Chemical composition, antioxidant and antimicrobial effects of Tunisian *Limoniastrum guyonianum* Durieu ex Boiss extracts

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Abstract: In the present investigation, extracts obtained from *L. guyonianum* Durieu ex Boiss. aerial parts were used to evaluate total phenolic, flavonoid and tannin contents. A study of antioxidant activities of the prepared samples was carried out on the basis of 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2-2'-azino-bis-(3-ethylbenz-thiazoline-6-sulfonic acid) ($ABTS^{+\cdot}$) and ferric reducing antioxidant power (FRAP) assays. Moreover, the efficiency of methanolic, chloroformic and petroleum ether extracts and the deriving fractions from the methanolic extract was tested against human bacterial and fungal pathogenic strains using micro dilution method in 96 multiwell microtiter plate. Furthermore, leaves and stems extracts were subjected to RP-HPLC for phenolic compounds identification. Results showed that polyphenolic contents and antioxidant activities varied considerably as function of solvent polarity. Moreover, antiradical capacities against DPPH, $ABTS^{+\cdot}$ and reducing power were maxima in methanol aerial parts extract which showed the highest polyphenol contents (134mg CE/g DW). The antimicrobial activities showed that methanolic, chloroformic and petroleum ether extracts were found to be most potent against *Pseudomonas aeruginosa* and *Staphylococcus aureus* with MIC values of 23 and 46µg.mL^{-1}, respectively. The fractions F_{13} and F_{16} have a great antifungal potential against *Candida glabrata*, *Candida krusei* and *Candida parapsilesis* (MIC=39µg.mL^{-1}). The RP-HPLC analysis lead the identification of gallic, procatechuic and trans-cinnamic acids, methyl-4-hydroxybenzoate, n-propyl-3,4,5-trihydroxybenzoate, epicatechin, naringin and myricetin in *L. guyonianum* Durieu ex Boiss. leaves and stems extracts.

Keywords: *Limoniastrum guyonianum* Durieu ex Boiss., extracts, phytochemical contents, antioxidant activities, antibacterial and antifungal activities.

INTRODUCTION

Extracts from medicinal plants are gaining increasing interest as safe sources of natural biological active products, having antibacterial (Nasir *et al.*, 2015; Ben Abdelkader *et al.*, 2010; Liouane *et al*., 2010), antifungal (Bakht *et al*., 2014; Bakht *et al*., 2015; Zardi-Bergaoui *et al*., 2010) and antioxidant properties (Saidana *et al*., 2014; Hammami *et al*., 2015; Ullah *et al.*, 2015).

Plumbaginaceae species are signaled in traditional medicine as an important part of health care for humans. *Limoniastrum monopetalum* (L.) Boiss. is an example of a medicinal plant belonging to Plumbagin aceae family cited for its anti-dysenteric effects against infectious diseases and parasites that cause painful and bloody diarrhea (Chaieb and Boukhris, 1998). *Limoniastrum feei* (Girard) Batt is used in Algeria as antibacterial for treatment of bronchitis and stomach infection (Cheriti, 2000).

L. guyoninum, *L. monopetalum* and *L. feei* are halophyte species tolerating hypersalin ecosystem. Halophyte plants are interesting species, known by their richness on polyphenols and other bioactive substances. Many studies demonstrated their wealth in phenolic components and bioactive substances known for their anti-trombotic, cardio protective and vasodilatrice effects (Menzel and Lieth, 1999; Balasundram *et al*., 2006; Siddhuraju, 2007).

Limoniastrum guyonianum Durieu ex Boiss. subject of the present study is widely used by local people to cure dysentery (Chaieb and Boukhris, 1998). Its root decoction is considerably known for applications as depurative (El Rhaffari, 1999, V. Fintelmann, 2004).

During this study, along with the phytochemical investigation, phenolic content and antioxidant properties of *L. guyonianum* halophyte, the antimicrobial activities of crude extracts from the aerial parts and different fractions deriving from methanol chromatographic separations, were investigated by the micro-dilution method (NCCLS, 2000) against some pathogenic bacteria and fungi.

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MATERIALS AND METHODS

Plant material

L. guyonianum was collected from Skanès region (Gouvernorat of Monastir, Tunisia) in October 2008 and the different organs were dried at room temperature.

Preparation of plant extracts

3kg of *L. guyonianum* aerial parts (stems and leaves) were air dried, then extracted by stirring with absolute methanol at room temperature. After filtration, the solvent was evaporated under vacuum to dryness giving 295g of methanolic extract, which was dissolved in distilled water. The aqueous solution obtained was successively extracted with petroleum ether then chloroform giving two extracts less complex than methanolic extract.

Chromatographic simplification of methanolic extract

Seven grams of the methanolic extract were simplified by chromatographic studies over silica gel column eluted with (chloroform/methanol) mixtures. 47 fractions of 200mL were collected then grouped in 21 groups having different compositions based on Thin Layer Chromatographic (TLC) analysis.

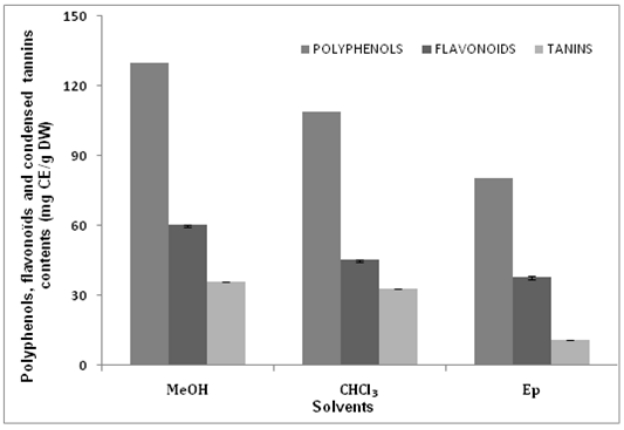

Fig. 1: Polyphenols, flavonoids and condensed tannins contents in different aerial parts extracts of *L. guyonianum* Data are given as mean of three replicates ± SD MeOH: Methanol; $CHCl_3$: Chloroform; Ep: Petroleum Ether

Phytochemical composition

Determination of total polyphenols contents

Total polyphenols were determined using the Folin-Ciocalteu procedure as described by Julkunen-Titto (1985). 50µl of extract solution were mixed with 1ml of distilled water, 0.5ml of Folin-Ciocalteu reagent (F-C) and 2.5ml of 20% Na_2CO_3. The absorbance of the resulting blue complex was then measured at 735nm (Spectro UV-vis, Dual Beam 8 Auto Cell UVS-2700, Labomed, Inc), after incubation for 20min at room temperature in dark. Total polyphenols contents were expressed as mg Gallic acid equivalents (GAE)/g Dry Weight (DW). All samples were analyzed in triplicate.

Flavonoids contents

Flavonoids contents were determined according to the method of Zhishen *et al*. (1999). 250µl of each extract were mixed with 1.25ml of distilled water and 75µl of 5% $NaNO_2$ solution. After 6min, 150µl of 10% $AlCl_3$ H_2O solution were added. After 5min, 0.5ml of 1M NaOH aqueous solution was added and then 225µl of distilled water, following by through mixing of the solution. The absorbance against blank was determined at 510nm. The results were expressed as mg Catechin Equivalents (CE)/ g DW.

Condensed tannins contents

Condensed tannins were determined according to the method of Julkunen-Titto (1985). An aliquot (50µl) of each extract was mixed with 1.5ml of 4% vanillin and then 750µl of concentrated HCl were added. The well-mixed solution was incubated at room temperature in the dark for 20min. The absorbance against blank was read at 500nm. The results were expressed as mg CE/g DW.

Antioxidant activities

DPPH assay

DPPH radical-scavenging activity was determined according to the method of Brand-Williams *et al*. (1995). Extracts having different concentrations were prepared. An aliquot of 25µl of a diluted sample was added to 975µl of 1,1-diphenyl-2-picrylhydrazyl (DPPH) methanolic solution and vortexed. The mixture was shaken and incubated at room temperature for 30min in darkness. The absorbance against blank was measured at 515nm. Ascorbic acid was used for comparison. The ability to scavenge the DPPH radical was calculated using the Inhibition Percentage **(**IP %) = $[(A_0 - A_1)/A_0] \times 100$

where A_0 and A_1 are respectively the absorbance of the control (DPPH) at t=0 and the sample at 30min. All samples were analyzed in triplicate.

$ABTS^{+\cdot}$ assay

$ABTS^{+\cdot}$ cation radicals scavenging activity was evaluated using an improved ABTS method as described by Re *et al*. (1999) and Cai *et al*. (2004). The 2-2'-azino-bis-(3-ethylbenz-thiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$) solution (7 mmol/L) was then diluted with 80% ethanol to obtain an absorbance of 0.7±0.02 at 734nm. An aliquot of 25µl of the test sample was added to 975µl of $ABTS^{+\cdot}$ solution and mixed vigorously. The absorbance at 734nm was recorded every 6 minutes. The ability to scavenge the $ABTS^{+\cdot}$ cation radicals was calculated using the following equation: IP= $[(A_0 - A_1)/A_0] \times 100$

Where A_0 and A_1 are respectively the absorbance of the control radical cation $ABTS^{+\cdot}$ at t = 0 and the sample at 6 min. Antioxidant activity was expressed as mmol Trolox Equivalents (TE)/g MS.

Reducing power against Fe^{3+}

The ability of aerial parts extracts of *L. guyonianum* to reduce Fe^{3+} was determined according to Oyaizu method

(1986). An aliquot of each sample solution prepared with MeOH (500µl) was mixed with 500µl of sodium phosphate buffer (0.2M, pH 6.6) and 500µl of 1% $K_3Fe(CN)_6$ incubated at 50°C for 20min. After adding 500µl of 10% trichloroacetic acid, the mixture was centrifuged at 7500 RPM for 10min. The supernatent (500µl) was then taken out and immediately mixed with 500µl of MeOH and 125µl of 0.1% ferric chloride. After incubation for 10min, the absorbance against blank was determined at 700nm. The Effective Concentration at which the absorbance was 0.5 (EC_{50} (µl/ml) value is that at which the absorbance is 0.5 of reducing power. Ascorbic acid was used as control.

Antimicrobial Activities

Microorganisms

Three crude extracts (Petroleum ether, chloroform and methanol) and 21 fractions of *L. guyonianum* aerial parts methanol extract were tested for their antibacterial activities using the micro-dilution method against four human pathogenic bacteria: *Escherichia coli* American Type Culture Collection (ATCC) 25922, *Staphylococcus aureus* ATCC 27853, *Pseudomonas aeruginosa* ATCC 25923 and *Enterococcus faecalis* ATCC 29212. The same samples were tested against four Candidal strains: *Candida albicans* ATCC 90028, *Candida glabrata* ATCC 90030, *Candida Krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019.

The microorganisms tested in this study were provided from the laboratory of Parasitology-Mycology and the laboratory of Bacteriology, CHU F. Bourguiba of Monastir, Tunisia.

Microbiological protocol

Antibacterial and antifungal activities were analyzed by the micro-dilution method (NCCLS, 2000). The Minimal inhibitory concentration (MIC) (May *et al*., 2000; Hammer *et al*., 1999; Delaquis *et al*., 2002) was measured in 96-well micro titer plates after 24h of samples incubation and of microbial suspensions (5.10^6 germs/ml) at 37°C for bacteria and 48h for fungi. MIC was defined as the lowest extract concentration inhibiting a visible growth of each microorganism.

Analysis of individual phenolic compounds by Analytical RP-HPLC

Phenolic compound analysis was carried out using an Agilent Technologies 1100 series liquid chromatograph (RP-HPLC) coupled with an UV-vis multiwavelength detector. The separation was carried out on a 250x4.6 mm, 4µm Hypersil ODS C18 reversed phase column. The mobile phase consisted of methanol with 0.2% formic acid (solvent A) and water with 0.2% formic acid (solvent B). The flow rate was kept at 1mL/min. The gradient program was as follows: 35% A/65% B 0-6 min, 60% A/40% B 6-9 min, 80% A/20% B 9-14min, 100% A/0% B 14-25min, 35% A/65% B 25-30min. The injection volume was 20µL and peaks were monitored at 280nm. Peaks were identified by congruent retention times compared with those of authentic standards. Phenolic compound contents were expressed in micrograms per gram of dry plant material weight.

STATISTICAL ANALYSIS

Determination of antioxidant contents and all antioxidant capacity assays were executed in triplicate and values were calculated. The data were subjected to analysis of variance and Duncan's multiple range tests were employed to gauge differences between means. A significant difference was judged to exist at a level of $p<0.05$.

RESULTS

Polyphenols, flavonoids and condensed tannins contents

The determination of polyphenol, flavonoids and condensed tannins contents indicate that the methanolic extract has the highest amount of antioxidant components with the values of 130, 60.3 and 35.7mg CE/g DW, respectively. The chloroformic extract, which is less polar than methanolic extract, contains respectively, 108.6mg CE/g DW polyphenols, 45.1mg CE/g DW flavonoids and 32.6 mg CE/g DW condensed tannins contents. However, petroleum ether extract had the lowest amount of polyphenols (80.4mg CE/g DW), flavonoids (38.1mg CE/g DW) and condensed tannins (10.8mg CE/g DW). According to Hertog *et al*. (1993) and Siddhuraju and Becker (2003), methanol is a good solvent to extract phenolic compounds.

Evaluation of antioxidant activities

DPPH radical-scavenging activity

Table 1 shows the highest ability of methanolic extract to inhibit DPPH (EC_{50}=2.3mg/ml), followed by chloroformic extract (EC_{50}=2.4mg/ml). The lowest antiradical capacity was found in the petroleum ether extract (EC_{50}= 4.5mg/ml).

$ABTS^{+\cdot}$ Assay

Results of $ABTS^{+\cdot}$ test (table. 1) demonstrated that the methanolic extract exhibited strong radical scavenging effect (14mmol TE/g DW) however chloroformic and petroleum ether extracts have respectively 21.1 and 21.7 mmol TE/g DW.

Reducing power against Fe^{3+}

The evaluation of *L. guyonianum* aerial parts extracts to reduce Fe^{3+} showed that the methanolic extract was more effective (EC_{50}=0.2mg/ml) than the chloroform and petroleum ether ones with respectively EC_{50}=0.32 and 0.39mg/ml.

Correlations established between antioxidant activities EC_{50}/TEAC values and antioxidant components contents

Table 1: Antioxidant activities against DPPH radicals and $ABTS^{+\cdot}$ cation radicals and reducing power against Fe^{3+} of aerial parts extracts of *L. guyonianum*

Extracts	DPPH (EC_{50} mg/ml)	$ABTS^{+\cdot}$ (mmol TE/g DW)	RP (EC_{50} mg/ml)
E_1	4.58±0.00a*	21.79±0.00	0.39±0.00a*
E_2	2.45±0.00b*	21.13±0.07	0.32±0.00b*
E_3	2.39±0.00c*	14.00±0.07	0.24±0.00c*
Ascorbic acid	2.01±0.02d*	-	0.01±0.00d*

Values (mean ± SD, *n*=3) in the same column followed by a different letter are significantly different ($p<0.05$) E_1: Petroleum ether extract, E_2: Chloroformic extract, E_3: Methanolic extract

Table 2: Correlations between antioxidant activities EC_{50}/TEAC values and antioxidant components contents

	Pearson correlations		
	Polyphenols	Flavonoids	Condensed tannins
DPPH	$r^2=0.914^{**}$ p=0.001	$r^2=0.761^{*}$ p=0.017	$r^2=0.996^{**}$ p<0.001
$ABTS^{+\cdot}$	$r^2=0.933^{**}$ p<0.001	$r^2=0.792^{*}$ p=0.011	$r^2=0.999^{**}$ p<0.001
RP	$r^2=0.993^{**}$ p<0.001	$r^2=0.983^{**}$ p<0.001	$r^2=0.904^{**}$ p=0.001

*The correlation is significant at $p<0.05$ **The correlation is significant at $p<0.01$

Pearson analysis (table. 2) showed a strong correlation between EC_{50} values of DPPH radical-scavenging activity and polyphenolic contents ($r^2=0.914$) and also between the EC_{50} values and condensed tannins contents ($r^2=0.996$). The flavonoids contents were moderately correlated with EC_{50} values of DPPH radical-scavenging activity ($r^2=0.761$). A strong correlation was observed between TEAC values and polyphenolic contents and also between the TEAC values and condensed tannins contents ($r^2=0.933$ and $r^2=0.999$ respectively). However, TEAC values were correlated moderately with flavonoids contents ($r^2=0.792$). A higher correlation was found also between the EC_{50} of reducing power and the antioxidant components (polyphenols, $r^2=0.993$; flavonoids, $r^2=0.983$ and condensed tannins contents, $r^2=0.904$).

Evaluation of the antimicrobial activities

Antibacterial activities

Our results showed that the methanolic (E_1), chloroformic (E_2) and petroleum ether (E_3) extracts inhibited microorganisms growth of the three bacteria species (*Pseudomonas aeruginosa*, *S. aureus* and *E. faecalis*) with MIC values of 23, 46 and 93µg.mL^{-1}, respectively (table 3). Methanolic and chloroformic extracts exhibited similar antibacterial effects towards *Escherichia coli* (MIC=46µg.mL^{-1}). From the 21 fractions collected the F_7 and F_{11} fractions had a slight similar antibacterial activity against *P. aeruginosa* and *S. aureus* (MIC=93µg.mL^{-1}).

Antifungal activities

Regarding the antifungal activities (table. 3), results proved that all crude extracts and the majority of the tested fractions inhibited *Candida* strains growth. Methanolic aerial parts extract had the most antifungal activity against *C. krusei* (MIC=78µg.mL^{-1}). While *C. parapsilosis* was observed the most sensitive Candida strain tested to the effect of chloroformic and petroleum ether extracts (MIC=78µg.mL^{-1}). Among all the studied fractions, only F_{13} and F_{16} (from methanolic extract), exhibited interesting antifungal activities against *C. glabrata*, *C. krusei* and *C. parapsilesis* (MIC=39µg.mL^{-1}). Methanolic extract and F_2 have a moderate ability to inhibit *C. albicans* (MIC=310µg.mL^{-1}).

Contents of Individual Phenolic Compounds (µg/g DW) of Limoniastrum gyuonianum leaves and stems extracts

The HPLC analysis revealed several phenolic compounds in *L. gyuonianum* leaves and stems extracts. As shown in table 4, phenolic composition varied as function of organ, in fact, five phenolic compounds were characterized from leaves and stems extracts. The common one was gallic acid. In leaves extract, three phenolic acids and two esters were identified (gallic, procatechuic, trans-cinnamic acids, methyl-4-hydroxybenzoate and propyl-3,4,5-trihydroxybenzoate). Gallic and procatechuic acids are the two predominant compounds in this extract with respectively 102.28 and 58.99µg/g DW. From stems extract, five phenolic compounds were identified: two phenolic acids: Gallic acid is the major compound (24.43 µg/g DW) and chlorogenic acid, and three flavonoids (epicatechine, naringin and myricetin).

DISCUSSION

Table 3: Minimal Inhibitory Concentrations (MIC µg.mL^{-1}) of extracts and fractions of *L. guyonianum* against human pathogenic fungi

	Bacteria strains				Candida strains			
	E. coli	S. aureus	P. aeruginosa	E. faecalis	C. albicans	C. glabrata	C. parapsilesis	C. krusei
E_1	93	46	23	93	620	>620	150	150
E_2	46	46	23	93	620	620	78	150
E_3	46	46	23	93	310	620	78	78
F_1	750	>750	750	>750	620	>620	>620	620
F_2	>750	750	>750	>750	310	>620	>620	620
F_3	375	375	375	187	>620	620	620	>620
F_4	750	187	750	187	620	620	620	310
F_5	187	750	375	375	620	620	>620	620
F_6	187	375	>750	750	620	>620	620	310
F_7	375	187	93	187	>620	>620	>620	>620
F_8	187	187	375	375	620	620	620	>620
F_9	750	375	187	>750	IN	IN	IN	IN
F_{10}	750	187	750	375	>620	>620	620	1250
F_{11}	750	93	187	750	620	620	620	620
F_{12}	750	375	750	375	>620	>620	>620	>620
F_{13}	>750	750	187	375	>620	39	39	39
F_{14}	750	750	750	>750	>620	>620	>620	>620
F_{15}	>750	375	750	>750	>620	>620	>620	>620
F_{16}	750	375	187	750	>620	39	39	39
F_{17}	>750	>750	375	>750	(-)	(-)	(-)	(-)
F_{18}	750	375	750	375	>620	>620	>620	>620
F_{19}	>750	750	>750	>750	>620	>620	>620	>620
F_{20}	750	375	>750	>750	IN	620	78	>620
F_{21}	750	750	750	>750	(-)	(-)	(-)	(-)

E. coli : Escherichia coli ; S. aureus : Staphylococcus aureus ; P. aeruginosa: Pseudomonas aeruginosa; E. faecalis: Enterococcus faecalis, E_1: Petroleum ether extract, E2: Chloroformic extrac, E3: Methanolic extract, F_1 to F_{21} : fractions of methanolic extract
(-) : not tested IN : Inactive

Several previous studies have proved the existence of correlation between total phenolic, flavonoid contents and antioxidant activity of plant extracts. According to Barreira *et al*. (2008), the polyphenol contents of chestnut water extract and EC_{50} antioxidant activity values for DPPH radical-scavenging activity and reducing power were correlated. Moreover, Marimuthu *et al*. (2008) observed that the antioxidant activity of *Chamaecyparis obtuse* var. Formosan ethanolic bark extract was correlated with its phenolic content. Although, some studies have demonstrated that there were no correlation between total phenolic content and antioxidant activity (Eberhardt *et al*., 2001). In the present study, our results showed that polyphenolic components would probably play an important role in the antioxidant capacity of different aerial parts extracts of *L. guyonianum*. Antimicrobial activities of *L. guyonianum* evaluated herein were determined by the micro-dilution method against some pathogenic bacteria and yeasts. As can be seen from the results, *L. guyonianum* may be considered as a natural preservative against these microorganisms which can explain the use of this halophyte in traditional medicine as anti-dysenteric against infectious diseases and parasites that cause painful and bloody diarrhea (Chaieb and Boukhris, 1998). A study reported by Hammami *et al*. (2011), showed that the antimicrobial assays of essential oil from leaves, flowers, seeds and roots of *L. gyonianum* prevented visible growth of all tested bacteria (*E. coli*, *M. luteus*, *S. epidermidis* and *S. aureus*) at a lower concentration (MIC=20µg.mL^{-1}). The presence of some phenolic compounds known for their higher antioxidant properties, such as benzoic acid derivatives (for example, gallic and procatechuic acids), may explain the antioxidant and antibacterial activities, which we have observed in *L. guyonianum* methanolic extract. In fact, many studies have demonstrated that good linear relationships exist between antibacterial activity and the high level of phenolic components. However we cannot exclude that components in lower amounts also contributed to the antimicrobial activity of plant extracts. It has been also observed that the minor components might be involved in some type of synergism with the other active compounds (Abbassi *et al.*, 2014).

Chemical composition of *L. monopetalum* has been identified previously (Trabelsi *et al*. (2010, 2012). These

Table 4: Contents of Individual Phenolic Compounds (µg/g DW ± SD) of *L. gyuonianum* leaf and stem extracts

	Leaves extract	Stems extract
Gallic acid	58.99±0.15	24.43±1.76
Procatechuic acid	102.28±0.29	-
Chlorogenic acid	-	5.72±0.16
Epicatechine	-	5.85±0.49
Trans-cinnamic acid	29.86±0.01	-
Naringin	-	7.29±1.52
n propyl-3,4,5-trihydroxybenzoate	5.23±0.42	-
Methyl-4-hydroxybenzoate acid	11.02±1.01	-
Myricetin	-	6.69±0.59

reports showed the presence of gallic and chlorogenic acids in *L. monopetalum* leaves and stems acetone extracts. Trans-cinnamic acid was identified only in leaves extract, which displayed the highest level of polyphenolic content. Moreover, these studies indicated that vanillic and gallic acids were the predominant phenolic compounds. In fact, the higher correlation between phenolic contents and antioxidant activities may be explained by the amounts of these two major compounds. From *L. guyonianum* growing in Oued Ran region, only four compounds were identified namely gallocatechin, catechin 3,4-dimethoxybenzoic acid and vanillic acid (Trabelsi *et al*., 2013). The difference observed between the two species *L. monopetalum* and *L. guyonianum* and the following one *L. guyonianum* collected from different regions may be explained by some factors. In fact, the biosynthesis of different constituents of a species depends of the following factors: the species, the region in which it grows and the climatic conditions (salinity, low rainfall, high radiation…).

CONCLUSION

In conclusion, our results showed that the polyphenols contents of different extracts from *L. guyonianum* aerial parts can be responsible for their antibacterial activities and antioxidant properties. Therefore, this medicinal plant may be used as a source of natural antioxidants and a preventing agent for many diseases caused by free radicals. Tunisian flora may represent an interesting source of crude extracts and fractions with biological and pharmacological activities. Further chromatographic studies of *L. guyonianum* to search for new pure natural substances is one of our objectives.

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