In vitro Antifungal Activity of Aegle marmelos, Syzygium cumini and Pongamia pinnata Extracts against Fusarium oxysporum f. sp. cicero

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More, et al.: Antifungal Efficacy of Medicinal Plants Extracts against Fusarium oxysporum f. sp. ciceri

The present investigation was carried out using acetone, ethanol, methanol and chloroform extracts of different plants and bio agents against Fusarium oxysporum f. sp. cicero. Effect of plant extracts and bio agents alone and in combination on wilt incidence of chickpea was assessed using pot culture method. Methanol extracts of Aegle marmelos was found most active (88.64%) followed by methanol extract of Pongamia pinnata (85.30%). Ability of these four solvents to produce maximum extraction yield from four different plants was also evaluated. Methanol extracts of A. marmelos at concentrations of 250, 500, 750 and 1000 µl was found compatible with Trichoderma viride and Pseudomonas fluorescens. Methanol also gave the highest percent extraction yield. Seed treatment with P. fluorescens 10 g/kg seed+T. viride 4 g/kg seed+methanol extract of A. marmelos 4% proved effective to reduce incidence of chickpea wilt disease caused by F. oxysporum f. sp. cicero (69.31%).

Key words: Extracts, antifungal activity, A. marmelos, S. cumini, P. pinnata, F. oxysporum f. sp. cicero

Fungal diseases of crop plants have always been one of the major constraints in successful crop production, which cause severe yield loss every year. Injudicious use of synthetic fungicides for controlling plant diseases have given rise to negative effects on human and animal health and agro-ecosystem. Researchers continue to strive to develop alternatives to chemical fungicides. Eco-friendly methods involving plant products and biological agents, which act directly on the pathogens or indirectly by inducing resistance in plants, have gained considerable importance as an alternative to using synthetic fungicides[1].

Chemical fungicides pollute the environment, soil

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Accepted 14 April 2017
Revised 02 January 2017
Received 15 June 2016
Indian J Pharm Sci 2017;79(3):457-462
and water besides being deleterious to human health and environment, necessitating a continuous search for an environmentally safe and economically viable strategy to control the fungal diseases and to reduce the dependence on synthetic agrochemicals. Natural products or plant extracts have the potential to be employed as leads for synthesis of new agrochemicals or directly as fungicides, which inspired biochemists to develop new bioassays capable of detecting other, structurally simpler compounds with similar modes of action. Medicinal plants may thus prove to be a new source for antibacterial, antifungal and antiviral agents with significant activity against microorganisms. As the synthetic fungicides are toxic to plants and have the potential to enter food chain leading to bioaccumulation, the focus must be to develop eco-friendly and effective methods to control plant diseases, and one such approach is employing medicinal plants.

Plant metabolites and plant-based pesticides could prove to be a better alternative with minimal environment impact and safer in contrast to synthetic pesticides. Active principles from medicinal plants have been tried as replacements for synthetic fungicides in management of plant diseases in organic farming system. Plant extracts could have unique antimicrobial properties with a holistic mode of action. There have been reports in the literature that plant extracts and their constituents such as alkaloids, terpenoids, glycosides and phenolic acids possessed antimicrobial activity.

Phytochemical analysis of Aegle marmelos revealed the presence of alkaloids, saponins, tannins, flavonoids and furanocoumarins. Employing aqueous extracts of many allelopathic plants and utilization of microbial antagonists as the environmentally safe alternative methods for plant disease management appears to be the need of the hour for efficient integrated disease management strategies.

The wilt of chickpea incited by Fusarium oxysporum f. sp. ciceri is one of the serious diseases. This soil and seedborne pathogen causes profound losses (20 to 100%) depending upon phase of illness and wilting. A systematic investigation was undertaken to screen the antifungal activity and explore the possibilities of utilizing these for management of wilt of chickpea under laboratory conditions against F. oxysporum f. sp. ciceri.

A virulent isolate of F. oxysporum f. sp. ciceri isolated from wilt-infested chickpea plants was used in this investigation. Chickpea variety JG-62 was used as a host crop. Two antagonists viz., Trichoderma harzianum and Pseudomonas fluorescens isolated from soil sample were collected from department of plant pathology, Dr. P. D. K. Vidyapeeth, Akola. The experiment was conducted in vitro and under greenhouse conditions during 2015 in the department of plant pathology, Dr. P. D. K. Vidyapeeth, Akola. The sand sorghum medium (SSM) was used for the mass multiplication of F. oxysporum f. sp. ciceri in the laboratory.

Fresh leaves of A. marmelos, Syzygium cumini and Pongamia pinnata were collected from various places nearby the institution. Identification and authentication of the plants was carried out at Nagarjun Medicinal Plants Garden, Dr. P. D. K. Vidyapeeth, Akola, India. The fresh plant leaves collected were thoroughly washed under tap water first followed by distilled water to remove dirt and impurities, and shade-dried separately with occasional shifting for about 3 to 4 w. The dried leaves were coarsely powdered with a sample grinder and stored in airtight container until further use.

Acetone, ethanol, methanol and chloroform were used as solvents for preparing leaf extracts. Forty gram powder of each leaf was separated soaked in 200 ml of acetone, ethanol, methanol and chloroform in 500 ml conical flasks plugged tightly with cotton wrapped in paper. All conical flasks were kept on a rotary shaker for 4 d and allowed to stand for 5 h for the marc to settle. Supernatants from each flask were filtered separately through Whatman No. 1 filter paper and evaporated at room temperature. The marc was extracted three times to harvest maximum from the leaf powder. Air dried extracts were weighed separately and transferred into small vials and kept in the refrigerator at 5° until further use. The percent extraction yield was calculated.

The efficacy of acetone, ethanol, methanol and chloroform extracts of A. marmelos, S. cumini and P. pinnata at 250, 500, 750 and 1000 µl concentration were tested against F. oxysporum f. sp. ciceri under in vitro condition following poisoned food technique on potato dextrose agar (PDA). One gram crude extract of all plants extracted with acetone, ethanol, methanol and chloroform were diluted in 10 ml dimethyl sulphoxide (DMSO) separately and from this 250, 500, 750 and 1000 µl suspension were added to conical flasks containing 60 ml sterilized melted PDA medium sufficient for 3 plates. The conical flasks were shaken well for uniform mixing, the contents poured in
Compatibility was the effect of medicinal plant extracts on test pathogens. Initially, these bio agents were tested in vitro and the promising bio agents were then tested in pot experiment individually as well as in combination with plant extracts as seed treatment. Antagonistic activity of *P. fluorescens* and *T. viride* against *F. oxysporum f. sp. ciceri* causing wilt of chickpea. Observations regarding antagonistic effect of all these bio agents against test pathogens were recorded at 3, 5 and 7 d after inoculation. The growth inhibition of each fungal pathogen was calculated.

Present investigation was carried out to study the antagonistic activity of bio agent’s *P. fluorescens* and *T. viride* against *F. oxysporum f. sp. ciceri* causing wilt of chickpea. Initially, these bio agents were tested in vitro and the promising bio agents were then tested in pot experiment individually as well as in combination with plant extracts as seed treatment. Antagonistic activity of *P. fluorescens* and *T. viride* was studied using the dual culture technique on PDA plates. The inoculated plates were incubated at 28±2°C for 7 d for *F. oxysporum f. sp. ciceri*. Observations regarding antagonistic effect of all these bio agents against test pathogens were recorded at 3, 5 and 7 d after inoculation. The growth inhibition of each fungal pathogen was calculated.

Pot culture experiment were carried out for studying antagonistic activity of *P. fluorescens*, *T. viride* and *A. marmelos* methanol extract alone or in combination as seed treatment against *F. oxysporum f. sp. ciceri*. Chickpea JG-62 seeds were surface disinfected in 2% sodium hypochlorite for 30 s, rinsed in sterile distilled water and dried overnight. Ten seeds were planted per pot filled with sterilized potting soil (1.5 kg) and inoculated with sand:sorghum medium was added to each pot at 1:20 (w/w) ratio of pathogen to soil. In every treatment, the talc-based formulation of *T. harzianum* and *P. fluorescens* was applied as a seed treatment at 4 and 10 g/kg of seed, respectively. In marigold water extract treatment, seeds were soaked in 2, 3 and 4% solutions separately for 3 h and air-dried overnight before sowing and inoculated pots with the pathogen alone served as control. Three replications were maintained for each treatment in a factorial completely randomized design (FCRD) in a glasshouse. Incidence of wilt in chickpea was recorded at 30 and 60 d after sowing.

Treatment details are as follows, S1P1 (acetone extract of *A. marmelos*), S1P2 (acetone extract of *S. cumini*), S1P3 (acetone extract of *P. pinnata*), S2P1 (ethanol extract of *A. marmelos*), S2P2 (ethanol extract of *S. cumini*), S2P3 (ethanol extract of *P. pinnata*), S3P1 (methanol extract of *A. marmelos*), S3P2 (methanol extract of *S. cumini*), S3P3 (methanol extract of *P. pinnata*), S3P4 (methanol extract of *P. pinnata*), S4P1 (chloroform extract of *A. marmelos*), S4P2 (chloroform extract of *S. cumini*) and S4P3 (chloroform extract of *P. pinnata*).

In vitro effect of medicinal plant extracts on test pathogen was done by using FCRD with three factors having four levels in each factor. The data was analysed statistically following the method reported by Panse and Sukhatme[16]. "F" test was used to determine which treatment effects were significant. Standard error (SE) and critical difference (CD) at 1% level of probability were calculated.

The results revealed that all extracts tested at each concentration inhibited the growth of *F. oxysporum f. sp. ciceri*. The rate of inhibition of growth is found to be proportional to the concentrations of the plant extracts tested. Using these medicinal plant wastes as a raw material for plant-derived fungicides, it is possible to manage the disease while creating an economical use for the medicinal plant waste.

Results in Table 1 indicated that the extraction yield was significantly influenced by the extraction solvent chosen, which depended on the polarity and capacity of the solvent to extract chemical constituents. Methanol was found to be most suited for extraction of many substances that are soluble in greater yields from *A. marmelos* (7.04%), *S. cumini* (8.12%) and *P. pinnata* (6.81%).

It was observed from the data in Table 2 that *F. oxysporum f. sp. ciceri* was sensitive to all the tested bio agents but *P. fluorescens* extract produced maximum mycelial inhibition (81.59%). These results were similar to those reported by Trivedi and Rathi[17], Mohmed and El-Hadidy[18] that the *Trichoderma* species exhibited greater potential in managing chickpea wilt under glasshouse and field conditions. Selected isolates of *P. fluorescens* were found to be effective in reducing the wilt incidence and increasing the plant growth as well as grain yield of chickpea[19,20]. *P. fluorescens* has revolutionised the field of biological control of soil-
borne plant pathogenic fungi\textsuperscript{[21]} due to the fact that it was reported to contain phenazin\textsuperscript{[22]}, pyroluterin\textsuperscript{[23]} phloroglucinol\textsuperscript{[24]} and siderophores\textsuperscript{[25]}, which might be involved in the supersession of the wilt pathogen\textsuperscript{[26]}. Muneeb \textit{et al}.\textsuperscript{[27]} observed 84.79\% mycelial inhibition of the \textit{F. oxysporum f. sp. ciceri} by \textit{T. viride} under \textit{in vitro} condition.

Observations on interaction effect of solvents, leaves and concentrations on dry mycelial weight of \textit{F. oxysporum f. sp. ciceri} were recorded and percent inhibitions were determined and presented in Table 3. Statistically analysed results clearly indicated the antifungal activity of the methanol extract of \textit{A. marmelos} plant and its ability to control mycelial growth of \textit{F. oxysporum f. sp. ciceri}. At 250 µl (C1) concentration, S3P1 showed a maximum of 59.86\% inhibition of test fungus followed by 58.89, 58.80\% reduction in S3P3 and S2P1, respectively and all these interactions were at par with each other. Lowest inhibition observed in the interaction of S4P2 with the test fungus at 250 µl concentration (C1).

At 500 µl (C2) concentration, 79.81\% inhibition of the test fungus was observed in S3P1, followed by 76.42\%, 75.43\% inhibition by S3P3 and S2P1. An inhibition of 64.32\% was recorded in S4P2. At 750 µl (C3) concentration, 83.56\% inhibition of test fungus was occurred in S3P1, 82.11\% inhibition occurred in S3P3 and 68.51\% inhibition was recorded at the interaction with S4P2. At the highest concentration of 1000 µl, 88.64\% inhibition of mycelial growth of test fungus was recorded in S3P1, 85.30\% inhibition observed in interaction S3P3 inhibition of test fungus and 68.83\% and 70.98\% were recorded in interactions S4P2 and S4P3, respectively.

Ahanjan \textit{et al}.\textsuperscript{[28]} reported 40.00 and 42.38\% inhibition of mycelial growth of \textit{F. oxysporum} by aqueous and methanol extract of \textit{P. persica}, respectively at 500 ppm concentration. Abdel-Monain \textit{et al}.\textsuperscript{[29]} tested extracts including \textit{Calotropis procera}, which suppressed growth of \textit{F. oxysporum f. sp. lupini}. Effects of methanol extract of \textit{A. marmelos} and bio agents alone and in combination on chickpea wilt caused by \textit{F. oxysporum f. sp. ciceri} under pot experiment was studied in the present investigation. Observations on per cent seed germination and wilt incidence at 30 and 60 d was noted and results are presented in Table 4.

Results in Table 4 revealed that, T9 (\textit{P. fluorescens} 10 g/kg seed+\textit{T. viride} 4 g/kg seed+methanol extract of \textit{A. marmelos}) gave maximum seed germination (94.33\%), which was significantly greater than all the other treatments. Lowest percent germination (68.67\%) was reported in T10 (control). Minimum wilt incidence (14.33\%) at 30 d was exhibited in T9 (\textit{P. fluorescens} 10 g/kg seed+\textit{T. viride} 4 g/kg seed+methanol extract of \textit{A. marmelos}) followed by 22.00\% in T6 (\textit{P. fluorescens} 10 g/kg seed+\textit{T. viride} 4 g/kg seed) and T7 (\textit{P. fluorescens} 10 g/kg seed+\textit{T. viride} 4 g/kg seed+methanol extract of \textit{A. marmelos}). Maximum wilt incidence (59.67\%) at 30 d was observed in the control, followed by 41.33\% in T3 (methanol extract of \textit{A. marmelos} 2\%). Similar trends were recorded at 60 d. Percent disease reduction was calculated and was found higher in T9 (69.31\%).

Abdel-Monain \textit{et al}.\textsuperscript{[29]} reported that the water

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**TABLE 1: EFFECT OF DIFFERENT SOLVENTS ON PER CENT EXTRACTION YIELD FROM DRY WEIGHT OF LEAVES**

| Sample and local name | Extraction yield (%) in solvents |
|-----------------------|---------------------------------|
|                       | Acetone | Ethanol | Methanol | Chloroform |
| \textit{A. marmelos (Beal)} | 1.42     | 1.61   | 7.04    | 1.42       |
| \textit{S. cumini (Jamun)} | 3.01     | 2.82   | 8.12    | 1.61       |
| \textit{P. pinnata (Karanj)} | 2.63     | 3.81   | 6.81    | 1.45       |

**TABLE 2: EFFICACY OF BIO AGENTS ON MYCELIAL GROWTH OF F. OXYSPORUM F. SP. CICERI**

| Treatment | Radial mycelial growth (mm) | Per cent inhibition |
|-----------|-----------------------------|---------------------|
|           | \textit{F. oxysporum ciceri} | \textit{F. oxysporum ciceri} |
| \textit{T. viride} | 20.43                      | 77.30 (61.58)*       |
| \textit{P. fluorescens} | 16.57                      | 81.59 (64.68)       |
| Control   | 90.00                      | 0.00 (0.00)         |
| F test    | Sig                        | Sig                 |
| SE(M)±    | 0.72                       | 0.57                |
| CD at (P=0.01) | 2.83           | 2.21                |

*Figures in parenthesis are arc sin transformed values, average of five replications.
TABLE 3: EFFECT OF INTERACTION MEANS OF SOLVENTS X PLANTS CONCENTRATIONS

| S×P×C  | C1 (250 µl) | C2 (500 µl) | C3 (750 µl) | C4 (1000 µl) |
|--------|-------------|-------------|-------------|-------------|
| S1P1   | 58.63 (49.97)* | 73.22 (58.84) | 76.60 (61.07) | 78.78 (62.58) |
| S1P2   | 56.42 (48.69) | 67.67 (55.35) | 73.10 (58.76) | 74.34 (59.57) |
| S1P3   | 57.69 (49.42) | 70.97 (57.40) | 74.49 (61.00) | 79.75 (63.26) |
| S2P1   | 58.80 (50.07) | 75.43 (60.29) | 79.78 (63.28) | 82.14 (65.00) |
| S2P2   | 57.52 (49.32) | 71.91 (57.99) | 76.49 (61.00) | 79.75 (63.26) |
| S2P3   | 57.45 (49.28) | 70.78 (57.28) | 74.13 (59.43) | 78.79 (62.58) |
| S3P1   | 59.86 (50.69) | 79.81 (63.31) | 83.56 (66.09) | 88.64 (70.30) |
| S3P2   | 58.53 (49.9)  | 75.38 (60.25) | 79.80 (63.30) | 82.04 (64.92) |
| S3P3   | 58.89 (50.12) | 76.42 (60.95) | 82.11 (64.98) | 85.30 (67.45) |
| S4P1   | 57.68 (49.42) | 67.35 (55.15) | 69.72 (56.61) | 72.15 (58.15) |
| S4P2   | 55.40 (48.10) | 64.32 (53.32) | 68.51 (55.87) | 68.83 (56.06) |
| S4P3   | 57.41 (49.26) | 66.34 (54.54) | 68.68 (55.97) | 70.98 (57.40) |

| Control | 0.00 | 0.00 | 0.00 | 0.00 |

Source: S.E (M)± C.D. at (P=0.01)
Solvent (S): 0.04 0.17
Plants (P): 0.03 0.13
Concentrations (C): 0.08 0.33
Solvent×plants (S×P): 0.07 0.29
Solvent×concentrations (S×C): 0.08 0.33
Plants×concentrations (P×C): 0.07 0.29
Solvent×plants×concentrations (S×P×C): 0.14 0.54

*Figures in parenthesis are arc sin transformed values, average of three replications. Solvents (S): S1-acetone, S2-ethanol, S3-methanol, S4-chloroform. Plant leaves (P): P1-A. marmelos leaf extract, P2-S. cumini leaf extract, P3-P. pinnata leaf extract. Concentrations (C): C1-250 µl, C2-500 µl, C3-750 µl, C4-1000 µl

TABLE 4: EFFECT OF P. FLUORESCENS, T. VIRIDE AND METHANOL EXTRACT OF A. MARMELOS ALONE AND IN COMBINATION ON CHICKPEA WILT CAUSED BY F. OXYSPORUM F. SP. CICERI

| S. No. | Treatment | Germination (%) | Wilt (%) | % disease control |
|--------|-----------|----------------|---------|------------------|
| T1     | P. fluorescens alone (10 g/kg) | 83.33 (65.91)* | 24.00 (33.42)* | 56.70 |
| T2     | T. viride alone (4 g/kg) | 86.67 (68.58) | 30.33 (29.33) | 48.38 |
| T3     | Methanol extract of A. marmelos alone 2% | 73.67 (59.13) | 41.33 (40.01) | 37.18 |
| T4     | Methanol extract of A. marmelos alone 3% | 76.33 (60.89) | 39.33 (38.84) | 37.18 |
| T5     | Methanol extract of A. marmelos alone 4% | 81.00 (64.16) | 44.33 (39.6) | 37.18 |
| T6     | P. fluorescens (10 g/kg) + T. viride (4 g/kg) | 90.67 (72.21) | 22.00 (27.97) | 65.33 |
| T7     | P. fluorescens (10 g/kg) + methanol extract of A. marmelos 4% | 89.67 (71.25) | 22.00 (31.09) | 64.98 |
| T8     | T. viride (4 g/kg) + methanol extract of A. marmelos 4% | 92.33 (73.93) | 26.67 (27.97) | 58.48 |
| T9     | P. fluorescens (10 g/kg) + T. viride (4 g/kg) + methanol extract of A. marmelos 4% | 94.33 (76.23) | 14.33 (22.25) | 69.31 |
| T10    | Control (pathogen inoculated) | 68.67 (55.96) | 59.67 (50.57) | 0.00 |

SE (M)± 1.41 0.72 1.06 -
CD P=0.01 5.22 2.65 3.90 -

extracts of E. jambonala leaves, C. colocynthis fruits and N. oleander leaves resulted in highest reduction in disease severity caused by F. oxysporum f. sp. lupini. Results obtained showed that all the tested plant extracts suppressed growth of F. oxysporum f. sp. Cicero to different degrees. Also, methanol extracts inhibited growth of the pathogen more than the other solvent extracts. A. marmelos plant extract showed the highest effect in reducing radial growth of the pathogen compared to other extracts. Several higher plants have been found to possess inhibitory effects against mycelial growth of different phytopathogenic fungi[30].

The present study revealed that methanol extract of A. marmelos in poisoned food technique inhibited highest growth of test pathogen at the concentration of 1000 µl. Methanol was the best solvent for extraction of
antifungal constituents from the medicinal plant leaves. Methanol extract of *A. marmelos* exhibited superior activity against *F. oxysporum f. sp. ciceri*. *T. viride* and *P. fluorescens* were found to be effective against *F. oxysporum f. sp. ciceri*. *T. viride* and *P. fluorescens* were compatible with each other as well as with methanol extract of *A. marmelos*. In pot culture study, combined application of *T. viride*, *P. fluorescens* and the methanol extract of *A. marmelos* as seed treatment was found effective. Thus, chickpea wilt could be managed by the integration of various practices like, seed treatment with bio agents and plant extract. Further field experiments are required to investigate the *in vivo* effects of these medicinal plants extracts in comparison with some chemical fungicides for the management of wilt of chickpea.

**Conflict of interest:**
All authors declare no conflict of interests.

**Financial support:**
Nil.

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