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Original article

Chemical composition and antibacterial properties of *Ocimum basilicum, Salvia officinalis* and *Trachyspermum ammi* essential oils alone and in combination with nisin

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Abstract

Background and objectives: Plant essential oils are sometimes considered for use as antimicrobial agents in foods and medicines and they could be combined with other antimicrobial agents to strengthen the effect and/or reduce the required dose. This study was conducted to determine the chemical composition of the *Ocimum basilicum*, *Salvia officinalis* and *Trachyspermum ammi* essential oils and evaluate their antibacterial efficiency, alone and in combination with nisin, against *Escherichia coli O 157* and *Staphylococcus aureus*. **Methods:** The chemical composition of three essential oils (*Ocimum basilicum*, *Salvia officinalis* and *Trachyspermum ammi*) were determined by gas chromatography/mass spectrometry. Further, their antibacterial properties and the synergistic effect of the combination of three essential oils and nisin were also assessed against *Escherichia coli* and *Staphylococcus aureus*. The antibacterial activity was determined by evaluation of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) by broth dilution method in 96-well microplates. The synergistic effects were tested by the checkerboard method and the fractional inhibitory concentration (FIC) index was calculated. **Results:** The major components of *O. basilicum*, *S. officinalis* and *T. ammi* were linalool (35.99%), 1,8-cineole (22.91%) and *p*-Cymene (35.5%), respectively. In general, all of the essential oils as well as nisin exerted more considerable antibacterial effects against Gram-positive bacteria than Gram-negative one. The essential oil of *T. ammi* showed the highest activity against *S. aureus* with MIC (≤0.078 mg/mL) and MBC (≤0.156 mg/mL). The combined application showed synergistic activity against *E. coli* but no change in activity was observed against *S. aureus*. The most synergistic effect was observed for the combination of nisin and *S. officinalis* (FIC 0.03). **Conclusion**: It can be concluded that nisin could enhance the antibacterial potential of the essential oils.

Keywords: antibacterial activity, nisin, *Ocimum basilicum, Salvia officinalis,* synergy, *Trachyspermum ammi*

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Introduction

Food materials from livestock such as meat and dairy products can be easily contaminated with microorganisms, leading to food spoilage, economic losses and more seriously public health problems. Food spoilage includes physical damage, chemical changes such as oxidation, color changes, or appearance of off-flavors and off-odors resulting from microbial growth and metabolism in the product [1]. Although, novel technologies for preservation and shelf life extension have been introduced recently, unfortunately, these steps do not completely eliminate undesirable pathogens from these products or delay microbial spoilage entirely. Hence, alternative preservation techniques such as novel non-thermal technologies and naturally derived antimicrobial ingredients are under investigation for their application to food products [2]. In the resent years, plant materials have been considered as an excellent alternative of currently available synthetic drugs and preservatives due to their lower or no side effects, broad spectrum antimicrobial activity and economic benefits [3]. Essential oils are rich sources of biologically active compounds and have been assessed to possess antimicrobial properties *in vitro* and *in vivo* [4-6]. Basil is the common name for the culinary herb *Ocimum basilicum* L. of the family Lamiaceae. It has been cultivated from many years ago and it is a well-known plant worldwide. It is half-hardy and annual, depending on the species and cultivar; the leaves may taste somewhat like anise, with a strong, pungent, often sweet smell. Basil oil has also found a wide application in perfumery, as well as in dental and oral products [7,8]. *Salvia officinalis* L. (sage) is a perennial, evergreen subshrub, with woody stems, grayish leaves, and blue to purplish flowers. It is a member of the family Lamiaceae and is native to the Mediterranean region, though it has been naturalized in many places throughout the world. The plant has been used for treatment of a number of diseases such as, obesity, diabetes, depression and cancer from ancient times [9,10]. *Trachyspermum ammi* L. belonging to Apiaceae family, commonly known as 'Ajwain', is widely grown in arid and semi-arid regions such as Egypt, Iraq, Iran, Afghanistan, Pakistan, and India [11]. There are a number of studies, which shows the potential anti-bacterial effects of the three mentioned plants against various bacteria including *Escherichia coli* and *Staphylococcus aureus* [8,12-16]. As a rule, herbal extracts show antimicrobial effects in considerably high concentrations. That is thought to be the major limitation for the application of the essential oils in foods. The high concentrations of essential oils can negatively influence the flavors and odors of foods and result in unacceptability. Therefore, research in this area should be focused on optimizing the essential oil combinations and applications to obtain effective antimicrobial activity at sufficiently low concentrations so as not to adversely affect the organoleptic acceptability of foods [2]. Bacteriocins from lactic acid bacteria such as nisin have been known as effective antibacterial peptides and have been proposed as food preservative since several years ago [2]. In the recent years, some researchers have demonstrated that the combined application of plants essential oils and bacteriocins exerts stronger antibacterial effects than the individual compounds [2,17]. The aim of the present study was to determine the chemical composition of the essential oils of three selected plants (*O. basilicum*, *S. officinalis* and *T. ammi*) and evaluation of the antibacterial efficiency of the essential oils, alone and in combination with nisin, against *Escherichia coli* O 157 and *Staphylococcus aureus*.

Experimental

Plant material

Ocimum basilicum and *Salvia officinalis* were purchased freshly from the local market at the end of spring 2013 and *T. ammi* seeds were collected from the rural area of West Azerbaijan at the same period. Pharmacognostic verification of the plants was performed by the Department of Botany and Herbal Medicine, Faculty of

Agriculture and Technology, Urmia University, Urmia, Iran. The voucher specimens were deposited at the institute Herbarium (No. 77231, No. 77286 and No. 77317, respectively).

Chemicals and reagents

Nisin was purchased from Sigma–Aldrich Chemical Co. (USA) that contained 2.5% nisin with minimum potency of 10^4 IU/g. Nisin (20 mg) dissolved in 1 mL 0.02 N HCl (Merck, Germany). It was centrifuged for 20 min at 1500 rpm. The supernatant was collected and sterilized and kept in 4 °C until analysis onset.

Extraction of essential oils

Arial parts of *O. basilicum and S. officinalis* as well as the seeds of *T. ammi* were air-dried and powdered (250 g), then subjected to hydro-distillation for 3 h using a Clevenger type apparatus. The oil was dried over anhydrous Na_2SO_4 and preserved in a sealed vial at 4 °C until further analysis.

Gas chromatography/mass spectroscopy (GC/MS) analysis of essential oils

GC/MS analysis was carried on a Varian Gas Chromatograph (Thermofinigan, USA) series 3800 fitted with a VF-5 ms fused silica capillary column (60 m × 0.25 mm, film thickness 0.25 µm) coupled with a 4000 series mass detector under the following conditions: injection volume 0.5 µL with split ratio 1:60, helium as the carrier gas at 1.0 mL/min constant flow mode, injector temperature 260 °C, oven temperature was programmed from 70 to 180 °C at 3 °C/min. Mass spectra: electron impact (EI+) mode, 70 eV and ion source temperature 290 °C. Mass spectra were recorded over 50–500 a.m.u range.

Identification of components

Identification of the essential oil constituents was performed on the basis of Retention Index [RI, determined with respect to homologous series of *n*-alkanes (C_8–C_{40}, Polyscience Corp., Niles IL) under the same experimental conditions], co-injection with standards and MS Library search (NIST 05 and Wiley) and by comparing with the MS literature data [18].

Preparation and maintenance of bacteria

The stock culture of *Escherichia coli* O 157 (ATCC 25922) and *Staphylococcus aureus* (ATCC 29213) were obtained from department of Food Hygiene & Quality Control, Faculty of Veterinary Medicine, the University of Urmia, Urmia, Iran. The bacteria were maintained on BHI agar (brain heart infusion) at 4 °C and was grown in BHI broth at 37 °C for 24 h.

Antibacterial assays

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the essential oils were assessed by the micro dilution method, recommended by the National Committee for Clinical Laboratory Standards (NCCLS) [19]. The oils were dissolved in dimethyl sulphoxide 5% (2.50 mg/mL) (Merck, Germany), added to the medium, and then diluted two fold to obtain concentrations in the range of 2.5-0.078 mg/mL. Inoculum suspension with a final concentration of 5×10^5 CFU/mL was added to a 96-well micro plate. The MIC was defined as the lowest concentration of the essential oil at which the microorganism did not demonstrate any visible growth after incubation at 37 °C for 24 h. The MBC was defined as the lowest concentration of the oils at which incubated microorganisms were completely killed [20]. All determinations were performed in triplicate.

Synergy studies, checkerboard method

In combination assays, a checkerboard procedure described by Davidson and Parish [21] was carried out. The checkerboard method was performed using 96-well microplates to obtain the fractional inhibitory concentration (FIC) index of the essential oils and nisin against each pathogenic bacterium. Briefly, the method allows different concentrations of each antimicrobial agent in each well of the assay plate in two axes (x and y) of an 8×8 matrix and it ensures that each well of the assay plate represents a different combination of two antimicrobial agents. Plates consisted of columns containing 25 µL of an

essential oil (a) which was two-fold diluted in a model medium along the x-axis. Rows of the same plates contained 25 µL of nisin (b) which was also two-fold diluted in the same medium along the y-axis. Subsequently, 90 µL of BHI broth media containing 2.0×10^{6} CFU/mL of the indicator strain was added to all wells. Plates were then incubated at 37 °C for 24 h and the optical density (600 nm) was measured. The tested compounds consisted of three essential oils of different plants (designated as antimicrobial 'a') (*O. basilicum, S. officinalis and T. ammi*) and nisin (designated as antimicrobial 'b').The MIC of each essential oil alone or in combination with nisin was taken as the lowest concentration that inhibited bacterial growth completely after 24 h [4].

The fractional inhibitory concentration (FIC) index was calculated by adding the FIC values of antimicrobial compounds (a) and (b) (FICa+FICb). The FICa and FICb values were represented as the lowest concentrations of the essential oil and nisin, respectively, that caused the inhibition of bacterial growth in the combination tests. Calculations were performed as follows:

FICa = (MICa combined/MICa alone)
FICb = (MICb combined/MICb alone)
FIC = FICa + FICb

For interpretation of the results, FIC ≤0.5 was assigned as a synergistic effect, 0.5 < FIC ≤1 represented an additive effect, 1< FIC ≤4 represented no interactive effect and FIC >4 showed antagonistic effect between the two tested antimicrobial agents [22].

Statistical analysis

All the experiments were performed twice in triplicate. The data were analyzed using one-way analysis of variance (ANOVA) tests using SPSS program (version 15, USA). Duncan's multiple range test was conducted to compare the mean values of the tested compounds alone or in combination. A significant difference was presumed at a p value <0.05.

Results and Discussion

Various chemical constituents were identified in the essential oil of *O. basilicum, S. officinalis* and *T. ammi* which have been shown in tables 1-3 respectively. As seen from table 1, the major components of *O. basilicum* were linalool (35.99%), estragole (28.56%) and eucalyptol (7.57%). Table 2 shows GC/MS analysis for *S. officinalis*; twenty-seven constituents were identified in the essential oil. The major component was 1,8-cineole (22.91%). The detailed chemical composition of *T. ammi* essential oil has been presented in the table 3; the principal chemical constituent was found to be *p*-cymene (35.5%). In general, the essential oils displayed similar antibacterial effects against Gram-negative bacterium. Besides, Gram-positive bacterium, *S. aureus* was found to be more sensitive to the essential oils than the Gram-negative one. The essential oil of *T. ammi* showed the highest activity against *S. aureus* with MIC (≤0.078 mg/mL) and MBC (≤0.156 mg/mL). Nisin exerted antibacterial activity in similar way to the essential oils. Table 4 also shows the antibacterial activity of *O. basilicum, S. officinalis* and *T. ammi* in combination with nisin. The FIC indices of nisin in combination with the essential oils against the bacteria can be found in table 4. The results show that the combined nisin and the different essential oils displayed a synergic activity against *E. coli* and an indifference effect against *S. aureus*.

Table 1. Chemical composition of *Ocimum basilicum* essential oil

No.	Compounds	KI	Percentage
1	β –pinene	980	0.25
2	**Eucalyptol**	**1043**	**7.57**
3	**linalool**	**1082**	**35.99**
4	Camphor	1121	1.8
5	**Estragole**	**1172**	**28.56**
6	Methyleugenol	1361	6.37
7	Eugenol	1392	2.2
8	Aromadendrene	1419	0.56
9	Caryophyllene	1440	6.54
10	γ-Muurolene	1455	2.24
11	β-Caryophyllene oxide	1557	0.64
12	Tau-Cadinol	1619	7.27

The dominant compounds are indicated in bold.

Table 2. Chemical composition of *Salvia officinalis* essential oil

No.	compounds	KI	Percentage
1	cis-Salvene	850	0.40
2	**a-Thujene**	**922**	**13.96**
3	Tricyclene	925	0.09
4	**α-Pinene**	**931**	**12.91**
5	Camphene	938	4.74
6	β- Thujene	967	8.91
7	β-Pinene	978	5.93
8	β-Myrcene	980	0.69
9	α-Terpinolene	1086	0.20
10	Camphor	1108	3.28
11	Borneol	1141	6.18
12	l-Phellandrene	1148	0.15
13	α-Terpinene	1155	0.31
14	**1,8-Cineole**	**1191**	**22.91**
15	β-Ocimene	1216	0.10
16	γ-Terpinene	1231	0.41
17	Bornyl acetate	1259	0.39
18	Endobornyl acetate	1283	0.77
19	Aromadendrene	1413	0.56
20	Trans-Caryophyllene	1419	7.41
21	α-Humulene	1430	3.19
22	α-Gurjunene	1432	0.10
23	Trans-Sabinene hydrate	1439	0.13
24	Δ-Cadinene	1499	0.24
25	Veridiflorol	1587	3.08
26	1-Naphthalenepropanol	2026	0.11
27	Others	-	1.03

The dominant compounds are indicated in bold.

Food material contamination with microorganisms is considered a major concern and can lead to public health problems such as poisoning and even death. Considering the different aspects of food spoilage, the development of novel preservatives with the ability of protection against wide spectrum of microorganisms, with no impact on odor and flavor, is attractive. In recent years, the demand for chemical preservatives has considerably decreased because of their undesired effects on food and also on human health. Plants essential oils have been interesting agents as excellent replacements for the synthetic preservatives. Since higher concentrations of essential oils are generally required to ensure their antimicrobial activity for food preservation as compared to *in vitro* system, their application may be limited due to changes in organoleptic and textural quality of food or interactions of essential oils with food components [22].

Table 3. Chemical composition of *Trachyspermum ammi* essential oil

No.	Compounds	KI	Percentage
1	α-Thujene	924	1.34
2	α-Pinene	930	1.75
3	Camphene	940	0.22
4	Sabinene	965	0.08
5	2-β-Pinene	976	5.10
6	β-Thujone	978	0.48
7	β-Myrcene	982	2.17
8	Cis-Sabinene Hydrate	1065	0.17
9	α-Terpinene	1080	0.42
10	**p-Cymene**	**1088**	**35.5**
11	α-Terpinolene	1096	0.12
12	Thujone	1105	0.30
13	Camphor	1109	0.09
14	Pulegone	1208	0.14
15	γ-Terpinene	1230	0.09
16	**γ-Terpinene**	**1235**	**32.52**
17	**Thymol**	**1259**	**18.32**
18	Trans-Caryophyllene	1405	0.29
19	Aromadendrene	1415	0.02
20	α-Humulene	1435	0.13
21	Caryophyllene Oxide	1553	0.017
22	Others	-	0.489

The dominant compounds are indicated in bold.

The current study targeted this limitation and was conducted to evaluate the synergistic effects of the essential oils and a well-known bacteriocin, nisin, as a promising natural alternative. The major components of *O. basilicum* were linalool and estragole. Our data confirmed previous study, which reported that the most abundant component of *O. basilicum* was linalool. Further, it has been claimed that the season of *O. basilicum* cultivation could influence the chemical composition and antimicrobial activity of the plant. Generally, essential oils from winter and autumn crops exhibited greater antimicrobial activity, which might be attributed to the high contents of linalool and other oxygenated compounds in these samples [23]. Based on a previous comprehensive study [24], four dominant constituents of *O. basilicum* essential oil are methyl chavicol, linalool, methyl eugenol and methyl cinnamate. The predominant constituents of *S. officinalis* were found to be 1,8-cineole and α-tujone. This finding is similar to the previous researches [16,25] but partly different with those of reported from Brazil [26].

Table 4. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of nisin (I.U/mL) and *Ocimum basilicum*, *Salvia officinalis* and *Trachyspermum ammi* essential oil (mg/mL) against *E. coli* and *S. aureus*. The fractional inhibitory concentration (FIC) was calculated for determination of the antibacterial relationship between the plants essential oil and nisin.

Test compound	Bacterium							
	Escherichia coli O 157				*Staphylococcus aureus*			
	MIC	MBC	FIC	Result	MIC	MBC	FIC	Result
O. basilicum	10	10<	-	-	0.62	1.25	-	-
S. officinalis	10<	10<	-	-	1.25	2.5	-	-
T. ammi	10	10<	-	-	≤0.078	≤0.156	-	-
Nisin	1000	1000	-	-	7.8≥	15.6≥	-	-
Nisin+ *O. basilicum*	31.25+0.31[a]	62.5+0.62[a]	0.06	S[*]	7.8+0.078[a]	15.6+0.156[a]	1.06	I
Nisin+ *S. officinalis*	15.6+0.156[b]	31.2+0.31[b]	0.03	S	7.8+0.078[a]	15.6+0.156[a]	1.06	I
Nisin+ *T. ammi*	31.25+0.31[a]	62.5+0.62[a]	0.06	S	7.8+0.078[a]	15.6+0.156[a]	2	I

*S: synergism, I: indifference within each column, the same small letters are not significantly different (p>0.05)

In this regard, Santos-Gomes and Fernandes-Ferreira have reported organ and season dependent variation in the essential oil composition of *S. officinalis* [27]. Our results show that *S. officinalis* essential oil showed great antibacterial activity against *S. aureus* but very little effects on *E. coli*. This aspect of the work is inconsistent with a previous study, which showed limited effects on both bacteria [16]. Phyto-chemical analysis of *T. ammi* essential oil revealed that the principal compositions were *p*-cymene and γ-terpinene. This was in agreement with previous works [28]. In our study, *T. ammi* possessed the highest antibacterial activity. It had been reported that, *T. ammi* essential oil had better or equal efficacy as compared to the standard antibiotics [29]. Our results showed that the essential oils were more active against the Gram-positive bacterium than the Gram-negative one. This aspect of the work is also consistent with the previous reports [2,30]. The different interactions, permeability and diffusion of various hydrophobic agents across the cell membrane may influence the extent of the antimicrobial effect. Hydrophobic components can usually diffuse across lipid bilayers easily, whereas hydrophilic agents are often transported passively via channels present in the membranes. In the case of Gram-negative bacteria, which are less susceptible to various antimicrobials, the presence of lipopolysacharide in the outer membrane protects against penetration of hydrophobic antimicrobial agents; thus, higher doses of antimicrobial agents are required compared to those needed for Gram-positive bacteria [2,20]. Nisin is effective against the Gram-positive bacteria and has also shown its efficacy against the Gram-negative bacteria when it is used as an additional technological barrier (in the application of multiple processes or barriers) for food safety and preservation. It is approved as a food preservative in more than 40 countries worldwide [4]. In the current work, nisin showed considerable antibacterial activity against *S. aureus*, however, it was ineffective against *E. coli*. In the recent years, the combined application of bacteriocin and essential oils has attracted great interest. As can be seen from table 4 all of the combination showed synergistic effect on *E. coli* (FIC≤0.5). The mechanisms responsible for the synergetic effects have not yet clearly been established, but it has been claimed that, the essential oils usually act on cell membrane and result in increase in permeability and subsequent the cell death [31,32]. The use of essential oils and bacteriocins together play an important role in the formation of membrane pores, which alter the permeability of the membrane, the proton motive force, the efflux of amino acid and the pH gradient of bacteria [4]. Taking together, the results show that the combined use of the essential oils and nisin, exert synergetic antibacterial effect on *E. coli*. This would allow the lower concentration use of

essential oils and thereby lower or no impact on food quality. Adjustment of precise minimal concentration of essential oils and investigation of efficacy of this method on shelf life extension should be investigated in further researches

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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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