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RESEARCH ARTICLE

Chemical composition and antibacterial activity of *Clinopodium nepeta* subsp. *glandulosum* (Req.) Govaerts essential oil

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Abstract

Clinopodium nepeta subsp. *glandulosum* (Req.) Govaerts is a natural perennial grass belonging to the Lamiaceae family, growing spontaneously on the Mediterranean coast. It is locally known as "kedi fesleğeni" in Turkey. This aromatic plant is used as a mint-like spice in food preparations and in the composition of some recipes during religious feasts. In folk medicine, it is used as stimulant, antiseptic and antispasmodic. Aerial parts of *C. nepeta* subsp. *glandulosum* were collected from Bilecik in July 2017, and was air dried. The essential oil was isolated by hydrodistillation using a Clevenger-type apparatus and the composition of the essential oil was simultaneously analysed by GC-FID and GC-MS. Piperitenone oxide (47.8%), limonene (18.6%) and piperitone oxide II (13.6%) were found as major components. Antibacterial activity of the essential oil was tested against *Escherichia coli* NRRLB-3008, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* ATCC 13311, *Bacillus cereus* NRRL-B3711 and *Streptococcus sanguinis* ATCC 10556 were used by broth microdilution method. Ciprofloxacin was used as control and Minimal Inhibitory Concentrations (MIC) were determined. The highest activity was found against *S. typhimurium* (1250 µg/mL). The essential oil is more effective against *B. cereus* (2500 µg/mL) and *S. sanguinis* (2500 µg/mL). The lowest activities were determined against *E. coli* (5000 µg/mL) and *P. aeruginosa* (10000 µg/mL).

Keywords: Lamiaceae, *Clinopodium nepeta* subsp. *glandulosum*, essential oil, chemical composition, antibacterial activity

Introduction

The genus *Clinopodium* (Lamiaceae) is represented by 39 taxa (Govaerts, 1999, Conforti et al., 2012). *Clinopodium* species are used in folk medicine like mints, mainly as stimulant, digestive, tonic, antiseptic (Baytop, 1999). *Clinopodium nepeta* subsp. *glandulosum (Req.)* Govaerts (syn. *Calamintha nepeta* subsp. *glandulosa*) is a perennial aromatic plant widespread in the Mediterranean region. *C. nepeta* occurs in Southern, Western and Southern Central Europe from South-eastern England to Crimea, Caucasus and Turkey, and is naturalised in North America (Conforti et al., 2012). This aromatical plant is effective as an antiseptic, antispasmodic and tonic. The essential oil of plant has antimicrobial, antifungal, antispasmodic, antioxidant and antitumor activities (Brankovic et al., 2009; Debbabi et al., 2020; Dzhambazov et al., 2002; Sarac & Uğur, 2009; Tepe et al., 2007).

Microorganisms develop resistance to antibiotics due to the unconscious and inappropriate use of antibiotics resulting in the difficult treatment of infectious diseases. Especially, strains of *Streptococcus sanguinis, Bacillus cereus, Escherichia coli, Pseudomonas aeruginosa,* and *Salmonella typhimurium* are among the resistant pathogens to various antibiotics. *P. aeruginosa* is an ubiquitously distributed opportunistic Gram-negative pathogen that inhabits soil fresh water, marine environments, colonizing plant, animal, and human hosts. It is an opportunistic pathogen that is naturally resistant to many antibiotics. It is one of the causes of hospital infections (Goldberg and Pier, 2000). *P. aeruginosa* is responsible for ventilator-associated pneumonia, contact lens keratitis, otitis externa, cystic fibrosis (Wolska & Szweda, 2009). *Salmonella* species are the most common causes of foodborne illness worldwide and *S. typhimurium* can cause infection in humans. It is a Gram-negative, facultative anaerobe bacterium and the leading cause of gastroenteritis

(Patricia et al.,1986). *E. coli* is a Gram-negative, rod-shaped bacterium that is commonly found in the lower intestine of warm-blooded organisms. Most *E. coli* strains are harmless, but some serotypes can cause serious food poisoning in humans (Jang et al., 2017). *S. sanguinis* is an indigenous Gram-positive bacterium that has been recognized for a long time as a key player in colonization of the human oral cavity. *S. sanguinis* is considered a benign, or even a beneficial, bacterium with regard to dental caries (Kreth et al., 2005). *B. cereus*, Gram-positive, an ubiquitous organism, commonly found in soil, hay, live trees, and other plant material, raw and processed foods, post-operative wound infections, local infections with necrosis, intravenous drug abuse, alcoholism, osteomyelitis and trauma caused by *B. cereus* (Kotiranta et al. 2000). Diseases caused by multidrug resistant microorganisms can be treated by plant sources, especially with the antimicrobial activity of essential oils.

In this study, essential oil obtained from aerial parts of *C. nepeta* subsp. *glandulosum* was evaluated for its *in vitro* antibacterial properties against food and human pathogenic standard bacterial strains. Besides, the volatile components of the essential oil were also investigated.

Materials and Methods

Materials

Aerial parts of *C. nepeta* subsp. *glandulosum* were collected from Bilecik in July 2017, herbarium samples were prepared. Voucher specimens were deposited in the Herbarium of the Faculty of Pharmacy in Ankara University with the herbarium number of AEF 27033 and was air dried. The essential oil was isolated by hydrodistillation for 3 h using a Clevenger-type apparatus. Antibacterial activities of the *C. nepeta* subsp. *glandulosum* essential oil against *Escherichia coli* NRRL B-3008 (Agricultural Research Service Culture Collection), *Bacillus cereus* NRRL B-3711, *Pseudomonas aeruginosa* ATCC 27853 (American Type Culture Collection), *Salmonella typhimurium* ATCC 13311, and *Streptococcus sanguinis* ATCC 10556 strains were screened. Standard antibiotic ciprofloxacin and resazurin were acquired from Sigma-Aldrich.

GC-FID and GC-MS analyses

Gas Chromatography-Flame Ionization Detection (GC-FID) and Gas Chromatography-Mass Spectrometry (GC-MS) analyses processes were performed with reference to Demirci et al. (2008).

Identification of the components

Identification of the essential oil components were carried out by comparison of their relative retention times with those of authentic samples or by comparison of their relative retention index (RRI) to series of *n*-alkanes. Computer matching against commercial (Wiley GC/MS Library, MassFinder Software 4.0) and in-house "Başer Library of Essential Oil Constituents" built up by genuine compounds and components of known oils.

Antibacterial activity

The antibacterial activity of the essential oil was evaluated by broth microdilution assay according to a modified Clinical and Laboratory Standards Institute (CLSI) method (CLSI, 2006). *Escherichia coli* NRRL B-3008, *Bacillus cereus* NRRL-B3711, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* ATCC 13311, and *Streptococcus sanguinis* ATCC 10556 were used as test microorganisms. Minimal Inhibitory Concentrations (MIC) of the samples were determined and given in Table 2, where ciprofloxacin was used as a positive control in the experiments. The tests were carried out in 96-well micro plates. The sample (100 µL per well) was diluted two-fold, with a final concentration range of 5000 to 9.76 µg/mL, respectively. Standard antibacterial agent ciprofloxacin (64 to 0.125 µg/mL) was used under the same conditions as positive

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controls. Strains were incubated in Mueller Hinton Broth (MHB) overnight at 37°C for 24h. Cultures, with a final inoculum size of 1 x 10^6 colonies forming units (CFU/mL) were used. Microbial growth was observed by adding 20 µL of resazurin of 0.01% with minor modifications of CLSI standards (Pfaller et al., 2008). A change from blue to pink indicated the reduction of resazurin and, therefore, microbial growth. The minimal inhibitory concentration (MIC) was determined as the lowest drug concentration that prevented the colour change. All experiments were repeated in triplicate, and average results were reported.

Results and Discussion

GC-FID and GC-MS analyses

The greenish-yellow essential oil was analysed by GC-FID and GC-MS, simultaneously to determine its chemical characterization. Piperitenone oxide (47.8%), limonene (18.6%) and piperitone oxide II (13.6%) were found as major components of the essential oil. Other components are given in Table 1.

Table 1. Volatile components of *Clinopodium nepeta* subsp. *glandulosum* essential oil

[a]RRI	Component	%
1018	Methyl-2-methyl butyrate	0.1
1032	*α*-Pinene	1.0
1035	*α*-Thujene	0.1
1063	Ethyl-2-methyl butyrate	[b]tr
1076	Camphene	0.1
1118	*β*-Pinene	1.0
1132	Sabinene	0.6
1174	Myrcene	0.9
1203	Limonene	18.6
1213	1,8-Cineole	0.4
1246	(*Z*)-*β*-Ocimene	tr
1255	*γ*-Terpinene	tr
1280	*p*-Cymene	tr
1393	3-Octanol	1.3
1474	*trans*-Sabinene hydrate	1.0
1494	(*Z*)-3-Hexyl-2-methyl butyrate	0.1
1497	*α*-Copaene	0.1
1535	*β*-Bourbonene	0.3
1532	Camphor	0.2
1553	Linalool	1.0
1556	*cis*-Sabinene hydrate	0.2
1571	*trans*-*p*-Ment-2-en-1-ol	tr
1590	Bornyl acetate	0.2
1612	*β*-Caryophyllene	0.9
1611	Terpinen-4-ol	1.1
1639	*trans*-*p*-Mentha-2,8-dien-1-ol	0.2
1668	(*Z*)-*β*-Farnesene	0.2
1678	*cis*-*p*-Mentha-2,8-dien-1-ol	0.1
1706	*α*-Terpineol	0.6

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1719	Borneol	0.2
1726	Germacrene D	0.6
1733	Piperitone oxide I	0.3
1755	Piperitone oxide II	13.6
1751	Carvone	0.3
1849	Calamenene	0.3
1864	*p*-Cymen-8-ol	0.1
1864	Isopiperitonone	0.6
1880	Benzyl-2-methyl-butyrate	0.2
1949	Piperitenone	0.6
1983	Piperitenone oxide	47.8
2006	8,9-Dehydrothymol	0.2
2016	4aβ, 7α, 7aα Nepatalactone	0.4
2069	4aα, 7α, 7aβ Nepatalactone	2.5
2198	Thymol	tr
	Total	**98.0**

[a]RRI: Relative retention indices calculated against *n*-alkanes, %: calculated from FID data, [b]tr: trace <0.1%

In a previous study of our group, forty-five components representing 91.65% of the oil were identified, piperitenone oxide (43.8%), *trans*-piperitone oxide (25.2%), and limonene (13.0%) were found as the major components *C. nepeta* essential oil (Kirimer et al, 1992). Marongiu et al. (2010) were found pulegone, piperitenone oxide and piperitenone as the main components (64.4–39.9%; 2.5–19.1%; 6.4–7.7%); conversely, the oil distilled from aerial parts of Portuguese *C. nepeta* is predominantly composed of isomenthone (35.8– 51.3%), 1,8-cineole (21.1–21.4%) and *trans*-isopulegone (7.8–6.0%) (Marongiu et al., 2010). Another study reported that, pulegone (40.5-54%) and menthone (23.6-16%) were found as the main components *C. nepeta* essential oil, respectively (Şarer & Pançalı, 1998; Demirci et al. 2011). Kitic et al. (2002) determined that pulegone (37.5%), menthone (17,6%), piperitenone (15.0%) and piperitone were as the main constituents in the oil (Kitic et al., 2002).

Antibacterial activity

The potential *in vitro* antibacterial activity of the essential oil was tested against *Escherichia coli* NRRLB-3008, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* ATCC 13311, *Bacillus cereus* NRRL-B3711 and *Streptococcus sanguinis* ATCC 10556 were used by broth microdilution method. Ciprofloxacin was used as control and Minimal Inhibitory Concentrations (MIC) were determined. The essential oil is more effective against *B. cereus* (2500 µg/mL), *S. sanguinis* (2500 µg/mL) and *S. typhimurium* (1250 µg/mL). The lowest activities were determined against *E. coli* (5000 µg/mL) and *P. aeruginosa* (10000 µg/mL). The results were given in Table 2.

Table 2. Minimal inhibitory concentration values (MIC) (µg/mL)

Bacteria	***C. nepeta* subsp. *glandulosum* essential oil**	**Ciprofloxacin**
Escherichia coli NRRLB-3008	5000	30
Pseudomonas aeruginosa ATCC 27853	10000	30
Salmonella typhimurium ATCC 13311	1250	30
Bacillus cereus NRRL-B3711	2500	<10
Streptococcus sanguinis ATCC 10556	2500	<10

Numerous studies exist on the *C. nepeta* essential oil antibacterial activity, where varying amounts of the tested pathogens were found to be susceptible to the oil were reported (Arantes et al., 2019; Miladinovic et al., 2012; Kitic et al., 2002). In previous study, MIC values of *C. nepeta* essential oil were determined against *S. aureus* (>2.0 µL/mL), *E. coli* (1.0 µL/mL), *S. typhimurium* (>2.0 µL/mL) and *P. aeruginosa* (1.0 µL/mL) (Arantes et al., 2019). According to other study, essential oil from *C. nepeta* have antimicrobial activity with a range of MIC values from 0.025 to 1.56 µL/mL (Miladinovic et al., 2012). In another study, antimicrobial activity of *C. nepeta* essential oil was determined by disk diffusion method. All tested microorganisms were found to be susceptible to essential oil (Kitic et al., 2002).

Conclusion

In the present study, piperitenone oxide (47.8%), limonene (18.6%) and piperitone oxide II (13.6%) were found as major components of the essential oil. Also, the essential oil of *C. nepeta* subsp. *glandulosum* showed different levels of activities against *E. coli*, *P. aeruginosa*, *S. typhimurium*, *B. cereus* and *S. sanguinis*. It was shown more effective against *B. cereus* (2500 µg/mL), *S. sanguinis* (2500 µg/mL) and *S. typhimurium* (1250 µg/mL). The lowest activities were determined against *E. coli* (5000 µg/mL) and *P. aeruginosa* (10000 µg/mL). More in detail evaluations on biological activity both on *in vitro* and *in vivo* levels are needed to exhaust the potential of essential oil from *C. nepeta*. Further work is ongoing.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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