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Antioxidant properties and total phenolic contents of *Asperula hirsuta* extracts

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Abstract

The current study aimed to determine the phenolic contents and antioxidant properties of methanol (Met. E) and aqueous (Aq. E) extracts of *Asperula hirsuta*. Met. E showed the highest levels of polyphenols (144.68 ± 10.87 µg GAE/mg extract) and flavonoids (32.78 ± 2.06 µg QE/mg extract). Both extracts exhibited potent scavenging activity with IC_{50} values of 19.54 ± 1.60 µg/mL and 42.14 ± 0.57 µg/mL, respectively. At 250 µg/mL, Aq. E exerted stronger chelating effect (97%) than Met. E (86%). However, Met. E antioxidant capacity (169.01 ± 13.25 µg AAE/mg extract) was more than that of Aq. E (125.84 ± 07.12 µg AAE/mg extract). Both extracts protected from β-carotene bleaching by 61% and 66%, respectively. The findings of this study suggest that *Asperula hirsuta* extracts may serve as a promising source of natural antioxidants.

Keywords: *Asperula hirsuta*, antioxidant activity, oxidative stress, phenolic compounds, medicinal plant.

Introduction

Humans are constantly exposed to free radicals generated by various endogenous and environmental sources. This exposure leads to an increase in reactive species, which can cause cellular damage to lipids, proteins, and DNA (Santharam *et al*., 2015). Free radicals are implicated in numerous human diseases, including aging, heart disease, atherosclerosis, diabetes, cancer, and other chronic conditions (Gafrikova *et al*., 2014). Additionally, reactive oxygen species (ROS), including both free radicals and non-radical species, are the primary

cause of food deterioration, leading to the formation of toxic compounds and a decrease in nutritional value (Raju, 2005).

Numerous studies have highlighted the potential of bioactive compounds derived from natural sources to prevent diseases and mitigate oxidative stress. Antioxidants, in particular, can exert protective effects by neutralizing free radicals or inhibiting the formation of ROS, thereby slowing the progression of various chronic diseases (Kada *et al*., 2016). Additionally, natural antioxidants can extend the shelf life of foods (Rubió *et al*., 2013).

The genus *Asperula* L., belonging to the Rubiaceae family, comprises approximately 200 species distributed worldwide (Özgen *et al*., 2018). *Asperula hirsuta* is a perennial herb that is most commonly found in the northern areas of Algeria. Several *Asperula* species have been employed in traditional medicine as diuretics, tonics, antidiarrheals, and agents to reduce blood pressure and inflammation (Kırmızıbekmez *et al*., 2017). Previous phytochemical investigations of the *Asperula* genus have identified the presence of iridoids, flavonoids, anthraquinones, and phenolic acids (Park *et al*., 2002; Özgen *et al*., 2006; Tzakou *et al*., 2011).

In this context, the aim of this study was to evaluate the antioxidant activity and total phenolic contents of methanol and aqueous extracts of *Asperula hirsuta*, in order to explore new sources for natural antioxidants in food and pharmaceutical formulations.

Materials and methods

Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), Ascorbic acid, EDTA, $FeCl_2$, Ferrozine, Follin-Ciocalteu, Gallic acid, Linoleic acid and Quercetin were obtained from Sigma-Aldrich (Darmstadt, Germany). Aluminium chloride ($AlCl_3$), Ammonium molybdate, β-carotene, Butylated hydroxytoluene (BHT), Sodium carbonate (Na_2CO_3), Sodium phosphate and Tween-40 were purchased from Prolabo (Paris, France). The other reagents were analytical grade and provided from Riedel-de Haën (Illkirch, France).

Plant material

Asperula hirsuta (*A. hirsuta*) was collected in May 2021 from Beni Ourtilane, Setif area (Algeria) and identified by Dr. Djamel Sarri from the department of natural sciences and life, University of M'sila, Algeria. A voucher specimen (AH 08/21) was deposited in the Herbarium of M'sila University. The aerial part was air dried at room temperature and then reduced to powder.

Preparation of *Asperula hirsuta* extracts

Methanolic extract (Met. E) of *A. hirsuta* was prepared by maceration of the aerial part powder with methanol/water (7:3 v/v) with agitation for 24 h at room temperature. After filtration, the filtrate was concentrated under reduced pressure at 40 °C. The residue was lyophilized to give a brown powder.

Aqueous extract (Aq. E) of *A. hirsuta* was prepared by boiling 50 g of powdered plant in 500 mL distilled water for 20 min, followed by filtration and centrifugation for 10 min. The supernatant obtained was lyophilized to give a dark brown powder. Both extracts were stored at -32 °C until use (Bouriche *et al*., 2016).

Determination of total polyphenol content

Total phenolic content of *A. hirsuta* Met. E and Aq. E extracts was determined using the Folin Ciocalteu assay (Li *et al*., 2007). Samples (100 µL) were mixed with 500 µL of Folin-Ciocalteu reagent (10%). After 4 min, 400 µL of Na_2CO_3 (7.5%) was added. The mixture was incubated for 2 h at room temperature, and the absorbance was measured at 765 nm. Gallic acid was used as a standard and all tests were performed in triplicate. The concentration of total phenolic compounds in both extracts was determined as microgram of gallic acid equivalent (µg GAE/mg extract).

Determination of flavonoid content

The flavonoid content of the extracts was determined by the aluminium chloride method (Bahorun *et al*., 1996). The sample solution (1 mL) was mixed with 1 mL of $AlCl_3$ (2%). After 10 min of incubation at room temperature, the absorbance was measured at 430 nm. Quercetin was used as a standard. Flavonoid content was expressed as microgram of quercetin equivalent (µg QE/mg extract).

Free radical scavenging activity

The free radical scavenging activity of *A. hirsuta* extracts was measured using 1,1-diphenyl-2-picryl-hydrazil (DPPH), according to the method described by Que *et al*. (2006). A volume of 500 µL of the DPPH solution (0.1 mM) was added to 500 µL of extracts solutions or standard (BHT) at different concentrations, the mixture was incubated in the dark for 30 min at room temperature, and then the absorbance was recorded at 517 nm. The DPPH scavenging activity was calculated as follows: $[(A_0 - A_1) / A_0 \times 100]$, where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the extract/standard (BHT).

Ferrous ions chelating activity

The ferrous ion chelating activity of Met. E and Aq. E extracts of *A. hirsuta* was estimated by the method of Le *et al*. (2007). Briefly, extract samples at different concentrations were added to a solution of 0.6 mmol/L $FeCl_2$ (50 µL). The reaction was initiated by the addition of 50 µL of ferrozine (5 mM) and the mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated. EDTA was used as a reference. The percentage of chelating activity was calculated as follows: $[(A_0 - A_1) / A_0 \times 100]$, where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the test sample.

Total antioxidant capacity (TAC)

The total antioxidant capacity of *A. hirsuta* extracts was evaluated by the phosphomolybdenum method (Yaici *et al*., 2019). In Brief, 0.1 mL of each extract sample was added to 0.9 ml of reagent solution (0.6 M sulphuric acid, 4 mM ammonium molybdate and 28 mM sodium phosphate). The mixtures were incubated at 95 °C for 90 min then cooled to room temperature. Absorbance of the solution was determined at 695 nm against a blank. Ascorbic acid was used as standard. The total antioxidant capacity was expressed in µg equivalent of ascorbic acid per mg of extract (µg AAE/mg extract).

β-Carotene bleaching method

The antioxidant activity of the extracts was determined according to the β-carotene bleaching method described by Tepe *et al*. (2006). A reagent mixture containing 1 mL of β-carotene solution (1 mg/mL in chloroform), 25 µL of linoleic acid and 200 mg of Tween 40. After removing the chloroform by using a rotary evaporator, 100 mL of oxygenated distilled water was added. The mixture was stirred vigorously to form a liposome solution. Aliquots (2.5 mL) of the liposome solution were transferred to a series of test tubes containing 500 µL of extract (2 mg/mL), 500 µL of BHT (2 mg/mL) or 500 µL distilled water (control). The absorbance was measured immediately (t=0 min) at 490 nm using a spectrophotometer. Subsequently, the reaction mixtures were incubated at 50 °C. The absorbance was measured again at time intervals of 20 min for 2 h (t=120 min). All samples were assayed in triplicate. BHT was used as standard antioxidant. A second emulsion consisting of 100 mL oxygenated distilled water, 25 µL of linoleic acid and 200 mg of Tween 40 was also prepared. Distilled water (500 µL) with 2.5 mL of this second emulsion was used to zero the spectrophotometer.

The antioxidant activity (%) was calculated in terms of percentage inhibition relative to the control, using the following equation:

$$\text{Antioxidant activity (\%)} = [1- (At_0 – At_{120}) \text{ test}/(At_0-At_{120}) \text{ control}] \times 100$$

Statistical analysis

Results were expressed as mean ± SD. The significance of differences between control and the various tests was determined by an ANOVA test followed by a Dunnett/Tukey tests for multiple comparisons using the Prism 9.00 computer software (GraphPad, Boston, USA). The differences were considered statistically significant at $P \leq 0.05$.

Results and discussion

Total polyphenol and flavonoid content

Total content of polyphenol and flavonoid of *A. hirsuta* extracts are presented in Table 1. Methanol extract contains the highest amount of polyphenols and flavonoids compared to aqueous one.

Table 1. Polyphenols and flavonoids content of *A. hirsuta* methanol (Met. E) and aqueous (Aq. E) extracts. Values are mean ± SD (n = 3).

Extract	Polyphenols (µg GAE/mg extract)	Flavonoids (µg QE/mg extract)
Met. E	144.68 ± 10.87	32.78 ± 2.06
Aq. E	109.36 ± 19.41	14.77 ± 1.23

Free radical scavenging activity

The free radical scavenging activity was determined by the DPPH assay. This test aims to measure the capacity of the extracts to scavenge the stable radical DPPH formed in solution by donation of hydrogen atom or an electron (Tepe *et al*., 2005). Results showed that both extracts exhibited concentration-dependent inhibition on DPPH radical (Figure 1). The best radical scavenging activity was exerted by Met. E with IC_{50}=19.54 ± 1.60 µg/mL flowed by Aq. E with IC_{50}=42.14 ± 0.57 µg/mL. These values are better than that obtained with BHT (IC_{50}=55.99 ± 1.42 µg/mL), used as standard. The radical scavenging activity of *A. hirsuta* extracts is probably attributed to the presence of phenolic compounds. The DPPH scavenging activity was found to

be highest in methanol extracts from five endemic *Asperula* taxa (*A. brevifolia*, *A. pseudochlorantha* var. *antalyensis*, *A. pseudochlorantha* var. *pseudochlorantha*, *A. purpurea* subsp. *apiculata* and *A. serotina*) (Minareci *et al*., 2011).

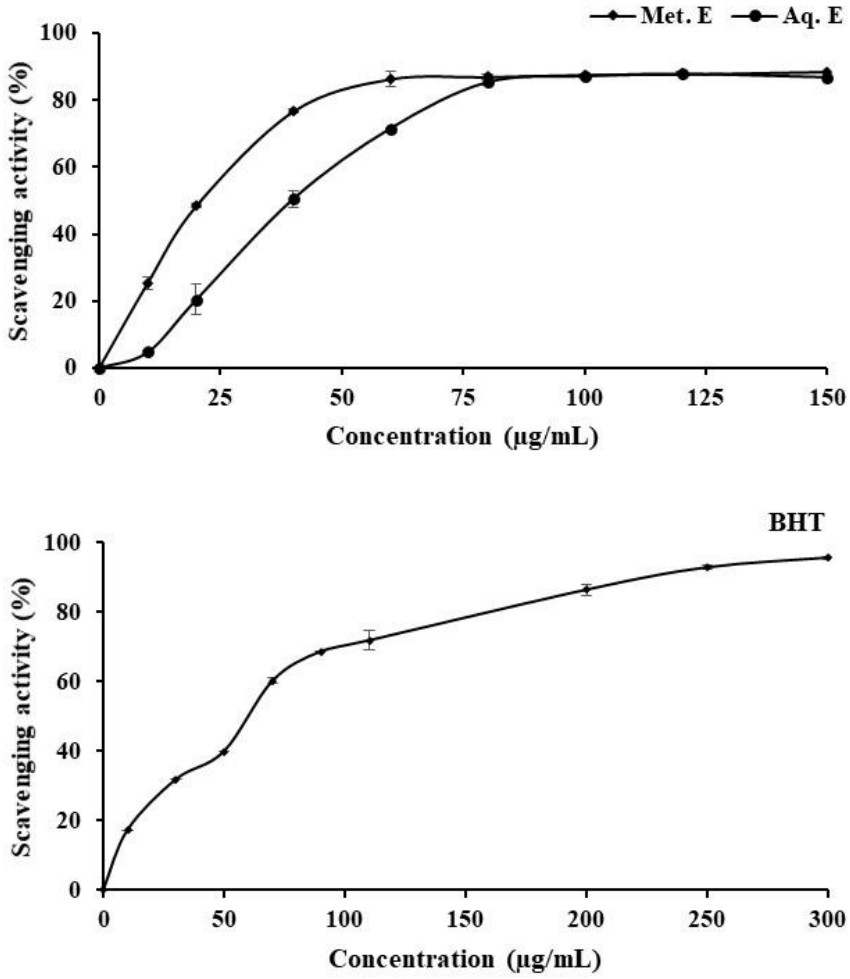

Figure 1. Free radical scavenging activity of methanol extract (Met. E), aqueous extract (Aq. E) of *A. hirsuta* and BHT. Values are mean ± SD (n = 3).

Ferrous ions chelating activity

The capacity to chelate or inactivate transition metals, particularly those capable of catalyzing hydroperoxide decomposition and Fenton-type reactions, is a fundamental mechanism underlying antioxidant activity. Given this, it was deemed essential to assess the iron(II) chelating ability of the extracts (Manian *et al*., 2008). The methanol and aqueous extracts of *A. hirsuta* were able to chelate ferrous ions in a concentration-dependent manner (Figure 2). However, Aq. E was more active (EC_{50}=76,39 ± 5,87 µg/mL) than Met. E (EC_{50}=180,86 ± 5,72 µg/mL). This activity was less than that obtained with EDTA (EC_{50}=7,62 ± 0,29 µg/mL), used as a standard. The different phenolic components present in methanol and

aqueous extracts, as for example, flavonoids, may have contributed to these results. Indeed, it has been reported that the flavonoids presented good chelating properties (Cherrak *et al*., 2016).

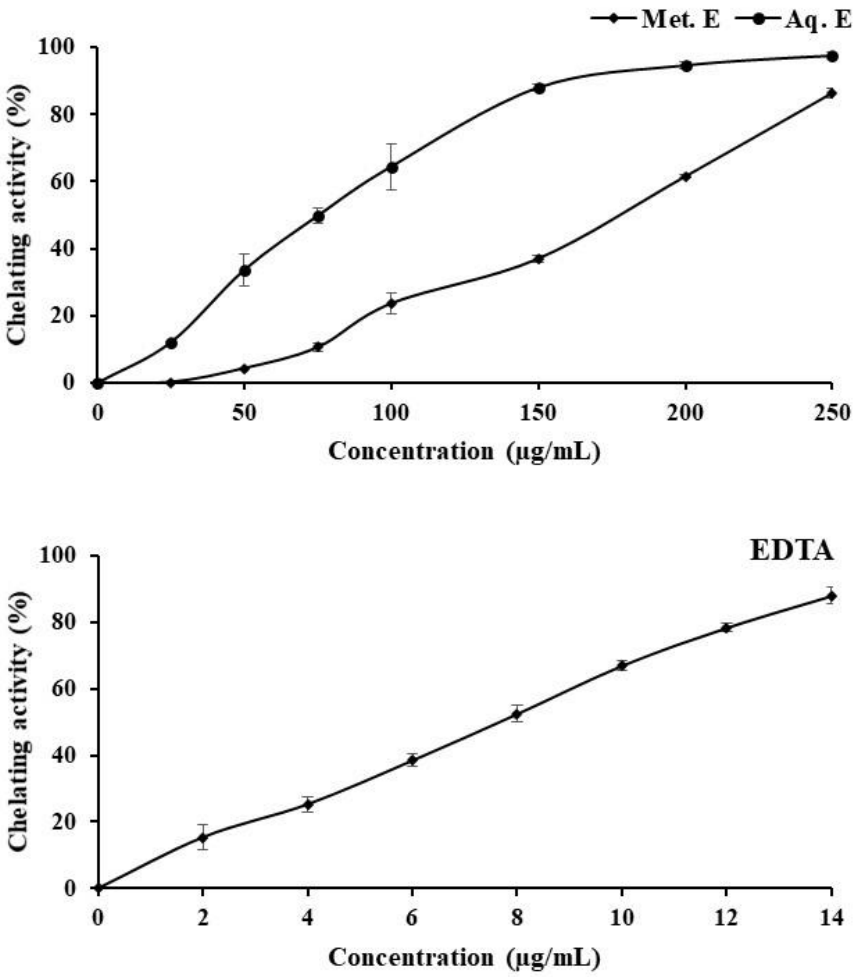

Figure 2. Ferrous ion chelating activity of methanol extract (Met. E), aqueous extract (Aq. E) of *A. hirsuta* and EDTA. Values are mean ± SD (n = 3).

Total antioxidant capacity (TAC)

The total antioxidant capacity was quantified using a colorimetric method based on the formation of phosphomolybdenum complexes. This assay involves the reduction of molybdate(VI) to molybdate(V) by the extract, resulting in the formation of a green-colored phosphomolybdenum complex (Aouam *et al*., 2019). The methanol extract significantly revealed the highest total antioxidant capacity compared to aqueous one (Table 2). Strong antioxidant capacity of *A. hirsuta* extracts indicates strong antioxidants in these extracts and these could be attributable to the presence of phenolic compounds.

Table 2. Total antioxidant capacity of *A. hirsuta* methanol (Met. E) and aqueous (Aq. E) extracts. Values are mean ± SD (n = 3).

Extract	TAC (µg AAE/mg extract)
Met. E	169.01 ± 13.25
Aq. E	125.84 ± 07.12

β-Carotene bleaching

This is one of the rapid method to screen antioxidants, which is mainly based on the principle that linoleic acid, which is an unsaturated fatty acid, gets oxidized by reactive oxygen species produced by oxygenated water. The products formed will initiate the β-carotene oxidation, which will lead to discoloration (Alam *et al*., 2013). The changes in the absorbance under the influence of *A. hirsuta* methanol and aqueous extracts compared to BHT during 120 min are presented in Figure 3. As shown in this figure, in the presence of both extracts of *A. hirsuta*, the absorbance was remained stable during all the incubation time. The inhibition of β-carotene bleaching exerted by 2 mg/mL of methanol and aqueous extracts of *A. hirsuta* were about 61% and 66%, respectively. This activity was less than that obtained with BHT (98%), used as a standard. The ability of the extracts to inhibit lipid peroxidation may be attributed, as cited before, to their constituents that are electron donors, which can react with free radicals to convert them to more stable products. Indeed, *Asperula* species are rich in phenolic compounds (Minareci *et al*., 2011). These compounds are potent antioxidants and inhibit strongly the lipid peroxidation (Lizcano *et al*., 2012).

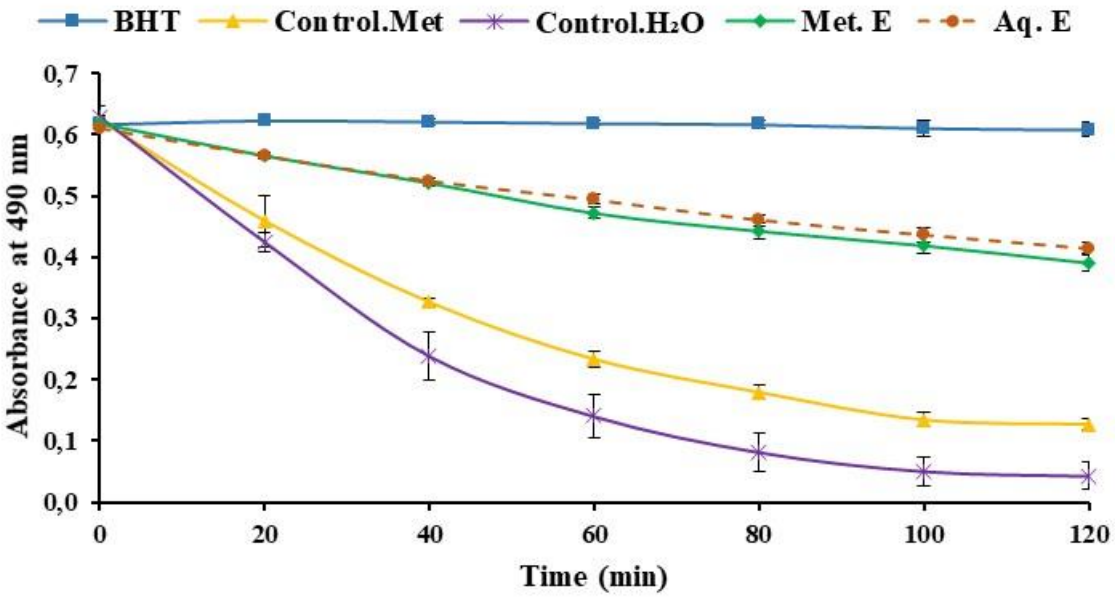

Figure 3. Kinetics of antioxidant activity of *A. hirsuta* methanol extract (Met. E), aqueous extract (Aq. E), and the standard antioxidant (BHT) in β-carotene-linoleic acid system. Values are expressed as means ± SD (n = 3).

Conclusion

In this investigation, we evaluated the phenolic content and antioxidant activities of *A. hirsuta* extracts. Our results indicate that both methanol and aqueous extracts of *A. hirsuta* demonstrate significant antioxidant properties. These effects were strongly correlated with the levels of polyphenols and flavonoids present in the extracts. These findings suggest that *A. hirsuta* could serve as a promising natural source of bioactive compounds for potential applications in food and medicinal products.

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Declaration of competing interest

Authors declare no conflict of interest.

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