Chemical Composition of the Essential Oil of *Mentha pulegium* L. and its Antimicrobial Activity on *Proteus mirabilis*, *Bacillus subtilis* and *Zygosaccharomyces rouxii*

M. A. Khosravi Zanjani*, N. Mohammadi*, M. Zojaji*, H. Bakhoda

*a* Ph. D. Research Student of the Department of Food Science and Technology, Tehran Science and Research Branch, Islamic Azad University, Tehran, Iran.  
*b* Research Laboratory Expert, Tehran Science and Research Branch, Islamic Azad University, Tehran, Iran.  
*c* Assistant professor of the Department of Agricultural Mechanization, Faculty of Agriculture, Tehran Science and Research Branch, Islamic Azad University, Tehran, Iran.

ABSTRACT: There is a growing interest in food industry to replace the synthetic chemicals by the natural products with bioactive properties from plant origin. The aim of this study was to determine the chemical composition of *Mentha pulegium* essential oil and to characterize the antimicrobial activities of the essential oil. The essential oil of *Mentha pulegium* was analyzed by GC-MS. The evaluation of the antimicrobial activity on *Proteus mirabilis* ATCC 15146, *Bacillus subtilis* ATCC 12711 and *Zygosaccharomyces rouxii* ATCC 14679 was determined by Minimum Inhibitory Concentration procedure. The sensitivity of microorganisms was also measured by disc diffusion and cup plate methods. The essential oil of *Mentha pulegium* revealed pulegone, cineole and piperitenone were the main constituents, comprising 19.89%, 19.38% and 15.14% of the essential oil, respectively. The results showed a significant antimicrobial activity against microorganisms especially *Bacillus subtilis*, while the least susceptible microorganism was *Zygosaccharomyces rouxii* (*P*<0.05). The minimum inhibitory concentration of the essential oil of *Mentha pulegium* was 0.5%, 1.25% and 1.5% for *Bacillus subtilis*, *Proteus mirabilis* and *Zygosaccharomyces rouxii* respectively. In this research work, *Bacillus subtilis* bacteria was more sensitive to the essential oil than *Proteus mirabilis*. In general, this study indicated that the essential oil of *Mentha pulegium* has remarkable antimicrobial activity on microorganism especially *gram positive bacteria*. Related researches are required to assess the efficacy of this essential oil in therapeutic applications.

Keywords: *Bacillus subtilis*, *Mentha pulegium* Essential Oil, *Proteus mirabilis*, *Zygosaccharomyces rouxii*.

Introduction

Synthetic chemical compounds are used as antimicrobial agents in food products to prevent microbial spoilage. However, the use of chemical preservatives might cause many environmental, medical and economic problems. Thus, it is necessary to provide an accessible and easy method without any toxicity to humans and plants (Nobakht *et al.*, 2011; Teixeira *et al.*, 2012). Since consumers are less willing to use products that contain synthetic preservatives or additives, natural compounds can be good alternatives for this purpose. These compounds increase the shelf-life of foods by preventing the growth of photogenic microorganisms and protecting food products against oxidizing agents (Diaz-Maroto *et al.*, 2007; Shirazi *et al.*, 2004).

Many researchers have used essential oils of aromatic plants to enhance the shelf-life
of foods (Nobakht et al., 2011; Erhan et al.,
2012; Ghalamkari et al., 2012) while others
have investigated the antioxidant and
antimicrobial properties of extracts and
essential oils (Kamkar et al., 2010; El-
ghorab, 2006; Mahboubi and Haghi, 2008).

Essential oils are a mixture of volatile
organic compounds extracted from non-
woody parts of plants through steam or
hydrodistillation (Teixeira et al., 2012; Riahi
et al., 2013). Therefore, more than 3000
essential oils have been identified. About,
300 commercially essential oils are used in
pharmaceutical, agricultural, food, health,
 cosmetics and perfumery industries (Kamkar
et al., 2010; El-ghorab, 2006; Mahboubi and
Haghi, 2008). The antimicrobial activity
of the essential oils is related to their
chemical structure. Essential oils are
characterized by two or three original
constituents with highest concentrations
(greater than 80%) as compared to those
found in lower concentrations (Mahboubi and
Haghi, 2008; Ait-Ouazzou et al., 2012). Mentha Pulegium
L. is a plant of Lamiaiceae or Labiatae
family which can be found throughout the
world. Mentha Pulegium is one of Menta
species which is typically called Pennyroyal.

Mentha pulegium is a herb native to
Europe, North Africa, Minor Asia and
the Middle East (Teixeira et al., 2012). Mentha
pulegium is traditionally used in the
treatment of colds, sinusitis, cholera, food
poisoning, bronchitis and tuberculosis (Diaz-
Maroto et al., 2007; Shirazi et al., 2004).
According to the literature, the essential
oil of Mentha pulegium can be regarded as a
good alternative to synthetic antioxidants
due to the prevention of polyunsaturated
fatty acids oxidation (Ait-Ouazzou et al.,
2012; Kamkar et al., 2010). Typically, the
leaves, flowers, and branches of Mentha
pulegium are used due to the antimicrobial
properties (Hassanpour et al., 2012; Kanakis
et al., 2012).

It is also used as an additive in tea,
commercial spices or is mixed with other
foods to create a flavor (Teixeira et al.,
2012; Nickavar et al., 2008). Many studies
have been carried out on the chemical
composition of Mentha pulegium essential
oil (Ozgen et al., 2006; Padmashree et al.,
2007). It has been proven that the essential
oil of Mentha pulegium has antimicrobial
activity (Riahi et al., 2013; Mahboubi and
Haghi, 2008). Nowadays, synthetic
preservatives are mainly used to maintain
and increase the shelf-life of food products.

However, the overuse of synthetic
preservatives in food products might cause
side effects in consumers. Therefore,
replacement of synthetic compounds with
natural preservatives such as Mentha
pulegium essential oil can play a significant
role in public health promotion.

The antimicrobial activity of the essential
oil of Mentha pulegium has been confirmed
on Proteus mirabilis and Bacillus subtilis
causing spoilage of food products. Various
species of Proteus, especially mirabilis and
Proteus vulgaris cause food poisoning of poultry
meat (Wang et al., 2010; Zhao et al., 2014).

Proteus species have been identified as
opportunistic etiological agents in infections
of respiratory system, ulcers, wounds, burns,
skin, eyes, ears, throat, and gastroenteritis
caused by consumption of contaminated
foods. Some Proteus subspecies cause the
same reaction as that occurred by typhus
agent in the human immune system (Wang
et al., 2010; Gul et al., 2013; Vinogradov
et al., 1991). There has not been a research
study on the antimicrobial effect of Mentha
pulegium essential oil on Zygosaccharomyces rouxii. The yeast can
tolerate high osmotic pressures and cause
spoilage of foods including soft drinks and
juices (Martorrell et al., 2007; Rojo et al.,
2014). The aim of the present study is to
determine the chemical composition of
Mentha pulegium essential oil using GC-MS
apparatus and evaluate its antimicrobial activity on the spoiling agents including *Proteus mirabilis*, *Bacillus subtilis* and *Zygosaccharomyces rouxii*.

**Materials and Methods**

- **Preparation of the plant**
  The required amount of *Mentha pulegium* was collected from certain areas of Tehran and its scientific name was approved. The collected plant was dried at ambient conditions for 10 days. The dried plant was used to extract the essential oil.

- **Preparation of cell suspension**
  *Proteus mirabilis* ATCC 15146, *Bacillus subtilis* ATCC 12711 and *Zygosaccharomyces rouxii* ATCC 14679 were purchased from Scientific and Industrial Organization Iran. The microbial suspensions were prepared and lyophilized cells were inoculated in nutrient agar for 24 h at 37°C according to McFarland method. To achieve the appropriate microbial load for inoculation, a relationship between the microbial suspension absorption and the number of microbes was calculated using a spectrophotometer (VARIAN, USA). For each suspension with known absorption value, microbial count was performed using nutrient agar medium at 37°C for 48 h (Mahboubi and Haghi, 2008; Zanjani et al., 2012). To prepare yeast microbial suspension, yeast lyophilized powder was dissolved in 20 ml of YPD broth culture medium and then was incubated at 24°C for 48 h. The chemical composition of the culture medium per liter consist of 40 g glucose, 5 g peptone, 5 g yeast extract and 20 g agar. Pour plate method was used to dilute suspensions. The prepared suspension (10⁶ CFU/ml) was stored at 4°C.

- **Essential oil preparation**
  Dried *Mentha pulegium* was milled using a crusher. The essential oil of *Mentha pulegium* was extracted by hydrodistillation in Clevenger apparatus for 4 h. The essential oil to dry weight ratio was 0.67% w/w. The extracted essential oil was stored in colored glass at 4°C.

- **The minimum inhibitory concentration (MIC)**
  The minimum inhibitory concentration of each species was determined by blotting method using 0.25, 0.5, 0.75, 0.8, 1, 1.25 and 1.5% concentrations of the essential oil. For bacteria in each plate, 15 mL of Mueller Hinton agar culture medium with the desired concentration of the essential oil and DMSO (Dimethyl sulfoxide) were used. It is important to note that DMSO was used for uniform dispersion of the essential oil over the surface of culture medium. The blot diameter was about 3 mm equivalent to 0.5 McFarland of the desired microorganism. A control plate (with a blot lacking the essential oil) and a plate (with a blot lacking the essential oil with 8% DMSO) were used to examine the possible effect of DMSO. The plates were transferred to an oven at 37°C. After 48 h of incubation, the plates were examined for microbial growth. The same procedure was used for *Zygosaccharomyces rouxii* using YPD agar culture medium. The blot diameter was about 2 micrometers containing 10⁶ CFU/ml of the yeast. To study the microbial growth, the plates containing the yeast was incubated at 24°C for 48 h. The minimum concentration of essential oil that inhibits the growth of *Bacillus subtilis*, *Proteus mirabilis* and *Zygosaccharomyces rouxii* was reported as minimum inhibitory concentration (Mahboubi and Haghi, 2008; Zanjani et al., 2012). The experiments were performed in three replications.

- **Sensitivity of microorganisms to Mentha pulegium essential oil**
  Disk diffusion and cup plate methods were used for more detailed comparison of the sensitivity of *Zygosaccharomyces rouxii,*
**Proteus mirabilis** and **Bacillus subtilis** to **Mentha pulegium** essential oil.

- **Disk diffusion method**

In this method, the sterile control discs were placed in the essential oil for 5 min to absorb the essential oil completely. A suspension equivalent to 0.5 McFarland was prepared from 24-h cultures of bacteria and a uniform culture was prepared by swab over the Mueller-Hinton agar medium. Discs containing essential oils were placed on the surface of the culture medium. The plates were incubated at 37 °C for 48 h. The same procedure was used for **Zygosaccharomyces rouxii** using YPD agar culture medium. The plate containing the yeast was incubated at 24 °C for 48 h to study the inhibition zone. Bacteria and yeast sensitivity or resistance against the essential oil was determined by measuring the inhibition zone diameter. If the diameter of inhibition zone is less than 12 mm, the microorganism is resistant, a diameter between 12 and 16 mm shows a relatively sensitive microorganism and a diameter greater than 16 mm was considered to be quite sensitive (Prasannabalaji et al., 2012; Mahboubi and Haghi, 2008; Indu and Hatha, 2006; Srinivasan et al., 2001). The experiments were performed in three replications.

- **The chemical composition of essential oil**

The constituents of the **Mentha pulegium** essential oil was identified by the GC apparatus equipped with a mass spectrometer (GC-MS, HP-6840/5973).

- **Statistical analysis**

A complete randomized factorial design was used for analysis of the results that were means of three replications. Data analysis was carried out using Statistical Package for Social Sciences (SPSS) Inc. software (20: SPSS Inc., Chicago, IL). The mean differences were analyzed by Duncan’s multiple range test.

**Results and Discussion**

- **Minimum inhibitory concentration (MIC)**

The results obtained from the plates cultured by three microorganisms at various concentrations of the essential oil showed that **Bacillus subtilis** was grown only in the plate containing 0.25% **Mentha pulegium** essential oil, while it was grown in the control sample and the sample containing DMSO. Accordingly, a minimum inhibitory concentration of 0.5% **Mentha pulegium** essential oil was reported for **Bacillus subtilis**. The minimum inhibitory concentrations of **Mentha pulegium** essential oil for **Proteus mirabilis** and **Zygosaccharomyces rouxii** were 1.25 and 1.5%, respectively.

- **Sensitivity of microorganisms**

Disk diffusion and cup plate methods were used to study the sensitivity and resistance of bacteria and yeast against **Mentha pulegium** essential oil. According to the results, **Mentha pulegium** essential oil has been able to affect all microorganisms.
therefore the diameter of inhibition zone for all 3 types of microorganisms was larger than 12 ml in disk diffusion method. Table 1 shows the diameter of inhibition zone measured by cup plate and disk diffusion methods. As can be observed, all the bacteria were sensitive to Mentha pulegium essential oil, but Proteus mirabilis showed a greater resistance as compared to Bacillus subtilis (P<0.05). Bacillus subtilis with an inhibition zone diameters of 17.2 mm (cup plate method) and 17.45 mm (disk diffusion method) was the most sensitive bacteria. Zygosaccharomyces rouxii showed the least sensitivity with an inhibition zone diameter of 12.2 mm.

- The chemical composition of Mentha pulegium essential oil

The chemical composition of Mentha pulegium essential oil is shown in Table 2 and the chromatogram obtained from GC-MS is presented in Figure 1.

Comparing the GC results (Table 2 and Figure 1) with those reported by others there are differences in the percentage of phenolic compounds despite the similarity of compounds. This might be due to different geographical conditions of the plants. In the present study, pulegone (19.89%), cineole (19.38%) and piperitenone (15.14%) were the predominant compounds present. Identification of new compounds for inhibition of pathogenic or spoiling microorganisms is of great importance. Natural compounds found in the plants might be important and potential source of new types of food preservatives. Despite increasing research in this field, further studies on antimicrobial activity and chemical composition of these compounds are required. The Mentha pulegium essential oil evaluated in this study showed different antimicrobial activity against three microorganisms. The chemical profile of the essential oil was different with that observed by other researchers. According to the literature, some of the compounds are found in all species but at different concentrations presumably due to differences caused by environmental factors (Ait-Ouazzou et al., 2012; Reis-Vasco et al., 1999; Kanakis et al., 2012). In general, different species of Mentha pulegium contain high contents of piperitone, piperitenone or pulegone. Given the chemical composition of Mentha pulegium, the samples were similar to the species found in Portugal (Teixeira et al., 2012), Kazeroon in Iran (Mahboubi and Haghi, 2008) and Morocco (Ait-Ouazzou et al., 2012). The antibacterial activity of Mentha pulegium essential oil has been attributed to the major constituents including pulegone, isomenthon, menthone and piperitenone (Hajlaoui et al., 2009) or increased concentration of piperitone and the synergistic effects of other constituents (Mahboubi and Haghi, 2008). High content

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Minimum inhibitory concentration (MIC)</th>
<th>Cup plate method (mm)</th>
<th>Disk diffusion (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteus mirabilis</td>
<td>1.25 %</td>
<td>14.3±0.23a</td>
<td>15.24±0.37a</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>0.5 %</td>
<td>17.2±0.32b</td>
<td>17.45±0.26b</td>
</tr>
<tr>
<td>Zygosaccharomyces rouxii</td>
<td>1.5%</td>
<td>12.2±0.12c</td>
<td>12.51±0.33c</td>
</tr>
</tbody>
</table>

*Means with different letter in a column are significantly different (P<0.05).
Table 2. The main constituents of the essential oil of *Mentha pulegium*

<table>
<thead>
<tr>
<th>Compounds</th>
<th>RTa</th>
<th>(%)</th>
<th>Compounds</th>
<th>RT</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Methylene-1-cyclohexanol</td>
<td>1217</td>
<td>4.99</td>
<td>Alpha-pinene</td>
<td>938</td>
<td>1.81</td>
</tr>
<tr>
<td>Pulegone</td>
<td>1239</td>
<td>19.89</td>
<td>Sabinene</td>
<td>976</td>
<td>0.91</td>
</tr>
<tr>
<td>1,2,3-Trimethyl Cyclohexane</td>
<td>1251</td>
<td>0.73</td>
<td>Beta-Pinene</td>
<td>979</td>
<td>3.00</td>
</tr>
<tr>
<td>Piperitone</td>
<td>1275</td>
<td>3.08</td>
<td>1,8-Cineole</td>
<td>1035</td>
<td>19.38</td>
</tr>
<tr>
<td>2-cyclohexen-2-one</td>
<td>1281</td>
<td>0.74</td>
<td>2-Methylproplidene</td>
<td>1078</td>
<td>1.67</td>
</tr>
<tr>
<td>Thymol</td>
<td>1290</td>
<td>0.57</td>
<td>Menthone</td>
<td>1140</td>
<td>4.42</td>
</tr>
<tr>
<td>Piperitenone</td>
<td>1350</td>
<td>15.14</td>
<td>Delta-Terpineol</td>
<td>1160</td>
<td>3.79</td>
</tr>
<tr>
<td>3-Ethoxy-4-Methoxyphenol</td>
<td>1375</td>
<td>0.70</td>
<td>Endo-Borneol</td>
<td>1178</td>
<td>3.54</td>
</tr>
<tr>
<td>Cis-Salvene</td>
<td>1389</td>
<td>4.29</td>
<td>Cis-iso Pulegone</td>
<td>1184</td>
<td>1.15</td>
</tr>
<tr>
<td>2-Cylopenten-1-One</td>
<td>1402</td>
<td>0.75</td>
<td>Terpineene-4-ol</td>
<td>1190</td>
<td>1.40</td>
</tr>
<tr>
<td>Minit Furanone</td>
<td>1580</td>
<td>1.19</td>
<td>Alpha-Terpineol</td>
<td>1205</td>
<td>6.68</td>
</tr>
</tbody>
</table>

a: Retention time

of cineole might justify the antimicrobial activity of *Mentha pulegium* essential oil (Teixeira et al., 2012; Ait-Ouazzou et al., 2012). Oxygenated monoterpene (which are significantly more active than hydrocarbon monoterpene) are largely found in *Mentha pulegium* essential oil (Ait-Ouazzou et al., 2012). The results also showed that *Mentha pulegium* essential oil can be an effective inhibitor for most microbial and yeast strains examined in this study. The previous results of disk diffusion and cup plate methods (Mahboubi and Haghi, 2008; Hajlaoui et al., 2009) suggested that *Mentha pulegium* essential oil shows a strong antimicrobial activity against microorganisms, especially gram-positive bacteria with an inhibition zone of 10-31 mm (Hajlaoui et al., 2009).

In addition, *Mentha pulegium* essential oil shows a strong bacteriostatic activity against all strains (Ait-Ouazzou et al., 2012). The use of *Mentha pulegium* essential oil can increase the shelf-life of most food products such as fresh meat and fish (Erhan et al., 2012). According to the Table 1, the gram-positive bacteria were more sensitive than gram-negative bacteria due to the protective effect of lipopolysaccharide layer on the outer wall of the gram-negative bacteria.

Gram-positive bacteria like *Staphylococcus aureus* and *Bacillus cereus* are more sensitive to *Mentha pulegium* essential oil than gram-negative *E. coli* bacteria (Oueslati et al., 2010; Mahboubi and Haghi, 2008).

Pulegone as a monoterpene phenolic compound penetrates the lipid wall of bacteria leading to the breakdown of the cell wall and bacterial cell death due to leakage of cell contents. The phenolic compounds cause the destruction of bacterial cell by affecting the transport of electrons in the cytoplasm, protein synthesis and cell enzymes (Hajlaoui et al., 2009; Erhan et al., 2012; Ait-Ouazzou et al., 2012). The results indicated that *Mentha pulegium* essential oil has a significant antimicrobial activity on *Proteus mirabilis* as a gram-negative bacteria. *Proteus* species cause infection, especially in people with immune problems. In this process, membrane polysaccharides of *Proteus* bacteria play an important role (Wang et al., 2010). *Proteus mirabilis* is known as an opportunistic etiologic factor.
causing infections in respiratory systems, ulcers and gastroenteritis resulting from the consumption of contaminated foods (Gul et al., 2013; Vinogradov et al., 1991). 

Proteus mirabilis causes food poisoning in many products such as chicken and is usually resistant to existing antibiotics (Zhao et al., 2014; Wang et al., 2010). Mentha pulegium essential oil with concentration of 1.5% had an inhibitory effect on Saccharomyces rouxii. Although several studies have been conducted on antifungal and antimicrobial
properties of *Mentha pulegium*, there is no study on *Zygosaccharomyces rouxii*. The results of disk diffusion and cup plate methods showed that *Saccharomyces rouxii* is less sensitive to *Mentha pulegium* essential oil, because it can tolerate high osmotic pressures in unfavorable environmental conditions and cause spoilage of foods including soft drinks and juices.

Most routine preservatives are not able to reduce the yeast in carbonated drinks (Martorell *et al.*, 2007; Rojo *et al.*, 2014). In addition, the spoiling yeasts such as *Zygosaccharomyces rouxii* are able to grow in an acidic environment with low water activity containing 18% alcohol (Rojo *et al.*, 2014).

**Conclusion**

The use of chemical preservatives cause many environmental, medical and economical problems. Therefore, methods should be provided without any toxicity and side effects to human and plants. The synthetic preservatives are mainly used to maintain and increase the shelf-life of food products. The overuse of such preservatives in food products is associated with dangerous side effects for consumers. Understanding the significant effect of *Mentha pulegium* essential oil on the microorganisms that contribute to food spoilage, the use of this essential oil in food and pharmaceutical industries might play important roles in improving the public standard of health.

**References**


