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Water-soluble polysaccharides from *Opuntia stricta* Haw. fruit peels: recovery, identification and evaluation of their antioxidant activities**

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A b s t r a c t. *Opuntia stricta* Haw. is considered as one of the most common cactus plant growing in Tunisia. Extracting valuable compounds from its fruit peel, considered as by-product, is drawing more and more attention, making it on the verge of commercialization. Water-soluble polysaccharides were extracted from *Opuntia stricta* Haw. peels, and their chemical composition assessed using thin layer chromatography. The antioxidant activities of the extracted polysaccharides were assessed using 2,2-diphenyl-1-picrylhydrazyl free radical scavenging activity, total antioxidant activity and reducing power capacity. The extraction yield of water-soluble polysaccharides was 7.53±0.86%. The chemical composition revealed the presence of rhamnose, arabinose, glucose, mannose, galactose and galacturonic acid. The infra-red spectroscopic analysis showed a similar structure to that of *Opuntia ficus-indica* polysaccharide peels. Additionally, the extracted polysaccharides exhibited high antioxidant activities. In fact, the free radical scavenging activity (half inhibition concentration = 6.5 mg ml^{-1} with 94.9% inhibition at 50 mg ml^{-1}), the total antioxidant activity (100 µg ascorbic acid equivalent at 50 mg polysaccharides) and the reducing power capacity (absorbance 700 nm = 0.7 at 50 mg ml^{-1}), appeared to be interesting compared to natural and synthetic antioxidants. Therefore, water-soluble polysaccharides from *Opuntia stricta* Haw. fruit peels could be a natural alternative to replace synthetic antioxidants.

K e y w o r d s: *Opuntia stricta* Haw. peels, polysaccharide extraction, water-soluble polysaccharides, antioxidant activity

INTRODUCTION

Plant extracts constitute natural sources of antioxidant compounds (Dent *et al.*, 2013; Polya, 2003; Šic Žlabur *et al.*, 2015). They are hence used in numerous applications ranging from phytotherapy to the modern food industries (Belton *et al.*, 2003). The extraction of antioxidant molecules represents an important way of agricultural waste valorisation, especially due to the technological advances in molecular separation and identification (Roselló-Soto *et al.*, 2015; Wijngaard *et al.*, 2012), as well as due to the uses of emerging technologies replacing thus the conventional ones (Galanakis, 2012, 2013). Numerous antioxidant molecules derived from plant extracts have been widely used as additives in food formulation (Sinha *et al.*, 2008), in part due to their biological activities (Roselló-Soto *et al.*, 2015). To address the problems of oxidation and contamination of foodstuffs, many synthetic antioxidant molecules are often added. However, regarding their potential toxicity and carcinogenicity, they have been restricted by legislation in many countries (Madhavi *et al.*, 1996). Hence, their replacement by natural molecules has been widely studied. The antioxidant potential of different polysaccharides has been demonstrated. Moreover, they are harmless and do not cause side effects (Warrand, 2006). In addition, these high molecular weight polymers may exhibit therapeutic properties *eg* anti-tumor, anti-inflammatory, and anti-microbial activities (Caili *et al.*, 2007; Chen *et al.*, 2008; Krichen *et al.*, 2015; Leung *et al.*, 2006; Mokni Ghribi *et al.*, 2015). All of these activities and others are closely related to the physicochemical properties of each polysaccharide molecule such as the type of the sugar residues, the chemical composition, the molecular weight and the degree of branching. Numerous research groups have been interested

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in extracting polysaccharides from agricultural and industrial by-products for their valorisation (Galanakis, 2011; Galanakis and Schieber, 2014; Sila *et al.*, 2014a).

Extracting polysaccharides from opuntia fruit peels, especially from the ficus-indica species, has been studied due to its high availability as agro-industrial by-product in arid and semi-arid regions (Habibi *et al.*, 2004; Majdoub *et al.*, 2010). In Tunisia, *Opuntia stricta* Haw. is the second widespread cactus plant growing after *O. ficus-indica*. Numerous studies have been conducted on *O. stricta* Haw. peels, such as the extraction and characterisation of polyphenols, flavonoids, betacyanins (Yeddes *et al.*, 2013) and dyes (Obón *et al.*, 2009), making it on the verge of commercialization.

In this work, chemical composition of *Opuntia stricta* Haw. peels were investigated. Water-soluble polysaccharides (WSP) were extracted from *O. stricta* Haw. peels, their structure was investigated using Fourier transform infrared spectroscopy, and their chemical composition was assessed using thin layer chromatography. DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging activity, total antioxidant activity, and reducing power capacity of the extracted WSP were also studied.

MATERIAL AND METHODS

Potassium ferricyanide was purchased from Loba Chemie (India). Sodium phosphate, sodium tetraborate, sodium dodecyl sulphate, ferric chloride, DPPH, trichloroacetic acid, trifluoroacetic acid, Tris, petroleum ether, glucose, arabinose, mannose, xylose, rhamnose, galactose, galacturonic acid, polygalacturonic acid, cellulose, citrus peel pectin and beechwood xylan were obtained from Sigma-Aldrich (France). Sulphuric acid and ethanol were obtained from Sharlab (Spain). Ammonium molybdate was obtained from NenTech Ltd (United Kingdom).

Opuntia stricta Haw. fruits were collected in the suburb of Sfax city, Tunisia, in February 2014. They were composed of 69% peel, 21% pulp and 10% seeds. The peels were separated manually from the pulps and the seeds, and then blended using a kitchen mixer. The obtained viscous peel juice was frozen at -20°C until analysis.

Dry matter was determined according to AFNOR standards (AFNOR, 1982). Total nitrogen content was determined by Kjeldahl method (AFNOR, 1977). Soluble proteins were extracted using 1 g of peel juice mixed with 1 ml of extraction buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl and 1% sodium dodecyl sulphate). The mixture was vigorously vortexted for 10 min, then centrifuged for 15 min at 13,000 r.p.m. The extraction was repeated four times and the supernatants were pooled together. The extracted proteins were quantified using Bradford (1976) method and BSA as standard curve. Lipid content was determined according to Soxhlet method (AFNOR, 1981), using 1 g of dried peels for 12 h at 105°C, and petroleum ether as solvent. Total sugar content was determined according to Dubois *et al.* (1956). Total ash was determined by the combustion of 5 g peel juice in a muffle furnace at 550°C for 4 h. After dry ashing, the mineral composition (Na, Mg and Ca) was determined by flame atomic absorption spectrometry (Analytic Jena ZEEnit 700 spectrometer, USA) (Jorhem, 2000).

Water-soluble polysaccharides (WSP) were extracted from *O. stricta* Haw. peels as described previously with slight modifications (Ding *et al.*, 2012). 100 g of *O. stricta* Haw. peel juice was mixed with 500 ml distilled water in 1 l round bottom flask. The mixture was then boiled with reflux for 4 h using heating mantle. The mixture was then recovered, filtered through Whatman paper using Büchner funnel. The pellet was then extracted a second time under the same conditions and the filtrates containing WSP were pooled and concentrated 20 times using a rotary evaporator system at 50°C. WSP were recovered overnight by adding 100 ml absolute ethanol at -20°C, followed by 15 min centrifugation at 5 000 r.p.m. Five washing steps were performed in order to remove the colorants from the extracted polysaccharides. Each step consisted of resolubilising the WSP in 20 ml distilled water, followed by adding one volume of absolute ethanol, an overnight precipitation and a centrifugation step as described above. A dialysis step was performed on the recovered WSP using bi-distilled water for 3 days, in order to remove the salts. The extracted WSP were freeze-dried overnight, and visualised using an XL30 ESEM scanning electron microscope.

Monosaccharide composition of the extracted polysaccharides was determined after acid hydrolysis. 2 mg of lyophilized WSP was mixed with 0.5 ml of trifluoroacetic acid (2 N) and heated at 120°C for 2, 4, and 6 h. The mixture was then completely dried under nitrogen flow, then solubilised in 100 µl distilled water. The obtained hydrolysate (5 µl from each solution) was then analyzed with thin layer chromatography using silica gel plate 60 F254 (Millipore) as previously described (Ben Jeddou *et al.*, 2014). Glucose, arabinose, mannose, xylose, rhamnose, galactose and galacturonic acid were used as standards for compounds identification (5 µl from each standard solution at 4 mg ml^{-1}). A mixture of chloroform / acetic acid / water (6:7:1 v/v) was used as the mobile phase. After the separation of the compounds, the plate was dried and pulverised with a mixture of absolute ethanol / concentrated sulphuric acid (95:5 v/v). Drying at 110°C for 10 min allowed the revelation of the different spots.

Uronic acid content was determined using the carbazole method (Bitter and Muir, 1962) with slight modifications. 200 µl of a solution of S1 (0.025 M sodium tetraborate in sulphuric acid) was mixed with 40 µl of WSP solution (10 mg ml^{-1}). The mixture was then vortexted and incubated for 15 min at 100°C, followed by cooling at 4°C. The second step consisted of adding 8 µl of 0.125% carbazole solution prepared in absolute ethanol. The mixture was

then vortexed and incubated as previously described. The absorbance was measured at 490 nm and uronic acid content was determined using a standard curve prepared under the same conditions with polygalacturonic acid standard.

The absorption spectrum of lyophilised WSP was obtained using FTIR (Fourier transform infrared) spectroscopy (Analect Instrument fx-6 160) and was compared to FTIR spectra of citrus peel pectin, cellulose, and beechwood xylan. 1 mg from each sample was mixed with 100 mg KBr and the transmission (%) was recorded between 450 and 4 000 cm^{-1}.

The water activity (Aw) of WSP was assessed using a Novasina AW SPRINT TH-500 instrument (Switzerland). The measurement consisted of introducing lyophilized WSP to the instrument capsule and determining the Aw at 25°C.

The ability of WSP to scavenge free radicals was determined using the synthetic free radical compound DPPH, according to (Bersuder *et al.*, 1998) with slight modifications. Different amounts of WSP (3 mg to 50 mg) were prepared in 500 µl of distilled water, and then mixed with 125 µl of DPPH solution (0.02 % in ethanol) and 375 µl of absolute ethanol. All tubes were then shaken and incubated for 60 min in the dark at room temperature. The scavenging activity was recorded at 517 nm using a Shimadzu UV/VIS mini 1240 spectrophotometer. The absorption at 517 nm of the DPPH in its radical form decreases in the presence of an anti-radical compound (A_{sample}). For each WSP concentration, a blank was performed using the same amount of WSP, without DPPH (A_{blank}). A control experiment was performed by mixing 125 µl of DPPH solution and 875 µl of ethanol under the same conditions, and the absorbance was recorded as ($A_{control}$). The free radical scavenging activity (% inhibition) was then calculated as follows:

$$Inhibition = \frac{A_{control} + A_{blank} - A_{sample}}{A_{control}} 100 .$$

Total antioxidant activity of WSP was assayed as described previously (Koubaa *et al.*, 2015), with slight modifications. Different amounts of WSP (0.1 to 50 mg) were mixed with 1 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The mixtures were adjusted to 1.1 ml with distilled water and incubated for 90 min at 90°C. After cooling to room temperature, the absorbance was measured at 695 nm and the total antioxidant activity was expressed as ascorbic acid equivalent using a standard curve previously established. A mixture containing 1 ml of reagent and 100 µl of distilled water was incubated under the same conditions and used as blank.

Reducing power capacity was measured according to Yildirim *et al.* (2001). A mixture of 0.5 ml of a solution containing 0.5 mg to 50 mg WSP, 1.25 ml phosphate buffer (0.2 M, pH 6.6) and 1.25 ml potassium ferricyanide (1% in water, w/v) was incubated at 50°C for 20 min. After cooling to room temperature, 1 ml of trichloroacetic acid (10% in water, w/v) was added to the reaction mixture. After 10 min centrifugation at 3000 r.p.m., 1.5 ml of the supernatant was mixed with 1.5 ml of distilled water and 100 µl of fresh ferric trichloride (0.1% in water, w/v). The reaction mixture was shaken and its absorbance was measured at 700 nm against a blank (water was used instead of WSP solution). The absorbance is proportional to the reduction capacity of the sample. As reference curves, the reducing capacities of butylated hydroxyanisole (BHA) and ascorbic acid (0.01 and 50 mg) were determined under the same conditions.

All experiments were carried out in triplicate, and average values with standard deviation (SD) errors are reported. Significant differences between the results were calculated by multiple sample comparison of the means (ANOVA), with a significance level of $p<0.05$, using the software SPSS Version 22 (IBM® SPSS® Statistics, USA).

RESULTS AND DISCUSSION

Table 1 shows the chemical composition of *O. stricta* Haw. fruit peels determined as described in the material and methods section. The obtained dry matter for *O. stricta* was similar to *O. ficus indica* (9.67%) (Nebbache *et al.*, 2009). Protein content, determined by Kjeldahl and Bradford methods, revealed no significant differences with an average of 3.7 ± 0.07%, dry matter basis. In general, opuntia has low protein content (3.2 to 5.0%, dry matter basis) (Tibe *et al.*, 2008), and our results were concordant with protein content found in *O. joconostle* (3.22%) (Reyes-Agüero *et al.*, 2006). However, lower amounts were recorded for *O. ficus indica* (1.45%) (Nebbache *et al.*, 2009) and *O. matudae* (1.65%) (Guzmán-Maldonado *et al.*, 2010). Lipid content in *O. stricta* fruit peels, determined by Soxhlet method (1.85 ± 0.37 %), was comparable to the

T a b l e 1. Chemical composition of *Opuntia stricta* Haw. fruit peels

Parameter	Value
Dry matter (%)	11.33 ± 0.47
Proteins (%)	
Kjeldahl method	3.77 ± 0.11
Bradford method	3.63 ± 0.13
Lipids (%)	1.85 ± 0.37
Total sugars (%)	27.25 ± 0.14
Ash (%)	3.115 ± 0.035
Minerals (mg100 g^{-1} dry matter)	
Magnesium	722.18
Calcium	6 280
Sodium	1 399

one obtained for *O. ficus indica* (2.43%) (El Kossori *et al.*, 1998). The total sugars content recorded for *O. stricta* fruit peels was 27.25 ± 0.14%. No data have been reported in literature describing the total sugar content in opuntia fruit peels. Total ash was not significantly different from the fruit peels of *O. ficus indica* (3.05%) (Nebbache *et al.*, 2009), however, higher mineral contents were found (Table 1). In fact, it has been shown that *O. ficus indica* fruit peels contain: 15.7, 15.2, and 1.1 mg ml^{-1} calcium, magnesium, and sodium, respectively (Nebbache *et al.*, 2009). For *O. matudae*, the mineral contents recorded were: 1.41, and 0.51 g ml^{-1} calcium and magnesium, respectively (Guzmán-Maldonado *et al.*, 2010).

Water-soluble polysaccharides (Fig. 1) were extracted from *O. stricta* Haw. fruit peels with a yield of 7.53 ± 0.86%. This yield was higher than that obtained for *O. ficus indica* – 0.48% (Majdoub *et al.*, 2010), and *O. milpaalta* – 0.7% (Cai *et al.*, 2008), which is probably related to the different extraction procedures.

Water activity is considered as one of the most important parameters in food preservation and processing. It is now generally accepted that aw is more closely related to the microbial, chemical, and physical properties of foods and other natural products than the total moisture content. The obtained aw of WSP was 0.399, indicating a low and delayed non-enzymatic browning reactions as well as total absence of enzymatic activities and microorganism development (Barbosa-Cánovas *et al.*, 2007).

FTIR spectroscopy was performed on the extracted WSP, between 450 and 4000 cm^{-1}, in order to characterize their structure and purity. As shown in Fig. 2, the characteristic transitions of WSP were observed at 3 000-3 700, 1 500-1 770, and 950-1 200 cm^{-1}. These features are characteristic of polysaccharidic structure. Similar results have been reported previously (Sila *et al.*, 2014b; Wu, 2009; Yao *et al.*, 2003). The peak at 3 398 cm^{-1} represents the stretching of the hydroxyl groups. The small band at 2 922 cm^{-1} is attributed to C-H stretching and bending vibrations, as previously reported (Sila *et al.*, 2014b). Furthermore, the peak observed at 1634 cm^{-1} is due to the stretching vibrations of C-O bonds. The bands at 1 073 cm^{-1} and 1 039 cm^{-1} indicate the presence of pyranose units (Hua *et al*., 2014; Sila *et al.*, 2014b; Zhao *et al.*, 2005). The comparison between WSP spectra and the performed standards – citrus peel pectin, cellulose and beechwood xylan – shows similar spectra, indicating the polysaccharidic structure of WSP as well as its purity. Similar spectrum was observed for O. ficus indica peel polysaccharides (Majdoub *et al.*, 2001).

The chemical composition of WSP was assessed with thin layer chromatography (Fig. 3), using monosaccharides as standards (glucose, arabinose, mannose, xylose, rhamnose, galactose and galacturonic acid). The WSP hydrolysates, after 2 h, 4 h and 6 h acidic hydrolysis, were analyzed. The results obtained show that WSP are mainly composed of rhamnose, galactose and galacturonic acid. Minor monosaccharides (glucose, arabinose and mannose) were also present even after 2 h hydrolysis. However, two

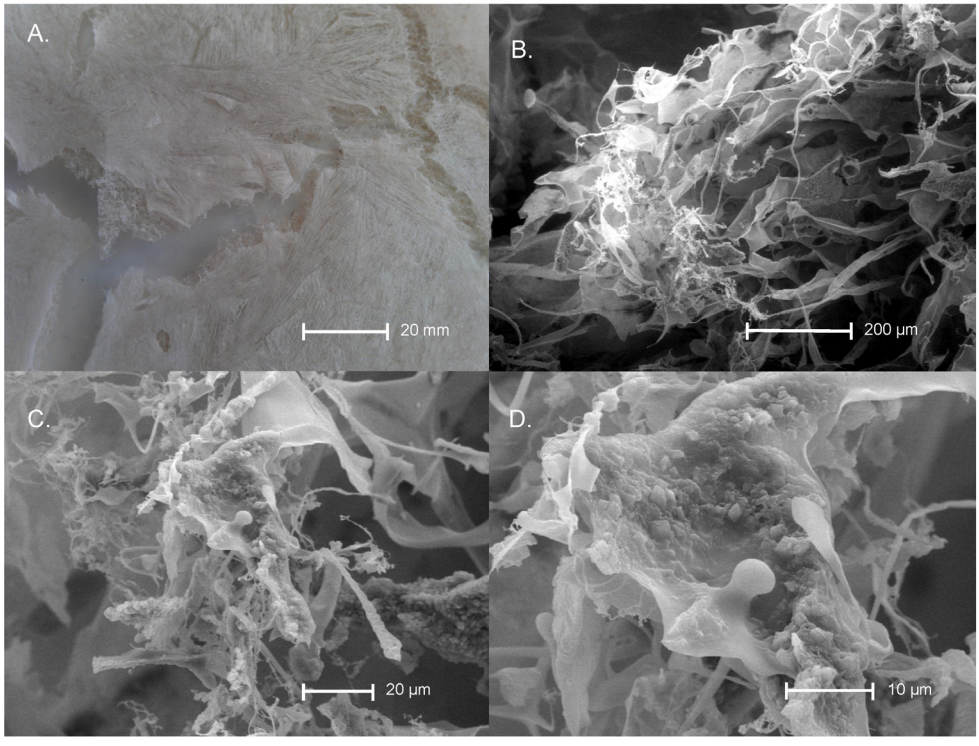

Fig. 1. A – lyophilised water-soluble polysaccharides extracted from *Opuntia stricta* Haw. fruit peels, B, C, D – scanning electron microscopy of the lyophilised WSP, at 150x, 1000x and 2500x magnification, respectively.

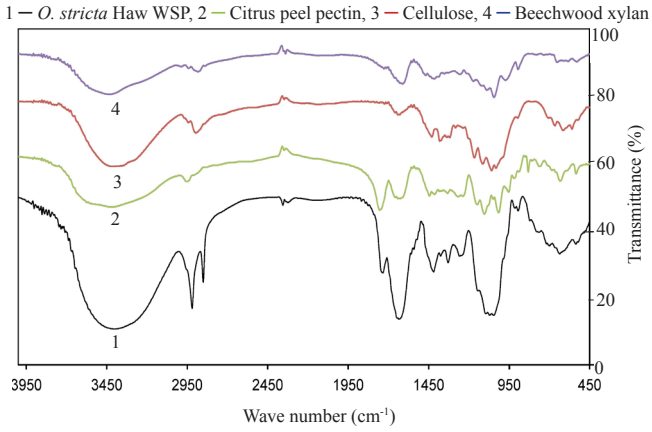

Fig. 2. FTIR spectroscopy of water-soluble polysaccharides extracted from *Opuntia stricta* Haw. fruit peels (WSP), citrus peel pectin, cellulose, and beechwood xylan. Spectra were performed in transmittance (%) between 450 and 4 000 cm^{-1}.

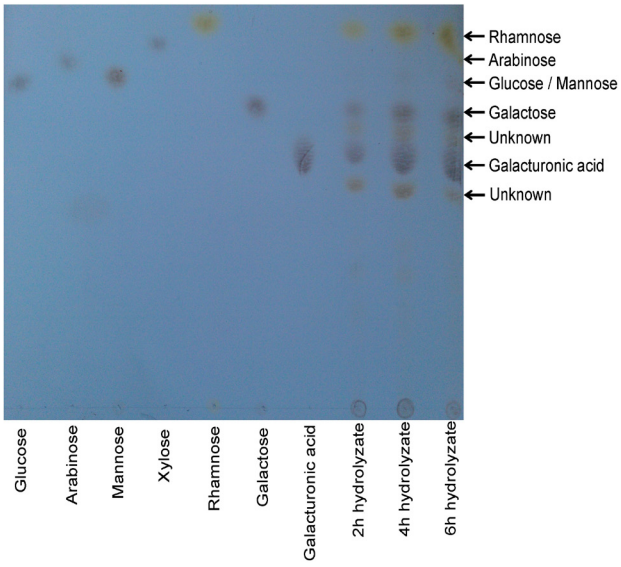

Fig. 3. Thin layer chromatography after 2, 4, and 6 h acidic hydrolysis of WSP. Spots were identified using sugar standards. The experiment was performed in duplicate.

spots were not identified, corresponding probably to other monosaccharides or oligosaccharides after partial hydrolysis. The WSP composition was similar to that obtained for *O. ficus* indica (Majdoub *et al.*, 2010). Uronic acid content in O. *stricta* Haw. WSP was 26.16 ± 4.33%, mainly composed of galacturonic acid (Fig. 3). This content was lower than that observed for *O. ficus indica* peel polysaccharides (65%) (Forni *et al.*, 1994), and higher than that found in dietary fibre containing material from olive mill wastewater (3.3 g /100 g) (Galanakis *et al.*, 2010).

DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging assayis the oldest indirect method for determining the antioxidant activity. In the DPPH assay, antioxidant molecules are able to reduce the stable radical DPPH to the yellow-coloured diphenyl-picrylhydrazine. In the presence of a hydrogen-donating antioxidant, the DPPH is reduced, leading to the formation of the non-radical form – DPPH-H (Gülçin, 2012). The antioxidant activity of WSP was determined by their DPPH radical scavenging activity (Fig. 4). The extracted polysaccharides showed concentration-dependent DPPH radical scavenging activity and good antioxidant capacities compared to BHA and ascorbic acid, used as reference molecules. The highest radical scavenging activity values were 94.9, 93 and 96.8% for WSP, BHA and ascorbic acid, respectively, at 50 mg ml^{-1}. The half inhibition concentration (IC_{50}) values of WSP, BHA and ascorbic acid were also determined. In general, the lowest IC_{50} corresponds to the highest DPPH scavenging activity. The IC_{50} values were 6.5 mg ml^{-1} for WSP and 3 mg ml^{-1} for BHA and ascorbic acid. The IC_{50} of WSP was lower than those of other polysaccharides reported in literature (*eg* polysaccharides extracted from guara fruits with IC_{50} of 10.8 mg ml^{-1}) (Hua *et al.*, 2014). These results suggest that WSP represent strong electron donors and could react with free radicals to convert them to more stable products and terminate the radical chain reaction.

The total antioxidant capacity (TAC) is based on the reduction of phosphomolybdate by the antioxidant molecule. The subsequent reaction is the formation of a green phosphate/Mo (V) complex, at acidic pH, which absorbs at

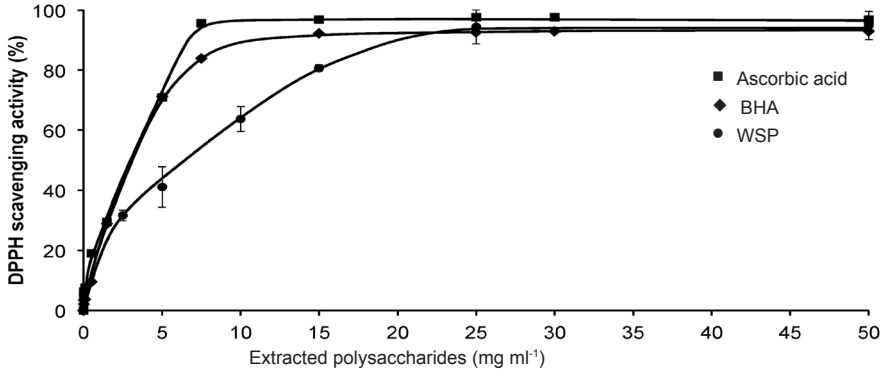

Fig. 4. DPPH free radical scavenging activity of the extracted polysaccharides from *O. stricta* Haw. fruit peels. BHA and ascorbic acid were used as reference molecules. Values are the average of triplicate experiments ± SD.

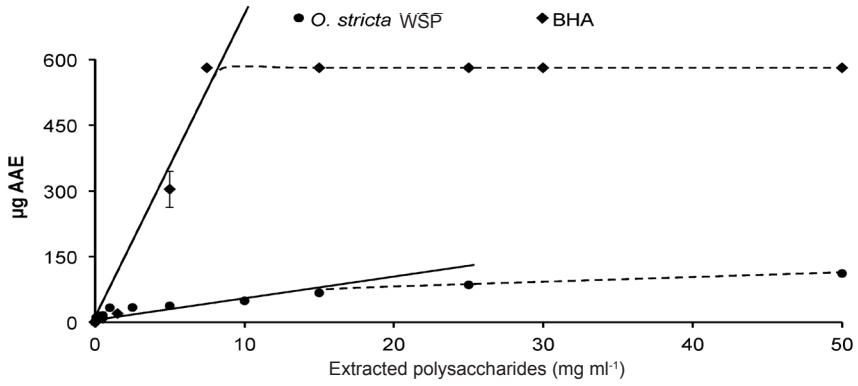

Fig. 5. Total antioxidant activity of the extracted polysaccharides from *O. stricta* Haw. fruit peels. Results are expressed in µg ascorbic acid equivalent (AAE) and BHA was used as a reference molecule. Values are the average of triplicate experiments ± SD.

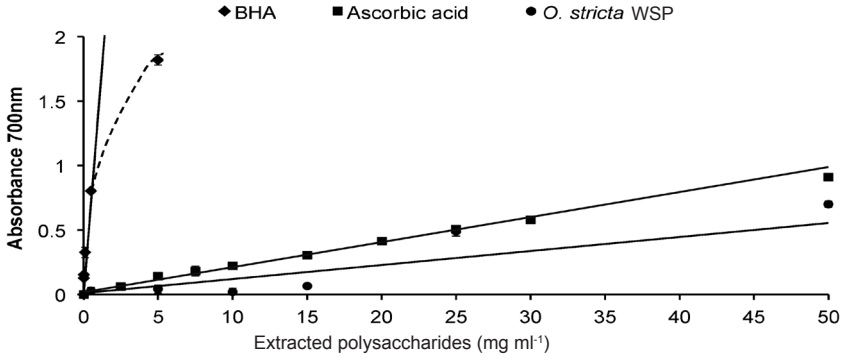

Fig. 6. Reducing power activity of the extracted polysaccharides from *O. stricta* Haw. fruit peels. BHA and ascorbic acid were used as reference molecules. Values are the average of triplicate experiments ± SD.

695 nm. The total antioxidant activities of WSP and BHA were determined and expressed as ascorbic acid equivalent (AAE) (Fig. 5). The results obtained showed an increase of the antioxidant activity proportionally to the concentration of the analyzed sample. 50 mg of WSP were equivalent to 100 µg ascorbic acid, in terms of TAC. At 7.5 mg BHA, the measured absorbance exceeded the quantification limits of the spectrophotometer and stabilised at 600 µg equivalent ascorbic acid. Despite the significant differences between WSP and BHA, these results show the efficiency of the extracted polysaccharides as a natural antioxidant.

The WSP ability to reduce Fe^{3+} to Fe^{2+} was determined by measuring the formation of Perl Prussian blue at 700 nm (Fig. 6). The reducing power of WSP was increasing proportionally to the sample concentration, from 13 mg ml^{-1}. At 50 mg ml^{-1}, the absorbance values of WSP and ascorbic acid were 0.7 and 0.91, respectively. The absorbance of BHA at this concentration exceeded 2, showing a saturation behaviour. Despite the presence of reducing capacity, the values obtained remain lower than those of other polysaccharides such as those extracted from almond (1.5 at 5 mg ml^{-1}) and pistachio (1.74 at 5 mg ml^{-1}) juice processing by-products (Sila *et al.*, 2014b), as well as from mushroom (3.4 at 20 mg ml^{-1}) (Kozarski *et al.*, 2012).

CONCLUSIONS

1. The structure and the chemical composition of *Opuntia stricta* Haw. peels were investigated, revealing similarities with extracted water-soluble polysaccharides compared to those obtained from *Opuntia ficus-indica* fruit peels.

2. Afterwards, it was demonstrated that the extracted polysaccharides were efficient natural antioxidants tested *in vitro* through 2,2-diphenyl-1-picrylhydrazyl free radical scavenging activity, total antioxidant activity and reducing power capacity.

3. The extracted water-soluble polysaccharides could therefore be used as natural food additives, replacing synthetic antioxidants, and resolving thus the environmental problems related with their disposal.

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