Phytochemical Composition and in Vitro Analysis of Nopal (O. Ficus-Indica) Cladodes at Different Stages of Maturity

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ABSTRACT
This study aimed to determine the phytochemical profile and nutraceutical properties of nopal cladodes (Opuntia ficus-indica) at different stages of maturity. Medium-age cladodes showed the highest total saponins, phytosterols, and indigestible fiber, as well as the highest in vitro antioxidant capacity and digestive enzymes inhibitory activity. Furthermore, these cladodes presented the highest content of p-hydroxybenzoic acid, p-coumaric acid, rutin, narcissin, nicotiflorin, ß-sitosterol, and sitosteryl-3-ß-glucopyranoside, as well as several amino acids, organic acids, and fatty acids. Whereas young cladodes contained the highest concentration of condensed and hydrolyzable tannins. These results demonstrated that maturity affects the nutritional and nutraceutical properties of nopal cladodes.

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Introduction
Nopal (Opuntia ficus-indica) is a drought-tolerant plant cultivated in arid and semi-arid regions. This plant produces edible stems known as cladodes, which are widely consumed in Mexico. Interestingly, its production and consumption is extending to other countries due to its high nutritional and nutraceutical value, since nopal cladodes are rich in pectin, mucilage, vitamins, polyphenols, and minerals.[1]

Mexican traditional medicine widely recommends the consumption of nopal cladodes for the treatment of several diseases, one being diabetes. Several studies have reported the antidiabetic effect of Opuntia, for instance, the administration of steamed (Opuntia ficus indica) cladodes to diabetic patients reduced postprandial blood glucose and serum insulin peaks.[2] Similar results were obtained with Opuntia streptacantha, which decreased the hyperglycemic peak in healthy rabbits.[3]

These beneficial effects have been associated to several phytochemical compounds.[4] For instance, a petroleum ether extract of nopal cladodes exerted hypoglycemic effects in streptozotocin (STZ)-induced diabetic mice, which was related to their high content of phytosterols, polyunsaturated fatty acids, and phytol.[4] Furthermore, polysaccharides of O. monacantha cladodes improved blood glucose and serum lipid levels in STZ-induced diabetic rats.[5] Similarly, isorhamnetin glycosides-rich extract of nopal cladodes stimulated insulin secretion and improved blood lipid profile in obese mice, preventing the development of metabolic abnormalities associated to diet-induced obesity.[6]
The profile of phytochemicals and nutrients in plants is not only variety-dependent, it also depends on other factors such as cultivation conditions and physiological stage. For instance, older cladodes showed a higher content of insoluble fiber, whereas soluble fiber decreases with increasing age. Additionally, it has been reported that protein and starch decreases in mature cladodes, whereas simple carbohydrates increase.

We have previously evaluated the acute hypoglycemic effect of nopal cladodes at different maturity stages in healthy and diabetic rats, and small and medium cladodes (40–70 g) exerted the greatest beneficial effect on postprandial blood glucose as compare to large cladodes (293 g). This effect was associated to the higher fiber content in cladodes of early maturity stage, which increases the ability to form suspensions with higher viscosity, promoting glucose entrapment. Nevertheless, a nutrimental and phytochemical profile is necessary to understand the differential hypoglycemic effect of nopal cladodes at different maturity stages. Therefore, the aim of this study is to determine the indigestible fraction content, as well as the phytochemical and low molecular weight metabolite profile of nopal cladodes at different maturity stages, and to associate their bioactive composition with their antioxidant and digestive enzymes inhibitory capacities.

**Materials and Methods**

**Plant Material**

*Opuntia ficus-indica* var. Milpa Alta was used in this study, which is the cactus specie of major economic relevance in the world. Fresh cactus cladodes were collected at September 2012 from a commercial orchard located at El Refugio, Guanajuato, Mexico, and were identified by C. Mondragon Jacobo from the Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP). Cladodes were classified according to their age (days) and size (weight and length) as young (12 days, 40 ± 10 g), medium (20 days, 74 ± 20 g), and old (30 days, 293 ± 70 g). All cladodes were in a maturity stage adequate for human consumption. Cladodes were washed with distilled water and disinfected using commercial 10% sodium hypochlorite solution, and thorns were manually removed with a stainless steel knife. Afterward, cladodes were diced (about 2 × 2 cm) and dehydrated in a tray drier at 40°C for 24 h. The dried material was ground using a mill with a mesh size of 80 (particle size <0.18 mm). Nopal flours were stored in sealed containers at room temperature and protected from light and oxygen to prevent oxidation.

**Determination of Total Polyphenols and Flavonoids**

Polyphenols extraction was performed according to Hassan. Briefly, 0.5 g of dry sample were mixed with 20 mL of methanol/water acidified with HCl (50:50 v/v; pH 2), the mixture was shaken for 1 h at room temperature, centrifuged at 4000 × g for 10 min, and supernatants were recovered. Afterward, 20 mL of acetone/water (70:30 v/v) were added to the residue, and the mixture was shaken and centrifuged as previously described. Methanol and acetone extracts were mixed and used for the quantification of total polyphenols and total flavonoids. Results were expressed as milligrams of gallic acid equivalent/g of dry sample (mg GAE/g) and mg of catechin equivalent/g of dry sample (mg CE/g), respectively.

**Determination of Condensed Tannins**

Condensed tannins content was determined as described by Zurita using the residue obtained in the methanol/acetone extraction. Samples (100 mg) were mixed with 6 mL of 95:5 n-butanol/HCl and 0.2 mL of iron reagent (2% w/v ferric ammonium sulphate in 2 mol/L HCl). Mixtures were incubated in a boiling water bath for 50 min. Then, the mixture was adjusted to 25 mL with n-butanol/HCl, and absorbances were measured at 450 and 555 nm. Results were expressed as mg of proanthocyanidins equivalent/g of dry sample (mg PAE/g).
Determination of Hydrolyzable Tannins

The residue obtained in the methanol/acetone extraction (20 mg) was hydrolyzed with 2 mL of methanol and 200 μL of sulphuric acid (20 h, 85°C). Then, the methyl gallate released from the reaction was oxidized with potassium iodate (pH 5.5, 30°C), and the chromophore was measured at 525 nm. Results were expressed as mg of methyl gallate equivalent/g of dry sample (mg MGE/g).[15]

Determination of Total Phytosterols

Samples (1 g) were saponified with 15 M KOH in methanol (100 mL). This solution was heated at 80°C for 60 min. Then, 20 mL of water and 40 mL of hexane were added. Samples were centrifuged at 5000 × g during 5 min, and supernatants were recovered. Solvents were removed at 45°C with N₂, and then 80 mL of isopropanol were added. An aliquot (300 μL) was mixed with 3 mL of free cholesterol kit reagent, containing cholesterol oxidase and peroxidase (Spinreact, Spain). Samples were incubated for 5 min at 37ºC, and absorbances were measured at 505 nm. Results were expressed as mg of β-sitosterol equivalents/g of dry sample (mg SE/g).

Determination of Total Saponins

Dry samples (5 g) were defatted with 50 mL of petroleum ether, then the solvent was evaporated to complete dryness, and samples were dissolved in 150 mL of 75% ethanol. Samples were subjected to reflux at 70°C for 4 h, and then the extract was filtered and evaporated at 40°C. The dried residue was extracted three times with 40 mL of n-butanol, the solvent was evaporated to dryness, and samples were dissolved in 25 mL of methanol. Finally, an aliquot (50 μL) was added with 0.2 mL of 5% vanillin (8% w/v), and 0.8 mL of sulfuric acid (72% v/v). The mixture was incubated at 70°C for 15 min, and then cooled on ice. Then, 5 mL of glacial acetic acid were added; and absorbances were measured at 550 nm. Results were expressed as mg sitosteryl 3β-D-glucopyranoside equivalents/g of dried sample (mg SGE/g).

Identification of Phytochemical Compounds

The phytochemical profile of nopal cladodes was assessed using an Agilent 1200 high performance liquid chromatography (HPLC)-diode array detector (DAD) system connected to a SL quadrupole mass spectrometer Agilent 1100 equipped with an electrospray interface, using a Phenomenex C18 column (250 mm × 4.6 mm, 5 μm). Mass spectrometer was operated in negative ion mode, using the following conditions: capillary voltage, 4000 V; nebulizer pressure, 40 psi; drying gas flow rate, 10 L/min; gas temperature, 300°C; skimmer voltage, 50 V; octapole, 150 V; and fragmentor voltage, 130 V. LC-MS accurate mass spectra were recorded across the range of m/z 50–1200. Two different systems were used for the separation of phytochemicals compounds.[16]

System I (Phenolic Acids and Flavonoids)

Samples were extracted with acidified methanol/water as previously described. Then, solvents were evaporated to dryness, and samples were dissolved in 200 μL of methanol. The mobile phase consisted of (A) acetic acid-water 2:98 and (B) acetic acid-water-acetonitrile 2:48:50 under gradient conditions. Absorbances were monitored at 280 nm (hydroxybenzoic acids and flavonols) and 320 nm (hydroxycinnamic acids and stilbenes).

System II (Phytosterols and Saponins)

Dried samples (0.5 g) were extracted with 4 mL of hexane during 6 h, and then the mixture was centrifuged at 19,000 × g for 5 min. The hexanic phase was recovered, evaporated to dryness, and dissolved in 200 μL of acetonitrile. The mobile phase consisted of (A) acetonitrile-acetic acid-methanol-water 48:2:25:25 and B) acetonitrile-acetic acid 98:2 under gradient conditions. Absorbances were measured at 203 nm.
Identification of Low Molecular Weight Metabolites

Samples (10 mg) were mixed with 1 mL of methanol, sonicated for 15 min, and centrifuged at 12,000 × g at 4°C for 10 min. Supernatants were recovered, filtered with 0.45 μm pore membranes, and concentrated with a nitrogen gas stream. Then, samples were derivatized with 50 μL of BSTFA (N,O-bis[trimethylsilyl]trifluoroacetamide + 1% TMCS [trimethylchlorosilane]).

Samples (1 µL) were injected into an Agilent GC Series 7890A (Wilmington, DE, USA) coupled to an Agilent single quadrupole MS detector (Agilent 5975C), with electron energy set at 70 eV, recording across a mass range of 50–800 m/z. A HP-5MS capillary column (30 m × 0.25 mm, 0.25 μm) was used. The injector temperature was set at 250°C in split-less mode. Initial oven temperature was 100°C, held for 1 min and raised at 6°C/min to 220°C, which was held for 1.23 min, then raised at 10°C/min to 290°C, after raised at 40°C/min to 310°C and held for 7.5 min. The flow rate of the carrier gas (helium) was maintained at 1 mL/min. Preliminary compound identification was assessed with the National Institute of Standards and Technology (NIST) and Wiley electron impact mass spectral library search using the ChemStation (Agilent Technologies) software. Peaks with similarity index ≥70% were assigned compound names.

Determination of Non-Digestible Fraction

Soluble and insoluble indigestible fractions were determined following the procedure of Saura-Calixto. Briefly, 300 mg of each sample were digested with 0.2 mL of pepsin (300 mg/mL solution in HCl-KCl 0.2 M buffer, pH 1.5, 1 h, 37°C) and 1 mL of α-amylase (120 mg/mL solution in tris-maleate buffer 0.1 M, pH 6.9, 16 h, 37°C). Samples were centrifuged (3000 × g for 15 min) and residues were dried and quantified gravimetrically. Supernatants were dialyzed against water for 48 h at 25°C. Then, dialysates were lyophilized and weighed to determine the content of soluble indigestible fraction. Results were expressed as g of fraction/kg of dry sample.

Free Radical Scavenging Assays

DPPH* and ABTS** free radical scavenging activities were measured in the polyphenolic extract as described by Brand-Williams and Re, respectively. Results were expressed as half maximum inhibitory concentration (IC₅₀, μg/mL).

Digestive Enzyme Inhibition Assays

α-Amylase, α-glucosidase, and pancreatic lipase inhibitory activities were evaluated in the polyphenolic extract according to the methods described by Kandra, Apostolidis, and McDougall, respectively.

Statistical Analysis

All results were expressed as mean values ± standard error (SE). Data were analyzed by one-way analysis of variance (ANOVA) and differences among treatments were determined by comparison of means using Tukey’s test. The level of statistical significance was considered at p < 0.05. Pearson correlation was used to evaluate associations between phytochemicals, antioxidant capacity, and digestive enzymes inhibitory activity. All statistical analyses were carried out with JMP software (v11.0, SAS Institute).
Results and Discussion

Phytochemical Profile

Phytochemicals exert anti-hyperglycemic properties through several mechanisms, such as protection against oxidative damage and inhibition of carbohydrate and lipid digestion. Nevertheless, their content in plants depend of several factors such as variety, maturity, and cultivation conditions. Therefore, we evaluated the phytochemical profile of O. ficus-indica cladodes harvested at different maturity stages.

The highest content of polyphenols and flavonoids were found in medium age cladodes, whereas old cladodes showed the lowest content of total polyphenols. Interestingly, medium-age cladodes presented 13 and 40% more polyphenols, and 33 and 41% more flavonoids than young and old cladodes, respectively (Table 1). The variation of polyphenols in fruit and vegetables during maturity may be attributed to changes in primary and secondary metabolism. Two different trends have been observed in polyphenols; a steady decrease or increase during maturation. However, these effects vary from plant to plant or even in different organs of the same plant.

The phytochemical profile of nopal cladodes at different maturity stages was assessed by HPLC-DAD-mass spectrometer detector (MSD). Regarding polyphenols, 15 phenolic acids and 13 flavonoids were identified (Table 2, Fig. 1b), from which only 11 have been identified previously in nopal cladodes. Interestingly, different polyphenol profiles were observed in nopal cladodes harvested at different maturity stages. For instance, young and medium cladodes presented a higher content of several phenolic acids and flavonoids as compared to old cladodes, such as p-hydroxybenzoic acid (42%), p-coumaric acid (50%), ferulic acid (17%), rutin (33%), narcissin (31%), and nicotiflorin (37%). Moreover, several polyphenols were only identified in young and medium age cladodes, such as chlorogenic acid, protocatechuic acid, sinapic acid, rosmarinic acid, ellagic acid, procyanidins B1 and B2, gallocatechin gallate, and epicatechin gallate.

Interestingly, several compounds identified in cladodes of early maturation have been associated to beneficial health effects. For instance, chlorogenic acid has been reported to exert antidiabetic effects by delaying intestinal glucose absorption and inhibiting hepatic gluconeogenesis, whereas gallocatechin gallate and epicatechin gallate-rich green tea extracts decrease triglyceride and insulin resistance in type 2 diabetic patients. On the other hand, tannins are polyphenols of high molecular weight with several health beneficial properties. These compounds are classified into two groups according to their monomer composition: hydrolysable tannins formed by phenolic acids, and condensed tannins or proanthocyanidins formed by flavonoids. Interestingly, nopal cladodes of early stage maturation showed a higher content of condensed and hydrolysable tannins as compared to old cladodes (25–63 and 17–31%, respectively; Table 1). Similar results were reported by Qudsieh, who studied the effect of maturity stages on yellow cane, and found that tannins decreased rapidly during maturity, reaching a reduction of about 90%.

In addition to polyphenols, the health benefits of nopal cladodes can be attributed to other phytochemicals such as phytosterols and saponins. Phytosterols are compounds that present similar structures to...
cholesterol, which vary only in carbon side chains and/or the presence or absence of a double bond. On the other hand, saponins are compounds of high molecular weight formed by triterpene or steroid aglycones with one or more sugar chains. Studies have shown that phytosterols and saponins exert health beneficial effects by decreasing blood lipids, improving blood glucose response, among others mechanisms.

The highest concentration of phytosterols and saponins was found in medium age cladodes, whereas old cladodes presented the lowest content. Interestingly, medium age cladodes presented 7 and 30% more saponins, and 27 and 44% more phytosterols than young and old cladodes, respectively (Table 1, Fig. 1c).

### Table 2. Phytochemical profile of O. ficus-indica cladodes at different stages of maturity assessed by HPLC-DAD-MSD.

| Proposed compound                | Retention time (min) | m/z | Young       | Medium stage | Old   |
|---------------------------------|----------------------|-----|-------------|--------------|-------|
| **Phenolic acids**              |                      |     |             |              |       |
| Chlorogenic acid*               | 3.8                  | 100 | 1.5 ± 0.0a  | 2.1 ± 0.1a   | LDL   |
| Gallic acid*                    | 5.5                  | 169 | 4.0 ± 0.2b  | 5.6 ± 0.3b   | 8.1 ± 0.5a |
| Protocatechuic acid*            | 12.6                 | 153 | 3.5 ± 0.2a  | 3.4 ± 0.1a   | LDL   |
| Caftaric acid                   | 14.0                 | 311 | 1.1 ± 0.0b  | LDL          | 1.9 ± 0.1a |
| p-Hydroxybenzoic acid*          | 17.7                 | 137 | 19.9 ± 1.2a | 17.1 ± 1.1a  | 11.0 ± 0.7b |
| Cautaric acid                   | 20.9                 | 295 | 2.7 ± 0.1b  | 4.1 ± 0.3b   | 3.7 ± 0.3a |
| Caffeic acid*                   | 23.5                 | 179 | 6.9 ± 0.4b  | 8.9 ± 0.3b   | 13.5 ± 0.6a |
| Syringic acid                   | 25.5                 | 197 | 5.1 ± 0.3a  | 6.1 ± 0.2b   | 6.3 ± 0.3a |
| p-Coumaric acid*                | 31.4                 | 163 | 28.5 ± 1.2b | 39.3 ± 1.5b  | 14.1 ± 0.7c |
| Sinapic acid*                   | 32.6                 | 223 | 8.1 ± 0.4a  | 12.3 ± 0.6a  | LDL   |
| Ferulic acid*                   | 33.0                 | 193 | 41.8 ± 2.0b | 50.3 ± 2.4a  | 33.9 ± 2.1c |
| Rosmarinic acid*                | 34.3                 | 360 | 10.3 ± 0.8b | 15.1 ± 0.7a  | LDL   |
| **Flavonoids**                  |                      |     |             |              |       |
| Procyanidin B1                  | 13.4                 | 577 | 3.7 ± 0.2b  | 5.6 ± 0.3a   | LDL   |
| Catechin*                       | 17.6                 | 289 | 8.1 ± 0.4c  | 12.1 ± 0.5b  | 16.5 ± 0.5a |
| Procyanidin B2                  | 21.2                 | 577 | 1.4 ± 0.1a  | 0.9 ± 0.1b   | LDL   |
| Epicatechin*                    | 25.6                 | 289 | 3.8 ± 0.3b  | 2.8 ± 0.2b   | LDL   |
| Rutin*                          | 35.1                 | 609 | 15.4 ± 0.6b | 19.2 ± 0.8a  | 10.4 ± 0.5c |
| Gallocaftaric gallate*          | 38.5                 | 457 | 9.4 ± 0.4a  | 8.9 ± 0.5a   | LDL   |
| Epigallocatechin gallate*       | 39.9                 | 457 | 12.3 ± 0.7ab | 15.4 ± 0.7a  | 9.9 ± 0.4b |
| Epicatechin gallate             | 41.5                 | 441 | 6.9 ± 0.3a  | 7.2 ± 0.3a   | LDL   |
| Quercetin-3-O-galactoside*      | 49.6                 | 463 | 12.0 ± 0.7b | 15.3 ± 0.8b  | 24.1 ± 1.2a |
| Quercetin-3-O-glucoside*        | 50.2                 | 463 | 27.1 ± 1.3b | 37.1 ± 1.8a  | 34.5 ± 1.5a |
| Coumestrol                      | 55.7                 | 268 | 3.5 ± 0.1a  | 2.9 ± 0.2b   | 3.5 ± 0.2a |
| Narassin                        | 60.3                 | 525 | 4.5 ± 2.0a  | 53.1 ± 2.2a  | 31.9 ± 1.6b |
| Nicotiflorin                    | 64.5                 | 595 | 35.1 ± 1.3a | 30.4 ± 1.5b  | 22.4 ± 1.0b |
| **Phytosterols**                |                      |     |             |              |       |
| β-Sitosterol                    | 3.6                  | 414 | 25.1 ± 1.2b | 36.9 ± 1.8a  | 12.7 ± 0.9c |
| β-Campesterol                   | 4.2                  | 400 | 5.6 ± 0.3a  | 5.8 ± 0.4a   | LDL   |
| Campestanol                     | 4.6                  | 402 | 9.1 ± 0.5a  | 7.8 ± 0.4a   | 2.3 ± 0.1b |
| Δ5-Avenasterol                  | 5.3                  | 412 | 8.8 ± 0.4a  | 9.3 ± 0.6a   | 3.7 ± 0.2b |
| Δ7-Stigmasterol                 | 5.8                  | 412 | 12.0 ± 0.7a | 15.4 ± 1.0a  | 7.4 ± 0.4b |
| Stigmastanol                    | 8.1                  | 416 | 19.3 ± 1.2a | 17.9 ± 1.4a  | 4.9 ± 0.3b |
| **Saponins**                    |                      |     |             |              |       |
| Campesterol 3β-D-glucopyranoside| 85.5                 | 562 | 23.9 ± 1.7a | 19.5 ± 1.3a  | 10.3 ± 0.6b |
| Stigmasteryl 3β-D-glucopyranoside| 88.7               | 574 | 25.6 ± 1.9a | 21.8 ± 1.4a  | 15.6 ± 1.0b |
| Sitosterol 3β-D-glucopyranoside | 92.9                 | 576 | 39.8 ± 2.3b | 48.1 ± 2.6a  | 29.4 ± 1.9c |
| Isorhamnetin 3-O-rutinoside      | 98.9                 | 625 | 45.2 ± 2.0a | 40.7 ± 1.7a  | 21.8 ± 1.3b |

Results are the average of three independent determinations ± SE.
Data is presented as areas under the curve (mA).
*Confirmation in comparison with authentic standards.
LDL: lower than detection limit.
Similar results were reported by Lima,\textsuperscript{[33]} who reported that steroidal saponins in \textit{Brachiaria} spp. decreased with maturation, whereas Le Fur\textsuperscript{[34]} found that young grape berries presented a higher content of phytosterols than older fruits. Decreased phytosterols content in old cladodes may be related to their conversion into steroidal hormones and vitamins, which regulate growth and development of immature tissues in plants.\textsuperscript{[35]}

Six phytosterols were identified in nopal cladodes, such as β-sitosterol, Δ7-stigmasterol, and stigmastanol (Table 2). Interestingly, this is the first study that identify campesterol in nopal cladodes. Regarding the maturity stage, young and medium cladodes presented the highest amount of all individual phytosterols. Furthermore, medium cladodes showed a higher content of β-sitosterol as compared to young and old cladodes (32 and 66%, respectively). Interestingly, β-campesterol was identified only in nopal cladodes harvested at early maturity stages.

Similarly, Luo\textsuperscript{[4]} tentatively identified sitosterol and stigmastanol in a petroleum ether extract of \textit{O. ficus-indica} var. Milpa Alta; however, the effect of maturity on the phytosterol profile of nopal cladodes has not been evaluated previously. The greater antidiabetic effect of nopal cladodes harvested at early maturity as compared to old cladodes\textsuperscript{[10]} may be related to their higher content of phytosterols, since Luo\textsuperscript{[4]} reported that a phytosterol-rich extract of \textit{Opuntia} cladodes decreased blood glucose levels in STZ-induced diabetic mice.

Additionally, four steroid saponins were identified in nopal cladodes, campesteryl-, stigmasteryl-, and sitosteryl-3-β-glucopyranoside, and isorhamnetin-3-O-rutinoside, which were found in greater amount in young and medium age cladodes (Table 2). Interestingly, the presence of saponins has been reported in a methanolic extract of \textit{O. monocantha} cladodes;\textsuperscript{[36]} nevertheless, the profile of these bioactive compounds has not been reported. Several studies have suggested that saponins exert several health benefits such as anti-carcinogenic, anti-hyperglycemic, and hypolypidemic effects.\textsuperscript{[31]}

### Low Molecular Weight Metabolites Profile

In addition to phytochemicals, nutritional compounds are affected during plants maturation.\textsuperscript{[37]} Therefore, the profile of low molecular weight metabolites, such as amino acids, carbohydrates, organic acids, and fatty acids of nopal cladodes was assessed by GC-MSD (Table 3, Fig. 1a). Several studies have reported that young nopal cladodes present high levels of several amino acids, such as glutamine, serine, and valine.\textsuperscript{[38]} However, the amino acid profile of older cladodes has not been reported previously.

In this study, 12 amino acids were identified in nopal cladodes (Table 3). Interestingly, young and medium cladodes showed several amino acids that were not detected in old cladodes, such as alanine, isoleucine, and asparagine, whereas threonine was detected only in old cladodes. Furthermore, cladodes harvested at early maturation stages presented a higher content of most amino acids than older cladodes. Similarly, it has been reported that young nopal cladodes present a higher amount of protein than older cladodes,\textsuperscript{[10]} which may be related to a higher metabolic activity in early maturity stages. In addition, nitrogen transport occurs from mature to young tissues.\textsuperscript{[39]}

Regarding carbohydrates profile, two compounds were identified in nopal cladodes, glucose and arabinose (Table 3). Accordingly, Nobel\textsuperscript{[40]} reported arabinose as the main sugar of \textit{O. ficus indica} mucilage. Interestingly, arabinose was detected in higher amounts in old cladodes, whereas glucose was found in greater amount in young cladodes (Table 3). The rate of accumulation of these carbohydrates in plants relays on photosynthesis and respiration, which are influenced by several factors among them, growth and development.\textsuperscript{[41]}

On the other hand, nine organic acids were identified in nopal cladodes (Table 3). Feitosa\textsuperscript{[42]} reported malic acid as the majoritarian organic acid of nopal cladodes. Young cladodes presented several organic acids that were not identified in medium and old cladodes, such as malonic acid, lauric acid, and tartaric acid. Furthermore, young and medium cladodes presented a greater amount of glycolic acid, threonic acid, and citric acid as compared to old cladodes (25–44, 41–52, and 65–82%, respectively). Similar results were reported by Stintzing\textsuperscript{[58]} who found a lower content of malonic, citric, and tartaric acid in old cladodes. Interestingly, medium and old cladodes showed a
higher amount of succinic acid and malic acid as compared to young cladodes (up to 19 and 22%, respectively).

It has been reported that organic acids are decreased in plants during advanced maturation stages due to an increased membrane permeability, allowing acids to be stored in respiring cells. Furthermore, altered membrane permeability reduces organic acids translocation from leaves, as well as their synthesis.\[43\]

Organic acids are primary metabolites that have been reported to exert beneficial effects against several oxidative diseases, such as diabetes, due to their antioxidant properties.\[44\]

Additionally, six fatty acids were identified in nopal cladodes, mainly 16 and 18 carbon-length compounds (Table 3). Young and medium cladodes presented a higher amount of palmitic acid as compared to old cladodes (22–26%). Interestingly, linoleic and α-linolenic acids were only identified in young and medium cladodes. Conversely, medium and old cladodes presented a higher amount of myristic and oleic acid as compared to young cladodes (3.7- and 1.8-fold, respectively). Accordingly, it has been reported that fat content decreases with increasing maturity.\[9\]
Indigestible Fraction Content

Nopal cladodes represent an important source of dietary fiber, mainly insoluble, which are considered partly responsible of nopal beneficial effects.\[^9\] Recently, the quantification of dietary indigestible fraction (DIF) has been proposed as substitute for dietary fiber, since DIF includes dietary fiber and other components that reach colon, such as resistant starch, resistant proteins, tannins, and other associated compounds. Altogether, these compounds show resistance to the action of digestive enzymes and contribute to the health beneficial effects reported to dietary fiber.\[^17\]
All nopal cladodes presented a higher content of insoluble DIF as compared to soluble DIF (Table 4). Interestingly, young and medium cladodes presented a higher content of soluble and insoluble DIF as compared to old cladodes (11–20 and 12–13%, respectively). It has been reported that the insoluble moiety reduces postprandial glycemia by several mechanisms, such as reduction of digestion rate and carbohydrates absorption. Furthermore, when DIF reaches colon, it serves as a substrate for fermentative microflora, producing short-chain fatty acids. These compounds have been reported to improve insulin sensitivity and to decrease hepatic glucose production.\(^{[45]}\) On the other hand, DIF decreases cholesterol intestinal absorption by linking with biliary acids.\(^{[46]}\)

**Antioxidant Capacity**

Antioxidant capacity is mainly related to compounds with the ability to counteract free radicals formation. ABTS and DPPH are assays widely used to determine foods antioxidant capacity, and these assays evaluate their ability to donate electrons and hydrogen atoms, respectively.\(^{[47]}\) It has been proposed that foods and raw plant extracts with IC\(_{50}\) values <50 µg/mL exert a high antioxidant capacity.\(^{[48]}\) Therefore, nopal cladodes could be considered with high antioxidant capacity (Table 4). Interestingly, nopal cladodes exhibited a higher capacity to donate electrons than hydrogen atoms, since lower IC\(_{50}\) values were observed in ABTS assay.

The antioxidant capacity of nopal cladodes may be related to their phytochemical composition; therefore, correlations between bioactive compounds and antioxidant capacity were assessed (Table 5). Total polyphenols presented a significant correlation with 2,2’-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay, whereas total flavonoids were correlated with 2,2- diphenyl-1-picrylhydrazyl (DPPH) and ABTS assays. Interestingly, no significant correlation was found between the antioxidant capacity and tannins. Conversely, it has been reported that tannins show a high radical scavenging activity due to their high number of hydroxyl groups.\(^{[49]}\)

Furthermore, nopal antioxidant capacity was correlated with several individual phenolic acids (chlorogenic, caftaric, coutaric, p-coumaric, sinapic, ferulic, and rosinmaric acids) and flavonoids (procyanidins B1, epicatechin gallate, rutin, epigallocatechin gallate, quercetin-3-O-glucoside, coumestrol, and narcissin). Additionally, several phytosterols, such as β-sitosterol and stigmasterol, and one saponin, sitosteryl 3β-glucopyranoside, were correlated with ABTS scavenging activity. Interestingly, several phytochemicals that showed a significant correlation with antioxidant capacity were found in greater concentration in young and medium cladodes.

| Parameter | Young (g/Kg) | Medium (g/Kg) | Old (g/Kg) |
|-----------|-------------|--------------|------------|
| Indigestible fraction | 325.6 ± 9.2 | 365.3 ± 0.4 | 291.3 ± 0.0 |
| Insoluble indigestible fraction | 54.6 ± 0.2 | 55.4 ± 0.4 | 48.3 ± 0.0 |
| Soluble indigestible fraction | 24.8 ± 1.2 | 22.7 ± 2.0 | 25.4 ± 0.9 |
| Antioxidant capacity (IC\(_{50}\), µg/mL) | 48.1 ± 1.5 | 42.3 ± 2.5 | 46.1 ± 3.1 |
| DPPH- | 28.7 ± 1.6 | 21.3 ± 0.9 | 22.1 ± 0.6 |
| ABTS+ | 15.6 ± 1.2 | 18.4 ± 1.3 | 25.4 ± 1.5 |

Results are the average of three independent determinations ± SE. \(^{a,b,c}\)Different letters in each row indicate significant differences (p < 0.05, Tukey’s test).


Table 5. Correlations between phytochemicals, antioxidant capacity, and digestive enzymes inhibitory activity O. ficus-indica cladodes at different stages of maturity.

| Phytochemicals | DPPH  | ABTS  | Amylase | Glucosidase | Lipase |
|----------------|-------|-------|---------|-------------|--------|
| Total phenols  | -0.471| -0.868| -0.022  | 0.111       | -0.869 |
| Total flavonoids| -0.872| -0.999| -0.557  | -0.441      | -0.466 |
| Condensed tannins| 0.667 | 0.170 | 0.932   | 0.972       | -0.763 |
| Hydrolizable tannins| 0.368 | -0.182| 0.748   | 0.830       | -0.939 |
| Chlorogenic acid| -0.442 | -0.851| 0.011   | 0.143       | -0.885 |
| Gallic acid     | -0.218 | 0.333 | -0.636  | -0.733      | 0.981  |
| Protocatechuic acid| -0.152 | -0.653| 0.311   | 0.435       | -0.984 |
| Caftaric acid   | 0.712  | 0.976 | 0.317   | 0.188       | 0.683  |
| p-Hydroxybenzoic acid| 0.135 | -0.412| 0.568   | 0.673       | -0.994 |
| Caffeic acid    | -0.122 | 0.423 | -0.558  | -0.663      | 0.995  |
| Syringic acid   | -0.655 | -0.154| -0.926  | -0.968      | 0.773  |
| p-Coumaric acid| -0.580 | -0.924| -0.149  | -0.016      | -0.799 |
| Sinapic acid    | -0.497 | -0.882| -0.051  | 0.082       | -0.854 |
| Ferulic acid    | -0.661 | -0.958| -0.250  | -0.119      | -0.732 |
| Rosmarinic acid | -0.474 | -0.869| -0.025  | 0.108       | -0.868 |
| Cinamic acid    | -0.307 | 0.245 | -0.704  | -0.792      | 0.960  |
| Ellagic acid    | -0.256 | -0.730| 0.209   | 0.337       | -0.960 |
| Vanillic acid   | -0.411 | 0.136 | -0.778  | -0.855      | 0.922  |
| Procyanidin B1  | -0.495 | -0.881| -0.048  | 0.085       | -0.856 |
| Catechin        | -0.393 | 0.323 | -0.709  | -0.796      | 0.958  |
| Procyanidin B2  | 0.182  | 0.368 | 0.607   | 0.707       | -0.988 |
| Epicatechin     | -0.903 | -0.537| -0.996  | -0.988      | 0.454  |
| Rutin           | -0.583 | -0.925| -0.153  | -0.020      | -0.797 |
| Gallocafechin gallate | -0.130 | -0.636| 0.332   | 0.455       | -0.988 |
| Epigallocatechin gallate | -0.699 | -0.972| -0.301  | -0.171      | -0.696 |
| Epicatechin gallate | -0.212 | -0.699| 0.252   | 0.378       | -0.971 |
| Quercetin-3-O-galactoside | -0.090 | 0.453| -0.530  | -0.638      | 0.998  |
| Quercetin-3-O-glucoside | -0.901 | -0.534| -0.995  | -0.988      | 0.457  |
| Coumestrol      | 0.941  | 0.977 | 0.686   | 0.583       | 0.314  |
| Narcissin       | -0.495 | -0.881| -0.049  | 0.084       | -0.855 |
| Nicotiflorin    | 0.196  | -0.354| 0.618   | 0.717       | -0.985 |
| Total phytosterols| -0.739 | -0.984| -0.355  | -0.227      | -0.654 |
| β-Sitosterol    | -0.634 | -0.948| -0.216  | -0.084      | -0.756 |
| β-Campesterol   | -0.206 | -0.694| 0.258   | 0.384       | -0.973 |
| Campestanol     | 0.004  | -0.528| 0.455   | 0.570       | -0.995 |
| Δ5-Avenasterol  | -0.255 | -0.729| 0.209   | 0.337       | -0.960 |
| Δ7-Stigmasterol | -0.577 | -0.922| -0.145  | -0.012      | -0.801 |
| Stigmastanol    | -0.089 | -0.604| 0.371   | 0.491       | -0.993 |
| Total saponins  | -0.398 | -0.824| 0.060   | 0.192       | -0.907 |
| Campesteryl 3β-D glucopyranoside | 0.145 | -0.402| 0.576   | 0.680       | -0.993 |
| Stigmasteryl 3β-D-glucopyranoside | 0.207 | -0.344| 0.627   | 0.725       | -0.984 |
| Sitosterol 3β-D-glucopyranoside | -0.594 | -0.930| -0.166  | -0.034      | -0.788 |
| Isofermentin 3-O-rutinoside | 0.005 | -0.527| 0.456   | 0.571       | -0.995 |

Bold values indicate significant correlation by Pearson’s test.

**Digestive Enzymes Inhibitory Activity**

Several phytochemicals identified in nopal cladodes, such as polyphenols and saponins, have been reported to inhibit digestive enzymes involved in the breakdown of lipids and carbohydrates, leading to positive effects on obesity and blood glucose control.[50,51] Therefore, the capacity of nopal cladodes to inhibit carbohydrate digestive enzymes, such as α-amylase and α-glucosidase, was evaluated. Pancreatic α-amylase is involved in starch hydrolysis to oligosaccharides, which are further hydrolyzed to glucose and other monosaccharides by intestinal α-glucosidases. Therefore, the inhibition of these enzymes may result in the delay of glucose intestinal digestion and absorption.[52]
All nopal cladodes showed inhibitory activity against both carbohydrate digestive enzymes, presenting a maximum inhibition of 55–65% against α-amylase and ~25% against α-glucosidase (data not shown), presenting IC\textsubscript{50} values of 13.5–28.7 µg/mL (Table 4). Interestingly, medium and old cladodes showed a greater inhibitory activity against α-amylase and α-glucosidase, as observed in lower IC\textsubscript{50} values than young cladodes (26–37 and 30–35%, respectively; Table 4). Acarbose, a drug widely used to inhibit these digestive enzymes, showed a maximum inhibition of 81 and 89%, respectively, presenting IC\textsubscript{50} values of 5.4 and 3.9 µg/mL, respectively (data not shown). Therefore, nopal cladodes exhibit a low inhibitory activity against these enzymes as compared to acarbose.

Becerra-Jiménez\cite{53} reported that water, water–ethanol, and acidified ethanol extracts of \textit{O. streptacantha} cladodes showed no inhibitory activity against α-glucosidase. These discrepancies may be related to the use of different plant species, growth conditions, and maturity stage during harvest, since these parameters affect the content and composition of bioactive metabolites.\cite{7,54}

Nopal cladodes inhibitory activity against pancreatic lipase was also evaluated. This enzyme hydrolyzes dietary triglycerides into fatty acids, which are further absorbed. Therefore, its inhibition may lead to decreased serum triglyceride levels.\cite{22} All nopal cladodes showed an inhibitory activity against pancreatic lipase, with IC\textsubscript{50} values of 16–25 µg/mL (Table 4), presenting a maximum inhibition of 38–45% (data not shown). This is the first study that reports the ability of nopal cladodes to inhibit this digestive enzyme. Interestingly, young and medium cladodes exhibited a higher capacity to inhibit this enzyme as compared to old cladodes (38 and 54%, respectively). Orlistat is a drug widely used to inhibit lipid absorption through lipase activity inhibition, and showed a maximum inhibition of 86% and IC\textsubscript{50} values of 9.06 µg/mL (data not shown). Therefore, nopal cladodes exert a low inhibitory activity against this enzyme as compared to orlistat.

The inhibitory activity of nopal cladodes against α-amylase and α-glucosidase showed a significant correlation with condensed tannins content, as well as several individual phenolic acids and flavonoids, such as coutaric acid, syringic acid, epicatechin, and quercetin-3-O-glucoside (Table 5). Regarding pancreatic lipase inhibition, significant correlations were observed with hydrolizable tannins, and most phenolic acids and flavonoids identified in this study. Furthermore, several phytosterols, such as β-campesterol, campestanol, Δ5-avenasterol, and stigmastanol, as well as all saponins identified in this work, showed a significant correlation with pancreatic lipase inhibition assay (Table 5).

**Conclusion**

The maturity stage of nopal (\textit{O. ficus-indica}) cladodes at harvest plays an important role in their nutrimental and phytochemical profile, which may be reflected in their health beneficial properties. Medium age nopal cladodes (20-days-old) showed a high inhibitory activity against α-amylase, α-glucosidase, and pancreatic lipase, which was related to their content of several polyphenols, phytosterols and saponins. On the other hand, young cladodes showed the highest concentration of tannins, as well as amino acids, organic acids, and fatty acids, whereas old cladodes showed the lowest content of bioactive compounds and exerted a low antioxidant capacity and \textit{in vitro} digestive enzymes inhibitory activity. Therefore, this study could be useful to determine the optimal maturity stage to harvest nopal cladodes to increase their nutraceutical potential.

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