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Comparison of phenolic content and antioxidant activity of methanolic and ethanolic extracts of *Limoniastrum guyonianum*

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Abstract

Limoniastrum guyonianum Durieu ex Boiss. (Plumbaginaceae) popularly known as "Zeta" is mainly mentioned to cure constipation, diabetes, leprosy, hypertension and other ailments. Its efficacy is widely acclaimed among communities in Southeastern Algeria. The present study is designed to determine the phenolic composition and the antioxidant activities of hydro alcoholic extracts of this raw material. The phenolic extracts of the dried and powdered aerial parts of *Limoniastrum guyonianum* were obtained by conventional maceration using 70% methanol and ethanol in water as solvent. The hydro alcoholic extracts were evaluated for their phenolic contents via colorimetric methods and were investigated for their antioxidant capacities using phosphomolybdate, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) tests. The highest amounts of total phenolic (121.51 mg GAE/g DW), flavonoids (8.65 mg RE/g DW), condensed tannins (44.57 mg EPE/g DW) and overall antioxidant activity (55.57 mg AAE/g DW) were found in the methanolic extracts. The IC50 values of methanolic and ethanolic extracts for DPPH radical scavenging activity were 31.10 and 23.25 µg/ml, respectively. As for ABTS (successively, 22.71 and 21.25 µg/ml), the top 26.97 and 08.60 % scavenging activity were observed in plant extracts. The trend observed for bioactive compounds was confirmed by antioxidant activity assays and high correlation values were obtained with all studied antioxidant compounds. Collectively, the findings suggested that *Limoniastrum guyonianum* is a natural source of antioxidant substances, which may be useful for curing diseases arising from oxidative deterioration.

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Introduction

Antioxidants refers to a group of compounds that when present in low concentration compared to those of the oxidants, prevent or delay oxidative processes by inhibiting the initiation or propagation of oxidising chain reactions caused by free radicals (Cui *et al.*, 2004 ; Azlim Almey *et al.*, 2010). A number of synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tert-butylhydroquinone (TBHQ) and propyl gallate (PG) have been extensively added to food products as a preservative to prevent lipid peroxidation. However, their use has begun to be restricted because of their carcinogenic effects (Canadanovic-Brunet *et al.*, 2006). Therefore, in recent years, extensive attention has been directed towards the identification of natural antioxidants from various plant sources (El-Hela and Abudullah, 2010). Among these phytochemical antioxidants, polyphenols are widely involved in these activities. Their antioxidant activity is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, singlet oxygen quenchers and metal chelators (Rice-Evans *et al.*, 1995).

The Plumbaginaceae is a cosmopolitan family, which comprises around 836 species classified into 27 genera. Only two of them, *Limonium* and *Limoniastrum*, are widespread in the Sahara. *Limoniastrum guyonianum* Durieu ex Boiss. is an endemic from North Africa (Ozenda, 2004). This endemic species are used in folkloric medicine of the Northeast of Algerian Sahara for the treatment of several ailments, including digestive, skin, circulatory, metabolic, liver and respiratory disorders (Hadjadj *et al.*, 2015). Its leaves, branches and galls decoctions are used to treat dysentery (Chehma and Djebar, 2008). In addition, it is employed for a hypoglycaemic activity, even if it was never studied from this point of view (Telli *et al.*, 2016). Owing to it frequent use in traditional medicine, we investigated the antioxidant capacity of *L. guyonianum*. The focus of this paper was to quantify the total phenolic, flavonoid and condensed tannin contents of methanolic and ethanolic extracts of *L. guyonianum* aerial parts and to investigate whether these compounds have an antioxidant activity toward free radical propagation in order to evaluate the interest of this plant.

Material and methods

Chemicals and reagents

Folin-Ciocalteu's reagent, gallic acid, sodium carbonate, ascorbic acid, sulfiric acide (H_2SO_4), ethanol, methanol, aluminum chloride ($AlCl_3$) and potassium persulfate ($K_2S_2O_8$) were purchased from Biochem Chemophorma (ZA Cosne sur loire, France). Hydrochloric acid (HCl) was obtained from (Prolabo-France). Rutin, ammonium molybdate ($(NH_4)_2MoO_4$), sodium phosphate (Na_3PO_4), 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH) were acquired from Sigma-Aldrich Co. (St. Louis, MO, USA). Ammonium iron (III) sulfate dodecahydrate ($NH_4Fe(SO_4)_2$ 12 H_2O) and butanol were obtained from VWR Chemicals (BDH Prolabo).

Plant material

The aerial part of *Limoniastrum guyonianum* (Plumbaginaceae) was collected in April 2016 from the region of Meggarine (Wilaya of Ouargla) (33° 12' 30.9" N, 006° 07' 59.6" E) Altitude 299 m, in southeastern of Algeria. The plant was identified using adapted flora of the Sahara (Ozenda, 1983) and by comparison with the Herbarium specimens available at the Herbarium of the Institute at the University Kasdi Merbah, Ouargla (Algeria). The fresh aerial part was air dried in the dark, at room temperature for two weeks, then crushed into a mortar and stored until further use.

Preparation of phenolic extracts

The dried powder of plant material (100 g) was extracted by maceration with 500 ml of 70% methanol and ethanol at room temperature for 24 h and filtered through filter paper (Wattman n°1). The residue on the filter paper was macerated again twice using the same volume of alcoholic solvents (2× 500 ml). The filtrates were combined and concentrated in a rotavapor under vacuum at 40°C until dryness. The dry residue was re-dissolved in methanol for further analysis in a ratio of 1 mg/ml.

Determination of extract yield

The yield of evaporated dried extracts based on dry weight basis was calculated from the following formula :

$$\%\ \text{discolouration} = [1 - (\text{Abs sample}/\text{Abs control})] \times 100. \quad \text{(Eq. 1)}$$

Where W1 was the weight of the extract after the solvent evaporation and W2 was the weight of the dry plant material.

Total phenolic content

The amount of total phenolic was determined with the Folin-Ciocalteu method (Lister and Wilson, 2001). 100 µl of extracts were combined with 500 µl of the Folin-Ciocalteu reagent (1/10 dilution) and 1000 µl of distilled water. After incubation 1 min at room temperature, 1500 µl of a 20% sodium carbonate (Na_2CO_3) solution were added. After incubation 2 h in the dark at room temperature, the absorbance was measured at 765 nm using a UV mini- 1240 UV–Vis spectrophotometer SHIMADZU and total phenolic content was determined on the basis of a standard calibration curve of gallic acid. The results were expressed as mg gallic acid equivalent per gram of dry weight of plant (mg GAE/g DW).

Total flavonoid content

The total flavonoid content of extracts was assessed spectrophotometrically using the aluminum chloride method as reported by Quettier-Deleu *et al.* (2000). 1 ml of extract was mixed with 1 ml of 2% aluminum chloride methanolic solution. After incubation at room temperature 10 min, the absorbance was measured with a UV mini- 1240 UV–Vis spectrophotometer SHIMADZU. The flavonoid content was quantified on the basis of a standard calibration curve of rutin. The results were expressed as mg rutin equivalent per gram of dry weight of plant (mg RE/g DW).

Total condensed tannin content

The quantitative condensed tannins or proanthocyanidin in plant extracts was assessed following the method developed by Porter *et al.* (1986).

100 µl of extracts were added to 2.5 ml of 95/5 (v/v) *n*-butanol-HCl. Then, 100 µl of 2% ferric ammonium sulfate ($NH_4Fe(SO_4)_2 12\ H_2O$) in 2M HCl were added. The tubes capped with a glass marble were shaken by a vortex mixer and heated to 95 °C in a water bath for 40 min. After cooling the tubes, the absorbance was recorded at 540 nm using a UV mini- 1240 UV–Vis spectrophotometer SHIMADZU and condensed tannin content was determined on the basis of a standard calibration curve of epicatechin. The results were expressed as mg epicatechin equivalent per gram of dry weight of plant (mg EPE/g DW).

Total antioxidant capacity

The total antioxidant capacity was evaluated by the phosphomolybdate test (PM) according to the procedure described by Prieto *et al.* (1999). 300 µl of hydroalcoholic extracts were added to the test tube containing 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4mM ammonium molybdate). The tubes were incubated at 95 °C for 90 min. After incubation, these tubes were normalized to room temperature for 20-30 min and the absorbance of the solution was measured at 695 nm using a UV mini- 1240 UV–Vis spectrophotometer SHIMADZU and total antioxidant activity was determined on the basis of a standard calibration curve of ascorbic acid. The results were expressed as mg ascorbic acid equivalent per gram of dry weight of plant (mg AAE/g DW).

DPPH radical scavenging activity

The antioxidant scavenging activity of sample extracts was measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical as described by Brand-Williams *et al.* (1995). The extracts were previously dissolved at different concentrations (5 to 100µg/ml) in methanol. 100 µl of antioxidant solution (sample or control) were added into the test tube individually containing 2900 µl of DPPH° methanolic solution (60 µM). The mixture was vigorously stirred and incubated 30 min at room temperature (25°C) in obscurity. The absorbance of the resulting solution was measured at 515 nm using a UV mini- 1240 UV–Vis spectrophotometer SHIMADZU.

The percent of DPPH discolouration of the extracts was calculated according to the equation:

$$\%\,\text{discolouration} = [1 - (\text{Abs}_{\text{sample}}/\text{Abs}_{\text{control}})] \times 100. \quad \text{(Eq. 2)}$$

Where $\text{Abs}_{\text{sample}}$ and $\text{Abs}_{\text{control}}$ are the absorbance of the test sample and the blank control (containing all reagents except the extract solution), successively.

The IC5 values are widely used parameter to measure the free radical scavenging activity and is defined as the concentration of substrate necessary to reduce by 50% the initial quantity of DPPH. A lower IC50 value indicates a higher antioxidant activity (Maisuthisakul *et al.*, 2007). The IC50 values were calculated from the regression equation for the concentration of extract and percentage inhibition. Trolox was used as a positive control.

ABTS radical scavenging activity

The radical scavenging capacity for 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical cation was performed according to the method employed by Re *et al.* (1999). The stock solutions included 7 mM ABTS and 2.45 mM potassium persulfate. The ABTS radical cation ($ABTS^{\bullet+}$) was generated using 5 ml of the ABTS stock solution and 88 µl of the potassium persulfate, in the dark, at room temperature for 12-16 h before use. The solution was then diluted in order to obtain an absorbance of 0.7± 0.02 at 734 nm. Fresh diluted $ABTS^{\bullet+}$ solution was prepared every day. 100 µl of antioxidant solution (sample or control) were mixed with 2900 µl of diluted $ABTS^{\bullet+}$ solution and incubated for 5 min in a dark condition. The absorbance of the resulting solution was measured at 734 nm using a UV mini- 1240 UV–Vis spectrophotometer SHIMADZU.

The percentage of inhibition of $ABTS^{\bullet+}$ was calculated using above formula (Eq. 2). The capacity of free radical scavenging was expressed by IC50 values calculated denote the concentration required to scavenge 50% of ABTS radicals. The IC50 values were determined using the same previously described equation for the DPPH method. Trolox was used as a positive control.

Statistical analysis

All analyses were conducted in triplicate and the results were expressed as means ± standard deviation (SD). The differences were tested for statistical significance with one way ANOVA with Tukey's test, assuming the differences to be statistically significant at $p < 0.05$. The correlation analysis of antioxidant activity versus the phenolic components content and their statistical significance were carried out using the Bravais-Pearson correlation test. All statistical analyses were performed using the statistical XLSTAT software.

Results and discussion

Extraction yield

Among the several parameters that can influence extract yields and the contents of phenolic compounds from the plant materials, solvent nature is known as the most controversial one (Peschel *et al.*, 2006). In this work, fifteen percent increase in recovery yields was obtained by extraction with 70% methanol (17.06 g/ 100 g of dry plant material) as compared to 70% ethanol (14.80 g/ 100 g of dry plant material), revealing the greater efficacy of this solvent to extract maximum amounts of components of interest (Table 1).

Table 1. Extraction yield, total phenolic, flavonoid and condensed tannin contents of methanolic and ethanolic extracts of *L. guyonianum* aerial parts.

Solvent	Extract yields (g/100 g of dry plant material)	Total phenolic (mg GAE/ g DW)	Flavonoids (mg RE/ g DW)	Condensed tannins (mg EPE/ g DW)
Methanol	17.06	121.51± 4.51[a]	8.65± 0.11[a]	44.57± 0.93[a]
Ethanol	14.80	98.62± 0.30[b]	7.74± 0.19[b]	27.91± 1.70[b]

Means of three replicates ± SD (standard deviation) followed by at least one same letter are not significantly different according to Tukey's test at $p < 0.05$.

Our results are in close agreement with that reported by Naqvi *et al.* (2013), they found a maximum amount of extract yields was obtained using 70 % methanol as the extraction solvent and corresponded to 10.3 g/100 g dried plant sample.

Total phenolic, flavonoid and condensed tannin contents

Plant phenolics are the widest spread secondary metabolite in the plant kingdom. These compounds have attracted considerable attention because of their broad spectrum of biological functions such as antibacterial, anti-inflammatory, antiallergic, hepatoprotective, antithrombotic, antiviral, anticarcinogenic and cardioprotective vectors (Wong *et al.*, 2006).

Table 2. Effects of *L. guyonianum* extracts in different antioxidant assays.

Solvent	PM (mg AAE/g DW)	DPPH (IC50 µg/ml)	ABTS (IC50 µg/ml)
Methanol	55.57 ±5.99a	31.10± 0.25a	22.71± 0.28a
Ethanol	45.59 ±3.04a	23.25± 0.62b	21.25± 0.42b
Trolox		4.13± 0.03	4.34± 0.08

Means of three replicates ± SD (standard deviation) followed by at least one same letter are not significantly different according to Tukey's test at $p < 0.05$.

Because of this, it is worthwhile to evaluate the total amount of phenolic content in the plant chosen for the study. Results from the quantitative analysis of total phenolic, flavonoids and condensed tannins of methanolic and ethanolic extracts of *L. guyonianum* aerial parts are summarized in Table 1. The level of total phenolic in the methanolic extracts was significantly higher (121.51± 4.51 mg GAE/g DW, p= 0.026) than obtained using ethanol (98.62± 0.30 mg GAE/g DW) and similar trends in concentration was observed while estimating total flavonoids and total condensed tannins, where their amounts in the methanolic extracts represent 7.9 and 36.7 % of the total phenolic content versus 7.1 and 28.3 % in the ethanolic extracts, respectively. It is clear from these results that the aerial parts of *L. guyonianum* within the same extracting solvent, showed more than four-fold higher flavonoids contents compared to tannins ones. Based on the results presented here, we conclude that the tannins components are the dominating phenolic group present in tested extracts.

Table 3. Correlation coefficient (*R values*) between different antioxidant assays and phenolic compounds content.

Phenolic components	PM		DPPH		ABTS	
	R	*p*	R	*p*	R	*p*
Total phenolic	0.85	0.009	0.80	0.016	0.89	0.004
Flavonoids	0.86	0.008	0.97	0.000	0.99	<0.0001
Condensed tannins	0.72	0.034	0.99	<0.0001	0.92	0.003

The differences in the level of phenolic compounds of *L. guyonianum* between extracting solvents might be attributed to the varying nature of the components present and the polarities of the solvent used for textraction process (Sarwar *et al.,* 2012). The solubility of polyphenols was observed to depend mainly on the extraction medium polarity, the presence and position of hydroxyl groups and the molecular size and the length of constituent hydrocarbon chains (Iloki-Assanga *et al.*, 2015*)*. Our results agreed with that reported by Naqvi *et al.* (2013), where methanol solvent was most effective in

extracting phenolic components from *Sewertia chirata*. In that study, the content of phenolic and flavonoids components extracted with methanol was respectively, 27.2±2.84 and 3.38±0.23 g/100g, which were about 30.51 and 6.21% higher than that extracted by ethanol. Hamdoon *et al.* (2013) assayed sequential extraction starting with chloroform and followed by solvents of increasing polarity on *L. guyonianum* and they showed that ethanol had the highest phenolic content as compared to ethyl acetate and chloroform. In fact, the contents of both total phenolic and total flavonoids extracted by ethanol were respectively superior by (88.9 and 98.2%) and (81.7 and 93.7%) than that extracted with ethyl acetate and chloroform. Also, in the previous study conducted by Trabelsi *et al.* (2010), the total condensed tannin content determined by the vanillin assay, reported the presence of this compound in the 80% hydromethanolic and hydroethanolic extracts of *L. monopetalum* leaves, at an amount of 2.4 and 1.48 mg catechin equivalent/g DW, respectively. corroborating thus, the data obtained in the present work.

Total antioxidant activity

The total antioxidant activity of the extracts was determined by the PM test (Table 2). The method is based on the reduction of molybdenum (VI) to molybdenum (V) by the antioxidant compounds and subsequent formation of a green phosphate/ Mo (V) complex at acid pH, which can be evaluated spectrophotometrically at 765 nm (Prieto *et al.*, 1999). The results of total antioxidant activity showed an analogous pattern as concluded with total phenol, flavonoid and condensed tannin contents. The total antioxidant activity of methanolic extracts was higher than those of ethanolic extracts for 17.96% (without significant differences among them, P=0.062).

The differences in antioxidant potencies among the both solvents used in this experiment may be explained by the difference in solvent selectivity for extracting certain phenolic groups. In fact, the total phenolics content does not incorporate all the antioxidants. In addition, the synergistic between the antioxidants in the mixture makes the antioxidant activity not only dependant on the concentration, but also on the structure and the interaction between the antioxidants (Djeridane *et al.*, 2006). Therefore, the global antioxidant property of a plant extract is generally considered as the result of the combined activity of a wide range of compounds (Gallardo *et al.*, 2006). Different from our results, Trabelsi *et al.* (2013), studied two Tunisian *Limoniastrum* species. The PM test showed significantly higher levels of total antioxidant activity from the acetonic extracts of aerial parts, 106.6 mg GAE/g DW for *L. guyonianum* and 70.76 mg GAE/g DW for *L. monopetalum*. Our results on *L. guyonianum* were 55.57 and 45.59 mg AAE/g DW in methanlic and ethanolic extracts, successively.

DPPH radical scavenging activity

The DPPH assay is a simple, rapid and sensitive method for free radical-scavenging assessments. DPPH is a stable free radical with an absorption band at 515 nm. It loses this absorption when accepting an electron or hydrogen radical, which results in a visually noticeable discoloration from the violet color to yellow (Hseu *et al.*, 2008). The antioxidant property of the plant extracts was determined by the decrease in absorbance power of DPPH induced by plant antioxidants (Table 2). According to the IC50 values, the ethanolic extracts showed a highly significant capacity to neutralize this radical when compared to methanolic extracts IC50 equal to 23.25± 0.62 and 31.10± 0.25 µg/ml, successively (p<0.0001). However, these scavenging activities dropped by 82.24 and 80.72%, respectively, when compared with the positive control (IC50 = 4.13± 0.03 µg/ml).

ABTS radical scavenging activity

The ABTS radical scavenging assay is based on the measurement of the reduction in the blue green ABTS•+ chromophore, which can be reduced by an antioxidant thereby resulting in a loss of absorbance at 734 nm and the discoloration of the ABTS (Rubalya Valantina and Neelamegam, 2015).

The ABTS assay is particularly interesting in plant extracts because the wavelength absorption at 734 nm eliminates color interference (Li *et al.*, 2008). Results from Table 2 demonstrated that the ethanolic extracts from *L. guyonianum* exhibited a *slightly lower* significantly *IC50 values* than methanolic extracts (respectively, 21.25± 0.42 and 22.71± 0.28 µg/ml, p= 0.008), reflected their better capacity on scavenge the ABTS•+ radical. However, these activities were approximately five-fold least relative to those of ascorbic acid (IC50= 4.34± 0.08 µg/ml).

It is interesting to highlight that the IC50 values estimated by DPPH test for methanolic and ethanolic extracts were notably higher as compared to those obtained by ABTS test. In general, 26.97 and 08.60 % had ABTS scavenging activity higher than those DPPH, successively. This is in line with previous published data of Samaniego Sanchez *et al.* (2007) showed that the best method for determining the antioxidant capacity of olive oil is ABTS, giving high-quality reproducibility and acceptable correlation coefficients. The difference in radical scavenging ability between the both systems could be attributed to the poor selectivity of ABTS to hydrogen donors, as it reacts with hydroxylated compounds independently of their antioxidant potential (Roginsky and Lissi, 2005). In contrast, DPPH is more selective as it does not react with flavonoids, which contain no hydroxyl groups in the B ring (Yokozawa *et al.*, 1998) as well as with aromatic acids containing only one hydroxyl group (Von Gadov *et al.*, 1997).

Relationships among the estimates of antioxidant capacities with PM, DPPH and ABTS assays and phenolic contents

Investigations of relationships between phenolic compounds and antioxidant capacity, give clear evidence for the effects of these constituents as antioxidants. In fact, the total phenolic, the flavonoid and the condensed tannin contents, all correlate positively to the antioxidant activity, ether it was evaluated by PM, DPPH or ABTS tests (Table 3).

The highly significant correlation coefficients were respectively ($0.72 \leq R \leq 0.86$), ($0.80 \leq R \leq 0.99$) and ($0.89 \leq R \leq 0.99$). Thus, these data suggest that phenolic components present in these extracts are the major contributors to the antioxidant capacity of this plant. Indeed, phenolic compounds comprise one or more aromatic rings, bearing one or more hydroxyl substituants, an ideal structure for quench free radicals by forming resonance-stabilized phenoxyl radicals, which makes it an important antioxidant agent (Sakihama *et al.*, 2002). The same correlation is established by Chaabi *et al.* (2008) by comparing total phenolic content and DPPH scavenging activity of *L. feei.*

Conclusion

In general, it is found that the extractability of the antioxidant phenolic compounds from *L. guyonianum* is significantly affected by type of extracting solvent. The hydromethanolic extracts showed the highest total phenolic, flavonoid and condensed tannin contents with consequent the most effective in the reducing molybdenum and in scavenging of free ABTS radical. But, the ethanolic ones proved the highest DPPH scavenging activity. In the light of these results, we can conclude that antioxidant activity not only dependant on the phenolic contents, but also on the phenolic compositions and the oxidant systems.

L. guyonianum is a natural source rich in bioactive substances and powerful antioxidant activities, which may be useful for curing diseases arising from oxidative deterioration.

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