IDENTIFICATION OF ANTIFUNGAL COMPOUNDS FROM LEAF EXTRACT OF *EUCALYPTUS CITRIODORA* AGAINST *ASCOCHYTA RABIEI*

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ABSTRACT: *Ascochyta rabiei* (Pass.) Lab. is a highly destructive pathogen of chickpea. In this study, leaf extract of *Eucalyptus citriodora* was assessed against this pathogen. Bioassays with methanolic extract (0, 05, 1.0, 1.5, …, 4.0%) extract revealed the remarkable antifungal potential of leaf extract where 69–94% reduction in biomass of *A. rabiei* was recorded. Chloroform fraction of this extract was separated by partitioning the extract in a separating funnel. Using a solvent system of chloroform: *n*-hexane (20:80), three compounds were detected on TLC plate which were separated through preparative TLC and purified on HPLC. GC-MS of the purified compounds lead to the identification of 3-cyclohexene- 1-ol, 4-methyl-1-(1-methylethyl)- **(1)**, 1-cyclohexene- 1-carboxaldehyde, 4-(1-methylethyl)- **(2)** and eucalyptol **(3)** that might caused antifungal activity of the extract.

Keywords: Antifungal activity, *Ascochyta rabiei*, chickpea blight, *Eucalyptus citriodora*, leaf extract, natural products.

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INTRODUCTION

Chickpea is the second most important spring-sown drought resistant leguminous crop plant grown widely in North Africa, West Asia, East Africa, South Asia, Australia, South and North America, and southern Europe (Merga and Haji, 2019). In Pakistan, it was grown on 867,000 ha with 319,000 tons production annually (Pakistan Economic Survey 2021-22). It has an impressive nutrition profile including proteins (16-24%), starch (18-35%), carbohydrates (45-58%) and a good quality of oligosaccharides (Dadon *et al.,* 2017). It is an essential constituent of human diet particularly for those who cannot afford proteins of animal origin or for the vegetarians by choice (Verma *et al.,* 2017). It not only plays important role in modern farming system but also improves nitrogen fixation in soil and increases the soil fertility (Garg and Singla, 2016). In Pakistan, chickpea production is very less than the demand due to many pathogenic fungal constrains responsible for leaf spot (*Alternaria* sp.), foot rot (*Sclerotium rolfsii*), powdery mildew (*Leviellula taurica*), gray mould (*Botrytis cinera*), rust (*Uromyces ciceris-arientini*) (Shurigin *et al.,* 2018; Motagi *et al.,* 2020), and blight (*Ascochyta rabiei*) (Javaid *et al.,* 2020a). Among these, ascochyta blight, a soil-borne fungal pathogen, is the major limiting constrain under favorable environmental conditions with 100% yield losses in chickpea growing areas (Javaid and Munir, 2012; Mengist *et al.,* 2019).

For the control of ascochyta blight, many foliar fungicides are in practice including maneb, ferbam, chlorothalonil, Bordeaux mixture, dithianon, propiconazole, penconazole, thiabendazole, sulfur and propineb (Ejeta *et al.,* 2017; Owati *et al.,* 2017). Whereas, the application of synthetic agro-chemicals should be discouraged as they are non-host specific, detrimental for beneficial microbes, having toxic effects and thus pollute the environment (Kumar, 2018). Therefore, there is a strong need to develop some plant derived, eco-friendly alternative management strategies to control diseases (Javaid *et al.,* 2018; Khan *et al.,* 2020; Jabeen *et al.,* 2021, 2022). *Eucalyptus citriodora* is a medicinal plant grown widely in Indonesia, Tasmania, Australia, Brazil, Africa, India and Pakistan (Franco *et al.,* 2016). The plant leaves possess secondary metabolites include eucalyptol, citronellal, phenolics, hyperoside, hyperin, flavonoids, tannins, rutin, quercitrin, ketones, aldehydes and sesquiterpenes enriched with antibacterial, antifungal, antiseptic, antimicrobial, antispasmodic, diuretic, deodorant and anti-inflammatory properties (Tolba *et al.,* 2018). Previously, *E. citriodora* leaf extracts were also tested against the pathogenic fungal strains namely *Colletotrichum gloeosporioides*, *Candida albicans*, *Rhizoctonia solani*, *Fusarium oxysporum*, *Microsporum gypsum*, *Trichophyton mentagrophytes*, *Helminthosporium oryzae* (Lee *et al.,* 2007; Musyimi and Ogur, 2008; Shafique *et al.,* 2015). The present investigation was undertaken to manage the *Ascochyta rabiei* pathogen responsible for ascochyta

blight in chickpeas by practicing the *Eucalyptus citriodora* leaf methanolic extracts and identification of compounds through TLC followed by GC-MS study.

MATERIALS AND METHODS

Leaf extract: Leaves were collected from a mature tree of *E. citriodora* from Lahore, Pakistan. After washing and air drying, leaves were cut into pieces, dried at 45 ºC and crushed. The crushed leaves (500 g) were extracted in methanol (2.0 L). After two weeks, solvent was separated from soaked material through filtration and evaporated at 45 ºC with the help of a rotary evaporator. Bioassay with 0.5, 1.0, 1.5, 2.0, …, 4% concentrations of methanolic extract was done in flasks in triplicate following procedure of Amin and Javaid (2013). After 10 days, fungal biomass was filtered, dried and weighed. Percentage reduction in fungal biomass over control was also calculated.

Isolation and identification of compounds: Crude methanolic extract was mixed with water (300 mL) and partitioned using *n*-hexane (5 × 500 mL) and then chloroform (400 mL). The last fraction was evaporated under reduced pressure. From this fraction, three compounds were detected on TLC eluting with chloroform: *n*-hexane (20:80). To isolate these compounds, preparative thin layer chromatography (PTLC) was employed using 20 × 20 cm^2 silica gel plates. With the help of a fine needle, compounds were separated from plates and dissolved in a 5:5 mixture of chloroform: methanol. The solvent was filtered and evaporated at 30 ºC.

The separated compounds were purified through HPLC using HiQ Sil C18, 4.6 × 250 mm, 5 micron column. A volume of 20 µL of each sample was used with twenty minutes run time. Detection was carried out at 270 nm wavelength and major peak were collected (Figure 1). Purified compounds were identified through GC-MS analysis.

Statistical analysis: Data were analyzed by ANOVA and LSD test using computer software Statistix 8.1.

RESULTS AND DISCUSSIONS

Antifungal activity of leaf extract: Leaf extract was highly inhibitory against *A. rabiei*. Different concentrations declined biomass of the pathogen by 69–94% (Figure 2A and B). There was a polynomial relationship between extract concentrations and *A. rabiei* biomass with $R^2 = 0.7734$ (Fig. 2C). Previously, Amin *et al.* (2012) evaluated the antifungal efficacy of *E. citriodora* bark and fruit methanolic extracts against the *A. rabiei.* The root-bark extract showed maximum growth inhibition by 72–89% of the tested pathogen. Earlier, *E. citriodora* fruit *n*-hexane and ethanolic extracts have been reported very effective in suppressing the growth of *A. rabiei* (Jabeen and Javaid 2008). Likewise, Iram *et al.* (2018) worked on *E. citriodora* leaf aqueous extracts and tested against *Aspergillus flavus* and *A. rabiei* with promising results. Moreover, Javaid *et al.* (2020b) observed a significant reduction against chili southern blight pathogen namely *Sclerotium rolfsii* by using *E. citriodora* leaf methanolic extracts.

Identification of compounds: Three compounds were isolated and identified in the present study. These were 3-cyclohexene- 1-ol, 4-methyl-1-(1-methylethyl)- **(1)** having formula $C_{10}H_{18}O$ and molecular weight (MW) 154; 1-cyclohexene- 1-carboxaldehyde, 4-(1-methylethyl)- **(2)** with formula $C_{10}H_{16}O$ and MW 152; and eucalyptol **(3)** having formula $C_{10}H_{18}O$ and MW 154 (Figure 3).

Compound **1** was previously identified in *Artemisia lavandulaefolia* extracts with fungicidal and insecticidal properties (Huang *et al.*, 2018). It was also isolated from the extracts of *Cupressocyparis leylandii* and tested against pathogenic fungi *viz. Fusarium oxysporum*, *Alternaria alternata*, *Candida albicans* and *Paecilomyces lilacinus* with promising results (Wang *et al.*, 2012; Johnson *et al.*, 2013). Likewise, compound **2** was identified from oil of *Artemisia lavandulaefolia* with potent antifungal activities against *Alternaria solani* (Huang *et al.*, 2019). It was also isolated from a medicinal plant *Cistus salviifolius* ethanolic extracts and tested against bacterial and pathogenic fungal strains. The compound showed maximum inhibition against the *Verticillium fungicola, Streptococcus anginosus and Aspergillus niger* (Soto *et al*., 2015). Moreover, the compound was also found in oil of *Artemisia nilagirica* with strong antifungal activities against *Aspergillus flavus* (Kumar *et al.* 2019). Similarly, previously compound **3** was tested against pathogenic fungal strains namely *Fusarium oxysporum, F. culmorum, F. verticillioides, F. subglutinans, F. sporotrichioides, F. cerealis,* and *A. alternata*. A maximum percent inhibition was checked in *F. culmorum* isolate (Morcia *et al.*, 2012). Previously, Rosello *et al.* (2015) isolated the compound from the extracts of *Cinnamomum zeylanicum* and tested against *Fusarium culmorum* and *F. verticillioides*. The compound showed excellent antifungal efficacies against the both pathogenic *Fusarium* spp. Previously, this compound was also found in different leaf extracts of *Alpinia allughas* and assessed against *R. solani, C. falcatum, S. rolfsii* and *S. sclerotium*. The studies showed that the compound showed maximum inhibitory effect against all the tested fungal pathogens (Sethi *et al.*, 2015).

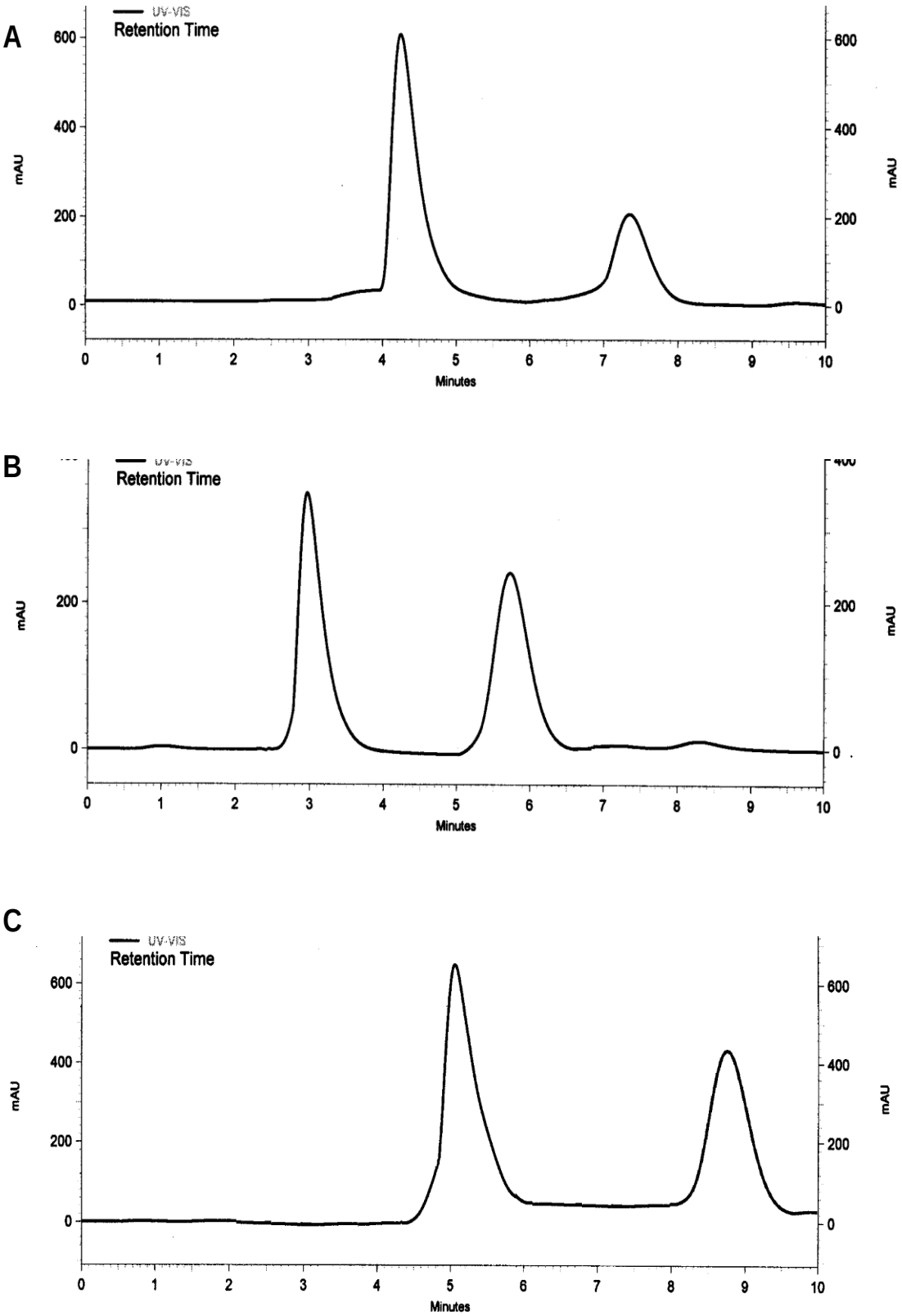


Fig. 1: HPLC chromatograms of sub-fractions of chloroform fraction of methanolic leaf extract of *Eucalyptus citriodora* isolated using PTLC.

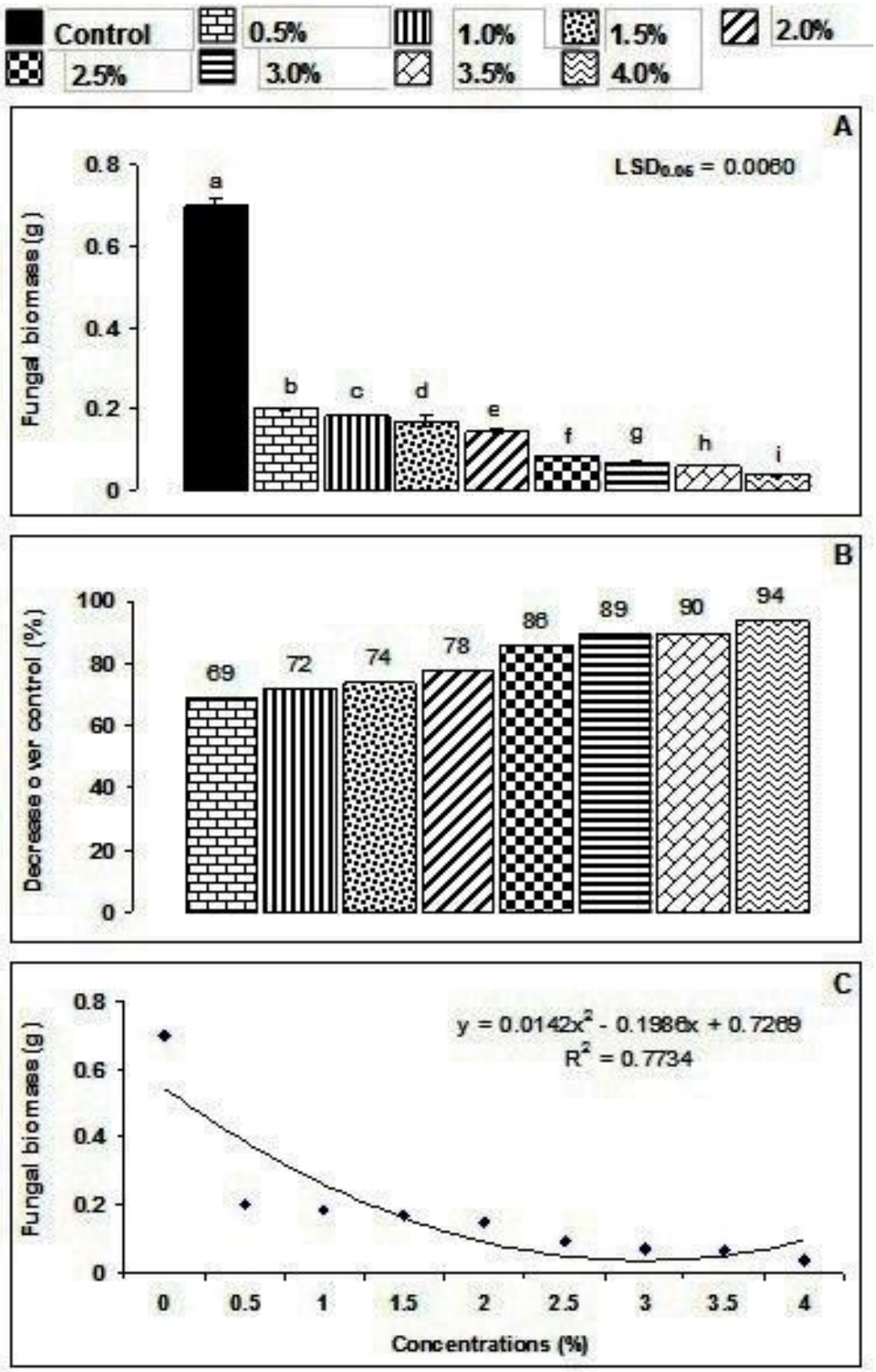
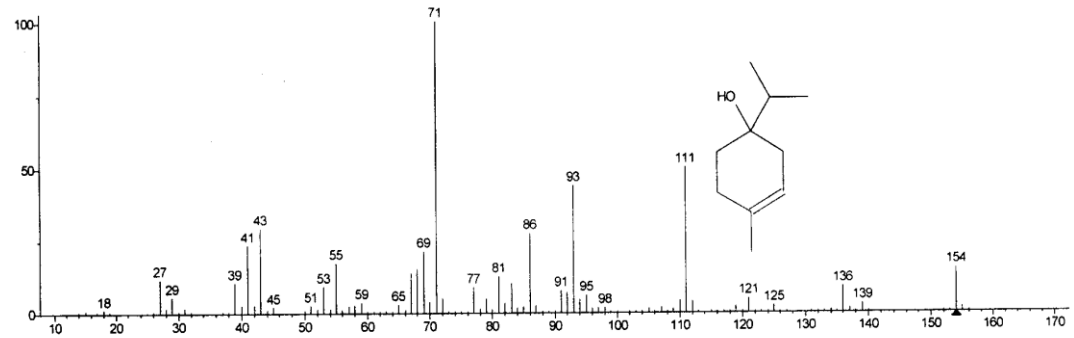
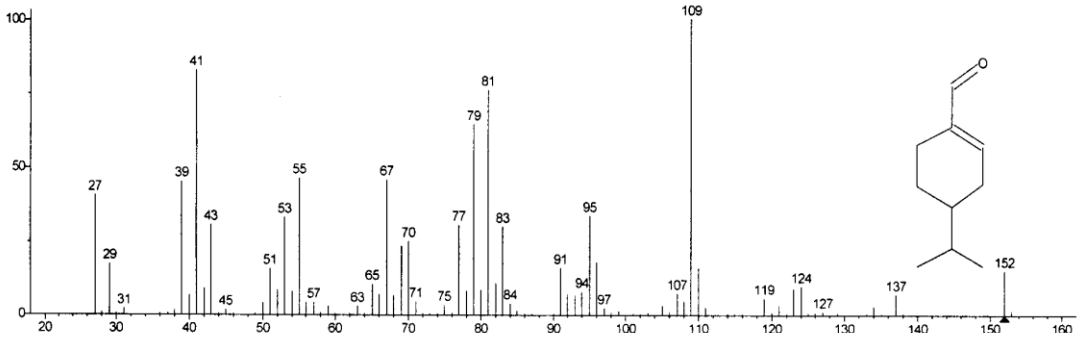


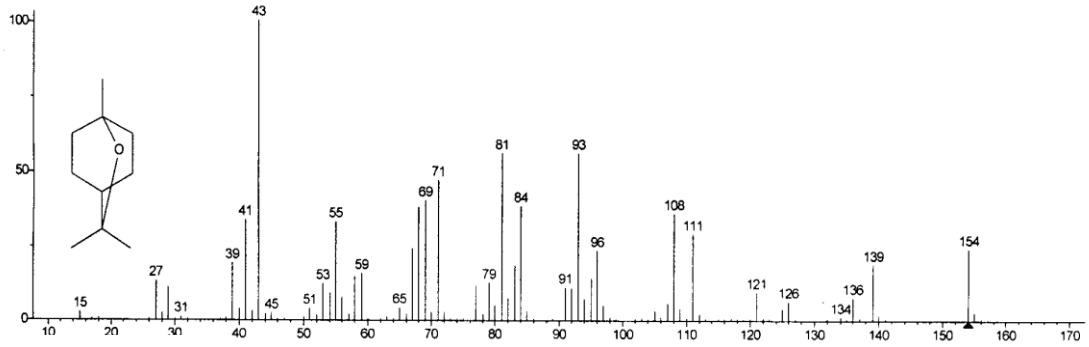
Fig. 2: Effect of methanolic leaf extract of *Eucalyptus citriodora* on biomass of *Ascochyta rabiei.* Vertical bars show standard errors of means of four replicates. Values with different letters at their top show significant difference (P≤0.05) as determined by LSD Test.



1. 3-Cyclohexene 1-ol, 4-methyl-1-(1-methylethyl)-



2. 1-Cyclohexene- 1-carboxaldehyde, 4-(1-methylethyl)-



3. Eucalyptol

Fig. 3: Structures of compounds isolated from chloroform fraction of methanolic leaf extract of *Eucalyptus citriodora*

Conclusions: Leaf extract of *E. citriodora* was very effective where a 4% concentration of the extract reduced fungal biomass up to 94%. The three identified compounds have been reported as antifungal agents against other fungal species in the previous literature and could also be responsible for inhibitory effect of *E. citriodora* extract against *A. rabiei* in this study.

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