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Article

EVALUATION OF ANTIFUNGAL POTENTIAL OF LEAF EXTRACT OF *Chenopodium murale* AGAINST *Fusarium oxysporum* F. SP. *lycopersici*

*Avaliação do Potencial Antifúngico do Extrato de Folha de **Chenopodium murale** contra **Fusarium oxysporum** f. sp. **lycopersici***

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ABSTRACT - The present study was performed to evaluate antifungal activity and GC-MS analysis of leaf extract of *Chenopodium mural* against *Fusarium oxysporum* f. sp. *lycopersici* (FOL), a highly problematic soil-borne pathogen of tomato. Dried leaves of *C. murale* were extracted with methanol for 2 weeks and after evaporating the solvent on a rotary evaporator, antifungal bioassay was carried out against FOL. All concentrations (1 to 5%) significantly reduced FOL biomass by 14-45%. The remaining methanolic extract was fractioned with *n*-hexane, chloroform and ethyl acetate and all these fractions were assayed for their antifungal potential. A 200 mg mL^{-1} concentration of various sub-fractions reduced fungal biomass significantly by 94-98% over control. All the sub-fractions were subjected to GC-MS analysis that revealed presence of 32 compounds in *n*-hexane, 2 compounds in chloroform and 13 compounds in ethyl acetate sub-fraction. The predominant compounds in *n*-hexane sub-fraction were hexadecanoic acid, methyl ester (14.64%), methyl linolenate (16.61%) and g-sitosterol (13.53%). In chloroform sub-fraction, bis (2-ethylhexyl) phthalate (92.31% and in ethyl-acetate sub-fraction, ethyl butyrate (19.57%), dihexyl phthalate (11.19%) and dioctyl phthalate (12.16%) were present in higher concentration.

Keywords: Fusarium wilt, GC-MS analysis, tomato.

RESUMO **-** *O presente estudo foi realizado para avaliar a atividade antifúngica e a análise por CG-EM do extrato de folhas de **Chenopodium murale** contra **Fusarium oxysporum** f. sp. **lycopersici** (FOL), um patógeno de tomate altamente problemático no solo. Folhas secas de **C. murale** foram extraídas com metanol por duas semanas e, após a evaporação do solvente em evaporador rotatório, foi realizado um bioensaio antifúngico contra a FOL. Todas as concentrações (1% a 5%) reduziram significativamente a biomassa de FOL em 14-45%. O restante do extrato metanólico foi fracionado com n-hexano, clorofórmio e acetato de etila, e todas essas frações foram analisadas quanto ao seu potencial antifúngico. Uma concentração de 200 mg* mL^{-1} *de várias subfrações reduziu significativamente a biomassa fúngica em 94-98%, em relação ao controle. Todas as subfrações foram submetidas à análise por GC-MS, que revelou a presença de 32 compostos em n-hexano, 2 compostos em clorofórmio e 13 compostos em subfração de acetato de etila. Os compostos predominantes na subfração n-hexano foram ácido hexadecanoico, metil éster (14,64%), metil linolenato (16,61%) e g-sitosterol (13,53%). Na subfração de clorofórmio, o bis (2-etil-hexil) ftalato (92,31%) e, na subfração etil-acetato, etilbutirato (19,57%), di-hexil ftalato (11,19%) e dioctilftalato (12,16%) estavam presentes em maior concentração.*

Palavras-chave: Fusariose, análise por GC-MS, tomate.

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INTRODUCTION

Tomato is an economically very important and major crop grown on the global scale. Tomatoes are very nutritious being full of vitamins like vitamin A, B and C. Other essential minerals such as phosphorus, lycopene and β-carotene are also abundantly found in tomatoes (Anitha and Rabeeth, 2009). Tomato is considered an important crop in Pakistan. It is used as main ingredient in salads and is cooked with other vegetables. In Pakistan, tomato is cultivated on an area of 54.23 thousand hectares with an average yield of 9.6 ton h^{-1} which is extremely low due to several pathological constraints (Government of Pakistan, 2012).

Under field conditions, tomato crop can be parasitized by a number of pathogens. Fungal pathogens are one of the major concerns in cultivation of tomato all over the world including Pakistan, as these pathogens cause huge economic losses (Goswami and Kistler, 2004). Fusarium wilt is a highly damaging disease of tomato, caused by *F. oxysporum* f. sp. *lycopersici* (Akhter et al., 2016). This fungus is generally soil-borne and can also be isolated from different infected parts of the plant including root and stem tissues (Summeral et al., 2003). It attacks on the vascular system of the plant causing vascular wilts. Chemical fungicides are mainly used for the control of wilt diseases. Some important fungicides are Fusaria, Prochloraz and Carbendazim (Song et al., 2004). However, vast use of these chemical fungicides causes harmful effects on human health and environment. It triggers environmental pollution and pathogen resistance against the specific synthetic fungicide (Oruc, 2010). Researchers and scientists all over the world are now seeking for the alternative disease management strategies mainly based on natural resources, which are environment friendly (Javaid et al., 2015). Alternative methods of controlling diseases have been studied highlighting the use of different types of extracts and antifungal compounds from plants (Sana et al., 2017; Akhtar and Javaid, 2018; Shoaib et al., 2018). Several allelochemicals and essential oils have been isolated from different parts of plants, which are now recognized as effective tool against many fungal pathogens (Bowers and Locke, 2000). These products are better alternatives to synthetic fungicides, as they are easily biodegradable in natural environmental conditions.

Chenpodium murale, commonly known as nettle-leave goose-foot, is native to Asia, Europe and North Africa. It belongs to family Chenopodiaceae. Members of this family are generally known for their antifungal activity against various fungal pathogens (Javaid and Amin, 2009; Arafat et al., 2015; Ali et al., 2017). In this study, efficacy of crude methanolic leaf extract of *C. murale* and its various organic fractions is reported for the management of *F. oxysporum* f. sp. *lycopersici.*

MATERIAL AND METHODS

Preparation of inoculum

For preparation of inoculum of FOL, the fungus was isolated from roots of a diseased tomato plant and cultured on malt extract agar medium, which is quite appropriate for its growth (Khurshid et al., 2016). After inoculation, plates were put into an incubator at 28 °C for 10 days. Thereafter, plates were kept in refrigerator at 4 °C for future experimental work.

Preparation of leaf extract

Fresh leaves of mature *C. murale* were collected from fields of Lahore and Jhelum districts, Pakistan, between February-March 2016. Leaves were rinsed thoroughly with water and were dried under shade. Then 200 g of these leaves soaked in 1000 mL of methanol for 14 days. The soaked leaf material was filtered using sterilized cheese cloth followed by filter papers in order to separate the extract from its undissolved residues. The solvent was removed in a rotary evaporator at 45 °C and 22 g leaf extract of *C. murale* was obtained.

Screening bioassays with leaf extract

Methanolic extract of leaves of *C. murale* (9 g) was dissolved in 5 mL of (DMSO (dimethyl sulphoxide)) and total volume (20 mL) was achieved by adding distilled autoclaved water for the

preparation of stock solution. Likewise, 5 mL of DMSO was mixed with 15 mL of distilled water in order to prepare control solution. In 250 mL volume flasks, 55 mL of malt extract was prepared and autoclaved. It was then allowed to cool for 10-15 minutes. Six working concentrations were made by mixing 5, 4, 3, 2, 1 and 0 mL of control solution with 0 1, 2, 3, 4 and 5 mL of stock solution respectively, in 55 mL autoclaved medium to make 60 mL in each flask. It was then sub-divided into four replicates of 15 mL each in 100 mL conical flasks. By using a 5 mm diameter cork borer, small discs of FOL were made from the edges of 5 days old actively growing culture plate and each disc was shifted to each flask. Flasks were then incubated at 28 °C for 14 days. Afterwards, the visible growth of fungus in all flasks was separated by using filter papers and were dried in a dry heating oven at 60 °C and were weighed on an electric weighing balance (Javaid et al., 2018).

Fractionation of methanolic leaf extract

For this study, 2 kg of crushed dried leaves were soaked in 5 L methanol for two weeks. Afterwards, leaf material was separated from methanolic extract through a sterilized autoclaved muslin cloth and the remaining residues were again dipped in methanol. Methanolic extract was filtered through filter paper and evaporated under rotary evaporator. Thick gummy mass of leaf extract was obtained by evaporating in rotary evaporator and later partially dried in dry heating oven at 45 °C. Two hundred milliliter of distilled water was added to crude methanolic leaf extract and compounds soluble in *n*-hexane were separated by several mixings of the aqueous phase with *n*-hexane and separating by using a 1 L volume separating funnel. Afterwards, the residual extract was sequentially partitioned with chloroform) (400 mL) and ethyl acetate) (400 mL). All the fractions in organic solvents were evaporated on a rotary evaporator to gain 30 g of *n*-hexane) fraction, 10 g of (chloroform) fraction and 2 g of (ethyl acetate fraction (Khurshid et al., 2018).

Bioassays with organic sub-fractions

Different sub-fractions acquired from methanolic leaf extract were assessed for their antifungal potential. For this purpose, 1.2 g of each methanolic sub-fraction was mixed in 1 mL of DMSO. For preparing 6 mL of stock solution, the mixed materials were added to 5 mL of malt extract broth and concentration was maintained at 200 mg mL^{-1}. Broth medium was then equally divided into two portions, one portion was utilized for the experimental study while the second portion was serially double diluted for preparing lower concentrations reaching up to 1.562 mg mL^{-1}. For the preparation of control, 5 mL of ME broth and 1 mL DMSO were mixed and serially double diluted by adding ME broth for preparation of control treatments. The concentration of DMSO in control was kept exactly same to the concentration used for extract treatments. Bioassays were carried out in sets of three test tubes of 10 mL volume. One milliliter of broth was poured in each test tube. Conidial suspension of FOL was made in autoclaved distilled water and 20 μL of the suspension was added to each test tube of every treatment and incubated at 28 °C. After seven days of incubation, fungal mycelia were harvested by filteration. Fungal biomass was dried at 60 °C and weighed (Javaid et al., 2017).

GC-MS analysis

The analysis of all sub-fractions *viz*. *n*-hexane, chloroform and ethyl acetate was performed using the GC-MS QP-2010. Compounds were separated on (30 × 0.25 mm × 0.25 μmdf capillary column. Split ratio of injection samples was 10:0 and flow rate of helium was 1.69 mL min^{-1}. Flow of column was 153.9 mL min^{-1} and the pressure was maintained at 100 kPa. Linear velocity was observed 47.2 cm sec^{-1}. One microliter of sample was injected. Other conditions including oven temperature was raised up to 50 °C for 3 min and the temperature was raised at the rate of 11 °C up to 320 °C. Injection temperature was kept at 200 °C. Total time period for GC running was estimated 27 min. All diagnosed compounds are presented in Tables 1, 2 and 3. Structures of major compounds from all the three sub-fractions are shown in Figure 1.

Table 1 **-** Compounds identified from *n*-hexane sub-fraction of methanolic leaf extract of *Chenopodium murale* through GC-MS analysis

Sr. No.	Names of compounds	Formula	Weight	Retention time (min)	Peak area (%)
1	Cyclopentanol	$C_5H_{10}O$	86	3.07	0.59
2	Dodecane	$C_{12}H_{26}$	170	10.32	0.58
3	Tridecane	$C_{13}H_{28}$	184	13.07	0.74
4	Hexadecane	$C_{16}H_{34}$	226	15.47	0.83
5	Octadecane	$C_{18}H_{38}$	254	17.62	0.45
6	3,7,11,15-tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	296	17.99	0.53
7	Tridecanal	$C_{13}H_{26}O$	198	18.05	2.30
8	Methyl linolenate	$C_{19}H_{32}O_2$	292	18.61	0.55
9	Methyl palmitoleate	$C_{17}H_{32}O_2$	254	18.82	1.28
10	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	18.86	14.64
11	Palmitic acid	$C_{16}H_{32}O_2$	256	19.20	2.35
12	Methyl linoleate	$C_{19}H_{34}O_2$	294	20.43	5.23
13	Methyl linolenate	$C_{19}H_{32}O_2$	292	2.48	16.61
14	Phytol	$C_{20}H_{40}O$	296	20.58	6.31
15	Octadecanoic acid, methyl ester	$C_{19}H_{38}O_2$	298	20.71	1.37
16	Oleic acid	$C_{18}H_{34}O_2$	282	20.83	3.54
17	Heneicosanoic acid,	$C_{21}H_{42}O_2$	326	22.39	0.81
18	Ethylbenzylamine	$C_9H_{13}N$	135	23.58	0.55
19	Docosonate	$C_{23}H_{46}O_2$	354	23.68	1.61
20	2-Palmitoylglycerol	$C_{19}H_{38}O_4$	330	23.88	1.32
21	Docosanoic acid, methyl ester	$C_{23}H_{46}O_2$	354	23.95	0.83
22	Dioctyl phthalate	$C_{24}H_{38}O_4$	390	24.01	2.61
23	Octadecyl vinyl Ether	$C_{20}H_{40}O$	296	25.17	0.65
24	Pentacosanoic acid	$C_{25}H_{50}O_2$	382	25.39	0.87
25	1-Eicosanol	$C_{20}H_{42}O$	298	26.55	2.99
26	Fantofarone	$C_{31}H_{38}N_2O_5S$	550	27.78	0.61
27	Ethyl 12-fluororentinoate	$C_{22}H_{31}FO_2$	346	27.83	0.84
28	Cholestrol	$C_{27}H_{46}O$	386	28.17	1.12
29	Stigma sterol	$C_{29}H_{48}O$	412	29.14	4.12
30	γ-sitosterol	$C_{29}H_{50}O$	414	29.64	13.53
31	4-Pyrimidinecarboxylic acid	$C_5H_4N_2O_2$	124	30.01	3.65
32	*β*-sitosterol	$C_{29}H_{50}O$	414	30.19	5.97

Table 2 **-** Compounds identified from chloroform sub-fraction of methanolic leaf extract of *Chenopodium murale* through GC MS analysis

Sr. No.	Names of compounds	Formula	Weight	Retention time (min)	Peak area (%)
1	Dioctyl phthalate	$C_{24}H_{38}O_4$	390	24.008	7.69
2	Bis(2-ethylhexyl)phthalate	$C_{24}H_{38}O_4$	390	25.508	92.31

Table 3 **-** Compounds identified from ethyl acetate sub-fraction of methanolic leaf extract of *Chenopodium murale* through GC-MS analysis

Sr. No.	Names of compounds	Formula	Weight	Retention time (min)	Peak area (%)
1	Ethyl butyrate	$C_6H_{12}O_2$	116	3.533	19.57
2	Ethyl benzene	C_8H_{10}	106	4.408	6.75
3	*o*-Xylene	C_8H_{10}	106	4.575	16.16
4	*p*-Xylene	C_8H_{10}	106	5.025	3.96
5	Nitrobenzene	$C_6H_5NO_2$	123	8.667	9.15
6	Dibutyl phthalate	$C_{16}H_{22}O_4$	278	18.292	3.63
7	Hexadecanoic acid	$C_{17}H_{34}O_2$	270	18.867	2.43
8	Dihexyl phthalate	$C_{20}H_{30}O_4$	334	19.208	11.19
9	5-Pentadecylresorcinol	$C_{21}H_{36}O_2$	320	20.492	3.35
10	Oleic acid	$C_{18}H_{34}O_2$	282	20.833	6.22
11	Octadecanoic acid	$C_{18}H_{36}O_2$	284	22.158	1.88
12	Dioctyl phthalate	$C_{24}H_{38}O_4$	390	24.008	12.16
13	5α-Androstane	$C_{19}H_{32}$	260	29.658	3.56

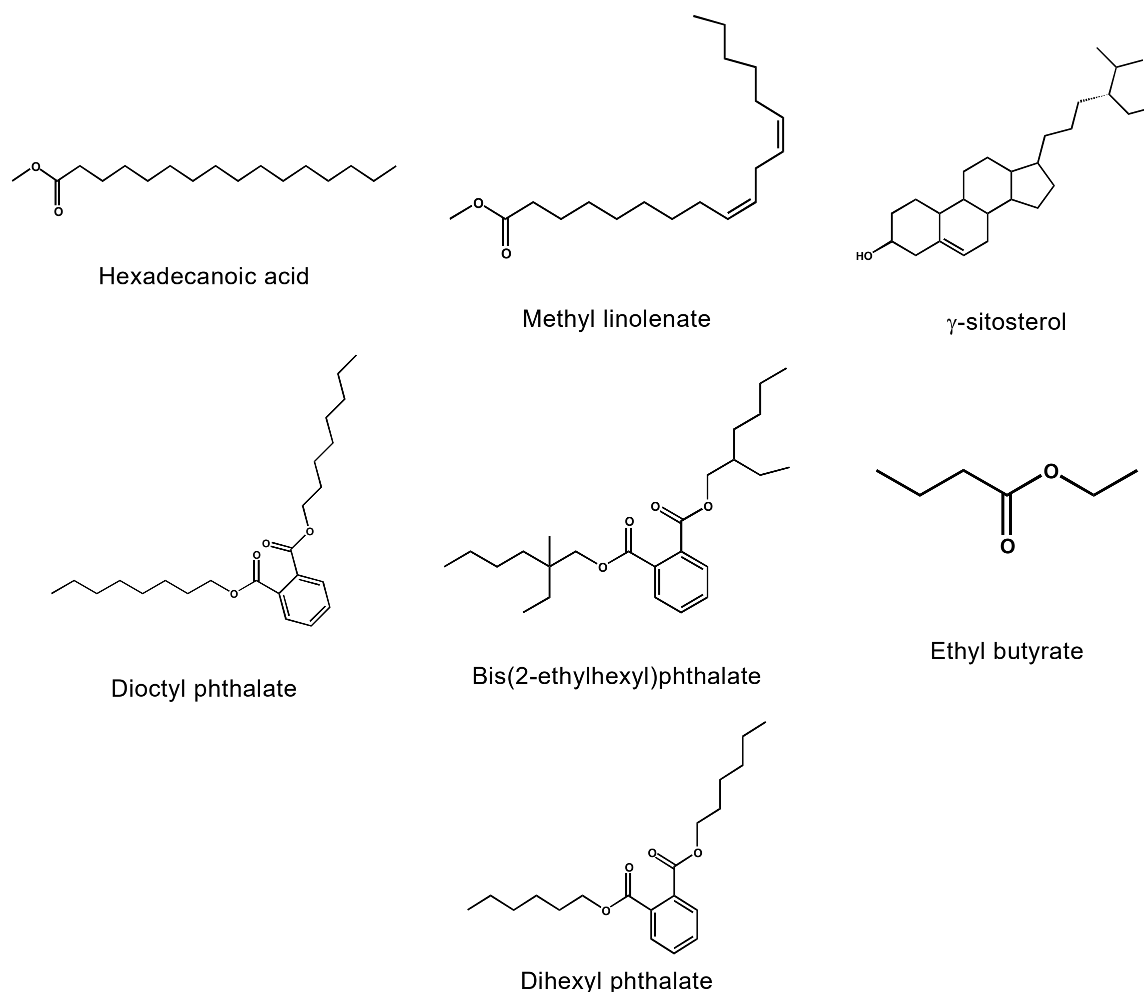


Figure 1 - Structures of major compounds identified in different sub-fractions of methanolic leaf extract of *Chenopodium murale.*

Statistical analysis

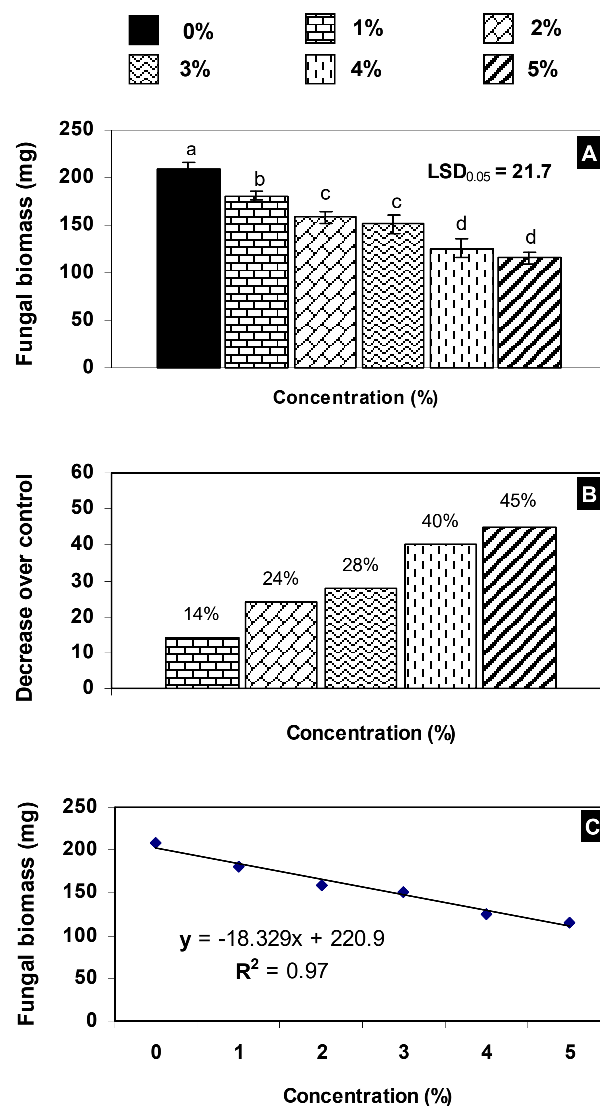
All the laboratory bioassays data were subjected to ANOVA and the mean were separated using LSD test at 5% level of significance using software Statistix 8.1.

RESULTS AND DISCUSSION

Data presented in Figure 2 shows that all concentrations of methanolic leaf extract significantly reduced biomass of FOL by 14-45% over control. Inhibitory effect of the extract was concentration dependant. Fungal biomass was linearly decreased by increasing extract concentration with $R^2 = 0.97$.

Data regarding antifungal activity of different concentrations of *n*-hexane, chloroform and ethyl acetate fraction of methanolic leaf extract of *C. murale* against FOL are presented in Figure 3. In general, all the sub-fractions significantly decreased fungal biomass over control. However, the effect of higher concentrations was more pronounced than lower ones in all the sub-fractions. The lower concentration *viz.* 1.562-6.25 mg mL^{-1} reduced fungal biomass just by 12-26%, 12-31% and 21-31% in *n*-hexane, chloroform and ethyl acetate sub-fractions, respectively, over control. Similarly, 12.5-50 mg mL^{-1} concentrations markedly reduced the fungal biomass by 27-96%, 24-83% and 55-65%, respectively, over control. Higher concentrations (100 and 200 mg mL^{-1}) almost completely arrested the fungal growth and limited its growth up to 96%, 98% and 94% in *n*-hexane, chloroform and ethyl acetate fractions, respectively, over control (Figure 4). Previously,

members of Chenopodiaceae are known to exhibit potent antimicrobial activity against many pathogens (Singh et al., 2011; Amjad and Alizad, 2012; Ali et al., 2017). Qasem and Abu-Blan (1995) reported that extracts of *C. murale* possess antifungal potential against *Penicillium digitatum* and *Alternaria solani*. Similarly, Amin and Javaid (2007) evaluated the antifungal potential of *C. murale* against *Macrophomina phaseolina*, the cause of charcoal rot of sunflower. Different concentrations of leaf extracts of *C. murale* markedly reduced the biomass of *M. phaseolina* by 57-68%.

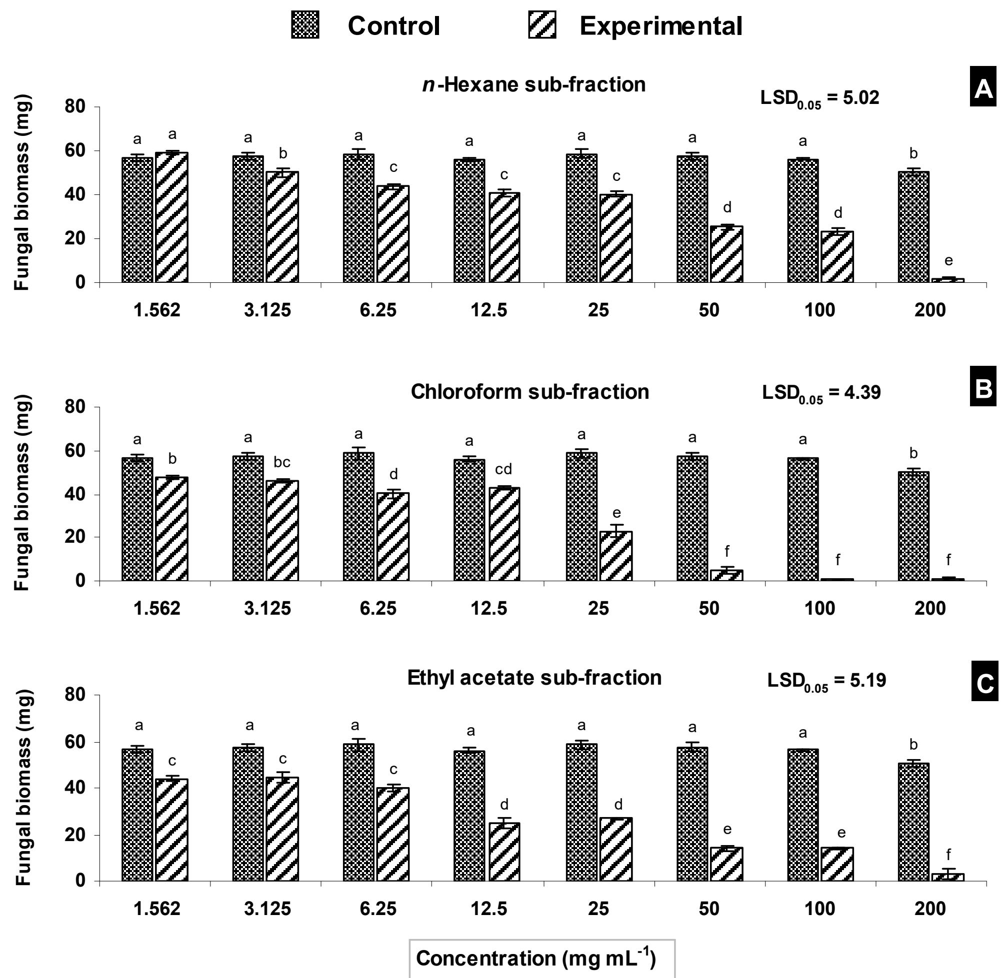


Vertical bars show standard errors of means of four replicates. Values with different letters at their top show significant difference ($P \leq 0.05$) as determined by LSD Test.

Figure 2 – **(A)** Effect of different concentrations of methanol leaf extract of *Chenopodium murale* on biomass of *Fusarium oxysporum* f. sp. *Lycopersici*; **(B)** Percentage reduction in fungal biomass due to different concentrations of methanolic leaf extract over control; **(C)** Relationship between extract concentration and fungal biomass.

GC-MS analysis of *n*-hexane fraction showed the presence of 32 organic compounds (Figure 5A). Names and formulas of these compounds are presented in Table 1. Most abundant compounds were methyl linolenate (16.61%), hexadecanoic acid, methyl ester (14.64%) and γ-sitosterol (13.53%). Previously, literature is filled with the antimicrobial activity of methyl linolenate and hexadecanoic acid, methyl ester. Till now, their presence has been detected in many allelopathic plants (Gopalakrishnan and Udayakumar, 2014; Jahirhussain et al., 2015). Sana et al. (2017) reported antifungal activity of leaf extract of *Melia azedarach against Sclerotium rolfsii* and identified methyl linolenate, hexadecanoic acid, methyl ester and phytol in the extract through GC-MS. Moderately abundant compounds in *n*-hexane sub-fraction were phytol (6.31%), β-sitosterol (5.97%), methyl linoleate (5.23%), stigmasterol (4.12%), 4-pyrimidinecarboxylic acid (3.65%) oleic acid (3.54%), 1-eicosanol (2.99%), dioctyl phthalate (2.61%), palmitic acid (2.35%), and tridecanal (2.30%). Palmitic acid has been reported previously in many plant species against fungal pathogens including *F. oxysporum* (Liu et al., 2008). The antimicrobial activity of phytol is attributed to its antioxidant nature (Pejin et al., 2014). Compounds found in least quantities in *n*-hexane sub-frction were docosonate (1.61%) followed by octadecanoic acid (1.37%), 2-palmitoylglycerol (1.32%), methyl palmitoleate (1.28%), cholestrol (1.12%), pentacosanoic acid (0.87%), ethyl 12-fluororentinoate (0.84%), hexadecane (0.83%), docosanoic acid (0.83%), heneicosanoic acid (0.81%), tridecane (0.74%), octadecyl vinyl ether (0.65%), fantofarone (0.61%), cyclopentanol (0.59%), dodecane (0.58%), methyl linoleate (0.55%) and ethylbenzylamine (0.55%). The use of fatty acids as antifungal agents is very advantageous. Liu et al. (2008) proposed that antifungal fatty acids can replace synthetic chemicals for the control of plant diseases worldwide.

GC-MS analysis of chloroform sub-fraction revealed the presence of only two compounds (Figure 5B). Names and formulae of these compounds are presented in Table 2. Highly abundant compound was bis (2-ethylhexyl) phthalate (92.31%) followed by dioctyl phthalate (7.69%). Bis (2-ethyl hexyl) phthalate has also been reported from the roots of *Euphorbia hylonoma* and seeds



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.

Figure 3 **-** Effect of different concentrations of sub-fractions of methanolic leaf extract of *Chenopodium murale* on biomass of *Fusarium oxysporum* f. sp. *lycopersici*.

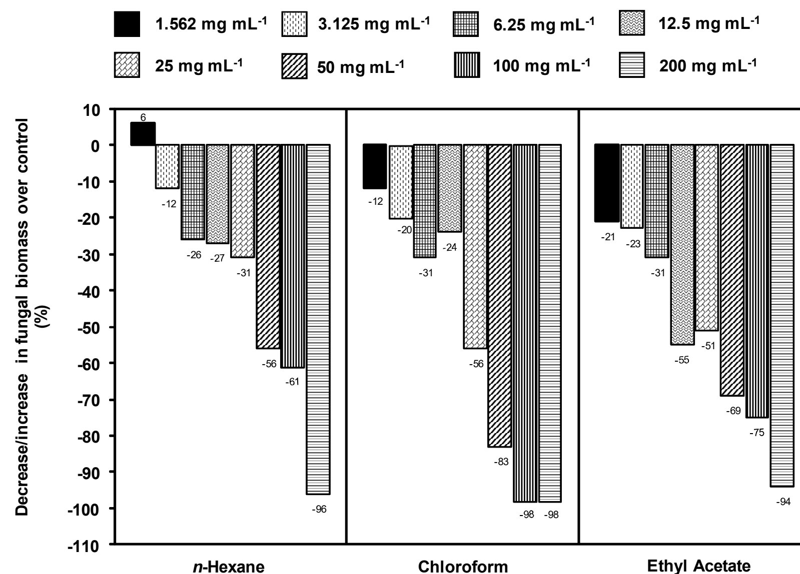


Figure 4 **-** Percentage increase/decrease in biomass of *Fusarium oxysporum* f. sp. *lycopersici* due to different concentrations of sub-fractions of methanolic leaf extract of *Chenopodium murale*.

of *Ricinus communis* (Sani and Pateh, 2009). Bis (ethyl hexyl) phthalate reported from *Streptomyces bangladeshiensis* showed antifungal activity against some pathogenic fungi (Al-Bari et al., 2006). In general, phthalates are known to possess antimicrobial activities. The essential oil of *Leea indica* showed phthalic acid esters (95.6%) as major constituents, which had potent antifungal activity (Srinivasan et al., 2009).

GC-MS analysis of ethyl acetate sub-fraction revealed the presence of 13 organic compounds (Figure 5C). Names and formulae of these compounds are presented in Table 3 along with their retention time and peak area percentages. Compound found in the highest concentration was ethyl butyrate (19.57%), followed by *o*-xylene (16.16%), dioctyl phthalate (12.16%), dihexyl phthalate (11.19%) and nitro benzene (9.15%). Al-Owaisi et al. (2014) isolated and tested antimicrobial activity of ethyl butyrate and *o*-xylene from crude extracts of *Moringa peregrine* leaves. Dioctyl phthalate has also been reported from *Limonium bicolour* and *Dracaena cochinensis* (Wei and Wang, 2006). Compounds found in lower concentrations were ethyl benzene (6.75%), oleic acid (6.22%), *p*-xylene (3.96%), dibutyl phthalate (3.63%), 5α-androstane (3.56%), 5-pentadecylresorcinol (3.35%), hexadecanoic (2.43%) and octadecanoic acid (1.88%). The GC-MS analysis of leaf extract of *Finlaysonia obovata*, a mangrove plant has showed the presence of octadecanoic acid, oleic acid and several other constituents to be responsible for antimicrobial and antifungal activity (Walters et al., 2004). Casuga et al. (2016) identified 5α-androstane from methanolic extract and hexadecenoic acid from ethyl-acetate extract of *Broussonetia luzonica* leaves. Fatty acids are widely present in natural fats and oils and they play vital role as nutritious substances and metabolites in living organisms (Cakir, 2004). Many fatty acids are known to have antimicrobial potential (Russel, 1991). Palmitic acid and oleic acid are known for their antifungal activity (McGaw et al., 2002; Seidel and Taylor, 2004). Agoramoorthy et al. (2007) determined the antifungal potential of fatty acids and methyl esters isolated from blind your eye mangrove (*Excoecaria agallocha*) against four fungal species.

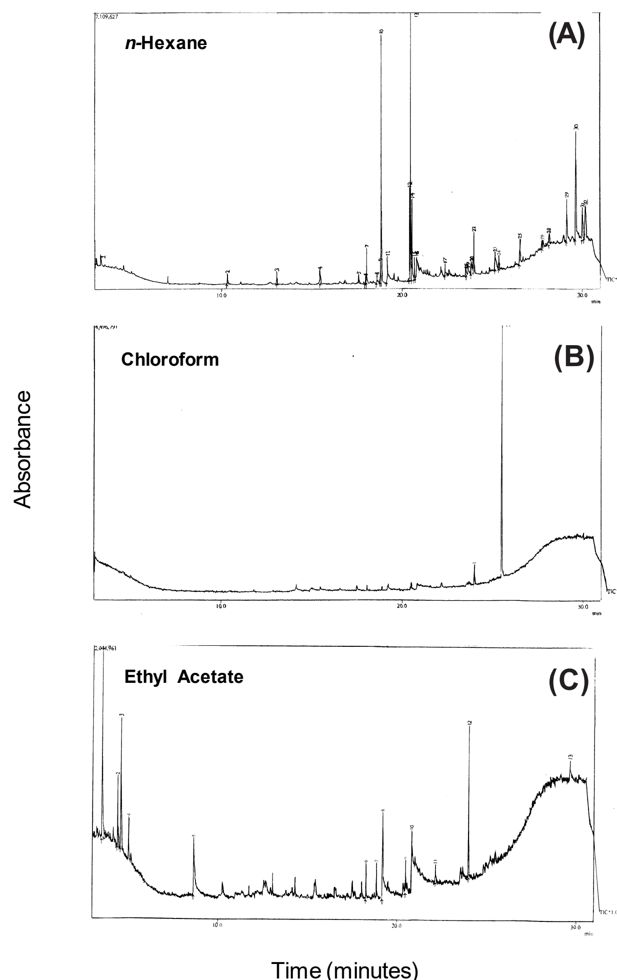


Figure 5 - GC-MS chromatograms of *n*-hexane **(A)**, chloroform **(B)**, and ethyl acetate **(C)** sub-fractions of methanolic leaf extract of *Chenopodium murale*.

The present study has been found useful in the identification of several constituents present in the methanolic extract of the leaves of *C. murale*. The presence of various bioactive compounds justifies the use of the various sub-fractions of the leaf extract of this plant for the management *F. oxysporum* f. sp. *lycopersici*.

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