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Investigation of Antibacterial and Antioxidant Activities of Rosemary Essential Oil from Algeria.

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ABSTRACT

The purpose of this study was to characterize the chemical constituents, antibacterial and antioxidant activities of *Rosmarinus officinalis* L. (Rosemary) essential oil from Algeria. The essential oil was obtained by hydrodistillation method. GC-MS investigation of the produced oil showed detection of thirty compounds. The main constituents identified were 1,8-cineole (50,1 %), camphor (11,1 %), α-pinene (8.7 %) as major compounds. Biological activity of essential oil was examined against eight bacterial species *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Salmonella* spp, *Bacillus subtilis, Staphylococcus aureus* and *Listeria monocytogenes*. The obtained results have shown that the essential oil exhibited moderate to strong antimicrobial activity against the tested microorganisms. The essential oil was also subjected to a biological screening for its possible antioxidant activities by means of DPPH radical scavenging test, the sample tested showed an average antioxidant activity in comparison with the positive control (Ascorbic acid). This results suggested that the *Rosmarinus officinalis* L. essential oil possesses a good antimicrobial and antioxidant properties, and is a potential source of active ingredients for food and pharmaceutical industry.

Keywords: *Rosmarinus officinalis* L., essential oil, chemical constituents, antibacterial property, antioxidant activity.

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INTRODUCTION

Rosemary (*Rosmarinus officinalis* L.) is an aromatic, medicinal and condiment plant that belongs to the family Labiatae. It is widely spread in Algeria and broadly used in traditional medicine [1]. Different parts of plants have been used to obtain essential oil. These include flowers, leaves, seeds, roots, stems, bark, and wood though secretionary parts. Rosemary essential oil is of immense medicinal importance for its powerful antimutagenic, antiphlogistic, antioxidant, chemo-preventive and antibacterial properties, antiinflammatory, antiseptic, antispasmodic and anti-diabetic [2-3]. The essential oil of *Rosmarinus officinalis* L. has been the object of several studies as antioxidant activity [4-5], antibacterial [6, 7- 8], toxicity insecticidal [9-10], antiinflammatory and Antinociceptive [11], antifungal [12-13] and recently as a pest control product.

MATERIALS AND METHODS

Plant material

Leaf sample of *Rosmarinus officinalis* L. growing wild in Azzaba located at Skikda city (North-east of Algeria) were collected on June 2013. The taxonomic identity of the plant was confirmed by the well-known Algerian flora of Quezel and Santa [14].

Isolation of the essential oil

The extraction of essential oil from the leaves of rosemary was performed by hydro distillation in a Clevenger-type apparatus [15]. We conducted three distillations by boiling 200 g of dry plant material. The extraction time was about 03 hours in average. Yields are expressed in ml per 100 g of dry matter. The essential oil obtained was dried by anhydrous sodium sulfate and then stored at low temperature (below 4°C) and dark before use.

GC-MS analysis

1 µl of the oil was diluted in 1ml hexane. 1 µl of the diluted oil was used for the analysis. GC-MS analysis was carried out on Gas Chromatography- Mass Spectrometer-Quadrupole (QP) -2010- SHIMADZU, with (30 m × 0.25 mm i.d, film thickness 0.25 µm) capillary column at flow rate 1.74ml/min. Injection temperature was 280°C and injection mode was split. The detector temperature was 280°C. Helium was used as the carrier gas with velocity 47.6 cm/sec. Oven temperature programmed at 45°C for 4min, then was raised to 240°C at rate 6°C/min, hold time 2min, then raised to 280°C at rate 39°C/min. The interface temperature was 260 °C. The quadrupole mass spectrometer scanning range was 40-350 m/z. Identification of the components of the oil was achieved by comparing their mass spectra with those from NIST library database (2008) and by comparison with the available references and published data.

Test organism

Microorganisms were obtained from the Bacteriology Laboratory, Faculty of Medicine, HCU of Dorban in Annaba (Algeria). Five strains of gram-negative bacteria [*Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 15380), *Pseudomonas aeruginosa* (ATCC 27853), *Proteus vulgaris* (MTCC 1771) *Salmonella* spp. strains (Samples (date))] and three strains of gram-positive bacteria [*Bacillus subtilis* (MTCC 441) and *Staphylococcus aureus* (ATCC 25923) *Listeria monocytogenes* (ATCC 19112)] were used. The cultures of bacteria were maintained in their appropriate agar slants at 4°C throughout the study and used as stock cultures.

Antibacterial assay

Screening of essential oil for antibacterial activity was done by the disk diffusion method, which is normally used as a preliminary check and to select between efficient essential oils [16]. The strains were reactivated using a 20 h culture growth at 37 °C and adjusted to 10^8 CFU/mL. The bacterial strains are sowed on the surface of the agar in radial spots form by means of swab and suspensions of young bacterial cultures

prepared according to the CLSI (committee for laboratory standards institute). The application is made by sterile filters paper disks (6 mm diameter, 06/limp) which were placed on the inoculated agar surfaces and impregnated with 10 µL of the solution; the plates were incubated during 24 h at 37 °C [17]. The reading of the results is made by the measurement of the inhibition diameter around the disk.

Antioxidant activity

The antioxidant activity of Rosemary essential oil was measured in terms of hydrogen-donating or radical scavenging ability, using the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) as a reagent Burits and Bucar (2000) [18]. Fifty microliters of each concentration (200, 400, 600, 800 and 1000 µg/ml) of each sample was diluted in methanol and were added to 5 ml of methanolic solution of DPPH (0,004 %). The inhibition of the DPPH by the ascorbic acid was even analyzed at the same concentration for comparison. Absorbance measurements were read at 517 nm, after 20 min of incubation time at room temperature. Absorption of a blank sample containing the same amount of methanol and DPPH solution acted as the negative control. All determinations were performed in triplicate. The inhibition percentage of the DPPH radical by the samples was calculated according to the formula presented by Sharififar et *al.*, (2007) [19]:

$$\% \text{ inhibition} = ([A_{White} - A_{Sample}] / A_{White})$$

where:

A White: the absorption of the blank sample (t = 0 min).

A Sample: the absorption of the tested oil or substance solution (t = 20 min).

The kinetic reactions of the essential oil and the ascorbic acid with DPPH, were mentioned for every concentration tested. The essential oil and the ascorbic acid of the inhibited DPPH, were recognized at the end of the reaction in order to reach the index IC_{50}. This parameter is defined as the antioxidant concentration required to reduce the concentration of the initial DPPH of 50 %.

Time balance determination of TEC_{50}

TEC_{50} parameter was defined when time reached balance with an antioxidant concentration equal to IC_{50}. This time is graphically calculated.

Determination of the antiradical efficiency (AE)

The two factors IC_{50} and TEC_{50} were combined in order to get the Anti-radical efficiency:

$$AE = AA/IC_{50} \times TEC_{50}$$

Statistics analysis

The classical methods of statistics were used to calculate the average and the standards deviations. All measurements were performed in triplicate, and results were presented as an average ± standard deviation. Analyses of variation were realized by ANOVA with the software «SPSS». The probability of p inferior to 0.05 was admitted as a criterion of a significant difference.

RESULTS AND DISCUSSION

Yield and chemical composition of essential oil

The essential oil yield was estimated according to the dry vegetal matter by using the following equation: R_{EO} %= m_{EO} / m_S x 100. where m_{EO} = essential oil mass (g), m_S = dry vegetal matter mass (g) and R_{EO} = essential oil yield (%) [1].

The average yield of essential oil extracted from the plant is studied in the range of 0.8%. This performance is comparable to that obtained for the same species studied in the region of Tlemcen (Algeria) whose yield was 0.8 % for the wildlife species and 0.6 % for the cultivated species [20]. In contrast, this performance was revealed lower than that given by the same species from different regions in Tunisia [21] and Sardinia [22] whose performance can reach 1.2 % to 1.75 %. This difference can be attributed to several factors

such as climate and geographical conditions and the period of harvesting and drying conditions. It has been shown that drying affects the performance of essential oil: a plant dried in non-optimal condition may lose all of its essential oil [23].

Overall, essential oil of Moroccan Rosmarinus officinalis L. have a relatively constant chemical composition comparable to works Studies on Rosmarinus officinalis L. harvested in Morocco [24, 25], Algeria [20, 26], Tunisia [21], Spain and France [24], Italy [27], Turkey [28] and Iran [29] showed variations in the composition of essential oil.

The percentage composition and modes of identification of the oil component are listed in **Table 1**. The chromatographic analyses resulted in the identification of 30 compounds, representing 98.8 % of the oil, 1,8-cineole (50,1 %), camphor (11,1 %), α-pinene (8.7 %) were the major components.

Table 1. Chemical composition of *Rosmarinus officinalis* L. essential oil identified by GC-MS.

N°	Compounds	Retention time (min)	Percentage %
01	**α-Pinene**	6.16	**8.7**
02	Camphene	7.00	0.5
03	β -Pinene	7.69	4.2
04	Myrcene	8.00	2.2
05	β -Phellandrene	8.10	0.1
06	*p*-Cymene	8.32	1.7
07	**1,8-Cineol**	9.38	**50,1**
08	γ -Terpinene	10.34	0.4
09	Sabinene hydrate	10.59	0.4
10	Terpinolene	11.37	0.2
11	Linalol	12.01	3. 1
12	**Camphor**	14.98	**11,1**
13	Borneol	15.85	3.1
14	Terpinene-4-ol	16.24	0.4
15	α-Terpineol	17.60	3.4
16	Bornyl acetate	19.80	3.4
17	Thymol	20.89	0.1
18	Carvacrol	22.19	0.1
19	Eugenol	23.89	0.1
20	α-Copaene	24.36	0.3
21	β -Caryophyllene	24.80	3.1
22	α-Humulene	25.27	0.2
23	Germacrene D	28.69	0.4
24	α-Muurolene	40.75	0.3
25	α-Farnesene	60.24	0.4
26	γ -Cadinene	60.76	0.1
27	Calamenene	68.29	0.1
28	δ -Cadinene	72.55	0.2
29	Calacorene	73.87	0.3
30	α-Cadinene	73.95	0.1
			98.8

The chemical composition of the essential oil *Rosmarinus officinalis* L. grown in the region of Azzaba, Skikda city (Algeria) shows some differences by some Work **Table 2**.

Table 2. Chemical composition of the essential oil *Rosmarinus officinalis* L. growing in different regions in Algeria.

Author's	Region	Major constituent
Boutekedjiret et *al*.,(1998) [26]	Bibans (Algeria).	1,8-cineol (52.4%), camphor (12.6%).
Benhabiles et *al*., (2001) [30]	Bordj-Bou-Arreridj (Algeria).	(E)-β-caryophyllene (13,9%), camphor (12,1%), borneol (10,1%), α- terpineol (9,5%), 7,5% cineol.
Atik Bekkari et *al*., (2007) [20]	Tlemcen (Algeria).	For spontaneous Rosemary: α-pinene (23.1%), followed by camphor (15.3%) et le β-pinene (12.2%). For cultivated Rosemary, the main compound is camphor (13.8%), followed by α-pinene (12.6%), cineole (11.8%), borneol (10.8%).

This deviation from the common chemotypes may be attributed to the effect of the factors that specifically affect the composition and yield of the essential oil, which include seasonal and maturity variation, geographical origin, genetic variation, growth stages, postharvest drying and storage [31, 32- 33].

Antimicrobial activity

Various publications have documented the antimicrobial activity of essential oil constituents and plant extracts. In recent years, several researchers have also reported mono- and esquiterpenoids as the major components of essential oils, which are phenolic in nature. It seems reasonable to assume that their antimicrobial mode of action might be related to the phenolic compounds present [34-35].

The antimicrobial activity of essential oil would be related to the respective composition and structural configuration of the plant volatile oils, their functional groups and possible synergistic interactions between components [36].

Data on the antimicrobial activity of the different dilutions of Rosmarinus officinalis L. essential oil against several bacteria are summarized in **Table 3**.

Table 3. Results of the antibacterial activity of the essential oil of *Rosmarinus officinalis* L.

Microorganisms	Sensitivity*
Escherichia coli	++
Klebsiella pneumoniae	+++
Pseudomonas aeruginosa	++
Proteus vulgaris	+++
Bacillus subtilis	+++
Staphylococcus aureus	+
Listeria monocytogenes	+
Salmonella sp	+++

Each value represents the mean of two replicates ± standard deviation

*The sensitivity to the different strains was classified by the diameter of the inhibition zone as follows [37]:

-: diameter less than 8 mm, not sensitive;

+: sensitive, diameter 9-14 mm;

++: very sensitive, diameter 15-19 mm;

+++: extremely sensitive for diameter larger than 20 mm.

All microbiological results obtained during the study shows that all the products tested have a very significant antibacterial activity, in which some strains seem to be distinguished by a very high sensitivity. 1,8 cineole would also include compounds providing Rosemary antimicrobial properties [38]. In fact, for ages, spices like Rosemary are used to extend the shelf life of food. Studies on Rosemary extracts showed today that it can help reduce the multiplication of several microorganisms.

The results are in agreement with Marzouk et *al*., 2006 [39] which relate that antimicrobial activities of rosemary essential oil are not related only to the major compounds but also the minor components of the oil. The results are similar to those documented by Mounchid and Mounchid et *al*., (2005) [40] *Escherichia coli* is resistant to several antibiotics is susceptible to the essential oil of rosemary.

Rosemary essential oil with a chemical composition (α-pinene 15.34%, Camphene 4.85%, β-pinene 6.52 %, Eucalyptol 56.85 %, Camphor 12.99 %, Trans - caryophyllene 3.45 %) exhibited significant activity against *Klebsiella pneumoniae* with inhibition zone of about 16 mm [41].

Prabuseenivasan et *al*., (2006) [42] report that essential oil of rosemary has strongly and consistent inhibitory effects against various pathogens (*Pseudomonas aeruginosa, Proteus vulgaris, Bacillus subtilis, Staphylococcus aureus*).

In the case of the bacteria *Listeria monocytogenes* essential oil from *Rosmarinus officinalis* L. were applied in pure oil by the disk diffusion method from showed microbicide effect appeared: inhibitory zone 9 mm [43].

On the other hand, the essential oil strongly inhibits the growth of *Salmonella* spp with an inhibition diameter greater than 20 mm [44].

Antioxidant activity

The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogendonating ability. DPPH radical is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule [45]. The scavenging ability of the essential oil and positive control are presented in **Table 4.**

Table 4. DPPH scavenging activity (%) of *Rosmarinus officinalis* L. essential oil and standard antioxidant.

Concentrations (µg/ ml)	*Rosmarinus officinalis* L. essential oil	Ascorbic acid
200	15,46±0 ,035	26,58±0 ,077
400	17,15±0 ,040	32,27±0 ,097
600	21,93±0 ,045	51,89±0 ,155
800	29,30±0 ,060	71,77±0 ,217
1000	40,56±0 ,070	94,93±0 ,286

Kinetic reaction

The kinetic reactions of the free radical DPPH obtained for each concentration of the ascorbic acid and of the essential oil are mentioned in **Figure 1** and **2**.

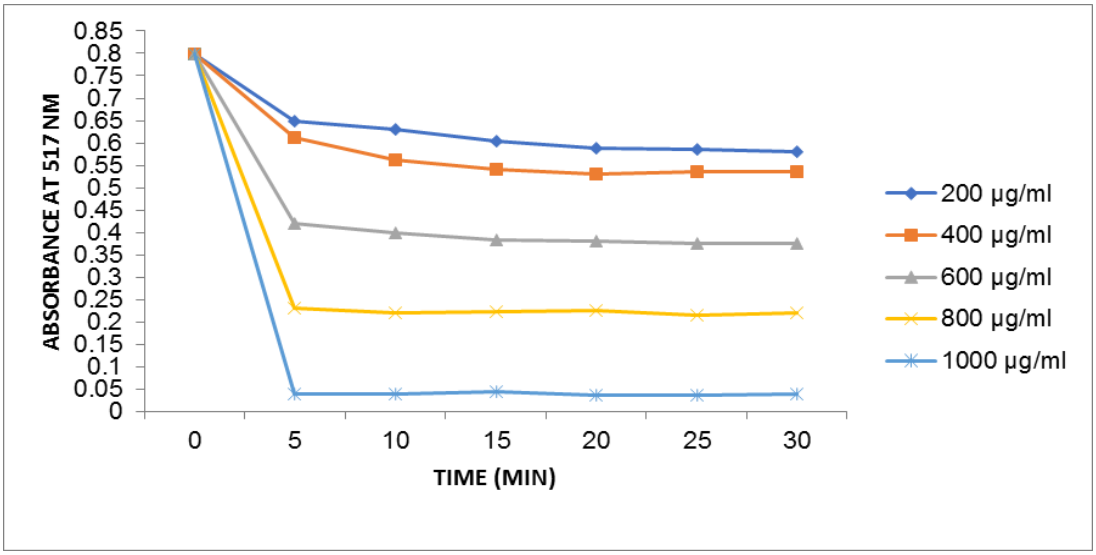

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Figure 1: Kinetic reduction of DPPH of ascorbic acid.

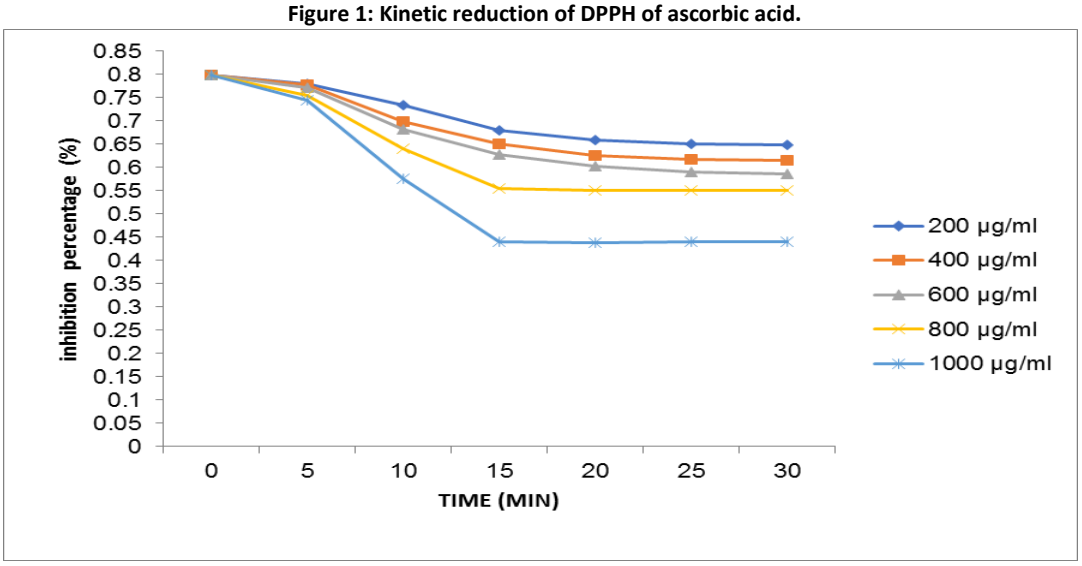

Figure 2: Kinetic reduction of DPPH of *Rosmarinus officinalis* L. essential oil.

From the results obtained, we found that the biphasic reaction has a quick weakening of absorbance during the first minutes, followed by a slow step till balance is reached, so we can distinguish two areas: area of strong kinetic of DPPH radical scavenging with radical scavenging absorbed during the first 5 minutes, as for the ascorbic acid for all concentrations during ten minutes for a concentration of 1000 µg/ml. This area is observed during the first fifteen minutes for the essential oil. A second area with a slow kinetic of DPPH radical scavenging a tendency zone towards a recognized balance after five minutes for all concentrations of the ascorbic acid except the concentration 1000 µg/ml. For the essential oil of this area, it is recognized after fifteen minutes. While making the reaction between DPPH and the ascorbic acid with hydrogen, we can recognize in this reaction that balance is reached in a short period of time compared to the essential oils. The antioxidant activity is dependent to the hydrogen atom movement of the hydroxyl group of the phenolic components of the essential oil. In presence of the free radical DPPH, the H atom is transferred to DPPH stable molecule. This induce a diminution in the concentration of the free radical and the absorbance during reaction till the weakening of the antioxidant capacity as a hydrogen donor. The inhibition percentage results of the radical DPPH are mentioned in Figure 3. We observe that the inhibition percentage of the free radical of the essential oil is low to those of the ascorbic acid for all concentrations used.

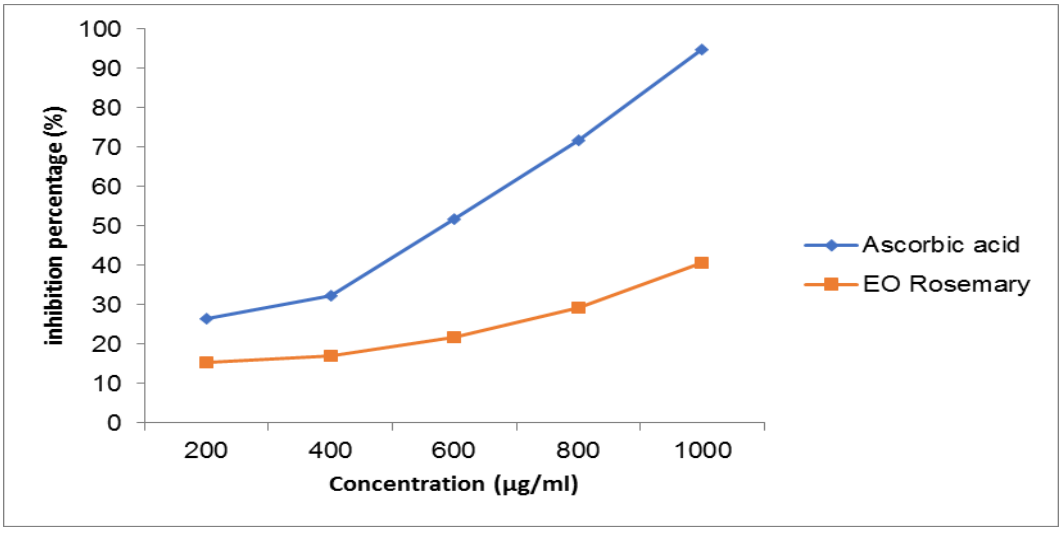

Figure 3: The inhibition percentage of essential oil and ascorbic acid.

We observe that the inhibition percentage of the free radical of the essential oil is low in comparison to those of the ascorbic acid for all concentrations used. For the highest concentration (1000 µg/ml), the essential oil revealed an inhibition percentage of 40,56 %, while the ascorbic acid is inhibited with 94,93 % of DPPH [46].

IC_{50} determination

The IC_{50} is the quantity of the antioxidant needed to reduce the concentration of the free radical DPPH to 50 %. We have chosen the state of balance as a period of measurement where growth reaction can't go further. Timing of balance state depends on the reactivity of the essential oil and the concentrations used. We recognize that the ascorbic acid reacts rapidly with DPPH. The IC_{50} for essential oil studied was 17±10 min, so the ascorbic acid needs just 08±0,6 min to reduce the concentration of the radical free to 50 %. It`s well known that not only the main components of the essential oil are responsible for the antioxidant activity, this activity may be attributed also to the minor components that may interfere in synergy and antagony to create this system against the free radicals [47].

CONCLUSION

Rosemary (*Rosmarinus officinalis* L.) has received special attention over the past few years both for its antibacterial properties, mainly attributed to the presence of phenolic compounds. The chromatographic analysis of the essential oil of *Rosmarinus officinalis* L. allowed the identification of 30 different compounds dominated by 1,8-cineole. Our results confirm that many essential oils possess antimicrobial activities against pathogens. The investigated essential oil may be used for the preservation of processed foods as well as pharmaceutical and natural therapies for the treatment of infectious diseases in humans and plants.

Therefore, studies concerned with the essential oils lie not only in the chemical characterization but also in the possibility of linking the chemical contents with particular functional properties. Our interest was at the same time based on the antioxidant activity of the essential oil of the *Rosmarinus officinalis* L. for the purpose to find new natural antioxidant in order to avoid the use of synthetically ones which may some of them be toxic or carcinogenic. The Results obtained confirm that the medium antioxidant potential of the essential oil of this plant according to others. These results keep an open perspective for research of formulations on the basis of essences of the *Rosmarinus officinalis* L.in place of other synthesis preservatives or antioxidant on the basis of plant used in the field of food industry, pharmaceutical and cosmetics industry.

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