Moroccan Journal of Chemistry
ISSN: 2351-812X
http://revues.imist.ma/?journal=morjchem&page=login
Sabbahi & al. / Mor. J. Chem. 8 N°4 (2020) 866-875

Altitude effect on the chemical composition and antioxidant activity of rosemary in the region of Talsint (Morocco).

Monsif Sabbahi*, Asmae El Hassouni, Abdessalam Tahani, Ali El Bachiri

Laboratory of Physical Chemistry of the Natural Resources and Environment, University Mohammed Premier, BP 717, 60000, Oujda, Morocco.

** Corresponding author:*
sabbahimonsif@gmail.com

Received 28 Oct 2019,
Revised 10 sept 2020,
Accepted 15 sept 2020

Abstract

The *Rosmarinus officinalis* (rosemary) is an evergreen shrub used worldwide for its aromatic and medicinal virtues. It plays an important role in the local economy of Talsint (Eastern Morocco). Even though, forest managers and decision makers are short of information and scientific indicators to accurately understand how the chemical composition of rosemary varies in the region. Hence, the aim of this work is to study the effect of altitude gradient over the chemical composition of the rosemary essential oil and its antioxidant activity. In this study, eight samples of wild population of rosemary were collected from different altitudes in the High Atlas Mountains in the region of Talsint in order to determine the chemical composition and antioxidant activity of their essential oils. The volatile profiles were determined by Gas chromatography–mass spectrometry (GC-MS), meanwhile, the antioxidant activity to scavenge the free radicals was pointed out by 1,1- diphenyl-1-picrylhydrazyl (DPPH) assay. Based upon our analysis, the major constituents are 1,8-Cineole (50.60-64.27%), Camphor (1.77-14.12%), α-Pinene (6.61-9.02%), and Borneol (1.98-6.20%). Except the 1,8-Cineole, the altitude effect remains unclear for the other constituents. On the other hand, the essential oils of Talsint rosemary showed considerable antioxidant activity.

Keywords: *Rosmarinus officinalis*; essential oil; antioxidant; Talsint.

1. Introduction

Rosmarinus officinalis (commonly named rosemary) is an aromatic plant of Lamiaceae family. It's an evergreen shrub reaching a height of up to 1.5 m. It grows spontaneously in many Mediterranean countries [1]. Rosemary is used widely since antiquity in culinary, cosmetics, and medicinal products [2,3]. Many studies have demonstrated that it is a very efficient plant as natural antioxidant [4,5]. In addition, it presents strong anti-inflammatory [6], and antimicrobial properties against yeast [7], mold [8] and gram-positive bacteria [9]. Moreover, rosemary's essential oil has been proven as a green anti-corrosion inhibitor [10]. Numerous researches have focused on the chemical composition of *Rosmarinus officinalis* essential oil. The main compounds detected were 1,8-cineole, camphor, α-pinene, borneol, verbenone, and camphene which show high variability as regard to their concentrations [11–14]. Several researches have tied the variability of the composition and the yield of the rosemary essential oil to different intrinsic factors as genetic background and plant age [15] or extrinsic factors such seasons [16], or extraction methods [17,18]. Nonetheless, the secret behind how the chemical composition of rosemary essential oils varies across the land cover distribution remains unlocked so that many studies explained chemical change by the genetic and the provenance variability rather than any ecological conditions [19–22]. In Morocco, rosemary, locally known "Azir", grows spontaneously in various regions especially the eastern parts of the country, where it covers approximately 500,000 ha in the three provinces of Taourirt, Jerada and Figuig. Just in Talsint (province of Figuig), rosemary's shrub is covering more than 200,000 ha [23]. Talsint is a remote locality situated in the southeast of the High Atlas Mountains series and characterized by an arid climate with a weak annual rainfall of 140 mm [24]. Actually, rosemary harvesting by local cooperatives and the essential oil commerce, play an important role in its local economy. Nevertheless, the means of essential oil production and marketing are still traditional due to a lack of scientific knowledge concerning the characteristics and the variability of the local rosemary leaves and its essential oil. Hence, given the lack of chemical information concerning Talsint's rosemary essential oil, and the importance that plays this plant on the local economy, this work aims to characterize the essential oil chemical composition and the antioxidant activity of *Rosemarinis offinalis* in the region a Talsint. Furthermore, we will investigate in the present study, with a new perspective, the effect of the altitude gradient over the chemical composition across a tiny study area. The selection of a restrained sampling zone is aiming to avoid the effect of the provenance that usually masks the ecological effects [20–22,25] . To the best of our knowledge, the elevation gradient effect across a confined area has never been studied. The same for Talsint's rosemary that has never been characterized since it's a remote locality. Besides, this information will represent an added value to rosemary's essential oil of the region as a commercial product, and an efficient tool for decision makers, cooperatives and industrials in order to participate in a sustainable rosemary management plan.

1. Material and methods

1.1. Plant Material

Eight samples of *Rosmarinus officinalis L*. leaves were collected from eight wild populations (Figure 1) from different altitudes of the eastern hillside of the High Atlas Mountains in Talsint (Province of Figuig, Morocco). Samples were collected on May 2017at bloom stage from new shoots of individual plants. Afterwards, the plant material was dried at room condition (25°C) for 7 days. The coordinates of the sampling locations are illustrated in table 1. We mention that the samples are identified by the Forest Management Studies Service of Oriental.

Table 1. Lambert coordinates altitude, and location name of samples of wild rosemary populations collected in 2017 from the forest of Ait Seghrouchen, Talsint (Province of Figuig, Morocco).

Sample	Location	Lambert coordinates		Altitude
		X	Y	
T01	El Loh	672760	255492	1447
T02	Ghezouane	663922	225465	1760
T03	Tamslemt	647714	227243	1635
T04	Skendis	668223	218339	1914
T05	Ouaezart	661912	217043	2143
T06	Maazer	658584	223526	2153
T07	Boujrad	674144	230096	1739
T08	Lamrija	667834	235577	1581

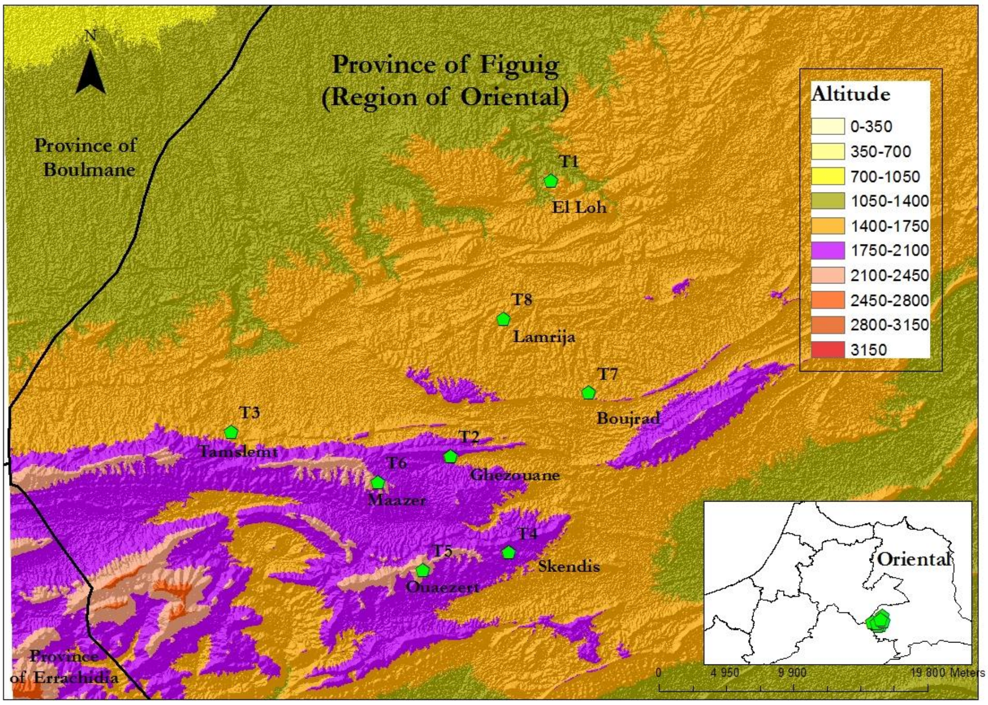

Fig. 1. Map of Samples' location in Talsint vicinity (Provinces of Figuig, Region of Oriental Morocco).

1.2Essential oil extraction

The leaves of *Rosmarinus officinalis* samples underwent hydrodistillation for 3 h with a Clevenger-type apparatus according to the European Pharmacopoeia (1996). The essential oil extracted was isolated from water , dried over anhydrous sodium sulphate and kept in amber vials at 4 C. The yield of each sample was calculated per 100 g of plant dry matter.

1.3Chromatography-mass spectrometry analysis

The essential oil extracted was analyzed by gas chromatography-mass spectrometry (GC-MS) using a Hewlett Packard 6890 mass selective detector coupled with a Hewlett Packard 6890 gas chromatograph with a 30 m× 0.25 mm HP-5 (cross-linked Phynel-methyl Siloxane) column with 0.25 µm film thickness (Agilent). The carrier gas is Helium and

the flow rate along the column was 1,4mL min-1. The temperature of the column was of 10°C min^{-1} and increased finally, at rate of 30°C min^{-1}, from 230 to 280. The parameters of the mass-spectrometry used in this study were as follows: ionization energy of 70 eV and ionization current was 2 A. The ion source temperature was 200°C and resolution was 1000. Mass unit were set from 30 to 450 m/z. The components of essential oil were identified based on relatives retention indices , Kovat index, WILEY 275 Library and by comparison to the literature data [26].

1.4DPPH radical-scavenging activity

The scavenging activity of the stable 1,1-diphenyl-2- picrylhydrazyl (DPPH) free radical, was carried out by the method reported by [27] with minor modifications. 0.6 Ml of essential oil solution with various concentrations (50 µl/ml, 100 µl/ml, 150 µl/ml and 20 µl/ml) were mixed with 2.4 mL of DPPH solution (diluted in methanol at 0.004%) and incubated afterwards for 30 minutes in dark at room temperature. Then, the absorbance is measured at 517 nm. Methanol is used as negative control meanwhile acid ascorbic (vitamin C) as standard antioxidant. The percentage of inhibition activity of DPPH radicals was calculated as:

$$\text{Inhibition Activity } (\%) = \left[\frac{(A0 - A1)}{A0}\right] \times 100$$

Where A0 was the absorbance of the negative control, and A1 was the absorbance of the essential oil solution. The minimal concentration to reduce 50% of DPPH radicals (IC50) was worked out from linear regression analysis.

1.5Statistical analyses

To assess the affinity between the essential oil belonging to different localities, a hierarchical cluster analysis (HCA) was conducted with the Euclidean distance as a measure of dissimilarity based on the main five components. All the analyses were conducted using SPSS software, version 19 (IBM SPSS, Chicago, IL, USA).In order to study the relationship between the altitude and the other variables such as the yield and chemical composition, we used the linear regression and Pearson coefficient (p).

2. Results and discussion

2.1Yield of the Essential Oils.

Table 2 reports the yield of the extracted essential oils. The yield ranges from 0.58 to1.67%. We notice that the lowest value belong to Boujrad and Mrija localities that have relatively medium altitude (1739 and 1581 respectively); meanwhile, the highest yields were found in the samples of Skendis, Ouaezart, and Maazer, characterized by high altitudes (more than 1900 m). However, we can't attribute the yield variability to the altitude since Tamslemt and Ghezouane, which are samples with the same range of altitude as Boujrad and Mrija; have higher yields (1.32% and 1.09% respectively). Those observations are confirmed by the regression line (Fig. 2) set between the essential oil yield and the altitude that showed a non-significant correlation ($R^2=0.37$; $p>0.1$). Many studies have investigated rosemary's essential oil yield in different regions. Thus, the rosemary studied in the region of Boulmane (Morocco) yielded 0.54% [28]. Meanwhile, the *Rosmarinus officinalis* of Taourirt (Morocco) yielded 1.8% [29]. Those values, that were registered in Morocco, are less than those found in Iran by [30], who reported a yield of 2.05 %. They are also less than those found in Brazil where it yielded 2.5% [1]. Nevertheless, according to [31], who studied Sicilian rosemary, and to [21], who studied Spanish rosemary, we can't attribute the yield variability to the bioclimatic factor. As a conclusion, we can assume that the yield is mainly influenced by the habitat origin and genetic background rather than the altitude which hasn't a clear effect.

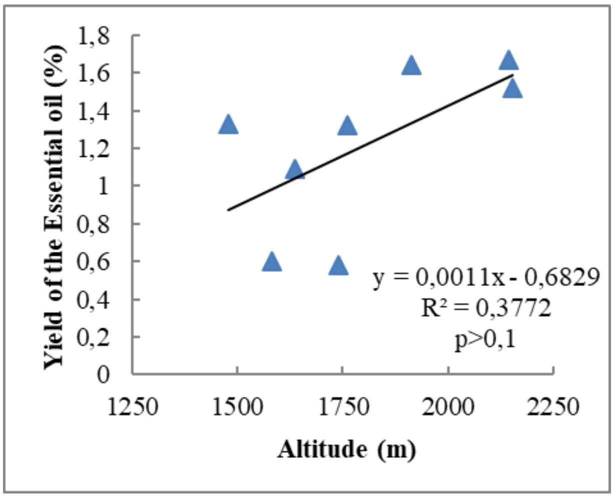

Figure 2: linear regression of the yield of the essential oil of the studied samples of rosemary along altitude.

2.2the chemical composition of the essential oil

The GC-MS analysis revealed 18 components, which represent from 99.5 to 100% of the total of the identified compounds in the extracted essential oil (Table 2). We report that the major compounds were 1.8-cineole, camphor, and α-pinene are present in all samples with variable concentrations depending to the location (Fig. 3): 1.8 cineole is ranging from 50.60% (Maazer) to 64.27% (El Loh); camphor change largely from 1.77% (Tamslemt) to 14.12 % (Boujrad); meanwhile the concentration of α-pinene goes from 6.61% to 9.02%. Other compounds as β-pinene (4.66 - 8.51%), borneol (1.98-6.20) and camphene (1.64-3.93%) are also present, with less importance, in all the analyzed samples.

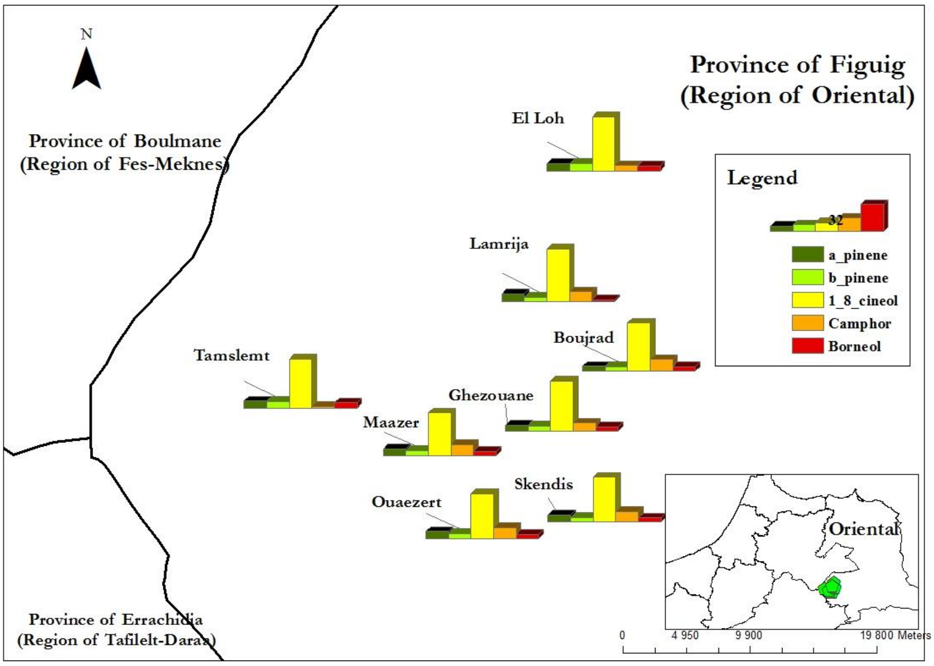

Figure 3: volatile profile of the main compounds in 8 populations of rosemary collected in the region of Talsint

Table 2: Chemical compositions of the Essential Oil of Rosemary Leaves of Talsint (Figuig, Morocco)

Locations	El loh	Ghezouane	Tamslemt	Skendis	Ouaezart	Mâazer	Boujrad	Mrija	RT[a]	IK[b]
Yield (g/100mg)	**1.33**	**1.32**	**1.09**	**1.64**	**1.67**	**1.52**	**0.58**	**0.60**	-	-
Compounds									-	-
Monoterpenes Hydracarbons	**20.7**	**19.28**	**24.77**	**21.13**	**21.65**	**21.11**	**18.87**	**19.11**	-	-
α-pinene	8.58	6.61	8.29	8.71	8.64	8.15	6.03	9.02	4.63	137
Camphene	3.61	3.93	3.78	2.93	3.04	2.8	1.64	3.04	5.06	189
β-pinene	8.51	5.96	8	5.3	5.97	5.96	5.89	4.66	5.613	254
β-myrcene	-	0.75	0.82	1.04	0.95	1.01	1.12	0.63	5.695	265
α-Terpinene	-	-	-	-	-	-	0.54	-	6.39	334
D-Limonene	-	1.3	2.99	1.39	1.59	1.78	1.91	1.28	6.49	360
β-terpinene	-	-	-	-	-	-	0.48	-	6.64	378
β-Cymene	-	-	-	0.8	0.79	0.69	-	-	6.74	391
δ-terpinene	-	0.73	0.89	0.96	0.67	0.72	1.26	0.48	7.10	433
Monoterpenes Oxigenated	**79.3**	**79.53**	**74.47**	**77.85**	**77.13**	**76.43**	**81.13**	**79.42**		
(+)-2-Carene	0	0.37	-	-	-	-	0.56	-	6.27	492
1,8 Cineol	64.27	58.81	58.24	53.16	53.35	50.6	57.24	61.73	6.81	399
Linalool	-	0.66	-	1.2	1	1.15	1.15	-	7.99	539
Camphor	6.39	9.36	1.77	12.82	12.96	13.33	14.12	11.47	9.62	736
Borneol	5.73	4.63	6.2	5.07	4.85	5.26	5.51	1.98	9.66	741
α-terpineol	-	3.71	4.65	4.39	3.87	4.97	1.08	3.11	10.00	781
Bornyl acetate	2.91	1.99	3.61	1.21	1.1	1.12	1.47	1.13	11.18	924
Sesquiterpenes	**-**	**1.19**	**0.76**	**1.02**	**1.22**	**1.43**	**-**	**0.98**		
Careophyllene	-	1.19	0.76	1.02	1.22	1.43	-	0.98	12.771	1113
Others	**-**	**-**	**-**	**-**	**-**	**1.03**	**-**	**-**		
Diethyl Phtalate	-	-	-	-	-	1.03	-	-	16.6	1504
Total identified	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**99.51**	-	-

a.RT, retention Time. b. IK, kovat index

Many studies conducted across the world concerning *Rosmarinus officinalis* essential oil reported a wild range of volatile profiles. Thus, the main constituent in Montenegrin and Albanian rosemary essential oil is camphor with a relative concentration of 24.4 and 17.3% respectively [32]. Whereas, Mexican rosemary essential oil is rich with α–pinene (31.07%) and Verbenone (15.26%). The literature related to Moroccan *Rosmarinus Officinalis* essential oil pointed out volatile profiles that are close or similar to our finding with 1,8-cineol as a major constituent with more than 40% [18,22,25,27,33]. However, we highlight that Talsint rosemary's essential oil is characterized by a less content of camphor and higher content of 1,8-cineole compared to the other regions of Morocco. The regression drawn between the four major components (α-pinene, β-pinene, 1,8 cineol and camphor) in the studied rosemary essential oil is illustrated in Figure 4 in order the emphasis the relationship between the altitude and the volatile composition. Excepted for 1,8-cineole content, which showed a significant relationship (R^2=0.88, $p<0.0005$) with a declining trend across the increasing altitude, the tree other compounds show a very week correlation with non-significant correlation. Thus, we can say that there is a negative impact of the elevation over 1,8-cineole molecule synthesis, meanwhile, the effect over the other molecules remains unexplained. Our results are congruent with the reports made by [34] who confirmed that the ecological effect over the volatile variability of *Rosmarinus officinalis* essential oil from Corsica and Italy are unclear. However, our findings are encouraging in term of understanding the altitude effect on the chemical composition.

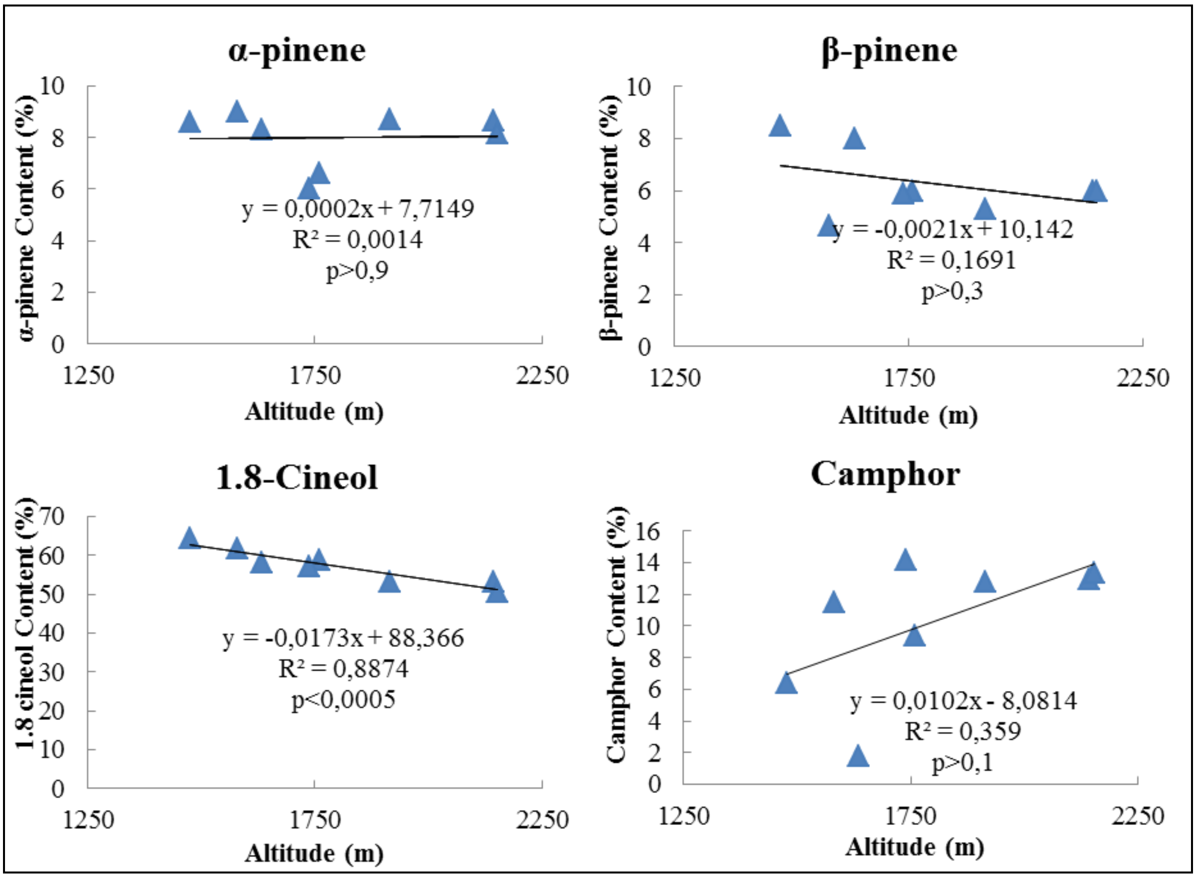

Figure 4: the regression analysis of the α-pinene, β-pinene, 1.8 cineol,and Camphor content along the altitude, p is the coefficient of Pearson and R^2 is coefficient of determination.

As regard to molecule groups, table 3 showed that the monoterpenes oxiginated are the dominant constituents of the essential oil of Talsint's rosemary. They range from 74.47 to 81.13% of the total of the constituents; meanwhile the monotepenes hydrocarbons represent from 18.87% to 24.77%. In contrast, the sesquiterpenes don't exceed 1.43%. Our finding are in agreement with the results of [27,35]. The dendrogram of the cluster analysis of the essential oil volatile profile is carried out in Figure 5. Therefore, we can split the studied samples to two main groups: A and B. The group A can be divided to two subgroups (A1 and A2). Group A is characterized by a relatively high content of camphor (9.36-14.12%). Actually, 1,8-cineol content is the one what makes the difference between Sub groups since in Sub Group A1 (Skendis, Ouaezrt and Maazer), it is ranging from 50.6 to 53.35% which is lower than the values registered in Sub Group A2 (Ghezouane, Boujrad and Lamrija) where 1,8-cineole percentage attains 57.24 to 61.73%. By contrast, the Group B (El Louh and Tamslemt) is characterized by a relatively weak content of camphor (1.77-6.39%) and high content of 1.8-cineole (58.28-64.27%). Based on this classification, we can say that samples of moderate altitude are richer of 1.8-cineol which confirms the regression analysis (Figure 4). However, there is a quite difference between SubGroub A1 and Group B concerning the content of camphor, even if they belonged to the same range of altitude, since we find a low content in Group B. As a consequence, we can report that the effect of altitude except for 1,8-cineole, remains unclear. This is can be explained by the interaction of the different factors influencing the chemical composition of the essential oil like the genetics and age.

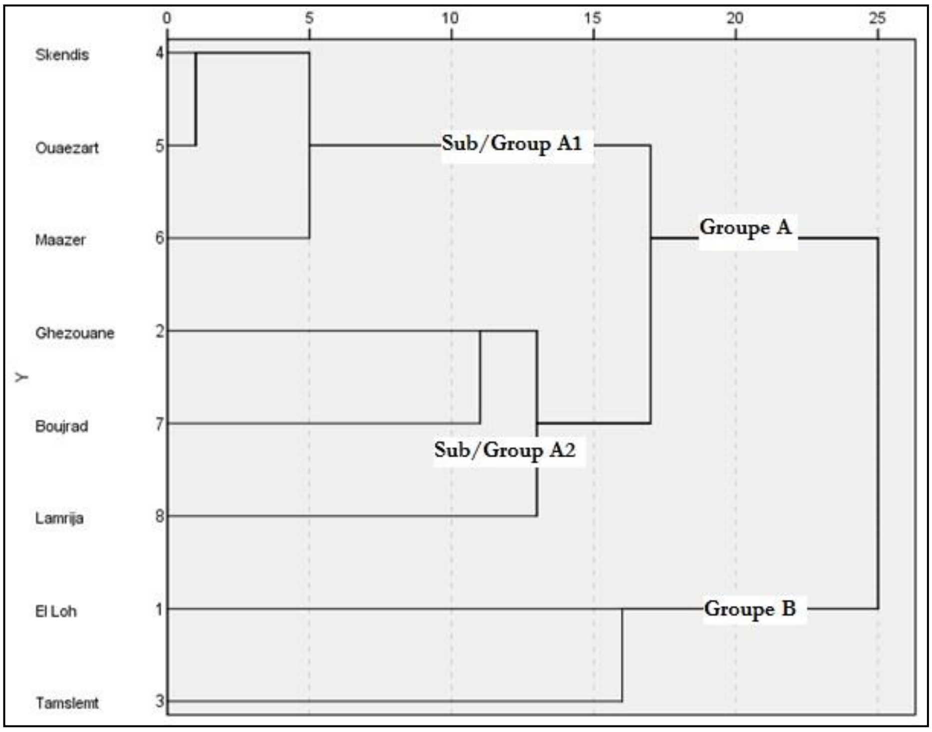

Figure 5: Cluster Dendrogram using the main five constituents of the eight populations of rosemary of Talsint (Province of Figuig)

2.3Anti-oxidant activity

The eight samples of the essential oil extracted were submitted to the test of radical scavenging capacity through the original DPPH assay [27]. The results of the DPPH test are summarized in Table 3.

Table 3. Free radical-scavenging activities (%) of rosemary essential oil of the Talsint region in Morocco and of acid ascorbic, and the total of monoterpenes hydrocarbons and monoterpenes oxiginated.

Sample	location	Concentration of essential oil (µl/ml)				R^{2a}	DPPH[b] IC50 (µl/ml)	total MH	Total MO
		20	40	60	80				
T01	El Loh	41.98	50.09	64.98	78.73	0.986	32.81	20.7	79.3
T02	Ghezouane	43.73	54.02	60.14	72.64	0.984	33.57	19.28	79.53
T03	Tamslemt	42.26	57.86	64.96	77.44	0.981	31.13	24.77	74.47
T04	Skendis	43.16	54.37	60.83	72.85	0.988	33.7	21.13	77.85
T05	Ouaezart	43.85	52.69	65.26	74.65	0.986	32.23	21.65	77.13
T06	Maazer	43.89	53.09	64.08	78.64	0.989	32.79	21.11	76.43
T07	Boujrad	43.08	55.16	61.07	73.12	0.983	33.26	18.87	81.13
T08	Lamrija	43.3	55.04	60.94	72.85	0.984	33.07	19.11	79.42
Acid Ascorbic[c]	-	46.61	63.34	71.16	82.77	0.97	22.65	-	-

a.R^2, the regression coefficient used to measure the linear relationship between the Essential oil concentration and the percentage of radical scavenging. b. Essential oil concentration required to scavenge 50% of DPPH solution. c. Acid ascorbic used as a reference antioxidant.

The Radical scavenging capacity of the analyzed samples showed an increase in line with the oil concentration: the most potent essential oil was the one of Skendis (IC50= 31.13%). However, the values are judged very close to each other's (31.13-33.70%). In addition, our samples show less antioxidant capacities compared to ascorbic acid (table 3), which was used as standard antioxidant (IC50= 22.65 ± 0.61 µl/ml with R^2= 0.97). Anyhow, according to those results, the essential oil of the rosemary of Talsint is judged as a very potent antioxidant to neutralize the free radicals.

Our findings are in congruent with the results of [27,36]. In order to investigate the relation between the content of molecules and the bio activity, we calculated the correlation of the IC50 and the percentage of hydrocarbons. Thus, we conclude that the antioxidant activity is negatively proportional to the percentage of monoterpenes hydracarbons (R^2=71%). Many studies pointed out that the capacity of an antioxidant component to scavenge DPPH is mainly depending to its ability to free hydrogen molecules, and this is too related the abundance of functional groups of monoterpenes hydrocarbons [27].

3. Conclusion

As regard to chemical composition, 1.8-cineol is the major component of *Rosmarinus officinalis L.* essential oil in region of Talsint. Besides, camphor is present with relatively less concentration than other regions in Morocco. Nonetheless, there is certain variability concerning the percentage of the chemical components among the studied rosemary essential oil. The origin of this variability remains unclear for the majority of the constituents except for the 1,8-cineole concentration which decrease in the high altitude. Finally, we mention that Talsint's rosemary essential oil showed an important anti-oxidant activity that could be used in food industry as non-synthetic anti-oxidant.

Acknowledgement:

We would to thank Regional Service of Forest of the Oriental for supporting our work during the samples collection.

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