ORIGINAL ARTICLE

Antimicrobial activity of essential oils of cultivated oregano (*Origanum vulgare*), sage (*Salvia officinalis*), and thyme (*Thymus vulgaris*) against clinical isolates of *Escherichia coli*, *Klebsiella oxytoca*, and *Klebsiella pneumoniae*

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Background: Oregano (*Origanum vulgare*), sage (*Salvia officinalis*), and thyme (*Thymus vulgaris*) are aromatic plants with ornamental, culinary, and phytotherapeutic use all over the world. In Europe, they are traditionally used in the southern countries, particularly in the Mediterranean region. The antimicrobial activities of the essential oils (EOs) derived from those plants have captured the attention of scientists as they could be used as alternatives to the increasing resistance of traditional antibiotics against pathogen infections. Therefore, significant interest in the cultivation of various aromatic and medicinal plants is recorded during the last years. However, to gain a proper and marketable chemotype various factors during the cultivation should be considered as the geographical morphology, climatic, and farming conditions. In this frame, we have studied the antimicrobial efficiency of the EOs from oregano, sage, and thyme cultivated under different conditions in a region of NE Greece in comparison to the data available in literature.
Methods: Plants were purchased from a certified supplier, planted, and cultivated in an experimental field under different conditions and harvested after 9 months. EOs were extracted by using a Clevenger apparatus and tested for their antibacterial properties (Minimum inhibitory concentration – MIC) against clinical isolates of multidrug resistant *Escherichia coli* ($n=27$), *Klebsiella oxytoca* ($n=7$), and *Klebsiella pneumoniae* ($n=16$) strains by using the broth microdilution assay.
Results: Our results showed that the most sensitive organism was *K. oxytoca* with a mean value of MIC of 0.9 μg/mL for oregano EOs and 8.1 μg/mL for thyme. The second most sensitive strain was *K. pneumoniae* with mean MIC values of 9.5 μg/mL for thyme and 73.5 μg/mL for oregano EOs. *E. coli* strains were among the most resistant to EOs antimicrobial action as the observed MICs were 24.8–28.6 μg/mL for thyme and above 125 μg/mL for thyme and sage. Most efficient were the EOs from thyme followed by those of oregano.
Conclusions: With MIC values above 150 μg/mL, sage EOs did not show any antibacterial efficiency against the majority of the strains. However, no significant differences were observed concerning the antimicrobial action of all EOs originating from irrigated versus non-irrigated cultivated aromatic plants.

Keywords: E. coli; K. oxytoca; K. pneumoniae; *essential oils*; *thyme*; *oregano*; *sage*

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*E*scherichia coli, *Klebsiella pneumoniae*, and *Klebsiella oxytoca* are common human and animal pathogens often responsible for causing mild to severe illness which are serotype dependent. Both pathogens are transmitted *via* the fecal–oral route and have been detected in various foodstuffs and food preparations.

Also, both pathogens are often resistant to common synthesized antibiotics, which compromise the treatment of the patients. This intrinsic or acquired resistance exhibited by various pathogens is by far one of the most significant problems today, so there has been a growing interest for alternative and natural antibacterial agents. Essential oils (EOs) which derived from aromatic plants have been proposed as alternative agents (1). In literature there are many articles concerned with the antimicrobial activity of EOs against a wide range of microorganisms (2, 3), and particularly against common food pathogens. The main advantage of these natural products is that they do not enhance antibiotic resistance as with the long-term use of synthetic antibiotics (4). On the other hand, the ability of plant EOs to protect foods against pathogenic and spoilage microorganisms with the benefit of being natural products and thus more acceptable by the consumers, has been reported by several researchers (5). It is known that Mediterranean countries are the biggest producers of native aromatic plants (6) due to the favorable climatic conditions, oil composition, and geographical morphology. Greece is also one country which produces hundreds of species of aromatic plants which are native (7). Among them oregano, sage, and thyme are widely used in Greek and European cooking, are endemic, and are therefore suitable for cultivation under the climatic conditions in Greece; various studies have shown that their EOs seem to possess significant antimicrobial properties. Therefore, they are ideal candidates for farmers seeking novel crop opportunities with a prospect of an increase economic profit.

Oregano (*Origanum vulgare* subsp. *hirtum*), a herb of the Labiatae family that has been used widely in cooking and folk healing, is the common oregano that thrives naturally in almost every region of Greece, especially on the edges of fields, dry and uncultivated or waste ground. Chemical analysis of the oregano EO revealed the presence of several ingredients, most of which possess important antioxidant and antimicrobial properties (8). Carvacrol and thymol, the two main phenols that constitute about 78–85% of oregano EOs, are principally responsible for the antimicrobial activity (9). In addition, other minor constituents such as the monoterpene hydrocarbons γ-terpinene and *p*-cymene also contribute to the antibacterial activity of the oil (10). In the literature, there are many reports relating the chemical composition and the antimicrobial properties of the EOs of various oregano species, and their application in various commercial preparations, as antimicrobials and antioxidants (11, 12).

Thyme (*Thymus vulgaris* L.) is also an aromatic plant of the Labiatae family. Its EO contains more than 60 ingredients, most of which possess important antioxidant and antimicrobial properties (13). The most important compounds of thyme EO are also the phenols thymol (44–60%) and carvacrol (2.2–4.2%), which constitute the

major and more active constituents (14), as well as the monoterpene hydrocarbons *p*-cymene (18.5–23.5%) and γ-terpinene (16.1–18.9%).

Salvia (sage), the largest genus of the Lamiaceae family, includes about 900 species, spread throughout the world, some of which are economically important since they are used as spices and flavoring agents in perfumery and cosmetics. The antimicrobial activity of *Salvia officinalis* was recognized decades ago and was attributed to the presence of 1,8-cineole, α-thujone and camphor (15). Sivropoulou et al. (16) reported the antimicrobial activity of sage collected in Greece. As with *S. officinalis*, its oil was also characterized by high concentrations of 1,8-cineole, α-thujone, and camphor.

Although some of the key components of EOs act in a similar way to the synthesized antibiotics (17), it is unlikely that they will be used soon in therapeutics or as food preservatives mainly because of the limited number of bacterial strains tested and the differences in their susceptibilities. Thus, the application of such compounds as either therapeutics or as food preservatives should be tested against a larger number of strains and different bacterial species to determine their usefulness. As most of the published reports were concerned with the antibacterial properties of EOs against common food pathogens, in this study we focused on three species of clinical origin with proven resistance to antibiotics. Additionally, our aim was to study if the antimicrobial action of EOs is differentiated by the cultivation method of aromatic plants and particularly with irrigation (irrigated versus non-irrigated farming) in order to promote their commercialization.

Materials and methods

Plant material
Seedlings of oregano (*Origanum vulgare* subsp. *hirtum*), sage (*Salvia officinalis*), and thyme (*Thymus vulgaris*) to ensure the same genotype per species were initially supplied from a certified institute (DIO, Thessaloniki – Greece). Plants were set up at an experimental field located at NE Greece during spring 2012. The cultivation consisted of two separated experimental blocks per species with the first block left under drought and the second with frequent irrigation. First harvesting took place 1 year later from June to July during the full blooming of plants. Aerial parts were air dried in well-ventilated rooms and away from sunlight for 20 days before EOs extraction. A voucher specimen of each plant is deposited in the *herbarium* of the Department of Agricultural Development with a reference number from DAD-LM-01 to DAD-LM-03.

EOs isolation
EO concentration of leaves and inflorescences was isolated from the air-dried material by a Clevenger apparatus (1, 5, 8) using 220 g of ground plant samples diluted in 1,500 mL

deionised water each time. The mixture was heated at 100°C for 3 h and the volume of the EOs produced was measured and expressed as mL/100 g dry weight. Dehydration was achieved by using anhydrous magnesium sulfate (Sigma). All EOs were stored in amber glass vials at −30°C until use.

Bacterial strains

The antibacterial assay was performed against gram-negative bacteria *Klebsiella oxytoca* (n = 7), *Klebsiella pneumoniae* (n = 16), and *Escherichia coli* (n = 27). All strains were collected, verified (Vitek 2, Biomerieux, France) and tested for resistance against nine antibiotics from the local University hospital (Alexandroupolis, Greece). Strains were cultured overnight on nutrient agar (Oxoid) at 37°C and isolated colonies were frozen at −20°C in Tryptic Soy Broth supplemented with 15% (v/v) glycerol until used. As reference strains *E. coli* NCTC 10410, *K. oxytoca* NCTC 49131, and *K. pneumoniae* subsp. *pneumoniae*, NCTC 11228 have been used.

Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of EO was determined by broth microdilution method according to National Committee for Clinical Laboratory Standards. All strains were cultured to Muller Hinton agar (Oxoid, UK) and incubated at 37°C for 24 h prior to MIC determination. An inoculum density of 150×10^6 cfu/mL equivalent to 0.5 McFarland units of each of the test organisms was prepared in sterile saline (0.84% NaCl). One hundred microliters (100 µL) of double strength Muller Hinton Broth (MHB) containing 5% dimethyl sulfoxide (DMSO) was dispensed into wells of 96-well micro titer plates. In the first column of wells, EOs were added at a final concentration of 512 µg/mL and then serially diluted (by two-fold) across the plate to a final concentration of 0.125 µg/mL. One hundred microliters (100 µL) of bacteria suspension was finally added to each well and the plates were incubated at 37°C for 16 h. The assay for each of the pathogens was repeated three times. Growth of bacterial cells in each of the wells was verified by the color formed after the addition of 20 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) prepared at a concentration of 5 mg/mL in PBS, and additional incubation for 30 min.

Statistical analysis

Average and standard deviations of MIC values were estimated. Mean MIC values (mg/L) between different groups were compared by using Analysis of Variance (ANOVA) with Fisher's least significance differences procedure at a significance level of 0.05. All statistical analyses were performed with SPSS v17 (SPSS Inc., USA).

Results and discussion

Antibiotics commonly used for therapeutic purposes, as well as antibiotics added to animal feedstuff for increasing animal flesh production, contribute to the extensive spread of resistance. Antibiotic resistance has also been shown in plant pathogenic bacteria. Furthermore, hospital, industrial, and domestic wastes worsen the global situation. Resistant microorganisms may pass on to other hosts in different ways or their mutations may give new multiplying bacterial generations.

Horizontal gene transfer between microorganisms has a great effect on increasing bacterial pathogens. Phages, plasmids, and pathogenicity islands (PIs) have been known as carriers of virulence associated gene clusters (18). In order to safeguard public health, an alternative interesting approach for reducing pathogen transmission should be to use EOs.

The antibiotic susceptibility profile of all strains is shown in Table 1. *K. pneumoniae* was the most resistant microorganism since almost all 16 strains were resistant to the various synthesized agents. *K. oxytoca* strains were less resistant since they were completely susceptible to three antibiotics (cefepime, imipenem, and meropenem) while *E. coli* strains were susceptible to four antibiotics (amikacin, ceftazidime, imipenem, and meropenem). However, approximately 30% of all strains (22.2–37.5%) tested were proven to be multiresistant to at least three without exhibiting an extended spectrum β-lactamase character. In all cases reference strains exhibited a lower resistance than the tested strains. Antimicrobial valor of *Origanum* EOs seems to be related to the membrane permeability for the microorganisms and linked to its components

Table 1. Susceptibility profile (%) of clinical pathogens

Organism (number of strains)	Antibiotic (susceptible breaking point, mg/L)[a]								
	AK (≤16)	AM (≤8)	AUG (≤8/4)	CAZ (≤4)	CPE (≤8)	CPT (≤0.5)	IMP (≤1)	MER (≤1)	P/T (≤16/4)
E. coli, (n = 27)	100	41.4	65.5	100	93	79.3	100	100	79.3
K. oxytoca, (n = 7)	71.4	0	57.1	57.1	100	57.1	100	100	57.1
K. pneumoniae, (n = 16)	81.6	0	65.8	60.5	65.8	57.8	65.8	65.8	68.4

[a]According to the CLSI breaking points (CLSI, 2011). AK: amikacin; AM: ampicillin; AUG: amoxicillin/clavulanic acid; CAZ: ceftazidime; CPE: cefepime; CPT: ceftaroline; IMP: Imipenem; MER: Meropenem; P/T: Piperacillin/Tazobactam.

carvacrol and thymol, which are then considered as membrane permeabilizers (19).

The antimicrobial activity of the EOs collected from *Origanum vulgare*, *Salvia officinalis*, and *Thymus vulgaris* to the above pathogenic microorganisms is shown in Table 2. The EOs of thyme exhibited strong antimicrobial activity against all the microorganisms. Results obtained by measurement of MIC indicated that EOs from irrigated thyme plants had an average MIC of 24.81 µg/mL against *E. coli*, 8.12 µg/mL against *K. oxytoca*, and 11.34 µg/mL against *K. pneumoniae*. These concentrations were comparable to some of the synthesized antibiotics tested. Additionally, a lot of our stains and particularly *Klebsiella* spp. were susceptible to even lower EOs concentrations (0.063 µg/L) indicating that thyme oil possesses a strong antibacterial action. Our results were among the lowest in comparison to other published reports also using the micro-dilution as a method of choice for the antibacterial assay. Burt and Reinders (20) reported a MIC value of 625 µg/mL for *E. coli*. Al-Bayati (21) reported values of 62.5 µg/mL against *E. coli* and 500 µg/mL for *K. pneumoniae*. Pei et al. (22) found MIC of 400 µg/L in their study and Da Costa et al. (23) reported MIC values of 250 µg/mL for both species. However, some early investigations from Greece also reported an increased antibacterial action of thyme EOs with MIC values between 0.28 and 3.35 µg/mL for *E. coli* and 0.72 µg/mL for *K. pneumoniae* (24).

Oregano EOs also exhibited some antibacterial action, which was more profound against *K. oxytoca* with average MICs of 0.90 and 2.11 µg/mL for irrigated and non-irrigated plants, respectively. In contrast, *K. pneumoniae* strains were less susceptible with average MICs of 43.5 and 102.7 µg/mL, while *E. coli* strains were the most resistant with mean MIC values of 219.9 and 236.1 µg/mL, accordingly. Our results are consistent with previously reported MIC values concerned with *E. coli* and ranged between 100 and 250 µg/mL (25, 26) although in other studies MIC values as low as 0.5 µg/mL (26) and 31.25–40 µg/mL (27, 28) have been observed. In reports, various *Klebsiella* species appeared as either susceptible to oregano EOs with MIC values of 0.5 µg/mL (27), moderately susceptible with MIC close to 250 µg/mL (28) or highly resistant and unaffected by oregano EOs (27, 28).

Sage EOs were lacking a noticeable antibacterial action since the MIC values recorded against all pathogens were above 150 µg/mL. MIC for *E. coli* was 370 µg/mL, MICs for *K. oxytoca* was 173.7–178.9 µg/mL for irrigated and non-irrigated plants respectively, and finally, MICs of 207.4–240 µg/mL were recorded for *K. pneumoniae*. These increased values are well above an efficient and applicable concentration. Accordingly, increased MIC values have also appeared in literature. It was reported (29) that EOs from *Salvia officinalis* had MIC values against *E. coli* between 5,000 and 10,000 µg/mL and 100 µg/mL against *K. oxytoca*.

Klebsiella spp. possess capsules composed of complex acidic polysaccharides. Capsules are associated with the virulence of *Klebsiella* spp. and protect the bacteria from phagocytosis or when exposed to bactericidal serum factors. However, some strains following the capsular serotype may be less virulent than others. This possibly implies other pathogenicity factors which may be involved such as fimbriae, siderophores or extracapsular polysaccharides. *K. oxytoca* has similar antibiotic resistance

Table 2. Minimum inhibitory concentration (MIC, mg/L) of essential oils against clinically isolated pathogens

Organism	Value	Thyme (irrigated)	Thyme (non-irrigated)	Sage (irrigated)	Sage (non-irrigated)	Oregano (irrigated)	Oregano (non-irrigated)
				MIC (mg/L)			
E. coli	N	27	27	27	27	27	27
	Mean	24.81[a1]	28.56[a1]	370.7[a2]	370.0[a2]	236.1[a3]	219.9[a3]
	Min	2	0.125	64	64	8	8
	Max	128	256	512	512	512	512
K. oxytoca	N	7	7	7	7	7	7
	Mean	8.12[a1]	4.7[a1]	173.7[b2]	178.9[b2]	0.90[b1]	2.11[b1]
	Min	0.063	0.063	64	32	0.063	0.063
	Max	32	16	512	512	2	8
K. pneumoniae	N	16	16	16	16	16	16
	Mean	11.34[a1]	9.51[a1]	240[b2]	207.4[b2]	102.7[c3]	73.5[c3]
	Min	0.063	0.063	16	8	0.063	4
	Max	32	32	512	512	256	256

Same superscript letters indicate non-significant differences ($p > 0.05$) in column groups. Same superscript numbers indicate non-significant differences ($p > 0.05$) among row groups according to the ANOVA with Fisher's least significant difference (LSD) procedure.

profiles to *K. pneumoniae*. Like *K. pneumoniae*, *K. oxytoca* can carry beta-lactamases which are divided into phylogenetic groups with different geographic breakdown. The physiological role of beta-lactamases enzymes is hydrolysis and destruction of *β*-lactam antibiotics. The antimicrobial action of EOs might be due to the impairment of a variety of enzyme systems, including those involved in energy production and structural component synthesis (29).

As already discussed, *K. pneumoniae* possesses a capsule as an innate defense, which seems to block EOs from accessing the fragile inner membrane. Moreover, complex enzyme systems should confer to this direction. The location of one or more functional groups on these molecules can affect their antimicrobial activity. Thymol is structurally analogous to carvacrol, but the locations of the hydroxyl groups differ between the two molecules.

Different cytochrome P450s (30) from thyme and oregano have been described to be involved in thymol and carvacrol biosynthesis and characterized from a heterologous expression. Namely, thymol and carvacrol formation from γ-terpinene occurs with the aid of P450s. Moreover, beta-lactamase genes are normally found on the chromosome of *Klebsiella* species. Beta-lactamases produced by gram-negative organisms are usually secreted, especially when antibiotics are present in the environment (31).

It is known that gram-negative bacteria are more resistant to antibiotics than the gram-positive bacteria. The use of terpenes as a therapeutic alternative combined with antibiotics could amplify their competence of income to the cell. Moreover, antibiotics could permit an effective transport of the latter until reaching its bacterial cell target owing to the lipidic nature of the terpene (32). This type of synergism raises the question if the presence of beta-lactamases should be discouraging by a potential drastic effect of EOs.

Ivanovic et al. (33) reported MIC values over 2,560 μg/mL for *E. coli*. Generalić et al. (34) reported MIC values of 550–990 μg/mL for *E. coli*. Miguel et al. (35) reported a very weak antibacterial activity of sage EOs despite the different method used in their assay. Similarly, no antibacterial action from sage EOs against *E. coli* was observed, in concentrations of 1,000 μg/mL or even as high as above 8,000 μg/mL for both *E. coli* and *K. pneumoniae* (2, 25–28). However, lower MICs (close to 20 μg/mL) have also been reported for *K. pneumoniae*; see Hammer et al. (36).

It is known that gram-positive bacteria carry a thick layer of peptidoglycan, which has the potential to inhibit the membrane-disrupting action of many of the EOs, possibly explaining the increased resistance. As a general rule, gram-negative bacteria are more resistant to EOs than gram-positive bacteria. In the present study, gram-positive bacteria were involved. Gram-negative bacteria are surrounded by a thin peptidoglycan cell wall, which itself is surrounded by an outer membrane containing lipopolysaccharide (LPS). LPS play a key role in the barrier function of their outer membrane. The 'non-fluid net' formed by the LPS is a very drastic barrier for hydrophobic molecules. Moreover, the lipidic nature of the terpene should act synergically and this explains the high volume of terpenes through the outer membrane in gram-negative bacteria. Thymol and carvacrol gain access to the periplasm and deeper portions of the cell.

Small hydrophilic substances are considered able to pass through the porin proteins matrix that serve as hydrophilic transmembrane channels, and this is one reason that gram-negative bacteria are relatively resistant to hydrophobic antibiotics and other drugs. In this vein, the outer membrane seems to be permeable to hydrophobic molecules, some of which can slowly traverse through porin proteins (37). This also explains the important capacity diffusion of the lipidic terpenes through the cell membrane and their high performance as antimicrobials.

No significant differences ($p < 0.05$) among the antibacterial action of irrigated versus non-irrigated plants were observed from the statistical analysis (Table 2). This is an indication that any expected variation into their EOs constitution does not signify an analogous antibacterial effectiveness. It is known that whole EOs tend to vary in their exact composition due to factors such as seasonal variation, climate conditions, farming, and even extraction methods (38). This has consequences for their actual chemotype and their antibacterial activity. Oregano and thyme are two of the most studied EOs with varied yet known chemotypes. They both contain carvacrol, thymol, γ-terpinene, and ρ-cymene with proposed modes of action against bacteria, the disruption of the membrane, enzyme inhibition, reduction in lipase and coagulase activity, and reduction of proton motive force (39–41).

Conclusion

E. coli strains were among the most resistant to EOs antimicrobial action.

The oils that performed best were thyme followed by those of oregano. Overall, we can conclude that thyme EO is a promising natural component suitable for use as an antimicrobial agent with a particular interest for the pharmaceutical industry as it represents an inexpensive compound. Moreover, a focus must be upon the bactericidal or bacteriostatic activity of the EOs which is tightly dependent on the concentration used.

In our study, despite other differences observed between irrigated and non-irrigated aromatic and medicinal plants, as for example in EOs yield (data not shown), it seems that cultivated plants possess an equal yet species-dependent antimicrobial efficiency as the wild ones, which gives them commercial opportunities.

Conflict of interest and funding

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