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Chemical Composition and Antibacterial Activity of Essential Oils of Two Species of *Lamiaceae* against Phytopathogenic Bacteria

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Abstract

In this study, we aimed to determine chemical composition and antibacterial activities of *Satureja hortensis* and *Calamintha nepeta* against to 20 phytopathogenic bacteria causing serious crop loss. The essential oils of *S. hortensis* and *C. nepeta* were isolated by the hydrodistillation method and the chemical composition of the essential oils were analyzed by GC-MS. The antibacterial properties of the essential oils were evaluated against 20 phytopathogenic bacteria through Disc diffusion assay and micro dilution assay. The results revealed that the essential oils of *S. hortensis* and *C. nepeta* have significant antibacterial activity. Furthermore, the findings of the study are valuable for future investigations focusing on the alternative natural compounds to control plant diseases.

Key words: *Calamintha nepeta*, *Satureja hortensis*, antibacterial activity, biopesticide, chemical composition

Introduction

In recent years, one of the most popular subjects is the increase of yield production because of starvation that threats millions of people (Fletcher *et al.*, 2006). Every year, substantial part of the yield has been lost due to plant diseases caused by fungi, bacteria and viruses. Bacteria can also cause undesirable effects on quality, reliability and preservation of crop. To solve these problems, synthetic chemicals have been mostly used for many years. However, due to indiscriminate use of antimicrobial synthetic chemicals in the treatment of infectious diseases, both human and plant pathogenic microorganisms have developed resistance to multiple drugs/chemical substances (Sahin *et al.*, 2003; Gormez *et al.*, 2012). In addition, these chemical compounds can cause undesirable effects on environment because of their slow biodegradation and several serious side effects on mammalian health because of toxic residues in agricultural products (McManus *et al.*, 2002; Horvath *et al.*, 2009; Kotan *et al.*, 2010). This situation forced the researchers to discover new natural antimicrobial substances from various sources like medicinal plants (Clark, 1996; Cordell, 2000). Among many plant products, essential oils are the most studied plant secondary metabolites. Essential oils such as biopesticide have some advantages, where pathogenic microorganisms are not likely to develop resistance against them, little or no mammalian toxicity and not accumulated in soils (Heisey and Heisey, 2003; Singh *et al.*, 2003, 2005; Cardile *et al.*, 2009; Grosso *et al.*, 2010; Tian *et al.*, 2011). Therefore, the present study was conducted to investigate alternative antimicrobial agents among essential oils of *Lamiaceae* species that can be used as biopesticide.

The *Lamiaceae* is a family of plants having about 233 genera and 6900 species (Heywood *et al.*, 2007). The phenolic compounds, such as rosmarinic acid, caffeic acid, ferulic acid, chlorogenic acid, luteolin, apigenin, genkwanin, quercitrin, rutin, epicatechin and catechin are rich in *Lamiaceae* (Moreno *et al.*, 2006; Ben Farhat *et al.*, 2009; Castro-Vazquez *et al.*, 2009). Due to its rich contents of plants, they have many biological activities, such as anti-inflammatory, anticancer, antifungal,

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antimicrobial activity (Sarer and Pancali, 1998; Cheung and Tai, 2007; Figueiredo *et al.*, 2008; Takaki *et al.*, 2008; Quave *et al.*, 2008). *C. nepeta* and *S. hortensis* are well known aromatic and medicinal plants which belong to *Lamiaceae*. They have been used in folk medicines to treat many illnesses because of their antispasmodic, expectorant, diuretic, antimicrobial activities as it is stated in the relevant literature (Sarer and Pancali, 1998; Baser *et al.*, 2000; Sahin *et al.*, 2003). However, there is no report concerning the antibacterial activity of these essential oils against these many phytopathogenic bacteria.

In the study, we aimed to determine chemical compositions of hydro-distilled essential oils of *S. hortensis* and *C. nepeta* by GC-MS system as their biological activities were connected to their chemical compositions and to evaluate their antibacterial potentials against plant pathogen bacteria which have not been evaluated in the previous studies.

Experimental

Materials and Methods

Plant materials. *C. nepeta* and *S. hortensis* were collected at the flowering stage in July 2010, from the eastern part of Erzurum in Turkey. Identification of the plant materials was confirmed by a plant taxonomist, Assoc. Prof. Dr. Ozkan AKSAKAL, in the Department of Biology, Ataturk University, Erzurum, Turkey. Plants herbarium samples were stored in the herbarium of the Science Faculty, Ataturk University, Erzurum.

Isolation of the Essential Oils. Plant samples were dried in a canopy room. The aerial parts (leaves, flowers and steams) of the plants were powdered with blender and then subjected to water distillation for 2–3 h in a Clevenger-type apparatus (Thermal Laboratory Equipment, Turkey). The essential oils were stored at +4°C for further studies.

GC-MS Analysis Conditions. The essential oils were analyzed by using a Thermofinnigan Trace GC/Trace DSQ/A1300, (E.I Quadrapole) equipped with a SGE-BPX5 MS capillary column (30 m X 0.25 mm *i.d.*, 0.25 µm). For GC-MS detection, an electron ionization system with ionization energy of 70 eV was used. Helium was the carrier gas at a flow rate of 1 ml/min. Injector and MS transfer line temperatures were set at 220°C and 290°C, respectively. The program was used at 50–150°C at a rate of 3°C/min. Diluted samples (1/100, v/v, in methylene chloride) of 1.0 µl were injected manually and in the splitless mode. The components were identified based on the comparison of their relative retention time and mass spectra with those of standards, Wiley 7N library data of the GC-MS system and literature data. The results were also confirmed by the comparison of the compounds elution order with their relative retention indices on non-polar phases reported in the literature.

Table I
Plant pathogenic bacterial species used in the study

Bacteria	Strain No	Host
Agrobacterium tumefaciens	Apricot	AA-685
Bacillus pumilus	Apricot	AA-479
Clavibacter michiganensis subsp. *michiganensis*	Tomato	AA-703
Enterobacter intermedius	Cherry	AA-184
Erwinia caratovora subsp. caratovora	Tomato	AA-687
Erwinia chrysanthemi	Apricot	AA-58
Pseudomonas cichorii	Peach	AA-234
Pseudomonas corrugate	Tomato	AA-684
Pseudomonas fluorescens	Apricot	AA-616
Pseudomonas syringae pv. *syringae*	Cherry	AA-218
Pseudomonas syringae pv. *syringae*	Apricot	AA-637
Pseudomonas syringae pv. *syringae*	Apricot	AA-638
Pseudomonas syringae pv. *syringae*	Apricot	AA-647
Pseudomonas syringae pv. *phaseolicola*	Beans	AA-652
Pseudomonas syringae pv. *pisi*	Peach	AA-237
Pseudomonas syringae pv. *tabaci*	Apricot	AA-704
Pseudomonas syringae pv. *tomato*	Cherry	AA-220
Ralstonia solanacearum	Apricot	AA-116
Xanthomonas axonopodis pv. *campestris*	Pepper	AA-705
Xanthomonas vesicatoria	Tomato	AA-683

Plant pathogenic bacterial strains. The essential oils of the plants were tested against 20 plant pathogenic bacterial strains which were shown in the Table I. All the bacterial strains were isolated from some fruits and vegetables exhibiting typical bacterial disease symptoms on their respective host plants. They were identified by using conventional methods such as morphological, biochemical, pathogenicity tests and microbial identification system (MIS) (Miller and Berger, 1985). The isolated and identified bacterial cultures were preserved in Luria broth and 30% glycerol solutions at –80°C prior to use.

Antimicrobial activity: *2 Disc diffusion assay*. Twofold serial dilutions of the essential oils were made by diluting %10 DMSO to prepare a decreasing concentration range from 500 µg/ml to 7.81 µg/ml. Antimicrobial tests were carried out by disc diffusion assay using 100 µl of suspension containing 10^8 cfu/ml of bacteria spread on tryptic soy agar (TSA) medium by a sterile swab (Murray *et al.*, 1995). The discs (6 mm in diameter) were individually impregnated with 10 µl of essential oils at all the prepared concentrations and placed on the inoculated agar. Negative controls were prepared using the same solvents employed to dilute the essen-

Table II
Antibacterial activities of the essential oils of *S. hortensis* and *C. nepeta*

Bacteria	*S. hortensis*			*C. nepeta*			Negative control	Positive control
	DD		MIC	DD		MIC	DMSO	Standart antibiotic discs
	500 µg	7.81 µg		500 µg	7.81 µg			
Agrobacterium tumefaciens	48	8	7.81	48	8	7.81	–	28 (SCF)
Bacillus pumilus	47	7	7.81	47	7	7.81	–	23 (OFX)
Clavibacter michiganensis ssp. *michiganensis*	48	9	7.81	48	8	7.81	–	26 (SCF)
Enterobacter intermedius	16	8	7.81	35	7	7.81	–	26 (SCF)
Erwinia caratovora ssp. *caratovora*	48	9	7.81	45	7	7.81	–	29 (OFX)
Erwinia chrysanthemi	48	8	7.81	46	8	7.81	–	25 (SCF)
Pseudomonas cichorii	10	–	31.25	36	8	7.81	–	25 (OFX)
Pseudomonas corrugate	48	10	7.81	48	7	7.81	–	26 (OFX)
Pseudomonas fluorescens	48	7	7.81	43	8	7.81	–	11 (OFX)
Pseudomonas syringae pv. *syringae****	48	9	7.81	48	8	7.81	–	25 (OFX)
Pseudomonas syringae pv. *syringae*	8	–	31.25	33	8	7.81	–	21 (OFX)
Pseudomonas syringae pv. *syringae*	8	–	31.25	39	8	7.81	–	20 (OFX)
Pseudomonas syringae pv. *syringae*	8	–	31.25	42	10	7.81	–	21 (OFX)
Pseudomonas syringae pv. *phaseolicola*	14	–	15.63	44	7	7.81	–	24 (OFX)
Pseudomonas syringae pv. *pisi*	14	–	15.63	41	8	7.81	–	24 (OFX)
Pseudomonas syringae pv. *tabaci*	10	–	31.25	45	9	7.81	–	23 (OFX)
Pseudomonas syringae pv. *tomato*	11	8	7.81	45	8	7.81	–	25 (OFX)
Ralstonia solanacearum	48	10	7.81	48	8	7.81	–	24 (SCF)
Xanthomonas axonopodis pv. *campestris*	48	–	31.25	47	10	7.81	–	23 (SCF)
Xanthomonas vesicatoria	47	7	7.81	47	8	7.81	–	21 (SCF)

DD, Inhibition zone in diameter (mm/sensitive strains) around the disks (6 mm); MIC, minimal inhibitory concentration; * DMSO; Dimethyl sulfoxide (%10); ** OFX, ofloxacin (10 µg/disc); SCF, sulbactam (30 µg/disc) + cefoperazone (75 µg) (105 µg/disc) were used as positive reference standart antibiotic discs (oxoid); *** from different host (cherry).

tial oils. Positive controls were prepared using the antibiotics as indicated in Table II. The bacterial cultures were incubated at 27°C for 48 h. Antimicrobial activities of the essential oils were evaluated by measuring the zone of inhibition against the bacteria. Each test assays were repeated in triplicate.

Micro dilution assay. The minimal inhibition concentration (MIC) values studied for the bacteria were determined as sensitive to the essential oils in disc diffusion assay. The inocula of the bacteria were prepared from 12 h broth cultures and cultures were adjusted to 0.5 McFarland Standard turbidity. The essential oils were prepared by diluting 10% DMSO to prepare a decreasing concentration range from 500 µg/ml to 7.81 µg/ml to be tested in 10 ml sterile test tubes containing tryptic soy broth. MIC values of the essential oils against bacterial strains were determined based on a micro-well dilution method (Zgoda and Porter, 2001). The 96-well plates were prepared by dispensing into each well 95 µl of tryptic soy broth and 5 µl of the inoculum. Then 100 µl from essential oils from all the prepared concentrations were individually added into the wells. A negative control was prepared as the last well containing 195 µl tryptic soy broth without essential oil and 5 µl of the inoculum. Maxipime (Bristol-Myers Squibb) at the concentration range of 500–7.81 µg/µl was prepared in tryptic soy broth and used as standard drug for positive control. The plate was covered with a sterile plate sealer, mixed on plate shaker at 300 rpm for 20 s, and then incubated at 27°C for 24 h. Bacterial growth was determined by absorbance at 600 nm using the EL × 800 universal microplate reader and confirmed by plating 5 µl samples from clear wells on tryptic agar medium. The essential oils were tested against all the bacteria for three times. The MIC was defined as the lowest concentration of the compounds to inhibit the growth of microorganisms.

Results and Discussion

Chemical composition of the essential oils. The essential oil compositions of Turkish *Satureja*, *Calamintha* and the relative amounts of the components are shown in the Table III. This table shows that the Turkish *Satureja* contains carvacrol (79.17%), γ-terpinene (9.05%), *p*-cymene (3.14%), thymol acetate (2.24%), β-caryophyllene (1.48%); *Calamintha* contains

Table III
Essential oil contents of *S. hortensis* and *C. nepeta*

RI*	*S. hortensis*			*C. nepeta*			Identification methods
	RT**	Components	(%)	RT	Components	(%)	
983	11.84	β-Pinene	0.33	–	–	–	GC, MS, RI
995	–	–	–	13.21	3-Octanol	0.70	GC, MS, RI
1023	13.75	α-Terpinene	0.55	–	–	–	GC, MS, RI
1034	14.24	*p*-Cymene	3.14	–	–	–	GC, MS, RI
1037	–	–	–	14.29	Limonene	13.51	GC, MS, RI
1067	15.72	γ-Terpinene	9.05	–	–	–	GC, MS, RI
1106	–	–	–	18.07	Linalool	0.51	GC, MS, RI
1172	21.61	Borneol	0.64	21.59	Borneol	0.14	GC, MS, RI
1178	21.99	Terpinen-4-ol	0.96	21.87	Terpinen-4-ol	4.55	GC, MS, RI
1190	–	–	–	22.82	α-Terpineol	0.38	GC, MS, RI
1255	–	–	–	25.65	cis-Piperitone epoxide	48.66	GC, MS, RI
1289	26.97	Thymol	0.10	–	–	–	GC, MS, RI
1296	27.43	Carvacrol	79.17	27.46	Carvacrol	2.13	GC, MS, RI
1313	–	–	–	28.33	Dihydrocarveol acetate	1.24	GC, MS, RI
1347	29.39	Thymol acetate	2.24	–	–	–	GC, MS, RI
1369	–	–	–	30.43	Piperitenone oxide	22.08	GC, MS, RI
1419	32.25	β-Caryophyllene	1.48	32.24	β-Caryophyllene	2.21	GC, MS, RI
1442	33.04	Aromadendrene	0.30	–	–	–	GC, MS, RI
1478	34.60	γ-Muurolene	0.25	–	–	–	MS, RI
1486	–	–	–	34.87	Germacrene D	0.42	GC, MS, RI
1494	35.24	Viridiflorene	0.35	–	–	–	GC, MS, RI
1513	36.33	γ-Cadinene	0.51	–	–	–	MS, RI
1574	39.40	Spathulenol	0.92	–	–	–	MS, RI
1579	–	–	–	39.59	Caryophyllene oxide	0.80	GC, MS, RI

RI*; Retention index relative to *n*-alkanes on SGE-BPX5 capillary column, **RT****; retention times, **GC**; identification was based on retention times of authentic compounds on SGE-BPX5 capillary column, **MS**; tentatively identified based on computer matching of the mass spectra of peaks with Wiley 7N and TRLIB libraries and published data, **RI**; identification was based on comparison of retention index with those of published data

cispiperitone epoxide (48.66%), piperitenone oxide (22.08), limonene (13.51%) and terpinen-4-ol (4.55%) as major components.

According to the previous studies, essential oil compositions of *S. hortensis* and *C. nepeta* from different origins showed varieties in terms of quality and quantity. The compositions of essential oils of *S. hortensis* were reported as γ-terpinene (40.9%) and carvacrol (39.3%) with 4.46% oil content by Gora *et al.* (1996); carvacrol (40–49%) and γ-terpinene (36–45%) by Svoboda (2003); carvacrol (46%), γ-terpinene (37.7%) and oil content of 0.93% by Sefidkon *et al.* (2006); carvacrol (42.0–83.3%), γ-terpinene (0.5–28.5%) and *p*-cymene (1.0–17.1%) with twenty nine components in the oils by Hadiana *et al.* (2010); γ-terpinene (35.5%), thymol (18.2%) and carvacrol (29.7%) from extracted oil through supercritical fluid extraction by Khajeh (2011). It can be concluded from all the previous studies that carvacrol, thymol, and their precursors, *p*-cymene and γ-terpinene are major components of *S. hortensis* oil. Carvacrol and thymol were determined as the major components in all *Satureja* from Turkey, too. The compositions of essential oils of *S. hortensis* from Turkey were reported: thymol (29.0%), carvacrol (26.5%), a total 22 constituents consisted of γ-terpinene (22.6%), and *p*-cymene (9.3%) by Gulluce *et al.* (2003); carvacrol (42.0–63.0%) with oil content ranged from 1.30% to 2.67% by Baser *et al.* (2004); *p*-cymene (40.6% and 35.9%), thymol (39.9% and 43.4%), carvacrol (5.7% and 16.0%) and γ-terpinene (3.7% and 3.2%) with oil content of 0.5% and 0.7% by Azaz *et al.* (2005); thymol (40.54%), γ-terpinene (18.56%), carvacrol (13.98%), and *p*-cymene (8.97%) by Adiguzel *et al.* (2007).

The main constituents of *C. nepeta* oils were determined in the previous studies as pulegone (about 50%); menthone (9.4%), limonene (7.0%), menthol (4.6%), piperitenone oxide (4.6%), piperitone oxide (3.9%), and piperitenone (3.4%) by Flamini *et al.* (1999); pulegone (41.0%), menthone (32.0%), piperitone (7.3%) and piperitenone (7.0%) by Couladis and Tzakou (2001);

pulegone (75.5%), piperitenone oxide (6.0%), menthone (5.3%) and menthol (4.3%) by Kitic *et al.* (2005); pulegone (76.5%) and piperitone (6.1%) by Schulz *et al.* (2005); pulegone, piperitenone oxide and piperitenone by Marongiu *et al.* (2010). According to previous studies, the essential oils of *S. hortensis* and *C. nepeta* contain similar major compounds in spite of differences in their quantity. These differences might have been derived from local, climatic, seasonal and experimental factors. Our results have generally confirmed the findings of the previous studies.

Antibacterial activities of essential oils. In this study, the essential oils at 7.81–500 µg/disk concentrations were also tested for antibacterial activities against 20 phytopathogenic bacterial strains isolated from fruit and vegetables origins (Table II). The inhibition zone above 7 mm in diameter was regarded as positive result. As shown in this table, the oils of *S. hortensis* and *C. nepeta* exhibited considerable antibacterial activities against most of the tested bacteria (7–48 mm inhibition zone). Both gram-positive and Gram-negative bacteria were sensitive to the tested essential oils. No significant difference in susceptibility was found between Gram-negative and Gram-positive bacteria. It was interesting to find that most of the essential oils had stronger MIC values than standard antibiotic. 10% DMSO was used as a negative control, it exhibited no inhibition zone (Table II).

In various studies, although the extracts or essential oils of *S. hortensis* and *C. nepeta* were tested for their antimicrobial activity, there are no satisfactory reports against plant pathogenic bacteria. There is only a few data about the antibacterial effectiveness of the essential oil of *S. hortensis* against to phytopathogenic bacteria, which were provided by Gulluce *et al.* (2003), Sahin *et al.* (2003), Kizil and Uyar (2006), Kotan *et al.* (2007), Mihajilov-Krstev *et al.* (2009). The findings of those studies are supported by our findings demonstrating strong antimicrobial activity of essential oil of *S. hortensis*. To our knowledge, there is no report about the antibacterial properties of essential oil of *C. nepeta* against phytopathogenic bacterial strains. So, this study is the first report on the antibacterial effectiveness of the essential oil of *C. nepeta* against phytopathogenic bacteria.

According to our results the antibacterial effect of oil of *S. hortensis* was found to be lower than the essential oil of *C. nepeta* according to inhibition zone. But, generally, it is clear that both of the essential oils have strong antibacterial activity against tested phytopathogenic bacteria. Furthermore, in our study, we detected bactericidal activity against the tested bacteria, especially at high concentrations of essential oil of SH. In our study, generally most of the tested organisms were also sensitive to many of the essential oils. The maximal inhibition zones and MIC values of *S. hortensis*, *C. nepeta* showed a significant difference in the range of 7–48 mm and 7.81–31.25, 7.81 µg/ml, respectively (Table II). *A. tumefaciens*, *B. pumilus*, *C. michiganensis* subsp. *michiganensis*, *E. intermedius*, *E. chrysanthemi*, *P. fluorescens*, *P. syringae* pv. *syringae* (from cherry), *P. syringae* pv. *tomato*, *R. solanacearum* and *X. vesicatoria* were the most sensitive organisms against to both of essential oils (MIC value 7.81 µg/ml). *P. cichorii*, *P. syringae* pv. *syringae* (isolated from apricot), *P. syringae* pv. *tabaci* and *X. axonopodis* pv. *campestris* were the most resistant microorganisms to the essential oil of *S. hortensis* with the MIC value (31.25 µg/ml). The other resistant microorganisms to essential oil of *S. hortensis* were *P. syringae* pv. *phaseolicola* and *P. syringae* pv. *pisi* (15.63 µg/ml). It is thought that the sensivity can be caused by the differences in host, virulent of pathogens, toxins produced by these pathogens. For example, although *P. syringae* pv. *syringae* isolated from cherry was determined as sensitive (7.81 µg/ml), *P. syringae* pv. *syringae* isolated from apricot was the most resistant microorganism to *S. hortensis* oil with MIC value (31.25 µg/ml). As shown in the Table II, *C. nepeta* showed promising inhibitory activity especially even at low concentration. All of the tested bacteria were sensitive against to the essential oil of *C. nepeta*, too.

According to these results, it is clear that the essential oils have a potential antibacterial effect on the tested bacteria. Many of the previous studies demonstrated that essential oils show a considerable antimicrobial activity due to the presence of chemical compounds containing mainly aromatic oxygenated monoterpenes and high phenolic contents; carvacrol, thymol, ketones, pulegone, piperitone and piperitenone. For example, the antimicrobial activity of the essential oil of *C. nepeta* can be explained with the high contents of ketones, pulegone, piperitone and piperitenone (Panizzi *et al.*, 1993). This claim is further supported by our findings (Table III). Therefore, in our study; a high antibacterial effect of essential oil of *C. nepeta* can be associated with the presence of many components. In addition, according to studies made very recently, the antibacterial effect against the microorganisms were associated with the main constituents of the oil. According to Flamini *et al.* (1999), pulegone among constituents of *C. nepeta* only showed antimicrobial activity. It is also reported that some components such as carvacrol and thymol have potentials for controlling certain important plant pathogenic bacteria and seed disinfectant (Kotan *et al.*, 2007, 2010). So, the high antimicrobial activity of *S. hortensis* essential oil could be explained through the high level of carvacrol, well known for having antibacterial activity; *C. nepeta* have cis-piperitone epoxide, piperitenone oxide. Furthermore, the synergistic and antagonistic effects of these chemicals and

minor components can also affect the antibacterial activity of essential oils. In this regard, it is very important to stimulate systemic resistance mechanisms of the plants through the natural stimulators, use of healthy seeds, and seed disinfection through natural antimicrobial substances. Therefore, it is necessary to test several different combinations in commercial formulations of volatile oils and extracts and to determine bio-formulations according to the results obtained from these tests. It showed that essential oils of these plants are more effective than the antibiotics produced commercially against many bacteria. So; these essential oils are alternative components for defeating plant diseases. High level of antimicrobial activity of certain species in the Eastern Anatolia Region in Turkey put forward the necessity to take their gene sources under control and to research the possibility to cultivate them before dying out. Furthermore, it is necessary to carry out serious studies on their cultivatability.

In conclusion, the development of natural antimicrobials will help to decrease the negative effects (residues, resistance, and environmental pollution) of synthetic drugs. In this respect, natural antimicrobials may be also effective, selective, biodegradable, and less toxic to environment. In conclusion, according to the results presented in this study, we suggest that the essential oil of these plants can be used as antimicrobial agents in the management of plant diseases. However, the safety and toxicity of these compounds will need to be addressed.

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