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Micromeria thymifolia Essential Oil Suppresses Quorum-sensing Signaling in *Pseudomonas aeruginosa*

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The chemical composition, antimicrobial and antiquorum sensing activity of the essential oil of *Micromeria thymifolia* (Scop.) Fritsch were investigated. Limonene, piperitone epoxide and piperitenone epoxide were found as the main constituents using a gas chromatography-mass spectrometry technique. *In vitro* antimicrobial activity of the oil was tested against six bacterial and seven fungal strains and high antimicrobial potential was noticed. Minimum inhibitory concentration varied from 0.031 mg/mL to 0.5 mg/mL for bacterial and 0.062 mg/mL to 0.5 mg/mL for fungal strains. The antiquorum properties of the essential oil were evaluated on *Pseudomonas aeruginosa* PAO1. The oil was tested at subMIC concentrations for anti-quorum sensing activity. The analyses on quorum-sensing functions have been carried out by evaluating twitching and swarming of bacterial cultures and the total amount of pyocyanin production produced by *P. aeruginosa*. This study showed that *M. thymifolia* essential oil exhibited antiquorum sensing activity and may be used as an antipathogenic drug.

Keywords: *Micromeria thymifolia*, Essential oil, Antimicrobial activity, Antiquorum effect, GC-MS.

Micromeria thymifolia (Scop.) Fritsch (thyme savory) belongs to the family Lamiaceae. It is distributed in the Balkan Peninsula throughout Serbia, Croatia, Bosnia and Montenegro [1], extending to north-eastern Italy and with a disjunctive area of distribution in Hungary [2]. Plant shoots are numerous, branched, upright, polished, up to 50 cm high. Flowers are 5-9 mm long, white and violet, in shortly branched clusters. The plant usually grows in the clefts of broken rocks [3].

M. thymifolia has been commonly used for a long time in the Balkans area as a medicinal plant, especially for the treatment of nervous system disorders such as hysteria and epilepsy [2,4]. It is also used for gastrointestinal and respiratory ailments [5]. This species is also known in the literature as *Satureja thymifolia* Scop. and more recently as *Clinopodium thymifolium* (Scop.) Kuntze [6]. *M. thymifolia* essential oil (EO) possesses a pleasant odor similar to that of *Mentha* and *Thymus* species [2].

Some bacteria, including *Pseudomonas aeruginosa*, use small signaling molecules in order to communicate between populations and coordinate gene expression in order to enhance their survival rates in the inhospitable environmental conditions. This mechanism is called quorum sensing (QS) and includes a whole set of behaviours such as: biofilm formation, bioluminescence, conjugation and virulence, antibiotic production, competence, conjugation, swarming, motility and sporulation [7]. Biofilm is a form of growth of microorganisms, an adhesion of microbial cells to abiotic surfaces. Maturation of biofilm structures is connected with changes in cell phenotype and manifested by new metabolic pathways, which increase resistance to toxic compounds and virulence [8]. Biofilm formation plays an important role in infectious disease and causes infections [9]. A major problem of antibiotic therapy is the emergence of drug-resistant bacteria. It has been reported that EOs of *M. thymifolia* possess antimicrobial properties against various microorganisms such as *Staphylococcus* sp., *Pseudomonas* sp., *Micrococcus* sp., *Salmonella* sp., *Escherichia coli*, and *Candida albicans* [10, 11]. Biofilm forming bacteria are resistant to disinfectants, antibiotics and the action of host immune defenses. Bacteria produce diffusible signal molecules known as autoinducers [12]. Adonizio *et al*. [13] highlighted antiquorum sensing (AQS) compounds that may be very promising in the treatment of chronic infections caused by biofilm forming microorganisms. AQS properties of natural compounds are well documented in the literature [13-17]. AQS treatment may be a reliable way to reduce bacterial virulence and attenuate the acquisition of drug resistance by pathogenic bacteria [12].

The aim of this study was to analyze the chemical composition of the EO of wild growing *M. thymifolia* from Serbia, determine its antimicrobial properties and to evaluate the quorum sensing (QS) inhibitory activities of the EO using *Pseudomonas aeruginosa* PAO1. To the authors best knowledge this is the first report on *M. thymifolia* AQS properties.

Table 1: Chemical composition of *M. thymifolia* essential oil.

Compounds	Rt (min)	RI	%
α-Pinene	5.81	933	0.8
Sabinene	6.90	973	0.4
β-Pinene	7.02	977	2.4
Myrcene	7.39	991	0.7
2-Octanol	7.50	995	0.3
Limonene	8.77	1031	20.8
β-(*Z*)-Ocimene	8.97	1036	1.0
β-(*E*)-Ocimene	9.35	1047	0.1
trans-*p*-Mentha-2,8-dien-1-ol	12.18	1121	0.1
Octanol-acetate	12.33	1124	0.1
cis-*p*-Mentha-2,8-dien-1-ol	12.78	1136	t
Menthone	13.59	1151	t
Terpinen-4-ol	14.60	1176	t
p-Cymen-8-ol	14.95	1184	t
α-Terpineol	15.18	1190	0.3
Myrtenal	15.34	1193	0.1
Coahuilensol, methyl ether	16.40	1217	0.5
Pulegone	17.34	1239	0.4
Piperitone epoxide	18.29	1260	38.9
S-isopiperitenone	18.83	1273	0.2
Thymol	19.88	1296	0.5
Piperitenone	21.89	1342	1.8
Piperitenone epoxide	23.34	1375	28.4
α-Copaene	23.46	1378	0.2
β-Bourbonene	23.86	1387	0.6
β-Copaene	25.73	1431	0.1
Germacrene D	27.96	1484	0.3
Germacratrien-1-ol	36.16	1688	0.1
total identified %			99.1

RI = Retention Index on HP-5MSI capillary column; t-traces (<0.1%)

A total of 30 components were identified in the EO of *M. thymifolia* on the basis of the comparison with MS data base spectra. The chemical composition of *M. thymifolia* EO is listed in Table 1. The major components were piperitone epoxide (38.9%), piperitenone epoxide (28.4%) and limonene (20.8%). Previous studies on *M. thymifolia* EO chemical composition demonstrated pulegone as the major component, in some of them with 50.4% of pulegone being found [10, 11]. The second major component was piperitenone. In our oil, the percentage of pulegone was only 0.35%. Different environmental factors could be the reason for the differences noted in the contents of *M. thymifolia* EO.

M. thymifolia EO showed a strong antimicrobial activity (Table 2). The most sensitive bacterium was *E. coli* with MIC = 0.062 mg/mL, while the most resistant one was *P. fulva* (MIC = 0.5 mg/mL). Among the yeasts, the highest MIC was shown by *G. klebanchii* with a value of 0.062 mg/mL and the most resistant was *C. humilis* with a MIC value > 0.5 mg/mL. The EO possesses an antibacterial activity against *B. cereus* (MIC = 0.0312 mg/mL) and *S. enteritidis* (MIC = 0.0312 mg/mL), similar and greater than streptomycin (MIC = 0.30 mg/mL and MIC = 0.05 mg/mL, respectively), which was used as a positive control. The experimental data indicate that the oil showed a wide spectrum of antimicrobial action.

The presence of a high amount of oxygenated monoterpenes (more than 80%) suggests strong antimicrobial activity of *M. thymifolia* EO [18]. Monoterpenes such as limonene could be responsible for the antifungal properties [19]. The results obtained in this work are in agreement with previous studies regarding antimicrobial activities of different *Micromeria* species [10, 20].

The effect of *M. thymifolia* EO on biofilm formation of *P. aeruginosa* was tested with concentrations corresponding to 1/2 MIC, 1/4 MIC, and 1/8 MIC. The oil reduced biofilm formation in the range of 22.8-26.5%, which indicated that biofilm was formed in the presence of *M. thymifolia* oil in the range of 73.6-77.2% at subMIC concentrations. Streptomycin and ampicillin reduced biofilm by 50.6% and 30.8%, respectively. Results showed that the oil reduced biofilm formation to a higher extent than streptomycin and ampicillin when tested at 1/8 MIC (Table 3).

Table 2: Antimicrobial activity of *M. thymifolia* essential oil.

	MIC (mg/mL)	**MBC/MFC (mg/mL)**	**Streptomycin/Cyclo-heximide (mg/mL)**
B. cereus ATCC 11966	0.0312	2.0	0.30
E. coli ATCC 25922	0.062	1.5	0.05
L. monocytogenes 56Ly	0.0312	1.0	0.02
S. enteritidis 155	0.0312	1.5	0.05
P. aeruginosa	0.25	1.0	0.15
P. fulva LV1	0.5	2.5	0.03
C. humilis LVL 1	>0.5	-	< 0.05
C. krusei LVL 12	0.25	0.5	< 0.05
G. klebanhii LVL 3	0.0625		<0.05
P. anomala OC70	0.25	0.5	0.04
P. anomala OC71	0.125	0.25	0.02
P. membranaefaciens CBS 5759	0.25	0.5	0.04
P. membranaefaciens DBVPG 3003	0.25	0.5	0.04

Table 3: Effects of *M. thymifolia* EO on biofilm formation of *P. aeruginosa* (PAO1).

Sample	**Biofilm formation***		
	1/2 MIC	**1/4 MIC**	**1/8 MIC**
M. thymifolia essential oil	77.2±1.2	77.2±1.2	73.6±1.5
Streptomycin	49.4±0.5	71.0±0.4	88.4±0.4
Ampicillin	69.2±0.6	56.5±0.5	92.2±0.4

* Biofilm formation values were calculated as: (mean A_{620} EO treated well) (mean A_{620} control well) ×100. Values are expressed as means ± SE.

In addition to QS, the initiation of biofilm formation by *P. aeruginosa* depends on two cell-associated structures, ie. the flagellum and type IV pili [21, 22]. The flagellum is responsible for swimming motility while the type IV pili are responsible for twitching motility [23]. Both types of motility are important in the initial stages of biofilm formation by *P. aeruginosa* [21, 22]. Therefore, we tried to determine if the EO can influence either one or both motilities. On swimming plates, the motile strain PAO1 was used as the 100% standard (control) for motility while Petri dishes with the same strain plus the EO were compared with the control.

Table 4: Twitching and motility activity of *M. thymifolia* EO

Agents	Colony diameter (mm ± SD)	Colony color	Protrusions diameter (μm)	Colony edge on microscope
M. thymifolia essential oil	15.00 ± 5.29	green	40-80	slightly reduced protrusion
Streptomycin	11.00 ± 1.00	white	16-56	slightly reduced protrusion
Ampicillin	13.33 ± 5.03	light green	27-56	slightly reduced protrusion
control PAO1	21.0 ± 3.60	green	80-240	regular protrusion

M. thymifolia oil reduced the twitching motility of *P. aeruginosa*. The normal colonies of *P. aeruginosa,* i.e. in the absence of the oil, were flat with a rough appearance displaying irregular colony edges (Figure 1B) and a hazy zone surrounding the colony. The protrusions were regular with sizes of 80-240 μm. The cells were in a very thin layer. After 2 days of incubation at ambient temperature, colony expansion occurred very rapidly due to twitching motility, the control *P. aeruginosa* isolates produced swimming zones corresponding to 100% (Table 4) and it was 21.0 mm. Bacteria that were grown with the oil solution were incapable of producing such a twitching zone and had almost round, smooth, regular colony edges. Moreover, the protrusions were reduced both in sizes (40-80 μm) and in numbers (Fig 1A), and the diameter of the swimming zones was also reduced (15.00 mm). On the contrary, streptomycin and ampicillin slightly reduced protrusions (Figure 1C, 2D).

The effects of *M. thymifolia* EO on the ability of *P. aeruginosa* (PAO1) to synthesize and secrete virulence-associated pigment pyocyanin was investigated (Figure 2). The oil reduced the production of pyocyanin (74.4%), exhibiting less activity than streptomycin and ampicillin (41.5% and 48.3, respectively). These results are in agreement with Sepahi *et al*. [24] reporting that Ferula (*Ferula asafoetida* L.) EO, from the Apiaceae family, exhibited anti-QS activity by decreasing pyocyanin, pyoverdine, elastase and

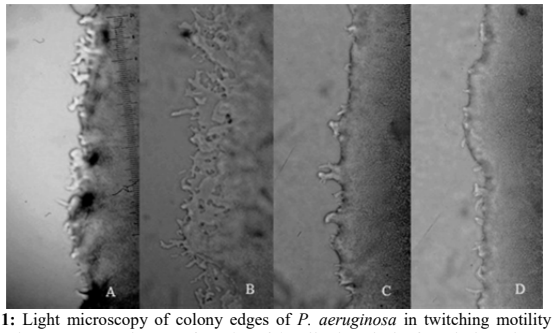

Figure 1: Light microscopy of colony edges of *P. aeruginosa* in twitching motility plates, grown in the presence or absence of *M. thymifolia* oil. The colonies from the bacteria grown with the oil in a concentration 0.5 MIC (**A**) were rounded, had a smooth domed shape, and lacked a hazy zone surrounding the colony. *P. aeruginosa* produced a flat, widely spread, irregularly shaped colony in the absence of oil (**B**); *P. aeruginosa* colony in the presence of streptomycin (0.5 MIC) showed reduced protrusion (**C**); *P. aeruginosa* colony in the presence of ampicillin slightly reduced protrusion (**D**); Magnification: (**A–D**) × 100.

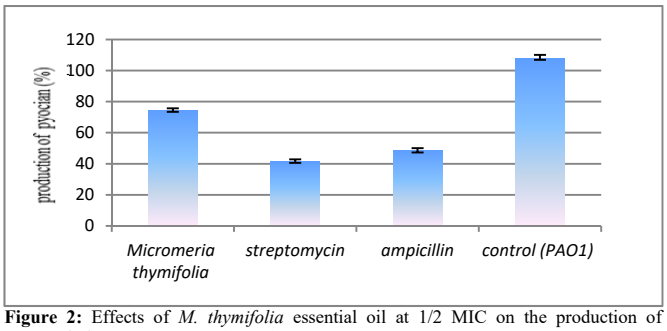

Figure 2: Effects of *M. thymifolia* essential oil at 1/2 MIC on the production of pyocyanin by *P. aeruginosa* (PAO1).

also biofilm production by *Pseudomonas aeruginosa* PAO1. Recently, Ahmad *et al*. [25] investigated the antimicrobial and anti-quorum properties of different mono- and sesqui-terpenes, which can be found in several EOs, against *Chromobacterium violaceum* and *P. aeruginosa* ATCC 27853. The finding that 18 of the 29 assayed compounds inhibited pyocyanin production could account for the significant reduction in the QS factor observed in our study.

This study exhibits strong *in vitro* antimicrobial activity of the EO of *M. thymifolia* and *P. aeruginosa* antiquorum-sensing activity, and it may have a great relevance in the prevention and therapy of disease caused by the tested microorganisms. The anti-quorum sensing property of this oil may play an important role in antibacterial activity and offers an additional strategy for fighting bacterial infections.

Experimental

Plant material and essential oil isolation: *Micromeria thymifolia* (Scop.) Fritsch was collected from Bačevci, Serbia, in August, 2013. A voucher specimen has been deposited in the Herbarium at the Institute of Botany and Botanical Garden "Jevremovac", University of Belgrade (BEOU). Material was dried at room temperature. The aerial parts of the plant (100 g) were dried at room temperature and hydrodistilled for 2 h, using a Clevenger-type apparatus. The oil yield was 1.3%. After hydrodistillation, the EO was stored at 4 ºC and protected against light.

GC and GC-MS analyses: These were performed using an Agilent 7890A GC equipped with inert 5975C XL EI/CI MSD and FID detector connected by a capillary flow technology 2-way splitter with make-up. A HP-5MSI capillary column (30 m × 0.25 mm × 0.25 µm) was used. The GC oven temperature was programmed from 60°C to 315°C at a rate of 3°C/min and held for 15 min. Helium was used as the carrier gas at 1.72 mL/min at 60°C (constant pressure mode). The sample was analyzed in the splitless mode. The injection volume was 1 µL. GC detector temperature was 300°C. MS data were acquired in EI mode with scan range 40-550 *m/z*, source temperature 230°C, and quadrupole temperature 150°C; solvent delay was 3 min. The components were identified based of their retention index and comparison with reference spectra (Wiley 07 and NIST 11 databases), as well as by the retention time lock (RTL) method and RTL Adams 04 data base. The retention indices were experimentally determined using the standard method [26] involving retention times of *n*-alkanes, injected after the essential oil under the same chromatographic conditions. The percentage (relative) of the identified compounds was computed from their GC peak area. The quantitative composition of the oil was GC (FID) analyzed by internal normalization assuming an identical mass response factor for all compounds. In this study, only those components present in the oils in amounts higher than 0.1% were taken into consideration.

Antimicrobial activity: The antimicrobial effect of the EO was evaluated against different yeast strains (*Candida humilis* LVL1, *C. krusei* LVL 12, *Geotrichum klebanhii* LVL 3, *Pichia anomala* OC70, *P. anomala* OC71, *P. membranaefaciens* CBS 5759, *P. membranaefaciens* DBVPG 3003), and bacterial strains (*Bacillus cereus* ATCC 11966, *Escherichia coli* ATCC 25922, *Listeria monocytogenes* 56Ly, *Salmonella enteritidis* 155, and *Pseudomonas fulva* LV1). All the strains were obtained from the microbial culture collection of the Department of Agricultural and Food Sciences, Alma Mater Studiorum – University of Bologna (Italy). Yeast strains were grown in Yeast extract Peptone Dextrose (YPD) at 27ºC for 48 h, while bacterial strains were grown in Tryptic Soy Broth (TSB) at 37ºC for 24 h. After harvesting, microbial cells were suspended in sterile saline solution and immediately used. The antimicrobial activity of the EO was determined according to a literature procedure [20]. Experiments were undertaken with *P. aeruginosa* PA01 (ATCC 27853). The strain is from the collection of the Mycoteca, Institute for Biological Research "Siniša Stanković", Belgrade, Serbia. The bacterium was routinely grown in Luria-Bertani (LB) medium (1%, w/v, NaCl, 1%, w/v, Tryptone, 0.5%, w/v, yeast extract) with shaking (220 rpm) and cultured at 37°C. Biofilm formation was analyzed using polystyrene flat-bottomed microtiter 96 well plates as described [27, 28], with some modifications. Briefly, 100 µL of an overnight culture of *P. aeruginosa* (inoculum size 1×10^8 CFU/mL) was added to each well of the plates in the presence of 100 µL of different concentrations of EO (ranging from 1/2, 1/4, 1/8 of MIC; MIC was 0.25 mg/mL) or 100 mL medium (control). After incubation for 24 h at 37°C, each well was washed twice with sterile PBS (pH 7.4), dried, and stained for 10 min with 0.1% crystal violet in order to determine the biofilm mass. The content of the wells was homogenized and the absorbance at 625 nm was read on a Sunrise™ - Tecan ELISA reader. The experiment was made in triplicate and repeated 2 times and values were presented as mean values and standard error.

Twitching and flagella motility: These were evaluated on tryptone plates (1%, w/v, tryptone, 0.5%, w/v, NaCl) containing 0.3%, w/v, agar as previously described [21]. After growth in the presence or absence of *M. thymifolia* oil (subMIC, 0.5 MIC - 0.125 mg/mL), streptomycin and ampicillin (subMIC), cells of *P. aeruginosa* PAO1 were washed twice with sterile PBS and resuspended in PBS at 1×10^8 cfu/mL (OD of 0.1 at 660 nm). Briefly, cells were stabbed into a nutrient agar plate with a sterile toothpick and incubated overnight at 37°C. The colony edges and the zone of motility were measured with a light microscope [21, 22]. The extent

of swimming was determined by measuring the area of the colony [29]; specifically, the colony diameters were measured 3 times in different directions. The experiment was made in triplicate and repeated twice.

Pyocyanin production was visualized by plating the bacteria on *P. aeruginosa* PA01, diluted to $OD_{600\ nm}$ 0.2 [29]. *M. thymifolia* EO (0.125 mg/mL) was added to *P. aeruginosa* (5.00 mL) culture and incubated at 37°C for 24 h. The treated culture was extracted with chloroform (3 mL), followed by mixing the chloroform layer with 0.2 M HCl (1 mL). Absorbance of the extracted organic layer was measured at 520 nm using a Shimadzu UV1601 spectrophotometer (Kyoto, Japan). The values were expressed as ratio (OD520/OD600) × 100.

All assays were performed in triplicate and the significance of the data was tested using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $p = 0.05$. This analysis was carried out using SPSS v. 18.0 programs.

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