica followed by bark extract, stem extract and root extract which is 81.29%, 74.83% and 72.25% respectively. Further more,as it is evident from the **Figure II** that, the comparatively less percent inhibition of mycelial growth is observed in methanolic extract of Azadirachta indica i.e. 84.51%, 84.51%, 78.06% and 50.96% respectively.The percent inhibition of mycelial growth observed for control of three extract of Azadirachta indicais 15.5%.

It is found from the **Figure III** that the promising fungal isolate VMB12 shows the largest percent inhibition of mycelial growth i.e. 88.38% to methanolic leaves extract of Ricinus communis followed by bark extract, stem extract and root extract which is 83.87%, 78.06% and 76.12% respectively.

Similarly, the promising fungal isolate VMB12 shows the comparatively less percent inhibition of mycelial growth i.e. 85.80% to aqueous leaves extract of Ricinus communis followed by bark extract, stem extract and root extract which is 78.06%, 77.41% and 66.45% respectively. Furthermore, as it is evident from the **Figure III** that, the less percent inhibition of mycelial growth is observed in ethanolic extract of Ricinus communis i.e. 83.87%, 79.35%, 76.12% and 67.74% respectively. The percent inhibition of mycelial growth observed for control of three extract of Ricinus communis is also 15.5%.

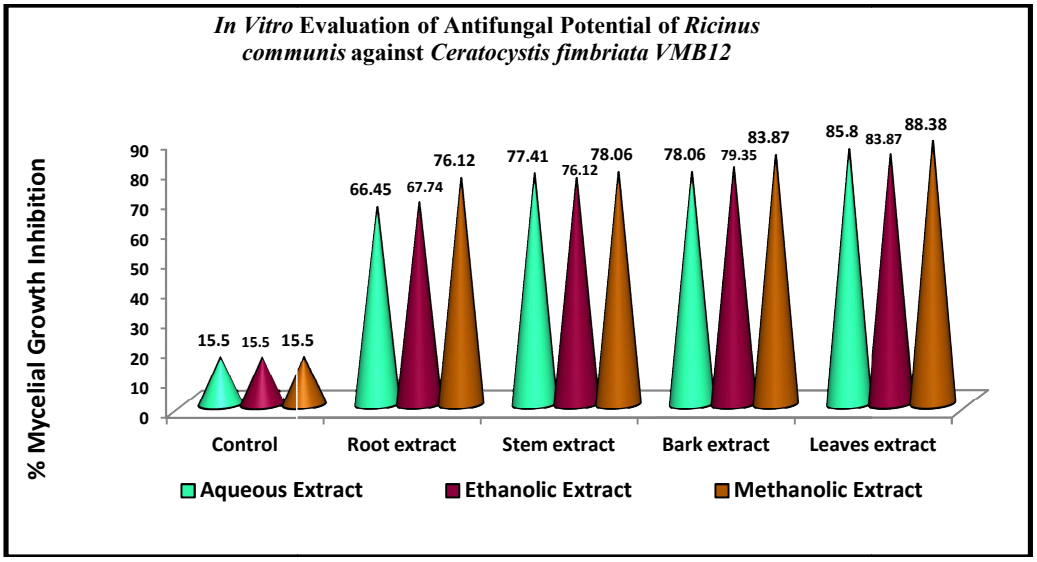

Figure III:In Vitro Evaluation of Antifungal Potential of Ricinus communis against Ceratocystis fimbriata VMB12

It is found from the **Figure IV** that the promising fungal isolate VMB12 shows the largest percent inhibition of mycelial growth i.e. 86.45% to aqueous leaves extract of Pongamia pinnata followed by bark extract, stem extract and root extract which is 80.64%, 77.41%, 70.32% respectively.

Similarly, the promising fungal isolate VMB12 shows the comparatively less percent inhibition of mycelial growth i.e. 85.80% to methanolic leaves extract of Pongamia pinnata followed by bark extract, stem extract and root extract which is 80.64%, 77.41% and 75.48% respectively. Furthermore, as it is evident from the **Figure IV** that, the less percent inhibition of mycelial growth is observed in ethanolic extract of Pongamia pinnata i.e. 83.87%, 80.64%, 80.00% and 76.12% respectively. The percent inhibition of mycelial growth observed for control of three extract of Pongamia pinnatais also 15.5%.

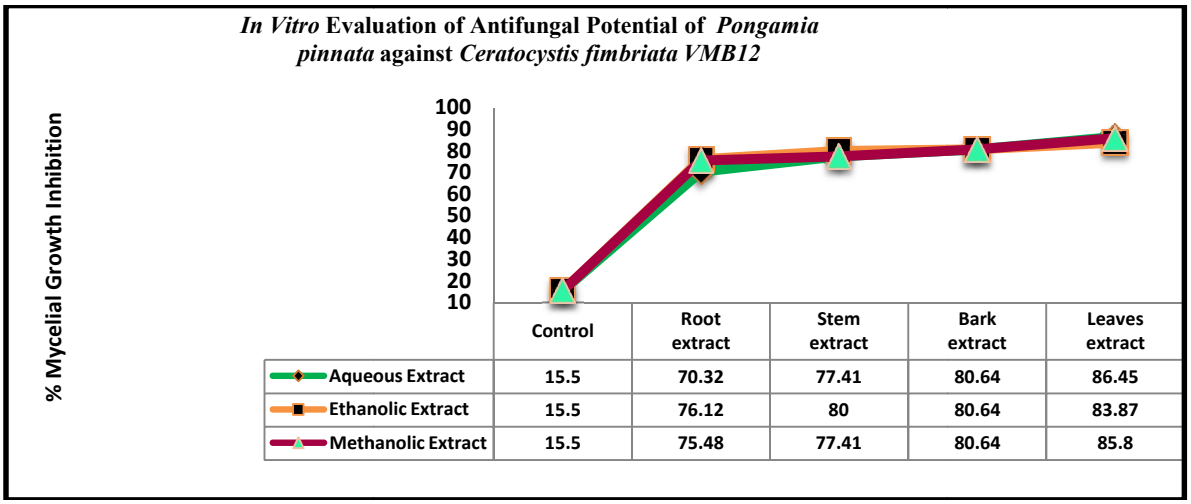

Figure IV:In Vitro Evaluation of Antifungal Potential of Pongamia pinnata against Ceratocystis fimbriata VMB12

All theextracts of leaves, bark, stem and root of Azadirachta indica, Ricinus communis and Pongamia pinnata provides preliminary information regarding its efficacy against a fungal pathogen within a shortest period of time and hence serve as guide for further field testing. Usually, all the medicinal plants under study showed significant increase in their inhibitory effect against the tested organism.From the result, the leaves extract has the highest activity than the bark, stem and root of the extract. The antifungal activityof the plant extract of Azadirachta indica, Ricinus communis and Pongamia pinnata differ according to the plant parts.

From the result it showed that the leaves extract of Azadirachta indica, Ricinus communis and Pongamia pinnata is more effective compared to the other extracts of parts of plant under study. In general, the extract Azadirachta indica show high activity compared to Ricinus communis and Pongamia pinnata.It was observed that the extracts showed strong activity on the fungus Ceratocystis fimbriata VMB12.

Discussions

The difference in the activities of the plant extract might be due to the fact that such types of variation might be due to the differences in the phytochemical composition of the medicinal plants which is influenced by the sever al environmental factors such as temperature, rainfall,vegetation and the climatic condition soil mineral(Dave Ganskopp and Dave Bohnert, 2003).The results of the present study is in strong agreement with the previous work reported by Mariod et al., (2010),in which they showed the methanolic leave extract of sclerocarya birrea have antimicrobial activities who further reported that the root of S. birrea inhibit the growth of Escherichia coli, Candida albican and Staphylococcus aureus.Although, all the methanolic plant extract of sclerocarya birrea from Yola and Kem showed varying degree of zone of inhibition.

Perumal et al., (2008) evaluated the antimicrobial activity of ethanol leaf extract of Azadirachta indica (Neem) and flower extracts of Nochi tested against phytopathogens, like Xanthomonas and Fusarium. However in the present study, the promising fungal isolate VMB12 shows the largest percent inhibition of mycelial growth i.e. 88.38% to aqueous leaves extract of Azadirachta indica.

Patel et al., (2015) in their study reported that the ethanol extracts of Azadirachta indica (Neem) and bio-control agent Trichoderma viride showed significant inhibitory effect at 50% concentration against phytopathovar of bacterial blight on pomegranate i.e. Xanthomonas axonopodis punicae,. Sukanya et al., (2009) demonstrated the methanol extracts of Chromolaena odorata having antimicrobial activity against Escherichia coli, Xanthomonas vesicatoria, Ralstonia solanacearum and Staphylococcus aureus by using agar disc

method in which diameter of inhibition zone of 10mm, 9mm, 12mm and 12mm respectively. The Minimum inhibitory concentration (MIC) value of these extract for these clinical bacteria were ranged between 0.35mg/ml to 4.0mg/ml and 0.25mg/ml to 4.0mg/ml for phytopathogenic bacteria.

Jamuna Bai et al., (2011) in their study evaluated antimicrobial activity of the crude methanol leaf extracts of Memecylon malabaricum Clarke, leaves extracts and flowers extracts of Cochlospermum religiosum Linn. and leaves extracts of Andrographis serpyllifolia Vahl. by using the standard disc diffusion assay against 08 strains of bacterial species, Viz. Xanthomonas axonopodis pv., Salmonella typhi, Staphylococcus aureus,Xanthomonas oryzae pv. oryzae, Entero bacter aerogenes, Pseudomonas aeruginosa, malvacearum, Micrococcus spp. and Bacillus cereus. The extracts of the plants at a concentration of 1.25mg / disc show ed minimum to moderate activity against both Gram positive and Gram negative bacteria which indicates a broad spectrum activity.

Hexane, Cow urine extract, ethyl acetate, chloroform, methanol, alcohol, aqueous fractions of Pongamia pinnata Linn.were effectively showed 10mm-13mm zone diameter of inhibition against Xanthomonas oryzae pv. Oryzae which is a causative agent of bacterial blightof paddy (Murugan et al., 2012) in the present study, the promising fungal isolate VMB12 shows the comparatively less percent inhibition of mycelial growth i.e. 85.80% to methanolic leaves extract of Pongamia pinnata followed by bark extract, stem extract and root extract which is 80.64%, 77.41% and 75.48% respectively.

Satish et al., (2007) reported that the aqueous extract of Datura stramonium, Acacia nilotica, Emblica officinalis, Achras zapota, Pelto phorum pterocarpum, Eucalyptus globules, Mimusops elengi, Prosopis juliflora, Polyalthia longifolia, Punica granatum, LawSonia inermis and Sygigium cumini have been recorded significant antifungal activity against one or the otherAspergillus species tested.

Results of our study coincidence with the works of Ouedrago et al., (2013) who stated that the stem back of Sterculia Setigera showed strong antifungal activity.

Conclusions

In Vitro studies showed that the plant extracts of Azadirachta indica, Ricinus communis and Pongamia pinnata showed significant antifungal activity against Ceratocystis fimbriata VMB12 of wilt in pomegranate. The Screening of various medicinal plants and their use in integrated disease management strategy is needed to manage wilt in pomegranate.The antimicrobial activities of the medicinal plant extract differ according to the plant part and location of the plants. The best activities of all the extract were observed. It was also observed that the extracts are more effective on the fungus under study.The outcome of present study would help farmers to employ an effective method by using indigenous medicinal plants for controlling wilt in pomegranate by cost effective and eco-friendly manner.

Acknowledgement

We would like to offer our sincere thanks to National Center for Cell Sciences, University of Pune Campus for the 18s rRNA sequencing of the isolate. Our sincere thanks to Principal, Lal Bahadur Shastri Mahavidyalaya, Department of Botany, Dharmabad, Nanded for providing the Laboratory facilities.and seven star friend group

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