

Original Article

ANTIBACTERIAL ACTIVITY OF *MENTHA PULEGIUM* ESSENTIAL OIL AGAINST AVIAN ISOLATED ESBL PRODUCING BACTERIA AND ITS SYNERGISTIC POTENTIAL WITH ANTIBIOTICS

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ABSTRACT

Objective: The aim of this study was to evaluate the antibacterial effect of Algerian *Mentha pulegium* essential oil against extended-spectrum β -lactamases (ESBL) producing bacteria isolated from avian livestock. The synergistic interactions between conventional antibiotics and *Mentha pulegium* essential oil were assessed.

Methods: Essential oil composition was determined by gas chromatograph-flame ionization detector (GC/FID), gas chromatograph-mass spectrometer (GC/MS) analysis.

Antibacterial activity of *Mentha pulegium* essential oil against ESBL producing bacteria was investigated by disc diffusion assay. Minimum inhibitory concentration (MIC) of essential oil and its synergistic interaction with conventional antibiotics were determined by micro-broth dilution method and checkerboard test, respectively.

Results: The results indicate that *Mentha pulegium* essential oil with a high amount of pulegone (88.78%) had high inhibitory activity against the tested strains and particularly displays a satisfactory action against the studied ESBL producing bacteria from animal origin, with a diameter ranging from 13 to 26 mm. Out of 51 combinations tested between essential oil and antibiotics 60.78 % showed total synergy, 13.72 % had presented a partial synergy. The best antibacterial activities were obtained with the combination of *Mentha pulegium* essential oil and cefazolin, cefotaxime and gentamycin.

Conclusion: This study allowed concluding that *Mentha pulegium* essential oil showed not only satisfactory antibacterial properties, but also acts synergistically combined with conventional antibiotics, which make it a promising alternative to antimicrobial drugs; beside that, it might reduce the minimum effective dose of the drugs which minimizes their possible side effects.

Keywords: *Mentha pulegium*, Essential oil, Pulegone, ESBL bacteria, Antibacterial, Synergy, Antibiotics

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INTRODUCTION

The extended-spectrum β -lactamases (ESBL) producing bacteria are plasmid mediated enzymes that are able to hydrolyze and inactivate a wide variety of beta-lactams including third generation cephalosporins, penicillins, and aztreonam [1]. Production of extended-spectrum β -lactamases (ESBLs) is one of the major causes of antibiotic resistance in these bacteria [2]. Their emergence in animal livestock is of a major medical and economic importance, indeed, antimicrobial resistance threatens public health and confronts humans at a therapeutic risk impasse, especially for zoonotic bacterial infections, and moreover, it causes several therapeutic failures in livestock which can lead to great economic losses.

The battle against bacterial infections involving antibiotic-resistant microorganisms has thus become a critical concern in the veterinary field and so, the development of alternative strategies becomes mandatory. The exploration of natural resources appears to be more promising, essential oil extracted from medicinal herbs and food plants have been highlighted as natural substances having a rich source of compounds with effective antimicrobial and antibiotic properties.

Mentha pulegium is one of medicinal *Mentha* species with good antimicrobial properties, it is commonly known as pennyroyal. It is native from Europe, North Africa and in minor and near East Asia [3]. In Algeria, where it is an endemic plant, leaves are widely used both as a tea brewing and as flavoring in salads or cooked food. Moreover, it is used in folk medicine in several ways, actually, it is known to be a good digestive tonic, it stimulates digestive juices,

relieves flatulence and colic. It is also a good remedy for headaches and for respiratory infections (cold, sinusitis, bronchitis, tuberculosis) and a powerful stimulant to the uterine muscle encouraging menstruation [4]. It can be used externally to relieve rheumatic conditions including gout [5]. Besides that, pennyroyal leaves, both fresh and dried, are especially noted for repelling insects [6].

Previous studies have reported satisfactory antibacterial activities of *Mentha pulegium* essential oil; actually, it shows a better activity on Gram positive bacteria [7]. Moreover, Teixeira *et al.* (2007) highlighted a good growth inhibition of Gram negative bacteria including *E. coli* [8]. To date, few studies have been conducted on its possible inhibitory effect on multidrug resistant bacteria especially ESBL producing one. Accordingly to this, the purpose of this study was to determine the antibacterial activity of *Mentha pulegium* essential oil against different multidrug resistant bacteria particularly on ESBL producing strains, isolated from chicken livestock, and to assess the antimicrobial possible synergistic effect of the association between classical antibiotics and *Mentha pulegium* essential oil.

MATERIALS AND METHODS

Reagents and chemicals

Muller Hinton agar and Muller Hinton broth were purchased from Conda, Spain, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and tween 80 were purchased from Sigma-Aldrich Chemical USA.

Plant material

Flowering aerial parts of *Mentha pulegium* were collected from Adekar area (N 36.7172164, E 4.6690730, Bejaia-ALGERIA) in summer 2013. The voucher specimen was identified by the Department of Botany of Ecole Nationale Supérieure d'Agronomie (ENSA, El Harrach), and deposited at the Herbarium of Santé et productions animales Laboratory research (ENSV, Algiers) under number 027/13. The samples were dried in the shade away from light at room temperature for 7 to 10 d.

Essential oil extraction procedure, GC/FID and GC/MS analyses

After drying, the samples were ground and hydrodistilled for 3 h using a clevenger-type apparatus. The essential oils were stored in sterile amber bottle at 4 °C until analyses. The *Mentha pulegium* essential oil analysis was performed at *Lexva analytique* laboratory (Saint-Beauzire, France) under the operating conditions described below:

The essential oil of *Mentha pulegium* was analysed on an agilent gas chromatograph-flame ionization detector (GC-FID) model 6890, equipped with an apolar column (DB5 MS: 20 m length 0,18 mm internal diameter 0,18 µm film thickness), programmed from 50 °C (3,2 min) to 300 °C at 10 °C/min. Injector temperature were 280 °C. The essential oil was diluted in acetone in 4% (v/v), and 2 µl was injected in split mode (1/120). Hydrogen was used as a carrier gas (1.1 ml/min) [9].

Mass spectrometry was performed on an agilent gas chromatograph-mass spectrometer (GC/MS) model 7890/5975 C, programmed with the same conditions as for GC-FID (as described above). The essential oil was diluted in acetone in 4% (v/v), and 2 µl was injected in split mode (1/150). Hydrogen was used as a carrier gas (1.1 ml/min). Mass spectral data were acquired in the scan mode in the mass range 33-550.

The percentage was calculated from the peaks area given by the GC/FID without the use of correction factor. The components of the oil were identified by a combined search of retention times (lab library) and mass spectra (Library NIST 225000 records).

Antibacterial activities and synergy test

Bacterial strains

Different bacterial strains from the American type culture collection (ATCC) standard strains (*E. coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 27853), obtained from Institute Pasteur of Algiers, were tested.

Besides that, thirty one multi-resistant veterinary clinical isolates of different bacterial strains were studied (table 1) including 18 ESBL producing bacteria; their resistance profiles have been previously established according to clinical and laboratory standards institute (CLSI) [10].

Disc diffusion assay

The disk diffusion method was employed as a qualitative screening test for the determination of antimicrobial activities of *Mentha pulegium* essential oil, according to the method described by Kirby and Bauer [11].

The bacterial cultures were first grown on nutrient agar plates at 37 °C for 18 to 24 h. Some colonies of each bacterial tested strain were transferred into physiological water and the obtained bacterial suspensions were adjusted to 1×10⁷CFU/ml (1/10, v/v; pattern 0.5 of McFarland standard). Then, it was spread into Muller Hinton agar, using a sterile cotton swab.

Subsequently, sterile disks of 6 mm diameter were placed in the surface of Petri dishes and impregnated with essential oil. Negative control was prepared using water and tween 80 at a concentration of 0.01%. Reference antibiotics were used as a positive control for each tested strain. The Petri dishes were incubated at 37 °C for 24 h. All determinations were performed in triplicate. Antibacterial activity was evaluated by measuring the radius of the inhibition zones.

Minimal inhibitory concentration (MIC), Minimal bactericidal concentration (MBC)

The sensitive bacteria to *Mentha pulegium* essential oil were selected for the micro dilution assay in order to determine the minimal

inhibitory and bactericidal concentrations. Moreover, the time kill assay was performed to establish the dynamic of action of *Mentha pulegium* essential oil against these same bacteria.

The MIC and MBC assays were performed using a serial microplate dilution method as described by Eloff with some variations [12]. Briefly, serial twofold dilutions of *Mentha pulegium* essential oil in Mueller-Hinton broth-tween 80 0.01 % was prepared in 96-well microplate, over the range 18.56 mg/ml to 0.036 mg/ml. 100 µl of an actively growing culture of the tested organism were added to each of the dilutions, the microplate was sealed and incubated 18 h at 37 °C.

After incubation, 40 µl of 0.2 mg/ml of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma Chemicals] solution, was added to each of the wells. Microplate was examined after additional incubation of 30-120 min. Bacterial growth was indicated by the purple color of the MTT reduced to formazan. The lowest concentration at which a decrease in the purple color was observed compared to the next dilution was taken as the MIC value.

The samples showing no bacterial growth were streaked on MHA agar plates, which were incubated for 24 h at 37 °C and finally examined for 99.9% killing. The minimal bactericidal concentration was thus deduced.

Time-kill assay

Time-kill assay was performed according to the method described by Viljoen *et al.* [13]. Activities of essential oils against *Staphylococcus aureus* ATCC 25923, *E. coli* ATCC 25922, ESBL producing bacteria including *E. coli*, *Proteus mirabilis*, *Salmonella pullorum* and *Enterobacter cloacae* strains were evaluated by measuring the reduction in the number of CFU/ml over 2 h.

An initial inoculum of 5 x 10⁶CFU/ml was prepared and 1 ml was added to 9 ml of MHB containing both the essential oils and tween 80 (tests) or tween 80 only (control).

Essential oils were used at a final concentration equal to their MIC and their MBC. Tween 80 was added to both tests and control at a final concentration of 0.01% (v/v). The test tubes were incubated with agitation at 37 °C and samples (100 µl), taken in duplicate at 0, 10, 30, 60, 90 and 120 min, were serially 10-fold diluted, plated onto MHA and the total viable counts were determined after overnight incubation at 37 °C.

Synergistic interaction between *Mentha pulegium* essential oil and antibiotic

Broth micro dilution checkerboard method was used to determine the potential synergistic interactions between *Mentha pulegium* essential oil and antibiotics on 96 well-plate [14, 15]. Nine conventional antibiotics have been used (table 4).

In this experiment, *Staphylococcus aureus* ATCC 25923, *E. coli* ATCC 25922 and 4 ESBL producing bacteria including *E. coli*, *Proteus mirabilis*, *Salmonella pullorum*, and *Enterobacter cloacae* were studied. The tested dilutions were based on the MIC of the two antimicrobial agents which are mixed together so that the wells of the plate could contain various concentration combinations of the two compounds.

Eight serial two-fold dilutions (from MIC to MIC/128) of each antimicrobial agent were prepared.

100 µl aliquots of the first antimicrobial agent dilution were added in a vertical orientation, and 100 µl aliquots of the second antimicrobial agent dilution were added in a horizontal orientation. 100 µl of fresh bacterial suspension (≈10⁶CFU/ml) were added to each well and cultured at 37 °C for 24 h.

The results of the checkerboard assay were used for calculation of the fractional inhibitory concentration (FIC) index for two antimicrobials in combination according to the following formula:

$$\text{FIC Index} = \frac{\text{MIC}_{\text{essential oil combination}}}{\text{MIC}_{\text{essential oil alone}} + \frac{\text{MIC}_{\text{antibiotic in combination}}}{\text{MIC}_{\text{antibiotic alone}}}}$$

RESULTS

Chemical composition

Chemical analysis of *Mentha pulegium* essential oil revealed 26 different compounds accounting for 97.71% of the essential oil composition (table 1). The major constituent of the studied *Mentha pulegium* essential oil is represented by pulegone (88.78%).

Antibacterial activities and synergy test

Disc diffusion assay

The disk diffusion method was employed as a qualitative screening test for the determination of antimicrobial activities of *Mentha pulegium essential oil*, antibacterial activity was evaluated by measuring the radius of the inhibition zones (mm) (table 2).

Table 1: *Mentha pulegium* essential oil composition

Compounds	GC/FID%	Compounds	GC/FID %
Alpha-Pinene	0.235	Neo-menthol	2.083
Camphene	0.016	Trans-dihydrocarvone	1.364
Sabinene	0.054	Pulegone	88.78
Beta-pinene	0.196	8-hydroxy-4(5)-para menthen3 one	0.453
Myrcene	0.073	Piperitenone	0.329
3-Octanol	1.207	Inknown MW	0.087
Para-cymene	0.026	Beta-caryophyllene	0.104
Limonene	0.372	Alpha-humulene	0.180
Eucalyptol	0.068	Germacrene-D	0.020
Trans-Iso-Limonene	0.020	Mintfuranone	0.029
Menth-3-ene8-ol-para	0.236	Oxyde de caryophyllene	0.187
Menthone	0.919	Humulene 1,5epoxyde	0.224
Menthofuran	0.044		
Iso-menthone	0.211		
		Total identified	97.71

Table 2: Diameter of inhibition zones (mm) obtained by the agar diffusion method

Bacterial strain	Antibiotic control IMP	Antibiotic control GTC	Negative control tween	<i>Mentha pulegium</i> essential oil
<i>Staphylococcus aureus</i> ATTC 25923	-	30±1.1	≤6	35±0.7
<i>E. coli</i> ATTC25922	-	25±0.5	≤6	17±1.41
<i>Pseudomonas aeruginosa</i> ATTC 27853	-	-	≤6	7±0
<i>Staphylococcus aureus</i>	-	24±0.4	≤6	16±0.7
<i>Staphylococcus epidermidis</i>	-	19±0.7	≤6	14±0
<i>Klebsiella pneumonia</i>	-	ND	≤6	12±0.7
<i>Salmonella dublin</i> ,	-	18±0.3	≤6	13±0
<i>Salmonella enteridis</i> ,	-	19±0.3	≤6	13±0
<i>Salmonella thyphi</i> ,	-	19±0.4	≤6	10±0
<i>Salmonella infantis</i> ,	-	19±0.3	≤6	12±0.7
<i>Salmonella gallinarum</i>	-	25±0.7	≤6	10±0
<i>Proteus mirabilis</i> (1)	-	ND	≤6	14±0.7
<i>Shigella</i> sp	-	23±0.4	≤6	12±1
<i>Yersinia</i> sp,	-	25±0.6	≤6	11±0.8
<i>Providencia</i> sp,	-	23±0.4	≤6	12±0.7
<i>Enterobacter cloacae</i> (1)	-	24±0.3	≤6	14±1
<i>Salmonella pulorum</i> ESBL	≥30	-	≤6	13±0.12
<i>E. coli</i> ESBL (1)	≥30	-	≤6	15.33±0.7
<i>E. coli</i> ESBL (2)	≥30	-	≤6	12.66±0.7
<i>E. coli</i> ESBL (3)	≥30	-	≤6	14±0.2
<i>E. coli</i> ESBL (4)	≥30	-	≤6	13.66±0.2
<i>E. coli</i> ESBL (5)	≥30	-	≤6	10±0.24
<i>E. coli</i> ESBL (6)	≥30	-	≤6	14.66±0.57
<i>E. coli</i> ESBL (7)	≥30	-	≤6	14.33±1.12
<i>E. coli</i> ESBL (8)	≥30	-	≤6	11.33±0.8
<i>E. coli</i> ESBL (9)	≥30	-	≤6	13±1
<i>E. coli</i> ESBL (10)	≥30	-	≤6	12.66±0.76
<i>E. coli</i> ESBL (11)	≥30	-	≤6	12.66±0.76
<i>E. coli</i> ESBL (12)	≥30	-	≤6	14±1
<i>E. coli</i> ESBL (13)	≥30	-	≤6	16.66±0.23
<i>Proteus mirabilis</i> ESBL (2)	≥30	-	≤6	26.66±0.76
<i>Proteus mirabilis</i> ESBL (3)	≥30	-	≤6	16.66±0.23
<i>Enterobacter cloacae</i> ESBL (2)	≥30	-	≤6	12±0.46
<i>Enterobacter cloacae</i> ESBL (3)	≥30	-	≤6	13.16±0.57

Values are given as mean±standard deviation (n=3), IMP= imipenem/GTC= gentamicin ND= not determined

The obtained results revealed that *Mentha pulegium essential oil* had wide antibacterial spectrum, it inhibited the growth of all tested bacteria with ranging magnitudes (10-35 mm), except for

Pseudomonas aeruginosa which shown a very low sensibility (table 2). No inhibition zone was observed for the negative control (0.01% tween 80).

In which concerns the other tested bacteria, our study has shown for the first time that *Mentha pulegium* essential oil displays a satisfactory action against the studied ESBL producing bacteria (12/18) from animal origin, with a diameter ranging from 13 to 26 mm.

Minimal inhibitory concentration (MIC), Minimal bactericidal concentration (MBC)

The sensitive bacteria to *Mentha pulegium* essential oil were selected to the micro dilution assay in order to determine the minimal inhibitory and bactericidal concentrations. Moreover, the time kill assay was performed to establish the dynamic of action of *Mentha pulegium* essential oil against these same bacteria.

The minimum inhibitory concentrations (MIC) were ranging from 0.14 mg/ml to 2.32 mg/ml (table 3).

MIC values are close to MBC values, so that the MBC/MIC ratios of the different strains are lower than 4 (table 3).

Time kill assay

Preliminary treatment of bacteria with *Mentha pulegium* essential oil used at a concentration equivalent to their MIC has shown a rapid decrease in the bacterial growth rate, the bactericidal end point was obtained after one hour at last, whereas, a total inhibition of growth was noted after 10 min when a concentration equivalent to the MBC were used, these observations are valuable for all tested bacteria including ESBL producing ones.

Synergistic interaction between *Mentha pulegium* essential oil and antibiotic

The results of the checkerboard assay are interpreted by calculating the fractional inhibitory concentration (FIC) index. FIC Index values were interpreted as following: Synergy (FICI \leq 0.5), additive effect (0.5 \leq FICI \leq 1), indifferent or antagonistic effect (1 \leq FICI \geq 2), [16, 17].

The results of synergistic effect between *Mentha pulegium* essential oil and antibiotics are given in table 4.

Table 3: Minimal inhibitory concentrations MIC (mg/ml), minimal bactericidal concentrations MBC (mg/ml) and MBC/MIC ratios of *Mentha pulegium* essential oil

	MIC (mg/ml)	MBC (mg/ml)	MBC/MIC
<i>Staphylococcus aureus</i> ATCC 25923	0.29±0.16	0.58±0.16	2.07
<i>Staphylococcus epidermidis</i>	0.58±0.16	1.16±0.33	2
<i>E. coli</i> ATCC25922	1.16±0.33	2.32±0.66	2
<i>Proteus mirabilis</i> (ESBL) 3	2.32±0.66	4.64±1.33	2
<i>Proteus mirabilis</i> (ESBL) 2	2.32±0.66	2.32±0.66	1
<i>E. coli</i> (ESBL) 3	1.16±0.33	2.32±0.66	2
<i>E. coli</i> (ESBL) 6	1.16±0.66	2.32±0.66	2
<i>E. coli</i> (ESBL) 7	1.16±0.66	2.32±0.66	2
<i>E. coli</i> (ESBL) 1	0.58±0.16	1.16±0.33	2
<i>E. coli</i> (ESBL) 12	1.16±0.66	2.32±0.66	2
<i>E. coli</i> (ESBL) 13	0.58±0.16	1.16±0.33	2
<i>Salmonella dublin</i>	2.32±0.66	2.32±0.66	1
<i>Salmonella enteridis</i>	0.58±0.16	1.16±0.33	2
<i>Salmonella pullorum</i> (ESBL)	2.32±0.66	2.32±0.66	1
<i>Enterobacter cloacae</i> (ESBL) 3	2.32±0.66	2.32±0.66	1

Values are given as mean±standard deviation, (n=3)

Table 4: Synergistic interaction between *Mentha pulegium* essential oil and antibiotics against selected bacteria, FIC^a Index of different combinations of antibiotics and MP^b essential oil

	<i>S. aureus</i> ATCC 25923	<i>E. coli</i> ATCC 25922	<i>E. coli</i> (ESBL1)	<i>P. mirabilis</i> (ESBL2)	<i>S. pullorum</i> (ESBL)	<i>E. cloacae</i> (ESBL3)
MP+amoxicillin	0.56±0.1 ^d	0.5±0.1 ^c	0.5±0.1 ^c	0.31±0.1 ^c	1.5±0.5 ^e	1.25±0.5 ^e
MP+penicillin	ND	ND	ND	0.06±0.01 ^c	1.5±0.5 ^e	0.75±0.15 ^d
MP+cefotaxime	0.5±0.1 ^c	0.093±0.01 ^c	0.13±0.06 ^c	0.5±0.1 ^c	0.25±0.1 ^c	0.093±0.01 ^c
MP+cefazolin	0.18±0.06 ^c	0.07±0.01 ^c	0.07±0.01 ^c	0.25±0.1 ^c	0.5±0.1 ^c	0.325±0.1 ^c
MP+tetracycline	0.18±0.06 ^c	0.5±0.1 ^c	0.37±0.1 ^c	0.25±0.1 ^c	1±0.5 ^d	0.75±0.15 ^d
MP+imipenem	0.18±0.06 ^c	0.5±0.1 ^c	0.187±0.06 ^c	0.06±0.01 ^c	0.625±0.15 ^d	1±0.5 ^d
MP+enrofloxacin	2±0.5 ^e	2±0.5 ^e	2±0.5 ^e	2±0.5 ^e	1.5±0.5 ^e	1±0.5 ^d
MP+marbofloxacin	2±0.5 ^e	2±0.5 ^e	2±0.5 ^e	2±0.5 ^e	1.5±0.5 ^e	0.325±0.1 ^c
MP+gentamycin	0.375±0.1 ^c	0.18±0.06 ^c	0.18±0.06 ^c	0.18±0.06 ^c	0.18±0.06 ^c	0.18±0.06 ^c

ND: not determined, Values are given as mean±standard deviation. (n=3), ^aFractional inhibitory Concentration Index, ^b*Mentha pulegium*, ^ctotal synergy, ^dadditive effect, ^eindifferent or antagonistic

DISCUSSION

Chemical analyses of the studied essential oil showed that it belongs to the pulegone type, it contained mainly oxygenated monoterpenes represented by pulegone with a rate of 88.78%, no study has reported before such high pulegone rate.

Worldwide, several studies on *Mentha pulegium* essential oil composition establish three chemotypes: pulegone type, piperitenone/piperitone type and isomenthone/neoisomenthol type [18].

Previously in Algeria, the compounds of *Mentha pulegium* essential oil have been identified by Beghidja et al., who showed a difference on its constituents depending on the region of cultivation; actually, it appears that Algerian oils can be classified following two

chemotypes: the first is the pulegone type with 52–87% yield of pulegone and some variations of the other constituents and the second chemotype which is poorer in pulegone and relatively rich in the non-oxygenated terpenic fraction [19].

Studies conducted in Bulgaria, Tunisia, Morocco, India, Serbia and Iran, have reported pulegone chemotype but in lower proportions of pulegone compared to the presently studied essential oil [18, 20-25]. The high amount of pulegone can be attributed to the harvest area which is located in high altitude.

It is known that variability in the chemical composition of *Mentha pulegium* essential oil through different studies is probably due to the high chemical variability of extracted oils from *Mentha spp*,

induced by various factors, such as the age of the plant, variety of species, geographic region, soil composition and processing conditions [26, 27]. Moreover, this variability confers to each essential oil different biological effects.

The antibacterial investigation on *Mentha pulegium* essential oil showed that it exhibits a potent antibacterial activity against a wide panel of bacteria including multi-resistant ones except for *Pseudomonas aeruginosa* which is known to be the least sensitive bacteria to essential oils [28, 29]. According to Longbottom *et al.*, its resistance appears to be due to its external membrane structure, particularly impermeable to essential oil molecules and the action of efflux mechanisms, which enhance the protection of the bacteria against the essential oil action [30].

Staphylococcus aureus was the most sensitive to essential oil, it was followed by *Staphylococcus epidermidis* and some other *Enterobacteria* (*E. coli*, *Enterobacter cloacae*, *Proteus mirabilis*, *Salmonella* strains: *S. dublin*, *S. enteritidis*, *S. pullorum*). Concerning the other *Enterobacteria* (*Salmonella gallinarum*, *Salmonella thyphi*, *Salmonella infantis*, *Providencia sp*, *Klebsiella sp*, *Shigella sp*, *Yersinia sp*) we noted a moderate response. Gram positive species are more sensitive to natural products than Gram-negative bacteria, because of the hydrophobic lipopolysaccharide in the outer Gram negative membrane's which provides protection against different agents [31].

Concerning EBSL strains represented by *E. coli*, *P. mirabilis*, *S. pullorum*, *E. cloacae*, our study shows for the first time that *Mentha pulegium* essential oil displays a satisfactory action against the studied ESBL producing bacteria (12/18) from animal origin, with a diameter ranging from 13 to 26 mm.

The reported differences in susceptibility may be due to the differences in the cell wall composition and/or genetic content of their plasmids [32].

Some studies have proved the antibacterial effects of essential oil on human ESBL producing bacteria, actually, it has been described an interesting activity of natural compounds including clove, cinnamon, oregano, green tea against *E. coli* and *Klebsiella pneumonia* ESBL producing bacteria [33-36].

It was reported through this study that *Mentha pulegium* essential oil displays a bactericidal activity [36].

The MIC values obtained in our study are lower than those reported by Teixeira *et al.* [8], who noted respectively a MIC of 3.2 and 3.8 mg/ml for *E. coli* and *S. typhimurium*.

However, it seems difficult to compare these results with ours, because of the difference in essential oil composition of the chemotypes studied and the different strains tested (ESBL producing bacteria from animal origin). Thereby, Mahboubi et Haggi [7] using piperitone chemotype have noted a significant activity against Gram-positive bacteria with inhibition zones ranging from 8–21 mm and no activity against *Escherichia coli* and *Salmonella typhimurium*. Furthermore, they noted the lowest MIC with values of 0.25–4 µl/ml. On the opposite, Teixeira *et al.* and Marzouk *et al.* reported a satisfactory inhibition of *E. coli* [8, 37].

The objective of time kill assay was to characterize the antibacterial kinetic activity of essential oil. The obtained results highlighted a rapid decrease in the bacterial growth rate, indeed the bactericidal end point was obtained after one hour at last, whereas, a total inhibition of growth was noted after 10 min when a concentration equivalent to the MBC were used, these observations mean that *Mentha pulegium* essential oil displays an immediate bactericidal action.

The bactericidal action of *Mentha pulegium* essential oil might be attributed to the high amount of pulegone (88.78%), Duru *et al.*, have previously demonstrated a strong antimicrobial activity of pulegone against a set of bacteria, including *S. typhimurium* and *E. coli* [38].

Moreover, because of the complex composition of essential oils, multiple nonspecific cellular targets might be implicated [39]. In bacteria, the permeabilization of the membrane is associated with loss of ions and reduction of membrane potential, collapse of the proton pump and depletion of the ATP pool. Furthermore, essential

oils can coagulate the cytoplasm and damage lipids and proteins generating thus damage to the cell wall and membrane which lead to the leakage of macromolecules and to bacteria lysis [40].

Out of 51 combinations tested between *Mentha pulegium* essential oil and nine antibiotics (enrofloxacin, gentamicin, amoxicillin, penicillin, cefazolin, cefotaxime, tetracycline, marbofloxacin, imipenem): 31 (60.78 %) showed total synergy, 7 (13.72 %) had partial synergistic interaction and 13 (25.49 %) had no effect. The best antibacterial activities were obtained with the combination of *Mentha pulegium* essential oil and cefazolin, cefotaxime and gentamicin in which FIC index ranged from 0.07 to 0.5 and the total synergy effect obtained with this combination was observed for all studied strains. The combination of *Mentha pulegium* essential oil with amoxicillin, tetracycline and imipenem showed total synergy against all the tested strains, excepting *Salmonella* and *Enterobacter* (FICI ≥ 0.75).

The ESBL producing bacteria are plasmid mediated enzymes that are able to hydrolyze and inactivate a wide variety of betalactams including third generation cephalosporins, penicillins, and aztreonam [41]. Their large emergence since last decades both in animals and humans threatens public health by many serious incurable infections because of their high resistance to conventional antibiotics.

Accordingly to this, the objective of our study was to explore the potential synergistic effect of *Mentha pulegium* essential oil with conventional antibiotics.

The obtained results showed that *Mentha pulegium* essential oil displays a bactericidal effect especially against ESBL producing bacteria and reported potential synergistic effects with conventional antibiotics like amoxicillin, cefazolin, cefotaxime, gentamicin.

We demonstrated for the first time synergy between *Mentha pulegium* essential oil and antibiotics tested on avian multi-resistant clinical isolates.

The synergistic effect of *Mentha pulegium* essential oil combined with amoxicillin, cefazolin and cefotaxime is promising, it can lead to recover these antibiotics efficiency against ESBL producing bacteria.

This synergy might be attributed to the action of the major compounds of the studied essential oil which is mainly represented by pulegone. Oumzil *et al.* have compared the antibacterial action of different monoterpenes (limonene, menthone, carvone and pulegone) known for their high antimicrobial properties and concluded that pulegone displays the most potent biocide activity [42]. However the mechanism of action of pulegone is still poorly known, Cox *et al.* suggested that monoterpenes disrupt the permeability barrier of cell membranes and inhibit respiration [43].

Many mechanisms pathways of essential oil/antibiotics synergism include sequential inhibition of common biochemical pathways, inhibition of protective enzymes, combination of membrane active agents, and use of membranotropic agents, these mechanisms leads to enhance the diffusion of other antimicrobials [44-47]. In addition, recent studies have demonstrated that some plant compounds can effectively inhibit the efflux pumps involved in antibiotic resistance mechanisms [48], which could lead to the restoration of sensitivity to antibiotics and reduce their minimum effective dose and thus their side effects and residues in animal products.

CONCLUSION

This study allowed concluding that the rich pulegone Algerian *Mentha pulegium* essential oil showed satisfactory bactericidal properties, and demonstrated that its association with classical antibiotics presents a real potential of synergistic interactions especially against threatening ESBL producing bacteria.

The use of these combinations is an efficient practice to reduce the minimum effective dose of the drugs reducing thus their possible toxic effects and the treatment cost.

Pulegone rich *Mentha pulegium* essential oil may have a huge potential as food preservative and pharmaceutical additive.

However, further work is necessary to explore the molecular action of pulegone and to understand the cellular mechanisms of their combinations with various antibacterial drugs.

AUTHORS CONTRIBUTION

All the authors have contributed in various degrees to conception and design, and/or acquisition of data, and/or analysis and interpretation of data, and/or writing present article.

CONFLICTS OF INTERESTS

All the author(s): YAHIAOUI Fatima, BENAMEUR Qada and BEN-MAHDI Meriem Hind declare that there is no conflict of interest regarding the publication of this paper.

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