Research Article

Phytochemical Analysis and Study of Antioxidant, Anticandidal, and Antibacterial Activities of *Teucrium polium* subsp. *polium* and *Micromeria graeca* (Lamiaceae) Essential Oils from Northern Morocco

Taoufiq Benali,1,2 Khaoula Habbadi,3 Abdelhakim Bouyahya,4 Abdelmajid Khabbach,5 Ilias Marmouzi,6 Tarik Aanniz,7 Houda Chtibi,2 Hanae Naceiri Mrabti,8 El Hassan Achbani,3 and Khalil Hammani2

1Environment and Health Team, Polydisciplinary Faculty of Safi, Cadi Ayyad University, Marrakesh, Morocco
2Laboratory of Natural Resources and Environment, Polydisciplinary Faculty of Taza, Sidi Mohamed Ben Abdellah University, B.P.: 1223, Taza-Gare, Taza, Morocco
3Laboratoire de Recherche et de Protection des Plantes URPP- INRA-Meknès, Meknès, Morocco
4Laboratory of Human Pathologies Biology, Department of Biology, Faculty of Sciences, and Genomic Center of Human Pathologies, Faculty of Medicine and Pharmacy, Mohammed V University in Rabat, Rabat, Morocco
5Laboratory of Materials, Natural Substances, Environment and Modeling, Polydisciplinary Faculty of Taza, Sidi Mohamed Ben Abdellah University, B.P.: 1223, Taza-Gare, Taza, Morocco
6University Mohammed V in Rabat, Faculty of Medicine and Pharmacy, Laboratory of de Pharmacology et Toxicology, Rabat Institutes, BP 6203, Rabat, Morocco
7Medical Biotechnology Laboratory (MedBiotech), Rabat Medical & Pharmacy School, Mohammed V University in Rabat, Rabat 6203, Morocco
8Laboratory of Pharmacology and Toxicology, Bio Pharmaceutical and Toxicological Analyzes Research Team, Faculty of Medicine and Pharmacy, Mohammed V University in Rabat, BP 6203, Rabat, Morocco

Correspondence should be addressed to Abdelhakim Bouyahya; boyahyaa-90@hotmail.fr

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The protection of agricultural crops and the preservation of the organoleptic and health qualities of food products represent a major challenge for the agricultural and agro-food industries. Essential oils have received greater attention as alternatives to replace the control strategies based on pesticides against phytopathogenic bacteria and synthetic compounds in food preservation. The aims of this work were to study the chemical composition of *Teucrium polium* subsp. *polium* and *Micromeria graeca* essential oils and to examine their antioxidant and antimicrobial effects. To carry out this work, the chemical composition of the essential oil was determined using gas chromatography (GC) with the detection feature of mass spectrometry (MS). Subsequently, the antioxidant activity was investigated by DPPH and FRAPs assays. The antimicrobial effect was studied against phytopathogenic and foodborne pathogenic bacteria using the disc and the microdilution methods. Our results showed that GC-MS analysis of EOs allowed the identification of 30 compounds in *T. polium* EO (TPpEO), while 5 compounds were identified in *M. graeca* EO (MGE). TPpEO had as major compounds β-pinene (19.82%) and germacrene D (18.33%), while geranial (36.93%) and z-citral (18.25%) were the main components of MGE. The most potent activity was obtained from MGE (IC₅₀ = 189.7 ± 2.62 µg/mL) compared to TPpEO (IC₅₀ = 208.33 ± 3.51 µg/mL). For the FRAP test, the highest reducing power was obtained from 1.32 ± 0.1 mg AAE/g of TPpEO compared to MGE 0.51 ± 0.13 mg AAE/g of EO. Both EOs exhibited varying degrees of antibacterial activities against all the tested strains with inhibition zones in the range of 9.33 ± 0.57 mm to >65 mm and MIC values from 0.19 to 12.5 mg/mL. However, MGE exhibits an interesting antifungal effect with inhibition zone 44.33 ± 0.57 mm. The findings of this research establish the riches of EOs on volatile compounds, their important antioxidant activity, and their antimicrobial effect against the bacteria tested.
1. Introduction

The security of agricultural crops and both organoleptic and health qualities of food products represent a main defiance for the agricultural and agro-food industries [1, 2]. The control of the problems caused by phytopathogenic bacteria is based on use of pesticides and antibiotics with potential side effects on the environment and living beings. Such chemicals are not very biodegradable and represent a risk of developing antibiotic resistance, which inspired the European Union to limit their use [3, 4]. On the other hand, the preservation of food products is assured by synthetic compounds called “food additives,” presented potential side effects on the consumer [5–7]. The plant extracts and essential oils as antioxidant and antimicrobial agents are focused to overcome these problems and to satisfy the improved demand for more natural solutions.

For a long time, different cultures and civilizations worldwide have been using plants as drugs to treat numerous diseases [8–10]. The essential oils (EOs) are among the natural products of great interest in food, cosmetic, and pharmaceutical industries due to their antioxidant and antibacterial [11–18], antifungal [19–25], antiparasitic [6, 26], insecticidal [27–31], and anticancer activities [32–34].

Morocco by its biogeographical position is characterized, on the one hand, by ecological and floristic diversities and, on the other hand, by a long tradition and expertise in the use of plant medicines [35–37]. Previous works in some regions of Morocco have shown that the Moroccan pharmacopoeia is dominated mainly by Lamiaceae [38–41]. A great economic importance is given to many of their species due to their EO production [42] and their traditional use [40, 43, 44]. In recent decades, in the goal to valorize the Moroccan Lamiaceae species, previous researchers have evaluated the antioxidant and antimicrobial activities of essential oils of many plants [45–49]. In this order, our study focused on two species of Lamiaceae, Teucrium polium subsp. polium and Micromeria graeca, locally known as “Jâada” and “Bakolt’nhal,” respectively. These species have been strongly used in Moroccan traditional medicine [38–40, 44, 50].

To the best of our knowledge, no reports on the variation of essential oil composition and biological activities of these plants collected from the Province of Taza, Northern Morocco, are available. Therefore, the objectives of this study were the identification of volatile compounds of hydrodistilled EOs of T. polium and M. graeca and the investigation of their antioxidant and antimicrobial activities.

2. Materials and Methods

2.1. Collection of Plants and Isolation of Essential Oils.

Both plants were collected in April 2016 from the Province of Taza, Northern Morocco (004°52.607′ N, 004°01.190′ W and 34°09.825′ N, 004°09.850′ W). The identification of plants was achieved by Pr. Ennabili Abdeslam and Dr Khabbach Abdelmajid in the Natural Resources and Environment Laboratory of the Polydisciplinary Faculty of Taza, Sidi Mohamed Ben Abdellah University of Fez, where a voucher plant specimen has been deposited for future reference (FPT-LRNE-73: Teucrium polium subsp. polium and FPT-LRNE-72: Micromeria graeca). The aerial parts of the plants were dried at room temperature. Then, the plant sample (100 g) was subjected to hydrodistillation using a Clevenger-type apparatus for 4 h. The essential oil was stored at 4°C until use.

2.2. Gas Chromatography-Mass Spectrometry (GC-MS) Analysis.

The chemical composition of EOs was analyzed according the conditions described in our previous works [51, 52]. For each compound, the Kovats retention index (RI) was calculated relative to a standard mix of n-alkanes between C9 and C31 (Sigma-Aldrich Co.). Identification of constituents was performed by comparison of RI and MS spectra with those reported in the literature and by computer matching with standard reference databases (NIST98, Wiley275, and CNRS libraries).

2.3. Antioxidant Activity

2.3.1. Free Radical Scavenging Activity. The radical effect of EOs was evaluated using the radical 2,2-diphenyl-1-picyrylhydrazyl (DPPH) as reported by Benali et al. [52] and Huang et al. [53], with some modifications. In brief, the DPPH solution (0.2 mM in methanol) was prepared. Then, 2.5 mL of test sample at different concentrations (2.5–100 μg/mL) was added to 0.5 mL of DPPH solution, and the absorbance of samples was measured at 517 nm after 30 min. Ascorbic acid and Trolox were used as positive controls.

The calculation of the antioxidant activity was done according to the following formula:

\[ DPPH \text{ scavenging activity (\%)} = \left( \frac{A_0 - A_s}{A_0} \right) \times 100, \]

where \( A_0 \) is the absorbance of the negative control and \( A_s \) is the absorbance of the test sample at 30 min. The test was carried out in triplicate, and the IC_{50} values were reported as mean ± SD.

2.3.2. Reducing Power of Ferric Ions. The reducing activity of EOs was determined according to Benali et al. [52] and Oyaiizu [54]. The mixture of the sample (1 mL), the phosphate buffer (2.5 mL, 0.2 M, pH 6.6), and the potassium ferricyanide (2.5 mL) was prepared. After incubation for 20 min at 50°C (water bath), 2.5 mL of trichloroacetic acid (10%) was added to the mixture. Then, the solution was centrifuged at 3000 g for 10 min. Finally, 2.5 mL of the supernatant was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride. Absorbance was measured at 700 nm.

Ascorbic acid (50–450 μg/mL) is used as a standard. The reducing power is expressed in milligram equivalence of ascorbic acid per gram of essential oil (mg AAE/g of EO).
2.4. Antimicrobial Activity

2.4.1. Microorganism Strains, Origin, and Growth Conditions. The foodborne pathogenic bacteria used including Gram-positive (Listeria innocua CECT 4030, Staphylococcus aureus CECT 976, and Bacillus subtilis DSM 6633) and Gram-negative (Pseudomonas mirabilis, Escherichia coli K12, and Pseudomonas aeruginosa CECT 118) bacteria were obtained from the Laboratory of Biology and Health, Sciences Faculty of Tetouan; Candida albicans ATCC 10231 was, also, used which was obtained from the Laboratory of Agri-Food and Health, Sciences and Technics Faculty of Settat, Morocco. Plant pathogenic bacteria were Clavibacter michiganensis subsp. michiganensis 1616-3 and Pseudomonas savastanoi pv. savastanoi (PSS2636-40) which were obtained from the Laboratory of Researches and Protection of Plants, URPP- INRA-Meknes, Morocco.

The pathogen bacterial strains were cultivated in Mueller-Hinton agar (MHA) or Mueller-Hinton Broth (MHB) at 37°C for 24 h as described by Benali et al. [52]. The fungi and the phytopathogenic bacteria were cultured in YPGA medium (5 g yeast extract, 5 g peptone, 10 g glucose, 15–18 g agar, in 1 liter) or YPG and incubated as following: 48 h at 37°C for Candida albicans ATCC 10231; 48 h at 25°C for Pseudomonas savastanoi pv. savastanoi 636-40; 72 h at 25°C for Clavibacter michiganensis subsp. michiganensis 1616-3. The inoculum test concentrations are 10^6 CFU/mL for bacteria, 10^5 CFU/mL for phytopathogenic plant, and 10^5 spores/mL for fungi.

2.4.2. Antimicrobial Activity. The antibacterial activity was evaluating using disc diffusion method as described by Benali et al. [52] and Rota et al. [55], with some modifications. In brief, sterile disks (6 mm diameter) containing 12.5 μL of pure essential oil were applied onto the surface of the agar medium which were previously spread by the test inoculum concentrations. Gentamycin (15 μg), vancomycin (30 μg), streptomycin (25 μg), and amphotericin (10 μg) were used as a positive control. Negative control consisted of 10% dimethylsulfoxide (DMSO). After incubation as described above, the antimicrobial activity was assessed by measuring the diameter of inhibition zones. Tests were performed in triplicate.

2.4.3. Determination of Minimum Inhibitory Concentration. MIC was determined only for strains considered very sensitive and essential oils considered very active leading to diameters larger than 15 mm [52–56]. Minimum inhibitory concentrations (MICs) were realized in sterile 96-well microplate as described by Güllüce et al. [22], with some modifications. First, 100 μL of MHB was distributed in all test wells, except the first well in which a volume of 200 μL containing the essential oil at a concentration of 25 mg/mL in 10% DMSO. A series of concentrations ranging from 0.097 to 25 mg/mL were prepared by the transfer of 100 μL by scalar dilutions from the first to the ninth well. Then, except the 10th well used as sterility control, 10 μL of the suspension from each well was removed and replaced by the test inoculum concentrations as described above. The eleventh well was considered as positive growth control containing only broth medium. The last well containing 10% DMSO (v/v), without oils, was used as negative control. Then, the plates were incubated at conditions of growth as described above. After incubation, a volume of 25 μL of an indicator of microorganism’s growth was added in each well, and tetrazolium (MTT: 3-(4,5-dimethylthiazol)-2-yl-2, 5-diphenyltetrazolium bromide (Sigma)) was prepared at a concentration of 0.5 mg/mL in sterile distilled water. The microplate was re-incubated for 30 min at temperature 25°C or 37°C. Where microbial growth was inhibited, the solution keeps the initial color of MTT. To determine the minimum bactericidal concentration (MBC) value, 10 μL of broth from the uncolored wells was inoculated and incubated at growth conditions.

2.5. Statistical Analysis. All experiments were done in triplicates and values of each were expressed as mean ± standard deviation (SD) and were subjected to analysis of variance (one-way ANOVA). The statistical analysis was performed using GraphPad Prism version 6.00 (GraphPad Inc., San Diego, California). Differences (between groups) were considered as statistically significant at p < 0.05.

3. Results

3.1. Chemical Composition. The essential oil yields (w/w) were 0.24 ± 0.02% and 0.18 ± 0.02%, for Micromeria graeca and Teucrium polium subsp. polium, respectively. Volatile compounds of both studied plants were separated by GC (Figures 1 and 2) and identified using MS analysis. The results obtained by GC-MS analysis of EOs are summarized in Table 1. As summarized, 29 and 5 compounds were identified in TPpEO and MGE representing 97.46% and 99.95% of the total, respectively. Our results showed that the major compounds in TPpEO are β-pinene (19.82%), germacrene D (18.33%), α-cadinol (6.83%), α-pinene (6.76%), limonene (5.71%), epi-bicyclesquiphellandrene (5.05%), delta-cadinene (4.51%), spathulenol (4.15%), bicyclogermacrene (3.21%), myrcene (2.9%), and camphor (2.45%). However, MGE contains geranial (36.93%) as a main component followed by z-citral (18.25%), 1,8-epoxy-p-menth-2-ene (13.01%), nerol (11.96%), and isoromadendrene epoxide (10.14%).

3.2. Antioxidant Activity. The essential oils were evaluated for their antioxidant effect using two methods, the DPPH free radical scavenging and the ferric ion reduction assay (FRAP). For the DPPH assay, as summarized in Table 2, the most potent activity was obtained from M. graeca (IC50 = 189.7 ± 2.62 μg/mL), followed by T. polium (IC50 = 208.33 ± 3.51 μg/mL), but they were all less potent than the standards used as positive controls, namely, Trolox and ascorbic acid (IC50 = 1.4 ± 0.04 μg/mL and IC50 = 1.82 ± 0.025 μg/mL, respectively). For the FRAP test, the results were expressed in milligram equivalence of ascorbic acid per gram of extract (mg AAE/g of EO), and the
Figure 1: GC analysis of *Micromeria graeca* essential oil.

Figure 2: GC analysis of *Teucrium polium* subsp. *polium* essential oil.
highest reducing power was obtained from TPpEO 1.32 ± 0.1 mg AAE/g of EO compared to MGEO 0.51 ± 0.13 mg AAE/g of EO.

3.3. Antimicrobial Activity. The in vitro antimicrobial activity of the essential oils against the tested microorganisms was qualitatively and quantitatively confirmed by diameter of inhibition zone and the MIC values. As shown in Tables 3 and 4, the essential oils exhibited varying degrees of antibacterial activity against all tested strains. For the essential oil of TPpEO, the inhibition zones were in the range from 7.33 to 52 mm, with MIC values of 0.19 mg/mL and 0.78 mg/mL. C. michiganensis was the most sensitive bacteria to TPpEO with inhibition...
The results indicated the possibility of the chemical composition difference in Micromeria EOs from one species to another. These results are different to the only one investigation of oil species from Saudi Arabia, Algeria, Jordan, Greece, Turkey, and Serbia identified the following compounds with a high content: β-pinene, limonene, germacrene D, α-pinene, bicyclogermacrene, and spathulenol [58]. The GC-MS analysis of Teucrium polium subsp. polium that demonstrated germacrene (14.8%) β-cadinol (7.2%) as main compounds [57], except for α-cadinol (6.83%), epi-bicyclosesquiphellandrene (5.05%), δ-cadinene (4.51%), and camphor (2.45%), which were not detected in the Algerian sample. However, the results of the volatile product analysis of Teucrium polium subsp. polium from the regions of Midelt, which indicated 3-carene (16.49%), γ-murolene (14.03%), α-pinene (9.94%), α-phellandrene (6.93%), and carophyllene (7.51%) as major constituents [58]. The results of the volatile product analysis of Teucrium polium species from Saudi Arabia, Algeria, Jordan, Greece, Turkey, and Serbia identified the following compounds with a high content: β-pinene, limonene, germacrene D, α-pinene, bicyclogermacrene, and spathulenol [59–64].

For M GEO from Morocco, this is the first study of their chemical composition. In Greece, EOs of two samples of this plant were characterized by the presence of carophyllene oxide (17.0%), epi-α-bisabolol (12.8%), linalool (18.1%), and β-chamigrene (12.5%) [65]. Compared with other species of the Microseria genus, the study of the chemical composition of Microseria ciliicola EO from Tukey showed that the major components characterized were pulegone, cis-p-menthone, and trans-p-methone [66]. In addition, Microseria fruticosa oil was characterized by a high content of γ-terpinene, β-caryophyllene, p-cymene, α-pinene, and β-bisabolene [67]. These results indicated the possibility of the chemical composition difference in Microseria EOs from one species to another. The qualitative and/or quantitative difference between the oil composition in our results and those noticed in

| Tested microbial strains       | Essential oils | MIC (μg) | MBC or MFC (μg) | MIC (μg) | MBC or MFC (μg) |
|-------------------------------|----------------|---------|-----------------|---------|-----------------|
| S. aureus CECT 976            |                | 0.39    | 3.12            | 1.56    | 1.56            |
| B. subtilis DSM 6633          |                | 0.98    | 12.5            | 1.56    | 1.56            |
| L. innocua CECT 4030          |                | 0.78    | 1.56            | 3.21    | 3.21            |
| E. coli K12                   |                | 0.78    | 1.56            | 3.21    | 3.21            |
| P. mirabilis                  |                | 0.78    | 1.56            | 3.21    | 3.21            |
| C. michiganensis 1616-3       |                | 0.19    | 0.19            | 0.78    | 0.78            |
| P. savastanoi PSS2636-40      |                | 0.19    | 0.19            | 0.78    | 0.78            |
| C. albicans ATCC 10231        |                | 0.19    | 0.19            | 0.78    | 0.78            |

zone of 52 ± 1 mm with MIC value of 0.78 mg/mL, followed by B. subtilis (23 ± 2 mm), P. savastanoi PSS2636-40 (22 ± 1 mm), and P. mirabilis (21.33 ± 2.08 mm). This oil has a low effect against the other bacteria. No antifungal activity is observed against C. albicans (7.33 ± 0.57 mm).

For M GEO, the inhibition zones varied from 9.33 to >65 mm, with MIC values from 0.19 to 12.5 mg/mL. C. michiganensis was the most sensitive bacteria with inhibition zone superior to 65 mm with MIC value of 0.19 mg/mL, followed by P. savastanoi PSS2636-40 (49 ± 1 mm), B. subtilis (28.33 ± 1.52 mm), S. aureus (22 ± 1 mm), P. mirabilis (20 ± 2 mm), L. innocua (19.33 ± 1.15 mm), and E. coli K12 (17.66 ± 1.52 mm). For antifungal activity, M GEO exhibits a good antifungal effect with an inhibition zone of 44.33 ± 0.57 mm and MIC value of 3.12 mg/mL compared to control positive amphotericin (18.66 ± 1.15 mm).
previous works may be attributed to the ecological factors, genetic differences, environment, geographical origins, and season of harvest [68–72].

As indicated above, β-pinene, germacrene D, and α-pinene were among the major compounds of TPpEO chemical composition and nerol and z-citral were for MGEO. Previous research studies showed the antioxidiant effect of β-pinene, germacrene D, and α-pinene tested individually [73–77]. Also, nerol and citral are known for their antioxidiant efficacy [78–80]. These proprieties can explain the antioxidiant activity of both essential oils. The small difference of antioxidiant activity between TPpEO and MGEO may be associated to the variability in chemical composition since the antioxidiant mechanisms of essential oils are generally caused by several compounds’ functional groups and their structure [81]. However, the difference observed between testing methods could be explained by the correlation between the chemical composition and/or each compound and the used method [82–84].

For the antibacterial activity, it is known that the Gram-negative bacteria are less sensitive to plant extracts than Gram-positive ones [85–87]. However, the present findings showed that essential oils of plants studied do not have selective antibacterial effects against microorganisms tested. This result may be related to the high level of β-pinene, germacrene D, and α-pinene (TPpEO) and z-citral and nerol (MGEO). Antibacterial and antifungal activities of these substances have been reported in other studies [75, 88–96]. On the other hand, previous research studies reported the synergic effect of minor compounds against bacteria [97, 98].

5. Conclusion

To the best of our knowledge, this is the first report contributing details on chemical composition and antioxidiant and antimicrobial activities of *Teucrium polium* subsp. *polium* and *Micromeria graeca* essential oils from Northern Morocco. Our findings have shown that both essential oils are rich by volatile compounds which could be responsible for the observed antibacterial and antioxidiant effects. TPpEO and MGEO may be proposed as natural antioxidiant and antibacterial product for application on food preservation and management against phytopathogenic bacteria. Further in vivo studies will be recommended to investigate their biological proprieties and negative effects before the practical applications.

Data Availability

The data used in this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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