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# **Oil composition and characterization of phenolic compounds of *Opuntia ficus-indica* seeds**

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## **Abstract**

The seed composition of four varieties of *Opuntia ficus-indica* growing in Algeria was investigated. Seeds ground into a fine powder were first, subjected to oil extraction and fatty acids analysis. The phenolic compounds were then extracted from the defatted powder of seeds in order to be quantified and characterized by liquid chromatography coupled to mass spectrometry (LC-MS<sup>n</sup>) and to nuclear magnetic resonance (LC-NMR) approaches. In addition, an evaluation of the antioxidant activity of the phenolic extracts was investigated. Gas chromatography analysis of the seed oil showed high percentages of linoleic acid in the four varieties ranging from 58 to 63%. The phenolic profile of the *Opuntia ficus-indica* seeds displayed a high complexity, with more than 20 compounds detected at 330nm after the LC separation. Among them, three isomers of feruloyl-sucrose were firmly identified and another was strongly supposed to be a sinapoyl-diglycoside. High correlations were found between phenolic content in the defatted seed extracts and their antioxidant activity. The data indicate that the defatted cactus seed wastes still contain various components that constitute a source for natural foods.

**Key words:** *Opuntia ficus-indica*, seeds, fatty acids, phenolic compounds, antioxidant activity.

## **1. Introduction**

*Opuntia ficus-indica* (OFI) belongs to the Opuntioideae subfamily among the Cactaceae. Several cultivars are found in the Mediterranean basin. They grow also throughout Algeria where they are used mainly for human food, exclusively as fresh fruit, but also for livestock forage and planted as ornamentals or for fencing.

The literature contains much information concerning the cactus pear, describing the chemical composition of the pulp, peel and seeds (El Kossori, Villaume, El Boustani, Sauvaire, & Méjean, 1998; Felker, Soulier, Leguizamon, & Ochoa., 2002; Ramadan & Morsel, 2003a,b; Matsuhira, Lillo, Sáenz, Urzúa, & Zárate, 2006), the antioxidant, anti-inflammatory and anti-ulcerogenic properties of the pulp (Galati, Monforte, Tripodo, d'Aquino, & Mondell, 2001; Ahmed, El Tanbouly, Islam, Sleem, & El Senousy, 2005; Livrea & Tesoriere, 2006; Maataoui, Hmyene, & Hilali, 2006) and, more generally, the nutritional significance of *Opuntia* sp. (Stintzing, Schieber, & Carle, 1999). However, among all this literature, seed has been the less studied part.

The edible part of the fruit contains a relatively large number of seeds, which amount can vary from 30% to 40% on a dry weight basis. These seeds are usually discarded while proper utilization of these waste products could lead to an important new source of oil and meal

(Habibi, Heux, Mahrouz, & Vignon, 2008). OFI seeds were effectively demonstrated to be rich in the health-promoting polyunsaturated fatty acids and vitamins and may potentially be included in animal and human diets (Ramadan & Morsel, 2003a). Hence, developing high value methods for utilizing the byproducts seeds are of great importance.

Recently, the research has been directed towards natural antioxidants in the seeds. Indeed, seeds from *Opuntia* sp. were shown to be rich in polyphenols, flavonoids and tannins, the concentrations of those molecules being always higher than in the fruit pulp (Cardador-Martínez, Jiménez-Martínez, & Sandoval, 2011; Morales, Ramírez-Moreno, Sanchez-Mata, Carvalho, & Ferreira, 2012).

Phenolic compounds are a large group of ubiquitous molecules synthesized by plants under different environmental factors and stress conditions (Boudet, 2007). They form a diverse group that includes phenolic acids, flavonoids and tannins. Phenolic compounds may arise in food plants as glycosides or esters with other natural compounds such as sterols, alcohols, glucosides and hydroxyl fatty acids (Ghasemzadeh & Ghasemzadeh, 2011).

The interest in plant materials containing phenolic compounds are increasing due to their high antioxidant potency, which may offer protection against cancer through the inhibition of oxidative damage, known to be a potential cause of mutation (Khomdram & Singh, 2011). The antioxidative properties of phenolic compounds 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. To our knowledge, the phenolic composition of OFI seeds is very badly documented, studies being often limited to global phenolic measurements. To date, only one study identified individual phenolic acids and flavonoids in *Opuntia* seeds by using HPLC separation coupled to retention time comparison with standards (Tounsi et al., 2011). This approach allowed notably to identify catechin, rutin and syringic acid, but was however limited to the identification of molecules for which a commercial standard was available. Determining the phenolic profile of OFI seeds could serve as a basis for their further utilization in addition to the oil extraction. This work was carried out on seeds of four OFI varieties (green, yellow, orange and red) growing in Algeria. The main objectives of this comparative study were (i) to determine the fatty acid composition of these seeds, (ii) to quantify phenolic contents and (iii) to characterize the phenolic composition in defatted seeds. For the third point a combined approach of LC-MS<sup>n</sup> and LC-NMR was used. In addition, this study was also designed to evaluate the antioxidant and reducing power of the defatted seed extracts. This study goes within the framework of the valorization of the OFI of local origin and the findings will

provide more details on bioactive substances from cactus seeds which are considered as by-products.

## **2. Material and methods**

### **2.1. Plant material**

The study was conducted on four varieties of OFI chosen according to the presence or the absence of spines, the color and the shape of the fruits. They have been picked on August 2008 from Bejaia (Algeria). For each variety, a representative sample comprised of 10 ripe berries has been taken.

### **2.2. Fruit preparation**

The fruits were washed with running water to remove glochids and impurities, air-dried and hand-peeled. The seeds were separated from the juicy pulp, washed abundantly with distilled water then dried at room temperature for 24H and weighed to be at end reduced into a fine powder using a blender type A11 basic (IKA, Germany).

### **2.3. Seed oil Characterization**

#### **2.3.1. Seed oil extraction**

OFI seed oil was extracted using the Soxhlet apparatus (Behr, Labor-Tecnick, Dusseldorf, Germany). Thirty grams of powder were mixed with 150ml of hexane for 9H at 120°C. The solvent was evaporated at 40°C using a Buchi rotavapor R-200 (Germany). The oil weight was determined as follows: Oil weight (%) =  $[(M_1 - M_0) / M_2] * 100$ , where  $M_0$  is the weight of the empty flask (g),  $M_1$  the weight of the flask after evaporation (g) and  $M_2$  the weight of the seeds powder (g). The resulting oil was kept away from light and at low temperature.

#### **2.3.2. Identification of fatty acids by gas chromatography**

The fatty acids composition of the four oil samples was determined through gas chromatography using a Perichrom TM 2000 gas chromatograph (Perichrom, Saulx Les Chartreux, France) equipped with a flame ionization detector and a fused capillary (50cm length, 0,25mm internal diameter) column of silica (Varian, North America). Carrier gas was hydrogen and the temperature for injection and detection was 260°C. The injection volume was 1µl and the analysis duration was 45min. The software WINLAB (Perichrom, Saulx Les Chartreux, France) was used for the chromatograms integration. Fatty acids were identified by comparison of their retention times with standard mixtures.

### **2.4. Seed phenolic compounds**

#### **2.4.1. Phenolic extraction**

The solid-liquid extraction method reported by Nawaz, Shi, Mittal, and Kakuda (2006) was used to extract the phenolic compounds from the defatted seed powder. Two grams of sample

were added to 20ml of ethanol 70% (v/v). The mixture was subjected to an agitation during 2H at room temperature in darkness and then filtered. A second extraction was performed following the same conditions. The two filtrates were combined, concentrated under vacuum using a rotary evaporator and finally reconstituted in 10ml of pure methanol and stored at -20°C.

#### **2.4.2. Determination of total phenolic contents**

The phenolic compounds content was determined according to the Velioglu method (Velioglu, Mazza, Gao, & Oomah, 1998). In summary, 0.5ml of extract was mixed with 1.5ml Folin-Ciocalteu reagent, diluted ten times. After 5min, 1.5ml of sodium carbonate (6%) were added. The mixture was incubated in darkness for 1H and then the absorbance was measured at 760nm against a blank. Gallic acid was used as standard for the calibration curve. The results are expressed as mg equivalent of gallic acid (GAE) per 100g of dry mater.

#### **2.4.3. Determination of condensed tannin (proanthocyanidins) contents**

The method based on butanol/HCl dosage described by Vermerris and Nicholson (2006) was followed with small modifications. It consisted to mix 250µl of the seed extract with 2.5ml of an acidic solution of ferrous sulfate [77mg of ferric ammonium sulfate:  $\text{Fe}_2(\text{SO}_4)_3$  dissolved in 500ml of (3:2 n-butanol:HCl)]. After mixing and incubating at 95°C for 50min, absorbance at 530nm was measured against a blank. Condensed tannins were calculated by using the following formula:  $[A_{530\text{nm}} * \text{DF} * \text{MW} / (\epsilon * l)]$ , where DF is the dilution factor, MW the molecular weight of the cyaniding (287g/mol) and  $\epsilon$  the molecular extinction coefficient (34700l/mol/cm). The condensed tannins were expressed as mg of cyanidin equivalent/100g dry matter (CE).

#### **2.4.4. Determination of flavonoid contents**

The method of Bahorun et al. (1996) was adopted to determine the flavonoids content in OFI seeds extracts. To the aliquot of 1.5ml was added 1.5ml of  $\text{AlCl}_3$  reagent (2%). After 30 min of incubation the absorbance was recorded at 430nm against a blank. The flavonoid contents were calculated using a standard calibration curve, prepared from quercetin. The amount of flavonoids was expressed as mg of quercetin/100g of dry matter (QE).

#### **2.4.5. HPLC-MS<sup>n</sup> analysis**

The phenolic compounds were analyzed on a HPLC-MS<sup>n</sup> system consisting in a binary solvent delivery pump (Surveyor MS, Thermo-Finnigan, USA) connected to a diode array detector (Surveyor PDA plus, Thermo-Finnigan, USA) and a LTQ Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany), equipped with an atmospheric pressure ionization interface and operated in ESI negative and positive ion modes. The LC separation

was realized on a C18 LichroCART (250mm × 4,6mm) column (Merck, Germany) by using a gradient elution from 5% to 20% MeOH in 120min, then to 60% MeOH in 60min with a flow rate at 700µl/min. For the mass spectrometer, the spray voltage was 5kV, and the temperature of the heated capillary was set to 300°C. The flow rates for sheath gas, auxiliary gas and sweep gas were set to 40, 10 and 10 arbitrary units min<sup>-1</sup>, respectively. The capillary voltage was 36V, the split lens was -44V, and the front lens was -3.25V. The data were processed using the XCALIBUR software program (version 2.1, <http://www.thermoscientific.com>).

#### **2.4.6. HPLC-NMR analysis**

The phenolic compounds were subjected to HPLC-NMR analysis using an experimental procedure already described in a previous report (Acevedo et al., 2012). Briefly, HPLC was performed on a Agilent 1200 (Agilent Technologies, USA) connected to a diode array detector (Bruker, Germany) and a fraction collector FOXY R1 (Teledyne ISCO, USA). The LC separation was realized on a ProntoSil C18 (5µm porosity, size 250mm × 4,6mm, Bischoff, Germany) column by using a gradient elution from 10% to 35% ACN (0.25% TFA) in 55min with a flow rate at 600µl/min. NMR analysis was realized on a Bruker Avance III 600 MHz spectrometer (Bruker, Germany) equipped with a <sup>1</sup>H-<sup>13</sup>C inverse-detection flow probe. <sup>1</sup>H-NMR spectra were acquired in stopped-flow mode. The residual solvent signal was suppressed using pre-saturation. For 2D-NMR experiments, the compounds were directly collected after on-flow analysis onto FOXY collector from Teledyne ISCO (Lincoln, USA), lyophilized and analyzed by using classical NMR experiments: COSY and ROESY.

#### **2.4.7. Phenolic quantification**

The phenolic compounds were separated on a U-HPLC system (Prominence, Shimadzu, Japan) consisting in a binary solvent delivery pump connecting to a diode array detector. The LC separation was realized on a C18 Kinetex (100mm × 2.1mm) column (Phenomenex, USA) by using a gradient elution from 1% MeOH to 20% in 10min, then 60% MeOH in 5min with a flow rate of 300µl/min. The compound quantification was based on the area under peak determined at 330nm and expressed relative to a ferulic acid calibration curve.

### **2.5. Determination of antioxidant activity**

#### **2.5.1. Reducing power assay**

The reducing power was determined using the method of Oyaizu (1986). Five hundred microliters of seed extract were added to 1.25ml of phosphate buffer (0.2M, pH 6.6), 1.25ml ferricyanide potassium and 1.25ml trichloro-acetic acid (10%). The mixture obtained was incubated at 50°C for 20min. Then, 1.25ml of the mixture was picked and added to 1.25ml of

distilled water and 0.25ml of ferric chloride (1%). The absorbance was measured at 700nm. The results are expressed as equivalent mg Ascorbic acid (AAE) per 100g of dry matter.

### **2.5.2. Antiradical activity (radical DPPH)**

The antiradical DPPH of the seed extract was determined using the method of Brand-Williams, Cuvelier and Berset (1995) with a few modifications. Different concentrations ranging from 0.08 to 0.33g/ml were prepared. Sixty microliters of extract were mixed with 2.44ml of DPPH solution ( $6 \times 10^{-5} \text{M}$ ). The mixture was first incubated in darkness for 60min then the absorbance was read at 515nm against a control. The percentage of the free radical inhibition was calculated according to the formula:  $[(A_c - A_e) / A_c] * 100$  where  $A_c$  is the absorbance of the control and  $A_e$  the absorbance of the seed extract sample. The extract concentration providing 50% of radicals scavenging activity ( $EC_{50}$ ) was calculated from the graph of DPPH inhibition percentage against extract concentration.

### **2.6. Statistical analyses**

All data represent the average of three tests. For results, the analysis of variance ANOVA (STATISTICA) was used, and the degree of meaning of data was taken to probability  $p \leq 0.05$ .

## **3. Results and discussion**

### **3.1. Physico-chemical analysis**

The comparative physical parameters of the four OFI fruits analyzed in this study are shown in table 1.

The four OFI fruits were easily distinguished by their color (green, yellow, orange, red) and also by their shape and the presence of spines.

The fruit properties differed significantly ( $p \leq 0.05$ ) among the varieties. The green variety comprised higher fruit and edible portion (106g and 57g respectively) followed by the orange and red ones, while the yellow variety produced the smallest fruits (64g/fruits). The weight of seeds was higher in the green cultivar (3.9g) but was not statistically different between the other cultivars. The percentage of OFI seeds to the fresh weight out came between 5.7% and 7.9%. The yellow variety presented the highest and the orange and the red ones the lowest. These results are in the same range than those registered for Moroccan and Tunisian cultivars (Habibi, 2004; El-Guizani, Tlili & Triki, 2012).

The physico-chemical data of the harvest fruits were also measured and are available in Chougui et al. (in press). According to the obtained results, these fruits were characterized by a high humidity (87.4-89.6%), high pH (6.2-6.6), low acidity (0.04-0.07%) and high amount of solid soluble compounds (12-15%). The most important difference was observed with the last parameter, which is higher in the orange variety.



The large variability observed may be due to different factors, among them, variety, fruit load, lighting period, climate, and harvesting season (Barbera, Inglese & La Mantia, 1994).

### **3.2. Characterization of oil**

Fats and oils are important storage forms of carbon in many seeds. The oil rate has been estimated for the four OFI seed powders and the results showed significant oil contents that varied among the different varieties from 7.3 to 9.3% DM (table 2). The seeds from the yellow variety fruits had the highest oil content, followed by the green one with 8.4% DM. The lowest value was found in the red variety.

These oils contents were in agreement with those found in the literature (Ramadan & Morsel, 2003a; Ennouri, Evelyne, Laurence & Hamadi, 2005). However, they were lower than the crude oil yields found in fruits of Turkish origin with contents rising up to 14.4% of DM (Coşkuner & Tekin, 2003; Matthäus & Özcan, 2011).

Compared to other oil-seed crops, the OFI studied presented lower oil content. Indeed, higher amounts were recovered from cotton seeds (15–24%), soybean (17–21%), grape seeds (6–20%), olive (20–25%) (Matthäus & Özcan, 2006).

The extracted oils were composed mainly by unsaturated fatty acids (UFA) (around 83%) including mono-unsaturated fatty acids (oleic and vaccenic) and the poly-unsaturated fatty acid (linoleic). The saturated fatty acids (SFA; palmitic and stearic fatty acids) were detected, but at lower rates (16-17%). These results are in agreement with those of Ramadan and Morsel (2003a) and Tlili, Bargougui, Elfalleh, Triki, and Nasri (2011). The ratios of SFA acids to UFA varied from 4.9 to 5.0.

In all the samples, linoleic acid was the dominating fatty acid ranging from 58.7 (Red) to 63.1% (Orange), followed by oleic (from 15.2% in orange OFI to 24.3% in red, palmitic acid (from 12.7% in red to 13.4% in orange) and stearic (from 3.3% in orange to 4.2% in red). Vaccenic acid was only present in the green and orange varieties.

The fatty acid contents obtained in this study were quite similar to those found in the OFI oil studied by Matthäus and Özcan (2011) and Ozcan and Al Juhaimi (2011). However higher levels of palmitic acid (>16%) and linoleic acid (>67%) have previously been reported (Coşkuner & Tekin, 2003; Ramadan & Morsel, 2003a; Ennouri et al., 2005; Pantelean et al., 2009). The observed differences in the concentrations of oil could be due to maturity of the fruit or the environmental condition (Coşkuner & Tekin, 2003).

The essential fatty acids raises a great interest because of the health potential of poly-unsaturated fatty acids. It is considered that the fatty acids play a natural preventive role in cardiovascular diseases and in alleviation of some other health problems (Hooper et al., 2001;

Faremi & Ekanem, 2011). Moreover, poly-unsaturated fats were shown to promote the reduction of both total and LDL cholesterol and a small but significant decrease in HDL cholesterol (Ajayi & Ajayi, 2009). OFI-seed oil presents higher proportion of poly-unsaturated fats (linoleic acid) compared to certain conventional edible vegetable oils such as olive oil (3.5-21%), soy oils (49.7%), corn oils (47.7%), sesame oils (44.5%), sunflower oils (49.7%), and cotton oils (50.0%) (Astiasarán & Candela, 2000; Mailer, 2006). These characteristics illustrate that OFI may be an interesting natural source of edible oil containing high amounts of healthy fatty acids.

### **3.3. Total phenolic acids, flavonoids and tannins**

The levels of phenolic acids, flavonoids and condensed tannins in the four OFI seed extracts are summarized in table 3.

The total phenolic acid contents, measured by the Folin-Ciocalteu method, varied from 48mg (red variety) to 89mg GAE/100g (orange variety). The total flavonoid contents varied from 1.55 to 2.64mg QE/100g and the total tannins from 4.1 to 6.6mg CE/100g. For the total phenolic, flavonoid and tannin contents, the ranking between the four OFI varieties was maintained, the orange being always the most concentrated and the red the less.

With regard to the literature, the total phenolic contents compared with those obtained from Mexican and Tunisian OFI varieties (Camarena-Ordonez, Gutierrez-Urbe, Hernandez-Brenes, & Mertens-Talcott, 2010; Tounsi et al., 2011). Other varieties from Mexico and Taiwan exhibited quite higher content in phenolic acids and also flavonoids (Cardador-Martinez et al., 2011; Chang, Hsieh, & Yen, 2008).

These differences could be attributed to the type of the cultivar, geographical origin of the fruits, degree of maturity and the storage conditions but also to the extraction protocols and analytic assays. Indeed, in most of the studies cited above, differences were noticed with regard to the extraction time and the solvent used for phenolic extraction. In our case, it must be stressed that extraction was conducted on powders of defatted seeds. Regarding the available literature, the defatting process does not generally affect the extraction of phenolic compounds (Mondor et al., 2009, Khoo, Azlan, Ismail, & Abas, 2012). However, some authors found an increase in the phenolic extraction efficiency after defatting in peanut and sorghum that was explained by a higher disruption of the cell wall through heating or a breakdown of insoluble phenolic compounds (Nepote, Grosso, & Guzman, 2002; Kayitesi, 2009).

### **3.4. Identification of seed phenolic compounds**

The nature of phenolic compounds contained in the OFI seeds was analyzed by a combined approach of HPLC-MS<sup>n</sup> and HPLC-NMR studies. The HPLC separation profile revealed a

high complexity in the seed phenolic composition as demonstrated by more than 20 peaks recorded at 330nm (Fig. 1). The phenolic extract from each variety displayed the same profile (data not shown). A majority of those peaks being too small to support structural analyses, a subset of 8 major peaks was selected for identification (Fig.1). The MS and MS<sup>2</sup> data in positive and negative modes are listed for the 8 molecules in table 4. The Peaks 1–3 exhibited the same calculated mass at 518 and the same fragmentation pattern in MS (-), indicating that those peaks corresponded to 3 isomers. The [M-H]<sup>-</sup> fragment at *m/z* 355 corresponded to the loss of an hexose (162) from the parent [M-H]<sup>-</sup> peak at *m/z* 517. The [M-H]<sup>-</sup> fragment ion at *m/z* 337 was the result of the loss of an hexose and a molecule of water. The significant peak at *m/z* 193 corresponded to a ferulic acid obtained after the loss of two hexoses, and the peak at *m/z* 175 was obtained after the loss of two hexoses and a molecule of water. These observations suggested that the compounds 1-3 were composed of one feruloyl moiety and two hexoses. Complementary structural informations were provided after NMR analysis of the compound 3 which was the most abundant in the extract. NMR spectra were recorded after collecting the compound 3 from three successive injections. NMR data for the compound 3 are listed in table 5.

The<sup>1</sup>H-NMR data showed the presence of a tri-substituted phenolic ring, a double bond, a glucose and a fructose. The ROE correlation between proton H-2 and the OCH<sub>3</sub> group indicated that it was located on the third position of the phenolic ring. The coupling constant (*J* = 16 Hz) indicated that double bond was in *trans* conformation. NOE correlations H-7/H-2 and H-8/H-6 indicated the double bond position on the cycle. This structure was characteristic of ferulic acid. The compound 3 was therefore identified as a feruloyl-sucrose. The compounds 1 and 2 were too diluted in the extract to support NMR analysis, even after collecting several injections. With regard to the MS data, we could assume that these two compounds were structural isomers of feruloyl-sucrose.

MS data revealed a calculated mass for the compound 4 of 548. The MS<sup>2</sup> data in negative mode led to fragments at *m/z* 164, 175, 205, 223, 367 and 385 which were characteristic for sinapoyl-glucose (Wolfram et al., 2010). The mass of sinapoyl-glucose (*m/z* 386), corresponded to the mass of the compound 4 (*m/z* 548) with the loss of one hexose (*m/z* 162). It is thus tempting to assume that the compound 4 corresponded to a sinapoyl-diglucoside. However, we couldn't confirm the data by NMR analysis due to the low concentration of the molecule in the extract.

From MS data, the compounds 5 and 6 were supposed to be isomers with a same mass of 1136. The masses of compounds 7 and 8 were established to 328 and 563 respectively.

Fragmentation pattern in negative mode of the compound 8 revealed  $[M-H]^-$  fragments at  $m/z$  175 and 193 which could indicate the presence of a feruloyl moiety. However, no additional information could be obtained from NMR analysis due to a too low content of those molecules in the extracts.

Oligo and polysaccharide-bound hydroxycinnamic derivatives, such as sinapic and ferulic acids, are commonly found in plants (Huang, Rozwadowski, Bhinu, Schäfer, & Hannoufa, 2008; Mesaik et al., 2012). Feruloyl-sucrose was firstly identified from root extracts of *Polygala arillata* and, as a consequence, is commonly named Arillatose B (Kobayashi, Miyase, Suzuki, Noguchi, & Chen, 2000).

The hydroxycinnamic acid esters are directly involved in plant protection against pathogens by their involvement in the cell wall structure (Ikegawa, Mayama, Nakayashiki, & Kato, 1996). 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 (Louw, 1997; Ou & Kwok, 2004).

As far as we know, this is the first time that phenolic esters were described in OFI seeds. However, other compounds such as catechin, rutin and quercetin derivatives, already identified in *Opuntia* seeds (Tounsi et al., 2011), were not detected in the four varieties analyzed. Although these differences could be linked to the variety or the plant environment, another explanation could be related to the extraction procedure used. Indeed, our solid/liquid extraction procedure contained small differences with the maceration procedure described by Tounsi and coworkers (2011) in term of solvent (ethanol 70% vs methanol 100%) and stirring time (2h vs 30min). Moreover, although the seed defatting process was generally shown not to modify phenolic extraction efficiency on the basis of total phenolic assays, only few things are known about its impact on the phenolic composition.

### **3.5. Quantification of individual phenolic compounds**

The feruloyl-sucrose isomers (1, 2 and 3), the putative sinapoyl-diglucoside and the putative feruloyl derivative were quantified in the seed extracts relatively to a ferulic acid standard. The quantification data are given in the table 6. The five compounds were present in the seed extracts of the four varieties studied. In all varieties, the dominant compound was the feruloyl-sucrose isomer 3 (105 to 717  $\mu\text{moles}/100\text{g}$ ) followed by the putative feruloyl derivatives (34 to 233  $\mu\text{moles}/100\text{g}$ ), the putative sinapoyl-diglucoside (12.6 to 23.4  $\mu\text{moles}/100\text{g}$ ) and, at almost the same level, the feruloyl-sucrose isomers 1 (6.7 to 34.3  $\mu\text{moles}/100\text{g}$ ) and 2 (5.6 to 33.1  $\mu\text{moles}/100\text{g}$ ).

The seed extract of the orange variety showed, significantly, the highest values for the all detected compounds. The red variety presented lower amounts except for the sinapoyl-diglucoside compound.

#### **4.6. Antioxidant and antiradical activities**

The reducing power and DPPH free radicals were used to evaluate the antioxidant and the antiradical activities of the OFI seeds extracts. The reducing power values expressed as mg ascorbic acid equivalent per 100g of dry seeds are presented in figure 2. The values varied from 32.3mg (Red) to 51.3mg AAE /100g (Orange). The antiradical activity of the defatted seed extracts were comprised between a short interval, EC<sub>50</sub> ranging from 68.4 (Red) to 49.6mg/ml (Orange) (figure 3).

With regard to literature, most authors have evaluated the antiradical activity of the seed extract using the DPPH free radical. Cardador-Martínez et al. (2011) tested seed extracts of three Mexican varieties and found that the green and the yellow varieties had better scavenging effect than the red one. Similar tendency was observed for peel and juicy pulp extracts (Butera et al., 2002; Cardador-Martínez et al., 2011). These results coincide with those obtained in the present study.

Correlation analysis highlighted significant correlations between the dosed antioxidants contents and the activities tested (table 7). The samples presenting the highest antioxidant activities have also the highest phenolics, tannins and flavonoids contents. Indeed, the seed extract of the orange fruits presented better activities, while the extract from the red one showed lower ones. No correlation was found however between the flavonoid content and DPPH inhibition.

Similar results were noticed in previous studies (Cardador-Martínez et al., 2011). Other authors have reported higher coefficients ( $r > 0.90$ ) not only between OFI juicy pulp chemical constituents (betalains, ascorbic acids and total phenolics) but also with their antioxidant activities (Cai, Sun, & Corke, 2003; Stintzing et al., 2005). Moreover, among the coefficient obtained, it appears that some correlations were less significant than others or does not exist; this is particularly observed between the two activities ( $r = 0.614$ ,  $p \leq 0.05$ ) and between the DPPH inhibition activity and the amount of tannins ( $r = -0.658$ ,  $p \leq 0.05$ ) and of flavonoids ( $r = -0.574$ ,  $p > 0.05$ ).

It is well known that phenolic compounds exert antioxidant activity. To estimate the inhibitory capacity of these compounds against oxidants, data correlating antioxidant activities and phenolics concentration are commonly reported. However, when the  $r$  values from these correlations are low, it is concluded that phenolic compounds are not responsible

of the antioxidant activity. Although valid in most cases, this interpretation does not consider factors such as the differences in the phenolic profiles that can be qualitatively (type of phenolics present) and quantitatively (the relative amounts or proportions of phenolics present) between samples that vary with genotypes (Chirinos et al., 2008), samples of different maturity stages (Castrejon, Eichholz, Rohn, Kroh, & Huyskens-Keil, 2008), or even samples exposed to different postharvest storage conditions (Rapisarda, Lo Bianco, Pannuzzo & Timpanaro, 2008). Moreover, the antioxidant activity of a phenolic compound is related with its chemical structure. It can act synergistically, additively, or antagonistically to inhibit reactive species. Additionally, the presence of glycosylations on the molecule may decrease its antioxidant activity (Velazquez & Zevallos, 2009). Therefore knowing and considering the phenolic profiles in addition to the factors cited below will provide information on the relation of the antioxidant activity with the phenolic compounds.

## **5. Conclusion**

This investigation showed that the seeds of the studied varieties had an appreciable amount of oil with high level of unsaturated fatty acids which are healthy benefic. The oil rate and composition varied slightly among the four varieties. The defatted seeds contained total phenolic, flavonoid and tannin levels comparable to already published contents on OFI seeds. The LC-NMR indicated a high complexity in the seed phenolic composition and allowed the identification of hydroxycinnamic acids esters among which three feruloyl sucrose isomers. The OFI seeds or defatted seeds are not waste worthless, they should be considered of great interest for promoting the OFI culture and better exploitation of the fruits compartments so that all the nutrients present are fully used.

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## Figures list:

**Figure 1:** HPLC chromatogram of phenolic compounds of the OFI seed extract at 330nm.

**Figure 2:** Reducing power of OFI seed ethanol extracts  
a,b: The letters indicate the statistical difference

**Figure 3:** Scavenging activity of OFI seed ethanol extracts on the DPPH radical  
a,b,c: The letters indicate the statistical difference

**Table 1:** Fruit characteristics

Cultivars	Fruit characteristics	Fruit (g)	Edible portion (g)	Seed (g)	Ratio of seed (%)
Green	Spineless, skin green clear, ovoid, edible portion green clear	106±16 <sup>a</sup>	57±9 <sup>a</sup>	3.9±0.8 <sup>a</sup>	6.8±0.6 <sup>ab</sup>
Yellow	With spins, skin yellow, elongated, edible portion yellow orange	64±8 <sup>c</sup>	37±5 <sup>c</sup>	2.9±0.8 <sup>b</sup>	7.9±3.2 <sup>a</sup>
Orange	With spins, skin orange, ovoid, edible portion orange	76±8 <sup>b</sup>	42±6 <sup>b</sup>	2.4±0.4 <sup>b</sup>	5.7±0.6 <sup>b</sup>
Red	With spins, skin red, elongated, edible portion red	77±13 <sup>b</sup>	48±10 <sup>b</sup>	2.8±0.7 <sup>b</sup>	5.7±1.1 <sup>b</sup>

The values having the same letter within the column did not differ significantly from each other according to LSD test at  $p \leq 0.05$ .

**Table 2:** Fatty acids content identified in the OFI seed oil.

Variety	Oil rate (%)	Fatty acids (%)							
		Palmitic acid (C16)	Stearic acid (C18)	Oleic acid (C18:1n-9)	Vaccenic acid (C18:1n-7)	Linoleic Acid (C18:2n-6)	Total SFA	Total UFA	UFA/SFA
Green	8,4	13.1±0.1 <sup>b</sup>	3.5±0.1 <sup>b</sup>	16.3±0.1 <sup>c</sup>	5.3±0.1 <sup>a</sup>	61.8±0.1 <sup>c</sup>	16.6±0.1 <sup>b</sup>	83.5±0.1 <sup>a</sup>	5.0±0.1 <sup>a</sup>
Yellow	9,3	13.4±0.1 <sup>a</sup>	3.6±0.1 <sup>b</sup>	20.9±0.1 <sup>b</sup>	0.0±0.0 <sup>c</sup>	62.1±0.1 <sup>b</sup>	16.9±0.1 <sup>a</sup>	83.1±0.1 <sup>c</sup>	4.9±0.1 <sup>c</sup>
Orange	7,7	13.4±0.1 <sup>a</sup>	3.3±0.0 <sup>c</sup>	15.2±0.1 <sup>d</sup>	5.1±0.1 <sup>b</sup>	63.1±0.1 <sup>a</sup>	16.7±0.1 <sup>b</sup>	83.3±0.0 <sup>b</sup>	5.0±0.0 <sup>b</sup>
Red	7,3	12.7±0.1 <sup>c</sup>	4.2±0.1 <sup>a</sup>	24.3±0.1 <sup>a</sup>	0.0±0.0 <sup>c</sup>	58.7±0.1 <sup>d</sup>	16.9±0.1 <sup>a</sup>	83.1±0.1 <sup>c</sup>	4.9±0.1 <sup>c</sup>

SFA: saturated fatty acid, UFA: unsaturated fatty acid.

The values having the same letter within the column did not differ significantly from each other according to LSD test at  $p \leq 0.05$ .

**Table 3:** Antioxidant contents of the OFI seed extracts.

	<b>Total phenolic compounds (mg GAE/100g)</b>	<b>Flavonoids (mg QE/100g)</b>	<b>Tannins (mg CE/100g)</b>
<b>Green</b>	61±9 <sup>c</sup>	1.5±0.1 <sup>c</sup>	4.5±0.3 <sup>c</sup>
<b>Yellow</b>	74±3 <sup>b</sup>	1.9±0.1 <sup>bc</sup>	4.8±0.1 <sup>b</sup>
<b>Orange</b>	89±5 <sup>a</sup>	2.6±0.2 <sup>a</sup>	6.6±0.1 <sup>a</sup>
<b>Red</b>	48±1 <sup>d</sup>	1.5± 0.1 <sup>c</sup>	4.1±0.1 <sup>d</sup>

The values having the same letter within the column did not differ significantly from each other according to LSD test at  $p \leq 0.05$ .

**Table 4:** Mass data on the phenolic acids of the OFI seed extract.

Peak	Rt (min)	Molecular ion		Fragments MS/MS-	Estimated mass	Molecule name
		MS+	MS-			
1	51	519.1645	517.1549	355/337/193/175	518.15	Feruloyl-sucrose isomer 1
2	77	519.1645	517.1549	355/337/193/175	518.15	Feruloyl-sucrose isomer 2
3	98	519.1645	517.1549	355/337/193/175	518.15	Feruloyl-sucrose isomer 3
4	109	549.1937	547.1653	385/367/223/205/175/164	548.179	Sinapoyl- diglucoside
5	142	1137.3348	1135.3694	-	1136.35	Unknown 1
6	147	1137.3348	1135.3694	-	1136.35	Unknown 2
7	150	329.0982	327.0877	-	328.09	Unknown 3
8	153	564.1636	562.1548	386/337/193/175	563.15	Feruloyl derivative

**Table 5:**  $^1\text{H-NMR}$  data of the molecule 3 in acetone- $d_6$ .

$^1\text{H-RMN}$	
1	-
2	7.38 (1H, d, $J = 2$ )
3	-
4	-
5	6.87 (1H, d, $J = 8$ )
6	7.17 (1H, dd, $J = 8, 2$ )
7	7.65 (1H, d, $J = 16$ )
8	6.49 (1H, d, $J = 16$ )
OCH <sub>3</sub>	3.91 (3H, s)
Glucose	
1'	5.45 (1H, d, $J = 3$ )
2'	3.52 (1H, dd, $J = 8, 4$ )
3'	3.77 (1H, m)
4'	3.39 (1H, m)
5'	4.12 (1H, m)
6a'	4.45 (1H, dd, $J = 12, 2$ )
6b'	4.30 (1H, dd, $J = 12, 5$ )
Fructose	
1a''	3.65 (1H, d, $J = 12$ )
1b''	3.58 (1H, d, $J = 12$ )
3''	4.21 (1H, d, $J = 8$ )
4''	4.05 (1H, dd, $J = 8, 8$ )
5''	3.80 (1H, m)
6a''	3.77 (1H, m)
6b''	3.74 (1H, m)

**Table 6:** Phenolic compound contents identified in the OFI seed extracts ( $\mu\text{mol}/100\text{g DM}$ ).

Variety	Feruloyl-sucrose isomer 1	Feruloyl-sucrose isomer 2	Feruloyl-sucrose isomer 3	Sinapoyl-diglucoside	Feruloyl derivative
<b>Green</b>	14.2±1.5 <sup>b</sup>	5.9±0.5 <sup>b</sup>	253±11 <sup>b</sup>	12.6±2.2 <sup>b</sup>	47.6±9.2 <sup>b</sup>
<b>Yellow</b>	9.3±0.7 <sup>c</sup>	5.6±1.9 <sup>b</sup>	161±5 <sup>c</sup>	17.4±3.8 <sup>b</sup>	51.7±7.8 <sup>b</sup>
<b>Orange</b>	34±9 <sup>a</sup>	33±22 <sup>a</sup>	717±260 <sup>a</sup>	23.4±3.7 <sup>a</sup>	233±37 <sup>a</sup>
<b>Red</b>	6.7±0.4 <sup>d</sup>	6.8±0.6 <sup>b</sup>	105±14 <sup>d</sup>	17.3±1.9 <sup>b</sup>	34.0±4.2 <sup>b</sup>

The values having the same letter within the column did not differ significantly from each other according to LSD test at  $p \leq 0.05$ .

**Table 7:** Correlation coefficients for phenolic compounds, flavonoids, tannins and the antioxidant activities for the OFI seeds

	Flavonoids	Tannins	DPPH inhibition (EC <sub>50</sub> )	Reducing power
Phenolic compounds	0.913***	0.874***	-0.753**	0.895***
Flavonoids		0.957***	-0.574	0.855***
Tannins			-0.658*	0.754**
DPPH inhibition (EC <sub>50</sub> )				-0.614*

\*, \*\*, \*\*\*: Significant at  $p \leq 0.05$ ,  $p \leq 0.01$  and  $p \leq 0.001$  respectively.







