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Original Research Article

In Vitro Inhibitory Effect of Four Essential Oils against Fluoroquinolone- Resistant *Enterobacteriaceae* Responsible for Community-Acquired Urinary Tract Infections

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ABSTRACT

The emergence of antibiotic resistance genes has highlighted the need to discover new drugs for the treatment of infections caused by multidrug-resistant bacteria. The study aims to investigate the antibacterial and anti-adhesive effect of four essential oils (EOs) from aromatic medicinal plants: *Ammoides verticillata*, *Origanum glandulosum*, *Thymus fontanesii*, and *Thymus capitatus*, against fluoroquinolones-resistant uropathogenic *Enterobacteriaceae*. The chemical composition of the oils was determined by gas chromatography-mass spectrometry GC/MS, the antibacterial activity was assessed using disc diffusion and micro-dilution methods, while the adhesion assay was carried out using light microscopy. The essential oils of *Thymus fontanesii* and *Thymus capitatus* were carvacrol chemotype, whereas *Ammoides verticillata* and *Origanum glandulosum* EOs were thymol chemotype. Sixteen *Enterobacteriaceae* with different fluoroquinolones-resistance profiles were isolated, belonging to *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis* species. The inhibition zone diameters ranged from 17.67 ± 0.58 to 31 ± 0.00 (mm), *Thymus capitatus* EO appears to be the most effective, with the minimum inhibitory concentrations ranging from 0.5 mg/mL to 7.5 mg/mL. The lowest MIC (0.5 mg/mL) was observed for *Thymus capitatus* EO against *Escherichia coli* resistant strains. The adhesion assay showed a good adhesion reduction of the test *Escherichia coli* for all EOs, varying from 79% to 90%. These findings indicate that the studied essential oils can be used as a promising drug in the prevention and treatment of infections caused by resistant *Enterobacteriaceae*, however, exploring their therapeutic use requires thorough investigations that involves rigorous clinical studies to ensure their safety and efficacy.

***Keywords*:** Essential oils, *Enterobacteriaceae*, Urinary tract infections, Fluoroquinolone-resistance, Antibacterial activity, Aromatic plants.

Introduction

The human body is a host of a large number of microorganisms, most of which are beneficial and not harmful, occasionally, some of these microbes start to invade the organs and the tissues, and evade the host immune response employing their virulence factors (toxins, adhesins...). The microbial colonization of the urinary tract by these pathogenic bacteria and its interaction with the host's defense determine the outcome of a urinary tract infection (UTI).[1] UTI is among the most common infectious diseases in outpatients but also it represents the majority of the hospital-acquired infections, *Escherichia coli* and other *Enterobacteriaceae* including *Klebsiella*, *Proteus*, *Enterobacter* species.., which represent around 75% of the isolates, constitute the most prevalent pathogens causing the uncomplicated UTIs in the community.[2]

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For a long time, multidrug resistant *Enterobacteriaceae* were previously confined to the hospital environment, however this is no longer the case as they have spread widely in the community setting. In human medicine, the use of antibiotics is the highest in the community representing more than 85% of antibiotics consumption.[3] The intensive use of these substances is responsible for selection pressure that increases community antibiotic resistance by reducing susceptible bacterial strains and shifting the competitive balance in favor of existing resistant bacteria.[4] In developing countries, the dissemination of antibiotic resistance in the management of urinary tract infections (UTIs) is a crucial subject in public health, particularly where many studies showed a high prevalence of antibiotic resistance of pathogens isolated from UTI samples.[5, 6]

Nalidixic acid was the first quinolone used clinically classified as a urinary antiseptic, after the appearance of nalidixic acid-resistant *Enterobacteriaceae*, quinolones have been fluorinated for more improvement of the antibacterial activity against pathogens. Since then, fluoroquinolones have become established for the treatment of urinary, respiratory, gastrointestinal, urogenital, and intra-abdominal infections in outpatients and hospitalized patients.[7] However, in the past few decades, the resistance to these antimicrobials has developed especially by *Enterobacteriaceae* species due to the spontaneous mutations in the quinolone resistance determining regions (*QRDR*) of *gyr A* and *par C* genes but also the propagation of plasmid-mediated quinolone resistance (*PMQR*) all over the world.[8]

The swift emergence of antimicrobial resistance genes highlights the urgent need to discover new drugs for managing infections induced by multi-resistant human and animal bacteria.[9] Thus, natural compounds from aromatic medicinal plants have been exploited to find new

remedies for human health problems. Recently, a wide range of these plants were screened for antimicrobial properties such as plant essential oils (EOs) that comprise a large reservoir of unrelated compounds with high antimicrobial potential.[10]

Thyme and oregano species are Mediterranean endemic plants that belong to the *Lamiaceae* family, they are commonly used as a condiment and as herbal teas in cuisine, but also as analgesic, diuretic, antiseptic, antipyretic, anticancer, and many other properties in traditional medicine. Moreover, they have a captivating antibacterial, antifungal and antioxidant properties, which are linked to the presence of phenolic compounds: carvacrol, thymol, *α*-terpinene and p-cymene. [11, 12, 13, 14] Another interesting herb that grows spontaneously in the matorrals of the western region of Algeria, is *Ammoides verticillata* (Desf.) Briq, belonging to the *Apiaceae* family, is an aromatic herb consumed extensively by the local population for its culinary and therapeutic purposes, the aerial parts of *A. verticillata* are used as antipyretic and antispasmodic. It is recommended as well for influenza and has therapeutic properties against hypertension and diabetes. [15, 16, 17]

The recent findings regarding essential oils from *Ammoides verticillata*, *Origanum glandulosum* Desf., *Thymus fontanesii* Boiss. et Reut. and *Thymus capitatus* (L.) Hoffmann and Link showed that they were strongly effective against resistant pathogens, [18, 19, 20, 21] the present study focused on their antibacterial and their anti-adhesive activity against fluoroquinolones-resistant uropathogenic *Enterobacteriaceae* strains, this can provide valuable insights into their potential as alternative or adjunctive therapies for UTIs. Assessing the anti-adhesive properties also is crucial, as the capacity to inhibit bacterial adhesion can help to prevent the initial colonization of uroepithelial cells, thereby reducing the risk of recurrent infections.

Materials and Methods

Collection and identification of plant material

Four medicinal plants were selected: *Ammoides verticillata*, *Origanum glandulosum*, *Thymus fontanesii* and *Thymus capitatus*, the choice of these species was based on a literature survey and their use by the local population. These plants have been harvested from the region of Tlemcen located in northwestern Algeria, in the period from April 2021 to June 2022. All species in this study were identified by Laboratory of Ecology and Management of Natural Ecosystems, University of Tlemcen, also were deposited in the laboratory. Data on the selected plants are summarized in Table 1.

Table 1: Details about the studied medicinal plants

Scientific name	Family	Studied organs	Harvest date	Harvest station		Voucher number
				Name	Altitude	
Ammoides verticillata	*Apiaceae*	Leaves, Stems & Flowers	Jun, 2022	Tirni (Tlemcen)	1190	Av0620LS
Origanum glandulosum	*Lamiaceae*	Leaves, Stems & Flowers	Jun, 2021	Tirni (Tlemcen)	1190	Og0620TLM
Thymus fontanesii	*Lamiaceae*	Leaves, Stems & Flowers	Jun, 2022	Mansoura (Tlemcen)	980	Tf0620TLS
Thymus capitatus	*Lamiaceae*	Leaves, Stems & Flowers	May 2022	Beni Snous (Tlemcen)	835	Tc0620LS

Essential oil extraction

The air-dried aerial parts (stems, leaves, and flowers) of the selected plants were extracted by hydrodistillation for 3 hours using a Clevenger-type apparatus. The obtained EOs were transferred to amber glass vials and stored at 4 °C until analyzed. According to the plant's dry weight, the yield of the extracted essential oils was determined per 100 g of each dried plant.

GC/MS analysis

A gas chromatography and gas chromatography-mass spectrometry GC/MS analysis were employed to characterize the chemical composition of the essential oils. A Perkin Elmer Autosystem GC-type chromatograph, equipped with two flame ionization detectors was used for the Gas chromatography (GC) analysis. To detect the volatile compounds, one injector/splitter, and two polar (Rtx-Wax, polyethylene glycol) and nonpolar (Rtx-1, polydimethylsiloxane) columns (60 m×0.22 mm inner diameter, film thickness 0.25 µm). The carrier gas was helium (1 mL/min) with a column head pressure of 25 psi. The injector temperature was 250 °C while the detector's was 280 °C. The temperature was set to rise from 60 to 230 °C at a rate of 2 °C/min, and was maintained at that temperature for 45 minutes. The split mode was used for the injection, with a split ratio of 1/50. The injected EO volume was 0.2 µL. Direct electronic integration of peak areas was used for quantification.

The GC/MS analysis was conducted by a Perkin Elmer Autosystem XL chromatograph, fitted with an automatic injector and two columns: one polar (Rtx-Wax) and one nonpolar (Rtx-1), both measuring 60 m in length, with an inner diameter of 0.22 mm, and a film thickness of 0.25 µm, the system was coupled with a Perkin Elmer TurboMass for mass spectrometric detection. Helium (1 mL/min) was served as the carrier gas with a column head pressure of 25 psi. The temperature of the injector was 250 °C. The temperature was programmed to increase from 60 to 230 °C at the rate of 2 °C/min, and then maintained constant for 35 minutes. A split ratio of 1/80 was used for the injection in split mode. The injected EO amount was 0.2 L. A quadrupole analyzer, which had an assembly of four parallel electrodes with cylindrical sections, was employed for the detection. The temperature at the source was 150 °C. The system performed in electron impact and fragmentation was conducted at a 70 eV electric field. A mass range of 35-350 Da was applied to obtain the resulting mass spectra. The identification of the studied EOs components was made using Kovats index, and a comparison of mass spectra with those of the computerized libraries.[22]

Bacterial strains
To focus on community-acquired UTI, the *Enterobacteriaceae* strains were isolated at three medical analysis laboratories at Tlemcen city-Algeria, samples coming from hospitals or healthcare facilities were excluded.[23] The isolated strains were streaked on MacConkey Agar (Sigma-Aldrich, Germany) and CHROMagar Orientation plates (CHROMagar company, Paris, France), then incubated for 24 h at 37 °C, to examine the macroscopic aspect of the colonies. The strains were identified by a standardized identification system, API 20E®, the Vitek 2 system (Biomerieux®, France) was used to confirm the identification. Two *E.coli* strains were also included in the study are American Type Culture Collection with codes ATCC 25922 and ATCC 27325. Finally, the total of *Enterobacteriaceae* strains were stored at -20 °C in a mixture of brain heart infusion broth (BHIB) and glycerol (Sigma-Aldrich, Germany) (7:3, v/v).

Fluoroquinolones-Resistant enterobacteriaceae screening
For the selection of fluoroquinolones-resistant strains, antibiotic susceptibility testing was performed using the disc diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) recommendations.[24] Briefly, a 0.5 MacFarland inoculum of each strain was streaked using sterile swabs on Mueller Hinton Agar (MHA) plates (Sigma-Aldrich, Germany), then the standard antibiotic discs (Liofilchem S.r.l, Italy) were placed on the inoculated MHA plates and incubated for 24 h at 37 ºC. The following standard fluoroquinolones discs were tested: nalidixic acid (30 µg), ciprofloxacin (5 µg), ofloxacin (5 µg), levofloxacin (5 µg), moreover, other antibiotic classes were used: amoxicillin (30 µg), amoxicillin-clavulanic acid, (30 µg), cefazolin (30 µg), cefoxitin (30 µg), ceftazidime (30 µg), imipenem (10 µg), aztreonam (30 µg), gentamicin (10 µg), the trimethoprim-sulfamethoxazole combination (30 µg) and fosfomycin (200 µg).

Antibacterial activity screening
The antibacterial potential of the test EOs was assessed using the disc-diffusion method. Muller Hinton agar (MHA) plates were seeded with a 0.5 McFarland suspension from each strain by sterile swab, Whatman No.4 paper discs (6 mm diameter) impregnated with 10 µL of EOs were placed aseptically on the surface of the agar. The plates were left for 30 min at room temperature, after incubation at 37°C for 24 h, the result was determined by measuring diameters of the inhibition zones in millimeters (mm). All assays were carried out in triplicate.

Determination of MIC and MBC
Broth micro-dilution method in 96-well microplates was employed to determine the minimum inhibitory concentrations (MICs) of EOs.[25] Initially, 12 distinct concentrations were prepared in external tubes by dilution of each EO by half in Mueller-Hinton broth (MHB) (Sigma-Aldrich, Germany) containing 2 mg of dimethylsulfoxide (DMSO) (Sigma-Aldrich, Germany), starting from 600 mg to 0.3 mg, after that, wells were imbued with 20 µL of every EO concentration mixed with 180 µL of 10^5 CFU/mL inoculum of the test strains, the final concentration of EOs and the DMSO in wells ranged from 60mg to 0.03 mg and 0.2 mg respectively. Negative (MHB and DMSO) and positive (MHB and bacterial suspension, free of EOs) controls were prepared for each microplate, finally, the microplates were incubated over the night at 37 °C. The lowest EO concentration that inhibits any discernible growth is known as the MIC.
The minimum bactericidal concentration (MBC) was determined by seeding 10 µL of wells with no bacterial growth on MacConkey agar plates and then incubated at 37°C for 24 h, the MBC represents the concentrations at which bacterial colonies are totally absent.

Adhesion assay
The *in vitro* adhesion assay was conducted using Zam & Khaddour method with a slight modification.[26]
Control slides preparation: A mixture of one milliliter of epithelial cell suspension and one milliliter of bacterial suspension were incubated at 37°C for 3 hours in a shaking water bath. Then it was washed three times and a fraction of the final cell suspension was placed on a slide, air dried, fixed with methanol (Honeywell, USA), and stained with Giemsa stain (10%) (Cypress Diagnostics, Belgium) for 30 minutes before being observed under a light microscope (X100) (Carl ZEISS, West Germany). Fifty cells were examined, and the average number of adhered bacteria per cell was counted.
Test slides preparation: The test strain *Escherichia coli 1* was grown for 36 h at 37°C in Brain Heart Infusion Broth with the addition of the four test essential oils at their minimum inhibitory concentration. Then one milliliter of the incubated bacterial suspension was mixed with one milliliter of epithelial cells and incubated at 37°C for 3 h. Finally, they were washed with PBS to remove any bacteria that had not been attached. The cells were then air dried, methanol fixed and stained with Giemsa stain (10%) for 30 minutes. After that, they were observed at the microscope (X100). The average number of adhering bacteria per cell was obtained from an examination of 50 cells. Each test was executed in triplicate.

Statistical analysis
The mean values and standard deviations (SD) of the experimental data along with the one-way ANOVA combined with Tukey's test were carried out to reveal significant differences ($P < 0.05$) between the means. The analyses were performed using the Microsoft® Excel2016 software.

Results and Discussion

Bacterial strains
Sixteen *Enterobacteriaceae* have been opted for this study, with different quinolones-resistance profiles, the predominant isolates belong to *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis* species. The dominant *Enterobacteriaceae* that were isolated in this study are concordant with several studies about bacteria responsible for the community-acquired urinary tract infections CA-UTI.[27, 28, 29] Quinolone resistance cases are frequently linked to resistance to other antibiotic classes, these are ß-Lactamines, sulfonamides, and aminoglycosides. [30]
The results of the antibiotic susceptibility testing showed different fluoroquinolones-resistance phenotypes of the studied strains (Table 3), as well as for the other test antibiotics (Table 4). These profiles reflect the high resistance rates to fluoroquinolones and beta-lactam antibiotics in community-acquired UTIs which is due to the worrisome dissemination of antimicrobials resistance genes. It might be also the result of the pressure of selection generated by the inadequate prescriptions, self-medication, and the overuse of broadspectrum antibiotics.

Compounds identified from GC/MS analysis
The studied essential oils were obtained from the aerial parts of *Ammoides verticillata*, *Origanum glandulosum*, *Thymus fontanesii* and *Thymus capitatus*, with yields of 1.9%, 2.1%, 1.6% and 1.7% respectively. The analytical results of these EOs are illustrated in Table 2, the oxygenated monoterpenes are copious in all of the test EOs, whereas the monoterpene hydrocarbons are less abundant for *Ammoides verticillata, Thymus fontanesii* and *Thymus capitatus.*
In *Ammoides verticillata* essential oil, 15 chemical compounds were identified accounting for 98.70% of the total oil composition. Oxygenated monoterpenes accounted for more than half of the components (59.30%). Thymol appeared to be the principal compound with a percentage of 50.10% followed by p-cymene (15.60 %) and limonene (15%). The monoterpenes hydrocarbons dominated *Origanum glandulosum* essential oil with the percentage of 54.4%, followed by oxygenated monoterpenes (44.9%). The rest are small amounts of Sesquiterpene hydrocarbons (1.8%) and oxygenated sesquiterpene (0.2%), all represented in 26 identified chemical compounds (99.3 % of the total oil composition),923 the main constituent was thymol (41.6 %) accompanied with a large proportion of γ-terpinene (27%) and p-cymene with 17.1% (monoterpenes hydrocarbons).

Various research have examined the chemical composition of the studied essential oils. For *Ammoides verticillata* EO, the results are extremely coherent with the report of Attou *et al.* (2019)[31] who found that the main constituent was thymol (45.77%), followed by p-cymene, limonene, and *γ*-terpinene (44.31%). As well as for Mami *et al.* (2021)[53] who confirmed the dominance of thymol (30.5%), p-cymene (13.1%), limonene (12.5%) and terpinene-4-ol (12.3%). Conversely, they disagree with the study of Senouci *et al.* (2020)[17] and Benyoucef *et al.* (2020)[21], where they reported that the EO was predominated by the monoterpene hydrocarbons (51, 3%) and oxygenated monoterpenes (45, 4%) and the main compound was carvacrol. For *Origanum glandulosum* EO, the outcomes are in great accordance with the reports of Bendahou *et al.* (2008)[32] where they identified 31 components that accounted for 99.6%, the main components were thymol c-terpinene and p-cymene. The study of Khalfi *et al.* (2008)[33] determined 18 compounds representing 92.6% of *O. glandulosum* oil, it contained an important amount of oxygenated compound, thymol and carvacrol (38.8%, 32.9%) were the major compounds, the monoterpenes hydrocarbons were less abundant represented by p-cymene (7.9%) and *γ* – terpinene with 5.1%.

Ruberto *et al.* (2002)[34] investigated the chemical composition of EOs from Algerian *Orig-anum glandulosum* that were collected from four different locations (Ouled Iyiche, Djebel Megriss, Aniniand Tafat). Two samples (Ouled Iyiche and Djebel Megriss) had a balanced proportion of carvacrol and thymol (the second was the major element), however, the two last oils showed a small amount of thymol and carvacrol was the main component. Belhattab *et al.* (2005)[35] also found that carvacrol was the principal compound, the other important constituents (>5%) were *γ*-terpinene, p-cymene and thymol.

In *Thymus capitatus* essential oil, 25 compounds have been determined, representing 99.01 % of the total oil composition. These components were grouped in five classes: monoterpene hydrocarbons, oxygenated monoterpenes, oxygenated sesquiterpenes, sesquiterpene hydrocarbons and phenylpropanoids. The amounts of the two first classes are very high (23.97%, 73.03% respectively), accounting for almost the totality of the oil, the proportions of the last three classes are very limited (2.01% combined). *T. capitatus* EO is characterized by a large amount of carvacrol (68.4%), with two monoterpene hydrocarbons, p-cymene (12.4%) and *γ* –terpinene (4.4%), and very poor in thymol (0.7%). *Thymus fontanesii* EO analysis enabled the identification of 24 chemical constituents, accounting for 99.34% of the total oil composition, oxygenated monoterpenes were highly abundant (74.45%), represented by a large amount of carvacrol 70.9%, together with a low percentage of Terpinene 4-ol (0.35%) and thymol (0.2%). The second highest predominant components are monoterpene hydrocarbons (21.86%) mainly constituted by: p-cymene (8.5%), *γ* – terpinene (6.2%) and linalool (2.1%), sesquiterpene hydrocarbons were present in a small portion (*β*–Caryophyllene 2.73%).

In the case of *Thymus capitatus* EO, the findings are in great agreement with the research of El Ouariachi *et al.* (2011)[36] they analyzed the chemical composition of Morrocon *Thymus capitatus* essential oil and they found the same principal constituents (carvacrol, p-cymene) with slightly different amounts (18.9%, 13.4% respectively). The report of El-Jalel *et al.* (2018)[20] analysed the EO of Libyan *Thymus capitatus* collected from two different altitudes. The results revealed the identification of 14 components corresponding to 91.99% of the total EO composition from the first altitude and 23 compounds corresponding to 96.54% from the second one, where carvacrol was the major compound in both essential oils, which is consistent with these outcomes. However, they identified *β*-caryophyllene and *γ*-terpinene as the second major components and this appears to be different to the obtained data.

The results about the analysis of *Thymus fontanesii* essential oil exhibit an excellent coherence with the research of Sidali *et al.* (2020)[37] who identified 27 of the Algerian *Thymus fontanesii* EO and the main compound was carvacrol (63.9%) followed by p-cymene (17.5%) and *γ*–terpinene (14.9%). Furthermore, Nabet *et al.* (2017)[54] found that the principal compounds were carvacrol (67.5%) and *γ*–terpinene (13%). Nonetheless, those results were discordant with the research of Benyoucef *et al.* (2018)[38] and Dob *et al.* (2006)[39] they noted that the main constituent was thymol (76.6%, 29.3%), with p-cymene (7.4%, 15.9%) and *γ*–terpinene (2.3%, 21.7%) as the second main components.

All these data showed a predominance of thymol, carvacrol, p-cymene and *γ*–terpinene, this is due to the common biosynthetic pathways of monoterpenes, where *γ*-terpinene and p-cymene are the precursors of the monoterpenes thymol and carvacrol.[40]The test essential oils were carvacrol and thymol chemotypes, the studies mentioned above revealed a chemotypic diversity within the same medicinal plant species. This chemical variation might be correlated to environmental factors (climatic conditions, geographic locations, type of soil), experimental factors (harvest time, drying and storage, extraction method) and the genetic diversity of these species.[41, 42]

Table 2: Chemical compositions of the four studied essential oils

					Chemical composition (%)				
No	**Species** / ***Components***	**LRIa**	**RIa**	**RIp**	**1**	**2**	**3**	**4**	**ID**
1	*α*-thujene	932	928	1023	0.30	1.00	0.17	1.18	RI, SM
2	*α*-pinene	936	931	1022	1.00	0.70	1.40	1.55	RI, SM
3	Camphene	950	943	1066	-	-	0.22	0.48	RI, SM
4	1-Octen-3-ol	962	959	1446	-	0.20	0.50	0.10	RI, SM
5	3-Octanone	981	963	1253	-	0.10	-	-	RI, SM
6	*β*-pinene	978	970	1110	0.10	-	0.10	0.26	RI, SM
7	Myrcene	987	979	1159	0.60	2.00	2.13	1.22	RI, SM
8	*α*-Phellandrene	1002	997	1164	-	0.30	0.21	0.19	RI, SM
9	Δ-3-Carène	1010	1005	1147	-	0.10	0.11	0.10	RI, SM
10	*α*-terpinene	1013	1008	1178	0.10	2.80	1.70	1.20	RI, SM
11	p-cymene	1015	1011	1268	15.60	17.10	12.40	8.50	RI, SM
12	Limonene	1025	1020	1202	15.00	0.60	-	0.40	RI, SM

13	1,8 cineol	1024	1020	1210	-	-	-	0.40	RI, SM
14	*cis*– *β*–Ocimene	1029	1024	1230	-	0.10	-	-	RI, SM
15	*trans*– *β*–Ocimene	1041	1034	1247	-	0.10	0.40	-	RI, SM
16	*γ*-terpinene	1051	1047	1243	6.60	27.00	4.40	6.20	RI, SM
17	*trans*-Sabinene hydrate	1053	1051	1451	-	0.20	-	0.10	RI, SM
18	Terpinolene	1082	1078	1280	0.10	0.10	0.23	0.20	RI, SM
19	Linalool	1086	1081	1544	0.10	0.70	1.60	2.10	RI, SM
20	*cis*-Sabinene hydrate	1087	1083	1345	Tr	-	0.10	-	RI, SM
21	Camphor	1123	1123	1651	-	-	0.10	-	RI, SM
22	Borneol	1150	1148	1698	-	0.10	0.60	0.11	RI, SM
23	Terpinene 4-ol	1162	1161	1600	0.20	0.10	1.12	0.35	RI, SM
24	*trans*-dihydrocarvone	1174	1172	1605	-	-	-	0.21	RI, SM
25	*cis*-dihydrocarvone	1175	1178	1625	-	-	-	0.18	RI, SM
26	*α*-Terpineol	1176	1172	1697	0.10	0.20	0.11	-	RI, SM
27	Thymol	1267	1266	2189	50.10	41.60	0.70	0.20	RI, SM
28	Carvacrol	1278	1278	2219	8.80	2.20	68.70	70.90	RI, SM
29	Eugenol	1331	1330	2171	-	-	0.10	-	RI, SM
30	*β*–Caryophyllene	1420	1419	1591	-	1.00	1.71	2.73	RI, SM
31	*α*-Humulène	1455	1456	1665	-	0.10	0.10	-	RI, SM
32	*β*–bisabolene	1503	1500	1720	-	0.10	-	-	RI, SM
33	*β*-Sesquiphellandrène	1516	1516	1765	-	0.60	-	-	RI, SM
34	Caryophyllene oxide	1578	1576	1960	-	0.20	0.10	0.30	RI, SM
	Total (%)				98.70	99.30	99.01	99.34	
	Monoterpene hydrocarbons				39.40	52.40	23.97	21.86	
	Oxygenated monoterpenes				59.30	44.90	73.03	74.45	
	Sesquiterpene hydrocarbons					01.80	1.81	2.73	
	Oxygenated sesquiterpenes					0.20	0.10	0.30	
	Phenylpropanoids						0.10		

Results are in percentage (%) of components for EOs of (1) *Ammoïdes verticillata*, (2) *Origanum glandulosum*, (3) *Thymus capitatus*, (4) *Thymus fontanesi*. RTa: Retention Time of components are given on nonpolar column Rtx-1; LIra: Retention index of components from library of Adams are given on nonpolar column Rtx-1; RIa and RIp: Retention index of compounds obtained experimentally are given on nonpolar coloumn (RTX-1) and on polar column (RT-Wax), respectively; RI: Retention indices; MS: Mass spectra.

Table 3: Quinolones-resistance profiles of the studied strains

Strains	**Quinolones-resistance phenotypes**			
	NA	**CIP**	**OFX**	**LVX**
Escherichia coli 1	R	R	R	R
Escherichia coli 2	R	I	I	S
Escherichia coli 3	R	S	S	S
Escherichia coli 4	I	S	S	S
Escherichia coli 5	S	S	S	S
Klebsiella pneumoniae 1	R	R	R	R
Klebsiella pneumoniae 2	R	I	I	S

Klebsiella pneumoniae 3	I	I	I	S
Klebsiella pneumoniae 4	I	I	S	S
Klebsiella pneumoniae 5	I	S	S	S
Klebsiella pneumoniae 6	S	S	S	S
Proteus mirabilis 1	R	R	R	R
Proteus mirabilis 2	R	I	I	S
Proteus mirabilis 3	R	S	S	S
Proteus mirabilis 4	I	S	S	S
Proteus mirabilis 5	S	S	S	S

NA : nalidixic acid, CIP : ciprofloxacin, OFX : ofloxacin, LVX : levofloxacin.

Table 4: Antibiotic- resistance profiles of the studied strains

Strains	Antibiotic-resistance phenotypes
Escherichia coli 1	AMX, AMC, KZ, CAZ, IMP, SXT
Escherichia coli 2	AMX, AMC, KZ, CAZ, IMP, ATM, SXT
Escherichia coli 3	SXT
Escherichia coli 4	AMX, AMC, KZ, CAZ, IMP
Escherichia coli 5	Sensitive
Klebsiella pneumoniae 1	AMC, KZ, FOX, CAZ, ATM, CN, FOS, SXT
Klebsiella pneumoniae 2	AMC, KZ, CAZ, IMP, CN, FOS, SXT
Klebsiella pneumoniae 3	AMC
Klebsiella pneumoniae 4	AMC, KZ, FOX, CAZ, ATM, CN, FOS, SXT
Klebsiella pneumoniae 5	AMC, KZ, CAZ, FOS
Klebsiella pneumoniae 6	AMX, AMC, KZ
Proteus mirabilis 1	AMX, AMC, FOS, SXT
Proteus mirabilis 2	AMX, AMC, KZ, FOX, CAZ, IMP, ATM, FOS, SXT
Proteus mirabilis 3	AMX, AMC, SXT
Proteus mirabilis 4	Sensitive
Proteus mirabilis 5	AMX, AMC

AMX : amoxicillin, AMC : amoxicillin-clavulanic acid, KZ : cefazolin, FOX : cefoxitin, CAZ : ceftazidim, IMP : imipenem, ATM : aztreonam, CN : gentamicin, FOS : fosfomycin, SXT : trimethoprim-sulfamethoxazole

Antibacterial activity

The disc diffusion method was used to assess the antibacterial activity, the results revealed that all the test strains (resistant or sensitive to quinolones) were susceptible to all the test EOs (Table 5), so any possible correlation between antibiotic (specifically fluroquinolones) and EOs resistance can be eliminated. The inhibition zone diameters ranged from 17.67±0.58 to 31±0.00 (mm). The essential oil of *Thymus capitatus* seem to be the most effective followed by *Ammoides verticillata, Origanum glandulosum,* and *Thymus fontanesii.*

Few publications have focused on the antimicrobial activity of the studied essential oils against uropathogenic resistant *Enterobacteriaceae*. It is noticed that all *Klebsiella pneumoniae* strains appeared to be the less sensitive to the totality of the test EOs, unlike *Escherichia coli* and *Proteus mirabilis* species where the four EOs were found to be strongly efficacious, this goes along with the research of El-Jalel *et al.* (2018)[20] for *thymus capitatus* EO, and also with the study of Bekhechi *et al.* (2007)[18] for *Thymus fontanesii* EO, but not in *Proteus mirabilis* case. Precedent studies have confirmed in the same way that *Klebsiella pneumoniae* is more resistant to *Origanum glandulosum* and *Ammoides verticillata* EOs. [31, 32]

MIC and MBC of the essential oils

The determination of the minimum inhibitory concentrations (MICs) of *Ammoides verticillata*, *Origanum glandulosum*, *Thymus fontanesii* and *Thymus capitatus* essential oils showed a good efficacy against the totality of the test strains (Table 6). The values ranging from 0.5 mg/ml to 7.5 mg/mL, the lowest values of MIC (0.5 mg/mL) belong to *Thymus capitatus* essential oil against *Escherichia coli* clinical strains. *Ammoides verticillata* and *Origanum glandulosum* essential oils exhibited the highest value of MIC (7.5mg/mL) against all the test fluoroquinolones-resistant *Klebsiella pneumoniae* uropathogenic strains. Furthermore, 7.5 mg/mL was the highest minimum bactericidal concentration (MBC) of *Ammoides verticillata*, *Origanum glandulosum* and *Thymus fontanesii* essential oils, these values were found against *Klebsiella pneumoniae*, but also against *Proteus mirabilis* quinolones-resistant strains, whereas *Thymus capitatus* essential oil revealed the lowest MBC with the value of 0.5 mg/mL. Interestingly, the lesser values of MIC and MBC were against *Escherichia coli* strains (*E. coli* is the most prevalent bacteria known to induce urinary tract infections).

A previous study showed that *Thymus capitatus* has a powerful bactericidal activity against pathogenic *Enterobacteriaceae.* The values were equal for the *Escherichia coli strain*, but lightly lower for

Klebsiella pneumoniae, this efficiency might be due mainly to the phenolic action of carvacrol.[43] Several studies explained that membranes instantly became more fluid in the presence of carvacrol, it disrupts the bacterial membrane, which produces leaks of intracellular ATP and potassium ions and inevitably, death of the bacteria.[44] Similar values were reported by Sidali *et al.* (2017)[45] for the MIC of *Thymus fontanesii* essential oil (1.95 mg/ml), they likewise suggested that this EO exhibited a broad-spectrum antimicrobial activity, proving effective against numerous microorganisms. This efficacy could be associated not only to the existence of carvacrol but also to a possible synergetic interaction between carvacrol and other molecules such as its precursor p-cymene.

Oregano (*Origanum glandulosum)* bears a ressemblance to thyme species, showcasing a wide spectrum of antibacterial properties, this can be credited to the presence of thymol as a major compound in the essential oil. According to Béjaoui *et al.* (2013)[19] research, the Tunisian *Origanum glandulosum* had a very low MIC (0,25 mg/ml) against *E.coli* reference strain, and MBC values ranging from 0.35 to 0.4 mg/ml, these values are lower than those found in this study, this difference in the antibacterial potential of the essential oils might be explained by the difference in the chemical composition, the proportions and the interactions between the EOs components, along with the test bacteria themselves (their genetic background, membrane composition, efflux pumps expression etc..).[46]

For *Ammoides verticillata* essential oil, only a few studies had investigated its antibacterial activity against *Enterobacteriaceae.* Benyoucef *et al.* (2020)[21] together with Attou *et al.* (2019)[31] have reported that the EO of *A. verticillata* had bactericidal effects against all *E.coli*, however, it was less effective against *K.pneumoniae*. This strong efficacy is correlated to the presence of thymol as a major compound, it interacts with the cell membrane proteins via many non-specific mechanisms. Briefly, it affects the bacterial membrane similarly to carvacrol.[47] All the studied essential oils were less active against the test *Klebsiella pneumoniae* strains due to the presence of a complex acidic polysaccharide called a capsule, it contains complex enzyme systems, that appear to prevent essential oils from reaching the delicate inner membrane. Additionally, it protects the cell from phagocytosis or when subjected to bactericidal serum factors.[48]

Table 5: Antibacterial activity of the four studied essential oils

Strains	Inhibition zones (mm± SD)			
	Ammoides	***Origanum***	***Thymus***	***Thymus***
Escherichia coli 1	29.67±0.58	27.33±0.58	27.33±0.58	29.67±0.58
Escherichia coli 2	29.67±0.58	26.33±0.58	26.33±0.58	28.67±0.58
Escherichia coli 3	29.00±0.00	27.33±0.58	27.00±0.00	30.00±0.00
Escherichia coli 4	28.67±0.58	27.67±0.58	26.33±0.58	28.67±0.58
Escherichia coli 5	26.67±0.58	27.00±0.00	26.00±0.00	28.67±0.58
Klebsiella pneumoniae 1	20.33±0.58	17.67±0.58	19.00±1.00	23.33±0.58
Klebsiella pneumoniae 2	23.67±0.58	18.67±0.58	20.67±0.58	21.67±0.58
Klebsiella pneumoniae 3	22.33±0.58	19.33±0.58	22.33±0.58	23.00±0.00
Klebsiella pneumoniae 4	22.33±0.58	20.67±0.58	21.33±0.58	22.67±0.58
Klebsiella pneumoniae 5	23.00±0.00	22.67±0.58	22.33±0.58	24.67±0.58
Klebsiella pneumoniae 6	20.33±0.58	22.33±0.58	20.33±0.58	24.67±0.58
Proteus mirabilis 1	29.33±1.15	29.67±0.58	28.33±0.58	31.00±1.00
Proteus mirabilis 2	25.33±0.58	27.33±0.58	23.33±0.58	25.67±0.58
Proteus mirabilis 3	26.67±0.58	25.33±0.58	19.67±0.58	27.67±0.58
Proteus mirabilis 4	18.33±0.58	24.33±0.58	23.67±0.58	24.33±0.58
Proteus mirabilis 5	28.67±0.58	26.33±0.58	27.00±1.00	28.00±0.00
Escherichia coli 25922	24.00±0.00	22.67±0.58	24.67±0.58	24.67±0,00
Escherichia coli 27325	23.00±0.00	25.33±0.58	23.67±0.58	25.67±0.58

Values are means ± Standard Deviation (SD) of triplicate determinations expressed in mm including 6 mm of the paper disc
P values of less than 5 % ($p < 0.05$), were considered to be significant.

Table 6: MIC and MBC values of the four studied essential oils.

Strains	MIC (mg/ml)				MBC (mg/ml)			
	1	**2**	**3**	**4**	**1**	**2**	**3**	**4**
Escherichia coli 1	1.87	1.00	1.87	0.50	1.87	1.00	3.75	0.50
Escherichia coli 2	1.87	1.87	1.87	1.00	3.75	3.75	3.75	1.00
Escherichia coli 3	1.87	1.00	1.87	0.50	1.87	1.87	3.75	1.00
Escherichia coli 4	1.87	1.00	1.87	1.00	3.75	1.00	1.87	1.00
Escherichia coli 5	1.87	1.00	1.87	1.00	1.87	1.00	1.87	1.00

Klebsiella pneumoniae 1	7.50	7.50	3.75	1.87	7.50	7.50	3.75	1.87
Klebsiella pneumoniae 2	1.87	7.50	3.75	3.75	7.50	7.50	7.50	3.75
Klebsiella pneumoniae 3	3.75	7.50	3.75	3.75	3.75	7.50	7.50	3.75
Klebsiella pneumoniae 4	3.75	7.50	3.75	3.75	3.75	7.50	3.75	3.75
Klebsiella pneumoniae 5	3.75	3.75	7.5	3.75	7.50	3.75	7.50	3.75
Klebsiella pneumoniae 6	3.75	3.75	3.75	1.87	3.75	3.75	3.75	1.87
Proteus mirabilis 1	1.87	1.00	1.87	1.00	1.87	1.00	3.75	1.00
Proteus mirabilis 2	3.75	1.87	3.75	1.87	3.75	1.87	3.75	3.75
Proteus mirabilis 3	3.75	3.75	7.5	1.00	7.50	7.50	7.50	1.00
Proteus mirabilis 4	3.75	375	3.75	3.75	3.75	3.75	7.50	3.75
Proteus mirabilis 5	1.87	1.87	1.87	1.00	1.87	1.87	1.87	1.00
Escherichia coli 25922	3.75	3.75	1.87	1.87	3.75	3.75	3.75	3.75
Escherichia coli 27325	3.75	3.75	3.75	1.87	3.75	3.75	3.75	3.75

Results are in (mg/ml) of (1) *Ammoïdes verticillata*, (2) *Origanum glandulosum*, (3) *Thymus fontanesii*, (4) *Thymus capitatus* essential oils
MIC: Minimum Inhibitory Concentration. MBC: Minimum Bactericidal Concentration
P values of less than 5% ($p < 0.05$), were considered to be significant

In vitro adhesion

The adhesion assay was performed using the uropathogenic *Escherichia coli 1* strain, the control was prepared by incubation of the bacteria with the uroepithelial cells without adding the studied essential oils. A clear adhesion reduction has been observed (Figure 1), the percentage of the adhesion reduction for all EOs varying from 79% to 90%. The results showed that approximately 117.87±5.22 bacteria had adhered to the uroepithelial cells before treatment with the essential oils (Table 7). Nevertheless only 24.35±1.85, 18.52±2.86, 20.47±1.13 and 11.99±1.43 *Escherichia coli 1* had adhered to the cells after treatment with *Ammoides verticillata*, *Origanum glandulosum*, *Thymus fontanesii* and *Thymus capitatus* essential oils at their minimal inhibitory concentration respectively. These findings indicate that some components of these essential oils interfere with the mechanisms of bacterial adhesion to human uroepithelial cells.

Bacterial adhesion presents the initial stage in the pathogenesis of urinary tract infections. Antibiotics were the common strategy to obstract the adherence to the human epithelial cells, however, in the last decades, scientists have immensely investigated the antimicrobial activities of essential oils and their components. Sasso *et al*. (2006)[49] have observed that thymol inhibits the bacterial adhesion to vaginal epithelial cells by a possible interference with *E.coli* fimbriation. The study of Tomičić *et al*. (2022)[50] revealed that the essential oils of *Origanum vulgare*, *Thymus vulgaris* and carvacrol reduced the adhesion of *Candida glabrata* with a range of 30-46%. Several experiments have investigated the anti-adhesive effect of thyme extract on bacteria responsible for intestinal diseases. The extracts showed a strong adhesion inhibition of *Salmonella enteritidis* and *Campylobacter jejuni* on the intestinal epithelial cells.[51, 52] these outcomes lead us to suggest that the essential oils constituents mainly carvacrol and thymol have the aptitude to reduce the bacterial fimbriation of *E.coli*, which blocks the adhesion (the initial step of the urinary tract and other infections).

Table 7: The adhesion reduction effect of the four studied essential oils on *Escherichia coli*

Essential oils	Control	Adherent *E. coli*	Adhesion reduction percentage
Ammoides verticillata	117.87±5.22	24.35±1.85	79%
Origanum glandulosum		18.52±2.86	84%
Thymus fontanesii		20.47±1.13	83%
Thymus capitatus		11.99±1.43	90%

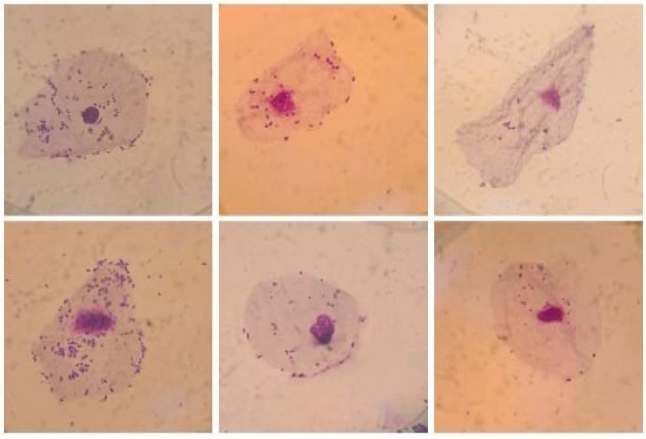

Figure 1: A control slide of *Escherichia coli1* adhesion to an uroepithelial cell (A, B). Reduction of *Escherichia coli1* adhesion to the uroepithelial cell after treatment with *Ammoides verticillata*, *Origanum glandulosum*, *Thymus fontanesii* and *Thymus capitatus* essential oils, respectively (C, D, E and F).

Conclusion

The emergence of antibiotic resistance genes within uropathogenic *Enterobacteriaceae* responsible for community-acquired UTIs has induced an alarming high resistance rates to fluoroquinolones and beta-lactam antibiotics. All the studied essential oils exhibited a high antibacterial potencies against all the test strains (resistant or susceptible to fluoroquinolones), this efficacy is due to the presence of thymol, carvacrol, p-cymene and *γ*–terpinene as major components, revealed that fluoroquinolones and EOs resistance cannot be possibly correlated. The anti-adhesive activity of the natural compounds can be used as a promising strategy to circumvent the begining of UTI. Further research is still required to investigate the possibility of using the isolated main components as oral therapy, this involves assessing their pharmacokinetics, toxicity, side effects and interactions with other medications, which will provide valuable insights into the therapeutic potential and safety profile of these compounds, paving the way for their future clinical use.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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