

**Antiproliferative and anti-inflammatory activity from aerial parts of
Psychotria cupularis (Rubiaceae)****Atividade antiproliferativa e anti-inflamatória das partes aéreas de *Psychotria
cupularis* (Rubiaceae)**

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ABSTRACT

The crude extract and fractions of aerial parts from *Psychotria cupularis*, collected at Camacan (Brazil), were tested for anti-inflammatory and antiproliferative activity. A phytochemical screening indicated the presence of tannins, anthraquinones, triterpenes, steroids and flavonoids. The crude extract and fractions inhibited the ear oedema in mice between 50.2 to 87.2% and the myeloperoxidase enzyme activity between 51.6 to 97.1%. The butanolic and ethyl acetate fractions was active against glioma, breast, ovary, kidney, colon and leukaemia cell line (IG₅₀ = 4.3 to 16.9 µg/mL).

Keywords: *Psychotria cupularis*, Rubiaceae, anti-inflammatory activity, antiproliferative activity, phytochemical screening, pharmacology.

RESUMO

O extrato bruto e as frações das partes aéreas de *Psychotria cupularis*, coletadas em Camacan (BA-Brasil), foram submetidas a avaliação de atividade antiproliferativa e anti-inflamatória. As mesmas frações, também, foram submetidas a uma prospecção fitoquímica que indicou a presença de taninos, antraquinonas, triterpenos, esteroides e flavonoides. O extrato bruto e as frações inibiram

o edema de orelha em camundongos entre 50,2 a 87,2% e a atividade da enzima mieloperoxidase entre 51,6 e 97,1%. As frações butanólica e acetato de etila da espécie foram ativas contra linhagens de glioma, mama, ovário, rim, cólon e leucemia (IG₅₀ = 4,3 a 16,9 µg/mL).

Palavras-chave: *Psychotria cupularis*, Rubiaceae, atividade anti-inflamatória, atividade antiproliferativa, prospecção fitoquímica, farmacologia.

1 INTRODUCTION

Psychotria cupularis (Müll. Arg.) Standl. is a endemic specie of Latin America, may be finded in Brazil at North, Northeast and Southeast seasonal forests and has shrub habit. *Psychotria* species are employed in folk medicine for treatment of different types of diseases in female breeding, respiratory and digestive systems and rural communities at Amazônia (Pará – Brazil) use various species as painkillers. These ethnobotanical survey motivated genus research that yielded bioactive extracts. Some examples include anti-inflammatory, antibiotic, antifungal and antiviral activity, due a great diversity of secondary metabolites also indolic alkaloids, iridoids, terpenoids, flavonoids, anthraquinones and other biological phenolic derivatives. (Porto et al. 2009; Farias et al. 2012; Calixto et al. 2016).

This present work describes the anti-inflammatory and antiproliferative potential of methanolic crude extract and fractions of *Psychotria cupularis* aerial parts.

2 MATERIALS AND METHODS

2.1 PLANT MATERIAL AND EXTRACTS PREPARATION

The aerial parts of *P. cupularis* were collected and identified by Prof. Dr. André Márcio Amorim in Private Reserve of the National Patrimony of Serra Bonita (PRNP – Serra Bonita – Camacan - Brazil) in Atlantic forest area (Bapeda Trail; 15° 23' 30''S, 39° 33' 55''W) on December 2013. A voucher specimen has been deposited at Santa Cruz State University Herbarium (CEPEC 141.211). Dried powdered vegetal material (893 g) was submitted to an exhaustive maceration at room temperature with 10 L distilled methanol (p.a.), for two days in four weeks, and concentrated under vacuum (38.5 °C) to yield the crude extract (**CE** – 63.8 g). A part of **CE** (26.5 g) was suspended in MeOH:H₂O solution (1:1) and partitioned with *n*-hexane (p.a) (**HF** – 4.90 g), chloroform (**CF** – 951.6 mg) and ethyl acetate (**AF** – 9.80). The remaining fraction was named hydromethanolic fraction (**HMF** – 10.8 g) and 9.0 g was partitioned with *n*-butanol making butanolic fraction (**BF** – 4.56 g) and aqueous fraction (**WF** – 4.14 g).

Phytochemical screening

The substances class tested on this study for crude extract and fractions of *P. cupularis*

were triterpenoids and steroids, tannins, anthraquinones, alkaloids, flavonoids and saponins. For all tests, the experiments used 2.0 mg of CE and each fraction (**HF, CF, AF, HMF, BF, WF**) in test tubes (Matos, 2009; Marques et al. 2016).

Triterpenes and steroids: Samples were solubilized in $\text{CHCl}_3:\text{Ac}_2\text{O}$ solution (1:1) with five drops of concentrated sulfuric acid (Lieberman-Burchard reaction). **Tannins:** Samples were solubilized in 2.0 mL of chloroform or water and were added 1.0 mL of $\text{FeCl}_3:\text{EtOH}$ (10% m/m), then 5.0 mL of icy acetic acid was added with 3.0 mL of lead acetate 1% for each sample. **Anthraquinones:** For each sample was added 3.0 mL of toluene and 2.0 mL of NH_4OH 10% under slight agitation. **Alkaloids:** Every samples were tested with 1.0 mL of three reactive: Bouchardt, Dragendorff and Mayer and 3.0 mL of HCl 5%. **Flavonoids:** For each tube were added 3.0 mL of methanol, 1.0 mL of concentrated HCl and 1.0 cm of magnesium tape (Shinoda reaction). **Saponins:** Each samples was solubilized by 3.0 mL of water and submitted to a strongly agitation by three minutes (persistent foam experiment).

2.2 ANTI-INFLMMATORY ASSAY

Male Swiss mice (30–40 g) were kept under controlled temperature (22°C) and a light/dark cycle (12 h), with free access to water and food. The experimental protocols were approved by the Ethics Committee on Animal Experimentation of the State University of Maringá (CEUA/UEM 9804/2016).

Ear oedema induced by croton oil assay: The ear oedema was induced by topic application of croton oil (CO – 200 µg/ear) diluted in acetone solution 70% on the left ear. Immediately after application of phlogistic agent, was administrated 20 µL of CE and fractions in solutions of 2.5 mg/ear and 5.0 mg/ear. On the right ear was applicated only vehicle (20 µL) used to dilute the CO. After six hours, every animals were anesthetized and sacrificed. The ears were sectioned in circle discs and weighted in analytical balance. The inhibition percentage was determined by the equation 1 (van Arman, 1974; De Moura et al, 2020).

$$\% \text{ inhibition} = [(W_{LE_{\text{control}}} - W_{LE_{\text{treated}}}) / (W_{LE_{\text{control}}} - W_{RE})] \times 100 \quad (1)$$

W = weight; LE = Left ear; RE = right ear

Myeloperoxidase (MPO) activity: Myeloperoxidase enzyme activity was evaluated in ear section homogenized supernatant (Bradley et al, 1982). The ear tissue was putted on in potassium phosphate buffer 50 mM, ph 6.0, contained 0,5% of hexadecyltrimethylammonium bromide (1

mL.50/mg of tissue) at Potter homogenizer. The homogenized was centrifuged for five minutes at 2500 rpm. In a part of supernatant (10 μ L) was added 200 μ L of a buffer containing 16.7 mg of o-anisidine dihydrochloride, 90 mL of water, 10 mL of potassium phosphate buffer and 50 μ L of H₂O₂ 1%. The reaction was interrupted by addition of 30 μ L of sodium acetate 1,46 M (pH 3.0) and the enzyme activity was determined by final point technique of absorbance measure (450 nm) in microplate reader (Lionhart Diagnostic ® - ELX 800). The inhibition percentage of MPO activity was determined by the same equation of oedema (1). The results was expressed as mean \pm standard error of the mean and were submitted to analysis of variance (ANOVA), followed by the Tukey test. P<0.05 was considered as significant level.

In vitro Antiproliferative Assay

The *in vitro* antiproliferative activity of **CE** and fractions of *P. cupularis* were evaluated with eight human tumor cell lines gived in by National Cancer Institute (NCI) of United States of America: glioma (U251), breast (MCF-7), ovarian-expressing phenotype multiple-drug resistance (NCI-ADR/RES), kidney (786-O), lung (NCI-H460), ovary (OVCAR-3), colon (HT-29) and leukaemia (K562). A non-tumoral cell line, HaCaT (human keratinocyte - normal immortalized cell), was ceded by Prof. Dr. Ricardo Della Coletta (University of Campinas – UNICAMP).

Cell lines were diluted in DMSO (1.0 mg/mL) and resulting in stock solutions for screening antiproliferative activity. In test were used 100 μ L of cell in experimental culture (RPMI/gentamicin/inactivated bovine physiological serum). The plates were incubated by 24 hours at 37 °C in CO₂ (5%) atmosphere and humid ambience.

The control plate was fixed by trichloroacetic acid (TCA) and to the other plates were added **CE** and fraction in concentration of 0.25; 2.5; 25 and 250 μ g/mL. After, the plates were incubated for 48 hours, centrifuged by 3 minutes at 2000 rpm and fixed with 50 μ L of TCA 50% for attached cells and 80% for suspension cells (leukaemia). Finally, the plates were incubated by 1 hour at 4 °C, washed with water and dried.

The plates were stained by addition of 50 μ L of protein dye (SRB 0.4% -weight/volume), dissolved in acetic acid 1%, incubated at 4 °C by 30 minutes and washed with acetic acid 1%. The dye bound to cell proteins was solubilized by Trizma Base (10 μ M and pH 10.5) by 5 minutes at ultrasound (Monks et al, 1991).

The absorbance spectrophotometric reading was realized in microplates reader at 560 nm. The mean absorbance and growth inhibition percentage were calculated; results were disposed in related graphs with growth inhibition percentage and test substance concentration. The positive

control was doxorubicin (chemotherapeutic). The graphs was obtained from sigmoidal non-linear regression by Origin 7.5 and 8.0 software.

3 RESULTS AND DISCUSSION

The crude extract (**CE**) of aerial parts from *P. cupularis* was submitted for a phytochemical screening to evaluate the presence of secondary metabolites in this specie. The classes of substances that were identified are triterpenes and steroids, tannins and flavonoids. The same tests with *P. cupularis* fractions indicate triterpenes and steroids in the *n*-hexane fraction (**HF**), anthraquinones and flavonoids in the ethyl acetate fraction (**AF**), tannins and flavonoids in the butanolic (**BF**) and hydromethanolic fraction (**HMF**), and only tannins in the aqueous fraction (**WF**) (**Table S1**).

Table 1. Phytochemical screening of *Psychotria cupularis*.

Substance Class	Sample ^a						
	CE	HF	AF	CF	BF	WF	HMF
Triterpenes and steroids	+	+	-	+	-	-	-
Tannins	+	-	-	-	+	+	+
Antraquinones	-	-	+	-	-	-	-
Alkaloids ^b							
(BR)	-	-	-	-	-	-	-
(DR)	-	-	-	-	-	-	-
(MR)	-	-	-	-	-	-	-
Flavonoids	+	-	+	+	+	-	+
Saponines	-	-	-	-	-	-	-

^aSamples: CE (crude extract), HF (*n*-hexane fraction), AF (ethyl acetate fraction), CF (chloroform fraction), BF (butanolic fraction), WF (water fraction), HMF (hydromethanolic fraction).

^bAlcaloides: BR (Bouchardat reactive), DR (Dragendorff reactive), MR (Mayer reactive).

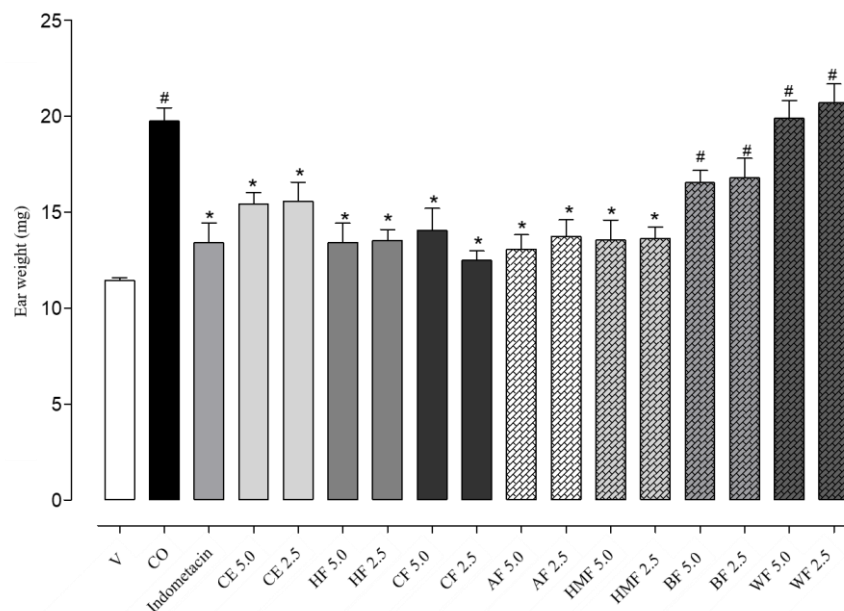
(+) Positive result; (-) Negative result.

The anti-inflammatory activity of **CE** and fractions was evaluated with croton oil (**CO**) induction on mice. In this test, the parameters measured were ear tissue topic effect and myeloperoxidase enzyme activity (**MPO**). The results were expressed in percentage of oedema inhibition (through ear weight) and MPO activity (absorbance) (**Figures S1 and S2, Table S2**).

Table 2. Inhibition (%) of ear oedema and MPO activity for the indomethacin, crude extracts and fractions of *P. cupularis*

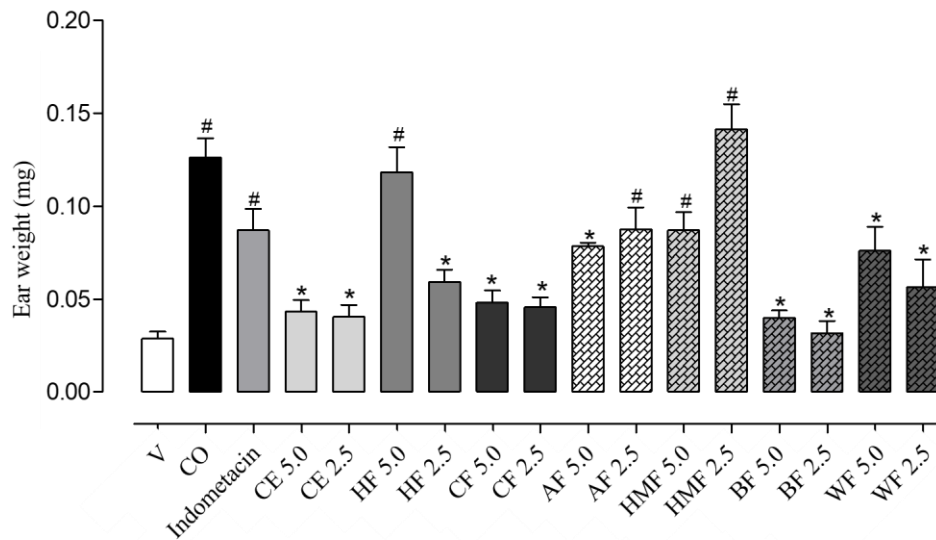
Samples	Ear oedema (%)		MPO (%)	
	5.0 mg/ear	2.5 mg/ear	5.0 mg/ear	2.5 mg/ear
INDO		76.3		N/R
CE	51,9	50,2	85,4	88,2
HF	76,2	74,7	N/R	68,9
CF	68,7	87,2	80,3	82,9
AF	80,4	72,6	49,3	N/R
HMF	74,5	73,5	N/R	N/R
BF	N/R	N/R	88,7	97,1
WF	N/R	N/R	51,6	71,7

N/R - no reduction compared with control group.

Figure 1- Effect of crude extract and fractions of *P. cupularis* over ear oedema induced by croton oil (CO) in Swiss mice (30-40 g).

Each column represents the average weight \pm mean standard error, 6 hours after the application of croton oil. # $p < 0.05$, compared with control group (V); * $p < 0.05$, compared with control group (CO); (ANOVA followed by Tukey test). Vehicle (acetone:water 7:3).

Figure S2 -Effect of crude extract and fractions of *P. cupularis* over myeloperoxidase activity in ear tissue of Swiss mice (30-40 g).



Each column represents the average weight \pm mean standard error, 6 hours after the application of croton oil. # p <0.05, compared with control group (V); * p <0.05, compared with control group (CO); (ANOVA followed by Tukey test). Vehicle (acetone:water 7:3).

The **CE** presented moderate inhibition (51.9 and 50.2 %; p <0.05) for two tested concentration (5.0 and 2.5 mg/ear). This extract was partitioned and new fractions were submitted to the test of ear oedema. **HF**, **CF**, **AF** and **HMF** fraction inhibited oedema between 68.7 to 87.2 %. The MPO activity evaluation indicates the polymorphonuclear leukocytes presence on ear tissue. **CE**, **CF**, **BF** and **WF** exhibited the greatest activity between 51.6 to 97.1 % (p <0,05) of MPO inhibition. The **HF** (2.5 mg/ear) and **AF** (5.0 mg/ear) fractions show an inhibition of 68.9 and 49.3 %, respectively, while the other fraction exhibited no MPO inhibition. The heme-enzyme myeloperoxidase is an enzyme present on neutrophils and it is employed as polymorphonuclear leukocytes migration marker. A decrease of enzyme action can indicates an anti-inflammatory response increase in injured area. (Medina et al. 2018)

The *in vitro* antiproliferative activity of **CE** and fractions were tested on eight cancer human cells and one non-tumoral cell line. The results were expressed through GI_{50} (μ g/mL) parameter that indicates what concentration the growth cell inhibition is 50% (**Table S3**).

Table 3. Antiproliferative activity (GI_{50} ; $\mu\text{g mL}^{-1}$) of doxorubicin, crude extract and fractions of *P. cupularis*

	Cell lines (GI_{50} $\mu\text{g/mL}$)									
	2	m	a	7	4	O	h	K	q	
DOX	<0.025	0.071	0.15	0.11	>0.025	<0.025	0.045	<0.025	0.026	
CE	26.8	15.2	35.5	41.1	43.9	37.0	38.5	10.7	32.4	
HF	33.4	16.23	25.6	38.5	77.7	5.9	36.5	25.6	30.7	
CF	28.0	28.5	31.7	36.4	40.5	11.1	41.3	36.4	31.7	
AF	5.0	5.2	27.1	25.2	42.7	38.4	16.6	7.3	16.9	
HMF	35.7	43.5	58.2	50.7	121.9	57.4	50.7	48.1	48.1	
BF	4.3	5.1	8.4	10.5	35.3	10.0	10.5	5.3	10.2	
WF	*	*	*	*	*	*	*	*	*	

2 = U251 (glioma, Central Nervous System); m = MCF-7 (breast adenocarcinoma); a = NCI-ADR/RES (ovary, multidrug resistance phenotype), 7 = 786-0 (kidney); 4 = NCI-H460 (lung, non-small cells adenocarcinoma); o = OVCAR-3 (ovary); h = HT-29 (colon); k = K562 (leukaemia); q = HaCat (keratinocyte human, immortalized normal cell).

CE exhibited activity against breast (MCF-7; $IG_{50} = 15.21 \mu\text{g/mL}$) e leukaemia (K562; $IG_{50} = 10.68 \mu\text{g/mL}$) cells. Among the fractions tested, the most active were **BF** and **AF** fractions. **BF** presented GI_{50} between 4.32 to 10.54 $\mu\text{g/mL}$ for cancer cell lines tested, except for lung line (NCI-H460; non-small cells; $GI_{50} = 35.26 \mu\text{g/mL}$), and **AF** was active for glioma (U251; $IG_{50} = 4.96 \mu\text{g/mL}$), breast (MCF-7; $IG_{50} = 5.23 \mu\text{g/mL}$), colon (HT29 = $IG_{50} = 16.61 \mu\text{g/mL}$) and leukaemia (K562; $IG_{50} = 7.33 \mu\text{g/mL}$) lineages. The **CF** was active and selective on ovary lineage (OVCAR-03; $IG_{50} = 11.11 \mu\text{g/mL}$) and **HF** exhibited activity against ovary (OVCAR-03; $IG_{50} = 5.88 \mu\text{g/mL}$) and breast (MCF-7; $IG_{50} = 16.23 \mu\text{g/mL}$) cell lines.

Ethnopharmacological and phytochemical studies of *Psychotria* described the genus with high pharmacological potential. The NCI-USA consider *Psychotria* a “hot genera” in citotoxic potential (Cragg et al. 2006). On genus studies, this activity has been attributed to a wide range of compound also anthraquinones, benzoquinones, flavonoids, peptides and alkaloids (Porto et al. 2009; Calixto et al. 2016). The antiproliferative activity observed on **CE** and fractions of *P. cupularis* may be partly attributed by the presence of tannins, anthraquinones, flavonoids, and triterpenes. Tannins and anthraquinones are known as melanoma and leukaemia potent agents. (Hayashi et al. 1987; Kashiwada et al. 1992; Dholwani et al. 2008). Flavonoids were widely related in literature and also presented antiproliferative activity against tumor human cells as gastric adenocarcinoma, promyelocromucemic cells and colon carcinoma (Moghaddan et al. 2012), and lung non-small cells (Santos et al. 2015). The selectivity of **HF** against ovary cell line may be

explained by the presence of triterpenes and steroids (Topçu et al. 2011; Dai et al. 2013; Zhang et al. 2014).

The anti-inflammatory character also are observed in the *Psychotria* (Moraes et al. 2010; Queiroz et al. 2017; Tran et al. 2017). *P. nigriensis* and *P. octusulcata* were evaluated for anti-inflammatory activity by paw oedema model; both of plants present 67,9 % and 73,5 % of antiphlogistic action, respectively (Iniyavan et al. 2012; Mariyammal and Kavimani. 2013).

The results of anti-inflammatory activity from CE and fractions of *P. cupularis* can be justified by presence of tannins, triterpenes, steroids and flavonoids. Flavonoids are metabolites that act altering the capillary permeability in inflamed cells, and this situation inhibits the leukocytes migration on the initial stage of inflammation. (Aquino et al. 2016). The tannins are widely related as potentials anti-inflammatory agents, evidenced by ear and path oedema experiments. This action may be responsible by adstringents properties that cause protein precipitation and affect cell functions likes leukocytes migration (Mota et al. 1985; Liu et al. 2015). Finally, the pentacyclic triterpenes have described anti-inflammatory activity, mainly for metabolites with ursane and oleanane skeleton that are usually isolated in *Psychotria* species. (Almeida et al. 2015; Calixto et al. 2016; Romero-Estrada et al. 2016; Huang et al. 2016). Triterpenes act in anti-inflammatory process by any aspects like inhibition of histamine liberation from mast cells, of elastase and lipoxygenase and cyclooxygenase activity. (Liu. 1995)

4 CONCLUSION

This is the first study about biological potential of crude extract and fractions of *P. cupularis*. This work allowed identifying the class of secondary metabolites also tannins, anthraquinones, triterpenes flavonoids and steroids that can be responsible for considerably anti-inflammatory and antiproliferative activity. These activities showed that chemical study of *P. cupularis* might be promising for new drugs or phytopharmaceutical development.

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