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Antibacterial Activity of Clove, Oregano, Thyme, Eucalyptus, and Tea Tree Essential Oils against *Escherichia coli* and *Klebsiella pneumoniae* strains

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Abstract

***Background**: In view of the high recurrence rate of urinary tract infections and the increasing number of germs resistant to multiple antibiotics, the aim of the present study was to evaluate the antibacterial properties of clove, oregano, thyme, eucalyptus, tea tree essential oils (EOs) against 32 isolates of Escherichia coli and 28 isolates of Klebsiella pneumoniae from patients with urinary tract infections (UTI). **Methods**: The agar disk diffusion method was used to assess the susceptibility of these isolates to essential oils and the minimal inhibitory concentration (MIC), and the minimal bactericidal concentration (MBC) were determined. **Results**: Our results suggest that volatile phenols (such as carvacrol in oregano EO, thymol in thyme EO, and eugenol in clove EO) are more efficacious as antibacterial than non-aromatic compounds (such as eucalyptol in eucalyptus EO and terpinene derivatives in tea tree EO). **Conclusion** : The oregano EO, followed by thyme appear to have the highest efficacy against Escherichia coli and Klebsiella pneumoniae isolates investigated.*

***Keywords**: Escherichia coli, essential oils, urinary tract infections, Klebsiella pneumoniae*

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Introduction

Given the high recurrence rate of urinary tract infections [1] and the increasing number of germs resistant to multiple antibiotics [2], therapeutic alternatives are needed [3]. The essential oils (EOs) of plant origin, which seem to act by altering cell membranes resulting in compromised osmo and chemostatic mechanisms (electrolyte leakage) and injured organelles [4] could be used as an alternative to synthetic chemical products, in the treatment of infectious diseases, caused by resistant strains. *Escherichia coli* is by far the microorganism most often involved in the etiology of UTI. A less frequent one is *Klebsiella pneumoniae* [5]. Several studies have proven the antibacterial efficacy against *E. coli* of EO extracted from: *Thymus vulgaris L*., thyme *(Lamiaceae*) [6–8], other plants in the genus *Thymus* [9–12], *Origanum vulgare L*., oregano (*Lamiaceae*) [13, 14], other plants in the genus *Origanum* [15], *Eucalyptus globulus Labill.*, eucalyptus (*Myrtaceae*) [16], other plants in the genus *Eucalyptus* [17], *Melaleuca alternifolia (Maiden and Betche) Cheel*, tea tree (*Myrtaceae*) [18, 19], *Syzygium aromaticum (L.) Merrill and Perry Clove*, clove (*Myrtaceae*) [20]. Several studies have proven the antibacterial efficacy against *K. penumoniae* of EO extracted from *Thymus vulgaris* [6], other plants in the genus *Thymus* [12], plants in the genus *Eucalyptus* [17], *Melaleuca alternifolia* [21], *Syzygium aromaticum* [22].

The aims of this study were: 1. to investigate the antibacterial activity of clove, oregano, thyme, eucalyptus, tea tree EOs against clinical strains of *E. coli* and *K. pneumoniae*, isolated from urine samples collected from patients with UTI; 2. to determine whether some EOs are more efficacious as antibacterials than others; 3. to establish whether a given EO is more efficacious on one of the two bacterial species than on the other.

Materials and methods

Essential oils

Five EOs (provided by Fares Bio Vital Laboratories ltd. Orastie, Romania) were used: thyme EO (Thy) *(Thymus vulgaris),* oregano EO (Ore) (*Origanum vulgare*), tea tree EO (Tea) (*Melaleuca alternifolia*), clove EO (Clo) (*Syzygium aromaticum*), and eucalyptus EO (Euc) (*Eucalyptus globulus*). Gas chromatography mass spectrometry was used to determine the composition of the essential oils employed in this study. The composition was in agreement with the standards imposed by the European Pharmacopeia, 9th edition and the active compounds in the EOs used in this study are as follows:

- for Thyme *(Thymus vulgaris)* EO: - thymol 45.2 %, p-cymene 17.5%, γ-terpinene 6.09%, β-linalool 4.97%, caryophyllene 6.45%, eugenol 3%, carvacrol 2.2%, camphen 1.54%, β-myrcene 1.58%, eucalyptol 1.52%, borneol 1.52%;
- for Oregano (*Origanum vulgare*) EO: - carvacrol 68.96%, caryophyllene 4.25%, γ-terpinene 5.61%, eucalyptol 2.05%, p-cymene 7%, D-limonene 1.03%, thymol 1.03%, β-pinene 1.11%, bergamiol (linalool acetate) 3.3%, camphor 1.15%, borneol 1.18%;
- for Tea tree (*Melaleuca alternifolia*) EO: - 1-terpinene-4-ol 44.2%, γ-terpinene 20.22%, α-terpinene 9.08%, eucalyptol 4.38%, terpinolene 3.3%, α-terpineol 3.23%, aromadendren 1.18%, α-pinene 2.39%, D-limonene 1.03%;
- for Clove (*Syzygium aromaticum*) EO: - eugenol 79.59%, eugenol acetate 11.47%, caryophyllene 8.25%, caryophyllene oxyde 0.47%, copaene 0.16%;
- for Eucalyptus (*Eucalyptus globulus*) EO: - eucalyptol 84.39%, D-limonene 6.08%, α-pinene 2.66%, p-cymene 3.4%, γ-terpinene 1.52%, β-pinene 0.32%, α-phellandrene 0.91%.

The densities of the employed EOs were in the range 0.9-1 g/mL [µg/µL]: 0.9220 [0.9000-0.9350] g/ml for thyme EO. 0.9460 [0.9300-0.9600] g/ml for oregano EO. 0.8920 [0.8800-0.9060] g/ml for tea tree EO. 1.0550 [1.0440-1.0570] g/ml for clove EO. 0.9080 [0.9000-0.9250] g/ml for eucalyptus EO.
Determining the composition and density of the investigated EOs was not part of our experiment, as the corresponding information was supplied, together with the EOs, by the provider of these EOs, Fares Bio Vital Laboratories ltd.

Bacterial Strains Culturing and Identification
A total of 60 clinical isolates of *E. coli* (32 strains) and *K. pneumoniae* (28 strains) from the urine of patients with UTI hospitalized in various wards of the Emergency University Hospital, Bucharest, Romania were investigated.
The urine samples were cultured on Columbia Blood agar medium (Biomerieux) and Bromthymol Lactose Blue Agar (Cantacuzino MMNIRD), by the quantitative culture technique using a standard loop, with incubation in aerobic facultative anaerobic conditions, at 37°C for 18 h. Growth of a single organism with a count of $\geq 10^5$ colony-forming units (CFU)/ml were considered to represent the infection. *E. coli* and *K. pneumoniae* strains were identified using the morphological appearance of the colonies, staining reactions and biochemical properties by means of the tests: Triple Sugar Iron (TSI), Motility Indole Urease (MIU), Simmons citrate (Cantacuzino MMNIRD) [23]. The ATCC 25922 strain *E. coli* was used as a control.

Assessing the antibacterial activity of antibiotics
The susceptibility to antibiotics of the bacterial strains was evaluated using the Kirby-Bauer disk-diffusion method on Mueller-Hinton Agar (Biomerieux). Cultures were incubated at 37°C for 18-24 hours. The results were interpreted according to Clinical and Laboratory Standard Institute guidelines [24].
According to their susceptibility to antibiotics (evaluated as the diameter of the growth inhibition area) the bacterial strains were classified as resistant (R), intermediate (I) or susceptible (S). The antibiotics to which the susceptibility of *E. coli* and *Klebsiella pneumoniae* strains was tested were: imipenem IPM (10µg) (R≤20, 20≤I ≤22 , S≥23), piperacillin/tazobactam (TZP; 100µg /10µg) (R17, 18≤I ≤20 , S≥21), ceftazidime (CAZ; 30µg) (R≤17, 18≤I ≤20 , S≥21), norfloxacin (NOR; 10 µg) (R ≤ 12, 13 ≤I ≤16, S ≥17), gentamicin (CN; 10 µg), (R≤12, 13≤I ≤14, S≥15), cefuroxime (CXM; 30µg) (R≤14, 15≤I ≤17, S18), amoxicillin/clavulanic acid (AMC; 20 µg/10µg), (R≤13, 14≤I ≤17, S≥18), trimethoprim/sulfamethoxazole SXT (1.25µg) /23.75µg)) (R≤10, 11≤I ≤15, S≥16), fosfomycin (FOT; 30µg) (R≤12, 13I ≤15, S≥16), tetracycline (TE; 30µg) (R≤11, 12≤I ≤14, S≥15), doxycycline (DO; 30µg) (R≤10, 11≤I 13, S≥14) .

Assessing the antibacterial activity of EOs
The antibacterial activities of the five EOs were evaluated by means of broth dilution method and a modified Kirby-Bauer disk-diffusion method on Mueller-Hinton Agar, the latter technique being called aromatogram.
Aromatogram. Bacterial suspensions with an optical density of 0.5 on the McFarland scale were prepared and inoculated on Petri plates with Muller Hinton Agar (Biomerieux.) All the oils were stored at room temperature in the dark, prior to testing. The blank filter paper discs of 6 mm diameter were placed on each plate, using a dispenser. Each disc was impregnated with 10 µL of a different EO: clove, oregano, thyme, eucalyptus, tea tree. The diameters of the inhibition zones (DIZ) were recorded after incubation of the Petri plates at 37°C for 18 h in aerobic-facultative anaerobic conditions.

Determining the MIC and MBC of the EOs by broth microdilution method. The antibacterial properties of the tested EOs were assessed by means of the MIC, which was determined by using successive broth dilutions of EO in a 96 well microtiter plate, starting from 10^{-1} (90 µL broth added to 10 µL EO) down to 10^{-5}, each level of dilution being obtained by adding 10 µL of the previous level of dilution to 90 µL of broth. For each level of dilution, a constant quantity of inoculum (90 µL) was added to the 100 µL of broth-diluted EO, the resultant concentrations of EO varying from 55 µL/mL down to 0.0055 µL/mL (which are roughly equivalent to 55 µg/mL to 0.0055 µg/mL, given all the tested EOs had densities in the range 0.9-1 g/mL). Positive controls (oil-free broth with bacterial inoculum) and negative controls were included in each assay. The minimal inhibitory concentration (MIC) was the lowest concentration that prevented the visible growth of bacteria and it was determined after 18 h of incubation in aerobic-facultative anaerobic conditions at 37°C. The minimal bactericidal concentration (MBC) was assessed by subculturing 10 µL broth-diluted EO from the last wells without visible bacterial growth on a Columbia agar plate. After incubation in aerobic-facultative anaerobic conditions at 37°C for 18 h, the MBC was recorded as the lowest concentration resulting in a negative subculture.

The antibacterial efficacy (ABE) was evaluated by both a continuous parameter (DIZ) and two discontinuous ones (MIC and MBC). The antimicrobials with an MBC / MIC ratio <4 were considered bactericidal, while those with an MBC / MIC ratio of 4 or higher (up to 16) were considered bacteriostatic [25].

Statistical Analysis

Mann-Whitney test with continuity correction was used for comparing continuous variables (namely DIZ), while either Chi-squared test with Yates' continuity correction, or Fisher's exact test were employed for comparing categorical variables, such as MIC and MBC. Fisher's exact test was used when the conditions for employing Chi-squared test [no more than 20% of the expected values (EVs) should be less than 5 and no individual EV should be less than 1 [26]] were not met.

The calculations were performed using the R language and environment for statistical computing and graphics. Given the large number of calculations made (35 using the data on MIC and MBC and 25 using the data on DIZ), the significance level had to be lowered according to Bonferroni criterion to an $\alpha_{corrected} = 0.05/35 \approx 0.001$ for the results yielded by MIC and MBC related data and to $\alpha_{corrected} = 0.05/25 \approx 0.002$ for the results yielded by DIZ related data.

Ethical considerations

The study was performed after gaining the approval of the Ethics Committee of the University Emergency Hospital Bucharest, Romania. The urine samples were used after obtaining the informed consent of the patients.

Results

Antibacterial activity of tested antibiotics as determined by agar disc diffusion method

The profiles of susceptibility to the 11 antibiotics of the 32 isolates of *Escherichia coli* and the 28 isolates of *Klebsiella pneumoniae* are summarized in Table 1.

Antibacterial activity of tested EOs as determined by the modified agar disc diffusion method (aromatogram)

The median (and first and third quartiles) of DIZ for each of the five EOs against the two bacterial species (as determined by aromatogram) were:

- against *E. coli*: 16 (14-16) mm for Clove EO, 24.5 (22.75-28) mm for Oregano EO, 28 (24-30) mm for Thyme EO, 23 (21.75-24) mm for Eucalyptus EO, and 20.5 (19.75-24.25) mm for Tea tree EO;

Table 1. Percentages of *Escherichia coli* and *Klebsiella pneumoniae* isolates susceptible, intermediate, or resistant to the various antibiotics.

Antibiotic concentration	*Escherichia coli*			*Klebsiella pneumoniae*		
	S	I	R	S	I	R
IMP (10μg)	83.87%	3.23%	12.90%	72.22%	5.56%	22.22%
TZP (100μg /10μg)	84.38%	9.38%	6.25%	30.00%	36.67%	33.33%
CAZ (30μg)	78.13%	3.13%	18.75%	34.48%	6.90%	58.62%
NOR (10 μg)	65.63%	0.00%	34.38%	56.67%	6.67%	36.67%
CN (10 μg)	84.38%	6.25%	9.38%	62.07%	0.00%	37.93%
CXM (30μg)	75.00%	3.13%	21.88%	44.83%	0.00%	55.17%
AMC (20 μg/10μg)	61.29%	19.35%	19.35%	26.67%	20.00%	53.33%
SXT (1.25μg/23.75μg)	46.88%	6.25%	46.88%	32.14%	10.71%	57.14%
TE (30μg)	40.00%	0.00%	60.00%	50.00%	0.00%	50.00%
FOT (30μg)	87.10%	3.23%	9.68%	36.36%	9.09%	54.55%
DO (30μg)	53.85%	0.00%	46.15%	37.50%	6.25%	56.25%

S: Susceptible, I: intermediate, R: resistant, IPM: imipenem, TZP: piperacillin/tazobactam, CAZ: ceftazidime, NOR: norfloxacin CN: gentamicin, CXM: cefuroxime, AMC: amoxicillin/clavulanic acid, SXT: trimethoprim/sulfamethoxazole, FOT: fosfomycin, TE : tetracycline, DO: docycycline.

- against *K. pneumoniae*: 12 (11-13.25) mm for Clove EO, 22 (20-24) mm for Oregano EO, 25.5 (20-29.5) mm for Thyme EO, 19 (17-22) mm for Eucalyptus EO, and 20 (18-21.25) mm for Tea tree EO.

The Mann-Whitney test revealed significantly higher DIZ against *E. coli* than against *K. pneumoniae* for clove and eucalyptus EOs, as shown in Table 2. The p-values for the other comparisons were greater than the 0.002 thresholds.

Regarding the association between the ABE (evaluated by means of DIZ) and the type of EO, Mann-Whitney test was used to compare each pair of EOs with respect to the DIZ on each of the two bacterial species (Table 3). The hierarchy for both *E. coli* and *K. pneumoniae* strains was Thyme (Thy) ≥ Oregano (Ore) > Eucalyptus (Euc) ≈ Tea tree (Tea) > Clove (Clo).

Assessment of the Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC)

For each EO, the number of bacterial strains was determined for which the EO was inhibitory at four levels of dilution: ≥E-1, E-2, E-3, and ≤ E-4. The results for all five EOs were gathered in

Table 2. Comparison (by Mann-Whitney test) of the antibacterial efficacy (ABE) (as evaluated by DIZ) of each of the five EOs against the two bacterial species.

EO	Compared ABE	MD [conf.int.]	p-value
Clove EO	E. coli > K. pn	3 [2-4]	5E-6
Oregano EO	E. coli > K. pn	3 [0.00002-4]	0.01
Thyme EO	E. coli ≈ K. pn	3 [(-0.00001)-7]	0.09
Eucalyptus EO	E. coli > K. pn	4 [2-5]	4E-5
Tea tree EO	E. coli ≈ K. pn	1 [(-0.00005)-3]	0.2

„>"sign : the higher ABE (against E. coli); „≈" sign: the lack of a significant difference between the ABEs against the two bacterial species; p-values calculated by Mann-Whitney test; „>" = more efficacious (for example, "E. coli > K. pn" in the "Clove EO"-row means that the clove EO is more efficacious against *E. coli* strains than against *K. pneumoniae* strains); „≈" = about as efficacious, no significant difference in the efficiencies; MD = median difference, i.e., the difference between EO1 median and EO2 median; conf.int. = 95% confidence interval for MD.

Table 3. Pairwise comparison (by Mann-Whitney test) between EOs regarding their antibacterial efficacy (ABE) (as evaluated by the DIZ) against the two bacterial species.

Escherichia coli			*Klebsiella pneumoniae*		
EO1~EO2	MD [conf.int.]	p-value	EO1~EO2	MD [conf.int.]	p-value
Clo < Ore	-10 [(-11)-(-8)]	7E-12	Clo < Ore	-10 [(-11)-(-9)]	2E-9
Clo < Thy	-12 [(-14)-(-10)]	2E-11	Clo < Thy	-13 [(-15)-(-9)]	2E-6
Clo < Euc	-8 [(-9)-(-6)]	1E-11	Clo < Euc	-6 [(-8)-(-5)]	2E-7
Clo < Tea	-6 [(-7)-(-4)]	6E-9	Clo < Tea	-8 [(-9)-(-6)]	1E-7
Ore ≤ Thy	-2 [(-5)-0]	0.02	Ore ≤ Thy	-3 [(-6)-1]	0.1
Ore ≥ Euc	2 [0-4]	0.06	Ore > Euc	3 [2-5]	0.001
Ore > Tea	3 [1-6]	0.002	Ore ≥ Tea	2 [0-4]	0.01
Thy > Euc	5 [2-7]	2E-4	Thy ≥ Euc	6 [2-9]	0.004
Thy > Tea	6 [4-9]	2E-5	Thy ≥ Tea	5 [1-8]	0.009
Euc ≥ Tea	2 [0-4]	0.07	Euc ≤ Tea	-1 [(-3)-1]	0.4

MD: median difference, i.e., the difference between EO1 median and EO2 median; conf.int.: 95% confidence interval for MD; „<" or „>" indicate a significantly lower or higher efficacy, respectively, corresponding to a negative or positive MD, respectively; „≤" and „≥" indicate the lack of a significant difference between the ABEs of the two EOs; EO1 ~ EO2: EO1 compared with EO2 (for example, in "Clo < Ore"-row, EO1 is clove EO, while EO2 is oregano EO).

a 5×4 table (5 rows corresponding to the 5 EOs and 4 columns corresponding the 4 levels of dilution) (called contingency table). There were two such contingency tables corresponding to the two bacterial species, *E. coli* and *K. pneumoniae*: sub-tables titled "MIC for *Escherichia coli*" and "MIC for *Klebsiella pneumoniae*" in Table 4. The same process was repeated for the number of bacterial strains for which the EO was bactericidal, the resultant sub-tables in Table 4 being titled "MBC for *Escherichia coli*" and "MBC for *Klebsiella pneumoniae*". Consequently, the cells of Table 4 contain the number of bacterial strains for which the corresponding EO (indicated by the row heading) had the corresponding MIC/MBC (indicated by the column heading). To evaluate the association between both MIC and MBC and the type of EO, Chi-squared test was applied to each of the four contingency tables condensed as Table 4, yielding highly significant p-values. Fisher's exact test was used to compare each pair of EOs with respect to the MIC and MBC against each of the two bacterial species. The efficacy hierarchy recorded was oregano > thyme ≈ clove > eucalyptus ≈ tea tree for both bacterial species (*E. coli* and *K. pneumoniae*). The MIC of oregano EO for most of *E. coli* strains was 0.055 µL/mL (27 strains), the MIC of thyme EO for most of *E. coli* strains was 5.5 µL/mL (12 strains) or 0.55 µL/mL (14 strains), and the MIC for clove was 5,5 µL/mL (6 strains) or 0.55 µL/mL (11 strains). The MIC of thyme EO and clove for most of *K.pneumoniae* strains were 5.5 µL/mL or 0.55 µL/mL.

As the concentrations of EO used in this study followed a geometric progression of ratio 1/10, the only possible values of MBC / MIC were 1, 10, 100, 1000, 10000, therefore the condition MBC / MIC < 4 was equivalent to MBC / MIC = 1, while for all the other possible values (10, 100, 1000, 10000), MBC / MIC > 4.

Comparing the MIC and MBC of each of the five EOs against the two bacterial species by means of Fisher's exact test yielded no significant results.

Discussion

One of the greatest medical challenges of the 21st century is the increasing number of infections with multi-drug resistant bacteria, especially hospital-acquired infections.

Table 4. The distribution of the MICs and MBCs in relation to the bacterial species and the type of EO, contingency tables for each combination of the two bacterial species (*E. coli* and *K. pneumoniae*) with either MIC or MBC. The cells of the table contain the number of bacterial strains for which the corresponding EO (indicated by the row heading) had the corresponding MIC/MBC (indicated by the column heading); e.g., in the contingency table "MIC for *Escherichia coli*", the value 2 in the "Clove EO"-row and "≥E-1"-column means that there were two *E. coli* bacterial strains for which clove EO had a MIC ≥E-1. The p-values were calculated by means of Chi-squared test. The ≥E-1, E-2, E-3, E-4, and E-5 dilutions for MIC/MBC correspond to 55, 5.5, 0.55, 0.055, and 0.0055 µL/mL (or µg/mL) concentrations, respectively.

	Frequency distribution of the MIC and MBC, i.e. the number of bacterial strains corresponding to each MIC/MBC and each EO			
MIC or MBC (as dilution)	**≥E-1**	**E-2**	**E-3**	**E-4 and E-5**
MIC for *Escherichia coli* (Chi-squared statistics = 131.82, df = 12, p-value = 3E-22)				
Clove EO	2	6	11	13
Oregano EO	0	3	2	27
Thyme EO	0	12	14	6
Eucalyptus EO	17	11	3	1
Tea tree EO	20	11	1	0
MBC for *Escherichia coli* (Chi-squared statistics = 146.84, df = 12, p-value = 2E-25)				
Clove EO	2	10	14	6
Oregano EO	0	5	4	23
Thyme EO	4	11	13	4
Eucalyptus EO	27	4	1	0
Tea tree EO	24	8	0	0
MIC for *Klebsiella pneumoniae* (Chi-squared statistics = 148.78, df = 12, p-value = 1E-25)				
Clove EO	3	4	14	7
Oregano EO	1	1	2	24
Thyme EO	0	11	14	3
Eucalyptus EO	16	11	1	0
Tea tree EO	21	7	0	0
MBC for *Klebsiella pneumoniae* (Chi-squared statistics = 145.16, df = 12, p-value = 5E-25)				
Clove EO	4	11	9	4
Oregano EO	1	2	4	21
Thyme EO	3	10	13	2
Eucalyptus EO	26	2	0	0
Tea tree EO	22	6	0	0

df: (number of) degrees of freedom.

Regarding the bactericidal properties, although tea tree seemed to be at the top of the hierarchy (if one considers the number of bacterial strains for which each EO was bactericidal), one should notice that tea tree EO was bactericidal at concentrations of 10^{-1} and 10^{-2}, while oregano, thyme, and clove EOs were bactericidal at lower concentrations: 10^{-4} and 10^{-5} for oregano EO, 10^{-2} and 10^{-3} for thyme EO, and 10^{-2} and 10^{-3} for clove EO. Among the investigated EOs, oregano EO, followed by thyme EO have the highest efficacy against *E. coli* and *K. pneumoniae* isolates, a conclusion also reached by other researchers [27, 28]. Clove EO was also demonstrated to have powerful antibacterial effect against uropathogens [29].

Our results regarding the MIC of thyme EO for *E. coli* strains isolated from patients are similar to those obtained by other researchers for the EO extracted from *Thymus vulgaris* [0.25-0.5 µl/mL][7] or from other plants in the genus *Thymus* [1±0.3 µg/mL][9], 0.63 µl/mL [10], 5 µL/mL (EOs were tested against reference strain of *E .coli*) [23].

The MIC of oregano EO for most of *E. coli* strains tested in our research was 0.055 µL/mL, an order of magnitude lower than the values reported in other studies: 0.5 µL/mL [13]. For *K. pneumoniae* the information is scarcer. One study found that 1.2 % (v/v) thyme EO concentration has the highest antimicrobial effect against *K. pneumoniae* [6], which is roughly equivalent to a MIC of 5.5 µL/mL as in our study.

None of the comparisons regarding the activity of the tested EOs against the two bacterial species yielded significant results, hence the conclusion: a given EO is approximately equally efficacious against *E. coli* and *K. pneumoniae*.

There is a wide range of values for the DIZ in the literature. For the EO extracted from *Thymus* species against *E. coli* the range of values extends from 17 mm [*T. Vulgaris*] [6], 18 mm [23], passing through 30 and 50 mm [*T. hirtus sp. Algeriensisn*] [11], 40 and 50 mm [*T. Kotschyanus*] [12], 51 and 62 mm [*T. persicus*] [12], up to 90 mm [*T. schimperi*] [10], with variations depending on the region of growth [11], on the developmental stage, and on the investigated strains [12].

A similar variability of the DIZ was found for *K. pneumoniae*: 75 mm and 57 mm (*T. kotschyanus*), 48 mm and 52 mm (*T. persicus*) [12]. In striking contrast, another study found a 19 mm inhibition zone for *T. vulgaris* EO against *K. pneumoniae* [6].

Oregano EO has also been shown to be active on standard *E. coli* strains [30] with a MIC of 66 ± 28.87 µg/mL [31]. Much higher (383 ± 57.74 µg/mL) was the MIC against *E. coli* for clove EO [31], a result in agreement with those produced by our experiments.

The dominant components in the oregano and the thyme EOs used in our study were carvacrol (68.96%) and thymol (45.2%), respectively. The only difference between carvacrol and thymol is the hydroxyl group position.

These EOs exert, beside anti-inflammatory effects, a strong antibacterial activity due to their ability to increase the permeability and induce the depolarization of the cytoplasmic membrane [32, 33], to inhibit microbial toxin production and biofilm formation, to decrease fimbriae production and swarming motility of uropathogenic *E. coli* [34–36]. The ABE hierarchy as reflected by MIC and MBC, indicated by our results suggests that volatile phenols (such as carvacrol in oregano EO, thymol in thyme EO, and eugenol in clove EO) are more efficacious as antibacterial than non-aromatic compounds (such as eucalyptol in eucalyptus EO and terpinene derivatives in tea tree EO).

The main limitations of our study are: 1. the relatively small number of investigated bacterial strains (nonetheless the results were quite sound from the statistical point of view); 2. the ABE of the EOs was only tested in vitro, so that the results obtained do not guarantee an in vivo ABE, as it is uncertain whether these EOs can be ingested in quantities large enough to provide a urine concentration sufficient to exert their antibacterial activity (issues such as oral tolerability, bioavailability, liver metabolism, blood protein binding, urine concentration of the ingested EOs come into play).

Future studies are warranted to determine whether the *in vitro* proven ABE is also exerted *in vivo*, so that these EOs qualify as viable alternatives to antibiotics in the treatment of UTI, especially of the recurrent ones and/or those caused by resistant strains. The molecular biology methods that should present how the tested strain virulence

factors are influenced in the presence of the essential oils must be evaluated.

Conclusions

The results of this *in vitro* study suggest that:
(1) Among the investigated EOs, oregano EO and thyme EO have the highest efficacy against *E. coli* and *K. pneumoniae* isolates.
(2) Volatile phenols are more efficacious as antibacterial than non-aromatic compounds.
(3) A given EO is approximately equally efficacious against *E. coli* and *K. pneumoniae*.

Abbreviations

ABE = antibacterial efficacy; Clo = Clove EO; conf.int. = 95% confidence intervals; DIZ = diameter of the inhibition zone; EO = essential oil; Euc = Eucalyptus EO; *K. pn = Klebsiella pneumoniae*; *E.coli = Escherichia coli;* MBC = minimal bactericidal concentration; MD = median difference; MIC = minimal inhibitory concentration; Ore = Oregano EO; Tea = Tea tree EO; Thy = Thyme EO; UTI = urinary tract infections; W = Mann-Whitney statistics.

Author contributions

Conceptualization, Methodology, A.B., D.I., D.D., Formal Analysis: A.B., F.C.M., A.M.P., R.S.D., D.I., D.D., Validation: A.B., D.D., Investigation: A. B., F.C.M., A.M.P., R.S.D., M.C.D., A.A.M., D.I., D.D., Writing original draft: A.B., D.D., Writing - Review & Editing, A B., F.C.M., D.I., D.D.

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Conflicts of Interest

The authors declare no conflict of interest.

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