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In vitro antioxidant activities of *Rhanterium suaveolens* extracts

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- ✓ Haemolysis inhibition.

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Abstract

To evaluate the antioxidant activity of the *n*-butanol and ethyl acetate extracts obtained from aerials part of *Rhanterium suaveolens*. Different extracts were tested for *in vitro* free radical scavenging assays, such as hydroxyl radical scavenging activity, inhibition of lipid peroxidation and inhibition of hydrogen peroxide-induced erythrocyte haemolysis. Further, total flavonoids content of *R. suaveolens* was analyzed. *R. suaveolens* extracts effectively scavenged free radicals at different concentrations and showed potent antioxidant activity in a dose dependent manner. The extracts also reduced hydrogen peroxide induced erythrocyte haemolysis and lipid peroxidation. Results were compared with vitamin C. In conclusion, *R. suaveolens* have strong antioxidant potential

1. Introduction

Oxidative stress is a fundamental principle in the pathophysiology of many diseases. It occurs when the production of reactive oxygen species exceeds the capacity of the cell defense system [1]. Antioxidants prevent free radical induced tissue damage by preventing the formation of radicals, scavenging them, or by promoting their decomposition. Synthetic antioxidants are recently reported to be dangerous to human health. Thus the search for effective, nontoxic natural compounds with antioxidative activity has been intensified in recent years. In addition to endogenous antioxidant defense systems, consumption of dietary and plant-derived antioxidants appears to be a suitable alternative [2].

In the search of plants as a source of natural antioxidants, some medicinal plants have been extensively studied for their antioxidant activity and radical scavenging among them plants from Asteraceae family [3].

The genus *Rhanterium* Desf (Asteraceae: Inuleae) is distributed over western North Africa (Algeria, Tunisia and morocco), the Arabian Peninsula, Iraq and Iran. Three species are recognized: *R. adpressum* Coss. & Durieu, *R. epapposum* Oliver and *R. suaveolens* Desf. A fourth previously accepted species, *R. intermedium* Coss. & Durieu ex Pomel, is here considered to be composed of hybrids between *R. adpressum* and *R. suaveolens* [4]. Many chemical compounds have been isolated and identified from *R. suaveolens* such as ceramides [5], ranthenone glucoside, 9-hydoxylinaloyl glucoside, sitosterol-3b-O-[6´-palmitoyl-b-D-glucopyranoside], scopoletin, fraxetin, and scopolin [6]. Fews tudies have demonstrated that different extracts (hexane, ethyl acetate, water and methanol) of *R. suaveolens* possesses antibacterial, anticholinesterase [7, 8] and antioxidant activities [9]. In this context, the present study aims to determine the total flavonoid contents of *n*-butanol and ethyl acetate extracts obtained from aerials part of *R. suaveolens* and to evaluate their antioxidant activities.

2. Experimental details

1.2. *Chemicals and reagents*

Solvents: Chloroforme, toluene, ethyl acetate and methanol reagent grade were purchased from VWR (Fontenay-sous-bois, France).

Chemicals: Trichloroacetic acid (TCA), Iron (II) sulfate, sodium sulfate, 2-Thiobarbituric acid, Aluminum chloride and vitamin C were purchased from Sigma–Aldricht (Steinheim, Germany). Sodium chloride was purchased from VWR (Fontenay-sous-bois, France); potassium chloride was purchased from Prolabo (Paris, France); Potassium dihydrogen phosphate and sodium hydrogen phosphate were purchased from Merck (Darmstadt, Germany).

2.2. *Plant material*

Rhanterium suaveolens Desf was collected during the flowering stage 2003 from the area of Ouargla southern Algeria and authenticated by Pr. M. Kaabeche (University of Sétif, Algeria)

2.3. *Extraction procedure*

Air-dried aerial parts of Rhanterium suaveolens (1290 g) were powdered and macerated at room temperature with EtOH–H_2O (8:2, v/v) for 48 h, three times. After filtration, the filtrates were combined, concentrated under vacuum (up to 35 °C), dissolved in distilled H_2O (600 ml) under magnetic stirring and maintained at 4 °C for one night to precipitate a maximum of chlorophylls. After filtration, the resulting solution was successively extracted with (3 x 400 ml) of chloroform, ethyl acetate and *n*-butanol. The organic layers were dried with Na_2SO_4, filtered using common filter paper and concentrated under reduced pressure (up to 35 °C) to obtain the following dry extracts: $CHCl_3$ (6.5 g), EtOAc (12 g) and *n*-butanol (19 g) [9].

2.4. *Determination of total flavonoids content*

Total flavonoids content was estimated according to the method described by Wang et al. (2008) [10]. Briefly, to 0.5 mL of sample, 0.5 mL of 2% $AlCl_3$ methanol solution was added. After 1 h incubation at room temperature, the absorbance was measured at 420 nm. Total flavonoid content was calculated as µg of quercetin equivalents (QE) per 1 mg of extract.

2.5. *Hydroxyl radical scavenging activity*

Hydroxyl radical Scavenger ability was measured following the method described by Wang et al. (2008) [10] with a few modifications Su et al. (2009) [11]. ·OH radicals were generated from $FeSO_4$ and H_2O_2 and evaluated by their ability to react with sodium salicylate. In this method 0.5 ml $FeSO_4$ (8 mM) was mixed with 0.8 mL H_2O_2 (6 mM), 0.5 mL distilled water, then varying concentrations of test samples and 0.2 mL sodium salicylate (20 mM) was added. The mixture was incubated for 1 h at 37°C; the absorbance of the mixture was recorded at 562 nm. Ascorbic acid was used as control positive.

2.6. *Assays of lipid peroxidation using vitellose*

A modified thiobarbituric acid-reactive species (TBARS) assay described by Cao and Ikeda, 2009 [12] was used to measure the lipid peroxide formed, using egg *vitellose* homogenates. Briefly 10% homogenate were incubated with different dose of *n*-butanol, ethyl acetate extracts or vitamin C in the presence of 50µL $FeSO_4$ (0.07 M) at 37 ◦C for 1 h. reaction was stopped by addition of 1mL trichloroacetic acid (TCA 20%), and 1.5mL thiobarbituric acid (TBA 1%) in succession, and the solution was then heated at 100 ◦C for 15 min. After centrifugation at 4000 rpm for 20min to remove precipitated protein the absorbance was detected at 532 nm.

2.7. *Inhibition of the haemolysis of erythrocytes induced by hydrogen peroxide*

The anti-haemolysis activity was assayed according to the method described by Li et al. (2009) [13]. Blood was collected from male rats eyeballs. The erythrocytes were separated from plasma by centrifugation at 1006g for 20 min. The erythrocytes were then washed five times with 10 mL of phosphate-buffered saline (PBS, pH 7.4). About 1 mL of 0.5% (v/v) suspension of erythrocytes in PBS was mixed with 0.1 mL different concentrations of different extracts of *R. suaveolens*. The incubation mixture was shaken gently in a water bath at 37 °C for 10 min. After incubation, 0.5 mL of 100 mM H_2O_2 was added to the mixture and incubated in a water bath at 37°C for 60 min. After that, 4 mL of PBS solution were added to the reaction mixture, followed by centrifugation at 1006 g for 5 min. The absorbance of the supernatant at 415 nm was recorded in a spectrophotometer.

2.8. *Statistical analysis*

All data are expressed as the mean ± SD of triplicate measurements. The statistically significant differences among mean values at the level of significance ($P < 0.05$) were evaluated with the paired *t* test in SPSS (version 19.0).

2. Results and Discussion

3.1 *Concentration of flavonoids*

The concentration of flavonoids in various extracts of *R. suaveolens* was determined by using a spectrophotometric method using $AlCl_3$. The values for concentrations of flavonoids are expressed in terms of Quercetin equivalents (standard curve equation: y = 0.034x + 0.015, R^2= 0.990) as µg QE/mg extract. The concentration of flavonoids in plant extracts ranged from 54.4µg/mg to 300µg/mg. A high concentration of flavonoids was measured in the *n*-butanol extract.

3.2 *Hydroxyl radical scavenging activity*

Scavenging of hydroxyl radical is an important antioxidant activity because of very high reactivity of the hydroxyl radical which can easily cross cell membranes; react with most biomolecules (such as sugars, amino acids, lipids, and nucleotides) and furthermore cause cell damage [14]. Although hydroxyl radical formation can occur in several ways, by far the most important mechanism *in vivo* is the Fenton reaction, where a transition metal is involved as prooxidant in the catalyzed decomposition of superoxide and hydrogen peroxide [10, 15]. With this assay, the IC_{50} value of ethyl acetate and *n*-butanol extracts were 19.33 µg/mL and 22.15 µg/mL respectively while the value of vitamin C was 10.53 µg/mL (Figure1). Generally, the scavenging activity of phenolic compounds might be due to the active hydrogen donating ability hydroxyl substitutions.

Figure1 showed that different extracts of *R. suaveolens* exhibited chelating effect on ferrous ions, suggesting that they decrease the concentration of metal in the Fenton reaction. The antioxidant effect of several polyphenols that acts as inhibitors of hydroxyl radical formation and lipid peroxidation has been correlated with iron chelating properties [12, 16].

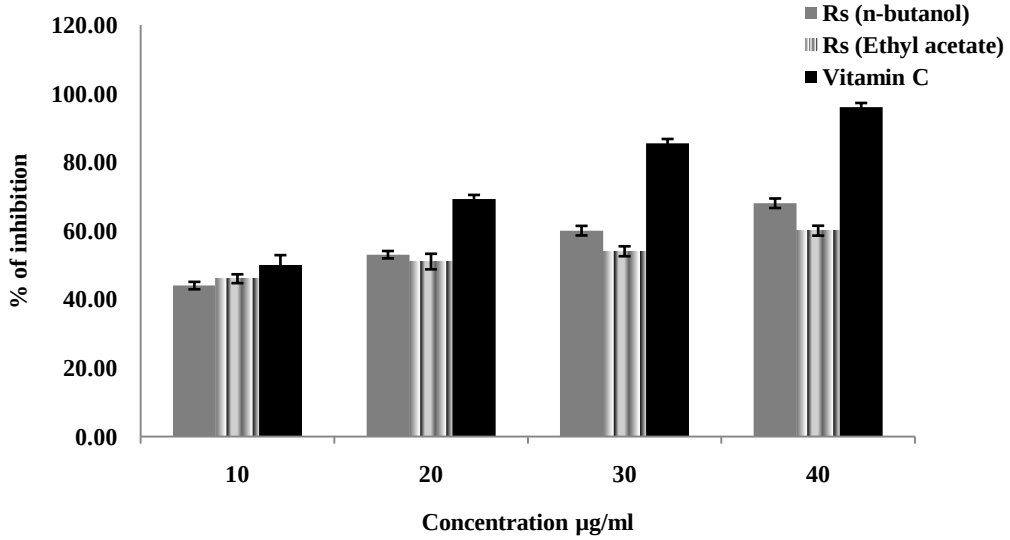

Figure 1: Scavenging activity of *n*-butanol, ethyl acetate extracts of *R. suaveolens* and vitamin C against hydroxyl radical measured with the system of Fenton (mean±SD, n=3).

3.3 *Inhibition of lipid peroxidation*

Lipid peroxidation is a process generated naturally in small amounts in the body, mainly by the effect of several reactive oxygen species (hydroxyl radical, hydrogen peroxide etc.). These reactive oxygen species readily attack the polyunsaturated fatty acids of the fatty acid membrane, initiating a self-propagating chain reaction. The destruction of membrane lipids and the end-products of such lipid peroxidation reactions are especially dangerous for the viability of cells, even tissues [17].

In the present study, we measured the potential of different extracts of *R. suaveolens* to inhibit non-enzymatic lipid peroxidation in egg *vitellose* homogenate, induced by the $FeSO_4$ system. The effects of different extracts of *R. suaveolens* on non-enzymatic peroxidation are shown in Figure 2. The inhibition of lipid peroxidation of ethyl acetate and *n*-butanol extracts were increased with increasing concentration. The percentage inhibition of lipid peroxidation by 200 µg/mL of ethyl acetate and *n*-butanol extracts were found to be 50.90% and 34.49 % respectively. The ratio at this concentration for vitamin C was found to be 97.5% inhibition of lipid peroxidation. While at 300 µg/mL the percentage inhibition of lipid peroxidation of ethyl acetate and *n*-butanol extracts were increased to be 57.53% and 66.73 % respectively.

Decrease in lipid peroxidation by ethyl acetate and *n*-butanol extracts may be a result of it scavenging free radicals produced by $FeSO_4$ in the reaction system and on the other hand, ethyl acetate and *n*-butanol extracts may be rich in phenolic compounds such as flavonoids that have many OH groups. The strong antioxidant effects of flavonoids have been highlighted by several studies [18, 19].

3.4 *In vitro erythrocyte haemolysis inhibition assay*

Red blood cells (RBCs) membrane is the widest and most used model for studying biomembrane oxidative damage. As most of the membranes, it plays a fundamental role in maintaining cellular homeostasis of RBCs. RBCs are especially susceptible to oxidation due to their high content of polyunsaturated lipids, their rich oxygen supply and the presence of transition metals such as Fe and Cu [20]. Invasion of the RBCs membrane by peroxidants may lead to cell hemolysis. [21]. Flavonoids, which are phytochemicals produced by various plants in high quantities, have been proposed as an effective approach for the prevention and treatment of multiple RBCs disorders *via* scavenging reactive oxygen species and inhibiting free-radical-induced membrane lipid oxidation [22]. The *in vitro* model of erythrocytes haemolysis was generally used to evaluate the antiradical and antioxidant activities by mimicking this situation. The assay principle of this experiment is that hydrogen peroxide, which crosses the erythrocytes membrane and acts on the intracellular moiety, forms ferryl radical or hydroxyl radical by interacting with hemoglobin and initiates a series of reactions, resulting in erythrocytes lysis (haemolysis) [23].

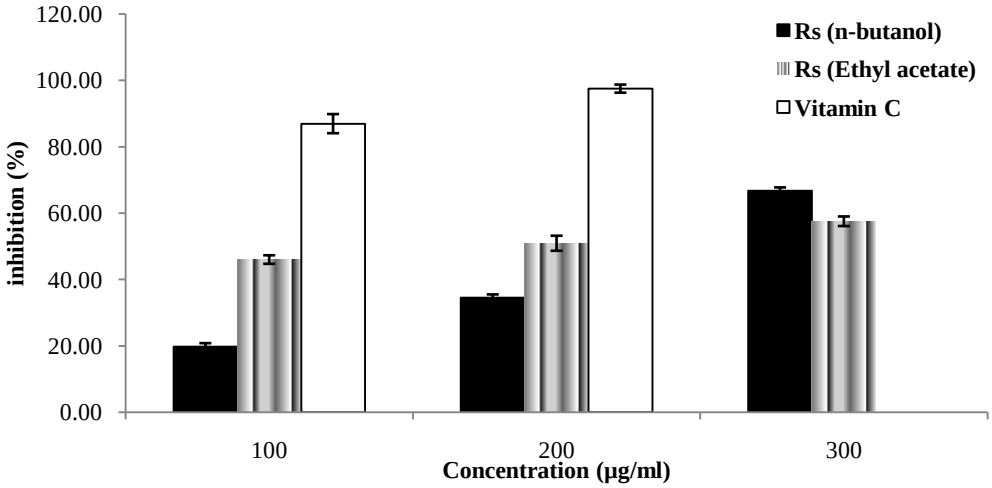

Figure 2: Inhibition of $FeSO_4$ induced lipid peroxidation of egg *vitellose* by *n*-butanol, ethyl acetate extracts of *R. suaveolens* and vitamin C (mean±SD, n=3).

The inhibition effects of *n*-butanol and ethyl acetate extracts of *R. suaveolens* on the erythrocytes haemolysis induced by H_2O_2 are shown in Figure 3.

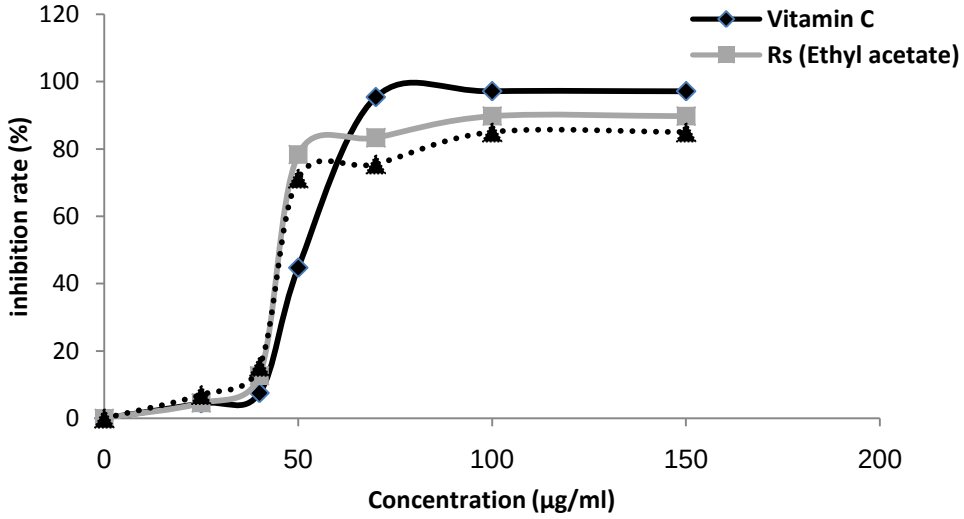

Figure 3: Inhibition effects of *n*-butanol and ethyl acetate extracts of *R. suaveolens* on the haemolysis of erythrocytes induced by H_2O_2 (mean±SD, n=3).

The results indicated that the *n*-butanol and ethyl acetate extracts of *R. suaveolens* could prevent erythrocytes haemolysis in a dose-dependent manner. At the dose 0.1 mg/mL, ethyl acetate and *n*-butanol extracts displayed a significant inhibition of erythrocytes haemolysis (89.72% and 85.01 % respectively while the ratio of vitamin C was 97.12%). The inhibitory effect may be attributed to their phenolic compounds such as flavonoids which can donate electrons to H_2O_2, thus neutralizing it to water molecule [24]. So *R. suaveolens* extracts are proved to be potentially valuable sources of natural antioxidants and bioactive materials.

Conclusions

The result of this study clearly indicate that *n*-butanol and ethyl acetate extracts obtained from aerials part of *R. suaveolens* have powerful antioxidants capacity against various antioxidant systems *in vitro* and the capacity was concentration dependent. Further studies are needed to further explain the results obtained in this work.

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