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Chemical Characterization and Biological Activity of *Macfadyena unguis-cati* (*Bignoniaceae*)

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Abstract

Macfadyena unguis-cati (L.) has been widely used in folk medicine as an anti-inflammatory, antimalarial and antivenereal. The purpose of this study was to chemically characterize the main plant components, and to evaluate the biological properties of some of the fractions derived from leaves (MACb) and liana (MACa) of this plant.

Chemical characterization allowed the identification of the compounds corymboside, vicenin-2, quercitrin, chlorogenic acid, isochlorogenic acid, lupeol, β-sitosterol, β-sitosterylglucoside, allantoin and lapachol.

The biological screening of fractions and/or purified substances derived from fractions revealed antitumoral and antitrypanosomal activities in fractions MACa/lapachol and MACb/MACb21, respectively. The anti-lipoxygenase and anti-cyclooxygenase effect seen in fractions MACa and MACb showed a partial correlation with the anti-inflammatory property attributed to this plant.

Macfadyena unguis-cati (L.) A. Gentry (*Bignoniaceae*) is a variable climbing plant, widespread in tropical America from Mexico to Rio de la Plata, and also in Western India (Pio Correa 1978). This species is used in folk medicine against snakebite (Houghton & Osibogun 1993), dysentery, inflammation and rheumatism (Pio Correa 1978). Also, there are reports on its use in the treatment of venereal disease and as a quinine substitute for malaria (Ferrari et al 1981).

Despite the therapeutic properties attributed to *M. unguis-cati*, the plant has not been investigated for its pharmacological activities, apart from an anti-protozoal screening using a Bolivian specimen. Under this assay condition, the extracts from *M. unguis-cati* (whole plant) did not show activity against *Leishmania spp* or *Trypanosoma cruzi* (Fournet et al 1994).

Few data are found in the literature about the chemical constituents of this species. A survey revealed that root extracts from *M. unguis-cati* contain lapachol, quinovic acid, 3-(O-fucosyl) alcohol, β-amyrin and β-sitosterol (Joshi et al 1985), while flower extracts show traces of cyanidin-3-glucoside (Scogin 1980). In addition, leaf extracts of another species of this genus (*M. cynchanoides*) contain the iridoids cynanchoside (Bonini et al 1981), macfadienoside (Bianco et al 1974) and 5,7-bisdeoxycynanchoside (Adriano et al 1982).

The purpose of this study was to chemically characterize the leaves and liana components from this plant, and to evaluate the antitumoral, trypanosomicidal and anti-inflammatory properties of these extracts.

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Material and Methods

Plant material

M. unguis-cati leaves and lianas were collected in Belo Horizonte, Minas Gerais, Brazil, during the summer season, 1995. A voucher specimen was deposited at the Herbarium of the Instituto de

Ciências Biológicas, UFMG, Belo Horizonte, Brazil (Lombardi 277).

The plant material was dehydrated in an oven at 40°C for four days. This was followed by mechanical grinding to a powder and extraction at room temperature with a mixture of ethanol–water (9 : 1), for one week. Solvent removal was carried out under vacuum, below 40°C, and yielded crude extracts from leaves (MACb, 17·8 g, 5·5%) and lianas (MACa, 100 g, 2·5%).

Chromatographic procedures

HPLC apparatus. A chromatograph HP1090 coupled to a UV-diode array detector and integrator HP3392A, with software HP79994A HPLC Chem-Station was used. The analysis was carried out in a LiChroCART 125-4 RP-18 (5 mm) Merck column linked to LiChroCART 4-4 RP-18 (5 μm) precolumn.

Samples. Authentic samples used as standards, crude extracts and partially purified fractions (1 mg) were dissolved in CH_3OH (1 mL) and filtered through a Millex-HV 0·45-μm filter (Millipore) before applying to the column.

The mobile phase was composed of acetonitrile and H_2O containing 1% (v/v) 0·1M phosphoric acid. Elution of sample was performed by stepwise increase in acetonitrile as follows: 1 min −5%; 20 min −20%; 30 min −30%; 40 min −80%; 50 min −95% at 1 mL min^{-1} flow rate.

Brine shrimp lethality bioassay

Artemia salina encysted eggs (10 mg) (*A. franciscana*) were a gift from Dr Eupídio Beltrani, Laboratório de Camarões, UFSC. They were incubated in artificial seawater (3·6 g NaCl, 1·5 g Mg_2SO_4, 0·5 g $NaHCO_3$ in 100 mL distilled water, pH 9) at 28°C under tungsten light. After incubation for 24 h, nauplii were collected with a Pasteur pipette and kept for an additional 24 h under the same conditions to reach the metanauplii stage. The samples (triplicate) to be assayed were dissolved in dimethylsulphoxide (DMSO) (2–120 mg/400 μL) and serially diluted (1, 2, 4 and 10 μL mL^{-1}) in artificial seawater. Between 10–20 metanauplii were added to each set of tubes containing the samples and the cultures further incubated at 28°C for 24 h (Meyer et al 1982). Controls containing DMSO were included on each set of experiments. Following scoring of survivors, the lethal concentration 50% (LC50) was calculated by Probit analysis.

Dosage of tannins and polyphenols

Tannins and polyphenols were evaluated by the protein precipitation test (Câmara 1967). The LC50 concentrations obtained for each fraction by the *Artemia salina* bioassay were incubated in holes (2 mm) drilled in a support of 1·5% gelatine and 1·5% agar at 56°C. After 24 h, the protein precipitation halo was measured. Several dilutions of tannic acid standards dissolved in DMSO were used as positive controls.

Dosage of saponin

Saponins were dosed by the haemolytic procedure (Câmara 1967). Erythrocytes from Holtzman rats were washed and suspended in saline (0·9%, pH 7·4) and added to 1·3% agar at 40°C in culture plates. The LC50 concentration determined for each sample was applied onto the gel and incubated for 24 h at room temperature, followed by measurement of the haemolytic halo. Serially diluted saponin standards (0·5–5·0 mg mL^{-1}) were used as positive controls.

Crown gall tumours on potato discs assay

One loop of *Agrobacterium tumefaciens* suspension was inoculated into a sterile culture containing 0·5% sucrose, 0·8% nutrient broth and 0·1% yeast extract, and incubated with stirring for 6 h at 30°C. In a laminar flow, washed potatoes were bored, cut into discs and five discs were placed on each Petri dish. To each disc, 20 μL bacterial suspension was added and incubated for 24 h at room temperature. This was followed by addition of 20 μL sample (triplicate) to each disc. After 21-days incubation of cultures at 27°C, the crown gall tumours were counted to determine the percent inhibition induced by each sample studied. Controls containing DMSO were assigned 100% growth (Ferrigni et al 1982).

Cyclooxygenase inhibition assay

The cyclooxygenase assay was a down scale modification of a previously described method (Wagner et al 1986) using cyclooxygenase isolated from sheep seminal vesicle microsomes. Plant extracts (10 μg) and controls were dissolved in 10 μL CH_3CH_2OH. The assay mix contained the enzyme protein (1 μg) in 190 μL 0·1M Tris–HCl (pH 8·0), 1 mM reduced glutathione, 1 mM epinephrine-hydrogentartrate and 0·05 mM Na_2EDTA. Following a 5 min pre-incubation of the mix at room temperature, the reaction was started by addition of 10 μL 1-[^{14}C]arachidonic acid (Amer-

sham; 4·5 mM, 0·05 μCi) and further incubated for 20 min at 37°C. To stop the reaction 10 μL 10% formic acid was added.

1-[^{14}C]Arachidonic acid and its labelled metabolites were separated by reversed-phase HPLC and further quantified by measuring the radioactive peak in a β-scintillation counter. The resulting activity obtained with each fraction was expressed as percent inhibition of prostaglandin E_2 formation, after subtracting the activity of controls (96% ethanol) without inhibitor.

The HPLC apparatus consisted of a Merck Hitachi L-6200 Intelligent Pump coupled to a Merck Hitachi AS-2000 autosampler and Berthold HPLC radioactivity monitor LB 506 C-1. The analysis was carried out using a LiChroCART 125-4 RP-18 (5 mm) Merck column and LiChroCART 4-4 RP-18 (5 μm) precolumn. The mobile phase was composed of acetonitrile and water containing 1% (v/v) 0·1 M phosphoric acid. The stepwise gradient started with 36% acetonitrile for 12 min, then 80% acetonitrile for 15 min at a flow rate of 1·0 mL min^{-1}.

5-Lipoxygenase assay

Briefly, the 5-lipoxygenase assay was performed according to a previously described method (Kuhl et al 1984). Porcine polymorphonuclear lymphocytes (PMNL) were isolated by dextran sedimentation and the remaining erythrocytes removed by cell lysis. The PMNL fraction was treated with eicosatetranoic acid, a lipoxygenase inhibitor, Ca^{2+} and Ca^{2+}-ionophore A23187 (Boehringer Mannheim) as inductor. After addition of plant extracts in 50 μL ethanol and 10 μL 9 μM (0·1 μCi) 1-[^{14}C]arachidonic acid, the mixture was incubated for 5 min at 37°C, followed by metabolite extraction with ethyl acetate. The solvent was then evaporated and the metabolites dissolved in ethanol before HPLC separation. HPLC fractionation was carried out on a reversed-phase column coupled to a β-scintillation detector. An estimate of 5-lipooxygenase inhibition was obtained by measuring the radioactivity of the metabolite [^{14}C]5-hydroxy-eicosatetraenoic acid produced by different amounts of sample. The uninhibited activity was determined in the presence of controls containing ethanol. The HPLC system used was similar to that described for the cyclooxygenase assay. The mobile phase was composed of acetonitrile and water containing 1% (v/v) 0·1 M phosphoric acid. The elution of the sample was by use of a linear gradient of acetonitrile between 50% and 90% for 20 min, then kept at 90% for an additional 10 min. The flow rate was 1·0 mL min^{-1}.

Results and Discussion

Chemical constituents

Crude extract from leaves (MACb) was partitioned into non-miscible phases according to the scheme shown in Figure 1. The resulting fractions (MACb1–3) were analysed by HPLC using a C_{18}-silica reverse phase column. A comparison of retention times (RT) and on-line UV spectra between *M. unguis-cati* fractions and authentic samples led to characterization of two C-glycosyl-flavonoids, corymboside (RT = 17·20 min) in the aqueous fraction and vicenin-2 (RT = 14·33 min) in the hydro-alcoholic fraction, along with detection of other non-identified substances.

A crude extract from liana (MACa) was also analysed by HPLC as described for leaf extracts. A comparison of retention times and on-line UV spectra of *M. unguis-cati* crude liana extracts together with authentic samples, led to identification of corymboside (RT = 17·20 min), the O-flavonol quercitrin (RT = 23·28 min) and two caffeic esters, chlorogenic acid (RT = 10·72 min) and isochlorogenic acid (RT = 23·0 min), as well as other unidentified substances.

The silica gel 60 column chromatography of MACa crude extract, using a hexane (100%)→ethyl acetate→methanol→methanol–water (50%) gradient led to isolation of lupeol, β-sitosterol, β-sitosterylglucoside, allantoin and lapachol. The identity of each of these compounds was confirmed by spectrometric analysis, with the aid of authentic standards. With the exception of lapachol and β-sitosterol, this is the first time that the remaining substances have been reported to occur in this species.

The presence of these compounds, to some extent, explains the uses of *M. unguis-cati* preparations in folk medicine. Lupeol showed in-

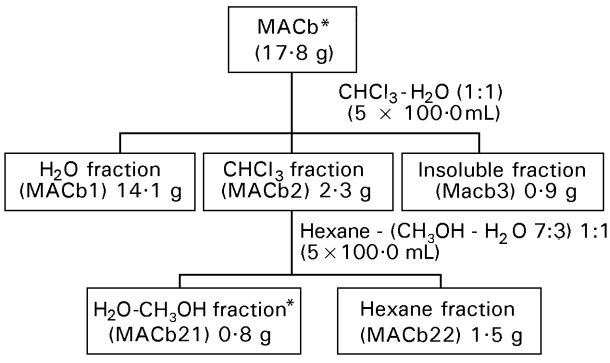

Figure 1. Bio-guided partition of *Macfadyena unguis-cati* leaves hydro-alcoholic extract against *Trypanosoma cruzi*. *Active against *T. cruzi*.

vitro activity against *Plasmodium falciparum* (Alves et al 1997), and lupeol, β-sitosterol and β-sitosteryl-glucoside showed anti-inflammatory and anti-pyretic effects (Kuhl et al 1984; Davis et al 1994). In addition, a mixture of β-sitosterol and β-sitosterylglucoside can be found as components of prescriptions used against prostate diseases, for example Harzol (Henneking & Heckers 1983).

Pharmacological activities

We evaluated the toxicity of the crude extracts, fractions and purified substances (Table 1). Meyer et al (1982) classified crude extracts and purified substances into toxic ($<1000\,\mu g\ mL^{-1}$) and non-toxic ($>1000\,\mu g\,mL^{-1}$) according to the levels required to attain the LC50 in the *Artemia salina* bioassay. Based on this classification, the MACa extract ($713{\cdot}6 \pm 14{\cdot}3\,\mu g\,mL^{-1}$) can be considered as toxic, while the MACb extract and its aqueous fraction MACb1 ($2288{\cdot}4 \pm 61{\cdot}3$ and $1510{\cdot}0 \pm 97{\cdot}3\,\mu g\,mL^{-1}$, respectively) are non-toxic. The alcoholic fraction MACb21 ($85{\cdot}0 \pm 12{\cdot}3\,\mu g\ mL^{-1}$) and the hexanic fraction MACb22 ($146{\cdot}0 \pm 15{\cdot}8\,\mu g\,mL^{-1}$) derived from MACb2 $CHCl_3$ fraction showed high toxicity. One of the substances purified from the MACa fraction (lapachol) shows high toxicity on *A. salina* ($LC50 = 16{\cdot}7 \pm 0{\cdot}9\,\mu g\,mL^{-1}$). Consequently, MACa, MACb21, MACb22 may contain potentially useful substances, for example lapachol (Table 1).

Among the fractions shown in Table 1, the crude extracts MACa, MACb and lapachol had their antitumoral activity studied using the potato disc assay (Table 2). MACa at the LC50 concentration inhibited crown gall tumour growth by $14{\cdot}4 \pm 1{\cdot}2\%$, while lapachol-mediated inhibition attained $64{\cdot}0 \pm 0{\cdot}5\%$ at LC50/100 i.e. $0{\cdot}16\,\mu g\,mL^{-1}$. The antitumoral activity of MACa can be partially explained by the presence of lapachol. The lapachol content of the MACa extract was estimated to be $0{\cdot}11\,\mu g\,mL^{-1}$, thus the expected crown gall tumour inhibition was 41%. The actual value of inhibition observed was 14%. This difference may be explained by the availability of lapachol in the extract or by the presence of other substances in the extract, which may interfere with the inhibitory effect. The antiproliferative activity exhibited by lapachol was studied further by using the MTT (3-[4,5- Dimethylthiazol- 2- yl]2,5- diphenyl tetrazolium bromide) colorimetric method (Bellamy 1992) on three tumour cell lines. In C6 (rat glioma) and Mewo (human melanoma) cell lines, lapachol at $16{\cdot}7\,\mu g\,mL^{-1}$ inhibited cell proliferation by $66{\cdot}7 \pm 5{\cdot}0\%$ and $55{\cdot}7 \pm 15{\cdot}2\%$, respectively. At a concentration range between 10^2- to 10^3-times lower, the inhibitory effect of lapachol on these cells was about 30%. In MDA MB 231 (human breast carcinoma) cells, the inhibitory effect reached 37% with the highest concentration used ($16{\cdot}7\,\mu g\,mL^{-1}$) (unpublished results). Some authors described, in-vivo, a higher antitumoral effect of lapachol on sarcomas Yoshida 180 and Walker 256 carcinosarcoma (Rao et al 1968; Santana et al 1968). In previous studies, lupeol, β-sitosterol and β-sitosterylglucoside were also identified as being responsible for the antitumoral activity exhibited by other plant extracts (Henneking & Heckers 1983; Mehta & Moon 1991; Moriarity et al 1998).

MACa and MACb extracts were tested in-vitro against blood forms of *Trypanosoma cruzi* using a procedure previously described by Chiari et al (1996). The samples considered as active were those that removed 100% of the trypomastigote form in blood samples from animals previously infected with the parasite. Unexpectedly, the MACb extract was not toxic for *A. salina*

Table 1. Determination of LC50 on *Artemia salina*, tannin and saponin contents from crude extracts, partially purified and purified samples from *M. unguis-cati*.

Sample	LC50 ($\mu g\,mL^{-1}$)	Haemolytic activity	Protein precipitation
Crude extracts			
Liana (MACa)	$713{\cdot}6 \pm 14{\cdot}3$	+ + +	No precipitation
Leaf (MACb)	$2288{\cdot}4 \pm 61{\cdot}3$	+ + + +	No precipitation
MACb fractions			
Aqueous (MACb1)	$1510{\cdot}0 \pm 97{\cdot}3$	+	No precipitation
Hydro-alcoholic (MACb21)	$85{\cdot}0 \pm 12.3$	+ + +	Visible precipitation
Hexanic (MACb22)	$146{\cdot}0 \pm 15{\cdot}8$	+ + +	Visible precipitation
MACa purified substance			
Lapachol	$16{\cdot}7 \pm 0{\cdot}9$	–	No precipitation

The LC50 values were determined by *Artemia salina* bioassay, saponin content was determined by haemolysis of rat red blood cells (saponin standards: $+ = 0{\cdot}5\,\mu g\,mL^{-1}$, $+ + = 1{\cdot}0\,\mu g\,mL^{-1}$, $+ + + = 2{\cdot}0\,\mu g\,mL^{-1}$; $+ + + + = 4{\cdot}0\,\mu g\,mL^{-1}$, $+ + + + + = 5{\cdot}0\,\mu g\,mL^{-1}$), and tannin content was measured by protein precipitation as described in Materials and Methods.

Table 2. Inhibition of crown gall tumour formation in potato discs by extracts and purified examples from *M. unguis-cati*.

Sample	Concentration			
	LC50	LC50/10	LC50/100	LC50/1000
Crude extracts				
Liana (MACa)	14·4 ± 1·2	nd	nd	nd
Leaf (MACb)	nd	nd	nd	nd
MACa purified substance				
Lapachol	89·2 ± 0·6	85·3 ± 1·5	64·0 ± 0·5	10·5 ± 0·6

The LC50 values were obtained by the *Artemia salina* bioassay. Results are expressed as percent inhibition of callus formation ± s.d. nd, not detected.

Table 3. The biological activity of crude extracts and partially purified samples from *M. unguis-cati*.

Sample	Trypanosomicidal activity[a]	5-Lipoxygenase inhibition[b]	Cyclooxygenase inhibition[c]
Crude extracts			
Liana (MACa)	Inactive	<25%	<25%
Leaf (MACb)	Active	<25%	<25%
MACb fractions			
MACb2	Inactive	–	–
Hydro-alcoholic (MACb21)	Active	–	–
Hexanic (MACb22)	Inactive	–	–

[a]The crude extract samples (100 $\mu g\,mL^{-1}$) and partially purified samples (50 $\mu g\,mL^{-1}$) were assayed for trypanosomicidal effect according to Chiari et al (1996). Active fractions were considered to be those that removed 100% of the trypromastigote form. [b,c]The crude extracts (1000 $\mu g\,mL^{-1}$) were assayed as described in Materials and Methods.

(LC50 = 2288·4 ± 61·3 $\mu g\,mL^{-1}$), but was active against the trypomastigote form of *T. cruzi* at 100 $\mu g\,mL^{-1}$ (Table 3). Upon partitioning of MACb into $CHCl_3/H_2O$ (Figure 1), the recovered fractions (50 $\mu g\,mL^{-1}$) were checked for their biological activity. The results showed that the hydro-methanolic fraction MACb21, but not MACb22, retained its trypanosomicidal activity (Table 3). The activity can be attributed to vicenin-2, as previous work showed that flavonoids in *Trixis vauthieri* were effective against *Trypanosoma* (Ribeiro et al 1997). The bioactivity of fraction MACb21 was also analysed on *A. salina*. The results revealed that upon partitioning this fraction became toxic (LC50 85·0 ± 12·3 $\mu g\,mL^{-1}$) by the Meyer criteria. The rise in toxicity of MACb21 can be explained by its activation upon fractionation with hexane/methanol/H_2O.

Despite *M. unguis-cati* containing lupeol, β-sitosterol and β-sitosterylglucoside, all anti-inflammatory substances, neither the MACa nor the MACb extract at 1000 $\mu g\,mL^{-1}$, the average LC50 value for these fractions, showed significant inhibitory effect (<25%) in-vitro on 5-lipoxygenase and cyclooxygenase. The weak inhibitory effects may be due to the low lipophilicity of the ethanolic extracts assayed (Panthong et al 1994). Alternatively, the weak inhibition observed could be explained by the use of isolated enzymes rather than the whole cell in the cyclooxygenase assay. Mitchell et al (1994) determined the inhibition of cyclooxygenase using several anti-inflammatory drugs, in three different models (intact cells, broken and purified enzymes) and demonstrated that broken cells and purified enzymes had higher IC50 values than intact cells.

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