Research Article

Antiplasmodial and Cytotoxic Activity of Piper Piedecuestanum Trel. and Yunck

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Abstract

Background and Objective: Plasmodium resistance to antimalarial drugs has expanded and intensified, making new and effective antimalarial drugs urgently. The objective of this work was the in vitro evaluation of antiplasmodial activity of extracts of different polarity and compounds of the species P. piedecuestanum. Materials and Methods: The plant materials were obtained through successive extractions using solvents of different polarity such as hexane (H), dichloromethane (D), ethyl acetate (A) and methanol (M) and separations techniques for fractionation and isolation of compounds. The antiplasmodial activities of the extracts and compounds were evaluated by SYBR Green I™ method and evaluated the cytotoxicity in the cell lines U-937, HUVEC by the MTT method. Results: The antiplasmodial and cytotoxic activity of the extracts of dichloromethane (PPD) and ethyl acetate (PPEA) with antiplasmodial activity of IC₅₀ = 17.93 μg mL⁻¹; IS = 2.093 and IC₅₀ = 19.5 μg mL⁻¹; IS = 0.791, respectively are reported for the first time. In addition, from P. piedecuestanum species were isolation and characterization five metabolites 5,8-Hydroxy-7-methoxyflavone(1), 6,7-dimethoxy-5,8-dihydroxyflavone(2), 6,7-dimethoxy-5-hydroxyflavone (mosloflavone) (3), 5,6-dihydroxy-7-methoxyflavone (nigletein) (4), 5-hydroxy-7-methoxyflavone (S) and a brominated derivative from (5) named 6,8 bromo-5-hydroxy-7-methoxyflavone(7). Compound (1) presented promising antiplasmodial activity with an IC₅₀ = 7.325 μg mL⁻¹ (25.69 μM); IS₅₀ = 13.65. Conclusion: Chemical analysis of extracts and compounds from P. piedecuestanum spices will play a central role in the development and modernization of an antimalarial herbal traditional in Colombia.

Key words: Piper piedecuestanum, antiplasmodial activity, cytotoxic activity, Plasmodium falciparum


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Data Availability: All relevant data are within the paper and its supporting information files.
INTRODUCTION

Malaria is a disease caused by protozoan parasites belonging to the family Plasmodiidae, genus Plasmodium, which are transmitted by female mosquitoes of the genus Anopheles. Currently, malaria is a pressing health problem in many parts of the world, particularly in Africa and Latin America, which are the regions with the highest mortality rates. Recent data indicate that malaria is present in 97 countries and an estimated 3.2 billion people are at risk of contracting the disease. In the year 2015, 214 million cases of malaria were reported and 438,000 people died most of them children under 5 years of age. Most of these cases occurred in Africa. At present, the situation is becoming even more complicated by the spread of drug resistant parasites, especially in areas where Plasmodium falciparum and Plasmodium vivax are endemic and of higher prevalence. Few alternative drugs are under development and urgent measures are required to identify new classes of antimalarial agents, many of which have their origins from natural products.

Piper, the nominal genus of the family Piperaceae, is one of the most diverse genera of basal angiosperms. It is currently considered to have about 1500 species and its greatest diversity is found in the humid forests of tropical regions around the world. Ecologically, they are important and dominant components in the humid forests, especially in the neotropics and they constitute an important part of the diet of some families of American bats, insects and birds. Few species of Piper are economically important, among them Piper nigrum from which the pepper is obtained, condiment popularly used all over the world. For the Piper genus, a wide range of traditional foods has been reported in the treatment of various diseases such as malaria, anemia, cholera, diabetes, asthma, bronchitis, pneumonia, influenza, rheumatism and arthritis. In this study, plants were used as condiments, aphrodisiacs, stimulants and hallucinogens. Chemically, in Piperaceae, lignans and neolignans have been found, tannins, saponins, phenolic compounds, terpenes, flavonoids and alkaloids among others. Many of these compounds, especially alkaloids, terpenes and lignans, are responsible for the antimalarial activity of many species reported in other parts of the world. A large variety of Piperaceae species are used by traditional medicine to treat malaria, some of which have been the subject of antimalarial activity both in vitro and in vivo. Regarding the antimalarial potential of extracts and components of P. piedecuestanan Trel. and Yunck., there are not reports in the literature and there is a single report investigating the antioxidant activity of these extracts.

Thus, the present study was aimed to obtain extracts of different polarity of the species P. piedecuestanan Trel. and Yunck, isolated and characterized their major components. Evaluate antimalarial activity in vitro on continuous cultures of P. falciparum chloroquine-sensitive strain NF-54 and evaluated the cytotoxicity activity of the extracts and compounds in the cell lines U-937 and HUVEC.

MATERIALS AND METHODS

All experiments were realized in Laboratory of Malaria Research Group. University Research Headquarters (SIU), University of Antioquia, Medellin, Colombia and Department of Inorganic and Organic Chemistry, Universitat Jaume I, Castellón, Spain.

Chemical and solvents: hexane, dichloromethane, ethyl acetate, methanol, chloroform, ethanol, dimethyl sulphoxide (DMSO), Silica-gel 60, sulfuric acid, N, N-Dimethylformamide (DMF), N-Bromosuccinimide (NBS), RPMI-1640, HEPES, SYBR Green I, 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide (MTT)and standard chloroquine were used. Analytical grade reagents/chemicals were also used in this experiment.

Collection and identification of plant material: Three types of samples were collected from the plant material: A sample for herbarium specimen, samples as controls of specimens collected and samples of leaves and stems to obtain the extracts. The specimen for herbarium was processed, deposited and taxonomically characterized in the herbarium of the University of Antioquia (HUA) and determined as Piper piedecuestanan Trel. and Yunck. (Voucher f. 191. 1950) collected in Piedecuesta, Santander-Colombia.

Preparation of extracts and isolation of compounds: Piper piedecuestanan (PP) plant material was subjected to a desiccation process at room temperature with aeration and without exposure to sunlight for 10 days. About 2.36 g of mixture of leaves and stems of the ground vegetable material was initially taken to a percolation process until exhaustion (5 days/3 times) using ethanol (E) that was then filtered and concentrated on a rotary evaporator. On the other hand, 0.36 kg of ground material was subjected to extraction with solvents of upward polarity (500 mL) by a percolation process to exhaustion with each of the following solvents: hexane (H), dichloromethane (D), ethyl acetate (A) and methanol (M). After 3 days, the extract was concentrated under reduced pressure in a rotary evaporator. The five extracts of the plant were
coded with the initials of the species name and the type of extract and according to the polarity of the solvent, starting with the petroleum ether (H) (PPH), dichloromethane (D) extract (PPD), ethyl acetate (EA) (PPE), methanol (M) (PPM) and ethanolic extract encoded as (PPE). All extracts were monitored by thin layer chromatography (CCD) supported with Merck® Silica-gel 60 GF254 using different elution systems. The extraction percentages of the extracts were calculated according to Eq. 1:

\[
de\text{Extraction} (\%) = \frac{\text{Extracts weight}}{\text{Weight of plant material}} \times 100\quad(1)
\]

The extracts considered as active were processed by fractionation and isolation of the major substances. The extract of dichloromethane (PPD) of the species of *P. pidecuestania* Trel. and Yunick. (4.89 g) was active and was fractionated by column chromatography using as eluent gradients of petroleum ether: ethyl acetate, EtOAc and MeOH. Thirty fractions were obtained from which fraction 10 was taken and column chromatography was performed using petroleum ether, petroleum ether:DCM (1:1), DCM, gradients of DCM: EtOAc and finally MeOH as eluent. Twenty-three fractions were obtained which were pooled between 1-7 and preparative plate chromatography was performed using DCM as the eluent and 20 mg of the compound designated as (4) were purified. On the other hand, to section 25 of the dichloromethane extract column, preparative layer chromatography was performed and two compounds were isolated, one of which was a yellow amorphous solid named (2) (31.8 mg) and the other a crystalline orange solid referred to as (1) (183.9 mg). The remaining fractions of the petroleum ether extract and dichloromethane were combined to perform column chromatography again using petroleum ether gradients: DCM, DCM: EtOAc, EtOAc: Methanol (MeOH) gradients and finally MeOH and obtained 14 fractions, fractions of 6-10 were taken and column chromatography using eluent petroleum ether, petroleum ether: DCM (1:1), DCM and DCM: MeOH gradients as eluents, whereby 30 fractions, fractions 1-9 and 15-17 were taken for preparative plate chromatography eluting with petroleum ether: DCM 25: 3 and DCM to isolate 39 mg of the compound (4), 59 mg of compound (3) and 58 mg of compound (5). Halogenated atoms were introduced into the isolated compounds by the production of brominated derivatives. Only a successful reaction was performed from compound (5). The experimental procedure is briefly described below: a solution of (5) (20 mg, 0.71 mmol) was added. The reaction mixture was left at 0°C for 2 h under nitrogen. Subsequently, the temperature was gradually raised to 80°C for 24 h. The crude mixture was diluted in dichloromethane (10 mL) and washed with saturated aqueous ammonium chloride (3 × 10 mL). The organic layer was dried (MgSO4) and concentrated under reduced pressure to give compound (6). All extracts and compounds were stored at room temperature for the biological assays.

**Structural characterization:** IR spectra were obtained by using KBr pellets on a Jasco FT/IR-6200 spectrometer, spanning the region 4000-600 cm⁻¹. Mass spectra were measured on a Q-TOF mass spectrometer (Waters, Manchester, UK) with electrospray-type combined ionization source and Z-spray design API; the capillary voltage of 3.5 KV was used in the positive direction and the cone voltage was set at 20V. NMR spectra were recorded on Varian Unity spectrometers of 300 and 500 (approximate operating frequencies, 300 and 500 MHz for 1H, 125 and 75 MHz for 13C). The nature of the carbon signals (C, CH, CH2, CH3) was determined using the APT or DEPT techniques. Signal assignments were performed using two-dimensional heteronuclear correlations (COSY and HMBC/HMBC). Unless otherwise indicated, the spectra were measured in CDCl3 solution. Chemical shifts (δ) are reported in ppm using the residual solvent signals (δ 7.27 ppm for 1H and 77.0 ppm for 13C) as reference. As far as the referencing of the spectra when it comes to multiplets, the range they occupy is included.

**Compound (1):** 5,8-Hydroxy-7-methoxyflavone, yellow amorphous solid. C16H12O5: TOF MS ES+ ([M+H]+): 285.0760. 1H-NMR-300 MHz δ (chloroform-d1) (ppm): 12.50 (1H, s, C1-OH), 7.89 (2H, dd, J = 2.2, 8.0 Hz, Ar-H), 7.56-7.52 (3H, m, Ar-H), 6.69 (1H, s, Cδ-H), 6.63 (1H, s, Cβ-H), 4.018 (3H, s, CH3O). 13C-NMR-75 MHz δ (chloroform-d1) (ppm): 183.06, 165.59, 162.15, 153.33, 146.04, 132.19, 131.88, 130.048, 129.49 (CH X 2), 126.67 (CH X 2), 105.91, 105.85, 90.95, 56.8817.

**Compound (2):** 6,7-dimethoxy-5,8-dihydroxyflavone, yellow amorphous solid. C17H14O5: TOF MS ES+ ([M+OH]+): 315.0869. 1H-NMR-300 MHz δ (chloroform-d1) (ppm): 12.63 (1H, s, C1-OH), 8.20 (2H, dd, J = 2.2, 8.0 Hz, Ar-H), 7.53-7.51 (3H, m, Ar-H), 6.82 (1H, bs, Cδ-OH), 6.56 (1H, s, Cβ-H), 3.98 (3H, s, CH3O), 3.94 (3H, s, CH3O). 13C-NMR-75 MHz δ (chloroform-d1) (ppm): 197.36, 174.85, 159.77, 152.88, 151.92, 136.96, 132.52, 130.72, 129.04 (CH X 2), 128.03 (CH X 2), 96.95, 91.36, 86.65, 61.37, 56.8017.
Compound (3): 6,7-dimethoxy-5-hydroxyflavone (mosloflavone), yellow amorphous solid. \( \text{C}_{13} \text{H}_{10} \text{O}_{5} \). TOF MS ES+ [M+H]+: 299.0919. \(^1\)H-NMR 500 MHz \( \delta \) (chloroform-d\(_{1}\)) (ppm): 12.68 (1H, s, C\(_5\)-OH), 7.88 (2H, dd, \( J = 2.2, 8.0 \) Hz, Ar-H), 7.54-7.52 (3H, m, Ar-H), 6.66 (1H, s, C\(_5\)-H), 6.56 (1H, s, C\(_6\)-H), 3.97 (3H, s, CH\(_3\)O), 3.93 (3H, s, CH\(_3\)O). \(^1\)C-NMR 125 MHz \( \delta \) (chloroform-d\(_{1}\)) (ppm): 182.66, 163.90, 158.87, 153.27, 152.99, 132.66, 131.80, 131.25, 129.05 (CH\(_2\)×2), 126.20 (CH\(_2\)×2), 106.24, 105.56, 90.62, 60.80, 56.29\(^{17}\).

Compound (4): 5,6-dihydroxy-7-methoxyflavone (negletein), yellow amorphous solid. \( \text{C}_{14} \text{H}_{10} \text{O}_{5} \). TOF MS ES+ [M+H]+: 285.0767. \(^1\)H-NMR 300 MHz \( \delta \) (chloroform-d\(_{1}\)) (ppm): 11.72 (1H, bs, C\(_3\)-OH), 8.20 (2H, dd, \( J = 2.2, 8.0 \) Hz, Ar-H), 7.53-7.48 (3H, m, Ar-H), 6.51 (1H, s, C\(_6\)-H), 6.39 (1H, s, C\(_3\)-H), 3.90 (3H, s, CH\(_3\)O). \(^1\)C-NMR 75 MHz \( \delta \) (chloroform-d\(_{1}\)) (ppm): 194.62, 166.54, 166.0, 161.55, 145.36, 131.97, 130.314, 129.49, 129.46 (CH\(_2\)×2), 129.08 (CH\(_2\)×2), 114.04, 104.95, 94.04, 57.60\(^{17}\).

Compound (5): 5-hydroxy-6-methoxy-7-hydroxyflavone, yellow amorphous solid. \( \text{C}_{14} \text{H}_{10} \text{O}_{5} \). TOF MS ES+ [M+H]+: 269.0810. \(^1\)H-NMR 500 MHz \( \delta \) (chloroform-d\(_{1}\)) (ppm): 12.72 (1H, s, C\(_5\)-OH), 7.88 (2H, dd, \( J = 2.2, 8.0 \) Hz, Ar-H), 7.53-7.52 (3H, m, Ar-H), 6.67 (1H, s, C\(_5\)-H), 6.50 (1H, s, C\(_6\)-H), 6.37 (1H, s, C\(_3\)-H), 3.88 (3H, s, CH\(_3\)O). \(^1\)C-NMR 125 MHz \( \delta \) (chloroform-d\(_{1}\)) (ppm): 182.46, 165.59, 162.15, 157.76, 131.80, 131.28 (CH\(_2\)×2), 129.05 (CH\(_2\)×2), 126.64, 105.83, 105.67, 98.17, 92.65, 55.78\(^{17}\).

Compound (6,7): 6,8 bromo-5-hydroxy-6-methoxyflavone, crystal yellow color. \( \text{C}_{16} \text{H}_{10} \text{O}_{5} \). TOF MS ES+ [M+H]+: 426.9007. \(^1\)H-NMR 500 MHz \( \delta \) (chloroform-d\(_{1}\)) (ppm): 12.72 (1H, s, C\(_5\)-OH), 7.89 (2H, dd, \( J = 2.2, 8.0 \) Hz, Ar-H), 7.53-7.52 (3H, m, Ar-H), 6.67 (1H, s, C\(_5\)-H), 3.88 (3H, s, CH\(_3\)O)\(^{17}\).

Biological activity test

**In vitro determination of antimalarial activity:** *In vitro* antimalarial activity assays were performed on the sensitive (NF-54) chloroquine strain. *P. falciparum* strains (NF-54) were cultured and maintained according to the method of Trager and Jensen\(^{18}\), using a suspension of 5% human A+erythrocytes in RPMI-1640 culture medium (Sigma R6504) dissolved in sterile water with 25 mM HEPES, 5.0% NaHCO\(_3\), 10% fresh human A+serum (inactivated at 56°C for 30 min) incubated in 5% O\(_2\), 5% CO\(_2\) and 90% N\(_2\) an atmosphere. Fresh red blood cells were added twice a week. *In vitro* antimalarial activity by the SYBR Green I method was performed in the Malaria Group, according to the methodology described by Smilkstein *et al.*\(^{19}\). Assays were performed on Falcon® 96-well flat bottom plates. A suspension of parasitized red blood cells with a hematocrit of 2.5% and a parasitemia of 1% were prepared. Cultivation with treatments and chloroquine (CQ) positive control were incubated at 37°C for 48 h in 5% CO\(_2\), 5% O\(_2\) and 90% of N\(_2\) atmosphere. Subsequently, the contents of each well were transferred to Greiner Pro one dishes and the parasites were labeled with a solution of SYBR® Green 1 2X in lysis buffer. The plates were incubated at room temperature in the dark for one hour and the relative fluorescence units (RFU) were read on a spectrofluorometer at 485 nm excitation wavelength and 538 nm emission wavelength. Treatments were prepared to a stock solution of 10 mg mL\(^{-1}\) in pure DMSO and sonicated to facilitate dissolution, first dilution was 1% and showed to be non-toxic to the parasite. From this solution, 50 μL were taken and adjusted to 1000 μL with complete RPMI-1640 medium, obtaining a final concentration of 0.5 mg mL\(^{-1}\). Seven concentrations of each extract were evaluated in a range between 100-1.56 μg mL\(^{-1}\). Each concentration was evaluated in triplicate on the plate and three independent assays were performed. The CQ control was evaluated in a range between 150-4.7 nM and the control of Peruvian quina extract (MeOH: H\(_2\)O: 70:30) was evaluated in the range of 0.01-10 μg mL\(^{-1}\).

Data from three trials were analyzed to find the inhibitory concentration in μg mL\(^{-1}\) (IC\(_{50}\)). Inhibitory concentrations 50 (IC\(_{50}\)±SD) were calculated for each compound from a non-linear logistic regression model. The data were analyzed and plotted using GraphPad Prism 4 for Macintosh version 4.0b which outputs the adjustment value (r) (GraphPad Software, San Diego, California, USA). To classify the antimalarial activity of an extract, the Malaria Group of the University of Antioquia established a consensus for the extracts evaluated: highly active <5 μg mL\(^{-1}\), promising 6-15 μg mL\(^{-1}\), moderate activity 16-30 μg mL\(^{-1}\), low activity 31-50 μg mL\(^{-1}\) and non-active>50 μg mL\(^{-1}\). To classify the antimalarial activity of a compound, the Malaria Group estimated that a compound is promising if the IC\(_{50}\) is ≤10 μM\(^{21}\).

**Cytotoxicity testing and calculation of the selectivity index (SI):** The method of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) according to Mosmann\(^{22}\), which reveals cellular damage at the mitochondrial level, to evaluate the cytotoxic activity of the extracts and compounds. U-937 and HUVEC cells lines were used, which were maintained in continuous cultures in the Malaria Group Laboratory. These cells were cultured at 37°C and 5% CO\(_2\) in RPMI medium supplemented with 10% inactivated Fetal Bovine Serum (FBS). The media changes were assessed every 48 h or according to
pH changes of the medium, with 10 min centrifugation at 1000 rpm and by replacement with fresh medium\textsuperscript{25}. In Neubauer's chamber, U-937 cells were counted and plated in a 96-well flat bottom plate, 200,000 cells mL\textsuperscript{-1} in RPMI 1640 medium with 10% FBS. For other the assay, the HUVEC cells are counted in a Neubauer chamber and seeded in a 96-well flat bottom plate 2×10\textsuperscript{4} cells well\textsuperscript{-1} in 100 μL of RPMI-1640 medium with 10% fetal bovine serum. They were incubated at 37°C with 5% CO\textsubscript{2} for 72 h in the presence of each of the seven concentrations of each extract and/or compound and evaluated in a range between 100-156 μg mL\textsuperscript{-1}. Each concentration was evaluated in triplicate in the dish and three independent trials were performed. Subsequently, mitochondrial dehydrogenase activity was measured by adding 20 μL well\textsuperscript{-1} of MTT to a concentration of 5 mg mL\textsuperscript{-1} and incubated for 3 h at 37°C under 5% CO\textsubscript{2}. To dissolve the formed crystals, 100 μL well\textsuperscript{-1} of a 50% solution of isopropanol and 10% SDS were added and the absorbance read at 595 nm in an ELISA reader (BioRad). For cytotoxicity assays, the positive control was the culture medium and the negative control was amphotericin which showed toxicity in the U-937 cells and HUVEG cell line. Data from three independent trials were analyzed using the GraphPad Prism 5 program to find the toxic concentration in μg mL\textsuperscript{-1} (IC\textsubscript{50}) using a non-linear logistic regression model. The coefficient of variation (% CV) was also estimated to estimate the degree of dispersion of the IC\textsubscript{50} obtained from the three independent assays\textsuperscript{16}. To classify the cytotoxicity of the extract, the Malaria Group of the University of Antioquia established a consensus for the samples evaluated: highly toxic <10 μg mL\textsuperscript{-1}, cytotoxic 10-40 μg mL\textsuperscript{-1}, moderately cytotoxic 41-100 μg mL\textsuperscript{-1} and no cytotoxic>100 μg mL\textsuperscript{-1}\textsuperscript{12}. In addition, the selectivity index (IS), which indicates selectivity towards the parasite, was calculated as the relationship between cytotoxic IC\textsubscript{50} activity and antiplasmodial activity IC\textsubscript{50}. For the U-937 cell line it was estimated that IS values above 2 are considered promising extracts to be evaluated in the HUVEC cell line (endothelial cells isolated from the human umbilical cord vein). For the HUVEC cell line, IS values above 5 were considered as promising extracts.

**Statistical analysis:** Measurements were performed in triplicate and the results were presented as the mean and its standard deviation (DS). The data were analyzed and plotted using GraphPad Prism 4 for Macintosh version 4.0b which outputs the adjustment value (r) (GraphPad Software, San Diego, California, USA) and all calculations were performed in the statistical program STATGRAPHICS CENTURION XVI.

**RESULTS AND DISCUSSION**

The percentages of extractable material, in vitro antiplasmodial activity in chloroquine-sensitive *Plasmodium falciparum* strain NF-54, cytotoxicity in U-937 promonocytes, HUVEC endothelial cells and determination of the selectivity index of extracts and compounds of *P. piedecuestaum* Trel. and Yunck were presented in Table 1 and 2. They were classified according to the antiplasmodial potential established by the Malaria Group of the University of Antioquia. The percentages of extractable material with the different solvents showed better yields for the dichloromethane extracts of the stems and leaves mixture of the *P. piedecuestaum* (PPD) species with 1.75%, however no association was found between extraction yields and the biological tests that were performed. For all extracts of the species *P. piedecuestaum* Trel. and Yunck, found an adequate concentration-response relationship with statistically significant correlation coefficients (R\textsuperscript{2}) for all the samples evaluated. The chloroquine positive control had a mean value of IC\textsubscript{50} = 26.1 ± 5.4 nM and the control of the Peruvian quinine extract (MeOH: H\textsubscript{2}O; 70:30) presented a mean value of IC\textsubscript{50} = 0.32 ± 0.16 μg mL\textsuperscript{-1}.

<table>
<thead>
<tr>
<th>Code</th>
<th>Extract by percolation, leaves and stems</th>
<th>Performance of extraction (g of extract/g of dry plant material) (%)</th>
<th>IC\textsubscript{50} (μg mL\textsuperscript{-1}) X±SD*</th>
<th>Strain of <em>P. falciparum</em> NF-54**</th>
<th>CC\textsubscript{50} (μg mL\textsuperscript{-1}) X±SD</th>
<th>Cell line U-937 X±SD</th>
<th>IS***</th>
<th>Cell line HUVEC**** I***</th>
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<tbody>
<tr>
<td>PPH</td>
<td>Petroleum ether, 25°C ZP*: 5 mm 10 days</td>
<td>0.7</td>
<td>&gt;50</td>
<td>24.9 ± 4.1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>PPD</td>
<td>Dichloromethane, 25°C ZP*: 5mm 10 days</td>
<td>1.7</td>
<td>17.9 ± 2.02</td>
<td>37.6 ± 1.7</td>
<td>2.1</td>
<td>101.4 ± 2.7</td>
<td>5.7</td>
<td>ND</td>
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<td>PPPE</td>
<td>Ethyl acetate, 25°C ZP*: 5 mm 10 days</td>
<td>1.1</td>
<td>19.5 ± 1.8</td>
<td>15.4 ± 2.6</td>
<td>0.8</td>
<td>80.7 ± 1.6</td>
<td>4.1</td>
<td>ND</td>
</tr>
<tr>
<td>PPM</td>
<td>Methanol, 25°C ZP*: 5 mm 10 days</td>
<td>1.6</td>
<td>&gt;50</td>
<td>42.3 ± 0.23</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>PPE</td>
<td>Ethanol 25°C ZP*: 5 mm 24 h</td>
<td>0.9</td>
<td>25.2 ± 0.3</td>
<td>12.4 ± 0.33</td>
<td>0.5</td>
<td>NE</td>
<td>NE</td>
<td>ND</td>
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\textsuperscript{*ZP: Particle size, **Data of a single replica, *X(Average)±SD (Standard deviation), ****Chloroquine positive control IC\textsubscript{50} = 26.10 ± 5.37 nM, peruvian machine extract 0.32 ± 0.16 μg mL\textsuperscript{-1}, *****Selectivity index (IS) = CC\textsubscript{50} (μg mL\textsuperscript{-1})/IC\textsubscript{50} (μg mL\textsuperscript{-1}), ND: Not determined value of IC\textsubscript{50} >50 μg mL\textsuperscript{-1}. Classification of antiplasmodial activity: highly active <5 μg mL\textsuperscript{-1}, promising 6-15 μg mL\textsuperscript{-1}, moderate activity 16-30 μg mL\textsuperscript{-1}, low activity 31-50 μg mL\textsuperscript{-1} and non-active activity>50 μg mL\textsuperscript{-1}. Classification of cytotoxic activity: highly toxic <10 μg mL\textsuperscript{-1}, cytotoxic 10-40 μg mL\textsuperscript{-1}, moderately cytotoxic 41-100 μg mL\textsuperscript{-1} and non-cytotoxic>100 μg mL\textsuperscript{-1}. |
**Table 2: Antiplasmodial activity of isolated compounds of *P. piecuestanum***

<table>
<thead>
<tr>
<th>Code</th>
<th>Structure</th>
<th>IC₅₀ (µg mL⁻¹) X±SD*</th>
<th>CC₅₀ (µg mL⁻¹) X±SD</th>
<th>Strain of <em>P. falciparum</em> NF-54**</th>
<th>Cell line HUVEC</th>
<th>IS***</th>
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<tbody>
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<td>1</td>
<td><img src="image1.png" alt="Structure" /></td>
<td>7.3±0.4 or 25.7 µM</td>
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<td>2</td>
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<td>&gt;100</td>
<td>ND</td>
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<tr>
<td>3</td>
<td><img src="image3.png" alt="Structure" /></td>
<td>&gt;50</td>
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* (*Average)+SD (Standard deviation), **Chloroquine positive control IC₅₀ = 0.008±2.78 µg mL⁻¹ or IC₅₀ = 26.1±5.4 nM, ***Selectivity index (IS) = CC₅₀ (µg mL⁻¹)/IC₅₀ (µg mL⁻¹) IS>2 confirms efficacy and safety, ND: Not determined. The definition of the antiplasmodial activity used was: IC₅₀<5 µg mL⁻¹-strong activity, 6-15 µg mL⁻¹-moderate activity, 16-30 µg mL⁻¹-slightly active and IC₅₀>30 µg mL⁻¹-inactive

The biological activity found for the extracts of the species *P. piecuestanum* Trel. And Yunck show that the petroleum ether extract (PPH) with non-polar components and the methanolic extract (PPM) with polar components did not present antiplasmodial activity (IC₅₀>50 µg mL⁻¹). However, extracts of dichloromethane and ethyl acetate showed moderate antiplasmodial activity with IC₅₀ = 17.9 µg mL⁻¹; IS₅₀ = 2.09 and IC₅₀ = 19.5 µg mL⁻¹; IS₅₀ = 0.79, even presenting better pharmacological activity than the ethanolic extract with IC₅₀ = 25.2 µg mL⁻¹; IS₅₀ = 0.49 (Fig. 1). It can be inferred that the effect of these extracts is due to components with moderately polar and non-toxic characteristics, since for the extract of dichloromethane, which was the most active and selective, a selectivity index>2 was presented, indicating the specificity of extract towards the parasite. The fact that the cytotoxicity of this extract will increase CC₅₀ = 37.63 µg mL⁻¹ with respect to the other extracts indicates that the components present in this extract are poorly cytotoxic.
addition, when these extracts were evaluated in HUVECs metabolically active cells an interesting IS was found, since it was shown that the extracts did not affect this type of cells, since their cytotoxic concentrations were CC50 = 101.35; IC50HУVЕG = 5.65 and CC50 = 80.7; ISHУVЕG = 4.14, for the extracts of dichloromethane and ethyl acetate respectively.

For species of Piper genus, promising activity against Plasmodium in P. capense L. species has been demonstrated. (Piperaceae) with an IC50 = 7.0 µg mL−1 activity in P. hostmannianum strain (W2) chloroquine resistant26 with an IC50 = 8.0 µg mL−1, P. umbellatum with 70% inhibition at 40 µg mL−1 and P. sammentosum with an IC50 = 0.05 µg mL−1.27. In vivo antimalarial activity of the Piper betle leaf methanolic extract was demonstrated in Plasmodium berghei (NK65) infected mice has been demonstrated over a range of concentrations (50-400 mg kg−1). The activity of (-)-methylenderatim was confirmed in vivo in mice infected with P. vinckei petteri, showing an 80% reduction in parasitemia at a dose of 20 mg kg−1 day−1. It is the first time that the metabolites have been reported 5,8-Hydroxy-7-methoxyflavone1, 6,7-dimethoxy-5,8-dihydroxyflavone2, 6,7-dimethoxy-5-hydroxyflavone (mosloflavone) (3), 5,6-dihydroxy-7-methoxyflavone (negletein) (4), 5-hydroxy-7-methoxyflavone (5) from P. pachycerasum species and a brominated derivative from (6) named 6,8 bromo-5-hydroxy-7-methoxyflavone(7) in the species P. pachycerasum. The compound (1) was the only one that presented antimalasmodial activity with an IC50 = 7.325 µg mL−1 (25.69 µM); ISHУVЕG = 13.65. The brominated derivative of (5) also did not exhibit antimalasmodial activity, indicating that for this compound the presence of halogenated groups does not significantly influence the therapeutic response. Other species of this genus have reported various flavonoids with antimalasmodial activity. The compound 6-prenyl-3’-methoxyethylidethion showed no activity on the P. falciparum strain. Analysis of the structure-activity ratio shows that the presence of adjacent methoxy and hydroxy groups or the absence of adjacent hydroxyl groups could contribute to the inactivity of the 6-prenyl-3’-methoxyethylidethion compound and the substituents in the flavanone backbone clearly influence the antimalasmodial activity28. The antimalasmodial activity of flavonoids is arousing a great interest in the chemical medicine, since they are orienting studies of structure relation SAR of chalcones and derivatives of flavonoids with diverse substituents in ring B, A and C. Chalcones are more selective than methoxyflavones, chalcone derivative compounds have a more selective activity (6.7-16.9µM) over P. falciparum (W2) than flavonoid derivatives (5-33 µM)22; methoxyflavones present the best activities, even these results complement the validity of the results obtained in this work for the isolated compound 5,8-hydroxy-7-methoxyflavone (1).

CONCLUSION AND RECOMMENDATION

The antiplasmodial and cytotoxic activity of the species P. pachycerasum was reported for the first time in which the moderately active extracts were those of dichloromethane and ethyl acetate with the best effect of antiplasmodial and cytotoxic activity with IC50 = 17.93 µg mL−1; IS = 2.093 and IC50 = 19.5 µg mL−1; IS = 0.791, respectively. The antiplasmodial effect of these extracts is due to components with mild polar characteristics and little toxicity, which motivates continuing with the study of the SAR structure-activity relationship of flavonoids with various substituents in ring B, A and C.

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REFERENCES


