Antioxidant capacity and polyphenolic content of the *Echinocystis lobata* (Michx.) Torr. et A.Gray flowers

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Abstract: *Echinocystis lobata* (Michx.) Torr. et A.Gray is a spontaneous species in the Romanian flora, lesser studied by scientific literature, but which has proved significant activities in traditional medicine. The present study is aimed to provide data on the polyphenolic compounds in the composition of the flowers of this species and to test their biological potential. Polyphenols were identified and quantified using an HPLC-MS method. Tested biological activities were the cytotoxic, anti-plasmodial and antioxidant ones. Methods used for testing the antioxidant activity were the DPPH, CUPRAC, FRAP, TEAC, EPR and SNPAC assays. Cytotoxic activity was tested on cancerous and healthy cell lines and anti-plasmodial activity was assesed on two strains of *Plasmodium falciparum*. Ethanolic extracts of the flowers of *E. lobata* proved to contain isoquercitrin, rutin, quercitrin, kaempferol, *p*-coumaric and ferulic acid. No cytotoxic and anti-plasmodial activity was found, but antioxidant assays showed an important antioxidant capacity. The obtained results show that flowers of *E. lobata* are important sources of antioxidant compounds. It is the first approach of the kind on the flowers of this species and it offers a new perspective on possible sources of antioxidant compounds.

Keywords: *Echinocystis lobata* (Michx.) Torr. et A.Gray., polyphenols, antioxidant.

INTRODUCTION

Cucurbitaceae family (order Cucurbitales) contains the most used plants for human alimentation, of worldwide importance, among which the melons, the watermelons, the cucumbers and the pumpkins are the most popular (Deshmukh *et al*. 2015). The plants which provide them belong to the genera *Citrullus*, *Cucumis* or *Cucurbita* and are found in crops all over the world (Deshmukh *et al*. 2015; Caili *et al*. 2006; Singh *et al*. 2016). They have been cultivated for years for their fruits or seeds, which represent the basis of numerous industries related to human nutrition, but also to economy (e.g. agriculture, cosmetic or pharmaceutical industry) (Ielciu *et al*. 2016). Cucurbits (plants belonging to the Cucurbitaceae family) have proved to be valuable not only for their content in nutrients, but also for their content in bioactive compounds, that have proven interesting biological activities and which made them significant research subjects for researchers worldwide (Dhiman *et al*. 2012; Rus *et al*. 2015). Despite the fact that their content in bitter principles and with relative toxicity is largely discussed (Kaushik *et al*. 2015; Bernard & Olayinka 2010; Tannin-Spitz *et al*. 2007), recent researches investigate cucurbits for their content in compounds with protective activities for different purposes, among which the hepatoprotective (Bartalis & Halaweish 2011) or antioxidant properties (Singh *et al*. 2016; Chekroun *et al*. 2015; Yasir *et al*. 2016; Irshad *et al*. 2014; Sharma *et al*. 2012; Tapkir *et al*. 2013).

Polyphenols are secondary metabolites that are widely spread in plant kingdom and are known for a series of activities such as: modulation of lipid peroxidation, free radical scavanging, inhibition of hydrolytic and oxidative enzymes (phospholipase A_2, cyclooxygenase) and anti-inflammatory activity (Lu *et al*. 2016). It appears that the antioxidant activity is the most important among all (Bouayed *et al*. 2011; Franco *et al*. 2008; Irshad *et al*. 2014), scientific evidence suggesting that it may be the one that is responsible for all the other pharmacological effects (Lu *et al*. 2016; Manach *et al*. 2004).

Echinocystis lobata (Michx.) Torr. et A. Gray is a naturalized species, that is found in Central and South Eastern Europe, North America and Canada, in moist soils, forests and open areas (Melymuka *et al*. 2005; Reaume 2010). It is an invasive species, spontaneous in the Romanian flora, on which scientific literature provides few data (Ielciu *et al*. 2017). Despite this fact, it is a species that is used in ethno-medicine as a poultice for headaches or for menstrual disorders, rheumatisms, chills,

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fevers, kidney disorders or for stomach troubles (Melymuka *et al*. 2005; Krauze-Baranowska & Cisowski 1996). It is a species that has no clear evidence of its composition in active principles. The existing studies on the species cite the presence of flavonoids: quercetin and isorhamnetin derivatives (Krauze-Baranowska & Cisowski 1996). A recent study assesses the polyphenolic content and shows the lack of cellular toxicity for the leaves and aerial parts of the species, showing that these parts may exhibit a selectivity for the antioxidant capacity (Ielciu *et al*. 2017). These studies are the only ones, to the best of our knowledge, that evaluate the content of polyphenolic compounds in the composition of the species and their biological activities. The species appears though as a species lesser studied by scientific literature, but with important potential as medicinal plant.

The present study aims to complete scientific litterature with important data regarding the content of polyphenols in the flowers of *Echinocystis lobata* (Michx.) Torr.et A.Gray and their biological activities. As this species appears to be lesser known and studied by researchers for its potential as a medicinal plant, the present study may bring important evidence to prove its promising biological potential.

MATERIALS AND METHODS

Plant material

The tested samples were collected from the spontaneous flora of Hunedoara County (Table 1) and their identification was performed at the Department of Pharmaceutical Botany of "Iuliu Haţieganu" University of Medicine and Pharmacy Cluj-Napoca, where vouchers nr. 105.8.1.1-18 are deposited. Samples were harvested at the beginning and at the end of the development of the species, in order to establish the possible differences between their polyphenolic composition and biological activities.

Extraction procedure

Flowers of *E. lobata* were grinded until reaching an appropriate degree of fineness. The powder was subjected to a 24h maceration with 50% ethanol and subsequently sonicated for 30minutes at 70^0C. The obtained solutions were filtered and completed with the extraction solvent until reaching the initial volume. Extracts were subjected to the HPLC analysis, after being filtered by 0,45mm membrane filters.

HPLC-MS analysis of polyphenols was performed using an Agilent 1100 HPLC Series system, coupled with an Agilent 1100 mass spectrometer (LC/MSD Ion Trap VL). A Zorbax SB-C18 analytical column (100 x 3.0 mm i.d., 3.5µm particle) was employed for the separation of compounds. Mobile phase consisted in a mixture of methanol and acetic acid 0.1% V/V. Linear gradient starting with 5% methanol and ending with 42% methanol, was performed for the elution of compounds, which was achieved in 35 minutes. Isocratic elution with 42% methanol was performed for another 3 minutes. Flow rate was set at 1mL/min and injection volume was 5µL. Temperature of the thermostat was set at 48°C. Detection of the polyphenols was performed both on UV and MS mode. UV detector was set at 330 nm until 17.5 minutes, then at 370 nm, until the end of the method. The MS system used an electrospray ion source in negative mode and provided the mass spectra of each polyphenol. Signals were used for the qualitative analysis. A spectra library was created with all the spectra obtained from a reference solution of each tested polyphenol and it allowed to compare later with the MS traces/spectra of the analysed samples, which allowed the identification of polyphenols, based on spectral match. UV signal was used for the quantification of identified compounds in the MS. Data obtained in the HPLC-MS analysis of polyphenols were processed using ChemStation and DataAnalysis software from Agilent. (Benedec, Hanganu, *et al*. 2016; Hanganu, N. K. Olah, *et al*. 2016; Hanganu, L. Filip, Olah, *et al*. 2016; Hanganu, N.-K. Olah, *et al*. 2016; Ibrahim *et al*. 2010). Results are expressed as mg polyphenol/100 g dried vegetal product (dvp).

Fig. 1: Macroscopic characters of *Echinocystis lobata* (Michx.) Torr. et A.Gray

Table 1: Samples of *E. lobata*, with the harvesting date and place

Sample	Harvesting date	Harvesting place
EL 05	05.08.2014	Simeria, Hunedoara
EL 23	23.08.2014	Deva, Hunedoara

HPLC-MS analysis of methoxylated flavones was achieved using the same chromatographic system. Separation was performed on a Zorbax SB-C18 reverse-phase analytical column (100 x 3.0mm i.d., 3.5µm particle), using a linear gradient, with the mobile phase that consisted in a mixture of methanol and acetic acid 0.1% V/V, starting with 45% methanol and ending with 50% methanol in 8 minutes. The work temperature was 48°C, injection volume was 5µl and the flow rate was set at 0.9 ml/min. Detection was achieved in MS/MS, in a MRM mode, using an electrospray ionisation source, in a negative mode. Detection was performed by monitoring

Table 2: Investigated polyphenols and their retention times (Rt)

Phenolic compound	Rt ± SD (min)	Phenolic compound	Rt ± SD (min)
Caftaric acid	2.10±0.06	Rutoside	20.20±0.15
Gentisic acid	2.15±0.07	Myricetin	20.70±0.06
Caffeic acid	5.60±0.04	Fisetin	22.60±0.15
Chlorogenic acid	5.62±0.05	Quercitrin	23.00±0.13
p-coumaric acid	8.7±0.08	Quercetol	26.80±0.15
Ferulic acid	12.2±0.10	Patuletine	28.70±0.12
Sinapic acid	14.3±0.10	Luteolin	29.10±0.19
Hyperoside	18.60±0.12	Kaempferol	31.60±0.17
Isoquercitrin	19.60±0.10	Apigenin	33.10±0.15

Table 3: Retention times (t_R), specific monitored fragments and mass of the investigated methoxylated flavones

Compound	t_R (min)	M	$M-H^-$	Ions/Monitored fragments
Jaceosidin	2.9±0.15	330.3	329.3	314
Hispidulin	4.2±0.07	300.2	299.2	284
Eupalitin	7.05±0.13	344.3	343.3	328
Eupatorin	7.6±0.09	344.3	343.3	328
Casticin	8.05±0.01	374.3	373.3	358
Acacetin	9.8±0.06	284.3	283.3	268

Table 4: Quantification of polyphenols in tested samples

	EL 05 (mg/100g dvp)	EL 23 (mg/100g dvp)
p-Coumaric acid	1.31±0.18	1.81±0.74
Ferulic acid	0.51±1.03	0.62±0.05
Isoquercitrin	231.86±1.92	238.22±0.54
Rutin	106.36±0.76	83.06±0.05
Quercitrin	67.84±0.74	56.81±0.83
Quercetin	ND	5.29±0.12
Kaempferol	2.18 ± 0.04	2.66±3.02

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

the specific ions of tested flavones (Mocan *et al*. 2016; Orodan *et al*. 2016) (Table 3). Results are expressed as mg methoxylated flavone/100g dried vegetal product (dvp).

Determination of Total Polyphenols and Flavonoids Content

Total polyphenolic content (TPC) was quantified by a spectrophotometric method, using the Folin-Ciocâlteu reagent. The method has some modifications compared with the classic one, which concerned especially the wavelength of the spectrophotometric measurements and the expression of the polyphenolic total. Results were expressed as mg gallic acid equivalents (GAE)/100g dvp. A spectrophotometric method based on the formation of the aluminium chloride complex was performed in order to assess total flavonoidic content. Results were expressed as mg rutin equivalents (RE)/100g dvp (Benedec, Filip, *et al*. 2016; Benedec, Hanganu, *et al*. 2016).

Antioxidant assays

Various *in vitro* systems were used to test the antioxidant potential of the samples: classical DPPH bleaching, TEAC, FRAP, CUPRAC, SNPAC and EPR. Results are expressed as IC_{50} values for the DPPH and TEAC methods and as μM Trolox equivalents (TE)/100ml extract for the rest of the methods (Olah *et al*. 2016; Hanganu, N. K. Olah, *et al*. 2016; Hanganu, L. Filip, Olah, *et al*. 2016).

DPPH free radical method is based on the neutralization of the 2,2-diphenyl-1-picrylhydrazyl free radical, reduced in the presence of an antioxidant. Spectrophotometric determination was performed at 517 nm. The inhibition of the radical was expressed as IC_{50} (μg/ml), the concentration of extracts required to cause a 50% DPPH inhibition (Hanganu, N.-K. Olah, *et al*. 2016; Benedec *et al*. 2013; Hanganu, D. Benedec, Vlase, *et al*. 2016).

TEAC assay is based on the ability of an antioxidant to reduce the 2,2-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid) free cationic radical ($ABTS^{\bullet+}$), which is obtained by adding a potassium persulfate solution. Spectrophotometric determination was performed at 734 nm. The percentage of inhibition was expressed as IC_{50} (μg/ml) and it was compared with a Trolox standard

(Thaipong *et al*., 2006a; Arnao *et al*., 2001; Hanganu, Filip, Olah, *et al*., 2016).

Table 5: Quantification of hispidulin in samples of *E. lobata*

Sample	Hispidulin (mg/100g dvp)
EL 05	82.10 ± 0.65
EL 23	59.93 ± 1.01

Note: Values are the mean ± SD (n = 3)

FRAP method uses the reduction of the ferric to the ferrous ion in a complex formed with iron of the radical 2,4,6-tripyridyl-s-triazine. According to the concentration of antioxidant compounds in the sample, the color of the complex is changed and absorbance is measured at 593nm. The results are expressed as mM Trolox equivalent/100 ml extract, on the basis of a calibration curve using a Trolox standard (Thaipong *et al*., 2006; Olah *et al*., 2016; Benzie & Strain 1996).

CUPRAC method assesses the reduction of the copper ion (II) to the copper iron (I) in the neocupreine (2,9-dimethyl-1,10-phenantroline), which determines a change in the color from light green to reddish-orange. The color change was correlated with the antioxidant capacity by measuring the absorbance at 450nm. The calibration curve was plotted using concentrations of the Trolox standard and the results are expressed as mM Trolox equivalent/100ml extract (Özyürek *et al*. 2012; Olah *et al*. 2016).

SNPAC method uses the spherical silver nanoparticles (SNPs), obtained from silver nitrate by reducing of Ag^+ ions and using as surface stabilizer the trisodium citrate. It is based on reduction of the silver ion to colloidal silver with fine silver nanoparticles suspended in solution, which occurs in the presence of the antioxidants. The absorbance correlated to the color change from pale yellow to brown was measured at 423nm and the calibration curve for standard was plotted using concentrations ranging of Trolox standard. Results are expressed as mM Trolox equivalent/100 ml extract (Benedec, Filip, *et al*. 2016; Özyürek *et al*. 2012).

EPR method uses the DPPH radical, which is added to the extract. EPR spectra is recorded after the sample is being mixed and transferred in a EPR quartz capillary. Measurements were performed on a Bruker Elexsys E500 spectrometer. The difference of reaction between antioxidant compounds in the samples and DPPH radical was expressed by the integral intensity (I) (Benedec, Hanganu, *et al*. 2016; Benedec, Filip, *et al*. 2016; Hanganu, N. K. Olah, *et al*. 2016).

Cytotoxic activity

Extracts tested for biological activity were evaporated and dissolved in DMSO in order to reach the starting concentration of 10mg/mL. Cytotoxic tests were performed on 3 cell lines, of which 2 cancerous cell lines and one healthy cell line. *A549* (lung cancer) and *HeLa* (cervical cancer) cell lines were used as cancerous cell lines and *WI38* (fetal lung fibroblasts) as a healthy cell line. The first step of the assay consisted in seeding the 96-well microplates with 200 µl suspension containing 8000 (A549, HeLa) and 9000 (WI38) cells per well. After 24h incubation, cells adhered to the bottom of each well and were treated with six dilutions of each sample in the culture medium. Tested concentrations were ranging between 3.2 and 100µg/mL. After another 48h incubation of the cells with the dilutions of the samples, cell viability was determined by a spectrophotometry. The cytotoxicity indicator consisted in the WST-1 tetrazolium salt, which was added to each well and after one hour the absorbance of the solutions was measured at 450nm, using a scanning multi well spectrophotometer (Stat Fax 2100, Awareness Technology Inc). Each sample was tested in triplicate and each set of tests was performed twice. The growth inhibition of cells was calculated by comparison with the negative control, which consisted in the non-treated cells. IC_{50} values (concentration of the sample needed to obtain 50% inhibition of cell growth) were calculated by linear regression of the six levels of concentrations (Ledoux *et al*. 2017; Jansen *et al*. 2012).

Anti-plasmodial activity was performed on *Plasmodium falciparum* strains (chloroquine resistant and sensitive). *3D7* (chloroquine sensitive) and *W2* (chloroquine resistant) were used. Samples were dissolved in DMSO, until reaching a concentration of 10mg/ml. Parasitic suspension was put in contact with eight two-fold dilutions of each sample in the culture medium on a 96-well microplate and incubated for 48h. Tested concentrations were ranging between 0.8 and 100µg/mL. The inhibition of parasite growth was assessed by a spectrophotometric determination, based on the evaluation of the plasmodial lactate dehydrogenase (pLDH) activity. Absorbances were measured at 630nm using a multi well scanner (Stat Fax 2100, Awareness Technology Inc). Artemisinin (Sigma–Aldrich) was used as positive standard for the inhibition of parasite growth. Erythrocytes infected and uninfected with the parasites were used as positive and negative controls. The values for the assessment of the parasite growth inhibition were calculated by comparison with the values obtained for the infected non-treated erythrocytes, which represent 100% growth. IC_{50} values (concentration of the sample needed to obtain 50% inhibition of parasite growth) were calculated by linear regression of the eight concentrations tested for each sample. Each sample was reproduced in double in each test and tests were performed three times for each strain (Ledoux *et al*. 2017; Jansen *et al*. 2012; Jonville *et al*. 2008).

Table 6: Quantification of total polyphenols and flavonoids in samples of *E. lobata*

Sample	Polyphenols (g GAE/100g dvp)	Flavonoids (g RE/100g dvp)
EL05	3.23 ± 0.39	3.75 ± 0.37
EL23	3.04 ± 0.18	2.89 ± 0.11

Table 7: IC_{50} of the tested *E. lobata* samples (anti-plasmodial and cytotoxic)

Sample	Anti-plasmodial 3D7 (µg/mL)	Anti-plasmodial W2 (µg/mL)	Cytotoxic A549 (µg/mL)	Cytotoxic HeLa (µg/mL)	Cytotoxic WI38 (µg/mL)
EL 05	> 50	> 50	> 50	> 50	> 50
EL 23	> 50	> 50	> 50	> 50	> 50

Note: Values are the mean ± SD (n = 3)

Table 8: Antioxidant activity results obtained for the tested *E. lobata* samples

Sample	DPPH method/ *IC50*	CUPRAC method/ µM TE/100 mL	FRAP method/ µM TE/100 mL	TEAC method/ *IC50*	SNPAC method/ µM TE/100 mL	EPR method/ *Integral intensity**
EL 05	91.9±0.84	196±0.85	294± 1.25	16.5± 0.43	684± 3.69	125.08±1.90
EL 23	74.3±0.65	226±0.72	308± 1.95	8.2± 0.05	492± 3.22	93.34±1.76

Note: Values are the mean ± SD (n = 3), *DPPH Integral intensity = 668.62 ± 0.18

STATISTICAL ANALYSIS

Data were presented as mean values ± standard deviation (SD). For each assay, samples were analyzed in triplicate. Averages and SD were calculated using the Excel software package (Hanganu, L. Filip, Olah, *et al*. 2016; Benedec, Hanganu, *et al*. 2016; Hanganu, NK Olah, *et al*., 2016; Olah *et al*., 2016; Benedec, Filip, *et al*., 2016; Hanganu,. Olah, *et al*., 2016).

RESULTS

HPLC-MS analysis of polyphenols

Among the compounds used as references, six were found in both tested samples: isoquercitrin, rutin, quercitrin, kaempferol, *p*-coumaric and ferulic acid. Quercetin was only found in the sample collected at the end of the development of the species. Table 4 contains the results of the quantification of polyphenols in the tested samples.

HPLC-MS analysis of methoxylated flavones

Of all the methoxylated flavones, only hispidulin was identified in tested samples (table 5).

Total Polyphenols and Flavonoids Content

Quantification of the flavonoidic and polyphenolic totals provided values that were in corelation with the quantification of compounds by HPLC-MS (table 6).

Biological assays

Samples were found inactive both on cancerous and healthy cell lines and on both parasitic strains, at a concentration of 100µg/mL (table 7). Only the antioxidant assays proved to have promising results, indicating the fact that the species has a selectivity for its antioxidant properties (table 8).

DISCUSSION

The amount of polyphenols in tested samples of flowers of *E. lobata* appears to be different. Isoquercitrin, rutin, quercitrin, kaempferol, *p*-coumaric and ferulic acid were found in samples collected at the beginning and at the end of the development. Quercetin was only found in the sample collected at the end of the development of the species. Among these compounds, only isoquercitrin is previously cited, but in the aerial parts of the species (Krauze-Baranowska & Cisowski 1996). In fact, the study performed by Krauze-Baranowska *et al*.cites the presence of a compound that changes identity in the study from kaempferol-3-O-glucoside (astragalin) to quercetin-3-O-glucoside (isoquercitrin), but it appears more as the kaempferol derivative, as following the acid hydrolysis it is transformed into kaempferol. A recent study cites the presence of these compounds in samples of leaves and aerial parts (Ielciu *et al*. 2017), but no studies were found on the flowers of this species.

Table 6 shows the quantification of the amount of polyphenols in the tested samples of *E. lobata*. Almost all compounds were found in highest amounts in the sample collected at the end of the flowering period, indicating that compounds accumulate during the development of the species. Only rutin and quercitrin were found in lower amounts in the sample collected at the end of the development. Quercetin is only found in the sample collected at the end of August. The compound which appeared in the highest concentration was isoquercitrin (231.86mg/100g dvp for EL 05 and 238.22mg/100g dvp for EL 23). It is followed by rutin and quercitrin. Other compounds were found in lesser amounts.

Polyphenolic total is in a strong correlation with the HPLC-MS quantification of the polyphenols and shows

an increase of the amount towards the end of the development of the species. Flavonoidic content shows though a decrease towards the end of August.

It is the first study of this kind on this species that brings scientific evidence on the presence of polyphenols in flowers of the species. Other studies were performed on the fresh aerial parts (Krauze-Baranowska & Cisowski 1996) or on the aerial parts and leaves (Ielciu *et al*. 2017), but flowers proved hereby to be richer in amounts of polyphenolic compounds. Taken into consideration the fact that tested samples were collected in different periods of time, this study shows that the quantities of different polyphenols differ depending on the harvesting period. The acumulation of polyphenols towards the end of development of the species is noticed. It is clear that the highest amount of polyphenols is found in the sample collected in the end of the month of August, when the species reaches its highest stage of development. Further studies are needed in order to establish a rigorous accumulation dynamics for the polyphenolic compounds of this species.

Table 5 contains the results of the quantification of the methoxylated flavones, of which only hispidulin could be quantified. The opposite trend is noticed, concerning the decrease of the amount of methoxylated flavones towards the end of the month of August.

Cytotoxicity assays showed no activity of the extracts at a concentration of 100μg/mL, both on cancerous cell lines and on healthy cell lines. In correlation, anti-plasmodial tests also showed no significant activity of the tested samples. Only the antioxidant assays showed a significant activity of the samples, indicating therefore a selectivity of the species for its antioxidant properties. Results for the antioxidant assays show that the EL23 is the most efficient sample in all the methods used for testing, except SNPAC. IC_{50} values for the DPPH and TEAC methods are lower for the sample collected at the end of the development, while for the CUPRAC and FRAP methods, the quantity of Trolox equivalents needed to reduce the corresponding ions increases for this sample. EPR measurements have shown similar results that show a better integral intensity for the same sample. Only SNPAC assay shows that the sample collected at the beginning of the development is more efficient.

If correlating all the above findings, it appears clear that an accumulation of the polyphenols in the flowers of *E.lobata* is noticed during the development of the species, which reflects clear evidence on the antioxidant activity of the species, which has also proved lack of toxicity at concentrations of 100μg/mL. Further studies are needed in orther to establish a more rigorous dinamics of accumulation of the main compounds. The present study is the first of a kind performed on the flowers of the species and it opens new opportunities for the study of a plant which has proved to have important potential as a medicinal plant.

CONCLUSION

Assessment of the polyphenolic profile and antioxidant activity of the flowers of *Echinocystis lobata* (Michx.) Torr. et A.Gray allowed to bring important information on the chemical composition of the flowers of this species and on its biological potential, in order to complete the lack of information of the scientific literature regarding this species. The novelty of this study consists in the fact that it is the first of the kind that evaluates the polyphenolic composition of the flowers of the species. Various polyphenols are found: isoquercitrin, rutin, quercitrin, kaempferol, *p*-coumaric and ferulic acid. Moreover, flowers proved important antioxidant capacity, showing, at the same time, lack of cellular toxicity. The present study managed though to emphasize the need to deepen the knowledge on the species, as it has proved to be an important source of compounds with antioxidant activity.

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