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Total Phenolic, Total Flavonoid, Tannin Content, and Antioxidant Capacity of *Halimium halimifolium* (Cistaceae)

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ABSTRACT

This study was carried out to evaluate the phytochemical constituents and antioxidant potential of the leaves and flowers extracts of *Halimium halimifolium* in order to validate the medicinal potential of this herb. The antioxidant activity of alcoholic and aqueous extracts was evaluated using 2, 2-diphenyl-l-picrylhydrazyl (DPPH), 2, 2'-azinobis- (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and ferric reducing antioxidant power (FRAP) assays. The total polyphenol, flavonoid and tannin content were determined according respectively to Folin-Ciocalteu method, Zhishen method and Broadhurst method. The leaves of *H. halimifolium* had greater antioxidant activity than flowers by DPPH and ABTS assays. The ethanol extract of the leaves exhibited the better antioxidant activity by DPPH assay (IC_{50} = 1.19 µg.mL^{-1}), the n-butanol by ABTS assays (IC_{50} = 1.73µg.mL^{-1}). As opposed to FRAP method the flowers had greater antioxidant activity as leaves. Ethyl acetate extract exhibited better antioxidant activity (IC_{50} = 15.5 mmol Fe^{2+}.g^{-1}). The obtained results showed that the extracting solvent significantly influenced the antioxidant property estimations of *Halimium halimifolium*. The ethanol is a recommended solvent for extracting antioxidants from this plant. The antioxidant activity determined by DPPH, ABTS and FRAP demonstrated a linear relationship with their polyphenols, flavonoids and tannins content.

INTRODUCTION

Plants have always been used by humans to relieve and cure many diseases (Ramawat *et al.*, 2008). Today, in many parts of the world traditional medicine replaces conventional medicine (Winslow *et al.*, 1998). With multiple biological activities, many medicinal plants have antioxidant activity that is attracting more and more the attention of several research teams for its role in the fight against several diseases such as cancer, the atherosclerosis, cerebral cardiovascular events, diabetes, hypertension, and Alzheimer's disease (Liu *et al.*, 2003; Devasagayam *et al.*, 2004).

In order to enhance the Tunisian forest resources and develop new products with high added value, we are interested in the family of Cistaceae and particularly the *H. halimifolium*. It is a common medicinal plant in the Mediterranean (Zunzunegui *et al.*, 2000; Ferraz., 2000) used as antispasmodic, to treat stomach pain and to expel intestinal gas (Zunzunegui *et al.*, 2000). It is also present in the south and west of the Iberian Peninsula, Italy, Greece and northern Morocco (Zunzunegui *et al.*, 2002). In Tunisia, *H. halimifolium* is distributed in the forest road of Ain Draham and in the dunes of Tabarka (Debazac., 1995). The selection of this plant was guided on the one hand by the indications of its traditional use and on the other hand by the fact that at present, there have been very little chemical and biological investigations done.

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The purpose of this study is to enhance the knowledge of the *H. halimifolium* collected in Tunisia. Various solvents with various polarity (ethanol, dichloromethane, n-butanol, ethyl acetate and water) were used to compare the extraction efficiency of antioxidants *H. halimifolium*. Ethanol was also included to test the possibility of replacing methanol with ethanol in order to reduce toxicity. The antioxidative properties of the extracts in rockrose were assessed by 3 in vitro methods such as DPPH (2, 2-dipheny-l-picrylhydrazyl), ABTS (2,2'-azinobis-3-ethylbenzo thiazoline-6-sulphonate) and FRAP (ferric reducing antioxidant potential).

MATERIALS AND METHODS

Plant Materials

The leaves and flowers of *H.halimifolium* were collected in June 2012 from Sidi Mechreg (Sejnen) located in the northern parts of Tunisia. *H. halimifolium*was botanically characterized by Ilham Mallak Maalej (botanist at the Faculty of Sciences, Bizerte, Tunisia) and according to the morphological description presented in Tunisian flora (Pottier-Alapetite., 1981). Herbaria of these plants are kept in the Department of chemistry, Faculty of Sciences, Bizerte under the code number 12/HH/cis. Plants were dried at room temperature for half month in a dry and airy environment. The dried samples were powdered and stored in the dark at a dry place until further use.

Chemical Reagents

Folin-Ciocalteu phenol, DPPH (2, 2-Diphenyl picrylhydrazyl) reagent were purchased from Sigma Aldrich (St. Louis, MO, USA). Sodium nitrite (≥ 99.0 % purity), Gallic acid and Catechin reagents were from Sigma Aldrich (St. Louis, MO, USA). Sodium carbonates is from Fluka Biochemika (Switzerland), concentrated hydrochloric acid (37%) and absolute methanol was purchased from Panereac Quimica Sa (Barcelone). Absolute ethanol (≥ 99.8 % purity) is from Scharlab S.L (European Union).

Extraction method

The dried leaves and flowers (400g) were macerated with absolute ethanol at room temperature for 24 h. The solution was filtered and concentrated under reduced pressure at 50°C, yielding 42.04 and 28.22 g of crude ethanolic extract of *H. halimifolium* leaves and *H. halimifolium* flowers respectively. The extracts were suspended in MeOH: H_2O (3: 7; v/v) and extracted successively with dichloromethane, n-butanol, ethyl acetate in this order.

DETERMINATION OF TOTAL ANTIOXYDANT COMPOUNDS

Total polyphenol content

The total phenolic content was estimated by Folin Ciocalteu method as described by Singleton et al., (1965) with slight modifications. The extract (1 mg. mL^{-1}) was mixed with 5 mL of distilled water, 1 mL of sodium carbonate (20%) and 1 mL of Folin Ciocalteu reagent. The mixture was allowed to stand in a water bath for 30 min at 40°C. The content of total phenolic compounds was expressed as mg of gallic acid equivalents per g dry matter (mg GAE. g^{-1}DM). The absorbance was measured at 765 nm using a UV-Vis spectrophotometer T60 U. All the experiments were run in triplicate. The mean values and standard deviations were calculated using the Microsoft Excel software (Microsoft Corporation, Redmond, WA).

Total flavonoid content

The flavonoids content was determined by aluminium trichloride method using catechin as reference compound (Zhishen *et al.,* 1999). A volume of 125µL of extract is added to 75 µL of a 5% $NaNO_2$ solution. The mixture was allowed to stand for 6 min, then 150 µL of aluminium trichloride (10%) was added and incubated for 5 min, followed by the addition of 750 µL of NaOH (1M). The final volume of the solution was adjusted to 2500 µL with distilled water. After 15 min of incubation the mixture turned to pink and the absorbance was measured at 510 nm. The total flavonoids content was expressed as g E catechin.$100g^{-1}$DM.

Total condensed tannin contents

The tannin contents or Proanthocyanidin were determined by method of Broadhurst *et al.,* 1978 with slight modification, using catechin as a reference compound. A volume of 400µL of extract is added to 3 mL of a solution of vanillin (4% in methanol) and 1.5 mL of concentrated hydrochloric acid. After 15 min of incubation the absorbance was read at 500 nm. The condensed tannin was expressed as g E.Catechin.$100g^{-1}$DM.

DETERMINATION OF ANTIOXYDANT ACTIVITY

Free radical-scavenging ability by the use of a stable DPPH radical

The DPPH radical-scavenging activity was determined using the method described by Brand-Williams *et al.,* 1995. A DPPH solution (0.070 $mg.mL^{-1}$) was mixed with sample solutions at different concentrations (2.5 to 50.0 $\mu g.mL^{-1}$). A control (Abs Control) containing methanol and DPPH solution was also realized. All solutions obtained were then incubated for 1 hour at room temperature. Absorbances were measured at 517 nm.

The standards used are: ascorbic acid (3.0-15.0 $\mu g.mL^{-1}$), gallic acid (0.5 to 5.0 $\mu g.mL^{-1}$), Trolox (2.5 to 15.0 $\mu g.mL^{-1}$)
The radical scavenging capacity using the free DPPH radical was evaluated by measuring the decrease of absorbance at 517 nm.

When the reading was complete, the percentage of inhibition of samples was calculated from obtained absorbance by the equation:

$$\% \text{ inhibition} = ((\text{Abs control} - \text{Abs test})/\text{Abs control}) \times 100$$

Then, curves were constructed by plotting percentage of inhibition against concentration in µg/mL. The equation of this curve allowed to calculate the IC_{50} corresponding to the sample concentration that reduced the initial $DPPH^{\bullet}$ absorbance of 50 %.

A smaller IC50 value corresponds to a higher antioxidant activity. All test analyses were realized in triplicate.

Free radical-scavenging ability by the use of a stable ABTS radical cation

The free radical-scavenging activity of samples was determined by ABTS radical cation decolorization assay described by Pellegrini *et al.,* 1999. The stock solutions included 7 mM ABTS solution and 4.9 potassium persulfate solutions. The working solution was then prepared by mixing the two stock solutions in equal proportions and allowing them to react for 12-16 hours. This solution was stored in a dark place at room temperature. Before use, the solution was diluted with ethanol to obtain absorbance between 0.800 and 1.000 nm. This solution was mixed with sample (2.5 to 50 µg.mL^{-1}) or standard solutions. A control containing methanol and $ABTS^{\bullet+}$ solution was also realized. The absorbance was read at 734 nm after 30 min incubation at 25°C. The percentage inhibition of free radical ABTS was calculated from the following equation.

% of inhibition =((Abs control-Abs test) /Abs control) X 100

Then, curves were constructed by plotting percentage of inhibition against concentration in µg/mL. The equation of this curve allowed to calculate the IC_{50} corresponding to the sample concentration that reduced the initial $ABTS^{+\bullet}$ absorbance of 50%.

Ferric reducing/antioxidant power (FRAP) assay

The total antioxidant potential of a sample was determined using the ferric reducing ability of plasma FRAP assay (Benzie *et al.,* 1996). The principle of this method was based on the reduction of a ferric-tripyridyltriazine complex to its ferrous colored form in presence of antioxidants. The FRAP reagent was freshly prepared by mixing together 10mM TPTZ (2, 4, 6-tripyridyl triazine) and 20 mM ferric chloride in 300 mM sodium acetate buffer (pH 3.6). The FRAP reagent (Fe^{3+}- TPTZ complex) was mixed with test sample (2.5 to 20 µg.mL^{-1}) or standard solutions The absorbance was read at 593 nm after 30 min incubation at 40°C. The antioxidant capacity was expressed in FRAP unit, in mmol $Fe^{2+}.g^{-1}$ calculated by linear regression curve of iron sulphate standard.

The equation obtained allow to convert concentrations in µg.mL^{-1}of sample in FRAP unit (mmol $Fe^{2+}.\ g^{-1}$).

Statistical analysis

Data were analysed using Microsoft Excel and reported as mean ± standard deviation of triplicate determination.

RESULTS AND DISCUSSION

Total Phenolic Content (TPC)

Phenolic compounds are ubiquitous secondary metabolites in plants. They are known to have antioxidant activity and it is likely that the activity of these extracts is due to this compounds (Okudu *et al.,* 1994; Tepe *et al.,* 2006). The results obtained in this study showed a significant level of phenolic compounds in ethanol, butanol and aqueous extracts of the leaves and flowers of *H. halimifolium* (Tab. 1). The contents of flavonoids and phenolic compounds were greater in leaves than in fruits and the ethanolic extracts presented higher contents than the others extracts. These results indicated the influence of the extraction solvent on the total content of phenolic compounds extracted. These results are similar to those reported by Alaliab et al (Alaliab *et al.,* 2007) that showed similar antioxidant activity in the *Helianthemum lippii* L (Cistaceae).

Table 1: Total phenolic compounds of *H. halimifolium* extracts.

	Total phenolics (g gallic acid eq.100g^{-1} DM)	
Extracts	**Leaves**	**Flowers**
Ethanol Ext	55.98±1.30	40.55±0.30
Water Ext	53.75±0.45	42.91±0.21
n-butanol Ext	46.78±0.2	42.05±0.2
Ethyl acetate Ext	12.32±1.14	10.8±0.50
DichloromethaneExt	11.11±0.50	9.45±0.05

Total Flavonoids Content (TFC)

Flavonoids are one class of secondary plant metabolites that are also known as Vitamin P. These metabolites are mostly used in plants to produce yellow and other pigments which play an important role in the colors of plants. In addition, Flavonoids are readily ingested by humans and they seem to display important anti-inflammatory, anti-allergic and anti-cancer activities (Crozier *et al.,* 2006). The total flavonoids content of *H. halimifolium* extract was also determined using aluminium chloride colorimetric method (Tab. 2). Leaves flavonoid content was higher than flowers. Polar extracts (ethanol, butanol and water extracts) showed more flavonoids than apolar extracts.

Table 2: Total flavonoids compounds of *H. halimifolium* extracts.

	Total flavonoid (g catechin eq. 100g^{-1} DM)	
Extracts	**Leaves**	**Flowers**
Ethanol Ext	20.11±1.02	15.50±0.47
Water Ext	18.52±0.80	14.01±0.50
n-Butanol Ext	17.32±0.71	14.00±0.82
Ethyl acetate Ext	6.54±0.54	3.20±1.12
DichloromethaneExt	4.23±1.30	3.00±0.11

Total Condensed Tannins (TCT)

We found that the plant *H. halimifolium* have high levels of flavonoids and low levels of tanins. By comparing the results obtained with various solvents (Tab.3), we can showed that the extraction values of polyphenols, flavonoids and tannins is greatly depending on the solvent polarity. The maceration with ethanol showed the highest TPC, TFC and TCT in the extract.

Table 3: Total condensed tannins of *H. halimifolium* extracts.

	Total tannins (g catechin eq. 100g^{-1} DM)	
Extracts	**Leaves**	**Flowers**
Ethanol Ext	2.20±0.20	1.99±0.02
Water Ext	0.91±0.03	1.30±0.02
n-butanol Ext	0.82±0.02	1.31±0.12
Ethyl acetate Ext	0.32±0.03	0.10±0.01
DichloromethaneExt	0.54±0.01	0.05±0.54

DPPH, ABTS radical scavenging activity, FRAP reducing ability

The result of the antioxidant activity of the samples varies according to the nature of the solvent used and particularly to the methods of analysis. Recent studies have shown that there is no universal method to evaluate antioxidant activity quantitatively and accurately (Prior *et al.*, 2005), therefore, the antioxidant activity of plants is evaluated using several methods.

Previous studies by Schlesier *et al.*, 2002 showed that when analyzing the antioxidant activity, it is preferable to use at least two methods. In their experiments, they used six methods to assess the antioxidant activity of tea and fruit juices: DPPH, ABTS, total radical-trapping antioxidant parameter assay, N, N-dimethylp-phenylendiamine assay, photo chemiluminescence assay and ferric reducing ability of plasma assay. Among the samples analyzed, blackcurrant juice showed the greatest antioxidant activity by all the methods, but the results of the other three samples (tea, apple juice and tomato juice) varied depending on the method used.

In this study, the analysis of the antioxidant activity of leaves and flowers of the *H. halimifolium* harvested in Tunisia was performed using three methods: DPPH, ABTS and FRAP. These methods are distinguished by their mechanism of action and would be complementary to the study of the antioxidant potential of plants.

The antioxidant proprieties of extracts were measured in terms of their efficient IC 50 concentration corresponding to the sample concentration that reduced the initial DPPH$^{\bullet}$ absorbance of 50%. These IC 50 values are given in table 4 for DPPH, table 5 for ABTS.

The results for the FRAP assay are presented in table 6. The antioxidant activity of the leaf extract measured by DPPH and ABTS method was significantly higher than the flower extract. This result can be attributed to the higher phenolic content of the leaf.

Measured by the DPPH method, the antioxidant activity of ethanol and water extracts were higher than all other extracts, whereas by the ABTS method (Tab. 5), the antioxidant activity of n-butanol extract was higher than all other extracts. In water extract, the flowers had an antioxidant activity higher than the leaf. With the FRAP method, the dichloromethane extract has the best antioxidant activity, and unlike the other two methods, the extracts of the flowers have the greatest antioxidant activity except in the case of n-butanol extract.

DPPH scavenging activities of both ethanolic and aqueous extracts were the most important. They were higher than any standards used (Tab.4).

This study confirmed the medicinal potential of the leaves and flowers of *H. halimifolium* and is in agreement with the medicinal potential of Cistaceae family showed by several authors (Zidane *et al.*, 2013; Enrique *et al.*, 2010).

Despite the various mechanisms of the methods used, the combined results of these in vitro tests gave us an idea of the relative antioxidant activity of different parts of *H. halimifolium* and its various extracts. In summary, the leaves have greater antioxidant activity than fruits, and ethanolic and aqueous extracts showed greater antioxidant activity than other.

Table 4: Antioxidant activity of *H. halimifolium* extracts against DPPH method.

DPPH	IC_{50} ($\mu g.mL^{-1}$)	
	Leaves	**Flowers**
Ethanol extract	1,19	15,09
Water extract	1,548	8,00
Ethyl acetateextract	35,87	61,30
Dichloro-methaneextract	>52	>52,38
n-butanolextract	11,78	14,17
Gallicacid	2,24	
Ascorbicacid	6,25	
Trolox	8,16	

Table 5: Antioxidant activity of *H. halimifolium* extracts against ABTS method.

ABTS	IC_{50} ($\mu g.mL^{-1}$)	
	Leaves	**Flowers**
Ethanol extract	10.40	13.99
Water extract	16.70	7.30
Ethyl acetateextract	37.26	47.82
Dichloro-methaneextract	>52	>52
n-butanolextract	1.73	11.69
Gallicacid	2.61	
Ascorbicacid	8.21	
Trolox	9.47	

Table 6: Antioxidant activity of *H. halimifolium* extracts against reduc method.

FRAP	(mmol Fe^{2+}/g)	
	Leaves	**Flowers**
Ethanol extract	55.63	30.82
Water extract	59.02	37.16
Ethyl acetateextract	19.07	15.50
Dichloro-methaneextract	16.33	15.71
n-butanolextract	45.30	45.86
Gallicacid	40.34	
Ascorbicacid	14.38	
Trolox	9,7	

Correlation between antioxidant activity and total polyphenols, and total flavonoid contents

Correlation analysis was used to explore the relationships between total phenolic, flavonoid and Proanthocyanidin content and the different antioxidant variables measured in *H. halimifolium*(Tab.7).

There was a significant linear correlation between the free radical scavenging activity determined by using the DPPH, ABTS and FRAP method, and total polyphenolic compounds (phenolic and flavonoids). However, no significant relationship between antioxydant activity (DPPH and ABTS) and total condensed tannins, in the leaves of *H. halimifolium*, were observed. (R^2 = 0.4602; R^2 = 0.3113, respectively). The strongest correlative value is obtained with ABTS and total phenolic compounds in the flowers of *H. halimifolium* (R^2 = 0.9937) followed of that of FRAP in the leaves (R^2 = 0.9806). The phytochemical study showed that the ethanol extract of *H. halimifolium* contained many bioactive compounds. This result justifies their usage in traditional medicines.

Table 7: Linear correlation of Trolox equivalent antioxidant capacity versus the total phenolic content in leaves and flowers of *H. halimifolium*extract.

		Correlation coefficients		
		DPPH	**ABTS**	**FRAP**
TP	Leaves	Y = -0.9769x+55.633 (R^2 = 0.9422)	Y = -0.8217x+53,191, (R^2 = 0.8019)	Y= 0.8911x+7,0014, (R^2 = 0.9806)
	Flowers	Y = -1.3979x+70.939 (R^2 = 0.9705)	Y = -1.2321x+62.477, (R^2 = 0.9937)	Y= 0.7105x+8,2978, (R^2 = 0.857)
TF	Leaves	Y = -3.0086x+60,624 (R^2 = 0.9677)	Y = -2.5892x+58,168, (R^2 = 0. 862)	Y= 2.6794x+3,315, (R^2 = 0.9601)
	Flowers	Y = -3.8272x+68.238, (R^2 = 0.9469)	Y = -3.3544x+59.909, (R^2 = 0.9586)	Y=1.8724x+10,394, (R^2 = 0.7747)
TT	Leaves	Y = -20.899x+40.499, (R^2 = 0.4602)	Y = -15.674x+38,634, (R^2 = 0.3113)	Y=70.381x-10,642, (R^2 = 0.8381)
	Flowers	Y = -26.395x+55.263, (R^2 = 0.8193)	Y = -23.074x+48,48, (R^2 = 0.8252)	Y=11.951x+17,656, (R^2 = 0.5741)

TP: Total phenolic content; TF: Total flavonoids content; TT: Tanins content

CONCLUSION

This study showed the presence of antioxydant compounds (phenolic acids, flavonoids and tannins) and demonstrated some level of antioxidant activities in *H. halimifolium*.Total amount of flavonoid, phenolic and tanins compounds were maximum in polar extracts (ethanol, water and butanol). High antioxidant activity was observed in ethanol extract and aqueous extract.

The leave extracts showed antioxidant activities quantitatively comparable to that of gallic acid. The total flavonoid content and the antioxidant activity of the leave extracts had significant correlation. Therefore, flavonoids are suggested to be a principal group of antioxidants in *H. halimifolium*. Based on the results of investigations, *H. halimifolium* is a potent source of novel bioactive compounds. Further investigations regarding more biological activities of the plant parts need to be conducted.

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