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Full Length Article

Comparative Antifungal Potential of Stem Extracts of Four Quinoa Varieties against *Macrophomina phaseolina*

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Abstract

Quinoa (*Chenopodium quinoa* Willd.) is native to South America and is recently introduced in Pakistan. Previous studies revealed that wild species of *Chenopodium* from Pakistan hold antifungal potential against a seed- and soil-borne notorious plant pathogen *Macrophomina phaseolina*. In the present study, quinoa stem was assessed for its antifungal potential against this destructive plant pathogen. Diseased mungbean plants were collected for the procurement of fungal pathogen and methanolic stem extracts (1, 2, 3, 4 and 5%) of four varieties of quinoa were examined *in vitro* for their antifungal properties. Different extracts showed profound antifungal activity and reduced fungal biomass by 48–89%. Quinoa variety V7 showed the best results against the pathogen and minimized its growth by 80–89%. Methanolic extract of this variety was fractionated with four organic solvents on the basis of increase in their polarities. Eight concentrations ranging from 1.562 to 200 mg mL^{-1} were used in antifungal bioassays for each fraction. Each concentration of *n*-hexane and chloroform fraction arrested growth of the pathogen by 100%. Ethyl acetate and *n*-butanol fractions were proved less antifungal with 66–100% and 52–100% decline in pathogen's biomass, respectively. GC-MS study of *n*-hexane and chloroform fractions revealed the presence of predominant antifungal compounds *viz.*, 9,12-octadecadien-1-ol, (Z,Z)- (22.23%) followed by 8,11-octadecadienoic acid, methyl ester (16.68%); 1,2-benzedicarboxylic acid, diisooctyl ester (12.48%); and hexadecanoic acid,2-hydroxy-1-(hydroxymethyl) ethyl ester (10.99%). To conclude, *n*-hexane and chloroform fractions of stem extract of V7 variety of quinoa possess effective antifungal constituents against the pathogen.

Keywords: Antifungal constituents; GC-MS analysis; *Macrophomina phaseolina*; Quinoa; Stem extract

Introduction

Macrophomina phaseolina (Tassi) Goid. is a necrotrophic fungal pathogen responsible for soil- and seed-borne diseases in up to 500 economically important host plants including mungbean, mashbean, soybean, sunflower, sorghum, maize, linseed, chickpea and alfalfa (Pawlowski *et al*. 2015). At initial stages of infection, symptom is not visible but later on they can appear as black lesions on plant stem, peduncle and branches which ultimately invade the vascular bundles and causes root rot, collar rot, seedling blight and damping off disease in plants (Arora 2017). It forms hard sclerotia, which can survive for long time in the soil and upon favorable environmental conditions become primary source of infection. The pathogen becomes more destructive under dry and humid environmental conditions. As the disease progresses, it provokes the root system destruction along with chlorosis, growth losses, withering, and ultimately death of a plant (Ullah *et al.* 2019).

Many of the fungicides are in practice for the control of fungal pathogens but no registered fungicide is available in market for the management of *M. phaseolina* due to the production of sclerotia. The fungicides have also hazardous effects on environment that makes their usage restricted (Kalsoom *et al.* 2019). In this regard scientists are making efforts to find out environment friendly and cheap bio-products derived from plants to control the soil-borne pathogens as they are non-phytotoxic and an excellent substitute to synthetic fungicides. Plants contain a large number of secondary metabolites such as tannin, terpenoids, alkaloids, flavonoids, phenols, volatile oils, glycosides and steroids, which manifest antifungal properties (Khan *et al*. 2019). Recently, various reports on different parts of plant extracts exhibited strong antifungal potential against plant pathogenic fungi under *in vitro* conditions. In this regard, the extracts of *Sisymbrium irio*, *Senna occidentalis*, *Azadirachta indica*, *Kochia indica* and *Sonchus oleraceous* have proved very effective to control *M. phaseolina* (Javed *et al.* 2018; Munir *et al.* 2018).

Members of Chenopodiaceae family such as *Chenopodium ambrosioides*, *C. album* and *C. murale* are known to possess several allelochemicals found very useful in obstructing the growth of phytopathogens especially *M. phaseolina* (Javaid and Amin 2009). *C. quinoa* also belongs

to this family and is known as a pseudo-cereal. It gained worldwide importance due to its diverse genetic characteristics and recently introduce in North America, Asia, Africa and Europe. It is commonly known as quinoa, became an excellent food crop for humans as an alternate to wheat because of its high nutritious values. In recent years, its cultivation has also been started in Pakistan on a large scale due to its remarkable tolerance to salinity, drought and heat (Hernandez-Ledesma 2019). Therefore, keeping in view that it is a member of family Chenopodiaceae, it was hypothesized that *C. quinoa* may also contain antifungal compounds with potent efficacies against plant pathogens. Therefore, stem extracts of four varieties of quinoa were explored for their potential to control *M. phaseolina* and the detection of antifungal compounds through GC-MS analysis.

Materials and Methods

Antifungal bioassays

For the collection of plant stem in appropriate quantity, four quinoa verities namely V1, V2, V7 and V9 were cultivated in winter 2017 in Lahore. Seeds of the four varieties were obtained from University of Agriculture, Faisalabad, Pakistan. Details regarding origin of these varieties are given in Table 1. At the time of maturity, the stems of each variety were collected, dried and thoroughly crushed. Methanolic extracts were prepared by macerating 200 g of crushed stems of each variety in methanol (1 L) and kept for two weeks at room temperature. Thereafter, the mixture was coarse filtrated by muslin cloth and the extract was concentrated by recovering the solvents on rotary evaporator at 45°C. Stock solution of 15 mL of each extract was prepared in dimethyl sulphoxide (5 mL) by dissolving 9 g of crude methanolic extracts with subsequent addition of autoclaved distilled water. Similarly, control solution was prepared without the addition of plant extract. Five concentration *viz.* 1, 2, 3, 4 and 5% were formulated by mixing control and stock solutions in suitable amounts with four replicates of each as reported by Javaid *et al.* (2017). *M. phaseolina* was procured from Biofertilizer and Biopesticide Lab, IAGS, Punjab University Lahore. Five-millimeter diameter mycelial plugs of 7-day-old *M. phaseolina* culture were added to each conical flask and left to stand at 28°C. After 7 days, fungal mats were collected on filter papers and dried in an electric oven at 70°C for data collection.

The variety named V7 was selected and fractionated with different solvents with increase in their polarities. For this, 3 kg of shade dried, powdered plant stem was dipped into 10 L of methanol for 15 days and filtered through a muslin cloth. After that, the thick gummy extract was suspended in autoclaved distilled water 200 mL and kept for 4 h. The mixture was successively fractionated beginning with *n*-hexane (5 × 500 mL) followed by chloroform (500 mL), ethyl acetate (500 mL) and *n*-butanol (500 mL) into a separating funnel. Among these solvents, chloroform and *n*-hexane fractions were evaporated to obtain their crude extract. The *in vitro* biological activity of *n*-hexane and chloroform fraction was assessed against *M. phaseolina*. Out of the selected extracts, 1 mL of dimethyl sulphoxide was added to each 1.2 g of the extract to dissolve in followed by the addition of malt extract 5 mL in order to prepare the sequential concentrations starting with 200 mg mL^{-1} and then it was divided into two aliquots. One aliquot was used for further serial dilution to make the lower concentrations *viz*. 100, 50, …, 1.562 mg mL^{-1} and the other one was used to evaluate extract bio-efficacy. A control was also prepared similarly in a series without extract addition to maintain the amount of dimethyl sulphoxide. Inoculum of *M. phaseolina* was prepared from 8-day-old culture in autoclaved distilled water. The assay was performed by adding 50 µL aliquots of the inoculum in each test tube and left to stand at 28°C for 7 days. The obtained fungal mats were filtered and weighed after seven days of incubation (Shafique *et al*. 2016). Three replicates of each treatment were run simultaneously.

GC-MS analysis

GC-MS analysis of *n*-hexane and chloroform fractions was carried out for compounds identification. Ten milligrams of each of the two fractions were dissolved in 1 mL of their respective solvents and filtered through Whatman® glass microfiber filters grade GF/C. Analysis was performed by using a Shimadzu GC-2010plus system coupled with an auto sampler AOC-20s, an auto injector AOC-20i, and a gas chromatograph. Using helium as a carrier gas, a volume of 1.0 µL sample was injected by setting injector temperature at 250°C. The interface temperature was adjusted at 320°C. After injection of sample, the initial column temperature was 100°C for 60 s that was enhanced from 100 to 200°C at 20°C min^{-1} and hold for 2.0 min, finally from 200°C to 300°C at 40°C min^{-1}. The total run time was 10.9 min.

Statistical analysis

Completely randomized design was selected for both the laboratory experiments and all the data were analyzed by ANOVA and LSD test ($P \leq 0.05$) using Statistix 8.1.

Results

Antifungal activity

ANOVA presented in Table 2 indicates that the effect of extract concentration (C), quinoa varieties (V) and V×C was found to be very effective ($P \leq 0.001$) for the production of fungal biomass. Among the quinoa varieties, V7 methanolic extract showed a remarkable antifungal activity causing 80–89% suppression of fungal biomass. V9 extract was ranked as the second most effective antifungal source against *M. phaseolina* where it reduced its growth by 69–88% over

Table 1: Details of four varieties used in the present study

Quinoa lines	Origin	Plant name
V1	Colorado, USA	Colorado 407D
V2	New Mexico, USA	IESP
V7	New Mexico, USA	2WANT
V9	Chile	Pichaman

Table 2: Analysis of variance (ANOVA) for the effect of different concentrations of methanolic stem extracts of four varieties of *C. quinoa* on biomass of *M. phaseolina*

Sources of variation	df	SS	MS	F values
Varieties (V)	3	143547	47849	328*
Concentration (C)	5	1305852	261170	1790*
V × C	15	47138	3143	21.5*
Error	72	10505	146	
Total	95	1507042		

*, Significant at P≤0.001

control by using different concentrations. Although methanolic leaf extracts of other two varieties significantly declined fungal growth but their antifungal potentials were less pronounced than V7 and V9 as extracts of V1 and V2 inhibited fungal growth by 45–71% and 45–80%, respectively (Fig. 1).

The highest activity was shown by V7 methanolic extract and was thus selected for further studies. Different fractions of the extract were effective against *M. phaseolina* (Fig. 2). The selected non-polar fraction *n*-hexane, and less polar fraction chloroform completely arrested the growth of fungal pathogen with 1.562 mg mL^{-1} the lowest MIC value (Fig. 2A–B). In comparison to the others, two more polar fractions *viz. n*-butanol and ethyl acetate were relatively less inhibitory in nature with MIC values of 25 and 12.5 mg mL^{-1}, respectively. There was 52–100% and 66–100% reduction in *M. phaseolina* biomass over control due to the *n*-butanol and ethyl acetate fractions (Fig. 2C–D). The aqueous fraction with the highest polarity showed the least antifungal efficacy by suppressing 46–100% fungal growth (Fig. 2E).

GC-MS analysis

GC-MS chromatogram of *n*-hexane indicates the presence of 15 constituents as given in Fig. 3A. The most prevailing compounds were 9,12-octadecadien-1-ol, (Z,Z)- (22.23%) followed by 9,12-octadecadienoic acid-(Z,Z)-, methyl ester (16.84%) and 1-(+)-ascorbic acid 2,6-dihexadecanoate (15.18%). Moderately abundant compounds were hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)-ethyl ester (10.99%) and hexadecenoic-acid, methyl ester (7.37%). Whereas, the least abundant compounds were 1,2-benzedicarboxylic acid, diisooctyl-ester, ar-tumerone, 6-hexadecenoic-acid, 7-methyl, methyl ester (Z), octadecanoic acid, phytol, tetradecanoic acid, curlone, 2-propenoic-acid,3-[4-(acetyloxy)-3-methoxyphenyl]-, methyl ester, octadecanoic acid, methyl ester and benzoic-acid,4-hydroxy-3,5-dimethoxy-, hydrazide with peak areas ranging from 4.62 to 1.18% (Table 3; Fig. 4).

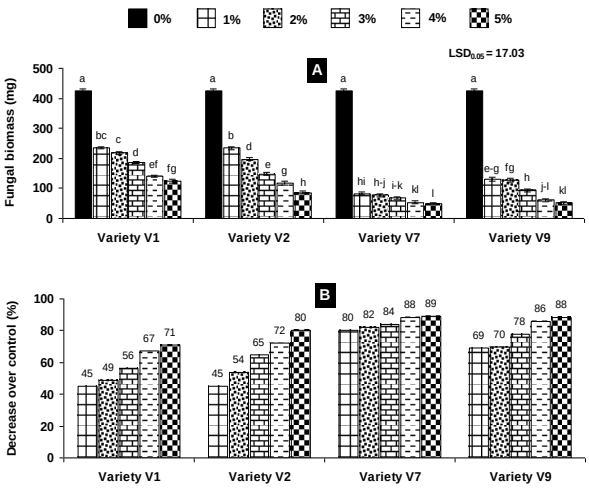


Fig. 1: Effect of different concentrations of methanolic stem extract of four varieties of *C. quinoa* on biomass of *M. phaseolina.* Vertical bars show standard errors of means of four replicates. Values with different letters at their top show significant difference (P≤0.05) as determined by LSD test.

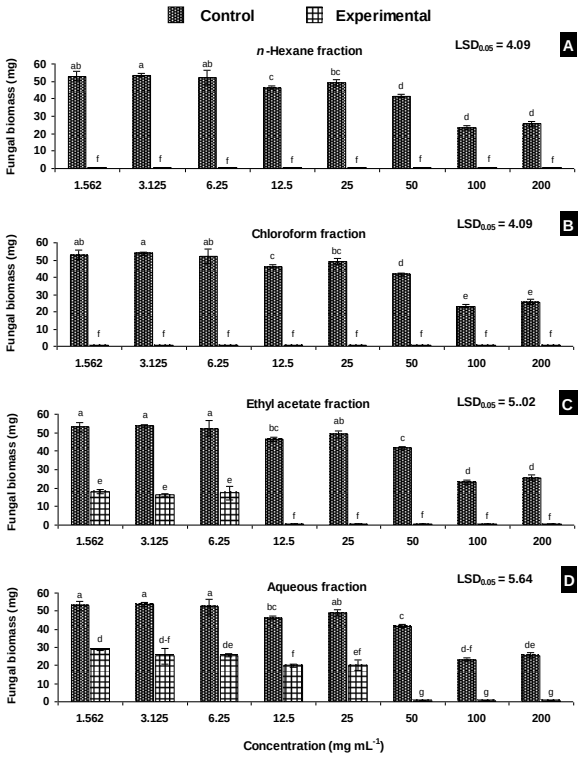


Fig. 2: Effect of different concentrations of *n*-hexane, chloroform and ethyl acetate fractions of methanolic stem extract of *C. quinoa* on biomass of *M. phaseolina.* Vertical bars show standard errors of means of four replicates. Values with different letters at their top show significant difference (P≤0.05) as determined by LSD test.

Chloroform fraction revealed the presence of 20 compounds (Table 4; Fig. 3B). 8,11-Octadecadienoic acid, methyl ester (16.68%) was present abunduntly followed by

Table 3: List of compounds in *n*-hexane fraction of methanolic stem extract of *C. quinoa* identified by GC-MS analysis

Names of compounds	Molecular formula	Molecular weight	Retention time (min)	Peak area (%)
Ar-tumerone	$C_{15}H_{20}O$	216	5.797	3.75
Curlone	$C_{15}H_{22}O$	218	5.996	2.50
Tetradecanoic Acid	$C_{14}H_{28}O_2$	228	6.177	2.53
Benzoic acid,4-hydroxy-3,5-dimethoxy-,hydrazide	$C_9H_{12}N_2O_4$	212	6.258	1.18
2-Propenoic acid,3-[4-(acetyloxy)-3-methoxyphenyl]-,methyl ester	$C_{13}H_{14}O_5$	250	6.608	2.07
Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	6.911	7.37
1-(+)-Ascorbic acid 2,6-dihexadecanoate	$C_{38}H_{68}O_8$	652	7.104	15.18
9,12-Octadecadienoic acid(z,z)-,methyl ester	$C_{19}H_{34}O_2$	294	7.637	16.84
Phytol	$C_{20}H_{40}O$	296	7.698	2.97
Octadecanoic acid, methyl ester	$C_{19}H_{38}O_2$	298	7.735	1.54
9,12-Octadecadien-1-ol,(z,z)-	$C_{18}H_{34}O$	266	7.837	22.23
Octadecanoic acid	$C_{18}H_{36}O2$	284	7.908	3.05
6-Hexadecenoic acid,7-methyl,methyl ester (z)	$C_{18}H_{34}O2$	282	8.492	3.18
Hexadecanoic acid,2-hydroxy-1-(hydroxymethyl) ethyl ester	$C_{19}H_{38}O4$	330	9.645	10.99
1,2-Benzedicarboxylic acid, diisooctyl ester	$C_{24}H_{38}O_4$	390	9.753	4.62

Table 4: List of compounds in chloroform fraction of methanolic stem extract of *C. quinoa* identified by GC-MS analysis

Names of compounds	Molecular formula	Molecular weight	Retention time (min)	Peak area (%)
Benzene, nitro-	$C_6H_5NO_2$	123	2.861	1.23
3-Acetoxy-3-hydroxypropionic acid,methyl ester	$C_6H_{10}O_5$	162	2.918	1.94
4-((1E)-3-Hydroxy-1-Propenyl)-2-methoxyphenol	$C_{10}H_{12}O_3$	180	6.168	3.28
1-Tetracosanol	$C_{24}H_{50}O$	354	6.208	3.36
1-Pentacosanol	$C_{25}H_{52}O$	368	6.255	6.28
2,4-Hexadienedioic acid,3,4-diethyl-,dimethyl ester,(z,z)-	$C_{12}H_{18}O_4$	226	6.436	1.81
3-Isopropoxy-4-methoxybenzamide	$C_{11}H_{15}NO_3$	209	6.538	2.01
Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	6.906	4.274
Piperine	$C_{17}H_{19}NO_3$	285	7.020	8.43
n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	7.066	3.95
4,8-Ethano-4H-1,3-benzodioxin,hexahydro-	$C_{10}H_{16}O_2$	168	7.142	1.99
Benzenemethanol,2,5-dimethoxy acetate	$C_{11}H_{14}O_4$	210	7.260	6.195
Dimethyl 1-(2-methoxyethyl)-5-methylpyrazole-3,4-dicarboxylate	$C_{11}H_{16}N_2O_5$	256	7.390	1.71
8,11-Octadecadienoic acid, methyl ester	$C_{19}H_{34}O_2$	294	7.629	16.68
9,12-Octadecadienoic acid(z,z)-	$C_{18}H_{32}O_2$	280	7.795	9.14
Cis-9-hexadecenal	$C_{16}H_{30}O$	238	8.617	1.08
gamma-Sitosterol	$C_{29}H_{50}O$	414	9.343	3.49
Hexadecanoic acid,2-hydroxy-1-(hydroxymethyl) ethyl ester	$C_{19}H_{38}O4$	330	9.624	2.07
1,2-Benzedicarboxylic acid, diisooctyl ester	$C_{24}H_{38}O_4$	390	9.752	12.48
1-Triacontanol	$C_{30}H_{62}O$	438	10.335	8.60

Table 5: Potential antifungal constituents in *n*-hexane and chloroform fractions of *Chenopodium quinoa* stem extract

Names of compounds	Property	Reference
9,12-Octadecadien-1-ol,(Z,Z)-	Antifungal	Wang *et al.* (2008)
Hexadecanoic acid,2-hydroxy-1-(hydroxymethyl) ethyl ester	Antifungal	Al-Marzoqi *et al.* (2015)
1,2-Benzedicarboxylic acid, diisooctyl ester	Antifungal	Rahman and Anwar (2006)
8,11-Octadecadienoic acid, methyl ester	Antifungal	Kianinia and Farjam (2018)
2,4-Hexadienedioic acid,3,4-diethyl-,dimethyl ester,(Z,Z)-	Antifungal	Chhouk *et al.* (2018)

1,2-benzedicarboxylic acid, diisooctyl ester (12.48%) and 9,12-octadecadienoic_acid_(Z,Z)- (9.14%). The moderately abundant compounds were 1-triacontanol (8.60%), piperine (8.43%), 1-pentacosanol (6.28%) and benzenemethanol,2,5-dimethoxy acetate (6.195%). However, the least abundant compounds were hexadecanoic_acid, methyl-ester; *n*-hexadecanoic acid; gamma-sitosterol; 1-tetracosanol; 4-((1E)-3-hydroxy-1-propenyl)-2-methoxyphenol; hexadecanoic_acid,2-hydroxy-1-(hydroxymethyl) ethyl_ester; 3-isopropoxy-4-methoxybenzamide; 4,8-ethano-4H-1,3-benzodioxin,hexahydro-; 3-acetoxy-3-hydroxypropionic acid, methyl ester; 2,4-hexadienedioic acid, 3,4-diethyl, dimethyl ester (Z,Z)-; dimethyl 1-(2-methoxyethyl)-5-methylpyrazole-3,4-dicarboxylate; benzene, nitro- and *cis*-9-hexadecenal with peak areas ranges from 4.274 to 1.08% (Table 5).

Discussion

In general, methanolic stem extracts of all the four quinoa varieties significantly reduced growth of *M. phaseolina*. Previously, literature regarding antifungal activity of *C. quinoa* is very limited. Woldemichael and Wink (2001) reported antifungal activity of *C. quinoa* against *Candida albicans*. Saponins, a diverse group of natural compounds containing steroid aglycone or triterpene and one or more

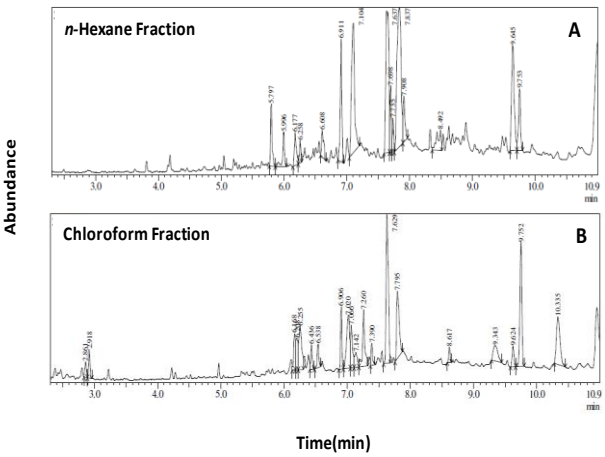
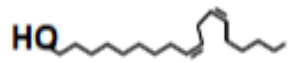
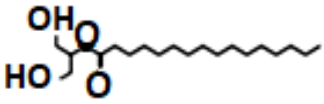


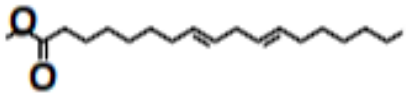
Fig. 3: GC-MS chromatograms of *n*-hexane and chloroform fractions of methanolic stem extract of *C. quinoa*



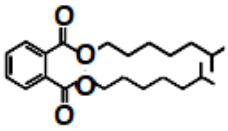
9,12-Octadecadien-1-ol,(z,z)-



Hexadecanoic acid,2-hydroxy-1-(hydroxymethyl) ethyl ester



8,11-Octadecadienoic acid, methyl ester



1,2-Benzedicarboxylic acid, diisooctyl ester

Fig. 4: Structures of potential antifungal compounds identified in *n*-hexane and chloroform fractions of stem extract of *C. quinoa* through GC-MS

chains of sugar in their structure (Guçlu-Ustundag and Mazza 2007), are well known for their antifungal activity (Tsuzuki *et al.* 2007). The plant contains at least 26 saponins (Madl *et al.* 2006), which might be the reason of its antifungal activity against *M. phseolina* (Woldemichael and Wink 2001). Besides saponins, a number of other components like eugenol, thymol, carvacrol, phenolics, linalool and flavonoides are also reported in quinoa that are known for their antimicrobial properties (Juneja *et al.* 2012).

Methanolic extracts showed a marked variation in different verities towards their antifungal potential. V7 possessed the greatest antifungal potential followed by V9. Similar varietals differences in antifungal activity have also been recorded among the extracts of varieties of *Vitis vinifera*, *Allium sativum* and *Cupressus arizonica* against a wide range of fungal pathogens (Fratianni *et al.* 2016; Jediyi *et al.* 2019). Varietals antifungal activity differences could be attributed to the difference in chemical composition among the varieties (Khouadja *et al.* 2015). Jediyi *et al.* (2019) reported that *V. vinifera* varieties were also different in phenols and flavonoids contents so provided a marked variation in antifungal activities among the selected varieties.

Chloroform and *n*-hexane fractions were highly antifungal and completely retarded the growth of the pathogen even at lower concentrations. To reveal the chemical composition of these fractions a GC-MS analysis was performed to identify the known antifungal compounds. Literature survey showed that these compounds might be responsive in inhibiting the growth of *M. phaseolina*. Wang *et al.* (2008) isolated 9,12-octadecadien-1-ol,(Z,Z)- as a major component from *Digitaria sanguinalis* and found it to be very effective against *Curvularia eragrostidis*. Similarly, Al-Marzoqi *et al.* (2015) stated that hexadecenoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester is antifungal in nature against *Aspergillus flavus* and *A. niger*. Likewise, diisooctyl phthalate also known as 1,2-benzedicarboxylic_acid, diisooctyl ester was previously isolated from the leaves of *Hugonia mystax* and *Plumbago zeylanica* roots as a major chemical constituent. This compound was very effective against *M. phaseolina, Alternaria alternata, Botryodiplodia theobromae* and *Fusarium equiseti* (Rahman and Anwar 2006). Moreover, a compound namely 8,11-octadecadienoic acid, methyl ester was also isolated from a medicinal plant *Arum maculatum* which was found to be effective in arresting the growth of *Penicillium digitatum* and *A. niger* (Kianinia and Farjam 2018). Similarly, Chhouk *et al.* (2018) identified 2,4-hexadienedioic_acid,3,4-diethyl-, dimethyl_ester (Z,Z)- from Khmer a medicinal plant and reported that this compound possessed antifungal activity against many pathogentic fungi (Table 5).

Conclusion

There were large differences among the four selected varieties of quinoa towards their antifungal potential. *M. phaseolina* was very susceptible to extracts of V7. The pathogenic growth was completely controlled when treated with chloroform and *n*-hexane fractions of methanic extract of this variety. Various possible antifungal compounds were identified through GC-MS.

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Author Contributions

IHK did experimental work and wrote the paper. AJ supervised the work and contributed in writing and finalizing the paper.

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