Pharmacogn. Res., 2024; 16(4):817-827.
https://www.phcogres.com

Original Article

Exploring the Antioxidant and Antiproliferative Properties of Four Medicinal Plants from Veracruz, Mexico for Urinary and Prostate Health

Oscar Antonio Sánchez-Aguirre[1], Cynthia Fernández-Pomares[2], Enrique Juárez-Aguilar[3], Alberto Sánchez-Medina[4], Leticia Margarita Cano-Asseleih[5,*]

[1]Centro de Investigaciones Biomédicas, Universidad Veracruzana, Xalapa, Veracruz, MÉXICO.
[2]Escuela Superior de Medicina, Laboratorio de Diseño y Desarrollo de Nuevos Fármacos e Innovación Biotecnológica, Instituto Politécnico Nacional, Ciudad de México, MÉXICO.
[3]Instituto de Ciencias de la Salud, Laboratorio de Cultivo Celular, Departamento de Biomedicina, Universidad Veracruzana, Xalapa, Veracruz, MÉXICO.
[4]Instituto de Química Aplicada, Universidad Veracruzana, Xalapa, Veracruz, MÉXICO.
[5]Centro de Investigaciones Tropicales, Universidad Veracruzana, Xalapa, Veracruz, MÉXICO.

ABSTRACT

Background: *C. scaber, P. aequale, S. oleraceus* and *S. racemosa* play a vital role in traditional medicine in Veracruz, Mexico, known for their effectiveness in treating a wide range of health issues. Particularly noteworthy is their significant use in traditional medicine in Mexico for addressing urinary and prostate-related ailments. **Objectives:** The current study assesses the antioxidant and anti-proliferative properties of these species on the androgen-independent prostate cancer cell line PC-3. **Materials and Methods:** Methanolic and aqueous extracts of the selected plants' parts were prepared and their chemical profiles were evaluated using ^{1}H-RMN. Antioxidant activity was assessed using the DPPH and FRAP methods. The total phenolic content was determined using the Folin-Ciocalteu method. The antiproliferative activity on PC-3 and HPrEC cells was evaluated by the MTT method. **Results:** *S. racemosa* showed the highest antioxidant activity (84.90% radical scavenging activity), attributed to the presence of flavonoids. However, the methanol extract of *P. aequale* had a better effect on the proliferation of PC-3 cells with an IC_{50} of 251.6 µg/mL, additionally, it showed cytotoxicity at high concentrations (above 500 µg/mL). The antiproliferative and cytotoxic effect observed is not associated with antioxidant activity or the presence of alkaloids. However, cytotoxicity also occurred in control cells (HPrEC) with an IC_{50} of 23.98 µg/mL. **Conclusion:** The methanolic extract of *P. aequale* showed the highest antiproliferative activity against PC-3 cell line, an effect not related to its antioxidant activity and attributed to the presence of aliphatic, aromatics and aldehydes compounds identified by ^{1}H-NMR in the extracts.

Keywords: *Costus scaber*, *Piper aequale*, *Sonchus oleraceus*, *Serjania racemosa*, antioxidant activity, antiproliferative activity.

Correspondence:
Dr. Leticia Margarita Cano-Asseleih
Centro de Investigaciones Tropicales, Universidad Veracruzana. José María Morelos 44, Zona Centro, Xalapa, Veracruz, C.P. 91000 Centro, MÉXICO.
Email: lecano@uv.mx
ORCID: 0000-0003-4216-349

Received: 17-05-2024;
Revised: 28-05-2024;
Accepted: 14-06-2024.

INTRODUCTION

Prostate cancer is the leading cause of death among men due to malignant tumors. It originates in the prostate gland and typically lack symptoms in its early stages, earning it the designation of a silent disease. Over 65% of cases of this type of cancer are diagnosed in men older than 65 years. In 2020, worldwide, there were 1,414,259 new cases of prostate cancer diagnosed, constituting 11.4% of all cancers affecting men. During the same period, 375,304 deads were attributed to this neoplasm.[1] Currently, chemotherapy is the main treatment. However, the use of this therapy generates short and long-term side effects, affecting both malignant cells and normal cells.[2] Currently, traditional medicine is recognized by the World Health Organization as a fundamental resource for the treatment of health problems, therefore, medicinal plants play a very important role in the health of populations without access to modern medicine.

The selection of species for study in this work started with the list of medicinal plants in the database of the 'Flora medicinal de Veracruz' project at the Centro de Investigaciones Tropicales of the Universidad Veracruzana, which documents 1,240 medicinal plants in the state of Veracruz, Mexico. Four of the most frequently

DOI: 10.5530/pres.16.4.94

used species in the traditional medicine within the state for the treatment of urinary and prostate problems are indicated below.

Costus scaber Ruiz and Pav (Costacea) popularly known as 'caña de jabalí', 'cañita agria' or 'beso francés', is distributed throughout Mexico, Central America and South America. In Mexico, it is used for the treatment of kidney problems, renal and gallstones, with the stem being consumed as a tea. Additionally, it has been indicated for prostate and urinary issues, diabetes, venereal diseases (gonorrhea), as a diuretic and for kidney cancer.[3] To our knowledge, this species lacks any reports regarding its phytochemical or pharmacological properties.

Piper aequale Vahl (Piperaceae), traditionally known as 'cordoncillo', is native to Central and South America. In Brazil, the leaves of this species are used by decoction for the treatment of rheumatism and inflammation.[4] In the Mexican state of Veracruz, it is used as anticrotalic, for treating bruises, in women's baths, for cold symptoms and also for prostate issues.[5,6] Phytochemical and pharmacological reports on the species are scarce. However, there is some available information on its essential oil, which contains major components, such as monoterpenes and sesquiterpenes like α-pinene, β-pinene, δ-elemene, β-atlantol, bicyclogermacrene, cubebol and sabinene, among others.[4,7] In addition, its antioxidant activity has been evaluated against the DPPH radical, exerting a weak inhibition of 25.9%.[4] On the other hand, the essential oil was found to have cytotoxic activity against cancer cell lines HCT-116 (colon), SKMEL19 (melanoma) and ACP-03 (gastric), with IC_{50} values of 8.69, 25 and 1.54 µg/ mL, respectively.[4]

Serjania racemosa Schumach (Sapindaceae), known as 'bejuco tres en uno', bejuco 'siete corazones' or 'vara de tres costillas', is native to Central America. In Mexico, in the state of Veracruz, this species' leaves are indicated for treating diabetes, kidney problems, reducing kidney inflammation, as a diuretic, for prostate disorders and kidney stones.[6,8,9] Phytochemical and pharmacological studies of this species have not been detected.

Sonchus oleraceus L. (Asteraceae), known as 'cerraja', 'lechuguilla', thistle or false dandelion, is currently of cosmopolitan distribution. It is used as food and the entire plant is employed for treating stomach pain, acting as a laxative and diuretic, depurative, aiding digeston, analgesic, ophthalmic, for the flu, cystitis, gout, high blood pressure, edema, hepatobiliary dyskinesias, dermatitis and wounds, otitis and ear pain, kidney and prostate problems. Phytochemical and pharmacological information on this species is extensive. Sesquiterpene lactones (eudesmanolides and guaianolides) and flavonoids (glycosylated flavones, flavonols and flavonoids) are known to be the main groups of secondary metabolites in *S. oleraceus*.[10-15] Regarding its antioxidant activity, studies have reported that hydroxycinnamic acids (chicoric, chlorogenic and caftaric) along with flavonoids (like kaempferol, quercetin and isoquercitrin) are responsible to its activity.[16] Additionally, sesquiterpene lactones including 1*β*- *O*-*β*-D-glucopyranosyl-15-*O*-(3,4-dihydroxylphenylacetyl)-5α,6βH-eudesma-3-en-12,6α-olide, loliolide and 15-*O*-β-glucopyranosyl-11β,13- dihydrourospermal A have been found to confer its antiproliferative and cytotoxic activity on lung cancer cell lines.[12,13]

This work aims to evaluate the antioxidant and antiproliferative activity in methanolic and aqueous extracts of *C. scaber* (stems), *P. aequale* (leaves), *S. racemosa* (leaves) and *S. oleraceus* (whole) on the androgen-independent cell line PC-3 from bone metastasis from prostate cancer.

MATERIALS AND METHODS

Species selection

For the selection of the four medicinal species to be studied in the present work, an ethnomedical approach was applied. For this purpose, the list of medicinal plants from the database of the project 'Flora Medicinal de Veracruz' of the Centro de Investigaciones Tropicales of the Universidad Veracruzana was consulted, considering the following selection criteria: species from the central zona of the state of Veracruz, commonly used of the treatment of urinary and prostate problems, frequently used species, located in accessible areas, abundance and present at any time of the year.

Vegetal material

Leaves of *P. aequale* and *S. racemosa* were collected on March 10th, 2020, in Xalapa, Veracruz, Mexico. *S. oleraceus* (whole plant) was collected on December 21st, 2020, in Orizaba, Veracruz, Mexico. Stems of *C. scaber* were collected in Atzacan, Veracruz, Mexico on December 22nd, 2020. All of them were identified and voucher specimens deposited at the CIB herbarium of the Instituto de Investigaciones Biológicas of the Universidad Veracruzana with the following herbarium codes: *P. aequale* (23295UV), *S. racemosa* (23296UV), *S. oleraceus* (23297UV) and *S. scaber* (23298UV).

Preparation of extracts

The collected plant material was dried at 45°C for five days in air circulating ovens (Yamato DX602C) and ground using an electric mill. Then, 2 g of plant material from each species were added to 40 mL of methanol and placed in a sonic bath (Branson 1800) for 50 min at room temperature. After this period, the extract was filtered through Whatman no.1 filter paper and this procedure was repeated. The solvent was removed under reduced pressure using a rotary evaporator (BÜCHI R-114) at 40°C. For the aqueous extract, 4 g of plant material were mixed with 500 mL of distilled water and heated to boiling. The water was subsequently removed from the resulting extract using a rotary evaporator at 45°C under reduced pressure. Both extracts were refrigerated and protected from light until use.

^{1}H-NMR profiles of methanolic and aqueous extracts

To obtain the chemical profile by ^{1}H-NMR, 20 mg of each extract was dissolved in 0.6 mL of deuterated dimethylsulfoxide (DMSO-*d6*) containing Tetramethyl Silane (TMS) at a concentration of 0.03%. The solutions were then transferred into 5 mm NMR tubes. Proton analysis was performed using an Agilent Technologies DD2 premium model 500 MHz spectrophotometer (model number 500154). The spectra obtained were processed using Mestrenova 12.0 software, with the phase and baseline corrections applied and referenced to the solvent signal at 2.5 ppm.

Determination of antioxidant activity

Determination of the total content of polyphenols

The total polyphenol content was determined using a modified Folin-Ciocalteu method as described by Cai and Luo.[17] Briefly, 2.5 mL of a 1:10 solution of the Folin-Ciocalteu reagent and 2 mL of 7.5% Na_2CO_3 were added to 50 µL of extract (1mg/mL). The samples were then placed in a water bath at 45°C for 15 min. Absorbance readings were recorded at 765 nm on a UV-VIS spectrophotometer (Thermo Electron Corporation Spectronic Helios α). The assay was carried out in triplicate. Total polyphenols content was quantified from a calibration curve (range 25-1000 µg/mL, R^2=0.9946), using gallic acid as primary standard.

$$\text{Absorbance} = 0.0014(\mu g/mL \text{ of gallic acid}) + 0.0487$$

2,2-Diphenyl-picrylhydrazyl free radical scavenging activity (DPPH)

First, a solution of DPPH at $9x10^{-5}$ M in methanol was prepared. Then, 2.9 mL of this solution was taken and 100 µL of the extract solutions were added at a concentration of 1 mg/mL. The samples were kept at 37°C in a water bath for 30 min, protected from light. After incubation, the absorbance was measured at 517 nm using a UV-vis spectrophotometer (Thermo Electron Corporation Spectronic Helios α). A solution containing 100 µL of methanol in 2.9 mL of the DPPH solution served as a blank and a 5 mM ascorbic acid solution was used as a positive control. The experiment was conducted in triplicate.[18,19] The antioxidant activity was determined by applying the following equation:

$$\% \text{ of inhibition} = [(A - A1)/A] x100$$

Where:

A: DPPH reagent absorbance,

A1: Sample Absorbances,

FRAP Iron Reducing Power Method (Ferric Reducing Antioxidant Power).

The reducing power of the extracts was evaluated according to the method described by Benzie and Strain.[20] The FRAP solution was prepared by mixing 100 mL of 300 mM acetate buffer (pH=3.6), 10 mL of 10 mM of TPTZ (Ferric Complex-2,5,6-tripyridy l-5-triazine) dissolved in a solution of 40 mM hydrochloric acid and 10 mL of 20 mM ferric chloride solution.

To 150 µL of the extract (concentration of 1 mg/mL), 150 µL of distilled water and 2.7 mL of FRAP solution were added. The solutions were placed in a water bath at 37°C for 4 min. Subsequently, the absorbance of the samples was read at 593 nm in a UV-vis spectrophotometer (Thermo Electron Corporation Spectronic Helios α), in which the FRAP reagent was used as a blank. The assay was carried out in triplicate. The results were expressed in µmol Fe^{2+}/L based on a calibration curve from different concentrations of $FeSO_4.7H_2O$ (range 50-750 mmol/L, R^2=0.9842).

$$\text{Absorbance} = 0.0027(Fe^{2+}/L) + 0.2725$$

Antiproliferative activity

Preparation of extracts for addition to cell culture

Three mg of each species' methanolic and aqueous extracts were weighed and dissolved in 3 mL of RPMI-1640 culture medium supplemented with 8% FBS (Fetal Bovine Serum) and 1% P/S (Penicillin-Streptomycin) to create a stock solution at a concentration of 1000 µg/mL. This solution was filtered using a sterile filtration unit with a pore size of 0.22 µm. After sterilization, a series of dilutions were performed to achieve the following concentrations: 100, 250, 500, 750 and 1000 µg/mL. The extracts dissolved in the culture medium were stored frozen until use.

Proliferation assay and MTT

The androgen-independent prostate cancer cell line PC-3 was used and it was seeded in a 96-well multiplate (0.32 cm^2, CORNING) at 12,500 cells/cm^2 in 100 µL of RPMI-1640 supplemented with 8% FBS and 1% P/S, incubated for 48 hr at 37°C and 5% CO_2 (Nuaire US Autoflow CO_2 Incubator) to allow cell adhesion. After this period, the culture medium was removed and the change was made with different concentrations of the extracts (0 to 1000 µg/mL). It's important to note that, at that time, the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was performed on cultures without the extracts to establish the zero Time (T_0) as a reference point for cell proliferation.

To carry out the MTT assay in the T_0 cultures, the culture medium was first removed and then 50 µL of MTT at 5 mg/mL was added. The cultures were then incubated for 4 hr at 37°C and 5% CO_2. After this time, the MTT was removed and 200 µL of DMSO (Dimethylsulfoxide) were added to dissolve the formazan crystals produced. Subsequently, the optical density of the cultures was measured in an ELISA microplate reader (Stat FAX 4200 Awareness Technology) at 630 nm.

The rest of the cultures were incubated with the extracts for 48 hr at 37ºC and 5% CO_2 and microscopically inspected at 25X at 24 and 48 hr. At the end of the incubation, cell viability was determined by the MTT assay. In total, three independent experiments with three replicates each were performed. From the data, dose-response curves were obtained (non-linear regression of the percentage of proliferation vs logarithm of the concentration) following the methodology of the National Cancer Institute of the United States,[21] with the purpose of determining the values of IC_{50} (Concentration at which 50% of cell proliferation is inhibited), TGI (Concentration at which 100% of cell proliferation is inhibited) and LC_{50} (Concentration at which 50% of cells die). The data obtained were normalized to the percentage of cell proliferation for the creation of the dose-response curves. For this calculation, the following equations were used depending on the absorbance values obtained in the treatment:

If the absorbance value of the sample is greater than that of T_0, the following expression applies:

$$\%= 100[(T-T_0)/(C-T_0)]$$

If the absorbance value of the sample is less than that of T_0, it is applied:

$$\%= 100[(T-T_0)/(T_0)]$$

Where:

T_0=Zero time, culture absorbance 48 hr after seeding.

T=Culture absorbance at 48 hr of treatment.

C=Absorbance of the control culture (without extracts) at 48 hr of treatment.

The results were statistically analyzed by developing a non-linear regression for the antiproliferative activity evaluated in the MTT assay, using the GraphPad Prism version 8 software (La Jolla California USA).

The species with the highest activity were evaluated against prostate epithelium cells HPrEC. Three mg of the extract with the highest antiproliferative activity were weighed and dissolved in 3 mL of ATCC Prostate epithelial cell basal medium (PCS-440-030), supplemented with the ATCC primary Prostate cell growth kit (PCS-440-040), in this way, the stock solution of 100 µg/mL was obtained and it was filtered in a sterile filtration unit with a pore size of 0.22 µm. Subsequiently, a dilution series was carried out to obtain concentrations of 0, 12.5, 25, 50, 75 and 100 µg/mL and the samples were kept frozen until use. HPrEC cells were seeded in a 96-well multiplate (0.32 cm^2, CORNING) at 6,250 cells/cm^2 in 100 µL of prostate epithelial cell basal medium supplemented with the ATCC primary prostate cell growth kit, incubated for 48 hr at 37ºC and 5% CO_2 (Nuaire US Autoflow CO_2 Incubator) to allow for cell adhesion. The antiproliferative activity was evaluated following the methodology of the National Cancer Institute of the United States.[21]

Qualitative identification of alkaloids

10 mL of methanolic extract of *P. aequale* was evaporated, the dry extract was resuspended with 5 mL of chloroform and acidified with a 5% H_2SO_4 solution. The mixture was homogenized and allowed to stand until the phases separated. The aqueous phase was separated and 5 mL of ammonia and 10 mL of ethyl ether

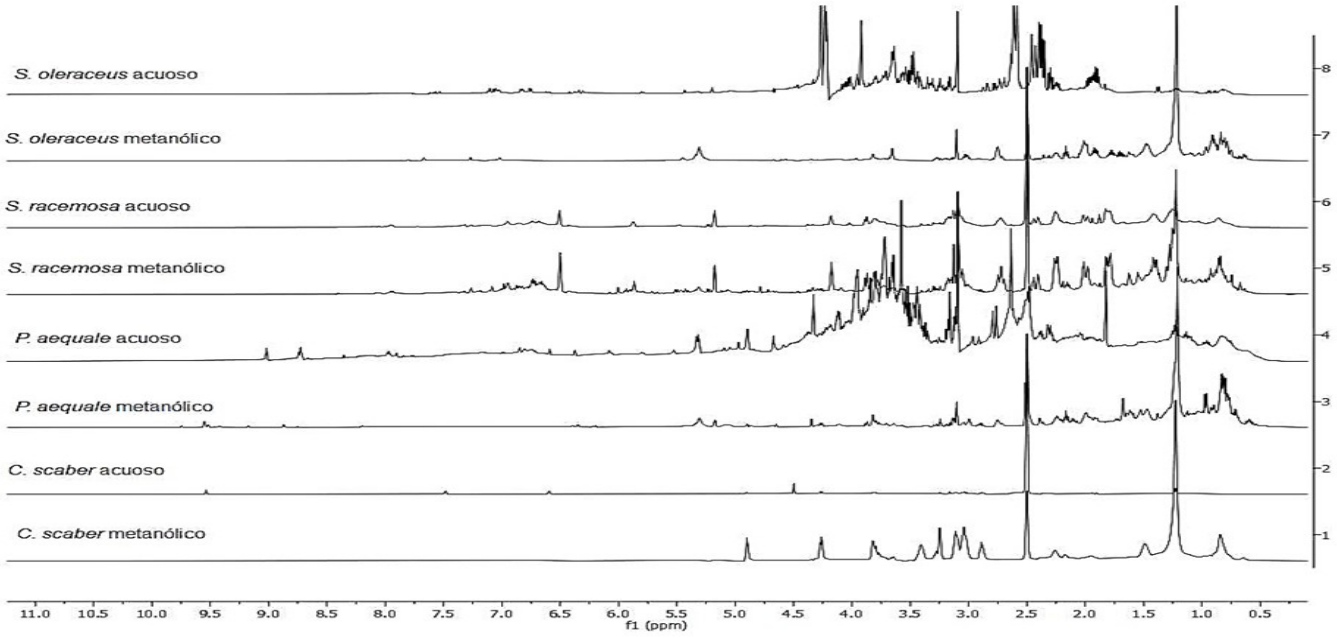

Figure 1: ^{1}H-NMR spectrum of methanolic and aqueous extracts of the selected species (500 MHz, DMSO *d6*).

were added. The excess solvent was removed under reduced pressure in a rotary evaporator at 45ºC. Finally, a direct phase thin layer chromatography was performed using an 80:15:5 ethyl acetate-isopropanol-ammonia mobile phase. Once the plate was eluted, it was developed with Dragendorff's reagent. The test is positive if an orange-brown color is seen.

RESULTS

Characterization of the metabolite profiling of extracts by ^{1}H-NMR

The ^{1}H-NMR analysis of the methanolic and aqueous extracts of the four species studied allowed us to identify signals of the different groups of secondary metabolites in the crude extracts. Figure 1 shows the ^{1}H-NMR spectra of each extract, which are stacked and referenced with the DMSO-*d6* signal (2.5 ppm).

The proton spectrum of the methanolic extract of *C. scaber*, at 1.24 ppm the characteristic signal of lipids is found, in the region of 0.5 to 3 ppm there are signals of methyls, methylenes and hydroxyl protons of alcohols. From 3 to 5 ppm correspond to double bonds. The spectrum of the aqueous extract of *C. scaber* shows aliphatic signals, from 6.59 to 8.26 ppm there are signals of aromatic compounds, in addition a signal is seen at 9.54 ppm that may be aldehydes or carboxylic acids.

In *P. aequale*, the proton spectrum of the methanolic extract shows the lipid signal at 1.24 ppm and in the region of 0.5 to 3 ppm it corresponds to aliphatic signals such as methyls, methylenes and methines, which are intense. From 3 to 5.5 ppm, double bond signals observed. From 6 to 8.25 ppm there are small signs of aromatics and finally signs of aldehydes or carboxylic acids of 8.75-9.75 ppm are observed. On the other hand, in the proton spectrum of the aqueous extract of *P. aequale*, from 0 to 3 ppm the aliphatic signals, the intensity of the signals is much lower compared to the methanolic extract. However, the intense representative signal of sugars stands out, ranging from 3 to 5 ppm. There are also signs of aromatics, aldehydes or carboxylic acids.

The proton spectrum of the methanolic extract of *S. oleraceus*, at 1.24 ppm, the lipid signal is presented. From 0 to 3 ppm there are signs of aliphatic components. From 3.5 to 5.5 ppm, signals of alkenes or esters are present and from 6.5 to 8 ppm, some signals of aromatics are present. In the case of the aqueous extract of *S. oleraceus*, the intense signal of lipids is not observed, however, there are signals of aliphatic compounds of 0 to 3 ppm and the signal that stands out the most in this sample is the intense signal of sugars from 3 to 5 ppm. From 5 to 5.5 ppm some signals can be observed that may correspond to double bonds and from 6.25 to 7.75 ppm aromatic signals are presented.

The proton spectra of the methanolic and aqueous extracts of *S. racemosa* show the intensity of 1.24 ppm of the lipids, which is more intense in the methanolic extract. In both spectra, the signals of aliphatics such as methyls and methylenes are present in the region. from 0 to 3 ppm. In the range of 3 to 6 ppm the region of double bonds is found and finally aromatic protons of 6 to 8 ppm aromatics are visualized.

Antioxidant activity

The antioxidant activity study reveals that the methanolic and aqueous extract of *S. racemosa* showed the highest Total phenolics content, which are commonly related to its ability to inhibit the DPPH radical compared to the ascorbic acid control and to reduce the Fe^{+3} of the reagent. FRAP (Table 1); then followed in antioxidant activity by the extracts of *P. aequale, S. oleraceus* and *C. scaber*, respectively, the latter being the least active.

Antiproliferative activity

The antiproliferative activity of the methanolic and aqueous extracts of *C. scaber, P. aequale, S. oleraceus* and *S. racemosa* was evaluated in the androgen-independent prostate cancer cell line PC-3. Figure 2A shows the percentage of cell proliferation of PC-3 vs the Log of the concentration in µg/mL of the

Table 1: Antioxidant activity of the methanolic and aqueous extracts of the studied species.

Extract	Species	mg gallic acid /g of sample	% Inhibition (DPPH)	µmol Fe^{2+}/L (FRAP)
Methanolic	*C. scaber*	0.656±0.059	21.23±1.81	251.88±9.71
	P. aequale	2.509±0.123	53.66±1.57	462.25±7.75
	S. racemosa	3.580±0.614	84.90±2.51	653.12±13.44
	S. oleraceus	0.428±0.016	25.56±4.24	204.60±6.38
Aqueous	*C. scaber*	3.140±0.876	21.77±4.16	153.12±24.30
	P. aequale	10.926±0.082	55.19±3.16	502.87±9.82
	S. racemosa	27.307±0.327	87.31±3.23	672.38±4.71
	S. oleraceus	4.497±0.532	28.39±1.03	284.97±11.34
Ascorbic acid		---	100	---

The results show the average of three replicates±standard deviation.

methanolic extracts, which allowed identifying the IC_{50} values and TGI. In addition, micrographs of the cultures were taken with the treatment of the extracts at 48 hr at 25X, which allows a comparison to be made with a control culture without treatment (0 µg/mL) (Figure 2B-2E).

The most active methanolic extract on PC-3 cells was that of *P. aequale*. Figure 2A shows a marked decrease in proliferation to negative values with an IC_{50} of 251.6 µg/mL and a TGI of 716.8 µg/mL, a concentration that inhibits cell proliferation by 100%. Figure 2B shows micrographs of the cultures in which fewer cells without apparent damage are seen from 250 µg/mL, while at 500 µg/mL, the cell density continues to decrease while the cell damage is more evident; higher concentrations (750-1000 µg/mL) are frankly cytotoxic.

The methanolic extract of *S. racemosa* showed a concentration-dependent decrease in PC-3 proliferation, in which an IC_{50} value of 642.9 µg/mL was determined (Figure 2A). According to the micrographs of the cultures (Figure 2C) from 500 µg/mL, the decrease of cells is apparent, showing cytotoxic cell damage between 750 and 1000 µg/mL.

The cultures incubated with the methanolic extract of *S. oleraceus* showed a slight decrease in cell proliferation (Figure 2A); however, this was very weak and the IC_{50} could not be calculated. In the

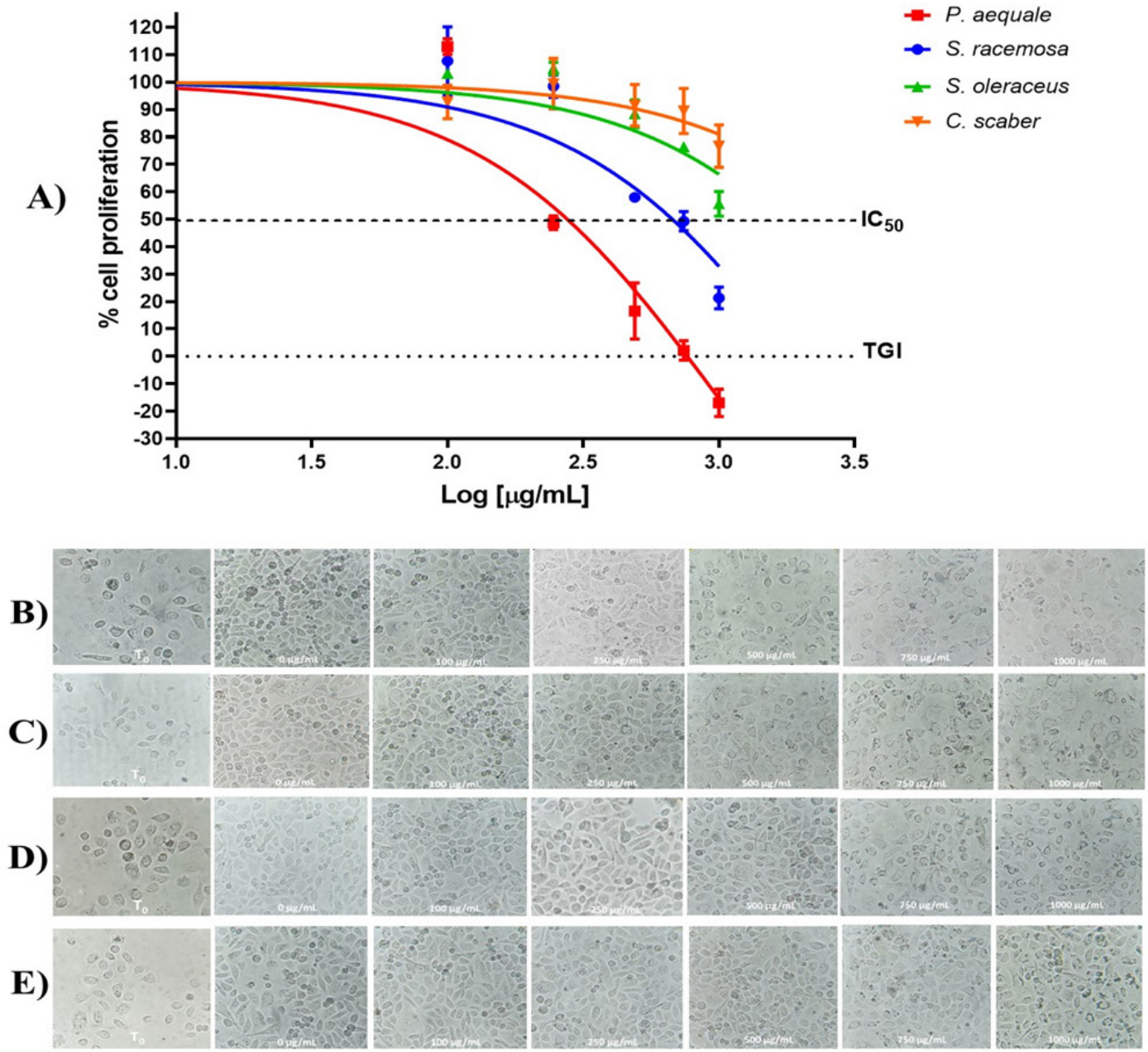

Figure 2: Anti-proliferative activity of the methanolic extracts of *C. scaber, P. aequale, S. oleraceus* and *S. racemosa* in PC-3 cells. A) Dose-response curve shows the average of three independent experiments±SEM at 48 hr of treatment with the methanolic extracts of the species studied. Micrographs of cultures incubated with different concentrations (100-1000 µg/mL) of the extracts of *P. aequale* (B), *S. racemosa* (C), *S. oleraceus* (D) and *C. scaber* (E).

micrographs of the cultures (Figure 2D), fewer cells can be seen at high extract concentrations compared to the control culture without treatment (0 µg/mL). However, these are observed intact and the dispersion between cell-cell is quite noticeable.

The methanolic extract of *C. scaber* was the least active against PC-3 cells. According to the dose-response curve in Figure 2A, cell viability was little affected. In this case, it was impossible to calculate the IC_{50} since 50% inhibition of appearance was not reached. The micrographs of the cultures presented in Figure 2E confirm the inactivity of this extract since no cell damage is observed. However, at 750 and 1000 µg/mL.

On the other hand, the aqueous extract of *P. aequale* was the most active against the PC-3 cell line, which showed a marked decrease in cell proliferation (Figure 3A) with an IC_{50} of 775.4 µg/

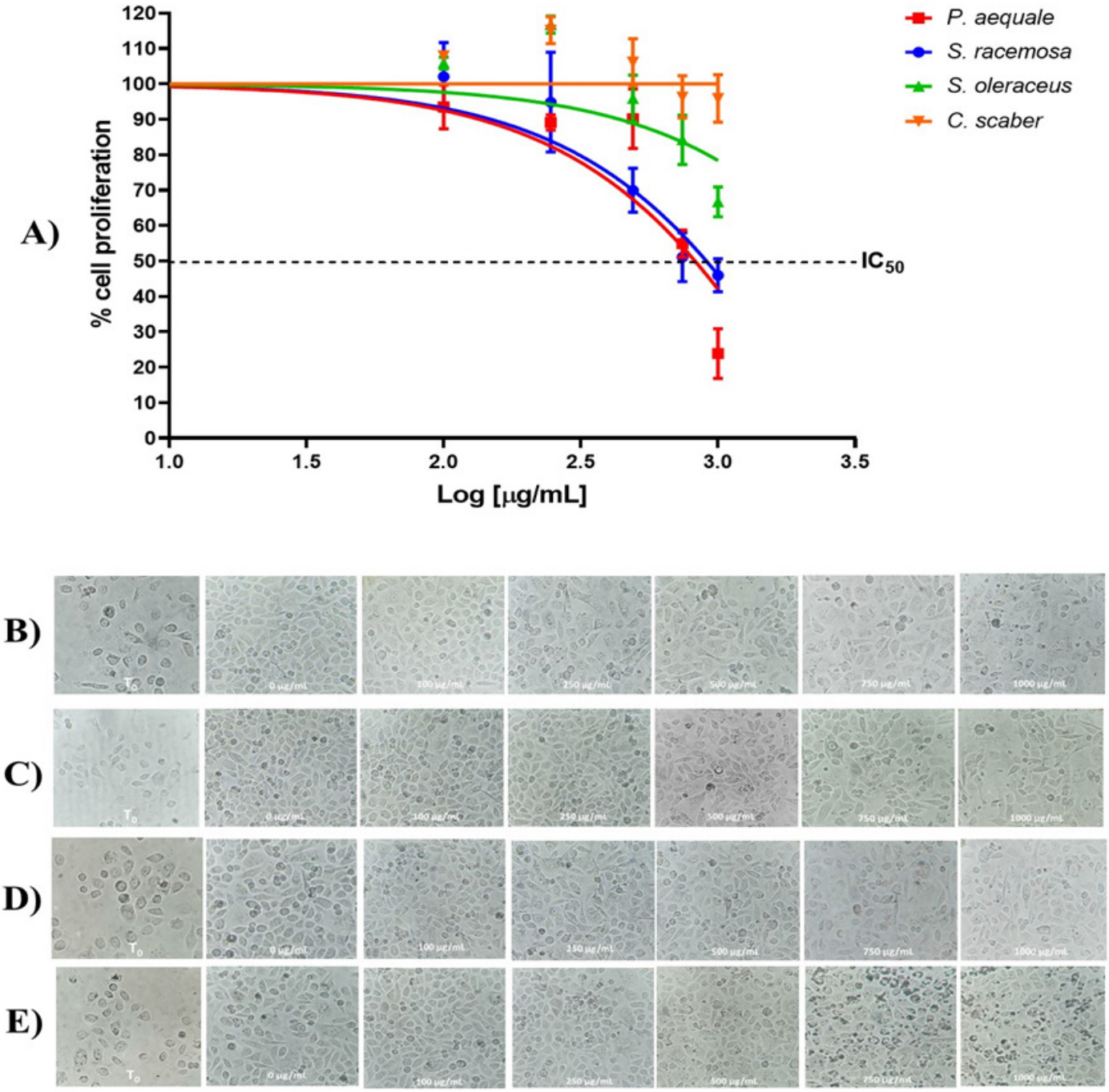

Figure 3: Anti-proliferative activity of the aqueous extracts of *C. scaber, P. aequale, S. oleraceus* and *S. racemosa* in PC-3 cells. A) Dose-response curve shows the average of three independent experiments±SEM at 48 hr of treatment with the methanolic extracts of the species studied. Micrographs of cultures incubated with different concentrations (100-1000 µg/mL) of the extracts of *P. aequale* (B), *S. racemosa* (C), *S. oleraceus* (D) and *C. scaber* (E).

mL. Figure 3B shows micrographs of cultures with a decreased cell density at 750 and 1000 µg/mL, with evident cell damage compared to the control culture without treatment (0 µg /mL), indicating cytotoxicity.

The aqueous extract of *S. racemosa* also exerted an antiproliferation effect with an IC_{50} of 829.3 µg/mL (Figure 3A). The microscopic analysis in Figure 3C shows the decrease of cell number without evident cell damage.

In *S. oleraceus* treated cultures, a slight decrease in proliferation is observed (Figure 3A). Figure 3D shows a slight decrease in cells at high concentrations with no apparent cell damage.

Finally, the aqueous extract of *C. scaber* was inactive on PC-3 cells since, according to the dose-response curve (3A), cell proliferation was not affected and micrographs (Figure 3E), showed intact cultures. However, the presence of a residue in the cultures at concentrations of 750 and 1000 µg/mL was more significant than observed in the methanolic extract, which suggests that this extract has more insoluble components in the culture medium.

The methanolic extract of *P. aequale*, the one showing the highest antiproliferative effect, was evaluated against the HPrEC cell line. It showed a higher effect compared to that in PC-3 cells at much lower concentrations (12.5 to 100 µg/mL), the decrease in the percentage of proliferation is noticeable until cell death occurs (Figure 4A). These results indicate that the methanolic extract of *P. aequale* has an effect on the proliferation of normal cells and is also cytotoxic at small concentrations.

The dose response graph allowed us to find that the IC_{50} was 23.98 µg/mL, the TGI was 44.66 and the LC_{50} was 70.79 µg/mL. Figure 4B shows the micrographs of the cultures treated with the *P. aequale* extract after 48 hr. It can be seen that from 25 µg/mL there are fewer cells, that are no longer attached and some are already damaged. After 50 µg/mL the cultures are already completely damaged as they are rounded and the presence of cellular debris is seen, which is indicative of cell death. Therefore, prostate epithelium cells HPrEC are more sensitive to methanolic extract treatment compared to PC-3 prostate cancer cells.

DISCUSSION

C. scaber, P. aequale, S. oleraceus and *S. racemosa* are used in traditional medicine in the state of Veracruz in Mexico for a wide variety of conditions, including prostate and urinary problems. Therefore, it was considered that these species would have therapeutic properties in prostate cancer. This is the first study reporting the effect of *C. scaber, P. aequale* and *S. racemosa* on prostate cancer cells. Moreover, in the case of *C. scaber* and *S. racemosa*, no phytochemical and pharmacological reports were found.

The ^{1}H-NMR analysis allowed us to characterize the major types of secondary metabolites present in the extracts supported by

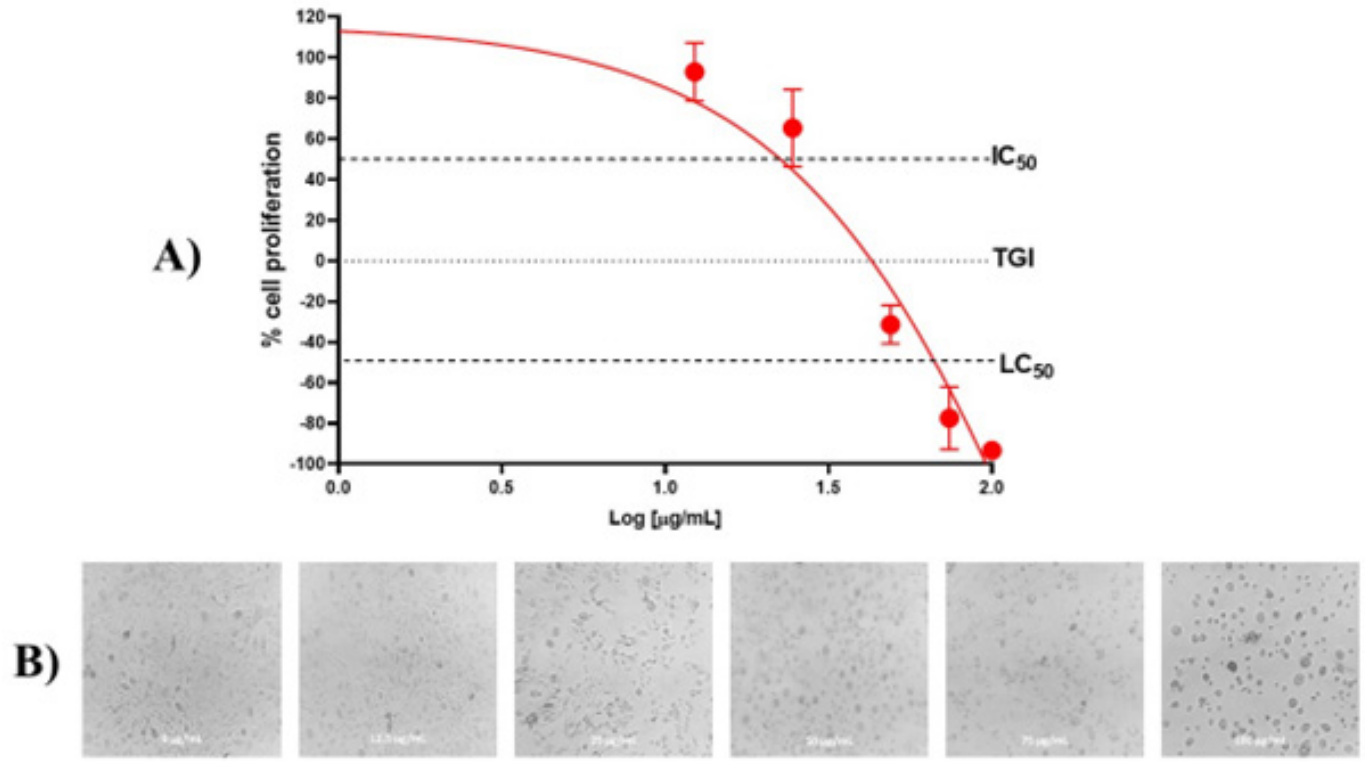

Figure 4: Anti-proliferative activity of the methanolic extracts of *P. aequale* in HPrEC normal cells. A) Dose-response curve shows the average of three independent experiments±SEM at 48 hr of treatment with the methanolic extract. B) Micrographs of cultures incubated with different concentrations (100-1000 µg/mL) of the extract of *P. aequale*.

previously reported secondary metabolites for the species or genus. Hence, in *C. scaber* triterpenes, phenolic compounds (6.59-8.26 ppm) and fatty acids (1.24 ppm) were the main components. *P. aequale* is contained terpenes (0.5-3.0 ppm), double bonds (3-5.5ppm), phenolic compounds (6.0-8.25 ppm) and aldehydes (9.75 ppm). In *S. oleraceus* the presence de of terpene lactones (3.5-5.5 ppm) and phenolic compounds (6.25-7.75 ppm) were identified and in *S. racemosa* the major secondary metabolites detected were flavonoids (6.0-8.0 ppm) and terpenes (0-3.0 ppm).

For the antioxidant evaluation, the methanolic and aqueous extract of *S. racemosa* were the most active extracts. The inhibition of free radicals and the iron reducing power of the aqueous extract (87.31±3.23% and 672.38±4.71 µmol Fe^{2+}/L) was slightly higher than that of the methanolic extract (84.90±2.51% and 653.12±13.44 Fe^{2+}/L), showing a strong activity at 1 mg/mL concentration. As mentioned earlier, flavonoids are the main secondary metabolites in this specie. It is known that flavonoids act as hydrogen donors to stabilize DPPH radicals. The antioxidant activity of this group of metabolites depends on many factors such as the number and position of hydroxyl groups in the molecules.[22] On the other hand, the aqueous extract (55.19±3.16% and 502.87± 9.82 Fe^{2+}/L) and methanolic extract (53.66±1.57% and 462.25±7.75 Fe^{2+}/L) of *P. aequale* presented moderate activity. For these extracts, alkaloids were the group of major chemical constituents in this species. However, neolignans were also identified, which could have contributed to the ability to inhibit free radicals and reduce the FRAP reagent.[23] Finally, the extracts of *S. oleraceus* and *C. scaber* showed aweakly activity. The antioxidant activity of the studied species was in the following order: *S. racemosa*>*P. aequale*>*S. oleraceus*>*C. scaber* and related to the content of total phenolic compounds.

The extracts of *P. aequale* were the most active in the antiproliferative activity against the PC-3, showing an IC_{50} of 251.6 µg/mL and 775.4 µg/mL for the methanolic and aqueous extracts, respectively. According to Atjanasuppat,[24] the antiproliferative and cytotoxic activity of plant extracts can be categorized into four groups because of their IC_{50} value: ≤ 20 µg/mL is active, between 20-100 µg/mL it is moderately active, between 100-1000 µg/mL it is weakly active and >1000 µg/mL is inactive. Based on this criterion, *P. aequale* extracts are considered weakly active against the PC-3 cell line.

According to the micrographs of the cultures of the methanolic extract of *P. aequale* at 250 µg/mL, fewer cells showed any damage compared to the control culture. However, from 500 µg/mL the cells begin to show indicating cytotoxicity. We can also see this behavior in the aqueous extract that, by increasing the concentration of extract, the cultures present cell damage. These results indicate that *P. aequale* extracts could exert a positive antiproliferative effect on prostate cancer cells in their early stages. Interestingly, HPrEC cells of epithelium prostate were more susceptible to methanol extract of *P. aequele* showing stronger antiproliferative and cytotoxic effect at lower concentrations with an IC_{50} of 23.98 µg/mL. These preliminary results suggest that care should be taken when consuming its extracts and that it is necessary to continue studying it to find the components responsible for its antiproliferative and cytotoxic effect in both cell lines. In addition, evaluating the extracts in animal models to guarantee the safety of their consumption.

Scientific reports of species of the genus *Piper* are extensive. However, very few report the compounds isolated from them that are responsible for the antiproliferative activity against prostate cancer cells. Among these reports we find the following. Alkaloids with biological activities against prostate cancer cells have been isolated from species of the *Piper* genus. From *P. nigrum* and *P. longum*, the piperine alkaloid with has been isolated; Ouyang *et al.*[25] reported that this compound inhibits prostate cancer cell lines LNCaP, DU-145 and PC-3 with IC_{50} values of 74.4 µM, 226.6 µM and 111.0 µM, respectively. the androgen-dependent LNCaP cell line is the most sensitive to treatment with piperine, which arrests the G_0/G_1 phase of the cell cycle and promotes autophagy. On the other hand, Lee *et al.*[26] evaluated the effect of pipernoline, an alkaloid from *P. longum*, against PC-3 and LNCaP prostate cancer cells. They found that PC-3 cells were more susceptible to treatment (IC_{50} of 40 µM) arresting cells in the G_0/G_1 phase by direct modulation of cyclin/CDK complexes and the generation of apoptosis via caspase-3 activation. Kim *et al.*[27] evaluated piperlongumine alkaloid from *P. logum* against DU-145, PC-3 and LNCaP prostate cancer cells that presented GI_{50} values (concentration to inhibit cell proliferation by 50%) of 7.1>10 and 9.2 µM, respectively, which showed higher activity in the androgen-independent cell line DU-145 and higher resistance to treatment in PC-3 cells. These reports suggest that possibly the compounds responsible for generating the antiproliferative and cytotoxic effect in prostate cancer PC-3 cells and prostate epithelial HPrEC could be alkaloids. However, according to the Dragendorff test carried out, these were not found.

In this study, we expected *S. racemosa* to show a high activity against PC-3 cells (IC_{50} of 642.9 µg/mL for the methanolic extract) due to the high antioxidant capacity. Now, considering what was described by Vue *et al.*[28] not all flavonoid subclasses have shown activity against PC-3 cells. For example, naringenin and myricetin have been reported with weak antiproliferative effect. On the other hand, this cell line has also shown resistance to other flavonoids such as luteolin, biflavone and Hydroxy-6,7-dimethoxyflavanone. Phytochemical and antiproliferative reports of the genus *Serjania* are scarce. However, Quintal-Novelo *et al.*[29] isolated goniocarpic acid sesterterpene from *S. goniocarpa* which was evaluated for its antiproliferative and cytotoxic activity in PC-3 cells with an identified a IC_{50} of 45.5 µg/mL. This is the only report found involving the study of species of the genus *Serjania* in prostate

cancer cells. Hence, suggests that terpenes from *S. racemosa* may be responsible for the antiproliferative activity in PC-3 cells in our study. However, future research is necessary to gain an in-depth understanding of the phytochemical constitution and antiproliferative activity in prostate cancer cells and non-cancerous cells in this specie.

S. oleraceus and *C. scaber* were not shown to be active against PC-3 cells. Ahmed *et al.*[30] evaluated the cytotoxic activity of the methanolic extract (80%) of the leaves of *S. oleraceus* against the PC-3 cell line in which they found an IC_{50} greater than 100 µg/mL, which they considered inactive. This report confirms that the methanolic extract of *S. oleraceus* does not generate an antiproliferative and cytotoxic effect on PC-3 cells. Finally, in *C. scaber* no pharmacological report was found for this species. However, Elkady[31] identified that the hexane fraction of the methanolic extract (75%) of the rhizomes of *C. speciosus* presented an IC_{50} of 2.3 µg/mL against the PC-3 cell line, generating an effect on cell proliferation by inducing apoptosis. This information suggests that in the case of *C. scaber*, in future research, it will be necessary to evaluate low polarity extracts and extracts from other parts of the plant to detect if there is activity against prostate cancer cells.

CONCLUSION

The methanolic extract of *P. aequale* presented the best results of antiproliferative activity in the prostate cancer cell line PC-3 compared to the other species studied with an IC_{50} of 251.6 µg/mL in addition to showing a cytotoxic effect from the 500 µg/mL. In addition, it has a greater effect on HPrEC cells with a IC_{50} of 23.98 µg/mL. While the methanolic extract of *S. racemosa* showed the best antioxidant activity (84.90% of DPPH inhibited) due to the presence of flavonoids that were detected. The antiproliferative effect observed in *P. aequale* is not related to antioxidant activity. According to previous scientific reports of species of the genus *Piper* against prostate cancer cell lines, alkaloids are the main group of secondary metabolites responsible for generating an antiproliferative and cytotoxic effect. However, in *P. aequale* they were not detected. Furthermore, it is necessary to investigate the phytochemical composition of the extract and identify the possible compounds responsible for the antiproliferative activity in PC-3 cells, as well as detect the compound(s) responsible for generating the cytotoxic effect in HPrEC cells. This work confirms that *P. aequale* affects prostate cancer cells, which might support their use in traditional Mexican medicine.

ACKNOWLEDGEMENT

This work was supported by the Consejo Nacional de Humanidades Ciencias y Tecnologías (CONAHCYT) of Mexico (grant number 743124). We thank Dr. Miguel Cházaro Basáñez of the Facultad de Biología campus Xalapa of the Universidad Veracruzana for his support in the botanical identification of the species studied.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

DMSO: Dimethylsulfoxide; **DPPH:** 2,2-Diphenyl-picrylhydrazyl; **FBS:** Fetal Bovine Serum; **FRAP:** Ferric Reducing Antioxidant Power; **GI_{50}:** Concentration to inhibit cell proliferation by 50%; **IC_{50}:** Concentration at which 50% of cell proliferation is inhibited; **LC_{50}:** Concentration at which 50% of cells die; **MTT:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **NMR:** Nuclear Magnetic Resonance; **P/S:** Penicillin-Streptomycin; **TGI:** Concentration at which 100% of cell proliferation is inhibited; **TPTZ:** Ferric Complex-2,5,6-tripyridyl-5-triazine.

REFERENCES

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, *et al.* Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2021;71(3):209-49. doi: 10.3322/caac.21660, PMID 33538338.
2. Alonso-Castro AJ, Villarreal ML, Salazar-Olivo LA, Gómez-Sánchez M, Domínguez F, García-Carranca A. Mexican medicinal plants used for cancer treatment: pharmacological, phytochemical and ethnobotanical studies. J Ethnopharmacol. 2011;133(3):945-72. doi: 10.1016/j.jep.2010.11.055, PMID 21146599.
3. Flora de Veracruz VA. Fascículo 78 Costaceae. Instituto de Ecología, A.C. 1994.
4. Da Silva JK, Pinto LC, Burbano RM, Montenegro RC, Andrade EH, Maia JG. Composition and cytotoxic and antioxidant activities of the oil of *Piper aequale* Vahl. Lipids Health Dis. 2016;15(1):174. doi: 10.1186/s12944-016-0347-8, PMID 27717404.
5. Mata S, Etnobotánico E de las Plantas Medicinales entre los Zoque-Popolucas de Piedra Labrada, Veracruz. ENEP Iztacala [bachelor thesis]. MEX: UNAM; 1983.
6. Burgos H. Flora Vascular con características potenciales para el aprovechamiento y conservación de los fragmentos de selva en el Municipio de Atzalán, Veracruz [masters thesis]. Instituto de Ecología. Veracruz: AC Xalapa; 2009.
7. Setzer WN, Park G, Agius BR, Stokes SL, Walker TM, Haber WA. Chemical compositions and biological activities of leaf essential oils of twelve species of Piper from Monteverde, Costa Rica. Nat Prod Commun. 2008;3(8):1367-74. doi: 10.1177/1934578X0800300823.
8. Del Amo Rodríguez S. Plantas medicinales del estado de Veracruz Instituto Nacional de Investigaciones sobre Recursos Bióticos, editor. Vol. 1ª. Xalapa, VER; 1979.
9. Ambrosio M, Avendaño S. Catálogo de plantas útiles del Municipio de Misantla, Veracruz. Cienc Hombre. 1991;5:31.
10. Bondarenko VG, Glyzin VI, Shelyuto VL. Flavonoids of the flowers of *Sonchus oleraceus*. Chem Nat Compd. 1983;19(2):228-9. doi: 10.1007/BF00580571.
11. Miyase T, Fukushima S. Studies on sesquiterpene glycosides from *Sonchus oleraceus* L. Chem Pharm Bull. 1987;35(7):2869-74. doi: 10.1248/cpb.35.2869.
12. Ehab E. Cytotoxic and antibacterial constituents from roots of *Sonchus oleraceus* L. growing in Egypt. Pharmacogn Mag. 2009;5:324-8. doi: 10.4103/0973-1296.58154.
13. Zhang H, Wang Y, Wang S, Wang Q, Wang T, Zhu J, *et al.* Cytotoxic sesquiterpenes from *Sonchus oleraceus*. Anti Cancer Agents Med Chem. 2020;20(6):709-14. doi: 10.2174/1871520620666200101152934, PMID 31894000.
14. Elsadig M, Rezk A, Alkhatim O, Yousif A, Ullrich A, Kuhnert N. Antimicrobial, antiparasitic and antioxidant activities of medicinal plants from Sudan. J Complement Med Alt Healthc. 2017;2:1-9. doi: 10.19080/JCMAH.2017.02.555597.
15. Yin J, Si CL, Wang MH. Antioxidant activity of flavonoids and their glucosides from *Sonchus oleraceus* L. J Appl Biol Chem. 2008;51(2):57-60. doi: 10.3839/jabc.2008.010.
16. Mawalagedera SM, Ou ZQ, McDowell A, Gould KS. Effects of boiling and *in vitro* gastrointestinal digestion on the antioxidant activity of Sonchus oleraceus leaves. Food Funct. 2016;7(3):1515-22. doi: 10.1039/C5FO01508A, PMID 26891707.
17. Cai Y, Luo Q, Sun M, Corke H. Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. Life Sci. 2004;74(17):2157-84. doi: 10.1016/j.lfs.2003.09.047, PMID 14969719.
18. Brand-Williams W, Cuvelier M, Berset C. Use of a free radical method to evaluate antioxidant capacity. Food Sci Technol. 1995;28:25-30. doi: 10.1016/S0023-6438(95)80008-5.

19. Dominguez-Ortiz M, Muñoz O, García R, Vázquez M. Antioxidant and anti-inflammatory activity of Moussonia deppeana. Bol Latinoam Caribe Plant Med Aromat. 2009;9:13-9.
20. Benzie IF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power: the FRAP assay. Anal Biochem. 1996;239(1):70-6. doi: 10.1006/abio.1996.0292, PMID 8660627.
21. Monks A, Scudiero D, Skehan P, Shoemaker R, Paull K, Vistica D, *et al*. Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured humal tumor cell lines. J Natl Cancer Inst. 1991;83(11):757-66. doi: 10.1093/jnci/83.11.757, PMID 2041050.
22. Cardoso CA, Coelho RG, Honda NK, Pott A, Pavan FR, Leite CQ. Phenolic compounds and antioxidant, antimicrobial and antimycobacterial activities of *Serjania erecta* Radlk. (Sapindaceae). Braz J Pharm Sci. 2013;49(4):775-82. doi: 10.1590/S1984-82502013000400017.
23. Peng Y, Lou LL, Liu SF, Zhou L, Huang XX, Song SJ. Antioxidant and anti-inflammatory neolignans from seeds of hawthorn. Bioorg Med Chem Lett. 2016;26(22):5501-6. doi: 10.1016/j.bmcl.2016.10.012, PMID 27765508.
24. Atjanasuppat K, Wongkham W, Meepowpan P, Kittakoop P, Sobhon P, Bartlett A, *et al. In vitro* screening for anthelmintic and antitumor activity of ethnomedicinal plants from Thailand. J Ethnopharmacol. 2009;123(3):475-82. doi: 10.1016/j.jep.2009.03.010, PMID 19473794.
25. Ouyang DY, Zeng LH, Pan H, Xu LH, Wang Y, Liu KP, *et al.* Piperine inhibits the proliferation of human prostate cancer cells via induction of cell cycle arrest and autophagy. Food Chem Toxicol. 2013;60:424-30. doi: 10.1016/j.fct.2013.08.007, PMID 23939040.
26. Lee W, Kim KY, Yu SN, Kim SH, Chun SS, Ji JH, *et al.* Pipernonaline from *Piper logum* Linn. induces ROS-mediated apoptosis in human prostate cáncer PC-3 cells. Biochem Biophys Res Commun. 2013;430(1):406-12. doi: 10.1016/j.bbrc.2012.11.030, PMID 23159637.
27. Kim YH, Yoon YJ, Lee YJ, Kim CH, Lee S, Choung DH, *et al*. Piperlongumine derivative, GC-06, inhibits STAT3 activity by direct binding to STAT3 and regulating the reactive oxygen species in DU-145 prostate cancinoma cells. Bioorg Med Chem Lett. 2018;28(14):2566-72. doi: 10.1016/j.bmcl.2018.05.025, PMID 29807795.
28. Vue B, Zhang S, Chen QH. Flavonoids with therapeutic potential in prostate cancer. Anti Cancer Agents Med Chem. 2016;16(10):1205-29. doi: 10.2174/1871520615666151008122622, PMID 26446382.
29. Quintal-Novelo C, Torres-Tapia LW, Moo-Puc R, Peraza-Sanchez SR. Bio-guided isolation of a new sesterterpene from *Serjania goniocarpa*. Nat Prod Commun. 2015;10(9):1513-6. doi: 10.1177/1934578X1501000906, PMID 26594746.
30. Ahmed M, Penech F, Ahmed M. Four select Sudanese medicinal plants induce anticancer and cytotoxic effects in prostate cancer cell line. Clin Med Biochem. 2017;3:1000134. doi: 10.4172/2471-2663.1000134.
31. Elkady AI. Targeting prostate cancer cell proliferation, stemness and metastatic potential using *Costus speciosus* derived phytochemicals. Am J Transl Res. 2019;11(4):2550-69. PMID 31105862.

Cite this article: Sánchez-Aguirre OA, Fernández-Pomares C, Juárez-Aguilar E, Sánchez-Medina A, Cano-Asseleih LM. Exploring the Antioxidant and Antiproliferative Properties of Four Medicinal Plants from Veracruz, Mexico for Urinary and Prostate Health. Pharmacog Res. 2024;16(4):817-27.