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Original article

Synergic antibacterial activity of some essential oils from Lamiaceae

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Abstract

Background and objectives: Despite the vast production of new antibiotics in the last three decades, resistance to these drugs by microorganisms has increased and essential oils (EOs) have been recognized to possess antimicrobial properties. **Methods**: In the present study, EOs obtained from aerial parts of *Thymus vulgaris* L., *Lavandula angustifolia* Mill., *Rosmarinus officinalis* L. and *Mentha piperita* L., were evaluated for their single and binary combined antibacterial activities against four Gram-positive and Gram-negative pathogenic bacteria: *Staphylococcus aureus*, *Bacillus cereus, Escherichia coli* and *Pseudomonas aeruginosa*. **Results**: The results exhibited that some of the tested essential oils revealed antibacterial activities against the examined pathogens using broth microdilution method. Maximum activity of the tested essential oils was obtained from the combination of *T. vulgaris* and *M. piperita* essential oils against *Staphylococcus aureus* (MIC= 0.625 mg/mL). **Conclusion:** Combinations of the essential oils in this study showed synergic action against some pathogenic microorganisms which could be considered in medical and food industries as preservatives.

Keywords: *Lavandula angustifolia*, *Mentha piperita*, *Rosmarinus officinalis* , synergic antibacterial activity, *Thymus vulgaris*

Introduction

Even though pharmaceurical industries have produced a number of new antibiotics in the last decades, microbial resistance to these drugs has grown increasingly [1,2]. Due to the number of patients with suppressed immunity and because of new multi-resistant bacterial strains, action must be taken to control the use of antibiotics, and to understand the genetic mechanisms of resistance and to continue studies to find new drugs, either synthetic or natural products [2]. For a long time, plants have been a valuable source of natural products for maintaining human health, especially in the last decades, with more intensive studies for natural therapies. Many plants have been used because of their antimicrobial activities [2,3] which are due to the compounds as the secondary metabolism of the plants such as essential oils (EOs) [2].

Essential oils which are also called volatile or ethereal oils are aromatic oily liquids obtained

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from different plant parts. In addition to the various pharmacological effects of EOs, it has been recognized that some EOs have antimicrobial properties [4,5]. Beside antibacterial activities, EOs or their components have shown to exhibit antiviral, antimycotic, antitoxigenic, antiparasitic and insecticidal properties [4].

The Lamiaceae family (Labiatae) is one of the largest and most distinctive families of flowering plants. Lamiaceae has great diversity and distribution in Iran with 46 genera and 410 species and subspecies, Lamiaceae species are best known for the essential oils [6]. Moreover, the family is a rich source of phenolic acids and oxygenated monoterpens which have demonstrated to possess antibacterial activity [6,7].

Thymus vulgaris L. (thyme) is an aromatic and medicinal member of Lamiaceae which is cultivated in large scale in Iran. Thyme volatile phenolic oil has been reported to be among the top 10 EOs, showing antibacterial [5,8], and antimycotic activity, and is a natural food preservative and mammalian age delaying agent [8]. It has been revealed that terpene type components of thyme oil such as myrcene, camphene and *p*-cymene possess antimicrobial activity [9]. Lavender oil, obtained from the aerial part of *Lavandula angustifolia* Mill. has been traditionally used as an antiseptic agent in swabbing of wounds, burns and insect bites [10]. Beside its relaxant and sedative effects, antibacterial, antiviral and antifungal effects of the oil have been revealed [11,12]. Moreover, lavender oil has exhibited *in vitro* activity against both MRSA (methicillin-resistant *Staphylococcus aureus*) and VRE (vancomycin-resistant *Enterococcus faecalis*) at a concentration of less than 1% [12]. *Rosmarinus officinalis* L. (rosemary), one of the most widely distributed species of the family Lamiaceae, contains a great quantity of essential oil which is largely used in traditional medicine for its tonic, stimulant and pulmonary antiseptic properties [13]. In addition to its antioxidant effect, the plant is known medicinally for its powerful antimutagenic antibacterial and chemopreventive properties [14]. Pintore *et al*. revealed that the essential oils from *R. officinalis* possess moderate antibacterial activity while the Gram-positive strains were more sensitive than the Gram-negative bacteria [13]. *Mentha piperita* L. (Peppermint), one of the well-known Lamiaceae species, is a perennial herb which is cultivated in many parts of the world. It’s essential oil has been used in food, cosmetic and pharmaceutical products [15,16]. Pattnaik *et al.* found that peppermint oil was effective against 22 different bacterial strains, including Gram positive cocci and rods and Gram-negative rods [17]. Moreover, peppermint oil has shown the ability to inhibit the production of *Staphylococcus aureus* toxin. The plant oil has also demonstrated antiviral and fungicidal activities [15]. The findings of Yadegarinia *et al*. suggest feasibility of application of *M. piperita* oil in treatment of the infections caused by *Candida albicans* and *Escherichia coli* [18].

Many studies have been carried out to extract various natural products for screening antimicrobial activity but attention has not been focused intensively on studying the combinations of these products for their antimicrobial activity [5].

In the present study, the antimicrobial activity of thyme, lavender, rosemary and peppermint essential oils was investigated against some pathogenic Gram positive and Gram-negative bacteria using microdilution method. Furthermore, the assessment of binary combinations of the EOs against tested microorganisms was performed to detect synergic or antagonist effects.

Experimental

Plant materials

The aerial parts of *Thymus vulgaris* L., *Lavandula angustifolia* Mill., *Rosmarinus officinalis* L. and *Mentha piperita* L. were prepared from Tehran suburbs, Iran and were identified by botanists at Traditional Medicine and Materia Medica Research Center (TMRC), Shahid Beheshti University of Medical Sciences, Tehran, Iran. The plant materials were powdered and stored separately in well-closed containers.

Chemicals

All materials, media and solvents were purchased from Merck, Germany.

Essential oil extraction

The plant powders were submitted to steam distillation in a Clevenger-type apparatus for 4 h. The obtained essential oils were dried over anhydrous Na_2SO_4 and stored at 4 °C until use.

Test organisms

Two Gram-positive bacteria (*Staphylococcus aureus* PTCC 1112 and *Bacillus cereus* PTCC 1015) and Two Gram-negative bacteria (*Pseudomonas aeruginosa* PTCC 1074 and *Escherichia coli* PTCC 1399) were purchased from Pasture Institute, Iran and were used in the experiment.

Antibacterial activity assay

The assay was performed according to Sobero´n with modifications [19]. Bacterial strains were cultured overnight at 37 °C on Mueller Hinton broth and adjusted to a final density of 0.5 McFarland standard (1.5 × 10^8 CFU/mL) and used as the inoculum. Stock solutions of the essential oils were prepared by dissolving material in dimethylsulfoxide (DMSO) 10% v/v at concentration of 10 mg/mL. Serial two-fold dilutions (0.078–10 mg/ml) of the essential oils were prepared in 96-well microplates; the microplates were prepared by dispensing 100 µL bacterial inoculum into each well (except for the first wells which were filled with 200 µL of the bacterial inoculums). A 40 µL (20 µL + 20 µL in case of combination) from each plant essential oil was added to the first wells. Then, 100 µL from their serial dilutions was transferred to seven consecutive wells of each plant essential oil. The final volume in each well was 100 µL. Clindamycin was used against *Bacillus cereus* and ciprofloxacin for *Staphylococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli* as positive controls, respectively. The wells containing the bacterial inoculum without plant essential oil were considered as negative controls. The microplates were incubated at 37°C for 24 h. Lack of turbidity, indicating the absence of bacterial growth, was interpreted as antibacterial activity. The MIC value was considered as the lowest concentration of the sample that caused complete inhibition (100%) of bacterial growth in experimental conditions [19].

Results and Discussion

Despite the vast production of new antibiotics in the last three decades, resistance to these drugs has increased by microorganisms. The problem of microbial resistance is growing and the outlook for the use of antimicrobial drugs in the future is still uncertain. It has been acknowledged that plant essential oils have been used for thousands of years. In addition to the fragrance industries, essential oils have been applied in food preservatives, pharmaceuticals, alternative medicine and natural therapies. The antimicrobial properties of some plant essential oils have been investigated by several studies. Hence, essential oils are potential sources of novel antimicrobial compounds especially against bacterial pathogens [2,5] which were the basis of our investigation.

In the present study, the antibacterial activities of *T. vulgaris*, *L. angustifolia*, *R. officinalis* and *M. piperita* essential oils were assessed *in vitro* by broth micro-dilution method against four pathogenic bacteria including *Staphylococcus aureus*, *Bacillus cereus*, *Pseudomonas aeruginosa* and *Escherichia coli*. Moreover, the effects of binary compounds of the essential oils against the mentioned pathogenic bacteria were evaluated. The microbial growth inhibition by each plant essential oil and their combination effects have been shown in tables 1 and 2.

According to the results, the essential oil of *T.*

vulgaris and *L. angustifolia* inhibited the growth of some examined bacteria but their effectiveness varied, while *Rosmarinus officinalis* and *Mentha piperita* essential oils didn't show any inhibitory effect on the tested pathogenic bacteria. The strongest individual antibacterial activity was observed against *Staphylococcus aureus* and *Escherichia coli* with MIC value of 1.25 mg/mL followed by *Bacillus cereus* (MIC 2.5 mg/mL).

Table 1. Individual antibacterial activity of *Thymus vulgaris, Lavandula angustifolia, Rosmarinus officinalis* and *Mentha piperita* essential oils against pathogenic microorganisms

Microorganism	MIC (mg/mL)			
	Thyme oil	**Lavander oil**	**Rosemary oil**	**Peppermint oil**
S. aureus	1.25	>10	>10	>10
B. cereus	2.5	2.5	>10	>10
P. aeruginosa	>10	>10	>10	>10
E. coli	1.25	>10	>10	>10

Biological activity of essential oils depends on their chemical composition, which is determined by the genotype and influenced by environmental and agronomic conditions [9]. In our study, *T. vulgaris* essential oil was found to be active against all examined pathogenic bacteria except *Pseudomonas aeruginosa*. The antibacterial activities of thyme essential oil could be associated with the presence of phenolic compounds like carvacrol, thymol, *γ*-terpinene and *p*-cymene, which are all reported to have antibacterial properties [5]. Thymol is structurally very similar to carvacrol, having the hydroxyl group at a different location on the phenolic ring. Both substances appear to make the cell membrane permeable. Studies on *B. cereus* have shown that carvacrol interacts with the cell membrane, where it dissolves in the phospholipid bilayer and is assumed to align between the fatty acid chains. It has been reported that carvacrol is able to inhibit the production of diarrheal toxin by *B. cereus* in broth and in soup [4]. *p*-cymene is a very weak antibacterial agent and it causes bacterial cell membranes to swell more than carvacrol. By this mechanism *p*-cymene probably enables carvacrol to be more easily transported into the cell so that a synergic effect is achieved when the two are used together [20].

Antibacterial evaluations in this research revealed that *L. angustifolia* essential oil exhibited antibacterial activity against *B. cereus* with MIC 2.5 mg/mL. This result is consistent with the former studies which demonstrated the antibacterial effect of linalyl acetate and linalool, the two major component of lavender, against some pathogenic bacteria [21]. Moreover, our study showed that *Pseudomonas aeruginosa* was resistant to all tested essential oils. It has been demonstrated that Gram-positive bacteria are more susceptible to essential oils than Gram-negative ones. The fact that Gram-negative organisms are less susceptible to the action of antibacterials has been ascribed to the presence of an hydrophilic outer membrane surrounding the cell wall membrane which blocks penetration of

Table 2. Antibacterial activity of *Thymus vulgaris, Lavandula angustifolia, Rosmarinus officinalis* and *Mentha piperita* essential oils against pathogenic microorganisms in binary combinations

Microorganism	MIC (mg/mL)					
	Thyme oil & lavander oil	**Thyme oil & rosemary oil**	**Thyme oil & peppermint oil**	**Lavander oil & rosemary oil**	**Lavander oil & peppermint oil**	**Rosemary oil & peppermint oil**
S. aureus	>5	>5	0.625	>5	5	>5
B. cereus	1.25	>5	>5	>5	>5	>5
P. aeruginosa	>5	>5	>5	>5	>5	>5
E. coli	2.5	5	>5	>5	>5	5

hydrophobic essential oils into target cell membrane [4,5]. *Pseudomonas*, and in particular *P. aeruginosa*, appear to be least sensitive to the action of essential oils [4]. The inherent activity of oils can be related to the chemical configuration of the components, the proportions in which they are present and to interactions between them. Synergism is observed when the effect of the combined substances is greater than the sum of the individual effects. Antagonism is observed when the effect of individual compounds is less than the time they are applied together [4]. In the present research, binary combination of the essential oils exhibited some synergic and antagonist effects against examined microorganisms. The strongest synergic activity was related to the combination of *T. vulgaris* and *M. piperita* essential oils (MIC 0.625 mg/mL) followed by *L. angustifolia* and *M. piperita* essential oils (MIC 5 mg/mL) against *Staphylococcus aureus*. Surprisingly, the mixture of *R. officinalis* and *M. piperita* essential oils exhibited synergic effect on *Escherichia coli* (MIC 5 mg/mL) whereas none of these essential oils showed antibacterial activity alone. The antagonist effects of the combinations resulted from association of the essential oils of *T. vulgaris* with *L. angustifolia* or *R. officinalis* against *S. aureus* as well as *T. vulgaris* with *R. officinalis* or *M. piperita* against *B. cereus*, respectively. There are some generally accepted mechanisms of antimicrobial interaction that produce synergism: sequential inhibition of a common biochemical pathway, inhibition of protective enzymes, combinations of cell wall active agents, and use of cell wall active agents to enhance the uptake of other antimicrobials. Mechanisms of antimicrobial interaction that produce antagonism are less known, although they include combinations of bactericidal and bacteriostatic agents, use of compounds that act on the same target of the microorganism and chemical interactions (direct or indirect) among compounds. For instance, non-oxygenated monoterpene hydrocarbons such as terpinene and *p*-cymene appear to produce antagonistic effects, since they reduce the aqueous terpene solubility and therefore the microbial availability of the active components. Specific modes of action of plant constituents with antimicrobial properties on the metabolic activities of microorganisms still need to be clearly defined even when the antimicrobials are used individually [22].

The present study enabled us to evaluate and compare the antimicrobial activity of *T. vulgaris*, *L. angustifolia*, *R. officinalis* and *M. piperita* essential oils individually and in binary combinations against some pathogenic bacteria. The results showed that a synergic effect could be achieved for some of the tested microorganisms which provided the first approach for developing an antimicrobial packaging with less concentrations of the active essential oils, whilst, this achievement could raise industrial interest in naturally produced preservatives.

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Declaration of interest
The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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