Seeds of *Opuntia* spp. as a novel high potential by-product: phytochemical characterization and antioxidant activity

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ABSTRACT

Most of the nearly 1500 species in *Opuntia* genus contain health-promoting components. These species are able to grow under adverse conditions, not suitable for other fruits/vegetables. Despite the acknowledge interest of their xoconostle fruits, especially due to their exquisite taste, other botanical parts from *Opuntia* are being scarcely studied. Herein, it was intended characterize *Opuntia* spp. seeds for their composition in soluble sugars, organic acids, tocopherols and phenolic compounds, besides evidencing their potential as sources of essential fatty acids. In addition, their antioxidant activity was assessed through different *in vitro* biochemical assays. Seeds of *Opuntia* spp. presented phytochemical profiles that allow their classification as a high-interest by-product. The levels of linoleic acid, γ-tocopherol and phenolic acids, as also their high antioxidant activity, are amongst the top characteristics that might endorse *Opuntia* spp. seeds in novel applications in food or pharmaceutical industries.

Keywords: *Opuntia* spp; seeds; by-products; phytochemicals; antioxidant activity; HPLC-DAD-ESI/MS.

1. Introduction

There are nearly 1500 species of cactus belonging to the *Opuntia* genus (*Cactaceae*), which are mainly distributed in Africa, Mediterranean countries and other areas with similar climate conditions (Nassar, 2008). One of the most well-known species is cactus pear, a plant grown wild in arid and semiarid regions, where the production of other food plants is severely limited. Interestingly, it is considered as a delicacy in Mexico, United States, Mediterranean countries and South Africa (Ramadan and Mörsel, 2003). Besides its organoleptic qualities, the *Opuntia* genus was previously reported as having antioxidant, anti-inflammatory, anti-ulcerogenic, anti-fungicide, anti-bacterial and anti-diabetic properties. Furthermore, different botanical components like the pulp, cladode or flower, have also been studied for their chemical composition and nutritional value (Maataoui et al., 2006; Ammar et al., 2012; Morales et al., 2012; Chahdoura et al., 2014a,b). The seeds of *Opuntia* spp., which are tightly packed together in a mucilaginous structure inside the endocarp, contain high amounts of polyunsaturated fatty acids, especially linoleic and linolenic acids, which are known as having a wide variety of health benefits due to their role as biosynthetic precursors of eicosanoids (Simopoulos, 2002; Mannoubi et al., 2009). In fact, the xoconostle fruits are considered as being a promising raw material for their nutritional composition and antioxidant properties, but the seeds could also be recovered for their high nutritional value, and as sources of polyunsaturated fatty acids (PUFAs), tocopherols and dietary fiber, instead of being discarded, as it currently occurs (Prieto-García et al., 2006). In addition, the edible part of the fruit contains a relatively large number of seeds, which represent an important percentage of total fruit mass, on a dry weight basis (Habibi et al., 2008). Besides the mentioned, the definition of seeds as a novel by-product might be eased by evaluating other classes of phytochemicals such as the soluble sugars, organic acids, or, especially, the phenolic compounds. Regarding the latter, the available information is rather scarce, since most studies

are limited to global phenolic measurements, and only a few identified individual phenolic acids and flavonoids in *Opuntia* seeds (Tounsi-Saidani et al., 2011; Chougui et al., 2013).

Accordingly, this research work was conducted to characterize the seeds of *Opuntia* spp. regarding their chemical composition and antioxidant activity, in order to find a phytochemical profile that allows their definition as a novel by-product with potential to be used in food or pharmaceutical applications.

All analytical tasks were carried out on the seeds of two red varieties of *Opuntia*: *Opuntia macrorhiza* (Engelm.) and *Opuntia microdasys* (Lhem.), grown in Tunisia. This study was set within the framework of the valorization of the seeds of local plants, since it is expected that the main findings will provide more details about the bioactive substances from cactus seeds.

2. Materials and methods

2.1. Standards and Reagents

Acetonitrile (99.9%), n-hexane (97%) and ethyl acetate (99.8%) were of HPLC grade from Fisher Scientific (Lisbon, Portugal). The fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U) was purchased from Sigma (St. Louis, MO, USA), as also were other individual fatty acid isomers and standards: L-ascorbic acid, tocopherols (α-, β-, γ- and δ-isoforms), sugars (D(-)-fructose, D(+)-melezitose, D(+)-sucrose and D(+)-glucose), organic acids and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). Phenolic compounds were purchased from Extrasynthèse (Genay, France). Racemic tocol, 50 mg/mL, was purchased from Matreya (Pleasant Gap, PA, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA). All other chemicals and solvents were of analytical grade and purchased from common sources.

2.2. Samples

Fruits of *Opuntia macrorhiza* (Engelm.) and *Opuntia microdasys* (Lhem.) were manually harvested in spring (2013) from the cliff of Monastir (Tunisia) when the characteristics of maturation (size and skin colors) were according to conventional standards for this product. The seeds were cleaned by removing any mucilaginous material or pulp. The seeds were dried under shade, grounded with a Warring blender (Phillips, France), reduced to a fine dried powder (20 mesh), mixed to obtain a homogenate sample and stored at -20 °C protected from light, until analysis.

2.3. Chemical composition in hydrophilic compounds

2.3.1. Soluble sugars

Soluble sugars were determined by high performance liquid chromatography coupled to a refractive index detector (HPLC-RI). Dried sample powder (1.0 g) was spiked with melezitose as internal standard (IS, 5 mg/mL), and extracted with 40 mL of 80% aqueous ethanol at 80 ºC for 30 min. The resulting suspension was centrifuged (Centurion K24OR refrigerated centrifuge, West Sussex, UK) at 15,000*g* for 10 min. The supernatant was concentrated at 60 ºC under reduced pressure and defatted three times with 10 mL of ethyl ether, successively. After concentration at 40 ºC, the solid residues were dissolved in water to a final volume of 5 mL and filtered through 0.2 µm nylon filters from Whatman (Pinela et al., 2012). The equipment of analysis consisted of an integrated system with a pump (Knauer, Smartline system 1000, Berlin, Germany), degasser system (Smartline manager 5000), auto-sampler (AS-2057 Jasco, Easton, MD) and a RI detector (Knauer Smartline 2300). Data were analysed using Clarity 2.4 Software (DataApex, Prague, Czech Republic). The chromatographic separation was achieved with a Eurospher 100-5 NH_2 column (4.6 × 250 mm, 5 mm, Knauer) operating at 30 ºC (7971 R Grace oven). The mobile phase was

acetonitrile/deionized water, 70:30 (*v/v*) at a flow rate of 1 mL/min. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was performed using the internal standard method and sugar contents were further expressed in g per 100 g of dry weight (dw).

2.3.2. Organic acids extraction and analysis

Organic acids were determined following a procedure previously optimized and described by the authors (Barros et al., 2013). Samples (~2 g) were extracted by stirring with 25 mL of meta-phosphoric acid (25 ºC at 150 rpm) for 45 min and subsequently filtered through Whatman No. 4 paper. Before analysis, the sample was filtered through 0.2 µm nylon filters. Analysis was performed by ultra-fast liquid chromatograph (UFLC) coupled to photodiode array detector (PDA), using a Shimadzu 20A series UFLC (Shimadzu Cooperation, Kyoto, Japan). Separation was achieved on a SphereClone (Phenomenex, Torrance, CA, USA) and detection was carried out in a PDA, using 215 and 245 nm as preferred wavelengths. The organic acids found in the seeds were quantified by comparison of the area of their peaks recorded at 215 nm with calibration curves obtained from commercial standards of each compound. The results were expressed in g per 100 g of dry weight (dw).

2.3.3. Phenolic compounds extraction and analysis

The powdered seeds (~1 g) were extracted by stirring with 30 mL of methanol:water 80:20 (*v/v*), at room temperature, 150 rpm, for 1 h. The extract was filtered through Whatman nº 4 paper. The residue was then re-extracted twice with additional portions (30 mL) of methanol:water 80:20 (*v/v*). The combined extracts were evaporated at 35 °C (rotary evaporator Büchi R-210, Flawil, Switzerland) to remove methanol. The aqueous phase was lyophilized and the extracts were re-dissolved in 20% aqueous methanol at 5 mg/mL and

filtered through a 0.22-μm disposable LC filter disk for high performance liquid chromatography (HPLC-DAD-MS) analysis.

Phenolic compounds were determined by HPLC (Hewlett-Packard 1100, Agilent Technologies, Santa Clara, USA) as previously described by the authors (Barreira et al., 2013). Double online detection was carried out in the diode array detector (DAD) using 280 nm and 370 nm as preferred wavelengths and in a mass spectrometer (MS) connected to the HPLC system via the DAD cell outlet. MS detection was performed in an API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) equipped with an ESI source and a triple quadrupole-ion trap mass analyser that was controlled by the Analyst 5.1 software. The phenolic compounds were characterized according to their UV and mass spectra and retention times, and comparison with authentic standards when available. For the quantitative analysis of phenolic compounds, a 5-level calibration curve was obtained by injection of known concentrations (2.5-100 μg/mL) of different standards compounds. The results were expressed in μg per g of extract (dw).

2.4. Chemical composition in lipophilic compounds

Before analysing individual molecules, the fat content was determined following the AOAC procedure (Horwitz and Latimer, 2005).

2.4.1. Fatty acids

Fatty acids were determined after a transesterification procedure as described previously by the authors (Pinela et al., 2012), using a gas chromatographer (DANI 1000) equipped with a split/splitless injector and a flame ionization detector (FID at 260 ºC) and a Macherey-Nagel (Düren, Germany) column (50% cyanopropyl-methyl-50% phenylmethylpolysiloxane, 30 m × 0.32 mm i.d. × 0.25 μm d_f). Fatty acid identification was made by comparing the relative

retention times of FAME peaks from samples with standards. The results were recorded and processed using CSW 1.7 software (DataApex 1.7, Prague, Czech Republic). The results were expressed in relative percentage of each fatty acid.

2.4.2. Tocopherols

Tocopherols were determined following a procedure previously optimized and described by the authors (Pinela et al., 2012). Analysis was performed by HPLC (equipment described above), and a fluorescence detector (FP-2020; Jasco, Easton, MD, USA) programmed for excitation at 290 nm and emission at 330 nm. The chromatographic separation was achieved with a Polyamide II (250 mm × 4.6 mm i.d.) normal-phase column from YMC Waters operating at 30 ºC. The mobile phase used was a mixture of n-hexane and ethyl acetate (70:30, *v/v*) at a flow rate of 1 mL/min, and the injection volume was 10 µL. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was based on the fluorescence signal response of each standard, using the IS (tocol) method and by using calibration curves obtained from commercial standards of each compound. The results were expressed in mg per 100 g of dry weight.

2.5. Antioxidant activity

The hydromethanolic extract (prepared according to subsection *2.3.3. Phenolic compounds extraction and analysis*) was re-dissolved in methanol (final concentration 5 mg/mL); the final solution was further diluted to different concentrations to be submitted to antioxidant activity evaluation by different *in vitro* assays as described by the authors (Pinela et al., 2012). DPPH radical-scavenging activity was evaluated by using an ELX800 microplate reader (Bio-Tek Instruments, Inc; Winooski, USA), and calculated as a percentage of DPPH discolouration using the formula: $[(A_{DPPH}-A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of

the solution containing the sample at 515 nm, and A_{DPPH} is the absorbance of the DPPH solution.

Reducing power was evaluated by the capacity to convert Fe^{3+} into Fe^{2+}, measuring the absorbance at 690 nm in the microplate reader mentioned above.

Inhibition of β-carotene bleaching was evaluated through the β-carotene/linoleate assay; the neutralization of linoleate free radicals avoids β-carotene bleaching, which is measured by the formula: β-carotene absorbance after 2h of assay/initial absorbance) × 100.

Lipid peroxidation inhibition in porcine (*Sus scrofa*) brain homogenates was evaluated by the decreasing in thiobarbituric acid reactive substances (TBARS); the color intensity of the malondialdehyde-thiobarbituric acid (MDA-TBA) was measured by its absorbance at 532 nm; the inhibition ratio (%) was calculated using the following formula: [(A - B)/A] × 100%, where A and B were the absorbance of the control and of the sample solution, respectively.

The results were expressed in EC_{50} value (sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay). Trolox was used as positive control.

2.6. Statistical analysis

All extractions were performed in triplicate and each replicate was also analysed in triplicate. The results are expressed as means±standard deviations. Results were classified using a *t*-test for equality of means (after checking the equality of variances through a Levene's test), since there were fewer than three groups. All statistical tests were performed at a 5% significance level using the SPSS software, version 22.0 (IBM Inc).

3. Results and Discussion

3.1. Chemical composition in hydrophilic compounds

In previous studies performed in the flowers and cladodes of the same species studied herein, the composition in sugars and organic acids included the same compounds (Chahdoura et al., 2014a,b). Nevertheless, seeds showed qualitative differences in their soluble sugars composition. As it can be concluded from **Table 1**, sucrose was the only sugar detected in *O. macrorhiza*, while *O. microdasys* presented also fructose and glucose. In both cases, the overall concentrations of soluble sugars were quite lower than those detected in the respective flowers and cladodes, which might indicate that soluble sugars were mobilized by anabolic pathways to synthesize storage polysaccharides in the seed. As far as we know, no previous results for soluble sugars have been reported for the seeds of these species, although they are in the same magnitude range of those obtained for other *Opuntia* species, like *O. joconostle* and *O. matudae* (Morales et al., 2012).

The profiles in organic acids (**Table 2**) were also simpler than those presented for the flowers and cladodes in both species (Chahdoura et al., 2014a,b) due to the absence of citric acid (present in flowers and cladodes) and ascorbic acid (found in cladodes). Besides the lower number of detected organic acids, the total amounts quantified in either case were also smaller when compared to the botanical compounds produced earlier in the phenological cycle (Chahdoura et al., 2014a,b). Quinic acid (0.30 g/100 g dw) and oxalic acid (0.32 g/100 g dw) were the major organic acids detected in *O. microdasys* and *O. macrorhiza*, respectively.

Regarding the phenolic compounds, data of the retention time, λ_{max}, pseudomolecular ion, main fragment ions in MS^2, tentative identification and concentration of phenolic acid derivatives and flavonoids are presented in **Table 3**. Two exemplifying HPLC phenolic profiles, recorded at 280 nm, are presented in **Fig. 1** for *O. microdasys* (A) and *O. macrorhiza* (B).

With the exception of a flavonol derivative in *O. microdasys*, the UV and mass spectra obtained by HPLC-DAD-ESI/MS analysis showed that all detected phenolic compounds were hydroxycinnamoyl derivatives. Sugar substituents consisted of hexoses and deoxyhexoses, as deduced from the losses of 162 mu and 146 mu, respectively.

Compound 1, with a pseudomolecular ion ($[M-H]^-$) at *m/z* 341, was tentatively identified as a caffeoyl hexoside, according to its characteristic UV spectrum, showing maximum wavelength around 324 nm, and to the ions at *m/z* 179 (-162 mu, loss of a hexosyl residue; [caffeic acid-H]$^-$), 161 ([caffeic acid-H_2O-H]$^-$) and 135 ([caffeic acid-CO_2-H]$^-$). Similar reasoning can be applied to assign compound 4 ($[M-H]^-$ at *m/z* 487) as a coumaroyl di-hexoside, owing to the maximum wavelength around 308 nm and the observation of fragment ions at *m/z* 163 (-324 mu, loss of a two hexosyl residues; [coumaric acid-H]$^-$) and 119 (coumaric acid-CO_2-H]$^-$).

Compounds 2, 3, 6 and 7 presented the same pseudomolecular ion ($[M-H]^-$ at *m/z* 517) and similar UV and mass spectral characteristics. Three compounds with the same pseudomolecular ion and similar fragmentation pattern were reported by Chougui et al. (2013) in seeds of *Opuntia ficus-indica* and assigned as feruloyl-sucrose isomers, one of them being identified based on ^{1}H NMR data. This identity could also be assumed for the compounds detected in our samples, although in our case the nature of the sugar residues could not be established. The identification finds support in the observed fragmentation pattern. The loss of a di-hexosyl residue (-324 mu) would yield the fragment ion at *m/z* 193 ([ferulic acid-H]$^-$) and further product ions at *m/z* 175 ([ferulic acid-H_2O-H]$^-$), 149 ([ferulic acid-CO_2-H]$^-$) and 134 ([ferulic acid-CH_3-CO_2-H]$^-$).The ion at *m/z* 355 would result from the loss of a hexosyl moiety (-162 mu), and would give place to the fragments at *m/z* 337, by further loss of a water molecule, and at *m/z* 295, 265 and 235, by cross-ring cleavage of the remaining sugar residue (losses of 60, 90 and 120 mu, respectively). This fragmentation pattern was also described by

Quéméner and Ralet (2004) for feruloyl mono- and di-saccharides, according to whom the higher relative abundance of the ions at *m/z* 193 and 175, resulting from ester-bond cleavage on either side of the linked oxygen atom of the ferulic acid, would support that the sugar residue is attached to it by an ester linkage. As an example, the structure of one compound of this type identified by Quéméner and Ralet (2004) in sugar beet (*i.e.*, *O*-[6-*O*-(*trans*-feruloyl)-β-D-galactopyranosyl]-(1,4)-D-galactopyranose) is shown in the **Fig. 2** together with the proposed fragmentation pattern. In our case, the observation of four isomers might be explained by the existence of different positions of attachment of the sugars to the ferulic acid, different hexoses and/or *cis/trans* isomers. All in all, peaks 2, 3, 6 and 7 were tentatively assigned as feruloyl di-saccharides isomers.

Compound 5 ($[M-H]^-$ at *m/z* 489) should correspond to a ferulic acid derivative as revealed by the characteristic fragments at *m/z* 193 ($[\text{ferulic acid-H}]^-$), 175 ($[\text{ferulic acid-H}_2\text{O-H}]^-$), 149 ($[\text{ferulic acid-CO}_2\text{-H}]^-$) and 134 ($[\text{ferulic acid-CH}_3\text{-CO}_2\text{-H}]^-$). Furthermore, this compound must be structurally related to compounds 2, 3, 6 and 7, as suggested by their common fragments. The same compound was previously found in the flowers and cladodes of these species (Chahdoura et al., 2014a,b); however, the exact nature of the substituent attached to the feruloyl-hexose could not be established.

Compound 8 would correspond to a flavonoid derived from isorhamnetin (MS^2 fragment at *m/z* 315). The loss of 454 mu corresponds to two deoxyhexosyl (2×146 mu) and one hexosyl (162 mu) moieties. In fact, flavonol bearing deoxyhexosylhexoside substituents have been reported to occur in different *Opuntia* species, namely rutinosides (*i.e.*, rhamnosyl-glucosides) of quercetin and isorhamnetin (Stintzing and Carle, 2005). Furthermore, this same compound had been reported in *Opuntia microdasys* flowers and cladodes in recent studies of our group (Chahdoura et al., 2014a,b). Based on this precedent, the substituting sugars in the compounds

detected in the present samples were speculated to be rhamnose and glucose. Therefore, compound 8 was tentatively identified as isorhamnetin-*O*-(rhamnosyl)rutinoside.

3.2. Chemical composition in lipophilic compounds

The seeds from both species presented fat percentages (*O. microdasys*: 9.2 g/100 g dw; *O. macrorhiza*: 11.3 g/100 g dw) significantly higher than those reported in other *Opuntia* species (Morales et al., 2012).

Regarding fatty acids composition, in addirion to the tabled ones (**Table 4**), caproic acid (C6:0), caprylic acid (C8:0), capric acid (C10:0), lauric acid (C12:0), myristoleic acid (C14:1), pentadecanoic acid (C15:0), heptadecanoic acid (C17:0), eicosadienoic acid (C20:2n6), eicosatrienoic acid + heneicosanoic acid (C20:3n3 + C21:0), eicosapentaenoic acid (C20:5n3), tricosanoic acid (C23:0), lignoceric acid (C24:0) and tetracosenoic acid (C24:1) were also quantified, but in amounts below 0.1% (C20:5n3 and C24:1 were detected only in *O. macrorhiza*). The fatty acid profiles detected in the seeds of both species were similar, with linoleic acid (C18:2n6) as the major fatty acid (71% in *O. microdasys*; 74% in *O. macrorhiza*), followed by palmitic acid (C16:0, 13.9% in *O. microdasys*; 13.0% in *O. macrorhiza*) and oleic acid (C18:1n9, 10.0% in *O. microdasys*; 7.9% in *O. macrorhiza*). Interestingly, these profiles are quite dissimilar from those found for the flowers and cladodes of these species (Chahdoura et al., 2014a,b), in which the saturated fatty acids were predominant. However, the prevalence in diene fatty acids, and particularly C18:2n6, was previously reported in other *Opuntia* species (Ramadan and Mörsel, 2003; Morales et al., 2012). These findings might be understood as an incentive to use the seeds of these *Opuntia* species in different applications, since polyunsaturated fatty acids are documented as having health-promoting properties (Lands, 2014).

When compared with the flowers and cladodes, *Opuntia* seeds showed higher contents in tocopherols (**Table 5**), being γ-tocopherol the isoform quantified in highest amount (7.4 mg/100 g dw in *O. microdasys*; 5.8 mg/100 g dw in *O. macrorhiza*), in line with the observed for *O. joconostle* and *O. matudae* (Morales et al., 2012). These increased tocopherol levels might have been triggered due to a biochemical response to the high PUFA percentages, since tocopherols are recognized as effective lipophilic antioxidants (Jiang, 2014).

3.3. Evaluation of bioactive properties

The EC_{50} values calculated for each of the performed assays (**Table 6**) are in the range of those reported for the cladodes and flowers of these *Opuntia* species. Also, *O. macrorhiza* showed higher antioxidant activity than *O. microdasys* (DPPH scavenging activity, *O. microdasys*: 1.00 mg/mL; *O. macrorhiza*: 0.89 mg/mL; reducing power, *O. microdasys*: 1.11 mg/mL; *O. macrorhiza*: 0.60 mg/mL; inhibition of β-carotene bleaching, *O. microdasys*: 0.13 mg/mL; *O. macrorhiza*: 0.09 mg/mL; TBARS inhibition capacity, *O. microdasys*: 0.11 mg/mL; *O. macrorhiza*: 0.06 mg/mL), in agreement with the observed for those botanical components (Chahdoura et al., 2014a,b). The higher antioxidant activity observed in the seeds of *O. macrorhiza* might be associated with its fairly higher levels of phenolic compounds (**Table 3**), since the influence of an extract phenolic composition in the antioxidant capacity is a well-known fact (Lien et al., 1999).

4. Conclusion

As it was initially proposed, the seeds of *Opuntia* spp. presented phytochemical profiles which may allow considering this botanical parts as high interest by-products. Besides the already known predominance of linoleic acid among the fatty acids, the levels of γ-tocopherol are also matter of interest, as it is the fairly high antioxidant activity shown by the EC_{50} values

obtained for each of applied assays. Furthermore, the phenolic composition gave interesting values in di-hexosyl-ferulic acid (different isomers). Overall, the clarification of their phytochemical profile establishes useful information to use *Opuntia* spp. seeds in novel food and/or pharmaceutical applications.

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Table1. Soluble sugars composition (g/100 g dw) of *Opuntia microdasys and Opuntia macrorhiza* seeds. Results are presented as mean± SD.

	Levene's test	*Opuntia microdasys*	*Opuntiamacrorhiza*	*t*-test (n = 9)
Fructose	-	0.46± 0.03	nd	-
Glucose	-	0.52±0.03	nd	-
Sucrose	$p = 0.527$	0.30±0.02	0.48±0.03	$p < 0.001$
Soluble sugars	$p = 0.281$	1.29±0.02	0.48±0.03	$p < 0.001$

nd (not detected)

Table 2. Organic acids composition (g/100 g dw) quantified in the seed of *Opuntia microdasys* and *Opuntia macrorhiza*. Results are presented as mean± SD.

	Levene's test	*Opuntia microdasys*	*Opuntia macrorhiza*	*t*-test (n = 9)
Oxalic acid	$p < 0.001$	0.23±0.01	0.32±0.01	$p < 0.001$
Quinic acid	$p < 0.001$	0.30±0.05	0.11±0.01	$p < 0.001$
Malic acid	$p = 0.010$	0.045±0.005	0.018±0.002	$p < 0.001$
Total organic acids	$p < 0.001$	0.58±0.05	0.44±0.01	$p < 0.001$

Table 3. Retention time (Rt), wavelengths of maximum absorption (λ_{max}), mass spectral data, relative abundances of fragment ions, tentative identification and quantification of the phenolic compounds in seeds of *Opuntia microdasys* (G1) and *Opuntia macrorhiza* (G2).

Peak	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Quantification (μg/g extract)		Levene's test	*t*-test (n = 9)
						G1	**G2**		
1	6.2	324	341	179(26), 161(13), 135(17)	Caffeoyl hexoside	46±3	33±2	$p = 0.024$	$p < 0.001$
2	7.2	326	517	355(2), 337(7), 295(3), 265(2), 235(2), 193(86), 175(54), 149(9), 134(18)	Feruloyl di-hexoside	35±3	47±2	$p = 0.010$	$p < 0.001$
3	7.5	324	517	355(3), 337(3), 295(3), 265(5), 235(5), 193(56), 175(34), 149(5), 134(10)	Feruloyl di-hexoside	45±2	64±3	$p = 0.016$	$p < 0.001$
4	9.3	308	487	163(54), 119(21)	Coumaroyl di-hexoside	24±1	37±1	$p = 0.013$	$p < 0.001$
5	10.0	330	489	337(4), 355(3), 295(11), 235(20), 193(55), 175(26), 149(4), 134(7)	Feruloyl hexoside derivative	25±2	77±3	$p = 0.088$	$p < 0.001$
6	10.5	326	517	355(3), 337(5), 295(5), 265(8), 235(14), 193(40), 175(19), 149(4), 134(6)	Feruloyl di-hexoside	26±1	41±3	$p = 0.009$	$p < 0.001$
7	11.5	328	517	355(3), 337(4), 295(9), 265(7), 235(31), 193(37), 175(27), 149(3), 134(6)	Feruloyl di-hexoside	225±4	718±13	$p = 0.002$	$p < 0.001$
8	19.3	356	769	315(100)	Isorhamnetin-*O*-(rhamnosyl)rutinoside	24±2	nd	-	-
					Phenolic compounds	**450±11**	**1016±19**	$\boldsymbol{p = 0.225}$	$\boldsymbol{p < 0.001}$

nd (not detected)

Table 4. Fatty acids composition (relative percentage) of *Opuntia microdasys* and *Opuntia macrorhiza* seeds. Results are presented as mean± SD.

Fattyacids	Levene's test	*Opuntia microdasys*	*Opuntia macrorhiza*	*t*-test (n = 9)
C14:0	$p = 0.278$	0.13±0.01	0.15±0.01	$p < 0.001$
C16:0	$p = 0.014$	13.9±0.4	13.0±0.3	$p < 0.001$
C16:1	$p = 0.170$	0.51±0.02	0.59±0.01	$p < 0.001$
C18:0	$p = 0.083$	2.8±0.1	2.9±0.1	$p = 0.581$
C18:1n9	$p = 0.022$	10.0±0.3	7.9±0.2	$p < 0.001$
C18:2n6	$p = 0.001$	71±1	74±1	$p < 0.001$
C18:3n3	$p < 0.001$	0.27±0.04	0.67±0.04	$p < 0.001$
C20:0	$p = 0.005$	0.27±0.03	0.27±0.02	$p = 0.477$
C20:1n9	$p = 0.332$	0.11±0.01	0.11±0.02	$p = 0.009$
C22:0	$p = 0.049$	0.18±0.01	0.17±0.01	$p = 0.270$
SFA	$p = 0.916$	17.6±0.4	16.7±0.3	$p < 0.001$
MUFA	$p = 0.175$	10.6±0.3	8.6±0.2	$p < 0.001$
PUFA	$p = 0.425$	72±1	75±1	$p < 0.001$

nd (not detected)
Myristic acid (C14:0); Palmitic acid (C16:0); Palmitoleic acid (C16:1); Stearic acid (C18:0); Oleic acid (C18:1n9); Linoleic acid (C18:2n6); α-Linolenic acid (C18:3n3); Arachidic acid (C20:0); Eicosenoic acid (C20:1n9); Behenic acid (C22:0).

Table 5. Tocopherols composition of *Opuntia microdasys* and *Opuntia macrorhiza* seeds. Results are presented in mg/100 g of dry weight as mean± SD.

	Levene's test	*Opuntia microdasys*	*Opuntia macrorhiza*	*t*-test (n = 9)
α-tocopherol	$p = 0.002$	2.0±0.1	0.42±0.02	$p < 0.001$
β-tocopherol	-	0.06±0.01	nd	-
γ-tocopherol	$p = 0.006$	7.4±0.3	5.8±0.1	$p < 0.001$
δ-tocopherol	$p = 0.559$	0.030±0.003	0.033±0.003	$p = 0.066$
Total tocopherols	$p = 0.018$	9.5±0.3	6.3±0.2	$p < 0.001$

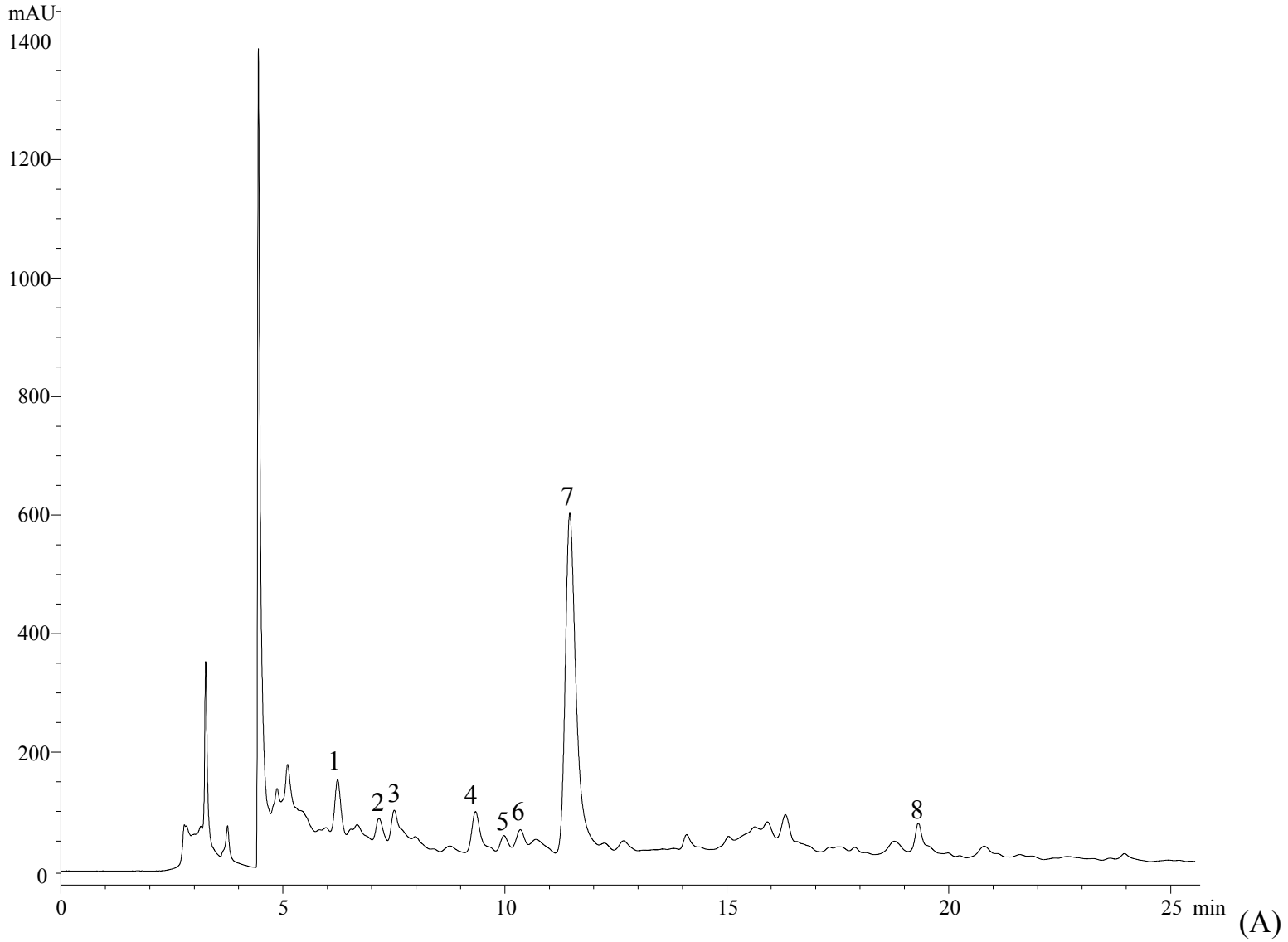
nd (not detected)

Table 6. Antioxidant activity (EC_{50}, mg/mL) of methanolic extracts obtained from the seeds of *Opuntia microdasys* and *Opuntia macrorhiza.* Results are presented as mean± SD.

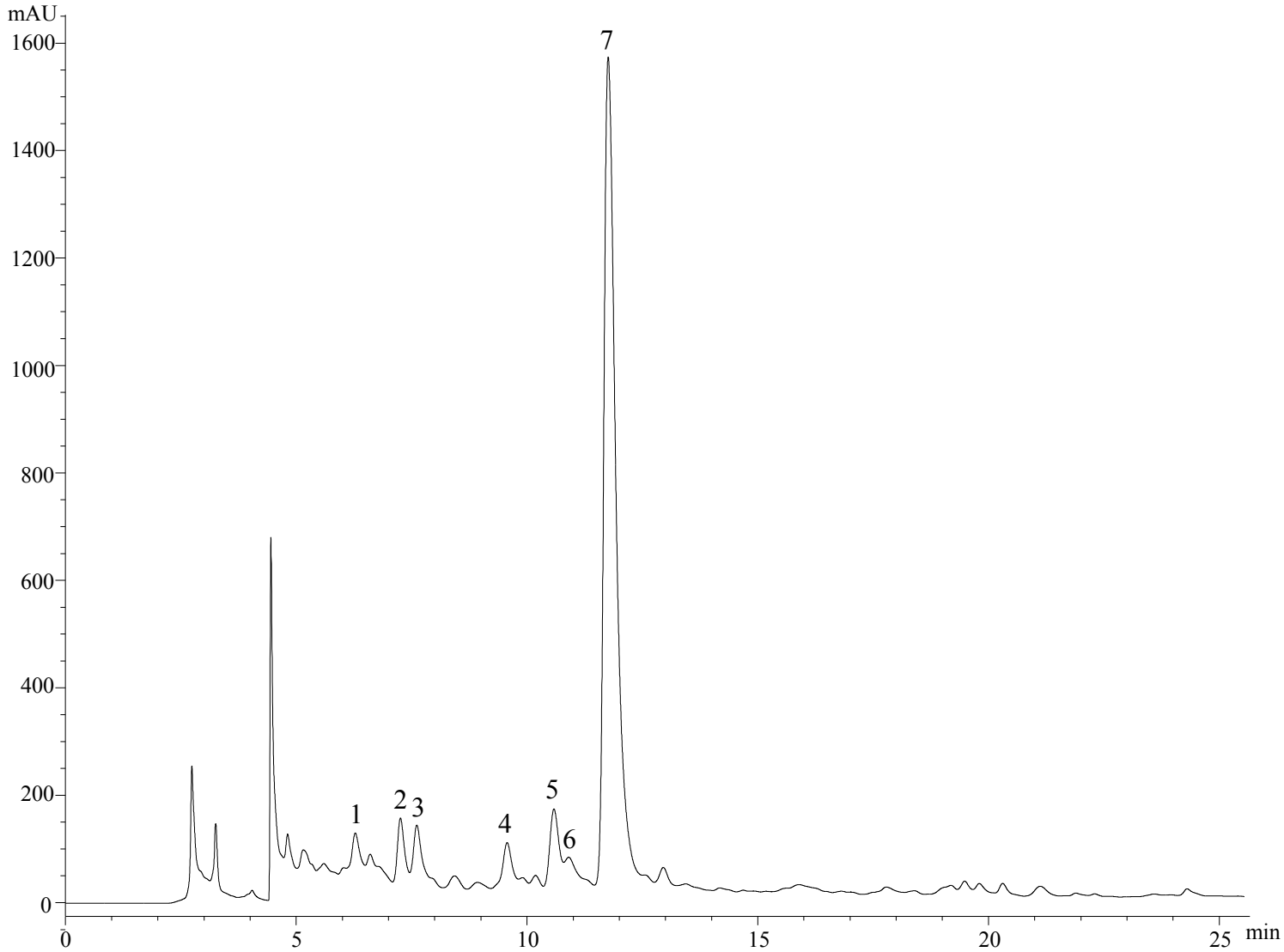
	Levene's test	*Opuntia microdasys*	*Opuntia macrorhiza*	*t*-test (n = 9)
DPPH scavenging activity	$p = 0.017$	1.10±0.01	0.60±0.01	$p < 0.001$
Reducing power	$p = 0.737$	0.53±0.01	0.39±0.01	$p < 0.001$
β-carotene bleaching inhibition	$p = 0.424$	0.31±0.01	0.18±0.01	$p < 0.001$
TBARS inhibition	$p = 0.007$	0.21±0.01	0.14±0.01	$p < 0.001$

Figure 1. HPLC chromatograms of the phenolic compounds of *Opuntia microdasys* (A) and *Opuntia macrorhiza* (B) seeds recorded at 280 nm. 1- Caffeoyl hexoside; 2- Feruloyl di-hexoside; 3- Feruloyl di-hexoside; 4- Coumaroyl di-hexoside; 5- Ferulic acid derivative; 6- Feruloyl di-hexoside; 7- Feruloyl di-hexoside; 8- Isorhamnetin-O-(rhamnosyl)rutinoside.

Figure 2. MS spectrum of feruloyl di-hexoside and chemical structure of an exeplifying isomer: *O*-[6-*O*-(*trans*-feruloyl)-β-D-galactopyranosyl]-(1,4)-D-galactopyranose.



(A)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(B)

Figure 1.

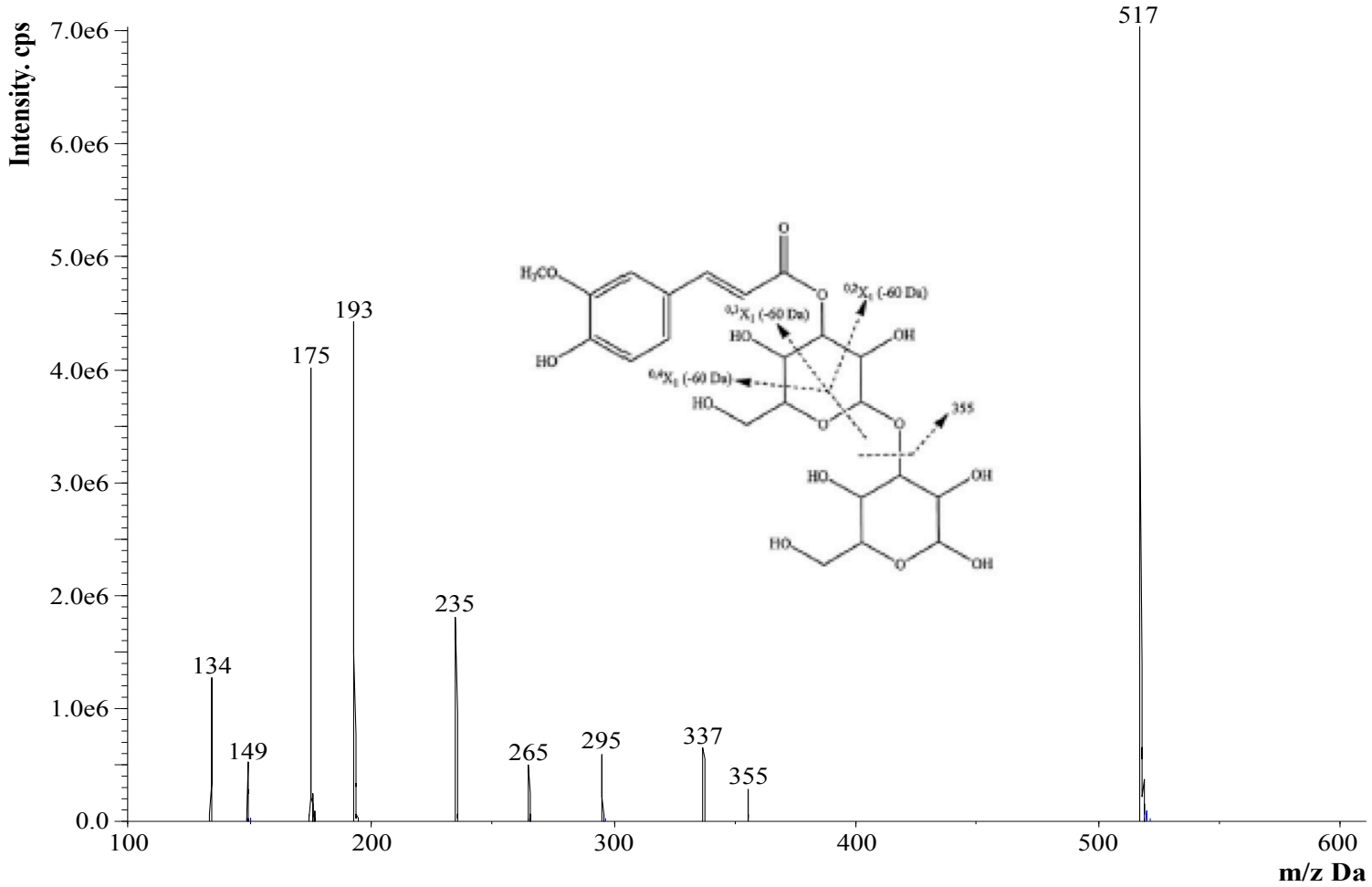

Figure 2. MS spectrum of feruloyl di-hexoside and chemical structure of an exeplifying isomer: **O**-[6-**O**-(**trans**-feruloyl)-_-**d**-galactopyranosyl]-(1,4)-**d**-galactopyranose.