

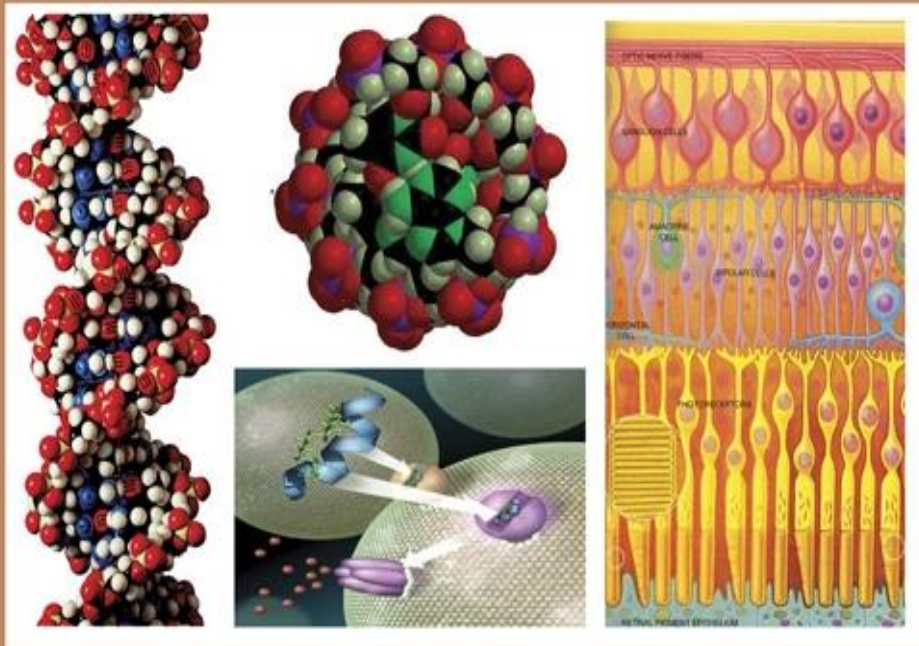


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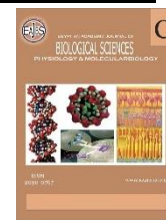
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## Antioxidant and Antibacterial Activities of *Rosmarinus officinalis* aqueous extract with Study of The Pharmacological Effect

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### ABSTRACT

The objective of the present work was to study the antioxidant activity of the *aqueous extract* and the antibacterial activity of the *Rosmarinus officinalis* aerial part essential oils. The Rosemary essential oil yield was equal to 1.47%. A great inhibitory power of Rosemary extract was revealed by the test of antioxidant activity (93.72%) by an IC<sub>50</sub> of 16.95 µg/ml, which was superior to that of Butylated hydroxy anisole (BHA a synthetic antioxidant) (6.01 µg/ml), and of ascorbic acid (1.27 µg/ml). The essential oil antibacterial activity was evaluated against five bacterial strains, using the microdilution method. Hence, a remarkable antibacterial activity was noted for Rosemary essential oil. Gram-positive bacteria were highly sensitive to the activity of this essential oil than the Gram-negative bacteria. Furthermore, pharmacological investigation of the plant aqueous extract revealed the plant had very important pharmaceutical effects (anti-inflammatory effect). The preliminary study of toxicity, according to oral gavage, revealed that the *Rosmarinus officinalis* leaves were not toxic.

### INTRODUCTION

Since antiquity, human beings have used aromatic plants (Bojase *et al.*, 2001). Today, the majority of the world's inhabitants use many plants, either for seasoning or as a remedy in traditional medicine. These plants represent a huge reservoir of potential compounds, attributed to secondary metabolites that possess a wide variety of biological activities (Dorman and Deans, 2000). Rosemary (*Rosmarinus officinalis*) is the subject of recent research in pharmaceutical, cosmetic and food fields. It's an aromatic herb of the *Lamiaceae* family, widely used in pharmaceuticals and traditional medicine and appreciated for its antimicrobial, antispasmodic, emmenagogues and anti-tumor properties, (Ait Bekkara *et al.*, 2007). Recent researches have focused on the study of the antioxidant, and antimicrobial molecules of polyphenols and essential oils of this herb. In the present work, we have objectified the study of antioxidant and antimicrobial activity of *Rosmarinus officinalis*, harvested from the region of Chlef (North- West of Algeria), as well as, determination of the anti-inflammatory activity of its *aqueous extract*.

## MATERIALS AND METHODS

Our study was focused on a plant species, *Rosmarinus officinalis* of the *Lamiaceae* family. Its aerial part was harvested in February 2019, in the Kremia region of Chlef town (North-West of Algeria), far from any pollution impact. To get rid of dust and other particles, the plant aerial parts were washed with running water and dried in the shade in a dry and ventilated place for 7 days. Before any extraction, the plant leaves were dried in the oven for 72 hours at +40°C to complete the drying.

The bacterial species that were tested for antibacterial activity with *Rosmarinus officinalis* essential oil included: two strains from the international collection ATCC (American Type Culture Collection): *Pseudomonas aeruginosa* ATCC 25763 and *Staphylococcus aureus* ATCC 25843, and three isolated strains from patients from Chlef Hospital (H): (*Escherichia coli* (H), *Bacillus sp* (H) and *Streptococcus sp* (H)).

The used animal material was Albinos mice strains from Naval Medical Research Institute of Swiss origin; male sex, weight was at 18 to 25 g, the animals were divided into 3 lots of 5 mice. All experimental protocols were conducted on mice in accordance with the standards established by the International Committee of Animals Ethics, consistent with those agreed upon by the European Community on 24 November 1986 (86/609/EEC).

### ***Rosmarinus officinalis* Extraction:**

The fresh aerial parts (20-200 g) of leaves, flowers, branches and flowering plants were chopped into small parts, with a blender and placed in a 3-litre round-bottom flask with 1 L of deionized water. The solution was steam-distilled for 1 h in a Clevenger type apparatus to oil isolation. The residue was extracted using methanol and acetone by a Soxhlet. The essential oil was stored at +4°C until be

used. The yield calculation was defined as the relationship between the mass of the essential oil obtained and the mass of plant matter to be treated (Belyagoubi, 2006).

- R - MH/ MS.100
- R: Fixed extract yield in g/100g
- MHE: The extract amount recovered in g
- MS: The plant material amount used for extraction, expressed in g.

### **Antioxidant Activity:**

The antioxidant activity test has been used (Annie *et al.*, 2006). DPPH was solubilized in methanol to 0.1 M. 1ml of this solution was added to 1 ml of the extract solution (1mg/ml) in distilled water, at different concentrations (05; 10; 20; 40 and 60 g/ml). After 30 min in the dark and at room temperature, the absorbance was measured at 517 nm, and the test was repeated 3 times. The same protocol was performed for positive control, by a standard antioxidant solution of ascorbic acid and BHA. The results were expressed in inhibition percentages according to the following formula: % scavenging radical DPPH =  $(CA-SA/CA) \times 100$ , Where: CA: Control absorption (DPPH-methanol); SA: Sample Absorption [Sample Absorption (Sample-DPPH) - Control test Absorption (Sample-methanol)]. % scavenging radical DPPH =  $(CA-SA/CA) \times 100$ , Where: CA: Control absorption (DPPH-methanol); SA: Sample Absorption [Sample Absorption (Sample-DPPH) - Control test Absorption (Sample-methanol)].

### **Antibacterial Activity:**

The purity of strains control has been assured by re-isolation of each used strains on the specific isolation medium. The strain transplanting was done from the growth medium, incubated at 37°C for 24 hours. The antibacterial activity evaluation was carried out by the discs diffusion method or aromatogram. The disc distribution applied method was described by (Gachkar *et al.*, 2007 and

Hussain *et al.*, 2010).

Rosemary essential oil was dissolved in methyl sulfoxide (DMSO) to prepare different concentrations of successive dilutions at half. DMSO is the solvent which was used by the majority of researchers, notably, (Gachkar *et al.*, 2007), who showed that DMSO has no antibacterial effect. To obtain a young bacterial culture and isolated colonies, used bacteria were seeded on Petri dishes containing nutrient agar (NA), and incubated at 37 °C for 24 hours. From these dishes, a few well-isolated and perfectly identical colonies are scraped with a platinum handle. In the case of *Streptococcus sp.*, a swab was used to remove more easily bacterial colonies. Then the handle or swab was resuspended in 5 to 10 ml of physiological water (0.9%). In the case of *Streptococcus sp.*, bacterial suspension was well homogenized. Its opacity must be equivalent to 0.5 (McFarland) or a D.O from 0.08 to 0.10, was read at 625 nm. The antibacterial activity of the Rosemary extracts was estimated qualitatively, by using the disk diffusion technique. The agar medium surface was inoculated with initial culture adjusted to 10<sup>6</sup> colony forming units (CFU) /ml. Filter paper disks (6 mm in diameter; Whatman paper No. 1, USA), were impregnated with 40 ml of extracts (concentration 6-18 mg/ml). The disks were dried at room temperature. Petri dishes were then incubated at 37°C for 24 h. The antibacterial activity was determined through the zone of bacterial growth inhibition around the disk. Controls were set up with solvents alone in corresponding amounts to the highest amount quantity present in the test.

#### **The Aqueous Extract:**

The extraction method used was the infusion which consists of pouring boiled water on the plant material for a necessary time. This time varied on the plant nature from 10 minutes to 1 hour.

This so, 1g of plant powder was mixed with 10 ml of boiling distilled water. The solution was left infused for 20 minutes and then filtered.

#### **Toxicity Study:**

This test includes abnormal toxicity according to the European Pharmacopeia, 2005. The purpose of this test is to define the toxicity of the used plant (Rosemary), regardless of the used dose. It's about the toxicological study, by testing the maximum concentration of our infusions; which can lead to mortality. We used 3 batches of 5 same-sex mice, with a weight varying from 18 g to 25 g. We tried different doses of Rosemary leaves aqueous extract. The used doses were increasing dilutions up to the value that gives mortality. The administration was carried out by gavage, at a rate of 0.5 ml/mouse. The observation time lasted over 48 hours.

#### **Anti-inflammatory Test:**

The reveal of anti-inflammatory activity was performed by using the Levy method (Levy, 1969), cited by (Berkan *et al.*, 1991). The general principle is to reduce experimentally in animals, an inflammatory process that will be antagonized by substances; which possess an anti-inflammatory activity, administered before the plant aqueous extract. This technique was performed on homogeneous mice subjected to fasting for 18 hours. This test consists of evaluating the aqueous extract anti-inflammatory effect at 10% and 16% on the edema of the hind legs, caused by the injection of 1% of carrageenin solution in mice. Injecting this solution under plantar aponeurosis of the mouse leg causes an inflammatory reaction that can be reduced by an anti-inflammatory product. The carrageenin solution preparation was made by a 20 mg latter dilution in 20 ml of physiological water. The aqueous extract preparation was carried out by infusion at the rate of 1g/10 ml and 1.6/10 ml of Rosemary powder. The animals

were divided into 3 batches.

- Lot control: each mouse received 0.5 ml of physiological water
- Lot test 1: Each mouse received 0.5 of 10% of infusion.
- Lot test 2: Each mouse received 0.5ml of 16% of infusion.

After 1 hour, the carraghenin solution (1%) was injected under plantar aponeurosis of the right hind legs, at a volume of 0.025 ml to all mice of the different experimentation batches.

These same mice received an injection of 0.025 ml of physiological water into the left hind legs.

The volume of the right and left hind legs of the 3 lots was measured after 30 minutes using a calliper. The percentage of edema reduction was calculated using the following formula: % Edema reduction = % of edema control - % of edema test / % of edema control (Berkan et al., 1991).

## RESULTS

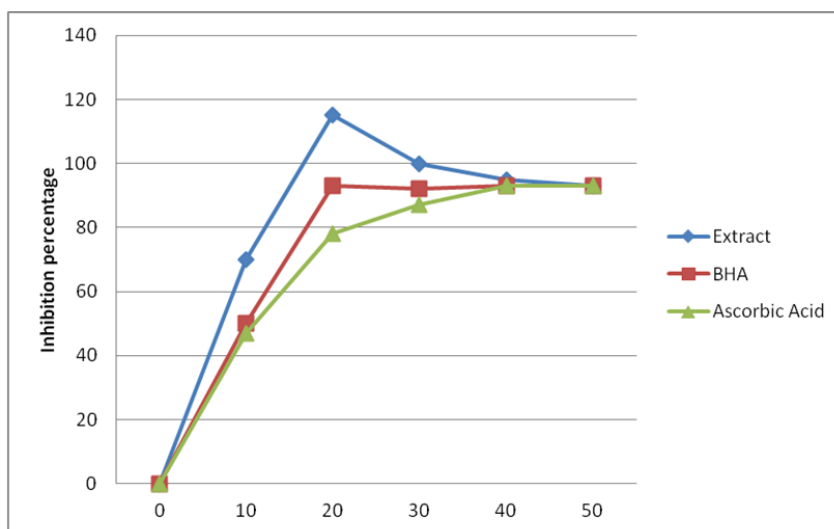
The *Rosmarinus officinalis*

essential oil obtained by hydrodistillation was a viscous liquid, clear with a yellowish coloration and a strong smell characteristic of Rosemary. The essential oil yield of local *Rosmarinus officinalis* was 1.47%.

### Antioxidant Activity:

The results were expressed as an antiradical activity percentage, according to the concentration of standards BHA and ascorbic acid; for the extract, it's also expressed, by using the IC<sub>50</sub> parameter, which is defined as the substrate concentration that causes a 50% DPPH activity loss. The results of anti-radical activity, towards the radical DPPH, standard and extract are represented in (Figure.1).

The results of the DPPH test showed that Rosemary extract has an inhibition percentage equal to 93.72% with an IC<sub>50</sub> of 16.95 µg/ml, which is higher than that of BHA (6.01µg/ml) and ascorbic acid (1.27 µg/ml).



**Fig. 1:** Inhibition percentage of radical DPPH based on the extract concentration, BHA and ascorbic acid

### Evaluation of Antibacterial Activity:

The antimicrobial power of this essential oil was obtained by calculating diameters of inhibition zones diameters in mm. The results presented in ( Table.1), show that Rosemary's essential oil had an

important activity, extending over all the tested strains; but it exerted a strong activity on gram-positive bacteria. Moreover, our results showed that the antibacterial activity, of *Rosmarinus officinalis* essential oils, increased

considerably with the extract concentration. According to the classification of (Ponce et al., 2003), inhibition zones, ranging from 15 to 19 mm, indicate that the *Bacillus sp.* strain was the most sensitive than the other tested strains (inhibition zone between 7-14 mm). The antibacterial effect of Rosemary essential oil persisted until

dilution 1/16 for *Escherichia coli* (H), *Streptococcus sp.* (H), and *Pseudomonas aeruginosa* ATCC 25763.

**Abnormal Toxicity Study:**

Even used doses of Rosemary aqueous extract administration (over 48 hours) in the abnormal toxicity test were very high; we didn't notice mice mortality in our experiments (Table .2).

**Table 1:** Inhibition zones diameters of Rosemary essential oils on the different tested bacterial strains.

Inhibition zones diameters (mm) Strains	Dilution of Rosemary essential oils					
	1/2	1/4	1/8	1/16	1/32	NG
<i>Escherichia coli</i>	10.29±0.565	10.27±1.620	10±1.515	8±1.613	7±1.513	6
<i>Bacillus sp.</i>	14±1.2	13.41±0.419	6±0	6±0	6±0	6
<i>Staphylococcus aureus</i>	12.52±0.611	11.21±0.591	7±0	6±0	6±0	6
<i>Streptococcus sp.</i>	13.5±0.611	11.05±0.519	9.01±2.304	8±1.307	7.51±1.161	6
<i>Pseudomonas aeruginosa</i> ATCC 25763	12.34±1	12±0	11.1±1	9±1.370	7±1	6

NG: negative control (DMSO); (1/2, 1/4, 1/8, 1/16, 1/32): different Rosemary essential oil dilutions.

**Table 2:** Results of the Rosemary aqueous extract administration

Doses (g/ml)	5 g /20ml	10 g /20ml	15 g /20ml
Mice number	10	10	10
Dead mice number	0	0	0

**Anti-Inflammatory Effect of Rosemary Aqueous Extract:**

The results of physiological water and plant aqueous extracts administration to mice, in which inflammation was caused by the injection of carraghenin (1%) into the plantar surface of the hind legs, and the thickness estimations of the hind legs (in mm) were mentioned in (Table.3) .

After the carraghenin administration at the mice hind legs, the edema formation and the thickness increase of the hind legs were observed in mice in the 3 replicats. After 30 minutes of the carraghenin administration, there

was a slight edema decrease in the control lot. The reduction of these edemas was very slow over time; they don't reach the stabilization point until after 201 minutes of the injection. For mice in lot 1 test, with a dose of 1g/ml of the administered extract; there was a significant drop in edema after 30 minutes. The stabilization time was 180 minutes from the injection. For mice in lot 2 test with a dose of 1.6 g/ml, a very significant drop in edema was observed and the edema reduction was very rapid. They reach stabilization after 120 minutes of injection (Tables .3 and Table .4).



**Table 3:** Evolution of the thickness average of the mice right legs in each batch

	Before Admin	After Admin	After 30 mn	After 60 mn	After 90 mn	After 120 mn	After 150 mn	After 180 mn	After 210 mn	After 240 mn
<b>Control:</b> Physiological water - carraghenin	2.25±0.05	3.27±0.18	3.52±0.08	2.92±0.12	2.71±0.18	2.063±0.13	2.51±0.14	2.42±0.13	2.34±0.05	2.34±0.05
<b>Test 1:</b> aqueous extract at 10%+ carraghenin	2.41±0.02	3.36±0.01	2.97±0.15	2.78±0.01	2.65±0.15	2.58±0.11	2.49±0.10	2.38±0.15	2.41±0.03	2.41±0.03
<b>Test 2:</b> aqueous extract at 16%+ carraghenin	2.32±0.01	3.15±0.12	2.63±0.07	2.43±0.14	2.32±0.15	2.27±0.13	2.27±0.13	2.27±0.13	2.27±0.13	2.27±0.13

Admin : Administration

**Table 4:** Reduction percentage in edema straight hind leg mice.

	After 30 mn	After 60 mn	After 90 mn	After 120 mn	After 150 mn	After 180 mn	After 210 mn	After 240 mn
<b>Reduction % of leg edema in the control lot</b>	5.6%	6.88%	7.52%	5.41%	4.97%	4.74%	4.17%	-
<b>Reduction % of leg edema in the control lot Test : 1</b>	13.71%	7.11%	6.78%	4.81%	3.82%	0.91%	-	-
<b>% of reduction of leg edema in the control lot Test: 2</b>	16.01%	8.21%	4.21%	0.98%	-	-	-	-

-: : Stabilization of the edema reduction

## DISCUSSION

This work was conducted to describe the antioxidant performance and effectiveness of *Rosmarinus officinalis* essential oils. The essential oil yield of our *Rosmarinus officinalis* was 1.47%. This yield was close to that cited by Karimi *et al.* (2006) at 1.5% and Hilan *et al.* (2006) at 1.52%, and lower than that cited by Hosny *et al.* (2011) at 1.79% and Ayadi (2011) at 85%. However, it was higher than that cited by Atik Bekkara *et al.* (2007) at 0.8% and Glick *et al.* (2007) at 1.18%. This differs depending on geographical location of *Rosmarinus officinalis* or used extracting method.

Our results showed a high radical scavenging activity using DPPH method. Indeed, the DPPH test results showed that Rosemary extract had an inhibition percentage equal to 93.72% with an IC<sub>50</sub> 16.96 µg/ml, which was higher than that of BHA (6.01 µg/ml) and ascorbic acid (1.27 µg/ml). These obtained values were higher than that reported by Athamena *et al.* (2015) in Constantine (East of Algeria), which was in the order of 80.50%. The IC<sub>50</sub> value was 16.96 µg/ml that obtained for the *Rosmarinus officinalis* aqueous extract exhibits a high antioxidant activity compared to the IC<sub>50</sub> (0.023 mg/ml) and (54 µg/ml) values;

obtained for the same species reported by Dorman *et al.* (2003) and Erkan *et al.* (2008) respectively. This may be due to several factors such as extraction method, the phenolic compounds nature in the extracts, the total phenolic compound content, the geographic localization and the plant's collection period (Stefanovits *et al.*, 2003).

Our extracts showed effective antibacterial activity of Rosemary essential oils fractions against all tested bacterial strains. According to Wu *et al.* (2005), the resistance results could be due to the outer membrane chemical composition of Gram-negative bacteria. In fact, they have a membrane that had selective permeability, the lipopolysaccharides surface, contains negative charges that prevent the spread of hydrophobic molecules and porins that block the passage of molecules with a high molecular weight. The results of Ayadi *et al.* (2011) revealed that the clinical *Pseudomonas aeruginosa* strain was resistant with a diameter of 07 mm and *Pseudomonas aeruginosa* ATCC 25763 was sensitive with an inhibition zone of 09 mm. Moreover, the disk charge influences antimicrobial activity. Rasooli *et al.* (2008) have noticed that the *Aspergillus parasiticus* growth inhibition was higher when the disc was more charged with *Rosmarinus officinalis* essential oils. Some studies didn't show any selective antibacterial activity against bacteria's Gram (Gram + or Gram-) (Guesmi and Boudabous, 2006). The *Streptococcus sp.* strain resistance can be attributed to the antibacterial molecules ability to spread evenly on the agar (Hayouni *et al.*, 2007). All our data suggested that the antibacterial Rosemary extracts efficacy was associated with their specific phenolic composition.

Considering a huge number of different groups of chemical compounds present in Rosemary essential oils, it's most likely that their antibacterial activity

is not attributable to one specific mechanism, but that there are several targets in the cell. An important characteristic of essential oils and their components is their hydrophobicity, which enables them to partition in the lipids of the bacterial cell membrane, disturbing the structures and rendering them more permeable. Ions leakage and other cell contents can then occur and causes the death of bacterial cells. Generally, the essential oils possessing the strongest antibacterial properties against food pathogens, contain a high percentage of phenolic compounds, such as carvacrol, eugenol and thymol. The mechanisms of the essential oils action include the cell wall degradation, the cytoplasmic membrane damaging, increased permeability leading to leakage of the cell contents, reducing the proton motive force, reducing the intracellular ATP (Adenosine Three Phosphate) pool via decreased ATP synthesis and augmented hydrolysis; that is separate from the increased membrane permeability and reducing the membrane potential via increased membrane permeability (Hayouni *et al.*, 2007).

The increased public sensibilities of the negative effects caused by overexposure to synthetic chemicals led to the search for "green solutions", such as organic and synthetic free-chemical food products. Rosemary is an available herb, inexpensive, and was shown to be relatively non-toxic in animal models and as antimutagenic. Our *in vivo* bioassays results showed that Rosemary aqueous extract contained a higher activity than previously described based on the obtained results, it's inferred that the Rosemary aqueous extract has an anti-inflammatory effect. Several studies have been conducted on the anti-inflammatory activity evolution of plant *aqueous extracts* using other methods. Dongmo *et al.* (2008) showed that the *Citrus scinensis* *aqueous extract* had a remarkable anti-



inflammatory effect. Our results are consistent with Cheung and Tai, (2007) who highlighted the anti-inflammatory effect of Rosemary leaves aqueous extract. The anti-inflammatory activity of the *Rosmarinus officinalis* aqueous extract can be explained by its richness in phenolic compounds, especially flavonoides. Indeed, Flavonoides inhibit inflammation by decreasing the release of certain mediators (Manunta *et al.*, 2004). Their pharmacological effectiveness as anti-inflammatory compounds depends on their basic structure of hydroxyl residues. They exert a suppressive effect on mediators at the level of inflamed tissues (Takano-Ishikawa *et al.*, 2006).

### CONCLUSION

*Rosmarinus officinalis* exhibited an effectiveness antioxidant, antibacterial and anti-inflammatory activity and was relatively non-toxic in animal models. More studies are essentially needed to highlight the different biologically active compounds of this available and inexpensive herb; which are related to its potential effects.

### Conflicts of Interest

The authors declare that they have no conflicts of interest with respect to the publication of this document.

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