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Chemical, metabolomic and proteomic analysis of young cladodes from *Opuntia* cultivars with different domestication grade

Tesis que presenta

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Para obtener el grado de

Doctora en Ciencias en Biología Molecular

Codirectoras

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Constancia de aprobación de la tesis

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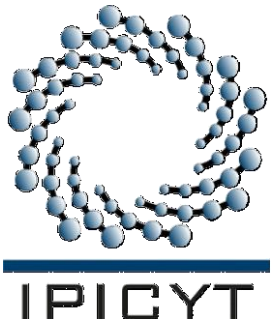

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Marizel Georgina Astello García

sobre la Tesis intitulada:

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que se desarrolló bajo la dirección de

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Dedications

A mis padres, Marcelino y Elma, he aquí la manifestación de mi imperecedero amor por ustedes, soy yo quien agradece, a Dios y a la vida, ser su hija.

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A la Sra. Sonia Bolaños

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ABSTRACT

"Chemical, metabolomic and proteomic analysis of young cladodes from *Opuntia* cultivars with different domestication grade"

Nutraceutical potential of *Opuntia* cladodes has been reported, therapeutical effects have been associated to antioxidant compounds reported in *Opuntia*, such phenolic acids and flavonoids. *Opuntia* species have been classified according to their domestication grade. This gradient started with wild and less domesticated *O. streptacantha* Lem., followed by *O. hyptiacantha* F.A.C. Weber, then the moderate domesticated *O. megacantha* Salm-Dyck, prior to *O. albicarpa*, to finish with *O. ficus-indica*, the most domesticated and humanized species. The aim of this work was to evaluate the effect of domestication grade on chemical and nutritional attributes, metabolomic profiles and proteomic analysis of taxonomically identified *Opuntia* species. Proximate composition, mineral content and chemical attributes were different between *Opuntia* species. However, no clear trend was observed among domestication grade and attribute. Total phenolic and total flavonoids content as well as antioxidant capacity were different among cultivars from collect 2010 and collect 2012. However, the content of total phenolics and antioxidant capacities increased cladodes from collect 2012. These differences were no related to domestication grade. The metabolomic analysis of *Opuntia* cladodes showed similar profiles. However some compounds were present in specific cultivars. Particularly compound 26' identified as Kaempferol 3-O-arabinofuranoside was detected only in *O. streptacantha,* a wild species. Similar 1D patterns were observed in proteomic analysis between cultivars. So far, shotgun analysis allowed the identification of 266 proteins from *O. megacantha* "Rubí reina" and *O. ficus-indica* "Rojo vigor", 38 and 61 specific proteins for each cultivar, respectively. Data from *O. streptacantha, O. hyptiacantha* and *O. ficus-indica* is still being analyzed. Shotgun analysis showed differences in protein expression due to domestication process. This is the first study of domestication effect on nutritional composition, chemical attributes, phenolic compounds profiling and proteomic analysis by shotgun proteomic in *Opuntia* cladodes.

Keywords: *Opuntia*, domestication, antioxidant, phenolics, flavonoids, proximate, LC-MS/MS, mineral, shotgun, proteomics.

RESUMEN

“Chemical, metabolomic and proteomic analysis of young cladodes from *Opuntia* cultivars with different domestication grade”

En este trabajo se analizaron las especies de *Opuntia* incluidas en un gradiente de domesticación que inició con *O. streptacantha Lem.* la especie más primitiva y menos domesticada, seguida de *O. hyptiacantha* FAC Weber, *O. megacantha* Salm- Dyck, moderadamente domesticada, luego *O. albicarpa*, y en el extremo más domesticado y humanizado se encuentra *O. ficus-indica*. Los efectos terapéuticos se han asociado a los compuestos antioxidantes reportados en *Opuntia*, tales como ácidos fenólicos y flavonoides. El objetivo de este trabajo fue evaluar el efecto del grado de domesticación en los atributos químicos y nutricionales, perfil metabolómico y proteómico de especies de *Opuntia* taxonómicamente identificados. La composición proximal, los atributos químicos y contenido de minerales fueron diferentes entre las variedades de *Opuntia*, aunque no se observó una tendencia en relación al grado de domesticación. Los contenidos de fenoles y flavonoides totales, así como la capacidad antioxidante se modificaron entre las variedades de las colectas 2010 y 2012. Sin embargo, se observó un incremento en el contenido de fenoles totales y capacidad antioxidante en las variedades 2012. Tales diferencias no están relacionadas al grado de domesticación. El análisis de metabolitos mostró perfiles de compuestos fenólicos similares, aunque el análisis diferencial demostró la presencia de compuestos específicos, como el 26’ (kaempferol 3 - O- arabinofuranósido) detectado únicamente en *O. streptacantha*, la especie más silvestre. El análisis proteómico reveló patrones similares, en primera dimensión, entre variedades. El análisis tipo ”shotgun” permitió la identificación de 266 proteínas en *O. megacantha* "Rubí reina " y *O. ficus -indica* "vigor Rojo", así como 38 y 61 proteínas específicas de cada variedad, respectivamente. Los datos obtenidos de *O. streptacantha, O. hyptiacantha y O. ficus –indica* aún están siendo analizados. El análisis tipo shotgun reveló diferencias relacionadas con el proceso de domesticación de *Opuntia*. Éste es el primer trabajo sobre el efecto de domesticación en el contenido nutricional, atributos químicos, perfil de compuestos fenólicos y análisis proteómico tipo “shotgun” en cladodios de *Opuntia*.

Palabras clave: *Opuntia*, domesticación, antioxidante, fenólicos, flavonoides, proximal, mineral, LC-MS/MS, shotgun, proteómica.

CHAPTER 1.

ANTECEDENTS

1.1 INTRODUCTION

In Mexico, cactus pear has been considered as a high value plant since pre-Columbian times. Ancient Aztecs used cactus as a source of food, for pigment production (carminic acid) and as a medicinal plant. The relevant role of this plant was described in documents such Codex Mendoza (Berdan and Anwalt, 1992), Florentine Codex and José Antonio Alzate's manuscript (1777) (Figure 1). After colonization, cactus pear was spread through trade into North and South America, Mediterranean (South Europe and North Africa), and subsequently into arid and semiarid regions of the world (Griffith, 2004). Additional uses were given to cactus such as fodder, natural fences, and an alternative for decreasing erosion (Stintzing and Carle, 2005). In the last few decades, the use of cactus pear for treatment of chronic-degenerative diseases has promoted a scientific interest to study its therapeutical properties and its nutraceutical potential.

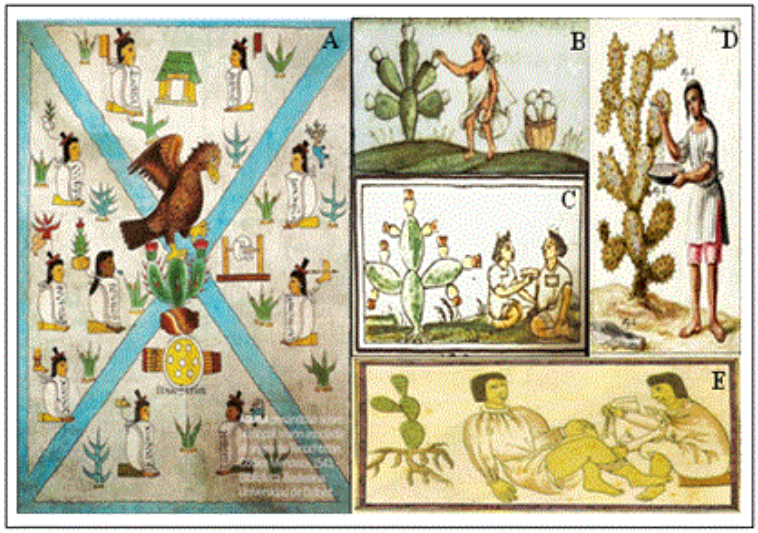

Figure 1. An eagle standing on a cactus pear, a sign for the establishment of Tenochtitlan (A); harvest of *O. albicarpa* (B); consumption of *O. ficus-indica* flower tea (C); production of carminic acid (D); medicinal use of *O. macrorrhiza* (E). Modified from Berdan and Anwalt (1992), Florentin Codex, and Alzate (1777).

1.2 *Opuntia* biology

Cactus pear belongs to *Opuntia* genus (family Cactaceae, subfamily Opuntioideae, subgenus *Opuntia*), which includes 189 species in America, from Canada to Argentina (Anderson, 2001) in. *Opuntia* is a dicotyledonous plant (today is a Magnoliophyta division) with segmented stems, also called cladodes or pads or phylloclades, and produces fruits or cactus pear and nopalitos (young cladodes). The cladodes are succulent organs consisted by white medullar parenchyma (core tissue) and the chlorophyll containing photosynthetically active parenchyma (cortex tissue). Their shape and length depend on type of species. The stems are modified structures for optimal water retention by producing mucilage, a mixture of hydrocolloids. In addition, the thick cuticle wax coating present in both cladodes and fruits minimizes water losses. On the surface of cladodes, the areoles are found. These structures contain spines (modified leaves), trichomes, glochids (short, sharp and retro-barbed spines) and meristematic tissue. Cladode size, shape and color depend of the species. In addition, cactus pear roots allow the optimal absorption of superficial water and nutrients. These features are very useful during dry seasons and, eventually drought conditions. Flowers (red, yellow, white or orange) are sessile, hermaphrodite and appear at the top of cladodes. The characteristics of the fruit also depend on species, including color (yellow, orange, red, green or purple), shape (ovoid, oblong or elliptical) and size (Anderson, 2001; Mondragón, 2001; Saenz *et al.,* 2006). *Opuntia* performs photosynthesis through the Crassulacean Acid Metabolism (CAM), characterised by an inverse opening of stomata. During the night, carbon dioxide (CO_2) is captured, metabolized to malate and stored in vacuoles. In the light phase, malate is integrated into the Calvin Cycle for carbohydrate biosynthesis (Hopkins, 1999). Due these physiological and biochemical modifications, *Opuntia* plants are suitable for arid and semiarid environments.

1.3 Economical value

México hosts 78 wild and cultivated species, located in arid and semiarid zones (corresponding to 51% of total national surface), where the widest

germplasm variability of *Opuntia* is found (Bellón *et al.,* 2005). Particularly, 29 species are distributed in the Meridional Highland Plateau, occupying approximately 3,000,000 Ha of territory, where *O. streptacantha* and *O. leucotricha* are the most predominant wild species found there (Guzmán, 2003; Reyes-Agüero *et al.,* 2005). Among cultivated species, *O. albicarpa* sp. Novo and *O. ficus-indica* are important for horticultural purposes (Mondragrón, 2001). *O. ficus-indica* is the cultivated species with highest economical value worldwide (Reyes-Agüero *et al.,* 2005). In Mexico, during 2012, *O. ficus-indica* cultures for production of tender cladodes or nopalitos (12,453.72 ha), fodder (17,747.75 ha) and cactus pear (55, 917.70 Ha) yielded 856,542.61 t, 133,913.83 t and 527,627.11 t, respectively (SIAP, 2012). Indeed, the economical importance of this domesticated species favored the interest about its ancestral specimens.

1.4 *Opuntia ficus-indica* and *O. albicarpa* domestication process

In Mexico, the highest richness of wild cultivars and cultivars of *Opuntia* is found. Due this, is considered the center of *Opuntia* domestication (Reyes-Agüero *et al.,* 2009). The domestication may be defined as the last phase in humanization process. In the first phase, the collection or pick up of abundant, persistent and useful species from wild environments is performed. Then, these species are cultured in human environments (phase two) and finally, genetic modifications occur (phase three). Thus, domestication refers to an evolutionary process in populations, generated by human selection. During this process, genetic, morphological and physiological changes occur in wild populations, generating domesticated populations with features more desirable for human requirements. In plants, humanization gradients consist in two extremes, wild species is in one, and domesticated species in the other (Perales and Aguirre, 2008; Arellano and Casas, 2003).

Reyes-Agüero *et al.,* (2005) elucidated a domestication gradient for *O. ficus-indica* and *O. albicarpa* by comparing 42 morphological characteristics of cladodes, fruits and flowers of 243 *Opuntia* variants collected (n=1458) from Meridional Highland Plateau of Mexico. Thus, *O. streptacantha* Lem. was assigned to the wild and less domesticated extreme, followed by *O.*

hyptiacantha F.A.C. Weber, then the moderate domesticated *O. megacantha* Salm-Dyck, prior to *O. albicarpa*, to finish with *O. ficus-indica*, the most domesticated and humanized species. Some of the morphological features of these species are briefly described as follows.

1.4.1 *Opuntia streptacantha*

O. streptacantha is a 1.2 to 4.5 m height arborescent with a defined and thick stem and abundant branches. Its dark green cladodes are obovate, rounded or oval. The length, width and thickness of mature cladodes vary from 19 to 43 cm, 13 to 31 cm and 1.0 to 3.7, respectively. Abundant areoles are found in surface and spines are partially flattened. The flowers are yellow, orange or red. The fruit is obovoid, globose, cylindrical or elliptical (2.9 to 5.5 cm diameter) and its peel has areoles with glochids but no spines. *O. streptacantha* is the wild species of major economic importance in San Luis Potosí, although also distributed in Zacatecas, Durango, Aguascalientes and Querétaro (Bravo, 1978; Reyes-Agüero *et al.,* 2009).

1.4.2 Opuntia hyptiacantha

O. hyptiacantha is a 1 to 5 m height shrubby plant. Its dark green cladodes are obovate, rounded or oval. The length, width and thickness of mature cladodes vary from 19 to 49 cm, 13 to 31 cm and 0.8 to 4, respectively. On surface, abundant areoles with partially flattened spines are found. The flowers are yellow, orange or red. The fruit is obovoid, globose, cylindrical or elliptical (1.3 to 6.8 cm diameter) and its peel has areoles with glochids. It is mainly distributed in Aguascalientes, Puebla, Oaxaca, México City and State of México (Bravo, 1978; Reyes-Agüero *et al.,* 2009).

1.4.3 Opuntia megacantha

O. megacantha is a 1 to 6.5 m height shrubby plant with a lignified stem (at the base). Its green cladodes are obovate, oblanceolate or oval. The length, width and thickness of mature cladodes vary from 15 to 63 cm, 10 to 34 cm and 0.3 to 4.7, respectively. The areoles contain from two to seven spines. The flowers are yellow or red. The fruit is obovoid, globose, cylindrical or elliptical

(3.2 to 13.5 cm diameter) and its peel has spineless areoles. It is mainly distributed in Meridional Highlands Plateau (Bravo, 1978; Reyes-Agüero *et al.,* 2009).

1.4.4 Opuntia albicarpa

O. albicarpa is a 1 to 6.5 m height shrubby or decumbent plant. Its light green cladodes are oblanceolate or oval. The length, width and thickness of mature cladodes vary from 10 to 81 cm, 12 to 42 cm and 0.32 to 5.1, respectively. The areoles contain from two to eight spines. The flowers are orange or yellow. The fruit is yellow and obovoid, globose, cylindrical or elliptical (2.4 to 12.4 cm diameter) and its peel has spineless areoles. It is mainly cultivated in Hidalgo and Zacatecas (Bravo, 1978; Reyes-Agüero *et al.,* 2009).

1.4.5 Opuntia ficus-indica

O. ficus-indica is a 0.7 to 3.0 m height plant with branches since base. Its green cladodes are oblanceolate or rhombic. The length, width and thickness of mature cladodes vary from 24 to 67 cm, 12 to 51 cm and 0.5 to 4.8, respectively. Spineless areoles in cladodes and fruit peel. The flowers are yellow. The fruit is red (2.4 to 12.4 cm diameter). *O. ficus-indica* is cultivated in North and Central México (Bravo, 1978; Reyes-Agüero *et al.,* 2005).

Due this domestication process, attributes such size, shape, color, texture and flavor of cladodes and fruits were improved. *O. streptacantha*, the wildest species, produces relatively small and fleshy cladodes, covered with a tough cuticle and areoles containing a large amount of thick spines; the fruits are small and contain higher amount of normal seeds (and few sterile seeds) to ensure the survival of the species. On the other extreme, *O. ficus-indica* cladodes and fruits features includes intermediate to large size, spineless areoles and a very resistant cuticle, and the fruit contains a small amount of seed, and some of them are sterile (Reyes-Agüero *et al.,* 2005).

1.5 Nutritional content

Nutraceuticals or functional foods have a nutritional role in diet and contains phytochemicals (secondary metabolites) with health promoting

properties associated to antiradical scavenging capacity of these metabolites (Briskin, 2000; Lizcano *et al.,* 2012). The nutraceutical potential of *Opuntia* has been evaluated and the nutritional content and medicinal properties of cladodes and fruits have studied (Feugang *et al.,* 2006). *Opuntia* contains high levels of important nutrients such organic acids, lipids, minerals, vitamins, aminoacids, including taurine, polysaccharides, betalains, and phenolic compounds (Salim and *et al.,* 2009; Zhang *et al.,* 2011). These attributes depend on maturity stage, harvest season, environmental conditions and the species (Hernandez-Urbiola *et al.,* 2011; Contreras-Padilla *et al.,* 2012; Ramírez-Tobías *et al.,* 2007). However, the effect of domestication on proximate composition of young cladodes has not been evaluated.

1.6 Medicinal properties

Therapeutical activities of *Opuntia* (cladodes, fruit, root, flowers) have been evaluated and anti-inflammatory, wound-healing, cytoprotective, neuroprotective, antispasmodic, chemopreventive, hypocholesterolemic, antigenotoxic, antioxidant, antiulcerogenic, hypoglycemic, antidiabetic, antitumoral, hypotensive, immunomodulatory and antiviral effects have been reported (Ahmad *et al.,* 1996; Alarcón-Aguilara *et al.,* 1998; Budinsky *et al.,* 2001; Park and Chun, 2001; Dok-Go *et al.,* 2003; Galati *et al.,* 2003; Saleem *et al.,* 2005; Ambriz *et al.,* 2006; Galati *et al.,* 2007; Shepetkin *et al.,* 2008; Yang *et al.,* 2008; Zorgui *et al.,* 2009; Baldassano *et al.,* 2010; Hahm *et al.,* 2010; Najm and Desiree, 2010; Alimi *et al.,* 2011; Brahmi *et al.,* 2011; Zhao *et al.,* 2011). These effects have been associated to secondary metabolites with antioxidant properties such ascorbic acid, α-tocopherol, carotenoids, glutathione, phenolic acids and flavonoids (Zhong *et al.,* 2010; Valente *et al.,* 2010).

1.7 Secondary metabolites

Plant secondary metabolites are low-molecular weight compounds synthesized as a defense mechanism against biotic and abiotic stress. The biological activities attributed to these compounds have been widely studied. Currently, 60% of anticancer drugs and 75% of drugs for infectious diseases

treatment are obtained from natural sources or bioactive compound analogues (Wilson and Roberts, 2012; Cragg and Newman, 2009). Secondary metabolites are also used in food, textile and cosmetic industry (Srivastava and Srivastava, 2007). According to their biosynthetic pathway, secondary metabolites can be classified into several major groups: terpenoids, alkaloids, other nitrogen compounds and phenolic compounds (Manach *et al.,* 2009). In this study we focused on phenolic compounds.

1.7.1 Phenolic compounds

Phenolic compounds are phenylpropanoids derivatives containing hydroxyl groups capable of reducing or scavenging reactive oxygen species and other organic or nonorganic compounds. This antiradical behavior generates the antioxidant properties of phenolic compounds associated to their health-promoting effects (Buchanan, 2002; Pérez-Jiménez *et al.,* 2010). These compounds can be classified into two major groups: flavonoids (flavonols, flavanols, flavones, flavanones, anthocyanins and isoflavones) and non-flavonoids (hydroxybenzoates, hydroxycinnamates, phenolic aldehydes, phenolic alcohols, stilbenes and lignans) (Manach *et al.,* 2009). More than 5000 different phenolic compounds have been reported, which is related to their broad spectrum of pharmacological effects and medicinal uses (Steinmann and Ganzera, 2011). Currently, metabolomic approaches, such metabolic profiling allow the

1.7.2 Metabolomic profiling

Metabolome refers to the complete complement of all the low molecular weight molecules present in cells in a particular physiological or developmental state (Goodacre, 2005). Metabolomics is defined as the comprehensive analysis in which all metabolites of a biological system were identified as quantified (Fiehn, 2001). The metabolomic profiling involves the identification and quantitation by a particular analytical procedure of a predefined set of metabolites belonging to a selected metabolic pathway (Ryan and Robards, 2006). Metabolomics has been widely used for medicinal plant research (Li *et*

al., 2013). Currently, liquid chromatography coupled to mass spectrometry (LC-MS^n) is commonly used for metabolomic analysis in plants.

In plants, the identification of phenolic compounds has represented an analytical challenge due to variability and complexity of chemical structures and the limited commercial availability of standards. Therefore LC-MS^n analysis is an optimal technique for characterization of phenolic compounds in complex matrices. Their MS^n fingerprint or fragmentation pattern allows the elucidation of backbone, the determination of substituents, type and position of glycosilation (Ferreres *et al*., 2012).

Several studies focused on metabolomic analyses of *Opuntia* have been reported. Guevara-Figueroa et al. (2010) reported six phenolic acids (gallic, coumaric, 3-dihydroxybenzoic, 4-benzoic, ferulic and salicylic acids) and five flavonoids (isoquercitrin, isorhamnetin 3-*O*-glicoside, nicotiflorin, rutin and narcissi) in wild and cultivated cladodes from Opuntia spp. Ginestra *et al.,* (2009) analyzed the phenolic profile of *O. ficus-indica* cladodes by LC-MS techniques and identified two phenolic acids (eucomic and psidic acids) and fourteen glycosilated flavonoids, mainly kaempferol and isorhamnetin glycosides (glucoside and rhamnoside). Santos-Zea *et al.,* (2011) reported nine flavonoids from *O. ficus-indica* extracts. According to data described above, most of the metabolomic analyses were performed in domesticated *O. ficus-indica* cultivars. In addition, data about phenolic profile of wild *Opuntia* species is scarce.

1.7.3 Production of secondary metabolites in *in vitro* cultures

Plant cell culture (PCC) has great potential as an alternative system for the production of phytochemicals of interest, commonly extracted from whole plants (Mulabagal and Hsin-Sheng 2004). Particularly, callus culture, an amorphous (non-differentiated) tissue generated from disorganized growth of plant cells cultured under suitable conditions, offers desirable features for biochemical studies and for secondary metabolites production (Jalali *et al.,* 2012). Advantages of plant cell culture include increase of production, recovery and isolation of metabolites (Wilson and Roberts, 2012). In addition, depletion of natural sources is avoided by using *in vitro* cultures. Particularly, several

strategies for increase of metabolite concentration have been described, such elicitation. Elicitation induces defense related genes and proteins, as well as secondary metabolites (Namdeo 2007). Application of jasmonic acid and chitosan have been widely used as elicitors in cell cultures of several species (Babar-Ali et al. 2007; Gadzovska et al. 2007; Pavlík et al. 2007; Kneer et al. 1999; Zhan et al., 2001; Santos-Díaz et al. 2005).

Several protocols for the establishment of *in vitro* culture from *O. ficus-indica* cultivars have been reported (Llamoca-Zárate *et al.,* 1998; Llamoca-Zárate *et al.,* 1999; Cruz *et al.,* 2009; Silos-Espino *et al.,* 2006). However there is no available data about *in vitro* culture from wild *Opuntia* species for metabolite production.

JUSTIFICATION

Lopez-Palacios *et al.,* (2011) evaluated the effect of domestication on polysaccharides and fiber contents in young cladodes of *Opuntia* species (similar gradient included in this study). According to their results, higher mucilage contents were determined in cultivated species, but pectins and hemicelluloses contents were higher in wild species. Thus, genetic modifications of *Opuntia* through domestication modified the nutritional and chemical composition of cladodes and fruits. However, the data about it is limited.

The metabolomic profiling of cladodes by LC-MS represents a great strategy for screening phenolic compounds and identification of biomarkers related to the domestication and therapeutical properties of *Opuntia*.

Since *Opuntia* is a non-sequenced genome plant, proteomic analysis is an optimal technique to evaluate the effect of domestication on protein expression patterns and the identification of differential proteins among cultivars.

This study will contribute to the knowledge and validation of *Opuntia* as nutraceutical and the identification of phenolic compounds will allow elucidating the action mechanisms associated to therapeutical properties of *Opuntia*. Since this work is included in the BIOPUNTIA Project, a México-France bilateral collaboration, with IPICyT, UASLP, INRA, INSERM and CNRS as partner institutions, the analytical protocols developed in this study, will be useful for analysis of phenolic compounds in *Opuntia* samples and *in vitro* cell cultures.

HYPOTHESIS

1. The chemical composition of *Opuntia* young cladodes grown under same environmental conditions (temperature, humidity and soil) and harvested at same maturity stage varies due to the domestication.
2. The domestication process modifies the content of phenolic compounds and the antioxidant capacity of *Opuntia* cladodes.
3. The protein expression patterns of cladodes are modified due to domestication.

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CHAPTER 2.
NUTRITIONAL AND CHEMICAL ANALYSIS OF CLADODES FROM FIFTEEN *Opuntia* CULTIVARS WITH DIFFERENT GRADE OF DOMESTICATION

1.1 INTRODUCTION

Cactus pear belongs to *Opuntia* genus (*Cactaceae*) which includes 189 species and 78 are native to México (Anderson, 2001). *Opuntia* is a dicotyledonous plant with flattened segmented stems, also called cladodes, with a great adaptation capacity to extreme environmental conditions (i.e. temperature, drought) and distributed in arid and semiarid regions (Mondragón, 2001; Yahia and Mondragón, 2011). *Opuntia* had been closed related to Mexican native people since 14000 years (Majure *et al.* 2012) as a source of food, building, natural pigment production (carminic acid), for ornamental uses and in traditional medicine (Betancourt-Domínguez *et al. 2006*). *Opuntia* plants produce tender cladodes, consumed as vegetable, and fruit, prickly pear, both highly appreciated in arid zones of México due its high nutritional value (Zhong *et al.* 2010; Shetty *et al.* 2012).

Opuntia is considered a medicinal plant due to its use in treatment of wounds and oxidative-stress related diseases, including diabetes, cancer, gastric and cardiovascular diseases (Galati *et al.,* 2003; Saleem *et al.,* 2005; Hahm *et al.,* 2010; Andrade-Cetto and Wiedenfeld, 2011). The therapeutical effects have been associated to antioxidant compounds found in *Opuntia*, such ascorbic acid, α-tocopherol, caroteinoids, glutathione and phenolic acids and flavonoids (Zhong *et al.,* 2010; Valente *et al.,* 2010). In addition, the nutritional composition of *Opuntia* has been investigated and is considered a good source of fiber and calcium (Stintizing and Carle, 2005). However, chemical composition of cladodes is modified by maturity stage, harvest season, environmental conditions, post-harvest treatment and type of species (Ramírez-Tobías *et al.,* 2007; Hernandez-Urbiola *et al.,* 2011; Contreras-Padilla *et al.,* 2012).

O. ficus-indica Miller is most domesticated and cultivated species. Domestication refers to the genotypic and phenotypic modifications that occur in wild plants, in order to improve its adaptation capacity to human environments (Perales and Aguirre, 2008). Reyes-Agüero *et al.,* (2005) elucidated a domestication gradient for *O. ficus-indica*, which started from wild and less domesticated *O. streptacantha* Lem., followed by *O. hyptiacantha* F.A.C. Weber, then the moderate domesticated *O. megacantha* Salm-Dyck, prior to *O. albicarpa*, to finish with *O. ficus-indica*. Due this process, attributes such size, shape, color, texture and flavor of cladodes and fruits were improved. Lopez-Palacios *et al.,* (2011) evaluated the effect of domestication on the polysaccharides content from fourteen *Opuntia* cultivars included in this gradient, and they found mucilage content was higher in the most domesticate but hemicelluloses and pectins contents were higher in wild species. Then, domestication process in *Opuntia* modified biochemical properties of cladodes. However, there is a lack of information about the effect of domestication on nutrimental attributes and antioxidant compounds.

The aim of this study was to evaluate the effect of domestication on nutritional and chemical composition and antioxidant capacity of cladodes from fifteen *Opuntia* species. In addition, the comparative analysis of phenolic compounds profiles and proteomic patterns of cladodes will provide scientific evidence of domestication effect on *Opuntia* at a metabolomic level.

2. OBJECTIVES

2.1 General

Evaluate the effect of domestication on nutritional and chemical composition and antioxidant capacity of *Opuntia* cladodes.

2.1 Specifics

1. To obtain the proximate composition of *Opuntia* samples.
2. To obtain the mineral profile of *Opuntia* samples by ICP-MS.
3. To standardize the protocol for phenolic compounds content determination in lyophilized samples and *in vitro* cultures from *Opuntia* by UV-Vis spectrophotometry.
4. To evaluate the antioxidant capacity of ethanolic extracts from *Opuntia* by DPPH assay.
5. To analyze the phenolic compounds contained in ethanolic extracts from *Opuntia* by thin layer chromatography (TLC)
6. To obtain the metabolomic profiles of *Opuntia* samples by LC-MS and comparative analysis of identified phenolic compounds.

3. MATERIAL AND METHODS

3.1 Reagents, chemicals and solvents

All chemicals and reagents were of analytical grade and were obtained from commercial sources. Gallic acid, Folin-Ciocalteau reagent, sodium carbonate, quercetin, aluminum chloride, 1,1-diphenyl-2-picrylhydrazil, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), rutin, chlorogenic acid, formic acid, acetic acid, ethyl acetate (EtoAc), dextrose, anthrone, sodium hydroxide were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). For LC-MS/MS analysis, all solvents were HPLC grade. Silica plates were purchased from Merck. Ultra-pure water was obtained from a Milli-Qplus purification system (Millipore Corp., Bedford, MA).

3.2 Plant material

Young cladodes or nopalitos were collected from donor plants grown under same environmental conditions (temperature, precipitation and soil) at the *Opuntia* Germoplasm Bank in the Centro Regional Universitario Centro Norte of the Universidad Autónoma de Chapingo (CRUCEN-UACh), located in Huertas del Orito, Zacatecas, México (22° 44.7' North latitude, 102° 36.4' west length) (Lopez-Palacios et al. 2011). Young cladodes (15–25 cm of length) showed in Figure 1, were washed, N_2 freeze, grinded (KRUPS GX4100 grinder) and stored at -80°C until analysis. Prior to the determinations reported in this work, samples were freeze-dried (LABCONCO USA) and sieved (Fisher Scientific Company, USA).

3.3 Proximate analysis

Total nitrogen content was determined by the micro-Kjeldahl method (12.960.52 method, AOAC 1990) and total protein content was calculated using a 6.25 factor. Fat content was determined by the Soxhlet method (996.01 method, AOAC 1997). Crude fiber and ash contents were obtained according to 991.43 and 900.02 methods, respectively (AOAC 2007). Carbohydrates content was obtained by difference. All determinations were analyzed by triplicates.

3.4 Mineral content determination by ICP-MS (Inductively Coupled Plasma Mass Spectrometry).

For mineral content analysis, hydrolysis of the samples was performed according to the optimized method in the National Laboratory of Medical and Environmental Biotecnology (LANBAMA). Samples (0.1 g, DW) were hydrolyzed using a HNO_3:HCl (1:3) mixture, on a hot plate, diluted with ultra-pure water and filtered (Whatman paper, 0.45 µm). Samples were analyzed in a Varian 820-MS ICP mass spectrometer (Agilent Technologies, USA).

3.5 Total sugars, titratable acidity and pH

Total soluble sugars were determined using the anthrone-H2SO4 reagent following the method reported by Irigoyen *et al.,* (1992). Dextrose was used as a reference compound (R^2=0.9824) and results were expressed as mg of dextrose per 100 g of sample. Titratable acidity and pH were measured according to the AOAC method (1999).For pH measurement, samples (1g, FW) were added to 10 ml of ultra-pure water, homogenized and measured with a pH meter 210 (Hanna instruments). Acidity of samples (1 g, FW) was titrated to a pH 8.2 using a 0.1 N NaOH solution. Results were expressed as citric acid percentage, using the formula % citric acid = [(N*V*mEq citric acid)/W], where N was the concentration of NaOH;V, the volume of NaOH used for titration; W, the weight of sample; and mEq, the citric acid milliequivalents (0.064).

3.6 Phenolic compounds extraction procedure

In this study, the extraction procedure of phenolic compounds from dried samples was standardized (Ginestra *et al.,* 2009). Briefly, samples (1 g, DW) were added to 100 ml of absolute ethanol (pre-chilled, -20°C), mixed (4°C, 2.5 h) and centrifuged (14 000 rpm, 4°C). Pellets were used for proteomic analysis and supernatants were filtered (Whatman paper, 0.45 µm) and used for phenolic compounds determinations. Independent extraction procedures (triplicates) were performed for each sample. All determinations were made within the 24 h after extraction.

3.7 Total phenolics contents

For total phenolics determination, Folin-Ciocalteau assay was used, following a methodology reported by Luximon-Ramma *et al.,* (2002) with brief modifications. In a test tube, 20 µl of sample (extract, standard or blank) were added to 1.58 ml of MilliQ water and mixed, followed by the addition 100 µl of Folin-Ciocalteau reagent. Then, 300 µl of 20 % Na_2CO_3 were added and the mixture was allowed to stand for 2 h (at room temperature). Absorption was read at 765 nm in a Cary UV-Vis spectrophotometer. Total phenolics were expressed as µmol Gallic acid g^{-1} of sample. All determinations were performed in triplicates.

3.8 Total flavonoids content

Total flavonoids quantification was performed following the method reported by Guevara-Figueroa *et al.,* (2010) with some modifications. Briefly, 15 µl of extract (or standard, for calibration curve) were added to 735µl of ultra-pure water and mixed with 750 µl of 2% $AlCl_3$ (aqueous). After ten minutes (at room temperature), the mixtures were read at 367 nm in a Cary UV-Vis Spectrophotometer. Quercetin was used as a reference standard and results were expressed as µmol Quercetin g^{-1} of sample. All determinations were performed in triplicates.

3.9 Antioxidant capacity

Free radical scavenging activity of *Opuntia* extracts was evaluated using the DPPH assay, following the methodology reported by Heimler *et al.,* (2007) with brief modifications. In a test tube, one milliliter of extract was added to one milliliter of 0.1 mM DPPH solution (in ethanol), mixed and incubated during 30 min (at room temperature). The absorption of each mixture was measured at 517 nm in a Cary UV-Vis spectrophotometer.The results were expressed as Trolox equivalents (µmol per 100 g^{-1} of sample), according to Ozgen *et al.,* (2006).

3.10 Thin layer chromatography (TLC) analysis

In this study, extraction method and solvent system for TLC analysis were standardized. Samples (0.05 g) were extracted with absolute ethanol (1 ml), filtered added to ere performed on pre-coated silica gel (Merck), using a solvent system recommended for polyphenolic compounds reported by Valente *et al.,* (2010) which consisted in a EtOAc:HCOOH:HOAc:H2O (100:11:11:27) mixture. Bands were visualized using UV irradiation (365 nm).

3.11 Phenolic compounds profiling by LC-MS analysis

3.11.1 Extraction procedure

For phenolic compounds extraction, a mixture of methanol:acetone:water (5:4:1) was added to samples (0.1 g) and stirred during 2.5 h (4°C). Samples were filtered (0.2 µm) and concentrated in a vacufuge (Labconco, USA) to dryness (45 °C). Extracts were resuspended in methanol:water prior to LC-MS analysis.

3.11.2 LC-DAD-ESI-MS^n analysis conditions

A Surveyor (Themo Scientific, USA) with a Diodo Array Detector system coupled to a Thermo Finnigan (San José, CA) LCQ Deca XP Max Ms^n ion trap mass spectrometer equipped with an ESI source were used. LC conditions: mobile phases consisted in acetonitrile/methanol (1:1, 0.5% formic acid, phase A) and water (0.5% formic acid, phase B). An isocratic elution gradient was applied at a flow rate of 200 µl min^{-1}. A Reverse-phase Phenomenex (Torrance, USA) Luna C_{18} 3µm) was used. MS and MS^2 acquisitions were performed on negative mode using a Xcalibur 1.3 software. ESI conditions: spay voltage 1.5 kV, capillary temperature 120 °C, capillary voltage 30 V and N_2 was used as a desolvation gas.

3.11.3 Identification of phenolic compounds

For assignation of identity, databases and literature were consulted. The ChemOffice program was used for structural formulas processing.

4. RESULTS AND DISCUSSION

In this study, young cladodes from fifteen different *Opuntia* cultivars were analyzed (Table 1). The species *O. streptacantha* Lem., *O. hyptiacantha* A. Weber, *O. megacantha* Salm-Dyck, *O. albicarpa* Scheinvar and *O. ficus-indica* (L.) Mill. were selected. Three different cultivars belonging to each species were studied (Reyes-Agüero et al. (2005). The young cladodes were harvested at similar maturity stage, according to Ramírez-Tobias et al. (2009). The donor plants were grown under similar environmental (temperature, soil, precipitation) conditions. Therefore, the variables considered for the comparative analysis among cultivars were the domestication grade and species. The young cladodes from *Opuntia* cultivars are shown in Figure 2. The wild species cladodes are smaller, with high density of areoles containing abundant spines. The domesticated species produce larger cladodes and the amount of areoles and spines decreases or disappear.

4.1 Nutritional composition of *Opuntia* cladodes

In this study, young cladodes from *Opuntia* species with different grade of domestication were analyzed (Table 1), according to the domestication gradient reported by Reyes-Agüero *et al.,* (2009).Proximate composition of *Opuntia* species is shown in Table 2. The cultivars with highest protein content were *O. streptacantha* “Cardona” (11.84 %), *O. hypticantha* “San pedreña” (12.57 %), *O. megacantha* “Rubí reina” (11.32 %) and *O. ficus-indica* “Atlixco” (12.56 %). The lowest contents were observed in *O. streptacantha* “Tuna loca” (11.00 %), *O. hypticantha* “Amarilla olorosa” (10.15 %), *O. megacantha* “Rojo lirio” (10.13 %) and *O. ficus-indica* “Copena V1” (12.56 %). In *O. albicarpa* cultivars, no differences were observed between protein contents (11.2 % to 11.97 %). Similar results were obtained by Ramírez-Moreno *et al.,* (2013) in *O. ficus-indica* “Milpa alta” (12.9 %) and *O. ficus-indica* “Atlixco” (13.84 %), but higher than total protein values (5.85 - 8.99 %) obtained in a study to evaluate the effect of maturity stage on nutritional content of *O. ficus-indica* cladodes (Hernández-Urbiola *et al.,* 2011).

Table 1. *Opuntia* species included in this study.

Species	Cultivar	Key	Domestication grade [a]
O. streptacantha Lem.	Cardona	Os-cr	Low, wild
	Tuna loca	Os-tl	
	Cardona de castilla	Os-cc	
O. hyptiacantha A. Weber	Memelo 1	Oh-m1	Moderated, close to wild
	San pedreña	Oh-sp	
	Amarilla olorosa	Oh-ao	
O. megacantha Salm-Dyck	Rojo lirio	Om-rl	Moderated, close to domesticated
	Rubí reina	Om-rr	
	Amarillo plátano	Om-ap	
O. albicarpa Scheinvar	Copena Z1	Oa-cz	Domesticated
	Naranjón legítimo	Oa-nl	
	Villanueva	Oa-vi	
O. ficus-indica (L.) Mill.	Copena V1	Ofi-cv	Very high, domesticated
	Rojo vigor	Ofi-rv	
	Atlixco	Ofi-at	

[a] *Opuntia* domestication gradient elucidated by Reyes-Agüero *et al.,* (2005).

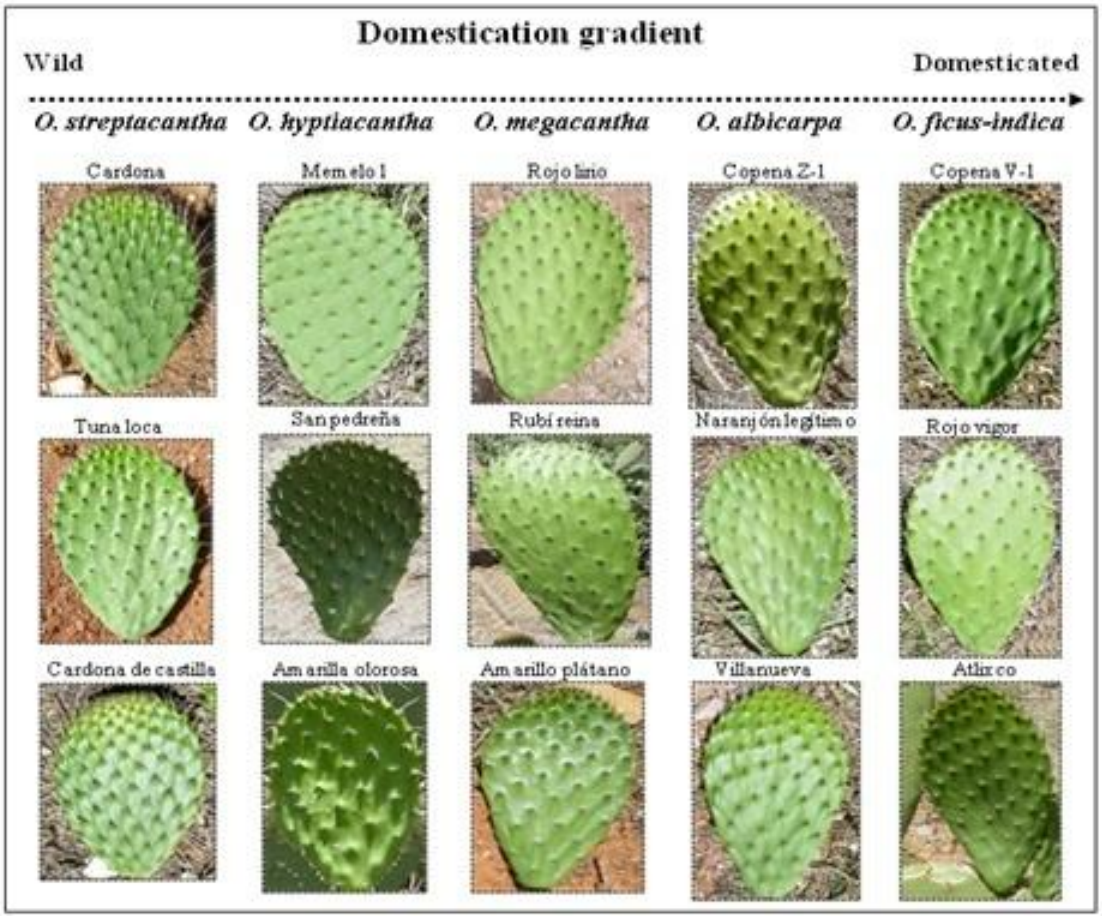


Figure 2. Cladodes of *Opuntia* cultivars analyzed in this study.

Table 2. Proximate composition of *Opuntia* cladodes with different grade of domestication

Cultivars	Composition[a] (%)				
	Protein	**Fat**	**Crude fiber**	**Carbohydrates**	**Ash**
***O. streptacantha* Lem.**					
Cardona	11.84 ± 0.29 b	0.82 ± 0.02 c	7.88 ± 0.20 a	67.38 ± 0.66 b	12.08 ± 0.14 i
Tuna loca	11.00 ± 0.13 d	0.62 ± 0.02 f	6.52 ± 0.03 d	69.20 ± 0.21 a	12.65 ± 0.03 h
Cardona de castilla	11.44 ± 0.14 c	0.77 ± 0.04 cd	7.52 ± 0.08 b	67.11 ± 0.33 b	13.16 ± 0.07 f
***O. hyptiacantha* A. Weber**					
Memelo 1	10.48 ± 0.04 e	0.94 ± 0.06 b	6.58 ± 0.03 d	66.21 ± 0.56 bc	15.78 ± 0.17 b
San pedreña	12.57 ± 0.06 a	0.80 ± 0.04 cd	7.16 ± 0.12 c	66.19 ± 0.47 c	13.27 ± 0.26 f
Amarilla olorosa	10.15 ± 0.46 ef	0.66 ± 0.04 ef	5.77 ± 0.18 e	67.21 ± 0.71 bc	16.21 ± 0.02 a
***O. megacantha* Salm-Dyck**					
Rojo lirio	10.13 ± 0.04 f	0.67 ± 0.02 ef	6.53 ± 0.06 d	69.40 ± 1.18 a	13.26 ± 0.91 efg
Rubí reina	11.32 ± 0.03 c	0.63 ± 0.03 f	5.99 ± 0.22 e	69.34 ± 0.28 a	12.73 ± 0.01 g
Amarillo platano	10.69 ± 0.17 e	0.79 ± 0.02 c	7.42 ± 0.34 abc	66.36 ± 0.94 bc	14.75 ± 0.41 c
***O. albicarpa* Scheinvar**					
Copena Z1	11.97 ± 0.45 b	0.77 ± 0.00 d	6.70 ± 0.04 d	67.90 ± 0.50 ab	12.66 ± 0.02 h
Naranjón legítimo	11.2 ± 0.41 bcd	0.77 ± 0.02 d	6.46 ± 0.07 d	68.47 ± 0.56 ab	13.11 ± 0.06 f
Villanueva	11.68 ± 0.16 b	0.70 ± 0.02 e	6.45 ± 0.31 de	67.36 ± 0.52 b	13.81 ± 0.04 e
***O. ficus-indica* (L.) Mill.**					
Copena V1	9.34 ± 0.03 g	1.14 ± 0.06 a	6.93 ± 0.23 cd	68.44 ± 0.92 ab	14.14 ± 0.60 cde
Rojo vigor	11.71 ± 0.43 bc	0.68 ± 0.04 e	5.63 ± 0.03 e	67.76 ± 0.58 ab	14.21 ± 0.09 d
Atlixco	12.56 ± 0.08 a	0.90 ± 0.03 b	5.35 ± 0.16 f	66.40 ± 0.45 bc	14.78 ± 0.18 c

[a]Media ± SD (n=3), dry weight basis. Different letter between rows in the same column are statistically different (Tukey test, $p<0.05$).

Ash content was different between species and cultivars, and varied from 12.64% (*O. streptacantha* “tuna loca”) to 16.21% (*O. hypticantha* “Amarilla olorosa”). Contreras-Padilla *et al.,* (2011) reported higher ash contents in *O. ficus- indica* cladodes with different stage of maturity. The ash content is related to the mineral content in vegetal samples.

4.2 Mineral Content

In *Opuntia* samples, K, Ca, Na, P, Fe and Mn were determined (Table 3). The most abundant minerals were potassium (1730 to 2870 mg 100 g^{-1}) and calcium (610 to 820 mg 100 g^{-1}). No statistical differences were observed between cultivars. Sodium content was similar (60 to 100 mg 100 g^{-1}) in all cultivars, except in *O. hypticantha* “Amarillo platano”, which contained the highest amount of Na (260 mg 100 g^{-1}). K, Ca and Na were also the major minerals in *O. ficus-indica* cladodes analyzed by Hernández-Urbiola *et al.,*(2011). Mn content values were ranged between 5.58 (*O. albicarpa* “Naranjón legítimo”) and 25.87 mg 100 g^{-1} (*O. hyptiacantha* “Amarilla olorosa”). Fe concentration varied from 0.28 (*O. albicarpa* “Copena Z1”) to 18.05 mg 100 g^{-1} (*O. ficus-indica* “Copena V1”). These minerals were also found in *O. ficus-indica* “Milpa alta” and “Atlixco” cladodes, however, the Mn and Fe contents reported were lower than our results (Ramírez-Moreno *et al.,* 2011). No statistical differences were found in P content (0.08 to 0.1 mg 100 g^{-1}) between cultivars. In general, no trend associated to domestication was observed on mineral content. It has been reported soil characteristics, such composition, water availability and pH, may change mineral content of cladodes (Hernandez-Urbiola et al., 2011).

Opuntia has been considered a good source of Ca and our results confirm *Opuntia* cladodes may be consumed in order to accomplish Ca daily dietary requirements. Dietary intake of vegetables with high content of calcium reduces the risk of osteoporosis and increases bone mineral density (Park et al., 2011). On the other hand, manganese supplementation and treatment increased insulin secretion and improved glucose tolerance under conditions of dietary stress in mice. These effects were associated to the mitochondrial manganese-

superoxide dismutase (MnSOD), a metalloenzyme that provides antioxidant protection against oxidative stress during homeostasis, and particularly diabetes (Lee et al., 2013). Therefore, Mn could be associated to antidiabetic effects reported in *Opuntia.* Further studies need to be performed.

4.3 Attributes: total sugars, titratable acidity and pH

These attributes are shown in Table 4. Total soluble sugars content was expressed as mg of Dextrose $g^{-1}$of sample, and the highest and lowest contents were found in *O. megacantha* “Rojo lirio” (103.67mg g^{-1}) and *O. ficus-indica* “Atlixco” (19.14 mg g^{-1}), respectively. Statistical differences were observed between species and cultivars. To estimate the organic acids content, titratable acidity, expressed as citric acid percentage, was determined. The highest and lowest citric acid contents were found in *O. streptacantha* “Cardona de castilla” (4.3) and *O. albicarpa* “Copena Z1” (1.9). The pH values were ranged between 4.25 (*O. megacantha* “Rojo lirio”) and 4.64 (*O. streptacantha* “Cardona”). Betancourt-Dominguez *et al.,* (2006) determined pH values in a range between 4.05 and 4.55 in *Opuntia spp.* Also Rodríguez-García *et al.,* (2007) determined a pH of 4.41 in *O. ficus-indica* “Redonda” cladodes. These data confirmed *Opuntia* cladodes are low acidic vegetables (Hernández-Pérez *et al.,* 2005). In *Opuntia*, the acidity and pH depend on the content of organic acids, which are synthesized through CAM pathway (Cushman and Bohnert, 1999).

Table 3. Mineral content in *Opuntia* cladodes.

Cultivar	Mineral [a] (mg 100 g^{-1})					
	Ca	**K**	**Na**	**P**	**Fe**	**Mn**
***O. streptacantha* Lem.**						
Cardona	640 ± 30 b	2260 ± 250 ab	70 ± 10 c	0.10 ± 0.01 a	1.16 ± 0.65 e	13.45 ± 1.83 b
Tuna loca	660 ± 40 b	2080 ± 290 ab	60 ± 10 c	0.08 ± 0.00 ab	2.25 ± 0.32 d	17.19 ± 2.41 ab
Cardona de castilla	700 ± 60 ab	2300 ± 390 ab	80± 10 c	0.09 ± 0.01 ab	5.40 ± 1.45 c	18.97 ± 3.85 ab
***O. hyptiacantha* A. Weber**						
Memelo 1	730 ± 60 ab	2710 ± 440 a	80 ± 10 c	0.08 ± 0.01 ab	4.51 ± 0.87 c	7.78 ± 1.06 d
San pedreña	670 ± 50 b	2490 ± 390 ab	70 ± 10 c	0.09 ± 0.01 ab	2.72 ± 0.67 d	16.10 ± 3.23 ab
Amarilla olorosa	820 ± 110 a	2870 ± 790 a	110 ± 10 b	0.09 ± 0.02 ab	4.47 ± 1.91 cd	5.58 ± 1.83 d
***O. megacantha* Salm-Dyck**						
Rojo lirio	740 ± 70 ab	2080 ± 460 ab	80 ± 10 c	0.08 ± 0.01 b	11.41 ± 1.76 b	11.08 ± 2.94 bc
Rubí reina	680 ± 50 ab	1730 ± 270 b	70 ± 00 c	0.08 ± 0.01 ab	2.84 ± 0.19 d	15.81 ± 3.53 c
Amarillo platano	630 ± 50 b	2070 ± 320 ab	260 ± 30 a	0.08 ± 0.01 ab	0.98 ± 0.84 ef	12.20 ± 1.32 bc
***O. albicarpa* Scheinvar**						
Copena Z1	630 ± 40 b	2010 ± 210 b	100 ± 10 bc	0.10 ± 0.01 ab	0.28 ± 0.11 f	20.80 ± 3.47 a
Naranjón legítimo	680 ± 80 b	1780 ± 350 b	70 ± 20 c	0.09 ± 0.02 ab	1.16 ± 0.68 e	25.87 ± 6.60 a
Villanueva	630 ± 40 b	2080 ± 260 ab	60 ± 10 c	0.08 ± 0.01 ab	0.74 ± 0.25 e	25.51 ± 3.27 a
***O. ficus-indica* (L.) Mill.**						
Copena V1	610 ± 60 b	2230 ± 380 ab	60 ± 10 c	0.09 ± 0.02 ab	18.05 ± 1.20 a	19.81 ± 2.30 d
Rojo vigor	640 ± 40 b	2310 ± 260 ab	60 ± 00 c	0.09 ± 0.02 ab	3.43 ± 1.25 cd	10.20 ± 0.85 c
Atlixco	630 ± 30 b	2670 ± 310 a	70 ± 10 c	0.09 ± 0.02 ab	4.42 ± 1.20 cd	11.48 ± 1.55 b

[a]Media ± SD (n=3), dry weight basis. Different letter between rows in the same column are statistically different (Tukey test, $p<0.05$).

Table 4. Total sugars, titratable acidity and pH of *Opuntia* cladodes.

Cultivar	Attribute[a]		
	Total sugars[b]	Titratable acidity[c]	pH
***O. streptacantha* Lem.**			
Cardona	66.08 ± 6.77 b	2.06 ± 0.48 c	4.64 ± 0.01 a
Tuna loca	60.99 ± 7.13 bc	3.00 ± 0.61 bc	4.34 ± 0.01 g
Cardona de castilla	37.13 ± 6.03 cd	4.30 ± 0.24 a	4.51 ± 0.02 bc
***O. hyptiacantha* A. Weber**			
Memelo 1	37.42 ± 3.40 d	3.70 ± 0.57 ab	4.41 ± 0.02 e
San pedreña	30.60 ± 6.45 de	3.76 ± 0.85 ab	4.68 ± 0.02 a
Amarilla olorosa	39.88 ± 11.42 cde	4.10 ± 0.77 ab	4.36 ± 0.01 f
***O. megacantha* Salm-Dyck**			
Rojo lirio	47.19 ± 3.39 c	2.21 ± 0.34 c	4.25 ± 0.01 h
Rubí reina	103.67 ± 4.65 a	2.03 ± 0.60 bc	4.44 ± 0.01 g
Amarillo platano	48.12 ± 6.53 cd	2.00 ± 0.50 c	4.50 ± 0.01 c
***O. albicarpa* Scheinvar**			
Copena Z1	27.49 ± 3.59 e	1.90 ± 0.34 c	4.53 ± 0.01 b
Naranjón legítimo	36.92 ± 7.11 cde	2.67 ± 0.93 bc	4.46 ± 0.01 d
Villanueva	20.34 ± 6.90 f	2.69 ± 0.10 b	4.66 ± 0.01 a
***O. ficus-indica* (L.) Mill.**			
Copena V1	38.55 ± 2.91 d	3.05 ± 1.33 abc	4.42 ± 0.02 e
Rojo vigor	56.21 ± 10.87 bc	2.47 ± 0.68 bc	4.51 ± 0.01 bc
Atlixco	19.14 ± 7.80 f	3.51 ± 1.16 abc	4.41 ± 0.02 e

[a]Media ± SD (n=3), dry weight basis. Different letters between rows in the same column are statistically different (Tukey test, $p<0.05$). [b]Expressed as mg Dextrose per gram of sample. [c]Expressed as percentage of citric acid per gram of sample.

CONCLUSIONS

No behavior directly associated to the domestication grade was observed in proximate and mineral analyses, and chemical attributes in Opuntia cultivars. These data suggest differences inherent to type of species and also to the evolutionary process, although no specific or linear trend was observed.

Protein, fat, crude fiber and ash content was different among cultivars. Mineral such Ca, K, Na, P, Fe and Mn were detected in all cultivars. Particularly, *Opuntia* cladodes may be considered as a good source of Ca and its consumption might accomplish the daily dietary requirements of this mineral. In addition, the Mn contents could be associated to antidiabetic effects reported in *Opuntia.* Total sugars, titratable acidity and pH are parameters associated to organoleptic features of cladodes and the results obtained changed among cultivars, although no clear behavior due to domestication grade was observed.

4.4 Phenolic compounds analysis

The health-promoting effects of *Opuntia* have been associated to the phenolic compounds content and their antioxidant potential. Therefore, total phenolics and total flavonoids contents were determined and results are shown in Table 5. Total phenolic compounds were expressed as gallic acid μmol g^{-1} of sample (DW). The highest gallic acid contents were determined in *O. streptacantha* "Tuna loca" (65.13 μmol g^{-1}) and *O. streptacantha* "Cardona" (59.48 μmol g^{-1}), *O. megacantha* "Rubí reina" (57.84 μmol g^{-1}) and *O. ficus-indica* "Rojo vigor" (56.68 μmol g^{-1}) and no statistical differences were observed between these cultivars. Since these cultivars belong to the wild, moderate and most domesticated species, these results suggest no differences associated to domestication grade. However, the lowest gallic acid was determined in *O. ficus-indica* "Atlixco" (22.51 μmol g^{-1}). This species is one of the most cultivated for young cladodes production. Among the domesticated *O. albicarpa* species, "Naranjón legítimo" (47.98 μmol g^{-1}) and "Copena Z-1" (40.24 μmol g^{-1}) showed the highest gallic acid content. In *O. hyptiacantha* cultivars total phenolics values were similar (31.75 to 36.06 μmol g^{-1}). Values obtained in this study were higher than gallic acid contents reported in *O. ficus-indica* Mill. "Milpa Alta" (12.28 μmol g^{-1}) and in *O. ficus-indica* "Saboten" (21.74 μmol g^{-1}) (Lee et al., 2002; Corral-Aguayo et al., 2008).

In order to evaluate the effect of domestication on total phenolic contents between species, the averages of gallic acid values were calculated (Figure 3). Considering absolute values, gallic acid contents were higher in wild *O. streptacantha* (56.77 μmol g^{-1}), followed by *O. megacantha* (44.66 μmol g^{-1}), then *O. albicarpa* (40.84 μmol g^{-1}) and the domesticated *O. ficus-indica* (40.08 μmol g^{-1}). These absolute values suggest phenolic compounds contents decrease as a function of domestication. The lowest value was determined in *O. hyptiacantha* (33.38 μmol g^{-1}), a wild (although moderately domesticated) species. Therefore, the behavior of *O. hyptiacantha* species could be associated to the specific synthesis of gallic acid (reference compound) and further analysis

is required. However, standard deviations are overlapped, suggesting no differences among species due to the domestication effect.

Total flavonoids content were expressed as quercetin μmol g^{-1} of sample (DW) and results are shown in Table 5. Quercetin values were ranged between 16.0 μmol g^{-1} (*O. albicarpa* "Copena Z1") and 20.41 μmol g^{-1} (*O. ficus-indica* "Rojo vigor"). Statistical differences among cultivars were observed. Guevara-Figueroa *et al.* (2010) reported quercetin values ranged from 6.6 to 11.91 μmol g^{-1} and 19.5 to 32.42 μmol g^{-1} in young cladodes from wild and commercial *Opuntia* species (*Opuntia spp.*), respectively. Our results were higher than values reported in wild species.

In order to determinate the effect of domestication between species, the averages of quercetin values from cultivars were calculated (Figure 3). Quercetin absolute values were higher in domesticated *O. ficus-indica* (19.37 μmol g^{-1}), followed by wild *O. streptacantha* (18.03 μmol g^{-1}), then *O. hyptiacantha* (17.08 μmol g^{-1}) and *O. albicarpa* (17.24 μmol g^{-1}). The lowest quercetin content was observed in *O. megacantha* species (16.78 μmol g^{-1}). Despite these differences in quercetin content, no behavior directly associated to the domestication grade was observed.

The effect of harvest season on phenolic compounds content was evaluated. Young cladodes collected on 2012 were analyzed. Results are shown in Table 5. The higher phenolics contents were in obtained in *O.albicarpa* cultivars, "Copena Z-1" (155.8 μmol g^{-1}), "Naranjón legítimo" (131.1 μmol g^{-1}) and "Villanueva" (147.9 μmol g^{-1}) and *O. streptacantha* "Cardona" (146.5 μmol g^{-1}). These values were 3.8, 2.7, 4.3 and 2.4 fold higher than gallic acid contents determined in same cultivars from 2010, respectively. The lowest gallic acid contents were determined in *O.ficus-indica* "Rojo vigor" (59.1 μmol g^{-1}) and *O.ficus-indica* "Atlixco" (56.4 μmol g^{-1}). Despite no direct association of phenolic compound and domestication grade was observed, the increase of gallic acid content in all species, except in *O.ficus-indica* "Rojo vigor", confirmed the synthesis of phenolic compounds in *Opuntia* depend on harvest season and environmental conditions.

Comparative analysis of total phenolics content between species also was performed and averages of gallic acid values from cultivars are shown in Figure 4. Considering absolute values, gallic acid contents were higher in *O. albicarpa* (144.94 µmol g^{-1}), followed by *O. streptacantha* (122.27 µmol g^{-1}), then *O. hyptiacantha* (101.87 µmol g^{-1}) and *O. megacantha* (99.94 µmol g^{-1}). The lowest gallic acid content was determined in domesticated *O. ficus-indica* (67.42 µmol g^{-1}). Absolute quercetin values from all species, except *O. albicarpa*, suggest possible association to domestication effect. However, quercetin content in *O. albicarpa* interferes with such hypothesis.

Total flavonoids contents are shown in Table 5. The highest quercetin values were determined in *O.ficus-indica* “Rojo vigor” (35.1 µmol g^{-1}), followed by *O.albicarpa* “Villanueva” (28.0 µmol g^{-1}), then *O.megacantha* “Rubí reina” (22.6 µmol g^{-1}), *O.hyptiacantha* “San pedreña” (22.6 µmol g^{-1}) and *O. streptacantha* “Tuna loca” (21.8 µmol g^{-1}). These values were 1.7, 1.5, 1.4, 1.4 and 1.1 fold higher than quercetin values determined in same cultivars from 2010, respectively. Interestingly, the lowest quercetin content was observed in *O. streptacantha* “Cardona” (16.0 µmol g^{-1}) and this amount was similar to quercetin content in cladodes from same cultivar collected in 2010.

Comparative analysis of total flavonoids contents between species also was performed and averages of quercetin values from cultivars are shown in Figure 4. Considering absolute values, quercetin contents were higher in *O. ficus-indica* (25.14 µmol g^{-1}), followed by *O. albicarpa* (22.15 µmol g^{-1}) and *O. hyptiacantha* (21.7 µmol g^{-1}), then *O. streptacantha* (19.35 µmol g^{-1}) and the lowest quercetin content was observed in *O. megacantha* (18.96 µmol g^{-1}). These data is not clear about association to domestication effect. In addition, as it is shown, standard deviations are overlapped, suggesting no differences among contents.

In general, no trend associated to domestication grade was observed. These results may be associated to the reference compounds used for total phenolics and flavonoids determinations. Since gallic acid and quercetin may not be the most adequate for these determinations, alternatives such

Chlorogenic acid and rutin represents a good alternative for further analysis. Total phenolics contents were similar to values reported in ginger, asparagus, onion and lettuce. Quercetin values were comparable to data obtained in lettuce and chickpea, and higher than values determined in asparagus (Llorach *et al.,* 2004; USDA, 2010; Lu *et al.,* 2010; Xiaoun *et al.,* 2009; Sreerama *et al.,* 2010; Khanam *et al.,* 2012; Haniadkca *et al.,* 2013). The nutritional properties and the antioxidant potential of these vegetables and legume are well documented and are associated to antioxidant compounds such vitamins, pigments and phenolic compounds. Antioxidant properties of onion and lettuce have been reported and the asparagus is considered a nutraceutical food with high antioxidant activity associated to antiradical compounds such ascorbic acid, flavones, phenols, glutathione, etc (Lu *et al.,* 2010). Chickpea is a valuable source of protein, mineral, vitamins, and its consumption has been linked to reduced risk of various diseases such as bronchitis, diabetes, cancer and cardio-vascular diseases (Sreerama *et al.,*2010). Therefore, the evaluation of antioxidant capacity of *Opuntia* extracts was the next aim in this study.

4.5 Antioxidant capacity by DPPH assay

To evaluate the anti-radical scavenging effect of *Opuntia* extracts, the DPPH assay was used. Currently, this assay is considered a valid colorimetric method for the assessment of antioxidant properties of plant extracts (Cheng *et al.,* 2006). According to our results (Table 5), among samples collected in 2010, the highest antioxidant capacity was showed by *O. streptacantha* “Tuna loca” (897.77 µmol TE 100 g^{-1}), followed by *O. megacantha* “Rubí reina” (749.5 µmol TE 100 g^{-1}) and *O. ficus-indica* “Rojo vigor” (659.38 µmol TE 100 g^{-1}). Since these cultivars belong to the wild, moderate domesticated and highly domesticated, our first attempt was to hypothesize that antioxidant capacity is higher in wild species and decreases as well as domestication increases. However, *O. hyptiacantha* “Memelo 1” (592.35 µmol TE 100 g^{-1}) and *O. albicarpa* “Naranjón legítimo” (556.74 µmol TE 100 g^{-1}) exhibited a similar antioxidant capacity and trolox values were lower than in the first described cultivars. Therefore, is not possible to confirm an association between

antioxidant capacity and domestication grade. Statistical differences were observed between species and cultivars ($p < 0.05$).

The averages of antioxidant capacities of cultivars from same species were calculated (Figure 3). Considering absolute values, the antioxidant capacity exhibited by *Opuntia* extracts was higher in *O. streptacantha* (629.83 µmol Trolox 100 g^{-1}), followed by *O. megacantha* (602.49 µmol Trolox 100 g^{-1}), *O. ficus-indica* (508.41 µmol Trolox 100 g^{-1}), then *O. albicarpa* (406.2 µmol Trolox 100 g^{-1}) and *O. hyptiacantha* (368.51 µmol Trolox 100g^{-1}). Despite these data suggest a trend between phenolics and antioxidant capacity, is not possible to confirm a direct variation of antioxidant capacity and domestication grade.

The antioxidant capacities from extracts of Opuntia cultivars collected in 2012 was also evaluated (Table 5). The cultivars with highest antioxidant capacity were *O. albicarpa* “Copena V1” (1685.5 µmol Trolox 100 g^{-1}) and “Villanueva” (1616.5 µmol Trolox 100 g^{-1}), followed by *O. megacantha* “Rubí reina” (1419.5 µmol TE 100 g^{-1}) and *O. streptacantha* “Cardona de castilla” (1289 µmol Trolox 100 g^{-1}), then *O. hyptiacantha* “Memelo 1” (986.9 µmol Trolox 100 g^{-1}) and *O. ficus-indica* “Rojo vigor” (488.9 µmol Trolox 100 g^{-1}). These values were 5.6, 4.5, 1.9, 2.2 and 1.7 fold, respectively, higher than values from cladodes collected in 2010. Despite the variation due to environmental conditions, these results suggest no trend between antioxidant capacity and domestication grade.

The averages of antioxidant capacities of cultivars from same species were calculated (Figure 4). Considering absolute values, the antioxidant capacity exhibited by *Opuntia* extracts was higher in *O. albicarpa* (1517.75 µmol Trolox 100 g^{-1}), followed by *O. megacantha* (1214.3 µmol Trolox 100 g^{-1}), *O. streptacantha* (1015.85 µmol Trolox 100 g^{-1}), then *O. hyptiacantha* (877.13 µmol Trolox 100 g^{-1}) and the lowest value was observed in *O. ficus-indica* (401.35 µmol Trolox 100g^{-1}). These data suggest no association between domestication grade and antioxidant activity.

In general, trolox equivalents were similar to values determined in ginger, pepper black and basil (Xiaoyun *et al.,* 2009; USDA, 2010). The antioxidant

properties of these have been studied. Particularly, Ginger (*Zingiber officinale* Roscoe) has been shown to possess free radical scavenging, antioxidant and inhibition of lipid peroxidation properties. These properties might have contributed to the health-promoting effects of ginger (Haniadka *et al.,* 2013).

Table 5. Phenolic compounds content and antioxidant capacity of *Opuntia* cladodes[a]

Cultivar	Total phenolics[b]		Total flavonoids[c]		Antioxidant capacity[d]	
	2010 collect	2012 collect	2010 collect	2012 collect	2010 collect	2012 collect
***O. streptacantha* Lem.**						
Cardona	59.48 ± 7.73 a	146.5 ± 9.0 ab	16.33 ± 1.05 bc	16.0 ± 1.2 f	402.82 ± 136.41 def	1109.0 ± 112.3 cd
Tuna loca	65.13 ± 4.02 a	111.9 ± 2.6 d	19.00 ± 1.04 a	21.8 ± 0.7 c	897.77 ± 45.81 a	649.6 ± 51.9 f
Cardona de castilla	45.69 ± 1.36 b	108.4 ± 4.5 d	18.76 ± 1.39 ab	20.3 ± 0.5 d	588.91 ± 43.04 cd	1289.0 ± 92.2 bc
***O. hyptiacantha* A. Weber**						
Memelo 1	36.06 ± 1.76 cd	142.2 ± 7.7 b	18.59 ± 0.19 a	20.8 ± 0.3 cd	592.35 ± 70.56 cd	986.9 ± 10.4 d
San pedreña	31.75 ± 4.79 d	61.6 ± 0.7 g	16.59 ± 0.38 c	22.6 ± 1.1 c	229.51 ± 55.25 f	767.4 ± 34.0 e
Amarilla olorosa	32.34 ± 5.28 cd	-	16.07 ± 1.34 bc	-	283.67 ± 72.27 ef	-
***O. megacantha* Salm-Dyck**						
Rojo lirio	36.72 ± 1.83 cd	96.7 ± 4.7 e	16.26 ± 1.50 bc	17.7 ± 0.7 ef	538.94 ± 52.90 d	1051.0 ± 152.3 cd
Rubí reina	57.84 ± 10.44 a	119.1 ± 3.3 c	16.53 ± 0.83 bc	22.6 ± 1.2 c	749.50 ± 47.08 b	1419.5 ± 51.5 b
Amarillo platano	39.41 ± 1.17 c	84.0 ± 5.7 f	17.54 ± 0.53 b	16.6 ± 1.6 ef	519.04 ± 17.03 d	1172.5 ± 164.0 c
***O. albicarpa* Scheinvar**						
Copena Z1	40.24 ± 2.89 c	155.8 ± 1.4 a	16.00 ± 1.65 bc	18.1 ± 0.5 e	300.46 ± 37.10 ef	1685.5 ± 37.1 a
Naranjón legítimo	47.98 ± 7.10 bc	131.1 ± 10.2 ab	16.80 ± 1.18 bc	20.3 ± 1.4 cd	556.74 ± 71.41 d	1251.3 ± 56.6 c
Villanueva	34.30 ± 1.87 d	147.9 ± 9.1 ab	18.92 ± 1.16 ab	28.0 ± 1.5 b	361.41 ± 39.96 e	1616.5 ± 71.4 a
***O. ficus-indica* (L.) Mill.**						
Copena V1	41.04 ± 3.64 bc	86.8 ± 4.9 f	17.56 ± 0.35 b	19.6 ± 2.1 cde	556.75 ± 51.51 d	472.3 ± 70.7 g
Rojo vigor	56.68 ± 4.45 a	59.1 ± 5.7 gh	20.41 ± 2.40 a	35.1 ± 1.8 a	659.38 ± 29.43 c	488.9 ± 72.7 g
Atlixco	22.51 ± 0.99 e	56.4 ± 0.4 h	20.14 ± 1.51 a	20.7 ± 2.7 cde	309.11 ± 59.71 ef	242.8 ± 46.7 h

[a]Media ± SD (n=3), dry weight basis. Different letters between rows in the same column are statistically different (Tukey test, $p<0.05$). [b]Expressed as µmol of Gallic Acid (GA) $g^{-1}$of sample. [c]Expressed as µmol of Quercetin (QE) $g^{-1}$of of sample. [d]Expressed as µmol of Trolox 100 $g^{-1}$of of sample.

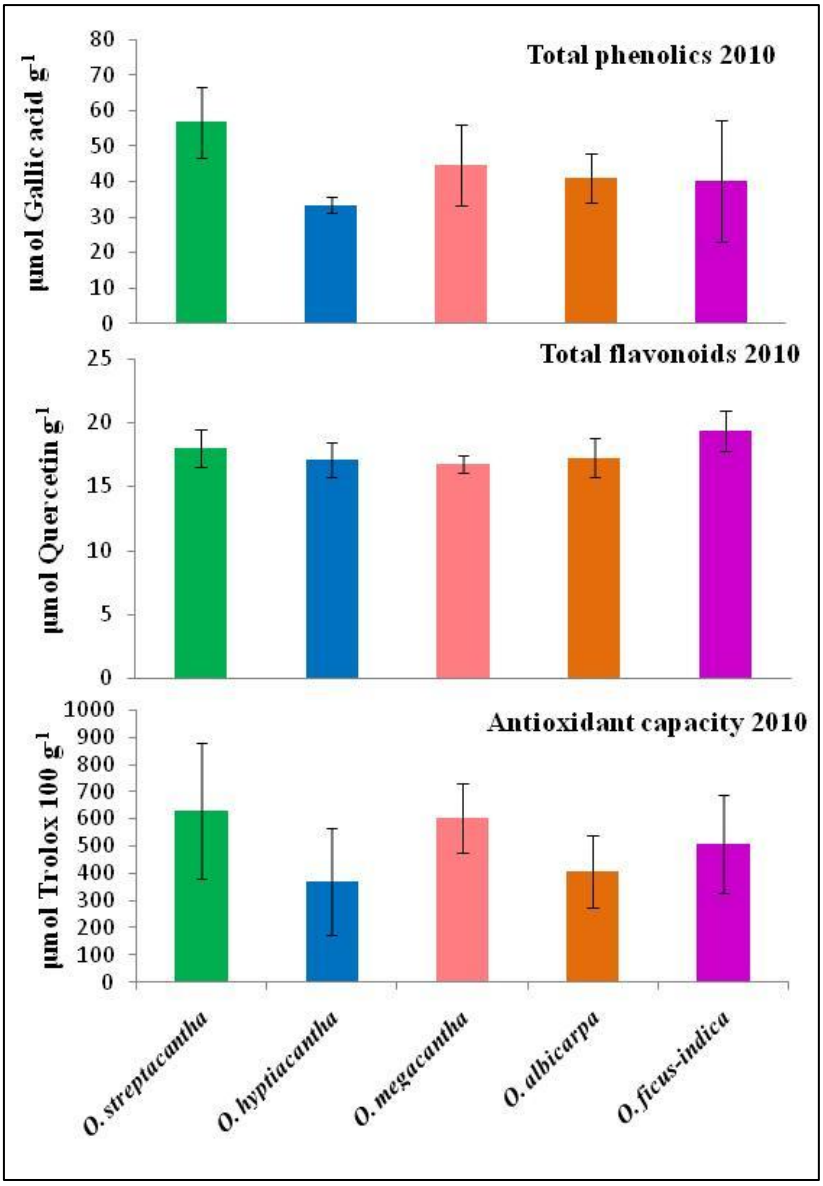


Figure 3. Average of total phenolics, total flavonoids and antioxidant activity values determined in young cladodes from fifteen cultivars belonging to five *Opuntia* species collected in 2010 (media + SD, n=15).

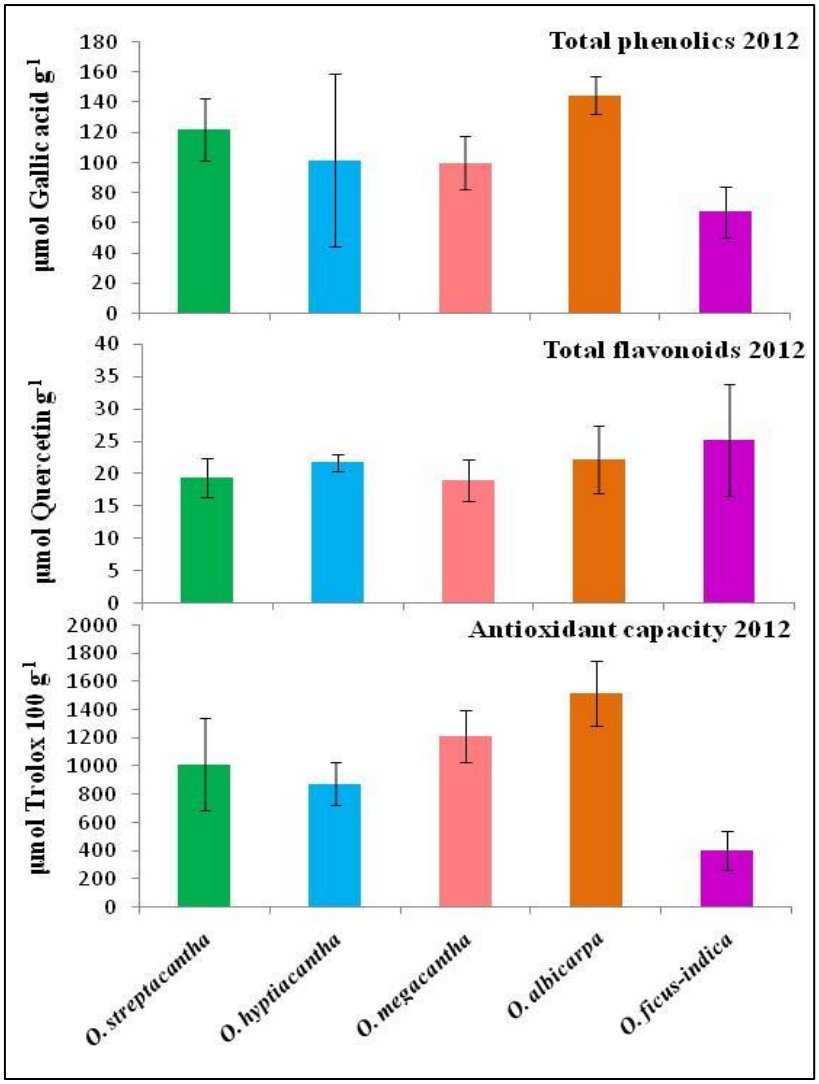


Figure 4. Average of total phenolics, total flavonoids and antioxidant activity values determined in young cladodes from fifteen cultivars belonging to five *Opuntia* species collected in 2012 (media + SD, n=15).

As it is shown in Figure 3, total phenolics content exhibited a similar behavior than antioxidant capacities in young cladodes collected in 2010. This behavior suggests phenolic compounds are responsible of antioxidant capacities exhibited by *Opuntia* species. In order to confirm this hypothesis, a linear correlation between gallic acid contents and antioxidant capacity of each cultivar was calculated and results are shown in Figure 5. In general, higher content of total phenolics resulted in a higher antioxidant capacity, and most of the cultivars showed a similar behavior. Interestingly, *O. albicarpa* "Naranjón legítimo" and *O. ficus-indica* "Rojo vigor", both domesticated, were fit into the trendline. In the wild *O. streptacantha* "Tuna loca" a higher antioxidant capacity was observed. However, "Cardona de castilla" showed a lower antioxidant capacity than *O. megacantha* "Rubí reina" and both gallic contents were similar. This also was observed between *O. hyptiacantha* "Memelo 1" and *O. albicarpa* "Naranjón legítimo".

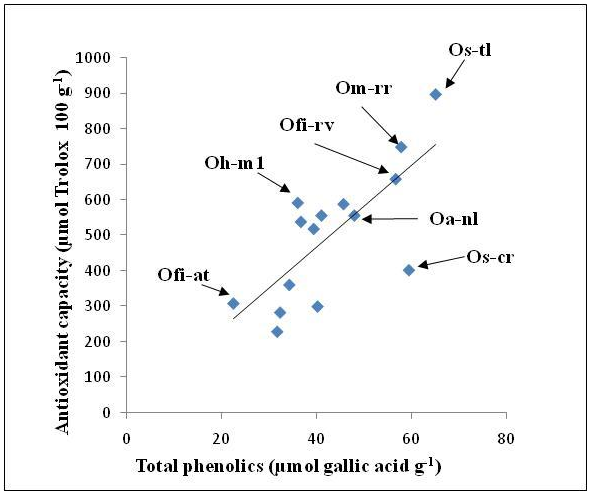


Figure 5. Linear correlation between total phenolics content and antioxidant capacity values determined in *Opuntia* cultivars (2010) with different domestication grade. Values represented were the average of each assay. Os-tl, *O. streptacantha* "Tuna loca"; Os-cr, *O. streptacantha* "Cardona"; Oh-m1, *O. hyptiacantha* "Memelo 1"; Om-rr, *O. megacantha* "Rubí reina"; Oa-nl, *O. albicarpa* "Naranjón legítimo"; Ofi-rv, *O. ficus-indica* "Rojo vigor"; Ofi-at, *O. ficus-indica* "Atlixco".

In order to determinate a link between total phenolics contents and antioxidant capacities in cladodes collected in 2012, a linear correlation was calculated (Figure 6). The effect of different harvesting season modified the pattern observed in previously (Figure 5). *O. albicarpa* “Naranjón legítimo” and *O. ficus-indica* “Rojo vigor” almost fit into the trendline higher content of total phenolics resulted in a higher antioxidant capacity, and most of the cultivars showed a similar behavior. Interestingly, *O. albicarpa* “Naranjón legítimo” and *O. ficus-indica* “Rojo vigor”, both domesticated, were fit into the trendline, but “Naranjón legítimo” showed a higher antioxidant capacity. In fact, *O. ficus-indica* cultivars were located in the lowest extreme of the trendline. *O. megacantha* “Rubí reina” exhibited the same behavior since both, total phenolics and antioxidant capacity increased 2 fold each (compared with same cultivar collected in 2010). Despite the 2 fold increase in gallic acid content, antioxidant capacity in wild *O. streptacantha* “Tuna loca” a lower antioxidant capacity was observed. In *O. hyptiacantha* “Memelo 1”, the gallic acid content detemined in *O. hyptiacantha* “Memelo 1” and *O. albicarpa* “Naranjón legítimo” but the antioxidant capacity was higher in “Naranjón legítimo”.

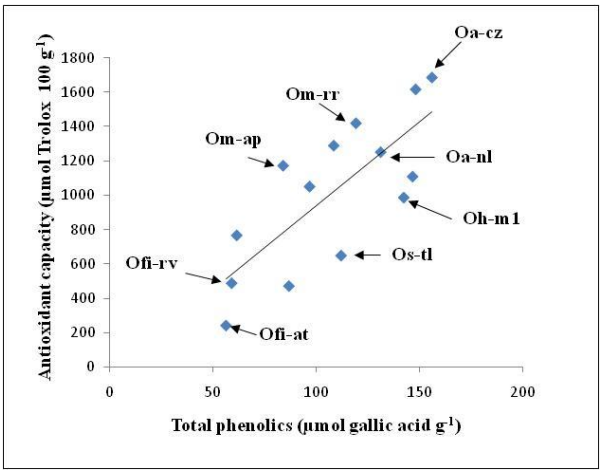


Figure 6. Linear correlation between total phenolics content and antioxidant capacity values determined in *Opuntia* cultivars (2012) with different domestication grade. Values represented were the average of each assay. Os-tl, *O. streptacantha* “Tuna loca”; Os-cr, *O. streptacantha* “Cardona”; Oh-m1, *O. hyptiacantha* “Memelo 1”; Om-rr, *O. megacantha* “Rubí reina”; Om-ap, *O. megacantha* “Amarillo plátano”; Oa-nl, *O. albicarpa* “Naranjón legítimo”.

These data suggest antioxidant capacity depends on total phenolics content in some cultivars (*O. megacantha* “Rubí reina”). However, in cultivars where no correlation was observated, antioxidant capacity could be associated to other antioxidant compounds such vitamins, carotenes, ascorbate or gluthation, also reported in *Opuntia* (Stintizing and Carle, 2005). Our results suggest a preponderant role of phenolic compounds in the antioxidant capacity exhibited by *Opuntia* extracts. However, the identification and isolation of phenolics compouns, as well as in vitro assays are required to confirme their participation in antioxidant capacity of *Opuntia.*

4.6 Analysis of *Opuntia* extracts by Thin Layer Chromatography (TLC)

Since antioxidant capacity may be associated to specific phenolic compounds synthesized in *Opuntia* cultivars, the next step was to perform a phenolic compounds screening. The TLC technique is a simple, precise and accurate method for phenolic profiling (Anandjiwala et al., 2007). In this study, the presence of phenolic acids and flavonoids was confirmed in the ethanolic extracts from *Opuntia* as it is shown in TLC patterns (Figure 7). Chlorogenic acid, rutin, quercetin and hesperidin were used as reference compounds. Defined bands were observed and good resolution was obtained. Differences in band intensities are due to different concentration, since same amount of extract was eluted. Chlorogenic acid band was observed in all cultivars except in *O. albicarpa* cultivars. Quercetin was detected in all cultivars. Possibly herperidin was also detected in these profiles. These results confirmed differences in phenolic compounds profiles among *Opuntia* cultivars. Thus, to perform the metabolomic profiling was the next step in this study.

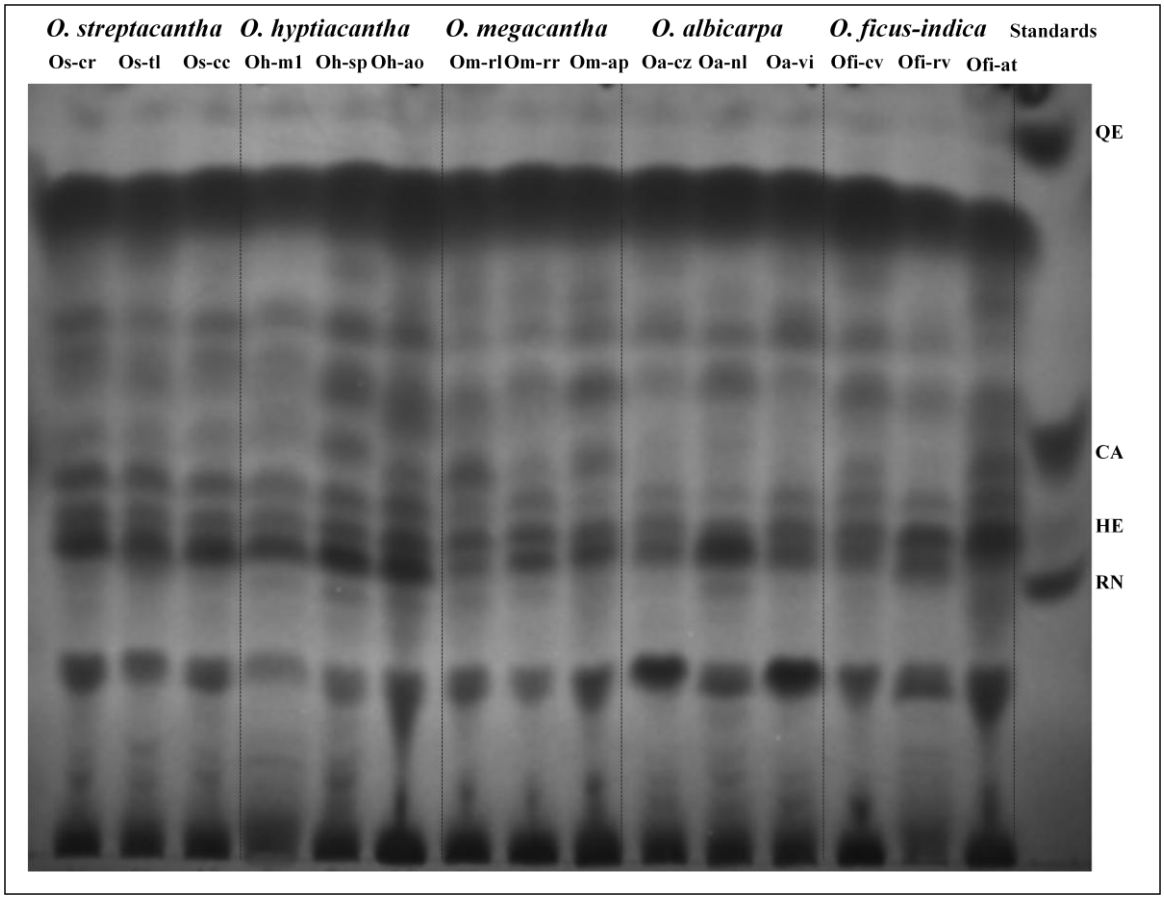


Figure 7. TLC of *Opuntia* extracts. QE, quercetin; CA, chlorogenic acid; HE, Hesperidin; RN, Rutin.

4.7 Phenolic compounds profile by LC-MS^n analysis

Metabolite identification is a critical and integral part of drug discovery, chemotaxonomic analysis, developmental and evolutionary process, and is particularly challenging due to its complexity and the level of expertise required. Liquid chromatography coupled with mass spectrometry (LC/MS) has become an important tool for metabolite identification (Yu *et al.,* 2011). According to total phenolics content and antioxidant capacity values, the cultivars *O. streptacantha* "Tuna loca", *O. hyptiacantha* "Memelo 1", *O. megacantha* "Rubí reina" *O. albicarpa* "Naranjón legítimo" and *O. ficus-indica* "Rojo vigor" were selected for the next step in this study, metabolomic analysis of *Opuntia*.

The chromatograms of *Opuntia* samples obtained from LC-DAD-MS analysis are shown in Figure 8, according to their domestication grade. As it is shown, the major number of peaks was detected in *O. megacantha* "Rubí reina" (30 peaks), followed by *O. albicarpa* "Naranjón legítimo" and *O. ficus-indica* "Rojo vigor" (26 peaks, each), then *O. hyptiacantha* "Memelo 1" and *O. streptacantha* "Tuna loca" (25 peaks, each). In general, eight major peaks detected were detected. These peaks were the 1, 2, 15, 16, 17, 18, 23 and 25. These peaks were selected for MS^2 analysis. The major in all samples was peak 15. Interestingly, the relative abundance of peak 16 was similar in all samples, but decreased in *O. hyptiacantha* "Memelo 1". Particularly, the relative abundance of peaks 23 and 25 increased as well as domestication decreased.

The MS and MS^2 fragmentation patterns, potential compounds and additional data are shown in Table 6. The identification of flavonoids was achieved by comparison of MS^2 fragmentation pattern, UV spectra (nm) and retention time (RT) with data reported in the literature. The MS^{2}data mining process began with the identification of aglycone fragment in order to determinate the flavonoid backbone of each compound, thus quercetin ([M-H] m/z 301 uma), isorhamnetin ([M-H] m/z 315 uma), kaempferol ([M-H] m/z 285 uma) and luteolin ([M-H] m/z 285 uma) were found. However, in this study, precursor or fragment ions showed an increase of one or two uma which can be related to the protonation of the molecule. The next step was to determinate the

number and type of sugar units attached to each flavonoid skeleton. In this work, mono, di and triglycosides were found. The type of sugar unit and sequence were determined according to the neutral losses showed in fragmentation patterns (Cuyckens and Claeys, 2004). Thus hexoses (galactose, glucose), deoxyhexoses (rhamnose) or pentoses (xylose, arabinose) were identified due to losses of 162 uma, 146 uma or 132 uma, respectively. Also, other neutral losses, such H_2O (18 uma), CO_2 (44) and CO (28 uma), were observed. Lithium $[(M-H) + 7]^-$ or sodium $[(M-H) + 23]^-$ adducts were detected (Kéki *et al.,* 2001). Finally, the compound structures were proposed according to the structural data available. The glycosylation sites and hydroxylation position were confirmed by the UV data.

Phenolic acids

Compounds **1** and **2** precursor ions were detected at m/z 240 $[M-H]^-$ and m/z 358 $[(M-H)-18+23]^-$, respectively. The MS^2 fragmentation pattern of compound **1** yielded ions at m/z 180.28 $[(M-H)-56]^-$ and 150.07 $[(M-H)-90]^-$. The MS^2 fragmentation pattern of compound **2** yielded ions at m/z 194.41 $[(M-H)-162]^-$ and 176.18 $[(M-H)-180]^-$ due to the caffeoyl group loss. Compound **1** was identified as eucomic acid, previously reported in *Opuntia* (Ginestra *et al.,* 2009). Antioxidant activity of this compound has been reported and may be used for treatment of age-related disorders such as skin aging (Simmler *et al.,* 2011). Compound **2** was identified as chlorogenic acid and the antioxidant properties of this compound have been widely studied. In addition, the antidiabetic effect of Chlorogenic acid on type II diabetic rats has been reported (Hunyadi *et al.,* 2012).

Quercetin *O*-glycosides

The MS^2 analysis of the precursor ions from **7a**, **7b**, **8**, **15**, **16** and **20** yielded fragments at m/z 302, thus, these compounds were tentatively identified as Quercetin *O*-glycosides. The fragmentation pattern of compound **7a**, at m/z 762 $[(M-H)+7]^-$, yielded ions at m/z 596.63 $[(M-H)-162]^-$, m/z 614.67 $[(M-H)-146]^-$ and m/z 302.63 $[(M-H)-162-146-146]^-$, and compound **7b**, at m/z 748 $[(M-H)+7]^-$,

showed fragments at m/z 596.5 $[(M-H)-146]^-$, m/z 614.74 $[(M-H)-132]^-$, m/z 469.16 $[(M-H)-132-146]^-$ and m/z 302.63 $[(M-H)-132-146-162]^-$. These tri-glycosides were identified as Quercetin 3-*O*-rhamnosyl-rutinoside (**7a**) and Quercetin 3-*O*-xylosyl-rutinoside (**7b**). The Quercetin 3-*O*-xylosyl-rutinoside (**7b**) fragment pattern reported by Ha *et al.,* (2010) is similar to data obtained in this study. This is first report of these compounds in *Opuntia* samples.

The compound **8**, precursor ion at m/z 600 $[(M-H)+7]^-$, generated fragments at m/z 448.9 $[(M-H)-146]^-$ and m/z 302.63 $[(M-H)-146-162]^-$, and the compound **15**, precursor ion at m/z 614 $[(M-H)+7]^-$, showed fragments at m/z 467.33 $[(M-H)-146]^-$ and m/z 303.54 $[(M-H)-146-162]^-$. Both compounds were classified as di-glycosides. Compound **15** was identified as Quercetin 3-*O*-rutinoside (Rutin), a well-known flavonoid, previously described in *Opuntia* species (Ginestra *et al.,* 2009, Guevara-Figueroa, 2011, Jiau *et al.,* 2012). Therapeutical effects of rutin have been widely studied, including antidiabetic effect on type II diabetic rats, antiangiogenic activity and reduction of fat accumulation, possibly associated to a hypolipidemic effect (Hunyadi et al., 2012; Bhattacharya et al., 2013; Kumazawa et al 2013).

The MS^2 analysis of compound **16**, precursor ion at m/z 467, and compound **20**, precursor ion at m/z 436, yielded the fragments at m/z 302.56 $[(M-H)-162]^-$ and at m/z 302.59 $[(M-H)-132]^-$, respectively. These compounds were assigned as mono-glycosides. The compound **16** was identified as Isoquercitrin, a Quercetin 3-*O*-glucoside, according to the fragmentation pattern reported by Sánchez-Rabaneda *et al.,* (2003). Therapeutical effects of isoquercitrin have been reported, including antioxidant activity, anti-inflammatory, immunomodulatory and α-glucosidase inhibitory effects. In addition, isoquercitrin induced a reduction on fat accumulation possibly associated to a hypolipidemic effect ntioxidant activity (Kim et al., 2011; Bhattacharya et al., 2013; Kim et al., 2013; Vongsak et al., 2013).

Kaempferol *O*-glycosides

The MS^2 fragmentation pattern of compounds **10**, **11**, **14**, **19**, **21, 23** and **26'** showed a fragment at m/z 286, allowing tentatively identify these

compounds as Kaempferol *O*-glycosides. The compounds **10** and **11**, both with same precursor ion at m/z 746 $[(M-H) + Li]^-$ but different RT, yielded similar fragments at m/z 580.4 [(M-H)-146-18]-, m/z 598.5$[(M-H)-146]^-$ and m/z 286.4 $[(M-H)-146-146-162]^-$, thus these compounds are isomers. Comparing these data with MS^2 fragments and UV spectra reported by Abad-García *et al.,* (2012) these tri-glycosideswere identified as Kaempferol 3-*O*-(rhamnosyl-hexoside)-7-*O*-rhamnoside I (**10**) and Kaempferol 3-*O*-(rhamnosyl-hexoside)-7-*O*-rhamnoside II (**11**). Ginestra *et al.,* (2009) reported similar Kaempferol 3-*O*-tri-glycosides in *O. ficus-indica* stems. The compound **14a**, precursor ion at m/z 732 $[(M-H)-18+23]^-$, yielded fragments at m/z 580.38 $[(M-H)-146]^-$, m/z 598.53 $[(M-H)-132]^-$ and m/z 286.45 $[(M-H)-146-132-162]^-$. According to these fragments, three different sugar units, such a hexose (162 uma), a deoxyhexose (146 uma) and a pentose (132 uma) are attached to a Kaempferol molecule. Thus compound **14a** was identified as Kaempferol 3-*O*-robinobioside-7-α-L-arabinofuranoside. This is the first report of these tri-glycosides in *Opuntia*.

The compounds **19** and **23**, with the same precursor ion at m/z 598 $[(M-H)+7]^-$ but different RT, showed similar fragments at m/z 287.41 $[(M-H)-146-162]^-$ and m/z 257.18 $[(M-H)-146-162-28]^-$, then these di-glycosides are isomers. The compound **19** was identified Kaempferol 3-*O*-rutinoside (Nicotiflorin), due to similar MS^2 fragments and UV spectra reported by Abad-Garcia *et al.,* (2012). Guevara *et al.,* (2011) previously reported the presence of Nicotiflorin in *Opuntia* samples. Therapeutical effects of nicotiflorin such antiangiogenic, antinociceptive and anti-inflammatory activities have been reported (Kumazawa *et al.,* 2013; Wang *et al.,* 2014). In addition, this compound has shown a reduction on fat accumulation, possibly associated to a hypolipidemic effect (Bhattacharya et al., 2013). The compound **23** was identified as Kaempferol 7-*O*-neohesperidoside, previouslycharacterized by LC-ESI-MS analysis in cocoa (*Theobroma cacao*) (Sánchez-Rabaneda *et al.,* 2003). This is the first report of Kaempferol 7-*O*-neohesperidoside in *Opuntia* samples. The in vitro antitumor activity against A549, LAC, Hep-G2, and HeLa cell lines of this compound has been evaluated (Xu *et al.,* 2011).

The compound **21**, precursor ion at m/z 451, showed a fragment at m/z 286.35 $[(M-H)-162]^-$ and m/z 257.5 $[(M-H)-162-28]^-$. This mono-glycoside was tentatively identified as Kaempferol 3-*O*-glucoside. This compound has exhibited immunomodulatory effect, antinociceptive and anti-inflammatory activities (Vongsak *et al.,* 2013; Wang *et al.,* 2014). This is the first report of Kaempferol 3-*O*-glucoside in *Opuntia* samples.

The compound **26'** with a precursor ion at m/z 420, yielded a fragment at m/z 286.32 $[(M-H)-132]^-$ and m/z 258.19 $[(M-H)-132-28]^-$. This compound, a mono-glycoside, was identified as Kaempferol 3-*O*-arabinofuranoside, according to the MS data and UV spectra reported by Lhuillier *et al.,* (2007). The antioxidant activity of Kaempferol 3-*O*-α-L-arabinofuranoside (juglanin) has been reported (Phan *et al.,* 2010; Nguelefack *et al.,* 2011). Therapeutical effects of juglanin have been reported, including neuroprotective effect against glutamate-induced toxicity in the mouse hippocampal neuronal cell line HT22 and cytotoxic activity against a human hepatoma cell line (HepG2) (Liu *et al.,* 2008; Yang *et al.,* 2011). Also juglaning showed an inhibitory effect against cellular senescence in human dermal fibroblasts, with potential for treatment and prevention of diverse age-related diseases and cancer. (Yang *et al.,* 2014). This is the first report about the detection of Kaempferol 3-*O*-arabinofuranoside in *Opuntia* and it was only detected in *O. streptacantha* "Tuna loca", a wild species.

Isorhamnetin *O*-glycosides

The compounds **14b**, **17**, **24**, **26** and **28** were tentatively identified as Isorhamnetin *O*-glycosides due to the presence of a fragment ion at m/z 316. The compound **14b**, precursor ion at m/z 776 $[(M-H)+7]^-$, yielded fragments at m/z 610.73 $[(M-H)-162]^-$and at m/z 317.73 $[(M-H)-162-132-162]^-$. This compound, a tri-glycoside according to these fragments, was identified as Isorhamnetin 3-*O*-(pentosyl-glucoside)-7-*O*-glucoside. The fragmentation pattern of this compound, a flavonoid present in honey was characterized by Truchado *et al.,* (2009) was similar to data obtained in this study. This is the first report of Isorhamnetin 3-*O*-(pentosyl-glucoside)-7-*O*-glucoside in *Opuntia*.

The compound **17**, precursor ion at m/z 630 $[(M-H)+7]^-$, yielded fragments detected at m/z 463.8 $[(M-H)-162]^-$and at m/z 318.9 $[(M-H)-146-162]^-$, showing this compound is a di-glycoside. The fragmentation pattern of compound **17** allowed identification of Isorhamnetin 3-*O*-rutinoside (Narcissin), according to the data obtained by García-Abad *et al.,* (2012). Previously, Narcissin have been reported in other *Opuntia* species (Park *et al.,* 2007; Yeddes *et al.,* 2013). Therapeutical properties of Isorhamnetin-3-*O*-rutinoside such an α-glucosidase inhibitory effect, antioxidant activity and a apoptosis promoting effect in human myelogenous erythroleukaemia cells have been reported (Boubaker *et al.,* 2011; Kim *et al.,* 2011).

The compounds **24** and **26** showed the same precursor ion at m/z 482 $[(M-H)-18+23]^-$ but different RT, and yielded similar fragments at m/z 316.71 $[(M-H)-146-162]^-$ and m/z 316.62 $[(M-H)-146-162]^-$, respectively. These di-glycosides were identified as isomers. These compounds, **24** and **26**, were identified as Isorhamnetin 3-*O*-galactoside and Isorhamnetin 3-*O*-glucoside, respectively, according to data generated by De Leo *et al.,* (2010) from LC-MS^2 analysis of *O.ficus-indica* flowers. The anti-inflammatory activity of Isorhamnetin 3-*O*-galactoside and Isorhamnetin 3-*O*-glucoside have been evaluated, and both compounds are considered as candidate therapeutic agents for the treatment of severe vascular inflammatory diseases (Kim *et al.,* 2013). In addition, Isorhamnetin 3-*O*-glucoside induced a reduction on fat accumulation possibly associated to a hypolipidemic effect (Bhattacharya *et al.,* 2013).

The compound **28**, precursor ion at m/z 451, showed a fragment at m/z 316.54 $[(M-H)-162]^-$ and m/z 272.03$[(M-H)-132-44]^-$. This mono-glycoside was tentatively identified as Isorhamentin 3-*O*-pentose (Lhuillier *et al.,* 2010).

So far, comparative analysis of data showed most of the compounds were present in all cultivars, except the following: compound **7a** (quercetin + rhamnose + rhamnose + hexose) present only in *O. megacantha* “Rubí reina” and *O. ficus-indica* “Rojo vigor”; compound **11** was detected only *in O. streptacantha* “tuna loca” and *O. megacantha* “Rubí reina”; compound **26’** (kaempferol 3-*O*-arabinofuranoside) was detected only in *O. streptacantha* “tuna

loca” and *O. hyptiacantha* “Memelo 1”; compound **7b** (Quercetin 3-*O*-Xylosyl-*O*-rhamnosyl-*O*-glucoside) was no detected in *O. hyptiacantha* “Memelo 1”. Compounds **10** (Kaempferol 3-*O*-rha-gal-7-*O*-rha), **20** (Quercetin 3-*O*-arabinoside) and **28** (Isorhamnetin + Xylose/Arabinose) were no detected in *O. albicarpa* “Naranjón legítimo”; compound **19** (Kaempferol 3-O-rutinoside) was no detected in *O. ficus-indica* “Rojo vigor”.

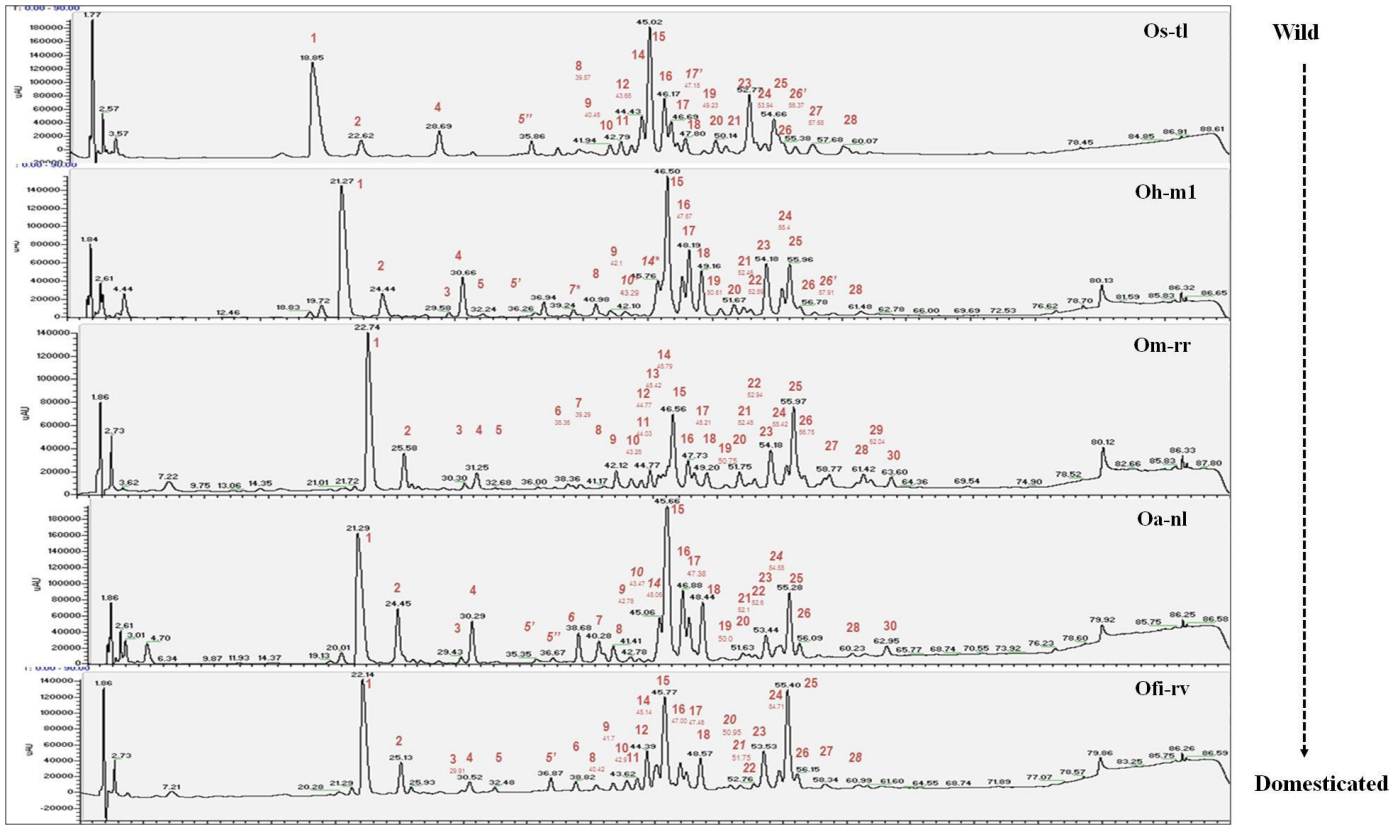


Figure 8. Chromatograms of phenolic [a] obtained from young cladodes of *Opuntia* species[b] by LC-MS^2. [a] Peak numbers correspond to the potential phenolic compounds identified as follows: 1, eucomic acid; 2, chlorogenic acid; 3, unknown; 7a, quercetin 3-*O*-rhamnosyl-(1→2)-[rhamnosyl-(1→6)]-glucoside; 7b, quercetin 3-*O*-xylosyl-rhamnosyl-glucoside; 8, quercetin 3-*O*-dirhamnoside; 9, unknown; 10, kaempferol 3-*O*-(rhamnosyl-galactoside)-7-*O*-rhamnoside; 11, kaempferol 3-*O*-(rhamnosyl-glucoside)-7-*O*-rhamnoside; 12, unknown; 14a, kaempferol 3-*O*-robinobioside-7-*O*-arabinofuranoside; 14b, isorhamnetin 3-*O*-rhamnoside-7-*O*-(rhamnosyl-hexoside); 15, quercetin 3-*O*-rutinoside; 16, quercetin 3-*O*-glucoside; 17, isorhamnetin 3-*O*-rutinoside; 19, kaempferol 3-*O*-rutinoside; 20, quercetin 3-*O*-arabinofuranoside; 21, kaempferol 3-*O*-glucoside; 22, unknown; 23, kaempferol 7-*O*-neohesperidoside; 24, isorhamnetin 3-*O*-galactoside; 26, isorhamnetin 3-*O*-glucoside; 26', kaempferol 3-*O*-arabinofuranoside; 28, isorhamnetin + Xyl/Ara (xylose/arabinose); 29, unknown; 30, unknown.[b] Os-tl, *O. streptacantha* "Tuna loca"; Oh-m1, *O. hyptiacantha* "Memelo 1"; Om-rr, *O.megacantha* "Rubí reina"; Oa-nl, *O. albicarpa* "Naranjón legítimo"; Ofi-rv, *O. ficus-indica* "Rojo vigor" collected in 2010.

Table 6. Potential phenolic acids and flavonoids analyzed by LC-MS/MS in young cladodes from *Opuntia* species[a]

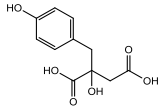
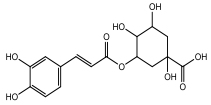
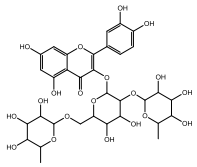
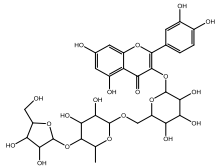
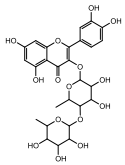
Peak No.	Systematic name	Structural formula	Molar mass (g mol^{-1})	Detection in LC-MS/MS[b]	Os-tl	Oh-m1	Om-rr	Oa-nl	Ofi-rv
1	Eucomic acid		240.2	240.91/180.28/150.07/178.41; RT 22.74 min	+	+	+	+	+
2	Chlorogenic acid		354.31	358/194.41/218.62/176.18/297.48; RT 25.58 min	+	+	+	+	+
3	Unknown			493/236.83/267.13/194.38/208.62; RT 30.30 min	-	+	+	+	*
7a	Quercetin 3-O-rhamnosyl-(1→2)-[rhamnosyl-(1→6)]-glucoside		756.66	762/302.59/596.63/614.67; RT 39.29 min	-	-	+	-	+
7b	Quercetin 3-*O*-xylosyl-rhamnosyl-glucoside		742.64	748/302.63/596.5/614.74; RT 39.29 min	+	-	+	+	+
8	Quercetin 3-O-dirhamnoside		594.52	600/302.56/448.90; RT 41.17 min	+	+	+	+	+
9	Unknown			501/343.09/350.14/334.12; RT 42.12 min	+	+	+	+	+

Table 6. (continuated)

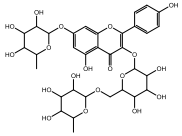
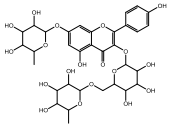
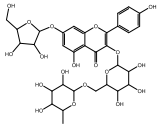
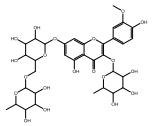
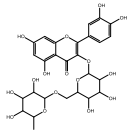
Peak No.	Systematic name	Structural formula	Molar mass (g mol^{-1})	Detection in LC-MS/MS	Os-tl	Oh-m1	Om-rr	Oa-nl	Ofi-rv
10	Kaempferol 3-*O*-(rhamnosyl-galactoside)-7-*O*-rhamnoside		740.66	746/286.45/580.40/598.54; RT 43.28 min	+	+	+	-	+
11	Kaempferol 3-*O*-(rhamnosyl-glucoside)-7-*O*-rhamnoside		740.66	746/580.42/286.46/598.55; RT 44.03 min	+	-	+	-	-
12	Unknown			822/481.66/653.57/800.74; RT 44.77 min	+	-	+	-	+
14a	Kaempferol 3-*O*-robinobioside-7-*O*-arabinofuranoside		726.64	732/580.38/286.45/598.53; RT 45.79 min	+	+	+	+	+
14b	Isorhamnetin 3-*O*-rhamnoside-7-*O*-(rhamnosyl-hexoside)		770.69	776/317.73/610.77; RT 45.79 min	+	+	+	+	+
15	Quercetin 3-*O*-rutinoside		610.52	614/303.54/302.58/345.88; RT 46.56 min	+	+	+	+	+

Table 6. (continuated)

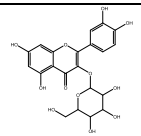
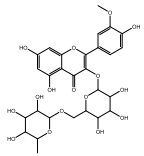
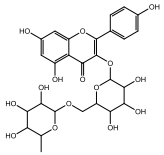
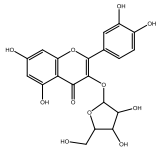
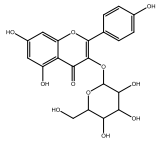
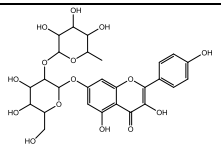
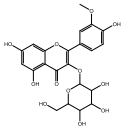
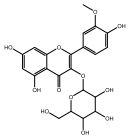
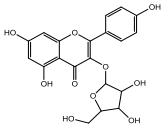
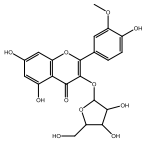
Peak No.	Systematic name	Structural formula	Molar mass (g mol^{-1})	Detection in LC-MS/MS	Os-tl	Oh-m1	Om-rr	Oa-nl	Ofi-rv
16	Quercetin 3-*O*-glucoside		464.38	467/303.51/302.61/180.38/151.98; RT 47.73 min	+	+	+	+	+
17	Isorhamnetin 3-*O*-rutinoside		624.55	630/318.9/463.8/317.78/599.52; RT 48.21 min	+	+	+	+	+
19	Kaempferol 3-*O*-rutinoside		579.49	598/287.36/286.39/329.76/257.16; RT 50.75 min	+	+	+	+	-
20	Quercetin 3-*O*-arabinofuranoside		434.35	436/302.59/303.59/349.9/180.42; RT 51.75 min	+	+	+	-	+
21	Kaempferol 3-*O*-glucoside		448.38	451/286.35/287.47/451.12/257.5; RT 52.48 min	+	+	+	+	+
22	Unknown			554/286.37/436.97/287.29; RT 52.94 min	+	+	+	+	+

Table 6. (continuated)

Peak No.	Systematic name	Structural formula	Molar mass (g mol^{-1})	Detection in LC-MS/MS	Os-tl	Oh-m1	Om-rr	Oa-nl	Ofi-rv
23	Kaempferol 7-*O*-neohesperidoside		594.52	598/287.41/286.55/257.21/329.87; RT 54.18 min	+	+	+	+	+
24	Isorhamnetin 3-*O*-galactoside		478.41	482/316.71/481.33/360.17; RT 55.42 min	+	+	+	+	+
26	Isorhamnetin 3-*O*-glucoside		478.41	482/316.62/317.72/287.43; RT 56.75 min	+	+	+	+	+
26'	Kaempferol 3-*O*-arabinofuranoside		418.35	420/286.32/329.8/287.52; RT 56.37 min	+	+	-	-	-
28	Isorhamnetin + Xyl/Ara (xylose/arabinose)		448.38	451/316.64/451.01/317.75/360.17; RT 61.42 min	+	+	+	-	+
29	Unknown			596/450.94/493.37/533.75/287.47; RT 62.04 min	-	-	+	-	-
30	Unknown			626/293.46/378.44; RT 63.6 min	-	-	+	+	-

[a] Os-tl, *O. streptacantha* "Tuna loca"; Oh-m1, *O. hyptiacantha* "Memelo 1"; Om-rr, *O.megacantha* "Rubí reina"; Oa-nl, *O. albicarpa* "Naranjón legítimo"; Ofi-rv, *O. ficus-indica* "Rojo vigor" collected in 2010. **+**, detected; -, not detected; *, undetermined.

[b] MS and MS^2 fragment ions (m/z), negative mode $[M-H]^-$; RT, retention time.

CONCLUSIONS

Total phenolics and total flavonoids contents varied between cultivars, but no as a function of domestication grade. The antioxidant potential of *Opuntia* extracts also depended on type of species. The effect of domestication was observed, although the expected behavior was not determined. However, data obtained in this study suggest preponderant role of phenolic compounds in the antioxidant capacity exhibited by *Opuntia* extracts. In addition, these results allowed the identification of cultivars with high antioxidant capacities and also high total phenolics content, such *O. streptacantha* “Tuna loca”, *O. hyptiacantha* “Memelo 1”, *O. megacantha* “Rubí reina”, *O. albicarpa* “Naranjón legítimo” and *O. ficus-indica* “Rojo vigor”.

This is the first report of metabolomic analysis and identification of phenolic compounds in wild *Opuntia* species. The chromatograms allowed the detection of major peaks, as follows: 15 (rutin), 1 (eucomic acid), 25 (unknown), 16 (quercetin 3-*O*-glucoside), 17 (isorhamnetin 3-*O*-rutinoside), 23 (kaempferol 7-*O*-neohesperoside) and 2 (chlorogenic acid) present in all cultivars. However, these results apparently not depended on domestication grade.

The MS^2 analysis allowed the identification of phenolic acids and flavonoids glycosides (mono-, di- and tri-glycosides) including derivates from quercetin, kaempferol and isorhamnetin. Particularly compound **26’** identified as Kaempferol 3-O-arabinofuranoside was detected only in *O. streptacantha,* a wild species. Therapeutical properties of this flavonoid include a cytotoxic activity, a neuroprotective and inhibitory effect against cellular senescence in human dermal fibroblasts, with potential for treatment and prevention of diverse age-related diseases and cancer.

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CHAPTER 3.
ESTABLISHMENT OF CALLUS FROM *Opuntia robusta* Wendl., A WILD AND MEDICINAL CACTUS, FOR PHENOLIC COMPOUNDS PRODUCTION

In this work, a protocol for the establishment of callus cultures from *Opuntia robusta* Wendl., a wild and medicinal cactus, was developed. The effects of plant growth regulators and culture media composition on callus development were evaluated. The best response was observed on Murashige and Skoog medium added with 2,4-dichlorophenoxyacetic acid, benzyladenine, biotin, casein hydrolysate and proline. The exposure of *O. robusta* callus to jasmonic acid increased 1.3-fold and 3-fold total phenolic acids and flavonoids concentration, respectively. The *in vitro* cultures from O. robusta could be a new approach for the obtainment of metabolites with pharmaceutical and/or nutraceutical value.

RESULTS

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Establishment of callus from *Opuntia robusta* Wendl., a wild and medicinal cactus, for phenolic compounds production

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In this work, a protocol for the establishment of callus cultures from *Opuntia robusta*, a wild and medicinal cactus, was developed. The effects of plant growth regulators and culture media composition on callus development were evaluated.The best response was observed on Murashige and Skoog medium added with 2,4-dichlorophenoxyacetic acid, benzyladenine, biotin, casein hydrolysate and proline. The exposure of *O. robusta* callus to jasmonic acid increased 1.3-fold and 3-fold total phenolic acids and flavonoids concentration, respectively. The *in vitro* culture from *O. robusta* could be a new approach for the obtainment of metabolites with pharmaceutical and/or nutraceutical value.

Key words: Callus, flavonoids, jasmonic acid, wild *Opuntia*, phenols.

INTRODUCTION

Cactus prickly pear belongs to *Opuntia* genus and has been used as a source of food, building material, natural pigment production and as a medicinal plant. *Opuntia* plants produce tender cladodes, consumed as vegetable and fruits (prickly pear) both highly appreciated due its high nutritional value and nutraceutical properties. Their therapeutic effects have been associated to its antioxidant constituents like vitamin C, vitamin E, carotenoids, glutathione and phenolic compounds such as flavonoids and phenolic acids (Santos-Zea, 2011).

It is known that metabolite and chemical profile in *Opuntia* depends on harvesting season, culture conditions, growth stage and species. In addition, production of young cladodes and prickly pear fruits is affected by plagues and diseases that attack both commercial crops and wild populations of *Opuntia* (Méndez-Gallegos et al., 2008). Plant cell culture (PCC) represents an alternative with biotechnological potential for metabolite production.

In addition, using PCC is possible to increase yield using strategies like optimization of culture conditions, selection of high-producing cell lines, bioreactor design and elicitation.

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Abbreviations: **BA**, Benzyladenine; **CH**, casein hydrolysate; **2,4-D**, 2,4-dichlorophenoxyacetic acid; **DICAMBA**, 3,6-dichloro-2-methoxybenzoic acid; **IAA**, indole-3-acetic acid; **IBA**, indole-3-butyric acid; **KIN**, kinetin; **NAA**, 1-napthaleneacetic acid; **PGR**, plant growth regulators; **PICLORAM**, 4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid; **PPM**, plant preservative mixture; **MS**, Murashige and Skoog medium.

During elicitation, *in vitro* cultures are exposed to compounds that induce metabolite synthesis as a defense mechanism like jasmonic acid (JA) and its methylated form (MeJA). These elicitors increase the expression of phenylpropanoid biosynthetic genes and enzymes and have been used in plant cell cultures to enhance the synthesis of health promoting metabolites (Karuppusamy, 2009). For the establishment of *in vitro* cultures, the participation of PGR is essential. It has been described that auxins affect development, cell growth, embryo formation and callus induction in different species. Kinetin (KIN) and benzyladenine (BA) are synthetic cytokinin commonly used in plant cell culture to generate somatic embryogenesis, organogenesis and callus formation in cacti (Shedbalkar et al., 2010). Addition of amino acids (proline and glutamine) and organic supplements (CH and peptone) also have a promoting effect on *in vitro* culture development stimulating cell proliferation. Vitamins, like biotin improves the physiological response of *in vitro* cultured recalcitrant species (Al-Khayri, 2001).

Previous reports of *in vitro* culture and micropropagation protocols of *Opuntia ficus-indica* and its cultivars have been described (Llamoca-Zárate et al., 1999; García-Saucedo et al., 2005;) but this is the first report of a protocol for *in vitro* culture establishment of *Opuntia robusta*, a wild species, as a potential metabolite production source. *O. robusta* has long and succulent cladodes, highly appreciated for human consumption, in both fresh and pickled forms and effects on glucose and lipid metabolism have been reported (Wolfram et al., 2002).

In this study, a protocol for the establishment of callus culture from *O. robusta* for the phenolic compounds production, was developed. An optimized sterilization protocol for *O. robusta* cladodes was designed and the effect of plant growth regulators (PGR) and different organic nitrogen sources (casein hydrolysate, glutamine, and proline) was evaluated. Since we are particularly interested in the production of flavonoid and phenolic acids (due to their antioxidant properties), the induction of these metabolites by elicitation with JA is also described.

MATERIALS AND METHODS

Collection of plant material

O. robusta donor plants were provided by the National Institute of Agricultural, Livestock and Forest Research at San Luis Potosí. After collection, plants were put in pots and maintained under greenhouse conditions for young cladodes production.

Surface sterilization protocol

Prior to collection of young cladodes, *O. robusta* donor plants were treated with a biocide mixture (containing 3 mg l^{-1} Captan, 3 mg l^{-1} Benlate, 1 ml l^{-1} Previcur, 0.5 g l^{-1} amoxicillin, and 0.4 g l^{-1} ketoconazol). Collected young cladodes were washed with commercial antibacterial detergent and running tap water for 45 min, followed by immersion in the biocide mixture (described above) for 36 h, rinsed in sterile water, and treated with 10% calcium hypochlorite [$Ca(ClO)_2$] during 10 min, followed by 8% $Ca(ClO)_2$ during 5 min. Finally, cladodes were rinsed in sterile water three times.

Culture conditions and callus induction

Sterile explants (1.5 cm^2) were cultured on Murashige and Skoog (MS) basal media (Murashige and Skoog, 1962) supplemented with116 µM myo-inositol, 1.2 µM thiamine-HCl and 30 g l^{-1}sucrose. To evaluate the auxin type effect on callus induction, explants were cultured on MS basal media with 2 mg l^{-1} (IAA), (IBA), (NAA) or (2,4-D). To evaluate the effect of PGR combinations on callus development, explants were cultured on MS basal media with 3 mg l^{-1} IAA or 2,4-D in combination with 1 mg l^{-1} Picloram, Dicamba, BA or KIN. The effect of media supplements (0.1 mg l^{-1} biotin/0.4 g l^{-1} CH, 0.1 mgl^{-1} biotin/500 µM glutamine or proline) on callus development was also tested.

In order to prevent or decrease oxidation of explant, all culture media contained 1% (w/v) activated charcoal (AC), 0.3% polyvinylpyrrolidone (PVP), 0.025 mg l^{-1}ascorbic acid and 0.025 mg l^{-1}citric acid. All media were adjusted to pH 6.7 with 0.1 N KOH solution, solidified with 4.5 g L^{-1} phytagel (SIGMA) and autoclaved at 121°C for 20 min (1.37 x 10^5 Pa). The explants were cultured under dark conditions and callus formation was evaluated three weeks later.

Induction of phenolic compounds production

To promote phenolic compounds production, *O. robusta* calli were cultured on MS basal media with 50 µM MeJA. Elicited and control calli cultures were maintained under same conditions during 15 days and then were lyophilized and stored at -20°C until analysis.

Total phenolic compounds content determination was performed according to Luximon-Ramma et al. (2002). Gallic acid (GA) was used as standard and results were expressed as µmol of GA equivalents (GAE) per g of sample (dry weight). Data correspond to mean value ± standard deviation (SD). Total flavonoids content was performed according to the method reported by Ottica et al. (2007) with brief modifications. Quercetin (Q) was used as standard and the results were expressed as µmol of quercetin equivalents (QE) per g of sample (dry weight). Data were reported as a mean value ± standard deviation (SD). All determinations were made in triplicate.

A completely random experimental design was selected using 20 to 30 explants per treatment. Statistical analysis were performed using the Instat III program the Tukey test (P=0.05) to compare differences between means.

RESULTS AND DISCUSSION

The sterilization of *O. robusta* cladodes surface was a challenging process due to the huge variety of microorganisms including insects, fungus, bacteria and viruses hosted in spines, areoles, hairs and waxes, which density is higher than in *O. ficus-indica* cladodes (Méndez-Gallegos et al., 2008). In order to obtain sterile explants, washing with detergent, immersion in a biocide solution, followed by the application of $Ca(ClO)_2$ solution were required. The sterilization efficiency was 90%.

Auxin type and concentration are determinant in callus development. Therefore, the effect of natural (IAA, IBA) and synthetic auxins (NAA, and 2,4-D) on callus induction from *O. robusta* was primarily evaluated. Callus formation was obtained in 65 to 75% of explants cultured in presence of IAA, IBA or 2,4-D (no significant differences,

Table 1. Effect of auxins on callus induction from cladodes of *Opuntia robusta*

Auxin (2 mg l^{-1})	Callus induction (%)	Callus morphology
IAA	75^{a}	Moderate friable callus
IBA	75^{a}	Green compact callus
NAA	33.3^{b}	Green compact callus
2,4-D	66.6^{a}	Green compact callus

Data with different letters are statistically different ($p<0.05$).

Table 2. Effect of PGR combinations and supplements on callus formation from *Opuntia robusta*.

PGR (mg l^{-1})	Callus Induction (%)	Size of callus (mm)
IAA3	100^{a}	5-10
IAA3-DICAMBA1	22^{c}	< 5
IAA3-PICLORAM1	100^{a}	< 5
IAA3- PICLORAM1-KIN1	100^{a}	5-10
IAA3- PICLORAM1-KIN1-Bio-gln	100^{a}	5-10
IAA3- PICLORAM1-KIN1-Bio-CH	100^{a}	10-13
2,4-D 3	66^{b}	5-10
2,4-D 3-PICLORAM1-BAP1-Bio-CH	20^{c}	6-14
2,4-D 3- BAP1-Bio-CH-pro	50^{b}	6-14

Bio, biotin (0.1 mg l^{-1}); CH, casein hidrolysate (0.4 g l^{-1}); gln, glutamine (500 µM); pro, proline (500 µM). Values with different letters are statistically different ($p<0.05$).

$p<0.05$). The lowest callus formation (33%) was observed in explants cultured in the presence of NAA (Table 1). However, IAA induced the generation of friable callus, which is a desirable feature in order to obtain suspension cultures. Therefore, sterile explants were cultured on media with 0.5, 1, 2 and 3 mg l^{-1} of IAA to evaluate callus formation. Callus formation occurred in100% of explants cultured with 3 mg l^{-1} IAA, nevertheless, development rate was slow.

It is well known that recalcitrant plants require combinations of PGR, higher concentration of macro-nutrients, additional organic nitrogen sources or vitamins to induce growth or development. To improve growth rate of callus, PGR combinations and addition of media supplements (biotin and CH, glutamine) were tested (Table 2). Callus induction was observed in 100% of explants maintained on media containing IAA alone or in combination with PICLORAM, KIN, biotin/glutamine and biotin/CH but callus formation decreased on medium with IAA/DICAMBA combination.The highest callus amount was obtained in media supplemented with CH/biotin (10 to 12 mm), however, after subcultures, the development of callus was arrested.

It has been shown that 2,4-D promote callus formation and increases growth rates in several cacti species.Thus, 3 mg l^{-1} 2,4-D in combination with PICLORAM and BA, and supplemented with biotin/CH or proline were tested (BA used instead KIN because higher oxidation rates were observed in presence of KIN). Data (Table 2) suggest that 2,4-D, PICLORAM and BA combination induced callus formation, but was lower than callus formation in explants cultured with 2,4-D alone (statistically different, $p<0.05$), however, biomass was similar.On the other hand, callus formation occurred in 50% of explants cultured on media containing 2,4-D and BA, biotin/CH and proline. This calli were friable and its development continued after subcultures. Then, this media was considered as the optimal for the establishment of callus culture of *O. robusta*. The beneficial effect of CH and proline have been attributed to a higher availability of organic nitrogen source, due to the ammonium, derived of transamination reactions of amino acids. CH is also a source of calcium, phosphate, micronutrients, vitamins, and several growth factors that improve shooting and callus development (Khaleda and Al-Forkan, 2006). In addition, proline is an energy source (the oxidation of one molecule of proline yields 30 ATP equivalents), has a reactive oxygen species (ROS) scavenging activity, an osmoprotective role, is considered as a molecular chaperone able to protect protein integrity, and enhance the activities of different enzymes (Szabados and Savoure, 2009). Biotin is a heterocyclic compound that binds covalently to specific carboxylases to facilitate the transfer of CO_2 during carboxylation and decarboxylation reactions, improves the physiological response of *in vitro* cultured recalcitrant species, and embryo development (Al-Khayri, 2001).

The content of phenolic compounds was determined in

Table 3. Production of phenolic acids and flavonoids by callus from *Opuntia robusta*[1].

Metabolite	Control callus (µmol g^{-1})	Elicited callus (µmol g^{-1})	Increment
Phenolic acids[2]	7.50 ± 0.33^{b}	9.81± 0.36^{a}	1.30
Flavonoids[3]	1.91± 0.08^{b}	5.84 ± 0.34^{a}	3.06

[1]Values between columns with different letters are statistically different ($p<0.05$).
[2]Phenolic acids content determined as gallic acid equivalents.
[3]Flavonoids content determined as quercetin equivalents.

O. robusta callus. The results show that callus produced 2.3 times more phenolics acids (GAE) than flavonoids (QE). Ellicitation with JA increased 1.3-folds the phenolic acids content and 3-folds the flavonoids conoentration in comparison to control callus (Table 3). JA has been widely used in promoting the biosynthesis of both inducible and constitutive secondary metabolites, including several anti-oxidants as α-tocopherol, cyanidineglucosides, resveratrol and flavonoids (Matkowski, 2008).

Comparing our results with other data from literature, the content of phenolic acids in *O. robusta* cultures was 11 times higher than in callus from *Lepidum meyenii* (Wang et al. 2007), while the flavonoids content was two times higher than in cellular suspensions of *Pueraria tuberose* (Goyal and Ramawat, 2008). Further studies must be done to identify and characterize the metabolites from *in vitro* cultures of *O. robusta* species and know if they correspond or not to those present in *O. robusta* plant.In addition, the levels of metabolites could be improved using biotic and abiotic stress.

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CHAPTER 4.
PROTEOMIC ANALYSIS OF *Opuntia* SPECIES WITH DIFFERENT GRADE OF DOMESTICATION

1. INTRODUCTION

Opuntia, as well as other xerophytes, grows in arid and semi-arid regions. These environments have generated, in organisms, cellular and molecular modifications in during their adaptation process, including the expression of proteins, with specific biological functions, but also with desirable features for industrial processes, nowadays. In *Opuntia*, most of the studies have been focused in temperature-stable and defense mechanisms proteins with potential applications in biotechnology and bioremediation. Particularly, two laccases and two endoglucanases were isolated from *O. vulgaris*, and some arylamidases, lipases, proteinases and glucosidases were characterized from *O. ficus-indica* (Kumar and Srikumar, 2011; Shyamala *et al.,* 2011;Teixeira *et al.,* 2000). In addition, enzymes related to biotic and abiotic stress defense such lectins and peroxidases have been isolated from *O. ficus-indica* cladodes (Paiva *et al.,* 2011; Khales and Baaziz, 2005). On the other hand, some proteins with therapeutical potential have been studied in cladodes (a 90 kDa glycoprotein with anti-allergy properties) and fruits (a nucleotide pyrophosphatase/phosphodiesterase) from *O. ficus-indica* (Kim, 2010; Spano *et al.,* 2011). However, data related to *Opuntia* proteome is limited.

Proteomics studies the expression pattern of proteins in organisms and, in non-sequenced genome plants provides a global view of the biochemical environment at a specific stage or condition (Haynes and Roberts, 2007). Electrophoretic separation in first (1D-SDS-PAGE) and second dimension (2D-SDS-PAGE) are as well as Shotgun proteomics represent optimal techniques for proteome screening and also for comparative analysis related to differential expression of proteins of interest. Several attempts have been made in order to analyze the protein pattern of *Opuntia* cladodes using these techniques but there are no reports of Shotgun proteomics data about this genus. However, cactus pear is considered as a recalcitrant plant due to its high content of

polysaccharides, phenolic compounds and pigments (Xie *et al.,* 2007). Since *Opuntia* genome remains un-sequenced, proteome data will lead to the understanding of protein expression, accumulation and organization in this plant and changes related to its domestication process.

2. OBJECTIVES

2.1 General

Evaluate the effect of domestication on protein expression patterns in *Opuntia* cladodes by proteomics approaches.

2.2 Specifics

a. Standardization of protein extraction procedure from *Opuntia* fresh tissue.

b. 1D-SDS-PAGE patterns of total protein from *Opuntia* samples.

c. 1D-SDS-PAGE patterns of total protein from *Opuntia* samples for shotgun proteomics

d. 2D-SDS-PAGE patterns of total protein from *Opuntia* samples.

3. MATERIALS AND METHODS

3.1 Reagents, chemicals and solvents

Polyacrylamide, Laemmli buffer, immobilized 4-7 pH gel strips (IPG), critterion cassettes, ammonium persulfate (PSA), N,N,N',N'-tetramethylethylenediamine (TEMED), Bradford reagent were purchased from BIO-RAD (BIO-RAD Laboratories Inc. USA). Ammonium acetate, hydroxymethyl-ammino-methane (TRIS), bovine serum albumin (BSA), dithiotrietol (DTT), Phenyl-methanesulfonyl fluoride (PMSF), 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate hydrate (CHAPS) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). All solvents (Methanol, acetone, ethanol) were ACS grade. For LC-MS analysis, all solvents were HPLC and LC-MS grade. Ultra-pure water was obtained from a Milli-Qplus purification system (Millipore Corp., Bedford, MA).

3.2 Plant material

Young cladodes or nopalitos were collected from donor plants grown under same environmental conditions (temperature, precipitation and soil) at the *Opuntia* Germoplasm Bank in the Centro Regional Universitario Centro Norte de la Universidad Autónoma de Chapingo (CRUCEN-UACh), located in Huertas del Orito, Zacatecas, México (22° 44.7’ North latitude, 102° 36.4’ west length) (Lopez-Palacios et al. 2011). Young cladodes (15–25 cm of length) showed in Figure 1,were washed, N_2 freeze, grinded (KRUPS GX4100 grinder) and stored at-80°C until analysis. Fresh samples were used for protein extraction.

3.3 Protein extraction method

In this work, a modified protein extraction method from fresh tissue was used. This method consisted in four steps: pre-washing, washing, extraction and precipitation. Pre-washing: 50 ml of ethanol (-20°C) were added to fresh tissue (1 g), this mixture was stirred (4°C, 2 h) and then centrifuged (14 000 rpm, 20 min, 4 °C). The supernatant was discarded and the pellet was reserved. Washing: 25 ml of acetone (containing 0.2% DTT, -20°C) was added to the pellet, mixed and centrifuged (14 000 rpm, 20 min, 4 °C). Extraction: 20 ml of

extraction buffer were added to treated tissue in a mortar (on ice bath), homogenized and centrifuged (14 000 rpm, 20 min, 4 °C). Precipitation: the supernatant was added to five volumes of ammonium acetate (in methanol, 0.1 mM, -20 °C), maintained at -20 °C (overnight) and then centrifuged (14 000 rpm, 20 min, 4 °C). Pellets were washed with ammonium acetate (twice) and acetone (+ 0.2% DTT, three times). Bradford assay (Bio Rad Protein Assay) was used for protein quantization, and serum albumin was used as standard reference.

3.4 1-D-SDS-PAGE conditions of fifteen cultivars of *Opuntia*

Samples were resuspended in Laemmli buffer and loaded in 12% polyacrylamide gels (Critterion Cassettes). Elecrophoretic conditions: 10 mA per gel. Colloidal Blue Coomassie stain was used. Images were acquired and some bands were selected for LC-MS analysis for peptide identification. For each sample, independent extractions were performed (n=3)

3.5 UPLC-ESI-MS/MS analysis

Selected bands were excised and gel matrix was removed. Proteins were reduced, alkylated and digested with Trypsin. Peptides were analyzed by UPLC-ESI-MS/MS using a WATERS NanoAcquity UPLC System (Milford, Massachusetts, USA) coupled to a WATERS Synapt HDMS (Milford, Massachusetts, USA), using an Electroionization source. Data was obtained using a PLGS 2.0 software. For protein identification, MASCOT (www.matrixscience.com)

3.6 Shot-gun Proteomics analysis

Protein extraction and quantification of *O. streptacantha* “Tuna loca”, *O. hyptiacantha* “Memelo 1“, *O. megacantha* “Rubí reina“, *O. albicarpa* “Naranjón legítimo” y *O. ficus-indica* “Rojo vigor” were made as previously described. 1D-SDS-PAGE conditions: 12 % polyacrylamide gels, 10 mA per gel. Colloidal Blue Coomassie stain was used. For each sample, independent extractions were

performed. All 1D profiles were performed in quadruplicates. Samples were cut into 10 fractions, digested using Trypsin and peptides were analyzed by LC-MS/MS (Stanislas *et al.,* 2009). MS data mining was achieved using MASCOT (www.matrixscience.com) for protein identification. Proteincenter Software (Thermo-scientific) was used for comparative analysis of identified proteins, according to Gene Ontology Classification.

3.7 2-D-SDS-PAGE conditions

Extraction and determination total protein from *O. streptacantha* “Tuna loca”, *O. hyptiacantha* “Memelo 1“, *O. megacantha* “Rubí reina“, *O. albicarpa* “Naranjón legítimo” y *O. ficus-indica* “Rojo vigor” were made as previously described. Samples were suspended in rehydration buffer and loaded onto11 cm lineal pH 4-7 IPG strips during overnight. Isoelectrofocusing conditions were selected according recommendations of manufacturer. Prior to second dimension separations, stripes were equilibrated in a buffer. Electrophoretic conditions: 12 % polyacrylamide gels, 10 mA per gel. Colloidal Blue Coomassie stain was used. For each sample, independent extractions were performed. Each 2D profile was performed in triplicates.

4. RESULTS AND DISCUSSION

4.1 1D-SDS-PAGE profiles

The modified extraction method allowed to obtain total protein from fresh *Opuntia* samples. The proteomic electrophoretic patterns (1D) from the fifteen cultivars studied in this work are shown in Figure 9. Good resolution was achieved and a similar abundance of major bands was observed between species and cultivars. Some of these bands (35, 50 and 100 kDa) were selected and analyzed by LC-MS/MS for protein identification. The results are shown in Table 7. Glyceraldehyde 3-phosphate dehydrogenase, malate dehydrogenase, aldolases, enolases, and PEP carboxylases were identified. These enzymes are involved in carbohydrates metabolic pathways (glycolysis) (Buchanan *et al.,* 2002). According to these results, the elucidation of differences in proteome of *Opuntia* samples, required more efficient proteomic approaches, like Shot-gun and 2D-SDS-PAGE patterns analysis.

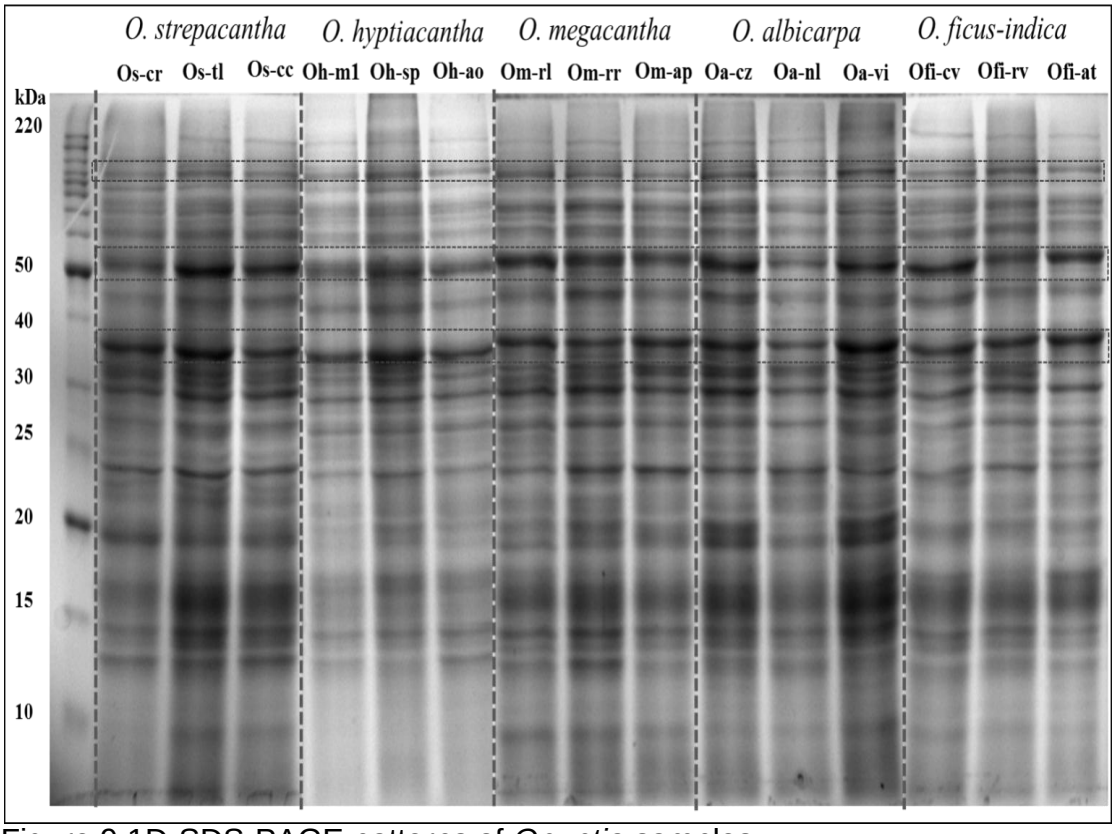

Figure 9 1D-SDS-PAGE patterns of *Opuntia* samples.

Table 7. Identified proteins from *Opuntia* young cladodes using LC-MS/MS analysis

Band (kDa)	Protein name (candidates)	Accession no.	DataBase	Peptides matched/ sequence coverage	Mascot score (p<0.05)	Theoretical mass (kDa)/ pI	Ortholog
35	Glyceraldehyde 3-phosphate dehydrogenase	G3PC_DIACA	SwissProt	8/23%	179(>35)	37.10/6.66	*Dianthus caryophyllus*
	Malate dehydrogenase	MDHC_BETVU	SwissProt	6/18%	85 (>35)	35.81/5.89	*Beta vulgaris*
	Fructose-bisphosphate aldolase	ALF_SPIOL	SwissProt	3/7%	51(<51)	38.67/5.96	*Spinacia oleracea*
	Aldolase	gi|281485510	NCBInr	5/13%	111(<50)	39.22/7.57	*Phyllostachy sedulis*
50	Enolase 2	ENO2_MAIZE	SwissProt	6/13%	176 (>36)	48.41/5.7	*Zea mays*
	Bifunctionalenolase 2/ transcriptionalactivator	ENO2_ARATH	SwissProt	8/12%	91 (>36)	47.97/5.54	*Arabidopsis thaliana*
100	Phosphoenolpyruvate carboxylase	gi|63053868	NCBInr	6/4%	118 (>50)	110.26/6.26	*Alternaria pungens*
	Pyruvate phosphate di-kinase 2	PPDK_ORYSJ	SwissProt	3/2%	35 (>35)	97.23/5.42	*Oryza sativa*

4.2 Shot-gun proteomic analysis

Based on their total phenolics content and antioxidant activity, previously discussed, the cultivars *O. streptacantha* “Tuna loca”, *O. hyptiacantha* “Memelo 1” , *O. megacantha* “Rubí reina” *O. albicarpa* “Naranjón legítimo” and *O. ficus-indica* “Rojo vigor” were selected for proteomic analysis.

For shot-gun analysis, the first step was to obtain an electrophoretic pattern of proteins from Opuntia cultivars. In Figure 10, 1D-SDS-PAGE profiles of *O. streptacantha* “tuna loca” (Os-tl), *O. hyptiacantha* “Memelo 1 (Oh-m1), *O. megacantha* “Rubí reina” (Om-rr), *O. albicarpa*“ Naranjón legítimo” (Oa-nl) y *O. ficus-indica* “Rojo vigor” (Ofi-rv) are shown.

Currently, only shot-gun results from *O. megacantha* “Rubí reina” y *O. ficus-indica* “Rojo vigor” will be discussed. From these cultivars, 255 proteins were identified, being 38 and 61 specific proteins detected only in *O. megacantha* “Rubí reina” and *O. ficus-indica* “Rojo vigor”, respectively. The identified proteins were classified according to gene ontology, in proteins involved in biological processes and molecular functions, as well as their distribution in cellular components.

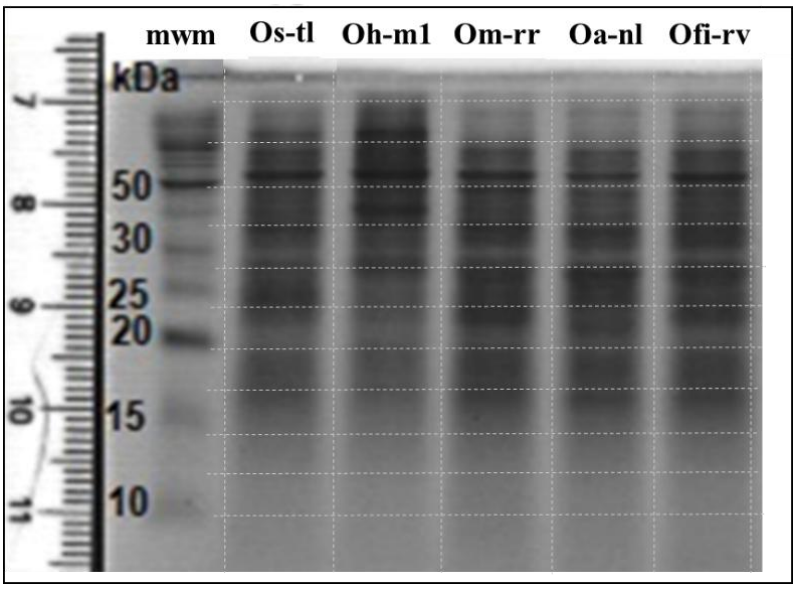

Figure 10. 1D-SDS-PAGE of *Opuntia* species for shot-gun analysis.

Biological processes distribution is shown in Figure 11. Particularly, defense response and development proteins were only detected in *O. megacantha* “Rubí reina”. Metabolic processes proteins were more abundant in *O. ficus-indica* “Rojo vigor” but the content of proteins involved in regulation of biological processes was higher *O. megacantha* “Rubí reina”. These results suggest a wide diversity in protein synthesis in moderate domesticated species, *O. megacantha* “Rubí reina” than in *O. ficus-indica* “Rojo vigor”.

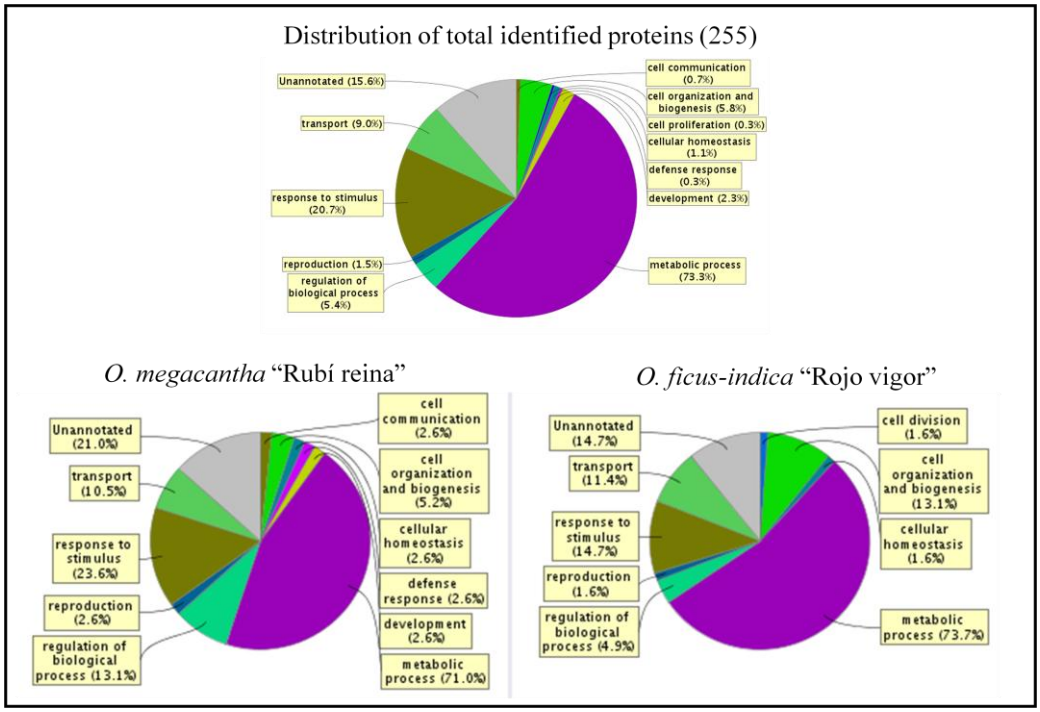

Figure 11. Biological processes classification of identified proteins from *O. megacantha* “Rubi reina” and *O. ficus-indica* “Rojo vigor”.

Cellular components distribution is shown in Figure 12. Proteins related to Golgi, endoplasmic reticulum, proteasome, vacuole, chromosome and cytoskeleton were detected only in *O. ficus-indica* “Rojo vigor”. These may suggest domesticated species requires synthesizing a higher diversity of protein components that less domesticated species. In addition, a higher abundance of cytoplasm proteins was observed in *O. ficus-indica* “Rojo vigor”. This may suggest the requirements of protein components are higher than in less domesticated species.

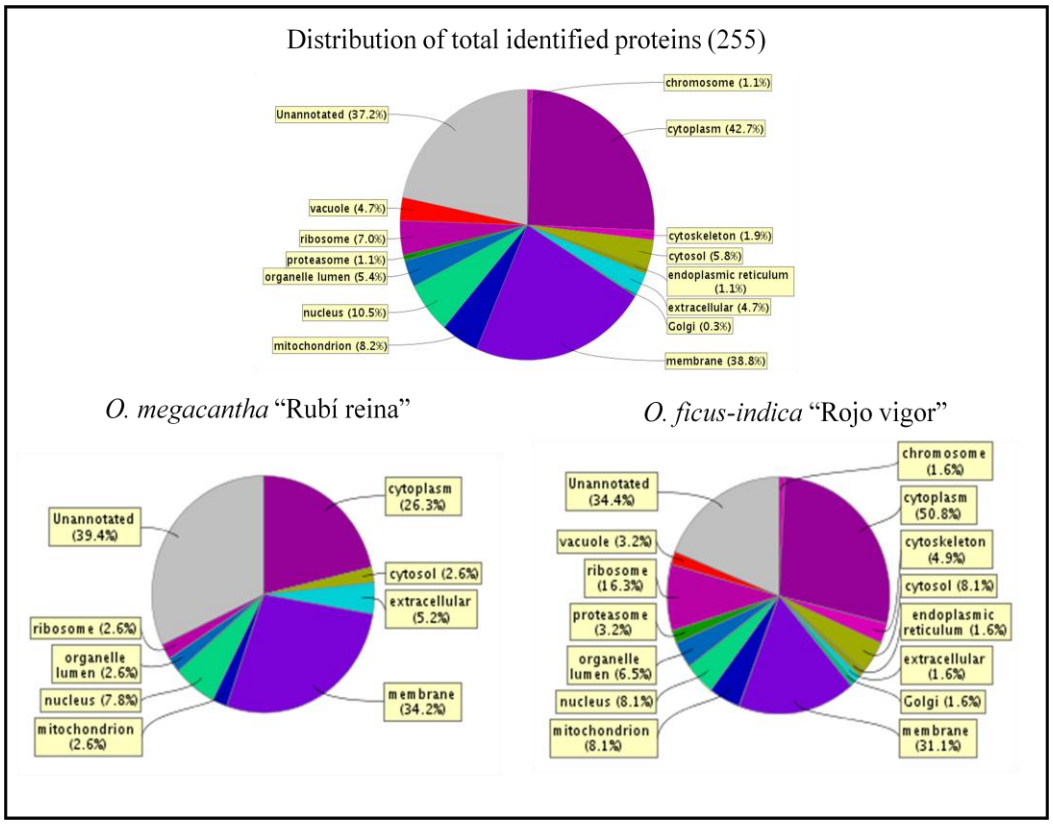

Figure 12. Cellular components distribution of identified proteins from *O. megacantha* “Rubi reina” and *O. ficus-indica* “Rojo vigor”.

Molecular functions distribution is shown in Figure 13. We were particularly interested in proteins related to antioxidant activity and in this study, the abundance of proteins associated to antioxidant activity was similar between these species. Catalytic activity and RNA binding proteins were more abundant in *O. ficus-indica* “Rojo vigor” but the content of metal ion binding proteins was higher in *O. megacantha* “Rubí reina”. these differences suggest modifications at a molecular level, associated to domestication grade.

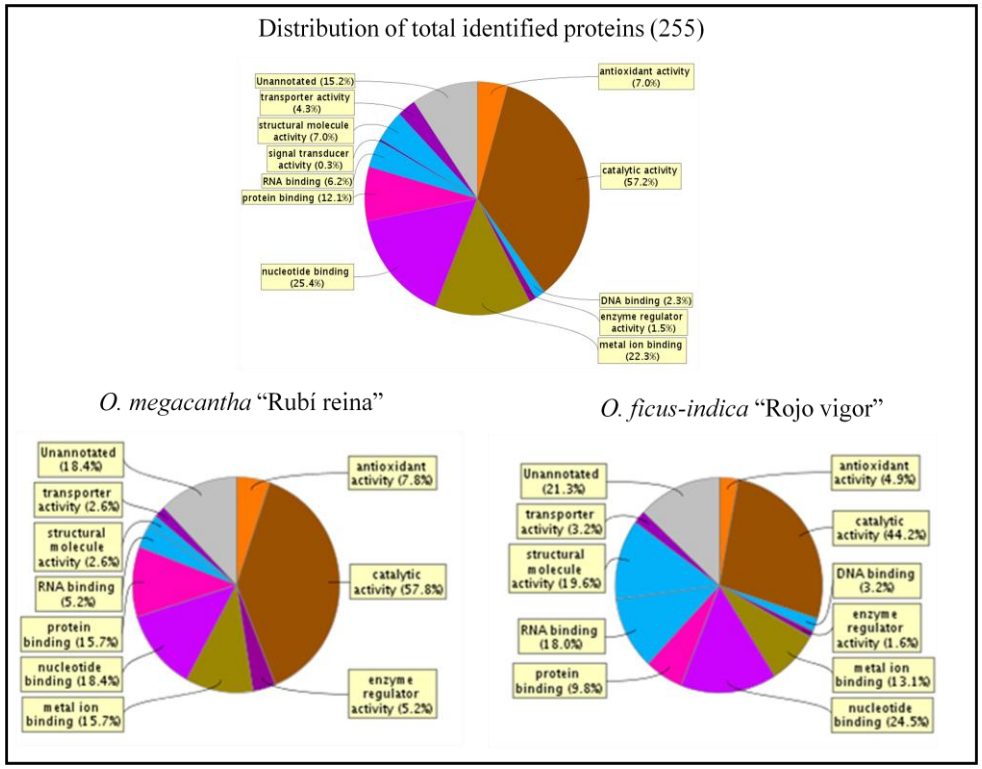

Figure 13. Molecular functions of identified proteins from *O. megacantha* “Rubi reina” and *O. ficus-indica* “Rojo vigor”.

4.3 PENDING ISSUES

4.3.1 2D-SDS-PAGE analysis

Two-dimensional gel electrophoresis (2D-PAGE) is a highly resolving separation technique often used to fractionate protein mixtures prior to identification of the proteins using mass spectrometry (Liu *et al.,* 2004). Proteins were separated according their isoelectric point (pI) through a pH-gradient gel matrix and then by molecular weight in the second dimension. 2D-PAGE patterns of *O. streptacantha* "tuna loca", *O. hyptiacantha* "Memelo 1", *O. megacantha* "Rubí reina", *O. albicarpa "*Naranjón legítimo" and *O. ficus-indica* "Rojo vigor" are shown in Figure 14. Although comparative analysis of these images is still pending, good resolution and defined spots were observed.

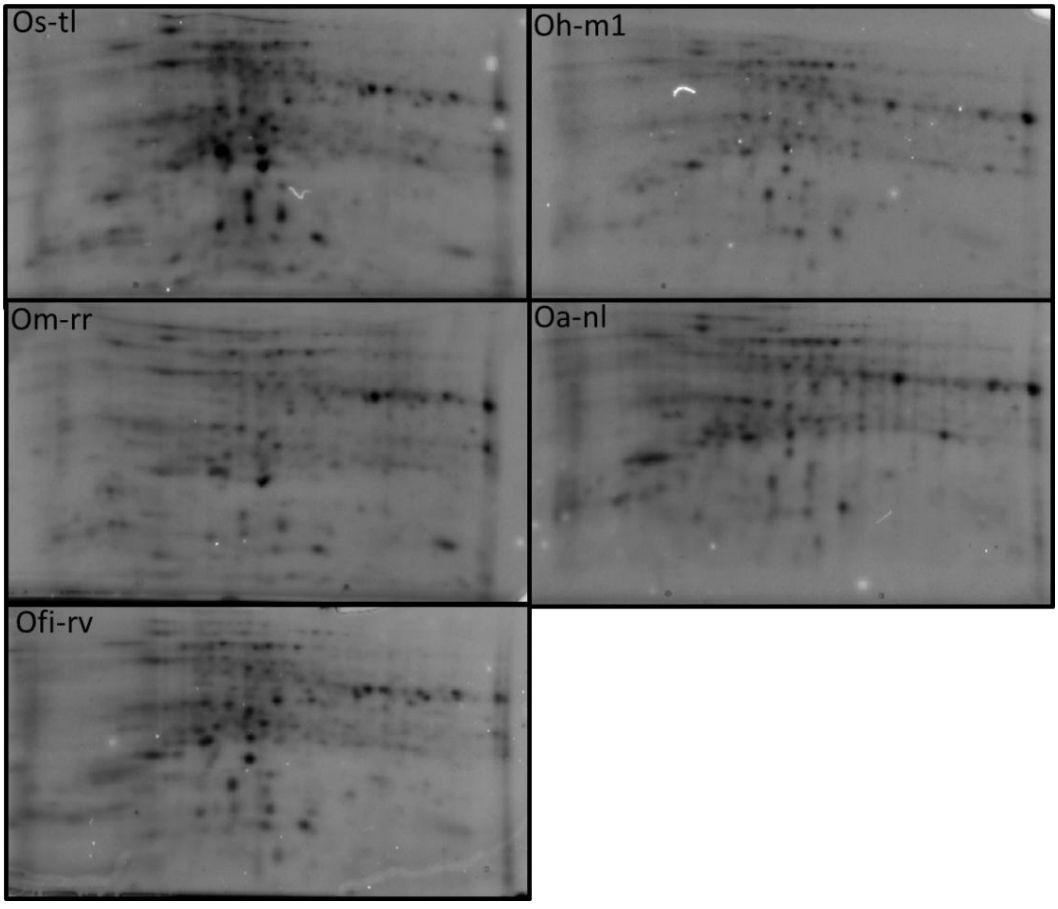

Figure 14. 2D-SDS-PAGE *Opuntia* species profiles. Os-tl, *O. streptacantha* "Tuna loca"; Oh-m1, *O. hyptiacantha* "Memelo"; Om-rr, *O. megacantha* "Rubí reina"; Oa-nl, *O. albicarpa "*Naranjón legítimo"; Ofi-rv, *O. ficus-indica* "Rojo vigor".

CONCLUSIONS

1. An extraction protocol for total protein from fresh tissue was designed

2. 1D- SDS-PAGE profiles were similar between cultivars, no differences related to domestication process were observed

3. Shotgun analysis allowed the identification of 255 proteins from *O. megacantha* “Rubí reina” and *O. ficus-indica* “Rojo vigor”, 38 and 61 specific proteins for each cultivar, respectively. Data from *O. streptacantha, O. hyptiacantha* and *O. ficus-indica* is still being analyzed. Shotgun analysis showed differences in protein expression due to domestication process.

4. 2D- SDS-PAGE profiles were obtained; however, analysis of images was not finished.

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APPENDIX 1. GALLIC ACID AND QUERCETIN CALIBRATION CURVES FOR TOTAL PHENOLIC AND TOTAL FLAVONOIDS DETERMINATION.

The protocols optimized in this work allowed the determination of total phenolics and total flavonoids contents in *O. robusta* Wendl. calli (control and elicited). In addition, this methodology was applied for analysis of Opuntia samples used by partners from INSERN, INRA and CNRS during therapeutical properties assays.

Gallic acid calibration curve

The determination of total phenolic compounds was performed by UV-Vis spectrophotometry, using the Folin-Ciocalteau regent (Folin, 1927). The methodology was optimized for *Opuntia* samples. Gallic acid (GA) was used as reference compound, and a calibration curve was obtained for phenolics content calculation in samples. In a test tube, 20 µl of standard were added to 1.58 ml of MilliQ water and mixed, followed by the addition of 300 µl of 20 % Na_2CO_3. Then, 100 µl of Folin-Ciocalteau reagent were added and the mixture was allowed to stand for 2 h (at room temperature). Absorption was read at 765 nm in a Carys UV-Vis spectrophotometer. Results were expressed as µmol of GA per ml^{-1}. Triplicates of standard solutions were measured.

Total flavonoids content

For determination of total flavonoids, the $AlCl_3$ reagent was used and in this study, methodology was optimized for *Opuntia* species analysis. Calibration curve was performed using Quercetin as a reference compound. Briefly, 15 µl of standard were added to 735µl of ultra-pure water and mixed with 750 µl of 2% $AlCl_3$ (aqueous). After ten minutes (at room temperature), the mixtures were read at 367 nm in a Carys UV-Vis Spectrophotometer. Results were expressed as µmol quercetin ml^{-1}. All determinations were performed in triplicates.

RESULTS

Gallic acid calibration curve

The calibration curve for phenolic compounds calculation was performed using 50, 100, 150, 200, 250 and 300 µg of gallic acid per ml^{-1} (R^2=0.9993).

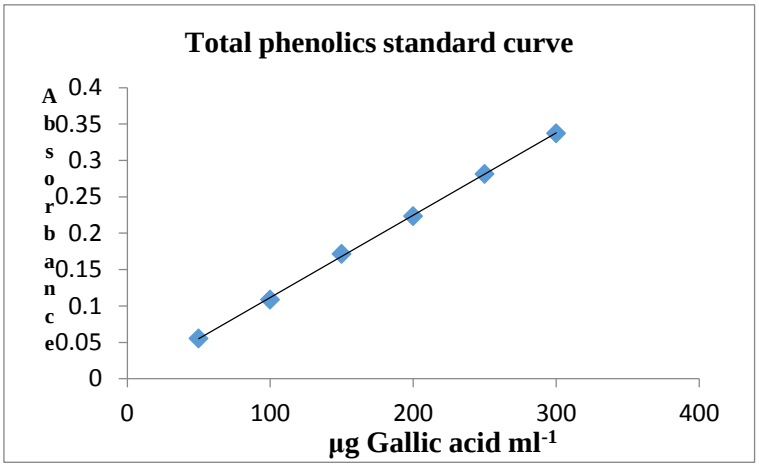

Figure 10. Gallic acid calibration curve.

Quercetin calibration curve

The calibration curve for total flavonoids calculation was performed using 50, 100, 150, 200 and 250 µg of Quercetin per ml^{-1} (R^2=0.992)

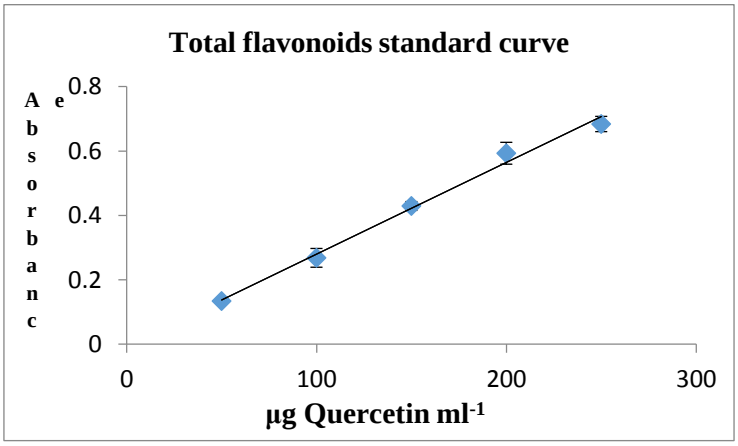

Figure 11. Quercetin calibration curve.

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APPENDIX 2.
CHAPTER IN ACS SERIES: *Opuntia spp*. AS A SOURCE OF BIOACTIVE COMPOUNDS

Opuntiaspp. as a source of bioactive compounds

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The genus *Opuntia* represents an important food and feed resource and gradually its economic importance has increased worldwide as a health-promoting food. *Opuntiaspp*. contains a number of potentially active nutrients and bioactive compounds with different therapeutic uses. Wild *Opuntia* species are a source of novel metabolites and cell culture system represents a potential renewable source of those compounds.

The cactus pear (*Opuntiaspp*.) represents the most symbolic native plant in Mexico that has been used since prehispanic times as documented in the Florentine Codex (Fig. 1). *Opuntia* species as well maize, amaranth and agave were important in the ancient agricultural economy and basic components of the diet (*1*).

Figure 1.*The Aztecs, led by the prophecies ofHuitzilopochtli(god ofsunand war), they ended their migrationthrough the construction ofTenochtitlanon an island ina lake wherean eagle witha snakeperched on acactusorcactusin bloom.*

The semiarid region of the central part of Mexico hosts the greatest diversity of this cactus in the world (*2*). The *Opuntia* genus includes 188 species, and 78 are native of Mexico (*3*).Most of wild and cultivated species are located in the Central and High Plateau zones distributed in an area of three 3 million hectareas (*4*). The most abundant wild species are *Opuntiastreptacantha*Lem.,*Opuntialeucotricha*DC., *Opuntiarobusta*H.L.Wendl., *Opuntiacantabrigiensis*Lynch, *O. rastrera*Weber, *Opuntiahyptiacantha* and *Opuntiachavena* (*5*).The cultivatedvarieties belongs to the domesticated *Opuntiaficus-indica* L. This specie is well adapted to extreme climate and edaphic conditions, growing in dry, hot climates of northern Mexico, south-western of the United States, Africa, Mediterranean countries and Europe.

Opuntia plants produceedible stems known as pads, vegetable, cladodes, nopales or pencas. The tender young part of the cactus stem, young cladode or "nopalito", is frequently consumed as vegetable in salads, while the cactus pear fruit is consumed as a

fresh fruit. In Mexico, 10,200 ha of*Opuntiaspp*. are cultivated for production of nopalito and 51,112 ha for cactus pear. Under optima conditions annual production can reach 50 tons of dry matter per hectare (*6*).

Cladodes chemical composition

Opuntiaspp.have a high nutritional value, mainly due to their mineral, protein, dietary fiber and phytochemical contents (*7*). The main constituent of *O. ficus-indica* cladodes is water (80-95%), followed by carbohydrates (3-7%), fiber (1-2%), and protein (0.5 – 1%).However, as shown in Table I, the chemical composition on dry weight of commercial and wild species depends on variety, maturation stage, environmental conditions and manufacturing techniques(*1,8*).

The carbohydrate fraction includes mucilaginous components containing polymers, such as chains of (1-4)-linked β-D-galactouronic acid and α(1-2)-linked L-rhamnose residues (*9*). Ginestra et al. (*10*) reported that glucose and galactouronic acid were the main sugars of *Opuntia* cladodes.

Therapeutic uses

Besides being a traditional source of vegetable, cladodes also have medical applications. The *O. ficus-indica* var. *saboten* is used for wounds, burns, edema, dyspepsia, and asthma in traditional medicine (*11-14*). The cladode of the plant is traditionally used to treat gastritis, intestinal colic and ulcers (*15*). The extracts of fruits and stems exhibit hypoglycemic (*16,17*), anti-allergic activities (*18*), antioxidant (*19*), and anti-inflammatory (*20*).

In Table II is shown a brief summary of some uses and the molecule that is claimed as responsible of the action.

Table I. Proximate composition of different commercial and wild *Opuntia* spp.

Variety	Component (%)				
	Protein	Fat	Fiber	Ash	CH
Blanco[1]	6.7	0.1	15.0	17.3	61.4
Amarillo[2]	15.1	0.6	6.2	15.9	63.2
Cristalino[2]	9.4	1.5	7.7	14.8	66.5
Duraznillo[2]	13.5	1.1	7.1	19.7	69.8
Tapon II[2]	17.4	1.8	20.4	19.5	42.4
Tempranillo[2]	13.4	nd	5.5	19.3	61.9
Tablets[3]	4.2	0	51.6	37.6	6.7

CH=Carbohydrates,nd=not detected, 1=domesticated specie, 2=wild species, 3=product found at commodity market. (*SOURCE: Modified from Reference8*).

Clinical studies led by report the effect of this plant on type 2 diabetic patients(*21-24*).Reports have suggested that the anti-hyperglycemic effect of Nopal may be due to its fiber and pectin content, which may decrease carbohydrate absorption (*25*). However it was confirmed that the plant and filter plant extracts from *O. streptacantha*, produce an anti-hyperglycemic effect on streptozotocin (STZ)-diabetic rats, then some other bioactive compounds more than fiber and mucilage are responsible of this beneficial activity (*24*). Also it was demonstrated that the petroleum ether extract from the edible part of *O. Milpa Alta* showed remarkable decrease of blood glucose levels, which may be a potential natural hypoglycemic functional ingredient (*26*).

A recent study by Godard et al. (*27*),have shown that the commercial preparation made from cladode extracts and fruit skins of *O. ficus-indica* (OpunDia™), was useful for the glucose lowering in blood. Other species such as *O. streptacantha* cladodes have been used as anti-diabetic foods (*28*).Theories, such as the stimulation in the insulin secretion observed in *O. ficus-indica* (*29*) should also applied to *O. streptacantha*; however, further studies

Table II. Therapeutic effecs of *Opuntiaspp.*

Specie	Effect	Plant organ	Extract	Metabolite	Model	Ref
O. humifusa	G1 arrest and proliferation inhibition	Cladode	Aqueous fraction	---	Glioblastoma-human cancer cell line (U87MG)	*36*
*O.ficus-indica*varMill.	Neuroprotective	Cladode /fruit	Ethyl acetate fraction	Flavonoids	Cortical cell cultures (from Sprague–Dawley rats)	*14*
*O.ficus-indica*varMill.	Hypoglycemic	Cladode	Raw cladode	---	STZ-induced diabetic rats	*22*
O. monacantha	Hypoglycemic	Cladode	Raw cladode	Polysaccharides	STZ-induced diabetic rats	*51*
O. Milpa Alta	Hypoglycemic.	Cladode	Petroleum ether fraction	---	STZ-induced diabetic rats	*26*
*O.ficus-indica*varMill.	Hypoglycemic	Cladode /Fruit	Raw cladode/fruit	---	Non-diabetic Wistar rats	*29*
O. streptacantha Lem.	Anti-hyperglycemic	Cladode	Raw cladode	Secondary metabolites	STZ-diabetic rats	*24*
*O.ficus-indica*varMill.	Antioxidant and antiulcerogenic	Fruit	Juice	Flavnoids	Male Wistar rats	*21*
*O.ficus-indica*varMill.	Cytoprotective	Cladode	Mucilage and pectin fractions	Mucilages, pectins	Male Wistar rats	*13*
Opuntia ficus indica f. inermis	Antioxidant and antiulcerogenic	Flower	Methanolic	Phenolics, flavonoids, polysaccharides	Male Wistar albino rats	*52*
*O.ficus-indica*varMill.	Antioxidant Antigenotoxic	Cladode	10 mM Tris–HCl homogenated	---	Balb/c mice	*53, 54*
*O.ficus-indica*varMill.	Chemopreventive	Cladode	Raw cladode	---	Balb/c male mice	*31*
*O.ficus-indica*varMill.	Healing	Cladode	Methanolic	β-sitosterol	Male ICR mice	*12*
*O.ficus-indica*varMill.	Antispasmodic	Fruit	Fresh juice	Indicaxanthin	Adult male mice (C57BL/10SnJ)	*55*
O. dillenii	Anti-diabetic	Cladode	Total polysaccharide extract	Polysaccharide (ODP)-Ia	STZ-induced diabetic mice	56
O. streptacantha	Hypoglycemic	Cladode	Fresh juice	Secondary metabolites	New Zealand adult male rabbits	*57*
O.robusta	Hypocholesterolemic Antioxidative	Cladode	Broiled cladode	---	Humans with hypercholesterolemia	*19*

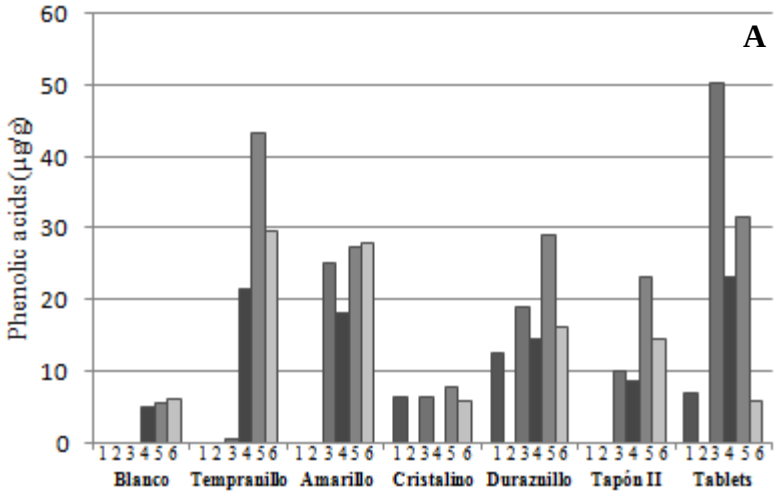
should be done toward the understanding of the molecular mechanisms of *Opuntia* extracts towards its anti-diabetic effects.

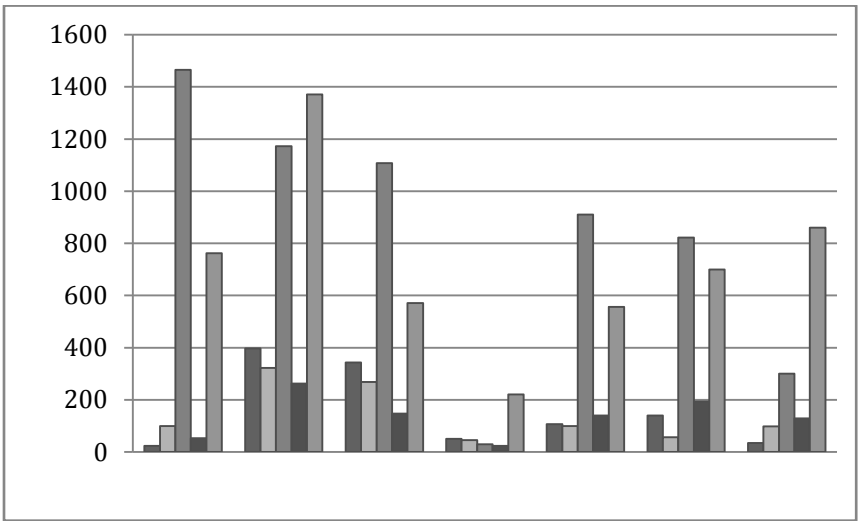
Also has been reported that *Opuntia* extracts have anti-tumoral (*30*) andhepatoprotective effects in mice (*31*). Park et al. (*32*) reported that the β-sitosterol as an active anti-inflammatory principle from the stem extract. The n-butanolicextracts of *O. ficus-indica* has beneficial effects on memory performance in mice (*33*).Two flavonoids (kaempferol and quercitin) were isolated from the *O.ficus-indica* var. *saboten*and demonstrated to exert a potent a potent antidepressant effect (*34*). An extract of the cactus plant *O.streptacantha* was able to inhibit the intracellular virus replication and toinactivate extracellular virus, but in this work, the active inhibitory components of the extract appeared to be protein in nature (*35*). The hexane, ethyl acetate, and water partitioned extracts from*O.humifusa*, were tested on proliferation, G1 arrest and apoptosis in U87MG human glioblastoma cells (*36*). In addition, *O. ficus-indica* fruits extracts have been found to increase apoptosis, and to inhibit the ovary-cancerous, vesicle, cervical and immortal epithelial cell growth. The inhibition has been shown to be doses-time dependent *(30).*

Phenolic and Flavonoids presents in *Opuntia* spp.

The use of natural phytochemicals present in fruits, vegetables, and herbs as antioxidants has increased. *Opuntia* seems to be a good source of those compounds (*37*). Then, the interest in the characterization of phytochemicals presents in cladodes and fruits has also increased, but most of the studies have been carried out using the *O.ficus-indica*commercial samples. Few studies have been focused on the characterization and quantification of phytochemicals using wild species (*8*). It was found six phenolic acids (Fig. 2A) and five flavonoids (Fig. 2B) in *Opuntia* samples which concentrations varies depending on the specie. Also, it was observed that processing affected the concentration of those compounds. Medina-Torres et al. (*38*) reported that using a

temperature drying of 45°C and an air flow rate of 3 m s^{-1} were the best conditions for bioactive compound preservation in Opuntia samples.





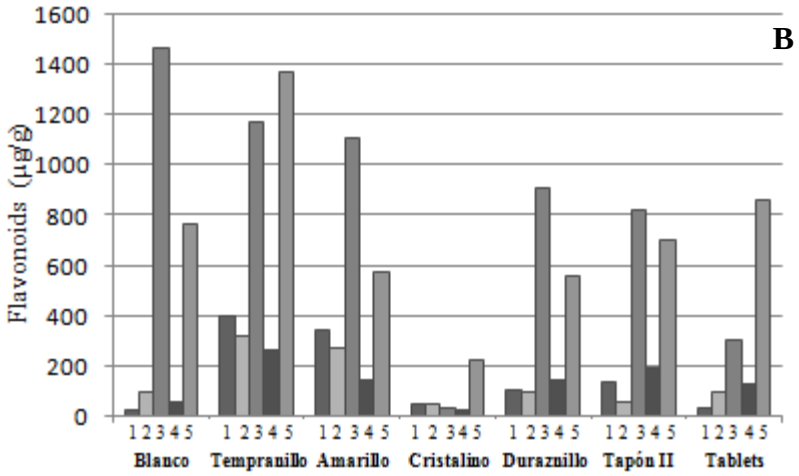


Figure 2.*A)Phenolic acids: 1=gallic, 2=coumaric, 3=3,4-dihydroxybenzoic, 4=4-hydroxybenzoic, 5=ferulic, 6= salicylic. B) Flavonoids: 1=isoquercitrin, 2=isorharmnetin,3-O-glucoside, 3=nicotiflorin, 4=rutin, 5=narcissin. (SOURCE: Modified from Reference 8).*

Some other works has ben focused in the characterization of novel compounds. Two alkaloids (indicaxanthin and neobetanin) and few flavonoids were isolated (*39-41*). As well, the isorhamnetin-3-*O*-(6"-*O*-*E*-feruloyl)-neohesperidoside, (6R)-9,10-dihydroxy-4,7-megastigmadien-3-one-9-*O*-β-D-glucopyranoside and (6S)-9,10-dihydroxy-4,7-megastigmadien-3-one-9-O-β-D-glucopyranoside were isolated from methanolic extract of *O.ficus-indica* var. *saboten* (*42*). Luo et al. (*43*) analyzed the petroleum ether extract of *O.Milpa Alta* by GC/MS, and reported the isolation of 26 compounds. The most abundant was phytosterol (30%), followed by polyunsaturated fatty acids (18.6%), phytol (12.1%), palmitic acid and palmitate (13.5%), vitamin E (4.5%) and other compounds (7.5%).

Cell culture systems for production of secondary metabolites

Plants are the tremendous source of new products with medicinal importance in drug development. Today several distinct biomolecules, mainly secondary metabolites (alkaloids, terpenoids, steroids, saponins, phenolic, flavonoids) and amino acids, derived from plants are important drugs, which are currently used in one or more countries in the world (*44*,*45*). Plant cell culture systems represent a potential renewable source of valuable medicinal, flavors, essences and colorants that cannot be produced by microbial cells or chemical syntheses (*44*). The isolation of the phytochemical can be rapid and efficient, when compared with extraction from complex whole plants. Thus, plant cell cultures represents an excellent biotechnological technique in production of secondary metabolites with therapeutical applications (*46*).

A protocol for the establishment of callus cultures from non-domesticated spcie*O.robusta* was developed (*47*). In this work, different disinfection procedures, type of plant growth regulators (Fig. 3), and culture media composition on callus development were evaluated.*O. robusta* callus induction was achieved on MS medium (Murashige and Skoog, 1962) containing 3 mg l^{-1}indol acetic acid, 1 mg l^{-1}picloram, and 1.2 mg l^{-1}kinetin.Addition of 0.4 g l^{-1}casein hydrolysate and 0.1 mg l^{-1} biotin to medium improved callus formation.

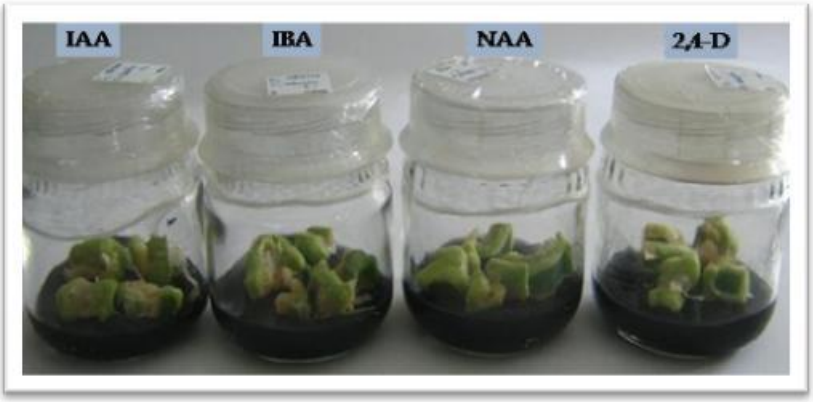


Figure 3*. Callus systems from O. robusta. IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; NAA, naphthalene acetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid (SOURCE: Reference 47).*

Accumulation of metabolites often occurs in plants subjected to stresses including various elicitors or signal molecules.It is well known that jasmonic acid (JA) and methyl jasmonate (MeJ) are signal molecules in biotic and abiotic stresses.Chitosan has been used to promoted the production of pigments in cactus *in vitro* cultures (*48*), taxol in *Taxuschinensis* (*49*), and phenolic acids in *Vitisvinifera* cell suspension cultures (*50*).

The effect of jasmonic acid and chitosan on phenolics compounds content in *O. robusta*cultures was also evaluated. The exposure of *O. robusta*callus to jasmonic acid increased 1.3-fold and 3-fold total phenolic acids and flavonoids concentration, respectively. Addition of chitosan to *O. robusta*cellular suspensions, also improve accumulation of total phenolic acids by 2.2 folds and flavonoids by 1.7 folds (Fig. 4).

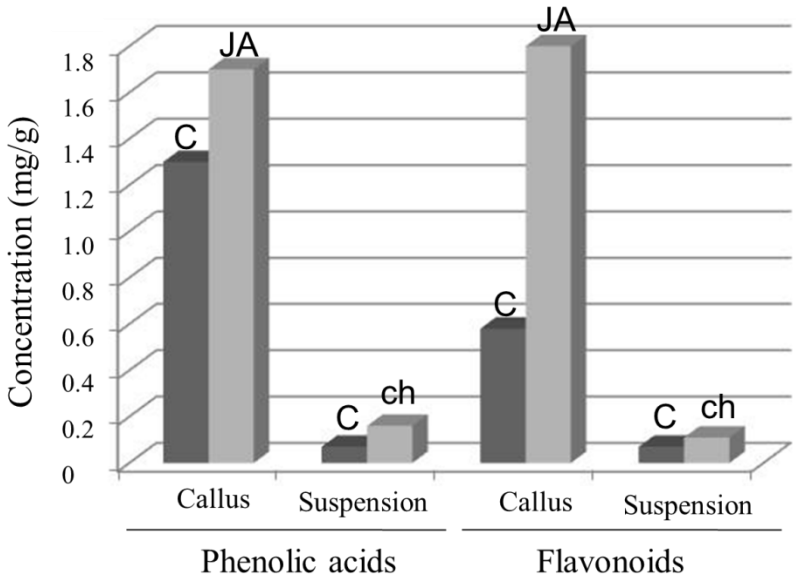


Figure 4. *In vitro cultures production of phenolic acidsand flavonoids.C, control samples; JA, induction with Jasmonic acid; ch, induction with chitosan.(SOURCE: Reference 47).*

Concluding remarks

Opuntiaspp.is a source of novel bioactive compounds that have a wide range of biological functions, among them the anti-diabetic effects are the most importants. However until date it is not clear which compounds is the responsible of the observed effects and further work must be done in order to get more insights abouth the molecular mechanisms of the action of *Opuntia* extracts.*In vitro* cell culture has been established and work must be done towards to obtain a large-scale bioreactor process for the production of valuable bioactive compounds in an active field.

Acknowledegments

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