

Antioxidant, cytotoxic and alpha-glucosidase inhibition activities from the Mexican berry “Anacahuita” (*Cordia boissieri*)

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SUMMARY: This study describes the total phenolic and flavonoid content as well as cytotoxic, alpha-glucosidase inhibition and antiradical/antioxidant potential of extracts obtained from the edible fruits of *Cordia boissieri*, which is widely distributed throughout northeastern Mexico. Phenolic and flavonoid content were evaluated by means of the Folin-Ciocalteu method and aluminum chloride colorimetric assay respectively. The antiradical/antioxidant activity was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging and Trolox Equivalent Antioxidant Capacity (TEAC) assays. Cytotoxic activity was assessed by means of human cancer cell lines (MCF-7 and HeLa), alpha-glucosidase inhibition was determined by colorimetric assay using p-Nitrophenyl α -D-glucopyranoside (PNPG) as a substrate. Results indicate that extract of *C. boissieri* fruit has a good antioxidant potential to show a EC_{50} : $137.76 \pm 35 \mu\text{g/mL}$ and $65 \pm 2 \mu\text{M/g}$ in the DPPH and TEAC assays respectively, inhibitor of the enzyme alpha-glucosidase involved in sugar uptake (IC_{50} : $215.20 \pm 35 \mu\text{g/mL}$), cytotoxic activities against MCF-7 (IC_{50} : $310 \pm 42 \mu\text{g/mL}$) and HeLa (IC_{50} : $450.4 \pm 21 \mu\text{g/mL}$) cancer cell lines as well as an important phenolic content with $230 \pm 23 \text{ mg/100g}$ and $54 \pm 11 \text{ mg/100g}$ of phenols and flavonoids totals respectively. These results point towards an interesting potential for the fruits of *C. boissieri* as chemopreventive properties and expand the possibilities for agro-industrial uses..

Key words: Fruits, antioxidants, alpha-glucosidase, chemopreventive.

RESUMEN: Actividad antioxidante, citotóxica e inhibidora de la alpha glucosidasa por la baya mexicana “Anacahuita” (*Cordia boissieri*). Este estudio describe el contenido de fenoles y flavonoides totales, el efecto citotóxico, la inhibición de la enzima alfa-glucosidasa y el potencial antirradical/antioxidante del extracto obtenidos a partir de los frutos de *Cordia boissieri*, especie distribuida por todo el noreste de México. El contenido de fenoles y flavonoides totales se determinó por medio de los métodos de Folin-Ciocalteu y cloruro férrico respectivamente. La actividad antirradical / antioxidante se determinó mediante el secuestro del radical libre 2,2-difenil-1-picrilhidrazil (DPPH) y el ensayo de Capacidad Antioxidante Equivalente al Trolox (CAET). La actividad citotóxica se evaluó sobre las líneas celulares de cáncer humano (MCF-7 y HeLa), para determinar la inhibición de la enzima alfa-glucosidasa se utilizó el ensayo colorimétrico utilizando como sustrato p-Nitrofenil- α -D-Glucopiranosido (PNPG). Los resultados indican que el extracto del fruto de *C. boissieri* tiene un buen contenido de antioxidantes al mostrar una CE_{50} de $137.76 \pm 35 \mu\text{g/mL}$ y de $65 \pm 2 \mu\text{M/g}$ en los ensayos de DPPH y CAET respectivamente, un efecto inhibitorio interesante sobre la enzima alfa-glucosidasa, implicadas en la absorción de azúcar (CI_{50} : $215.20 \pm 35 \mu\text{g/mL}$), efecto citotóxico contra las células cancerosas MCF-7 (CI_{50} : $310 \pm 42 \mu\text{g/mL}$) y HeLa ($450.4 \pm 21 \mu\text{g/mL}$), así como un importante contenido compuestos fenólicos con $230 \pm 23 \text{ mg / 100g}$ y $54 \pm 11 \text{ mg / 100 g}$ de fenoles y flavonoides totales, respectivamente. Estos resultados sugieren el potencial del fruto de *C. boissieri* como una fuente importante de compuestos quimiopreventivos y amplían las posibilidades para su aprovechamiento agroindustrial.

Palabras clave: Frutos, antioxidantes, alpha-glucosidasa, quimiopreventivo.

INTRODUCTION

In recent years there has been a growing trend of studies related to the bioactive properties and compounds present in plant foods; this is mainly due to scarce reports about deleterious side effects associated with their consumption (1,2). In fact, different investigations have shown that increased consumption of fresh fruits is related with a lower incidence of disease, particularly of degenerative ailments associated to the ageing process, including cancer prevention (3, 4).

Consumption of berries has increased over the last years, and different kinds of berries are being widely consumed across many countries, given their high contents of bioactive compounds and their health-beneficial effects (5). Their protective mechanisms are attributed to the presence of natural antioxidants, such as flavonoids, which help to scavenge free radicals and reactive oxidants in the body (6). In this context, the genus *Cordia* has been screened for biological activities (7, 8), however, the fruits of *Cordia boissieri* (Mexican anacahuita) remains unstudied, and in spite of they are used in traditional medicine to treat diseases such as coughs and colds (9). This is of particular interest, given that the species is common in the northeast regions of Mexico and it is not exploited. *Cordia boissieri* is a native North American evergreen tree which reaches 6 meters in height with a 3 to 4.5- meters spread, has silvery green leaves with a velvety texture, and white flowers appear year- round, these are followed by yellow-green fruits with a sweet flesh relished by birds and other wildlife; one of the major forms of human consumption is like jellies (10). The main objectives of this study were to evaluate the total phenolic and flavonoid content, antioxidant and α -Glucosidase inhibitory potential, as well as cytotoxic activity of the fruits from *C. boissieri*.

MATERIALS AND METHODS

Preparation of the fresh fruit extract

The fruits of *Cordia boissieri* were obtained from trees located in the municipality of San Nicolás de los Garza, Nuevo León (México) in a random sampling between the months of October and November of 2014. Taxonomic identification was provided by Dr. Marco Antonio Guzmán Lucio and a voucher specimen (Accession Number: 26307) was deposited in the FCB-UANL herbarium.

The fresh fruits of *C. boissieri* were ground to a pulp and mixed with water 10% w/v. The juice (300 mL) was passed through an Amberlite XAD-7 column, then the column was washed with distilled water and eluted with 900 mL methanol, the organic extract was concentrated in vacuo to dryness; the Amberlite-retained namely *C. boissieri* methanolic fruit extract (1.7 g) was stored at 4° C until use (11).

Chemicals and reagents

All reagents were of analytical grade and obtained from Sigma Aldrich Chemical Co. (Saint Louis, MO, USA), except the minimal essential medium (MEM) and fetal bovine serum, both were obtained from Invitrogen (Grand Island, NY, USA).

Determination of total phenolics

Total phenolic content was determined using the Folin-Ciocalteu reagent as described by Singleton and Rossi (12) with some modifications. One hundred μ L of each sample, 250 μ L of Folin-Ciocalteu's reagent (1 N), 1250 μ L of sodium carbonate (20%) and 400 μ L of distilled water were placed into test tubes. The contents of each tube were homogenized and incubated for 2 h. The absorbance of each mixture was measured at 760 nm, with gallic acid as a standard. Total phenolic content was expressed as mg of gallic acid eq./100g of fresh fruit.

Determination of the flavonoid content

Flavonoid content was determined by use of the aluminum chloride colorimetric assay (+). The fruit extract (250 μ L) or standard solution of catechin (50–500 mg/L) was mixed with 1,250 μ L deionized water and 150 μ L NaNO₂. After standing at room temperature for 5 min, 150 μ L of water solution of AlCl₃ (2%) was added to the solution, followed by 500 μ L of 1 M of NaOH after another 5 min. The absorbance in the reaction mixture was measured at 415 nm. Results were expressed as mg (+) Catechin eq. /100g of fresh fruit (13).

Free radical scavenging activity

The Trolox equivalent antioxidant capacity (TEAC) of the extracts was determined by the ABTS [2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)] cation radical discoloration assay (14) and the values are reported as μ M Trolox/g of extract. The method is based on the consumption of the preformed ABTS^{•+} in the presence of potassium persulfate followed at the maximum absorption of 734 nm. Addition of antioxidants to ABTS^{•+} reduces it to ABTS. The assay was performed on 96 wells microplates, the absorbance of ABTS^{•+} was adjusted to 0.70 ± 0.02 . The decrease of the absorption was measured after 6 min. To determine the antiradical/antioxidant activity, 100 μ L of DPPH[•] (2 mg/L) were mixed with 100 μ L of serial dilutions of the test solution in 96-well microplates; MeOH and Trolox® were used as a negative and positive controls, respectively. The decrease in absorbance at 517 nm was measured (13).

Cytotoxic Assay

The cancer cell lines MCF-7 and HeLa (American Type Culture Collection No. HTB-22 and CCL-2 respectively) were grown in minimal essential medium (MEM) with 10% fetal bovine, penicillin [100 U/mL], streptomycin [100 μ g/mL] and incubated in an atmosphere of 5% CO₂

at 37°C. Cytotoxic assays were performed in 96-well microplates containing 5×10^3 cells/well. Cell cultures were exposed to different concentrations of the fruit methanolic extract for 48 h. Cell viability was measured by WST-1 assay after treatment, to do this, 10 μ L of WST-1 were added to each well and after 90 min of incubation, and the absorbance was measured at 450 nm. Taxol was used as positive control, and cell culture medium, and 1% DMSO were used as negative controls.

Alpha-Glucosidase Inhibition Assay

A modified version of the protocol described by Yang et al. (15) was employed in this study, wherein *Saccharomyces cerevisiae* α -Glucosidase (E.C.3.2.1.20) was suspended in 0.25 M phosphate buffer (pH 6.5). A separate 5 mM p-Nitrophenyl α -D-glucopyranoside (pNPG) solution was prepared using the same buffer. A sample of fruit methanolic extract was added to the enzymatic solution and allowed to incubate for 10 minutes at 37°C. Afterwards, the pNPG solution was added to the enzymatic-plant extract solution and allowed to incubate for additional 45 minutes at 37°C. Once the incubation period was over, the reaction was stopped by the addition of a 1 M solution of Na₂CO₃. Acarbose was used as positive control. The test solution was then analyzed using a spectrophotometer adjusted at 405 nm in order to evaluate enzymatic activity.

Statistical analysis

All data are given as the mean \pm SD of three measurements. The concentration of the samples that inhibited 50% of cell growth (IC₅₀), enzymatic reaction or half maximal effective concentrations (EC₅₀) in DPPH radical scavenging activity was calculated from the log-dose inhibition growth curve obtained by a nonlinear regression algorithm, all values were compared by paired t tests using SPSS (Version 10 for Windows, SPSS Inc., Chicago, IL), $p < 0.05$ was considered significant.

RESULTS

Phenolic compounds are extremely diverse in the plants. In the fruits, the phenolic compounds are related with sensory quality and in recent years they have gained interest for its beneficial effect protector to prevent damage by free radicals (16). The structures of this secondary metabolites contain a polyphenol structure with numerous hydroxyl groups that can donate electrons and thus stabilize free radicals. Exist numerous rapid techniques quantification of phenolic compounds by colorimetric assays, in this work it was used an extrapolation of our results obtained with phosphomolybdic-phosphotungstic acid reagent for total phenolics, the calibration equation was $y = -0.0023+0.0057x$ ($n = 3$, $r^2 = 0.996$) for gallic acid and a optimize spectrophotometric method based on flavonoid-aluminum chloride

($AlCl_3$) complexation to determine the total flavonoid content, the calibration equation was $y= 0.1187x-0.2218$ ($n = 3$, $r^2 = 0.843$) for catechin. Table 1 shows the phenolic content and free radical scavenging capacity of *C. boissieri* fruit extract, based in DPPH assay although there is a significant difference between the antioxidant activities of the fruit methanolic extract compared with trolox (water-soluble vitamin E analogue), it is important to mention that the trolox is a pure compound, while the extract is a mixture of compounds not determined. While for the cytotoxicity assay, the viability of each cell type was examined by the WST-1 reduction assay method. We have previously examined the relationship between culture cell density and its ability to form formazan (data not shown). The inhibition

TABLE 1. Phenolic content and free radical scavenging capacity of *C. boissieri* methanolic fruit extract.

Assay	Fruit Extract	Control
DPPH	137.75 ± 35 µg/mL	12 ± 2* µg/mL
ABTS	65 ± 2.8 µM/g	—
Phenolics	230 ± 23 mg/100g	—
Flavonoids	54 ± 11 mg/100g	—

Trolox was used as positive control in DPPH assay ($p < 0.05$); methanol was used as a negative control and no showed effect.

TABLE 2. Antidiabetic and cytotoxic activities in vitro of *C. boissieri* methanolic fruit extract..

Enzyme/ Cell line	Extract [µg/mL]	Control [µg/mL]
Alpha-glucosidase	215.20 ± 35	52.3 ± 25*
MCF-7	310 ± 42	0.5 ± 0.1*
HeLa	450.4 ± 21	0.9 ± 0.2*

Acarbose was used as positive control on enzymatic inhibition, and Taxol in cytotoxicity assays, $p < 0.05$..

rate in Figure 1 displays the cytotoxicity of *C. boissieri* fruit extract at various concentrations after 48 h, which was estimated from the cell population of the control and cell populations after treatment. The cytotoxicity show a marking effect against on MCF-7 compared with HeLa cells, the taxol (paclitaxel), a natural antitumor compound, was more cytotoxic against cancer cell lines. The inhibition enzymatic assay showed that the fruits extract of *C. boissieri* against alpha glucosidase enzyme (Figure 1) depended on their concentration, the acarbose an anti-diabetic drug used to treat type 2 diabetes mellitus have more significant inhibition activity against alpha glucosidase than the fruit extract, but again, it is important note that this is a pure substance.

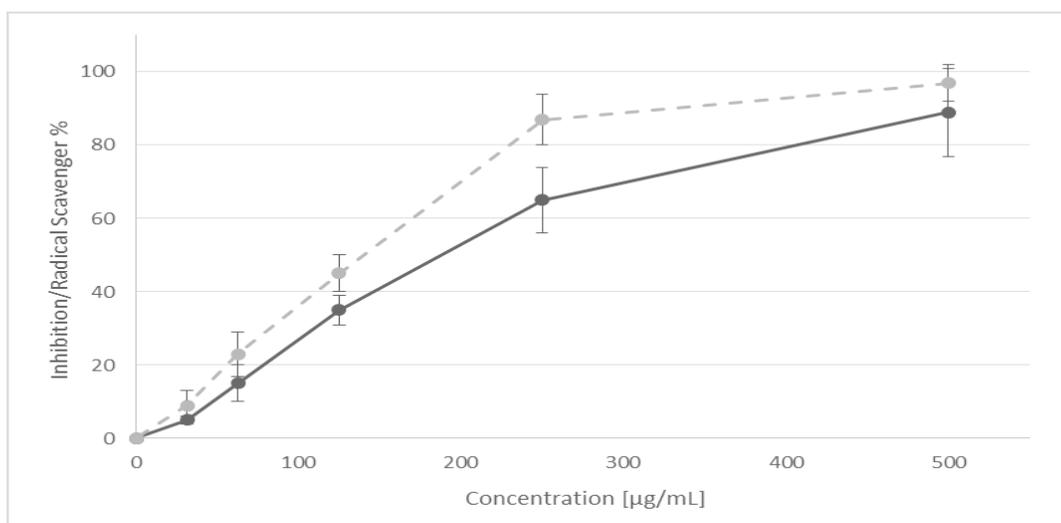


FIGURE 1. Percent of inhibition of the α -glucosidase enzyme (dotted line) and DPPH radical scavenging effect (solid line) of *C. boissieri* fruit extract.

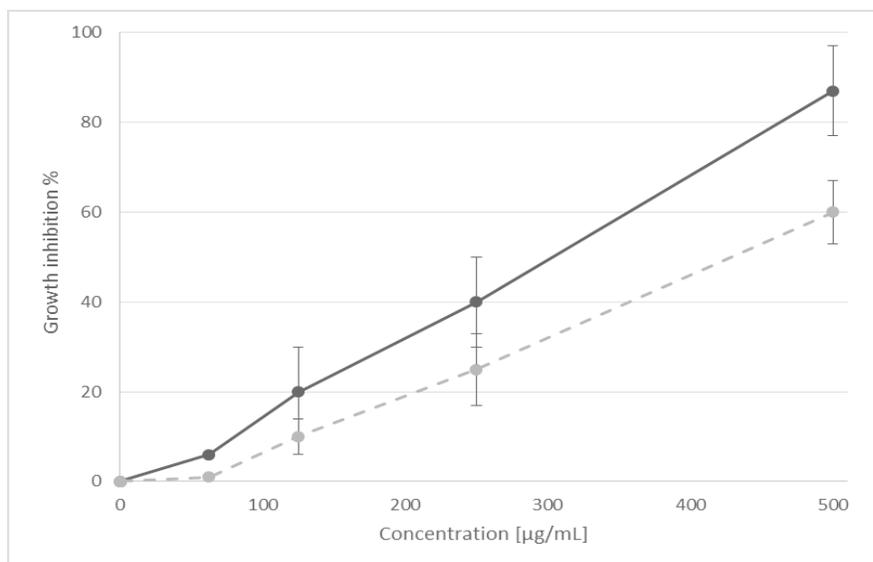


FIGURE 2. Percent inhibition of cellular proliferation MCF-7 (solid line) and of HeLa (dotted line) cancer cell lines exposed for 48h to *C. boissieri* fruit extract.

DISCUSSION

Many methods are used to evaluate antioxidant activities from natural compounds in foods. Two free radicals which are commonly used to assess antioxidant activity in vitro are 2, 2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2, 2-diphenyl-1-picrylhydrazyl (DPPH),

both methods employ the principle of a synthetic colored free radical, and the ability of a sample to scavenge the radical monitored by spectrophotometer. The ABTS assay is interesting in organic extracts because the wavelength absorption at 734 nm eliminates color interference, this assay is based on the decreasing absorbance changes

of a blue/green to a colorless solution; whereas the DPPH assay is based on the reduction of the purple to yellow (17). According to our results, the “Mexican anacahuita” has good antioxidant potential with $EC_{50} = 137.75 \pm 35 \mu\text{g/mL}$ when compared with other wild un-common fruits like: *Litchi chinensis* (102 $\mu\text{g/mL}$), *Lonicera caerulea* (76.14 ± 4.04 to $134.92 \pm 4.62 \mu\text{g/mL}$), *Physalis alkekengi* (248 $\mu\text{g/mL}$) and vegetables like: *Moringa oleifera* (376 $\mu\text{g/mL}$), *Chenopodium album* (454.7 $\mu\text{g/mL}$) *Caralluma tuberculata* (695.7 $\mu\text{g/mL}$) (18- 21). ABTS results, on the other hand, were low ($6.5 \pm 0.28 \mu\text{M/g}$) when compared to the Brazilian berries (6.3 ± 0.2 to $125 \pm 9.7 \mu\text{M/g}$). The same standing applies when phenolic content is compared to those same berries, rating a medium content according to the standards they employed. Total phenolic content is similar or superior to that exhibited by wild berries and cultivars (22, 23). On the other side, alpha-glucosidase is one of the enzymes responsible for breaking down carbohydrates to smaller sugar particles, like glucose, to facilitate their absorption. Alpha-glucosidase inhibitors block by competitive and reversible interaction these intestinal enzymes; they slow the digestion of carbohydrates and delay glucose absorption. This results in a smaller and slower rise of blood glucose levels following meals, and throughout the day, for this reason it is important to study alpha glucosidase inhibitors as possible nutraceuticals (24); in this regard, the “anacahuita” showed moderate inhibitory activity (Figure.1) when compared to the blueberries used in the previously mentioned report, however inhibition was on par or superior when compared to that displayed by root extracts of four species of *Flemingia* (25), as well as when compared to controls. The cytotoxic effects of *C. boissieri* fruits against HeLa cells it was good (Figure.2) if is compared with previous reports of non-traditional fruits and analyzed under similar conditions, like: *Pyracantha coccinea* (IC 500 $\mu\text{g/mL}$), *Zosima absinthifolia* (IC 1 mg/mL), *Cipadessa baccifera*

(133 $\mu\text{g/mL}$), *Solanum erianthum* (142 $\mu\text{g/mL}$) (26-29) on the other side, the methanolic fruit extract of *C. boissieri* was poorly active against MCF-7 cell line in comparative with fruit extracts of *B. racemosa* (57 $\mu\text{g/mL}$) and *H. sabdariffa* (112 $\mu\text{g/mL}$), but good when compared to malay apple (*Syzygium malaccense* with $IC_{50}=632.3 \mu\text{g/ml}$) (30,31). Taking into account the above the cytotoxicity of methanolic extracts from *C. boissieri* (Mexican anacahuita) is moderate compared to other uncommon fruits, however, it could be a good source of compounds with antioxidant activity and potential alpha-glucosidase inhibitory effect

CONCLUSION

Perspectives of utilization of Latin American native fruits are relatively big. The research accomplished indicated the fruits of *C. boissieri* (Mexican anacahuita) as potential source of bioactive substances that could be considered like a possible nutraceutical product.

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