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ORIGINAL ARTICLE

Comparative HPLC, GC-MS Analysis and *In vitro* Antifungal Activity of Walnut Kernels against *Alternaria mali* in Apple

Ruhee Jan[*1], Tabassum Ara[1], Javid Iqbal Mir[2]

[1]*Department of Chemistry NIT- Srinagar, JK, India*

[2]*ICAR-Central Institute of Temperate Horticulture, Srinagar, 191132, India*

A R T I C L E I N F O

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A B S T R A C T

Natural plant products are suitable, eco-friendly, and toxicologically safe for using in integrated disease management approaches. Present work was carried out to decipher the potential of walnut kernels against *Alternaria mali*, the causal agent of leaf and fruit blotch on apples. Bioactive compound profiling of extracts was also done through HPLC and GC-MS to reveal the potential role of the compounds for disease management. In the present study, a comparative analysis of the anti-fungal activity and concentration of active substances using HPLC and GC-MS in different walnut extracts has been carried out on the inhibition of *A. mali.* The extracts of walnut kernels belonging to different eco-geographical regions viz. CS (Char-e-Sharief), KG (Kulgam), TM (Tangmarg), and KW (Kupwara) of Kashmir valley were prepared. The extracts prepared were added to potato dextrose agar (PDA) @ 1000, 2000, and 3000 ppm for evaluation against *A. mali*. The results revealed the efficacy of the different walnut extracts, with a maximum inhibition of mycelia growth in the range of 60%, 62.2%, and 71.1% at 3000ppm. Among them, the TM (Tangmarg) extract showed the highest inhibition (71.1%). The bioactive compounds were identified by using GC-MS chromatographic technique. The Quercetin content was quantified in the kernels of walnut, collected from four locations in Kashmir valley using HPLC. Among the selected genotypes, the highest Quercetin content was observed in samples collected from TM (Tangmarg) (0.765mg g^{-1}) followed by KW (Kupwara) (0.705 mg g^{-1}). The major phenolic components identified by GC/MS were methyl-7,8-Octadecadienoate, hexadecanoic acid, linoleic acid, epicatechin, and tocopherol. The results of the present study can be utilized further in the development of formulation from walnut kernels for evaluation under field conditions.

Introduction

The fungal pathogens are mostly managed by chemical fungicides. Their continuous use still leads to negative impacts both on the environment and human health, together with the rapid development of resistance among plant pathogens against these chemicals (Wianowska *et al*., 2016). Although synthetic chemicals are being employed to manage plant diseases, the use of natural plant products or botanicals is environmentally benign and toxicologically safe for use in integrated disease management (Mohidin *et al*., 2019; Habibi *et al.*, 2023). One nut that is frequently included in the diets of many nations is walnut (Chatrabnous *et al*., 2018a). The Persian walnut (*Juglans regia* L.) is the most

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widely grown and economically significant walnut species (Vahdati 2000; Hassankhah *et al*., 2017; Farsi *et al*., 2018; Akca and Sahin, 2022). Utilizing natural active ingredients to preserve diverse items after harvest is becoming increasingly widespread recently (Di Venere *et al*., 2016; Habibie *et al.*, 2019). The presence of various phenolic compounds can be attributed to the bioactivity of plant extracts (Gatto *et al*., 2011; Di Venere *et al*., 2016; Chatrabnous *et al.*, 2018b; Ghorbani *et al*., 2022; Habibi *et al.*, 2022). The growers have remained unaware of the utility of these plant products, although researchers have realized the use of these plant products. With the discovery of chemical pesticides, it was thought, that a reliable and permanent solution for plant disease management has been achieved. Growers used higher quantities of these chemical fungicides. Soon, it was realized that the intensive and indiscriminate use of these fungicides is not safe in agriculture, as the toxicants cause environmental degradation and eliminate non-target organisms (Stangarlin *et al*., 2011). Considering the ill effects caused by synthetic fungicides, there is a serious need for alternatives; that is safe for both humans and the environment (Ghassan Al-Samarrai *et al*., 2012). Among various apple diseases, Alternaria leaf spot (ALS), caused by different *Alternaria* species, pose a severe threat to both production and productivity of apples (*Malus domestica* Borkh) (Filajdic *et al*., 1991). Several workers have reported various plant products/botanicals effective against various *Alternaria* spp. Examples of such products are the extracts of *Pongamia glabra* Vent. (Pan *et al*., 1985) Eucalyptus leaf extracts (Mate *et al*., 2005) Essential oils of *Salvia hydrangea* DC. ex Benth. (Kota *et al*., 2008) leaf extracts of Neem (*Azadirachta indica* L.) (Hassanein *et al*., 2008) the bark and leaf extracts of the Indian spice plant *Cinnamomum zeylanicum* Blume (Mishra *et al*., 2009), and leaf extracts of Zimmu (6 *Allium sativum* L.) alone and in combination with several bacterial bioagents (Latha *et al*., 2009). Walnut (*J. regia*) Juglandaceae, contains phenolic compounds; natural antioxidants; which are of much importance due to their benefits in improving the health of plants (Cheniany et al., 2013; Habibi *et al.*, 2021; Oliveira *et al*., 2008; Sarikhani *et al*., 2021). In the present study, a comparative investigation of the anti-fungal activity and concentration of active ingredients in various walnut extracts has been done to suppress *A. mali*, causing leaf blotch on apples.

Materials and Methods

Chemicals and reagents

All chemicals and reagents were of analytical grade or high-performance liquid chromatography (HPLC) (99.9%) and gas chromatography (>99.5%) grade from Merck Germany. Water used for the analysis was obtained from an SG water purification system (Germany). Sigma (Germany) provided Quercetin (HPLC-grade).

Plant material

The walnut samples were collected in September 2020-2021 from different eco-geographical areas of Jammu and Kashmir, India, covering varied altitude regions like KW (Kupwara) /(Sogam), TM (Tangmarg) / (Dhobiwan), KG (Kulgam) / (Munad) and CS (Char-e-Sharief) / (Nagam) (Table1). In 2020-21, all walnut samples were taken from mature trees that had been grown in the specified zones. Three trees were selected as replicas of each other to collect nuts. Within two hours after being gathered, nuts were immediately sent to the lab. The shelled walnuts were immediately washed with water after the green husks were peeled. The cleaned walnuts were dried in the open air and the sun for about four days. The hard shells of the nuts were manually cracked after drying. The samples were visually examined to look for flaws like bug damage, fungus growth, unusual color, or dangerous conditions. Only healthy kernels that were macroscopically free of diseases were selected for analysis, and subpar samples were excluded. The chosen nuts were stored in polyethylene bags between

3-5°C and 65-75% relative humidity for subsequent usage.

Morphological analysis

Nearly 20 to 30 nuts from mature trees were haphazardly collected during the harvest season, and their characteristics, such as shell color, texture, shell integrity, thickness, and strength, nut length, weight, diameter at the suture, diameter at the cheek, nut shape, weight, color, and percentage were assessed following the international descriptor for walnuts (Eriksson 1998).

Extraction Preparation

The kernels were crushed in a mixer grinder and immediately subjected to the extraction protocol. The procedure described by Tian *et al* 2013 with some modifications was used for the extraction. Total of 5 grams of the crushed walnut kernels of each sample were immersed for four days in methanol at room temperature in the dark and after that placed into an ultrasonic bath (Transsonic T 420/HElma) for 25-30 min at 50°C and then centrifuged at 8000 rpm for 10 min. The extract was further filtered through Whatman no. 4 paper and using a rotary vacuum evaporator (Buchi R II rota vapour, 40°C), the solvent was evaporated.

Table 1. Sample collection from four different locations in Kashmir, India.

Sample sites	Latitude	Longitude	Altitude (m)
Kupwara (Sogam)	$34^0\ 07'\ 92.5''$ N	$74^0\ 72'\ 86''$ E	1615
Char-e-Sharief (Nagam)	$33^0\ 55'\ 29''$ N	$74^0\ 47'\ 23''$ E	1933
Tangmarg (Dhobiwan)	$34^0\ 05'\ 35.2''$ N	$74^0\ 32'\ 41.6''$ E	2080
Kulgam (Munad)	$33^0\ 44'\ 21.08''$ N	$74^0\ 57'\ 45.75''$ E	1639

Isolation and identification of the pathogen

During the growing season of 2020-21, apple leaf samples with disease symptoms were collected from the germplasm bank of ICAR-CITH Srinagar. Small leaf bits with symptomatic and healthy portions were surface sterilized with 1% sodium hypochlorite solution followed by rinsing in sterilized distilled water and placed on potato dextrose agar (PDA) plates. The PDA plates were incubated for two weeks at 25 ± 2ºC (Galvez *et al*., 2016). The isolated fungal culture was purified using a single spore technique (Johnston *et al.*, 1983) and was later identified by comparing it with available literature on *Alternaria mali* (Soleimani *et al*., 2007).

Antifungal activity assay

The kernel extract diffuses were evaluated using the food poison technique (FPT) (Ali-Shtayeh *et al*., 1999). The extracts were mixed with PDA @ 500, 1000, and 2000 ppm and the PDA medium containing methanol served as control. The Petri plates containing these extracts were inoculated with a 5 mm plug of a pure isolate of *Alternaria mali* isolated from apple leaves. For each treatment three replicates were made and were incubated at 25 ± 2°C. After one week, the diameter of the growth of fungi was measured by taking an average of three diameters taken for each colony. The percentage growth inhibition of the fungal colonies was measured by using the formula (Balouiri *et al*., 2016):

Inhibition of growth (%) = $[(C - T) \times C^{-1}] \times 100$

Where: C = Diameter of the colony mycelium on the control (mm); T = the colony diameter of the mycelium on the treatment (mm).

Identification of various components using GC/MS

GC-MS analysis was performed using Agilent Triple Quadrupole QQQ 7000 mass spectrometer with a capillary column (HP-5MS, Phenyl Methyl

Siloxane: 30m × 250µm × 0.25µm), EI-mode was 70eV, mass selective detector (HP 5977) with mass range 10-1050 m z^{-1}. Hydrogen gas was used a carrier at 1ml min^{-1} with initial column temperature as 100°C for 1 minute, then the temperature was increased at the rate of 25°C min^{-1} till 350°C and finally, this temperature was maintained for 10 minutes. The ionization source temperature was kept at 300°C and 1µl of each extract was injected in the splitless mode (Li *et al*., 2016).

High-performance liquid chromatography (HPLC) analysis

HPLC analysis was conducted on walnut kernels of four genotypes collected from four different locations to quantify the Quercetin content in them. Fresh samples were preserved by freezing at –40°C. The methanolic extract was prepared and supernatants were pooled and evaporated and the dried residues were stored at -80°C until HPLC analysis was performed. The samples extracted were dissolved in methanol of HPLC grade at a final concentration of 100mg ml^{-1} and were analyzed in triplicates. The analysis was carried out in a Shimadzu HPLC equipped with quaternary pumps and a degasser coupled to a photo-diode-array detector. Separation was carried out with an injection volume of 20 µl extract, a flow rate of 1ml min^{-1} with 30-40 minutes of run time. The standard of Quercetin (purity>98%) was obtained from Sigma. Chromatographic separations were performed on C18 (250mm×4.6mm), 5µm column using a solvent system consisting of 75% acetonitrile and 25% methanol. The optimum resolution was obtained by using an isocratic elution program. Before analysis, the mobile phase was filtered through a 0.45µm membrane filter (Millipore, Bedford, MA, USA) (Kalogiouri *et al*., 2021). The software Class WP (version 6.1) from Shimadzu was used for instrument control, data acquisition, and data processing.

Results

Identification of pathogens based on morphological characteristics

The cultural characters of fungus recorded on the 7th day of inoculation revealed olive green to brown colony color with circular concentric mycelium with or without fluffy growth (Fig. 1). Morphological characters viz; conidia were large, dark brown, multi-celled, single, ovoid with beak and longitudinal and transverse septa. The characters were similar to those of *Alternaria mali* (Soleimani *et al*., 2007).

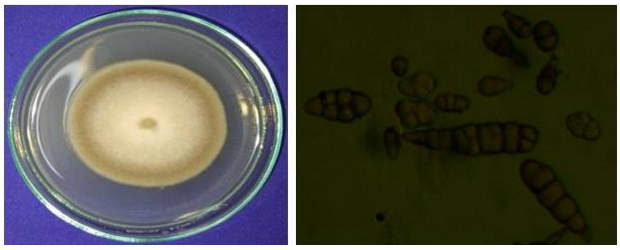

Fig. 1 Cultural and morphological features of *Alternaria mali.*

Efficacy of walnut extracts against *Alternaria mali*

The efficacy of four different walnut extracts against *A. mali* at three different concentrations revealed maximum inhibition of 60%, 62.2%, and 71.1% at 3000 ppm collected from different locations. Among the locations, TM (Tangmarg) showed the highest inhibition of 71.1% at 3000ppm. The data revealed that a significant reduction in the growth of *A. mali* was observed in respect of all the walnut extracts tested. The inhibition level was directly proportional to the concentration of the extract. The overall inhibition of *A. mali* at different concentrations of walnut extract collected from different Kashmir locations are shown in (Figs. 2 and 3).

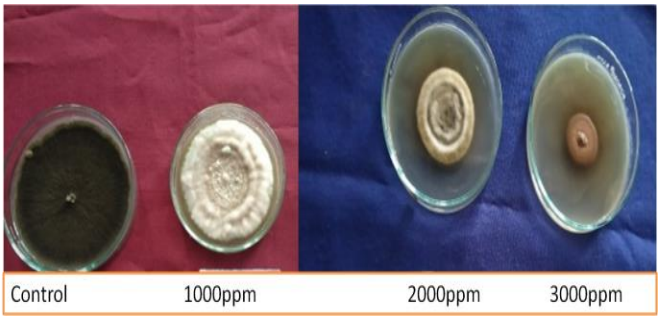

Fig. 2. Mycelium inhibition of *Alternaria mali* at three different concentrations of walnut kernel extract.

GC-MS analysis

GC-MS chromatograms were used to identify a variety of phytochemical constituents based on charge-to-mass ratio spectra. All GC/MS-detected components from each of the chosen genotypes are displayed in (Tables 2- 5).

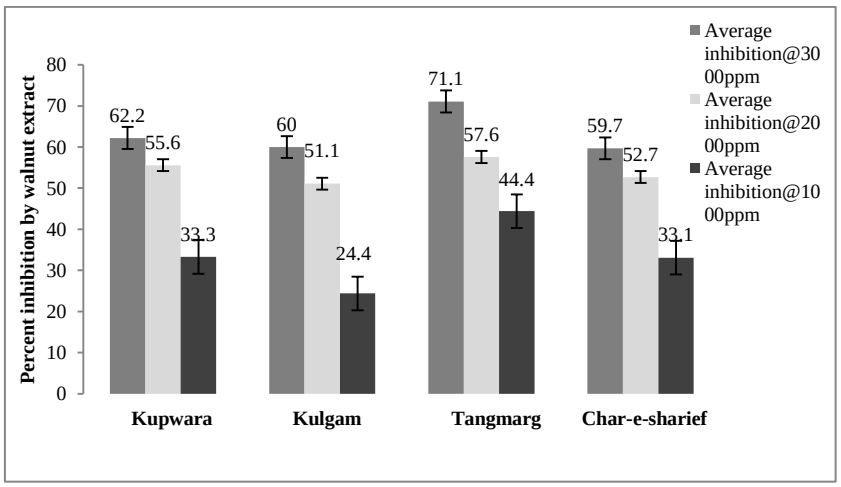

Fig. 3. Percentage of mycelium inhibition of *Alternaria mali* using different walnut extracts at three concentrations.

Statistical analysis

The results are presented as the mean values ± SD. All the measurements with $P < 0.01$ were assumed to the statistically significant.

Quantification of Quercetin via HPLC (High-Performance Liquid Chromatography)

In the present investigation, Quercetin was quantified in the kernels of walnut collected from four locations in the Kashmir valley. The results revealed that the highest Quercetin content, among selected genotypes, was present in samples collected from TM (Tangmarg) (0.765mg g^{-1}) followed by KW (Kupwara) 0.705 mg g^{-1}. The Quercetin content present in all genotypes is shown in (Fig. 4). The graphs obtained from HPLC analysis are presented as supplementary files.

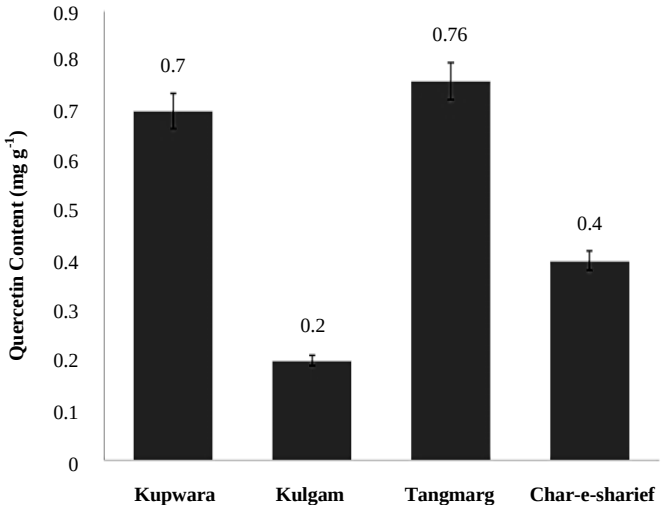

Fig.4. Quercetin content (mg g^{-1} fw) present in walnut genotypes collected from four locations. Data are shown as mean ± SD (Standard Deviation) of three replicates from one independent experiment.

Table 2. Identification of compounds in GC-MS presented in walnut kernel samples of Kulgam.

Peak	R.time	Area	Area%	Name	Formula
1	3.924	381735223	0.39	Cyclohexylmethyl S-2-(diisopropyl amino)ethyl propylphosphonothiolate	$C_{18}H_{38}NO_2PS$
2	4.03	425188000	0.44	2-Isobutoxy-4-methyl-[1,3,2]dioxaborinane	$C_8H_{17}BO_3$
3	4.219	1599533581	1.65	.delta.2-Tetrazaboroline, 5-ethyl-1,4-dimethyl-	$C_4H_{11}BN_4$
4	4.267	466611279	0.48	Methane, trichloroisocyanato-	C_2Cl_3NO
5	4.824	1057785353	1.09	2-Naphthyl-.beta.-D-galactopyranoside	$C_{16}H_{18}O_6$
6	5.735	6281424291	6.49	1,3,2-Oxazaborolane-4-carboxylic acid, 2-butyl-,methyl ester, L-	$C_8H_{16}BN\ O_3$
7	8.014	2710548824	2.8	1,2,3-Benzenetriol	$C_6H_6O_3$
8	8.163	1914153739	1.98	Pyrazine, methoxy-4-oxide	$C_5H_6\ N_2O_2$
9	13.587	655817048	0.68	Demeton-S-methyl sulfone	$C_6H_{15}O_5PS_2$
10	14.131	1057149175	1.09	Demeton-S-methyl sulfone	$C_6H_{15}O_5PS_2$
11	18.273	463816468	0.48	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$
12	19.164	8767814065	9.07	n-Hexadecanoic acid	$C_{16}H_{32}O_2$
13	21.422	1797501414	1.86	Methyl 9,10-octadecadienoate	$C_{19}H_{34}O_2$
14	21.531	1337770713	1.38	Methyl 8,9-octadecadienoate	$C_{19}H_{34}O_2$
15	22.576	36715608163	37.96	Methyl 8,9-octadecadienoate	$C_{19}H_{34}O_2$
16	22.648	8457150751	8.74	Methyl 7,8-octadecadienoate	$C_{19}H_{34}O_2$
17	22.866	2394451125	2.48	Octadecanoic acid	$C_{18}H_{36}O_2$
18	28.31	648547832	0.67	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	$C_{19}H_{38}O_4$
19	31.028	4238203944	4.38	Methyl 8,9-octadecadienoate	$C_{19}H_{34}O_2$
20	31.141	1641957867	1.7	Methyl 8,9-octadecadienoate	$C_{19}H_{34}O2$
21	34.561	421564749	0.44	delta.-Tocopherol	$C_{27}H_{46}O_2$
22	35.98	3462718528	3.58	delta.-Tocopherol, O-methyl-	$C_{28}H_{48}O_2$
23	39.315	5777399655	5.97	gamma.-Sitosterol	$C_{29}H_{50}O$
24	39.496	970088032	1	Tris(tertiary-butyl dimethyl silyl oxy) arsane	$C_{18}H_{45}AsO_3Si_3$
25	40.19	3072955538	3.18	Arsenous acid, tris(trimethylsilyl)ester	$C_{18}H_{45}AsO_3Si_3$

Table 3. Identification of compounds in GC-MS presented in walnut kernel samples of Tangmarg.

Peak	R.time	Area	Area%	Name	Formula
1	3.929	375712850	0.51	cyclohexylmethyl S-2-(diisopropyl amino)ethyl propylphosphonothiolate	$C_{18}H_{38}NO_2S$
2	3.969	338631210	0.46	Benzyltri-n-propylammonium chloride	$C_{16}H_{28}ClN$
3	4.034	561149868	0.77	2-Isobutoxy-4-methyl-[1,3,2]dioxaborinane	$C_8H_{17}BO_3$
4	4.223	1645207152	2.25	.delta.2-Tetrazaboroline, 5-ethyl-1,4-dimethyl-	$C_4H_{11}BN_{14}$
5	4.263	372117906	0.51	Methane, trichloroisocyanato-	C_2Cl_3NO
6	4.401	292136806	0.4	2-Azido-2,4,4,6,6,8,8-heptamethylnonane	$C_{16}H_{33}N_3$
7	4.816	874621358	1.2	2-Naphthyl-.beta.-D-galactopyranoside	$C_{16}H_{18}O_6$
8	5.223	453717950	0.62	Trimethylene borate	$C_9H_{18}B_2O_6$
9	5.735	5154895600	7.06	5-Hydroxymethylfurfural	$C_6H_6O_3$
10	6.526	225463918	0.31	1,2-Pentadiene, 4-(tertiary-butyl dimethyl silyl)oxy-	$C_{11}H_{22}O$ Si
11	8.006	3764711580	5.16	1,2,3-Benzenetriol	$C_6H_6O_3$
12	10.074	232965489	0.32	1-.beta.-d-Ribofuranosyl-3-[5-tetraazolyl]-1,2,4-triazole	$C_8H_{11}N_7O_4$
13	18.273	611234629	0.84	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$
14	19.112	6033572113	8.26	n-Hexadecanoic acid	$C_{16}H_{32}O_2$
15	21.422	2266520430	3.1	Methyl 9,10-octadecadienoate	$C_{19}H_{34}O_2$
16	21.527	1172034800	1.61	Methyl 8,9-octadecadienoate	$C_{19}H_{34}O_2$
17	22.451	22254963491	30.48	Methyl 9,10-octadecadienoate	$C_{19}H_{34}O_2$
18	22.511	5849758306	8.01	Methyl 7,8-octadecadienoate	$C_{19}H_{34}O_2$
19	22.773	1324526827	1.81	Octadecanoic acid	$C_{18}H_{36}O_2$
20	28.306	407711190	0.56	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	$C_{19}H_{38}O_4$
21	31.004	2745712120	3.76	Methyl 8,9-octadecadienoate	$C_{19}H_{34}O_2$
22	31.125	898043306	1.23	Methyl 8,9-octadecadienoate	$C_{19}H_{34}O_2$
23	34.561	357663183	0.49	delta.-Tocopherol	$C_{27}H_{46}O_2$
24	35.988	4397768771	6.02	.delta.-Tocopherol, O-methyl-	$C_{28}H_{48}O_2$
25	37.004	518237794	0.71	.alpha.-Tocopherol acetate	$C_{31}H_{52}O_3$
26	38.17	419310620	0.57	Tris (tertiary-butyl dimethyl silyl oxy) arsane	$C_{18}H_{45}AsO_3Si_3$
27	39.319	7064020586	9.67	.gamma.-Sitosterol	$C_{29}H_{50}O$
28	39.496	840323534	1.15	Tris (tertiary-butyl dimethyl silyl oxy) arsane	$C_{18}H_{45}AsO_3Si_3$
29	40.174	1566473526	2.15	Tris (tertiary-butyl dimethyl silyl oxy) arsane	$C_{18}H_{45}AsO_3Si_3$

Table 4. Identification of compounds in GC-MS presented in walnut kernel samples of Char-e-Sharief.

Peak	R.time	Area	Area%	Name	Formula
1	3.933	400484038	0.43	Cyclohexylmethyl S-2-(diisopropyl amino)ethyl propylphosphonothiolate	$C_{18}H_{38}NO_2PS$
2	3.981	326579743	0.35	Benzyltri-n-propylammonium chloride	$C_{16}H_{28}ClN$
3	4.038	491741163	0.53	2-Isobutoxy-4-methyl-[1,3,2]dioxaborinane	$C_8H_{17}BO_3$
4	4.227	1804513181	1.95	.delta.2-Tetrazaboroline, 5-ethyl-1,4-dimethyl-	$C_4H_{11}BN_4$
5	4.272	463704771	0.5	Benzamide, 4-fluoro-N-(5H-tetrazol-5-yl)-	$C_8H_6FN_5O$
6	4.824	1035449031	1.12	1-Methyl-5-fluorouracil	$C_5H_5FN_2O_2$
7	5.735	5108481644	5.52	o-Ethylo-3-methyl cyclohexyl methyl phosphonate, trans-	$C_{10}H_{21}O_3P$
8	8.01	3961263815	4.28	1,2,3-Benzenetriol	$C_6H_6O_3$
9	17.031	436385174	0.47	3-Hydroxy-6-(N,N-dimethylamino)methyl pyridazine-1-oxide	$C_7H_{11}N_3O_2$
10	18.277	691754785	0.75	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$
11	19.132	7261802692	7.85	n-Hexadecanoic acid	$C_{16}H_{32}O_2$
12	21.426	2404307098	2.6	Methyl 8,9-octadecadienoate	$C_{19}H_{34}O_2$
13	21.535	1989611121	2.15	Methyl 8,9-octadecadienoate	$C_{19}H_{34}O_2$

14	21.991	415378123	0.45	Methyl 12,13-tetradecadienoate	$C_{15}H_{26}O_2$
15	22.499	27372897423	29.59	Methyl 9,10-octadecadienoate	$C_{19}H_{34}O_2$
16	22.568	7829123899	8.46	Methyl 8,9-octadecadienoate	$C_{19}H_{34}O_2$
17	22.81	1900248736	2.05	Methyl 12,13-tetradecadienoate	$C_{15}H_{26}O_2$
18	24.193	393665980	0.43	d-Gulopyranoside, 2,3:4,6-di-O-(ethylboranediyl)-1-O-methyl-	$C_{11}H_{20}B_2O_6$
19	24,685	378961581	0.41	Cyclononasiloxane, octadecamethyl-	$C_{18}H_{54}O_9Si_9$
20	27.032	52669554	0.57	Tetracosamethyl-cyclododecasiloxane	$C_{24}H_{72}O_{12}Si_{12}$
21	28.31	603193817	0.65	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	$C_{19}H_{38}O_4$
22	29.246	620109469	0.67	Tetracosamethyl-cyclododecasiloxane	$C_{24}H_{72}O_{12}Si_{12}$
23	29.516	1142438970	1.24		
24	31.016	3092674358	3.34	Methyl 8,9-octadecadienoate	$C_{19}H_{34}O_2$
25	31.129	1009613643	1.09	Methyl 8,9-octadecadienoate	$C_{19}H_{34}O_2$
26	31.331	925503002	1	Tetracosamethyl-cyclododecasiloxane	$C_{24}H_{72}O_{12}Si_{12}$
27	33.278	827776808	0.89	Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-	$C_{16}H_{50}O_7$ Si_8
28	34.561	532311303	0.58	.delta.-Tocopherol	$C_{27}H_{46}O_2$
29	35.111	808860458	0.87	Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-	$C_{16}H_{50}O_7Si_8$
30	35.984	3692061307	3.99	.gamma.-Tocopherol	$C_{28}H_{48}O_2$
31	36.242	307970665	0.33	Tris (tertiary-butyl dimethyl silyl oxy) arsane	$C_{18}H_{45}AsO_3Si_3$
32	36.828	656576885	0.71	Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-	$C_{16}H_{50}O_7Si_8$
33	37.008	417732468	0.45	Methanamine, N-(diphenyl ethenylidene)-	$C_{15}H_{13}N$
34	38.17	464897459	0.5	Tris (tertiary-butyl dimethyl silyl oxy) arsane	$C_{18}H_{45}AsO_3Si_3$
35	38.452	452782555	0.49	Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-	$C_{16}H_{50}O_7Si_8$
36	39.323	7662407087	8.28	.gamma.-Sitosterol	$C_{29}H_{50}O$
37	39.5	1168355716	1.26	Tris (tertiary-butyl dimethyl silyl oxy) arsane	$C_{18}H_{45}AsO_3Si_3$
38	40.186	2920856877	3.16	Tris (tertiary-butyl dimethyl silyl oxy) arsane	$C_{18}H_{45}AsO_3Si_3$

Table 5. Identification of compounds in GC-MS presented in walnut kernel samples of Kupwara.

Peak	R.time	Area	Area%	Name	Formula
1	3.913	225029482	0.32	4-Methylpentyl S-2-(diisopropyl amino)ethylpropylphosphothiolate	$C_{17}H_{38}NO_2PS$
2	4.026	266759504	0.37	1H-1,2,3,4-Tetrazole-1-propanamide, N-(4,5-dimethyl-2-thiazolyl)-	$C_9H_{12}N_6OS$
3	4.219	1487218666	2.09	Methane, trichloroisocyanato-	$C_2C_{13}NO$
4	4.272	325832892	0.46	Methane, trichloroisocyanato-	$C_2C_{13}NO$
5	4.812	764995434	1.07	2-Napthyl-,beta,-D-galactopyranoside	$C_{16}H_{18}O_6$
6	5.748	5374766867	7.54	1,3,2-Oxazaborolane-4-carboxylic acid, 2-butyl-,methyl ester, L-	$C_8H_{16}BNO_3$
7	6.526	242518292	0.34	Ethanone, 1-(2,5-diethyl-4-methyl-1,3,2-dioxa borolan-4-yl)-	$C_9H_{17}BO_3$
8	6.707	265179747	0.37	1,3,2-Oxazaborolane-4-carboxylic acid, 2-butyl-,methyl ester, L-	$C_8H_{16}BNO_3$
9	6.856	285005614	0.4	Tetrazole, 1-(3,4-dimethoxy benzylide amino)-	$C_{10}H_{11}N_5O_2$
10	7.945	1199320916	1.68	1,2,3-Benzenetriol	$C_6H_6O_3$
11	8.006	2228554061	3.12	1,2,3-Benzenetriol	$C_6H_6O_3$
12	9.413	431178259	0.6	4,5,6,7-Tetrahydroxydecyl isothiocyanate	$C_{11}H_{21}NO_4S$
13	9.941	577723104	0.81	.beta.-D-glucosyloxyazoxymethane	$C_8H_{16}N_2O_7$
14	14.075	231082913	0.32	(-)-(2S,8Ar)-(camphorsulfonyl)oxaaziridine	$C_{10}H_{15}NO_3S$
15	18.273	578888712	0.81	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$
16	19.108	5770746790	8.09	n-Hexadecanoic acid	$C_{16}H_{32}O_2$
17	21.418	1782693824	2.5	Methyl 8,9-octadecadienoate	$C_{19}H_{34}O_2$
18	21.531	1236576356	1.73	Methyl 8,9-octadecadienoate	$C_{19}H_{34}O_2$
19	22.443	20824942810	29.2	Methyl 9,10-octadecadienoate	$C_{19}H_{34}O_2$
20	22.515	7961866540	11.16	Methyl 7,8-octadecadienoate	$C_{19}H_{34}O_2$

21	22.773	1576332600	2.21	Methyl 12,13-tetradecadienoate	$C_{15}H_{26}O_2$
22	28.298	217992839	0.31	Methyl 3,4-tetradecadienoate	$C_{15}H_{26}O_2$
23	29.512	231522625	0.32		
24	31	1986293887	2.78	Methyl 8,9-octadecadienoate	$C_{19}H_{34}O_2$
25	31.117	797113475	1.12	Methyl 8,9-octadecadienoate	$C_{19}H_{34}O_2$
26	34.56	537086312	0.75	.delta.-Tocopherol	$C_{27}H_{46}O_2$
27	35.98	3552709739	4.98	.delta.-Tocopherol, O-methyl-	$C_{28}H_{48}O_2$
28	37.005	386495092	0.54	Methanamine, N-(diphenyl ethenylidene)-	$C_{15}H_{13}N$
29	38.166	324569834	0.46	Tris (tertiary-butyl dimethyl silyl oxy) arsane	$C_{18}H_{45}AsO_3Si_3$
30	39.315	6511728207	9.13	.gamma.-Sitosterol	$C_{29}H_{50}O$
31	39.496	899971960	1.26	Tris (tertiary-butyl dimethyl silyl oxy) arsane	$C_{18}H_{45}AsO_3Si_3$
32	40.178	2243135136	3.14	Tris (tertiary-butyl dimethyl silyl oxy) arsane	$C_{18}H_{45}AsO_3Si_3$

Discussion

The economic loss due to damage of crops by various fungi and health risks to the consumers is the area of utmost care and concern. The random and indiscriminate use of pesticides has invariably resulted in resistance development among pathogens and increased residual toxicity in food products. Therefore, to minimize the use of these synthetic chemical pesticides, the search for alternative chemicals at present is of great concern (Mohidin *et al*., 2019). Another potential threat caused by the widespread use of synthetic chemicals is the contamination of soil and water, which might harm the environment. In recent years, a remarkable growing interest is observed in exploring plant extracts as an alternative and natural chemical to fight plant diseases because of being safe, easily decomposable, nature-friendly, and have non-phytotoxic effects (Wianowska *et al*., 2016). It has been observed that plant extracts are rich in biologically active compounds when obtained with different solvents. So, systematic research is the need of the hour to explore the possibilities of disease management in this area (Khodadadi *et al.*, 2020; Mohidin *et al*., 2019). The disease suppression by the natural products suggests that the bioactive molecules present in them may either act directly on the pathogen or may induce systemic resistance in host plants (Khodadadi *et al.*, 2016). The main aim of our study was to know the antifungal activity of methanolic extracts of different walnut kernels collected from four different areas of Kashmir valley against *Alternaria mali*. The antifungal activity studies of walnut kernels against *A. mali* are scarce or non-existent. However, various botanicals viz., methanol extracts of peppermint, eucalyptus *Tagetes patula* L., *Mentha* sp. *Allium sativum L.*, and *Rosmarinus officinalis* L., have been found effective against various *Alternaria* species (Zaker *et al*., 2010, Sesan *et al*., 2015). A defense mechanism against fungi that cause disease has been established for walnut extracts. It is well established that walnut extracts can protect against three dermatophytes, *Microsporum canis,* E. Bodin (S14, S20, and SH41), *Trichophyton mentagrophytes,* Blanchard (SH13, SH1, SH8), and *T. rubrum*, Castell., the causative agent of chalkbrood disease in bees, *Ascophaera apis*, Maasen *Candida albicans*, Berkhout *Cryptococcus neoformans*, Sanfelice *Microsporum canis*, E. Bodin and *Trichophyton violaceum*, Sabour. ex E. Bodin (S5, SH32, SH38) (Ebrahimi *et al*., 2018). Additionally, walnuts include a variety of bioactive substances that are known to promote health, including plant sterols, polyphenols, and bioactive peptides (Jahanbani *et al.*, 2021). These bioactive substances have been shown in several studies to be protective against aging, cancer, metabolic syndrome, diabetes, and cardiovascular illnesses (Ma *et al*., 2021). Antioxidants in walnuts appear to offer anti-

cancer capabilities and boost the immune system. (Jahanbani *et al*., 2016, Milind and Deepa 2011). Walnut bioactive peptides also seem to be useful for treating and preventing hypertension (Jahanbani *et al*., 2018). Recent studies have demonstrated the potential of such inexpensive natural resources as a source of phytochemicals and phenolic compounds with antibacterial and antiradical properties (Fernández - Agullóa *et al*., 2013). Knowing that green walnut husk contains significant amounts of catechin, juglone, gallic acid, ellagic acid, protocatechuic acid, caffeic acid, and chlorogenic acid (Stampar *et al*., 2006), an aqueous extract of green walnut husk may inhibit the development of gram-positive bacteria (Oliveira *et al*., 2008). Walnut kernel extracts were shown to contain hydrolyzable tannins, which are phenolic substances characterized by their hexahydroxy-diphenoyl (HHDP) group. Methyl octadecadienoate, a conjugated linoleic acid (CLA), an isomer of linoleic acid, was another significant phenolic molecule found. It is renowned for having anti-atherogenic and anti-carcinogenic properties. Although CLA itself is known to lack antioxidant properties, it is believed to produce compounds that shield cells from the damaging effects of peroxides (MacDonald 2000). Some authors have also established the presence of these chemicals in walnut kernels (Grace *et al*., 2014, Reguerio *et al*., 2014). In addition to these, several hydroxyl benzoic acids have been found, as stated in the tables, including gallic, ellagic, and tocopherol. These identified molecules are well known for their positive effects on health, including their anti-inflammatory and anti-carcinogenic properties, ability to fight depression and anxiety, support brain growth, and lower risk of heart disease. (Anderson *et al*., 2001, Ahad *et al*., 2020).

Our objective in the present study was to evaluate the inhibitory effects of methanolic extracts of four walnut genotypes on the mycelial growth of *Alternaria mali* under *in-vitro* conditions to confirm its potential application as an alternative non-toxic antifungal agent in apples against ALS disease. The simplest and best way to evaluate the antifungal activity of the examined extracts is to compare the different concentrations for inhibition of mycelial growth. From our data presented in Fig. 3, all extracts exhibited antifungal activity. Different walnut green extracts from hulls have been also reported to show antifungal activity against *Rhizoctonia solani*, Kuhn *Alternaria alternata*, Keissl *Fusarium culmorum*, Sacc. *Botrytis cinerea*, Pers. and *Phytophthora infestans*, Montagne (Wianowska *et al*., 2016). In the present study, the Quercetin content was quantified from all kernel extracts and the presence of higher Quercetin content in the extracts gave a clear indication of the inhibitory nature of extracts. Previous studies have also proved the antifungal activity of Quercetin against *Fusarium* infection in barley (Skadhauge *et al*., 1997) and *Neurospora crassa*, Shear & B.O. Dodge in *Arabidopsis thaliana* L. (Parvez *et al*., 2004). The Phloridizin and Quercetin (phytochemical constituents) of apple waste had been successfully tested as potential antifungal agents against four pathogens *Neosartorya fischeri*, D.Malloch and R.F. Cain *Fusarium oxysporum*, Synder and Hansen *Botrytis sp*. and *Petriella setifera*, Curzi (Oleszek *et al*., 2019).

Conclusions

The presence of Quercetin is one of the compounds with antifungal activity besides other compounds in the extracts. The present study shows that the extracts from four different walnut genotypes can inhibit the pathogen up to various levels. The extract obtained from the Tangmarg genotype inhibited the *Alternaria mali* up to a significant extent. Considering the recent increasing interest in the biological management of plant diseases, the results of this study, which presents both the Quercetin content and the antifungal potential of walnuts, maybe a significant addition to our contemporary knowledge of the biological potential of walnuts.

The present study results are in good agreement with the previous findings from other researchers.

However, in future studies, field trials are necessary to ascertain the practical applicability of botanical pesticides. They should be incorporated with other management strategies used for the management of plant diseases. Biosafety studies should be conducted to ascertain their toxicity to humans, animals, and crop plants. Findings from this study supported the use of plant extracts as less dangerous natural fungicides to control plant pathogenic fungi, hence lowering the need for synthetic fungicides. Methanol extracts of walnut kernels might be promising materials for natural formulations in controlling the Alternaria leaf spot of apples in the field. This work becomes the base for future studies on the role of identified bioactive compounds in disease management and the preparation of plant-derived formulations for the control of fungal diseases to replace chemical fungicides.

Supporting information

The GC-MS (Figs.2S-5S) and HPLC (5S-8S) spectra of the different walnut genotypes showing a variety of phytochemical constituents are provided as Supporting Information.

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Conflict of interest

The authors declare no conflict of interest.

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