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Roots from mulberries (*Morus alba*) natural and hybrids varieties: phenolic contend and nutraceutical potential as antioxidant

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ABSTRACT

Fruits of *Morus alba* have been described as source of phytochemicals with antioxidant activity, which is of interest for human consume, and food industry. However, differences in antioxidant capacity among varieties of this species, as well as the potential of other parts of this plant has been little explored. In this work, extracts of roots from twelve (natural or hybrid) varieties of *M. alba* were evaluated. There were observed differences between varieties, as the range of total phenol content among hexane, ethanol and aqueous extracts (0.55 - 1.59; 2.72 - 6.61; 5.09 - 9.85 gallic acid /100 g of extract, respectively). Antioxidant action of *M. alba* roots extracts was proved in different assays as radical scavenger and iron reduction capacity. Ethanol was found to be the best solvent to extract antioxidant compounds. Among all extracts assayed, the best association between high phenol and flavonoid content associated to free radicals scavenger and iron reducing power activities were shown by ethanol root extract from hybrid variety IZ 64. This work demonstrated that *M. alba* roots possess great potential as source of phytochemicals with antioxidant activity and pointed variety IZ 64 as the best cultivar to be used due to its functional properties or as source of natural antioxidants.

INTRODUCTION

Morus alba is a plant species that belongs to *Moraceae* family and has high economic interest worldwide (Donno *et al.*, 2015). Its fruits are source of alkaloids, flavonoids, anthocyanins and phenolic acids, being used as human food while its foliage contains unique nutrients for silkworm development (Butt *et al.*, 2008; Chan *et al.*, 2016). This plant species is native to Asia, but due to its wide adaptation in the tropics (Europe, north and south America, Africa and India), it has been considered cosmopolitan nowadays (Noda *et al.*, 2004; Khan *et al.*, 2013). Sericulture was the main process that drove the improvement of *M. alba* species leading to the development of varieties that represent valuable plant genetic resources. In Brazil, for example, there are three

collections of M. alba which bears 90 varieties (Medina et al., 2009). In Cuba, the Estacion Experimental de Pastos y Forrajes Indio Hatuey (EEPF-IH) is one of the centers for genetic improvement of *M. alba* for sericulture holding a gene bank with varieties obtained from countries like Costa Rica, South Korea, China and Spain (Martín et al., 2014). Among the varieties available at EEPF-IH, it is worth mentioning Cuban, Indonesia, Murcia, Tigreada, University, YU-12, YU-62 (natural ones) and IZ 13/6, IZ15/7, IZ 40, IZ 56/4 and IZ 64 (hybrid ones) which have been developed by different agronomic studies. Besides the nutritional value of Morus fruits, other parts of this plant have been related to possess biological activities such its stems, with antioxidant (Nam et al., 2012), antiulcer (Ahmad et al., 2013) and anti-inflammatory activities (Rivière et al., 2014). For M. alba leaves, vasodilator (Kurniati et al., 2014) and hyperglycemia reducing effects (Kim et al., 2011) have been reported while antityrosinase activity (Chang et al., 2011) was reported for M. alba twigs.

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The fruits were also pointed to carry on macrophage activation (Kim *et al.*, 2013) and to bear antioxidant activity (Donno *et al.*, 2015). Lyophilized extract of *Morus* fruits displayed significant wound healing activity (Akkol *et al.*, 2015). Root barks also have shown biological activity as hypocholesterolemic, antioxidant (El-Beshbishy *et al.*, 2006), anti-inflammatory and anti-cancer agents (Eo *et al.*, 2014). It is important to note also that there are already proving the absence of toxicity studies on oral administration of extracts of *Morus alba* (Oliveira *et al.*, 2016).

Phenolic compounds are secondary metabolites that protect plants against damage from pathogens and environmental stress (Paredes-López et al., 2010). The consumption of these substances has been correlated with minimizing damaging effects of uncontrolled free radicals production, due to their antioxidant activity (Yangthong et al., 2009). Mechanisms of antioxidant action are very complex, including electrons donation, which act accelerating lipids oxidation (El-Beshbishy et al., 2006; Huang et al., 2005). Other mechanisms include hydrogen donation to reduce reactive oxygen species (Villaño et al., 2007) related to alterations in DNA and cancer. Phenolic compounds concentration and, consequently, antioxidant activity of any plant is greatly affected by environmental conditions, storage, geographic region, variety and the part of plant used in the biological evaluation (Paredes-López et al., 2010). In the literature there are only a few reports regarding biological activities of M. alba roots extracts (El-Beshbishy et al., 2006; Eo et al., 2014), since the studies on M. alba roots usually concentrated in their barks and not in the whole roots (Wei et al., 2016). The use of roots in food as a source of metabolites with biological activity is underexploited in the literature although of great interest. Zingiber officinale rhizome, for instance, is greatly used to compose the taste of soft drinks and beverages and has been proved to possess antioxidant activity higher than butylhydroxyanisole, a synthetic compound used to prevent food oxidation (Singh et al., 2008).

Due to toxicity associated to synthetic antioxidants used as preservatives in some foods, legal restrictions have been imposed to their use. On the other hand, the growing knowledge of healthy effects of nutraceuticals in the daily diet has been increasing the search for novel natural antioxidants (Butt *et al.*, 2008; Egea *et al.*, 2010). In this context, the aim of the present work was to evaluate and compare phenolic and flavonoid contents in extracts prepared from roots of different natural and hybrid *M. alba* varieties, and to determine their antioxidant activities by different mechanisms aiming at determining the antioxidant potential of *M. alba* roots.

MATERIALS AND METHODS

Chemicals

All chemicals used in this work were of analytical grade. Solvents (ethanol, methanol and hexane), acids and salts were obtained from Vetec Química fina LTDA (Rio de Janeiro, Brazil), except for ascorbic acid, quercetin, 2,2'-azinobis-3etilbenzotiazolin-6-sulfonic (ABTS), 1, 1-Diphenyl-2picrylhydrazyl (DPPH), hidroquinone, and gallic acid, that were purchased from Sigma-Aldrich (São Paulo, Brazil). Folin-Ciocalteu reagent was purchased from Merck (Darmstadt, Germany).

Plant material and extracts preparation

All plants used at this work were obtained from *Estacion Experimental de Pastos y Forrajes* "Indio Hatuey". Twelve varieties of *M. alba* were used: Cubana, Indonesia, IZ 13/6, IZ 15/7, IZ 40, IZ 56/4, IZ 64, Murcia, Tigreada, Universidad, YU 12 and YU 62. The dried roots of each variety (1 kg) were milled and successfully extracted from hexane, ethanol and distilled water by percolation. After exhaustive extraction, the solvent was removed under vacuum. There were obtained 36 extracts that were labeled and stored at 4 °C.

Total antioxidant activity (phosphomolybdate method)

Phosphomolybdate assay was used to evaluate extracts capacity to reduce Mo⁺⁶ (yellow) to Mo⁺⁵ (green) in acid pH (Prieto *et al.*, 1999). Extracts were dissolved in hexane, ethanol or water (0.1 mL) and 3 mL o reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, 4 mM ammonium molybdate) were gently mixed and incubated for 90 min at 95 °C. Samples were cooled to room temperature and absorbance was read at 695 nm against a blank containing only the solvent and reagent solution. Ascorbic acid was used as reference standard and total antioxidant activities of the extracts from the different varieties assayed were expressed as ascorbic acid equivalents (mmol ascorbic acid/g dry weight of the root extracts).

Iron reducing power

Iron reducing antioxidant capacity was evaluated by mixing 0.25 mL of extracts at concentrations varying from 12.5 to 100 µg/mL to phosphate buffer (0.2 M, pH 6.6) and 0.625 mL of potassium ferrocyanide 1 % (w/v). After homogenization, samples were incubated for 20 min at 50 °C. Then 0.625 mL of trichloroacetic acid 10 % (w/v) was added and the contents were centrifuged (3000 rpm, 10 min). Supernatant (0.18 mL) was added to 1.8 mL water and 0.36 mL ferric chloride (0.1%, w/v). Absorbance reading was taken at 700 nm and results were expressed as mean \pm standard deviation. Ascorbic acid was used as reference standard (Oyaizu, 1986).

Radicals' scavenger antioxidant activities (ABTS and DPPH)

Evaluation of radicals' scavenger antioxidant activities followed two methodologies. Scavenger ABTS method (Johnstone *et al.*, 2006; Olajuyigbe and Afolayan, 2011) consisted into preparation of an ABTS solution (7 mM ABTS and potassium persulfate 2.4 mM incubated for 12 h in dark). This solution was diluted with methanol until absorbance at 734 nm reached 0.708 \pm 0.001. Then 1 mL of this solution was mixed with 1 mL of extracts solution in different concentrations (2.5-125 µg/mL) and the reaction took place for 7 min in dark. Absorbance readings at 734 nm were taken. Scavenger DPPH method (Brand-Williams *et al.*, 1995; Re *et al.*, 1999) used consisted in gently mixing the extracts solutions in different concentrations (100-1000 μ g/mL) with DPPH 0.3% in methanol. The reaction was allowed to take place for 30 min in dark. Then, absorbance at 517 nm was measured. In both assays, ascorbic acid was used as positive control, a blank was made with metanol/ethanol instead of the extracts and antioxidant activity was expressed as inhibition percentage, calculated using the formula: % inhibition = (blank absorbance - sample absorbance) / blank absorbance x 100. Sample concentration required for capturing 50% of DPPH or ABTS radicals (IC₅₀) was obtained from an inhibition (%) vs. concentration curve.

Total phenolic compounds

Concentration of total phenolic compounds on the extracts from roots of *M. alba* varieties was evaluated by adding the extracts (0.5 mL, 1000 μ g/mL) to 2.58 mL of Folin-Ciocalteu reagent (Gao *et al.*, 2000). After 3 min, saturated sodium carbonate solution (0.3 mL) was added and the materials were incubated for 20 min at room temperature. Gallic acid was used as reference standard. Absorbance reading at 760 nm was taken and the results were expressed as gallic acid equivalents (g gallic acid /100 g dry weight of the root extracts).

Total flavonoids content

Flavonoids content was evaluated by aluminum chloride method (Yang *et al.*, 2007). Extracts (0.5 mL), water (2.5 mL) and sodium nitrite 5 % (w/v) (150 μ L) were combined, and strongly agitated by vortex (10 s). After 5 min, 300 μ L of aluminum chloride 10 % (w/v), 1 mL of NaOH (1 mM) and 550 μ L of water were added. This solution was homogenized and incubated at room temperature for 15 min. Samples absorbance at 510 nm were taken and results were expressed in quercetin equivalents (μ g quercetin/g dry weight of the root extracts).

Statistical analysis

The results were analyzed by ANOVA (one-way), Tukey's and Dunnett's multiple comparison tests. Microsoft Excel 2010 (Roselle, IL, USA) was used in data evaluation. Correlations between phenolic compound contents and antioxidant activities were evaluated by Pearson correlation coefficient (r) with $P \leq 0.05$.

RESULTS AND DISCUSSION

Hexane, ethanol and aqueous extracts of roots from natural and hybrid varieties of *M. alba* were prepared resulting in a total of thirty six extracts. This approach enabled the comparison of extracts activity according to the polarity and to the cultivar variety. In fact, antioxidant activity varied significantly. Ethanol extracts were, in general, far more active than the respective aqueous extracts, while hexane ones were only weakly active. In fact, when extracts were screened using phosphomolybdate assay, the most active ones were those prepared from ethanol. Hybrid *M*. *alba* varieties IZ 15/7 and IZ 13/6 showed the better activities (891 and 815 mmol ascorbic acid/g extract, respectively) (Figure 1) followed by the natural cultivars Tigreada, Murcia e Universidad. Aqueous extracts showed intermediary activity but none of the aqueous extracts was more active than the ethanol ones. Although they were not as active as the ethanol extracts, relevance of their activities is high since they indicate the possibility of their use in the form of teas, which can be a straightforward way to benefit from their antioxidant activity. None of the hexane extracts was significantly active in this assay.



Fig. 1: Total antioxidant activity of extracts obtained by different solvents from roots of *Morus alba* varieties

On ferric ion reducing capacity, ethanol extracts were again the most active ones. Reducing power assay relies on the reduction of unstable Fe⁺³ to stable and Fe⁺² (Umamaheswari and Chatterjee, 2008). The latter is more stable and unable to start or propagate lipid oxidation. Varieties IZ 64 (47.4%), YU-62 (46.2%) and IZ 13/6 (43.2%) showed highest antioxidant activities, while natural cultivar Murcia and hybrid cultivar IZ 56/4 were much less active (28.8 and 28.4 respectively) (Table 1). Interestingly, aqueous extracts of varieties Universidad and Murcia were more active than the corresponding ethanol extracts. These data show the importance of assaying different cultivars and different extracts, since a straight correlation was not observed regarding this biossay. Although the standard used in this assay (ascorbic acid) was twice more active than the extracts, it is important to point that extracts are a mixture of many metabolites present in small yields. Therefore, extracts activities found in this work are very relevant and higher than ferric ion reducing capacity activity reported for extracts of M. indica L. leaves (10.37 %) (Arabshahi-Delouee and Urooj, 2007). Among the aqueous extracts, besides Universidad and Murcia, the varieties IZ 64, YU-12 and Cubana were the most active for reducing ferric iron power activity (Table 1). Extracts able to avoid the action of iron ions present in food can prevent lipid oxidation process, being potential natural preservatives. The high activity of varieties IZ 64, YU-62, IZ 13/6 (as ethanol extracts), Murcia (33.2 %), IZ 64 (32.6 %), YU-12 (32.2 %) and Cubana (30.2 %) (as aqueous extracts) make these extracts good targets for development of natural food preservatives.

Table 1: Ferric reducing power antioxidant activity of <i>M. alba</i> varieties roots extracts.
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M	Absorbance at 700 nm							
<i>M. alba</i> varieties	hexane	% ^a	ethanol	% ^b	aqueous	% ^c		
Ascorbic acid	1.65	100	1.95	100	2.59	100		
Cubana	0.15	9.3	0.81	41.4	0.78	30.2		
Indonesia	0.18	11.1	0.66	33.6	0.68	26.1		
IZ 13/6	0.09	5.6	0.84	43.2	0.65	25.0		
IZ 15/7	0.06	3.7	0.69	35.3	0.68	26.4		
IZ 40	0.04	2.4	0.67	34.3	0.64	24.7		
IZ 56/4	0.04	2.2	0.56	28.4	0.60	23.2		
IZ 64	0.10	6.3	0.92	47.4	0.85	32.6		
Murcia	0.05	2.7	0.56	28.8	0.86	33.2		
Tigreada	0.06	3.8	0.80	40.9	0.75	28.8		
Universidad	0.11	6.6	0.74	37.8	1.01	38.8		
YU-12	0.12	7.0	0.67	34.4	0.84	32.2		
YU-62	0.11	6.6	0.90	46.2	0.71	27.3		
Mean		5.6		37.6		29.0		

^{a,b,c} Percentages calculated considering the absorbance of ascorbic acid in each solvent, as 100%.

Table 2: Radicals scavenger activities (ABTS/DPPH) and phenol contents of <i>M. alba</i> varieties

M. alba		DPPH ^a			ABTS ^b			Phenol ^c	
varieties	hexane	ethanol	aqueous	hexane	ethanol	aqueous	hexane	ethanol	aqueous
Cubana	1824.2	429.0	812.1	326.3	23.8	87.4	0.64	7.49	3.70
Indonesia	1916.6	733.6	1014.0	91.2	34.8	75.1	1.00	5.67	4.13
IZ 13/6	1215.0	642.2	866.8	509.0	29.1	112.3	0.55	7.29	6.61
IZ 15/7	563.4	599.4	654.3	158.6	36.5	87.4	1.59	6.90	3.50
IZ 40	539.6	568.8	791.5	89.9	28.6	69.0	1.25	7.09	2.89
IZ 56/4	1191.3	792.6	839.0	178.2	34.3	83.0	1.09	6.15	2.73
IZ 64	808.8	378.1	717.1	204.6	13.2	53.8	1.29	9.85	4.21
Murcia	593.2	667.7	912.9	169.2	37.8	94.4	1.31	5.09	3.53
Tigreada	1344.9	591.8	615.1	187.7	29.5	85.0	1.53	7.48	3.44
Universidad	2013.5	826.5	842.8	315.2	33.2	85.9	0.91	6.19	5.88
YU-12	1210.0	934.6	1069.8	290.1	32.2	96.6	1.10	5.63	5.00
YU-62	828.5	668.3	1181.2	180.6	26.0	120.5	1.28	7.48	4.10
Ascorbic acid	63.7	59.2	59.7	48.0	6.3	11.6			
							nd	nd	nd

^{a,b} extract concentration (µg/mL) capable of causing 50 % inhibition of DPPH/ABTS radical, in assay conditions (respectively). ^cµg gallic acid /100 g.

DPPH and ABTS radical scavenger assays allows evaluating ability of the extracts in stabilizing free radicals (Huang et al., 2005). In this way, M. alba extracts were also screened by these assays. The first one was conducted to observe DPPH radical scavenger activity and the concentration of each extract (µg/mL) able to inhibit 50% of DPPH radicals, under the conditions assayed, is shown in Table 2. The variety more active in this assay among the ethanol extracts was IZ 64, followed by Cubana variety (Table 2). These two cultivars presented extracts far more active than the other in general. It is noteworthy that one of them is a natural variety while the other one (IZ 64) is a hybrid one. Varieties Tigreada and IZ 15/7 presented the highest DPPH scavenging activities among the aqueous extracts, a value comparable to the activities of the corresponding ethanol extracts, showing that, in some case, extracting solvent is secondary in respect to the cultivar type on a bioassay response. It is worth mentioning that hexane extracts from varieties IZ 40, IZ 15/7 and Murcia were as active as some ethanol and aqueous extracts, reinforcing the previous observation on the predominance of the variety properties over solvent extraction, in some cases.

Radical ABTS scavenging activity of ethanol extracts was also assayed and the results are shown in Table 2, indicating that radical inhibition was dose dependent (Figure 2). In this assay, IC_{50} values found are inversely related to the antioxidant capacity,

therefore, variety IZ 64 presented the best activity in this assay among all varieties, when either water or ethanol were used in the extraction process, being more active when ethanol was used. Again, hexane extracts were only weakly active.



- ◆ - Ascorbic acid - ■ IZ 64 - ▲ - Cubana - ■ YU-62 · · ■ · · IZ 13/6
 Fig. 2: ABTS radical inhibition by *M. alba* roots ethanol extracts

Total phenolic compounds present in the extracts were evaluated revealing that such compounds were present in higher concentration in ethanol, followed by aqueous and then, by the hexane extracts (Table 1). The lack of phenolic compounds in the hexane extracts is related to the high polarity of such compounds and explain why activity predominates in the extracts prepared from more polar extracting solvents, ethanol and water. Ethanol extracts of IZ 64 variety showed the highest concentration of phenolic compounds (9.85 g gallic acid /100 g extract). Among aqueous extracts, variety IZ 13/6 presented the highest concentration of phenolic compounds, followed by varieties Universidad and YU-12 (6.61; 5.88 and 5.0 g gallic acid /100 g extract, respectively).

Variety IZ 64 presented outstanding highest flavonoids concentrations being far more active than all other extracts, regardless the extracting solvent used for extracts preparation (Figure 3). This high flavonoid content explains the high antioxidant presented by the extracts of this variety and is also an indicative on the improvements occurred as results of obtaining new hybrid cultivars. Three natural cultivars also presented high flavonoids contents (cubana, tigreada and YU-62), as well as another hybrid cultivar (IZ 40). In some cases, aqueous extracts presented comparable flavonoid contents in relation to the corresponding ethanol extracts; varieties IZ 13/6, IZ 64, IZ 15/7 and Indonesia were the ones more rich in flavonoids, ranging from 234.68 to 204.36 μ g quecertin /g extract, without significant difference (p < 0.05).



hexane ethanol aqueous
 Fig. 3: Total flavonoids content in *M. alba* roots ethanol extracts.

Total phenolic concentration of aqueous extracts studied in this work showed a positive correlation with total antioxidant activity (r = 0.78). Besides, total phenolic concentration of ethanol extracts presented strong positive correlation with ferric reducing power (r = 0.82). A strong negative correlation between total phenolic of ethanol and hexane extracts with the IC₅₀ ABTS inhibition (r = -0.91; -0.72) and DPPH (r = -0.77; -0.61) radicals was also observed.

In total antioxidant activity, ethanol root extracts showed to be 1.4 fold more active than aqueous ones. The later, in turn, showed activity 4.6 fold higher than hexane extracts. These values found to ethanol (708.7 mmol ascorbic acid/g extract) and aqueous extracts (493.4 mmol ascorbic acid/g extract) are much higher than values related for grape (*Vitis vinifera*) seeds, studied as methanol extracts (233.0 µmol ascorbic acid/g extract) (Jayaprakasha et al., 2003).

The extracts most efficient for DPPH radical scavenging among the varieties tested was IZ 64 (378 μ g/mL). Other *Morus* extracts have also been reported to be active in this assay (Arabshahi-Delouee and Urooj, 2007). In the present study, activity was detected for extracts from *M. alba* roots, showing that antioxidant compounds are thoroughly distributed in the plant, not relying only in the fruits. Polyphenols have been reported as potential antioxidants, protecting lipids and proteins against oxidative damage through their ability to scavenger metal ions and oxygen and nitrogen free radicals (Andallu *et al.*, 2014). The correlations found between phenolic compounds of ethanol extracts suggest that they act by different mechanisms of action.

All results corroborate that factors such as plant genetic variety affect the composition and concentration of phenolic compounds and, consequently, its antioxidant activity (Paredes-López *et al.*, 2010). At this context, among all extracts evaluated, ethanol extract of variety IZ 64 presented best antioxidant activity by iron reducing power (Table 1), free ABTS and DPPH radical scavenger (Table 2). Considering the high ABTS free-radical scavenging observed for all assayed extracts (ethanol, aqueous and hexane) of *M. alba* roots from variety IZ 64, it is possible to conclude that this variety has phytochemicals with different polarities possessing antioxidant capacity.

In addition, activities presented by IZ 64 were consistent with its high concentration of phenolic components (Table 2) and flavonoids (Figure 3), which were notably higher than the other ethanol extracts of *M. alba* varieties. Besides, the aqueous extract of the same variety IZ 64 presented the smallest IC₅₀ in ABTS assay (IC₅₀ = 53.81), the second higher concentration of flavonoids among the aqueous extracts (212.37 μ g quecertin/g extract), high values for DPPH inhibition (IC₅₀ = 717.11), total antioxidant activity (532 mmol ascorbic acid/g extract) and ferric reducing power (32.6% compared to ascorbic acid activity). Knowledge of antioxidant activity of the plants is crucial in exploiting their potential. In this way, these results suggest that among the varieties of *M. alba* evaluated, IZ 64 is the most promising as source of natural antioxidant benefits, as nutraceuticals or in food composition as preservatives.

CONCLUSION

This work showed the great potential of roots from *M. alba* as antioxidants, as well as detected differences between natural and hybrid varieties of this species related to their antioxidant activities. These differences were, at great extension, also dependent on the solvent used for extracting natural components present in the roots of the cultivars studied. In this way, hexane extracts, which contains mainly non polar compounds such as fatty acids and long-chain alkanes, were consistently the less active ones. Ethanol extracts were always the most active ones, independent on the variety assayed, probably due to the high levels of phenolic and flavonoids compounds that can be extracted

with ethanol. Antioxidant activity was corroborated by different assays, showing that the compounds present in the extracts act by different mechanisms (radical scavenger and iron reducing power). ethanol being the best solvent to extract compounds with antioxidant activity. Phytochemicals extracted from Morus alba roots with water also showed good antioxidant activities. These results suggest that the making of teas, soft drinks and juices from the roots of this plant could provide antioxidant benefits. Nevertheless, the activity of aqueous extracts was consistently smaller than activity of corresponding ethanol extracts, the former ones can be readily prepared and used by the population, being a good alternative for supply antioxidants to human diet. M. alba variety IZ 64 presented total phenolic and flavonoids concentration bigger than the other varieties screened. This was correlated to its great activity of capturing radicals DPPH, as well as its ability to reduce transition metals. Its ability to capture radical ABTS was also high, independent of the solvent used in the extraction. This shows that in addition to phenolic compounds, there are other components with different polarity and antioxidant activity in roots of M. alba.

This work broadens the scope of use of *M. alba* as human food with antioxidant activity (Khan *et al.*, 2013) showing the huge potential of roots from this species for applications in nutraceuticals and food preservatives industry.

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