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Research Article

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HPLC Profile and *In vitro* Antioxidant Properties of the *n*-butanol Extract of *Linaria tingitana* Boiss. and Reut.

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ABSTRACT

The study was performed to assess the antioxidant properties of the n-butanol extract of the Algerian endemic specie Linaria tingitana Boiss. and Reut., using different tests (DPPH• radical, β-carotene-linoleic acid, ferric-reducing power and ferrous-chelating ability). The phytochemical investigation was based on total phenolic and flavonoids contents together with the HPLC analysis which led us to identify twelve constituents (gallic acid 1, chlorogenic acid 2, hydroxycafeic acid 3, caffeic acid 4, vanillic acid 5, syringic acid 6, rutin 7, luteolin 8, ferulic acid 9, coumaric acid 10, quercetin 11 and chrysin 12). Results showed that the n-BuOH extract exhibited an antioxidant activity as reflected by the DPPH• scavenging effect, ferric reducing power, and ferrous-chelating ability, (IC50: 122.26, 19.39 and 113.13 µg/mL, respectively) and β-carotene inhibition (59.358 %). In addition, the quantity of the compounds (total phenolic and flavonoids contents) confirmed the obtained data. These results indicate that L. tingitana can be used as functional products.

Keywords: *Linaria tingitana*; HPLC; Flavonoids; Phenolic acids; Antioxidant activity

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INTRODUCTION

The use of many *Linaria* species (Plantaginaceae), in folk medicine for the treatment of various diseases such as vascular problems, or as tonic, diuretic and antidiabetic [1], leads searchers to investigate their biological properties such as antidiabetic [2], anti-inflammatory [3], antioxidant [4] and antitumor [5]. These activities were linked to the chemical composition of the genus which is known for the presence of iridoids [6], flavonoids [2], terpenoids [7] and phenolic acids [8].

Therefore, in continuation of our research on *Linaria tingitana* [3,4,9,10], this study was conducted to investigate the chemical constituents of the *n*-BuOH extract of *L. tingitana* and its antioxidant properties by using 1,1-diphenyl-

2-picrylhydrazyl (DPPH$^{\bullet}$) scavenging effect, *β*-carotene inhibition, ferric-reducing power and ferrous-chelating ability.

MATERIALS AND METHODS

Chemicals and Reagents

1,1-diphenyl-2-picrylhydrazyl (DPPH$^{\bullet}$), potassium ferricyanide ($K_3Fe(CN)_6$), gallic acid, ethylenediamine tetra acetic acid (EDTA), *β*-carotene, linoleic acid, Tween 20, were purchased from Sigma Aldrich (Germany). Ferrozine, Folin-Ciocalteus's phenol reagent, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxilic acid (Trolox), L-ascorbic acid andtert-butyl-4-hydroxyanisole (BHA), Aluminium chloride ($AlCl_3$), ferrous chloride ($FeCl_2$), ferric chloride ($FeCl_3$) and sodium carbonate (Na_2CO_3), were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Plant Material

The aerial parts of *L. tingitana* Boiss. and Reut. were collected during the flowering phase in May 2010 from the national park of El-Kala, Algeria. The plant was authenticated by Dr. Djamel Sarri on the basis of Quezel and Santa [11]. A voucher specimen (No. 08/2009/CCN12) has been deposited in the Herbarium of the VARENBIOMOL unit research, University of Mentouri Brothers- Constantine, Algeria.

Preparation of Plant Extract

The dried aerial parts of *L. tingitana* (1285 g) were macerated using a hydroalcoholic solution containing MeOH/H_2O (80/20) at room temperature for 72 h four times. After filtration, the filtrate was concentrated and dissolved in water (514 mL). The resulting solution was extracted successively with petroleum ether (1×200 ml), $CHCl_3$ (3×200 mL), EtOAc (3 × 200 mL) and *n*-BuOH (11 × 200 mL). Combined solutions were concentrated under reduced pressure and dried (PE: 3 g, $CHCl_3$: 8 g, EtOAc: 7 g, *n*-BuOH: 61 g). In our investigation we are interested by the *n*-BuOH extract.

Preliminary Phytochemical Screening

The dried aerial parts of *L. tingitana* were subjected to preliminary phytochemical screening to identify the various active chemical constituents present in this spice such as alkaloids, coumarins, terpenoids, triterpenoids, unsaturated sterols, saturated sterols, anthocyanins, leucoanthocyanins, flavonoids, saponins and tannins according to standard phytochemical methods as described by Harborne [12] and Khandelwal [13].

Determination of Phytochemical Constituents

Determination of Total Phenolic Content (TPC)

The total phenolic content of the *L. tingitana* was measured using a modified colorimetric Folin-Ciocalteu method [14]. The results were expressed as Gallic acid equivalents (GAE)/g extract dry weight through the calibration curve of Gallic acid.

Determination of Total Flavonoids Content (TFC)

Aluminum chloride calorimetric method was used for flavonoid determination [15]. The absorbance was measured at 420 nm along with standard quercetin. Results were expressed as mg quercetin equivalents (QE)/g extract. The amount of flavonoids in the *L. tingitana* was calculated according to following formula:

$$X=(A\ .m_0)/(A_0.\ m)$$

Where X is the flavonoids content in mg/mg extract in QE. A_0 is the absorption of a standard quercetin solution, m is the weight of extract in mg and m_0 is the weight of quercetin in the solution in mg.

HPLC Profile of the n-BuOH

The HPLC profile was performed using a VP Shimadzu liquid chromatograph. The polyphenols were separated under the following conditions: Agilent Zorbax SB-C18-column (150 mm × 4.6 mm i.d.; 5 µm particle diameter, end-capped), the Photo Diode Array (PDA) detector was set at 254 nm, The mobile phase consisted of acetone/acetonitrile (60/40; V/V). Prior to use the solutions were degassed in ultrasonic bath and filtered through 0.45 µm membranes. Elution was carried out at 1 mL/min in isocratic conditions. The injection volume was 20 µL. All separations are performed at ambient temperature.

***In vitro* Antioxidant Assays**

Each sample was dissolved in 95% methanol to make a concentration of 1 mg/mL and then diluted to prepare lower concentrations for antioxidant assays. Reference chemicals (EDTA, Trolox, BHA) were used for comparison in all assays. For each sample of antioxidant activity experiments were carried out in triplicate.

DPPH$^{\bullet}$ Scavenging Activity

The effect of *L. tingitana* on DPPH$^{\bullet}$ was assayed using the method of Ohinishi et al. [16] with minor modifications. 1 mL of a methanolic solution of DPPH$^{\bullet}$ (0.2 mM) was added to 1 mL of different concentrations of *L. tingitana* and allowed to react in the dark at room temperature for 30 min. The absorbance of the mixture was measured at 517 nm. Trolox and BHA were used as references. Results were expressed as percentage of inhibition of the DPPH$^{\bullet}$ according to the following equation:

$$\% \text{ Inhibition} = [(\text{Abs C} - \text{Abs S})/\text{Abs C}] \times 100$$

Where Abs C was the absorbance of the control, and Abs S was the absorbance of the extract or standards.

***β*-carotene Bleaching Assay**

The antioxidant activity was also evaluated using the *β*-carotene-linoleic acid model system [17] with small modifications. First, *β*-carotene (0.5 mg) was prepared in 1 mL of chloroform, then 25 µL of linoleic acid and 200 mg of Tween 40 were added. After evaporation of chloroform, 100 mL of distilled water saturated with oxygen were added. An aliquot of this mixture was transferred into different test tubes containing the sample. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 490 nm. The emulsion system was allowed to stand at room temperature (20-23 °C) for 48 h. A blank control, devoid of *β*-carotene, was prepared for background subtraction. BHA and Trolox were used as standards. The antioxidant activity of the extract was evaluated in terms of bleaching of *β*-carotene using the following formula:

$$\% \text{ Inhibition} = [\text{Abs : 48 h (sample)}/\text{Abs : 48 h (standard)}] \times 100.$$

Ferric-Reducing Power Assay

The total reducing power of *L. tingitana* was determined according to the method of Oyaizu [18]. Various concentrations of extract were mixed with 1 mL of phosphate buffer (0.2 M, pH 6.6) and 1 ml of 1% $K_3Fe(CN)_6$. The mixture was incubated at 50 °C for 20 min and then 1 ml of 10% Trichloroacetic acid (TCA) was added. After centrifugation at 650 rpm for 10 min, 1.5 mL of upper layer was mixed with 1.5 mL distilled water and 0.3 mL of

$FeCl_3$ (0.1%) and measured at 700 nm. Higher absorbance of reaction mixture indicated greater reducing power. Trolox and BHA were used as references.

Ferrous-Chelating Ability

The method of Dinis et al [19] was used for the assay. Briefly, 50 µL of $FeCl_2$ (2 mM) was added to extract (1 mL). The reaction was initiated by the addition of 0.2 mL of ferrozine solution (5 mM). The mixture was vigorously shaken and left to stand at room temperature for 10 min. The absorbance was measured at 562 nm. EDTA was used as a positif control and the chelating capacity was calculated as following:

$$(\%)\ \text{Chelating}=[(\text{Abs C}-\text{Abs S})/\text{Abs C}]\times 100$$

Statistical Analysis

Experiment data were carried out in triplicates and results expressed as means ± SD. IC_{50}-value (µg extract/mL), was calculated for each assay. The level of significance between different groups was evaluated by the Student's t-test and values were considered to be highly significant at $P < 0.01$ and significant at $P < 0.05$.

RESULTS AND DISCUSSION

Plantaginaceae family has a wide variety of natural compounds with pharmacological properties [3]. The Algerian endemic specie *Linaria tingitana* Boiss. & Reut. (Plantaginaceae) is one of the known genera of this botanic family largely distributed in Mediterranean basin and Eastern Asia [1].

Preliminary Phytochemical Screening

The phytochemical screening results of the aerial parts of *L. tingitana* showed the high presence of alkaloids and sterols, good presence was detected for terpenoids, flavonoids and saponins with a traces of leucoanthocyanins and tannins, while no coumarins, triterpenoids, saturated sterols and anthocyanins could be observed (Table 1).

Table 1: Chemical composition of *L. tingitana* (+ indicates Presence; - indicatesAbsence)

Chemical groups	Aerial parts
Alkaloids	+++
Coumarins	-
Terpenoids	++
Unsaturated sterols	+++
Triterpenoids	-
Saturated sterols	-
Anthocyanins	-
Leucoanthocyanins	+
Flavonoids	++
Saponins	++
Tannins	+

Phytochemical Constituents, TPC and TFC

The phytochemical constituent analysis carried out on *L. tingitana* revealed the percentage composition of total polyphenol and flavonoids. TPC and TFC were found to be 113.98 ± 2.56 mg GAE/g extract and 52.41 ± 1.63 mg QE/g extract, respectively.

Identified Compounds from the HPLC Profile

The analysis of HPLC profile obtained from the *L. tingitana n*-BuOH extract led to the detection of many compounds in which eight phenolic acids: gallic acid 1, chlorogenic acid 2, hydroxycafeic acid 3, caffeic acid 4, vanillic acid 5, syringic acid 6, ferulic acid 9 and coumaric acid 10 together with four flavonoids: rutin 7, luteolin 8, quercetin 11 and chrysin 12 were identified. All these compounds are new for the studied plant while compounds 3, 6 and 7 are new for the genus.

Antioxidant Activity

DPPH• scavenging effect

The effect of antioxidants on DPPH• is thought to be due to their hydrogen donating ability. The UV absorbance decreases indicates the amount of DPPH• scavenged which signifies the scavenging activity of natural products [18]. As shown in Figure 1, *L. tingitana* showed a gradual increase in DPPH• scavenging activity in a concentration-dependent manner. At 700 µg/mL, the DPPH• scavenging activity of *L. tingitana* was found to be 85.17 ± 0.89%. The maximum inhibition value was observed at 1600 µg/mL (88.82 ± 0.48%). The IC_{50}-value of *L. tingitana was* (122.26 ± 8.55 µg/mL), lower than those of standard references, BHA (11.44 ± 0.32 µg/mL) and Trolox (4.60 ± 0.08 µg/mL) (Table 2). These results showed that the extract has the proton-donating ability and could serve as free radical inhibitor or scavenger, acting possibly as primary antioxidants.

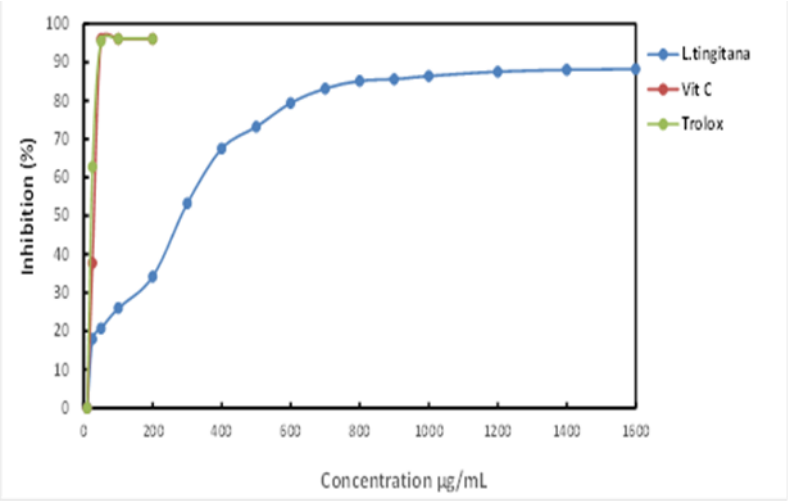

Figure 1. DPPH• scavenging activity of *L. tingitana n*-BuOH extract and standards. Values are means ± SD (n=3) P < 0.05.

Table 2: The IC_{50}-values of antioxidant activities of *L. tingitana* (Each value represents a means ± SD (n=3))

Extract and standards references	IC50 (µg/mL)		
	DPPH°	Reducing power	Iron-chelating
BELT	122.26 ± 8.55	19.39 ± 5.72	113.13 ± 1.19
Trolox	4.60 ± 0.08	1.79 ± 0.84	/
BHA	/	1.91 ± 0.78	/
Vit C	11.44 ± 0.32		
EDTA	/	/	14.66 ± 0.15

***β*-carotene bleaching effect**

The oxidation of linoleic acid produces hydroperoxide derived free radicals which attack the chromophore of *β*-carotene resulting in bleaching of the reaction emulsion and the antioxidant activity was expressed as percent inhibition relative to the control [16]. It can be observed from (Figure 2A) that the absorbance decrease rapidly in blank control from 1.14 ± 0.01 to 0.05 ± 0.01, while the decrease was slower for *L. tingitana* from 1.14 ± 0.008 to 0.68 ± 0.002. At 100 µg/mL, *L. tingitana* was able to inhibit *β*-carotene oxidation by 59.36 ± 0.41%, while the positive controls BHA and Trolox inhibited 87.72 ± 0.20% for 87.57 ± 0.26%, respectively (Figure 2B). These results implied that the potential antioxidant capabilities in *L. tingitana* were attributed to the phenolic compounds in this plant species.

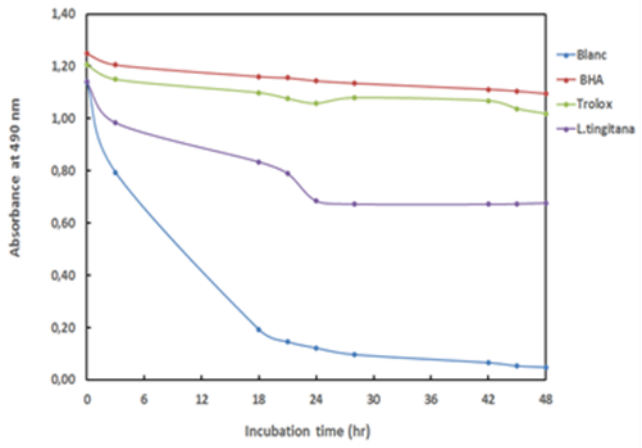

Figure 2A. β-carotene bleaching assay: Changes of absorbance at 490 nm with time in β-carotene-linoleic acid emulsions added with *L. tingitana n*-BuOH extract and standards

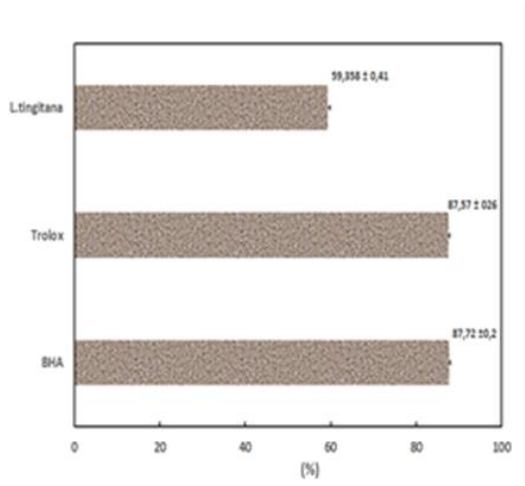

Figure 2B. β-carotene bleaching assay: Antioxidant activity of *L. tingitana* analyzed by β-carotene bleaching method. Values are means ± SD (n=3) P<0.05

Reducing power

In presence of reductants, the Fe^{3+}/ferricynide complex reduced to the ferrous forms which have been exhibiting antioxidative potential by breaking the free radical chain and donating a hydrogen atom [21]. The yellow color of

the test solution changes to green depending on the reducing power of the test specimen and Fe^{2+} can be monitored by absorbance measurement at 700 nm. Increasing absorbance at 700 nm indicates an increase in reducing ability. Figure (3) shows that *L. tingitana* is able to reduce the Fe^{3+}/ferricynide as evidenced by the concentration dependent elevation of the absorbance value. However, *L. tingitana* was less effective (0.39 ± 0.008) than the reference compounds at 300 µg/mL as compared with the values obtained with BHA (1.86 ± 0.05) and Trolox (1.90 ± 0.01) at the same concentration. For reaching its maximal effect (1.35 ± 0.01 of OD value), *L. tingitana* needed 1200 µg/mL. Regarding to IC_{50}-values the reducing capacity followed the order of Trolox (1.79 ± 0.84 µg/mL), BHA (1.91 ± 0.78 µg/mL) and *L. tingitana* (19.39 ± 5.72 µg/mL) (Table 2). The antioxidants present in *L. tingitana n*-BuOH extract caused this reduction and thus proved the reducing power.

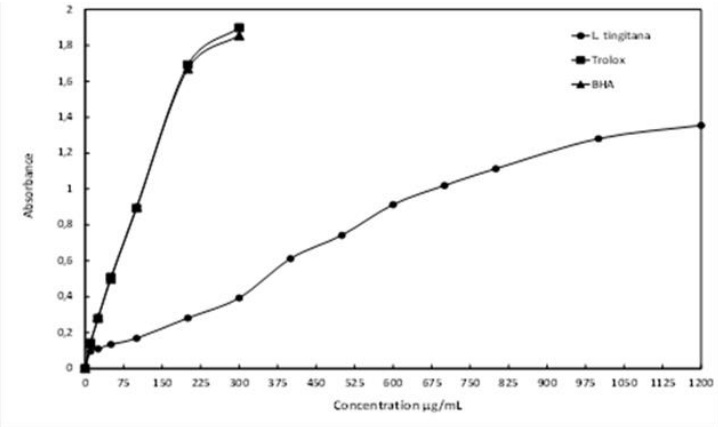

Figure 3. Reducing power of *L. tingitana n*-BuOH extract and standards. Values are means ± SD (n=3) P<0.05

Ferrous-chelating ability

The iron-chelating capacity assay measures the ability of antioxidants to compete with ferrozine in chelating ferrous ion [22]. As it can be deduced from Figure 4, *L. tingitana* displayed a ferrous ion chelating activity (65.8 ± 0.9%) at a concentration 200 µg/mL. At the same concentration the positive control EDTA exhibited a maximal effect 96.98 ± 1.8%. The maximal effect of *L. tingitana* (80 ± 0.14%) was obtained at 600 µg/mL. The IC_{50}-values of *L. tingitana* was found to be 113.13 ± 1.19 µg/mL as compared to EDTA 14.66 ± 0.15 µg/mL (Table 2).

According to recent reports, these data are in agreement with previous studies indicating the presence of some secondary metabolites classes in this specie, especially iridoids, diterpenoids, flavonoids, alkaloids and phenylethanoids with a wide variety of biological activities [1,9,10]. Many of these phytochemicals possess significant antioxidant capacities and it is likely that the activity of the extract is due to these compounds. These antioxidants present in *L. tingitana n*-BuOH extract defined and indicated by the phytochemical screening and HPLC caused the reduction and thus proved the reducing power.

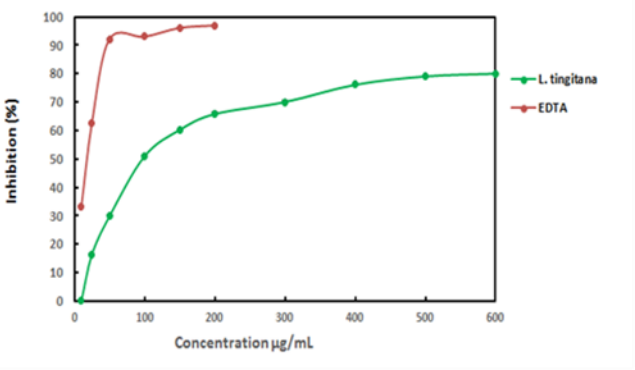

Figure 4. Ferrous ion chelating ability of *L. tingitana n*-BuOH extract and standards. Values are means ± SD (n=3) P<0.05

CONCLUSION

In conclusion, our data showed the capability of the *n*-BuOH extract of *L. tingitana* to scavenge different free radicals in different systems also be to the presence of wide range of defined constituents such as terpenoids, tannin and polyphenoles as indicated by the present phytochemical screening results that may exert a synergistic effect, which indicat that they may be useful therapeutic agents for treating radical-related pathological damage.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgment

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