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Short Communication

Antiplasmodial and antileishmanial activities of compounds from *Piper tuberculatum* Jacq fruits

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Abstract

Introduction: This study assessed the activity of compounds from *Piper tuberculatum* against *Plasmodium falciparum* and *Leishmania guyanensis*. **Methods**: The effects of compounds from *P. tuberculatum* fruits on *P. falciparum* and *L. guyanensis* promastigote growth *in vitro* were determined. Hemolytic action and cytotoxicity in HepG2 and J774 cells were measured. **Results**: Three compounds showed strong antiplasmodial activity and one compound showed strong antileishmanial activity. Two compounds were non-toxic to HepG2 cells and all were toxic to J774 cells. The compounds showed no hemolytic activity. **Conclusions**: The tested compounds from *P. tuberculatum* exhibited antiparasitic and cytotoxic effects.

Keywords: Malaria. Leishmaniasis. *Piper*. Bioactivity.

Neglected tropical diseases disproportionately affect poor populations throughout the world, resulting in a severe burden within endemic regions. Among these diseases, malaria and leishmaniasis are protozoal infections with the highest number of cases and deaths[1]; therefore, studies involving new methods of interventions for these diseases are highly relevant to public health. *Piper tuberculatum*, an Amazonian medicinal plant, is a species from which many amide alkaloids have been isolated and it is used as a traditional medicine for the treatment of gastric disorders[2]. The species has a broad spectrum of biological activities, including insecticidal[3], antileishmanial[4], and trypanocidal actions[5]. Owing to its pharmacological potential, this study aimed to evaluate the antiplasmodial and antileishmanial potential of extracts, fractions, subfractions, and an isolated compound from *P. tuberculatum* against *Plasmodium falciparum* and *Leishmania guyanensis*.

Piper tuberculatum fruits (1.3kg) were collected from a central area in Porto Velho City, State of Rondônia, Brazil, and the plant material was subsequently identified at the herbarium of *Instituto Nacional de Pesquisa da Amazônia* (INPA), where an exsiccata was deposited (number 211724). The crude extract of *P. tuberculatum* fruits (40g), named PTFCE (*Piper tuberculatum* fruits crude extract), was obtained by percolation with ethanol (99%) for 3 days, followed by solvent evaporation. Some of the dried extract (38.2g) was subjected to silica gel column chromatography and eluted with hexane, chloroform, ethyl acetate, and methanol, yielding the PTFHF (*Piper tuberculatum* fruits - hexane fraction), PTFCF (*Piper tuberculatum* fruits - chloroform fraction), PTFEAF (*Piper tuberculatum* fruits - ethyl acetate fraction extract), and PTFMF (*Piper tuberculatum* fruits - methanol fraction) fractions, respectively[6]. PTFHF was then fractioned and eluted in a hexane/chloroform gradient of increasing polarity, yielding the fractions HF-1 (hexane fraction 1), HF-2 (hexane fraction 2), HF-3 (hexane fraction 3), HF-4 (hexane fraction 4), HF-5 (hexane fraction 5), HF-6 (hexane fraction 6), and HF-7 (hexane fraction 7). HF-6 presented a solid white precipitate that was dissolved in chloroform and recrystallized. The 1D and 2D ^{1}H-NMR and ^{13}C-NMR data and mass spectrum of the purified compound matched that of pellitorine, a molecule previously isolated from fruits of *P. tuberculatum* (**Figure 1**).

Human erythrocytes were used for the *P. falciparum* W2 (cloroquine-resistant Indochine strain) strain culture. The

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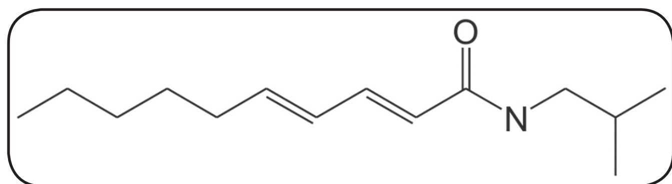

FIGURE 1: Pellitorine structure.

parasite was cultured with complete Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HEPES, 22.8mM; glucose 11.1mM; hypoxanthine, 0.36mM (50µg.mL^{-1}); $NaHCO_3$, 23.8mM), supplemented with 1% albumax and 5% hematocrit. The parasites were maintained in an incubator at 37ºC under an atmosphere of 5% O_2, 5% CO_2, and balanced N_2. The culture was subsequently synchronized with sorbitol (0.5%)[7] to maintain only ring forms and the hematocrit was adjusted to 1.5% for the tests, in which the parasitemia was 0.05%. The culture was then incubated for 48h with the *P. falciparum* culture. Triplicate experiments with concentrations between 1.56 and 100µg/mL were conducted. The negative control consisted of infected erythrocytes without treatment and the positive control consisted of serial dilutions of artemisinin from 50 to 1.56ng/mL. For all biological assays, 0.5% dimethyl sulfoxide [(DMSO) Sigma-Aldrich)] was used as the negative control. To assess the effect of the test compounds on *P. falciparum* growth, an anti-HRPII (histidine-rich protein) assay was performed[8]. Two 96-well plates were prepared: a test plate, containing the parasites and the test compounds, and another plate precoated with monoclonal antibodies against the *P. falciparum* HRPII antigen. To sensitize the plates, 100µL of primary antibody (MPFM-*Plasmodium falciparum* antibody-55A ICLLAB®, EUA) at 1.0µg/mL was added to each well. The test plates were incubated for 24h; subsequently, the background (control culture) was withdrawn and frozen at -20ºC for later use. The plate was incubated again and subjected to two freeze-thaw cycles at -80ºC to lyse the erythrocytes. After the plates were incubated and washed, 100µL of the secondary antibody (MPFG55P ICLLAB®, EUA; 1:5,000 dilution) was added to each well. The plate was incubated further, washed again three times, at which point 100µL 3,3',5,5'-tetramethylbenzidine (TMB) was added to each well. The absorbance at 450nm was measured by using a microplate spectrophotometer (BIOCHRON Model: Expert plus).

The IC_{50}, the concentration at which a compound kills 50% of the parasite population, was obtained by nonlinear curve fitting of the serial concentrations computed by Origin software (OriginLab Corporation, Northampton, MA, USA). Compounds with an IC_{50} below 10µg/mL were considered active; values of 10-25µg/mL were considered partially active and values of ≥25µg/mL were considered inactive. The percentage of parasite growth inhibition for each concentration was calculated from the following formula:

Activity (%) = 100 – [(test compounds – positive control)/(negative control – positive control)] × 100

Leishmania guyanensis promastigotes (IOCL 565) were obtained from the *Leishmania* Collection of the Oswaldo Cruz Institute – CLIOC/FIOCRUZ and cultured *in vitro* at 24°C in RPMI 1640 (Sigma) supplemented with 10% inactivated fetal bovine serum [(FBS); Gibco/Invitrogen], 2mM L-glutamine, 20mM HEPES (N-2-hydroxyethylpiperazine-N'-22, ethanesulfonic acid), and 40µg/mL gentamicin (Sigma). The promastigotes (1×10^6 parasites/180µL) were then introduced into each well of a 96-well plate containing the test compounds from *P. tuberculatum* (1.56-100µg/mL). The negative and positive controls were DMSO (0.5%) and pentamidine, respectively. The plates containing the parasites and test compounds were incubated at 24°C for 72h. After incubation, 10µL/well MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added and the plate was incubated for 4h. Subsequently, the plates containing the cultures were centrifuged for 10 min and the supernatant was discarded. DMSO (100µL/well) was then added and the plate was incubated for 1h at 24ºC[9]. The experiments were performed twice, with all samples tested in triplicate in each experiment, and a mean value was calculated. The absorbance at 570nm was measured by using a spectrophotometer. The calculated IC_{50} values were classified as previously described.

For the cytotoxicity analysis, HepG2 and J774 cells were cultured as recommended by Calvo-Calle et al.[10] and the MTT assay was used to assess cell viability[11]. The cells were seeded at 2×10^4/well and after treatment (24h for HepG2 and 72h for J774) with the test compounds (4.68-100µg/mL), 10µL MTT was added to each well. The plates were incubated with MTT for 4h at 37ºC. After incubation, the supernatant was aspirated and 100µL DMSO was added to each well. The optical density at 540nm was then determined. The negative control comprised cells in the absence of any test compound and the positive control comprised cells treated with 1% DMSO. The tests were performed twice, with samples in triplicate in each experiment, and subsequently, the CC_{50} (50% cytotoxicity concentration) was obtained by non-linear curve fitting of the serial concentration data of the tested compounds computed using Origin. The cytotoxic status of the compounds was determined based on their selectivity index (SI), which was calculated from the ratio of the CC_{50} and IC_{50} (IS = CC_{50}/IC_{50}). Compounds with an SI of < 10 were considered non-selective/toxic; compounds with an SI of ≥10 were considered selective/non-toxic. The cytotoxic effect of each compound concentration was generated by the formula:

Cytotoxicity % = 100 – [(test compounds – positive control)/(negative control – positive control)] × 100

The hemolysis assay was performed according to the method of Wang et al.[12]; the test compounds were serially diluted from 100 to 1.56µg/mL with 0.05% DMSO.

The crude extract of *P. tuberculatum* was not active against *P. falciparum* (**Table 1**). Of all fractions, only PTFCF (IC_{50} = 9.81µg/mL) was considered active. The assessment of the cytotoxicity of the compounds by the MTT colorimetric method revealed that PTFCE was toxic (SI ≤ 0.4). The previously reported isolation of β-sitosterol, stigmasterol,

TABLE 1: Biological activity of *P. tuberculatum* compounds against the W2 strain of *P. falciparum* and cytotoxic evaluation in the HepG2 cell line.

Compounds	*P. falciparum* IC_{50} (µg/mL) ± SD	HepG2 CC_{50} (µg/mL) ± SD	SI
PTFCE	≥ 100	40.5 ± 9.8	≤ 0.4
PTFHF	10.72 ± 0.3	23.05 ± 4.5	2.1
PTFCF	9.81 ± 1.9	≥ 100	≥ 10.2
PTFEAF	34.9 ± 0.2	≥ 100	≥ 2.9
PTFMF	46.46 ± 2.6	≥ 100	≥ 2.1
HF-1	20.86 ± 3.8	≥ 100	≥ 4.8
HF-2	≥ 100	30.42 ± 6.7	≤ 0.3
HF-3	16.26 ± 3.2	≥ 100	≥ 6.1
HF-4	≥ 100	6.75 ± 5.1	≤ 0.06
HF-5	7.03 ± 1.2	≥ 100	14.2
HF-7	4.13 ± 0.3	7.2 ± 0.8	1.7
Pellitorine	21.8 ± 1.7	≥ 100	≥ 4.6
Artemisinin	0.0026 ± 0.4	≥ 1,000	≥ 384.6

P. tuberculatum: *Piper tuberculatum;* ***P. falciparum***: *Plasmodium falciparum;* **W2:** cloroquine-resistant Indochina clone; **HepG2:** liver hepatocellular carcinoma; **IC_{50}**: inhibition of 50% of parasite growth; **CC_{50}:** 50% cytotoxicity concentration; **SD**: standard deviation; **DMSO:** dimethyl sulfoxide; **CC_{50}**: 50% cytotoxic concentration in mammalian cells. **SI**: selectivity index (CC_{50}/IC_{50}). The IC_{50} value of the positive control, artemisinin, was 2.6ng/mL. The solvent (0.5% DMSO) was not toxic to HepG2 cells nor to *P. falciparum* (data not shown). **PTFCE**: crude extract of *P. tuberculatum* fruits; **PTFHF**: hexane fraction of *P. tuberculatum* fruits; **PTFCF**: chloroform fraction of *P. tuberculatum* fruits; **PTFEAF**: ethyl acetate fraction of *P. tuberculatum* fruits; **PTFMF**: methanol fraction of *P. tuberculatum* fruits; **HF-1**: hexane fraction 1; **HF-2:** hexane fraction 2**; HF-3**: hexane fraction 3; **HF-4**: hexane fraction 4; **HF-5:** hexane fraction 5**; HF-7**: hexane fraction 7.

3-(3,4,5-trimethoxyphenyl) propanoic acid, piplartine, and dihydropiplartine from the chloroform fraction of *P. tuberculatum* (6) suggested that the activity of PTFCF may be attributable to one of these compounds. The analysis of the cytotoxicity also indicated that PTFCF was the only fraction considered non-toxic to HepG2 cells (SI ≥ 10.2).

Of the hexane subfractions, only HF-5 (IC_{50} = 7.03µg/mL) and HF-7 (IC_{50} = 4.13µg/mL) were considered active; HF-7 had the highest antiplasmodial activity of the test compounds (IC_{50} = 4.13µg/mL). HF-2, HF-4, and HF-7 were toxic to HepG2 cells, with SI values of ≤0.3, ≤0.06, and 1.7, respectively, and were therefore non-selective for *P. falciparum*. HF-5 was the only non-toxic subfraction and the most selective compound against *P. falciparum* (SI =14.2).

The compound pellitorine (**Figure 1**) was partially active against *P. falciparum* (IC_{50} = 21.8µg/mL), with CC_{50} ≥100µg/mL for HepG2; it may either be selective or not against this parasite, as its exact SI is unknown (SI ≥ 4.6). Weenen et al.[13] reported an IC_{50} of 20µg/mL for pellitorine on the K10 strain of *P. falciparum*; however, as cytotoxicity assays were not conducted, the authors could not assess the selectivity of this compound. Similar actions in the W2 (chloroquine-resistant) and K10 (mefloquine-resistant) strains suggested a common mode of action in both strains. Heme formation, protein synthesis, and PfDHFR (*P. falciparum* dihydrofolate reductase) activity inhibition[14] are possible molecular targets for pellitorine, as these are common mechanisms of action of antimalarial compounds.

Hemolytic assays were also performed to investigate whether the compounds inhibited *P. falciparum* growth via erythrocyte lysis. However, it was found that none of the compounds resulted any degree of hemolysis in human erythrocytes (data not shown).

To the best of our knowledge, this is the first study to report the anti-*L. guyanensis* activity of compounds from *P. tuberculatum*. The crude extract was not active against this parasite (**Table 2**) and was considered toxic to the J774 cell line (SI ≤ 1.58); the only partially active fraction was PTFCF (IC_{50} = 19.98µg/mL), which was also toxic to J774 (SI = 0.21). Ferreira et al. (4) described the isolation of 3-(3,4,5-trimethoxyphenyl) propanoic acid, obtained from the hexane/ethyl acetate (35:65) extraction of the fruits of *P. tuberculatum*, and reported an IC_{50} of 145µg/mL for this molecule against *L. amazonensis* promatigotes. In the present study, the ethyl acetate fraction was unable to inhibit the growth of *L. guyanensis*, probably owing to the antagonism of other compounds present in the fraction or to the absence or low concentration of 3-(3,4,5-trimethoxyphenyl) propanoic acid. A more detailed analysis of the phytochemical profile of this fraction is needed to confirm the content of this compound and the presence of other substances with antiparasitic action

TABLE 2: Biological activity of *P. tuberculatum* compounds against *L. guyanensis* and cytotoxicity evaluation in the J774 cell line.

Compounds	*L. guyanensis* IC_{50} (µg/mL) ± SD	J774 CC_{50} (µg/mL) ± SD	SI
PTFCE	≥ 100	63.2 ± 5.2	≤ 1.58
PTFHF	93.89 ± 8.4	≥ 100	≥ 1.06
PTFCF	19.98 ± 1.3	4.2 ± 1.3	0.21
PTFEAF	≥ 100	48.94 ± 0.9	≤ 0.48
PTFMF	≥ 100	≥ 100	---
HF-1	≥ 100	≥ 100	---
HF-2	14.4 ± 0.7	75.77 ± 0.1	5.26
HF-3	≥ 100	≥ 100	---
HF-4	10.15 ± 1.9	3.8 ± 0.9	0.37
HF-5	≥ 100	≥ 100	---
HF-7	2.75 ± 0.5	1.6 ± 0.07	0.58
Pellitorin	26.84 ± 9.4	67.8 ± 9.3	2.52
Pentamidine	0.87 ± 0.8	6.13 ± 0.9	7.04

P. tuberculatum: *Piper tuberculatum;* ***L. guyanensis****: Leishmania guyanensis;* **IC_{50}**: inhibition of 50% of parasite growth; **CC_{50}:** 50% cytotoxicity concentration; **SD**: standard deviation; **SI**: selectivity index (CC_{50}/IC_{50}); **---:** selectivity index not calculated; **CC_{50}**: 50% cytotoxic concentration in mammalian cells;**DMSO:** dimethyl sulfoxide; The IC_{50} value of the positive control, pentamidine, was 0.87µg/mL. The solvent DMSO (0.5%) showed neither antileishmanial activity nor toxicity towards J774 (data not shown). **PTFCE**: crude extract of *P. tuberculatum* fruits; **PTFHF**: hexane fraction of *P. tuberculatum* fruits; **PTFCF**: chloroform fraction of *P. tuberculatum* fruits; **PTFEAF**: ethyl acetate fraction of *P. tuberculatum* fruits; **PTFMF**: methanol fraction of *P. tuberculatum* fruits; **HF-1**: hexane fraction 1; **HF-2:** hexane fraction 2; **HF-3:** hexane fraction 3; **HF-4:** hexane fraction 4; **HF-5**: hexane fraction 5; **HF-7**: hexane fraction 7.

observed in this study. Of the subfractions, only HF-7 was considered active against the parasite (IC_{50} = 2.75µg/mL), although its effect on J774 cell viability (CC_{50} = 1.6µg/mL) led to its characterization as a toxic compound (SI = 0.58).

The purified compound pellitorine was considered inactive against *L. guyanensis* (IC_{50} = 26.84µg/mL), although the SI (2.52) suggested that the molecule was non-selective for *L. guyanensis* and the J774 cell line; however, the potential of pellitorine against amastigote forms of *L. guyanensis* should be determined, as it is the parasitic form normally found in this vertebrate organism and it is possible that the low selectivity of this molecule observed in this study for both *P. falciparum* and *L. guyanensis* could be improved with structural modifications of the molecule by using a semi-synthetic approach.

A remarkable fact observed in the present experiments was the greater susceptibility of *P. falciparum* than *L. guyanensis* to the tested compounds. This phenomenon may result from genetic plasticity, a constitutive feature of the *Leishmania* genus. It has been demonstrated that *Leishmania* spp. vary the number of chromosomal copies with changing environmental conditions, a feature that possibly plays an important role in drug resistance[15].

In conclusion, the subfraction HF-7 was the most active against the evaluated parasites, and HF-5 was the most selective for *P. falciparum*. Although the compounds exhibited activity against the parasites and were not hemolytic, there was some degree of toxicity in mammalian cell lines. This study has expanded our knowledge of the antiparasitic potential of *P. tuberculatum* and has highlighted the importance of identification of the individual substances present in the subfractions that are responsible for the observed antiprotozoal and cytotoxic effects.

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Conflict of interest

The authors declare that there is no conflict of interest.

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REFERENCES

1. Bhutta ZA, Sommerfeld J, Lassi ZS, Salam RA, Das JK. Global burden, distribution, and interventions for infectious diseases of poverty. Infect Dis Poverty. 2014;3(1):21-7.
2. Burci LM, Pereira IT, Silva LM, Rodrigues RV, Facundo VA, Militão JSLT, et al. Antiulcer and gastric antisecretory effects of

dichloromethane fraction and piplartine obtained from fruits of *Piper tuberculatum* Jacq. in rats. J Ethnopharmacol. 2013;148(1):165-74.

3. Scott IM, Jensen H, Nicol R, Lesage L, Bradbury R, Sánchesvindas LP, et al. Efficacy of *Piper* (Piperaceae) extracts for control of common home and garden insect pest. J Econ Entomol. 2004;97(4):1390-403.
4. Ferreira MGPR, Kayano AM, Silva-Jardim I, Silva TO, Zuliani JP, Facundo VA, et al. Antileishmanial activity of 3-(3, 4, 5-trimethoxyphenyl) propanoic acid purified from Amazonian *Piper tuberculatum* Jacq., Piperaceae, fruits. Rev Bras Farmacogn. 2010;20(6):103-6.
5. Regasini LO, Cotinguiba F, Passerini GD, Bolzani VS, Cicarelli RMB, Kato MJ, et al. Trypanocidal activity of *Piper arboreum* and *Piper tuberculatum* (Piperaceae). Rev Bras Farmacogn. 2009;19(1B):199-203.
6. Facundo VA, Polli AR, Rodrigues RV, Militão JSLT, Stabelli RG, Cardoso CT. Constituintes químicos fixos e voláteis dos talos e frutos de *Piper tuberculatum* Jacq. e das raízes de *P. hispidum* H. B. K. Acta Amazon. 2008;38(4):733-42.
7. Lambros C, Vanderberg J. Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. J Parasitol. 1979;65(3):418-20.
8. Noedl H, Wernsdorfer WH, Miller RS, Wongsrichanalai C. Histidine-rich protein II: a novel approach to malaria drug sensitivity testing. Antimicrob Agents Chemother. 2002;46(6):1658-64.
9. Pal D, Bhattacharya S, Baidya P, Dey BK, Pandey JN, Moulisha B. Antileishmanial activity of *Polyalthia* leaf extract on the *in vitro* growth of *Leihmaniose donovani* promastigotes. Global J Pharmacol. 2011;5(2):97-100.
10. Calvo-Calle JM, Moreno A, Eling WMC, Nardin EH. *In vitro* development of infectious liver stages of *P. yoelii* and *P. berghei* malaria in human cell lines. Exp Parasitol. 1994;79(3):362-73.
11. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 1983;65(1):55-63.
12. Wang C, Qin X, Huang B, He F, Zeng C. Hemolysis of human erythrocytes induced by melamine-cyanurate complex. Biochem Biophys Res Commun. 2010;402(4):773-7.
13. Weenen H, Nkuya MHH, Bray DH, Mwasumbi LB, Kinabo LS, Kilimali VAEB, et al. Antimalarial compounds containing an α, β-unsaturated carbonyl moiety from Tanzanian medicial plants. Planta Med. 1990;56(4):371-3.
14. Burrows JN, Chibale K, Wells TNC. The state of the art in anti-malarial drug discovery and development. Curr Top Med Chem. 2011;11(10):1226-54.
15. Mannaert A, Downing T, Imamura H, Dujardin J. Adaptive mechanisms in pathogens: universal aneuploidy in *Leishmania*. Trends Parasitol. 2012;28(9):370-6.