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

Vivian F. Zelada¹, João Ernesto de Carvalho², Armando Cáceres³

¹ Universidad Galileo, Guatemala C.A. Email: viv.fzg@gmail.com
² Universidad de Campinas, Campinas, Brasil. Email: carvalho@fcf.unicamp.br
³ Universidad de San Carlos de Guatemala, Guatemala C.A. Email: acaceres@farmaya.net

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 micro-metric 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 (IC₅₀ 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 (DL₅₀ 0.26 ± 0.02 mg/mL) and dichloromethane (DL₅₀ 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: 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: 31.0 µg/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.

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-
mine antioxidant activity are important in evaluating anti-
neoplastic potential.

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

Species of Piper genus are basal angiosperms used by hu-
mans since ancient times, and have demonstrated promisso-
ry activity in different pharmacological models.\(^4\) Piper patu-
lum 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.\(^5\) Previous studies have demon-
strated that its leaves have antioxidant activity.\(^5\) The essential
oil contains as major components 1,3,5-trimethoxy-2-pro-
pylene (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 frac-
tion of \textit{P. patulum} with cytotoxic activity, for the future de-
velopment of a molecule as a potential accessible drug with
fewer side effects. This research is relevant for Guatemalan
phytochemical studies because \textit{P. patulum} is within the na-
tive 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 Suchite-
pequez, 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 Labora-
tories under number 1075. The material was collected in the
earby hours of the morning and transported to Farmaya Lab-
oratories (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 Neoclevergan
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 Clevergan-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 or-
der: 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 chro-
matography (TLC). The solution and the standards to be
analyzed (5 µL) was placed at a specific position in a silica
gel 60F\textsubscript{254} chromaticographic plate, then the plate was placed
vertically in a saturated chromatographic chamber with the
specific mobile phase for six metabolites (alkaloids, flavo-
noids, coumarins, anthraquinones, saponins and sesquiter-
enolactones) an developed with specific reagents.\(^10\)

Evaluation of the antioxidant activity of plant extracts
Qualitative antioxidant activity by 2,2-diphenyl-1-picryl-
hydrazyl (DPPH) was evaluated by TLC; 10 µL of sample
and 5 µL of the antioxidant standards (gallic acid and ascor-
bic acid, 1 mg/mL) were applied to a silica gel 60F254 chro-
maticographic 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 antioxi-
dant 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 ex-
tract 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 methyl alcohol; and well
6, test 2, 15 µL of sample, 35 µL of methanol and 150 µL 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.\(^12\)

\[
\text{Inhibition percentage} = \left(1 - \frac{A_{\text{test}}}{A_{\text{control}}} \right) \times 100
\]

Total phenolics were evaluated by the method of Folin-Ci-
ocalteu in a plate spectrophotometer (BioTek) at 630 nm us-
ing a standard curve to express the concentration as equiva-
 lent of gallic acid/g dry weight.\(^13\)

Biologic (cytotoxicity) assays
\textit{A. salina} test
The positive results of this bioassay indicate that the frac-
tion has a cytotoxic activity and might correlate with cyto-
toxicity to normal or malignant cell lines. In addition, be-
ing 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 solu-
tion. Then, 100 µL of the dissolved fraction and 100 µL of
seawater containing 10 to 15 nauplii (in triplicate) were add-
ed to a sterile 96-well microplate (flat bottom). As a nega-
ative 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. 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 LD₅₀ 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 gentamycin (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. 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. 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% (IC₅₀) 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 (LD₅₀) 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 Dunnet’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>
<thead>
<tr>
<th>Root (%)</th>
<th>Leaves (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane</td>
<td>0.77</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>0.93</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>0.18</td>
</tr>
<tr>
<td>Methanol</td>
<td>1.45</td>
</tr>
<tr>
<td>Butanol</td>
<td>0.10</td>
</tr>
<tr>
<td>Essential oil</td>
<td>-</td>
</tr>
</tbody>
</table>

Extracts were obtained by fraction extract and the essential oil by neoclevenger method. The best yield was obtained 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. 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. It was evident that the leaves of *P. patulum* contains eugenol, α-pine, finalool, 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

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

<table>
<thead>
<tr>
<th>Extract</th>
<th>Root (%)</th>
<th>Leaves (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane</td>
<td>0.77</td>
<td>1.62</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>0.93</td>
<td>2.42</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>0.18</td>
<td>0.68</td>
</tr>
<tr>
<td>Methanol</td>
<td>1.45</td>
<td>9.29</td>
</tr>
<tr>
<td>Butanol</td>
<td>0.10</td>
<td>0.12</td>
</tr>
<tr>
<td>Essential oil</td>
<td>0.59</td>
<td>-</td>
</tr>
</tbody>
</table>

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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 acetate leaves with an IC_{50} of 3.70±0.01 mg/mL.

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.

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 deviation, 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, 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. Dunnnett’s test was performed where fractions showed no significant difference from the negative control. The fractions had a lowering effect reducing the mitotic index against the cell line MCF-7.

### 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<sub>50</sub> 0.26 ± 0.02 mg/mL), followed by methanol root (LD<sub>50</sub> of 0.26 ± 0.02 mg/mL).

The dichloromethane root extract had the best antioxidant activity by the DPPH (IC<sub>50</sub> 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* apices of organic fractions from roots and leaves from *P. patulum***

<table>
<thead>
<tr>
<th>Fraction/organ</th>
<th>Mitotic index</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane/leaf</td>
<td>62.5</td>
<td>26.47</td>
</tr>
<tr>
<td>Dichloromethane/leaf</td>
<td>80</td>
<td>5.88</td>
</tr>
<tr>
<td>Ethyl acetate/leaf</td>
<td>72</td>
<td>15.29</td>
</tr>
<tr>
<td>Methanol/leaf</td>
<td>82</td>
<td>3.53</td>
</tr>
<tr>
<td>Hexane/root</td>
<td>70</td>
<td>17.65</td>
</tr>
<tr>
<td>Dichloromethane/root</td>
<td>80</td>
<td>5.88</td>
</tr>
<tr>
<td>Ethyl acetate/root</td>
<td>65</td>
<td>25.53</td>
</tr>
<tr>
<td>Methanol/root</td>
<td>73</td>
<td>14.12</td>
</tr>
<tr>
<td>Paraquat&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30</td>
<td>64.70</td>
</tr>
<tr>
<td>Negative Control</td>
<td>85</td>
<td>0</td>
</tr>
</tbody>
</table>

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 and NCI-H460 of organic extracts from roots and leaves from *P. patulum***

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

Results from Unicamp from fractions sent from Guatemala.

### Acknowledgments

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### References


