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Antioxidant, anti-inflammatory and antimicrobial activities of aqueous and methanolic extract of *Rosmarinus eriocalyx* Jord. & Fourr.

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ABSTRACT

Rosmarinus eriocalyx Jord. & Fourr is a well-known aromatic and medicinal plant whose consumption serves to remedy a number of disorders, evergreen bush endemic to Algeria. The present study aimed it investigating the *in-vitro* anti-inflammatory and antimicrobial for two extracts of *Rosmarinus eriocalyx.* The antioxidant activity was evaluated by DPPH and the reducing power essay, anti-inflammatory activity with the Human Red Blood Cell (HRBC) membrane stabilization method. However, the antimicrobial activity was tested with three bacterial strain *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC25923 and *Bacillus subtilis ATCC6633.* The results show an excellent antioxidant and an interesting inflammatory activities but a weak power against the used strains therefore.

Keywords: *Rosmarinus eriocalyx* Jord. & Fourr, DPPH, reducing power, HRBC, antimicrobial.

INTRODUCTION

Rosmarinus eriocalyx Jord. & Fourr. (Rosemary) is a medicinal plant native to the Mediterranean region. This plant belongs to Lamiaceae family. Rosemary is a perennial evergreen herb with fragrant needlelike leaves (Bousbia et al., 2008).

Lamiaceae (Labiatae) is a family in the Lamiales order. It has important medicinal and aromatic plants, very important in the honey and cosmetic industry. It has famous culinary herbs like sage, mint, thyme and savory. Their chemical constituents, including terpenoids and flavonoids, are the important agents for their aromatic, antioxidant, antibacterial and antifungal properties. There are also many well-known ornamental plants in the family which are widely used in horticulture and landscaping. The family contains 236 genera and about 7,173 species and is almost cosmopolitan, but absent from the coldest regions of high altitude or latitude (Harley et al., 2004).

Rosemary extracts have been used in the treatment of diseases, due to its hepatoprotective potential (Raškovi et al., 2014) the therapeutic potential for Alzheimer's disease (Habtemariam, 2016) and its antiangiogenic effect (Kayashima and Matsubara, 2012). On the other hand, they have been used in food preservation, because they prevent oxidation and microbial contamination (Djenane, 2002).

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Plant antioxidants are very significant as their presence in the human diet can help the body to neutralize free radicals and reduce the oxidative stress damage. On the contrary, synthetic antioxidants have possible activity as promoters of carcinogenesis (Suhaj, 2006).

Inflammation is the response of living tissues to injury. It involves a complex array of enzyme activation, mediator release, extravasations of fluid, cell migration, tissue breakdown and repair (Vane and Bolting, 1995; Perianayagam et al., 2006). Inflammatory diseases are becoming common in aging society throughout the world. Recent studies indicate that the mediators and cellular effectors of inflammation are important constituents of the local environment of tumors (Albert et al., 2008).

The present study focused on the *R. eriocalyx* to determine the antioxidant, anti-inflammatory and antimicrobial activities of two extracts of Algerian endemic rosemary.

MATERIALS AND METHODS

Plant material

The areal parts of *R. eriocalyx* were taken from the massif of Boutaleb (X1 E: 5° 30' 2.46" Y1 N: 35° 44‟ 41.74"; X2 E: 5° 25' 17.69" Y2 N: 35° 50‟ 1.85"; X3 E: 5° 8' 35, 98" Y3 N: 35° 43‟ 12.47"; X4 E: 5° 18' 45.10" Y4 N: 35° 41‟ 3.56") in mars 2017.

Preparation of aqueous extracts

The method for preparing aqueous extracts from dried plant has been already described by (Predrag et al., 2005). Briefly, dried plant material (10 g) was stirred in 100 ml of distilled water for 15 min at 90 °C followed by rapid filtration through four layers of gauze and then by a more delicate filtration through Whatman filter paper № 1. The resulting filtrate evaporated to dryness under vacuum. The powder was stored at −10°C until required.

Preparation of methanol extract

The areal parts of the two species were powdered and macerated in 80% methanol for 24, 48 and 72 h, at the laboratory temperature (1:10 w/v, 10 g of dried herb). After maceration, the extracts were collected, filtered and evaporated to dryness under vacuum (Lakić et al., 2010). The dry extracts were stored at a temperature of -18 °C for later use.

Determination of total phenolic content

For total polyphenol determination, the Foline Ciocalteu method was used (Li et al., 2007). The samples (0.2 mL) were mixed with 1 mL of the Folin-Ciocalteu reagent previously diluted with 10 mL of deionized water. The solutions were allowed to stand for 4 min at 25 °C before 0.2 mL of a saturated sodium carbonate solution (75 mg/mL) was added. The mixed solutions were allowed to stand for another 120 min before the absorbances were measured at 765 nm. Gallic acid was used as a standard for the calibration curve. The total phenolic compounds content was expressed as mg equivalent of Gallic acid per gram of extract (mg EAG/GE).

Determination of total flavonoids contents

The flavonoids content in our extracts were estimated by the Aluminium chloride solution according to the method described by (Bahorun et al., 1996). Briefly, 1 mL of the methanol solution of the extracts was added to 1 mL of 2 % $AlCl_3$ in methanol. After 10 min, the absorbances were determined at 430 nm. Quercetin was used as a standard. Results were expressed as mg equivalent Quercetin per gram of extract (mg EQ/GE).

DPPH assay

The donation capacity of extract was measured by bleaching of the purple-coloured solution of 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) according to the method of (Hanato et al., 1988). One millilitre of the extracts at different concentrations was added to 0.5 mL of DPPH-methanol solution. The mixtures were shaken vigorously and left standing at the laboratory temperature for 30 min in the dark. The absorbances of the resulting solutions were measured at 517 nm. The antiradical activity was expressed as IC_{50} (micrograms per millilitre). The ability to

scavenge the DPPH radical was calculated using the following equation:

$$DPPH\ scavenging\ effect\ (\%) = [(A_0 - A_1)/A_0] \times 100$$

Where:

A_0: the absorbance of the control at 30 min 111

A_1: is the absorbance of the sample at 30 min. Butylated hydroxytoluene (BHT) was used as standard (Bettaieb et al., 2012).

Reducing power

The reducing power was determined according to the method of (Oyaizu, 1986) 2, 5 mL of the extracts were mixed with 2.5 mL of sodium phosphate buffer (pH 6.6; 200 mmol/L) and 2.5 mL of potassium ferricyanide (10 mg/mL). The mixtures were incubated at 50 °C for 20 min. After cooling, 2.5 mL of trichloroacetic acid (100 mg/mL) were added; the mixtures were centrifuged at 200g for 10 min. The upper layer (5 mL) was mixed with 5 mL of deionized water and 1 mL of 1 mg/mL ferric chloride, and the absorbance was measured at 700 nm against a blank. EC_{50} value (mg extract/mL) is the effective concentration at which the absorbance was 0.5 for reducing power and was obtained by interpolation from linear regression analysis. Ascorbic acid was used as a reference standard (Huang Mau, 2006).

The Human Red Blood Cell (HRBC) membrane stabilization method

To prepare the HRBC suspension, fresh completely human blood (10 mL) was collected and transferred into the centrifuge tubes. These lasts were centrifuged at 3000 rpm for 10 min thrice and washed with an equal volume of normal saline each time. The volume of blood was measured and reconstituted as 10 % v/v suspension with normal saline.

The principle involved here was stabilization of human red blood cell membrane by hypo-tonicity induced membrane lysis. The mixtures contain 1mL phosphate buffer (pH 7.4, 0.15 M), 2 mL hypo saline (0.36%), 0.5 mL HRBC suspension (10% v/v) and 0.5 mL of plant extracts or standard drug (diclofenac sodium) at various concentrations (10, 50, 100, 250, 500 µg/mL). The control was distilled water instead of hyposaline to produce 100 % hemolysis.

The mixtures were incubated at 37 °C for 30 min and centrifuged at 2500 rpm for 5 min. The absorbance of hemoglobin content in the suspensions was estimated at 560 nm. The percentage of hemolysis of HRBC membrane can be calculated as follows:

$$Haemolysis\ (\%) = (Optical\ density\ of\ Test\ sample / Optical\ density\ of\ Control) \times 100$$

However, the percentage of HRBC membrane stabilization can be calculated as follows:

$$Protection\ (\%) = 100 - [(Optical\ density\ of\ Test\ sample / Optical\ density\ of\ Control) \times 100]$$ (Seema et al., 2011).

Antimicrobial activity

Bacteria Strains were obtained from the American Type Culture Collection: Gram-positive bacteria (*Staphylococcus aureus* ATCC25923 and *Bacillus subtilis* ATCC6633), Gram-negative bacteria (*Klebsiella pneumonia* ATCC700603) and one yeast: *Candida albicans* ATCC1024. Muller Hinton agar was used for bacteria culture and Sabouraud for yeast.

Anti-bacterial Activity

Agar disc diffusion method was employed for the determination of antibacterial activities of the extract (NCCLS, 1999) (NCCLS, 1997). Briefly, a suspension of the tested microorganism (10^8 CFU / mL) was spread on the solid media plates. Filter paper discs (6 mm in diameter) were impregnated with 10 µL (100 mg/mL) of the extract and placed on the inoculated plates. These plates were incubated at 37 °C for 24 hours. Gentamicin (10 µg/disc) was used as a standard and dimethylsulfoxide DMSO as a control.

The antibacterial activity was determined by measuring of inhibition zone diameters (mm) and was evaluated according

to the parameters suggested by Alves et al. (2000)

- <9 mm, inactive;
- 9–12 mm, less active;
- 13–18 mm, active;
- >18 mm, very active.

Statistical analysis

Results were expressed as the mean ± standard deviation. Data was statistically analysed using one-way ANOVA and Newman-Keuls Multiple Comparison to determine whether there were any significant with the criterion of P values < 0.05 between methanol extracts of the two species and standards, using Graphpad prism 5 Demo Software.

RESULTS

R. eriocalyx to contain an important quantity of polyphenols and flavonoids (Table 1) and even the yield of extract. Phenolic are the largest group of phytochemicals and have been touted as accounting for most of the antioxidant activity of plants or plant products. Higher content of total polyphenols was found in the Aqueous extracts (18,90 ± 1,06mg EAG/GE) thane the méthanolic extract (4,47± 2,99 mg EAG/GE). The total flavonoid content of extracts was 4,10 ± 0,05mg EQ/GE for the methanolic *extract* and 1,15 ±0,05mg EQ/GE for the aqueous extract.

The result of DPPH scavenging activity in the present study indicates that the plant is potentially active. The antioxidant capacity is also expressed as 50% inhibitory concentration (IC_{50}). The aqueous extract of *R. eriocalyxto* (IC_{50}value 74,62 µg/ml) exhibited little stronger scavenging efficacy than methanolic leaf extract (IC_{50} value 247,21µg/ml) and BHT (IC_{50} value 8,37µg/ml) was used as a standard as shown in figure. 1et figure 2.

Protein denaturation is a process in which proteins lose their tertiary structure and secondary structure by application of external stress or compound, such as strong acid or base, a concentrated inorganic salt, an organic solvent or heat. Most biological proteins lose their biological function when denatured. Denaturation of proteins is one of the well-defined causes of inflammation (Leelaprakashand and Mohan Dass, 2011). The methanol extract and aqueous extract of *Rosmarinus eriocalyx* significantly ($p \leq 0.001$) inhibited lysis induced by water. This is confirmed by the high percentage inhibition of hemolysis obtained for doses from 10, to 500 µg/mL (Figure 3). However, Diclofenac Sodium was weaker than the tested extracts even at high concentrations.

The antibacterial activity of the plant extracts was tested against two Gram-positive bacteria, *Bacillus subtilis (*ATCC6633) and *Staphylococcus aureus* (ATCC25923) and one Gram-negative bacteria *Pseudomonas aeruginosa* (ATCC 27853). Table 2 portrayed the zones of methanol extract and aqueous extract of *R. eriocalyx* inhibition. The methanol extracts showed more antimicrobial activity than the aqueous extract of *R. eriocalyx.*

Table 1: Yield of the two extracts of *R. eriocalyx* to and their quantification of polyphenols and flavonoids.

	Aqueous extract	**Methanolic extract**
Polyphenols(MGEAG/GE)	18,90± 1,06	4 ,47±2,99
Flavonoids (MG EQ/GE)	1,15 ±0,05	4,10±0,05

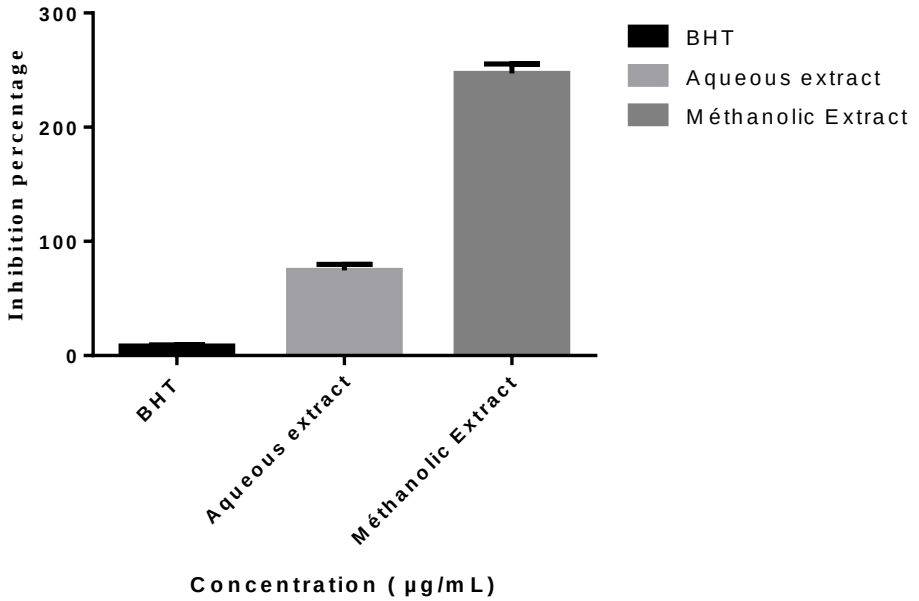

Figure 1: DPPH free radical scavenging activity of different IC_{50} of two extract of *R. eriocalyx* Jord. & Fourr.

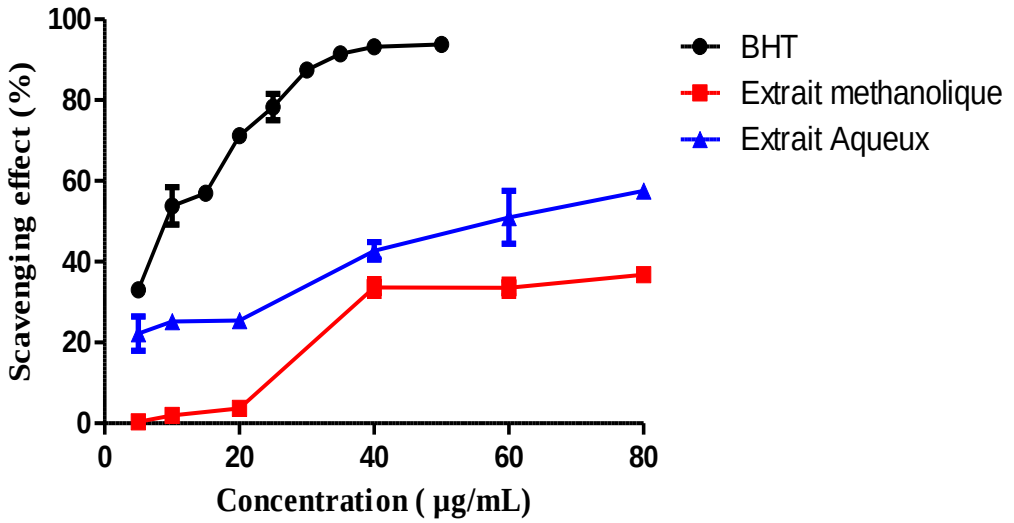

Figure 1: DPPH test of two extracts *Rosmarinus eriocalyx* Jord. & Fourr.

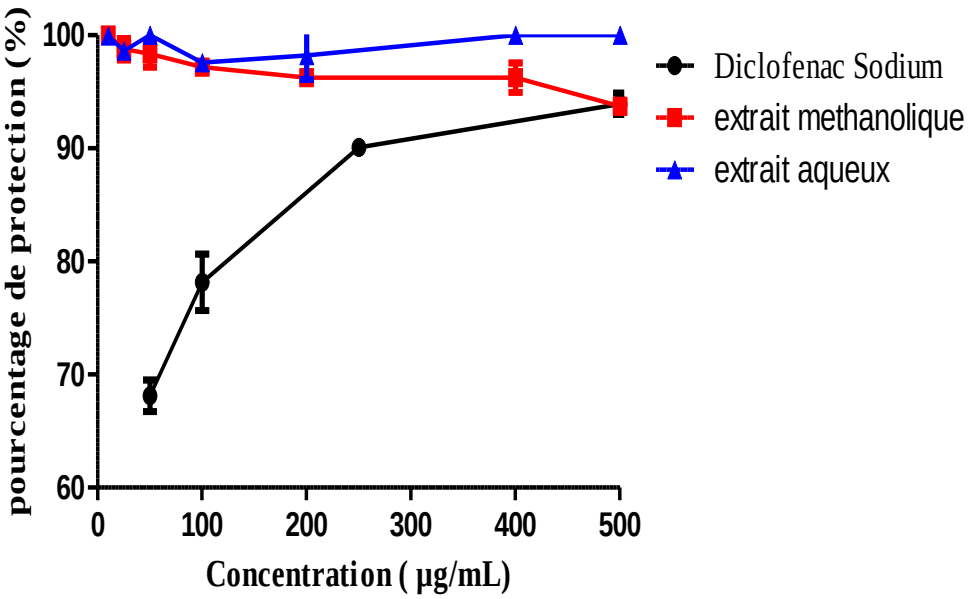

Figure 3: The percentage inhibition of hypotonicity induced haemolysis of HRBCs (%) of standard and methanol extract and aqueous extract of *R. eriocalyx.*

Table 2: The inhibition zone of methanol extract and aqueous extract of *R. eriocalyx* with positive and negative controls.

Organism test	**Aqueous extract**	**Methanolic extract**	**Gentamicin**	**Control negative**
PSEUDOMONAS AERUGINOSA ATCC 27853,	7,75±0,63	9,12±0,26	40,29±0,15	NI
BACILLUS SUBTILIS ATCC6633	8,45±0,25	8,59±0,39	29,14±0,16	NI
STAPHYLOCOCCUS AUREUS ATCC25923	6±0	8,34±0,21	25,85±0,10	NI

All the results are mean ±SD (n=3).

DISCUSSION

The assay is based on the reduction of DPPH, a stable free radical; any molecules that can donate an electron or hydrogen to DPPH can react with it and thereby breach the DPPH absorption. As the odd electron of the radical becomes paired off in the presence of a hydrogen donor, that is a free radical scavenging antioxidant, the absorption strength is decreased and the resulting decolorization is stoichiometric with respect to the number of electrons captured (Halliwell and Gutteridge, 1999). The slightly higher free radical scavenging activity of the aqueous extract presumably indicates the presence of a higher content of protic flavonoids in the aqueous extract than the methanolic extract, facilitating HAT (hydrogen atom transfer) to take place. Steric inaccessibility of the large molecules may also be a factor (Prior et al., 2005).

It is relevant from the present study that secondary metabolites of *extracts of R. eriocalyx* protected the human erythrocyte membrane against lysis induced by hypotonic solution and heat. The erythrocyte membrane resembles to lysosomal membrane and as such the erythrocyte could be extrapolated to the stabilization of lysosomal membrane (Omale and Okafor, 2008). The vitality of cells depends on the integrity of their membranes, exposure of RBC's to injurious substances such as hypotonic medium(Augusto et al ., 1982). The process of acute inflammation is initiated by the blood vessels local to the injured tissue, which alter to allow the exudation of plasma proteins and leukocytes into the surrounding tissue. The increased flow of fluid into the tissue causes the characteristic swelling associated with inflammation (Deraedt et al., 1980).

Agar disc diffusion method was employed for the determination on antimicrobial activities of secondary metabolites of *Galium tunetanum* Poiret (NCCLS,1999). Antimicrobial activity in plant extracts depends not only on the presence of phenolic compounds but also on the presence of various secondary (Ćetković et al., 2007).

Conclusion

The results obtained in the present study showed that the two extracts of the *R. eriocalyx* show important sources of phenolic compounds, an excellent antioxidant extract, and a perfect anti-inflammatory substance. However, further investigations are required to isolate the active constituents responsible for the observed effect, and to elucidate the possible mechanisms of action responsible for the inflammatory activities of the plant extract.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

All authors contributed seriously to this work. The manuscript was read and approved by all.

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