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The Chemical compositions and the Antioxidant and Antimicrobial Activities of the Essential Oil of *Rosemary* Leaves from Eastern Morocco

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Abstract

The essential oils compositions of *Rosmarinus. tournefortii* wild plant, *Rosmarinus. tournefortii* domesticated plant, and *Rosmarinus officinalis.L* wild plant growing in different bio climates from eastern Morocco, was determined by GC-FID and GC–MS. Oils were assessed for their antimicrobial and antioxidant activities. A variation of chemical compositions attributed to varieties rather than to bio climates was revealed. α-Pinene (0.637% ; 44.22% ; 5.74%), Camphene (11.62% ; 6.52% ; 2.21%), ß-Pinene (14.72% ; 1.14% ; 3.71%), 1,8-Cineole (10.1% ; not identifying ; 56.51%) and Camphor (39.27% ; 7.64% ; 13.56%) were identified as the main constituents of *R. tournefortii* wild plant, *R. tournefortii* domesticated plant, and *R. officinalis*. L wild plant respectively. This study is based on the determination of the diameter of inhibition to moderate antimicrobial and antioxidant activities of oils revealed to be against eight bacteria tested. This was determined by 1,1-diphenyl-1-picrylhydrazyl (DPPH) assay. The highest antimicrobial and antioxidant activities were found in oils from *Rosmarinus. tournefortii* domesticated plant.

Keywords: Rosmarinus officinalid.L, Rosmarinus. Tournefortii, wild plant, domesticated plant, antioxidant activity, antibacterial activity, GC/MS , GC/FID, Morocco,

1. Introduction

An antioxidant may be roughly defined as "any substance that when present at low concentrations, lower than the oxidizable compound to be protected, significantly delays or inhibits its oxidation". There are two basic categories of antioxidants, natural and synthetic. The second one has been found to cause long-term toxicological effects, including carcinogenicity [1, 2]. Consequently, there is an increasing interest in finding naturally occurring antioxidants for food and medicinal applications.

The extraction of natural substances to replace synthetic food preservatives has become increasingly more important [3-10].

Rosemary plants grow worldwide and have been cultivated since a long time ago for its strong antioxidant and antimicrobial activities. This plant species also has many other beneficial activities such as antiviral, anti-inflammatory and anticarcinogenic [11-15] activity. This species is considered to be one of the most important sources of both volatile and non-volatile bioactive compounds [16, 17]. Significant variations in the chemical composition of rosemary essential oils have been reported in relation to the geographic origin [16]. Moreover, variations in the antioxidant and antimicrobial properties of rosemary oils from natural populations were also detected. The latter variations were found to be due to regional, environmental and agronomic conditions, the time of harvest, the stage of development of plants, the method of extraction and methodologies used to evaluate their biological activities [18-22]. Although many works have dealt with the antimicrobial and antioxidant activities of the essential oils, the correlation between the presence and content of specific compounds and their activity and mechanisms of action has not been investigated [23-25].

The Herbs and spices have been used for many centuries to improve sensory or flavour characteristics and to extend the shelf life of foods. As a result, considerable research has been carried out assessing the antioxidant

activity of many herbs, herbal extracts and essential oils when added to a variety of foods and food model systems. The advantage of essential oils is their bioactivity in the vapour phase, a characteristic that makes them useful as possible fumigants for stored commodity protection. Antimicrobial packaging containing essential oils is a form of active packaging that could prolong the shelf-life of food product and provides microbial safety for consumers. It exerts its effect by reducing, inhibiting, or retarding the growth of microbial pathogens in packed foods and packaging material [26, 27]. It is this trade mark of the essential oils that makes them attractive targets for future research in food industry. The present work was undertaken to determine the compounds responsible for the antioxidant and antibacterial activities of essential oils from two phenotypes of rosemary growing in eastern Morocco.

2. Materials and methods

2.1. Plant material

Herbarium information of the plant species, which are individually numbered, is listed below:

- *Rosmarinus. officinalis.L* wild plant: location in the region of Jerada (el aounat) in eastern Morocco.
- *Rosmarinus Tournefortii* wild plant: location in the region of Tafoughalt in eastern Morocco.
- *Rosmarinus Tournefortii* domesticated plant: location in same region of Tafoughalt in eastern Morocco.

The plants were dried in the laboratory away from sunlight. Thereafter, the dried aerial parts were submitted to Hydrodistillation for 3 h using Clevenger type apparatus, according to the European Pharmacopoeia (1996). The essential oil was collected, dried over anhydrous sodium sulphate and stored at 4°C until used. The identification of the species was confirmed and a voucher specimen was preserved in Laboratory of Chemistry Bio Analytical, Toxicology and Physical Applied chemistry, Institute of Pharmacy, libre University of Brussels, Brussels, Belgium.

2.2. Gas chromatography

Essential oil samples (0,1µL) were injected neat into an HP 6890 gas chromatography equipped with a flame ionisation detector (FID) and a 30 m× 0,25 mm HP-5 (cross-linked Phynel-methyl Siloxane) column with 0,25 µm film thickness (Agilent), was used for the study. Helium was used as carrier gas, the flow through the column was 1,4mL min^{-1} and the splitless mode was used. The column was of 10°C min^{-1} and finally raised from 230 to 280 at rate of 30°C min^{-1}.

2.3. Chromatography-mass spectrometry analysis

The oil was analysed by gas chromatography-mass spectrometry (GC-MS) using a Hewlett Packard 6890 mass selective detector coupled with a Hewlett Packard 6890 gas chromatograph. The MS operating parameters were as follows: ionisation potential, 70 eV; ionisation current, 2 A; ion source temperature, 200°C, resolution, 1000. Mass unit were monitored from 30 to 450 m/z. Identification of components in the oil was based on retention indices relatives to n-alkanes and computer matching with the WILLEY 275. Library as well as by comparison of the fragmentation patterns of mass spectra with those reported in the literature [28]. The chromatographic conditions were identical to those used for GC/FID analysis.

2.4. Antibacterial Activity of Oils

Antibacterial activity of essential oils was screened using the wet disc diffusion method [29]. Agar cultures of Gram-negative bacteria of Salmonella sp, Klebsiella sp, Pseudomonas sp and Escherichia coli, and Gram- positive bacteria of Streptococcus sp and Staphylococcus aureus were prepared. This method can be explained as following: A 16-h culture was diluted with sterile physiological saline solution with reference to the MC Farland 0.5 standard to achieve an inoculum of approximately 10^6 CFU/ml a suspension was swabbed in three directions on 4 mm thick Mueller Hinton agar (MHA) (Oxoid, England) with a cotton swap. Sterile, 6mm diameter stainless steel cylinders were placed on plats of MHA, were impregnated with 10µl of the oil and were placed on the inoculated plates (one disc per box). Then, these plates were incubated for 24h at 37°C. The diameters of the inhibition zone were measured in millimetres.

2.5. Free radical-scavenging activity: DPPH test

Radical scavenging using the DPPH radical is the main antioxidant assay used to investigate the mechanisms by which antioxidants act in food. We studied the free radical-scavenging activity of the EOs by the original method of [30] with some modification. We made the final test solution (3 mL) by adding 0, 6 ml of various concentrations (11 µl/ml, 20 µl/ml, 40 µl/ml and 60 µl/ml) of the each sample were diluted in methanol and mixed with 2,4 ml of a 0.004% methanol solution of DPPH(101.44µM). After a 30 min incubation period at room temperature, the absorbance was read against a blank at 517 nm. Inhibition of free radical DPPH in percent (I%) was calculated in following way:

$$I\,\% = \frac{(A_{blank} - A_{sample})}{A_{blank}} \times 100$$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. Extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotting inhibition percentage against extract concentration. Tests were carried out in double.

3. Results and discussion

3.1. Chemical composition of the essential oil

The results obtained by GC-FID and GC–MS analyses of the essential oils *Rosmarinus officinalis.L* wild plant, *Rosmarinus tournefortii* wild plant and *Rosmarinus Tournefortii* domesticated plant are presented in Table 1:

Table 1 Chemical compositions of the Essential Oil of Rosemary Leaves from Eastern Morocco

				Rosmarinus. tournefortii	*R.tournefortii*. domesticated	*Rosmarinus. officinalis.L*
N°	COMPOUNDS	RT	RI	% Compound	% Compound	% Compound
	Monoterpene hydrocarbons			**35.79**	**63.88**	**12.42**
1	Tricyclene	4.59	813	2.04	0.37	--
2	α-Pinene	6.78	916	0.63	44.21	5.74
3	Camphene	7.08	929	11.62	6.51	2.21
4	β-Pinene	7.44	944	14.72	1.13	3.71
5	Myrcene	8.10	971	3.30	3.28	0.75
6	α -phellandrene	8.46	986.7	--	1.35	--
7	α-Terpipene	9.28	1020	1.22	0.28	
8	1-methyl-4-(1-methylethyl) benzene	9.32	1020.9	--	0.25	--
9	Limonene	9.36	1022.5	--	3.85	--
10	Trans-β- ocimene	9.38	1024	--	0.33	--
11	γ-Terpinene	9.39	1025	2.24	1.52	--
12	Terpinolene	9.40	1027	--	0.75	--
	Monoterpenes oxygenated			**62.68**	**17.63**	**77.65**
13	1.8-Cineole	9.47	1028	10.09	--	56.50
14	(+)-2-Carene	10.12	1054	0.27	--	--
15	Linalool l	11.15	1096	--	2.28	1.70
16	Bicyclo[2.2.1]heptan-2-ol. 1.3.3-trimethyl-. (1r-endo)	11.62	1116.4	--	0.54	--
17	Allo-ocimene	11.78	1123	0.88	0.27	--
18	Camphor	12.33	1146	39.27	7.64	13.56
19	Pinocarvone	12.69	1161	2.42	--	--
20	1-Borneol	12.78	1165	3.98	6.52	2.92
21	Terpinen-4-ol	13.03	1175	1.78	0.36	--
22	(-)α.-Terpineol	13.35	1188	1.10	--	2.95
23	Nopol	13.66	1201	1.49	--	--
24	Verbenone	14.59	1242	0.58	--	--
25	Bornyl acetate	15.53	1283	0.78	--	--
	Sesquiterpene hydrocarbon			**0.73**	--	--
26	α-Copaene	18.44	1418	0.73	--	--

Nineteen, Eighteen and Nine compounds were identified in the essential oils of *R. tournefortii wild plant, R. tournefortii domesticated plant and R. officinalis.L wild plant*, respectively. As a result of GC-FID and GC–MS analyses, *R. tournefortii wild plant, R. tournefortii domesticated plant and R. officinalis.L wild plant*. contained α-Pinene (0.637% ; 44.22% ; 5.74%), Camphene(11.62% ; 6.52% ; 2.21%), ß-Pinene (14.72% ; 1.14% ; 3.71%), 1,8-Cineole (10.1% ; not identifying ; 56.51%) and Camphor (39.27% ; 7.64% ; 13.56%) respectively, as the major compounds. In addition 1,8-Cineole (56.51%) was present in oil of *R. officinalis.L wild plant.* The result obtained is similar to that previously reported by different authors from different countries: in Iran [31], Morocco [32], France [33], china [34], Serbia and Montenegro [35], Tunisia [36] and Turkey [37], while Camphor (39.27%) was an additional compound in oil *of Rosmarinus tournefortii wild plant*, the result obtained is similar to that found by O.O. Okoh & al in South Africa [21] and for α-Pinene (44.22%) was an additional compound in *R. tournefortii domesticated plant*, the result obtained is similar to that previously reported by different authors from different countries: in Algeria [38], Iran [39] and in Serbia [40]. Differences in oil composition of Rosemary have already been reported [41, 42].

The monotepenes hydrocarbons (35.8% ; 63.88% ; 12.42%) respectively, represented mainly by α-Pinene, Camphene, ß-Pinene, Myrcene, formed the major group. There were some reports of the presence of alpha-Pinene, 1,8- cineole, Camphor, Verbenone and Borneol, constituting about 80% of the total *Rosmarinus officinalis L. plant* oil [25]. The major components, alpha-Pinene, Borneol, Camphene, Camphor, Verbenone and Bornyl-Acetate, were also reported to be present in Sardinian R. Officinalis L. oil [43]. Compounds, such as Camphene, Camphor, Verbenone and Borneol, reported as the major compounds, were also present in our oil at a total contribution of 55.46% for *Rosmarinus tournefortii wild plant* (Table 1), for *R. Tournefortii domesticated plant* present in our oil at a total contribution of 20, 67% and *R. officinalis.L wild plant* present in our oil at a total contribution of 18.7%. These variations in chemical compositions of rosemary could be attributed to climatic effects on the plants that are growing in different habitats [41].

3.2. Antimicrobial activity

The antibacterial activities of essential oils from rosemary leaves growing in eastern Morocco against the microorganisms, was qualitatively and quantitatively assessed by the presence or absence of inhibition zones. Table 2 reports the inhibition zone of essential oils determined for 6 of Gram positive and Gram negative bacteria using the diffusion technique on solid media.

Table 2 Antibacterial activity of rosemary leaves essential oils as determined by diffusion technique on solid media

Micro-organisms	Zone of inhibition of the essential oils (mm)		
	R. officinalis.L wild plant	*R. tournefortii* wild plant	*R. Tournefortii* domesticated plant
Bacteria Gram -			
Salmonella sp	9	9	19
Klebsiella sp	15	9	32
Pseudomonas sp	10	**0**	11
Escherichia coli	14	9	20
Bacteria Gram +			
Streptococcus sp	10	9	30
Staphylococcus aureus	15	10	**40**

The results showed that the essential oil had a substantial inhibitory effect on all assayed bacteria strains noted by large growth inhibition halos.

The data indicated that Gram-positive Staphylococcus aureus was the most sensitive strain tested to the oil of *R.Tournefortii* domesticated plant with the largest inhibition zone (40 mm). Also the Streptococcus sp, in general, found to be the most sensitive among Gram-positive bacteria of *R.Tournefortii* domesticated plant with inhibition zone of 30 mm. The Modest activities of *R. tournefortii* wild plant were observed against Streptococcus sp, with inhibition zone of 9 mm. Gram-negative strains also displayed variable degree of susceptibility against investigated oil. Maximum activity of *R.Tournefortii* domesticated plant was observed against Escherichia coli with inhibition zone of 20 mm. Modest activity were observed against Pseudomonas sp by essential oil *R. tournefortii* wild plant with inhibition zone of 0 mm.

The essential oil from *R. officinalis* has been reported to be weakly inhibitory against E. coli, S. aureus and L. monocytogenes as compared to other oils [44]. Inhibition zones of E. coli and L. monocytogenes, on exposure to *R. officinalis* oil-rich fractions, were about 17 mm [25] and about 16 mm [41]. And for ten (10) bacteria selected by A.I. Hussain and al [45] the inhibition zones from has been included (14 – 24.4 mm).

The results of *R. officinalis* in this study are little different from that of the above report. Under equal conditions, the difference in the diameter of zones of inhibition can be attributed to the techniques employed. The major components of this oil, 1,8-cineole, has been known to exhibit antimicrobial activity against the bacterial strains (E. coli, P. aeruginosa, S. typhi, S. aureus, rhizobium leguminosarum, and bacillus subtilis) [46]. Although Terpinen-4-ol represents a minor constituent in the oil under study, it is known to have very efficient antibacterial properties [47].

3.3. Antioxidant Activity of Essential Oils:

The DPPH radical scavenging activity of the essential oil might prevent reactive radical species from damaging biomolecules such as PUFA, DNA, protein and sugars in susceptible biological and food systems [48]. First, we studied the radical scavenging capacity (RSC) of the three essential oils from rosemary leaves by the original DPPH test of [30]. Antioxidant activities of essential oils from aromatic plants are mainly attributed to the active compounds present in them. This can not only be due to the high percentage of main constituents, but also to the presence of other constituents in small quantities or to synergy among them. In this study, the antioxidant activity of essential oils of rosemary leaves growing in eastern Morocco compared with Ascorbic acid (IC50= 24.88 ± 0.48 µl/ml with r^2= 0.98 and in triplicate (n=3)) as a reference anti-oxidant compound were determined by the method of DPPH radical scavenging assay and the results are summarized in table (3). The EO from the *R.Tournefortii* domesticated plant was the most active with (20.17 ± 1.04 µl/ml), followed by the oils from the *R. tournefortii* wild plant (28.97 ± 0.86 µl/ml) and then from *R. officinalis.L* wild plant with (37.95 ± 1.11 µl/ml). It was found that the essential oils of *R.Tournefortii* domesticated plant and the *R. tournefortii* wild plant showed good antioxidant capacities compared with Ascorbic acid.

Table 3 Increase in the DPPH scavenging ability increasing the EO concentration.

concentrations (µl/ml)	(DPPH) RSA% (n=2)		
	R. officinalis.L wild plant	*R. tournefortii* wild plant	*R. Tournefortii* domesticated plant
11	32.06± 0.82	33.28± 1.01	39.76± 1.25
20	36.81± 1.13	44.73± 1.06	51.50± 0.88
40	55.13± 1.1	63.06± 0.98	69.83± 1.09
60	62.41± 1.67	70.34± 1.23	77.11± 0.74
Calibration	y=0.6527x + 25.226	y=0.7548x + 28.134	y=0.7592x + 34.689
Curve	r^2=0.9673	r^2=0.948	r^2=0.9471
IC_{50} *(µl/ml)*	37.95 ± 1.11	28.97± 0.86	20.17± 1.04

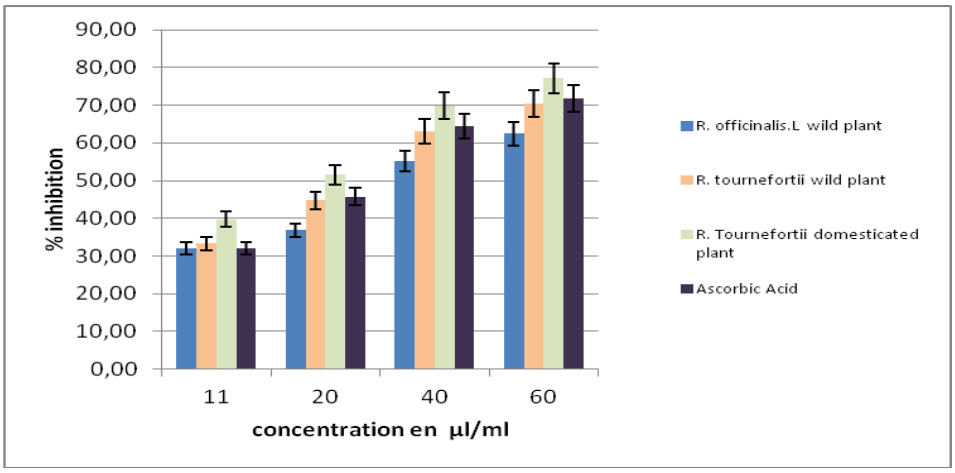

Figure 1: Free radical-scavenging activities (%) of rosemary leaves growing in eastern Morocco essential oil and Ascorbic acid measured by DPPH assay.

Fig. 1 shows the scavenging effect of the essential oil of rosemary leaves growing in eastern Morocco on the DPPH radical. DPPH is a stable radical that loses its purple colour when it accepts an electron from an antioxidant molecule. Ascorbic acid was used as the reference standard. The essential oil exhibited a concentration dependant scavenging of DPPH radicals comparable to the reference standard. In this study, the essential oils of rosemary leaves are found to possess remarkable radical-scavenging and antioxidant activities. The obtained herein were found to be in agreement with the findings of several authors who reported that the efficiency of an antioxidant component to reduce DPPH essentially depends on its hydrogen donating ability, which is directly related to the presence of the abundance of monoterpenes hydrocarbons [46] and oxygenated monoterpenes [49]. Then, the results obtained are very similar to that found by [46] who reported that the monoterpene hydrocarbons had a significant protective effect due to the different functional groups.

Conclusion

In conclusion, we have developed a strategy to isolate the essential oil from the leaves of rosemary growing in eastern Morocco and analyse it by GC-FID and GC–MS. The major components were α-pinene (44.22%) in *R.Tournefortii* domesticated plant; camphor (39.27%) , β-pinene (14.72%) , camphene (11.62%) , 1,8 cineol (10.1%) in R. tournefortii wild plant; and 1,8 cineol (56.5%) , camphor (13.56%) in *R. officinalis.L* wild plant. The biological evaluation in this study suggested that the essential oils of the leaves of rosemary growing in eastern Morocco, particularly the *R.Tournefortii* domesticated plant, exhibited a potent broad spectrum of antimicrobial and antioxidant activities which could be a natural alternative to synthetic preservatives to enhance the safety and the shelf life of food.

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