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

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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

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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). *Menta Pulegium L.* is a plant of *Lamiaceae* 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 the 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 over use 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. Therefore, research concerned with the antimicrobial effects of *Mentha pulegium* essential oil on *Proteus mirabilis* and *Bacillus subtilis* as spoiling agents of food products has not been carried out. *Bacillus subtilis* causes bacterial spoilage of food products in bakery and cereal industries. 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 etiologic 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 tessential 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 (10^6 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 10^6 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.

- *Cup plate method*

In this method, a bacterial suspension equivalent to 0.5 McFarland was uniformly cultured on the surface of Mueller Hinton agar medium. Then, the cup plates with a diameter of 6 mm was created using a cork borer. About 100 ml of the essential oil was added to the cup plate using a sampler. The cup plate was 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 (Prasannabalaji *et al*., 2012; 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 1. Minimum inhibitory concentration and diameter of inhibition zone measured by cup plate and disk diffusion methods for three microorganisms

Microorganisms	Minimum inhibitory concentration (MIC)	Cup plate method (mm)	Disk diffusion (mm)
Proteus mirabilis	1.25 %	14.3±0.23 [a*]	15.24±0.37 [a]
Bacillus subtilis	0.5 %	17.2±0.32 [b]	17.45±0.26 [b]
Zygosaccharomyces rouxii	1.5%	12.2±0.12 [c]	12.51±0.33 [c]

*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*

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

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

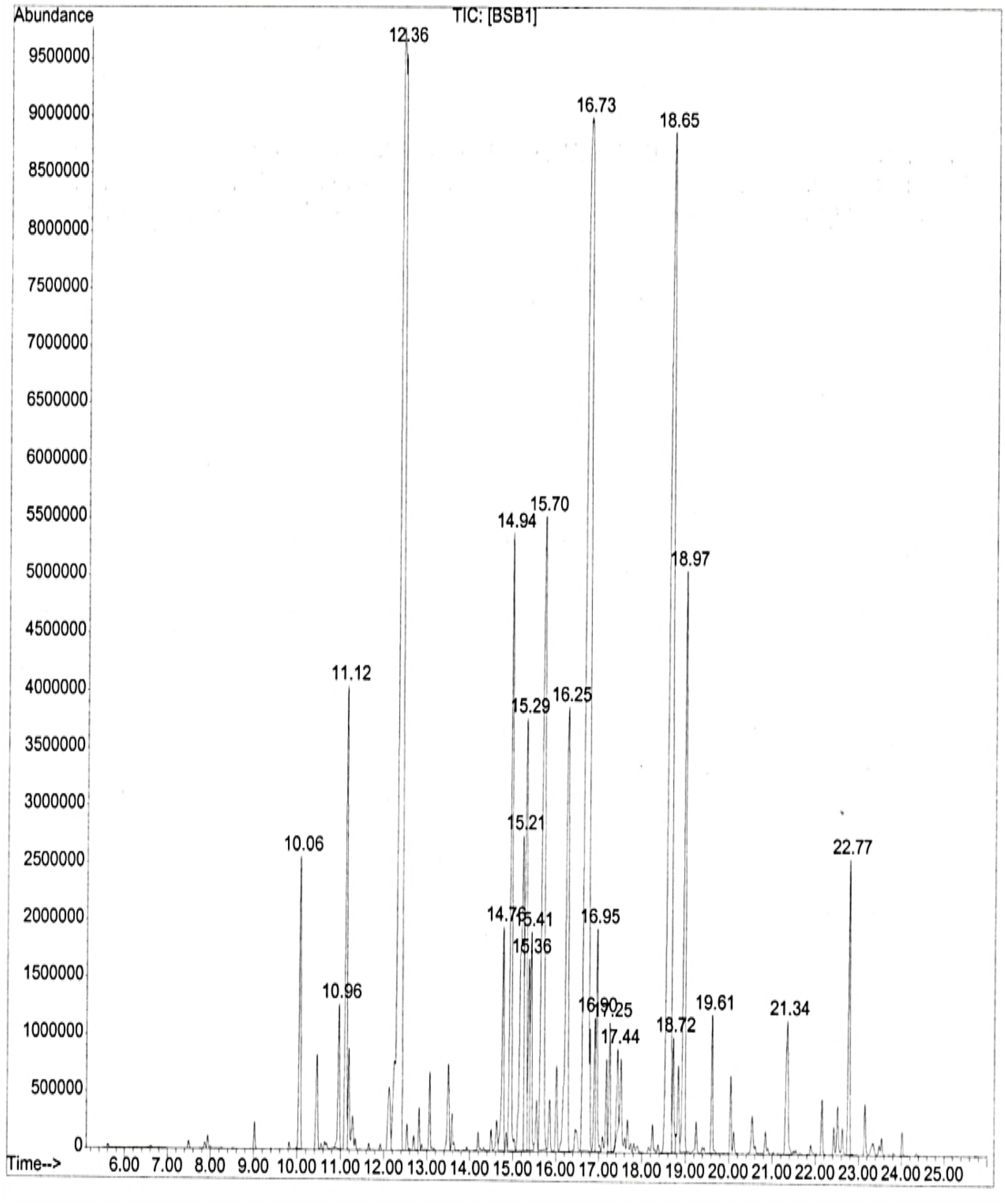

Fig. 1. *Chromatogram* of the essential oil of *Mentha pulegium*

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.

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