Phenolic composition, antioxidant and anti-inflammatory activities of extracts from Moroccan *Opuntia ficus–indica* flowers obtained by different extraction methods

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ABSTRACT

The flowers of cactus *Opuntia ficus-indica* are by-products generally discarded by separation of the fruit and might represent an important source of polyphenols. The objective was to compare the phenolic extraction efficiency of four extraction procedures consisting of different solvents (80% acetone or 50% methanol both in water) and extraction techniques (maceration and accelerated solvent extraction, ASE). The phenolic profile of Moroccan flower extracts obtained in the best conditions was studied by HPLC-DAD-ESI-MS. Antioxidant and anti-inflammatory activities of flower extracts were also evaluated. ASE with 80% acetone in water at 80ºC gave rise to extracts with higher yields of total polyphenols, procyanidins and flavonoids. The qualitative analysis of the phenolic composition showed that extracts obtained by ASE were composed mainly by flavonol glycosides (80.56% of total polyphenols with quercetin and isorhamnetin aglycons. Moreover, several hydroxycinnamic acids (12.85% of total polyphenols) were detected in the extracts for first time. Our study also revealed the high antioxidant (272.76 μmol Trolox Equivalents/g) and anti-inflammatory potential of cactus pear flower extracts (0.19 mg/mL inhibited 50% nitric oxide production in in macrophages RAW267.4). In light of the obtained results, cactus flowers are a good source of polyphenols that could be used in foods, cosmetics or pharmaceutical products, thus contributing to diminish the environmental impact of cactus by-products and to its revalorization.

KEYWORDS: *Opuntia ficus-indica*, flowers, accelerated solvent extraction, phenolic acids, flavonols, antioxidant, anti-inflammatory.

INTRODUCTION

Phenolic compounds are secondary metabolites found ubiquitously in plants. They display a large range of structures and are responsible for the major organoleptic characteristics of plant-derived-foods and beverages, particularly color and taste properties. In addition, many studies have reported that their regular consumption impact positively on health reducing the risk of cardiovascular diseases, neurodegenerative disorders and cancer (Vauzour, Rodriguez-Mateos, Corona, Oruna-Concha, & Spencer, 2010). Among polyphenol compounds, flavonoids have been strongly linked with beneficial effects in many human and animal studies (Vauzour, Rodriguez-Mateos, Corona, Oruna-Concha, & Spencer, 2010). *In vitro* and animal studies have shown that flavonoids can exert multiple activities such as anti-inflammatory (Larrosa, González-Sarrías, Yáñez-Gascón, Selma, Azorín-Ortuño, Toti, et al., 2010), anti-hypertensive (Hodgson & Croft, 2006), anti-oxidant (Jeong, Choi, Kwon, Kang, Park, Lee, et al., 2005), anti-atherosclerotic (Fuhrman, Volkova, Coleman, & Aviram, 2005), anti-proliferative (Mantena, Meeran, Elmets, & Katiyar, 2005) and anti-angiogenic (Piao, Mori, Satoh, Sugita, & Tokunaga, 2006) among others.

Cactus (*Opuntia* spp.) belongs to *Cactaceae* family which is cultivated in both hemispheres and all continents. Among all species identified *Opuntia ficus-indica* L. also known as cactus pear is the most common. Native to Mexico, this plant is widely distributed in arid and semi-arid regions of South and Central America, Africa and the Mediterranean area (Mohamed-Yasseen, Barringer, Splittstoesser, & Schnell, 1995). Cactus pear has a rapid growth, good adaptation to poor soils and low water requirement.

Different parts of *O. ficus-indica* have increased its economic importance being exploited in food and pharmaceutical areas (Feugang, Konarski, Zou, Stintzing, & Zou, 2006). Cactus fruits (prickly pear) have been used for the manufacture of food products such as juices, jams, jellies alcoholic beverages, etc while cladodes are commercialised mainly as minimally processed fresh products (Stintzing & Carle, 2005). Cactus pear cladodes, fruits and infusions of cactus pear flowers

have also been used in traditional folk medicine for treatment of a number of diseases and pathological conditions, including inflammatory conditions, diabetes, stomach ulcers, renal diseases, etc (Feugang, Konarski, Zou, Stintzing, & Zou, 2006; Kaur, Kaur, & Sharma, 2012).

More recently, cactus pear has attracted the interest of nutraceutical and healthy food areas. Cactus pear fruits and their by-products (peel and seeds) as well as cladodes are valuable sources of polyphenols (Moussa-Ayoub, El-Samahy, Kroh, & Rohn, 2011) which make them perfect candidates for the production of health-promoting foods and food supplements. Cactus pear fruit is a unique source of isorhamnetin glycosides, especially isorhamnetin-3-O-rutinoside and isorhamnetin tryglicosides and phenolic acids such as fukic acid, piscidic acid and eucomic acid (Moussa-Ayoub, El-Samahy, Kroh, & Rohn, 2011; Serra, Poejo, Matias, Bronze, & Duarte, 2013). The occurrence of these polyphenols in cactus pear fruits depends on many factors such as stage of maturity, growing region and post-harvest practices affecting consequently to their biological activity (Coria Cayupán, Ochoa, & Nazareno, 2011). Moreover, quercetin, (+)-dihydroquercetin, and quercetin 3-methyl ether were isolated from cactus pear plant and reported as active neuroprotectants against oxidative stress (Dok-Go, Lee, Kim, Lee, Lee, Song, et al., 2003). More recently, polyphenolic-rich extracts from cactus pear by-products have reported to display antioxidant and antiproliferative activities in human colon carcinoma (Serra, Poejo, Matias, Bronze, & Duarte, 2013; Yeddes, Chérif, & Ayadi, 2014).

The *Opuntia ficus-indica* flowers constitute one of the by-products in cactus industry. The flowers are generally discarded by separation of the fruit. To the best of our knowledge, the polyphenolic profile and biological activity of *Opuntia ficus-indica* flowers are very scarcely documented. Previous studies identified isorhamnetin glycosides being the major phenolic compounds followed by quercetin and kaempferol glycosides in methanolic extracts of cactus pear flowers grown in Italy and Tunisia obtained by maceration (De Leo, Abreu, Pawlowska, Cioni, & Braca, 2010; Yeddes, Chérif, Guyot, Baron, & Trabelsi-Ayadi, 2014). Regarding health-promoting

potential of flower extracts, an animal study has demonstrated their antioxidant and antiulcerogenic activity (Alimi, Hfaiedh, Bouoni, Hfaiedh, Sakly, Zourgui, et al., 2010).

The aim of this study was to completely identify phenolic profile of *Opuntia-ficus indica* flowers from Moroccan region, in order to evaluate these flowers as a possible raw material for infusions or nutraceutics. To provide accurate data on phenolic composition of cactus pear flower extract and its biological activity (antioxidant and anti-inflammatory) we have compared different organic solvents as well as conventional (maceration) and an advanced green extraction technology (accelerated solvent extraction, ASE). ASE has attracted increasing interest due to its economic, fast and automatic features which besides improving extraction yield, decreases time and solvent consumption (Hossain, Barry-Ryan, Martin-Diana, & Brunton, 2011). Furthermore, the set up in ASE equipment provides protection for oxygen and light sensitive compounds, which is prominent for extraction of bioactive compounds such as polyphenols.

MATERIALS AND METHODS

Chemicals

Unless otherwise stated, all chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA). (+)-catechin (≥99%, HPLC grade) and gallic acid (≥95%, tritation), standards were purchased from Sigma-Aldrich and cyanidine chloride from Extrashynthese (Lyon, France). HPLC grade methanol and acetic acid were supplied from Panreac (Barcelona, Spain). Water was deionized by using a Milli-Q system (Millipore, Beldford, MA, USA). Macrophage RAW 264.7 cell line was obtained from American Type Culture Collection (Rockville, MD, USA). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), trypsin-EDTA (1x), penicillin (105 U/L), streptomycin (0.1 g/L) were from GIBCO (Madison, WI, USA).

Plant material

The yellow coloured *Opuntia ficus indica* flowers were collected in the Rif region located in the north of Morocco during summer 2010. The fresh flowers were then air-dried at room temperature, powdered and stored at - 20°C until use.

Phenolics extraction

Phenolic compounds of the *O. ficus-indica* flowers were extracted using four different procedures summarized in Table 1 and described below:

Maceration

First, 0.5 g of dried flowers was defatted by vigourosly agitation with n-hexane twice at room temperature. Phenolic compounds were then extracted from the defatted residue using 10 ml of two different solvents: (A) acetone/HCl/water (79/1/20 v/v/v) and (B) methanol/HCl/water (49/1/50 v/v/v). The extractions were performed in an orbital shaker for 24 h at 25 ºC. Samples were filtered through a Whatman No.1 filter paper and the filtrate was evaporated using a rotary evaporator. The obtained residue was dissolved in 2 ml of methanol/water (50/50, v/v), filtered through a 0.45 μm PVDF membrane and finally kept at -20°C until further analysis.

Accelerated Solvent Extraction (ASE)

An Accelerated solvent extraction unit ASE 200 (Dionex Co., Sunnyvale, CA, USA) was also used for phenolics extraction. The unit was an automated system with temperature and pressure limits of 200ºC and 10,342 kPa, respectively. The ASE consists of stainless steel extraction cells (11 mL) with electronically controlled heaters, a pump and a solvent controller. The system included and autosampler carousel and a collection bottle tray holding 12 collection bottles and one rinse bottle. Extraction was performed in extraction cells containing 0.5 g of powdered cactus

flowers and using the conditions reported on the US EPA 3545 method (EPA, 1998). Hexane was used first to remove lipids. The further used solvents were: acetone/water (80/20, v/v), and methanol/water (50/50, v/v), the temperature was 80 °C and the pressure was 1500 psi (10.3 MPa). The extraction was performed during three cycles including 5 min (heating) and 8 min (static time). The extraction cell was flushed with solvent (60 % cell volume) and purged with nitrogen gas (120 s). Three replicate extractions for each experimental condition were performed (n = 3). At the end of each extraction, a total extract volume of 24 ml was obtained. The extracts were dried under nitrogen at 40 ºC, using Turbovap^{R} LV (Caliper, Lifescience). Finally, dried extracts were dissolved in 2 ml methanol/water (50/50, v/v), filtered through 0.45 µm PVDF membrane, and finally kept at -20°C until further analysis.

Determination of total phenolics content

Total phenolic compounds content was determined using the Folin–Ciocalteu reagent (Singleton & Rossi, 1965) with some modifications. Briefly, 0.1 ml of each extract was mixed with 0.1 ml of Folin–Ciocalteu reagent during 5 min at room temperature. The reaction was then neutralized with 10 ml of 7,5% sodium carbonate, and the mixture was further incubated at room temperature for 1 h. The absorbance was then measured at 750 nm with a DU800 Beckman coulter spectrophotometer (Barcelona Spain). A gallic acid standard curve with a linear range (0-225 µg gallic acid/mL) was prepared from a freshly made 1 mg/mL gallic acid stock solution. Results were expressed as mg of gallic acid equivalents (GAE)/g extract on dry weight basis (d.w.).

Determination of procyanidins content

Procyanidin content (PC) was estimated by spectrophotometry after acid hydrolysis and colour formation(Ribéreau-Gayon & Stonestreet, 1966). Briefly, 0.1 ml of each extract was mixed with 20 ml of 0.15 g of iron sulphate ($FeSO_4$, $7H_2O$) in 500 ml of HCl/n-butanol. Samples were

incubated in a water bath at 90 °C for 1 h. A control sample without heating was used. After incubation, samples were cooled and absorbance readings were performed at 550 nm. Quantification was carried out by external calibration using cyanidin chloride as standard. Results were expressed as mg of cyanidin chloride equivalents (CCE)/g extract in d.w.

Determination of flavonoids content

The method of Swain and Hillis based on the condensation ability of some phenols in a strongly acidic medium with compounds containing carbonyl groups such as vanillin was used (Swain & Hillis, 1959). A volume of 0.1 ml of each extract was added to 2 ml of 1 % methanolic vanillin solution. The reaction mixture was mixed with 70 % HCl. Absorbance was measured 20 min later at a wavelength of 500 nm. (+) catechin was used as standard. Results were expressed as mg of catechin equivalents (CE)/g extract in d.w.

Analysis of phenolic compounds by HPLC-MS-ESI-MS

Analysis of selected extract was carried out on a Waters 996 HPLC system equipped with vacuum degasser, autosampler, binary pump ultraviolet-visible diode array detector (Waters). The HPLC system was coupled with a quadrupole mass spectrometer Hewlett Packard series 1100MSD with an electrospray interface (ESI) (Agilent Technologies, Aldbronn, Germany). The separation of phenolic compounds was performed on a reversed phase Nova-Pak C_{18} column (Waters, Milford, USA) (250 x 4 mm, 4 µm particle size) operating at room temperature and a flow rate of 1 ml/min. A volume of 10 µl was injected. The mobile phases used consisted of 2% (v/v) acetic acid in water (solvent A) and 2% (v/v) acetic acid in acetonitrile (solvent B). The following solvent gradient was used: 0 min, 100% B; 0 to 55 min, 0-80 % B; 55 to 57 min, 80-90 % B; 57 to70 min 90 % B; 70 to 80 min, 90-95 % B, 80 to 90 min, 95-100 % B followed by a washing and equilibration of the

column during 30 min. The UV-Vis spectra were acquired in the range of 220-380 nm. Flavan-3-ol monomers were monitored at 280 nm, phenolic acids at 320 nm, and flavonols at 360 nm.

In the mass spectrometry detection, nitrogen was used as the drying and nebulising gas. The ESI parameters were flow rate 10 L/min, dry gas temperature 350 °C, nebulizer pressure 55 psi. Mass spectrometry data were acquired in the negative isonisation mode. The capillary voltage was 4 kV. Mass Scan and MS-MS spectra were measured in the range of m/z from 100 to 3000 using the following fragmentation program: from *m/z* 0 to 200 (100 V) and from *m/z* 200 to 3000 (200 V).

The identification of the compounds was carried out by means of their elution order in the HPLC chromatograms, UV-Vis spectra, molecular weight, their MS/MS fragments, and whenever possible chromatographic comparison with authentic standards.

Biological assays

Antioxidant activity

In order to investigate the antioxidant activity of the studied *Opuntia ficus-indica* flower extracts, Oxygen Radical Absorbance Capacity (ORAC) technique was used. This assay is based on the original method reported by Cao and Prior using fluorescein as substrate and AAPH (2,2´-azobis (2-amidinopropane) dihydrochloride as oxidant generator (Cao & Prior, 1998). Extract diluted in buffer (30 µl) was added to each microplate well. This was followed by addition of 120 µl of disodium fluorescein 116.66 nM, 20 µl of 0.075 M phosphate buffer (pH 7.4) solution. The mixture was incubated at 37 °C for 15 min. A volume of 60 µl of free radical generator (AAPH) in 10 ml phosphate buffer was then added to each well. Microplates were read using an HTS 7000 plus fluorometer (Perkin Elmer, Shelton, CT). Samples and Trolox standard solutions were always analyzed in duplicate. The final ORAC values were calculated using a regression equation between the Trolox concentration and the net area under the FL curve. Results were expressed as µmoles Trolox equivalents/g extract d.w.

Cell viability assay

In order to study the cytotoxicity of flower extracts, macrophages cell line RAW 264.7 were cultured in Dubelcco´s modified Eagle Medium (DMEM) containing 1 % penicillin/streptomycin, and 10 % fetal bovine serum (ATCC) at 37 ºC in 5% CO_2 atmosphere. The cell proliferation assay was conducted using the Cell Titer 96 Aqueous One Solution Proliferation assay kit using the novel tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS), and an electron coupling reagent, phenazine ethosulfate (PES) (Promega Biotech Iberica, Madrid, Spain). Briefly, 5 x 10^4 cells/well were seeded in a 96-well plate and the total volume was adjusted to 200 µL with DMEM. The cells were allowed to grow for 24 h at 37 ºC in 5% CO_2. After 24 h incubation, they were treated with different concentrations of flower extracts (0-2.25 mg/mL) for 24 h. After treatment, DMEM was replaced by 100 µL fresh medium and 20 µL MTS/PES was added to each well. The plate was incubated for 2 h at 37 ºC and the absorbance read at 490 nm. The percentage of viable cells was calculated with respect to cells treated with vehicle (0.1% DMSO) as follows:

$$\% \text{ cell viability} = A_{\text{treatment 490 nm}}/A_{\text{control 490 nm}} * 100$$

Anti-inflammatory activity

Anti-inflammatory activity of the studied extract was investigated through determination of nitric oxide production. Approximately, 5 x 10^4 cells/well were seeded in a 96-well plate and allowed to grow to its 80-90% confluence. The cells were treated with 1 µg/mL lipopolysaccharide (LPS) from *Escherichia coli* O55:B5 with or without different concentrations of flower extracts (0-2.25 mg/mL) for 24 h. The medium was then collected and NO production analyzed. Nitrite accumulation, and indicator of NO synthesis, was measured in the culture medium by Griess reaction (Green, Wagner, Glogowski, Skipper, Wishnok, & Tannenbaum, 1982). Briefly, 100 µL of

Dulbecco's modified Eagle's medium (DMEM) were plated in 96-well plate and an equal amount of Griess reagent constituted by 1% (w/v) sulfanilamide and 0.1% (w/v) N-1-(naphthyl)ethylenediamine-diHCl in 2.5% (v/v) H_3PO_4, was added. The plate was incubated for 5 min and the absorbance measured at 550 nm in a microplate reader (Biotek, Winooski, VT, USA). The amount of NO was calculated using a sodium nitrite standard curve. Potency was determined by dose–response curves in which the range of concentrations was distributed in a logarithmic scale and the IC_{50} values were calculated using non-linear regression sigmoidal curve fit functions in GraphPad Prism 4.00 (Graphpad Software Inc., San Diego, CA, USA).

Statistical analysis

Data shown are the average ± standard deviation of three replicated extractions. Data were subjected to one-way analysis of variance (ANOVA) by Statgraphics Centurion XVI software, version 16.1.17 (Statistical Graphics Corporation, Rockville, Md). Differences between mean values were compared by using a Duncan´s multiple-range test at $P \leq 0.05$ probability levels.

RESULTS AND DISCUSSION

Efficiency of different extraction procedures on phenolic extraction from cactus pear flower

The polarities of phenolic compounds vary significantly rendering difficult the use of one extraction method for all phenolic compounds. In order to investigate the total phenolic content of the studied cactus flowers, two different solvents and extraction techniques were assayed and compared. The results of the effect of extraction procedure on the extraction yields, expressed on a dry matter basis are gathered in Table 1. Regarding maceration extractions, Table 1 showed that hydro-acetonic extract had higher yield than the methanolic extract, while the latter showed higher extractive yield in the ASE extraction technique. Methanol extract from ASE was found to contain

significantly higher yield (34 %) than methanolic extract of maceration method (14 %) of dried *Opuntia* flower. The yields and dried mass weights of acetonic extract were shown similar in two methods (18 %) of *Opuntia* flower. The comparison of the two used techniques showed that the ASE method significantly improved the extractive yields of the methanolic solvent which increases from 14 % (maceration) to 34 % (ASE), while no effect has been observed in the acetonic solvent. Comparison with literature showed that the obtained results are similar to those obtained for Opuntia ficus-indica f. inermis flowers where a percentage of 23.64 % was obtained by using 50 % methanol for the extraction (Alimi, Hfaiedh, Bouoni, Sakly, & Ben Rhouma, 2011).

After solid liquid extraction, the contents of total phenolic compounds, procyanidins and catechins of extracts were measured (Table 2). The results showed that, in both used extraction methods, the acetonic solvents gave extracts with the highest amounts of total phenolic compounds (2.85 and 3.18 mg/g d.w.) compared to the methanolic solvents (2.34 and 2.40 mg/g d.w.). Comparison between the two used methods showed that the ASE method allow to extract higher amount of phenolics than maceration. This difference may be due to the solvent polarity and the solubility of phenolic compounds in the used mixtures.

With regard to literature, few studies have been focused on cactus flowers compared to fruits and cladodes. A study conducted on *Opuntia ficus-indica f. inermis* showed quite higher content in phenolic compounds (Alimi, Hfaiedh, Bouoni, Sakly, & Ben Rhouma, 2011). This could be obviously attributed to the type of the cultivar, geographical origin, flowering stage but also to the extraction protocols and analytical assays.

Table 2 also showed that procyanidins contents in the studied dried flower extracts were very low in the two solvents methods and ranging from 0.025 to 0.074 mg/g d.w. Finally the contents of flavonoids were slightly higher than procyanidins and range from 0.78 mg/g d.w. to 1.58 mg/g d.w. In both extraction methods the acetonic extracts were richer in flavonoids than the methanolic extracts.

Identification of phenolic compounds of cactus pear flowers

Based on previous results the best conditions to extract phenolics from cactus pear flower were ASE with acetonic solvent. For these reason, extract C was selected for further identification of individual phenolic compounds.

Figure 1 shows HPLC-UV chromatogram of cactus pear flower extracted with 80% acetone in water (extract C) and recorded at 280 nm. The HPLC chromatographic profile revealed the presence of ten major peaks (**1**–**10**). Some other peaks were also observed however, they were too small to allow structural analyses. Chromatographic peaks identification was carried out by means of their retention time in the HPLC chromatogram, UV spectra, molecular weight, their MS/MS fragments and by comparison with literature data (Chougui, Tamendjari, Hamidj, Hallal, Barras, Richard, et al., 2013; De Leo, Abreu, Pawlowska, Cioni, & Braca, 2010; Gouveia & Castilho, 2011; Hossain, Rai, Brunton, Martin-Diana, & Barry-Ryan, 2010). Using all the mentioned information, a group of 10 compounds, belonging to two families were identified. Table 3 summarizes the phenolic compounds identified in the cactus pear flower extract.

On line UV-visible spectra of the observed phenolic compounds were typical of phenolics acids and flavonol glycosides. Indeed, their spectra showed two major absorption bands in the UV region. The observed compounds could be divided into two different groups of phenolics compounds. Compounds **1** to **6** showed a typical UV-Vis spectra of cinnamic acids with maximum absorption around 255–265. Compounds **7** to **10** showed a typical maximum absorption of flavonols glycosides in the UV-Vis spectra from 350 to 355 nm (Table 3) (Wollenweber, 1982).

Phenolic acids

Different hydroxycinnamic acid derivatives were detected in *Opuntia* flowers (Table 3).

Compound **1** which was eluted at 3.40 min presents a molecular ion signal at *m/z*: 517 corresponding to the deprotonated ion $[M-H]^-$. Its MS spectrum also showed an ion signal at *m/z*: 371 corresponding to the loss of 146 Da. Another ion signal was observed at *m/z*: 209 corresponding to the further loss of 162 Da. The two loosed groups were tentatively identified as sugar unites, rhamnoside (146 Da) and a hexoside (162 Da), rather than hydroxycinnamic acids moieties due to the low retention time of this compound. Therefore, compound **1** was characterized as 5-hydroxyferulic acid-rhamnoside-hexoside.

Compound **2** which eluted at 5.78 min presents a signal ion at *m/z*: 513 corresponding to the deprotonated $[M-H]^-$. The data MS showed an ion signal at *m/z*: 191 with a high relative intensity which is in agreement with the presence of a quinic acid moiety. Another ion signal was shown at *m/z*: 377 corresponding to the loss of a 136 Da which is a caffeoyl residue. This could indicate the presence of a caffeoyl moiety. The ion signal observed at *m/z*: 191 correspond to the loss of 322 Da. This is compatible with a loss of caffeoyl and methoxycinnamoyl moieties. This means that this compound could be caffeoyl methoxycinnamoyl quinic acid.

Compound **3** which eluted at 11.02 min presents the deprotonated molecular ion signal at *m/z*: 549 indicating a molecular weight of 550. The obtained mass spectrum did not present enough fragmentations allowing its unambiguous characterization. However a tentative structure consisting of 1, 4-diferuloyl syringic acid is compatible with the obtained data.

Compound **4** was observed with a retention time of 25.43 min. Its UV spectra are in agreement with a hydroxycinnamic acid. Its mass spectrum presents a deprotonated molecular $[M-H]^-$ ion at *m/z*: 325. This is compatible with a structure consisting either of p-coumaroyl caffeic acid derivative or p-coumaroyl hexoside. Examination of the obtained MS data showed that their in favour of the cinnamic acid hypothesis and ruled out the hexoside hypothesis. Indeed, typical hexoside fragmentations consisting of losses of 60, 90 and 120 Da are not observed in this case. The MS data showed a signal ion at *m/z:* 255 $[M-H-70]^-$ corresponding to the loss of coumaroyl residue

in ortho-position ($C_3O_2H_3$) and another fragments of a ion *m/z:* 167 $[M-H-156]^-$ formed by the loss of $C_3H_2O_3$ unit (86 Da). These spectral features suggested that this compound was 4-*p*-coumaroyl caffeic acid

Compound **5** which eluted at 27.55 min presents UV spectral characteristics of phenolic acids. Its mass spectrum presents a deprotonated ion signal at *m/z*: 517 Da indicating a molecular mass of 518. This is compatible with a tentative structure consisting of two caffeoyl units and ferulic acid. This compound was then supposed to be 1, 5-dicaffeoyl ferulic acid.

Compound **6** which eluted at 39.57 min presents a UV spectrum similar to that of *p*-coumaric acid (λ_{nm} 310). The MS data showed a deprotonated ion signal at *m/z*: 681. A fragment ion signal was observed at *m/z*: 485 $[M-H-196]^-$ with low relative intensity, indicating the loss of syringic acid. Another signal ion was observed at *m/z*: 323 formed through the further loss of 162 Da $[(M-H)-196-162]^-$, corresponding the loss of caffeic acid. The presence of coumaric acid indicated above was confirmed by the presence of a signal at *m/z*: 163 with a relatively high intensity. All these data are compatible with a structure consisting of 1,4-syringicferuloyl 4-coumaroyl caffeic acid.

Hydroxycinammic derivatives are commonly found in plants and are directly involved in plant protection against pathogens by their involvement in the cell wall structure (Huang, Rozwadowski, Bhinu, Schäfer, & Hannoufa, 2008; Ikegawa, Mayama, Nakayashiki, & Kato, 1996; Mesaik, Jabeen, Halim, Begum, Khalid, Asif, et al., 2012). These molecules have also been reported to exhibit a wide range of important biological and therapeutic properties including anti-inflammatory, anti-bacterial, anti-diabetic, anti-carcinogenic, anti-aging, and neuro-protective effects (Ou & Kwok, 2004).

As far as we know, this is the first time that hydroxycinnamic acids were described in *Opuntia ficus indica* flowers.

Flavonol derivatives

Four peaks (**7**, **8**, **9** and **10**) present UV spectra similar to those of flavonols with two absorption maxima in the ranges 256-258 nm (band I), and 350-364 nm (band II). Based on absorption of band II, compounds **7** and **9** were concluded to be quercetin derivatives, while compounds **8** and **10** were based of isorhamnetin skeleton. However, the glycosylation position was terminated by comparison of the relative intensity of the fragments ions (Sakushima & Nishibe, 1988).

Compound **8** presents a molecular ion at *m/z*: 769. The mass spectrum also showed a fragment ion signal at *m/z*: 609 $[(M-H)-160]^-$ and another fragment ion at *m/z*: 463 $[(M-H)-160-146]^-$. The obtained UV and mass spectral data are compatible with a structure consisting of an isorhamnetin trisaccharide compound. Compound **8** was supposed to be isorhamnetin 3-*O*-rhamnosyl 7-*O*-rutinoside.

Compound **9** which eluted at 63.02 presents a molecular ion at *m/z*: 609. A fragment ion signal was observed at *m/z*: 463 which is in agreement with the presence of a rhamnose unit. This was also confirmed by the neutral loss fragments of 88 Da ($C_4H_6O_2$) and 42 Da ($C_2H_2O_9$**)**. The obtained data are compatible with a disaccharide compound consisting of quercetin, rhamnose and glucose. Compound **9** was then identified as rutin which was confirmed by comparison with a standard purchased from Extrasynthese.

Compound **10** eluted at 69.14 min presents a deprotonated ion signal at *m/z*: 623 indicating a molecular mass of 624. The mass spectrum also showed a fragment signal at *m/z*: 477 corresponding to a loss of 146 Da and indicating the presence of a rhamnose unit. The Compound **10** represents thus the same fragmentation pattern as compound **9** which showed that compound **10** was a disaccharide compound consisting of isorhamnetin as aglycon. According to the obtained data, compound **10** was concluded to be isorhamnetin rutinoside.

Compound **10** was finally identified as isorhamnetin 7-*O*-rutinoside by comparison with a standard purchased from Extrasynthese. Moreover, the main fragmentation pathways of compound 9 and 10 showed in Figure 3.

With regard to literature, few studies have been focused on cactus flowers compared to seeds. On the basis of HPLC–PDA-ESI-MS analysis seven compounds have been identified as kaempferol, quercetin, and isorhamnetin glycosylated derivatives from Italian sample of cactus flowers (De Leo et al., 2010). Studies on the biological activities of flavonol glycosides showed that isorhamnetin derivative was a testosterone 5a-reductase inhibitor (Yang, Yoneda, Oide, & Sakae, 1998).

Quantitative analysis of the identified individual phenolic compounds

After having studied the qualitative phytochemical composition of the cactus flower simple, we were interested in the quantitative analysis of the individual identified phenolic compounds. The obtained results, expressed as percentage deduced from the identified peak areas are gathered in Table 4.

The obtained results showed that the identified compounds are presents at different percentages. The quantitative phytochemical composition of the studied cactus flower sample was thus dominated by isorhamnetin 3-*O*-rutinoside (47.72 %) followed by isorhamnetin 7-*O*-rhamnosyl 3-*O*-rutinoside (32.84 %). These two flavonol glycosides were by far the major metabolites of the studied sample extract representing 80.56 % of the total identified compounds. The other compounds including phenolic acids and the two quercetin derivatives were presents at percentages ranging from 0.63 to 3.65 %.

The flowers extract of the *Opuntia ficus-indica* sample showed thus significantly higher values of flavonol glycoside derivatives compared to phenolic acids which were detected at lower amounts.

Antioxidant and anti-inflammatory activity of extracts from cactus pear flower

The antioxidant activity of the obtained flower extracts has been investigated through ORAC technique and the obtained results are gathered in table 2. In this method, the obtained values reflect the ability of hydrogen-donating antioxidants to scavenge the AAPH radical. The obtained results showed the influence of the used extraction method on the observed antioxidant activity. Thus the ORAC value of ASE methanol extract was lower (263.72 µmo TEl/g) than that of maceration methanol extract (326.44 µmol/g), and that of the acetonic extract of ASE method was higher (272.76 µmol/mg) than that of maceration acetonic extract (243.68 µmol/mg).

In the literature few reports have been focused on antioxidant activity study of Opuntia flowers compared to cladodes and fruits (Valente, da Paixão, do Nascimento, dos Santos, Scheinvar, Moura, et al., 2010). The recent report was tested by DPPH method of flowers hexane extract(Ammar, Ennouri, Khemakhem, Yangui, & Attia, 2012). Another study reported in vitro antioxidant activity of methanol extract(Alimi, Hfaiedh, Bouoni, Sakly, & Ben Rhouma, 2011).

With regard to the total phenolic content of the different studied flower extracts, the obtained results showed that a high content of phenolic compounds does not correspond automatically to a high antioxidant activity. Indeed the obtained results showed that the three extracts A, B and C which contain similar total phenolic contents present different antioxidant activities. Extract C with the highest total phenolic content did not show the highest antioxidant activity. In the other hand, the extract D which was the poorest extract in total phenolics was the weaker in term of antioxidant activity. These results could be explained due to the polyphenolic composition which differs from one extract to the other. This may be also due to synergy phenomena which may occur within each extract.

The observed antioxidant activity could obviously due, at least partially, to the presence of the phenolic acids and flavonol glycosides identified above. According to previous reports, these

compounds have demonstrated high antioxidant activity, which may offer protection against cancer (Khomdram & Singh, 2011). The antioxidative properties of flavonol derivatives are a predominant feature of their radical-scavenging capacity (Cotelle, 2001). This activity is attributed to their ability to scavenge free-radical and to chelate metal ions involved in their production. In general, antioxidant activity of flavonoids depends on the structure and substitution pattern of hydroxyl groups. The essential requirement for effective radical scavenging is the 3´, 4´-orthodihydroxy configuration in ring B and 4-carbonyl group in ring C. The presence of 3-OH or 3-and 5-OH groups, giving a catechol-like structure in ring C, is also beneficial for the antioxidant activity of flavonoids. The presence of the C2-C3 double bond configured with a 4-keto arrangement is known to be responsible for electron delocalisation from ring B and it increases the radical-scavenging activity. In the absence of the o-dihydroxy structure in ring B, a catechol structure in ring A can compensate for flavonoid antioxidant activity (Yeşilada, Tsuchiya, Takaishi, & Kawazoe, 2000).

Among the flavonoids identified in our study, quercetin and isorhamnetin derivatives various hydroxyl groups and also contains the 2,3-double bond in the C ring and the 4-oxo function, allowing for delocalisation of the phenoxyl radical electron. This may increase and explain the observed antioxidant activity.

In addition to flavonols, phenolic acids are also recognized for their antioxidant activity which depends on the number of hydroxyl groups in the molecule (Dziedzic & Hudson, 1983). The predominant hydroxycinnamic found in ours extracts have been reported to have good antioxidant activities (Andreasen, Kroon, Williamson, & Garcia-Conesa, 2001). The presence of the CH=CH-COOH group in the hydroxycinnamic acids is considered to be the key for the significantly higher antioxidative efficiency than the COOH (quinic acids) in the hydroxybenzoic acids (White & Xing, 1997). Thus, the diphenolics, caffeic acid, apparently have a higher radical scavenging ability than monophenolics (p-coumaric acid) consistent with the chemical criteria applied to diphenolics (Bors

et al., 1990). Methoxylation of the hydroxyl group in the ortho position of the diphenolics, as in ferulic acid, results in a decrease in the scavenging reaction (Shahidi & Wanasundara, 1992).

In order to investigate the anti-inflammatory activity of the studied extract, macrophages activated with LPS (1 μg/mL) were treated for 24 h with flower extracts. NO released by activated macrophages is considered as a marker of active proinflammatory responses (Fiebich, Lieb, Engels, & Heinrich, 2002). The cytotoxicity of the flower extracts was evaluated using the MTS assay. All samples did not show significant effect on the macrophage RAW 264.7 cell proliferation (data not shown) at the concentrations tested (0.18-2.25 mg extract/mL of medium) which suggests that concentration range used to treat the cells did not exert any cytotoxic effect.

The obtained results (Figure 2) showed that the tested flower extracts presents a significant dose-dependent inhibition of the NO production in LPS-activated macrophages. The concentrations required for inhibiting the production of NO by 50% (IC_{50} value) were 0.19 mg/mL for both extracts, respectively.

The present results provide strong evidence that extracts of *Opuntia ficus indica* flowers can suppress the inflammatory processes which are related to various chronic diseases such as diabetes type-2, obesity, hypertension and the metabolic syndrome. For protection from NO-related damage, several biological processes can be suggested such as scavenging of NO, inhibition of inducible nitric oxide synthase (iNOS) enzymatic activity and iNOS expression. In consistency with our results, the extract of the stem of *Opuntia ficus indica* have shown to reduce NO production(Lee, Kim, Yoon, Lim, Kim, Jin, et al., 2006). This could be due through the inactivation of the nuclear transcription factor B (NF-κB) and subsequent down-regulation of iNOS gene expression as previously reported (Lee, et al., 2006).

CONCLUSION

This study showed that the flowers of *Opuntia ficus-indica* growing in the north of Morocco could be considered as a source of bioactive phenolic compounds. The qualitative analysis allowed the identification of phenolic compounds pertaining to hydroxycinnamic acids esters and flavonol glycosides. The latter have been identified as quercetin and isorhamnetin glycosylated derivatives. The exploration of the biological activities of the studied extracts showed that *Opuntia ficus-indica* flowers have antioxidant and anti-inflammatory effects. In light of the results obtained, it would seem possible to use cactus flowers as a source of natural compounds which could be incorporated in foods, cosmetics or pharmaceutical products, thus contributing to diminishing the environmental impact of cactus by-products and to its revalorization.

REFERENCES

Alimi, H., Hfaiedh, N., Bouoni, Z., Hfaiedh, M., Sakly, M., Zourgui, L., & Rhouma, K. B. (2010). Antioxidant and antiulcerogenic activities of Opuntia ficus indica f. inermis root extract in rats. *Phytomedicine, 17*(14), 1120-1126.

Alimi, H., Hfaiedh, N., Bouoni, Z., Sakly, M., & Ben Rhouma, K. (2011). Evaluation of antioxidant and antiulcerogenic activities of Opuntia ficus indica f. inermis flowers extract in rats. *Environmental Toxicology and Pharmacology, 32*(3), 406-416.

Ammar, I., Ennouri, M., Khemakhem, B., Yangui, T., & Attia, H. (2012). Variation in chemical composition and biological activities of two species of Opuntia flowers at four stages of flowering. *Industrial Crops and Products, 37*(1), 34-40.

Andreasen, M. F., Kroon, P. A., Williamson, G., & Garcia-Conesa, M. T. (2001). Esterase activity able to hydrolyze dietary antioxidant hydroxycinnamates is distributed along the intestine of mammals. *Journal of Agricultural and Food Chemistry, 49*(11), 5679-5684.

Cao, G., & Prior, R. L. (1998). Comparison of different analytical methods for assessing total antioxidant capacity of human serum. *Clinical Chemistry, 44*(6), 1309-1315.

Coria Cayupán, Y. S., Ochoa, M. J., & Nazareno, M. A. (2011). Health-promoting substances and antioxidant properties of Opuntia sp. fruits. Changes in bioactive-compound contents during ripening process. *Food Chemistry, 126*(2), 514-519.

Cotelle, N. (2001). Role of flavonoids in oxidative stress. *Current topics in medicinal chemistry, 1*(6), 569-590.

Chougui, N., Tamendjari, A., Hamidj, W., Hallal, S., Barras, A., Richard, T., & Larbat, R. (2013). Oil composition and characterisation of phenolic compounds of Opuntia ficus-indica seeds. *Food Chemistry, 139*(1-4), 796-803.

De Leo, M., Abreu, M. B. D., Pawlowska, A. M., Cioni, P. L., & Braca, A. (2010). Profiling the chemical content of Opuntia ficus-indica flowers by HPLC-PDA-ESI-MS and GC/EIMS analyses. *Phytochemistry Letters, 3*(1), 48-52.

Dok-Go, H., Lee, K. H., Kim, H. J., Lee, E. H., Lee, J., Song, Y. S., Lee, Y. H., Jin, C., Lee, Y. S., & Cho, J. (2003). Neuroprotective effects of antioxidative flavonoids, quercetin, (+)-dihydroquercetin and quercetin 3-methyl ether, isolated from Opuntia ficus-indica var. saboten. *Brain Research, 965*(1-2), 130-136.

Dziedzic, S. Z., & Hudson, B. J. F. (1983). Polyhydroxy chalcones and flavanones as antioxidants for edible oils. *Food Chemistry, 12*(3), 205-212.
EPA. (1998). Method 3545A (SW-846): Pressurized Fluid Extraction (PFE). In). http://www.epa.gov/sam/pdfs/EPA-3545a.pdf.
Feugang, J. M., Konarski, P., Zou, D., Stintzing, F. C., & Zou, C. (2006). Nutritional and medicinal use of Cactus pear (Opuntia spp.) cladodes and fruits. *Frontiers in Bioscience, 11*(SUPPL. 2), 2574-2589.
Fiebich, B. L., Lieb, K., Engels, S., & Heinrich, M. (2002). Inhibition of LPS-induced p42/44 MAP kinase activation and iNOS/NO synthesis by parthenolide in rat primary microglial cells. *Journal of Neuroimmunology, 132*(1-2), 18-24.
Fuhrman, B., Volkova, N., Coleman, R., & Aviram, M. (2005). Grape powder polyphenols attenuate atherosclerosis development in apolipoprotein E deficient (E0) mice and reduce macrophage atherogenicity. *Journal of Nutrition, 135*(4), 722-728.
Gouveia, S., & Castilho, P. C. (2011). Characterisation of phenolic acid derivatives and flavonoids from different morphological parts of Helichrysum obconicum by a RP-HPLC-DAD-(-)-ESI-MS n method. *Food Chemistry, 129*(2), 333-344.
Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S., & Tannenbaum, S. R. (1982). Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Analytical Biochemistry, 126*(1), 131-138.
Hodgson, J. M., & Croft, K. D. (2006). Dietary flavonoids: Effects on endothelial function and blood pressure. *Journal of the Science of Food and Agriculture, 86*(15), 2492-2498.
Hossain, M. B., Barry-Ryan, C., Martin-Diana, A. B., & Brunton, N. P. (2011). Optimisation of accelerated solvent extraction of antioxidant compounds from rosemary (Rosmarinus officinalis L.), marjoram (Origanum majorana L.) and oregano (Origanum vulgare L.) using response surface methodology. *Food Chemistry, 126*(1), 339-346.
Hossain, M. B., Rai, D. K., Brunton, N. P., Martin-Diana, A. B., & Barry-Ryan, A. C. (2010). Characterization of phenolic composition in lamiaceae spices by LC-ESI-MS/MS. *Journal of Agricultural and Food Chemistry, 58*(19), 10576-10581.
Huang, J., Rozwadowski, K., Bhinu, V. S., Schäfer, U., & Hannoufa, A. (2008). Manipulation of sinapine, choline and betaine accumulation in Arabidopsis seed: Towards improving the nutritional value of the meal and enhancing the seedling performance under environmental stresses in oilseed crops. *Plant Physiology and Biochemistry, 46*(7), 647-654.
Ikegawa, T., Mayama, S., Nakayashiki, H., & Kato, H. (1996). Accumulation of diferulic acid during the hypersensitive response of oat leaves to Puccinia coronata f.sp. avenae and its role in the resistance of oat tissues to cell wall degrading enzymes. *Physiological and Molecular Plant Pathology, 48*(4), 245-255.
Jeong, Y. J., Choi, Y. J., Kwon, H. M., Kang, S. W., Park, H. S., Lee, M., & Kang, Y. H. (2005). Differential inhibition of oxidized LDL-induced apoptosis in human endothelial cells treated with different flavonoids. *British Journal of Nutrition, 93*(5), 581-591.
Kaur, M., Kaur, A., & Sharma, R. (2012). Pharmacological actions of Opuntia ficus indica: A review. *Journal of Applied Pharmaceutical Science, 2*(7), 15-18.
Khomdram, S. D., & Singh, P. K. (2011). Polyphenolic Compounds and Free Radical Scavenging Activity in Eight Lamiaceae Herbs of Manipur. *Notulae Scientia Biologicae, 3*, 108-113.
Larrosa, M., González-Sarrías, A., Yáñez-Gascón, M. J., Selma, M. V., Azorín-Ortuño, M., Toti, S., Tomás-Barberán, F., Dolara, P., & Espín, J. C. (2010). Anti-inflammatory properties of a pomegranate extract and its metabolite urolithin-A in a colitis rat model and the effect of colon inflammation on phenolic metabolism. *Journal of Nutritional Biochemistry, 21*(8), 717-725.
Lee, M. H., Kim, J. Y., Yoon, J. H., Lim, H. J., Kim, T. H., Jin, C., Kwak, W. J., Han, C. K., & Ryu, J. H. (2006). Inhibition of nitric oxide synthase expression in activated microglia and

peroxynitrite scavenging activity by Opuntia ficus indica var. saboten. *Phytotherapy Research, 20*(9), 742-747.

Mantena, S. K., Meeran, S. M., Elmets, C. A., & Katiyar, S. K. (2005). Orally administered green tea polyphenols prevent ultraviolet radiation-induced skin cancer in mice through activation of cytotoxic T cells and inhibition of angiogenesis in tumors. *Journal of Nutrition, 135*(12), 2871-2877.

Mesaik, M. A., Jabeen, A., Halim, S. A., Begum, A., Khalid, A. S., Asif, M., Fatima, B., Ul-Haq, Z., & Choudhary, M. I. (2012). In Silico and In Vitro Immunomodulatory Studies on Compounds of Lindelofia stylosa. *Chemical Biology and Drug Design, 79*(3), 290-299.

Mohamed-Yasseen, Y., Barringer, S. A., Splittstoesser, W. E., & Schnell, R. J. (1995). Rapid propagation of tuna (Opuntia ficus-indica) and plant establishment in soil. *Plant Cell, Tissue and Organ Culture, 42*(1), 117-119.

Moussa-Ayoub, T. E., El-Samahy, S. K., Kroh, L. W., & Rohn, S. (2011). Identification and quantification of flavonol aglycons in cactus pear (Opuntia ficus indica) fruit using a commercial pectinase and cellulase preparation. *Food Chemistry, 124*(3), 1177-1184.

Ou, S., & Kwok, K. C. (2004). Ferulic acid: Pharmaceutical functions, preparation and applications in foods. *Journal of the Science of Food and Agriculture, 84*(11), 1261-1269.

Piao, M., Mori, D., Satoh, T., Sugita, Y., & Tokunaga, O. (2006). Inhibition of endothelial cell proliferation, in vitro angiogenesis, and the down-regulation of cell adhesion-related genes by genistein combined with a cDNA microarray analysis. *Endothelium: Journal of Endothelial Cell Research, 13*(4), 249-266.

Ribéreau-Gayon, P., & Stonestreet, E. (1966). Dosage des tannins du vin rouges et determination du leur structure (Content of tannins in red wine and determination of their structure). *Chemical Analysis, 48*, 188–196.

Sakushima, A., & Nishibe, S. (1988). Mass spectrometry in the structural determination of flavonol triglycosides from Vinca major. *Phytochemistry, 27*(3), 915-919.

Serra, A. T., Poejo, J., Matias, A. A., Bronze, M. R., & Duarte, C. M. M. (2013). Evaluation of Opuntia spp. derived products as antiproliferative agents in human colon cancer cell line (HT29). *Food Research International, 54*(1), 892-901.

Shahidi, F., & Wanasundara, P. K. (1992). Phenolic antioxidants. *Critical Reviews in Food Science and Nutrition, 32*(1), 67-103.

Singleton, V. L., & Rossi, J. (1965). Colorimetry of total phenolics with phosphomolybdic phosphotungstic acid reagents. . *American Journal of Enology and Viticulture, 16*, 144-158.

Stintzing, F. C., & Carle, R. (2005). Cactus stems (Opuntia spp.): A review on their chemistry, technology, and uses. *Molecular Nutrition and Food Research, 49*(2), 175-194.

Swain, T., & Hillis, W. E. (1959). The phenolic constituents of Purmus domestica. I. The quantitative analysis of phenolic constituents. *Journal of the Science of Food and Agriculture, 10*, 63-68.

Valente, L. M. M., da Paixão, D., do Nascimento, A. C., dos Santos, P. F. P., Scheinvar, L. A., Moura, M. R. L., Tinoco, L. W., Gomes, L. N. F., & da Silva, J. F. M. (2010). Antiradical activity, nutritional potential and flavonoids of the cladodes of Opuntia monacantha (Cactaceae). *Food Chemistry, 123*(4), 1127-1131.

Vauzour, D., Rodriguez-Mateos, A., Corona, G., Oruna-Concha, M. J., & Spencer, J. P. E. (2010). Polyphenols and human health: Prevention of disease and mechanisms of action. *Nutrients, 2*(11), 1106-1131.

White, P. J., & Xing, Y. (1997). *Antioxidants from Cereals and Legumes.* . Champaign, IL: AOCS Press.

Wollenweber, E. (1982). Flavones and flavonols. . In J. B. Harborne & T. J. Mabry (Eds.), *The Flavonoids-Advances in Researches.*, (pp. 240–242). London: Chapman and Hall.

Yang, S. K., Yoneda, F., Oide, H., & Sakae, M. (1998). Isorhamnetin 3-O-robinobioside, its manufacture, and testosterone 5a-reductase inhibitors and therapeutics containing the flavonoid derivative. *Japanese Kokai Tokkyo Koho*, 7.

Yeddes, N., Chérif, J. K., & Ayadi, M. T. (2014). Comparative study of antioxidant power, polyphenols, flavonoids and betacyanins of peel and pulp of three Tunisian Opuntia forms. *Pakistan Journal of Biological Sciences*, *17*(5), 650-658.

Yeddes, N., Chérif, J. K., Guyot, S., Baron, A., & Trabelsi-Ayadi, M. (2014). Phenolic profile of tunisian opuntia ficus indica thornless form flowers via chromatographic and spectral analysis by reversed phase-high performance liquid chromatography-UV-photodiode array and electrospray ionization-mass spectrometer. *International Journal of Food Properties, 17*(4), 741-751.

Yeşilada, E., Tsuchiya, K., Takaishi, Y., & Kawazoe, K. (2000). Isolation and characterization of free radical scavenging flavonoid glycosides from the flowers of Spartium junceum by activity-guided fractionation. *Journal of Ethnopharmacology, 73*(3), 471-478.

Figure captions

Figure 1. Inhibitory effect of flower extracts A and C on NO production in LPS-induced macrophages RAW 264.7. Data represent the mean ± standard deviation of three independent experiments.

Figure 2. Chromatogram of the selected *O. ficus-indica* flower extract C obtained by HLPC-DAD detector at 280 nm. Numbers correspond to the main compounds detected and are reported in Table 4.

Figure 3. The main fragmentation pathway of (a) compound **9,** rutin (m/z=609) and (b) compound **10,** Isorhamnetin-7-O-rutinoside (m/z=623)

Table 1. Extraction conditions and yields of obtained extracts per g of dried cactus pear flower

Extract Code	Extraction method	Solvent	Solvent ratio (%)	Temperature (ºC)	Yield (g)*
A	Maceration	acetone/HCl/water	79:1::20	25	0.18±1.63[a]
B	Maceration	methanol//HCl/water	49:1::50	25	0.14±2.82[a]
C	ASE	acetone/water	80:20	80	0.18±0.0[a]
D	ASE	methanol/water	50:50	80	0.34±4.89[b]

Table 2. Effect of extraction method on total phenolics, procyanidins, flavonoids and antioxidant activity of extracts from *O. ficus-indica* flowers

Extraction method	Total phenolics (mg GAE/g)	Procyanidins (mg CCE/g)	Flavonoids (mg CE/g)	ORAC (µmol TE/g)
A	2.85 ± 0.00^{c}	0.05 ± 0.00^{b}	1.58 ± 0.01^{c}	243.11 ± 4.55^{a}
B	2.34 ± 0.01^{a}	0.03 ± 0.00^{a}	0.78 ± 0.01^{a}	326.44 ± 4.99^{c}
C	3.18 ± 0.01^{d}	0.07 ± 0.00^{c}	1.48 ± 0.03^{b}	272.84 ± 6.63^{b}
D	2.4 ± 0.00^{b}	0.07 ± 0.00^{c}	0.80 ± 0.02^{a}	263.72 ± 4.99^{b}

Data indicate mean value ± standard deviation of three independent extractions. Different uppercase letters within column are significantly different ($P<0.05$, Duncan´s test).

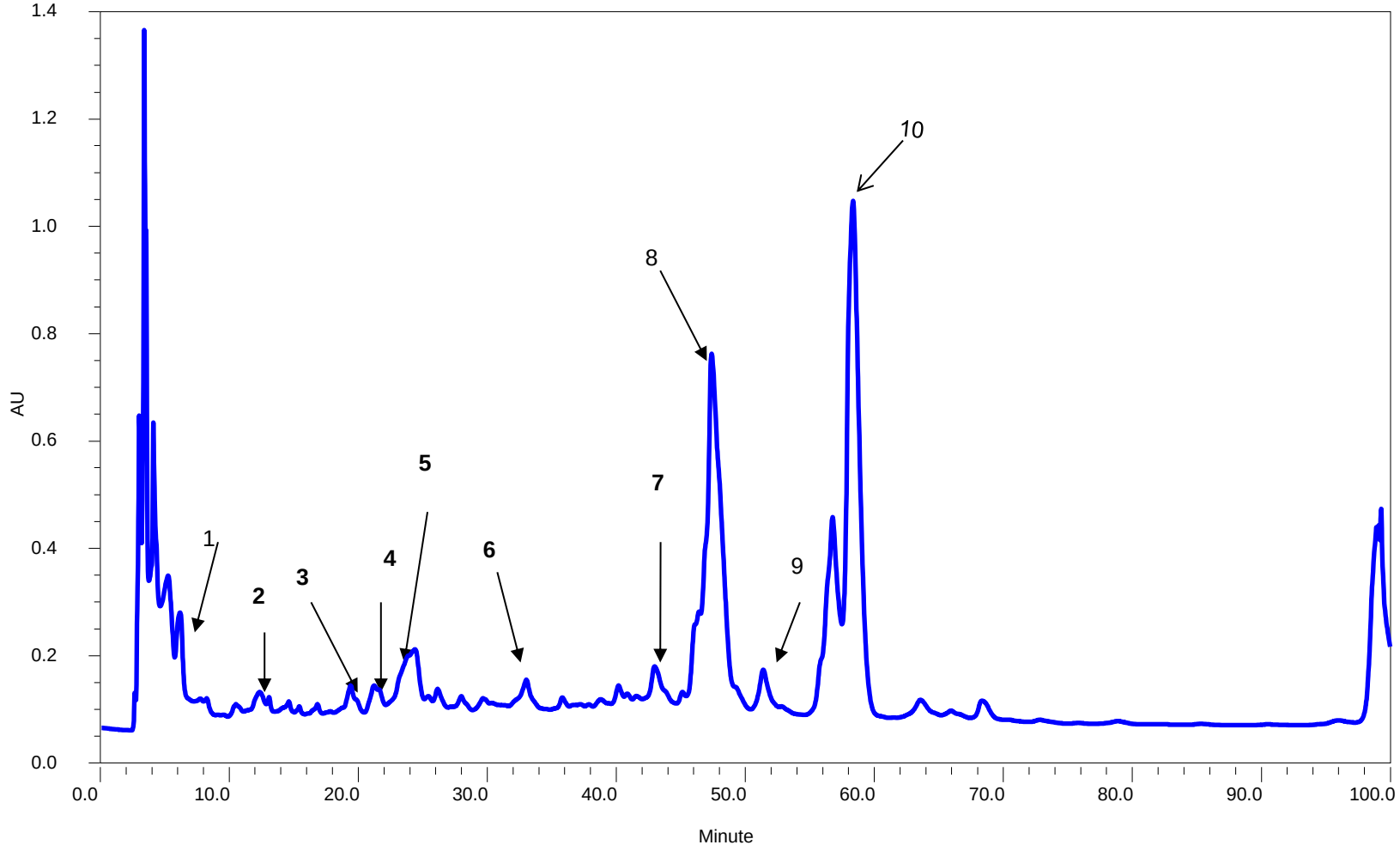
Table 3. Identification of phenolic compounds from *Opuntia ficus-indica* flowers by using high performance liquid chromatography (HPLC) coupled to ultraviolet-visible diode array detector (UV-Vis-DAD) and mass spectrometry detector (ion trap).

Peak	Rt (min)	λmax (nm)	$[M-H]^-$	Fragments (% intensity)	Neutral fragments loss	Assignment
Phenolic acids						
1	3.40	238, 282	517	371 (14%), 209 (53%), 195 (100%), 193 (49%), 179 (24%), 135 (16%)	146, 162	5-hydroxyferulic acid-rhamnoside-hexoside
2	5.78	230, 266	513	195 (14%), 191 (55%), 167 (100%), 161 (43%), 133 (24%), 128 (21%)	30, 322	caffeoyl methoxycinnamoyl quinic acid
3	11.02	232, 274	549	255 (100%)	294	1,4-diferuloyl syringic acid
4	25.43	230, 264, 292	325	305 (100%), 255 (5%), 239 (35%), 167 (14%)	70, 72	4-*p*-coumaroyl caffeic acid
5	27.55	232, 274	517	239 (100)	278	1,5-dicaffeoyl ferulic acid
6	39.57	232,310	681	485 (21%), 391 (22%), 383 (100%), 323 (7%), 255 (12%), 191 (16%), 163 (62%), 119 (15%)	28, 44, 64, 60, 68, 196, 102	1,4-syringicferuloyl 4-coumaroyl caffeic acid
Flavonols						
7	54.81	256, 352	1231	1085 (20%), 925 (4%), 785 (11%), 610 (90%), 595 (100%), 542 (11%), 119 (8%)	140, 146, 160, 175, 423	quercetin-derivative
8	57.74	258, 364	769	609 (100%), 463 (54%)	146, 160	isorhamnetin 3-*O*-rhamnosyl 7-*O*-rutinoside
9	63.02	256, 352	609	593 (25%), 505 (19%), 463 (5%)	104, 42	quercetin 7-*O*-rutinoside
10	69.14	256, 350	623	477 (35%), 417(12%)	146,60	isorhamnetin 7-*O*-rutinoside

Table 4. Phenolic compound content in *Opuntia ficus-indica* flowers

Compound	Identification	% total identified phenolics
1	5-hydroxyferulic acid-rhamnoside-hexoside	3.50±0,03
2	caffeoyl methoxycinnamoyl quinic acid	3.65±0.04
3	1,4-diferuloyl syringic acid	1.02±0.022
4	4-*p*-coumaroyl caffeic acid	0.63±0.04
5	1,5-dicaffeoyl ferulic acid	1.51±0.051
6	1,4-syringicferuloyl 4-coumaroyl caffeic acid	2.54±0.032
7	quercetin-derivative	3.40±0.022
8	isorhamnetin 3-*O*-rhamnosyl 7-*O*-rutinoside	32.84±1.07
9	quercetin 7-*O*-rutinoside	3.19±0.016
10	isorhamnetin 7-*O*-rutinoside	47.72±1,32

Figure 1


1.4
1.2
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Minute
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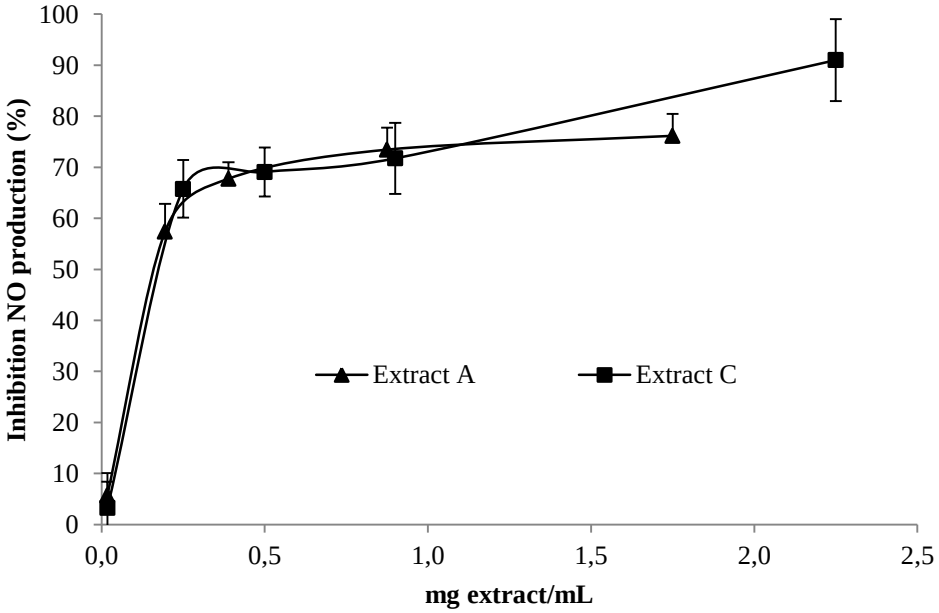
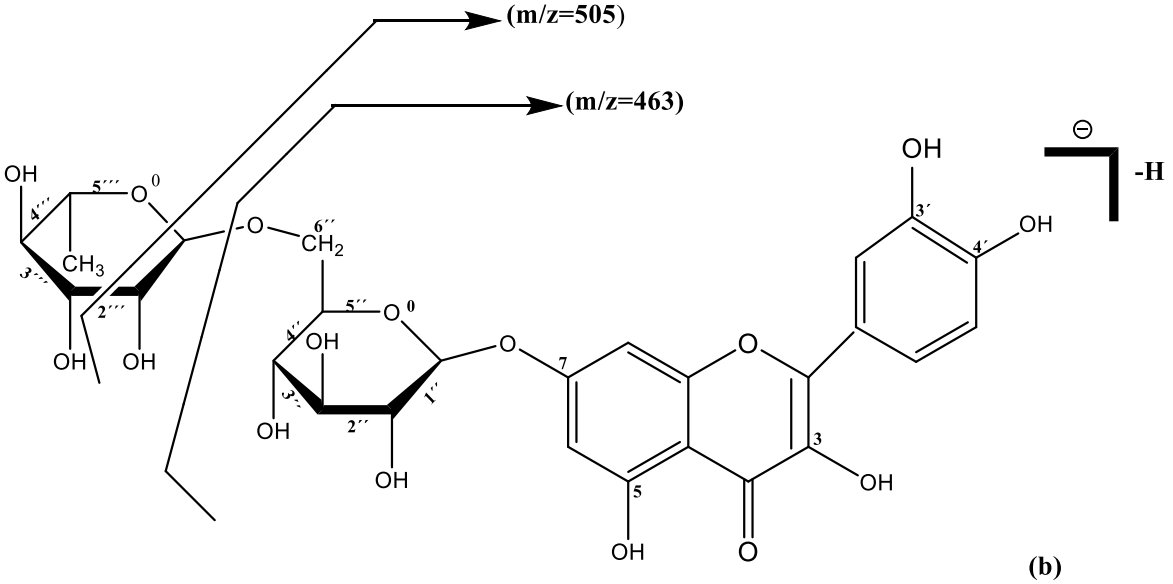
Figure 2


Inhibition NO production (%)
100
90
80
70
60
50
40
30
20
10
0
0,0
0,5
1,0
1,5
2,0
2,5
mg extract/mL
Extract A
Extract C

(a)



(b)