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Composition of Secondary Metabolites in Mexican Plant Extracts and their Antiproliferative Activity towards Cancer Cell Lines

Armida Andrea Gil Salido[1], Simon Bernard Iloki Assanga[1], Lidianys María Lewis Luján[1], Daniela Fernández Ángulo[1], Claudia Lizeth Lara Espinoza[1], Ana Lílian Acosta Silva[1], José Luis Rubio Pino[1]

[1]Laboratorio de Investigaciones en Bioactivos y Alimentos Funcionales, Rubio Pharma y Asociados S.A. de C.V. Blvd. Garcia Morales Km. 6.5 # 330. El Llano. Hermosillo, Sonora, Mexico. C.P. 83210

Abstract

Background: Throughout the years humanity has used plants to treat different illnesses. Plants have several secondary metabolites such as phenol compounds, which have important biological activities. In this work, we evaluated the phytochemical screening, the phenol content and the antiproliferative activity of nine methanolic plants extract: *Bucida buceras*, *Haemotoxylon brasiletto*, *Bursera hindsiana*, *Bursera microphylla*, *Ambrosia ambrosioides*, *Phoradendron californicum*, *Annona muricata*, *Morinda citrifolia*, and *Larrea tridentata*, *in murine cell lines:* RAW 264.7 and L929; and human cell lines: A549, HeLa, 22Rv-1, BxPc-3, LS-180 and ARPE-19.

Methods: The type metabolites in the sample were evaluated in a phytochemical screening. The phenols content present in the plant was evaluated by the Folin-Ciocalteu´s method and the antiproliferative activity wad determinate by MTT method, searching the IC_{50} value in each extract for each cell line.

Results: The most abundant secondary metabolites in these plants were lactonic groups, saponins, phenols/tannins and flavonoids. The phenolic content fell in a range from 43.11 ± 6.22 to 827.74 ± 3.48 µgGAE/mg, the order from best to worst was: *P. californicum* (oak) > *L. tridentata* > *B. buceras* > *H. brasiletto* > *B. microphylla* > *B. hindsiana* > *P. californicum* (mesquite) > *A. ambrosioides* > *A. muricata* > *M. citrifolia*. While the better extracts in antiproliferative activity were: *A. muricata, B. buceras, L. tridentata, H. brasiletto* (range from 13.35 ± 0.74 to 163.73 ± 8.42 µg/mL), showing IC_{50} value similars to the cisplatin drug in the different cell lines.

Conclusions: In this study was possible observe that the richest plants in secondary metabolites were *B. buceras, H. brasiletto, B. hindsiana, M. citrifolia* and *P.* californicum. The plant extract with the highest phenolic content was *P. californicum* of oak. While in the antiproliferative activity the best extracts were: *A. muricata* and *L. tridentata* in murine and human cell lines, and also *B. buceras* and *H. brasiletto* only in the case of human cell lines.

Keywords: Antiproliferative activity, Medicinal plants, Cancer, Phenolic content, Phytochemical screening.

Background

Plants have been used for centuries for medicinal purposes. Mexico has a rich plant biodiversity with a long tradition in folk medicine among indigenous communities**.** Now a days the interest in traditional medicine as a major from treatment for different diseases has increased due to the important role that these practices play in primary health care and the high percentage of the world population that uses them. Also, historically, thousands of herbs and their derivatives have been utilized in the treatment of numerous illnesses, including cancer, allergy and diabetes. Over the years, the widespread use of herbal medicine has grown and recently their medicinal

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Armida Andrea Gil-Salido (Correspondence)
andrea.gil@libaf.com
+52 662 2 36 01 01

properties are under extensive interest and investigation, and have become a major part of complementary and alternative medicine. However, in the wide world of plants, only a small percentage has been subjected to scientific research [1-5].
Plants synthesize a vast range of organic compounds that are traditionally classified as primary and secondary metabolites. Primary metabolites are compounds that have essential roles associated whit photosynthesis, respiration, growth and development. These include phytosterols, acyl lipids, nucleotides, amino acids and organic acids. Secondary metabolites have a key role in protecting plants from herbivores, pathogens, other plants like parasitism, and other factors such as pollution, nutrient deficiency, excess of nutrients and salts, climate change, soil compaction and radiation. These compounds are divided into three major groups: terpenes, phenolics and nitrogen-containing alkaloids and sulphur containing compounds [6,7].

In recent years secondary metabolites have become of great interest for the scientific community because they have important properties and are widely use in the fabrication of dyes, fibers, glues, oils, waxes, flavoring agents, drugs and perfumes, and also are viewed as potential sources of new natural drugs, antibiotics, insecticides and herbicides [6]. One of the most interesting metabolites are the polyphenols; in human, a lot of studies suggest that high dietary intake of polyphenols is associated with decreased risk of a range of diseases including cardiovascular disease, specific forms of cancer and neurodegenerative diseases [8].

Cancer is a multi-step disease that has continued to receive the attention of many researchers in the world, and is one of the principal causes of death. This disease involves environmental, chemical, physical metabolic and genetic factors, which play a direct and/or indirect role in the induction and deterioration of cancers. Today, many drugs have been used to improve the quality of life of patients with some kind of cancer; however they have unbearable side effects and have a high cost. Because of this, in the last years, researchers have been focusing on plant compounds and their activities. At the cellular level, the polyphenols present in tea, red wine, cocoa, fruit juices and olive oil influence carcinogenesis and tumor development [8-11]. Success in finding plants or plants compounds with anti-cancer effects with fewer side effects coupled with less cost is a priority in the world of research.

For these reasons, the objective of this study was to evaluate the antiproliferative effects of secondary metabolites in cancerous and normal cell lines, as well as the polyphenol content of methanolic extracts of some Mexican plants.

Methods

Materials

All the Chemicals used in this work were analytical grade reagents. Methanol, hydrochloric acid (HCl) and isopropanol were purchased from J.T.Baker (USA). Folin-Ciocalteu´s reactive, dimethylsulfoxide (DMSO), sodium carbonate, gallic acid, Dulbecco´s Modified Eagle´s Medium (DMEM), Roswell Park Memorial Institute (RPMI-1640) Medium, fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), Penicillin/Streptomycin, 5-fluorouracil, Glutamine, Sodium Pyruvate and Trypan blue were obtained from Sigma-Aldrich Co (USA). Deionized water was obtained from Ultrapure Water System (Milli-Q).

Plant material: Collection

In this work, nine species of plants selected from different zones of Mexico were employed. *Bucida buceras*, *Haematoxylon brasileto*, *Bursera microphylla*, *Bursera hindsiana*, *Ambrosia ambrosioides*, *Phoradendrom californicum* of *Quercus ilex* (oak tree) *and Phoradendron californicum* of *Proposis glandulosa Torr* (mesquite tree), *Annona muricata*, *Morinda citrifolia* and *Larrea tridentata*. The plants *H. brasileto and A. ambrosiosides* were commercial. The plants *B. microphylla*, *B. hindsiana* and *P. californicum* of mesquite tree were recollected in Ures, Sonora. The plant *P. californicum* of oak tree was recollected in Mulatos, Sonora. *L. tridentata* was from Coahuila State. The plants *A. muricata* and *M. citrifolia* were recollected in Tepic, Nayarit. And the plant *B. buceras* was recollected in Hermosillo, Sonora and its main use is ornamental. The plants were taxonomically identified by Dr. Jose Cosme Guerrero Ruiz at the Agronomy Department of Universidad de Sonora and a voucher of classification was assigned. These plants are used by different communities on the country to cure some diseases (Table 1).

Table 1. Plants with medicinal properties used by Mexican communities

Scientific name (Family)	Common name	Traditional use	Parts of plant used in our work	Voucher
Bucida buceras (Combretaceae)	Black olive	Ornamental, antitumor, antifungal, antibacterial, cytotoxic [12-16].	Leaves	19325
Haematoxylon brasileto (Fabaceae)	Palo de Brasil	Hypertension, stomach upsets, mouth infections, diarrhea, gastric ulcers and diabetes [17,18].	Stems	18421
Bursera microphylla (Burseraceae)	Torote blanco	Heal wounds, headache, lung and venereal diseases and throat infections [18].	Stems	19584
Bursera hindsiana (Burseraceae)	Torote prieto or rojo	Respiratory diseases and dyspnoea [18].	Stems	19621
Ambrosia ambrosioides (Asteraceae)	Chicura	Stomachache, placental expulsion, sores, menstrual symptoms, hair diseases, wounds and rheumatism [18, 19].	Stems	15230
Phoradendron californicum (Santalaceae) of mesquite	Toji mesquite	Diarrhea, stomach polyps, venereal disease, in-body diseases [18, 20].	Stems	22431
Phoradendron californicum (Santalaceae) of oak	Toji oak	Diarrhea, stomach polyps, venereal disease, in-body diseases [18, 20].	Stems	22445
Annona muricata (Annonaceae)	Guanabana	Cancer, intestinal parasites, urinary tract infection, skin disease, increase mother´s milk after child birth, as pesticide [21, 22].	Leaves	15128
Morinda citrifolia Liin *(Rubiaceae)*	Noni	Respiratory infections, arthritis, diabetes, muscle aches, menstrual difficulties, heart disease, cancers, gastric ulcer, blood vessel problems, digestive disorders, tuberculosis, stimulation of appetite, depression and drug addiction [23-25].	Fruits	18945
Larrea tridentata (Zygophyllaceae)	Gobernadora	Sexually transmitted diseases, tuberculosis, chicken pox, wounds, rheumatism, gallstones and kidney stones, intestinal parasites, to treat tumors, contraceptive, dysmenorrhea and snakebite [2,18,26].	Leaves	17396

Extract preparation

Different parts of plants (Table # 1) were died at room temperature for 7 days, finely ground and used for extraction. The power obtained (500 g) was mixed with 1.5 L of methanol solvent and was shaken mechanically for 7-10 days in an orbital shaker DS-500 (VWR, USA) at room temperature. Three continuous extraction processes were carried out. All extracts were filtered through a Whatman No. 41 filter paper and the filtrates were concentrated with a vacuum rotary evaporator (Buchi R-210, Switzerland) at 36°C, and were dried in a lyophilizer to powder. The extracts were prepared in solution at know concentration with DMSO as solvent and were stored at 4ºC for later use.

Preliminary phytochemical screening

Phytochemical screenings of the plant extracts were carried out to identify the secondary metabolites such as alkaloids (Mayer´s and Draggendorff´s test, carotenes (Carr-Price test), triterpens, steroids (Liebermann-Burchard test), lactonic groups (Baljet test), lipids and essential oils (Sudan III test), reducing compounds (Fehling test), flavonoids (Shinona test), saponins (Frothing test) and anthraquinones (Borntrager´s test) according to standard phytochemical methods described by Chhabra *et al.* [27] with some modifications [28]. Initially the sample used was the vegetal material before of the methanolic extraction, 1 to 5 gr was collocated in a thimble of the soxhlet system and the sample was extracted with 150 mL of the different solvents in the next order: hexane, methanol and water. The tests above mentioned were carried out with each solvent employed.

Determination of Total Phenols

The quantification of polyphenols was realized according to Singleton and Rossi [29] by the Folin-Ciocalteu´s method with some modifications to carry

out the method using reagents in minor volume [30]. Aliquots measuring 500 µl were taken from the different extracts and mixed with 500 µl of Folin-Ciocalteu reagent diluted 1:4 in water plus 500 µl of 10 % sodium carbonate. The samples were incubated during 2 hrs and the absorbance was read at 760 nm in a microplate reader (MultiSkan-Spectrum). The results were expressed as µg of gallic acid equivalent per mg of extract.

Cell Cultures

The following cell lines were used: from mouse RAW 264.7 (ATCC TIB-71) (macrophage, transformed by Abelson murine leukemia virus) and L-929 (ATCC CCL-1) (subcutaneous connective tissue); from human A-549 (ATCC CCL-185) (lung carcinoma), LS-180 (ATCC CL-187) (Colorectal Adenocarcinoma), HeLa (ATCC CCL-2) (Cervix Adenocarcinoma), 22Rv-1 (ATCC CRL-2505) (Prostate Carcinoma), purchased from the American Type Culture Collection (ATCC) and BxPc-3 (ATCC CRL-1687) (Pancreas Adenocarcinoma) and ARPE-19 (ATCC CRL-2302) (retinal pigmented epithelium) were kindly provided by Horacio L. Rilo, MD (University of Arizona). The adherent cell-lines RAW 264.7, L-929, A549, LS-180 and HeLa, were cultured in bottles of 25 cm^3 from Corning with DMEM medium in a range of 7.2 to 7.4 pH. The medium was supplemented with 5 % with fetal bovine serum (FBS) for all cell lines except ARPE-19 for which it was supplemented with 10% FBS. DMEM was also supplemented with: Penicillin/Streptomycin (1 %), Glutamine (0.75 %) and Sodium Pyruvate (1 %). The cell-lines 22Rv-1 and BxPc-3 were cultured in bottles of 25 cm^3 from Corning with RMPI-1640 Medium supplemented at 10 % with FBS. Supplemented media were filtered with 0.45 µm Millipore membranes before use. All cell-lines were maintained in a 5% CO_2 flux incubator (Binder) at 37 ºC [28].

Antiproliferative assay

Cell proliferation was determined using the standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay, which is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases [31]. Briefly, cells were seeded in triplicate at a density of 10×10^3 cells/well in 96 well plates and allowed to adhere for 24 h at 37°C in an atmosphere of 5% CO_2. 50 µL of the samples at several concentrations (0.1 to 800 µg/mL) were added to the cells, and the incubation continued for another 48 hours. The cytotoxic drug cisplatin (1.8 to 60 µg/mL) was used as positive control in the antiproliferative assays**.** In the last 4 hours of incubation, 10 µL of MTT solution (5 mg/mL) were added to each well. Formed formazan crystals were dissolved with 0.05 N HCl in isopropanol (100 µL), and the absorbance was read at 570nm on a microplate reader (Thermo Scientific Multiskan Spectrum), using a reference wavelength of 655 nm. Data were processed using SkanIt Software 2.4.2. All measurements were carried out in triplicate in three different experiments. The results were expressed as IC_{50} values; which is the required amount of extract to kill 50% of the cells.

To determine the citotoxic selectivity of the tested substances the selectivity index (SI) was calculated, using the formula: IC_{50} non-cancerous cell line/IC_{50} cancerous cell line. If this value is >1, it indicates that the substance is more cytotoxic to cancer cells than to non-cancerous cells and vice versa [32].

Statistical analysis.

All the results were expressed as mean ± standard errors (SE) for at least three experiments in each. All the grouped data were statistically evaluated with NCSS software (version 2007). Hypothesis testing methods included one way analysis of variance (ANOVA) followed by Tuckey test. P values of less than 0.05 were considered to indicate statistical significance. The IC_{50} value was determined using linear regression. The figures were realized with GraphPad Prism software (version 5.01).

Results and Discussion

Preliminary phytochemical screening.

The studied Mexican plants were subjected to several tests to determine the presence of secondary metabolites. The results from our phytochemical screening are summarized on table 2.

Table 2. Phytochemical screenings of medicinal Mexican plants.

B.B.: *Bucida buceras*, H.B.: *Haemotoxylon brasiletto*, B.H.: *Bursera hindsiana*, B.M.: *Bursera microphylla*, A.A.: *Ambrosia ambrosioides*, P.C.: *Phoradendron californicum*, A.M.: *Annona muricata*, M.C.: *Morinda citrifolia*, L.T.: *Larrea tridentata*.

-, Absent of secondary metabolite; +, Little presence of secondary metabolite; ++, Moderate presence of secondary metabolite; +++, Abundant presence of secondary metabolite; ? Doubtful; ND: Not determined.

As can be seen on table 2, Lactonic Groups, Saponins and Phenols/Tannins are the most abundant secondary metabolites in these plants of which Phenols/Tannins are important natural antioxidants [9]. In many of the plants studied, the least abundant secondary metabolites were alkaloids, followed by quinones and reducing compounds. In general, the plants with higher secondary metabolites content were *B. buceras, H. brasiletto, B. hindsiana, M. citrifolia and P. californicum*, which contain many of the studied metabolites.

Very few studies have been done with *Bucida buceras*, but there are some reports of plants of the same family Combretaceae. One of the studies characterizing the plant *Combretum roxburghii* reported the presence of flavonoids, tannins and saponins [33] in its extracts. On the other hand, Hayashi *et al.* [15] reported the presence of diterpenes and flavonoids in *B. buceras*, results that are similar to those shown in this work. Another poorly studied specimen is *Haemotoxylon brasiletto.* This plant was analyzed by Rivero-Cruz where he isolated flavonoids with antimicrobial effects [17].

Other poorly studied plants are *Bursera hindsiana* and *Bursera mcicrophyllla*. However there are other studies*;* about plants in the same genus like *B. graveolens* and *B. simaruba*. *B. graveolens* has shown the presence of cumarins, cardiac glycosides, flavonoids, and quinones. And *Bursera simaruba* showed the presents of alkaloids, flavonoids, saponins, triterpenes, steroids and quinones [34]. In this work both plants show a similar presence of secondary metabolites and they are similar to those reported in *B. graveolens* and *B. simaruba.*

There are few reports realized in *Phoradendrum californicum*, one of which is the study carried out by Iloki *et al.* [28], who reported presence of carotenes, reducing compounds, quinones, flavonoids, saponins, tannins, amines and lactonic groups in this plant. Our observations agree with the results reported in his paper.

In her study in leaves of *Annona muricata*, Vimala *et al.* [35] reported the presence of metabolites (such as alkaloids, quinones and flavonoids), which were not detected in our experiments. However, Vijayameena *et al.* [36] reported results very similar to ours. It's important to note that the collection site of *Annona muricata* in each study was different which could be a reason for the different results of these phytochemical screenings.

Secondary Metabolite / Sample	B. B.	H. B.	B. H.	B. M.	A. A.	P.C.	A.M	M.C.	L.T.
Alkaloids	-	-	-	-	-	-	-	+	-
Carotenes	+++	++	+++	-	-	+	-?	ND	-
Triterpenes/Steroids	++	+	++	++	+	ND	ND	ND	-
Quinones	-	+	++	-	-	-	-	+	-
Lactonic Groups	+++	+++	+++	+++	-	+++	+++	++	-
Lipids and/or Essential oils	-	++	++	++	-	-	-	++	+
Amines and/or Amino Acids	+?	-	++	+	+	+++	-	++	+++
Reducing Compounds	+	+	-	-	-	++	-	+++	++
Saponins	+++	++	++	++	++	+++	-	++	+++
Phenols/Tannins	+++	++	++	+++	-	+++	+	+	+++
Cardiac glycosides	+	++	++	+	+	ND	ND	ND	ND
Flavonoids/Anthocyanins	+	+++	+	+	-	+++	-	+	++

Table 3. Total phenol content in methanolic plant extracts

Methanolic Plant extracts	Total Phenols Content (µgGAE/mg)
***Phoradendron californicum* (oak)**	827.74 ± 3.98 [a]
Larrea tridentata	541.71 ± 9.19 [b]
Bucida buceras	378.39 ± 33.25 [c]
Haemotoxylon brasiletto	276.35 ± 19.58 [d]
Bursera microphylla	202.42 ± 6.44 [e]
Bursera hindsiana	171.44 ± 21.44 [f]
***Phoradendron californicum* (mesquite)**	130.85 ± 2.34 [g]
Ambrosia ambrosioides	48.33 ± 3.37 [h]
Annona muricata	43.63 ± 1.38 [h]
Morinda citrifolia	43.11 ± 6.22 [h]

Means with different letters differed significantly ($p < 0.05$).
µgGAE/mg: Micrograms equivalent of gallic acid per milligram of extract

Nagalingam *et al*. [37] reported similar results for the presence of secondary metabolites in the *Morinda citrifolia* fruit, which was extracted with different solvents like ethanol, methanol and water. These authors reported the presence of steroids, cardiac glycosides, phenols, tannins, terpenoids, alkaloids, carbohydrates, flavonoids, reducing sugar, saponins, protein and lipids. In our results the same metabolites were found, additional to the presence of lactonic groups. Lewis *et al.* [30] quantified proteins, carbohydrates, and phenolic compounds and others physical-chemical properties in the same fruit.

In the case of *Larrea tridentata,* differents results than ours were reported by Garcia *et al.* [38] and Ruiz-Martinez *et al*. [39]. Both authors agree with the presence of triterpenes in this plant, which was not observed in our study. The presence of lactonic groups in this plant was also reported by Garcia *et al*. [38], however in our results these metabolites were absent. Other very important metabolites in this plant are phenols. Several studies have been carried out to isolate them, like nordihydroguaiaretic acid (NDGA), kaempferol and quercetin that can be found at considerable high concentrations [26].

Determination of total Phenolic Content

The results of the total phenolic content of the plant extracts examined, using Folin-Ciocalteu method, are presented in Table 3. Total phenolic content expressed as gallic acid equivalents ranged between 43.11 to 827.74 µg GAE/mg extract. *P. californicum* of oak contained the highest concentration of phenolic compound with a significant difference ($p < 0.05$) from the rest of the extracts. The extract of *M. citrifolia* contained the lowest concentration of phenolic compound. However most of the medicinal plants used conventionally in folkloric medicine have lower phenolic content than that reported in this paper. With respect to the phytochemical screening results, all the plants presented phenolic compounds, which agree with what we found in our phenolic content quantification.

P. californicum is a hemiparasitic plant found in leguminous and woody trees. In our work *P. californicum* found in oak and mesquite trees was evaluated. The information available on this parasite is limited; a previous scientific study about *P. californicum* in mesquite tree only, reports its phenolic content to be approximately 200 µgGAE/mg of extract [20]. Our results showed a concentration of phenolic compounds in mesquite of 130.85 ± 2.34 µgGAE/mg of plant extract. However, it is important to note that *P. californicum* of oak tree showed much greater concentration of phenolic compound (827.74 ± 3.98 µgGAE/mg of plant extract), which indicates that metabolites in *P. californicum* may vary depending of the source tree.

In the case of *L. Tridentata* Martins *et al.* [26] reported that the phenolic content in methanolic leaves extract was 263.60 ± 25.78 µg GAE/mg dry weight plant, which is different from the result found in our investigation (541.71 ± 9.19 µg GAE/mg of extract). This plant contains high quantities of phenols, its leaves are covered with a resin that contains multiple flavonoid aglycones, essential oils, halogenic alkaloids as well as several lignans; in fact the lignan nordihydroguaiaretic acid (NDGA) has been isolated from this plant and amply studied due to its antioxidant properties. This metabolite is currently used in the treatment of multiple diseases, like antineoplastic, antiviral or anti-inflammatory agent [2].

Little research has been conducted on *B. buceras*, which is mainly an ornamental plant. However, there are reports that show that *B. buceras* has antifungal activity against *A. fumigates* [16] and that flavonones isolated from this plant present antitumor activity [15]. In our study we have found a considerable amount of phenolic compounds (378.39 ± 33.25 µg

GAE/mg of extract) in *B. buceras*, which to our knowledge has not been previously reported. Nonetheless, other members of the Combrataceae family, such as *Terminalia arjuna*, have been reported to contain a high amount of phenolic compounds (817.49 ± 8.108 µg/mg, GAE) in their methanolic extracts [40]. Another study of plants from this family is that of Hazra *et al*. [41]. These researchers evaluated the 70% methanolic crude extracts from two plants: *Termiania chebula* and *Terminalia belerica*. They report a value of 1276.0 ± 0.010 and 1333.0 ± 0.003 µg/mL gallic acid per mg plant extract respectively, which in both cases is three times higher than the values obtained in our study. The Combrataceae family is rich in polyphenolic components such as punicalagin, punicalin, chebulagic acid, corialgin, gallic acid, ellagic acid, isovitexin, vitexin, and rutin [42], this abundance in terms of quantity, have also been observed in *B. buceras* in our study.

In the plant *H. brasiletto* two neoflavonoids, heatoxylin and brazilin, have been isolated [17]. In our results, it was found that this plant has a high concentration of phenolic compounds (276.35 ± 6.44 µg GAE/mg). Additionally, we evaluated the phenolic content of B*ursera microphylla* and B*ursera hindsiana* which yielded an amount of 202.42 ± 6.44 and 171.44 ± 21.44 µg GAE/mg of extract respectively. There are no previous studies for these two plants, but studies with other species in the same genus have been realized, like *B. graveolens, which has been shown* to have high anti-inflammatory activity in ears of albino mice (OF1 strain) inhibiting close to 90 % of inflammation [43].
Other plants with low phenolic content are *A. ambrosioides, A. muricata* and *M. citrifolia* (48.33 ± 3.37, 43.63 ± 1.38 and 43.11 ± 6.22 µg GAE/mg of extract respectively). The information and scientific works on *A. ambrosioides* are nearly null; our study is one of the first reports to evaluate it. We have shown that its quantity of phenols is low compared with other plants in this paper.

A. muricata is a plant with a good biological activity but its phenolic content is low, this plant has other secondary metabolites responsible for this activity, such as alkaloids, essential oils, and acetogenins. Acetogenins have been reported to present antitumor activity *in vitro*, cytotoxicity, pesticide activity, anti-parasitic activity and activity as immune-suppressors [32]. Several studies have been conducted to evaluate the cytotoxic activity of acetogenins of *A. muricata* in different cancer cell lines: human lung carcinoma (A549), human lung adenocarcinoma (H 460), mouse gastric adenocarcinoma (C-678), (human pancreatic tumor cell line) (PACA-2), human lung cancer (NCI-H292), human prostate adenocarcinoma (PC-3) and human hepatoma carcinoma carcinoma (Hep G2) [22,44,45]. Furthermore, other studies have been realized to evaluate the phenolic content in *A. muricata*, one of them, carried out by do Santo *et al.* [46], reported a phenol content of 19 ± 0.6 µg pyrogallic acid/mg dry weight.
Many studies have been realized regarding *Morinda citrifolia*, which have mainly focused on its fresh fruit. One of these studies is the one reported by Lewis *et al.* [30] in which it was found that the total phenolic content in noni differed between different ripeness stages and seasons (9.9 – 19.094 µg GAE/mg dry weight). As expected in our work the phenol content is favored in extract form (43 ± 6 µg GAE/mg of extract).

Antiproliferative activity

Today cancer has become a real problem for our society being one of the most important causes of death worldwide. For this reason, the search of compounds, extracts or natural substances that improve the quality of life of cancer patients is an international priority. In this paper, the antiproliferative activity of plant extracts was evaluated with different cancer cell lines. We aimed to find those that offer the best effect against the proliferation of these kinds of cells *in vitro*. The results from our study are shown in the table 4 and figure 1. Table 4 shows the results of the effect of plant extracts on cell lines of murine origin and the figure 1 shows the effect of plant extracts on cell lines of human origin.

Table 4. Antiproliferative Activity (IC_{50})*of plants in murine cell lines.

Plants / Cells Lines	RAW 264.7	L929	SI
Bucida buceras	$52.88 \pm 3.84^{A,b,c}$	$84.83 \pm 3.95^{B,c}$	1.60
Haemotoxylon brasiletto	$63.35 \pm 1.06^{A,c,d}$	$90.91 \pm 7.02^{B,c}$	1.43
Bursera hindsiana	$304.55 \pm 13.94^{A,f}$	$405.32 \pm 11.99^{B,e}$	1.33
Bursera microphylla	$353.97 \pm 0.74^{A,f}$	$504.02 \pm 3.51^{B,f}$	1.42
Ambrosia ambrosioides	$725.48 \pm 27.9^{A,g}$	$> 800^{B,h}$	> 1.10
***Phoradendron californicum* (mesquite)**	$103.21 \pm 3.01^{A,e}$	$178.43 \pm 3.32^{B,d}$	1.73
***Phoradendron californicum* (oak)**	$99.54 \pm 4.44^{A,d,e}$	$160.78 \pm 7.75^{B,d}$	1.62
Annona muricata	$13.35 \pm 0.74^{A,a,b}$	$23.29 \pm 1.72^{B,a,b}$	1.74
Morinda citrifolia	$>600^{h}$	$>600^{h}$	ND
Larrea tridentata	$19.85 \pm 0.12^{A,a,b}$	$62.84 \pm 2.83^{B,b,c}$	3.17
Cisplatin	$4.99 \pm 0.8^{A,a}$	$10.27 \pm 0.84^{B,a}$	2.06

SI: Selectivity Index. *IC_{50} value of extracts or compound (µg/mL) is representative of at least three independent experiments ± standard error. Means with different lowercase letters in the same column differed significantly ($p<0.05$), means with different capitals letter in the same row differed significantly ($p<0.05$). Cisplatin was used as positive control in the antiproliferative assays.

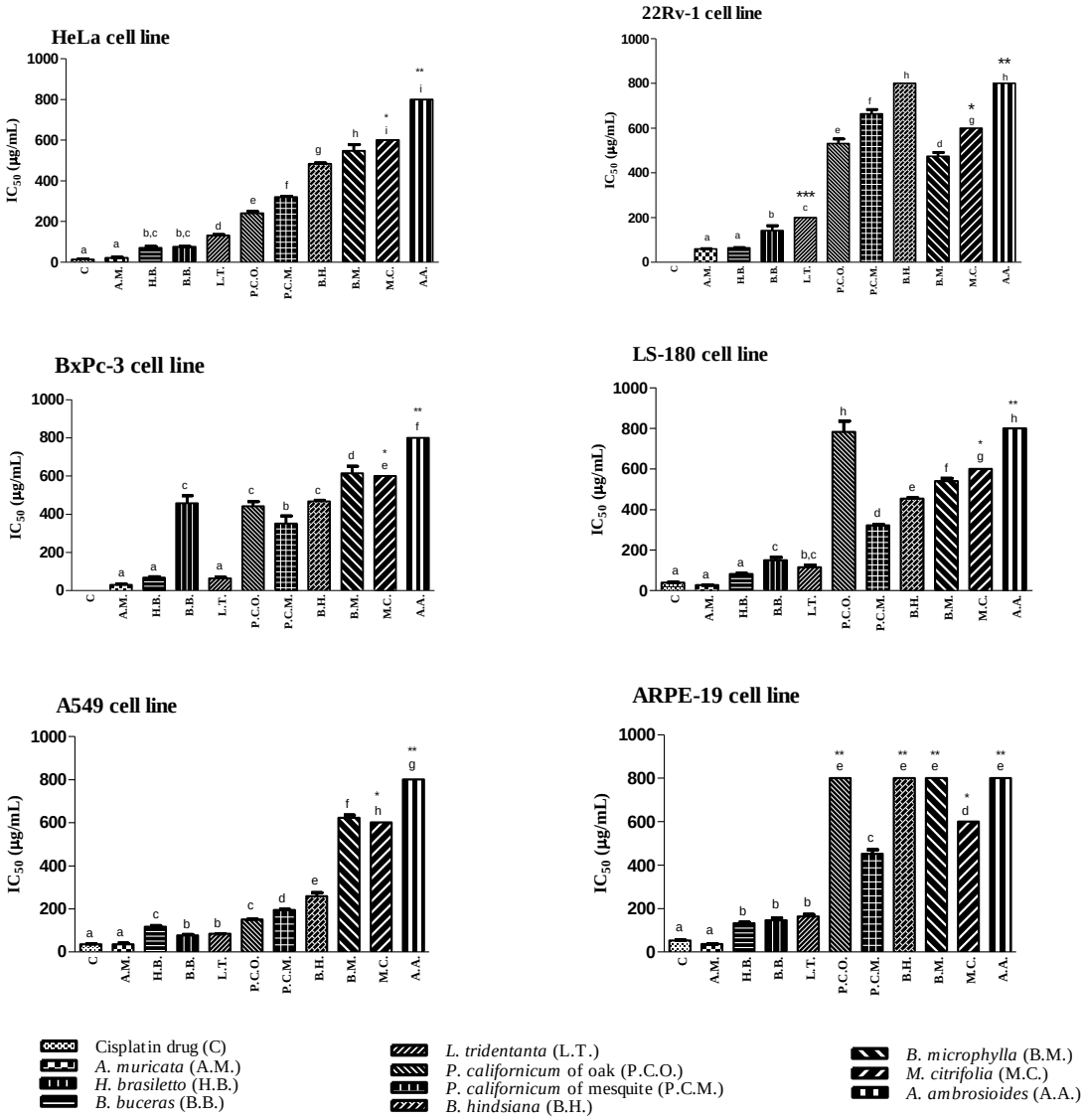
The antiproliferative effect of the different extracts evaluated showed the following behavior: *A. muricata*, *L. tridentata*, *B. buceras* and *H. brasiletto* were the best extracts evaluated. While for the most of others extracts the antiproliferative activity was moderate, except to *A. ambrosioides* and *M. citrifolia*, these two extracts showed the worst antiproliferative activity in the RAW 264.7 cell line.

The L929 cell line was studied as a normal murine cell line control for this assay, and it was expected that the antiproliferative activity of the studied extracts on this cell line would be less than on the cancer cell line. The activity trend for the different extracts is the same in L929 cells than in RAW 264.7 cell, however the different extracts are less effective at killing cells in the L929 cells than in RAW 264.7 cells. Another important parameter to analyze is the selective index (SI), which is a measure of the selectivity of the extract for eliminating cancer cells line, rather than normal cells. When the SI> 1, the extract evaluated is considered to be more effective in a cancer cell line than in normal cell line. Considering these results, the best extract was *L. tridentata* (SI = 3.17), which is more selective than cisplatin (SI=2.0). These results indicate that *L. tridentata* would be an excellent candidate for cancer treatment. In this experiment, the worst extracts were *A. ambrosioides* (SI> 1.10) and *B. hindsiana* (SI = 1.33); even so they still offering present selectivity for the cancer cell line RAW 264.7.

Cisplatin was used as a positive control drug, due to the fact that it's one of the most widely used chemotherapeutic agents for the treatment of several human malignancies [47,48]. In our studies cisplatin showed the lowest IC_{50} value (4.99 ± 0.8 and 10.27 ± 0.84 in RAW 264.7 and L929 cell line respectively), compared with the plant extracts, although some of them, such as *L. tridentata* and *A. muricatta*, showed similar IC-50 values, in RAW 264.7 cell line, and *A. muricatta* in L929 cells. It's Important to mention that the drug cisplatin has severe side effects among which are: ototoxicity, gastrotoxicity, myelosupression, allergy reactions, and high nephrotoxicity [47-50], and that our extracts could be as effective as this drug, but without its undesirable side effects.

Moreover, the antiproliferative effect of the different extracts was also tested on human cell lines. These results are shown in figure 1. The secondary metabolites responsible for the antiproliferative activity are various in the different plants, therefore different results were expected, and also some cell lines are known to be more sensitive than others [28].

Figure 1 Antiproliferative Activity in human cell lines.



* Sample was evaluated up to 600 µg/mL and it did not showed antiproliferative activity (IC_{50} > 600 µg/mL)

** Sample was evaluated up to 800 µg/mL and it did not showed antiproliferative activity (IC_{50} > 800 µg/mL)

*** Sample was evaluated up to 200 µg/mL and it did not showed antiproliferative activity (IC_{50} > 200 µg/mL)

Antiproliferative activity on HeLa, 22Rv-1, BxPc-3, LS-180, A549 and ARPE-19 cells lines of methanolic extracts of Annona muricata, Bucida buceras, Haematoxylum brasiletto, Larrea tridentata, Phoradendron californicum of oak, Phoradendron californicum of mesquite, Bursera hindsiana, Bursera microphylla, Morinda citrifolia and Ambrosia ambrosioides, evaluated by MTT assay. IC50 value of extracts or compounds (mg/mL) is representative of at least three independent experiments ± standard error. Differents lowercase letters differed significantly ($p<0.05$). HeLa: Cervix Adenocarcinma, 22Rv-1: Prostate Carcinoma, BxPc-3: Pancreas Adenocarcinoma, LS-180: Colorectal Adenocarcinoma, A549: Lung Carcinoma and ARPE-19: Retinal Pigmented Ephitelium. Cisplatin was used as positive control in the antiproliferative assays.

As is show in the figure 1 the extract with the best antiproliferative effect was *A. muricata* with the lowest IC_{50} values for all human cell lines: 21.79 ± 3.1 for HeLa, 26.43 ± 2.6 for LS-180, 30.17 ± 3.68 for BxPc-3, 36.21 ± 1.06 for ARPE-19, 38.65 ± 3.94 for A549 and 58.95 ± 1.78 µg/mL for 22Rv-1. All of these IC_{50} values were not significantly different to cisplatin´s IC_{50} (range: 13.91 ± 1.62 to 52.97 ± 1.56 µg/mL). Other extracts with good activity in all the evaluated cells are *H. brasiletto* (range from 64.4 ± 1.65 to 133.31 ± 3.38 µg/mL), *L. tridentata* (range from 64.28 ± 5.15 to 163.73 ± 8.42 µg/mL) and *B. Buceras* (range from 76.17 ± 1.56 to 457.2 ± 39.4

µg/mL). In general in the figure could be appreciate the best activities and the most sensitive cell line, which are the HeLa and the A549 cell line, with the lowest IC_{50} values.
In summary, the extracts of *A. muricata*, *B. buceras*, *H. brasiletto* and *L. tridentata* had the best activity regardless of the cell line (except the *B. buceras* extract for BxPc-3 cell line, IC_{50} = 457.2). Of the plants studied, only *A. muricata* had poor phenol content (44.63 ± 1.38 µgGAE/mg), therefore the metabolites responsible for this activity are the acetogenins [22,32] and not the phenols. On the other hand the rest of the plants analyzed had high phenolic contents (541.71 ± 9.19, 378.39 ± 33.25 and 276.35 ± 19.58 µgGAE/mg for *L. tridentata*, *B. buceras* and *H. brasiletto* respectively), and it´s reported that these compounds are responsible for their antiproliferative activity.

L. tridentata has been amply studied, it is the source of the phenolic lignan, NDGA and it also contains high concentrations of kaempferol and quercetin, which are flavonols with important biological activities. NDGA, the main metabolite of *L. tridentata*, has been shown to have promising applications in the treatment of multiple diseases. Several medicinal properties have been reported in cell culture and animal studies as well as historical reports. Among these properties, one of the most studied is its antioxidant effect. Over the years this compound has been studied and has gained popularity and interest due to its antineoplastic, antifungal, antiviral and anti-inflammatory characteristics. NDGA has been identified to have a significant role in cancer therapy including breast, prostate, lung, esophageal and skin cancers. Models of carcinogenesis have demonstrated the capacity of NDGA to inhibit the growth of several human cancer types both in cell cultures and in animal models [2,26,51,52]. Among these studies Soriano *et al.* [53] tested combinations of several drugs against non small cell lung cancer (NSCLC) (A549, H460 and H157) and small cell lung cancer (SCLC) (SHP77, H69 and H345 cell lines) NDGA showed an IC_{50} value in the range of 10 – 65 µM for the different cell lines; particularly the A549 cell line, for which they reported an IC_{50} value of 65 µM (19.65µg/mL) and a good synergic effect with 13-cis retinoic acid for all cells tested. In this study we found an IC_{50} value of 83.46 µg/mL for this cell line, but in a crude extract. These encouraging results suggest a possible therapeutic chemotherapy role for NDGA.

Also, the plant *Bucida buceras* showed a good antiproliferative activity for all the cells (Range: 52.88 ± 3.84 to 150.4 ± 14.0 µg/mL) except for BxPc3 (457.2 ± 39.4 µg/mL) in which the IC_{50} value is higher than the others cells. In a study realized by Hayashi *et al.* [15] they isolated some flavanones: Buceracidins A and B, minimiflorin, 3-hydroxyminimiflorin, 3-methoxyminimiflorin and mundulinol. They showed the stronger cytotoxicity activity of the compounds minimiflorin, 3-methoxyminimiflorin and mundulinol against human tumor cell replication, in several cell lines: between theses A549 cell line (8.0, 7.6 and 7.5 µg/mL respectively), in this cell line we found an IC_{50} value of 76.71 ± 3.28 µg/mL for the crude extract of *B. buceras*. Also, in another study realized by Hayashi *et al.* [14] with the same purpose, they evaluated the flavonones isolated from *B. buceras*, buciradasin A, B, C y D. All of these extracts (except the buciradasin D) showed cytotoxicity in different cancer cell lines among which were A549, KB (nasopharyngeal), IA9 (ovarian), CAKI (kidney), MCF-7 (breast), HO8 (bone). The IC_{50} values for these compounds are in a range from 0.2523 µg/mL to 0.5551 µg/mL for buciradasin A; 0.5186 to 0.9854 µg/mL for buciradasin B; 0.2443 µg/mL to 0.8795 µg/mL for buciradasin C, these compounds showed lowest IC_{50} values but are pure compounds and our sample is a crude extract with a promising antiproliferative activity.

Another plants whose extracts showed a good antiproliferative activity (range: 63.35 ± 1.06 to 133.31 ± 3.38 µg/mL) was *H. brasiletto*; this plant is a large tree which is very abundant in the south-east of Mexico and is used as a tea for treating hypertension, stomach upsets, mouth and kidney infections, diarrhea, gastric ulcers and diabetes [17,18]. Despite its important uses in traditional medicine, this plant has not been thoroughly studied. In our research *H. brasiletto* was tested in methanolic extract against some cells lines in antiproliferative activity and we showed that this is one of the best extracts evaluated. Two neoflavonoids have been isolated from this plant: hematoxylin and brazilin, with antimicrobial activities versus *Salmonella typhosa*, *Straphylococcus aureurs*, *Brucella suis* and *Shigella flexneri* [17, 54]. Quintanilla-Licea *et al.* [55] realized a study with several plants used in Mexican traditional medicine for the treatment of parasitic infections, one of these plants *H. brasiletto* Karsten whose methanol crude extract show an IC_{50} value of 96.38 µg/mL against *Entamoeba histolytica*. *H. brasiletto* has been previously been reported to have antimicrobial effects on bacteria and parasites. In this scientific work we have also observed an antiproliferative effect on cancer cell lines. Additionally to the flavonoids previously isolated from *H. brasiletto* by Sanchez-Marroquin *et al.* [54], we quantified phenol content of 276.35 ± 19.58 µgGAE/mg. These compounds are possibly responsible of the "antigrowth effect" of parasites, bacteria and cancer cell lines.

Curiously, the *P. californicum* of oak extract with the

higher content of phenols (827.74 ± 3.98 µgGAE/mg) is not the best in antiproliferative activity (rank: 99.54 ± 4.44 to > 800 µg/mL). We expected that the extract with the higher phenol content would be the best in antiproliferative activity, but this is not a rule and in nature there are many other secondary metabolites that give plants their biological activities. In fact, in our phytochemical screening we were able to detect the presence of lactonic groups, amines and/or amino acids, saponins and reducing compounds, additional to phenols, tannins, flavonoids and anthocyanins. There is a study in which the antiproliferative activity of *P. californicum* was evaluated, showing more remarkable antiproliferative action on RAW 264.7 and L929 murine cell lines than on the ARPE-19 and A549 human cell lines [28]. A similar effect was found by through our experiments, with the murine lines being more sensitive to this extract. Iloki *et al.* [28] reported an IC_{50} value for the A549 cell line of 56.2 and 37.52 µg/mL for *P. californicum* of oak and mesquite ethanolic extract respectively; and an IC_{50} value for ARPE-19 of 354.67 and 220.50 µg/mL for *P. californicum* of oak and mesquite ethanolic extract respectively. These results are different from ours but, the extracts used in this study were made in methanol solvent. Additionally, other work in which *P. californicum* of mesquite, in methanolic extract form were used, reported poor antiproliferative activity, with IC_{50} values of 500 µg/mL in HeLa cells, > 800 in RAW 264.7 cells and L929 cells and 800 µg/mL to $M12A^{k}$.C3.F6 cells [20], we found better activities for all these cell lines and others also evaluated in this work (table 4 and figure 1).

To our knowledge, the plants *Bursera hindsiana* and *Bursera microphylla*, have been poorly studied, there are only a few known studies are on plants within this genus. We could say that this one of the first reports on the antiproliferative activity of these plants. They present a moderate activity (range: 249.12 ± 10.72 to >800 µg/mL and 473.72 ± 17.34 to >800 µg/mL respectively for human cell lines; and 304.55 ± 13.94 to 405.32 ± 11.99 µg/mL for *B. hindsiana*, and 353.97 ± 0.74 to 504.02 ± 3.51 for *B. microphylla* in murine cell lines) but their phenol content is considered (202.42 ± 6.44 µgGAE/mg for *B. microphylla* and 171.44 ± 21.44 µgGAE/mg *B. hindisiana*) responsible for other biological activities in these plants. *B. microphylla* have mono and sesquiterpenes on the essential oil obtained from the resin and of burseran, a lignan that showed antitumor activity [56].

Morinda citrifolia L. is a highly researched plant, which has been used medicinally for over 2000 years. It contains a great number of biological compounds such as glycosides, polysaccharides, phenolic compounds, alkaloids, lignans, fatty acid esters, organic acids, vitamins and minerals [29,57,58]. There have been many reports on the antioxidant activity of the fruit, roots and leaves of *M. citrifolia* L, [23,25]. Its antiproliferative activity has also been studied, and it has been reported to have an effect in human laryngeal epithiloma (HEp2) with an IC_{50} value of 12.5 mg/mL [24]. Unfortunately, we in this work did not find an antiproliferative activity appreciable in none of the cell lines evaluated (IC_{50} > 600 µg/mL). But in other studies, Hirazumi and Furusawa [59] isolated a polysaccharide–rich substance from *M. citrifolia* which shows antitumor activity in the lewis lung (LLC) peritoneal carcinomatosis model. In this study mice were inoculated intraperitoneally with tumour homogenate (LLC) and 1 day after, the ethanol insoluble fraction of *M. citrifolia* fruit extract was administered intraperitoneally every day to complete of 4 – 5 injections. And they observed that the life span of tumour bearing mice treated with extract was prolonged by more than 75%, showing its antitumor activity. Additionally, the evaluation of the antiproliferative activity in cancer cell lines was realized by Arpornsuwan and Punjanon [60]. They evaluated methanol extracts from *M. citrifolia* in several cell lines, and obtained IC_{50} values of 2.5, 3 and 5 mg/mL for the BHK (normal cells from baby hamster kidney), Vero (African green monkey kidney) and Hep2 (human laryngeal carcinoma) cells respectively. Also the MCF7 (breast cancer) and the LAN5 (neuroblastoma) cells were evaluated and their IC_{50} value was of 1.5 mg/mL in both; however in low concentrations (0.1 mg/mL), methanol extract of *M citrifolia*, showed more selective for the cancer cell lines.

Of all de samples tested in this study only *A. ambrosioides* did not show antiproliferative activity (IC_{50} value > 800 for all cell lines). Results opposite from ours were shown by Booth *et al.* [61] who evaluated different parts of this plant against HeLa cell line proliferation and bacterial cells. Stem and root methanol extracts showed actives in cell antiproliferation with IC_{50} values of 466 and 439 µg/mL respectively, but did not show inhibition of bacterial growth.

Cisplatin, a chemotherapy drug, was used as positive control, in the case of the human cells this drug presented IC_{50} values of 13.91 ± 1.62 µg/mL to HeLa line, 34.7 ± 2.77 µg/mL to A549 cell line, 39.66 ± 0.97 µg/mL to LS-180 line, in the three cancerous cell line, and 52.97 ± 1.56 µg/mL in ARPE-19 normal cell line. Cisplatin yields a putatively high toxicity (mainly nephrotoxicity, ototoxicity and emesis). This can be problematic in the more fragile and elderly patients or in patients with cardiac or renal comorbidity [50]. However and in spite of harmful side effects to health, today this drug is

amply used in the fight versus different cancer types like: non-small cell lung cancer, head and neck squamous cell carcinoma, sarcoma, germ cell tumor, lymphoma, ovarian cancer, cancer of soft tissue, bones, muscles and blood vessels [44,62,63]. The goal of our work was to find plant extracts with a promising effect against cancerous cell lines, for which we achieved very good promising results. An example of this is the methanolic extract of *A. muricata*, which presents the same antiproliferative effect as cisplatin but maybe without undesirable side effects. Additionally, as mentioned before, the *B. buceras*, *L. tridentata* and *H. brasiletto* extracts promise to become important alternatives to fight different kinds of cancer, especially since the results here shown pertain to crude extracts only, which have the potential of being further purified.

We also evaluated the selectivity index (SI), in human cell lines, with respect to the normal cell line: ARPE-19. As can be seen in table 5 most of the plant extracts have an SI was greater than one, remembering that when this occurs, the extract or sample is selective to the cancerous cell line.

Table 5. Selectivity index of human cell lines.

Plants / Cells Lines	A549	HeLa	22Rv-1	BxPc-3	LS-180
Bucida buceras	1.91	1.93	1.04	0.32	0.97
Haemotoxylon brasiletto	1.14	1.90	2.07	1.95	1.61
Bursera hindsiana	>3.21	> 1.65	≥ 1	>1.71	>1.76
Bursera microphylla	>1.29	> 1.46	>1.69	>1.3	>1.49
Ambrosia ambrosioides	NQ	NQ	NQ	NQ	NQ
***P. californicum* (mezquite)**	2.32	1.41	0.68	1.29	1.41
***P. califronicum* (oak)**	>5.33	>3.32	>1.51	>1.81	>1.02
Annona muricata	0.94	1.66	0.61	1.2	1.37
Morinda citrifolia	NQ	NQ	NQ	NQ	NQ
Larrea tridentata	1.96	1.24	<0.82	2.54	1.42
Cisplatin	1.53	3.81	ND	ND	1.34

ND: Not determinated. NQ: Not quantified

The extracts with the best selection power were *H. brasiletto*, *B. microphylla* and *B. hindsiana*, which were effective against most cell lines. *L. tridentata*, was effective mainly against A549, BxPc3 and LS-180 lines, *P. californicum* of mesquite and *A. muricata* against HeLa, BxPc3 and LS-180 cell lines. The other samples, despite showing antiproliferative activity, were poor at selecting between cancerous and non-cancerous cell lines.

It's important to mention that some samples have better selective behavior than the cisplatin drug, as is the case of *B. hindisiana* in the A549 and LS-180 cell lines; *L. tridentata* in the A549 and the BxPc-3 cell lines; *P californicum* of mesquite in the LS-180 cell line; and *H. brasiletto* in the LS-180 cell line.

Few researchers have reported SI values, for the nine plant extracts evaluated. The SI of *P. californicum*, *A. muricata* and *M. citrifolia* have been reported by other authors, Iloki *et al.* [28], reported the SI of *P. californicum* of oak and mesquite for: the A549 and RAW 264.7 cell lines, showing similar SI values to ours. While the study by Quispe *et al.* [32] showed that the muricin H (acetogenin isolated from *A. muricata*) is selective in the H460 cell line (Carninoma, large cell lung cancer), in our results *A. muricata* was selective in the RAW 264.7, HeLa, BxPc-3 and LS-180 cell lines. Additionally, the methanol extract of *M. critrifolia* has also been shown to be selective in several cell lines: Hep2, MCF7 and LAN5, when it was probed at low concentrations (0.1 mg/mL) [59]. Unfortunately, we did not find good results for this plant.

Conclusions

The finding of this study showed that among the nine plant extracts evaluated, the richest in secondary metabolites were *B. buceras*, *H. brasiletto*, *B. hindsiana*, *M. citrifolia* and *P. californicum*. While the plant extract with the highest phenolic content was *P. californicum* of oak. Regarding antiproliferative activity the best extracts were: *A. muricata* and *L. tridentata* in murine and human cell lines, and also *B. buceras* and *H. brasiletto* in the case of human cell lines, probably this activity is due to the presence of phenols or other secondary metabolites not quantified in this research.

Abbreviations

HCl: hydrochloric acid; DMSO: dimethylsulfoxide sodium; DMEM: Dulbecco´s Modified Eagle´s; RPMI-1640: Roswell Park Memorial Institute, FBS: fetal bovine serum; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-

tetrazolium bromide; SI: selectivity index; SE: standard errors; ANOVA: analysis of variance; B.B.: *Bucida buceras*, H.B.: *Haemotoxylon brasiletto*, B.H.: *Bursera hindsiana*, B.M.: *Bursera microphylla*, A.A.: *Ambrosia ambrosioides*, P.C.: *Phoradendron californicum*, A.M.: *Annona muricata*, M.C.: *Morinda citrifolia*, L.T.: *Larrea tridentata*. NDGA: nordihydroguaiaretic acid; µgGAE/mg: Micrograms equivalent of gallic acid per milligram of extract; NSCLC: non small cell lung cancer; SCLC: small cell lung cancer; LLC: lewis lung peritoneal carcinomatosis.

Competing interests

The authors declare that there is no conflict of interests.

Authors´ contributions

S.B.I.A. conducted the searching and selection of the nine plants used in this study. A.A.G.S., A.L.A.S., DF carried out the extract preparation. A.A.G.S. and A.L.A.S. realized the cell culture and the antiproliferative assays. L.M.L.L. realized the determination of phenols, L.M.L.L. and C.L.L.E. carried out the phytochemicals screening. A.A.G.S., L.M.L.L. and S.B.I.A. were involved with drafting of the manuscript. C.L.L.E. was participated in the revision of the manuscript and analysis of data, J.L.R.P. conducted the scientific work. All the authors read and approved the final manuscript.

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