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Phenolic content and Antioxidant activities of Algerian *Echium plantagineum* Extracts

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ABSTRACT

Objective: This study aims to explore the phenolic composition of aqueous (Aq E) and methanolic (Met E) extracts of Algerian *Echium plantagineum* aerial part and evaluate their antioxidant capacity. **Methods**: Total phenolics, flavonoids and tannins contents were determined by spectrophotometric methods using Folin-Ciocalteu, aluminium chloride and hemoglobin precipitation assays, respectively. The antioxidant activities were evaluated using several *in vitro* tests including reactive oxygen species ($ABTS^{\cdot}$, $OH^{\cdot}$, $O_2^{\cdot}$ and H_2O_2) scavenging assays, ferrous ions chelating test, reducing power and anti-hemolytic assays. **Results**: The phytochemical study revealed a predominance of tannins, total polyphenols and flavonoids in both extracts. Aq E showed more efficiency to scavenge $ABTS^{\cdot}$, $O_2^{\cdot}$ and $OH^{\cdot}$ radicals and to chelate ferrous ions. However, Met E was more efficient (IC_{50}=193.87µg/mL) in scavenging H_2O_2 than Aq E (IC_{50}=262.46µg/mL). In the same way, Met E inhibited AAPH induced hemolysis (HT_{50}=179.79±5.82 min) more than Aq E (HT_{50}=161.62±5.87 min). On the other hand, both Met E and Aq E exhibited very high reducing power (IC_{50}=117µg/mL and 128µg/mL, respectively). **Conclusion**: This study shows that *Echium plantagineum* extracts possess strong antioxidant activity, which is probably due to their phenolic composition. Therefore, this plant can constitute a promising source of natural antioxidants.

Key words: Antioxidant, *Echium plantagineum*, Flavonoids, Phenolic compounds, ROS.

INTRODUCTION

Reactive oxygen species (ROS) have been implicated in the oxidative deterioration of food products as well as in the pathogenesis of several human diseases such as atherosclerosis, diabetes, chronic inflammation, neurodegenerative disorders and even certain types of cancer [1, 2]. Many synthetic antioxidants, such as butylatedhydroxytoluene (BHT), are used as additives in food industry to prevent deterioration. However, there is concern about their safety on human health [3]. Medicinal plants seem to be one of the main sources of safe antioxidants and healthcare supplements. It has been reported that polyphenolic compounds are among the active substances in these plants having multiple biological activities.

Echium plantagineum, known as purple flower, is an annual herbaceous species which belongs to the Boraginaceae family. Native from Europe and Northern Africa, it is indicated for ornamental,

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melliferous and medicinal purposes [4]. Findings showed that the various parts of *Echium* species could be used for their antibacterial, anti-inflammatory, anti-proliferative, antidepressant, antioxidant, antiviral, anxiolytic and cytotoxic properties [5, 6]. *E. plantagineum* bee pollen as well as its seeds oil have been extensively studied, however, very few studies on phenolic composition and antioxidant activities of the aerial part of this plant are available. Therefore, the present study was designed to explore the phenolic content as well as the *in vitro* antioxidant activities of *E. plantagineum* aerial part extracts.

MATERIALS AND METHODS

Chemicals

Folin–Ciocalteau reagent, gallic acid, quercetin, tannic acid, Butylatedhydroxtoluene (BHT), 2,2-diphenyl-1-picryl-hydrazyl (DPPH$^{\bullet}$), EDTA, $FeCl_2$, ferrosine, potassium persulfate, 2,2′azinobis (3-ethylbenzothiozoline-6-sulfonic acid) disodium salt (ABTS), Trolox, potassium ferricyanide, tricholoroacetic acid, $FeCl_3$, hydrogen peroxide, ferrous sulfate, ascorbic acid, phenazinemethosulfate (PMS), nitro blue tetrazolium (NBT), NADH, ferrous ammonium sulfate, 1, 10-phenanthroline and 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) were obtained from Sigma (Germany). Sodium bicarbonate, Aluminum chloride and sodium salicylate were purchased from Prolabo (France). All other chemicals used in this study were of analytical grade.

Plant material

Echium plantagineum L. was collected in June 2017 from Babor region (Northern of Setif, Algeria). It was identified and authenticated taxonomically by Pr. Laouar H., Laboratory of Valorization of Natural Biological Resources, University of Setif 1, Algeria. A specimen was conserved at the same laboratory. The aerial part was cleaned, air-dried at room temperature and then reduced to powder.

Preparation of the plant extracts

E. plantagineum aqueous extract (Aq E) was prepared by boiling 50 g of powdered plant in 1000mL of distilled water for 25 min followed by filtration through Whatman filter paper N°3. The filtrate was dried and the obtained powder (yield 40%) was stored at -32°C until required.
Methanolic extract (Met E) was prepared by maceration of 50g of the powdered plant material, twice for 24h,with 500mL of 80% methanol and then with 50% methanol at room temperature, with frequent agitation. After filtration, the filtrate was concentrated under reduced pressure at 40 °C (Buchi, Flawil, Switzerland) and then dried. The obtained powder (yield 22%) was stored at -32 °C until use.

Determination of total polyphenols

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The content of total polyphenolic compounds in *E. plantagineum* extracts was determined using Folin-Ciocalteu assay [7]. Samples (100 µl) were mixed with 500 µL of 1:10 diluted Folin-Ciocalteu reagent. After 4 min., 400 µL of 7.5% sodium carbonate (Na_2CO_3) solution were added. After 90 min. of incubation at room temperature, the absorbance was measured at 765 nm. Gallic acid was used as astandard. The concentration of total polyphenolic compounds was determined as mg of Gallic acid equivalents per 1 g of extract (mg GAE/g extract).

Determination of total flavonoids

The total flavonoid content was determined by the aluminum chloride method [8]. Briefly, 1 ml of 2% aluminium trichloride ($AlCl_3$) in methanol was mixed with the same volume of extracts. After 10 min of incubation at room temperature, the absorbance was measured at 430 nm. Quercetin was used for the standard calibration curve. The concentration of total flavonoid compounds was determined as mg of quercetin equivalents per 1 g of extract (mg QE/g extract).

Determination of total condensed tannins

Tannins content of *E. plantagineum* extracts was determined using the hemoglobin precipitation assay [9]. Tannic acid was used as standard. A volume of 1mL of each extract was mixed with 1mL of hemolyzed bovine blood (absorbance = 1.6). The mixture was incubated for 20 min, and then centrifuged at 4000rpm for 10min. The absorbance of the supernatant was measured at 756nm and tannins content was expressed as mg tannic acid equivalent per 1 g of extract (mg TAE/g extract).

ABTS radical scavenging activity

The ability of *E. plantagineum* extracts to scavenge the ABTS radical was evaluated according to Re et al. [10]. A volume of 2.5 mL of ABTS (7 mM) was mixed with 2.5mL of potassium persulfate (2.45 mM) and the mixture was allowed to stand in the dark at room temperature for 12–16 h to produce ABTS radical cations ($ABTS^{\bullet+}$). The $ABTS^{\bullet+}$ solution was diluted with ethanol to obtain an absorbance of 0.70 (±0.02) at 734 nm. The scavenging activity was estimated by mixing 50µL of diluted *E. plantagineum* extracts (0-500µg/mL) or Trolox (0-100µg/mL) with 1mL of $ABTS^{\bullet+}$ solution. The absorbance was read at 734 nm after 30min. of incubation at room temperature. The antioxidant activity was calculated as followed:

$$ABTS^{\bullet+} \text{ scavenging activity (\%)} = (A_0 - A_1 / A_0) \times 100$$

Where A_0 is the absorbance of the control without test sample and A_1 is the absorbance of the test sample.

Hydroxyl radical scavenging activity

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The scavenging ability of hydroxyl radicals was evaluated as described by Smirnoff and Cumbes [11]. A volume of 1 mL of ferrous sulfate (1.5 mM) was added to 700 µL of hydrogen peroxide (6 mM) and 300 µL of sodium salicylate (20 mM). Then, 1 mL of *E. plantagineum* extracts, at different concentrations (0-2000µg/mL), or ascorbic acid (standard antioxidant) was added. The mixture was incubated at 37°C for 1h before measuring absorbance at 562 nm. The antioxidant activity was calculated as following:

$$\text{Hydroxyl radical scavenging activity (\%)} = [1– (A_S – A_B/ A_C)] \times 100$$

A_C: absorbance without sample. **A_B:** absorbance of the blank (without sodium salicylate).
A_S: absorbance of the sample.

Superoxide anion scavenging activity

The superoxide anion scavenging activity was measured as described by Robak and Gryglewski [12]. The superoxide anions generating system contains 0.5mL of Tris–HCl buffer (16mM, pH 8.0), 1 mL of NBT (0.3mM) solution, 0.5mL of NADH (0.936mM) solution and 1 mL of *E. plantagineum* extracts at different concentrations (0-3000µg/mL) or ascorbic acid. A volume of 0.5 mL of phenazinemethosulfate (PMS) solution (0.12 mM) was added to start the reaction. After 5 min. of incubation at 25°C, the absorbance was measured at 560 nm and the antioxidant activity was calculated using the following equation:

$$\text{Superoxide anion radical scavenging activity (\%)} = (A_C – A_S / A_C) \times 100$$

A_C: absorbance of the control. **A_S:** absorbance of the sample.

Hydrogen peroxide scavenging activity

This method was carried out according to Mukhopadhyay et al. [13]. A volume of 750 µL of *E. plantagineum* extracts at different concentrations (0-800µg/mL) was added to 125 µL of ferrous ammonium sulfate (1mM) and 35 µL of H_2O_2 (5mM). The mixture was incubated at room temperature for 5min. Then, a volume of 750 µL of 1,10-phenanthroline (1mM) was added and the absorbance was read at λ =510nm. The ability of the extracts to scavenge hydrogen peroxide was calculated using the following formula:

$$H_2O_2 \text{ scavenging activity (\%)} = (A_{Test} / A_{Blank}) \times 100\%$$

A_{blank}= absorbance of the solution containing only ferrous ammonium sulphate and 1,10-phenanthroline.
A_{test}= absorbance of the solution containing ferrous ammonium sulphate, hydrogen peroxide along with test compound and 1,10-phenanthroline.

Ferrous ions chelating activity

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The chelating ability of ferrous ions by *E. plantagineum* extracts was evaluated by the method described by Le et al. [14]. The reaction mixture contained 250 µLof the extracts at different concentrations (0-1200µg/mL) or EDTA (standard chelator), 50 µL of $FeCl_2$ (0.6 mM) and 450 µL of methanol. After well shaking and incubation for 5 min at room temperature, 50 µL of ferrozine (5 mM) were added. The mixture was shaken and incubated again for 10 min at room temperature before reading absorbance at 562 nm.
The inhibition of ferrozine–Fe^{2+} complex formation was calculated using the following formula:

$$\text{Ferrous ions chelating activity (\%)} = [(A_0 - A_1)/A_0] \times 100$$

Where A_0 is the absorbance of the control and A_1 is the absorbance of the test compound.

Reducing power

The reducing power of *E. plantagineum* extracts was determined according to Topçu et al. [15]. A volume of 2.5 mL of *E. plantagineum* extracts solutions at different concentrations (0-300µg/mL) was added to 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide (1%). After 20 min of incubation at50 C°, 2.5 mL of TCA (10%) were added and the mixture was centrifuged at 3000 rpm for 10min. The upper layer (2.5 mL) was mixed with 2.5 mL of deionized water and 0.5 mL of $FeCl_3$ (0.1%), and the absorbance was measured at 700 nm. Higher absorbance indicates higher reducing power. Data were expressed as Effective Concentration corresponding to a 0.5 absorbance (EC_{50}).

Blood total antioxidant capacity

The peroxidation of erythrocytes membranes was induced with AAPH according to Takebayashi et al. [16]. Heparinized male rat blood was centrifuged at 6000rpm for 10min and washed three times with PBS buffer (pH 7.4). Then, 100µL of the red blood cells were suspended in 4.9mL of PBS to obtain a 2% dilution. On the other hand, 120µL of the 2% blood cells solution was pre-incubated for 15min at 37°C on microplates with 60µL of *E. plantagineum* extracts (150µg/mL) or Trolox, used as standard antioxidant. Finally, 120µL of AAPH solution (300mM) were added and the reaction mixtures were then incubated at 37°C for 4 - 5h.The degree of hemolysis was determined spectrophotometically by measuring absorbance every 30min at 620nm. The inhibition percent of hemolysis was calculated as following:

$$\text{Hemolysis inhibition (\%)} = (A_0 - A_X \,/\, A_0 - A_{Final}) \times 100$$

A_0: absorbance at t_0. **A_X:** absorbance at a specific time. **A_{Final}**: absorbance at the end of the experience.

Data are also represented by the necessary time for 50% of hemolysis (HT_{50}): The presence of an antioxidant (Trolox or extracts) is supposed to increase the half time of hemolysis which influences a good resistance of erythrocytes.

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Statistical analysis

All data were expressed as means ± SD of three replications. IC_{50} values were calculated by regression analysis. One-way ANOVA test was used for statistical analysis. The values were considered to be significantly different when the p value was less than 0.05.

RESULTS ANS DISCUSSION

Total polyphenols, flavonoids and tannins contents

Phenolic compounds such as phenolic acids, flavonoids and tannins are considered to be major contributors to the antioxidant capacity of plants [7]. Results showed that both *E. plantagineum* extracts contained phenolic compounds. However, methanolic extract contains the highest amount of polyphenols, flavonoids and tannins compared to aqueous extract (Table 1). Tannins represent the major compounds in both extracts.

Table 1. Polyphenols, flavonoids and tannins contents of *E. plantagineum* extracts

Extracts	Polyphenols (mg GAE/g extract)[a]	Flavonoids (mg QE/g extract)[a]	Tannins (mg TAE/g extract)[a]
Aq E	61.89 ± 1.89	7.11 ± 0.65	173.36 ± 62.37
Met E	122 ± 11.39	12.14 ± 0.67	239.47 ± 68.99

GAE: Galic Acid Equivalent; **QE:** Quercetine Equivalent. **TAE:** Tannic acid Equivalent,[a]mean±SD,*n* = 3.

Arumugam et al. [17] have reported that Met E of *Echium angustifolium* aerial part, a species from Boraginaceae family, presents an amount of flavonoids close to that of *E. plantagineum*, but lower amount of polyphenols. Another study, carried on by Aouadi et al. [18], showed that Met E and Aq E of *Echium humile* (Boraginaceae) presented higher amounts of polyphenols and flavonoids than *E. plantagineum* extracts, but lower amounts of tannins.

ABTS radical scavenging activity

The ABTS assay is based on the production of the blue/green $ABTS^{\bullet+}$ chromophore through the reaction between ABTS and potassium persulfate. The presence of an antioxidant eliminates ABTS radicals by reducing them, resulting in discoloration of the radical solution [10]. The advantage of this method is that measuring antioxidant capacity of plant extracts at long

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wavelength maximum absorption of 734 nm eliminates color interference [19]. Both *E. plantagineum* extracts showed a concentration dependant scavenging activity of the ABTS radicals (Figure 1). However, Aq E was more powerful (IC_{50} = 167.03µg/mL) than Met E (IC_{50} = 261.45µg/mL). These results are not concurring with the amounts of polyphenolic compounds present in each extract. This is probably due to the fact that the active principals responsible for this activity are more available in Aq E than Met E. These results are very powerful comparing to those found with Aq E and Met E of *Echium humile* (Boraginaceae) [18].

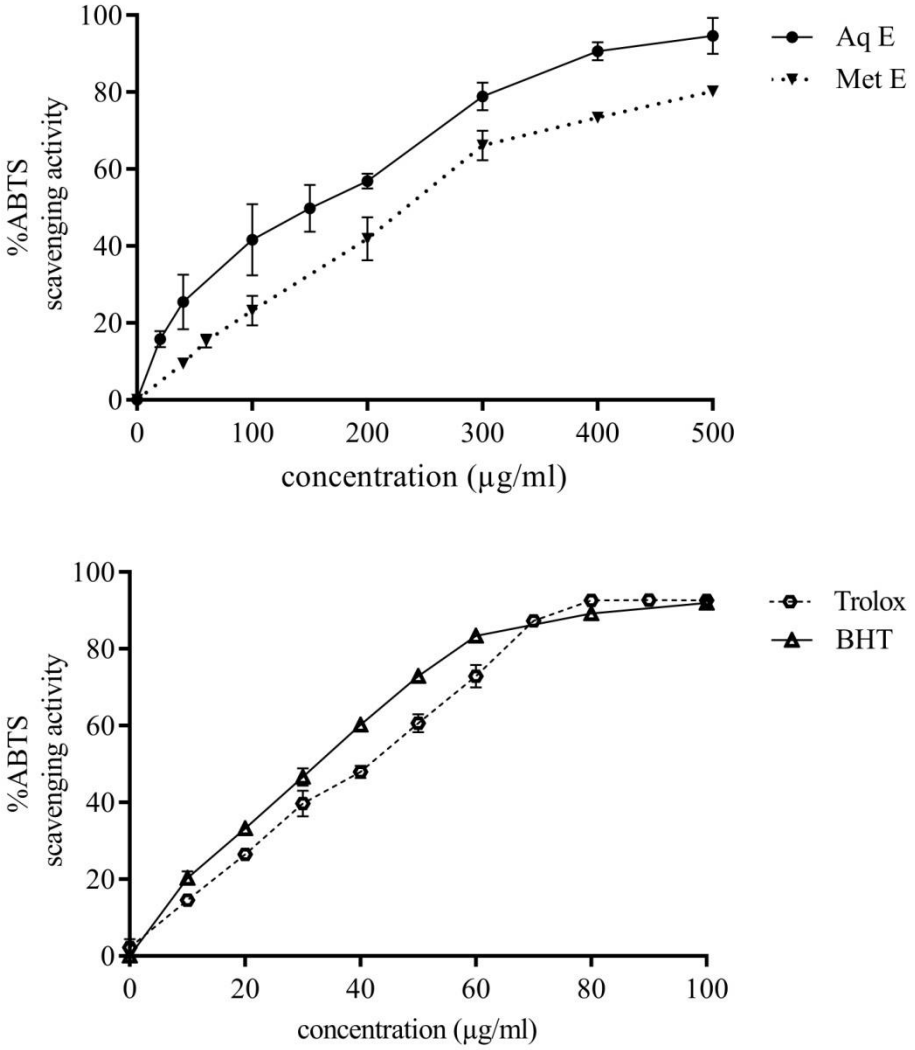

Figure 1. ABTS scavenging activity of *E. plantagineum* extracts, Trolox and BHT. The values are means ± SD (n=3).

Hydroxyl radical scavenging activity

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Hydroxyl radical is generated both *in vivo* and in foods and can undergo several reactions, including dismutation to give H_2O_2. Hydroxyl radical is the most damaging molecule among ROS, being highly reactive, able to damage almost any organic molecule and cannot be eliminated by an enzymatic reaction [20]. *E. plantagineum* extracts showed concentration dependant scavenging activity towards hydroxyl radicals as represented in figure 2. Aqueous extract is more effective (IC_{50} = 103.44µg/mL) than both methanolic extract (IC_{50} = 261.86µg/mL) and vitamin C (IC_{50} = 311.96 µg/mL). Phenolic compounds may be responsible for this antioxidant potential. In fact, the antioxidant activities of phenolic compounds are mainly due to their redox properties, making them free radical quenchers, hydrogen donators and reducing agents [21].

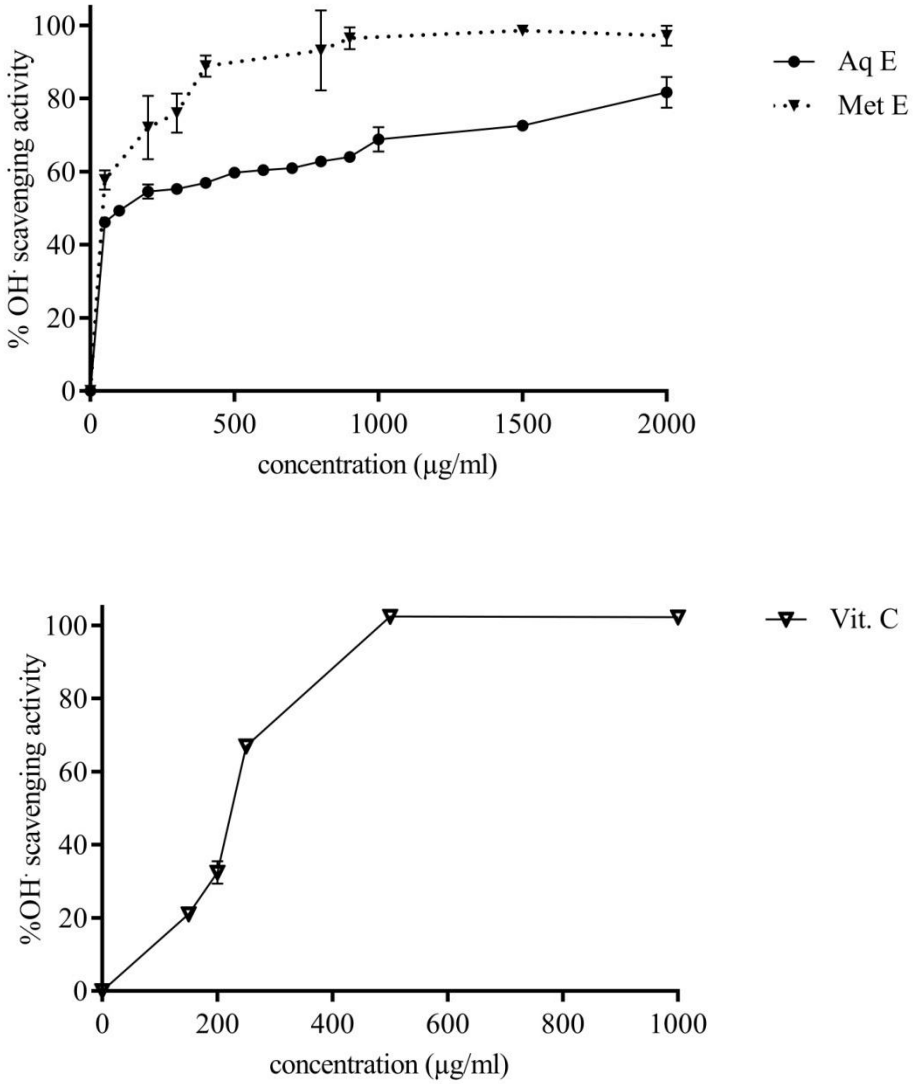

Figure 2. OH· scavenging activities of *E. plantagineum extracts* and vitamin c (Vit. C).Values are means±SD (n=3).

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Superoxide anion scavenging activity

The aqueous and methanolic extracts of *E. plantagineum* act against $O_2^{\cdot}$ in concentration dependent manner (Figure 3). Results show that Aq E was more efficient (IC_{50}=563.78 µg/mL) than Met E (IC50=830.825 µg/mL), but both of them were less powerful than ascorbic acid (IC_{50}=24.34µg/mL). Zemmouri et al. [22] showed that *Borago officinalis* (Boraginaceae) extracts were more efficient in scavenging superoxide anion than *E. plantagineum* extracts, even though these lasts are richer in phenolic compounds. This may be explained by a difference in the nature of the phenolic composition between the two plants. Another study carried on by Amudha and Rani [23] showed that ethanolic extract of *Cordia retusa* (Boraginaceae) exhibited a superoxide anion scavenging activity compared to that found in the present study. In fact, studies concerning the phenolic composition and antioxidant activities of *E. plantagineum* aerial part are very scanty. Thus, it was necessary to compare our results with those found with species that belong to the same family.

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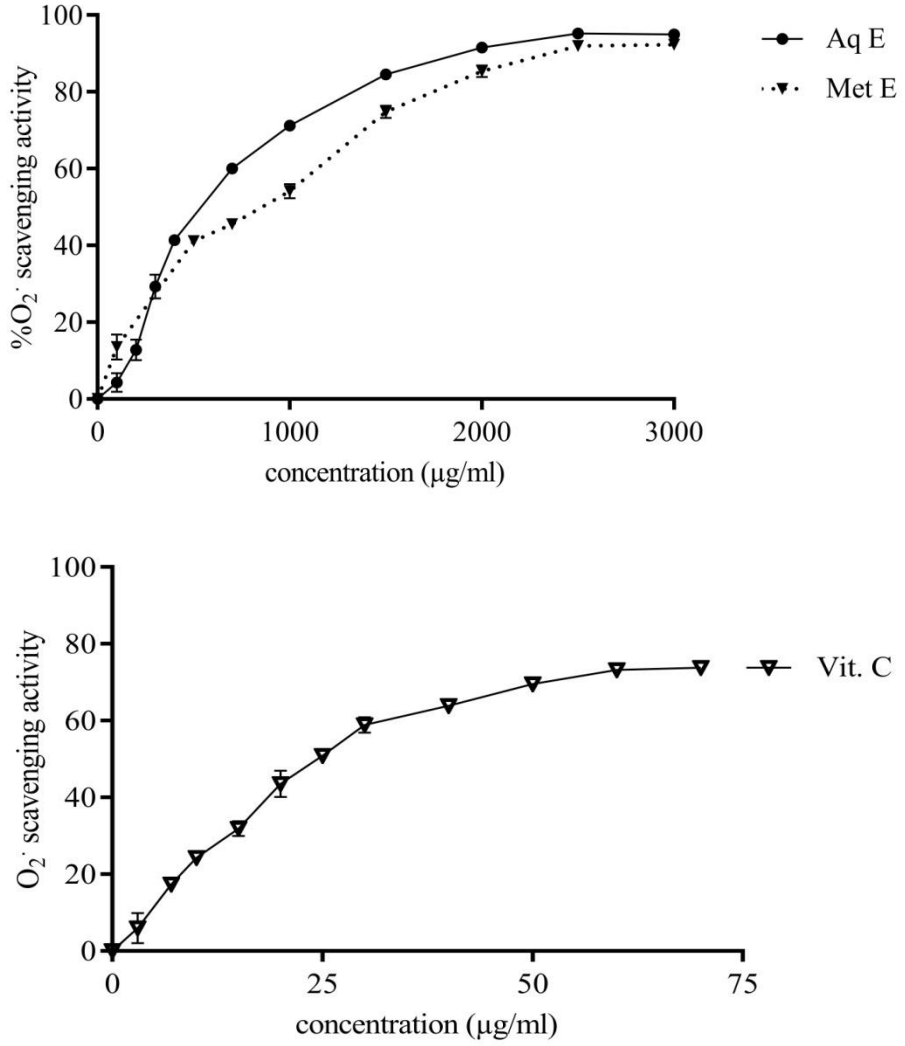

Figure 3. $O_2^{\cdot}$ scavenging activity of *E. plantagineum extracts* and ascorbic acid. The values are means±SD (n=3).

Hydrogen peroxide scavenging activity

Hydrogen peroxide is not a free radical itself, but leads to free radicals formation like $OH^{\cdot}$, which then do the damage [20]. The principle of the used test is based on the formation of a red-orange ferrous ions (Fe^{+2})-1,10-phenanthroline complex. The addition of H_2O_2 causes oxidation of all ferrous ions to ferric ions (Fe^{3+}) unable of forming the red-orange complex. However, the addition of hydrogen peroxide scavenger to ferrous ions before adding H_2O_2 itself forbids the ferrous to ferric conversion, and the addition of phenanthroline yields the chromophore complex. Therefore, higher absorbance indicates higher hydrogen peroxide scavenging activity. Figure 4 represents the peroxide hydrogen scavenging activity of Aq E and Met E of *E. plantagineum*.

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Both extracts exerted a good hydrogen peroxide scavenging activity. However, the effect of Met E (IC_{50}=193.87µg/mL) was better than the effect of Aq E (IC_{50} =262.46µg/mL). Standard antioxidant (ascorbic acid) was better than both of them (IC_{50} = 51.93µg/mL). In a study carried on by Bekhradnia and Ebrahimzadeh [24], polyphenolic extract of *Echium amoenum* petals exhibited better hydrogen peroxide scavenging activity (IC_{50}= 110.5 µg/mL), but still close to that of *E. plantagineum* extracts. Methanolic extract of another member of Boraginaceae family, *Trichodesma zeylanicumm*, showed a scavenging activity of hydrogen peroxide also close to that found with *E. plantagineum* extracts [25].

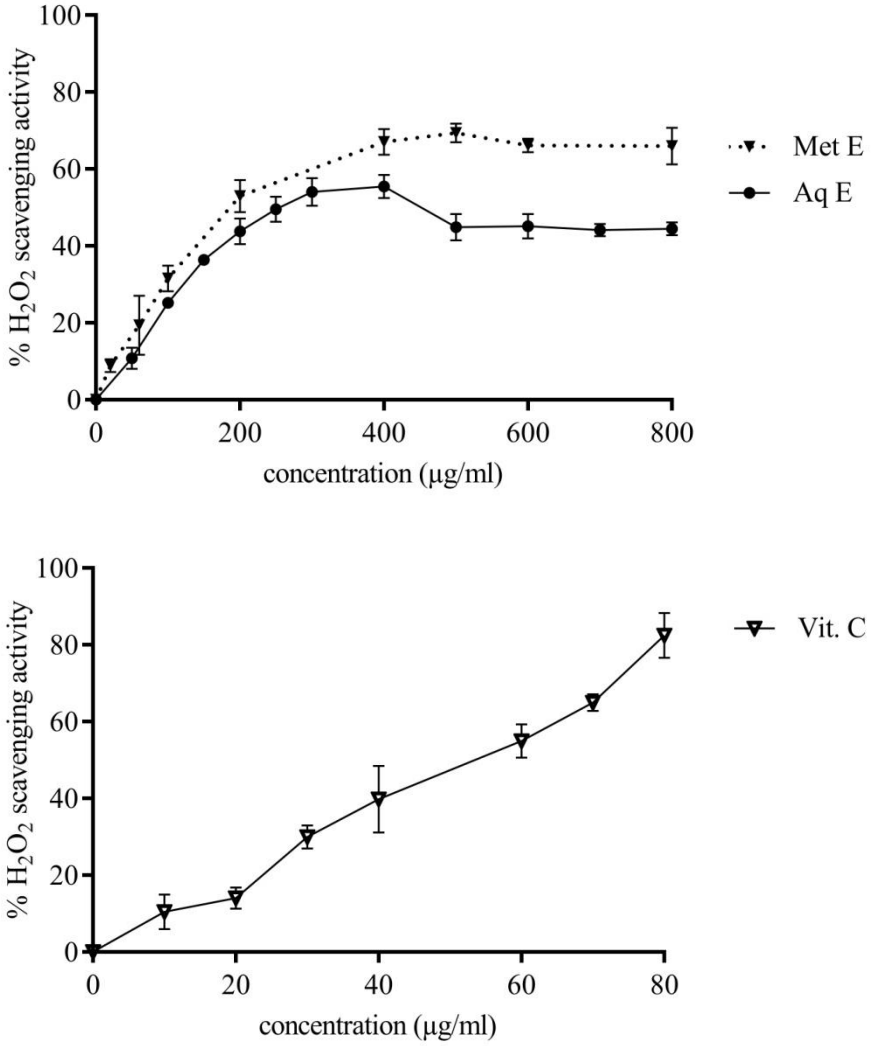

Figure 4. H_2O_2 scavenging activity of *E. plantagineum* extracts and vitamin C. The values are means±SD (n=3).

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Ferrous ions chelating activity

Ferrous ions chelating test is based on the ability to form a complex with Fe^{2+} leading to a decrease in the amount of Fe^{2+}-ferrosine red complex, therefore a decrease in absorbance [14]. Fe^{2+} catalyzes the decomposition of hydrogen or lipid peroxide into the highly reactive and biologically damaging hydroxyl radical. Thus, the ability of an antioxidant to chelate Fe^{2+} or other transition metal in human body is such important property to measure [26]. Methanolic and aqueous extracts of *E. plantagineum* exerted a ferrous ions chelating activity which increased with increasing concentration (Fig. 5). However, Aq E showed more activity than Met E with IC_{50} values of 164.91µg/mL and 247.57µg/mL, respectively. These values are lower than that obtained with EDTA, known to be a very powerful chelator. Bekhradnia and Ebrahimzadeh [24] have reported that polyphenolic extract of *Echium amoenum,* from Boraginaceae, showed more activity in iron chelating assay. However, the ethanolic extract of another Boraginaceae belonging species, *Cordia retusa,* showed less activity [23].

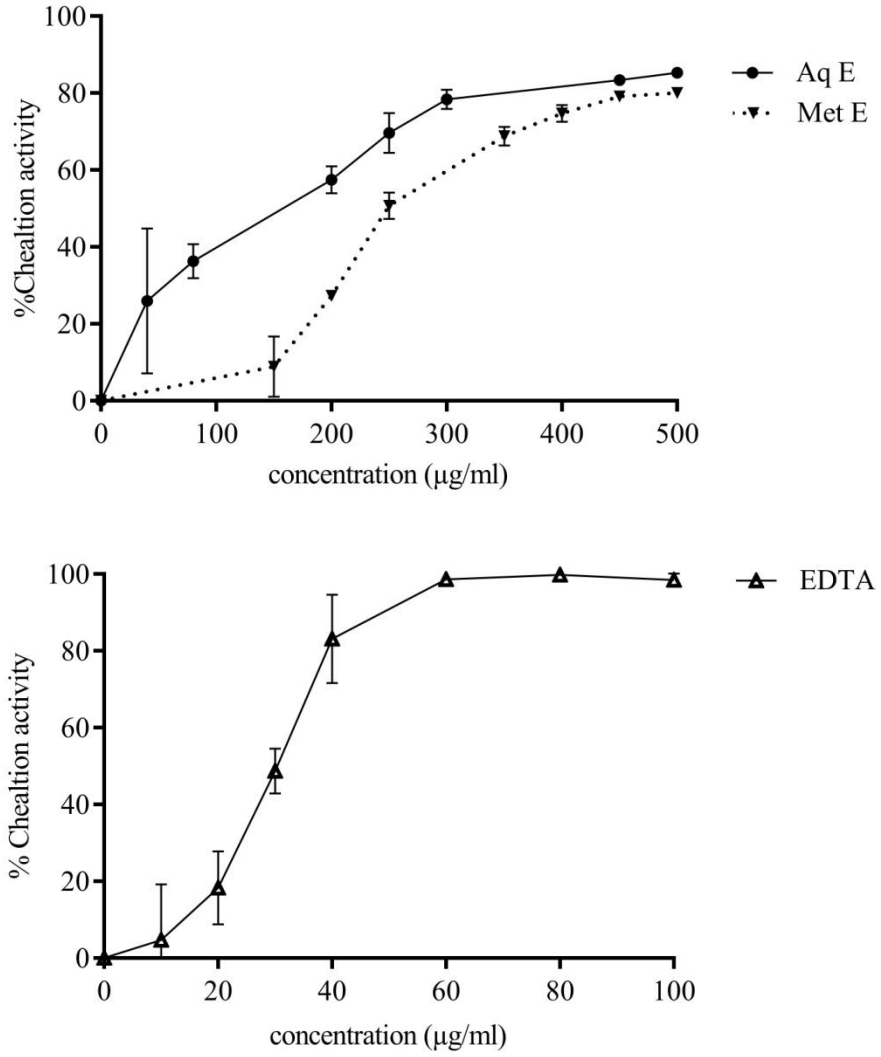

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Figure 5. Chelation activity of *E. plantagineum* extracts and chelator standard (EDTA).The values are means±SD (n=3).

Reducing power

Reducing power test consists of direct reduction of $Fe^{3+}(CN^{-})^{6}$to $Fe^{2+}(CN^{-})^{6}$ leading to an increase in the amount of the red complex $Fe_4^{3+}[Fe^{2+}(CN^{-})_6]^{3+}$, and consequently an increase in absorbance [14]. The reductive activity of aqueous and methanolic extracts of *E. plantagineum* compared with BHT has been illustrated in Figure 6. Met E and Aq E exerted similar and remarkable reducing activity with IC_{50} values of 117µg/mL and 128 µg/mL, respectively. This reductive activity remains less important than that of BHT (IC_{50}= of 61.29µg/mL), but much stronger than that of Aq E and Met E of *Echium humile* aerial part [18]. The results of the reducing power test as well as those of iron chelating test indicate that both *E. plantagineum* extracts can deal with ferrous ions either by binding (chelating) them or reducing them.

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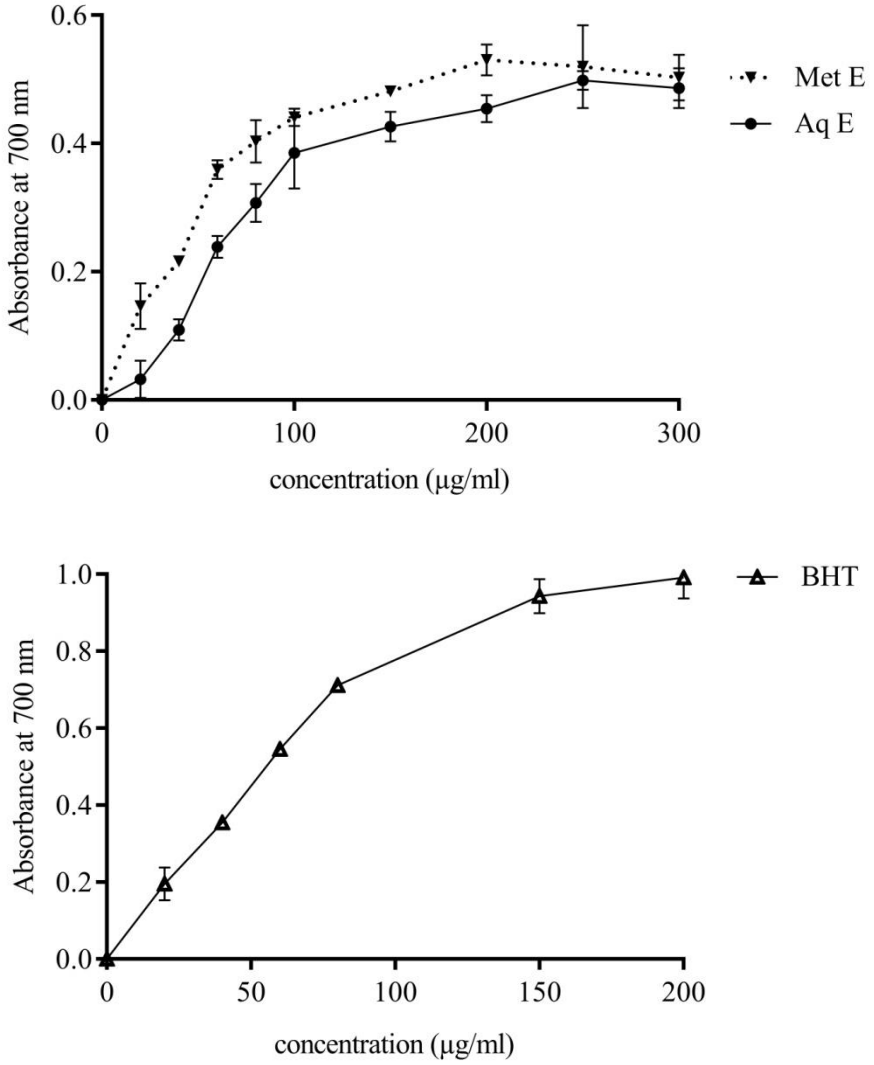

Figure 6. Reducing power of *E. plantagineum* extracts and BHT. The values are means ± SD (n=3).

Blood total antioxidant capacity

The ability of the extracts to protect biological membranes against radical attacks was elucidated in the present study using the AAPH test. The decomposition of AAPH in physiological environment to alkyl radicals (R·) can form peroxyl radicals (ROO·) in presence of oxygen. These lasts will attack erythrocytes lipids and proteins to induce the oxidation chain, disturbing membranes organization and eventually leading to hemolysis [26, 27]. The obtained sigmoid hemolysis curves (figure 7) show that both of Aq E and Met E could be the raison of a delay of red blood cells hemolysis, which appears in a shift of the curves to the right. The HT_{50} values (Figure 8) show that Aq E exhibited a strong anti-hemolytic activity (HT_{50}=161.62±5.87 min) at

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150 µg/mL, statistically similar to that of Trolox ($HT_{50=}$ 153.17±3.95 min), when Met E was more efficient ($HT_{50=}$179.79±5.82 min) than both of Trolox and Aq E at the same concentration. This can be explained by the fact that the extracts may contain molecules such as polyphenolic compounds able to either inhibit AAPH radical formation or penetrate the lipid bilayer of the red blood cells enhancing their integrity, and consequently their resistance against radicals aggression.

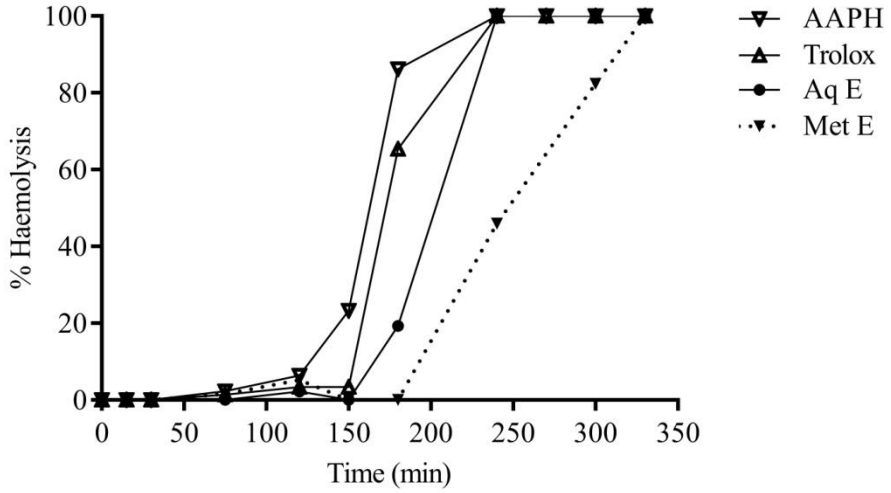

Figure 7. Kinetics of the red blood cells hemolysis caused by AAPH. The tested concentration of aqueous and methanolic extracts of *E. plantagineum* as well as Trolox is 150µg/mL. The negative control contains only AAPH. The values are means ± SD (n= 3**).**

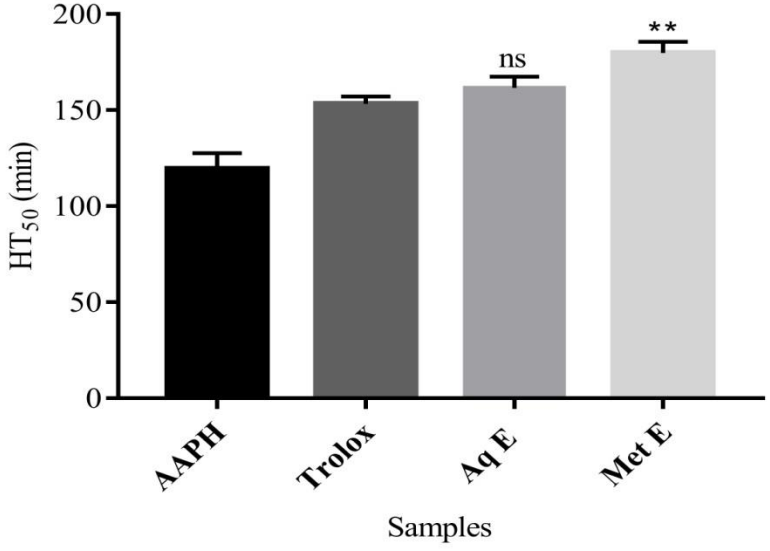

Figure 8. Half-Hemolysis Time (HT50) of *E. plantagineum* extracts and Trolox (150µg/mL). Values are expressed as means±SD. **P<0.01, ns: not significant versus the standard (Trolox).

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CONCLUSION

In the present study, the antioxidant activity of aqueous and methanolic extracts of *E. plantagineum* was evaluated through several antioxidant tests. Results revealed that both extracts acted as good antioxidants in different ways, which can be explained by their important phenolic content. Therefore, this plant could be a promising source of natural antioxidants considered as good alternatives for synthetic ones.

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STATEMENT OF CONFLICT OF INTEREST

Authors declare no conflict of interest.

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