EVALUATION OF THE ANTIFUNGAL AND PISCICIDAL ACTIVITIES OF COMPOUNDS ISOLATED FROM *PIPER OVATUM VAHL*.

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**Abstract:** The species *P. ovatum* Vahl is a plant piperaceae family, known as João-Burandi, which is used in folk medicine in various regions of Brazil. The species has two active molecular compounds, piperovatine and piperlonguminine. The objective of the research was to investigate the antifungal activity against *Trichophyton rubrum* (TM) *Trichophyton mentagrophytes* and (MC) *Microsporum canis*, antipiscidal activity and cytotoxicity in macrophages and Vero cells. The spores were observed during inhibition tests that piperovatine associated with fluconazole was more efficient against TR (MIC 1.2 µg/mL), with a concentration of piperovatine only (5.2 µg/mL). In the fungal inhibition test MIC was 16.1 µg/mL to 2.1 µg/mL piperovatine and association piperovatine + fluconazole respectively. The cytotoxicity test against piperovatine Vero cells showed 52.5 µg/mL, already a mixture with piperovatine + fluconazole was less cytotoxic 823 µg/mL, both formulations were not cytotoxic for macrophages. In antipiscidical (*Biomphalaria glabrata*) tests, piperovatine inhibited growth at about 0.62 ppm, and approximately 9 times more toxic than the snail piperlonguminine. So this confirms piperovatine has high biological activity against fungi and snails, it is expected that in the future this will become a drug molecule or is a precursor to development of new drugs.

**Keywords:** *Piper ovatum*, Piperovatine and Piperlonguminine
1.0 Introduction

Brazil has an enormous potential to be exploited in the field of herbal medicines due to its great biodiversity. The use of medicinal plants in the treatment and cure of diseases is as old as the human species. Early civilizations noted that vegetables existed that when tested for disease, revealed their curative potential, the medicinal plants in Brazil have been shown to be an effective and low cost alternative for the control of several diseases, mainly for the developing countries. Despite advances in the synthetic drug industry, herbal medicines have not lost their place in therapeutics. The species *P. ovatum* Vahl, distributed throughout Atlantic forests, and growing in the understory layer of the dense forest, commonly known as João-Burandi is used in folk medicine for the treatment of toothache, bursitis and as an anti-infective remedy. There has been about 700 species of the genus Piper cataloged in the tropical and subtropical regions, some of the species are commonly used as medicine [1]. Several studies have reported the biological activities isolated in the compounds of the genus Piper [2], the presence of amides in the species *Piper hispidum* and *P. tuberculatum*, to which these showed antifungal activity against *Cladosporium sphaerospermum*. Conducted research for [3], demonstrated that in the *P. alatabaccum*, there are piplartin, *N*-3′,4′,5′-trimetóxidihidrocinamol-Δ^3^-piridin-1, piperovatine, 5,5′,7-trimetóxi-3′,4′metilenodioxiflavone substances [4], isolated five amides isobutilinic of plant species *Ottonia martiniana* the piperlongumine, piperovatine, isopiperlonguminine, corcovadine and isocorcovadine. Leaves of *P. arboretum* presented two active amides against *C. sphaerospermum* e *C. cladosporioides*, *N*-[10-(13,14-metilenodioxifenil)-7-(E)-pentenoil]-pirrolidin e *N*-[10-(13,14-metilenodioxifenil)-7-(E), 9 (E)-pentadienoil]-pirrolidin, presented antifungal activity of 0,1 µg/ml, whereas other amides, which were obtained from the seeds of *P. tuberculatum*, showed moderate antifungal activity in the concentration between 5 e 10 µg/ml [5]. The plant species *Piper ovatum* (Figure 1) is a bush of approximately two meters in height. The leaves are oval-elliptic, measuring from 11-14 cm long by 3-7 cm wide, grayish-green in color with smooth texture. The flowers are dark green in color and the leaves are located opposite. The root system is very branched with no distinction of a major root. The roots have secondary growth, the epidermis is unstratified with anticline divisions and presents suberified cells. In the outermost persistent cortex was observed a continuous ring of sclerenchyma consisting of 2 to
3 strata of cells. Below the layers of cortex cells are crystalline idioblasts containing prismatic calcium oxalate crystals and parenchyma cells containing starch grains and endoderm with *Casparystriae* (SILVA, 2015). The piperovatine and piperlonguminine amides isolated from the leaves of this species *P. ovatum* by Silva et al., (2008), showed important activity against promastigotes and axenic amastigotes of *Leishmania amazonensis*. The essential oil of *P. ovatum* leaves also showed antifungal activity for *C. tropicalis*. In addition, the amides presented low toxicity to Vero cells and macrophages [2]. Studies carried out with several Piper showed the antileishmanial and antibacterial activities of essential oils in different species [9-10-11]. The *P. alamago*, presented activity against *Leishmania amazonenses* while other studies indicate that isolated constituents of *P. nigrum* produced development of antimicrobial biocomposite films to preserve the quality of bread [12].

![Image of Piper ovatum](image)

URE 1 *Piper ovatum* in detail: inflorescence, leaves and stems.

2.0 Results and Discussion
The evaluation of the antifungal activity according to Table 1, revealed that piperovatine is active against TR with the minimum inhibitory concentration (MIC) of 5.2 µg/mL in spore germination tests, whereas the MIC fungal growth was 16.1 µg/mL. In piperovatine combination with fluconazole, it was possible to show a probable synergism with the combination, considerably reducing the MIC for 1.2 µg/mL at TR for spore germination and 2.1 µg/mL of the fungus growth, while fluconazole used alone produced an MIC of 1.6 µg/mL and 3.2 µg/mL in inhibiting growth of fungus and spores, respectively. In piperovatine with fluconazole, it was possible to ensure the presence of TM in inhibiting growth and MC. The piperlonguminine with fluconazole, showed better activity compared to only fluconazole + piperovatine MC 4.8 µg/mL for spore germination and 4.6 µg/mL in the fungal growth. The fungi TR, TM, and MC showed high sensitivity to ketoconazole.

Table 1: Antifungal activity of compounds isolated from leaves of *P. ovatum* Vahl against (TR) *T. rubrum* (TM) and *T. mentagrophytes* (CM) *M. canis*.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Minimal concentration required for inhibition of spore germination (/ml)</th>
<th>Minimal inhibitory concentration for fungal growth (/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TR</td>
<td>TM</td>
<td>MC</td>
</tr>
<tr>
<td>Piperovatine</td>
<td>5.2</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>Piperlonguminine</td>
<td>10.7</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>M</td>
<td>5.3</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>Pp + F</td>
<td>1.2</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>Pg + F</td>
<td>8.1</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>1.6</td>
<td>4.6</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>0.2</td>
<td>3.8</td>
</tr>
</tbody>
</table>

M: Piperovatine + Piperlonguminine, F + Pp: Fluconazole + Piperovatine (50/50 v / v), Pg + F, Fluconazole + Piperlonguminine (50/50 v / v).

The compounds isolated from *P. reginelli*, demonstrated antifungal activities of neolignans (eupomatenoid-3 and eupomatenoid-5) reducing synthesis of fungal ergosterol [13]. This experiment showed synergism of compounds isolated from *Piper*
In *gaudichaudianum* Kuntze, other researchers reported that bioactive compounds of this type can provide prototype molecules for the synthesis of more potent, selective analogs, less toxic and high activities [14].

The cytotoxicity test results as shown in Table 2, revealed that the isolated Piperovatine showed a larger CC\(_{50}\) in Vero cells than in Macrophages, being 52.5 µg/mL and > 1000 µg/mL respectively. However, when Piperovatine was combined with fluconazole (50/50 v/v), showed the CC\(_{50}\) in Vero cells, increased to 823 µg/mL, and macrophages remained greater than >1000 µg/mL, demonstrating that the combination reduced the cytotoxic effect. The results will assist the pharmaceutical industry’s goal to produce molecules with low cytotoxicity and high activities. Studies prove if analogues pipiplartine have cytotoxic action against glioblastoma [15].

Table 2. Effects of the Piperovatine, Piperlonguminine, M (Piperovatine + Piperlonguminine) Piperovatine + Fluconazole (50/50 v/v), Piperlonguminine + Fluconazole (50/50 v/v), fluconazole, ketoconazole, cytotoxicity to Vero cells (VERO) and Macrophages (MF).

<table>
<thead>
<tr>
<th></th>
<th>Cytotoxicity Concentration (CC(_{50})) - µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vero cells</td>
</tr>
<tr>
<td>Piperovatine</td>
<td>52.5</td>
</tr>
<tr>
<td>Piperlonguminine</td>
<td>426</td>
</tr>
<tr>
<td>M (Piperovatine + Piperlonguminine)</td>
<td>61.3</td>
</tr>
<tr>
<td>Fluconazole + Piperovatine (50/50 v/v)</td>
<td>823</td>
</tr>
<tr>
<td>Fluconazole + Piperlonguminine (50/50 v/v)</td>
<td>752</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>&gt; 1000</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>&gt; 1000</td>
</tr>
</tbody>
</table>

The evaluation of the molluscicidal activity (Table 3) shows that different concentrations of piperovatine showed efficacy in the control of mollusc *Biomphalaria glabrata* over 24 hour. The results also showed molluscicidal activity Piperlonguminine, however, Piperovatine was about 9-fold or 87.52 % more efficient in this activity.
Table 3. Molluscidal activity on *Biomphalaria glabrata* of Piperovatine and Piperlonguminine isolated from *Piper ovatum* Vahl. Positive control (niclosamide) in 24 hours of action

<table>
<thead>
<tr>
<th>Concentration (ppm)</th>
<th>10</th>
<th>5</th>
<th>2.50</th>
<th>1.25</th>
<th>0.62</th>
<th>0.31</th>
<th>0.16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Piperovatine</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Piperlonguminine</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>niclosamide</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

(+) Death of snail and (-) did not kill the snail.

Table 4 indicates that the piperovatine has lower levels of median lethal dose (LD) of 50% and 90% of the tested experimental model. This result suggests that, in model in vivo, piperovatine presents greater adverse effect when compared to the snail piperlonguminine. The schistosomiasis is a neglected disease and has victimized many people in Brazil. This is caused by worms of the *Schistosoma mansoni*, whose intermediate host is the snail the *B. glabrata*. Therefore, to control this species is an effective way to control the disease which has about 200 million people infected worldwide [16-17].

Table 4: LD$_{50}$ and LD$_{90}$ (ppm) values is piperovatine and piperlonguminine at the 24 hour exposure time.

<table>
<thead>
<tr>
<th>Substance</th>
<th>DL 50</th>
<th>DL 90</th>
</tr>
</thead>
<tbody>
<tr>
<td>Piperovatine</td>
<td>0.92</td>
<td>2.33</td>
</tr>
<tr>
<td>Piperlonguminine</td>
<td>&gt; 10</td>
<td>&gt; 10</td>
</tr>
</tbody>
</table>

This data suggests that the same molluscicide effect can be achieved using a lower dose of the active piperovatine and less cytotoxic effect (Table 2). The analysis of variance (ANOVA) were performed with the aid of software GraphPadPrism® with 95% confidence. Studies with mammea A/BB substance, show an effect against snails [18]. According to HPLC analysis, it was possible to detect the presence of amides piperovatine and piperlonguminine in the crude extract of leaves of *P. ovatum*. (Figure 2).
Figure 2: Chromatogram of the leaf extract of *P. ovatum* obtained under the following conditions: ODS column Metasil; mobile phase: mixture of acetonitrile / water starting with acetonitrile 0 to 60 gradient for 35 minutes; flow: 1.0 ml / min; detection: 280 nm.

This analysis was carried out to find what wavelength each substance absorbed better. The piperlonguminine has better absorption at 339 nm (Figure 3), while in piperovatine was observed with an optimum wavelength at 280 nm (Figure 4). Once defined optimal length of analysis has been determined for the piperovatine and piperlonguminine, permitting subsequent analysis in HPLC (Figure 2).
Figure 3: Scanning spectrum of piperlonguminine ultraviolet in wavelength 200 in 400 nm (sample 2)

Figure 4: Scanning spectrum of piperovatine ultraviolet in wavelength 200 in 400 nm (sample 1)

3.0 materials and methods
Molluscicidal assay

The molluscicidal assay was performed with piperovatine and piperlonguminine substances. They were used for each concentration of the three snails *Biomphalaria glabrata* species of uniform size. The samples were diluted in filtered aquarium water without chlorine, with the aid of 100 of DMSO in 10 concentrations; 5; 2.5; 1.25; 0.625; 0.312e 0.156 ppm at room temperature. Each snail was separately in contact with 50 ml of this solution. a blank test was used only with DMSO and a positive control and niclosamide. readings at 24 h after this time were conducted heartbeat were observed through a magnifying glass to check their mortality. To obtain LD50 and LD90 of the pure substance were used ten adult *Biomphalaria glabrata* snails of the species at each concentration of the sample, according to the recommendations of the World Health Organization [19] for the analysis of plant molluscicides. Data were analyzed by Probit Program Version 1.516.

Microorganism used and growth conditions

The test specie used for this investigation was *T. rubrun, T. mentagrophytes e M. canis*. The fungi was maintained on Sabouraud dextrose agar (SDA) slants at 28 °C and subcultured monthly throughout this study.

Microbroth dilution assay

Culture was grown on Sabouraud dextrose agar (SDA, Difco Laboratories, Detroit, MI, USA) tubes for 7–14 days, after which time spores were harvested from sporulating colonies and suspended in sterile ion solution. The concentration of spores was adjusted to 1.0 × 105 spores/mL using a hemocytometer. The antifungal assay was performed by the microdilution technique in sterile flat bottom microplates. Each well contained appropriate test samples, Sabouraud dextrose broth and approximately 2 × 103 –3 × 103 spores in a total volume of 100 µL. The plates were incubated at 28 °C for 72 h. Two susceptibility endpoints were recorded for each isolate. The MIC was defined as the lowest concentration of compounds at which the microorganism tested did not demonstrate visible growth. For this experiment the
piperovatine, piperlonguminine, fluconazole and ketoconazole at concentrations between 0.2 to 100 µg/mL as standard drugs were used. For comparative purposes, the plates were incubated at 28 ºC for 20–30 h and then examined for spore germination under an inverted microscope. For quantification, spores were considered germinated if they had a germ tube at least twice the length of the spore.

Cytotoxicity assay

A suspension of 5 × 104 J774G8 macrophage cells in RPMI1640 medium or Vero cells in DMEM supplemented with 10% FBS was added to each well in 96-well microplates. The plates were incubated in a 5% CO₂-air mixture at 37ºC to obtain confluent growth of the cells. After 24 h, the medium was removed and several concentrations of purified compound and fractions (0.1 to 1000 µg/ml) were added to each well containing the cells, and the plates were incubated for 48 h. The nonadherent cells were removed by washing with the medium, and the adhered cells were fixed with 50 µl/well of 10% trichloroacetic acid at 4ºC for 1 h; after that, the well plates were washed with water, and 50 µl/well of sulforhodamine B (0.4% w/v in 1% acetic acid solution was added); the microplate was then maintained at 4ºC for 30 min. Next, the sulforhodamine B was removed and the microplate was washed 5 times with 1% acetic acid, then 150 µl/well of 10 mM unbuffered Tris-base solution (Sigma) was added, and this was homogenized for 15 min. The absorbance of each individual well, minus the blank value, was calculated automatically. Each experiment was performed in triplicate on three different occasions, and the percentage of viable cells was calculated in relation to controls cultured in medium alone. The 50% Cytotoxicity Concentration (CC₅₀) was determined by logarithm regression analysis of the data.

Plant material

Leaves of *Piper ovatum* Vahl were collected in December 2006 in Monte Formoso (16º51’45.6”S 41º15’03.6”W), state of Minas Gerais, Brazil, and were identified by Dr. Elsie Franklin Guimarães. A voucher specimen was deposited in the herbarium of the Department of Botany, University of Maringá (HUM 10.621).
Plant extraction and purification

Leaves were dried at room temperature and powdered. The extract was prepared by exhaustive maceration in ethanol-water (9:1 v/v) at room temperature, filtration, concentration under C to obtain a hydroalcoholic extract, and then lyophilization, which yielded of vacuum at extract. The hydroalcoholic extract was chromatographed and purification on according [7-20].

HPLC analysis

The HPLC analyses were carried out using a GILSON apparatus equipped with a quaternary pump (Pump 321), automatic injector valve (234) with 20 µL loop, degasifier (865), CTO-10Avp oven and a UV/visible detector model 152, controlled by a BOWTER computer program. In the chromatographic analysis, we used a reverse-phase column Metasil ODS, 5 µm, 150.0 x 4.6 mm, kept in an oven set at ambient temperature. HPLC conditions were as follows: solvent A, acetonitrile, and solvent B, 1.0 % acetic acid. A gradient elution used was 0–35 min, 0-60% acetonitrile/water. Flow rate was 1.0 mL/min, and detection was at 280 nm. All the samples were prepared in triplicate. The reagents used to prepare the mobile phase were acetonitrile (HPLC grade from OmniSolv EM Science, Gibbstown, NJ), ultrapure water (Milli-Q system, Millipore, Bedford, USA), acetic acid (analytical grade, Merck, Darmstadt, Germany), and methanol (HPLC grade from OmniSolv EM Science, Gibbstown, NJ). The stock solutions of extracts of the leaves, stems and roots from *P. ovatum* were prepared in methanol at a concentration of 1000 µg/mL. The solutions were filtered through a 0.45 µm membrane filter (Millipore, São Paulo, Brazil) [21].

4.0 Conclusion

The piperovatine amides and extracted piperlongumimine *P. ovatum* showed excellent activity against filamentous fungi *T. rubrum*, *M. canis* and *T. mentagrophytes*. With a combination of piperovatine and fluconazole, the effect was enhanced and produced a reduction of cytotoxicity. The piperovatine presented piscicidal activity against the snail *B.*
*glabrata*, suggesting that this molecule may be an alternative to control the intermediate host of *S. mansoni*, which causes infection of thousands of victims every year in Brazil. Currently there is limited research regarding this disease. The results of this study indicate that piperovatine has high pharmacological potential and could be used in future development of a drug or precursor base for synthesis of new innovative drugs.

Conflicts of Interest: The authors declare no conflict of interest.

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