Evaluation of the Antibacterial Effect of *Salvia Officinalis* Essential Oil and its Synergistic Effect with Meropenem

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Abstract: Warnings by WHO concerning the antibacterial resistance was an encouragement to investigate the antibacterial effects of extracted and purchased *Salvia officinalis* essential oils against *S. aureus*, *L. monocytogenes*, *S. pyogenes*, *E. coli*, *P. aeruginosa*, and *K. pneumonia* by the agar well diffusion method. Besides, the checkerboard method evaluated the potential synergistic antibacterial activity of volatile sage oil and meropenem against multi-drug resistant *E. coli*. Results showed that the purchased oil did not have antimicrobial activity against any of the tested pathogens, while the extracted oil of *S. officinalis* inhibited the growth of all the tested microorganisms except the MDR *K. pneumonia*. The extracted oil showed the highest activity against the *E. coli* with an IZ of 18±0.4 mm and MIC of 6.25±0.2 mg/ml, and the lowest activity was recorded against *P. aeruginosa* with an IZ of 10±0.2 mm and MIC of 25 mg/ml. In addition, SoEO showed a promising synergistic effect with meropenem against MDR *E. coli*. Results suggest that the EOs of *S. officinalis* possess antimicrobial activity, and therefore, they can be used to treat infections caused by some pathogens.

Keywords: *Salvia officinalis*; meropenem; synergistic effect; *Escherichia Coli*; plant extracted oil; antibacterial activity; essential oil.

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1. Introduction

*Salvia officinalis*, sage, or locally called “merameya”. The name is derived from the Latin term “Salvia” which means to cure [1,2]. It is a perennial plant that originates from the family *Lamiaceae* or mint, a large family containing more than 900 species [3,4]. The *Lamiaceae* plant family is one of the largest families among the dicotyledons. Many species belonging to the family are highly aromatic due to the presence of external glandular structures that produce volatile oil [5]. People were using marameya as a tea to benefit from its extract. It grows in the Mediterranean regions and is one of the most popular plants in Arabian countries. Biologically it has many effects such as antiseptic [6], antibacterial [7-9], antioxidant [10,11], astringent, anti-inflammatory [12,13], antiviral [14], antimutagenic [15,16], cytotoxic [17,18], spasmolytic, antifungal [19], anticonvulsant [20,21] and antimycobacterial [22]. Traditionally it was commonly used in folk medicine [23]. Anciently, it was thought that it helps treat
circulation disturbances, bronchitis, diabetes, cough, asthma, angina, mouth and throat inflammation, depression, skin diseases, and many other diseases [24,25]. In addition, it was claimed that its essential oil is useful in treating excessive sweating [26] and nervous system diseases, heart and blood circulation problems, respiratory system diseases, digestive system diseases, metabolic and endocrine disorders, and sexual debility [27]. Recently, studies have shown that sage oil is rich in biologically active, mainly phenolic compounds [28]. The principal components in the sage oils were 1,8-cineole, camphor, α-thujene, β-thujone, borneol, and Viridiflorol [29-32]. It is worth mentioning that essential oil contents differ in composition depending on the time of collection and area of planting [33,34]. With the increased danger of antibiotic resistance and the emergence of new resistance mechanisms, the need for developing new antibacterial agents has increased dramatically. Moreover, the World Health Organisation (WHO) has raised the threat regarding antibiotic resistance, which encouraged researchers to dig deeper in natural resources as synthetic agents have proven to be risky and sometimes lethal with their adverse effects over the years. Natural resources can be a game-changer in the medical field when fighting antibiotic resistance due to their lipophilic nature and different mechanisms.

2. Materials and Methods

2.1. Materials.

500g of Pakistani *S. officinalis* leaves and 250ml of pre-extracted oil were purchased from a local store at Al-Bab, Bahrain. Extraction of *S. officinalis* essential oil (SoEO) was performed using Clevenger type apparatus, and distilled water was used as a solvent. Component analysis was determined by gas chromatography from Merck company. Six different bacterial strains and antibiotics were used throughout the current work. The microbiology research lab kindly provided these isolates at the Faculty of Science, Alexandria University, Egypt. The media used throughout the present work are described below. All the media’s composition is given in g/l unless otherwise indicated. These culture media were prepared according to the manufacturer’s guidelines (Oxoid Ltd., UK) with distilled water and sterilized by autoclaving for 20 min at a pressure of 15 lb/inch² to raise the temperature to 121°C. The nutrient broth was used for inoculum preparation and standardization and had the following composition: beef extract, 3 and peptone, 5. While, Müller-Hinton agar was used for testing the antimicrobial activity and had the following composition: dehydrated beef infusion, 300ml; casein hydrolysate, 17.5; soluble starch, 1.5 and agar, 17. Müller-Hinton broth was used for testing the minimum inhibitory concentration (MIC) and had the following composition: acid hydrolysate of casein, 17.5; beef extract, 2 and starch, 1.5. Chemicals used in this work like dimethyl sulfoxide (DMSO) were obtained from Sigma Chemicals, GlaxoSmithKline, and AstraZeneca UK.

2.2. Methodology.

2.2.1. Plant material.

The Pakistani *Salvia officinalis* leaves and the oil bottle were purchased from a local store at bab al-Bahrain on July 3rd, 2019. Specimen were identified at the Faculty of Science, Alexandria University, Egypt.

2.2.2. Essential oil extraction.
Salvia officinalis essential oil was extracted using Clevenger type apparatus through steam hydro-distillation technique for 2.2 hrs. Extracted oil and the purchased oil were then dehydrated using anhydrous sodium sulfate (Na₂SO₄).

2.2.3. Gas chromatography-mass spectrometry (GC-MS) analysis of essential oil.

Both extracted oil and the purchased oil were subjected to GC-MS analysis. GC-MS analysis was evaluated using a capillary column CBP5 (30 m x 0.25 mm x 0.25 μm) installed in a Hewlett-Packard 5890 Series II instrument interfaced with an HP-5971 mass selective detector operated in scanning mode (m/z 40-400). GC-MS analysis was previously programmed from 50°C (0.3 min hold) to 285°C (15 min hold) at a rate of 6°C/min. Samples were injected with an auto-injector using a splitless injection technique (0.6 μl injection volume), and the carrier gas Helium (He) flow was set at 1.0 ml/min. The identification was accomplished using computer search user-generated reference libraries (Wiley 138 and Nist 98 libraries), incorporating mass spectra. Peaks were examined by single-ion chromatographic reconstruction to confirm their homogeneity. In some cases, when identical spectra have not been found, only the structural type of the corresponding component was proposed based on its mass spectral fragmentation [35].

2.3. Antibacterial screening.

2.3.1. Microorganisms and growth conditions.

Bacterial strains were obtained from the microbiology lab at the Faculty of Pharmacy, Alexandria University, Egypt, as standard bacteria like Escherichia coli ATCC 25922, Staphylococcus aureus ATCC 6538, Pseudomonas aeruginosa ATCC 2785, Listeria monocytogenes, Streptococcus pyogenes, and MDR clinical isolate Klebsiella pneumonia, and E.coli.

2.3.2. Agar diffusion method.

The antibacterial activity of the extracted SoEO, the purchased oil, and the ceftriaxone against the indicator strains was assessed in vitro using the agar well diffusion method [31] with some modifications. Briefly, all the indicator strains (E. coli ATCC 25922, S. aureus ATCC 6538, P. aeruginosa ATCC 2785, L. monocytogenes, S. pyogenes, and MDR K. pneumonia) were grown in a nutrient broth overnight to attain the colony-forming unit (CFU) of ~10⁶/ml. Aliquots of the solution of the tested compounds in DMSO (100 µl) were placed in 6 mm diameter wells that were cut in Müller-Hinton semi-solid agar plates previously seeded with the bacterial indicator pathogens. The plates were incubated at 37 °C for 24 h. After incubation, the diameters of the resultant inhibition zone (IZ) were accurately measured. The experiments were run in triplicate, and the mean value of the inhibition zone was determined.

2.3.3. Determination of minimum inhibitory concentration.

The minimum inhibitory concentration (MIC) values of the extracted SoEO, ceftriaxone, and meropenem against the indicator strains (E. coli ATCC 25922, S. aureus ATCC 6538, P. aeruginosa ATCC 2785, L. monocytogenes, S. pyogenes, and MDR K. pneumonia) were determined according to the Clinical and Laboratory Standards Institute (CLSI) with minor modifications. [36] An overnight culture at 37 °C in Müller-Hinton broth
medium of the tested indicator strains was adjusted to the logarithmic-phase growth, yielding approximately 10^6 CFU/ml. Using the indicator strains, the broth dilution method was carried out in 96-well microtitre plates. A stock solution of the SoEO was prepared aseptically in DMSO (80, 20%, v/v), ceftriaxone, and meropenem and then transferred to sterile 96-well microtitre plates containing the Müller-Hinton broth followed by a diluted by two-fold serial dilution. Ten microliters of each strain inoculum were added to each well. The final concentrations of the SoEO in wells were 6.25, 12.5, 25, 50 mg/ml of the oil. After that, all samples were incubated at 37 °C for 24 h then the MIC values were obtained. Tubes not containing the extracted oil were used as a negative control. The MIC values were determined visually as the minimum concentration that led to bacterial growth inhibition. The experiments were performed in triplicate [37].

2.3.4. Dilution checkerboard method.

The broth microdilution checkerboard method was used to evaluate the interaction between the extracted SoEO and the meropenem (Sigma Aldrich, USA) against MDR E. coli.

The MIC values of the extracted SoEO and the meropenem against the MDR E.coli were briefly measured using the broth microdilution (BMD) method. The synergism between different combinations was evaluated according to the fractional inhibitory concentration index (FICI), which was calculated as the sum of the FICs of stock solutions of both the SoEO dissolved in DMSO the meropenem. The FICs of each substance was calculated as follows: FIC of a substance = MIC of the substance in combination when it is used with the other agent /MIC of individual substance alone. The FICIs were interpreted as follows: synergy, FICI ≤0.5; additivity, FICI >0.5 to ≤1; indifference, FICI >1 to ≤4 and antagonism, FICI >4 [38].

3. Results and Discussion

3.1. Gas chromatography analysis.

Samples of both the extracted SoEO and purchased oil were subjected to GC-MS analysis to identify their chemical composition. The percentages of components identified in both samples are listed and compared (Table 1). High EO quantities were detected in the extracted SoEO. The major chemical composition found in the Extracted SoEO were camphor (23.12%), α-thujone (19.66%), 1,8-cineole (15.16%), and borneol (8.6%). These essential oils were also detected in the purchased oil but in relatively low quantities. The major components were camphor (8.18%), α-thujone (2.24 %), borneol (2.2%) and viridiflorol (1.84%). Other important components were also detected in both samples, which include β-thujone, manoyl-oxide, humulene, caryophyllene, γ-selinene, limonene, borneol, α-terpinyl acetate, α-terpineol, caryophyllene, caryophyllene oxide, humulene epoxide.

Identifying the chemical composition of S. officials is important in understanding the various activities the essential oil possesses. Changes in the phytochemicals can affect the activities drastically. Therefore, in this study, samples of both the Extracted SoEO and Purchased SoEO were subjected to GC-MS analysis to identify and compare their components. In this study, the extracted SoEO showed remarkable quantities of the important aromatic essential oils. The dominant fraction was the oxygenated monoterpenes fraction; the main compounds in this fraction were camphor (23.12%), α-thujone (19.66%), 1,8-cineole (15.16%), and borneol (8.6%). Some sesquiterpenes were also identified; those include β-caryophyllene.
(2.90 %) and viridiflorol (6.89 %). The most commonly reported constituent in the literature which tested positive for antibacterial activity were camphor, α-thujone, β-thujone, and 1,8-cineole [39,40]. These findings supported our results but with minor variations in constituent concentrations. Moreover, the GC-MS analysis illustrated very low quantities of the main SoEO constituents compared to the extracted oil. The major constituents were Camphor (8.18%), α-thujone (2.24 %), Borneol (2.2%) and Viridiflorol (1.84%). Thus, it can be deduced that the purchased SoEO was adulterated.

Table 1. Components percentages of both purchased and extracted SoEO.

| Component       | Extracted SoEO | Purchased SoEO |
|-----------------|----------------|----------------|
| Camphor         | 23.12 %        | 8.18 %         |
| α-thujone       | 19.66 %        | 2.24 %         |
| 1,8-cineole     | 15.16 %        | 1.14 %         |
| Viridiflorol    | 6.89 %         | 1.84 %         |
| β-thujone       | 5.16 %         | 0.94 %         |
| β-caryophyllene | 2.90 %         | 0.0 %          |
| Borneol         | 8.6%           | 2.2%           |

3.2. Antibacterial activity.

The *S. officinalis* extracted oil and the purchased oil were screened against six bacterial strains (E. coli ATCC 25922, S. aureus ATCC 6538, P. aeruginosa ATCC 2785, L. monocytogenes, S. pyogenes, and MDR K. pneumonia). It is worth mentioning that the purchased oil did not show any antimicrobial activity against the tested microorganisms, and this fact was supported by the GC-MS analysis that revealed the absence of the major constituents related to SoEO. Moreover, the extracted oil showed antimicrobial activity against all the tested microorganisms except the clinical isolated MDR K. pneumoniae (Table 2). The results showed that the extracted oil of *S. officinalis* had the highest activity on *E. coli* with a MIC of 6.25 mg\/mL and an IZ of 18 mm. However, *P. aeruginosa* was the least inhibited with a MIC of 25 mg\/mL and an IZ of 10 mm. Moreover, *S. aureus*, *L. monocytogenes*, and *S. pyogenes* also demonstrated susceptibility to SoEO. The activity was determined and compared to the reference standard of ceftriaxone.

Table 2. Antibacterial activity of extracted oil and reference standard ceftriaxone and meropenem.

| Strain           | IZ parameter (mm) | MIC |
|------------------|-------------------|-----|
|                  | SoEO Sol.(mg/mL)  | Ceftriaxone | SoEO Sol. (mg/mL) | Ceftriaxone (µg/mL) | Meropenem (µg/mL) |
| S. aureus        | 15±0.5            | 38±0.2 | 12.5 | 0.06 | 0.06 |
| E. coli          | 18±0.4            | 42±0.8 | 6.25± 0.2 | 0.06 | 0.06 |
| P. aeruginosa    | 10±0.2            | 36±0.4 | 25 | 0.5 | 0.04 |
| L. monocytogenes | 16±0.8            | 48±0.6 | 12.5 | 0.12 | 0.08 |
| S. pyogenes      | 14±0.4            | 42±0.2 | 25 | 0.06 | 0.04 |
| K. pneumoniae    | NA                | NA     | NA | NA | NA |

Results showed that the highest antibacterial activities exhibited by the extracted SoEO were observed against the standard *E. coli* and *L. monocytogenes* strains, respectively. The IZ of the extracted SoEO against the *E. coli* strain was observed to be 18±0.4 mm compared to the ceftriaxone, which had an IZ of 42±0.8 mm. The MIC of *S. officinalis* against *E. coli* was 6.25± 0.2 mg\/mL, the lowest concentration between all strains, whereas the MIC of ceftriaxone against *E. coli* was only 0.06 µg\/mL. At the same time, the lowest antibacterial activity was observed against *P. aeruginosa* with an IZ of 10±0.2 mm compared to the ceftriaxone with an IZ of 36±0.4 mm. The MIC of *S. officinalis* against *P. aeruginosa* was 25 µg\/mL, the highest concentration between all strains, whereas the MIC of ceftriaxone against *P. aeruginosa* was
0.5 µg/mL. In a study that was conducted in Syria [39], there results revealed that the extracted SoEO was more effective against the Gram-positive bacteria (S. aureus and Streptococcus group D) than the Gram-negative bacteria (E. coli, S. typhi, and P. aeruginosa). E. coli and S. typhi resumed their optimum growth after 24 h, even when the highest concentration of S. officinalis essential oil was used [39]. Moreover, P. aeruginosa was proven to be a resistant bacteria to SoEO; the number of the inhibited bacteria after 10 min and 1 h of contact at any essential oil concentration was limited, which indicates the limitations of SoEO activity against P. aeruginosa [39].

In another study that was performed in Tunisia, they tested the antibacterial effect of SoEO that was dissolved in ethanol/water (v/v) against Gram-positive bacteria (Bacillus subtilis, Bacillus cereus ATCC 14579, Staphylococcus aureus ATCC 25923, Micrococcus luteus ATCC 1880) and Gram-negative bacteria (Salmonella enterica serotype Enteritidis, E. coli ATCC 25922, Agrobacterium tumefaciens) [40]. They found that both Gram-positive and Gram-negative bacteria were inhibited by the SoEO [40]. Their results can be attributed to the antibacterial activity of the used solvent (ethanol). However, in the current study, the SoEO was dissolved in DMSO, which did not show any antibacterial activity against the used microorganisms.

Differences in results between various studies can be attributed to different factors, such as the difference in concentrations and percentages of the components, which in turn depends on several factors, including the stage of development, cultivation season, and extraction methods. Besides, the difference in the extraction procedure causes qualitative and quantitative differences in the essential oil composition.

3.3 Synergistic effect.

The checkerboard method was used to determine the FIC index value of both the SoEO and meropenem against MDR E. coli. SoEO and meropenem were combined, and the synergy of their combination was recorded. This study showed that the FICI value was less than 0.5 (table 3).

Previous studies have confirmed the synergistic effect of S. officinalis extracts when combined with antibiotics [41]. The resulting antibacterial abilities of S. officinalis against different drug-resistant bacteria can be attributed to their high content of phenolic and terpene compounds, increasing bacterial membrane permeability. Increased levels of antibiotics inside the bacterial cell would result [42].

Table 3. The minimum inhibitory concentration of SoEO, meropenem, and their combination in checkerboard test.

|          | MIC (mg/ml) | FIC   | FICI  | Interpretation |
|----------|-------------|-------|-------|---------------|
| SoEO     | Single drug | Combined drug | 0.25  | 0.375 | Synergy       |
| Meropenem| 320         | 40    | 0.125 |       |               |

4. Conclusions

In conclusion, by using gas chromatography-mass spectrometry, it was found that the major components of Pakistani Salvia officinalis essential oil are: Camphor, α-thujone, 1,8-cineole, and Borneol respectively. Other important components include β-thujone, manoyl oxide, humulene, caryophyllene, limonene, α-terpineol, and others. These components are found in the extracted oil in a higher percentage and concentration than the purchased oil,
proving the adulteration of the ready-made (trade) oil, which shows only trace quantities of these components. Extracted *Salvia officinalis* essential oil has demonstrated an efficient antibacterial activity against the studied microorganisms (*E. coli* ATCC 25922, *S. aureus* ATCC 6538, *P. aeruginosa* ATCC 2785, *L. monocytogenes*, and *S. pyogenes*), with the highest antimicrobial activity against *E. coli* ATCC 25922 and the lowest activity was observed against *Pseudomonas aeruginosa* ATCC 2785, unlike the readily purchased oil that did not show any activity against any of the tested microorganisms. It also showed a synergetic antibacterial activity when combined with meropenem against MDR clinical isolate *E. coli* applying the checkerboard method. *Salvia officinalis* essential oil exhibits an effective antibacterial activity. Thus, it is a promising agent in fighting bacterial infections in the future. It can be used alone as a single agent or in combination with other antibiotics, which leads to the production of more effective antibiotics at lower costs and fewer side effects than the production of novel synthetic agents that are more expensive and may possess more serious side effects. More future studies can be done to evaluate the antimicrobial effects of SoEOs on wider varieties of microorganisms, besides assessing their synergistic activity with more commercial antibacterial agents other than the one stated in this study to produce more efficient therapeutic agents.

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**Conflicts of Interest**

The authors declare no conflict of interest.

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