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Study of Antibacterial and Synergistic Activities of *Cinnamomum verum*, *Eucalyptus camaldulensis* and *Zataria multiflora* Boiss. in Persian Medicine Against Some Gram-Negative and Gram-positive Pathogenic Bacteria

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ANTIBACTERIAL activities of essential oils (EOs) of three widely used herbal elements of cinnamon (*Cinnamomum verum* J.Presl), eucalyptus (*Eucalyptus camaldulensis* Dehnh.), and thyme (*Zataria multiflora* Boiss.), whose antimicrobial effects are indirectly described in the old literature of Persian medicine as antiseptic agents and treatments of diseases caused by Havaye Vabaee (e.g., air-borne infectious diseases), were investigated. Antibacterial activities of the EOs were evaluated against some important Zoonosis and medicine pathogenic gram-negative (*Escherichia coli*, *Klebsiella pneumonia*, *Listeria monocytogenes,* and *Pseudomonas aeruginosa*) and gram-positive (*Staphylococcus aureus*, *S. epidermidis*, and *Streptococcus pyogenes*) bacteria. These pathogens cause disease in animals and humans.To this end, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and double-diffusion assay (DDA) tests were utilized using a microdilution method. While MIC values for cinnamon, eucalyptus, and thyme ranged between 0.25-1, 8-64, and 0.5-16 µg mL^{-1}, respectively, the range of MBC values were 1-4, 32-256, and 2-64 µg mL^{-1}. In DDA tests, a concentration of 0.5 µg mL^{-1} of the three EOs induced inhibition zones ranging between 20-35, 9-25, and 0-35 mm in diameter in the culture media of the above bacteria. Antibacterial effects of double combinations of the EOs against *E. coli* and *S. aureus* were also investigated by checkerboard test and fractional inhibitory concentration (FIC) index. Combinations of the EOs resulted in FIC index values of 1.06-2, indicating no interaction between most of the compounds combined. Our study also suggested the use of thyme oil with caution in combination with cinnamon or eucalyptus oils against *E. coli* because of their possible antagonistic effects (FIC index = 4).

Keywords: Medicinal plants, Essential oils, Antibacterial, Antagonism, Animal pathogen, Human, Zoonosis, Human

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Introduction

Infectious diseases are one of the most serious health challenges nowadays. With the advent of various antibiotics and the development of disinfection methods in recent decades, the problem of infectious diseases seemed to be completely solved. However, the emergence of antibiotic-resistant microbial strains, side effects of drugs, lack of public access to antibiotics, production and storage limitations, and even international issues have revealed the fact that there are still challenges to overcome microbial epidemics and the treatment of infectious diseases; therefore, further studies are needed in this area of science [1]. For these reasons, World Health Organization (WHO) has recommended using traditional and complementary medicine to improve health care systems in the world [2]. So human has been facing lots of health issues, that attracted researchers' attention to the importance of biodiversity and its high potential benefits, including medicinal plants. These plants contain a variety of natural compounds with different benefits and properties [3-5]. Applications of such plants have been frequently described in Persian medicine, and their benefits are confirmed in recent literature as well. Therefore, Persian medicine and its capacity with a very rich scientific and experimental background could be one of the best choices in order to solve health issues [6].

Although the words bacterial, fungal, and viral diseases, or generally, microbial diseases, have not been mentioned in the original books of Persian medicine as it is spoken in contemporary medicine, the term Havaye Vabaee shares many similarities with contagious diseases [7]. However, this term is not limited to infectious diseases and includes a wider range of diseases, including those caused by environmental pollutions [9]. Particularly, a range of air-borne infectious diseases transmitted through inhalation falls into this category. For example, diseases such as smallpox, measles, and tuberculosis are well known in Persian medicine, and several articles and treatises, including Razi's Al-Jazari and Al-Hasbeh, described the symptoms and treatment of smallpox and measles. Moreover, topics related to the diagnosis and treatment of some infectious diseases such as Nazleh (sinus and allergy) and jaundice can be easily found in original books of Persian medicine [9]. On the other hand, several herbal elements utilized in the prevention and treatment of diseases caused by Havaye Vabaee are now classified into antioxidant, antimicrobial, and bolstering immune system categories [10].

Here, we describe some herbal elements in Persian medicine with antimicrobial activities. Thyme (*Zataria multiflora* Boiss., Lamiaceae) is an indigenous plant of Iran, and its leaves and flowers are used as spice and drug. Several useful properties have been mentioned for this plant in Persian medicine, such as strengthening the gastrointestinal tract and eliminating respiratory infections. Thyme essential oil (EO) is composed mostly of phenols, monoterpenes, hydrocarbons, and alcohols. Thymol, the main phenolic compound in thyme, is responsible for its antibacterial activities [11]. Eucalyptus (*Eucalyptus camaldulensis* Dehnh., Myrtaceae) tree has hard wood which its brown color appears after its bark is peeled off [12]. Eucalyptus leaves extract is commonly applied as an antibiotic in herbal medicine [13]. This plant has been described as an effective medicine for the treatment of many diseases, including infections of various parts of the body, especially the respiratory system and urinary tract, and as an antiseptic agent in some Persian medicine literature [14]. Cinnamon (*Cinnamomum verum* J.Presl, Lauraceae) is an evergreen plant. EOs of cinnamon barks contain mucilage, cinnamaldehyde, resin, tannin, and oxalate, of which cinnamaldehyde is the main compound with antibacterial activities [15]. In Persian medicine, cinnamon is used in many medicinal concoctions and applied in the treatment of various diseases, including cough, sore throat, and pneumonia as a result of lung infections [16].

In this study, EOs of three common herbal medicines used as single or combination drugs, i.e., cinnamon, eucalyptus, and thyme, whose antimicrobial activities have been indirectly described in Persian medicine literature, were selected and their antibacterial properties against seven important human pathogenic Gram-negative and -positive bacteria were investigated. For this purpose, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and double-diffusion assay (DDA) tests were applied using a microdilution method. On the other hand, one of the well-known therapeutic principles in traditional and herbal medicine is the use of combination drugs composed of several herbal elements [8]. However, the effects of different components of the combined drug could be additive, synergistic, and even antagonistic. For this reason, antibacterial activities of double

combinations of the three EOs were also studied in this work. The results will hopefully help us understand more about the scientific roots of Persian medicine.

Materials and Methods

Essential oils

Three EOs, including cinnamon, eucalyptus, and thyme, were used in this study. These EOs were obtained from Tabib Daru Co. (Kashan, Iran) and the Department of Pharmacognosy, School of Pharmacy, Shahid Beheshti University of Medical Sciences (Tehran, Iran). The EOs were selected based on literature review and their common use in Persian medicine. Stem bark, leaves, and aerial parts of these plants, respectively, were subjected to steam distillation method for essential oils.

Bacterial strains

Seven important Zoonosis and medicine pathogenic bacterial strains were prepared from the bacterial collection of the Iranian Biological Resource Center (Tehran, Iran). The strains used in this study were four Gram-negative bacteria: *Escherichia coli* (ATCC 25922), *Klebsiella pneumonia* (ATCC 13883), *Listeria monocytogenes* (ATCC 7644) and *Pseudomonas aeruginosa* (ATCC 27853), and three Gram-positive bacteria: *Staphylococcus aureus* (ATCC 25923), *S. epidermidis* (ATCCC 12228) and *Streptococcus pyogenes* (ATCC 19615) (Table 1).

Standardization of inoculum

The bacterial strains were first revived in Nutrient Broth media (BBL, Baltimore, USA) at 37°C for 24 h, according to standard procedures. The bacterial concentration in the inoculum was subsequently adjusted at 0.5 on the McFarland turbidity scale, equivalent to 1.5×10^8 colony-forming unit (CFU) mL^{-1}, with normal saline solution (0.9% w/v sodium chloride in water). Inoculums of 10^6 CFU mL^{-1} were also prepared by transferring aliquots of this suspension to test tubes containing Muller-Hinton Broth (MHB) medium (Mir-Media, Khuzestan, Iran).

Minimum inhibitory concentrations

Each essential oil was dissolved in dimethyl sulfoxide (DMSO) (Merck, Germany). The concentration of DMSO was less than or equal to 5% The suspension was mixed thoroughly with a shaker to obtain a clear solution. Serial twofold dilutions of each essential oil stock were made with MHB to yield final concentrations ranging from 256-0.0625 µg mL–1. MIC assays were performed using 96-well microplates (SPL Life Sciences Co., Ltd., Gyeonggi-do, Korea) according to a broth microdilution (BMD) procedure [17]. Briefly, 100 µl of MHB was added to all the plate wells. Different concentrations of EOs, and subsequently 100 µl of bacterial suspension with a concentration of 106 CFU mL–1 were added to the wells. The negative controls were composed of sterile MHB either alone or with DMSO (at concentrations used in the dilutions). Control wells containing sterile MHB but without inoculum were also used to ascertain aseptic conditions. The microplates were incubated at 37°C for 24 h under aerobic conditions, and the bacterial growth in each well was then checked. The lowest concentration without bacterial growth, i.e., clear wells, was considered as the MIC (µg mL-1). All assays were performed in triplicate

Minimum bactericidal concentrations

MBC determinations were performed by inoculating 100 µl of the assay mixtures from the wells showing no bacterial growth onto the surface of sterile Muller-Hinton Agar (MHA) medium. The plates were incubated under aerobic conditions for 24-48 h at 37°C and subjected to visual inspection. The lowest concentration without bacterial growth on the plates was considered as the MBC (µg mL^{-1}). In other words, the presence of bacterial growth on the medium indicated that the EO had bacteriostatic activity, while the absence of the growth indicated bactericidal activity of the EO sample.

TABLE 1. Human pathogenic bacterial strains used in this study.

Bacterial pathogen	ATCC Code	Gram
Staphylococcus aureus	25923	Positive
Staphylococcus epidermidis	12228	Positive
Streptococcus pyogenes	19615	Positive
Listeria monocytogenes	7644	Negative
Klebsiella pneumonia	13883	Negative
Pseudomonas aeruginosa	27853	Negative
Escherichia coli	25922	Negative

Double-diffusion assay

Antibacterial activities of the three EOs were also investigated by DDA tests. The tests were performed by using an agar well diffusion method described earlier [17]. The agar plate surface was first inoculated by streaking the bacterial inoculum (0.5 McFarland or 1.5×10^8 CFU mL^{-1}) over the entire agar surface using a sterile swab. Then, holes were made in each plate aseptically using a sterile punching device, and 100-µL of the EOs (0.5 mg mL^{-1}) was then introduced into the wells. In this procedure, Gentamycin was employed as a positive control. For this purpose, an agar disk-diffusion method [17, 18] and the Gentamycin-treated disks containing 10 µg of the antibiotic (PadtanTeb Diagnostic Co., Ltd., Tehran, Iran) were utilized. Negative controls were composed of the sterile medium either alone or with normal saline solution. The plates were then incubated at 37°C for 24 h under aerobic conditions; after this period, the diameter of inhibition zones was measured and recorded.

Checkerboard test

Antibacterial effects of double combinations of the three EOs against *E. coli* and *Staph. aureus* were also tested by the checkerboard method. This procedure is a two-dimensional array of serial concentrations of test compounds that is frequently utilized to evaluate antimicrobial combinations *in vitro* [19]. The dilutions tested in this study were originally selected based on the MIC of the EOs. The result of a checkerboard test was applied as input to calculate a fractional inhibitory concentration (FIC) index [19] based on the formulas: $FIC_A = MIC_{A+B}/MIC_A$, $FIC_B = MIC_{B+A}/MIC_B$, and FIC index = FIC_A+FIC_B. In these formulas, the value of MIC_{A+B} is the MIC of compound A in the presence of compound B, and *vice versa* for MIC_{B+A}. FIC index values were interpreted according to an earlier recommendation [20]: synergy (FIC index $\leq$ 0.5), antagonism (FIC index > 4.0), and no interaction (FIC index > 0.5–4.0). This procedure was carried out in 96-well microtiter plates using bacterial suspensions (10^6 CFU mL^{-1}), MHB medium and eucalyptus-thyme, eucalyptus-cinnamon, and cinnamon-thyme in twofold serial concentrations. Plate wells were finally checked for bacterial growth after 24 h incubation at 37°C under aerobic conditions.

Results

Minimum inhibitory concentrations (MIC), Minimum bactericidal concentration (MBC), and DDA (Double-Diffusion Assay) tests

In the present work, the antibacterial activities of three EOs, including cinnamon, eucalyptus, and thyme, were assessed using MIC and MBC tests. Based on the results, MICs of the EOs ranged from 0.25-1 µg mL^{-1}, 8-64 µg mL^{-1}, and 0.5-16 µg mL^{-1}, respectively. Similarly, the lowest MBCs were obtained for cinnamon (1-4 µg mL^{-1}), followed by thyme (2-64 µg mL^{-1}), and eucalyptus (32-256 µg mL^{-1}). While the reactions of the three Gram-positive bacteria to EOs were almost similar, MIC and MBC values evaluated for the four Gram-negative bacteria were almost considerably different. Among the bacteria tested, the best effect were found for *P. aeruginosa*. The results are shown in Table 2 and Fig. 1.

TABLE 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) (µg mL^{-1}) of three essential oils against seven important human bacterial pathogens[a].

Bacterial pathogen	Thyme		Cinnamon		Eucalyptus	
	MIC	MBC	MIC	MBC	MIC	MBC
Staphylococcus aureus	0.5[a]	4	0.25	1	8	32
Staphylococcus epidermidis	1	8	0.25	1	8	128
Streptococcus pyogenes	1	4	0.5	2	16	256
Listeria monocytogenes	1	4	0.25	1	16	128
Klebsiella pneumonia	1	8	0.5	4	32	128
Pseudomonas aeruginosa	16	64	1	4	64	256
Escherichia coli	0.5	2	0.25	2	16	64

[a] A broth microdilution (BMD) procedure (Clinical and Laboratory Standards Institute, 2012) was employed to estimate MIC and MBC values.

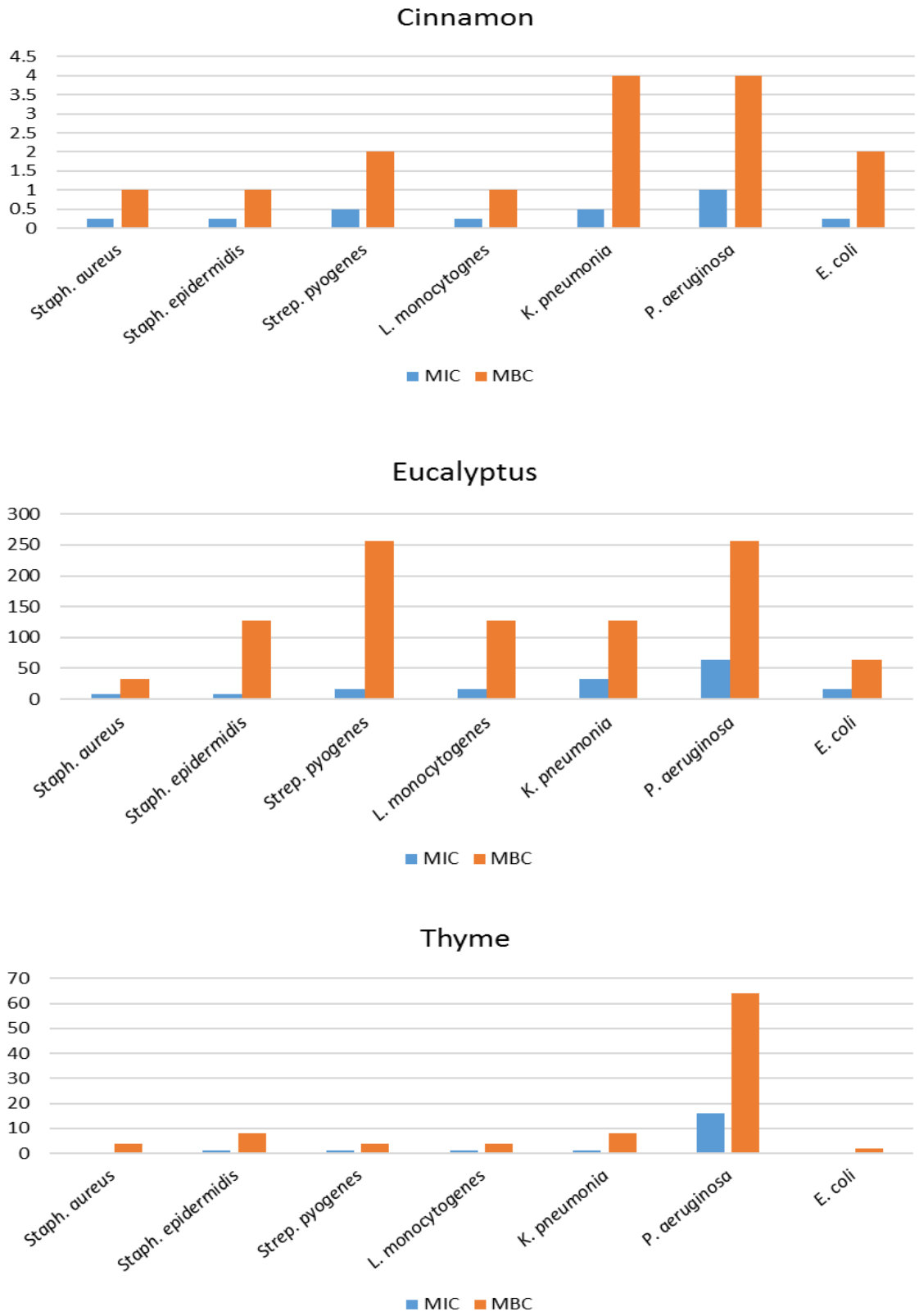

Fig. 1. Minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations of three essential oils against seven important human bacterial pathogens. A broth microdilution (BMD) procedure (Clinical and Laboratory Standards Institute, 2012) was employed to estimate MIC and MBC values.

The inhibition zones of the EOs against different pathogenic bacteria ranged from 20-35 mm, 9-25 mm, and 0-35 mm for cinnamon, eucalyptus, and thyme, respectively. Thyme followed by cinnamon showed the highest inhibition zones in almost all EO-bacterium combinations. The values obtained were comparable with those obtained for the positive control used in this study, i.e., Gentamycin, except for thyme-*S. pyogenes* where no inhibition zone was produced. The lowest inhibition zones were related to eucalyptus essential oil, except for those obtained for *S. pyogenes* where the value was quite greater than that of thyme and was equal to that measured for cinnamon. The results are shown in Table 3 and Fig. 2.

TABLE 3. Inhibitory diameters (mm) of three essential oils against seven important human bacterial pathogens[a]

Bacterial pathogen	Thyme	Cinnamon	Eucalyptus	Gentamycin
Staphylococcus aureus	35	35	18	20
Staphylococcus epidermidis	35	30	19	23
Streptococcus pyogenes	0	20	20	19
Listeria monocytogenes	30	28	13	21
Klebsiella pneumonia	35	30	11	22
Pseudomonas aeruginosa	20	25	9	17
Escherichia coli	35	30	25	20

[a] Agar well diffusion (Valgas et al., 2007) and agar disk-diffusion (Clinical and Laboratory Standards Institute, 2012) methods were utilized in the double-diffusion assays of the three essential oils and Gentamycin, respectively.

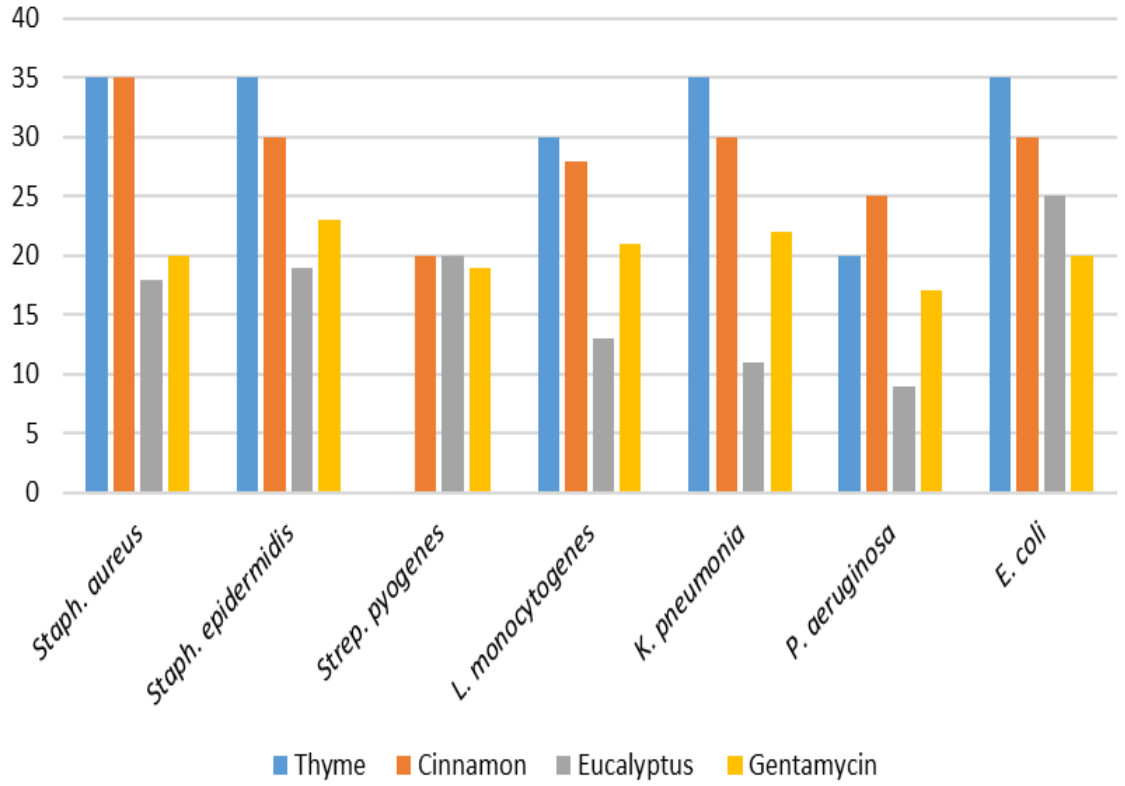

Fig. 2. Inhibitory diameters (mm) of three essential oils against seven important human bacterial pathogens. Agar well diffusion (Valgas et al., 2007) and agar disk-diffusion (Clinical and Laboratory Standards Institute, 2012) methods were utilized in the double-diffusion assays of the three essential oils and Gentamycin, respectively.

Checkerboard test and FIC index

In the checkerboard assay, different double combinations of cinnamon, eucalyptus, and thyme were tested for *S. aureus* and *E. coli*, ranging from several dilutions below the MIC to twice the MIC. FIC index values were calculated by considering all combinations of the EOs in which there was no visible growth. The best combination of the EOs, i.e., the one giving the lowest FIC index value, was shown in Table 4. The FIC index values ranged from 1.0625 to 2 for *S. aureus*, and from 2 to 4 for *E. coli*. The lowest and the highest values were recorded for cinnamon-eucalyptus/*S. aureus*, and for cinnamon-thyme/*E. coli* and eucalyptus-thyme/*E. coli* combinations, respectively. The FIC index values were greater than 0.5 and less than 4, indicating "no interaction" between components of the combinations. The values calculated for cinnamon-thyme/*E. coli* and eucalyptus-thyme/*E. coli* combinations were 4, close to the cut-off value for antagonistic interaction (Table 4).

TABLE 4. Checkerboard test and fractional inhibitory concentration (FIC) index.

Bacterial pathogen	Minimum inhibitory concentration (MIC)[a]				Fractional inhibitory concentration (FIC)[b]		
	MIC_A	MIC_B	MIC_{A+B}	MIC_{B+A}	FIC_A	FIC_B	FIC index
	Cinnamon	Eucalyptus	Cinnamon	Eucalyptus	Cinnamon	Eucalyptus	
Staphylococcus aureus	0.25	8	0.25	0.5	1	0.0625	1.0625
Escherichia coli	0.25	16	0.25	16	1	1	2
	Cinnamon	Thyme	Cinnamon	Thyme	Cinnamon	Thyme	
Staphylococcus aureus	0.25	0.5	0.25	0.5	1	1	2
Escherichia coli	0.25	0.5	0.5	1	2	2	4
	Eucalyptus	Thyme	Eucalyptus	Thyme	Eucalyptus	Thyme	
Staphylococcus aureus	8	0.5	4	0.5	0.5	1	1.5
Escherichia coli	16	0.5	32	1	2	2	4

[a] A broth microdilution (BMD) procedure (Clinical and Laboratory Standards Institute, 2012) was employed to estimate MIC values.

[b] FIC index values were calculated according to an earlier recommendation (Pillai *et al.*, 2005). The values were interpreted as synergistic (FIC index ≤ 0.5), antagonistic (FIC index > 4.0), and no interaction (FIC index > 0.5–4.0) (Odds, 2003).

Discussion

A variety of herbal substances have been indirectly described in traditional and herbal medicine as antimicrobial agents. Here, the antibacterial activities of three routinely used herbal elements in Persian medicine, i.e., cinnamon, eucalyptus, and thyme, were investigated. The results clearly indicated significant antibacterial properties of the three EOs under study [11,13,15]. This characteristic can be attributed to the major chemical components of the EOs, e.g., carvacrol, 1,8-cineole, thymol, *p*-cymene, cinnamaldehyde, and cinnamic acid [21,22]. However, there is some evidence showing that minor components have a crucial role in the antibacterial properties of EOs, possibly by making synergistic effects with other constituents. This may be one of the possible reasons for the higher antimicrobial properties of EOs than those of their major components reported in some studies [23-25]. Interestingly, some of the minor components were shown to have no antimicrobial activity on their own [26, 27]. On the other hand, the hydrophobicity of EOs and their constituents is an important characteristic as it facilitates the accumulation of these components in microbial cell membranes followed by an increase of cell permeability and leakage of intracellular constituents; this results in disturbing cell function and finally, cell death [28-30]. Therefore, the variation observed in the antibacterial activities of the EOs against different bacterial strains tested could be attributed to the differences in the rate of penetration of the EOs in the cell membranes and their subsequent interferences and disruption [31].

In the current work, cinnamon, eucalyptus, and thyme oils exhibited significant antibacterial activities against a range of human pathogenic

Gram-negative and -positive bacteria. Based on MIC and MBC values, cinnamon oil followed by thyme oil had the highest antibacterial properties; the lowest activities were found for eucalyptus (Table 2). However, the values related to Gram-negative bacteria were almost higher than those of Gram-positive counterparts, similar to those reported earlier [33-35]. The differences observed in bacterial resistance and susceptibility to EOs have morphological roots, i.e., the variation in bacterial cell membranes [35]. In contrast to the structure of cell membrane in Gram-positive bacteria, which allows the readily penetration of hydrophobic molecules, Gram-negative counterparts have an outer bilayer membrane containing several proteins and lipopolysaccharides, which make their membrane more resistant to EOs [36]. Amongst Gram-negative bacteria tested, *P. aeruginosa* showed the highest resistance to the EOs; our results are consistent with those reported previously.

In DDA tests, cinnamon and thyme oils almost produced larger inhibition zones against the bacterial strains as compared with those obtained with eucalyptus oil, indicating their higher levels of antibacterial activities (Table 3). These findings, in agreement with the results of MIC and MBC assays, confirmed the antibacterial activities of the EOs against a range of human pathogenic Gram-negative and -positive bacteria. However, different from MIC and MBC assays, the values obtained for Gram-negative bacteria were almost similar to those of Gram-positive counterparts [37]. This could be due to the higher titer of bacteria applied in DDA tests (1.5×10^8 CFU mL^{-1}) than that used in MIC and MBC assays (10^6 CFU mL^{-1}) [38]. Moreover, thyme oil did not induce an inhibition zone against *S. pyogenes,* which probably means that the EO cannot act on high titers of the bacterium. Our findings indicated that the antibacterial activities of the three EOs against different bacterial strains were tested, except for thyme-*S. pyogenes* combination were comparable with those of an antibiotic [39].

Application of herbal elements, including EOs, and their combinations are the main strategies in Persian medicine against different diseases, including those caused by Havaye Vabaee [8, 9]. Such combinations possibly affect a variety of biochemical and physiological processes in the human body and produce a plethora of interactions on the target microbes, e.g., bacterial pathogens [39]. For this reason, examining the interactions of EO-based antibacterial complexes with the bacterial strains is of great importance as combined treatments may alter the actual effects of each compound. The combined effects evaluated in this study varied based on the EO-EO combination and the target bacterium ($1.0 < \text{FIC index} \leq 4$) (Table 4), which is in agreement with previous reports [40]. Although no interaction effects were found for most of the combined EOs, and the target bacteria, the values obtained for thyme-cinnamon and thyme-eucalyptus combinations against *E. coli* (FIC index = 4) seem to indicate possible antagonistic effects. Antagonism has been previously reported for various EOs or their constituents' combinations against different bacterial pathogens [40]. Interestingly, recent investigations on antibacterial complexes have mainly found synergistic or antagonistic effects rather than no interaction effects [41]. Little is known about the mechanisms of interaction that induce antagonistic effects. One reason could be chemical (direct or indirect) interactions among compounds. Other studies have attributed this phenomenon to the combinations of compounds with bactericidal and bacteriostatic activities or the application of compounds acting on the same target of the microbe [42, 43].

Some of the herbal medicines commercially available to treat infectious diseases such as infections of the respiratory system are formulated from different plants with antibacterial properties. Based on the results of the present study, it is highly recommended to investigate the interactions of other compounds before formulating them as medicinal concoctions. For example, our study suggests that thyme oil must be used with caution as an antimicrobial in combination with cinnamon or eucalyptus oils because antagonism may predominate. It is also recommended to warn patients in the brochures of herbal medicines to avoid the simultaneous use of them with chemical drugs without consulting with their physician because of the possible adverse interactions, e.g., antagonistic effects of EOs and antibiotics [44]. In general, it can be said that the active ingredients in plants have effective antibacterial properties [44]. Finally, we should try to change the general belief saying that the use of herbal medicines is completely safe and healthy, without specialized considerations.

Conclusion

Thyme and cinnamon showed the highest inhibitory compounds in almost all EO-bacterial compounds and can be used for antibiotic purposes.

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Conflict of interest

The authors declare no conflict of interest

Authors'contribution

All authors contributed to the design and implementation of the research, analysis of the results, and the writing of the manuscript.

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