Introduction

Staphylococcus aureus as one of the most common bacteria is associated with some worldwide incidents of foodborne intoxication (1). The primary causes of staphylococcal food poisoning have been traced to the personnel who contaminated food products during improper handling and preparation (2). Other sources of contamination in meat industry could be attributed to the utilised equipment and materials, such as improperly cleaned and sanitised meat grinders, knives, saw blades, cutting boards and food storage containers (3).

Meat products can be considered as a potential environment for the growth of pathogens; therefore, in the meat industry application of the natural and synthetic preservatives is unavoidable (4). However, the incorpora-
tion of essential oil as an alternative to synthetic antimicrobials in food encountered some drawbacks and limitations. For instance, to inhibit spoilage in food matrices, the addition of high concentration of antimicrobial agents is required, which often exceeds the acceptable flavour threshold of consumers (5).

The plant Echinophora platyloba belongs to the family Apiaceae (or Umbelliferae), which is commonly used as a flavouring agent in traditional cheese and yogurt products, particularly in Iran (6). Echinophora platyloba is also added to traditional beef stews due to the unique and delicate flavour. Some studies have been carried out to analyse the extracted essential oils from different geographical origins and with various morphologies and genetics (7). These studies have demonstrated several biological properties such as antimicrobial, antioxidant and anti-inflammatory activities. β-Ocimene has been identified as the main constituent of the investigated E. platyloba from various origins (8).

The smoking of foods, in particular meat, has been introduced as a common method for centuries (9). Liquid smoke application has been increasingly widespread as a suitable replacer for traditional wood smoking due to several advantages, such as more efficient control of polycyclic aromatic hydrocarbon (PAH) content, in which their constituents can be determined by rapid methods (10), better applicability to different food systems and lower environmental pollution (11).

In order to extend the shelf life and improve the food safety, food manufacturers are using different combinations of technologies such as smoking, vacuum packaging, cold storage and natural preservatives such as essential oils (12). The antimicrobial activity of two essential oils has been quantified in a previous study by the microdilution checkerboard method (13).

To our knowledge, no study has been conducted to determine the synergistic or antagonistic interactions between the extracts of essential oils and liquid smoke. Also, despite the vast application of both fresh and dried aerial parts of E. platyloba plant in the Iranian cuisine, no research has been carried out on essential oil applications in food with the evaluation of its acceptable sensory level. The aim of this study is to investigate the chemical composition of E. platyloba essential oil and the PAH content. Also, in vitro antimicrobial properties of E. platyloba essential oil and liquid smoke, alone and in combination, against Staphylococcus aureus in minced meat samples were evaluated taking into consideration their acceptable sensory levels. The treated cultures were spectrophotometrically monitored in order to determine the mechanism of antimicrobial action and bacterial growth kinetics.

Materials and Methods

Materials

Lyophilised culture of Staphylococcus aureus ATCC 29213 was obtained from the Pasteur Institute of Iran, Tehran, Iran.

The Echinophora platyloba plant was collected from Hamadan in a temperate mountainous region of Iran, taxonomically verified and the voucher specimen was deposited (TMRC no. 3720) in the herbarium of Traditional Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran. The aerial parts (leaves and stems) were collected in the spring season (21 and 22 May 2014) and were exposed to air in a well sheltered and ventilated area for ten days until the moisture was removed.

The commercial liquid smoke was supplied by Red Arrow® Company, Tehran, Iran. Total acidity was 11.2, pH=2.8, ω(carbonyl)=12.4 % and γ(phenols)=7.6 mg/mL.

Minced beef samples (200 g) obtained from a local butcher’s shop were prepared as follows: 0.03, 0.04, 0.05 or 0.06 g of E. platyloba and 0.5, 0.6, 0.7 or 0.8 g of liquid smoke were added per 100 g of meat. Meat samples without the added essential oil and liquid smoke were used as control.

Essential oil extraction

Dried aerial parts of Echinophora platyloba were pulverised to obtain fine powder, which was then hydrodistilled for 4 h. In each distillation, 100 g of E. platyloba powder were hydrodistilled in a round-bottomed flask (2000 mL) fitted to the Clevenger-type apparatus (built by R&D section of Shahid Beheshti University, Tehran, Iran) with an ice-water-cooled condenser on top of it. Anhydrous sodium sulphate (Fisher Scientific®, Loughborough, UK) was used to absorb moisture to avoid any possible change of essential oil. Essential oil yield (Y(%)) was calculated as follows:

\[ Y = \frac{V}{m} \times 100 \]

where \( V \) is the volume of the obtained essential oil (mL), and \( m \) is the mass of the sample (100 g of dried plant).

Plant chemical composition analysis

The essential oil composition was analysed by using gas chromatograph/mass selective detector (GC/MSD) (models 7890 GC and 5975 MSD; Agilent Technologies, Santa Clara, CA, USA) with an HP-5 capillary column (30 m×250 μm, 0.25 μm film thickness), equipped with a split-splitless injection port. The temperature cycle included an initial temperature of 50 °C (isothermal, 5 min) first increased by 5 °C/min to 150 °C and then by 10 °C/min to 300 °C. The 270 °C temperature (isothermal) was kept for 5 min. The helium as the carrier gas was maintained at a constant flow of 0.5 mL/min. The essential oil was diluted in n-hexane. Injection volumes were 2 μL. The components were identified by matching their mass spectra with standards of principal components in Wiley and NIST library data, mostly from the literature (14,15). The component concentration was obtained by semi-quantification of peak area integration from GC peaks and by applying the correction factors.

Determination of liquid smoke chemical composition

The liquid smoke used in the conducted experiments was analysed using the same GC-MS as mentioned above and according to the recommended temperature program (10): first at 150 °C for 2 min and then the temperature
was increased by 7 °C/min to 200 °C, held for 1 min, and then increased by 5 °C/min to 250 °C and held for 1 min. Finally, it was increased rapidly by 20 °C/min to 290 °C and kept for 10 min. The helium was used as the carrier gas at a constant flow of 0.8 mL/min. The injector and auxiliary temperatures were set to 290 and 280 °C, respectively. Liquid smoke was diluted in methanol. Injection volumes of 2 μL were injected in a split mode with the split ratio of 1:50. Finally, the quantification of the compounds was carried out in the selected ion monitoring (SIM) mode and a qualifier ion was picked for each compound.

Minimum inhibitory concentration and minimum bactericidal concentration

The minimum inhibitory concentration (MIC) values as the lowest concentration of the antimicrobial agent that can inhibit the bacterial growth was determined by the lack of visual turbidity (16). Stock solutions of *E. platyloba* and liquid smoke were prepared individually. The highest concentration was diluted to 100 000 ppm. Fractional dilutions of stock solution from 1000 to 30 000 ppm were prepared using tryptic soy broth (TSB; Merck Millipore, Darmstadt, Germany) and Tween 80 (Sigma-Aldrich, St. Louis, MO, USA). To prepare the stock solution, the pure essential oil and liquid smoke were dissolved in 5 % (by volume) of Tween 80, which was used as an emulsifying agent for dispersing essential oil and liquid smoke in the culture medium. Turbidity was measured in two 100-well honeycomb microplates using Bioscreen C analyzer (Oy Louis, MO, USA). To prepare the stock solution, the pure essential oil and liquid smoke were dissolved in 5 % (by volume) of Tween 80, which was used as an emulsifying agent for dispersing essential oil and liquid smoke in the culture medium. Turbidity was measured in two 100-well honeycomb microplates using Bioscreen C analyzer (Oy Growth Curves Abt Ltd, Helsinki, Finland). Changes in absorbance vs. time (18 h) were at λ=260 nm (wavelength range) and microbiological growth curves were obtained. A homogenous mixture in individual wells was provided by linear shaking every 15 min before measurements. Population density of 10^5 cells/mL of *S. aureus* was obtained by adding serial dilutions from overnight cultures to TSB medium. Triplicate plates with 5·10^6 CFU/mL of *S. aureus* were prepared by adjusting the absorbance to A_{\text{abs}}=0.3. The bacterial suspension was diluted to 10^7 cells/mL, and then 50 μL were added to 300 μL of solution in each well. The last two wells served as positive and negative controls, which confirmed the viability of *S. aureus* culture and the stability of working conditions and solutions, respectively. Experiments were repeated for minimum bactericidal concentration (MBC) determination. A loop full of the content of each clear tube that did not show visible growth, and of the control tube (which contained only TSB and Tween 80 without any antibacterial agent) were spread over a quarter of the plate on tryptic soy agar (TSA; Merck Millipore) medium. The plate was incubated at 37 °C overnight.

Antimicrobial activity of oil and liquid smoke assayed by disk diffusion method

Disk diffusion method was performed as the preliminary assay for the determination of antibacterial activity of essential oil in combination with liquid smoke. The test was carried out in sterile Petri dishes (100 mm in diameter) containing 20 mL of Mueller-Hinton agar medium (HiMedia, Mumbai, India). The combinations of different volumes of each, essential oil and liquid smoke (5, 10, 15 and 20 μL) were applied separately to sterile filter paper discs (6 mm in diameter; HiMedia). The essential oil and liquid smoke fixed on sterile paper discs were placed on the surface of the medium on which 100 μL of microbial suspension (10^6 CFU/mL) incubated overnight were distributed uniformly in TSB. A single filter paper disc was placed over the agar in a Petri dish to avoid any possible additional bacterial activity. All samples were incubated aerobically for 18 h at 37 °C, as this is the optimum growth temperature for *Staphylococcus aureus*. The antimicrobial activity was evaluated by measuring the diameter of inhibition zones against *S. aureus* formed around the disc expressed in mm. Antibiotic discs of vancomycin (30 mg/disc) were used as positive control, whereas distilled water was used as a negative control. All the tests were performed in duplicates (36 plates), and values were expressed as mean with standard deviation.

Determination of antimicrobial activity of oil and liquid smoke by checkerboard assay

To investigate the combined effect of essential oil and liquid smoke against *S. aureus*, initial inocula mentioned in the previous section were used, and the method described by White et al. (17) was applied using checkerboard microtiter plates in triplicate. The MIC value of each antimicrobial agent in combination varied from 1/32 to 4 times. A volume of 300 μL of each dilution was added to the wells of two honeycomb microplates. Afterwards, each well was inoculated with 50 μL of *Staphylococcus aureus* bacterial suspension (10^6 CFU/well) and cultivated at 37 °C for 24 h. Fractional inhibitory concentration (FIC) of each antimicrobial agent was calculated as the MIC of the combination of *E. platyloba* essential oil (EO) with liquid smoke (LS), divided by the MIC of essential oil and liquid smoke alone. The used formulae to calculate the FIC values were as follows:

\[
\text{FIC(EO)} = \frac{\text{MIC(EO+LS)}}{\text{MIC(EO)}} \\
\text{FIC(LS)} = \frac{\text{MIC(EO+LS)}}{\text{MIC(LS)}} \\
\text{FIC_{checker} = FIC(EO) + FIC(LS)}
\]

The FIC_{checker}, which represents a combined effect of both antimicrobial agents, was interpreted as follows: if the FIC_{checker} was <1.0 (on average ±0.5) the effect of essential oil and liquid smoke was synergistic; when FIC_{checker}=1 (on average ±0.5) the effect was additive; when FIC_{checker}=2.0 the effect was indifferent and at FIC_{checker}=2.0 (on average ≥0.4) or ≤4.0) it was antagonistic. The generation (doubling) time (t_g) of *S. aureus* in meat samples treated with the combination of essential oil and liquid smoke was assessed using growth curves. Doubling of turbidity was detected as an increase of the absorbance from 0.4 to 0.8. Each of the selected absorbances (18) was plotted against time. The doubling time in the exponential growth phase was calculated as follows:

\[
t_g = \frac{t_2 - t_1}{\log_{10}(2)}
\]

where t_1 is time at A_{\text{abs}}=0.8 and t_2 is time at A_{\text{abs}}=0.4.
Cytoplasmic material release

The method proposed by Rhayour et al. (19) was followed to measure the absorbance of the released Staphylo-
coccus aureus cell constituents into the supernatant at 260
nm. Viable cells from 100 mL of S. aureus culture in the
exponential growth phase were obtained by centrifuga-
tion (model Symphony 4417R; VWR International, Radi-
nor, PA, USA) for 15 min at 8000×g, then washed two to
three times, and resuspended in 0.1 M phosphate buffer
saline solution (pH=7.0) (Merck Millipore). A volume of
25 mL of cell suspension with 5 % Tween 80 was incubat-
ed under agitation for 1 h at 37 °C with the addition of
essential oil and liquid smoke at 16 different volume ra-
tios, ranging from 1/2 MIC to 2 MIC. After incubation, 10
mL of samples were collected and centrifuged at 11 000×
g for 15 min. The absorbance of the supernatant was deter-
mined using UV-Vis spectrophotometer (Cecil Instru-
mments Ltd, Cambridge, UK).

Sensory analysis

The organoleptic evaluation of the treated meat sam-
ple was done by nine trained persons who were selected
among the students and staff of Shahid Beheshti Univer-
sity of Tehran, Iran. Four training sessions were designed
to improve the ability of the panellists to recognise and
quantify the sensory perception. All meat samples treated
with different mass fractions of essential oil and liquid
smoke were labelled with three digits, placed in alumini-
um foils, and disk di-

Statistical analysis

All of the assays were performed in triplicate. All
growth curves were recorded and plotted in Microsoft Of-
fice Excel 2010 v. 14 (Microsoft Corporation, Redmond,
WA, USA). The obtained results are expressed as the
mean value±standard deviation. The results of sensory
and disk diffusions assays were subjected to statistical
analysis of variance using the general one-way ANOVA
followed by Tukey’s test. With the help of the SPSS soft-
ware v. 17 (SPSS Inc, Chicago, IL, USA), signifi-
cant differences between the control and the samples with
added essential oil and liquid smoke were evaluated (p<0.05).

Results

Echinophora platyloba oil yield and chemical composition

The obtained oil yield was (0.80±0.05) %. The highest
yield was obtained immediately after grinding the dried
plant material, but the pulvserised plant samples used for
oil extraction did not have any significant effect on oil
yield.

The main components of E. platyloba oil are listed in
Table 1, which shows that 25 compounds were identified,
representing 97.26 % of the oil. β-Ocimene (73.26 %) was
found as the major constituent, besides other constituents
with relatively low fractions including p-cymene (3.79 %),
α-pinene (3.33 %) and α-phellandrene (2.57 %). These re-
sults suggest that the extracted essential oil has a high
level of antimicrobial activity.

Content of polycyclic aromatic hydrocarbons in liquid
smoke

The content of polycyclic aromatic hydrocarbons (PAH) in
liquid smoke is shown in Table 2. Liquid smoke used for
sensory evaluation contained less than 2 μg/kg of ben-
zo[a]pyrene as an indicator (highly carcinogenic).

Antistaphylococcal activity of essential oil and liquid
smoke alone

MIC values of E. platyloba essential oil and liquid smoke
against Staphylococcus aureus are 7200 and 3500 mg/L, and
MBC values are 8500 and 8000 mg/L, respectively. MBC
values of E. platyloba essential oil and liquid smoke were
almost similar, and they were 1.5 times higher than MIC
values. Liquid smoke MIC value is lower than of E. platy-
loba, which can be interpreted as a higher antimicrobial
activity of the former against Staphylococcus aureus.

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>RI</th>
<th>Peak area/%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>n-Nonane</td>
<td>900</td>
<td>0.21</td>
</tr>
<tr>
<td>2</td>
<td>α-Tujene</td>
<td>930</td>
<td>0.08</td>
</tr>
<tr>
<td>3</td>
<td>α-Pinene</td>
<td>939</td>
<td>3.33</td>
</tr>
<tr>
<td>4</td>
<td>Camphene</td>
<td>953</td>
<td>0.25</td>
</tr>
<tr>
<td>5</td>
<td>Sabine</td>
<td>975</td>
<td>0.37</td>
</tr>
<tr>
<td>6</td>
<td>β-Pinene</td>
<td>979</td>
<td>0.21</td>
</tr>
<tr>
<td>7</td>
<td>Myrcene</td>
<td>991</td>
<td>1.18</td>
</tr>
<tr>
<td>8</td>
<td>α-Phellandrene</td>
<td>1003</td>
<td>2.57</td>
</tr>
<tr>
<td>9</td>
<td>p-Cymene</td>
<td>1025</td>
<td>3.79</td>
</tr>
<tr>
<td>10</td>
<td>β-Phellandrene</td>
<td>1030</td>
<td>0.46</td>
</tr>
<tr>
<td>11</td>
<td>(Z)-β-Ocimene</td>
<td>1037</td>
<td>73.26</td>
</tr>
<tr>
<td>12</td>
<td>γ-Terpinene</td>
<td>1060</td>
<td>1.13</td>
</tr>
<tr>
<td>13</td>
<td>Linalool</td>
<td>1097</td>
<td>0.55</td>
</tr>
<tr>
<td>14</td>
<td>Spathunenol</td>
<td>1132</td>
<td>0.60</td>
</tr>
<tr>
<td>15</td>
<td>p-Menth-2-en-1-ol</td>
<td>1139</td>
<td>1.48</td>
</tr>
<tr>
<td>16</td>
<td>p-Mentha-1,5-dien-8-ol</td>
<td>1143</td>
<td>0.17</td>
</tr>
<tr>
<td>17</td>
<td>α-Terpineol</td>
<td>1176</td>
<td>0.13</td>
</tr>
<tr>
<td>18</td>
<td>cis-3-Hexenyl-2-methyl butanoate</td>
<td>1214</td>
<td>0.71</td>
</tr>
<tr>
<td>19</td>
<td>cis-3-Hexenyl isovalerate</td>
<td>1216</td>
<td>1.18</td>
</tr>
<tr>
<td>20</td>
<td>α-Terpineyl acetate</td>
<td>1335</td>
<td>0.21</td>
</tr>
<tr>
<td>21</td>
<td>Methylene</td>
<td>1369</td>
<td>0.50</td>
</tr>
<tr>
<td>22</td>
<td>4-Decanole</td>
<td>1429</td>
<td>0.25</td>
</tr>
<tr>
<td>23</td>
<td>2-Furanone</td>
<td>1466</td>
<td>2.57</td>
</tr>
<tr>
<td>24</td>
<td>cis-3-Hexenyl benzoate</td>
<td>1568</td>
<td>0.67</td>
</tr>
<tr>
<td>25</td>
<td>Spathunenol</td>
<td>1577</td>
<td>1.40</td>
</tr>
</tbody>
</table>

RI=retention index
Antistaphylococcal activity of essential oil and liquid smoke in combination

In Table 3 the antimicrobial activity (inhibition zone diameter) of different combinations of the essential oil and liquid smoke against Staphylococcus aureus is demonstrated. The values given in the table are classified into two groups: one in which the value of liquid smoke is constant and of the essential oil is varied, and the other in which the volume of essential oil is constant and that of liquid smoke is varied. Results are reported as mean values±standard deviation of triplicate experiments (p<0.05).

As the volume of the essential oil and liquid smoke was increased, the inhibition zone increased in all of the samples (p<0.05). The discs containing 20 μL of liquid smoke and 5 μL of essential oil, and 15 μL of essential oil and 5 μL of liquid smoke had the highest and lowest zone of inhibition, respectively (p<0.05).

The FICindex of the essential oil combined with the liquid smoke was measured to be 2.031 (data not shown), which indicates that the mixture constituents had an antagonistic effect. The results of S. aureus generation time measurement during its exponential growth phase are shown in Table 4. The generation time of S. aureus treated with essential oil and liquid smoke at different MIC values was significantly increased (p<0.05). Double MIC of the mixture did not inhibit the S. aureus growth but had a positive effect on generation time (p<0.05). The generation time was significantly increased when higher volumes of antimicrobial agents were used, except at the MIC value.

Table 2. Mass concentrations of 12 polycyclic aromatic hydrocarbons (PAH) in liquid smoke

<table>
<thead>
<tr>
<th>No.</th>
<th>PAH</th>
<th>t50/μmin</th>
<th>γ/L/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Naphthalene</td>
<td>8.063</td>
<td>0.216</td>
</tr>
<tr>
<td>2</td>
<td>Acenaphthylene</td>
<td>12.362</td>
<td>1.320</td>
</tr>
<tr>
<td>3</td>
<td>Acenaphthene</td>
<td>12.825</td>
<td>0.678</td>
</tr>
<tr>
<td>4</td>
<td>Fluorene</td>
<td>14.554</td>
<td>0.106</td>
</tr>
<tr>
<td>5</td>
<td>Phenanthrene</td>
<td>18.761</td>
<td>0.178</td>
</tr>
<tr>
<td>6</td>
<td>Anthracene</td>
<td>19.026</td>
<td>0.346</td>
</tr>
<tr>
<td>7</td>
<td>Fluoranthene</td>
<td>25.427</td>
<td>0.548</td>
</tr>
<tr>
<td>8</td>
<td>Pyrene</td>
<td>26.467</td>
<td>0.684</td>
</tr>
<tr>
<td>9</td>
<td>Benz[a]anthracene</td>
<td>31.760</td>
<td>1.284</td>
</tr>
<tr>
<td>10</td>
<td>Benzo[b]fluoranthene</td>
<td>31.955</td>
<td>1.482</td>
</tr>
<tr>
<td>11</td>
<td>Benzo[a]pyrene</td>
<td>38.222</td>
<td>1.908</td>
</tr>
<tr>
<td>12</td>
<td>Dibenzo[a,h]anthracene</td>
<td>40.852</td>
<td>2.400</td>
</tr>
</tbody>
</table>

Table 3. Inhibition of Staphylococcus aureus growth by Echinophora platyloba essential oil (EO) and liquid smoke (LS) in combination

<table>
<thead>
<tr>
<th>V(LS+EO) μL</th>
<th>d(inhibition) mm</th>
<th>V(EO+LS) μL</th>
<th>d(inhibition) mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>5+5</td>
<td>(21.0±0.0)</td>
<td>5+5</td>
<td>(21.0±0.0)</td>
</tr>
<tr>
<td>5+10</td>
<td>(20.5±0.7)</td>
<td>5+10</td>
<td>(25.0±0.0)</td>
</tr>
<tr>
<td>5+15</td>
<td>(20.0±0.0)</td>
<td>5+15</td>
<td>(28.5±0.7)</td>
</tr>
<tr>
<td>5+20</td>
<td>(21.5±0.7)</td>
<td>5+20</td>
<td>(32.5±0.7)</td>
</tr>
<tr>
<td>10+5</td>
<td>(25.0±0.0)</td>
<td>10+5</td>
<td>(20.5±0.7)</td>
</tr>
<tr>
<td>10+10</td>
<td>(25.0±0.0)</td>
<td>10+10</td>
<td>(25.0±0.0)</td>
</tr>
<tr>
<td>10+15</td>
<td>(26.0±0.1)</td>
<td>10+15</td>
<td>(27.0±0.0)</td>
</tr>
<tr>
<td>10+20</td>
<td>(25.5±0.7)</td>
<td>10+20</td>
<td>(30.0±0.0)</td>
</tr>
<tr>
<td>15+5</td>
<td>(28.5±0.7)</td>
<td>15+5</td>
<td>(20.0±0.0)</td>
</tr>
<tr>
<td>15+10</td>
<td>(27.0±0.0)</td>
<td>15+10</td>
<td>(26.0±0.1)</td>
</tr>
<tr>
<td>15+15</td>
<td>(29.5±0.7)</td>
<td>15+15</td>
<td>(29.5±0.7)</td>
</tr>
<tr>
<td>15+20</td>
<td>(29.5±0.7)</td>
<td>15+20</td>
<td>(31.5±0.7)</td>
</tr>
<tr>
<td>20+5</td>
<td>(32.5±0.7)</td>
<td>20+5</td>
<td>(21.5±0.7)</td>
</tr>
<tr>
<td>20+10</td>
<td>(30.0±0.0)</td>
<td>20+10</td>
<td>(25.5±0.7)</td>
</tr>
<tr>
<td>20+15</td>
<td>(31.5±0.7)</td>
<td>20+15</td>
<td>(29.5±0.7)</td>
</tr>
<tr>
<td>20+20</td>
<td>(29.0±1.4)</td>
<td>20+20</td>
<td>(29.0±1.4)</td>
</tr>
</tbody>
</table>

Inhibition zone represents the mean values±standard deviation of the antimicrobial activity of liquid smoke and essential oil in combination.

Mean values with different letters within the same group are significantly different at p<0.05. Control values are 23.5±0.5 for positive control (vancomycin) and 0.0±0.0 for negative control (distilled water).

Table 4. Staphylococcus aureus generation time after the treatment with essential oil (EO) and liquid smoke (LS) mixture

<table>
<thead>
<tr>
<th>MIC(EO)</th>
<th>MIC(LS)</th>
<th>t50/μmin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1/2</td>
<td>1/4</td>
</tr>
<tr>
<td>2</td>
<td>195±</td>
<td>165±</td>
</tr>
<tr>
<td>1</td>
<td>165±</td>
<td>120±</td>
</tr>
<tr>
<td>1/2</td>
<td>120±</td>
<td>90±</td>
</tr>
<tr>
<td>1/4</td>
<td>120±</td>
<td>90±</td>
</tr>
<tr>
<td>1/8</td>
<td>120±</td>
<td>90±</td>
</tr>
<tr>
<td>1/16</td>
<td>165±</td>
<td>90±</td>
</tr>
<tr>
<td>1/32</td>
<td>135±</td>
<td>105±</td>
</tr>
</tbody>
</table>

MIC=minimum inhibitory concentration
*p<0.05 as compared to the control

Results of sensory analysis

The sensory properties of minced beef treated with essential oil and liquid smoke are given in Table 5. The sensory attribute was acceptable by the panellists when 0.05 g of essential oil and 0.6 g of liquid smoke were added to 100 g of meat. However, unacceptable odour related to the presence of essential oil in minced meat was reported when 0.06 g or more were added.

Cell constituent release

The results of A260 nm values of S. aureus cells (amino acids, nucleotides and ions) when treated with E. platyloba essential oil and liquid smoke at four concentrations (i.e. 0 MIC, 1/2 MIC, and 2 MIC value) are 0.0, 0.36, 0.72 and 1.44, and 0.0, 0.275, 0.55 and 1.1, respectively (Fig. 1). In total, there are 16 combinations of essential oil and liquid smoke values.

It can be concluded that with increasing the volume of essential oil and liquid smoke (Fig. 1), alone and in combination, absorbance was increased. Higher antimicrobial agents were used, except at the MIC value.
Table 5. Overall acceptance evaluation of minced beef treated with essential oil (EO) and liquid smoke (LS)

<table>
<thead>
<tr>
<th>w(EO)/%</th>
<th>Score</th>
<th>w(LS)/%</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.03</td>
<td>(1.9±1.0)(^a)</td>
<td>0.5</td>
<td>(3.2±1.4)(^a)</td>
</tr>
<tr>
<td>0.04</td>
<td>(2.4±1.3)(^a)</td>
<td>0.6</td>
<td>(3.8±0.9)(^a)</td>
</tr>
<tr>
<td>0.05</td>
<td>(2.4±1.3)(^a)</td>
<td>0.7</td>
<td>(3.3±1.0)(^a)</td>
</tr>
<tr>
<td>0.06</td>
<td>(2.1±0.8)(^b)</td>
<td>0.8</td>
<td>(3.2±1.0)(^a)</td>
</tr>
</tbody>
</table>

Scores represent the mean values\(\pm\)standard deviation, \(N=9\).
Mean values with different letters within the same column are significantly different compared to controls (p<0.05).
Control values are 3.8±1.0 for essential oil and 3.7±1.1 for liquid smoke.

Microbial activity was found in the samples treated with higher volumes of essential oil or liquid smoke (e.g. 0.1:1 and 1.44:0) than with lower volumes of both essential oil and liquid smoke when they are combined (e.g. 0.36:0.275 or 0.36:0.55).

Discussion

The yield of *Echinophora platyloba* oil obtained after hydrodistillation was 0.8%, which is higher than previously reported values of 0.55 (6), 0.67 (14) and 0.7% (20), perhaps due to the variation of sowing date and water stress (21), sampling time, geographical origin or genetic differences (22).

Essential oils are natural products. Despite the genetic factors, all environmental conditions and geographical origin have an influence on their chemical constituents (23). Results showed that essential oil mainly consists of hydrocarbon monoterpenes which, unlike phenolic compounds, generally accumulate in higher amounts in cooler and damper areas (24). The concentrations of hydrocarbon monoterpenes in the current study are comparable to the results reported by Hassanpourghadam et al. (15), as in both cases similar damp and cool geographical origin of *E. platyloba* plant can be a possible reason for their higher concentrations than in other studies. Based on the experiments of Ghanii et al. (25), the high monoterpene ratio is constant during three different growth and developmental stages.

It is interesting to note that the same *E. platyloba* species harvested in Kermanshah (200 km west of Hamadan, Iran) but treated with a different extraction method (microwave distillation) had a slightly different composition (26).

However, more than one compound of essential oils possesses the overall antibacterial activity (27). The extracted essential oil from *E. platyloba* has a high concentration of \(\beta\)-ocimene, which is responsible for its antimicrobial activity. Also, \(\alpha\)-pinene can be considered as an active antibacterial agent against *Staphylococcus aureus* (28).

Benzo[a]-pyrene, a marker of the carcinogenic PAHs in food, was found in the analysed liquid smoke below the maximum recommended level by European Scientific Committee on Food (2 \(\mu\)g/kg) (29). Therefore, the acceptable concentration of liquid smoke (according to sensory evaluation) is considered to be safe.

Both *E. platyloba* essential oil and liquid smoke exhibited a significant antibacterial activity against *Staphylococcus aureus*. The liquid smoke tested in this study was found to be more effective than the essential oil.

Antagonistic effect between constant volumes of liquid smoke (5, 10 and 20 \(\mu\)L) and different volumes of essential oil was determined by disk diffusion method, whereas there was a significant increase of antibacterial activity when constant volume of liquid smoke was added to 5, 10 or 15 \(\mu\)L of essential oil. As shown in Table 3, the lowest volumes of essential oil and liquid smoke did not show the minimum antimicrobial effect, while higher volumes resulted in a decrease of antimicrobial activity. Previously, the synergic effect of the mixture of the essential oil and acetic acid was demonstrated. The acid dissociation results in the release of H\(^+\) ions, which lower the pH and damage cell membranes of bacteria, thus increasing the effectiveness of essential oil components such as phenols (30).

Antagonistic interactions between the oil and the liquid smoke flavour were explored by checkerboard method. The possible explanations of antagonistic interactions were not well demonstrated (31,32). The antagonistic effect has been attributed to the interaction between non-oxygenated and oxygenated monoterpene hydrocarbons (13).

The result of generation time assays of *Staphylococcus aureus* treated with essential oil and liquid smoke mixture showed that the growth was inhibited by liquid smoke at 2 MIC, whereas essential oil did not show the same effect. Therefore, liquid smoke in the presence of the essential oil was expected to prolong doubling time but not to inhibit the growth completely.

The increase in \(A_{\text{abs}}\) is consistent with the release of amino acids, nucleotides, and ions from *S. aureus* cells due to cell membrane damage when treated with *E. platyloba* and liquid smoke at four different concentrations. The release of cellular content of the treated bacteria led to the hypothesis that the primary effect of an essential oil is membrane disruption. *E. platyloba* contains terpenes which can penetrate or disrupt lipid structures. The hy-
The sensory properties of minced beef treated with essential oil showed that the addition of 0.05 g of *E. platyloba* essential oil to 100 g of meat has a positive effect on the acceptance of the product. Also, the liquid smoke at higher mass fraction of 0.6 g per 100 g of meat (more than ten times of that of the essential oil) did not affect organoleptic properties of minced meat.

**Conclusions**

The combination of *Echinophora platyloba* essential oil and liquid smoke could be applied to minced meat products without any off-taste. According to the results of sensory evaluation, the added mass fractions of essential oil and liquid smoke (0.05 and 0.6 g per 100 g of meat, respectively) to minced beef had no adverse effect on the overall acceptance. The conducted experiments showed that liquid smoke had a better antimicrobial activity against *Staphylococcus aureus* than *E. platyloba* essential oil. Moreover, we observed that both antimicrobial agents showed a weak antimicrobial effect against *S. aureus* when used alone, and that the combination of essential oil with liquid smoke did not improve its antibacterial effect, although it may increase the minimum effective dose of these compounds. Therefore, further investigations are recommended to find ways for improving the antimicrobial effect of this essential oil in combination with other antimicrobial agents.

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