International Journal of Food Microbiology 145 (2011) 464–470

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Chemical composition and antifungal activity of essential oil from *Cicuta virosa* L. var. *latisecta* Celak

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ARTICLE INFO

Article history:
Received 28 August 2010
Received in revised form 31 December 2010
Accepted 19 January 2011

Keywords:
Essential oil
Cicuta virosa L. var. *latisecta* Celak
Chemical composition
Antifungal
Aflatoxin
Cherry tomato

ABSTRACT

The essential oil extracted from the fruits of *Cicuta virosa* L. var. *latisecta* Celak was tested *in vitro* and *in vivo* against four foodborne fungi, *Aspergillus flavus*, *Aspergillus oryzae*, *Aspergillus niger*, and *Alternaria alternata*. Forty-five different components accounting for 98.4% of the total oil composition were identified by gas chromatography–mass spectrometry. The major components were γ-terpinene (40.92%), *p*-cymene (27.93%), and cumin aldehyde (21.20%). Antifungal activity was tested by the poisoned food technique against the four fungi. Minimum inhibitory concentration against the fungi was 5 μL/mL and percentage inhibition of mycelial growth was determined at day 9. The essential oil had a strong inhibitory effect on spore production and germination in all tested fungi proportional to concentration. The oil exhibited noticeable inhibition on dry mycelium weight and synthesis of aflatoxin B_1 (AFB_1) by *A. flavus*, completely inhibiting AFB_1 production at 4 μL/mL. The effect of the essential oil on inhibition of decay development in cherry tomatoes was tested *in vivo* by exposing inoculated and control fruit to essential oil vapor at a concentration of 200 μL/mL. Results indicated that the essential oil from *C. virosa* var. *latisecta* (CVEO) has potential as a preservative to control food spoilage.

1. Introduction

Fungal contamination of food products is a chronic problem in developing countries and results in a decline in quality and quantity. According to an investigation, pathogenic fungi alone cause a nearly 20% reduction in the yield of major food and cash crops (Agrios, 2000). Fresh fruits and vegetables are highly susceptible to attack by fungi in conditions of high moisture content and high temperature (Boyraz and Ozcan, 2006). Some *Aspergillus* and *Alternaria* species are responsible for many cases of food spoilage. Cherry tomato (*Lycopersicon esculentum*) is an important food crop in China and many other countries. It is susceptible to attack by various microorganisms such as *Aspergillus* and *Alternaria* species because of the warm, humid climate in China. Apart from their potential to cause yield losses and food decay, many species represent a serious risk for consumers because of production of dangerous secondary metabolites. Aflatoxins are a group of extremely hazardous and common mycotoxins, carcinogenic metabolites produced by some species of *Aspergillus*, especially *Aspergillus flavus* and *A. parasiticus*. About 4.5×10^9 people are subjected to uncontrolled amounts of aflatoxin in developing countries (Williams et al., 2004). Aflatoxins are carcinogenic, teratogenic, hepatotoxic, mutagenic, and immunosuppressive, and can inhibit of several metabolic systems (Joseph et al., 2005) causing food safety concerns and economic losses in many food industries.

Because of these concerns, investigators are seeking new sources of materials to control spoilage fungi in food. However, the application of synthetic fungicides has led to a number of environmental and health problems because they are themselves carcinogenic, teratogenic, and highly and acutely toxic with long degradation periods (Lingk, 1991). Accordingly, the public demands more acceptable compounds that are biodegradable and safe to humans, as well as the environment. The antimicrobial properties of plant products have been recognized and used for food preservation and in medicine in China since ancient times. Among the different groups of plant products, essential oils are especially recommended as one of the most promising groups of natural products for the formulation of safer antifungal agents (Varma and Dubey, 2001). Many essential oils are classified as "generally regarded as safe" (GRAS) by the United State Food and Drug Administration (FDA), so are potential targets for developing natural antifungals due to their safety on eukaryotic systems (Tolouee et al., 2010). In recent years, numerous studies have documented the antifungal effects of plant essential oils to control food spoilage fungi *in vitro* and *in vivo* (Shahi et al., 2003; Anthony et al., 2004; Feng and Zheng, 2007; Amiri et al., 2008; Dikbas et al., 2008; Dubey et al., 2008; Tzortzakis, 2007, 2009).

C. virosa var. *latisecta* is mainly distributed in Russia, Japan, and China, particularly in the provinces of Jiangsu and Jilin of China, where the fruit is native to some regions. It has been used as a spice and folk medicine for the treatment of dyspepsia since antiquity (Li et al.,

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doi:10.1016/j.ijfoodmicro.2011.01.023

2010). However, to our knowledge and according to literature survey, there are no reports on the chemical composition of the essential oil from *C. virosa* var. *latisecta* (CVEO), or its antifungal and anti-aflatoxin properties. For this reason, efforts have been made to develop CVEO as a potential source of eco-friendly antifungal agent.

In this study, the chemical composition of essential oil extracted from the fruits of *C. virosa* var. *latisecta* was investigated and the oil evaluated for its effects on mycelial growth, spore production and spore germination in all tested fungi, and mycelium weight and AFB_1 content in *A. flavus*. The potential application of essential oil to control postharvest spoilage on stored cherry tomatoes was also assessed.

2. Materials and methods

2.1. Plant material

The fruit of *C. virosa* var. *latisecta* (Umbelliferae) were harvested from Xinghua, Jiangsu Province, China in May 2009. Identification of plant material was initially made using morphological features and then confirmed by Prof. Youwei Wang at the College of Pharmacy, Wuhan University. A voucher specimen number (no. 593) has been deposited in the herbarium of the Institute of the Traditional Chinese Medicine and Nature Products, College of Pharmacy, Wuhan University.

2.2. Isolation of the essential oil

A total of 200 g of the powdered fruits (800 mesh size) were hydrodistilled for approximately 5 h using a Clavenger-type apparatus. The essential oil yield was 3.9% (v/w). It was dried over anhydrous sodium sulfate. After filtration, it was stored in air-tight sealed glass vials covered with aluminum foil at approximately 4 °C for testing and chemical analysis.

2.3. Gas chromatography–mass spectrometry (GC–MS) analysis

The chemical composition of the essential oil was analyzed using GC–MS. The essential oil (10 μL) was dissolved in acetone (100 μL) and 1 μL of the solution was injected into a GC–MS (QP-2010, Shimadzu Co., Kyoto, Japan). The capillary column was Rtx-5MS (length = 30 m, i.d = 0.25 mm, thickness = 0.25 μm). Helium was used as the carrier gas at a flow rate of 0.94 mL/min. The column inlet pressure was 55.8 kPa. The GC column oven temperature was increased from 60 to 280 °C at a rate of 10 °C/min, with a final hold time of 10 min. Injector and detector temperatures were maintained at 280 °C. EI mode was at 70 eV, while mass spectra were recorded in the 45–450 amu range and ion source-temperature was 200 °C. Essential oil components were quantified by relative percent peak area of TIC from the MS signal and identified by comparing their mass fragmentation pattern with those stored in the spectrometer database using NIST05.LIB and NIST05s.LIB (National Institute of Standards and Technology).

2.4. Fungal strains used

Four filamentous fungal strains, *A. flavus* CCAM 080001, *Aspergillus oryzae* CCAM 080006, *Aspergillus niger* CCAM 080002 and *Alternaria alternata* CCAM 080014 were obtained from the Culture Collection of State Key Laboratory of Agricultural Microbiology (CCAM), China. The fungal strain cultures were maintained on a Potato Dextrose Agar (PDA) slant at 4 °C.

2.5. Antifungal activity assay

Antifungal activity of the essential oil was tested against the four fungal strains reported above following the poisoned food technique (Singh et al., 2008). Aliquots of the essential oil dissolved separately in 0.5 mL of 5% (v/v) Tween-20 were pipetted aseptically onto glass Petri dishes (9 cm × 1.5 cm) containing 9.5 mL PDA medium at a temperature of 45–50 °C to produce concentrations of 1, 2, 3, 4, and 5 μL/mL. Control plates (without essential oil) were inoculated following the same procedure. A fungal disc (9 mm in diameter) of mycelium, cut from the periphery of a five-day-old culture using a cork borer, was inoculated aseptically into the center of each Petri dish. The plates were sealed with polyethylene film and incubated at a temperature of 28 ± 2 °C. The efficacy of the treatment was evaluated daily for nine days by measuring the average of two perpendicular diameters of each colony. All tests were performed in triplicate. The percentage inhibition of the radial growth of the four tested fungi by the oils, compared with the control, was calculated at day 9, using the following formula (Albuquerque et al., 2006):

$$\text{Percentage mycelial inhibition} = [(dc - dt)/dc] \times 100$$

where *dc* is the mean colony diameter for the control sets and *dt* is the mean colony diameter for the treatment sets. The lowest concentration that completely inhibited the growth of the fungus was considered the minimum inhibitory concentration (MIC).

2.6. Spore production and spore germination assay

Fungal spore production and spore germination were tested using the modified method of Tzortzakis and Economakis (2007). Spores from colonies incubated for 6 to 10 days (until spore formation) of *A. niger*, *A. flavus*, *A. oryzae*, and *A. alternata* previously exposed to CVEO enrichment (1, 2, 3, and 4 μL/mL), were harvested by adding 5 mL sterile water containing 0.1% (v/v) Tween-20 to each Petri dish and gently scraping the mycelial surface three times with a sterile L-shaped spreader to free spores. The suspension was collected and then centrifuged at room temperature at 2000 × *g* for 5 min. The supernatant was discarded and the remainder was recentrifuged until 1 mL of the highly concentrated spore solution remained. Spore concentration was estimated using a haemocytometer slide (depth 0.1 mm, 1/400 mm^2) under a light microscope (Olympus IX51, Tokyo, Japan).

Spores from 6 to 10-day cultures of *A. niger*, *A. flavus*, *A. oryzae*, and *A. alternata*, previously exposed to CVEO enrichment (1, 2, 3, and 4 μL/mL), were collected as described above. Control (requisite amount of sterilized distilled water in place of oil) was subjected to the same procedure. Spore suspensions were inoculated into fresh PDA medium in depression slides with an inoculating loop. Depression slides containing the spores were assembled with the cover slip and then incubated in a moisture chamber at 28 °C for 20 h in six replicates. For each treatment, 100 spores were examined and the extent of spore germination assessed by looking for germ tube emergence. The number of spores germinated was scored and reported as a percentage of spore germination.

2.7. A. flavus growth and analysis of aflatoxin B_1

The anti-aflatoxigenic efficacy of CVEO on *A. flavus* was studied following Kumar et al. (2007). A spore suspension (100 μL) of *A. flavus* containing 10^7 spores/mL prepared in 0.1% (v/v) Tween-20 was added to 20 mL Potato Dextrose Broth (PDB) medium in an Erlenmeyer flask. The requisite amounts of CVEO dissolved in 5% (v/v) Tween-20 were transferred to PDB medium to produce 1, 2, 3, 4, and 5 μL/mL concentrations. The control sets contained the medium without oil. The flasks were incubated at 28 ± 2 °C for 10 days. Three replicates of each treatment were performed, and the experiment was repeated three times. After incubation, the mycelia produced in liquid cultures were filtered and washed. The dry weight of each mycelium was determined after drying at 60 °C for 24 h.

AFB_1 in the filtrate was extracted twice with 25 mL chloroform in a separating funnel. The chloroform extracts were combined, evaporated to dryness, and the residue redissolved in chloroform up to 1 mL in a volumetric flask. Silica gel-G thin layer plate was used for analysis of the AFB_1. Fifty microliters of each sample spotted onto the TLC sheets was developed in the solvent system comprised of toluene: isoamyl alcohol: methanol (90:32:2 v/v/v) (Reddy et al., 1970). The identity of AFB_1 was detected under UV lamp at 365 nm and confirmed chemically by spraying trifluoroacetic acid (Bankole et al., 2005). For the quantification of AFB_1, amethyst fluorescent spots of AFB_1 on the TLC were scraped out and dissolved in 5 mL cold methanol then centrifuged at 2000×*g* for 5 min. The absorbance of the supernatant was made using a UV–visible spectrophotometer (UV-1240, Shimadzu, Japan) at 360 nm wavelength. The amount of AFB_1 present in the sample was calculated according to the formula by Sinha et al. (1993):

$$AFB_1 \text{ content } (\mu g / mL) = (D \times M) / (E \times l) \times 1000$$

where D is the absorbance, M is the molecular weight of aflatoxin (312), E is the molar extinction coefficient (21, 800), and l is the path length (1 cm cell was used).

In addition, AFB_1 inhibition was calculated as follows:

$$\text{Inhibition } (\%) = (1-X / Y) \times 100,$$

where X is the mean concentration of AFB_1 in the treatment and Y is the mean concentration of AFB_1 in the control.

2.8. Effect of CVEO in the conservation of cherry tomatoes

To assess the potential of CVEO in the control of fungal decay of cherry tomatoes caused by food spoilage fungi, the fruits were treated with CVEO following the modified technique of Feng and Zheng (2007) and Tzortzakis et al. (2009).

2.8.1. Antifungal effect of CVEO on wound-inoculated cherry tomatoes

Selected mature and healthy fresh fruits collected from Hubei Province, China were divided into three replicates (12 fruits per replicate). The fruits were washed in running water, dipped in 70% ethanol for 2 min, and then washed twice with double distilled water (5 min each). Surface-sterilized fruits were wounded with a sterilized cork borer to produce uniform wounds (4 mm diameter, 2 mm deep). Each fruit was separately inoculated with fungi by placing 10 μL of a spore suspension containing 1×10^6 spores/mL of *A. niger*, *A. flavus*, *A. oryzae*, and *A. alternata*. Three independent replicate batches of fruits inoculated with the same fungi were placed into 0.9 L polystyrene containers with snap-on lids. CVEO dissolved separately in 0.5 mL of 5% (v/v) Tween-20 were pipetted aseptically onto filter paper discs (DX102, Xinhua Paper Co., Ltd., Hangzhou, China) of 4 cm diameter respectively placed into individual weighing bottles (φ 40×25 mm) without lids to produce the requisite concentration of 200, 100, and 50 μL/mL. Filter paper moistened with 0.5 mL sterilized water was placed into each container, maintaining high relative humidity (90%–95%) during the storage period. The essential oil was vaporized inside the containers spontaneously at 18 °C. Controls were prepared similarly with the exception of the volatile treatment. All the containers were then transferred to storage at 18 °C for 9 days. The percentage of infected fruits was recorded after 9 days of incubation. All treatments consisted of 3 replicates with 12 fruits per replicate. The entire experiment was repeated twice.

2.8.2. Antifungal effect of CVEO on healthy cherry tomatoes

A random selection of cherry tomatoes without any treatment were divided into three replicates (12 fruits per replicate) and then placed into 0.9 L polystyrene containers with snap-on lids. Four different concentrations (200, 150, 100, and 50 μL/mL) of CVEO solution and sterilized water were prepared as described above. The percentage of infected fruits was recorded after 21 days at 18 °C. All treatments consisted of 3 replicates with 12 fruits per replicate. The entire experiment was repeated twice.

2.9. Statistical analysis

All data are reported as means ± standard deviations. The significant differences between mean values were determined by Duncan's Multiple Range test ($p<0.05$), following one-way ANOVA. The statistical analysis was performed using statistical software (SPSS, 13.0; Chicago, USA).

3. Results

3.1. Chemical composition of essential oil

A total of 45 different components of the essential oil, accounting for 98.4% of the total oil composition, were identified by GC–MS analyses. The identified chemical composition, retention time, and percentage composition are given in Table 1. The oil mainly contained

Table 1
Chemical composition of the essential oil isolated by hydrodistillation from *Cicuta virosa* L. var. *latisecta* Celak.

Number	Retention time (min)	Compound	Composition (%)
1	3.92	α-Phellandrene	0.48
2	4.03	α-Pinene	0.13
3	4.23	Camphene	0.01
4	4.51	Sabinene	0.74
5	4.58	β-Pinene	0.41
6	4.67	β-Myrcene	0.97
7	4.73	2,3-dehydro-1,8-cineole	0.08
8	4.85	Octanal	0.01
9	4.91	α-Thujene	0.02
10	5.00	3-Carene	0.08
11	5.08	α-Terpinene	0.62
12	5.18	p-Cymene	27.93
13	5.27	β-Phellandrene	0.66
14	5.30	Eucalyptol	0.32
15	5.46	β-*cis*-Ocimene	0.01
16	5.65	γ-Terpinene	40.92
17	6.09	Terpinolene	0.09
18	6.24	4-Isopropyl-1-methyl-2-cyclohexen-1-ol	0.06
19	6.43	1,3,8-p-Menthatriene	0.02
20	6.83	Carveol	0.04
21	6.99	Thujol	0.03
22	7.18	*trans*-2-Caren-4-ol	0.07
23	7.22	(2E)-1-Cyclohexyl-2-buten-1-ol	0.07
24	7.39	4-Terpineol	0.20
25	7.48	p-Cymen-8-ol	0.04
26	7.61	Phellandral	0.09
27	8.00	Verbenol	0.23
28	8.13	Thymol methyl ether	0.07
29	8.28	Cumin aldehyde	21.20
30	8.45	Methyl citronellate	0.09
31	8.62	cis-p-Menth-2-en-7-ol	0.18
32	8.83	Isothymol	0.07
33	8.91	Safranal	1.88
34	9.07	Thymol	0.23
35	9.51	Myrtenol	0.03
36	9.71	Citronellyl acetate	0.05
37	9.81	2,6-dimethyl-1,3,5,7-octatetraene	0.03
38	10.12	Geranyl acetate	0.03
39	10.47	Ocimene	0.02
40	10.69	p-Menth-1-en-9-ol	0.02
41	11.57	(Z,E)-α-Farnesene	0.05
42	12.54	Citronellyl propionate	0.02
43	12.94	Neryl propionate	0.01
44	13.48	α-Santalol	0.02
45	13.98	1-Phenylundecane	0.02

a complex mixture of monoterpene hydrocarbons (73.09%) and oxygenated monoterpenes (24.84%). The most abundant components of the essential oil were γ-terpinene (40.92%), *p*-cymene (27.93%), and cumin aldehyde (21.20%). Several other components such as safranal (1.88%), β-myrcene (0.97%), sabinene (0.74%), β-phellandrene (0.66%), α-terpinene (0.62%), α-phellandrene (0.48%), β-pinene (0.41%), eucalyptol (0.32%), verbenol (0.23%), and thymol (0.23%) were in less amounts. However, oxygenated sesquiterpenes, sesquiterpene hydrocarbons, esters, aldehydes, hydrocarbons, and others were also found as trace or minor components.

3.2. Antifungal activity assay

The growth of the four fungal species over the nine days is shown in Fig. 1. The results showed that growth increased with incubation time but mycelial growth was considerably reduced with increasing concentration of CVEO. Growth was delayed by five days for *A. oryzae* and *A. niger*, six days for *A. flavus*, seven days for *A. alternata* at 4 μL/mL concentration. A MIC of 5 μL/mL was obtained after nine days of incubation. The percentage inhibition of mycelial growth was determined at day 9. The oil produced a significant reduction in mycelial growth with the four fungi species at 1, 2, 3, and 4 μL/mL concentrations with percentage reduction ranges of 19.9%–31.2%, 35.8%–43.4%, 60.0%–65.7%, and 70.6%–87.7%, respectively.

3.3. Spore production and spore germination assay

The effects of the essential oil on spore production of each of the tested fungi are shown in Fig. 2. Results showed that spore production was significantly inhibited by the different concentrations of essential oil, with spore production reduced by 23.2% for *A. flavus*, 39.2% for *A. oryzae*, 38.9% for *A. niger*, and 45.6% for *A. alternata* at 1 μL/mL concentration. At 3 and 4 μL/mL concentrations the essential oil showed a strong inhibitory effect, reducing spore production to less than 10%, except in *A. flavus*.

Fig. 3 shows the effects of the essential oil on the percentage of spore germination. As the essential oil concentration increased, a reduction in the percentage of spore germination was observed. The spores of the control all germinated after 20 h incubation at 28 °C in PDA medium. The most pronounced reduction in spore germination for all the tested fungi was at the highest essential oil concentration (4 μL/mL): 79.7% for *A. flavus*, 88.2% for *A. oryzae*, 91.3% for *A. niger*, and 84.0% for *A. alternata*.

3.4. Efficacy of the essential oil on dry mycelium weight and aflatoxin B_1 content

The efficacy of the essential oil on *A. flavus* aflatoxin production and dry mycelium weight in PDB medium are in Table 2. The five different concentrations of essential oil caused different degrees of

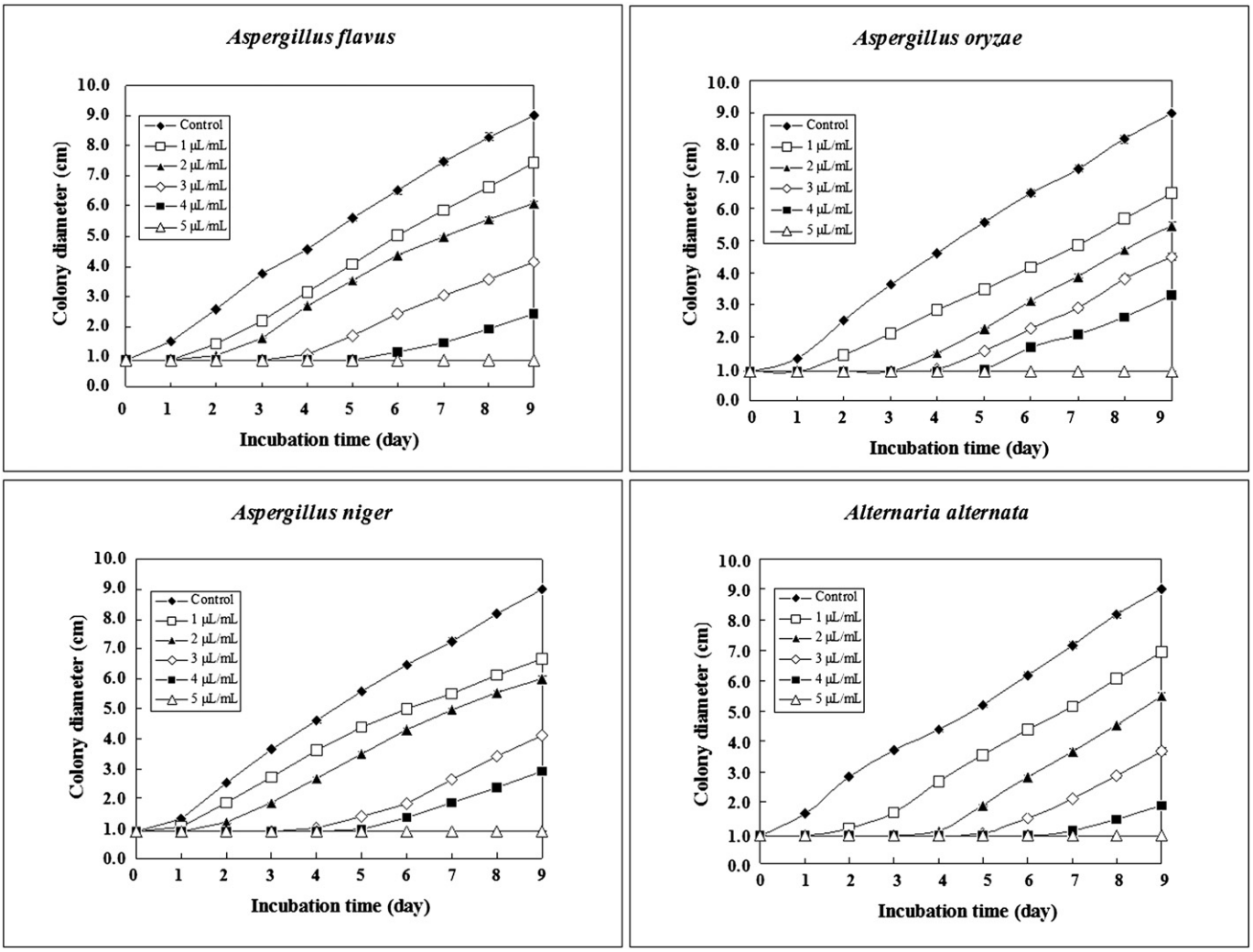

Fig. 1. Effects of the different concentrations of CVEO on colony diameter (cm) growth of *Aspergillus flavus*, *Aspergillus oryzae*, *Aspergillus niger*, and *Alternaria alternata* raised in PDA. Plates were incubated at a temperature of 28 ± 2 °C for nine days. Values are means (n = 3) ± standard deviations.

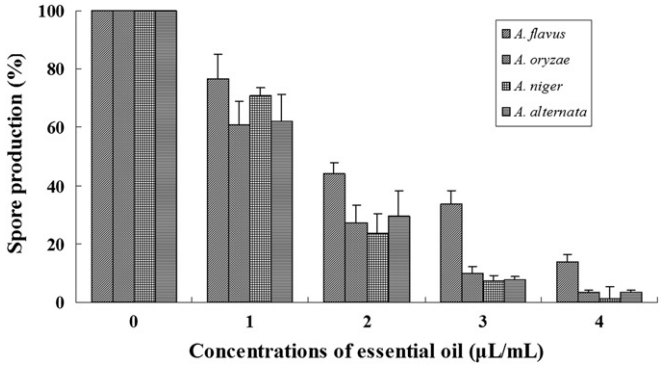

Fig. 2. Effects of different concentrations of CVEO on the spore production of four food spoilage fungi. Values are means (n = 6) ± standard deviations.

Table 2
Efficacy of the different concentrations of CVEO on dry mycelium weight and aflatoxin B_1 synthesis by *A. flavus*.

Oil concentration (μL/mL)	DMW (mg)	AFB_1 (μg/mL)	Inhibition of AFB_1 synthesis (%)
Control	395.8 ± 6.8^a	353.4 ± 12.0^a	0.0
1	292.2 ± 4.0^b	240.5 ± 6.9^b	31.9
2	271.1 ± 4.2^c	162.0 ± 12.0^c	54.2
3	137.1 ± 3.2^d	49.1 ± 6.9^d	86.1
4	53.3 ± 1.1^e	0.0 ± 0.0^e	100.0
5	0.0 ± 0.0^f	0.0 ± 0.0^e	100.0

DMW = Dry mycelium weight (mg); AFB_1 = Aflatoxin B_1 content (μg/mL).
Values are mean (n = 3) ± standard deviations.
Values followed by the same letter in each column are not significantly different in ANOVA and Duncan Multiple Range Test ($p<0.01$).

inhibition in terms of dry mycelium weight and AFB_1 synthesis ($p<0.01$). The oil completely inhibited mycelial production at 5 μL/mL. However, mycelial growth was observed at 4 μL/mL though AFB_1 production was completely inhibited. The AFB_1 content was reduced to about half that of the control at 2 μL/mL.

3.5. Effect of CVEO in the conservation of cherry tomatoes

3.5.1. Antifungal effect of CVEO on wound-inoculated cherry tomatoes

The effects of CVEO vapor on fungal infection development in wound-inoculated cherry tomatoes are shown in Fig. 4. The percentages of decayed cherry tomatoes were significantly ($p<0.01$) reduced in all three treatment groups compared with the control groups and also significantly ($p<0.01$) reduced with increasing concentration of CVEO. Almost all the cherry tomatoes were spoiled the absence of essential oil. CVEO showed the lowest percentages of decayed cherry tomatoes for all fungi compared with the control at 200 μL/mL with values of 11.1% for *A. flavus*, 11.1% for *A. oryzae*, 8.6% for *A. niger*, and 2.8% for *A. alternata*.

3.5.2. Antifungal effect of CVEO on healthy cherry tomatoes

The results obtained using essential oil in unwounded cherry tomatoes are shown in Fig. 5. The results indicated the percentage of infected fruits is significantly ($p<0.01$) reduced by essential oil at 18 °C for 21 days. In this case, the oil at 200 μL/mL concentration showed the highest inhibition of fungal infection with a value of 72.2% compared with the control.

4. Discussion

In recent years, consumer demand for effective, safe natural products to control food spoilage without chemical residues has increased. Essential oils, aromatic volatile products of plant secondary metabolism, have formed the basis of many applications in food flavoring and preservation industries (Rahman and Kang, 2009; Tsigarida et al., 2009). *C. virosa* var. *latisecta*, named *shiluozi* in Chinese, incorrectly treated locally as dill, is completely different from dill. Though belonging to genus *Cicuta*, it is quite different from some highly poisonous plant such as water hemlock (*Cicuta virosa*). Some poisonous compounds belonging to the class of conjugated polyacetylenes such as cicutoxin, isocicutoxin and virol have been isolated from genus *Cicuta* (Anet et al., 1953; Uwai et al., 2000). However, *C. virosa* var. *latisecta* is a popular spice plant in China with a long history (Li et al., 2010), and it is the only plant in genus *Cicuta* which can be used as a spice. The main components of CVEO are three stable compounds, γ-terpinene, *p*-cymene, and cumin aldehyde, which are very similar to those of cumin (*Cuminum cyminum*) oil (Viuda-Martos et al., 2007). It has a similar odor to cumin which is one of the commonly used spices in food preparations around the world. To the best of our knowledge, there is no report of poisonous compound being isolated from *C. virosa* var. *latisecta*. In particular, there were no poisonous compounds detected in CVEO by GC–MS analysis (Table 1). Furthermore, the yield of essential oil is 3.9% (v/w), which is relatively high. Thus, CVEO is suitable for development into a food preservative.

Most chemical components of essential oils are terpenoids, including monoterpenes, sesquiterpenes, and their oxygenated derivatives. The active antimicrobial compounds of essential oils also are generally terpenes. The mechanism of action of this class of compounds is not fully understood, but it is speculated to involve membrane disruption by these lipophilic compounds (Cowan, 1999). However, it would seem possible that the antimicrobial or antifungal mode of action of essential oils may be due to other compounds (Bajpai et al., 2008). The low-molecular weight, highly lipophilic compounds of essential oils easily pass through cell membranes to

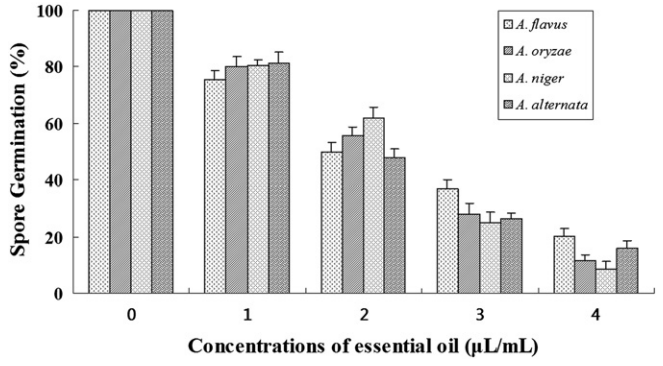

Fig. 3. Effects of the different concentrations of CVEO on the spore germination of the four food spoilage fungi. Values are means (n = 6) ± standard deviations.

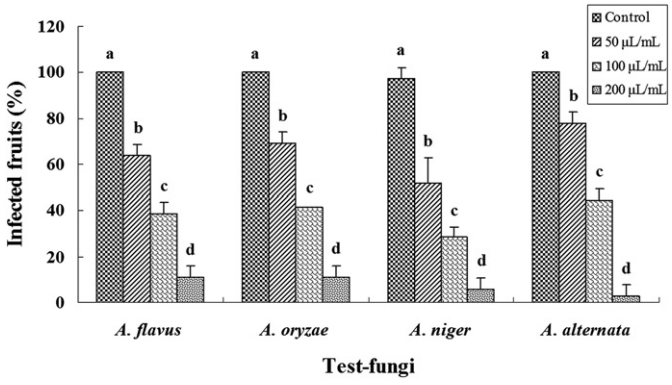

Fig. 4. Efficacy of CVEO on fungal development in wound-inoculated cherry tomatoes (significant difference at $p<0.01$, ANOVA test). Values are means (n = 3) ± standard deviations.

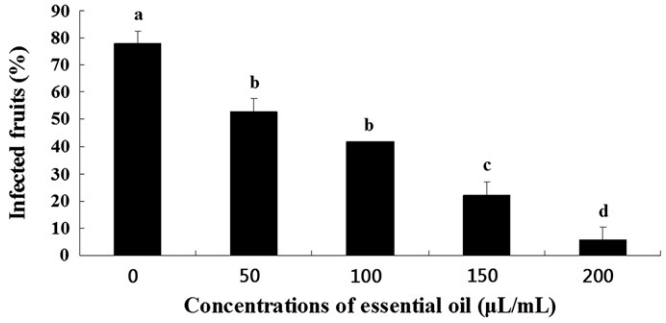

Fig. 5. Efficacy of CVEO on fungal development in unwounded cherry tomatoes. Significant differences ($p<0.01$) between means are indicated by letters above histogram bars. Values are means ($n=3$) ± standard deviations.

induce biological responses (Chao et al., 2005). CVEO exhibited antifungal activities which might be attributed to the presence of γ-terpinene, *p*-cymene, and cumin aldehyde. These three compounds have been shown to possess strong antifungal properties (Singh and Upadyay, 1991; Filipowicz et al., 2003; Terzi et al., 2007). However, whole essential oil has greater antifungal activity, which may be attributed to some minor components that have a synergistic effect with the major components.

In our study, CVEO showed pronounced antifungal efficacy against all tested fungi. Mycelium growth was reduced with increasing concentrations of the oil. However, for *A. alternata*, at 4 μL/mL, fungal colonies began to develop seven days after inoculation whereas the other test fungi started growing earlier than seven days, indicating that the hyphae of *A. alternata* may be more sensitive to CVEO than the other tested fungi.

The essential oil was also effective in inhibition of sporulation and germination of food spoilage fungi. The inhibitory effect of the essential oil on sporulation of different fungi has been previously reported (Kuate et al., 2006; Pawar and Thaker, 2006; Mahanta et al., 2007; Tzortzakis and Economakis, 2007; Gandomi et al., 2009). As shown in Fig. 2, the percentage of spore production of *A. flavus* was visibly higher than that of other tested fungi, which is attributed to greater resistance than the other fungi to the essential oil. Moreover, the absolute number of spores produced differed greatly among all tested fungi. In this study, *A. alternata* produced 3×10^6 spore/mL without oil treatment; for *A. niger*, however, spore production was up to 1.9×10^8 spore/mL without oil treatment (data not presented). The mycelia were completely inhibited at 5 μL/mL, which resulted in the failure of spore production. The effects of essential oil on sporulation might reflect the effects of volatile components produced by oil on surface mycelial development and/or the perception/transduction of signals involved in the switch from vegetative to reproductive development (Tzortzakis and Economakis, 2007). In addition, the partial inhibition of spore production could be attributed to mycelial destruction or inhibition of fungal growth (Tataoui-Elaraki et al., 1993). This study also describes the effect of essential oil on fungal spore germination. As the essential oil concentration increased, a visible reduction in the percentage of spore germination was observed in the test fungi. Sharma and Tripathi (2006) found that the *Citrus sinensis* oil is extremely toxic to spore germination; completely inhibiting spore germination in *A. niger* and *A. alternata* at 400 ppm. Yenjit et al. (2010) found that fernenol, arundoin, and the mixture of stigmasterol and β-sitosterol greatly inhibit spore germination and germ tube elongation in *Colletotrichum gloeosporioides* with EC_{50} values of 45.8, 62.3, and 86.9 mg/L. However, Al-Burtamani et al. (2005) reported that the *Haplophyllum tuberculatum* oil also affects the mycelial growth of *A. alternata*, *Curvularia lunata*, and *Fusarium oxysporium* in a dose-dependent manner but has no effect on the germination of their spores.

A. flavus can produce AFB_1, so we examined the effect of CVEO on AFB_1 production. Our results showed that CVEO can effectively reduce dry mycelium weight and inhibit the synthesis of AFB_1 in *A. flavus*. The essential oil exhibited anti-aflatoxigenic properties at concentrations lower than its fungitoxic concentration, and similar types of results were also reported by Shukla et al. (2009) and Rasooli et al. (2008). Some studies have shown that there is a direct correlation between fungal growth and AFB_1 production (Kumar et al., 2008, 2010). However, the inhibition of AFB_1 production cannot be completely attributed to reduced fungal growth, but was attributed to the inhibition of carbohydrate catabolism in *A. flavus* by acting on some key enzymes, thus reducing its ability to produce AFB_1 (Tatsadjieu et al., 2009). The mechanism of the inhibition of AFB_1 production is not clear. CVEO may interfere with some steps in the metabolic pathways which control the biosynthesis of AFB_1 in *A. flavus*. Determining the mechanism of AFB_1 suppression requires further investigations on CVEO.

In vitro studies on CVEO indicate their potential as an ideal antifungal agent against food spoilage fungi. Thus, further investigation into its efficacy as a botanical pesticide for the control of rotting in fruits *in vivo* is necessary. Results clearly demonstrated that CVEO significantly reduced decay both in artificially inoculated cherry tomatoes and in unwounded cherry tomatoes. *In vivo* experiments showed that exposure to vapors of the essential oil may lower resistance to fungal infections (Tzortzakis, 2009). However, higher concentrations of plant essential oils are required in foods than in laboratory media (Farbood et al., 1976), possibly because nutritional and moisture conditions in food that are better than in laboratory media making microbial growth easier. The fungal inhibition observed under vapor treatments may be a result of hydroxyl groups in antimicrobial compounds forming hydrogen bonds with active enzymes resulting in deactivation (Juglal et al., 2002). Generally, whole essential oils have greater antifungal activity due to a synergistic effect of some of the active components; thus, whole essential oils are more promising in commercial application than single compounds. Moreover, the use of vapor treatments is ideal for controlling food spoilage because it leaves no residual essential oils. Thus, essential oils have potential as food preservatives that may enhance shelf life.

In conclusion, the results of *in vitro* and *in vivo* studies indicate that using CVEO as a fumigant during routine storage or extended transport is very promising. The use of essential oils can improve food safety by eliminating fungal spread, and they also leave no detectable residues after storage. Hence, CVEO would be economical in application, with considerable commercial significance and worthy of further investigation when used as fumigant in storage containers. In addition, some further experiments are required to standardize organoleptic characteristics, such as fruit color, aroma, or firmness with CVEO.

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