Chemical Profiling, Antioxidant and Antibacterial Activities of Essential Oil From *Englerastrum gracillimum* Th. C. E. Fries Growing in Niger

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**Abstract**

In the present study, the chemical composition, antioxidant and antibacterial activities of the essential oil obtained by hydrodistillation of the aerial parts of *Englerastrum gracillimum* Th. C. E. Fries growing in Niger were investigated. Gas chromatography-mass spectrometry (GC–MS) analysis of the essential oil resulted in the identification of 42 compounds representing 97.9% of the total oil constituents. The major compounds of the essential oil were: α-humulene (30.5%), followed by cubenol (19.8%), γ-muurolene (14.0%), (E)-β-caryophyllene (5.8%), β-gurjunene (5.2%), and curzerene (4.9%). The antioxidant activity of the essential oil was determined by using the free radical-scavenging activity (2,2-diphenyl-1-picrylhydrazyl: DPPH˙) and ferric reducing antioxidant power assays. The essential oil showed good antioxidant potential with both methods. The antibacterial activity was evaluated against multi-resistant *Acinetobacter baumannii* P1483, extended-spectrum β-lactamase (ESBL)-*Escherichia coli* Bu8566, *Salmonella* spp. H1548, *Proteus mirabilis* Bu190, *Enterobacter cloacae* Bu147, *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 700603), methicillin-resistant *Staphylococcus aureus* P1123, *Enterococcus faecium* H3434, and *Staphylococcus aureus* (ATCC 25923) using the agar disc diffusion and microdilution methods. The essential oil exhibited good antibacterial activity. The highest antibacterial effect was observed against *Staphylococcus aureus* ATCC 25923 and methicillin-resistant *Staphylococcus aureus* P1123 with minimal inhibitory concentration (MIC) values of 0.03 mg/mL and 0.06 mg/mL, respectively. These data are of scientific importance for the valorization of aromatic and medicinal plants of Niger, especially *E. gracillimum*. This study reports for the first time the antioxidant and antibacterial activities of this essential oil.

**Keywords**

*Englerastrum gracillimum*, essential oil, chemical composition, antioxidant activity, antibacterial activity

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Recently, essential oils (EO) have been widely investigated by researchers as valuable sources of bioactive compounds, and for their diverse applications in industrial fields. Essential oils are complex mixtures of volatile compounds obtained most commonly by steam or hydro-distillation. There has been great research interest in the assessment of the chemical composition and biological properties of essential oils from plants. *Englerastrum gracillimum* Th, C. E. Fries, known as *Coleus gracillimus* (T.C.E. Fr)¹ and *Plectranthus gracillimus* (T.C.E.Fr)² is an annual herb of the Lamiaceae family present in Niger. This plant grows in the Sudanese zone of Africa and is traditionally used as an insect repellent and love filter by nomads.³ However, to the best of our knowledge, there are no data published in the literature on the biological activities of *E. gracillimum* essential oil. Therefore, the purpose of this study was to characterize the chemical composition of the essential oil isolated from the aerial parts of this plant by hydrodistillation, and to evaluate its antioxidant and antibacterial activities. Then, the results could be used for further applications of this plant as a source of valuable products.
Results and Discussion

Chemical Composition of the EO

The essential oil yield obtained from the aerial parts of *E. gracillimum* by hydrodistillation was 0.4%; the chemical composition of the oil, determined by GC-MS, is presented in Table 1. A total of 42 components were identified, representing 97.95% of the total oil constituents. The major compounds were: α-humulene (30.5%), cubenol (19.8%), γ-muurolene (14.0%), (E)-β-caryophyllene (5.8%), β-gurjunene (5.2%), and curzerene (4.9%). The EO’s main classes of compounds were sesquiterpene hydrocarbons (61.2%) and oxygenated sesquiterpenes (32.7%). In contrast, monoterpene hydrocarbons and oxygenated monoterpenes were found in small quantities, with amounts of 3.2% and 0.2%, respectively. Our results are in agreement with those of Djibo et al.,3 who also found sesquiterpene hydrocarbons (32.4%) and oxygenated sesquiterpenes (29.4%) as the main classes of compounds in the essential oil obtained from aerial parts of *E. gracillimum* from Niger. They identified 28 compounds, the major ones being humulene 1,2-epoxide (8.6%), δ-cadinene (7.5%), α-cadinol (7.0%), α-humulene (6.6%), caryophyllene oxide (6.4%), and β-caryophyllene (4.9%). There are minor differences in the chemical composition in terms of the major constituents of the two essential oils of *Englerastrum gracillimum* from Niger. Also, we found more components in our study. These differences could be due to the different habitat, sample collection times, genetic factors, soil composition, environmental conditions, age of the plant, and other factors that can influence the quality and quantity of oil constituents. It has been reported that variation in the EO chemical composition could be due to the developmental stage of growth,4 culture and harvest time,5 soil composition, environment,6,7 genetic factors,8 and geographical location.9

Antioxidant Activities

Antioxidant activities of the essential oil were evaluated by using the DPPH• method and ferric reducing antioxidant power assay at concentrations ranging from 0.1 to 1 mg/mL. DPPH• is a stable free radical, which by accepting a hydrogen radical becomes a stable diamagnetic molecule.10 The DPPH• reduction capacity was evaluated by the decrease in absorbance at 517 nm induced by the essential oil of *E. gracillimum*. The IC50 value obtained for the essential oil was 3.12 mg/mL, while that of ascorbic acid, the positive control, was 0.044 mg/mL. The antioxidant activity of the essential oil was also evaluated by the ferric reducing antioxidant power assay. The result for this method is expressed in terms of an EC50 value, which is the concentration of the essential oil giving an absorbance of 0.5 for reducing power. The EC50 value obtained for the oil was 1.02 mg/mL and for ascorbic acid, the positive control, 0.028 mg/mL. The essential oil exhibited good antioxidant activities in both assays. In the DPPH• test, the IC50 value of 3.12 mg/mL suggested a moderate free radical-scavenging

**Table 1.** Chemical Composition of *Englerastrum gracillimum* Essential Oil.

| No. | RIa | RIb | Compound | Percentage (%) |
|-----|-----|-----|----------|----------------|
| 1   | 948 | 939 | α-Pinene | 0.5            |
| 2   | 956 | 954 | Camphene | 0.1            |
| 3   | 973 | 979 | β-Pinene | 0.9            |
| 4   | 1024 | 1025 | p-Cymene | 0.1            |
| 5   | 1038 | 1029 | Limonene | 0.8            |
| 6   | 1040 | 1037 | (Z)-β-Ocimene | 0.6        |
| 7   | 1056 | 1050 | (E)-β-Ocimene | 0.1        |
| 8   | 1058 | 1059 | γ-Terpine | 0.08           |
| 9   | 1168 | 1177 | Terpinen-4-ol | 0.2        |
| 10  | 1343 | 1351 | α-Cubebe | 0.1            |
| 11  | 1374 | 1376 | α-Copaene | 0.08           |
| 12  | 1382 | 1381 | Geranyl acetate | 0.08       |
| 13  | 1398 | 1390 | β-Elemene | 0.9            |
| 14  | 1419 | 1409 | α-Gurjunene | 0.1        |
| 15  | 1417 | 1419 | (E,β)-Caryophyllene | 5.8      |
| 16  | 1435 | 1433 | β-Gurjunene | 5.2            |
| 17  | 1430 | 1434 | trans-α-Bergamotene | 0.4      |
| 19  | 1445 | 1436 | γ-Elemene | 1.2            |
| 20  | 1459 | 1454 | α-Humulene | 30.5           |
| 21  | 1469 | 1479 | γ-Muurolene | 14.0          |
| 22  | 1480 | 1498 | α-Amorphene | 0.3            |
| 23  | 1486 | 1490 | β-Selinene | 0.1            |
| 24  | 1494 | 1498 | α-Selinene | 0.6            |
| 25  | 1492 | 1499 | Curzerene | 4.9            |
| 26  | 1507 | 1509 | Germacrene A | 0.1          |
| 27  | 1526 | 1522 | β-Sesquiphellandrene | 0.5      |
| 28  | 1565 | 1561 | Germacrene B | 1.0          |
| 29  | 1564 | 1563 | (E)-Nerolidol | 0.8            |
| 30  | 1570 | 1568 | Palustrol | 0.4            |
| 31  | 1576 | 1578 | Spathulenol | 0.6            |
| 32  | 1580 | 1583 | Caryophyllene oxide | 1.5      |
| 33  | 1637 | 1646 | Cubenol | 19.8           |
| 34  | 1644 | 1650 | β-Eudesmol | 1.9            |
| 35  | 1660 | 1654 | α-Cadinol | 1.2            |
| 36  | 1675 | 1685 | α-Bisabolol | 0.2           |
| 37  | 1684 | 1693 | Germacrone | 0.7            |
| 38  | 1697 | 1700 | Eudesm-7(11)-en-4-ol | 0.2      |
| 39  | 1854 | 1845 | Phytone | 0.10           |
| 40  | 1955 | 1959 | Geranyl benzoate | 0.09          |
| 41  | 2109 | 2100 | α-Henicosane | 0.2            |
| 42  | 2407 | 2400 | α-Tetracosane | 0.1            |

Sub-totals (%)

- Monoterpene hydrocarbons: 3.2
- Oxygenated monoterpenes: 0.2
- Sesquiterpene hydrocarbons: 61.2
- Oxygenated sesquiterpenes: 32.7
- Other components: 0.6

Total identified (%): 97.9

RIa: experimental retention indices.
RIb: retention indices from literature.
*Compounds are listed in order of elution from the column.
capacity. In the ferric reducing antioxidant power assay, the EC₅₀ value indicated a good reducing power capacity of the essential oil. This antioxidant potential of *E. gracillimum* essential oil can be attributed to its chemical constituents, such as γ-terpinene, α-copaene, limonene, carophyllene oxide, α-pinene, p-cymene, and β-selinene, which have been reported to have antioxidant properties. The essential oil antioxidant activities represent the product of synergistic, additive, and/or antagonistic effects, because they are complex combinations of several classes of compounds. To our best knowledge, this is the first report of antioxidant activities of the essential oil of *E. gracillimum*.

**Antibacterial Activity**

The EO of *Englerastrum gracillimum* aerial parts was examined for its antibacterial activity against eleven pathogenic bacterial strains: Gram-negative: multi-resistant *Acinetobacter baumannii* P1483, extended-spectrum β-lactamase (ESBL)-*Escherichia coli* Bu8566, *Salmonella* spp. H1548, *Proteus mirabilis* Bu190, *Enterobacter cloaceae* Bu147, *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 700603) and Gram-positive: methicillin-resistant *Staphylococcus aureus* P1123, *Enterococcus faecium* H3434 and *Staphylococcus aureus* (ATCC 25923).

**Agar Disc Diffusion Assay**

The antibacterial activity of the EO was screened against various human pathogenic Gram-positive and Gram-negative bacteria by measuring the inhibition zone diameter of bacterial growth around the discs by the agar disc diffusion method. The inhibitory capacity of the EO and chloramphenicol as a positive control was estimated as described by Ponce et al. The results from the agar disc diffusion assay are presented in Figure 1. The EO demonstrated a variable level of antibacterial capacity against the micro-organisms tested. *Staphylococcus aureus* ATCC 25923 was the most sensitive strain to the EO of *E. gracillimum*, with the strongest inhibition zone of 44 mm, followed by methicillin-resistant *Staphylococcus aureus* P1123, and *Enterococcus faecium* H3434, with inhibition zones of 35 and 25 mm, respectively. The EO was strongly active against all the Gram-negative bacteria tested. However, only a weak inhibitory effect was observed against multi-resistant *Acinetobacter baumannii* P1483, with an inhibition zone of 9 mm. Also, seven Gram-negative bacterial strains: *Salmonella* spp. H1548, extended-spectrum β-lactamase (ESBL)-*Escherichia coli* Bu8566, *Enterobacter cloaceae* Bu147, *Proteus mirabilis* Bu190, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, and *Klebsiella pneumoniae* ATCC 700603, were resistant to the EO of *E. gracillimum*. Chloramphenicol was more active than the EO of *E. gracillimum* against Gram-negative bacteria. A higher inhibition zone for chloramphenicol was obtained for *Salmonella* spp. H1548 (30 mm). *Enterococcus faecium* H3434, *Pseudomonas aeruginosa* ATCC 27853, and *Enterobacter cloaceae* Bu147 were not sensitive to chloramphenicol. It is important to notice that the EO proved to be more active than chloramphenicol against Gram-positive bacteria. These findings indicate the susceptibility of the Gram-positive bacteria to the EO investigated, and are in accordance with many other studies, which have reported that Gram-positive bacteria are more susceptible to the inhibitory effects of essential oils than Gram-negative ones.

According to the literature, the resistance of Gram-negative bacteria to essential oils has been attributed to the presence of a hydrophobic external lipopolysaccharide membrane that blocks the penetration of hydrophobic molecules of oil into the cells.

**Microdilution Assay**

The determination of the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) was assessed by the microdilution assay. The MIC was determined for the bacterial strains that were sensitive to the essential oil in the agar disc diffusion assay. The MBC was determined from the inoculate aliquots of culture for the wells that showed no visible growth of bacteria from the MIC assessment. The MIC and MBC assessments are summarized in Table 2. The antibacterial activity of *E. gracillimum* EO was variable according to the micro-organism tested. The highest antibacterial activity was obtained for *Staphylococcus aureus* ATCC 25923, reflected by the lowest MIC value of 0.03 mg/mL. Also, the EO showed strong antibacterial activity against methicillin-resistant *Staphylococcus aureus* P1123, evidenced by a MIC value of 0.06 mg/mL. The lowest MIC value for Chloramphenicol was obtained for
salmonella spp. H1548 (0.03 mg/mL), indicating the strongest antibacterial activity of chloramphenicol against this strain. The MBC values were identical to or greater than the MIC values. The significant antibacterial effect of E. gracillimum EO might be attributed to constituents such as α-humulene, terpinen-4-ol, α-pinene, γ-terpinene, (E)-β-caryophyllene, p-cymene, β-cadinol, β-pinene, and limonene, which have been reported to possess antibacterial properties.9,23-27 However, essential oils are a mixture of various active compounds, therefore, the synergistic and additive effects of these compounds should be taken into account for the evaluation of their antibacterial activities.28-31 We have not found any data reported on the antibacterial activities of E. gracillimum essential oil.

**Conclusions**

In this study, the chemical composition analysis of Englerastrum gracillimum essential oil led to the identification of 42 compounds, with a predominance of sesquiterpene hydrocarbons (61.2%) and oxygenated sesquiterpenes (32.7%). The essential oil showed good antioxidant potential. These results suggest that the EO of E. gracillimum could be considered as a significant source of natural antioxidant agents. Also, the EO demonstrated a good antibacterial effect against the human pathogenic bacteria tested, especially against the Gram-positive ones. These results showed that the EO of E. gracillimum is a good source of bioactive compounds with potential antioxidant and antibacterial activities. Therefore, E. gracillimum could be a possible alternative for the treatment of diseases related to oxidative stress and to treat some infectious diseases caused by human pathogenic bacteria. This report is the first study on the chemical composition and biological activities of E. gracillimum essential oil from Niger, and provides a scientific basis for the use of this plant in traditional medicine.

**Materials and Methods**

**Antibiotics, Broth, and Chemical Reagents**

Dimethyl sulfoxide (DMSO), 2,2 diphenyl-1-picrylhydrazyl (DPPH•), ethanol, ascorbic acid, iron III chloride (FeCl3), potassium ferricyanide (K3Fe(CN)6), and all others chemical reagents used in this study were obtained from Sigma-Aldrich. Standard antibiotics were purchased from OXOID (UK), while the sterile 96-well microtiter plates were purchased from CITOTEST (CHINA). Sterile paper discs and culture media (Nutrient broth, Nutrient agar) were products of Becton, Dickinson & Co (New Jersey, USA).

**Plant Material and Essential Oil Extraction**

The aerial parts of Englerastrum gracillimum were collected from Lido, located in the South of Niger (altitude 232 m, latitude (N) 12°53'058'', longitude (E) 003°44'446'') during October 2018. The plant materials were air-dried at room temperature away from light. The plant was identified by a botanist at Abdou Moumouni University, Niamey (Niger), and a voucher number (NA/06) was given to the plant. The essential oil (EO) was obtained by hydrodistillation using a Clevenger type apparatus.32 One hour's sundry of dried aerial parts were hydrodistilled for 3 hours. The obtained essential oil was stored at 4°C until analysis.

**GC-MS Analysis of Essential Oil**

The essential oil (EO) chemical composition was analyzed by a GC-MS technique, which used a GCMS-QP2010SE Gas Chromatograph/Mass Spectrometer (SHIMADZU CORPORATION, Columbia-USA) equipped with two columns, a Zeborn ZB-5ms (20 m × 0.18 mm × 0.18 µm) and Zeborn ZB-WAX (20 m × 0.18 mm × 0.18 µm), and coupled with a Mass Analyzer single quadrupole system, operating in Scan/SIM mode. The initial temperature of the columns was maintained at 50°C for 3 minutes and increased at 2°C/min until 280°C, and then maintained at 280°C for 30 minutes. The sample was injected by using split mode (1/30), and the volume injected was 1 µL. The injector temperature was 280°C and helium was used as the carrier gas (1 mL/min). The system operated in the electron impact (EI) mode, and data were collected over a mass range of 20 to 500; scan time 0.2 s. GC/FID analysis was performed under the same experimental conditions as described for the GC-MS. Constituents were identified by comparing their retention indices (RI) determined by using a homologous series of n-alkanes and mass spectral fragmentation patterns with those stored in the NIST08.LIB mass spectral libraries of the GC–MS data system and from literature.

**Table 2. MIC and MBC Values for Englerastrum gracillimum Essential Oil and Chloramphenicol Against the Pathogenic Bacteria Tested.**

| Bacteria                        | Chloramphenicol MIC (mg/mL) | Essential oil MIC (mg/mL) | Essential oil MBC (mg/mL) |
|--------------------------------|----------------------------|---------------------------|---------------------------|
| Methicillin-resistant          | 0.90                       | 0.06                      | 0.06                      |
| Staphylococcus aureus P1123    | -                          | 0.08                      | 0.5                       |
| Enterococcus faecium H3434     | 0.08                       | 0.03                      | 0.03                      |
| Staphylococcus aureus ATCC 2592 | 1.12                       | 3                         | 4                         |
| Multi-resistant Acinetobacter baumannii P1483 | -                          | 0.05                      | -                         |
| Proteus mirabilis Bu190        | 0.03                       | -                         | -                         |
| Salmonella spp. H1548          | 0.03                       | -                         | -                         |
| E. coli ATCC 25922             | 1.09                       | -                         | -                         |
| ESBL-E. coli Bu8566            | 0.06                       | -                         | -                         |
| Klebsiella pneumonia ATCC 700603 | 0.12                     | -                         | -                         |
| Pseudomonas aeruginosa ATCC 27853 | 0.12                   | -                         | -                         |
| Enterobacter cloacae Bu147     | 0.05                       | -                         | -                         |

- “not active”. Abbreviations: ATCC, American Type Culture Collection; E. coli, Escherichia coli; EO, Essential oil; ESBL, Extended-spectrum β-lactamase; MBC, minimum bactericidal concentration; MIC, minimum inhibitory concentration.
Ascorbic acid was used as a standard compound.

Antioxidant Activity

DPPH• Radical-Scavenging Activity. The 2,2-diphenyl-1-picrylhydrazyl radical-scavenging activity of the essential oil was evaluated according to the method reported by Blois, with slight modifications.34 The samples were diluted to prepare a range of concentrations from 0.1 to 1 mg/mL. One mL of each concentration of the different samples was added to 2 mL of DPPH• (0.2 mmol) ethanolic solution. The mixtures were left in the dark at room temperature. After 30 minutes of incubation, the absorbance of the test sample.

Finally, 2.5 mL of the upper layer solution was mixed with 2.5 mL of distilled water and 0.5 mL of FeCl3 (0.1%). The mixture was left at room temperature, 2.5 mL of tri-chloroacetic acid (10%) was added, and then centrifuged (HERMLE Z323K, Germany) at 3000 rpm for 10 minutes. The mixture was incubated at 50 °C in a water bath (DIGITERM 100, J.P. SELECTA, S.A; Spain) for 30 minutes. After incubation, the mixture was left at room temperature, 2.5 mL of tri-chloroacetic acid (10%) was added, and then centrifuged (HERMLE Z323K, Germany) at 3000 rpm for 10 minutes. The absorbance was measured at 700 nm (A700) with a Uviline 9400 spectrophotometer (SECOMAM). The antioxidant activity of all the test samples was expressed as EC50 (mg/mL). The EC50 value corresponds to the effective concentration of the essential oil antioxidant activity of all the test samples was expressed as EC50 (mg/mL). The EC50 value of the different samples was added to 2 mL of DPPH• (0.2 mmol) ethanolic solution. The mixtures were left in the dark at room temperature. After 30 minutes of incubation, the absorbance of the blank solution (1 ml of ethanol + 2 ml of DPPH• solution). Ascorbic acid was used as a positive control. The antioxidant activity of all the test samples was expressed as IC50 (mg/mL), defined as the concentration of the antioxidant needed to scavenge 50% of DPPH• present in the test solution. The percentage of inhibition of DPPH• free radical-scavenging activity was calculated by the followed equation:

\[
\text{DPPH}^* \text{ scavenging effect (\%)} = \left(\frac{A_0 - A_1}{A_0}\right) \times 100
\]

Where A0 is the absorbance of the blank solution and A1 is the absorbance of the test sample.

Ferric Reducing Antioxidant Power Assay

The capacity of the essential oil to reduce Fe3+ was assayed with the method reported by Yıldırım et al.35 Different concentrations of the essential oil were prepared in absolute ethanol: 0.1, 0.2, 0.4, 0.6, 0.8, and 1 mg/mL. One mL of the different sample concentrations was added to 2.5 mL of phosphate buffer (0.2 M, pH = 6.6) and 2.5 mL of K3Fe(CN)6 solution (1%). The mixture was incubated at 50 °C in a water bath (DIGITERM 100, J.P. SELECTA, S.A; Spain) for 30 minutes. After incubation, the mixture was left at room temperature, 2.5 mL of tri-chloroacetic acid (10%) was added, and then centrifuged (HERMLE Z323K, Germany) at 3000 rpm for 10 minutes. The mixture was incubated at 50 °C in a water bath (DIGITERM 100, J.P. SELECTA, S.A; Spain) for 30 minutes. After incubation, the mixture was left at room temperature, 2.5 mL of tri-chloroacetic acid (10%) was added, and then centrifuged (HERMLE Z323K, Germany) at 3000 rpm for 10 minutes. The absorbance was measured at 700 nm (A700) with a Uviline 9400 spectrophotometer (SECOMAM). The antioxidant activity of all the test samples was expressed as EC50 (mg/mL). The EC50 value corresponds to the effective concentration of the essential oil giving an absorbance of 0.5 for reducing power and was determined from linear regression analysis of the graphs of A700 against the corresponding essential oil concentrations.36 Ascorbic acid was used as a standard compound.

Antibacterial Activity

The evaluation of the essential oil antibacterial activity was performed at the Laboratory of Microbiology and Virology of Mohamed VI University Hospital Center in Marrakech, Morocco.

Bacterial Strains

The essential oil antibacterial activity was evaluated against the eleven pathogenic bacterial strains including Gram-negative: multi-resistant Acinetobacter baumannii P1483, extended-spectrum β-lactamase (ESBL)-Escherichia coli Bu8566, Salmonella spp. H1548, Proteus mirabilis Bu190, Enterobacter cloacae Bu147, Pseudomonas aeruginosa (ATCC 27853), Escherichia coli (ATCC 25922), Klebsiella pneumoniae (ATCC 700603) and Gram-positive: methicillin-resistant Staphylococcus aureus P1123, Enterococcus faecium H3434 and Staphylococcus aureus (ATCC 25923), as reported in our previous published work.37 The reference strains (ATCC: American Type Culture Collection Center, Manassas, Virginia, USA) were obtained from the culture collection of the Laboratory of Microbiology and Virology of Mohamed VI University Hospital Center in Marrakech (Morocco), and all the clinical strains were isolated and identified at the same Laboratory.

Agar Disc Diffusion Assay

The agar disc diffusion assay was used to perform the antibacterial activity of the essential oil according to the method reported by Ali et al.38 To obtain a young culture of bacterial colonies, the test bacteria were seeded on Petri dishes containing Mueller-Hinton agar and incubated for 24 hours at 37 °C. After incubation, these colonies were taken and placed in 3 mL of sterile 0.9% NaCl solution to prepare a bacterial suspension adjusted to 0.5 McFarland (108 CFU/mL) standard turbidity. The microdilution method was used to determine the minimum inhibitory concentration (MIC), as described by Chrysargyris et al., and Selim et al.,39,40 with slight modifications. A range of concentrations of the essential oil from 0.03 to 10 mg/mL was prepared in dimethyl sulfoxide (DMSO). The inoculum of the bacterial strains was prepared from 24 hours broth cultures and suspensions were adjusted to 0.5 McFarland (108 CFU/mL) standard turbidity. The microdilution assay was carried out using sterile 96-well microtiter plates. The 96 well-plates were prepared by putting 90 µL of...
Mueller-Hinton broth (MHB) and 10 μL of the inoculum into each well. One hundred μL of each concentration of the serial dilution of the essential oil was added and the final volume in each well was 200 μL. The negative control was prepared containing 90 μL of Mueller-Hinton broth (MHB), 10 μL of the inoculum and 100 μL of chloramphenicol. The positive control was also prepared containing 90 μL of Mueller-Hinton broth (MHB), 10 μL of the inoculum, and 100 μL of DMSO in the last wells of each strip. After mixing the wells, they were covered and incubated at 37 °C for 24 hours. The MIC value was defined as the lowest concentration of the essential oil that inhibited the visible growth of the microorganisms tested after 24 hours of incubation. To determine the minimum bactericidal concentration (MBC), the content of each well without any visible growth of bacteria was seeded on Petri dishes containing the Mueller-Hinton agar and incubated for 24 hours at 37 °C. The MBC was considered as the concentration of the essential oil that did not exhibit any viable organism in the culture after 24 hours of incubation.

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