

Artículo Original / Original Article

**Antioxidant, antimicrobial and antiproliferative activities of alcoholic extracts from *Piper aequale* Vahl leaves**[Actividad antioxidante, antimicrobiana y antiproliferativa de extractos alcohólicos de hojas de *Piper aequale* Vahl]Oscar Antonio Sánchez-Aguirre<sup>1</sup>, Marina Guevara-Valencia<sup>2</sup>, Enrique Juárez-Aguilar<sup>3</sup>, Ninoska Flores<sup>4</sup>, Omar Germán Malagón-Avilés<sup>5</sup>, Alberto Sánchez-Medina<sup>6</sup> & Leticia Margarita Cano-Asseleih<sup>7</sup><sup>1</sup>Centro de Investigaciones Biomédicas, Universidad Veracruzana, Xalapa, Veracruz, México<sup>2</sup>Facultad de Ciencias Químicas, Universidad Veracruzana, Orizaba, Veracruz, México<sup>3</sup>Instituto de Ciencias de la Salud, Laboratorio de Cultivo Celular, Departamento de Biomedicina, Universidad Veracruzana, Xalapa, Veracruz, México<sup>4</sup>Instituto de Investigaciones Fármaco Bioquímicas, Facultad de Ciencias Farmacéuticas y Bioquímicas, Universidad Mayor de San Andrés, La Paz, Bolivia<sup>5</sup>Departamento de Química, Universidad Técnica Particular de Loja, Loja, Ecuador<sup>6</sup>Instituto de Química Aplicada, Universidad Veracruzana, Xalapa, Veracruz, México<sup>7</sup>Centro de Investigaciones Tropicales, Universidad Veracruzana, Xalapa, Veracruz, México**Reviewed by:**Lyudmyla Symochko  
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**Abstract:** *Piper* is a large plant genus containing essential oils rich in mono and sesquiterpenes and other secondary metabolites showing different biological activities. *Piper aequale*, cordoncillo, is used in México for urinary and prostate ailments, suggesting a potential therapeutic effect against prostate cancer. Due to the lack of chemical and pharmacological information on this species, in this work antimicrobial and antiproliferative activities were evaluated. Leaves ethanol and methanol extracts were used to assess antioxidant activity (DPPH, FRAP), antimicrobial activity (Kirby-Bauer) against clinically relevant strains and antiproliferative activity on prostate cancer cell line PC-3 (MTT assay). Methanolic extract exhibited the highest antioxidant activity, with 69.08% (DPPH) and inhibitory effects on pathogenic bacterial strains associated with urinary tract infections. Ethanol extract displayed moderate antiproliferative activity (IC<sub>50</sub> 81.28 µg/mL), showing cytotoxicity from 100 µg/mL. This study demonstrates *P. aequale* exhibits inhibitory effects against bacteria associated with urinary problems and antiproliferative properties in prostate cancer cells.

**Keywords:** *Piper aequale*; Traditional mexican medicine; Profile 1H-NMR; Prostate cancer; Urinary ailments

**Resumen:** *Piper* es un género numeroso de plantas con aceites esenciales ricos en mono y sesquiterpenos y otros metabolitos bioactivos. *Piper aequale*, cordoncillo, se usa en México para afecciones urinarias y prostáticas, sugiriendo un posible efecto terapéutico contra el cáncer de próstata. Con poca información química y farmacológica, en este trabajo se evaluaron las actividades antimicrobiana y antiproliferativa. En los extractos etanólico EE y metanólico EM de hojas se determinaron la actividad antioxidante (DPPH, FRAP), antimicrobiana (Kirby-Bauer) frente a cepas clínicamente relevantes y antiproliferativa en la línea celular PC-3 de cáncer de próstata (ensayo MTT). EM exhibió la mayor actividad antioxidante, 69,08% (DPPH) y efecto inhibidor de bacterias asociadas a infecciones urinarias. EE mostró actividad antiproliferativa moderada (IC<sub>50</sub> 81,28 µg/mL) y citotoxicidad a partir de 100 µg/mL. Este estudio demostró que *P. aequale* ejerció actividad antimicrobiana contra bacterias presentes en afecciones urinarias y actividad antiproliferativa en células de cáncer de próstata.

**Palabras clave:** *Piper aequale*; Medicina tradicional mexicana; Perfil 1H-NMR; Cáncer de próstata; Afecciones urinarias

## INTRODUCTION

Piperaceae family includes eleven genera with around 3,500 species of pantropical distribution: *Piper*, *Peperomia*, *Trianaeopiper*, *Ottonia*, *Arctotonia*, *Macropiper*, *Manekia*, *Pothomorphea*, *Sarcorachis*, *Verhuellia*, and *Zippelia*. *Piper* is the most representative of this family with approximately 2000 registered species (Wang *et al.*, 2014). They are usually found abundantly in Southeast Asia, southern Mexico, the Andes, Chocó, the Amazon and the Atlantic Forest of Brazil (Jaramillo & Callejas, 2004). In traditional medicine they are used worldwide to treat urological, cutaneous, hepatic, gastric ailments, wound healing, as well as fever and inflammation (Salehi *et al.*, 2019).

Phytochemically research has been made in only 10% of all species of the genus and approximately 667 secondary metabolites have been found such as alkaloids/amides, propenylphenols, lignans, neolignans, terpenes, steroids, kavapyrones, piperolides, chalcones/dihydrochalcones, flavones, flavonones and compounds that are not found within the main groups of metabolites (Dyer *et al.*, 2004). Noteworthy is the extraction of essential oils from different parts of the plants where primarily monoterpenes and sesquiterpenes have been identified (Xiang *et al.*, 2017). Many of these metabolites are responsible of some of the pharmacological properties such as anxiolytic, analgesic, anti-inflammatory, vasodilatory, cytotoxic, immunomodulatory, antibacterial, antifungal, and antitumor activities to species of this genus (Lima *et al.*, 2020), related to their traditional uses (Salehi *et al.*, 2019).

Within these species, *Piper aequale* Vahl commonly known as “cordoncillo” in the state of Veracruz, Mexico, is a plant whose leaves are traditionally used as an anti-venom remedy and for treating bruises, women's baths, chills, urinary and prostate ailments. Scientific information about this species is scarce. However, benzofuran-type neolignans have been reported in the aerial parts (Maxwell *et al.*, 1999). Additionally, some major mono- and sesquiterpenes have been identified in the essential oil of the leaves, including  $\alpha$ -pinene,  $\beta$ -pinene,  $\delta$ -elemene, sabinene, limonene,  $\beta$ -elemene, germacrene D, valerianol, cubebol,  $\beta$ -atlantol, and bicyclogermacrene (Setzer *et al.*, 2008; Da Silva *et al.*, 2016).

Regarding the pharmacological properties of

the species, the essential oil has shown weak antioxidant activity with 25.9% inhibition against the DPPH radical. On the other hand, its antibacterial effect against strains of *B. cereus*, *S. aureus*, and *E. coli* has been reported at concentrations ranging from 156 to 625  $\mu\text{g/mL}$  (Setzer *et al.*, 2008). However, its cytotoxic properties against cancer cell lines such as ACP-03 gastric cancer, HCT-116 colon cancer, and SKMEL 19 melanoma have been highlighted, with  $\text{IC}_{50}$  values of 1.54, 8.69, and  $> 25$   $\mu\text{g/mL}$ , respectively. Da Silva *et al.* (2016), suggest that this strong cytotoxic activity is due to the high content of  $\delta$ -elemene (19%),  $\alpha$ -pinene, and  $\beta$ -pinene (15.6% and 12.6%, respectively) present in the essential oil, as sesquiterpenes like  $\delta$ -elemene have been identified to induce apoptosis and affect the cell cycle in malignant cells.

Because *P. aequale* is used in mexican traditional medicine to treat urinary and prostate ailments, we consider that this species may have a positive effect against urinary infections and prostate cancer. Therefore, the aim of this study is to evaluate the antioxidant, antimicrobial, and antiproliferative activities of methanolic and ethanolic extracts of *P. aequale* leaves.

## MATERIALS AND METHODS

### *Vegetal material*

The leaves of *P. aequale* were collected on March 10, 2020, in the city of Xalapa, Veracruz, Mexico. A specimen was deposited in the herbarium of the Center for Biological Research (CIB) at the Universidad Veracruzana for identification and botanical classification (Voucher 23295UV).

### *Preparation of extracts*

The leaves were placed in a forced-air oven (Yamato DX602C) at 45°C. Subsequently, the dried plant material was ground using an electric mill. 2 g of powdered material were mixed with 40 mL of ethanol, and separately, 2 g of powdered material were mixed with 40 mL of methanol. Both mixtures were sonicated (Branson 1800) for 50 minutes at room temperature. After this period, the extract was filtered through Whatman No.1 filter paper. Excess solvent was removed under reduced pressure using a rotary evaporator (BÜCHI R-114) at 40°C until dryness. The extracts were stored protected from light and refrigerated until use.

**Chemical profile by <sup>1</sup>H-NMR of the crude extracts**

20 mg of each extract was dissolved in 0.6 mL of deuterated dimethyl sulfoxide (DMSO-*d*<sub>6</sub> 99.9%) and transferred to a 5 mm NMR tube. A <sup>1</sup>H-NMR analysis was performed on a BRUKER 500 MHz Nuclear Magnetic Resonance (NMR) spectrometer, model Magnet System 500'54 Ascend ULH. The analysis consisted of 1024 scans with a 2.75-second acquisition time and a spectral width of 11904.8 Hz. The data from the analysis were processed using MestReNova 12.0 software. Once the spectra of the extracts were obtained, the signals were analyzed based on their chemical shifts and correlated with different groups of secondary metabolites previously reported in the literature.

**Antioxidant activity****Total polyphenol content**

The total polyphenol content was determined using the Folin-Ciocalteu method, in which 50 μL of extract (1 mg/mL) was mixed with 2.5 mL of a 1:10 solution of Folin-Ciocalteu reagent and 2 mL of 7.5% Na<sub>2</sub>CO<sub>3</sub> solution. The samples were incubated at 45°C for 15 minutes. After incubation, absorbance readings were taken at 765 nm using a UV-VIS spectrophotometer (Thermo Electron Corporation Spectronic Helios α). Quantification of the total polyphenol content was performed using a calibration curve (25-1000 μg/mL, R<sup>2</sup> = 0.9946) with gallic acid as the standard (Cai *et al.*, 2004). The experiment was conducted in triplicate.

**Free radical scavenging assay by DPPH**

A solution of DPPH at 9 x 10<sup>-5</sup> M in methanol was prepared, then 2.9 mL of this solution were taken and 100 μL of the extract solutions at a concentration of 1 mg/mL were added. The samples were incubated for 30 minutes at 37°C protected from light, and then the absorbance was measured at 517 nm using a UV-VIS spectrophotometer. A solution of ascorbic acid at 5 mM was used as a positive control. The assay was conducted in triplicate. (Brand-Williams *et al.*, 1995; Domínguez-Ortiz *et al.*, 2009). Antioxidant activity was determined using the following equation:

$$\% \text{ of inhibition} = \frac{A - A_1}{A} \times 100$$

Where:

A: DPPH reagent absorbance

A<sub>1</sub>: Average absorbances of the samples

**FRAP iron reducing power**

The FRAP solution was prepared using 100 mL of acetate buffer (300 mM, pH 3.6), 10 mL of 10 mM TPTZ solution (Ferric Reducing Antioxidant Power) dissolved in a 40 mM hydrochloric acid solution, and 10 mL of 20 mM ferric chloride solution.

To determine the reducing power of the extracts, 150 μL of the extract (1 mg/mL) was mixed with 150 μL of distilled water and 2.7 mL of FRAP solution. The solutions were incubated at 37°C for 4 minutes. Subsequently, the absorbance of the samples was read at 593 nm using a UV-VIS spectrophotometer. The results were expressed in μmol Fe<sup>2+</sup> based on a calibration curve from different concentrations of FeSO<sub>4</sub>·7H<sub>2</sub>O (50 to 750 mmol/L, R<sup>2</sup> = 0.9842). The FRAP assay was conducted in triplicate (Benzie & Strain, 1996; Domínguez-Ortiz *et al.*, 2009).

**Antimicrobial activity**

Strains of *Burkholderia cepaceae*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Sphingomonas paucimobilis*, *Staphylococcus aureus*, *Staphylococcus saprophyticus*, *Streptococcus pneumoniae*, *Candida albicans*, *Candida tropicalis* and *Candida krusei* were characterized and donated by the microbiology laboratory of the Regional Hospital of the Mexican Social Security Institute (IMSS) in the city of Orizaba, Veracruz, Mexico.

The determination of the antimicrobial activity of *P. aequale* extracts was carried out according to the Kirby-Bauer method (Clinical Laboratory Standards Institute, 2015). For the preparation of the inoculum, 2 to 3 colonies of the strain were deposited in a tube with 15 mL of saline solution, and then the absorbance was read at 595 nm until an absorbance in the range of 0.08-0.1 was obtained, corresponding to a 0.5 McFarland inoculum (1x10<sup>8</sup> CFU/mL). To test the extracts on the microbial cultures, 6 mm diameter Whatman No. 1 filter paper discs were used, which were impregnated with each of the extracts at a concentration of 10 mg/mL. Additionally, ceftriaxone was used as a positive control in the antibacterial activity test while nystatin was used in the antifungal activity, both to 25 μg. Solvents used in the extraction processes were the negative controls.

The inoculum 0.5 of the McFarland scale of

each strain was sown on the surface of Mueller-Hinton agar contained in Petri dishes by streaking. Subsequently, the SensiDiscs were placed on the surface of the agar. The cultures were incubated at

37°C for 24 h, after this period measurements of the inhibition zones formed were made. The tests were done in triplicate and the inhibition value (mm) was calculated according to the following equation:

$$\text{Inhibition value} = (\text{inhibition diameter} - \text{SensiDisk diameter})/2$$

### Antiproliferative activity

For the study using the androgen-independent prostate cancer cell line PC-3 of bone metastasis, 3 mg of the methanolic and ethanolic extracts were weighed and dissolved in 3 mL of RPMI culture medium supplemented with 8% FBS (fetal bovine serum) and 1% P/S (penicillin-streptomycin) to obtain a stock solution at a concentration of 1000 µg/mL. This solution was sterilized using a sterile 0.22 µm filtration unit. Subsequently, a series of dilutions of the extracts were performed to obtain the following concentrations: 0, 100, 250, 500, 750, and 1000 µg/mL, respectively. The extracts dissolved in the culture medium were stored at -20°C until use.

The PC-3 cell line was seeded in a 96-well multiplate (0.32 cm<sup>2</sup>, CORNING) at a density of 12,500 cells/cm<sup>2</sup> in 100 µL of RPMI culture medium supplemented with 8% FBS and a mixture of penicillin and streptomycin. at 1%, incubating for 48 h at 37°C and 5% CO<sub>2</sub>.

After this period, the culture medium was removed, and the different concentrations of the extracts were immediately added. The cultures were incubated for 48 h at 37°C and 5% CO<sub>2</sub>. At the time of the change of conditions, the viability of cultures without extracts was determined using the MTT assay. This value was called T<sub>0</sub> (cell viability at the beginning of treatment).

After 48 h of incubation, micrographs were taken with the 25X objective. Forty-eight hours later, the cell viability of the treated cultures was determined using the MTT assay.

A total of three independent experiments with three replicates each were carried out. From the absorbance data obtained from the MTT assay, dose-response curves were prepared (non-linear regression between the percentage of proliferation vs log concentration). Following the methodology of the National Cancer Institute of the United States (Monks

et al., 1991), the values of IC<sub>50</sub> (Inhibitory concentration at which 50% of cell proliferation is inhibited), TGI (Concentration at which inhibits 100% of cell proliferation) and LC<sub>50</sub> (Concentration at which 50% of the cell population dies). To prepare the dose-response curves, the data obtained were normalized to the percentage of cell proliferation. 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 T<sub>0</sub>, the following is applied:

$$100[(T-T_0)/(C-T_0)]$$

If the absorbance value of the sample is less than that of T<sub>0</sub>, the following is applied:

$$100[(T-T_0)/(T_0)]$$

Where:

T= Absorbance of the sample with the treatment.

T<sub>0</sub>= Absorbance of the cell culture without extract at the beginning of the experiment

C= Absorbance of the cell culture without treatment (control).

The results obtained were analyzed statistically, by developing a non-linear regression using the GraphPad Prism version 8 program, La Jolla California USA.

## RESULTS

### Identification of secondary metabolites by <sup>1</sup>H-NMR

The <sup>1</sup>H-NMR analysis allowed the identification of the different groups of secondary metabolites that constitute the ethanol and methanol extracts of *P. aequale* by detecting signals due to their chemical shifts, Figure No. 1.

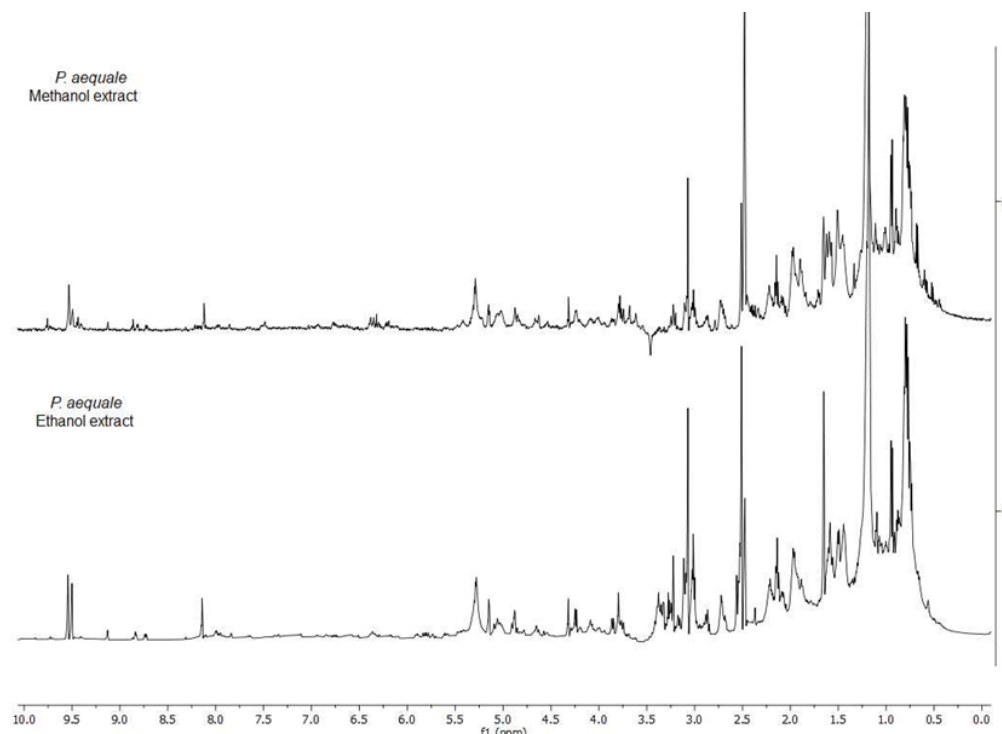


Figure No. 1

<sup>1</sup>H-NMR of methanolic and ethanolic extract of *P. aequale* (500 MHz, DMSO d<sub>6</sub>)

The proton spectrum of both extracts presents signals 0.42 to 1.9 ppm that correspond to methyls, methylenes and methines, the intense signal of 1.24 ppm represents lipids. In the region from 2.72 to 3.24 amine signals are shown, while from 3.3 to 4.0 ppm ether signals were identified. On the other hand, from 4.30 to 5.28 ppm double bonds are present. From 6.28 to 8.89 ppm there are signals of aromatic compounds or amides; and, finally from 9.12 to 9.75 ppm signals of aldehydes. The signals from 0.75 to

1.94 ppm that correspond to aliphatic compounds were those that appeared with the greatest intensity.

#### Antioxidant activity

The results of antioxidant activity reveal that the methanolic extract of *P. aequale* has a better free radical capture effect and reducing power. This effect was considered moderate compared to the ascorbic acid control, Table 1. The observed activity is related to the total phenol content.

Table No. 1

#### Antioxidant activity of alcoholic extracts of *P. aequale*

Sample	mg gallic acid/g of sample	% of DPPH inhibition	μmol de Fe <sup>+2</sup>
Methanolic extract	57.66 ± 4.60	69.08 ± 4.16	410.79 ± 25.41
Ethanolic extract	39.36 ± 2.60	20.91 ± 0.72	159.93 ± 16.48
Ascorbic acid	---	100	---

The table shows the average of three repetitions ± standard deviation

#### Antimicrobial activity

According to Table No. 2, the evaluation of antibacterial activity reveals that Gram (+) bacteria such as *S. aureus* and *S. saprophyticus* are more sensitive to the methanolic extract of *P. aequale* with

inhibition zones greater than 13 mm at 10 mg/mL; while Gram (-) bacteria such as *K. pneumoniae* and *P. mirabilis* are more resistant to treatment. On the other hand, interestingly it is observed that the ethanolic extract does not affect most of the strains studied;

however, *S. paucimobilis* and *S. aureus* were sensitive to this extract. In this sense, the results indicate that the methanolic extract affects both Gram

(+) and Gram (-) strains. In the evaluation of antifungal activity, none of the extracts presented antifungal activity against *Candida* strains.

**Table No. 2**  
**Antimicrobial activity of alcoholic extracts of *P. aequale***

Bacteria	Inhibition diameter (mm)		
	Methanolic extract	Ethanollic extract	Positive control
<i>Burkholderia cepacea</i>	---	---	---
<i>Enterococcus faecalis</i>	---	---	14.83 ± 1.10*
<i>Escherichia coli</i>	---	---	30.55 ± 0.72*
<i>Klebsiella pneumoniae</i>	1.33 ± 0.57	---	17.01 ± 0.9*
<i>Proteus mirabilis</i>	1.66 ± 0.57	---	13.94 ± 1.10*
<i>Salmonella typhi</i>	---	---	18.33 ± 1.0*
<i>Pseudomona aeruginosa</i>	---	---	16.11 ± 1.45*
<i>Sphingomonas paucimobilis</i>	11 ± 0.5	12.16 ± 0.76	35.33 ± 1.00*
<i>Staphylococcus aureus</i>	16.16 ± 2.30	8.16 ± 0.57	19.33 ± 1.10*
<i>Staphylococcus saprophyticus</i>	13.16 ± 1.04	---	13.33 ± 0.80*
<i>Streptococcus pneumoniae</i>	8.16 ± 0.57	---	34 ± 0.30*
<i>Candida albicans</i>	---	---	19.1 ± 0.90**
<i>Candida tropicalis</i>	---	---	15.7 ± 1.10**
<i>Candida krusei</i>	---	---	14.6 ± 1.10**

The table shows the average of three replicates ± standard deviation.

\*Bacterial positive control: ceftriaxone; \*\*Fungal positive control: nystatin

#### Antiproliferative activity

The antiproliferative activity of the methanolic and ethanolic extracts of *P. aequale* was evaluated in the PC-3 prostate cancer cell line. Figure No. 2a depicts the dose-response curve of this study, showing a significant decrease in proliferation with the ethanolic extract, which exhibited IC<sub>50</sub> values of 81.28 µg/mL, TGI of 177.82 µg/mL, and LC<sub>50</sub> of 501.18 µg/mL. According to the culture micrographs (Figure No. 2c), morphological changes in cells, such as

vacuolization and increased size, were observed starting from 100 µg/mL. This effect was notable up to 500 µg/mL, where fewer cells were observed compared to the untreated control culture (0 µg/mL). Concentrations of 750 µg/mL and 1000 µg/mL were detrimental to the cells, as evidenced by the presence of cellular debris. These results suggest that the ethanolic extract exerts an effect on PC-3 cell proliferation through a cytotoxic mechanism.

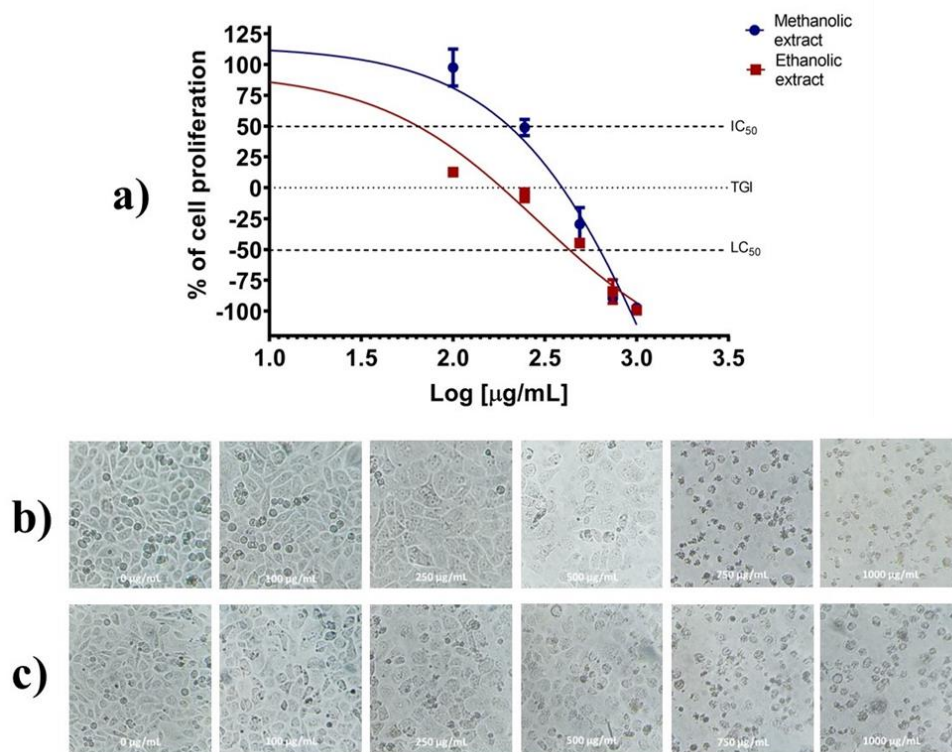


Figure No. 2

**Anti-proliferative activity of the methanolic and ethanolic extract of *P. aequale* leaves against the PC-3 cell line. a) Dose-response curve of the methanolic and ethanolic extract of *P. aequale* after 48 h of treatment, the graph shows the average of three independent experiments  $\pm$  standard error of the mean. b) Micrographs of the cultures treated with the different concentrations of the methanolic extract of *P. aequale*. c) Micrographs of the cultures treated with the different concentrations of the ethanolic extract of *P. aequale***

On the other hand, the methanolic extract of *P. aequale* also exhibited an effect on PC-3 prostate cancer cell line proliferation. However, according to the dose-response curve (Figure No. 2a), it was less active compared to the ethanolic extract, as it presented higher IC<sub>50</sub> (239.88 µg/mL), TGI (371.53 µg/mL), and LC<sub>50</sub> (549.54 µg/mL) values than those observed with the ethanolic extract. The culture images in Figure No. 2b show an increase in size and cytoplasmic vacuolization starting from 250 µg/mL, contrasting with the untreated control cultures. Cultures incubated with higher concentrations (500 µg/mL) showed lower cell density, while concentrations of 750 µg/mL and 1000 µg/mL were markedly cytotoxic, as evidenced by the presence of cellular debris in the culture. Thus, our results suggest that the methanolic extract of *P. aequale* possesses antiproliferative effects on PC-3 cells

through a cytotoxic mechanism.

## DISCUSSION

The *Piper* genus comprises approximately 2000 species, out of which 10 have been used in traditional medicine for cancer treatment or related symptoms. Among them are *P. aduncum*, *P. boehmeriifolium*, *P. capense*, *P. cubeba*, *P. gibbilimum*, *P. guineense*, *P. logum*, *P. nigrum*, *P. sylvaticum*, and *Piper sp.* Some studies have reported that 35 extracts and 32 isolated compounds from species of this genus exhibit cytotoxic properties against cancer cell lines, with amide-type alkaloids representing 53% of the main groups of bioactive secondary metabolites (Wang et al., 2014).

*Piper aequale* is native to Mexico and is prescribed in Mexican traditional medicine for a variety of diseases, particularly for urinary and



prostate conditions. Despite this, there are no reports on the effect of extracts of this species on human prostate cancer cell lines. Phytochemical reports on the species have only documented the presence of benzofuran-type neolignans (Maxwell *et al.*, 1999) and the presence of monoterpenes and sesquiterpenes in the essential oil (Setzer *et al.*, 2008; Da Silva *et al.*, 2016). Analysis of the <sup>1</sup>H-NMR spectra of both extracts identified signals corresponding to lipids (1.24 ppm), terpenes (0.42-1.94 ppm), alkaloids (3.3-4.0 and 9.12-9.75 ppm), and phenolic compounds (6.28-8.89 ppm), with terpene signals being the most abundant. Phytochemical research on the genus has been extensively studied, reporting a wide variety of compounds including amide-type alkaloids, lignans, neolignans, terpenes, steroids, kawapirones, piperolides, chalcones, dihydrochalcones, flavones, and flavanones (Parmar *et al.*, 1997; Wang *et al.*, 2014).

In this regard, the ethanolic and methanolic extracts of *P. aequale* used in this study contain characteristic metabolites of the genus. On the other hand, the evaluation of antioxidant activity in these extracts showed that the ethanolic extract does not have lower activity compared to the methanolic extract. In both cases, antioxidant activity is related to the presence of phenols, suggesting that the antioxidant components of *P. aequale* are highly polarity.

Regarding the studied antibacterial activity, the results suggest that the methanolic extract is more active than the ethanolic extract, inhibiting a greater number of strains. However, both extracts inhibit strains of *S. aureus* and *S. paucimobilis*. Specifically, *S. aureus* is known for causing infections in the skin, respiratory tract, bloodstream, and urinary tract, while *P. paucimobilis* is associated with respiratory, urinary tract, and bloodstream infections. The methanolic extract also showed inhibitory effects on other strains such as *S. saprophyticus*, which causes urinary tract infections, and exhibited an effect on *S. pneumoniae*, a bacterium that causes respiratory diseases. Lastly, the methanolic extract exhibited slight inhibition against *K. pneumoniae*, which causes urinary tract infections, pneumonia, bloodstream infections, and wounds and soft tissue infections, as well as against *P. mirabilis*, which causes urinary and wound infections. Importantly, neither of the extracts showed activity against *Candida* strains.

Patients suffering from urinary tract

infections are commonly treated with antibiotics; these treatments can cause long-term alterations in the normal microbiota and in the gastrointestinal tract that can generate microbial resistance to multiple drugs (Flores-Mireles *et al.*, 2015). Furthermore, prostate cancer is known to be associated with urinary tract infections such as prostatitis, cystitis, and pyelonephritis (Pan *et al.*, 2023). In this way, the results of the present work suggest a possible effect of the methanolic extract of *P. aequale* in the treatment of urinary conditions in patients with or without prostate cancer.

Medicinal plants chemistry is complex, as they contain hundreds or thousands of bioactive metabolites. The synergistic interactions between individual compounds of its extracts play an important role in antimicrobial therapeutic efficacy. In this sense, the activity of a medicinal plant extract is the combined effect of the action of multiple compounds with synergistic, additive, or antagonistic activity, depending on the concentration and phytochemical constitution of the extracts (Vaou *et al.*, 2022). This mixture of compounds can affect the microbial cell in various ways. Wherein, the main site is the plasma membrane, affecting its structure and integrity, permeability, or functionality. Some studies even indicate that plant extracts may contain efflux pump inhibitors in their composition (Vaou *et al.*, 2021).

In our study, we identified a relationship between the antioxidant and antimicrobial activity of the methanolic extract, which was the most active in relation to ethanolic. This could be explained because of the higher concentration of the active metabolites in the methanolic extract which can be seen in total phenolic content of 57.66 milligrams of gallic acid per gram of dry sample, compared to 39.36 mg of the ethanolic extract in our study (Table No. 1). Therefore, it is important to identify the responsible metabolites in *P. aequale* since the highest activity occurred in a high polarity extract.

Extracts from *Piper* genus species have shown interesting effects on strains of Gram (+) and Gram (-) bacteria, which gives the opportunity to search for new antimicrobial compounds. However, many studies report the antimicrobial activity due to essential oils in which  $\alpha$ - and  $\beta$ -pinene are associated with their toxic effects on cell membranes. In general, many terpenoids can inhibit microbial vital processes such as oxygen consumption and oxidative



phosphorylation (Santos *et al.*, 2023). While in extracts, terpenoids, alkaloids and phenolic compounds have shown greater antimicrobial potential (Heliawati *et al.*, 2022).

Some authors have identified that in species of the genus *Piper*, certain monoterpenes such as limonene and *cis*- $\beta$ -ocimene have been the compounds that have had the greatest correlation in bacterial inhibition, mainly against species of the genus *Staphylococcus* (Perigo *et al.*, 2016). Also, there is a report of approximately 275 neolignans detected in species of this genus which attribute to them various pharmacological properties such as antimicrobial, anti-inflammatory, neuroprotective, antioxidant, antiplatelet aggregation, antiproliferative, cytotoxic, among others (Fan *et al.*, 2023).

Regarding the antiproliferative activity, the ethanolic extract exhibited an  $IC_{50}$  three times lower than that of the methanolic extract at 81.28  $\mu\text{g/mL}$ . Considering the criteria established by Atjanasuppat *et al.* (2009), the antiproliferative and cytotoxic activity of plant extracts can be categorized into four groups based on their  $IC_{50}$  value:  $\leq 20$   $\mu\text{g/mL}$  is active, 20-100  $\mu\text{g/mL}$  is moderately active, 100-1000  $\mu\text{g/mL}$  is weakly active, and  $>1000$   $\mu\text{g/mL}$  is inactive. According to this criterion, the ethanolic extract of *P. aequale* is moderately active against the PC-3 cell line. The  $IC_{50}$  value of the ethanolic extract is not very high, and considering that, based on the micrographs, damage begins to occur in the cultures from 100  $\mu\text{g/mL}$ , it is of interest to identify the bioactive components of this species.

Although scientific research on species of the *Piper* genus is abundant, few species have been evaluated and even few secondary metabolites have been identified with antiproliferative and/or cytotoxic effects against prostate cancer cells. Among them we find the work carried out by Ouyang *et al.* (2013), who evaluated the antiproliferative activity of piperine, an alkaloid isolated from *P. nigrum* and *P. logum* against prostate cancer cell lines LNCaP, PC-3 and DU-145. They found that the  $IC_{50}$  values of this compound were 74.4, 111, and 226.6  $\mu\text{M}$  in LNCaP, DU-145, and PC-3, respectively. This compound has shown better results in LNCaP which arrests the G0/G1 phase of the cell cycle and induces autophagy. On the other hand, Lee *et al.* (2013), found

piperinoline in *P. logum* which inhibits the proliferation of the PC-3 cell line with an  $IC_{50}$  of 40  $\mu\text{M}$  that affects the G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle by negative regulation of cyclins. This compound has been reported to have apoptotic properties through the production of Reactive Oxygen Species (ROS) as it has been found to cause alterations in mitochondrial function. Finally, Kim *et al.* (2018), found that piperlongumine, an alkaloid isolated from *P. logum*, presented a strong inhibitory effect on the DU-145, PC-3 LNCaP prostate cancer cell lines with  $GI_{50}$  values (concentration to inhibit proliferation by 50%) of 7.1,  $> 10$  and 9.2  $\mu\text{M}$ , respectively.

## CONCLUSION

*P. aequale* stands out for its greater frequency of use for the treatment of urinary and prostate conditions. The present work confirmed that this species can inhibit various strains of bacteria that cause urinary problems and prostate cancer cells that are associated to an advanced stage of this disease, which is related to its use in traditional Mexican medicine. We consider that this species may have a positive effect in the treatment of prostate cancer, however, it is important to be very careful when consuming it because it is unknown if it has any toxic effects or generates drug interactions that could lead to unwanted complications and side effects. Also, studies that evaluate the fractions of the ethanolic extract in prostate cancer cells are important, to know the polarity of the possible compounds and identify the bioactive metabolites, as well as evaluate them against normal cells. In general, this work supports the knowledge of traditional Mexican medicine and the *Piper* genus.

## CONFLICT OF INTEREST DECLARATION

The authors report having no conflict of interest.

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