Efficacy of organic and inorganic extracts of *Nerium indicum* against pathogenic fungi of *Aegle marmelos*

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Abstract: A bioassay was conducted to determine the potential of organic and inorganic solvents for antifungal activity against fungal pathogens. Among the organics were ethanol and methanol, whereas, aqueous was considered as the inorganic solvent for the bioassay. Extracts from leaf, shoot, and flowers were taken of *Nerium indicum* for the experiment. These extracts were tested against two fungal pathogens isolated from the medicinal plant *Aegle marmelos*. *Alternaria* and *Fusarium* species were the tested fungal isolates. Among the three solvents analyzed, ethanol showed higher antifungal activity compared to methanol and aqueous. However, all three solvents showed positive results against the isolated fungal genera. Aqueous showed the lowest effect compared to the three solvents. The zone of inhibition was measured for evaluations. Commercial fungicide fluconazole was considered as a control for comparative evaluations.

Keywords: antifungal, bioassay, inorganic, organic.

I. INTRODUCTION

The agriculture sector in the modern era has gained more importance due to advanced techniques and population increase. Due to these two factors, more pressure has been focused on rapid crop production and quality management. India is well known for its variety of edible products including cereals, pulses, vegetables, staples, and many more. A huge percentage of the Indian population is engaged in farming. In contrast, India is also well known for its 1.3 billion population. This encumbrance the farmers for regular crop production both qualitatively and quantitatively. Not only crops and vegetables, but a more valuable treasure of India is its medicinal plants. Though worldwide use of medicinal plants has been observed for the treatment of various diseases, India has a great history of Ayurveda and utilization of herbal medicines since ancient history [1]. Medicinal plants have a variety of active compounds that not only provide support to the plant itself but have great value to the environment as well. From a defensive nature towards insects, and pests to a stress-resistant nature including temperature, water, and nutrients, medicinal plants owe all [2,3,4,5]. Therefore, it is of prime importance to protect medicinal plants from serious environmental hazards including plant diseases caused by microbial communities. In the last few years, the use of chemical fertilizers including pesticides and fungicides has increased. The harsh effects of these chemicals not only destroy the plant's active compounds and nutrients but also impact the whole ecosystem [6]. [7] investigated the antifungal activity of plants including *Argemone mexicana* and *Parthenium hysterophorus.* The botanical extracts inhibited the growth of *Alternaria alternata* found in onion plants. The research showed a positive effect of the extracts on pathogen growth retardation [7]. Similarly, [8] studied the effect of the weed plant *Tridax procumbens* against the phytopathogen isolated from *Luffa acutangula*. Three solvents prepared in leaf extract revealed great antifungal activity [8]. Biocontrol is described as a natural way to suppress plant diseases. [9] gave a three-way mechanism of the host plant, pathogens, and biocontrol agents. This was described as a three-way interaction between the three components and portraying a balance between each other to suppress the diseases in a natural phenomenon [9].

II. STUDY LOCATION AND RESEARCH

Ajmer City is centrally located in the state of Rajasthan, India. The city is located north-eastern in the state and is the fifth-largest city in the population of Rajasthan. This beautiful city is well known for its beauty and nature due to its mountain surroundings known as Aravalli's. The Aravalli mountains along with the historical background make the city a major hub of botanical resources. The semi-arid climate provides a great opportunity for the growth of a vast

category of plants. A huge reservoir of medicinal plants can be found in the city which can be useful for several purposes including homeopathy, naturopathy, allopathy, and Ayurveda. *Aegle marmelos* (Rutaceae) is a traditional medicinal plant known for curing several diseases. The tree is indigenous to India but is found in several Asian countries including Pakistan, Nepal, Bangladesh, and many more [10]. This plant has been studied for the plant diseases associated and how botanical fungicides can be useful in eliminating the phytopathogens from it. For the natural fungicides, *Nerium indicum* (Apocynaceae) which is again a medicinal plant has been studied for its antifungal activity. *N. indicum* has been reported to have a pharmacological nature by [11]. Similarly, the plant is known to have several phytochemical constituents including alkaloids, phenols, flavonoids, and others that structure it as a great antimicrobial agent [12].

The objective of the research focuses on the botanical fungicides prepared by organic and inorganic solvents. Two medicinal plants have been studied for plant microbial disease and antifungal activity. Ethanol and methanol as organic and aqueous as the inorganic solvent have been determined for the bioassay. The in-vitro study was conducted which was not only natural but a cheaper, ecofriendly, and time-consuming technique to minimize the use of harsh chemicals on plants and utilize the medicinal properties of plants for the amelioration of the environment.

III. MATERIAL AND METHODS

i. COLLECTION AREA: Ajmer City has been considered the prime location for the collection of infected and curing medicinal plants. The collection has been conducted at five random places in the city for better evaluation and analysis. The infected diseased plant parts of *Aegle marmelos* were collected from summer to winter (May-December). During this period, several adverse conditions including extremely hot and rainy seasons, make the plant more exposed to the pathogens. This is the best time for the growth of several microorganisms, specifically in plants. The plant selected for biocontrol was *Nerium indicum*. The plant was collected from S.P.C. Government College, Ajmer, India.

ii. MICROSCOPY: The plant materials were studied primarily for microscopic examination. Small fine sections were stained in 0.05% (w/v) of toluidine solution for a few minutes [13]. Water was applied for excess stain removal. The stained plant parts were examined for primary identification of fungal pathogens.

iii. CULTURE, ISOLATION, AND PURIFICATION: A potato dextrose agar medium in sterile Petri plates was prepared for inoculation of sterile plant materials. Antibiotic streptomycin (50mg/L) was used for the growth prevention of unwanted microorganisms. The test plant materials were inoculated in PDA + A and incubated for 4-5 days at 25±2°C. The Petri plates were regularly checked for any contamination and required pathogen growth. The isolated fungal strains were isolated and identified. Sub-culturing was done for purification. The fungal genera were identified according to their microscopic and macroscopic characters [14,15,16,17,18,19,20]. The two fungal isolates identified were *Alternaria* and *Fusarium* species. For the antifungal bioassay, the mycelium of each fungus was inoculated in Potato dextrose media (absence of agar, PD + A). This process was done 6-7 days before the final day for quick multiplication of the mycelium without solidification. This will allow the pathogenic fungus to grow and stay in liquid form for the bioassay.

iv. ORGANIC AND INORGANIC EXTRACTS: Leaves, stems, and flowers of *N. indicum* were collected randomly from the campus area of S.P.C. Government College, Ajmer, Rajasthan, India (Fig. 1). The plant parts were aseptically collected in sterilized zip-lock bags and processed. Thorough washing, cleaning, and drying were done. The plant parts were weighed 25 g each and soaked in respective three solvents (95% ethanol, 95% methanol, and autoclaved distilled water). 100 ml of each solvent was measured, and the plants were soaked overnight. The overnight soaked plant parts were crushed in mortar and pestle and filtered through sterilized Whatman's paper. The extract thus obtained was then centrifuged for 15 minutes and the clear supernatant was obtained. The supernatant obtained at the end was stored in a

sterilized bottle in a refrigerator at 6°C until needed [21,22,23].

Fig. 1. *Nerium indicum*. Shoot, leaves, and flowers.

v. DISC DIFFUSION ASSAY: The disc diffusion method was implemented for the antifungal bioassay [24]. The paper disc (Whatman filter paper) of 5 mm was made of equal size with a sterilized cork borer for all the treatments. They were dry autoclaved before final use to prevent unwanted microbial contamination. Around 50 discs were soaked individually in each solvent for 72 hours. Later, for about 10-15 minutes, the disc was dipped and dried in respective solvents for complete impregnation of plant extracts. Similar steps were followed for commercial fungicide, Matco (Metalaxyl 8%, Mancozeb 54% WP; systemic and contact fungicide, Indofil Chemicals Company, Mumbai, India) considered as controls. The disc when changed in its colors marks the impregnation stage complete. The discs were stored separately in sterilized Petri dishes with labels and covered with aluminum foils until final usage. They were kept in the refrigerator at 6°C.

vi. BIOASSAY:

a) Day one : For the antifungal bioassay, Czapek dox agar media (CDA) was prepared. Streptomycin was used as an antibiotic for the prevention of unhealthy microorganisms' growth. CDA + A was poured in 100×15 mm and allowed to solidify. Later, the bottom of the Petri plates was marked with a point for the placement of the impregnated botanical disc. The Petri plates were carefully stored in the refrigerator.

b) Day two: The fungal broth of individual pathogens was spread evenly on the Petri plates in a clock and anti-clockwise direction with the help of autoclaved cotton buds (7.6 x 30.5 x 7.6 cm). The cotton buds allow the homogenized spread of the microbes. The botanical disc of individual solvents was placed at the center of each Petri plate. A similar procedure was followed for controls. Each treatment was set in triplicates for result analysis. The Petri plates were incubated for 6-8 days at 25±2°C.

vii. STATISTICAL ANALYSIS: Colony diameter in millimeters of each treatment was studied for evaluation. The zone of inhibition was determined, and the average was taken [21,25,26]. Analysis of Variance (ANOVA) was used to analyze the significant difference of each treatment. Moreover, Tukey's HSD test was conducted to analyze the significant difference among all the treatments tested with $P<0.05$ level of significance [27]. The statistical data were analyzed by SPSS software.

IV. RESULTS

Among the three organic and inorganic solvents examined, ethanol showed the highest impact against the two isolated fungal pathogens (Fig. 2, 3). Fig. 1 shows the graphical representation where *Alternaria spp*, shoot, and flower extracts show the highest response with an average of 26±2 mm and 23±3 mm respectively against the pathogen. However, leaf extract also showed a positive response with an average of 17±2 mm zone of inhibition. However, with ethanol extracts against *Fusarium spp*, leaf extract showed remarkably the greatest effect with an average of 28±2 in comparison to stem and flower with 22±2 and 18±2 respectively (Table 1). The response of all three plant parts in ethanol extract showed a much stronger effect as an antifungal substitute against the selected microbial community.

The second effective inhibitory activity was exhibited by botanical extracts prepared in a methanol solvent. Contrary to ethanol results, flower extract showed the highest impact with an average of 20±1 and 25±0 mm colony diameter against *Alternaria spp.* and *Fusarium spp.* respectively. The least inhibitory zone was 14±0 mm of stem extract against *Alternaria spp.* (Table 1). In comparison to the organic and inorganic solvents for antifungal activity, aqueous as an inorganic solvent showed the least response towards the growth of the isolated fungal strains. This result can be supported by one negative result of flower extract prepared in an

aqueous solvent against *Fusarium spp*. However, the same extract showed a positive response to the growth retardation of *Alternaria spp.* (Fig. 3). Leaf extract in aqueous showed a consistent average measurement of 20±0 mm and 20±3 mm for both pathogens.

A significant difference (P<0.05, ANOVA) was observed among maximum botanical extracts compared to the commercial fungicide used as control. The highest inhibitory zone formed by the control was an average of 13±3 mm against *Fusarium spp*.

Fig. 4 shows the comparative pie chart of all the three solvents used for the bioassay. Among the three, ethanol showed a 38% mean inhibitory activity compared to methanol and aqueous with 34% and 28% respectively. This shows a huge gap between the organic solvent ethanol and the inorganic solvent aqueous. These results were shown against *Alternaria spp.* however, for *Fusarium spp*. the results are quite transparent. Both the organic solvents (ethanol and methanol) showed similar inhibitory activity of 35% against an inorganic solvent (aqueous) of 30% (Fig. 4). The results show the efficacy of organic solvents against isolated pathogens of *Aegle marmelos* to be more effective in comparison to the inorganic solvent.

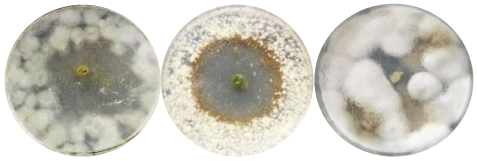


Fig. 2. Antifungal bioassay. Growth retardation of the fungal pathogen by botanical extracts against fungal pathogens. A. infected plant parts of *Aegle marmelos*. B. treatment of *Fusarium spp* C. treatment of *Alternaria spp*. D. control (commercial fungicide).

V. DISCUSSION

Metabolites play a very important role in the functioning and processing of plants. These metabolites are broadly categorized into two including primary and secondary. Primary metabolites are required necessarily for plant growth and development whereas secondary plays a role in the hormonal actions and interaction of the plant with the environment [28]. Research shows the role of secondary metabolites to be positive for growth inhibition of fungal mycelium [29]. Several secondary bioactive compounds including alkaloids, phenols, terpenoids, flavonoids, and several others contribute to the inhibitory potential of the plants. [30], researched the antibacterial activity of *Nerium indicum*. Three plant parts including leaf, root, and bark prepared in benzene, alcohol, and chloroform showed antibacterial potential against *Bacillus subtilis*, *Staphylococcus aureus*, and several others. Phytochemical analysis was also done which showed the presence of alkaloids, terpenoids, tannins, carbohydrates, saponins, and cardiac glycosides. These data were supported by another phytochemical research conducted by [12] on *Nerium indicum*. Based on investigations, the leaf, shoot, and flower of *N indicum* are a rich source of secondary metabolites including alkaloids, flavonoids, terpenoids, carbohydrates, and tannins [30,12,31,32]. The presence of these secondary compounds might have the ability to inhibit the growth of isolated phytopathogens. Flavonoids and phenols are known to possess an effect based on their organic structure. The presence of hydroxy or methyl group might have a greater contribution as antifungal potential. These two metabolites retard the cellular functioning of the pathogens and restrain the enzymatic activity. Additionally, alkaloids promote major changes to the cells and their components like mitochondria and membranes which subsequently damage the growth of the fungal mycelium [29,33,34]. These data support our findings of plant parts of *N. indicum* showing antifungal activity in different solvents against the isolated phytopathogens.

Table 1. Effect of organic and inorganic solvents prepared with plant parts of *N. indicum* against *Alternaria* and *Fusarium spp.* isolated from *A. marmelos*. Colony diameter in millimeters was studied for final evaluations.

Solvent	Concentration (mg/ml)	Test fungi with plant parts					
		Colony Diameter (mm)					
		Alternaria			*Fusarium*		
		Leaf	Shoot	Flower	Leaf	Shoot	Flower
Ethanol	100	17±2	26±2	23±3	28±2	22±2	18±2
Methanol	100	18±0	14±0	20±1	19±2	18±2	25±0
Aqueous	100	20±0	17±1	0±0	20±3	22±2	11±1
Control	100	10±3	13±1	5±0	13±3	6±0	7±1

*N=9

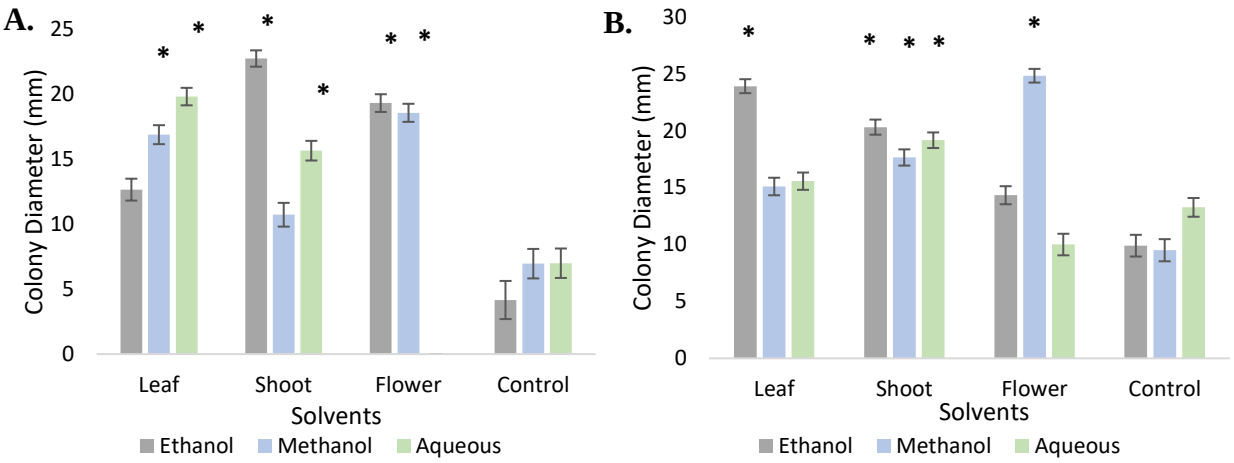
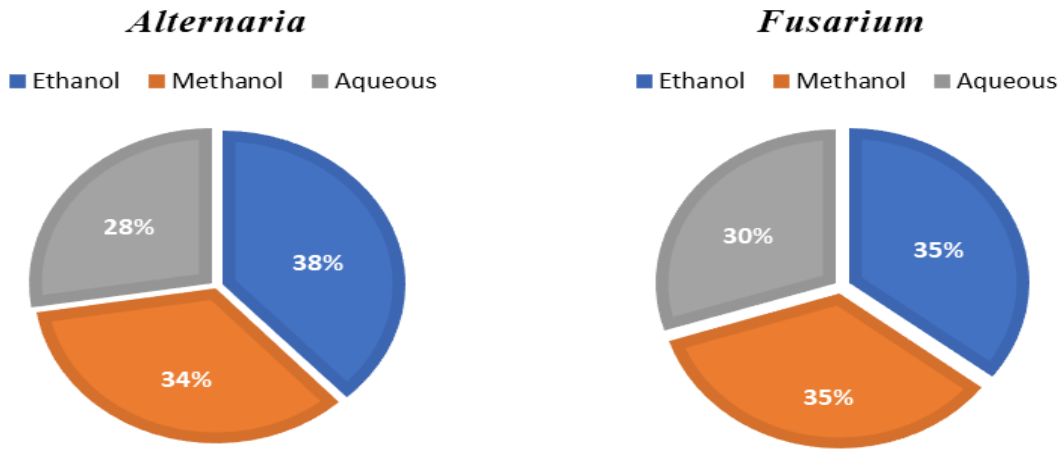


Fig. 3. Effect of organic (ethanol and methanol) and inorganic (aqueous) solvents prepared with plant parts (leaf, shoot, and flower) of *Aegle marmelos* against phytopathogen A. *Alternaria spp.* B. *Fusarium spp*. Commercial fungicide (Matco) was considered as a control. * indicates $P<0.05$ based on ANOVA and Tukey's HSD test. The values are Mean ± SE of triplicates ($P<0.05$).



A B

Fig. 4. Effect of different solvents on fungal pathogens. A. *Alternaria*. B. *Fusarium*. A combined average of the three plant parts (leaf, shoot, and flower) has been taken as a percentage for evaluation. The percentage indicates the effect of the respective solvent on the growth of the fungus.

I. CONCLUSION

Botanical extracts have been proven to be highly important in the agriculture sector due to their environment-friendly and chemical-free nature. They can be so far seen to be the best alternative to the chemicals possessing harm not only to humans, but to the whole ecosystem including animals, plants, food webs, soils, and all the living and non-living entities. This research shows the investigation results of organic and inorganic solvents as antifungal agents. Ethanol, methanol, and aqueous have been tested against the phytopathogens isolated from the medicinal plant *A. marmelos*. All three solvents have shown great results for the growth inhibition of fungal mycelium. Moreover, all three organic and inorganic botanical extracts have shown a significant difference in comparison to the selected commercial fungicide. Future research is recommended for the implementation of the botanical extracts on other plant diseases and practical implementation at the commercial level. This research could play a vital role in crop maintenance and quality improvement.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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