Comparative studies on antioxidant activity and polyphenolic content of *Lycium barbarum* L. and *Lycium chinense* Mill. leaves

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Abstract: The purpose of this research was to bring new data regarding the phenolic composition and the antioxidant activity of *L. barbarum* L. and *L. chinense* Mill. leaves. The determination of the main polyphenolic compounds was performed using a HPLC-UV-MS method. The dominant compound found for both species was rutin, with its highest amount registered in *L. chinense* (24141.90±21.3µg/g plant material) leaves. Among the flavonoidic aglycones, quercetin was found in both samples, being quantified in a higher amount in *L. chinense*. In the antioxidant assays, both extracts exhibited important antioxidant activities, as witnessed by the three methods, both correlated with their total polyphenolic content.

Keywords: *Lycium barbarum*, *Lycium chinense*, phenolic compounds, antioxidants, EPR.

INTRODUCTION

The importance of plants belonging to the *Lycium* L. genus has increased lately due to their usage in the traditionally Chinese medicine but also to their wide acceptance as functional foods (Danech *et al.,* 2013; Dong *et al.,* 2009). *Lycium barbarum* L. is one of the most important traditional Chinese medicinal plant species possessing vital biological activities, such as anti-aging, neuroprotection, anti-fatigue, hypoglycemic, anti-proliferative activity and cytoprotection, immunomodulation and antioxidant properties (Amagase and Farnsworth, 2011; Potterat 2010; Wang *et al.,* 2010; Yu *et al.,* 2005). *L. chinense* Mill. or the Chinese desert thorn is also a well known traditional Chinese medicinal plant species considered an ingredient for eternal youth and long life, a tonic that reduces the risk of arteriosclerosis and arterial hypertension. The species draw attention to specialists due to its fruits and roots bark which exhibit several important biological activities like liver-protective, antioxidant and anti-inflammatory, respectively (Potterat 2010; Qian *et al.,* 2004; Terauchi *et al.,* 1997). Considering that only few reports deal with the chemical composition of the leaves from these two species, the aim of this paper was to evaluate the polyphenolic composition and *in vitro* antioxidant activity of Romanian cultivated *L. barbarum* and *L. chinense* leaves.

MATERIALS AND METHODS

Plant material and sample preparation

The leaves of the two species (vouchers no. 3574 and 3575) were harvested in the summer of 2013 from Romanian cultivators. Leaves were air-dried in the shade, at room temperature. The vegetal material was grinded into a fine powder and macerated with 70% ethanol for 24 h and then percolated. In order to obtain more accurate data on flavonoid glycosides and aglycones concentration, each extract (2mL) was treated with 2M hydrochloric acid (2mL) and ascorbic acid solution (0.2mL, 100mg/mL) and the mixtures were heated at 80◦C on a water bath for 30min (Barakat and Rohn, 2014; Toiu *et al.,* 2011).

HPLC determinations

Apparatus and chromatographic conditions

An Agilent 1100 HPLC Series equipped with a degaser G1322A, HP 1100 Series binary pump, a Zorbax SB-C18 reversed-phase analytical column 100mm x 3.0mm i.d., 3.5µm particle (Agilent technologies, USA) were used and operated at 48◦C. HPLC system was coupled with MS detection (Agilent 1100 MSD Ion Trap VL). The mobile phase consisted in a binary gradient: methanol and acetic acid 0.1% (v/v). The elution started with a linear gradient, beginning with 5% methanol and ending at 42% methanol, for 35min; then 42% methanol for the next 3 min and the flow rate was 1 $mL \cdot min^{-1}$ with an injection volume of 5µL (Bucur *et al.,* 2009; Vlase *et al.,* 2013).

Identification and quantification

The MS signal was used only for qualitative analysis based on the specific mass spectra of each compound. The MS spectra obtained from a standard solution of polyphenols were integrated in a mass spectra library and the MS traces/spectra of the analyzed samples were compared to spectra from library, which allowed positive identification of compounds, based on spectral match. The UV trace was used for quantification of identified compounds from MS detection (Vlase *et al.,* 2013).

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Determination of total polyphenols, flavonoids and caffeic acid derivatives

The total phenolic content (TPC) was determined by the Folin-Ciocâlteu assay and expressed as gallic acid equivalents (GAE). The determination was assessed by using an equation obtained from calibration curve of gallic acid (R^2=0.999). The spectrophotometric aluminium chloride method was used for flavonoids determination. The total flavonoids content values was calculated by using an equation obtained from calibration curve of the rutin graph (R^2=0.999). The total content of caffeic acid derivatives was determined by using the spectrophotometric method with Arnow's reagent. The percentage of phenolic acids, expressed as caffeic acid equivalent on dry material plant (mg CAE/g plant material), was determined using an equation that was obtained from calibration curve of caffeic acid (R^2=0.994) (Vlase *et al.,* 2014; Slah *et al.,* 2015).

Antioxidant activity assays

The extracts were tested for their antioxidant activities using three *in vitro* assays, the DPPH assay, FRAP method and an EPR radicals detection. DPPH free radical method is an antioxidant assay based on electron-transfer that produces a violet solution in ethanol. The experiment was carried out after the protocols of Benedec, Singh and Tian (Benedec *et al.,* 2013; Singh *et al.,* 2014; Tian *et al.,* 2014). In the FRAP assay antioxidants are evaluated as reducers of Fe^{3+} to Fe^{2+}, which is chelated by TPTZ to form Fe^{2+}-TPTZ complex (Bunea *et al.,* 2011). EPR measurements were performed on a Bruker Elexsys E500 spectrometer operating in X band (~9.4 GHz) with 100 kHz modulation frequency, at room temperature after the protocol described in our previous paper (Mocan *et al.,* 2014; Espinoza *et al.,* 2009).

RESULTS

The quantitative determination of the polyphenols was performed using the external standard method. In order to obtain more accurate data on flavonoid glycosides and aglycones concentration, and to estimate the nature of hydrolyzed compounds, each sample was analyzed before and after acid hydrolysis. The concentrations of identified compounds were organized in order of their retention times and are presented in table 1.

Gentisic, caffeic, chlorogenic, *p*-coumaric and ferulic acids were identified in all ethanolic extracts. Sinapic acid was present only in *L. chinense* extract and in both hydrolysed samples, the highest amount being obtained for the *L. chinense* sample. Two quercetin glycosides were quantified only in the unhydrolysed samples; the dominant coumpound being rutin with its highest amount in *L. chinense* sample (24141.90±21.3µg/g plant material). Regarding the presence of the free aglycones, patuletin was present only in the hydrolysed sample of *L. barbarum* (table I). Quercetin was the main compound being present in both *L. chinense* samples, with the highest amount registered in the hydrolyzed one (1230.04±2.13µg/g plant material). Considering the 19 standard compounds used for this study, some other peaks were not identified.

Determination of phenolic compounds content

The extract of *L. chinense* contained the highest amount of polyphenols, flavonoidic compounds and caffeic acid derivatives (5.3±0.05, 4.37±0.06 and 2.88±0.07g/100g respectively), as seen in table 2.

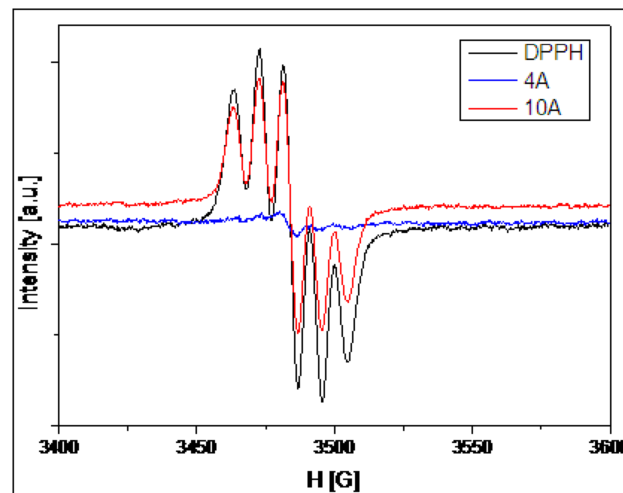

Fig. 1: The rate of reaction between antioxidant compounds and DPPH radical.

Note: 4A: *L. barbarum* leaves extract; 10A: *L. chinense* leaves extract

Determination of antioxidant activity by several assays

Antioxidant activities of both *L. barbarum* and *L. chinense* are shown in table 3. In the DPPH assay quercetin was used as a positive control. The values of IC_{50} represent the concentration of the test extract or compound where the inhibition of test activity reached 50%. As we can observe, the *L. chinense* extract exhibits higher antioxidant activity than *L. barbarum.* The results can be correlated with the content in polyphenolic compounds. In the FRAP assay, the antioxidant capacity registered for the *L. chinense* extract was also higher than for *L. barbarum*; the results were expressed in µmol TE/100mL extract (table 3).

EPR spectroscopy using stable free radicals is a simple and common method used for the study of qualitative antioxidant properties. A mixture of free radical (DPPH) and antioxidant extract was made and the rate of reaction between antioxidant compounds and DPPH was monitored by using normalized double integrated residual EPR signal, which is correlated with the number of paramagnetic species (fig. 1).

Table 1: Polyphenolic compounds content in the studied samples (μg/g plant material)

Polyphenolic compound	*m/z*	R_T ± SD (min)	*L. barbarum*	*L. barbarum* hydrolysed	*L. chinense*	*L. chinense* hydrolyed
Gentisic acid	153	3.69±0.03	<0.02	<0.02	<0.02	<0.02
Caffeic acid	179	6.52±0.04	<0.02	<0.02	<0.02	<0.02
Chlorogenic acid	353	6.43±0.05	<0.02	<0.02	<0.02	<0.02
p-coumaric acid	163	9.48±0.08	<0.02	33.90±0.02	<0.02	10.43±0.02
Ferulic acid	193	12.8±0.10	107.19±0.32	485.87±0.93	261.90±0.67	824.62±2.03
Sinapic acid	223	15.00±0.10	NF	192.02±0.25	100.39±0.31	228.68±0.98
Isoquercitrin	463	20.29±0.10	12.75±0.02	NF	25.08±0.06	NF
Rutin	609	20.76±0.15	15839±8.75	NF	24141.90±21.3	NF
Quercetin	301	27.55±0.15	NF	853.46±1.75	3.94±0.01	1230.04±2.13
Patuletin	331	29.41±0.12	NF	27.62±0.08	NF	NF
Kaempferol	285	32.48±0.17	NF	14.11±0.03	NF	24.06±0.04

Note: NF-not found, below limit of detection. Values are the mean ± SD (n = 3).

Table 2: Total phenolic compounds content in the analyzed samples

Samples	TPC (g GAE/100g)	Flavonoids (g RE/ 100 g)	Caffeic acid derivatives (g CAE/ 100 g)
L. barbarum	3.17±0.03	2.77±0.01	2.20±0.02
L. chinense	5.3±0.05	4.37±0.06	2.88±0.07

Each value is the mean ± SD of three independent measurements. TPC: Total polyphenols content; GAE: Gallic acid equivalents; RE: rutin equivalents; CAE: caffeic acid equivalents.

Table 3: Antioxidant activity by several assays

Samples	DPPH IC_{50} (μg/mL)	FRAP (μmol Trolox/100 mL)	EPR
L. barbarum	124.06±5.34	1344±50.32	28.21±1.23
L. chinense	51.01±2.54	2190±76.39	664.48±32.18
Quercetin	5.47±0.03	-	-
DPPH	-	-	797.01±43.64

Each value is the mean ± SD of three independent measurements.

DISCUSSIONS

The employed HPLC method was developed for the identification and quantification of nineteen phenolic compounds: eight phenolic acids and eleven flavonoids, allowing a simultaneous target analysis of different polyphenolic classes by a single column pass (Vlase *et al.,* 2013). Rutin was found as dominant compound in both of the analyzed species, the best source of rutin being in this case *L. chinense* leaves. Dong *et al.* reported lower amounts of flavonoids, but higher amounts of rutin, as main flavonoidic compound from cultivated *L. barbarum* (Dong *et al.,* 2009). Regarding the phenolic acids pattern, in the unhydrolyzed extract sinapic acid was found just in *L. chinense*, but however the increased quantity of this compound in both hydrolyzed samples suggests the presence of several glycosylated structures. This applies also regarding the increased amounts of quercetin and kaempferol after acidic hydrolysis, the aglycones being released from their glycosylated forms. Referring to the total phenolic, flavonoids and caffeic acid derivatives content, these findings are in line with the results of HPLC analysis and as one can already notice, flavonoids represent the major polyphenolic fraction from both species (table 2).

The antioxidant capacity of the two extracts was tested by two electron transfer methods and EPR spectroscopy. The radical scavenging capacity of the two extracts against the stable synthetic DPPH radical indicated *L. chinense* as having a superior antioxidant potential (IC_{50} = 51.01±2.54μg/mL), the results being also correlated with the FRAP assay.

Information about antioxidant activity of *L. barbarum* and *L. chinense* leaves using the electron paramagnetic resonance (EPR) spectroscopy assay is not available, so far. This technique was used in the present study to support results obtained by the traditionally used DPPH and FRAP assays and might give additional information about the antioxidant capacity of the investigated plant tissues. One can observe that integral intensity of the

DPPH mixture with the different antioxidant extracts decreases compared with the DPPH standard solution (Mocan *et al.,* 2014). The EPR spectra presented in fig. 1, which represents the oxido-reduction rate of the DPPH radical, show that we have a smaller intensity of the signal function for the antioxidant extracts. Surprisingly, comparing the calculated rates of both analysed samples, one can observe that, in this case the *L. barbarum* extract exhibits a higher antioxidant capacity than the *L. chinense* sample.

CONCLUSIONS

A phytochemical investigation was carried out on two extracts obtained by maceration of *L. barbarum* and *L. chinense* leaves, completing the literature data with new information regarding polyphenolic compounds and their bioactivities. The identification and quantification of the polyphenolic compounds were carried out using a HPLC method assisted by mass spectrometry. The dominant flavonoid was rutin, being present in the highest amount in the *L. chinense* extract (24141.90±21.3μg/g plant material) and among the flavonoidic aglycones, quercetin was the main compound. The antioxidant activity was evaluated using the DPPH, FRAP assays and also by an EPR spectroscopy method indicating both species as valuable sources of antioxidants related with their total polyphenolic and flavonoidic contents.

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