Chemical composition, antibacterial activity and mechanism of action of essential oil from seeds of fennel (Foeniculum vulgare Mill.)

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ABSTRACT

Fennel (Foeniculum vulgare Mill.) is widely cultivated and used as a culinary spice. In this work, the chemical composition of the essential oil obtained by hydrodistillation of fennel seeds was analyzed by gas chromatography-mass spectrometry (GC-MS), and 28 components were identified. Trans-anethole (68.53%) and estragole (10.42%) were found to be the major components. The antibacterial activity, minimum inhibitory concentration (MIC), and minimum bactericide concentration (MBC) of essential oil against several food-borne pathogens were evaluated. The results showed that the gram positive and gram negative strains of bacteria had different sensitivities to essential oil of fennel seeds, the essential oil exhibited antibacterial activity against Staphylococcus albus, Bacillus subtilis, Salmonella typhimurium, Shigella dysenteriae and Escherichia coli according to the results of MIC and MBC. Among these bacteria, S. dysenteriae was the most sensitive to essential oil, showing the lowest MIC and MBC values of 0.125 and 0.25 mg/mL respectively. In addition, kill-time assay also showed that the essential oil had a significant effect on the growth rate of surviving S. dysenteriae. We concluded that the mechanism of action of the essential oil against S. dysenteriae might be described as essential oil acting on membrane integrity according to the results of the leakage of electrolytes, the losses of contents (proteins, reducing sugars and 260 nm absorbing materials) assays and electron microscopy observation.

1. Introduction

Food poisoning and food spoilage caused by microorganisms are still the most important issues facing the food industry and consumers (Sokmen et al., 2004; Walker, 1988), even in developed countries (Sokmen et al., 2004). To avoid food contamination the food industry has used synthetic additives to diminish microbial growth or inhibit microorganisms (Al-Reza, Rahman, Lee, & Kang, 2010; Bajpai, Baek, & Kang, 2012). However, because of consumers’ growing concerns over the safety of foods containing synthetic chemicals, much attention has been paid to use natural antibacterial products for food preservation (Alzoreky & Nakahara, 2003). Recently, spices have also received attention in their useful physiological functions and antimicrobial activity. There are a lot of reports about antimicrobial activity of spice extracts and its essential oils, and use of natural essential oils as antimicrobial agents in food systems may be considered as additional intrinsic determinant to increase the safety and shelf life of foods (Sagdic, Karahan, Ozcan, & Ozkan, 2003; Salgueiro, Martins, & Correia, 2010).

Fennel (Foeniculum vulgare Mill.) is a small genus of annual, biennial or perennial herbs distributed in central Europe and Mediterranean region. It is widely cultivated throughout the temperate and tropical regions of the world for its aromatic fruits, which are used as a culinary spice (Díaz-Maroto, Díaz-Maroto, Esteban, & Sanz, 2006; Rather, Dar, Sofi, Bhat, & Qurishi, 2012). Mature fennel fruit and its essential oil are used as flavoring agents in food products such as liqueurs, bread, pickles, pastries, and cheese. They are also used as a constituent in cosmetic and pharmaceutical products (Rather et al., 2012; Telci, Demirtas, & Sahin, 2009). There have been several reports on fennel oils, including reports on the relative concentration of fennel oil compounds considerably depending on the geographical origins (Díaz-Maroto et al., 2006), extraction methods (Díaz-Maroto, Díaz-Maroto, Sánchez-Palomo, & Pérez-Coello, 2005), maturation stages (Telci et al., 2009), and parts of the fennel (Díaz-Maroto et al., 2006; Guillén & Manzanos, 1996), and reports on various biological activities of the essential oil, such as hepatoprotective effect (Özbek et al., 2009).
et al., 2003), antioxidant activity (Ruberto, Baratta, Deans, & Dorman, 2000; Singh, Maurya, de Lampasona, & Catalan, 2006), antithrombotic activity (Tognolini et al., 2007), anti-inflammatory activity (Choi & Hwang, 2004), antiabetic activity (El-Soud et al., 2011), antitumour activity (Pradhan et al., 2008), acaridal activity (Lee, 2004). In addition, the essential oil of fennel seeds had significant antifungal activity (Singh et al., 2006; Soylu, Vigitbas, Soylu, & Kurt, 2007) as well as antibacterial activity (Dadadioglu & Evrendilek, 2004; Lo Cantore, Iacobellis, De Marco, Capasso, & Senatore, 2004; Ruberto et al., 2000). Although the chemical compositions and antimicrobical properties of essential oil from fennel have been studied, these informations are still limited. Most importantly, to the best of our knowledge, little work has been reported on the mechanism of action of essential oil from fennel seeds on the growth of microorganisms. Therefore, the aim of the present study was conducted to investigate the chemical composition and antibacterial activity of essential oil from fennel seeds on several food-borne pathogens, and to further evaluate the possible mechanism of action responsible for the antibacterial activity against sensitive strains by kill-time analysis, the permeability and integrity of cell membrane assays as well as scanning electron microscopy observation.

2. Material and methods

2.1. Plant materials

The *Foeniculum vulgare* grows in Yaodu County of Sichuan Province on 2012. Fennel seeds were harvested, and then obtained as a commercial product from the local market in mid-October, 2012. Dried whole fennel seeds were stored at −20 °C until analysis. The moisture content was 7.6% for fennel seeds, which was determined according to the method described by Xu, Hu, Duan, and Tian (2010) with some modifications. Briefly, fennel seeds about 10 g were dried using a laboratory oven at 110 °C for 4 h.

2.2. Chemicals

Dimethyl sulfoxide (DMSO), n-alkanes C8–C22 was purchased from Sigma (USA). Nutrient agar (NA), nutrient broth (NB) and tryptone soy agar mediums were from Beijing Aoboxing Bio-tech Co. Ltd. (Beijing, China). Other chemicals used were all of analytical grade.

2.3. Microbial strains and culture

The antimicrobial activity of the essential oil was tested against seven different microorganisms. Three Gram-positive strains were *Staphylococcus aureus* ATCC 25923, *Staphylococcus albus* ATCC 8799, *Bacillus subtilis* ATCC 6051, Four Gram-negative bacteria were *Salmonella typhimurium* ATCC 19430, *Pseudomonas aeruginosa* ATCC 9027, *Shigella dysenteriae* CMCC (B) 152252, and *Escherichia coli* ATCC 25922. These strains were provided by the College of Life Science, Shanxi Normal University, and stored with liquid paraffin wax at 4 °C. All strains were cultured at 37 °C on Nutrient agar (NA) or nutrient broth (NB) mediums.

2.4. Extraction of essential oil

The essential oil was extracted according to the method described by Viuda-Martos et al. (2011) with minor modifications. Briefly, the dried fennel seeds (500 g) were ground and hydrodistilled for 4 h using a Clevenger-type apparatus. The oily layer obtained on top of the aqueous distillate was separated and dried with anhydrous sodium sulfate. The oil obtained was stored in tightly closed dark vials and covered with aluminum foil at 4 °C until further analysis. The essential oil was obtained as a light yellow transparent liquid and had specific fennel aroma with a 1.74% (v/v) yield.

2.5. GC-FID analysis

The essential oil was analyzed using Hewlett-Packard 5890 II GC equipped with flame ionization detector (FID) and DB-5 capillary column (30 m × 0.25 mm; film thickness, 0.25 μm), whose injector and detector temperatures were maintained at 250 °C. The oven temperature was programmed from 40 °C for 2 min, raised to 250 °C at a rate of 3 °C/min, and isotherm at 250 °C for 5 min. Helium was the carrier gas, at a flow rate of 1 mL/min. A sample of 0.1 μL of essential oil was injected manually (in split mode 50:1).

2.6. GC–MS analysis

The analysis of the essential oil was performed using a Hewlett-Packard 5890 II GC, equipped with a DB-5 MS capillary column (30 m × 0.25 mm; film thickness, 0.25 μm) and a HP 5972 mass selective detector for the separation. The mass selective detector was operated in electron-impact ionization (EI) mode with a mass scan range from m/z 50 to 350 at 70 eV. GC conditions were the same as described above. The retention indices were calculated, for all volatile constituents using a homologous series of n-alkanes C8–C22. The essential oil constituents were identified by comparing their GC retention indices, mass spectra with publish data (Adams, 2001, pp. 1–40) and National Institute of Standards and Technology mass spectra library data provided by the software of GC–MS system. Essential oil components are reported as a relative percent of the total oil by peak area.

2.7. Antimicrobial activity

The essential oil was dissolved in DMSO and sterilized by filtration through 0.22 μm Millipore filters. Antimicrobial tests were then carried out by the Oxford cup method (Wang, Tang, Jiang, Fang, & Liu, 2006) using 100 μL of suspension containing 1 × 107 colony forming units (CFU)/mL of bacteria determined by blood count assay spread on nutrient agar (NA) medium. Oxford cups (6 mm in diameter) were placed on the inoculated agar, and then 100 μL of essential oil was added with a micropipette. The diameter of inhibition zone (DIZ) was measured after 24 h of incubation at 37 °C, and DMSO was used as a negative control. Tests were performed in triplicate.

2.8. Minimum inhibitory concentration (MIC) and minimum bactericide concentration (MBC)

MIC and MBC were determined according to the method described by Kubo, Fujita, Kubo, Nihei, and Ogura (2004) with minor modifications. Briefly, stock solution of essential oil was prepared in DMSO. Two fold serial dilutions of essential oil were filtered through 0.22 μm Millipore filters and prepared in sterile NB medium ranging from 0.125 to 10 mg/mL. To each tube, 50 μL of the inoculum containing approximately 1 × 107 CFU/mL microorganisms determined by blood count assay were added. A control test containing inoculated broth supplemented with only DMSO was also performed. The tubes were then incubated at 37 °C and examined for evidence of the growth. The MIC was determined as the lowest concentration of the essential oil that demonstrated no visible growth for incubating for 24 h, while the MBC was the lowest concentration of the test essential oil that showed no visible growth in the culture incubating at 37 °C for 48 h.
2.9. Kill-time analysis

The kill-time curve assay method was used to investigate the bactericidal effects of the essential oil according to the method described by Kong et al. (2008). After incubated at 37 °C for 10 h, S. dysenteriae strains were separated by centrifugation at 5000 rpm for 10 min. Then the bacteria were washed with 5% of glucose until their electric conductivities were near to that of 5% glucose, and they were the case for isotonic bacteria. The essential oils at two different concentrations (MIC, and 2 × MIC) were added to 5% glucose and the electric conductivities of the mixtures were marked as L2. Then different concentrations of essential oils were added into the isotonic bacteria solution. After completely mixed, the samples were incubated at 37 °C for 8 h, and then the conductivities were measured and marked as L2. The conductivity of bacteria in 5% glucose treated in boiling water for 5 min was served as the control and marked as L0. The permeability of bacteria membrane is calculated according to the formula, the relative electric conductivity (%) = 100 × (L2 − L1)/L0.

2.10. Membrane cell permeability

The permeability of bacteria membrane is expressed in the relative electric conductivity and determined according to the method described by Du, Sun, Liang, Han, and Yu (2012), with slight modifications. Cells from the 100 ml working culture of tested microorganisms were collected by centrifugation for 15 min at 5000 rpm, washed three times, and resuspended in 0.1 M phosphate buffer solution (PBS, pH 7.4). One hundred milliliters of cell suspension were incubated at 37 °C under agitation for 4 h in the presence of essential oil at three different concentrations (control, MIC and 2 × MIC). Then, 25 mL of samples were collected and centrifuged at 11,000 g for 5 min. And then the concentrations of proteins and reducing sugars in supernatant were determined according to the method described by Xu, Hu, Wang, et al. (2010). In addition, to determine the concentration of the released constituents consisting largely of nucleic acids, 3 mL supernatant was used to measure UV absorption at 260 nm. Correction was made for the absorption of the suspension with the same PBS containing the same concentration of essential oil after 2 min of contact with tested strains. The untreated cells were corrected with pH 7.4 PBS.

2.11. Integrity of cell membrane

The cell integrity of S. dysenteriae strains is examined by determining the release of cell constituents into supernatant according to the method described by Du, Sun, Liang, Han, and Yu (2012), with slight modifications. Cells from the 100 ml working culture of tested microorganisms were collected by centrifugation for 15 min at 5000 rpm, washed three times, and resuspended in 0.1 M phosphate buffer solution (PBS, pH 7.4). One hundred milliliters of cell suspension were incubated at 37 °C under agitation for 4 h in the presence of essential oil at three different concentrations (control, MIC and 2 × MIC). Then, 25 mL of samples were collected and centrifuged at 11,000 g for 5 min. And then the concentrations of proteins and reducing sugars in supernatant were determined according to the method described by Xu, Hu, Wang, et al. (2010). In addition, to determine the concentration of the released constituents consisting largely of nucleic acids, 3 mL supernatant was used to measure UV absorption at 260 nm. Correction was made for the absorption of the suspension with the same PBS containing the same concentration of essential oil after 2 min of contact with tested strains. The untreated cells were corrected with pH 7.4 PBS.

2.12. Scanning electron microscope (SEM)

To determine the efficacy of the essential oil and the morphological changes of S. dysenteriae strains, SEM observation was performed on the tested bacteria. The bacteria cells were incubated in nutrient broth at 37 °C for 10 h. The suspensions were added 0 ×, 1 × and 2 × MIC of essential oil, respectively; control culture was left untreated. Next the suspensions were incubated at 37 °C for 4 and 8 h respectively, and then the suspensions were centrifuged. The precipitated cells were washed twice with 0.1 M PBS (pH 7.4) and fixed with 2.5% (v/v) glutaraldehyde in 0.1 M PBS overnight at 4 °C. After this, the cells were dehydrated using sequential exposure per ethanol concentrations ranging from 30 to 100% and the ethanol was replaced by tertiary butyl alcohol at last. Then, cells after centrifugation were dried at “critical point” in liquid CO2 under 95 bar pressure, and samples were gold-covered by cathodic spraying. Finally, morphology of the bacterial cells was observed on a scanning electronic microscope (JSM-7500F, JEOL Ltd., Japan).

2.13. Statistical analysis

One-way analysis of variance (ANOVA) and Duncan’s multiple range tests were carried out to determine significant differences (p < 0.05) between the means by Data Processing System (DPS, version 7.05) and EXCEL program.

3. Results

3.1. Chemical compositions of the essential oil

The essential oil was obtained by hydrodistillation of air-dried sample with a yield of 1.74% (v/w). The chemical compositions of essential oil were analyzed by GC and GC–MS and the result was presented in Table 1. In total, 28 components were identified, representing 95.8% of the total amount. Trans-anethole (68.53%), a phenylpropanoid, was found as the main component. Estragole (10.42%) was the second major phenylpropanoid detected in fennel oil, followed by limonene (6.24%), fenchone (5.45%), and others were found to be the minor components in the essential oil of fennel seeds. The profile obtained in the present study was very similar to the previous results reported by Viuda-Martos et al. (2011) who identified 20 components and also found that trans-anethole (65.59%), estragole (13.11%), limonene (8.54%) and fenchone (7.76%) were the predominant components.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>RI*</th>
<th>RI*</th>
<th>Peak area (%)</th>
<th>Id*</th>
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</thead>
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<tr>
<td>Styrene</td>
<td>893</td>
<td>893</td>
<td>0.05</td>
<td>GC–MS, KI</td>
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<tr>
<td>6-Thuene</td>
<td>928</td>
<td>927</td>
<td>Trace</td>
<td>GC–MS, KI</td>
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<tr>
<td>6-Pine</td>
<td>939</td>
<td>938</td>
<td>0.42</td>
<td>GC–MS, KI</td>
</tr>
<tr>
<td>Camphene</td>
<td>952</td>
<td>950</td>
<td>Trace</td>
<td>GC–MS, KI</td>
</tr>
<tr>
<td>Sabine</td>
<td>975</td>
<td>972</td>
<td>Trace</td>
<td>GC–MS, KI</td>
</tr>
<tr>
<td>6-Pine</td>
<td>979</td>
<td>976</td>
<td>0.35</td>
<td>GC–MS, KI</td>
</tr>
<tr>
<td>Myrcene</td>
<td>991</td>
<td>990</td>
<td>0.21</td>
<td>GC–MS, KI</td>
</tr>
<tr>
<td>6-Phellandrene</td>
<td>1003</td>
<td>1006</td>
<td>0.12</td>
<td>GC–MS, KI</td>
</tr>
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<td>o-Cymene</td>
<td>1026</td>
<td>1026</td>
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<td>GC–MS, KI</td>
</tr>
<tr>
<td>Limonene</td>
<td>1029</td>
<td>1031</td>
<td>6.24</td>
<td>GC–MS, KI</td>
</tr>
<tr>
<td>6-3-Carene</td>
<td>1031</td>
<td>1032</td>
<td>1.21</td>
<td>GC–MS, KI</td>
</tr>
<tr>
<td>Fenchone</td>
<td>1087</td>
<td>1090</td>
<td>5.45</td>
<td>GC–MS, KI</td>
</tr>
<tr>
<td>2, 4, 6-Octatriene, 3, 4-dimethyl</td>
<td>1130</td>
<td>1130</td>
<td>0.14</td>
<td>GC–MS</td>
</tr>
<tr>
<td>Camphor</td>
<td>1146</td>
<td>1146</td>
<td>0.16</td>
<td>GC–MS, KI</td>
</tr>
<tr>
<td>p-Menth-1-en-4-ol</td>
<td>1177</td>
<td>1177</td>
<td>0.27</td>
<td>GC–MS, KI</td>
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<tr>
<td>Estragole</td>
<td>1200</td>
<td>1202</td>
<td>10.42</td>
<td>GC–MS, KI</td>
</tr>
<tr>
<td>Fenchyl acetate</td>
<td>1233</td>
<td>1230</td>
<td>0.08</td>
<td>GC–MS, KI</td>
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<tr>
<td>p-Anisaldehyde</td>
<td>1250</td>
<td>1252</td>
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<tr>
<td>cis-anethole</td>
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<td>1258</td>
<td>0.47</td>
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<tr>
<td>Trans-anethole</td>
<td>1285</td>
<td>1288</td>
<td>68.53</td>
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<td>1-(p-Methoxyphenyl)-2-propanone</td>
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<td>GC–MS, KI</td>
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<td>(E)-β-Farnesene</td>
<td>1457</td>
<td>1458</td>
<td>0.09</td>
<td>GC–MS, KI</td>
</tr>
<tr>
<td>1-(3-methoxyphenyl)-1-propanone</td>
<td>1462</td>
<td>1462</td>
<td>Trace</td>
<td>GC–MS</td>
</tr>
<tr>
<td>A germacrene</td>
<td>1509</td>
<td>1502</td>
<td>0.21</td>
<td>GC–MS, KI</td>
</tr>
<tr>
<td>Cadina-3(2), 4-diene</td>
<td>1435</td>
<td>1537</td>
<td>0.04</td>
<td>GC–MS, KI</td>
</tr>
<tr>
<td>(E)-Nerolidol</td>
<td>1563</td>
<td>1562</td>
<td>Trace</td>
<td>GC–MS, KI</td>
</tr>
</tbody>
</table>

* RL, the retention index published by Adams.  
  b RI, the retention index was calculated for all volatile constituents using a homologous series of n-alkanes C8–C22 on DB-5 column.  
  c Peak area obtained by GC-FID.  
  d Identification methods: Trace (≤0.01%).
major compounds in the essential oil from Egyptian fennel. Telci et al. (2009) reported a higher value for trans-anethole (84.12%) and lower for estragole, limonene and fenchone (4.19%); 2.96–4.69%; 1.17–2.65%, respectively) of sweet fennel cultivated in Turkey. Díaz-Maroto et al. (2005) reported that fennel oil extracted by simultaneous distillation-extraction (SDE) and supercritical fluid extraction (SFE) showed similar compositions, with trans-anethole, estragole, and fenchone as the main components; but there were significant differences in the contents between SDE and SFE. However, Napoli, Curcuruto, and Ruberto (2010) reported that the main constituents from wild Sicilian fennel were estragole, followed by oxygenated monoterpene, fenchone, which was very different with the chemical compositions obtained in this study. These differences in components and its content of essential oil from fennel may be concerned in the geographical origins (Díaz-Maroto et al., 2006), cultivated varieties, and maturity of fennel fruits, as well as extraction methods (Díaz-Maroto et al., 2005) and analysis conditions of the essential oil.

3.2. DIZ, MIC and MBC of the essential oil

The DIZ, MIC, and MBC values of the essential oil from fennel seeds are presented in Table 2. The results showed that the essential oil had certain antibacterial activity on all of the tested food-borne pathogens, including both Gram-positive and Gram-negative bacteria. The DIZ values for all tested bacterial strains were in the range of 11.5–20.2 mm. The DIZ was the maximum value for S. typhimurium, followed by E. coli, S. dysenteriae, and S. albus, however, no difference in DIZ values was found among these bacterial strains. In contrast with above strains, although the DIZ values were lower for B. subtilis, P. aeruginosa and S. aureus, we could not deny that the essential oil from fennel seeds had a better antibacterial activity on these food-borne pathogens because the DIZ values were much higher than that of negative control. The MIC and MBC values for tested bacterial strains were in the range of 0.125–0.25 mg/mL and 0.25–0.50 mg/mL, respectively. Unfortunately, the MIC and MBC values of the essential oil for P. aeruginosa and S. aureus strains have not been gained when the concentration of essential oil reached the maximum in method system tested. Of these bacteria, the essential oil from fennel seeds performed both a minimum MIC of 0.125 mg/mL and a minimum MBC of 0.25 mg/mL against S. dysenteriae, which indicated it was the most effective bacterial inhibitor and bactericide against S. dysenteriae. Therefore, the antibacterial properties and mechanism of action of essential oil from fennel seeds against S. dysenteriae will be further investigated in this study.

3.3. Kill-time analysis

Based on the sensitivity of the tested food-borne pathogens, one Gram-negative bacterium (S. dysenteriae CMCC (B) 51252) was selected as the model organisms for further study to confirm the antibacterial mode of action of essential oil from fennel seeds. The effect of the essential oil on the viable counts of tested bacterial pathogen is shown in Fig. 1. As observed in Fig. 1, compared to the control, susceptible S. dysenteriae treated with the essential oil at the MIC value showed a slow decrease in the number of viable cells over the first 12 h period of the test, and the number of viable cells decreased by 11.67% from 6.0 to 5.3 log_{10} CFU/mL at the cultivation time of 12 h. Subsequently, an increase in cell numbers was detected and increased to 6.3 log_{10} CFU/mL, which showed that concentrations of the essential oil lower than MIC did not completely inhibit growth of S. dysenteriae but did increase the lag time in the growth curve (Fig. 1). Unlike the changing trend of the number of viable cells at 1 × MIC, in treatments at 2 × MIC, the number of viable cells decreased obviously from the first hour after cultivation and decreased by 97.5% to 0.15 log_{10} CFU/mL over 24 h of incubation. These results showed that the treatment time and concentration of essential oil had great influences on antibacterial effects.

3.4. Cell membrane permeability

Further antibacterial mode of action of essential oil against the tested food-borne pathogens was confirmed using the assay of cell membrane permeability. Fig. 2 showed the effect of essential oil from fennel seeds on the membrane permeability of S. dysenteriae. There was little change in the relative electric conductivity of the control during the first 12 h period of the test, and then an increase in the relative electric conductivity was found, which may be due to normal lysis and death of bacteria, resulting in the rise in the relative electric conductivity. Compared to the control, the relative electric conductivity of the suspensions increased immediately after the addition of essential oil at greater than or equal to MIC concentration and it also increased rapidly with the increasing treatment time and concentration of essential oil. It meant that the permeability of bacteria membrane would be increased correspondingly, which caused the leakage of intracellular ingredient, especially losses of electrolytes including K⁺, Ca²⁺, Na⁺ and so on.

3.5. Integrity of cell membrane

The integrity of cell membrane were determined by the measurement of the release of cell constituents including protein, reducing sugar and the absorbance at 260 nm of the supernatant of tested bacteria. Table 3 showed the results when S. dysenteriae were
treated with different concentrations of essential oil from fennel seeds for 4 h, respectively. The results indicated that after adding the corresponding essential oil to strains, the cell constituents’ release increased significantly with the increased concentration of the essential oil. Compared to control, the concentration of proteins, reducing sugars and cell constituents (OD260nm) in suspensions treated with 1 × MIC essential oil increased by 8.11, 2.43, 10.63 times respectively, while they increased by 13.03, 4.07, 16.64 times respectively when treatment at 2 × MIC. These results indicated that the irreversible damage to the cytoplasmic membranes might occur, which led to the losses of cell constituents such as protein and some essential molecules and to cell death.

3.6. Electron microscope observation

The S. dysenteriae bacteria were treated with the essential oil from fennel seeds at 1 × and 2 × MIC for 4, 8 h respectively, and then the morphological and physical changes of treated S. dysenteriae bacteria were observed by SEM. Fig. 3 showed the SEM images of the treated and untreated bacteria. These images directly illustrated the destructive effects of the essential oil on the tested bacteria. The surfaces of the treated strains underwent obvious morphological changes compared with the untreated controls. Untreated cells were rod shaped, regular, and intact (Fig. 3, a, and b), while some bacterial cells treated with the essential oil became deformed, pitted, shriveled, adhesive to each other and parts of the cell were broken (Fig. 3, A4, A8, B4, and B8), which may give rise to the leaching out of nutrient and genetic materials. And the changes were more frequent, evident with the increase of concentration and treatment time of essential oil. This supported the results of the kill-time study, permeability and integrity of cell membrane assays, and indicated that the essential oil from fennel seeds may have severe effects on the cell wall and cytoplasmic membrane. However, these changes in more details still need to be further observed by transmission electron microscopy (TEM).

4. Discussion

It is generally known that the growth of food spoilage and food-borne pathogens can decrease nutritional quality of the food by consuming nutrients including fat, protein and carbohydrate that are present in the food, subsequently causes food discoloration, mustiness, biochemical changes, weight loss and toxicity, which can adversely affect the health of humans. The method of microbial growth inhibition most appropriate to food is the use of food preservatives. Essential oils are volatile and odorous principles of plant secondary metabolism which have wide applications in food flavoring and preservation industries (Bajpai et al., 2012). Recently, some researchers have reported that monoterpenic or sesquiterpene hydrocarbons and their oxygenated derivatives, which are the major components of essential oils, exhibit potential antimicrobial activities (Cakir, Kordali, Zenghin, Izumi, & Hirata, 2004). These findings strongly supported the results of this study as the essential oil from fennel seeds was also found to contain these components, which confirmed its efficacy as natural antimicrobial agent.

The results from the Oxford cup method, followed by measurement of MIC and MBC indicated that the essential oil from fennel seeds had strong and consistent inhibitory effects against all of the tested food-borne pathogens including both Gram-positive and Gram-negative bacteria as confirmed by the inhibitory effect of essential oil showing different susceptibility rate against the tested food-borne pathogens, and S. dysenteriae was found to be the most sensitive microorganism tested, showing larger inhibition zone and the lowest MIC and MBC values. Some studies reported that the essential oil extracted from fennel seeds exhibited antibacterial effect against food-borne pathogens such as E. coli, Bacillus megaterium, S. aureus (Dadalioglu & Evrendilek, 2004; Mohsenzadeh, 2007); Ozcan, Chalchat, Arslan, Ates, and Unver (2006) also found that fennel essential oils possessed an inhibitory effect against a wide range of Bacillus species, which supported our results in the present study, indicating that essential oil of fennel seeds was a potent bacterial inhibitor with a broad antibacterial spectrum. Furthermore, the effects of essential oil on the growth of S. dysenteriae were investigated by measuring the viable cell counts and the results revealed that exposure of essential oil had a rigorous effect on the cell viability of the tested bacterial pathogens. A minor amount of essential oil could prolong the lag phase of S. dysenteriae, while the essential oil at 2 × MIC exerted its maximum bactericidal activity as evident by the significant reduction in microbial counts and complete inhibition of S. dysenteriae cell viable counts at 24 h exposure, which indicated that the essential oil have perfect antibacterial activity. Besides, the effects of essential oil on the growth of other bacterial strains except for S. aureus and P. aeruginosa were also investigated and the results were similar to that on S. dysenteriae, where they differed was that the extent of changes in the number of viable cells and the time of controlling the lag phase were different for different bacteria treated by essential oil (not shown). Similar to our findings, other essential oils from plants also exhibited inhibitory effects against various food-borne pathogens (Al-Reza et al., 2010; Bajpai, Sharma, & Baek, 2013).

A number of authors have mentioned the antimicrobial activity of essential oils, however, the mechanism of action has not been studied in great detail (Tajkarimi, Ibrahimia, & Cliver, 2010). The previous findings showed that terpenes, phenols, aldehydes and ketones are the major components of essential oils (Ceylan & Fung, 2004), and it is generally believed that essential oils principally
permeability barrier to the passage of small ions such as K⁺ concentrations. The bacterial plasma membrane provides a

motility (Cox et al., 2001). Therefore, even relatively slight changes to the structural integrity of cell membranes can detrimentally affect cell metabolism and lead to cell death (Cox et al., 2001). The results in this study showed that the relative electric conductivity of the suspension increased rapidly with the increasing treatment time and concentration of essential oil, which meant that the permeability of bacteria membrane would be increased correspondingly, causing the leakage of electrolytes and leading to cell death.

Another apparent antimicrobial mode of action of essential oil was visualized by the confirmation on the leakage of protein, reducing sugar and 260 nm absorbing materials when the tested food-borne pathogens exposed to the essential oils at 1 × and 2 × MIC concentrations. The macromolecules of a bacterial cell including protein, nucleic acids which reside throughout the interior of the cell, in the cytoplasm, are the key structural components. The transfer of cellular information through the processes of translation, transcription and DNA replication occur within the same compartment and can interact with other cytoplasmic structures (Kohanski, Dwyer, & Collins, 2010). Measurement of specific cell leakage markers including proteins and 260 nm absorbing materials is an indicator of membrane integrity to specific antimicrobial agent in relationship to unexposed cells (Bajpai et al., 2013). Our experimental results showed that the essential oil of fennel seeds apparently caused rapid losses of proteins, reducing sugars and 260 nm absorbing materials from the treated bacteria, which indicated that irreversible damage to the cytoplasmic membranes occurred, which was in accordance with the results of SEM. So it could be conferred that integrity of cell membrane would also be an important factor to inhibit the bacterial growth. But it is still a mystery where the damage takes place, on the lipopolysaccharide or membrane proteins in cell wall.

The SEM micrograph of S. dysenteriae cells treated with the essential oil of fennel seeds showed that severe morphological alterations appeared in the cell wall and membrane, and the SEM micrograph of treated cells showed many fragmentary bacteria, which have also been observed for various kinds of tested organisms when treated with different essential oils (Bajpai et al., 2013; Du et al., 2012). The changes in physical and morphological of bacterial cells might have occurred due to the effect of essential oil on the permeability and integrity of membrane, thereby resulting in the lysis of bacterial cell wall followed by the losses of intracellular dense materials on the surface of treated cells. However, as state before, a more detail cell wall changes still need to be further observed by TEM.

5. Conclusion

Based on the present research, the essential oil from fennel seeds, which was rich in trans-anethole, possessed good...
antibacterial activity against selected food-borne pathogens in this study. We concluded that the mechanism of action of essential oil from fennel seeds against S. dysenteriae may be described as essential oil making firstly a break through the permeability of cell membrane associated with generalized the integrality of membrane-disrupting effects, leading to the leakage of electrolytes as well as losses of proteins, reducing sugars, and 260 nm absorbing materials. These changes resulted in cell decomposition and death eventually, and this corresponded to a simultaneous reduction in the number of viable bacteria. Moreover, the SEM observation also supported the above hypothesis. However, because of the heterogeneous compositions of essential oils, it seems unlikely that there is only one mechanism of action or that only one component is responsible for the antimicrobial action. Therefore, further research is still necessary to fully understand the mechanisms involved including against other food-borne pathogens, TEM observation as well as the interactions with other food ingredients in order to justify the real applications of essential oil from fennel seeds in food practices as a natural antibacterial agent.

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References
antibacterial activities of essentials oils obtained from Egyptian aromatic plants.


