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CHEMICAL COMPOSITION AND BIOLOGICAL ACTIVITIES OF THE LEAF EXTRACT AND ESSENTIAL OIL FROM *Piper alatabaccum* (Piperaceae) FRUITS

COMPOSIÇÃO QUÍMICA E ATIVIDADES BIOLÓGICAS DO EXTRATO DE FOLHAS E ÓLEO ESSENCIAL DE FRUTOS DE *Piper alatabaccum* (Piperaceae)

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ABSTRACT

This study aimed to obtain the chloroform leaf extract, evaluate its biological activity, and assess the chemical composition and activities of the essential oil from the fruit. The crude leaf extract from *Piper alatabaccum* was obtained using maceration (chloroform), followed by rotaevaporation. The fruit's essential oil was obtained through steam distillation using a system adapted for 3h and analyzed using GC/MS. The major constituents identified in the leaf chloroform extract of the species were the amides piperovatine and pipercallosidine. The major compounds of the oil were sesquiterpenes (45.83%), phenylpropanoids (20.83%), and monoterpenes (16.67%). Following the recommendations found in the literature, the crude extract was considered partially active, non-toxic on HepG2 and VERO cell lines, and selective against *P. falciparum*. It did not present antimicrobial activity; neither was it toxic against cancer cell lines. The essential oil was considered inactive, non-toxic at the highest concentration tested against HepG2 and VERO cell lines, and was non-selective regarding antiplasmodial activity. It did not present significant activity against the bacteria *P. mirabilis*, but was considered toxic against PC3 (prostate cancer cells) and HL60 (leukemia cells) cancer cell lines.
Keywords: Piperaceae; biological activity; *Piper*.

RESUMO

Este trabalho teve como objetivo obter o extrato clorofórmico das folhas, avaliar sua atividade biológica e analisar a composição química do óleo essencial dos frutos e suas atividades. O extrato bruto das folhas de *Piper alatabaccum* foi obtido por maceração (clorofórmio), seguido por rotoevaporação. O óleo essencial foi obtido por destilação a vapor usando um sistema adaptado por 3h e foi analisado por CG/EM. Os constituintes majoritários identificados no extrato clorofórmico da espécie foram às amidas piperovatina e pipercalosidina. As

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principais classes encontradas no óleo foram sesquiterpenos (45,83%), fenilpropanóides (20,83%) e monoterpenos (16,67%). Seguindo as recomendações da literatura, o extrato bruto foi considerado parcialmente ativo, não tóxico para células HepG2 e VERO, e seletivo contra *P. falciparum*. O mesmo não apresentou atividade antimicrobiana e nem foi tóxico para as linhagens celulares. O óleo essencial foi considerado inativo, não tóxico na maior concentração testada contra as células HepG2 e VERO, e não foi seletivo em relação à atividade antiplasmodial. O mesmo não apresentou atividade contra a bactéria *P. mirabilis*, mas foi considerado tóxico para as linhagens celulares PC3 (células de câncer de próstata) e HL60 (células leucêmicas).

Palavras-chave: Piperaceae; atividade biológica; *Piper*

1. INTRODUCTION

Piper alatabaccum is popularly known as "joão brandinho" and can be found in the Brazilian Amazon, in the states of Amazonas, Amapá, Pará and Rondônia [1,43]. Its roots are commonly used as local anesthetics, probably due to the presence of amides in its chemical composition [3,4]. The inflorescence is erect, spike, with triangular, peltate bracts [5].

In the phytochemical study of *P. alatabaccum* leaves and stalks, it was possible to isolate and identify five compounds: three amides (piperovatine, 8,9-dihydropiplartine and piplartine) and two flavones (5,5',7-trimethoxy-3', 4'-methylenedioxyflavone and 3', 4', 5, 5', 7-pentamethoxyflavone) [6].

A literature review of the species *P. alatabaccum* showed that the following biological assays have already been carried out: larvicidal (*Anopheles darlingi*) with extracts and isolated substances (piplartine, dihydropiplartine and 5,5',7-trimethoxy-3', 4'-methylenedioxyflavone); insecticidal (against *Hypothenemus hampei*) with the acetone extract; evaluation of the larvicidal activity of the leaf's ethanolic extract, ethyl acetate fraction of the leaves, ethanolic extract from the roots, and the leaf's essential oil; the substances isolated from the leaves (piplartine and 5,5', 7-trimethoxy-3',4'-methylenedioxyflavone) and the major constituent of the leaf's essential oil, dillapiole, were tested against *Aedes aegypti*. An antifungal activity assay of the leaf's essential oil was also carried out [7-8; 3-4]. This study aimed to characterize the essential oil from the fruits, prepare the leaf chloroform extract, carry out phytochemical screening of the extract, and evaluate the biological activities of the samples.

2. MATERIALS AND METHODS

2.1 PLANT COLLETION AND EXTRACTION

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Fruits and leaves of *P. alatabaccum* were collected in the municipality of Porto Velho - Rondônia, in February (Amazonian winter) at BR 319, Km 15.5 towards Porto Velho - Humaitá (08º38'44.38" south latitude, 63º59'42.55" west longitude). A specimen of the species is deposited in the INPA herbarium under number 211.711. Extraction of the essential oil was carried out in the Laboratory of Organic Chemistry and Natural Products at IFRO - Porto Velho-Calama Campus. The fruits (189 g) were subjected to steam distillation using a system adapted for 3 h. The hydrolate was separated and subsequently subjected to filtration in a glass funnel with cotton and anhydrous sodium sulfate. A volume of 2.2 mL was obtained. The oil was stored in a 13 mL glass flask, wrapped in foil to protect it from light, sealed, and preserved [26].

1.22 Kg of plant material (leaves) was dehydrated, macerated, placed in an Erlenmeyer flask and subsequently extracted using the solvent chloroform for approximately three days at room temperature. The extracts were concentrated through rotaevaporation. The extract, upon concentration, weighed about 28.9 g. Aliquots of the oil and extract were sent for chromatographic analysis to Fundação Oswaldo Cruz (FIOCRUZ-RJ), Rio de Janeiro-RJ, Brazil.

2.2 ANALYSIS OF THE CHEMICAL COMPOSITION OF THE ESSENTIAL OIL AND CRUDE EXTRACT

Ionization detector by gas chromatography

Qualitative and quantitative analyses were performed on a Shimadzu GC 2010 gas chromatograph with a DB-5MS fused silica capillary column (30 m x 0.25 mm x 0.25 µm film thickness). The operating parameters were as follows: injector temperature: 280 °C; detector: 290 °C and oven temperature: 60-290 °C (10 °C/min). Split ratio: 20:1. Hydrogen was used as the carrier gas at 1.0 mL min^{-1}. Percentages of the compounds were obtained using the relative peak areas in the chromatogram.

Gas Chromatography-Mass Spectrometry

Qualitative analyses were performed on a Shimadzu GC-QP2010 PLUS apparatus with a ZB-5MS fused silica capillary column (30 m x 0.25 mm x 0.25 µm film thickness) under the experimental conditions reported for GC-FID analysis. The constituents of the essential oil were identified by comparing their retention indices and mass spectra with published data, along with computer matching with WILEY 275 and the National Institute of

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Standards and Technology (NIST 3.0) libraries provided by a GC-MS system controlled by a computer.

2.3 PHYTOCHEMICAL SCREENING

Phytochemical screening aims to assess the presence of classes of substances in plant extracts, orienting further steps of chromatographic fractioning. The analysis was performed following Barbosa's [9] guidelines for the presence of alkaloids, cardiac glycosides, catechins, flavonoids, phenols, tannins, saponins, and triterpenoids.

2.4 ANTIPLASMODIAL ACTIVITY

Antiplasmodial assays were carried out at Plataforma de Bioensaios de Malária e Leishmaniose – PBML – FIOCRUZ-RO, in the municipality of Porto Velho, state of Rondônia, Brazil. The project was approved by the local Ethics and Research Committee (CEP) under protocol number: 83791418.8.0000.5300, case: 2.541.143.

P. falciparum Culture

Erythrocytic forms of *P. falciparum* (W2 strain) were used in the antiplasmodial assays. The parasites were cultured in O^+ red blood cells in RPMI 1640 medium (Gibco) supplemented with 25 mM HEPES, 300 μM hypoxanthine, 11 mM glucose, 40 μg/mL 10% (v/v) gentamicin, and O^+ plasma or 5% albumax (Gibco) under conditions established by Trager, Jensen [42]. The erythrocytes added to the culture were obtained from a single volunteer donor, with a final hematocrit of 4%. The culture flasks were maintained in an incubator at 37° C with the addition of a gaseous mixture (5% CO_2 + 5% O_2 + 90% N_2). After a period of 48 hours, the culture medium was replaced.

Determination of 50% parasite growth inhibition (IC_{50})

The substances were diluted in dimethylsulfoxide (DMSO), not exceeding a concentration of 0.5%. The serial dilution started from an initial concentration of 200 μg/mL in the first well. Cultures were synchronized until they reached the desired minimal parasitemia of 8% of young trophozoites, as described by Lambros, Vanderberg [10].

Subsequently, the parasite culture with a predominance of ring forms was adjusted to 0.5% parasitemia and 2% hematocrit. This way, both the oil and extract were diluted 10x, starting with serial concentrations (200 to 3.125 μg/mL). Three controls were used: artemisinin (50 ng/mL) was used as the killing control, untreated infected erythrocytes were

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used a growth control and uninfected erythrocytes were used as a blank (whose fluorescence was discounted from all other values obtained).

Plates were incubated at 37° C with 5% CO_2 for 48 hours. Thereafter, the supernatant was discarded without suspending the red cells. To the latter, 200 µL/ well of 1x PBS was added, and the suspension was centrifuged at 1,500 rpm for 10 minutes. In parallel, 200 µL of 1x PBS was added to a flat bottom 96-well culture plate for fluorescence reading. After the plate was centrifuged, the supernatant was discarded again. In the plate containing the culture, 200 µL of lysis buffer (20 mM Tris-HCl, 5 mM EDTA, 0.08% Triton X-100, 0.008% saponin in 1x PBS, pH 7.5) containing SYBR Green I (Invitrogen) was added at the following ratio: 2 µL of Sybr green I dye to 10 mL of the lysis buffer solution. The mixture was homogenized and 200 µL of it transferred to the reading plate where 200 µL of 1x PBS were previously added.

The plate was incubated for 30 minutes at room temperature. Afterwards, a UV/ visible spectrophotometer reading (Synergy HT (BioTek) was performed, with excitation and emission centered at 485 nm and 535 nm, respectively, and a gain parameter equal to 100. The concentrations at which the compounds caused 50% parasite growth inhibition (IC_{50}) were determined by the dose-response curve of the triplicate mean for each compound tested [11].

2.5 CYTOTOXICITY ASSAY USING THE MTT METHOD

The HepG2 cell line (human hepatocarcinoma), which is related to drug metabolism, was used to assess the cytotoxicity of the test compounds. Cells were grown in RPMI medium supplemented with 10% Fetal Bovine Serum (Gibco) and 40 mg/L gentamicin (complete medium) in a 5% CO_2 incubator at 37° C. The culture was monitored daily through light microscopy, and the medium was exchanged whenever necessary until 90% confluence was obtained, a percentage that indicates initiation of the cytotoxicity assay [12].

After reaching the desired confluence, the HepG2 cells were detached from the bottom of the flask using 1x Trypsin (Sigma-Aldrich). They were then centrifuged (1500 rpm for 10 minutes), the supernatant removed, and the pellet resuspended in 1 mL of complete medium.

An aliquot of 10 µL from this volume was removed for a cell count in Neubauer's chamber under optical microscopy and at the volume was adjusted to $1x10^4$ cells/well. Afterwards, the cells were plated in 96-well plates and incubated for 24 h at 36° C. The oil and extract were distributed separately on the plate in triplicate using the serial dilution

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method (400 to 3.125 µg/mL). The culture was incubated again (48 h). Cells treated with a 20 mM Tris lysis buffer (Sigma Aldrich), 5 mM EDTA (Dynamic), saponin 0.008% w/v, and Triton x-100 0.08% v/v (Sigma-Aldrich). After the treatment period, the assay was revealed using the MTT technique ([3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide]) [13]. This technique evaluates cell viability through mitochondrial dehydrogenase, present only in metabolically viable cells. After the addition of MTT (Sigma-Aldrich) at 5 mg/mL, the plate was incubated for 4 h, the supernatant discarded immediately, and 100 µL of dimethyl sulfoxide DMSO (Dynamic) was added to each well for dissolution of the formazan crystals formed (violet color). A spectrophotometer (Biochrom, Asys Expert Plus) was used to read the absorbance (570 nm) of the test plate. Then, Origin 9.1 software (Origin Lab Corporation, Northampton, MA, USA) was used to determine the cytotoxic concentration for 50% of the cell population (CC_{50}). This cytotoxicity assay was carried out at PBML – FIOCRUZ-RO, Brazil.

2.6 CYTOTOXICITY ASSAY USING THE ALAMAR BLUE METHOD

The oil was assessed for cytotoxicity using the VERO (ATCC® CCL-81™), HELA (ATCC® CCL-2) and THP-1 (ATCC® TIB-202 ™) cell lines; this assay was carried out at the Leônidas and Maria Deane Institute (ILMD) – Fundação Oswaldo Cruz (FIOCRUZ), Manaus-AM. Brazil. VERO and HELA cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) while THP-1 cells were cultured in RPMI-1640 medium (Sigma), supplemented with 10% inactivated fetal bovine serum (Gibco) and the antibiotics penicillin and streptomycin (50 µg/mL) (Gibco). The assays were carried out using the Alamar Blue method according to Ahmed et al. [14].

Cells were plated at a concentration of 1.0 x 10^4 cells/well in 96-well plates. The oil and extract were distributed separately on the plate, in triplicate, using the serial dilution method (200 to 25 µg/mL). The plates were maintained in a CO_2 incubator for 24 hours at 5% CO_2 and 37°C. After this period, 10 µL of 0.4% Alamar blue (dilution 1:20) was added to each well. After the 2-hour metabolization period, the fluorescence was monitored on a microplate reader (Multimode DTX 800 Beckman Coulter (USA) at the following wavelengths: 540 nm for emission and 585 nm for excitation).Cell growth was used as the positive control; DMSO 0.1% was the negative control; doxorubicin (Sigma-Aldrich, Brazil) was tested as a positive control at the same concentrations as those of the samples. Percentage of cell viability was calculated according to the following formula: %Viability = Ft x 100 /

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Fb, where Ft= (cell fluorescence + medium + sample + Alamar blue) and Fb = (cell fluorescence + medium + Alamar blue). Then, Origin 9.1 software (Origin Lab Corporation, Northampton, MA, USA) was used to determine the cytotoxic concentration of 50% of the cell population (CC_{50}).

2.7 DETERMINATION OF THE SELECTIVITY INDEX (SI): CRITERIA FOR ANTIPLASMODIAL ACTIVITY

Since they are a mixture of substances, crude extracts and essential oils followed the criteria proposed by Valdés et al. [15]: if they exhibited an IC_{50} > 100 µg/mL for *P. falciparum*, they were considered inactive. An extract and essential oil showing an IC_{50} value of <100 µg/mL against *P. falciparum* were classified as follows: inactive if SI <4, partially active if SI = 4-10 and active if SI> 10.

2.8 ANTIMICROBIAL ACTIVITY

The antimicrobial activity assays were performed at the Leônidas and Maria Deane Institute (ILMD) – Fundação Oswaldo Cruz (FIOCRUZ), Manaus-AM, Brazil. The strains used belong to the microbial culture of the Biotechnological Bioassay Platform (RPT11H). The oil and the extract were solubilized in 10% dimethylsulfoxide (DMSO) at an initial concentration of 5 mg/mL.

For the evaluation of activity against Gram-negative bacteria, the following strains were used: *Acinetobacter baumanii* (ATCC 19606-143), diffusely adherent *Escherichia coli* DAEC (BUTANTÃ F1845), enteroaggregative *E. coli* EAEC (CDC EDL- O42), enterohemorrhagic *E. coli* EHEC (CDC EDL-933-171), enterotoxigenic *E. coli* ETEC (ST8), enterotoxigenic *Escherichia coli* ETEC (BUTANTÃ LT 2871), enteropathogenic *E. coli* EPEC (ATCC E234869), enteroinvasive *E. coli* EIEC (ATCC 1381), non-diarrheagenic *E. coli* (ATCC 10536), *Klebsiella pneumoniae* (ATCC 4352-083), *Pseudomonas aeruginosa* (ATCC 29336), *Proteus mirabilis* (ATCC 15290-095), and *Serratia marcescens* (ATCC 14756-131). The Gram-positive strains tested were: *Staphylococcus aureus* (ATCC 80958), methicillin-resistant *Staphylococcus aureus* (ATCC 33591) and *Staphylococcus simulans* (ATCC 27851).

2.9 STANDARDIZATION OF BACTERIAL INOCULA

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The strains were previously cultured in Brain Heart Infusion (BHI) broth (HIMEDIA). The microbial cultures were then diluted in culture medium according to the 0.5 McFarland scale (1.5×10^8 CFU mL). All assays were performed in triplicate.

Antibacterial activity using the agar well diffusion method

Determination of the antibacterial activity was performed using the agar and well diffusion techniques, according to Grove and Randall [16], with modifications. The samples tested were solubilized in 10% dimethylsulfoxide (DMSO). The culture medium Müller Hinton Agar (AMH) (HIMEDIA) was used for the experiments. As a positive control, 500 µg/mL of the drug TIENAM (imipenem + cilastatin sodium) was used. The plates were incubated at 37° C for 24 hours, and then a 0.01% triphenyl tetrazolium chloride (CTT) dye solution plus 0.1% bacteriological agar was added to the plates, with reincubation for 30 minutes. After the staining conversion in the overlay, the inhibition halos were measured.

The presence of inhibition halos ≥ 10 millimeters was used as a criterion to determine the antimicrobial activity of the oil and the extract [17-18]. The minimum inhibitory concentration was only performed for the samples whose activity in the agar diffusion test was ≥ 10 mm.

2.10 DETERMINATION OF THE MINIMUM INHIBITORY CONCENTRATION (MIC) USING THE BROTH MICRODILUTION METHOD

The MIC values of the active samples in the agar diffusion test were determined using the microdilution method in 96-well plates, according to CLSI [19], with some modifications. Each well received a standardized bacterial inoculum according to the 0.5 MacFarland scale, and decreasing concentrations of treatments ranging from 500 to1.95 µg/mL were evaluated. 20 µL of 0.01% Alamar blue solution (Sigma-Aldrich, Brazil) (INLAB) was added, totaling a volume of 100 µL/well. Bacterial inoculum was used as a microbial growth control; 10% DMSO was used as a negative control, since it was the solvent used in the treatments. The drug used as a positive control was TIENAM, which was tested at the same concentrations as the extract and the oil. The plates were incubated at 37° C for 24 h. The plates were then incubated at 37° C for 24 h. The MIC was defined as the lowest concentration of the samples capable of inhibiting bacterial growth, indicated by the persistence of the blue color of Alamar blue, without conversion to the pink coloration, demonstrating non-cell viability and the consequent lack of conversion of the dye to resazurin.

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2.11 CRITERIA FOR ANTIMICROBIAL ACTIVITY

The criteria suggested by Holetz et al. [20] for plant extracts were used for essential oils since they are a mixture of substances just like crude plant extracts. The criteria according to the authors are: less than 100 μg/mL - strong antimicrobial activity, between 100 and 500 μg/mL - moderate antimicrobial activity, between 500 and 1000 μg/mL - weak antimicrobial activity and above 1000 μg/mL - inactive. They conclude that plant extracts with antimicrobial activity at concentrations ≥ 1000 μg/mL in the MIC tests are considered inactive and of low interest in the development of antibacterial drugs.

2.12 *IN VITRO* CYTOTOXIC ACTIVITY AGAINST FOUR CANCER CELL LINES

The tests were performed at the National Laboratory of Experimental Oncology, Federal University of Ceará - UFC, Fortaleza-CE, Brazil. The HCT-116 (human colon cancer cells), SNB-19 (Glioblastoma cells), PC3 (Prostate cancer cells), HL60 (Leukemia cells) and MCF-7 (breast cancer cells) lines were donated by the National Cancer Institute (USA) having been cultured in RPMI 1640 medium, supplemented with 10% fetal bovine serum, and 1% antibiotics, maintained in an incubator at 37° C and an atmosphere containing 5% CO_2. The samples (chloroform extract and essential oil) were diluted in DMSO in order to create stock concentrations at 20 mg/mL. For the single concentration test, the samples were tested at 100 μg/mL.

Cells were plated at concentrations of 0.7 x 10^5, 0.1 x 10^6, 0.3 x 10^6 cells/mL for the HCT-116, SNB-19/PC3 and MCF-7/HL60 cell lines, respectively. The plates were incubated with the substance for 72 hours in a 5% CO_2 incubator at 37° C. At the end of this process, they were centrifuged and the supernatant was removed. Then, 150 μL of the MTT solution (tetrazolium salt) was added and the plates were incubated for 3 h. After incubation, the plates were centrifuged again for removal of the MTT solution. Absorbance was read after the dissolution of the formazan precipitate with 150 μL of pure DMSO in a plate spectrophotometer at 595 nm. Single concentration experiments were analyzed according to the mean ± standard deviation (SD) of the percentage of cell growth inhibition using GraphPadPrism software.

3. RESULTS AND DISCUSSION

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3.1 PHYTOCHEMICAL SCREENING AND COMPOSITION ANALYSIS OF THE EXTRACT

The results of the phytochemical screening of the chloroform extract indicated the presence of the following classes of compounds (Table I)

Table I. Phytochemical screening of the chloroform extract from *P. alatabaccum*

CLASSES OF SUBSTANCES	PRESENCE/ ABSENCE	COLOR
Alkaloids		
Mayer reagent	Negative	
Wagner reagent	Positive	Light orange
Dragendorff reagent	Positive	Bright orange
Cardiotonic Glycosides		
Kedde reagent	Negative	
Catequines		
	Negative	
Flavonoids		
	Positive	Pink
Phenols/Tannins		
Phenols	Positive	Red
Hydrolysable tannins	Negative	
Condensed tannins	Positive	Green
Saponins		
	Negative	
Triterpenoids		
	Positive	Green

The major chemical constituents identified in the *P. alatabaccum* leaf extract were piperovatine (retention time = 21.296 min, Area = 8480870, and % Area = 1.04) and pipercallosidine (retention time = 22.143 min, Area = 11781133, and % Area = 1.44) (Figure S1). In the phytochemical investigation on the leaf crude extract from a previous study, three amides (piperovatine, 8,9-dihydropiplartine, and piplartine) and two flavones (5,5', 7-trimethoxy-3', 4'-methylenedioxyflavone and 3',4',5,5',7-pentametoxiflavone) were isolated [6], which are also present in roots and fruits [2].

3.2 COMPOSITION OF THE FRUIT'S ESSENTIAL OIL

The chemical composition of the essential oil from the *P. alatabaccum* fruit was analyzed using GC-MS. The presence of sesquiterpenes (45.83%), phenylpropanoids (20.83%), and monoterpenes (16.67%) were observed (Figure S2 and Table S1). Some of the

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constituents described have already been identified in the essential oil from leaves of *P. alatabaccum* and other species of *Piper*, with a higher yield of some substances and a lower yield of others [8,4]. This is the first report on the constituents of the essential oil from *P. alatabaccum* fruit. Interestingly, two of the major compounds of the fruit's essential oil were dillapiole (34.81% area) and myristicin (26.48% area). Myristicin is a phenylpropanoid which was not identified in the essential oil of *P. alatabaccum* leaves previously investigated by Nascimento [8] and Santana [4]. Myristicin has already been identified in some *Piper* species; for example, in *P. aduncum* leaves [20] and in *P. hispidinervum* leaves and stems [21]. In the study carried out by Schindler [22], the author points out that myristicin was observed only in the reproductive organs and fruits (6.2 to 11.9%) of *P. gaudichaudianum*.

Dillapiole is not cited as the major compound by Nascimento [8], and in a study by Santana [4] it appears in a lower amount than in the present study. According to Dudareva et al. [23], Gobbo-Netto and Lopes [24], factors such as soil, seasonality, temperature, ultraviolet radiation, altitude, air pollution, pathogens, etc. might influence the variation, yield, and chemical composition of the compounds present in essential oils. These changes reflect the differences found when comparing different studies and different species.

3.3 ANTIPLASMODIAL ACTIVITY - INHIBITORY CONCENTRATION AND CYTOTOXIC CONCENTRATION (IC_{50} and CC_{50})

Following the recommendations in the literature, the crude extract was considered partially active, non-toxic (at the highest concentrations) against the HepG2 and VERO cell lines. The extract was also considered partially selective for antiplasmodial activity. The essential oil was considered inactive (>100 µg/mL), nontoxic at the highest concentration tested against HepG2 and VERO, and not selective for antiplasmodial activity (Table II).

Table II. IC_{50} and CC_{50} of the crude extract and essential oil from *Piper alatabaccum* (µg/mL)

	IC_{50} *P. falciparum* 48h	**CC_{50} HepG2 48h**	**SI**	**CC_{50} VERO 24h**	**SI**	**CC_{50} HELA 24h**	**SI**	**CC_{50} THP-1 24h**	**SI**
Crude extract	33.0	254.95	7.72	>200	> 6	ND	-	ND	-
Essential oil	146.8	≥400	>2.72	>200	> 1.36	26.30	> 0.17	ND	-
Artemisinin	12.7 ng/mL	≥ 1,000	>78.74	ND	-	ND	-	ND	-

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ng/mL

ND: Not determined. (-) No data to determine.

Oliveira et al. [25] analyzed the antiplasmodial activity with crude extracts of *Piper tuberculatum*. The investigation revealed that only the extract named PTFCF (chloroform fraction) was considered active (IC_{50} = 9.81 μg/mL). The chloroform fraction was also considered the only one that was not toxic to HepG2 cells (SI ≥ 10.2).

A study carried out by Dill [26] evaluated the antiplasmodial activity of the essential oils from *P. hispidum* stalks at the concentrations of 12.5 μg/mL, 25 μg/mL, 50 μg/mL, and 100 μg/mL. The oil inhibited the parasite growth by about 7.61% at 12.5 μg/mL. At 25 μg/mL the inhibition rose to 17.17%. At 50 μg/mL, the percentage of inhibition increased to 58.31%, characterizing a dose-dependence of exponential nature.

Lima [27] showed that essential oils from the *Piper* species under investigation were cytotoxic to the HepG2 and HL60 cell lines. It is possible that the cytotoxicity of some essential oils may be related to their concentrations and the time of exposure, which can often produce toxic effects since they comprise a mixture of compounds acting in combination.

3.4 ANTIMICROBIAL ACTIVITY - DIFFUSION IN AGAR AND MINIMUM INHIBITORY CONCENTRATION

The extract showed no activity in the agar diffusion test. It was possible to observe only a mild effect on microbial growth in the agar diffusion test at 5 mg/mL for the oil against *P. mirabilis* (Table III).

Table III. Results of the susceptibility test from substances and oil tested against six species of Gram-negative bacteria and two species of Gram-positive bacteria*, evaluated using the agar diffusion method.

BACTERIA	OEFPA	CHLOROFORM CE	POSITIVE CONTROL TIENAM
K. peneumoniae	-	-	21.33±0.57
P. mirabilis	10±2.00	-	26.33±0.57
S. marcescens	-	-	28±0.0
A. baumannii;	-	-	30.33±0.57
P. aeruginosa	-	-	19.33±0.57
Diffusely adherent *E. coli* (DAEC);	-	-	33±1.00
Enterotoxigenic LT	-	-	32±1.00

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E. coli Enterotoxigenic ST	-	-	31±1.00
E. coli INV- enteroinvasive	-	-	32±0.1
E. coli enteropathogenic *E. coli* (EPEC)	-	-	32±0.1
enteroaggregative *E. coli*	-	-	31±0.9
Non-diarrheagenic *E. coli*	-	-	32±0.1
enterohemorrhagic *E. coli*	-	-	31±0.1
Methicilin resistant *S. aureus**	-	-	32±1.0
*S. aureus**	-	-	35±1.0
*S. simulans**	-	-	34±1.0

OEFPA: Essential oil from *P. alatabaccum* fruits; CE: Chloroform crude extract from *P. alatabaccum* leaves. (-) There was no inhibition.

The essential oil did not present activity against the bacteria *P. mirabilis* at the initial concentration of 500 µg/mL in the minimum inhibitory concentration test (Table IV).

Table IV. Minimum Inhibitory Concentration results from the essential oil tested against *P. mirabilis*, at an initial concentration of 500 µg/mL.

Species	**Samples**	*P. mirabilis*
Piper alatabaccum	OEFPA	0
Positive control	TIENAM	250 µg/mL

OEFPA: Essential oil from *P. alatabaccum* fruits; (-): Not evaluated

Henriques, Danielli, Apel [28] cite a review by Janssem et al. [29], on the antimicrobial activities of essential oils and the aspects of testing methods. The authors state that the liposolubility of the essential oils may influence the tests, preventing their diffusion in the agarose medium. They also point out that several factors may influence the results of antimicrobial activity observed for the essential oils, such as the volatility of the compounds during the period of time they remain in the greenhouse, which might result in false positives and false negatives.

3.5 *IN VITRO* CYTOTOXIC POTENTIAL IN FOUR TUMOR CELL LINES

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The extract did not show a significant percentage of cell growth inhibition. The essential oil showed a percentage of inhibition above 90% against the evaluated cell lines. Details of the tested samples' cytotoxic activity are shown in Table V.

Table V. Percentage of cell cytotoxic concentration (CC) and the standard deviation (SD) of the samples at 100 µg/mL

SAMPLES	MCF-7		HCT116		PC3	
	CC (%)	SD	CC (%)	SD	CC (%)	SD
OEFPA	96.42	0.86	98.87	0.083	97.76	0.67
CHLOROFORM CE	43.14	2.71	51.40	2.59	ND	ND

OEFPA: Essential oil from *P. alatabaccum* fruits; CE: Chloroform crude extract from *P. alatabaccum* leaves; ND: Not determined.

IC_{50} analysis was performed only for the samples with a percentage above 75% of cell growth inhibition. For the IC_{50} tests, the sample was tested in serial dilution against HL60, MCF-7, HCT-116, SNB-19, and PC3 cell lines at a maximum concentration of 100 µg/mL (Table VI).

TABLE VI. IC_{50} values of OEFPA for tumor cell lines.

MTT - IC_{50} µg/mL (confidence interval)					
Sample	**HL60**	**MCF-7**	**HCT-116**	**SNB-19**	**PC3**
OEFPA	32.39 (26.82 – 39.11)	50.79 (44.97 – 57.35)	65.89 (59.87 – 72.53)	50.30 (43.51 – 58.14)	29.99 (25.12 – 35.82)

OEFPA: Essential oil of *P. alatabaccum* fruits. The IC_{50} value was obtained by non-linear regression from two independent experiments, performed in duplicate.

It was possible to observe that the oil presented cytotoxic potential against the PC3 and HL60 cell lines. For the prostate cancer cells, the IC_{50} was 29.99 µg/mL, while for the leukemia cell line, the value obtained was 32.39 µg/mL. For the other cell lines, IC_{50} values ranged from 47.68 to 70.36 µg/mL (Table VI).

Essential oils have been widely used in the food, agronomic, sanitary, and cosmetic industries. In recent years, interest by the pharmaceutical industry has also been evidenced

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since a variety of tests have demonstrated interesting biological activities. The major compounds arouse the greatest interest since there is an evident relation between the biological activity and this compound [30].

There are several references that also describe the biological properties attributed to essential oils, some of them which are astringent, antimicrobial, analgesic, antidepressant, antipyretic, antiviral, stimulant, immunostimulating and others. With respect to terpene compounds, the most commonly investigated activities are herbicidal, antimicrobial, cytotoxic, cytostatic and antitumor [31-41].

4. CONCLUSIONS

The phytochemical screening of the chloroform extract showed positive results for alkaloids, flavonoids, phenolic compounds, and tannins. The chromatographic profile revealed the presence of two amides. The antiplasmodial activity was considered partial, and no antimicrobial nor cytotoxic activity against the cancer cell lines was observed.

The chemical composition of the essential oil from *P. alatabaccum* fruits showed a great variety of compounds. Some of these compounds have not been identified in samples of this specie's leaf essential oil in previous investigations. The tests indicated that the essential oil from *P. alatabaccum* fruits showed no significant antiplasmodial and antimicrobial activity against the evaluated microorganisms. The oil was shown to be cytotoxic against prostate and leukemia cell lines.

Future studies are suggested to isolate, purify, and evaluate the biological activity of the major compounds present in the chloroform extract and the essential oil.

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SUPPLEMENTARY MATERIAL

Figure S1. CG-MS spectra of the chloroform extract from *P. alatabaccum* leaves.
Figure S2. CG-MS spectra of *P. alatabaccum* fruits.
Table S1. Results obtained through the GC-MS analysis of the essential oil from *P. alatabaccum* fruits.

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BIOLOGICAL ACTIVITIES OF *Piper alatabaccum*

SUPPLEMENTARY MATERIAL

FIGURE S1

CG-MS spectra of the *P. alatabaccum* chloroform extract

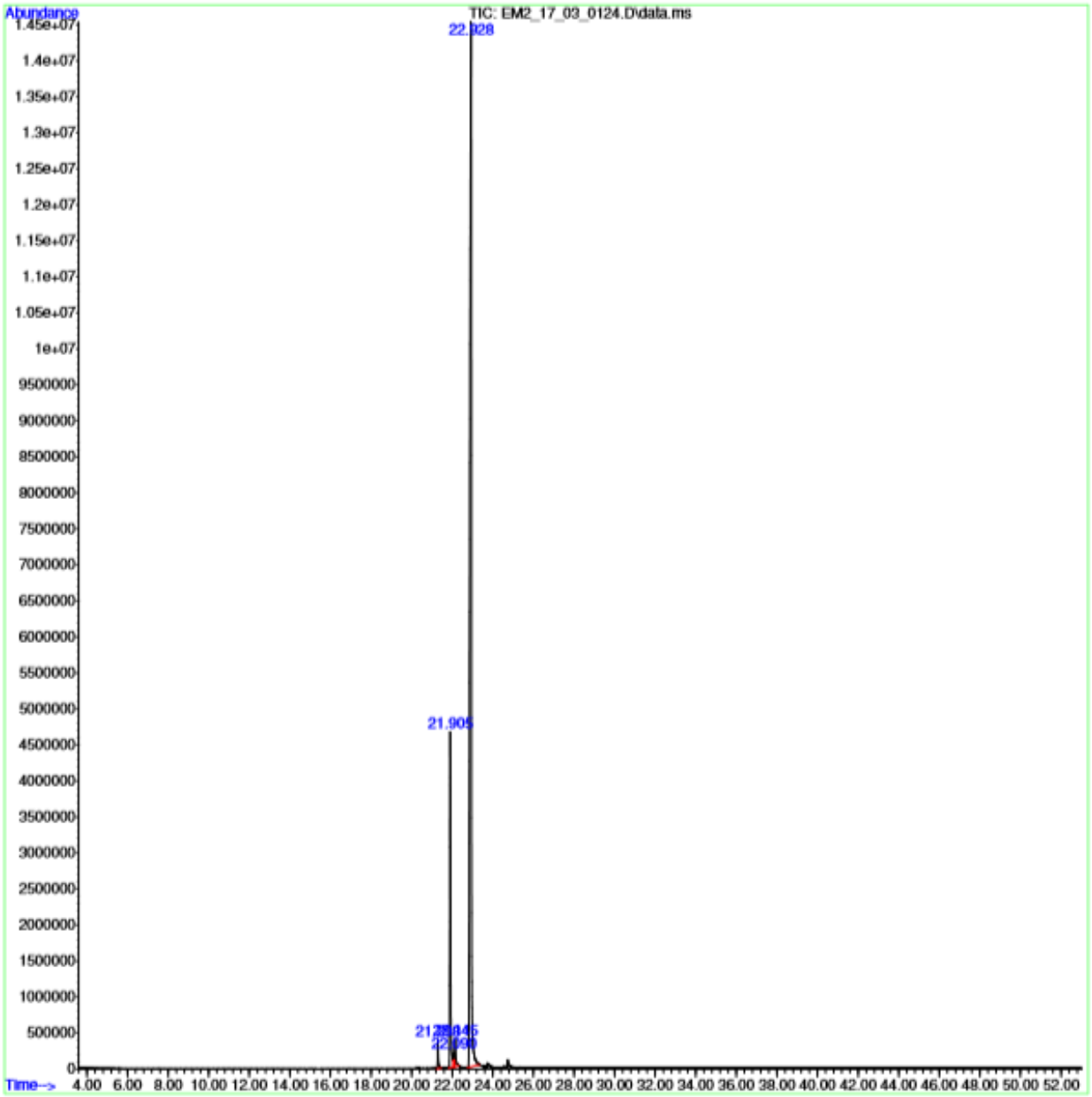

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FIGURE S2

CG-MS spectra of *P. alatabaccum* fruits

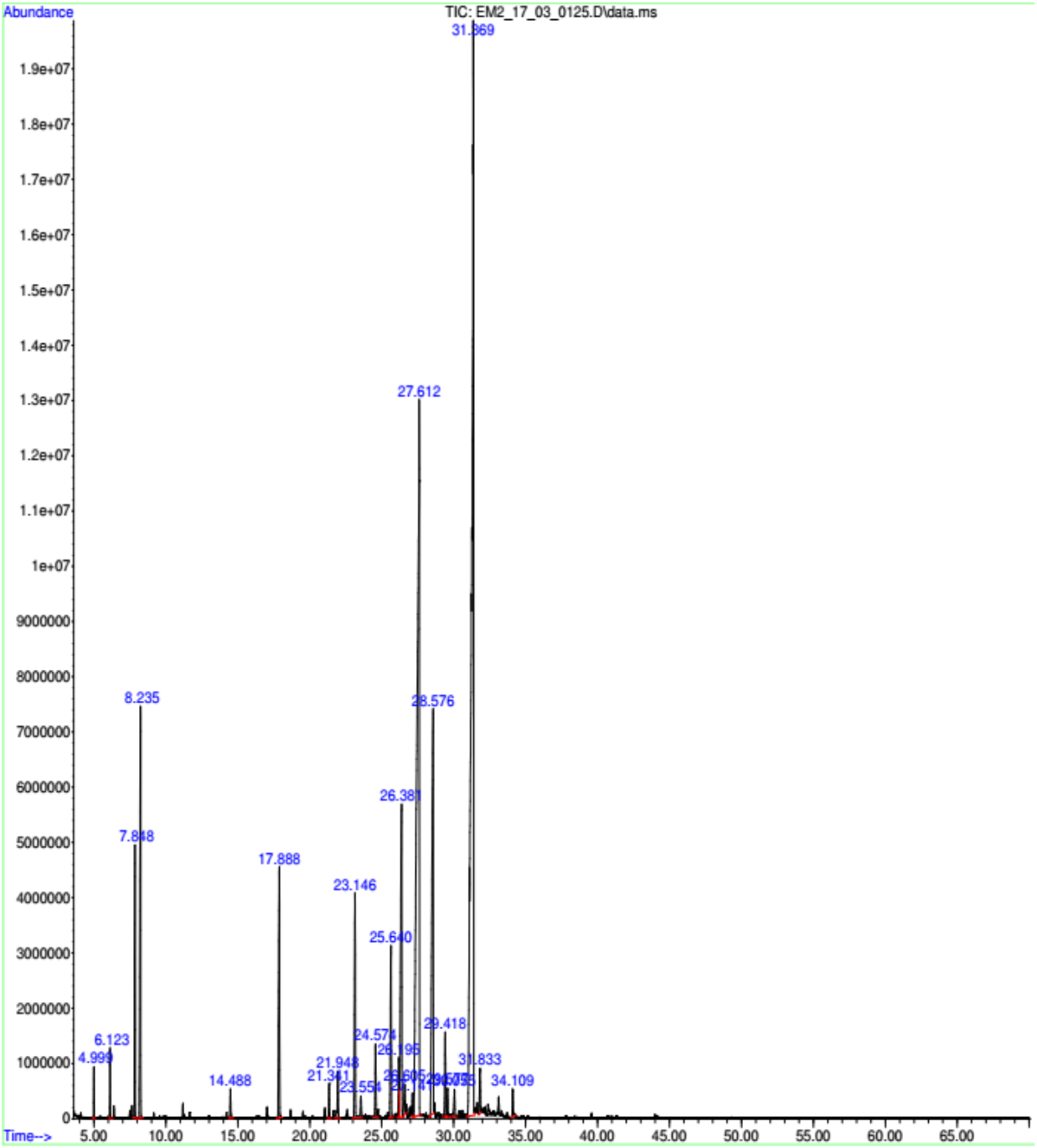

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Table S1

Results obtained from GC-MS analysis of the essential oil from *P. alatabaccum* fruits – 24 identifiable and unidentifiable constituents.

MONOTERPENES – 16.67%				
CONSTITUENTS	**R.T.[a]**	**% Area**	**Peak**	**CAS**
Alpha-pinene	4,999	0.32	1	000080-56-8
Beta-pinene	6,123	0.51	2	018172-67-3
Cis-ocimene	7,848	2.56	3	027400-71-1
Trans-beta-ocimene	8,235	5.17	4	003779-61-1
		8.56		
SESQUITERPENES – 45.83%				
CONSTITUENTS	**R.T.[a]**	**% Area**	**Peak**	**CAS**
Alpha-copaene	21,341	0.40	7	003856-25-5
Beta-elemene	21,948	0.61	8	000515-13-9
Beta-caryophyllene	23,146	3.07	9	000087-44-5
Germacrene-D	23,554	0.29	10	023986-74-5
Alpha-humulene	24,574	0.87	11	006753-98-6
Beta-cubebene	25,640	2.26	12	013744-15-5
Bicyclogermacrene	26,195	0.74	13	100762-46-7
Delta-cadinene	27,138	0.28	16	000483-76-1
Spathulenol	29,418	1.12	19	006750-60-3
Caryophyllene oxide	29,576	0.36	20	001139-30-6
Viridiflorol	30,057	0.37	21	000552-02-3
		10.37		
PHENYLPROPANOIDS – 20.83%				
CONSTITUENTS	**R.T.[a]**	**% Area**	**Peak**	**CAS**
Safrole	17,888	3.66	6	000094-59-7
Croweacin	26,383	5.82	14	000484-34-4
Myristicin	27,612	26.48	17	000607-91-0
Elemicin	28,576	8.59	18	000487-11-6
Dilapiol	31,369	34.81	22	000484-31-1
		79.36		
Unidentifiable – 16.67%				
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----------	----------	0.46	15	
----------	---------	0.60	23	
----------	---------	0.35	24	
TOTAL		**1.71**		
		100%		

[a] Retention time in minutes.

Enviado em: 12/08/2019 - Aprovado em: 14/12/2019