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MANAGEMENT OF *FUSARIUM OXYSPORUM F. SP. CAPSICI* BY LEAF EXTRACT OF *EUCALYPTUS CITRIODORA*

SHAZIA SHAFIQUE*, MUHAMMAD ASIF AND SOBIYA SHAFIQUE

Institute of Agricultural Sciences, University of the Punjab, Lahore 54590, Pakistan
**Corresponding author's e-mail: shazia.iags@pu.edu.pk*

Abstract

Fusarium wilt of chili (*Capsicum annuum* L.) is an important disease in Pakistan that causes significant yield losses. In the present study, pathogenicity test was conducted using four strains of *Fusarium oxysporum f.sp. capsici* and ten chili varieties. It revealed that strain B was the most pathogenic strain and variety sky red was the most susceptible while variety Anchal was the most resistant against *F. oxysporum* strain B. Antifungal bioassays were conducted to find out antimycotic effect of extracts of fruit, bark and leaves of *Eucalyptus citriodora* (Hook.) against *F. oxysporum*. Ten concentrations (0, 1.0, 1.5, 2.0, 2.5 and 5%) of methanolic extracts of each plant part were employed against the target pathogen. Leaf extract imparted the maximum (up to 98%) and significant suppression in fungal growth while fruit and bark extracts proved less effective exhibiting only 50–60% reduction in fungal mycelial growth. The work concludes that methanolic extract of leaves of *E. citriodora* have potential to restrain the disastrous effects of the pathogenic fungus as the plant extracts of *Eucalyptus* conferred about 85% disease control in chilli plants with significantly high intensity of defense related enzymes under pathogenic stress.

Key words: Antifungal bioassays, *Capsicum annuum, Fusarium oxysporum, Eucalyptus citriodora,* Methanolic extract.

Introduction

Chili (*Capsicum annuum* L.) is an important vegetable and spice crop belonging to the nightshade family *Solanaceae*, cultivated in about 130 countries, mostly in the tropical regions of the world (Anonymous, 2003a). These are full of vitamin C and vitamin A containing beta-carotenoids which are potent antioxidant. It is very high in potassium, magnesium, calcium, and iron (Serra *et al*., 2002).

Major producers of chili include Asia, Latin America, Africa, Europe, and North America (Anonymous, 2003b). In Pakistan; chili production has great contribution in its economy (Khalid *et al*., 2000). Its total area under cultivation is about 91800 hectare, with total annual production of 115,000 tons (Govt. of Pak., 2001). Worldwide yield of chili is now decreased up to 50%. There are many biotic and abiotic factors which are decreasing chili production. Among these factors the most devastating are fungal diseases which lower the yield per acre annually. Chili plants are liable to attack by several fungal diseases among which damping-off, root rots and wilt are widespread (Abada, 1994). The chili wilt has been found as the most frequently encountered disease problem (Skaggs *et al*., 2000; Siddiqui & Akhtar, 2007). Wilt of chili is a fungal disease caused by the F*usarium oxysporum* also known as Fusarium wilt. In Pakistan Fusarium wilt of chili causes 15 to 20% yield losses in dry areas during the last few years (Siddiqui & Akhtar, 2007).

In order to reduce the economic loss and to protect the crop from pathogen different control methods are used. One very common method is to use a resistant cultivar. Besides, soil fumigants and solarization are practiced to minimize pathogens in soil. Carbendazim and Bordeaux mixture are also the most commonly used chemicals but increasing use of pesticides in past has led to several problems, such as environmental degradation, health hazards, pest resistance and decrease in population of beneficial insects (Groenewald, 2005). Thus it is a universal requisite to implement the practice of sustainable agriculture, using strategies that are environment-friendly and less damaging to soil and water resources. Biological and environmental innocuous substitutes, such as biocontrol agents, natural plant metabolites, and cultural methods, are being explored for possible use in integrated disease management platforms and to improve the foreign exchange on the basis of the efficacy of plant extract. Plants extracts and essential oils show antifungal activity against a large no of fungal diseases (Shafique & Shafique, 2012; Zaheer *et al*., 2012; Javaid & Iqbal, 2014; Javaid & Rauf, 2015). The plants are rich in a wide variety of secondary metabolites such as tannins, terpenoids, alkaloids, flavonoids, glycosides, etc., which have been found *In vitro* to have antimicrobial properties (Dahanuker *et al*., 2000). Numerous studies conducted in Pakistan revealed a wide spectrum prospects of using extracts of plants for biological control of pathogenic fungi (Bajwa *et al*., 2001; Ahmad & Abdelgalie, 2005; Shafique & Shafique 2012; Zaheer *et al.,* 2012). By keeping all these intricacies in view it is suggested that biological control using plant extracts provides an effective and overall fungal management program, and represents an alternative to reliance on fungicides. Therefore, the present study was conducted to find the effective control of Fusarium wilt of chili through *Euclaptus citriodora* fruit, bark and leaves methanol extracts.

Eucalyptus Citriodora also called lemon-scented eucalyptus is a diverse genus of family Myrtaceae. Of the more than 600 species that comprise this genus, most are endemic to Australia (Marzoug *et al*., 2011). The natives of Australia traditionally use eucalyptus leaves to heal wounds and fungal infections. In recent times, medicinal assets of the extracts have been engrossed. In supplement, the trees have been known to fabricate several natural substances having antagonistic activities against several microorganisms (Lee, 2007).

Material and Method

Selection of Chili Varieties: A total of 10 chili varieties namely Golden hot, Revival, Hot chili, Saim hot, Sky red, P6, Neelam, Sitara, Anchal and Kundri were selected which were acquired from NARC, and Fruit and Vegetables Market of Allama Iqbal town, Lahore, Pakistan.

Procurement and maintenance of cultures: Four pure cultures of *Fusarium oxysproum f.sp. capsici* were isolated from the diseased chilli plants on PCNB medium and labeled as *F. oxysproum* **A**, *F. oxysproum* **B**, *F. oxysproum* **C** and *F. oxysproum* **D**. They were maintained on 2% malt extract agar (MEA) medium, sub cultured monthly and stored at 4ºC.

Pathogenicity Test: Pathogenicity test was performed according to modified procedure of Grogan *et al.* (1975). Pots of 8cm diameter and 11cm deep were filled with 350 g soil per pot that was sterilized at 45°C for 24 h in hot air oven. Conidial suspension of 4 x 10^5 conidia mL^{-1} was prepared by using the protocol of French & Hebert (1982). Three seeds of selected varieties were sown in pots and thinned to one plant per pot after 20 days. One month old chili plants were sprayed with adjusted spore suspension. Control received same quantity of distilled water. The plants were kept covered with polythene bags for 48 hours. Greenhouse temperature ranged from 28°C to 30°C and watered when required. Each treatment was replicated five times. Disease rating scale was developed for the estimation of disease severity. Disease incidence was observed as the symptoms appeared on the plant and disease index was calculated using formula:

$$\text{Disease Severity} = \frac{\text{Area of Plant Part Affected}}{\text{Total Area}} \text{ x } 100$$

$$\text{Disease Index} = \frac{\text{Number of plants in Particular Category}}{\text{Total Number of Plants}} \text{ x } 100$$

The isolate causing maximum disease severity was selected as the Most Virulent/pathogenic Isolate and selected for further experimentation. Moreover, chili varieties were also evaluated for their individual resistance against fungal pathogen on the basis of disease index and screened as the representative ones for subsequent studies.

Phytochemical Control of the Most Pathogenic Isolate.

Synthesis of methanolic plant extract: Fresh samples of leaves, bark and fruits of *E. citriodora* were collected from Quaid-e-Azam campus, University of the Punjab, Lahore in December, 2012. The plant materials were washed, dried and grinded to make powder. Then methanolic extract of each part was prepared by using the protocol of Javaid & Munir (2012). Hundred grams (100 g) of dried powder of each plant part was soaked in 1000 mL methanol for one month. After 1 month materials were filtered through muslin cloth followed by filter paper filtration and evaporated under vaccum in rotary evaporator at 45°C. Then methanolic extracts (16.2 g) of each part were dissolved in sterilized water to prepare 60 mL of stock solutions of each plant part separately.

Antifungal bioassays: Aqueous solution of each plant part of methanolic residues was prepared in sterilized distilled water to make the final concentration of 0.6 g mL^{-1}. Methanol residues were first dissolved in dimethoxy sulfoxide (DMSO) and then sterilized distilled water was added to obtain the final concentration of 0.6 g per mL. To check the bioactivity of extract 10 concentrations viz., 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0% of the organic solvent residue solution were formed. In 100 mL flasks, 55 mL malt extract broth was sterilized by autoclaving at 121°C and cooled at room temperature. Ten concentrations viz. 5.0, 4.5, 4.0, 3.5, 3.0, 2.5, 2.0, 1.5 and 1.0 g 100 mL^{-1} were prepared by adding 5.0, 4.5, 4.0, 3.5, 3.0, 2.5, 2.0, 1.5 and 1.0 mL stock solutions, and 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mL sterilized distilled water, respectively to each flask to make the total volume up to 60 mL, while control received 5 mL of distilled water. The 60 mL medium of each treatment was divided into four equal potions in 100 mL conical flasks to serve as replicates. An inoculum of 0.3 mL of adjusted conidial suspension (4 x 10^5 conidia mL^{-1}) was given to all treatments and incubated at 28±2°C for 10 days. There were four replicates of each treatment (Javaid & Munir, 2012).

Dried mycelial biomass of all treatments was determined after 10 days according to (Bajwa *et al.*, 2006). Percentage of fungal biomass inhibition in response to all concentrations of the extracts over control was deliberated by applying the following formula:

$$\text{Growth inhibition (\%)} = \frac{\text{Growth in control - Growth in treatment}}{\text{Growth in control}} \times 100$$

Statistical analysis: The differences among the treatment means were compared by using Duncan's Multiple Range Test (DMRT) (Steel & Torrie, 1980). All the data were analyzed by analysis of variance (ANOVA) using computer software COSTAT followed by Duncan's Multiple Range (DMR) test to separate the treatment means.

Results

Pathogenicity assay: Pathogenicity test was executed using one month old chili plants to determine the pathogenicity of 4 strains of *Fusarium oxysporum* on ten different varieties of chili plant. Infection and visible characteristic symptoms were evident after 10 days of inoculation (Tables 1 & 2). Symptoms of wilt disease observed on different varieties of chili plants were found to be varied with different strains of *F. oxysporum* (Fig. 1). Symptoms were started to develop from the leaf tips and along the margins of the leaf petiole. Strain B of *F. oxysporum* was proved to be the most pathogenic as it induced symptoms within 7 days. Initially the plants displayed yellowing on the leaves that was turned into chlorosis and necrosis; slowly it was converted to wilting and eventually the death of the whole plant. The severely infected plants showed the stunted growth. Data analysis revealed that all the strains of *F. oxysporum* demonstrated maximum disease severity in sky red. On the other hand

Sitara, Anchal and Kundri exhibited less severity (30-35%) against the same pathogen (Fig. 1). *F. oxysporum* strain B induced maximum disease index of about 50% in Sky red (Fig. 2). By the evaluation of percentage infected area, the variety Sky red having the accession 020699 was proved the most susceptible and Anchal was most resistant against *Fusarium oxysporum*.

Table 1. Disease rating scale exhibiting disease severity.

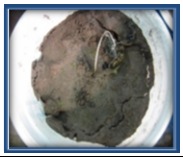
Percentage infected area	score	Status
01-20 %	1-2	Highly resistant
21-40 %	3-4	Resistant
41-60 %	5-6	Moderately susceptible
61-80 %	7-8	Susceptible
81-100 %	9-10	Highly susceptible

Thus the disease progress curve was plotted for two chili varieties i.e., Sky red and Anchal (Fig. 3). This disease progress curve ascertained Sky red (variety 020699) as the most susceptible and Anchal as the most resistant variety. As Sky red exhibited maximum percentage disease incidence so this susceptible variety was used for further lab experimentation.

Phytochemical control of *Fusarium oxysporum*: *F. oxysporum* strain B induced maximum pathogenicity in chili plants so it was selected for subsequent studies. The methanol extracts of leaves, bark and fruit of *E. citriodora* were used to evaluate the antifungal potential against the target pathogen.

Antifungal activity of leaf extract of *E. citriodora*: The *In vitro* antifungal potential of methanolic Leaf Extract of *E. citriodora* was recorded against *F. oxysporum* strain B. The employed concentrations of leaf extract significantly decreased the fungal biomass as compared to control treatment. The pattern of gradually lower production of biomass in response to increasing concentrations of methanolic extract was relatively more sharp (98 to 99%) in 4-5% concentrations. Arrest in biomass production was least in the lowest concentration (1%) but it was still significant in comparison to control as it induced about 47% suppression in biomass. Among all the employed concentrations, the most promising effect on the growth of fungal biomass was observed at the highest concentration (5%) as it caused maximum reduction of 99% in fungal biomass (Fig. 4).

Table 2. Pictorial representation of disease rating scale on the basis of symptom development.

Key scale	Disease description		Disease severity
0	No symptom		0
1	Yellowing start at the margin of the leaves		10
2	Wilting will start on the whole chili plant		30
3	Plant show reduced vigor and growth		50
4	Whole plant is infected necrosis on all the tissues		70
5	Complete death and dieback of the plant		100

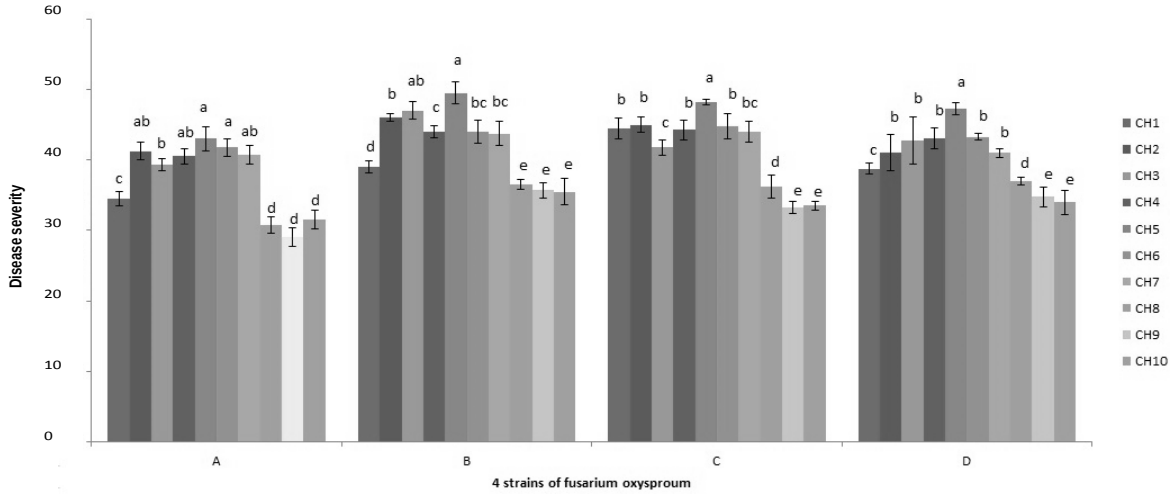


Fig. 1. Disease severity of different strains of *Fusarium oxysporum* against the selected chili varieties.
Vertical bars indicate standard error of means of three replicates. Values with different letters show significant difference ($p \leq 0.05$) as determined by Duncan's multiple range (DMR) test.

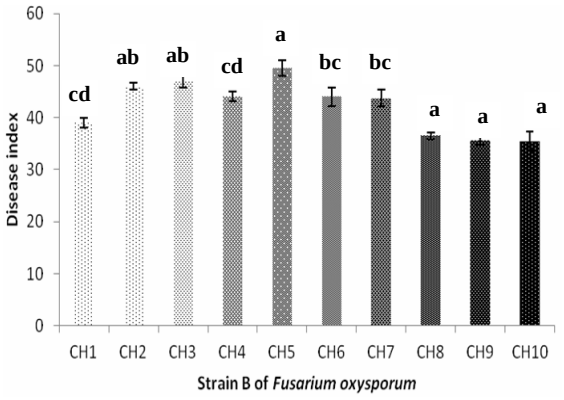


Fig. 2. Disease index of selected *Fusarium oxysporum* strain B with reference to all chili varieties.
Vertical bars indicate standard error of means of three replicates. Values with different letters show significant difference ($p \leq 0.05$) as determined by Duncan's multiple range (DMR) test.

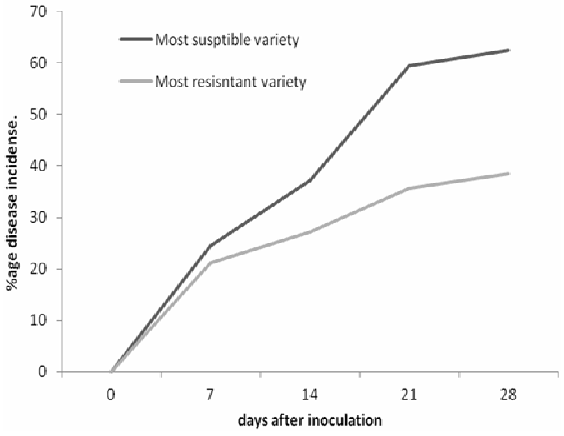


Fig. 3. Disease progress curve of the most resistant and the most susceptible chili varieties.

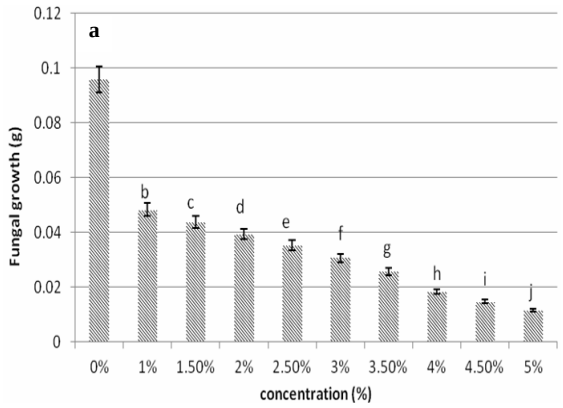


Fig. 4. Effect of different concentrations of methanolic leaf extract of *Eucalyptus citriodora* on growth of *Fusarium oxysporum* strain B.
Vertical bars indicate standard error of means of three replicates. Values with different letters show significant difference ($p \leq 0.05$) as determined by Duncan's multiple range (DMR) test.

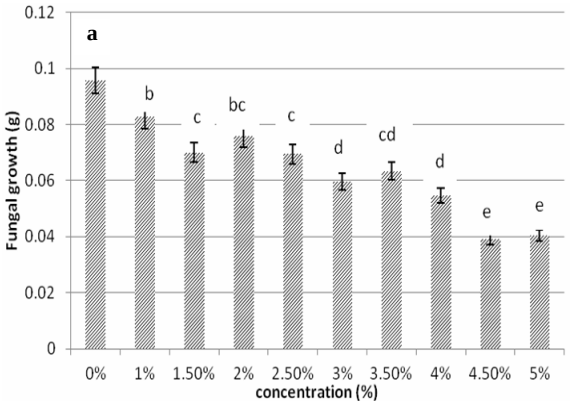


Fig. 5. Effect of different concentrations of methanolic bark extract of *Eucalyptus citriodora* on growth of *Fusarium oxysporum* strain B.
Vertical bars indicate standard error of means of three replicates. Values with different letters show significant difference ($p \leq 0.05$) as determined by Duncan's multiple range (DMR) test.

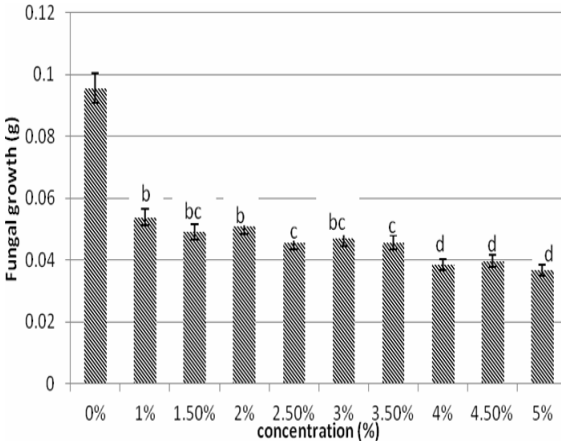


Fig. 6. Effect of different concentrations of methanolic fruit extract of *Eucalyptus citriodora* on growth of *Fusarium oxysporum* strain B. Vertical bars indicate standard error of means of three replicates. Values with different letters show significant difference ($p \leq 0.05$) as determined by Duncan's multiple range (DMR) test.

Antifungal activity of bark extract of *E. citriodora*: The growth assays of *F. oxysporum* strain B in terms of dry biomass production against exposure to methanolic bark extract of *E. citriodora* revealed an excessive interference as presented in Fig. 5. Control treatment was used to compare the difference between the treatments. It was evident from the results that with increasing the extract concentration; the significant reduction in biomass was more visible with an erratic pattern. The 1% concentration depicted less negative effect on biomass inhibition (12.63% decline) but significantly lower than control. The treatments 1.5-3.5% were comparatively insignificant among themselves as these inclined about 20-36% cessation in fungal growth. Among all the concentrations 4.5 and 5% concentrations were proved to be the most effective against the fungal pathogen as these persuaded around 57.8 and 60% subjugation in dry biomass production, respectively.

Antifungal activity of fruit extract of *E. citriodora*: In general the methanolic fruit extract of *E. citriodora* was found to be less effective in terms of its antimycotic potential against test fungal growth than leaf extracts. A variable response of dry biomass production in *F. oxysporum* strain B was recorded to methanol fruit extract of *E. citriodora* in different concentrations (Fig. 6). All the treatments presented a piercing and significant imprisonment in dry biomass production as compared to control but the suppression rate among the concentrations was negligible. There was about 45-50.5% inhibition in fungal growth due to all the employed concentrations. Among the three plant parts of *E. citriodora* tested, performance of leaf extract was the best in suppressing the growth of target pathogen followed by fruit and bark extracts.

Discussion

In the present study, pathogenecity test was performed to determine the pathogenic potential of four strains of *F. oxysporum*. The results depicted that all the strains had the potency to induce wilt as all of the strains caused more or less typical symptoms on plants. Among these, *F. oxysporum* strain B induced maximum characteristic symptoms of wilt and plants died within few days of inoculation. These results were found in agreement with the work conducted by Mahmood (2010) who reported the same trend of disease development in tomato by *A. alternata*.

To evaluate the antifungal potential of methanol extracts of leaves, bark and fruit of *E. citriodora,* ten different concentrations of extract of each plant part exhibited markedly variable antagonistic activities against the pathogen *In vitro* conditions. The relative intensity of this effect however varied with the extract type, as well as the particular concentration of the extract employed. There are other evidences showing similar effects against known pathogenic fungi (Khan *et al*., 1998; Shafique & Shafique, 2008; Baraka *et al*., 2011), which further confirm the presence of antifungal agents in the test species. Similarly, Shafique *et al*. (2006) reported 60% reduction in incidence of *Alternaria alternata* on wheat due to aqueous leaf extract of *C. album.* In contemporary studies, Bajwa *et al*. (2007) evaluated antifungal activity of aqueous and *n*-hexane shoot extracts of *Aloe vera* against few pathogenic species viz., *Alternaria alternata*, *A. citri* and *A. tenuissima*. They reported that the inhibitory effect was found to be variable with the applied concentrations and caused a significant inhibition in biomass production of test fungi.

Presently, among the three plant parts of *E. citriodora* tested, leaf extract was evidenced as the best in subduing the growth of *F. oxysporum* up to 98 to 99% followed by fruit and bark extracts. The difference in the fungicidal activity of the extracts of different parts of *E. citriodora* could probably be due to the different types and/or different amounts of chemical constituents in the varied types of extracts. A high-performance liquid chromatography analysis by Kowthar *et al*. (2011) showed that the following acids; caffeic, ferulic, coumaric, benzoic, vanelic, chlorogenic, and hydroxybenzoic were present in Eucalyptus extracts which may be responsible for the fungicidal activity of the plant. Another phytochemical analysis of the leaf extracts revealed the presence of anthraquinones, cardiac glycosides, flavonoids, saponins and tannins (Bhagat, 2012). Among the various components of eucalyptus, 1, 8-cineole is the most important one and, in fact, a characteristic compound of the genus *Eucalyptus*, and is largely responsible for a variety of its pesticide properties (Duke, 2004). Su *et al*. (2006) demonstrated the antifungal activity of essential oils from *Eucalyptus grandis*, *E. camaldulensis*, and *E. citriodora* against the mildew and wood rot fungi *viz. Aspergillus clavatus*, *A. niger*, *Chaetomium globosum*, *Cladosporium cladosporioides*, *Myrothecium verrucaria*, *Penicillium citrinum*, *Trichoderma viride*, *Trametes versicolor*, *Phanerochaete chrysosporium*, *Phaeolus schweinitzii* and *Lenzites sulphureus*. Based on the study, the authors opined that essential oil from *E. citriodora* could be an excellent choice as a wood preservative and preservation of leather goods and wood artifacts. In a similar kind of research work conducted by Suleiman & coworkers (2008) they mentioned that vegetative growth of *Fusarium* sp. at different concentrations of *Senna alata* extract were generally low compared to control. All the leaf extracts were effective in the reduction of the incidence of soil-borne pathogenic fungi tested except extracts from spearmint that did not give

significant reduction of the fungi when compared with untreated control seedlings. This result indicates that the leaf extracts probably have some fungicidal properties that inhibit the growth of the soil-borne fungi.

Thus the evidence obtained from present study advocated that plant metabolites of *Eucalyptus* reduced the growth of pathogen up to 98% and acted as defense weapons as 85% disease control was observed in protective assays. Further the high intensity of defense related enzymes indicated higher expression of plant defense metabolism under pathogenic stress.

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