Antimicrobial Activity of Essential Oils of *Cinnamomum zeylanicum*, *Mentha piperita*, *Zataria multiflora Boiss* and *Thymus vulgaris* Against Pathogenic Bacteria

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ABSTRACT

Background and Objective: Considering the increasing tendency of public towards green consumption and the dangers of artificial additives, this study aimed to assess antibacterial activity of essential oils of *Cinnamomum zeylanicum*, *Mentha piperita L.*, *Zataria multiflora Boiss and Thymus vulgaris* against three important pathogenic and spoilage bacteria (*Pseudomonas fluorescens*, *Erwinia carotovora* and *Escherichia coli*).

Methods: After obtaining the essential oils from Magnolia Co., their antimicrobial activity was assessed using broth microdilution method by determining the minimum inhibitory concentration (MIC50, MIC90) and minimum bactericidal concentration (MBC). All experiments were performed in triplicate and the data were analyzed using the GraphPad software and Duncan's new multiple range test.

Results: All essential oils showed antimicrobial activity in a concentration-dependent manner. Increasing the concentration of essential oils from 0.01% to 4% (v/v) significantly enhancing the antibacterial activity. The statistical calculations and comparison of data showed that the essential oils of *C. zeylanicum* and *Z. multiflora* performed better compared to the other two essential oils, due to having lower values of MIC50 (≤0.1%), MIC90 (≤0.4%) and MBC (≤1%) ($P < 0.05$).

Conclusion: Considering the high antimicrobial activity of essential oils of *C. zeylanicum* and *Z. multiflora*, they can be used as effective food additives with fewer side effects. However, further studies are being conducted on the effectiveness of essential oils on the growth of other microorganisms and their results will be published soon.

Keywords: Essential Oils, Antimicrobial, Pathogenic Bacteria, *Cinnamomum Zeylanicum*, *Zataria Multiflora Boiss.*

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INTRODUCTION

Considering the increasing tendency of public towards green consumption and the risks of artificial additives, the plant sources such as spices, herbs, essential oils and other compounds with antibacterial properties are among the best options that can increase the shelf life of foodstuff (1, 2).

Essential oils are the volatile liquids that have antimicrobial properties. Many studied have reported the antibacterial, antifungal and insecticidal effects of essential oils and plant extracts (3, 4). Given that essential oils have long been traditionally used to make pleasant taste in food, the simultaneous presence of the antimicrobial properties can encourage their use for this purpose (5). The U.S. Food and Drug Administration have also certified essential oils as generally recognized as safe (GRAS) (6). The antimicrobial effect of essential oils of four plants were evaluated and compared in this study.

Cinnamon (*Cinnamomum zeylanicum*) is an aromatic herb from the family Lauraceae that has been widely used for thousands of years as a food flavoring and seasoning ingredient (7). Essential oil of *C. zeylanicum* is one of the most important medicinal parts of this herb which is rich in compounds such eugenol and cinnamaldehyde (1, 8). The antifungal (9), antibacterial (10, 11), antioxidant and antimutagenic (12) activities of these compounds in this essential oil have been reported. Peppermint (*Mentha piperita L*., *Labiatae* family) has long been known to have several pharmaceutical, food and health applications. The essential oil of peppermint is among the most important medicinal parts of this plant that can be extracted from different parts of the plant such as aerial organs and leaves (13). Menthol is a main constituent of this essential oil and is reported to have antibacterial (14, 15) and antifungal (16, 17) properties.

Thymus vulgaris (family *Lamiaceae*) has long been traditionally used for its special features as a sputum inducer, antitussive, anticonvulsant, vermicide, carminative and diuretic agent. The antibacterial and antifungal properties of *T. vulgaris* essential oil constituents including thymol and carvacrol have been reported (18, 19).

Zataria multiflora Boiss (family *Lamiaceae*) grows wild in southern and central regions of Iran, Pakistan and Afghanistan. Some studies reported the phenolic compound of carvacrol as the main constituent of this essential oil, while other studies reported thymol (carvacrol's isomer) (20). The antiseptic, anesthetic, antioxidant, antifungal and antibacterial properties of these compounds are well-demonstrated (21, 22). This study aimed to compare the antibacterial effect of the mentioned essential oils on different stages of growth of the following three important pathogenic and spoilage bacteria:

1. Pseudomonas fluorescens, as the cause of spoilage in meat products (13) and head rot in agricultural products (23).

2. Erwinia carotovora, as the cause of various diseases in crops (24,25).

3. Escherichia coli, as one of the most dangerous pathogens of the gastrointestinal tract and cause of spoilage in meat products (26).

In addition, the appropriate essential oils with specific concentrations were determined to deal with the above bacteria.

MATERIAL AND METHODS

The essential oils used in this study (*C. zeylanicum*, *M. piperita*, *T. vulgaris* and *Z. multiflora*) were purchased from Magnolia Co. the qualitative parameters of each essential oil such as color, odor, appearance, purity, solubility and chemical properties (pH, acidity, brix, etc.) were described in the specifications form along with their analysis.

The studied microbial strains including *E. coli* (O157: H7 ATCC 25922), *P. fluorescens* (ATCC 17482) and *E. carotovora* (PTCC No: 1675) were obtained in the form of lyophilized ampoules from the Iranian Industrial and Scientific Research Center. To activate the microbial strains, the ampules were opened under sterile conditions and primary culture was done followed by secondary culture. The cultured bacteria were stored for the next experiments and in order to maintain the viability of the bacteria, reculturing was performed every 14 days.

The spectrophotometric method of broth microdilution was used, according to the

Clinical and Laboratory Standards Institute (CLSI) standards, to investigate the effects of essential oils on the growth of bacteria and to plot growth curves (27). The Mueller Hinton Broth (MHB) was autoclaved for about 15 minutes at 120 °C and all stages of the experiments were performed under Class II biological safety cabinet. In this study, 96-well plates (8 x 12) were used to investigate the antimicrobial activity of the essential oils. The final concentration of essential oils in the well was 0.01% to 4% (v/v), so that 200 µl of each essential oil solution with the highest concentration was added to the first column along with Tween 80 (Merck) and distilled water. Then, 100 µl of cation-adjusted MHB was added to the remaining columns. Next, the serial dilutions were made from the essential oils by transferring 100 µl of the solution from the first column to the next column and this was continued until the last column. At the end, the volume in each well reached 200 µl by inoculating 100 µl of each bacterial inoculum into each well of the microplate. The inoculum was prepared by 16-hour culture of the bacteria and matching its absorbance with the standard solution (equivalent to 0.5 McFarland standards) at 600 nm, and preparing serial dilutions in the MHB to achieve a dilution of 1.25 x 10^6 CFU/ml. Finally, final concentration of 10^5 CFU/well was achieved by adding the suspension to the wells. Three controls were designed for each sample. Positive controls contained bacterial suspension and lacked essential oils, while negative controls contained essential oils and lacked microbial suspension. After inoculation, the plates' caps were completely covered with Parafilm and the contents were mixed by shaking. The plates were incubated for 24 hours at 28 °C (*Pseudomonas* and *Erwinia*) and 37 °C (*Escherichia*). The light absorbance of each well at 600 nm was recorded by a spectrophotometer after the incubation and shaking. Finally, the antibacterial activity of essential oils was calculated according to the following formula (28):

$$\text{Antibacterial activity (\%)} = \frac{ODc - ODs}{ODs} \times 100$$

In this formula, OD_s and OD_c represent the optical density of the samples and the controls, respectively. The minimum inhibitory was determined by observing lack of increased optical density (OD) after 24 hours and based on the lowest concentration that would prevent the growth of strains (lack of growth in the wells). The minimum bactericidal concentration (MBC) values of the extracts was determined by subculturing 10 µl of the contents of each well into the plate containing Mueller Hinton agar medium, incubation at 37 °C for 48 hours and counting colonies. The MBC is the lowest concentration of treated samples that causes the death of 99.9% of the inoculated suspension. The GraphPad software was used to calculate the MIC50 values (concentration that causes 50% growth inhibition) of the essential oils and to plot absorbance at different concentrations. Microsoft Excel was used for plotting curves and calculation of standard deviation (SD) and error bars for each point of the curve. All experiments were performed in triplicate for each sample and the obtained results were recorded by repeating the entire experiment. Analysis of variance (ANOVA) was used in order to evaluate significant differences between the effects of various concentrations of essential oils. The Duncan's multiple range tests was applied to compare the means, if there was a significant difference. P-value <0.05 was considered as the statistical significance level. The statistical analysis was performed using GraphPad software (version 6).

RESULTS

The antimicrobial activity of the tested essential oils against each bacterium is presented in Figures 1-3. The results showed no significant difference between repetitions of a certain concentration, which indicates the accuracy of sampling.

In assessment of the antimicrobial activity of essential oils against *Pseudomonas* (Figure 1), the bacterial density was reduced to less than 50% in the presence of ≤ 0.08% dilution of *C. zeylanicum*, *M. piperita* and *Z. multiflora* in comparison with the controls. Moreover, 1% concentration of the mentioned essential oils completely inhibited the growth of *Pseudomonas* bacteria ($P<0.05$). Concentrations of ≥ 0.2% *T. vulgaris* showed more than 50% antimicrobial effect.

Figure 1- Percentage of inhibition for different concentrations and various types of essential oils against *Pseudomonas.* AB: *T. vulgaris*, AS: *Z. multiflora*, NF: *M. piperita* and DA: *C. zeylanicum*.

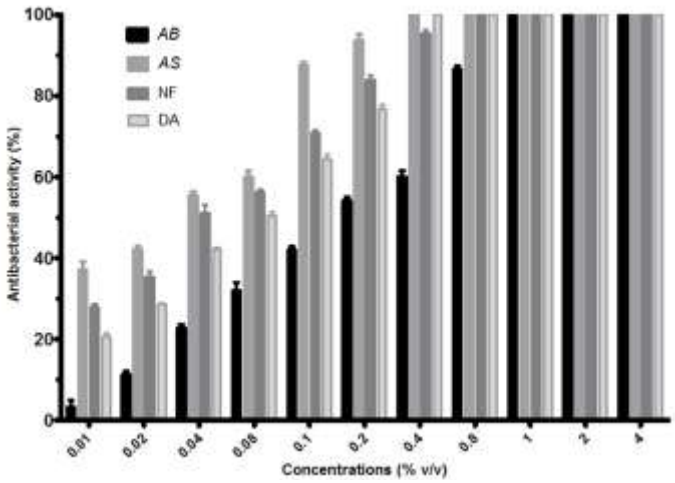

Table 1-Analysis of variance and effect of concentration and type of essential oils on growth of *Pseudomonas*

Result	probability level of higher than F	F-value	mean squares	degrees of freedom	sum of squares	Source
****	***P*<0.0001**	203.82	214.9	30	6447	Essential oil × Concentration ($X_1 \times X_2$)
****	***P*<0.0001**	2693	2841	3	8522	Essential oil (X_1)
****	***P*<0.0001**	9698	10228	10	102282	Concentration (X_2)
			1.055	88	92.81	Error

It is significant at a level of *P*-value <0.05. Symbol meaning; ns P > 0.05; * P ≤ 0.05, ** P ≤ 0.01,* P ≤ 0.001, **** P ≤ 0.0001.**

Figure 2- Percentage of inhibition for different concentrations of essential oils against *Erwinia*. AB: *T. vulgaris*, AS: *Z. multiflora*, NF: *M. piperita* and DA: *C. zeylanicum*

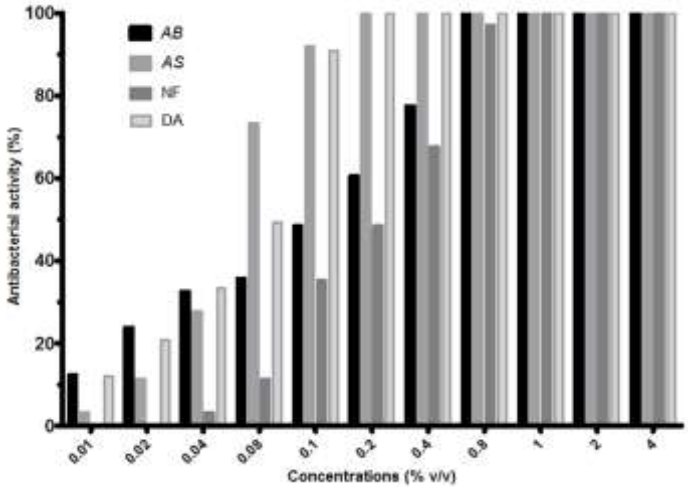

Table 2- Analysis of variance and effect of concentration and type of essential oils on growth of *Erwinia*

Result	probability level of higher than F	F-value	mean squares	degrees of freedom	sum of squares	Source
****	***P*<0.0001**	576.7	518.2	30	15545	Essential oil × Concentration ($X_1 \times X_2$)
****	***P*<0.0001**	4110	3692	3	11077	Essential oil (X_1)
****	***P*<0.0001**	18061	16228	10	162275	Concentration (X_2)
			0.8985	88	79.07	Error

It is significant at a level of *P*-value <0.05. Symbol meaning; ns P > 0.05; * P ≤ 0.05, ** P ≤ 0.01,* P ≤ 0.001, **** P ≤ 0.0001.**

The antimicrobial activity of the essential oils against *Erwinia* can be divided into three different categories (Figure 2). The first category is activity of less than 50% that was observed in concentrations of ≤ 0.1% *T. vulgaris* and *M. piperita* as well as concentrations of <0.08% *C. zeylanicum* and *Z. multiflora* (P<0.05). The second category contains concentrations of ≥ 0.08% of *C. zeylanicum* and *Z. multiflora*, as well as ≥ 0.02% concentration of *T. vulgaris* and ≥ 0.4% concentration of *M. piperita*, which had antibacterial activity of 50-90%. The third category with complete antimicrobial activity (100%) can be observed at concentrations of ≥ 1% of all essential oils. Moreover, the concentration of ≤ 0.2% *C. zeylanicum* and *Z. multiflora* could completely inhibit the growth of *Erwinia* (100%). As demonstrated in the *E. coli* growth curve (Figure 3), the essential oils of *T. vulgaris* and *M. piperita* were less effective at low concentrations. The concentration of ≥ 0.04% *T. vulgaris* and ≥ 0.2% *M. piperita* caused more than 50% inhibition in the growth of *E.coli* (P<0.05). This effect was observed at concentration of ≥ 0.1% *C. zeylanicum* and *Z. multiflora*. The growth of this bacterium was completely inhibited at concentration of ≥ 0.8 for all essential oils. The analysis of variance showed the significant effect of both factors of essential oil type and concentration at 99% confidence level (Table 1-3).

The most effective essential oil with highest antimicrobial activity

In order to identify the most effective essential oil, the values of MIC50, MIC90 and MBC were determined for each essential oil (Table 4). The results of this table and the statistical calculations showed that the essential oils of *C. zeylanicum* and *Z. multiflora* have better efficiency against all bacterial samples compared to the other two essential oils (p <0.05). In addition, the comparison of MIC50, MIC90 and MBC values indicated that these values are the lowest in essential oils of *C. zeylanicum* and *Z. multiflora*. Lower value of MIC50 for an antimicrobial substance certainly indicates higher antimicrobial properties for inhibition of bacterial growth at lower amounts. In conclusion, the essential oils of *C. zeylanicum* and *Z. multiflora* showed better performance.

Figure 3- Percentage of inhibition for different concentrations of essential oils against *E.coli*. AB: *T. vulgaris*, AS: *Z. multiflora*, NF: *M. piperita* and DA: *C. zeylanicum*.

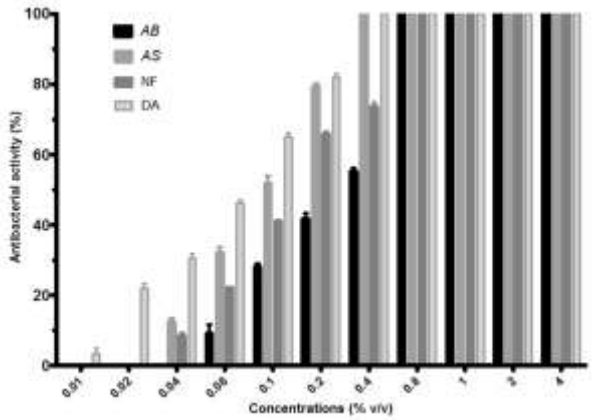

Table 3- Analysis of variance and effect of concentration and type of essential oils on growth of *E.coli*

Result	probability level of higher than F	F-value	mean squares	degrees of freedom	sum of squares	Source
****	***P*<0.0001**	340.3	258	30	7740	Essential oil × Concentration ($X_1 \times X_2$)
****	***P*<0.0001**	2351	1782	3	5347	Essential oil (X_1)
****	***P*<0.0001**	26345	19974	10	199741	Concentration (X_2)
			0.7582	88	66.72	Error

It is significant at a level of *P*-value <0.05. Symbol meaning; ns P > 0.05; * P ≤ 0.05, ** P ≤ 0.01,* P ≤ 0.001, **** P ≤ 0.0001.**

Table 4- Values of MIC90, MIC50 and MBC for the essential oils against the strains tested

P. fluorescens % (v/v)			*E. carotovora* % (v/v)			*E. coli* % (v/v)			Essential oil
MBC	MIC_{90}	MIC_{50}	MBC	MIC_{90}	MIC_{50}	MBC	MIC_{90}	MIC_{50}	
4%	1%	0.27%	2%	0.8%	0.16%	4%	0.8%	0.33%	*T. vulgaris*
1%	0.4%	0.04%	0.8%	0.2%	0.06%	1%	0.4%	0.1%	*Z. multiflora*
1%	0.4%	0.06%	0.8%	0.2%	0.06%	1%	0.4%	0.1%	*C. zeylanicum*
4%	0.8%	0.08%	2%	1%	0.26%	2%	0.8%	0.16%	*M. piperita*

DISCUSSION

The antibacterial effects of essential oils and plant extracts for controlling the growth of food-borne pathogens and spoilage microorganisms have been reported in several studies (3, 4, 29). According to previous studies, the antimicrobial activity of these compounds is exerted through damaging the structure and function of bacterial cell membrane. These studies also show that the composition, structure and functional groups play an important role in determination of the antimicrobial activity (30).

Based on the results of the present study, the essential oils of *C. zeylanicum* and *Z. multiflora* exhibited the highest antimicrobial activity. The antimicrobial properties of these two essential oils against many spoilage bacteria and fungi have been reported (9, 31). For example, Amini et al. (2012) and Abdolmaleki et al. (2010) have reported the antimicrobial properties of *Z. multiflora* against some plant pathogenic microorganisms (31, 32). Furthermore, several studied have demonstrated the antimicrobial activity of *C. zeylanicum* essential oil against various microorganisms. This essential oil is highly effective against Gram-positive and Gram-negative bacteria (33). Matan et al. demonstrated that the essential oil of *C. zeylanicum* is capable of inhibiting the growth of main spoilage microorganisms in food with average moisture (34). In this study similar to the other studies, the antimicrobial activity of this essential oil has been evident against microorganism tested. This substance was able to inhibit the growth of *E. coli*, *P. fluorescens* and *E. carotovora* to a great extent. The results of the previous studies showed that the antimicrobial activity of the plant extracts is associated with the content of secondary metabolites (e.g. alkaloids, phenolics, flavonoids and terpenoids) (35).

The Phenolic compounds (such as carvacrol), which are found in the hydrophobic part of the essential oil, may be dissolved in the hydrophobic cytoplasmic membrane and cause collapse and loss of adenosine triphosphate that will eventually lead to cell death (36). The antimicrobial activity of *C. zeylanicum* is related to its large quantities of eugenol (1) and cinnamaldehyde (8), while cinnamaldehyde has been regarded as the main compound of this essential oil, responsible for its antibacterial activity (37, 38). Cinnamaldehyde exerts its antimicrobial activity by binding to bacterial proteins through its carbonyl group and inhibiting the carboxylation of amino acids (1). In other studies, it is suggested that cinnamaldehyde and eugenol prevent the production of a necessary enzyme by the bacteria that may inhibit the growth of bacteria by damaging the bacterial cell wall (39).

According to the results of various studies, the antimicrobial activity of *Z. multiflora* is mainly because it contains high percentages of thymol and carvacrol, which are considered as antibacterial agents (40, 41). In this regard, Ponce et al. (2008) reported that carvacrol can easily inhibit the growth of *Listeria monocytogenes* (42). In another study, the inhibitory effect of carvacrol on pathogenic bacteria including *E. coli* (O157: H7) and *Salmonella enterica* has been reported (43).

However, considering the large number of different chemical compounds in this essential oil, the antimicrobial activity cannot be easily attributed to a specific component (44). P-cymene is among other biologically effective compounds in thyme that facilitates carvacrol's transfer through the cytoplasmic membrane of bacteria and increases the antibacterial activity. This has been well demonstrated by Ultee et al. in foodborne

pathogens (*Bacillus cereus)* (45). According to studies of Talebzadeh et al. and Jirovetz et al., the other constituents of *Z. multiflora* essential oil such as γ-terpinene, α-terpinene and Myrcene are also capable of inhibiting the growth of some bacteria (44, 46). These compounds can be different depending on age, phases of growth (vegetative growth, early flowering, full flowering and fruit maturation), climate, soil composition and organ of the plant (47, 48). Accordingly, the antimicrobial activity of this essential oil should be assessed considering these variables. Similar to the present study, El-Zemity et al. and Al-Ani et al. investigated the effectiveness of *C. zeylanicum* essential oil on *E. carotovora* (49, 50). However, the mentioned studies used the pour plate method, and the values of MIC, MBC and MIC50 were not reported. Calculating the amount of MIC, MBC and MIC50 can determine the scope of antimicrobial activity as well as the exact amount of antimicrobial substances required to eliminate pathogenic bacteria. The results of the present study using the broth microdilution method, showed higher antimicrobial effect of the essential oil compared to the mentioned studies. In our study, the antimicrobial activity of *C. zeylanicum* essential oil at concentrations of 0.1% and 0.2% were 90% and 100%, respectively. While, the other studies indicated that these compounds were capable of inhibiting the bacterial growth only at high concentrations (0.5%). The possible important reasons for the results' inconsistencies include organ type, height and geographical location of the plant used as well as the tested microorganisms. Furthermore, the other internal and external factors that affect the antimicrobial performance of essential oil such as temperature, pH and methods used should not be neglected (51). About the present study, the method used (broth microdilution) to assess the antimicrobial properties may partly explain the differences in the results. It seems that the antimicrobial performance of essential oils can be better revealed by implementing the microdilution method in which the essential oil is in contact with the liquid medium. Moreover, the temperature of the medium in the pour plate method (used in the mentioned studies) can significantly reduce the antimicrobial properties. Therefore, it is suggested using and comparing different methods for assessing the antimicrobial activity of essential oils.

CONCLUSION

The results indicate the antibacterial effect of the essential oils, particularly *C. zeylanicum* and *Z. multiflora* against some pathogenic bacteria. These essential oils can be used as natural antibiotics with fewer side effects than chemical antibiotics to deal with bacterial pathogens.

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CONFLICT OF INTEREST

All contributing authors declare no conflicts of interest.

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