Management of Strawberry Anthracnose Using Plant Essential Oils as Bio-fungicides, and Evaluation of Their Effects on Quality of Strawberry Fruit

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Abstract: *Colletotrichum nymphaeae* is the causal agent of strawberry anthracnose, which is one of the most important disease affecting strawberry plant in Iran. This research aimed to apply the selected plant essential oils (EOs) such as *Achillea millefolium*, *Mentha longifolia*, and *Ferula kuma* to the management of strawberry anthracnose disease under *in vitro*, *in vivo*, and greenhouse conditions. *In vitro* tests indicated that all the EOs and fungicide were able to inhibit mycelial growth and conidial germination of the pathogen. Scanning electron microscopy (SEM) revealed that EOs significantly suppressed the mycelia growth and caused a change in morphology of fungal mycelia. The severity of strawberry anthracnose disease was significantly ($p \leq 0.05$) reduced by all EOs under *in vivo* and greenhouse conditions. Results of all experiments showed that *M. longifolia* EO was the best EO to control *C. nymphaeae*. Also, EOs almost reduced weight loss and preserved firmness, ascorbic acid, total phenol, antioxidant activity (DPPH), and enzyme peroxidase activity in treated fruit. Moreover, EOs preserved the sensory quality of strawberry fruit during the storage period so that there were no significant differences between treatments (EOs) in their appearance, flavor, odor attributes, and overall evaluation compared to the control. Our results indicate that EOs are excellent bio-fungicides for the management of strawberry anthracnose.

Key words: Captan, *Colletotrichum nymphaeae*, DPPH, sensory quality

1 Introduction

Kurdistan Province (west of Iran) is the most important habitat of strawberry (*Fragaria × ananassa* Duch.) in Iran. Strawberry anthracnose disease, caused by *Colletotrichum nymphaeae*, is one of the main pre-harvest and post-harvest diseases that affect strawberry production in Iran. Several species of *Colletotrichum* such as *C. acutatum* J.H. Simmonds, *C. fragariae* Brooks, *C. gloeosporioides* (Penz), and *C. nymphaeae* (Pass.) cause strawberry anthracnose disease¹. *C. nymphaeae* is one of the important pathogens causing strawberry anthracnose diseases in Kurdistan province, Iran. This widespread, aggressive species² is a limiting factor in strawberry production. The symptoms appear on the aerial parts of the strawberry plant (i.e., stolon, petiole, flower, fruit, leaf, and crown), especially fruit decay such as sunken lesions and conidial ooze on fruits¹. Resistance cultivar to this fungus has not been found and reported in Iran yet. Application of chemical fungicides on fruit against the disease is limited and

harmful for human health. Also, public demand to reduce fungicides use and the development of fungicide resistant has increased the need for alternative control strategies. In this regard, applications of fungicides are not permitted as postharvest treatments under organic production rules³. Therefore, an alternative to fungicides is needed for managing the strawberry anthracnose. Physical, chemical and biological methods have been evaluated in the management of strawberry fruit production⁴. The use of plant essential oils (EOs) is a promising alternative to chemical products and has the potential for postharvest diseases management⁵. The plant EOs have antimicrobial activities and induce the host defenses system⁶. Several plant EOs have been evaluated and reported as effective management of postharvest disease on citrus^{5,7}, pepper⁸, avocados⁹, gray mold of table grapes¹⁰ and banana¹¹. Application of plant EOs allows controlling the plant diseases and preserving the fruits during postharvest¹². Moreover, they do not pose any risk to human health and rather improve the fruit

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quality^{11, 13}).

The potential of EOs to control strawberry anthracnose by *Achillea millefolium*, *Ferula kuma*, and *Mentha longifolia* has not been reported yet. The objective of this research is to evaluate the potential of EOs of these plants to control the strawberry anthracnose under *in vitro*, *in vivo*, and greenhouse conditions. Also, the effect of EOs on quality of strawberry fruit after harvest is evaluated.

2 Experimental Procedures

2.1 Fungal pathogen and inoculum preparation

C. nymphaeae isolate CCh32 (GenBank Acc. No. CCTUCCh32) causing anthracnose strawberry used in this research was obtained from the culture collection of the Plant Protection Department of University of Tabriz in Iran¹. The isolate was previously isolated from infected strawberry plants in Kurdistan Province. The pathogen was cultured on Potato dextrose agar (PDA) for 10 days at $25 \pm 2^\circ\text{C}$ and was kept on PDA at 4°C . The conidia of the pathogen were collected from 14-days-old cultures by filtration and were rinsed with sterile water. Next, the concentration of conidial suspension was adjusted to 1×10^6 CFU/ mL using a hemocytometer.

2.2 Plant and fruits

The healthy strawberry plants cv. Paros (5-weeks old) were used in experiments. Plants were grown in greenhouse conditions at 24 to 27°C , 60-70% relative humidity, 16 h light, and 8 h darkness. Healthy strawberry fruits (c.v. Paros) of uniform size shape, maturity and free from any indication of mechanical injury and pathogenic infection were collected for the experiments. The fruits were transferred to the laboratory on June 2016, washed with distilled water, and air-dried by sterile paper under the laminar hood for 15 min.

2.3 Essential oil extraction

The specimens of *Achillea millefolium*, *Ferula kuma* and *Mentha longifolia* were collected from the growing field in their natural habit (Marivan and Ghorveh, Kurdistan Province, Iran) at the flowering stage on June 2016. The plant specimens were shade dried. The dried plant materials (300 g) were selected and extraction of EOs was carried out through Clevenger-type apparatus and hydrodistilled for 5 h¹⁴. The extracted essentials oil was dried in anhydrous sodium sulfate (Na_2SO_4). Then, the oils were preserved in a sealed amber bottle at 4°C in the refrigerator until use.

2.4 GC-MS analysis

The chemical analysis of EOs were performed on an Agilent 7890A gas chromatography (GC) coupled with

Agilent 5975C mass spectrometry (MS) (Agilent Technologies, USA), using a HP-5 MS capillary column (30 m length $\times$ 0.25 mm internal diameter, film thickness 0.25 μm) on July 2016 and their constituents were measured¹⁵. GC oven temperature was kept at 50°C for 5 min and programmed to 240°C with a rate of $3^\circ\text{C}/\text{min}$. The volume of sample injection was 1 μL and helium was used as the carrier gas with a flow rate of 0.8 mL/min. Injection and detector temperature were set as of 290 and 250°C , respectively. The mass spectrometer parameters were recorded at electron energy of 70 eV¹⁵. The components of EOs were identified by their inhibition index and compared with those reported in the literature and of their mass spectra with those in the WILEY/ NBS^{16, 17}.

2.5 Antifungal activity of EOs on mycelial growth

Antifungal activity of EOs on mycelial growth was calculated using contact and vapor methods on July 2016¹⁸. In the contact method, the antifungal assay of EOs and commercial fungicide of Captan (50% a.i. WP) were carried out in the plates (9-cm-diameter) containing a PDA medium. Each concentration of EOs and fungicide was dissolved in Tween[®] 80 (0.05% v/v) (Merck, Darmstadt, Germany). The solution was added to autoclaved PDA media at 40°C at plates (9-cm-diameter). Six different concentrations of EOs and fungicide were selected by pre-test for the pathogen (Fig. 1). In order to evaluate the antifungal ability of EOs and fungicide, a disc of 5-mm in diameter (7-days old stock cultures) of the pathogen was prepared by cork borer and placed in the center of the plates. Plates containing PDA medium, Tween[®] 80 and pathogen were used as control. Three replicates per each treatment were used. All plates were incubated at $25 \pm 2^\circ\text{C}$ for 10 days. The diameter of fungal colonies of *C. nymphaeae* was measured (mm) and the inhibition percentage was calculated using the following formula: inhibition percentage = [(control – treatment)/ control] $\times$ 100. Next, the median effective inhibitory concentration (EC_{50}) was measured for EOs and fungicide ($\mu\text{L}/\text{L}$) on *C. nymphaeae*. The Probit analysis was used to evaluate EC_{50} with the SPSS software versions 16.0.

In the vapor method, the effect of the volatile phase of EOs and Captan fungicide was investigated using a filter paper (6-mm-diameter) in plates (9-cm-diameter). First, one mycelial disc plug of the pathogen (5-mm-diameter) was taken from the margin of a 7-day-old culture and was placed in the center of plates containing PDA medium. Then, the filter paper was dipped in different concentration of the EOs as well as Captan and placed in the inner space of the inverted lid of the plates. Afterward, the plates were immediately sealed with parafilm to prevent any loss of the volatile compounds. In the next step, the plates were incubated at $25 \pm 2^\circ\text{C}$ for 10 days. Then, radial mycelial growth was measured and the inhibition percentage was measured using the formula mentioned above.

Fig. 1 Antifungal activity of EOs on mycelial growth of *C. nymphaeae* after 10 days using two contact and vapor methods. Data with different letters indicate significantly different ($p \leq 0.05$) according to statistics analysis using SAS software version 8.2 with Duncan multiple-ranges test (DMRT).

2.6 Antifungal activity of EOs on conidial germination

Effect of EOs of *Achillea millefolium*, *Mentha longifolia*, and *Ferula kuma* at concentrations 1500, 600, and 1850 $\mu\text{L/L}$ respectively and fungicide at the concentration of 2100 $\mu\text{L/L}$ were evaluated on conidial germination of the pathogen using two contact and vapor methods on PDA plates on July 2016. In the contact method, one mL of different concentrations of EOs and fungicide ($\mu\text{L/L}$) supplemented by 0.05 % of Tween[®] 80 (0.05% v/v) (Merck, Darmstadt, Germany) were pipetted into micro tube (5-mL-volume). Then, one mL of the suspension of the pathogen (10^6 conidia/mL) was added to it and was placed on the shaker in 120 rpm at $25 \pm 2^\circ\text{C}$ for 24 h. Afterward, 50 μL of resulting solutions were pipetted on water agar medium (WA) in plates (9-cm-diameter) and distributed evenly with a spreader. In the next step, 50 μL of a conidial suspension of the pathogen (10^6 conidia/mL) alone supplemented by 0.05 % of Tween[®] 80 (0.05% v/v) (Merck, Darmstadt, Germany) was added to WA medium as a control treatment. Three replicates per each treatment were prepared. Plates were incubated at $25 \pm 2^\circ\text{C}$ for 48 h under a 12-h photoperiod. Then, 200 conidia from each replicate were evaluated to assess the percentage of conidial germination (out of the total number of evaluated spores) using an optical microscope (40 $\times$ magnification). A conidium was adverted to have germinated if the length of the germ tube was larger than or equal to the conidial diameter. The percentage of inhibition of conidial germination of *C. nymphaeae* by EOs

and fungicide was calculated and compared to control with the above-mentioned formula¹⁹.

In the vapor method, following the above method, 10 mL of WA medium was added to each plate (9-cm-diameter). A total of 50 μL of suspension of the pathogen (10^6 conidia/mL) was spread onto WA medium in plates. Then, sterile filter paper disc (5-mm-diameter) was dipped into one mL of each concentration of EO and fungicide for one min, was placed in the inner space of the inverted lid of the plates. The plates containing spore solution of the pathogen were placed upside the EOs containing plates. Plates containing filter paper discs dipped in sterile water were used as the control. Then, plates were sealed with parafilm, incubated at $25 \pm 2^\circ\text{C}$ for 48 h and tried similarly discussed previously. The inhibition percentage was evaluated with the above-mentioned formula²⁰.

2.7 Morphological changes in presence of dill EOs

Mycelia of the pathogen were treated with EOs of *Achillea millefolium*, *Ferula kuma*, and *Mentha longifolia* at concentration of 1500, 1850 and 600 $\mu\text{L/L}$, respectively, based on contact phase for 7 days. Then, the mycelia of *C. nymphaeae* was freeze-dried. Morphological changes of mycelia were observed using scanning electron microscope (SEM) on August 2016.

2.8 Antifungal activity tests *in vivo* and greenhouse conditions

Effect of EOs and fungicide on fruit decay development and strawberry anthracnose was evaluated *in vivo* and greenhouse conditions, respectively on September 2017. The concentrations of EOs of *Achillea millefolium* (1500 $\mu\text{L/L}$), *Ferula kuma* (1850 $\mu\text{L/L}$), *Mentha longifolia* (600 $\mu\text{L/L}$), and Captan (2100 $\mu\text{L/L}$) were selected based on total inhibitory effects *in vitro*.

2.9 Fruit decay test

To evaluate the antifungal efficacy of the volatile fraction of the EOs on fruit decay development, healthy strawberry fruits were surface sterilized by dipping in 70% ethanol for 3 s, rinsed in sterile distilled water (three times), and dried by sterile paper under a laminar hood for 10 min. After that, 20 μL conidial suspensions of *C. nymphaeae* (10^6 conidia/mL) amended with Tween[®] 80 (0.05% v/v) (Merck, Darmstadt, Germany) were pipetted onto the fruit surface. The fruits inoculated with distilled water with Tween[®] 80 (0.05%, v/v) and fungal spore were used as the negative and positive control, respectively. Inoculated fruits were put in plastic boxes, followed by adding 2 mL of different concentrations of EOs and fungicide of Captan to the dish (2 cm diameter and 1.5 cm high) and placing it in the center and at the bottom of each plastic box. A total of 20 healthy fruits were selected for each treatment and distributed in four boxes. In the control treatment, plastic boxes were put distilled water. All plastic boxes were immediately sealed with layers of parafilm to avoid any loss of volatile compounds and incubated at $25 \pm 2^\circ\text{C}$ under a regime of 12 h of light and 12 h of darkness for 7 days. After one week, the fruit decay development (disease severity) was measured.

2.10 Greenhouse studies

To determine the inhibition effect of EOs in greenhouse condition, the strawberry plants cv. Paros (five weeks old) were used. The strawberry plants were sprayed with selected concentrations of EOs amended by Tween[®] 80 (0.05% v/v) (Merck, Darmstadt, Germany) until all plant was wetted to run-off using a hand-held atomizer. The plants were individually covered with a 0.1-mm-thick transparent plastic film to maintain moisture and incubated at $24\text{--}27^\circ\text{C}$, 60–70% RH for 24 h in the dark. After that, 5 mL of the pathogen inoculum at a concentration of 1×10^6 conidia/mL were sprayed onto each plant with a hand-held atomizer. The plants were sprayed with distilled water and fungal spore, which were utilized as the negative and positive control, respectively. The plants were incubated in plastic bags for 48 h in the dark as before to stimulate infection^{21, 22}. Then, plants were incubated for 60 days in greenhouse conditions at $24\text{--}27^\circ\text{C}$, 60–70% relative humidity, 16 h light, 8 h darkness. After 2 months, the disease

severity was measured.

2.11 Disease severity

Disease severity on strawberry fruits was scored after seven days using a scale of 0 to 5 through the method described by²³ with some modification. A rating of 0 was scored when there was no symptom of anthracnose disease on the surface of the fruits (healthy fruit). The rates of 1, 2, 3, 4 and 5 were scored when <20 , 20.1 to 40, 40.1 to 60, 60.1 to 80, and 80.1 to 100% of the fruit surface showed anthracnose symptom, respectively.

Disease severity in greenhouse conditions after 2 months was scored on petioles of strawberry plants by the method described by²⁴ using a scale of 0 to 5. A rating of 0 was scored when there was no symptom of lesions on petiole (healthy petiole or without lesions). Ratings of 1, 2, 3, and 4 were scored when <3 mm, 3 to 10 mm, 10.1 to 20 mm and >20 mm of the petiole showed lesions symptom, respectively. If lesions entirely appeared on petiole and the plant is dead, a rating of 5 was scored. Finally, efficacy percent was measured using the following formulas:

$$\text{Efficacy \%} = [(\text{disease index of control}) - (\text{disease index of treated})] / \text{disease index of control} \times 100.$$

2.12 Effect of EOs on fruit quality

Healthy and mature strawberry fruits of similar size and color (70% red stage of ripening) were harvested from a local farm on October 2016 and immediately (within 1 h) transported to the laboratory and incubated at 1°C . At first, the fruits were rinsed in sterile distilled water and were dried under a laminar hood for 15 min. They were sprayed separately with EOs of *Achillea millefolium*, *Ferula kuma*, and *Mentha longifolia* at concentrations ($\mu\text{L/L}$) of 1500, 1850, and 600, respectively, and sterile distilled water as the control. The inoculated fruits were dried at 25°C for one hour and then the fruits were placed into polyethylene bags and incubated in cold storage (1°C and 75% RH) for 10 days. Six fruits per replicate and three replicates per treatments were used. The storage quality parameters of the treated strawberry fruit were recorded as described below. It was indexed before storage (i.e., harvesting time) and eight days after the storage. All tests were performed duplicate. At last, treatments (with different combinations of factor levels) were analyzed in a factorial experiment with three replications in the frame of a Completely Randomized Design (CRD). Means of treatments were compared using Duncan's multiple range test.

2.12.1 Weight loss

The weight loss percentage of the treated strawberry fruits were recorded using the formula $[(M1 - M2) / M1] \times 100$, where M1 denotes the weight of the strawberry fruit before storage and M2 is the final weight (after storage).

2.12.2 Fruit firmness

Fruit firmness was calculated using a TA.XT2i texture

analyzer (Santam, STM-1, Iran) by performing a penetration test with an 5-mm diameter cylindrical probe. The speed of the probe was 3.0 mm/min and a distance of 10 mm was used. The fruit firmness was recorded at two locations in the middle of the strawberry fruit. Finally, firmness was expressed as the maximum penetration force (N).

2.12.3 Ascorbic acid

Ascorbic acid (vitamin C) was recorded described by Kevers *et al.*²⁵⁾. The results were expressed as mg ascorbic acid per 100 g of fruit fresh weight (FFW) basis.

2.12.4 Total phenol content

The total phenol content (TPC) of fruit juice was measured based on the Folin-Ciocalteu total phenolic assay²⁶⁾. For this work, 20 µL fruit extracts were dissolved in 250 µL distilled water, 750 µL solution of 10% Folin-Ciocalteu (v/v) and 800 µL 7.5% Na₂CO₃ (w/v). Samples were placed at room temperature in the dark for 60 min at 25°C. Then, the absorption was measured at 765 nm by a spectrophotometer. The results were expressed as mg gallic acid per 100 g fruit fresh weight (FFW) basis.

2.12.5 Antioxidant activity

The antioxidant activity was measured according to the 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging capacity method described by Patras *et al.*²⁷⁾. For antioxidant activity assay, 200 µL of fruit sample extracts were added to 500 µL DPPH (0.238 mg per one ml methanol). The resulting reaction mixture was kept at room temperature for 30 min at 25°C. The absorbance was recorded at 515 nm. The inhibition percentage of DPPH was measured as follows: DPPH Inhibition % = $[(A_b - A_s)/A_b] \times 100$, where A_b is the absorption in DPPH control and A_s is absorption in treatment.

2.12.6 Peroxidase (POD) activity

The peroxidase (POD) activity was recorded according to the guaiacol method described by Ghanati *et al.*²⁸⁾, with slight modifications. The unit of enzyme activity was defined to be equivalent to a 0.01 per minute increase in the absorbance at 470 nm for two min. The result (enzyme activity) was expressed in U per gram fresh weight.

2.13 Postharvest sensory quality analysis

This research was performed on strawberry fruits without pathogen on October 2016. The generic descriptive analysis method was employed to determine whether there was any detectable difference in the flavors, odor, taste (sweet and sour), and appearance of fruit. Moreover, the overall assessment of the strawberry fruits exposed to the EOs vapors was compared to the control (inoculated to distilled water)²⁹⁾. The strawberry fruits were removed from storage room 60 min prior to evaluation in order to equilibrate to room temperature. The sensory analyzes were performed in a sensor panel room of the Department of Plant protection in University of Kurdistan on three different days at 10:00 am at 24-26°C. Sensory evaluation of the fruit inoculated by EOs was performed on the 10th days

of storage at 4°C by 11 assessors (5 males and 6 females within the age range of 20-30 years). There was a five min interval between testing and water was provided between assessors. All assessors were trained at the beginning of the experiment in order to become familiar with the experimental conditions. The qualitative score was performed on the basis of a 5-point hedonic scale (1, very weak; 2, weak; 3, intermediate; 4, good; and 5, very good) for the sensory evaluation. Then, the data for sensory evaluation were tested for normality. Due to the lack of a normal distribution of the entire data, they were subjected to non-parametric analysis using the Kruskal-Wallis test in SPSS ver. 16.0.

2.14 Statistical analysis

Experiments were designed in a completely randomized design (CRD). Data were statistically analysed by standard analysis of variance (ANOVA) using the SAS software (Version 8.2; SAS Institute, Cary, NC, USA, 2013). Differences among different treatments were measured using Duncan's Multiple Range Tests (DMRT). All data were expressed as mean values $\pm$ standard error (SE). To measure EC₅₀, Probit analysis was performed in the SPSS ver. 16.0. All experiments were performed in duplicate.

3 Results and Discussion

3.1 Chemical composition of the EOs

The chemical composition of the EOs of *A. millefolium*, *F. kuma*, and *M. longifolia* was analyzed by GC-MS (Tables 1, 2, and 3). In the present research, GC-MS analysis of *A. millefolium* EO identified 44 constituents, explaining more than 94.58% of total EOs. The major components of the *A. millefolium* EO were acetaldehyde, (3,3-dimethyl cyclohexylidene), (Z)- (20.152%), eucalyptol (13.384%), and 2-cyclohexen-1-ol, 4-ethyl-1,4-dimethyl- (5.715%). The main components of the *F. kuma* EO in the present study were bicyclo[2.2.1]heptan-2-ol, 1,7,7-trimethyl-, acetate, (1S-endo)- (23.154%), fenchyl acetate (18.993%), 1R-.alpha.-pinene (18.764%), and camphene (13.915%). The Bicyclo[3.1.1]hept-3-en-2-one, 4,6,6-trimethyl (43.412%), cyclohexanone, 5-methyl-2-(1-methylethylidene)- (35.391%), and eucalyptol (14.569%) were identified as the major components of the *M. longifolia* EO by GC-MS analysis.

3.2 Effect of EOs on Hyphal growth and conidial germination

All EOs significantly ($p \leq 0.05$) reduced the mycelial growth (Fig. 1) and conidial germination (Table 4) of *C. nymphaeae* *in vitro* to compare the fungicide of Captan and control in contact and vapor phases. There were significant differences between their effects against *C. nym-*

Table 1 Chemical composition of the EOs of *A. millefolium*.

Number	Compounds	Molecular formula	Area (%)	Confirmed by	CAS #
1	1-Butanol, 3-methyl-, acetate	C ₇ H ₁₄ O ₂	0.229	STD, MS	123-92-2
2	Santolina triene	C ₁₀ H ₁₆	0.169	STD, MS	2153-66-4
3	Tricyclo[2.2.1.0(2,6)]heptane, 1,7,7-trimethyl-	C ₁₀ H ₁₆	0.269	RI, MS	508-32-7
4	1R-.alpha.-Pinene	C ₁₀ H ₁₆	5.114	STD, MS	7785-70-8
5	Camphene	C ₁₀ H ₁₆	3.998	RI, MS	79-92-5
6	Benzaldehyde	C ₇ H ₆ O	0.153	STD, MS	100-52-7
7	Bicyclo[3.1.0]hexane, 4-methylene-1-(1-methylethyl)	C ₁₀ H ₁₆	2.520	STD, MS	387-41-5
8	Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene	C ₁₀ H ₁₆	2.286	RI, MS	18172-67-3
9	Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)-	C ₁₀ H ₁₆	0.234	STD, MS	18172-67-3
10	3,5-Heptadien-2-ol, 2,6-dimethyl-	C ₉ H ₁₆ O	0.144	RI, MS	77411-76-8
11	3,3,6-Trimethyl-1,4-heptadien-6-ol	C ₁₀ H ₁₈ O	0.606	STD, MS	30458-12-9
12	1,3-Cyclohexadiene, 1-methyl-4-(1-methylethyl)	C ₁₀ H ₁₆	1.239	STD, MS	99-86-5
13	Benzene, 1-methyl-2-(1-methylethyl)-	C ₁₀ H ₁₄	2.365	RI, MS	527-84-4
14	Eucalyptol	C ₁₀ H ₁₈ O	13.384	STD, MS	470-82-6
15	Benzaldehyde, 2-hydroxy	C ₇ H ₆ O ₂	0.170	RI, MS	90-02-8
16	1,4-Cyclohexadiene, 1-methyl-4-(1-methylethyl)	C ₁₀ H ₁₆	0.427	STD, MS	99-85-4
17	Terpineol, cis-.beta	C ₁₀ H ₁₈ O	0.519	STD, MS	7299-41-4
18	3,3,6-Trimethyl-1,5-heptadien-4-ol	C ₁₀ H ₁₈ O	1.574	RI, MS	57590-19-9
19	Bicyclo[2.2.1]heptane-2,5-diol, 1,7,7-trimethyl-, (2-endo,5-exo)-	C ₁₀ H ₁₈ O ₂	0.234	STD, MS	10359-41-8
20	Butanoic acid, 2-methyl-, 3-methylbutyl ester	C ₁₀ H ₂₀ O ₂	0.466	RI, MS	27625-35-0
21	Butanoic acid, 2-methyl-, 2-methylbutyl ester	C ₁₀ H ₂₀ O ₂	1.223	STD, MS	2445-78-5
22	2-Cyclohexen-1-ol, 4-ethyl-1,4-dimethyl	C ₁₀ H ₁₈ O	5.715	STD, MS	55162-55-5
23	Acetaldehyde, (3,3-dimethylcyclohexylidene)-, (Z)-	C ₁₀ H ₁₆ O	20.152	RI, MS	26532-24-1
24	2-(2-Isopropenyl-5-methylcyclopentylmethoxy)tetrahydropyran	C ₁₅ H ₂₆ O ₂	0.814	STD, MS	35158-25-9
25	Bicyclo[2.2.1]heptan-2-ol, 1,7,7-trimethyl-, (1S-endo)	C ₁₀ H ₁₈ O	4.690	RI, MS	464-45-9
26	3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)	C ₁₀ H ₁₈ O	1.751	STD, MS	562-74-3
27	3-Cyclohexene-1-methanol, .alpha.,.alpha.4-trimethyl	C ₁₀ H ₁₈ O	4.227	STD, MS	98-55-5
28	2-Cyclohexen-1-ol, 3-methyl-6-(1-methylethyl)-, trans	C ₁₀ H ₁₈ O	2.691	RI, MS	16721-39-4
29	Fenchyl acetate	C ₁₂ H ₂₀ O ₂	0.811	STD, MS	13851-11-1
30	3,6-Octadien-1-ol, 3,7-dimethyl-, (Z)	C ₁₀ H ₁₈ O	1.449	RI, MS	5944-20-7
31	Bicyclo[3.1.1]hept-2-en-4-ol, 2,6,6-trimethyl-, acetate	C ₁₂ H ₁₈ O ₂	0.958	RI, MS	33522-69-9
32	Bicyclo[3.1.1]heptane-2-methanol, 6,6-dimethyl	C ₁₀ H ₁₈ O	1.119	STD, MS	514-99-8
33	Acetic acid, 1,7,7-trimethyl-bicyclo[2.2.1]hept-2-yl ester	C ₂₁ H ₂₀ O ₂	0.193	RI, MS	92618-89-8
34	Phenol, 2-methyl-5-(1-methylethyl)	C ₁₀ H ₁₄ O	0.310	STD, MS	499-75-2
35	2-Cyclohexen-1-ol, 2-methyl-5-(1-methylethenyl)-, acetate, cis	C ₁₂ H ₁₈ O ₂	0.636	RI, MS	1205-42-1
36	Phenol, 2-methoxy-5-(1-propenyl)-, (E)-	C ₁₀ H ₁₂ O ₂	0.188	STD, MS	19784-98-6
37	Bicyclo[3.1.0]hexane, 6-isopropylidene-1-methyl	C ₁₀ H ₁₆	0.545	STD, MS	24524-57-0
38	2,6-Octadien-1-ol, 3,7-dimethyl-, acetate	C ₁₂ H ₂₀ O ₂	0.249	RI, MS	16409-44-2
39	Bicyclo[3.1.1]hept-3-en-2-one, 4,6,6-trimethyl	C ₁₀ H ₁₄ O	0.772	STD, MS	80-57-9
40	2-Isopropylidene-3-methylhexa-3,5-dienal	C ₁₀ H ₁₄ O	0.782	RI, MS	99172-18-6
41	Caryophyllene	C ₁₅ H ₂₄	4.211	STD, MS	87-44-5
42	1H-Cycloprop[e]azulene, decahydro-1,1,7-trimethyl-4-methylene-, [1aR-(1a.alpha.,4a.beta.,7a.alpha.,7a.beta.,7b.alpha.)]-	C ₁₅ H ₂₄	0.048	STD, MS	25246-27-9
43	Caryophyllene oxide	C ₁₅ H ₂₄ O	3.573	RI, MS	1139-30-6
44	beta.-Guaiane	C ₁₅ H ₂₄	1.389	STD, MS	88-84-6
Total	—	—	94.580	—	—

STD: confirmed by injection of standard and by mass spectra library; MS: tentative identification, confirmation only by mass spectra library; RI: confirmed by *n*-alkanes retention index by mass spectra library.

Table 2 Chemical composition of the EOs of *F. kuma*.

Number	Compounds	Molecular formula	Area (%)	Confirmed by	CAS #
1	Tricyclo[2.2.1.0(2,6)]heptane, 1,7,7-trimethyl	C ₁₀ H ₁₆	1.332	STD, MS	508-32-7
2	1R-.alpha.-Pinene	C ₁₀ H ₁₆	18.764	RI, MS	7785-70-8
3	Camphene	C ₁₀ H ₁₆	13.915	STD, MS	79-92-5
4	Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)-	C ₁₀ H ₁₆	3.116	RI, MS	18172-67-3
5	beta.-Myrcene	C ₁₀ H ₁₆	3.319	STD, MS	123-35-3
6	Limonene	C ₁₀ H ₁₆	3.105	STD, MS	138-86-3
7	Cyclohexanol, 1-methyl-4-(1-methylethenyl)-, acetate	C ₁₂ H ₂₀ O ₂	3.214	STD, MS	10198-23-9
8	Bicyclo[3.1.1]hept-3-en-2-ol, 4,6,6-trimethyl-, [1S-(1.alpha.,2.beta.,5.alpha.)]-	C ₁₀ H ₁₆ O	2.210	MS	18881-04-4
9	Fenchyl acetate	C ₁₂ H ₂₀ O ₂	18.993	RI, MS	13851-11-1
10	Bicyclo[2.2.1]heptan-2-ol, 1,7,7-trimethyl-, acetate, (1S-endo)-	C ₁₂ H ₂₀ O ₂	23.154	RI, MS	5655-61-8
11	2,6-Octadiene, 2,6-dimethyl-	C ₁₀ H ₁₈	1.320	STD, MS	2792-39-4
12	Caryophyllene	C ₁₅ H ₂₄	3.758	STD, MS	87-44-5
13	Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-	C ₁₅ H ₂₂	1.240	RI, MS	644-30-4
14	Caryophyllene oxide	C ₁₅ H ₂₄ O	5.775	RI, MS	1139-30-6
Total	—	—	96.55	—	—

STD: confirmed by injection of standard and by mass spectra library; MS: tentative identification, confirmation only by mass spectra library; RI: confirmed by *n*-alkanes retention index by mass spectra library.

Table 3 Chemical composition of the EOs of *M. longifolia*.

Number	Compounds	Molecular formula	Area (%)	Confirmed by	CAS #
1	Eucalyptol	C ₁₀ H ₁₈ O	14.569	STD, MS	470-82-6
2	Cyclohexanone, 5-methyl-2-(1-methylethyl)-	C ₁₀ H ₁₈ O	3.852	STD, MS	10458-14-7
3	Cyclohexanone, 5-methyl-2-(1-methylethylidene)-	C ₁₀ H ₁₆ O	35.391	RI, MS	15932-80-6
4	Bicyclo[3.1.1]hept-3-en-2-one, 4,6,6-trimethyl-	C ₁₀ H ₁₄ O	43.412	STD, MS	80-57-9
5	Caryophyllene oxide	C ₁₅ H ₂₄ O	2.766	RI, MS	1139-30-6
Total	—	—	99.990	—	—

STD: confirmed by injection of standard and by mass spectra library; MS: tentative identification, confirmation only by mass spectra library; RI: confirmed by *n*-alkanes retention index by mass spectra library.

phaeae. The inhibition effect of mycelial growth was positively correlated with the concentration of EOs (Fig. 1). The results showed that EOs of *M. longifolia* (EC₅₀ = 26.08 µL/L) appear to be more toxic than *A. millefolium* (EC₅₀ = 60.31 µL/L), Captan fungicide (EC₅₀ = 62.97 µL/L), and *F. kuma* (EC₅₀ = 65.06 µL/L) against *C. nymphaeae* (Table 5). The EOs of *F. kuma* showed the lowest inhibitory effects against pathogen. Antifungal activity of EOs against different plant pathogens using contact and vapor phase has been reported in previous works^{30–33}. The mycelial growth of *C. musae* causal agent of anthracnose of banana fruit was inhibited by Cinnamon oil and clove oil³⁴. Application of EOs on subtropical fruit such as avocado and mango de-

creased the anthracnose incidence caused by *C. gloeosporioides* on these fruits^{9, 35}.

The results of the *in vitro* experiment indicated that all EOs strongly inhibited the conidial germination of *C. nymphaeae* after 48 h in both methods. In contact and vapor methods, the reduction of germinated conidial of *C. nymphaeae* was 79.2% to 84.6% and 67.2% to 90%, respectively, compared to the control treatment (Table 4). In the control treatment, spores of *C. nymphaeae* germinated and produced the germ tube that gradually developed to long hyphae (data are not shown). In the contact method, there was no significant difference in the treatment of *M. longifolia*, *A. millefolium*, *F. kuma* EOs, and Captan. The

Table 4 *In vitro* conidial germination inhibition of essential oils against *C. nymphaeae* using contact and vapour methods after 7 days.

Treatments	Contact method		Vapour method	
	Number of germinated conidia	Inhibition percent (%)	Number of germinated conidia	Inhibition percent (%)
Control pathogen	193.75 ± 2.28 a	—	187.50 ± 2.39 a	—
<i>A. millefolium</i>	29.75 ± 0.29 b	84.6	18.75 ± 3.16 c	90.0
<i>M. longifolia</i>	36.25 ± 0.94 b	81.3	29.25 ± 2.75 d	84.4
<i>F. kuma</i>	40.25 ± 1.03 b	79.2	49.25 ± 1.25 c	73.7
Captan	34.75 ± 0.71 b	82.1	61.5 ± 1.75 b	67.2

Concentration of *Achillea millefolium*, *Mentha longifolia*, *Ferula kuma* EOs and fungicide was used at 1500, 600, 1850 and 2100 µL/L respectively. Means ± SE in the same column with different letters are significantly different ($p \leq 0.05$) according to Duncan multiple-ranges test (DMRT).

Table 5 EC₅₀ of EOs and fungicide on *C. nymphaeae* (at Confidence limit 95%).

Treatment	EC ₅₀ (µL/L)	a	b ²	X ²	R ²
<i>M. longifolia</i>	26.08	1.83 ± 0.16	− 4.43 ± 0.41	18.51	0.90
<i>A. millefolium</i>	60.31	1.46 ± 0.15	− 4.08 ± 0.42	21.20	0.81
<i>F. kuma</i>	65.06	2.30 ± 0.18	− 6.48 ± 0.52	44.30	0.85
Captan	62.97	2.14 ± 0.17	− 6.01 ± 0.52	4.71	0.98

EC₅₀ = median effective inhibitory concentration; a = Slope; b² = Intercept; X² = Chi-Square; R² = Correlation coefficient.

EOs of *A. millefolium* had the most effect on reduction of conidial germination of the pathogen after 48 h in contact and vapor methods (Table 4). The conidial germination of *C. coccodes* on infected tomato fruits was decreased by Origanum oil treatment *in vitro*³⁶⁾. Inhibition of the mycelial growth and conidial germination of *C. gloeosporioides* causal agent of pepper fruit anthracnose was inhibited by vapor treatment with EOs⁸⁾. Also, the conidial germination and mycelial growth of *B. cinerea* and *Fusarium proliferatum* were inhibited using cinnamon oil³⁷⁾.

3.3 Scanning electron microscopy analysis

The EOs in the culture medium revealed that mycelia of *C. nymphaeae* were different from those of the control treatment. The mycelia morphology of *C. nymphaeae* in control treatment was regular, smooth and uniform. In comparison, the mycelia in treatments by EOs were abnormal due to its thick, irregular swelling. In addition, structures of fungal mycelia appeared depressed and collapsed (Fig. 2). Similarly³⁸⁾, the investigation of the mycelium of *Alternaria alternate* treated with *Syringa ablata* EO, showed considerable morphological alterations. The SEM analysis indicated that mycelium treated with eugenol was shrunken compared to the control³⁹⁾.

3.4 Antifungal activity tests *in vivo* and greenhouse conditions

The efficacy of the EOs in decay rate in strawberry fruits *in vivo* presented in Table 6. The disease index in control treatment after 10 days reached 4.75. The postharvest decay was significantly decreased when strawberry fruit was treated with EOs as compared to the control ($p \leq 0.05$). Among these results, *M. longifolia* EO was most effective with over 98.7% reduction in disease severity (Table 6). Anthracnose, caused by *C. nymphaeae*, is the most aggressive disease of strawberry and limits good fruit quality¹⁾. The results obtained in this work were reflected in other investigations, where it was reported that EOs attributes to the decreased postharvest decay in several fruit such as apricots and plums⁴⁰⁾, strawberries⁴³⁾, bananas¹¹⁾, pomegranates²²⁾ and peppers⁸⁾. The pepper fruit anthracnose was reduced by application of EOs on pepper fruits⁸⁾. Thyme oil reduced postharvest decay in several fruits such as apricots and plums⁴⁰⁾, citrus⁴²⁾, strawberries⁴¹⁾ and oranges⁷⁾. The EOs increased the phenolic compounds in plants and prevents the growth of pathogens as well as declining the severity of fruit decay⁷⁾.

The results of the greenhouse test are reported in Table 6. Disease index was measured after two months. In greenhouse conditions, symptoms of anthracnose on petioles appeared 40 days after inoculation in control pathogen. All EOs and fungicides significantly ($p \leq 0.05$) decreased the

Fig. 2 Morphological changes of *C. nymphaeae* hyphae treated with *A. millefolium* EO (B), *M. longifolia* EO (C) and *F. kuma* EO (D) observed through Scanning electron microscopy. Structures of fungal mycelia treated with EOs appeared depressed and collapsed. Mycelia in control treatment (A) showed a regular, linear and uniform shape.

Table 6 Effect of EOs and fungicide on fruit decay development *in vivo* using vapour method after 7 days and disease severity in greenhouse conditions after 60 days.

Treatments	<i>In vivo</i>		Greenhouse	
	Disease severity	Control efficacy (%)	Disease severity	Control efficacy (%)
Control pathogen	4.75 ± 0.10 a	—	4.33 ± 0.22 a	—
<i>A. millefolium</i>	0.31 ± 0.62 b	93.5	0.75 ± 0.14 bc	82.7
<i>M. longifolia</i>	0.06 ± 0.06 c	98.7	0.33 ± 0.22 cd	92.4
<i>F. kuma</i>	0.40 ± 0.07 b	91.6	1.08 ± 0.83 b	75.1
Captan	1.05 ± 0.06 b	77.9	0.50 ± 0.14 c	88.4

Concentration of *Achillea millefolium*, *Mentha longifolia*, *Ferula kuma* EOs and fungicide was used at 1500, 600, 1850 and 2100 µL/L respectively. Data were based on Mean ± standard error (SE) in the same column with different letters are significantly different ($p \leq 0.05$) according to Duncan multiple-ranges test (DMRT).

disease severity and increased control efficacy compared with the untreated control. Fungicide of Captan reduced the disease severity of 88.4%. EO of *M. longifolia* was the most effective with over 92.4% reduction in disease severity, followed by *A. millefolium* and *F. kuma* with 82.7% and 75.1% reduction, respectively (Table 6). Application of

EOs against soil born pathogen^{15, 43}, foliar fungal disease^{8, 38}, and postharvest disease^{7, 10, 11, 20} was reported by other researchers. The high rate of phenolic compounds including thymol and carvacrol in plant essential oils prevents the growth of a fungal colony of *Alternaria citri* causal agent of orange black rot and reduces the severity of decay⁷.

Table 7 Effect of EOs and fungicide on strawberry fruit quality after 8 days storage period.

Paros cultivar	Treatments	Spray time	D8
Weight loss %	Control	0 ± 0 b	0.41 ± 0.14 b
	<i>A. millefolium</i>		0.43 ± 0.12 b
	<i>M. longifolia</i>		0.41 ± 0.02 b
	<i>F. kuma</i>		0.82 ± 0.23 a
	Captan		0.38 ± 0.10 b
Firmness (N)	Control	4.56 ± 0.49 a	3.20 ± 0.20 c
	<i>A. millefolium</i>		2.99 ± 0.58 d
	<i>M. longifolia</i>		3.92 ± 0.48 bc
	<i>F. kuma</i>		3.25 ± 0.72 c
	Captan		3.25 ± 0.72 c
Vitamin C (mg/100 g FFW)	Control	25.59 ± 2.78 a	21.13 ± 3.01 a
	<i>A. millefolium</i>		22.96 ± 1.41 a
	<i>M. longifolia</i>		24.13 ± 1.11 a
	<i>F. kuma</i>		24.37 ± 0.43 a
	Captan		23.30 ± 1.28 a
Total phenol content (mg/100 g FFW)	Control	156.32 ± 20.27 b	219.96 ± 8.88 a
	<i>A. millefolium</i>		226.12 ± 10.09 a
	<i>M. longifolia</i>		209.30 ± 10.38 a
	<i>F. kuma</i>		239.37 ± 10.52 a
	Captan		215 ± 24.03 a
Antioxidant activity (DPPH %)	Control	76.73 ± 1.83 a	71.04 ± 0.26 ab
	<i>A. millefolium</i>		67.25 ± 2.07 b
	<i>M. longifolia</i>		76.77 ± 0.98 a
	<i>F. kuma</i>		69.42 ± 0.38 b
	Captan		76.04 ± 2.30 a
Peroxidase activity (Unit/g FFW)	Control	0.094 ± 0.06 c	0.60 ± 0.03 ab
	<i>A. millefolium</i>		0.72 ± 0.08 a
	<i>M. longifolia</i>		0.54 ± 0.02 b
	<i>F. kuma</i>		0.65 ± 0.09 ab
	Captan		0.63 ± 0.01 ab

The values are means ± standard error (SE). Data in the same column with different letters represent significant difference ($p \leq 0.01$) according to Duncan multiple-ranges test.

3.5 Fruit quality analyses

The effect of EOs and fungicide on fruit quality is shown in **Table 7**.

3.5.1 Weight loss

The weight loss of strawberry fruit in any of the treatments (except *F. kuma* EOs) was not affected by EOs and increased gradually throughout the storage period. There was no significant difference between the treatments of EOs (except *F. kuma* EOs) and control. EOs of *F. kuma* decreased weight loss (0.82%) than control significantly ($p \leq 0.01$) after 8 days of storage (**Table 7**). The results ob-

tained in this study are in line with those of other works, where weight loss of lettuce was not affected by thyme EO⁴⁴. Thyme oil prevented weight loss of banana and grape berries^{11, 45}. Moreover, it has been shown that a high temperature leads to an increase in the respiration rate of the fruit and weight loss⁴⁶.

3.5.2 Fruit firmness

Fruit firmness is one of the most important quality deterioration characters during storage time, which is caused by the degradation of pectin in the fruit cell wall and hydrolysis of starch to sugar associated with fruit ripening⁴⁷.

The preservation of fruit firmness is an important factor for increasing storage life of fresh products and reductions in fruit firmness lead to the declined quality of fruit⁴⁸⁾. Results of this study indicated that fruit firmness was decreased through storage time in all treatments ($p \leq 0.01$) (Table 7). All fruits treated with the EOs (except *A. millefolium*) were firmer than the control fruit. The highest and the lowest strawberry firmness values were found in *M. longifolia* EO and *A. millefolium* with 3.92 N and 2.99 N, respectively (Table 7). Similar values were reported by Anthony *et al.*¹³⁾, using the effect EOs of *Cymbopogon nardus* and *Ocimum basilicum* on the firmness of banana fruit. Plant EOs reduced the activity of the softening enzymes in the cell wall components including polygalacturonase and galactosidase and preserved the fruit firmness during the storage⁷⁾.

3.5.3 Ascorbic acid

The ascorbic acid (vitamin C) rate was reduced in all treatments after 8 days, but no significant differences ($p \leq 0.01$) were found between the treatments of EOs and control treatment at the first and end of storage period. The vitamin C content in EOs was more than control treatment until the end of the cold storage (Table 7). This study showed that EOs caused maintains vitamin C content in fruit during storage probably due to the antioxidant effects of EOs⁷⁾.

3.5.4 Total phenol content (TPC)

A general increase in TPC was observed for all treatments ($p \leq 0.01$) while no significant differences were found between the treatments and control at end of the storage period (Table 7). The TPC rate in control treatment was 219.96 mg gallic acid per 100 g fruit fresh at the end of storage period. Thus, there were significantly higher levels of TPC in fruit inoculated with EOs compared to spray time after 8 days storage. The most and the least total phenol was found in fruit treated with *F. kuma* (239.37 mg/100 g) and *M. longifolia* (209.30 mg/100 g), respectively, after 8 days of cold storage. The phenol compounds can be found in EOs, which play a very good role in plant health and are an important quality factor in fruits and vegetables⁴⁷⁾. They have a direct relationship at reduction decay severity of fruits and the management of postharvest diseases⁴⁹⁾. Results indicated that phenolic compounds in *Ocimum basilicum* increased the antioxidant activity in plant and then induced the defense system of the plant against plant pathogens⁵⁰⁾.

3.5.5 Antioxidant activity

After 8 days of storage, a DPPH value was decreased in all treatments, except for *M. longifolia* and Captan Fungicide. The DPPH value of strawberry fruits in control treatment at end of storage period was 71.04%. *M. longifolia* EO (76.77%) and Captan (76.04%) gave the higher antioxidant values, respectively, but no significant difference was observed between them and control at first days of the

storage (Table 7). A previous study has shown that EOs possess antioxidant properties⁵¹⁾. It has been well documented that reduction of vitamin C and anthocyanin in fruits through time leads to a decrease in the antioxidant activity in plants⁵²⁾ and, at least, may induce the fruit decay⁵³⁾.

3.5.6 Peroxidase (POD) activity

During the whole storage period, a more rapid increase in POD activity was observed in all treatments. The POD activity was different in the treatments. The POD activities of the strawberry fruits treated by *A. millefolium* EO and *F. kuma* EO were higher compared to the other treatments (Table 7). POD is an important resistance-related enzyme in fruit as a plant defense mechanism and plays an important role during postharvest storage in the protection of fruits against postharvest disease^{54–56)}.

3.6 Sensory evaluation

The sensory evaluation showed that sensory parameters except the taste were not affected by treatments. The greatest and the lowest scores in the taste attribute were 3.09 and 2.09, which belong to *M. longifolia* and *F. kuma* treatment, respectively. The *A. millefolium* and control scores in the taste attributes were 2.90 and 2.63, respectively (data are not shown). *M. longifolia* EO at 600 $\mu\text{L/L}$ was the best treatment in reducing the decay produced by *C. nymphaeae* in strawberry fruit and maintaining the physicochemical parameters during cold storage. It has been reported that use of thyme oil in avocados and organic bananas retained the texture, flavor, overall appearance, and odor of the fruits^{9,11)}. In addition, the study showed that EO did not affect the appearance, flavor and overall acceptability in strawberry fruits⁵⁷⁾.

4 Conclusion

In conclusion, the results of this research showed a positive effect of EOs on reduction of fruit decay during the storage period. In addition, EOs were effective in maintaining the physicochemical and sensory qualities of strawberry fruit. Therefore, these results are an important step toward the goal of using EOs of *Achillea millefolium*, *Ferula kuma*, and *Mentha longifolia* as bio-fungicide to the management of the pathogen *C. nymphaeae* of strawberry. However, further research is needed to confirm the findings and combination of EOs with other methods may be hopeful for integrated control of strawberry anthracnose disease.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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