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ESSENTIAL OIL COMPOSITION AND *IN VITRO* ANTIBACTERIAL ACTIVITY OF *CHENOPODIUM ALBUM* SUBSP. *STRIATUM*

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The objective of this study was to identify the bioactive compounds of essential oil and evaluate the antibacterial activity of the essential oil extracted from *Chenopodium album* subsp. *striatum* against multidrug-resistant bacterial strains (MDR) which were isolated from clinical specimens by conventional methods. Furthermore, eight different Gram-negative and Gram-positive multidrug-resistant bacterial strains were used to investigate the antibacterial potential of the essential oil. The antibacterial activity was tested using MIC and MBC microdilution method, well and disc diffusion in different concentration. The hydro-distillation of aerial parts powder yield was 0.466% (v/w). Essential oil showed bactericidal activity against both MDR Gram-negative and Gram-positive bacterial strains. MIC and MBC results were ranged from 0.31 to 2.5 and 0.62 to 5.0 mg/mL. The inhibition zones in well-diffusion method were ranged from 7 ± 0.6 mm to 15 ± 1.0 mm. Disc diffusion method was ranged from 7 ± 0.0 mm to 16 ± 0.6 mm depending on the type of bacteria strain and essential oil concentration. Essential oil of *Ch. album* had the greatest potential to be considered as an antibacterial agent against MDR bacteria strain. This potential was due to different biological and bioactive compounds like phytol, linalool, α-terpineol and linolenic acid in the plant.

Keywords: GC analysis – multidrug-resistant bacterial strains – *Chenopodium album* subsp. *striatum*

INTRODUCTION

In recent years, there is a significant interest in medicinal plants and their metabolites because most of them have several advantages such as efficacy, cultural acceptability, and better compatibility with human body, as well as lesser side effects. One-quarter of all prescribed pharmaceuticals in developed countries contain compounds derived directly or indirectly from plants [15, 26]. Many compounds of plant origin have been used for centuries as remedies for human diseases. Nowadays, there is a broad inter-

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est in plant remedies. Green medicine is reported as efficacy and reliable practice because of secondary metabolites which also have therapeutic properties, and the formulation of plants for standardization and the regulation of phytomedicinal products are the most alternative way recently [1, 25, 42]. On the other hand, infections due to pathogenic microorganism especially bacterial strain can cause serious clinical problems [32, 35]. In recent years, antibiotic resistance has become a serious and widespread problem in developing countries because of inappropriate usage, abusive and over prescription of antibiotics causing mortality each year [29, 39]. Global emergence of resistant bacteria is the result of the ineffectiveness of current antibiotics and drugs causing treatment failure [10]. Hence, there is a growing interest in alternative therapy and therapeutic use of natural products especially medicinal plants [1]. Essential oil of plants may inhibit the growth of broad spectrum of pathogenic microorganisms drawing the attention for their biological and bioactive compounds with antimicrobial activity [3]. The term of "essential oil" has been used in the 16th century for the first time by Paracelsus von Hohenhem. Essential oils are a mixture of natural, volatile and complex constituents which are produced by plant organs especially in aromatic plants considered as secondary metabolites. They were characterized by their strong fragrance, solubility in organic and lipid solvents, colorless, etc. As Bassole stated among the 100,000 known secondary metabolites, essential oils account for over 3000, of which about 300 have commercial purposes and are used by the food, cosmetic and pharmaceutical industries [4, 16, 17, 34]. Essential oils contain two main constituents which responsible for diverse chemical, biochemical and pharmaceutical activity: terpenes (monoterpenes and sesquiterpenes) and terpenoids (isoprenoids), and another group of aliphatic and aromatic compounds (e.g. aldehydes, phenols).

A wide range of plant including *Chenopodium album* subsp. *striatum* is discovered to present therapeutic effects. *Chenopodium album* subsp. *striatum* belonging to the family Chenopodiaceae (goosefoot family) which is one of the largest families of the flowering and annual plants including 104 genus and more than 1400 species [23, 33]. They widely spread worldwide from the moderate and subtropical zone to arid and saline regions. Iran with the surface area of 1,648,000 km^2 has a large area of saline and arid rangelands. The harsh and halophyte ecosystems provide a suitable condition for growing and cultivation of many plant species such as *Ch. album* subsp. *striatum* [11]. *Chenopodium album* is known to be a rich source of flavonoid, glucosides, terpenoids, and phenolic acid. The leaves are rich in carotenoids and the seeds in proteins and fats [24, 30]. Many *Chenopodium* species were reported to have numerous medicinal properties such as antipruritic, antibacterial, antifungal, and anticancer effect [6, 13, 19].

Ch. album subsp. *striatum* has been locally used for its traditional and medicinal properties, however, its efficacies against MDR bacteria have not been studied. Hence, in this study we were intended for retrieving the attention of scientific community on the antibacterial activity of the essential oil and provide to develop new drug from natural products. Their constituents can be presumably considered in the future for more clinical investigations and as adjuvants to current medications.

The present study was designed to determine the role of the essential oil of *Ch. album* subsp. *striatum* for potential antibacterial activity according to the standard protocols by agar-based methods and MIC, MBC tests against some selected MDR Gram-positive and Gram-negative bacteria like *Staphylococcus aureus, Escherichia coli*, *Shigella flexneri*, *Sh. sonnei*, *Sh. dysenteriae*, *Salmonella typhimurium*, *S. enteritidis*, and *S. infanti*s. The aim of this study was to screen the *in vitro* antimicrobial activity of the plant as potential sources of natural antimicrobial agents.

MATERIALS AND METHODS

Plant material by tissue culture

Ch. album subsp. *striatum* was obtained from tissue culture. The basal medium was made up with Murashige and Skoog Salt, vitamins supplements, 3% sucrose without any hormones and also solidified with 0.7% (w/v) plant agar. The medium was adjusted to pH 5.8 before adding plant agar, and then it was sterilized by autoclaving at 121 °C for 20 min [21]. The primary plant sample was identified by Plant Physiology Laboratory and voucher specimens were confirmed and deposited in the Herbarium (No. 2565) at the Department of Pharmacy, Faculty of Pharmacy, Shahid Beheshti University of Medicinal Sciences. Aerial parts of the plant were washed thoroughly 2–3 times with running tap water and then once with distilled water, then they were dried in shady place for a period of 3–4 days at room temperature, and finally they were subsequently ground into a fine powder using grinding mill and kept in air tight amber glass vials.

Essential oil procedure

The shade-dried plant material (500 g) were subjected to hydrodistillation for 4 h using Clevenger-type apparatus. Afterwards, the essential oil was dried over anhydrous sodium sulfate. The hydrodistilled essential oil yield was 0.466% (v/w). Finally, it was preserved at 4 °C in a sealed vial for further analysis, i.e. GC-MS and antimicrobial activity.

GC-MS analysis

GC-MS analysis of essential oil obtained from aerial parts was carried out using an Agilent 7890B/5975C GC-MSD on HP-5MS capillary column (30 m × 0.25 μm, i.d. 0.25 mm) and a 5975C mass selective detector. The 70 eV ionization energy was used for electron ionization. Diluted sample (1 μL, 1/10 v/v in methanol) was manually injected with a split ratio of 1:10. Inert helium gas was used as carrier gas at constant

flow rate of 1 mL/min. The first oven temperature was held at 50 °C for 2 min, then it gradually increased to 290 °C at 5 °C/min rate and held at 290 °C for 10 min. Injector and mass transfer line temperatures were set at 220 °C and 300 °C, respectively. The relative percentage of the essential oil constituents was expressed as the percentage by peak area normalization. Identification of compound was based on the comparison of the retention time and mass spectra with NIST GC-MS library and also those obtained from literature data.

Bacterial preparation

The used Gram-positive and Gram-negative species were *Staphylococcus aureus, Escherichia coli*, *Shigella flexneri*, *Sh. sonnei*, *Sh. dysenteriae*, *Salmonella typhimurium*, *S. enteritidis*, and *S. infantis*. Bacteria species were taken from isolated specimens which exhibited resistance to some antibiotics in hospitalized patients. They were taken based on ethical clearance approval from the ethical committee in hospital. They were cultured over night at 37 °C on nutrient broth for the preparation of cell suspensions. The bacteria cell suspensions were homogenized and adjusted to 0.5 McFarland standards. Commercial antibiotic discs of Ampicillin (10 µg/disc), Amikacin (30 µg/disc), Amoxicillin-clavulanic acid (30 µg/disc), Azithromycin (30 µg/disc), Cefazoline (30 µg/disc), Cefixime (5 µg/disc), Cefotaxime (30 µg/disc), Cefoxitin (30 µg/disc), Cefpiramide (30 µg/disc), Ceftazidime (30 µg/disc), Ceftizoxime (30 µg/disc), Ceftriaxone (30 µg/disc), Cephalothin (30 µg/disc), Chloramphenicol (30 µg/disc), Ciprofloxacin (5 µg/disc), Clindamycin (2 µg/disc), Doxycycline (30 µg/disc), Erythromycin (15 µg/disc) Gentamycin (10 µg/disc), Imipenem (10 µg/disc), Kanamycin (30 µg/disc), Nalidixic acid (30 µg/disc), Norfloxacin (10 µg/disc), Piperacillin (100 µg/disc), Rifampin (5 µg/disc), Streptomycin (10 µg/disc), Tetracyclin (30 µg/disc), Ticarcillin (75 µg/disc), Tobramycin (10 µg/disc) and Trimethoprim-sulfamethoxazole (25 µg/disc) were used for assessment of their activity and sensitivity against the tested bacteria strains. Each antimicrobial assay was performed in triplicate for more accuracy.

Determination of antibacterial activity of essential oil

The hydrodistilled essential oil was first dissolved up to 5% v/v of total essential oil in DMSO (dimethyl sulfoxide) to the final concentration of 10 mg/mL for MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) assay and then they were sterilized through filtration system using 0.45 µm membrane filters. The antibacterial activity was determined by agar well and disc diffusion methods also MIC and MBC assay [13]. The protocol of the study was based on CLSI guidelines.

Agar well-diffusion method

The antimicrobial activity of the essential oil was screened by agar well diffusion method as described by Pérez et al. [28]. Bacterial strains were grown on nutrient agar at 37 °C for 18 h and then they were suspended in LB broth adjusted to a turbidity of 0.5 MacFarland standards [~10^8 Colony Forming Units (CFU)/mL]. Fifty µL inoculum suspension was swabbed uniformly to solidified 25 mL Mueller-Hinton Agar (MHA) for bacteria. Afterwards, the inoculum was allowed to dry for 5 min. Wells with 6 mm of diameter were punched in the agar and finally filled with 50 µL of 35, 40, 45, 50, 55 and 60 mg/mL essential oil solution. The plate was allowed to stand on the bench for 1 h for proper diffusion and then they were incubated at 37 °C for 24 h. The antibacterial activity was evaluated by measuring the inhibition zone diameter observed. The experiments were performed in triplicate.

Agar disc diffusion

The antibacterial activity was also tested by disc diffusion method. The method for growing of bacterial strains as same as the one which was described in well diffusion method but instead of creating wells, we used blank discs with 6 mm of diameter. Each disc was soaked with about 10 µL of 35, 40, 45, 50, 55 and 60 mg/mL of essential oil solution. Six discs were placed on each Petri dish. The plates were incubated at 37 °C for 24 h and finally, the inhibition zones were measured in mm. The test was carried out in triplicate.

Minimum inhibitory concentration

In order to determine MIC, serial twofold dilutions plant essential oil was made in a concentration from 10 mg/mL to 0.005 mg/mL in sterile 96-well plates as described by Clinical and Laboratory Standards Institute (CLSI). These dilutions were added to tubes containing 100 µL Muller Hinton broth and 5 µL of bacterial suspension. Microplates were incubated at 37 °C for 24 h. The lowest concentration of the essential oil in broth medium inhibited the growth of the tested microorganisms was considered as MIC. DMSO was used as a control and Mueller–Hinton broth as a negative control [18, 40].

Minimum bactericidal concentration

To determine the MBC, about 10 µL of broth from tubes, which did not exhibit any visible growth in the MIC assay, were cultured on freshly prepared sterile Muller-Hinton agar, and then they were incubated at 37 °C for 18–24 h. After incubation, the highest dilution (least concentration) inhibited colony formation on solid medium was considered as MBC [27, 41].

Statistical analysis

Measured data were expressed as means ± SEM (n = 3).

RESULTS

Chemical composition of essential oil

GC-MS analysis of the essential oil of *Ch. album* subsp. *striatum* resulted 36 different organic and bioactive compounds which represented 97.09% of the total oil. The result of the GC-MS analysis is listed in Table 1 based on their elution orders. The essential oil contained complex mixture mainly consisted of higher hydrocarbons (71.99%), oxygenated (6.58%) and bicyclic mono-, di- and sesquiterpenoids (6.56%), and also fatty acids (8.51%). Phytol was the most oxygenated diterpene in the oil (3.07%), while linalool (1.94%) and α-terpineol (1.57%) were the most abundant oxygenated monoterpene. Linolenic acid (2.53%) as fatty acid was also found in appreciable proportion in the sample.

Antibacterial activity of essential oil

The antibacterial activity against MDR bacteria obtained by agar well, disc diffusion and microdilution assay (MIC and MBC) are shown in Tables 2, 3 and 4. The results of the present research revealed considerable antibacterial activity against MDR microorganism. The inhibition zones were in the range of 7.0±0.6 mm to 15.0±1.0 mm in well diffusion method and 7.0±0.0 mm to 16.0±0.6 mm in disc diffusion method (Tables 2, 3).

The MIC value ranged from 0.31 mg/mL to 2.5 mg/mL and the MBC value of the essential oil ranged from 0.62 mg/mL to 5 mg/mL (Table 4). The essential oil showed greater inhibitory effect on the growth of *S. typhimurium* and *Sh. dysenteriae*, while it had no antimicrobial effect against *S. enteritidis* by microdilution methods. It is important to mention that discrimination of the endpoint bacteria growth in MIC and MBC methods was based on the determination of OD (optical density measured by Elisa reader), so that OD of the wells has to be near the OD of blank (without bacteria). On the other hand, the solvent (up to 5% DMSO) did not inhibit the growth of the tested bacteria strains.

The results of panel test of *Staphylococcus aureus*, *Escherichia coli*, *Shigella flexneri*, *Sh. sonnei, Sh. dysenteriae, Salmonella typhimurium, S. enteritidis* and *S. infantis* are summarized in Table 5, in points of resistance to antibiotics for *in vitro* antibacterial screening.

Table 1
Chemical composition of *Ch. album* subsp. *striatum* essential oil

No	Chemical compounds	Formula	Kovats Retention Index	Peak area (%)
1	Cyclohexane, methyl-	C_7H_{14}	755	9.55
2	Heptane, 3-methyl-	C_8H_{18}	775	8.61
3	Octane	C_8H_{18}	800	8.32
4	α-Pinene	$C_{10}H_{16}$	933	1.21
5	β-Pinene	$C_{10}H_{16}$	976	0.87
6	Decane	$C_{10}H_{22}$	999	8.11
7	Linalool	$C_{10}H_{18}O$	1098	1.94
8	α-Terpineol	$C_{10}H_{18}O$	1198	1.57
9	Ascaridole	$C_{10}H_{16}O_2$	1237	1.44
10	2(1H)-Naphthalenone, octahydro-8a-methyl-, trans-	$C_{11}H_{18}O_2$	1279	2.66
11	Carvacrol	$C_{10}H_{14}O$	1298	0.82
12	Tetradecane	$C_{14}H_{30}$	1399	7.01
13	β-Caryophyllene	$C_{15}H_{24}$	1467	1.44
14	β-Ionone	$C_{13}H_{20}O$	1494	0.59
15	Isoaromadendrene epoxide	$C_{15}H_{24}O$	1590	1.32
16	Geranyl isovalerate	$C_{15}H_{26}O_2$	1599	1.41
17	4-(6,6-Dimethyl-1-cyclohexen-1-yl)-3-buten-2-one	$C_{12}H_{18}O$	1609	0.91
18	4-Acoren-3-one	$C_{15}H_{24}O$	1649	0.23
19	Tetradecane, 2,6,10-trimethyl-	$C_{17}H_{36}$	1700	8.49
20	Octadecane	$C_{18}H_{38}$	1800	4.11
21	Phytane	$C_{20}H_{42}$	1812	0.78
22	Methyl palmitate	$C_{17}H_{34}O_2$	1908	0.61
23	Dibutyl phthalate	$C_{16}H_{22}O_4$	1922	0.23
24	2-Piperidinone, N-[4-bromo-n-butyl]-	$C_9H_{16}BrNO$	1974	1.32
25	10-Octadecenal	$C_{18}H_{34}O$	1977	3.36
26	Eicosane	$C_{20}H_{42}$	2000	1.78
27	12-Methyl-E,E-2,13-octadecadien-1-ol	$C_{19}H_{36}O$	2052	2.06
28	Oleyl alcohol	$C_{18}H_{36}O$	2060	1.493
29	Methyl oleate	$C_{19}H_{40}$	2085	0.71
30	Phytol	$C_{20}H_{40}O$	2105	3.07
31	Linolenic acid	$C_{18}H_{30}O_2$	2122	2.53
32	Glyceryl linolenate	$C_{21}H_{36}O_4$	2161	0.93
33	Tetrahydro-2H-Pyran, 2-(7-heptadecynyloxy)	$C_{22}H_{40}O_2$	2566	0.63
34	Meadowlactone	$C_{20}H_{38}O_2$	2573	0.83
35	Heptacosane	$C_{27}H_{56}$	2700	4.84
36	Disogenin	$C_{27}H_{42}O_3$	3220	1.31
Total				97.09

Table 2
Antibacterial effect of *Ch. album* subsp. *striatum* essential oil by agar well diffusion in six different concentration (35, 40, 45, 50, 55 and 60 mg/mL, respectively)

Studied bacteria	Inhibition zone (mm)					
	Concentrations (mg/mL)					
	60	55	50	45	40	35
Escherichia coli	12±0.6	12±0.0	10±0.0	8±1.0	–	–
Shigella flexneri	10±0.0	8±0.6	7±0.6	–	–	–
Shigella sonnei	12±0.6	9±0.6	–	–	–	–
Shigella dysenteriae	13±1.0	10±0.0	7±0.6	–	–	–
Salmonella infantis	11±0.6	8±0.6	8±0.0	–	–	–
Salmonella enteritidis	7±0.6	–	–	–	–	–
Salmonella typhimurium	15±1.0	11±0.6	11±1.0	9±0.0	–	–
Staphylococcus aureus	12±0.6	10±0.0	8±0.0	7±1.0	–	–

Table 3
Antibacterial effect of *Ch. album* subsp. *striatum* essential oil by agar disc diffusion in six different concentration (35, 40, 45, 50, 55 and 60 mg/mL, respectively)

	Inhibition zone (mm)					
	Concentrations (mg/mL)					
	60	55	50	45	40	35
Escherichia coli	13±0.6	13±1.0	11±0.6	10±0.0	–	–
Shigella flexneri	12±0.6	11±1.0	9±1.0	9±0.6	8±0.6	–
Shigella sonnei	14±0.0	12±0.6	11±0.6	10±0.6	8±0.0	–
Shigella dysenteriae	14±1.0	11±0.0	9.± 0.6	8±0.0	–	–
Salmonella infantis	12±1.0	11±1.0	9±0.6	8±0.0	8±0.0	–
Salmonella enteritidis	9±0.0	8±0.6	8±0.6	–	–	–
Salmonella typhimurium	16±0.6	14±0.0	12±1.0	10±0.6	8±0.6	–
Staphylococcus aureus	14±1.0	13±1.0	11±0.6	9±0.6	7±0.6	7±0.0

Table 4
MIC and MBC of *Ch. album* subsp. *striatum* essential oil against the tested bacteria

Studied bacteria	MIC	MBC
Escherichia coli	1.25	2.5
Shigella flexneri	2.5	5
Shigella sonnei	1.25	2.5
Shigella dysenteriae	0.62	1.25
Salmonella infantis	2.5	5
Salmonella enteritidis	Nd*	Nd*
Salmonella typhimurium	0.31	0.62
Staphylococcus aureus	1.25	2.5

*Not detected.

Table 5
Panel of test organisms for *in vitro* antibacterial screening

Species	Antibiotic resistance pattern
Staphylococcus aureus	AN, AZM, FOX, CP
Escherichia coli	AZM, CPM, CRO, CAZ, CTX, AM
Shigella flexneri	AMC, NA, CAZ, AM, TIC, CTX, CRO, TE, S, SXT, CF
Shigella sonnei	AMC, AM, TOB, TIC, CTX, CRO, TE, S, SXT
Shigella dysenteriae	AMC, AM, TIC, CTX, CRO, K, GM
Salmonella infantis	AMC, NA, CAZ, AM, TIC, CTX, CRO, CT, PIP, D, TE, S, SXT, CF
Salmonella enteritidis	AMC, NA, AM, PIP, D, TE, SXT
Salmonella typhimurium	AMC, AM, PIP, TE, S, C

AN: Amikacin, AZM: Azithromycin, FOX: Cefoxitin, CPM: Cefpiramide, CP: Ciprofloxacin, CRO: Ceftriaxone, CAZ: Ceftazidime, CTX: Cefotaxime, AM: Ampicillin, AMC: Amoxycillin-clavulanic acid, NA: Nalidixic acid, TIC: Ticarcillin, TE: Tetracycline, S: Streptomycin, SXT: Trimethoprim-sulfamethoxazole, CF: Cephalothin, TOB: Tobramycin, K: Kanamycin, GM: Gentamycin, CT: Ceftizoxime, PIP: Piperacillin, D: Doxycyllin, C: Chloramphenicol

DISCUSSION

Recently, searching for new and effective antibacterial agents has become a very important and serious global concern, considering escalating levels of antibiotic resistance among pathogenic bacteria species which continuously challenges the scientific community [8, 9]. Hence, many studies are directed to discover more organic and natural compounds for this solution [37]. One of the efforts in this research is focused on the use of essential oil extracted from plants, which has been applied for human diseases for a long time because of components having therapeutic value [2]. Essential oils contain diverse classes of bioactive compounds which are responsible, in turn, for various pharmacological properties [14, 21, 40]. A number of reports are available on the antifungal, antiviral, antibacterial, and anti-inflammatory properties of plant extracts, fractions and essential oils, thus therapeutic properties of medicinal plants are well recognized [5, 31].

In our study, a total of 36 compounds that exhibit 97.9% of the oil were identified in *Ch. album* subsp. *striatum* by GC-MS, like hydrocarbons and oxygenated and bicyclic mono-, di- and sesquiterpene hydrocarbons, and fatty acids. Usman et al. [36] reported that α-pinene was the most abundant hydrocarbon monoterpene, but β-pinene (6.2%) and limonene (4.2%) were also found in higher amount in the oil of *Ch. album* [36]. The most abundant oxygenated monoterpene in the oil was pinane-2-ol (9.9%), and α-terpineol (6.2%) and linalyl acetate (2.0%) also occurred in detectable quantities in this earlier report. Our results about some chemical and bioactive of compounds of *Ch. album* subsp. *striatum* essential oil are in accordance with the previous study. The differences in chemical compositions and content could be attrib-

uted to several factors such as the different methods of the extraction of the essential oil, stage of plant growth and lots of other factors [7, 20].

It is imperative that less expensive antibacterial agents should be developed to cure patients, regardless of financial status. So, medicinal plants can be the best option. As we mentioned before, some medicinal plants have been known for their antibacterial properties, but their efficacies against MDR bacteria have not been well-documented in the medicinal literature. Agar well, disc diffusion and also microdilution method were used in the present study as antibacterial tests because it is a quantitative reference method routinely used in clinical laboratories. Compared with agar-based methods, broth microdilution can decrease much labor and time [38]. In this study, high concentration of essential oil showed antibacterial activity against the tested MDR bacteria by all applied methods. The low value of the concentration was not as effective as high-value concentration and they did not show any antibacterial activity. There were exceptions, for example, against *S. aureus* in disc diffusion method, small concentration of essential oil was effective. Generally, it has been surmised that the antibacterial activities of plant extracts and essential oils are due to the presence of various bioactive compounds [12]. *Ch. album* subsp. *striatum* showed significant antagonist activities against MDR Gram-positive and Gram-negative bacteria. Varying agar well and disc diffusion, MIC and MBC values could be attributed to the reinforced defense mechanism acquired by MDR bacteria. The data presented in this study describe the broad-spectrum antimicrobial activity of *Ch. album* subsp. *striatum* essential oil as a novel and cost-effective antibacterial agent against MDR bacteria. In addition, it also provide a basis for reviving investigation on the biopharmaceutical diversity of essential oils. Additional and complementary studies are required concerning phytochemical screening, physiological analysis, isolation, purification and quantification of bioactive components of the plant for its *in vivo* assessment.

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