Journal of Ethnopharmacology 170 (2015) 218–225

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

Involvement of monoaminergic systems in the antidepressant-like properties of *Lafoensia pacari* A. St. Hil

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ARTICLE INFO

Article history:
Received 2 December 2014
Received in revised form
5 May 2015
Accepted 5 May 2015
Available online 14 May 2015

Keywords:
Lafoensia pacari
Medicinal plant
Antidepressant-like
Forced swimming test
Cerrado

ABSTRACT

Ethnopharmacological relevance: *Lafoensia pacari* A. St.-Hil. (Lythraceae), known popularly as “pacari” or “mangaba-brava” is popularly used in the state of Goiás, Brazil. The stem bark or leaves are used to treat cancer, gastric disorders, inflammation and as a tonic to treat loss of enthusiasm.

Aim of the study: Previous results suggest that the ethanol:water 7:3 extract of the stem bark of *L. pacari* (PEx) has antidepressant-like activity in male mice. Our aim was to perform the PEx's bioguided fractionation and evaluate the monoaminergic system involvement in the antidepressant effect as well as progress in the study of *L. pacari* mechanism of action.

Material and methods: Mice (30–35 g) orally treated (24, 5 and 1 h) with PEx (100, 300 or 1000 mg/kg), chloroform (ChloF—70 mg/kg), ethyl acetate (180 mg/kg), *n*-butanol (370 mg/kg) and aqueous (1 g/kg) fractions were submitted to the forced swimming test. To assess the mechanism of action, different groups of mice were pretreated with *p*-chlorophenylalanine (PCPA—100 mg/kg, 4 days, i.p.) and alpha-methyl-*p*-tyrosine (AMPT—100 mg/kg, 4 h, i.p.) to assess the involvement of serotoninergic and catecholaminergic systems in the ChloF effects, respectively. A putative *in vitro* inhibition of monoamine oxidase (MAO) activity as well as the *ex vivo* hippocampal brain-derived neurotrophic factor (BDNF) quantification were carried out. Phytochemical screening, spectroscopy and chromatography analysis were used for identification of compounds present in ChloF.

Results and discussion: After the fractionation, the ChloF 70 mg/kg was the most active fraction, reducing the immobility time by 22%. Pre-treatments with both PCPA and AMPT abolished the ChloF effects, suggesting that ChloF antidepressant-like effect is dependent on serotonergic and catecholaminergic systems. ChloF did not inhibited MAO-A or MAO-B activity, excluding this as possible mechanism of action. ChloF augmented hippocampal BDNF level, which could be accounted for its antidepressant-like effect. Phytochemical screening showed the presence of saponins, tannins, steroids and triterpene in the PEx, and the presence of triterpene and steroids in ChloF. The spectroscopy and chromatography analysis identified lupeol, β-sitosterol and stigmasterol in ChloF.

Conclusion: ChloF is the fraction that better retained the crude extract active constituents. ChloF presents antidepressant-like effect that involves both serotonergic and catecholaminergic systems without inhibiting MAO enzymatic activity; this fraction also increases the hippocampal BDNF levels.

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1. Introduction

The use of plants as medicines for humankind is connected to its own development. In Brazil, the traditional medicine is a rich mixture of various cultures, mainly African (mostly Yoruba and Bantu), European (mainly Portuguese), and Native South America medical traditions (Sá and Elisabetsky, 2012). Among the many

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http://dx.doi.org/10.1016/j.jep.2015.05.015
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plants used in folk medicine by the population of the states of Goiás and Mato Grosso, it is the herbal *Lafoensia pacari* A. St. Hil. (Lythraceae family).

Lafoensia pacari is a tree plant of up to 5 m height that grows in Brazilian Cerrado and altitude forest ecosystems being also present in Paraguay and Bolivia (Silva, 1998). It is known popularly as "pacari" which in Tupi-Guarani Amerindian language means "precious wood", but has other popular names, among them is "mangaba-brava" (Proença et al., 2000). It has the botanical synonyms *Lafoensia sessilifolia* Klotzch and *L. pacari* Saint-Hilaire *ssp. petiolata* Koehne (Carvalho, 1994; Lorenzi, 2002).

In folk medicine, the *L. pacari* stem bark or leaves are widely used after decoction in water or maceration in alcoholic drinks such as "cachaça" or white wine (known as "garrafada"). This medicinal plant is used to treat gastric ulcers, wounds, pain, local and systemic inflammation, cancer, itch, diarrhea, kidney problems, besides being used as antipyretic, slimming, healing and, of the special interest to us, as a tonic for the loss of energy (Cabral and Pasa, 2009; Corrêa, 1984; Pott and Pott, 1994).

The literature has shown the pharmacologic potential of this plant. Different extracts from this plant has shown antimicrobial (Lima et al., 2006; Müller et al., 2007; Pereira et al., 2011; Silva et al., 2010; Queiroz-Silva et al., 2012), antinociceptive (Guimarães et al., 2010; Matos et al., 2008; Nascimento et al., 2011), anti-inflammatory and anti-pyretic (Guimarães et al., 2010; Matos et al., 2008; Rogerio et al., 2006, 2008), besides gastroprotective (Menezes et al., 2006, Tamashiro-Filho et al., 2012) effects and antioxidant and anti-cancer activities (Solon et al., 2000).

Our group have studied the *L. pacari* central effects in rodent behavioral models, where we showed that the stem bark crude ethanol:water 7:3 extract has anxiolytic-like activity (Galdino et al., 2010); and when administered for 21 days by oral route, the PEx has antidepressant-like effect (Galdino et al., 2009). Depressive disorders are among the most prevalent lifetime mood disorders, affecting approximately 16% of North Americans (Kessler et al., 2005) and 18.4% of Brazilians (Bromet et al., 2011). In this context, our aim was to evaluate the involvement of monoaminergic system in the antidepressant effect of the active fraction and phytochemically characterize the extract and its active fraction.

2. Material and methods

2.1. Animals

Experiments were conducted using male *Swiss* mice, weighing 30–35 g, provided by the Central Animal House of Federal University of Goiás (Universidade Federal de Goiás—UFG). Animals were maintained at constant room temperature of 21–23 °C with free access to water and food, under a 12:12 h light:dark cycle (lights on at 7:00 h). Animals were acclimatized for 4 days before the beginning of the experiments. All experimental protocols were developed in accordance with the principles of ethics and animal welfare designated by Brazilian Society of Science of Laboratory Animals (Sociedade Brasileira de Ciência em Animais de Laboratório—SBCAL) and the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. All the protocols were approved by the Ethics Commission of the UFG (Protocol number 104/08). All behavioral experiments were conducted from 1:00 p.m. to 5:00 p.m.

2.2. Plant material

The stem bark of *L. pacari* was collected (May 2011) from plants in natural habitat in the savannah region of Bela Vista, Goiás, Brazil (837 m, 16°58′54.2″ S, 40°55′45.1″ W). Samples were authenticated by Prof. Dr. José Realino de Paula, and a voucher specimen was deposited at the Herbarium of the Federal University of Goiás (UFG) under the number 27031/UFG.

2.3. Extraction and fractionation

The stem bark was cut into small pieces, dried at 40 °C for 48 h, powdered and stored under refrigeration until its use in the extractive process. The stem bark maceration in alcoholic drinks is the form widely used by Goiás population, then the pacari ethanol:water 7:3 extract (PEx) was obtained by maceration in 70% (v/v) hydro-alcoholic solution for 72 h, followed by filtration and evaporation in rotary evaporator (yield = 16.1%, w/w). Part of the PEx (20 g) was dried and dissolved in 400 mL of methanol/water solution (1:9), and successively partitioned with chloroform, ethyl acetate and *n*-butanol. The yields of the chloroformic (ChloF), ethyl acetate (EAF), n-butanol (BuF) and aqueous (AqF) fractions were 3, 9, 18 and 61% (w/w), respectively. ChloF were suspended in Tween 80® 2% (w/w) such as all other treatments (PEx, EAF, BuF and AqF).

2.4. Drugs

Clorgyline, pargyline, *p*-chlorophenylalanine (PCPA), α-methyl para-tyrosine (AMPT), tyramine, vanillic acid, 4-aminoantipyrine, peroxidase, polyoxyethylenesorbitan monooleate (Tween 80®) were purchased from Sigma-Aldrich (Brazil). Imipramine (Trofanil®—Novartis, Brazil), fluoxetine (EMS, Brazil). PCPA, AMPT was suspended in a 0.9 w/v% NaCl solution containing 2% Tween 80 for oral treatment. Imipramine and fluoxetine were dissolved in a 0.9 w/v% NaCl solution for oral treatment.

2.5. Methods

2.5.1. Extract, fraction and drugs effects on open field test (OFT)

In order to investigate the PEx effects in locomotor activity in male mice, animals (n = 10–12) were treated (p.o) with vehicle (Tween 80® 2%) 10 mL/kg, PEx 100, 300 and 1000 mg/kg or imipramine 30 mg/kg (positive control). The same animal received the same treatment by gavage at the times of 24, 5 and 1 h before the open field test. In the bio-guided fractionation, animals (n = 9) were treated (p.o.) with vehicle, ChloF 70 mg/kg, EAF 180 mg/kg, BuF 370 mg/kg, AqF 1 g/kg or imipramine 30 mg/kg (positive control) (24, 5 and 1 h) before the OFT. In a separate series of experiments, to assay the ChloF dose-effect curve, animals (n = 9–11) were treated (p.o.) with vehicle, ChloF 35, 70 and 140 mg/kg or imipramine 30 mg/kg (positive control) (24, 5 and 1 h) before the OFT. To avoid possible false-positive results due to alteration in animal's ambulation in the mechanism of action study; the mice (n = 8–11) pretreated (i.p.) with PCPA (100 mg/kg, a tryptophan hydroxylase inhibitor) or vehicle (2% Tween 80), once a day, for 4 consecutive days; in addition, the animal were orally treated with vehicle or ChloF 70 mg/kg (24, 5 and 1 h) and submitted to the OFT. The animals (n = 8–11) pretreated (i.p.) with AMPT (100 mg/kg, a tyrosine hydroxylase inhibitor) or vehicle (2% Tween 80) 4 h, and after treated (p.o.) with vehicle or ChloF 70 mg/kg (24, 5 and 1 h) were also submitted to the OFT. The apparatus consisted of a circular arena measuring 36 (diameter) × 20 cm (height), with the bottom divided into eight equal areas. Animals were individually placed at the center of the open field arena and allowed to explore it for 5 min (Archer, 1973). During this time, the locomotor activity: defined as the number of total squares crossed/5 min, was videotaped. The arena was cleaned with a 10% ethanol solution after each animal.

2.5.2. Crude extract and fractions antidepressant-like effect

In order to investigate the PEx antidepressant-like effect in male mice, animals (n=10–12) were treated (p.o) with vehicle (Tween 80® 2%) 10 mL/kg, PEx 100, 300 and 1000 mg/kg or imipramine 30 mg/kg (positive control). The same animal received the same treatment by gavage at the times of 24, 5 and 1 h and immediately after the OFT the animals were submitted to the forced swimming test (FST). In the bioguided fractionation, animals (n=9) were treated (p.o.) with vehicle, ChloF 70 mg/kg, EAF 180 mg/kg, BuF 370 mg/kg, AqF 1 g/kg or imipramine 30 mg/kg (positive control) (24, 5 and 1 h) before the OFT followed by the FST. In a separate series of experiments, to assay the ChloF dose-effect curve, animals (n=9–11) were treated (p.o.) with vehicle, ChloF 35, 70 and 140 mg/kg or imipramine 30 mg/kg (positive control) (24, 5 and 1 h) before the OFT followed by the FST. Other group of animals that received the same treatments was evaluated in tail suspension test (TST) to confirm the ChloF antidepressant-like effect. In the FST, each mouse was individually placed in a plastic cylinder (diameter 18 cm, height 42 cm) filled with water at a height of 30 cm. Each session was recorded by a videocamera. The immobility time (floating while animal makes only the necessary movements to keep the nostrils above the surface level) was scored during 6 min (Porsolt et al., 1978). In the TST, the mice were individually suspended by the tail 50 cm above the floor and fixed with adhesive tape placed approximately 1–2 cm from the tip of the tail. The immobility time was recorded for 6 min (Stéru et al., 1985).

2.5.3. ChloF antidepressant-like mechanism of action

In order to investigate a possible contribution of the 5-HT (serotonin) system to the ChloF antidepressant-like effect, animals (n=8–11) were pretreated (i.p.) with PCPA (100 mg/kg, a tryptophan hydroxylase inhibitor) or vehicle (2% Tween 80), once a day, for 4 consecutive days. In addition, animals were treated (p.o.) with vehicle and ChloF 70 mg/kg, 24, 5 and 1 h before the OFT and FST as described in Section 2.5.3. Fluoxetine 30 mg/kg was used as positive control of serotoninergic system involvement. To investigate a possible contribution of the catecholaminergic system to the ChloF antidepressant-like effect, animals (n=8–11) were pretreated (i.p.) with AMPT (100 mg/kg, a tyrosine hydroxylase inhibitor) or vehicle (2% Tween 80) 4 h before OFT and FST. In addition, animals were treated (p.o.) with vehicle and ChloF 70 mg/kg, 24, 5 and 1 h before the OFT and FST as described in Section 2.5.3. Imipramine 30 mg/kg was used as positive control of catecholaminergic system involvement.

2.5.4. Biochemical procedures

2.5.4.1. In vitro MAO assay. Three naïve mice were sacrificed by cervical dislocation and the liver was removed and used as source of crude mitochondrial pellets. The dissected tissue was washed in ice-cold potassium phosphate buffer (0.2 M, pH 7.6) and homogenized (Turrax®) in 40 vol 0.32 M sucrose solution in potassium phosphate buffer. The homogenates were centrifuged (1200 × g, 7 min, 4 °C) and the supernatants collected and centrifuged (12,500 × g, 15 min, 4 °C). The pellets obtained were centrifuged again (12,500 × g, 15 min, 4 °C) in 0.32 M sucrose solution in potassium phosphate buffer. The crude mitochondrial pellets were suspended in 1 ml of 3.6 mM KCl solution in potassium phosphate buffer. Protein contents of crude mitochondrial solution were measured by the Bradford's method (1976), with bovine serum albumin as standard. At the time of use, the protein concentration was adjusted with phosphate buffer (0.2 M; pH 7.6) to 0.2 mg protein per mL. The continuous peroxidase-linked photometric assay was carried out in the 96-well microtiter format modified from Holt et al. (1997) and Stafford et al. (2007). To differentiate type A and B activities, the mitochondrial fraction of mouse liver were pre-incubated with 250 nM clorgyline or pargyline at 37 °C during 5 min, respectively. Each test well contained 120 μL amino substrate (2.5 mM tyramine in potassium phosphate buffer), 40 μL chromogenic solution (2 mM vanillic acid, 1 mM 4-aminoantipyrine, 8 U/ml peroxidase in potassium phosphate buffer), 40 μL enzyme (rat liver homogenate) and 40 μl of sample. Background wells contained potassium phosphate buffer (0.2 M, pH 7.6) in place of chromogenic solution. The plate was incubated at 37 °C during 30 min and read at 498 nm.

2.5.4.2. Ex vivo ELISA measurements of BDNF protein. In order to investigate if ChloF alter BDNF protein concentration on mice hippocampus, animals were treated (p.o.) with vehicle, ChloF 70 mg/kg and imipramine 30 mg/kg, 24, 5 and 1 h before FST. Immediately after the behavioral test, mice were sacrificed by cervical dislocation and their hippocampi dissected to BDNF ELISA assay. After brain dissection (5–6 per group), the hippocampi were rapidly dissected, weighed, frozen in liquid nitrogen, and stored at −80 °C. Hippocampi were suspended in 1:40 (w:v) of lysis buffer (100 mM Tris–HCl, 1 mM NaCl, 4 mM EDTA, 2% albumin, 2% Triton-X 100, 0.01% thimerosal, and protease inhibitors cocktail (GE Life Sciences), pH 7.0) and homogenized using a Potter homogenizer, the tube was submersed in an ice bath. The homogenates were centrifuged at 16.800 × g for 35 min, 4 °C, and the supernatant was used in ELISA assay according to kit's instructions (ELISA Kit for BDNF—Uscn Life Science Inc.®, China). Protein contents were measured by Bradford's methods (1976), with bovine serum albumin as standard.

2.5.5. Phytochemical screening

2.5.5.1. Thin-layer chromatography analysis (TLC). The assay of chemical constituents presents in PEx and fractions were performed by TLC in silica gel plates (Whatman 20 cm × 20 cm, UV 254, 0.25 mm), according to procedures described by Wagner and Bladt (1995).

2.5.5.2. Nuclear magnetic resonance (NMR). The active fraction was analyzed by nuclear magnetic resonance (NMR) using ^{1}H (500 MHz) and ^{13}C (125 MHz) and bi-dimensional techniques Heteronuclear Multiple Quantum Correrence (HMQC) in a Bruker spectrometer, model DRX-500 MHZ, with deutered chloroform $CDCl_3$ as solvent.

2.5.5.3. High performance liquid chromatography (HPLC-PDA). HPLC of ChloF was performed on Waters equipment (Massachusetts, USA) equipped with quaternary pump, separation module e2695, photodiode array detector (PDA) 2998 and data processing system Enpower 2.0. We used Phenomenex C-8 column (250 mm × 4.6 mm, 5 μm) at a temperature of 25 °C. The detection system used was monitored at 202 nm. The injection volume was 30 μL and the run in isocratic mode, used as mobile phase acetonitrile/water (9:1) at a constant rate of 1.0 mL/min. The maximal running time was 40 min. Lupeol (Santa Cruz) and β-sitosterol (Sigma) were used as reference.

2.5.6. Statistical analysis

Data were presented as mean ± standard error of the mean (SEM). Statistical analysis were done using Student's t test (control × positive control) or by one-way ANOVA followed by Newman–Keuls' test (control × treated groups). When appropriated two-way ANOVA followed by Bonferroni's post-hoc test were used. Statistical difference was set at $P < 0.05$. All graphics were drawn using GraphPad Prism® software version 5.0.

3. Results

3.1. Overall effects of the treatments in the locomotor activity (OFT)

All treatment were tested in the open field test in order to avoid false-positive or false-negative results related to alteration in the locomotors activity. As Table 1 shows, none of the treatments tested in this study altered the number of areas crossed when compared with its respective control group.

3.2. Antidepressant-like effects of crude extract and fractions

In FST, male mice treated with PEx 100, 300 and 1000 mg/kg reduced the in immobility time ($P<0.05$ to all tested doses, Fig. 1); imipramine 30 mg/kg also reduce the immobility time ($P<0.001$; Fig. 1), as expected.

When the experiment was conducted with the fractions, only the ChloF 70 mg/kg reduced the immobility time ($P<0.05$; Fig. 2), whereas with the EAF 180 mg/kg was observed a tendency in reducing this parameter ($P=0.06$). Imipramine reduced the immobility time ($P<0.001$; Fig. 2), as expected.

In an attempt to assess the ChloF dose-effect relationship, three doses of this fraction were tested in the forced swimming test: 35, 70 and 140 mg/kg, and all doses reduced the immobility time ($P<0.05$, $P<0.001$ and $P<0.05$, respectively, Fig. 3A), as well as imipramine 30 mg/kg ($P<0.001$; Fig. 3A). ChloF 35, 70 mg/kg also increased the climbing time ($P<0.05$ and $P<0.01$, respectively, Fig. 3D). Moreover, ChloF 70 and 140 mg/kg increased the swimming time ($P<0.01$, Fig. 3C). Imipramine 30 mg/kg also increased the climbing ($P<0.05$; Fig. 3D) and swimming time ($P<0.01$; Fig. 3C). This dose-effect relationship study was also observed in the tail suspension test, where ChloF 70 and 140 mg/kg reduced the immobility time ($P<0.05$, Fig. 3B), as well as imipramine 30 mg/kg ($P<0.01$; Fig. 3B).

Table 1
Locomotor activity (number of squares crossed) in the open field after different pharmacological treatments.

Experimental groups	Mean ± SEM
Crude *L. pacari* extract	
Vehicle 10 mL/kg p.o.	127.7 ± 10.68
PEx 100 mg/kg p.o.	147.2 ± 14.34
PEx 300 mg/kg p.o.	122.9 ± 16.79
PEx 1000 mg/kg p.o.	97.59 ± 14.93
Imipramine 30 mg/kg p.o.	108.2 ± 10.06
***L. pacari* extract fractions**	
Vehicle 10 mL/kg p.o.	108.3 ± 5.07
ChloF 70 mg/kg p.o.	97.4 ± 5.44
EAF 180 mg/kg p.o.	109.7 ± 4.47
BuF 370 mg/kg p.o.	98.8 ± 8.84
AqF 1 g/kg p.o.	100.8 ± 6.91
Imipramine 30 mg/kg p.o.	97.0 ± 7.07
ChloF dose × effect curve	
Vehicle 10 mL/kg p.o.	162.6 ± 7.62
ChloF 35 mg/kg p.o.	161.2 ± 8.26
ChloF 70 mg/kg p.o.	160.2 ± 11.05
ChloF 140 mg/kg p.o.	162.5 ± 12.03
Imipramine 30 mg/kg p.o.	153.7 ± 9.41
Interaction of fluoxetine with PCPA	
Vehicle 10 mL/kg i.p.+Vehicle 10 mL/kg p.o.	128.5 ± 9.81
Vehicle 10 mL/kg i.p.+Fluoxetine 30 mg/kg p.o.	140.0 ± 7.25
PCPA 100 mg/kg i.p.+Vehicle 10 mL/kg p.o.	140.8 ± 10.86
PCPA 100 mg/kg i.p.+Fluoxetine 30 mg/kg p.o.	155.6 ± 10.41
Interaction of ChloF with PCPA	
Vehicle 10 mL/kg i.p.+Vehicle 10 mL/kg p.o.	130.0 ± 10.50
Vehicle 10 mL/kg i.p.+ChloF 70 mg/kg p.o.	125.6 ± 8.02
PCPA 100 mg/kg i.p.+Vehicle 10 mL/kg p.o.	128.1 ± 10.59
PCPA 100 mg/kg i.p.+ChloF 70 mg/kg p.o.	115.4 ± 6.66
Interaction of imipramine with AMPT	
Vehicle 10 mL/kg i.p.+Vehicle 10 mL/kg p.o.	116.2 ± 9.91
Vehicle 10 mL/kg i.p.+Imipramine 30 mg/kg p.o.	120.9 ± 6.68
AMPT 100 mg/kg i.p.+Vehicle 10 mL/kg p.o.	98.5 ± 12.70
AMPT 100 mg/kg i.p.+Imipramine 30 mg/kg p.o.	99.2 ± 10.62
Interaction of ChloF with AMPT	
Vehicle 10 mL/kg i.p.+Vehicle 10 mL/kg p.o.	137.0 ± 9.36
Vehicle 10 mL/kg i.p.+ChloF 70 mg/kg p.o.	121.6 ± 12.46
AMPT 100 mg/kg i.p.+Vehicle 10 mL/kg p.o.	106.9 ± 8.63
AMPT 100 mg/kg i.p.+ChloF 70 mg/kg p.o.	122.2 ± 8.17
ChloF and imipramine effect before BDNF assay	
Vehicle 10 mL/kg p.o.	110.9 ± 10.78
ChloF 70 mg/kg p.o.	105.3 ± 12.05
Imipramine 30 mg/kg p.o.	99.33 ± 7.64

3.3. Contribution of the serotonergic and catecholaminergic system to ChloF antidepressant-like effect

Pretreatment with the 5-HT synthesis inhibitor PCPA 100 mg/kg prevented the fluoxetine 30 mg/kg ($P<0.01$; Fig. 4A) and ChloF 70 mg/kg antidepressant-like effects ($P<0.01$; Fig. 4B). Pretreatment with the non-selective blocker of catecholamine synthesis AMPT 100 mg/kg also prevented the ChloF 70 mg/kg ($P<0.05$; Fig. 4D) as well as imipramine 30 mg/kg antidepressant-like effects ($P<0.01$; Fig. 4C).

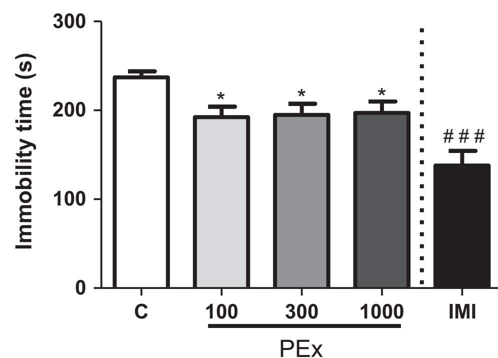

Fig. 1. Effect of oral treatment with ethanol extract of *L. pacari* stem bark (PEx) at 24, 5 and 1 h before the forced swimming test (6 min), at doses of 100, 300 and 1000 mg/kg in mice. Bars represent mean ± sem of total immobility time (in seconds) of 10–12 animals. *$P<0.05$ compared to control group by one-way ANOVA followed by Newman–Keuls' test (control × treated groups). #$P<0.05$ compared to control group by Student's *t* test (control × positive control). C: control group – vehicle (2% Tween) 10 mL/kg p.o., IMI: imipramine 30 mg/kg p.o.

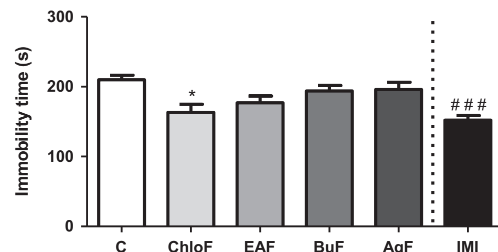

Fig. 2. Effect of oral treatment with fractions from the ethanol extract of *L. pacari* stem bark at 24, 5 and 1 h before the forced swimming test (6 min) in mice. Bars represent mean ± sem of total immobility time (in seconds) of 9 animals. *$P<0.05$ compared to control group by one-way ANOVA followed by Newman–Keuls' test (control × treated groups). #$P<0.05$ compared to control group by Student's *t* test (control × positive control). C: control group – vehicle (2% Tween) 10 mL/kg p.o., ChloF 70 mg/kg p.o. – chloroformic, EAF 180 mg/kg p.o. – ethyl acetate, BuF 370 mg/kg p.o. – n-butanol p.o. and AqF 1 g/kg p.o. – aqueous fractions, IMI: imipramine 30 mg/kg p.o.

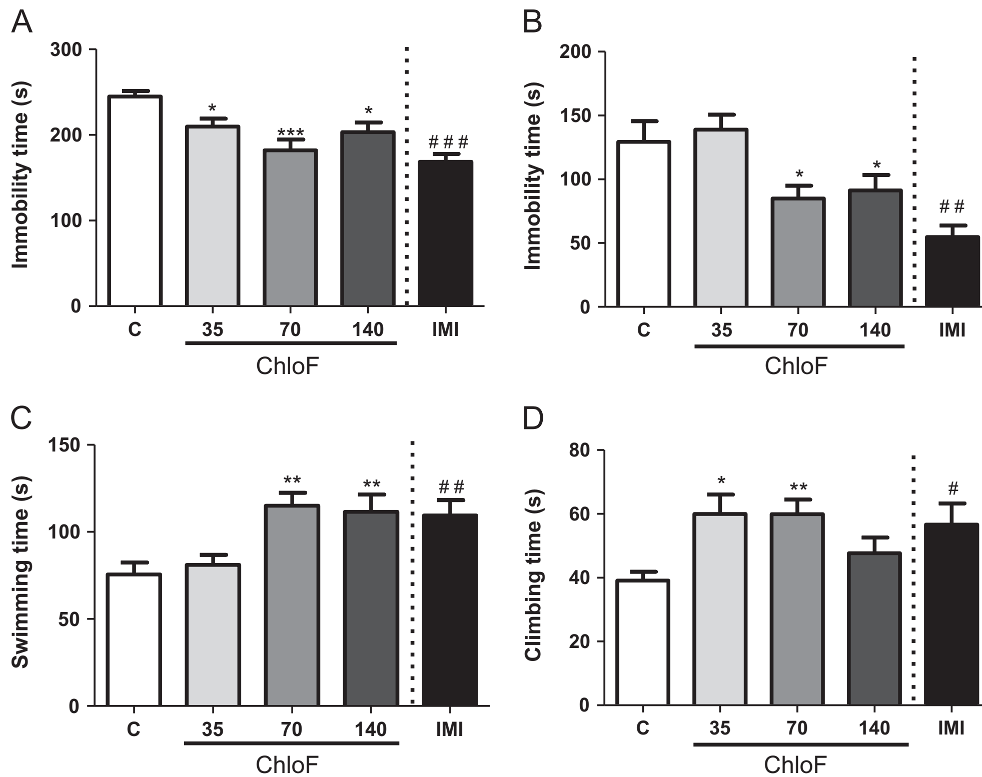

Fig. 3. Effect of oral treatment with chloroformic fraction (ChloF 70 mg/kg) from ethanol extract of *L. pacari* stem bark at 24, 5 and 1 h before the forced swimming (A, C and D) and tail suspension (B) tests (6 min), at doses of 35, 70 and 140 mg/kg in mice. Total immobility (A), swimming (C) and climbing (D) time in the forced swimming test and, total immobility time in the tail suspension (B) test. Bars represent mean ± sem of 9–11 animals. *$P < 0.05$ compared to control group by one-way ANOVA followed by Newman–Keuls' test (control × treated groups). #$P < 0.05$ compared to control group by Student's t test (control × positive control). C: control group – vehicle (2% Tween) 10 mL/kg p.o., IMI: imipramine 30 mg/kg p.o.

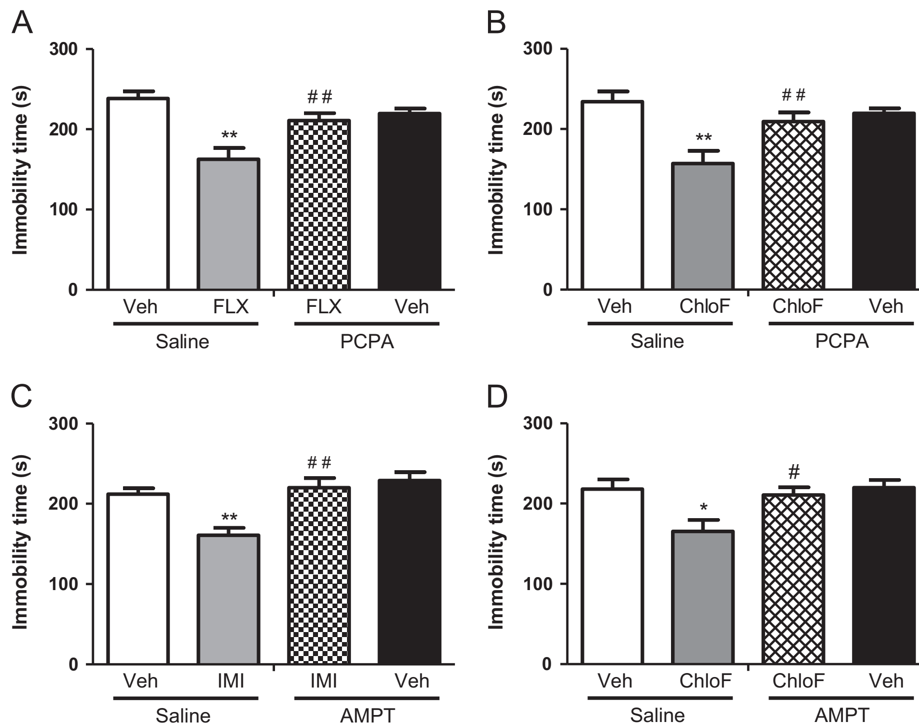

Fig. 4. Effect of pretreatment with PCPA (100 mg/kg i.p.–4 days; A and B) and AMPT (100 mg/kg i.p.—4 h; C and D) in fluoxetine (FLX—30 mg/kg, p.o, 1 h; A), chloroformic fraction (FClor—70 mg/kg, p.o., 24, 5 and 1 h, B and D), or imipramine (IMI—30 mg/kg, p.o., 1 h, C) effects on the total immobility time of forced swimming test. Bars represent mean ± sem of 8–11 animals. *$P < 0.05$ compared to control group; and #$P < 0.05$ compared to FLX, IMI or ChloF groups by two-way ANOVA followed by Bonferroni's test. C: control group—vehicle (saline 0.9%) 10 mL/kg i.p. + vehicle (2% Tween) 10 mL/kg p.o.

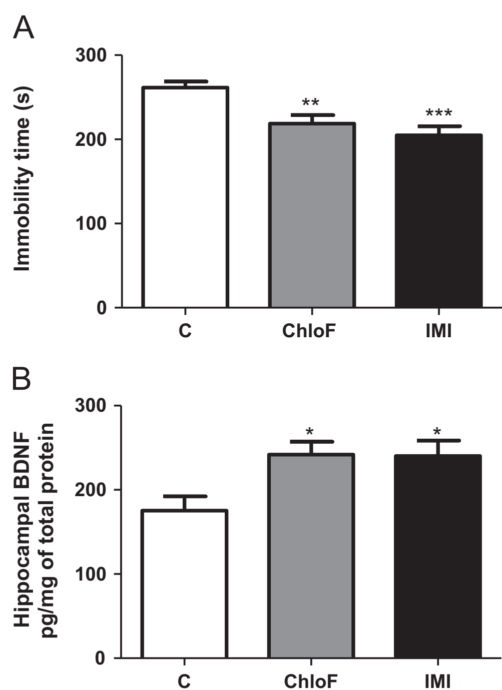

Fig. 5. Effect of oral treatment (24, 5 and 1 h) with chloroformic fraction (ChloF—70 mg/kg) and imipramine (IMI—30 mg/kg) on total immobility time (in seconds) of forced swimming test (6 min) in mice. Each group consisted of 9–10 animals (A). Effect of these treatments on hippocampal BDNF protein levels of 5–6 animals (randomly chosen) previously submitted to the forced swimming test (B). Bars represent the mean ± sem. *$P<0.05$ compared to control group by Student's *t* test. C: control group – vehicle (2% Tween) 10 mL/kg p.o.

3.4. *In vitro MAO assay*

ChloF was not able to inhibit neither MAO-A nor MAO-B enzymatic activity, assayed *in vitro*, whereas, as expected, both positive controls, clorgyline—an iMAO-A, and pargyline—an iMAO-B, were able to inhibit the MAO subtype A (IC_{50}: 76 nM [49–115.7 nM]) and B (IC_{50}: 0.3 μM [0.19–0.49 μM]), respectively (data not shown).

3.5. *Ex vivo ELISA measurements of BDNF protein*

Treatment with ChloF 70 mg/kg and imipramine 30 mg/kg, at doses that reduced the immobility time ($P<0.01$ and $P<0.001$; Fig. 5A), increased the hippocampal BDNF levels ($P<0.05$; Fig. 5B).

3.6. *Phytochemical screening and identification of compounds in CholF by NMR and HPLC-PDA*

Phytochemical screening of PEx and fractions had shown the presence of tannins, steroids, triterpenes and traces of flavonoids in PEx, and the presence of steroids and triterpenes in ChloF. In the ethyl acetate fraction were detected steroids, triterpenes and flavonoids, while in the n-butanol and aqueous fraction, only tannins were detected.

In the TLC, in ChloF analysis was seen the presence of steroids and triterpenes and with RF compatible with the authentic standards of β-sitosterol, lupeol, oleanolic and ursolic acid and α-amyrin.

NMR analysis showed characteristic data for aliphatic compounds. ^{13}CNMR and HMQC of ChloF showed chemical shifts to β-sitosterol ($C_{29}H_{50}O$) with lupeol ($C_{29}H_{50}O$) and stigmasterol $m/z=412$ Da ($C_{30}H_{50}O$) (Mahato and Kundu, 1994; Lemes et al., 2011). The other chemical shifts are characteristics of ^{1}H and ^{13}C of the aliphatic compounds and were not confirmed its structures. The analysis by HPLC-PDA of ChloF and of authentic standards confirmed the presence of β-sitosterol and lupeol at $Rt=18.6$ min and 24.7 min, respectively. However, the presence of oleanolic and ursolic acid and α-amyrin were not confirmed.

4. Discussion

In this study, we have evaluated the effect of *L. pacari* in mice forced swimming test, as well as its fractions, and the possible involvement of monoaminergic system in the effect of the active fraction (ChloF), and phytochemically characterized the extract and active fraction.

FST is the most commonly behavioral predictive test used in preclinical phase of evaluation for antidepressant potential drugs, since it has a good predictive validity. The acute or chronic antidepressants administration increases the time that the animal tried to escape of this situation, thus reducing its immobility (Porsolt et al., 1978).

Based in our previous results, where PEx reduced the immobility time on FST after 21 days of treatment but not after acute administration (1 h) (Galdino et al., 2009), we assessed the antidepressant-like PEx effect using the treatment protocol proposed by Porsolt et al. (1978), where three injections are performed at an interval of 24 h (24, 5 and 1 h prior to FST), because this protocol is supposed to optimize the antidepressants effect on animal predictive tests (Porsolt et al., 1978). Using this protocol PEx reduced the immobility time in the FST, suggesting an antidepressant-like effect.

After this initial behavioral assay, the crude extract was subjected to liquid–liquid fractionation to give the chloroform (ChloF), ethyl acetate, n-butanol and aqueous fractions. Subsequently, we evaluated what fraction was able to better retain the neuroactive constituents and induce the same behavioral changes observed with the crude extract. The fractions doses were estimated based on previous results (Galdino et al., 2009) regarding its yield after partition. As result, the ethyl acetate fraction showed a trend in reducing the immobility time, while the ChloF best mimicked the PEx effect, reducing the immobility time by 22.3%, probably by retaining a greater concentration of the constituents responsible for the behavioral effect. Thus, we continued this study only with this fraction, performing the dose-response relationship on the FST and tail suspension test (TST) (Stéru et al., 1985), another common behavioral predictive test.

Treatment with ChloF reduced the immobility time on FST at all tested doses, and on TST at the doses of 70 and 140 mg/kg, reinforcing our suggestion that this fraction retaining the neuroactive compounds, with possible antidepressant activity. Detke et al. (1995) suggested that in rats serotonergic drugs increase swimming, while noradrenergic drugs increase climbing time, and this correlation are widely used in the study of the mechanism of action of new drugs. Evaluating these parameters, swimming and climbing, we suggest that ChloF are capable to increase serotonergic and noradrenergic transmission, since that ChloF at the doses of 70 and 140 mg/kg increased swimming and at the doses of 35 and 70 mg/kg increased climbing. Imipramine, the positive control, a serotonin and norepinephrine reuptake inhibitor, besides the reduction in immobility, increased both swimming and climbing behaviors.

However, to better infer about the possible mechanisms involved in the behavioral changes induced by ChloF, we also used a pharmacological approach, in which animals were pretreated with two drugs that deplete monoamines: *p*-chlorophenylalanine (PCPA—100 mg/kg for 4 days- an inhibitor of the enzyme tryptophan hydroxylase) and α-methyl-*p*-tyrosine (AMPT—100 mg/kg, 4 h before - an inhibitor of tyrosine

hydroxylase), to reduce serotonin and catecholamines endogenous levels, respectively (Machado et al., 2012, Rodrigues et al., 2002).

Pretreatment with PCPA blocked the ChloF and fluoxetine effects, whereas AMPT blocked ChloF and imipramine effects which suggest that both endogenous serotonin as well as catecholamines are involved in the behavioral changes induced by ChloF. Because of the involvement of both catecholaminergic and serotonergic pathways, we assessed whether the ChloF could act as MAO-A inhibitors. Several medicinal plants popularly used to improve mood have active ingredients that inhibit the of MAO-A activity (Stafford et al., 2007). However, ChloF neither inhibit the MAO-A nor MAO-B enzymes activity. This is an advantage since it reduces the chances of a “cheese reaction” for ChloF.

Antidepressant drugs or antidepressant approaches, such as electroconvulsive therapy, increases the BDNF expression, a possible common pathway by which antidepressants exert their therapeutic effect (Nestler et al., 2002). ChloF and imipramine increased the hippocampal BDNF protein concentration, suggesting that in this fraction there are active constituents capable of increasing the amount of BDNF, with potential to exert antidepressant therapeutic effects.

In the phytochemical analysis, we observed the presence of tannins, steroids, triterpenes and traces of flavonoids in the crude extract; in the chloroform fraction only steroids and triterpenoids were observed, whereas the ethyl acetate fraction presented steroids, triterpenes and traces of flavonoids. In the n-butanol and aqueous fractions only tannins were detected. In the ChloF, we had identified, for the first time in *L. pacari* extracts, the presence of triterpene lupeol and steroids β-sitosterol and stigmasterol in the stem bark of this plant.

5. Conclusions

The ethanol:water 7:3 extract and chloroform fraction, which is rich in triterpenes, such as lupeol, showed positive results on rodent predictive antidepressant tests. In the active fraction, the effect showed may be involving the endogenous monoaminergic systems, without inhibiting the MAO-A enzyme activity. Moreover, this fraction increased hippocampal BDNF levels, suggesting that these fraction constituents induce neuronal neuroplasticity. For the first time in the literature, it was reported the presence of lupeol and the steroids β-sitosterol and stigmasterol in the stem bark of this plant species. These results taken together support the tonic use in the local traditional medicine and the antidepressant-like effects in earlier preclinical studies besides shedding more light on the general mechanisms by which *L. pacari* exhibits its antidepressant-like effects.

Conflict of interest

The authors declare that there are no conflict of interest.

Role of the funding source

This work was supported by CNPq, which provided a research grant to Dr. JR de Paula and Dr. TCM de Lima. CAPES and CNPq that provided Ph.D. and Master scholarships to PM Galdino, AAV Carvalho, IF Florentino, JLR Martins and AC Gazola.

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