ISBN: 978-628-96613-3-0. ISSN: 2414-6390. Digital Object Identifier: https://dx.doi.org/10.18687/LACCEI2026.1.1.627

Differential Cytotoxic Effects of *Opuntia ficus-indica* Fractions Against Colon Cancer Cells

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Abstract*– Opuntia ficus-indica (OFI) is a source of diverse phytochemicals with reported antitumoral activities; however, the specific compounds responsible for cytotoxicity in colorectal cancer (CRC) cells and their underlying mechanisms remain unclear. This study evaluated the cytotoxic, redox-modulating, and mitochondrial effects of different OFI extracts obtained via sequential exhaustive extraction (SEE) with hexane, petroleum ether, ethyl acetate, acetone, butanol, ethanol, and water on human colorectal adenocarcinoma Caco-2 cells. Ethyl acetate (EA) and water (W) extracts exhibited the strongest cytotoxicity at 100 µg/mL, reducing cell viability to 43.07% and 63.80%, respectively, whereas the remaining extracts showed no significant effects. EA extract decreased mitochondrial membrane potential and simultaneously reduced intracellular reactive oxygen species (ROS), indicating a cytotoxic mechanism independent of oxidative stress. The phytochemical profile showed a high concentration of flavonoids in the EA extract, consistent with the observed antioxidant effect. These results demonstrate that the antitumoral activity of OFI is strongly solvent-extraction-dependent, highlighting the EA extract as the most promising candidate for the development of antitumoral therapies. These findings support OFI as a source of potential antitumoral molecules and encourage future evaluation of mechanistic pathways and selectivity toward healthy colon cells.

Keywords*-- Opuntia ficus-indica; colorectal cancer; cytotoxicity; flavonoids; oxidative stress.

I. Introduction

Cancer is a multifaceted disease characterized by the uncontrolled proliferation and survival of abnormal cells, driven by genetic and epigenetic changes. This process enables malignant cells to evade normal cellular constraints and to invade other parts of the body, a process known as metastasis [1].

Colorectal cancer is a prominent subtype of this pathology that arises from the inner lining of the colon or rectum, typically preceded by the abnormal development of glandular epithelial cells, which can result in polyps (Fig 1). [2].

Colorectal cancer (CRC) represents a significant global public health burden, being the third most diagnosed cancer and the second cause of cancer death worldwide, with more than 900,000 deaths reported in 2020 [3]. Worldwide, this type of cancer is more frequent in males, specifically, men aged 50 and over had the highest incidence (56.84%) and mortality (57.32%) in 2022, compared to females [4]. Nevertheless, there has been an increase in CRC cases among younger individuals in the last century [5].

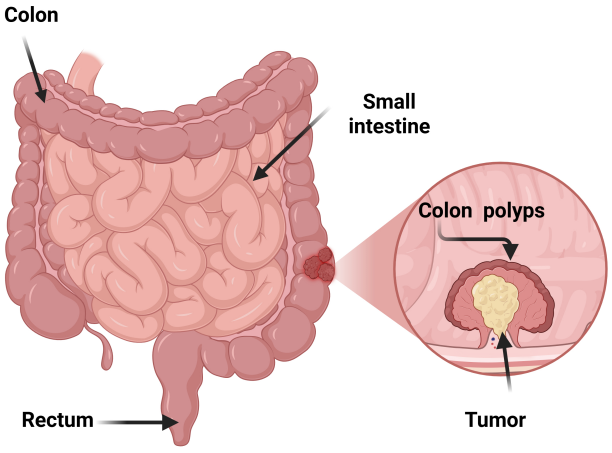


Fig. 1. Representative colonic polyps formed in the initial stages of colorectal cancer. *Image created with BioRender.com*

Surgery, chemotherapy, and radiotherapy have been the main treatments against CRC for many years. However, these treatments have disadvantages, like systemic toxicity and low tumor-specific selectivity, causing fatigue, hair loss, and anemia, which significantly affect the patient's quality of life [2].

In this context, natural products have gained importance due to their proven antitumor effects and the synergistic benefits of their complex phytochemical profiles, which have sparked interest in exploring their potential as a biotherapeutic strategy against CRC.

Opuntia ficus-indica (L.) Mill. (OFI), commonly known as the prickly pear, is a species of the Cactaceae family widely distributed in arid and semi-arid regions worldwide. *In vitro* and *in vivo* studies with various *Opuntia sp.* extracts have demonstrated their potential antitumoral effects against different cancer cell lines, particularly colon cancer cells. Among the changes induced by *Opuntia sp*. extract in these cells are caspase-dependent cell death, cell cycle arrest,

mitochondrial damage, and ROS production in metastatic human colorectal adenocarcinoma cells (HT-29) [6],[7], highlighting their potential as an alternative treatment for colorectal cancer.

To date, it has not been possible to associate the antitumoral activity of Opuntia sp. extract on colon cancer cells with the presence or concentration of any compound or compound family.

Moreover, the biological activities of the extracts may change depending on the extraction method used to isolate bioactive molecules, growing conditions, species, and the part of the plant used to prepare the extracts [8].

This work aimed to evaluate the potential of different extracts obtained from the sequential fractionation of *Opuntia ficus-indica* (OFI) cladode flour to induce cell death in human colon cancer (Caco-2) cells and to determine the phytochemical profile of the OFI cladode fraction with the highest cytotoxic potential.

II. Materials and Methods

A. Biological Material

Opuntia ficus-indica cladode flour was obtained from the northeastern region of Mexico (provided by Alimentos Funcionales S. de R.L. M.I.). The flour was produced according to [9], as follows: fresh cladodes with spines were sanitized in water containing sodium hypochlorite (200-400 ppm, 10 min). After that, the cladodes were cut, dehydrated, and ground into flour.

B. Sequential Exhaustive Extraction (SEE)

The OFI flour was fractionated into seven different extracts using solvents from least polar to most polar, as shown in Table I.

TABLE I
SOLVENT ORDER USED IN SEQUENTIAL EXHAUSTIVE EXTRACTION (SEE) OF *OPUNTIA FICUS-INDICA* FLOUR

Order	Solvent	ID
1	*n*-Hexane	HEX
2	Petroleum ether	PE
3	Ethyl acetate	EA
4	Acetone	AC
5	Butanol	BUT
6	Ethanol 80%	ETH
7	Water	W

Fractionation began by mixing the OFI flour with HEX (1:10 w/v), shaking the samples (250 rpm, 30 °C, 30 min), and then centrifuging (5000 × xg, 10 min) to separate the pellet from the supernatant. The supernatant was removed, dried in a rotary evaporator (below 40 °C), and then freeze-dried. The remaining OFI flour pellet was mixed with the successive solvent (PE), and the procedure was repeated with each solvent until all extracts were obtained.

C. Cell Culture

The human colorectal adenocarcinoma (Caco-2) cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in Dulbecco's Modified Eagle's Medium F12 (DMEM-F12, Cat.12400-016, Gibco, Grand Island NY, USA) containing 5% of fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and incubated at 37 °C and 5% CO_2. A stock solution of each OFI extract (40 mg/mL) was prepared and then sterilized using 0.22 µm nylon sterile filters.

D. Cell Viability

Human colorectal adenocarcinoma (Caco-2) cells were seeded in a 96-well plate (1×10^5 cells/mL). After 24 hours of adherence, treatments were prepared by diluting the OFI extract stock solutions into DMEM-F12 cell culture medium. Then, 100 µL of each treatment was added to their respective wells. The plate was then incubated for 48 hours at 37 °C and 5% CO_2. CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) was used to determine cell viability by adding 10 µL of this reagent to each well of the plates.

The absorbance was measured at 490 nm in a microplate reader (Synergy HT, Bio-Tek, Winooski, VM, USA). The percentage (%) of cell viability was calculated using (1).

$$\text{Cell viability (\%)} = \frac{\text{Abs S with cells} - \text{Abs S blank}}{\text{Abs C cells} - \text{Abs C blank}} \times 100 \quad (1)$$

Where *Abs S with cells* is the absorbance obtained at 490 nm of cells treated with each OFI extract, *Abs S blank* corresponds to the absorbance of culture media + each OFI extract without cells, *Abs C cells* is the absorbance of untreated cells or control cells, and *Abs C blank* is the absorbance of culture media without cells.

E. Intracellular Reactive Oxygen Species (ROS) Production

Intracellular reactive oxygen species (ROS) were measured using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) (Sigma-Aldrich, St. Louis, MO, USA). Cells were seeded into a black, clear-bottom 96-well plate (1×10^5 cells/mL). Treatments were prepared using the OFI extracts and DMEM-F12 with DCFH-DA (60 µM). The plate wells were rinsed with phosphate-buffered saline (PBS) before treatment. Also, PBS was added to the wells along the plate edge to prevent edge effects. The plate was incubated at 37 °C and 5% CO_2 for 48 hours. The plate wells were then rinsed with PBS again before reading.

The fluorescence was measured at 485/528 nm for 10 minutes, with readings every 1 minute, using the microplate reader mentioned earlier. The fluorescence under the curve over time was integrated, and the fluorescence from the blank

cells was subtracted. ROS production was calculated using (2).

$$\text{ROS Production (\%)} = \frac{\int SA}{\int CA} \times 100 \quad (2)$$

Where *SA* is the integrated area of the samples over time, and *CA* is the integrated area of the positive control. Cells cultured in DMEM-F12 alone served as a negative control. Cells with DMEM-F12 and DCFH-DA were used as positive control.

F. Membrane Potential Loss

Membrane potential loss was measured using Safranin O (HYCEL, Zapopan, Jalisco, Mexico). Cells were seeded into a black, clear-bottom 96-well plate (1×10^5 cells/mL). The OFI extracts were diluted with DMEM-F12 to get a final concentration of 100 µg/mL. The content of the wells was removed before the treatments were added to their respective wells. Also, PBS was added to the plate's border wells to prevent edge effects. The plate was incubated at 37 °C and 5% CO_2 for 48 hours. After that, the content of the plate was removed, and before Safranin O at 10 µM (100 µL) was added to each well. The plate was incubated for another 30 minutes under the same conditions. After that, wells were washed with PBS, and 100 µL of PBS were added again to measure absorbance at 540 nm in the microplate reader. Cells cultured in DMEM-F12 alone served as a negative control.

G. Phytochemical profile analysis of Opuntia ficus-indica extract by Ultra-Performance Liquid Chromatography (UPL-PDA-QDa)

The phytochemical profile of the ethyl acetate OFI extract was analyzed by Ultra-Performance Liquid Chromatography Waters Acquity UPLC system coupled with photodiode array/Quadrupole Dalton Analyzer (PDA/QDa) (Waters Corp., Milford, MA, USA). The sample was prepared at 1 mg/mL in methanol and water (50/50 v/v) and filtered with a 0.22 µm filter. The separation was performed after injection of 5 µL of sample using a Phenomenex biphenyl UPLC column (100 mm × 2.1 mm, 2.6 µm) that was maintained at 30°C. The gradient consisted of water acidified with 0.1% formic acid (A) and acetonitrile (B), starting at 98% water and gradually increasing acetonitrile content. The following gradient was used: 0 min, 98% (A); 1 min, 98% (A); 3 min, 95% (A); 24 min, 65% (A); 28 min, 0% (A); 31 min, 0% (A); and 35 min, 98% (A). Chromatograms were recorded at 280, 320, and 365 nm.

H. Statistical Analysis

Results were expressed as mean ± standard deviation. Data were analyzed using the software JMP Pro 14 (SAS Institute, Inc., Cary, NC, USA). Statistical analysis was performed using analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons ($\alpha = 0.05$).

III. Results and Discussion

A. Cell Viability

Opuntia ficus-indica (OFI) extracts differentially affected cell viability in human colon cancer (Caco-2) cells when treated at 100 µg/mL (see Fig. 2). OFI ethyl acetate (EA) and OFI water (W) extracts showed the highest cytotoxicity, reducing cell viability to 43.07% and 63.80%, respectively. On the other hand, OFI extracts obtained with HEX, PE, AC, BUT, and ETH did not exhibit cytotoxicity against colon cancer cells at the evaluated concentration.

Results suggested the presence of promising cytotoxic compounds in the ethyl acetate (EA) and water (W) extracts, which may differ in nature due to the polarity of the extraction solvents. Ethyl acetate is widely used as a solvent for the extraction and purification of organic compounds, particularly esters [10]. On the other hand, water is a polar solvent that dissolves other polar substances, such as salts and sugars [11].

In agreement with our results, [12] demonstrated that the ethyl acetate extract of *Opuntia littoralis* cladodes showed the best antitumoral effect against human breast cancer (MCF-7) cells than diethyl ether, chloroform, 70% ethyl alcohol 96%, ethyl alcohol, or water extracts from the same plant.

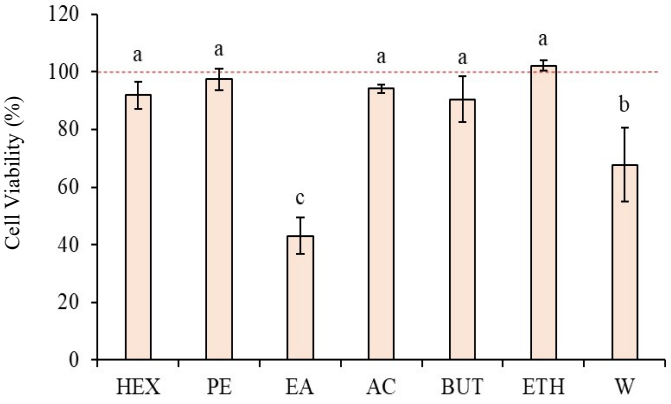


Fig 2**.** Effect of *Opuntia ficus-indica* (OFI) extracts on cell viability of human colon cancer (Caco-2) cells. Cells were treated with OFI extracts (100 µg/mL) for 48 h. HEX: hexane; PE: petroleum ether; EA: ethyl acetate; AC: acetone; BUT: butanol; ETH: ethanol; W: water. [abc] Different letters indicate statistical differences by the Tukey test (p-value < 0.05).

B. Intracellular Reactive Oxygen Species (ROS) Production

As shown in the Fig. 3, ethyl acetate (EA) extract reduced ROS production, indicating that its compounds have antioxidant properties that help cells survive by activating alternative pathways [13],[14], and suggesting that the cell death mechanism induced by the ethyl acetate (EA) extract in colon cancer cells is probably independent of ROS production. The same behavior was observed for the hexane (HEX) and water (W) extracts, which exhibited significant cytotoxicity despite low ROS reduction.

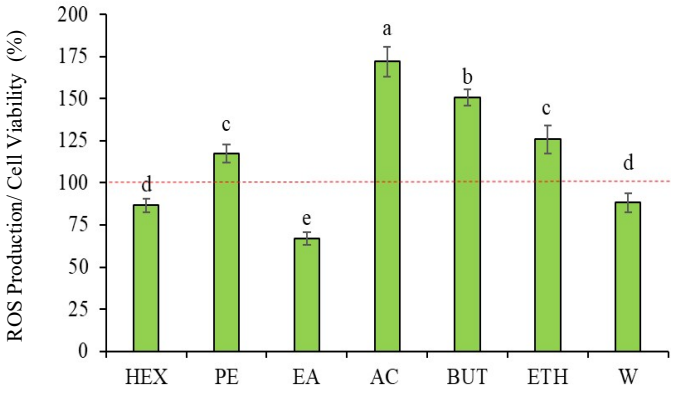


Fig 3. Effect of *Opuntia ficus-indica* (OFI) extracts on intracellular reactive oxygen species (ROS) production of human colon cancer (Caco-2) cells. Cells were treated with OFI extracts (100 µg/mL) for 48 h. HEX: hexane; PE: petroleum ether; EA: ethyl acetate; AC: acetone; BUT: butanol; ETH: ethanol; W: water. [abc] Different letters indicate statistical differences by the Tukey test (p-value < 0.05).

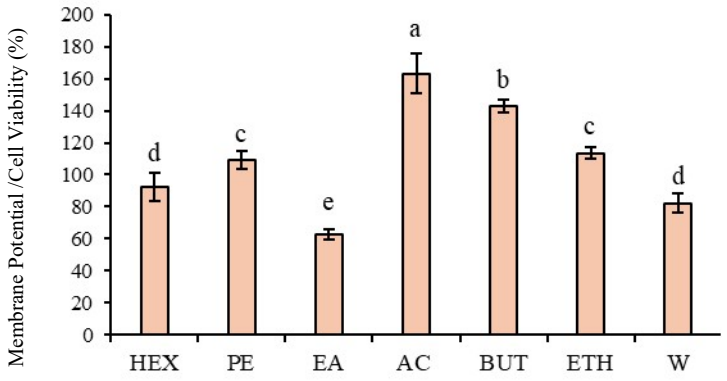


Fig 4. Effect of *Opuntia ficus-indica* (OFI) extracts on membrane potential of human colon cancer (Caco-2) cells. Cells were treated with OFI extract (100 µg/mL) for 48 h. HEX: hexane; PE: petroleum ether; EA: ethyl acetate; AC: acetone; BUT: butanol; ETH: ethanol; W: water. [abc]Different letters indicate statistical differences by Tukey test (p-value <0.05).

C. Membrane Potential Loss

A significant reduction in membrane potential was observed in cells treated with ethyl acetate (EA) extract, as shown in Fig. 4. Ethyl acetate (EA), hexane (HEX), and water (W) OFI extracts reduced membrane potential by 7.7%, 37.17%, and 17.75%, respectively, compared with untreated cells (control). These reductions in membrane potential are described as hyperpolarization of the cellular membrane and may be associated with cell cycle arrest. Acetone (AC), butanol (BUT), ethanol (ETH), and petroleum ether (PE) extracts increased mitochondrial membrane potential by 63.24%, 45.58%, 13.52%, and 9.18%, respectively. These increases suggested a depolarization of the membrane and possibly the onset of apoptosis.

Mitochondria play significant roles in cellular processes, including apoptosis and cell death [15]. Dysfunction in this organelle leads to the generation of reactive oxygen species (ROS) and loss of energy through ATP release. It has been reported that the methanolic extract from OFI cladode induced a loss of membrane potential in Caco-2, HT-29, and CT-26 cells, a process associated with redox reactions and interactions between mitochondria and the benzene ring of phenols [8].

On the other hand, alkylphospholipids (APLs), including miltefosine and perifosine, have been shown to decrease cell viability in CRC cell lines (HT29 and SW620) by modulating mitochondrial function and altering mitochondrial pool size. These APLs increased mitochondrial mass and decreased levels of oxidative phosphorylation (OXPHOS) complexes, leading to a higher mitochondrial membrane potential and an imbalance in mitochondrial function [16]. This means that high ROS production can be achieved regardless of the membrane potential level in certain colon cancer cells.

D. Phytochemical profile analysis of Opuntia ficus-indica extract by Ultra-Performance Liquid Chromatography

The chromatographic profile of ethyl acetate (EA) extract from OFI flour showed a variety of phenolic compounds, mainly phenolic acids detected at 280 nm and flavonols detected at 365 nm (see Fig. 5). These results agree with previous research and suggest that these compounds are the main compounds involved in cell death in colon cancer cells [6],[7],[8],[9].

In addition, mass analysis of the ethyl acetate (EA) extract identified the phenolic acids and flavonols that are most representative and potentially responsible for the observed biological activity. As shown in Fig. 6, piscidic acid (*m/z* 257 $[M+H]^+$) and its isomer (1 and 2), along with eucomic acid (*m/z* 241 $[M+H]^+$) (3), were identified in the ethyl acetate fraction. These compounds have been previously identified in *Opuntia sp.* and are associated with antioxidant, anti-steatotic, and antiproliferative effects [6],[7,[8].

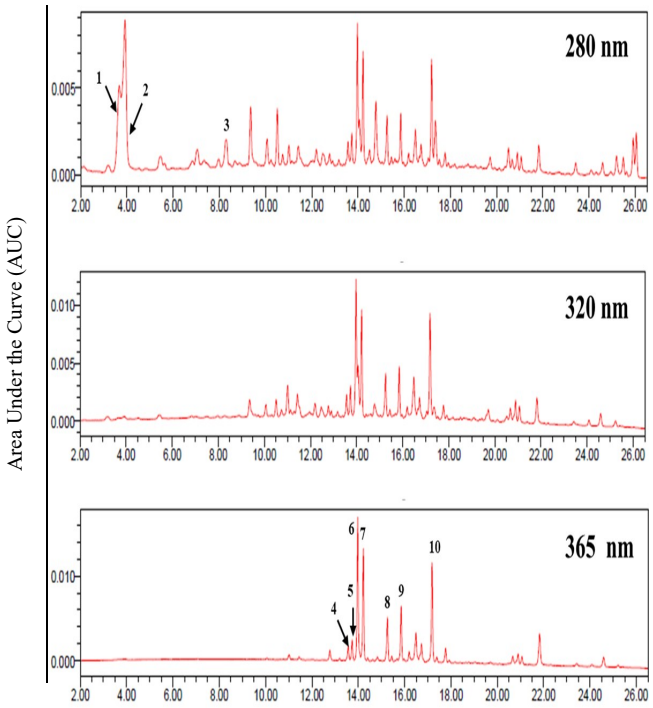


Fig 5. Phenolic profile of ethyl acetate (EA) extract obtained from *Opuntia ficus-indica* flour detected by ultra-performance liquid chromatography (UPLC) at 280, 320, and 365 nm.

Similarly, Fig 7 shows the UV spectrum and the assigned identities of the main flavonols present in the ethyl acetate (EA) extract. Kaempferol-rhamnosyl-rhamnosyl-hexoside (*m/z* 741 $[M+H]^+$) and its isomer (4 and 5), isorhamnetin-glucosyl-rhamnosyl-rhamnoside (*m/z* 771 $[M+H]^+$) and its isomer (6 and 7), isorhamnetin-glucosyl-pentoside (*m/z* 611 $[M+H]^+$) (8), isorhamnetin-glucosyl-rhamnoside (*m/z* 625 $[M+H]^+$) (9), and isorhamnetin-di-glycoside (*m/z* 595 $[M+H]^+$) (10) were the flavonoids identified in ethyl acetate (EA) extract. These compounds have also been reported in extracts of different *Opuntia sp.* and are considered the chemical fingerprint of this plant.

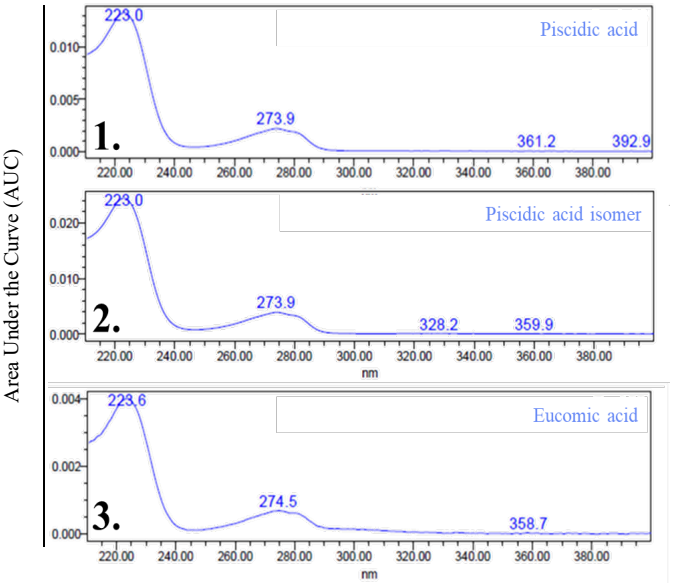
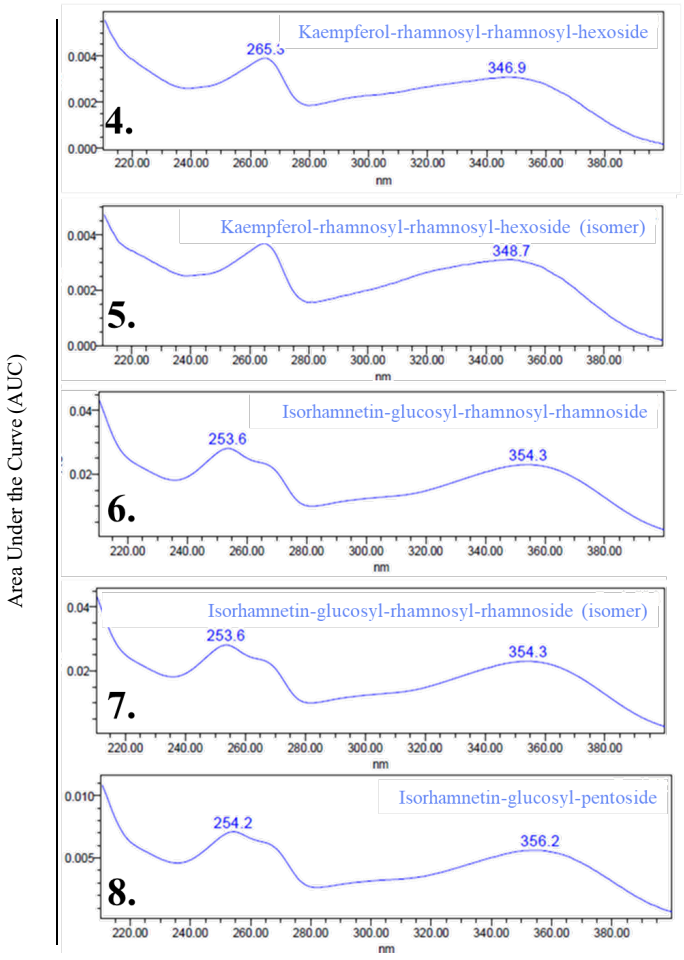


Fig 6. Chromatographic profile of phenolic acids present in the ethyl acetate (EA) extract obtained from *Opuntia ficus-indica flour* using a UPLC-PDA at 280n nm. Piscidic acid and its isomer (1 and 2), eucomic acid (3).



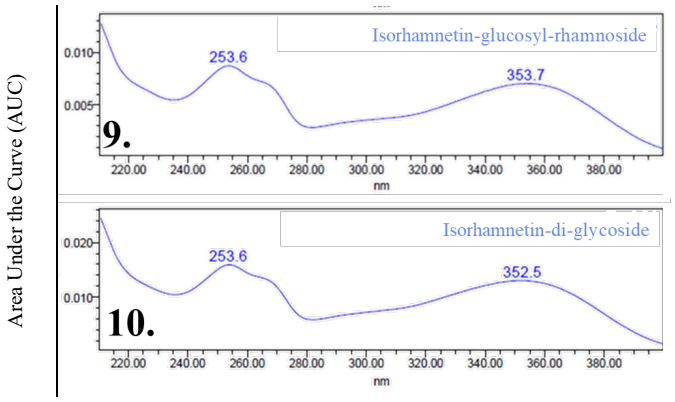


Fig 7. Phenolic profile of ethyl acetate (EA) extract obtained from *Opuntia ficus-indica flour* using a UPLC-PDA at 365 nm. Kaempferol-rhamnosyl-rhamnosyl-hexoside and its isomer (4 and 5), isorhamnetin-glucosyl-rhamnosyl-rhamnoside and its isomer (6 and 7), isorhamnetin-glucosyl-pentoside (8), isorhamnetin-glucosyl-rhamnoside (9), and isorhamnetin-di-glycoside (10).

IV. Conclusion

The present study evaluated the effects of different *Opuntia ficus-indica* extracts on cytotoxicity, ROS production, and membrane potential loss in Caco-2 cells. The ethyl acetate (EA) extract significantly reduced cell viability across all evaluated doses. This extract also showed a loss of membrane potential, which may be implicated in the decrease in cell viability. Nevertheless, the EA extract led to a significant reduction in ROS, suggesting that ROS did not directly mediate its cytotoxicity. Furthermore, this indicates that the compounds in this extract possess antioxidant properties, such as flavonoids, as confirmed by UPLC-PDA-Qda analysis. Further experiments are needed to elucidate the specific mechanisms of cell death induced by the EA extract at different doses in Caco-2 cells and to determine its IC50 value. In addition, the effects of the EA extract need to be evaluated in healthy human colon cells to determine its selectivity and viability for further applications.

Acknowledgment

The authors acknowledge the Secretaria de Ciencia, Humanidades, Tecnología e Innovación (SECIHTI) for financial support for this work through the Ciencia de Frontera 2023 grant (CF-2023-G-669). The authors express their gratitude to the Centro de Biotecnologia FEMSA of the Tecnologico de Monterrey for providing the facilities and equipment for the development of this project, as well as to Alimentos Funcionales Sociedad de Responsabilidad Limitada Microindustrial for donating *Opuntia ficus-indica* (L.) flour.

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