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Increased Resistance of Epiphytic Microorganisms Isolated from Aromatic and Medicinal Plants to the Essential Oils of Oregano (*Origanum vulgare*) and Thyme (*Thymus vulgaris*)

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Abstract

The epiphytic microbiota from 224 samples of 32 different aromatic-medicinal plants sold dried in local markets or by producers in Greece was investigated by using classical microbiological techniques. The occurrence of total mesophiles, yeasts and molds, *E. coli*, *Staphylococcus* spp., *Pseudomonads* spp., *Enterococcus* spp., *C. perfringens* and *Bacillus* spp. was observed in most of the samples and in counts reaching up to 5.5 Log cfu/g of dry weight. Despite the variabilities in their counts between the different plants or within the same plant species, a uniform rather than a lognormal distribution was revealed. Additionally, the sensitivity of the bacteria isolated from the surface of aromatic and medicinal plants was examined against the essential oil of oregano and thyme through the estimation of minimum inhibitory concentration with micro dilution technique. Our results indicate a decreased sensitivity in comparison to previous studies probably as a result of adaptation to the epiphytic micro environment. If bacteria are capable to overcome the antimicrobial action of essential oils then we should reconsider their use as alternatives to synthesized antibiotics in medicinal use.

Keywords: Epiphytic microbiota; Aromatic plants; Essential oils; Antimicrobial; Sensitivity

Introduction

The above-ground parts constitute the largest surface of plants and one of the most extensive ecological habitats on the planet with a total area estimated by Morris (2003) at 400x106 Km² [1]. Although it is an oligotrophic and variable environment, it is naturally colonized by microorganisms whose role is the subject of research for over one hundred years [2-5]. Despite the above efforts, even today the role of microorganisms that colonize the phyllosphere has not been studied as extensively as the rhizosphere [6]. We do know that, some species are causing infestation to crops with important economic losses [7-8] but we are also aware that epiphytic bacteria play a crucial role in plants biology since they a) contribute to the defense against pathogens and other abiotic stressors, b) are involved in plant biodiversity, c) degrade environmental pollutants, d) synthesize phytohormones and plant cytokines [6,9].

Concerning their populations, it seems that bacteria are abundant in all plant species, particularly in young ones and on those growing under constant humid conditions as in tropical environments and rainforests [10]. Additionally, there are reports suggesting that bacterial populations within a set of leaves usually vary only by a factor of 10 and generally follow the lognormal distribution [3].

On the other hand, aromatic and medicinal plants comprise a vast number of species with particular aromatic or medicinal properties [11]. These properties are mainly the result of Essential Oils (EOs) production which are actually complex mixtures of volatile secondary metabolites and initially play an important role in plant's defense and signaling [12]. Additionally, EOs traditionally

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have been used by humans in cooking, food preservation, cosmetology and folk medicine since among other properties are natural antibacterial agents [13]. According to FAO, approximately 30% of the drugs sold worldwide contain compounds derived from plants [11]. In contemporary research about the antimicrobial properties of EOs, it is often suggested that EOs could replace synthetic antibiotics and eliminate the problem of antibiotic resistance from pathogens [14-15]. However, there are scarce reports which suggest that bacteria are capable to increase their resistance to EOs [16-19] and rising questions against a successful exploitation of EOs in medical treatment of infectious diseases [20].

Based on the above, the aim of this research was to study the species and numbers of epiphytic bacteria on the surface of aromatic-medicinal plants and also to investigate the hypothesis of increased resistance of those bacteria to the EOs, probably as a result of adaptation to the micro environment of those plants.

Materials and Methods

Plant species

Two hundred twenty-four dried samples corresponding to thirty-two different species of aromatic and medicinal plants were acquired from local markets and producers from various cities in Greece over a period of 2 years. All samples, packaged in plastic perforated bags by the retailers, were transported to the laboratory where a voucher specimen of each species was deposited in the Department's herbarium.

Isolation of epiphytic bacteria

In order to isolate the epiphytic flora, sub-samples of 10 g were weighted in sterilize stomacher bags equipped with a filter and 90 ml of sterile phosphate buffered saline (Sigma-Aldrich) was added. Samples were left to rehydrate for 15 min and then homogenized for additional 5 min in a stomacher apparatus (Bag Mixer-400, Interscience, France). From this initial suspension, a series of 1:10 dilutions was prepared in test tubes and then 100 μ l from each dilution was spread on the surface of the following agar plates: plate count agar (Oxoid, UK), TBX agar (HiMedia Co.), Sabouraud dextrose agar with chloramphenicol (Oxoid, UK), Mannitol salt agar (Oxoid, UK), Slanetz & Bartley agar (Oxoid, UK), Perfringens agar base (Oxoid, UK) with the addition of SFP agar containing egg yolk (Oxoid, UK), *Bacillus cereus* agar (Oxoid, UK) and *Pseudomonas* agar base with CFC supplement (Oxoid, UK), which corresponded to the isolation of total mesophilic count, *E. coli*, Yeasts-molds-fungi, *Staphylococcus* spp., *Enterococcus* spp., *C. perfringens*, *Bacillus* spp., and *Pseudomonas* spp., respectively. After proper aerobic or anaerobic incubation (AnaeroGen, Oxoid UK.) as in the case of *C. perfringens*, all-petri dishes with 20-300 colonies were used for enumeration and the bacterial populations were calculated as Log₁₀ cfu/g of dry plant material. Representative colonies of each bacterial species from each one of the plant samples were isolated and dispensed in 2 ml Eppendorf tubes contained Tryptone soya broth fortified with 30% glycerol and stored in -40°C until needed.

Essential oils

Essential Oils (EOs) were isolated from oregano (*Oregano vulgare*) and thyme (*Thymus vulgaris*) dried material during a previous study [21] by using hydro distillation in a Clevenger apparatus. Essential oils were dehydrated by using anhydrous sodium thiosulphate (Sigma-Aldrich) and stored at -40°C in sealed amber glass vials until use. The composition of EOs in active ingredients had been determined

previously with Gas chromatography - Mass spectrometry analysis [21].

Sensitivity to essential oils

The susceptibility of isolated bacteria against Essential Oils (EOs) of oregano and thyme was determined by the broth micro-dilution method according to Clinical Laboratory Standards Institute [22]. All frozen strains were initially revived with culturing in their respective culture media and incubation conditions as described above. Then, an inoculum density of 1.5x10⁸cfu/ml to confront with 0.5 of the McFarland turbidity units scale, was prepared in sterile saline (0.9% NaCl). One hundred microliters (100 μ l) of double strength Mueller-Hinton Broth (Oxoid, UK) with 5% dimethyl sulfoxide (Sigma-Aldrich) was dispensed into wells of a 96-well sterile microplate. In the first column 100 μ l of a mixture of EOs with Mueller-Hinton broth were added to achieve an initial concentration of 2048 μ g/ml and then serially diluted up to 10th column to a final concentration of 2 μ g/ml. Finally, 50 μ l of bacterial suspension was added in all wells except column 12 (negative control) while column 11 served as a positive control since it contained only bacteria but not EOs. Three rows in each microplate were used per strain and the assay was repeated with increased or decreased concentration of EOs in order for the Minimum Inhibitory Concentration (MIC) to be determined accurately. Growth of bacterial cells in each well was verified either visually through the presence of sediment or by the color development after the addition of 20 μ l of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at a concentration of 5 mg/ml in Phosphate Buffered Saline (Sigma-Aldrich), and additional incubation for 30-45 min. *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 11778, *Pseudomonas aeruginosa* ATCC 27853, *Clostridium perfringens* NCTC 3180 and *Enterococcus faecalis* ATCC 29212 served as reference strains either during the epiphytic micro-flora analysis or the EOs sensitivity determination.

Statistical analysis

Counts of epiphytic micro-flora are presented as Log₁₀ cfu/g of dry plant tissue $\pm$ their corresponding standard deviation. Results of minimum inhibitory concentration are presented as μ g/ml $\pm$ standard deviation. Comparison of the means from sub-samples or species was performed with the Analysis of variance (ANOVA) with Tukeys HSD post hoc procedure at a significance level of 0.05. All statistical analyses were conducted with SPSS v17 statistical software (SPSS Inc, USA).

Results and Discussion

An abundant epiphytic micro-flora was recorded in almost all plant species (Table 1). Total mesophiles, yeasts and molds, staphylococci, *E. coli* and *C. perfringens* spore forms were always observed in contrast to *pseudomonads*, *bacilli* and *enterococci* which often were absent or below the detection limit of the isolation method.

Mean counts of total mesophiles ranged from 1.5 $\pm$ 2.1 Log cfu/g to 4.9 $\pm$ 1.2 Log cfu/g with *Angelica archangelica*, *Artemisia absinthium*, *Malva sylvestris* and *Matricaria chamomilla* exhibiting the highest of counts (>4.4 Log cfu/g). Yeasts and molds populations ranged less considerably from 1.6 $\pm$ 0.9 to 3.2 $\pm$ 1.7 Log cfu/g with a more pronounced relative abundance in *Achillea millefolium*, *Rosmarinus officinalis* and *Lippia citriodora*. Staphylococci were recorded in populations of 0.9 $\pm$ 0.1 to 2.9 $\pm$ 1.4 Log cfu/g, *E. coli* from 0.8 $\pm$ 0.9 to 2.7 $\pm$ 0.9 Log cfu/g and *C. perfringens* from 0.3 $\pm$ 0.3 to 2.4 $\pm$ 0.1 Log cfu/g. *Pseudomonas*, *bacilli* and *enterococci* were recorded in

Table 1: Epiphytic bacterial counts (Log cfu/g dry weight) of the 32 aromatic - medicinal plant species studied (n=224).

Plant species	N	TMC	Yeasts-Molds	<i>Staphylococcus spp</i>	<i>Pseudomonas spp</i>	<i>E. coli</i>	<i>C. perfringens</i>	<i>Bacillus spp</i>	<i>Enterococcus spp</i>
<i>Angelica archangelica</i>	2	4,1±0,7	2,8±0,3	2±0,8	0,6±0,8	2,2±0,3	0,7±0,9	0,3±0,4	0,6±0,9
<i>Sedum telephium</i>	8	3,3±1,4	2,7±1,3	1,7±1,2	0,2±0,4	1,8±1,7	0,8±0,5	0,7±0,6	0,1±0,3
<i>Rutagraveolens</i>	2	3,4±0,6	2,3±1,8	1,8±1,1	0,6±0,9	0,8±1	0,5±0,7	0,3±0,4	0,3±0,4
<i>Artemisia absinthium</i>	2	4,9±1,2	2±0,4	1,6±0,2	Nd	1±0	1,2±1,7	Nd	Nd
<i>Achillea millefolium</i>	2	3,8±0,3	3,1±0,1	2,1±1,5	Nd	2±1	1,8±1,1	0,7±0,8	Nd
<i>Valeriana officinalis</i>	2	1,5±2,1	2,9±0,6	1,6±2	Nd	1,7±1	2,4±0,1	0,6±0,8	Nd
<i>Hypericum perforatum</i>	12	2,9±1,6	2,5±1,6	1,1±1,2	0,1±0,3	1,9±1,3	1,7±0,7	0,7±0,6	0,6±0,1
<i>Ocimum basilicum</i>	4	3,4±1,2	2,7±0,4	2±1,7	Nd	1,3±0,9	1,6±1,1	1,1±0,3	Nd
<i>Pimpinella anisum</i>	4	2,9±0,6	2,3±1,9	1,3±1	0,3±0,6	2,3±1,7	1,4±1	0,8±0,8	0,4±0,7
<i>Laurus nobilis</i>	10	2,8±1,8	2,1±1,3	1±0,8	0,4±0,6	0,9±0,8	0,8±0,4	0,5±0,6	0,3±0,5
<i>Rosmarinus officinalis</i>	11	3,6±1,3	3,2±1,7	1,4±0,9	0,2±0,4	1,6±1,4	1,3±0,7	0,6±0,6	0,6±1,1
<i>Origanum dictamnus</i>	8	3,9±1	2,9±0,6	1,9±1,2	0,5±0,7	1,8±1,4	1,3±0,7	0,9±0,6	0,4±0,7
<i>Mentha longifolia</i>	8	3±0,9	2,5±1,2	1,9±0,6	0,2±0,4	1,4±1,6	1,1±0,4	1,1±0,5	0,3±0,9
<i>Eucalyptus globulus</i>	11	3,1±1,6	2,4±0,5	1,8±0,8	0,1±0,3	1,9±1,2	0,3±0,3	0,7±0,6	0,1±0,1
<i>Satureja montana</i>	11	3,1±1,9	2,4±0,8	2,4±1,3	0,3±0,6	1,7±1	1,5±0,8	0,5±0,6	0,5±0,8
<i>Thymus vulgaris</i>	12	3,4±1	1,9±0,7	1,7±1	0,2±0,5	1,1±1,1	1,3±0,4	0,5±0,5	0,5±0,6
<i>Lavandula officinalis</i>	4	3,2±1,2	1,7±0,6	0,9±1	Nd	1,5±1,7	0,6±1,1	Nd	Nd
<i>Lippia citriodora</i>	4	3,3±1	3,2±1,7	1,5±1,1	Nd	2,5±1,7	1,8±0,5	0,7±0,3	0,4±0,8
<i>Origanum majorana</i>	2	3,6±1,4	1,6±0,9	1,6±0,1	Nd	1,3±1,7	2±0,3	0,6±0,8	0,3±0,4
<i>Foeniculum vulgare</i>	12	3,5±1,4	2,2±1	1,9±1,7	0,1±0,4	1,5±1,5	1±0,1	0,7±0,6	0,2±0,7
<i>Melissa officinalis</i>	4	3,5±0,8	2,6±1,7	2,4±0,7	Nd	1,3±0,4	1,2±0,8	0,6±0,7	Nd
<i>Mentha spicata</i>	3	2,8±1,1	2,2±1,1	2,7±0,7	0,6±0,6	1,7±1,6	0,8±0,7	0,9±0,8	Nd
<i>Malva sylvestris</i>	9	4,5±1,1	2,8±1,5	2,3±1,4	Nd	2±1,5	0,7±0,6	0,4±0,6	Nd
<i>Micromeria juliana</i>	3	2,6±0,9	2,2±1	2±0,5	0,4±0,7	2,7±0,9	0,9±0,8	1±0,3	Nd
<i>Origanum vulgare</i>	13	3,1±0,9	2,5±1	2,2±0,8	0,2±0,3	1,4±1,4	0,7±0,5	0,7±0,5	0,7±0,7
<i>Sideritis raeseri</i>	16	3±0,7	1,7±0,9	1,6±0,9	0,5±0,7	0,8±0,9	0,9±0,7	0,7±0,6	0,3±0,6
<i>Asplenium ceterach</i>	4	3,6±1,3	2,2±0,7	1,8±1,3	Nd	1,6±1,1	0,5±0,4	1,1±0,7	0,3±0,6
<i>Urtica dioica</i>	8	3,5±1	2,2±1	1,7±1,3	Nd	2,2±0,9	0,4±0,4	0,7±0,4	1,1±1,7
<i>Salvia officinalis</i>	18	3,4±1,2	2,5±1,1	1,7±0,9	0,2±0,5	1,5±1,2	1,4±0,7	0,8±0,6	0,7±0,3
<i>Tilia platyphyllos</i>	8	3,4±1,2	2,6±0,8	2,3±1,2	Nd	1,8±1,2	1,8±0,3	0,2±0,5	0,2±0,4
<i>Mentha pulegioides</i>	3	1,7±1,6	2,5±0,9	1±0,8	Nd	1,2±2,1	1,2±0,1	0,4±0,7	Nd
<i>Matricaria chamomilla</i>	4	4,4±1,3	2,7±2	2,9±1,4	Nd	1,4±1,8	1,4±0,3	0,7±0,7	1,6±1,9

TMC: Total Mesophilic Count; Nd: Non-detected

densities of up to 0.6±0.8, 0.9±0.8 and 1.6±1.9 Log cfu/g respectively. However, counts ranged considerably among the samples of the same plant species as revealed by the increased standard deviation values. Such highly variable results have been also reported in previous studies. Karamanoli et al., (2000) [23] in an investigation of the epiphytic bacterial populations on aromatic-medicinal plants, reported significant differences of up to 107 cfu/g even within the same species or within the same set of samples. In a similar study by Yadav et al., (2004) [24], the average total population values ranged between 4.12 and 5.12 Log cfu/g which are much higher than in our study but this difference is easily explained by the fact that, in the previous study, fresh plants were used instead of dried which is more common in the trade of such products. Gekenidis et al., (2017) reported 3.6 to 3.7 total mesophilic Log cfu/g in marjoram, basil and thyme most of which comprised of *Bacillus* spp. and *E. coli*, while *Enterococcus* spp. detected only in few marjoram samples [25]. Also, during the above investigation, an increase in term of numbers and

species of the epiphytic bacteria was recorded at the stage of which these products were delivered to the consumer probably as the result of multiple handling.

Due to our culture depending approach we believe that there is an underestimation of the true or active epiphytic bacteria in our study. Laforest-Lapointe and Whitaker (2018) [6] and others [26,27] supported that, the assessment of phyllosphere community diversity and structure would greatly be enhanced due to the improvements in culture-independent sequencing technologies. In this way, it would be possible not only to distinguish between culturable and non-culturable microbes but also between dormant and active ones placing them in a context of spatial and temporal heterogeneity [6].

Since 1982, Hirano et al., [3] suggested that bacterial populations within a set of leaves usually varied by a factor of 10 and generally followed lognormal distribution. This was also supported by the findings of Gekenidis et al., [25] but haven't being able to be verified

Table 2: Minimum inhibitory concentration (in $\mu\text{g/ml}$) of various epiphytic microbial species isolated from the surface of corresponding aromatic - medicinal plants.

Plantspecies	Oregano essential oil sensitivity in MIC ($\mu\text{g/ml}$)						Thyme essential oil sensitivity in MIC ($\mu\text{g/ml}$)				
	<i>Staphylococcus spp.</i>	<i>Pseudomonas spp.</i>	<i>E. coli</i>	<i>C. perfringens</i>	<i>Bacillus spp.</i>	<i>Enterococcus spp.</i>	<i>Staphylococcus spp.</i>	<i>Pseudomonas spp.</i>	<i>E. coli</i>	<i>C. perfringens</i>	<i>Enterococcus spp.</i>
<i>Angelica archangelica</i>	256 $\pm$ 0	1152 $\pm$ 1267	1280 $\pm$ 1086	192 $\pm$ 90	24 $\pm$ 11	-	192 $\pm$ 90	512 $\pm$ 0	1152 $\pm$ 1267	130 $\pm$ 178	144 $\pm$ 158
<i>Sedum telephium</i>	512 $\pm$ 0	384 $\pm$ 181	1152 $\pm$ 1267	528 $\pm$ 701	192 $\pm$ 90	1024 $\pm$ 0*	272 $\pm$ 339	640 $\pm$ 543	272 $\pm$ 339	288 $\pm$ 316	96 $\pm$ 45
<i>Ruta graveolens</i>	512 $\pm$ 0	1152 $\pm$ 1267	384 $\pm$ 181	32 $\pm$ 0	24 $\pm$ 11	-	256 $\pm$ 0	1152 $\pm$ 1267	2048 $\pm$ 0*	16 $\pm$ 0	64 $\pm$ 0
<i>Artemisia absinthium</i>	384 $\pm$ 181	1536 $\pm$ 724*	1024 $\pm$ 0	256 $\pm$ 0	24 $\pm$ 11	-	272 $\pm$ 339	256 $\pm$ 0	384 $\pm$ 181	128 $\pm$ 0	160 $\pm$ 135
<i>Achillea millefolium</i>	768 $\pm$ 362	192 $\pm$ 90	1280 $\pm$ 1086	16 $\pm$ 0	128 $\pm$ 0	192 $\pm$ 90	256 $\pm$ 0	768 $\pm$ 362	1152 $\pm$ 1267	256 $\pm$ 0	512 $\pm$ 0
<i>Valeriana officinalis</i>	512 $\pm$ 0	576 $\pm$ 633	1024 $\pm$ 0	24 $\pm$ 11	256 $\pm$ 0	136 $\pm$ 169	640 $\pm$ 543	1024 $\pm$ 0	384 $\pm$ 181	4 $\pm$ 0	160 $\pm$ 135
<i>Hypericum perforatum</i>	384 $\pm$ 181	128 $\pm$ 0	1152 $\pm$ 1267	544 $\pm$ 678	64 $\pm$ 0	144 $\pm$ 158	272 $\pm$ 339	512 $\pm$ 0	1152 $\pm$ 1267	32 $\pm$ 0	-
<i>Ocimum Basilicóçicum</i>	384 $\pm$ 181	1280 $\pm$ 1086	1152 $\pm$ 1267	256 $\pm$ 0	-	288 $\pm$ 316	72 $\pm$ 79	256 $\pm$ 0	640 $\pm$ 543	256 $\pm$ 0	-
<i>Pimpinella anisum</i>	512 $\pm$ 0	2048 $\pm$ 0	2048 $\pm$ 0	192 $\pm$ 90	24 $\pm$ 11	160 $\pm$ 135	192 $\pm$ 90	384 $\pm$ 181	1280 $\pm$ 1086	288 $\pm$ 316	-
<i>Laurus nobilis</i>	640 $\pm$ 543	1280 $\pm$ 1086	768 $\pm$ 362	1024 $\pm$ 0*	48 $\pm$ 22	288 $\pm$ 316	136 $\pm$ 169	768 $\pm$ 362	544 $\pm$ 678	128 $\pm$ 0	-
<i>Rosmarinus officinalis</i>	256 $\pm$ 0	384 $\pm$ 181	1536 $\pm$ 724	256 $\pm$ 0	256 $\pm$ 0	256 $\pm$ 0	1024 $\pm$ 0	1536 $\pm$ 724*	1040 $\pm$ 1425	32 $\pm$ 0	-
<i>Origanum dictamnus</i>	384 $\pm$ 181	1280 $\pm$ 1086	640 $\pm$ 543	640 $\pm$ 543	144 $\pm$ 112	96 $\pm$ 45	72 $\pm$ 79	640 $\pm$ 543	1056 $\pm$ 1402	288 $\pm$ 316	-
<i>Mentha longifolia</i>	768 $\pm$ 362	1152 $\pm$ 1267	1280 $\pm$ 1086	4 $\pm$ 0	144 $\pm$ 112	192 $\pm$ 90	520 $\pm$ 712	768 $\pm$ 362	1152 $\pm$ 1267	16 $\pm$ 0	-
<i>Eucalyptus globulus</i>	512 $\pm$ 0	192 $\pm$ 90	1152 $\pm$ 1267	516 $\pm$ 718	32 $\pm$ 0	16 $\pm$ 0	272 $\pm$ 339	272 $\pm$ 339	1152 $\pm$ 1267	-	-
<i>Satureja montana</i>	256 $\pm$ 0	2048 $\pm$ 0	384 $\pm$ 181	16 $\pm$ 0	-	128 $\pm$ 0	640 $\pm$ 543	1536 $\pm$ 724	144 $\pm$ 158	-	-
<i>Thymus vulgaris</i>	512 $\pm$ 0	640 $\pm$ 543	1280 $\pm$ 1086	132 $\pm$ 175	-	-	528 $\pm$ 701	1152 $\pm$ 1267	1088 $\pm$ 1357	-	-
<i>Lavandula officinalis</i>	512 $\pm$ 0	640 $\pm$ 543	768 $\pm$ 362	4 $\pm$ 0	80 $\pm$ 67	-	144 $\pm$ 158	640 $\pm$ 543	272 $\pm$ 339	-	-
<i>Lippia citriodora</i>	384 $\pm$ 181	384 $\pm$ 181	768 $\pm$ 362	520 $\pm$ 712	48 $\pm$ 22	-	24 $\pm$ 11	640 $\pm$ 543	96 $\pm$ 45*	40 $\pm$ 33	-
<i>Origanum majorana</i>	512 $\pm$ 0	1280 $\pm$ 1086	768 $\pm$ 362	288 $\pm$ 316	64 $\pm$ 0	-	192 $\pm$ 90	1024 $\pm$ 0	288 $\pm$ 316	72 $\pm$ 79	-
<i>Foeniculum vulgare</i>	512 $\pm$ 0	384 $\pm$ 181	1152 $\pm$ 1267	544 $\pm$ 678	160 $\pm$ 135	-	192 $\pm$ 90	640 $\pm$ 543	192 $\pm$ 90	130 $\pm$ 178	-
<i>Melissa officinalis</i>	512 $\pm$ 0	640 $\pm$ 543	1280 $\pm$ 1086	64	512	-	384 $\pm$ 181	256 $\pm$ 0	160 $\pm$ 135	4	-
<i>Mentha spicata</i>	320 $\pm$ 271	1536 $\pm$ 724*	384 $\pm$ 181	384 $\pm$ 181	-	-	136 $\pm$ 169	640 $\pm$ 543	528 $\pm$ 701	-	-
<i>Malva sylvestris</i>	384 $\pm$ 181	1024 $\pm$ 0	1280 $\pm$ 1086	-	-	-	640 $\pm$ 543	1536 $\pm$ 724	384 $\pm$ 181	-	-
<i>Microme raijuliana</i>	768 $\pm$ 362	576 $\pm$ 633	2048 $\pm$ 0	136 $\pm$ 169	-	-	192 $\pm$ 90	1152 $\pm$ 1267	1152 $\pm$ 1267	72 $\pm$ 79	-
<i>Origanum vulgare</i>	384 $\pm$ 181	320 $\pm$ 271	1536 $\pm$ 724	264 $\pm$ 350	320 $\pm$ 271*	-	40 $\pm$ 33	640 $\pm$ 543	1152 $\pm$ 1267	34 $\pm$ 42	-
<i>Sideridis raeseri</i>	320 $\pm$ 271	192 $\pm$ 90	2048 $\pm$ 0	-	384 $\pm$ 181*	-	128 $\pm$ 0	1536 $\pm$ 724*	272 $\pm$ 339	-	-
<i>Asplenium ceterach</i>	320 $\pm$ 271	1536 $\pm$ 724	640 $\pm$ 543	640 $\pm$ 543	-	-	640 $\pm$ 543	1280 $\pm$ 1.086	528 $\pm$ 701	144 $\pm$ 158	-
<i>Urtica dioica</i>	512 $\pm$ 0	768 $\pm$ 362	1280 $\pm$ 1.086	68 $\pm$ 84	-	-	528 $\pm$ 701	640 $\pm$ 543	1536 $\pm$ 724	-	144 $\pm$ 158
<i>Salvia officinalis</i>	512 $\pm$ 0	1280 $\pm$ 1.086	2048 $\pm$ 0	-	-	-	128 $\pm$ 0	1280 $\pm$ 1.086	80 $\pm$ 67*	-	384 $\pm$ 181
<i>Tilia platyphyllos</i>	640 $\pm$ 543	384 $\pm$ 181	640 $\pm$ 543	40 $\pm$ 33*	-	-	256 $\pm$ 0	256 $\pm$ 0	640 $\pm$ 543	36 $\pm$ 39	384 $\pm$ 181
<i>Mentha pulegium</i>	384 $\pm$ 181	1152 $\pm$ 1.267	640 $\pm$ 543	264 $\pm$ 350	-	-	544 $\pm$ 678	384 $\pm$ 181	1040 $\pm$ 1.425	160 $\pm$ 135	192 $\pm$ 90
<i>Matricaria chamomilla</i>	384 $\pm$ 181	192 $\pm$ 90	1536 $\pm$ 724*	-	-	-	544 $\pm$ 678	256 $\pm$ 0	144 $\pm$ 158	-	320 $\pm$ 271
Reference strains	48 $\pm$ 27*	170 $\pm$ 74*	213 $\pm$ 74*	43 $\pm$ 19*	27 $\pm$ 9*	43 $\pm$ 19*	64 $\pm$ 55*	213 $\pm$ 74*	256 $\pm$ 0	85 $\pm$ 37*	85 $\pm$ 37*

*Statistically significant differences among the average MIC value of each column (ANOVA, $p < 0.05$).

by others [23]. In our study, no statistical differences (ANOVA, $p > 0.05$) between the counts of the various bacterial species among the aromatic and medicinal plants were observed which is an indication of a uniform distribution of the epiphytic micro-flora over dried plant material.

The data concerning the sensitivity of the epiphytic microbial species isolated from the aromatic-medicinal plant strains are presented in Table 2. As observed, MIC values against *E. coli* are ranging from 384-2048 $\mu\text{g/ml}$ while from previous studies in our laboratory (Alexopoulos 2011; Alexopoulos 2017, Fourmoniti, 2015; Fournomiti, 2020) and mainly from bibliographical reports, the corresponding range of values for environmental or clinical strains of *E. coli* is much lower and specifically between 0.4-750 $\mu\text{g/ml}$. This difference is statistically significant and much larger than the

expected experimental variability. Same strains when tested for MIC in thyme oil which had been used in previous laboratory research, showed an analogous and also statistically significant increase in MIC (96-2048 $\mu\text{g/ml}$) compared to our previous or other published studies which ranged from 1 to 62.5 $\mu\text{g/ml}$. Equally, statistically increased MIC values were also observed for *Pseudomonas* in oregano oil (128-2048 $\mu\text{g/ml}$ vs. 0.5-1648 $\mu\text{g/ml}$) and thyme (213-1536 $\mu\text{g/ml}$ vs. 1-500 $\mu\text{g/ml}$), *C. perfringens* in oregano oil (4-1024 $\mu\text{g/ml}$ vs. 2.12-750 $\mu\text{g/ml}$) as well as *Bacillus* spp. in oregano oil (24-512 $\mu\text{g/ml}$ vs. 25-160 $\mu\text{g/ml}$) and *Enterococcus* spp. in oregano oil (16-1024 $\mu\text{g/ml}$ vs. 26-190 $\mu\text{g/ml}$). In contrast, the differences between experimental and literature values in staphylococci regarding oregano oil and also in staphylococci, *C. perfringens* and *Enterococcus* spp. in thyme essential oil, where were not statistically significant. Overall, our observations

are a strong indication of the resistance of microbes isolated from the surface of aromatic and medicinal plants in relation to similar environmental (food, water) or clinical strains where especially the latter are expected to show resistance to antimicrobial agents [16,19,20,28]. Alternatively, the observed variation in the MIC values of this study with those in the literature could be interpreted on the basis of the different content of essential oils in active compounds such as carvacrol (in oregano) and thymol (in thyme). Indeed, it is known that the composition of essential oils and especially the content of the above compounds has been used to explain their differentiated antimicrobial activity [21,29-35]. In a study published by Maksimov (2017) which included both experiments with thyme essential oils with a high thymol content but also comparing his own data with those of other researchers (meta-analysis), he concluded that the high thymol content (>40%) leads consistently to lower values of MIC and therefore higher activity [36]. Other researchers reported similar conclusions about carvacrol, where concentrations above 50-55% are considered particularly active elucidating morphological changes in the cells of microorganisms, or diffusion of the intracellular matrix as the result of microbial membrane rupture [37-40]. In any case, the essential oils of oregano and thyme used to study the inhibitory concentration in our study, contained 77% carvacrol and 47.4% of thymol [21]. Therefore, this high content of active substances would be expected to further reduce rather than increase the observed inhibitory concentration or otherwise increase, rather than decrease, the activity of the oils. The last observation further strengthens the view expressed above for the possible emergence of resistance by microorganisms isolated from the surface of aromatic and medicinal plants. In order to definitively answer the above question, further research is needed at both chemical, morphological and molecular level, but certainly, the possible emergence of resistance of microorganisms to essential oils, should change our way of thinking about their future use as alternative antimicrobial agents.

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