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Original Article

Evaluation of the antioxidant, genotoxic and cytotoxic activity of organic fractions from leaves and roots of *Piper patulum* Bertol. from Guatemala

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Abstract

Piper patulum Bertol., is a native Mesoamerican species. Previous studies have demonstrated that its leaves have antioxidant activity and the essential oil has shown cytotoxic activity against brine shrimp (*Artemia salina*) at a concentration of 0.5 mg/mL. The aim of this research was to demonstrate the antioxidant, cytotoxic and genotoxic activity of fractions of the vegetal material.

Fractions were obtained by separate sequential extraction of leaves and roots. Phytochemicals were evaluated to determine the qualitative composition, suggesting a possible pattern to continue the study of cytotoxic activity. Qualitative antioxidant activity was evaluated by TLC based on 2,2-diphenyl-1-picrylhydrazyl (DPPH) test, and quantitative DPPH and total phenolics by spectrophotometry. Cytotoxic activity against *A. salina* was evaluated macrometrically, and towards cancer cell lines [U251 (glioma), MCF-7 (mammary) and NCI-H460 (lung)] by micrometric sulforhodamine B assay, estimating the Total Growth Inhibition (TGI). Genotoxicity was tested by the *Allium cepa* assay.

The dichloromethane fraction from the roots showed the best antioxidant activity (IC_{50} of 0.61 ± 0.04 mg/mL) and the greatest concentration of total phenolics (848.42 ± 9.89 µg of gallic acid equivalents/mL). Cytotoxic activity was determined against *A. salina* and neoplastic cell lines. For *A. salina*, the highest activity was observed in the methanol (DL_{50} 0.26 ± 0.02 mg/mL) and dichloromethane (DL_{50} 0.29 ± 0.02 mg/mL) organic fractions from the roots. In cytotoxic assays against cancer cell lines, the best TGI were obtained in the dichloromethane (U251: 68.9 µg/mL, MCF-7: 23.5 µg/mL, NCI-H460: 74.4 µg/mL) and ethyl acetate leaf fractions (U251: 53.8 µg/mL, MCF-7: 42.0 µg/mL, NCI-H460: 71.6 µg/mL), and the dichloromethane root fraction (U251: 94.2 µg/mL, MCF-7: 31.0µg/mL, NCI-H460: 88.6 µg/mL). By means of the *A. cepa* assay, no genotoxic activity was demonstrated.

The dichloromethane fraction of the leaf from *P. patulum* shows interesting cytotoxic activity, which deserves further investigation of a responsible molecule by bioguided fractionation.

Keywords: Antioxidant, genotoxic, *Artemia salina*, *Allium cepa* assay

Introduction

Cancer is an uncontrolled process of growing and dissemination of malignant cells. It can appear in almost any part of the body. The growths often invade surrounding tissues and can metastasize to distant sites.[1] It is one of the leading causes of death worldwide. Approximately 12.7 million new cases of cancer are diagnosed worldwide each year; without substantial improvement in cancer control, it is expected that this figure will rise globally to 21.3 million new cancer cases and deaths up to 13.1 million for 2030. For Latin America and the Caribbean, it is estimated that 1.7 million cases of cancer will be diagnosed in 2030 and more than 1 million cancer deaths may occur annually.[2]

The involvement of free radicals in the different stages of carcinogenesis is well documented. Some of the reactive oxygen and nitrogen species and signaling could facilitate cancer development by damage to the deoxyribonucleic acid (DNA) and other biomolecules.

Chemotherapy is considered the most important treatment for cancer, however, this treatment has many side effects. Therefore it is important to continue researching natural medicine as a treatment for some cancers. Phytochemicals with antioxidant activity have demonstrated the ability to inhibit carcinogenesis. That is why assays to deter-

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mine antioxidant activity are important in evaluating antineoplastic potential.

Considering that cancer is a disease with the highest rate of mortality worldwide, it is relevant to search for new extracts or molecules with cytotoxic activity against neoplastic cells.

Species of *Piper* genus are basal angiosperms used by humans since ancient times, and have demonstrated promissory activity in different pharmacological models.[3] *Piper patulum* Bertol., is a Mesoamerican native species. In Guatemala there are about 88 species of *Piper*, 11 species distributed in seven to nine areas, 24 with average distribution and 53 with limited distribution.[4] Previous studies have demonstrated that its leaves have antioxidant activity.[5] The essential oil contains as major components 1,3,5-trimethoxy-2-propenylbenzene (33.7%), methyl eugenol (24.7%) and -caryophyllene (16.2%),[6] has shown cytotoxic activity against brine shrimp (*Artemia salina*) at a concentration of 0.5 mg/mL,[7] and contain distinctive features to characterize micro and macroscopically.[8] This research seeks to find an organic fraction of *P. patulum* with cytotoxic activity, for the future development of a molecule as a potential accessible drug with fewer side effects. This research is relevant for Guatemalan phytochemical studies because *P. patulum* is within the native flora of this country and its distribution is limited only to the national territory.[4,8]

Materials and methods

Collection of plants

The collect was performed in the department of Suchitepequez, Ecoparcela El Kakawatal, Samayac, located 14°33'5.83" North latitude and 91°27'58.47" West longitude at an altitude of 480 m; voucher sample was deposited in CFEH Herbarium from Farmaya Natural Products Laboratories under number 1075. The material was collected in the early hours of the morning and transported to Farmaya Laboratories (Guatemala City) for its identification and shade drying, at a constant temperature (40°C) for 5-7 days.

Preparation of plant extracts

Extraction of essential oils by Neoclevenger

The plant material was grinded and 30-50 g were placed in a distillation flask. Then, 400-500 mL of water was added to cover the plant material and submitted to hydrodistillation in a Clevenger-type apparatus for 3h in triplicate, followed by evaporation in a desiccator with anhydrous silica, mea-sured, transferred to glass vials and kept at a temperature of -18 °C for further analysis.

Fractionated extraction

The following solvents were used in the described order: hexane, dichloromethane, ethyl acetate, methanol and butanol. The plant material was grinded and placed into a container where it was humidified with the first solvent. The wet plant material was placed in a stainless steel percolator and covered with the amount of solvent required to achieve the workload; the material was allowed to stand for 24 h. The liquid phase was recovered and concentrated by rotary evaporation (Buchi). The same procedure was performed with each of the solvents.[9]

Phytochemical screening methods

Phytochemical screening was done by thin layer chromatography (TLC). The solution and the standards to be analyzed (5 µL) was placed at a specific position in a silica gel 60F_{254} chromatographic plate, then the plate was placed vertically in a saturated chromatographic chamber with the specific mobile phase for six metabolites (alkaloids, flavonoids, coumarins, anthraquinones, saponins and sesquiterpenolactones) an developed with specific reagents.[10]

Evaluation of the antioxidant activity of plant extracts

Qualitative antioxidant activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH) was evaluated by TLC; 10 µL of sample and 5 µL of the antioxidant standards (gallic acid and ascorbic acid, 1 mg⁄mL) were applied to a silica gel 60F254 chromatographic plate. The plate was placed in a glass chamber saturated with ethyl acetate:acetic acid:formic acid:water (100:11:11:26), after running and drying it was sprayed with DPPH (1 mg/mL in methanol). The extracts with antioxidant activity showed DPPH discoloration.[11]

Quantitative antioxidant activity by DPPH was evaluated macrometrically. Series of wells was prepared with sample, methanol and a solution of DPPH 500 µM. 0.1 g of dry extract was diluted in 5 mL of methanol. Before making the reading curve, a direct reading of the extract was performed as follows: well 1, blank 50 µL of methanol; well 2, control 150 µL of DPPH solution; well 3, test 1, 50 µL of sample; well 4, test 1, 50 µL of sample and 150 µL of DPPH solution; well 5, test 2, 15 µL of sample and 35 µL of methanol; and well 6, test 2, 15 µL of sample, 35 µL of methanol and 150 mL of DPPH.[11]

Blank was performed to each well, which included the same amount of sample and methanol. The microplate was stirred in a vortex for plates for 30 sec, and incubated at 20-25 °C for 30 min protected from light. It was read in a plate spectrophotometer (BioTek ELX800) at 490 nm. The percentage of inhibition in each well was calculated using the following formula.[12]

$$100 \text{ X } \frac{\text{(Absorbance of the control - Absorbance of the test)}}{\text{(Absorbance of the control)}}$$

Total phenolics were evaluated by the method of Folin-Ciocalteu in a plate spectrophotometer (BioTek) at 630 nm using a standard curve to express the concentration as equivalent of gallic acid/g dry weight.[13]

Biologic (cytotoxicity) assays

A. salina test

The positive results of this bioassay indicate that the fraction has a cytotoxic activity and might correlate with cytotoxicity to normal or malignant cell lines. In addition, being a simple and reproducible technique, it could serve to continue the bioassay-guided fractionation and isolation in pursuit of structural elution of the responsible molecules.[14,15]

40 mg of the test fraction was weighed and dissolved in 2000 µL of seawater, and homogenized to a uniform solution. Then, 100 µL of the dissolved fraction and 100 µL of seawater containing 10 to 15 nauplii (in triplicate) were added to a sterile 96-well microplate (flat bottom). As a nega-

tive control, 100 µL of fresh seawater and 100 µL of seawater containing 10 to 15 nauplii (in triplicate) was used, and as a positive control 100 µL of a solution of furosemide (2 mg/mL) dissolved in seawater and 100 µL of fresh seawater. Microplates were incubated at room temperature for 24 h with artificial light and then the number of dead nauplii per well was counted with the aid of a stereoscope.[16] All fractions were tested at a concentration of 2 mg/L. If the result of dead nauplii was greater than 50% it is considered that the LD_{50} is > 2 mg/mL.

Cytotoxicity assays in cell lines (performed in Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas (CPQBA) from the University of Campinas, Brazil).

Assays were performed in three cell lines, MCF7 (breast), NCI-H460 (lung) and U251 (glioblastoma), provided by the National Cancer Institute (NCI) of United States. The procedure was to spread the stock cultures for growth in 5 mL of medium RPMI 1650 (GIBCO BRL) supplemented with fetal bovine serum 5% and gentamicin (50 µg/mL). The cells in 96 well plates were exposed to the samples in DMSO / RPMI (0.25, 2.5, 25, and 250 µg/mL) at 37°C, 5% of CO2 in air for 48 h. The final concentration of DMSO does not affect cell viability. Subsequently, the cells were fixed with 50% trichloroacetic acid and cell proliferation was determined estimating the cell protein content spectrophotometrically (540 nm) in a microplate reader by the sulforhodamine assay. Doxorubicin hydrochloride (0.1 mg/mg; Europharma) was used as a positive control. The concentration response curve for each cell line was used to determine the total growth inhibition (TGI) by nonlinear regression analysis using the program ORIGIN 7.5.

A. cepa test

Allium cepa is a good experimental model to evaluate *in vivo* toxicity and genotoxicity of substances and complex mixtures.[17] Fractions for evaluation were prepared at a concentration of 1 mg/mL in distilled water; tap water was used as a negative control and Paraquat® (Germany) as positive control dilutions in distilled water (40, 4, 0.4, 0.04, 0.004 and 0.0004 mg/ml). After the solutions were prepared, the tubes were filled with each of the extracts and controls (five replicates per sample), and clean bulbs were placed over the mouth of the tubes ensuring that the root zone was immersed in the solution to be evaluated. Tubes with bulbs were stored in a cool, dry place, free from vibration and direct lighting for 48 h. The volume of each solution was recovered at least twice a day during the trial period and ensured that the roots were always immersed in the solution to be evaluated.[18] Mitotic index and the percentage inhibition was calculated by analyzing the data using ANOVA and Dunnett's test, where the only difference was shown by the positive control.

Statistical analysis

In the determination of antioxidant activity by DPPH and total phenols, measures of central tendency, mean, measures of dispersion and standard deviation were calculated. Inhibition concentration of 50% (IC_{50}) was calculated by linear regression of the DPPH antioxidant activity.

In total phenol antioxidant capacity equivalent of Trolox was compared in each extract for descriptive analysis. For cytotoxic activity with *A. salina* the mean lethal dose (LD_{50}) was calculated by nonlinear regression with Probit transformation, with 95% confidence interval using the statistical program Statgraphics.

In evaluating the genotoxic activity, an analysis of variance (ANOVA) was performed. If it was found with significant difference, extracts and negative control were compared to positive control by Dunnett's test.

Results and discussion

Plant extracts, essential oil and fractional extraction

Fractions were obtained by the sequential fractional extraction method using hexane, dichloromethane, ethyl acetate, methanol and butanol as solvents. The highest yield was obtained in the methanol fraction from leaves (9.29%), followed by dichloromethane fractions from leaves (2.42%). In the case of the methanol fraction, a medium yield was obtained (1.45%). The butanol fraction of both organs produced the lowest yield (0.10%-0.12%). The essential oil ex-tracted from leaves by neoclevenger yielded 0.59 ±0.08% (Table 1).

Table 1: Yields of extracts from *P. patulum* roots and leaves

	Root (%)	Leaves (%)
Hexane	0.77	1.62
Dichloromethane	0.93	2.42
Ethyl acetate	0.18	0.68
Methanol	1.45	9.29
Butanol	0.10	0.12
Essential oil	-	0.59

Extracts were obtained by fraction extract and the essential oil by neoclevenger method. The best yield was ob-tained with methanol leaves.

Phytochemical screening

Piper patulum contains all chemical groups evaluated within its chemical structure (alkaloids, flavonoids, coumarins, anthraquinones, saponins and sesquiterpenolactones); such results have been reported previously.[19] It was observed that not all extracts contain all the groups evaluated: alkaloids and saponins were absent from roots; saponins were found only in the dichloromethane fraction.

The presence of volatile oils in leaves was evaluated by TLC in triplicates, where that of references is reportedly higher.[6,7] It was evident that the leaves of *P. patulum* contains eugenol, α-pinene, linalool, geraniol and 1-8 cineol. The evidence obtained is only qualitative; no quantitative analysis was performed.

Evaluation of the antioxidant activity of plant extracts

The results of the qualitative and quantitative antioxidant activity of the fractions are shown in Table 2. The qualitative assessment was conducted by TLC with DPPH. Five standards were used in the evaluation and the best antioxidant activity was shown by vitamin C. The methanol and butanol fractions of both plant organs also had intense discoloration. Micrometric method of DPPH was used for quantitative assessment, and the antioxidant capacity of four standards was evaluated. The best antioxidant capacity was displayed by

the root dichloromethane fraction (IC_{50} of 0.61±0.04 mg/mL), which is better than the IC_{50} of quercetin and TBHQ, but not better than rutin (IC_{50} of 0.03±0.01 mg/mL). The fraction with less antioxidant activity was that of ethyl ace-tate leaves with an IC_{50} of 3.70±0.01 mg/mL.

Table 2: DPPH scavenging activity of organic fractions from roots and leaves from *P. patulum*

Fraction/organ	Qualitative	Quantitative (IC_{50} mg/ml)
Hexane / leaf	+	1.19 ± 0.01
Dichlorometane / leaf	+	1.37 ± 0.01
Ethyl acetate / leaf	+	3.70 ± 0.01
Methanol / leaf	++++	1.50 ± 0.13
Butanol / leaf	+++++	-
Hexane / root	++	1.47 ± 0.02
Dichloromethane / root	++	0.61 ± 0.04
Ethyl acetate / root	++	1.69 ± 0.01
Methanol / root	+++++	1.99 ± 0.02
Butanol / root	++++	-
Standards		
Quercitin	+	0.64 ± 0.01
Rutin	++	0.03 ± 0.01
TBHQ	++	1.16 ± 0.01
Trolox	++	-
Vitamin C	++++	0.14 ± 0.01

* Decoloration = +: poor ++: weak +++: moderate ++++: abundant +++++

Inhibition concentration of 50% (IC_{50}) was calculated by linear regression of the DPPH antioxidant activity.
The fraction with the best quantitative activity was dichloromethane roots which had better IC_{50} compared to the quercetin and TBHD standards.

The Folin-Ciocalteu was used for the determination of total phenols (Table 3). The extract that showed the highest content of phenols expressed as µg equivalent of gallic acid/mL was the dichloromethane root with 840.42 ± 9.89 µg/mL, and the fraction with less content of phenols was the methanol leaves with 0.62. ± 01 µg equivalents of gallic acid/mL.

Table 3: Phenol content (Folin-Ciocalteu) of organic fractions from roots and leaves from *P. patulum*

Fraction/organ	µg equivalents of gallic acid/ml
Hexane / leaf	77.01 ± 0.98
Dichlorometane / leaf	61.80 ± 0.06
Ethyl acetate / leaf	21.32 ± 0.12
Methanol / leaf	0.62 ± 0.02
Dichloromethane / root	848.42 ± 9.89
Ethyl acetate / root	21.65 ± 0.13
Methanol / root	0.72 ± 0.01

*The antioxidant capacity equivalent of Trolox was compared to each extract for descriptive analysis.
The fraction that showed the best phenol content was the dichloromethane root.

The evaluation by DPPH and total phenol showed that the root dichloromethane fraction is the best candidate as an antioxidant; it demonstrated the best activity by DPPH test and the largest amount of total phenol.

Biological tests

The ethyl acetate of leaves fraction was the only one with a concentration >2 mg/mL in the *A. salina* test. Other fractions were evaluated at concentrations of 1.00, 0.50 and 0.25 mg/mL. The results obtained after the analysis by the Statgraphics program are shown in Table 4. The fraction with the lowest LD_{50} was methanol root with 0.26 ± 0.02 mg/mL followed by dichloromethane root with an LD_{50} of 0.29 ± 0.02 mg/mL.

Table 4: Cytotoxic activity of organic fractions from roots and leaves from *P. patulum* by *Artemia salina*

Fraction/ organ	DL_{50} mg/ml	LC lower 95.0%	LC upper 95.0%
Hexane / leaf	0.79 ± 0.01	0.74	0.84
Dichloromethane / leaf	0.72 ± 0.02	0.67	0.76
Ethyl acetate / leaf	> 2	-	-
Methanol / leaf	1.46 ± 0.01	1.38	1.55
Butanol / leaf	1.24 ± 0.01	1.17	1.32
Hexane / root	0.49 ± 0.02	0.46	0.52
Dichloromethane / root	0.29 ± 0.02	0.28	0.31
Ethyl acetate / root	0.68 ± 0.01	0.64	0.73
Methanol / root	0.26 ± 0.02	0.25	0.27
Butanol / root	0.93 ± 0.03	0.86	0.99
Oil / leaf	0.32 ± 0.02	0.29	0.35

The mean lethal dose (LD_{50}) was calculated by nonlinear regression with Probit transformation, with an interval of confidence of 95% using the statistical program Statgraphics.
The analysis resulted in a $p < 0.05$ for all fractions. This means that the model significantly reduced the devi-ation, which predicts good probability of the results.

A good TGI for the three cell lines was obtained in the dichloromethane and ethyl acetate leaf fractions, and dichloromethane root, the best fractions were hexane leaves, and ethyl acetate root against the cell line MCF-7.

There is evidence that the *Piper* genus has cytotoxic activity against the cell line MCF-7. A study in 2012 showed that *P. imperiale* is active against this cell line,[20] so the results are validated and suggests further research on MCF-7 cell line.

Genotoxic activity was evaluated by the test of *A. cepa*. In performing the ANOVA, it was determined that the means are significantly different since the F value is greater than the critical value. Dunnett's test was performed where fractions showed no significant difference from the negative control. The fractions had a lowering effect reducing the mitotic index (MI), where Paraquat® had a reducing effect on the MI (64.71%). The analysis of variance and Dunnett's test indicated that only Paraquat® presented genotoxic effect and that fractions studied had no significant genotoxic effect.

Conclusions

The phytochemical characterization showed that the leaves and root of *P. patulum* contains some molecules of

interest in the study. In determining the overall cytotoxic activity for *A. salina*, the best activity was presented by the dichloromethane root extract (LD_{50} 0.26 ± 0.02 mg/mL), followed by methanol root (LD_{50} of 0.26 ± 0.02 mg mL).

The dichloromethane root extract had the best antioxidant activity by the DPPH (IC_{50} 0.61 ± 0.04 mg/mL) and total phenolic techniques (848.42 ± 9.89 µg equivalent gallic acid/mL).

None of the extracts showed genotoxic activity evaluated by *A. cepa* test.

Based on the obtained results, it is suggested that the best candidate for further studies of isolation and purification of novel bioactive molecules from the Guatemalan *P. patulum*, is the root dichloromethane fraction, because of its good antioxidant and cytotoxic activity (*A. salina*) and did not present genotoxic effects. The best TGI was evidenced in the leaf hexane fraction (TGI of 17.0 µg/mL) followed by dichloromethane root (TGI 31.0 µg/ mL), so for inhibition of MCF-7 it would be interesting to study these two fractions.

Table 5: Genotoxic activity in *A. cepa* apexes of organic fractions from roots and leaves from *P. patulum*

Fraction/ organ	Mitotic index	% inhibition
Hexane / leaf	62.5	26.47
Dichloromethane / leaf	80	5.88
Ethyl acetate / leaf	72	15.29
Methanol / leaf	82	3.53
Hexane / root	70	17.65
Dichloromethane / root	80	5.88
Ethyl acetate / root	65	25.53
Methanol / root	73	14.12
Paraquat®	30	64.70
Negative Control	85	0

ANOVA = $p < 0.05$. If it was found with significant difference, extracts and negative control were compared to positive control by Dunnett's test. Only the positive control showed genotoxicity.

Table 6: Total Growth Inhibition (TGI) of extracts in cell lines U251, MCF-7 y NCI-H460 of organic extracts from roots and leaves from *P. patulum.*

	TGI (µg/mL)		
Fraction/Organ	U251	MCF-7	NCI-H460
Hexane leaf	178.8	17.0	105.3
Dichloromethane / leaf	68.9	23.5	74.4
Ethyl acetate / leaf	53.8	42.0	71.6
Methanol / leaf	> 250	> 250	> 250
Butanol / leaf	190.3	114.9	> 250
Hexane / root	110.1	43.2	159.5
Dichloromethane / root	94.2	31.0	88.6
Ethyl acetate / root	121.9	39.7	96.6
Methanol / root	> 250	114.0	> 250
Butanol / root	242.0	87.5	> 250
Doxorubicina	> 25	02.4	5.8

Results from Unicamp from fractions sent from Guatemala.

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