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Table: 3, Figs: 4

Antifungal potential of *Chenopodium quinoa* root extract against *Macrophomina phaseolina* (Tassi) Goid.

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ABSTRACT

Quinoa (*Chenopodium quinoa* Willd.) is a newly introduced crop in Pakistan. We evaluated the antifungal efficacy of root extracts of 4-quinoa varieties (PI 596293, Ames 13730, Ames 13737 and PI 634919) against the most harmful soil inhabiting fungal pathogen *Macrophomina phaseolina* (Tassi) Goid. Methanolic root extracts (1 to 5 %) of these varieties reduced the fungal biomass by 33-70 %. Due to its higher antifungal activity, Ames 13737 variety was selected for further studies and fractionated with 4-organic solvents (*n*-hexane, chloroform, ethyl acetate and *n*-butanol) based on their increased polarities. All concentrations of chloroform and the lowest concentration of *n*-butanol (1.562 mg mL^{-1}) completely inhibited the pathogen growth. These fractions were further subjected to GC-MS analysis that revealed the presence of 7-antifungal phytoconstituents *(*decane; undecane; oleic acid; benzene, 1,2,3-trimethyl; cycloheptasiloxane, tetradecamethyl-; cyclohexasiloxane, dodecamethyl- and 9-octadecanoic acid (Z)-, methyl ester). This study concluded that chloroform and *n*-butanol fractions of root extract of quinoa possess strong antifungal potential against *M. phaseolina.*

Keywords: Antifungal activity, *Chenopodium quinoa*, chloroform fraction, GC-MS, *Macrophomina phaseolina*, *n*-butanol fraction, phytochemicals, Quinoa, root extract.

INTRODUCTION

Macrophomina phaseolina (Tassi) Goid. causes charcoal rot disease in many crops in subtropical and tropical areas (6,17,38). It is a soil- and seed-inhibiting pathogen that produces sclerotia as the prime source of its survival. Depending upon the soil conditions, its sclerotia can survive in the soil up to 3-years even in the absence of host plant (27). The pathogen severity increases in high temperature and low moisture contents (8). Pathogen management is difficult task despite the physical, biological and cultural approaches (23). These measures are effective, only when used prior to pathogen attack. Once the pathogen develops, none is effective and practical. Hence farmers mostly rely on chemical fungicides for its control (4), but in the recent decades the use of such chemicals has been discouraged due to their serious health hazards (20). Besides, they cause toxicity, persist in soil and pollute the environment. Hence researchers are searching the bio-products to minimize the dependency on present pesticides to save the environment (19,21). Different plants extracts [*Nigella sativa* L. (1), *Sonchus oleraceous* L. (5), *Monotheca buxifolia* (Falc.) A. DC. (14), and *Ageratum conyzoides* L. (6)] have been studied to manage this devastating pathogen.

The genus *Chenopodium* [family Chenopodiaceae (presently Amaranthaceae)], has broad medicinal and antifungal properties (35). Extracts of *Chenopodium album* L., *C. murale* L., *C. ambrosioides* (L.) and *C. botrys* L. are highly effective against many pathogens such as *M. phaseolina*, *Sclerotium rolfsii* Sacc., *Fusarium oxysporum* (Schlecht.) Snyder & Hansen and *Alternaria alternata* (Fr.) Keissl. (11,28,35). *C. quinoa* (Figure 1) is native to Andean states of South America and has recently been introduced in Pakistan (9).

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Figure 1. *Chenopodium quinoa* growing in a field.

It is a highly nutritious multipurpose agro-industrial crop that can be grown under adverse climatic conditions such as drought, salinity and frost (22). It is a rich source of saponins, used as potential antibiotics, pesticides, fungicides, insecticides and in pharmaceutical industry (10). Recently, Khan and Javaid reported the antifungal activity of its leaf and stem extracts against *M. phaseolina* (17,18). However, studies on antifungal activity of *C. quinoa* root extract are rare. Hence, this study aimed (i). to evaluate the antifungal efficacy of root extracts of 4 quinoa varieties against *M. phaseolina* and (ii). to identify the possible antifungal compounds through GC-MS analysis.

MATERIALS AND METHODS

Methanolic extraction

The seeds of 4-quinoa varieties (PI 596293, Ames 13730, Ames 13737 and PI 634919) were obtained from the University of Agriculture, Faisalabad, Pakistan (Table 1). The seeds were sown in November 2020 under field conditions at University of the Punjab Lahore, Pakistan (31°15 - 31°45′ N and 74°01′- 74°39′ E, altitude 217 m, annual rainfall 838.8 mm) using recommended agronomic practices. When the crops reached maturity (120 days after sowing), the plants were uprooted carefully and roots were washed in tap water to remove the adhering soil debris. The roots were dried in shade and then crushed with mechanical grinder. Root sample (200 g) of each variety was extracted for 2 weeks in methanol (1 L) at room temperature. Next, the mixture was filtered through Whatman No. 1 filter paper and the filtrate was evaporated on a rotary evaporator until a gummy residue remained. A stock solution of each variety was prepared by dissolving 9 g crude methanolic extract of each variety in 5 mL of dimethyl sulphoxide (DMSO) and

Table 1. Origin of test varieties used in this study.

Code	G. Line	Origin
PI 596293	V1	Colorado, USA
Ames 13730	V2	New Mexico, USA
Ames 13737	V7	New Mexico, USA
PI 634919	V9	Chile

raising the volume to 15 mL with the addition of distilled water. Similarly, a control solution was also prepared without methanolic extracts. Different concentrations (1, 2, 3, 4 and 5 %) of each quinoa extract were prepared in autoclaved malt extract (ME) broth (55 mL) by adding 5 mL mixtures of stock and control solutions in different proportions (1:4, 2:3, 3:2, 4:1 and 5:0) in conical flasks. All concentrations were divided in 4-equal aliquots and each served as a replicate. Then media containing flasks were inoculated with 8-day-old actively growing *M. phaseolina* (isolated from mungbean) culture discs of 5 mm size and kept at 28 °C for one week. Thereafter, the fungal mats were collected on pre-weighed filter papers and air dried at 60 °C in an electric oven to get their dry weights (13).

Fractionation

Further experimental studies were done on Quinoa variety 'Ames 13737' due to its highest antifungal activity in bioassays. Two kilograms root material was extracted for 15 days in methanol (6 L). The filtrate was evaporated to obtain crude extract that was dissolved in 300 mL distilled water. The resultant mixture was fractioned using 4-organic solvents (*n*-hexane, chloroform, ethyl acetate and *n*-butanol) of different polarities. First, the mixture was mixed with *n*-hexane (500 mL) and left for 3 h in a separating funnel for the separation of two phases. This procedure was repeated several times for complete separation of *n*-hexane soluble components from aqueous phase followed by a successive partitioning with chloroform, ethyl acetate and *n*-butanol. All fractions were then evaporated under vacuum and the gummy mass obtained was used for further antifungal bioassays.

For the antifungal bioassays, 1.2 g of each of the 4-organic solvent fractions of methanolic root extract was dissolved in DMSO (1 mL) and the volume was raised to 6 mL with the addition of ME (malt extract) broth to obtain a stock solution of 200 mg mL^{-1}. It was subsequently double diluted to prepare lower concentrations *viz.*, 1.562, 3.124, 6.25, 12.5, 25, 50 and 100 mg mL^{-1}. A control was prepared similarly without extract fractions. Next, the *M. phaseolina* suspension was prepared from 8-day-old colonies in autoclaved distilled water. Each tube was inoculated with 20 µL of the fungal inoculum and left for one week at 28 °C. Thereafter, the fungal mats were separated, dried and weighed (1).

GC-MS analysis

GC-MS analyses of chloroform and *n*-butanol fractions (showing the best antifungal activity) were carried out on a Shimadzu GC-2010 plus chromatographic system. The capillary column was 0.25 µm × 0.25 mm × 30 m. A carrier gas helium was used in its highly pure form in accordance with split-less injection system (1.0 µL volume), developed for operating the chromatograph with 1 cm^3 min^{-1} spill count at 250 °C. Sample running time was set for 17 min for the recognition of compounds and compared with a library established in computer. Mass spectral library Version 2.70, Shimadzu Co. was used in this study.

Statistical analysis

ANOVA was performed on all the experimental data followed by LSD test ($P \leq 0.05$) by using Statistix 8.1 software.

RESULTS AND DISCUSSION

Antifungal activity of methanolic root extracts

Extracts of all 4-varieties significantly suppressed growth of the pathogen by 33-70 %. Extracts of quinoa varieties PI 596293, Ames 13730, Ames 13737 and PI 634919 reduced biomass of *M. phaseolina* by 45-66 %, 43-70 %, 41-66 % and 33-60 % over control, respectively (Figure 2). The genus *Chenopodium* is known for its antifungal properties but the studies regarding *C. quinoa* antifungal efficacy are rare. In the present study, methanolic extracts of four selected quinoa varieties inhibited the *M. phaseolina* growth. However, the various extracts were not equally antifungal in nature against the pathogen. Earlier, Aluwi *et al.* (3) described how quinoa varieties differ in their physical and chemical properties. Likewise, Miranda *et al.* (24) worked on ethanolic extracts of 6-quinoa varieties and reported variable antimicrobial efficacy by using agar disk method. Therefore, findings of the present investigation are in agreement with those of previously recorded literature. Quinoa root's antifungal properties may be attributed to the saponins found in it (30).

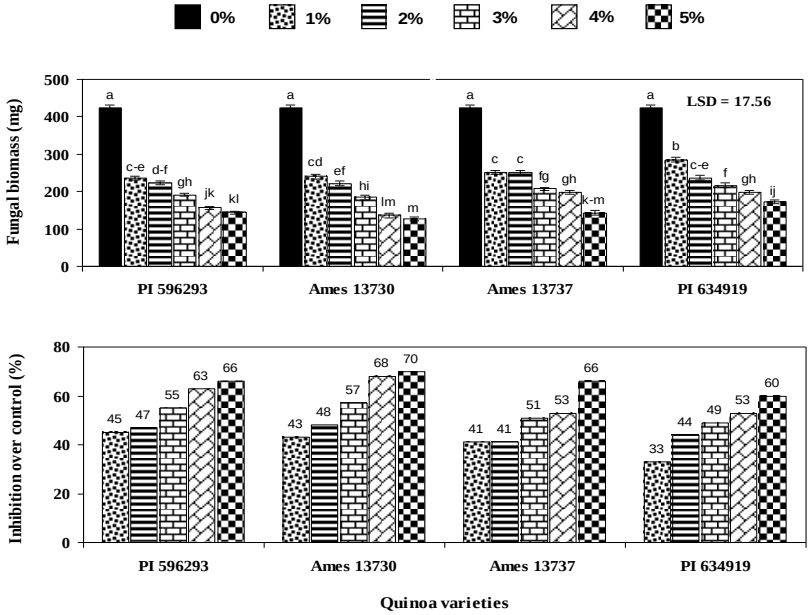

Figure 2. Inhibitory effects of methanolic root extract concentrations of 4-varieties of *Chenopodium quinoa* on biomass of *Macrophomina phaseolina*. Vertical bars show standard errors of means of four replicates. Values with different letters at their top show significant difference ($P \leq 0.05$) as determined by LSD Test

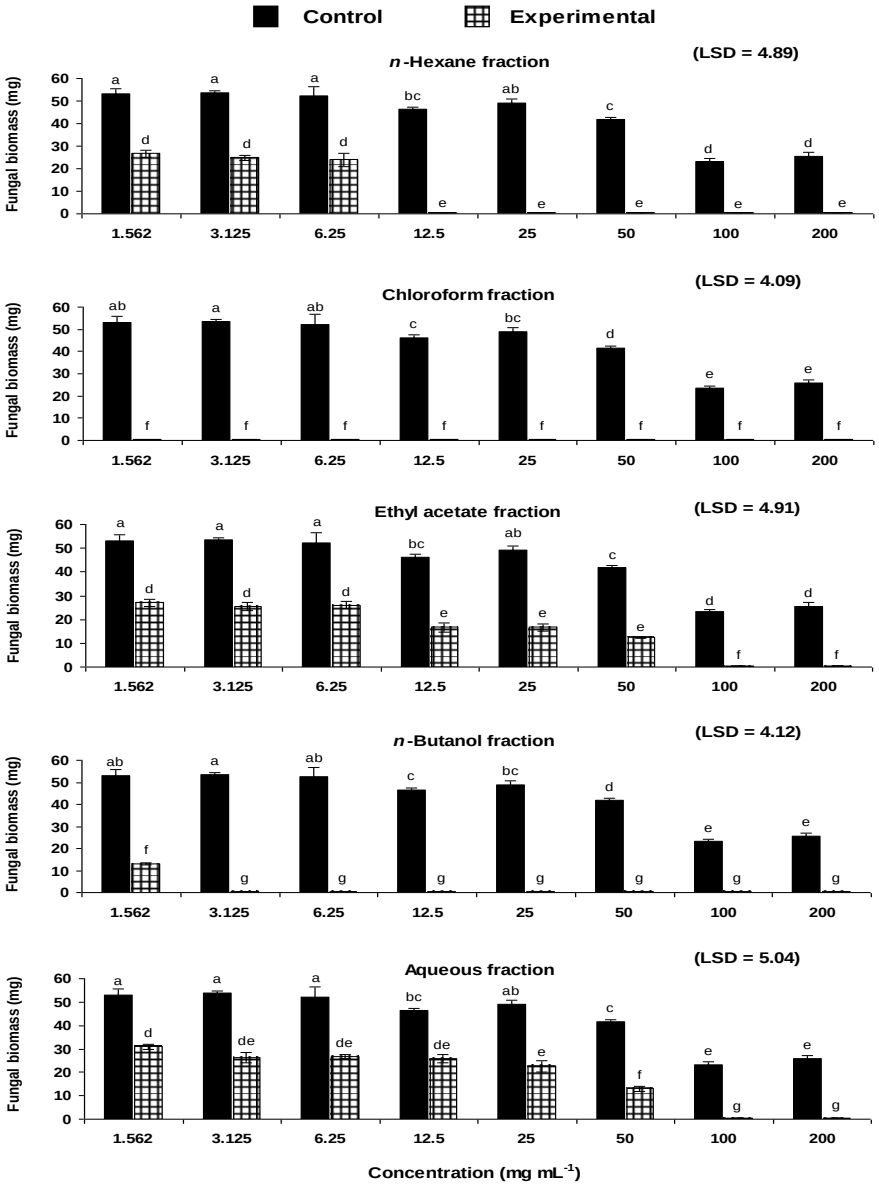

Figure 3. Effects of different concentrations of *n*-hexane, chloroform, ethyl acetate, *n*-butanol and aqueous extracts of methanolic root extract of *Chenopodium quinoa* variety 'Ames 13737' on biomass of *Macrophomina phaseolina.* Vertical bars show standard errors of means of four replicates. Values with different letters at their top show significant difference ($P \leq 0.05$) as determined by LSD Test.

Yao *et al.* (39) worked on four saponin fractions of *C. quinoa* and reported that triterpene and monodesmosidic were predominant with supposed antifungal properties. Quinoa associated saponins have some biological properties that are unfortunately still unknown. Previously, it was reported that *C. quinoa* isolated saponins have antifungal potential against the pathogenic fungal spores of *Botrytis cinerea*. They function by adhering with fungal membranes and cause damage to their spore formation and integrity (33,37).

Antifungal activity of fractions of methanolic root extracts

Different fractions of quinoa variety 'Ames 13737' root extract had variable antifungal activity against *M. phaseolina* (Figure 3 and 4). The chloroform fraction followed by *n*-butanol fraction were the most antifungal. All concentrations of chloroform fraction and all *n*-butanol fractions except the lowest concentration (1.562 mg mL^{-1}) completely inhibited the pathogen. The *n*-hexane fraction at lower concentration (1.562 to 6.25 mg mL^{-1}) remarkably reduced the fungal biomass (50-54 %) while the higher concentration (12.5 mg mL^{-1}) completely arrested the pathogen growth. Lower concentrations (1.562 to 50 mg mL^{-1}) of ethyl acetate and aqueous fractions were less effective in suppressing the fungal biomass (49-70 % and 42-69 %), respectively. At 200 and 100 mg mL^{-1} concentrations, the fungal growth was completely controlled.

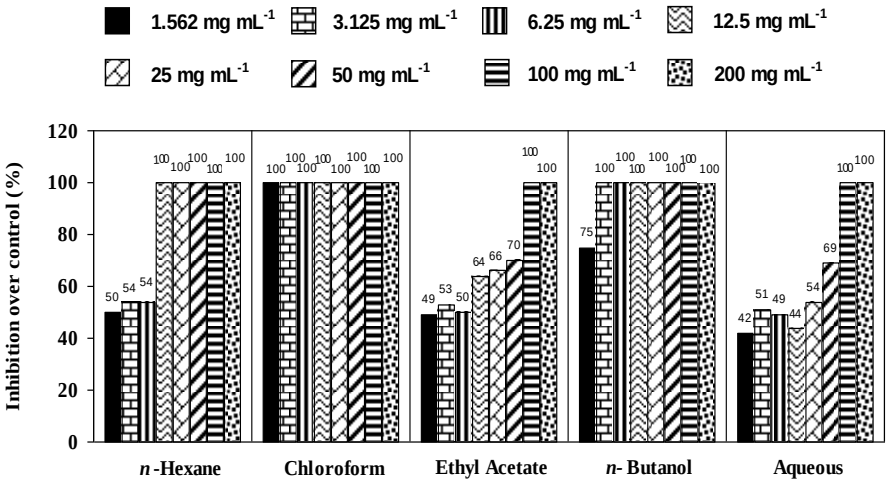

Figure 4. Percentage increase/decrease in biomass of *Macrophomina phaseolina* due to different concentrations of *n*-hexane, chloroform, ethyl acetate, n-butanol and aqueous fractions of methanol root extract of quinoa over control.

'Ames 13737' methanolic extract was fractionated using different organic solvents with variable polarities, allowing compounds in the extract to be separated into different groups. The bioassays with these fractions of methanolic extract, showed high activity against the target pathogen. Chloroform fraction was most effective, followed by *n*-butanol and *n*-hexane fractions. The *C. quinoa n*-hexane fraction of inflorescence extract exhibited strong antifungal activities (15). Similarly, Khan and Javaid (16) worked on *C. quinoa* ethyl acetate fraction of stem extract and found various antifungal constituents through GC-MS

analysis. Rauf and Javaid (32) reported that different fractions of *C. album* were variable in their antifungal activities against the targeted soil-borne fungal pathogen *Fusarium oxysporum.* Sherazi *et al*. (36) found that *Ascochyta rabiei* can be managed by *Chenopodium album* extracts prepared in *n*-hexane extract. The *Sisymbrium irio* weed was fractionated in different solvents based on increase in polarities and tested against *M. phaseolina,* where, *n*-hexane and chloroform extracts controlled the fungal growth (12).

GC-MS analysis

(i). Chloroform fraction: The GC-MS chromatogram of chloroform fraction revealed the presence of 9 constituents (Table 2). The most prevailing compounds were decane; undecane; oleic acid; benzenemethanol,2,5-dimethoxy acetate; benzene, 1,4-diethyl and benzene, 1,2,3-trimethyl with peak areas of 24.52 %, 16.13 %, 11.60 %, 11.25 %, 10.04 % and 10.00 %, respectively. The compounds namely 4-((1E)-3-hydroxy-1-propenyl)-2-methoxyphenol; heptane,3,3,5-trimethyl and octadecanoic acid, 9,10-dihydroxy-, methyl ester were less abundant with peak areas of 7.01 %, 4.77 % and 4.67 %, respectively, were recorded as less abundant ones.

Table 2. List of compounds identified in chloroform fraction of methanolic root extract of quinoa by GC-MS analysis.

Sr. No.	Names of compounds	Molecular formula	Molecular weight	Retention time (min)	Peak Area (%)
1	Decane	$C_{10}H_{22}$	142	2.374	24.52
2	Benzene, 1,2,3-trimethyl	C_9H_{12}	120	2.423	10.00
3	Heptane,3,3,5-trimethyl	$C_{10}H_{22}$	142	2.467	4.77
4	Benzene,1,4-diethyl	$C_{10}H_{14}$	134	2.667	10.04
5	Undecane	$C_{11}H_{24}$	156	2.801	16.13
6	4-((1E)-3-Hydroxy-1-Propenyl)-2-methoxyphenol	$C_{10}H_{12}O_3$	180	6.169	7.01
7	Benzenemethanol,2,5-dimethoxy acetate	$C_{11}H_{14}O_4$	210	7.258	11.25
8	Oleic acid	$C_{18}H_{34}O_2$	282	7.806	11.60
9	Octadecanoic acid,9,10-dihydroxy-methyl ester	$C_{19}H_{38}O_4$	330	9.418	4.67

(ii). *n*-butanol fraction: The GC-MS chromatogram of *n*-butanol fraction revealed the presence of 14 phytoconstituents (Table 3). The most abundant compounds were decane and 1H-indene, octahydro-, *cis*- with peak areas of 42.17 % and 23.34 %, respectively. The compounds namely 1H-indene, octahydro-5-methyl-; cycloheptasiloxane, tetradecamethyl- and cyclohexasiloxane, dodecamethyl- were present in moderate concentrations with peak areas of 8.01 %, 5.04 % and 3.65 %, respectively. The least abundant compounds were 9-octadecanoic acid (Z)-, methyl ester; cyclohexane, butyl-; cyclooctasiloxane, hexadeca-methyl-; cyclononasiloxane, octadecamethyl-; oxirane,[(1-methylethoxy)methyl]-; benzene, nitro-; cyclodecasiloxane, eicosamethyl-; undecane and hexadecanoic acid, methyl ester with respective peak areas of 2.89 %, 2.84 %, 2.55 %, 1.85 %, 1.83 %, 1.72 %, 1.65 %, 1.23 % and 1.15 %, respectively.

Table 3. Compounds identified in *n*-butanol fraction of methanolic stem extract of *Chenopodium quinoa* by GC-MS analysis.

Sr. No.	Names of compounds	Molecular formula	Molecular weight	Retention time (min)	Peak area (%)
1	Decane	$C_{10}H_{22}$	142	2.367	42.17
2	1H-Indene,octahydro-,*cis*-	C_9H_{16}	124	2.447	23.34
3	Cyclohexane, butyl-	$C_{10}H_{20}$	140	2.566	2.84
4	1H-Indene,octahydro-5-methyl-	$C_{10}H_{18}$	138	2.658	8.01
5	Undecane	$C_{11}H_{24}$	156	2.793	1.23
6	Benzene, nitro-	$C_6H_5NO_2$	123	2.854	1.72
7	Oxirane,[(1-methylethoxy)methyl]-	$C_6H_{12}O_2$	116	3.105	1.83
8	Cyclohexasiloxane, dodecamethyl-	$C_{12}H_{36}O_6Si_6$	444	3.735	3.65
9	Cycloheptasiloxane, tetradecamethyl-	$C_{14}H_{42}O_7Si_7$	518	4.635	5.04
10	Cyclooctasiloxane, hexadecamethyl-	$C_{16}H_{48}O_8Si_8$	592	5.468	2.55
11	Cyclononasiloxane, octadecamethyl-	$C_{18}H_{54}O_9Si_9$	666	6.194	1.85
12	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	6.901	1.15
13	9-Octadecanoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$	296	7.640	2.89
14	Cyclodecasiloxane, eicosamethyl-	$C_{20}H_{60}O_{10}Si_{10}$	740	9.198	1.65

Chloroform and *n*-butanol fractions were highly effective in reducing the *M. phaseolina* growth even at lower concentrations. GC-MS analysis of these fractions was done to identify the antifungal compounds (Table 4). GC-MS identifies the diverse range of fatty acids, esters, alkanes, branched chain and long chain hydrocarbon constituents (2). In the present study, decane was identified as a major component of quinoa root. Fernando *et al*. (7) isolated decane from *Phyllanthus amarus* leaf methanolic extract and tested against *Sclerotinia sclerotiorum* and *Colletotrichum truncatum.* The compound effectively reduced the test pathogen growth at 150 µL concentration. Antifungal activity of undecane, a prominent compound in the present study, was previously tested against the barley net blotch pathogen *Pyrenophora teres* with promising results (26,34). Presher and Dhanda (31) identified the compound cyclohexasiloxane, dodecamethyl from a fungus *Nigrospora sphaerica* ethyl acetate extract. These compounds were tested against *Candida* spp. and *Drechslera halodes* with excellent antifungal properties. Likewise, antifungal efficacies of oleic acid; benzene, 1,2,3-trimethyl; cycloheptasiloxane, tetradecamethyl and 9-octadecanoic acid (Z)-, methyl ester are reported in literature against, *Botrytis cinerea*, *C. albicans* and *A. niger* with notable results (25,29).

CONCLUSIONS

The root extracts of all the 4-varieties (PI 596293, Ames 13730, Ames 13737 and PI 634919) of *Chenopodium quinoa* possess remarkable antifungal efficacy against *M. phaseolina.* Methanolic extract of ‘Ames 13737’ was fractionated using four organic

solvents based on their polarity. Among these, chloroform and *n*-butanol fractions gave the extraordinary performance in controlling *M. phaseolina* growth. GC-MS analyses of these fractions numerous compounds among them decane; undecane; cyclohexasiloxane, dodecamethyl; cycloheptasiloxane, tetradecamethyl and 9-octadecanoic acid (Z)-, methyl ester were responsible for antifungal activity of root extract against *M. phaseolina*.

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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