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Research Article

A Contribution to the Valorization of Two Medicinal Plants: *Atriplex Halimus* Sub. Sp. *Schweinfurthii* and *Bunium Incrassatum*, Growing in the Region of M'sila (North-East Algeria).

DEHIMI KHADIDJA[1, 3]*, DJOUDI ZOUINA[2], BOULAOUAD ANIS[2], MAADADI ABD RAOUF[2], DAHAMNA SALIHA[3], KHENNOUF SEDDIK[3]

[1] Faculty of Sciences, University Mohamed Boudiaf, M'sila, Algeria.

[2] Faculty of Nature and Life Sciences, University Mohamed El Bachir El Ibrahimi, Bordj Bou Arrerdj, Algeria.

[3] Laboratory of Phytotherapy Applied to Chronic Diseases, Faculty of Nature and Life Sciences, University of Setif-1, Algeria.

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ABSTRACT

Atriplex halimus and *Bunium incrassatum* were extracted using solvents from different polarities (water, methanol, acetone and hexane). To evaluate the antioxidant abilities of the extracts, three *in vitro* test systems were employed: DPPH scavenging assay, β- carotene bleaching test and the reducing power assay. Extractions using acetone were the richest in polyphenols, while hexane fractions from both plants were found to contain the highest amounts of flavonoids. Tannins were more frequent in acetone fraction from AH. The best scavenging activity against DPPH radical was obtained by hexane extract from AH, followed by acetone and methanol extracts from the same plant. BI fractions showed a weak scavenging activity with IC_{50} values higher than 20 $mg.mL^{-1}$. Acetone fractions showed the best iron reducing activity in both plants. In the BCB assay, hexane fraction from AH showed an excellent activity which was very close to that of butylated hydroxytoluene.

INTRODUCTION

Oxidative stress, the consequence of an imbalance of prooxidants and antioxidants in the organism, is a key phenomenon in chronic diseases. Although free radicals can be scavenged by the in vivo produced antioxidant compounds the endogenous antioxidants are insufficient to completely remove them, which demonstrate the importance of dietary antioxidants. Polyphenols are present in a variety of plants used as components of the human diet and also in traditional medicine. Polyphenols exhibit a wide range of biological effects as a consequence of their antioxidant properties [1].

Atriplex halimus sub. sp. Schweinfurthii (commonly called Guettaf in Algeria) is a perennial halophytic shrub belonging to the Chenopodiaceae family, it is native to arid and semi-arid zones in the Mediterranean basin [2, 3]. It is used as livestock forage and has many applications in folk medicine particularly in the treatment of diabetes, heart conditions, thyroid disease and different types of cysts [4, 5]. *Bunium incrassatum* is a medicinal plant belonging to the Apiaceae family; it is widely distributed in Algerian east and locally called "Talghouda". The tubers of this plant are traditionally used as antidiarrheal, in the treatment of inflammatory hemorrhoids, to relieve bronchitis and thyroid disease; they are also very nutritious and thus used as food [6].

The aim of the present study is to test different extracts from *A. halimus* and *B. incrassatum* for their antioxidant activity in three in vitro models, including DPPH scavenging test, reducing power and β-carotene bleaching assays; besides, estimating the content of phenolic compounds in the studied extracts.

Author for Correspondence:
Email: khadidja.dehimi@univ-msila.dz

MATERIALS AND METHODS

Chemicals

Reagents used in this study were purchased from Sigma-Aldrich, Fluka and BioChem. Solvents

were obtained from Sigma-Aldrich and AnalaR NORMAPUR.

Plant Materials

Atriplex halimus sub. sp. *Schweinfurthii* (Fig. 1) was collected from the region of Draa El-Hadjja in M'sila (Algeria) in December 2017. The plant was air-dried in shade and then leaves and flowers were separated from stems and used for extraction. The plant *Bunium incrassatum* (Fig. 2) was collected at the end of February 2018 from a culture field near Ksob dam in M'sila. Tubers were separated, washed, peeled and dried to be used for extraction. The species were identified by botanists from the University of Bordj Bou Arreridj and the University of Setif (Algeria).

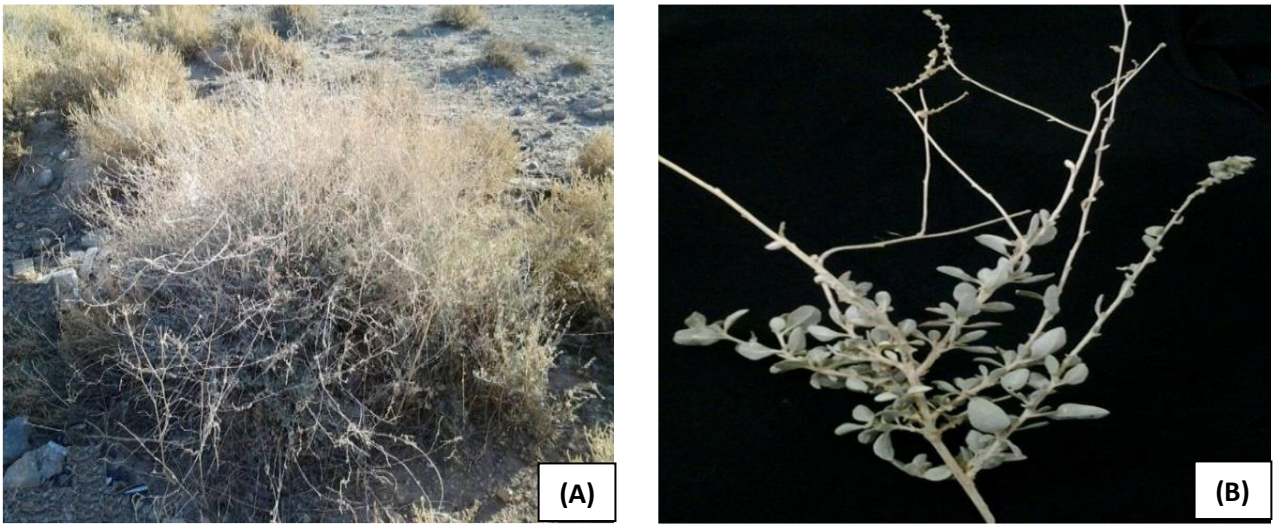

Figure 1: *Atriplex halimus* ssp. *Schweinfurthii*. **A :** Plant in environment. **B:** Aerial part of the plant.

Figure 2: *Bunium incrassatum*. **A:** Tuber and long white roots in winter. **B:** Plant after flowering in spring (extended aerial part).

Extraction

Four extracts were prepared from each plant following the procedures described by Gnanaprakash *et al* [7] and Siracusa *et al* [8]. Aqueous extracts were prepared as a decoction by heating the mixture of 10 g of plant material and 100 mL of distilled water at 70°C for 1 hour under agitation. To obtain organic extracts, 10 g of each plant were defatted with n-hexane; the plant residue was then extracted with acetone, and after filtration, the material was further extracted with methanol. All extractions were performed in dark and left for two days under occasional stirring. After filtration and removal of solvents at reduced pressure, extracts were kept at +4°C until analysis in which all fractions were dissolved in the appropriate volume of dimethyl sulfoxide (DMSO).

Phytochemical Screening

The preliminary phytochemical screening of various active compounds in both plants' extracts was carried out using standard methods as described by Ganatra *et al* [9]. All extracts were prepared at a concentration of 4 mg. mL^{-1} in DMSO.

Test for Saponins

Each extract was diluted with an equal volume of distilled water. The mixtures were shaken vigorously and observed for a stable persistent frothing.

Test for Flavonoids

Few drops of dilute sodium hydroxide (NaOH) were added to 1 mL of each extract. The formation of a yellow coloration indicated the presence of flavonoids.

Test for Tannins

One milliliter of 1% ferric chloride (FeCl3) was added to each extract. The formation of a bluish-black or greenish-black color indicated the presence of tannins in extracts.

Test for Quinones

Equal volumes of each extract and concentrated sulphuric acid (H2SO4) were mixed and observed for the appearance of red coloration.

Test for Terpenoids

A volume of 5mL of each extract was mixed with 2mL of chloroform. 3mL of concentrated sulphuric acid was then added slowly to form a layer. The formation of a reddish-brown ring in the interface indicated the presence of terpenoids.

Total Phenols Content

Total phenols content was determined spectrophotometrically, using the Folin–Ciocalteu reagent according to the method described by Li et al [10]. Briefly, a volume of 200 µL of each extract (2 mg.mL-1) was mixed with 1 mL of Folin–Ciocalteu reagent (diluted 10 times in distilled water). After 4 min, 800 µL of sodium carbonate solution (Na2CO3, 75 g.L-1) was added to the mixture which was incubated for 2 h in darkness, at room temperature. Absorbance was measured at 765 nm (Shimadzu UV mini-1240 spectrophotometer) and gallic acid (20-100µg.mL-1) was used as a standard for the calibration curve. Total polyphenols content was expressed as µg of gallic acid equivalents per mg of extract (µg GAE/mg E). All samples were performed in triplicate.

Flavonoids Content

The content of flavonoids in the studied extracts was determined using the aluminum chloride method as described by Bahorun et al [11]. Briefly, 1 mL of 2% AlCl3 aqueous solution was mixed with 1 ml of each extract (2mg.mL-1). After 10 min of incubation in darkness at room temperature, absorbance was recorded at 430 nm. Quercetin (10-50µg.mL-1) was used as standard and total flavonoid content was expressed as µg of quercetin equivalents per mg of extract (µg QE/mg E). Each determination was repeated three times.

Total Tannins Content

The total content of tannins in extracts was estimated using Folin–Ciocalteu reagent and tannic acid as standard, according to the method described by Prasanth et al [12]. Aliquots of 350 µL of each extract at a concentration of 3mg.mL-1 were added to 1.5 mL of Folin-Ciocalteu reagent (10%). After agitation, 1.5 mL of sodium carbonate solution (75g.L-1) was added and the final mixture was incubated at 45 °C for 45 min in darkness. Absorbance was then measured at 765 nm and the calibration curve was established using different concentrations of tannic acid (37.5-300 µg.mL-1). Total tannin content was expressed as µg of tannic acid equivalents per mg of extract (µg TAE/mg E). Each determination was carried out in triplicate.

Antioxidant Activity

DPPH Test

Studied extracts were tested for their scavenging effect on DPPH radical according to the method of Brand Williams et al [13]. Fifty µL of extracts solutions at different concentrations were added to 1250 µL of methanolic solution of DPPH (0.04 mg.mL-1). The mixture was shaken and then kept in the dark for 30 minutes at room temperature. Absorbance was measured at 517 nm using a spectrophotometer. Quercetin dissolved in methanol at different concentrations was used as the positive control. All determinations were performed in triplicate. The DPPH radical-scavenging activity was calculated using the following equation:

Radical scavenging activity (%) = $\{(A_{blank} - A_{sample}) / A_{blank}\} \times 100$

Where A_{blank} is the absorbance of the blank control (containing all reagents except the extract solution) and A_{sample} is the absorbance of the test sample.

Ferric Reducing Antioxidant Power (FRAP)

The reducing power was determined as described by Vijayalakshmi and Ruckmani [14]. Briefly, 500 µL of each extract at different concentrations were added to 1250 µL of phosphate buffer (0.2 M, pH 6.6) and 1250 µL of potassium ferricyanide (1%). The reaction mixture was stirred well and then incubated at 50°C for 20 minutes in a water bath. After incubation, 1250 µL of trichloroacetic acid (10%) was added and the mixture was centrifuged at 3000 rpm for 10 minutes. 1250 µL of the supernatant were then mixed with 125 µL of distilled water and 250 µL of ferric chloride (0.1%) and absorbance was determined at 700 nm. High values of absorbance indicate strong reducing power. Ascorbic acid was used as a positive control and each solution was repeated three times.

β-Carotene Bleaching Assay

Antioxidant activity of the studied extracts was evaluated by the spectrophotometric β-carotene bleaching test as described by Dapkevicius et al [15]. The β-carotene solution was prepared by dissolving 0.5 mg of β-carotene in 1 ml of chloroform. One milliliter of this solution was added to 25 µL of linoleic acid and 200 mg of tween 40. The chloroform was then removed using a rotary evaporator at 40°C and 100 mL of distilled water saturated with oxygen was added with vigorous shaking. Aliquots of 2.5 mL of this mixture were added to 350 µL of the extracts prepared at a concentration of 3 mg.mL-1. Absorbance at 490 nm was determined at t0 and then after 1h, 2h, 3h, 4h, 24h, and 48h of incubation at room temperature. As a positive control, synthetic antioxidant BHT was used at the same concentration of extracts (3 mg.mL-1). DMSO, methanol and distilled water were used as negative controls. Each determination was repeated three times. The percentage of inhibition was calculated using the following equation:

$$\% \text{ of inhibition} = (A_t / A_{t0}) \times 100$$

Where A_t is the absorbance of the test sample at time (t) and A_{t0} is the absorbance of the test sample at a time t_0.

Statistical Analysis

All tests were conducted in triplicate. The results are expressed as means ± SD. The significance of difference was tested by the one-way ANOVA using GraphPad Prism 7.04. Values of $P < 0.05$ were regarded as significant. IC50 values were calculated by linear regression method using Microsoft Excel 2010.

RESULTS

Phytochemical Study

Extracts from the studied plants were prepared using solvents with different polarities. The yields of the extractions expressed as percentage are shown in Table 1. The highest yields in both plants are obtained using distilled water as solvent, followed by methanol for A. halimus and hexane for B. incrassatum. The yields for the other extractions were much lower.

Table 1: Yields of extractions from *B. incrassatum* and *A. halimus*.

Extract	Yield (%)	
	Bunium incrassatum	*Atriplex halimus* ssp. *Schweinfurthii*
Aqueous	24.48	19.87
Methanol	03.36	10.92
Acetone	00.52	01.56
Hexane	11.28	00.88

The preliminary phytochemical investigation confirmed the presence of flavonoids and tannins in all tested extracts. Quinones tested positive in all extracts except the aqueous fraction from AH. Terpenoids are reported present in all extracts except aqueous fractions and methanol extract from AH, while saponins tested negative in all extracts (Table 2).

The total contents in phenols, flavonoids and tannins of AH and BI extracts were examined and results are presented in Table 3. Acetone extracts showed the highest amounts of total phenols (47.10 µg GAE/mg E for BI and 28.75 µg GAE/mg E for AH), followed by aqueous extract from AH (28.10 µg GAE/mg E). Methanolic extracts from both plants showed close values (14.44 µg GAE/mg E for AH and 13.01 µg GAE/mg E for BI) together with hexane fraction from AH (13.36 µg GAE/mg E), whereas the contents obtained with aqueous and hexane extracts from BI were much lower. Flavonoids content in extracts was inversely proportional to the polarity of solvents used in their preparation for both plants (Hex>Ace>MeOH>Aq).

Table 2: Phytochemical screening of *B. incrassatum* and *A. halimus* extracts.

	Bunium incrassatum				***Atriplex halimus* ssp. *Schweinfurthii***			
Extract	Aq	MeOH	Ace	Hex	Aq	MeOH	Ace	Hex
Flavonoids	+	+	+	+	+	+	+	+
Tannins	+	+	+	+	+	+	+	+
Saponins	-	-	-	-	-	-	-	-
Terpenoids	-	+	+	+	-	-	+	+
Quinones	+	+	+	+	-	+	+	+

(+) : Presence, (-) : Absence. Aq : Aqueous, MeOH : Methanol, Ace : Acetone, Hex : Hexane.

Table 3: Total phenols, flavonoids and tannins contents in *B. incrassatum* and *A. halimus* extracts.

Extract		**Total phenols content (µg GAE/mg E)**	**Total flavonoids content (µg QE/mg E)**	**Total tannins content (µg TAE/mg E)**
Atriplex halimus* ssp. *Schweinfurthii	Aq	28.10 ± 9.66	17.70 ± 0.65	32.30 ± 0.97
	MeOH	14.44 ± 0.82	22.79 ± 0.32	29.47 ± 4.59
	Ace	28.75 ± 10.78	31.94 ± 0.00	76.18 ± 19.79
	Hex	13.36 ± 0.41	43.96 ± 0.25	19.67 ± 0.70
Bunium incrassatum	Aq	06,92 ± 0,00	06,91 ± 0,01	04,72 ± 0,00
	MeOH	13,00 ± 0,09	16,32 ± 0,05	17,23 ± 0,01
	Ace	47,10 ± 0,04	27,60 ± 0,02	22,50 ± 0,01
	Hex	05,68 ± 0,00	32,84 ± 0,01	32,06 ± 0,02

Aq : Aqueous, MeOH : Methanol, Ace : Acetone, Hex : Hexane, GAE : Gallic acid equivalents, QE : Quercetin equivalents, TAE : Tannic acid equivalents, E : extract. Each value represents the mean ± SD of three repetitions.

The highest amounts were found in hexane fractions with 43.96 µg QE/mg E for AH and 32.84 µg QE/mg E for BI, while the lowest content was determined in aqueous extract from BI with 6.92 µg QE/mg E. Tannins amounts in extracts ranged from 4.72 µg TAE/mg E (BI aqueous extract) to 76.18 µg TAE/mg E (AH acetone fraction). For BI extracts, a polarity-depending order was observed (Hex>Ace>MeOH>Aq), while for AH extracts the following order was obtained: Ace>Aq>MeOH>Hex.

Antioxidant Activity

The Antioxidant abilities of the studied extracts were evaluated using different methods. In the DPPH assay, the reducing ability is evaluated by measuring the loss of DPPH color at 517 nm after reaction with extracts, the color intensity is inversely related to the test antioxidant concentration. Results obtained in this assay for both plants are represented in Fig. 3. All tested extracts showed a dose-dependent weak activity against DPPH radical. AH extracts exhibited IC_{50} values ranging from 7.24 to 21.90 mg.mL^{-1}, where scavenging activity was proportional to flavonoid content in these extracts (Hex>Ace>MeOH>Aq). Similarly, extracts from BI showed weak reducing activity against DPPH radical, where IC_{50} values varied from 21.11 to 38.12 mg.mL^{-1} (Potency order: MeOH>Ace>Hex), while for aqueous extract no activity was recorded even for the highest dose tested (21 mg.mL^{-1}). The highest scavenging activity was obtained by quercetin with IC_{50} of 0.12 mg.mL^{-1}.

In the FRAP assay, the studied extracts exhibited different degrees of electron-donating capacities in a concentration-dependent manner (Fig. 4). Among extracts from AH, acetone fraction showed the best activity (1.05) at the dose of 6 mg.mL^{-1}, knowing that it contains also the highest amounts of total polyphenols and tannins. The potency order observed for this plant's extracts was Ace>MeOH>Hex>Aq for the dose of 6 mg.mL^{-1}. However, fractions from BI presented a reducing capacity proportional to their content of total polyphenols (Ace>MeOH>Aq>Hex). Moreover, ascorbic acid used as standard exhibited better reducing power in comparison with the studied extracts giving an absorbance value of 0.61 at a concentration of 50 µg.mL^{-1}

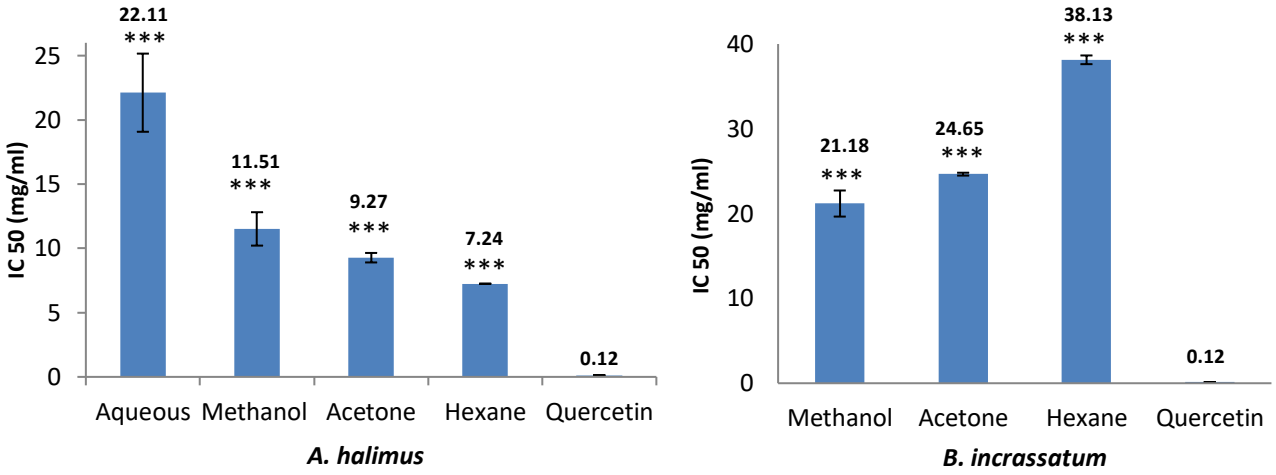

Figure 3: Scavenging activity of ***A.*** *halimus* and ***B.*** *incrassatum* extracts against DPPH radical. Data are expressed as mean ± SD of three experiments. *** ($p < 0.001$) versus quercetin.

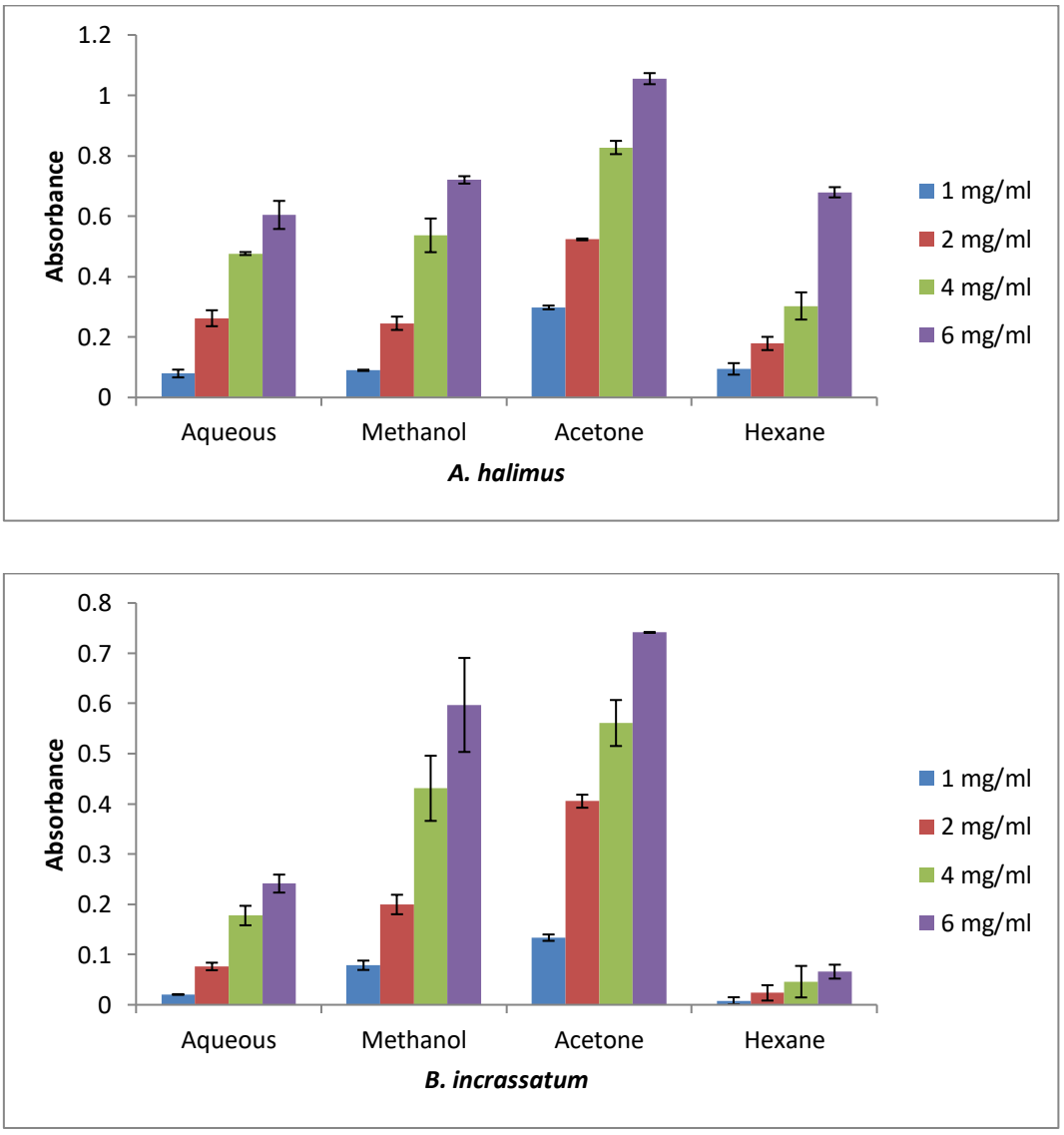

Figure 4: Reducing power of extracts from ***A.*** *halimus* and ***B.*** *incrassatum* measured by FRAP assay. Data are expressed as mean ± SD of three experiments.

The obtained results in BCB assay are presented in Fig. 5. In this assay, extracts from both plants showed a good antioxidant effect, especially AH hexane extract with an inhibition percentage of 92.47% after 24h of incubation. This value was very close to that obtained using BHT (99.88%) at the same concentration (3 $mg.mL^{-1}$). The potency of AH extracts was inversely proportional to their polarity and proportional to their flavonoids content. For BI extracts, interestingly, aqueous decoction showed good activity in this assay, unlike previous ones. The potency order for BI fractions was Aq>Hex> MeOH>Ace.

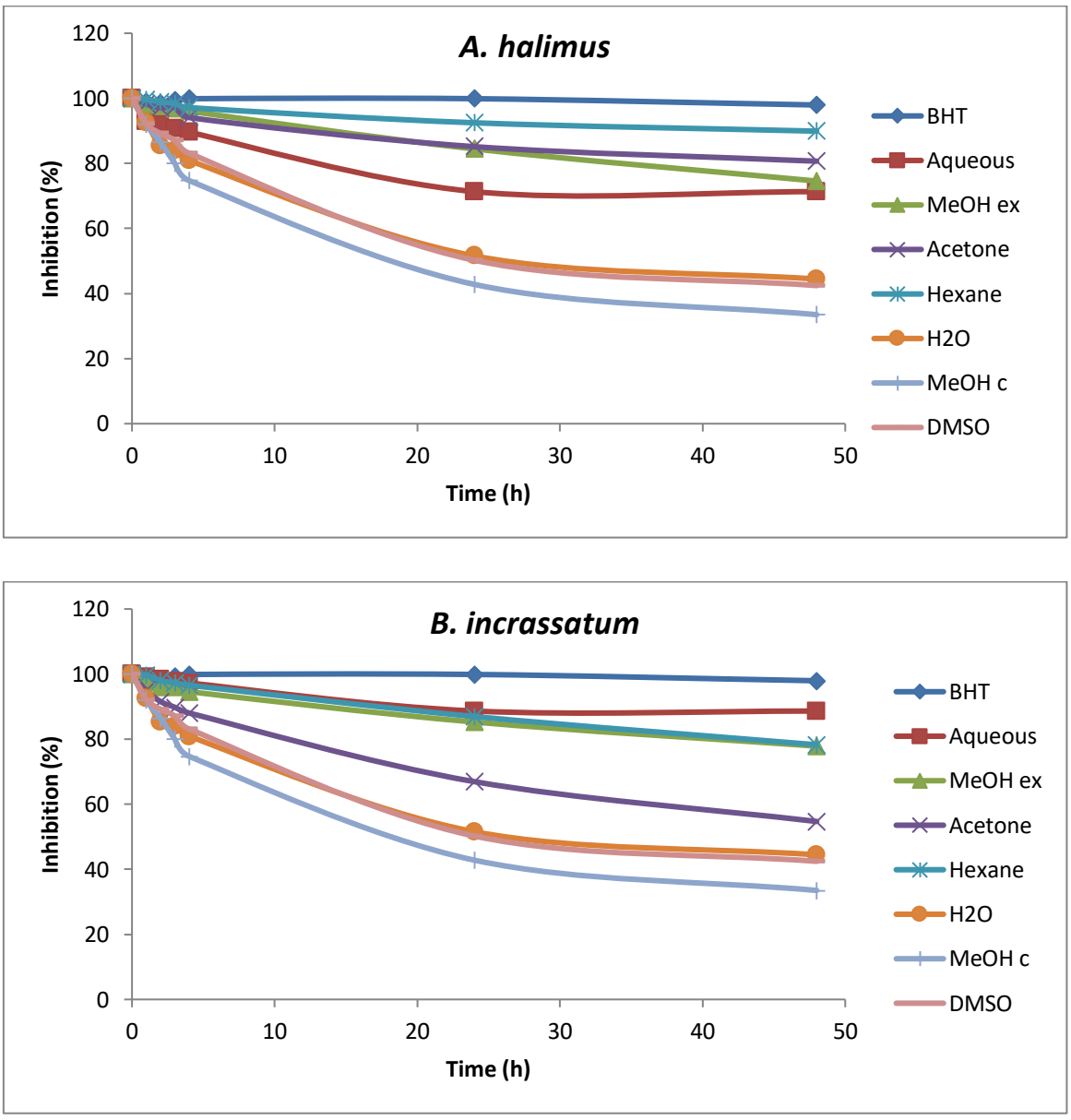

Figure 5: β-carotene bleaching kinetic in the presence of AH and BI extracts (Aqueous, MeOH ex, Acetone and hexane) and controls (BHT, H_2O, $MeOH_c$ and DMSO). Each value represents the mean of three repetitions

DISCUSSION

Medicinal plants are well known, nowadays, as an important source of bioactive molecules. In the present study, AH and BI are investigated as a possible source of active compounds with the aim of the valorization of these plants since only a few studies exist in literature about these two species. For this purpose, a simple chemical screening was performed and the results confirmed the presence of active molecules including flavonoids, tannins, quinones, and terpenoids in the extracts. These findings are in agreement with previous studies about AH, which reported the presence of flavonoids,

tannins, alkaloids, and resins in the plant [2, 3, 5]. Only one study was realized about BI tuber's chemical composition, in which two coumarins, β-sitosterol, sucrose, and oleic acid were isolated [6]. Furthermore, the studied fractions were found to contain considerable amounts of phenols, flavonoids, and tannins; the results for AH are comparable to those described in a study realized by Benhammou et al [16], in which methanolic fraction contained 10.12 µg GAE/mg E. Only one study carried out by ElKolli et al [17] reported that the phenolic content of BI in aerial parts of the plant (236.6 µg GAE/mg E) is much considerable in comparison with tubers.

It is well known that the measurement of antioxidant activity in plants' extracts must be conducted using tests based on different mechanisms view that each assay can reflect only the chemical reactivity under the specific conditions applied in it. In this work, three different simple redox-based tests differing in the mechanisms involved and the chemical environment were used. The DPPH assay is based on the measurement of the scavenging ability of antioxidants towards the stable radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH). The free radical DPPH, which bears a deep purple color, is reduced to the yellow-colored diphenylpicrylhydrazine when it reacts with hydrogen donors. The DPPH assay is usually classified as electron transfer (ET) reaction although this radical may be neutralized either by direct reduction via ETs or by radical quenching via H atom transfer (HAT) [18]. The obtained results for this assay are in accordance with those mentioned by Benhammou et al [16], where high IC50 values were obtained by testing methanolic extracts from leaves and stems from AH against DPPH radical (31.83 and 20.58 mg.mL-1, respectively), while for BI tubers, the antioxidant activity is evaluated for the first time in this work. The FRAP assay can be used to measure the total antioxidant activity of extracts from medicinal plants [14]. Antioxidants have the ability to reduce ferric ions (Fe3+) to ferrous form (Fe2+) by donating an electron, leading to changing the reaction medium color from yellow to blue measured at 700 nm, where absorbance intensity is proportional to antioxidant capacity [19]. The reducing potential of extracts is linked to the presence of molecules capable of donating electrons; in this study, AH acetone fraction which is the richest in polyphenols gave the best activity, also extracts from BI showed a capacity proportional to their content in polyphenols which are known to sequester metal ions. In the β-carotene bleaching assay (BCB), the antioxidant capacity of extracts was determined by measuring the inhibition of the oxidative degradation of β-carotene (discoloration or bleaching) by the oxidation products of the linoleic acid. Unlike the two previous assays, the BCB assay is performed in a heterogeneous system; in fact, the presence of two phases, one hydrophilic and one hydrophobic, might limit the antioxidant power of compounds unable to reach the organic phase (where lipoperoxidation occurs) and/or to interact with lipid micelles/biomembranes. This test is proton-transfer based, given that the scavenging of the lipo-peroxyl radicals by an antioxidant is thought to be a proton-transfer based reaction, and thus it allows us to evaluate the capacity of a compound to act as a chain-breaking antioxidant in the process of lipid peroxidation [20]. Similarly, in this assay, AH acetone fraction showed a very good activity in comparison with the synthetic antioxidant BHT; For this plant, the potency was proportional to flavonoids content in extracts, which are known to have a good capacity to terminate the chain reaction of lipid peroxidation by trapping peroxyl radical [21].

CONCLUSION

In the present investigation, extracts from *A. halimus* and *B. incrassatum* exhibited a good antioxidant activity, especially in the lipid peroxidation prevention. It is evident that these activities are related to the extracts composition rich in phenolic compounds including flavonoids and tannins. For *A. halimus*, acetone and hexane were more effective as solvents for the extraction of phenolic compounds, and their extractions showed the best activities in the different assays used. However, for *B. incrassatum*, acetone and methanol extractions seem to be more active and contained the highest amounts of total phenols, besides the aqueous extraction which showed a good effect in the β-carotene bleaching assay. These findings prove that the solvent effect changes from a plant to another depending on the plant composition.

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