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Article

ANTIFUNGAL ACTIVITY OF AERIAL PARTS OF *Cenchrus pennisetiformis* AGAINST *Fusarium oxysporum* f. sp. *lycopersici*

*Atividade Antifúngica da Parte Aérea de **Cenchrus pennisetiformis** contra **Fusarium oxysporum** f. sp. **lycopersici***

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ABSTRACT - Antifungal potential of aerial parts of an allelopathic grass *Cenchrus pennisetiformis* (Hochst. & Steud.) Wipff. was evaluated against *Fusarium oxysporum* f. sp. *lycopersici* Snyder & Hansen, a fungal pathogen causing wilt disease in tomato (*Solanum lycopersicum* L.). Different concentrations (1% to 6%) of methanolic leaf, stem and inflorescence extract of the grass significantly reduced fungal biomass by 40-88%, 13-89%, and 26-76%, respectively. Methanolic shoot (leaf + stem) extract was fractionated using four organic solvents viz. *n*-hexane, chloroform, ethyl acetate and *n*-butanol. All the sub-fractions of methanolic shoot extract showed remarkable antifungal potential to variable extents. Different concentrations (1.56-200 mg mL^{-1}) of ethyl acetate sub-fraction exhibited the best antifungal activity resulting in 49-100% suppression in the fungal biomass. GC-MS analysis of ethyl acetate sub-fraction showed the presence of 10 compounds. Phenol, 2,4-bis{1,1-dimethlethyl}- was the major compound (30.99%) followed by hexadecanoic acid, ethyl-ester (21.72%), benzofuran 2,3-dihydro (10.65%), 1-propanol-2-2-hydroxypropxy (10.60%) and 1-eicosene (8.32%).

Keywords: antifungal activity, GC-MS analysis, methanolic extracts.

*RESUMO - Foi realizada uma avaliação do potencial antifúngico da parte aérea da gramínea alelopática **Cenchrus pennisetiformis** (Hochst. & Steud.) Wipff. contra **Fusarium oxysporum** f. sp. lycopersici Snyder & Hansen, um patógeno fúngico que causa a doença de murcha no tomate (**Solanum lycopersicum** L.). Diferentes concentrações (1% a 6%) do extrato metanólico da folha, do caule e da inflorescência da gramínea reduziram significativamente a biomassa fúngica em 40-88%, 13-89% e 26-76%, respectivamente. O extrato metanólico (folha + caule) foi fracionado, utilizando-se quatro solventes orgânicos: n-hexano, clorofórmio, acetato de etila e n-butanol. Todas as subfrações de extrato metanólico da parte aérea apresentaram significativo potencial antifúngico em graus variáveis. Concentrações diferentes (1,56-200 mg mL^{-1}) da subfração do acetato de etila exibiram a melhor atividade antifúngica, com supressão de 49-100% na biomassa fúngica. A análise CG-EM da subfração do acetato de etila revelou a presença de 10 compostos. O principal composto (30,99%) foi o fenol, 2,4-bis{1,1-dimetiletil}, seguido de ácido hexadecanoico, éster etílico (21,72%), 2,3-di-hidrobenzofurano (10,65%), 1-propanol-2-2-hidroxipropil (10,60%) e 1-eicoseno (8,32%).*

Palavras-chave: atividade antifúngica, análise por CG-EM, extratos metanólicos.

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INTRODUCTION

Tomato (*Solanum lycopersicum*) is an important horticultural crop around the globe. The importance of tomato as a food item is due to presence of dietary fibers, vitamins A, B and C, sugar, amino acids and various nutrient elements including N, K, P, Ca, Mg, Fe (Kumar et al., 2008). Pakistan ranks 35th in the world's tomato production; this crop is cultivated in 58.2 thousands hectares with total production of 574.1 thousand tonnes. The average yield of tomato in Pakistan is 9.9 ton ha^{-1} (Pakistan, 2013), which is very low. Various biotic and abiotic factors are responsible for this low yield of tomato in Pakistan. Among the various biotic factors, fungal diseases are the most important in damaging this crop. One of the important fungal diseases is Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *lycopersici* (Abdel-Fattah and Al-Amri, 2012). The pathogen has three races (Morid et al., 2012), among which races 1 and 2 have worldwide distribution while race 3 has a limited geographical range (Reis and Boiteux, 2007).

Chemical fungicides, namely prochloraz and carbendazim, have been proved highly effective against *in vitro* growth of *F. oxysporum* f. sp. *lycopersici* (Song et al., 2004). Likewise, Amini and Sidovich (2012) reported that prochloraz and bromuconazole were highly effective against this pathogen both *in vitro* and *in vivo*, followed by carbendazim and benomyl. However, because of hazardous effects of synthetic pesticides on health and the environment, scientists are focusing on alternative environment-friendly disease management strategies, including the use of natural antifungal compounds from plants (Javaid et al., 2015; Sana et al., 2016a). Many recent studies have shown that extracts of allelopathic grasses, namely *Dicanthium annulatum* (Forssk.) Stapf., *Sorghum halepense* (L.) Pers. and *Imperata cylindrica* (L.) P. Beauv, exhibit antifungal activity against *Macrophomina phaseolina* and *F. oxysporum* f. sp. *cepae* (Javaid et al., 2012; Naqvi et al., 2012; Javaid et al., 2015). *C. pennisetiformis* is a drought tolerant, allelopathic grass commonly growing along roadsides and open places in Pakistan. It is known to possess herbicidal activity against parthenium (*Parthenium hysterophorus* L.) and antifungal activity against *M. phaseolina* (Javaid and Anjum, 2006; Javaid and Naqvi, 2012). Given the above, the present study was carried out to evaluate the antifungal activity of its shoot gainst *F. oxysporum* f. sp. *lycopersici.*

MATERIALS AND METHODS

Preparation of methanolic extracts

Stem, leaves and inflorescence of *C. pennisetiformis* were collected from different areas of Lahore, Pakistan. Each part was rinsed with tap water and dried in an electric oven at 45 °C. Two hundred grams of each of the ground plant parts were soaked in 2 L methanol in air tight glass jars separately for 15 days at room temperature. Afterwards, the soaked materials were passed through cheese cloth to separate debris and then filtered through Whatman No. 1 filter paper and methanol was evaporated on a rotary evaporator at 45 °C under reduced pressure. Finally, thick pastes of 13.1 g, 14.1 g and 12.9 g of leaf, stem, and inflorescence extracts, respectively, were obtained (Banaras et al., 2017).

Evaluation of antifungal activity of methanolic extracts

Methanolic extracts (12.6 g) of each of the three plant parts were dissolved in 6 mL dimethyle sulphoxide (DMSO) and distilled autoclaved water was added to prepare 21 mL of the stock solutions. Similarly, the control solution was prepared by adding 6 mL DMSO in 15 mL distilled water. Seventy-four milliliters of malt extract was autoclaved in a 250 mL flask and cooled at room temperature. Six concentrations viz. 1%, 2%, 3%, 4%, 5% and 6% were prepared by adding 1, 2, 3, 4, 5 and 6 mL of stock solution along with 5, 4, 3, 2, 1 and 0 mL of control solution, respectively, to produce final volume of 80 mL for each concentration. The 80 mL volume of each treatment was divided into four equal portions in 100 mL flask to serve as replicates. The control treatment was prepared by adding 6 mL control solution to 74 mL of malt extract. The purpose of the control solution was to maintain the same quantity of DMSO in all the treatments. The flasks were inoculated with 500 µL of conidial suspension (1 x 10^{9} conidia mL^{-1}) of *F. oxysporum* f. sp. *lycopersici.* Flasks were

incubated for 10 days in an incubator at 27 °C. Fungal mycelium was harvested by filtering the fungal mat through pre-weighed filter papers followed by oven drying at 60 °C to get dry biomass from each flask (Javaid and Akhtar, 2015).

Bioassays with sub-fractions of methanolic shoot extract

C. pennisetiformis shoot (leaf + stem) extract was prepared by soaking 2 kg crushed plant material in 7 L methanol for 15 days. After filtration and evaporation on a rotary evaporator, the extract was mixed in 300 mL sterilized distilled water and partitioned in a separating funnel using *n*-hexane, chloroform, ethyl acetate and *n*-butanol. After partitioning, the solvents were evaporated on a rotary evaporator to obtain *n*-hexane (15.74 g), chloroform (12.15 g), ethyl acetate (10.9 g), *n*-butanol (9.8 g) and aqueous (25.21 g) sub-fractions. These fractions were evaluated for their antifungal activity using the serial dilution method as described by Javaid et al. (2015). The inoculum was prepared by suspending fungal conidia in distilled water. For this purpose, 1.2 g of each of the five sub-fractions of methanolic extract was dissolved in 1 mL DMSO and added to 5 mL autoclaved malt extract broth. This stock solution (200 mg mL^{-1}) was serially double diluted by adding malt extract broth to prepare lower concentrations viz., 100, 50, 25, 12.5, 6.25, 3.125 and 1.15 mg mL^{-1}. For control, 1 mL of DMSO was dissolved in 5 mL malt extract broth and serially double diluted to prepare controls corresponding to various extract concentrations. Bioassays were conducted in 10 mL volume glass test tubes each containing 1 mL of growth medium. Test tubes were inoculated aseptically with 15 µL of conidial suspension of *F. oxysporum* f. sp. *lycopersici*. Each treatment was replicated three times. Test tubes were incubated at 27 °C for 7 days and then fungal biomass in each test tube was filtered, dried to constant weight and weighed. The inhibitory effect of different sub-fractions of methanolic shoot extracts against *F. oxysporum* f. sp. *lycopersici* was calculated in terms of fungal biomass produced in each treatment and compared with fungal biomass in the corresponding control treatment.

GC-MS analysis of ethyl acetate sub-fraction of the shoot

GC-MS analysis was performed in a Perkin Elmer Turbo Mass Spectrophotometer (Norwalk, CTO6859, and USA). Helium was used as a carrier gas with a flow rate of 0.5 mL min^{-1}. Inlet temperature of the instrument was 250 °C. Oven was preset at 110 °C for 4 min, raised up to 280 °C and run time was finished in 90 minutes. Temperature of the MS transfer line was 200 °C and that of the source was 180 °C. One microliter of sample was used in injection. Electron impact ionization (70 eV) was used for identification of compounds. For measurement of peak areas and data processing, the Turbo-Mass-OCPTVS-Demo SPL software was used.

Statistical analysis

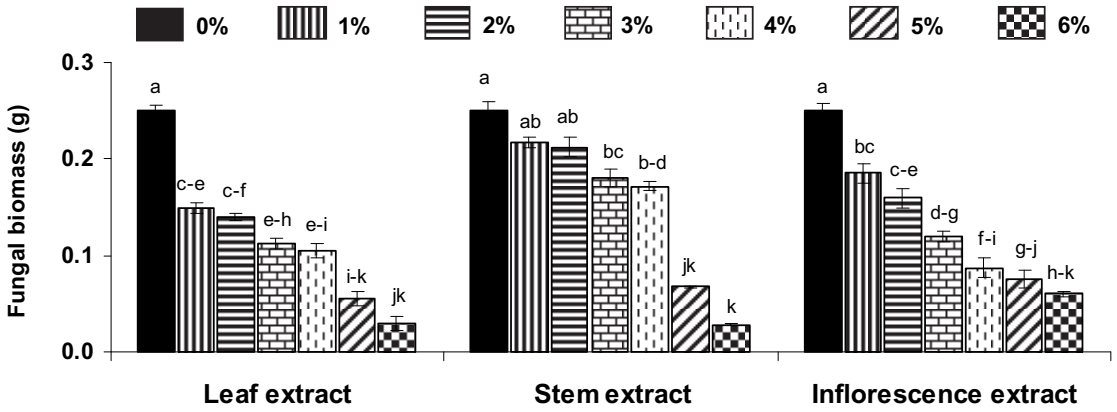
All the data of laboratory bioassays were subjected to ANOVA followed by separation of treatment means by Tukey's HSD test at 5% level of significance using the computer software Statistix 8.1.

RESULTS AND DISCUSSION

Antifungal activity of mathanolic extracts

Methanolic extracts of all the three parts of *C. pennisetiformis* were found highly effective in suppressing *in vitro* growth of *F. oxysporum* f. sp. *lycopersici.* Different concentrations (1-6%) of leaf, stem, and inflorescence extracts significantly ($P \leq 0.05$) reduced fungal biomass by 40-88%, 13-89%, and 26-76%, respectively (Figure 1). There was a polynomial relationship between fungal biomass and concentrations of methanolic extract of leaf and stem with $R^2 = 0.9236$ and 0.9577, respectively. On the other hand, the relationship between fungal biomass and different concentrations of inflorescence extract was linear with $R^2 = 0.9468$ (Figure 2). Previous studies showed that *C. pennisetiformis* has antifungal potential against *M. phaseolina*

(Javaid and Naquvi, 2012). The use of methanolic extracts is a very useful technique for screening different parts of plants for their antifungal activity (Javaid and Bashir, 2015; Sana et al., 2016b). In the present study, screening trials showed that the leaf and stem extracts possessed greater antifungal activity than the inflorescence extract; therefore, the leaf + stem extracts were selected for further experiments to assess antifungal activity of sub-fractions of the methanolic extract.



The vertical bars show standard errors of means of four replicates.

Values with different letters at their top show significant difference ($p \leq 0.05$) as determined by Tukey's HSD test.

Figure 1 - Effect of different concentrations of methanolic extracts of aerial parts of *Cenchrus pennisetiformis* on growth of *Fusarium oxysporum* f. sp. *lycopersici*.

Antifungal activity of sub-fractions of the methanolic shoot extract

Among the five sub-fractions of the methanolic shoot extract, the ethyl acetate sub-fraction exhibited the highest antifungal activity against *F. oxysporum* f. sp. *lycopersici* (Figure 3). Lower concentrations (1.56-6.25 mg mL^{-1}) of this sub-fraction significantly ($P \leq 0.05$) reduced fungal biomass by 50-60%, and further increase in concentrations (12.5-200 mg mL^{-1}) resulted in 100% growth inhibition. The other highly effective antifungal sub-fractions were chloroform and *n*-hexane, where different concentrations generally showed a significant adverse effect, resulting in 40-100% decline in fungal biomass. The *n*-butanol sub-fraction also proved effective in inhibiting fungal biomass by 24-50% in the concentrations of 1.56-25 mg mL^{-1}, and by 100% in the rest of the concentrations. The aqueous fraction was found to be effective at higher concentrations (100 and 200 mg L^{-1}). A similar variable antifungal potential of different organic solvent fractions of methanolic extracts of *Withania somnifera, Coronopus didymus, Chenopodium album* and *Datura metel* has also been reported against various fungal pathogens including *F. oxysporum* (Iqbal and Javaid, 2012; Javaid and Munir, 2012; Javaid and Saddique, 2012; Javaid et al., 2015). Variability in antifungal activity of different sub-fractions possibly happened because of different polarity compounds in different organic solvents (Rauf and Javaid, 2013). It could be speculated that some compounds could be more active in the ethyl acetate sub-fraction as compared to other organic solvent fractions. Increase in inhibition of fungal growth with increased concentrations of the extract could result from the intensification of antioxidant potential of secondary metabolites in the extract (Pandey et al., 2010).

GC-MS analysis of the ethyl acetate sub-fraction

A total of 10 phytoconstituents were recorded in the ethyl-acetate sub-fraction of the methanolic shoot extract of *C. pennisetiformis* (Figure 4 and 5; Table 1). These compounds were phenol, 2,4-bis{1,1-dimethlethyl}-(30.99%); hexadecanoic acid, ethyl-ester (21.72%); 2,3-dihydro 1-benzofuran (10.65%); 1-propanol-2-2-hydroxypropxy (10.60%); 1-eicosene (8.32%); E-15-heptadecenal (5.80%); heptanal, 2 [phenylmethylene]- (3.59%); ethanone 1-[2,4,5-trimethoxypenyl]-(3.05%); benzene, 1 {1,1-dimethylethyl]-3,5-dimethyl-2,4,6-trinitro (2.90%) and

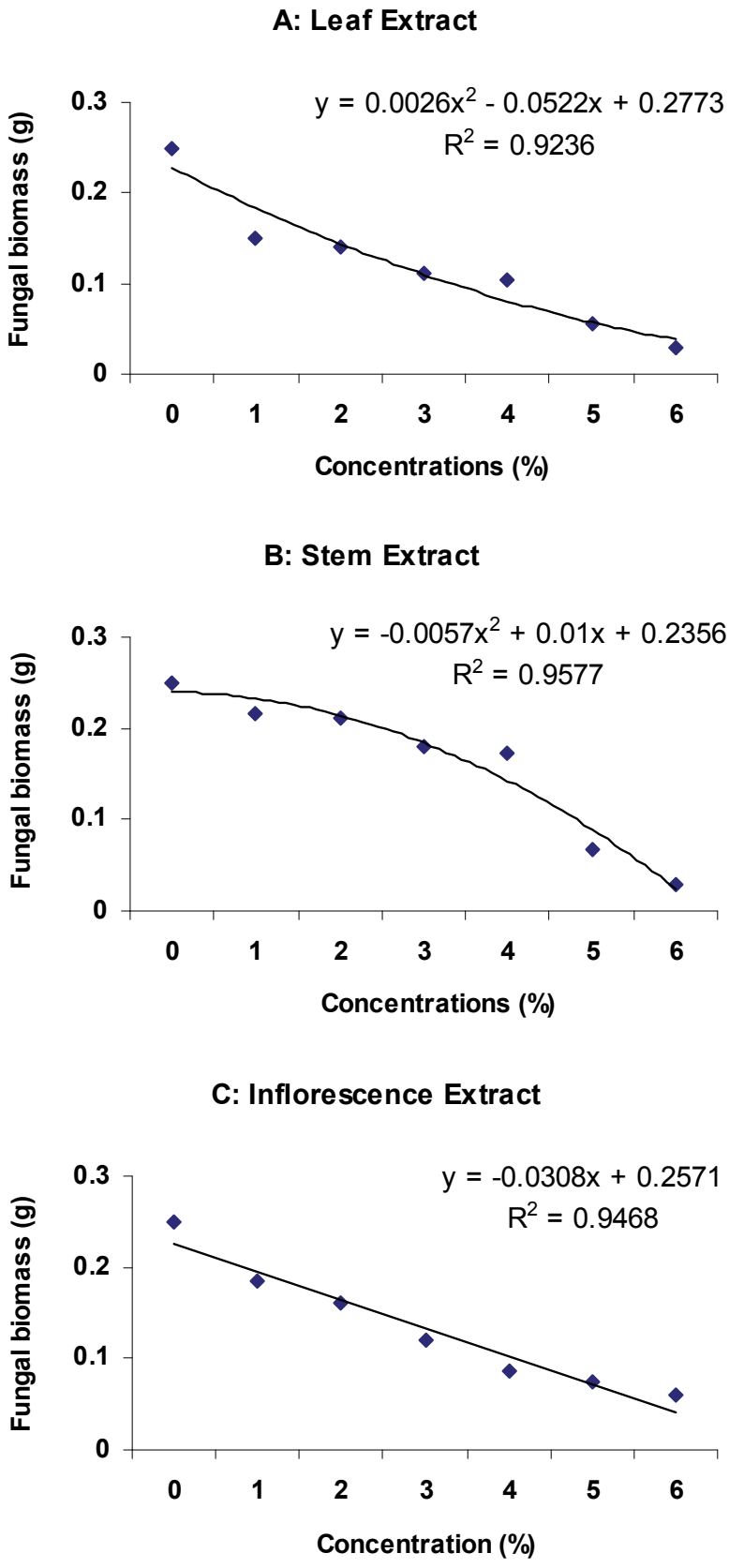
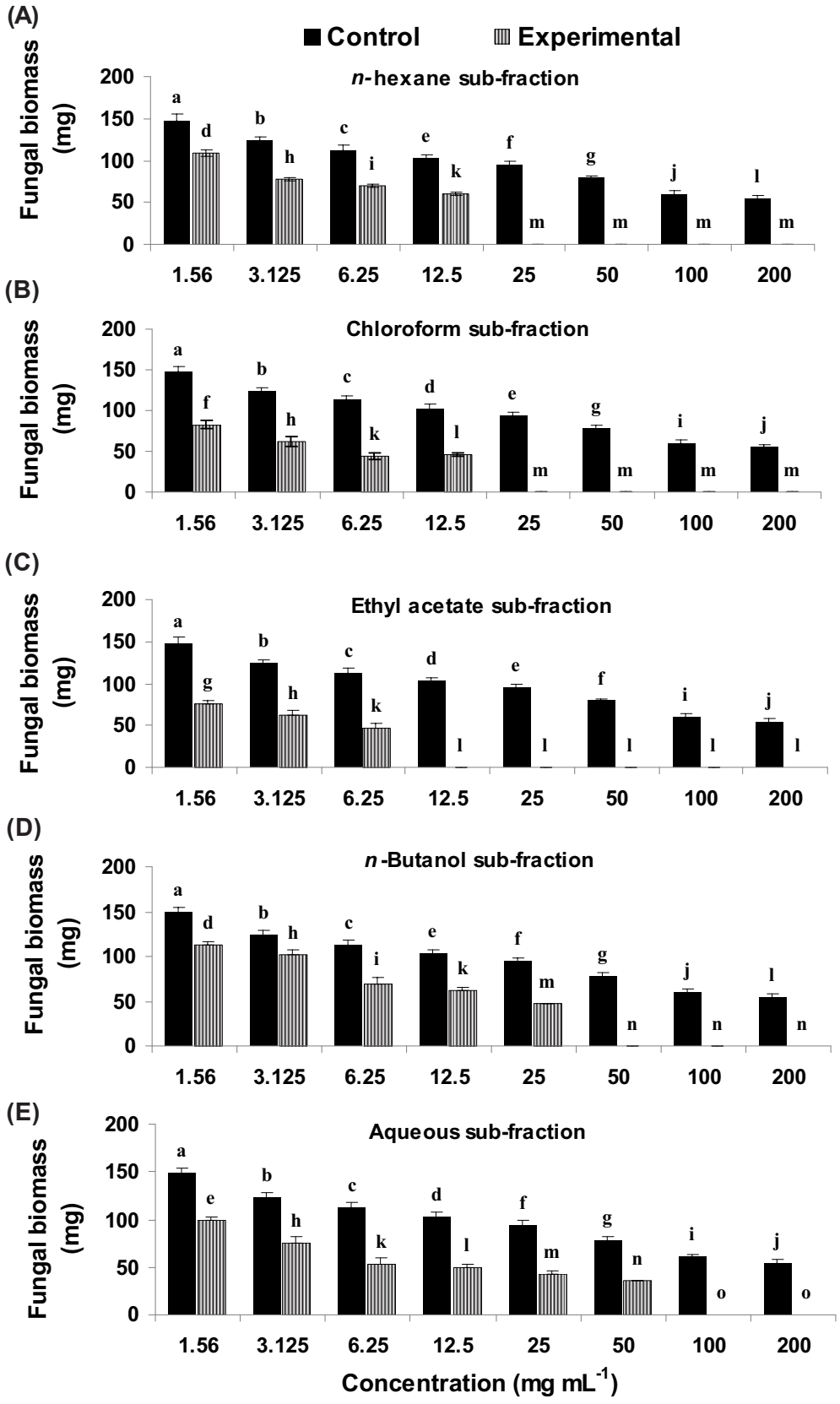


Figure 2 - Relationship between concentrations of different methanolic extracts of *Cenchrus pennisetiformis* and biomass of *Fusarium oxysporum* f. sp. *lycopersici*.



The vertical bars show standard errors of means of four replicates. The values with different letters at their top show significant difference ($p \leq 0.05$) as determined by Tukey's HSD Test.

Figure 3 - Effect of different concentrations of sub-fractions of the methanolic shoot extract of *Cenchrus pennisetiformis* on the growth of *Fusarium oxysporum* f. sp. *lycopersici*.

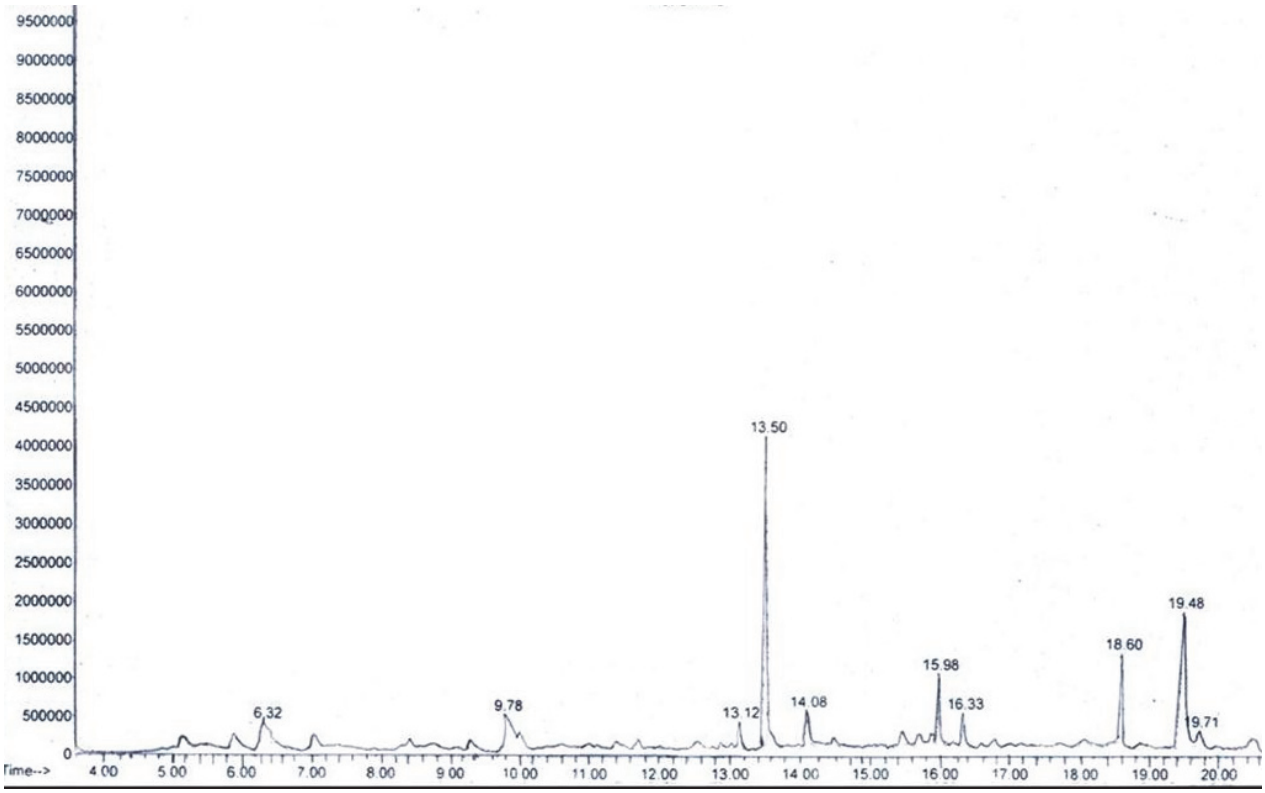


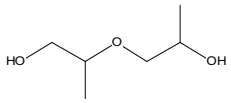
Figure 4 - GC-MS chromatogram of the ethyl acetate sub-fraction of the methanolic shoot extract of *C. pennisetiformis.*

Table 1 - Compounds identified from the ethyl-acetate sub-fraction of the methanolic shoot extract of *Cenchrus pennisetiformis* through GC-MS analysis

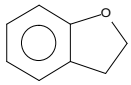
Comp. No.	Names of compounds	Molecular formula	Molecular weight	Retention time (min)	Peak area (%)
1	1-Propanol-2-2-hydroxypropxy	$C_6H_{14}O_3$	134	6.137	10.60
2	Benzofuran 2,3-dihydro	C_8H_8O	120	9.779	10.65
3	1-hexadecanol, 2 methyl-	$C_{17}H_{36}O$	256	13.120	2.39
4	Phenol, 2,4-bis{1,1-dimethlethyl}-	$C_{14}H_{22}O_2$	206	13.504	30.99
5	Ethanone, 1-[2,4,5-tri ethoxypenyl]-	$C_{11}H_{14}O_4$	210	14.082	3.06
6	E-15-heptadecenal	$C_{17}H_{32}O$	252	15.976	5.80
7	Heptanal, 2-[phenylmethylene]-	$C_{14}H_{18}O$	202	16.330	3.59
8	1-Eicosene	$C_{20}H_{42}$	280	18.596	8.32
9	Hexadecanoic acid, ethyl-ester	$C_{18}H_{36}O_2$	284	19.478	21.72
10	Benzene, 1-{1,1-dimethylethyl]-3,5-dimethyl-2,4,6-trinitro	$C_{12}H_{15}N_3O_6$	297	19.706	2.90

hexadecanol, 2 methyl- (2.39%). Presence of phenol, 2,4-bis{1,1-dimethlethyl} in the highest amount could provide the basis of great fungicidal activity in *C. pennisetiformis*. Phenol, 2,6-bis(1,1-dimethylethyl)-4-methyl, commonly known as butylated hydroxytoluene, is an antioxidant and has been demonstrated as an antimicrobial agent (Sova, 2012). 1-Eicosene has been isolated from many plants of medicinal values and hold significant antimicrobial potential (Nwodo et al., 2015). 1-[2,4,5 triethoxyphenyl] ethanone is widespread in plants and its fungicidal action has been reported against many devastating phytopathogens such as *Colletotrichum capsici* and *Rhizoctonia cerealis* (Yuqin et al., 2015). Organic compounds, e.g., hexadecanoic acid, ethyl-ester and hexadecanol, 2 methyl are well-known for their antiviral, insecticidal and antibacterial activity (Sujatha et al., 2014; Mihigo et al., 2015). Benzofuran, also known as methylcoumaran, is an important class of heterocyclic compounds; it has a significant position in medical chemistry

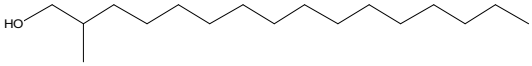
and has shown biological activity against a wide spectrum of bacteria and fungi (Kossakowski et al., 2010). Many compounds such as 1-propanol-2-2-hydroxypropxy; benzene and 1-{1,1-dimethylethyl]-3,5-dimethyl-2,4,6-trinitro have been isolated from the methanolic extract of *Aegle marmelos* (golden apple or bael) and hold antimicrobial activity (Mujeeb et al., 2014). Therefore, the presently reported antifungal potential of *C. pennisetiformis* against *F. oxyporum* f. sp. *lycopersici* could be attributed to the occurrence of many important compounds belonging to phenolics.



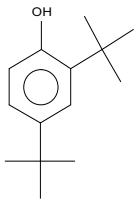
1. 1-propanol-2-2-hydroxyproppxy



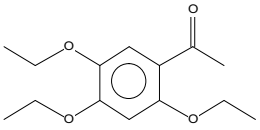
2. Benzofuran 2,3-dihydro



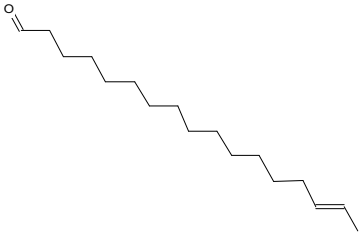
3. 1-hexadecanol, 2 methyl



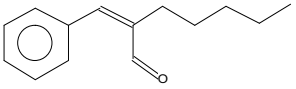
4. Phenol,2,4–bis{1,1-dimethlethyl}-



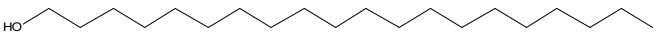
5. Ethanone, 1-[2,4,5-triethoxypenyl]-



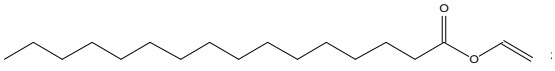
6. E-15-heptadecenal



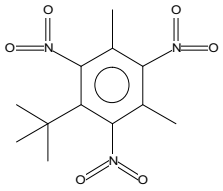
7. heptanal, 2[phenylmethlene]-



8. 1-Eicosene



9. Hexadecanoic acid, ethyl-ester



10. Bezene , 1-{1,1-dimethylethyl]-3,5-dimetyl-2,4,6-trinitro-

Figure 5 - Structures of compounds identified from the ethyl acetate sub-fraction of the methanolic shoot extract of *Cenchrus pennisetiformis* through GC-MS analysis.

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