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PREVENTION OF FUNGI BY NATURAL ANTIMICROBIAL AGENTS

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ABSTRACT
Plants and plant based products are good source of bio-control agents. Antimicrobial compounds present in the plants can be considered as most potent alternate of synthetic fungicides due to their less negative impacts on the environment, animals and plants. In the present study *Eucalyptus globulus* Labill leaf extracts were evaluated against *Alternaria solani* which is a causal organism of early blight of tomato. The dried and powdered leaves were successively extracted by cold extraction as well as hot extraction method. The antifungal activity assay was done by poison food technique. 100% alcohol crude extract and petroleum ether extract of *E. globulus* leaves showed highest activity against test fungi. The inhibitory effect was significant and better than the synthetic fungicides used as mancozeb and bavistin. Inhibitory action of the extract was concentration dependent. Minimum inhibitory concentration (MIC) was found to be 2.5 mg/ml and Minimum fungicidal concentration (MFC) was 5 mg/ml for *A. Solani*. The results obtained in this study clearly suggest the possible use of leaf powder as eco-friendly herbal fungi toxicant to prevent serious damage to the economically important crops.

INTRODUCTION
Synthetic fungicides, insecticides and herbicides have negative effects on whole ecosystem if use in inappropriate manner and also possesses a possible carcinogenic risk on human health as well as animals so that it is essential to search for an environmentally safe and economically viable strategy to overcome the diseases and to reduce the dependence on the synthetic agrochemicals.[1]. In the direction of safe and economically viable and ecofriendly search, plants are naturally gifted with bioactive compounds which form the backbone of traditional medicines and this is the only reason for increasing interest worldwide on therapeutic values of natural products from plants.[2]

Plants and plant based products are good source of bio-control agents and large number of antimicrobial compounds have been isolated from plants which can be natural alternative to synthetic fungicides due to their less negative impacts on the environment.[3]. for screening of

antimicrobial properties of plant against pathogenic bacteria, fungi and protozoa, there is strong need for preparation of extract or decoction. Plant extracts is usually prepared with cold and hot extraction methods. [4-5]All the active principles present in plants are usually saturated organic compounds so they get extracted in ethanol or methanol. Initial antimicrobial screening with crude extract is followed by screening of extracts prepared in various organic solvents by hot extraction method.[6] These extracts are studied to search for various phytochemicals, responsible for antimicrobial activity. Phytochemical constituents are responsible for medicinal and antimicrobial activity of plant species.[7] Plants are rich in wide variety of secondary metabolites viz. tannins, terpenoids, alkaloids, and Flavonoids.[8] which possess enormous antimicrobial properties. Antifungal agents based on natural products have always been promising in the control of fungi.

Numerous plants used in traditional medicines are effective in treating various ailments caused by oxidative stress, bacterial and/or viral infections.[9] Since ancient time plants have been used in various treatments because of their antimicrobial properties which are due to compounds synthesized in the secondary metabolism of the plant. These products are known by their active substances e.g. the phenolic compounds which are part of the essential oils, as well as tannin.[10] Moreover, products are not toxic and are decomposed easily.[11] Numerous literatures have highlighted the inhibition effect of plants and their possible utilization for control of plant diseases.[12]

In the present study *Eucalyptus globulus* Labill leaves were used for detection the antifungal activity against *Alternaria solani*. E. globulus belonging to the family Myrtaceae, commonly called Tasmanian blue gum is a fast-growing species native to Australia. [13] *Eucalyptus* is an evergreen tree of 24-40 m in height, leaf is petiolated expstipulate simple, lanceolated ovate smooth aromatic with oil glands. Inflorescence is umbellate in cluster of three. The flower of eucalyptus is pedicillate, complete actinomorphic and epigenous. Placentation is agile.[14] The essential oil extracted from the leaves of *Eucalyptus globulus* Labill is known to be a rich source of traditional medicines with a variety of biological activities.[15-16], To the best of our knowledge, there is little or no information on the antifungal activity of *E.globulus* against *A. solani* which is our test pathogen. The objective of the current study was, therefore, to determine the antifungal activities of *E. globulus* cold and hot extracts using *in vitro* antimicrobial screening methods.

MATERIALS AND METHODS

Collection and Identification of Plant Material

The healthy infection free leaves of *Eucalyptus globulus* were collected in the month of February 2014 from the campus of University College of science, Mohanlal Sukhadia University, Udaipur, Rajasthan, India. The herbarium specimen was identified by by Dr. Amit Kotia, In charge Herbarium, Department of Botany, and University college of Rajasthan, Jaipur, India where it was deposited as specimen and voucher no. RUBL 211446 was given.

Preparation of Extract

Test Plant material leaves were dried in shade at room temperature and grounded with electric grinder. The ground material was passed through sieve of mesh size 60 to obtain a fine powder which was used to prepare crude extract and partially purified extract. Cold extractions as well as hot extraction procedure were followed to obtained crude and partially purified fractions respectively.

Cold Extraction

This extraction was done in two universal solvents i.e. water and alcohol. Cold extract was prepared according to modified method.[17] 100% alcoholic, 50% alcoholic as well as 100% aqueous extract of leaves was prepared by dissolving 20 gram dried and powdered plant material in 100 ml of solvent (alcohol/ water) for 24 h. The mixture was then filtered and supernatant was evaporated under reduced pressure using a rotary evaporator. The dried residue was used as extract, which was stored in an airtight jar in refrigerator.

Hot Extraction

Hot extraction method is serial exhaustive method which involves successive extraction with solvents for the separation of different phytochemical constituents from plant part.[18-19] 40 gm dry leaf powder of test plant was used for hot extraction. Solvent series used for successive separation was non-polar to polar i.e. in Soxhlet extraction unit.

Pet. ether → Benzene → Chloroform → Acetone →Alcohol→ Methanol → Water

Isolation, Identification and Development of Pure culture of test fungi

The infected parts of leaves and fruits of tomato were cut into small pieces, surface sterilized with 0.1% mercuric chloride solution for 30 seconds, washed three times by distilled water and transferred on to Petri plates containing solid PDA (Potato dextrose agar) media by Potato dextrose Agar plate method[20] and single spore technique. The inoculated plates were incubated at 25°c for 4-6 days. Pure culture of test pathogen was stored on agar slant at 4°C and regularly sub culture after 7 days. Characters of *Alternaria solani* shows in table 1

Identifying Characters of *Alternaria solani*

Colony Morphology on PDA	Mycelium	Conidiophores	Conidia
Dark Black to brown Circular	Brown, septate branched	Dark,unbranched broder than vegetative hyphae bearing chain of conidia	Dark brown transversely and longitudinally septate have distinct beak

Inoculum Preparation

1.0×10^7 spores /ml spore suspension prepared from 7 day old culture of test fungi maintained on PDA (Potato Dextrose agar) at 27 ± 2^{o} C by washing the surface of cultures with 5 ml sterile distilled water was used as inoculum in broth dilution method. The number of spores/ml was counted with the help of haemocytometer. 5 mm disc of 7 day old culture of test fungi was used as inoculum in poison food technique.

Assay of Antifungal Activity of Crude Extracts

The inhibitory activity of crude 100% alcohol, 50% alcohol and 100%aqueous extracts, partially purified extracts and column fraction of *Eucalyptus globulus* against *A. solani* was tested using poison food technique.[21] 100 mg of extract was dissolved in 10 ml acetone to prepare 10mg/ml concentration of stock solution. 2 ml of stock solution was mixed with 18 ml molten sterile culture medium and this mixture was poured into pre-sterilized petri-plates (9 cm diameter) and allowed to solidify at room temperature. In the control set no extract was used. The plates were then incubated at 28±2°C for seven days. Culture control and acetone control were also maintained along with test samples. Antifungal activity was measured as a function of increase in growth of 6 mm disc of inoculum. The average diameter of the fungal colonies was measured on the 7th day of incubation and percentage of Mycelial growth inhibition was calculated by the following formula given below.

Percent Mycelial growth inhibition = gc-gt/gc×100

Where-

gc = growth of mycelia colony after incubation period in control set subtracting the diameter of inoculums's disc

gt= growth of mycelia colony after incubation period in treatment set subtracting the diameter of inoculums's disc

Estimation of Minimum inhibitory concentration (MIC) of selected plant extract

Minimum inhibitory concentration (MIC) was determined by broth dilution method.[22] Potato dextrose broth (PDB) was used for determining inhibitory activity. 200 mg of the extract was dissolved in 10 ml of acetone to prepare stock

solution of 20mg/ml. Two fold serial dilution method was used for the preparation of 10 mg/ml, 5 mg/ml, 2.5 mg/ml, 1.25 mg/ml, 0.625 mg/ml, 0.312 mg/ml, 0.156 mg/ml, 0.078 mg/ml, 0.039 mg/ml, 0.019 mg/ml. concentration from the stock solution and subsequently autoclaved. The final concentration was serially diluted with sterile potato dextrose broth medium to attain final concentration 1000 µg/ml, 500µg/ml, 250µg/ml, 125µg/ml, 62.5µg/ml, 31.2µg/ml, 15.6µg/ml, 7.8µg/ml, 3.9µg/ml, 1.9µg/ml, 0.9µg/ml and 0.04µg/ml. All these tubes were than respectively inoculated with 10 µl of spore suspension (1×10^6 spores/ml) and incubated at 27± 2°C for 72 h. One tube containing extract free autoclaved medium was used as control. Three replicates of each concentration were maintained and experiment was repeated thrice.

Estimation of MFC of Selected Plant Extract

A loopful of fungal biomass from each tube containing 9ml broth medium and MIC as well as all concentrations were streaked onto the extract free medium PDA slants and incubated at 27± 2°C for 72 h. Presence or absence of growth was observed after respective incubation time. Appearance of growth indicates that the extract concentration is just fungi static and absence of growth indicates that extract concentration is fungicidal.

RESULTS AND OBSERVATIONS

Antifungal activity of crude extract of leaves

100% alcohol, 50% alcohol and 100% aqueous extract of leaves were assayed for their antifungal activity against *Alternaria solani* and results of antifungal activity of crude extract are presented in table 1. Among all three type of crude extract 100% alcohol showed best antifungal activity with percent mycelia growth inhibition of 63.14%.The second highest inhibition was observed with 50% alcohol crude extract with 58.4% mycelia inhibition and lease inhibition was showed by 100% aqueous solution that was 51.61%. All data were compared with control of water in which no plant extract and fungicides were used (table 3)

Antifungal activity of partially purified extract

Results of antifungal activity of partially purified extract fraction of *E.globulus* leaf and standard chemical fungicides against *Solani* are given in table 2. .Among them comparative inhibitory activity of mancozeb and bavistin, mancozeb significantly active against *A.solani* .Significant inhibition of *A.solani* was observed with all partially purified fractions but petroleum ether fraction showed maximum inhibition with 79.26%followed by benzene (61.28%) chloroform (59.44%), acetone (65.90%), alcohol (46.08%), methanol (44.69%) and aqueous (37.78%) fraction.

MIC and MFC of petroleum ether fraction

Petroleum ether fraction was assayed as this fraction was found to be most effective. MIC of petroleum ether of leaf extract of *E.globulus* was assayed to find out the minimum inhibitory concentration and minimum fungicidal concentration. 0.019mg/ml to 10mg/ml of PE extract was assayed and MIC was found to be 2.5 mg/ml for *Alternaria solani* and MFC for this fungus was 5 mg/ml observed (table 4)

DISCUSSION

Chemical fungicides impose the adverse effect not only on humans but also on animal and the whole ecosystem. Hence, there is a great demand for novel antifungal with fewer side effects. One approach might be the testing of plants traditionally used for their antifungal activities. It is important to investigate scientifically novel antimicrobial compounds from the plants. [23] Medicinal plants are not only important to the millions of people for whom traditional medicine is the only opportunity for health care and to those who use plants for various purposes in their daily lives. Natural products, either as pure compounds or as standardized plant extracts, provide unlimited opportunities for new drug leads because of the matched less availability of chemical diversity.[24]

Results suggests that 100% alcohol crude extract of *E.globulus* and partially purified petroleum ether extract has given best inhibitory activity against *A. solani*. Similar results of antifungal

activity by extracts were reported by several workers.[25-27] Screening results of the present study suggests that aqueous extract did not show any strong activity against *Alternaria solani* whear as petroleum ether extract showed significant inhibition against *A.solani*. It can be due to better solubility of active metabolites in the petroleum ether like terpenoid, flavanoids as compare to water. Secondary metabolites found in plants are the main reason for their antimicrobial potential.[28]

Unsystematic use of commercial antimicrobial drugs applied in the treatment of infectious diseases causes multiple resistances in human pathogenic microorganisms so this has forced scientist to search for new natural antimicrobial substances. [29]

CONCLUSION

On the basis of results obtained it can be concluded that 100% alcohol crude extract, partially purified petroleum ether fraction of *Eucalyptus globulus* leaf were showed maximum inhibition against *Alternaria solani* as compare to standard fungicides. This will be used to develop an eco-friendly natural fungicide which will not leave any toxic substance in the environment.

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EXPERIMENTAL TABLE AND IMAGES

Table 1: Antifungal Activity of Crude Extract of *Eucalyptus globulus* against *Alternaria solani*

S. No	Type of Extract	Growth Diameter after 7 days(mm)±SD	% Mycelial Growth Inhibition
1	100% Alcohol	26.66±1.15	63.14
2	100% Aqueous	35±1	51.61
3	50%Alcohol	32.66±1.52	54.84

Table 2: Antifungal activity of partially purified fraction of *Eucalyptus globulus* leaf extract against *A.solani*

S.No	Type of Extract	Growth Diameter after 7 days (mm) ±SD	% Mycelial Growth Inhibition
1	Petroleum ether	15±1	79.26
2	Benzene	28±1.73	61.28
3	Chloroform	29.33±0.57	59.44
4	Acetone	24.66±0.57	65.90
5	Alcohol	39.56±0.57	46.08
6	Methanol	40.66±0.57	44.69
7	Aqueous	45±1.52	37.78

Table 3: Antifungal activity of Standard fungicides with water control against *A.solani*

S.No	Standard fungicides and water control	Growth Diameter after 7 days(mm)±SD	% Mycelial Growth Inhibition
1	Mancozeb	15.33±0.57	78.80
2	bavistin	34±1.73	52.99
3	water	72.33±2.51	

Table 4: MIC and MFC of petroleum ether fraction of *Eucalyptus globulus* leaf extract

S.No	Test pathogen	MIC (mg/ml)	MFC (mg/ml)
1.	*Alternaria solani*	2.5	5

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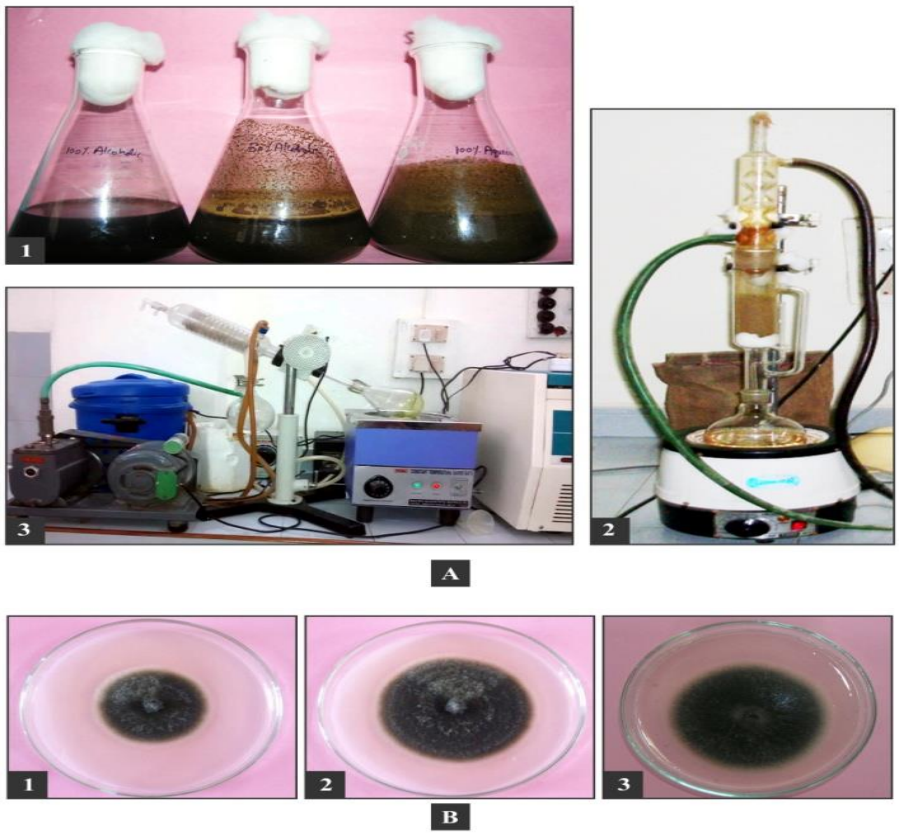

Fig. 1: Preparation of Extracts of *Eucalyptus globulus* Leaves

A: 1. Crude Extracts (100% alcohol; 100% aqueous; 50% alcohol)

2. Soxhlet Assambly

3. Rotary Evaporator

B: Assay of Antifungal activity of Crude extracts of *E. globulus* against *A. solani*

1. 100% alcohol Crude Extract
2. 100% aqueous Crude Extract

50% alcohol Crude Extract

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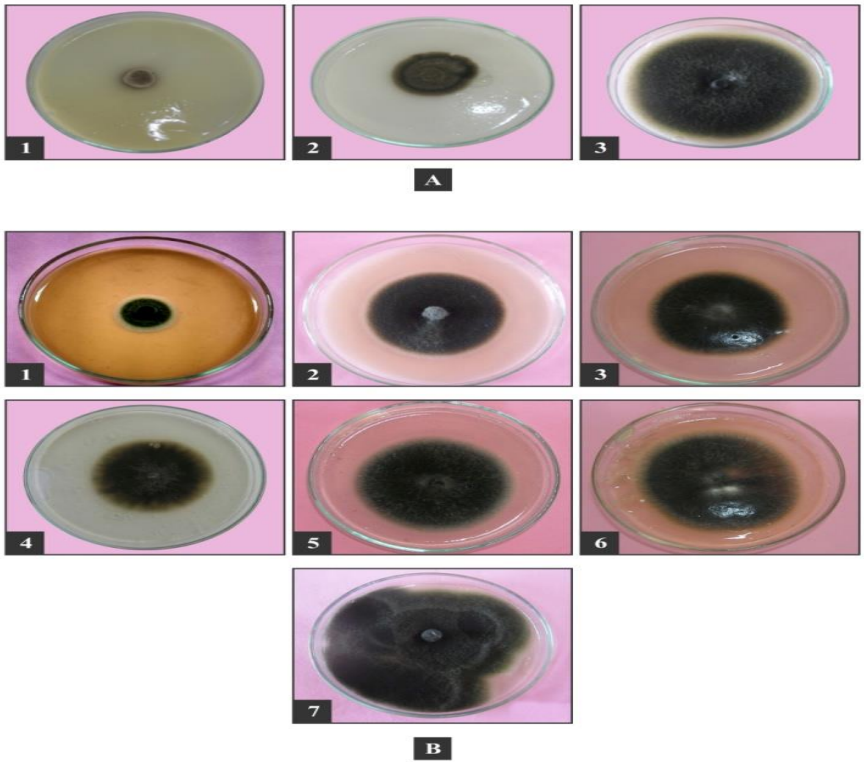

Fig. 2: Assay of Antifungal Activity of Standard Fungicides and Partially Purified Fractions of *E. globulus* Leaves against *A. solani*

A: Antifungal Activity of Standard Fungicides with Water Control
1. Mancozeb
2. Bavistin
3. Water

B: Antifungal Activity of Partially Purified Fractions
1. Petroleum Ether Fraction
2. Benzene Fraction
3. Chloroform Fraction
4. Acetone Fraction
5. Alcohol Fraction
6. Methanol Fraction
7. Aqueous Fraction