

Research Article

Muhammad Farooq Aslam*, Gulshan Irshad, Farah Naz and
Muhammad Azam Khan



Evaluation of the antifungal activity of essential oils against *Alternaria alternata* causing fruit rot of *Eriobotrya japonica*

<https://doi.org/10.1515/tjb-2021-0225>

Received September 21, 2021; accepted February 24, 2022;
published online April 11, 2022

Abstract

Objectives: The study was aimed to evaluate the effect of essential oils against fungal pathogens including the chemical analysis (GC-MS) of the most effective essential oil.

Methods: The antifungal effect of essential oils was assessed after morpho-molecular identification of *Alternaria alternata*. Mycelial growth inhibition (%) in *in vitro* treatment of eight essential oils at different concentrations was observed against *A. alternata*. Fungitoxicity assay was done following chemical composition analysis by Gas chromatography-mass spectrometry. Direct application of thyme oil was performed on healthy fruits against *A. alternata*.

Results: All eight essential oils showed mycelial growth inhibition at different levels, whereas thyme oil was found more efficacious against *A. alternata*. Chemical composition analysis detected 32 components and thymol was found in a higher percentage. Direct application of the most significant concentration of thyme oil was found effective against *A. alternata* with a varying decrease in decaying percentage.

Conclusions: Investigation of this study proved thyme oil as a potential and eco-friendly botanical fungicide effectively used against *A. alternata* on loquat fruit. The current

study explored that thyme oil could be potentially used against *A. alternata* and its compounds could be further investigated for the development of eco-friendly approaches for the control of postharvest fruit rots.

Keywords: essential oil; fruit decay; management; post-harvest; thyme.

Introduction

Loquat (*Eriobotrya japonica* Lindl.) belongs to the family Rosaceae which consists of over 3,000 species from 100 genera, and it is the third economically important family among plants in temperate regions including pome fruits. Fruits from this family are highly nutritious and provide the raw material for potential industrial and aesthetic products [1]. It is predominantly cultivated in tropical and subtropical regions worldwide. Loquat cultivation is improved and getting importance commercially in Turkey, Spain, Pakistan, Brazil, and India. Etiology and disease incidence depends on preharvest, harvest, and postharvest factors [2]. Loquat being a perishable fruit is highly exposed to mechanical abrasions and damages and bears a very short postharvest life at ambient temperature. Loss of nutrients and moisture and fruit decay occurs in a very short time after harvesting [3]. Postharvest losses of fruits and vegetables are the key problem in Pakistan. Fungal decay is the main reason causing significant postharvest losses in fruits which is a big loss to farmers. The shelf life of loquat fruit is very short and the quality of fruit after harvest deteriorates rapidly [4]. Browning of fruit is the most common phenotypic expression of decay which ultimately determines fruit acceptability for table use. Postharvest diseases destroy 40 percent of the total crop yield while in the case of perishable fruits, the amount is more than 30% of the yield especially in developing countries [5]. Moisture content in loquat fruit makes it prone to decay, therefore, it is highly susceptible to postharvest decaying caused by

***Corresponding author: Muhammad Farooq Aslam**, Department of Plant Pathology, PMAS Arid Agriculture University Rawalpindi, Rawalpindi, Pakistan, E-mail: farooqch.96@gmail.com.
<https://orcid.org/0000-0002-1864-0992>

Gulshan Irshad and Farah Naz, Department of Plant Pathology, PMAS Arid Agriculture University Rawalpindi, Rawalpindi, Pakistan. <https://orcid.org/0000-0003-0448-4826> (G. Irshad). <https://orcid.org/0000-0003-2648-5678> (F. Naz)

Muhammad Azam Khan, Department of Horticulture, PMAS Arid Agriculture University Rawalpindi, Rawalpindi, Pakistan.
<https://orcid.org/0000-0002-2730-9636>

microorganisms, which is the major reason influencing the postharvest fruit quality and storage life. Various postharvest pathogens attacking loquat fruit have been reported mainly fungal pathogens [6] in which *Alternaria* spp. is one of the major postharvest fungal pathogens infecting various fruits and vegetables. It has been reported to cause fruit rot on loquat in many countries including Spain, Greece, Iran, and Taiwan [7]. Additionally, *Alternaria alternata* has been reported as a causal agent of leaf spot diseases and fruit rots of loquat in Palestine [8]. For the identification, symptomology and morphological tools are used which are considered as a traditional method for the identification of fungal pathogens at the species level. Modern molecular techniques including PCR amplification and DNA sequence analysis have been proved effective for the diagnoses and confirmation of this species [9]. The internal transcribed spacer (ITS) rDNA sequence is most used which is a universal sequence marker for the identification of fungi up to the species level [10].

A. alternata is one of the major postharvest fungal pathogens infecting loquat fruit. Synthetic fungicides are mainly used for the management of postharvest diseases of fruits and vegetables which have serious concerns related to residual effects on consumer health. Synthetic antifungal formulations are contaminating fruits and their existence and non justify use is increasing resistance to pathogens. Related to fresh fruits and vegetables, food safety is a major concern being faced nowadays [11]. Globally synthetic chemical use for the management of postharvest losses is not encouraged especially in European countries where no conventional chemical fungicides are presently permitted for the postharvest treatments of the loquat fruits [12]. Biochemical, and botanicals are the most innovative approaches being used for the management of postharvest such as plant essential oils (EOs) [13]. Essential oils are green products sourced from various medicinal and aromatic compounds that have potential antimicrobial and antioxidant activity. Essential oils are the best alternative to synthetic chemicals used for the management of postharvest decay in fruits and vegetables and the use of these plant products especially essential oils for the control and management of postharvest diseases is an effective method [14]. Essential oils are non-hazardous and non-toxic and hence recommended as they do not leave toxic residues on human and animal health.

The study was designed for the identification of *A. alternata* involved in causing decay in loquat fruit. And to evaluate the antifungal activity of essential oil against pathogens including the chemical analysis of the highly effective essential oil to study the components present in it, and to

develop a novel approach for the control of this pathogen infecting loquats by application of EOs. These EOs have a non-hazardous effect on consumer health and are eco-friendly in nature which provides the alternative to synthetic chemicals and increases the shelf life of fruit after harvest.

Materials and methods

Sample collection, isolation, and morphology

Infected fruit samples were collected from five local fruit markets of Rawalpindi during the years 2017 and 2018. Fruits exhibiting the specific *Alternaria* Rot symptoms soft brown to black watery sunken lesions (see Figure 1) were taken for isolation of associated pathogenic species. Isolation was performed by cutting the small portion (3–5 mm²) of surface sterilized (2% sodium hypochlorite solution for 2 min) symptomatic fruit from the margins of the lesion. After surface sterilization, samples were rinsed thrice in sterilized distilled water and dried on sterilized filter paper for the removal of excessive moisture. Two to three dried segments were placed on sterilized potato dextrose agar (PDA plates), sealed with parafilm, and placed in an incubator at 25 ± 2 °C for 5 days. Petri dishes were observed regularly for hyphal growth. The isolates were purified by plating hyphal tips on fresh sterilized PDA plates. After 5 days of incubation, purified fungal isolates were examined employing cultural and morphological characteristics by consulting the fungal taxonomic keys.

Pathogenicity testing

A pathogenicity test was conducted for the confirmation and screening of the highly virulent isolates. For this purpose, asymptomatic loquat fruits were initially washed with running tap water and surface sterilized with 2% sodium hypochlorite solution for 1 min and left to dry on sterilized filter paper. Fungal pathogens isolated from fruit samples were inoculated using the mycelial disc plug method in which 5 mm of disc plug [15] from 7 days old culture was inoculated by making a wound of the same size on the fruits and were placed in a glass chamber and incubated at 25 ± 2 °C. Eight fruits were taken and three replicates were used for each treatment and experiments were repeated thrice. Rotting was categorized to check the virulence of the different fungal pathogens of loquat fruits by using a 0–5 disease rating scale where, 0=no rotting, 1=1–10%, 2=11–20%, 3=21–30%,

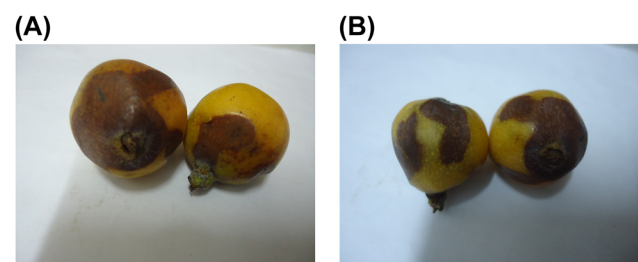


Figure 1: Symptomology of fruit rot of loquat associated with *Alternaria* species.

4=31–50%, and 5=more than 50%, given by [16] with modification for checking the virulence of the fungal isolates depending upon the mean rotting area developed on the fruits. For control, fruits were only wounded without inoculation and placed in glass chambers and incubated 25 ± 2 °C. To confirm Koch's postulates, fungal pathogens were reisolated from the artificially inoculated loquat fruits samples by plating the segments on PDA media. Cultural and morphological characters were compared with original cultures.

Molecular identifications

Genomic DNA of fungal isolates was extracted by using the standard protocol specified in the PrepMan[®] Ultra sample preparation reagent. Mycelium of fungal isolates was used for DNA extraction. DNA amplification was performed by PCR using the primers specific for ITS for molecular identification of *Alternaria* sp. The polymerase chain reaction was carried out in MJ Research PTC-100™ Programmable Thermal Controller. Initial denaturation was done at 95 °C for 5 min followed by 34 cycles of denaturation at 94 °C for 40 s, annealing at 60 °C for 1 min, extension for 60 s at 72 °C, and then final extension cycle at 72 °C for 7 min. Amplified PCR product was examined on 1% agarose gel in 1X TAE buffer and by using a gel documentation system, fragments were visualized. The amplified PCR product was purified using the standard protocol of the Thermo-fisher Scientific PCR Purification Kit. The purified DNA of fungal isolates was sent to the DNA Facility of Iowa State University, Ames, Iowa, United States of America for DNA sequencing. The sequenced DNA was further subjected to manipulation and alignment using BioEdit software in sense and antisense direction and was submitted to the GenBank of NCBI and accession numbers were obtained. After the confirmation of the isolates, the neighbor-joining method was performed in MEGA version-7.0 for phylogenetic analysis.

Preparation of essential oils

Matured plant leaves of the eucalyptus (*Eucalyptus globulus*), thyme (*Thymus vulgaris*), lemongrass (*Cymbopogon citratus*), moringa (*Moringa oleifera*), tea tree (*Melaleuca alternifolia*), and peel of lemon (*Citrus x limon*), pomegranate (*Punica granatum*), and rhizome of ginger (*Zingiber Officinale*) were obtained from the herbal stores of National Agriculture Research Centre (NARC) Islamabad and Rawalpindi. Collected plants material was dried under shade at room temperature to obtain the required form. Dried materials were crushed with Grinder (Pascall Motorised Pestel & Mortar, Machine No. 20069) to the fine powder. The extraction of essential oils was accomplished using Soxhlet's apparatus. Essential oils concentrations (0.2, 0.4, 0.6 mg/mL) were prepared by dissolving the essential oils in 1% tween 80 solutions in distilled water, and required prepared concentrations were preserved in 20 mL of sterile plastic tubes and stored at 4 °C.

In vitro screening of essential oils against highly virulent postharvest pathogens

In vitro screening and determining the efficacy of essential oils against the most highly virulent fungal isolates of *A. alternata* was performed using the Poisoned Food Technique described by [17]. In

the method, essential oils concentrations of 0.2, 0.4, and 0.6 mg/mL were incorporated in autoclaved PDA media at 40–50 °C. Petri plates were poured with 20 mL solution in each plate and placed for 24 h pre-incubation. Disc of 5 mm diameter from the 7-days-old culture of *A. alternata* was placed in the center of the Petri plates and sealed with parafilm. Petri plates with only PDA media were used as the control set. Petri dishes were incubated at 25 ± 2 °C for 4 days and mycelial growth inhibition percentage (%) was calculated by using the formula [18].

$$\text{MGI (\%)} = [(D_c - D_t)/D_c] \times 100$$

where, MGI=mycelial growth inhibition (%), D_c =fungal growth of pathogen in control, D_t =fungal growth of pathogen in treatment.

The data were subjected to statistical analysis using software Statistix 8.1 by CRD (Complete Randomized Design) 2-factor factorial. The significant difference between the mean values was determined by LSD (Least Significant Difference) at the $p \leq 0.05$, following two-way ANOVA.

Fungitoxicity assay of *T. vulgaris* EO

After *in vitro* screening of essential oils, a fungitoxicity assay was performed to check the toxicity (fungicidal/fungistatic) of the most effective essential oil *T. vulgaris* by the procedure described by [19]. An experiment was carried out in which the inhibited fungal discs from the *T. vulgaris* oil-treated sets were reinoculated on fresh PDA media and revival of the mycelial growth was observed. Fungal discs from all three concentrations were transferred with the control sets.

Chemical composition analysis of thyme oil through gas chromatography-mass spectrometry

Gas chromatography-mass spectrometry analysis was carried out by (Agilent7890A, USA) equipped with the Flame Ionization Detector (FID), narrow capillary column (30 × 0.25 mm with film thickness 0.25 mm). The column temperature was kept at 60°C and was set to increase in intervals from 60 to 250 °C at 5°C per min. Helium gas was used as a carrier gas at 1 mL per min. The split ratio was maintained at 1:30, interface temperature was at 250 °C. Mass spectra were attained in scan mode (electron energy 70 eV) in the range 30–655 atomic mass units. Two mL of the thyme oil diluted in hexane was injected. Quantitative data from the FID area percentage was obtained and the components were separated and identified by comparison of Retention Index (RI) of the volatile components with the (RI) of the saved data of Wiley Library and mass spectra with already reported in the literature [20].

Application of plant essential oil on loquat fruit

Application of the most effective essential oil concentration on the loquat fruits against *A. alternata* was done. For this purpose, loquat fruits were surface sterilized and dipped in prepared essential oil concentration for 2 min, and finally, loquat fruits were then inoculated by spraying with 50 µL fungal spore suspension and incubated at room temperature. There were three replicates for each treatment with 25 fruits and the experiments were repeated twice. Fruits inoculated with a pathogen but without thyme oil treatment were taken as a control

treatment. Data was recorded after every 3 and 6 days and by using 0–5 disease rating scale, 0=no rotting, 1=1–10%, 2=11–20%, 3=21–30%, 4=31–50%, and 5=more than 50%, for measurement of the percent severity index by using formula:

$$\text{Percent severity index} = \frac{\text{Sum of numerical ratings}}{\text{Total number of fruits examined} \times \text{maximum grade}} \times 100$$

Results

Isolation, morphology, and pathogenicity

Overall, 14.2% of the fruits were found infected in which 33.8% of the infected fruits were due to *Alternaria* rot. After isolation, 10 isolates of *A. alternata* were recovered causing *Alternaria* rot of loquats from the five different locations of main fruit markets of Rawalpindi. Based on their morphology and diversity for the locations from which the isolates were obtained, the isolate number, cultural and morphological characteristics are listed (see Table 1, Figure 2). Based on the cultural and morphological tools, 10 isolates were identified as *A. alternata* species: AltR1, AltR2, AltR3, AltR4, AltR5, AltR6, AltR7, AltR8, AltR9, AltR10. Pathogenicity test

revealed that among these 10 isolates of *A. alternata*, three isolates Viz. AltR3, AltR6, AltR7 were found highly virulent which showed more than 50% fruit rotting and fall in the fifth as per the disease rating scale used in pathogenicity testing (see Figure 3).

Molecular confirmation and phylogenetic analysis of *A. alternata*

For amplification of DNA, an internal transcribed spacer (ITS1/ITS4) primers set was used to amplify the ITS region from the most highly virulent isolates of *A. alternata*. Amplified sequences were submitted to the GenBank of NCBI, and their accession numbers were obtained, listed in (see Table 2). The initial species identification was largely confirmed by sequence analysis by BLAST (Basic Local Sequence Alignment Tool). Furthermore, phylogenetic analysis was done, and an evolutionary tree was made with the ITS gene region (see Figure 4). In the phylogenetic tree, CLUSTALW alignment was made between 10 sequenced isolates of *A. alternata* amplified with ITS gene regions

Table 1: *Alternaria* sp. isolate ID with morphological features including colony characteristics (color, texture, margin underside color) and conidia sizes and shapes (length, width, transverse and longitudinal septa) of 10 isolates of *Alternaria* sp.

Code	Colony characteristics				Conidia			
	Colony color	Colony texture	Colony margin	Underside color	Length $\pm$ SD	Width $\pm$ SD	Transverse septa	Longitudinal septa
AltR1	Dark olivaceous green	Greenish, appressed, velvety	Irregular light green	Dark grey to greenish	26.7 $\pm$ 0.16	14.57 $\pm$ 0.2	4	3
AltR2	Dark olivaceous green	Greenish, appressed, velvety	Irregular light green	Dark grey to greenish	28.6 $\pm$ 1.31	15.65 $\pm$ 0.22	4	2
AltR3	Green olivaceous	Cottony centered growth	Slight olivaceous green	Smoky greyish	29.2 $\pm$ 0.48	14.57 $\pm$ 0.2	5	3
AltR4	Dark olivaceous green	Greenish, appressed, velvety	Irregular light green	Dark grey to greenish	20.65 $\pm$ 0.06	11.43 $\pm$ 0	4	1
AltR5	Dark olivaceous green	Greenish, appressed, velvety	Irregular light green	Dark grey to greenish	23.36 $\pm$ 0.5	16.17 $\pm$ 0.6	5	3
AltR6	Greenish to grey	Greenish cottony center growth	Brownish with white rim	Dark grey to greenish	25.06 $\pm$ 0.7	15.4 $\pm$ 0.65	6	3
AltR7	Greenish to grey	Greenish cottony center growth	Brownish with white rim	Dark grey to greenish	38.45 $\pm$ 1.69	15.48 $\pm$ 0.2	4	3
AltR8	Greenish to grey	Greenish cottony center growth	Brownish with white rim	Dark grey to greenish	20.9 $\pm$ 0.78	15.52 $\pm$ 0.25	1	0
AltR9	Dark olivaceous green	Greenish, appressed, velvety	Irregular light green	Smoky greyish	30.23 $\pm$ 0.7	12.97 $\pm$ 0.5	6	3
AltR10	Green olivaceous	Cottony centered growth	Slight olivaceous green	Dark grey to greenish	25.5 $\pm$ 0.04	15.1 $\pm$ 0.8	5	2

along with previously identified reference sequences of *A. alternata*.

***In vitro* screening of essential oils against highly virulent postharvest pathogens**

Effect of eight plant essential oils were tested at three different concentrations viz. 0.2, 0.4, and 0.6 mg/mL against *A. alternata* isolate: AltR3. It was revealed in the *in vitro* application of EOs that among eight selected EOs, thyme EO was found to be highly effective and showed maximum efficacy to inhibit the mycelial growth of highly virulent *A. alternata* isolate (AltR3) at all three concentrations (0.2, 0.4, and 0.6 mg/mL) after the 4 days of incubation and reduced the growth inhibition (44.44, 57.03, and 70.81%) followed by lemon EO with mycelial growth inhibition (42.30, 53.63, and 61.70%) in comparison to control which showed none of the growth inhibition (see Figures 5 and 6). However, pomegranate EO was found to be the least effective and showed the minimum reduction in mycelial growth inhibition.

Fungitoxicity assay of most effective EO (*T. vulgaris*)

The results revealed that at lower concentrations (0.2 mg/mL and 0.4 mg/mL) of thyme oil, the essential oil showed weak and hyaline mycelial growth without sporulation indicated the fungistatic effect of the oil at lower concentrations. However, at a higher concentration (0.6 mg/mL), no proper growth was observed on Petri plates and the effect of thyme oil at 0.6 mg/mL concentration was found fungicidal as compared to the control set which showed normal growth after 4 days of incubation.

Chemical composition analysis of thyme oil through gas chromatography-mass spectrometry

The gas chromatography-mass spectrometry analysis depicted the presence of 32 chemical compounds in the thyme essential oil which represented about 96.56% of the total detected constituents (see Table 3). Among which

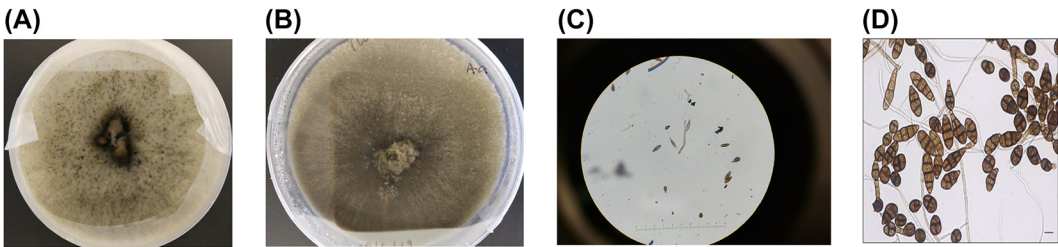


Figure 2: Cultural and morphological characteristics of *Alternaria* sp. (A) lower side view of the growth of the pathogen in Petri plates, (B) upper side view of the growth of the pathogen in Petri plates, (C) and (D) microscopic view of the spores of the fungus identified as *Alternaria alternata*.

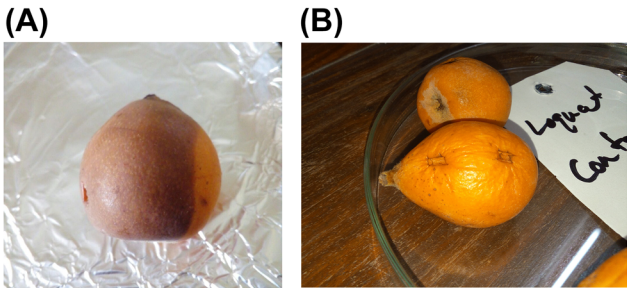


Figure 3: Pathogenicity test of the pathogen. (A) Shows the symptoms of fruit rot confirming the pathogenicity however, (B) fruits are symptomless in the control set.

Table 2: Accession numbers of the amplified nucleotide sequences of *Alternaria alternata*.

Pathogen	Isolate ID	Accession number (ITS)
<i>Alternaria alternata</i>	AltR1	MZ208145.1
<i>Alternaria alternata</i>	AltR2	MZ209285.1
<i>Alternaria alternata</i>	AltR3	MZ209279.1
<i>Alternaria alternata</i>	AltR4	MZ209280.1
<i>Alternaria alternata</i>	AltR5	MZ209284.1
<i>Alternaria alternata</i>	AltR6	MZ209282.1
<i>Alternaria alternata</i>	AltR7	MN031262.1
<i>Alternaria alternata</i>	AltR8	MZ209281.1
<i>Alternaria alternata</i>	AltR9	MZ209287.1
<i>Alternaria alternata</i>	AltR10	MZ209283.1

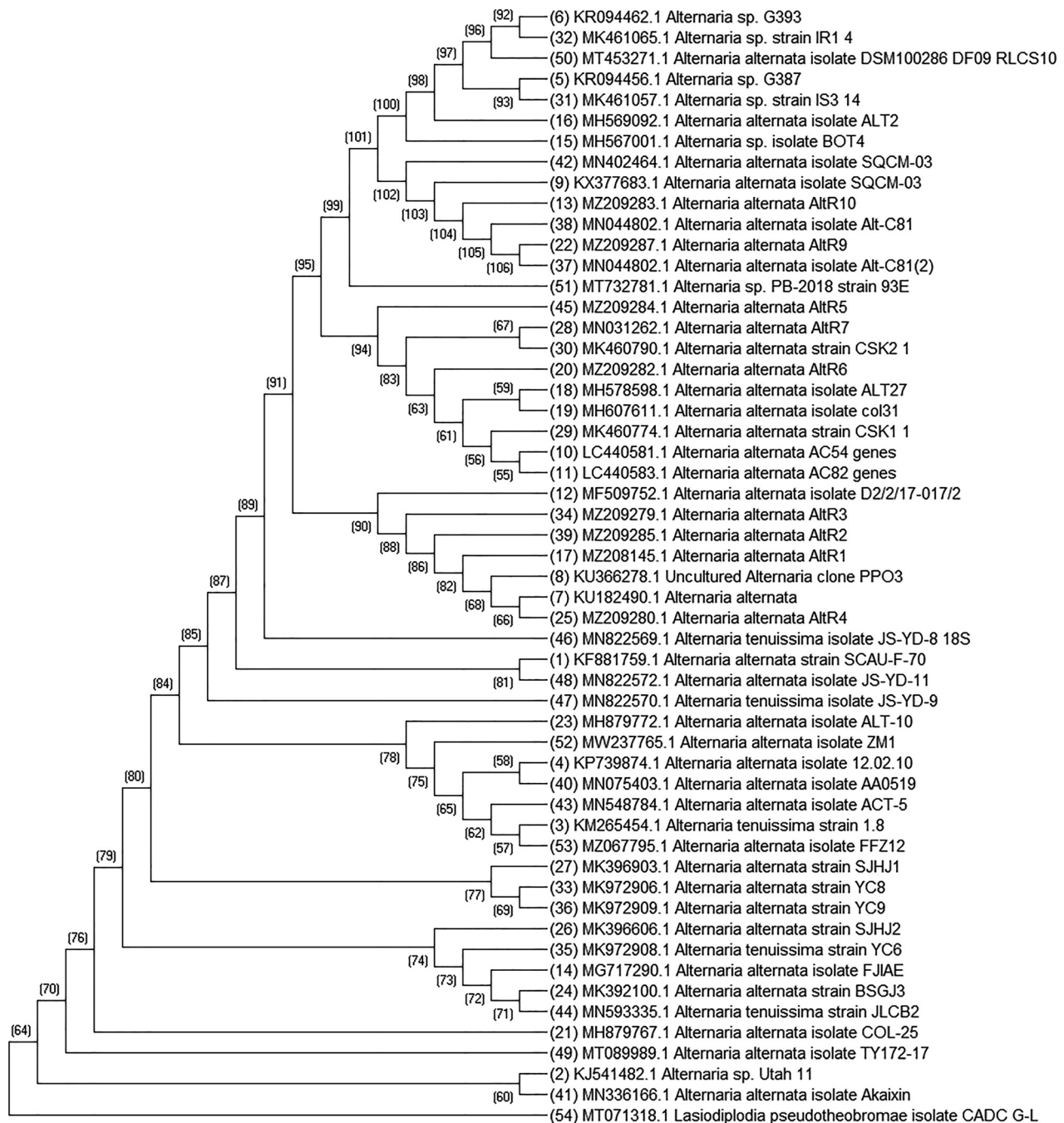


Figure 4: Phylogenetic tree based upon CLUSTALW alignment of internal transcribed spacer region nucleotide of *Alternaria alternata* isolates obtained from loquat fruits.

The evolutionary history was inferred using the neighbor-joining method. The optimal tree with the sum of branch length = 0.45855314 is shown. The percentage of replica trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. This analysis involved 54 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 1,343 positions in the final dataset. Evolutionary analyses were conducted in MEGA X. The tree is rooted with *Lasiodiplodia pseudotheobromae* isolate (MT071318.1).

the components with the highest percentage were Thymol (57.15%), followed by γ -terpinene (8.84%), *p*-cymene (8.45%), carvacrol (5.60%), and terpinolene (3.60%).

However, the other components showed less than 2% of the Relative area percentage. Thymol was found the major component in the GS-MS analysis of thyme oil.

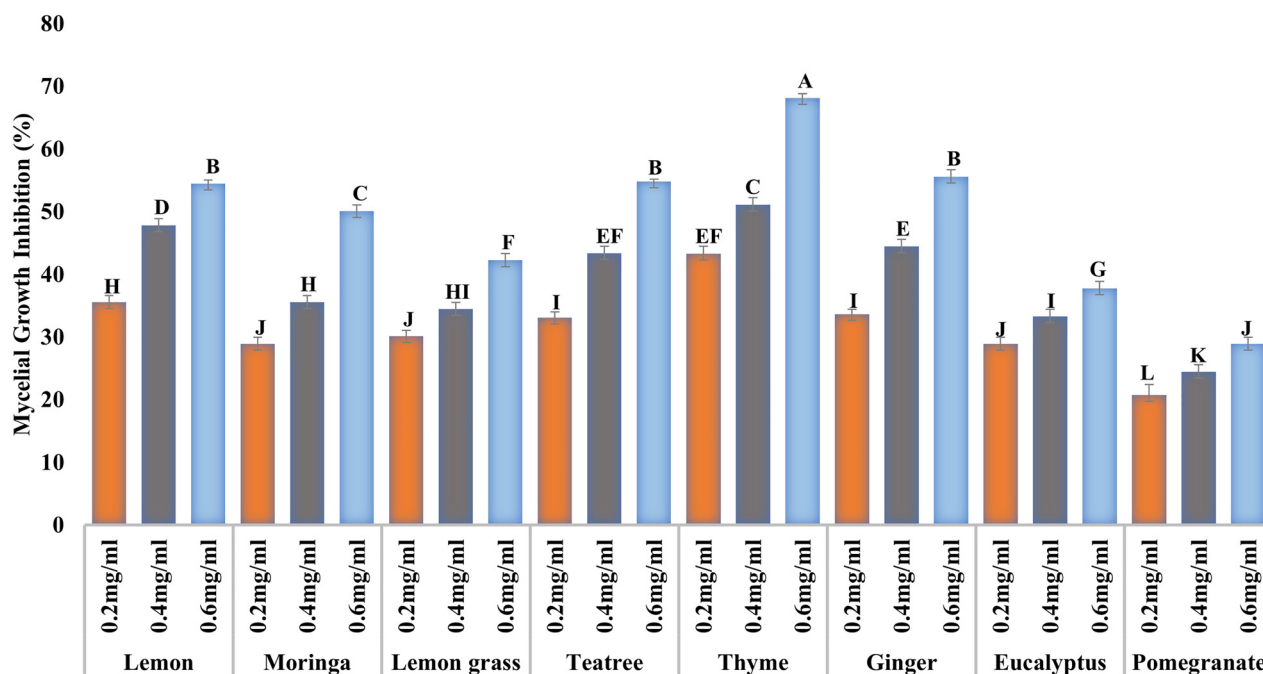


Figure 5: Evaluation of eight selected essential oils against *Alternaria alternata* in *in vitro* conditions showing the mycelial growth inhibition (%) of EOs in which thyme oil found most effective against *Alternaria alternata*.

Data with different letters are significantly different according to least significant difference test ($p < 0.05$).

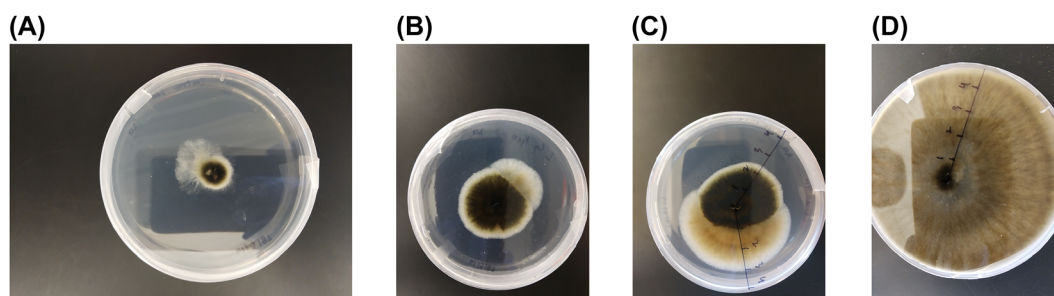


Figure 6: Growth of *Alternaria alternata* in Petri plates showing the mycelial growth inhibition.

(A) Growth at concentration of 0.6 mg/mL, (B) growth at a concentration of 0.4 mg/mL, (C) growth at a concentration of 0.2 mg/mL, (D) growth in control set showing no inhibition in growth of the fungus.

Application of thyme oil on loquat fruit against *A. alternata*

Thyme oil coating was done on loquat fruits at the most significant concentration (0.6 mg/mL) and the percent severity index was calculated using a disease rating scale. Results revealed that the percent severity index on thyme oil against a highly virulent isolate of *A. alternata* on loquat fruits after 3 days of incubation at room temperature was 10.4% compared to the control sets which showed a 46.4% of percent severity index value after 3 days. However, it was observed that the treated loquat fruits showed 18.4% after the 6 days of incubation whereas, the control set showed

71.2% of the percent severity index which can be considered as a complete decaying of the fruits (see Table 4).

Statistical analysis

Among all eight essential oils with three concentrations tested against *A. alternata* to check mycelial growth inhibition (%) and it was found that all essential oils exhibit antifungal activity whereas thyme oil was found most effective against pathogen. However, the concentration of 0.6 mg/mL of thyme oil showed the most significant results.

Table 3: Chemical composition of essential oil of *Thymus vulgaris* analysed by GC-MS (gas chromatography-mass spectrometry) from Pakistan.

No.	Components	^a RA, %	^b RI
1	α -Thujene	0.88	926
2	α -Pinene	0.55	934
3	β -Pinene	0.37	977
4	Camphene	0.43	948
5	Sabinene	0.10	975
6	Myrcene	1.59	992
7	α -Phellanderene	0.34	1,006
8	<i>p</i> -Cymene	8.45	1,024
9	Limonene	0.14	1,030
10	(<i>Z</i>)- β -Ocimene	0.10	1,034
11	(<i>E</i>)- β -Ocimene	0.21	1,045
12	α -Terpinene	1.80	1,016
13	γ -Terpinene	8.84	1,059
14	Terpinolene	3.64	1,088
15	Camphor	0.14	1,045
16	Borneol	1.37	1,070
17	<i>cis</i> -Dihydrocarvone	0.20	1,201
18	Thymol	57.15	1,292
19	Thymol methyl ether	0.38	1,233
20	Carvacrol	5.60	1,306
21	Carvacrol methyl ether	0.50	1,242
22	Eugenol	0.05	1,359
23	δ -Cadinene	0.25	1,518
24	γ -Cadinene	0.20	1,514
25	Linalool	0.10	1,105
26	Aromadendrene	0.10	1,437
27	α -Cadinol	0.16	1,650
28	Terpineol	0.47	1,206
29	Caryophyllene	1.58	1,420
30	Spathulenol	0.11	1,577
31	Germacrene D	0.54	1,480
32	α -Humulene	0.22	1,454
	Total	96.56	

^aRA(%), relative area percentage values. ^bRI, retention index.**Table 4:** Effect of thyme oil (0.6 mg/mL) concentration against *Alternaria alternata* after 3 and 6 days.

Treatments	Days	Disease severity index, %
T0 Control ^a	3	46.4
	6	71.2
T1 Thyme oil 0.6 mg/mL + <i>Alternaria alternata</i>	3	10.4
	6	18.4

^aControl (the only inoculum of *Alternaria alternata* applied).

Discussion

In the present study, cultural and morphological characteristics and phylogenetic analysis were used to identify the

10 isolates on symptomatic loquat fruits from five different locations of Rawalpindi city, Punjab, Pakistan. The species causing *Alternaria* rot of loquat was *A. alternata*. Initially, *Alternaria* sp. isolates showed white colonies having slight variation and regular to irregular margins. After seven days, *Alternaria* species showed the three distant colony colors. From 10 isolates, three isolates showed greenish to grey colony color, five isolates recorded dark olivaceous green color while one was found green olivaceous surface colony color. However, colony texture and margins showed variation in the growth pattern among isolates of *Alternaria* sp. Colony texture was found greenish, appressed, and velvety while some showed cottony to greenish cottony centered growth on the Petri plates. Whereas colony margins were irregular light green, slight olivaceous green, or brownish with a white rim [21]. Latinović, Radišek, and Latinović (2014) also demonstrated the ellipsoid to ovoid conidial microscopic observation, 3 μ m wide and 30 μ m in length with 1–3 longitudinal and 2–5 transverse septations [22]. Cuervo-Parra et al. (2011) also described the muriform shape of conidia in *A. alternata* and septations were 0–5 & 1–9 in longitudinal and transverse, respectively. Our results in contracts with many of the similar findings of morphological identification of *Alternaria* sp. using the cultural and microscopic tool were found and supported by several researchers [23]. However, Identification based on symptomology and morphological characteristics is considered to be the traditional approach for fungal species identification, therefor molecular techniques like Polymerase Chain Reaction (PCR) and sequence analysis are proven to be effective tools for fungal species identification [24]. [25] Capote et al. (2012) also illustrated that the PCR techniques are effective, reliable, and simple techniques for the fungal characterization which provides the results in a short time. So, selected isolates were further subjected to molecular identification for the reconfirmation of the results described earlier in the current study. The internal transcribed spacer region was used for the amplification of fungal isolates of *Alternaria* sp. After getting the PCR product, the gel electrophoresis resulted in the gel bands of amplified products. Nucleotide sequences were analyzed and further used for the phylogenetic analysis. The results revealed that the isolates showed 99–100% of similarity to the submitted different selected isolates and homology to the previous isolates of *A. alternata* confirming the *Alternaria* sp. isolated from the different locations [26]. Kakalikova et al. (2009) revealed that the ITS regions sequence analysis is sufficient which provides the confirmation of *A. alternata* species in molecular identification and characterization [25]. Capote et al. (2012) also revealed that DNA amplification and phylogenetic analysis have become the most specific and

accurate methods for the characterization of fungal species and developing the phylogenetic relationship among them. The ITS region of DNA has been widely applied for the phylogenetic studies of fungi due to its being highly conserved and can be easily investigated using PCR amplification [27].

Previous studies illustrated the frequent use of synthetic chemicals and fungicides in postharvest disease management as a primary means of controlling the pathogens but due to fungicidal resistance among the fungal population, high development cost, and residual effects on consumer health, this approach has some limitations and researchers have successfully introduced the botanicals as a replacement of synthetic chemicals due to their non-toxic residual effect, ecofriendly in nature and public acceptance [28].

The current study depicted that thyme oil (EO) was found significantly ($p < 0.05$) effective against major post-harvest fungal pathogens of loquat showing the Maximum growth inhibition against *A. alternata* (68.15%), at the concentration of 0.06%. Essential oils have an antifungal ability due to the presence of chemical compounds which act as a natural fungicide against various fungal pathogens inhibiting fungal activity [29]. The most active oil was found to be the thyme oil among all eight essential oils used. Thyme oil consists of the major active component thymol which might have a fungicidal activity that probably resulted in extensive damage to the cell wall and cell membrane of the fungi. The chemical composition of the *T. vulgaris* and found the presence of thymol 46.65, linalool 3.8%, α -pinene 3%, camphene 1% which showed the fungitoxic effect against fungal pathogens [30].

[31] Imelouane et al. (2009) extracted the two major chemical compounds from the thyme plant leaves which can be considered as an effective antifungal agent to provide permanent damage to the mycelial growth of fungi. The use of thyme oil in food preservatives and medicinal drugs has been already reported due to the presence of thymol as an essential chemical component of thyme [32]. [33] Abdolahi et al. (2010) reported that the thyme oil exhibit strong antifungal activity against several post-harvest fungal pathogens including *Botrytis cinerea* and *Mucor* spp.

The GS-MS analysis of the thyme oil detected the presence of 32 chemical compounds in which thymol showed the highest percentage value followed by terpinene, *p*-cymene [34]. Negahban and Saeedfar (2015) also identified a similar result with a higher percentage of thymol (49%) in the chemical analysis of thyme oil [35]. Miladi et al. (2013) also showed a higher percentage of thymol (52.19%) in chemical analysis of *T. vulgaris*. The

studies showed the different percentage quantities of these chemical components due to variation in climate conditions and geographical regions of the world [36].

The thyme oil contains thymol (33%), and 1,8-cineole (11.3%) as a major component and three times stronger inhibition as thyme oil exhibited by pure thymol according to Šegvic Klaric et al. (2007) [37]. The antifungal activity of thyme oil was reported against *A. alternata* in *invitro* studies and found growth inhibition from 68.5 to 74.8% [38]. The essential oils extracted from the leaves of thyme (*T. vulgaris*) were used as a potential agent to protect several food commodities from microbial deterioration caused by food pathogenic fungi. Thyme oil is an effective compound to control the black rot of grapes by using relatively low concentrations [39]. The results of present studies provide agreement to the previous studies of chemical compositions of thyme oil showed the presence of active component thymol 38.7, 47.59, and 38.6% in the thyme (*T. vulgaris*) oil which is a high concentration as compared to the other chemical components presents. The presence of thymol as an active compound in thyme oil was found to be the main reason for the high antifungal activity of thyme oil against a variety of fungal pathogens [40].

The essential oil of *T. vulgaris* has shown significant fungitoxic activity against *A. alternata* and inhibits the mycelial growth of the fungi ultimately increasing the shelf life of the fruit for consumption. However, the oil did not show any adverse symptoms on the fruit peel. The most promising performance of the thyme oil was observed against the pathogen. Therefore, the use of essential oil as an antifungal agent can be an interesting and promising approach for the management of postharvest diseases of fruits and vegetables. The essential oil of *T. vulgaris* with its strong fungitoxic effect, non-hazardous, high inhibitory action, fungistatic/fungicidal against the tested fungus, and in addition, the increase in fruit shelf life which are the desired characters of an ideal fungicide and can be recommended as a botanical fungitoxicant.

As loquat fruit is a perishable fruit and is available for a very short time in markets, hence maintenance of the healthy fruit was the major limitation in the execution of the study. As loquat fruit is very perishable, so contamination was one of the major problems other than *A. alternata*. In the study, direct application of thyme oil was done on the loquat fruits and the major components present in thyme oil could also be used separately to check the effect against the postharvest disease but due to financial constraints effect of thymol could not be used to explore its potential against *A. alternata*. To explore the safety of thyme oil concentration, a biological study should be carried out.

Conclusions

The result from the current research provides a comprehensive depiction of *Alternaria* rot of loquat from Pakistan through identification of *A. alternata* which is the first time reported on loquat as a postharvest disease in Pakistan. In conclusion, all essential oils showed antifungal activity whereas thyme oil was the most active inhibitor and found fungicidal/fungistatic at different concentrations against the tested fungal pathogen in both *in vitro* and through direct application on fruit. In the chemical composition analysis of thyme oil, thymol compound was found in higher percentage and which is the main reason for the high antifungal ability of the thyme oil. These natural compounds have all the desired properties of an ideal fungicide and could be recommended as a botanical fungicide that can replace hazardous synthetic chemicals. The current study explored that thyme oil could be potentially used against *A. alternata* and its compounds could be further investigated for the development of eco-friendly approaches for the control of postharvest fruit rots. EOs can provide effective technological hurdles against wide spectrum postharvest fungal pathogens and can be considered for the development of protective products for the food industry with no residual effects and can be eco-friendly in nature and also increase the shelf life of the fruits.

Research funding: None declared.

Author contribution: MFA and GI: Designed research, conducting experiments, data curation, manuscript writing; FN and MAK: Data analysis, review, editing, and proofreading. All the authors have read and agreed to the manuscript for publication.

Competing interests: Authors state no conflict of interest.

Informed consent: Not applicable.

Ethical approval: Not applicable.

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