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Chemical Composition and *in vitro* Cytotoxic and Antileishmanial Activities of Extract and Essential Oil from Leaves of *Piper cernuum*

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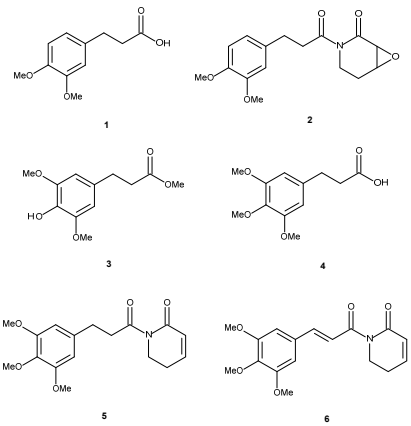
Fractionation of the MeOH extract from leaves of *Piper cernuum* Vell. (Piperaceae) afforded six phenylpropanoid derivatives: 3',4'-dimethoxy-dihydrocinnamic acid (**1**), piplaroxide (**2**), methyl 4'-hydroxy-3',5'-dimethoxy cinnamate (**3**), 3',4',5'-trimethoxydihydrocinnamic acid (**3**), dihydropiplartine (**5**), and piplartine (**6**). The structures of isolated metabolites were characterized by NMR and MS spectral data analysis. The chemical composition of essential oil from the leaves was determined using GC/LREIMS followed by the determination of Kovats indexes. This procedure allowed the identification of nineteen terpenoids, with *β*-elemene (**7**), bicyclogermacrene (**8**), germacrene D (**9**), and *(E)*-caryophyllene (**10**) as the main compounds. Compounds **1** and **3**–**6** displayed no *in vitro* cytotoxicity against cancer cell lineages B16F10-Nex2, U87, HeLa, HL-60, HCT, and A2058 while **2** showed moderate activity against B16F10-Nex2 and HL-60 lines. Otherwise, compounds **7**–**10** displayed high cytotoxic activity. Evaluation against non-tumorigenic HFF cells indicated a reduced selectivity of compounds **7**–**10** to tumoral cells. No antileishmanial activity on macrophages infected with *L. (L.) amazonensis* was found for the crude MeOH extract and compounds **1**–**6**. The crude essential oil and compounds **7**–**10** reduced parasitism and eliminated the majority of infected and non-infected cells at 50 μg/mL.

Keywords: *Piper cernuum* Vell., Phenylpropanoid derivatives, Essential oil composition, Cytotoxic, Antiparasitic.

Piper cernuum Vell. (Piperaceae), known as "pariparoba", is a native plant of the Atlantic rain forest and has been commonly used in folk medicine [1]. This species produces cinnamic acid derivatives and lignoids [2], and the essential oil is mostly monoterpenes and sesquiterpenes [3-5]. No information on antiparasitic and cytotoxic activities of this plant has been reported. As part of our studies of bioactive compounds from Brazilian plants [6-9], the MeOH extract and essential oil from the leaves of *P. cernuum* displayed cytotoxic activity against cancer cell lines and antileishmanial potential against amastigote forms of *Leishmania amazonensis*. Compounds **1-6** were isolated from the MeOH extract, this being the first occurrence of **2**, **4-6** in *P. cernuum*.

Compounds **1**–**5** were characterized as dihydrocinnamic derivatives by analysis of their ^{1}H NMR spectra, in which were observed typical triplets at δ 2.88 – 3.25 (J ~ 7 Hz) and δ 2.62 – 2.93 (J ~ 7 Hz), assigned to H-2 and H-3, respectively. The ^{1}H NMR spectrum of **6** showed two doublets (J = 15.0 Hz) at δ 7.68 and 7.65 indicating a cinnamic acid derivative. The ^{1}H NMR spectra of **1** and **2** displayed three aromatic hydrogens at δ 6.73 – 6.79, suggesting a 1,3,4-trisubstituted system containing two methoxyl groups based on singlets at δ 3.85 and 3.86 (3H each). The ^{1}H NMR of **2** also had two multiplets at δ 2.40 and δ 3.18, assigned to H-4" and H-5" of a piperidone amide, as well as one doublet at δ 3.56 (J = 4.1 Hz, H-2"), characteristic of epoxide hydrogens [10]. The epoxide ring at positions C-2" and C-3" was confirmed by analysis of ^{13}C NMR spectrum, which showed two signals at δ 52.3 and 53.4.



Comparison of spectroscopic data, including NMR, LRESIMS andLREIMS with those reported in the literature, allowed the identification of these compounds as 3',4'-dimethoxydihydro-cinnamic acid (**1**) [11] and piplaroxide (**2**) [12].

The ^{1}H NMR of **3**–**6**, had 2-proton singlets at δ 6.45 – 6.81 suggesting 1,3,4,5-tetrasubstituted aromatic rings. Compounds **4**–**6** had **s**inglets at δ 3.81 – 3.88 suggesting the presence of three aromatic methoxyl groups which was confirmed by peaks at δ 60.7 – 61.3 in the ^{13}C NMR. The ^{1}H NMR of **3** had an additional peak at δ 3.69 (3H) corresponding to a methyl ester as confirmed by

LREIMS data. Finally, the ^{13}C NMR spectra of **5** and **6** indicated the occurrence of amide derivatives due to carbonyl groups at δ 175.4 and 168.1, respectively, similar to **2**. Instead of epoxide carbons at approximately δ 55, sp^2 carbons at δ 125.8 and 145.5 were observed and assigned to C-2" and C-3", respectively. Finally, comparison of NMR data with that reported in the literature [13,14] identified methyl 4'-hydroxy-3',5'-dimethoxy cinnamate (**3**), 3',4',5'-trimethoxydihydrocinnamic acid (**4**), dihydropiplartine (**5**), and piplartine (**6**).

The essential oil from the leaves of *P. cernuum* was obtained by hydrodistillation using a Clevenger-type apparatus. Its yield, based on the weight of the fresh leaves, was 0.06%. The oil was analyzed by GC-LREIMS and the compounds identified by comparison of the Kovats indexes (determined relative to the retention times of a series of *n*-alkanes) on a non-polar column as well as the literature mass spectra [15]. The crude oil contained nineteen compounds, corresponding to 96.3% of the identified volatiles (Table 1).

The characterized compounds were grouped in four classes: hydrocarbon monoterpenes (4.2%), oxygenated monoterpenes (2.6%), hydrocarbon sesquiterpenes (82.0%), and oxygenated sesquiterpenes (7.5%). The major constituents were *β*-elemene (30.0%), bicyclogermacrene (19.9%), (*E*)-caryophyllene (16.3%), and germacrene D (12.7%) as hydrocarbons sesquiterpenes and *α*-terpenyl acetate (2.6%) and *β*-pinene (2.2%) as hydrocarbon monoterpenes. This differed from previously analyzed Brazilian specimens of *P. cernuum* since in the oil from leaves obtained in Santa Catarina [5] was composed mainly of the sesquiterpenes β-dihydroagarofuran (31.0%), elemol (12.0%), 10-*epi*-γ- eudesmol (13.0%) and β-eudesmol (2.6%), while that obtained from Rio de Janeiro [4] was composed mainly of *cis*-dihydroagarofuran (32.3%), α-pinene (10.2%), β-pinene (7.4%), 10-*epi*-γ-eudesmol (7.1%), and elemol (6.7%). Furan derivatives were not detected in the leaves collected in São Paulo State [3] but α- and β-pinenes (7.2 and 6.2%), germacrene D (6.7%), (*E*)-caryophyllene (20.7%) and bicyclogermacrene (21.9%) were.

Fractionation of crude essential oil by $SiO_2/AgNO_3$ column chromatography afforded pure β-elemene (**7**), bicyclogermacrene (**8**), germacrene D (**9**) and (*E*)-caryophyllene (**10**). The *in vitro* cytotoxicity of the MeOH extract, crude essential oil and compounds **1**–**10** obtained from leaves of *P. cernuum* as well as of the positive control (cisplatin) was evaluated against a panel of six tumor cell lines, including murine (B16F10-Nex2) and human (A2058, HTC, U87, HeLa, and HL-60) as well as a non-tumorigenic cell (HFF). The IC_{50} values are shown in Table 2

Compounds **1, 3–6** displayed no significant activity since IC_{50} values were higher than 200 μg/mL. Compound **2** showed moderate activity against B16F10-Nex2 (86±12 μg/mL) and HL-60 (107±23 μg/mL) lineages, suggesting that it might be responsible, at least in part, for the activity of the crude MeOH extract.

The crude oil showed strong activity against all tested cell lines with IC_{50} values from 16 to 30 μg/mL, mainly to HL-60 (16 ± 4 μg/mL), HCT (16.1 ± 0.8 μg/mL) and U87 (19.1 ± 0.2 μg/mL) approximately twice that of the positive control (cisplatin). Based on the criteria of the American National Cancer Institute that considers active a crude extract/essential oil which displays IC_{50} values below 30 μg/mL [16], the essential oil from the leaves of *P. cernuum* has promising activity against human and murine tumor cell lines, mainly leukemia and colon carcinoma, higher than the positive control (cisplatin). After chromatographic fractionation, compounds **7 – 10** showed strong activity against tested cells with germacrene D (**9**) and (*E*)-caryophyllene (**10**) being the most active in agreement with the literature [8] and explaining the activity of the crude oil from leaves of *P. cernuum*. A comparison of the IC_{50} values of the MeOH extract, essential oil and compounds **7–10** against non-tumorigenic HFF indicated reduced selectivities similar to those determined for tumoral cells.

Table 1: Chemical composition of essential oils from the leaves of *P. cernuum* Vell.

KI	Constituents	Relative amount %
932	*α*-Pinene	1.7
974	*β*-Pinene	2.2
993	NI	0.1
1032	*(Z)-β*-Ocimene	0.2
1054	*γ*-Terpinene	0.2
1219	NI	0.1
1346	*α*-Terpenyl acetate	2.6
1362	NI	0.1
1374	*α*-Copaene	0.6
1381	NI	0.6
1389	*β*-Elemene	30.0
1417	*(E)*-Caryophyllene	16.3
1420	NI	0.6
1428	NI	0.3
1452	*α*-Humulene	0.9
1465	NI	0.7
1481	Germacrene D	12.7
1500	Bicyclogermacrene	19.9
1522	*δ*-Cadinene	1.6
1541	NI	0.1
1561	*(E)*-Nerolidol	0.6
1577	Spathulenol	0.3
1590	Globulol	0.4
1602	Ledol	0.3
1612	NI	0.4
1628	NI	0.7
1645	Torreyol	4.9
1652	*α*-Cadinol	0.6
1665	Intermediol	0.6
	Hydrocarbon monoterpenes	4.2
	Oxygenated monoterpenes	2.6
	Hydrocarbon sesquiterpenes	82.0
	Oxygenated sesquiterpenes	7.5
	Non-identified compounds (NI)	3.7
	TOTAL	100.0

Table 2: IC_{50} values obtained for different tumor cell lines after their incubation with MeOH extract, crude essential oil and compounds **2** and **7 - 10** obtained from *P. cernuum* and positive control (cisplatin).

	IC_{50} (μg/mL)[a]						
	B16F10 -Nex2	*U87*	*HeLa*	*HCT*	*HL-60*	*A2058*	*HFF*
ME[b]	98±3	>200	133±5	188±16	>200	>200	>200
EO[c]	30±2	19.1±0.2	23±1	16.1±0.8	16±4	24±1	24±3
2	86±12	>200	152±1	171±18	107±23	>200	>200
7	19±2	9.8±0.8	17±2	23±4	9.1±0.3	10±3	13±1
8	2.8±0.1	6.7±0.4	12.4±0.3	4.0±0.2	4.4±0.5	8.1±0.2	2.4±0.3
9	6.8±0.8	6.3±0.3	14.5±0.7	7±1	4.4±0.2	3.2±0.3	8.1±0.7
10	4.3±0.4	3.5±0.8	10±1	6.4±0.8	3.9±0.1	4.1±0.6	9.6±0.3
PC[d]	53±4	45±6	20±1	> 60	21±2	43±3	nd

[a]B16F10-Nex2: murine melanoma; U87: human glioblastome; HeLa: human cervical carcinoma; HCT: human colon carcinoma; HL-60: human leukemia; A2058: human melanoma; HFF: human foreskin fibroblast (non-tumorigenic); [b]MeOH extract; [c]crude essential oil; [d]positive control (cisplatin). nd: not determined.

The anti-amastigote effects of the crude MeOH extract, essential oil and compounds **1–10** from *P. cernuum* against *L. (L.) amazonensis* were evaluated in the interaction among host cell and parasite. Compounds **1–6** displayed no antileishmanial effect on infected macrophages at 50 μg/mL. Macrophages treated at 2 and 10 μg/mL of essential oil showed significantly less parasitism, presenting infection indexes of 131 ± 15 and 115 ± 13, respectively, while untreated macrophages had an infection index of 198±12 ($p < 0.05$), as showed at Table 3. The effect of the crude essential oil could be associated with the infection index of the bioactive sesquiterpenes **7–10**. On the other hand, the essential oil and compounds **7–10** at a concentration of 50 μg/mL were toxic to J774 macrophages, eliminating the majority of infected and non-infected cells.

Table 3: Infection index of infected J774 macrophage from *L. (L.) amazonensis* treated with crude MeOH extract, essential oil and compounds **7 – 10** from leaves of *P. cernuum*.

	Infection index[a]		
	2 μg/mL	*10 μg/mL*	*50 μg/mL*
ME[b]	207 ± 9	212 ± 12	235 ± 32
EO[c]	131 ± 15	115 ± 13	-
7	122 ± 16	104 ± 12	-
8	108 ± 9	102 ± 10	-
9	110 ± 12	108 ± 6	-
10	105 ± 16	101 ± 7	-

positive control: amphotericin: 34 ± 5 at 0.1 μg/mL; [a]$p < 0.05$ compared to control. [b]MeOH extract; [c]crude essential oil;

Previous studies showed that the essential oil from *P. demeraranum* and *P. duckei* that eliminated amastigote forms of *L. (L.) amazonensis* had as one of the major constituents sesquiterpene hydrocarbons such as β-elemene [17]. Other essential oils with high amounts of sesquiterpenes, such as β-elemene, bicyclogermacrene and (*E*)-caryophyllene also were active against intracellular forms of *L. (L.) amazonensis* [18,19], corroborating our results. Although the amounts of hydrocarbon monoterpenes were less than sesquiterpenes, essential oils with high concentrations of pinene derivatives also eliminated *L. (L.) amazonensis* promastigotes [20]. The presence of known leishmanicidal compounds in essential oils can therefore account for the anti-amastigote effect found in the present work.

Experimental

Plant material: Leaves of *P. cernuum* were collected at Parque Ecológico do Pereque, Cubatão, São Paulo State, Brazil (Latitude: 23° 53′ 42″ South and Longitude: 46° 25′ 30″ West) in July, 2012. The botanical identification of the plant was made by Prof. Euder G. A. Martins (USP, São Paulo/SP) and the voucher specimen was deposited in the Herbarium of Instituto Florestal de São Paulo – SP, Brazil.

Extraction and isolation: Dried and powdered leaves of P. *cernuum* (123 g) were exhaustively extracted with MeOH at room temperature using an accelerated solvent extractor system (Dionex ASE-350). After solvent removal under reduced pressure, the 10.2g of syrupy green extract was chromatographed over SiO_2 using hexane with increasing amounts of EtOAc as eluent. This procedure afforded eight groups (A1 – A8). Groups A1 to A3 were composed of fatty material. Groups A4 (182 mg) and A7 (118 mg) were individually purified by prep. TLC (SiO_2) using $CHCl_3$:MeOH 99:1 as eluent. These afforded **3** (8 mg) and **6** (14 mg) from group A4 as well as **1** (27 mg) and **4** (9 mg) from group A7. Group A5 (89 mg) was purified over Sephadex LH-20 eluted with MeOH to give 7 mg of **5**. Group A6 (103 mg) was subjected to SiO_2 column chromatography using hexane:EtOAc 1:1 as eluent to afford 16 mg of **2**. All isolated compounds displayed purity higher than 97% (HPLC analysis).

Extraction of the essential oil: Fresh leaves of *P. cernuum* (227 g) were extracted by a steam distillation in a Clevenger type apparatus for 4 h, to give 128 mg of crude essential oil (yield 0.06%). The oil was dried at room temperature and stored in a sealed vial under refrigeration prior to analysis.

Compound–identification: The crude oil was obtained by steam distillation and analyzed by GC/LREIMS. The identification of the components was based on the comparison of their mass spectra with those in the spectrometer data base (Willey 229 library) and confirmed by determination of their Kovats indexes, which were determined relative to the retention times of a series of *n*-alkanes [25].

Isolation of main compounds from essential oil: Part of the essential oil extracted from leaves of *P. cernuum* (100 mg) was subjected to CC on silica gel soaked with $AgNO_3$ (15%) and eluted with CH_2Cl_2 and CH_2Cl_2-MeOH (95: 5 and 9:1) to afford 58 fractions (5 mL each), which were individually analyzed by FID-GC. These fractions were pooled in 12 groups (B1 – B12). Prep. TLC (SiO_2/$AgNO_3$ 15% – CH_2Cl_2:acetone 99:1) separation of group B5 afforded **8** (3 mg) and **10** (2 mg) while groups B8 and B11 gave **9** (2 mg) and **7** (5 mg), respectively. All isolated compounds displayed purity higher than 98% (FID-CG analysis).

General procedures: ^{1}H NMR spectra were recorded at 300 MHz and ^{13}C NMR at 75 MHz on a Bruker Avance 300 spectrometer using $CDCl_3$ as solvent. Silica gel (Merck, 230–400 mesh) was used for column chromatographic separation, while silica gel 60 PF_{254} (Merck) was used for analytical (0.25 mm) and preparative (1.0 mm) TLC. LC-ESIMS analyses were performed on a UFLC-XR Prominence liquid chromatograph coupled to a LCMS-8040 triple-quadrupole mass spectrometer (Shimadzu) equipped with electrospray source operating in positive polarity (ESI+). MS spectra were recorded in full scan and product ion scan modes (argon CID). The mobile phase composition was ACN/$HCOONH_4$ 20 mmol/L (50:50, v/v) at a flow of 100 μL/min and the ion source was set as follows: nebulizer gas = 3 L/min, desolvation gas = 15 L/min, DL = 150°C, heat block = 300°C and voltage = 3.5 kV. GC/LREIMS analysis was carried out in a Shimadzu AOC-20i chromatograph interfaced with a MS-QP-5050A mass spectrometer. Helium was used as the carried gas, using an RtX-5 capillary column (5% phenyl, 95% polydimethylsiloxane, 30 m × 0.32 mm × 0.25 μm film thickness). These analyses were performed by injecting 1.0 μL of a 1.0 mg/mL solution of volatile oil in CH_2Cl_2 in a split mode (1:30) employing helium as the carrier gas (1 mL/min) under the following conditions: injector and detector temperatures of 250 °C and 280 °C, respectively; oven programmed temperature from 60–280 °C at 3 °C/min, holding 10 min at 280 °C. Component concentrations were calculated from GC peak areas in the order of RtX-5 column elution.

Cell lines: The murine melanoma cell line B16F10 was originally obtained from the Ludwig Institute for Cancer Research (São Paulo, Brazil). The murine melanoma B16F10-Nex2 sub-line is characterized by low immunogenicity and moderate virulence. Human melanoma (A2058), glioblastome (U87), colon carcinoma (HCT), leukemia (HL-60), and human foreskin fibroblast (HFF) cell lines were obtained from the Ludwig Institute for Cancer Research. Human cervical carcinoma (HeLa) was acquired from Dr. Hugo Pequeno Monteiro, UNIFESP. Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2, in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (Hepes) (Sigma, St. Louis, MO), 24 mM sodium bicarbonate (Sigma), 40 mg/L gentamycin (Schering-Plough, São Paulo, Brazil), pH 7.2, and 10% fetal calf serum (Invitrogen).

In vitro cytotoxic activity: Compounds **1 – 10** as well as the crude MeOH extract and essential oil obtained from the leaves of *P. cernuum* were suspended in dimethylsulfoxide (DMSO) at a final concentration of 10 mg/mL, and then added to complete RPMI medium supplemented with 10% fetal calf serum. Different concentrations of the compounds **1 – 10** and extract/essential oil, ranging from 100 to 0 μg/mL, were incubated with 1×10^4 cells in a 96-well plate at 37 °C and 5% CO_2. After 24 h of incubation, cell viability was assessed using the Cell Proliferation Kit I (MTT) (Sigma), an MTT-based colorimetric assay as previously described [21,22]. Readings were made in a plate reader at 570 nm with a

reference of 650 nm. All experiments were performed in triplicates using cisplatin (Sigma) and DMSO 1% as positive and negative controls, respectively.

In vitro L. (L.) amazonensis (MHOM/BR/73/M2269) was classified on the basis of monoclonal antibodies and isoenzymes at the Evandro Chagas Institute, Belém, PA, Brazil. This strain was maintained in BALB/c mice to keep the infectivity. *L. (L.) amazonensis* were cultured in RPMI 1640 medium (R10) supplemented with 10% heat-inactivated fetal bovine serum, 0.25 mM HEPES, gentamicin (10 μg/mL), and penicillin (100 IU/mL). The experiments were performed using aomastigote forms in stationary phase of growth.

L. (L.) amazonensis macrophage interaction assay: J774 macrophages were plated in a 24-well plate on round coverslips (10^5 macrophages per well) containing R10 and allowed to adhere for 2h at 34 °C, at 5% CO_2. After this time, the wells were washed with warm R10 and *L. (L.) amazonensis* amastigotes (10 promastigote/macrophage) were added in the wells. After 24 h of infection the wells were washed with R10 medium, and 2.0, 10.0 and 50.0 μg/mL of crude MeOH extract, essential oil and compounds **1** – **10** were added in the cell culture. These experiments were conducted in triplicate. Amphotericin B (0.1 μg/mL) was added as positive control, and in negative control only PBS plus DMSO (never exceeded 1% of volume) was added in culture. After 24h of treatment the plate was washed three times with warm PBS and the macrophages were fixed using MeOH, followed by Giemsa staining to quantify the infection index (II), using the following expression: % infected macrophage X amastigote/macrophage [23].

Statistical analysis: The data obtained represent the means and standard deviations from three independent experiments. Differences among infection index of treated macrophages and control were statistically validated using ANOVA statistical test.

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