Antioxidant activity profiling by spectrophotometric methods of aqueous methanolic extracts of *Helichrysum stoechas* subsp. *rupestre* and *Phagnalon saxatile* subsp. *saxatile*

Farah Haddouchi 1,*, Tarik Mohammed Chaouche 1, Riadh Ksouri 2, Faten Medini 2, Fatima Zohra Sekkal 3, Abdelhafid Benmansour 1

1 Laboratory of Natural Products, Department of Biology, Faculty of Sciences, Abou Bekr Belkaid University, B. P 119, Tlemcen, 13000, Algeria;
2 Laboratory of Plant Adaptation to Abiotic Stresses, Biotechnologic Center in Borj-Cedria Technopol (CBBC), B. P 901, 2050 Hammam-Lif, Tunisia;
3 Department of Biotechnology, Faculty of Natural Science and Life, Abdelel Hamid Ibn Badiss University, Mostaganem, 27000, Algeria

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[ABSTRACT]

AIM: The aqueous methanolic extracts of two plants from Algeria, *Helichrysum stoechas* subsp. *rupestre* and *Phagnalon saxatile* subsp. *saxatile*, were investigated for their antioxidant activity.

METHOD: Total phenolics, flavonoids, and tannins were determined by spectrophotometric techniques. *In vitro* antioxidant and radical scavenging profiling was determined by spectrophotometric methods, through: Total antioxidant capacity, and radical scavenging effects by the DPPH and ABTS methods, reducing and chelating power, and blanching inhibition of the β-carotene.

RESULTS: All of the extracts showed interesting antioxidant and radical scavenging activity. The highest contents in phenolics, tannins, and the highest total antioxidant capacity as gallic acid equivalents of 97.5 ± 0.33 mg GAE/g DW was obtained for the flowers of *H. stoechas* subsp. *rupestre* extract in the phosphomolybdenum assay. An extract of the leafy stems of *P. saxatile* subsp. *saxatile* revealed the highest content of flavonoids, and the highest antioxidant activity by the radical scavenging and β-carotene assays when compared with standards. The best activity was by the scavenging radical DPPH with an IC₅₀ value of 5.65 ± 0.10 µg·mL⁻¹.

CONCLUSION: The studied medicinal plants could provide scientific evidence for some traditional uses in the treatment of diseases related to the production of reactive oxygen species (ROS) and oxidative stress.

(KEY WORDS) *Helichrysum stoechas* subsp. *rupestre*; *Phagnalon saxatile* subsp. *saxatile*; Phenolic compounds; Antioxidant activity

[Introduction]

When oxygen is supplied in excess, or its reduction is insufficient, reactive oxygen species (ROS) are generated. It is generally accepted that free radicals play an important role in the development of tissue damage and pathological events [1].

In order to prolong the storage stability of foods, and to reduce damage to the human body, synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), are commonly used. However, restrictions on the use of these compounds are being imposed because of their carcinogenicity and some side effects [2]. Thus, evaluation of the antioxidant activity of naturally occurring substances has been the focus of interest in recent years [1-4]. The antioxidant activity of plants, which contain a diverse group of phenolic compounds, is mainly due to their redox properties which make them act as reducing agents, hydrogen do-
nors, and singlet oxygen quenchers. They also may have a metal chelating potential [5].

The Helichrysum genus (Asteraceae) includes more than 500 species that are widespread around the world. A great number of biological activities are usually attributed to this genus, such as anti-inflammatory, anti-allergic, antioxidant, antimicrobial, cough relief, and treatment of colds and wounds [6]. The chemistry of the Helichrysum genus is complex, with a wide variety of chemical classes, among which are flavonoids, chalcones, phloroglucinol derivatives, essential oils, α-pyrones, and diterpenes [7]. The genus Phagnalon (Asteraceae) is represented by about thirty species distributed worldwide, six of which are typical of the Mediterranean region [8]. Different Phagnalon species are used in traditional medicine, and a variety of extracts have been examined [9]. Phytochemical investigations on Phagnalon species are mainly devoted to the study of P. rupestre, and report the presence of terpenoids [10], flavonoids [11], hydroquinone glycosides, and caffeoylquinic acid derivatives [12]. A survey of the literature shows the presence in the aerial parts of P. saxatile of an essential oil [13] and flavonoids [10].

However, scientific information on the antioxidant properties of various plants, particularly those that are less widely used in the culinary arts and medicine is still rather scarce. There are any reports of the Helichrysum and Phagnalon species belonging to Algerian flora. The aim of the present work is to study the phenolic content and antioxidant activity of aqueous methanolic extracts of Helichrysum stoepchas subsp. rupestre auct. and Phagnalon saxatile (L.) Cass. subsp. saxatile, collected from Algeria.

Material and Methods

Preparation of plant extracts

The flowering aerial parts of the plants were collected in March 2011, from two different regions of Tlemcen in the west of Algeria. H. stoepchas subsp. rupestre was collected from Honaine (35°10′35″ N, 1°39′18″ W) and P. saxatile subsp. saxatile from Fellaoucen (35°02′06″ N, 1°36′21″ W). They were identified in the Laboratory of Natural Products, Department of Biology, University of Tlemcen, Algeria. Voucher specimens were deposited at the Herbarium of the Laboratory.

The plants were dried at room temperature for two weeks. The flowers were separated from the leafy stems and the extracts were obtained separately by the magnetic stirring for 24 h of the plant powder (2 g) with aqueous methanol 80 : 20 (25 mL). The aqueous methanolic extracts were evaporated at 45 °C under reduced pressure, re-dissolved in methanol at a concentration of 10 mg·mL⁻¹, and stored at 4 °C for further use.

Quantification of phenolic classes

Total polyphenols

The amounts of total phenolics in the aqueous methanolic extracts were determined with the Folin–Ciocalteu reagent using the method described by Vermerris and Nicholson [14]. To each sample extract (100 µL) suitably diluted, Na₂CO₃ (2 mL, 2%, W/V) was added, and the mixture shaken and allowed to stand for 5 min. Folin–Ciocalteu reagent (100 µL, 1 mol·L⁻¹) was added. After incubation for 30 min at room temperature in the dark, the absorbance versus a prepared blank was read at 750 nm. Total phenolic content was expressed as mg gallic acid equivalents per gram of dry weight (mg GAE/g DW) using a calibration curve for gallic acid (0–400 µg·mL⁻¹).

Total flavonoids

Measurement of flavonoid concentration in different aqueous methanolic extracts was based on the method described by Dewanto et al [15]. An aliquot (250 µL) of the samples was added to test tubes containing 5% NaNO₂ solution (75 µL), and mixed for 6 min. Then, freshly prepared 10% AlCl₃ solution (150 µL) was added, and after 5 min at room temperature, 1 mol·L⁻¹ NaOH (0.5 mL) was added. The final volume was adjusted to 2.5 mL with distilled water and thoroughly mixed. The absorbance of the mixture was determined at 510 nm against the same mixture without the sample as a blank. Total flavonoid content was expressed as mg catechin per gram of dry weight (mg CE/g DW), through the calibration curve of (+)-catechin (0–400 µg·mL⁻¹).

Total condensed tannins

The content of condensed tannins were determined by the procedure of Sun et al [16]. Fifty microliters of different aqueous methanolic extracts was mixed with 4% vanillin-methanol solution (3 mL) and 1 mol·L⁻¹ hydrochloric acid (1.5 mL). The mixture was allowed to stand for 15 min and the absorbance was measured at 500 nm. The amount of total condensed tannins is expressed as mg catechin per gram of dry weight (mg CE/g DW). The calibration curve range of (+)-catechin was 0–400 µg·mL⁻¹.

Determination of antioxidant activities

Total antioxidant capacity

The assay is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acidic pH [17]. An aliquot (0.1 mL) of aqueous methanolic extract was combined to 1 mL of reagent solution (0.6 mol·L⁻¹ sulfuric acid, 28 mmol·L⁻¹ sodium phosphate and 4 mmol·L⁻¹ ammonium molybdate). The tubes were incubated in a thermal block at 95 °C for 90 min. After the mixture had cooled to room temperature; the absorbance of each solution was measured at 695 nm against a blank. The antioxidant capacity was expressed as mg gallic acid equivalent per gram dry weight (mg GAE/g DW). The calibration curve range was 0–500 µg·mL⁻¹.

DPPH assay

The antioxidant activity of the aqueous methanolic extracts of the plants was measured using the stable DPPH method based on the reduction of an alcoholic DPPH solution in the presence of a hydrogen donating antioxidant due to the formation of the non-radical form DPPH-H by the reaction [18]. The sample was diluted in pure extraction solvent at different
concentrations (0–3 mg·mL\(^{-1}\)), then each diluted plant extract (50 μL) was added to a 6.34 × 10\(^{-5}\) mol·L\(^{-1}\) DPPH methanolic solution (1 950 μL). The mixture of different extract concentration and DPPH was placed in the dark at room temperature for 30 min. The absorbance of the resulting solution was then read at 517 nm. BHT was used as a positive control. The ability to scavenge the DPPH radical was calculated using the following equation:

\[
\text{DPPH scavenging effect (\%) = } \left( \frac{A_0 - A_t/A_0}{} \right) \times 100
\]

where \(A_0\) is the absorbance of the control at 30 min, and \(A_t\) is the absorbance of the sample at 30 min. The antiradical activity was expressed as the concentration of an antioxidant (μg·mL\(^{-1}\)) needed to decrease the initial DPPH concentration by 50% (IC\(_{50}\)).

**Iron reducing power**

The capacity of the plant extracts to reduce Fe\(^{3+}\) was assessed by the method of Oyaizu [19]. In the reducing power assay, the presence of antioxidants in the sample would result in the reduction of Fe\(^{3+}\) to Fe\(^{2+}\) by donating an electron. Aqueous methanolic extract (1 mL) was mixed with sodium phosphate buffer (2.5 mL, 0.2 mol·L\(^{-1}\), pH 6.6) and 1% potassium ferricyanide (2.5 mL), and the mixture was incubated at 50 °C for 20 min. After that, 10% trichloroacetic acid (2.5 mL) was added, and the mixture was centrifuged at 650 × g for 10 min. The upper layer fraction (2.5 mL) was mixed with distilled water, shaken vigorously, and left standing at room temperature for 10 min. The absorbance of the solution was measured at 700 nm. Ascorbic acid and BHT were used as a positive control. A higher absorbance indicates a higher reducing power. The IC\(_{50}\) value (μg·mL\(^{-1}\)) is the effective concentration giving an absorbance of 0.5 for reducing power, and was obtained from linear regression analysis.

**ABTS assay**

The ABTS radical-scavenging activity of extracts was determined according to Re et al [20]. The ABTS\(^+\) cation radical was produced by the reaction between 14 mmol·L\(^{-1}\) ABTS\(^+\) solution (5 mL) and 4.9 mmol·L\(^{-1}\) potassium persulfate (K\(_2\)S\(_2\)O\(_8\)) solution (5 mL), stored in the dark at room temperature for 16 h. Before use, this solution was diluted with ethanol to obtain an absorbance of 0.700 ± 0.020 at 734 nm. In a final volume of 1 mL, the reaction mixture comprised ABTS\(^+\) solution (950 μL) and the plant extract (50 μL) at various concentrations. The reaction mixture was homogenized, and its absorbance was recorded at 734 nm. Ethanol blanks were run in each assay, and all measurements were done after at least 6 min. Similarly, the reaction mixture of standards group was obtained by mixing ABTS\(^+\) solution (950 μL) and BHT and Trolox (50 μL). The inhibition percentage of ABTS\(^+\) radical was calculated using the following formula:

\[
\text{ABTS\(^+\) scavenging effect (\%) = } \left( \frac{A_0 - A_t/A_0}{} \right) \times 100
\]

where \(A_0\) and \(A_t\) have the same meaning as in Eq. (1).

As for the antiradical activity, the ABTS\(^+\) scavenging ability was expressed as IC\(_{50}\) values (μg·mL\(^{-1}\)).

**Chelating effect on ferrous ions**

The ferrous ion chelating activity of the extracts was assessed as described by Zhao et al [21]. Different concentrations of plant extracts were added to FeCl\(_2\) (4H\(_2\)O) solution (50 μL, 2 mmol·L\(^{-1}\)) and left for incubation at room temperature for 5 min. Then, the reaction was initiated by adding ferrozine (0.1 mL, 5 mmol·L\(^{-1}\)), and the mixture was adjusted to 3 mL with distilled water, shaken vigorously, and left standing at room temperature for 10 min. The absorbance of the solution was measured at 562 nm. The percentage of inhibition of ferrous-Fe\(^{2+}\) complex formation was calculated using the formula given below:

\[
\text{Metal chelating effect (\%) = } \left( \frac{A_0 - A_t/A_0}{} \right) \times 100
\]

where \(A_0\) and \(A_t\) have the same meaning as in Eq. (1). The results were expressed as IC\(_{50}\) values (mg·mL\(^{-1}\)).

**β-Carotene bleaching test**

A slightly modified Koleva et al [22] method was employed to estimate the aqueous methanolic extracts capacity to inhibit β-carotene bleaching. Two milligrams of β-carotene was dissolved in chloroform (20 mL) and to this solution (4 mL), linoleic acid (40 mg) and Tween 40 (400 mg) were added. The chloroform was evaporated under vacuum at 40 °C and oxygenated water (100 mL) was added, then the fresh emulsion was vigorously shaken. An aliquot (150 μL) of the β-carotene/linoleic acid emulsion was distributed in 96-well microtitre plates and aqueous methanolic solutions of the test samples (10 μL) or authentic standard (BHA) were added. The microtitre plates were incubated at 50 °C for 120 min, and the absorbance was measured using a model EAR 400 microtitre reader at 470 nm. Readings of all samples were performed immediately (t = 0 min) and after 120 min of incubation. The antioxidant activity of the extracts was evaluated in terms of bleaching inhibition of the β-carotene using formula:

\[
\text{β-Carotene bleaching inhibition (\%) = } \left( \frac{S - C_{120}/C_0 - C_{120}}{C_0} \right) \times 100
\]

where \(C_0\) and \(C_{120}\) are the absorbance values of the control at 0 and 120 min, respectively, and S is the sample absorbance at 120 min. The results were expressed as IC\(_{50}\) values (μg·mL\(^{-1}\)).

**Correlation analysis**

Values shown in the Tables were \(\bar{x} \pm s\) of three parallel measurements. The IC\(_{50}\) and EC\(_{50}\) values were calculated from linear regression analysis. The correlation coefficients (R) between the total phenolic contents and the methods of antioxidant activity were demonstrated by employing EXCEL (2010).

**Results**

In this study, the total extract yield, total phenolics, flavonoid and tannin contents and the antioxidant activity of the aqueous methanolic extracts of H. stoechas subsp. rupestre and P. saxatile subsp. saxatile collected from Algeria were determined.

**Extraction yield, total phenolic, flavonoid, and condensed tannin contents**

The method of extraction, based on liquid–solid ex-
traction, was used to obtain the aqueous methanolic extracts of the plants. The yield percentages obtained are shown in Table 1. *H. stoechas* subsp. rupestre had the highest extract yields with a higher yield for the flowers, unlike *P. saxatile* subsp. saxatile, whose yields were close for the two plant parts.

<table>
<thead>
<tr>
<th>Yields (%)</th>
<th>Polyphenol Content (mg of GAE/g DW)</th>
<th>Flavonoid content (mg of CE/g DW)</th>
<th>Tannin content (mg of CE/g DW)</th>
<th>Total antioxidant capacity (mg of GAE/g DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FHS</strong></td>
<td>18.70 ± 0.33</td>
<td>31.34 ± 1.92</td>
<td>10.48 ± 0.28</td>
<td>97.50 ± 0.33</td>
</tr>
<tr>
<td><strong>LSHS</strong></td>
<td>13.50 ± 0.40</td>
<td>18.62 ± 0.41</td>
<td>9.10 ± 0.87</td>
<td>29.43 ± 0.38</td>
</tr>
<tr>
<td><strong>FPS</strong></td>
<td>10.00 ± 0.13</td>
<td>04.80 ± 0.20</td>
<td>00.00 ± 0.00</td>
<td>01.89 ± 0.12</td>
</tr>
<tr>
<td><strong>LSPS</strong></td>
<td>09.00 ± 0.33</td>
<td>18.82 ± 0.21</td>
<td>13.08 ± 0.28</td>
<td>53.55 ± 0.36</td>
</tr>
</tbody>
</table>

**Table 1** Total antioxidant capacity, total polyphenol, flavonoid, and condensed tannin contents of aqueous methanolic extracts of *H. stoechas* subsp. rupestre and *P. saxatile* subsp. saxatile

In order to establish a relationship between the chemical content and the antioxidant activity, the total phenol, flavonoid, and tannin contents of the aqueous methanolic extracts were determined. The total phenolic contents of the extracts, as estimated by the Folin-Ciocalteau reagent method with reference to the standard curve (*Y* = 0.002 53 *x* + 0.013 44), ranged from 4.80 ± 0.20 and 31.34 ± 1.92 mg GAE/g DW (Table 1). The highest level of phenolics was found in the extract of *H. stoechas* subsp. rupestre flower, while the lowest level was in the extract of *P. saxatile* subsp. saxatile flowers.

Flavonoids are one of the most numerous and widespread group of phenolic compounds in higher plants [23]. The content of flavonoids in the extracts was determined with reference to the standard curve (*Y* = 0.003 55 *x* + 0.099 03). The highest level of flavonoids was found in *P. saxatile* subsp. saxatile leafy stem extract (13.08 ± 0.28 mg CE/g DW), while the flowers are devoid of these compounds. The flavonoid contents of extracts of *H. stoechas* subsp. rupestre leafy stem and flowers are close and correspond to 9.10 ± 0.87 and 10.48 ± 0.28 mg CE/g DW, respectively.

It has been reported that condensed tannins possess various biological activities, such as antioxidant activity [24]. The content of condensed tannins was expressed with reference to the standard curve (*Y* = 0.000 76 *x* − 0.007 6). Levels of tannins are less important than flavonoids (Table 1). They are close in the extracts of leafy stem and flower of *H. stoechas* subsp. rupestre (3.21 ± 0.19 and 3.47 ± 0.21 mg CE/g DW, respectively). The extract of *P. saxatile* subsp. saxatile leafy stem is richer in tannins than the flower extract (2.08 ± 0.28 and 0.9 ± 0.20 mg CE/g DW, respectively).

**Antioxidant activity of aqueous methanolic extracts**

There are several methodologies widely used to measure the antioxidant capacity of extracts. In this study, the total antioxidant activity, DPPH* and ABTS* radical scavenging assays, β-carotene bleaching method, ferric reducing power and metal chelating activity were examined in order to have an indication of antioxidant capacity of the test samples.

| **Table 2** Antioxidant activities, expressed as IC₅₀ values for DPPH, ABTS, β-carotene, and iron chelating effect, and as EC₅₀ value for iron reducing power, for the aqueous methanolic extracts and standards |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
|                            | IC₅₀DPPH (µg·mL⁻¹) | EC₅₀ from reducing power (µg·mL⁻¹) | IC₅₀ABTS (µg·mL⁻¹) | IC₅₀iron chelation (mg·mL⁻¹) | IC₅₀β-carotene (µg·mL⁻¹) |
| **FHS**                    | 6.57 ± 0.25              | 466.52 ± 10.50               | 94.54 ± 1.30             | 10.48 ± 0.10                | 121.81 ± 0.55             |
| **LSHS**                   | 8.72 ± 0.20              | 697.14 ± 5.50                | 337.71 ± 2.50            | 10.26 ± 0.15                | 191.46 ± 1.05             |
| **FPS**                    | 129.77 ± 0.55            | 3335.4 ± 16.95               | n.t.                     | n.t.                        | n.t.                      |
| **LSPS**                   | 5.65 ± 0.10              | 355.24 ± 15.00               | 74.84 ± 1.05             | 04.62 ± 0.10                | 51.53 ± 0.40              |
| Standards                  | 10.50 ± 0.40 ±        | 99.64 ± 1.80 ±             | 73.1 ± 1.70 ±            | 58.14 ± 2.50 ±              | 0.0465 ± 0.0003 ±         | 48 ± 0.90 ±             |

**Total antioxidant capacity**

The phosphomolybdenum method is based on the formation of green Mo(V) complexes with a maximum absorption at 695 nm [17]. The results indicated that the aqueous methanolic extract of *H. stoechas* subsp. rupestre flowers tested had strong total antioxidant activity (97.5 ± 0.33 mg of GAE/g DW). The extract of *P. saxatile* subsp. saxatile flowers has a very low activity (Table 1). Table 3 shows a correlation between the total antioxidant capacity of extracts and levels of flavonoids (*R* = 0.64) and tannins (*R* = 0.72), and a significant relationship between this capacity and polyphenol content (*R* = 0.95).

**DPPH radical-scavenging activity**

The DPPH assay has been widely used to provide basic information on the antioxidant ability of plant extracts, because this method has shown to be rapid and simple [18]. The benefit of employing the DPPH assay is due to the high

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Table 3 Correlation coefficients (R) for relationships between assays and levels of polyphenols, flavonoids and tannins

<table>
<thead>
<tr>
<th></th>
<th>TAC</th>
<th>IC50/ABTS</th>
<th>EC50-iron reducing power</th>
<th>IC50/DPPH</th>
<th>IC50/Fe2+</th>
<th>EC50/Iron chelation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyphenols</td>
<td>0.95</td>
<td>–0.83</td>
<td>–0.84</td>
<td>–0.45</td>
<td>–0.01</td>
<td>0.51</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>0.64</td>
<td>–0.96</td>
<td>–0.98</td>
<td>–0.8</td>
<td>–0.98</td>
<td>–0.93</td>
</tr>
<tr>
<td>Tannins</td>
<td>0.72</td>
<td>–0.85</td>
<td>–0.82</td>
<td>0.4</td>
<td>0.76</td>
<td>0.99</td>
</tr>
</tbody>
</table>

stability of the DPPH radical and its commercial form being ready to use [25]. The free radical scavenging activity of the aqueous methanolic extracts of *H. stoechas* subsp. *rupestre* and *P. saxatile* subsp. *saxatile*, tested were determined through the DPPH method and the results are presented in Table 2. IC50 is a parameter widely used to measure antioxidant activity. As the IC50 value of the extract decreases, the free radical scavenging activity increases. The scavenging effect of aqueous methanolic extracts and standard (BHT) on the DPPH radical expressed as IC50 values was in the following order: leafy stems of *P. saxatile* subsp. *saxatile* (5.65 ± 0.10 µg·mL⁻¹), leaves of *H. stoechas* subsp. *rupestre* (6.57 ± 0.25 µg·mL⁻¹), leafy stems of *H. stoechas* subsp. *rupestre* (8.72 ± 0.20 µg·mL⁻¹), BHT (10.5 ± 0.40 µg·mL⁻¹), and flowers of *P. saxatile* subsp. *saxatile* (129.77 ± 0.55 µg·mL⁻¹) (Table 2).

Results reveal that *H. stoechas* subsp. *rupestre* extracts (flower and leafy stem) and *P. saxatile* subsp. *saxatile* leafy stem extract have a stronger effect of scavenging free radical than the positive control (BHT), which is not the case for *P. saxatile* subsp. *saxatile* flower extract.

Table 3 shows good correlations between the total polyphenols, flavonoids, condensed tannin contents and DPPH IC50 of extracts, with significant coefficients correlation *R* of –0.83, –0.96 and –0.85, respectively.

**Iron reducing power**

The reducing capacity of the extract, another significant indicator of antioxidant activity, was also found to be appreciable. Table 2 shows the reducing power potentials of the aqueous methanolic extracts of the test plants in comparison with standards (ascorbic acid and BHT). The *P. saxatile* subsp. *saxatile* leafy stem extract showed stronger reducing power (EC50 = 355.24 ± 15.00 µg·mL⁻¹) than did other extracts, but not stronger than the standards. *P. saxatile* subsp. *saxatile* flower extract was clearly less important. This less significant activity confirms the result of the total antioxidant and the DPPH radical-scavenging activities, and the antioxidant activity of *P. saxatile* subsp. *saxatile* flower extract was not determined by the other methods.

The results showed a correlation between the levels of total phenolic, flavonoids, and condensed tannins and the reducing power of the extracts with values of *R* equal to –0.84, –0.98 and –0.82, respectively (Table 3).

**Scavenging ability of ABTS**

ABTS is another synthetic radical, and more versatile than DPPH, because the ABTS model can assess the scavenging activity for both the polar and non-polar samples [20], and has the advantage of the working solution being soluble in aqueous and organic solvents over a large range of pH values, and the reaction time is lower than the DPPH assay [25]. Therefore, it was considered necessary to further assess the extracts against the synthetic ABTS free radical. Trolox and BHT were the reagents used as standards.

The scavenging effect of aqueous methanolic extracts and standards on the ABTS radical, expressed as IC50 values (Table 2), was in the following order: Trolox BHT and leafy stems of *P. saxatile* subsp. *saxatile* flowers of *H. stoechas* subsp. *rupestre* leafy stems of *H. stoechas* subsp. *rupestre*. The leafy stems of *P. saxatile* subsp. *saxatile* extract exhibited interesting antioxidant activity (IC50 = 74.84 ± 1.05 µg·mL⁻¹) compared to BHT (IC50 = 73.1 ± 1.70 µg·mL⁻¹). The establishment of a correlation shows a correlation between ABTS IC50 and the content of flavonoids (*R* = –0.8) and a low correlation with polyphenols (*R* = –0.45) and condensed tannins (*R* = –0.4) (Table 3).

The same order of activity observed in the ABTS method was found in the DPPH method, confirmed by the very good correlation (*R* = 0.97) between these two methods (Table 4). However, the DPPH values are lower than those obtained from the ABTS assay.

Table 4 Correlation coefficients (R) for relationships between assays

<table>
<thead>
<tr>
<th></th>
<th>DPPH</th>
<th>Iron reducing power</th>
<th>β-Carotene</th>
<th>Iron chelation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron reducing power</td>
<td>0.99</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>β-Carotene</td>
<td>0.97</td>
<td>0.98</td>
<td>1</td>
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<tr>
<td>Iron chelation</td>
<td>0.70</td>
<td>0.72</td>
<td>0.85</td>
<td>1</td>
</tr>
<tr>
<td>ABTS</td>
<td>0.97</td>
<td>0.97</td>
<td>0.90</td>
<td>0.53</td>
</tr>
</tbody>
</table>

**Iron(II) chelation**

The ability to chelate transition metals can be considered as an important antioxidant mode of action. The IC50 values show that the *P. saxatile* subsp. *saxatile* leafy stem extract had the highest ferric ion-chelating capacity (IC50 = 4.62 ± 0.10 mg·mL⁻¹), followed by the extracts of flowers and leafy stem of *H. stoechas* subsp. *rupestre* (IC50 of 10.26 ± 0.15 and 10.48 ± 0.10 mg·mL⁻¹, respectively) (Table 2). None of the aqueous methanolic extracts were as effective as the positive control EDTA (IC50 = 0.046 ± 0.000 3 mg·mL⁻¹). The ferrous ion chelating abilities were highly correlated with the flavonoid and tannin contents with values of *R* equal to –0.93 and 0.99, respectively. The data of the correlation (*R*) with total polyphenolic content was 0.51.

Chelating activity and synthetic free radical scavenging
models are valuable tools to assess the potential antioxidant activity of plant extracts. However, these systems do not use a food or biologically relevant, oxidizable substrate, so no direct information on the protective effects of the plant extracts can be determined [26]. In this assay, the peroxy radicals formed when linoleic acid is oxidised attack the highly unsaturated \( \beta \)-carotene molecules that consequently undergo fast discoloration [27]. Antioxidant activity, which was demonstrated by the ability of the samples to inhibit the bleaching of \( \beta \)-carotene, was measured and compared to the positive control (BHA). As shown in Table 2, the aqueous methanolic extracts showed antioxidant activity expressed as IC\(_{50}\) values, in the following order: BHA and leafy stems of \( P. \) saxatile subsp. saxatile flowers of \( H. \) stoachas subsp. rupestre leafy stems of \( H. \) stoachas subsp. rupestre. The aqueous methanolic extract of the leafy stems of \( P. \) saxatile subsp. saxatile inhibited the oxidation of linoleic acid (IC\(_{50} = 51.53 \pm 0.40 \) \( \mu \)g·mL\(^{-1}\)) compared to BHA (IC\(_{50} = 48 \pm 0.90 \) \( \mu \)g·mL\(^{-1}\)). The results of the relationship between inhibition of bleaching of \( \beta \)-carotene and the phenolic compound contents of different extracts show a correlation between the flavonoids and condensed tannins with values of \( R \) equal to –0.98 and 0.76, respectively (Table 3). There is no correlation between this method and the polyphenol contents (\( R = –0.01 \)).

**Correlation analysis between assays**

The correlation analysis shows a very good correlation between DPPH, iron reducing power, ABTS, and \( \beta \)-carotene (\( R \) between 0.9 and 0.99), and an average correlation between these methods and the chelating power (\( R \) between 0.53 and 0.85) (Table 4).

**Discussion**

No work on the determination of the levels of phenolic compounds was found on Phagnalon species, except for \( P. \) graecum [28], wherein the extraction was carried out successively by different solvents. Few references concerning the content of phenolic compounds of Helichrysum species could be found [6,29-31], with contents that change from one sample to another. The differences in phenolic compound contents of Helichrysum species may be due to the difference in their chemical composition, extraction methods, collection time, collection area, and season. Some authors reported that phenolic compounds are unstable and readily become non-oxidative under heating [32-33].

To completely elucidate a full profile of the antioxidant capacity of extracts, different antioxidant capacity assays (total antioxidant capacity, DPPH, iron reducing power, ABTS, \( \beta \)-carotene, and Iron (II) chelation) were used in this study. Of the four extracts examined, the antioxidant capacity of \( P. \) saxatile subsp. saxatile leafy stem extract was significantly higher than other extracts assayed by the \( \beta \)-carotene and iron (II) chelation methods. However, there were no significant differences in the extracts of the leafy stems and flowers of \( H. \) stoachas subsp. rupestre, except for the total antioxidant capacity and the ABTS methods. With the exception of the extract of \( P. \) saxatile subsp. saxatile flowers, all extracts had an activity greater than that of BHT by the DPPH method and an activity similar to that of BHT and BHA by the ABTS and \( \beta \)-carotene methods. Evaluated by the iron reducing and chelating powers methods, all extracts have a much lower activity than the standard compounds.

There are a few previous studies on the antioxidant capacity of Phagnalon species. According Conforti et al [14], the methanolic extract of \( P. \) saxatile (\( L. \)) Cass., preceded by two successive extractions with petroleum ether and chloroform, showed significant activity by DPPH with an IC\(_{50}\) value of 25 \( \mu \)g·mL\(^{-1}\), much less than ascorbic acid. The aqueous methanolic extract of \( P. \) saxatile subsp. saxatile leafy stems is much more important.

The results of the *in vitro* antioxidant potential of extracts of Helichrysum species showed that methanolic extracts of the sixteen Helichrysum species collected in Turkey had a strong total antioxidant capacity expressed as mg of ascorbic acid equivalents (AAE)/g dry extract. \( H. \) noeanum was the most active [6]. \( H. \) monizii had the highest antioxidant potential by the DPPH, ABTS, ferric reducing and \( \beta \)-carotene assays, due to the presence of dicafeoylquinic acids [25]. Some species had comparable activity to that of the standard compound BHT, namely \( H. \) longifolium aqueous extract using tests involving inhibition of superoxide anions, DPPH, and ABTS [25], \( H. \) stoachas subsp. barellieri (IC\(_{50} = 7.95 \) \( \mu \)g·mL\(^{-1}\)) by the DPPH method [6], \( H. \) chionophilum, \( H. \) noeanum, \( H. \) plicatum subsp. plicatum, and \( H. \) arenarium subsp. acheri, in the \( \beta \)-carotene/ linoleic acid test system [23]. \( H. \) stoachas from Italy had very high antioxidant activity comparable to that of Trolox and vitamin E, by radical scavenging method in cellular model [36]. However, \( H. \) stoachas of Portugal has shown low activity in comparison with Trolox by DPPH (IC\(_{50} = 520 \) \( \mu \)g·mL\(^{-1}\)), reducing power (IC\(_{50} = 140 \pm 10 \) \( \mu \)g·mL\(^{-1}\)), and \( \beta \)-carotene bleaching inhibition (IC\(_{50} = 250 \pm 80 \mu \)g·mL\(^{-1}\)) methods, respectively [25]. By the DPPH method, \( H. \) chasmeyucum extract (IC\(_{50} = 920 \mu \)g·mL\(^{-1}\)) [37], and methanolic extracts of \( H. \) chionophilum (IC\(_{50} = 40 \mu \)g·mL\(^{-1}\)) [6,23] showed less activity than the other Helichrysum species. When compared with those results, the other Helichrysum species showed less activity than \( H. \) stoachas subsp. rupestre, except for \( H. \) stoachas subsp. barellieri from Turkey, by the DPPH method and \( H. \) stoachas from Portugal, by reducing power.

Since there are a large number of different types of antioxidant compounds, it is not clear which components are responsible for the observed antioxidant capacity. Some authors have shown that high total phenol content increases the antioxidant activity [38], and the key role of phenolic compounds as scavengers of free radicals is emphasized in several reports [39]. To explore the influence of the phytochemical constituents on antioxidant capacity, the correlation between the phenolic contents and antioxidant activity was determined. The antioxidant capacity of the extracts appears to be largely influ-
enced by the polyphenol, flavonoid, and tannin levels. Highly significant linear correlations \( R = 0.9 \) were observed between the polyphenol content and total antioxidant capacity, between the flavonoid content and DPPH, iron reducing power, \( \beta \)-carotene, ABTS, and iron chelation, and between the tannin content and iron chelation.

The main phenolic compounds of the genus *Helichrysum* are flavonoids which have remarkable antioxidant activity \[^{[40]}\]. Conforti et al \[^{[34]}\] observed that the extract of *P. saxatile* could be a good source of phenolic compounds, which are one of the most bioactive groups of secondary metabolites in plants. Based on these reports, the antioxidant potential of the extracts studied here could be attributed to the flavonoids for *H. stoechas* subsp. *rupestre*, and in a general way, to the phenolic compounds for *P. saxatile* subsp. *saxatile*.

The ABTS method is weakly correlated with the polyphenol and tannin contents. However, a weak correlation of iron chelating activity with the total polyphenol content was observed, and this may indicate that they might not be the main chelators of iron, if they did not have suitable groups that could chelate the cations.

There is no correlation between the polyphenol content and \( \beta \)-carotene. Amarowicz et al \[^{[41]}\] demonstrated previously that there was no correlation between the levels of these compounds and the results of the \( \beta \)-carotene bleaching assay. The polyphenol content measurement indicates the level of both lipophilic and hydrophilic compounds. The \( \beta \)-carotene bleaching assay only provides an indication of the level of lipophilic compounds because the \( \beta \)-carotene bleaching test is similar to an oil-in-water emulsion system \[^{[42]}\].

**Conclusions**

The results reveal that the studied plants contain a considerable amount of phenolic compounds, and possess interesting antioxidant properties when compared with standards, except for iron reducing and chelating power methods. This study provides useful chemical analysis information in many areas, such as food, phytopharmacology, phytotherapy, and phytotoxicology. Further studies should be carried out for the evaluation of the *in vivo* potential of these extracts in animal models, and for the isolation and identification of the individual active phenolic compounds.

**References**

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