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STUDY OF THE INDIVIDUALITY EFFECT ON THE CHEMICAL COMPOSITION AND ANTIBACTERIAL ACTIVITY OF ESSENTIAL OILS OF *ROSMARINUS OFFICINALIS VAR. PROSTRATUS* GROWN IN THE MOROCCAN EXPERIMENTAL GARDEN

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ABSTRACT

The present article has been devoted to the performance identification and comparison of the chemical composition and antibacterial properties of the essential oil extracted from seven *Rosemary officinalis* var. *prostratus*. The individual's I5 and I7 have higher performance 1.79% and 1.93% respectively. The antibacterial activity study results indicated that the individual 5 and 7 have a much higher antibacterial effect than the other individuals as regards the three tested strains. GC and GC/MS chemical analysis identified that more than 97.21% of total volatile products (97.21% and 98.43% for I5 and I7 respectively). Both individuals I5 and I7 may be selected for future vegetative propagation to get clones having the capacity to provide a standard production. This production will provide an essential oil with significant activity as regards the tested strains. This could help resolve bacterial infections problem.

Key words: *Rosmarinus officinalis var. prostrates*, Essential oil, Chemical composition, Antibacterial activity.

INTRODUCTION

The growing infection rates with pathogenic microorganisms caused by the emergence of resistant bacteria have become a major concern in the health field (Maugein *et al.*, 2003). *Mycobacterium tuberculosis*, the principal tuberculosis agent, for instance, has developed many resistance mechanisms to the majority of known antituberculosis drugs (Chan & Iseman 2008).

This resistance often leads to extended diseased condition and an increased mortality rate. Almost 2 million people die each year because of TB with 150 000 deaths from multidrug-resistant tuberculosis (WHO, 2010).

The spread of this resistance phenomenon and the limited amount of antibiotics in the development phase make the discovery of new antibacterial agents absolutely imperative. These substances must be available and less toxic than antibiotics obtained chemically.

Several studies have been carried out on aromatic plants extract as a source of useful antimicrobial agents throughout the world: Australia (Seenayya & Prasad, 2000), Iran (Bonjar GHS 2004), Italy (Tampieri *et al.*, 2005), Turkey (Dulger *et al.*, 2006) and Morocco (Sqalli *et al.*, 2009).

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In Morocco there is a large variety and abundance of medicinal and aromatic plants (MAP) with therapeutic qualities. However, there is no rational use of the medicinal and aromatic plants in Morocco. Massive and repeated collections in natural habitats will be run out quickly the spontaneous MAP that could threaten their settlements survival, sustainability and may affect their gene pool. Domestication and cultivation of medicinal and aromatic plants are proving to be relevant solution to counteract this damaging phenomenon.

It is recognized that spontaneous medicinal and aromatic plants are more efficient than those grown with natural substances in term of performance. However, improved cultivation techniques are a promising way to enhance the cultivated medicinal and aromatic plants performance and yield. The expression in terms of quantity and / or quality of a natural substance in a plant remains under the control of three main components: genotype, environment and interaction between these two components.

The present work generally aimed at investigating the individuality effect on the content and chemical composition of cultivated Rosemary "Prostrates" essential oils species.

Rosmarinus (rosemary) is a natural plant that is known for its medicinal properties and fragrance since antiquity in Morocco. Recent research in the pharmaceutical and agro- food sectors has highlighted its excellent antibacterial properties (Zaouali *et al.,* 2010), anti- inflammatory (Gianmario *et al.,* 2007), Antidiabetic properties (Faixov Z & Faix, S 2008), antispasmodic (Al-Sereiti *et al.,* 1999) and antioxidant (Zaouali *et al.,* 2010). Our study identifies several aspects, namely the qualitative study including evaluation of the antibacterial effect of the essential oil of seven individuals (I1 to I7) of *R. officinalis* var. prostratus, a quantitative study which allowed the determination of the MIC of these individuals and identification of the chemical composition of different individuals using gas chromatography.

MATERIALS AND METHODS

Plant material

Samples of the aerial part (stems, leaves and flowers) of seven individuals (I1 to I7) of *Rosmarinus officinalis var. prostratus* were collected, from the experimental garden of the National Institute of Medicinal and Aromatic Plants of Taounate (NIMAP) during February 2012, when the plants were in full bloom. It should be noted that no agronomic treatment was imposed when the cultivation of this species.The Botanical identification and the Authenticated voucher specimens deposited in the Herbarium of the National Institute of Medicinal and Aromatic Plants (code: FA/RP/INPMA/003), University of Sidi Mohamed Ben Abdellah, Fez, Morocco.

BACTERIAL STRAINS

The essential oils antibacterial activity of different individuals was evaluated at all three strains: *M. smegmatis (MC^2 155)*: non pathogenic atypical mycobacterium, it has a similar sensitivity to that of *M.tuberculosis* (Mitscher, & Baker (1998)).

Escherichia coli (ATCC 25922): is a pathogen Gram-negative bacteria known for its strong antibiotic resistance and its toxic and invasive power to human. It causes intestinal diseases which vary in severity from benign to serious forms or even life threatening. *Bacillus subtilis* (ATCC 23857): is a non-pathogenic Gram-positive bacterium to human, but may contaminate food and may exceptionally cause food poisoning. It is regarded as an excellent model for the pathogenic bacteria study, such as *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Bacillus anthracis.*

These bacteria belong to the fungus culture collection of the Microbial Biotechnology Laboratory at the Faculty of Sciences and Techniques (FST) in Fez, Morocco.

Extraction and chromatographic analysis of essential oils

Extraction of essential oils

Essential oils extraction was done by hydrodistillation by means of Clevenger type hydrodistillation system. Two distillations have been made by boiling each individual for 2 h 30 of 150g of fresh plant material with 1 liter of water into a flask of 2l of a column with 60 cm in length connected to a condenser. The essential oil yield was identified in relation with the dry matter, and was evaluated from three samples of 25 g dried in an oven at 100°C within 24 hours. The essential oil was stored in the dark at 4°C. Every oil extraction was divided into two parts: The first was used for chemical analysis while the second was used for antibacterial in vitro tests. Oils, after extraction, are recovered and kept in small opaque flasks and stored at 4°C before their use.

Chromatographic analysis

GC: GC analysis were performed on a Hewlett-Packard (HP 6890) gas chromatograph (FID), equipped with a HP-5 capillary column (5 % phenyl methyl silicone). The characteristic of this column were: 30 m of length, 0.25 mm of diameter and 0.25 mm of film thickness. The temperature was programmed from 50°C (initial waiting 5min) to 200°C at 4°C/min. Gas chromatography conditions were as follows: N_2 as carrier gas (1.8 ml/min); split mode (Flow: 72.1 ml/min, ratio: 1/50) was used; temperatures of injector and detector were 275°C and 250°C respectively. Diluted samples (1/20 in Hexane) of 1 µl were injected manually. The

machine was led by a computer system type ″HP ChemStation″.

GC/MS

The chemical like 163 composition of essential oils were analyzed using a gas chromatograph (TRACE GC Ultra) fitted to a mass spectrometer (Polaris Q-Ion Trap MS). Operating in electron-impact EI (70 eV) mode. VB-5 (Methylpolysiloxane 5% phenyl) and a column (30m × 0.25mm × 0.25µm thickness) were used (National Centre of Scientific and Technical Research – (NCSTR), Rabat, Morocco). The chromatographic conditions were as follows: Injector and detector temperatures at 220 and 300°C respectively; carrier gas, helium at flow rate of 1.4 ml/min; temperature program ramp from 40 to 300°C with gradient of 4°C/min (holding the initial and final temperature for 4min). The relative amount of individual components of the total oil was expressed as a percentage peak area relative to total peak area. Library search was carried out using the combination of NIST MS. Oils constituents were identified by their retention indices relatives to n-alkanes (C8-C24) and by comparison of their mass spectral fragmentation patterns with those reported in literature (Adams 1995).

ANTIBACTERIAL ACTIVITY

Disc diffusion method

To highlight the essential oils antibacterial and antimycobacterial activity, the disk diffusion method (Bayoub *et al.,* 2010; Bauer *et al.,* 1966) was used because of its simplicity and efficiency to test bacteria sensitivity (Rozman and Jersek, 2009).

For this purpose, sterile Whatman paper discs of 6 mm diameter were placed in the center plate of 90 mm in diameter containing 30 ml of LB medium previously inoculated with 100µl liquid cultures of bacterial test strains. The discs are then impregnated with 10µl of essential oil. The control corresponds to a disc impregnated with 10µl of sterile distilled water. After incubation, the inhibition zones formed around the disks were measured.

Three replicates were performed for each strain.

Test statistics

The results statistical analysis is obtained by analyzing variance using the Light software.

The MIC Determination

The essential oils minimum inhibitory concentrations (MIC) were determined according to the method reported by Zaouali *et al.,* (2010) with a slight modification at the dilution. In fact, DMSO was substituted by 0.2% agar.

A dilution range was prepared in the agar solution, to obtain the following concentrations: 1/ 2, 1/ 4, 1/ 5, 1/ 8 and 1/ 10 (v/v) and 10 ml of each dilution was placed on sterile paper discs Watthman (6mm diameter) placed on the surface of Petri plates of 90mm in diameter containing 30ml of LB medium previously inoculated with 100 µl liquid cultures of tested strains. The control corresponds to a disc impregnated with sterile agar solution at 0.2%. After incubation, the inhibition zones formed around the disks were measured. Three repetitions were performed for each strain.

RESULTS

The Yield Essential Oil

The average yield of essential oil has been expressed in ml/100g dry plant material. The obtained results are collated in table 1.

Chemical composition

The chemical composition of different essential oils has been determined by GC and GC / MS analysis. The results of this analysis are presented in table 2 and Figure 1, 2 & 3.

Disc Method used in the Antibacterial activity

In vitro antimicrobial activity of the essential oils is estimated by the diameter of inhibition varied to according individual plant and bacteria strains. The method disc results (± standard deviation) are presented in Table 3 and Figure 3.

Analysis of variance

An analysis of (ANOVA) variance was used with a factor in order to check if there are significant differences between the average inhibition diameter across the 7 various levels of the *rosemary officinalis var. prostratus* individual tested on the three strains (*Escherichia coli, Bacillus subtilis, and smegmatis*). The results of this testing are set out in Table 4.

Minimal inhibitory concentration

The minimal inhibitory concentration (MIC) antimicrobial activity is appreciated by the inhibition zone. The MIC is determined based on this zone diameter. The results are set out in Table 5.

Table 1. Yields essential oils obtained by hydrodistillation from seven individuals *R. officinalis* Var. *prostratus*

Individual Selection of Rosemary prostrates	Yields (%)
I1	1.36 ± 0,02
I2	1.66 ± 0,02
I3	1.76 ± 0,03

I4	1.82 ± 0,02
I5	1.79 ± 0,03
I6	1.72 ± 0,01
I7	1.93 ± 0,01

Table 2. Chemical composition of seven individuals of Rosemary prostrates essential oils

RI	Compounds	I1%	I2%	I3%	I4%	I5%	I6%	I7%
931	α-Thujene	0.48	0.51	0.21	0.21	0.22	0.21	0.11
939	α-pinene	**12.76**	**18.82**	**28.04**	**30.14**	**39**	**37.31**	**16.31**
953	Sabinene	**7.17**	**7.05**	**13.86**	**12.7**	**18.66**	**18.03**	**8.64**
976	Camphene	**12.47**	**14.59**	**6.46**	**7.12**	**7.48**	**7.27**	**3.48**
1011	δ-3-Carene	4.54		1.8	-	--	1.95	-
1033	1,8- cineole	-	1.48	-	1.03	1.82	-	**38.24**
1068	Trans-Sabinene hydrate	0.36	--	0.49	0.36	0.42	0.86	0.57
1097	Cis-Sabinene hydrate	-	-	-	0.18	-	-	-
1125	α-Campholene	-	2.81	-	-	-	-	-
1143	Camphor	**4.66**	**1.97**	**4.62**	**3.02**	**5.54**	**5.53**	**14.81**
1165	Borneol	5.46	-	1.3	3.47	1.06	1.13	2.84
1204	Verbenone	0.55	-	-	0.23	-	0.29	-
1227	Trans-2-Caren-4-ol	-	-	-	1.36	0.29	0.79	-
1185	α-Terpineol	0.63	3.75	1.7	1.78	-	-	0.61
1194	Myrtenol	1.23	1.08	1.95	**10.54**	**6.18**	9	**5.01**
1235	Myrtenyl acetate	0.22	-	-	0.95	-	-	-
1285	Bornyl acetate	**29.88**	**32.55**	2.71	**8.8**	0.63	0.52	0.13
1351	α-Cubebene	0.15	-	0.36	-	-	-	-
1376	α-Copaene	0.10	0.10	0.18	-	-	-	-
1384	β-Bourbonene	0.16	0.16	-	-	-	-	-
1418	β-Caryophyllene	**3.8**	**4.18**	**11.3**	**8.39**	**7.23**	**7.39**	1.45
1473	γ-Gurjunene	0.2	-	1.80	0.4	0.37	0.39	-
1480	Germacrene-D	0.42	-	1.69	-	-	-	-
1493	Ledene	3.92	3.80	1.64	-	0.37	-	-
1499	α-Muurolene	0.32	0.3	0.75	-	-	-	0.15
1513	γ-Cadinene	0.8	0.79	1.88	-	-	-	0.64
1581	Caryophyllene oxide	**5.75**	**4.01**	**14.21**	**7.56**	**9.16**	**6.81**	4.22
1584	copaen-4-α-ol	1.43	-	0.45	1.01	-	-	-
1590	Viridiflorol	-	-	0.14	-	-	-	-
1611	Tetradecanal	0.21	0.21	0.43	-	-	-	-
1653	τ-Cadinol	0.19	-	-	-		-	-
Total (%)		97.86	98.16	97.97	99.25	98.43	97.48	97.21

RI: Retention index, I1-I7: Individual selection of *R. officinalis* var. *prostratus*

Table 3. Antibacterial activity estimated by diameter of inhibition according individual's *R. officinalis var. prostratus* essential oils

Samples of essential oils	Diameter of inhibition (mm)*		
	Escherichia coli	***Bacillus subtilis***	***M. smegmatis***
I1	6 ± 0	10.46 ± 0.5	7.16 ± 0.28
I2	12.23 ± 0.41	19.5 ± 0.5	15.56 ± 0.3
I3	13.16 ± 0.28	22 .5 ± 0.5	19.1 ± 0.36
I4	8.53 ± 0.05	14.33 ± 0.28	25.1 ± 0.85
I5	17.1 ± 0.26	31.23 ± 0.92	46.5 ± 1.5
I6	15 ± 0.2	24.6 ± 0.52	40.33 ± 0.35
I7	17.1 ± 0.26	28.2 ± 0.19	49.46 ± 0.61

*The average results available after three repetitions, I1-I7: Individuals of R. *officinalis var. prostratus*

Table 4. Variance Analysis of ANOVA with a Factorial combination of treatment

Strains	Variance analysis					
	Source of Variation	**Sum of Squares**	**Ddl**	**Average Square**	**F**	**Probabilities**
E. coli	Inter- groups	190.91	6	31.81	321.25	0
	Intra-groups	1.38	14	0.092		
B. subtilis	Inter- groups	1330.22	6	221.704	809.7	0
	Intra-groups	3.833	14	0.279		
M. smegmatis	Inter- groups	4871.82	6	811.97	1502.32	0
	Intra-groups	7.566	14	0.540476		

Table 5. Concentration of Minimal inhibitory of *R. officinalis var. prostratus* oils

Strains	CMI v/v						
	I1	I2	I3	I4	I5	I6	I7
E coli	0.5	0.25	0.25	0.25	0.2	0.25	0.2
B. subtilis	0.25	0.2	0.2	0.2	0.125	0.2	0.125
M. smegmatis	0.25	0.2	0.2	0.125	0.125	0.125	0.125

Figure 1. Chromatographic profiles of essential oils extracted from I1, I2

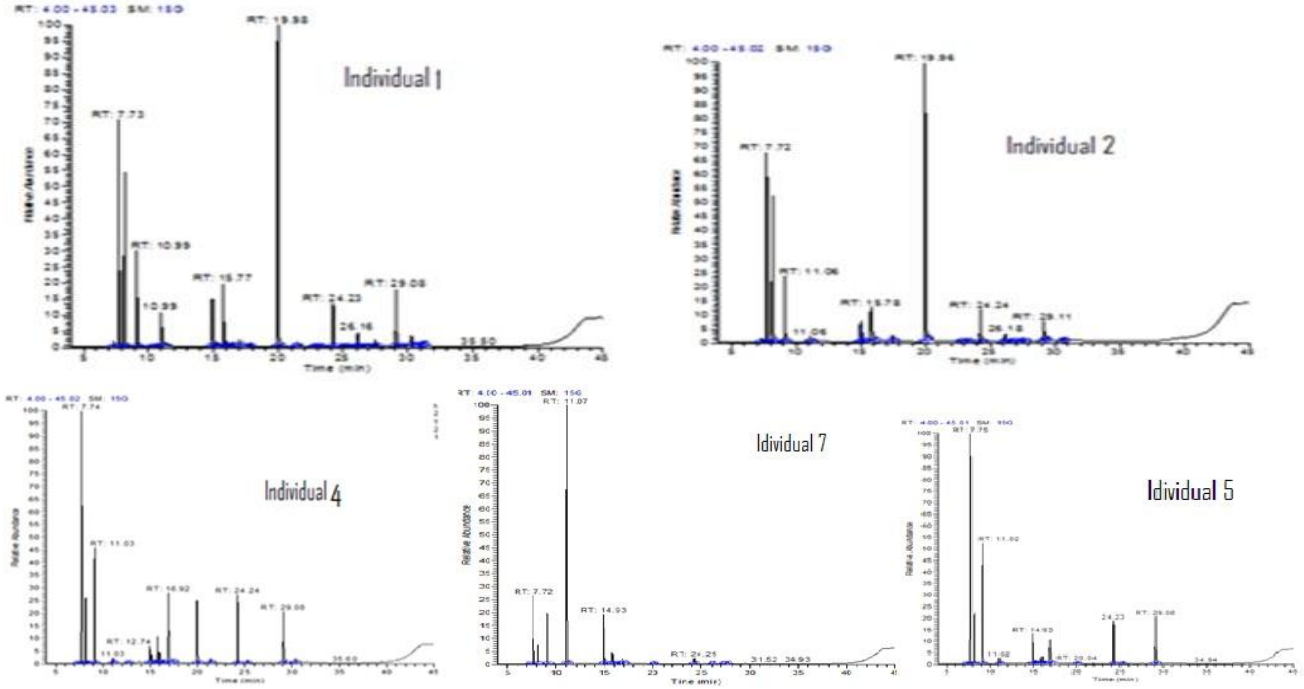


Figure 2. Chromatographic profiles of essential oils extracted from I3, I4, I5, I6 and I7

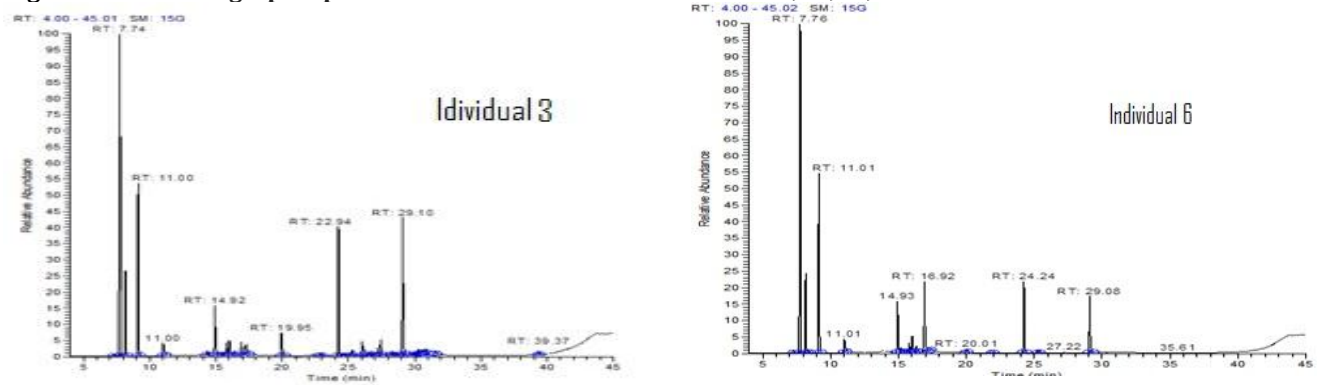
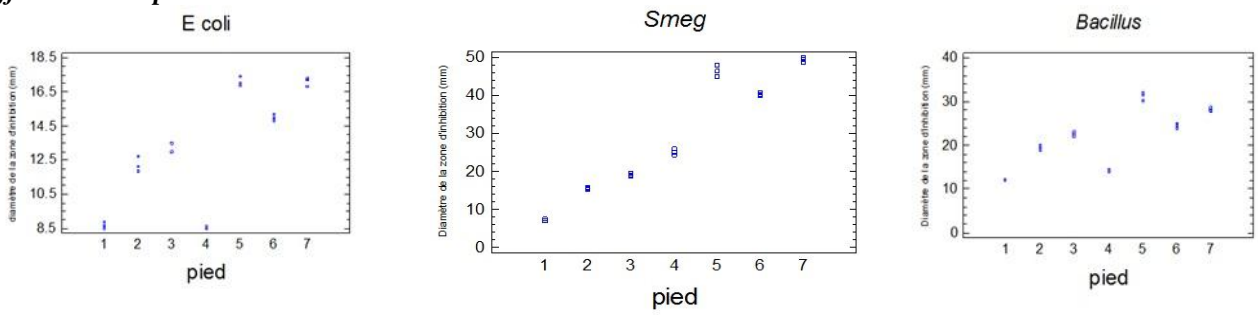


Figure 3. Antibacterial activity estimated by diameter of inhibition of the seven individuals of *Rosmarinus officinalis* var. *prostratus oils*



DISCUSSION

The seven individuals from *R. officinalis var. prostratus* provided essential oil yields ranging between 1.36 % and 1.93% (Table 1). These yields are higher than those mentioned on a number of studies. In fact, Ayadi *et al.,* (2011) were obtained variable yields depending on the harvest Tunisian area, 1.35% in the region of Sidi Bouzid and 1.25% in Bizerte. However, the yield obtained for Rosemary in Zaghouan Region is 1.27 % (Ayadi *et al.,* 2011). Whereas other studies have reported a lower yield than that obtained in our study with an essential oil content of 0.52% (Zekovic P *et al.,* 2009). At the same time, a yield of 0.8% for the Rosemary from Honaine station and 0.6 % for the Algerian Rosemary from Tlemcen station (Tomi *et al.,* 1995). However, our yields are lower than those obtained by (Jashmidi *et al.,* 2009), which are 2.1 and 2.6% respectively in the Iranian regions of Kerman and Lalehzar (Jamshidi *et al.,* 2009).

In general, the chemical composition varies according the individuals plant. The α-pinene, Camphene, Bornyl acetate and caryophyllene oxide are the major constituents. These components content varies from individual to other. The I7 has a different chemical composition from other individuals with the α-pinene (16.31%) (Table 2), the 1, 8-cineole (38.24%), and the camphor (14.81%) which represent the main constituents of the oil.

Concerning the chemical composition, individuals I1, I2 have similar chromatographic profiles (Figure 1). The individuals I5 and I6 are characterized by the presence of the myrtenol compound (10.54 and 6.8% respectively). The β-caryophyllene is also present in the essential oils extracted from different samples (1.45% for I7 to 11.3% for I3). For the caryophyllene oxide, the best rate is achieved with the I3.

Furthermore, no similar study has been carried out previously on this variety of rosemary. *Rosmarinus officinallusvar.prostratus* oil is characterized by the presence of the α-pinene, the 1.8-cineole and the camphor as the main components. (El Amarani *et al.,* 2000, chachat *et al.,* 1993).

In the table 4, the ANOVA variance is divided into two components: inter-groups and intra- groups. As the probability value for the F test is less than 0.05, there is a statistically significant difference among the inhibition average diameters from an individual level to the next at the 95.0% confidence level for the three used strains.

The antibacterial activity results of the seven individuals *R. officinalis* var. *Prostratus* essential oils have showed an inhibiting effect on all the studied strains. This activity varies from one individual to another. In fact, the highest antibacterial activity was found in the I5 and I7 and the lowest activity was found in the I1 (Table 2 and Figures 1 and 2) for the three used strains.

For individuals I5 and I7, the highest activity was found against *M. smegmatis* with respectively 46.5 and 49.46 mm inhibition diameters, while the lowest activity was observed against *Escherichia coli* with respectively (7 and 8 mm) inhibition diameters. The same results were reported for the I1. In fact, the inhibition diameter zones were obtained for *M. smegmatis* are higher than those obtained for *B. subtilis* and *E. col*i. These diameters are respectively 6; 10, 46 and 7, 16 to, *E. coli, B. subtilis* and *M. smegmatis*. The disc test has showed that I5 and I7 are higher than the other individual's antibacterial effect; this may be because of the essential oil composition of both individuals.

Concerning CMI results (Table 5), essential oils have shown an inhibiting effect on the studied microorganisms. In fact, all the microbial strains were inhibited at a concentration of 1/4v/v with the exception of one individual where the strains are inhibited from 1/2 v/v. The most sensitive micro-organism to all essential oils is *M. smegmatis* whose growth was stopped at the low concentration of 1/8v/v.

The results have also showed that the MIC of I5 and I7 are the lowest compared to other individuals for *M. smegmatis, E. coli* and *B. subtilis*, while individual one presented the highest MIC. This shows that the essential oils of the I5 and I7 exhibit interesting antibacterial characteristics of the tested micro- organisms. These results are in accordance with those of the disk test.

Variations in the nature of the components and their content induced a variation of the microbiological activity. The presence of a number of chemical constituents in the composition promotes this activity.

In fact, several studies have shown antibacterial properties of some components such as: α-pinene (Wei Wang *et al.,* 2012; Martin *et al.,* 2000). The 1,8-cineole (Prudent *et al.,* 1993) camphor and its derivatives (Felice *et al.,* 2004), Caryophyllene oxide (Ulubelen *et al.,* 1994) Borneol (Felice *et al.,* 2004). In addition (Magiatis *et al.,* 2002) have found that the caryophyllene oxide is the most effective, followed by camphor and 1,8-cineole.

The oil of the I7 and I5 has presented a significant antibacterial activity especially against *M. smegmatis*. This activity could be mainly due to the dominant components. The high antibacterial activity of the I7 can be connected to the presence of 1,8-cineole, α-pinene, and camphor that are dominant components, for the I5 this activity is mainly due to dominants components such as: sabinene, caryophyllene oxide and α-pinene. These compounds are in fact known for their antimicrobial properties.

CONCLUSION

This study has been devoted to identifying and comparing the performance of the chemical composition and antibacterial properties of the essential oil extracted from seven individual's *R. officinalis* var. *prostratus*. The I7 and I5 are the individuals who had the best yield respectively 1.93% and 1.79%.

The chemical analysis by GC and GC/MS, allowed identifying 97.21% over total volatile products (97.21% and 98.43% for I7 and I5 respectively).

The study results of the antibacterial activity showed that the individuals 5 and 7 are higher than the other individuals against the three tested strains. This difference in antimicrobial activity may be attributed to the presence of dominant components such as the α-pinene, camphor, and 1,8-cineole.Both individuals (I5 and I7) may be selected for future propagation clones capable of ensuring a standardized production. This production will provide an essential oil with significant activity as regards to the tested strains. This would help to solve bacterial infection problems.

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