EVALUATION OF ANTIMYCOTIC ACTIVITY OF Eucalyptus globulus, Datura stramonium AND Tagetes patula AGAINST THREE ECONOMICALLY IMPORTANT PLANT PATHOGENS

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ABSTRACT
The use of chemical pesticides poses a serious risk to health as well as the environment. Studies showing the hazardous effects of conventional pesticides led to a ban on several pesticides while also indicating the need of exploring unconventional options. Biopesticides have proved to be one such viable option as they are inherently less toxic than conventional pesticides. They are also more target specific, effective in much smaller quantities and quick to decompose leading to lower exposures and lesser pollution problems with high yield. Extracts from plants having biocidal properties can be developed, tested for proliferation of plants pathogens and can further be developed as bio-pesticides. Therefore, we undertook a comparative study of extracts from three plants Eucalyptus globules, Datura stramonium, Tagetes patula along with Azadirachta indica as a control due to its proven biocidal properties. We further chose three fungal plant pathogens (Fusarium oxysporium, Macrophomina phaseolina and Sclerotium rolfsii) that cause considerable economic losses to crop plants to be challenged with the plant extracts. In vitro bioassays (ring test and dry weight analysis) were performed where individual plant pathogen was challenged with a known amount (ppm) of all four biocidal plant extracts respectively. Both from the ring test and dry weight analysis, it was observed that eucalyptus extract has more biocidalability than neem and was more effective in controlling the growth of both M. phaseolina and F. oxysporium. The difference in biocidal efficiency of the two oils was more than 50% viz. (dry weight of pathogen when challenged with neem was 0.241 gm whereas eucalyptus brought down the dry weight to 0.112gm). There was also marked difference in the extent of growth (measured as radial growth diameter) and sporulation in pathogen when challenged with different plant extracts and eucalyptus gave uniformly better results amongst all four. Our study reports the effectiveness of Tagetes for the first time for its antimycotic nature. Further experiments are underway to determine the dosage and time of exposure of these plant extracts that would help in developing new phyto-biopesticides.

Key Words: Phyto-biopesticides, Antimycotic, Biocidal, Macrophomina, Fusarium

INTRODUCTION
Advancements in agriculture technologies are resulting in increased crop production at a significant rate to fulfil the growing requirements of human population. Nonetheless with these advancements more and more chemical inputs are added to agriculture soils which have been a cause of major concern. Various disadvantages associated with the use of chemical pesticides include genetic variations in plant populations, shift in soil microbial diversity, reduction in plant beneficial microbes, toxicity/pollution to the environment/water bodies, migration of toxins to food chain and adverse effects on human health. These detrimental effects arise mainly due to the persistence of pesticide residues present in agricultural commodities. Equally worrying is the development of resistance in pests to pesticides. Use of biopesticides can prove to be a safer solution towards this major distress and lessen the pesticide risk. Biopesticides are materials or products of plants (neem), microbes (Bacillus thuringiensis, Trichoderma, nuclearpoly hydrosis virus etc.), biochemical compounds (chitosin, pheromones, fatty acids, potassium bicarbonate, plant growth regulators...
etc.), or plant incorporated-protestants (Bt pesticidal protein) valued for their pesticide properties. An estimated total of 3,000 tons/year of biopesticides are produced all over the world. Phytopesticide materials range from whole fresh plants to isolated pure bioactive phytochemicals or their formulations which are effective against pests and pathogens. The use of plant derivatives for pest control was common in the tropics before the advent of synthetic pesticides where use of neem cake and other neem derived products was a tradition in practice. In a survey it has been found that 34 plant species belonging to 18 families are used in traditional agriculture practices for pest management in South Uganda. Also botanical products have been considered imminent in ancient China, Greece and India. United States and some European countries predominantly used botanical insecticides before the innovation of chlorinated hydrocarbons and organophosphorous insecticides in late 1930s. Presently plants with insecticidal properties are reported from the families Rutaceae, Lamiaceae, Meliaceae, Asteraceae, Annonaceae, Malvaceae and Labiatae. In traditional agriculture different preparations and parts of plants were used like dust, oils, powdered seeds, roots, leaves and stems etc. Concentrated water or organic extracts of insecticidal component of plants were also proposed and were reported to be more effective in their action. The biocidal nature of these plants is due to the presence of some bioactive compounds or secondary metabolites like terpinoids, phenolics, alkaloids etc. However most of the plant derived pesticides were targeted towards controlling insect pests. For instance, himalayan cedar wood oil (Cedrus deodara) to control the pulse be etle, housefly, mosquitoes, Neem (Azadirachta indica) against white flies, aphids, various caterpillars etc. Though leaves of Allium sativum L., Artemisia annua L. and Bidens pilosa L., bark of Eucalyptus globulus, Delbergia saxatilis, Clausena anisata etc. have been studied for their biocidal properties, less emphasis was laid on using phytopesticides to control fungal diseases in crop plants. Pathogenic fungi rank second only to insects as a cause of plants diseases and results in heavy loss in the yield of major food and cash crops (20% reduction). Fungal diseases like leaf spot, root wilt etc., cause serious damage to plant life. Various fungi like Phyllosticta musarum, Guignardia musae, Mycosphaerella fijiensis, Fusarium mangiferae and other Fusarium spp., Puccinia psidii severely affect cash crops like banana and mango. Though some experiments are available in literature showing that extracts of some higher plants exhibit antifungal properties under laboratory trails not much work has been done to test their effect on fungal growth. More-over other than neem and its derivatives not many commercial formulations exist with any other plant species for controlling fungal diseases in crop plants in modern agriculture systems. This presents a very good opportunity to go back to examples from traditional agriculture practice select most efficient plants to derive phytop-esticides and develop them as commercial form-ulations for controlling fungal pathogens.

**Selection of plants with biocidal properties**

Neem (Azadirachta indica Family Meliaceae) is a genus of tall, evergreen and magnificent trees cultivated world over. Neem oil has been docum-mented to possess biocidal properties attributed to their secondary metabolites triterpenoids and non-terpenoids. Also neem was reported to show efficacy against a broad range of pests minimal impact on pollinators minimal natural enemies and mammalian toxicity and rapid disappearance from the environment. Most importantly neem has been reported to control a variety of fungus by its action. Thus neem was taken as a reference isolate for our experiments. Three test plants which we have selected have some evidences regarding their biocidal activities and hence included in our selection.

Eucalyptus (Eucalyptus globules Family : Myrt-aceae) is a woody perennial tree native of Austra-lia but grown in many tropical and subtropical countries. The essential oils extracted from Euc-alyptus were reported to possess a wide spectrum of biological activity including antimicrobial, fungicidal, insecticidal/insect repellent, herbici-dal, acaricidal and nematocidal properties. There are some studies where the active role of Eucalyptus oils from few species (E. Gran-dis, E. camaldulensis and E. citriodora) was also checked against certain phyto pathogenic fungi and found effective. Datura (D. stramonium Family : Solanaceae) is a woody annual plant and is known to contain tropane alkaloids such as hyoscyamine, scopolamine and atropine in seeds and flowers. Datura is reported to have antimicrobial activity. Not much work has been done on controlling plant pathogens using Datura and hence not much information exists for its range of efficacy as a
biopesticide. This motivated our research to look for biocidal properties of this plant against the selected detrimental fungi. Marigold (Tagetes sp. Family: Asteraceae) a commonly occurring plant all over the world and a commercially important horticulture plant for India. The foliar parts of this plant possess essential oils known for antibacterial and insecticidal properties. Thiophenes from Tagetes have a marked biocidal activity due to its photodynamic activation acting as antibiotics, insecticides, nematicides and fungicides.17 Due to the high degree of chemodiversity observed within essential oils of Tagetes sp., biological activities also vary. However even though various properties of the Tagetes plant are well known less attention was devoted to study biocidal activity of these in controlling important phytopathogens.18 Therefore the present work was aimed at developing an antifungal formulation from tagetes flower extracts.

**Fungal pathogens included in the study**

*Fusarium oxysporium*: A fungus belonging to Phylum Ascomycota, it is abundantly present in soil and organic matter worldwide.19 formae speciales (F. sp.) of *Fusarium* (specialized pathogenic forms of this fungus) are responsible for causing diseases such as vascular wilt, corm rot, root rot or damping-off in a very broad range of hosts at the species level.20, 21 It is responsible for huge economic losses caused by severe vascular wilt in tomato banana and also damages many crops from the Solanaceae family (including potato and pepper) and other commercially important plants.22,23 Use of resistant varieties soil sterilization and mixing of seedlings with chemical fungicides are used as effective strategies against *Fusarium* wilt but as the application of chemical fungicides induces other problems such as environmental concerns and toxicity issues public attention is focused on alternative methods of pest control such as biopesticides which are less harmful than conventional pesticides.24 *Macrophomina phaseolina*: An anamorphic *Ascomycete* of the family Botryosphaeriaceae, it is one of the most destructive plant pathogens in the tropics and subtropics, causing diseases in a wide range of host plants with the most common diseases being charcoal rot, damping-off, dry rot, wilt, leaf blight and ashy stem blight.25,26 It is primarily a root inhabiting fungus and produces tuber or cushion shaped black sclerotia.27 Its hosts include more than 500 plant species including several crops of economic importance such as legumes, vegetables, sunflowers, cow pea, *Phaseolus vulgaris*, soybean, sesame, olives, sorghum and effects fiber yield of jute.28-30 Disease management strategies for this fungus include seed treatment and genetic host resis tance.31 Further use of chemicals like carba-mate and fungicides like vitavax 200 and azadirachtin are also effective in controlling its growth and sclerotial survival.32 *Sclerotium rolfsii*. It is a ubiquitously present polyphagous, omnivorous and very destructive soil borne fungus of phylum Basidiomycota. It infects several plant parts from the seedling stage to the fruit stage. It causes diseases in more than 500 plant species including peanut, potato, tomato, chilli and also many woody ornamentals herbaceous annuals and perennials. A potential threat to groundnut, it also causes up to 95.0% mortality in chickpea seedlings. Disease management strategies involve practices such as isolation of infected plants, removal of infected soil and plants, crop rotation and use of resistant varieties. Sclerotium rolfsii infections 33-36. However control efforts have often met with limited success due to the broad range of hosts and prolific growth of this fungus and its ability to form persistent sclerotia. Hence this provides an ample reason to look for more safe and effective biotechnological alternatives like phytosticidals to control this pathogen and hence included in our study. Therefore we undertook a comparative study of extracts from three test plants: *Eucalyptus globulus, Daturastrum monium* and *Tagetes patula* along with *Azadira chat indica* as a reference isolate due to its proven biocidal properties. We tested the efficacy of these plant extracts against three highly damaging fungal plant pathogens: *Fusariumoxysporium, Macrophomina phaseolina* and *Sclerotium rolfsii*. Different quantitative and qualitative assays have been conducted using organic extracts and oils of these plant species to check their activity on the above mentioned fungi. Observation from this experimental study may form the basis for devising more effective phytosticidal formulations that are safe and economical.
MATERIAL AND METHODS

We analysed the biocidal activity of *Eucalyptus globulus*, *Datura stramonium*, *Tagetes patula* and *Azadirachta indica* extracts against the three fungal isolates by means of ring test assay and dry weight analysis.

**Biocidal fractions used**

Ethanolic extracts of *Eucalyptus globules*, *Da-tura stramonium*, *Tagetes patula*, *Azadiracht a indica* in addition to neem and eucalyptus oil.

**Preparation of plant extracts**

Plant parts (Petals of *Tagetes* plant and leaves of neem and *Datura*) were dried and powdered. Ethanolic extracts were prepared using the dried plant material following the standard protocol. All extracts / oils tested in this study were applied as parts per million (ppm) concentration (e.g. bioactive fraction in mg suspended in known volume in (ml) of solvent).

**Maintaining fungal cultures**

Strains of the three fungal species were obtained from the Central Research Institute for Dryland Agriculture (CRIDA) Hyderabad, India. The fungal isolates were grown at 30°C on Potato Dextrose Agar (PDA). Mycelia plugs were cut from the growing edges of the fungal culture using a sterilized cork borer after one week of incubation at 30°C. The radial growth of the fungi was measured periodically and radial growth achieved by different fungi was measured after 7 days of incubation in case of *Fusarium* and *Sclerotium* spp. and 4 days for *Macrophomina* spp. and tabulated in (Table 1). Fungal plugs from such actively growing culture plates were then inoculated onto fresh PDA plates in all the following experiments and the radial growth was measured.

<table>
<thead>
<tr>
<th>S/N</th>
<th>Fungus</th>
<th>Mean area of growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Macrophomina phaseolina</td>
<td>50.64 cm sq</td>
</tr>
<tr>
<td>2</td>
<td>Sclerotium rolfsii</td>
<td>15.63 cm sq</td>
</tr>
<tr>
<td>3</td>
<td>Fusarium oxysporum</td>
<td>7.272 cm sq</td>
</tr>
</tbody>
</table>

**Dry weight analysis**

Fungal plugs were added to conical flasks containing 25ml of Potato Dextrose Broth (PDB). Three different concentrations of extracts/oils were then pipette into these flasks (100 ppm, 250 ppm and 500 ppm). The flasks were then incubated at 30°C in a shaker incubator. After 7 days the fungus containing media was filtered using sterilized Whatman filter paper. The filter paper is then oven-dried again and weighed till the constant weight is achieved. The difference in weights of the filter paper (e.g. initial and post drying weight is taken as the fungal dried biomass).

**Ring test for determination of biocidal activity**

Antifungal activity was measured by a quantitative ring test assay. The method is a simple effective and quantitative method where there is a provision to measure and compare various concentrations of a plant extract in exactly similar setup. The procedure for ring test is represented as a pictogram in Fig. 1.

![Fig. 1](image-url)
Fresh PDA plates were indented using sterilized hot metal loops to create ring-shaped grooves. Fungal plugs were taken from the tips of the growing mycelia on the fungal culture plates using a sterilized cork borer. They were inoculated at the centre of the ring-shaped grooves using sterilized toothpicks. Extracts/oils in increasing concentrations (100 ppm, 250 ppm and 500 ppm) were pipette into the grooves. Control plates were made by filling the grooves with the different concentrations (100 ppm, 250 ppm and 500 ppm) of the respective solvent in which the extract has been prepared. The petri plates were then incubated at 30°C for a week. All assays for antifungal activity were carried out at least in triplicate. After 7 days the radial growth of the fungi was measured and mean area of growth from the triplicates was taken at all the 3 different concentrations.

RESULTS AND DISCUSSION

In this paper we have summarized the effect of three plant extracts (Neem, Eucalyptus, Tagetes and Datura) and two oils (Neem and Eucalyptus) against three fungi (Fusarium, Macrophomina and Sclerotium) on the basis of calculating their radial growth when challenged by the oil/extract in a ring test assay with increasing concentrations of neem and eucalyptus oil (100ppm, 250ppm and 500ppm) (Table 2). For this radial growth of the above three fungi were used as a positive control to check and compare the activity of the negative controls so prepared.

Table 2: Ring test with phytopathogens challenged with phytopesticides

<table>
<thead>
<tr>
<th>S/N</th>
<th>Fusarium (7 days)</th>
<th>Control with no. oil only PDA (cm sq)</th>
<th>Neem oil mean area(cm sq)</th>
<th>Eucalyptus oil mean area(cm sq)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100 ppm</td>
<td>7.065</td>
<td>5.63</td>
<td>4.24</td>
</tr>
<tr>
<td>2</td>
<td>250 ppm</td>
<td></td>
<td>4.72</td>
<td>2.92</td>
</tr>
<tr>
<td>3</td>
<td>500 ppm</td>
<td></td>
<td>4.9</td>
<td>1.43</td>
</tr>
<tr>
<td></td>
<td>Macrophomina (4 days)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>100 ppm</td>
<td>52.28</td>
<td>51.35</td>
<td>48.24</td>
</tr>
<tr>
<td>5</td>
<td>250 ppm</td>
<td></td>
<td>51.83</td>
<td>37.24</td>
</tr>
<tr>
<td>6</td>
<td>500 ppm</td>
<td></td>
<td>51.92</td>
<td>1.25</td>
</tr>
<tr>
<td></td>
<td>Sclerotium (7 days)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>100 ppm</td>
<td>11.94</td>
<td>8.12</td>
<td>2.64</td>
</tr>
<tr>
<td>8</td>
<td>250 ppm</td>
<td></td>
<td>6.76</td>
<td>1.98</td>
</tr>
<tr>
<td>9</td>
<td>500 ppm</td>
<td></td>
<td>6.57</td>
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</table>

Based on radial diameter in ring test assay, we understand that eucalyptus oil acts as a better fungicide than neem oil (Table 2 and Fig.1). At the maximum concentration (500ppm) the antifungal activity of Eucalyptus oil was maximum and prominent for all the three fungi and % growth inhibition for Macrophomina, Fusarium and Sclerotium was calculated to be 97.61%, 79.73% and 100% (Fig. 2). However neem oil failed to show any considerable growth inhibition on Macrophomina (maximum of 1.77% growth inhibition at
100mg/ml) and for *Fusarium* and *Sclerotium* it showed maximum % growth inhibition of 33.12% and 44.97% at 250 ppm and 500 ppm respectively (Fig. 2 and Fig. 3).

**Fig. 2**: A effect of neem and eucalyptus oil on phytopathogens (100 ppm N/E), B : Effect of neem and eucalyptus oil on phytopathogens (250 ppm N/E), C : Effect of neem and eucalyptus oil on phytopathogens (500 ppm N/E)

Also when dry weight analysis was done, the difference in biocidal efficiency between neem oil and eucalyptus oil was more than 50% on *Macrophomina*. In case of neem oil weight of dried biomass was 0.241 gm where as *Eucalyptus* oil brought it down to 0.112gm. This activity of eucalyptus oil can be attributed to the presence of the bioactive components present in it. 1,8-cineole is present in majority (>70%) of eucalyptus oils along with some major and minor components. But evidences suggested that pure 1, 8-cineole did not exhibit inhibition activity as

<table>
<thead>
<tr>
<th>Phytopathogens</th>
<th>100 ppm N</th>
<th>100 ppm E</th>
<th>250 ppm N</th>
<th>250 ppm E</th>
<th>500 ppm N</th>
<th>500 ppm E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophomina</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Fusarium</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Sclerotium</td>
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</table>

NA = Concentration of neem oil (100 ppm), NB = Concentration of neem oil (250 ppm); NC = Concentration of neem oil (500 ppm), EA = Concentration of eucalyptus oil (100 ppm), EB = Concentration of eucalyptus oil (250 ppm), EC = Concentration of eucalyptus oil (500 ppm)

**Fig. 3**: Effect of increasing concentrations of plant oils on phytopathogens
much as Eucalyptus oils in totality (mainly from E. urophylla S. T. Blake, E. grandis, E. Camaldulensis, E. citriodora and E. globulus) which contain both major and minor components and hence it can be assumed that minor chemical components of the oils contribute to the growth inhibition of the tested fungi. Also a report of anti-fungal activity of Sri Lankan oils of E. microcorys, E. Grandis and E. robusta and ethanol extract of E. microcorys against F. solani and S. rolfii showed that three Eucalyptus species (E. grandis, E. microcorys and E. robusta) depicted considerable inhibitory effect against S. rolfii and F. solani. Thus this formed the sole basis of our experimentation with complete oils. Although not effective enough but neem oil does show some antifungal activity as mentioned earlier. This biocidal activity can be because of the presence of bioactive components along with the key active ingredient Azadirachtin a tetranortriterpenoid which is known to exhibit classical Insect Growth Regulatory (IGR) effects. Since the IGR effect is related to controlling insects, its implications for fungicidal activity cannot be elucidated as the molecular mechanisms of insects and fungi are vastly different.

Evidences in the literature suggest that aqueous extracts of four Datura sp are effective in reducing the growth of two plant pathogenic fungi A. solani and F. oxysporum. sp. Udum at 10%, 15% and 20% concentration levels. Comparatively, leaf extract of D. stramonium was found more inhibitory against F. Oxysporum. All the extracts of D. Stramonium have shown significant antifungal activity against Saccharomyces cerevisiae, Aspergillus fumigates and Aspergillus niger with maximum antifungal activity against S. cerevisiae and zone inhibition of about 16±0.2mm by ethanol extract, 15±0.3mm by chloroform and 14±1.6 mm by benzene extract while minimum antifungal activity was observed against A. niger.

Keeping this as the foundation we tested the three fungi with Datura extract in a comparative analysis with Tagetes and eucalyptus extract which also have proven biological activity including fungicidal, anti-microbial, herbicidal, insecticidal/insect repellent, acaricidal and nematicidal in the literature. Observations were made of the inhibitory activity of ethanolic extracts of datura, tagetes and eucalyptus using increasing extract concentrations (100ppm, 250ppm and 500ppm) against Macrophomina, the fastest growing fungus among the three fungi used (Table 3).

**Table 3 : Ring test with Macrophomina challenged with ethanolic plant extracts**

<table>
<thead>
<tr>
<th>S/N</th>
<th><strong>Macrophomina</strong></th>
<th>Control (with ethanol) (cm sq)</th>
<th><strong>Tagetes</strong> ethanolic extract (cm sq) mean area</th>
<th><strong>Datura</strong> ethanolic extract (cm sq) mean area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100 ppm</td>
<td>46.54</td>
<td>40.17</td>
<td>37.735</td>
</tr>
<tr>
<td>2</td>
<td>250 ppm</td>
<td>42.98</td>
<td>33.88</td>
<td>34.324</td>
</tr>
<tr>
<td>3</td>
<td>500 ppm</td>
<td>41.71</td>
<td>19.94</td>
<td>20.719</td>
</tr>
</tbody>
</table>

It was observed that tagetes and datura both demonstrated almost similar amount of % growth inhibition in area. At maximum concentration of 500ppm, datura and tagetes showed percentage inhibition of 50.33% and 52.19% respectively (Fig. 4 and Fig. 5) Tagetes being slightly more effective. Evidences attribute this activity to induced alterations caused by Tagetespatula extract on cell fungal membranes with a photo activation mechanism possibly involving the production of free radicals and lead -ing to a premature aging of the mycelium.
Fig. 4 : Effect of increasing concentrations of plant oils and ethanolic extracts on *Macrophomina* plant oils on phytopathogens

A = Concentration of extract (100 ppm), B = Concentration of extract (250 ppm), C = Concentration of extract (500 ppm), Control = Concentration of ethanol (500 ppm)

Fig. 5 : Effect of ethanolic plant extracts on *Macrophomina*

CONCLUSION

By observing the above data it can be concluded that the activity of eucalyptus oil is significant against all the three fungi tested. Thus eucalyptus oil based formulations can be developed and applied as a potent bio pesticide. *Tagetes* and *Datura* extracts also showed significant although lesser bio-control activity than eucalyptus oil. This could also form the basis for further research where various different and more effective formulations comprising a combination of phyto extracts along with eucalyptus oil can be designed to achieve enhanced results. It will be an interesting research proposition to explore if combinations of plant extract or oils can be formulated which may show a synergic effect as enhanced fungicides. This research will prove to be a best alternative to the chemical pesticides which are being used and causing massive detrimental effects to the environment.

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