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Article

Rosmarinus Officinalis: *Phytochemical* analysis and biological activities

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ABSTRACT

Rosemary (*Rosmarinus officinalis*), a very abundant species in Algeria, is a medicinal plant belonging to the *Lamiaceae* family, used for its various therapeutic effects. The present study was conducted to determine the bioactive compounds and biological activities (antioxidant and antibacterial activities) of the aqueous extract of the plant (EQRO). The sensitivity of the tested bacterial strains varies according to dilutions and bacterial nature (*Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*), which was determined using the agar diffusion method. Meanwhile, the *in vitro* antioxidant activity was assessed using DPPH radical scavenging. EQRO showed high levels of polyphenols and flavonoid contents (455.10 µg EAG/mg extract; 7.33 µg EAQ / mg extract, respectively) with a yield of 14.47%. In addition, the plant extract revealed a significant antioxidant activity as evidenced by the DPPH (IC_{50}=0.128 mg/ml), which is close to that obtained by BHT. Results showed a remarked antimicrobial effect against gram-positive bacteria (*Staphylococcus aureus*). At the same time, there was no significant effect on gram-negative bacteria (*Pseudomonas aeruginosa* and *Escherichia coli*), which explains the difference in susceptibility of the tested bacterial strains. *Rosmarinus officinalis* is suggested as an effective therapeutic medicinal plant because of its antioxidant and antibacterial properties.

Keywords: Antibacterial activity, Antioxidant activity, Aqueous extract, bioactive compounds, *Rosmarinus officinalis*.

INTRODUCTION

Oxidative stress is an imbalance between radical species oxygen (ROS) production and cellular antioxidant capacities[1]. ROS has long been considered to be a toxic by-product of normal oxygen metabolism and is involved in many pathologies. The effects of free radicals in biology are now well documented; not only have living organisms adapted and coexisting in the presence of ROS, but they have also developed mechanisms to build defenses against them [2].

Bacteria are responsible for several diseases. Their resistance to antibiotics is becoming more pronounced [3]. The situation is even more worrying because of the appearance of strains of antibiotic-resistant microorganisms. It is necessary to seek another approach to reduce or eliminate the effects without using synthetic products so that solutions can be found using plant-based bioactive molecules [4].

According to the WHO, 14 to 28% of plants worldwide are listed as having medicinal use [5]. Surveys carried out at the beginning of the 21st century reveal that 3 to 5% of patients in Western countries, 80% of rural populations in developing countries and 85% of populations of south Sahara use medicinal plants as the primary treatment [6]. Medicinal plants are valuably used in traditional medicine to treat diseases [7; 8]

Algeria is very rich in plants, which grow spontaneously [9]. *Rosmarinus officinalis* (family of *Lamiacea*e), also known as rosemary (in Arabic, 'IKLIL ELDJABEL'), is a medicinal plant native to the Mediterranean region. The dried flower heads, leaves, and an essential oil are used in herbal therapy[10]. Traditional healers often use this plant effectively in treating various infectious diseases [11]. Rosemary contains several active agents responsible for different activities: carnosol, romano, carnosic acid, methyl carbonate, and some flavonoids such as cirsimaritin and genkwanin [12]**.** So, it is a natural antioxidant used as one of the spices with the highest antioxidant activity due to their components [13].

To the best of our knowledge, there are few studies regarding the biological effects of *Rosmarinus officinalis*. Thus, in this paper, we investigate the potential of *Rosmarinus officinalis* aqueous extract (EQRO) to enhance the antioxidant activity and antibacterial effect on three strains of bacteria (*Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*) and to estimate as well as the amount of phenolics acids.

MATERIALS AND METHODS

Plant Material

The aerial part of *Rosmarinus officinalis* was collected in June 2021 in the region of Tessala Lemtai, in the extreme north of the wilaya of Mila in eastern Algeria. The town is characterized by a mountainous agricultural character, cold in winter and moderately hot in summer, especially in the community's north. The harvested parts are freed of impurities, dried away from the sun, and placed in a dry, ventilated, shaded place.

Microorganisms

The microbial strains that were the subject of this study were provided by the Microbiology laboratory at the University of M'sila. Algeria. They are maintained by subculturing on nutrient agar favorable to their growth for 24 h at 37 °C. One Gram-positive bacteria, *Staphylococcus aureus* ATCC 22923, and two Gram-negative bacteria, *Escherichia coli* ATCC 22922 and *Pseudomonas aeruginasa* ATCC 27853, were tested.

Preparation of the extract

The aqueous extract of the aerial part of *Rosmarinus officinalis* is obtained from the decoction method. This was prepared according to [14]. 10g of the plant extract is put in 250 ml of distilled water until boiling (100°C) for 15 to 20 minutes, then cooled before being filtered. Then, the extract was stored at -20 °C until use. The yield in dry extract expressed as a percentage is calculated according to the following formula:

Yield% = $\boldsymbol{M\ extract\ /M0\ sample \times 100}$
Yield%: percentage yield,
M: dry extract weight (g)
M0: powdered sample weight (g)

Determination of total polyphenols

The total polyphenols content in aqueous extract was determined using the method of [15] with slight modifications. Briefly, a volume of 0.1ml of the extract was added, and their volume made up to 4.5ml of distilled water to give a mixture of 4.6ml and then mixed with 0.1ml of Folin-Ciocalteu reagent (diluted 3 times by distilled water) and 0.3ml of a 2% sodium carbonate solution. After incubation for 2h, the absorbance is read at 760nm. Phenols were expressed as gallic acid equivalents (µg gallic acid/mg dried extract).

Determination of total flavonoid contents

The total flavonoid content in aqueous extract was determined using the method of [16]. In brief, 0.25ml of extract with 1.25ml of distilled water was mixed with 0.75ml of $NaNO_2$ solution (5%). After 6 minutes, 150ml of $AlCl_3$ solution (10%) was added. A volume of 0.5 ml of M NaOH was added, and the mixture was made up to 2.5 ml with distilled water. The absorbance is read immediately at 510 nm. Results were expressed as equivalent quercetin (mg quercetin /g dried extract).

Quantification of tannins

The tannin content was estimated using the method of [17]. A volume of plant extract was diluted to obtain a concentration of total polyphenols of approximately 500 µg/ml and mixed with an equal volume of hemolyzed sheep blood (absorbance equal to 1.6). After 10 minutes, this solution was centrifuged for 20 minutes, and the absorbance of the supernatant was measured at 576nm. The precipitation efficiency of the tested solutions is expressed in µg of tannic acid equivalent/g of extract.

Antioxidant Activity Assays

DPPH radical scavenging assay

DPPH reactions have often been used to estimate the antiradical activity of the natural products [18] because of the ability of the extract to donate an H atom to free radical by the decrease in its absorbance at 517nm. According to Chevallier [19], 50µL of different extract concentrations was added to 5ml of the DPPH solution (0,004%). After 30 minutes of incubation, the absorbance is read at 517 nm. The following formula (1) was used to calculate the percentage of free radical DPPH inhibition (I%):

Inhibition % = (A control - A test) x 100 / ABS control$_1$.

With: A Control: is the absorbance of the blank solution; A test is the absorbance of the sample compound.

Antibacterial activity

Conserved bacterial strains were seeded into test tubes containing nutrient broth and then incubated at 37°C for 24 hours to stimulate their development. After bacterial growth, these strains were subcultured on an agar nutrient poured into a Petri dish and then incubated at 37°C for 24 hours. Its opacity must be equivalent to 0.5

Mc Ferland, which corresponds to 108 CFU/ml (Colony Forming Units), then diluted to obtain an inoculum of 106 CFU/ml (Neggaz, 2010)

Antibacterial activity was evaluated by the agar diffusion method known as the disc diffusion method [20]. The discs of 9mm in diameter, impregnated with 30µL of the 3 concentrations of the aqueous extract 200, 400 and 600 mg/ml of the EQRO and a disk containing DMSO or Distilled water as a negative control placed in the center of each plate. The medium poured into Petri dishes is inoculated by swabbing from a 106 CFU/ml bacterial suspension. This operation is repeated 3 times. The plates were incubated at 37 °C/24 h. The microbial growth is assessed by measuring the diameters of the zone of inhibition (mm) around the disks.

- Not sensitive (-) or resistant: diameter< 8mm
- Sensitive (+): diameter between 9 to 14 mm
- Very sensitive (++): diameter between 15 to 19 mm
- Extremely sensitive (+++): diameter• > 20 mm [21].

Four synthetic Antibiotics, namely Streptomycin (S: 10µg/disk), Gentamicin (GEN: 10µg/disk), Colistin sulfate (C.S.: 10µg/disk), Cefazolin (CZL: 30µg/disk) were used by the agar diffusion method to detect the sensitivity of the bacterial strains.

Statistic study

All experiments were performed in triplicate. For each test or method, the averages and standard deviations of the tests and the graphic representations were carried out using Excel 2007 software.

RESULTS

Phytochemical analysis

The extraction yield of the aqueous extract of *Rosmarinus officinalis* by the decoction method was estimated to be 14.49%

As indicated in Table 1, the EQRO revealed high values of total polyphenols content (455.10 ± 0.07 µg EAG/mg of extract) since the high tannins content (135.91±0.020 µg TAE/mg) was noticed in PC AQE. Whereas the total phenolic content in terms of mg GAE/g of the dry weight of extract was 7.33±0.04

Extract	[C] of polyphenols (µg EAG/mg of extract)	[C] of flavonoids (µg EAG/mg of extract)	[C] of tannins (µg EAT/mg of extract)
Aqueous	455.10±0.07	7.33±0.04	135.91±0.020

Table 1: Total polyphenol, flavonoids and tannins content in *Rosmarinus officinalis* extract.

Antioxidant activity

DPPH radical scavenging assay

The antioxidant activity of the extract was evaluated *in vitro* by the reduction method of free radical DPPH. The results can be expressed using the parameter IC_{50}, defined as the substrate concentration that causes a 50% loss of DPPH activity [22].

The results of the antiradical action of EQRO show an IC_{50} of the order of 0.128mg/ml. Our extract is less active than BHT (0.031 mg/ml).

Extract	BHT	EAQ (mg/ml)
IC_{50} (mg/ml)	0.031	0.128

Table 2. IC_{50} values of aqueous extract and BHT.

Antibacterial activity

The antibacterial activity was determined by measuring the diameter of the zone of inhibition, which was determined by the different concentrations of the EQRO around the discs. The results are recorded in Table 3 and Figure 1. This result shows that the aqueous extract only inhibits Gram (+) bacterial strains at different concentrations (A: 200, B: 400 and C: 600 mg/ml) with diameters of 10mm, 13mm, and 12mm, respectively. Rosemary is found to be inactive against *Escherichia coli* and *Pseudomonas aeruginasa*.

	[C] Extract(mg /ml) / Diameters of the inhibition zones (mm)		
Srains	**200 mg/ml**	**400 mg/ml**	**600 mg/ml**
Staphylococcus aureus	10	13	12
Pseudomonas aeruginosa	0	0	0
Escherichia Coli	0	0	0

Table 3: Antibacterial test results.

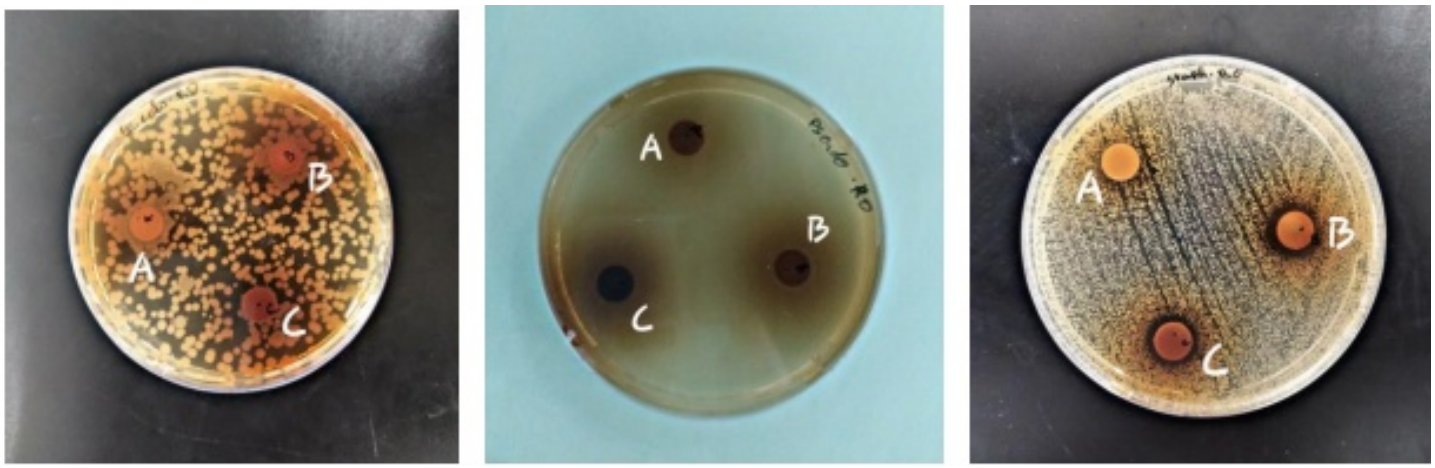
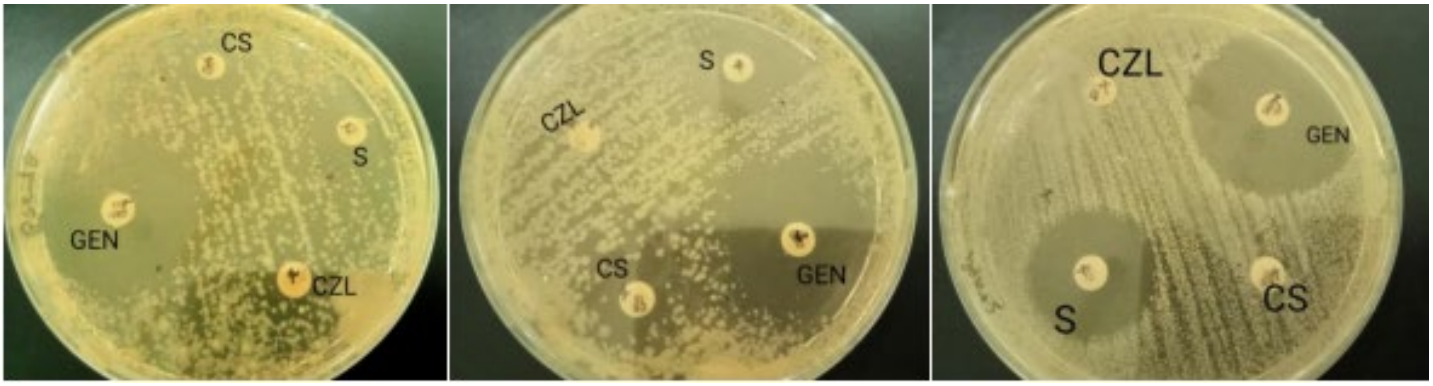


Figure 1: Growth inhibition zone of the three bacteria towards different concentrations of EQRO.

	Diameters of the inhibition zones(mm) / Antibiotics				
Strains	**Negative Control**	**Gentamicin (GEN)**	**Streptomycin (S)**	**Colistin sulfate (C.S.)**	**Cefazolin (CZL)**
Pseudomonas aeruginosa	0	31	21	12	0
Staphylococcus aureus	0	34	24	0	0
Escherichia Coli	0	30	23	11	0

Table 4: Diameters of the antibiogram inhibition zones of the three strains tested (*S. aureus*, *P. aeruginosa*, *E. coli*).



a) *P. aeruginosa* b) *E. coli* c) *S. aureus*

Figure 2: Antibiogram of strains: *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*.

It is observed that the bacterial strains react differently to the antibiotics tested; it is noted that the strains tested present an upper zone between 30 and 34 mm, which explains their sensitivity to gentamicin.
Cefalozine does not express any zone of inhibition on the three bacterial strains tested, and for Colistin sulfate, no zone of inhibition was found on *S. aureus*. This means that the bacterial strains are resistant to these antibiotics.

DISCUSSION

The aqueous extract (EQRO) is prepared by decoction (distilled water as a polar solvent). According to [23], temperature affects the yield of extraction (yield increases with heat), which shows that the higher the temperature, the greater the process of cell penetration and the solubilization of molecules by the solvent is easy [24]. However, heat can lead to the degradation of thermolabile molecules [25], so the decoction was performed briefly.
In this study, the yield of this extraction is of a value of 14.49%. One similar study was performed by [26], who recorded a yield of 14.49%. Also, there has been a high yield of 24.3% of aqueous extract of *Rosmarinus officinalis* originating from Sudan [27], equal to 24% in a study in Finland[28].
In general, the yield of the extraction method depends on several factors such as extraction time, temperature, geographical location, harvest time, climate and storage time [23]. It also depends on the nature of the solvent

used [29] and the extraction method applied [30]. Although ethanol and methanol were better solvents than others in extracting phenolic compounds due to their polarity and good solubility for these compounds, the results proved that ethanol was the best solvent to extract the phenolic compounds, followed by methanol and, finally, water, which could explain the difference [31].

Our results revealed that the aqueous extract is rich in polyphenols and flavonoids. Studies conducted on the contents of total phenolic compounds from three regions of Turkey, which varied between 70.3 and 147.3 mg EAG/g, differ from our results. The high polyphenol content in the aqueous extract is related to the high solubility of phenols in polar solvents [32].

The content of polyphenols in EQRO is similar to that reported by [33], which is 162 mg EAG/g, and [34], which is 127 ± 3 mg AEG/g, but are superior to those obtained by [35], which is 58.1 ± 0.9 mg AEG/g and [36] which is 39.1 ± 3.6 mg AEG/g. Furthermore, [37] and [38] recorded low polyphenol content in the leaves of *Rosmarinus officinalis* from France with 200 mg EAG/g and 211 mg EAG/g of extract, respectively. According to the results obtained by [36], the methanolic extract of rosemary contains 60.7±1.1mg /g, which supports our results. Thirty-nine recorded that the total flavonoid content of extracts of 28 plants is related to the content of total phenolic compounds. Likewise, we found that the flavonoid content of rosemary extracts is significantly correlated with the content of polyphenols (R2 = 0.996).

Tannins are proton donors to lipid free radicals produced during peroxidation. Forty revealed an absence of tannins from the methanol extract of *Rosmarinus Officinalis* in all parts of the plant.

Forty-one showed the effect of pre-extraction treatment (irradiation ionizing) and the extraction solvent on the concentration of total phenolic compounds in rosemary extracts. In addition, this difference can also be explained by the extraction method of the aerial part of the plant used to determine flavonoids [42].

The antioxidant activity is determined by a decrease in the absorbance produced by the antiradical substances [43]. The results are in agreement with [44,45].

Our results are lower than those reported by [46], where the IC_{50} values are 0.001 mg/ml and 0.05 mg/ml, respectively, for the methanolic extract and the ascorbic acid. In another study, [47] confirmed the antioxidant power of this plant in the Bechar region with inhibition percentages of around 80.70% and 79.62% for the two methanolic and aqueous extracts, respectively.

Furthermore, [48] reported that the concentration of total polyphenols is significantly correlated with the antioxidant capacity generally evaluated by the test DPPH. It is known that the antioxidant activity is solid and practical. That is, the value of IC_{50} is weak.

According to Turkmen [49], polyphenols are influential hydrogen donors to the DPPH radical due to their ideal structural chemistry. For the past 10 years, rosemary and its constituents (carnosol, acid carnosic acid, ursolic acid, rosmarinic acid, and caffeic acid) have been extensively studied [50]. Carnosic acid and carnosol are responsible for 90% of the antioxidant activity of rosemary and together represent about 5% of the dry weight of its leaves [51,52].

The results of the antibacterial action of the rosemary extract showed a difference in the diameters of the zone of inhibition of *Escherichia Coli* ATCC (0.00 mm) compared to the results obtained by [53] of the ethanolic extract (16.62 mm).

Our results agree with the scientific work of [54], who mentioned that the aqueous extract of *Rosmarinus officinalis* is inactive on all Gram – (*E. coli*, *P. aeruginosa*) strains.

The antimicrobial activity depends not only on the presence of the phenolic compounds but also on the presence of various secondary metabolites [55], location, and number of hydroxyl groups [42].

Several classes of polyphenols, such as phenolic acids, flavonoids and tannins, serve as a defense mechanism of plants against microorganisms, insects, and pathogenic herbivores [42]. Polyphenols, tannins and flavonoids such as epigallocatechin, catechin, myricetin, quercetin, [56] and Luteolin [57] are important antibacterial substances.

CONCLUSIONS

In conclusion, the EQRO of the leaves had a high content of total polyphenols, flavonoids, and tannins, which also had an antibacterial effect. This suggests that the EQRO plays a protective role from oxidative stress. Data from this study indicates that *Rosmarinus officinalis* can either increase antioxidant power, reduce oxidative stress, or do both. These results support the beneficial utilization of this plant as a natural antioxidant in food and folk medicine, which prevents excessive production of free radicals and is used as an antibacterial agent.

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Author contribution:
BENCHEIKH Dalila: Supervise, conceptualize, validate, visualize, perform formal analysis, write reviews and edits, and write original drafts.
Khawla laichi: Data curation; Formal analysis; Investigation; Methodology; Software; Writing-original draft
Chemseddine herizi: Data curation; Formal analysis; Investigation; Methodology; Software; Writing-original draft; Writing-review & editing.
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Seddik khennouf: Project administration; Validation; Visualization; Writing-review & editing
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