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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 11 (17): 2054-2061, 2008
ISSN 1028-8880

The Potential Application of Plant Essential Oils as Natural Preservatives Against *Escherichia coli* O157:H7

[1]A. Fazlara, [2]H. Najafzadeh and [2]E. Lak
[1]Department of Food Hygiene,
[2]Department of Basic Sciences, Faculty of Veterinary Medicine,
Shahid Chamran University, Ahvaz, Iran

Abstract: Investigation were carried out to compare the efficiency of three plant essential oils; *Zataria multiflora*, *Carum carvi* and *Mentha piperita* as natural food preservatives. The effect of these plant essential oils at concentrations of 0.0, 0.3, 0.6 and 1% was studied against inoculated *Escherichia coli* O157:H7 (10^5 cfu mL^{-1}) in prepared commercial chicken soup stored at 8 and 35°C over seven (168 h) and three (72 h) days, respectively by plate count technique on CT-SMAC agar. *Zataria multiflora* was the most effective essential oil against the bacterium in all concentrations, followed by *Mentha piperita* and *Carum carvi*. The maximum inhibitory effects of all essential oils were seen at 1% concentration. The inhibitory effects were affected by the incubation temperature as well as by the type and concentrations of essential oils. The 1% concentration of *Mentha piperita* and 0.6 and 1% concentrations of *Zataria multiflora* essential oil showed bacteriostatic effect on growth of *Escherichia coli* O157:H7 at 35°C. Also 1% concentration of *Carum carvi*, 0.6 and 1% concentrations of *Mentha piperita* and 0.6% concentration of *Zataria multiflora* essential oil had bacteriostatic effect while 1% concentration of *Zataria multiflora* essential oil showed bactericidal effect on the bacteria during the incubation period at 8°C. It is concluded that selected plant essential oils have promising inhibitory effects on *Escherichia coli* O157:H7 in chicken soup and could be considered as natural food preservatives. This is especially relevant at a time when there is an increasing interest in finding more natural alternatives to many existing preservatives.

Key words: *Escherichia coli* O157:H7, *Zataria multiflora*, *Carum carvi*, *Mentha piperita*, essential oil, natural preservative

INTRODUCTION

Escherichia coli O157:H7 is the well known strain of *E. coli* associated with foodborne diarrhoea in humans (Renter *et al*., 2003). The illness caused by *Escherichia coli* O157:H7 is typically severe and may be characterised by a spectrum of conditions, including mild diarrhea, haemorrhagic colitis, hemolytic-uremic syndrome and thrombotic thrombocytopaenic purpura (Coia, 1998; McClure, 2000; Shekarforoush *et al*., 2008). The relatively recent emergence of this bacterium as foodborne pathogen has had a significant impact on the food industry. This serovar possesses a number of undesirable characteristics that combine to make it one of the most serious threats to food safety (Sagdic *et al*., 2002). Epidemiological investigations have clearly linked *Escherichia coli* O157:H7 infection to the consumption of contaminated or improperly cooked foods (Hofmann, 1993; Kudva *et al*., 1996). In order to control and minimize foodborne disease by this organism, it has been necessary to change primary production, processing, retailing and consumer-handling practice. Despite these new control measures, foodborne disease outbreaks caused by this microorganism continue to occur and are accompanied by an increase in the number of cases of illness in many countries (Law, 2000; McClure, 2000). About 30% of people in developed countries at least once a year experience a foodborne disease. Therefore, there is a need for new methods to prevent the growth of foodborne pathogens or decrease the number of them in foods (Basti *et al*., 2007). Also the food industry has tended to reduce the use of chemical preservatives in their products due to increasing pressure from consumers or legal authorities, to either completely remove or to adopt more herbal alternatives for the maintenance or extension of product shelf life (Dillon and Board, 1994; Nychas, 1995). One of the methods is to use plant essential oils and antimicrobial additives in foods. To establish the

Corresponding Author: Ali Fazlara, Department of Food Hygiene, Faculty of Veterinary Medicine, Shahid Chamran University, P.O. Box 61355-145, Ahvaz, Iran Tel: 0098-9123091813 Fax: 0098-611-3360807

usefulness of herbal essential oils, they must be evaluated alone or in combination with other preservation factors (such as temperature, storage time and ...) in foods (Basti *et al.*, 2007).

The antibacterial activities of plant essential oils have been known for a long time and a number of researches on the antibacterial effect of plant essential oils and their derivatives have been reported by Betts (2000), Hsieh *et al.* (2001), Sagdic and Ozcan (2003), Delgado *et al.* (2004), Nasar-Abbas and Kadir Halkman (2004), Basti *et al.* (2007) and Fazeli *et al.* (2007). Also the antimicrobial effect of several plants and essential oils has been studied on *E. coli* and *Escherichia coli* O157:H7 (Dorman and Deans, 2000; Nostro *et al.*, 2000; Skandamis and Nychas, 2000; Marino *et al.*, 2001; Ozcan and Erkmen, 2001; Salvat *et al.*, 2001; Sagdic *et al.*, 2002; Dadalioglu and Evrendilek, 2004).

Contamination of the cooked and ready to eat foods such as commercial soups by this bacterium because of their high consumption in public food services have a high potential for foodborne illness. The contamination of these foods at the time of preparation by food handlers or poor sanitary condition and the growth of the organism in the time/temperature abuse conditions will cause foodborne illness in consumers. *Zataria multiflora*, *Carum carvi* and *Mentha piperita* are native traditional Iranian herbs which have strong and pleasant aromas and are currently added into foods to impart desirable flavors and aromas to the Iranian foods. This study was conducted to evaluate the comparative effects of various concentrations of these herbs essential oils as natural antimicrobial preservatives on growth pattern of *Escherichia coli* O157:H7 in commercial Iranian chicken soup.

MATERIALS AND METHODS

This study was conducted from October, 2007 to March 2008.

Test bacteria: The *Escherichia coli* O157:H7 ATTC 35150 was used as the test strain in this study kindly supplied by Asre Enghelab Research Complex of Iranian Research Organization for Science and Technology (IROST).

Commercial Iranian chicken soup: The commercial chicken soups in 100 g powder packs were bought from the market in attention to their batch number and product and expire dates. All packs were belonged to one batch number and their ingredients were including chicken meat, salt, starch, hydrogenize vegetable oil, fat and chicken extract, vegetable protein, carrot, onion, spice, citric acid and monosodium glutamate.

Table 1: Plants used in experiments for the extract of essential oils

Common plant name	Species name	Family name	Parts used
Avishane-e-Shirazi (Persian thyme, Zaatar)	*Zataria multiflora* Boiss.	Labiatae	Leaves
Caraway	*Carum carvi* Linn.	Umbelliferae	Seeds
Peppermint	*Mentha piperita* Linn.	Labiatae	Leaves

Plant samples: The *Zataria multiflora*, *Carum carvi* and *Mentha piperita* were selected as their essential oils had previously been shown to have bacteriostatic and bactericidal activity at low concentrations against *Escherichia coli* (Rasooli and Rezaei, 2002; Moreira *et al.*, 2005; Dakhili *et al.*, 2006; Fazeli *et al.*, 2007; Mohsenzadeh, 2007; Rasooli *et al.*, 2008). These plants were purchased from local botanical market and their taxonomy and identification were confirmed by the Botany Department of Faculty of Agriculture Shahid Chamran University in Khuzestan Province of Iran. The commercial and scientific names of the investigated plants are given in Table 1.

Extraction of essential oils: Portions of 500 g of each dried plant were cut into small pieces and placed in a flask (2 L) and distilled water (1.5 L) added. A continuous steam distillation extraction head was attached to the flask. After steam distillation for approximately 3 h, the oil was collected and stored at 4°C until use (Koutsoumanis *et al.*, 1999; Tassou *et al.*, 2000).

Preparation of bacterium and inoculum: The stock culture of *Escherichia coli* O157:H7 was kept in 20% glycerol PBS (phosphate buffered saline) at -70°C. Active culture was generated by inoculating 100 μL of the thawed microbial stock suspension into 5 mL sterile nutrient broth (Merck, Germany) and incubated at 37°C for 24 h. Freshly synchronized culture of bacterial strain for initial inoculum was prepared after two times overnight cultures (24 h) of bacterium by successively transferring 100 μL of the vegetative cells into Brain Heart Infusion (BHI) broth (Merck, Germany). Then the bacterial count was performed by preparing the serial dilution method and plating out on standard plate count agar (Merck, Germany) at 37°C for 24 h. The maximum population of bacteria was 9×10^8 Colony Forming Unit (cfu) mL^{-1}. Then according to this result, the optical density of active freshly synchronized culture was adjusted to 0.1 at 620 nm using fresh sterile BHI broth to give a standard inoculum of 10^7 cfu mL^{-1}. Also the enumeration of bacteria was confirmed by plate count technique on standard plate count agar at 37°C for 24 h.

Experiment procedure: First of all, the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of prepared essential oils on

Escherichia coli O157:H7 were determined with broth dilution method (Mohsenzadeh, 2007). The MIC values of *Zataria multiflora*, *Carum carvi* and *Mentha piperita* essential oils were 0.2±0.07, 0.3±0.04 and 0.3±0.02% and the MBC values of mentioned essential oils were 0.4±0.05, 0.5±0.06 and 0.5±0.01%, respectively. In present study, the prepared plant essential oils were used in values close to their MIC and also in higher concentrations (0.3, 0.6 and 1%). Then, a three-way analysis of variance experiment was designed. Three type of herbs (*Zataria multiflora*, *Carum carvi* and *Mentha piperita*) in four concentrations of essential oils (0.0, 0.3, 0.6 and 1%) and two storage temperatures (8 and 35°C) were studied. The commercial chicken soups were prepared as instruction for use and dispensed (100 mL final volume) into sterile sealed 250 mL glass containers and different amounts of essential oils were separately added to above portions to give final concentrations of 0.0, 0.3, 0.6 and 1% and finally heated for 20 min and cooked at room temperature. The final pH of the soups was 5±0.1. Inoculation of soups was adjusted using 1 mL of BHI culture having 10^7 cells of *Escherichia coli* O157:H7 and inoculated to 100 mL volumes of soups to reach inoculum level of 10^5 cfu mL^{-1} of organism in the experimental soups. After inoculation, the glass containers were shaken and incubated at 8 and 35°C for seven (168 h) and three (72 h) days, respectively. The growth of *Escherichia coli* O157:H7 for each treatment was monitored with time by periodic sampling in various time intervals. In each sampling, duplicate samples of 1 mL from each glass containers were taken. Microbial counts were carried out in a biosecurity chamber with vertical laminar flow. Samples were diluted in sterile normal saline and the count of the organism per mL was determined by plate count technique on Sorbitol MacConkey Agar plus Cefixime and Potassium Tellurite (CT-SMAC) (Merck, Germany) at 37°C for 24 h. All treatments and experiments were conducted at least 3 times. The growth pattern of the organism was plotted by Excel 2003 software with the results average values of three times repetition.

Data Analysis: Statistical analysis of results and obtained data were conducted by SPSS software (version 10.0).

RESULTS AND DISCUSSION

The effects of studied herb essential oils on growth of *Escherichia coli* O157:H7 in commercial chicken soup at 8 and 35°C for seven (168 h) and three (72 h) days, respectively is shown in Fig. 1-3. It was evident that the addition of these essential oils, regardless of concentration had some inhibitory effects on growth of *Escherichia coli* O157:H7 in comparing to controls. The activity of the herb essential oils appeared to depend on incubated temperature, type and concentration of essential oils.

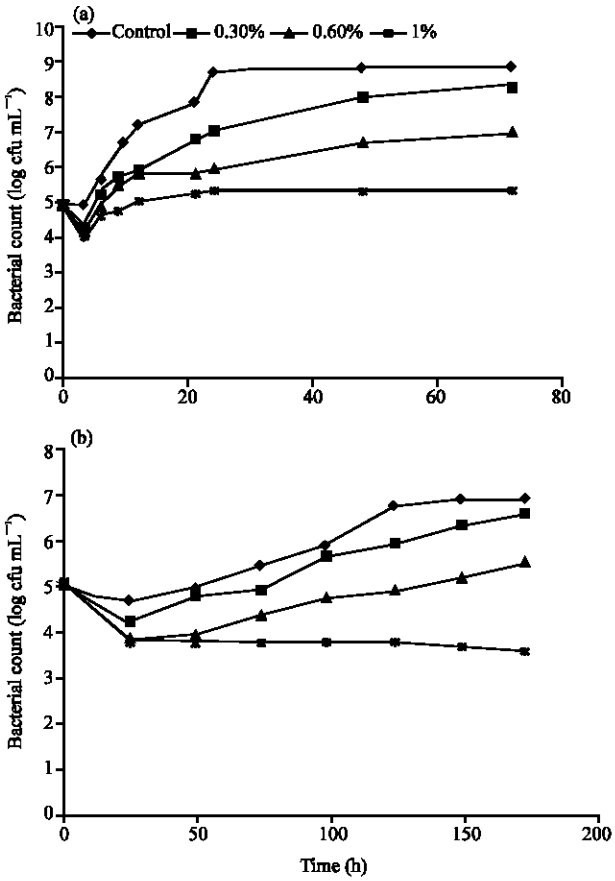

Fig. 1: Growth curves of *Escherichia coli* O157:H7 in commercial chicken soup in various concentration of *Carum carvi* essential oil at 35°C (a) and 8°C (b)

In control treatments (without adding essential oils) at 35°C, the bacterium which was inoculated at a level of 10^5 cfu mL^{-1} started to grow exponentially after a small delay and increased to a level of 10^8 cfu mL^{-1}. In chicken soups with *Carum carvi* essential oil added (0.3, 0.6 and 1%) and incubated in 35°C for 72 h, the bacterial level decreased about 1 log within first 3 h at all concentrations. All concentrations caused a delay of growth but did not stop the growth finally and the levels of bacteria reached to the 10^8, 10^7 and 10^5 cfu mL^{-1}, respectively (Fig. 1). In the simulatory situations after a primary 1 to 1.5 log reduction of bacteria, the levels of bacteria in the 0.3 and 0.6% concentrations of *Mentha piperita* and 0.3% concentration of *Zataria multiflora* essential oils increased to 10^7, 10^6 and 10^6 cfu mL^{-1}, respectively while

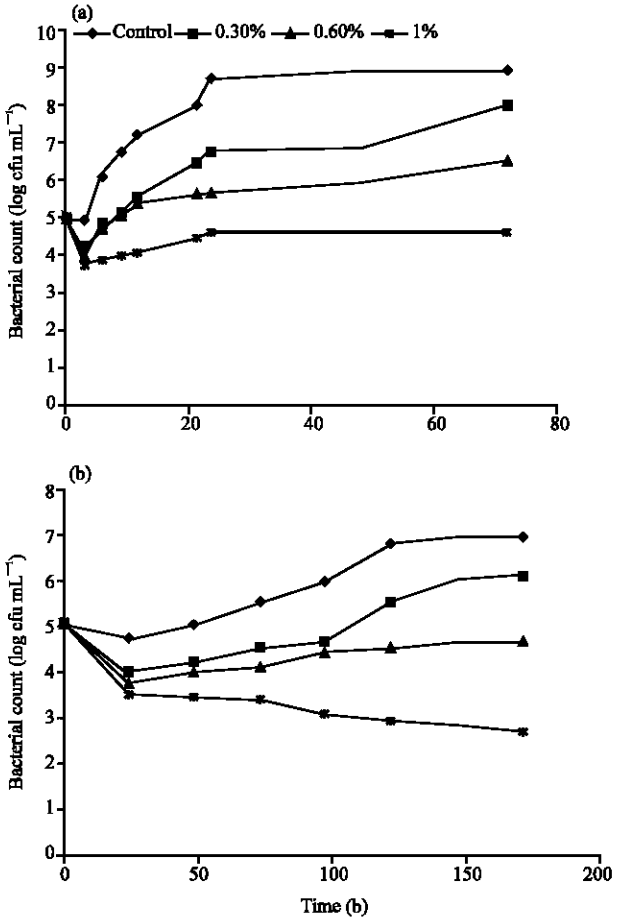

Fig. 2: Growth curves of *Escherichia coli* O157:H7 in commercial chicken soup in various concentration of *Mentha piperita* essential oil at 35°C (a) and 8°C (b)

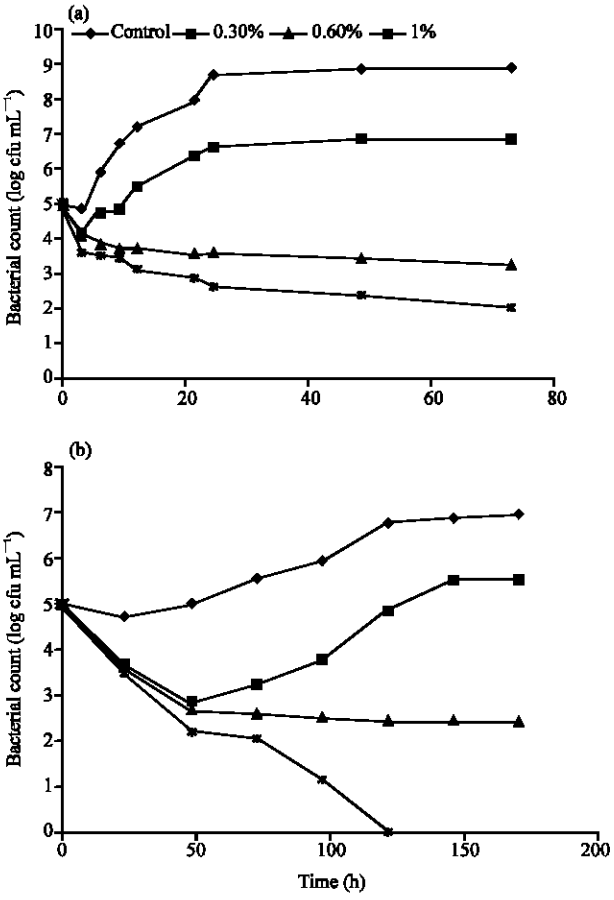

Fig. 3: Growth curves of *Escherichia coli* O157:H7 in commercial chicken soup in various concentration of *Zataria multiflora* essential oil at 35°C (a) and 8°C (b)

the 1% concentration of *Mentha piperita* and 0.6 and 1% concentrations of *Zataria multiflora* essential oils had bacteriostatic effects on the growth of *Escherichia coli* O157:H7 and the population of bacteria reached to levels of 10^4, 10^3 and 10^2 cfu mL^{-1}, respectively (Fig. 2, 3).

As shown in Fig. 1-3, in control treatments at 8°C, the population of inoculated bacteria gradually increased to the level of 10^6 cfu mL^{-1} of chicken soup during 168 h (one week). Among the three essential oils added in various concentrations, *Zataria multiflora* had the most inhibitory effect to *Escherichia coli* O157:H7. At low temperature storage (8°C), a quick 1 to 2 log repression of the inoculated bacteria was observed in soups treated with *Carum carvi* and *Mentha piperita* essential oils during 1 day (24 h) while about treated soups with *Zataria multiflora* essential oil, it was nearly 3 log reduction of bacteria during 2 days (48 h). Finally after one week at 8°C the levels of bacteria in chicken soups treated by 0.3 and 0.6% concentrations of *Carum carvi* reached to 10^6 and 10^5 cfu mL^{-1}, respectively while in the 1% concentration decreased to 10^3 cfu mL^{-1}. Also the population of bacteria in 0.3% concentrations of *Mentha piperita* and *Zataria multiflora* essential oils reached to 10^6 and 10^5 cfu mL^{-1}, respectively while the 0.6 and 1% concentrations of *Mentha piperita* and 0.6% of *Zataria multiflora* essential oils had bacteriostatic effects on the growth of *Escherichia coli* O157:H7 in chicken soup and the population of bacteria reached to levels of 10^4, 10^2 and 10^2 cfu mL^{-1}, respectively. The most inhibitory or bactericidal action was observed in *Zataria multiflora* essential oil in 1% concentration after 5 days storage of chicken soups at 8°C and the viable bacterial count were reduced less than detection limit (less than 10 cfu mL^{-1}).

In compared to controls, the applied *Carum carvi* essential oil at 0.3, 0.6 and 1% concentrations led to reduction in final population of bacteria about 0.6, 1.7 and 3.6 log after 72 h at 35°C and 0.5, 1.5 and 3.5 log after 168 h at 8°C, respectively. In the simulatory

concentrations, *Mentha piperita* essential oil showed reduction about 0.9, 2.5 and 5.3 log after 72 h at 35°C and 0.8, 2.1 and 4.5 log after 168 h at 8°C, respectively while *Zataria multiflora* exhibited more reduction about 2.1, 5.7 and 6.7 log after 72 h at 35°C and 1.6, 4.7 and 6 log after 168 h at 8°C, respectively. It is obvious that the maximum inhibitory effects of all essential oils were seen at 1% concentration. The results indicate that in compared to controls the *Zataria multiflora* was the most effective essential oil against the bacterium in all concentrations, followed by *Mentha piperita* and *Carum carvi* essential oils.

In present study, according to the results and in comparing to primary inoculated bacteria, 1% concentration of *Mentha piperita* and 0.6 and 1% concentrations of *Zataria multiflora* essential oil showed bacteriostatic effect on growth of *Escherichia coli* O157:H7 at 35°C. Also 1% concentration of *Carum carvi*, 0.6 and 1% concentrations of *Mentha piperita* and 0.6% concentration of *Zataria multiflora* essential oil had bacteriostatic effect while 1% concentration of *Zataria multiflora* essential oil showed bactericidal effect on the bacteria during the incubation period at 8°C.

In present study, the addition of *Zataria multiflora*, *Carum carvi* and *Mentha piperita* essential oils reduced significantly the population of the inoculated bacteria at both 8 and 35°C ($p<0.05$). Also the measurements of the maximum population of bacteria and comparing to controls showed that the inhibitory effects of all essential oils were affected by the incubation temperature as well as by the type of essential oils added in the chicken soups. The inhibitory effects of all studied essential oils in various concentrations on growth of *Escherichia coli* O157:H7 at 8°C were statistically significant ($p<0.05$). Also statistical evaluation of the results at 35°C showed that inhibitory effects of *Zataria multiflora* essential oil proved to be more effective in inhibiting the bacterial growth in chicken soup than other essential oils ($p<0.05$) while there was no significant difference between *Carum carvi* and *Mentha piperita* essential oils against growth of *Escherichia coli* O157:H7 at 35°C ($p>0.05$).

In comparing to controls, the reduction in the final population of bacteria at 35°C was more than 8°C in all concentrations of essential oils. The present study demonstrated that increased storage temperature to 35°C enhanced the inhibitory effect of all plant essential oils tested on chicken soup. According to these observations, plants essential oils may be more effective in case of abused storage temperatures. These results are also in accordance with those of Kotzekidou *et al.* (2008) who have also reported that *Escherichia coli* O157:H7 to be the more sensitive to various plant essential oils at 20°C or even higher temperatures in comparing to 7°C in chocolate. Friedman *et al.* (2004) reported that the antimicrobial activity of thyme essential oil against *Escherichia coli* O157:H7 at 37°C was about two to three times greater than at the lower temperatures (4 and 21°C).

In present study, by decreasing the storage temperature (from 35 to 8°C), the bactericidal effect of *Zataria multiflora* was exhibited. Also the more decreasing in growth of inoculated microorganism was observed. Recent study of Basti *et al.* (2007) showed the similar results about the effects of *Zataria multiflora* essential oil on the growth of *Staphylococcus aureus* in commercial barley soup. In other words, the spice and herb extracts or essential oils can enhance the destructive effects of low temperature to the bacterium (Yano *et al.*, 2006). It is well established that extrinsic factors such as temperature as well as intrinsic factors (e.g., proteins, fat, salt and ...) of the food affect the behavior of bacteria in food ecosystems and may act synergistically with preservatives such as natural antimicrobial agents (Nychas, 1995). Indeed the result of the present study shows an additive effect of storage temperature (8°C) with *Zataria multiflora* essential oil in 1% concentration to occur bactericidal effect. Ting and Deibel (1991), Koutsoumanis *et al.* (1999), Burt and Reinders (2003) and Yano *et al.* (2006) have reported that temperature effects varied with the kind of herbs and bacteria tested.

Kim and Fung (2004) proved antibacterial effects of *Puerariae radix* in ground beef and especially liquid foods like mushroom soup on *Escherichia coli* O157:H7. Different antimicrobial activity were reported with extracts or essential oils of *Zataria multiflora* (Rasooli and Rezaei, 2002; Dakhili *et al.*, 2006; Basti *et al.*, 2007; Fazeli *et al.*, 2007), *Mentha piperita* (Tassou *et al.*, 1995, 2000; Ozkan *et al.*, 2003; Moreira *et al.*, 2005; Rasooli *et al.*, 2008) and *Carum carvi* (Iacobellis *et al.*, 2005; Boniadian, 2006; Vasinauskiene *et al.*, 2006) against different pathogenic bacteria including *Escherichia coli* and *Escherichia coli* O157:H7. When comparing data obtained in different studies, most publications provide generalization about whether or not a plant essential oil or extract possesses activity against Gram-positive or Gram-negative bacteria and fungi. However, not all provide detail about the extent or spectrum of this activity. First, the variation in the antimicrobial activities of extracts or essential oils between different reports may be attributed to the different environmental growth condition and growth stage of plant, ecological conditions, differences in oil extraction methods, microbial species, food ingredients,

pH, storage temperature, essential oil component and concentration (Tassou *et al*., 1995; Nostro *et al*., 2000; Sagdic *et al*., 2002; Moreira *et al*., 2005). Furthermore, some essential oils with the same common name may be derived from different plant species. Secondly, the method used to assess antimicrobial activity and the choice of test organism (s) varies between publications (Hammer *et al*., 1999).

In present study in comparing to controls, all the essential oils showed various inhibitory effects on *Escherichia coli* O157:H7 and the inhibitory effects were augmented with increase in essential oil concentrations. Statistically, important variations were found among the inhibitory effects of all studied essential oils at 8°C and also *Zataria multiflora* with the other essential oils at 35°C. Generally, *Zataria multiflora* exhibited higher inhibitory effects than the others. Also the investigations of Rasooli and Rezaei (2002) showed that the bactericidal effect of *Zataria multiflora* essential oil was more effective than *Mentha longifolia* on *Escherichia coli* and *Staphylococcus aureus*. Rasooli and Rezaei (2002) reported the high carvacrol content of *Zataria multiflora* accounts for its strong antimicrobial activity. These results suggest that the *Zataria multiflora* essential oil can be considered as a practical natural preservative in some foods especially at low temperatures, although further research is needed.

The inhibitory effects of studied essential oils in 0.3, 0.6 and 1% concentrations on *Escherichia coli* O157:H7 in chicken soup were lower than their inhibitory concentrations as MIC and MBC in broth media. This is in agreement with other finding which reported that, the need to use plant essential oils at higher concentration in food than in laboratory media is believed to be due to the more complex growth environment in food, which provides microbial cells with greater protection from antimicrobial agents. During the application of essential oils in foods, the lower water content of food compared to laboratory media may hamper the progress of antimicrobial agents to the target site in the bacterial cell (Smith-Palmer *et al*., 2001). Carbohydrates in foods do not appear to protect bacteria from the action of essential oils as much as fat do (Kotzekidou *et al*., 2008). Fat in food could form a protective coat around bacteria, thereby protecting them from antimicrobial agents. Also the protein content of the food may also have been an influencing factor in the effectiveness of the essential oils. Indeed, complex formation between phenolic compounds in the essential oils and protein in food, prevent any similar complex formation between phenolic components and proteins or other components of the cell envelope especially the cell membrane which is widely regarded as one of the primary target sites for plant essential oils (Cox *et al*., 1998; Ultee *et al*., 1998). Also due to changes in the organoleptic properties of food caused by high level of plant essential oils, it may not be possible to add concentration high enough to cause bacterial cell death. However, in many cases, a concentration sufficient to result in the stasis of growth may be all that is required to achieve a safe product, provided that the initial pathogen is low. In this situation, the concentrations required for stasis can be lower than those for killing microorganisms (Smith-Palmer *et al*., 1998; Tassou *et al*., 2000).

It should be taken into consideration that the inhibition effects reported here were for commercial chicken soup and therefore may not be a true reflection of inhibition achieved in all kinds of soups or foods. Similarly different results may be achieved with various kinds of soups such as barley, mushroom etc. which are more readily associated with foodborne illness. Despite this, the research has shown the potential application of plant essential oils as natural food preservatives. The low bacteriostatic and bacteriocidal concentrations of some plant essential oils against some of the most important causes of bacterial food poisoning provides an exciting potential for the future, especially in the light of the shift away from artificial preservatives and the move towards more natural alternatives. Further studies are needed to investigate the essential oils incorporation into appropriate food formulations and evaluate flavor, chemical changes and antimicrobial effect in the whole food systems for extension of shelf life as well as prevention of food deterioration and foodborne pathogens.

ACKNOWLEDGMENT

This study is result of research sponsored by Shahid Chamran University of Ahvaz, Faculty of Veterinary Medicine.

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