In vitro antifungal activity and mechanism of essential oil from fennel (Foeniculum vulgare L.) on dermatophyte species

Hong Zeng,⁎ Xinping Chen⁎ and Jingnan Liang‡

⁎Key Laboratory of Protection and Utilization of Biological Resources, College of Life Science, Tarim University, Alar, 843300, Xinjiang, PR China
‡Instrument Center, Institute of Microbiology, Chinese Academy of Sciences, 100000 Beijing, PR China

Fennel seed essential oil (FSEO) is a plant-derived natural therapeutic against dermatophytes. In this study, the antifungal effects of FSEO were investigated from varied aspects, such as MIC and minimum fungicidal concentration, mycelia growth, spore germination and biomass. The results indicated that FSEO had potent antifungal activities on Trichophyton rubrum ATCC 40051, Trichophyton tonsurans 10-0400, Microsporum gypseum 44693-1 and Trichophyton mentagrophytes 10-0060, which is better than the commonly used antifungal agents fluconazole and amphotericin B. Flow cytometry and transmission electron microscopy experiments suggested that the antifungal mechanism of FSEO was to damage the plasma membrane and intracellular organelles. Further study revealed that it could also inhibit the mitochondrial enzyme activities, such as succinate dehydrogenase, malate dehydrogenase and ATPase. With better antifungal activity than the commonly used antifungal agents and less possibility of inducing drug resistance, FSEO could be used as a potential antidermatophytic agent.

INTRODUCTION

Fungal infections of skin, hair and nails are a common global problem, and about 20–25% of the world’s population are affected by superficial fungal infections (Havlickova et al., 2008). Dermatophytes are the most common form of fungal infections in most countries. They can be classified according to their usual habitats into anthropophilic and zoophilic organisms. In general, pathogens responsible for skin mycoses are Trichophyton, Microsporum and Epidermophyton. Dermatophytes are able to invade human and animal keratinized tissues, causing lesions in the skin, hair and other body parts (Hainer, 2003; Ameen, 2010). In recent years, there has been an increasing requirement for new antifungal compounds due to the lack of efficacy, the side effects and the resistance associated with existing drugs.

Plant-derived essential oils are natural compounds that show potential effects against bacteria and fungi (Park et al., 2007). Essential oils as antimicrobial agents have two main advantages: firstly, most essential oils are safer for consumers; secondly, there is low risk of the micro-organisms developing resistance to essential oils (Cardile et al., 2009).

In recent years, numerous studies have documented the antifungal effects of plant-derived essential oils on dermatophytes (Bajpai et al., 2009a, b; Behravan et al., 2004; Romagnoli et al., 2010; Shahi et al., 2007; Shin, 2004). Fennel (Foeniculum vulgare L.), a member of the Apiaceae, is a popular aromatic herb and spice. Its seeds are used as flavourings in baked goods, ice cream, meat and fish dishes, herb mixtures and alcoholic beverages. Fennel is also used in local and traditional medicine due to its therapeutic effects. It is widely used as a carminative, digestive, lactogogue and diuretic, and in treating respiratory and gastrointestinal disorders (Editor Committee of National Chinese Medical Manage Bureau, 2005). The essential oils obtained by steam distillation of the seeds show antioxidant (Ruberto et al., 2000), antithrombotic (Tognolini et al., 2007), antidiabetic (El-Soud et al., 2011) and acaricidal (Lee, 2004) activities. Fennel seed essential oil (FSEO) showed broad-spectrum antibacterial effects against pathogens such as Escherichia coli, Bacillus spp., Staphylococcus aureus, Micrococcus luteus, Pseudomonas spp., Listeria innocua CECT 910, Serratia marcescens CECT 854 and Pseudomonas fluorescens CECT 844 (Mohsenzadeh, 2007; Singh et al., 2006; Mahady et al., 2005; Kazemi et al., 2012). It also exhibited antifungal effects against pathogens such as Aspergillus spp., Fusarium spp., etc.
spp., Candida spp., Sclerotinia sclerotiorum and Trichophyton mentagrophytes (Roby et al., 2013; Viuda-Martos et al., 2011; Inouye et al., 2006).

In this study, the in vitro antidermatophytic activity of FSEO was evaluated and a possible mechanism of action against Trichophyton rubrum was proposed. The high antidermatophytic activity suggests that FSEO is a potential new antifungal agent from a natural product.

**METHODS**

**Plant materials.** The seeds of F. vulgare were obtained from Tarim University, Xinjiang, China. The seed was initially identified by its morphological features and finally confirmed by Professor Jianwei Hu at the College of Life Science, Tarim University.

**Extraction of essential oil.** The air-dried fennel (F. vulgare) seeds (200 g) were subjected to hydrodistillation for 4 h using a Clevenger apparatus. The essential oil was dried over anhydrous Na2SO4 and preserved in a sealed vial at 4 °C. The yield of FSEO was 6 % (v/w).

**GC-MS analysis.** The chemical composition of FSEO was analysed using GC-MS. The essential oil (20 μl) was dissolved in acetone (200 μl) and 1 μl of the solution was injected into a GC mass spectrometer (7890A-5975C; Agilent). The capillary column was HP-MS (length 30 m, thickness 0.25 μm, i.d. 0.25 mm). Helium was used as the carrier gas at a flow rate of 1 ml min−1. The GC column oven temperature was increased from 50 to 300 °C at a rate of 10 °C min−1, with a final hold time of 10 min. Injector and detector temperatures were maintained at 280 °C. El (electronic impact) mode was at 70 eV, while mass spectra were recorded in the 30-600 amu range and ion source-temperature was 230 °C. FSEO components were quantified by relative percentage peak area of Total ion current (TIC) from the MS signal and identified by comparing their mass fragmentation pattern with those stored in the spectrometer database using NIST05LIB and NIST05sLIB (National Institute of Standards and Technology).

**Micro-organisms.** One American Type Culture Collection (ATCC) type strain (T. rubrum ATCC 40051) and five clinical isolates of dermatophytes were used. T. rubrum ATCC 40051 was obtained from the ATCC. The five dermatophyte clinical strains, T. rubrum 10-0982, T. rubrum 10-0403, Trichophyton tonsurans 10-0400, T. mentagrophytes 10-0060 and Microsporum gypseum 44693-1, were isolated from fingernail, foot, hair, skin and hair respectively. The fungal isolates were identified by standard microbiological methods and stored in Sabouraud dextrose broth (SDB) with glycerol at −80 °C for further use.

**Antifungal activities of FSEO against dermatophytes.** Six fungal strains were grown on Sabouraud dextrose agar (SDA) plates in the dark at 28 ± 2 °C for 14 days, and then spores were harvested from sporulating colonies and suspended in sterile distilled water. The concentrations of spores were determined using a haemocytometer and adjusted to 1.0 × 10⁸ spores ml⁻¹. FSEO (40 μl) was dissolved in 1 ml 0.01 % Tween 20 to obtain a working concentration.

Broth microdilution protocols based on those of the CLSI (2008) with minor modifications were used to determine MIC values for filamentous fungi. Briefly, tests were performed in sterile U-bottom 96-well plates. FSEO was serially diluted twofold with RPMI 1640 (without sodium bicarbonate and l-glutamine, pH 7.0), and 100 μl drug solution at each concentration was used. The concentrations of FSEO ranged from 10 to 0.019 μl ml⁻¹. The concentrations of fluconazole (FCZ) and amphotericin B (AmB) ranged from 100 to 0.19 μg ml⁻¹. The inoculum suspension was diluted 10 times with RPMI 1640 broth to obtain a working suspension (approx. 1 × 10⁴ spores ml⁻¹), and 100 μl of the diluted suspension was added to each well. Wells without inocula were used as positive controls, while wells with 0.01 % (v/v) Tween 20 but without FSEO were used as negative growth controls. The flasks were incubated at 28 ± 2 °C for 4 days. MICs (the lowest concentrations of the test substances that prevented visible growth of micro-organisms) were determined.

To evaluate minimum fungicidal concentrations (MFCs; the lowest concentrations yielding negative subcultures or only one colony) of the tested drugs, 100 μl solution from each negative well and the positive growth control were transferred onto SDA, and colonies were counted after 4 days’ incubation at 28 ± 2 °C. All experiments were performed in triplicate.

**Antifungal effects of FSEO on mycelial growth.** The inhibition of mycelial growth of dermatophyte strains (T. tonsurans 10-0400, T. mentagrophytes 10-0060, M. gypseum 44693-1, T. rubrum ATCC 40051) by FSEO was determined with a method modified from that reported by de Albuquerque et al. (2006). Aliquots of FSEO separately dissolved in 1.0 ml 0.1 % (v/v) Tween 20 were pipetted aseptically to glass Petri dishes containing 9.0 ml melted potato dextrose agar (PDA) to make the requisite concentrations of 0.019, 0.039, 0.078, 0.156, 0.312 and 0.625 μl ml⁻¹. Control plates (with 0.01 % Tween 20 but without FSEO) were inoculated following the same procedure. A fungal disc (6 mm in diameter) of mycelial material, taken from the edge of the 9-day-old fungal culture, was placed on the centre of each Petri dish. Each dish was then sealed with polyethylene film and incubated at 28 ± 2 °C. The diameters of the fungal clones were measured every day for a period of 15 days and then the reduction in fungal mycelium growth was calculated.

Mycelium growth reduction (%) = [(D1 − D2)/D1] × 100, where D1 is the diameter of hyphal growth on the Petri dish treated with FSEO, and D2 is the diameter of hyphal growth on the control plate (untreated). All experiments were performed in triplicate.

**Determination of mycelial weight.** Varied volumes of FSEO were dissolved in 1 ml 0.01 % (v/v) Tween 20 and added to 48 ml SDB culture medium to make final concentrations of 0.019, 0.039 and 0.078 μl ml⁻¹, and then inoculated with 1 ml spore suspension of each fungal pathogen, containing 1.0 × 10⁸ spores. Control plates (with 0.1 % Tween 20 but without FSEO) were inoculated by the same procedure. The final concentrations of AmB and FCZ (positive control) were 0.156 and 50 μg ml⁻¹, respectively. The flasks were then incubated for 10 days with shaking (200 r.p.m.) at 28 °C. After incubation, the mycelium was filtered with sterile Whatman paper and washed twice with sterile distilled water. The mycelium samples were dried at 60 °C for 6 h, then at 40 °C overnight, and weighed. Percentage growth inhibition on the basis of dry weight was calculated as: [(control weight – sample weight)/control weight] × 100 (Rasooli & Abyaneh, 2004). All experiments were conducted in triplicate.

**Spore germination assay.** Fungal spore germination was tested by a modification of the method reported by Bajpai et al. (2009a), using different dermatophytic fungi, including T. rubrum ATCC 40051, T. tonsurans 10-0400, M. gypseum 44693-1 and T. mentagrophytes 10-0060. FSEO was dissolved in 0.1 % Tween 20 to final FSEO concentrations of 0.019, 0.039 and 0.078 μl ml⁻¹, with final concentrations of Tween 20 of 0.01 %. Controls contained 0.01 % Tween 20 without FSEO. The final concentrations of AmB and FCZ (positive control) were 0.156 and 50 μg ml⁻¹, respectively. The drug solutions were inoculated with spore suspension of each fungal pathogen containing 1.0 × 10¹ spores ml⁻¹, and then homogenized by inverting the test tubes three or four times. One hundred microlitres
of each mixture was plated on separate glass slides and incubated in a moisture chamber at 28 °C for 8 h. The slides were then observed under the microscope for spore germination. About 200 spores were counted and percentage spore germination was calculated.

**Determination of the ergosterol content in the plasma membrane of dermatophytes.** The ergosterol content in the plasma membrane of *T. rubrum* ATCC 40051 was determined by a published method with slight modifications (Arthington-Skaggs et al., 1999). Briefly, the fungal discs (9 mm in diameter, of mycelial material taken from the edge of 9-day-old fungal culture) were inoculated into 60 ml SDB containing different concentrations of FSEO. [The FSEO was dissolved in 0.1% (v/v) Tween 20 to final concentrations of 0.019, 0.039 and 0.078 µl ml^-1]. FCZ (50 µg ml^-1) was used as a positive control, and the negative control contained only 0.01% (v/v) Tween 20. The cultures were incubated for 10 days with shaking (200 r.p.m.) at 28 °C. After incubation, the mycelia were filtered through sterile Whatman paper and washed twice with sterile distilled water, and then the wet weights were determined. Ten milliliters of 25% alcoholic potassium hydroxide solution was added to each pellet and vortexed for 10 min. Cell suspensions were transferred into clean glass tubes and incubated in an 85 °C water bath for 4 h. Then, sterols were extracted from each sample by adding a mixture of 1 ml sterile distilled water and 3 ml n-heptane, followed by vigorous vortex mixing for 10 min. The n-heptane layer was transferred into a clean tube and stored at -20 °C for 24 h. Prior to analysis, a 1 ml aliquot of sterol extract was diluted twofold with 100% ethanol and the spectrum between 230 and 300 nm was scanned with a UV-1700 spectrophotometer (Shimadzu). The presence of ergosterol (282 nm) and the late sterol intermediate 24,28-dehydroergosterol (DHE; 230 and 282 nm) in the n-heptane layer led to a characteristic curve. The ergosterol content was calculated as a percentage of the weight of the cells, based on the absorbance and wet weight of the initial pellet, as follows: %ergosterol = [24,28-DHE + A24,28-DHE × F]/pellet weight; %24,28-DHE = [A24,28-DHE × F]/pellet weight; %ergosterol = [%ergosterol + %24,28-DHE] × 100; where F is the factor for dilution in ethanol, and 290 and 518 are the E values (as percentages cm^-1) determined for crystalline ergosterol and 24,28-DHE, respectively.

**Determination of plasma membrane damage.** Membrane damage was studied following a published procedure with slight modifications (Chen et al., 2013). Spore suspension of *T. rubrum* ATCC 40051 was obtained from 14-day-old cultures, which were harvested by adding 10 ml sterile water to each Petri dish and gently scraping the mycelial surface with a sterile L-shaped spreader to free the spores. The spore suspension (6 × 10⁸ spores ml⁻¹) of *T. rubrum* ATCC 40051 was then added into each glass tube. FSEO was added to the tubes to final concentrations of 0.019, 0.039 and 0.078 µg ml⁻¹. Samples containing 0.01% Tween 20 only were used as negative controls, and samples containing 50 µg FCZ ml⁻¹ were used as positive controls. The mixtures were then incubated at 28 ± 2 °C for 12 h in an incubator shaker (200 r.p.m.). The spores were washed and resuspended in 500 µl PBS, and stained with 1 µg propidium iodide (PI) ml⁻¹ (Sigma) in PBS for 30 min at 28 ± 2 °C. Unstained cell suspensions were always included as autofluorescence controls. For each sample, a scattergram analysis was performed to evaluate morphological changes, and the percentage of PI-positive cells was determined using FACSCalibur (BD Biosciences) in the FL2 channel (620 nm). All tests were performed in triplicate.

**Transmission electron microscopy (TEM).** Ten-day-old fungal material from *T. rubrum* ATCC 40051 treated with 0.078 µg FSEO ml⁻¹ were observed under TEM to study the mode of action of FSEO (Ghahfarokhi et al., 2004). Small segments (3 × 5 mm) were excised at the margin of the colony growing on the SDA plates, and then promptly placed into vials containing 2.5% glutaraldehyde in 0.1 M PBS (pH 7.2) at 4 °C and fixed overnight. The fixed samples were rinsed with the same buffer three times for 10 min each. Afterwards, the samples were dehydrated in a graded series of ethanol (70, 80, 90, 95 and 100%) for 20 min in each solution. The last step was performed for 30 min and repeated three times. The dehydrated specimens were then embedded and polymerized in Spurr’s resin at 65 °C for 72 h. Ultrathin sections (approx. 50 nm thickness) were hand trimmed with a diamond knife using an LKB-V Ultratome (Jel-1400) for TEM observations.

**Isolation of mitochondria.** The mitochondria of *T. rubrum* ATCC 40051 were isolated according to the method of Wu et al. (2009) with slight modifications. Briefly, six fungal discs (9 mm diameter) of mycelial materials taken from the edge of 9-day-old fungal cultures were used to inoculate 60 ml SDB containing various concentrations (0.019, 0.039 and 0.078 µg ml⁻¹) of FSEO. The cultures were incubated for 10 days with shaking (200 r.p.m.) at 28 ± 2 °C. The mycelium was filtered through sterile Whatman paper, washed twice with sterile distilled water, suspended and homogenized in medium (50 mM Tris pH 7.5, 2 mM EDTA, 1 mM PMSF). Then, 2% glucose was added to the mixture and centrifuged at 2000 g for 5 min to remove the cell debris and broken cells. The supernatants were collected and centrifuged at 10 000 g for 30 min, and the pellets were collected as mitochondria. The pellets were resuspended in 50 mM Tris (pH 7.5), 2 mM EDTA, 20% (v/v) glycerol and stored at -80 °C. Protein was estimated according to the method of Lowry et al. (1951) using BSA as a standard.

**Measurement of the succinate dehydrogenase (SDH), malate dehydrogenase (MDH) and ATPase activities in the mitochondria of *T. rubrum* ATCC 40051.** The effect of FSEO on the activities of mitochondrial SDH and MDH of *T. rubrum* ATCC 40051 was measured by the methods reported by Hill et al. (1984) and Brämer & Steinbuechel (2002), respectively. Assay kits were obtained from the Institute of Biological Engineering of Nanjing Jianchen (Nanjing, PR China).

The effect of FSEO on the mitochondrial ATPase activity of *T. rubrum* ATCC 40051 was measured by the method reported by Tian et al. (2012). The micro-ATPase assay kit was obtained from the Institute of Biological Engineering of Nanjing Jianchen. Measurement followed the manufacturer’s protocol. OD₅₆₀ was determined after 5 min reaction; 20 nM inorganic phosphate was used as a control. One unit of ATPase activity was defined as 1 mmol inorganic phosphorus produced by 1 mg enzyme in 1 h [mmol Pᵢ (mg protein)^⁻¹ h⁻¹].

**Statistical analysis.** The statistical analysis was performed using SPSS 13.0 software (SPSS). All data are reported as means ± SD. The significant differences between mean values were determined by Duncan’s multiple range test, following one-way ANOVA. A P value of < 0.05 was considered as statistically significant.

**RESULTS AND DISCUSSION**

**Chemical composition of the essential oil**

A total of 29 components of the essential oil, accounting for 98.96% of the total oil composition, were identified by GC-MS (Table 1). The most abundant components of the FSEO were *trans*-anethole (63.30%), pinene (11.11%) and fenchone (8.32%). Several other components such as 2, 3-cyclohexene-1-methanol (2.58%), apiole (2.01%), 3-carene (1.44%), 1-methyl-4-(1-methylethyl)benzene (1.32%),
methyl chavicol (1.28 %) and limonene (1.09 %) were present in lower amounts. However, oxygenated sesquiterpenes, sesquiterpene hydrocarbons, hydrocarbons and other compounds were only found in trace amounts.

Compared with the composition of FSEO reported by other researchers, the presence of trans-anethole and fenchone is in agreement but in different proportions. Pinene was detected but estragole was not in the FSEO examined in this paper. A literature search revealed trans-anethole (62.0 %), estragole (4.90 %), fenchone (20.3 %) and limonene (3.15 %) to be the main constituents of essential oils from wild-growing fennel seed native to the Podgorica region, central south Montenegro (Damjanovic et al. 2005). Roby et al. (2013) reported that the major components of FSEO were trans-anethole (65.4 %), fenchone (8.26 %), estragole (5.2 %) and limonene (4.2 %). Özcan et al. (2006) reported fenchone 23.46 estragole 61.08 and limonene 8.68 as the major constituents in the essential oil of ripe fruit from bitter fennel (F. vulgare ssp. piperitum) grown in Turkey. Kazemi et al. (2012) reported that the major components of FSEO were trans-anethole (17.19 %) and fenchone (4.03 %). Such variations in the chemical constituents of the essential oils might be attributed to the varied agroclimatic (geographical, climatic, seasonal) conditions of the regions, adaptive metabolism of plants and stage of maturity.

### Antifungal effects of FSEO

The MIC and MFC values indicated that FSEO was active against all the strains tested (Table 2), and exhibited strong antifungal activity. The MIC and MFC levels of FSEO against the five tested dermatophytes ranged from 0.039 to 0.078 µl ml⁻¹ and 0.078 to 1.56 µl ml⁻¹, respectively. For *T. rubrum* (10-0982, 10-0403, ATCC 40051) and *T. tonsurans* (10-0400), the MIC and MFC values were similar (Table 2). For *M. gypseum* 44693-1 and *T. mentagrophytes* 10-0060 strains, the MIC and MFC values were at the same level (Table 2). In recent years, the increasing social and health implications caused by dermatophytes have resulted in a constant striving to develop safe and new natural antifungal agents to cure human fungal disorders. In this study, FSEO showed pronounced antifungal efficacy against all the dermatophytes tested.

### Table 1. Chemical composition of the essential oil isolated by hydrodistillation from fennel seeds

<table>
<thead>
<tr>
<th>Peak no.</th>
<th>Compound</th>
<th>Composition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3-Methylbutanal</td>
<td>0.23</td>
</tr>
<tr>
<td>2</td>
<td>Phellandrene</td>
<td>0.21</td>
</tr>
<tr>
<td>3</td>
<td>Myrcene</td>
<td>0.16</td>
</tr>
<tr>
<td>4</td>
<td>Pinene</td>
<td>11.11</td>
</tr>
<tr>
<td>5</td>
<td>Limonene</td>
<td>1.09</td>
</tr>
<tr>
<td>6</td>
<td>3-Carene</td>
<td>1.44</td>
</tr>
<tr>
<td>7</td>
<td>(E,E,Z)-2,6-dimethyl-2,4,6-octatriene</td>
<td>0.59</td>
</tr>
<tr>
<td>8</td>
<td>Bicyclo[3.1.1]hept-3-en-2-ol, 4,6,6-trimethyl-</td>
<td>0.83</td>
</tr>
<tr>
<td>9</td>
<td>1,3-Cycloheptadiene</td>
<td>0.61</td>
</tr>
<tr>
<td>10</td>
<td>1-Methyl-4-(1-methylethyl)benzene</td>
<td>1.32</td>
</tr>
<tr>
<td>11</td>
<td>1-Octanol</td>
<td>0.15</td>
</tr>
<tr>
<td>12</td>
<td>Tetradecyloxiarane</td>
<td>0.59</td>
</tr>
<tr>
<td>13</td>
<td>Apiole</td>
<td>2.01</td>
</tr>
<tr>
<td>14</td>
<td>Fenchone</td>
<td>8.32</td>
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<tr>
<td>15</td>
<td>α-Terpineol</td>
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<tr>
<td>16</td>
<td>Camphor</td>
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<tr>
<td>17</td>
<td>Terpinen-4-ol</td>
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<td>18</td>
<td>Methylchavicol</td>
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<tr>
<td>19</td>
<td>2,3-Cyclohexen-1-methanol</td>
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</tr>
<tr>
<td>20</td>
<td>1,4-Dimethoxybenzene 1</td>
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<tr>
<td>21</td>
<td>1-Methoxy-4-(1-propenyl)benzene</td>
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<td>22</td>
<td>2-Caren-10-al</td>
<td>1.20</td>
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<tr>
<td>23</td>
<td>trans-Anethole</td>
<td>63.30</td>
</tr>
<tr>
<td>24</td>
<td>2-Methyl-5-(1-methylethyl)-2-cyclohexen-1-ol</td>
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</tr>
<tr>
<td>25</td>
<td>2-Methyl-5-(1-methylethyl)phenol</td>
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</tr>
<tr>
<td>26</td>
<td>Allantoic acid</td>
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</tr>
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<td>27</td>
<td>2-Cyclohexen-1-ol</td>
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<td>28</td>
<td>1,2-Dimethoxy-4-(1-propenyl)benzene</td>
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<tr>
<td>29</td>
<td>1-(3-Methoxyphenyl)-1-propanone</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>98.96</td>
</tr>
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Bold shows that main component in the FSEO.
FSEO showed inhibition of mycelial growth of dermatophytes in a dose-dependent manner (Fig. 1). In tests on *T. tonsurans* 10-0400, *T. rubrum* ATCC 40051, *T. mentagrophytes* 10-0060 and *M. gypseum* 44693-1 over a period of 15 days, FSEO exhibited significant effects when the concentration was above 0.312 μl ml⁻¹. However, the inhibition effect of FSEO lasted for different lengths of time on the four dermatophytes. For example, when using 0.312 μl FSEO ml⁻¹, the mycelia regrew at the 11th day for *T. rubrum* ATCC 40051, at the 7th day for *T. tonsurans* 10-0400, at the 8th day for *T. mentagrophytes* 10-0060 and at the 9th day for *M. gypseum* 44693-1 (Fig. 1). This indicated that the hyphae of *T. rubrum* ATCC 40051 may be more sensitive to FSEO than those of the other dermatophytes tested. The percentage inhibition of the mycelial growth was determined at the 15th day. FSEO in the range 0.019–0.312 μl ml⁻¹ showed 10.58–93.40, 9.04–93.17, 5.11–73.49 and 15.42–78.05 % inhibition of the mycelium growth of *T. rubrum* ATCC 40051, *T. tonsurans* 10-0400, *T. mentagrophytes* 10-0060 and *M. gypseum* 44693-1, respectively. FSEO at 0.625 μl ml⁻¹ exhibited 100 % inhibition of all fungi tested.

**Table 2.** MIC and MFC values of FSEO, FCZ and AmB against dermatophyte strains

<table>
<thead>
<tr>
<th></th>
<th>FCZ (μg ml⁻¹)</th>
<th>AmB (μg ml⁻¹)</th>
<th>FSEO (μl ml⁻¹)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>MIC</td>
<td>MFC</td>
<td>MIC</td>
</tr>
<tr>
<td><em>T. rubrum</em> ATCC 40051</td>
<td>50</td>
<td>100</td>
<td>0.156</td>
</tr>
<tr>
<td><em>T. rubrum</em> 10-0403</td>
<td>50</td>
<td>100</td>
<td>0.156</td>
</tr>
<tr>
<td><em>T. rubrum</em> 10-0982</td>
<td>50</td>
<td>100</td>
<td>0.156</td>
</tr>
<tr>
<td><em>T. tonsurans</em> 10-0400</td>
<td>25</td>
<td>50</td>
<td>0.312</td>
</tr>
<tr>
<td><em>M. gypseum</em> 44693-1</td>
<td>50</td>
<td>100</td>
<td>0.156</td>
</tr>
<tr>
<td><em>T. mentagrophytes</em> 10-0060</td>
<td>100</td>
<td>200</td>
<td>0.312</td>
</tr>
</tbody>
</table>

**Fig. 1.** Effects of different concentrations of FSEO on the colony growth of (a) *T. rubrum* ATCC 40051, (b) *T. tonsurans* 10-0400, (c) *T. mentagrophytes* 10-0060 and (d) *M. gypseum* 44693-1 in SDA. Plates were incubated at a temperature of 28 ± 2 °C for 15 days. Values plotted are means (n=3) ± SD.
Spores are usually difficult to kill, which results in repeated infection. Therefore, the effect of FSEO on spore germination was tested (Fig. 2). FSEO showed inhibitory effects on spore germination compared with the controls ($P < 0.01$). The inhibition calculated on the 10th day was found to be 97.02, 72.82 and 24.76% for *M. gypseum* 44693-1, 96.70, 70.73 and 24.67% for *T. rubrum* ATCC 40051, and 96.61, 74.78 and 23.35% for *T. tonsurans* 10-0400, while it was 96.46, 66.04 and 22.75% for *T. mentagrophytes* 10-0060, at the FSEO final concentrations.

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**Fig. 2.** Effects of different concentrations of FSEO, 0.156 μg AmB ml$^{-1}$ and 50 μg FCZ ml$^{-1}$ on spore germination of various fungal pathogens. Bars with different letters (a–d) within the same fungal pathogen differ significantly ($P < 0.01$). Values plotted are means ($n=3$) ± SD.

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**Fig. 3.** Effects of different concentrations of FSEO, 0.156 μg AmB ml$^{-1}$ and 50 μg FCZ ml$^{-1}$ on the mycelial growth of dermatophytes. Bars with different letters (a–d) within the same fungal pathogen differ significantly ($P < 0.05$). Values plotted are means ($n=3$) ± SD.
of 0.019, 0.039 and 0.078 µl ml⁻¹, respectively. The positive controls FCZ and AmB exhibited inhibition of spore germination at 50 and 0.156 µg ml⁻¹, respectively, while FSEO showed comparable activities at 0.039 µl ml⁻¹ and higher activities at 0.078 µl ml⁻¹ (Fig. 2).

Inhibition of fungal growth by FSEO was also estimated by measuring the mycelium dry weight (Fig. 3). FSEO significantly inhibited the growth of all four fungal pathogens in a dose-dependent manner ($P<0.05$). Similar to the results of MIC, MFC, radial mycelial growth and spore germination experiments, FSEO at 0.039 µl ml⁻¹ showed comparable activity to the positive controls FCZ (50 µg ml⁻¹) and AmB (0.156 µg ml⁻¹). The inhibitory rates of FSEO based on biomass were slightly higher than

**Fig. 4.** Efficacies of FSEO and FCZ in damage of the plasma membrane of *T. rubrum* ATCC 40051 cells. (a–f) Sequence of density plots showing *T. rubrum* ATCC 40051 cell size (forward scatter, FL2-H) analysed by flow cytometry in the FL2 channel (620 nm), and the respective percentages of PI-stained cells (upper right quadrant) for a series of samples treated with increasing concentrations of FSEO and FCZ. (a) Autofluorescence of non-treated cells; (b) fluorescence of non-treated cells stained with 1 mg PI ml⁻¹ for 30 min; (c) cells treated with 50 µg FCZ ml⁻¹; (d–f) cells treated with FSEO at 0.019 (d) 0.039 (e) and 0.078 µl ml⁻¹ (f); (g) effects of the FSEO and FCZ on the percentages of PI-stained *T. rubrum* ATCC 40051 cells analysed by flow cytometry for 12 h compared with untreated control. Bars with different letters (a–e) differ significantly ($P<0.05$). Values plotted are means ($n=3$) ± SD.
those calculated from the radial mycelial growth experiment, possibly because FSEO was more effective in liquid than in solid medium.

To sum up, FSEO significantly restricted the mycelial growth, spore germination and biomass of mycelium of the dermatophytes tested in a dose-dependent manner. In vitro studies on FSEO indicated its potential as an antifungal agent against dermatophytes. FSEO exhibited antifungal activities, which may be attributable to the presence of trans-anethole, pinene and fenchone (Dobrikov et al., 2014; Fujita & Kubo, 2004; Naeini et al., 2014).

**Mechanism of action of FSEO against dermatophytes**

Flow cytometry was used to investigate the membrane integrity of *T. rubrum* ATCC 40051 cells after treatment with FSEO, with PI as the fluorescent marker (Fig. 4). Substantial morphological changes were observed in the scattergrams of *T. rubrum* ATCC 40051 cells after 12 h incubation with 0.019, 0.039 and 0.078 μl ml⁻¹ FSEO (Fig. 4d–f), and the percentage of PI-stained *T. rubrum* ATCC 40051 cells increased along with the concentration. The percentages of stained cells with 0.078, 0.039 and 0.019 μl FSEO ml⁻¹ were 98, 87.3 and 18.4 %, respectively, suggesting that the cell membrane structure of *T. rubrum* ATCC 40051 was disrupted by FSEO in a dose-dependent manner. Under the same experimental conditions, 50 μg FCZ ml⁻¹ induced only 5.02 % cell death (Fig. 4c). These results indicated that the mode of action of FSEO involved primary damage of the cell membrane, leading to cell death. Furthermore, the effect of FSEO on the cell membrane might be different from that of FCZ.

Changes in the ultrastructure of *T. rubrum* ATCC 40051 were observed under TEM after treatment with FSEO (Fig. 5). For the control hyphae (Fig. 5a, b), the cell wall was uniform and completely surrounded by an intact fibrillar layer, the plasma membrane was uniform with a smooth surface, and the cytoplasmatic matrix was abundant. The main organelles, such as mitochondria and the nucleus, had normal and uniform structures. By contrast, in the FSEO-treated hyphae (Fig. 5c, d), ultrastructural alterations were conspicuous in the plasma membrane and cytoplasm. The plasmalemma became rough with continuous folding into the cytoplasm and detached from the cell wall. A decreased cytoplasmic matrix was also observed (Fig. 5d). Some mitochondria suffered extensive disruption of the internal structure, with a decrease in the mitochondrial cristae (Fig. 5c).

The above results indicated that the plasma membrane is a major target of the FSEO, and ergosterol is known to be the major sterol component of the fungal cell membrane and to maintain the cell function and integrity (Rodriguez et al., 1985); so the total ergosterol content in the plasma membrane of *T. rubrum* ATCC 40051 was determined after treatment with different concentrations of FSEO (0–0.078 μl ml⁻¹) (Fig. 6). The resulting ergosterol contents were 0.36 ± 0.21, 0.28 ± 0.19, 0.17 ± 0.06 and 0.13 ± 0.02 %, respectively, while the total ergosterol content of the positive control (FCZ at 50 μg ml⁻¹) was 0.24 ± 0.04 %. This demonstrated that the ergosterol content of the plasma membrane of *T. rubrum* ATCC 40051 was significantly reduced in a dose-dependent manner after treatment with different concentrations of FSEO. The decrease in ergosterol content after treatment with 0.039 μl FSEO ml⁻¹ (42.57 % decrease) was comparable to that with the positive control, 50 μg FCZ ml⁻¹ (49.49 % decrease), and ergosterol content was further decreased after treatment with 0.078 μl FSEO ml⁻¹ (76.16 % decrease) (Fig. 6). Other studies have also shown that plant-derived essential oils can cause a considerable reduction in the quantity of ergosterol (Khan et al., 2010; Pinto et al., 2009), which is consistent with this study. Thus, the plasma membrane of *T. rubrum* ATCC 40051 should be an important antifungal target of FSEO.

It could also be observed from the TEM images that the mitochondria of *T. rubrum* ATCC 40051 were damaged by FSEO. In order to confirm this, the effect of FSEO on the...
mitochondria of *T. rubrum* ATCC 40051 was assessed. The activities of mitochondrial SDH, MDH and ATPase of *T. rubrum* ATCC 40051 were significantly reduced (*P*<0.05) when treated with FSEO at all concentrations (Fig. 7). All of these reductions in activity by FSEO were dose-dependent.

Mitochondria are the main organelles for ATP synthesis. Mitochondrial dehydrogenases, including MDH and SDH, are very important enzymes in the biosynthesis of ATP (Lippold, 1982). MDH catalyses the interconversion of malate and oxaloacetate in the TCA cycle, and SDH catalyses the oxidation of succinate to fumarate in the TCA cycle and transfers the electrons from succinate to ubiquinol (Balietti et al., 2005). In this study, the mitochondrial MDH and SDH activities were measured using assay kits and the results showed that the mitochondrial MDH and SDH activities of *T. rubrum* ATCC 40051 were reduced after FSEO treatment. This suggested that the mitochondria of *T. rubrum* ATCC 40051 are another important antifungal target of FSEO. These results indicated that FSEO could cause damage to the plasma membrane of *T. rubrum* ATCC 40051 and also affect the enzyme activities of mitochondria, which might be the antifungal mechanism of FSEO.

In summary, FSEO was successfully extracted and analysed; its major components were trans-anethole, pinene and fenchone. FSEO showed antifungal activity against four dermatophytes tested, *T. tonsurans* 10-0400, *T. rubrum* 1.2

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**Fig. 6.** UV spectrophotometric profiles of sterol in *T. rubrum* ATCC 40051 treated with FSEO (0.019–0.078 µl ml⁻¹) and 50 µg FCZ ml⁻¹.

**Fig. 7.** Effects of FSEO on the activities of the SDH, MDH and T-ATPase of *T. rubrum* ATCC 40051. Bars with different letters (a–d) differ significantly (*P*<0.05). Values plotted are means *(n=3)* ± SD.
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ATCC 40051, *T. mentagrophytes* 10-0060 and *M. gypseum* 44693-1. It could inhibit the radial mycelial growth, spore germination and biomass increase of the fungi. Its mechanism of action was to cause damage to the plasma membrane and inhibit the enzyme activities of the mitochondria. With better antifungal activity than the commonly used antifungal agents and less likelihood of inducing drug resistance, FSEO is a potential natural antidermatophytic agent.
fennel (Foeniculum vulgare ssp. piperitum) fruit oils obtained during different vegetation. *J Med Food* 9, 552–561.


