Total polyphenolics, antioxidants, and cytotoxic activity of infusions from soursop (*Annona muricata*) leaves from two Mexican regions

Polifenoles totales, antioxidantes y actividad citotóxica de infusiones de hojas de guanábana (*Annona muricata*) de dos regiones de México

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ABSTRACT

Infusions of soursop or graviola (*Annona muricata* L.) leaves have been used as alternative medicine for their phytochemical composition, pharmacological and cytotoxic activity that are related with antibacterial, antioxidant, and anticancer activities. Infusions of soursop leaves were obtained at 5, 10, and 15 min in samples collected from the Chiapas and Nayarit regions of Mexico. Total soluble phenols (TSP), flavonoids (FC), condensed tannins (CT), total anthocyanins (AC), antioxidant activity, attenuated total reflectance – Fourier transform infrared spectroscopy analysis (FTIR-TRA), cytotoxic activity in MCF7 and HT-29 cell lines were evaluated. The average contents of TSP, FC, CT and AC were as follows: 0.229±0.006 mg gallic acid equivalents ml^{-1}, 0.177±0.003 mg catechin equivalents ml^{-1}, 0.298±0.012 mg cyanidin 3-glucoside equivalents ml^{-1}, and 0.189±0.003 mg catechin equivalents ml^{-1}, respectively. The FTIR-ATR analysis determined carbonyl, hydroxyl, ester, and carboxylic acid groups. The antioxidant activity of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were 0.446±0.014 and 3.53±0.515 mM Trolox equivalents ml^{-1}, respectively. The extraction yields of TSP, FC, CT, and AC by infusion were 88.41, 66.12, 34.93, and 56.09%, respectively. The antioxidant activity was 50.77% for ABTS and 21.44% for DPPH. Cytotoxic activity was present against the MCF7 (80%) cell line. The infusions had high polyphenol contents, antioxidant and cytotoxic activities.

Key words: antioxidant capacity, cell viability, tannins, flavonoids, anthocyanins.

RESUMEN

Las infusiones de hojas de guanábana (*Annona muricata* L.) se han utilizado como medicina alternativa por su composición fitoquímica, actividad farmacológica y citotóxica, que les confieren actividad antibacteriana, antioxidante y anticancerígena. Las infusiones de hojas de guanábana se obtuvieron a 5, 10 y 15 min en muestras colectadas de las regiones de Chiapas y Nayarit en México; se evaluaron fenoles solubles totales (FST), flavonoides (F), taninos condensados (TC), antocianinas totales (AT), actividad antioxidante, reflectancia total atenuada por análisis de espectroscopia infrarroja transformada de Fourier (FTIR-ATR) y actividad citotóxica en líneas celulares MCF7 y HT-29. El contenido promedio de FST, F, TC y AT fue de 0.229±0.006 mg equivalentes de ácido gálico ml^{-1}, 0.177±0.003 mg equivalentes de catequina ml^{-1}, 0.298±0.012 mg equivalentes de cianidina 3-glucósido ml^{-1} y 0.189±0.003 mg equivalentes de catequina ml^{-1} respectivamente. El análisis FTIR-ATR determinó grupos carbonilo, hidroxilo, éster y ácidos carboxílicos. La actividad antioxidante por 2,2-difenil-1-picrilhidracilo (DPPH) y 2,2'-azino-bis-3-etilbenzotiazolina-6-ácido sulfónico (ABTS) fue de 0.446±0.014 y 3.53±0.515 mM equivalentes de Trolox ml^{-1} respectivamente. Los rendimientos de extracción de FST, F, TC y AT mediante infusión fueron 88.41, 66.12, 34.93 y 56.09 respectivamente. La actividad antioxidante fue 50.77% de ABTS y 21.44% de DPPH. Se presentó actividad citotóxica contra la línea celular MCF7 (80%). Las infusiones presentaron un alto contenido de polifenoles, actividad antioxidante y citotóxica.

Palabras clave: capacidad antioxidante, viabilidad celular, taninos, flavonoides, antocianinas.

Introduction

Soursop belongs to the *Annona* genus, which contains more than 160 species. The soursop tree is distributed in tropical regions around the world, most of the year it tends to bloom and is evergreen (Terán-Erazo *et al.,* 2019). Nowadays, soursop is an important economic crop in Mexico, Venezuela, Brazil, and Colombia because of the fruits. The main growing regions in Mexico are the tropical areas, located in Nayarit, Chiapas, Colima, Michoacán, and Veracruz states (Jiménez-Zurita *et al.,* 2016; Escobedo-López *et al.,* 2018; SADER, 2020). In 2019, the Mexican government reported

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a national production of 30,790 t of soursop, with a commercial value of 248,170 million Mexican peso (SIAP, 2019).

Many plant species are rich sources of natural bioactive compounds and could have a diverse variety of biological effects; they have long been employed in alternative medicine because of their therapeutic potential and health advantages (Etheridge & Derbyshire, 2019). Due to the pharmacological, phytochemical, and toxicological effects as well as for their pharmacokinetics, most therapies in Mexican folk medicine are administrated in the form of infusions. These beverages are the first alternative usually suggested by "yerberos" or traditional healers for health care (Alonso-Castro *et al.,* 2017). The infusions consist of an aqueous extraction prepared by pouring boiling water on the leaves and then letting them steep for 5-15 min (Coz-Bolaños *et al.,* 2018).

Several studies report the phenolic characterization of methanolic (Saklar *et al.,* 2015; Nam *et al.,* 2017; Olugbuyiro *et al.,* 2018) and ethanolic (Roduan *et al.,* 2019) infusions of the leaves of soursop; however, due to their easy preparation, infusions are extensively used to treat various diseases and this nutraceutical potential is associated with their phenolic compounds including flavonoids, tannins, and anthocyanins (Coria-Téllez *et al.,* 2019; Balderrama-Carmona *et al.,* 2020; Nguyen *et al.,* 2020). An analytical method used to study important bioactive compounds, such as phenols, is the Fourier transform infrared spectroscopy-attenuated total reflectance (FTIR-ATR) (Daud *et al.,* 2016; Hidalgo *et al.,* 2019; Ibrahim *et al.,* 2021) that allows identifying functional groups that absorb in the mid-infrared and produce particular signal vibrations of the molecular bonds. The resulting spectrum is known as the sample fingerprint (Grijalva-Verdugo *et al.,* 2018).

Soursop leaf infusions show antibacterial (Iyanda-Joel *et al.,* 2019), antifungal (Folorunso *et al.,* 2019), antiprotozoal (Calzada *et al.,* 2020), and antiviral activity (Balderrama-Carmona *et al.,* 2020); and they protect against oxidative stress that could prevent hypertension (Ola-Davies *et al.,* 2019), diabetes (Rahayu *et al.,* 2019), inflammation (Cercato *et al.,* 2021), obesity (Sasso *et al.,* 2019), and cancer (Coria-Téllez *et al.,* 2019; Hassan *et al.,* 2019).

Some cytotoxic studies of *A. muricata* extract have been conducted against HeLa (Coria-Téllez *et al.,* 2019), MDA, and SKBR3 (Gavamukulya *et al.,* 2014) cell lines. The anticancer activity of *A. muricata* is attributed to acetogenin content that are fatty acid metabolic derivatives of long chains (35 to 37 carbon atoms). These compounds contain a terminal α-β-unsaturated or saturated γ-lactone group, together with their central regions with one to three rings of tetrahydrofuran or tetrahydropyran (Aguilar-Hernández *et al.,* 2020; Grba *et al.,* 2022).

The concentrations of phenolic compounds in leaves are influenced by the time of harvest, the growth environment, the soil type, the solar exposure, and the site of production (Guzmán-Maldonado *et al.,* 2020). These factors influence the content of phenolic compounds in the infusions; however, the main factor is the amount of leaves used and the steeping time of the aqueous preparation (Pérez-Burillo *et al.,* 2018).

Many studies approached the relationship of steeping time and migration of polyphenolic compounds into herbal infusions (Rusak *et al.,* 2008; Ramalho *et al.,* 2013; Pérez-Burillo *et al.,* 2018). In this sense, Hardoko *et al.* (2015) and Hardoko *et al.* (2018) assayed the effect of soursop leaves processed as black and green tea with times of extraction for 15, 30, and 45 min; and they characterized the content of phenols, tannins, and flavonoids. Several studies report the phenols and antioxidants in soursop leaf infusions using 10 min of steeping time and 90°C temperature (Innocent-Ukachi & Onukwugha, 2019). Irawan and Mahmudiono (2018) used 70°C water and a steeping time of 4 min, and Cercato *et al.* (2021) used water at 100°C and 15 min of steeping. But few studies have reported the polyphenolic compounds and antioxidant activity of the aqueous extracts of soursop leaves in Mexico (Coria-Téllez *et al.,* 2019; Balderrama-Carmona *et al.,* 2020).

However, there are limited reports about the relationship between production site, phenolic content, antioxidant capacity, and steeping time for soursop leaf infusions from Mexico. The aim of this study was to evaluate the polyphenolic composition, antioxidant activity, cytotoxic activity, and the effect of steeping time of infusions from Mexican soursop leaves collected from the regions of Chiapas and Nayarit.

Materials and methods

Plant samples

Annona muricata leaves were collected from two Mexican states, Nayarit (Tepic, 21°30'0" N, 104°54'0" W) and Chiapas (Cantón el Carmen, 14°46'24" N, 92°13'24" W), in July-August 2020. The selection of the plant material was made considering their healthy phytosanitary characteristics. The leaves were freeze-dried (Labconco, LYPH Lock 4.5, USA), milled (electric mill, NutriBullet, Los Angeles,

USA) and stored (two weeks) in closed plastic bags (Ziploc) at room temperature in the absence of light until analyses.

Infusion preparation

Tea bags for herbal teas were used for the infusion preparations. Each bag of tea, containing 3 g of lyophilized soursop leaves, were infused in 240 ml of boiling water and allowed to steep for 5 min, 10 min, and 15 min; afterwards the infusions were allowed to cool to room temperature and TSP, FC, CT, AC and antioxidant activity were determined (Coz-Bolaños *et al.,* 2018).

Methanolic extraction yields

The polyphenolic compounds from soursop leaves were extracted using solutions of water/methanol (ME), ratio 70:30 and 40:60 (v:v) for total soluble phenols and flavonoids, and absolute methanol was used for tannins. The mixtures were prepared in a ratio 10:1 (m:v), then stirred for 10 min, centrifuged at 5000 rpm for 10 min, and the supernatant were filtered using a Whatman filter paper and the same analyses as for infusions were made (Deshpande & Cheryan, 1985; Singleton *et al.,* 1999; Dewanto *et al.,* 2002).

Total soluble phenols

Total soluble phenols (TSP) were determined by spectrophotometric methods using Folin-Ciocalteu's reagent (Hycel), according to Singleton *et al.* (1999) with some modifications as follow: an aliquot (60 µl) of soursop infusion was mixed with 240 µl of distilled water and 60 µl of Folin-Ciocalteu reagent were added. The samples were then stirred and left to steep for 6 min. Finally, 600 µl of 7% (w/v) Na_2CO_3 (Meyer) solution and 480 µl of distilled water were added and maintained in dark conditions for 90 min at room temperature. Absorbance was read at 750 nm using a Multiskan GO (Thermo Fisher Scientific, 51119200, USA). A gallic acid (Fermont) standard curve (acid gallic = $0.2643(Abs_{750})$ - 0.006, R^2=0.9898) was elaborated using known concentrations (0.200 – 0.020 mg ml^{-1}). The results were expressed as milligrams of gallic acid equivalents per milliliter (mg GAE ml^{-1}).

Flavonoid content

The flavonoid content (FC) was determined following the procedure described by Dewanto *et al.* (2002) with some modifications: 150 µl of soursop infusion was mixed with 45 µl of 5% (w/v) $NaNO_2$ (J. T. Baker) solution, 90 µl of recent prepared 10% (w/v) $AlCl_3$ (J. T. Baker) and 300 µl of 1 M NaOH (J. T. Baker) were added. The mixture was brought to 1.5 ml with distilled water, mixed and left to repose for 5 min. Absorbance was measured against the blank at 510 nm using a Multiskan GO. Catechin (Sigma-Aldrich®, USA) was used as a standard (catechin=$0.5803(Abs_{510})$ + 0.0538, R^2=0.9817), and the solutions were prepared at 0.300-0.062 mg ml^{-1}. The results were expressed as milligrams of catechin equivalents per milliliter (mg CE ml^{-1}).

Condensed tannins

The methodology reported by Deshpande and Cheryan (1985) was used with some modifications to determine condensed tannins (CT) in the infusions. A total of 200 µl of sample was reacted with 1 ml of a solution prepared in proportion 1:1 (v/v) from 1% vanillin (Sigma-Aldrich®, USA) (w/v, dissolved in methanol) and 8% HCl (J. T. Baker) (v/v, dissolved in methanol) and incubated at a temperature of 30°C for 20 min. Absorbance was read at 500 nm against the blank using a Multiskan GO. To estimate the concentration of tannins, a calibration curve with catechin (Sigma-Aldrich®, USA) (catechin=$5.5325(Abs_{500})$ - 0.1577, R^2 =0.9866) was obtained using known concentrations of catechin (0.750-0.031 mg ml^{-1}). Condensed tannin contents were expressed as milligrams of catechin equivalents per milliliter (mg CE ml^{-1}).

Total anthocyanins

The anthocyanin contents (AC) were studied according to the Abdel-Aal and Hucl (1999) method with modifications. The infusion was measured and adjusted to pH 1 with 4N HCl. The sample was read in a Multiskan GO at 535 nm against the blank. Total anthocyanin content per sample (mg ml^{-1}) was calculated as cyanidin 3-glucoside:

$$C = \left(\frac{A}{\varepsilon}\right) \times \left(\frac{vol}{1000}\right) \times MW \times \left(\frac{1}{sample\ wt}\right) \times 10^6$$

where C is the concentration of total anthocyanin (mg ml^{-1}), A is the absorbance reading, ε is the molar absorptivity (cyanidin 3-glucoside=25,965 cm^{-1}), *vol* is the total volume of infusion, and MW is molecular weight of cyanidin 3-glucoside=449.

Antioxidant capacity (ABTS and DPPH)

The method of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS) to evaluate the antioxidant capacity was carried out according to Re *et al.* (1999). The radical $ABTS^+$ was prepared dissolving 3.8 mg of the reactive ABTS (Sigma-Aldrich®, USA) in 1 ml of 2.45 mM potassium persulphate (Fermont), and the mixture was kept in the dark at 4°C for 12 h before use. Then 0.15 ml of the $ABTS^+$ solution was diluted in 14 ml of phosphate buffer at pH 7.4 to obtain an absorbance of 0.7+0.020 at 734 nm using a spectrophotometer (Hach DR3900, USA). The

phosphate buffer was prepared by adding 8 g NaCl, 0.2 g KCl and 1.44 g KH_2PO_4 in 1 L of distilled water. First, the absorbance at initial time (t_0) was measured in 990 µl of the diluted radical $ABTS^+$ solution, and 10 µl of the soursop infusion was immediately added. The mix was kept in the dark at room temperature for 6 min; after that time the absorbance was measured at 734 nm (t_6). The antioxidant capacity was calculated by comparing the absorbance values of the infusion to a Trolox (Sigma-Aldrich®, USA) standard curve (Trolox equivalent=0.0253($Abs_{(t0-t6)}$) + 0.3864, R^2=0.9878). The curve was obtained using known concentrations of Trolox (3000-500 mM). The result was expressed as the equivalent Trolox per milliliter (mmol TE ml^{-1}).

The DPPH antioxidant activity of infusions was determined using Brand-Williams *et al.* (1995) with some modifications using 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich®, USA) as a free radical. A 200 µM DPPH solution in methanol (Reasol) was prepared. The solution was prepared on the same day of the analysis and the mixture was kept in the dark. A 180 µl aliquot of the DPPH radical solution was mixed with 20 µl of the infusion in wells of a 96-well plate and kept in the dark for 30 min. Absorbance was read at 515 nm in a Multiskan GO. The antioxidant activity was calculated using a Trolox calibration curve (Trolox equivalent= 0.0117($Abs_{(t0-t30)}$) – 0.0286, R^2=0.9748). The antioxidant capacity value was expressed as Trolox equivalents per milliliter (mmol TE ml^{-1}).

FTIR-ATR spectroscopy analysis

Two g of milled leaves from each region were mixed with 40 ml of absolute methanol; the mix was sonicated for 10 min, and was allowed to rest for 24 h, after which it was filtered. This procedure was repeated twice, and the filtrates were mixed, then evaporated using a rotary evaporator (Yamato Scientific, RE201) until a dark brown syrup was obtained. A small amount of ME syrup was analyzed using FTIR (PerkinElmer, Dynascan® brand spectrum 100 model) equipped with an attenuated total reflectance interferometer (ATR) at a temperature of 25±2°C. The spectra were obtained from 16 points with a resolution of 4 cm^{-1} in a region of 4000-500 cm^{-1} (Ramírez-Hernández *et al.,* 2020).

Preparation of leaf extract for cytotoxic activity assay

The procedure of Coria-Téllez *et al.* (2019) was used with some modifications; specifically, 630 mg of lyophilized and crushed soursop leaves were mixed with 15 ml of boiling water and the mixture was ground for 10 min, then allowed to steep until reaching room temperature. The mixture was centrifuged at 10,000 rpm for 10 min, and the supernatant was filtered through a 0.45 µm membrane.

Cytotoxic activity assay

The tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) assay was used to measure the cytotoxic activity of the extracts against MCF7, and HT-29 cells line according to the procedure described by Coria-Téllez *et al.* (2019). MCF7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma Aldrich); and the HT-29 cells were cultured in McCoy's Medium (Gibco), both containing 10% (v/v) fetal bovine serum, 1% penicillin–streptomycin antibiotic solution, and 5 ml trypsin. The cells were cultured in a CO_2 incubator with 5% CO_2 at 37°C. The cells were seeded at a density of $1x10^4$ cells per well, incubated for 24 h in darkness and exposed to various concentrations of aqueous concentrated extract (21, 10.5, and 5.25 mg ml^{-1}). The cells were also treated with 50 µl of dimethylsulfoxide (DMSO) (negative control). At the end of the treatment, the supernatant was removed in each well and replaced with 150 µl of MTT (3.33 mg ml^{-1}) followed by incubation at the same conditions for 4 h. The reaction was stopped by adding 100 µl DMSO to each well. The absorbance of each solution was read in a microplate spectrophotometer at 595 nm.

Statistical analysis

All experiments were conducted in triplicate and data were presented as the means ± standard deviations. Statistical analysis was performed using Minitab 17 for Windows (Minitab Statistical Software, USA). Significant differences between the samples were tested using analysis of variance (ANOVA) followed by Tukey's test comparison (P<0.05).

Results and discussion

Total soluble phenols (TSP)

TSP of samples ranged from 0.182 to 0.246 mg GAE ml^{-1} (Tab. 1). TSP in the extracts from Nayarit region correlated with extraction time, the longer infusion time the higher the phenolic content, but not for the Chiapas region. In leaves recollected in Chiapas, at 10 min of extraction the phenolic content decreased; and it increased at 15 min of extraction, even though the infusion from Chiapas reposed for 15 min and had the highest TSP (0.246±0.009). Hardoko *et al.* (2015) analyzed soursop leaf samples at different time extractions and reported the highest concentration of TSP at 45 min of extraction (0.321 mg GAE ml^{-1}), while the lowest concentrations were at 15 min of extraction (0.200 mg GAE ml^{-1}). In accordance with the results obtained in this study, Pérez-Burillo *et al.* (2018) reported for white tea

that increasing the extraction time in the infusions also improved the phenolic extraction (about 10 min).

The increase in TSP with respect to extraction time is related to the polarity and hydrophilicity of the bioactive compounds (Kelebek, 2016). In addition, the water temperature used in the infusions (100°C) influences the extraction efficiency, because the heating process opens the vacuoles of the cells (Hardoko *et al.,* 2015) facilitating the migrations and solubility of polyphenols due to diffusion coefficients of the components to be extracted (Saklar *et al.,* 2015).

Flavonoid content (FC)

For Chiapas, the FC of soursop leaf infusion increased with time, reaching the highest content at 15 min, while Nayarit had the highest concentration at 10 min (0.178 mg CE ml^{-1}). After this time the content decreased, but it was not significant. Several authors found the same effect for long infusion times. For example, Saklar *et al.* (2015) report for green tea elevated catechin concentrations at the beginning of the infusion using water at 75°C and after this time the concentration increased. However, using water at 95°C the catechin content did not change significantly during steep time. Therefore, increasing the steeping time of the infusion beyond 20 min would not result in a greater extraction of flavonoids (Saklar *et al.*, 2015). Ramalho *et al.* (2013) report that the catechin flavonoid content decreased after 10 and 15 min for Indian and British brands of black teas, but the Brazilian brand tea continued increasing up to 30 min. The levels of flavonoids in infusions are liable to change depending on the sample preparation conditions (Jin *et al.*, 2019). For example, long infusion times suggest that the flavonoids as catechins can suffer epimerization (the flavonoids are converted to their corresponding non-epi isomers) (Saklar *et al.,* 2015); and the high temperatures used in the extraction process could induce the flavonoid oxidation resulting in damage to the flavonoid compounds (Hardoko *et al.*, 2015).

Anthocyanin content (AC)

The AC in Chiapas infusions increased with the infusion time (Tab. 1), meanwhile, Nayarit infusions did not show the same behavior because at 10 min of infusion the AC decreased and increased at 15 min. The AC of infusions for the two regions ranged from 0.148 to 0.330 mg C3GE ml^{-1}, and Nayarit showed the highest content of AC at the three-repose time. A wide range of AC values has been reported for different infusion types, ranging from 0.126 to 1.645 mg C3GE ml^{-1} for *Hibiscus sabdariffa* infusion (Salmerón-Ruiz *et al.*, 2019), 0.119 mg C3GE ml^{-1} for *Hibiscus sabdariffa* mixed with bilberry tea that contained berry fruits of aronia, black currant, rose hip, raspberry, bilberry, and blueberry (Bratu *et al.*, 2018). In this sense, the variables that have the greatest impact on anthocyanin extraction are the number of leaves, volume of water, water temperature, and repose time used in the infusion preparation. Therefore, fewer leaves and a lower proportion of water allow for greater extraction efficiency, but it may take longer (Talib *et al.*, 2020). In addition, the use of high temperature water increases the extraction process that improves both anthocyanin solubility and diffusion coefficients (Paraíso *et al.*, 2021). However, high temperatures may not be suitable for anthocyanins, since they are susceptible to thermal degradations and could cause them to break down and lose their antioxidant activity and, consequently, their bioactivity (Salmerón-Ruiz *et al.*, 2019).

Condensed tannins (CT)

The CT content was increased according to the infusion time (Tab. 1) that ranged from 0.131 mg to 0.219 mg CE ml^{-1}. Previous research reports the concentrations ranging

TABLE 1. Phenolic contents and antioxidant activity of infusions from soursop leaves harvested in two Mexican states.

Region	Time (min)	TSP (mg GAE ml^{-1})	FC (mg CE ml^{-1})	AC (mg C3GE ml^{-1})	CT (mg CE ml^{-1})	ABTS (mM TE ml^{-1})	DPPH (mM TE ml^{-1})
Nayarit	5	0.182 ± 0.020^{b}	0.176 ± 0.011^{ab}	0.318 ± 0.028^{a}	0.151 ± 0.013^{de}	1.21 ± 0.065^{b}	0.405 ± 0.042^{ab}
	10	0.205 ± 0.024^{ab}	0.178 ± 0.002^{a}	0.247 ± 0.030^{ab}	0.199 ± 0.006^{ab}	1.33 ± 0.075^{ab}	0.440 ± 0.041^{ab}
	15	0.221 ± 0.008^{ab}	0.175 ± 0.019^{ab}	0.330 ± 0.052^{a}	0.219 ± 0.009^{a}	1.24 ± 0.073^{b}	0.492 ± 0.006^{a}
	Mean	0.202 ± 0.006^{B}	0.177 ± 0.003^{A}	0.298 ± 0.012^{A}	0.189 ± 0.003^{A}	1.26 ± 0.031^{A}	0.446 ± 0.014^{A}
Chiapas	5	0.235 ± 0.012^{a}	0.148 ± 0.006^{b}	0.148 ± 0.022^{c}	0.131 ± 0.011^{e}	1.19 ± 0.007^{b}	0.377 ± 0.051^{b}
	10	0.207 ± 0.012^{ab}	0.168 ± 0.005^{ab}	0.175 ± 0.027^{bc}	0.166 ± 0.011^{cd}	1.28 ± 0.060^{b}	0.381 ± 0.018^{b}
	15	0.246 ± 0.009^{a}	0.171 ± 0.007^{ab}	0.219 ± 0.029^{bc}	0.185 ± 0.003^{bc}	1.47 ± 0.082^{a}	0.357 ± 0.043^{b}
	Mean	0.229 ± 0.006^{A}	0.162 ± 0.003^{B}	0.181 ± 0.012^{B}	0.161 ± 0.003^{B}	1.31 ± 0.031^{A}	0.372 ± 0.019^{B}

Total soluble phenols (TSP); Flavonoid content (FC); Anthocyanin content (AC); Condensed tannins (CT); Antioxidant capacity (ABTS and DPPH). Mean is the average of the three repose times for each region for each phenolic and antioxidant activity. For each column, different lowercase letters indicate significant differences ($P<0.05$) between times measured by Tukey's multiple range test. For each column, different capital letters indicate significant differences ($P<0.05$) measured by Tukey's multiple range test.

from 0.199 to 0.519 mg CE ml^{-1} (Hardoko *et al*., 2015; Hardoko *et al*., 2018). The variations in the concentrations reported in the literature can be attributed to the maturity and treatment of leaves and the temperature and time used for drying (Jimenez-Garcia *et al*., 2020). The time, water temperature, and type of extraction are also related to extraction conditions. This may explain the prevention of tannin oxidation by increasing the concentration of these compounds in the extract (Castiglioni *et al*., 2015; Silva-Ramírez *et al*., 2020).

Antioxidant activity

The results of ABTS assays show that the infusions of Chiapas have the higher antioxidant activity (1.47 mmol TE ml^{-1}) (Tab. 1). The infusion time of 5 min has the lowest antioxidant activity with a significant increase only after 15 min from Chiapas. This behavior is similar to that observed for TSP, AC, and CT. The ABTS results obtained in this study are lower than those obtained by Kelebek (2016) for black tea (1.96 mmol TE ml^{-1}). Almajano *et al*. (2008) report that the antioxidant activity of peppermint infusion and red tea was equal to 0.315 and 0.825 mmol TE ml^{-1}, respectively. This indicates that the antioxidant capacity of the soursop infusion is similar to that of the commercial teas.

The DPPH assays found that Nayarit leaves infused for 15 min produce the highest DPPH values (0.492 mmol TE ml^{-1}). There were significant statistical differences ($P<0.05$) in antioxidant activity depending on infusion time for Nayarit, but not for Chiapas. The behavior of antioxidant activity with respect to time of extraction for Chiapas and Nayarit was similar to TSP and CT behavior. Jin *et al*. (2019) point out the influence of temperature and time of extraction on the antioxidant activity through DPPH method, and they report a maximum at 10 min and 95°C, while after 30 min the activity decreased; however, at 65°C after 10 min the value increased significantly.

It is important to note that no one method determines all the antioxidant compounds of a sample. For this reason, in this study, two methodologies were used to evaluate the antioxidant activity of the infusions obtained. The difference in the results between the ABTS and DPPH was explained by the different compounds with antioxidant activity in the samples. These compounds have different reaction mechanisms and kinetics, due to their chemical structure and functional groups. However, both methods show a decrease in absorbance due to the reduction of radicals. On the one hand, the cationic form of the ABTS radical is reduced by the interaction with hydrogen or electron donor species, while the DPPH radical is reduced in the presence of hydrogen donor antioxidants (Brand-Williams *et al*., 1995; Re *et al*., 1999; Grijalva-Verdugo *et al*., 2018; Guzmán-Maldonado *et al*., 2020).

The high antioxidant activity of the infusions is due to the presence of phenolic compounds and secondary metabolites, such as alkaloids, vitamins, terpenoids, saponins, and essential oils produced for the high metabolic activity of the leaves; and all these compounds in the infusions of soursop leaves play an important role over their antioxidant activity (Kelebek *et al*., 2016; Menezes *et al*., 2019; Roduan *et al*., 2019; Balderrama-Carmona *et al*., 2020; Mannino *et al*., 2020; Silva-Ramírez *et al*., 2020).

The reports about the antioxidant activity shows variations between the different authors, because the results are reported using different units (mg L^{-1} or μmol L^{-1}). However, the use of these practices makes it difficult to understand the real contribution in terms of consumption by portion size (Urías *et al*., 2020). In this research, the consumption of a portion (240 ml) of soursop leaf infusion contributes to antioxidant activity of 229 mM TE per portion. The same activity is proportioned by drinking the same portion of apple juice. Meanwhile, the same portion of red wine gives 1.8 times more antioxidant activity than soursop leaf infusion (Park *et al*., 2018).

Methanolic extraction yields

Table 2 shows the polyphenolic contents and antioxidant activity of methanolic extract of soursop leaves. The results of methanolic extracts did not show significant differences between regions in the variables tested. However, the infusions showed significant differences (Tab. 1) for TSP, FC, AC, CT and DPPH, where a higher content of FC, AC, CT and DPPH in the infusions was also observed

TABLE 2. Phenolic contents and antioxidant activity of methanolic extracts from soursop leaves harvested in two Mexican states.

Region	TSP (mg GAE ml^{-1})	FC (mg CE ml^{-1})	AC (mg C3GE ml^{-1})	CT (mg CE ml^{-1})	ABTS (mM TE ml^{-1})	DPPH (mM TE ml^{-1})
Nayarit	0.336 ± 0.076^{a}	0.290 ± 0.072^{a}	0.853 ± 0.255^{a}	0.423 ± 0.266^{a}	3.53 ± 0.515^{a}	2.08 ± 0.256^{a}
Chiapas	0.259 ± 0.041^{a}	$0.245\pm.053^{a}$	1.077 ± 0.065^{a}	0.287 ± 0.163^{a}	2.58 ± 0.540^{a}	2.56 ± 0.164^{a}

Total soluble phenols (TSP); Flavonoid content (FC); Anthocyanin content (AC); Condensed tannins (CT); Antioxidant capacity (ABTS and DPPH). The results are the mean ± standard deviations of n=3. For each column, different letters indicate significant differences at $P<0.05$ as measured by Tukey's multiple range test.

with Nayarit leaves, while TSP was higher for infusions with Chiapas leaves. The ABTS assays of the infusions did not show significant differences between Chiapas and Nayarit. Chiapas and Nayarit have different climatic and geographical conditions (temperatures, air humidity, and soil composition), inducing differences in the biochemical and physiological responses of the plants (Syed-Najmuddin *et al*., 2017; Ovando-Domínguez *et al*., 2019).

The infusion extraction yields for Chiapas at 15 min showed values of 91.7%, 70%, and 64.5% for ME, TSP, FC and CT, respectively. The yield for the Nayarit region showed results of 34.7% and 23.6% for AC and DPPH, respectively. Coz-Bolaños *et al*. (2018) report yields of 62.4% and 40% for TSP and FC for moringa infusions. Balderrama-Carmona *et al*. (2020) point out yields of 71.97% and 12.57% of antioxidant activity of ABTS and DPPH in soursop leaf infusions, compared to the yield of the extractions with acidified alcohol. Also, Nam *et al*. (2007) report yields of 20% in extractions for TSP in soursop leaves using water, in comparison to the methanol extractions. An accurate comparison could be difficult between the results previously reported by different authors. Due to a lack of uniformity in the conditions of infusion preparation and the properties of the leaves such as leaf age, leaf size and harvesting season, it is difficult to compare accurately the results obtained in the present work with other studies (Saklar *et al*., 2015).

FTIR-ATR analysis of *A. muricata* extracts

The FTIR spectrometry is a physico-chemical analytical technique that reveals the functional groups of the components separated based on their peak ratio. Figure 1 provides the functional groups present in *A. muricata* leaves extract.

In the FTIR, the broad and strong band between 3700 and 3000 cm^{-1} is due to overlapping stretches of vibrations of functional -OH groups, showing the presence of phenolic compounds and the methanol residual in the extracts (Daud *et al*., 2016). The peak at 2,924 cm^{-1} is due to the carboxylic acid group (Ibrahim *et al*., 2021). The peaks observed at 2,853 cm^{-1} are assigned to asymmetric stretching of the -CH_3 groups of the lactone rings, meanwhile the 1,740 cm^{-1} band is attributed to the -C=O stretch vibrations of the γ-lactone ring of acetogenins (Hidalgo *et al*., 2019). The absorptions at 1,656, 1,513, and 1,450 cm^{-1} correspond to the C=C stretch vibration characteristics of the flavonoid bonds (Grijalva-Verdugo *et al*., 2018). The bands between 1,376-1,320 cm^{-1} are assigned to the -CH_3 groups of the alkanes and alkenes (Ibrahim *et al*., 2021). The peaks observed at 1,286, 1,248, and 1,205 cm^{-1} are attributed to the functional group -OH out of plane torsion of carboxylic acids, C-O stretching vibration and C-O-C asymmetric stretching, respectively, belonging to the pyran ring structure of tannins (Daud *et al*., 2016; Grijalva-Verdugo *et al*., 2018). The signals observed at 1,161, 1,071, and 1,027 cm^{-1} correspond to the O=C-O ester functional group attributed to coumarins (Ibrahim *et al*., 2021). Strong absorptions are observed in the region 828-719 cm^{-1}, assigned to the C-O stretching vibrations of α and β pyranose compounds present in anthocyanins (Grijalva-Verdugo *et al*., 2018).

The FTIR spectra of the infusion from the two regions showed slight differences in the intensity and width of the bands and peaks; the differences may be due to the variation in the number of functional groups that produce particular vibrational signals of molecular bonds corresponding to that specific wavenumber. The resulting spectrum is a fingerprint of the *A. muricata* leaf extract (Farooq & Sehgal, 2019).

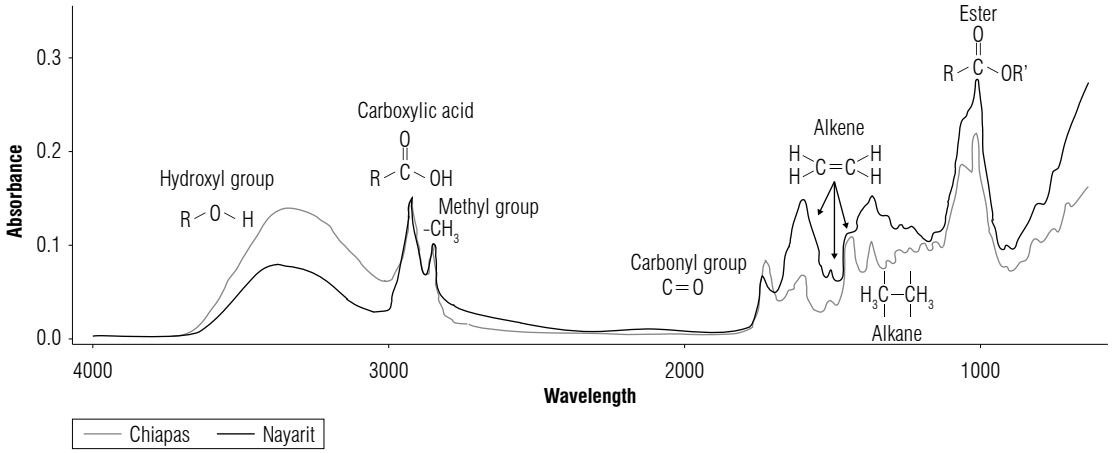

FIGURE 1. FTIR spectra in the region of 4000-850 cm^{-1} of *A. muricata* leaves extracts from Chiapas and Nayarit.

Cytotoxic activity assays

Figure 2 shows the results of cytotoxic activity assays of aqueous extract from Nayarit and Chiapas regions against MCF7 and HT-29 cells. The high cytotoxic activity was found for MCF7 cells with aqueous extract from Chiapas. Also, the results for HT-29 cells did not show statistical differences.

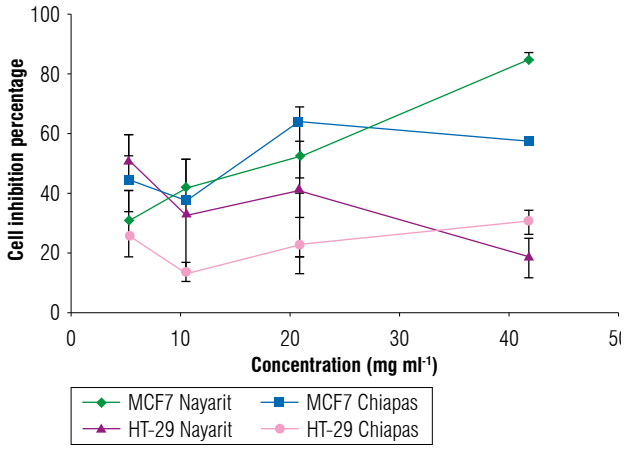

FIGURE 2. Cytotoxic effect of the aqueous extracts from Nayarit and Chiapas against MCF7 and HT-29 cells. Vertical bars correspond to the standard deviation.

Coria-Téllez *et al*. (2019) report that aqueous extracts of *A. muricata* from Nayarit reached a viability of 50% against HeLa cells with a dose extract of 2.42 mg ml^{-1}. Hadisaputri *et al*. (2021) mention a viability cell of 20% against MCF7 cell line using an aqueous fraction of *A. muricata* leaves at 250 µg ml^{-1}; the results show that the survival rate of the cells decreased with increased concentration. In the same way, Syed Najmuddin *et al*. (2016) evaluated the cytotoxic activity of water extracts of leaves of *A. muricata* against breast cells lines, MDA-MB231, MCF7, MCF-10A and 4T1 and reports a varied anti-breast cancer activity, around 50% for MCF-10A, 40% for MCF7, 30% for the cell lines MDA-MB231 and 4T1. The cytotoxic activity has been attributed to acetogenins in the leaves of *A. muricata*, from which more than 45 have been identified (Hadisaputri *et al*., 2021). These secondary metabolites are responsible for the cytotoxicity activity because they inhibit the complex I of the mitochondrial respiratory chain, reducing the production of ATP, bringing on cellular apoptosis (Aguilar-Hernández *et al*., 2020; Grba *et al*., 2022).

Conclusions

The soursop leaves from the Chiapas region showed the highest TSP at 15 min of infusion. The highest TF value appeared in the leaves from Nayarit at 10 min of infusion. For AC and CT, the leaves from Nayarit region at 15 min had the highest values. The higher antioxidant activity of ABTS was obtained for Chiapas and DPPH was highest for the Nayarit region, both at 15 min. The comparative analysis showed significate differences between the regions for TSP, FC, AC, CT and DPPH, but not for ABTS and methanolic extracts. The infusions had the best cytotoxicity activity against MCF7 compared to HT-29 cells. The FTIR-ATR analysis showed important functional groups as carbonyl, hydroxyl, ester, and carboxylic acid, related to the active compounds in the extracts. The use of soursop leaves as infusions can be recommended as a source of antioxidants due to their composition in polyphenolics; furthermore, its secondary metabolites offer cytotoxic activity against breast cancer cell lines. However, the presence and biological activity of its molecules from soursop leaves must be analyzed more deeply.

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Conflict of interest statement

The authors declare that there is no conflict of interests regarding the publication of this article.

Author's contributions

CGV, DMA and MGRC worked in the formal analysis, research, and methodology. JRRN and CLAM worked in the conceptualization, project administration, funding acquisition, writing, and preparing of the original draft. JMVF and RBM contributed with the sampling in the Chiapas and Nayarit regions. CANC was responsible for data curation and formal analysis. All authors have read and approved the final version of the manuscript.

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