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Table: 3, Figs: 5

Fungicidal activity of stem extract of *Chenopodium murale* L. against the pathogen of Fusarium wilt of tomato

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ABSTRACT

Fusarium oxysporum f. sp. *lycopersici* (FOL) is a highly destructive pathogen of tomato causing wilt disease. In search of an alternative strategy to synthetic fungicides for the control of this pathogen, methanolic stem extract of a winter weed *Chenopodium murale* L. was investigated against this pathogen. The methanolic stem extract (1 to 5 %) significantly ($P \leq 0.05$) suppressed the fungal growth by 39-74 %. This extract was partitioned using 3-organic solvents of different polarities. Bioassays with different concentrations (1.562 to 200 mg mL^{-1}) showed the best antifungal activity was of chloroform > *n*-hexane > ethyl acetate sub-fraction causing 51-100 %, 52-98 % and 29-98 % reduction in biomass of FOL, respectively. GC-MS analysis of chloroform and ethyl acetate fractions showed 20 and 8 compounds, respectively. Acetic acid, butyl ester (22.72 %), cyclopentanol (20.15 %), 2-hexanol (21.36 %), oleic acid (13.92 %), morphine (12.97 %) and β-sitosterol (11.79 %) were abundant compounds in these two sub-fractions.

Keywords: Antifungal activity, *Chenopodium murale*, chloroform fraction, ethyl acetate fraction, *Fusarium oxysporum*, Fusarium wilt, GC-MS, methanol extract, natural fungicides, stem extract

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is a major vegetable grown worldwide. It is used as salad, and also used in cooked food and ketchup to improve the taste. It is an important source of vitamins A and C, lycopene, β-carotene, phosphorus and iron. *Fusarium oxysporum* f. sp. *lycopersici* Snyder & Hansen is an important fungal pathogen causing wilt disease in many vegetable crops including tomato (13). It is quite common when air and soil temperatures are high such as under warm climatic conditions (35). It survives in the soil as dormant propagules up to 5-years (21). The application of broad-spectrum synthetic fungicides may not be very effective because it is a soil-borne and new pathogen races also emerge (25). Furthermore, the fungicides are not easily biodegradable, environmentally damaging and pose severe public concerns for their residues in food having harmful effects on human health (24). Growing awareness of the hazardous effects of these agrochemicals led scientists to search for new effective alternative control measures that are environmentally safe and practically effective (5,16,32). Hence, the use of plant-based products provides the potential approach to control the Fusarium wilt pathogen (23,26). The phytochemicals of plant origin are cost-effective, ecologically acceptable and offer a safer alternative to synthetic chemicals (7).

Chemical investigation to find the antifungal activities of plants are being done since long (18). Several plant species [*Chenopodium*, *Archontophoenix*, *Atriplex*, *Datura*, *Dodonaea* and *Callistemon*] have antifungal activity against many fungal pathogens [*Rhizoctonia solani* Kühn, *Fusarium oxysporum* (Schlecht.), *Verticillium dahlia* Kleb.,

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Thielaviopsis basicola and *Sclerotium rolfsii* Sacc.] (12,14,23,34). Extracts and compounds of plants of Chenopodiaceae (now Amaranthaceae) family such as *Chenopodium album* L., *C. quinoa* Willd. and *C. ambrosioides* L. were found highly effective against *Alternaria alternata* (Fr.) Keissl., *Alternaria solani* Sorauer, *Colletotrichum graminicola* Politis, *R. solani, V. dahlia, Macrophomina phaseolina* (Tassi) Goid. and *F. oxysporum* (11,17,22).

Figure 1. A plant of *Chenopodium murale*

Chenopodium murale is an annual weed plant native to South Asia (Fig. 1). It is rich in flavonoids, glycosides, saponins, terpenoids, sesquiterpenes, diterpenes, monoterpenes, sterols, alkaloids, coumarins and phenolic compounds (9). Studies regarding antifungal activities of stem extract of *C. murale* against FOL are lacking. Therefore, this study was done to evaluate the antifungal properties of *C. murale* methanolic stem extract against FOL and to determine its potential antifungal phytoconstituents through GC-MS analysis.

MATERIALS AND METHODS

Experimental site

The experiments were done during March- April 2019 at Punjab University, Lahore, Pakistan (31°15 - 31°45′ N and 74°01′- 74°39′ E, at an altitude of 217 m). During the study period, the lowest and the highest temperatures were 9-14 ºC and 20-28 ºC, respectively.

Culturing of the fungal pathogen

The pathogenic fungus was isolated from roots of an infected tomato plant. Diseased samples were immediately transferred to plastic bags and transported to the laboratory. The infected root portions were surface sterilized with 1 % sodium hypochlorite solution to avoid bacterial and fungal contaminations. The infected roots were cut into small portions and transferred to Petri plates containing malt extract agar medium under aseptic conditions. After inoculation, plates were incubated for 10 days at 28 °C. The isolated fungus was identified as FOL on morphological as well as molecular basis. Pathogenicity of the isolated

fungus was also confirmed through Koch's postulates. Plates were then kept in a refrigerator at 4 °C for further experimentation.

Extraction of plant material

Plants of *C. murale* were collected from Lahore and Jehlum region. The stems were separated from the leaves, washed thoroughly with sterilized water and dried in a dry heating oven at 45 °C for three days. Afterward, dried stem material was ground with mechanical grinder.

For the extraction, 150 g of dried and crushed stem material of *C. murale* was soaked in 1.0 L of 80 % methanol for 14 days in jars. Afterwards, the extract was filtered through filter paper and was evaporated by a rotary evaporator at 45 °C to obtain thick mass of stem extract. The yield of stem extract was recorded and preserved in the sterilized beaker to use further in bioassays and for fractionation.

Bioassays with methanolic stem extract

To prepare a stock solution, methanolic stem extract of *C. murale* was mixed with 5 mL of dimethyl sulphoxide (DMSO) and the volume was raised to 20 mL with autoclaved distilled water. To prepare control solution, DMSO (5 mL) was dissolved in 15 mL of distilled water. Fifty-five milliliters of malt extract (2 % w/v) was prepared, autoclaved in flasks and allowed to cool at room temperature. In autoclaved flasks, different concentrations of the extracts were prepared by adding a mixture of control and stock solutions in different proportions (5 mL) to make a 60 mL volume. Each flask was further divided into 4 replicates (15 mL each). Small discs of FOL were removed from the periphery of a 7-days-old pure culture plate and put in each flask. All the flasks were incubated at 28 °C. After 14-days, fungal biomass from all the flasks was separated by filter papers. These pre-weighed filer papers were kept in an oven at 45 °C and weighed again to get biomass of the fungus (22).

Fractionation of stem extract

Two kilograms of crushed stems were soaked in 5.0 L methanol. After 15-days, methanol was separated with the help of sterilized muslin cloth followed by filter papers. It was evaporated in a rotary evaporator and then dried in an oven at 45 ºC to obtain a thick paste of stem extract. To the stem extract, 200 mL of distilled water was added and mixed vigorously. This mixture was fractionated with *n*-hexane (4 × 400 mL). The aqueous phase was further partitioned using chloroform (400 mL) and ethyl acetate (400 mL). The solvents were evaporated to yield 7.5 g of *n*-hexane, 3 g of chloroform and 2 g of ethyl acetate sub-fraction.

Bioassays with sub-fractions

All the sub-fractions of stem extract were evaluated for their antifungal activity. To prepare stock solution, 1.2 g of each sub-fraction was mixed in DMSO (1 mL) and 5 mL of malt extract broth (MEB) was added to it. The concentration was 200 mg mL^{-1} which was equally divided into two proportions, one was used for experimental study and the second part was serially diluted to prepare lower concentration (1.562, 3.125, 6.25, 12.5, 25, 50 and 100 mg mL^{-1}). To make control, 5 mL MEB and 1 mL DMSO were mixed and serially diluted by adding only MEB. Three replicates were made for each concentration by taking 1 mL of medium in each 10-mL volume test tube. The conidial suspension (20 µL) of FOL

was added in each test tube under aseptic conditions. All test tubes were put into an incubator at 28 °C for 7 days and thereafter fungal biomass was weighed (2).

GC-MS analysis

The GC-MS analysis of chloroform and ethyl acetate sub-fractions were performed on GC-MS QP-2010 following the procedure of Naqvi *et al.* (22). A volume of 1 µL of each sub-fraction was injected into the gas chromatographic machine with the help of an auto injector. The column used had the dimensions 30 m × 0.25 mm × 0.25 µm. Helium was used a carrier gas with a flow rate of 1.69 mL per min. Flow of column was 153.9 mL min^{-1} at 100 kPa pressure. Oven temperature was raised up to 50 °C for 3 min and then raised to 320 °C at a rate of 11 °C.

Statistical analysis

Both the laboratory bioassays were done in a completely randomized design with four replications. Standard errors of means were calculated using MS Excel program. Data of both the experiments were analyzed by ANOVA and the means were separated by applying the LSD test at $P \leq 0.05$ using software Statistix 8.1.

RESULTS AND DISCUSSION

Antifungal activity of extracts

All concentrations of stem extract of *C. murale* significantly suppressed biomass of FOL (Fig. 2). Different concentrations of methanolic extract reduced biomass of the fungus by 39-74 %. Regression analysis showed a polynomial regression curve for the relationship between extract concentration and fungal biomass. A little work has been done on antifungal activity of *C. murale*. Abdel-Aziz *et al.* (1) found that *C. murale* contains phenolic compounds that may directly suppress the growth of fungal pathogens. Many other members of Chenopodiaceae have been studied for their antifungal activities. Shah and Khan (31) reported the antifungal potential of 3-species of *Chenopodium* (*C. album, C. quinoa* and *C. ambrosioides*). Recently Khan and Javaid (17) found that methanolic extracts of *C. quinoa* were very effective against *M. phaseolina.* Similarly, Javed *et al.* (15) observed that leaf extract of *Kochia indica* possesses fungicidal properties against *M. phaseolina.* Methanolic extract of *C. album* significantly suppressed the growth of *F. oxysporum* f. sp. *cepae* and *S. rolfsii* (3,29). Plants in Chenopodiaceae family are rich in saponins, terpenoids, flavonoids, phenolic, fatty acid methyl esters compounds that are well known for their antifungal activities (3,11,36).

The antifungal effects of different sub-fractions of the stem methanolic extract were variable. In general, there was little difference among the three organic sub-fractions in their antifungal behavior towards FOL. There was 52-98 % suppression in FOL biomass due to *n*-hexane, 51-100 % due to chloroform and 29-98 % due to ethyl acetate sub-fractions over their respective control treatments. By contrast the effect of aqueous sub-fraction were non-significant (Fig. 3 and 4). The three solvents used for the fractionation were of different polarities. During this process different groups of compounds were separated from one another based on their polarity nature. Similar antifungal activities of these solvent fractions in this study showed that antifungal compounds present in the stem of *C. murale* belonged to diverse groups of organic compounds with highly variable polarities. By contrast, in most

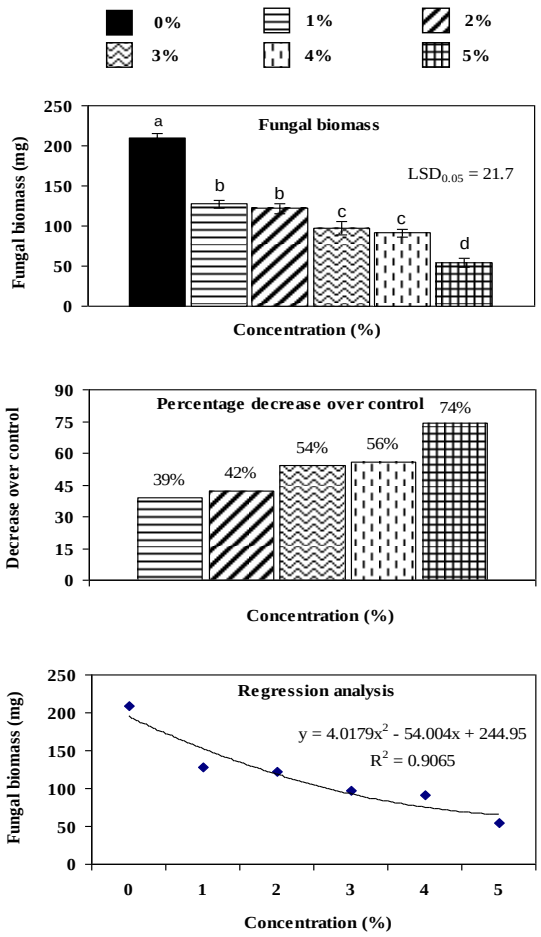

Figure 2. Effects of different concentrations of methanol stem extract of *Chenopodium murale* on biomass of *Fusarium oxysporum* f. sp. *lycopersici,* Values with different letters at their top show significant difference (P≤0.05) as determined by LSD Test.

of the previous studies, generally different fractions of the methanolic extracts showed highly variable antifungal activities against different fungal species showing that those plants had only specific types of antifungal compounds with less diversity (22). Sherazi *et al.* (33) worked on different organic fractions prepared from *C. album* methanolic extracts and found that *n*-hexane fraction more remarkably arrested the growth of *Ascochyta rabiei* than other fractions. Khan and Javaid (17) studied the antifungal activity of 4-fractions of *C. quinoa* stem extract against *M. phaseolina*, the *n*-hexane and chloroform fractions proved more antifungal than ethyl acetate and *n*-butanol fractions.

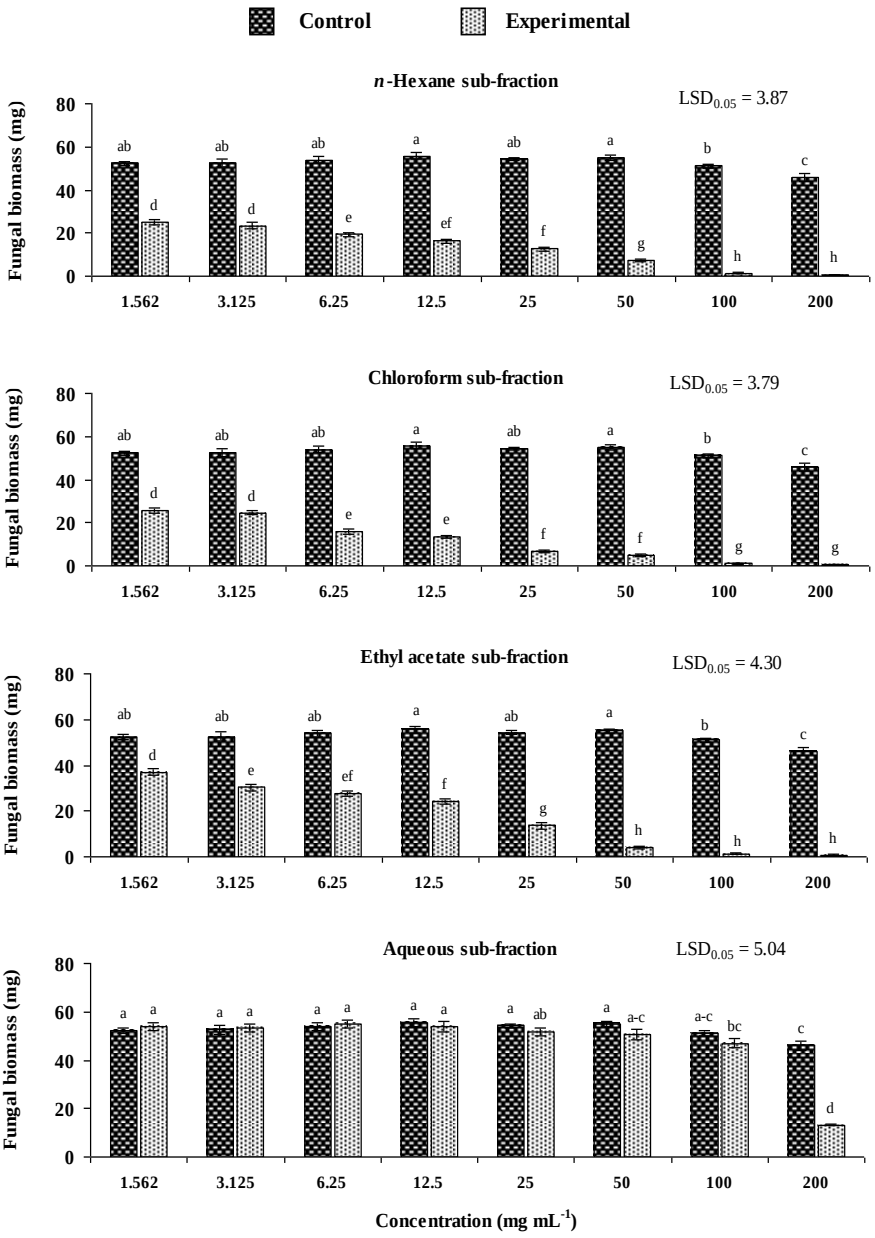

Figure 3. Effects of different concentrations of sub-fractions of methanolic stem extract of *Chenopodium murale* on biomass of *Fusarium oxysporum* f. sp. *lycopersici.* Vertical bars show standard errors of means of four replicates. Values with different letters at their top show significant difference (P≤0.05) as determined by LSD Test.

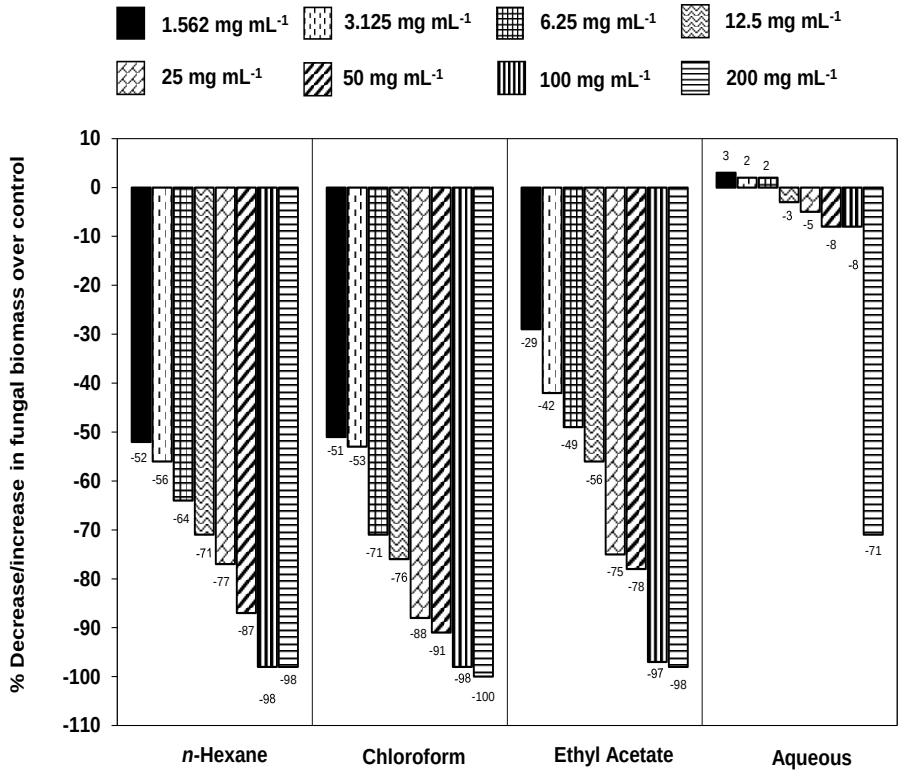

Figure 4. Effects of different concentrations of sub-fractions of methanolic stem extract of *Chenopodium murale* on % Inhibition/stimulation of biomass of *Fusarium oxysporum* f. sp. *lycopersici*

GC-MS analysis

Chloroform and ethyl acetate sub-fractions were analyzed by GC-MS. The analysis of chloroform fraction revealed the presence of 20 constituents (Table 1). The most prevailing compounds were oleic acid; morphine and β-sitosterol with 13.92 %, 12.97 % and 11.79 % peak areas, respective. Palmitic acid; hentriacontane; benzoic acid and hexadecanoic acid, methyl ester with 8.29 %, 8.28 %, 6.01 % and 5.96 % peak areas, respectively, were moderately abundant. The less abundant compounds were dioctyl phthalate; piperine; methyl oleate; monopalmitin; stigmasterol; γ-sitosterol; linoleic acid; nonacosane; 2-pentadecanone; arachidic acid; indole-3-acetic acid; 5-octadecene and methyl linoleate ranging from 1.17 to 3.78 %.

Ethyl acetate sub-fraction contained 8-compounds (Table 2). Acetic acid, butyl ester; 2-hexanol; cyclopentanol; *p*-xylene; γ-sitosterol and *o*-xylene with peak areas of 22.72 %, 21.36 %, 20.15 %, 13.67 %, 10.09 % and 7.18 %, respectively were present in higher concentrations. On the other hand, the compounds in less concentration were nitrobenzene and palmitic acidwith peak areas ranging between 3.57 and 1.21 %, respectively.

Table 1. Compounds identified from chloroform sub-fraction of methanolic stem extract of *Chenopodium murale* through GC-MS analysis.

Sr. No.	Names of compounds	Molecular formula	Molecular weight	Retention time (min)	Peak Area (%)
1	Oleic acid	$C_{18}H_{34}O_2$	282	20.833	13.92
2	Morphine	$C_{17}H_{19}NO_3$	285	26.858	12.97
3	β -Sitosterol	$C_{29}H_{50}O$	414	29.650	11.79
4	Palmitic acid	$C_{16}H_{32}O_2$	256	19.208	8.29
5	Hentriacontane	$C_{31}H_{64}$	436	25.200	8.28
6	Benzoic acid	$C_7H_6O_2$	122	25.825	6.01
7	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	18.858	5.96
8	2-Pentadecanone	$C_{15}H_{30}O$	226	18.050	1.96
9	Dioctyl phthalate	$C_{24}H_{38}O_4$	390	24.008	3.78
10	Piperine	$C_{17}H_{19}NO_3$	285	25.725	3.74
11	Methyl oleate	$C_{19}H_{36}O_2$	296	20.492	3.21
12	Monopalmitin	$C_{19}H_{38}O_4$	330	23.875	3.07
13	Stigmasterol	$C_{29}H_{48}O$	412	29.142	3.01
14	γ -Sitosterol	$C_{29}H_{50}O$	414	30.192	2.77
15	Linoleic acid	$C_{19}H_{34}O_2$	294	20.433	2.39
16	Nonacosane	$C_{29}H_{60}$	408	26.517	2.38
17	Arachidic acid	$C_{20}H_{40}O_2$	312	21.217	1.88
18	Indole-3-acetic acid	$C_{10}H_9NO_2$	175	23.542	1.75
19	5-Octadecene	$C_{18}H_{36}$	252	22.150	1.59
20	Methyl linoleate	$C_{19}H_{34}O_2$	294	22.217	1.17

Table 2. Compounds identified from ethyl acetate sub-fraction of methanolic stem extract of *Chenopodium murale* through GC-MS analysis.

Sr. No.	Names of compounds	Molecular formula	Molecular weight	Retention time (min)	Peak Area (%)
1	Acetic acid, butyl ester	$C_6H_{12}O_2$	116	3.533	22.72
2	2-Hexanol	$C_6H_{14}O$	102	3.308	21.36
3	Cyclopentanol	$C_5H_{10}O$	86	3.042	20.15
4	*p*-Xylene	C_8H_{10}	106	4.575	13.67
5	γ-Sitosterol	$C_{29}H_{50}O$	414	29.667	10.09
6	*o*-Xylene	C_8H_{10}	106	5.017	7.18
7	Nitrobenzene	$C_6H_5NO_2$	123	8.650	3.57
8	Palmitic acid	$C_{17}H_{34}O_2$	270	18.875	1.21

Table 3. Antifungal activity of components of chloroform and ethyl acetate fraction of methanolic stem extract of *Chenopodium murale* as reported in literature.

#	Names of compounds	Target fungi	Reference
1	Morphine	*C. albicans*	27
2	Oleic acid	*Candida albicans* and *Aspergillus fumigatus*	36
3	2-Hexanol	*C. albicans* and *A. niger*	17
4	Cyclopentanol	*C. albicans* and *A. niger*	8
5	Palmitic acid	*Candida tropicalis*	25
6	Acetic acid, butyl ester	*C. krusei, C. glabrata* and *C. albicans*	29
7	β-Sitosterol	*Cladosporium cladosporioides* and *A. niger*	6
8	Hexadecanoic acid, methyl ester	*A. niger, A. flavus* and *F. oxysporum*	19
9	Hentriacontane	*F. oxysporum* and *A. solani*	10
10	Benzoic acid	*F. oxysporum, F. moniliforme* and *F. solani*	4

Some of the identified phytoconstituents are known to possess antifungal properties that might be responsible for suppressing the growth of FOL in the present study (Table 3). Oleic was a major compound in chloroform sub-fraction with 13.92 % peak area. Zhu *et al.* (37) worked on the fungicidal activity of oleic acid and found that it significantly reduced the growth of *Candida albicans* and *Aspergillus fumigatus.* Similarly, 2-hexanol and cyclopentanol, the second and third most abundant compounds in ethyl acetate sub-fraction with 21.36 % and 20.15 % peak areas, were previously isolated from sunflower (18) and the essential oil of a medicinal plant *Helichrsyum italicum* (8), respectively. Both these compounds were very inhibitory to growth of *C. albicans* and *A. niger*. Likewise, morphine has also been isolated from the medicinal plant *Withania somnifera* extracts and showed a remarkable antifungal effect to *C. albicans* (28). Recently, Prasath *et al.* (27) reported the antifungal efficacy of palmitic acid against the *Candida tropicalis.* Acetic acid, butyl ester (22.72 %), the most abundant compound in ethyl acetate sub-fraction, was found in the extracts of a cyanobacterium *Oscillatoria* sp., arrested the growth of *Candida. krusei, C. glabrata* and *C. albicans* (30). The *n*-hexane root extract of *Dipsacus asper* contained β-sitosterol in higher concentrations and tested against a wide range of fungal pathogens, it was most inhibitory to *Cladosporium cladosporioides* and *A. niger* (6). Hentriacontane has also been reported as a fungicidal compound against *F. oxysporum* and *Alternaria solani* (10). Marrez and Sultan (20) isolated hexadecanoic acid, methyl ester from a cyanobacterium *Microcystis aeruginosa* with strong inhibitory potential against *Aspergillus flavus, A. niger* and *F. oxysporum.* Benzoic acid was found effective against *Fusarium moniliforme, F. oxysporum* and *F. solani* (4). Structures of potential antifungal compounds are shown in Fig. 5.

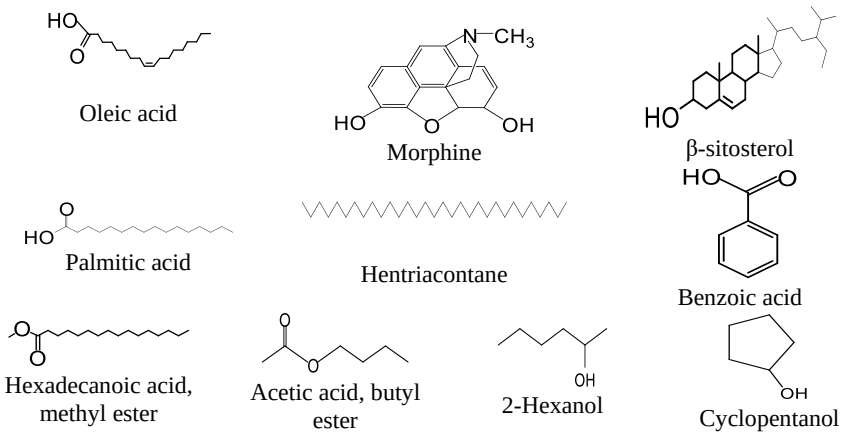

Figure 5. Structures of possible antifungal compounds in chloroform and ethyl acetate sub-fractions of methanolic stem extract of *Chenopodium murale.*

CONCLUSIONS

The methanolic stem extract of *C. murale* had remarkable antifungal potential to control the *Fusarium oxysporum* growth. The methanolic stem extract of *C. murale* at 5 % concentration, reduced the fungal growth up to 74 %. Fractionation of this extract using solvents of different polarity natures showed that most of the antifungal compounds were present in chloroform fraction that completely controlled the fungal growth at 200 mg mL^{-1} concentration. The stem extract contained many well-known antifungal compounds (oleic acid; morphine; palmitic acid; β-sitosterol; 2-hexanol hentriacontane; acetic acid, butyl ester and cyclopentanol).

DECLARATION

We declare that all authors of this Ms. have made substantial contributions. We did not exclude any author who substantially contributed to this Ms. We have followed our ethical norms established by our respective institutions.

CONFLICT OF INTEREST

The authors announce that they have no conflict of interest.

ETHICAL APPROVAL

The authors declare that the study was carried out following scientific ethics and conduct. However, this study did not involve any use of animals, hence no ethical approval has been obtained from the concerned committee.

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