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# Chemical Analysis of Volatile Constituents of *Pulicaria Mauritanica* Isolated by Hydrodistillation and Headspace Solid-phase Micro-extraction Techniques. Antimicrobial Activity of its Essential Oil

Ali Lboumhamdi[a], Mohamed Znini[a,*], Julien Paolini[b], Jean Costa[b] and Lhou Majidi[a]

[a]*Moulay Ismail University of Meknes, Laboratoire des Substances Naturelles & Synthèse et Dynamique Moléculaire, Faculté des Sciences et Techniques, Errachidia, Morocco*

[b]*Université de Corse, UMR CNRS 6134, Laboratoire de Chimie des Produits Naturels, Faculté des Sciences et Techniques, Corse, France*

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The objective of this study is the comparative chemical analysis of essential oil (EO) obtained by hydrodistillation (HD) isolated from *Pulicaria mauritanica* using Gas Chromatography-Retention Indices (GC-RI) and GC-Mass Spectrometry (GC-MS). The volatile fraction (VF) was detected by headspace solid-phase micro-extraction (HS-SPME) The antimicrobial activity of *P. mauritanica* EO against three bacterial strains referenced by the *ATCC* (American Type Culture Collection): *Escherichia coli* (*ATCC 25922*), *Staphylococcus aureus* (*ATCC 25923*) and *Pseudomonas aeruginosa* (*ATCC 27853*) and a one yeast *Candida albicans* was also evaluated *in vitro* using the paper disc diffusion and minimum inhibitory concentration (MIC) assays. The chromatographic profile indicates the quantitative and semi qualitative differences between the chemical compositions of both analyzed samples. The antimicrobial activity tested by two methods indicates that this EO is more effective against three strains tested except *P. aeruginosa* which is the most resistant strain. Indeed, the results of the report MBC (Minimum Bactericidal Concentration)/MIC show that the EO has a bactericidal effect on *S. aureus* and a bacteriostatic and fungistatic effect on *E. coli* and *C. albicans*, respectively. *P. mauritanica* EO seems to be a promising source of natural products with potential antimicrobial activity.

**Keywords:** *Pulicaria mauritanica*, Essential oil, Carvotanacetone, Antimicrobial activity, Headspace solid-phase micro-extraction, Microbial strains

## INTRODUCTION

Infectious diseases caused by pathogenic microorganisms, such as bacteria, viruses or fungi, represent a real public health problem. They are the second leading cause of morbidity and mortality in the world with about 15 million deaths per year, more than 25% of overall mortality (43% of deaths in developing countries compared to 1% in industrialized countries) [1]. To reduce the mortality attributable to these diseases, antibiotic therapy has been an original means of a treatment since the discovery of antibiotics in the second half of the twentieth century.

*Corresponding author. E-mail: m.znini@fste.umi.ac.ma

However, the massive and repeated use of these synthetic molecules generates an increase in bacterial resistance and the appearance of side effects on the human body giving rise the majority of these chemical molecules to lose their effectiveness [1]. Faced with this imperfectly resolved situation, it becomes urgent to develop new antibiotic solutions that can stop the spread of bacterial resistance and thus better prevent infections. Indeed, natural products from aromatic and medicinal plants, such as essential oils (Eos), can be considered as a good therapeutic alternative [2]. The first demonstration of the action of EOs against bacteria was carried out in 1881 by Delacroix [3]. Since then, many oils have been defined as antibacterial [4,5].

The antimicrobial activity of the EOs is attributed to their lipophilic properties and the strength of their functional groups. This activity is due to the major compounds, or those susceptible to be active. However, the minor compounds would act with a synergistic effect [5]. Thus, because of the variability of the amounts and the profiles of the components of essential oil, their mode of action is rather complex and difficult to determine from the molecular point of view, indicating that their activity is not probably attributed to a unique mechanism, but several mechanisms and different sites of action [6].

There are various techniques available for extracting volatile components from aromatic plants. Hydrodistillation (HD) is a conventional method used to extract EOs, because it can be easily implemented in industry and has no chemical pollution. However, it has certain disadvantages, particularly, the consumption of energy and time, the deterioration of heat-sensitive compounds and the low extraction yields of EOs. Thus, developing an alternative rapid, sensitive, safe, and energy-conserving extraction technique is highly desirable. Thereby, headspace solid-phase microextraction (HS-SPME) is an easy, fast and modern sample preparation technique to characterize the volatile fraction (VF) of aromatic and medicinal plants [7].

*Pulicaria*, established in 1791 by Gaertner, is a genus of flowering plant of the *Asteraceae* family, containing more than 77 species available in the world [8]. P. mauritanica Coss**.** is an aromatic and endemic plant growing wild in Southeast of Morocco. It is an herb branched, woolly-rooted perennial and creeping; its leaves are less broad, oval-lanceolate, endowed with a very strong odor; the flowers are yellow where it blooms in March-May. The plant abounds woodlands, rockeries on dry rocky pastures and in the plains and low mountains up to 1500 m (Fig. 1). This Moroccan medicinal plant, locally known as "Ifanzi oudaden", is endowed with a very strong and very pleasant smell making it usable as a flavoring of tea and as an ingredient for perfuming baths. It is also used in traditional local medicine thanks to these antispasmodic properties, hyperglycemic and repellent properties against insects [9] and in the treatment of dysentery [10,11].

Recently, we evaluated the antifungal potential of the EO of this plant, using the method of direct contact and the microatmosphere method, on the mycelial growth of three molds responsible for the rot of apples in posharvest (*Alternaria* sp*., Penicillium expansum* and *Rhizopus stolonifer*). Analysis of the results obtained with the direct contact method shows that the EO exhibits a fungistatic effect on the mold *Alternaria* sp., at 0.2% (v/v). In contrast, the use of the microatmosphere method allowed the observation of the fungicidal effect on *Alternaria* sp., at 40 µl/disc and on *P. expansum* and *R. stolonifer* at 80 µl/disc [12]. These encouraging results motivated us to study other biological activities. Indeed, the objective of this paper is twofold. Firstly, to report, for the first time, the comparative chemical analysis of volatile compounds of *Pulicaria mauritanica* using HD and HS-SPME. Secondly, to evaluate, *in vitro,* the antimicrobial activity of the essential oil against three reference bacterial strains: *Escherichia coli* (Gram +), *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Gram -) and a yeast strain, namely *Candida albicans*, by means of paper disc diffusion method and minimum inhibitory concentration (MIC) assays.

## EXPERIMENTAL

### Plant Material and EO Isolation

The aerial parts of *P. mauritanica* were harvested in March 2009 (full bloom) in the mountains of Amellagou located in the south-east of Errachidia (Morocco). The identification of this botanical species was confirmed by Mr. Jalal El Oualidi of the Scientific Institute of Rabat and voucher specimens were deposited in the herbarium of Faculty of Sciences and Technique of Errachidia. The EO used in this study was the same as used in our previous study [12]. It was prepared by hydrodistillation for 3 h using a Clevenger type apparatus and analyzed by gas chromatography equipped with dual flame ionization detectors (FID) (GC/FID) and gas chromatography/mass spectroscopy (GC/MS). The EO yield was approximately 0.45%.

### Volatile Compounds by HS-SPME

The dried and pulverized aerial parts of *P. mauritanica* were subjected directly to HS-SPME. The SPME fiber (Supelco) coated with divinylbenzene/carboxen/ polydimethylsiloxane (DVB/CAR/PDMS, 2 cm-50/30 µm)


**Fig. 1.** *P. mauritanica* in its native habitat to Amellagou (Southeast of Morocco).

was used for extraction of the plant volatiles. Optimization of conditions was carried out using fresh aerial parts of the plant (1.2 g in a 20 ml vial) and based on the sum of total peak areas measured on GC-FID. The temperature and the equilibration time were selected, respectively, after three different experiments at 50, 70 and 90 °C, and after three different experiments at 60, 90 and 120 min. The extraction time was selected after three different experiments at 15, 30 and 60 min. After sampling, SPME fiber was inserted into the GC and GC-MS injection ports for desorption of volatile components (5 min), both using the splitless injection mode. Before sampling, each fiber was reconditioned for 5 min in the GC injection port at 260 °C. HS-SPME and subsequent analyses were performed in triplicate. The coefficient of variation (9.6% < CV < 13.4%) calculated based on the total area obtained from the FID-signal for the samples indicated that the HS-SPME method produced reliable results. In the same way, the CV of the major compounds was always less than 15%.

## GC-RI Analysis

GC analyses were carried out using a Perkin-Elmer Auto system (Waltham, MA, USA) XL GC apparatus equipped with dual flame ionization detectors (FID) and fused-silica capillary columns (60 m × 0.22 mm i.d.; film thickness 0.25 µm) coated with Rtx-1 (polydimethyl-siloxane) and Rtx-wax (polyethylene glycol). The oven temperature was programmed for 60 to 230 °C at 2 °C $min^{-1}$ and kept at 230 °C for 35 min. Injector and detector temperatures were maintained at 280 °C. Samples were injected in the split mode (1/50) using helium as a carrier gas (1 ml $min^{-1}$); the injection volume of pure oil was 0.1 µl. For HS-SPME-GC analysis, only Rtx-1 (polydimethylsiloxane) column was used and volatile components were desorbed in a GC injector with a SPME inlet liner (0.75 mm. I.D., Supelco).

## GC-MS Analysis

Samples were analyzed with a Perkin-Elmer Turbo mass detector (quadrupole), coupled to Perkin-Elmer Auto system XL chromatograph equipped with Rtx-1 and Rtx-wax fused-silica capillary columns. The carrier gas was helium (1 ml $min^{-1}$), the ion source temperature was 150 °C, the oven temperature was programmed for 60 to 230 °C at

2 °C $min^{-1}$ and kept at 230 °C for 35 min, the injector was operated in the split (1/80) mode at a temperature of 280 °C, the injection volume was 0.2 µl of pure oil, the ionization energy was 70 eV, EI/MS were acquired over the mass range 35-350 Da. The volatile fractions sampling by HS-SPME were analyzed only on a Rtx-1 capillary column and volatile components were desorbed in a GC injector with a SPME inlet liner (0.75 mm. I.D., Supelco).

## Components Identification

Identification of the components was based: (i) on the comparison of their GC retention indices (RI) on non polar and polar columns, relative to the retention time of a series of n-alkanes with linear interpolation, with those of authentic compounds or literature data [13], and (ii) on computer matching with commercial mass spectral libraries [14] and comparison of spectra with those of our personal library.

## Evaluation of the Antimicrobial Activity of Essential Oil

**Microbial strains tested and growth conditions.** The micro-organisms used were provided by the microbiology laboratory of Moulay Ali Chérif Hospital in Errachidia. These are two gram-negative bacteria: *Escherichia coli* (*ATCC 25922*) and *Pseudomonas aeruginosa* (*ATCC 27853*), gram-positive bacteria: *Staphylococcus aureus* (*ATCC* 25923) and a yeast strain: *Candida albicans* which were collected under the nails of a patient. The growth is carried out at 37 °C for 24 h in the dark in Müeller-Hinton Broth (MHB, Oxoid) and on Müeller-Hinton agar (MHA bioMérieux) for the bacteria and in a Sabouraud dextrose agar (SDA) for yeast for seven days at 37 °C in the dark.

**Inocula preparation.** After incubation, suspensions were taken and shaken well using the vortex then diluted for standardizing. Inocula were set to 0.5 McFarland or an optical density from 0.08 to 0.13 at 625 nm wavelength corresponding to $10^8$ CFU/ml [15].

**Disc diffusion method.** This test was performed by the method of Hayes and Markovic (2002) with some modifications [16]. Indeed, four filter paper discs (Whatman disc, 6 mm diameter) are impregnated with 15 µl of the EO and placed onto the inoculated Petri dishes containing Mueller Hinton Agar. After incubation at 37°C during 24 h for bacteria and at 28 °C during 48 h for yeast, the diameters of inhibition zones were measured in mm. A disc of sterile distilled water was used as negative control, and Ciprofloxacin (5 µg) and Amphotericin B (100 µg) were used as positive controls for bacteria and yeast, respectively. Each test was performed in triplicate on at least three separate experiments.

**Minimum inhibitory concentration (MIC) assays.** The MIC assays were determined by the liquid macro-dilution method described by Wiegand et al., (2008) with slight modifications [17]. 400 µl of EO tested are placed in a sterile tube containing 4.6 ml of liquid medium (MHB for bacteria and SDB for yeast) supplemented with Tween 80 (0.01%, v/v). A serial dilution was performed to have solutions concentrations ranging from 80-0.3 mg $ml^{-1}$. The inoculum to be tested (13 µl, $10^8$ CFU/ml) was added to each of the test tubes which were incubated at 37 °C during 24 h for bacteria and at 28 °C during 48 h for yeast. When incubation was completed, all tubes were centrifuged at 5000 rpm for five minutes. All determinations were performed in triplicate and a negative control, consisting of MHB with 0.01% (v/v) tween 80, was included. The MIC is defined as the lowest antimicrobial concentration that will inhibit the visible growth of a microorganism after a period of incubation.

**Determination of minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC).** The MBC and MFC were determined for each of the extracts by sub-culturing the media from each tube showing no visible growth onto MHA for bacteria and SDA plates for yeast. The MBC/MFC was defined as the concentrations required for killing 99.9% of the cells [18]. The solutions from which no deposit was obtained after centrifugation were used to determine the MBC (MFC) [16]. Briefly, after homogenization, a loop (≈ 8-10 µl) of the suspension was lawn on agar (MHA for bacteria and SDA for yeast). This culture was incubated aerobically at 37 °C during 24 h for bacteria and at 28 °C during 48 h for yeast. The experiment was repeated three times. Thus, bactericidal (fungicidal) or bacteriostatic (fungistatic) effect of the EO was determined by the ratio MBC (MFC)/MIC. An antibacterial product is considered as bactericidal if MBC/MIC $\leq$ 4 and bacteriostatic if MBC/MIC > 4 [19].

## RESULTS AND DISCUSSION

### Essential Oil Analysis

The analysis of the *P. mauritanica* EO was carried out by GC-RI and GC-MS, using the methodologies described in Section 2. A total of twenty-five components, representing 94.3% of the total oil content, were identified by comparison of their electron ionization mass spectra (EI-MS) and their retention indices (RI) with those of our authentic compound library (Table 1 and Fig. 2).

The EO was characterized by a large amount of monoterpenic fraction with 92.1% of the total oil, in which the oxygenated monoterpenes account for 91.8%. This fraction was dominated by carvotanacetone 21 accounting for 87.3%. The other two most abundant compounds (≥ 1%) were linalool 10 (1.2%) and carvacrol 26 (1.0%). However, the oxygenated sesquiterpenes (1.9%) were represented only slightly in the EO and the sesquiterpene hydrocarbons were absent. It should be noted that this EO was characterized by the presence of four other oxygenated compounds: 3, 27, 30 and 32 with a percentage 1.1%.

These results were in agreement with those previously reported in the literature referring to carvotanacetone as the major component of EO of *P. mauritanica* growing wild in Morocco and Algeria. Indeed, the GC-MS analysis of EO from aerial parts of *P. mauritanica* collected in another site of Errachidia revealed the identification of 22 compounds representing 91.56% of the total oil, in which carvotanacetone is the major compound with 55.12% [20]. Besides, 22 compounds, representing 91.56% of the total oil, were identified using GC-MS in EO from aerial parts of *P. mauritanica* collected in the oasis of Tata in the south-east of Morocco. The most abundant components were carvotanacetone (55.12%) [21]. Moreover, the GC-MS analysis of aerial part EO of *P. mauritanica* from Algerian Sahara revealed fifteen compounds representing 97.0% of the total oil containing carvotanacetone (89.2 %) [22]. Also, the analysis by GC (RI) and $^{13}C$ NMR of thirty sex EO samples from aerial parts of *P. mauritanica* harvested at two flowering periods in three locations from Western Algeria revealed that carvotanacetone was the most abundant compound with 89.2-96.1% [23]. However, the analysis of root EO of *P. mauritanica* collected from western Algeria allowed the identification of 38 compounds accounting for 90.4% of the whole composition. The oil composition was dominated by thymyl derivatives (2,5-dimethoxy-p-cymene (37.2%), 6-methoxythymyl isobutyrate (14.2%), 10-isobutyryloxy-8,9-dehydrothymyl isobutyrate (4.8%) and thymyl isobutyrate (3.1%)) [24].

### Volatile Fraction by HS-SPME

The optimization of the HS-SPME sampling parameters was carried out using the aerial parts of *P. mauritanica,* based on the sum of the total peak areas obtained by GC-FID. The maximum sum of the total peak area was obtained at a temperature of 70 °C, an equilibrium time of 60 min, and an extraction time of 30 min. The sum of the total peak area increased according to the increase in the temperature until 70 °C. These results were in agreement with those recently reported for the volatile components of *salvia aucheri mesatlantica* [25]. The GC-RI and GC-MS analyses allowed the identification of 29 components, representing 82.2% of the total VF composition (Table 1 and Fig. 3).

The VF obtained was characterized by a large amount of oxygenated monoterpenes which amounted to 67.1% and the amount of oxygenated sesquiterpenes was 10.6%. In this analysis (HS-SPME), the carvotanacetone 21 (44.3%) was always the major compound of the VF followed by 2-methoxy-4-ethyl-6-methylphenol 36 (11.2%) and 6-oxo-cyclonerolidol 37 (9.4%). The other components present at amounts above 1% were menthone 13 (3.1%), carvomenthone 18 (1.7%), carvenone 22 (1.3%) and carvacrol 26 (1.8).

### Comparison of Two Methods

For comparison purposes, differences found in volatile compounds of plants isolated with HS-SPME and conventional methods such as HD are reported in the literature. Sometimes HS-SPME provides a larger scope of compounds [26]. In other cases, HS-SPME detects a lesser amount of compounds [27] and in some other cases, differences found are quantitative but not qualitative [28]. In fact, 25 compounds were characterized in EO extracted by HD, representing 94.4% of the total oil, while 29 volatile constituents have been detected by HS-SPME, accounting for 82.2% of the total VF. Moreover, the data summarized in Table 1, show some quantitative and semi qualitative differences between the chemical composition of both

**Table 1.** Chemical Composition of EO and VF for *P. mauritanica*

| N° [a] | Components | RIa[b] | RIp[c] | %HD[d] [11] | %SPME[e] |
|---|---|---|---|---|---|
| 1 | Hexanal | 761 | | | 0.1 |
| 2 | E-2-Hexenal | 817 | | | 0.3 |
| 3 | Isobutyl isobutyrate | 900 | 1083 | 0.1 | |
| 4 | *α*-Pinene | 930 | 1021 | 0.1 | |
| 5 | *β*-Pinene | 971 | 1103 | 0.1 | |
| 6 | *para*-Cymene | 1012 | 1253 | 0.1 | 0.2 |
| 7 | 1.8-Cineol | 1021 | 1202 | 0.2 | 0.2 |
| 8 | P-Cymenene | 1069 | 1380 | | 0.2 |
| 9 | Nonanal | 1081 | 1343 | | 0.2 |
| 10 | Linalol | 1084 | 1526 | 1.2 | 0.3 |
| 11 | Camphor | 1121 | 1490 | 0.5 | 0.2 |
| 12 | *(Z)*-Verbenol* | 1129 | 1656 | 0.2 | |
| 13 | Menthone* | 1129 | 1459 | | 3.1 |
| 14 | Isomenthone | 1137 | 1479 | | 0.7 |
| 15 | Borneol | 1150 | 1675 | 0.2 | 0.8 |
| 16 | Terpinen-4-ol | 1162 | 1579 | 0.2 | |
| 17 | *para*-Cymen-8-ol | 1165 | 1818 | 0.6 | |
| 18 | Carvomenthone | 1171 | 1498 | | 1.7 |
| 19 | Decanal | 1191 | 1478 | 0.2 | |
| 20 | Pulegone | 1214 | 1632 | | 0.7 |
| 21 | Carvotanacetone | 1230 | 1656 | 87.3 | 44.3 |
| 22 | Carvenone | 1233 | 1685 | 0.2 | 1.3 |
| 23 | *cis*-Chrysanthenyl acetate | 1242 | 1548 | | 0.1 |
| 24 | Ascaridol | 1254 | 1712 | | 0.5 |
| 25 | Thymol | 1272 | 2145 | 0.3 | 0.2 |
| 26 | Carvacrol | 1281 | 2171 | 1 | 1.8 |
| 27 | Dihydroedulan | 1284 | 1472 | 0.2 | |
| 28 | a-Copaene | 1368 | 1485 | | 0.5 |

**Table 1.** Continued

| | | | | | |
|---|---|---|---|---|---|
| 29 | b-Bourbonene | 1374 | 1496 | | 0.3 |
| 30 | 2.5-Dimethoxycymene | 1400 | 1835 | 0.7 | |
| 31 | *trans*-Caryophyllene | 1406 | 1419 | | 0.7 |
| 32 | Geranyl acetone | 1428 | 1826 | 0.1 | |
| 33 | b-Bisabolene | 1498 | 1710 | | 0.8 |
| 34 | g-Cadinene | 1502 | 1765 | | 0.5 |
| 35 | d-Cadinene | 1508 | 1744 | | 0.7 |
| 36 | 2-Methoxy-4-ethyl-6-methylphenol | 1540 | | | 11.2 |
| 37 | 6-oxo-cyclonerolidol | 1546 | 1954 | 0.5 | 9.4 |
| 38 | Caryophyllene oxyde | 1567 | 1945 | 0.6 | |
| 39 | τ-Cadinol | 1624 | 2133 | 0.1 | 0.9 |
| 40 | β-Eudesmol | 1633 | 2236 | 0.2 | 0.3 |
| 41 | α-Cadinol | 1637 | 2194 | 0.2 | |
| 42 | *(E)*-Phytol | 2098 | 2586 | 0.2 | |
| | Total | | | 94.4 | 82.2 |
| | Monoterpene hydrocarbons | | | 0.3 | 0.5 |
| | Sesquiterpene hydrocarbons | | | 0 | 3.5 |
| | Oxygenated monoterpenes | | | 91.9 | 67.1 |
| | Oxygenated sesquiterpenes | | | 1.9 | 10.6 |
| | Others | | | 1.1 | 0.5 |

[a]The numbering refers to elution order on apolar column (Rtx-1). [b]RIa = retention indices measured on the apolar column (Rtx-1). [c] RIp = retention indices measured on the polar column (Rtx-Wax). [d]% = relative percentages of components are given on the apolar column except for components with an asterisk (*) (percentages are given on the polar column). [e]% = relative percentages of components obtained by GC-FID (on RTX-1: apolar column) with peak-area normalization under optimized HS-SPME parameters: temperature: 70 °C; equilibrium time: 90 min; extraction time: 30 min.

analyzed samples. The current investigation revealed that higher amounts of oxygenated monoterpenes are found in EO obtained by HD (91.9%) as compared to the HS-SPME (67.1%) while, oxygenated sesquiterpenes were detected in lower concentrations in the hydro-distilled oil as compared to the HS-SPME (1.9% *vs* 10.6%, respectively). Also, we noted the absence of sesquiterpene hydrocarbons in EO, while they were isolated by HS-SPME with an appreciable amount of 3.5%. Carvotanacetone 21 was found to be the principal constituent with approximately double concentrations in the EO than HS-SPME (87.3 and 44.3%, respectively). It should be noted that among the 29

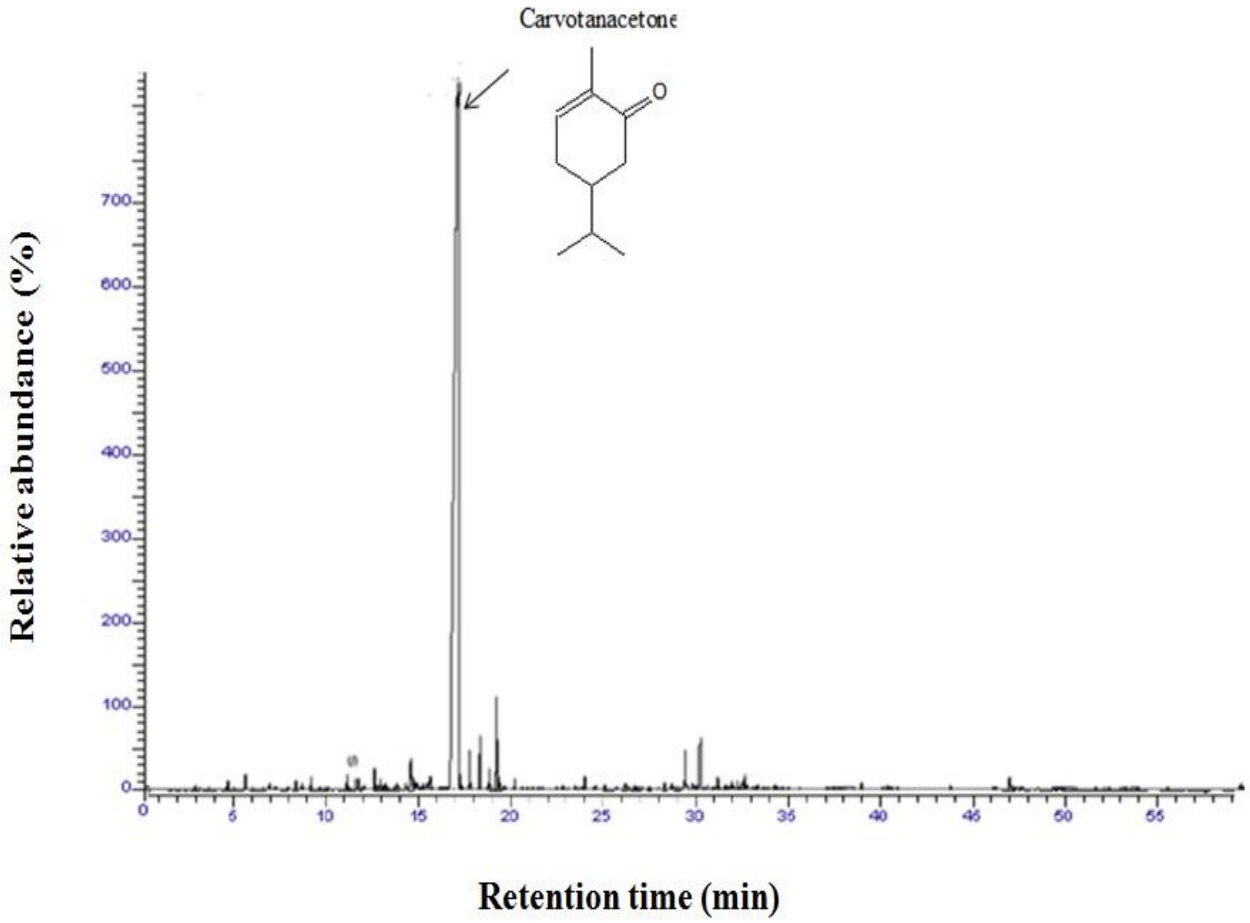


**Fig. 2.** Chromatographic profile of the aerial parts of EO for *P. mauritanica* obtained by HD. The separation was carried out on apolar column (Rtx-1).

**Table 2.** Comparison of HS-SPME and HD for Separation of the Volatile Components of *P. mouritanica*

| Characteristic | HS-SPME | HD |
|---|---|---|
| Amount of sample required (g) | 1.2 | 100 |
| Extraction time (min) | 30 | 180 |
| Extraction temperature (°C) | 70 | ~ 100 |
| Separation time by GC-MS (min) | 5 | 35 |
| Major compound identified | Carvotanacetone (44.3%) | Carvotanacetone (87.3%) |
| Total number of components identified | 29 (82.2%) | 25 (94.4%) |

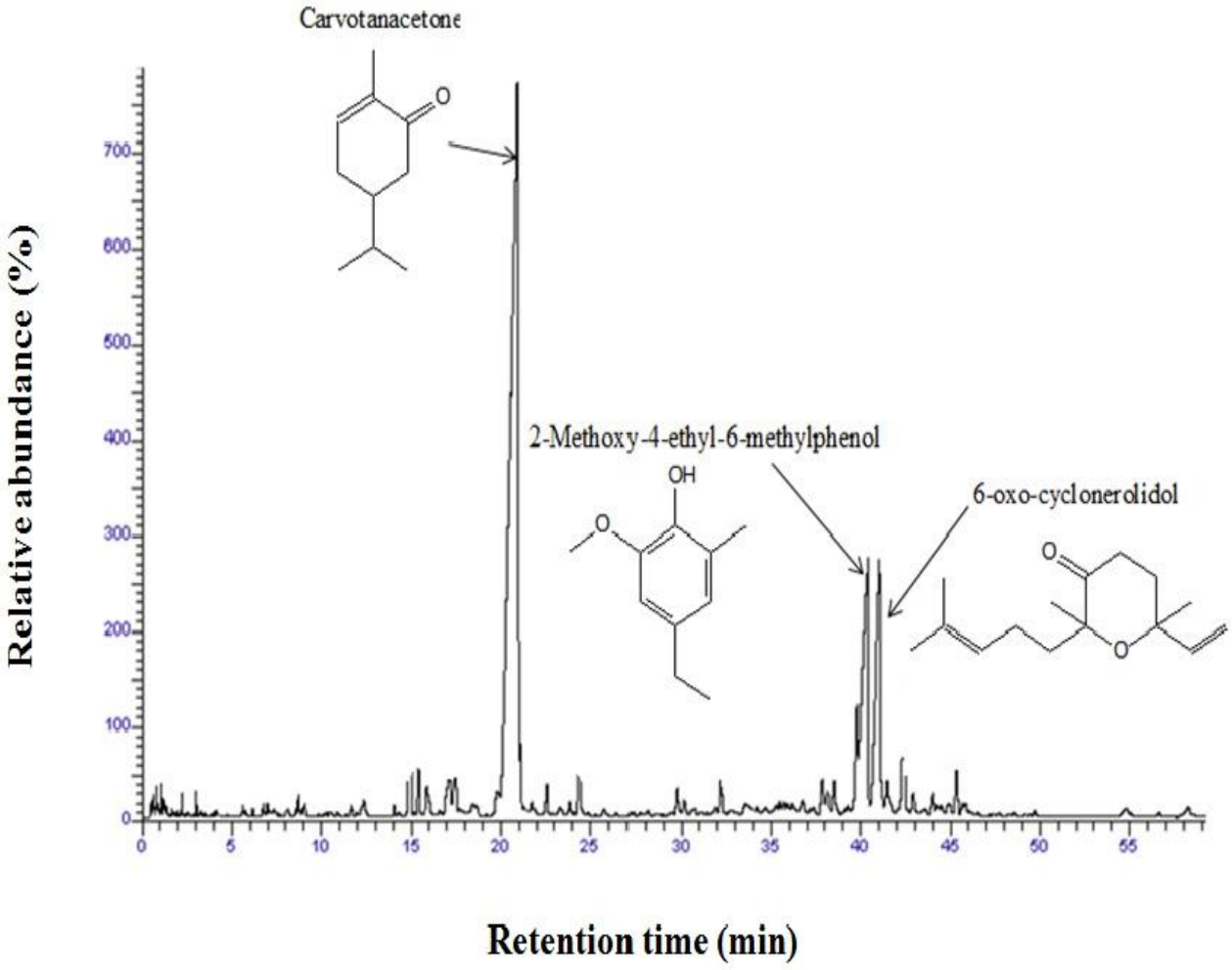


**Fig. 3.** Chromatographic profile of VF from aerial parts of *P. mauritanica* detected by HS-SPME. The separation was carried out on apolar column (Rtx-1).

**Table 3.** Antimicrobial Activity of *P. mauritanica* EO Expressed by the Diameter Inhibition Zones and MIC Methods

| | Diameter inhibition zones (mm) | | | |
|---|---|---|---|---|
| Microbial strains | 15 µl of EO | 10 µg of Ciprofloxacin | 100 µg of Amphotericin B | MIC (mg $ml^{-1}$) |
| *E. coli* (*Gram*-) | 25 | 41 | - | 0.3 |
| *P. aeruginosa* (*Gram*-) | na | 27 | nt | nt |
| *S. aureus* (*Gram* +) | 35 | 45 | nt | 0.6 |
| *C. albicans* (*yeast*) | 23 | nt | 26 | 0.6 |

(nt): not tested. (na): not activated.

compounds previously detected in the HS-SPME, only 12 of them were identified in the EO. Conversely, 13 components identified in the EO detected were absents in HS-SPME. Indeed, the main difference was found to be the relative percentage (11.2%) of 2-methoxy 4-ethyl-6-methylphenol in HS-SPME (not found in the EO) and 6-oxo-cyclonerolidol (0.5% for HD *vs* 9.4% for HS-SPME).

In general, it was difficult to establish a direct correlation between the chemical compositions of HD and HS-SPME techniques since the first technique is based on the liquid quasi-total extraction of plant volatiles and the latter technique is controlled by a solid/gas equilibrium step and a competition between interfering molecules at binding sites on the fiber. However, the presence and/or absence of certain compounds in the two samples can be explained also by the influence of the temperature and the extraction time (Table 2). Indeed, with HS-SPME extraction at 70 °C for 30 min, it is the fiber affinity of each compound that monitors the sampling of the volatiles limiting or favoring their extraction. Normally, the quantities of low boiling and high volatility compounds could be extracted by HS-SPME. However, during HD (180 min at 100 °C), the most volatile compounds and water-soluble compounds are lost in the gaseous phase and the hydrosol under the effect of heat and acid pH, respectively. Also, some of the fragile and thermosensitive constituents may get decomposed resulting in artifacts due to heating. In the same way, the amount of plant material used for sample preparation might probably be one of the major reasons which explain the difference between chemical HS-SPME and HD data. Indeed, the amount of plant material used for the HS-SPME analysis was smaller (1.2 g), while the production of hydrodistilled EO needed the use of 100 g of plant material (Table 2). HS-SPME analysis allowed a qualitative estimate of volatile compounds using a small quantity of material [29].

## Evaluation of the Antimicrobial Activity

The antimicrobial activity of *P. mauritanica* EO was studied *in vitro* against three reference bacterial strains: *Escherichia coli (Gram-: ATCC 25922), Staphylococcus aureus (Gram +: ATCC 25923)* and *Pseudomonas aeruginosa (Gram-: ATCC 27853)* and a yeast strain *Candida albicans* using the paper disc diffusion method and MIC assays. These methods are based on measuring the inhibition zone diameter and the determination of the Minimum Inhibitory Concentration (MIC), respectively. The results are summarized in Table 3.

Using disc diffusion method, it has been reported that according to the width of the inhibition zone diameter expressed in mm, the sensitivity of a given strain to an EO or a molecule varies as follows: not sensitive (-) to the diameter equal to or below 8.0 mm, moderately sensitive (+) to the diameter between 8.0 and 14.0 mm, sensitive (++) to the diameter between 14.0 and 20.0 mm, and extremely sensitive (+++) to the diameter ≥ than 20.0 mm [30]. Thus, the EO tested exhibited a stronger antimicrobial activity on the germs tested, but lower in comparison with the control negatives. The largest diameter was observed with *S. aureus* with 35 mm followed by *E. coli* and *C. albicans* with diameters of 25 and 23 mm, respectively. In contrast, *P. aeruginosa* was the most resistant strain to the oil because no inhibition has been detected. The inhibition zones were less potent than standard antimicrobial drugs, Ciprofloxacine and Amphotericin B. In addition, our sample was more effective than those reported previously in the literature. Indeed, using the agar diffusion method, Bammou *et al.* reported that 15 µl of EO tested showed an inhibition zone of 23 and 18 mm against *E. coli* and *S. aureus*, respectively [20]. Likewise, Gherib *el al.* (2016) reported that the 15 µl/disc EO was active against *S. aureus*, *E. coli*, and *C. albicans* with diameters of inhibition 18, 10.06 and 20.3 mm, respectively [23].

Besides, using the MIC assay, it was reported that according to the MIC values expressed in mg $ml^{-1}$, the sensitivity of a given strain to an EO or a molecule varies as follows: not sensitive (-) to the values between 50.0 or above 25.0 mg $ml^{-1}$, moderately sensitive (+) to the values between 12.5 and 3.0 mg $ml^{-1}$, sensitive (++) to the values between 2 and 0.4 mg $ml^{-1}$ and extremely sensitive (+++) to the values between 0.2 and 1 mg $ml^{-1}$ [30,31]. Thus, all strains tested were extremely sensitive to EO tested. Maximum activity was observed against *E. coli* (MIC = 0.3 mg $ml^{-1}$) followed by *S. aureus* and *C. albicans* with a MIC of 0.6 mg $ml^{-1}$. However, the same profile as a disc diffusion method was observed for the *P. aeruginosa*, appeared as the most resistant to this EO. Compared to the results previously cited using MIC assay, our sample is more efficient than that reported by Gherib *et al.* [23] and

**Table 4.** Determination of Ratio MBC (MFC)/MIC

| Microbial strains | MBC (MFC) (mg $ml^{-1}$) | MIC (mg $ml^{-1}$) | MBC (MFC)/MIC |
|---|---|---|---|
| *E. coli* (*Gram*-) | 2.5 | 0.3 | 8.33 |
| *P. aeruginosa* (*Gram*-) | na | nt | nt- |
| *S. aureus* (*Gram* +) | 1.25 | 0.6 | 2.08 |
| *C. albicans* | 5 | 0.6 | 8.33 |

(nt): not tested.

slightly less than that reported by Bammou *el al*. [20]

To clarify the bacteriostatic or bactericidal nature of the EO studied, we determined the ratio of MBC to MIC (MBC/MIC). An antibacterial product is considered as a bactericidal product if MBC/MIC ≤ 4 and bacteriostatic if MBC/MIC > 4 [19]. The results are indicated in Table 4.

The MBC (MFC)/MIC ratios of the *P. mauritanica* EO are equal to 8.33 for *E. coli* and *C. albicans*, indicating that it is bacteriostatic and fungistatic towards these two microbial strains, respectively. However, the CMB/MIC ratio is equal to 2.08 for *S. aureus*, indicating the bactericidal character of EO against this bacteria. Regarding the bactericidal activity, Gram (+) bacteria were more susceptible than Gram (-) ones, and no bactericidal effect was observed against *P. aeruginosa.* In general, the EOs were therefore active against Gram + bacteria having a structure more susceptible to these EOs. However, no effects were detected in *P. aeruginosa*, a Gram - bacteria. This latter is known for its resistance to any kind of antimicrobial agents which is directly related to the structure of its cell wall which constitutes an effective permeability barrier making it less sensitive to the action of EO [32].

Generally, the antimicrobial activity of the EOs is attributed to their lipophilic properties and the strength of their functional groups. This activity is due to the major compounds, or those susceptible to be active. However, the minor compounds would act with a synergistic effect [5]. In the present study, *P. mauritanica* EO showed antimicrobial activity against the tested microorganisms. This activity is related to its high levels of oxygenated monoterpenes, especially its major constituent, carvotanacetone 21 (87.3%). Previous studies of the antimicrobial activity of other *Pulicaria* species with carvotancetone as the major component have been reported. Indeed, *P. undulata* EO of Yemen origin, with carvotancetone constituting 91.4%, produces inhibition diameters of 16 mm, 32 mm and 26 mm against *E. coli*, *S. aureus*, and *C. albicans*, respectively, while *P. aeruginosa (Gram-)* is still the most resistant strain [33]. Similarly, *P. jaubertii* flower EO from southern Yemen, dominated by carvotanacetone (93.5%), inhibits the growth of *E. coli*, *S. aureus* and *C. albicans* with inhibition diameters of 15, 30 and 25 mm and MICs of the order of 1, 0.25 and 0.3 mg $ml^{-1}$, respectively [34]. Otherwise, the EO of the leaves of *P. jaubettri* originating from Saudi Arabia whose carvotancetone constitutes 98.59% presents a MIC value of the order of 1 mg $ml^{-1}$ towards *S. aureus* and *C. albicans*, whereas *E. coli* (*Gram*-) exhibits resistance even at 2 mg $ml^{-1}$ [35]. Furthermore, the bioactivity of monoterpenoid ketones derived from para-menthane such as pulegone, carvone, piperitone, and piperitenone has been extensively studied and demonstrated against several microorganisms [36-38]. The activity of these oxygenated monoterpenes results from their interference with enzymatic reactions during cell-wall synthesis, causing changes in cell permeability by disrupting lipid packing and changes to membrane properties and functions [39]. However, the presence of two phenolic isomers, thymol 25 and carvacrol 26, even as minor constituents (0.3% and 0.9%, respectively), may contribute to the antifungal activity of *Pulicaria* EO, involving some type of synergism. Generally, the phenolic compounds in EOs are reported to be

predominantly responsible for their biological properties [40]. The antimicrobial activity of these phenolic compounds can be attributed to the presence of an aromatic nucleus and a phenolic OH group, which are known to be reactive and can form hydrogen bonds with -SH groups at the active sites of the target enzymes resulting in the deactivation of the microbial enzymes [40].

## CONCLUSIONS

Volatiles isolated from *P. mauritanica* by HS-SPME and hydrodistillation were investigated using GC-RI and GC-MS. EO and VF were mainly composed of oxygenated monoterpenes and dominated by carvotanacetone. Moreover, the study of the volatiles sampled by two techniques showed quantitative and semi qualitative differences between the chemical compositions of both analyzed samples. The main difference was found to be the relative percentage of 2-methoxy 4-ethyl 6-methylphenol in HS-SPME (not found in the EO) and 6-oxo-cyclonerolidol (0.5% for HD *vs*. 9.4 for HS-SPME). It is interesting to note that the sample preparation method impacted quantitatively on the GC profile of *P. mauritanica* volatiles. The antimicrobial properties of *P. mauritanica* EO tested against three bacterial strains and one yeast showed that oil expresses a bactericidal effect against *S. aureus* and a bacteriostatic and fungistatic effect against *E. coli* and *C. albicans*, respectively. This activity can probably be attributed to the richness of the oil in carvotanacetone (87.3%). All the results obtained may suggest that *P. mauritanica* EO could be a viable alternative to synthetic antimicrobial agents in fighting against the recurring problem of microbial resistance.

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