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Zanthoxylum molle Rehd. essential oil as a potential natural preservative in management of *Aspergillus flavus*

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ABSTRACT

Essential oils are attracting attention owing to their potential as natural antimicrobial agents, and therefore, they find many applications in the food and pharmaceutical industries. This investigation aimed to evaluate the antifungal activity of *Zanthoxylum molle* essential oil (ZMEO) against *Aspergillus flavus*, a food contaminant. Gas chromatography–mass spectrometry detected 51 compounds representing 95.72% of the ZMEO. 2-Undecanone (26.81%), limonene (20.12%), and terpinen-4-ol (13.09%) were the main components. The mycelial growth of *A. flavus* was totally inhibited at concentrations of 8 μL/mL and 1 μL/mL ZMEO in the air at contact and vapor conditions, respectively. The ZMEO also had an inhibitory effect on spore germination in *A. flavus* proportional to its concentration. Moreover, the ZMEO presented evident inhibition on dry mycelium weight and synthesis of aflatoxin B_1 (AFB_1) by *A. flavus*, completely restraining AFB_1 production at 6 μL/mL. This study explores the possible mechanism of the ZMEO against *A. flavus* by transmission electron microscopy based on changes in the cell ultrastructure. Based on the promising antifungal activity of ZMEO, the effect of the oil on resistance to decay development in cherry tomatoes (*Lycopersicon esculentum*) was studied *in vivo* by exposing inoculated and control fruit groups to essential oil vapor at 0.4 μL/mL air concentration. The results showed that ZMEO can potentially be used as an antifungal agent for preservation purposes in the food and pharmaceutical industries.

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1. Introduction

The fungal contamination of foodstuffs reduces their shelf life and causes various safety issues in the food and agriculture industries worldwide. Foodstuffs are susceptible to contamination by various microorganisms such as the *Aspergillus* species, leading to severe economic losses (Cotty and Jaime-Garcia, 2007; Tian et al., 2012a). *Aspergillus flavus* is the most common species mainly responsible for spoilage and the production of aflatoxins, a group of extremely hazardous and common secondary metabolites. Aflatoxin is the most toxic form for mammals and presents carcinogenic, teratogenic, hepatotoxic, mutagenic, and immunosuppressive properties and can inhibit several aspects of the metabolic system (Joseph et al., 2005). The cherry tomato is one of the important crops in the world, but it is generally susceptible to various pathogens such as *A. flavus* due to the warm and humid climate in which it grows (Tian et al., 2011a). There is a need for safe and effective methods to control contamination of microorganisms in cherry tomato.

Over the last few decades, various synthetic chemicals have been used to avoid post-harvest losses of foodstuffs (Kumar et al., 2013). However, the increasing development of resistance, presence of chemical residues in the food chain, as well as limitations in the use of synthetic fungicides have led to an urgent need for safer alternative natural preservatives to replace synthetic chemicals (Zuzarte et al., 2013). The antimicrobial properties of plant products have been well known and used for food preservation and in medicine for centuries (Tiwari et al., 2009). Plant products, especially essential oils, have been used for their aroma and flavor, and they are recommended as one of the most promising groups of

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natural products (Cava-Roda et al., 2012). Essential oils, which are volatile in nature, are advantageous as botanical fumigants against the decay of stored foodstuffs from molds and mycotoxin contamination (Mishra et al., 2012).

Zanthoxylum molle Rehd., an endemic plant in China, belongs to the Rutaceae family. It is mainly grown in the provinces of Hubei, Anhui, Zhejiang, Jiangxi, Hunan, and Guizhou. It is locally named *Duohuajiao* in Chinese and traditionally used for medicinal purposes for gastritis, abdominal pain, ascariasis, eczema, and toothache (Chinese Herbal Medicine Compilation Group, 1975). To date, to our knowledge and according to literature survey, this is the first report on the chemical composition, antifungal, and anti-aflatoxin properties of the *Z. molle* essential oil (ZMEO).

In this study, the chemical composition of the ZMEO was investigated and evaluated for its effects on the mycelial growth, spore germination, mycelium weight, and aflatoxin B_1 (AFB_1) content in *A. flavus*. The possible modes of action of ZMEO against *A. flavus* were also investigated using transmission electron microscopy (TEM). Moreover, the practical applicability of the ZMEO to control postharvest spoilage on stored cherry tomatoes was also assessed. This investigation explores the potential use of ZMEO as an antifungal agent for preservation purposes in the food and pharmaceutical industries.

2. Material and methods

2.1. Plant material

The leaves of *Z. molle* plants were harvested from the Wuhan Botanical Garden, in the Hubei Province, in China, in July 2012. The identification of the plant material was initially performed based on its morphological features and was finally confirmed at the School of Pharmaceutical Sciences in Wuhan University. A voucher specimen number (No. 709) has been deposited in the herbarium of Institute of Traditional Chinese Medicine and Natural Products, School of Pharmaceutical Sciences, Wuhan University.

2.2. Extraction of ZMEO

Air dried leaves (500 g) were ground using a mill, passed through a mesh screen to obtain a uniform 40-mesh size, and submitted to hydrodistillation for approximately 5 h using a Clevenger-type apparatus. The ZMEO yield was 1.2% (v/w). It was dehydrated over anhydrous sodium sulfate and stored in airtight sealed glass vials for further testing covered with aluminum foil at approximately 4 °C.

2.3. Gas chromatography–mass spectrometry analysis of ZMEO

The chemical composition of the ZMEO was analyzed using gas chromatography–mass spectrometry (GC–MS) (QP-2010, Shimadzu Co., Kyoto, Japan). The amount injected was 1.0 μL in split mode (50:1). 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 1.00 mL/min. 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. The injector and detector temperatures were maintained at 280 °C. The EI mode was at 70 eV, while mass spectra were recorded from 45 to 400 amus with the ion source-temperature set to 200 °C. The relative percentage of oil constituents was expressed as percentages obtained by peak area normalization. The identification of the compounds was performed by comparing their retention indices based on a homologous series of *n*-alkane indices on the Rtx-5MS capillary column, referring to known compounds from the literature (Adams, 2007) and by comparing their mass spectra with those stored in the spectrometer database using NIST05.LIB and NIST05s.LIB (National Institute of Standards and Technology).

2.4. Fungal strains and culture condition

A. flavus CCAM 080001, isolated from stored cherry tomatoes was provided by 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. The old cultures were transferred to fresh slant every two months to avoid a decline in strain viability.

2.5. Effect of ZMEO on mycelial growth of A. flavus

The antifungal activity of ZMEO was evaluated to assess its contact and vapor phase effects on the mycelial growth of *A. flavus* with some modifications (Lopez et al., 2005; Soylu et al., 2006). For the determination of the contact phase effect of ZMEO, which inhibits hyphal growth, aliquots of the ZMEO dissolved separately in 0.5 mL of 5% (v/v) Tween-20 were pipetted aseptically onto glass Petri dishes (9 × 1.5 cm) containing 9.5 mL PDA medium at a temperature of 45–50 °C to procure the required concentrations of 1, 2, 4, 6, and 8 μL/mL. Agar discs (9 mm diameter) from the edge of a 5-day old *A. flavus* culture were placed at the center of each Petri plate, which was sealed with parafilm. The control plates (without ZMEO) were inoculated following the same procedure. Then, they were incubated at 28 ± 2 °C.

In the vapor phase, 20 mL PDA was pipetted aseptically onto glass Petri dishes (9 × 1.5 cm, with 80 mL air space after addition of 20 mL of PDA media), and one disk (9 mm) from the edge of a 5-day old *A. flavus* culture was placed on PDA in each plate. The Petri plates were inverted and essential oils with 1 mL difference in concentrations [10, 20, 40, 60 and 80 μL/mL; each pure essential oil was diluted in Tween-20 (5%, v/v)] were prepared to obtain serial dilutions. The diluted solutions were added to 70 mm sterile blank filter disks and placed on the medium-free cover of each Petri plate to obtain final concentrations of 0.125, 0.25, 0.5, 0.75, and 1 μL/mL air. The Petri plates were then sealed using parafilm and inoculated as described above.

The efficacy of the treatment was evaluated each day for 9 days by measuring the average of 2 perpendicular diameters of each colony. All tests were performed in triplicate. The percentage inhibition of the radial growth of the tested strain by oils, compared with the control group, was calculated at day 9, using the following formula (Albuquerque et al., 2006):

$$\text{Percentage mycelial inhibition} = \left[\frac{dc - dt}{dc}\right] \times 100,$$

where *dc* (cm) is the mean colony diameter for the control sets and *dt* (cm) is the mean colony diameter for the treatment sets.

2.6. Effect of ZMEO on spore germination of A. flavus

Fungal spore germination and growth kinetics were tested by a previous method with some modifications (Bajpai et al., 2008). Requisite amount of ZMEO was added to the glass tube containing 1 mL 0.1% (v/v) Tween-20 to obtain the final concentrations of 1, 2, 4, 6, and 8 μL/mL. A spore suspension of *A. flavus* was prepared from the 3-day old cultures, harvested by adding 5 mL of sterile water containing 0.1% (v/v) Tween-20 to each Petri dish and gently scraping the mycelial surface 3 times with a sterile L-shaped spreader to free spores. The homogenous spore suspension of *A. flavus* containing 10^7 spores/mL was then inoculated into each of the above tubes. From this, 10 μL aliquots of the spore suspension were incubated into fresh PDA media in separate depression slides. Depression slides containing the spores were assembled with the

cover slip and incubated in a moisture chamber at 28 °C for 20 h in 3 replicates. For each treatment, 200 spores were examined; the extent of spore germination was assessed by looking for the emergence of germ tubes. The number of germinated spores was calculated and reported as a percentage of spore germination.

A. flavus was also tested in a kinetic study to assess its antifungal activity. Aliquots (10 μL) of the spore suspension of *A. flavus* containing 10^8 spores/mL prepared in 0.1% (v/v) Tween-20 was aseptically transferred to different concentrations (2, 4, and 8 μL/mL) of 5 mL ZMEO solution, and then the homogenous suspension (about 2×10^5 spore/mL) was mixed vigorously by vortex (TS-1, Kylin-Bell Lab Instruments Co., Ltd., Shanghai, China) for 1 min. Samples without any oil treatment were considered controls. After specific time intervals, *i.e.*, 30, 60, 90, 120, 150, and 180 min, the reaction mixtures were collected and centrifuged at 6000 × *g* (TGL-16C, Anting Scientific Instrument Factory, Shanghai, China) for 5 min at room temperature. The supernatant was discarded and the remainder was resuspended in 10 mL of sterilized distilled water. From this, 10 μL aliquots of the spore suspension were taken to the depression slides, which were handled as described above. About 200 spores were examined and the percentage of spore germination was calculated.

2.7. Effect of ZMEO on AFB_1 production by A. flavus

The anti-aflatoxigenic efficacy of ZMEO was studied on *A. flavus* following Kumar et al. (2008). 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 required amounts of ZMEO dissolved in 5% (v/v) Tween-20 were transferred to the PDB medium to obtain 1, 2, 4, 6, and 8 μL/mL concentrations. The control groups 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 through filter paper (DX102, Xinhua Paper Co., Ltd., Hangzhou, China). The weight of the dry mycelia for each mycelium was determined after drying them 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 and evaporated to dryness and the residue was redissolved in chloroform up to 1 mL in a volumetric flask. A silica gel-G thin layer plate was used for the AFB_1 analysis. Fifty microliters of each sample spotted onto the TLC sheets were developed in the solvent system comprising toluene:isoamyl alcohol:methanol (90:32:2, v/v/v) (Reddy et al., 1970). The identity of AFB_1 was detected under a UV lamp at 365 nm and confirmed chemically by spraying trifluoroacetic acid (Bankole and Joda, 2004). For the quantification of AFB_1, amethyst-fluorescent spots of AFB_1 on the TLC were scraped out, dissolved in 5 mL cold methanol, and centrifuged at 2000 × *g* (TGL-16C, Anting Scientific Instrument Factory, Shanghai, China) for 5 min. The absorbance of the supernatant was made using a UV–visible spectrophotometer (UV-2802, Unico Co., Ltd., Shanghai, China) at a wavelength of 360 nm. The amount of AFB_1 present in the sample was calculated according to a formula by Sinha et al. (1993):

$$\text{AFB}_1 \text{ content}\,(\mu\text{g/mL}) = \frac{D \times M}{E \times l} \times 1000,$$

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).

AFB_1 inhibition was calculated as follows:

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

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. Transmission electron microscopy (TEM) observation of fungal mycelium

Fungal material obtained from 5-day-old cultures of *A. flavus* on PDA exposed to 2, 4, and 6 μL/mL of ZMEO and the control without oil were used for TEM observations to investigate the possible mode of action of ZMEO. Small fragments of mycelium (5 mm × 5 mm) were from the margins of the colonies of cultures growing on the PDA plates and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer saline (PBS) (pH 7.2) at 4 °C and fixed overnight, followed by a thorough washing with phosphate buffer (3 times, 10 min each). Afterwards, samples were dehydrated in a graded acetone series (70, 80, 90, 95, and 100%, one time at 20 min for each dilution and 3 times at 30 min at last step) and then embedded and polymerized in Spurr's resin at 65 °C for 72 h. Blocks were sectioned with a diamond knife (Leica EM UC7 Ultratome). Sections approximately 50 nm thick were collected for direct examination under an electron microscope (Tecnai G2 T12, FEI Company, Hillsboro, USA).

2.9. Effect of ZMEO on fungal decay of cherry tomatoes

To assess the potency of ZMEO in controlling fungal decay of cherry tomatoes caused by *A. flavus*, the fruits were treated with ZMEO following our previously published method (Tian et al., 2011a).

2.9.1. Effects of ZMEO on wound-inoculated fungal decay of cherry tomatoes

Cherry tomatoes in commercial maturation stage were collected from a local market and selected for mature fresh fruits without signs of mechanical damage or deterioration. The fruits were divided into 3 replicates (12 fruits per replicate) the surface sterilized dipped in 70% ethanol for 2 min, washed twice with double distilled water (5 min each) and then left for 1 h in safe cabinet to dry. Surface-sterilized fruits were wounded with a sterilized cork borer to obtain uniform wounds (4 mm diameter, 2 mm deep). Each fruit was separately inoculated with fungi by placing 10 μL of a conidial suspension of *A. flavus* (1×10^6 spores/mL). Three independent replicate batches of fruits inoculated with the test fungi were placed into 0.9 L polystyrene containers with snap-on lids. ZMEO with concentrations of 80, 160, 240, and 320 μL/mL dissolved separately in 0.5 mL of 5% (v/v) Tween-20 was pipetted aseptically onto filter paper discs of 4 cm diameter and respectively placed into individual weighing bottles (φ 40 × 25 mm) without lids to obtain final concentrations of 0.05, 0.1, 0.15, and 0.2 μL/mL air. At the same time, the another filter paper moistened with sterilized water was placed in each container, maintaining a high relative humidity (90–95%) during the storage period. Controls were prepared similarly without ZMEO. 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. The entire experiment was repeated twice.

2.9.2. Antifungal effects of ZMEO on healthy cherry tomatoes

A random selection of cherry tomatoes without any handling were divided into three replicates (12 fruits per replicate), and then placed in 0.9 L polystyrene containers with snap-on lids. Four different final concentrations (0.05, 0.1, 0.15, and 0.2 μL/mL air) of ZMEO and along with control sets 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.

2.10. Consumer evaluation of cherry tomato fruits stored

Consumer evaluation was conducted by according to the methods of Guillevic et al. (2009) and Huang et al. (2011) with a little modification. Cherry tomato fruits stored were subjected to a consumer test, using 12 consumers (50% female and 50% male, ranging from 16 to 54 years old). Twelve panelists scored the samples for overall acceptability in artificially inoculated groups at 3, 6, and 9 days of storage, and in naturally infected groups at 7, 14, and 21 days of storage. Panelists were also asked to provide additional qualitative comments for each sample. A questionnaire was used to record the data. A 9-point hedonic scale (9 = like extremely; 8 = like very much; 7 = like moderately; 6 = like slightly; 5 = neither like nor dislike; 4 = dislike slightly; 3 = dislike moderately; 2 = dislike very much; 1 = dislike extremely) was used to score the samples.

2.11. Statistical analysis

The SPSS, 13.0 (Chicago, USA) statistical software was used to calculate the analysis of variance (ANOVA). Significant differences between the mean values were determined by Duncan's multiple range test at the $p < 0.05$ level. All the measurements were replicated three times for each treatment, and the results expressed as mean ± standard deviations.

3. Results

3.1. Chemical composition of ZMEO

Fifty-one different components of the ZMEO, accounting for 95.72% of the total oil composition, were identified by GC–MS analyses. The identified chemical components, retention indices, and percentage composition are presented in Table 1. The oil contained a complex mixture of monoterpene hydrocarbons (28.41%), ketones (26.81%), oxygenated monoterpenes (21.36%), and oxygenated sesquiterpenes (10.50%). The most abundant components were 2-undecanone (26.81%), limonene (20.12%), and terpinen-4-ol (13.09%). Several other components such as sabinene (4.78%), β-eudesmol (4.22%), caryophyllene (3.25%), linalool (2.86%), *o*-cymene (2.45%), elemol (2.14%), α-terpinyl acetate (1.90%), pentadecane (1.70%), α-terpineol (1.42%), α-cadinol (1.18%) and T-muurolol (1.05%) were present in lesser amounts. However, sesquiterpene hydrocarbons, alcohols, ethers and others were also found as trace or minor components.

Table 1
Chemical composition of the ZMEO isolated by hydrodistillation from *Zanthoxylum molle* Rehd.

Number	RI[a]	Compound	Percentage[b]
1	930	α-Thujene	0.12
2	939	α-Pinene	0.32
3	977	Sabinene	4.78
4	981	β-Pinene	0.06
5	991	β-Myrcene	0.35
6	1024	*o*-Cymene	2.45
7	1029	Limonene	20.12
8	1039	*cis*-β-Ocimene	0.10
9	1046	*cis*-β-Terpineol	0.55
10	1062	γ-Terpinene	0.11
11	1074	*cis*-Linalool oxide	0.13
12	1082	Linalool	2.86
13	1097	*trans*-Sabinene hydrate	0.72
14	1099	*trans*-*p*-2-menthen-1-ol	0.56
15	1118	*cis*-4-Isopropyl-1-methyl-2-cyclohexen-1-ol	0.36
16	1175	Terpinen-4-ol	13.09
17	1189	α-Terpineol	1.42
18	1204	*trans*-Piperitol	0.21
19	1242	Carvone	0.08
20	1257	Linalyl acetate	0.17
21	1291	2-Undecanone	26.81
22	1347	α-Terpinyl acetate	1.90
23	1353	Citronellyl acetate	0.07
24	1361	Neryl acetate	0.11
25	1391	β-Elemene	0.28
26	1418	Caryophyllene	3.25
27	1437	γ-Elemene	0.04
28	1455	α-Humulene	0.61
29	1491	β-Selinene	0.14
30	1500	Pentadecane	1.70
31	1506	α-Muurolene	0.13
32	1514	γ-Cadinene	0.15
33	1523	δ-Cadinene	0.16
34	1530	Elemol	2.14
35	1537	Germacrene B	0.11
36	1563	*trans*-Nerolidol	0.03
37	1574	Caryophyllene oxide	0.09
38	1578	Spathulenol	0.71
39	1585	Globulol	0.03
40	1589	Guaiol	0.40
41	1593	Viridiflorol	0.16
42	1609	Humulene epoxide II	0.15
43	1612	Isoaromadendrene epoxide	0.05
44	1630	γ-Eudesmol	0.88
45	1643	T-Muurolol	1.05
46	1651	9-Aristolen-α-ol	0.24
47	1653	α-Cadinol	1.18
48	1656	β-Eudesmol	4.22
49	1660	α-Eudesmol	0.14
50	1672	Bulnesol	0.06
51	1702	2-Hexadecanol	0.17

[a] Retention indices relative to a series of *n*-alkanes on Rtx-5 capillary column.
[b] The relative proportions of the essential oil constituents.

3.2. Antifungal effects of ZMEO on mycelial growth and spore germination of A. flavus

ZMEO activity is evident in Fig. 1 where inhibition of radial colony growth and spore germination by ZMEO was detected for *A. flavus*. The contact and vapor phase effects of different concentrations of ZMEO on the mycelial growth of *A. flavus in vitro* are presented in Fig. 1A and B. Results show that mycelia growth was considerably reduced with the increasing concentration of ZMEO but it increased with incubation time. In contact phase testing, the mycelial growth was delayed by 4 days for *A. flavus* at 6 μL/mL concentration; and a 8 μL/mL could completely inhibit the growth of fungus after 9 days of incubation (Fig. 1A). The oil produced a significant reduction in mycelium growth with *A. flavus* at 1, 2, 4, and 6 μL/mL concentrations with reduction percentages of 25.6%, 49.9%, 76.0%, and 87.2%, respectively. Vapor phase effects of ZMEO were greater on mycelial growth than contact inhibitory effects were. Results of the vapor phase effect of ZMEO are indicated in Fig. 1B. In the vapor phase test at 1 μL/mL oil concentration in air, fungal growth was completely inhibited after 9 days of incubation. A significant reduction was also observed in mycelium growth of *A. flavus* at 0.125, 0.25, 0.5, and 0.75 μL/mL air concentrations with reduction percentages of 28.6%, 46.1%, 72.5%, and 89.7%, respectively.

The effects of ZMEO on spore germination of *A. flavus* are presented in Fig. 1C and D. Fig. 1C indicates that the percentage of spore germination was significantly ($p < 0.05$) inhibited by the different concentrations of ZMEO. All *A. flavus* spores germinated after 20 h of incubation at 28 °C in PDB without the ZMEO. However, ZMEO completely inhibited the germination of spores at 8 μL/mL. Observations revealed inhibitory effects on the spore germination of *A. flavus* within the range of 18.7–89.0% at concentrations ranging

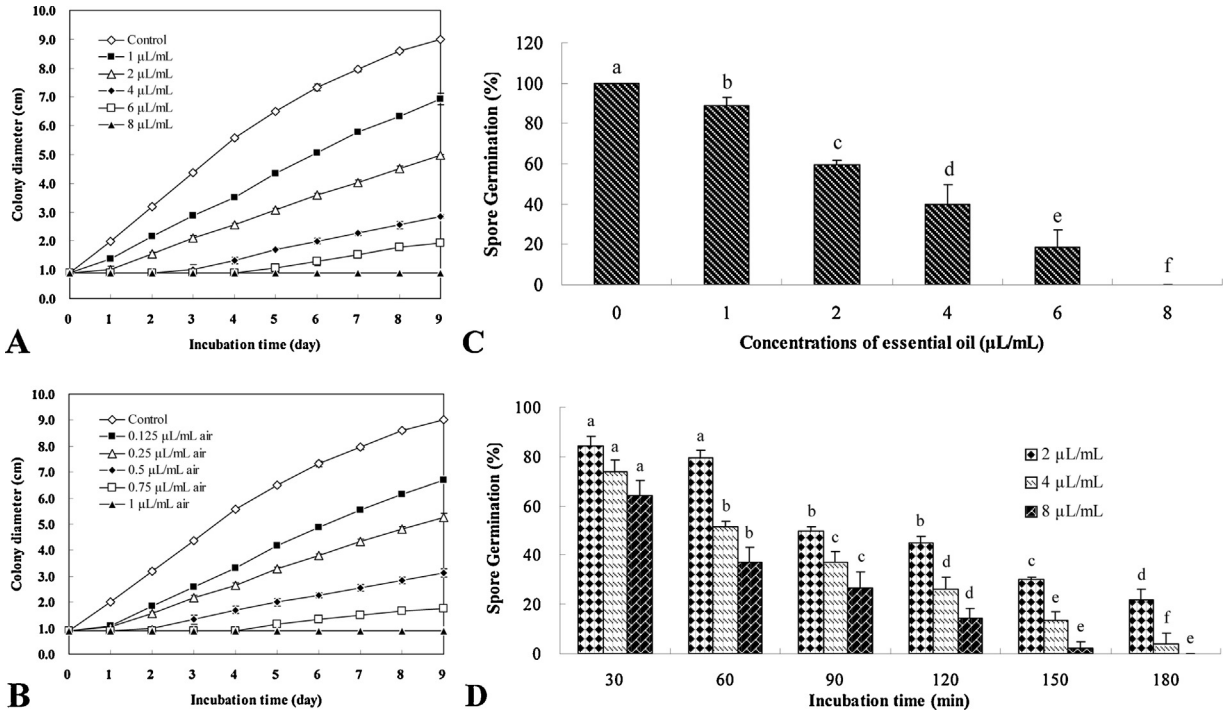

Fig. 1. *In vitro* control of mycelial growth and spore germination of *A. flavus*. The contact (A) and vapor (B) phase effects of different concentrations of ZMEO on the mycelial growth of tested fungi. (C) Effects of different concentrations of ZMEO on spore germination of tested fungi. (D) Growth kinetics of the inhibition of the tested fungi by ZMEO. Values are means ($n = 3$) ± standard deviations.

from 1 to 6 μL/mL. The inhibition growth kinetics of *A. flavus* by the ZMEO is presented in Fig. 1D. Exposure of the *A. flavus* spores to different concentrations of the ZMEO for a period of 0–180 min caused varying degrees of spore germination inhibition. Results exhibit that spore germination was reduced with increasing exposure time and ZMEO concentration. The ZMEO at 2 and 4 μL/mL showed antifungal activity but not rapid killing, and about 40–50% inhibition was observed at an exposure time of 90 min. However, there was a visible increase in the killing rate at 8 μL/mL after 30 min of exposure, and complete inhibition of spore germination was observed at 180 min of exposure.

3.3. Antifungal effects of ZMEO on dry mycelium weight and AFB_1 production by A. flavus

The effects of ZMEO on *A. flavus* aflatoxin synthesis and mycelial dry weight in PDB medium are shown in Fig. 2. The five different concentrations of ZMEO efficiently caused different degrees of inhibition of dry mycelium weight and AFB_1 synthesis ($p < 0.05$).

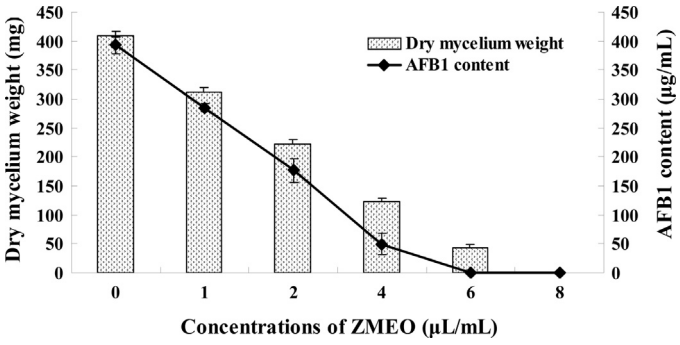

Fig. 2. Effects of the different concentrations of ZMEO on dry mycelium weight and AFB_1 production by *A. flavus*. Values are mean ($n = 3$) ± standard deviations.

The results indicated that ZMEO strongly inhibited AFB_1 production in a dose-dependent manner. ZMEO totally inhibited the mycelial production at 8 μL/mL. However, mycelial growth was observed at 6 μL/mL, although AFB_1 production was completely inhibited. The ZMEO caused an apparent inhibition in AFB_1 content at 1, 2, 4, and 6 μL/mL concentrations with inhibition percentages of 27.5%, 55.0%, 87.5%, and 100.0%, respectively (data not shown).

3.4. Effects of ZMEO treatment on the ultrastructure of A. flavus mycelium

To explore the mode of ZMEO against *A. flavus*, alterations in the hyphal ultrastructure of *A. flavus* observed by TEM are shown in Fig. 3. The uniform cell wall, intact fibrillar layer, smooth plasma membrane, and abundant cytoplasmic matrix were observed in the nontreated hyphae (Fig. 3A). The main organelles, such as mitochondria, vesicles, and nucleus also had normal and uniform structures in control. By contrast, ultrastructural changes were observed in the plasma membrane, fibrillar layer, and cytoplasm in the ZMEO-treated hyphae (Fig. 3B–D). At 2 μL/mL of ZMEO, the plasmalemma became uneven with some invagination and festooned with small lomasomes (Fig. 3B). The cell ultrastructure damage was aggravated in the presence of 4 μL/mL concentrations of ZMEO (Fig. 3C). The major alterations were observed by a decrease in the fibrillar layer, extensive disruption of the internal structure with an irregular tangle in the mitochondrial crist, the appearance of more lomasomes with folding, and the elution of the karyolymph (Fig. 3C). As presented in Fig. 3D, the fibrillar layers gradually lost their integrity, becoming thinner, and eventually failing to deposit on the cell wall. The cytoplasmic matrix was absent and some organelles were not recognizable. Furthermore, complete lysis and severe disorganization of membranous organelles including the nuclei and mitochondria were observed.

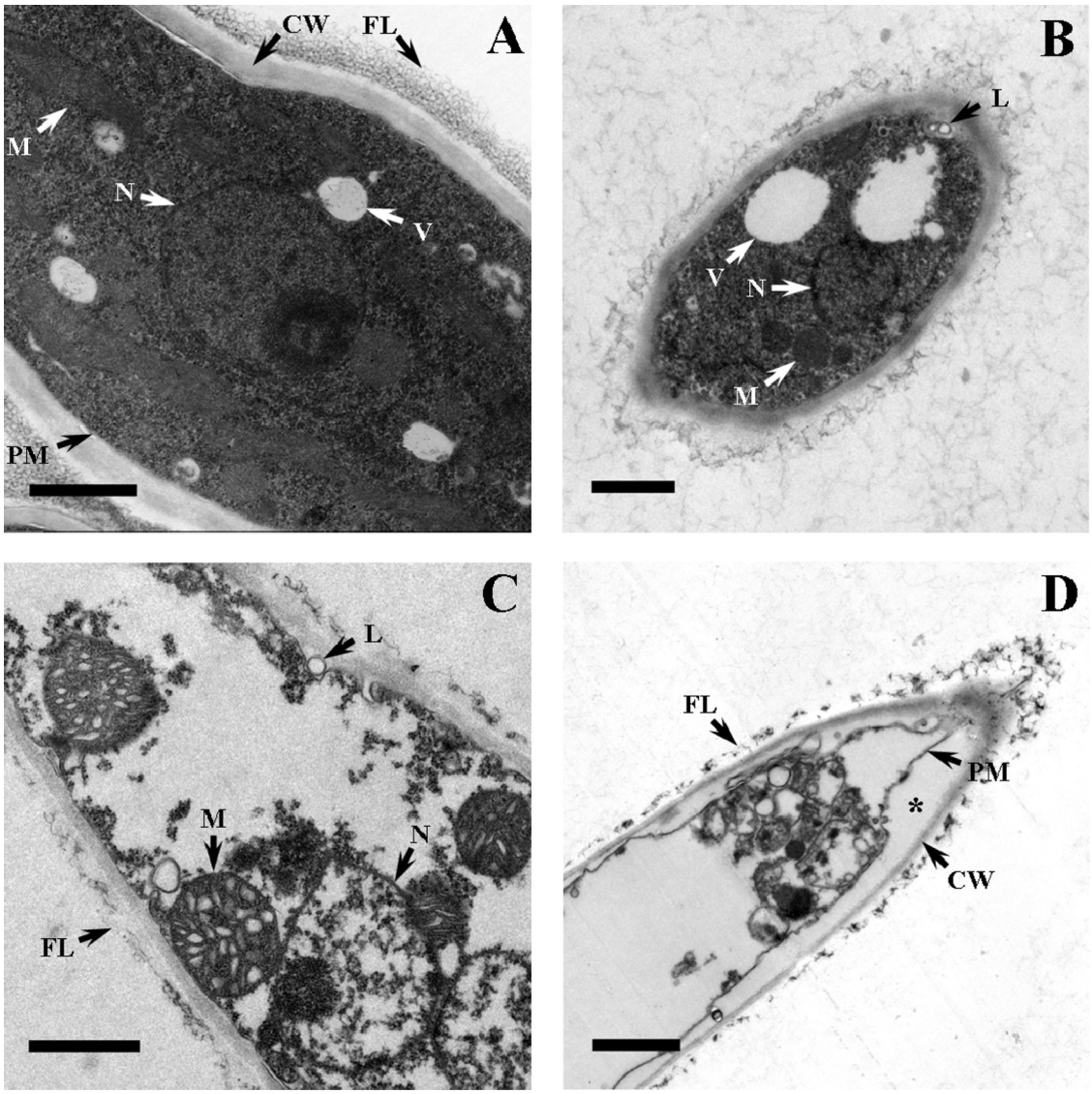

Fig. 3. TEM images of treated and untreated hyphae of *A. flavus*. (A) Control mycelia are homogenous and their cells contain abundant cytoplasmic matrix. The cell wall (CW), plasma membrane (PM), fibrillar layer (FL), and intracellular organelles including the mitochondria (M), vesicles (V), and nucleus (N) have usual and uniform structures. (B) Treatment exposed to 2 μL/mL of ZMEO. The PM was slightly invaginated and formation of small lomasomes (Ls). Swelling of the vesicles was observed. (C) Treatment exposed to 4 μL/mL of ZMEO. The FL was thinner, the PM was also becoming partly obscured and the appearance of some Ls. M and V were losing their structures. The karyolymph was elutropic. (D) Complete cell depression and disorganization signs, such as extensive destruction, clear separation of the PM from the cell wall (*), disruption of fibrillar layer, severe absence of cytoplasmic matrix, massive vacuolation of cytoplasm with vacuole fusion and lysis of hyphae membranous organelles. Bar = 1 μm.

3.5. *Effects of ZMEO in the preservation of cherry tomatoes from fungal decay*

The ZMEO vapor treatment can reduce fungal growth in wound-inoculated cherry tomatoes caused by *A. flavus* and natural decay development in unwounded cherry tomatoes (Fig. 4). After 9 days of storage at 18 °C, the artificial decay incidence of the control groups for *A. flavus* was 100%. It was found that the percentages of infected fruits were significantly ($p < 0.05$) reduced in the treatment group compared with the control group and also significantly ($p < 0.05$) reduced with increasing concentrations of ZMEO. The most effective concentration of 0.2 μL/mL air produced the greatest reduction in the percentages of decayed fruits for *A. flavus* compared with that of the control group, with percentage reductions of 91.7%. After 21 days of storage at 18 °C, the results indicated that the percentage of infected fruits was significantly ($p < 0.05$) reduced by ZMEO. In this case, the concentration of 0.2 μL/mL air exhibited the highest inhibition of fungal development compared with that of the control group, at 72%. As seen in Fig. 4, the protective effect of ZMEO on artificially inoculated fruits is obviously higher than naturally infected healthy fruits after oil vapor treatment.

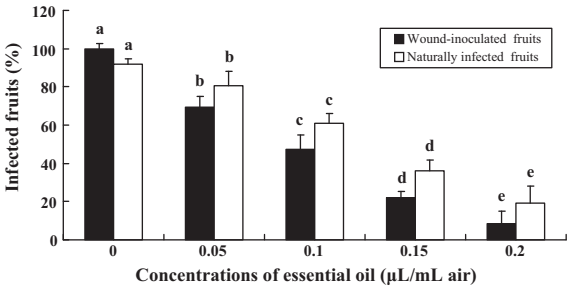

Fig. 4. Effects of ZMEO on fungal development in wound-inoculated and naturally infected fruits. Different letters above bars indicate significant differences ($p < 0.05$) in each group. Values are mean ($n = 3$) ± standard deviations.

3.6. Consumer evaluation of cherry tomato fruits stored

The overall acceptability scores of cherry tomatoes as affected by ZMEO are shown in Table 2. As presented in Table 2, the scores for ZMEO-treated fruits were significantly higher than those of control fruits, except for days 3 in artificially inoculated groups and days 7 in naturally infected groups. Overall, scores for ZMEO-treated fruits and the controls were considerably increased with storage time and the increasing concentration of ZMEO. The concentration of 0.2 μL/mL air indicated significantly higher scores compared with that of the control in both wound-inoculated and naturally infected fruits.

4. Discussion

Essential oils, which are complex mixtures of volatile compounds with strong odor that are synthesized in several plant organs, have formed the basis of many applications in the food flavoring and preservation industries (Ballester-Costa et al., 2013). Generally, whole essential oils have greater antifungal activity due to a synergistic effect with active components; thus, they are more promising in commercial application than single compounds (Tian et al., 2012b). Today, special attention has been paid to active products from inexpensive or residual sources from agricultural industries. *Z. molle* is a kind of deciduous tree, and its leaves are abundant as residual sources; thus ZMEO is a readily available and inexpensive antifungal agent. Moreover, the yield of its ZMEO is about 1.2%, which is relatively high. This makes ZMEO suitable for development of a food preservative.

Generally, most components of essential oils are terpenoids, such as monoterpenes, sesquiterpenes, and their oxygenated derivatives, and the active antimicrobial compounds of essential oils are terpenes. However, the antifungal mechanism of terpenes is not fully understood; it could be involved in membrane disruption through lipophilic compounds (Cowan, 1999). The low-molecular weight, highly lipophilic compounds of essential oils easily pass through cell membranes to produce biological responses (Chao et al., 2005). ZMEO exhibited antifungal properties which could be attributed to the presence of 2-undecanone (26.81%), limonene (20.12%), and terpinen-4-ol (13.09%). 2-Undecanone is commonly used in the perfume and flavoring industries, and also as an insect repellent (Singh et al., 2013). Merghache et al. (2008) reported that the essential oil from *Ruta chalepensis* L. showed significant antifungal activities against *A. flavus* due to 2-undecanone (79.06–82.74%) as the major constituent. Limonene and terpinen-4-ol also have been proved to possess strong antifungal activities (Singh et al., 2010; Terzi et al., 2007). However, whole ZMEO has greater antifungal activity, which may be attributed to some minor components that has a synergistic effect with the major components.

In our study, ZMEO showed a pronounced antifungal efficacy against the tested fungi. Mycelium growth was reduced with increasing concentrations of the ZMEO. The colony diameter was expanding with increasing incubation days. Nevertheless, in contact phase testing, relatively higher concentrations than the vapor phase testing are required to inhibit mycelial growth. Similar to the present findings, volatile phase effects of essential oils from oregano, thyme, rosemary, lavender, fennel, and laurel were also consistently found to be more effective on fungal growth than contact phase effect (Soylu et al., 2006). Some investigators revealed that antifungal activity results from a direct effect of essential oil vapors on fungal mycelium, and they postulated that the lipophilic nature of essential oils allow them to be absorbed into fungal mycelia (Edris and Farrag, 2003). Based on our results, the ZMEO showed high antifungal activity against *A. flavus* as its inhibitory effect on mycelial growth was stronger than that of some earlier reported essential oils *viz. Sesuvium portulacastrum*, *Cinnamomum zeylanicum* and *Hyptis suaveolens* (Magwa et al., 2006; Moreira et al., 2007, 2010).

The spore is another important structure for the survival and spread of fungi (Rabea et al., 2009). ZMEO was also efficacious inhibiting the germination of *A. flavus* spores. A significant decrease in the percentage of spore germination was observed on *A. flavus* with increasing concentrations of the oil. The effects of some compounds similar to major components of ZMEO on fungal spore germination were also conducted. Droby et al. (2008) showed that limonene as the major citrus fruit volatiles had a pronounced stimulatory effect on germination and germ tube elongation of both *Penicillium digitatum* and *Penicillium italicum*. The kinetic study of *A. flavus* revealed that the exposure time of the ZMEO had a little effect on the antifungal activity at 2 μL/mL. However, spore germination was considerably restrained at 6 and 8 μL/mL. The results are consistent with previous findings by Bajpai et al. (2008).

AFB_1, produced by *A. flavus*, is the most dangerous toxic metabolite among all classes of aflatoxin. Hence, a measurement of the content of AFB_1 was conducted in the present study. In light of our results, the ZMEO showed a remarkable effect in restraining dry mycelium weight and the AFB_1 production of *A. flavus*. The ZMEO displayed anti-aflatoxigenic properties at concentrations lower than its fungitoxic concentration; similar findings were also observed by Prakash et al. (2012). This could be explained by different modes of actions of the oil against fungal growth and aflatoxin production. Several research workers have demonstrated that there is a direct correlation between fungal growth and AFB_1 production (Kumar et al., 2010, 2008). However, the inhibition of AFB_1 synthesis cannot be completely attributed to the insufficient fungal growth, but it may be a result of the inhibition of carbohydrate catabolism in *A. flavus* by acting on some key enzymes leading to the reduction of its ability to produce AFB_1 (Tatsadjieu et al., 2009). No clear mechanism of AFB_1 synthesis of essential oil has been available in the scientific literature until now. Based on the present study, it could be concluded that ZMEO may interfere with some steps in the metabolic pathways of the *A. flavus*, which controls the biosynthesis of AFB_1. Therefore, further experiments on ZMEO are required to understand the AFB_1 suppression mechanisms.

TEM was seen to be a useful tool to elucidate the effects of antimicrobial substances. In the present study, the TEM of ZMEO-treated *A. flavus* in comparison with the control samples clearly appeared to be dose-dependent changes of fungal hyphae, especially on membranous structures. The complexity of the composition of essential oils indicated multiple mechanisms of action that commonly affect several targets at the same time, and no particular resistance or adaptation to essential oils has been described (Bakkali et al., 2008). ZMEO can pass not only through the cell wall but also the plasma membrane and then interact with the membranous structures of cytoplasmic organelles. However, the results clearly showed that ZMEO exerts its effect directly on the plasma membrane without any obvious damage to the cell wall. In contrast, the essential oils from cinnamon and thyme exhibited the strongest antifungal activity by rupturing the cell wall, which may prevent the correct arrangement of different parietal compounds, such as chitin, glucans and glycoproteins, during cell wall construction (Lu et al., 2013). Changes in the cell permeability due to the breakdown of the plasma membrane at variable intervals result in the loss of the normal shape of fungal mycelia and the formation of membrane bound vesicles inside the cells. Khan and Ahmad (2011) believed that the lipophilic properties of oils may assist in the penetration of the plasma membrane; and the accumulation of polysaccharides on stress condition may result in the disruption of the cell membrane in fungal cells. In light of our results, the plasma membrane and the organelles may be important targets of the ZMEO's active components, especially the mitochondria, indicated as a key target

Table 2
Mean scores of overall acceptability of stored cherry tomatoes treated with ZMEO.

Conc. of oil (μL/mL air)	Wound-inoculated fruits			Naturally infected fruits		
	Day 3	Day 6	Day 9	Day 7	Day 14	Day 21
Control	7.17 ± 0.83[a]	3.33 ± 0.65[a]	1.25 ± 0.45[a]	7.17 ± 1.03[a]	3.50 ± 1.38[a]	1.17 ± 0.40[a]
0.05	7.25 ± 0.87[ab]	4.17 ± 0.72[b]	2.58 ± 1.00[b]	7.08 ± 0.67[a]	5.08 ± 1.16[b]	2.67 ± 0.89[b]
0.1	7.33 ± 1.07[ab]	4.42 ± 1.08[b]	2.83 ± 1.27[b]	7.42 ± 0.90[ab]	5.67 ± 1.30[bc]	3.75 ± 0.97[c]
0.15	7.83 ± 1.03[ab]	6.00 ± 0.95[c]	5.58 ± 1.44[c]	7.67 ± 0.89[ab]	6.17 ± 1.11[c]	5.08 ± 1.08[d]
0.2	8.08 ± 0.90[b]	7.25 ± 1.14[d]	6.25 ± 0.87[c]	8.17 ± 1.03[b]	7.75 ± 1.14[d]	7.17 ± 0.94[e]

Conc.: concentration.
[a–e] 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 ($p < 0.05$).

of ZMEO. The antifungal activity of ZMEO could be explained by its ability to disrupt the permeability barrier of the plasma membrane and the mitochondrial dysfunction-induced ROS accumulation in *A. flavus* based on our previous investigation (Tian et al., 2012b), which eventually leads to fungal cell death.

Although *in vitro* tests of essential oils is an important first step in selecting plants with antifungal potential, *in vivo* tests are needed to check whether the positive results of the *in vitro* tests can be obtained too (Tegegne et al., 2008). The present *in vivo* results indicated that the ZMEO had a positive effect on storage life and reduced cherry tomato decay, with 0.2 μL/mL air ZMEO treatment giving the longest storage life. Our observations are consistent with previous findings by Shao et al. (2013), who reported tee tree oil reduced the main fungal disease in strawberries. The use of vapor treatments is ideal for controlling food spoilage because it leaves no residual essential oils (Tian et al., 2011b). Moreover, as ZMEO were applied with the filter paper discs, the volatility of the ZMEO was reduced, and thus persistence of the ZMEO on fruit surface could be prolonged. The *in vivo* experiment shows that exposure of produce to the vapors may induce higher resistance to fungal challenges. 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).

A sensory analysis was also carried out to determine the viability of the ZMEO using concentrations determined in this work. The control group of cherry tomatoes had the lowest sensory acceptability after storage, which could be explained by the high decay incidence and the change in carbohydrates, proteins, amino acids, lipids, and phenolic compounds that can influence the fresh fruits (Malundo et al., 1997). As was expected, the application of ZMEO as a promising botanical preservative improved the sensory aspects of overall acceptability throughout storage period.

5. Conclusions

The present results of *in vitro* studies suggest that significant antifungal activity against *A. flavus* growth, aflatoxin production, and ultrastructure alterations. Moreover, *in vivo* studies also indicate that ZMEO can produce growth inhibitory effects against *A. flavus* and a broad spectrum of fungal microbiota of cherry tomatoes. Employing ZMEO as a significant fumigant during routine storage or extended transport is very promising. The use of essential oils cannot only improve food safety by eliminating fungal spread but also leaves no detectable residues after storage. Hence, ZMEO would be economical, with considerable commercial significance in these industries, and it is worthy of further investigation for use as a preservative in storage containers.

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