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Chemical Composition and Antimicrobial Activity of Essential Oil of Wild and Cultivated *Rosmarinus Officinalis* from Two Moroccan Localities

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ABSTRACT

This research aimed to measure the impact of provenance on the yield, chemical profile and antimicrobial activity of *Rosmarinus officinalis* essential oil (EO) collected from cultivated and wild plants from two different regions in Morocco (Fez and Figuig). The chemical composition analysis was accomplished utilizing the GC-MS techniques. Sixteen compounds were identified in the EO of both samples, dominated by 1,8-cineole (32.18%), camphor (16.20%) and α-pinene (15.40%) in cultivated type. The α-pinene (51.19) presents the majority compound in the rosemary samples collected from the wild populations.The antimicrobial activity was investigated by using the broth dilution methods against yeast, four bacterial strains and two molds. Rosemary aerial part provided EO with the highest yield and comes from Figuig. The GC-MS analysis demonstrated the existence of two chemotypes of oils: α-pinene as well as 1,8-cineole/camphor/α-pinene. Both EOs showed good antimicrobial activity against all microbial strains. The essential oil produced by Fez plants was the most effective against the selected microorganisms having MIC values of 0.315–2.5 mg/L.

Keywords: essential oil, *Rosmarinus officinalis*, antimicrobial activity, chemotype.

INTRODUCTION

Essential oils (EOs) are volatile combination of organic compounds acquiring oily consistency, usually generated by plants. They are synthesized by all plant parts comprisingseeds, leaves, flowers, roots, peel, stem, bark and fruits, and their storage can be accomplished in different locations, including secretory and epidermic cells, glandular trichomes, canals, and cavities (Bakkali et al., 2008). Numerous extraction approaches, including distillation (steam, water, and microwave-assisted), extraction (organic solvent, supercritical CO_2, ultrasonic, and solvent-free microwave), as well as microwave hydrodiffusion and gravity, were employed for extracting EOs from various aromatic plant sections (Stratakos and Koidis, 2016).

In general, the chemical constituents of essentials oils are classified into two different groups; hydrocarbons that are almost entirely composed of terpenes (monoterpenes, diterpenes, and sesquiterpenes), as well asoxygenated terpenoids,typicallyalcohols, phenols, aldehydes, ketones, esters and oxides (Moghaddam and Mehdizadeh, 2017).

Essentials oils and their phyto-constituents exhibit several biological functions such as antibacterial, antiviral, insecticidal as well as antifungal activities. Given their hydrophobicity, EOs and their constituents can penetrate cell membrane and mitochondria lipid components

causing cell structure disturbance and contributing to a high permeability and leakage of essential molecules and ions, eventually causing cell death (Dhifi et al., 2016; Wani et al., 2021).

Rosmarins officinalis L. (Family Lamiaceae) is an evergreen shrub prevalently recognized as rosemary, native to the Mediterranean region and commonly grown world wide as an ornamental, aromatic and medicinal plant and can be deployed in traditional as well as complementary alternative medicine as an analgesic, anti-inflammatory, antirheumatic, antispasmodic in renal colic and dysmenorrhoea, carminative and choleretic (Hamidpour et al., 2017). Such plant species possess several additional advantageous activities, comprising antidiabetic, anticarcinogenic (Hamidpour et al., 2017), antinociceptive (González-Trujano et al., 2007), in addition to antitumorigenic (González-Vallinas et al., 2015). The European Union (EU) has actually endorsed rosemary extract (E392) to be a safer and efficient natural anti-oxidant towards food conservation (EFSA, 2015).

Rosemary essential oils demonstrated strong changes in their biological as well as anti-oxidant activity, mainly associated with chemical composition differences. The varying qualitative and quantitative chemical composition of EO originates from its geographical origin (Celiktas et al., 2007; Gohari et al., 2009), environmental, in addition to agronomic settings (Yeddes et al., 2018), growth stages and harvest time (Hassanzadeh et al., 2017; Salido et al., 2003). Definitely, 13 various rosemary oil chemotypes were recognized, dependingon essential oil compositions comprising: 1,8-cineole, camphor, verbenone, α-pinene, bornyl acetate, myrcene, in addition to 1,8-cineole with camphor or α-pinene in equivalent quantities (Satyal et al., 2017).

In the present work, the authors aimed to study the chemical composition and antimicrobial activities of two essential oil chemotypes of *R. officinalis* growing under different climatic conditions in Morocco; one wild, growing spontaneously in the region of Figuig, and another cultivated in the city of Fez.

MATERIALS AND METHODS

Plant material

The fresh aerial parts of rosemary were harvested at the flowering stage during May 2018 in two different locations. Wild aerial components as well as cultivated *R. officinalis*were harvestedin May 2018 from two different locations. First, the wild rosemary samples were taken from the province of Figuig (1344 m, 32°31’28.5’’ Latitude North and 3°47’29.4’’ Longitude West) with a semi-arid climate, distinguished by low precipitation (annual average pluviometry oscillates in the range of 25–150 mm). Second, the cultivated rosemary samples were collected from Saïs located in the city of Fez (406m, 34°01’59’’ Latitude North and 5°00’01’’ Longitude West) characterized by a semi-arid climate of 5–6 months dry season and a mean yearly rainfall (<500 mm). Room-temperature air-dryness of plants was conducted during seven days in a shady place.

Isolation and GC-MS of essential oil

Microwave Assisted Hydrodistillation was carried out with a microwave oven (MWD 119 WH, whirpool, China, 20L, 2.45 GHz) coupled to a Clevenger appliance and a cooling system to continually condense the distillate. The microwave oven consumes 1100 watts of power and produces 700 watts of output power when powered by a 230v–50Hz power source. Its cavity measures 216 x 302 x 277 mm.

Microwave-assisted hydrodistillation (MAHD) was accomplished under optimum settings, comprising time of extraction, microwave power, as well as ratio water/plant material (Elyemni et al., 2020). The dried leaves and and flowering tops were separately subjected to MAHD for 20 min at 600 W microwave power and 2 mL/g water-to-plant ratio.

Under experimental conditions as previously stated, the chemical ingredients of extracted EOs were calculated by means of GC/MS (gas chromatography coupled with mass spectrometry) (Elyemni et al., 2019). A comparative study between the EOs extracted by MAHD and those obtained by conventional hydrodistillation has already been reported before (Elyemni et al., 2019).

Microbial strains

Eight microbial organisms were subjected to antimicrobial activities of rosemary EO, comprising Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923 as well as *Bacillus subtilis* ATCC 6633), Gram-negative bacteria (*Escherichia coli* ATCC 8739 as well as *Pseudomonas aeruginosa* ATCC 9027), a yeast (*Candida albicans* 90028

ATCC) in addition to two molds (*Aspergillus niger* and *alternaria alternata*).The molds used in this study were isolated from rotten apple fruits.

The stock strains of Tryptic Soy Broth (TSB) were maintained at –80°C with 20% v/v glycerol supplementing all bacterial strains before use, while Sabouraud Dextrose Agar slant at 4°C has preserved fungi including yeasts.

Inoculums preparation

The bacterial strains were revived by streaking the frozen culture onto tryptic soy agar plates, and following by one-day incubation at 37°C, and several colonies produced on surface were suspended in tryptic soy broth, followed by overnight incubation at 37°C. The overnight culture (100 µL) was then transferred into TSB and incubated at the same temperature for 2 h to obtain bacteria in an exponential growth phase. The cultures were diluted and adjusted in order to achieve a density of 1.5×10^8 CFU/mL (0.5 McFarland turbidity standards).

The cultures of the molds (*Aspergillus niger* and *Alternaria alternata*) were initially grown on Sabouraud dextrose agar at 28°C for 7 days, and then the conidia were recovered by flooding the surface of the medium with sterile distilled water containing 0.1% Tween 80 and gently shaking the plate to dislodge the spores. The spores concentrations were determined by hemocytometer counting and adjusted with sterile saline to 5×10^4 conidia/mL.

Revivification of *candida albican* was made by subcultures in Sabouraud Dextrose plates at 25°C for 7 days. After preparing the stock inoculums through suspending three yeast colonies in five milliliters of sterile 0.85% NaCl and adjusting its concentration to $1–5\times10^6$ cells/mL using a hemocytometer.

Antimicrobial activities

Minimum inhibitory and minimum bactericidal concentrations (MIC and MBC)

For each EO, the MIC values were determined to wards bacterial strains examined using the broth microdilution method accomplished in 96-well microplates (Güllüce et al., 2003) by means of resazurin as an indicator of viability (Mann and Markham, 1998).

Dissolved in dimethylsulfoxide (DMSO, 10%), each essential oil was first subjected to dilution to 50 mg/mL for testing, followed by serial two-fold dilutions from 0.31–10 mg/mL for oils in sterile test tubes (5 mL) comprising nutrient broth.

A sterile 96-well microplate was formed through dispersing nutrient broth (95 µL) as well as bacterial inoculum (5 µL) into each well. One hundred µL of maximum oil dilution was transferred to the first wells. After that, 100 µL from each dilution were added to consecutive wells. Uninoculated medium (200 µL of TSB supplemented with 1% DMSO) was involved as a sterility control. Besides, growth controls were applied (195 µL of nutrient broth supplemented with 1% DMSO and 5 µL of bacterial inoculums without essential oil). Each well growth was compared with that of control well, and sealed (parafilm) plates were subjected to one-day incubation at 37°C.

For MIC determination, resazurin (30 µL, 0.02% (w/v)) was transferred to each well, followed by 2–4 hoursof further incubation. Bacterial growth was indicated through resazurin reduction (blue/purple) to resorufin (pink), and the lowest EO concentrations indicate that growth inhibition was recorded as the MIC value.

For MBC determination, 10 µL was removed from each culture medium of each well without apparent growth and inoculated in TSB plates. Following one-day incubation at 37°C, MBC was determined as the lowest essential oil concentration without observed bacterial development. All experiments were repeated three times, and the collected data were displayed as mean values ± standard deviation.

Minimum fungal concentrations (MFC)

Antifungal activities were evaluated using the broth microdilution method based on Clinical and Laboratory Standards Institute (CLSI) reference documents; M27-A3(CLSI, 2008a) and M38-A2(CLSI, 2008b), for yeast and filamentous fungi, respectively.

Serial EO dilutions (0.08–10 mg/mL) in 96-well microtiter were prepared as described for the antibacterial activity in microtitre trays using Roswell park memorial institute (RPMI) 1640 medium (with l-glutamine, without sodium bicarbonate) buffered at pH 7.0 with 0.165 mol 3-(N-morpholino) propane sulfonic acid (MOPS), with a maximum DMSO concentration (1%, v/v).

Fungal inoculum in a volume of 100 µL was added to each well, and the microplates were incubated for 48 h at 35°C for *Candida albican* and for 7 days at 28°C for *A.niger*as well as *A.alternata*. Sterility and growth controls in RPMI 1640 medium alone or with DMSO (1%, v/v) were included in each treatment. After incubation, MICs were determined visually as recommended by CLSI. A 10 µL aliquot from each negative well was transferred to Sabouraud dextrose agar plates, and minimum fungicidal concentrations were assessed after 72 hours of incubation at 28°C.

RESULTS AND DISCUSSION

Yield and chemical composition

The EOs isolated using microwave-assisted hydrodistillation for both regions was light yellow, with a yield of 1.35 ± 0.04% w/w based on dry sample weight for the samples originating from Fez and 2.24 ± 0.05% w/w for those from Figuig.

In fact, several studies show varying essential oil yield of rosemary according to geographical plant origin. Angioni et al. (2004) indicated variations of Sardinian rosemary essential oil yield collected from various natural stations. The yields produced by the northern as well as eastern samples were almost twice higher than the southern and central ones. Ben Abada et al. (2020) also revealed a variation in Tunisian rosemary essential oil yields collected from eight different collection sites, the yields ranged from 1.13% (Cap Zbib) to 1.69% (Thala).

Table 1 lists the chemical composition and Kovats' indices of the rosemary EO collected from two different Moroccan regions and obtained by microwave-assisted hydrodistillation.

Regarding the volatile characteristics of essential oils, chromatographic analysis can identify 16 main components, representing 99.75 and 99.86% of rosemary from Fez and Figuig, respectively.

The oil extracted from cultivated plants (Fez) contained the following major constituents: 1,8-cineole (32.18%), camphor (16.20%), α-pinene (15.40%), camphene (9.16%), in addition to α-terpineol (7.36%), as shown in Table 1. This oil could be classified as 1,8-cineole/camphor/ α-pinene chemotype (chemotype I). The oil

Table 1. Chemical composition of essential oils of the two chemotypes of rosemary obtained by MAHD

No.	Compounds	Kovat's index	Chemotype I(Fez)	Chemotype II(Figuig)
Monoterpene hydrocarbons			35.84	56.00
1	α-Pinene	939	15.40	51.19
2	Camphene	954	9.16	2.76
3	β-Pinene	979	3.72	0.22
4	α-Terpinene	1017	2.49	0.37
5	para-Cymene	1025	4.15	1.09
6	Limonene	1028	0.92	0.37
Oxygenated monoterpenes			63.03	43.78
7	1.8-Cineole	1030	32.18	28.97
8	β-myrcene	1048	4	2.07
9	Linalool	1097	1.37	0.08
10	Camphor	1146	16.2	10.01
11	Borneol	1169	1.64	0.73
12	α- Terpineol	1199	7.36	1.88
13	Verbenone	1205	0.28	0.04
Sesquiterpene hydrocarbons			0.27	0.08
14	β-Caryophyllene	1419	0.12	0.08
15	α-Caryophyllene	1423	0.15	Tr
Other oxygenated compounds			0.61	
16	Bornyl acetate	1289	0.61	Tr
Total oxygenated compounds			63.64	43.78
Total non-oxygenated compounds			36.11	56.08
Total			99.75	99.86

provided by wild plants (Figuig) is distinguished by its high content of α-pinene (51.19) with relatively considerable amounts of 1,8-cineole (28.97%),camphor (10.01%)as well as camphene (2.76), and can be categorized as α-pinene chemotype (Chemotype II).

Such results agree with the preceding research on Moroccan rosemary. Elamrani and coworkers explored the chemotaxonomy of rosemary EO in various regions (Rabat, Taforalt and Elateuf) and observed three chemotypes: 1,8-cineole (58–63%), camphor (41–53%), as well asα-pinene (37–40%) (Elamrani et al., 2000). Lahlou and coworkers have also indicated in their works the existence of two chemotypes of rosemary; α-pinene chemotype (34.0%) for the sample from Rabat and 1,8 cineol chemotype (40–50%) for the samples from Errachidia and Oujda (Lahlou and Berrada, 2003).

A careful examination of oil compositions demonstrated that the essential oil isolated from cultivated rosemary contains higher oxygenated monoterpenes amounts, involving 1,8-cineole, camphor, borneol, terpene-4-ol, linalool, β-myrcene,α-terpeneol, and verbenone (63.03%) thanthe oil extracted from wild plants (43.78%).

The variability in yield and EO composition between the cultivated and wild *R.officinalis* plants could be mainly attributed to genetic variation and environmental setting, including climate and soil properties. The wild populations do not have fertilization or irrigation, they depend on rain and dew in their water requirements (Ben Jemia et al., 2015; Moretti et al., 1998; Tawfeeq, 2017; Yeddes et al., 2018).

Zaouali and colleagues in 2010 studied the essential oil composition of two varieties of Tunisian *Rosmarinus officinalis* (var. typicus and var. troglodytorum) growing wild in different bioclimates and they found a large variation in the chemical composition of the essential oils attributed to genetic difference, rather than bioclimates (Jiang et al., 2011).

A survey of the literature from different regions in the world reveals a wide change in the chemical constituents of rosemary essential oil. For instance, Algerian rosemary essential oil comprises1,8-cineole (52.4%), followed by camphor (12.6%), then β-pinene (5.7%) as well as α-pinene (5.2%) (Kheiria et al., 2013).

Kheiria and colleagues in 2013 analyzed the essential oils provided by rosemary based on different geographic origins in Tunisia. The essential oil samples mainly comprise1,8-cineol (33.08–37.75%), followed by camphor (13.55–18.13%), then α-pinene (8.58–9.32%) and α-terpineol (6.79–8.17%) (Kheiria et al., 2013).

Pintore and colleagues in 2002 reported α-pinene (20.2%), verbenone (15.7%), camphor (11.5%), 1.8 cineole (11.3%) and bornyl acetate (11.3%) as major constituents in Sardinian rosemary oil, while the rosemary grown in Corsica showed high verbenone (20.3%), bornyl acetate (17.0%), α-pinene (13.7%) and borneol (6.7%) (Pintore et al., 2002).

The Brazilian rosemary leaves provide essential oil that contains 1,8-cineole (52.2%), followed by camphor (15.2%) as well as α-pinene (12.4%) as major constituents (da Silva Bomfim et al., 2015).

Regarding Turkish EOs of *R. officinalis,* it mainly includes p-cymene (44.02%), followed by linalool (20.5%), then γ-terpinene (16.62%), as well as thymol (1.81%) as investigated by Özcan and chalchat,2008 (Özcan and Chalchat, 2008).

The oils extracted from Chinese rosemary leaves, according to GC as well as GC-MS analyses comprise 1,8-cineole (26.54%), followed by α-pinene (20.14%), then camphor (12.88%), camphene (11.38%) as well as β-pinene (6.95%) as major components (Jiang et al., 2011).

In Erbil, Iraq, *Rosmarinus officinalis L.* leaves provide the essential oil composition (GC/MS) including verbenone (23.46%), followed by 1,8-cineol (15.96%), then α-pinene (12.10%), camphor (10.98%), as well as bornyl acetate (5.78%) (Ahamad et al., 2019).

Antibacterial and antifungal activity of rosemary EO

Rosmarinus officinalis oils chemotypes exhibited varying antimicrobial activity towards four bacteria and three fungi species tested, as is shown by the MIC and MBC/MFC values (Table 2).

MICs of cultivated rosemary EOs are in the range of 0.315–2.5 mg/L and those of wild rosemary are between 0.625 and 5 mg/L. MICs, as well as the MBC/MFC values of the two essentials oils were corresponding for *staphylococcus aureus* and *Alternaria alternata*, while chemotypes I show higher antimicrobial activity than chemotype II for all other microorganisms tested.

The difference observed in the antibacterial efficacy of the two chemotypes oils can be correlated with chemical configuration of their components, proportions in which they exist, and their synergistic interactions. Santoyo et

Table 2. Antimicrobial activities of EOs chemotypes of Rosmarinus officinalis

Microorganisms	Chemotype I		Chemotype II	
	MIC(mg/L)	MBC/MFC (mg/L)	MIC (mg/L)	MBC/MFC (mg/L)
Gram positive				
Staphylococcus aureus ATCC 25923	0.625	1.25	0.625	1.25
*Bacillus subtilis*ATCC 6633	0.315	0.625	1.25	2.5
Gram negative				
Escherichia coli ATCC 8739	1.25	2.5	2.5	5
Pseudomonas aerugenosa ATCC 9027	2.5	5	5	>10
Yeast and fungi				
Candida albican 90028 ATCC	0.315	1.25	0.625	1.25
Aspergillusn niger	1.25	2.5	2.5	5
Alternaria alternata	1.25	2.5	1.25	2.5

al. 2005 demonstrated that the EO antimicrobial property of *R. officinalis*is associated with α-pinene, 1,8-cineole, camphor, verbinone in addition to borneol, where borneol exhibited the most potency, then camphor and verbenone (Santoyo et al., 2005). Other researchers have also reported the strong antimicrobial activities of borneol and camphor (Chen et al., 2013; Tabanca et al., 2001).

The higher antimicrobial activity of chemotype I may be also correlated with high percentage of oxygenated monoterpenes in this oil. In fact, previous research exhibited that oxygenated terpenoids, comprising alcoholic as well as phenolic terpenes show case higher antimicrobial activities over hydrocarbons such as *R*-(–)-limonene, terpinene, camphene, in addition to(+)-α-pinene (Bassolé and Juliani, 2012; Koroch et al., 2007). It has been described that, in general, aromatic nuclei with polar functional groups provide the antimicrobial activity for essential oil (Guimarães et al., 2019).

Among different microorganisms screened, *P. aeruginosa* exhibited the least sensitive microorganism to rosemary EO with 2.5 and 5 mg/mL MIC values for chemotype I and chemotype II respectively, and the MBC values of 5 mg/mL for chemotype I and above 10 mg/ml for chemotype II.

For cultivated rosemary essential oil, the greatest activity was detected toward *Bacillus subtillus* and *Candida albican* with 0.315 mg/mL MIC value and 0.625 mg/mL MBC value for *bacillus subtilus* and 1.25 mg/mL for *Candida albican*, meanwhile Chemotype I shows the most important activity with 0.625 mg/mL MIC value for *staphylococcus aureus* and *Candida albican.*

The Pakistan *Rosmarinus officinalis* oil possesses a moderate antibacterial activity (MIC values ranging from 0.2 to 1.72 mg/ml), it contains 1,8-cineole (52.2%), followed by camphor (15.2%) as well as α-pinene (12.4%) as major constituents (Hussain et al., 2010). In turn, low antibacterial activity was reported for the oils extracted from Turkish rosemary leaves (MIC values ranged from 5 to 20 mg/mL), which was composed mainly of camphor (16.1%), α-pinene (14.2%), 1,8-cineol (12.1%) and verbenone (11.1%) (Celiktas et al., 2007).

Several researches have attempted to explain the mechanism of essential oils against microorganisms. The complexity of this mechanism is related to the chemical composition of EOs, which presents a diversity of molecules that can each act on a different target. The mechanisms of action include cell wall destruction, disturbance of the cytoplasmic membrane, disrupting of proton motive force, coagulation of cell contents and hydrolysis of ATP and decrease of its synthesis leading to a reduction of the intracellular pool of ATP (Bhavaniramya et al., 2019; Nazzaro et al., 2013).

The results manifested that the examined essential oil indicate high antibacterial activity toward Gram-positive bacteria (MIC 0.315–1.25 mg/mL) over Gram-negative bacteria (MIC 1.25–5 mg/mL).Such finding agreed with many other studies (Hussain et al., 2010; Lodhia et al., 2009). The Gram-negative bacteria resistance toward EOs can be because the of outer membrane that surrounds cell wall, restricting diffusion of hydrophobic compounds through its lipopolysaccharide (Burt, 2004).

CONCLUSIONS

This comparative study of essential oil composition extracted by microwave-assisted hydrodistillation of wild *Rosmarinus officinalis* collected from the region of Figuig, and the other cultivated in Fez, shows a great qualitative and quantitative difference. The yields of EOs of the Rosemary from Fez (central north of Morocco) and Figuig (Eastern High Atlas) were respectively 1.35 ± 0.04% and 2.24 ± 0.05%, respectively. The GC and GC-MS analyses revealed that α-pinene (51.19–51.19%), 1,8-cineole (32.18–28.97%), camphor (16.20–10.01%) and camphene (9.16–2.76%) are themajor compounds of oils. Twochemotypes, namely α-pinene as well as1,8-cineole/camphor/α-pinene were identified.

The EOs examined showed marked antimicrobial activity towardsall microorganisms examined, and that of the rosemary collected from Fez was the most active.These findings support the potential of using the EOs of *Rosmarinus officinalis* as natural antimicrobial in food and pharmaceutical industries.

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