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Assessment of the chemical/biological activities of extracts and essential oil of Rosmarinus Officinalis L. from the Oriental region of Morocco

Ab. Oussaid[(a)]* , M. Azzouzi[(a)], A. Ibn Mansour[(b)], M. Azouagh[(a)], M. Koudad[(a)], Ad. Oussaid[(a)]

[(a)] *Laboratory of Molecular Chemistry, Materials and Environment, Multidisciplinary Faculty of Nador, B.P. 300, Selouane, Nador 62700, Mohammed First University Oujda, Morocco.*

[(b)] *Laboratory of Applied Organic Chemistry, Faculty of Sciences of Tétouan, Abdelmalek Essaadi University, Morocco.*

** Corresponding author: aoussaid69@gmail.com*

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Abstract

The interest in natural conservatives, antimicrobial and antioxidants have slightly increased due to their advantageous effects. In this work bioactive compounds were obtained from Rosmarinus officinalis L. At first, the extractions of the essential oils by hydrodistillation was performed. The obtained essential oils from rosemary were yielded of about 1.8%. The GC-MS revealed that the rosemary oil was dominated by 1,8-cinéol (52.440 %), Camphor (11.241 %), and α-pinene (8.225%). The extraction of the dry powders using maceration and soxhlet with solvents of different polarity showed a significant yield for ethanol compared to that of hexane. In the same context, the phytochemical screening revealed the richness of the studied plant with secondary metabolites.The antioxidant activity was assessed in vitro using scavenging activity of the free radical DPPH. It revealed that the essential oil and all ethanolic extracts tested using ascorbic acid as a reference, exhibit anti-radical activities. The ethanolic extract of Rosemary leaves showed the highest anti-radical activity (IC50 = 64.27µg/ml), followed by the extract of the latter's stems with an IC50 = 64.74µg/ml. The microbiological results showed that the antimicrobial activity was variable from one bacteria to another, this is due to the variability of the chemical composition of each extract.

Keywords: *Essential oil, Gas chromatography, Phytochemical screening, Antioxidant activity, Antimicrobial activity, Rosmarinus officinalis L.*

1. Introduction

Currently, the development of microbial resistance to antibiotics and the negative effects of synthetic antioxidants, encourage their substitution by natural agents. This led researchers to take an interest in the plant world and especially medicinal plants in search for effective natural molecules with fewer side effects.

The Mediterranean basin is characterized by a large quantity of aromatic plants, producing essential oils and metabolites with low molecular weight. These plants belong to different botanical families and their presence is important in deciding the potential interference within the ecosystem [1]. The Moroccan flora have a considerable biodiversity, it has many aromatic and medicinal plants rich in secondary metabolites with significant therapeutic and pharmacological characteristics. In order to promote the natural resources of our region, we have been interested in studying the Rosmarinus officinalis L. from the Oriental region of Morocco. Rosemary (Rosmarinus officinalis L.) has a considerable importance in term of its great important medicinal and aromatic value [2-9]. This plant belongs to Lamiaceae family, common plant in the wild spread on all the shores of the Mediterranean especially in arid and rocky scrubland, on calcareous grounds. The plants of this family are herbaceous (or more or less woody) of the family Lamiaceae (or labiae) [10-14]. In Morocco, the plant grows spontaneously in the forests of the Rif, the great and Middle Atlas. It is also grown in gardens as an aromatic and ornamental plant. Noting that rosemary is the most exported herb by Morocco (12.70% of all dried PAM). The United States of America is the largest customer of this product (45%). They are followed by France (15%) and Spain (10%) (SNDS des PAM, 2008) [15, 16]. Rosemary is an evergreen shrub with straight stems, very ramose, 50 cm to 1 meter and more, whose long, slender branches bear numerous sessile and opposite leaves about 2.5 cm in length, with a hard-upper surface and green, while the underside is woolly, whitish, and glandular. The edges are rolled up and the central rib makes a strong projection on the underside. Rosemary has whorls of purple flowers. The upper edge of the corolla has two lobes and the lower edge three; only the pair of anterior stamens develops. Flowering begins in the months of January-February and continues until April – May [17]. Rosemary herbs have been widely used in the traditional medicine and cosmetics [2-9]. They are also used as flavoring agents in foods [18-20]. R. officinalis essential oil is also important for its medicinal uses and its powerful antibacterial, cytotoxic, antioxidant, antichloristic and chemopreventive properties [21-23]. The purpose of this study is to evaluate the antioxidant and the antimicrobial activity of essential oils and organic extracts of various solvents and extraction techniques.

2. Materials and methods

2.1. ***Collection of Plant Materials***

Rosmarinus officinalis L. was collected from the city of Taourirt (34.3984° N, 2.8935° W) of the oriental of Morocco. The plant was harvested at the beginning of its flowering period.

2.2. ***Essential Oil Extraction by Hydrodistillation***

The fresh aerial parts of the plant (100 g) was mixed with 1L of distilled water and submitted to hydrodistillation for 3 hours using a Clevenger-type. The essential oils were dried over anhydrous sodium sulfate, and stored without exposure to light at a temperature 4°C until analyses [24, 25].

2.3. ***GC-MS Analysis of Essential oils***

The essential oil was analyzed on GC-MS Hewlett Packard equipped with mass spectrometry of the same model using HP-5-MS (cross-linked Phynel-methyl Siloxane) capillary column (30 m x 0.25 mm internal diameter x 0.25 µm film thickness). Helium was used as the carrier gas with a rate of 1,4ml/min. 1 µl oil was introduced according to the

splitless mode. The mass spectra were recorded in electron ionization mode at 70 eV with scanning from 50 to 600 m/z and ion source temperature was set at 200 °C. Essential oils components were identified based on their retention indices, and by comparison of their mass spectral fragmentation patterns with those reported in literature.

2.4. ***Preparation of organic extracts***

The first step in the preparation of the organic extracts is the grinding of the dried plant material (stems, leaves of rosemary were dried for 4 weeks) using an electric grinder, the powder obtained was stored in paper bags at room temperature in a dry place until it is used. The choice of the extraction solvent is based on the nature of the species to be extracted in a given solvent by its penetration capacity in the plant material, its solubilization power and its material transfer properties. In our study we chose to work with two organic solvents of very different polarity: hexane and ethanol. The extraction process in our work is shown schematically in the following figure 1.

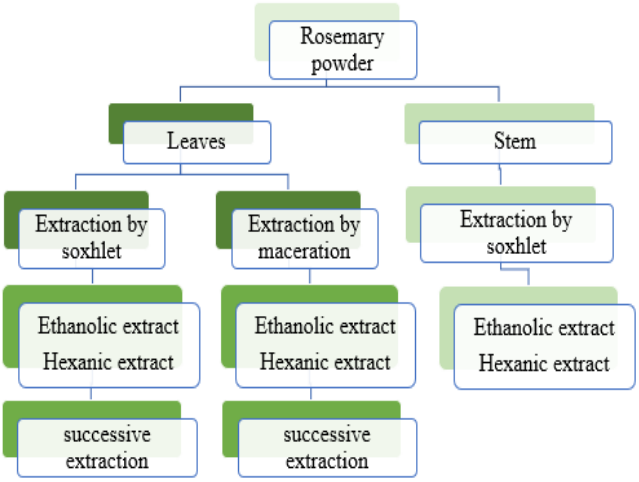


Figure 1. Flowchart of the essential oil extraction process.

2.4.1. Extraction by soxhlet system

25g of plant powder are weighed and put in Soxhlet cartridges. The solvent (250 ml) is introduced into the flask and then heated to start extraction. The extraction is generally stopped after 24 hours when the liquid surrounding the cartridge becomes clear the extract was filtered through Whatman No. 4 paper and finally the filtrate is concentrated in a rotavapor at 50°C and stored in the dark at -14°C until analysis. The successive extraction by two solvents carried out from 30 g of the powdered plant material that were extracted with hexane for 24 hours, after filtration, this filtrate extracted a second time with ethanol for 24 hours under the same conditions. At the end of the extraction, the ethanolic extract was concentrated on a rotary evaporator and then preserved with the other extracts already prepared.

2.4.2. Extraction by maceration

30 g of powder are macerated in 300 ml of solvent under mechanical stirring for 24 hours at room temperature. The macerate was then filtered through Whatman No. 4 paper and then concentrated by rotary evaporator and stored in the dark at -14° C until analysis. The successive maceration with two organic solvents were prepared successively by maceration 30 g of the powdered plant material with hexane for 24 hours, after filtration, this filtrate extracted a second time with ethanol for 24 hours under the same conditions. The macerate was then filtered through Whatman No. 4 paper and finally the filtrate is concentrated and stored until used.

2.5. ***Phytochemical screening***

Preliminary qualitative phytochemical analysis was carried out to have initial information on the chemical composition, such as polyphenols, alkaloids, flavonoids, tannins and steroids. The detection of these compounds is attested by the formation of a precipitate, change of color or turbidity.

The results are expressed using the symbols + and - as follows.

- the + symbol indicates a positive test
- the - symbol indicates a negative test

2.5.1. Flavonoids test

0.05g of extract dissolved in 5 ml of hydrochloric alcohol + (4 ml ethanol + 1 ml concentrated HCl), 2-3 magnesium or zinc chips are added. The release of heat then the appearance of a pink, orange or red color indicates the presence of flavonoids [26-28].

2.5.2. Quinones test

The presence of quinones is confirmed by the addition of a few drops of 1/10 NaOH, when the aqueous phase turns yellow, red or purple [29].

2.5.3. Tannins test

The reaction carried out from 1 ml of extract placed in a tube in which the addition of $FeCl_3$ at 1% makes it possible to detect the presence or absence of tannins. The color changes to black blue in the presence of gallic tannins and greenish brown in the presence of catechin tannins [29].

2.5.4. Steroids Test

To 5 ml of extracts, 5 ml of acetic anhydride and 3 ml of concentrated H_2SO_4 are added. Steroids give with this reaction a red coloring[30].

2.5.5. Polyterpenes test

To 5 ml of extracts, 5 ml of acetic anhydride and a few drops of concentrated H_2SO_4 are added. Polyterpenes give a green color[30].

2.5.6. Alkaloids test

The alkaloids are also demonstrated by Mayer's reagent (10 g of KI and 2.70 g of $HgCl_2$ dissolved in 20 ml of water). Adding a few drops of this reagent to 2 ml of the extract solution causes the formation of a white or yellow-white precipitate in the presence of alkaloid [29].

2.6. ***Antioxidant activity***

Radical scavenging activity was determined by a spectrophotometric method based on the reduction of the methanolic samples of 2,2-diphényl-1-picrylhydrazyl. We have prepared a range of concentrations by dilutions the extracts in methanol: 200 µg/ml, 150 µg/ml, 100 µg/ml, 50 µg/ml and 20 µg/ml. DPPH free radical scavenging activity was measured by combining the extracts with DPPH solution (4 mg of DPPH in 100 ml of methanol) at a ratio of 1:3 for 30 min at 25 °C in the dark. The mixture was shaken vigorously and then kept in the dark at laboratory temperature for 30 min. The absorbance was measured against a blank at 517 nm. The positive control is represented by a solution

of a standard antioxidant, ascorbic acid whose absorbance was measured under the same conditions as the samples studied. The ability to scavenge the DPPH radical was calculated using the following equation: DPPH scavenging effect (%) = [(A0 - A1)/A0]* 100 Where A0 is the absorbance of the control at 30 min, and A1 is the absorbance of the sample at 30 min. The antiradical activity was expressed as IC50 (µg/ml), (inhibitory concentration 50%) is the concentration values to inhibit or reduce 50% of the initial concentration of DPPH radicals were determined graphically by linear regression [31-33].

*2.7. **Antimicrobial Activity***

The antibacterial activity of Essential Oil and organic extracts was determined by disc diffusion method. The used bacteria were Staphylococcus aureus, Micrococcus luteus and Bacillus cereus. The organic extracts were dissolved in pure ethanol to obtain 50 mg/mL. A sterile paper disc (6mm diameter) was aseptically placed on the inoculated Petri dishes on which 10 µL of extracts were added to each well. Petri dishes were incubated 30°C for 24 h before measuring the diameter of the inhibition zone around discs. The diameters of inhibition zones were measured in millimeters [34].

3. Results and Discussions

*3.1. **Yield and composition of R. officinalis essential oil***

The essential oil isolated by water distillation were obtained in yield 1.8 % based on dry weight, the components determined in the essential oil are given in Table 1 and Figure 2, in which 16 identified compounds are listed according to their elution order.

Table 1. ***Chemical composition of Rosmarinus officinalis essential oil.***

N⁰	Components	RT	%
1	α-Pinene	5.000	8.225
2	Camphene	5.242	1.929
3	β-Pinene	5.675	4.675
4	β-Myrcene	5.833	1.114
5	(+)-4-Carene	6.267	0.707
6	m-Cymene	6.392	1.325
7	D-Limonene	6.458	1.907
8	Eucalyptol	6.517	52.440
9	γ-Terpinen	6.925	0.963
10	Terpinolen	7.392	0.358
11	Linalol	7.517	0.830
12	Camphor	8.308	11.241
13	Borneol	8.617	3.494
14	p-Menth-1-en-4-ol	8.775	1.378
15	Terpinéol	8.958	5.203
16	Caryophyllene	12.217	4.210

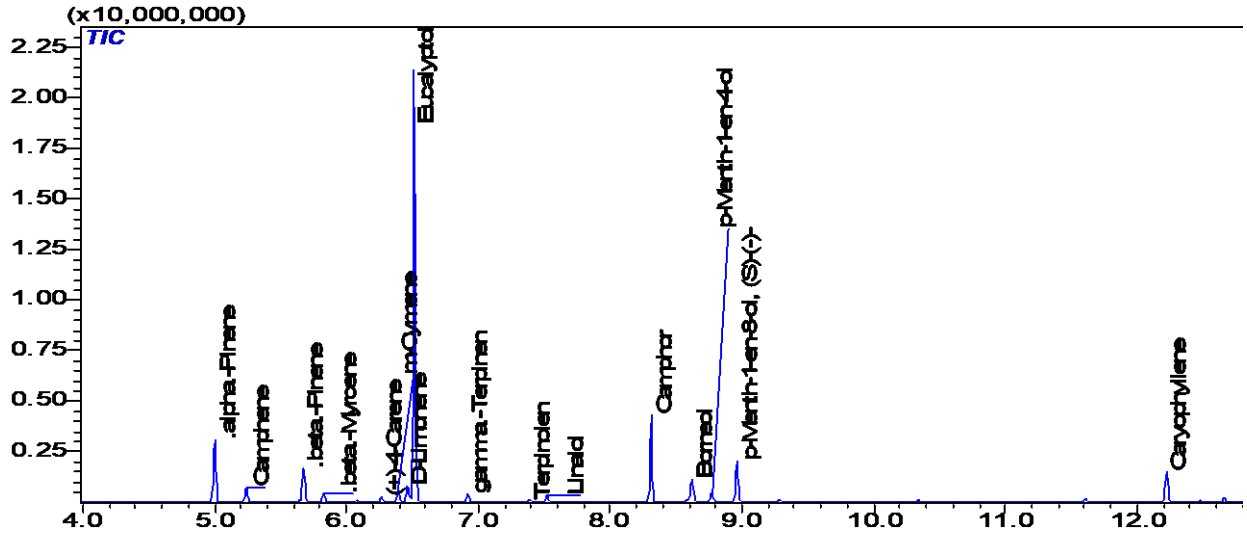
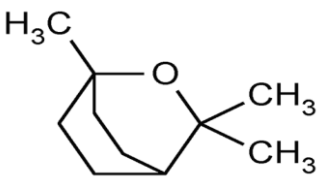
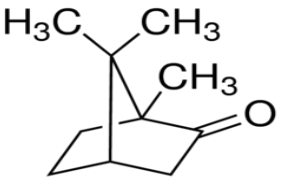


Figure 2. GC-MS chromatograms of the extracted Rosmarinus officinalis essential oil.

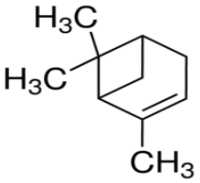
The analysis of essential oil extracted from Rosemary showed the predominance of 1.8-cineol (52.440%) as the major constituent. Other components are present with relatively high percentages such as Camphor (11.241%), α-pinene (8.225%) and Terpineol (5.203%). In contrast, Linalol (0.830%), (+) - 4-Carene (0.707%) and Terpinolen (0.358%) were found with much lower values.



Eucalyptol



camphor



α-pinene

Several recent studies have shown that variability in composition and yield of rosemary essential oil is due to intrinsic factors (genetics, subspecies and age of planting) or to extrinsic factors such as climate and growing conditions. (Geographic origin) or the extraction method. In comparison to similar studies conducted in Morocco, 1,8-cineole, camphor and α-pinene emerged as the major components of R. officinalis in Oujda region [35]. and Meknes-Tafilalet region [36]. In Addition, Yields and chemical composition of essential oils extracted from rosemary of both Middle Atlas and Loukkos origins were different, likely due to varietal and geographic variations, which influence soil. The main constituents were 1,8-cineole followed by camphor, borneol and α-and β-pinene. The oxygenated and hydro-carbonated sesquiterpenes were composed mainly of caryophyllene and caryophyllene oxide. The oxygenated monoterpenes dominated in the essential oils of rosemary from the Middle Atlas, while all other compounds were more abundant in the oils of samples from the Loukkos content [37]. In a similar study, the essential oil of R. officinalis from Spain [38] and Turkey [21]. composed of camphor and α-pinene, whereas the essential oil of R. officinalis from Italy [18]. consisted mainly of verbanone α-pinene, 1,8-cineole and camphor, The major components, α-pinene, borneol, camphene, camphor, verbenone and bornyl-acetate, were also reported to be present in Sardinian essential oil [39].

3.2. ***Yield and phytochemical characterization of R. officinalis organic extracts***

The organic extracts are recovered after evaporation of the solvents to dryness under reduced pressure. They were weighed to determine their weight. Each organic extract was characterized by its yield relative to the weight of the plant extracted. The results are summarized in the table 2 below.

Table 2. ***Yield and phytochemical characterization of R. officinalis organic extracts.***

Sample				**Yield %**	**Flavonoids**	**Quinones**	**Tannins**	**Steroids**	**Polyterpenes**	**Alkaloids**
Parts of the plant	Extraction technique	Solvent	Symbol							
Leaves	Soxhlet	Ethanol	R_L-S/Et	24.71	+	+	-	+	+	+
		Hexane	R_L-S/Hx	9.98	-	-	-	+	+	-
		Ethanol (2)*	R_L-S/Et(2)	9.55	+	+	-	+	+	+
	Maceration	Ethanol	R_L-M/Et	20.47	+	+	-	+	+	+
		Hexane	R_L-M/Hx	6.60	-	-	-	+	+	-
		Ethanol (2)*	R_L-M/Et(2)	12.83	+	+	-	+	+	+
Stem	Soxhlet	Ethanol	R_L-S/Et	9.14	+	-	+	+	-	+
		Hexane	R_S-S/Hx	1.12	-	-	-	+	+	-

Ethanol (2)*: Each sample was submitted to two successive extractions by hexane then ethanol. (+): Presence of compounds ; (-): Absence of compounds The yields vary according to the extraction procedure and the nature of the metabolites present in the extracts. In general, the yields of ethanolic extracts are higher compared to hexane extracts, the extraction yields observed in the table 2, shows that the highest yield is obtained from Rosemary Leaves using soxhlet and maceration obtaining respectively of 24.71 and 20.47% when the Stems extract is obtained with a yield of 9.14%. Alcoholic solvents are able to increase the permeability of cell walls facilitating the extraction of a greater number of polar molecules, of medium and low polarity, Ethanol is the polar solvent and it allows the extraction of a large fraction of organic compounds from plant material including antioxidants. This explains the yields of the polar extracts are superior to those obtained by hexane. The phytochemical tests carried out on the extracts consist in detecting the different families of compounds existing in the plant extracts by qualitative characterization reactions. These reactions are generally simple identity, quick to implement, performed mostly in test tubes, they appear either precipitation or appearance of turbidity, flocculation or a color change that tells us, which can give an idea about the nature of these families. The qualitative examination of the organic extracts was summarized in the table 2. Different varieties of phytochemical were included such as Flavonoids, Quinones, Tannins, Steroids, Polyterpenes, and Alkaloids. The phytochemical examination revealed the presence of Flavonoids in the ethanolic extracts of the leaves and stem, confirmed by the appearance of a red color. The existence of Quinones in the extracts is justified by the appearance of a yellow coloration, we observed the absence of Quinones in the stem and their presence in the leaves, while the Tannins are present in the leaves and they are absent in the stem, there presence confirmed by the appearance of a greenish-brown color. Steroids and Polyterpenes are present in the leaves and the stem, their presence is confirmed by the appearance of a red coloration for Steroids and green

for Polyterpenes. We observed the presence of Alkaloids in the ethanolic extracts of the leaves and stem, a white precipitate was formed on the top of the solution which indicates their presence.

*3.3. **Antioxidant activity***

To test the antioxidant activity of essential oil and organic extracts, we used the DPPH method because of its stability (in radical form) and simplicity of analysis. The antioxidant capacity of our extracts is determined from the IC50, the concentration of extract needed to reduce 50% of the DPPH radical. The IC50 and the antioxidant activity of the extract tested are inversely proportional. The DPPH is initially purple, following the reduction of this radical which is accompanied by its passage from the violet color (DPPH•) to the yellow color (DPPH-H). The IC50 values of organic extracts calculated graphically by linear regression of the plotted graphs found for all the extracts are shown in the table 3 and Figure 3 below.

Table 3. ***Free radical-scavenging capacities (IC50) of Rosmarinus officinalis organic extracts.***

Sample					
Species	**Part of the plant**	**Extraction technique**	**Solvent**	**Symbol**	**Inhibitory concentration IC_{50} in µg/ml**
Rosemary	Leaves	Soxhlet	Ethanol	R_L-S/Et	77.79
			Ethanol (2)*	R_L-S/Et(2)	107.15
		Maceration	Ethanol	R_L-M/Et	64.27
			Ethanol (2)*	R_L-M/Et(2)	67.6
	Stem	Soxhlet	Ethanol	R_S-S/Et	64.74
Hexanic extracts					>200
Reference		Ascorbic acid			44,63

Ethanol (2)*: Each sample was submitted to two successive extractions by hexane then ethanol.

The results obtained showed that ethanolic extracts of Rosemary, were able to reduce the stable free radical DPPH which demonstrates that the antioxidant potential of the plant is proven. The ethanolic extracts of leaves R_L-M/Et using Maceration and the ethanolic extracts of stem R_S-S/Et using Soxhlet presented the lowest (IC50= 64.27 µg/ml and 64.74 µg/ml Respectively) therefore the most important antioxidant activities. Ascorbic acid was used as positive control (IC50 = 44,63 µg/ml). The results described reveal a significant influence of the extraction method on the antioxidant power of the extracts. The maceration extract has a greater antioxidant activity than those obtained by soxhlet however, the successive extractions led to a decline in the antioxidant power. In addition, hexanic extracts are less effective in trapping the DPPH radical. Free radical scavenging activity of the R. officinalis essential oil, measured by DPPH assay are presented in Figure 4 and Table 4. These results showed that the essential oil was able to reduce the stable free radical DPPH with an IC50 of 44.79 µl/ml. The essential oil also exhibited remarkable antioxidant activities but lower than those reported for the organic extracts. The presence of phenolic compounds (Flavonoids, Coumarins ...), Alkaloids and Terpenoids would probably the origin of the antioxidant activity of the species. Flavonoids, recognized as excellent anti-oxidants, could perform an important role in the defense system. These metabolisms are further known for other diverse biological properties.

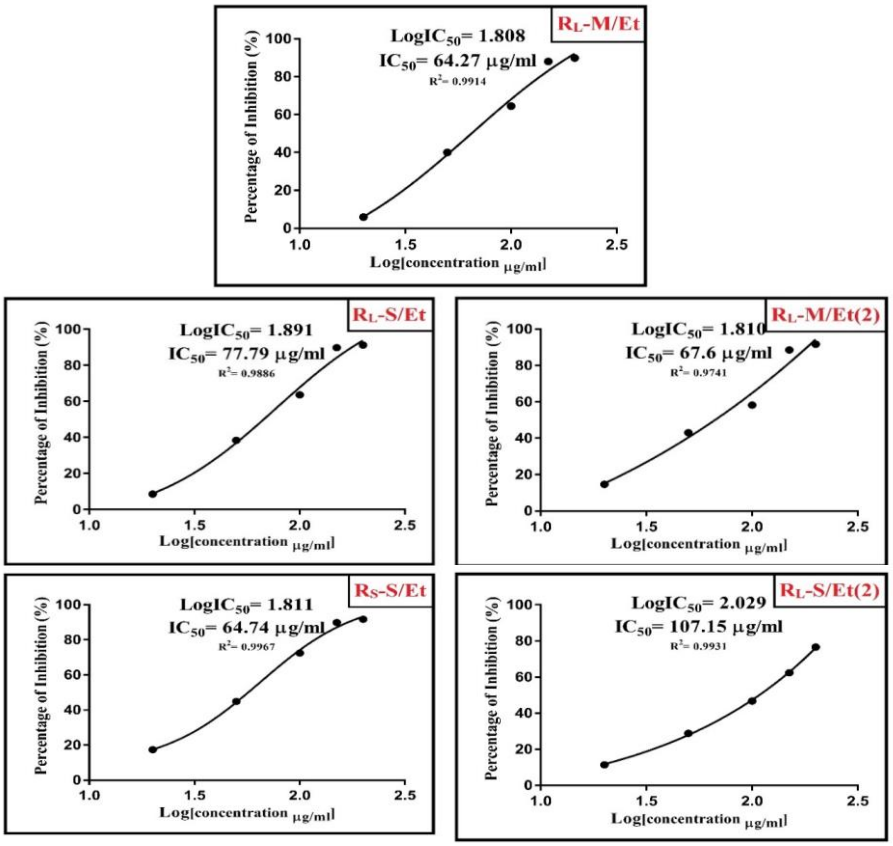


Figure 3. Free radical-scavenging capacities (%) of Rosmarinus officinalis organic extracts.

Table 4. ***Free radical-scavenging capacities (IC50) of Rosmarinus officinalis essential oil.***

Sample				Inhibitory concentration IC_{50}
Species	**Part of the plant**	**Extraction technique**	**Symbol**	**in µl/ml**
Rosemary	Leaves	Hydrodistillation	R_{EO}	44.79

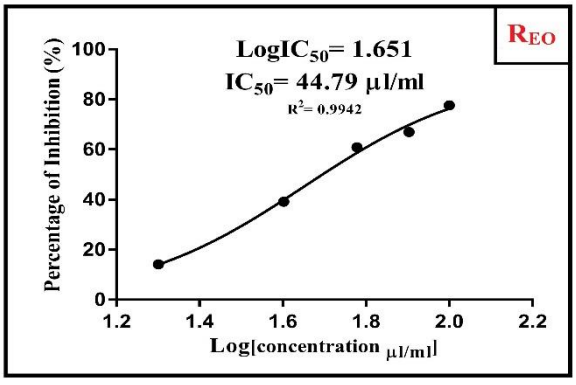


Figure 4. Free radical-scavenging capacities (%) of Rosmarinus officinalis essential oil.

3.4. ***Antimicrobial Activity***

The main objective of this study is to evaluate the antibacterial activity of organic extracts and essential oil and also to check if there is a difference in efficiency between different extraction methods. Plants contain many compounds with an antimicrobial action, these constituents include Phenolic compounds, Alkaloids, essential oils and Triterpenoids,

the antimicrobial potential of plant extracts is dependent on their chemical compositions. The antibacterial activity, when it exists, is manifested by zones of inhibition around the disks, the diameter of these zones is proportional to the intensity of the antibacterial activity. The diameters of the zones of inhibition observed are presented in the following table 5.

Table 5. ***Free radical-scavenging capacities (IC_{50}) of Rosmarinus officinalis essential oil.***

Sample			**Inhibition diameters (mm)**		
Species	**Symbol**		**Bacillus cereus**	**Staphylococcus aureus**	**Micrococcus luteus**
Reference (penicillin)			28	30	12
Rosemary	leaves	R_{EO}	9	10	8
		R_L-S/Et	10	36	11
		R_L-S/Hx	9	10	8
		R_L-M/Et	7	7	7
		R_L-M/Hx	7	7	8
	stem	R_S-S/Et	10	7	11
		R_S-S/Hx	11	8	7

The antimicrobial activities of Rosemary were evaluated against three bacterial strains, the Table 5 showed variable antimicrobial activity against all tested strains. The diameter of inhibition zones ranged from 7 mm to 11 mm for the Bacillus cereus in which the diameter of inhibition of penicillin was 28.These results confirm that essential oil and organic extract are more likely ineffective against Bacillus cereus.The inhibition activity was slightly less pronounced against Staphylococcus aureus (8–10 mm), except for the extract RL-S/Et who exhibited the most significant inhibition effect on the growth of Staphylococcus aureus than other extracts (36 mm) even more than the penicillin (30 mm). The inhibition zones were in the range of 7-11 mm for Micrococcus luteus. These bacteria shown to be more resistant to the Rosemary extracts as for the penicillin. These results confirm the antimicrobial activity exhibited by Rosemary oil and extract. Several major Terpenoids or Phenolic compounds such as 1,8 cineole, camphor could be the main responsible of this activity [40, 41]. In several studies, the antioxidant and antimicrobial effects are explained by the presence of phenolic derivatives in extracts or isolated [42-45].

4. Conclusion

In the present work, we realized the extractions of the essential oils by hydrodistillation. The essential oils obtained from rosemary (collected from Taourirt of the oriental of Morocco) were obtained with a yield of about 1.8%, the GC-MS revealed that the rosemary oil was dominated by 1,8-cinéol (52.440 %), Camphor (11.241 %), and α-pinene (8.225%). The phytochemical screening carried out, revealed the richness of the plants studied in secondary metabolites, where we found the presence of Flavonoids, Quinones, Tannins, Steroids, Polyterpenes and Alkaloids. In the second part, we were interested in studying the antioxidant properties of the organic extracts and essential oil of these plants by the free radical reduction method (DPPH). The results reveal that the essential oil and all extracts tested exhibit anti-radical activities. The ethanolic extract of rosemary leaves exhibited the highest anti-radical activity (IC50 = 64.27 µg/ml), followed by the extract of the stem with an IC50 = 64.74 µg/ml. All this show that there is a potential antioxidant activity in this plant and reveals that the polar extracts of this species is promising sources for the search for new compounds that are useful in the prevention or treatment of diseases associated with oxidative stress.

In this study we also performed an antimicrobial test. The microbiological results showed that the antimicrobial activity is variable from one strain to another; this is due to the variability of the chemical composition of each extract.

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