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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 (PPAE) with antiplasmodial activity of $IC_{50} = 17.93 \mu\text{g mL}^{-1}$; $IS = 2.093$ and $IC_{50} = 19.5 \mu\text{g mL}^{-1}$; $IS = 0.791$, 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 (negletein) (4), 5-hydroxy-7-methoxyflavone (5) and a brominated derivative from (5) named 6,8 bromo-5-hydroxy-7-methoxyflavone(7). Compound (1) presented promising antiplasmodial activity with an $IC_{50} = 7.325 \mu\text{g mL}^{-1}$ ($25.69 \mu\text{M}$); $IS_{HUVEC} = 13.65$. **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 Anopheles¹. 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 disease². 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 Africa³. 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 products⁴.

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 world⁵. 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 birds⁶⁻⁸. 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 hallucinogens⁹⁻¹¹. Chemically, in Piperaceae, lignans and neolignans have been found, tannins, saponins, phenolic compounds, terpenes, flavonoids and alkaloids among others¹². Many of these compounds, especially alkaloids, terpenes and lignans, are responsible for the antiplasmodial activity of many species reported in other parts of the world^{13,14}. A large variety of Piperaceae species are used by traditional medicine to treat malaria, some of which have been the subject of antiplasmodial activity both *in vitro* and *in vivo*¹⁵. Regarding the antiplasmodial potential of extracts and components of *P. piedecuestanum* Trel. and Yunck., there are not reports in the literature and there is a single report investigating the antioxidant activity of these extracts¹⁶.

Thus, the present study was aimed to obtain extracts of different polarity of the species *P. piedecuestanum* Trel. and Yunck, isolated and characterized their major components. Evaluate antiplasmodial 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 piedecuestanum* Trel. and Yunck. (Voucher f. 191. 1950) collected in Piedecuesta, Santander-Colombia.

Preparation of extracts and isolation of compounds: *Piper piedecuestanum* (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) (PPAE), methanol (M) (PPM) and ethanolic extract encoded as (PPE). All extracts were monitored by thin layer chromatography (TLC) supported with Merck® Silica-gel 60 GF₂₅₄ using different elution systems. The extraction percentages of the extracts were calculated according to Eq. 1:

$$\text{deExtraction (\%)} = \frac{\text{Extracts weight}}{\text{Weight of plant material}} \times 100 \quad (1)$$

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. piedecuestanum* Trel. and Yunck. (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 dissolved in N, N-Dimethylformamide (DMF) and N-Bromosuccinimide NBS

(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 (Mg₂SO₄) 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 APCI; 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 HMQC/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, C⁵-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, C⁶-H), 6.63 (1H, s, C³-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×2), 126.67 (CH×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, C⁵-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, C⁸-OH), 6.56 (1H, s, C³-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×2), 128.03 (CH×2), 96.95, 91.36, 86.65, 61.37, 56.80¹⁷.

Compound (3): 6,7-dimethoxy-5-hydroxyflavone (mosloflavone), yellow amorphous solid. $C_{17}H_{14}O_5$. TOF MS ES+ [M+H]: 299.0919. 1H -NMR-500 MHz δ (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^3 -H), 6.56 (1H, s, C^8 -H), 3.97 (3H, s, CH_3O), 3.93 (3H, s, CH_3O). ^{13}C -NMR-125 MHz δ (chloroform- d_1) (ppm): 182.66, 163.90, 158.87, 153.27, 152.99, 132.66, 131.80, 131.25, 129.05 ($CH \times 2$), 126.20 ($CH \times 2$), 106.24, 105.56, 90.62, 60.80, 56.29¹⁷.

Compound (4): 5,6-dihydroxy-7-methoxyflavone (negletein), yellow amorphous solid. $C_{16}H_{12}O_5$. TOF MS ES+ [M+H]: 285.0767. 1H -NMR-300 MHz δ (chloroform- d_1) (ppm): 11.72 (1H, bs, C^5 -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^3 -H), 6.39 (1H, s, C^8 -H), 3.90 (3H, s, CH_3O). ^{13}C -NMR-75 MHz δ (chloroform- d_1) (ppm): 194.62, 166.54, 166.0, 161.55, 145.36, 131.97, 130.314, 129.49, 129.46 ($CH \times 2$), 129.08 ($CH \times 2$), 114.04, 104.95, 94.04, 57.60¹⁷.

Compound (5): 5-hydroxy-7-methoxyflavone, yellow amorphous solid. $C_{17}H_{14}O_5$. TOF MS ES+[M+H]: 269.0810. 1H -NMR-500 MHz δ (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^3 -H), 6.50 (1H, s, C^6 -H), 6.37 (1H, s, C^8 -H), 3.88 (3H, s, CH_3O). ^{13}C -NMR-125 MHz δ (chloroform- d_1) (ppm): 182.46, 165.59, 162.15, 157.76, 131.80, 131.28 ($CH \times 2$), 129.05 ($CH \times 2$), 126.64, 105.83, 105.67, 98.17, 92.65, 55.78¹⁷.

Compound (6,7): 6,8 bromo-5-hydroxy-7-methoxyflavone, crystal yellow color. $C_{16}H_{11}O_4Br_2$. TOF MS ES+[M+H]: 426.9007. 1H -NMR-500 MHz δ (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^3 -H), 3.88 (3H, s, CH_3O)¹⁷.

Biological activity test

In vitro determination of antiplasmodial activity: *In vitro* antiplasmodial 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 Jensen¹⁸, 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 atmosphere. Fresh red blood cells were added twice a week. *In vitro* antiplasmodial 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 I 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 $\mu g mL^{-1}$. 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_2O ; 70:30) was evaluated in the range of 0.01-10 $\mu g mL^{-1}$. Data from three trials were analyzed to find the inhibitory concentration in $\mu g mL^{-1}$ (IC_{50}). Inhibitory concentrations 50 ($IC_{50} \pm SD$) 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 antiplasmodial activity of an extract, the Malaria Group of the University of Antioquia established a consensus for the extracts evaluated: highly active <5 $\mu g mL^{-1}$, promising 6-15 $\mu g mL^{-1}$, moderate activity 16-30 $\mu g mL^{-1}$, low activity 31-50 $\mu g mL^{-1}$ and non-active >50 $\mu g mL^{-1}$ ²⁰⁻²². To classify the antiplasmodial activity of a compound, the Malaria Group estimated that a compound is promising if the IC_{50} is $\leq 10 \mu M$ ²³.

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 Mosmann²⁴, 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 medium²⁵. In Neubauer's chamber, U-937 cells were counted and plated in a 96-well flat bottom plate, 200.000 cells mL⁻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 2×10⁵ cells well⁻¹ in 100 µL of RPMI-1640 medium with 10% fetal bovine serum. They were incubated at 37°C with 5% CO₂ for 72 h in the presence of each of the seven concentrations of each extract and/or compound and evaluated in a range between 100-1.56 µg mL⁻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⁻¹ of MTT to a concentration of 5 mg mL⁻¹ and incubated for 3 h at 37°C under 5% CO₂. To dissolve the formed crystals, 100 µL well⁻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 HUVEC cell line. Data from three independent trials were analyzed using the GraphPad Prism 5 program to find the toxic concentration in µg mL⁻¹ (CC₅₀) using a non-linear logistic regression model. The coefficient of variation (% CV) was also estimated to estimate the degree of dispersion of the CC₅₀ obtained from the three independent assays²⁶. 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⁻¹, cytotoxic 10-40 µg mL⁻¹, moderately cytotoxic 41-100 µg mL⁻¹ and no cytotoxic >100 µg mL⁻¹²⁷. In addition, the selectivity index (IS), which indicates selectivity towards the parasite, was calculated as the relationship between cytotoxic CC₅₀ activity and antiplasmodial activity IC₅₀. 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 CENTURIUN 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. piedecuestanum* 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. piedecuestanum* (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. piedecuestanum* Trel. and Yunck. found an adequate concentration-response relationship with statistically significant correlation coefficients (R²) for all the samples evaluated. The chloroquine positive control had a mean value of IC₅₀ = 26.1±5.4 nM and the control of the Peruvian quinine extract (MeOH: H₂O; 70:30) presented a mean value of IC₅₀ = 0.32±0.16 µg mL⁻¹.

Table 1: Cytotoxicity and antiplasmodial activity of the extracts of different polarities of *P. piedecuestanum* Trel. and Yunck

Code	Extract by percolation, leaves and stems	Performance of extraction (g of extract/g of dry plant material) (%)	IC ₅₀ (µg mL ⁻¹) X±SD*	CC ₅₀ (µg mL ⁻¹) X±SD	CC ₅₀ (µg mL ⁻¹) X±SD		IS***	IS****
			Strain of <i>P. falciparum</i> NF-54**	Cell line U-937	Cell line HUVEC*****			
PPH	Petroleum ether, 25°C ZP*: 5 mm 10 days	0.7	>50	24.9±4.1	ND	NE	ND	ND
PPD	Dichloromethane, 25°C ZP*: 5mm 10 days	1.7	17.9±2.02	37.6±1.7	2.1	101.4±2.7	5.7	5.7
PPAE	Ethyl acetate, 25°C ZP*: 5 mm 10 days	1.1	19.5±1.8	15.4±2.6	0.8	80.7±1.6	4.1	4.1
PPM	Methanol, 25°C ZP*: 5 mm 10 days	1.6	>50	42.3±0.23	ND	NE	ND	ND
PPE	Ethanol 25°C ZP*: 5 mm 24 h	0.9	25.2±0.3	12.4±0.33	0.5	NE	NE	NE

*ZP: Particle size, **Data of a single replica, ***X (Average)+SD (Standard deviation), ****Chloroquine positive control IC₅₀ = 26.10±5.37 nM, peruvian machine extract 0.32±0.16 µg mL⁻¹, *****Selectivity index (IS) = CC₅₀ (µg mL⁻¹)/IC₅₀ (µg mL⁻¹), *****ND: Not determined value of IC₅₀>50 µg mL⁻¹, Classification of antiplasmodial activity: 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 activity>50 µg mL⁻¹. Classification of cytotoxic activity: highly toxic <10 µg mL⁻¹, cytotoxic 10-40 µg mL⁻¹, moderately cytotoxic 41-100 µg mL⁻¹ and non-cytotoxic>100 µg mL⁻¹

Table 2: Antiplasmodial activity of isolated compounds of *P. piedecuestanum*

Code	Structure	IC ₅₀ (µg mL ⁻¹) X±SD*	CC ₅₀ (µg mL ⁻¹) X±SD	IS***
		Strain of <i>P. falciparum</i> NF-54**	Cell line HUVEC	
1		7.3±0.4 or 25.7 µM	>100	13.7
2		>50	>100	ND
3		>50	60.57	ND
4		>50	>100	ND
5		>50	>100	ND
6		>50	>100	ND

*X (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

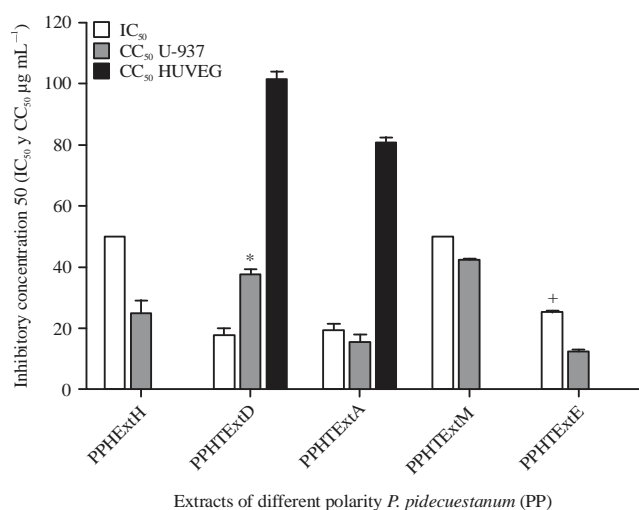


Fig. 1: Comparison of the antiplasmodial and cytotoxic activity of extracts of different polarity of *P. piedecuestanum*

The biological activity found for the extracts of the species *P. piedecuestanum* 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; IS_{HUVEC} = 2.09 and IC₅₀ = 19.5 µg mL⁻¹; IS_{HUVEC} = 0.79, even presenting better pharmacological activity than the ethanolic extract with IC₅₀ = 25.2 µg mL⁻¹; IS_{HUVEC} = 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. In

addition, when these extracts were evaluated in HUVEG 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 $CC_{50} = 101.35$; $IS_{HUVEG} = 5.65$ and $CC_{50} = 80.7$; $IS_{HUVEG} = 4.14$, 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 $IC_{50} = 7.0 \mu\text{g mL}^{-1}$ activity in *P. hostmannianum* strain (W2) chloroquine resistant²⁸ with an $IC_{50} = 8.0 \mu\text{g mL}^{-1}$, *P. umbellatum* with 70% inhibition at $40 \mu\text{g mL}^{-1}$ and *P. sarmentosum* with an $IC_{50} = 0.05 \mu\text{g mL}^{-1}$ ²⁹. *In vivo* antimalarial activity of the *Piper betle* leaf methanolic extract evaluated in *Plasmodium berghei* (NK65) infected mice has been demonstrated over a range of concentrations (50-400 mg kg^{-1})³⁰. The activity of (-)-methylenderatin was confirmed *in vivo* in mice infected with *P. vinckei petteri*, showing an 80% reduction in parasitemia at a dose of 20 mg $\text{kg}^{-1} \text{ day}^{-1}$ ³¹. It is the first time that the metabolites have been reported 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 (negletein) (4), 5-hydroxy-7-methoxyflavone (5) from *P. piedecuestanum* species and a brominated derivative from (6) named 6,8 bromo-5-hydroxy-7-methoxyflavone(7) in the species *P. piedecuestanum*. The compound (1) was the only one that presented antiplasmodial activity with an $IC_{50} = 7.325 \mu\text{g mL}^{-1}$ (25.69 μM); $IS_{HUVEG} = 13.65$. The brominated derivative of (5) also did not exhibit antiplasmodial 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 antiplasmodial activity. The compound 6-prenyl-3'-methoxyethylidethiol showed no activity on the *P. falciparum* strain. Analysis of the structure-activity ratio shows that the presence of adjacent methoxy and hydroxyl groups or the absence of adjacent hydroxyl groups could contribute to the inactivity of the 6-prenyl-3'-methoxyethylidethiol compound and the substituents in the flavanone backbone clearly influence the antiplasmodial activity³². The antiplasmodial 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 (6.7-16.9 μM) over *P. falciparum* (W2)

than flavonoid derivatives (5-33 μM)³²; 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. piedecuestanum* 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 $IC_{50} = 17.93 \mu\text{g mL}^{-1}$; $IS = 2.093$ and $IC_{50} = 19.5 \mu\text{g mL}^{-1}$; $IS = 0.791$, 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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