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Inhibición del Crecimiento de Células Cancerosas Caco-2 por Extractos de Prunus Serotina, Obtenidos Mediante Sonicación

Inhibition of Caco-2 Cancer Cell Growth by *Prunus Serotina* Extracts Obtained by Sonication

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Resumen
El fruto y la semilla del cerezo negro o Capulín (*Prunus serotina*) son consumidos en el centro y sur de México. Ambos contienen altos niveles de antioxidantes y se le atribuyen propiedades anti-inflamatorias, anti-cancerígenas y anti-parasitarias. Métodos de extracción como la sonicación pueden ser utilizados para extraer la mayor cantidad de estos compuestos antioxidantes. Se hicieron extractos metanólicos de pulpa sola y pulpa con semilla, y se sometieron a ultrasonicación ó sin ultrasonicación. Con los extractos obtenidos, se evaluó la muerte de células de cáncer de colon Caco-2, el perfil de antioxidantes por DPPH, Azúcares y Fenoles Totales y el perfil de compuestos por cromatografía en capa fina (TLC). La pulpa molida con semilla, sin el proceso de sonicación, presenta 1.2-1.3 más antioxidantes que pulpa y semilla sonicadas. La muestra que contiene sólo pulpa tiene comportamiento similar, donde se observan 1.5 veces más de fenoles totales que la pulpa no sonicada. En los ensayos de citotoxicidad, corroboran lo observado en antioxidantes, donde hay más muerte de las células Caco-2 con los extractos de pulpa con semilla y sin sonicación. La identificación de compuestos por TLC, indica mayor cantidad de compuestos fenólicos en los extractos con semillas. Al moler el hueso con la pulpa, aumenta la cantidad de compuestos fenólicos. La sonicación en baño, no es un método muy eficiente para extraer más compuestos que la extracción por agitación de los frutos *P. serotina*.
Palabras clave: Capulín, cáncer de colon, sonicación, compuestos fenólicos.

Abstract
The fruit and seed of the black cherry or Capulin (*Prunus serotina*) are traditionally consumed in central and southern Mexico. Black cherries contain high antioxidants with anti-inflammatory, anti-cancer, and anti-parasitic properties. Sonication can be used to extract higher amounts of these antioxidant compounds compared to classical methods. Methanolic extracts were made from pulp alone and with seed with or without sonication. The antioxidant capacity of the extract by DPPH, total sugars, and phenols, as well as the compound profile by thin layer chromatography (TLC), was characterized. The cytotoxic activity of the extracts was evaluated in colon cancer cells Caco-2. Without the sonication process, the ground pulp with seed presents a 1.2-1.3 fold increase in antioxidants than the sonicated pulp and seed. The sample containing only pulp exhibits similar behavior, increasing 1.5 times more the content of total phenols. The increase in antioxidant capacity and total phenolic contents positively correlated with their antiproliferative effect in Caco-2 cells, where pulp extracts with seed and without sonication showed a marked decrease in cell viability. Identifying compounds by TLC indicates more phenolic compounds in the extracts with seeds. Grinding the seed with the pulp increases the content of phenolic compounds, while sonication in a bath is not a very efficient method for extracting more phenolic compounds than extraction by agitation from *P. serotina* fruits.
Keywords: Capulin, colon cancer, sonication, phenolic compounds

INTRODUCTION

The black cherry or Capulín (Prunus serotina) is a fruit tree belonging to the Rosaceae family that grows in cool, temperate, and humid climates, preferably in the presence of optimal and distributed rainfall. We can find 5 subspecies distributed throughout the American continent within its species. Prunus serotina is characteristic of regions such as Mexico, Ecuador, Colombia, Guatemala, and the Ecuadorian Andes (Pathania et al., 2022, Rios-Corripio & Guerrero-Beltrán, 2020). Capulín is consumed in Mexico by separating the pulp from the kernel (seed), consuming them together, or consuming the kernel alone or toasted. This fruit makes jams, sweetened waters, tamales, syrups, and alcoholic beverages. Its consumption has significant health benefits due to its high protein (37.95%) and fat content (40.37%), which includes oleic acid (35%) and linoleic acid (27%) (Pathania et al., 2022, Guzmán et al., 2018).

Capulín is rich in antioxidants, some of which are phenolic compounds (flavonoids, tannins, and terpenoids), which provide significant antioxidant activity (Rios-Corripio & Guerrero-Beltrán, 2020; Brozdowski et al, 2021). The plant also has antidepressant, antiparasitic, and antimicrobial properties; furthermore, it is rich in α-eleostearic acid, which has antiproliferative effects on tumor cells (Brozdowski et al., 2021; Gallardo-Rivera et al, 2021).

Oxygen reactive species (ROS) within the cell form highly reactive hydroxyl radicals species that damage DNA and produce mutations that initiate tumors while maintaining their progression (Brown & Bicknell, 2001). Several studies aim to prevent this oxidative damage using the antioxidant capacity of various phytochemical compounds (carotenoids, phenolics, alkaloids, and compounds with nitrogen, sulfur, and selenium), which contribute to preventing damage caused by reactive oxygen species (García-Solis et al., 2009). Colon cancer is one of the leading causes of death worldwide as well as nationally, ranking third and fourth, respectively. The increase in its incidence is associated with various factors (environmental, genetic, and dietary, among others), and the majority of cases occur after the age of 50 (Campo-Sánchez et al., 2018; Quezada-Gutiérrez et al., 2020). Caco-2 cells are widely used in colon cancer research and intestinal absorption studies (Schulz, 2021).

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There are different results regarding the best solvent combination for extracting phenolic compounds from some Prunus species. Hernandez and colleagues (Hernández-Rodríguez, 2016) compared different combinations for extracting phenolic compounds from Prunus serotina Ehrh, such as acetone/water, methanol/water, or ethanol/water; the acetone/water combination yielded the highest amount of phenolic compounds. Gallardo-Rivera et al. (2021) reported that the highest yield of phenolic compounds from the seed is obtained with methanol. Vasco et al. (2008) obtained 331±56 mg GAE/100 g of phenolic compounds from the pulp with acetone: water mixture (70:30 v/v). Meanwhile, Brozdowski et al. (2021) reported that up to 11,394 mg per kg of the sample was obtained from fresh fruit of Prunus serotina.

Using ultrasound for extracting bioactive compounds has been indicated as a Green Extraction technique because it reduces the amount of solvent used while reducing contamination and extraction times (Yusoff et al., 2022; Chaves et al., 2024). However, there are no reports on using such technologies to extract phenolic compounds from Prunus serotina.

In this study, the extraction of phenolic compounds from Prunus serotina fruit was carried out employing a factorial experimental design (2k) using organic solvents in two different samples (pulp with and without seeds), and with sonication or simple agitation. The extract's antioxidant activity, total reducing sugars, and phenolic contents were characterized. Cytotoxicity assays were conducted on Caco-2 cells to evaluate the effect antiproliferative effects of the extracts.

MATERIALS AND METHODS

Preparation of plant material and extraction

Two kilograms of moist P. serotina material with a red/purple hue were collected in June 2023 in Tlaxcala, Mexico (19.3610360, -98.1037970). The collected fruits were separated by ripeness, discarding overripe fruits (see Figure 1A) and green fruits (Figure 1C and 1D). The

selected fruits (Figure 1B) were washed, dried, and subsequently ground in a blender to generate two types of samples: pulp with and without seeds. The samples with seeds were passed through a sieve to remove seed residues. All samples were lyophilized (LABCONCO) until dryness. They were stored protected from light and refrigerated at 4°C until use. The extracts were prepared with 60% methanol (J.T. Baker, Xalostoc, State of Mexico) with 3% formic acid (J.T. Baker, Xalostoc, State of Mexico) using the method described by Brozdowski et al., 2021; with the following modifications: 2 g of lyophilized sample and 10 ml of extraction solution, placing duplicates of each condition in sonication (Branson 5510) for 45 minutes; and duplicates without sonication, only agitation (IKA KS 3000i control) at 250 rpm and 30°C for 45 minutes.

The extracts were centrifuged at 4400 rpm at 4°C for 10 minutes in three cycles (Centrifuge 5702 R Eppendorf); recovering the supernatants and centrifuging again for another 2 cycles under the same conditions, recovering the supernatants. The solutions were placed in the extraction hood (LABCONCO, Protector Laboratory Hood) for 24 h and then in the incubator (Memmert IFP 500) at 41.7°C for one week, obtaining a semi-solid solution from each sample, which was weighed to determine the yield obtained. The samples were resuspended in 2 ml of extraction solution and stored in the refrigerator at 4°C until use—the abbreviation to indicate the conditions is shown in the following Table.

Table 1. Conditions are used to extract compounds from the pulp and/or seed of Prunus serotina.

Sample	Process	Keyword/ abbreviation
Pulp + seed	with sonication bath	+Seed/+Sonication
Pulp + seed	without sonication bath	+ Seed /- Sonication
Pulp	with sonication bath	- Seed /+ Sonication
Pulp	without sonication bath	-Seed/- Sonication

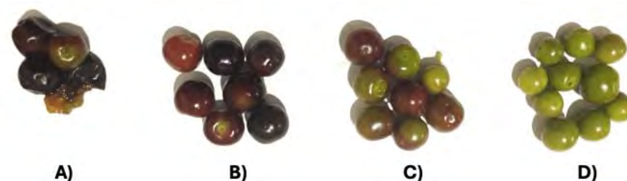

Figure 1. Fruits of *Prunus serotina* at different stages of ripeness. Overripe fruits (1a) and still green fruits (1C and 1D) were discarded. Only ripe fruits (1B) were used for subsequent tests.

Characterization of the extracts

The total content of reducing sugars, total phenols, antioxidant capacity, and presence of phenolic compounds was analyzed using colorimetric methods and Thin Layer Chromatography (TLC).

Reducing Sugars

To measure reducing sugars (RS), the method described by Miller 1959 was employed, with modifications. Briefly, 300 µl of diluted extracts in Milli-Q water (1:50) were mixed with 300 µl of DNS reagent, heated at 100°C for 5 minutes using a thermomixer (Eppendorf Thermomixer compact), and then cooled in an ice bath for 5 minutes. Finally, a 1:6 dilution with Milli-Q water was performed, triplicates of each sample were placed in a 96-well plate, and absorbance was read at 540 nm using a plate reader (Biotek CYTATION|[5] imaging reader). RS were calculated as equivalents of D-glucose and fructose using standard curves (25-800 µg/ml) of the sugar used, with the following corresponding equations ($y = 0.0031x + 0.0453$ $R^2 = 0.988$) and ($y = 0.0033x + 0.0622$ $R^2 = 0.9913$). Results were presented in milligrams of D-glucose and fructose per gram of lyophilized extract (mg G/g LE and mg F/g LE).

Total Phenolic Content

The content of total phenolic compounds (TPC) was determined using the colorimetric method described by Ainsworth & Gillespie, 2007, with some modifications. 100 µl of extract (1:50 in Milli-Q water) was mixed with 200 µl of 10% (v/v) Folin-Ciocalteu reagent. After a 3-minute reaction, 800 µl of Na_2CO_3 (700 mM) was added, and the mixture was kept in the dark for two hours at room

temperature. Absorbance was measured at 765 nm using a plate reader (Biotek CYTATION|5 imaging reader). TPC was calculated as gallic acid equivalents (GAE) using a standard curve (25-200 µM) of gallic acid ($y = 0.0055x + 0.1778$ $R^2 = 0.9487$). Results were expressed in milligrams of gallic acid equivalents per gram of lyophilized extract (mg GAE/g LE).

DPPH Assay

The antioxidant capacity was assessed with the DPPH assay, following the method described by Brand-Williams et al., 1995, with some modifications. A stock solution of DPPH (150 µmol) was prepared using 80% ethanol and sonicated for 20 minutes. 92 µl of diluted samples (1:10 in Milli-Q water) were mixed with 908 µl of the DPPH stock solution and allowed to react for 30 minutes at room temperature. Absorbance was measured at 517 nm using a plate reader (Biotek CYTATION|5 imaging reader), and DPPH activity was reported as the percentage of inhibition calculated using the following equation:

$$\%\, Inhition = \frac{(Abs\ control) - (Abs\ Sample)}{(Abs\ control)} * 100$$

The IC50 was calculated using the regression equation ($y = 1.547x + 2.2912$ $R^2 = 0.9885$) from the Trolox standard curve (5-60 µM). Expressing the results in micromoles of Trolox equivalents (TE) per gram of lyophilized extract (µM TE/g LE).

Thin-Layer Chromatography (TLC)

The presence of phenolic compounds, terpenes, and alkaloids was analyzed using the TLC colorimetric method, with a mobile phase of methanol: chloroform (9:1) and silica gel plates (Master leav) as the stationary phase. 3 µl of undiluted extracts were placed on the stationary phase, which was placed inside a 250 ml beaker containing the mobile phase (10 ml), covered with a watch glass to prevent contamination and excessive evaporation of the stationary phase. After the capillary migration process, the silica gel plate was placed on aluminum foil to remove the excess mobile phase at room temperature. Once the stationary phase was dry, it was sprayed with 2-aminoethyl diphenylborinate using an atomizer and allowed to dry again at room temperature before visualization of migration using UV light on the transilluminator (MultiDoc.It Digital Imaging System UVP).

Cell Viability Assay

The human colon cancer cell line Caco-2 was cultured in DMEM medium (Sigma-Aldrich India) with 5% fetal bovine serum (Gibco, USA) and 1% Antibiotic-Antimycotic (Gibco, USA) and incubated at 37°C with 95% relative humidity and 5% CO_2. Caco-2 cells were seeded in 96-well plates at a density of $2.5x10^5$ cells/well. The plates were incubated for 24 hours prior to treatment. The extracts were diluted in PBS (Gibco, USA) to obtain a homogeneous concentration (600 µg/ml) of each sample and then filtered (0.22 µm Labfil filter) individually. Dilutions were in DMEM medium (1:10) to obtain a working concentration (300 µg/ml). Cells without cytotoxic compounds were used as control, representing 100%, while Cisplatin (100 µg/ml and 10 µg/ml) was used as a positive control. Caco-2 cells were incubated with the extracts for 48 hours, and cell viability was measured using the CellTiter 96® Aqueous One solution (Promega, Madison WI, USA) proliferation assay following the manufacturer's instructions. Absorbance was measured using a microplate reader (Biotek CYTATION|5 imaging reader) at 490 nm, and cell viability was calculated relative to control cells. The assay was performed in triplicates with three independent experiments on different days.

Statistical Analysis

Statistical analysis of the results obtained from the sample characterization and cytotoxicity assays was done using factorial regression modeling and Pareto charts in Minitab 21 Statistical Software (State College, PA, USA).

RESULTS AND DISCUSSIONS

Characterization of the extracts

The results of the characterization of the extracts are shown in Table 2. The highest antioxidant activity was obtained with the sample containing seeds and was not treated with sonication (5.46 ± 0.15 µM TE/g LE). Total phenols (55.47 ± 14.83 mg GAE/g LE) and reducing sugars (367.40 ± 30.57 mg glucose/g LE and 345.15 ± 28.76 mg fructose/g LE) showed a positive correlation

with antioxidant activity. The same sample had the highest values in all characterization assays.

Table 2. General data of the different assays used to characterize the obtained samples. Factorial regression analysis and Pareto diagram of standardized effects

Condition	DPPH µM TE/g LE	D-glucose mg G/g LE	Fructose mg F/g LE	Total phenols mg GAE/g LE
+Seed/-Sonication	5.46 ±0.15**	367.40 ±30.57**	345.15 ±28.76**	55.47 ±14.83**
+Seed/+Sonication	4.53 ±0.66	231.13 ±127.99	217.11 ±120.24	40.83 ±9.75
-Seed/-Sonication	5.38 ±0.14*	331.23 ±48.67*	311.14 ±45.71*	53.92 ±8.90*
-Seed/+Sonication	4.86 ±0.74	216.04 ±86.55	202.93 ±81.30	36.74 ±14.68

* $p < 0.000$-0.001, **Highest value obtained in the assay.

According to the statistical analysis, sonication did not increase the antioxidant capacity, total reducing sugar and phenolic contents. Although the seed is not a significant factor in the results obtained, the highest value among all the samples contains seeds. According to Sharma et al. (2013) and Babaoğlu et al. (2022), implementing a sonication process to extract bioactive compounds does not lead to a decrease in them. However, a comparison with the methodology applied in this project reveals that strict control over operating conditions such as temperature, time, wavelength amplitude, solvent concentration, and equipment is necessary to obtain favorable results. According to Aznar-Ramos et al. (2022), sonication is commonly performed using ultrasonic bath and probe equipment. The ultrasonic bath is more commonly used because it is more economical and readily available, but it has the limitation of intensity attenuation caused by the water bath. The probe equipment is more powerful as it directly applies sonication, resulting in less energy loss. Therefore, a higher yield of bioactive compounds is observed when using the probe equipment (Aznar-Ramos et al., 2022).

Regarding the yield of the extracts, this parameter could not be adequately measured due to gelatinization in the samples. Using a temperature of 41°C to evaporate the solvent may have affected the sugars present in the sample, leading to gelatinization. For fruits with sugar content, an additional step to remove sugars is recommended, as mentioned by Brozdowski et al. (2021).

Thin Layer Chromatography (TLC)

The results of the thin-layer chromatography can be seen in Figure 2, where letter A indicates a characteristic yellow color of terpenes, found in greater proportion in the samples without seeds, while letter B indicates a blue color attributed to phenolic compounds, which are present in the samples containing the seed.

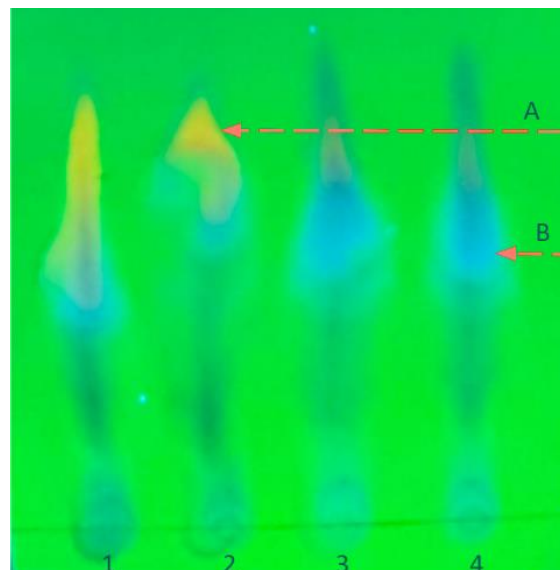

Figure 2. Thin-layer chromatography for the extracts. Numbers below the image 1) Seed/-Sonication 2) Seed/+Sonication 3) +Seed/-Sonication 4) +Seed/+Sonication A) Arrow indicating terpenes in yellow B) Arrow indicating phenolic compound in blue.

Similarly to what was observed in Table 2, thin-layer chromatography confirms that the sample with seeds without sonication has a higher content of phenolic compounds. Comparing our result with those obtained by Brozdowski et al., 2021 (11.399 mg GAE/g) and Andrea et al., 2020 (2.43 mg GAE/g MF), who used the same species as in this study, we obtained 4 times their reported amount of phenolic compounds. Similarly, phenolic

compounds have been found in the seeds of *other Prunus species*

(Gomaa, E. 2013, and Abraão et al., 2023), with a concentration of 0.0838 mg/g extract and 162.29 mg/g dry product. This suggests a higher yield of phenolic compounds in *Prunus serotina* than in *Prunus armeniaca L.* but lower than in *Prunus lusitanica L.*

Cytotoxicity assay on Caco-2 cell line

The cytotoxicity assay shown in Figure 3 depicts the percentage of viability of colon cancer cells (Caco-2) exposed to hydroalcoholic extracts and cisplatin controls of 100 and 10 µg/ml. Samples with seeds had a greater antiproliferative effect than those containing seeds, especially the one treated with sonication, reducing viability by 32.7% ($p \leq 0.003$). This data supports the findings of thin-layer chromatography, where an increase of phenolic compounds results in the death of colon cancer cells.

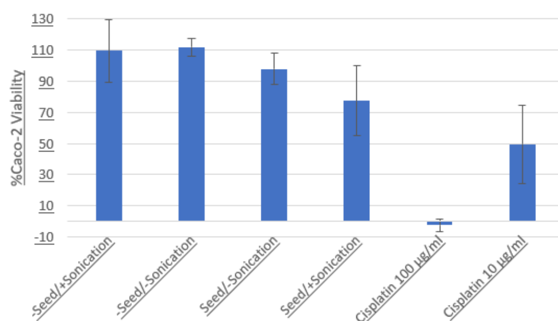

Figure 3. Representation of the percentage of cell viability of each of the extracts (300 µg/ml) and cisplatin controls against the Caco-2 cell line. *p <0.003

Sonication is a widely used technique for releasing nutraceutical compounds in plant samples. However, we found that sonication in a bath does not increase the release of antioxidant compounds. Furthermore, the seed contains more antioxidant compounds than the pulp, and the antioxidant compounds can decrease the viability of Caco-2 cells by approximately 12%. Extracts of *Prunus armeniaca L*. can decrease cell viability by approximately 90.9% in liver cancer (HepG2), 46% in breast cancer (MCF-7), and 54.3% in colon cancer (HCT-116), using a concentration of 50 µg/ml of the obtained extracts (Gomma, 2013); while *Prunus lusitanica L*. can decrease cell viability by approximately 75% in liver cancer (HepG2), 55% in transformed macrophages cancer (RAW 264.7), and 30% in colon cancer (Caco-2), using a concentration of 750 µg/ml of the obtained extracts (Abraão et al, 2023). Compared to extracts from other variants of *Prunus.* The viability obtained in this study is acceptable because the concentrations used in the different studies and the cell lines vary, placing our concentration in the acceptable range for conducting a cytotoxic assay.

CONCLUSIONS

The obtained extracts from *P. serotina* pulp were evaluated with and without sonication for their effect on the viability of Caco-2 colon cancer cells, where the viability of these cells was reduced by 12% with the pulp+seed condition without sonication. Additionally, the antioxidant activity through DPPH, total sugars, and total phenols assays was characterized, revealing that the sample with the highest amount of these compounds corresponded to pulp+seed without sonication. Thin-layer chromatography showed that pulp+seed samples contained the highest amount of phenolic compounds. Sonication in a bath may not be ideal for obtaining antioxidant compounds because the sonication conditions are not fully controlled. On the other hand, *P. serotina* seed contains a high amount of antioxidants, which promote the death of Caco-2 cells in greater quantity than pulp alone.

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