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Antimicrobial essential oil of *Origanum boissieri* Ietswaart

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ABSTRACT: Apart from being a popular spice, *Origanum* species is very important taxa as an herbal medicine for their several pharmacological activities. Essential oils of *Origanum* species bearing phenolic monoterpenoids such as carvacrol and thymol demonstrated strong antimicrobial properties. In the present study hydrodistilled essential oil of endemic species *Origanum boissieri* Ietswaart were analysed by GC and GC/MS techniques simultaneously. Thirty compounds representing 95.1 % of the total oil were identified. Carvacrol (39%) and *p*-cymene (32%) were detected as the main components of the oil. Total methanol extract and essential oil of the flowering aerial parts of the plant with the carvacrol and *p*-cymene were screened for its antibacterial and anticandidal properties by using microdilution broth assay. *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Staphlylococcus epidermidis* and *Listeria monocytogenes* were strongly inhibited by the oil between the concentrations of 15,6 - 62.5 µg/mL. *O. boissieri* extract and the oil showed moderate effects against all tested *Candida* strains having MIC values of 125-1000 µg/mL.

KEYWORDS: *Origanum boissieri*, essential oil, MIC, antimicrobial, GC/MS.

1. INTRODUCTION

Origanum L. (Lamiaceae) is an important medicinal and aromatic plant genus comprises of 42 species and 49 taxa divided into ten sections, mainly found in the Mediterranean region [1, 2]. In Turkey, *Origanum* species known as "Kekik" and represented by 23 species and 31 taxa of which 21 are endemic. Five of them have a great economic importance; *Origanum onites*, *O. majorana* (syn. *O. dubium*), *O. vulgare* subsp. *hirtum*, *O. minutiflorum* and *O. syriacum* var. *bevanii* [3-5].

Genus *Origanum* is very important herbal medicine for its several bioactivities such as antioxidant, antiviral, antimicrobial, antiparasitic, analgesic, hypotensive, antimutagenic, antihyperglycaemic and anticancer [4, 6-11].

Some members of *Origanum* taxa are extensively used around the world as a spice with the name of "oregano". Besides being a popular spice, many studies have been reported that *Origanum* essential oils (OEo) are important products for pharmaceutical purposes. OEo contain several phenolic monoterpenes and some sesquiterpenes that are known to demonstrate strong antimicrobial activity. The main compounds of them are generally carvacrol, thymol, *p*-cymene and *γ*-terpinene. According to chemo-types, proportions of these compounds in the same species vary between the specimens [12-15].

The major compounds of the oil carvacrol and thymol have been classified as GRAS and the antimicrobial effects of them have been studied many times by different authors, which is possess remarkable inhibition effects on pathogenic bacteria and fungi [16-25].

The endemic species *Origanum boissieri* Ietswaart belongs to Amaracus (Gleditsch) Bentham section. It is characterized by 1 or 2-lipped sepals without teeth, purplish bracts, and saccate corollas. The combination of a saccate corolla and distinctly toothed upper and lower calyx lips distinguishes this species from the other three Turkish species of Sect. Amaracus [26]. There was only one work on *O. boissieri* essential oil studied previously by our group [27].

In this study, chemical composition of hydrodistilled essential oil of *O. boissieri* was determined by GC-FID and GC/MS techniques simultaneously. Essential oil and the crude methanol extract of the aerial parts of *O. boissieri* have been evaluated for their antibacterial and anticandidal effects comparing with the major compounds and standard antimicrobials for the first time here.

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2. RESULTS AND DISCUSSION

Chemical composition of hydrodistilled essential oil of flowering aerial parts *O. boissieri* were determined by GC-FID and GC/MS techniques simultaneously (Figure 1). Oxygenated monoterpenes (49.3%), monoterpene hydrocarbons (36.5%) and sesquiterpene hydrocarbons (4.6%) were the main groups among the chemicals in the oil (Table 1). Thirty components were characterized, representing 95.1% of the total components detected. The main constituents were identified as carvacrol (39%) and *p*-cymene (32%). Similar results were reported in previous study, which were determined *p*–cymene (43%) and carvacrol (18%) as main components of the *O. boissieri* oil [27].

Table 1. The chemical composition of the essential oil of *O. boissieri.*

RRI[a]	Main Group Code[b]	Compound	(%)[c]	IM[d]
1032	1	α-Pinene	0.7	tR, MS
1035	1	α-Thujene	0.9	MS
1076	1	Camphene	1.1	tR, MS
1118	1	β-Pinene	0.2	tR, MS
1188	1	α-Terpinene	0.9	tR, MS
1203	1	Limonene	0.3	tR, MS
1218	1	β-Phellandrene	0.3	tR, MS
1265	5	3-Octanone	0.3	MS
1280	**1**	***p*-Cymene**	**32.1**	**tR, MS**
1393	5	3-Octanol	0.2	tR, MS
1451	2	β-Thujone	0.5	MS
1474	2	*trans*-Sabinene hydrate	1.5	MS
1556	2	*cis*-Sabinene hydrate	0.5	MS
1611	2	Terpinen-4-ol	1.4	tR, MS
1612	3	β-Caryophyllene	3.0	tR, MS
1624	2	*trans*-Dihydrocarvone	0.5	MS
1645	2	*cis*-Isodihydrocarvone	0.3	MS
1662	2	Pulegone	0.6	tR, MS
1687	3	α-Humulene	0.4	tR, MS
1706	2	α-Terpineol	Tr[e]	tR, MS
1719	2	Borneol	3.8	tR, MS
1741	3	β-Bisabolene	1.2	MS
1864	2	*p*-Cymen-8-ol	0.4	tR, MS
1940	5	4-Isopropyl salicylaldehyde	0.8	MS
2008	4	Caryophyllene oxide	2.5	tR, MS
2113	2	Cumin alcohol	0.2	tR, MS
2144	4	Spathulenol	0.2	MS
2198	2	Thymol	0.3	tR, MS
2239	**2**	**Carvacrol**	**39.3**	**tR, MS**
2392	4	Caryophylla-2(12),6-dien-5β-ol (=*Caryophyllenol II*)	0.7	MS
		Monoterpene Hydrocarbones (1)[b]	36.5	
		Oxygenated Monoterpenes (2) [b]	49.3	
		Sesquiterpene Hydrocarbones (3) [b]	4.6	
		Oxygenated Sesquiterpenes (4) [b]	3.4	
		Others (5) [b]	1.3	
		Total	**95.1**	

[a] RRI: Relative retention indices calculated against *n*-alkanes;
[b] Main group code
[c] %: calculated from FID data
[d] IM: Identification Method; tR, identification based on the retention times (tR) of genuine standard compounds on the HP Innowax column; MS, tentatively identified on the basis of computer matching of the mass spectra with those of the Wiley and MassFinder libraries and comparison with literature data.
[e] tr: Trace (< 0.1 %)

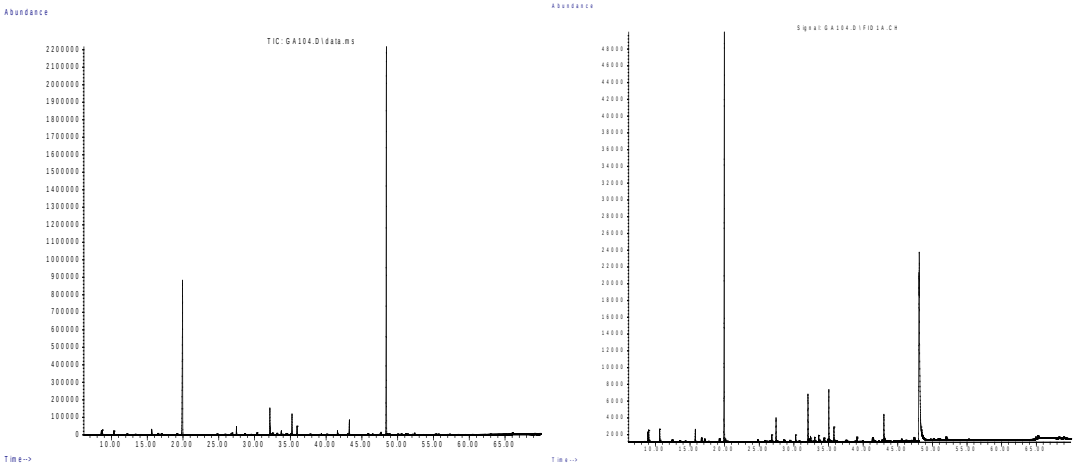

Figure 1. GC/MS and GC/FID chromatograms of the oil.

For the first time here antimicrobial properties of the *O. boissieri* essential oil and herbal methanol extract were studied in comparison with the standard agents. Antimicrobial effects of the OEo, carcavrol, *p*-cymene and total methanol extract were screened against five Gram (+), five Gram (-) bacteria and five strains of pathogenic *Candida* with the standard antimicrobial agent simultaneously (Table 2). The oil showed inhibition on the growth of tested bacteria between the concentrations of 31.25 to 1000 µg/mL.

Remarkably, known as a skin pathogen *Staphylococcus epidermidis* was inhibited by the oil having a MIC value of 31.25 µg/mL. All tested bacteria were inhibited by the carvacrol similar MIC values with OEo. However, oil was found more active against *S. aureus, L. monocytogenes* and *S. epidermidis* when compared with pure carvacrol, which may support synergistic contributions of other terpenoids in the oil. Other main compound *p*-cymene was showed weak effects against all tested bacteria species (4000-16.000 µg/mL). Except for *S. epidermidis*, methanol extract was stopped the growth of tested bacteria having MIC values of 1000 and 2000 µg/mL. According to antibacterial results (Table II) all Gram (+) bacteria were more susceptible to OEo and carvacrol.

Table 2. Minimum inhibitory concentrations of samples (MIC, µg/mL).

Bacteria strains		***Gram***	**OEo[a]**	**C[b]**	**PC[c]**	**ME[d]**	**St-1[e]**	**St-2**
B. cereus	NRRL B-3711	**+**	**62.5**	**62.5**	4000	1000	0.125	8
B. subtilis	NRRL B-4378	**+**	**62.5**	**62.5**	4000	1000	0.25	2
S. marcescens	NRRL B-2544	-	1000	250	8000	2000	1	1
E. coli	ATCC 8739	-	1000	125	8000	1000	2	2
S. typhimurium	ATCC 14028	-	1000	125	16000	1000	1	2
S. aureus	ATCC 43300	**+**	**62.5**	125	4000	1000	0.125	2
E. coli O157:H7	RSSK 234	-	500	250	8000	1000	2	2
P. aeruginosa	ATCC 10145	-	1000	500	8000	1000	16	32
L. monocytogenes	ATCC 19111	**+**	**62.5**	125	4000	1000	0.125	2
S. epidermidis	ATCC 14990	**+**	**31.25**	125	4000	250	0.25	2
***Candida* strains**							**St-3**	**St-4**
Candida albicans	ATCC 10231	na[f]	125	125	500	1000	0.25	0.25
C. utilis	NRRL Y-900	na	125	250	250	250	0.5	0.5
C. tropicalis	ATCC 1369	na	250	250	250	1000	0.25	0.25
C. albicans	ATCC 24433	na	250	125	250	1000	0.5	0.25
C. parapsilosis	ATCC 22019	na	125	250	1000	250	0.25	0.25

[a] OEo: *Origanum boissieri* essential oil
[b] C: Carvacrol
[c] PC: *p*-Cymene
[d] ME: Methanol extract
[e] St-1: Ampicillin, St-2: Chloramphenicol St-3: Amphotericin-B, St-4: Ketoconazole
[f] na: Not applicable

Similar to our results, previous studies support our findings that the phenolic monoterpenes are more effective against Gram (+) cell wall rather than sophisticated Gram (-) wall [20, 36, 37]. The most susceptible *S. epidermidis* chosen for bioautography assay as a test bacterium. Thanks to bioautography assay, bioactive component of the oil was determined undoubtedly as carvacrol because of the uncoloured inhibition zones on the TLC plate after overnight incubation (Figure 2).

It is well known that, as a phenolic monoterpene carvacrol possess strong inhibition effects against several pathogenic microorganisms [18-20, 24, 36]. All tested samples were exhibited moderate to weak anticandidal effects in comparison with Ketokonazol and Amphotericin-B, in the range of 125 to 1000 µg/mL (MIC). Souza et al. (2010) found that the essential oil from *Origanum vulgare* and *O. majorana* showed inhibition of the growth of *C. albicans, C. crusei* and *C. tropicalis* cells between the concentration of 80 to 160 µL/mL similar to our results [38].

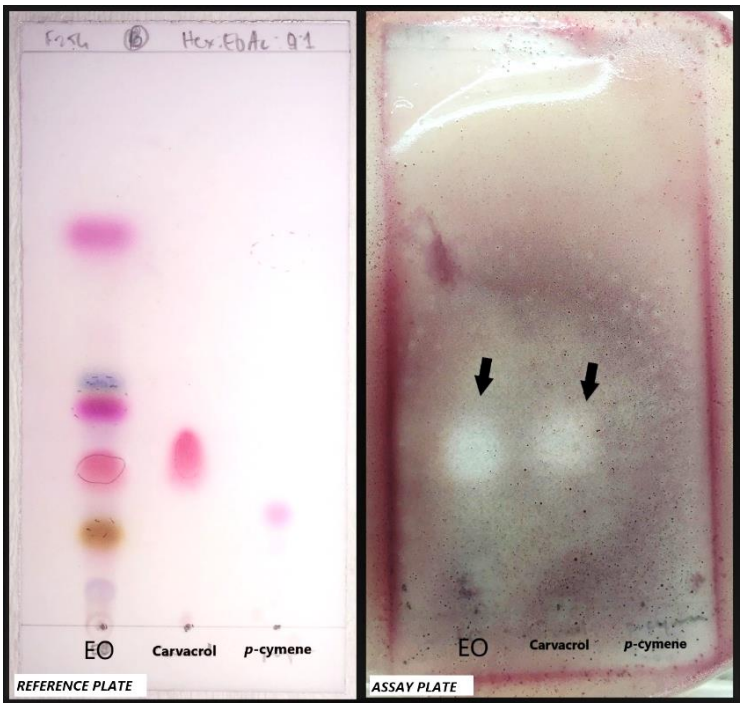

Figure 2. TLC-Bioautography assay result

3. CONCLUSION

According to all activity results, *O. boissieri* essential oil showed strong antibacterial effects against tested bacteria panel especially Gram positives, while it was displayed moderate effects against *Candida*. Carvacrol was found bioactive component of the oil according to TLC-bioautographic assay. TLC-bioautographic assay is useful tool for detection of the bioactive components of essential oils. *O. boissieri* oil has promising antibacterial natural product for dermal pathogen *S. epidermidis*.

4. MATERIALS AND METHODS

4.1. Plant material and extraction process

Plant material was collected from Namrun Province, İçel, Turkey in June, 2016. The plant specimens were authenticated and voucher specimen is kept at the Herbarium of Anadolu University, Faculty of Pharmacy (ESSE No:15490). Shade-dried aerial parts (50 g) of the plant were hydrodistilled for 3 h using a Clevenger-type apparatus to yield 2.0% of the oil. The essential oil was dried over anhydrous sodium sulphate and stored at +4°C in amber vial for further analysis.

To obtain crude methanol extract, air dried plant samples were chopped into small pieces and used brass mortar to pound a coarse powder. Plant material extracted with methanol by using orbital shaker at 150 rpm for 24 h. The extract was filtered through a standard filter paper and concentrated using a rotary evaporator with the water bath (40°C). The dry residue was kept at 4°C until antimicrobial assay.

4.2. Gas chromatography mass spectrometry (GC/MS) analysis

The GC/MS analysis was carried out with an Agilent 5975 GC-MSD system. 60 m Innowax FSC column (0.25 mm inner diameter., 0.25 µm film thickness) was used with helium as carrier gas (0.8 ml/min). GC oven temperature was set 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.

4.3. Gas chromatography (GC) 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 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 3 Library) [28, 29] 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 [30, 31] was used for the identification.

4.4. Microorganisms

Standard strains of *Bacillus cereus* NRRL B-3711, *Bacillus subtilis* NRRL B-4378, *Serratia marcescens* NRRL B-2544, *E. coli* ATCC 8739, *Salmonella typhimurium* ATCC 14028, *Staphylococcus aureus* ATCC 43300, *E. coli* O157:H7 RSSK 234, *Pseudomonas aeruginosa* ATCC 10145, *Listeria monocytogenes* ATCC 19111, *Staphylococcus epidermidis* ATCC 14990, *Candida albicans*, ATCC 10231, *Candida utilis* NRRL Y-900, *Candida tropicalis* ATCC 1369, *Candida albicans* ATCC 24433 and *Candida parapsilosis* ATCC 22019 were used as microorganism test panel.

4.5. Antimicrobial assay

Antibacterial and anticandidal properties of methanol extract, essential oil and its major compounds carvacrol (Aldrich) and *p*-cymene (Aldrich) were tested by using slightly modified CLSI protocols M7-A7 and M27-A2 [32, 33]. Five *Candida* strains that cause invasive fungal infections and ten different pathogenic bacteria strains were used as test microorganisms. Unlike the standard CLSI protocols, test solutions of the essential oil, authentic compounds and the extract were prepared between the concentration range of 16.0 to 0.015 mg/mL. Serial two fold dilutions of the samples were made on 96-well microtitration plates. Standard antimicrobial agents were diluted according the CLSI protocols. Amphotericin-B (Sigma-Aldrich) and Ketoconazol (Sigma-Aldrich) were used as standard antifungal agents where chloramphenicol (Sigma-Aldrich) and ampicillin (Sigma-Aldrich) were used as antibacterial agents. *Candida parapsilosis* ATCC 22019 was used for quality control strain for the protocol named M27-A2 susceptibility testing of yeasts.

4.6. TLC bioautography assay

According to antimicrobial assay results, OEo were subjected to Bioautography assay for the determination of active compounds in the oil. For this purpose, precoated silica gel 60_{F254} aluminium plates (~0.2 mm, Merck) were used (50x100mm). 15 µL of OEo, carvacrol and *p*-cymene solutions (10 mg/mL in *n*-hexane) were applied using Drummond micro-capillaries onto two equal TLC plates and developed (9:1 v/v, *n*-hexane/ethyl acetate) in the same time.

In one plate, the separated compounds were visualized under UV light (365 and 254 nm) and sprayed with anisaldehyde/H_2SO_4 reagent followed by heating to 110°C. The other plate was used for the bioautography assay, to determine the separated bioactive compounds. Overnight culture of liquid Mueller Hinton Broth with *S. epidermidis* ATCC 14990 were adjusted to McFarland No.05 turbidity standard (approximately 10^8CFU/mL). Additionally, 9 mL sterilized Mueller Hinton Broth in addition of agar (7.5%) was used as molten agar and kept at 45±1°C water bath. 1 mL of the liquid culture solution was added into the Molten Agar and vortexed for 15 seconds. Finally, the inoculated medium was poured onto the TLC plate and incubated at 37°C for 24 h. The plate was sprayed using a 1% (w/v, EtOH) tetrazolium violet reagent (2,5-diphenyl-3 [α-naphthyl] tetrazolium chloride, Sigma) and incubated at 37°C for further 4 h. After incubation, inhibition zones were visualized against the coloured background oil [34, 35].

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