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FACULTAD DE FARMACIA Y NUTRICIÓN

Cactus (*Opuntia ficus-indica* Mill.) cladodes as a dietary source of bioaccessible (poly)phenols: effect of heat treatment, gastrointestinal digestion and human gut microbiota action, and bioactivity in colon

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Elsy Gabriela De Santiago Castanedo

Pamplona, diciembre de 2018

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Memoria presentada por **Dña. Elsy Gabriela De Santiago Castanedo** para aspirar al grado de Doctora por la Universidad de Navarra.

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En Pamplona, diciembre de 2018

Dra. Mª Paz de Peña Fariza

Dra. Mª de la Concepción Cid Canda

Facultad de Farmacia y Nutrición

Departamento de Ciencias de la Alimentación y Fisiología

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Todos tenemos sueños, pero para convertirlos en realidad, se necesita determinación, esfuerzo y dedicación.

Jesse Owens

ABSTRACT

Cactus (*Opuntia ficus-indica* Mill.) cladodes, commonly eaten as a fresh or cooked vegetable, present a great amount of (poly)phenolic compounds which can be modified by cooking treatments, gastrointestinal digestion and the human gut microbiota. Likewise, (poly)phenols could be able to exert their biological activity into the colon cells. Therefore, the main aim of this work was to evaluate the impact of heat treatment (boiling, microwaving, griddling, and frying in olive and soybean oils) on nutritional composition, antioxidant capacity and (poly)phenols profile of cactus cladodes, as well as their bioaccessibility after an *in vitro* gastrointestinal digestion and colonic fermentation. Moreover, the potential biological activity of colonic fermented cactus cladodes in HT29 colon cells was assessed. Previously, the extraction method of (poly)phenolic compounds with several solvents and conditions was optimized, selecting a combination of methanol, acetone and water as the best one. In addition, acid hydrolysis conditions were also optimized in order to identify and quantify (poly)phenol aglycones by HPLC-DAD analysis.

Culinary processes, except boiling, increased soluble and insoluble fibre up to 5.0 g/100 g, becoming cactus cladodes a good fibre source. Likewise, heat treatments increased the total phenolic content measured by Folin-Ciocalteu in cactus cladodes, except in boiled ones due to the leaching losses. A total of 45 (poly)phenols were identified and quantified in raw and cooked cactus cladodes by UHPLC-PDA-HR-MS, predominating flavonoids (60−68% total), mainly isorhamnetin derivatives, and phenolic acids (32−40%) with eucomic acids as the predominant ones. Microwaving (1.4-fold) and griddling (1.2-fold) increased the total amount of compounds while both frying (0.6-fold) and boiling (0.9-fold) produced a decrease. Additionally, antioxidant capacity was measured, which was also increased after cooking treatments applied.

Gastrointestinal digestion significantly ($p<0.05$) decreased (poly)phenolic compounds showing flavonoids a higher retention or degradation (37−63% bioaccessibility) than phenolic acids (56−87% bioaccessibility). Furthermore, gastrointestinal digestion induced isomerizations among piscidic and eucomic acids. The human gut microbiota also produced a higher degradation in flavonoids than in phenolic acids. However, some compounds still remained after 24 h of colonic fermentation, being eucomic acid as the most relevant. Cactus cladodes after the action of colonic microbiota showed an antigenotoxic effect on HT29 colon cells, reducing the H_2O_2-induced DNA damage.

RESUMEN

El nopal (*Opuntia ficus-indica* Mill.), comúnmente consumido como vegetal fresco o cocinado, presenta una gran cantidad de compuestos (poli)fenólicos que pueden ser modificados por los tratamientos culinarios, así como por la digestión gastrointestinal y la acción de la microbiota intestinal. Asimismo, los (poli)fenoles podrían ejercer su actividad biológica en las células de colon. Por lo tanto, el objetivo principal de este trabajo fue evaluar el impacto del tratamiento térmico (hervido, al microondas, a la parrilla y fritura en aceites de oliva y de soja) sobre la composición nutricional, la capacidad antioxidante y el perfil de los compuestos (poli)fenólicos en el nopal, así como su bioaccesibilidad después de la digestión gastrointestinal *in vitro* y fermentación colónica. Además, se evaluó la potencial actividad biológica del nopal posterior a la fermentación colónica en células de colon HT29. Previamente, se optimizó el método de extracción de compuestos (poli)fenólicos con distintos solventes y condiciones, seleccionando una combinación de metanol, acetona y agua como el mejor. Además, se optimizaron las condiciones de hidrólisis ácida con el fin de identificar y cuantificar las agliconas de los (poli)fenoles mediante el análisis por HPLC-DAD.

Los procesos culinarios, excepto el hervido, incrementaron la fibra soluble e insoluble hasta 5.0 g/100 g, siendo el nopal una buena fuente de fibra. Los tratamientos térmicos también incrementaron el contenido total en compuestos fenólicos medido por Folin-Ciocalteu en el nopal cocinado, excepto en el hervido, debido a las pérdidas por lixiviación. Un total de 45 (poli)fenoles fueron identificados y cuantificados en el nopal crudo y cocinado por UHPLC-PDA-HR-MS, predominando los flavonoides (60-68% en total), principalmente derivados de isorhamnetina, así como ácidos fenólicos (32-40%) siendo los ácidos eucómicos los mayoritarios. La cocción al microondas (1.4 veces) y a la parrilla (1.2 veces) aumentaron la cantidad total de compuestos, mientras que la fritura (0.6 veces) y el hervido (0.9 veces) produjeron una disminución. Se evaluó también la capacidad antioxidante, la cual se incrementó después de los tratamientos de cocción aplicados.

La digestión gastrointestinal disminuyó significativamente ($p<0.05$) los compuestos (poli)fenólicos, mostrando una mayor retención o degradación de los flavonoides (37-63% bioaccesibilidad) que de los ácidos fenólicos (56-87% bioaccesibilidad). Además, la digestión gastrointestinal indujo isomerizaciones en los ácidos piscídico y eucómico. La microbiota intestinal produjo también una mayor degradación en los flavonoides que en los ácidos fenólicos. Sin embargo, algunos polifenoles aún permanecieron después de 24 h de fermentación colónica, siendo el ácido eucómico el más relevante. El nopal

después de la acción de la microbiota intestinal, mostró un efecto antigenotóxico sobre las células de colon HT29, reduciendo el daño en el ADN inducido por H_2O_2.

LIST OF ABBREVIATIONS

ABTS•	+2,2'-Azinobis (3-ethylbenzothiazonile-6-sulfonic acid) diammonium salt
ANOVA	Analysis of Variance
CID	Collision-Induced Dissociation
DAD	Diode-Array Detector
dm	Dry matter
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
DPPH•	2,2-Diphenyl-1-picrylhydrazyl
EDTA	Ethylenediaminetetraacetic acid
ESI	Electrospray Interface
FACS	Fluorescence-Activated Cell Sorting
FAME	Fatty Acid Methyl Esters
FBS	Fetal Bovine Serum
FC	Folin-Ciocalteu
FDA	Food and Drug Administration
FID	Flame Ionization Detector
FRAP	Ferric Reducing Antioxidant Power
GA	Gallic Acid
GIT	Gastrointestinal Tract
H_2O_2	Hydrogen Peroxide
HCl	Hydrochloric Acid
HPLC	High Performance Liquid Chromatography
HESI	Heated Electrospray Ionization

MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MUFA	Monounsaturated Fatty Acids
nd	Not detected
$\bullet O_2-$	Superoxide Radicals
•OH−	Hydroxyl Radicals
ORAC	Oxygen Radical Absorption Capacity
PBS	Phosphate Buffered Saline
PCA	Principal Component Analysis
PDA	Photodiode Array
Pen Strep	Penicillin Streptomycin
PUFA	Polyunsaturated Fatty Acids
RDA	Recommended Dietary Allowance
ROS	Reactive Oxygen Species
SD	Standard Deviation
SFA	Saturated Fatty Acids
TFC	Total Flavonoid Compounds
TPC	Total Phenolic Compounds
tr	Traces
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
UHPLC	Ultra High Performance Liquid Chromatography
WHO	World Health Organization

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INTRODUCTION

Cactus (*Opuntia ficus-indica* Mill.) cladodes

The cactus (*Opuntia ficus-indica* Mill.) is a native plant of the American continent, member of the family *Cactaceae,* which produces edible seeds, fruits and photosynthetic stems (cladodes) (Figure 1). Cactus can grow in arid and semi-arid climates and is also cultivated in the Mediterranean countries (Spain, Italy, Greece, Egypt, Turkey), Central and South Africa, as well as in the Middle East (Israel, Jordan), Australia and India (Stintzing & Carle, 2005).

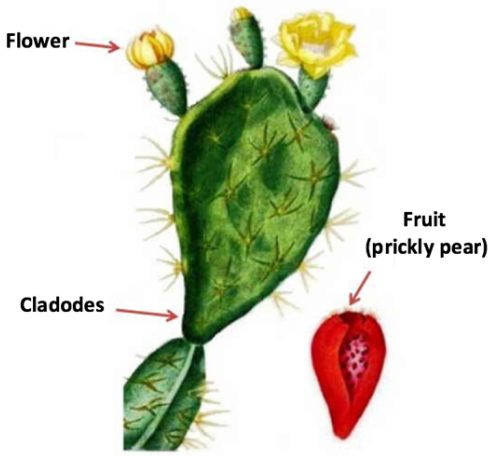

Figure 1. Cactus (*Opuntia ficus-indica* Mill.)

Cactus cladodes have been used to prepare different products such as jams, juices, body lotions, shampoos, creams, etc., as well as functional constituents for food and traditional medicine for several health problems as metabolic diseases, diabetes, arteriosclerosis and inflammatory diseases (Osuna-Martínez, Reyes-Esparza, & Rodríguez-Fragoso, 2014; Patel, 2014).

The interest in cactus cladodes has been increasing because of their great nutritional composition including a high content of fibre such as pectin, lignin, mucilage, cellulose and hemicellulose involved in health benefits as hypoglycemic and hypolipidemic action (Hernández-Urbiola, Pérez-Torrero, & Rodríguez-García, 2011; Jun, Cha, Yang, Choi, & Kim, 2013; López-Romero et al., 2014; Méndez, Flores, Martín, Rodríguez Rodríguez, & Díaz Romero, 2015). Likewise, it has been shown that cactus cladodes contain bioactive compounds such as (poly)phenols which provide potential antioxidant activity related to anti-inflammatory effects (Alimi et al., 2010; El-Mostafa et al., 2014; Feugang, Konarski, Zou, Stintzing, & Zou, 2006).

(Poly)phenolic compounds are secondary metabolites of plants which have structures characterized by the presence of at least one aromatic ring with one or more hydroxyl groups attached. (Poly)phenols can be classified by the number and position of their carbon atoms in two main groups: flavonoids and non-flavonoids such as phenolic acids (Crozier, Jaganath, & Clifford, 2009; Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004). Epidemiological studies have investigated the role of (poly)phenols in health benefits including cancer prevention, incidence, or mortality, as well as protection against diabetes, neurodegeneration and cardiovascular diseases (Crozier, Del Rio, & Clifford, 2010; Del Rio et al., 2013; Tresserra-Rimbau et al., 2014). The beneficial effects of (poly)phenolic compounds are attributed to their antioxidant activity that reduce the free-radical oxidative stress, which is responsible for cellular damage (Crascì, Lauro, Puglisi, & Panico, 2018).

Previous studies in raw cactus cladodes have been identified the presence of (poly)phenolic compounds such as phenolic acids: eucomic, piscidic, ferulic, 4-hydroxy benzoic, malic, citric and salicylic acids; as well as flavonoids, predominating isorhamnetin, kaempferol and quercetin, most of them usually in their glucosides, rutinosides and xylosides forms (Astello-García et al., 2015; Ginestra et al., 2009; Guevara-Figueroa et al., 2010; Mena et al., 2018). In addition, antioxidant capacity has been evaluated in raw cactus cladodes by DPPH, FRAP and ORAC assays, showing a great correlation with the (poly)phenols content (Astello-García et al., 2015; Corral-Aguayo, Yahia, Carrillo-Lopez, & González-Aguilar, 2008; Santos-Zea, Gutiérrez-Uribe, & Serna-Saldivar, 2011). Enzymatic (superoxide dismutase, ascorbate peroxidase and catalase) and other non-enzymatic (carotenoids) systems might also contribute to the antioxidant capacity of cactus cladodes (Ventura-Aguilar, Rivera-Cabrera, Méndez-Iturbide, Pelayo-Zaldívar, & Bosquez-Molina, 2013).

The extraction of (poly)phenols from the food matrix is an important process to obtain the compounds efficiently in order to identify and quantify those that contribute to the health benefits and also to incorporate them into different functional products. The extraction methods depend on the technique conditions applied, the polarity of solvents and the extraction time. Studies in *O. ficus-indica* extracts have been obtained using methanol (Medina-Torres et al., 2011; Mena et al., 2018; Sánchez et al., 2014), ethanol (Guevara-Figueroa et al., 2010; Lee, Kim, Kim, & Jang, 2002) or a combination of several solvents such as acetone, methanol and water (Avila-Nava et al., 2014; Ramírez-Moreno, Córdoba-Díaz, Sánchez-Mata, Díez-Marqués, & Goñi, 2013).

In addition, after the extraction, cactus cladodes are in most cases hydrolysed to release the aglycones from their respective glycosides prior to analysis by HPLC-DAD. The most

common method for breakdown flavonoid glycosides is the acid hydrolysis with hydrochloric acid (HCl) under continuous heating (Khoddami, Wilkes, & Roberts, 2013; Moussa-Ayoub, El-Samahy, Kroh, & Rohn, 2011).

Although there are different conditions reported, there is not a consensus on the optimal methodology and conditions to follow for the extraction of (poly)phenolic compounds and the hydrolysis of aglycones for cactus cladodes.

In America, especially in Mexico, cladodes known as "nopales", are part of the traditional diet and are commonly eaten as a fresh or cooked vegetable including boiling, griddling and frying (Stintzing & Carle, 2005). Several factors such as heat treatment, gastrointestinal digestion and the gut microbiota affect the content of (poly)phenols and their consequent bioaccesibility and biological activity (D'Archivio, Filesi, Varì, Scazzocchio, & Masella, 2010).

Heat treatment

Heat treatment can induce changes in chemical and nutritional qualities of foods such as water loss, changes in energy, fat content and consequently in the fatty acid profile, as well as in the amount of bioactive compounds such as (poly)phenols (Miglio, Chiavaro, Visconti, Fogliano, & Pellegrini, 2008) and also producing some beneficial aspects, improving palatability, texture, flavour and digestibility (Guillén, Mir-Bel, Oria, & Salvador, 2017; van Boekel et al., 2010). Likewise, heat treatments produce the release of (poly)phenolic compounds from the cell and walls plants leading to obtain better extractability and higher concentrations in cooked than in fresh samples. Depending on the vegetable and the cooking techniques applied, as well as temperature and time, (poly)phenolic compounds and consequently their antioxidant capacity, could be increased or degraded (Palermo, Pellegrini, & Fogliano, 2014; Pellegrini et al., 2009; Ramírez-Anaya, Samaniego-Sánchez, Castañeda-Saucedo, Villalón-Mir, & De La Serrana, 2015).

The high temperature applied in heat treatments as griddling and frying can favour the inactivation of oxidative enzymes and the hydrolization of phenolics glycosides, inducing the formation of Maillard reaction products, which besides giving sensorial characteristics, have also shown high antioxidant properties (Nunes & Coimbra, 2010; Pérez-Martínez, Caemmerer, De Peña, Cid, & Kroh, 2010).

The effect of heat treatments on (poly)phenolic compounds as well as on their antioxidant activity are controversial. Previous studies in cooked vegetables such as griddled and fried onion, pepper, cardoon (Juániz, Ludwig, Huarte, et al., 2016b),

griddled eggplant (Lo Scalzo et al., 2016) and microwaved broccoli (Turkmen, Sari, & Velioglu, 2005) have demonstrated the increase of (poly)phenols after cooking treatments. Nonetheless, decreases in squash, peas, leek, (Turkmen et al., 2005), kale and red cabbage (Murador, Mercadante, & De Rosso, 2015) after boiling and microwaving have been reported.

Even though the nutritional composition, identification of some (poly)phenolic compounds and antioxidant capacity in raw cactus cladodes have been studied, research about the effect of heat treatment on them is very limited. Ramírez-Moreno et al. (2013) and Jaramillo-Flores et al. (2003) found that the total content of phenolic compounds and consequently their antioxidant capacity decreased significantly after boiling. However, changes in the (poly)phenols profile in cactus cladodes after cooking treatments still remain unknown.

Bioaccessibility of (poly)phenolic compounds

Bioaccessibility has been defined as the amount or fraction of a food compound, which is released from the food matrix in the gastrointestinal tract (GIT), becoming available for absorption (Cilla, Bosch, Barberá, & Alegría, 2018).

Bioaccessibility of bioactive compounds depends on both the characteristics of the food matrix and the gastrointestinal digestion conditions, leading to the structural breakdown of cell wall matrix and their release them from fibres and proteins. The stability of the compounds can be also modified, tending to be more susceptibilities to degradation and isomerization (Acosta-Estrada, Gutiérrez-Uribe, & Serna-Saldívar, 2014; Alminger et al., 2014).

In vitro gastro intestinal digestion methods are widely used to evaluate bioaccessibility of food components, simulating the physiological conditions taking place in the human GIT throughout the mouth, stomach and intestine, including enzymatic, pH and temperature parameters (Cardoso, Afonso, Lourenço, Costa, & Nunes, 2015; Hur, Lim, Decker, & McClements, 2011; Minekus et al., 2014). The complex physicochemical and physiological conditions such as digestive enzymes, pH changes and temperature of the GIT can modify (poly)phenols and, consequently, their bioaccessibility (Saura-Calixto, Serrano, & Goñi, 2007).

Studies in vegetables after an *in vitro* gastrointestinal digestion applied are controversial, showing that (poly)phenolic compounds decrease in artichoke (Garbetta et al., 2014), pepper (Juániz, Ludwig, Bresciani, et al., 2016a) and cardoon (Juániz et al., 2017), whereas others reports demonstrate that compounds are not affected by

digestion process (D'Antuono, Garbetta, Linsalata, Minervini, & Cardinali, 2015; Kamiloglu & Capanoglu, 2014). However, the evaluation of (poly)phenols of both raw and cooked cactus cladodes after gastrointestinal digestion and their antioxidant activities have not yet been reported.

Most phenolic compounds have covalent interactions with the fibre from the cell wall, forming ester linkages which are not hydrolysed by phase I and II enzymes, limiting their release into the small intestine and can reach the colon to be metabolized by the microbiota. Likewise, the fibre can protect (poly)phenols from the digestive conditions favouring their bioaccessibility and maintaining their antioxidant properties (Domínguez-Avila et al., 2017).

Most of the (poly)phenols present in their glycoside forms are not absorbed during the gastrointestinal tract and reach the colon to be hydrolysed by colonic microbiota before absorption. The colonic microflora, inhabited by a diverse population of microorganisms, hydrolyses glycosides into aglycones and degrades them producing metabolites to smaller and more absorbable molecules (Rodriguez-Mateos et al., 2014; Serra et al., 2012; Williamson & Clifford, 2017).

(Poly)phenols biotransformations by the large intestine microbiota enzymes include hydrolysis, dehydroxylation, deconjugation, demethylation, ring cleavage and decarboxylation activities. Therefore, gut microbiota metabolism can also modulate the effects of dietary (poly)phenolic compounds by altering their absorption and their biological activity (Espín, González-Sarrías, & Tomás-Barberán, 2017; Scalbert, Morand, Manach, & Rémésy, 2002). In fact, studies in Ileostomy patients have demonstrated that, despite changes during the GIT, a great amount of (poly)phenolic compounds in berries are not absorbed into circulation from the small intestine and are able to pass into the colon to be degraded by the colonic microbiota (Brown et al., 2012; Brown, Nitecki, et al., 2014).

In vitro colonic fermentation models have performed using anaerobic, stirred, pH and temperature controlled faecal bacterial cultures to simulate the conditions located in the proximal region of the human large intestine (Low, Hodson, Williams, D'Arcy, & Gidley, 2016; Rechner et al., 2004; Vollmer et al., 2017). Effects of colonic microbiota on the phenolic profile have demonstrated in some vegetables as pepper (Juániz et al., 2016a), cardoon (Juániz et al., 2017), fruits as apples (Koutsos et al., 2017) and in coffee (Ludwig, de Peña, Cid, & Crozier, 2013), showing a degradation of most of the compounds, but at the same time, the formation of new metabolites. Hence, antioxidant and beneficial properties could be attributed not only to the native compounds, but also to their metabolites. Furthermore, metabolites generated might

depend on the food matrix, culinary processes, as well as the specific (poly)phenolic composition of each food.

Biological activity of (poly)phenolic compounds

The remaining bioaccessible (poly)phenols which reach the colon could exert their antioxidant activity and modulate metabolic pathways related to the gut microbiota and cell processes, as inflammation, immunity, cell proliferation and oxidative stress (Espín et al., 2017; Heyman-Lindén et al., 2016; Marín, Miguélez, Villar, & Lombó, 2015).

Potential antioxidant effects of (poly)phenols maybe due to the chelating redox active metals or by directly scavenging ROS (including H_2O_2), hydroxyl radicals (•OH−) and superoxide radicals (•O_2−), by being effective electron donors and modulating biological processes (Cemeli, Baumgartner, & Anderson, 2009).

In vitro cell culture studies are one of the most effective way to investigate the impact of the gut microbiota on the transformations of (poly)phenols, and therefore, on their biological activity. Some researches have focused on the effect of (poly)phenolics against colon cancer cell proliferation, intestinal inflammation and oxidative stress using intestinal cell models such as colonic HT29 and Caco2 cells (Brown, Latimer, et al., 2014). Antigenotoxicity of (poly)phenolic compounds in cell cultures has been also evaluated to measure the DNA damage after challenging with hydrogen peroxide (H_2O_2), to show if the compounds have a potential protection reducing the oxidative damage (Azqueta & Collins, 2016).

Studies in some fruits such as apples, berries and cherries (Olsson, Gustavsson, Andersson, Nilsson, & Duan, 2004) and in flavonoids such as rutin (ben Sghaier et al., 2016) and quercetin (Min & Ebeler, 2009) have reported anticytotoxic and antigenotoxic effects on HT29 and Caco2 cells, demonstrating the potential antioxidant properties involved. Although studies have shown the biological activity of (poly)phenols, few investigations have considered the effect of intestinal digestion and the action of the human microbiota. Recently, studies in berries after previous gastrointestinal digestion and colonic fermentation, have shown the effect of (poly)phenols in the reduction of H_2O_2-induced DNA damage (Brown et al., 2012; Coates et al., 2007; López de las Hazas, Mosele, Macià, Ludwig, & Motilva, 2016; McDougall et al., 2017). However, the biological activity could be different depending on the food extracts or isolated components.

Regarding to *O. ficus-indica*, studies have suggested that the cactus pear fruit have a potential antioxidant effects against cervical, ovarian and bladder cancer cell lines (Osuna-Martínez et al., 2014). Likewise, extracts from juices of cactus pear fruits (Serra,

Poejo, Matias, Bronze, & Duarte, 2013). have also demonstrated anti-proliferative effects on HT29 cells. Nevertheless, gastrointestinal digestion and gut microbiota action were not considered.

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OBJECTIVES / OBJETIVOS

Cactus cladode is one of the native foods of the traditional Mexican diet proposed to prevent diseases related to oxidative stress, such as cancer, diabetes and cardiovascular diseases due to the presence of bioactive antioxidant compounds. Among the main bioactive components present in cactus cladodes, (poly)phenolic compounds are found. However, the bioaccessibility of these compounds depends on several factors such as the interaction with other food components (proteins, carbohydrates, fats and fibre), as well as the culinary technological processes applied. Moreover, after ingestion, the structure of (poly)phenols could be modified along the gastrointestinal tract. Likewise, upon reaching the colon, these compounds could be transformed by the intestinal microbiota into smaller and more absorbable metabolites which could exert their biological activity at least in the colon cells.

Therefore, the main aim of this PhD thesis was to evaluate the bioaccessibility of (poly)phenolic compounds of raw and cooked cactus cladodes using simulated gastrointestinal digestion model and colonic microbiota action, as well as to evaluate the potential biological activity in colon cells.

To achieve this aim, the following partial objectives in cactus (*Opuntia ficus-indica* Mill.) cladodes were established:

1. To optimize the methodological conditions for extraction of (poly)phenolic compounds.

2. To evaluate the influence of heat treatment (boiling, microwaving, griddling, frying in olive and soybean oils) on nutritional composition, antioxidant capacity and (poly)phenolic compounds.

3. To evaluate the bioaccessibility of (poly)phenolic compounds and their antioxidant capacity after *in vitro* gastrointestinal digestion.

4. To evaluate the action of colonic microbiota on (poly)phenolic compounds.

5. To evaluate the biological activity of colonic fermented cactus cladodes on HT29 human colon cancer cells.

El nopal es uno de los alimentos autóctonos de la dieta mexicana tradicional sugeridos para prevenir enfermedades relacionadas con el estrés oxidativo, como cáncer, diabetes y enfermedades cardiovasculares debido a la presencia de compuestos bioactivos antioxidantes. Entre los principales componentes bioactivos presentes se encuentran los compuestos (poli)fenólicos. No obstante, la bioaccesibilidad de estos compuestos depende de diversos factores como la interacción con otros componentes de los alimentos (proteínas, hidratos de carbono, grasas y fibra), así como de los procesos tecnológicos culinarios aplicados. Por otra parte, la estructura de los compuestos (poli)fenólicos se puede modificar a lo largo del tracto gastrointestinal. Asimismo, al llegar al colon, estos compuestos podrían ser transformados por la microbiota intestinal en metabolitos, que son moléculas más pequeñas y más absorbibles capaces de ejercer su actividad biológica al menos en las células del colon.

Por lo tanto, el objetivo general de esta Tesis Doctoral fue evaluar la bioaccesibilidad de los compuestos (poli)fenólicos del nopal tratado térmicamente en un sistema modelo de digestión gastrointestinal y acción de la microbiota, así como evaluar su potencial actividad biológica a nivel del colon.

Para ello se propusieron los siguientes objetivos parciales en el nopal *(Opuntia ficus-indica* Mill.*)*:

1. Optimización de la metodología de análisis para la extracción de sus compuestos (poli)fenólicos.

2. Evaluación de la influencia del tratamiento térmico (hervido, microondas, plancha, fritura en aceite de oliva y de soja), sobre su composición nutricional, capacidad antioxidante y compuestos (poli)fenólicos.

3. Evaluación de la bioaccesibilidad de sus compuestos (poli)fenólicos y su capacidad antioxidante tras la digestión gastrointestinal *in vitro*.

4. Evaluación de la acción de la microbiota intestinal sobre sus compuestos (poli)fenólicos.

5. Evaluación de la actividad biológica de nopal posterior a la fermentación colónica en células de cáncer de colon HT29.

EXPERIMENTAL DESIGN

Cactus cladodes
(*Opuntia ficus-indica*)

Objective 1

Extraction methodology

Solvents Successive extractions

Total (poly)phenolic content
Total flavonoid content
Antioxidant capacity (DPPH, ABTS)
(Poly)phenolic compounds (HPLC-DAD)

Paper 1 (in preparation)

Objective 2

Heat treatment

Raw Boiled Microwaved Griddled Fried in olive oil Fried in soybean oil

Moisture
Protein
Ash
Fat (Fatty acids)
Carbohydrates
Fibre (soluble and insoluble)
Energy

Total phenolic compounds
Antioxidant capacity (DPPH, ABTS)
(Poly)phenolic compounds (HPLC-DAD)

Paper 2

Objective 3

Bioaccessibility

In vitro gastrointestinal digestion

(Poly)phenolic compounds (UHPLC-ESI-MSn)
- Flavonoids
- Phenolic acids
Antioxidant capacity (DPPH)

Paper 3

Objective 4

Effect of colonic microbiota

In vitro faecal fermentation

(Poly)phenolic compounds (UHPLC-ESI-MS/MS)
- Flavonoids
- Phenolic acids

Objective 5

Biological activity

HT29 human colon cancer cells

Cytotoxicity (MTT assay)
Geno-protective effect (Comet assay)
Cell cycle analysis

Paper 4 (under review)

RESULTS

Objective 1

To optimize the methodological conditions for extraction of (poly)phenolic compounds of cactus (*Opuntia ficus-indica* Mill.) cladodes.

Optimización de la metodología de análisis para la extracción de los compuestos (poli)fenólicos de nopal (*Opuntia ficus-indica* Mill.).

Paper 1

Extraction of (poly)phenolic compounds of cactus (*Opuntia ficus-indica* Mill.) cladodes: impact of solvents and conditions

Elsy De Santiago, Isabel Juániz, Concepción Cid and María-Paz De Peña. (In preparation).

TITLE: Extraction of (poly)phenolic compounds of cactus (*Opuntia ficus-indica* Mill.) cladodes: impact of solvents and conditions

AUTHORS: Elsy De Santiago[a], Isabel Juániz[a], Concepción Cid[a] and María-Paz De Peña[a]*

[a] Universidad de Navarra, Facultad de Farmacia y Nutrición, Departamento de Ciencias de la Alimentación y Fisiología, C/ Irunlarrea 1, E-31008 Pamplona, Spain.
IdiSNA, Navarra Institute for Health Research. Pamplona, Spain.

*Corresponding author: María-Paz de Peña. Tel: +34 948 425600 (806580); Fax: +34 948 425740. E-mail address: mpdepena@unav.es

ABSTRACT

Research in cactus (*Opuntia ficus-indica* Mill.) cladodes as a functional food is increasing because of their (poly)phenolic compounds and health-promoting ability. Extraction conditions are the basis to obtain the highest amount of bioactive compounds with antioxidant properties. Therefore, several extraction solvents and conditions were carried out. Even ethanol (80 and 100%) extracts high amount of (poly)phenols, successive extraction with methanol, acetone and water, favoured the extraction of flavonoids and antioxidant capacity (DPPH and ABTS). Additionally, in order to quantify the most abundant (poly)phenols by HPLC-DAD, aglycones were released from flavonoid glycosides by acid hydrolysis through a reflux at 90 °C at different times (2h and 3h) and HCl concentrations (0.6, 1.2, 1.5 and 1.7M). After hydrolysis, 3 flavonoids aglycones (isorhamnetin, quercetin and kaempferol) and 2 phenolic acids (ferulic and hidroxybenzoic acids) were identified and quantified. Comparing different procedures, acid hydrolysis with 1.5M HCl during 2h was the best for the release of flavonoid aglycones, but a less concentration of HCl in a shorter time of reflux showed better results for phenolic acids.

KEYWORDS: Phenolics, flavonoids, phenolic acids, antioxidant capacity, cactus, extraction, hydrolysis, aglycones.

1. Introduction

Studies have demonstrated that a consumption of fruits and vegetables is associated with a lower risk of chronic diseases such as cancer, diabetes or cardiovascular diseases due to the antioxidant properties of bioactive compounds that inhibit reactive oxygen species which are responsible for oxidative stress and cellular damage (Del Rio et al., 2013).

The cactus (*Opuntia ficus-indica* Mill.), an endemic American plant, has been used fresh or processed for human consumption, as well as a functional constituent for food and pharmaceutical products due to the important content of bioactive compounds like (poly)phenols (El-Mostafa et al., 2014).

Cactus stems, known as cladodes, have been characterized by the presence of phenolic acids and flavonoids such as isorhamnetin, kaempferol, quercetin and isoquercitrin, which most of them, are usually in their glucosides, rutinosides and xylosides forms (Astello-García et al., 2015; Ginestra et al., 2009; Weirong, Xiaohong, & Tang, 2010).

The (poly)phenolic compounds extraction is the most important step before analysis and, consequently, characterization of bioactive compounds of cactus cladodes. A common issue regarding to (poly)phenols extraction is the linkages with different fibres structures such as pectin, lignin, mucilage, cellulose, hemicellulose where the compounds can be retained (Hernández-Urbiola, Pérez-Torrero, & Rodríguez-García, 2011). Several studies with *O. ficus-indica* have used different solvents for their extraction like methanol (Sánchez et al., 2014), ethanol (Guevara-Figueroa et al., 2010) or a combination of several solvents such as acetone, methanol and water (Avila-Nava et al., 2014; Ramírez-Moreno, Córdoba-Díaz, Sánchez-Mata, Díez-Marqués, & Goñi, 2013). It is also important to control the conditions as temperature, time, and concentrations used in the extraction methods to avoid the degradation of the compounds or unwanted reactions like oxidation.

In addition, after the extraction, the glycosides of cactus cladodes are in most cases hydrolysed to their respective aglycones prior to analysis by HPLC-DAD because of the lack of pure standards. The most common method for breakdown the flavonoid glycosides is the acid hydrolysis with hydrochloric acid (HCl) under continuous heating (Moussa-Ayoub, El-Samahy, Kroh, & Rohn, 2011, Khoddami, Wilkes, & Roberts, 2013). Although there are different conditions reported, there is not a consensus on the optimal methodology and conditions to follow for the extraction and hydrolysis for cactus cladodes.

Therefore, the main aim of this work was to optimize the extraction conditions of (poly)phenolic compounds and antioxidant capacity from cactus cladodes using different solvents. After selecting the best extraction method, different conditions of acid hydrolysis were developed to release phenolic aglycones and to identify and quantify them by HPLC-DAD.

2. Material and methods

2.1 Chemical and reagents

Ethanol, methanol, acetone and hydrochloric acid (HCl) were of analytical grade from Panreac (Barcelona, Spain). The acetonitrile and formic acid (HPLC grade) were also purchased from Panreac (Barcelona, Spain). Folin–Ciocalteu reagent, trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), 2,2'-azinobis (3-ethylbenzothiazonile-6-sulfonic acid) diammonium salt (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH·) and rutin; as well as the standards used for identification and quantification of phenolic compounds (isorhamnetin, kaempferol, quercetin, ferulic acid and hydroxybenzoic acid), were purchased from Sigma-Aldrich (Steinheim, Germany).

2.2 Samples preparation

Fresh cactus (*O. ficus-indica*) cladodes were obtained from BioArchen Company located in Murcia, Spain. Cladodes were washed and the thorns were removed manually. Then, were cut into equal small pieces, mixed well and lyophilized in a freeze dryer Cryodos-80 (Telstar, Terrasa, Spain), and stored at -18 °C until analysis.

2.3 Extraction methods

Four different extracts were prepared as follows:

a) 100% water: The extract was prepared according to Siddiq method (Siddiq, Roidoung, Sogi, & Dolan, 2013) with some modifications. Briefly, 30 mL of distilled water was added to 2 g of lyophilized cactus cladodes. The content was mixed on a mechanical shaker for 1 h at room temperature and then centrifuged at 4,000 rpm for 10 min. Supernatant was collected and residues were re-extracted twice using 10 mL of distilled water by vortexing (1 min) and centrifuged at 4,000 rpm for 5 min. All three supernatants were combined and frozen at -18 °C before analysis.

b) 80% ethanol (EtOH): A second extract was performed as previously described, but instead of adding 100% of distilled water, thirty mL of ethanol/water (80/20) was used. Likewise, after mixing the three supernatants, the extract was frozen at -18 °C.

c) 100% EtOH: Lee et al. (2002) method was used for this extract. Five g of lyophilized cladodes were mixed with 50 mL of EtOH and shaken at room temperature for 72 h.

Then, it was filtered and centrifuged at 4,000 rpm 10 min. Supernatant was stored at -18 °C before analysis.

d) Successive extractions: The last extract was prepared based on method of Avila-Nava et al. (2014) with modifications. A mix of 50 mL of methanol/water solution (50/50) was added to 4 g of lyophilized cladodes. This mixture was stirred for two h and then vacuum filtered. The resulting filtrate was saved and stored. The residue was subjected to a second extraction with 50 mL of acetone/water (70/30) solution and agitation for two h. The mixture was vacuum filtered and stored. A third extraction was performed stirring the residue in 50 mL of distilled water for thirty min and also vacuum filtered. Resulting filtrates were mixed together and stored at -18 °C before analysis.

2.4 Total phenolic compounds (TPC)

TPC were measured using the Folin–Ciocalteu reagent according to Singleton's method (Singleton & Rossi, 1965). Each extract was properly diluted in demineralized water and 100 µL were mixture with 500 µL of Folin–Ciocalteu reagent and 7.9 mL of demineralized water. After 2 min, 1.5 mL of a 7.5% sodium carbonate solution was added. Then, samples were incubated in darkness for 90 min at room temperature. The absorbance of the sample was measured at 765 nm in a spectrophotometer Lambda 25 UV/VIS (Perkin Elmer Instruments, Madrid, Spain). Gallic Acid (GA) was used as reference, and the results were expressed as milligrams of GA equivalent per gram of dry matter (mg GA/g dm).

2.5 Total flavonoid content (TFC)

The aluminum chloride method (Lamaison & Carnet, 1990) was used for estimation of TFC of the extracted samples. An aliquot of 100 µL of each extract properly diluted was added to 1 mL of a 2% $AlCl_3 \cdot 6H_2O$ methanol solution. The mixture was shaken, and after 10 min of incubation at room temperature, the absorbance was read at 430 nm in a spectrophotometer Lambda 25 UV/VIS (Perkin Elmer). Rutin was used for calibration curve and results were expressed as milligram of rutin equivalent per gram of dry matter (mg rutin/g dm).

2.6 Antioxidant capacity by ABTS assay

The ABTS antioxidant capacity was performed according to the method of (Re et al., 1999). The radicals $ABTS^{\bullet+}$ were generated by the addition of 0.36 mM potassium persulfate to a 0.9 mM ABTS solution prepared in phosphate buffered saline (PBS) (pH 7.4), and the $ABTS^{\bullet+}$ solution was stored in darkness for 12 h. The $ABTS^{\bullet+}$ solution was adjusted with PBS to an absorbance of 0.700 (±0.020) at 734 nm in a 3 mL capacity cuvette (1 cm length) at 25 °C (Lambda 25 UV–VIS spectrophotometer, Perkin-Elmer

Instruments, Madrid, Spain). An aliquot of 100 µL of each extract sample properly diluted in demineralized water, was added to 2 mL of ABTS•+ solution. After 18 min, the absorbance was measured at 734 nm. Calibration curve was performed with Trolox solution (a water-soluble vitamin E analog), and results were expressed as micromoles of Trolox equivalent per gram of dry matter (µmol Trolox/g dm).

2.7 Antioxidant capacity by DPPH assay

The antioxidant capacity was also measured using 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) decolorization assay (Brand-Williams, Cuvelier, & Berset, 1995) with some modifications. A 6.1×10^{-5} M $DPPH^{\bullet}$ methanolic solution was prepared immediately before use. In a 3 mL capacity cuvette (1 cm length) the $DPPH^{\bullet}$ solution was adjusted with methanol to an absorbance of 0.700 (±0.020) at 515 nm (Lambda 25 UV–VIS spectrophotometer, Perkin-Elmer Instruments, Madrid, Spain). All the extracts were properly diluted in demineralized water and 50 µL of samples were added to 1.95 mL of the $DPPH^{\bullet}$ solution. The absorbance was measured at 515 nm after 18 min. Calibration was performed with Trolox solution and expressed as micromoles of Trolox equivalent per gram of dm (µmol Trolox/g dm).

2.8 Acid Hydrolysis

For the identification and quantification of (poly)phenolic aglycones by HPLC-DAD, previous acid hydrolysis was carried out through a reflux at 90 °C with different concentrations of HCl and times: 0.6M for 3 h, 1.2M for 3 h, 1.5M for 2 and 3 h, and 1.7M for 2 and 3 h. The resulting extract was filtered and frozen at -18 °C for chromatographic analysis.

2.9 (Poly)phenolic compounds by HPLC-DAD

(Poly)phenolic compounds were analysed by HPLC following the method described by Juániz et al. (2016) with some modifications. HPLC analysis was performed with an analytical HPLC unit model 1200 (Agilent Technologies, Palo Alto, CA, USA) equipped with a binary pump and an auto-sampler injector. The column used was a C18 5U Kinetex 100A (250 x 4.60 mm) and the volume of each sample injection was 20 µL. Chromatographic separation was carried out using a gradient of acid water with formic acid 1% (solvent A) and acetonitrile (solvent B) at a constant flow of 1 mL/min starting with 95% solvent A. Then solvent A was decreased to 60% within 55 min, maintained until 60 min and decreased to 10%. Then, it remained until 67 min and returned to the initial conditions (solvent A: 95% solvent B: 5%). Detection was accomplished with a diode array detector (DAD), at 360 nm for flavonoids; at 325 nm for ferulic acid, and at 256 nm for hydroxybenzoic acid. Chromatograms were analysed using the Agilent

ChemStation software and the identification of phenolic compounds was performed by comparison of retention times and UV spectra with reference standards. A calibration curve with reference standards of identified compounds was carried out to quantify (poly)phenols. Results were expressed as milligrams of each compound per gram of dry matter (mg/g dm).

2.9 Statistical analysis

Each parameter was analysed in triplicate. Results are shown as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was applied for each parameter. A Tukey test was applied as *a posteriori* test with a level of significance of 95%. All statistical analyses were performed using the STATA v.12.0 software package.

3. Results and discussion

3.1 Total Phenolic Content (TPC) and Total Flavonoid Content (TFC)

Figure 1 shows the TPC and TFC of the different extracts obtained from cactus (*O. ficus-indica)* cladodes. The highest amount of TPC corresponded to the 80% EtOH extract with 39.26 mg GA/g dm (250.86 mg GA/100 g), followed by the successive extractions extract with 36.62 mg GA/g dm (234.01 mg GA/100 g). The extract with the least amount of phenolic compounds was the 100% water extract with 14.18 mg GA/g dm (90.62 mg GA/100 g). There is a great variation in the results reported by other authors due in part to the different expression of results (fresh weight, dry matter, mililiter of extract) and the methodology applied. Nevertheless, the amount of TPC found in 80% EtOH and successive extractions extracts were higher than those previously reported in the literature. Sánchez et al. (2014) reported a total phenolic amount of 4.27 mg GAE/g dm using 70% methanol, whilst Guevara-Figueroa et al. (2010) reported a total content of 19.9 mg GAE/g with 100% ethanol used for extraction. Similar processes with successives extractions were carried out by Dib, Beghdad, Belarbi, Seladji, & Ghalem (2013) and Ramírez-Moreno, Córdoba-Díaz, de Cortes Sánchez-Mata, Díez-Marqués, & Goñi (2013). However, they reported less amount than in the present study (26.7 and 4.58 mg GA/g dm, respectively), may be due to the methodology applied but also to samples differences in the cladode maturity, cultivation region and climate.

On the other hand, the extract wih the highest amount of TFC was these obtained by successive extractions with 4.83 mg rutin/g dm (30.89 mg rutin/100 g). In this case, the 80% EtOH extract presented the lowest amount of TFC with 2.85 mg rutin/g dm (18.22 mg rutin/100 g). Flavonoid results are also difficult to compare because of different methodologies and standard compounds used such as rutin, quercetin and cathechine for reporting results.

3.2 Antioxidant capacity

Antioxidant capacity measured by ABTS and DPPH radicals of cactus cladodes extracts are shown in Figure 2. In both cases, the extract obtained by successive extractions presented the highest antioxidant capacity with 778.03 and 72.17 µmol trolox/g dm (4791.63 and 461.14 µmol trolox/100g) respectively. The extract with the least antioxidant capacity was that obtained with 100% water, accounted for 1.13 µmol trolox/g dm (7.25 µmol trolox/100g) for ABTS and 1.44 µmol trolox/g dm (9.21 µmol trolox/100g) for DPPH radical. In contrast, the amount found in successive extractions extract is higher than that reported by others authors: 13.97 µmol trolox/g dm for ABTS radical (Ramírez-Moreno, Córdoba-Díaz, Sánchez-Mata, Díez-Marqués, & Goñi, 2013) and 90 µmol trolox/100g for DPPH (Corral-Aguayo, Yahia, Carrillo-Lopez, & González-Aguilar, 2008).

Polysaccharides present in cactus cladodes, such as pectins, form a water-soluble complex with (poly)phenols, as well as cellulose with the non-covalent hydrogen bonds and hydrophobic interactions making extraction more difficult (Bordenave, Hamaker, & Ferruzzi, 2014).

Results suggest that the use of different solvents facilitates the antioxidant extraction process, obtained an optimal polarity conditions, because of the retention of the compounds in the cell walls of the cactus. Hence, the combination of ethanol, acetone and water could improve the extraction of bioactive compounds like (poly)phenols.

3.3 (Poly)phenolic compounds by HPLC-DAD

Because of the highest DPPH and ABTS antioxidant capacity and the high amount of total phenolic and flavonoid content, successive extraction method was selected as the best to further identify and quantify (poly)phenolic compounds by HPLC-DAD previous acid hydrolysis. Five acid hydrolysis were carried out through a reflux at 90 °C with different concentrations of HCl and times: 0.6M for 3 h, 1.2M for 3h, 1.5M for 2 and 3 h, and 1.7M for 2 and 3 h.

After acid hydrolysis, 3 flavonoids aglycones (quercetin, kaempferol and isorhamnetin) and 2 phenolic acids (ferulic and hidroxybenzoic acids) were identified and quantified in cactus cladodes (Table 1). Results from HPLC-DAD measurements showed the (poly)phenolic aglycones, indicating that after acid hydrolysis, compounds were released from the glycosides, but also from the food matrix bound to or associated with polysaccharides such as the fibre. Chromatograms obtained by HPLC-DAD before and after hydrolysis show the release of the quercetin, kaempferol and isorhamnetin aglycones (Figure 3).

Flavonoids were the main compounds accounting for around 85% of total (poly)phenolics. Isorhamnetin was the most abundant flavonoid compound, which is in agreement with literature (Avila-Nava et al., 2014; Ginestra et al., 2009; Jun, Cha, Yang, Choi, & Kim, 2013), followed by smaller amount of quercetin and kaempferol. Previous studies without hydrolysis have identified isorhamnetin glycosides as the main flavonoid glycosides in cactus cladodes (Ginestra et al., 2009; Moussa-Ayoub et al., 2014), although kaempferol and quercetin glycosides are also present (Guevara-Figueroa et al., 2010). Santos-Zea, Gutiérrez-Uribe & Serna-Saldivar (2011) have reported less amount of isorhamnetin (0.6 mg/g dm) and higher amount of kaempferol (0.4 mg/g dm) than in this study, but they did not find quercetin.

Otherwise, ferulic acid was the predominant phenolic acid, followed by 4-hydroxybenzoic acid. Protocatechuic, chlorogenic, salicylic, gallic, and caffeic acids have been previously reported in *Opuntia* cactus cladodes (Guevara-Figueroa et al., 2010; Jun, Cha, Yang, Choi, & Kim, 2013), but they were not detected in the present study.

Comparing different hydrolysis conditions, the highest amount of total (poly)phenolic compounds was that obtained after 2 h of reflux at 90 °C with 1.5M HCl (3.97 mg/g dm). Likewise, the deglycosylation of flavonoids and thus the highest amount of aglycones was also observed with 1.5M HCl during 2 h of reflux. Results show that less acidity (0.6 M HCl), does not completely release the aglycones. Otherwise, a higher acidity (1.7 M) and a longer time of reflux (3h), resulted in the degradation of flavonoid aglycones. A previous study in cactus cladodes which performed an acid hydrolysis with higher concentration of HCl (3.2M) during 20 min, showed less amount than in this study (Santos-Zea, Gutiérrez-Uribe, & Serna-Saldivar, 2011), demonstrating the degradation of the compounds. Likewise, Moussa et al (2014), identified isorhamnetin as the only aglycone presented after performing an enzymatic hydrolysis; but the amount was much smaller (0.003 mg/g dm) than that obtained in the present study.

Regarding to total phenolic acids content, the 0.6M HCl hydrolysis, showed the highest amount, following by the 1.2M HCl concentration. It can be observed that when the acid concentration and the reflux time were increased, ferulic acid was degraded. However, the highest content of 4-hydroxybenzoic acid was measured after 3h of 1.5M HCl hydrolysis, and starting to decrease after longer time and higher acid concentration applied. In any case, the amount of each phenolic acid found in the present study was higher than that found by Guevara-Figueroa et al. (2010). A similar work carried out in onion and spinach showed that the 2 h reflux at 80 °C with 1.2 M HCl gave the best results for quercetin and kaempferol aglycones, whereas phenolic acids were degraded under the same conditions (Nuutila, Kammiovirta, & Oksman-Caldentey, 2002).

Therefore, because phenolic acids are more susceptible than flavonoids to be degraded, it is necessary to choose the acid hydrolysis conditions that favoured the release of aglycones without put at risk the identification and quantification of phenolic acids.

(Poly)phenols are bound to the polymeric compounds like fibre, carbohydrates or proteins, thus the stability of the glycosides depends on the kind and position of the sugar, as well as the compound union. Likewise, the number of aromatic moieties and hydroxyl groups present in phenolic compounds are important factors, because more aromatic rings tended to bind more strongly to cellulose than those compounds with a single aromatic ring (Bordenave, Hamaker, & Ferruzzi, 2014). Even though the release of aglycones from the cell structure enhance the antioxidant capacity, if time and HCl concentrations are not the optimal and oxidation reactions could take place degrading the compounds.

4. Conclusions

The present study evidenced that the methodology and conditions of extraction are crucial to obtain bioactive compounds and therefore the potential antioxidant capacity of cactus cladodes. Results suggests that a mixture of methanol, acetone and water are required for an optimal polarity conditions to extract biological compounds like (poly)phenols. On the other hand, to allow the identification of phenolic compounds by HPLC-DAD, it is often necessary to carry out a previous acid hydrolysis, demonstrating that 1.5M HCl during 2 h showed the highest amount of (poly)phenols, especially flavonoids aglycones. This study also showed that acid hydrolysis at long time periods or at high temperature leads to the degradation of (poly)phenolic compounds. Therefore, the choice of the best method of extraction is very important for obtaining the highest amount of bioactive compounds, especially antioxidants, from the plant material, but toxicological and sustainable criteria should also be considered in order to use cactus cladodes extracts for the development of functional foods.

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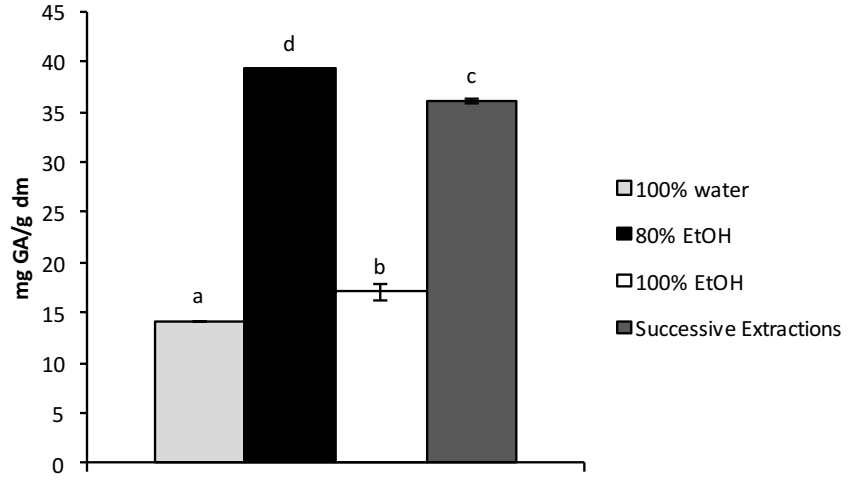
Figure 1. Total phenolic (a) and flavonoid (b) concentration of cactus cladodes extracts. Different letters indicate significant differences ($p \leq 0.05$).

a)



b)

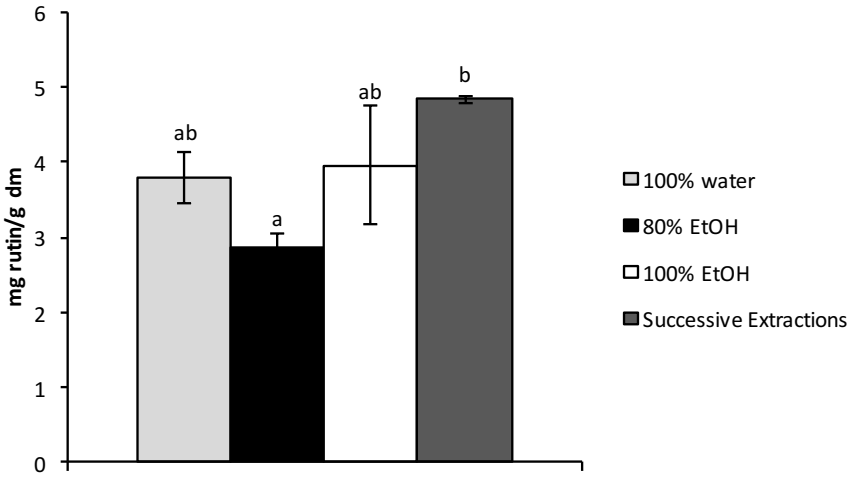
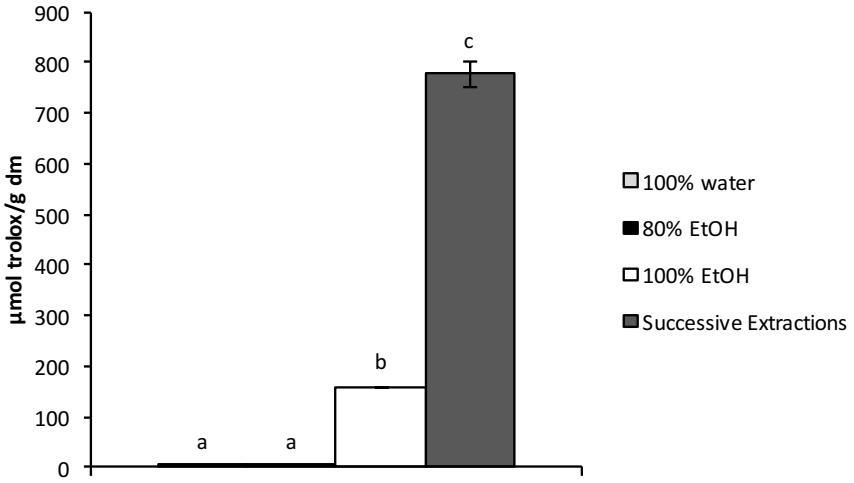


Figure 2. Antioxidant capacity measured by ABTS (a) and DPPH (b) of cactus cladodes extracts. Different letters indicate significant differences ($p \leq 0.05$).

a)



b)

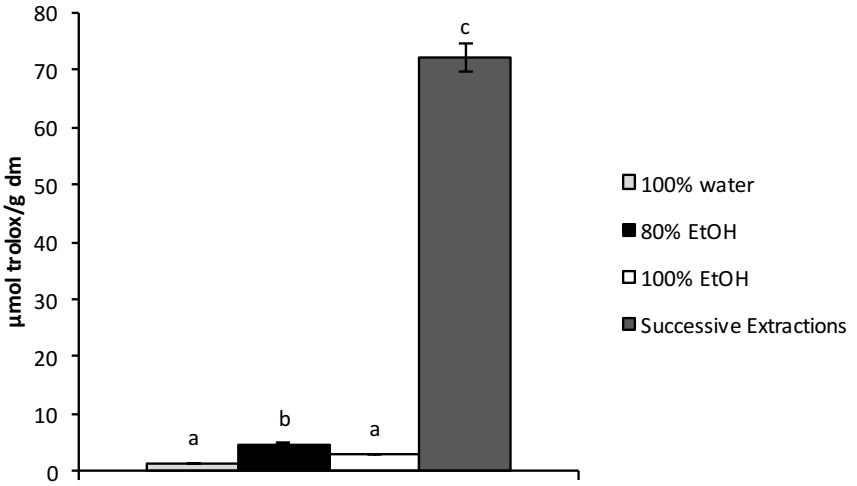
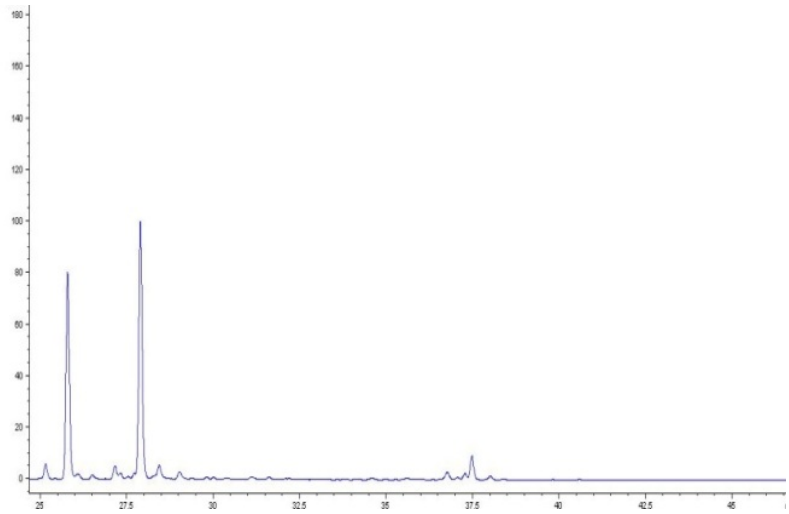
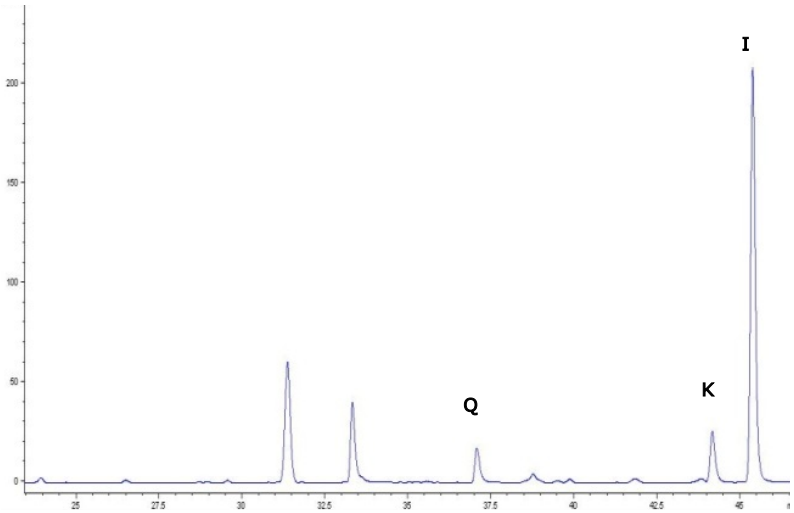


Figure 3. HPLC-DAD chromatograms of cactus cladodes extracts a) before and b) after acid hydrolysis (1.5M HCl 2h) at 360 nm.

a) Unhydrolysed



b) Hydrolysed



Q: quercetin, K: kaempferol, I: isorhamnetin.

Table 1. (Poly)phenolic compounds in cactus cladodes after different hydrolysis (HCl) conditions. Results are expressed as mean ± standard deviation (mg/g dm).

Compound	0.6M 3h	1.2M 3h	1.5M 2h	1.5M 3h	1.7M 2h	1.7M 3h
Isorhamnetin	0.92 ± 0.07a	1.34 ± 0.03b	2.58 ± 0.27d	2.15 ± 0.16c	2.08 ± 0.03c	1.29 ± 0.13ab
Kaempferol	0.25 ± 0.00b	0.11 ± 0.00a	0.36 ± 0.07c	0.29 ± 0.01bc	0.32 ± 0.00bc	0.06 ± 0.01a
Quercetin	0.36 ± 0.04c	0.19 ± 0.00b	0.42 ± 0.03c	0.37 ± 0.01c	0.38 ± 0.01c	0.11 ± 0.00a
Total flavonoids	**1.53 ± 0.04a**	**1.64 ± 0.04a**	**3.36 ± 0.29c**	**2.81 ± 0.18b**	**2.78 ± 0.05b**	**1.47 ± 0.15a**
Ferulic acid	0.63 ± 0.03c	0.58 ± 0.01c	0.43 ± 0.07b	0.41 ± 0.03ab	0.34 ± 0.00ab	0.32 ± 0.02a
4-Hydroxybenzoic acid	0.16 ± 0.05c	0.15 ± 0.00abc	0.18 ± 0.01c	0.15 ± 0.00abc	0.12 ± 0.00ab	0.10 ± 0.00a
Total phenolic acids	**0.79 ± 0.08d**	**0.73 ± 0.01cd**	**0.61 ± 0.08bc**	**0.56 ± 0.03ab**	**0.46 ± 0.00a**	**0.42 ± 0.02a**
Total compounds	**2.32 ± 0.07a**	**2.37 ± 0.05a**	**3.97 ± 0.37c**	**3.37 ± 0.17b**	**3.24 ± 0.05b**	**1.89 ± 0.17a**

Different letters for each row indicate significant differences ($p \leq 0.05$) among samples.

Objective 2

To evaluate the influence of heat treatment (boiling, microwaving, griddling, frying in olive and soybean oils) on nutritional composition, antioxidant capacity and (poly)phenolic compounds of cactus (*Opuntia ficus-indica* Mill.) cladodes.

Evaluación de la influencia del tratamiento térmico (hervido, microondas, plancha, fritura en aceite de oliva y de soja), sobre la composición nutricional, capacidad antioxidante y compuestos (poli)fenólicos de nopal (*Opuntia ficus-indica* Mill.).

Paper 2

Impact of cooking process on nutritional composition and antioxidants of cactus cladodes (*Opuntia ficus-indica*)

Elsy De Santiago, Maite Domínguez-Fernández, Concepción Cid and María-Paz De Peña

Food Chemistry, 2018, (240) 1055–1062.
https://doi.org/10.1016/j.foodchem.2017.08.039.

Quality indices:

- Impact factor (JCR, 2017): 4.946
- Journal Rank in categories:
    - Food Science & Technology: 7/133 (Q1) (D1)
    - Chemistry, applied: 7/71 (Q1) (D1)
    - Nutrition & Dietetics: 11/81 (Q1)

De Santiago E, Domínguez-Fernández M, Cid C, De Peña MP. Impact of cooking process on nutritional composition and antioxidants of cactus cladodes (Opuntia ficus-indica). Food Chemistry, 2018, 240:1055-1062.
https://doi.org/10.1016/j.foodchem.2017.08.039

Objective 3

To evaluate the bioaccessibility of (poly)phenolic compounds and its antioxidant capacity after *in vitro* gastrointestinal digestion in cactus (*Opuntia ficus-indica* Mill.) cladodes.

Evaluación de la bioaccesibilidad de sus compuestos (poli)fenólicos y su capacidad antioxidante tras la digestión gastrointestinal *in vitro* en el nopal (*Opuntia ficus-indica* Mill.).

Paper 3

Digestibility of (poly)phenols and antioxidant activity in raw and cooked cactus cladodes (*Opuntia ficus-indica*)

Elsy De Santiago, Gema Pereira-Caro, José Manuel Moreno-Rojas, Concepción Cid and María-Paz De Peña

Journal of Agricultural and Food Chemistry, 2018, 66, 5832−5844.
https://doi.org/ 10.1021/acs.jafc.8b01167.

Quality indices:

- Impact factor (JCR, 2017): 3.412
- Journal Rank in categories:
    - Agriculture, multidisciplinary: 2/56 (Q1) (D1)
    - Food Science & Technology: 18/133 (Q1)
    - Chemistry, applied: 17/71 (Q1)

De Santiago E, Pereira-Caro G, Moreno-Rojas JM, Cid C, De Peña MP. Digestibility of (Poly)phenols and Antioxidant Activity in Raw and Cooked Cactus Cladodes (Opuntia ficus-indica). Journal of Agricultural and Food Chemistry, 2018, 66, 5832-5844. http://doi.org/10.1021/acs.jafc.8b01167

SUPPLEMENTARY INFORMATION

TITLE: Digestibility of (poly)phenols and antioxidant activity in raw and cooked cactus cladodes (*Opuntia ficus-indica*)

AUTHORS: Elsy De Santiago[a], Gema Pereira-Caro[b], José Manuel Moreno-Rojas[b], Concepcion Cid[a], M. Paz de Peña[a*]

[a] Universidad de Navarra, Facultad de Farmacia y Nutrición, Departamento de Ciencias de la Alimentación y Fisiología, C/ Irunlarrea 1, E-31008 Pamplona, Spain.

IdiSNA, Navarra Institute for Health Research. Pamplona, Spain.

[b] Department of Food Science and Health. Andalusian Institute of Agricultural and Fisheries Research and Training (IFAPA). Alameda del Obispo, Avda. Menéndez Pidal, s/n, 14071, Córdoba. Spain

*Corresponding author: María-Paz de Peña. Tel: +34 948 425600 (806580); Fax: +34 948 425740. E-mail address: mpdepena@unav.es

Table S1. Concentrations of electrolytes Simulated Salivary Fluid (SSF), Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF)

	Stock solution		SSF (250mL)	SGF (250mL)	SIF (250mL)
Constituent	g/50mL	mol/L	mL	mL	mL
KCl	1.87	0.5	9.44	4.31	4.25
KH_2PO_4	3.40	0.5	2.31	0.57	0.50
$NaHCO_3$	4.20	1	4.25	7.81	26.56
NaCl	5.90	2	-----	7.38	6.00
$MgCl_2(H_2O)_6$	1.53	0.15	0.31	0.25	0.69
$(NH_4)_2CO_3$	2.40	0.5	0.04	0.31	0.69

Table S2. Mass spectrometric characteristics of native (poly)phenolic compounds identified in this study.

Compound	Chemical formula	R_t (min)	$[M-H]^-$ (m/z)	δppm	Fragment ions under low collision energy (m/z)
Piscidic acid I	$C_{11}H_{12}O_7$	5.4	255.0501	0.78	193.0499 / 179.0340 / 165.0546
Piscidic acid II	$C_{11}H_{12}O_7$	6.1	255.0501	0.78	193.0499 / 179.0340 / 165.0546
Piscidic acid III	$C_{11}H_{12}O_7$	6.6	255.0501	0.78	193.0499 / 179.0340 / 165.0546
Eucomic acid I	$C_{11}H_{12}O_6$	11.8	239.0550	-0.46	195.0658 / 179.0342 / 149.0599 / 107. 0490
Eucomic acid II	$C_{11}H_{12}O_6$	12.8	239.0550	-0.46	195.0658 / 179.0342 / 149.0599 / 107. 0490
Eucomic acid III	$C_{11}H_{12}O_6$	13.4	239.0550	-0.46	195.0658 / 179.0342 / 149.0599 / 107. 0490
Ferulic acid derivatives					
Ferulic acid	$C_{10}H_{10}O_4$	27.8	193.0497	1.04	
1-*O*-feruloylglucose I	$C_{16}H_{20}O_9$	15.1	355.1037	3.94	239.0558 / 193.0503 / 175.0391
1-*O*-feruloylglucose II	$C_{16}H_{20}O_9$	16.4	355.1037	3.94	239.0558 / 193.0503 / 175.0391
1-*O*-feruloylglucose III	$C_{16}H_{20}O_9$	17.5	355.1037	3.94	239.0558 / 193.0503 / 175.0391
1-*O*-feruloylglucose IV	$C_{16}H_{20}O_9$	18.6	355.1037	3.94	239.0558 / 193.0503 / 175.0391
Dihydroferulic acid -*O*-glucuronide I	$C_{16}H_{20}O_{10}$	9.4	371.0982	2.69	239.0558 / 179.0554 / 133.0135
Dihydroferulic acid -*O*-glucuronide II	$C_{16}H_{20}O_{10}$	9.9	371.0982	2.69	239.0558 / 179.0554 / 133.0135
Isorhamnetin derivatives					
Isorhamnetin	$C_{16}H_{12}O_7$	72.0	315.0504	1.59	151.0027
Isorhamnetin hexose rhamnose hexoside	$C_{34}H_{42}O_{21}$	32.4	785.2152	2.29	503.1777 / 371.0984 / 315.0503 / 151.0025
Isorhamnetin di-hexoside	$C_{28}H_{32}O_{17}$	34.6	639.1574	2.97	477.2342 / 361.1868 / 315.0503
Isorhamnetin rutinoside rhamnoside	$C_{34}H_{42}O_{20}$	36.4	769.2199	1.82	315.0503 / 145.0495
Isorhamnetin hexose pentoside	$C_{27}H_{30}O_{16}$	39.1	609.1462	1.97	477.1982 / 315.0508
Isorhamnetin rutinoside I	$C_{28}H_{32}O_{16}$	42.1	623.1627	1.76	477.2346 / 315.0501
Isorhamnetin rutinoside II	$C_{28}H_{32}O_{16}$	43.5	623.1627	1.76	477.2346 / 315.0501
Isorhamnetin 3-*O*-beta-(6-*O*-coumaroylglucoside)-7-*O*-beta-glucoside I	$C_{37}H_{38}O_{19}$	59.2	785.1940	2.16	315.0507 / 179.0554 / 145.0496
Isorhamnetin 3-*O*-beta-(6-*O*-coumaroylglucoside)-7-*O*-beta-glucoside II	$C_{37}H_{38}O_{19}$	61.8	785.1940	2.16	315.0507 / 179.0554 / 145.0496
Isorhamnetin 3-*O*-beta-(6-*O*-coumaroylglucoside)-7-*O*-beta-glucoside III	$C_{37}H_{38}O_{19}$	62.5	785.1940	2.16	315.0507 / 179.0554 / 145.0496
Isorhamnetin 3-*O*-beta-(6-*O*-coumaroylglucoside)-7-*O*-beta-glucoside IV	$C_{37}H_{38}O_{19}$	64.4	785.1940	2.16	315.0507 / 179.0554 / 145.0496
Isorhamnetin 3-*O*-beta-(6-*O*-coumaroylglucoside)-7-*O*-beta-glucoside V	$C_{37}H_{38}O_{19}$	66.1	785.1940	2.16	315.0507 / 179.0554 / 145.0496
Isorhamnetin 3-ferulylrobinobioside	$C_{38}H_{40}O_{19}$	63.9	799.2096	2.00	315.0509
Quercetin derivatives					
Quercetin	$C_{15}H_{10}O_7$	56.4	301.0354	3.98	178.9978 / 151.0027
Quercetin hexosyl pentosyl rhamnoside	$C_{32}H_{38}O_{20}$	30.4	741.1895	3.10	301.0351 / 151.0391
Quercetin hexose pentoside	$C_{26}H_{28}O_{16}$	31.5	595.1311	3.02	463.0881 / 433.2078 / 415.0884
Quercetin 3-*O*-rutinoside (rutin)	$C_{27}H_{30}O_{16}$	35.6	609.1470	3.27	301.0353 / 145.0496
Quercetin hexose dirhamnoside	$C_{33}H_{40}O_{20}$	36.8	755.2040	1.46	609.1467 / 301.0349
Kaempferol derivatives					
Kaempferol	$C_{15}H_{10}O_6$	68.9	285.0404	3.85	
Kaempferol hexoside dirhamnoside I	$C_{33}H_{40}O_{19}$	34.3	739.2102	2.98	431.2286 / 285.0402
Kaempferol hexoside dirhamnoside II	$C_{33}H_{40}O_{19}$	35.3	739.2102	2.98	431.2286 / 285.0402
Kaempferol hexose pentose rhamnoside	$C_{32}H_{38}O_{19}$	35.9	725.1945	3.03	285.0401
Kaempferol hexose pentoside	$C_{26}H_{28}O_{15}$	37.5	579.1362	3.11	496.2458 / 285.0402
Kaempferol rutinoside I	$C_{27}H_{30}O_{15}$	41.7	593.1520	3.22	496.2455 / 285.0403
Kaempferol rutinoside II	$C_{27}H_{30}O_{15}$	47.3	593.1520	3.22	496.2455 / 285.0403
Kaempferol rutinoside III	$C_{27}H_{30}O_{15}$	48.0	593.1520	3.22	496.2455 / 285.0403

Kaempferol acetyl arabinopyranosyl hexoside	$C_{28}H_{30}O_{16}$	42.5	621.1450	2.74	503.2504 / 285.0402
Methoxy kaempferol hexoside	$C_{22}H_{22}O_{12}$	44.6	477.1027	2.52	314.0434 / 285.0406
Kaempferol acetyl hexoside	$C_{23}H_{22}O_{12}$	45.8	489.1039	2.46	445.1141 / 285.0406
Kaempferide 3,7 – dirhamnoside	$C_{28}H_{32}O_{14}$	58.3	591.1708	2.37	285.0402
Kaempferol coumaryl glucoside glucoside I	$C_{36}H_{36}O_{18}$	60.5	755.1834	2.25	285.0404 / 179.0554 / 161.0446
Kaempferol coumaryl glucoside glucoside II	$C_{36}H_{36}O_{18}$	61.4	755.1834	2.25	285.0404 / 179.0554 / 161.0446

R_t, retention time; m/z, mass-to-charge ratio; $[M-H]^-$, Negatively charged molecular ion

Objective 4

To evaluate the action of colonic microbiota on (poly)phenolic compounds of cactus (*Opuntia ficus-indica* Mill.) cladodes.

Evaluación de la acción de la microbiota intestinal sobre los compuestos (poli)fenólicos del nopal (*Opuntia ficus-indica* Mill.).

Objective 5

To evaluate the biological activity of colonic fermented cactus (*Opuntia ficus-indica* Mill.) cladodes on HT29 human colon cancer cells.

Evaluación de la actividad biológica de nopal (*Opuntia ficus-indica* Mill.) posterior a la fermentación colónica en células de cáncer de colon HT29.

Paper 4

Digestion and colonic fermentation of raw and cooked *Opuntia ficus-indica* impacts bioaccessibility and bioactivity

Elsy De Santiago, Chris I. R. Gill, Ilaria Carafa, Kieran M. Touhy, María-Paz De Peña and Concepción Cid.

Journal of Agricultural and Food Chemistry. (Under review).

TITLE: Digestion and colonic fermentation of raw and cooked *Opuntia ficus-indica* impacts bioaccessibility and bioactivity

AUTHORS: Elsy De Santiago[a], Chris I. R. Gill[b], Ilaria Carafa[c], Kieran M. Touhy[c], María-Paz De Peña[a] and Concepción Cid[a]*

[a] Universidad de Navarra, Facultad de Farmacia y Nutrición, Departamento de Ciencias de la Alimentación y Fisiología, C/ Irunlarrea 1, E-31008 Pamplona, Spain.

IdiSNA, Navarra Institute for Health Research. Pamplona, Spain.

[b] Nutrition Innovation Centre for Food and Health, Centre for Molecular Biosciences, University of Ulster, Cromore Road, Coleraine, Northern Ireland, BT52 1SA, United Kingdom.

[c] Nutrition & Nutrigenomics Unit, Department of Food Quality and Nutrition, Research and Innovation Centre, Fondazione Edmund Mach (FEM), Via E. Mach 1, 38010, San Michele all'Adige, Trento, Italy.

*Corresponding author: María-Paz de Peña. Tel: +34 948 425600 (806580); Fax: +34 948 425740. E-mail address: mpdepena@unav.es

ABSTRACT

The bioactivity of (poly)phenols from a food are interplay between the cooking methods applied and its interaction with the gastrointestinal tract. (Poly)phenolic profile and biological activity of cactus (*Opuntia ficus-indica* Mill.) cladodes following *in vitro* digestion and colonic fermentation were evaluated. Twenty-seven (poly)phenols were identified and quantified by HPLC-ESI-MS, with piscidic acid the most abundant. Throughout the colonic fermentation, flavonoids showed more degradation than phenolic acids, remained eucomic acid the most relevant after 24 h. The catabolite 3-(4-hydroxyphenyl)propionic acid was generated after 24 h of fermentation incubation. Cytotoxicity, genotoxicity and cell cycle analyses were performed in HT29 cells. Cactus colonic fermentates showed higher cell viability (80%) compared to the control fermentation with no cactus. Cactus colonic fermentates significantly ($p<0.05$) reduced H_2O_2-induced DNA damage in HT29 cells, comparing to the control one. Results suggest that, although phenolic compounds were degraded during the colonic fermentation, the biological activity is retained in colon cells.

KEYWORDS: Opuntia ficus-indica, Polyphenols, Gut microbiota, Antioxidant activity, DNA damage, cytotoxicity.

CHEMICAL COMPOUNDS STUDIED IN THIS ARTICLE: Ferulic acid (PubChem CID: 445858); Piscidic acid (PubChem CID: 6710641); Eucomic acid (PubChem CID: 23757219); Kaempferol (PubChem CID: 5280863); Isorhamnetin (PubChem CID: 5281654).

1. Introduction

Cactus (*Opuntia ficus-indica* Mill.) cladodes is an American native plant, which farming is slowly extending throughout the World. In Mexico, its annual consumption is 6.4 kg per capita [1], which could be eaten as different cooking styles (i.e., boiling, griddling, frying and microwaving). It is being recognized as a great source of (poly)phenols, predominating flavonoids as isorhamnetin, kaempferol and quercetin derivatives, as well as phenolic acids like piscidic and eucomic acids [2–4].

The dietary intake of (poly)phenolic compounds has been studied for decades because of their beneficial and protective effects on different chronic diseases such as cancer, diabetes and cardiovascular disease due to their antioxidant properties to reduce inflammation caused by oxidative stress [5]. After consumption, digestive conditions and enzymes affect (poly)phenols bioaccessibility of cactus cladodes [6]. Then, bioaccessible compounds can be absorbed through the stomach and small intestine, or most of them could reach the colon and be extensively catabolised by the human gut microbiota [7].

(Poly)phenols biotransformations can occur in the colon, in which the enzymes of the gut microbiota act to breakdown complex polyphenolic structures by hydrolysis, dehydroxylation, deconjugation, demethylation, ring cleavage and decarboxylation reactions. Some studies have shown the influence of colonic human microbiota on the phenolic profile and catabolites produced in some vegetables as pepper [8], and cardoon [9] as well as in coffee [10] and lingonberries [11] .

Otherwise, the remaining polyphenols which reach the colon could modulate metabolic pathways related to the gut microbiota and cell processes, as inflammation, immunity, cell proliferation and oxidative stress [12]. Recently, studies have reported the biological and antioxidant activity of (poly)phenols from raspberries after colonic fermentation on HT29 human colon cells reducing DNA damage [13]. Furthermore, extracts from *O. ficus-indica* juices of cactus pear fruits have also demonstrated antiproliferative effects on HT29 cells [14], even intestinal digestion and gut microbiota action were not considered.

In light of the scientific evidence about the influence of gut microbiota on (poly)phenolic compounds, and subsequently their biological activity, as well as the increasing interest on cactus cladodes potential bioactivity, the aim of the current study was to evaluate the effect of the human colonic microbiota on (poly)phenolic compounds of raw and cooked cactus *(O. ficus-indica)* cladodes, as well as their biological activity on HT29 colon cells.

2. Material and methods

2.1 Chemical and reagents

O. ficus-indica cactus cladodes were purchased from BioArchen company located in Murcia, Spain (August 2015). Olive and soybean oils were obtained from local markets. Methanol, acetone, acetonitrile (HPLC grade) and formic acid (HPLC grade) were purchased from Panreac (Barcelona, Spain). Potassium chloride and sodium chloride were obtained from Merck (Darmstadt, Germany). Human saliva α-amylase (852 U/mg protein), pepsin (674 U/mg), pancreatin (4xUPS), bile salts (for digestion), sodium hydrogen carbonate, potassium phosphate monobasic, magnesium sulfate monohydrate, were purchased from Sigma-Aldrich (Steinheim, Germany).

Chemicals for the batch culture nutrient medium for the *in vitro* fermentation as bile salts, yeast extract, buffered peptone water, sodium chloride, Tween 80, hemin, vitamin K, L-cystein hydrochloride monohydrate, resazurin redox indicator, sodium hydrogen carbonate, potassium phosphate monobasic, magnesium sulfate monohydrate, potassium hydrogen phosphate, calcium chloride hexahydrate were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Applichem (Darmstadt, Germany).

Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS) and penicillin streptomycin (Pen Strep) were obtained from Gibco Life Technologies Ltd (Paisley, Scotland, UK). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was acquired from Promega (Madison, USA). All other chemicals for tissue culture and biological activity assays were purchased from Sigma-Aldrich Company Ltd (Dorset, England, UK) unless otherwise specified.

2.2 Samples preparation

Cactus cladodes were washed and the thorns were manually removed. Then, they were cut into small pieces and divided into six portions (300 g for each one). One portion was used as raw sample and the other five were processed by the different cooking methods as shown in Table 1. Afterwards, each raw and cooked sample was lyophilized in a freeze dryer Cryodos-80 (Telstar, Terrasa, Spain) and stored at -18 °C.

2.3 In vitro gastrointestinal digestion and colonic fermentation

A simulated digestion model was performed according to Minekus et al. (2014) [15] and Monente et al. (2015) [16] adapted to our laboratory. Three steps were carried out to simulate the oral (with α-amylase), gastric (with pepsin at pH 3) and small intestine (with pancreatin and bile salts at pH 7) conditions. Details of the *in vitro* gastrointestinal process are described in De Santiago et al. (2018) [6]. Each cactus sample was digested in duplicate and then the two repetitions were mixed and homogenized. After intestinal digestion, samples were lyophilized in a freeze dryer Cryodos-80 (Telstar), and stored at -18 °C until *in vitro* fermentation process.

The digesta raw and cooked cactus cladodes were subjected to an *in vitro* fermentation with human faecal samples to simulate the condition present in the colon following the method described by Koutsos et al. 2017 [17] and briefly described below.

The composition for 1 L of growth medium was 2 g of peptone, 2 g of yeast extract, 0.1 g of NaCl, 0.04 g of K_2HPO_4, 0.04 g of KH_2PO_4, 0.01 g of $MgSO_4$ x $7H_2O$, 0.04 g of $CaCl_2$ x $6H_2O$, 2 g of $NaHCO_3$, 2 mL of Tween 80, 0.05 g of hemin dissolved in 1 mL of 4 M NaOH, 10 mL of vitamin K, 0.5 g of L-cysteine HCl, 0.5 g of bile salts and 4 mL of resazurin solution (0.025%, w/v) as an anaerobic indicator. The growth medium was sterilized at 121 °C for 15 min glass vessels (280 mL) before sample preparation.

The fermentation process of digesta raw and cooked cactus as well as the control (with no cactus cladodes) was developed using anaerobic, stirred, pH (5.3-5.7) and temperature controlled faecal batch cultures. Glass water-jacketed vessels were sterilized and filled aseptically with 67.5 mL of pre-sterilized basal nutrient medium. The medium was then gassed overnight with oxygen free nitrogen to maintain anaerobic conditions. The following day and before the inoculation, each vessel was dosed with 0.75 g of digesta cactus, for a final concentration of 1% (w/v). Fresh human faecal samples were collected in an anaerobic jar from three healthy faecal donors who were free of any known metabolic and gastrointestinal diseases, were not taking probiotic or prebiotic supplements, followed a polyphenol-free diet for 2 days and had not taken antibiotics 3 months before faecal collection. Faecal slurry was prepared by homogenizing the faeces in pre-reduced phosphate buffered saline (PBS). The temperature was set to 37 °C using a circulating water-bath and the vessels were inoculated with 7.5 mL faecal slurry (10% w/v of fresh human faeces) for a period of 24 h, during which samples were collected at 4 time points (0, 5, 10 and 24 h). Samples were centrifuged at 13,500 rpm at 4 °C for 5 min and stored at −80 °C for further analysis.

2.4 Identification and quantification of (poly)phenolic compounds by HPLC-ESI-MS

(Poly)phenolic compounds from fermented raw and cooked cactus cladodes were extracted following the method described in Juániz et al. (2017) [9]. Briefly, 0.1 mL of methanol/acidified water (0.1% formic acid) (80:20 v/v) was added to 0.1 mL of fermented samples, vortexed and centrifuged at 14,000 rpm for 10 min. Then, supernatants were transferred to vials to carry out HPLC-ESI-MS analysis, which was performed using an HPLC unit model 1200 (Agilent Technologies, Palo Alto, CA, USA) equipped with a Triple Quadrupole Linear Ion Trap Mass Spectrometer 3200 Q-TRAP

(AB Sciex, Framingham, MA, USA). The column used was a CORTECS® C18 (3x75 mm, 2.7 µm) from Waters (Milford, MA, USA).

For HPLC separation, mobile phase A was 0.1% (v/v) formic acid in water and mobile phase B was acetonitrile. Separations were carried out with an injection volume of 4 µL, column oven temperature of 30 °C and elution flow rate of 0.35 mL/min. The mobile phases comprised a program of 0–1.20 min, 5% B; 1.20–8.80 min, 5–11.4% B; 8.80-10 min, 11.4–11% B; 10–30 min, 11-30% B; 30-32 min, 28-100% B and then return to 5% B in 2 min and maintained isocratic until the end of the analysis (38 min) to re-equilibrate the column. Mass analyses were performed in negative ionization mode, with the turbo heater maintained at 500 °C and Ion Spray voltage set at -3500. Nitrogen was used as nebulizing, turbo heater and curtain gas and was set at the pressure of 40, 50 and 35 psi, respectively. Chromatograms were acquired using Analyst software 1.6.3 (AB Sciex, Framingham, MA, USA).

For the identification of the phenolic compounds, a preliminary analysis was carried out in a full scan from 100 to 1000 m/z, and a consecutively selective product ion mode analysis with specific m/z. Identification was achieved by multiple reaction monitoring (MRM). Two transitions were studied for each phenolic compound. The first transition, corresponding to the most abundant fragment, was used as quantifier ion, and the second as qualifier ion. Details of the (poly)phenols identification are shown in the Supporting Information Table S1 (Supplementary Material).

Phenolic acids were expressed as ferulic acid equivalents, whereas isorhamnetin and kaempferol derivatives were quantified with their respective aglycones using calibration curves. Results were expressed as milligrams of each compound per millilitre of batch culture (µg /mL).

2.5 Biological activity assays

2.5.1 Tissue culture

Biological activity of fermented cactus cladodes was performed using HT29 human colorectal adenocarcinoma cells acquired from the European Collection of Cell Cultures (ECACC). Cells were grown in tissue culture flasks and maintained in DMEM supplemented with FBS (10%) and Pen Strep (1%). They were incubated at 37 °C with 5% CO_2 and sub-cultured every 2 days by the addition of trypsin (0.25% trypsin-EDTA) for 10 min at 37 °C and centrifuged at 1,200 rpm for 3 min. After that, the supernatant was decanted and cells were re-suspended in the appropriate medium. Twenty-four h was selected as the exposure time for all *in vitro* studies with fermented samples, as it is generally considered to reflect the average colonic transit time.

2.5.2 Cytotoxicity assay

Raw and cooked cactus cladodes colonic fermentates were diluted in DMEM at 10% and 20% (v/v) to evaluate cytotoxicity by the MTT colorimetric assay, following the method described by McDougall et al. (2017) [13]. First, HT29 cells were seeded in 96 multi-well plates (Costar, Cambridge, MA, USA) at a concentration of 1.5 x 10^4 cells per well. After 2 days of incubation at 37 °C, media was replaced with fermented raw and cooked cactus samples at 10% and 20% (v/v) and then incubated for 24 h. The wells were washed and refreshed with media. Thereafter, 15 μL of MTT (5 mg/ mL) were added to each well. After 4 h at 37 °C, lysis was carried out with 100 μL of solubilizing solution and measured using a plate reader (Alpha, SLT Rainbow Thermo, Antrim, UK) at a wavelength of 570 nm, reference 650 nm. Each sample was measured in octuple and results are presented as percentage cell viability compared to untreated cells. The experiment was repeated independently three times.

2.5.3 Genotoxicity assay (Comet assay)

The effect of colonically fermented raw and cooked cactus on colonocyte DNA damage was determined using the Comet assay according to the method described by McDougall et al. (2017) [13]. HT29 cells were pre-incubated with the samples for 24 h. The anti-genotoxic effect was assessed by treating pre-incubated HT29 cells with hydrogen peroxide (75 μM H_2O_2), or with PBS as their respective control. Briefly, the cells were reconstituted in 85 μL of 0.85% low melting point agarose in PBS and maintained in a water bath at 37 °C. This suspension was mixed with 1% normal agarose to previously prepared gels on frosted slides and coverslips were added. The slides were subjected to lysis buffer (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM TRIS) for 1 h at 4 °C and placed in electrophoresis buffer for 20 min at 25 V 300 mA. Subsequently, slides were washed (3 × 5 min) in neutralisation buffer (0.4 M TRIS HCl, pH = 7.5) at 4 °C. All slides were stained with ethidium bromide (20 μL of 20 μg/mL) prior to scoring. Images were analysed at 400 × magnification using a Nikon eclipse 600 epifluorescence microscope. The percentage (%) tail DNA was recorded using Komet 5.0 image analysis software (Kinetic Imaging Ltd, Liverpool, UK). For each slide, 50 cells were scored and the mean was calculated. Results are presented as mean percentage (%) tail DNA. The experiment was repeated independently 3 times and positive (H_2O_2) and negative controls (PBS) were included in all experiments, as well as cells without fermented treatment.

2.5.4 Cell cycle analysis

Cell cycle analysis of fermented raw and cooked cactus was determined using propidium iodide staining and measured using flow cytometry (Omerod, 2000). Briefly, HT29 cells

were seeded at a density of 1 x 10^6 cells per mL for 24 h before treatment with each fermented extract at 10%. After fermentate treatment exposure, the suspension was centrifuged at 1,200 rpm for 5 min, the supernatant discarded and the pellet re-suspended in 1 mL of ice cold PBS. Then, the suspension was centrifuged at 4,000 rpm for 5 min, the supernatant was discarded and the cells were re-suspended in 1 mL of ice cold ethanol/PBS (90:10) and incubated at -20 °C overnight. After incubation, cells were washed twice with ice cold PBS and the staining buffer (100 μL/mL of RNase A, 10 μL/mL of propidium iodide, 0.1% NP40 and 50 μL/mL tri sodium citrate in PBS) was added. The cells were then incubated at 37 °C, 5% CO_2 for 30 min before being analysed on a FACS Calibur flow cytometer (Becton Dickinson, UK) and the fluorescence emission spectra of propidium iodide was collected at 585 nm, using Beckman-Coulter Gallios flow cytometer. Subsequently these emission spectra were analysed for DNA content using WinMDI software (J. Trotter, Scripps Inst.). The experiment was performed as triplicate independent experiments and results are presented as a percentage (%) of cell count.

2.11 Statistical analysis

The mean of each data set was used for statistical analysis. The Shapiro-Wilk test was used to test for normality. Analysis of variance was applied to test for significant differences between means using one-way analysis of variance (ANOVA, Dunnett T and T3 tests). A Bonferroni test was applied as *a posteriori* test for significant differences among fermentation time points. Significance was accepted at $p < 0.05$. Analysis was carried out using SPSS (version 20 for Windows).

3. Results and discussion

The effect of the human gut microbiota on (poly)phenolic compounds of raw and cooked cactus *(O. ficus-indica)* cladodes, as well as their biological activity on colonic cells, has been evaluated for the first time, to the best of our knowledge.

3.1 Colonic catabolism of (poly)phenolic compounds

The (poly)phenolic composition of fermentates over time is reported in Table 2. Twenty-seven (poly)phenolic compounds were identified and quantified in baseline (0 h) of fermented cactus cladodes, being phenolic acids the most abundant in all samples, accounted for 78-88% of the total content. Piscidic acid was the main compound (52-65% total (poly)phenolic compounds), followed by eucomic acid (15-36% total (poly)phenolic compounds). Griddled cactus cladodes presented the highest amount of piscidic acid, and microwaved samples had higher amount of eucomic acid than the rest of cooking treatments. Flavonoids were also present in cactus cladodes colonic fermentates, being kaempferol derivatives (6-13% total) and isorhamnetin derivatives (4-7% total) mainly in their glycosides forms. Griddled cactus cladodes presented the

highest amount of kaempferol derivatives whilst microwaved samples contributed with the highest isorhamnetin derivatives content at the beginning of colonic fermentation (0 h). These (poly)phenolic profiles are consistent with those of previous studies in *O. ficus-indica* cactus cladodes without gastrointestinal digestion [2,4]. In a previous work in cactus cladodes after gastrointestinal digestion, quercetin derivatives were also identified [6]. Nevertheless, in this study they were not detected at any time point of the fermentation, may be due to the rapid metabolism of quercetin glycosides at the starting of colonic fermentation. It has been previously demonstrated a fast degradation within the first 15 min after colonic fermentation in quercetin derivatives from raw and cooked pepper [8] as well as in apples [17].

Changes in the amount of (poly)phenols throughout the faecal fermentations were observed. Even though a degradation of the compounds was shown, some (poly)phenols were still present after 24 h of faecal fermentation. Both kaempferol and isorhamnetin derivatives were rapidly catabolised by human microbiota, showing low amounts, traces or even no detected at the end of the 24 h of colonic fermentation (Fig. 1a). In particular, isorhamnetin hexose rhamnose hexoside II and III, isorhamnetin di-hexoside II, isorhamnetin hexose rhamnose hexoside I, kaempferol rutinoside III and kaempferol glucoside II were completely degraded in most of the samples after 24 h of colonic fermentation, except in griddled and microwaved, in which remained available or in traces.

Since the first step of the faecal incubation, the hydrolysis of flavonoid glycosides and the consequent formation of aglycones took place, especially from the isorhamnetin derivatives. The highest amount of isorhamnetin was detected in microwaved samples after 10 h of faecal incubation but then it decreased. Kaempferol derivatives were also degraded after 24 h fermentation, but their respective aglycone was not detected due to the low amount of its native (poly)phenols. Some increases in flavonoids during the fermentation were also observed, may be due to the release from the food matrix. Particularly, methoxy kaempferol hexoside I increased after 5 and 10 h of incubation in all samples, except in both fried samples. Moreover, although at the end of the 24 h fermentation it decreased, the final amount was higher than in the beginning in raw, microwaved and griddled cactus cladodes.

With respect to phenolic acids, eucomic acid was the most stable during the 24 h of faecal incubation (Fig. 1b). Although it was degraded, it still remained in high amounts in all samples, especially in microwaved and griddled ones (Table 2). Conversely, piscidic acid increased after 5 h of incubation in all samples; but was almost completely degraded after 24 h. However, the degradation was lower in griddled samples. Likewise,

feruloylglucose I and II were rapidly degraded after 24 h of faecal fermentation, but remained in traces in griddled and microwaved cactus cladodes.

Eucomic acid were more stable under colonic conditions probably due to the resistance to gastrointestinal and colonic microflora enzymes, as well as the release from the cell walls by the microbiota action [18]. However, ferulic acid derivatives were rapidly degraded in all samples may be due to the catabolism related to microbial feruloyl esterases which are present in the colonic inoculum and degraded the compounds [19].

After 24 h colonic fermentation, the catabolite 3-(4-hydroxyphenyl)propionic acid was found in trace amounts maybe produced via demethoxylation from ferulic acid derivatives [10], as well as via dehydroxylation of 3-(3,4-dihydroxyphenyl)propionic acid derived from ferulic acid and the ring fission of quercetin, isorhamnetin and kaempferol derivatives [8,9,20].

Metabolic products depend on (poly)phenolic compounds present and the interindividual variation in the gut microbial composition and enzymatic capacities leading to different catabolite profile [10]. However, in the present study 3-(3,4-dihydroxyphenyl)propionic acid was present in all the volunteers, even in traces. Further investigation is needed to evaluate the absorption of bioactive compounds from cactus cladodes taking into account the gastrointestinal digestion and microbiota conditions.

3.4 Biological activity in colon cells

The biological activity of cactus cladodes submitted to the most common culinary techniques (boiling, griddling and frying) in human colorectal cells was evaluated after gastrointestinal digestion and the action of gut microbiota in HT29 cells to simulate physiological conditions. Raw samples and control (with no cactus) fermentates were used as positive and negative controls.

No cytotoxic activity was observed in HT29 cells treated with fermented raw and cooked cactus cladodes at 10% and 20% (v/v) after 24 h of incubation, compared to untreated cells (Fig. 2). Likewise, no significant differences ($p<0.05$) were observed among cooking treatments. Nevertheless, cell viability was significant lower ($p<0.05$) in control fermentation (with no cactus cladodes) at 20% (v/v) than in untreated cells and did not present a cell viability higher tan 80%. Therefore, a 10% (v/v) concentration was used for the comet and cell cycle assays. Up to our knowledge, cytoxicity of cactus cladodes after colonic fermentation has not been previously evaluated. Similarly, Serra et al. (2013) [14] reported no cytotoxic activity of *O. ficus-indica* fruit juices in a Caco-2 human colon cell model.

The effect of cactus cladodes colonic fermentates against DNA damage induced by oxidation in colonocytes was assessed by the comet assay (Fig. 3). The percentage of DNA in the tail induced by H_2O_2 was significantly ($p<0.05$) reduced by raw and cooked cactus cladodes colonic fermentates (34-38%) in comparison with the control fermentate (with no cactus cladodes) (45%). The reduction in the H_2O_2-induced DNA damage of fermented cactus cladodes may be attributed to the (poly)phenolic compounds which still remained after colonic fermentation, especially eucomic acids. Similarly, studies in digested blackcurrant [21] and elderberry [22] have observed a reduction in H_2O_2-induced DNA damage in NCM460 (non-tumorigenic colon cell line) and in Caco-2 cells. However, the role of fermentation on the bioactivity of the extracts was not considered. A similar consideration to this study was those reported with colonic fermented raspberry, strawberry and blackcurrant which exerted a significant ($p<0.05$) reduction of H_2O_2-induced DNA damage in HT29 cell line [23,24], demonstrating that antioxidant activity, and specifically potential antigenotoxic activity of (poly)phenols is retained after colonic fermentation.

Finally, the effect of cactus cladodes colonic fermentates on the HT29 cell cycle was evaluated (Fig. 4). No significant effect ($p<0.05$) was observed in each stage of the HT29 cell cycle after 24 h of incubation with colonic fermented cactus cladodes. No significant differences ($p<0.05$) were also observed among fermented samples. Results are consistent with Coates et al. (2007) who reported not significant changes in cell cycle proliferation of HT29 cells pre-treated with colonic fermented berries. In contrast, extracts from *O. ficus-indica* fruit juice induced a cell cycle arrested in the G1 phase in HT29 cells [14], attributing this effect to the betalains.

In summary, results of the *in vitro* colonic fermentation of raw and cooked cactus cladodes showed that flavonoids tended to be degraded more than phenolic acids, but they are still available after 24 h of colonic incubation, especially eucomic acid. Results also suggest that remained (poly)phenols after the action of colonic microbiota still have antioxidant and genoprotective properties which can exert into the intestinal cells. Therefore, the current study provides a basis for further investigations about the cactus cladodes bioactive components potentially available to be absorbed, and to exert their beneficial effects in colon and other target organs after ingestion and to understand their mechanism of action *in vivo*.

Acknowledgments

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Figure caption

Figure 1. Degradation of (poly)phenolic compounds of griddled cactus cladodes during 24 h colonic fermentation. a) Total kaempferol derivatives, total isorhamnetin derivatives and isorhamnetin aglycone. b) Total phenolic acids, piscidic acid and eucomic acid.

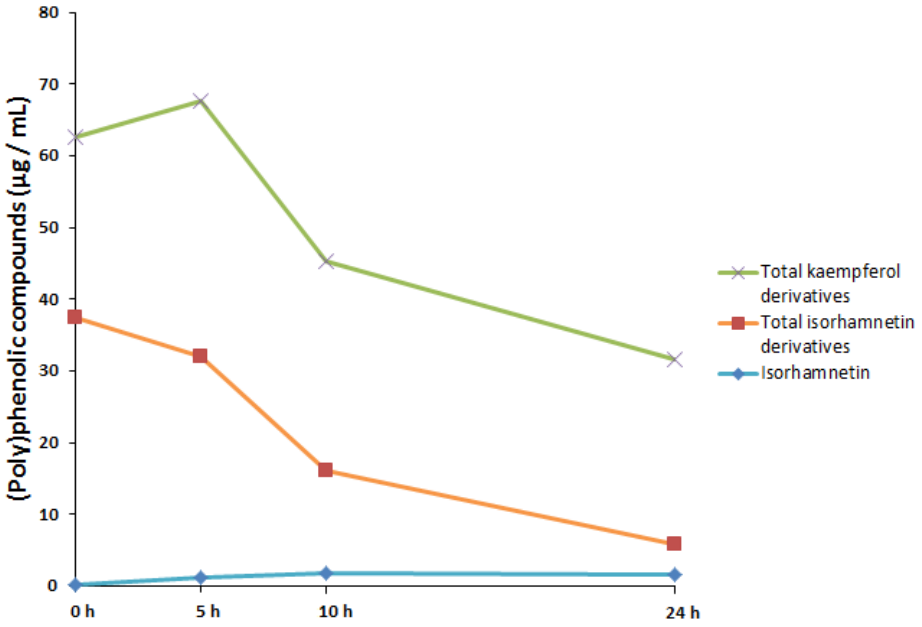
Figure 2. The cytotoxic effect of 20% (v/v) and 10% (v/v) fermented cactus cladodes on HT29 cells. Data are presented as means of 3 independent experiments +/- SD compared to the untreated cells as a control. One-way ANOVA and *Post Hoc* test Dunnett's T *p<0.05.

Figure 3. Antigenotoxic activity of fermented cactus cladodes 10% (v/v) on DNA damage in HT29 cells challenged with 75 µM H_2O_2. Data are presented as means of 3 independent experiments +/- SD compared to the control fermentation. One-way ANOVA and *Post Hoc* test Dunnett's T *p<0.05.

Figure 4. Effects of fermented cactus cladodes 10 % (v/v) on HT29 cell cycle. Data are presented as means of 3 independent experiments +/- SD compared to the untreated cells as a control.

Figure 1

a)


(Poly)phenolic compounds (µg / mL)
80
70
60
50
40
30
20
10
0
0 h
5 h
10 h
24 h
Total kaempferol derivatives
Total isorhamnetin derivatives
Isorhamnetin

b)

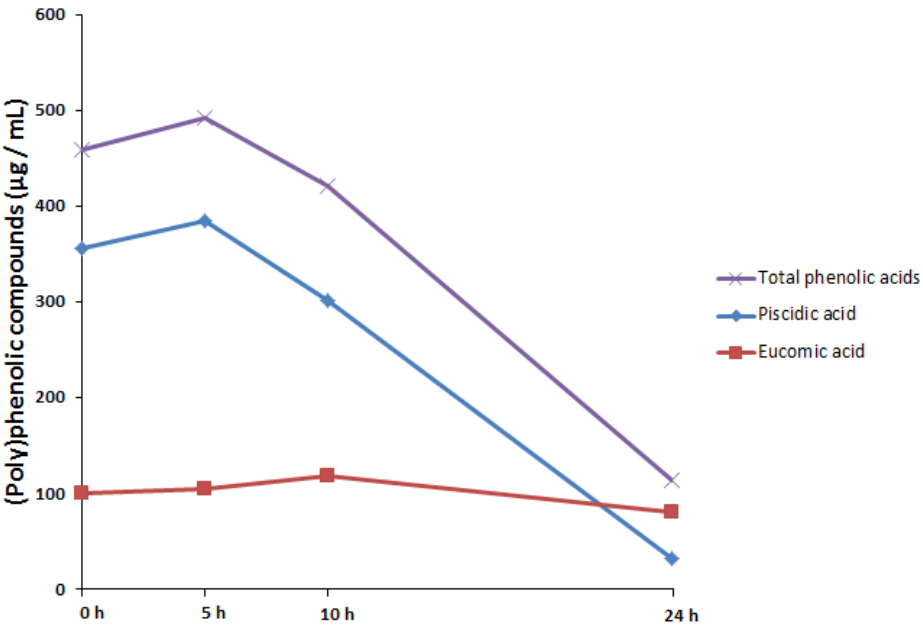

(Poly)phenolic compounds (µg / mL)
600
500
400
300
200
100
0
0 h
5 h
10 h
24 h
Total phenolic acids
Piscidic acid
Eucomic acid

Figure 2

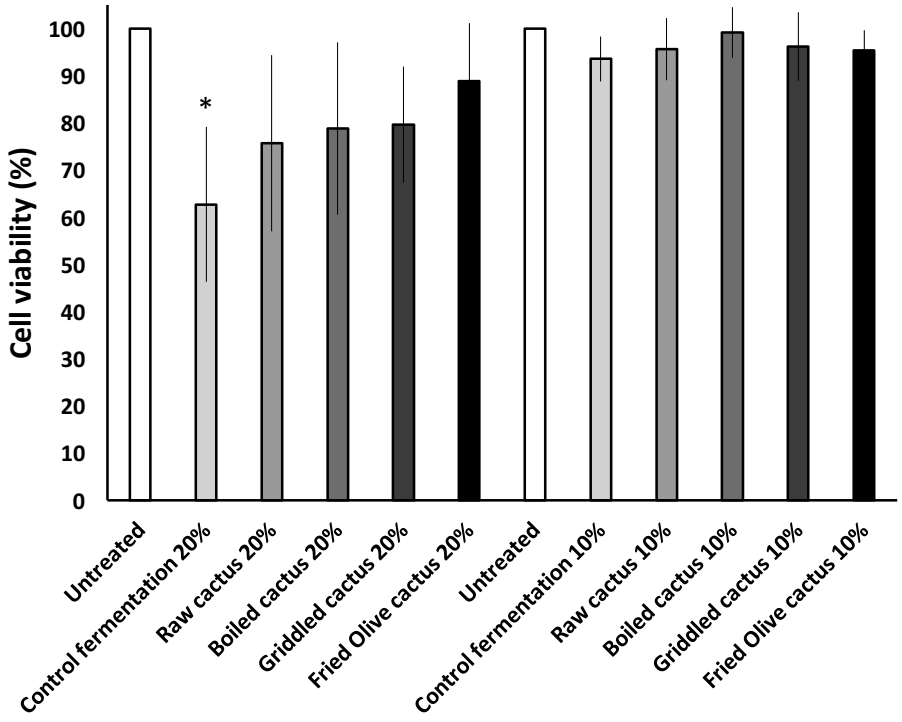

Cell viability (%)
100
90
80
70
60
50
40
30
20
10
0
*
Untreated
Control fermentation 20%
Raw cactus 20%
Boiled cactus 20%
Griddled cactus 20%
Fried Olive cactus 20%
Untreated
Control fermentation 10%
Raw cactus 10%
Boiled cactus 10%
Griddled cactus 10%
Fried Olive cactus 10%

Figure 3

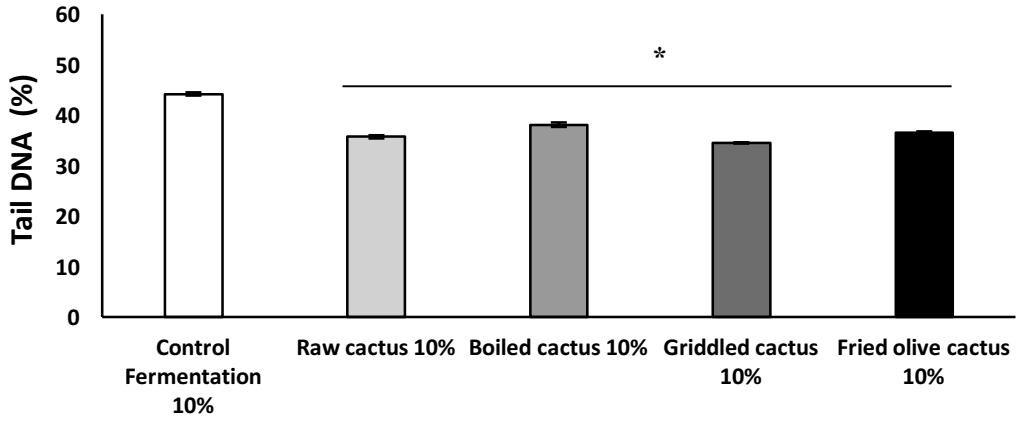

60
50
40
30
20
10
0
Tail DNA (%)
*
Control Fermentation 10%
Raw cactus 10%
Boiled cactus 10%
Griddled cactus 10%
Fried olive cactus 10%

Figure 4

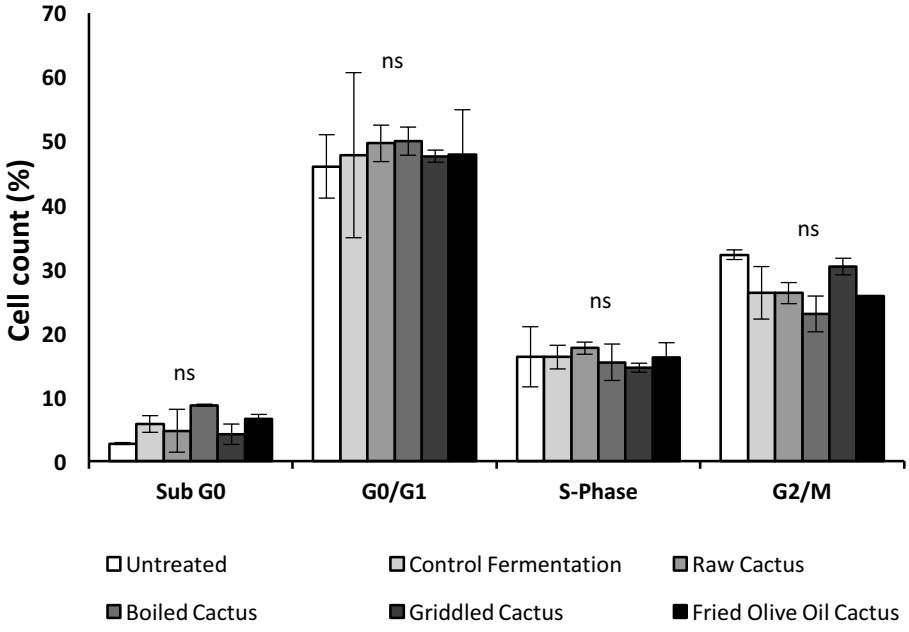

70
60
50
40
30
20
10
0
Cell count (%)
ns
ns
ns
ns
Sub G0
G0/G1
S-Phase
G2/M
Untreated
Control Fermentation
Raw Cactus
Boiled Cactus
Griddled Cactus
Fried Olive Oil Cactus

Table 1. Cooking Conditions Applied to Cactus Cladodes.

Cooking technique	Time	Temperature	Amount of water or oil	Equipment
Boiling	10 min	100 °C	600 mL	Stainless steel pot
Microwaving	5 min	-	-	Silicone case (Lékué, Barcelona, Spain) and microwave oven (Whirlpool, Michigan, USA) at 900W
Griddling	5 min & 5 min	150 °C & 110 °C	-	Non-stick griddle (Jata Electro, Vizcaya, Spain)
Frying in olive/ soybean oil	10 min & 5 min	100 °C & 90 °C	30 mL	Non-stick frying pan

Table 1. Native (Poly)phenolic Compounds and Catabolites Produced During Colonic Fermentation of Raw and Cooked Cactus Cladodes. Results are Expressed as Mean ± Standard Deviation (µg (Poly)phenolic Compound/mL) (n=3).

Compounds	Raw	Boiled	Microwaved	Griddled	Fried in olive oil	Fried in soybean oil
	µg /mL	µg /mL	µg /mL	µg /mL	µg /mL	µg /mL
Phenolic acids						
Piscidic acid						
0 h	250.59 ± 76.17	140.53 ± 24.75	333.09 ± 32.90	355.75 ± 11.86	182.60 ± 8.31	177.79 ± 46.77
5 h	265.01 ± 33.27	159.95 ± 23.06	422.39 ± 21.15	384.77 ± 17.70	205.54 ± 12.26	212.97 ± 96.91
10 h	249.05 ± 86.67	140.77 ± 73.26	431.91 ± 11.54	302.27 ± 93.99	137.83 ± 85.08	147.98 ± 98.80
24 h	0.46 ± 0.15	0.12 ± 0.05	1.47 ± 0.56	32.31 ± 6.42	0.16 ± 0.09	0.45 ± 0.12
Feruloylglucose I						
0 h	0.95 ± 0.51	0.54 ± 0.30	1.17 ± 0.29	0.84 ± 0.13	0.41 ± 0.18	0.54 ± 0.29
5 h	0.15 ± 0.20	0.07 ± 0.03	0.76 ± 0.03	0.28 ± 0.20	0.06 ±0.09	tr
10 h	0.01 ± 0.00	nd	0.03 ± 0.01	0.60 ± 0.23	tr	tr
24 h	nd	nd	tr	tr	tr	tr
Feruloylglucose II						
0 h	1.14 ± 0.43	0.70 ± 0.27	1.12 ±0.19	1.33 ± 0.25	0.77± 0.17	0.81 ± 0.24
5 h	0.48 ± 0.57	0.28 ± 0.36	1.06 ± 0.41	0.77 ± 0.43	tr	0.16 ± 0.21
10 h	0.01 ± 0.00	tr	0.24 ± 0.29	0.60 ± 0.23	nd	nd
24 h	nd	nd	tr	tr	nd	nd
Eucomic acid						
0 h	178.06 ± 51.20	33.01 ± 17.84	161.75 ± 12.80	100.91 ± 15.31	54.86 ± 3.96	64.91 ± 10.16
5 h	163.38 ± 43.33	56.99 ± 27.89	184.41 ± 19.23	105.63 ± 30.81	57.13 ± 13.20	55.89 ± 15.63
10 h	105.72 ± 16.66	58.21 ± 25.39	208.89 ± 11.54	118.23 ± 25.60	52.14 ± 16.07	53.66 ± 10.49
24 h	71.50 ± 30.68	42.98 ± 2.31	154.08 ± 2.88	81.09 ± 18.66	35.29 ± 12.68	36.31 ± 19.81
TOTAL PHENOLIC ACIDS						
0 h	**430.75 ± 126.65**	**174.78 ± 66.34**	**497.12 ± 158.46**	**458.83 ± 167.45**	**238.64 ± 85.86**	**244.05 ± 83.53**
5 h	**429.01 ± 130.27**	**217.29 ± 75.34**	**608.63 ± 199.85**	**491.44 ± 181.50**	**259.72 ± 104.41**	**269.02 ± 110.35**
10 h	**354.92 ± 117.91**	**198.98 ± 58.37**	**641.07 ± 206.10**	**421.18 ± 142.59**	**189.97 ± 60.59**	**201.64 ± 66.69**
24 h	**71.96 ± 50.23**	**43.10 ± 30.31**	**155.55 ± 107.92**	**113.40 ± 34.50**	**35.45 ± 24.84**	**36.77 ± 25.36**
Isorhamnetin derivates						
Isorhamnetin						
0 h	0.11 ± 0.14	0.18 ± 0.21	0.28 ± 0.42	0.22 ± 0.35	0.20 ± 0.28	0.15 ± 0.26
5 h	1.37 ± 0.86	1.30 ± 0.86	0.78 ± 0.80	1.17 ± 0.64	0.75 ± 0.35	0.75 ± 0.56
10 h	0.84 ± 0.69	1.38 ± 0.58	4.74 ± 2.01	1.85 ± 1.50	1.58 ± 1.68	1.08 ± 0.34
24 h	0.73 ± 0.93	0.68 ± 0.55	2.89 ± 2.49	1.63 ± 0.26	1.77 ± 1.24	0.49 ± 0.46

Isorhamnetin hexose rhamnose hexoside I						
0 h	0.02 ± 0.00	tr	0.02 ± 0.01	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
5 h	tr	tr	0.03 ± 0.00	0.01 ± 0.00	tr	tr
10 h	tr	nd	0.01 ± 0.00	tr	tr	tr
24 h	nd	nd	tr	tr	tr	nd
Isorhamnetin hexose rhamnose hexoside II						
0 h	tr	tr	tr	tr	tr	tr
5 h	tr	tr	tr	tr	tr	tr
10 h	tr	nd	tr	tr	tr	tr
24 h	nd	nd	nd	nd	nd	nd
Isorhamnetin hexose rhamnose hexoside III						
0 h	tr	tr	0.01 ± 0.00	0.01 ± 0.00	tr	tr
5 h	tr	tr	tr	tr	tr	tr
10 h	tr	tr	tr	tr	tr	tr
24 h	nd	nd	nd	nd	nd	nd
Isorhamnetin di-hexoside I						
0 h	0.15 ± 0.09	0.12 ± 0.06	0.24 ± 0.025	0.22 ± 0.02	0.12 ± 0.01	0.13 ± 0.05
5 h	0.14 ± 0.12	0.13 ± 0.04	0.35 ± 0.02	0.22 ± 0.07	0.10 ± 0.06	0.15 ± 0.01
10 h	0.09 ± 0.11	0.07 ± 0.07	0.27 ± 0.07	0.14 ± 0.10	0.06 ± 0.05	0.14 ± 0.01
24 h	0.06 ± 0.08	0.01 ± 0.02	0.15 ± 0.12	0.01 ± 0.01	0.04 ± 0.05	nd
Isorhamnetin di-hexoside II						
0 h	tr	tr	0.01 ± 0.00	0.01 ± 0.00	tr	tr
5 h	tr	tr	tr	tr	tr	tr
10 h	tr	tr	tr	tr	tr	tr
24 h	nd	nd	nd	nd	nd	nd
Isorhamnetin rutinoside rhamnoside						
0 h	0.17 ± 0.10	0.10 ± 0.03	0.25 ± 0.11	0.24 ± 0.08	0.14 ± 0.05	0.13 ± 0.07
5 h	0.09 ± 0.11	tr	0.35 ± 0.00	0.20 ± 0.08	0.10 ± 0.03	0.08 ± 0.07
10 h	0.08 ± 0.07	tr	0.25 ± 0.07	0.10 ± 0.07	tr	0.06 ± 0.05
24 h	tr	nd	0.07 ± 0.05	tr	tr	nd
Isorhamnetin rutinoside rhamnoside II						
0 h	0.18 ± 0.11	0.12 ± 0.04	0.23 ± 0.10	0.22 ± 0.07	0.12 ± 0.03	0.13 ± 0.09
5 h	0.14 ± 0.12	0.12 ± 0.09	0.38 ± 0.01	0.23 ± 0.09	0.08 ± 0.07	0.15 ± 0.00
10 h	0.09 ± 0.11	tr	0.34 ± 0.01	0.19 ± 0.09	0.03 ± 0.03	0.15 ± 0.00
24	0.02 ± 0.03	tr	0.20 ± 0.11	0.04 ± 0.06	tr	nd
Isorhamnetin hexose pentoside						
0 h	2.36 ± 1.83	2.29 ± 1.40	4.63 ± 2.12	4.39 ± 1.88	2.57 ± 1.19	2.77 ± 2.06
5 h	1.73 ± 1.57	1.63 ± 1.85	8.08 ± 1.22	3.92 ± 1.76	1.95 ± 1.60	3.40 ± 1.26
10 h	1.25 ± 1.48	0.85 ± 1.16	6.54 ± 1.71	1.69 ± 0.27	0.49 ± 0.63	2.84 ± 1.13
24 h	0.30 ± 0.48	tr	2.71 ± 0.22	0.94 ± 0.77	tr	tr

Isorhamnetin rutinoside I						
0 h	0.71 ± 0.52	0.83 ± 0.65	0.58 ± 0.13	0.60 ± 0.17	0.42 ± 0.14	0.42 ± 0.15
5 h	0.82 ± 0.76	0.72 ± 0.20	1.39 ± 0.56	1.06 ± 0.52	0.80 ± 0.36	0.66 ± 0.67
10 h	0.86 ± 1.18	0.53 ± 0.01	2.02 ± 1.12	1.17 ±1.01	0.59 ± 0.53	0.91 ± 0.78
24 h	0.30 ± 0.48	tr	2.11 ± 0.01	1.03 ± 0.83	0.22 ± 0.23	0.02 ± 0.03
Isorhamnetin rutinoside II						
0 h	19.12 ± 9.09	12.00 ± 6.72	34.58 ± 7.56	30.53 ± 9.75	13.26 ± 3.40	17.02 ± 5.31
5 h	6.15 ± 5.72	1.97 ± 0.95	30.61 ± 8.67	24.51 ± 11.16	8.36 ± 2.98	7.57 ± 3.53
10 h	1.76 ± 1.05	0.20 ± 0.07	12.80 ± 10.27	10.65 ± 4.06	0.79 ± 0.28	3.42 ± 3.13
24 h	0.07 ± 0.01	tr	0.51 ± 0.51	2.02 ± 1.41	tr	tr
Unknown isorhamnetin derivative						
0 h	0.75 ± 0.53	0.41 ± 0.26	1.00 ± 0.58	0.89 ± 0.49	0.54 ± 0.32	0.56 ± 0.44
5 h	0.33 ± 0.42	0.20 ± 0.27	1.37 ± 0.15	0.71 ± 0.36	0.40 ± 0.03	0.49 ± 0.30
10 h	0.22 ± 0.13	tr	1.07 ± 0.22	0.31 ± 0.15	0.05 ± 0.03	0.35 ± 0.20
24 h	nd	nd	0.24 ± 0.17	0.16 ± 0.08	tr	nd
TOTAL ISORHAMNETIN DERIVATES						
0 h	**23.57 ± 6.23**	**16.04 ± 3.90**	**41.81 ± 10.29**	**37.33 ± 9.09**	**17.36 ± 4.32**	**21.32 ± 5.56**
5 h	**10.78 ± 2.04**	**6.06 ± 0.77**	**43.34 ± 9.99**	**32.02 ± 7.95**	**12.53 ± 2.68**	**13.25 ± 2.62**
10 h	**5.19 ± 0.63**	**3.03 ± 0.53**	**28.03 ± 4.29**	**16.11 ± 3.40**	**3.61 ± 0.55**	**8.97 ± 1.31**
24 h	**1.49 ± 0.26**	**0.70 ± 0.48**	**8.87 ± 1.24**	**5.84 ± 0.80**	**2.03 ± 0.95**	**0.52 ± 0.33**
Kaempferol derivates						
Kaempferol hexoside dirhamnoside I						
0 h	0.24 ± 0.16	0.18 ± 0.09	0.40 ± 0.21	0.32 ± 0.14	0.20 ± 0.07	0.20 ± 0.12
5 h	0.16 ± 0.21	0.16 ± 0.21	0.65 ± 0.06	0.30 ± 0.15	0.23 ± 0.05	0.23 ± 0.00
10 h	0.17 ± 0.18	tr	0.58 ± 0.08	0.17 ± 0.13	0.05 ± 0.04	0.05 ± 0.04
24 h	nd	nd	0.32 ± 0.18	0.12 ± 0.16	tr	nd
Kaempferol hexoside dirhamnoside II						
0 h	0.14 ± 0.10	0.09 ± 0.05	0.17 ± 0.11	0.15 ± 0.07	0.10 ± 0.05	0.10 ± 0.06
5 h	0.07 ± 0.07	tr	0.29 ± 0.02	0.13 ± 0.08	0.06 ± 0.04	0.09 ± 0.01
10 h	0.05 ± 0.07	tr	0.20 ± 0.01	0.06 ± 0.05	0.13 ± 0.22	0.05 ± 0.04
24 h	tr	nd	0.04 ± 0.05	0.04 ± 0.05	nd	nd
Kaempferol hexose pentoside						
0 h	1.28 ± 0.88	0.83 ± 0.49	1.91 ± 1.08	1.38 ± 0.66	0.77 ± 0.36	0.88 ± 0.67
5 h	0.69 ± 0.71	0.51 ± 0.67	2.63 ± 0.12	1.09 ± 0.50	0.50 ± 0.44	1.02 ± 0.34
10 h	0.35 ± 0.41	0.26 ± 0.36	2.06 ± 0.05	0.55 ± 0.36	0.20 ± 0.20	0.68 ± 0.26
24 h	0.08 ± 0.14	0.01 ± 0.02	0.93 ± 0.18	0.12 ± 0.16	tr	tr
Kaempferol rutinoside I						
0 h	0.10 ± 0.07	0.07 ± 0.06	0.11 ± 0.06	0.09 ± 0.04	0.07 ± 0.05	0.08 ± 0.04
5 h	0.18 ± 0.07	0.19 ± 0.01	0.20 ± 0.11	0.14 ± 0.08	0.10 ± 0.06	0.11 ± 0.09

10 h	0.21 ± 0.21	0.16 ± 0.14	0.33 ± 0.24	0.15 ± 0.14	0.06 ± 0.07	0.16 ± 0.09
24 h	tr	nd	0.58 ± 0.01	0.07 ± 0.10	0.08 ± 0.11	tr
Kaempferol rutinoside II						
0 h	0.06 ± 0.03	0.07 ± 0.02	0.05 ± 0.00	0.06 ± 0.03	0.10 ± 0.05	0.06 ± 0.04
5 h	0.13 ± 0.10	0.16 ± 0.08	0.23 ± 0.13	0.13 ± 0.08	0.06 ± 0.04	0.16 ± 0.08
10 h	0.11 ± 0.166	0.02 ± 0.00	0.23 ± 0.18	0.15 ± 0.16	0.13 ± 0.22	0.28 ± 0.08
24 h	tr	nd	0.08 ± 0.08	0.08 ± 0.11	tr	tr
Kaempferol rutinoside III						
0 h	0.17 ± 0.05	0.20 ± 0.14	2.58 ± 3.69	1.53 ± 1.80	0.64 ± 0.91	1.28 ± 1.93
5 h	0.28 ± 0.30	0.04 ± 0.03	2.51 ± 2.82	1.91 ± 2.51	0.14 ± 0.12	1.43 ± 1.99
10 h	0.02 ± 0.02	nd	0.19 ± 0.24	0.35 ± 0.14	0.04 ± 0.05	0.63 ± 0.90
24 h	nd	nd	0.01 ± 0.02	0.08 ± 0.07	nd	nd
Kaempferol rutinoside IV						
0 h	4.20 ± 4.02	2.58 ± 2.67	8.18 ± 6.62	6.34 ± 5.21	1.78 ± 1.46	3.25 ± 2.90
5 h	1.10 ± 1.36	tr	14.04 ± 2.49	5.55 ± 4.31	1.23 ± 0.26	2.08 ± 1.13
10 h	0.53 ± 0.24	tr	2.44 ± 3.27	1.45 ± 0.85	0.17 ± 0.08	0.96 ± 1.13
24 h	0.01 ± 0.01	nd	0.11 ± 0.14	0.34 ± 0.57	nd	tr
Kaempferol glucoside I						
0 h	0.22 ± 0.23	0.21 ± 0.17	0.27 ± 0.24	0.26 ± 0.14	0.11 ± 0.08	0.10 ± 0.08
5 h	0.14 ± 0.20	0.19 ± 0.27	0.46 ± 0.43	0.32 ± 0.18	0.15 ± 0.10	0.19 ± 0.15
10 h	0.08 ± 0.13	0.14 ± 0.20	0.54 ± 0.50	0.32 ± 0.18	0.07 ± 0.09	0.11 ± 0.012
24 h	0.06 ± 0.09	nd	0.38 ± 0.21	0.19 ± 0.06	0.05 ± 0.03	nd
Kaempferol glucoside II						
0 h	0.10 ± 0.10	0.10 ± 0.09	0.22 ± 0.19	0.23 ± 0.02	0.06 ± 0.04	0.07 ± 0.09
5 h	0.05 ± 0.06	tr	0.15 ± 0.20	0.22 ± 0.09	0.05 ± 0.06	0.02 ± 0.01
10 h	0.04 ± 0.06	tr	0.01 ± 0.01	0.12 ± 0.07	tr	0.04 ± 0.05
24 h	nd	nd	tr	0.02 ± 0.03	nd	nd
Methoxy kaempferol hexoside I						
0 h	11.71 ± 7.36	15.45 ± 12.40	22.13 ± 16.07	24.01 ± 17.08	10.04 ± 7.48	4.99 ± 1.36
5 h	14.89 ± 21.47	18.79 ± 25.62	35.27 ± 25.54	32.53 ± 16.65	4.03 ± 2.03	3.61 ± 2.40
10 h	18.08 ± 27.29	15.47 ± 19.71	43.48 ± 35.16	31.11 ± 27.48	2.09 ± 0.53	2.35 ± 0.82
24 h	13.84 ± 21.34	0.51 ± 0.08	36.44 ± 15.75	30.42 ± 15.00	1.37 ± 0.58	1.51 ± 0.94
Methoxy kaempferol hexoside II						
0 h	12.41 ± 10.60	12.30 ± 9.94	25.96 ± 26.44	28.14 ± 23.52	8.81 ± 6.11	7.56 ± 10.30
5 h	4.08 ± 5.52	1.71 ± 2.39	21.44 ± 28.51	25.31 ± 19.48	6.29 ± 6.70	1.97 ± 2.34
10 h	0.29 ± 0.26	0.50 ± 0.70	0.60 ± 0.07	10.93 ± 12.62	1.20 ± 0.96	0.33 ± 0.29
24 h	0.02 ± 0.03	tr	0.26 ± 0.37	0.16 ± 0.12	tr	0.03 ± 0.04
TOTAL KAEMPFEROL DERIVATES						
0 h	**30.63 ± 4.75**	**32.08 ± 5.51**	**61.98 ± 9.44**	**62.51 ± 10.28**	**22.67 ± 3.69**	**18.56 ± 2.53**
5 h	**21.77 ± 4.44**	**21.75 ± 6.52**	**77.89 ± 11.68**	**67.64 ± 11.49**	**12.87 ± 2.06**	**10.92 ± 1.17**
10 h	**19.92 ± 5.40**	**16.55 ± 6.23**	**50.66 ± 12.92**	**45.35 ± 9.50**	**4.04 ± 0.69**	**5.83 ± 0.67**
24 h	**14.01 ± 5.63**	**0.53 ± 0.35**	**39.16 ± 10.91**	**31.63 ± 9.14**	**1.51 ± 0.66**	**1.55 ± 0.87**

3-(4-hydroxyphenyl)propionic acid						
0 h	nd	nd	nd	nd	nd	nd
5 h	nd	nd	nd	nd	nd	nd
10 h	nd	nd	nd	nd	nd	nd
24 h	tr	tr	tr	tr	tr	tr
TOTAL (POLY)PHENOLIC COMPOUNDS						
0 h	**484.95 ± 60.93**	**222.90 ± 28.98**	**600.91 ± 70.90**	**558.67 ± 71.30**	**278.67 ± 38.15**	**283.94 ± 37.80**
5 h	**461.56 ± 63.22**	**245.10 ± 38.03**	**729.85 ± 91.64**	**591.10 ± 79.95**	**285.13 ± 43.11**	**293.19 ± 46.12**
10 h	**380.03 ± 55.29**	**218.55 ± 40.59**	**719.75 ± 95.72**	**482.64 ± 64.89**	**197.61 ± 32.26**	**216.44 ± 33.60**
24 h	**87.46 ± 18.51**	**44.32 ± 17.44**	**203.58 ± 34.00**	**150.86 ± 19.76**	**38.99 ± 11.63**	**38.83 ± 13.58**

nd = no detected
tr = traces

SUPPLEMENTARY INFORMATION

TITLE: Digestion and colonic fermentation of raw and cooked *Opuntia ficus-indica* impacts bioaccessibility and bioactivity

AUTHORS: Elsy De Santiago[a], Chris I. R. Gill[b], Ilaria Carafa[c], Kieran M. Touhy[c], María-Paz De Peña[a] and Concepción Cid[a]*

[a] Universidad de Navarra, Facultad de Farmacia y Nutrición, Departamento de Ciencias de la Alimentación y Fisiología, C/ Irunlarrea 1, E-31008 Pamplona, Spain.

IdiSNA, Navarra Institute for Health Research. Pamplona, Spain.

[b] Nutrition Innovation Centre for Food and Health, Centre for Molecular Biosciences, University of Ulster, Cromore Road, Coleraine, Northern Ireland, BT52 1SA, United Kingdom.

[c] Nutrition & Nutrigenomics Unit, Department of Food Quality and Nutrition, Research and Innovation Centre, Fondazione Edmund Mach (FEM), Via E. Mach 1, 38010, San Michele all'Adige, Trento, Italy.

*Corresponding author: María-Paz de Peña. Tel: +34 948 425600 (806580); Fax: +34 948 425740. E-mail address: mpdepena@unav.es

Table S1. Mass spectrometric characteristics of (poly)phenolic compounds identified in this study.

Compound	Chemical formula	R_t (min)	$[M-H]^-$ (m/z)	Fragment ions
Phenolic acids				
Piscidic acid	$C_{11}H_{12}O_7$	3.1	255.1	165 / 179
Feruloylglucose I	$C_{16}H_{20}O_9$	7.7	355	193 / 179
Feruloylglucose II	$C_{16}H_{20}O_9$	11.08	355	193 / 179
Eucomic acid I	$C_{11}H_{12}O_6$	9.5	239.2	179 / 195
Isorhamnetin derivatives				
Isorhamnetin	$C_{16}H_{12}O_7$	33	315	151
Isorhamnetin hexose rhamnose hexoside I	$C_{34}H_{42}O_{21}$	17.5	785	315 / 151
Isorhamnetin hexose rhamnose hexoside II	$C_{34}H_{42}O_{21}$	27.9	785	315 / 151
Isorhamnetin hexose rhamnose hexoside III	$C_{34}H_{42}O_{21}$	28.15	785	315 / 151
Isorhamnetin di-hexoside I	$C_{28}H_{32}O_{17}$	18.19	639	477 / 315
Isorhamnetin di-hexoside II	$C_{28}H_{32}O_{17}$	19.25	639	477 / 315
Isorhamnetin rutinoside rhamnoside I	$C_{34}H_{42}O_{20}$	18.8	769	315/ 145
Isorhamnetin rutinoside rhamnoside II	$C_{34}H_{42}O_{20}$	19.1	769	315/ 145
Isorhamnetin hexose pentoside	$C_{27}H_{30}O_{16}$	20.25	609	477 / 315
Isorhamnetin rutinoside I	$C_{28}H_{32}O_{16}$	20.1	623	315 / 299
Isorhamnetin rutinoside II	$C_{28}H_{32}O_{16}$	21.99	623	315 / 299
Unknown Isorhamnetin derivaive		19	755	315 / 299
Kaempferol derivatives				
Kaempferol hexoside dirhamnoside I	$C_{33}H_{40}O_{19}$	18	739	285
Kaempferol hexoside dirhamnoside II	$C_{33}H_{40}O_{19}$	18.5	739	285
Kaempferol hexose pentoside	$C_{26}H_{28}O_{15}$	19.7	579	285 / 255
Kaempferol rutinoside I	$C_{27}H_{30}O_{15}$	18.98	593	285 / 255
Kaempferol rutinoside II	$C_{27}H_{30}O_{15}$	19.45	593	285 / 255
Kaempferol rutinoside III	$C_{27}H_{30}O_{15}$	20.03	593	285 / 255
Kaempferol rutinoside IV	$C_{27}H_{30}O_{15}$	21.5	593	285 / 255
Kaempferol glucoside I	$C_{22}H_{22}O_{12}$	21	447	285 / 255
Kaempferol glucoside II	$C_{22}H_{22}O_{12}$	22	447	285 / 255
Methoxy kaempferol hexoside	$C_{22}H_{22}O_{12}$	22	477	314 / 285
Methoxy kaempferol hexoside II	$C_{22}H_{22}O_{12}$	22.55	477	314 / 285
Catabolite				
3-(4-hydroxyphenyl)-propionic acid	$C_9H_{10}O_3$	12.18	165	93

R_t, retention time; m/z, mass-to-charge ratio; $[M-H]^-$, Negatively charged molecular ion

GENERAL DISCUSSION

Extraction and HPLC analysis of (poly)phenols

Cactus (*Opuntia ficus-indica* Mill.) cladodes have been proposed as a potential source of (poly)phenolic compounds with health related properties (El-Mostafa et al., 2014; Stintzing & Carle, 2005). Their extraction is a crucial step from the analytical point of view for the characterization of (poly)phenolic compounds profile, but also for a further development of ingredients for functional foods. Polysaccharides present in cactus cladodes, such as pectins, form a water-soluble complex with (poly)phenols. Also cellulose with non-covalent hydrogen bonds and hydrophobic interactions makes (poly)phenols extraction more difficult (Bordenave, Hamaker, & Ferruzzi, 2014). Therefore, several individual solvents as ethanol and methanol, as well as successive extractions with methanol (50%), acetone (80%) and water were used for the extraction of cactus cladodes (poly)phenolic compounds. Even ethanol (80%) extracted the highest total phenolic content, successive extraction with methanol, acetone and water favoured the extraction of antioxidants (measured by DPPH and ABTS radicals), and specifically flavonoids.

Once selecting successive extractions as the best extraction method, the (poly)phenolic compounds were extracted and analysed by HPLC-DAD. Even though the analysis by HPLC-MS is widely extended, the availability in many laboratories is limited due to the high price. Other limitation in the analysis of (poly)phenolic compounds is the lack of standards or their high price. For these reasons, a chromatographic method for HPLC-DAD was developed.

The analysis of the cactus cladodes extract by HPLC-DAD showed the presence of several unidentified peaks, but the absence of the free flavonoid aglycones and free phenolic acids. In fact, subsequent analyses in HPLC-MS (De Santiago, Pereira-Caro, Moreno-Rojas, Cid, & De Peña, 2018b) confirmed the absence of flavonoid aglycones in cactus cladodes. Therefore, acid hydrolysis was applied to hydrolyse the flavonoids in their corresponding aglycones and thus be able to quantify them. After acid hydrolysis with different concentrations of HCl (0.6-1.7M) and times (2-3h), three flavonoids aglycones (isorhamnetin, quercetin and kaempferol) and two phenolic acids (ferulic and hidroxybenzoic acids) were identified and quantified by HPLC-DAD using the corresponding standards. Acid hydrolysis with 1.5M HCl during 2h was shown as the optimal for deglycosilation of flavonoids without a degradation of phenolic acids.

After the optimization of (poly)phenols extraction and HPLC analysis, the research study about the content of (poly)phenolic compounds in cactus cladodes before and after culinary techniques, as well as their bioaccessibility was performed.

Influence of heat treatment

Cactus cladodes can be consumed both raw and after cooking techniques such as boiling, microwaving, griddling and frying. The influence of different culinary heat treatments on the nutritional and fatty acid composition, antioxidant capacity and (poly)phenolic profile of cactus cladodes was evaluated for the first time. The obtained results were published in Food Chemistry and in the Journal of Agricultural and Food Chemistry (De Santiago, Domínguez-Fernández, Cid, & De Peña, 2018a; De Santiago et al., 2018b).

Cactus cladodes were characterized by a high moisture content (79.7-93.7 g/100g), low protein (1.1-2.0 g/100 g) and mineral content (0.5 g/100 g) and high dietary fibre content (3.1-5.0 g/100 g), showing higher amount of insoluble fibre than soluble one. Dietary fibre is an essential part of the composition of cactus cladodes, including cellulose, hemicelluloses, pectin, lignin and gums (Ayadi, Abdelmaksoud, Ennouri, & Attia, 2009; Bensadón, Hervert-Hernández, Sáyago-Ayerdi, & Goñi, 2010). Recommended Dietary Allowance (RDA) suggests an intake between 20 and 25 g of fibre per day (WHO, 2003). A portion of 100 g of cactus cladodes provides between 15% (for raw and boiled samples) and 20–25% (for microwaved, griddled and fried samples) of the RDA of fibre per day. According to the Food and Drug Administration (FDA), foods can be considered as a high source of fibre if its contribution is at least 20% of the daily value per serving (Food and Drug Administration, 2013). Thereby, raw and cooked cactus cladodes are a good source of fibre.

After cooking processes, soluble and insoluble fibre, and consequently total fibre content, increased, except in boiling. Heat treatment induces depolymerisation and the rupture of some glycosidic linkages in dietary fibre polysaccharides that favour the observed increase in carbohydrates (Ramírez-Moreno, Córdoba-Díaz, Sánchez-Mata, Díez-Marqués, & Goñi, 2013).

Cactus cladodes can be considered as a fat-free vegetable, except when they were submitted to a frying process (8.3–11.0 g fat/100 g). In consequence, fried vegetables also had the highest caloric value. When cactus cladodes are submitted to frying, the use of olive oil is preferable due to its well-known healthy characteristics and a lower ω-6/ω-3 ratio (4.586) than soybean oil (10.224).

Heat treatment increased the total phenolic content in cactus cladodes, except in boiled ones due to the leaching losses favoured by the breakdown of cellular structures caused at high temperatures (Ramírez-Anaya, Samaniego-Sánchez, Castañeda-Saucedo, Villalón-Mir, & De La Serrana, 2015; Turkmen, Sari, & Velioglu, 2005). Griddling and frying apparently higher increased the total phenolic content measured by Folin-

Ciocalteu method than microwaving. However, when (poly)phenolic compounds profile was deeply analysed by HPLC, microwaving and griddling were shown as the culinary techniques that favoured the release of (poly)phenolic compounds from the food matrix.

The identification and quantification of (poly)phenolic profile of raw and cooked cactus cladodes by HPLC-DAD showed the predominance of flavonoids (80-85%) *versus* phenolic acids (20-25%). Three flavonoid aglycones (isorhamnetin, quercetin and kaempferol) and two phenolic acids (ferulic and 4-hydroxybenzoic acids) were found. The analysis performed by UHPLC-PDA-HR-MS showed a total of 45 (poly)phenolic compounds, with flavonoids accounted for 60-68% and phenolic acids for 32−40%. Among flavonoids, 13 isorhamnetin derivatives, 5 quercetin and 14 kaempferol derivatives were identified. Regarding phenolic acids, 3 stereoisomers of eucomic acid were the most abundant, followed by 3 of piscidic acids and 7 ferulic acid derivatives in lower amounts. Results of total (poly)phenolic compounds by UHPLC-PDA-HR-MS (72.77-158.21 mg/g dm) are much higher than HPLC-DAD (1.42-5.76 mg/g dm) analysis maybe due to (1) a partial degradation of (poly)phenols by acid hydrolysis, even though it was previously optimized; and (2) the higher detection capacity of UHPLC-PDA-HR-MS than HPLC-DAD. Then, besides a greater number of compounds identified (45), smaller amounts could be quantified by UHPLC-PDA-HR-MS than by HPLC-DAD. Specifically, eucomic and piscidic acids could be identified and quantified only by UHPLC-PDA-HR-MS.

Although there are no data related to (poly)phenols in cooked cactus cladodes, similar profiles have been reported in raw cactus cladodes of *O. ficus-indica* and other *Opuntia* cultivars with isorhamnetin glycosides as predominant flavonoids (Avila-Nava et al., 2014; Ginestra et al., 2009; Guevara-Figueroa et al., 2010).

Piscidic and eucomic acids have been previously identified in *O. ficus-indica* extracts as unique compounds (Ginestra et al., 2009; Mena et al., 2018). In the present study, eucomic acids were the most abundant (50−60% total phenolic acids), in contrast with Ginestra et al. (2009) who reported piscidic acid as the major one. Piscidic acid is rarely found in nature and is restricted to those with crassulacean acid metabolism, being *Opuntia* species one of these succulent plants (Stintzing & Carle, 2005).

(Poly)phenolic amount in cactus cladodes can vary depending on the maturity stage, origin place, harvest season and environmental conditions. In early stages phenolic compounds are higher because they are a defence mechanism against environment since the product has not developed thorns to protect themselves (Figueroa-Pérez, Pérez-Ramírez, Paredes-López, Mondragón-Jacobo, & Reynoso-Camacho, 2018;

Ventura-Aguilar, Bosquez-Molina, Bautista-Baños, & Rivera-Cabrera, 2017). Additionally, the season of the year and the moment for harvesting also influence, because early in the morning and during the winter, cladodes are more acid due to their crassulacean acid metabolism (Ventura-Aguilar et al., 2017). In the present study cactus cladodes were already mature because they contained thorns and they were harvested in summer.

Heat treatment applied to vegetables induces several structural and chemical changes, which turn into (poly)phenolic compounds losses and gains depending on the cooking technique, technological parameters, as well as the food matrices. Increases after microwaving (1.4-fold) and griddling (1.2-fold) processes could be due to the release of (poly)phenolic compounds from the cell walls and subcellular compartments caused by thermal destruction, but also due to their liberation from pectins, mucilages, and other dietary fibre compounds (Jaramillo-Flores et al., 2003). This thermal hydrolytic action might explain the detection in traces or in low amount of aglycones only after culinary heat treatments. Furthermore, both cooking techniques are carried out without the addition of water, avoiding the leaching, or at least minimized it using faster cooking time (5 min) in microwaving than in boiling treatment (10 min). Additionally, Maillard reactions can be favoured by the high temperatures reached during griddling (110−150 °C), and the melanoidins formed could retain (poly)phenolic compounds into their structures. Besides, the inactivation of the enzyme systems (as polyphenoloxidases) leads to inhibit degradation of the (poly)phenolic compounds (Girgin & El, 2015). Similarly, frying process induces a decrease probably due to a longer cooking time (15 min) than in the other heat treatments (5−10 min) making it a more deteriorative process.

Regarding the (poly)phenolic compounds profile, isorhamnetin derivatives showed a higher increment when cactus cladodes were submitted to microwaving, while quercetin and kaempferol derivatives increased higher after griddling. Microwaved cactus cladodes also present the highest amount of total phenolic acids, particularly in piscidic and ferulic acid derivatives. These results are in agreement with those previously reported in microwaved broccoli and cauliflower (Ramos Dos Reis et al., 2015) as well as in griddled onion, pepper, and cardoon (Juániz, Ludwig, Huarte, et al., 2016b).

In other cactus species like Xoconostle (*Opuntia joconostle*) pear fruit, boiling and griddling have shown significantly decreases in flavonoids and phenolic acids, whereas microwaving has preserved them (Cortez-García, Ortiz-Moreno, Zepeda-Vallejo, & Necoechea-Mondragón, 2015). However, the phenolic profile in this cactus pear fruit is

quite different from that shown in the present work, because isorhamnetin derivatives were not detected.

The increase (or even decrease) of antioxidant capacity after heat treatments in cactus cladodes is the result of the predominant effects (release, hydrolysis, degradation, leaching, etc) on different antioxidants (phenolic and non-phenolic like ascorbic acid). Individual compounds may respond in a different manner to different radicals, and they can also be differently affected by heat treatment. Thermal chemical changes in (poly)phenols structures might be crucial to overall antioxidant capacity. In fact, it is known that the glycosylation of flavonoids reduces their activity when compared to the corresponding aglycones (Buchner, Krumbein, Rohn, & Kroh, 2006). Then, their release from the cell structure, or their thermohydrolyzation into more simple glycosides resulting triglycosides into diglycosides and monoglycosides might enhance the antioxidant capacity of cooked cactus cladodes and counterbalance the losses of thermolabile and water soluble antioxidants. In addition, the antioxidant capacity of phenolic acids and their esters mainly depends on the number of hydroxyl groups in the molecule (Rice-Evans, Miller, & Paganga, 1996).

Bioaccesibility of (poly)phenolic compounds

(Poly)phenolic compounds must be released from the food matrix into the gastrointestinal tract to be absorbed and further reach the organs to exert their health benefits. Therefore, bioaccesibility as defined as the amount or fraction of a food compound which remain available for absorption in the gastrointestinal tract, was evaluated in raw and cooked cactus cladodes samples after the application of a simulated 3 steps (oral, gastric and intestinal) digestion *in vitro* model previously adapted in our laboratory (Minekus et al., 2014; Monente et al., 2015).

Gastrointestinal digestion significantly ($p<0.05$) decreased both flavonoids and phenolic acids of cactus cladodes, remained bioaccessible 55−64% of the total (poly)phenolic compounds of cooked cactus cladodes, while final bioaccessibility was 44% in raw samples (De Santiago et al., 2018b).

Again, microwaved cactus cladodes contributed with the highest amount of total (poly)phenolic content, after the *in vitro* gastrointestinal process, followed by griddled samples while cactus cladodes fried with soybean or olive oil had the lowest. Other authors have also demonstrated a higher bioaccessibility of total (poly)phenolic compounds after heat treatment in boiled and steamed cauliflower (Girgin & El, 2015), griddled green pepper (Juániz, Ludwig, Bresciani, et al., 2016a) and cardoon (Juániz et

al., 2017). Likewise, Antunes-Ricardo, Gutiérrez-Uribe & Guajardo-Flores (2017) also observed a significant decrease in the bioaccessibility of isorhamnetin glycosides after the intestinal digestion of raw *O. ficus-indica* extracts.

The high amount of pectins and mucilages in *Opuntia* species, which includes bound (poly)phenolic compounds, along with those attached to the melanoidins formed by Maillard reactions after intensive heat treatment like griddling, might favour a protective effect against enzymatic action (Prior, Wu, & Schaich, 2005).

Flavonoids and phenolic acids were unevenly affected, being the first more sensitive to gastrointestinal conditions than the latter. In this way, digestive enzymes and conditions higher retained or degraded flavonoids (37−63% bioaccessibility) than phenolic acids (56−87% bioaccessibility), changing the ratio between them by increasing the percentage of phenolic acids (40-54 % of the total content).

Flavonoid aglycones were detected in traces or very low amount in cooked cactus cladodes after *in vitro* gastric digestion and undetected after the intestinal phase. This confirms that the amylases added to simulate the salivary action and those present in pancreatin in the intestinal phase, which normally cleave α-linkages, are not able to break the β-glycosidic linkage between the flavonoid aglycones and their glycosidic moieties (Gonzales et al., 2015). The deglycosilation of flavonoids takes place in the brush border cells of the small intestine and also because of the action of the human microbiota, so the degradation of flavonoids could be mainly because of the affinity with digestive enzymes (Day et al., 2000; Németh et al., 2003).

Moreover, isomerization reactions of ferulic, piscidic and eucomic acids were induced through the gastrointestinal digestion, especially after the gastric phase, maybe due to the acid pH and digestive conditions.

In addition, antioxidant capacity evaluated by DPPH radical decreased after both gastric and intestinal phases in all cactus cladodes samples. However, in microwaved and griddled ones, the decrease was lower than in other cooking treatments, which is in accordance with the (poly)phenolic profile remained after the gastrointestinal digestion.

Therefore, the current study confirms that heating processes may significantly influence the digestibility of dietary (poly)phenols of cactus cladodes from the food matrix. Thus, even (poly)phenols are retained or degraded by digestive enzymes and pH conditions during gastrointestinal digestion, most of them remain bioaccessible when cactus cladodes are cooked, especially phenolic acids after microwaving and griddling.

Effect of colonic microbiota

Because most (poly)phenolic compounds are not absorbed in the small intestine and reach the colon, they can be metabolised by the action of human gut microbiota inducing the formation of (poly)phenolic catabolites (Espín, González-Sarrías, & Tomás-Barberán, 2017; Juániz et al., 2017; Juániz et al., 2016a; Ludwig, de Peña, Cid, & Crozier, 2013). Hence, to evaluate the effect of the human gut microbiota on (poly)phenolic compounds of digesta cactus cladodes, a 24 h *in vitro* colonic fermentation was developed, simulating the anaerobic conditions located in the human large intestine (Koutsos et al., 2017).

At the starting of colonic fermentation (0 h), 27 (poly)phenolic compounds were identified and quantified in fermented cactus cladodes, being phenolic acids the most abundant with 78-88% of the total content. Piscidic acid was the main compound (52-65% total (poly)phenols), followed by eucomic acid (15-36% total (poly)phenols). Flavonoids such as kaempferol derivatives (6-13% total (poly)phenols) and isorhamnetin derivatives (4-7% total (poly)phenols) were mainly found in their glycosides forms. No quercetin derivatives were detected at any time point of the colonic fermentation maybe due to a rapid catabolism of quercetin glycosides, even within the first 15 min after faecal incubation, which have been previously reported (Juániz et al., 2016a).

Most of (poly)phenols were degraded during colonic fermentation, although some of them were still present after the 24 h. Intestinal microbiota produced a higher degradation of flavonoids than phenolic acids. Eucomic acid was the most stable during the colonic fermentation may due to the resistance to the human microbiota, while piscidic acid and both kaempferol and isorhamnetin derivatives were rapidly catabolised. At the end of the 24 h of colonic fermentation, the catabolite 3-(4-hydroxyphenyl)propionic acid was detected in trace amounts maybe produced via demethoxylation from ferulic acid derivatives (Ludwig et al., 2013), as well as via dehydroxylation of 3-(3,4-dihydroxyphenyl)propionic acid derived from ferulic acid and the ring fission of quercetin, isorhamnetin and kaempferol derivatives (Juániz et al., 2017; Juániz et al., 2016a; Kay, Pereira-Caro, Ludwig, Clifford, & Crozier, 2017).

Biological activity

Even many (poly)phenolic compounds or their catabolites in cactus cladodes can be bioaccessible to cross through the intestinal barrier, they might also exert their health benefits into the colon cells. Therefore, the biological activity of colonic fermented cactus cladodes cooked by the most common culinary techniques (boiling, griddling and frying) was evaluated in HT29 human colon cells, including cytotoxicity (MTT assay), antigenotoxicity (Comet assay) and cell cycle analyses.

No cytotoxicity was observed in HT29 cells after 24 h of incubation with colonic fermented raw and cooked cactus cladodes at 10% and 20% (v/v), compared to untreated cells. However, cell viability was less than 80% in most samples at 20% (v/v) concentration. Based on that, a 10% (v/v) concentration was used for the Comet and cell cycle assays.

Antigenotoxicity effect of fermented cactus cladodes was evaluated for the first time, showing a significant reduction ($p<0.05$) in the H_2O_2-induced DNA damage in HT29 cells, comparing to the control fermentate (with no cactus cladodes). The reduction in the DNA damage was observed in all the cactus cladodes samples, maybe due to the presence of (poly)phenolic compounds after colonic fermentation.

Eucomic acid was the main compound after the faecal incubation and could still have antioxidant properties contributing to the tendency to reduce the DNA damage (Cemeli, Baumgartner, & Anderson, 2009). Similarly, colonic fermented raspberry, strawberry and blackcurrant presented a significant ($p<0.05$) reduction of DNA damage in HT29 cell line (Brown et al., 2012; Coates et al., 2007).

Finally, the fermented raw and cooked cactus cladodes at 10% (v/v) showed no significant ($p<0.05$) effects at any stage of the HT29 cell cycle. Results are consistent with Coates et al. (2007), who observed not significant changes in cell cycle of HT29 cells when they were pre-treated with fermented berries. However, Serra et al. (2013) observed that extracts from *O. ficus-indica* fruit juice induced a cell cycle arrested in the G1 phase in HT29 cells, attributing this effect to the betalains, which are absent in cactus cladodes.

Even the antigenotoxicity and antioxidant activity of cactus cladodes in colon cells, further investigations are needed in a living system or by human intervention studies to corroborate the role of (poly)phenolic compounds present in cactus cladodes.

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CONCLUSIONS / CONCLUSIONES

1. Successive extractions with methanol, acetone and water are required for an optimal extraction of (poly)phenolic compounds of cactus cladodes, as well as for the antioxidant capacity measured by DPPH and ABTS.

2. Acid hydrolysis with 1.5M HCl during 2h is the optimal condition for the release of flavonoid aglycones (isorhamnetin, quercetin and kaempferol) without putting at risk the identification and quantification of phenolic acids (ferulic and hydroxybenzoic acid) by HPLC-DAD.

3. Cooking treatments (boiling, microwaving, griddling and frying) induce changes on nutritional composition of cactus cladodes, increasing soluble and insoluble fibre up to 5.0 g/100 g becoming a good fibre source, as well as losses and gains on (poly)phenolic compounds and antioxidant capacity depending on the cooking technique.

4. Microwaving and griddling processes increase 1.4-fold and 1.2-fold the amount of the 45 (poly)phenolic compounds identified in cactus cladodes by UHPLC-PDA-HR-MS, respectively, due to their release from the cell walls caused by thermal destruction, whereas both frying in soybean and olive oils decrease 0.6-fold and boiling 0.9-fold the total compounds.

5. After *in vitro* gastrointestinal digestion, 55−64% of the total (poly)phenols of cooked cactus cladodes remains bioaccessible *versus* 44% in raw samples. Furthermore, digestive conditions and enzymes degrade or retain more flavonoids (37−63% bioaccessibility) than phenolic acids (56−87% bioaccessibility).

6. Microwaved cactus cladodes contribute to the highest amount of (poly)phenols (143.54 mg/g dm) after gastrointestinal digestion, followed by griddled samples (133.98 mg/g dm), showing also the highest antioxidant capacity. Additionally, gastrointestinal digestion induces isomerizations among the 3 stereoisomeric forms of piscidic and eucomic acids.

7. The human gut microbiota produces a higher degradation of (poly)phenolic compounds in flavonoids than in phenolic acids. However, some compounds are still remained after 24 hours of colonic fermentation, being eucomic acid as the most relevant.

8. (Poly)phenolic compounds of cactus cladodes that remain in the colon after the action of gut microbiota might exert their antigenotoxic effect by reducing the H_2O_2-induced DNA damage in the HT29 colon cells.

In conclusion, microwaving and griddling are the preferable culinary techniques in order to increase bioaccessible flavonoids and phenolic acids, as well as the antioxidant capacity in cactus cladodes. Despite of the degradation of (poly)phenols after the gastrointestinal digestion and the action of the human gut microbiota, some compounds are still available in the colon where they can exert their biological activity. However, further research is needed to evaluate the bioavailability of (poly)phenolic compounds using *in vivo* models or human intervention studies.

1. Se requieren extracciones sucesivas con metanol, acetona y agua para obtener las condiciones óptimas para la extracción de compuestos (poli)fenólicos del nopal, así como para la medición de la capacidad antioxidante por DPPH y ABTS.

2. La hidrólisis ácida con HCl 1,5 M durante 2 h es la condición óptima para la liberación de agliconas de flavonoides (isorhamnetina, quercetina y kaempferol) sin poner en riesgo la identificación y cuantificación de los ácidos fenólicos (ácido ferúlico e hidroxibenzoico) por HPLC-DAD.

3. Los tratamientos de cocción (hervido, al microondas, a la parrilla y fritura) producen cambios en la composición nutricional del nopal, aumentando la fibra soluble e insoluble hasta 5.0 g/100 g convirtiéndose en una buena fuente de fibra, así como pérdidas y ganancias en compuestos (poli)fenólicos y su capacidad antioxidante dependiendo de la técnica de cocción empleada.

4. Los procesos de microondas y a la parrilla aumentan 1.4 veces y 1.2 veces la cantidad de los 45 compuestos (poli)fenólicos identificados en el nopal por UHPLC-PDA-HR-MS, respectivamente, debido a su liberación de las paredes celulares causada por la destrucción térmica, mientras que ambos fritos en aceite de soja y oliva disminuyen 0.6 veces y el hervido 0.9 veces el total de compuestos.

5. Después de la digestión gastrointestinal *in vitro*, el 55-64% del total de (poli)fenoles de nopal cocinado permanece bioaccesible frente al 44% en nopal sin cocinar. Además, las condiciones enzimáticas y digestivas degradan o retienen más flavonoides (37-63% de bioaccesibilidad) que ácidos fenólicos (56-87% de bioaccesibilidad).

6. Nopal cocinado al microondas contribuye a una mayor cantidad de (poli)fenoles (143.54 mg / g dm) después de la digestión gastrointestinal, seguido de nopal a la parrilla (133.98 mg / g dm), que también muestra la mayor capacidad antioxidante. Además, la digestión gastrointestinal induce isomerizaciones entre las 3 formas estereoisoméricas del ácido piscídico y eucómico.

7. La microbiota intestinal produce una mayor degradación de compuestos (poli)fenólicos en los flavonoides que en los ácidos fenólicos. Sin embargo, algunos compuestos aún permanecen después de 24 horas de fermentación colónica, siendo el ácido eucómico el más relevante.

8. Los compuestos (poli)fenólicos del nopal que permanecen en el colon después de la acción de la microbiota intestinal pueden ejercer su efecto antigenotóxico reduciendo el daño del ADN inducido por H_2O_2 en las células de colon HT29.

En conclusión, el microondas y la parrilla son las técnicas culinarias preferibles para aumentar la bioaccesibilidad de los compuestos flavonoides y ácidos fenólicos, así como la capacidad antioxidante en el nopal. A pesar de la degradación de (poli)fenoles después de la digestión gastrointestinal y de la acción de la microbiota intestinal, algunos compuestos están aún disponibles en el colon donde pueden ejercer su actividad biológica. Sin embargo, se necesitan más investigaciones para evaluar la biodisponibilidad de los compuestos (poli)fenólicos usando modelos *in vivo* o estudios de intervención en humanos.

RESEARCH DISSEMINATION

Publications

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E. De Santiago, I. Juániz, C. Cid, M.P. De Peña. Extraction of (poly)phenolic compounds of cactus (*Opuntia ficus-indica* Mill.) cladodes: impact of solvents and conditions. In preparation.

Conference communications

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ANNEX

VIII JORNADA DE INVESTIGACIÓN EN CIENCIAS EXPERIMENTALES Y DE LA SALUD

UNIVERSIDAD DE NAVARRA

Libro de resúmenes

PAMPLONA, 26 DE MARZO DE 2015

VIII Jornada de Investigación en Ciencias Experimentales y de la Salud de la Universidad de Navarra

Póster nº 41

Alimentación, Estilo de Vida y Salud

SELECCIÓN DE UN MÉTODO DE EXTRACCIÓN DE COMPUESTOS FENÓLICOS EN EL NOPAL

Elsy Gabriela De Santiago Castanedo, Mª Concepción Cid Canda, Mª Paz de Peña Fariza

Departamento de Ciencias de la Alimentación y Fisiología, Universidad de Navarra

e-mail: edesantiago@alumni.unav.es

La OMS insta a un mayor consumo de vegetales por ser fuente de compuestos bioactivos implicados en la prevención de enfermedades relacionadas con el estrés oxidativo. El nopal (*Opuntia ficus-indica*) es uno de los alimentos de mayor consumo en México y además un alimento rico en compuestos antioxidantes. Sin embargo, la extracción de dichos compuestos puede verse dificultada por la capacidad de retención de agua propia de un cactus. El objetivo de este trabajo fue comparar la eficacia de extracción de antioxidantes de 3 métodos:

1. 100% etanol
2. 100% etanol con posterior evaporación y redisolución en agua
3. Extracciones sucesivas con metanol al 50%, acetona al 70% y agua al 100% y posterior hidrólisis ácida.

En todos los extractos se midió la capacidad antioxidante mediante DPPH y ABTS, así como el total de compuestos fenólicos y de flavonoides.

Los diferentes procesos empleados extrajeron cantidades semejantes de compuestos fenólicos totales. Sin embargo, la cantidad de flavonoides extraídos con el método de extracciones sucesivas resultó inferior, probablemente debido a la hidrólisis ácida aplicada. Por el contrario, la capacidad antioxidante fue significativamente superior, hasta un 87% en el ABTS y un 98% en el DPPH. Esto podría deberse a la ruptura de los glucósidos de flavonoides dando lugar a su forma aglicona, pudiendo ser estas estructuras más bioactivas.

En conclusión, el método de extracciones sucesivas y posterior hidrólisis ácida resultó el más efectivo para la extracción de los compuestos antioxidantes del nopal.

SELECCIÓN DE UN MÉTODO DE EXTRACCIÓN DE COMPUESTOS FENÓLICOS EN EL NOPAL

E. De Santiago, C. Cid, M. P. De Peña *
Dpto. Ciencias de la Alimentación y Fisiología, Facultad de Farmacia.
Universidad de Navarra, 31008 Pamplona España. * mpdepena@unav.es

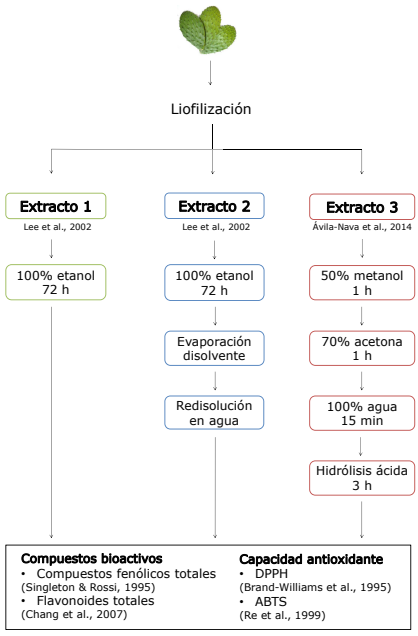
INTRODUCCIÓN

La Organización Mundial de la Salud insta a un mayor consumo de vegetales por ser fuente de compuestos bioactivos implicados en la prevención de enfermedades relacionadas con el estrés oxidativo (OMS, 2004). El nopal *(Opuntia ficus-indica)* es uno de los alimentos de mayor consumo en México y además un alimento rico en compuestos antioxidantes. Sin embargo, la extracción de dichos compuestos puede verse dificultada por la capacidad de retención de agua propia de un cactus.

OBJETIVO

El objetivo de este trabajo fue comparar la eficacia de tres métodos de extracción con la finalidad de seleccionar aquel que permitiera obtener la mayor cantidad de antioxidantes.

MATERIAL Y MÉTODOS



AGRADECIMIENTOS

Este trabajo ha sido financiado por el Plan de Investigación de la Universidad de Navarra (PIUNA). E. De Santiago agradece a la Asociación de Amigos de la Universidad de Navarra por la beca recibida.

RESULTADOS Y DISCUSIÓN

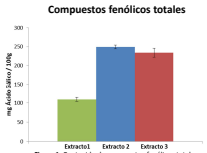


Figura 1. Contenido de compuestos fenólicos totales en los extractos de nopal

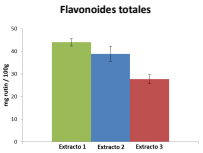


Figura 2. Contenido flavonoides totales en los extractos de nopal

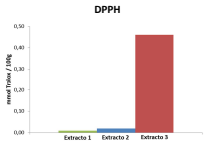


Figura 3. Actividad reductora del radical DPHH del los extractos de nopal

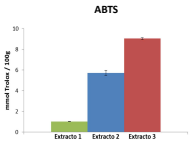


Figura 4. Actividad reductora del radical ABTS de los extractos de nopal

Los extractos de nopal con mayor cantidad de compuestos fenólicos totales fueron el 2 (249 mg AG/100g) y el 3 (234 mg AG/100g) (Figura 1). La cantidad de flavonoides totales extraídos con el método 3 resultó inferior, probablemente debido a la ruptura de parte de estos compuestos por la hidrólisis ácida aplicada. Sin embargo, la capacidad antioxidante de este extracto fue significativamente superior a los otros dos, hasta un 98% en el DPPH y un 87% en el ABTS. Lo anterior puede deberse a la ruptura de los glucósidos de flavonoides que dan lugar a su forma aglicona, pudiendo ser estructuras más bioactivas, o también a la presencia de otros compuestos diferentes a los flavonoides.

CONCLUSIONES

En conclusión, el método de extracciones sucesivas y posterior hidrólisis ácida resultó ser el más efectivo para la extracción de los compuestos antioxidantes del nopal.

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99%) than flavonoids, which were even increased in some cooked samples. Thus, the higher bioaccesibility of phenolic compounds in cooked vegetables suggests that changes during cooking process, including Maillard Reaction Products formation, might have a protective effect against digestive enzyme activity.

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Optimization of the methodology for identification and quantification of (poly)phenolic compounds in cactus cladodes (Opuntia ficus-indica)

E. De Santiago[1], I. Juaniz[1], C. Cid[1], M.P. de Peña[1*]

[1] Department of Nutrition, Food Science and Physiology, School of Pharmacy. University of Navarra. IdiSNA, Navarra Institute for Health Research. 31008,Pamplona, Spain.

**mpdepena@unav.es*

Consumption of vegetables is related with lower risks of chronic diseases associated with oxidative stress such as cancer, cardiovascular diseases or diabetes. The cactus *Opuntia ficus-indica* is one of the most consumed foods in Mexico and a vegetable rich in antioxidant compounds, like (poly)phenolics. Most (poly)phenolic compounds, particularly flavonoids, are in their glycosides forms, so that for their quantification with HPLC-DAD it is necessary to apply a previous hydrolysis to release their respective aglycones. The acid hydrolysis under continuous heating is the traditional method, but it should be standardized and optimized for each food because it is dependent on the binding sites of the glycosides in the flavonoid nucleus. The aim of this work was to optimize hydrolysis conditions for a better identification and quantification of (poly)phenolic aglycones. Additionally, antioxidant capacity was evaluated in the selected extract.

Cactus cladodes were successive extracted with methanol, acetone and water. Hydrolysis was carried out using HCl at 90ºC at different concentrations (0.6, 1.2, 1. and 1.7M) and times (2 and 3 hours). After hydrolysis, (poly)phenolic aglycones and phenolic acids were identified and quantified using HPLC–DAD. Antioxidant capacity was measured by DPPH and ABTS spectrophotometric assays.

Three flavonoids (isorhamnetin, quercetin and kaempferol) and two phenolic acids (ferulic and hidroxybenzoic acids) were identified and quantified. The 1.5M HCl hydrolisis during 2 hours showed the disappearance of glycosides peaks yielding the highest amount of (poly)phenols, especially flavonoids aglycones. Furthermore, there was an increase in the antioxidant capacity measured by DPPH after hydrolisis, which might release the flavonoids aglycones from their glycosides forms in the food matrix.

Financial support; Spanish Ministry of Economy and Competitiveness (AGL2014-52636-P) and PIUN(Plan Investigación Universidad de Navarra). E. De Santiago and I. Juaniz thank to''Asociación de Amigos de la Universidad de Navarra'' for the grants.

Optimization of the methodology for identification and quantification of (poly)phenolic compounds in cactus cladodes (*Opuntia ficus-indica)*

E. De Santiago[1], I. Juániz[1], C. Cid[1], M. P. De Peña[1]*
[1]Department of Nutrition, Food Science and Physiology, School of Pharmacy.
University of Navarra. IdisNA, Navarra Institute for Health Research. 31008, Pamplona Spain.
*mpdepena@unav.es

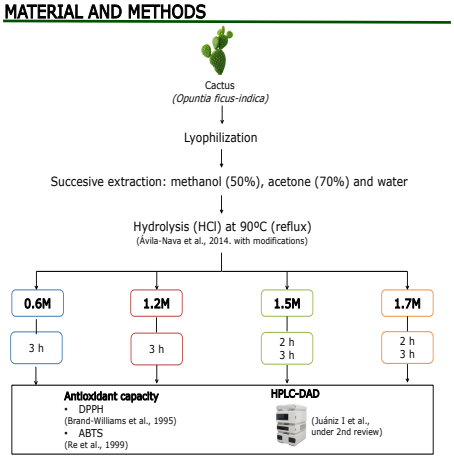
INTRODUCTION

Consumption of vegetables is related with lower risks of chronic diseases associated with oxidative stress such as cancer, cardiovascular diseases or diabetes. The cactus (*Opuntia ficus-indica*) is one of the most consumed foods in Mexico and a vegetable rich in antioxidant compounds like (poly)phenols, particularly flavonoids, which are in their glycosides forms. For their quantification with HPLC-DAD it is necessary to apply a previous hydrolysis to release their respective aglycones. The acid hydrolysis under continuous reflux heating is the traditional method, but it should be standardized and optimized for each food because it is dependent on the binding sites of the glycosides in the flavonoid nucleus.

OBJECTIVE

The aim of this work was to optimize the hydrolysis conditions for a better identification and quantification of (poly)phenolic aglycones and phenolic acids. Additionally, antioxidant capacity was evaluated in the selected extract.

MATERIAL AND METHODS

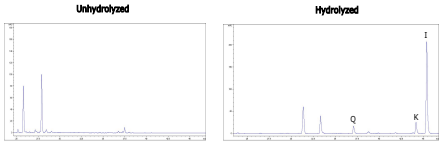


RESULTS AND DISCUSSION

Table 1. (Poly)phenols of cactus cladodes before and after different hydrolysis (HCl) conditions (mg phenolic compound/100g vegetable).

Compound	0.6M 3h	1.2M 3h	1.5M 2h	1.5M 3h	1.7M 2h	1.7M 3h
Isorhamnetin	5.91 ± 0.46	8.57 ± 0.30	16.49 ± 0.71	13.73 ± 1.01	13.31 ± 0.23	8.24 ± 1.20
Kaempferol	1.57 ± 0.06	0.68 ± 0.03	2.28 ± 0.48	1.89 ± 0.08	2.05 ± 0.06	0.41 ± 0.10
Quercetin	2.30 ± 0.28	1.19 ± 0.02	2.71 ± 0.21	2.39 ± 0.07	2.44 ± 0.08	0.72 ± 0.07
Ferulic acid	4.02 ± 0.21	3.69 ± 0.07	2.75 ± 0.21	2.63 ± 0.21	2.16 ± 0.03	6.54 ± 0.40
Hidroxibenzoic acid	1.05 ± 0.47	1.01 ± 0.01	1.17 ± 0.08	0.96 ± 0.03	0.77 ± 0.02	1.25 ± 0.02
Total	14.84 ± 0.09	15.13 ± 0.26	25.41 ± 1.39	21.60 ± 1.11	20.72 ± 0.38	17.16 ± 1.01

Figure 1. HPLC-DAD chromatograms of cactus cladodes extracts before and after acid hydrolysis (1.5M HCl 2h) at 360 nm.



Q: quercetin, K: kaempferol, I: isorhamnetin.

Figure 2. DPPH radical activity of cactus cladodes extracts.

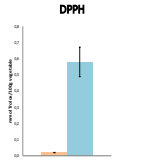
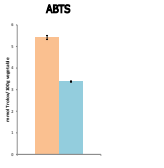


Figure 3. ABTS radical activity of cactus cladodes extracts.



Results from HPLC-DAD measurements indicate that after acid hydrolysis, (poly)phenolic aglycones were released from the glycosides forms of the food matrix and could be identified and quantified.

After acid hydrolysis, three flavonoids aglycones (isorhamnetin, quercetin and kaempferol) and two phenolic acids (ferulic and hidroxybenzoic acids) were identified and quantified in cactus cladodes extracts *(Opuntia ficus-indica*), being isorhamnetin the most abundant.

The 1.5M HCl hydrolysis during 2 hours showed the disappearance of glycosides yielding the highest amount of (poly)phenols, especially flavonoids aglycones. This may be partially explained by the release of those phenolics bound to cell structures during reflux. Higher concentrations of HCl and longer time of reflux could cause the degradation of the (poly)phenolic aglycones.

Cactus cladodes have antioxidant capacity due to the presence of phenolic compounds. An increase in DPPH and a decrease in ABTS after acid hydrolysis (1.5M HCl 2h) are shown (Figure 2 and 3). The release of (poly)phenolic aglycones probably seems favored the DPPH radical scavenging, whereas their glycosides forms are more active against ABTS radical.

CONCLUSIONS

An acid hydrolysis with 1.5M HCl during 2h reflux has been selected in order to further identify and quantitate (poly)phenolic aglycones and phenolic acids by HPLC-DAD. Moreover, scavenging capacity should be measured by using different radicals due to the fact of the similar behavior of (poly)phenolic compounds against DPPH and ABTS radicals.

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Antioxidant activity of (poly)phenolic extracts from raw and cooked cactus cladodes (*Opuntia ficus-indica*) after faecal fermentation in human colon carcinoma HT29 cells

De Santiago, E.[1]; Gill, C.[2]; Touhy, K.M.[3]; Cid, C.[1]; De Peña, M.P.[1]

[1] Universidad de Navarra, Dept. Ciencias de la Alimentación y Fisiología. IdiSNA. Pamplona, Spain; [2]University of Ulster, NICHE, Coleraine, UK; [3]Nutrition & Nutrigenomics Unit, Fondazione Edmund Mach, Trento, Italy.

edesantiago@alumni.unav.es

Cactus cladode (*Opuntia ficus-indica*) is a plant eaten as a fresh or cooked vegetable, which contains bioactive compounds, such as flavonoids and phenolic acids providing antioxidant capacity. (Poly) phenolic compounds could be modified by cooking treatments and gastrointestinal conditions. They are not efficiently absorbed in the small intestine and passing into the colon and exert their biological activity into the colon cells. For this purpose, biological activity of (poly)phenolic extracts from raw and cooked cactus cladodes after *in vitro*-simulated upper intestinal tract digestion and subsequent faecal fermentation (24 h) was evaluated in human colon carcinoma HT29 cell line. Cytotoxic activity was measured by MTT assay, showing no cytotoxicity in fermented raw and cooked cactus cladodes at 10 and 20% v/v, except in control fermentation at 20%. Then, a 10% v/v concentration was used for the evaluation of protection against DNA oxidative damage using the Comet assay. Fermented raw and cooked cactus cladodes decreased the % tail DNA (34.5–38.5%) in hydrogen peroxide (75 μM H_2O_2) challenged HT29 cells compared to the control fermentation (44.2%). No significant differences among cooking treatments were found. When cells were treated with PBS (negative control), the DNA damage was also less in fermented cooked cactus samples than control fermentation. Additionally, cell cycle analysis with fermented samples was carried out, showing no alterations during the cell cycle phases. Therefore, results suggest that colonic (poly) phenolic metabolites of cactus cladodes formed by the action of human gut microbiota potentially protect against DNA oxidative damage.

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