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Original article

CHEMICAL CHARACTERIZATION AND ANTIMICROBIAL EVALUATION OF *STACHYS CITRINA* SUBSP. *CITRINA* ESSENTIAL OIL

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Abstract

Flowering aerial parts of Stachys citrina Boiss. et Heldr. subsp. citrina (Lamiaceae) being endemic for Turkey were subjected to hydrodistillation to obtain the essential oil which was further analysed by GC-FID and GC-MS, simultaneously. 36 constituents were determined in the oil, where α-pinene (29.8%) and β-phellandrene (13.6%) were identified as the main components. The anticandidal and antibacterial effects of the oil were evaluated by using microdilution method. The oil showed weak inhibitory effects against the tested bacteria panel (1.25 to 5.0 mg/mL, MIC) whereas the Candida strains were inhibited at lower concentrations of the oil. Especially C. glabrata was inhibited at a concentration of 0.078 mg/mL of the oil.

Key words: *Stachys citrina subsp. citrina, Lamiaceae, Essential oil, Antibacterial, Anticandidal*

Stachys citrina subsp. *citrina* Uçucu Yağının Kimyasal Karakterizasyonu ve Antimikrobiyal Değerlendirilmesi

Türkiye için endemik bir tür olan Stachys citrina Boiss. et Heldr. subsp. citrina (Lamiaceae) bitkisinin çiçekli toprak üstü kısımlarından su distilasyonu ile elde edilen uçucu yağın GC-FID ve GC-MS sistemleri ile eş zamanlı analizleri gerçekleştirilmiştir. Yağda 36 bileşik tespit edilmiş, α-pinen (%29.8) ve β-fellandren (%13.6) ana bileşikler olarak belirlenmiştir. Uçucu yağın antikandidal ve antimikrobiyal etkileri mikrodilüsyon yöntemi ile değerlendirilmiştir. Uçucu yağ yağ, test edilen bakterilere karşı daha etkisiz kalmakla birlikte (MİK; 1.25-5.0 mg/mL), Candida serisi yağın nispeten daha düşük konsantrasyonlarında inhibe olmuştur. Özellikle C. glabrata'nın, uçucu yağın 0.078 mg/mL'lik konsantrasyonunda inhibe olduğu belirlenmiştir.

Anahtar kelimeler: *Stachys citrina subsp. citrina, Lamiaceae, Uçucu yağ, Antibakteriyal, Antikandidal*

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INTRODUCTION

The genus *Stachys* consists of about 300 species throughout the world and is one of the largest genera of the Lamiaceae family. Turkey is one of the richest countries for *Stachys* diversity and it is represented by 83 species with a level of 48% endemism in Turkey (1-3).

Stachys species are widely used in folk medicine and they are known as sedative, antispasmodic, diuretic, emmenagogue, stomachic, digestive, carminative,tonic and throat pain reliever(4-5). Several *Stachys* species have also been reported for uses on genital tumors, sclerosis of the spleen, inflammatory tumors and cancerous ulcers(6).

In the present work, flowering aerial parts of *Stachys citrina* Boiss. et Heldr. subsp. *Citrina* were hydrodistilled to obtain an essential oil that was then analysed simultaneously by GC-FID and GC/MS systems. The essential oil was tested for its *in vitro* antibacterial and anticandidal activities. To the best of our knowledge, *Stachys citrina* subsp. *Citrina* essential oil and its antimicrobial activity have been investigated for the first time here.

EXPERIMENTAL

Plant material and isolation of essential oil

Plant material was collected from, Çakılı 0Yaylası, Gündoğmuş, Antalya (Turkey) in July 2008 at an altitude of 2100 m. Voucher specimen is kept at the herbarium of Faculty of Pharmacy of Anadolu University, Turkey (ESSE 14456). The essential oil was obtained by hydrodistillation using a Clevenger-type apparatus for 3h, from flowering air dried aerial parts. The essential oil yield was calculated on dry weight basis as 0.75 %. The obtained oils were dried over anhydrous sodium sulphate and stored at +4°C in the dark until analysed and tested.

GC–MS analysis

The GC-MS analysis was carried out with an Agilent 5975 GC-MSD system. Innowax FSC column (60 m x 0.25 mm, 0.25 μm film thickness) was used with helium as carrier gas (0.8 ml/min). GC oven temperature was kept at 60°C for 10 min and programmed to 220°C at a rate of 4°C/min, and kept constant at 220°C for 10 min and then programmed to 240°C at a rate of 1°C/min. Split ratio was adjusted at 40:1. The injector temperature was set at 250°C. Mass spectra were recorded at 70 eV. Mass range was from *m/z* 35 to 450.

GC-FID analysis

The GC analysis was carried out using an Agilent 6890N GC system. FID detector temperature was 300°C. To obtain the same elution order with GC-MS, simultaneous auto-injection was done on a duplicate of the same column applying the same operational conditions. Relative percentage amounts of the separated compounds were calculated from FID chromatograms. The analysis results are given in Table 1.

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, Mass Finder 3 Library) (7, 8) and in-house "Başer Library of Essential Oil Constituents" built up by genuine compounds and components of known oils, as well as MS literature data (9, 10) was used for the identification.

Microorganisms and culture conditions

Microorganisms were stored at -85 °C in sterile glycerol solution. Cultures were refreshed in Mueller Hinton Broth (MHB-Merck) at 35-37°C and inoculated on Mueller Hinton Agar (MHA-Merck) for checking purity. Three strains of *C. albicans* (NRRL Y-12983, ATCC 90028 and a clinical isolate-Osmangazi University, Faculty of Medicine, Department of Micrtobiology, Eskisehir, Turkey), *C. utilis* (NRRL Y-900), *C. tropicalis* (NRRL Y-12968), *C. krusei* (NRRL Y-7179), *C. parapsilosis* (NRRL Y- 12696) and *C. glabrata* (Clinical isolate-Osmangazi University, Faculty of Medicine, Department of Microbiology, Eskisehir, Turkey), *Escherichia coli* (NRRL B-3008), *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 27853), *Enterobacter aerogenes* (NRRL 3567), *Salmonella typhimurium* (ATCC 13311), *Bacillus cereus* (NRRL B-3711), Methicillin resistant *Staphylococcus aureus* (Clinical isolate- Osmangazi University, Faculty of Medicine, Department of Microbiology, Eskisehir, Turkey) were used as pathogen microorganism panel in the microdilution assay.

Determination of antibacterial and anticandidal activities

Microdilution susceptibility assay (11-13) was used for the antimicrobial evaluation of the oil. A stock solution of the oil was prepared in dimethylsulfoxide (DMSO, Carlo-Erba, France). Dilution series were prepared in sterile distilled MHB in 96-well microtiter plates. Overnight grown microorganismsuspensions in Mueller-Hinton Broth were standardized to 10^6 (for *Candida* panel) and 10^8 CFU/mL (for bacteria) by using suspension turbidity detector (Biosan, Latvia) adjusted to McFarland No: 0.5 standard. 100 µl of each culture suspension was then added into the wells. The last row without microorganism was used as sterility control. Microorganism and the MHB medium served as a positive growth control in a different row. After incubation at 37°C for 24 h the first well without turbidity was determined as the minimal inhibitory concentration (MIC). For the best visualization 20µl of Tetrazolium Violet 1% (w/v, EtOH) (2,5-diphenyl-3-[α-naphthyl] tetrazolium chloride, TTC, Sigma) reagent was transferred to plates and incubated at 37^{o}C for 3 h. Ketoconazole (Sigma) and Chloramphenicol (Merck) were used as standard antifungal and antibacterial agents respectively.

RESULTS AND DISCUSSION

The essential oil of the *Stachys citrina* subsp. *citrina* was analysed by GC and GC/MS systems intriplicate. The oil has a high content of monoterpene hydrocarbons in which α-pinene (29.8 %) and β-phellandrene (13.6%) were detected as majorcomponents. 35 constituents representing 77.3% of the oil were characterized. Additionally, an unidentified compound (sesquiterpene alcohol, mw: 122, RRI: 2140, 8.3%) was detected and its mass fragmentations (*m/z*) are also given in Table 1.

Table 1. The composition of the essential oil of *S. citrina* subsp. *citrina*[1]

RRI[2]	Compound	%[3]	Identification method
1032	α-Pinene	29.87±0.12	a,b
1035	α-Thujene	1.13±0.06	a
1118	β-Pinene	4.13±0.06	a,b
1132	Sabinene	0.80±0	a,b
1174	Myrcene	0.50±0	a,b
1176	α-Phellandrene	1.80±0	a,b
1203	Limonene	1.40±0	a,b
1218	β-Phellandrene	13.60±0	a,b
1246	(*Z*)-β-Ocimene	0.27±0.06	a
1255	γ-Terpinene	tr	a,b
1266	(*E*)-β-Ocimene	tr	a
1280	*p*-Cymene	0.17±0.06	a,b
1452	1-Octen-3-ol	0.33±0.06	a
1497	α-Copaene	0.70±0	a
1535	β-Bourbonene	0.20±0	a
1639	Cadina-3,5-diene	0.20±0	a
1661	Alloaromadendrene	0.27±0.06	a
1688	Selina-4,11-diene (*=4,11-Eudesmadiene*)	0.30±0.17	a
1704	γ-Curcumene	2.13±0.21	a
1726	Germacrene D	3.63±0.15	a
1726	α-Zingiberene	2.20±0.10	a
1745	Selina-4(15),7(11)-diene	0.47±0.15	a
1755	Bicyclogermacrene	1.70±0.10	a,b
1755	β-Curcumene	0.67±0.15	a
1773	δ-Cadinene	0.50±0	a
1776	γ-Cadinene	tr	a
1785	7-*epi*-α-Selinene	1.23±0.06	a
1786	*ar*-Curcumene	0.77±0.06	a
1786	Aromadendra-1(10),4(15)-diene	2.37±0.06	a
2069	Germacrene D-4β-ol	0.37±0.06	a
2140	Unknown[4]	8.27±0.15	a
2209	T-Muurolol	2.17±0.06	a,b
2255	α-Cadinol	0.33±0.06	a,b
2264	Intermedeol	0.37±0.06	a
2380	8α,13-Oxy-14-en-epilabdane (*=epi-Manoyloxide*)	1.07±0.06	a
2438	Kaur-16-ene	1.67±0.06	a
	Identified %	77.3±0.27	
	Identified compound	35	

[1] The analysis were carried out in triplicate, [2] RRI Relative retention indices calculated against *n*-alkanes, % calculated from FID data,[3]% area ± SD, tr: Trace (< 0.1 %), a: comparison of mass spectra with the Wiley and Mass Finder libraries and retention times, b: comparison with genuine compounds on the HP Innowax column, [4] Mass spectra (*m/z*) of unidentified compound; RRI 2140: 220(M^+, 0.4), 43(100), 96(59), 79(54), 69(46), 159(45), 107(44), 119(34), 187(30), 131(25), 55(24), 202(12).

In a previous study, germacrene-D (2.9-45.3%), β-caryophyllene (2.3-62.3%), caryophyllene oxide (trace to 7.8%), spathulenol (trace to 7.8%) and α-cadinene (1.4-8.5%) were reported as main compounds of 22 *Stachys* essential oils (14). In another study α-pinene (1-19%), β-linalool (1-34), dihydroedulane I (tr-16%), α-copaene (1-12%), germacrene D (1-34%), δ-cadinene (1-11%) and pimaradiene (0-19%) have been characterized as major compounds of 8 different *Stachys* species(15). Belonging to Swainsonianeae subsection six different *Stachys* essential oils were found to be rich in sesquiterpene hydrocarbons and their oxygenated derivatives (1).

The essential oil was subjected to *in vitro* antibacterial and anticandidal assays by using microdilution methods. The MIC results of the oil and the standard antimicrobial agents were shown in Table 2.

Table 2. The MIC (mg/mL) results of *S. citrina* subsp. *citrina* essential oil

Microorganisms	**Source**	**Eo**	**St**
Candida albicans	Clinical isolate	0.1875	0.062[a]
Candida albicans	ATCC 90028	0.3125	0.0156 [a]
Candida albicans	NRRL Y-12983	0.3125	0.06 [a]
Candida utilis	NRRL Y-900	0.625	0.03125 [a]
Candida tropicalis	NRRL Y-12968	0.3125	0.03125 [a]
Candida krusei	NRRL Y-7179	0.625	0.06 [a]
Candida parapsilosis	NRRL Y- 12696	0.625	0.06 [a]
Candida glabrata	Clinical isolate	0.0781	0.03125 [a]
Escherichia coli	NRRL B-3008	5	0.007 [b]
Staphylococcus aureus	ATCC 6538	5	0.003[b]
Pseudomonas aeruginosa	ATCC 27853	5	0.25[b]
Enterobacteraerogenes	NRRL 3567	5	0.003[b]
Salmonella typhimurium	ATCC 13311	1.25	0.007[b]
Bacillus cereus	NRRL B-3711	2.5	0.007[b]
MRSA	Clinical isolate	2.5	0.007[b]

Eo: *S. citrina* subsp. *citrina* essential oil, **St**: Standart Antimicrobial agent, [a]: Ketoconazole, [b]: Chloramphenicol, MRSA: Methicilline resistant *Staphylococcus aureus*

The oil showed a moderate anticandidal activity within the range of 0.625 to 0.078 mg/mL in comparison with standard anticandidal agent ketoconazole. Particularly *C. glabrata* was inhibited by the oil having a MIC value of 0.078 mg/mL. The oil showed weak inhibitory effects (1.25 to 5.0 mg/mL, MIC) against tested bacteria panel in comparison with standard agent chloramphenicol. In a previous study 22 *Stachys* species have been subjected to disc diffusion assay for determination of antimicrobial effects. *Stachys* essential oils had generally shown weak to moderate effects on bacteria and candida cultures similar to our findings (14). In another study, eight different *Stachys* essential oils were tested against several pathogenic bacteria that were inhibited at the concentrations of 0.1 to 0.7 mg/mL of essential oils (15).

As a conclusion; the essential oil composition of *Stachys citrina* subsp. *citrina* and its antimicrobial properties have never been reported previously. Monoterpene hydrocarbons (α-pinene, β-pinene, β-phellandrene) and sesquiterpene alcohols bearing essential oil of *S. citrina*

has been found to be active against *Candida*. Particularly clinically isolated *Candida glabrata* was inhibited by the oil showing a promising anticandidal activity (MIC, 0.078 mg/mL).

REFERENCES

1. Skaltza HD, Mavrommati A, Contantinidis T, A chemotaxonomic investigation of volatile constituents in *Stachys* subsec. Swainsonianeae (Labiatae), Phytochemistry 57, 235-244, 2001.
2. Şerbetçi T, Demirci B, Güzel ÇB, Kültür Ş, Ergüven M, Başer KHC. Essential oil composition, antimicrobial and cytotoxic activities of two endemic *Stachys cretica* subspecies (Lamiaceae) from Turkey, Nat Prod Comm 5(9), 1369-1374, 2010.
3. Davis PH, Flora of Turkey and Aegean Islands, Vol. 11**,** pp. 199-262, Edinburgh, 2000.
4. Dulger B, Ugurlu E, Aki C, Suerdem TB, Camdeviren A, Tazeler G. Evaluation of antimicrobial activity of some endemic *Verbascum*, *Sideritis*, and *Stachys* species from Turkey, Pharm Biol 43(3), 270-274, 2005
5. Baytop T, Türkiye'de Bitkiler ile Tedavi Geçmişten Bugüne, pp.348-349, Nobel Tıp Basımevi, İstanbul, 1999.
6. Morteza-Semnani K, Akbarzadeh M, Changizi S, Essential oils composition of *Stachys byzantina*, *S. inflata*, *S. lavandulifolia* and *S. iaxa* from Iran, Flavour Frag J 21, 300-303, 2006.
7. McLafferty FW, Stauffer DB, The Wiley/NBS Registry of Mass Spectral Data, John Wiley and Sons, New York, 1989.
8. Koenig WA, Joulain D, Hochmuth DH, Terpenoids and related constituents of essential oils, Mass Finder 3, In: Convenient and Rapid Analysis of GCMS, Ed: DH Hochmuth, Hamburg, 2004.
9. Joulain D, Koenig WA, The Atlas of Spectra Data of Sesquiterpene Hydrocarbons, EB-Verlag, Hamburg, 1998.
10. ESO 2000, The Complete Database of Essential Oils, Boelens Aroma Chemical Information Service, The Netherlands, 1999.
11. Winn WC, Allen SD, Janda WM, Koneman E, Procop G, Schreckenberger P, Woods G, Antimicrobial susceptibility testing, In: Color Atlas and Textbook of Diagnostic Microbiology, 6th edition, Ed(s): WC Winn, Lippincott, NewYork, 2005.
12. Amsterdam D, Susceptibility Testing of Antimicrobials in Liquid Media, In: Antibiotics in Laboratory Medicine, Ed(s): V Lorian, pp.60-104, Williams &Wilkins Maple Press, 2005.
13. İşcan G, Kırımer N, Kürkçüoğlu M, Başer KHC, Demirci F, Antimicrobial screening of *Mentha piperita* essential oils, J Agric Food Chem 50(14), 3943-3946, 2002.
14. Goren AC, Piozzi F, Akcicek E, Kılıc T, Çarkıcı S, Mozioğlu E, Setzer WN, Essential oil composition of twenty-two *Stachys* species (mountain tea) and their biological activities, Phytochem Lett, doi:10.1016/j.phytol.2011.04.013, 2011.
15. Skaltsa HD, Demetzos C, Lazari D, Sokovic M, Essential oil analysis and antimicrobial activity of eight *Stachys* species from Greece, Phytochemistry 64, 743-752, 2003.

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