Evidence-Based Complementary and Alternative Medicine

Antimicrobial Properties of Essential Oils

Lead Guest Editor: Mozaniel de Oliveira
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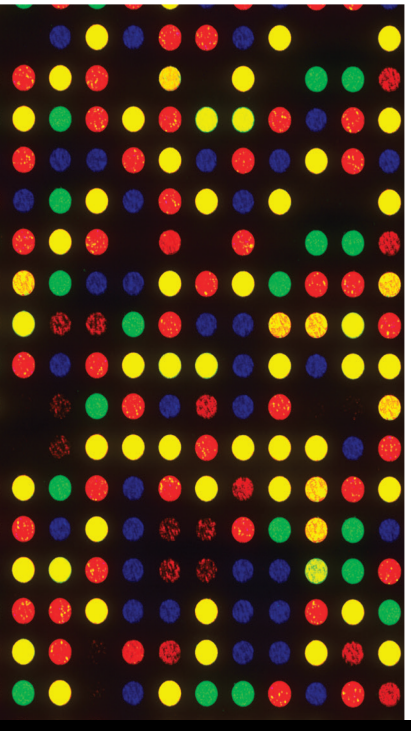

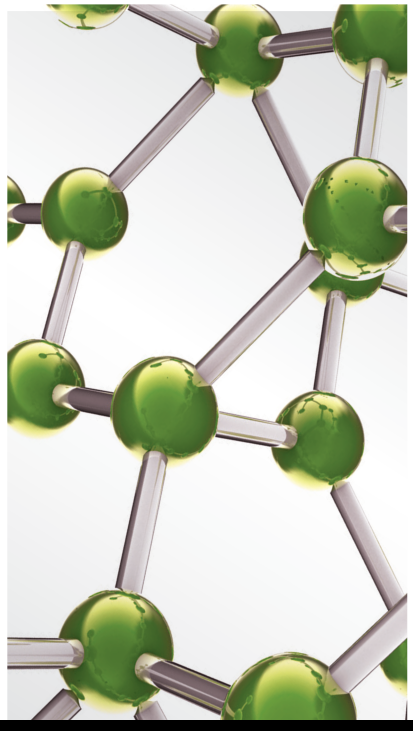


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Research Article

In Vitro Antibacterial Experiments of Qixingjian Decoction and Its Synergistic Interaction with Oxacillin against Clinical Isolates of Methicillin-Resistant *Staphylococcus aureus*

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Background. With the widespread use and abuse of antimicrobial drugs, the problem of bacterial resistance is becoming increasingly prominent. The clinical detection rate of drug-resistant bacteria is increasing year by year, so there is an urgent need to develop new antimicrobial drugs. Qixingjian Decoction (QXJT) is a formula commonly used in Chinese medicine for the treatment of sepsis caused by acute purulent infections of the face, hands, and feet. There are many compounds with antimicrobial effects that are available, but little is known about their mode of action. In this study, we mainly evaluated the antimicrobial activity of QXJT and explored its synergistic interaction with oxacillin (OX) and the mechanism of its antimicrobial activity. *Methods*. The antimicrobial activity of QXJT against methicillin-resistant *Staphylococcus aureus* (MRSA) was determined by the microdilution method, the broth macrodilution method, and the time-kill curve method. The main compounds in QXJT were analyzed by ultra-performance liquid chromatography. The synergistic interaction of QXJT and oxacillin (OX) was determined by checkerboard assay, and the antimicrobial mechanism of QXJT, OX, and QXJT + OX was evaluated by transmission electron microscopy (TEM) technique. The expression of MRSA superantigen virulence factors (sea, seb, and tst), and drug resistance gene (mecA) was detected to provide a new strategy for new antibiotic drugs. *Results*. QXJT exhibited antimicrobial activity against both clinical isolates of MRSA, MICs ranging from 18.75 to 37.5 mg/mL. Active substances such as *Scutellarein, Scutellarin, Apigenin*, and *Wogonin 7-O-glucuronide* were detected in the phytochemical analysis that may be associated with the antimicrobial activity of QXJT. The synergistic effect of QXJT and OX was determined by checkerboard assay (FICI = 0.5), and TEM images showed that QXJT could cause the disruption of MRSA cell wall, and QXJT + OX could produce greater disruption of MRSA cell wall, elucidating the synergistic effect of the two together on cell wall disruption by microscopic mechanisms. Our study shows that the combination of QXJT and OX can inhibit the expression of MRSA virulence factor, reduce the virulence of MRSA, and have no significant effect on the expression of MRSA resistance gene mecA. *Conclusion*. The results of this study provide scientific experimental data for the traditional application of QXJT and initially explore the mechanism of action of QXJT combined with OX.

1. Introduction

Since ancient times, nature plants have been used in medicine and are still used today [1]. Nature plants have been the most important source of antibiotic lead compounds and they also can be used to respond to the growing antibiotic resistance crisis [2]. The major strength of nature plants lies in their various antibacterial modes of action and the proven clinical effectiveness of plant extracts from which they are isolated [3].

Traditional Chinese medicine (TCM) has been practiced for thousands of years, and many of the herbal drugs and decoctions, particularly classical TCM decoctions, are still being used in modern Chinese medicine [4]. Qixingjian decoction (QXJT) has been clinically used in traditional Chinese medicine for more than 700 years [5, 6]. It was first

recorded in 1617 in The Authentic Book of Surgery and was used for treating sepsis caused by acute infections.

Staphylococcus aureus has emerged as a leading etiologic agent of sepsis, owing to its propensity to cause deep-seated tissue infection and bacteremia [7]. The advent of antibiotics reduced *S. aureus* bacteremia mortality from 80% to a still unacceptable 15–50% [8]. In 1961, shortly after the introduction of methicillin for treating infections of *S. aureus*, Jevons et al. [9] identified drug-resistant *S. aureus* strained in the United Kingdom and named them MRSA.

Since 1990, the widespread of MRSA in hospitals and communities and the increasing resistance to commonly used antibiotics have made MRSA a severe threat to public health worldwide [10]. Thus, novel antimicrobials and/or new approaches to combat these problems are urgently needed [11]. The use of combination therapy can broaden the spectrum of antimicrobial activity, minimize the emergence of resistant microbial variants, and sometimes result in synergistic interaction, thereby exhibiting antimicrobial activity greater than would be expected from each antimicrobial drug individually [7]. We made a lot of efforts in the preexperiment, combining QXJT with more than 10 antibiotics with different mechanisms of action, and found that QXJT and OX was the most ideal combination. This study also tried to explore the synergistic mechanism of QXJT + OX.

The diversity and severity of MRSA diseases are partly due to the pathogen's ability to regulate the expression of multiple virulence factors [12]. The development of anti-virulence strategies that interfere with bacterial toxins or virulence factors is currently attracting the attention of many researchers in various fields [13]. This study explored this perspective and may provide new strategies for exploring new antibiotic drugs.

2. Materials and Methods

2.1. Drugs. QXJT was purchased from Beijing TRT Group (Beijing, China) and identified by Professor Wang Chengxiang of Beijing University of Chinese Medicine. QXJT consists of *Wild Chrysanthemum* (9 g), *Fructus Xanthii* (9 g), *Herba Siegesbeckiae* (9 g), *Scutellaria Barbata* (9 g), *Herba Violae* (9 g), *Ephedra* (3 g), and *Paris polyphylla* (6 g). The above herbs were refluxed and extracted with a 10-fold quantity of deionized water for 1 h in a 1 L electric heating jacket and then filtered through 8 layers of gauze. The drug residue was further refluxed and extracted with an 8-fold quantity of deionized water for 1 h and then filtered through 8 layers of gauze. The two filtrates were combined and concentrated to 300 mg/mL by rotary evaporator and stored in a refrigerator at −60°C. Before the in vitro experiment, the product was sterilized in an autoclave at 0.1 MPa and 120°C for 15 min. The quality of the herbs was consistent with the standards of Chinese Pharmacopoeia (2020). The chemical fingerprint of QXJT (ultraperformance liquid chromatography-Q Extract hybrid quadrupole orbitrap high-resolution accurate mass spectrometry [UHPLC-Q-orbitrap HRMS]) was analyzed (Figure 1) (Table 1), and the detailed method of UHPLC-Q-orbitrap HRMS is provided in the supplemental data.

Oxacillin (product name: Oxacillin sodium capsule, serial number: 210503, Sichuan Pharmaceutical Preparation Co., LTD.) was purchased from the Third Affiliated Hospital of BUCM and configured to a concentration of 64 mg/ml using phosphate-buffered saline (PBS) as a positive drug control. Before the experiment, the positive drug was used as 0.22 μl membrane for bacteria removal.

2.2. Bacterial Strains and Culture Conditions. The tested MRSA strains (2007118 and 2008043) were kindly provided by Prof. Lin Ying isolated from clinical patients (Dongzhimen Hospital, Beijing University of Chinese Medicine, Beijing, China). All bacterial strains were cultured according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [14].

2.3. Growth Curve of MRSA by Microplate Reader Measure. The growth curve of MRSA was performed according to Xu et al. [15] with some modifications. The optimum detection wavelength of MRSA absorbance was selected at 630 nm. 50 ml sterile centrifuge tubes were taken; we added 1 ml of bacterial solution (bacterial solution concentration 5×10^5 CFU/mL) and incubated it for 24 h at 37°C and 200 r/min in a shaker. The bacterial growth was monitored every two hours until 24 h. The OD 630 nm value was analyzed by statistical software. Then growth curve of MRSA was plotted to determine the optimal culture time in an in vitro bacteriostatic test.

2.4. Evaluation of Minimum Inhibitory Concentrations (MICs). For the determination of MICs, the broth macrodilution method and the broth microdilution method were approved by Clinical and Laboratory Standard Institute (CLSI) with some modifications [14]. According to the preexperiment results, the wells around the 96-well plate were left empty because evaporation would affect optical density (OD) value, and the control (QXJT) group was added to exclude the effect of the excessive dark color of QXJT. QXJT was diluted with twofold serial dilutions to a final concentration of 2.34–75 mg/mL. Oxacillin (OX) was diluted with twofold serial dilutions to a final concentration of 0.125–8 mg/mL. Bacteria were collected, washed, and diluted with phosphate-buffered saline (PBS) to 5×10^5 CFU/mL. MIC analysis was performed in microtitration plates with 100 μl QXJT or OX, 10 μl bacterial suspension, and 100 μl Mueller–Hinton broth followed by incubation at 37°C for 24 h (Figure 2(a)). Each experiment was performed three times. MIC was defined as the lowest concentration, which prevented visible (naked eye) bacterial growth.

For broth macrodilution method test, QXJT was diluted with twofold serial dilutions to a final concentration of 2.34–75 mg/mL. Tubes were inoculated with the test microbes. Tubes were incubated using a shaker incubator at 37°C for 24 h.

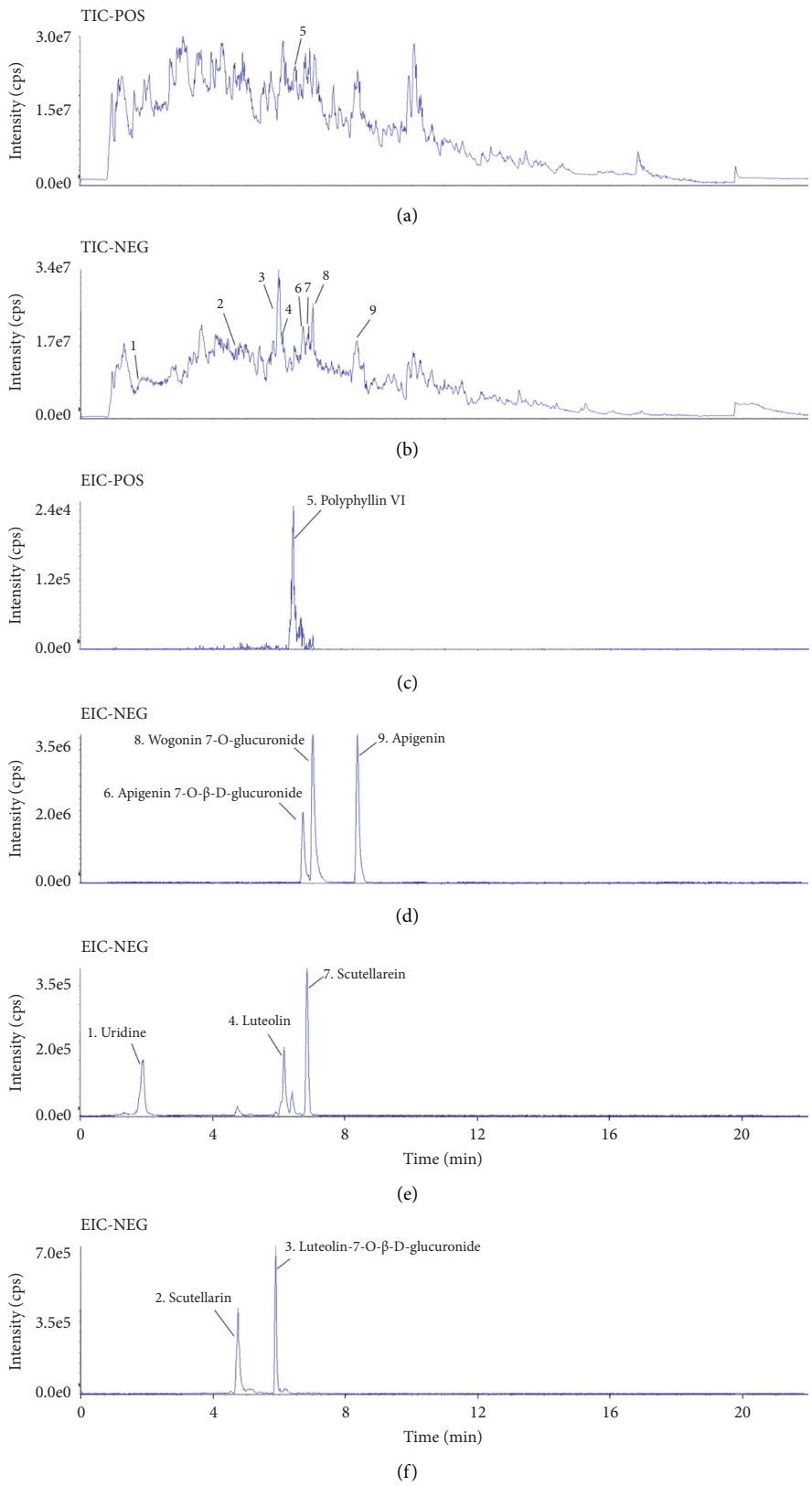
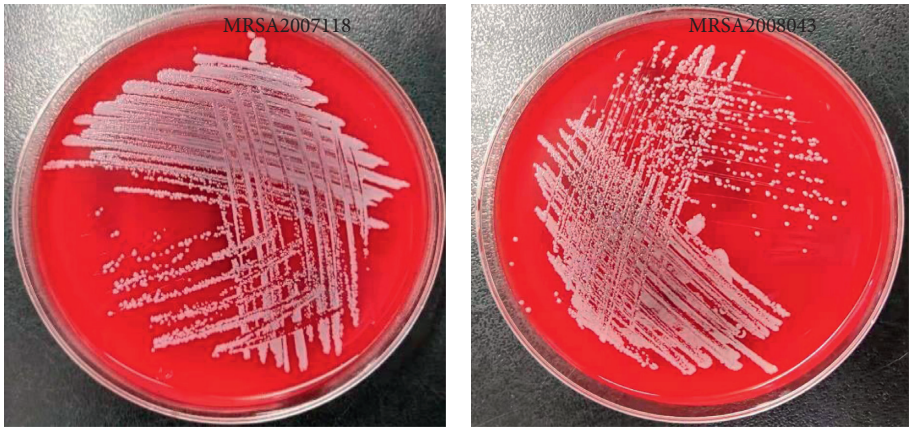


Figure 1: Total ion chromatography (TIC) on positive (a) and negative (b) and extraction ion chromatography (EIC, (c)–(f)).

Table 1: Chemical identification.

No.	RT (min)	Name	Formula	Ion	Cal. m/z	Mea. m/z	Error (ppm)	MS/MS
1	1.78	Uridine	$C_9H_{12}N_2O_6$	[M-H]	243.0622	243.062	3.445	243.062, 110.0271
2	4.744	Scutellarin	$C_{21}H_{18}O_{12}$	[M-H]	461.0725	461.0733	4.007	461.0733, 285.0403
3	5.886	Luteolin-7-O-β-D-glucuronide	$C_{21}H_{18}O_{12}$	[M-H]	461.0725	461.0731	3.574	461.0731, 285.0398
4	6.155	Luteolin	$C_{15}H_{10}O_6$	[M-H]	285.0404	285.0398	1.528	175.0409, 151.0047, 133.0307
5	6.41	Polyphyllin VI	$C_{39}H_{62}O_{13}$	[M + H]	739.4263	739.4256	-3.091	721.4129, 577.2560
6	6.708	Apigenin 7-O-beta-D-glucuronide	$C_{22}H_{20}O_{11}$	[M-H]	459.0932	459.0933	2.423	459.0933, 268.0372
7	6.85	Scutellarein	$C_{15}H_{10}O_6$	[M-H]	285.0404	285.0408	5.036	285.0408, 255.0243
8	7.007	Wogonin 7-O-glucuronide	$C_{22}H_{20}O_{11}$	[M-H]	459.0932	459.0923	0.244	459.0923, 283.0610, 268.0372
9	8.355	Apigenin	$C_{15}H_{10}O_5$	[M-H]	269.0455	269.0461	6.133	225.0551, 151.0555



(a) (b)

Figure 2: (a) MRSA2007118 image of blood plate with three streaking method. (b) MRSA2008043 image of blood plate with three streaking methods.

2.5. A Time-Kill Assay. A time-kill assay was performed according to CLSI guidelines (15). MRSA was cultured overnight and was adjusted to a 5×10^5 CFU/mL suspension with phosphate-buffered saline (PBS) (pH 7.4). Inocula were treated at 37°C with prescribed concentrations (1/4 MIC~8 MIC) of QXJT and with normal saline as a negative control. The OD630 nm value was monitored every two hours until 24 h and was analyzed by statistical software. Each experiment was performed three times.

2.6. Checkerboard Assay. The checkerboard assay, commonly used for measuring interactions [16], was used to determine synergy between the OX and QXJT. The range of drug concentrations used in the checkerboard assay was the dilution range included the MIC for each drug used in the analysis. The fractional inhibitory concentration (FIC) was derived from the lowest concentrations of the OX and QXJT in combination, permitting no visible growth of the test organisms in the Mueller–Hinton broth after incubation for 24 h at 37°C. The FIC index was calculated as FICI = MIC A drug combination/MIC A drug alone + MIC B drug combination/MIC B drug alone. In antimicrobial combination, Schelz et al. [17] defined synergy as FICI ≤ 0.5, additivity as $0.5 <$ FICI ≤ 1, indifference as $1 <$ FICI ≤ 4, and antagonism as FICI > 4.

2.7. Transmission Electron Microscopy. MRSA was cultured overnight and was adjusted to a 5×10^5 CFU/mL suspension with phosphate-buffered saline (PBS) (pH 7.4) and then was treated by 1/2 MIC QXJT, 1/2 MIC OX (preexperiment proved that it is not easy to collect a sufficient amount of Samples by the concentration above 1/2 MIC), incubated at 37°C for 24 h. To collect a sufficient amount of samples, the tubes were centrifuged at 2000 r/min for 6 minutes. Samples were rinsed two times with PBS (operated gently before fixing samples and minimized the number of rinses to avoid damage) and added 2.5% glutaraldehyde to fix at 4°C for 3 hours. Samples were rinsed again with PBS 3 times and were dehydrated with a graded acetone series and embedded in Spurr's resin. Thin slices were cut with an RMC MT-7000 ultramicrotome (Ventana) and stained with 1% uranyl acetate and Reynold's lead citrate before being seen on a transmission electron microscope at 80 kV (H-7500; Hitachi).

2.8. Quantitative Reverse-Transcription PCR. To compare the effect of 1/2MIC as well as MICs QXJT, OX, and QXJT + OX (derived from the checkerboard dilution method) on the expression of MRSA drug-resistant genes (mecA) as well as superantigen virulence genes (tst, sea, and seb), 1 ml of the above concentrations was added to test tubes, respectively, and 0.1 ml of MRSA bacterial solution and 1 ml of broth were added in each tube and incubated for 24 h. Quantitative reverse-transcription PCR (RT-qPCR) was performed as previously described by Li et al. [18] with some modifications. Total RNA was extracted using a bacterial RNA extraction kit for qRT-PCR according to the manufacturer's instructions. Total RNA (0.2 μg) was reverse-transcribed into cDNA using EasyScript First-Strand cDNA Synthesis SuperMix (TransGene, Beijing, China). Reactions were performed in triplicate using a Power SYBR®Green PCR Master Mix (Applied Biosystems, Warrington, UK) and the Applied Biosystems 7500 system. The mRNA expression levels of the genes were normalized to the 16S rRNA gene, compared with 0.9% NaCl-treated bacterial, and quantified by the $2^{-\Delta\Delta CT}$method. The sequences of the primers are presented in Table 2.

3. Statistical Analysis

All data were representative of the results of at least three independent experiments. For in vitro experiments, the MIC values were presented as number. Data for the growth curve of MRSA and the time-kill assay were presented as the mean ± standard deviation (S.D.). Quantitative reverse-transcription PCR randomized design with three replications was performed. Comparison between two groups with normal distribution and homogeneity of variance was analyzed using the unpaired *t*-test. A value of $p < 0.05$ was regarded as statistically significant. All data were analyzed using SPSS 25.0 software (IBM, Armonk, NY, USA) or GraphPad Prism software.

4. Results

4.1. Growth Curve of MRSA by Microplate Reader Measure. After isolation and purification, the clinical strains were identified as MRSA (Figure 2) by Biomeere's VITEKAMS30 identification system after culture. The growth curves of MRSA strains were plotted in three independent experiments. As shown in Figure 3, within 24 h, OD 630 nm value and MRSA concentration gradually increased as the culturing time increased. MRSA2007118 and MRSA2008043 had little difference. It could be preliminarily judged that the growth rule of MRSA is th following: 0~10 h slow period, 10~14 h logarithmic growth period, 15~18 h stability period (plateau), and >18 h decline period. According to the growth curve of MRSA, the optimal culture time of in vitro bacteriostatic test was determined to be 18~24 h.

4.2. Evaluation of Minimum Inhibitory Concentrations (MICs). QXJT had antimicrobial effects on MRSA. According to Figure 4, the liquid in the MIC well was straightforward and without bacterial growth, which was not different from the control (QXJT). The MICs of QXJT against MRSA2007118 and MRSA2008043 were 18.75 mg/ml and 37.5 mg/ml, respectively. QXJT exhibited significant antimicrobial activity against the tested MRSA. Each experiment was independently repeated three times.

4.3. A Time-Kill Assay. To further verify the inhibitory effect of QXJT on MRSA, a time-kill curve was made by detecting the OD values after treatment of MRSA with different concentrations of QXJT. The data were analyzed as shown in Figure 5. The time-killing curves of the 2 MRSA strains were similar. When MRSA was treated with 1/4 MIC, the OD630 nm value did not decrease significantly after 24 hours, but it served to delay the logarithmic growth period of MRSA. When MRSA was treated with 1/2 MIC, the OD630 nm value after 24 h could be significantly reduced. When the bacteria were treated with concentrations of MIC, 2 MIC, and 4 MIC, the OD630 of MRSA was almost unchanged between 0 and 24 h. These data suggested that QXJT could inhibit MRSA growth with a significant concentration dependence.

4.4. Checkerboard Assay. The MICs of OX against MRSA2007118 and MRSA2008043 were 4 mg/ml and 1 mg/ml, respectively. The synergistic effect of QXJT in conjugation with OX was evident in the FIC indices shown in Table 3. Overall, test microorganisms showed FIC index (=0.5) on the checkerboard assay, which indicates a synergism effect in the combination of QXJT and OX.

4.5. Transmission Electron Microscopy. To further observe the damage of QXJT on bacteria and the synergistic effect of QXJT + OX and explore the possible mechanism of action, we observed MRSA2007118 treated with QXJT, OX, and QXJT + OX at 1/2 MIC by TEM, respectively. Untreated cells are shown in Figures 6(a) and 6(b). Normal MRSA organisms were intact, almost round, with continuous cell walls, well-defined edges, and uniform cytoplasmic distribution. MRSA organisms treated with 1/2 MIC QXJT for 24 h showed deformation of the organism, vacuoles in the cytoplasmic region, slight cytoplasmic consolidation, incomplete cell wall, increased surface projections, severe damage to the organism, and cytoplasmic leakage (Figures 6(c) and 6(d)). After 24 h treatment with 1/2 MIC OX, the cytoplasmic consolidation of MRSA bacteriophage was not obvious. The cytoplasm was more uniform, and the cell wall grossness was seen to be damaged to different degrees (Figures 6(e) and 6(f)). After treatment with 1/2 MIC QXJT + OX for 24 h, it was observed that the MRSA bacteriophage was deformed, the cell wall was thin and discontinuous, the bacteriophage was severely damaged, and the cytoplasm was leaked. This indicated that QXJT + OX produced great damage to the cell wall of MRSA organisms, and this damage was more severe than that caused by either OX or QXJT alone. The synergistic nature of the cell wall damage was observed from the microscopic mechanism of the combination of the two (Figures 6(g) and 6(h)), and

Table 2: Primer sequences for real-time RT-PCR.

Target genes	Primer sequences (5′-3′)	
	Forward primer	Reverse primer
tst	CCGGGATCCCATTTGAATGAAGGAGA	GCCCTCGAGTATTGAGTTAGTGAGGAT
sea	ATGGTGCTTATTATGGTTATC	ATGGTGCTTATTATGGTTATC
seb	TGTTCGGGTATTTGAAGATGG	CGTTTCATAAGGCGAGTTGTT
mecA	GTTGTAGTTGTCGGGTTT	TTTATCGGACGTTCAGTC
16s rRNA	GTTCGCAAGAATGAAACTCA	CCCAACACCTTACGGCAC

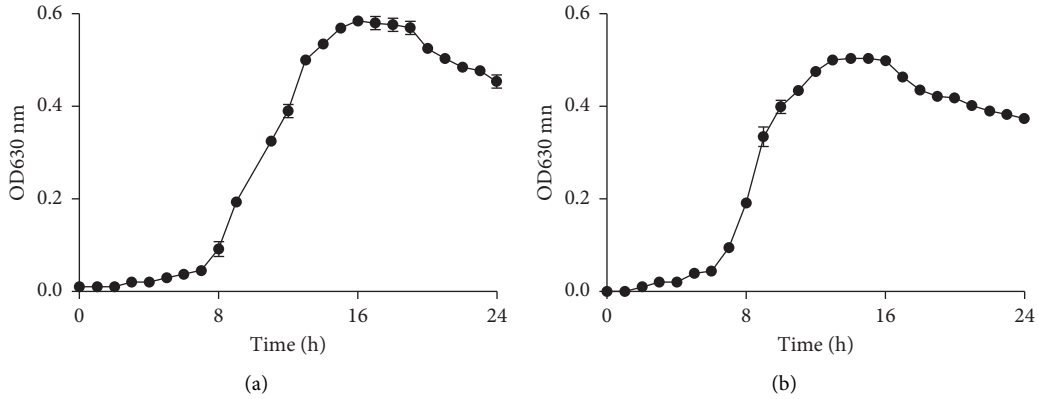


Figure 3: (a) Growth curve of MRSA2007118 (OD630 nm values) at different culture time points. (b) Growth curve of MRSA2008043 (OD630 nm values) at different culture time points.

surprisingly, we observed that, after being treated by QXJT + OX, MRSA adhered together similar to "rose." This is a unique form of disruption, and it is speculated that the combination of QXJT + OX may have influenced the division of MRSA organisms.

4.6. Quantitative Reverse-Transcription PCR. To further understand the interaction between QXJT and MRSA, The gene expression of superantigens was examined. The effect of different concentrations of QXJT, OX, and QXJT + OX on the transcription of sea, seb, tst, and mecA of MRSA2007118 and MRSA2008043 genes was examined by fluorescence quantitative PCR technique, and the results are shown in Figure 7. There was no significant decrease in the transcription of sea, seb, and tst under the addition of 1/2 MIC QXJT, but the transcription of sea, seb, and tst was a statistically significant decrease under the addition of MIC QXJT compared with the control group. This inhibition showed a dose-dependent manner: the higher the dose, the stronger the inhibition. The transcriptions of sea, seb, and tst were also significantly decreased by 1/2 MIC, MIC OX, but the decrease was less than that of 1/2 MIC, MIC QXJT + OX, and the difference was statistically significant.

5. Discussion

Natural plants represent an almost unlimited (multitarget) source of active ingredients, and because of the wide variety, we usually rely on clinical or life experiences summarized by the ancients to screen drugs with possible antimicrobial effects for the extraction and study.

QXJT is composed of different kinds and quantities of Chinese medicinal herbs with optimal therapeutic efficacy for a comprehensive regimen, according to the principles of traditional Chinese medicine (TCM). It may have more than one target and disturb bacterial physiology in several different pathways, which is beneficial for inflicting severe damage on the bacterial cell. The phenomenon is similar to polypharmacology [19, 20]. The antibacterial mechanism of QXJT is still unclear. We tried to reveal part of the mechanism by in vitro antibacterial experiment.

Due to the abuse of antibiotics and other factors, bacterial resistance has become more and more serious, which has become a century-old problem that needs to be solved in the future. Because of the high morbidity and mortality associated with MRSA, MRSA infection is considered one of the most challenging infectious diseases, resulting in a worse prognosis of sepsis and higher morbidity and mortality [21, 22]. Takesue et al. [23] demonstrated that the failure rate of vancomycin as first-line drug therapy against life-threatening MRSA disease is increasing. The successive reports of vancomycin heterogeneous resistant SA (hVRSA), vancomycin-mediated resistant SA (VISA), and vancomycin-resistant SA (VRSA) detected worldwide [24] remind us that we may be gradually losing the last line of defense against MRSA infection. The search for novel therapeutic agents that are effective against MRSA is therefore critical to maintaining public health in the future.

In our study, combining MIC and time-kill curve results, we found that QXJT showed significant bacterial inhibition against both clinical isolates of MRSA in a concentration-dependent manner. Active substances such as *Scutellarein*, *Scutellarin, Apigenin*, and *Wogonin 7-O-glucuronide* were

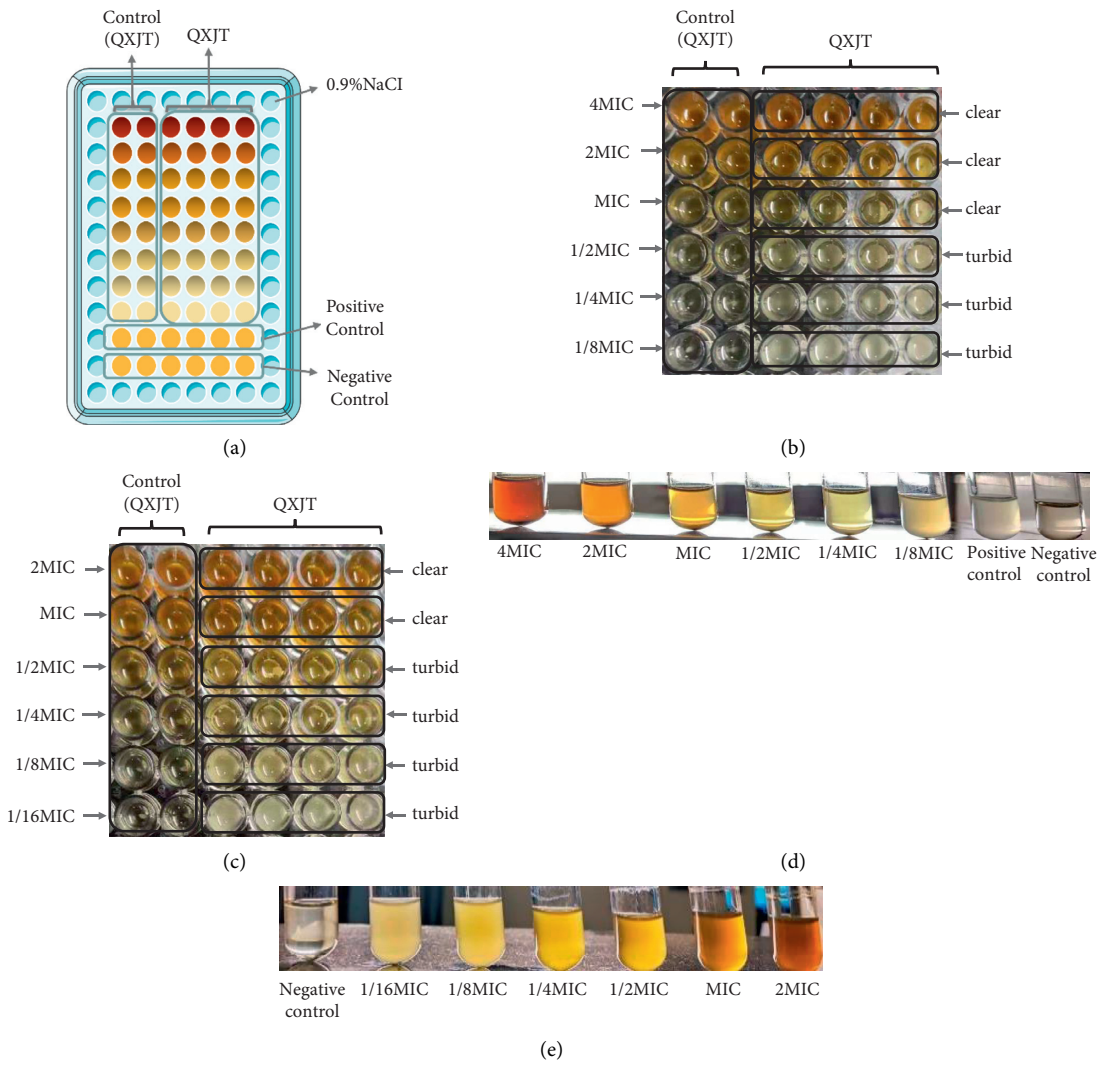


Figure 4: (a) 96-well plate plus sample: control (QXJT) means QXJT was continuously twofold diluted without adding bacteria liquid as control; positive control meant broth is added with bacteria without drugs; negative control meant broth is added without bacteria without drugs. (b) QXJT against MRSA2007118 (broth microdilution method results); MIC = 18.75 mg/ml. (c) QXJT against MRSA2008043 (broth microdilution method results); MIC = 37.5 mg/ml. (d) QXJT against MRSA2007118 (broth macrodilution method results); MIC = 18.75 mg/ml. (e) QXJT against MRSA2008043 (broth macrodilution method results); 37.5 mg/ml.

detected in the phytochemical analysis that may be associated with the antimicrobial activity of QXJT. Perhaps this is because although the MRSA cell wall has a thick layer of peptidoglycan, this barrier is unable to block these tiny molecular compounds. These compounds on the cell wall of MRSA produce damage [25]. We also found that the MIC of MRSA2007118 to OX was about 4 mg/ml, which is four times higher than that of MRSA2008043, but MRSA2007118 was more sensitive to QXJT than MRSA2008043. MRSA is resistant to penicillin drugs by altering the structure of its cell wall through the mecA resistance gene [26]. But this resistance mutation did not show an advantage in resisting the damage caused by QXJT.

From the results of the checkerboard assay, we conclude that QXJT and OX have a significant synergistic effect (FICI = 0.5). We analyze the results of qPCR which show that QXJT and OX do not inhibit the mecA gene, so the synergistic mechanism of the two is not related to inhibiting the expression of drug resistance genes. By TEM images we observe that QXJT and OX are able to disrupt the cell wall, and QXJT + OX can produce greater damage to the cell wall. Through these results and our speculation that the effects of QXJT + OX combinations against MRSA may arise from two distinct types of mechanisms, the first of these is due to QXJT originally having a certain destructive effect on the bacterial cell wall, and this destructive effect can be subtly synergized with the destruction of the cell wall by OX, but the specific target of action is not clear; the second mechanism may arise from the ability of QXJT to inhibit the β-lactamase hydrolysis and restore MRSA sensitivity to OX.

Earlier studies focused more on the anti-inflammatory, antioxidant, and antibacterial effects of natural drugs [27–30]. Until recently, several researchers in diverse domains have expressed interest in strategies that target pathogen virulence factors [31]. It contradicts the antimicrobial effect of traditional antibiotics. Because virulence

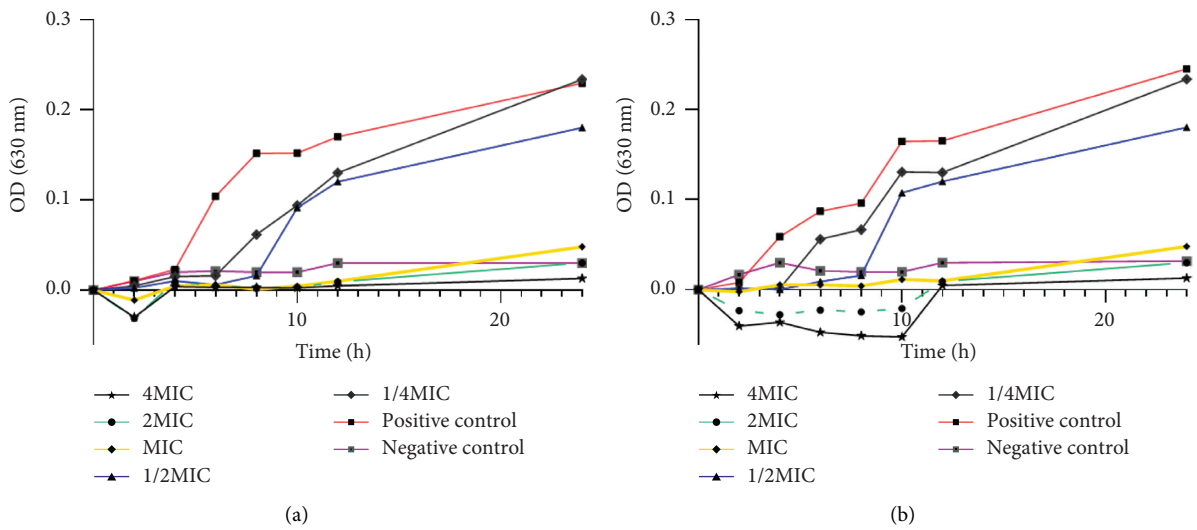


Figure 5: MRSA was incubated at different concentrations of QXJT from 1/4MIC to 4MIC. OD630 values were measured at 0, 2, 4, 8, 10, 12, and 24 h after treatment. Each experiment was repeated twice. (a) A time-kill assay of MRSA2007118. (b) A time-kill assay of MRSA2008043.

Table 3: QXJT and OX against MRSA.

MIC strains	QXJT(mg/mL)		OX(mg/mL)	
MRSA2007118	18.75		4	
MRSA2008043	37.5		1	
	CIC	FIC_{QXJT}	FIC_{OX}	FICI
QXJT + OX	4.69 mg/mLQXJT + 1 mg/mLOX	0.25	0.25	0.5, synergism
	9.38 mg/mLQXJT + 0.25 mg/mLOX	0.25	0.25	0.5, synergism

MIC, minimum inhibitory concentration; CIC, combined inhibitory concentration; FIC, fractional inhibitory concentration; FICI, fractional inhibitory concentration index.

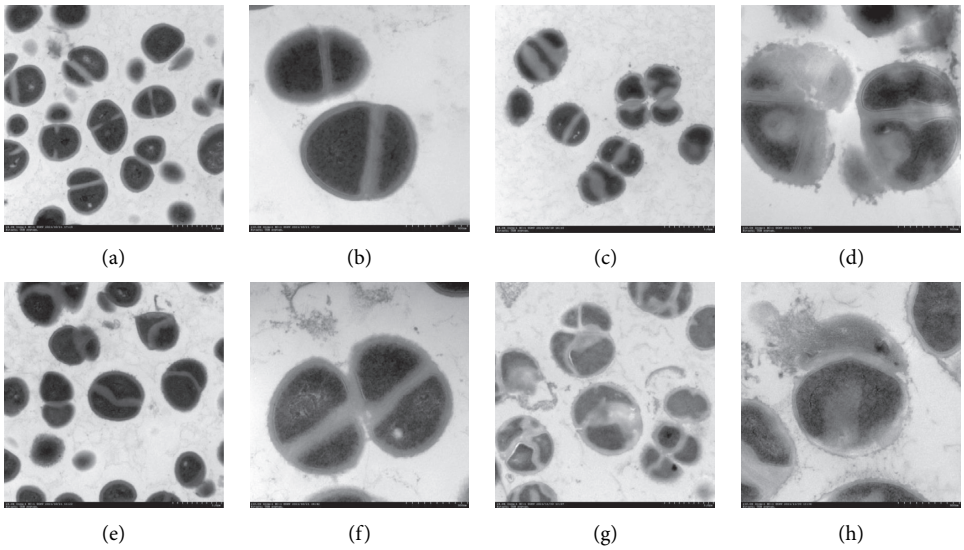


Figure 6: (a, b) Transmission electron micrographs of untreated MRSA cells showing normal cell shapes. (c, d) Transmission electron micrographs of treated with 1/2 MIC of QXJT for 24 h. (e, f) Transmission electron micrographs of treated with 1/2 MIC of OX for 24 h. (g, h) Transmission electron micrographs of treated with 1/2 MIC of QXJT + OX for 24 h.

factors are not always required for bacterial survival, the antiviral method is under less pressure to target bacteria and is less vulnerable to bacterial resistance [13, 32].

Studies have shown that the protective effect of natural drugs such as baicalin against lethal infection with enterohemorrhagic *Escherichia coli* may be through direct interaction

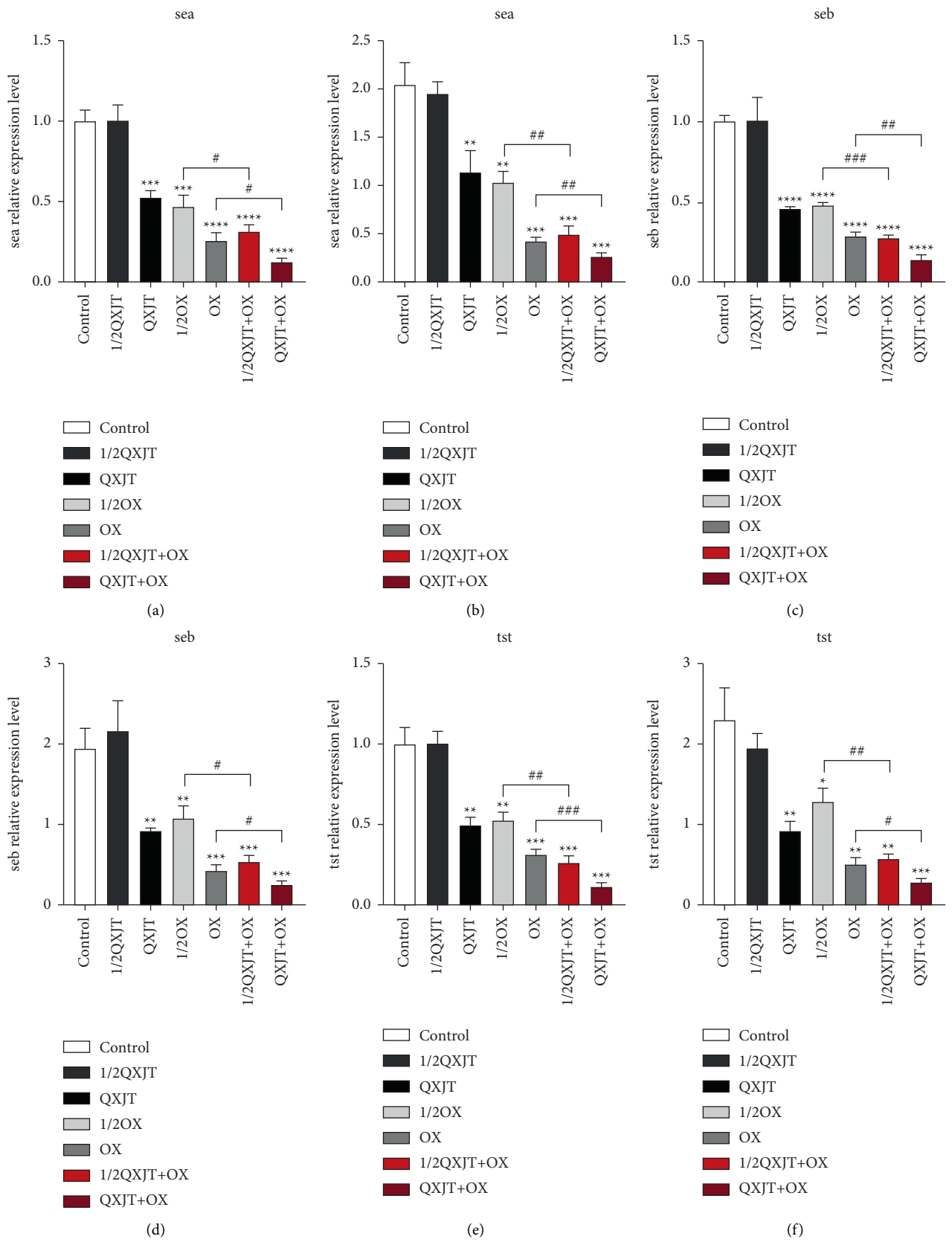


Figure 7: Continued.

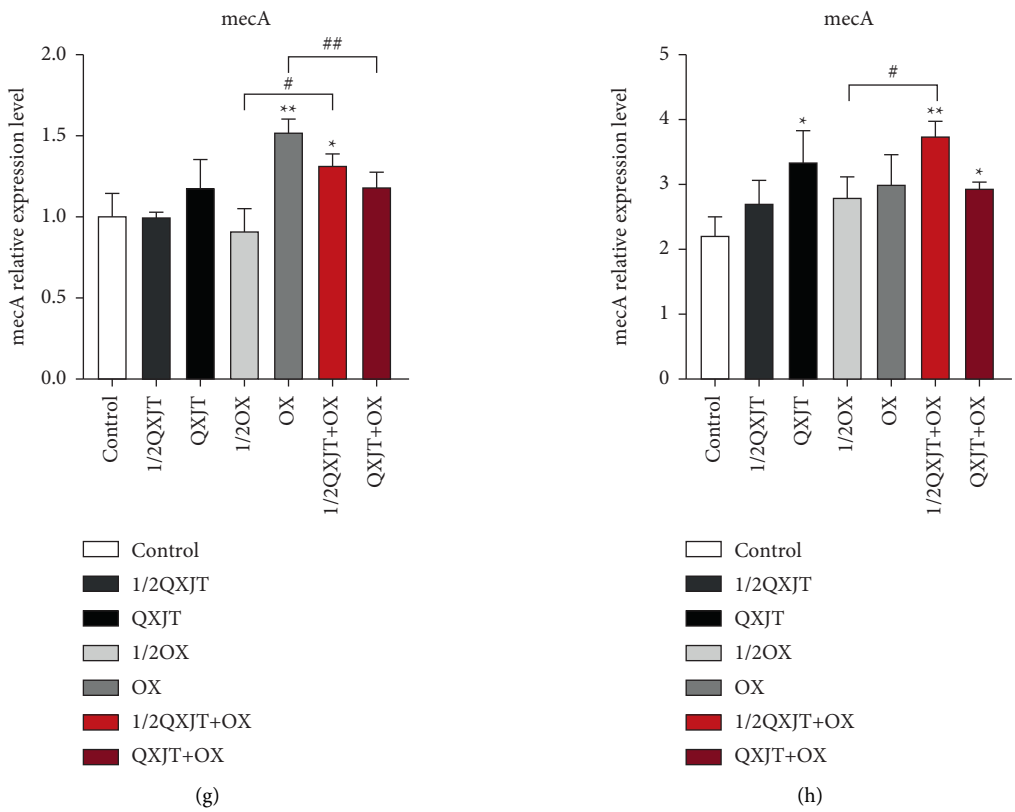


Figure 7: Different concentrations of QXJT, OX, and QXJT + OX were added to the final concentration of 5 × 105 CFU/mL and incubated at 37°C for 24 h. The expression levels of sea, seb, and tst were compared. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P \leq 0.0001$ vs. control group; $^{\#}P \leq 0.05$, $^{\#\#}P \leq 0.01$, $^{\#\#\#}P \leq 0.001$, $^{\#\#\#\#}P \leq 0.0001$ vs. 1/2OX or OX group. (a) Relative gene expression of MRSA2007118 strain sea under different concentrations of drug treatment. (b) Relative gene expression of MRSA2008043 strain sea under different concentrations of drug treatment. (c) Relative gene expression of MRSA2007118 strain seb under different concentrations of drug treatment. (d) Relative gene expression of MRSA2008043 strain seb relative gene expression. (e) MRSA2002118 strain tst relative gene expression under different concentrations of drug treatment. (f) MRSA2008043 strain tst relative gene expression under different concentrations of drug treatment. (g) Relative gene expression of mecA in strain MRSA2007118 under different concentrations of drug treatment. (h) Relative gene expression of mecA in strain MRSA2008043 under different concentrations of drug treatment.

with Shiga-like toxin 2 [33]. Baicalin showed a significant inhibitory effect on the pathogenic factor hla of *Staphylococcus aureus* [34]. In our study, we found that QXJT + OX significantly inhibited the virulence factors such as sea, seb, and tst of MRSA in a concentration-dependent manner, and QXJT + OX could inhibit the virulence factors such as sea, seb, and tst more significantly. The exact mechanism remains unclear, but this regulatory role in virulence factors may provide new strategies for exploring new antibiotic drugs.

6. Conclusions

This article is based on a famous formula for treating sepsis caused by acute purulent infections of the face, hands, and feet: QXJT (a combination of natural drugs), created by Chen Shigong, a leading TCM surgeon with over 700 years of clinical application. In this study, QXJT showed good antimicrobial activity against a clinical isolate of MRSA. We found a significant synergistic effect of QXJT and OX from the combined form of traditional Chinese medicine and Western medicine, which may be related to the mechanism of action of QXJT to disrupt the cell wall of MRSA. However, its effect on the cell membrane cannot be excluded and still needs further study. We found that QXJT and QXJT in combination with OX could inhibit the expression of MRSA virulence factor but had no significant effect on MRSA resistance gene expression without significant effects, providing a new strategy for exploring new antibiotic drugs.

Data Availability

Article data or supplementary data may be requested via e-mail to the author Siyuan Lv (lvsiyuan2008@126.com) and will be shared with applicants.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

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Research Article

Chemical Composition and Insecticidal, Antiplasmodial, and Anti-Leishmanial Activity of *Capparis spinosa* Essential Oil and Its Main Constituents

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Background. This investigation was designed to evaluate the insecticidal, antiplasmodial, anti-leishmanial, and cytotoxic effects of *Capparis spinosa* essential oil (CSEO) and its main components, methyl isothiocyanate, hexadecanoic acid, and limonene. *Methods*. Insecticidal activity of CSEO and its main components, methyl isothiocyanate, hexadecanoic acid, and limonene, was determined against *Aedes aegypti* 4th-instar larvae at $25 \pm 2^{\circ}$C. Antiplasmodial and anti-leishmanial effects of CSEO and its main components were carried out against chloroquine-resistant *Plasmodium falciparum* K1 strain and *Leishmania major* amastigotes based on the Malstat method and the macrophage model, respectively. We also performed the cytotoxic activity of CZEO and its main components against J774A1 macrophage cells using the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. In addition, the plasma membrane permeability and caspase-3-like activity CSEO and its main components were evaluated against *L. major*. *Results*. CSEO and its main components showed considerable ($p < 0.001$) larvicidal activity against *Ae. aegypti* larva. The 50% lethal concentration values for CSEO, methyl isothiocyanate, hexadecanoic acid, and limonene were 21.6, 30.9, 41.6, and 35.3 μg/mL, respectively. By antiplasmodial effects, the 50% inhibitory concentration (IC_{50}) values for CSEO, methyl isothiocyanate, hexadecanoic acid, and limonene were 7.4, 14.5, 19.6, and 21.3 μg/mL, respectively, while these values for their anti-leishmanial effects were 9.1, 20.7, 23.3, and 18.6 μg/mL, respectively. The 50% cytotoxic concentration values for CSEO, methyl isothiocyanate, hexadecanoic acid, and limonene were 93.7, 216.2, 199.4, and 221.3 μg/mL, respectively. Different concentrations of CSEO and its main components significantly ($p < 0.05$) increased the plasma membrane permeability and caspase-3-like activity against *L. major* promastigote level as dose-dependent response. *Conclusion*. Based on the obtained results, *C. spinosa* essential oil and its main components, methyl isothiocyanate, hexadecanoic acid, and limonene, displayed insecticidal, antiplasmodial, and anti-leishmanial activity against healthy 4th-instar larvae of *A. aegypti*, chloroquine-resistant P. falciparum K1 strain, and L. major amastigotes, respectively. However, further surveys are required to display the mechanisms of action mode of tested drugs and their efficacy in animal model and clinical settings.

1. Introduction

Mosquitoes are considered as the main vectors of a wide range of important human infectious diseases including malaria, dengue, filariasis, encephalitis, and yellow fever, which cause high mortality around the world [1]. Among the mosquitoes important in medicine, *Aedes aegypti* L., as an anthropophilic and domicile mosquito, is broadly observed in the most tropical and subtropical regions worldwide [2]. This mosquito is well known as a vector of a number of important vector-borne infections such as malaria, dengue, chikungunya, Zika, and yellow fever [3].

Today, according to the recommendations of the World Health Organization (WHO), the main strategies for

controlling vector are used as growth regulator insecticides such as diflubenzuron for immature forms of mosquitoes and the employment of some insecticides such as alpha-cypermethrin, malathion, and deltamethrin for controlling adult mosquitoes [4, 5]. In recent year, the excessive and constant use of these synthetic insecticides has led to a decrease in their efficacy and also some ecological worries such as emerging of drug resistance in mosquitoes, ecological imbalance, and outcome to animals [6, 7]. Hence, there is an rising concern to find new greatly selective and eco-friendly substitutes of insecticides around the world.

Malaria is a global public health problem, which estimated approximately 430,000 deaths annually, mostly in African children [8]. Although the most common *Plasmodium* species are *Plasmodium vivax* and *P. falciparum*, however, the most important species that causes high mortality in infected people is *P. falciparum* [9]. Meanwhile, the incidence of drug resistance in malaria, especially in *P. falciparum* malaria, is increasing to some synthetic antimalarial agents, such as chloroquine and mefloquine [10]. Thus, it is an urgent need for novel antimalarial agents, especially from natural resources with the least toxicity and the highest efficiency.

Leishmaniasis is one of the most significant protozoan infections triggered by the parasitic species *Leishmania,* which infects about 12 million people each year in 98 countries around the world [11]. *Leishmania* species in humans can cause cutaneous leishmaniasis, cutaneous-mucosal leishmaniasis, and visceral leishmaniasis [12]. A number of synthetic agents are applied to treat leishmaniasis; however, recent reviews have proven that most of the synthetic and chemical anti-leishmanial compounds have some limitations (e.g., drug resistance and long-term treatment), toxicity, and side effects [13].

Natural products and their derivatives as an inexpensive, accessible, and useful alternative medicine are broadly applied for the treatment of a wide range of diseases such as infectious ones [14]. Today, insecticides originating from plant extracts and essential oils have increased the overall interest, to replace or substitute synthetic insecticides [15]. Nowadays, there has been a growing development of research on the efficiency of medicinal herbs and their derivatives on various diseases, including parasitic diseases [14]. Although, in recent years, several laboratory and experimental studies have reported the significant antiparasitic effects of various medicinal herbs against *Leishmania* spp. and *Plasmodium* spp. 12,13, however, their efficacy and their toxicity are still debatable and vague [16].

Capparis spinosa L. (Capparaceae family) is an aromatic herb, which broadly grows in the Mediterranean area [17]. This plant is a multipurpose crop with unique properties in agro-biodiversity and agroecosystems such as resistance to drought and harsh environmental conditions. However, many biological aspects of this plant such as food and medicinal usages, phytochemistry, ethnopharmacology, and cultivation have not yet been studied [18].

In traditional medicine, *C. spinosa* was utilized as diuretic, appetizer, and anti-diarrheic and was used for the treatment of rheumatism, ulcers, ganglions, headache, and toothache [18, 19]. In addition to traditional uses, the *C. spinosa* has various ethnopharmacology and biological and chemical activity properties such as antioxidant, anticarcinogenic, anti-inflammatory, antidiabetic, and antimicrobial effects [18, 20]. The current investigation was designed to evaluate the insecticidal, antiplasmodial, anti-leishmanial, and cytotoxic effects of *C. spinosa* essential oil and its main components, methyl isothiocyanate, hexadecanoic acid, and limonene.

2. Materials and Methods

2.1. Compounds. In this study, methyl isothiocyanate, hexadecanoic acid, and limonene were obtained from Sigma-Aldrich (St. Louis, MO).

2.2. Plant Collection. *C. spinosa* aerial parts were provided in June 2021 from a market selling fresh herbs in Tabuk Market, Saudi Arabia, and they were identified by a botanist, and a voucher specimen (UT-2021-254) was deposited on the Herbarium of Department of Biology, Faculty of Science, University of Tabuk.

2.3. Preparing Essential Oil. To isolate the essential oil, 200 g of dried and powdered materials was put into the hydrodistillation technique for 180 min by means of a glass Clevenger-type device. The attained essential oil was then dehydrated by over anhydrous sodium sulfate and kept in darkness at 4°C in glass tubes until testing [21].

2.4. Gas Chromatography-Mass Spectrometry (GC-MS). To recognize the compounds in CSEO, a Hewlett Packard 6890 (Palo Alto, CA, USA) device was used to perform the GC analysis equipped with a HP-5ms column ($30\,m \times 0.25$ mm, film thickness 0.25 mm). To do this, 0.1 μL of essential oil was injected into the gas chromatography apparatus. The initial temperature was set at 50°C for 5 min and then increased to 300°C at a rate of 5°C/min. Helium gas was used at a rate of 1.1 mL/min, and ionization energy of electronvolt (EV) was used whereas the split ratio was 1:30, and injector and detector temperature was 280°C with split of 1/100. The components were identified according to the comparison of their mass spectra with those of NIST mass spectral library [22] and those explained by Adams and by comparison of their retention indices either with those of authentic compounds or with literature values [23].

2.5. Insecticidal Activity. Insecticidal activity of CSEO was performed according to the method explained by Huong et al. [24]. Briefly, *Ae. aegypti* eggs were prepared from the Department of Biology, Faculty of Science, University of Tabuk, Saudi Arabia, for further experiments. To do this, the various concentrations of CSEO and its main components, methyl isothiocyanate, hexadecanoic acid, and limonene (6.25, 12.5, 25, 50, and 100 μg/mL), were dissolved in DMSO (1% stock solution) and were put in a 500-mL beaker and added to 150 ml water with 20 healthy 4th-instar larvae at 25 ± 2°C. Larval mortality was determined after 24 h of

incubation; 50% lethal concentration (LC_{50}) was calculated via the probit test in SPSS software for each drug. All tests were carried out in triplicate, and during experiments, no nutritional complement was added; however, DMSO was considered as the control group.

2.6. Antiplasmodial Activity. The antiplasmodial effects of various concentrations (3.125, 6.25, 12.5, 25, 50, and 100 μg/mL) of CSEO and its main components, methyl isothiocyanate, hexadecanoic acid, and limonene, against chloroquine-resistant *P. falciparum* K1 strain were performed based on the Malstat method [25]. In brief, parasites were exposed to the human erythrocytes (red blood cells, RBC) in RPMI 1640 medium improved with 10% human serum and were incubated at 37°C with low oxygen atmosphere (3% O_2, 4% CO_2, and 93% N_2). The infected human RBC (0.2 mL, 1% parasitaemia, and 2% hematocrit) was added to each well with various concentrations of CSEO and incubated for 3 days. Then, the tested plates were frozen at −20°C. In the next step, in a new plate, 0.1 mL of Malstat reagent was mixed with 0.02 mL of suspension of hemolyzed parasite and incubated for 15 min at 21°C. After this time, 0.02 Ml of NBT/PES solution was added to the plates and was incubated again for 120 min in the dark. Finally, the absorbance of each well was determined using the light absorption at 655 nm with the ELISA reader. The 50% inhibitory concentrations (IC_{50}) were also calculated via the probit test in SPSS software.

2.7. Anti-Leishmanial Effects. To evaluate the anti-leishmanial effects of CSEO and its main components, methyl isothiocyanate, hexadecanoic acid, and limonene (3.125, 6.25, 12.5, 25, 50, and 100 μg/mL), against the intracellular amastigote of *L. major* (MRHO/IR/75/ER), J774A1 macrophage cells (5×105 cells/ml) were poured in sterile 6-well plates (with 1 cm^2 coverslips implanted on their floor) and incubated at 37°C for 24 hours with 5% CO_2 to adhere to macrophages. After 24 hours, the plates were removed from the incubator and washed with sterile warm saline phosphate buffer. Then, 1 ml of RPMI 1640 enriched medium containing 5×10^6 L. *major* promastigotes in the stationary phase was added into plates and kept warm at 37°C for 4 hours, and then, the wells were washed with RPMI 1640 medium to remove free promastigotes. In the next step, one ml of RPMI 1640 medium containing different concentrations of essential oil and MA was added to the wells for 48 hours, the slides were then fixed with methanol, and staining was then done with Giemsa dye diluted with water in a ratio of 1 : 10. The results were estimated by calculating the number of amastigotes inside 100 macrophages and the number of infected macrophages in each well. The IC_{50} values were also calculated via the probit test in SPSS software. All examinations in this study were carried out in triplicate [26].

2.8. Plasma Membrane Permeability. In this study, the effects of CSEO and its main components, methyl isothiocyanate, hexadecanoic acid, and limonene, on the permeability of plasma membrane of parasites were evaluated. To do this, *L. major* promastigotes of 1×10^6 cells/ml were treated with different concentrations of CSEO, methyl isothiocyanate, hexadecanoic acid, and limonene (3.125, 6.25, and 12.5 μg/mL) and then SYTOX Green stain was utilized based on the kit instructions. Parasites with no drug and those treated with 2.5% of Triton X-100 (Sigma-Aldrich) were determined as the negative control and positive control, respectively. The plasma membrane permeability was calculated by means of a microplate reader (BMG Labtech, Germany) for 4 h [26].

2.9. Evaluating the Caspase-3-Like Activity of Extract-Treated Promastigotes. The effects of CSEO, methyl isothiocyanate, hexadecanoic acid, and limonene on the induction of apoptosis were evaluated by the colorimetric protease (Sigma, Germany) method according to the manufacturer recommendations. In this way, the caspase-3-like activity level was measured based on the rate of color spectrophotometric produced through the release of a molecule (pNA attached to the substrate) under the enzyme caspase-3 activity. In brief, the promastigotes (1×10^6) were incubated with CSEO, methyl isothiocyanate, hexadecanoic acid, and limonene at the concentrations of 6.25, 12.5, and 25 μg/mL for 24 h and were centrifuged at 700 rpm for 5 minutes at 4°C. Next, the cell residue was lysed, and the cell lysate was centrifuged again at 20,000 rpm for 10 minutes. Lastly, the supernatant of reaction (5 μl) was added to the 85 μl of buffer and 10 μl of caspase-3 (pNA-DEVD-Ac) solution and the mixture was incubated for 120 min at 37°C. The caspase-3-like activity was determined through the light absorption at 405 nm with the ELISA reader [27].

2.10. Cytotoxic Effects. We determined the cytotoxic activity of CSEO and its main components, methyl isothiocyanate, hexadecanoic acid, and limonene, against J774A1 macrophage cells, using the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay based on the method described elsewhere. To perform it, the J774A1 macrophage cells (5×10^5) were treated with various concentrations of CSEO (0 to 200 μl/mL) and its main components, methyl isothiocyanate, hexadecanoic acid, and limonene (25, 50, 100, 200, and 400 μg/mL), at the concentrations for 48 h in microplates at 37°C with 5% CO_2. The 50% cytotoxic concentration (CC_{50}) values were calculated by means of the probit test in SPSS software [26].

2.11. Statistical Analysis. To analyze the results, we used the SPSS statistical package, version 22.0 (SPSS, Inc.). To compare the results among tested groups, we applied the unpaired samples *t*-test and one-way analysis of variance (ANOVA), and Dunnett's test. $p < 0.05$ was considered statistically significant.

3. Results and Discussion

Medicinal herbs have been applied for centuries as appreciated resources of bioactive and beneficial materials with medical, industrial, and agricultural goals [28]. Herbal

medicines, and their products such as essential oils and extracts, have been assessed for several strategies in the pest control and ovicidal activity and by evaluating the repellent [29]. In recent years, the present insecticidal agents commonly originated from a single active component, and herbal insecticides comprising combinations of chemical ingredients may affect both behavioral and physiological routes [30]. It seems that looking for bioinsecticides, which are effective, and being appropriate and adaptive to ecological situations, is necessary for gaining adequate insect control [31]. Essential oils are recognized to be complex combinations of secondary metabolites that may be acquired at low costs by means of updated technology, frequently demonstrating higher activities than the single isolated ingredients [32]. The current investigation was designed to evaluate the insecticidal, antiplasmodial, anti-leishmanial, and cytotoxic effects of *C. spinosa* essential oil. We found that the essential oil yielded 1.13% w/v; as shown in Table 1 of the obtained results in GC/MS, thirty-four compounds were identified, demonstrating 96.4% of the entire essential oil. The major components were methyl isothiocyanate (31.6%), hexadecanoic acid (18.5%), and limonene (11.6%), respectively, whereas the most chemical classes were aldehyde (48.4%), sesquiterpenes (19.4%), and monoterpene (12.9%), respectively.

In line with our results, Kulisic Bilusic (2010) has demonstrated that methyl isothiocyanate (92.06%) is the main constituent of essential oil of aerial parts of *C. spinosa* [33]. Ramdani et al. have reported that *C. spinosa* produced low yield (0.03%), whereas the main constituents of *C. spinosa* leaf essential oils obtained from six places in Algeria were hexadecanoic acid (38.19%), nonanal-n (12.61%), and cymene-2,5-dimethoxy-para (8.94%), respectively [34]. On the other hand, the results of the study conducted by Al-Mnaser demonstrate that the main constituents of the *C. spinosa* leaf essential oils are thymol (17%), octanoic acid (16%), methyl isothiocyanate (12%), and 2-hexenal (8.23%), respectively [35]. Esmaeilzadeh Bahabadi and Najafi have also reported that thymol (24%) and isothiocyanates (29%) are the most components of *C. spinosa* leaf essential oils [36]. Based on the previous reports, the chemical composition of essential oils is relatively different depending on several reasons including the place where the plant grew, the part of the herbs that is used, time of harvesting the herbs, and the technique of isolating the essential oil from the herbs [37, 38].

As shown in Figure 1, CSEO and its main components showed considerable ($p < 0.001$) larvicidal activity against *Ae. aegypti* larva. Based on the obtained LC_{50} results, the larvicidal effects of the components were found as CSEO > methyl isothiocyanate > limonene > hexadecanoic acid against *Ae. aegypti* larva, with the LC_{50} values for CSEO, methyl isothiocyanate, hexadecanoic acid, and limonene as 21.6, 30.9, 35.3, and 41.6, μg/mL, respectively (Table 2).

Due to the lack of a unique and specific standard criterion in the guidelines of the WHO for evaluating the larvicidal activity of medicinal herbs, a number of researchers have established specific criteria to illustrate the efficacy of insecticides originated from herbal medicines [39, 40]. Based on the study conducted by Komalamisra et al. [41], medicinal herbs with LC_{50} value of less than 50 μg/mL are promising and active; the products with LC_{50} values

Table 1: GC/MS analysis of *C. spinosa* essential oil

No.	Compound	RI_C	RI_L	Area (%)
1.	Tert-butanol	676	669	0.6
2.	Methyl isothiocyanate	703	704	31.6
3.	Cyclohexane	712	717	0.3
4.	Hexanal	802	804	0.3
5.	Heptanal	892	894	2.1
6.	Butyl isothiocyanate	947	959	1.1
7.	β-Pinene	974	980	1.2
8.	Octanal-n	1003	1001	1.1
9.	p-Cymene	1026	1027	0.7
10.	Limonene	1030	1036	11.6
11.	β-Phellandrene	1034	1043	0.8
12.	Benzeneacetaldehyde	1043	1044	0.5
13.	Octen-1-ol-2e	1064	1066	1.3
14.	Nonanal-n	1102	1102	7.6
15.	Benzeneacetonitrile	1140	1145	0.8
16.	Methyl salicylate	1198	1195	0.9
17.	Decenal-z-4	1263	1265	1.6
18.	Decanol-n	1272	1279	1.8
19.	Tetradecane	1400	1393	0.5
20.	β-Caryophyllene	1417	1421	0.7
21.	l-Octen-3-ol	1456	1458	0.6
22.	2-Tridecanone	1492	1497	0.7
23.	Pentadecane	1501	1509	0.7
24.	Tridecanal	1510	1512	0.6
25.	Germacrene B	1551	1556	0.4
26.	3-Hexenyl benzoate	1566	1558	0.6
27.	Caryophyllene oxide	1581	1580	0.5
28.	Tetradecanal	1613	1611	1.0
29.	Hexadecanoic acid	1962	1970	18.5
30.	Docosane	2211	2200	4.3
31.	Tetracosane-n	2416	2400	1.4
	Total			**96.4**

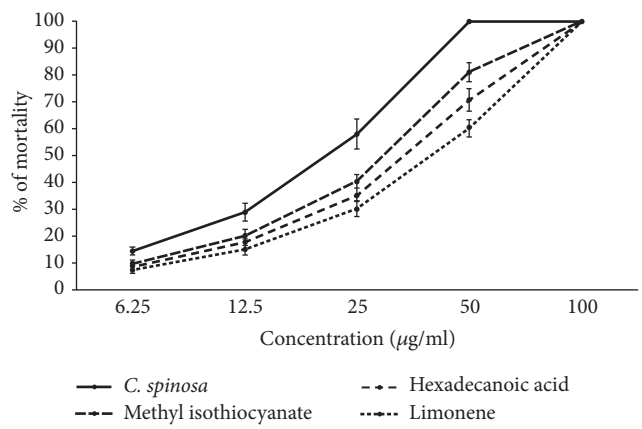

Figure 1: Insecticidal activity of the essential oil of *C. spinosa* and its main components, methyl isothiocyanate, hexadecanoic acid, and limonene, against *Ae. aegypti* larva. The results showed that among the main components of CSEO, methyl isothiocyanate and hexadecanoic acid displayed the highest and lowest larvicidal effects against *Ae. aegypti* larva. Data are presented as the mean ± SD ($n = 3$).

Table 2: Total content of compounds of *C. spinosa* essential oil by GC/MS analysis.

Chemical class	Percent (%)
Alcohol	9.6
Aldehyde	48.4
Monoterpene	12.9
Organosulfur	6.5
Sesquiterpenes	19.4
Fatty acid	3.2

between 500 and100 μg/mL were moderately active, whereas medicinal herbs with LC_{50} values between 100 and 750 μg/mL were effective and those with LC_{50} values higher than 750 μg/mL were inactive. The other study conducted by Kiran et al. demonstrated that natural products with LC_{50} less than 100 µg/mL are considered as a potent larvicidal effect [42]. It should be noted, however, that these criteria are dependent on exposure duration and larval source, which may alter the LC_{50} values of natural compounds examined [43]. Therefore, our finding revealed the promising and potent insecticidal effects of CSEO, based on the criterion reported by Komalamisra et al. [41] and Ravi Kiran et al. [42].

The essential oil of *C. spinosa* and its main components, methyl isothiocyanate, hexadecanoic acid, and limonene, exhibited relevant ($p < 0.001$) effects against *P. falciparum* (Figure 2). The IC_{50} value for CSEO was 7.4 μg/mL. Among the main components of CSEO, the highest to the lowest antiplasmodial effect was observed in methyl isothiocyanate > hexadecanoic acid > and limonene, with IC_{50} values of 14.5, 19.6, and 21.3 μg/mL, respectively (Table 2). As shown in Figure 3, the essential oil of *C. spinosa* and its main components, methyl isothiocyanate, hexadecanoic acid, and limonene, displayed activity property ($p < 0.001$) against intracellular amastigotes of *L. major*. The IC_{50} value for CSEO was 9.1 μg/mL; based on the obtained LC_{50} values, the antileishmanial effects of the main components were found as limonene > methyl isothiocyanate > and hexadecanoic acid, with IC_{50} values of 18.6, 20.7, and 23.3 μg/mL, respectively (Table 2).

Previous investigations have demonstrated that the rupture and/or cross-plasma membrane is recognized as one of the main action modes to inhibit the growth of intracellular pathogens [25]; therefore, we evaluated the plasma membrane permeability of the *L. major* promastigotes treated with CSEO, methyl isothiocyanate, hexadecanoic acid, and limonene. The findings of relative fluorescent units demonstrated that the promastigotes treated with CSEO, methyl isothiocyanate, hexadecanoic acid, and limonene changed the permeability of plasma membrane by SYTOX Green as dose-dependent response (Figure 4).

Apoptosis is considered as a form of programmed cell death process that happens in multicellular organisms. The process is a complex cascade of protease pathways designed to cellular death in a selective mode [44]. Among the main mediators of apoptosis, caspases and especially caspase-3 are considered as one of the crucial caspases that principally activated apoptosis-related proteases and consecutively

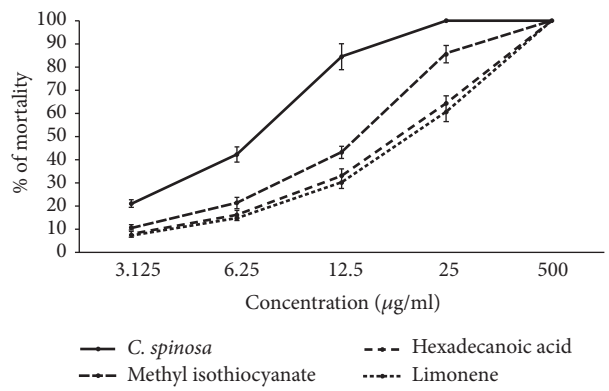

Figure 2: Antiplasmodial effects of various concentrations of CSEO and its main components, methyl isothiocyanate, hexadecanoic acid, and limonene, against chloroquine-resistant *P. falciparum* K1 strain based on the Malstat method. Among the main components of CSEO, the highest to the lowest antiplasmodial effect was observed in methyl isothiocyanate > hexadecanoic acid > and limonene. Data are presented as the mean ± SD ($n = 3$).

prompt cell death [45]. The findings of the present investigation exhibited that we found that CSEO, methyl isothiocyanate, hexadecanoic acid, and limonene significantly induced caspase-3 activation as dose-dependent response ranging from 8.6 to 29.3% in comparison with the control (Figure 5).

We found that the main components of CSEO are organosulfur (methyl isothiocyanate), fatty acid (hexadecanoic acid), and monoterpenoid (limonene) compounds. Considering the antimicrobial mechanisms of organosulfur compounds, previous investigations reported that these compounds displayed their antimicrobial mechanisms through disruption of DNA, RNA, and protein synthesis, reacting with sulfhydryl groups of the enzymes and proteins of microbes, damaging the cell wall and membrane, and subsequently integrity of cell membrane integrity [46–49]. Fatty acids also showed their antimicrobial mechanisms through the cell membrane damage, by disruption of the electron transport chain and oxidative phosphorylation of microbes [50]. With respect to the antimicrobial action mode of monoterpenoid, previous reports exhibited that these compounds affect cell membrane permeability of microbes and interact with intracellular targets [51–53].

Babanezhad Harikandei et al. (2020) have demonstrated that the synthesized isothiocyanate derivatives significantly reduced the growth rate of *Trypanosoma brucei rhodesiense* strain STIB900 (with IC_{50} values ranging from 1 to 46.6 μg/mL), *T. cruzi* strain Tulahuen C4 (with IC_{50} values ranging from 1.9 to 10.6 μg/mL), *L. donovani* axenic amastigote strain MHOM-ET-67/L82 (with IC_{50} values ranging from 0.4 to 7.1 μg/mL), and *P. falciparum* strain NF54 (with IC_{50} values ranging from 1.1 to 10.3 μg/mL) [54]. Hamdi et al. (2018) have reported that limonene significantly reduced and killed the *L. mexicana* promastigotes with IC_{50} value of 16.59 μg/mL [55]. Wang et al. have demonstrated the insecticidal activity of limonene against *Tribolium castaneum*

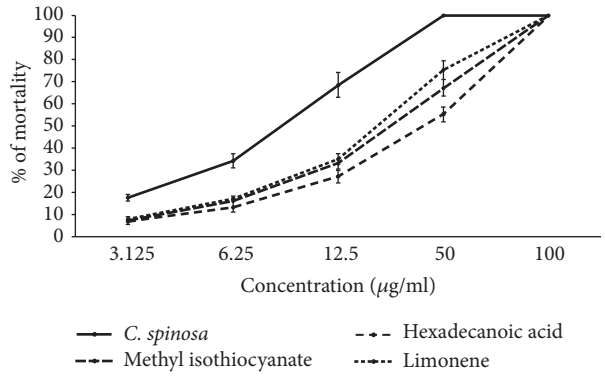

Figure 3: Anti-leishmanial effects of various concentrations of CSEO and its main components, methyl isothiocyanate, hexadecanoic acid, and limonene, against intracellular amastigote forms of *L. major* by the macrophage model, whereas among the main components of CSEO, the highest to the lowest anti-leishmanial effect was observed in limonene > methyl isothiocyanate > and hexadecanoic acid. Data are presented as the mean ± SD ($n = 3$).

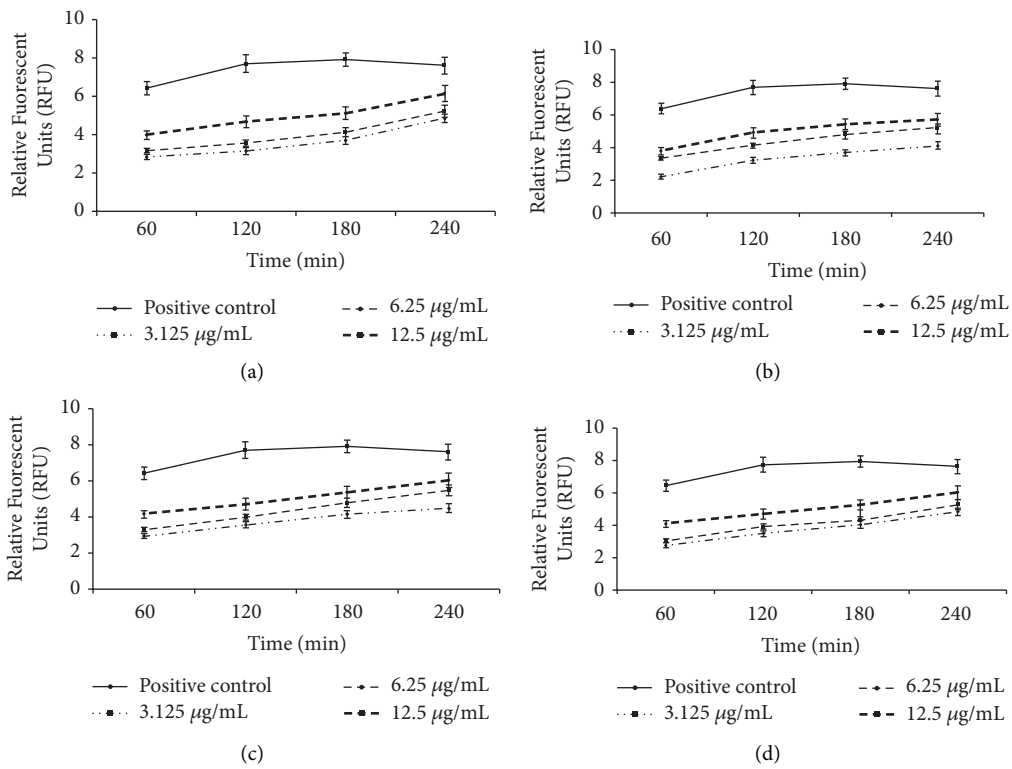

Figure 4: Plasma membrane permeability of the *L. major* promastigotes treated with CSEO (a), hexadecanoic acid (b), methyl isothiocyanate (c), and limonene (d). The results that exhibited relative fluorescent units revealed that the promastigotes treated with CSEO, methyl isothiocyanate, hexadecanoic acid, and limonene as dose-dependent response changed the permeability of plasma membrane by SYTOX Green. Data are presented as the mean ± SD ($n = 3$).

(Herbst) and *Lasioderma serricorne* (Fabricius) with LC_{50} values of 14.97 and 13.66 μg/mL, respectively [56]. Hence, it may be proposed that in addition to the high activity of individual compounds the activity of essential oil is influenced by the synergism of the compounds in lower concentration.

The results of MTT assay in this study exhibited that the essential oil of *C. spinosa* and its main components, methyl isothiocyanate, hexadecanoic acid, and limonene, showed no significant cytotoxicity against macrophage cells (Figure 6). The CC_{50} values for CSEO, methyl isothiocyanate, hexadecanoic acid, and limonene, were 93.7, 216.2, 199.4, and 221.3 μg/mL, respectively (Table 3). Previously, Kulisic Bilusic et al. (2012) have revealed that the essential oil of *C. spinosa* collected from Split City, Croatia, reduced the displayed cytotoxic effects against the HT-29 cells with the CC_{50} values of 261.3 and 373.6 μg/mL, respectively [57].

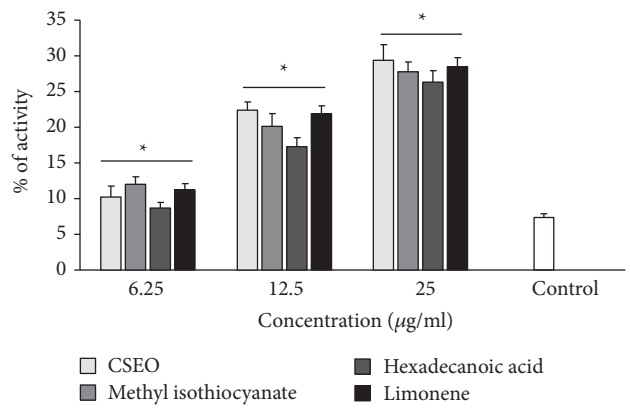

Figure 5: Caspase-3-like activity of *L. major* promastigotes treated with CSEO, methyl isothiocyanate, hexadecanoic acid, and limonene using the colorimetric protease methods. The results exhibited that CSEO, methyl isothiocyanate, hexadecanoic acid, and limonene significantly induced caspase-3 activation as dose-dependent response. $^{*}p < 0.05$ shows that the difference was statistically significant in comparison with control. Data are presented as the mean ± SD ($n = 3$).

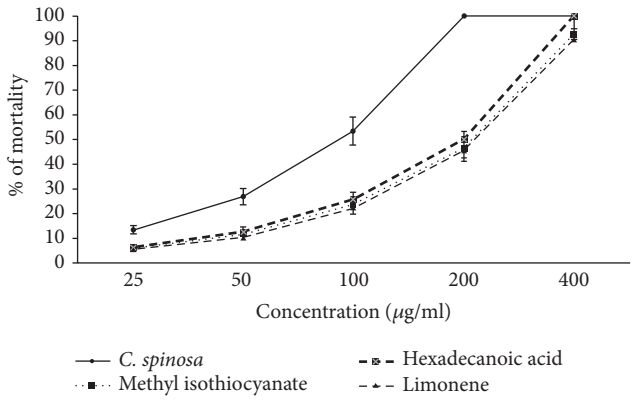

Figure 6: Cytotoxicity effects of various concentrations of CSEO and its main components, methyl isothiocyanate, hexadecanoic acid, and limonene against J774A4 macrophage cells by the MTT assay. Data are presented as the mean ± SD ($n = 3$).

Table 3: Insecticidal, antiplasmodial, and anti-leishmanial activity of the essential oil of *C. spinosa* and its main components, methyl isothiocyanate, hexadecanoic acid, and limonene.

Drug	Insecticidal activity LC_{50} (μg/mL)	Anti-leishmanial effect IC_{50} (μg/mL)	Antiplasmodial activity IC_{50} (μg/mL)	Cytotoxicity effect CC_{50} (μg/mL)
C. spinosa essential oil	21.6 ± 2.15	7.4 ± 0.89	9.1 ± 1.12	93.7 ± 4.54
Methyl isothiocyanate	30.9 ± 3.12	14.5 ± 2.51	20.7 ± 2.54	216.2 ± 8.65
Hexadecanoic acid	41.6 ± 3.62	19.6 ± 3.05	23.3 ± 3.14	199.4 ± 7.58
Limonene	35.3 ± 4.23	21.3 ± 2.15	18.6 ± 2.19	221.3 ± 9.87

4. Conclusion

Based on the obtained results, *C. spinosa* essential oil and its main components, methyl isothiocyanate, hexadecanoic acid, and limonene, displayed insecticidal, antiplasmodial, and anti-leishmanial activity against healthy 4th-instar larvae of *A. aegypti*, chloroquine-resistant *P. falciparum* K1 strain, and *L. major* amastigotes, respectively. In addition, the various concentrations of CSEO, methyl isothiocyanate, hexadecanoic acid, and limonene significantly ($p < 0.05$) increased the plasma membrane permeability and caspase-3-like activity level as dose-dependent response with no consideration of the cytotoxicity against J774A1 macrophage cells. However, further surveys are required to display the mechanisms of action mode of tested drugs and their efficacy in animal model and clinical settings.

Data Availability

The data presented in this study are available in this article.

Conflicts of Interest

The authors declare that there are no conflicts of interest in this study.

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Review Article

Lamiaceae Essential Oils, Phytochemical Profile, Antioxidant, and Biological Activities

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Medicinal and aromatic plants present important active compounds that have potential for use in food, pharmaceutical, and agriculture industries. In this sense, the present work aimed to conduct a literature review on the potential applications of essential oils from *Lamiaceae* species. Antioxidant, anti-inflammatory, and antimicrobial activities were evaluated. The importance of this study is demonstrated as a way to theoretically provide information on the use of different plants belonging to the *Lamiaceae* family, especially with regard to the physical, chemical, and biological properties of its essential oils.

1. Introduction

Several studies have shown that plants have bioactive compounds, such as terpenoids, alkaloids, glycosides, phytohormones, phenolic, and phenylpropanoids, that assist in the development of phytotherapeutic; in addition, natural products can be a viable alternative for the development of new drugs to control microorganisms resistant to traditional antibiotics [1–6]. *Lamiaceae* family has several species of aromatic plants that are applied in traditional medicine and in the pharmaceutical and food industries because of their biological properties [7]. They are used as stimulant for blood circulation and digestion, to strengthen the central nervous system, and as expectorant, antispasmodic, antiseptic [8], diuretic, carminative, and tonic [9]. The most popular plants in this family are oregano, rosemary, thyme, and sage [7].

The biological applications of the *Lamiaceae* are mainly related to its essential oils, which have various activities such as antioxidant, antitumor, anti-inflammatory, antiviral, analgesic, antitussive, antiasthmatic, antipyretic [9], antimicrobial, antiemetic, antifungal [10], insecticidal (against *Aedes aegypti*) [11], antidiabetic, antihypertensive [12], antipruritic, decongestant [13], antinociceptive [14], carminative, antirheumatic, antidepressant, neuroprotective, cholinergic [15], sedative, antiseptic, antiparasitic,

anthelmintic, immunoregulatory [16], antiallergic [17], antiangiogenic, anti-hepatotoxic [18], anticancer [19], and others.

According to Pires et al. [20], medicinal plants began to be used both in traditional medicine (*in natura*) and in vegetal products, such as essential oils, enhancing the investigations of plant species and, consequently, their natural pharmacological agents, considering the different perspectives of rural and urban areas.

According to data from the WHO, more than 70% of the population uses herbal medicines as the main form of medication to treat diseases [21]. This growing interest for less industrialized products with functional ingredients stimulated the use of essential oils in several industrial sectors (food, cosmetics, hygiene, and agriculture), which are applied in product conservation and control of microorganisms [22–24]. The advantage of volatile oils over synthetic preservatives lies in the lower development of toxic side products and economic viability [25, 26].

The essential oils (EOs) are aromatic and volatile substances found in different plant parts (leaves, flowers, seeds, roots, and fruits) [26]. Also, according to the de Oliveira et al., [27], they are extracted by steam distillation, dry distillation, mechanical extraction, or other processes such as supercritical CO_2 extraction. Essential oils are capable of undergoing physical processes which do not significantly alter their chemical compositions. The extraction methods vary according to the species, the plant part used, and the way the raw material is presented: fresh, partially dehydrated, or dried [28–30].

In general, EOs are formed by base elements (oxygen, hydrogen, and carbon), which generate aldehydes, esters, phenols, ketones, alcohols, organic acids, and substances with nitrogen/sulfur, hydrocarbons, and terpenes [31]. These functional groups are responsible for the properties of the oils. Those commonly found come from terpenoids and phenylterpenoids, with monoterpenes being the most frequent [32–34].

For the plant, EOs represent an adaptive advantage, being able to function as an attractant for pollination and as a natural defense against predator attacks [35]. The chemical composition of essential oils can vary within the same species because conditions such as cultivation site, collection method, environmental factors, and material storage can interfere in the production of metabolites [36].

The family to which plants belong can be important to make inferences about the composition of aromatic oils. For instance, *Rutaceae* family presents citrus species; *Myrtaceae* has as representative the eucalyptus; and *Oleaceae*, the jasmine [37–39]. *Lamiaceae* is one of the plant families that presents great interest regarding obtaining essential oils, which will be described in the next topics.

Thus, this paper is organized, besides introduction and final considerations, in three parts: (1) biological presentation and identification of *Lamiaceae* species; (2) chemical structures of biosynthesized molecules present in its EOs; and (3) different properties of these species.

2. *Lamiaceae* Family

The plant family *Lamiaceae*, formerly called Labiatae, for its flowers are characterized by a bilabiate corolla [40], *Lamiaceae* presents more than 7000 species that are grouped in about 240 genera; in Brazil, it is distributed in approximately 524 species belonging to 52 genera [41], and some of these species present aromatic properties, which confers great economic relevance to the *Lamiaceae* [42], being applied in cosmetics and herbal medicines. As examples of genera with such properties, *Mentha, Ocimum, Salvia, Clerodendrum,* and *Plectranthus* stand out.

Lamiaceae species are widely distributed around the globe, with various heights and habitats and greater abundance in the Mediterranean region [43]. They prefer hot areas; however, they can also be found in regions with low temperatures [41, 44]. In Brazil, *Lamiaceae* species are distributed throughout the country, with higher incidence in south, north, and northeast regions, comprising the Atlantic Forest, Amazon, and especially the Brazilian Cerrado [45–48].

The species of the *Lamiaceae* family have diversified morphological characteristics and may be herbs, herbaceous plants, shrubs, or tree species [41]. Nowadays, this is one of the biggest botanical families with flowers of different sizes, with warm and showy colors depending on the species. They are bisexual, with well-defined floral parts, apparent sepals and petals, inflorescence, and bilateral symmetry (zygomorphs), and the corolla tube is divided into two distinct parts, providing a "lip" shape, which is the main characteristic of the Labiatae family. Their leaves are normally simple, and their fruits are dry and multiple that become separated when ripe (schizocarpic fruits) [40, 49, 50].

This family presents many species rich in flavonoids and terpenes, with diterpenoids being the most abundant [51]. They are also rich in other substances that in addition to providing medicinal use have also assisted in taxonomic classifications [52]. Among the spices with aromatic properties, the six best-known vernacular names are thyme, basil, oregano, rosemary, sage, and lemon balm [16]. This variety of bioactive compounds confers *Lamiaceae* properties such as antioxidant, insecticidal, fungicidal, and bactericidal [53], which can result in an aggregation of potential economic and pharmacological value.

3. *Lamiaceae* Species Rich in Essential Oils

Species of the *Lamiaceae* family produce large amounts of secondary metabolites, including the compounds present in essential oils in plants with biological activities and therapeutic potential [41, 44]. Some examples include species *B. officinalis, G. hederacea, H. pectinata. Lavandula. Lamium, M. officinalis, Mentha, M. vulgare, Origanum, Ocimum, R. officinalis, Salvia, S. hortensis, S. lavandulifolia, S. lateriflora, Sideritis, Teucrium, Thymus,* and *Ziziphora tenuior* [54].

The genus *Plectranthus* is considered one of the richest in species diversity and essential oils, with monoterpenes and sesquiterpenes as the main constituents [55]. According to Crevelin et al. [56], the essential oils of *Plectranthus*

neochilus and *Plectranthus barbatus* have antimicrobial effects against *Streptococcus mutans*. Besides antibacterial activity, *Plectranthus* also has antifungal action on *Rhizopus stolonifer* [57] and showed *in vitro* antischistosomal activity attributed to boldo essential oil, which exterminated 100% of *Schistosoma mansoni* adult worms [58]. It also caused reduction in female eggs of B-type *Bemisia tabaci* in tomatoes [59, 60]. *Plectranthus amboinicus* exhibited anti-inflammatory and good digestion activities, as well [61].

Among the herbaceous plants of the *Lamiaceae* family, the genus *Ocimum* is the most important due to its application in several areas [62], such as folk medicine, cooking, plant marketing, and perfumery industry [63]. Approximately 30 species compose this genus [63]. Among them, some are *Ocimum gratissimum, O. basilicum L., O. micranthum,* and *O. campechianum*. The extracts are applied in traditional medicine to treat rheumatism, epilepsy, some mental conditions, and respiratory tract infections [64–66]. Studies also have verified fungicidal, nematicidal, and larvicidal properties [67–70].

Additionally, the antifungal action of essential oils from *Lamiaceae* species has been used to improve food preservation. Isolated essential oils derived from thyme and oregano (thymol), clove (eugenol), and mint (menthol) were tested in strawberry preservation [71]. As a result, the treatment reduced strawberry degradation when compared with the control sample. Thymol oil showed better results, with a decay of 0% on day 1 to 20 on day 14, with better results than the control sample, and the authors concluded that in addition to antimicrobial activity, treatment with essential oils also conferred antioxidant protection.

The essential oil of *O. gratissimum* L. was able to inhibit the growth of species such as *Klebsiella* sp., *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus,* and *Salmonella enteritidis*, even when used at different concentrations [72]. Pereira et al. [73], evaluated the antibacterial activity of *O. gratissimum L.*, *Cymbopogon citratus* (DC) Stapf., and *Salvia officinalis* L. on microorganisms isolated from urinary tract infections. *Salvia officinalis* L. showed the best results, inhibiting the growth of more than 75% of all microorganisms evaluated. Species such as *Salvia santolinifolia L, Salvia hydrangea L, Salvia mirzayanii L, Salvia triloba L, Salvia repens L,* and *Salvia runcinata L.* also stand out.

The genus *Hyptis* (*Hyptis ovalifolia* Benth, *Hyptis suaveolens* L, and *Hyptis pectinata* L.) is predominant in the semiarid region of Northeastern Brazil, with prevalence of herbs but also with shrubby representatives and small trees [74]. Its EO has antiseptic, insecticidal, and fungicidal activities in addition to treating gastrointestinal infections and muscle pain [72, 75]. In addition, the genus *Perilla*, whose main representative is *Perilla frutescens* L., has insecticidal activity, which is given by the isolated compound [76].

Genus *Mentha*, popularly known as mint, has menthol terpenes as main constituents of its essential oil. The greater yield is found in its leaves, presenting a considerable economic potential for food and pharmacological purposes [77, 78]. This genus has a small diversity of plants all over the globe, containing only 25 species [79]. The biological activities presented by these *Lamiaceae* species are varied, e.g., antihypertensive, antioxidant, antimicrobial, antiallergic, biopesticidal, antitumor, anticancer, anti-inflammatory, and antiviral [80], which may be associated with the presence of compounds such as menthol, menthone, 1,8-cineole, carvone, limonene, *ß*-caryophyllene, and pulegone, among others [81].

Research on the genus *Satureja* L. reports that it is distributed in 30 species around the globe, and that it has beneficial properties for human health, such as in the treatment of pain caused by oxidative stress. Therefore, the essential oils from leaves and stems of *Satureja spicigera* L, *Satureja cuneifólia* L, and *Satureja hortensis* L. have compounds that work as antioxidants [82, 83].

Genus *Thymus* presents about 200 species. Most of them have antibacterial action due to the high content of phenolic compounds. *T. caespititius, T. camphoratus*, *T. pectinatus* Fihch, *T. mastichina* L., and *Thymus vulgaris* L. [84] are great examples, whereas *T. numidicus* and *T. fontanesii* have bactericidal activity [85].

4. Chemical Composition of *Lamiaceae* Essential Oils

Essential oils are volatile, lipophilic, and odoriferous substances produced by the secondary metabolism of plants. Due to their aromatic properties and chemical composition, they are used in cosmetics and folk medicine, with antiseptic, antifungal, and insecticidal actions [7].

In general, the biological activities that essential oils present are observed by the major substances present in their chemical composition. Their bioactivity is shown synergistically or by the isolated substances [86, 87].

In addition, the chemical composition of an essential oil can vary depending on the species, seasonality, circadian rhythms, plant age, and geographic location [36, 88]. As an example, the chemical profile of *Hesperozygis myrtoides* essential oil, which is a subshrub native to Cerrado and Atlantic Forest of Brazil, depends on altitude [89].

Essential oils are characterized by two or three major constituents. For instance, *Mentha arvensis* L. presented as major compounds menthol (86.1%), menthone (4.3%), and isomenthone (3.7%) [90]; *O. gratissimum* L. showed as major constituents 1,8-cineole (30.04%), eugenol (27.58%), and terpineol-4 (14.45%) [91]; *Origanum vulgare* L. presented 4-terpineol (18.4%), sabinene hydrate (15.6%), and thymol (13.6%) [92]; and in the species *Plectranthus ornatus* Codd, the major compounds identified were α-thujene (12.7–32.7%), α-pinene (5.5–23%), sabinene (7.51–17.8%), β-pinene (3.5–11.6%), 1-octen3-ol (0.6–11.1%), 3-carene (0.84–5.6%), (E)-β-ocimene (1.5–8.4%), α-terpinyl acetate (1.3–13.2%), β-caryophyllene (3.9–13.6%), and germacrene D (0.3–18.5%) [93].

Giatropoulos et al. [94] evaluated 12 species of different plants of the *Lamiaceae* family and found high insecticidal action in the essential oils of *T. vulgaris* and *O. vulgare*. Such properties can be attributed to the high toxicity of its major constituents such as thymol (75.6%), carvacrol (74.08%), and p-cymene (7.9%). The considerable toxicity of *Satureja thymbra* essential oil is also observed probably due to its

major constituents carvacrol (32.4%) and γ-terpinene (32.4%).

Thus, the number of studies that seek applicability of the compounds present in *Lamiaceae* essential oils has increased since they have natural origin and present advantages when compared with synthetic substances [7]. Table 1 lists species rich in essential oils and their main constituents. Figures 1 and 2 show the main monoterpenes and sesquiterpenes identified in *Lamiaceae* essential oils.

5. Antioxidant Activity

Antioxidants are substances capable of retarding or preventing lipid oxidation caused by excessive oxygen radicals due to environmental factors or pathogens [110, 111]. Such compounds, which can be natural or synthetic, have great importance in the food industry because they are used as preservatives in several products, delaying or preventing deterioration caused by the action of oxygen. Besides this, antioxidants have great relevance in biochemical and medical fields because they are able to neutralize the harmful effects of oxidation in animal tissues [112].

In recent years, there has been an increasing search for natural products with antioxidant properties due to the toxic side problems that synthetic products may cause [110]. Aromatic and medicinal plants are considered natural sources of antioxidant substances since their secondary metabolites act by inhibiting the formation of free radicals [113]. The aromatic and medicinal species of the *Lamiaceae* family have been constantly studied regarding their antioxidant activities, as shown in Table 2.

There are several techniques that determine the antioxidant capacity of essential oils and their components, among them are FRAP (ferric reducing antioxidant power), CUPRAC (cupric ion reducing antioxidant capacity), ABTS (2,20-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)), DPPH (2,2-diphenyl-1-picrylhydrazyl), quantification of products formed during lipid peroxidation (TBARS, LDL oxidation, and co-oxidation of β-carotene), and other methods [131].

The chemical composition and antioxidant activity of *O. vulgare* essential oil were studied by Morshedloo et al. [124]. After being analyzed by the DPPH method, all oils presented antioxidant activity, which was correlated with the high concentration of carvacrol. The essential oil from *O. vulgare* flowers showed the highest capacity to eliminate DPPH radicals ($EC_{50} = 0.68$ mL/mL), while the stem oil showed the lowest capacity ($EC_{50} = 1.82$ mL/mL). Regarding phenological stages, flowering showed the highest antioxidant activity ($EC_{50} = 0.86$ mL/mL) [123]. The authors also pointed out the strong antioxidant action of the essential oil from *Origanum vulgare* aerial parts using ABTS radical scavenging technique with $IC_{50} = 14.00$ μg/mL.

Besides *O. vulgare*, other species of the genus *Origanum* are reported in the literature to possess antioxidant activity. The essential oil of *O. dictamnus* flowers showed antioxidant activity by the DPPH method ($IC_{50} = 0.0459 \pm 0.0042\%$ (v/v)) that was attributed to its main compound, carvacrol [121]. The oils from aerial parts of *O. floribundum* were studied by Hadjadj et al. [122] regarding their antioxidant potential by DPPH and ABTS assays. They presented better antioxidant activity by the ABTS method (33.6–95.5 μg/mL).

Zorzetto et al. [114] evaluated the antioxidant activity of *Cedronella canariensis* aerial parts using three methodologies (DPPH, ABTS, and FRAP). The authors demonstrated that the essential oil showed better antioxidant activity against the ABTS radical with $IC_{50} = 10.5$ mg/mL, which was about 20 times lower than Trolox. Although the DPPH method is similar to ABTS, *C. canariensis* oil presented low antioxidant activity ($IC_{50} = 615.5$ mg/mL), about 500 times lower than Trolox. In addition, EOs of *O. basilicum* were shown to possess antioxidant activity by DPPH ($IC_{50} = 0.21$–4.04 mg/mL) and β-carotene (bleaching content = 23.8–85.3%) [120].

Rosmarinus officinalis is known to possess several biological properties. Aerial parts of this species were collected in southeastern Anatolia (Turkey), and its essential oil showed antioxidant activity by DPPH and TBARS techniques with $IC_{50} = 10.08 \pm 0.15$ μg/mL and 1.76 ± 0.02 μg/mL, respectively, which may be related to polyphenols and phenylpropanoids found in the oil [125]. Moghadam [126] showed that the essential oil of *R. officinalis* collected in Kermanshah (Iran) presented antioxidant activity, by DPPH assay (13.00 ± 0.51 μg/mL), which was related to the presence of camphene and 1,8-cineole. Table 2 shows the relationship between *Lamiaceae* species and their antioxidant potential.

6. Anti-Inflammatory Activity

Inflammation is a sequential process produced by various biological stimuli, physical injuries, infectious agents, and antigen-antibody type interactions. Within the inflammatory process, there are reactive oxygen species (ROS) responses, which include superoxide anions, hydroxyl radicals, and hydrogen peroxides. These are released by activated macrophages, neutrophils, and dendritic cells [132].

The inflammatory process and its chain of development have presented relevance, and in this sense, intending to restrain this aggressive action on the organism, search for new anti-inflammatory agents, mainly of vegetable origin, is necessary [133]. It is worth emphasizing that within this branch, species such as *Hyptis spicigera,* which is used in folk medicine, have anti-inflammatory properties [134].

Essential oils from *O. basilicum* and *O. gratissimum* were obtained by hydrodistillation and hexane extraction. *O. basilicum* EO extracted by hydrodistillation presented eucalyptol and eugenol acetate, and the solvent extract presented 2-methylbenzyl and eugenol acetate. Regarding *O. gratissimum* EO, linalool, 1-terpinen-4-ol, alpha-caryophyllene, and trans-longipinocarveol were the major compounds. In this study, both EOs were analyzed for anti-inflammatory potential on induced edema in rat ears, and the results showed that at doses of 50 µg/ear, they exhibited significant anti-inflammatory effect ($p < 0.05$), with inhibitions of up to 80%. According to the authors, these results were in accordance with the 100 μg/ear hydrocortisone dose, which showed 54.8% of edema inhibition [118].

Table 1: Chemical composition and yield of essential oils from *Lamiaceae* species.

Species	Plant part	Essential oil yield	Main compounds	Reference
Aeollanthus suaveolens Mart. ex Spreng	Leaves	1.6%	Massoia lactone, linalool,(*E*)-B-farnesene, geraniol, 2,5-dimethoxy-p-cymene	[95]
Calamintha nepeta (L.) Kuntze	Leaves	*	Pulegone, menthone, limonene	[96]
Clinopodium macrostemum (Moc. and Sessé ex Benth.) Kuntze	Leaves	0.80%	linalool, nerol, caryophyllene, menthone, geranyl acetate, terpineol, pulegone	[97]
Hyptis dilatata Benth.	Leaves	*	Fenchone, 3-carene, α-pinene, β-caryophyllene, limonene, β-pinene, and camphor	[98]
Hyptis martiusii Benth.	Leaves	0.34%	1,8-cineole, d-carene, camphor, limonene, germacrene B	[99]
Lavandula dentata L.	Leaves and stems	*	1,8-cineole, isolimonene, thuj-3-en-10-al, trans-pinocarveol	[100]
Melissa officinalis L.	Leaves and flowers	0.10%	Citral, caryophyllene oxide, citronellal, geraniol, geranyl acetate, β-caryophyllene	[101]
Mentha arvensis L.	Leaves and flowers	*	Citronellal and nerol	[102]
M. piperita L	Leaves and flowers	*	Menthone, menthol, pulegone and menthyl acetate	[103]
Minthostachys mollis (Benth.) Griseb.	Leaves	0.98%	Menthone, pulegone, cis-dihydrocarvone, carvacryl acetate, linalyl acetate, and linalool	[104]
Ocimum basilicum L.	Leaves	$1.56 \pm 0.15\%$	linalyl acetate and linalool	[105]
O. gratissimum L.	Leaves	*	1,8-Cineole, eugenol, 4-terpineol	[91]
O. gratissimum	Leaves and flowers	*	Thymol, eugenol, 1,8-cineole, E-caryophyllene, β-selinene	[106]
Origanum scabrum Boiss. and Heldr.	Leaves	1.5%	Carvacrol, thymol, p-cymene,γ-terpinene	[107]
Origanum vulgare L.	Leaves	*	4-terpineol, sabinene hydrate, thymol	[92]
Plectranthus amboinicus (Lour.) Spreng.	Leaves	0.009%	Thymol, β-pinene, γ-terpinene, caryophyllene	[108]
Plectranthus barbatus var. grandis (L.H. Cramer) Lukhoba and A.J. Paton	Leaves	*	β-caryophyllene, α-copaene, germacrene	[109]

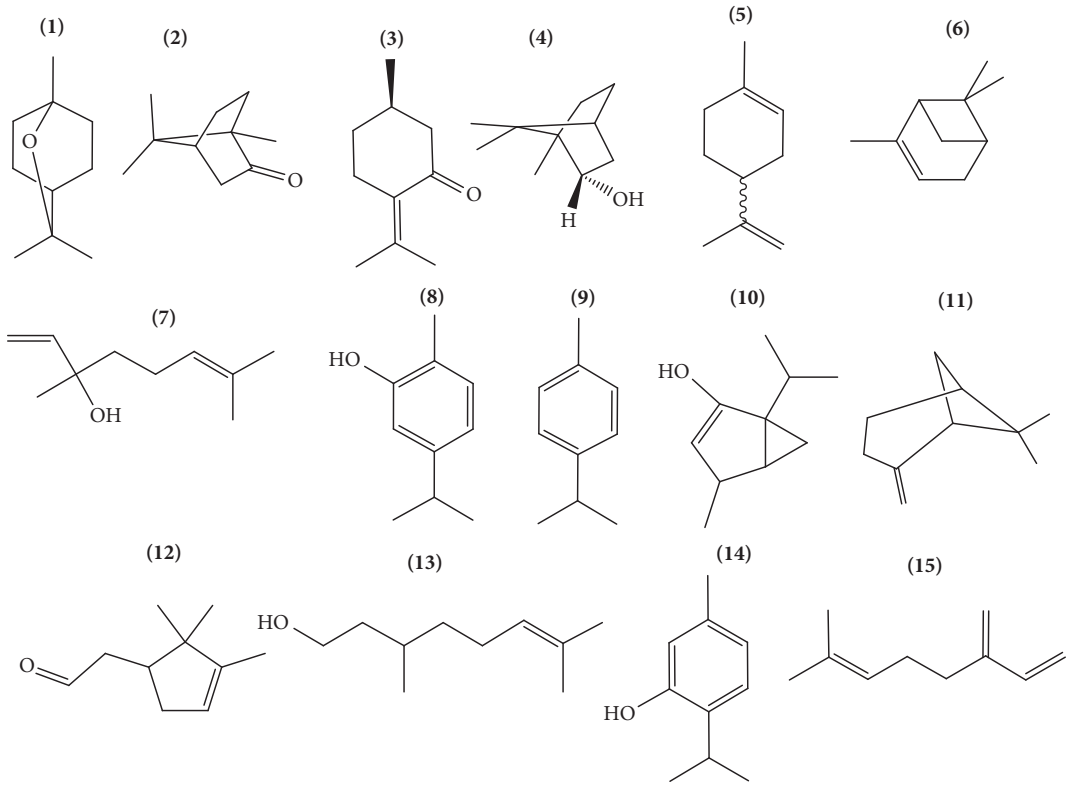

Figure 1: Monoterpenes: (1) = 1,8-cineole, (2) = camphor, (3) = pulegone, (4) = borneol, (5) = limonene, (6) = α-pinene, (7) = linalool, (8) = carvacrol, (9) = p-cymene, (10) = thujanol, (11) = β-pinene, (12) = α-campholenal, (13) = citronellol, (14) = thymol, and (15) = β-myrcene.

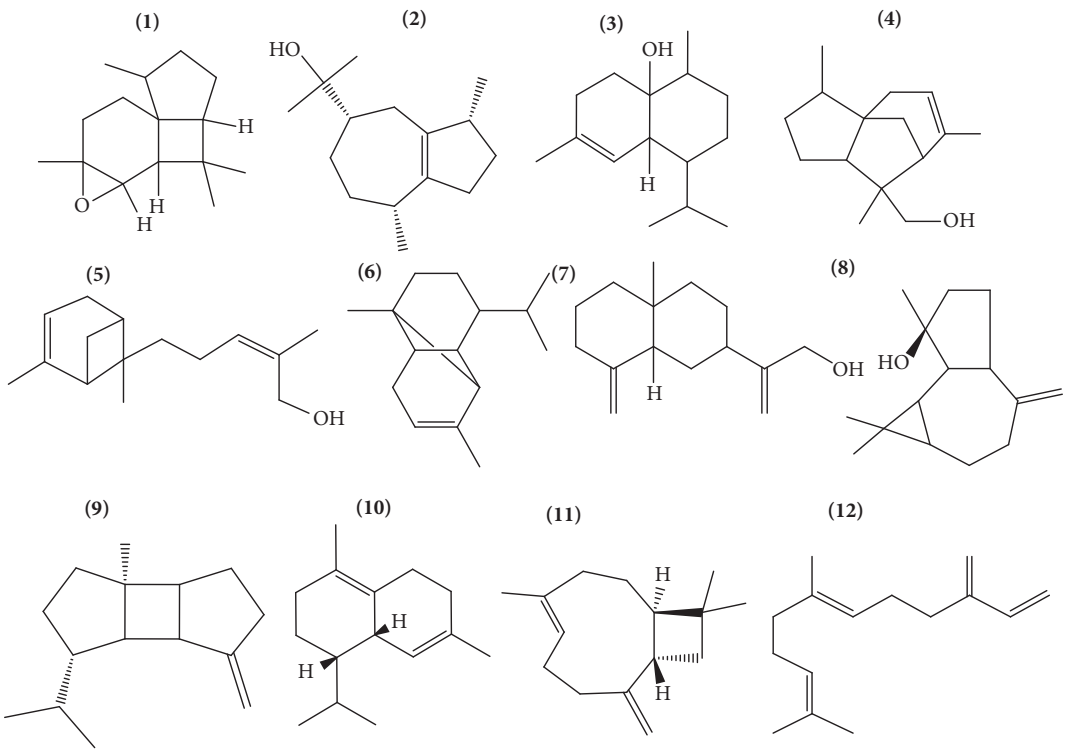

Figure 2: Sesquiterpenes: (1) = Italicene epoxide, (2) = guaiol, (3) = 1,10-di-epi-cubenol, (4) = 8-cedren-13-ol, (5) = (Z)-α-trans-bergamotol, (6) = α-copaene, (7) = β-costol, (8) = spathulenol, (9) = ß-bourbonene, (10) = δ-cadinene, (11) = ß-caryophyllene, and (12) = ß-farnesene.

Six EOs from *Lamiaceae* family (*Perilla frutescens*, *Mentha haplocalyx*, *Pogostemon cablin*, *R. officinalis*, *Lavandula angustifólia,* and *Scutellaria baicalensis)* were studied regarding their anti-inflammatory potential. The major compounds found were linalool (0.05–46.55%), α-pinene (0.12–45.35%), *o*-cymene (0.91–41.20%), patchouli alcohol (28.27%), dl-menthol (21.12%), isobornyl acetate (22.52%), D-limonene (0.01–18.42%), α-terpineol (0.07–4.88%), and β-pinene (0.08–2.03%). The anti-inflammatory tests were performed on the ears of rats of 6–8 weeks of age and bodyweight of 18 ± 2 g. They were induced by 12-O-tetradecanoyl phorbol-13-acetate (TPA), and the drug ibuprofen was used as positive control. All six essential oils exhibited anti-inflammatory activity, and the essential oils isolated from *P. cablin* remarkably inhibited the formation of ear edema (29.87–81.25% inhibition). Similarly, *R. officinalis* and *Scutellaria baicalensis* EOs worked better than ibuprofen (positive control) [135].

Thymol was the major compound found in *Thymus vulgaris* essential oil from two different regions of Algeria (Mostaganem-EO.TM and Tlemcen-EO.TT), with contents of 59.5% and 67.7%, respectively. The anti-inflammatory activity was evaluated *in vivo* based on the inhibition of paw edema induced by carrageenan injection. As a result, both EO samples showed anti-inflammatory activities after 6 hours of administration (400 mg/kg), reducing paw edema by 58.4% for EO.TT and 50.4% for EO.TM [136]. In the study by Avola et al. [137], *Origanum vulgare* EO presented as major compounds carvacrol (35.95–0.22%), thymol (25.2–0.27%), *p*-cymene (21.54–0.35%), and linalool (4.26–0.05%). This essential oil was tested to characterize the level of oxidative stress and evaluate the changes in intracellular ROS levels caused by IFNγ and histamine in the presence or absence of 25 μg/mL of oil. Confluent NCTC 2544 cells were treated with H2DCFDA 72 h after stimulation. This ROS levels can cause inflammation-induced cellular damage. In this study, the results pointed out that cells pretreated with *O. vulgare* essential oil at 25 μg/mL or indomethacin at 10 μM significantly reduced IFNγ- and histamine-induced ROS production.

The chemical profile of *Stachys lavandulifolia* essential oil (EOSL) was characterized by the main compounds (-)-α-bisabolol (56.4%), bicyclogermacrene (5.3%), δ-cadinene (4.2%), and spathulenol (2.9%). And the anti-inflammatory activity of (-)-α-bisabolol (BIS) and EOSL (50 mg/kg) was evaluated using carrageenan-induced inflammatory response in rats (2% in 0.2 mL). The results showed that both EOSL and BIS possessed significant inhibitory effects ($p < 0.05$ or $p < 0.01$ or $p < 0.001$) on different orofacial pain tests, but BIS proved to be more effective, significantly reducing nociceptive behavior in all tests [138].

7. Antimicrobial Activity

7.1. Antibacterial Activity. *Lamiaceae* family has great importance in the economic scenario, especially in the gastronomic sector, in which they are used as culinary herbs. Thus, there has been an incessant search for new antimicrobial agents from the secondary metabolism of plants [8], which according to Nieto (2017) can increase the shelf life of food products [7].

Table 2: Antioxidant activity of essential oils from *Lamiaceae* species.

Species and plant part	Technique	Results	Reference
Cedronella canariensis (aerial parts)	DPPH	$IC_{50} = 615.5 \pm 76.5\ \mu g/mL$	[114]
	ABTS	$IC_{50} = 10.5 \pm 0.6\ \mu g/mL$	
	FRAP	$IC_{50} = 3.8 \pm 1.4\ 6\ \mu mol$ TE/g	
Mentha piperita (leaves)	DPPH	Radical scavenging = $92.6 \pm 6.86\%$	[115]
	Reducing power	Reducing power = 0.9 ± 0.3	
M. pulegium (aerial parts)	DPPH	$IC_{50} = 321.41 \pm 2.53\ \mu g/mL$	[116]
	FRAP	$IC_{50} = 58.27 \pm 2.72\ \mu g/mL$	
M. rotundifolia (leaves)	DPPH	$IC_{50} = 2222.2 \pm 25.2\ \mu g/mL$	[117]
	ABTS	$IC_{50} = 133.8 \pm 4.8\ \mu g/mL$	
	Reducing power	$IC_{50} = 166.6 \pm 1.9\ \mu g/mL$	
	Phosphomolybdate	$IC_{50} = 45.2 \pm 1.2\ \mu g/mL$	
M. spicata (aerial parts)	DPPH	$IC_{50} = 3450 \pm 172.5\ \mu g/mL$	[118]
	ABTS	$IC_{50} = 40.2 \pm 0.2\ \mu g/mL$	
	FRAP	$IC_{50} = 215 \pm 4.50\ \mu g/mL$	
M. spicata (leaves)	DPPH	$IC_{50} = 41,\ 23\ \mu g/mL$	[119]
O. basilicum (aerial parts)	DPPH	$IC_{50} = 4.04 \pm 0.09–0.21 \pm 0.02$ mg/mL	[120]
	β-carotene	Bleaching = $23.8 \pm 0.6–85.3 \pm 1.0\%$	
Origanum dictamnus (flowers)	DPPH	$IC_{50} = 0.0459 \pm 0.0042\%$ (v/v)	[121]
O. floribundum (aerial parts)	DPPH	$IC_{50} = 369.9 \pm 3.1– 1091.7 \pm 4.5\ \mu g/mL$	[122]
	Reducing power	$IC_{50} = 230 \pm 5.2– 315 \pm 3.9\ \mu g/mL$	
	ABTS	$IC_{50} = 33.6 \pm 0.3– 95.5 \pm 2.2\ \mu g/mL$	
O. vulgare (aerial parts)	ABTS	$IC_{50} = 14{,}00257$ mg/mL	[123]
O. vulgare (flowers)	DPPH	$EC_{50} = 0.68$ mL/mL	[124]
O. vulgare (stems)	DPPH	$EC_{50} = 1.82$ mL/mL	[124]
R. officinalis (aerial parts)	DPPH	$IC_{50} = 523.41 \pm 8.25\ \mu g/mL$	[116]
	FRAP	$IC_{50} = 85.74 \pm 7.57\ \mu g/mL$	
R. officinalis (aerial parts)	DPPH	$IC_{50} = 10.08 \pm 0.15\ \mu g/mL$	[125]
	TBARS	$IC_{50} = 1.76 \pm 0.02\ \mu g/mL$	
R. officinalis (aerial parts)	DPPH	$IC_{50} = 13.00 \pm 0.51\ \mu g/mL$	[126]
Satureja hortensis (aerial parts)	DPPH	$IC_{50} = 13.45 \pm 0.35\ \mu g/mL$	[127]
Scutellaria immaculate (aerial parts)	DPPH	$IC_{50} = 82.8 \pm 3.1\ \mu g/mL$	[128]
	ABTS	$IC_{50} = 37.8 \pm 0.9\ \mu g/mL$	
	FRAP	$IC_{50} = 720.19 \pm 4.8\ \mu g/mL$	
S. ramosissima (aerial parts)	DPPH	$IC_{50} = 82.8 \pm 3.1\ \mu g/mL$	[128]
	ABTS	$IC_{50} = 93.6 \pm 0.8$ µg/mL	
	FRAP	$IC_{50} = 837.23 \pm 3.2\ \mu g/mL$	
S. schachristanica (aerial parts)	DPPH	$IC_{50} = 57.6 \pm 2.7\ \mu g/mL$	[128]
	ABTS	$IC_{50} = 66.6 \pm 1.2\ \mu g/mL$	
	FRAP	$IC_{50} = 779.64 \pm 8.6\ \mu g/mL$	
Teucrium flavum (aerial parts)	DPPH	$IC_{50} = 31.5 \pm 1.8\ \mu g/mL$	[129]
Thymus capitatus (Leaves)	DPPH	$IC_{50} = 0.619 \pm 0.11\ \mu g/mL$	[130]
	FRAP	$IC_{50} = 2{,}13 \pm 0.07\ \mu g/mL$	
	TAC	$IC_{50} = 0.78 \pm 0.14\ \mu g/mL$	

Essential oils from aerial parts (leaves) of *Teucrium africanum* and *T. trifidum* were characterized by the sesquiterpene hydrocarbons α-cubebene and β-cubebene, respectively. In this study, they were evaluated for their antimicrobial potential. *T. africanum* EO showed minimum inhibitory concentration (MIC) equal to 0.16 mg/mL against Gram-positive bacterium *Streptococcus pyogenes* (ATCC 25923). Similarly, *T. trifidum* EO demonstrated remarkable antimicrobial activity with the MIC of 2 mg/mL against Gram-positive bacterium *Staphylococcus aureus* (ATCC 8668) [139].

In another study, the essential oil from the leaves and flowers of *Origanum compactum*, collected in six regions of Morocco was characterized by the major compounds carvacrol (2.18–63.65%), *p*-cymene (6.69–42.64%), and thymol (0.16–42.37%). The antimicrobial activity of *O. compactum* EO was quite effective, being most active against *Escherichia coli*, *Listeria innocua*, and *Staphylococcus aureus* with inhibitory zones of 29.00 ± 0.35 mm, 49.00 ± 1.00 mm, and 43.00 ± 0.35 mm, respectively [140].

The major compounds such as citronellal (14.40%), isogeraniol (6.40%), and geranyl acetate (10.20%)

Table 3: Antibacterial activity of *Lamiaceae* essential oils.

Species	Bacteria	Method applied	Results	Reference
Mentha spicata	*E. coli*	Disc-diffusion	11.8–21 mm	[119]
	S. enterica		8–18 mm	
	P. aeruginosa		10–16 mm	
	S. aureus		8–13 mm	
	S. epidermidis		10.1–11.2 mm	
	B. subtilis		9–11.5 mm	
Melissa officinalis	*P. aeruginosa*	Agar-disc-diffusion	16.0 ± 1.2 mm	[141]
	K. pneumonia		3.0 ± 0.6 mm	
	S. aureus		20.0 ± 1.6 mm	
	C. koseri		14.0 ± 1.0 mm	
Origanum compactum	*E. coli* K12	Microdilution	29.00 ± 0.35 mm	[140]
	L. innocua 4030		49.00 ± 1.00 mm	
	S. aureus 25.923		43.00 ± 0,35 mm	
O. vulgare	*M. luteus*	Microdilution	*270 mg/mL*	[142]
	S. aureus		*263 mg/mL*	
	E. coli		*214 mg/mL*	
	P. aeruginosa		*383 mg/mL*	
Salvia ringens	*E. coli*	Microdilution	14.25	[144]
	S. typhimurium		14.25	
	S. enteritidis		11.40	
	P. tolaasii		14.25	
	P. aeruginosa		17.10	
	P. mirabilis		17.10	
	S. aureus		9.50	
	B. cereus		9.50	
	M. flavus		9.50	
	S. lutea		11.40	
	L. monocytogenes		9.50	
Teucrium africanum	*S. pyogenes* (ATCC)	Microdilution	0.16 mg/mL	[139]
T. trifidum	*S. aureus*	Microdilution	2 mg/mL	
Thymus pulegioides	.	Turbidity measurements	0.5 mg/mL	[143]
		CFU	27.500 bacterial/mL	
T. serpyllum	S. mutans	Turbidity measurements	0.9 mg/mL	
		CFU	1.750.000 bacterial/mL	
T. vulgaris	S. mutans	Turbidity measurements	0.75 mg/mL	
		CFU	3500 bacterial/mL	
T. zygis	S. mutans	Turbidity measurements	0.5 mg/mL	
		CFU	4500 bacterial/mL	

characterized the leaf essential oil of *Melissa officinalis*. It showed significant antimicrobial activity against *Pseudomonas aeruginosa*, *Klebsiella pneumonia, Staphylococcus aureus,* and *Citrobacter koseri* when compared with the conventional antibiotics cefaclor, oxacillin, and vancomycin [141].

Khan et al. [142] evaluated the chemical composition of the leaf essential oil of *O. vulgare*, which presented carvacrol (70.2 ± 1.37%) and γ-terpinene (5.6 ± 0.11%). In this study, *O. vulgare* EO was evaluated for its antimicrobial potential against Gram-positive (*Micrococcus luteus* and *Staphylococcus aureus*) and Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) bacteria in comparison with its purified compound carvacrol. The results showed that carvacrol was more effective and completely inhibited the growth of *E. coli* at 200 mg/mL and also retarded the growth of *P. aeruginosa*, with IC_{50} value of 151 mg/mL. The essential oil, on the other hand, inhibited bacterial growth at concentrations of 270, 263, 214, and 383 mg/mL for *M. luteus*, *S. aureus, E. coli,* and *P. aeruginosa,* respectively.

In the study conducted by Niksic et al. [119], major compounds carvone (56,4%), limonene (16,2%), 1,8-cineole (7%), β-pinene (2,4), and α-terpinene (2,3%) characterized Mentha spicata essential oil. It exhibited significant bactericidal activity against both Gram-positive and Gram-negative microorganisms, with M. spicata essential oil being more sensitive and showing greater zone of inhibition against *Escherichia coli* (11.8–21 mm), *Salmonella enterica* (8–18 mm), and *Pseudomonas aeruginosa* (10–16 mm). Gram-positive bacteria, on the other hand, showed moderate antimicrobial activity at concentrations of 1%, 5%, and 10%, against *S. aureus* (8–13 mm), *Staphylococcus* epidermidis (10.1–11.2 mm), and *Bacillus* subtilis (9–11.5 mm). According to the authors, Mentha spicata antibacterial

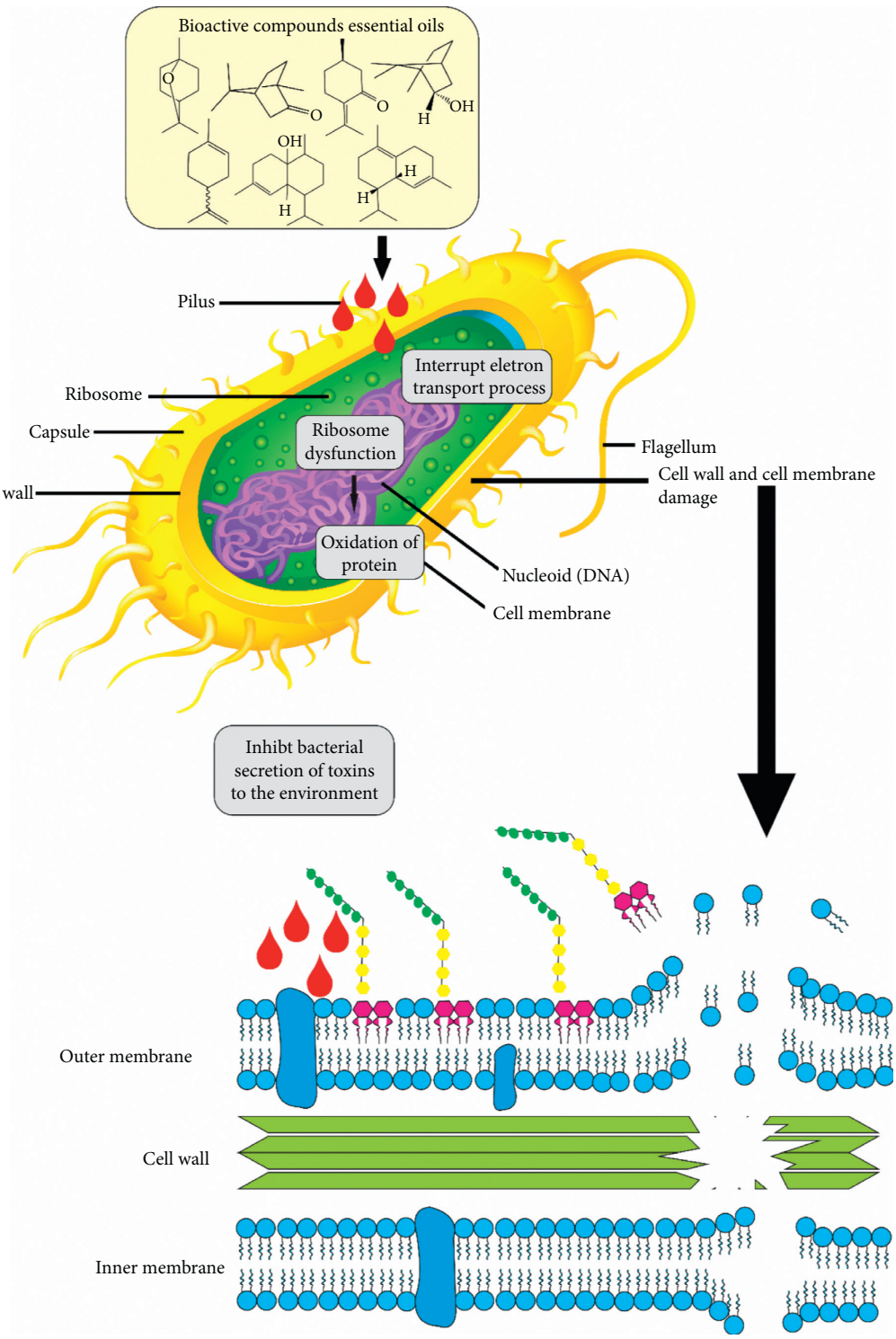

Figure 3: Potential mechanism of action of essential oils on bacteria, adapted from [145,146].

activity can be attributed to the presence of several chemical groups, such as oxygenated monoterpenes and hydrocarbons, which favors the use of M. spicata essential oil as an antiseptic agent in the pharmaceutical and food industries.

The essential oils of four species of the genus Thymus (*T. vulgaris*, T. zygis, T. serpyllum, and T. pulegioides) were analyzed, and their chemical profile was characterized by oxygenated monoterpenes and thymol, which

Table 4: Antifungal activity of *Lamiaceae* essential oils.

Species	Fungi	Method applied	Results	Reference
Lepechinia mutica	*C. albicans*	Broth microdilution	MIC >9 mg/mL	[148]
	M. canis		2.2 < MIC ≤4.5 mg/mL	
	T. rubrum		2.2 < MIC ≤4.5 mg/mL	
	F.graminearum		MIC >9 mg/mL	
	P.oryzae		MIC >9 mg/mL	
O. basilicum	*A. flavus*	Potato dextrose agar (PDA)	500 ppm: 30% 750 pp : 50% 1000 ppm: 70%	[150]
O. basilicum	*C. albicans*	Sabouraud dextrose agar (SDA)	MIC: 1.25 μL/mL MLC: 2.5 μL/mL	[151]
	C. tropicalis		MIC: 2.5–1.25 μL/mL MLC: 2.5 μL/mL	
	C. krusei		MIC: 1.25 μL/mL MLC: 2.5 μL/mL	
	C. guilliermondii		MIC: 1.25 μL/mL MLC: 1.25 μL/mL	
	C. parapsilosis		MIC: 1.25 μL/mL MLC: 2.5 μL/mL	
	C. neoformans		MIC: 0.16–0.32 μL/mL MLC: 0.64–0.32 μL/mL	
	T. mentagrophytes		MIC: 0.64 μL/mL MLC: 1.25 μL/mL	
	T. mentagrophytes var. interdigitale		MIC: 0.64–0.32 μL/mL MLC: 1.25 μL/mL	
	T. rubrum		MIC: 0.64 μL/mL MLC: 1.25 μL/mL	
	T. verrucosum		MIC: 0.64 μL/mL MLC: 1.25 μL/mL	
	M. canis		MIC: 0.64 μL/mL MLC: 1.25 μL/mL	
	M. gypseum		MIC: 0.64 μL/mL MLC: 1.25 μL/mL	
	E. floccosum		MIC: 0.64 μL/mL MLC: 0.64 μL/mL	
	A. niger		MIC: 0.64 μL/mL MLC: 5 μL/mL	
	A. fumigatus		MIC: 1.25 μL/mL MLC: 5 μL/mL	
	A. flavus		MIC: 1.25 μL/mL MLC: 2.5 μL/mL	
O. tenuiflorum	*C. albicans*	Sabouraud dextrose agar (SDA)	MIC: 0.64 μL/mL MLC: 1.25 μL/mL	
	C. tropicalis		MIC: 0.64 μL/mL MLC: 1.25 μL/mL	
	C. krusei		MIC: 0.64 μL/mL MLC: 2.5 μL/mL	
	C. guilliermondii		MIC: 0.64 μL/mL MLC: 1.25 μL/mL	
	C. parapsilosis		MIC: 0.64 μL/mL MLC: 2.5 μL/mL	
	C. neoformans		MIC: 0.16 μL/mL MLC: 0.64 μL/mL	
	T. mentagrophytes		MIC: 0.32 μL/mL MLC: 0.32 μL/mL	
	T. mentagrophytes var. interdigitale		MIC: 0.32 μL/mL MLC: 0.64 μL/mL	
	T. rubrum		MIC: 0.32 μL/mL MLC: 0.64 μL/mL	
	T. verrucosum		MIC: 0.32 μL/mL MLC: 0.64 μL/mL	
	M. canis		MIC: 0.32 μL/mL MLC: 0.64 μL/mL	
	M. gypseum		MIC: 0.32 μL/mL MLC: 0.32–0.64 μL/mL	
	E. floccosum		MIC: 0.32 μL/mL MLC: 0.32 μL/mL	
	A. niger		MIC: 0.64 μL/mL MLC: > 10 μL/mL	
	A. fumigatus		MIC: 0.64 μL/mL MLC: > 10 μL/mL	
	A. flavus		MIC: 0.64 μL/mL MLC: > 10 μL/mL	

Table 4: Continued.

Species	Fungi	Method applied	Results	Reference
Origanum vulgare	*Penicillium*	Potato dextrose agar (PDA)	0 μL–3 cm^2 12.5 μL–2 cm^2 25 μL–2 cm^2 50 μL–1 cm^2	[147]
	A. niger		0 μL–3 cm^2 12.5 μL–2 cm^2 25 μL–2 cm^2 50 μL–1 cm^2	
Satureja thymbra	*Penicillium*		0 μL–4 cm^2 12.5 μL–3 cm^2 25 μL–2cm^2 50 μL–1 cm^2	
	A. niger		0 μL–3 cm^2 12.5 μL–3 cm^2 25 μL–2 cm^2 50 μL–1 cm^2	
S. montana	*V. dahliae* *Pe. aurantiogriseum*	CYGA (chloramphenicol-yeast-glucose-agar)	0.25 mg/L–18% 0.25 mg/L–37%	[149]
Thymus capitatus	*Penicillium*	Potato dextrose agar (PDA)	0 μL–4 cm^2 12.5 μL–3 cm^2 25 μL–2 cm^2 50 μL–1 cm^2	[147]
	A. niger		0 μL–3 cm^2 12.5 μL–3 cm^2 25 μL–2 cm^2 50 μL–1 cm^2	
T. vulgaris	*V. dahliae* *P. aurantiogriseum*	CYGA (chloramphenicol-yeast-glucose-agar)	0.25 mg/L–10% ****	[149]
T. serpyllum	*V. dahliae* *P. aurantiogriseum*	CYGA (chloramphenicol-yeast-glucose-agar)	0.25 mg/L–30% 0.25 mg/L–99%	[149]

was the major chemical constituent. It presented the following contents: 37.7%, 41.7%, 13.7%, and 44.5% for *T. vulgaris*, *T. zygis, T. serpyllum,* and *T. pulegioides*, respectively. The determination of their antibacterial activity against the Gram-positive bacterium *Streptococcus mutans* was performed by turbidity measurement, determination of colony-forming units (CFUs), and the live/dead staining method. In the turbidity test, essential oils of *T. zygis* and *T. Pulegioides* had the highest minimum inhibitory concentration (MIC equal to 0.5 mg/mL), followed by *T. vulgaris* (MIC = 0.75 mg/mL) and *T. serpyllum* (MIC = 0.9 mg/mL). Regarding CFU, all four essential oils significantly affected S. *mutans* growth. The lowest CFU value was found for T. serpyllum (1,750 CFU [bacterial/ml]), followed by *T. vulgaris* (3,500 CFU [bacterial/ml]), *T. zygis* (4,500 CFU [bacterial/ml]), and *T. pulegioides* (27,500 CFU [bacterial/ml]). Regarding the live/dead staining method, *T. vulgaris* essential oil had the strongest *in vitro* antimicrobial activity against S. *mutans*, followed by *T. pulegioides* and *T. serpyllum.* In contrast, the essential oil of *T. zygis* had the weakest effect [143].

Leaf essential oil of *Salvia ringens* was characterized by 1.8-cineole (31.99%), camphene (17.06%), borneol (11.94%), and α-pinene (11.52%). It was tested against six Gram-negative bacteria: *E. coli* (ATCC25922*), Salmonella typhimurium* (ATCC14028), *Salmonella enteritidis* (ATCC13076), *Pseudomonas tolaasii* (NCTC387), *Pseudomonas aeruginosa* (ATCC27853), and *Proteus mirabilis* (ATCC14273), and five Gram-positive bacteria: *Staphylococcus aureus* (ATCC25923), *Bacillus cereus* (ATCC10876), *Micrococcus flavus* (ATCC14452), *Sarcina lutea* (ATCC10054), and *Listeria monocytogenes* (ATCC15313). The results showed that *S. ringens* EO showed the strongest antibacterial activity with MIC equal to 9.50–17.10 mg/mL [144]. In Table 3, results of the antibacterial activity of *Lamiaceae* essential oils are shown. Figure 3 shows a probable mechanism of action of essential oils in bacteria.

7.2. Antifungal Activity. Fungal infections can be very dangerous for humans, especially when it concerns food, because fungi have the ability to produce mycotoxins and also reduce or destroy the nutritional value of grains during storage. Thus, it is important to mention the numerous studies with *Lamiaceae* essential oils with antimicrobial properties against fungi [7]. EOs of *O. vulgare*, *Thymus capitatos,* and *Satureja thymbra* were analyzed and showed the following major constituents: carvacrol (82.48%), p-cymene (5.00%), and γ-terpinene (2.62%). They were tested against two phytopathogenic fungi (*Aspergillus Niger* and *Penicillium* spp.) isolated from slices of bread left outdoors at room temperature. Results showed that the addition of essential oils had significant effect ($p < 0.05$) on decreasing their colony surface area. Thus, oregano (*O. vulgare*), thyme (*Thymus capitatus*), and pink savory (*S. thymbra*) can be incorporated into bread recipes and be used in the food industry, as they have antimicrobial properties [147].

In the study by Niksic et al. [148], *Lepechinia mutica* EO was characterized by shyobunol (10.80%), 3-carene (8.69%), δ-cadinene (6.96%), and globulol (5.91%), and it was tested against three serious human pathogenic fungi: *Candida*

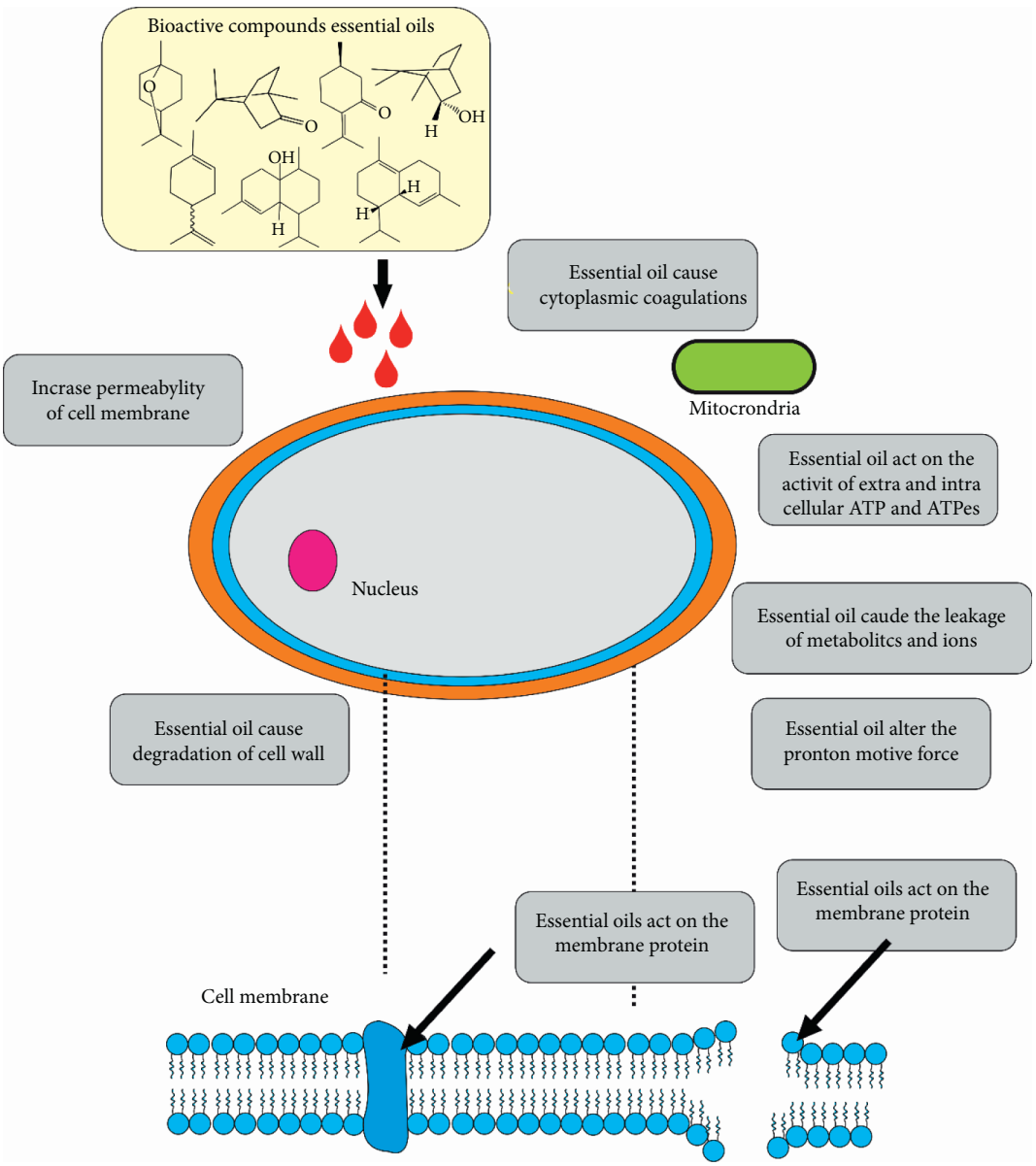

Figure 4: Potential mechanism of action of essential oils in fungi, adapted from [152].

albicans, *Trichophyton rubrum,* and *Microsporum canis*, and two potent plant pathogens: *Pyricularia oryzae* and *Fusarium graminearum*. Compared with the positive controls amphotericin B and voriconazole, *L. mutica* EO exhibited moderate activity against *M. canis* and *T. rubrum*, having MIC values ranging from 2.2 to 4.5 mg/mL.

Rus et al. [149] evaluated the chemical composition of EOs from three species of the *Lamiaceae* family (*T. vulgaris, T. serpyllum,* and *Satureja montana*), which showed the following major compounds: p-cymene, γ-terpinene, and carvacrol. Their antifungal activity was evaluated against *Verticillium dahliae* and *Penicillium aurantiogriseum* at concentrations ranging from 0.25–15 mg/L. The essential oils of *T. vulgaris, T. serpyllum,* and *Satureja montana* exhibited mycelial growth inhibition (MGI) equal to 10%, 30%, and 18%, respectively, against *V. dahlia,* and 0%, 99%, and 37% against *P. aurantiogriseum* at 0.25 mg/L. At the other concentrations, growth was almost nonexisting. These results show that *T. vulgaris* EO was the most effective, especially against *P. aurantiogriseum*, which was highly sensitive.

The major compounds linalool (48.4%), 1,8-cineole (12.2%), eugenol (6.6%), methyl cinnamate (6.2%), α-cubebene (5.7%), caryophyllene (2.5%), β-ocimene (2.1%), and α-farnesene (2.0%) characterized the chemical profile of the essential oil from *O. basilicum* leaves. It was tested against *Aspergillus flavus* at concentrations of 500, 750, and 1000 ppm, and the results showed that at 500 ppm, this EO showed inhibition rate of 30%; at 750 ppm, 50%; and at 1000 ppm, 70%. These results are promising for curing

mycotic infections and as a pharmaceutical preservative against *A. flavus* growth. It may also be used for aflatoxin B1 production [150].

Ocimum tenuiflorum essential oil was characterized by methyl eugenol (84.7%) and *ß*-caryophyllene (7.4%), whereas *O. basilicum* EO had its chemical profile characterized by the major constituents linalool (35.1%), eugenol (20.7%), and 1,8-cineole (9.9%). In this study, they were tested against *C. albicans, C. tropicalis, C. krusei, C. guilliermondii, C. parapsilosis, Cryptococcus neoformans, Trichophyton mentagrophytes, T. mentagrophytes var. interdigitale, Trichophyton rubrum, T. verrucosum, Microsporum canis, M. gypseum, Epidermophyton floccosum, Aspergillus niger, A. fumigatus,* and *A. flavus*, with significant results. The essential oil of *O. tenuiflorum* exhibited prominent antifungal activity against *C. neoformans* (MIC = 0.16 μL/mL) and dermatophyte fungi (0.32 μL/mL). However, it had no fungicidal effect against *Aspergillus niger* (MLC> 10 μL/mL), while *O. basilicum* EO hindered the development of this kind of fungus, presenting fungicidal activity (MLC = 2.5–5 μL/mL) and MIC equal to 0.64–1.25 μL/mL [151]. In Table 4, the antifungal activity of *Lamiaceae* essential oils is shown. Figure 4 shows the possible mechanism of action of essential oils on fungi.

8. Conclusion

Lamiaceae species and, consequently, their essential oils may have peculiarities according to their cultivation system, climate, and location. Thus, some chemical and biological properties tend to change, showing great potential, especially regarding medicinal applications.

They have been used to fight certain diseases due to their antioxidant, antifungal, antibacterial, and anti-inflammatory actions. Additionally, there are other *Lamiaceae* species that act against insects, as well as on environmental remediation (phytoremediation) and thermal protection (green roofs).

Because this botanical family is extremely versatile, more studies on its compounds must be conducted since it has great pharmacological potential, with a promising future. Therefore, this review contributes to future studies on *Lamiaceae* and encourages the use of alternative natural resources for different purposes.

Data Availability

The datasets generated and analyzed during the current study are available in the databases, such as PubMed, Google Scholar, Web of Science, Scopus, and Science Direct (datasets can be requested from the corresponding author upon formal request).

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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Research Article

Chemical Composition and Antibacterial Activity of the *Lippia origanoides* Kunth Essential Oil from the Carajás National Forest, Brazil

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Species of the genus *Lippia* are rich in essential oils and have shown antibacterial properties, which may be related to oils' chemical composition. The present work aimed to evaluate the antimicrobial potential of *Lippia origanoides* Kunth against two bacteria strains: *Escherichia coli* and *Staphylococcus aureus*. Leaf essential oils were obtained by hydrodistillation in a modified Clevenger-type apparatus, and their chemical composition was determined by gas chromatography coupled to mass spectrometry (GC/MS) and flame ionization detection (GC/FID). We identified 28 compounds, representing 98.87% of the total concentration of the essential oil. The compounds identified at the highest concentrations were 1,8-cineole (35.04%), carvacrol (11.32%), p-cymene (8.53%), α-pinene (7.17%), and γ-terpinene (7.16%). The leaf essential oil of *L. origanoides* showed antibacterial action on biological isolates of *Escherichia coli* and *Staphylococcus aureus*. For *Escherichia coli*, the oil presented bactericidal action at concentrations of 5–20 μL/mL. Regarding *Staphylococcus aureus*, the bactericidal effect was noted at 20 μL/mL and the bacteriostatic action was noted around 2.5–10 μL/mL. Given the results obtained, *L. origanoides* essential oil showed promising biological potential against Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacteria, thus encouraging further studies on substances isolated from this species to contribute to the development of new antimicrobial drugs.

1. Introduction

The Verbenaceae family comprises approximately 34 genera and 1,200 species. The most representative genera are *Verbena*, *Lippia*, *Citharexylum*, *Stachytarpheta*, *Glandularia*, and *Duranta* [1, 2]. This family is widely distributed throughout the tropical and temperate zones of the Americas, Africa, and India. South America, Mexico, and the Andes specifically concentrate most of the diversity of species [3, 4].

Belonging to the Verbenaceae family, *Lippia origanoides* is native to some countries of Central America and northern South America, especially the Amazon region. It grows to approximately three meters in height and is rich in essential oils with potential medicinal properties [5–8]. Traditionally, medicinal and aromatic plants of the *Lippia* genus have shown several properties, such as analgesic, anti-inflammatory, antipyretic, sedative, antifungal, antihypertensive, larvicide, repellent, and antimicrobial

activities. These plants have been used in the treatment of skin, gastrointestinal, and liver diseases [9–12].

The occasional development of microorganism resistance to commercially available drugs has encouraged studies on the antimicrobial potential of essential oils, which search for compounds that can prevent and treat diseases [13, 14]. Regarding bacteria, the effect of essential oils depends on some factors, such as the technique applied and the period of plant collection, which can influence the concentration of the compounds that will act on bacteria [15, 16].

The *Escherichia coli* bacterium belongs to the family of Enterobacteriaceae. It is a Gram-negative rod-shaped bacterium, nonsporulating, nonmotile, or motile by peritrichous flagella. Its ideal growth temperature is 37°C. *E. coli* is transmitted through contaminated food, as a result of inadequate handling and hygiene practices [17]. It causes a variety of diseases, such as diarrhea, which is a major cause of infant mortality [18]. Recently, this bacterium has been developing resistance through genetic mutations, hence hindering its control [19, 20].

Staphylococcus aureus is part of the human microbiota [21] but can cause local diseases, such as skin infection, metastatic abscess formation, sepsis, peritonitis, and pneumonia [22]. Due to its adaptability and resistance, *S. aureus* has become one of the most important species in hospital and community-acquired infections [23, 24]. In this context, considering the Amazon biodiversity and the need to promote its sustainable use, the present work aimed to evaluate the chemical composition and *in vitro* antimicrobial potential of *L. origanoides* Kunth essential oil against *E. coli* and *S. aureus*.

2. Materials and Methods

2.1. Raw Material Collection. The collection of the botanical material *L. origanoides* Kunth was carried out during the flowering period at the Carajás National Forest, an environmental protection area (geographic coordinates: 05°52′, 06°33′S; 49°53, 50°45′W). Samples were provided by Chico Mendes Institute for Biodiversity Conservation (ICMBio) (collection authorization number 24852-1). Specimens of *L. origanoides* Kunth were collected using botanical techniques and deposited in the João Murça Pires Herbarium of the Museu Paraense Emílio Goeldi, in Belém (Pará, Brazil), under the registration number MG 201029.

2.2. Essential Oil Isolation. To isolate the *L. origanoides* essential oil, we used 20 g of samples in a modified Clevenger-type apparatus coupled to a refrigeration system that maintained condensed water at 12°C. The essential oil obtained was centrifuged, and the residual moisture was removed with anhydrous sodium sulfate. Samples were stored in amber glass ampoules in the absence of oxygen and kept in a refrigerated room at −5°C. The oil yield was calculated by relating the volume of oil obtained and the material mass used in the extraction process on a dry basis [25, 26].

2.3. Chemical Composition Analysis. Chemical compositions were evaluated using a gas chromatograph (GC) coupled to a DSQ-II single quadrupole mass spectrometer (MS) (Thermo Fisher Scientific, Waltham, Massachusetts, USA) equipped with a silica capillary column DB-5MS (30 m × 0.25 mm × 0.25 μm) (Agilent Technologies, Santa Clara, CA, United States). The evaluation conditions were the following: the temperature increased from 60 to 240°C at 3°C/min; the injector temperature was 240°C; helium was the carrier gas (linear velocity of 32 cm/s, measured at 100°C); a 2 : 1000 aqueous solution of *n*-hexane was injected (0.1 μL); and the temperature of the ion source and other parts was 200°C. The quadrupole filter was scanned in the range of 39–500 Da per second. Ionization was achieved using the electronic impact technique at 70 eV. The retention index of all volatile compounds was calculated using a homologous series of n-alkanes (C_8–C_{40}) (Sigma-Aldrich, San Luis, AZ, USA) according to Van Den Dool and Dec Kratz [27]. The components were identified by comparison of (i) the experimental mass spectra with those existing in reference libraries and (ii) their retention indices with those found in the literature [28, 29]. Volatile components were quantified by peak area normalization using FOCUS GC/FID, which was operated under the same conditions as GC/MS, except for the carrier gas, which was nitrogen, as previously reported by our research group [30].

2.4. Analysis of In Vitro Antimicrobial Activity. The antimicrobial activity of *L. origanoides* oil was evaluated by the microdilution method, as described by Pinheiro et al. [31, 32]. Tests were performed at the Bioassay and Microorganism Chemistry Laboratory (LaBQuiM) of the Federal University of Pará, using strains provided by the Evandro Chagas Institute (IEC). Two bacteria were used: *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923). For 1 liter of broth, 37 g of brain heart infusion (BHI) agar was used. Then, 5 g of bacteriological agar was added to 100 mL of BHI broth. After these dilutions, all media were autoclaved at 121°C for 15 minutes to ensure complete sterility and dispensed onto dishes while still warm, before solidification. In the preparation of the antibiotic control, 1 mg of ampicillin was dissolved in 1 mL of distilled water. Then, 5 μL of this solution was diluted in 995 μL of BHI broth in an Eppendorf tube. A concentration of 5 μL/mL was thus obtained.

2.4.1. Bacteria Activation. The bacteria tested in the assays were activated in a 9 cm diameter Petri dish containing BHI agar for 24 h. After this period, approximately three colonies of each bacterium were transferred, with the aid of a sterile swab stick, to a test tube containing 3 mL of BHI broth, and then incubated for another 24 h. Their concentrations were standardized to obtain a culture medium with approximately 1.0×10^4 CFU/mL [33].

2.4.2. Standardization of the Culture Media. A barium sulfate suspension was obtained by mixing solutions of H_2SO_4 1% (9.95 mL) and $BaCl_2$ 1% (0.05 mL). Then, the turbidity of the test tube containing the bacteria was

compared with the barium sulfate standard. After this standardization, the tube presented a concentration of approximately 1.0×10^4 CFU/mL [33].

2.4.3. Sample Preparation. For sample preparation, 20 μL of *L. origanoides* essential oil was dissolved in 80 μL of dimethyl sulfoxide (DMSO). Then, 900 μL of sterilized and properly homogenized BHI broth was added to this solution.

2.4.4. Determination of the Minimum Inhibitory Concentrations (MICs). Minimum inhibitory concentrations (MICs) were determined by the microdilution method, using 96-well plates arranged in twelve columns (1–12) and eight rows (A-H), with 100 μL of BHI broth added to each well. Then, 100 μL of the essential oil solution was poured into the first well of each column and homogenized. After that, successive dilutions were performed, removing 100 μL from the first well and transferring this volume to the next well, always homogenizing the final solution. This procedure was repeated until the penultimate well of each plate was filled, from which 100 μL was removed and discarded. The last row was used as control of the medium, and no essential oil was added to this solution. Finally, 5 μL of the bacterial suspension was poured into each well, and the plates were incubated at 37°C for 24 hours. Assays were performed in triplicate, and the results (concentrations) were expressed in μL/mL.

After the incubation time, the presence of microbial growth in the plates was verified by the presence of turbidity (red), so clear wells corresponded to no microbial growth. Results were verified with the aid of TTC dye (2,3,5-triphenyltetrazolium chloride). Plates without red coloration were re-inoculated and incubated at 37°C for 24 hours [34].

2.4.5. Determination of the Minimum Bactericidal Concentrations (MBCs). After checking MIC, we verified the type of activity presented in each concentration of *L. origanoides* oil (bacteriostatic or bactericidal). The determination of the minimum bactericidal concentration (MBC) was performed by inoculation of Petri dishes containing BHI agar. Then, they were incubated at 37°C for 24 hours [35–37].

3. Results and Discussion

3.1. Yield and Chemical Composition of the Essential Oil. Approximately 0.6 mL of leaf essential oil was obtained, corresponding to an yield of 3%, which was higher than that obtained by Mar et al. [8]. The yield of *L. origanoides* may vary from 1 to 4.4% according to its geographical origin, extraction technique used, seasonal period, and rainfall rates [38–40]. Figure 1 shows the ion-chromatogram relative to the compounds identified in the essential oil of *L. origanoides*.

The chemical composition of *L. origanoides* essential oil is shown in Table 1. Oxygenated monoterpenes and monoterpene hydrocarbons were the major substances, which represented 56.57 and 35.73% of the compounds identified in this study, respectively. This result was similar to that observed by Mar et al. [8], in which the predominant class was also oxygenated monoterpenes (65%). Andrade et al. [41] also identified monoterpenes as the class with the highest concentration (90.3%) in the essential oil of *L. origanoides* collected in Minas Gerais (Brazil).

Ribeiro et al. [40] analyzed the chemical compositions of *L. origanoides* collected at different seasons of the year and found that the compound classes present in its essential oil may vary. For instance, monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, oxygenated sesquiterpenes, and phenylpropanoids (cinnamates) may be present in the ranges of 9.4–46.5%, 13.5–62.2%, 17.5–31.0%, 3.3–52.8%, and 0.1–28.8%, respectively.

In the present study, we identified 28 compounds, whereas Stashenko et al. [38] found 139 substances in oils and extracts of *L. origanoides*. The major compounds found were 1,8-cineole (35.04%), carvacrol (11.32%), p-cymene (8.53%), α-pinene (7.17%), and β-terpinene (7.16%). Similarly, Tozin et al. [42], in samples of *L. origanoides* collected during the flowering period in the state of São Paulo (Brazil), identified 1,8-cineole as the main component. Also, in the work published by Da Silva et al. [43], the main substances found were 1,8-cineole (64.1%) and α-terpineol (12.0%), a result similar to that obtained by da Silva et al. [44].

In contrast, the chemical composition found in the present study was different from that of other publications. Carvacrol, for instance, was the major component found in *L. origanoides* collected in the city of Jardinópolis (São Paulo, Brazil), with a concentration of 26.28% [45]. In the essential oil of *L. origanoides* collected at Embrapa Western Amazon in Manaus (Amazonas, Brazil), the major compound was thymol (76.6%), whereas in *L. origanoides* collected in the city of Oriximiná (Pará, Brazil), the major compounds were carvacrol (38.6%) and thymol (18.5%) [46].

According to Rojas et al. [47], compound concentrations in the essential oils of *L. origanoides* may vary according to the collection period. For example, in June (rainy season), thymol and carvacrol had concentrations of 61.9 and 7.9%, respectively, whereas in February (dry season), their concentrations were approximately 44.7% and 16.8%, respectively. Also, Santos et al. [5] identified the following compounds at the highest concentrations in *L. origanoides* collected in the state of Piauí (Brazil): carvacrol (33.5–42.9%), γ-terpinene (8.0–10.5%), thymol (5.1–8.4%), methyl thymol (6.1–8.7%), and *p*-cymene (11.9–15.8%).

3.2. Antimicrobial Activity. The greatest antimicrobial activity was observed at the lowest values of MIC. Other authors also reported this behavior: MIC values $\leq 100\,\mu$g/mL indicate strong antimicrobial activity [48, 49]. Our best result for antimicrobial activity was 2.5 μL/mL against *S. aureus* strain, while the weakest was 5 μL/mL against *E. coli*. Several studies [5, 46, 50] have shown that the essential oil of *L. origanoides* presents activity against the microorganisms *C. albicans*, *C. parapsilosis*, *C. guilliermondii*, *C. neoformans*, *T. rubrum*, *S. aureus* (MRSA BMB9393), *S. aureus*, *E. coli*, *L. casei*, *S. mutans*, *S. typhimurium*, *P. aeruginosa*, *B. cereus*, and *B. subtilis*. However, they report that these activities may be related only to the presence of carvacrol and thymol.

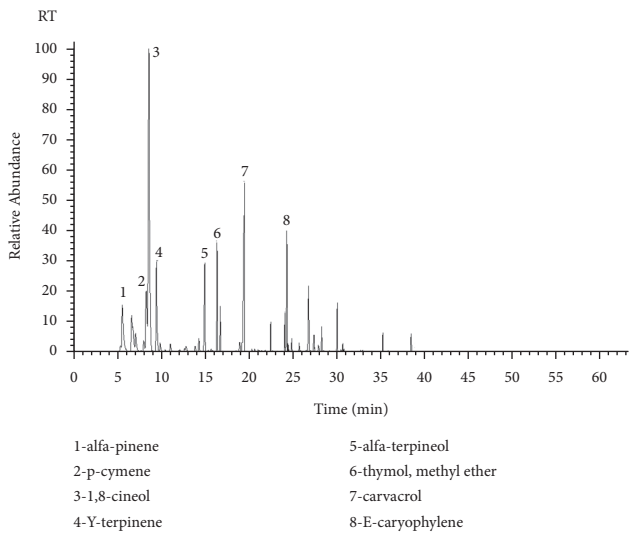

Figure 1: Ion-chromatogram (GC/MS) of the *L. origanoides* essential oil.

Table 1: Volatile constituents identified in *L. origanoides* Kunth essential oil.

RT	RI_C	RI_L	Constituents	Molecular formula	(%)
5.60	925	924	α-Thujene	$C_{10}H_{16}$	0.49
5.84	932	932	α-Pinene	$C_{10}H_{16}$	7.17
6.89	971	969	Sabinene	$C_{10}H_{16}$	3.69
7.03	976	974	ß-Pinene	$C_{10}H_{16}$	3.74
7.31	987	988	Myrcene	$C_{10}H_{16}$	3.73
8.35	1017	1014	α-Terpinene	$C_{10}H_{16}$	1.22
8.56	1024	1020	*p*-Cymene	$C_{10}H_{14}$	8.53
8.76	1032	1026	1,8-Cineole	$C_{10}H_{18}O$	35.04
9.79	1057	1054	γ-Terpinene	$C_{10}H_{16}$	7.16
15.23	1197	1186	α-Terpineol	$C_{10}H_{18}O$	4.36
17.20	1228	1232	Thymol, methyl ether	$C_{11}H_{16}O$	5.58
17.55	1242	1239	Carvone	$C_{10}H_{14}O$	0.12
20.15	1298	1298	Carvacrol	$C_{10}H_{14}O$	11.32
20.20	1321	1289	*p*-Cymen-7-ol	$C_{10}H_{14}O$	0.10
22.42	1356	1349	Thymol acetate	$C_{12}H_{16}O_2$	0.05
23.51	1373	1374	α-Copaene	$C_{15}H_{24}$	0.80
25.10	1413	1410	α-Cedrene	$C_{15}H_{24}$	0.07
25.38	1416	1417	(*E*)-Caryophyllene	$C_{15}H_{24}$	3.15
26.09	1420	1434	γ-Elemene	$C_{15}H_{24}$	0.25
26.12	1430	1432	α-trans-Bergamotene	$C_{15}H_{24}$	0.36
26.84	1452	1452	α-Humulene	$C_{15}H_{24}$	0.23
27.98	1478	1479	ar-Curcumene	$C_{15}H_{22}$	0.18
28.60	1492	1493	α-Zingiberene	$C_{15}H_{24}$	0.46
28.86	1495	1500	α-Muurolene	$C_{15}H_{24}$	0.10
29.04	1505	1505	ß-Bisabolene	$C_{15}H_{24}$	0.14
29.42	1507	1514	(*Z*)-γ-Bisabolene	$C_{15}H_{24}$	0.07
19.80	1515	1522	σ-Cadinene	$C_{15}H_{24}$	0.52
32.18	1578	1582	Caryophyllene oxide	$C_{15}H_{24}O$	0.24
	Hydrocarbon monoterpenes				35.7
	Oxygenated monoterpenes				56.5
	Hydrocarbon sesquiterpenes				6.33
	Oxygenated sesquiterpenes				0.24
	Total				98.87

RT: retention time; RI_C: retention index (on DB-5MS column); RI_L: retention index from literature (Adams [28]).

The bacteria tested showed variable susceptibility to the different concentrations of essential oil (Table 2). *L. origanoides* showed bacteriostatic action in the concentration range of 2.5–10 μL/mL and bactericidal action against *S. aureus* beginning at 20 μL/mL. Regarding *E. coli*, the essential oil showed bactericidal action at concentrations

Table 2: Result of the antimicrobial assays against *Staphylococcus aureus* and *Escherichia coli*.

Concentrations (μL/mL)	*Staphylococcus aureus*	*Escherichia coli*
20.00	=	=
10.00	−	=
5.00	−	=
2.50	−	+
1.25	+	+
0.62	+	+
0.31	+	+
Control	+	+

+, no activity; −, bacteriostatic; =, bactericidal.

starting from 5 μL/mL. Therefore, the MBC was 20 μL/mL for *S. aureus* and 5 μL/mL for *E. coli*. Studies on the chemical composition of *L. origanoides* essential oils have shown a great variety of components, such as thymol, β-caryophyllene, p-cymene (*E*)-nerolidol, trans-α-bergamotene, α-alaskene, α-pinene, α-humulene, caryophyllene oxide, and linalool [51]. This variety may be related to biological properties of the oil [52, 53], such as antimicrobial activity [54, 55].

According to Barreto et al. [56], *L. origanoides* essential oil in association with aminoglycosides may present a synergistic effect and be an appropriate alternative for antibiotic chemotherapy against diseases caused by methicillin-resistant *Staphylococcus aureus* (MRSA). Similarly, in a study on the potential antimicrobial effect of *L. origanoides* essential oil rich in thymol (76.6%) and ortho-cymene (6.3%) against *Aeromonas hydrophila,* Majolo et al. [54] obtained a MIC of 2500 μg/mL and a MBC of 2500 μg/mL.

Finally, the results obtained in this study indicated that one of the compounds responsible for the antimicrobial activity of *L. origanoides* essential oil may be 1,8-cineole, since several scientific publications have reported the antimicrobial properties of this substance [57–61]. For instance, Hendry et al. [57] obtained the following results using 1,8-cineole: MIC values of 16 μg/L (suspension) and 512 μg/L (biofilm) and MBC values of 256 μg/L (suspension) and >512 μg/L (biofilm) for *S. aureus*; MIC values of 64 μg/L (suspension) and 128 μg/L (biofilm) and MBC values of 64 μg/L (suspension) and 256 μg/L (biofilm) for *E. coli*.

Other compounds have had their antimicrobial activity reported. Studies have demonstrated that α-pinene also has antimicrobial activity against *S. aureus* and *E. coli* [62–64]. De Sousa et al. [65], for instance, obtained a MIC of 1.25 μl/mL and 2.5 μl/mL for α-pinene against *S. aureus* and *E. coli*, respectively. p-Cymene, another compound identified in the essential oil of *L. origanoides*, also has antimicrobial activity [66]. Namiecińska et al. [67] studied the antimicrobial effects of p-cymene on different strains. They obtained a MIC of 1000 μg/mL for *S. aureus* ATCC 29213, 62.5 μg/mL for *S. epidermidis* ATCC 12228, and 500 μg/mL for *E. faecalis* ATCC 29212. γ-Terpinene, in the work by Krist et al. [68], showed good antimicrobial activity, since the germ count decreased by 40%. In addition, carvacrol acted against several strains, such as *E. coli, P. fluorescens, S. aureus, L. plantarum, B. subtilis, S. cerevisiae,* and fungus *B. cinerea* [69]. Guarda et al. [70] studied the antimicrobial potential of carvacrol and obtained MIC values of 225, 225, 375, 75, and 225 μg/mL for *S. aureus*, *L. innocua*, *E. coli, S. cerevisiae,* and *A. niger,* respectively.

4. Conclusion

The chemical composition of the essential oil of *L. origanoides* Kunth collected during the flowering period proved to be rich in 1,8-cineole, indicating a possible correlation between the collection period and the biosynthesis of such a compound. We also observed a potential antimicrobial activity against Gram-positive and Gram-negative bacteria, suggesting a possible association of this behavior with the concentration of 1,8-cineole. Given the results, the essential oil of *L. origanoides* Kunth showed promising biological potential against pathogenic bacteria, thus encouraging further studies on substances isolated from this species to contribute to the development of new antimicrobial drugs.

Data Availability

The data sets used and/or analyzed during the current study are available from the corresponding author and will be delivered to responsible bodies on reasonable request.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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Supplementary Materials

This section contains data regarding collection and experiments. (*Supplementary Materials*)

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Research Article

Chemical Characterization and Antioxidant, Antimicrobial, and Insecticidal Properties of Essential Oil from *Mentha pulegium* L.

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The chemical composition and antibacterial, insecticidal, and antioxidant properties of the essential oil from *Mentha pulegium* L. (*M. pulegium*) growing in Morocco were investigated in this work. To achieve this goal, the oils were obtained by using hydrodistillation before being characterized by GC-MS. The antibacterial and antifungal activities were conducted against pathogenic strains using the disc diffusion and MICS bioassays. The insecticidal activity was carried out versus *C. maculatus* using contact and inhalation tests. The antioxidant activity was performed by using DPPH and total antioxidant capacity bioassays. The chemical analysis of the oil showed that 20 compounds were identified, which represented 98.91% of the total oil. In the oil, the main components detected were R-(+)-pulegone (76.35%), carvone (5.84%), dihydrocarvone (5.09%), and octanol-3 (2.25%). The essential oil has moderate-to-strong broad-spectrum antibacterial and antifungal properties; the results showed that *B. subtilis* was the most sensitive strain to *M. pulegium* oil, with the largest inhibition diameter (25 ± 0.33). For the antifungal activity, the results obtained indicated that *Aspergillus niger* was the most sensitive fungal strain to *M. pulegium* oil with an inhibition percentage up to 100%. Regarding the insecticidal activity, the inhalation test showed a high efficacy (100% mortality), and a lethal concentration of $LC_{50} = 1.41 + 0.48\,\mu L/L$ air was obtained after 24 hours of exposure. Moreover, the contact test showed that a total reduction in fertility and emergence was obtained with a dose of $20\,\mu L/mL$ of acetone. Regarding the antioxidant activity, the sample concentration necessary to inhibit 50% of HE radicals (IC_{50}) was 7.659 mg/mL (DPPH) and 583.066 57.05 mg EAA/g EO (TAC).

1. Introduction

Morocco's geographical location provides a diverse spectrum of bioclimates, allowing for the formation of diverse flora [1, 2]. The floral wealth is largely related to the ecological heterogeneity of the biotopes. Indeed, it can go from the desert to the high mountains, which allows the development of various species with different bioclimatic stages. The country possesses ancient know-how [3], which has been conserved throughout the ages, in addition to this especially favorable natural setting. In this sense, herbal medicine has witnessed large use by the indigenous people in flavoring and preserving foodstuffs. In Morocco, more than 400 plant species have been accounted for medicinal use [4].

Essential oil (EO) from plants is an important resource of natural products and their components are mainly used as food flavors. Additionally, EO has also non-food applications including antifungal, antimicrobial, antioxidant, and insecticidal activity [5–9]. This confirms the importance of these natural products to develop new alternative solutions in several areas such as health, food safety, and agriculture.

Mentha pulegium L. (*M. pulegium*) is an indigenous perennial plant found in Europe, North Africa, and the Middle East [10]. Species among *Mentha* (Labiatae) are widely used against several diseases with a wide spectrum of use, which varies from one region to another. Various research has also revealed that the plant extracts are frequently used as an anti-inflammatory, antispasmodic, carminative, antitussive, diaphoretic, antiemetic, analgesic, stimulant, and emmenagogue and in the form of powders, infusions, and decoctions [11]. The presence of numerous secondary metabolites, including phenolic chemicals, flavonoids, and essential oils, is primarily responsible for these characteristics [12–14].

M. pulegium is an important source of essential oils with pharmacological activities including allelopathic properties [15]. In addition, we found that the essential oil of *M. pulegium* leaves exhibited significant insecticidal activity against individuals of *Oryzaephilus surinamensis*, causing a total population reduction at the highest dose [16]. It would be interesting to discover whether *M. pulegium* essential oil and its components have insecticidal properties against other species. As a result, we studied the chemical composition of *M. pulegium* essential oils, as well as their insecticidal activities against *Callosobruchus maculatus*, one of the most common pests of chickpea grains in Morocco along with antioxidant activity, and antimicrobial activity against some pathogenic strains in this study.

2. Material and Methods

2.1. Plant Material and Oil Extraction. M. pulegium was collected from the Moroccan region of Ouazzane (34°47′49 N, −5°34′56 W). The botanical identification was carried out by a botanist at Sidi Mohamed Ben Abdellah University, where the reference specimen number DM01/02501 was deposited. Thereafter, the aerial part of the plant was dried in the shade in a dry and ventilated area of the laboratory at a temperature between 25 and 32°C. Briefly, 100 g of *M. pulegium* leaves and buds was subjected to essential oil extraction by using hydrodistillation for 3 hours.

2.2. GC-MS of Essential Oils. The identification of the essential oil composition was carried out by gas chromatography-mass spectrometry (GC-MS). Analysis was performed using GC system with a flame ionization detector and an HP-5MS capillary column. Temperature programmed gas chromatography was set to 35°C and 250°C with a gradient of 5°C/min. Gas chromatography with two fused silica capillary columns (30 m 0.25 mm) was used to determine retention indices. The operating temperature was set to 35 and then 250°C with a rate of 5°C/minutes, alongside lower and upper temperatures held for 3 and 10 minutes, respectively. The carrier gas (helium) flow rate was 1.0 mL/min. In split mode, a 1.0 L sample was injected (split ratio, 1 : 100). The essential oil constituents were identified by comparing their mass spectra with those of the NIST02 GC/MS library data and the Adams.

2.3. Antimicrobial Activity

2.3.1. Antibacterial Activity. Three filamentous fungi, *Aspergillus niger*, *Aspergillus flavus*, and *Fusarium oxysporum*, and one yeast strain *Candida albicans* were used in this study for testing. All fungal strains chosen are pathogenic and belong to drug-resistant microbes. They are among the most contaminating microbes of dried vegetables and cereals and the main producers of mycotoxins. *Candida albicans* is frequently implicated in nosocomial infections. All microbial strains were provided by Sidi Mohamed Ben Abdellah University, Fez, Morocco. Spore suspensions were prepared in a tube containing 0.9% NaCl from seven-day-old cultures on a potato dextrose agar (PDA) medium. The number of spores in suspension was counted using a Malassez cell and the suspensions were diluted to obtain an inoculum concentration of approximately 10^6 spores/mL [17].

2.3.2. Antibacterial Activity. In this study, the antibacterial activity of *M. pulegium* EO was tested against four bacterial strains including *Escherichia coli* (ATB: 57) B6N, *Staphylococcus aureus*, *Escherichia coli* (ATB: 97) BGM, and *Bacillus subtilis*. All these strains are pathogenic and provided by Hassan II University Hospital Center's Laboratory of Bacteriology in Fez, Morocco. Müller-Hinton Broth (MHB) and Müller-Hinton Agar (MHA) (provided by VWR Chemicals) were used as growth media for bacteria [1]. Isolated colonies from a fresh culture turn 18h to 24 h were transferred to a 0.9% NaCl solution to prepare the microbial suspension. Next, the optical density of the suspensions was checked with a UV-Visible spectrophotometer at 625 nm and adjusted to be between 0.08 and 0.1 nm, which corresponded to suspensions containing a 10^7 to 10^8 CFU/mL according to McFarland [18].

2.3.3. Determination of the Inhibition Zone on Solid Mediums. The disc diffusion technique was used to assess the antibacterial and antifungal activities of *M. pulegium* EO

[19]. Briefly, Petri dishes containing BN (nutrient broth) medium were seeded with the tested bacterial strains (*Escherichia coli* (ATB: 57) B6N; *Escherichia coli* (ATB: 97) BGM; *Staphylococcus aureus*; and *Bacillus subtilis*) whilst the MEA (Malt Extract Agar) medium was seeded with *C. albicans*, *A. niger, A. flavus,* and *F. oxysporum*. Next, Whatman paper discs (6 mm in diameter) were placed on the surface of inoculated culture media after being impregnated with 20 μL of EO from *M. pulegium* [20]. After that, the inoculated Petri plates were incubated in the darkness at 30°C for fungal strains and 37°C for bacterial strains, respectively. After 24 hours of incubation for bacterial strains and 48 hours for *C. albicans*, inhibitory diameter and percent inhibition were assessed. After 7 days of incubation, the inhibition diameter and percent inhibition of *A. niger*, *A. flavus,* and *F. oxysporum* were determined [21, 22].

In this study, the negative control was 10 μL of 0.2% agar, whilst fluconazole was used as a positive control with 5 mg/mL in the presence of fungal strains, and streptomycin (0.02 mg/disc) was used as a positive control in the presence of bacterial strains [23].

2.3.4. Determination of Minimum Inhibitory Concentration in the Liquid Medium. The microdilution method was used to determine the lowest inhibitory concentration of *M. pulegium* essential oils against bacterial and fungal strains, according to the method reported by [24]. After 18 hours of incubation for bacteria, 48 hours for yeast, and 7 days for fungi at 30°C [24, 25], the MIC was determined by using the colorimetric method (TTC 0.2% (w/v)) [25, 26].

2.4. Insecticidal Activity

2.4.1. Insect Rearing. The chickpea pest *Callosobruchus maculatus* (*C. maculatus)* was used for insecticidal activity testing. This species was maintained by mass rearing at the laboratory LGME, Department of Chemistry, USMBA, Fez, Morocco. Rearing of *C. maculatus* bruchid was carried out in glass jars with *Cicer arietinum* chickpea seeds. For numerous generations, the jars were kept at a constant temperature of 25°C, relative humidity, and a photoperiod of 14 h (light)/10 h (dark).

2.4.2. Toxicity of Essential Oil against C. maculatus

(1) Assessment of Essential Oil Toxicity by Contact. Several preliminary tests were conducted to determine the best doses for testing. Afterward, four doses were prepared by dilution including 0.016, 0.079, 0.157, and 0.315 μL/cm^2, respectively. Filter paper disks with 9 cm diameter (63.62 cm^2) (Whatman No. 1) were impregnated into EO before being placed in a glass Petri dish of the same diameter, which served to contain the insect. Only acetone was used to treat the disk for the control. In each Petri dish containing 20 g of seeds and a treated washer, a batch of 10 adult insects (5 males and 5 females) were introduced, freshly collected from their rearing environment and no more than 24 hours old (after emergence from seeds). The dishes were then immediately resealed. For each dose, three replicates were used, and dead insects were counted every 24 hours for four days.

In order to calculate the mortality rate, the number of dead insects was counted each day after the experiment ended. Eggs deposited on the walls of boxes and seeds were counted with a binocular loupe to demonstrate the importance of oviposition. The number of eggs of the treated insect was compared to that of the control. The rate of reduction of oviposition was also calculated [26–30].

(2) Toxicity of EO by Inhalation. Briefly, a small amount of the cotton was suspended into glass jars. Next, doses of 1 μL, 5 μL, 10 μL, and 20 μL of *M. pulegium* essential oil were deposited into the cotton using a micropipette. Afterward, ten bruchids of *C. maculatus* (male and female) whose ages ranged from 0 and 48 h were placed in each jar and then closed tightly. For each dose, three replications were carried out. The comparison was made with a control sample (cotton without test solutions).

2.4.3. Calculation Methods. The observed mortality rate was corrected by the following formula:

$$Pc = 100 \times \frac{Po - Pt}{100 - Pt}, \quad (1)$$

where Pc = percent corrected mortality, Po = observed mortality in the trial, and Pt = observed mortality in the control.

The following formula was used to calculate the egg-laying reduction rate:

$$Tx = 100 \times \frac{Nt - Ne}{Nt}, \quad (2)$$

where Tx = rate of reduction relative to the control, Nt = number of eggs in the control jar, and Ne = number of eggs in the trial.

2.5. Antioxidant Activity

2.5.1. Scavenging of the Free Radical DPPH. In the present study, the protocol used was that described by [31]. Briefly, 0.5 mL of different concentrations of methanol was used to prepare 0.004% DPPH solution. The reaction mixture was stirred immediately before being kept at room temperature (25°C) for 30 minutes in the dark. The absorbance of the reaction medium was measured at 517 nm against a blank containing only methanol. After then, the absorbance was measured and the ascorbic acid was used as a reference. The proportion of DPPH free radical inhibition was estimated using the following method:

$$\%\text{inhibition} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100, \quad (3)$$

where Abs control is control absorbance (including all reagents except the test substance) and Abs sample is

absorbance of the test compound. The percentage of inhibition was used to calculate the value of IC_{50}.

2.5.2. Total Antioxidant Capacity Determination. Three hundred microliters of selected doses of EO was mixed with 3 mL of liquid reactive solution constituted of sulphuric acid, ammonium molybdate, and sodium phosphate. The absorbance was measured using a spectrophotometer set at 695 nm after a 90-minute incubation period at 95°C. The negative control was a blank containing 300 methanol, while the positive control was ascorbic acid [32]. The antioxidant potential of the extracts was measured in mg EAA/g essential oil.

2.6. Data Analysis. The results were presented as arithmetic mean values with standard deviation. One-way ANOVA followed by a Tukey test was used to achieve analysis. SPSS version 21.0 was used, and significant values were considered when P was less than 0.05.

3. Results and Discussion

3.1. Extraction Yield and Chemical Composition of Essential Oil. The yield of EO recovered was 2.14 ± 0.22 mL/100 g dry matter. The EO was dried with anhydrous sodium sulfate before being stored in a refrigerator at 4°C until further use. The results of GC/MS analysis of essential oil extracted from *M. pulegium* leaves collected from Ouazzane region are presented in Table 1 and Figure 1. The identified compounds are listed according to the elution order of their retention index.

The yield of EO from *M. pulegium* leaves was 2.14 ± 0.22 mL/100 g. In this mass, 20 compounds were identified, which represented 98.91% of the total recovered oil. The oil was majority constituted of compounds among R-(+)-pulegone 76.35%, carvone 5.84%, dihydrocarvone 5.09%, and octanol-3 2.25% (Figure 2).

The chemical composition of the studied oils shared some similar compounds with other studies like the pulegone compound [15, 33–35]. The pulegone was also found in EOs from *M. pulegium* belonging to the Mediterranean countries with different proportions.

M. pulegium indigenous to Tunisia showed pulegone (61.11%) and isomenthone (17.02%) [36]. *M. pulegium* ingenious to Egypt was found to be also rich in pulegone (43.50%) and piperitone (12.2%) [37]. Environmental factors, the portion of the plant employed, the age of the plant, the phase of the vegetative cycle, and even genetic factors may all play a role in the chemical composition of our sample when compared to that recorded in similar species from other regions [38, 39]. The extraction method can also affect the yield and chemical composition of essential oils, and could therefore, explain the differences in bioactivity [40, 41].

The different biological activities of *M. pulegium* plants were caused by the majority compounds in the essential oils. Pulegone (Figure 2) is the most distinctive chemical of *M. pulegium*, and there are also piperitone (B), menthol (C), menthone (D), and piperitone oxide chemotypes (E) [42, 43]. Furthermore, the EO often contains a high percentage of oxygenated monoterpenes [37], with these chemicals accounting for more than 60% of the total oil [44]. These are known by their activities as biopesticides [45] and the principal contributors to *M. pulegium* EOs' antioxidant activity [46, 47].

Table 1: Constituents of the essential oil of *M. pulegium* identified by GC-MS analysis.

Compounds	Components	RI	Area (%)
1	α-Pinene	937	0.69
2	Cyclohexanone-3-methyl	952	0.37
3	β-Pinene	974	0.52
4	Myrcene	992	0.14
5	Octanol-3	995	2.25
6	D-2-Carene	1003	Tr
7	Limonene	1030	1.41
8	*p*-Mentha-3.8-diene	1071	1.95
9	Menthone	1150	0.08
10	Pinocarvone	1166	1.76
11	Isomenthol	1182	0.28
12	Menthol	1171	0.62
13	Dihydrocarvone	1193	5.09
14	R-(+)-pulegone	1236	76.35
15	Carvone	1240	5.84
16	α-Peperitone	1251	0.36
17	Caryophyllene	1418	0.18
18	Germacrene D	1475	0.09
19	γ-Eudesmol	1630	0.37
20	α-Eudesmol	1649	0.56
Total identified			98.91

(R)-(+)-pulegone is a ketone monoterpene that is found in the essential oils of a variety of plants. It has many bioactivities in cells and animals [48, 49]. According to Damião et al. [50], (R)-(+)-pulegone possessed analgesic therapeutic profile. Roy et al. [51] showed that pulegone reduces LPS-induced inflammation by reducing the effects of NF-κB suggests that pulegone could be used to treat and prevent a variety of inflammatory illnesses. Also, at 40 mM, R-(+)-pulegone reduced *R. dominica* and *L. serricorne* AChE activity by 69.0 percent and 88.0 percent, respectively. Therefore, pulegone can be considered as a good insecticide against these cereal seed pests [52].

3.2. Antimicrobial Activity. The antimicrobial activities of EO of *M. pulegium* against pathogenic and phytopathogenic microorganisms were investigated in the present research by the disk diffusion method (Figure 3). The results obtained are presented in Table 2.

The results showed that the essential oils of *M. pulegium* had a significant inhibitory effect against the tested bacterial and fungal strains. The results indicated that *B. subtilis* was the most sensitive strain tested to *M. pulegium* oil with the highest inhibition diameter (25 ± 0.33). *M. pulegium* oil also showed strong antibacterial activity against *E. coli* (ATB: 57) B6N (10.33 ± 0.44), *E. coli* (ATB: 97) BGM (12 ± 0.66), and *S. aureus* (13.16 ± 0.22). For the antifungal activity, the essential oils of *M. pulegium* also showed an important activity

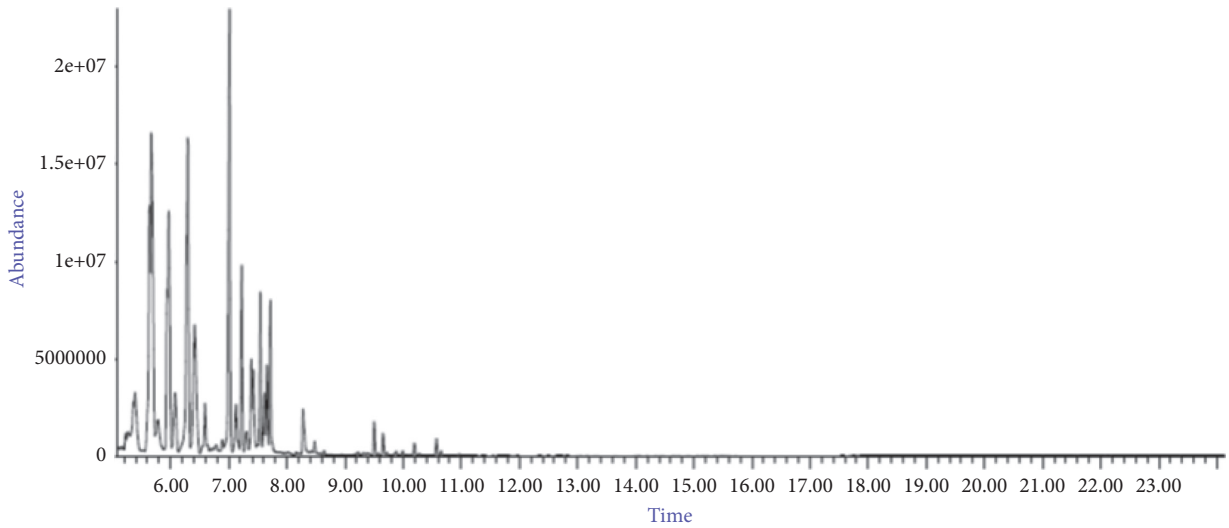

Figure 1: Chromatogram of essential oil from *M. pulegium* leaves.

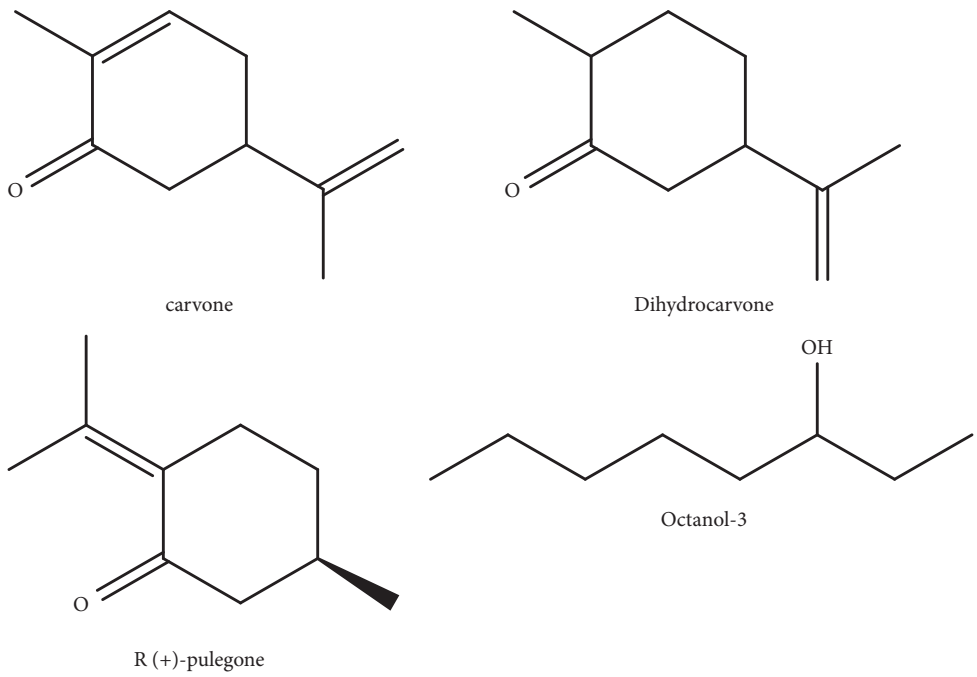

Figure 2: Chemical structure of the main compounds of *M. pulegium* EO determined by the ChemBioDraw software (Ultra 11.0).

against the tested strains, this activity varied from one strain to another, and the obtained results indicate that *Aspergillus niger* is the most sensitive fungal strain to the oil of *M. pulegium* with a percentage of inhibition up to 100%.

Table 3 shows the MIC findings of *M. pulegium* essential oils against the investigated bacterial and fungal strains. According to the findings, the essential oils of *M. pulegium* have different antibacterial properties. MIC values for bacterial strains (*E. coli* (TBA: 57) B6N, *E. coli* (TBA: 97) BGM, *Staphylococcus aureus*, and *Bacillus subtilis*) ranged from 0.704 to 2.812 g/mL. It can therefore be concluded that low doses of *M. pulegium* oil can inhibit bacterial growth of the tested strains. On the other hand, MIC values for fungal strains (*A. niger*, *A. flavus*, *F. oxysporum*, and *C. albicans*) were between 11.25 and 22.5 μg/mL, which means that the fungal strains tested were more resistant than bacterial strains to essential oils. In addition, the oils of *M. pulegium* showed bactericidal and fungicidal activity against all bacterial and fungal strains tested, respectively. This suggests that the essential oil extracted from *M. pulegium* can be valorized in many fields, especially in food safety thanks to its important antimicrobial activity and its low MIC values against pathogenic and phytopathogenic microorganisms. *M. pulegium* oil has been given high interest to fight resistant bacteria [53]. The essential oils of *M. pulegium* from Ouazzane provide antibacterial effects on a wide spectrum of bacterial strains with different MICs (Gram+: *S. aureus* MBLA: MIC 0.25 mg/mL, *S. Aureus* 976: CMI 1 mg/mL,

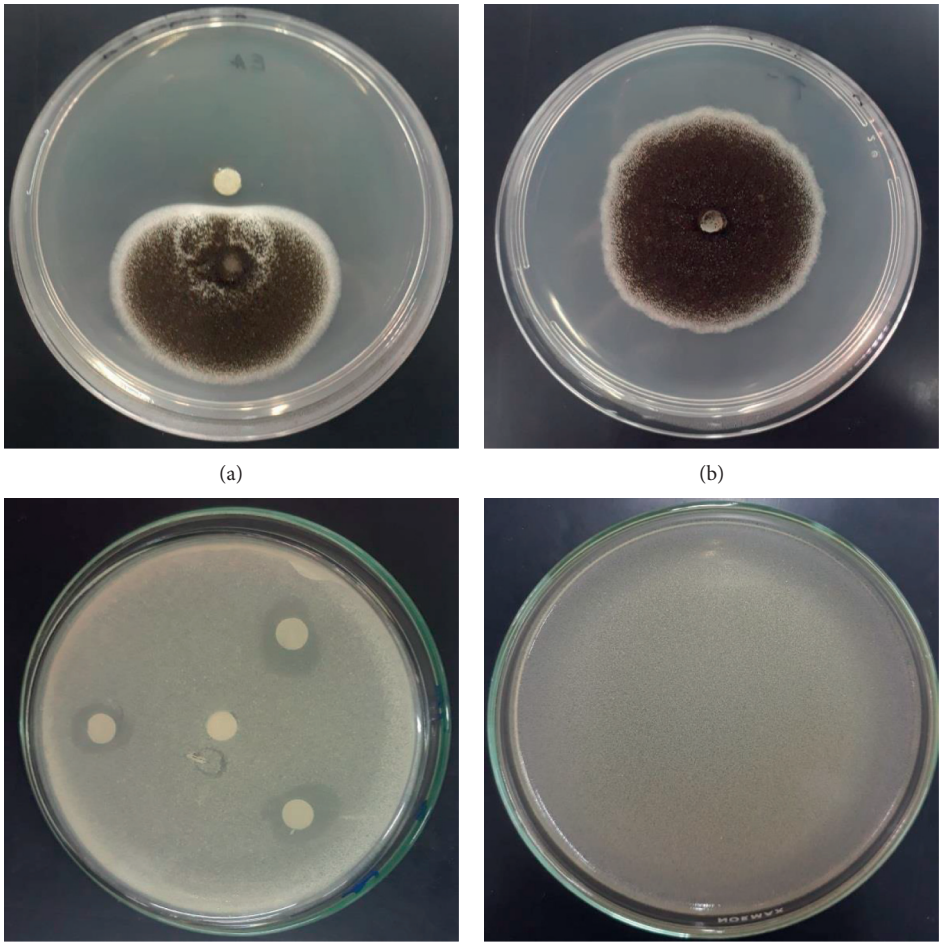

Figure 3: Pictures showing the antifungal and antibacterial activities of the studied essential oils. (a) Antifungal activity; (b) negative control for antifungal activity; (c) antibacterial activity; (d) negative control for antibacterial activity.

Table 2: Antibacterial and antifungal effect of *Mentha pulegium* EOs against bacterial fungal strains.

Antibacterial activity (inhibition diameter in mm)			Antifungal activity (percentage of inhibition)		
Bacterial strains	EOs	SPM	Fungal strains	EOs	FLU
E. coli (ATB:57)	10.33 ± 0.44	—	*A. niger*	100 ± 0.00%	89.75 ± 0.41%
E. coli (ATB:97)	12 ± 0.66	—	*A. flavus*	87.91 ± 0.08%	94.42 ± 0.92%
S. aureus	13.16 ± 0.22	9.73 ± 0.23	*F. oxysporum*	92.91 ± 0.09%	91.91 ± 0.9%
B. subtills	25 ± 0.33	10.52 ± 0.41	*C. albicans*	23 ± 0.66 %	95.81 ± 0.76%

EOs: essential oils of *Mentha pulegium*; SPM: streptomycin; FLU: fluconazole; and (−): non-inhibition.

Table 3: Minimal inhibitory concentration of *Mentha pulegium* essential oils against the four bacterial and four fungal strains.

Antibacterial activity		Antifungal activity	
Bacterial strains	Minimal inhibitory concentration (MIC) (μg/mL)	Fungal strains	Minimal inhibitory concentration (CMI) (μg/mL)
E. coli (ATB:57)	0.704	*A. niger*	11.25
E. coli (ATB:97)	1.406	*A. flavus*	22.5
S. aureus	1.406	*F. oxysporum*	22.5
B. subtills	2.812	*C. albicans*	11.25

Table 4: Effects of essential oils of *M. pulegium* tested by inhalation on the mortality of *C. maculatus*.

Dose (μm)	Percentage of mortality per treatment day			
	24 h	48 h	72 h	96 h
Control	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
1	43.3 ± 4.4^{b}	63.3 ± 5.7^{b}	86.7 ± 5.7^{b}	93.3 ± 11.5^{b}
5	63.3 ± 4.4^{c}	73.3 ± 5.7^{b}	100 ± 0^{c}	100 ± 0^{c}
10	80 ± 6.6^{d}	90 ± 10^{c}	100 ± 0^{c}	100 ± 0^{c}
20	100 ± 0^{e}	100 ± 0^{c}	100 ± 0^{c}	100 ± 0^{c}

Columns with the same letter did not differ significantly according to ANOVA analysis.

S. aureus 994: CMI 2 mg/mL, *L. monocytogenes*: CMI 0.50 mg/mL; Gram−: *P. Aeruginosa*: CMI 2.00 mg/mL, *Bacillus subtilis*: CMI>2.00 mg/mL, *P. mirabilis*: CMI 0.50 mg/mL, and *Escherichia coli* K12: CMI 0.50 mg/mL) [54]. The essential oils of *M. pulegium* responded differentially to the growth of the bacterial and fungal strains evaluated in our study and elsewhere. In this way, different components may have distinct modes of action, or the metabolism of specific bacteria may be able to counteract the effects of *M. pulegium* oil more effectively. Several studies have shown that the antimicrobial activity of EO can be attributed to its major compounds, such as pulegone (61.10%), isomenthone (17.00%), menthone (5.90%), and piperitone (2.60%) [33], or to the high concentration of piperitone (38%) without excluding any potential synergistic effects of constituents [14].

Mentha pulegium possesses antibacterial effects that are effective against a variety of pathogens for hens [55, 56]. Pulegone, menthone, menthol, and piperitone oxide are responsible compounds for the antibacterial activity of *M. pulegium* [55, 57], while antifungal activity is attributed to aldehydes, alcohols, and ketones (pulegone, menthone, and neo-menthol) [42]. Yeasts are sensitive to EO from *M. pulegium*; however, the effect varies depending on the species and strain [58–60].

3.3. Insecticidal Activity. The increasing need for fight-stored chickpea pests has led to an interest in the toxicity of plant-derived EOs. In the present work, we tested the essential oil toxicity at different concentrations against *C. maculatus* for 12, 24, 48, and 72 h. We also calculated LC_{50} values for each EO concentration at the respective treatment times. In this sense, the toxicity of the essential oils was tested by inhalation (Table 4). The 20.0 μL/L air dose showed 100% efficacy against *C. maculatus* after 24 h. Meanwhile, the 5 and 10 μL/L air doses killed all pollution after 72 h. Moreover, all oil concentrations showed more than 70% reduction in oviposition, and more than 90% emergence (Table 5). A total absence of emergence was recorded in batches treated with 10 and 20 μL/mL of EO (Table 6). Previous studies demonstrated EO from closer plant species tested by inhalation against *Sitophilus granarius* (L.) weevils (Coleoptera: Curculionidae); generated 100% mortality after 24 h of treatment with doses of 5, 10, 20, and 40 μL EO/mL acetone [58].

The contact toxicity test showed lower efficacy when compared to the inhalation test; the 20.0 μL/L dose showed 100% mortality after 72 h of exposure. For doses of 5 and 10 μL/L of air, the total mortality was not achieved until beyond 96 h of exposure. The lethal dose of the 24-hour inhalation test LC_{50} was 1.99 mL/L air, with a 95% confidence interval (0.27–4.127) over 48 h was 0.83 mL/L air (Table 7). For the contact test, the LC_{50} was 6.51 mL/L of air over 24 h of exposure; this value became lower after 48 and 72 h of exposure (Table 8). It is thus fitting that EOs of *M. pulegium* tested by inhalation can be a promising source of active compounds to fight chickpea pests. The 20.0 mL/L dose was the most active and could be an interesting ecological alternative to eliminate *C. maculatus* from stored seeds.

The insecticidal activity of *M. pulegium* oil has been tested against some insects in previous studies. These studies were mainly classified according to the life stage of the target insect (i.e., adult, larva, and other closer species). Our results showed that *M. pulegium* EOs exhibited efficacy against *Callosobruchus maculatus*. These results are in agreement with those reported in earlier work [12, 13, 15, 58, 61].

The chemical composition of *M. pulegium* oil, in general, and the monoterpenes that function as insecticidal agents in particular, are responsible for its efficiency [62–64]. Monoterpenes, particularly pulegone, are abundant in our plant (76.35 percent). These active ingredients had significant insecticidal efficacy against a variety of pests [62, 65]. Pulegone has the ability to enter the lipophilic cuticular tissue layer of insects, resulting in the suppression of respiration, growth, and fecundity. The mechanism of action of the responsible compound can also include the acetylcholinesterase inhibition [66, 67]. Interference with octamine action, gamma-aminobutyric acid (GABA), modulation of chlorine channels has also been reported in previous works [68, 69]. In addition, it should be noted that the method of application (inhalation or contact) of *M. pulegium* EO on *C. maculatus* showed differences in the percentage of mortality, fecundity rate, and emergence rate.

3.4. DPPH Free Radical Scavenging. The DPPH bioassay was used to assess the antiradical activity of *M. pulegium* essential oils. Ascorbic acid (vitamin C) was employed as a standard reference to attain this purpose. Figure 4 depicts the antiradical action of *M. pulegium* essential oil. The essential oils of *M. pulegium* had an IC_{50} of 7.659 mg/Ml for antioxidant activity against the DPPH radical. This finding is consistent with prior research, which found that oil from *M. pulegium*, a native of Iran, had an antioxidant activity with an IC_{50} of 14736 g/mL [11]. When compared to the

Table 5: Effects of essential oils from *M. pulegium* tested by contact on oviposition and emergence of *C. maculatus*.

Dose (μm)	Number of eggs and emergence	
Number of eggs laid emergence	Number of eggs adults emergence	
Control	0^{a}	0^{a}
1	74.64 ± 21.2^{b}	13.67 ± 2.51^{b}
5	83 ± 1.86^{bc}	6.33 ± 1.84^{b}
10	98.33 ± 0.41^{c}	100 ± 0^{b}
20	100 ± 0^{c}	100 ± 0^{b}

Columns with the same letter did not differ significantly according to ANOVA analysis.

Table 6: Lethal concentration values of *M. pulegium* essential oil tested by inhalation on *C. maculatus*.

Treatment (h)	df	Slope + SD	LC_{50} (CI95%)	LC_{95} (CI95%)	Intercept ± SE	*p* value	X^2
24	2	1.41 + 0.48	1.99 (0.27;4.127)	28.81 (10.93;1900.8)	−0.424 + 0.382	0.409	1.79
48	2	1.17 + 0.51	0.83 (0.0;2.298)	20.8 (7.03; 661438)	0.96 + 0.379	0.430	1.69
72	2	—	—	—	—	—	
96	2	—	—	—	—	—	—

(—): data are absent because the insects died within the first hour of the experiment.

Table 7: Effects of essential oils of *M. pulegium* tested by contact on the mortality of adults from *C. maculatus*.

Dose (μm)	Percentage of mortality per treatment day			
	24 h	48 h	72 h	96 h
Control	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
1	16.67 ± 4.44^{b}	33.33 ± 4.44^{b}	53.33 ± 4.44^{b}	90 ± 0^{b}
5	26.67 ± 4.44^{b}	53.33 ± 4.44^{c}	76.67 ± 4.44^{c}	100 ± 0^{b}
10	60 ± 6.67^{c}	73.33 ± 4.44^{d}	93.33 ± 4.44^{d}	100 ± 0^{b}
20	86.67 ± 4.44^{d}	96.67 ± 4.44^{e}	100 ± 0^{d}	100 ± 0^{b}

Columns with the same letter did not differ significantly according to ANOVA analysis.

Table 8: Lethal concentration values of *M. pulegium* essential oil tested by contact on *C. maculatus*.

Treatment (h)	df	Slope + SD	LC_{50} (CI95%)	LC_{95} (CI95%)	Intercept ± SE	*p* value	X^2
24	2	1.56 ± 0.5	6.51 (2.95; 15.06)	74.18 (25.2; 5759.5)	−1.268 + 0.457	0.004	2.01
48	2	1.38 ± 0.51	2.74 (0.53; 0.69)	42.71 (14.9; 4580.6)	−0.603 + 0.389	0.002	2.009
72	2	1.47 ± 0.55	1.026 (0.032; 2.34)	13.43 (5.58; 806.95)	−0.017 ± 0.381	0.007	0.832
96	2	—	—	—	—	—	—

(—): data is absent because the insects died within the first hour of the experiment.

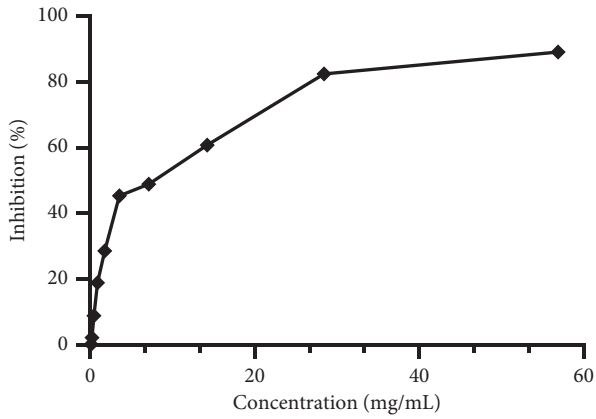

Figure 4: Results of the DPPH antioxidant test for *Mentha pulegium* essential oils.

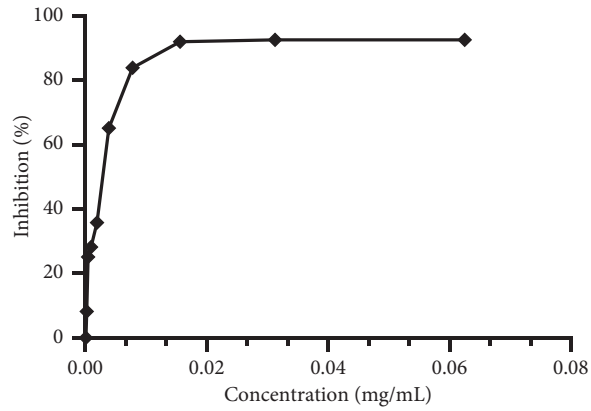

Figure 5: Results of the antioxidant test by the DPPH method for ascorbic acid.

standard synthetic antioxidant (Figure 5), ascorbic acid ($IC_{50} = 2.815\,\mu g\,mL$) (P0.0001), these essential oils have a moderate antioxidant potential. The chemical composition of *M. pulegium* essential oils accounts for their antioxidant action [70]. Pulegone (61.11%) may be implicated in *M. pulegium's* antioxidant action [70]. The obtained results are supported by those reported elsewhere, which showed that essential oil from *M. pulegium* from different collection areas had antioxidant power [36].

3.5. Total Antioxidant Capacity (TAC). The measurement of the total antioxidant capacity revealed the presence of important antioxidant agents in the studied oil (583.066 ± 57.05 mg EAA/g EO) (Figure 4). Our results were in agreement with those reported by Ahmed et al. [7], who showed antioxidant activity ($IC_{50} = 20.17 \pm 1.88$ mg/mL) in essential oils from *M. pulegium* collected from the different collection areas. In addition, the same authors reported that the studied EOs were potent when compared to reference antioxidants, butylated hydroxytoluene (BHT).

Ahmed et al. [7] discovered that the modest variations in components, mainly pulegone and menthone concentrations, can be attributable to the varying degrees of antioxidant capabilities found for EOs isolated from *M. pulegium* dried by different procedures. In this sense, it was reported that pulegone and menthone identified in *M. pulegium* may be the responsible compounds for the antioxidant effect [11, 62–73].

The active molecules in essential oils of aromatic plants are primarily responsible for their antioxidant properties, according to the literature. The monoterpene ketones menthone and isomenthone are the most powerful molecules [74]. Minor molecules in essential oils, rather than large compounds, are more likely to have a substantial role in antioxidant activity [75]. Similarly, previous works showed the presence of very important antioxidant activity of several essential oils including genus *Mentha* essential oil [76].

4. Conclusion

The chemical composition and antioxidant, antibacterial, and insecticidal activities of *M. pulegium* L. were studied in this work. In conclusion, the essential oil of *M. pulegium* L. was found to be very rich in R-(+)-pulegone, which remains the main contributor to the biological activities of this oil. This study revealed that the essential oil of *M. pulegium* was active against the tested microbes, insect pests of legume seeds along with antioxidant effect so that the plant oil can be used as natural drugs to serve health and food crops. Therefore, the essential oil of *M. pulegium* can be exploited in the development of antibiotics, bioinsecticides, and food preservatives. However, on a large-scale practical level, it is necessary to better understand the effect of sublethal doses of essential oils on nontarget organisms, as well as potential toxicities to humans. It is thus fitting that further studies on the potential toxicities of the tested essential oils are needed for safety purposes.

Data Availability

Data used to support the findings are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the publication of this study.

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Research Article

Antifungal Activity and Acute and Repeated-Dose Toxicity Study of Geranyl Cinnamate Ester in Mice

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In the present study, the antifungal activity and toxicity of the geranyl cinnamate ester (GCE) were investigated. The GCE showed antifungal activity at a minimum concentration of 0.16 μL/mL against *Candida albicans* and at concentrations greater than 2.5 μL/mL against *Aspergillus niger*. In acute toxicity studies, the administration of GCE (2.000 mg/kg) affected the body weight gain and food intake but did not induce the mortality of the animals studied. After the investigation of repeated-dose toxicity of GCE at 2 and 4 mg/kg, the hematological and biochemical parameters were changed. In addition, the adrenal weight of male mice treated with GCE at 4 mg/kg was affected. In conclusion, according to the Organization for Economic Cooperation and Development (OECD) acute toxicity parameters, the geranyl cinnamate ester can be classified into safety category number 5. The results of this study suggested that the geranyl cinnamate ester may be a source of natural antifungals.

1. Introduction

Natural antimicrobial compounds have been used by the food industry as preservatives in industrialized foods. These compounds control and reduce the growth of bacteria and fungi [1, 2]. However, many studies indicate that some natural compounds can cause cancer and allergies in humans [3]. Despite health hazards, these compounds are essential in prolonging the storage time of food, and the use of natural antimicrobials is an attractive opportunity for food preservation.

Natural antimicrobials can be obtained from different sources, including plants, animals, bacteria, algae, and fungi. Several studies with antimicrobial compounds obtained from plants have demonstrated their efficacy when applied for food preservation [4, 5]. In this sense, several studies have evaluated the efficacy of essential oils derived from aromatic plants, such as thymol, carvacrol, allicin, geraniol, limonene, among others, which showed inhibitory activity on the growth of pathogenic bacteria of food origin [6].

Geraniol is an acyclic monoterpene alcohol and is primarily extracted from different essential oils, namely,

palmarosa, ninde, and rose oils [7, 8]. In particular, geraniol has a high capacity to inhibit and kill Gram-positive and Gram-negative bacteria, as well as some types of fungi and some types of yeast [8]. The antifungal activity of geraniol is also cited. In the study of Frias and Kozusny-Andreani [9], essential oils extracted from lemon and citronella were tested in four pathogenic fungi (*Candida albicans*, *Nannizzia gypsea*, *Sporothrix schenckii*, and *Aspergillus niger*) and showed high antifungal activity. In addition, in a study by Tang et al. [10], the compounds geraniol and citral showed excellent antifungal effects against common grain pathogens, such as *Aspergillus flavus* and *Aspergillus ochraceus*, in in vitro and in situ tests.

Cinnamic acid, also known as 3-phenyl-2-propenoic acid, consists of a naturally occurring aromatic fatty acid originated from higher plants and found in Estoraques, cinnamon oils, and coca leaves, has low toxicity and a broad spectrum of biological activities against numerous microorganisms. Cinnamic acid is the main constituent of clove oil, which constitutes of approximately 70 to 80% followed by eugenol (4 to 7%) [11].

However, some essential oils are volatile, unstable to light and heat, and easily decomposed depending on the antimicrobial application. Generally, the esterification of some essential oils improves specific substrate properties such as emulsification, dispersion, and overall quality of the consumer products. In this sense, the microbiological and toxicity study of the geranyl cinnamate ester, which was obtained by the esterification reaction between geraniol and cinnamic acid, becomes interesting.

In the literature, no research presents the antifungal activity of the geranyl cinnamate ester. There are works that have studied the antibacterial activity of other esters such as eugenyl acetate against Gram-positive *Staphylococcus aureus* (ATCC 9763) and *Listeria monocytogenes* (ATCC 15117) and Gram-negative bacteria *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) [12].

There are no reports evaluating the toxicological effects of geranyl cinnamate ester (GCE) and its antimicrobial effect against the fungi *Candida albicans* and *Aspergillus niger*. Thus, the aim of the present study is to investigate the antifungal activity of geranyl cinnamate ester and the toxicity of acute and repeated doses (28 days) in mice.

2. Materials and Methods

2.1. Geranyl Cinnamate Ester Production. The preparation of the geranyl cinnamate ester was carried out according to Zanetti et al. [13] by the esterification reaction of cinnamic acid with geraniol (≥99%) from Sigma-Aldrich (Brazil), under the following reaction conditions: 70°C, 15 wt% of immobilized *Candida Antarctica* NS88011, and 3 : 1 geraniol to cinnamic acid molar ratio. The reaction was carried out in Erlenmeyer glass flasks (250 mL) using an orbital shaker (150 rpm), 10 mL n-heptane as solvent, and 2 h reaction time for all experiments. To purify the geranyl cinnamate ester and to remove the unreacted reagents (enzyme *Candida Antarctica*, cinnamic acid, and geraniol) after all experiments, the final product was filtered with membranes and then evaporated on a rotary evaporator with a maximum temperature of 40°C. For this reaction, a 97% conversion of geraniol to geranyl cinnamate ester was obtained.

2.2. Experimental Design

2.2.1. Antifungal Activity of Geranyl Cinnamate Ester. Antifungal activity of the geranyl cinnamate ester was evaluated according to the antifungal susceptibility testing method described by the NCCLS (2004), with adaptations and with two genera of fungi: yeast *Candida albicans* (ATCC 24433) and *Aspergillus niger* (ATCC 16888). The strains of *Candida albicans* and *Aspergillus niger* were obtained from the Laboratory of Mycology of the Community University of the Region of Chapecó—Unochapecó.

The strains were reactivated with Sabouraud dextrose broth, and for the study, the fungal suspensions were prepared by choosing five colonies with a diameter of approximately 1 mm after incubation of 24 h of the *Candida* species. The colonies were suspended in 5 mL of sterile saline (0.90% saline), and the resultant suspension was homogenized on a vortex shaker for 15 seconds. Subsequently, a saline solution was added to obtain the turbidity equivalent to the standard solution of the McFarland 0.5 scale to obtain a standard yeast suspension containing approximately 10^5 microorganisms per 1 mL.

Assays were performed using 20 mL of sterile Sabouraud dextrose agar culture medium at 65°C in Petri dishes (50 × 10 mm) and were allowed to solidify. Different amounts of geranyl cinnamate were added to the agar in different plates, obtaining different concentrations as shown in Table 1.

A volume of 10 μL of the microorganism suspension was then inoculated onto the agar and spread with the aid of a Drigalski loop over the entire surface of the plate. The plates were incubated at 36 ± 1°C in a greenhouse (J Prolab, model B3) for 48 h for the *Candida albicans* and for 5 days at 27 ± 1°C for Aspergillus *niger*. After this time, antifungal activities were evaluated by the presence or absence of colony formation. To verify the growth of the microorganisms, a control plate was prepared with the microorganism without the addition of antifungal compounds.

2.2.2. In Vivo Assays

(1) Animals. Male and female (nulliparous and nonpregnant) mice (20–30 g) from Unochapecó bioterium (Chapecó-SC) were used. Animals were housed in groups of five mice in plastic cages (28.0 × 12.5 × 19.0 cm) at constant room temperature (22 ± 2°C) and humidity (40–60%), under a 12 h light/dark cycle with free access to food (Biobase®) and water *ad libitum*. Experiments were approved by Animal Care Local Ethical Committee (CEUA-UNOCHAPECÓ; Protocol 008/2018). Animal care and experiments were conducted in accordance with Brazilian law (Brazil, 2008; CONCEA, 2018) and EU Directive 2010/63/EU for animal experiments.

Table 1: Concentration of geranyl cinnamate ester for the solid medium diffusion test.

Sample	Geranyl cinnamate volume (μL)	Geranyl cinnamate concentration in the plate (μL/mL)
a	50.00	10.00
b	25.00	5.00
c	12.50	2.50
d	6.25	1.25
e	3.12	0.62
f	1.56	0.31
g	0.78	0.16
i (control)	0	0

(2) Treatments. Geranyl cinnamate ester (GCE) is a water-insoluble compound and, therefore, it was dissolved in corn oil (0.1, 0.2, 0.4, or 200.0 mg/mL), according to the OECD Guidelines 423 [14] and 407 [15] and was administered by gavage. In the acute toxicity studies, the GCE was orally administered to mice at a dosage of 2000.0 mg/kg and for the repeated-dose toxicity studies at 1.0, 2.0, or 4.0 mg/kg (p.o.) for 28 days. All treatments were with respect to the dosage of 10 mL/kg body weight. To determine the concentration to be used in the tests, the maximum percentages of addition of active compounds in packaging (4%) and the minimum amount of ester necessary to inhibit microbial growth were considered. It was considered for the calculation of 100% release of the active compound from the packaging to the product and, thus, a value of 1 mg/kg is obtained. The euthanasia was performed with thiopental sodium (50 mg/kg, i.p.) preceded by hydrochloride lidocaine (10 mg/kg, i.p.).

(3) Toxicity Studies. The toxicity studies were based on the guidelines of the Organization for Economic Cooperation and Development (OECD). The acute oral toxicity studies were performed according to Guideline 423 [14], and the repeated-dose (28-day oral administration) toxicity studies followed the Guideline 407 [15]. These OECD guidelines are the worldwide reference for chemical testing.

Regarding the acute toxicity test (OECD 423), female mice received a single GCE dose (2000 mg/kg, p.o.) ($n = 3$ mice/experimental step). The animals were fasted before administration (food but not water was withheld for 3 h). After the administration, animals were observed with special attention during the first 4 hours and 12 hours later, and every day for 14 days. The body weight gain and food intake were registered every two days during the experimental period. Observations of the abnormal behavior of female mice such as piloerection, palpebral ptosis, abdominal writhing, muscular tonus, motor activity, hypothermia, shacking, posterior paw paralysis, salivation, bronchial secretion, lethargy, diarrhea, and convulsions were considered. Moreover, the number of deaths was registered.

For repeated-dose toxicity tests (OECD 407), the GCE was administered in three different doses by gavage once a day for 28 days. Female ($n = 20$) and male ($n = 20$) mice were divided into four groups containing five animals by gender, according to the OECD Guideline 407 [15]: group I: control, treated with vehicle (corn oil, 10.0 mL/kg); group II: GCE 1.0 mg/kg, p.o.; group III: GCE 2.0 mg/kg, p.o.; and group IV: GCE 4.0 mg/kg, p.o. The 1.0 mg/kg dose was chosen according to the profile of GCE release from the package to food. Considering that the preservative would be ingested at 1.0 mg/kg, the doses of 2.0 and 4.0 mg/kg were defined in accordance to the OECD 407 (2 to 4 fold intervals for setting the dose levels). The same toxicity signs described in the OECD 423 were observed. Food intake and body weight gain were registered every two days throughout the experiment.

The euthanasia of animals occurred at the end of the experimental protocols on the 15th and 29th days (acute and repeated-dose toxicity study, respectively). Mice were euthanized with thiopental sodium (50.0 mg/kg, i.p.) preceded by lidocaine hydrochloride (10.0 mg/kg, i.p.). Blood and urine were collected from the hepatic portal vein and bladder, respectively, from mice submitted to the repeated-dose toxicity study.

The organs (brain, heart, thymus, spleen, adrenals, kidney, and liver (OECD 2008)) were removed (both after the acute and subacute study) and weighed for statistical analysis and further histopathological studies. The relative organ weight was calculated considering the body weight of the mouse by using the following equation:

$$\text{relative organ weight}\,(\%) = \frac{\text{organ weight} * 100}{\text{mouse body weight}}. \quad (1)$$

2.2.3. Biochemical Parameters. Serum investigations were made for sodium (Na), potassium (K), glucose (GLU), total cholesterol (COL)and fraction (LDL), triglycerides (TRI), uric acid (UAC), creatinine (CRE), total protein (PRO), albumin (ALB), and two enzymes indicative of hepatocellular effects: alanine aminotransferase (ALT) and alkaline phosphatase (AP). The analyses were performed with Labtest® kits using a spectrophotometer BTS-310® (Biosystems®). Considering that the blood volume collected from the mice varied from animal to animal, the final number of animals used in the tests was between 3 and 5 per group.

2.2.4. Hematological Parameters. Blood was collected into EDTA tubes (0.5 mL), and some parameters were evaluated: hemoglobin (Hb), red cell distribution width (RDW), haematocrit (HCT), mean corpuscular volume (MCV), mean cell hemoglobin (MCH), mean cell corpuscular hemoglobin concentration (MCHC), white blood cell counts (WBC), erythrocyte count, reticulocytes, eosinophils (E), monocytes (M), neutrophils (N), lymphocytes (L), and

platelet counts. The analyses were performed on the ABX Micros 60® equipment. Considering the fast blood coagulation, the final number of mice haemogram varied between 3 and 5 per group.

2.2.5. Urinalysis. This analysis was performed using Uriquest Plus® (Labtest®, Brazil) semiquantitative fast determination reagent strips for urobilinogen, glucose, ketone bodies, bilirubin, total protein, ascorbic acid, blood, nitrite, leucocytes, pH, and density.

2.2.6. Histopathology. Five animals (two males and three females) from each treatment group were randomly chosen for the histological analysis. Brain, heart, thymus, spleen, adrenals, kidneys, and liver were fixed in neutral buffered 10% formalin. The samples were dehydrated with alcohol, cleared with xylene, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Samples were then processed and examined by optical microscopy.

2.3. Statistical Analysis. Two-way repeated-measures analysis of variance (ANOVA) followed by the Bonferroni test was used to evaluate the relative body weight and food intake of mice. One-way ANOVA *post hoc* Bonferroni was performed for the evaluation of the hematological and biochemical analysis and relative weight of organs (repeated-dose study). The relative weight of organs in the acute toxicity study was analysed with the unpaired *t*-test. GraphPad Prism® 5.0 software was used to perform the statistical analysis. Data were expressed as mean ± SEM. The level of significance was set as $p < 0.05$.

3. Results

3.1. Results Obtained for the Antifungal Activity of the Geranyl Cinnamate Ester. Antifungal activity of the geranyl cinnamate ester was carried out with yeast *Candida albicans*. The tests were performed with seven different concentrations of geranyl cinnamate ester, and the diameter values of *Candida albicans* growth inhibitor halos were compared with the control sample, which contained only pure agar without the active compound. The results are presented in Figure 1.

Geranyl cinnamate ester was active for all concentrations tested, and the minimum concentration was 0.16 μL/mL (letter f) against *Candida albicans*. It is possible to observe that, in the control sample, there was no inhibition of the growth of the fungus.

Antifungal activity of the geranyl cinnamate ester was also evaluated against the fungus *Aspergillus niger*. The tests were performed with different concentrations of geranyl cinnamate ester, and the results are shown in Figure 2.

The geranyl cinnamate ester showed antifungal activity against *Aspergillus niger* fungus when used at a concentration of up to 2.5 μL/mL (letter c).

3.2. Acute Toxicity. On the first day of the experiment (during 4 h after administration), GCE-treated mice presented lethargy and sedation, without the loss of reflexes and respiratory depression. No deaths occurred during the experiment. The body weight of the GCE-treated mice increased at the 6th ($p < 0.01$), 12th ($p < 0.01$), and 15th day after treatment ($p < 0.001$). The results indicate a weight gain of the mice treated with corn oil ($p < 0.001$) on the 12th and 15th day, when compared to the beginning (day 0) of the treatment and there was a significant ($p < 0.05$) body weight gain of the GCE-treated mice in comparison with the vehicle-treated group at the 15th day after the oral administration (Figure 3(a)). However, the food intake of the GCE-treated mice was significantly ($p < 0.001$) lower than the control group food intake from the 3rd to the 12th day after the treatment (Figure 3(b)). The oral administration of GCE induced a significant increase ($p < 0.05$) in the relative weight of the kidney when compared to the corn oil-treated mice (Figure 4). There were no significant changes in the relative weight of brain, heart, thymus, liver, and adrenals between the groups (data not shown).

3.3. Repeated-Dose Toxicity. Gross behavior of the animals was observed during the 28 days of administration. One male mouse treated with GCE 4 mg/kg group presented diarrhea at the first week of administration. Female mice did not present any sign of toxicity.

3.3.1. Body Weight Gain. All female mice treated with GCE (1, 2, and 4 mg/kg, p.o.; Figure 5(a)) presented a significant body weight gain when compared to the 1st day of treatment. At the 22nd and 28th day of observation, the body weight of GCE 1 mg/kg-treated female mice was significantly ($p < 0.05$) increased in comparison with the first (day 1) measurement taken in the same group. The body weight of GCE 2 mg/kg-treated female mice was significantly higher at the 25th ($p < 0.01$) and 28th ($p < 0.05$) day when compared to the 1st day of treatment. The body weight of GCE 4 mg/kg-treated female mice was significantly ($p < 0.001$) increased at the 25th day of treatment. The body weight of the vehicle-treated female mice (corn oil, 10 mL/kg) was significantly ($p < 0.05$) increased at the last day (28th) of the experiment when compared to the 1st day of treatment.

The body weight of male mice (Figure 5(b)) was not affected by the GCE administration. There were no significant differences between the weight gain of the GCE-treated groups in comparison with the vehicle group at the same day of treatment.

3.3.2. Food Intake. Figure 3 demonstrates the food intake of the GCE and vehicle-treated female (Figure 6(a)) and male (Figure 6(b)) mice. The food intake of female mice did not present any significant variation between the vehicle group and the GCE-treated groups (Figure 6(a)); while the food intake of the male mice (Figure 6(b)) was significantly affected by the GCE treatment: food consumption of GEC 1 mg/kg-treated group was significantly ($p < 0.05$) higher at week 2 in relation to the vehicle-treated group (corn oil-treated, 10 mg/mL) in the same week. The food consumption

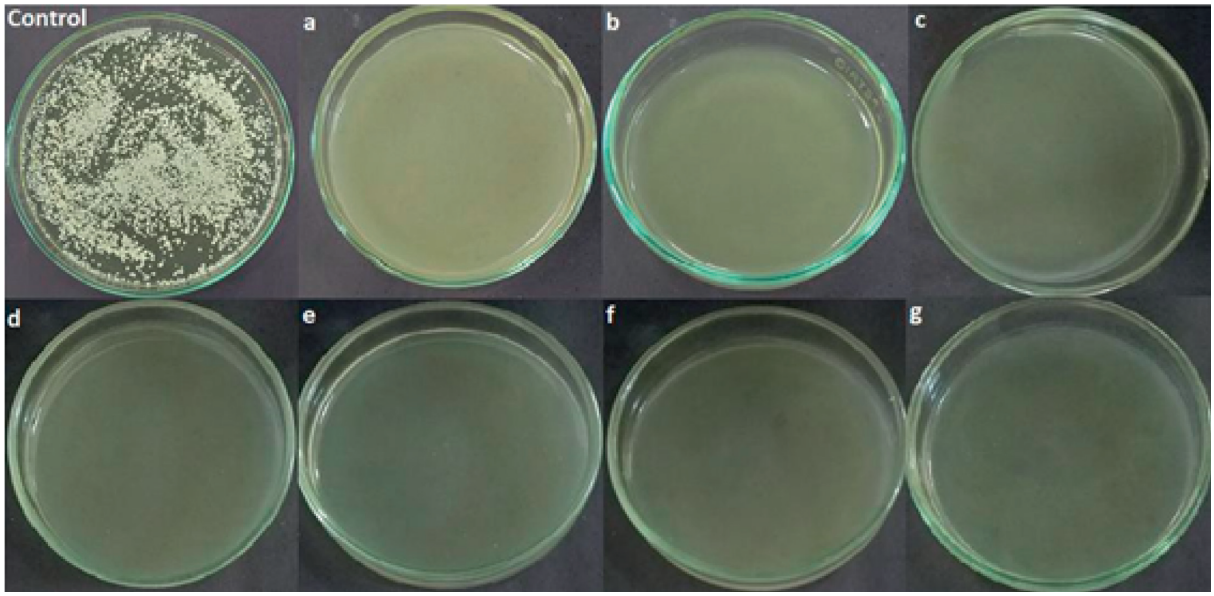

Figure 1: Results of the agar diffusion assays for the antifungal activity of geranyl cinnamate ester with yeast *Candida albicans* of different concentrations: (a) 10.00 μL/mL, (b) 5.00 μL/mL, (c) 2.50 μL/mL, (d) 1.25 μL/mL, (e) 0.62 μL/mL, (f) 0.31 μL/mL, and (g) 0.16 μL/mL.

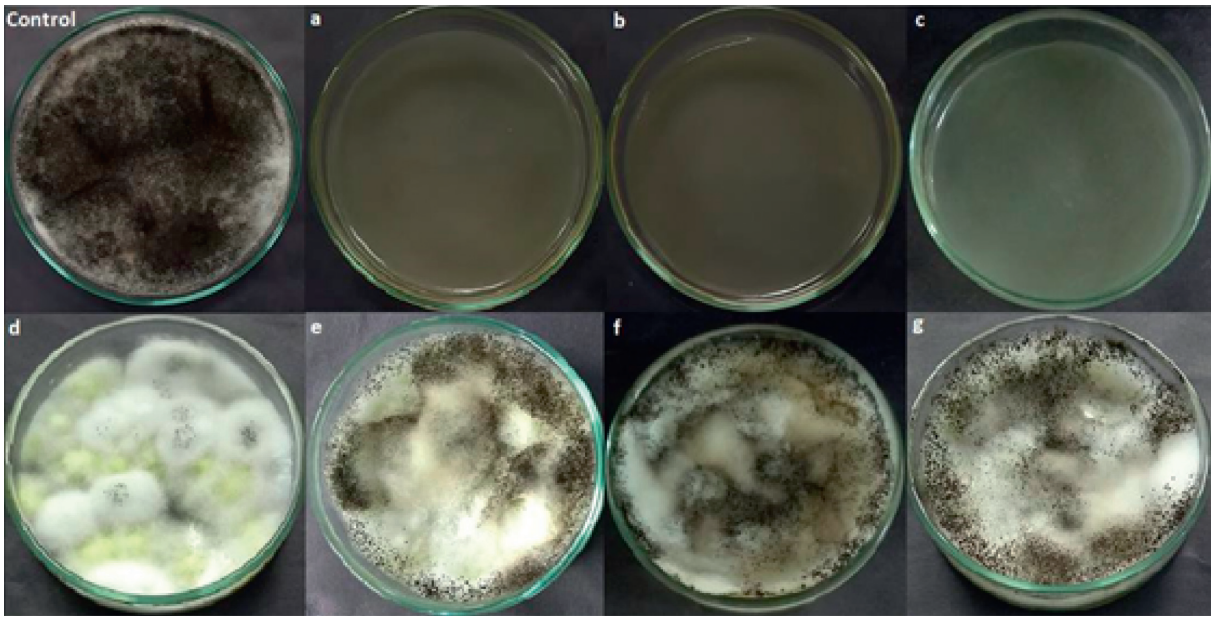

Figure 2: Results of the agar diffusion assays for the antifungal activity of geranyl cinnamate ester with yeast *Aspergillus niger* of different concentrations: (a) 10.00 μL/mL, (b) 5.00 μL/mL, (c) 2.50 μL/mL, (d) 1.25 μL/mL, (e) 0.62 μL/mL, (f) 0.31 μL/mL, and (g) 0.16 μL/mL.

of GCE 4 mg/kg-treated group was significantly ($p < 0.05$) decreased at week 3 in relation to the GCE 2 mg/kg-treated group in the same week, and there was a significant ($p < 0.05$) decrease at week 3 in relation to week 1 of the same treatment group.

3.3.3. Haematological Parameters. Haematological data of male and female ECG (1, 2, 4 mg/kg, p.o.) and vehicle-treated mice are presented in Table 2. Mice (female and male) treated with GCE at 4 mg/kg presented a significantly ($p < 0.05$) lower number of reticulocytes (%) when compared to GCE 1 mg/kg-treated group.

Lymphocytes (%) of the GCE 4 mg/kg-treated male mice presented a significant ($p < 0.05$) decrease in lymphocytes when compared to the vehicle-treated group (corn oil, 10 mg/mL). No changes were detected between the female mice groups. GCE 4 mg/kg-treated male mice presented a significant ($p < 0.05$) increase in neutrophils (%) compared to the vehicle-treated group, while no changes were detected between the female mice groups.

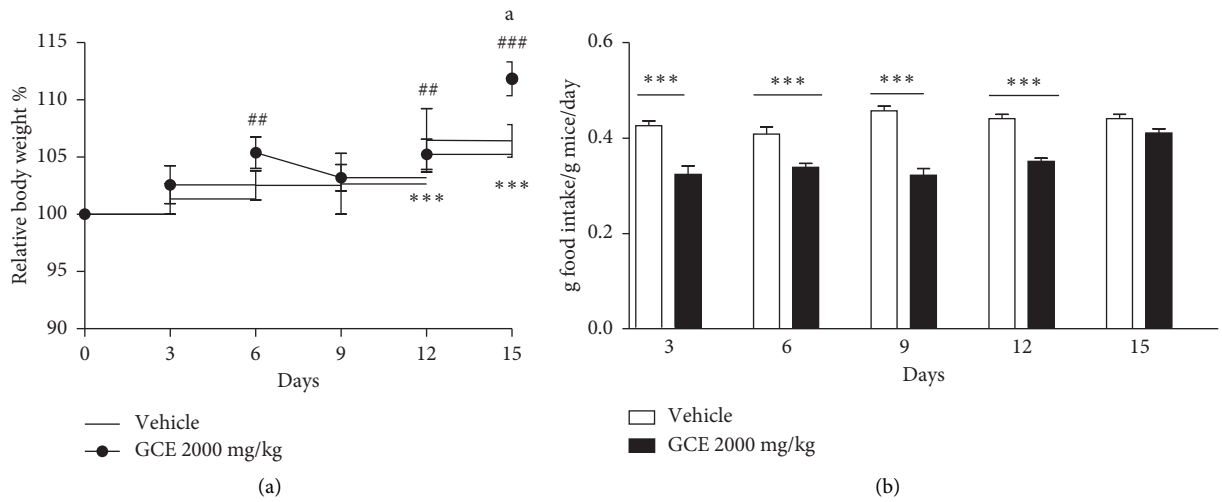

Figure 3: Effect of geranyl cinnamate ester (GCE) acute treatment (2000 mg/kg, p.o.) on relative body weight (%) of female mice ($n = 3$–6 mice/group) (a) and food intake (g food intake/g mice/day) (b). Data are expressed as mean + SEM. Two-way repeated-measures ANOVA *post hoc* Bonferroni. Relative body weight (a): different from the initial weight (day 0). $^{***}p < 0.001$ (corn oil-treated, 10 mL/kg, p.o.), $^{\#\#}p < 0.01$ (GCE-treated, 2000 mg/kg, p.o.), and $^{\#\#\#}p < 0.01$ (GCE-treated, 2000 mg/kg, p.o.), and $p < 0.05$ different from the vehicle group (corn oil-treated, 10 mL/kg, p.o.) in the same day of measurement. Food intake (b): $^{***}p < 0.001$ different from the vehicle group (corn oil-treated, 10 mL/kg, p.o.).

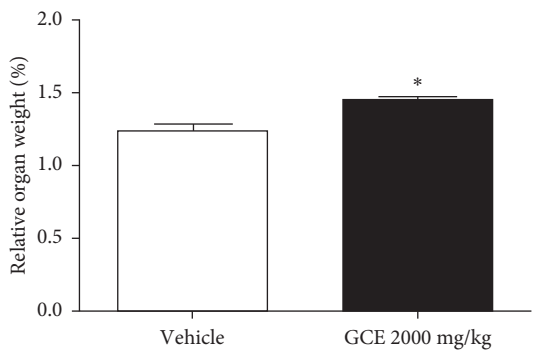

Figure 4: Effect of the geranyl cinnamate ester (GCE) acute treatment (2000 mg/kg, p.o.) on the relative weight of female mice ($n = 3$–6 mice/group) kidney. Data are expressed as mean ± SEM. Unpaired t-test: $^{*}p < 0.05$ compared to the vehicle-treated group (corn oil, 10 mL/kg, p.o.).

Platelets ($\times 10^3/mm^3$) of the male mice treated with GCE at 4 mg/kg were significantly decreased when compared to the GCE 2 mg/kg-treated group ($p < 0.01$), GCE 1 mg/kg-treated group ($p < 0.05$), and vehicle-treated group ($p < 0.05$). The female mice platelet number was not altered between the groups.

Hemoglobin (Hb), red cell distribution width (RDW), haematocrit (HCT), mean corpuscular volume (MCV), mean cell hemoglobin (MCH), mean cell corpuscular hemoglobin concentration (MCHC), white blood cell counts (WBC), erythrocytes counts, and monocytes did not change significantly between groups.

3.3.4. Biochemical Parameters. Several biochemical parameters were affected by the mice treatment with GCE. These data are depicted in Figure 4 (female mice) and Figure 5 (male mice).

The Na^+ (mEq/L) serum level in the GCE 1 mg/kg-treated female mice (Figure 4(a)) was significantly ($p < 0.05$) decreased in relation to the vehicle group. No variations in Na^+ (mEq/L) levels were detected in the serum of male mice (Figure 5(a)).

The K^+ serum levels (mEq/L) in the female (Figure 4(b)) and male (Figure 5(b)) GCE 4 mg/kg-treated groups were significantly ($p < 0.05$) increased in female mice and decreased in male mice when compared to the GCE 2 mg/kg-treated groups.

Treatment of female mice (Figure 4(c)) with GCE at 4 mg/kg induced a decrease in PRO (g/dL) serum levels when compared to the vehicle-treated animals; GCE 1 mg/kg-treated male mice (Figure 5(c)) presented a significant ($p < 0.05$) increase in PRO levels in relation to the GCE 2 and 4 mg/kg-treated groups.

Female mice that received GCE at 2 and 4 mg/kg (p.o.) showed significantly ($p < 0.05$) decreased ALB (g/dL) serum levels (Figure 4(d)) when compared to the vehicle-treated group, while male mice that were orally treated with GCE at 2 mg/kg presented a significant ($p < 0.05$) decrease in the ALB serum levels (Figure 5(d)) in relation to the vehicle and GCE 1 mg/kg-treated groups.

The treatment of female mice with GCE at the highest dose elicited a significant ($p < 0.05$) increase in GLU (mg/dL) serum levels (Figure 4(e)) in relation to the vehicle-treated group; in male mice, there was a significant ($p < 0.05$) increase in GLU serum levels (Figure 5(e)) of the GCE 2 mg/kg-treated group in comparison with the GCE 1 mg/kg-treated animals.

COL (mg/dL) levels in the serum of female (Figure 7(f)) and male (Figure 8(f)) GCE 4 mg/kg-treated mice were significantly ($p < 0.05$) increased in relation to the GCE 2 mg/kg-treated group.

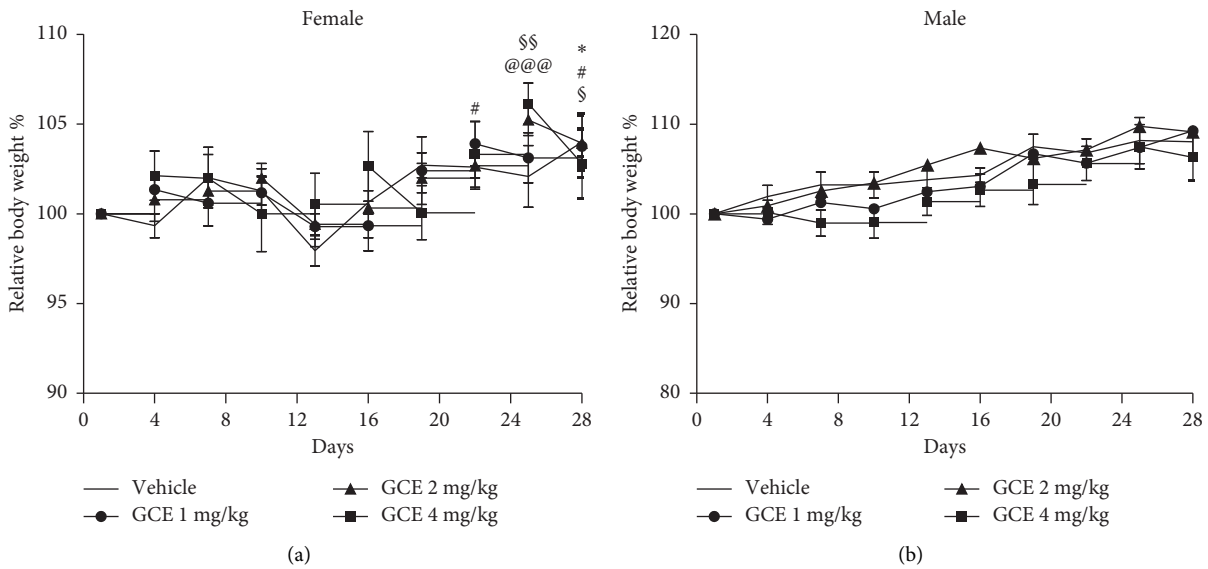

Figure 5: Effect of geranyl cinnamate ester (GCE) repeated-dose treatment (1, 2, and 4 mg/kg, p.o.) on the relative body weight of female (a) and male (b) mice ($n = 5$ mice/group). Data are expressed as mean + SEM. Two-way repeated-measures ANOVA *post hoc* Bonferroni. Symbols represent differences in relation to the first measurement (day 1) in the same treatment group ($^{*}p < 0.05$: vehicle group; $^{\#}p < 0.05$: GCE 1 mg/kg; $^{\S\S}p < 0.01$: GCE 2 mg/kg, and $^{@@@}p < 0.001$: GCE 4 mg/kg).

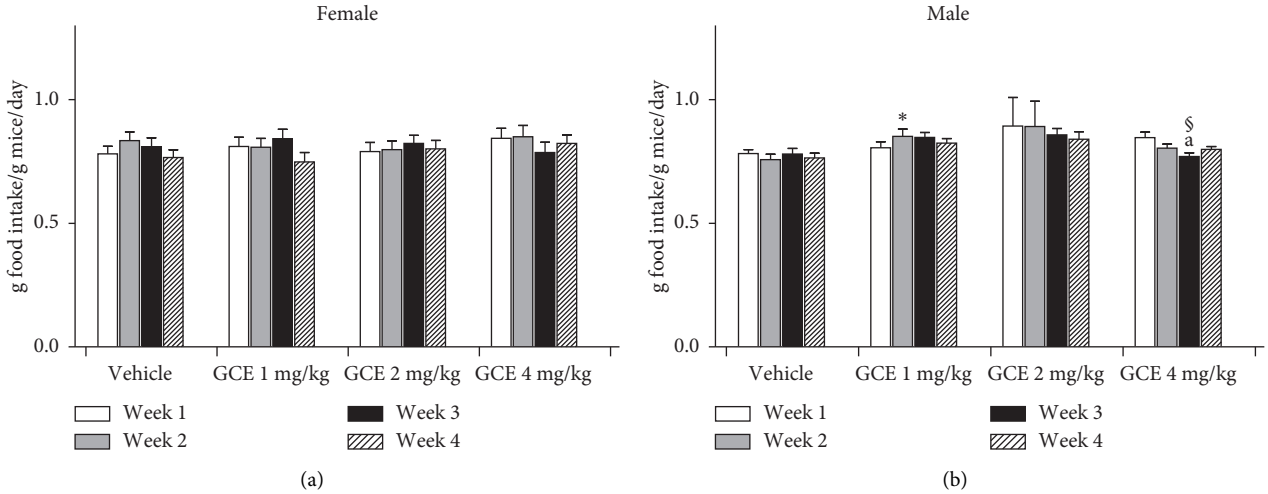

Figure 6: Effect of geranyl cinnamate ester (GCE) repeated-dose treatment (1, 2, and 4 mg/kg, p.o.) on food intake by female (a) and male (b) mice (g food intake/g mice/day). Data are expressed as mean + SEM ($n = 5$ mice/group). Two-way repeated-measures ANOVA *post hoc* Bonferroni. $^{*}p < 0.05$ different from the vehicle group (corn oil-treated, 10 mL/kg, p.o.) in the same week; $^{\S}p < 0.05$ different from the GCE 2 mg/kg-treated group in the same week; $^{a}p < 0.05$ different from the week 1 of the same treatment group.

LDL serum levels (mg/dL) were affected in the animals that received the highest ECG dose: in both sexes (Figures 4(g) and 5(g)), there was a significant ($p < 0.05$) decrease in comparison with the GCE 2 mg/kg-treated group.

TRI (mg/dL) levels in the serum of female mice (Figure 4(h)) treated with GCE at 4 mg/kg were significantly ($p < 0.05$) increased in relation to the vehicle-treated group; male mice (Figure 5(h)) that received GCE at 2 mg/kg presented a significant ($p < 0.05$) decrease in serum TRI levels in relation to the GCE 1 mg/kg-treated group.

ALT (mg/dL) levels in the serum of female (Figure 4(i)) and male (Figure 5(i)) mice treated with GCE at 2 mg/kg were significantly ($p < 0.05$) decreased in relation to the vehicle-treated group.

Female (Figure 4(j)) and male (Figure 5(j)) mice treated with GCE at 4 mg/kg presented significantly ($p < 0.05$) increased AP (U/L) serum levels when compared to the GCE 1 mg/kg-treated group.

CRE (mg/dL) serum levels of female (Figure 4(k)) and male (Figure 8(k)) GCE 4 mg/kg-treated groups were

Table 2: Effect of the geranyl cinnamate ester (GCE) repeated-dose treatment (1, 2, and 4 mg/kg, p.o.) on female and male mice haemograms.

	Vehicle	GCE 1 mg/kg	GCE 2 mg/kg	GCE 4 mg/kg
		Male		
WBC ($\times 10^3$/mm^3)	1.65 ± 0.37	2.16 ± 0.52	3.10 ± 0.38	2.94 ± 0.87
Hb (g/dL)	12.83 ± 1.34	11.78 ± 1.23	13.73 ± 1.34	12.88 ± 1.52
HCT (%)	38.20 ± 5.61	31.94 ± 4.67	41.63 ± 5.59	38.36 ± 4.86
MCV (μm^3)	48.50 ± 1.44	47.20 ± 0.20	47.20 ± 0.62	47.20 ± 0.44
MCH (pg)	16.15 ± 0.27	17.88 ± 1.21	16.23 ± 1.02	15.86 ± 0.16
MCHC (g/dL)	33.55 ± 1.08	37.96 ± 2.71	33.70 ± 1.75	33.84 ± 0.49
RDW (%)	16.75 ± 0.73	16.26 ± 0.17	16.73 ± 0.64	16.32 ± 0.19
Platelets ($\times 10^3$/mm^3)	373.80 ± 60.64	358.50 ± 60.33	475.50 ± 27.84	112.70 ± 5.55$^{*/\#/\S\S}$
Erythrocytes ($\times 10^6$/mm^3)	6.76 ± 0.89	5.85 ± 1.52	6.93 ± 0.93	7.15 ± 0.34
Reticulocytes (%)	0.90 ± 0.08	1.20 ± 0.08	1.00 ± 0.08	0.80 ± 0.08$^{\#}$
Neutrophils	6.00 ± 0.86	7.00 ± 0.86	7.00 ± 0.86	10.00 ± 0.86*
Lymphocytes	93.00 ± 1.03	91.00 ± 1.03	91.00 ± 1.03	88.00 ± 1.03*
Monocytes	1.00 ± 0.29	0.00 ± 0.29	1.00 ± 0.29	0.00 ± 0.29
		Female		
WBC ($\times 10^3$/mm^3)	2.13 ± 0.14	1.00 ± 0.20	1.95 ± 0.37	2.27 ± 0.60
Hb (g/dL)	13.60 ± 1.40	14.84 ± 0.49	15.26 ± 0.29	12.84 ± 0.71
HCT (%)	40.32 ± 5.03	45.18 ± 1.38	46.54 ± 1.05	36.72 ± 2.68
MCV (μm^3)	48.00 ± 0.57	47.20 ± 0.37	47.60 ± 0.24	47.20 ± 0.58
MCH (pg)	16.56 ± 0.70	15.44 ± 0.16	15.66 ± 0.10	16.58 ± 0.57
MCHC (g/dL)	34.28 ± 1.26	32.82 ± 0.28	32.82 ± 0.13	35.10 ± 0.86
RDW (%)	17.74 ± 1.37	16.04 ± 0.15	16.50 ± 0.21	16.12 ± 0.30
Platelets ($\times 10^3$/mm^3)	164.60 ± 44.89	137.60 ± 32.52	316.00 ± 115.5	253.00 ± 57.09
Erythrocytes ($\times 10^6$/mm^3)	6.71 ± 0.83	7.52 ± 0.23	7.75 ± 0.17	6.11 ± 0.44
Reticulocytes (%)	1.10 ± 0.11	1.50 ± 0.11	1.10 ± 0.11	1.00 ± 0.11$^{\#}$
Neutrophils	10.00 ± 0.47	11.00 ± 0.47	12.00 ± 0.47	12.00 ± 0.47
Lymphocytes	86.00 ± 0.57	88.00 ± 0.57	88.00 ± 0.57	86.00 ± 0.57
Monocytes	2.00 ± 0.48	3.00 ± 0.48	1.00 ± 0.48	3.00 ± 0.48

Data are expressed as mean + SEM. One-way ANOVA *post hoc* Bonferroni ($n = 5$ mice/group). $^{*}p < 0.05$ different from vehicle-treated group; $^{\#}p < 0.05$ different from GCE 1 mg/kg-treated group; $^{\S}p < 0.05$ and $^{\S\S}p < 0.01$ different from GCE 2 mg/kg-treated group. Hb: hemoglobin, RDW: red cell distribution width, HCT: haematocrit, MCV: mean corpuscular volume, MCH: mean cell hemoglobin, MCHC: mean cell corpuscular hemoglobin concentration, WBC: white blood cell counts, erythrocyte count, reticulocytes, E: eosinophils, M: monocytes, N: neutrophils, L: lymphocytes, and platelet counts.

significantly ($p < 0.05$) decreased in relation to the GCE 2 mg/kg-treated groups.

Treatment of female mice (Figure 4(l)) with GCE at 2 mg/kg significantly ($p < 0.05$) decreased UAC (mg/dL) serum levels in relation to the GCE 4 mg/kg-treated animals; while male (Figure 5(l)) GCE 4 mg/kg-treated mice UAC levels were significantly ($p < 0.05$) decreased in relation to the vehicle-treated group.

3.3.5. Relative Organs' Weights (%). Data of the relative organs' weight (%) of male and female GCE-treated mice (1, 2, and 4 mg/kg, p.o.) are shown in Table 3. The adrenal gland relative weights of the GCE 4 mg/kg-treated male animals were significantly ($p < 0.05$) increased when compared to the vehicle-treated group (corn oil 10 mg/mL, p.o.). The other organs (liver, kidney, spleen, heart, thymus, and brain) from the ECG-treated male and female mice did not show any significant differences in the relative weight (%) in comparison with the vehicle-treated animals.

3.3.6. Urinalysis. Urinary analysis did not present any significant variation in the parameters evaluated in the groups treated with the GCE when compared to the vehicle-treated group (data not shown).

3.3.7. Histopathological Parameters. Organs from the male and female mice treated during 28 days with GCE did not present significant anatomic or histopathological variations at any dose in comparison with the organs from the vehicle-treated group.

4. Discussion

In the present study, it was demonstrated that the geranyl cinnamate ester (GCE) was active for all concentrations tested in the *Candida albicans* and for the *Aspergillus niger* fungus when used with a concentration of up to 2.5 μL/mL. In the scientific literature, there were no studies related to the GCE, only some related to the geraniol activity, but without the optimization of the concentrations in the synthesis reactions. Therefore, the data obtained in this stage of the work confirm the antimicrobial activity of the GCE with the fungi tested, showing the importance that this compound may have in an area where few studies are published for growth control, using natural compounds.

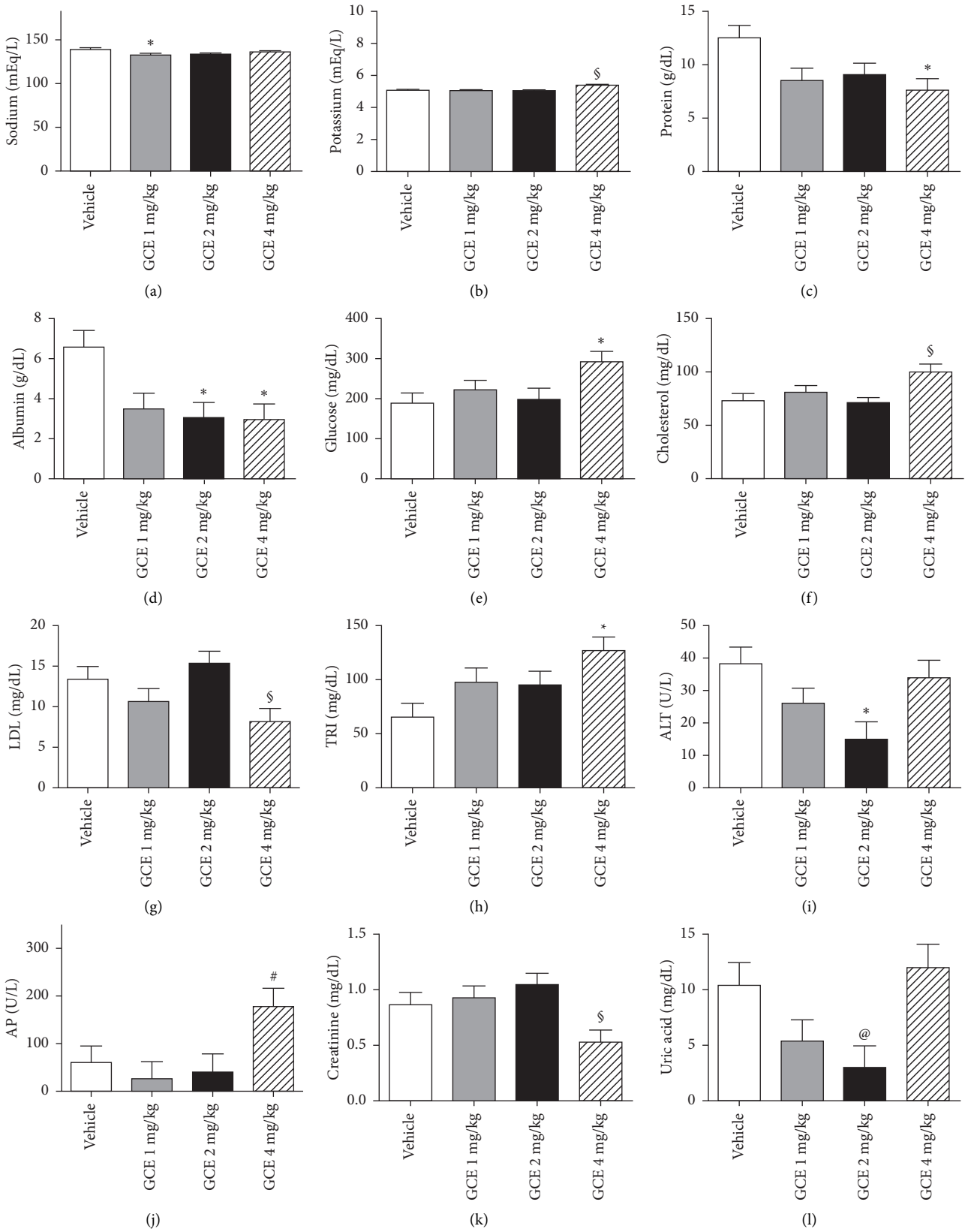

Figure 7: Effect of the geranyl cinnamate ester (GCE) repeated-dose treatment (1, 2 and 4 mg/kg, p.o.) on female mice biochemical parameters. Data are expressed as mean + SEM. One-way ANOVA *post hoc* Bonferroni ($n = 3$–5 animals/group). $^{*}p < 0.05$ different from vehicle group; $^{\#}p < 0.05$ different from GCE 1 mg/kg-treated group; $^{\S}p < 0.05$ different from GCE 2 mg/kg-treated group; and $^{@}p < 0.05$ different from GCE 4 mg/kg-treated group. (a) Serum sodium (Na); (b) potassium (K); (c) total protein; (d) albumin; (e) glucose; (f) total cholesterol; (g) cholesterol fraction (LDL); (h) triglycerides; (i) alanine aminotransferase (ALT); (j) alkaline phosphatase (AP); (k) creatinine, and (l) uric acid were evaluated.

In a study of the antimicrobial activity of an essential oil containing in its composition thymol, carvacrol, and geraniol (that is the compound used to obtain our ester), against the fungus *Candida albicans*, Botelho et al. [16] demonstrated that the essential oil exhibited an antifungal activity. Additionally, in the work of Marcos-Arias et al. [17], the authors reported that geraniol showed antifungal activity against strains of *Candida albicans*. Moreover, in the work carried out by Wang et al. [18], it was observed that geraniol showed antifungal activity against *A. flavus*, *A. carbonarius*, and *P. viridicatum* with values of minimum inhibitory concentration (MIC) above 5.00 μL/mL.

Ternus et al. [8] evaluated the antimicrobial activity of geraniol essential oil against different microorganisms. In the agar diffusion test for *Staphylococcus aureus*, the mean diameter of the zone of inhibition halo was 35.3 ± 0.08 mm and for *Escherichia coli*, the mean diameter of the halo was (25.5 ± 0.05) mm. For cinnamic acid to bacteria of type *Staphylococcus aureus*, the average diameter of the inhibition halo was (16.5 ± 0.10) mm and for the bacteria of the *Escherichia coli* halo average diameter was (11.0 ± 0.06) mm.

The work carried out by Zanetti et al. [13] brings the data on the antimicrobial activity of the geranyl cinnamate ester against the same bacteria, and it is possible to observe that it is bacteria of type *Staphylococcus aureus* the inhibition zone had an average diameter halo of 22.7 ± 0.60 mm and for *Escherichia coli*, the zone of inhibition had an average diameter of 17.2 ± 0.32 mm. These works [8, 13] show that the junction of geraniol with cinnamic acid produced the geranyl cinnamate ester, which is a compound microbiologically very active for the bacteria *Staphylococcus aureus* and *Escherichia coli*.

The mechanism of action of esters against bacteria and fungi is possibly the same as that of essential oils. The constituents of the oils destroy the cytoplasmic membrane and the cell wall of bacteria and fungi. This effect results in the extravasation of the cytoplasm and its coagulation, in addition to inhibiting cellular respiration [19].

This study also presented for the first time the acute and repeated-dose toxicity of GCE. The OECD guidelines 423 and 407 used to perform the toxicity tests are worldwide accepted and considered the standard model to assess the toxicity of chemical compounds [20]. Considering that the GCE is a candidate to be used as an additive for the food industry, our results are considerably relevant.

Acute toxicity study demonstrated that mice treatment with GCE at 2000 mg/kg (p.o.) decreased food intake and did not affect weight gain. Therefore, the decreased food consumption was not sufficient to affect mice weight gain and might be related to the sedation elicited by the GCE administration. Furthermore, GCE evoked an increase in the relative weight of the mice kidneys, suggesting a possible acute toxicity to this organ. Nevertheless, GCE acute treatment did not induce mice death; therefore, this compound is classified in the safety category 5 of the Global Harmonized Classification System (GHS), and its LD50 (median lethal acute dose) is above 2000 mg/kg [14].

Repeated-dose toxicity tests provided data about persistent or cumulative toxic effects on target organs, dose-response relationships, and the no-observed-adverse-effect level (NOAEL) [15], where both sexes of mice were used for the repeated-dose toxicity study, since toxicological studies demonstrated some differences in the sensitivity between females and males [15].

Gross behavior of the female GCE-treated (1, 2, and 4 mg/kg, p.o.) mice groups was considered normal, and no toxicity symptoms were noted during the 28 days of treatment. However, diarrhea was observed at the first week of administration in only one male mice treated with the highest dose of GCE, which could represent an adverse effect of the treatment. Moreover, no animals died during the experimental period, and significant hematological and biochemical variations were observed in animals treated with GCE at the highest doses (2 mg/kg and 4 mg/kg) only.

Treatment of female mice with GCE did not impact the food intake, neither body weight gain, since all groups presented a significant weight gain during the experiment. However, male mice treated with GCE did not present a significant increase in body weight, which could be related to the decreased food consumption elicited by the GCE at 4 mg/kg. Considering that the food intake of the vehicle-treated group did not change along the experimental period, we may infer that the stress caused by the repeated orogastric gavage [21] did not affect the animals' food intake. Therefore, the decrease in food intake of GCE 4 mg/kg-treated male mice could indicate adverse effects [22, 23] or might represent an anorexigenic effect [24] of the compound.

No macroscopic lesions, abnormal anatomic aspects, and no variations in the relative weight of the organs were observed in the female mice after the administration of GCE. Nevertheless, the treatment of male mice with GCE 4 mg/kg elicited an increase in the adrenal glands' relative weight in comparison with the vehicle group. Adrenal hyperplasia may be related to hypersecretion of corticosterone by the adrenal cortex as a consequence of adenohypophysis stimulation, which secrets a large amount of ACTH (adrenocorticotropic hormone) [25]. Therefore, we may speculate that the GCE at the highest dose could cause a dysfunction in the hypothalamic-pituitary-adrenal (HPA) axis in male mice.

This hypothesis is consonant to the neutrophilia and lymphopenia found in male mice treated with GCE 4 mg/kg. These abnormalities characterize a corticoid-mediated leukogram variation [26], which could be attributed to changes in the adrenal function. It is known that high levels of cortisol influence the distribution of leukocytes in the blood, causing lymphopenia by inducing the migration of lymphocytes from the peripheral circulation [27] and neutrophilia [27, 28] by inhibiting the apoptosis of these cells [28].

Biochemical analysis revealed that the liver could be a target organ of the GCE toxicity, since the levels of alkaline phosphatase (AP) were significantly increased in the groups (male and female) treated with GCE at the highest dose (4 mg/kg, p.o.), and other biochemical parameters that could be related to liver toxicity were also altered in the plasma of the GCE-treated mice. AP is present in several tissues, but is particularly concentrated in the liver. Therefore, increased

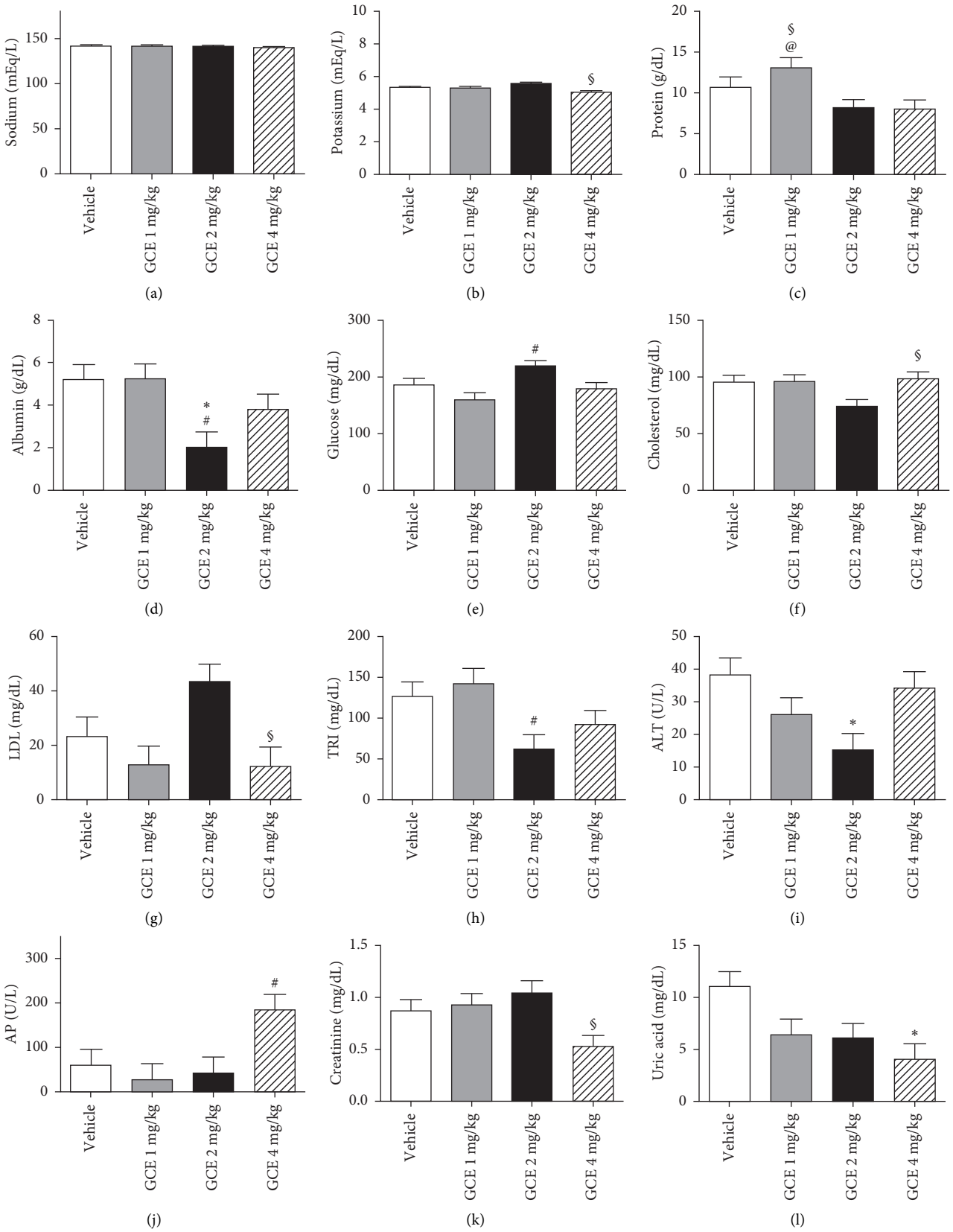

Figure 8: Effect of the geranyl cinnamate ester (GCE) repeated-dose treatment (1, 2, and 4 mg/kg, p.o.) on male mice biochemical parameters. Data are expressed as mean + SEM. One-way ANOVA *post hoc* Bonferroni ($n = 3$–5 animals/group). $^{*}p < 0.05$ different from vehicle group; $^{\#}p < 0.05$ different from GCE 1 mg/kg-treated group; $^{\S}p < 0.05$ different from GCE 2 mg/kg-treated group; $^{@}p < 0.05$ different from GCE 4 mg/kg-treated group. (a) Serum sodium (Na); (b) potassium (K); (c) total protein; (d) albumin; (e) glucose; (f) total cholesterol; (g) cholesterol fraction (LDL); (h) triglycerides; (i) alanine aminotransferase (ALT); (j) alkaline phosphatase (AP); (k) creatinine and (l) uric acid were evaluated.

Table 3: Relative organs' weight (%) (brain, heart, thymus, spleen, adrenals, kidney, and liver) of the male and female mice treated with the geranyl cinnamate ester (GCE) at 1, 2, and 4 mg/kg (p.o.) for 28 days.

	Vehicle	GCE 1 mg/kg	GCE 2 mg/kg	GCE 4 mg/kg
		Male		
Brain	1.18 ± 0.04	1.17 ± 0.01	1.16 ± 0.03	1.21 ± 0.02
Heart	0.39 ± 0.01	0.41 ± 0.01	0.43 ± 0.02	0.39 ± 0.01
Thymus	0.23 ± 0.01	0.21 ± 0.03	0.24 ± 0.01	0.23 ± 0.01
Spleen	0.29 ± 0.02	0.29 ± 0.01	0.31 ± 0.01	0.31 ± 0.01
Adrenals	0.020 ± 0.005	0.030 ± 0.004	0.030 ± 0.003	0.040 ± 0.004*
Kidney	1.55 ± 0.02	1.60 ± 0.06	1.58 ± 0.07	1.69 ± 0.07
Liver	4.63 ± 0.26	4.84 ± 0.07	4.81 ± 0.07	4.73 ± 0.05
		Female		
Brain	1.35 ± 0.07	1.36 ± 0.07	1.39 ± 0.0	1.46 ± 0.03
Heart	0.39 ± 0.02	0.37 ± 0.01	0.42 ± 0.02	0.41 ± 0.02
Thymus	0.33 ± 0.02	0.32 ± 0.02	0.28 ± 0.02	0.30 ± 0.03
Spleen	0.51 ± 0.08	0.38 ± 0.02	0.35 ± 0.02	0.40 ± 0.01
Adrenals	0.050 ± 0.007	0.060 ± 0.004	0.070 ± 0.008	0.080 ± 0.006
Kidney	1.44 ± 0.13	1.29 ± 0.11	1.27 ± 0.09	1.26 ± 0.05
Liver	4.71 ± 0.13	4.08 ± 0.18	4.20 ± 0.17	4.16 ± 0.23

Data are expressed as mean + SEM. One-way ANOVA *post hoc* Bonferroni. * $p < 0.05$ different from the vehicle-treated group (corn oil, 10 mg/mL, p.o.).

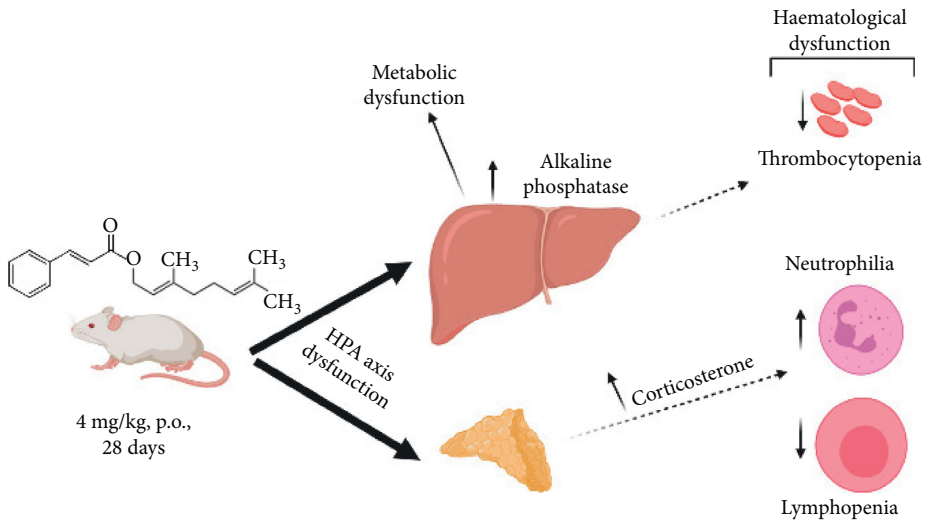

Figure 9: Diagram of the mechanisms involved in the toxic effect of GCE (4 mg/kg, p.o., 28 days) in mice. Dotted arrows indicate a possible effect (created with Biorender.com).

AP levels are related to liver injury rather than other reasons [24].

Generally, individual elevations of AP levels with no variations in ALT levels are related to hepatic cholestasis [29]. AP serum levels can be elevated due to the obstruction of the bile ducts, which is related to increased canalicular synthesis of AP with subsequent translocation to the hepatic sinusoid [30]. Therefore, cholestatic liver diseases are associated with increases in the synthesis and release of AP [31]. In this sense, we may suggest that GCE, at the highest dose, induced cholestatic hepatic injury in mice.

Additionally, other biochemical parameters, such as glucose, total cholesterol, LDL, triglycerides, total protein (in female mice), and albumin, were significantly affected in animals treated with GCE, mainly at 4 mg/kg. As the liver is the main organ related to metabolism (and the changed parameters are related to metabolism) [29, 32], we suggest that these variations could be related to liver damage. Nevertheless, no differences in the histopathological analysis of the liver were detected in the present study, indicating that the tissue damage was not sufficient to change its histological structure.

Hemostasis is directly associated with liver function, since several coagulation factors are synthesized by the liver [33]. In this sense, thrombocytopenia is a hematological change frequently found among patients with chronic liver disease [34]. Considering that the liver is the organ responsible for the activation of the coagulation cascade, and once damaged, it compromises the coagulation homeostasis [35], the variations in biochemical markers of hepatic function in mice treated with the highest GCE dose could be related to the thrombocytopenia [26] found in these animals.

The serological decrease in uric acid levels at the highest GCE dose and the discreet hyponatremia observed in the GCE 1 mg/kg-treated group may be related to the uricosuric action of plants from Poaceae family, such as *Cymbopogon martinii*. Interestingly, corn silk, *Zea mays* L. (Poaceae), presents uricosuric, diuretic, antilithogenic, and antiseptic properties [36] and is traditionally used worldwide for the treatment of edema, as well as for cystitis, gout, nephrolithiasis, nephritis, and prostatitis [37].

From the above discussion of the findings obtained in this study, it can be therefore suggested that the GCE, at 4 mg/kg, p.o., exerts its toxic effects on the animals by the following mechanisms: (i) liver injury (evidenced by the serological increase in the ALP enzyme), which leads to metabolic dysfunction (variations in serum glucose, proteins, and lipid levels) and, possibly, reduced production of coagulation factors, which are important for normal platelet function; (ii) adrenal hyperplasia, which might be related to GCE-induced dysfunction of the HPA axis, resulting in increased production of corticosterone, which impacts on the leukogram of the animals, causing lymphopenia and neutrophilia. These hypotheses are illustrated in Figure 9.

In conclusion, the GCE showed activity against *Candida albicans* and *Aspergillus niger* at very low concentrations as a very active compound for the tested fungi. Furthermore, this study comprises the first analysis on the toxicity of the geranyl cinnamate ester in experimental animals. The acute toxicity study demonstrated that the GCE can be classified into safety category 5, according to the OECD acute toxicity parameters. The study of repeated doses revealed that the lowest GCE dose is devoid of toxicity, which is extremely significant for the food industry, considering its application as a food preservative. Last, the biochemical and hematological variations observed in animals treated with GCE at the highest dose point to the liver as the target organ of potential GCE toxicity.

Data Availability

Article data or supplementary data may be requested via email to the author Micheli Zanetti (eng.miche@unochapeco.edu.br) and will be shared with applicants.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Research Article

Chemical Analysis and Antioxidant and Antimicrobial Activity of Essential oils from *Artemisia negrei* L. against Drug-Resistant Microbes

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Background. *Artemisia negrei* L. (*A. negrei*) is a medicinal and aromatic plant belonging to the family Asteraceae that is more widespread in the folded Middle Atlas Mountains, Morocco. *Materials and Methods*. This study was run to investigate the phytochemical composition and antioxidant, antibacterial, and antifungal activities of *Artemisia negrei* L. essential oil. This oil was extracted from the fresh plant material by using the Clevenger apparatus. The phytochemical composition was characterized by GC-MS. The antioxidant activity was evaluated using different methods including DPPH, β-carotene bleaching, and total antioxidant capacity. The antibacterial activity was tested vs. multidrug-resistant bacteria including both Gram-negative and Gram-positive using inhibition zones in agar media and minimum inhibitory concentration (MIC) bioassays. The antifungal activity was conducted on *Candida albicans*, *Aspergillus niger*, *Aspergillus flavus,* and *Fusarium oxysporum* using a solid medium assay. *Results*. The chromatographic characterization of essential oils of *A. negrei* revealed the presence of 34 compounds constituting 99.91% of the total essential oil. The latter was found to have promising antioxidant activity by all bioassays used such as DPPH, β-carotene bleaching, and total antioxidant capacity. The results obtained showed that our plant oils had potent antibacterial activity towards Gram-negative (*E. coli* 57, *E. coli* 97, *K. pneumonia*, and *P. aeruginosa)* and Gram-positive (*S. aureus*), so that the maximum inhibition zones and MIC values were around 18–37 mm and 3.25 to 12.5 mg/mL, respectively. The oil also showed antifungal activity towards *Candida albicans*, *Fusarium oxysporum,* and *Aspergillus Niger* except for flavus species. *Conclusion*. The findings obtained in the work showed that *A. negrei* can serve as a valuable source of natural compounds that can be used as a new weapon to fight radical damage and resistant microbes.

1. Introduction

Herbal medicine has become a more popular way of fighting against diseases and producing pharmaceutical medicines [1–3]. The use of herbal medicine for seeking potentially active compounds has been proven to promote scientific output [4]. Many synthesized drugs have come from natural sources including medicinal plants which can be available in the form of food supplements, nutraceuticals, and alternative and complementary medicines [5]. Plants are an

important source of natural substances with great antioxidant potential [6]. Modern medicines place in priority the development of effective antioxidant substances from a natural source for being applied in the medical field for medication purposes [7]. Natural antioxidant agents received full consideration in the food industry to prevent oxidative deterioration of food by free radicals. These agents have been placed in priority for being used as an alternative to synthetic antioxidant agents such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tertbutylhydroquinone (TBHQ) that are suspected of having serious side effects including carcinogenic and toxic effects [8].

Species among the genus *Artemisia* are used in traditional medicines for therapeutic purposes as antispasmodic, antirheumatic, antiinflammatory, antimicrobial, antihelminthics, and antiveinous agents [9, 10]. *Artemisia negrei* L. is a medicinal plant endemic to Morocco lands, which is distributed in the region of the Moulouya Basin, and the folded Middle Atlas Mountains of Morocco. This plant is commonly used in Moroccan traditional medicines in treating diseases including digestive genital tracts and dermatological infections by using powder and infusion forms [9–11].

Antimicrobial resistance is a phenomenon where microbes evolve strategies to fight against drugs planned to attack them, so that the germs, which are not defeated, continue to develop powerfully as never before [7]. A few years ago, antimicrobial resistance has become one of the biggest problems that overburden the health care system and is classified as among the greatest challenge by the World Health Organization for 2019 [12].

In this study, the studied bacterial strains belong to drug-resistant microbes such as *P. aeruginosa, K. pneumonia, S. aureus,* and *E. coli* pathogens. It is well known that these species can be multidrug-resistant [13]. Some of the fungal species studied in this work belong to drug-resistant microbes such as *Candia* spp., which was involved in the infection of more than 90% of people with AIDS in an earlier time. *Candia* spp. has developed unprecedented resistance due to excessive use of synthesized drugs to fight fungal infections caused by these microbes and continues to be a greatest growing health burden [14].

The current research study aimed to investigate the phytochemical composition and antioxidant, antifungal, antibacterial, and antifungal activities of *A. negrei* essential oil since no other studies have attempted this objective up to the time of writing this article.

2. Materials and Methods

2.1. Selection and Identification of Plant Material. *A. negrei* was harvested in June 2019 from the Middle Folded Atlas, Morocco (2100 m, 33.539648, −3.894474). The authentification was done by a botanist with reference # BPRN/04/18 that was deposited at the herbarium of Sidi Mohammed Ben Abdallah University, Fez, Morocco. Next, aerial parts of *A. negrei* were subjected to dry in a ventilated place for 10 days before extraction.

2.2. Extraction of Essential Oil. In the present work, the oil was extracted from the fresh plant material by using the Clevenger apparatus. In brief, a total of 200 g of aerial parts (leaves) were cut into small pieces before being placed into a flask with 750 mL of distilled water. Afterward, the whole solution was boiled for 3 h to maximize the essential oil extraction. The essential oil yield was obtained by using the following formula:

$$\mathrm{RHE} = \frac{M'}{M} \times 100, \tag{1}$$

where RHE is essential oil yield in %; M' is essential oil mass recovered in grams; M is plant material mass in grams.

2.2.1. Analysis of the Phytochemical Composition of the Oil. The phytochemical characterization of essential oil was effectuated by GC-MS using a nonpolar silica column. To fulfill this goal, the operating conditions of the analysis were run as follows: the initial temperature was set to 40°C/2 min along with speed 2°C/min, while the final and injector temperatures were set to 260°C/10 min and 250°C, respectively. In this analysis, helium gas was used as a vehicle (1 mL/min) with "split" mode injection. The ionization energy and ion source temperature were 70 eV and 200°C, respectively, and the scan mass range m/z is 40–650. The oil was diluted in hexane solvent (10 : 100) before being injected with 1 μL. The chemical identification was done by using retention indices (RI) along with comparison with ADAMS database [15].

2.3. Antioxidant Activity. In this study, the antioxidant power of the oil from *A. negrei* was evaluated using three bioassays including DPPH, β-carotene bleaching, and total antioxidant capacity [16].

2.3.1. DPPH Radical Scavenging Activity. DPPH bioassay was carried out using protocols as reported by Tepe et al. [17]. Both the essential oil (EO) and the positive control (BHT) were used at different concentrations including 1, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, and 1/512 mg/mL. The anti-free radical activity was evaluated by mixing 100 μL of each previously prepared concentration (EO and BHT) with 750 μL of DPPH (0.004%). Afterward, the solution was incubated at ambient temperature for 30 minutes before reading the absorbance. The DPPH scavenging ability was expressed as inhibition percentage as follows:

$$\mathrm{PI}\,(\%) = (A0 - A/A0) * 100, \tag{2}$$

where PI is the percentage of inhibition, $A0$ is the absorbance of DPPH without the sample (control), and A the absorbance of DPPH with the sample.

2.3.2. Total Antioxidant Capacity Test (TAC). The TAC test was carried out according to the protocol reported in the earlier work [18]. In brief, 25 μL of the sample test (1 mg/mL) was mixed with one milliliter of reagent solution constituted

of sodium phosphate, sulphuric acid, and ammonium molybdate. Next, the whole solution was placed for incubation at 95°C for 90 min before measuring the absorbance at 695 nm using a spectrophotometer [18]. BHT and Quercetin were used as standard references. The TAC has expressed in mg EAA/g HE.

2.4. ß-Carotene Bleaching Assay. This assay was performed to study the antioxidant power of essential oil from *A. negrei* using the protocol as reported in the literature [19, 20]. In brief, 1 mL of *ß*-carotene chloroform solution was added to 10 μL of solution constituted of linoleic acid and 100 mg of Tween 80. Next, the chloroform was retrieved using a vacuum rotary evaporator before adding 25 mL of hydrogen peroxide to the residue. Afterward, 2.5 mL of the obtained mixture was added to 100 μL of the sample test (1 mg/mL) and then maintained in the water bath at 51°C for 2h. BHT was used as a standard reference (1 mg/mL). The absorbance was measured at 470 nm. The antioxidant power was calculated as a percentage of antioxidant activity relative to the control as follows:

$$AA\% = (AE/ABHT) * 100. \quad (3)$$

AA% is the antioxidant property percentage, and ABHT is the absorbance of the positive control, while AE is the absorbance of the negative control.

2.5. Antibacterial Activity. The evaluation of the antimicrobial activity of the essential oils was carried out according to the previously reported data elsewhere [16]. The essential oil of *A. negrei* was tested vs. Gram-negative bacteria *Pseudomonas aeruginosa, Escherichia coli* ATB:57; *Klebsiella pneumoniae*, and *Escherichia coli* ATB:97 and Gram-positive bacteria (*Staphylococcus aureus* (*LM*, *FMP*, and *Fez*)). The strains tested in the current study were clinically isolated and have been reported as multidrug-resistant as reported in earlier work [13, 21, 22]. The bacterial suspension was prepared from fresh culture. To achieve this goal, few colonies from the culture were aseptically seeded in 0.9% of physiological water at a density of 0.5 McFarland, which corresponded to 10^7 to 10^8 CFU/mL [23].

The antibacterial activity was studied using the disc diffusion method. In brief, a volume of 10 μL of *A. negrei* essential oil (1 mg/mL) was used for testing purposes, while ampicillin 1.68 mg/disc and streptomycin 0.020 mg/disc were used as drug references as reported in earlier work [20].

The minimum inhibitory concentrations (MICs) were studied by using the microdilution assay [24]. In brief, MIC was assessed by using the microdilution method in 96-well plates. The concentrations of the oil were prepared in a 0.2% agar suspension. The concentrations were obtained by successive dilutions (25 to 0.02 mg/mL). Finally, the plates were placed for incubation at 37 C for 18 h. Next, the bacterial growth was visualized after adding 20 μL of triphenyltetrazolium in 5 mg/mL wells before further incubation for 30 min at 37°C [23].

2.6. Antifungal Activity of Essential Oils from Artemisia negrei. The antifungal activity of the studied oil was conducted using four fungal species including *Candida albicans* ATCC 10231, *Aspergillus niger* (LBEAH/FS/19), *Aspergillus flavus* (LBEAH/FS/18), and *Fusarium oxysporum* (LBEAH/FS/17). The disk diffusion method was used to achieve this goal as described elsewhere [25]. In brief, Petri dishes with MEA medium were inoculated with *C. albicans*, *A. niger, A. flavus,* and *F. oxysporum*. Next, Whatman paper disks (6 mm in diameter) impregnated with 10 μL of essential oils were placed on the surface of Petri dishes before being incubated at 30°C in the darkness. The inhibition diameter, as well as inhibition percentage, was determined after 48 h of incubation for *C. albicans* and after 7 days for *A. niger, A. flavus,* and *F. oxysporum* [26, 27].

2.7. Statistical Analysis. The obtained results were expressed as means ± SEM of triplicate assays. Statistical analysis was conducted using the ANOVA test. A significant difference was statistically considered when $p < 0.05$.

3. Results and Discussion

3.1. Phytochemical Compounds of Essential Oil. The obtained results showed that the yield of essential oil of *A. negrei* was 1.2%. The highest percentage of essential oil of the genus *Artemisia* was recorded for *Artemisia cana* (1.3%) and *Artemisia frigida* (1.5%). However, the essential oil yield of the aerial part of *Artemisia absinthium*, *Artemisia biennis*, *Artemisia dracunculus,* and *Artemisia ludoviciana* ranges from 0.3% to 0.5%, which is lower than that of *A. negrei* [28]. This yield can be considered important in comparison with some plants that are industrially exploited as a source of essential oils such as rose (0.1–0.35%), rosemary (1–2.5%), peppermint (0.5–1%), neroli (0.5–1%), lavender (0.8–2.8%), aniseed (1–3%), and thyme (2–2.75%) [29].

The chromatographic analysis of essential oil of *A. negrei* from the folded Middle Atlas revealed the presence of 34 volatile constituting 99.91% of the total essential oil recovered from fresh material (Figure 1; Table 1). The chemical analysis showed that the characterized essential oil possessed many potentially bioactive substances including thujone (29.02%), 2-bornanone (14.68%), octacosane (14.02%) eucalyptol (5.60%), endoborneol (3.78%), bicyclo (3.1.0) hexan-3-on (3.63%), pentacosane (3.07%), and camphene (2.38%). Some compounds identified in the current oil (β-thujone, α-thujone, borneol, camphor, and 1.8-cineol) were also identified in closer plant species including *Artemisia herba-alba* L., *Artemisia pontica L.*, *Artemisia absinthium L.* [25–27]. Thujone as a major element in the studied oil has been largely identified in essential oils of plants that are used for food and/or medicinal purposes [30].

3.2. DPPH Free Radical Scavenging Activity. The results obtained showed that the studied oil exhibited a potent DPPH free radical scavenging activity (IC50 = 0.0164 ± 0.0011 mg/mL) when compared to BHT (0.0082 ± 0.002 mg/mL) (Figure 2). Our oil with $IC_{50} = 0.0164 \pm 0.0011$ mg/ml is

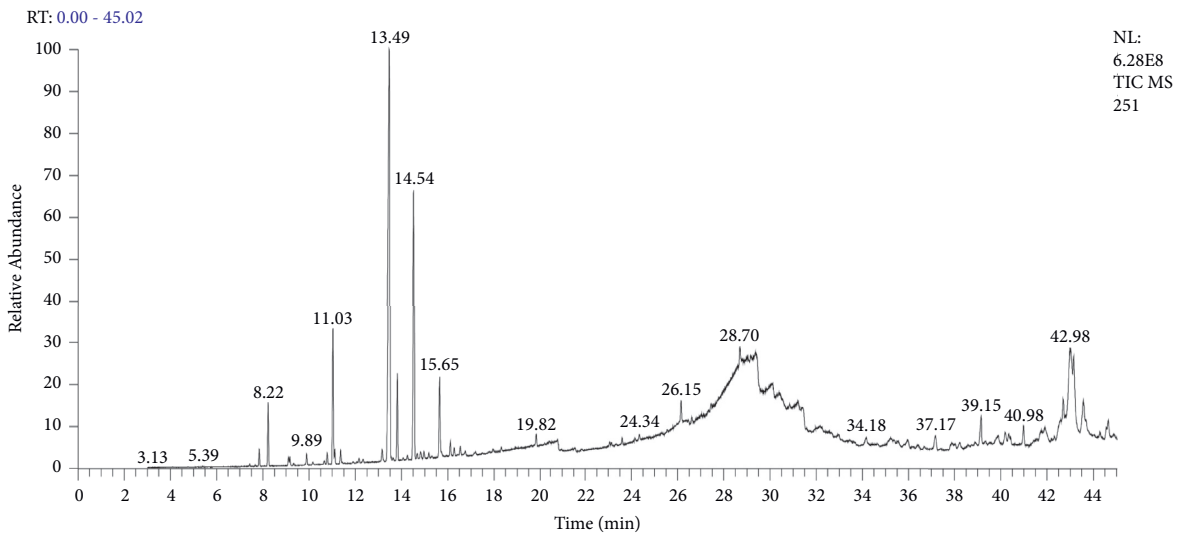


Figure 1: GC-MS chromatographic profile of essential oil from *A. negrei*.

relatively better than that found for oil extracted from *Artemisia dranculus* growing in Turkey, which showed $IC_{50} = 100.200$, 400, and 1000 μg/ml [31], and *Artemisia herba alba* from southwest Tunisia ($IC_{50} = 50.00\,\mu$g/mL) [32]. As shown in Table 1, *A. negrei* essential oil contains a higher amount of oxygenated monoterpenes, so that it can be a promising source of radical scavenging agents [33].

3.3. Total Antioxidant Capacity. In the current research work, the obtained findings showed that the essential oil from *A. negrei* had promising total antioxidant capacity with a value of 867.71 ± 30.21 mg/g when compared to 472.29 ± 6.19 mg/g of BHT and 307.65 ± 13.08 mg/g of quercetin. It has been indicated that the antioxidant activity of essential oils is closely related to compounds with hydroxyl functions such as alcohols, and phenolic compounds [30, 31].

3.4. ß-Carotene Discoloration Test. The evaluation of the antioxidant activity of the extract by using different assays is largely appreciated for comparison purposes [34]. It is thus fitting that the *ß*-carotene bleaching method was used to achieve this goal. A follow-up of the *ß*-carotene oxidation reaction in the presence of the oil and the standard reference (BHT) was performed by measuring the intensity of *ß*-carotene color at a wavelength of 470 nm. As shown in Figure 3, the results reported in percentages indicate that *A. negrei* oil exhibited potent inhibitory capacity (74.1428%) when compared to BHT (100%). The test used showed that *A. negrei* oil was a good hydrogen donor so that it is capable of being a free radical scavenger to control oxidation [35].

The investigated results in this work showed that *A. negrei* exhibited strong antioxidant power that may result from thujone as a major compound detected in the studied oil by GC-MS analysis [36]. In this sense, thujone (α and β) has been reported to have anti-free radical activity as reported elsewhere [37]. The findings obtained in this work are consistent with those reported elsewhere [38], which demonstrated that essential oil from genus *Artemisia* revealed antioxidant activity of DPPH and ABTS assays. Many works have investigated the relationship between the phytochemical content of the essential oil and the antioxidant potential. In this sense, it was reported that antioxidant power is closely related to the presence of chemicals with hydroxyl function [39]. Therefore, the oil higher in phenolic compounds along with terpene alcohols can have a strong antioxidant effect [40]. According to the results obtained, the essential oils of *Artemisia* species showed a very high antioxidant efficacy even at the lowest concentration tested, so that we can confirm that this potent activity is explained by the richness of the oil in oxygenated monoterpenes.

3.5. Antibacterial Activity of Essential Oils of Artemisia negrei. Faced with the problems of antimicrobial resistance to synthetic antibiotics, much work has been conducted on the antimicrobial power of natural products including essential oils of certain plants. In this research study, the antimicrobial power of *A. negrei* essential oil was tested vs. five strains including *E. coli 57*, *E. coli 97*, *K. pneumoniae*, *P. aeruginosa,* and *S. aureus* as multidrug-resistant bacteria with a high frequency of contamination and pathogenicity [22]. In this study, the antibacterial activity was evaluated by using inhibition zone diameter (Table 2) and MIC bioassays (Table 3). The results obtained showed that our plant oil had potent antibacterial activity towards *E. coli 57*, *E. coli 97*, *K. pneumoniae*, *P. aeruginosa,* and *S. aureus* since the maximum inhibition zones and MIC values were around 18–37 mm and 1.56–12.5 mg/mL, respectively, towards these species (Figure 4). 1.56 μg/mL was sufficient to inhibit the growth of *P. aeruginosa* (Gram negative), which was the most sensitive to the studied oil followed by *E. coli 9, E. coli 57*, and *K. pneumonia* (Gram negative), which were completely inhibited by 6.25 μg/mL. On the other hand, *S. aureus* (Gram+) was seriously inhibited at a concentration of 12.5 μg/mL. All strains were found to be resistant to the

Table 1: Phytochemical components identified in *A. negrei* essential oil by GC-MS.

Peak	RT (min)	Compound Name	RI		Molecular Formula	Area (%)
			Obs	Lit		
1	7.84	α-Pinene	938	939	C10H16	0.61
2	8.22	Camphene	965	959	C10H16	2.38
3	9.17	β-Pinene	976	979	C10H16	0.29
4	9.89	Myrcene	988	990	C10H16	0.37
5	10.78	Cymene	1024	1024	C10H14	0.44
6	11.03	Cineole	1031	1031	C10H18O	5.60
7	11.11	Limonene	1029	1029	C10H16	0.50
8	11.36	Fenchone	1086	1086	C10H18O	0.50
9	13.16	Artemisia alcohol	1073	1083	C10H18O	0.50
10	13.49	β-Thujone	1111	1114	C10H16O	29.02
11	13.82	α-Thujone	1100	1102	C10H16O	3.63
12	14.54	Camphenol	1110	1113	C10H16O	14.68
13	15.65	Borneol	1169	1169	C10H18O	3.85
14	16.11	Limonen-4-ol	1662	1667	C10H18O	0.56
15	16.54	α-Terpineol	1132	1133	C10H18O	0.35
16	19.85	Bornyl acetate	1286	1288	C12H20O2	0.51
17	20.81	Geranyl formate	1291	1298	C11H18O2	0.88
18	26.15	α-Copaene	1376	1376	C15H24	1.00
19	28.70	Aromadendrene epoxide	1641	1641	C15H24O	1.20
20	29.43	Cycloisolongifol-5-ol	1510	1513	C15H26O	2.88
21	30.12	α-Acoreno	1630	1633	C15H26O	1.30
22	31.44	γ-Muurolene	1476	1479	C15H24	1.17
23	37.17	Curcuphenol	1716	1718	C15H22O	1.09
24	39.15	Hexadecanoic acid	1957	1960	C16H32O2	1.72
25	40.19	Coumarin	1793	1434	C17H28O2	0.65
26	40.99	Trihydroxy benzaIdehyde	1818	1819	C7H6O4	1.05
27	41.77	Isopropyltetradecanoate	1823	1829	C17H34O2	0.56
28	41.91	Isotorquatone	1884	1845	C15H22O4	1.43
29	42.56	Lanceol acetate	1854	1855	C17H2602	0.95
30	42.69	Thujopsenic acid	1863	1864	CI5H2202	1.54
31	43.02	Octacosane	2798	2800	C28H58	14.02
32	43.57	Pentacosane	2497	2500	C25H52	3.07
33	44.56	Octadecanoic acid, ethylester	2122	2125	C20H40O2	0.60
34	44.64	Hexadecanol	1874	1875	CI6H34O	1.01
		Total identified				99.91%
		Monoterpene hydrocarbons				4.59%
		Oxygenated monoterpenes				58.69%
		Sesquiterpene hydrocarbons				2.17%
		Oxygenated sesquiterpenes				9.44%
		Others				25.02%

RT: retention time (min); RI: retention indices; Obs: calculated retention indices of phytochemicals found in *A. negrei* essential oils; Lit: retention indices of phytochemicals found in the literature.

tested antibiotics except for *S. aureus* (Gram+), which was found to be highly sensitive to *Streptomycin* with an inhibition zone diameter of 9.32 ± 0.84 mm and resistant to ampicillin. The antibacterial effect of the oil from *A. negrei* can be explained by the presence of oxygenated monoterpenes identified in oil particularly thujone, eucalyptol, endoborneol, 2-bornanone, and *ß*-terpineol reported to possess interesting pharmacological activities [41]. These findings were proven by Kordali [31], who showed that the oils of closer species such as *Artemisia santonicum* and *Artemisia spicigera* possessing a high level of bioactive oxygenated monoterpenes. On the other hand, a wormwood *A. campestris*, which is mainly composed of hydrocarbon monoterpenes, revealed a weak antimicrobial activity against pathogenic germs such as *E. coli* and *S. aureus* [42]. For getting the antimicrobial effect, antibiotics need to reach and interact with specific target sites. However, the antimicrobial agent is frequently interrupted due to the intervention of different mechanisms in bacteria, which lead to the failure of antimicrobial agents, so that bacteria continue to develop strongly [43]. The low sensitivity of Gram-negative bacteria to antibiotics may result in an outer membrane covering the cell wall, which interacts with the diffusion of hydrophobic agents through the lipopolysaccharide coating. Essential oil from natural sources can successfully cross the cell walls of bacteria and the cytoplasmic membrane inducing disorders of macromolecules (fatty acids, polysaccharides, and phospholipids) [44]. In this work, essential oil from *A. negrei* has almost closer activity vs. Gram-positive as much as Gram-negative bacteria. Hence, we could confirm that

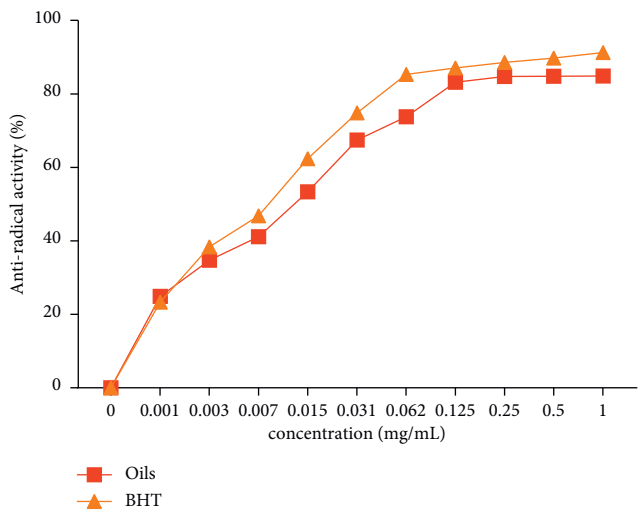


Figure 2: DPPH radical scavenging activity of *A. negrei* essential oil.

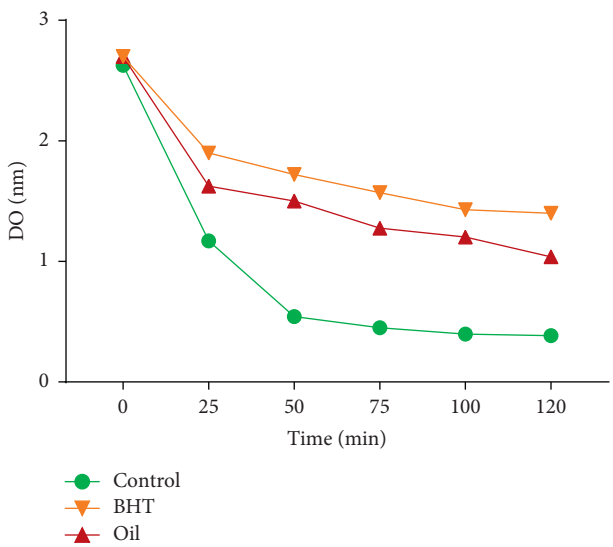


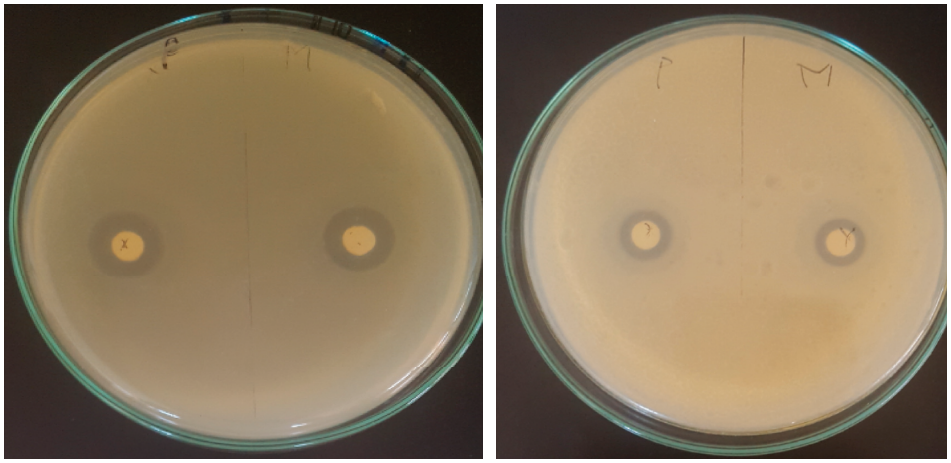
Figure 3: Antioxidant activity of *A. negrei* oil by *ß*-carotene discoloration test.

Table 2: Diameter of the zone of inhibition in mm by the agar diffusion method.

Compound	Gram-negative bacteria				Gram-positive bacteria
	E. coli 57	*E. coli 97*	*K. pneumoniae*	*P. aeruginosa*	*S. aureus*
EO	37.21 ± 1.24	28.37 ± 3.21	19.05 ± 2.01	18.51 ± 0.91	23.41 ± 2.36
Streptomycin	—	—	—	—	9.32 ± 0.84
Ampicillin	—	—	—	—	—

Table 3: Minimum inhibitory concentration (MIC in mg/mL).

Compound	Gram-negative bacteria				Gram-positive bacteria
	E. coli 57	*E. coli 97*	*K. pneumoniae*	*P. aeruginosa*	*S. aureus*
EO	6.25 ± 0.7	6.25 ± 0.7	6.25 ± 0.61	1.56 ± 0.20	12.5 ± 1.03
Streptomycin	4.51 ± 0.04	5.27 ± 0.23	3.38 ± 0.01	—	6.21 ± 0.04
Ampicillin	—	—	—	—	—



E. coli *S. aureus*

Figure 4: Photograph of Petri dishes showing inhibition zone of essential oils from *A.negeri* against bacterial strains.

essential oil from *A. negrei* is a potent weapon to fight multidrug-resistant strains.

The obtained findings demonstrated that the bacterial strains tested were found to be resistant to antibiotics ampicillin and streptomycin. These results were in accordance with those investigated in previous literature [43], which revealed that *Enterobacter* spp., *S. aureus*, *P. aeruginosa, K. pneumoniae*, *A. baumannii* along with *E. coli* pathogens were too drug-resistant microbes. Moreover, the strains tested in this research work are classified belonging to multidrug-resistant as reported in previous works [38, 40].

3.6. Antifungal Activity of Oil from A. negrei. Regarding the *in vitro* evaluation of the antifungal activity of *A. negrei* essential oil, the disk diffusion method revealed antifungal activity with a percentage inhibition of $32.93 \pm 0.53\%$, $33.80 \pm 1.27\%$, and 33.66 ± 0.44 against *F. oxysporum*, *A. niger,* and *C. albicans*. However, the essential oils did not show antifungal activity against *A. flavus* when compared to other strains. These results are in agreement with investigated elsewhere [45], which showed that oil from *Borojoa patinoi* Cuatrec exhibited an inhibitory effect towards *C. albicans* since both share some common chemicals. Moreover, numerous studies place priority natural products especially essential oil for controlling fungal strains including *F. oxysporum*, *A. niger*, *A. flavus,* and *C. albicans* [26]. Several epidemiological studies have been conducted on yeast infections indicated that *Candida* is responsible for many diseases [46]. Thus, the use of alternative treatment from a natural source can serve society to control fungal diseases at low cost.

Previously reported literature on the mechanism of action of essential oil towards fungi showed that essential oils are higher in thymol, and p-cymene penetrates cells inducing membrane damage [47, 48]. The reported activities in this work were consistent with the chemical composition of monoterpenes, which are the most potentially responsible for cell membrane damage. In previous works, it was reported that the fungicidal effect of thymol and p-cymene oil on *Candida* spp., resulting in indirect damage to the cytoplasmic membrane of target bacteria [49].

4. Conclusion

The present work aimed to shed light on the chemical composition and antioxidant, antibacterial, and antifungal activities of essential oil from *A. negrei* growing in the folded Middle Atlas, Morocco. The results obtained showed that the oil recovered from the studied plant was rich in potentially active compounds. The oil had potent antioxidant, antibacterial, and antifungal activities. Therefore, the oil from *A. negrei* can be used as a valuable natural source for further research that may lead to developing a new weapon to fight free radical damage and microbial resistance.

Data Availability

Data used to support the findings are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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