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Lafoensia pacari A. St.-Hil.: Wound healing activity and mechanism of action of standardized hydroethanolic leaves extract

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ABSTRACT

Ethnopharmacological importance: Lafoensia pacari A. St.-Hil., (Lythraceae) is a native tree of Brazilian Cerrado and commonly known in Brazil as "mangava-brava". Its leaves are used in Brazilian folk medicine in wound healing, cutaneous mycoses, and in the treatment of gastritis and ulcers.

Aim of the study: The present study was designed to evaluate the wound healing activity and mechanism of action of the hydroethanolic extract of *Lafoensia pacari* A. St.-Hil. leaves (HELp), and to advance in its chemical profiling.

Materials and methods: HELp was prepared by maceration in 70% hydroethanolic solution (1:10, w/v). The phytochemical analyses were investigated using colorimetry and electrospray ionization/mass spectrometric detection (ESI-MS^n). Its *in vitro* cytotoxicity was evaluated in CHO-K1 and L929 cells, while the *in vivo* acute toxicity was performed in mice. The potential *in vivo* wound healing activity was assessed using excision and incision rat models and histopathology of the wounded skin (excision model) was carried out. The *in vitro* wound healing activity of HELp was demonstrated by scratch assay in L-929 cells, by measuring proliferation/migration rate and p-ERK 1/2 protein expression using western blot analysis. HELp's *in vivo* anti-inflammatory activity was evaluated by lipopolysaccharide (LPS) induced peritonitis in mice, along with the determination of nitric oxide (NO) and cytokines (TNF-α and IL-10) in the peritoneal lavages. Its potential *in vitro* antibacterial activity was performed using microbroth dilution assay, while *in vitro* antioxidant activities was by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, and ferric reducing antioxidant power (FRAP) assays.

Results: The phytochemical analysis of HELp revealed the presence of polyphenols with ellagic acid, punicalagin, punicalin, kaempferol, quercetin-3-*O*-xylopyranoside and quercetin-3-*O*-rhamnopyranoside being the most prominent. HELp showed no toxicity on CHO-k1 and L929 cell lines. Topical treatment with HELp (10 and 30 mg/g of gel) presented increased rates of wound contraction at all the days evaluated with complete wound re-epithelialization at 22.0 ± 1.5 ($p < 0.05$) and 21.7 ± 1.6 ($p < 0.01$) days, respectively. Topical application of HELp (10, 30 or 100 mg/g of gel) in incised wounds caused an increase in tensile break strength at all concentrations resulting in moderate re-epithelialization and neovascularization, increased cell proliferation an accelerated remodeling phase of the wound, in a manner comparable to standard drug (Madecassol®, 10 mg/g). In the scratch assay with L929 cells, HELp (0.1 and 0.03 mg/mL) and PDGF (5 ng/mL) resulted in the increased proliferation/migration rate of fibroblasts and higher expression of p-ERK 1/2 protein. In LPS-induced peritonitis, HELp (100 and 200 mg/kg p.o.) decreased total leukocyte migration, comparable to the dexamethasone (0.5 mg/kg p.o.). In RAW 264.7 macrophages activated by LPS, HELp produced anti-inflammatory activity dependent on increased concentrations of IL-10, reduction in NO production, without altering the TNF-α levels. HELp also presented potent antioxidant activity in the DPPH and FRAP, but lacks *in vitro* antibacterial activity.

Conclusion: The present study results support the popular use of the leaves of *L. pacari* in the treatment of wounds. Its wound healing activity is multi-targeted and involves inhibition of the proliferative and anti-inflammatory phases, antioxidant and positive modulation of the remodeling phase that might be involved

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different secondary metabolites, with emphasis on the ellagic acid, punicalagin, punicalin, kaempferol, quercetin-3-*O*-xylopyranoside and quercetin-3-*O*-rhamnopyranoside.

1. Introduction

Cutaneous wound healing is a dynamic process that can be divided into four phases: hemostasis, inflammation, proliferation or repair, and remodeling or resolution (Guo and Dipietro, 2010). Immediately after wounding, there is occurrence of vasoconstriction, fibrin clot formation with the release of pro-inflammatory cytokines, and chemotactic growth factors, which facilitate the sequential influx of leukocytes (neutrophils, macrophages and lymphocytes) and subsequent fibroblast proliferation in the wound area (Guo and Dipietro, 2010).

Neutrophils represent the body's primary line of defence and play a role in the clean-up of the tissue through phagocytosis, as well as contribute to the death of invading pathogens by expressing major histocompatibility complex class II (MHCII), releasing free radicals, proinflammatory cytokines, eicosanoids and chemokines, cationic peptides and proteases at the site of the lesion (Wright et al., 2014).

Macrophages are the most important inflammatory cell in wound healing. Besides phagocytic activity (including neutrophils), they also play a key role in the transition of the exudative stage to the proliferative phase of healing by producing and releasing several substances involved in the inflammatory and skin healing processes. Among these substances, PDGF, TGF-β, FGF, and VEGF are the most prominent and are considered the main cytokines capable of stimulating the formation of granulation tissue. The T-lymphocytes migrate into wounds following the neutrophils and macrophages, but their role in wound healing is still under investigation (Guo and Dipietro, 2010).

Fibroblasts and endothelial cells are the most prominent cell types during the proliferative phase, which generally follows and overlaps with the inflammatory phase, and is characterized by fibroplasia, granulation, epithelialization and angiogenesis. Epidermal stem cells and bone-marrow derived cells gives rise to the keratinocytes that migrate, re-epithelialize wounds, and contribute to neovascularization (Morton and Phillips, 2016). Thus, wound healing enters the final phase characterized by regression of many of the newly formed capillaries, extracellular matrix remodeling, physical contractions of the wound, and conversion of type III collagen fibres into a strong network of type I collagen, resulting in the scar tissue (Guo and Dipietro, 2010).

Commonly used medications that have a significant impact on wound healing, encompass anti-inflammatories (steroidal and non-steroidal), and chemotherapeutics (antiseptics and antibiotics) drugs (Guo and Dipietro, 2010). However, they are expensive, sometimes fail and side effects have been frequently reported. Therefore herbal therapy emerges as an alternative strategy for treatment of wounds (Guo and Dipietro, 2010; Budovsky et al., 2015).

Lafoensia pacari A. St.-Hil. (*L. pacari*), Lythraceae, is a native tree present in the Brazilian Cerrado, ciliary and altitude forests, being present from Amapá to Rio Grande do Sul, and it is also found in Paraguay and Bolivia. It is popularly known as *mangava-brava* (Mato Grosso and Mato Grosso do Sul), *pacari* (Goiás), *dedaleiro* (São Paulo), and *louro-da-serra* (Santa Catarina) (Carvalho, 1994; Proença et al., 2000). Its leaves decoctions in water are widely used in the form of bath (50 g per 1 L water) by the population that inhabit the Central Region of Brazil to treat cutaneous and scalp mycoses and in the forms of the infusion (bath and tea) and ointment to treat cutaneous wound (Ortencio, 1997).

There are relatively few studies that have addressed the pharmacological properties of the leaves extracts of *L. pacari* and these relate to its antibacterial (de Lima et al., 2006; Porfírio et al., 2009), antiviral (Müller et al., 2007), anti-inflammatory and analgesic activities (Guimarães et al., 2010).

Sampaio et al. (2011) demonstrated the presence of phenols, tannins, flavonoids and ellagic acid. Recently, Carneiro et al. (2016) reported that punicalagin is the major ellagitannin constituent found in the leaves of *L. pacari*. A remarkable number of studies has already confirmed a wide range of biological activities for this compound, such as anti-inflammatory, antimicrobial, antidiabetic and antioxidant (Carneiro et al., 2016).

Despite the widespread use of *L. pacari* leaves homemade preparations in wound healing by the traditional population that inhabits the central region of Brazil there is no scientific publication that supports its use in this regard. Additionally, the phytochemical study referred is too preliminary. Our preference to study the leaves extract of *L. pacari* in place of its most used part, the stem bark, is significant, given that *L. pacari* is currently classified as vulnerable (IBAMA, 2006). That is because it is at risk of extinction, due to indiscriminate and unsustainable exploitation of its bark for therapeutic purposes, in addition to the destruction of its habitat (Fachim and Guarim, 1995). In the popular Brazilian medicine, its stem bark is often collected by stem ringing, which has led to severe decline in the population of this species (Tonello, 1997).

Thus, this work aims to evaluate the wound healing activity and mechanism of action of the hydroethanolic extract of *Lafoensia pacari* A. St.-Hil. leaves, and to advance in the chemical profiling of this extract for the purpose of its standardization.

2. Material and methods

2.1. Plant material

The leaves (3.8 kg) of *L. pacari* were harvested from Poconé, Mato Grosso, Brazil, February 2013, coordinates GPS 52°94'949" S and 82°25'837" W, after authorization by the proprietor of the area.

Botanical identification was done by Professor Germano Guarim Neto of Herbário – UFMT where a voucher specimen (no. BI 19,400) was deposited. The plant name was checked with www.theplantlist.org, on the 5th of May 2017. In order to access the traditional knowledge associated with genetic resources for research purposes from the traditional communities, an ethical clearance (approval number 247) from the Brazilian Ministry of Environment, under the auspices of the Council for Genetic Heritage Management (CGEN/MMA), was obtained.

2.2. Experimental animals

Albino mice *Mus musculus*, Swiss-Webster strain (25–30 g) and rats *Rattus norvegicus*, Wistar strain (180–200 g) were obtained from the Animal House of UFMT. Animals were maintained in propylene cages at 26 °C in a 12 h light-dark cycle, with free access to standard laboratory feed and water. Groups of six to eight animals were used for each experiment. The experimental protocol followed the International Principles for the Biomedical Research Involving Animal and was approved by the Ethical Committee on the Use of Animal (CEUA/UFMT) with protocol number 23108.042769/12-6.

2.3. Cell lines

For the *in vitro* assays RAW 264.7 murine macrophages (code: 0212) and Chinese hamster ovary epithelial cells (CHO-K1, code: 0069) and L929 murine fibroblasts (code: CRL-2148) purchased from Rio de Janeiro Cell Bank were used. After thawing, cells were maintained in

DMEM (Dulbecco's modified Eagle's Medium) plus 10% fetal bovine serum, supplemented by penicillin (100 U/mL) and streptomycin (100 μg/mL), under a temperature of 37 °C, and atmosphere of 5% of CO_2 and 90% humidity.

2.4. Micro-organisms

For evaluated antibacterial activity were used microorganisms from the American Type Culture Collection (ATCC, Rockville, MD, USA). *Staphylococcus aureus* 25923, *Staphylococcus epidermidis* 12228, *Streptococcus pyogenes* 19615, *Enterococcus faecalis* 29212, *Salmonella typhimurium* 14028, *Pseudomonas aeruginosa* 27853, *Shigella flexneri* 12022, *Klebsiella pneumoniae* 13883, *Escherichia coli* 25922. The strains were maintained on slopes of skim milk (OXOID) in freezer and subcultured two days before to assays to prevent pleomorphic transformations.

2.5. Plant extract preparation

The fresh leaves of *L. pacari* were cleaned and dried in the oven at 40 ± 1 °C, for a period of 72 h, milled using electric miller (model TE-625 Tecnal, São Paulo, Brazil) and thereafter sieved (mesh size no. 40). The powdered plant material was extracted by maceration in 70% hydroethanolic solution (1:10, w/v) for 7 days at 25 °C in order to obtain the aqueous ethanolic extract. After extraction, the macerate was partially evaporated under reduced pressure (600 mmHg) at 40 °C in a rotary evaporator (model 801, Fisatom, São Paulo, Brazil). The residual solvent was removed in an oven at 40 °C for 24 h, thus obtaining the hydroethanolic extract from the leaves of *L. pacari* (HELp), which was lyophilized (Lyophilizer model LL 1500, Heto, Italy), bottled and kept in a fridge (model 350 L, Brastemp, São Paulo, Brazil) at 4 ± 1 °C. For topical use in animals, HELp was solubilized in 2% propylene glycol and incorporated into Sepigel®. When used for systemic purposes, HELp was dissolved in distilled water using 2% Tween 80 and for *in vitro* experiments it was dissolved in DMEM culture. The extract was dissolved in DMSO (0.04%) prior to use in antimicrobial assays.

2.6. Drugs, reagents and culture medium

Methanol Chromasolv LC–MS-grade was acquired from Sigma-Aldrich (São Paulo, Brazil). Ultrapure water was produced using a Milli-Q system (Millipore, Massachusetts, USA). Ethyl Acetate, Thiobarbituric acid, Trichloroacetic Acid, Bovine serum albumin, Butyl alcohol, Absolute ethyl alcohol, methyl alcohol, Amphotericin B, Carbacol-934, Sodium citrate dihydrate, Dexamethasone, O-dianasidin dihydrochloride, Sodium carbonate, Dichloromethane, Dimethyl sulfoxide, doxorubicin, Eosin, Streptomycin, monobasic sodium phosphate, Griess reagent, indomethacin, DMEM, Nutrient blend F-10 HAM, penicillin, copper sulphate pentahydrate, Tris cap (Trizma®), triethanolamine, Trypsin, 1,1,3,3-tetramethoxypropane, 5.5 dithiobis (2-nitrobenzoic) – DTNB, Chloramphenicol (Sigma®, São Paulo, Brazil), anhydrous sodium acetate, hydrochloric acid, perchloric acid, acetic anhydride, Ferric chloride, Chloroform, silica GF254 chromatography, potassium chloride (Merck, Rio de Janeiro, Brazil), Glacial acetic acid, Sodium Chloride, ketamine, Sodium bicarbonate, ethyl ether, Formaldehyde, Xylol, Xylazine (Synth, São Paulo, Brazil), Alamar Blue, 4,6-diamino-2-phenylindole (Invitrogen, Waltham, United States of America), Clarithromycin (MEDLEY, Sao Paulo, Brazil), EDTA disodium salt (DYNAMICS, SP, Brazil), Folin reagent (QEEL, SP, Brazil), BHI broth, Giemsa colouring (Newprov, Parana, Brazil) Hematoxylin (Reagen, São Paulo, Brazil) Iruxol (Abbot, São Paulo, Brazil), reconstituted titrated dry extract of *Centella asiatica* (Madécassol®, Bayer, São Paulo, Brazil) Histological paraffin, Propylparaben, Amino Acid Propylene Glycol (Alchemy, SP, Brazil) PDGF, Hydrogen peroxide (Proquimios, Rio de Janeiro, Brazil), Fetal bovine serum (Cultilab, São Paulo, Brazil).

2.7. Phytochemical analysis

2.7.1. Preliminary phytochemical analysis

The preliminary phytochemical analysis was carried out according to literature procedure, such as colourations and precipitations tests (Matos et al., 2008). The secondary metabolites classes detected were confirmed by thin layer chromatography (TLC) with authentic standards.

2.7.2. Quantitative analysis of selected phytochemical constituents

2.7.2.1. Quantification of total phenolics content. Quantification of the total phenols was performed by the Folin-Ciocalteu method as described by Amorim et al. (2011) using tannic acid as a standard. Total phenols were determined by interpolation of the absorbance of the samples against a calibration curve constructed with different concentrations of tannic acid standard and expressed as mg tannic acid equivalents (TAE) per gram of lyophilized extract (TAE mg/g). All experiments were performed in triplicate.

2.7.2.2. Quantification of total flavonoid content. The quantification of total flavonoids was performed according as previously described (Peixoto-Sobrinho et al., 2008) with slight modifications. The total flavonoid contents were determined using a calibration curve constructed with different concentrations of rutin standard and expressed as mg of pinocembrin equivalents (PE) per gram of lyophilized extract (mg PE/g).

2.7.3. Fractionation and mass spectrometry analysis

2.7.3.1. Clean-up of the extracts. The solid-phase extraction (SPE) cartridge (500 mg), Macherey-Nagel, Chromabond C18 ec (Düren, Germany) was first preconditioned by consecutive passing of 5 mL of methanol and then 5 mL of pure water. The leaves extract (20 mg) of *L. pacari* was solubilized in MeOH/H_2O 1:9 (q.s.), loaded to the cartridge and first eluted with 5 mL of MeOH/H_2O 1:9. The cartridges were eluted again with 5 mL of MeOH/H_2O 1:1 and finally with 5 mL of MeOH/H_2O 9:1. Fractions were transferred into clean tubes and dried under compressed air at room temperature. The samples were redissolved in pure methanol to a concentration of 10 ppm and analyzed by mass spectrometry.

2.7.3.2. Mass spectrometry ESI-MS^n analysis. Direct flow infusion of the samples was performed on a Thermo Scientific LTQ XL linear ion trap analyzer equipped with an electrospray ionization (ESI) source, in negative mode (Thermo, San Jose, CA, USA). Stainless steel capillary tube at 280 °C, spray voltage of 5.00 kV, capillary voltage of − 35 V, tube lens of − 100 V and a 5 μL min^{-1} flow was used. Full scan analysis was recorded at *m/z* range from 150 to 1200. Multiple-stage fragmentations (ESI-MS^n) were performed using the collision-induced dissociation (CID) method against helium for ion activation. The first event was a full-scan mass spectrum to acquire data on ions at the *m/z* range. The second scan event was an MS/MS experiment performed by using a data-dependent scan on the $[M\text{-}H]^-$ molecules from the compounds of interest at a collision energy of 30% and an activation time of 30 ms.

2.8. Evaluation of toxicity

2.8.1. Cytotoxicity evaluation

Potential cytotoxicity of HELp was evaluated following a described method (Nakayama et al., 1997). Briefly, CHO-K1 cells were exposed to different concentrations of HELp (3.12–200 μg/mL) for up to 72 h. Thereafter, the cells were washed and the cell viability was assessed by using alamar blue. Aliquots of 200 μL of stock alamar blue solution were added to each well [containing 200 μL of medium (10% final solution)] and incubated for 5 h. The absorbance at 540 nm and 620 nm was read on a plate reader (MultiskanEX, Thermo Scientific,

Tewksbury, Massachusetts, USA) Doxorubicin (100–0.20 μg/mL) was used as a positive control.

2.8.2. Cell viability assay

In the assessment of cell viability of HELp on mouse L929 fibroblast cell line, similar experimental procedures as noted in the Section 2.8.1 were conducted to select the non-cytotoxic concentrations used in the *in vitro* assays involving L929 fibroblast cells.

2.8.3. In vivo acute toxicity test

The effect of HELp on the general behaviour of conscious animals was evaluated in mice, as previously described (OECD/OCDE, 2001). Briefly, male and female mice received by gavage (p.o.) the HELp at doses of 500, 1000, or 2000 mg/kg body weight (b.w.). Five control animals per group, received the vehicle, 2% tween 80 in distilled water (10 mL/kg). Animals were observed individually in open field at 5, 10, 15, 30, 60, 120 and 240 min and once a day, for a period of fourteen days, noting any clinical signs or mortality, according to established criteria. Animals were weighed before and at the end of the 14th day treatment periods.

2.9. Evaluation of in vivo wound healing activity

2.9.1. Excision wound healing model

This model was employed to have information about wound contraction and wound closure time of the HELp. The selection of the doses used in this study was based on a pilot study as well as the results of toxicity studies that showed that higher dose (2000 mg/kg b.w.) treatment had no toxic effects on the treated animals. Procedures reportedly described by Saha et al. (1997) were adopted with few modifications. Briefly, female rats weighing of 180–200 g were anesthetized by intraperitoneal (ip.) injection of xylazine/ketamine (30/180 mg/kg) at a volume of 0.2 mL/100 g b.w.

After the dorsal flanks were depilated, animals were then placed in ventral decubitus and immobilized for the surgical procedure. The depilated regions were cleaned with 70% ethanol and then, with the help of surgical scissors, the fragment in the dorsal region was cut out to expose muscle fascia (full area of 500 mm^2). The dorsal region was chosen to prevent the animal from biting the injured area and thus excercibate the trauma and damage to the area. The wounds were precleaned with 0.9% saline, and daily topical treatment with the vehicle (Sepigel®), HELp (10, 30 and 100 mg/g of gel) or Madecassol® (triterpene extract of *Centella asiatica* at 10 mg/g ointment), an amount that is sufficient to cover the injured area. Rats were kept in individual cages until the day the wound was completely healed.

2.9.2. Determination of the rate of contraction and re-epithelialization period

The rate of wound contraction was measured on alternating days using transparent paper and a permanent marker. Transparencies were scanned, and the areas were calculated with the use of Image J® software (version 1.47) where the perimeter was established and the contraction rate calculated by the formula: (Saha et al., 1997).

$$\text{Rate of contraction} = \frac{\text{Area on day } 0 - \text{Area on day evaluated}}{\text{Area on day } 0} \times 100$$

The re-epithelization period was calculated by the number of days required to complete healing of wounds (Shrimanker et al., 2013).

2.9.3. Incision wound model

Female rats were prepared for the incision as done in Section 2.9.1. Approximately 6 cm long incision was made down to the fascia in a cranio-caudal direction in the dorso-lumbar region. Immediately afterwards, the edges of each wound were held together by three surgical stitches held at a distance of 1 cm from one another.

Immediately after, the wounds were cleaned with saline 0.9%, and thereafter daily topical treatment was initiated as done in Section 2.9.1.

On day 9, animals were sacrificed with an overerdose of xylazin (60 mg/kg) and ketamine (200 mg/kg) i.p. and the dorsal skin was excised, 3 samples were used for the determination of the tensile strength, while a sample from each of the animals was preserved for histological analysis using a slightly modified procedure of Mustoe et al. (1987).

2.9.4. Determination of tensile strength of the skin samples

The tensiometer was used for determination of the tensile strength of the wound (Fig. 1). The excised strips of skin with the scar perpendicular to length were laid down on massive clamps 1 cm from its edge. The clamps were assembled on a tensiometer, with one of the clamps attached to a fixed hook and the other fixed to a drive system by gravity to which was attached a bucket containing water at a constant volume (1 L/30 s) until the scar ruptured. The volume of water required to rupture the scar was measured and considered the tensile strength (g/mm^2) the scar can supports. Three determinations were carried out in each incision and the average of the three measurements taken for each wounds on each side of the animal was used as the tensile strength of the wound. The measurements were performed on the 9th day after the surgery (Nagy and Zingg, 1971) slightly modified.

2.9.5. Histopathological analyses

The cross-sectional full-thickness skin specimens from each group were collected at the end of the experiment to evaluate for the histopathological alterations. Samples were fixed in 10% buffered formalin, processed and blocked with paraffin and then sectioned into 5 μm and stained with hematoxylin & eosin (HE), Van Gieson's (VG) and toluidine blue (TB) stains. Sections were analyzed and scored as mild (+), moderate (++) and severe (+++) for epidermal or dermal re-modeling. Re-epithelization or ulcus in epidermis; fibroblast proliferation, mononuclear and/or polymorphonuclear cells, neovascularization and collagen depositions in dermis were analyzed to score the epidermal or dermal remodeling. Van Gieson's stained sections were checked for collagen deposition and toluidine blue stained sections checked for

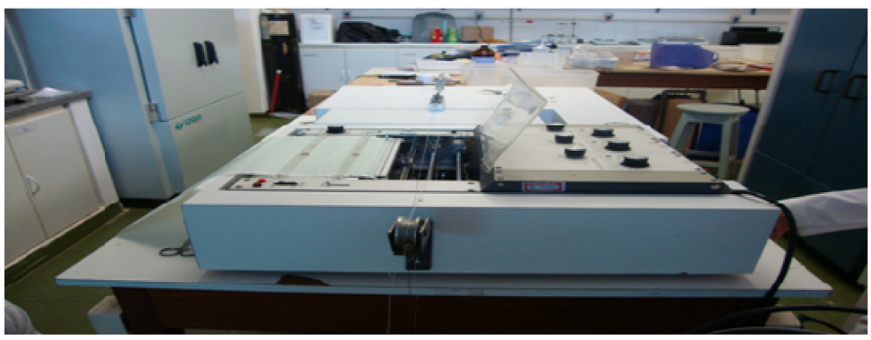


Fig. 1. Tensiometer used in the incision wound model. Source: locally made.

metachromatic staining of mast cells. At the end of the examination, all the wound healing processes were combined and staged for wound healing phases as inflammation, proliferation and remodeling in all groups (Akkol et al., 2011).

2.9.6. Evaluation of in vitro wound healing activity

2.9.6.1. Scratch wound healing assay. The effect of HELp on the spreading and migration capabilities of L929 fibroblasts were assessed using a scratch wound assay which measures the expansion of a cell population on surfaces. The cells were seeded into 24-well tissue culture dishes containing 3×10^5 cells/mL and cultured in medium containing 10% FBS to nearly confluent cell monolayers. Then, a linear wound was generated in the monolayer with a sterile 100 µL plastic pipette tip. Remnants of cell debridement were removed by washing with phosphate buffered saline (PBS). DMEM medium with dimethyl sulfoxide (0.25%) (control group), platelet derived growth factor (2 ng/mL) (as positive control) and HELp (0.01, 0.05 and 0.1 µg/mL) were incubated for 12 h at 37 °C and 5% CO_2. These concentrations were chosen were based on scientific literature and have been shown not to present toxicities. The contraction rate was calculated using three images per well captured with inverted optical microscope with a coupled digital camera (Cyber-shot S730, Japan) and analized with ImageJ® software (a modified method of Fronza et al., 2009).

2.9.6.2. Western blot analysis of p-ERK½ expression. With the purpose of exploring the role of phosphorylating cascades in the action mechanism of HELp, we examined by westernblot the activation of ERK1/2 MAP kinases.

To this end, L929 fibroblasts were treated with the vehicle, HELp at concentrations of 0.03, 0.1 and 0.3 µg/mL, and the respective standards (10 µM PD9805 and 5 ng/mL PDGF). After 30 min of cells incubation at 37 °C and 5% CO_2, supernatants were discarded, the cell pellets were resuspended in 500 µL of lysis buffer (50 mM Tris–HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl, 0.5 mM PMSF, 1% Triton, 0.5% protease inhibitor cocktail, 0.1% SDS, 10 mM NaF, 10 mM Na_3VO_4) and centrifuged to yield whole-cell lysates. The supernatants were collected and centrifuged at 1988 × *g* for 20 min at 4 °C and collected for total protein determination using protein standard curve obtained with bovine serum albumin (Bradford, 1976).

An amount of 40 µg of protein was applied to denaturing SDS-PAGE (10–12%) and later transferred to a nitrocellulose membrane (BioRad). The monoclonal primary antibodies for p-ERK (Santa Cruz Biotechnology, CA, United States of America) and the specific peroxidase-conjugated secondary antibody were added for 2 h at room temperature. Immunoreactive bands were revealed over 5 min after exposure with enhanced chemiluminescence (ECL) kit (Amersham Prime, NJ, United States of America) using photo documentation for the Chemidoc apparatus (Biorad, XRS, United States of America) according to a modified methodology of Kim et al. (2010).

2.10. In vivo anti-inflammatory assay

2.10.1. Peritonitis induced by lipopolysaccharide

In order to evaluate the effect of HELp on leukocyte recruitment into the peritoneal cavity, the mice were orally pre-treated with vehicle (0.9% saline solution), HELp (30, 100, or 200 mg/kg) or dexamethasone (0.5 mg/kg). After 1 h, lipopolysaccharide (LPS, 250 ng/cavity/0.2 mL), derived from *Escherichia coli* dissolved in sterile saline solution was administered intraperitoneally (i.p.). Six hours after, mice were anesthetized using xylazin/ketamin (30/180 mg/kg, ip.) and the cells from the peritoneal cavity were collected through injection of 3 mL saline solution containing ethylenediamine tetraacetic acid (EDTA). The abdomens were slightly massaged and the cell suspension was aspirated using a syringe. The peritoneal lavage collected was used for cellular counting in Neubauer chamber, while an aliquot of the lavage was used to make smear for differential counting (Orlandi et al., 2011).

2.11. In vitro nitric oxide determination

Nitrite, a stable product from nitric oxide (NO), was used as an indicator of NO production in the culture medium. Nitrite released in the culture medium was measured according to the Griess reaction (Minghetti et al., 1997). In summary, RAW 264.7 cells (1.0×10^6 cells/dish) were plated in a 24-well plate overnight. Cells were pre-treated with HELp at the concentrations of 10, 30 or 100 µg/mL for 1 h and incubated at 37 °C and 5% CO_2. Next, the cells were stimulated with LPS (0.5 µg/mL) for 24 h, in the presence or absence of HELp at the same condition. Dexamethasone (10 µg/mL), was used as a standard drug. For negative control, the same amount of medium was used in the microplate well. Supernatant from cell culture were measured for nitrite concentration and 100 µL of it was mixed with the same volume of Griess reagent for 10 min at room temperature. Absorbance was measured at 540 nm using a microplate reader and nitrite concentration was determined using a standard curve of sodium nitrite prepared in RPMI-1640 free of phenol red.

2.12. Determination of the cytokines

The concentrations of cytokines (TNF-α and IL-10) in the supernatant of RAW 264.7 treated cells (as done in Section 2.11) were determined using ELISA kit (eBioscience or and R&D, CA, United States of America), in accordance with manufacturer's instructions Microplate reader Multiskan® (Thermo Scientific, MA, United States of America) was used for reading the absorbance.

2.13. Antibacterial activity

2.13.1. Microbroth dilution method

The antibacterial activity was evaluated by determining the minimal inhibitory concentration (MIC) according guidelines established by Clinical and Laboratory Standards Institute (CLSI, 2012). Stock solution of HELp in distilled water were diluted to provide serial twofold dilutions which were added to each medium, resulting in concentrations ranging from 6.25 to 800 µg/mL for HELp. Bacterial inocula were adjusted to 0.5 MacFarland scale, corresponding to 1×10^8 CFU/mL were used. Chloramphenicol was used as standard drug. Plates were incubated for 24 h at 37 °C. MIC was expressed as the lowest concentration which inhibited growth, judged by lack of turbidity in the well. The turbidity in each well was then determined in spectrophotometer at 450 nm (Multiskan, RC/MS/EX, NA, United States of America). Extract with MIC values ≤ 100 µg/mL were considered active (Kuete, 2010). All antibacterial assays were tested in triplicate.

2.14. In vitro antioxidant assays

2.14.1. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay

The DPPH scavenging activity of HELp was evaluated using the method described by Karki et al. (2011). 100 µL of different concentrations (12.5–400 µg/mL) of HELp were mixed with an equal amount of DPPH (200 µM) in a 96-well microplate, both dissolved in absolute methanol. After 30 min, absorbances were read in a spectrophotometer at 540 nm. Ascorbic acid (32.0–0.25 µg/mL) was used as standard drug.

2.14.2. Ferric ion reducing power

The reducing power of HELp was determined according to the method of Moyo et al. (2010). Different amounts of extract (12.5–400 µg/mL) in water were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 mL, 1%). The mixture was incubated at 50 °C for 20 min. A portion (2.5 mL) of trichloroacetic acid (10%) was added to the mixture, which was then

centrifuged for 10 min. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

2.15. Data analyses

Results of parametric tests were expressed in terms of mean ± SEM. For comparison of means, a one-way analysis of variance (ANOVA) was employed, followed by the Dunnett's test when statistical difference was detected among the groups. Values of $p < 0.05$ were considered significant. The determination of half maximal inhibitory concentration (IC_{50}) was made from a linear regression relating the percentage of inhibition *versus* the logarithm of the concentrations tested and using confidence interval of 99% ($p < 0.01$) for the straight line obtained. For *in vitro* assays not involving statistical analysis, we used the mean ± SEM of three independent experiments performed in duplicates.

3. Results

3.1. Phytochemical analysis

3.1.1. Preliminary phytochemical analysis

Phytochemical analysis of HELp revealed the presence of flavonols, alkaloids, coumarins, phenols, catechin, flavonones and saponins.

3.1.2. Quantitative analysis of selected phytochemical constituents

3.1.2.1. Quantification of total phenolics and flavonoids contents. Quantitative analysis demonstrate high concentration of phenolics in HELp (22.40 ± 4.4 TAE mg/g) accounting for 22.4%, w/w of HELp and of flavonoids 19.7 ± 7.3 PE mg/g of HELp (19.7%, w/w).

3.1.2.2. Fractionation and mass spectrometry analysis. Compounds detected in the extract of *L. pacari* were submitted to MS^n fragmentation and identified based on their fragmentation pattern. All compounds are summarized in Table 1.

The chemical marker of the species, ellagic acid**,** was detected along with its ellagitannin derivatives punicalin and punicalagin. Those compounds were previously detected in the leaves of *L. pacari*, except for the punicalin. Nevertheless, since punicalin is a derivative of punicalagin and the fragmentation pattern corroborates with previous mass spectrometry studies of punicalin, the presence of this compound is suggested. Three flavonols were also identified in the leaf extract of *L. pacari.* These are kaempferol, which has been reported earlier for this species and two others not previously reported in the leaves of *L. pacari*: quercetin-3-*O*-xylopyranoside and quercetin-3-*O*-rhamnopyranoside.

3.2. Evaluation of toxicity

3.2.1. Cytotoxicity

Treatment of CHO-k1 cells with increasing concentrations of HELp had no cytotoxic effect on these cells ($IC_{50} > 800$ µg/mL) up to 72 h. By contrasts, doxorubicin used as a standard was highly cytotoxic to CHO-k1 cells, presenting $IC_{50} < 0.4 \pm 0.0$ µg/mL and 1.7 ± 0.1 µg/mL for the 24 h and 72 h, respectively.

3.2.2. Cell viability assay

Treatment of L929 cells with increasing concentrations of HELp had no effect on the cell survival with $IC_{50} = 414.0 \pm 0.3$ µg/mL up to 24 h, while doxorubicin presented IC_{50} of 41.7 ± 0.5 µg/mL.

3.2.3. Hippocratic screening test

Single oral administration of HELp to female mice at doses of 500, 1000, or 2000 mg/kg did not cause behavioural changes. There was no death in any of the treated animals and therefore it was not possible to determine the LD_{50} of HELp.

3.3. Evaluation of in vivo wound healing activity

3.3.1. Excision wound healing

3.3.1.1. Determination of wound contraction. In Fig. 2 and Table 2 the percentage values of the period of contraction and re-epithelialization of excisional wounds in rats subjected to the treatments can be seen. In all the evaluated periods, topical treatment with HELp (10 and 30 mg/g of gel) increased the wound contraction rates, with the effect noticeable from the second day of induction, with contraction of 44.5 ± 4.1% ($p < 0.05$) and 47.7 ± 2.6% ($p < 0.01$), respectively. The peak effect of the wound healing occurred on the 6th day of treatment at 30 mg/g of gel (57.5 ± 2.6%, $p < 0.01$) compared to the vehicle (Sepigel®). However, treatment with HELp at higher dose (100 mg/g of gel) was effective in increasing the rate of wound contraction only in the 2nd and 6th days with 46.0 ± 2.5% ($p < 0.05$) and 50.6 ± 4.0% ($p < 0.05$) contraction rate, respectively.

Treatment of the wound area with Madécassol® (10 mg/g ointment), the standard drug caused increase in the rate of wound contraction in all of the days evaluated, with onset right from the 2nd day (40.3 ± 1.5%, $p < 0.5$) and reaching maximum effect on the 6th day (55.7 ± 2.3%, $p < 0.01$), when compared to animals treated topically with the vehicle.

With respect to re-epithelization, only the HELp (100 mg/g of gel) treated group failed to show complete re-epithelization of wounds over the 24 days of the test. On the other hand, in the HELp (10 and 30 mg/g gel) and Madecassol® (10 mg/g) groups, the complete re-epithelization occurred on 22.0 ± 1.5 ($p < 0.05$); 21.7 ± 1.6 ($p < 0.01$) and 22.0 ± 1.0 ($p < 0.05$) days, respectively.

3.3.2. Incision wound model

3.3.2.1. Tensile strength determination. The results of the tensile force of wounds are shown in Fig. 3. The group that received Sepigel®, wound

Table 1
Compounds identified from hydroethanolic extract of the leaves of *Lafoensia pacari* (HELp) by electrospray ionization and mass spectrometry (ESI-MS^n) analysis.

No.	$[M-H]^-$	MS^2 fragments	SPE fraction	Compound
Ellagic acid and derivatives				
1	301	257, 229, 185	MeOH/H_2O 1:1	Ellagic Acid
2	781	601	MeOH/H_2O 1:9	Punicalin
3	1083	781, 601, 449	MeOH/H_2O 1:1	Punicalagin
Flavonoids				
4	285	269, 257, 241, 217	MeOH/H_2O 1:1	Kaempferol
5	433	301	MeOH/H_2O 1:1	Quercetin-3-*O*-xylopyranoside
6	447	301	MeOH/H_2O 1:1	Quercetin-3-*O*-rhamnopyranoside

MS – Multiple-stage fragmentations; SPE – solid-phase extraction.

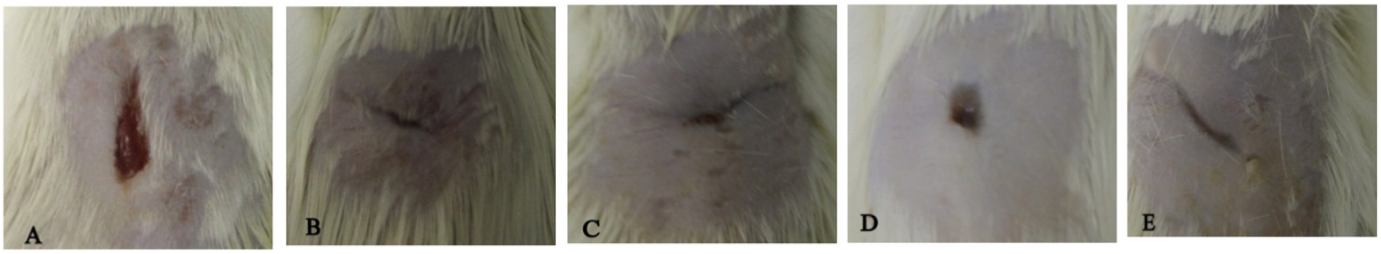


Fig. 2. Effect of topical administration of the hydroethanolic extract of the leaves of *Lafoensia pacari* (HELp) on skin wounds closure in 24-day excision wound model in rats. (A) vehicle; (B) HELp 10 mg/g of gel; (C) HELp 30 mg/g of gel, (D) HELp 100 mg/g gel (E) Madecassol® (10 mg/g).

Table 2
Effect of topical application of the hydroethanolic extract of the leaves of *Lafoensia pacari* (HELp) on the rate of wound contraction (%) and on re-epithelization time (days) in the excision wound model in rats.

Day	Rate of contraction (%)				
	Vehicle	HELp (mg/g of gel)			Madecassol® (mg/g)
	–	10	30	100	10
2	31.1 ± 3.6	44.5 ± 4.1*	47.7 ± 2.6**	46.0 ± 2.5*	40.3 ± 1.5*
4	30.4 ± 2.9	46.3 ± 3.2*	45.6 ± 2.8*	40.8 ± 4.0	40.3 ± 3.2
6	37.4 ± 5.0	55.2 ± 3.5**	57.5 ± 2.6**	50.6 ± 4.0*	55.7 ± 2.3**
8	66.7 ± 5.3	72.7 ± 3.5*	72.7 ± 3.8*	65.9 ± 5.0	73.9 ± 2.8*
10	71.0 ± 3.1	83.5 ± 2.9*	84.1 ± 2.9*	75.4 ± 3.7	84.4 ± 1.9*
12	79.4 ± 1.9	89.2 ± 2.2**	89.7 ± 1.7**	82.9 ± 2.8	88.7 ± 1.4**
14	84.1 ± 1.6	92.3 ± 1.3**	93.1 ± 1.6**	88.3 ± 1.3	91.7 ± 1.25**
16	87.8 ± 2.6	94.6 ± 0.9**	95.7 ± 0.8**	90.3 ± 1.6	95.3 ± 0.5**
18	90.5 ± 2.2	96.4 ± 0.7**	97.2 ± 0.8**	93.5 ± 1.5	98.8 ± 0.7**
20	93.7 ± 1.9	98.3 ± 0.5*	98.1 ± 0.8**	95.5 ± 0.9	98.5 ± 0.3*
22	95.8 ± 1.6	99.7 ± 0.2**	100.0 ± 0**	98.0 ± 0.7	100.0 ± 0**
24	95.8 ± 1.3	99.3 ± 0.4**	99.8 ± 0.1**	97.3 ± 1.0	99.8 ± 0.1**
RE	23.7 ± 0.2	22.0 ± 0.5*	21.7 ± 0.5**	23.7 ± 0.2	22.0 ± 0.3*

RE: re-epithelization. The results were expressed as mean ± SEM for 8 animals. One way ANOVA, followed by Dunnett's test.
* $p < 0.05$ *versus* vehicle.
** $p < 0.01$ *versus* vehicle.

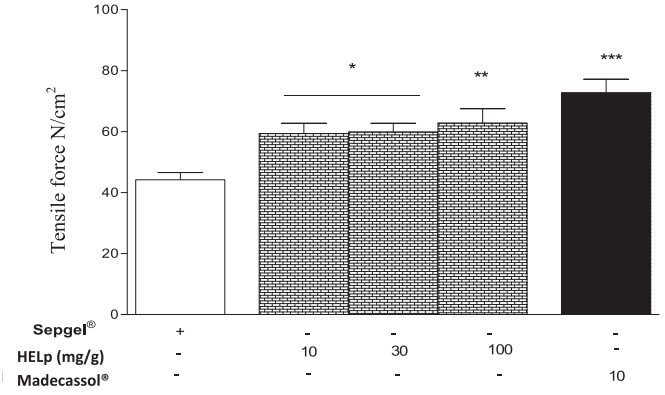


Fig. 3. Effect of topical application of vehicle (Sepigel®), hydroethanolic extract of the leaves of *Lafoensia pacari* (HELp 10, 30 and 100 mg/g of gel) and Madécassol® (10 mg/g) on tensile strength (N/cm^2) of incised wounds in rats, measured on the 9th day. The results were expressed as mean ± SEM for 8 animals. One-way ANOVA, followed by Dunnett's test. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ *versus* vehicle.

tensile strength was 44.1 ± 2.4 N/cm^2. Topical application of HELp (10, 30, or 100 mg/g of gel) of incised wounds caused increased tensile strength at all doses in a non dose-dependent manner, reaching the highest activity at the dose of 100 mg/g of gel (62.7 ± 4.7 N/cm^2, $p < 0.01$) compared to the vehicle group. In the animals treated with the standard (Madécassol®, 10 mg/g), the tensile strength reached 72.8 N/cm^2 ($p < 0.001$) compared to the vehicle group.

3.3.3. Histopathological analysis

The results of evaluation of the wound healing evolution obtained from the analysis of the representative samples of the rat skin sections in the incision wounds models are presented in Table 3 and Fig. 4(A–E).

A slight re-epithelization was observed in the vehicle group. HELp treated groups at the dose of 10 and 30 mg/gel caused moderate re-epithelization and neovascularization. In the healing phase, treatment with HELp at 10 and 30 mg/g of gel and standard Madécassol® accelerated the remodeling phase of the wound, when compared to the vehicle treated group.

As demonstrated in Fig. 4A, the vehicle treated animals presented diffuse hypertrophy, granuloma and severe edema of the epidermis. There are also signs of fibroblast proliferation, and neovascularization with moderate organization and a predominant mononuclear inflammatory reaction in the dermis.

In the animals treated with HELp 10 (Fig. 4B) and 30 mg/g per gram of gel (Fig. 4C), mildly disorganized basal cells with intraepidermal projection, neovascularization and connective tissue proliferation in the dermis were both observed, both occurring in an organized manner.

At the highest dose of HELp (100 mg/g of gel) (Fig. 4D) we observed that the epidermis was replaced by a layer of material containing cellular debris, fibrin and neutrophils (crust), directly over a layer of connective tissue with moderate neovascularization, and a marked presence of inflammatory infiltrates, predominantly mononuclear cells.

In the Madecassol® (10 mg/g) treated group (Fig. 4E), a moderate diffuse hypertrophy, with slight cellular edema presenting no alterations at the dermoepidermal junction was observed. Proliferation of connective tissue and new blood vessels were observed in the dermis, in addition to slight mononuclear cells infiltration.

3.3.4. Evaluation of in vitro wound healing activity

3.3.4.1. Scratch wound healing assay. The proliferation/cell migration activity of HELp was assessed in fibroblasts of L929 strain and the results are shown in Fig. 5. The cells that were not subjected to any kind of stimulus (baseline), representing the baseline group, demonstrated 55.5% rate of proliferation/migration.

In this assay, HELp presented activity at 0.1 and 0.03 μg/mL concentrations, in a non-concentration dependent manner, with increased rate of proliferation/migration of fibroblasts of 23.1% ($p < 0.05$) and 35.3% ($p < 0.001$), respectively, when compared to the baseline. PDGF at 5 ng/mL, accelerated the rate of proliferation/migration by 71.2% ($p < 0.001$) compared to the baseline, an effect significantly higher than HELp treated group (0.1 μg/mL).

3.3.4.2. Evaluation of the expression of p-ERK1/2. The p-ERK1/2 expression was not altered in vehicle (DMEM + 10% of SBF) treated cells. Whereas, HELp treatment caused increase in the p-ERK1/2 expression by 99.8% and 133.8% ($p < 0.001$) at concentrations of 0.01 and 0.3 μg/mL, respectively, when compared with the vehicle group. The effect of HELp decreases with the increase in the

Table 3
Events and phase of the healing process of rats treated with vehicle (Sepigel®), hydroethanolic extract of the leaves of *Lafoensia pacari* (HELp 10, 30 or 100 mg/g of gel) and Madécassol® (10 mg/g).

Groups	Wound healing events								Wound healing phases		
	ED	NE	RE	FP	CD	MN	PMN	NV	I	P	R
Vehicle (Sepigel®)	+	+	+ +	+	+ +	+ +	+	+ +	+	+ +	+ +
HELp											
10 mg/g	–	+	+ + +	+	+ + +	+ +	+	+ + +	+	+ +	+ + +
30 mg/g	–	+	+ + +	+	+ + + +	+ +	+	+ + +	+	+ +	+ + +
100 mg/g	–	+	+ + +	+ +	+ +	+ +	+ +	+ + +	+ +	+ + +	+
Madecassol®	–	–	+ + +	+	+ + +	+	+	+ + +	+	+ + +	+ + +

Skin sections with wounds stained with hematoxylin/eosin were classified as absent (-), mild (+), light (+ +), moderate (+ + +), severe (+ + +) for ED: edema, NE: necrosis, RE: reepithelialization, FP: fibroblast proliferation, CD: collagen deposition, MN: mononuclear cells, PMN: polymorphonuclear, NV: neovascularization. I: inflammatory phase, P: proliferative phase, R: remodeling phase.

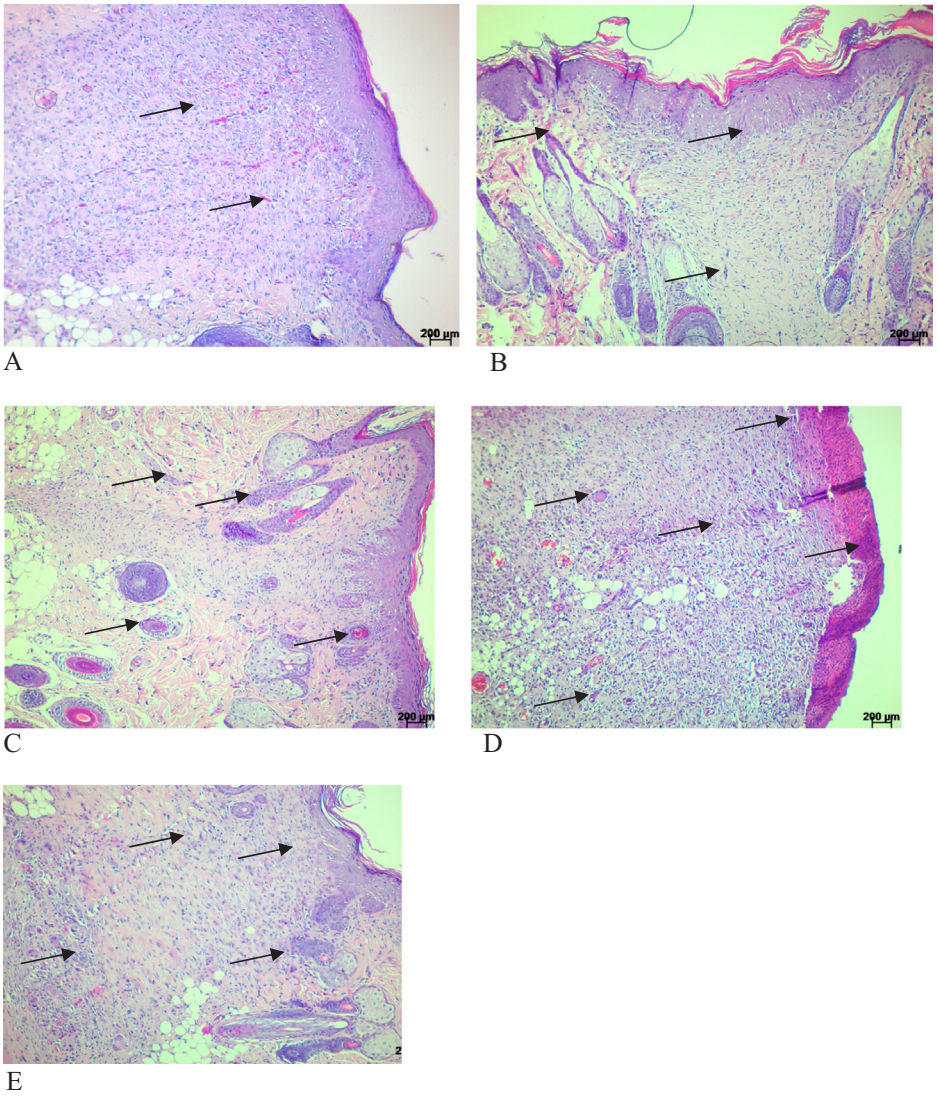


Fig. 4. Histopathological aspect of wound healing in rats treated topically for 14 days. Granulation tissue of animals treated with Sepigel® (vehicle) showing with less collagen and more macrophages (4A). Section of granulation tissue of animals treated with hydroethanolic extract of the leaves of *Lafoensia pacari* (HELp, 10 mg/g of gel) showing with less collagenation, less monocytes, fibroblasts and capillaries (4B). Granulation tissue of animals treated with HELp (30 mg/g of gel) showing moderate collagen and moderate macrophages (4C). Histological section of granulation tissue of animals treated with HELp (100 mg/g of gel) showing increased collagenation, few macrophages and capillaries (4D). Granulation tissue of animals treated with 10 mg/g of Madecassol® (standard drug) showing collagenation, lesser fibroblasts and capillaries (4E). Sections of skin were stained with hematoxylin and eosin (HE). The original magnification was 200× and the bars represent 250 μm. The figures are representative of eight animals per group.

concentration.

In the case of PDGF (5 ng/mL), the positive control for this assay, p-ERK1/2 expression was increased by 96% ($p < 0.01$). In contrast, cells treated with 10 μM of PD 98059 (standard inhibitor drug), presented a drastically reduced p-ERK expression by 76% ($p < 0.01$), in relation to the vehicle group (Fig. 6).

3.4. Anti-inflammatory activity

3.4.1. LPS-induced peritonitis in mice

Intraperitoneal injection of LPS accentuated the numbers of leukocytes in the peritoneal cavity by 73.1% ($p < 0.001$) in relation to the sham group (normal control). Treatment with HELp attenuated increases in the number of total leukocytes, by 39.4% ($p < 0.05$) and 44.9% ($p < 0.01$) at the dose of 100 mg/kg and 200 mg/kg,

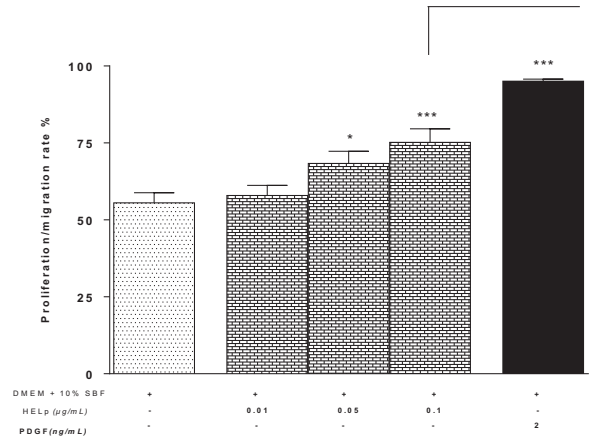


Fig. 5. Effect of the hydroethanolic extract of the leaves of *Lafoensia pacari* (HELp) on the proliferation/migration of L929 cells in scratch wound healing assay, assessed after 12 h of incubation (37 °C, 5% CO_2) in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The factor derived from platelets (PGDF) was used as positive control. The bars represent the mean ± SEM of three independent assays in duplicates (n = 6). One-way ANOVA followed by the Dunnett's test. *$p < 0.05$ and ***$p < 0.001$ *versus* vehicle. $^{++}p < 0.01$ *versus* HELp.

respectively, in relation to the vehicle group. Dexamethasone (0.5 mg/kg), standard drug for this assay also caused a reduction in the number of leukocytes by 52.3% ($p < 0.01$) (Fig. 7).

3.4.2. Determination of nitric oxide (NO)

In RAW 264.7 macrophages that were not stimulated with LPS the baseline concentration of nitrite was 3.3 ± 0.1 μM. Stimulation of the macrophages with 1 μg/mL of LPS caused a significant increase (34.5%, $p < 0.001$) in the production of nitrite in the sham (baseline) group, as seen in Fig. 8. Pre-treatment of the RAW 264.7 cells with HELp (100 μg/mL) caused reduction of 34.4% ($p < 0.001$) in the production of nitrite, in relation to the LPS-stimulated group. Dexamethasone (10 μM), as expected caused reduction by 89.1% ($p < 0.001$) in the production of nitrite when compared with the LPS group (Fig. 8).

3.4.3. Determination of cytokines

The concentrations of TNF-α and IL-10 in cell culture supernatants of LPS-stimulated RAW 264.7 cells increased significantly by 77.1% ($p < 0.001$) and 52.0% ($p < 0.001$) respectively when compared to the control group (Fig. 9).

The concentration of IL-10 significantly increased when the cells were treated with HELp (100 μg/mL, 66.5%, $p < 0.01$) in relation to the LPS group. However, HELp did not have any significant effect on the levels of TNF-α ($p < 0.05$). The treatment of RAW 264.7 cells with dexamethasone (10 μM) significantly reduced the concentrations of TNF-α (89.6%, $p < 0.001$) and the IL-10 by 56.9% ($p < 0.05$).

3.5. Antibacterial activity

The HELp showed no antibacterial activity in any of the strains tested by broth microdilution.

3.6. In vitro antioxidant activity

3.6.1. DPPH radical scavenging activity

The results of the quantitative evaluation of the antioxidant activity (%) of HELp and the positive control ascorbic acid, as determined by the DPPH scavenging assay, are shown in Fig. 10. HELp presented DPPH scavenging activity with IC_{50} value of 8.5 ± 0.1 μg/mL, while ascorbic acid, the positive control in this assay presented IC_{50} of 3.7 ± 0.02 μg/mL.

3.6.2. Reducing power of ferric ion

The antioxidant activity of HELp and ascorbic acid as reduction of ferric ion was determined as shown in Fig. 11. The HELp (200 μg/mL) presented ferric ion reducing activity, similar to that of ascorbic acid (3.2 ± 1.3), with maximum absorbance of 3.4 ± 0.2.

4. Discussion

Due to the usefulness and possibly of the efficacy of preparations of *Lafoensia pacari*, it uses have been cited in several ethnobotanical and ethnopharmacological studies from Brazil (Bieski et al., 2015, 2012; Firmo et al., 2016; Ribeiro et al., 2017). In fact, in a recent ethnobotanical field studies that was conducted by our research group, *L. pacari* featured as one of the most cited medicinal plant by the Ribeirinhos

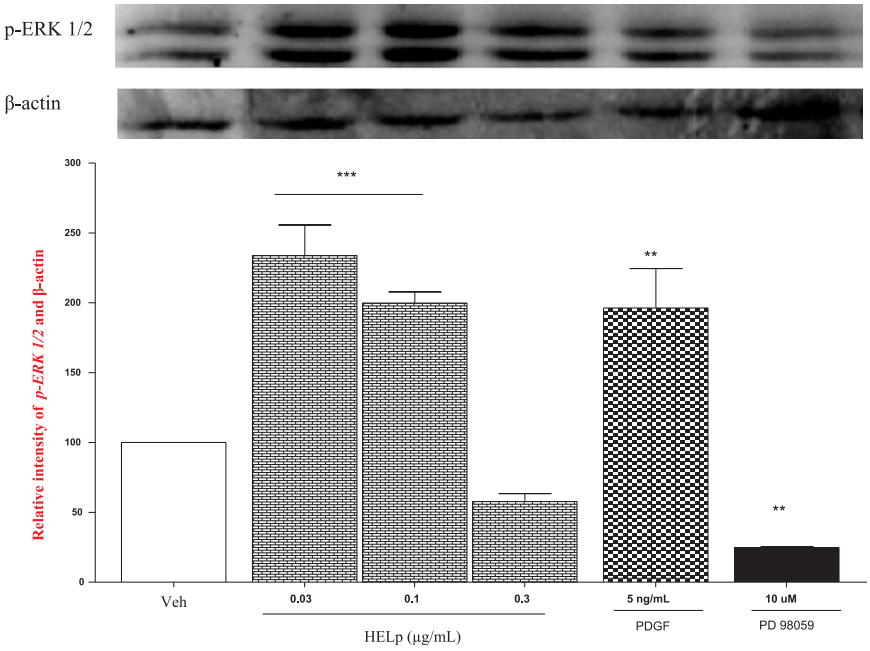


Fig. 6. Effect of 30-min exposure to vehicle (L929 fibroblasts), different concentrations of hydroethanolic extract of the leaves of *Lafoensia pacari* (HELp, 0.03, 0.1 or 0.3 μg/mL) on the phosphorylation of ERK 1/2 in fibroblasts of L929 lineage. Platelets derived growth factor (PDGF, 5 ng/mL) was used as positive control. PD 98059 (10 μM) was used as inhibitory of p-ERK 1/2 expression. The levels of phosphorylation were obtained from the ratio between the optical densities of the bands of phosphorylated form and load control (β-actin). The data are mean ± SEM of three independent assays in duplicates (n = 6). One-way ANOVA followed by the Dunnett's test. **$p < 0.01$ and ***$p < 0.001$ *versus* vehicle (Veh).

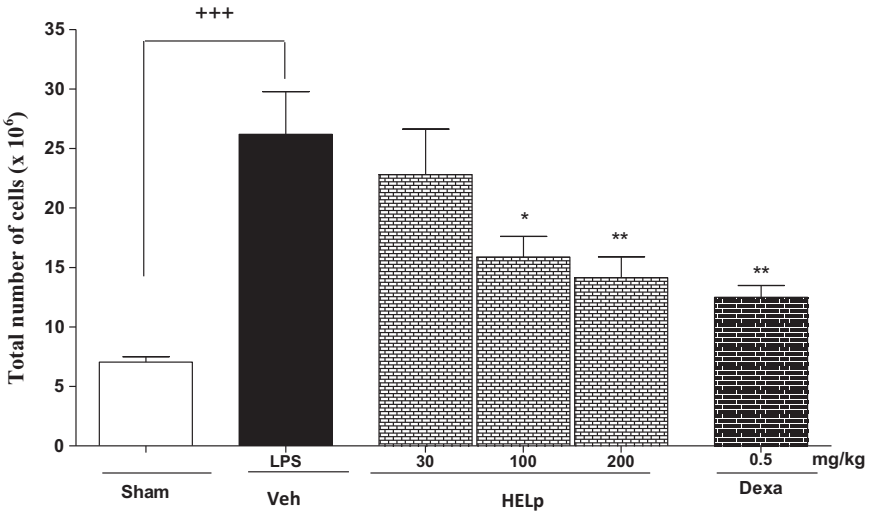


Fig. 7. Effect of oral administration of vehicle (0.9% saline solution), the hydroethanolic extract of the leaves of *Lafoensia pacari* (HELp, 30, 100, or 200 mg/kg) and dexamethasone (Dexa, 0.5 mg/kg) on the total number of leukocytes present in the peritoneal lavage of mice with lipopolysaccharide (LPS)-induced peritonitis (250 ng/0.2 mL/well). The sham group received vehicle (1 mL water/10 g, p.o) and intraperitoneal injection of 0.9% sterile saline solution (0.1 mL/10 g). The total number of cells was expressed as Mean ± SEM for 7 animals. One-way ANOVA followed by Dunnett's test. $^{+++}p < 0.001$ *versus* Sham $^{*}p < 0.05$ and $^{**}p < 0.01$ *versus* vehicle (Veh). $^{+++}p < 0.001$ *versus* Sham.

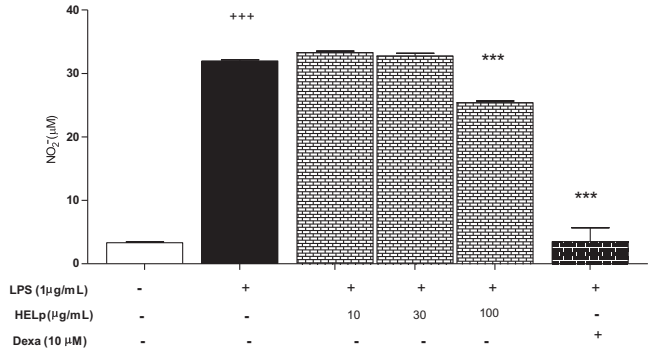


Fig. 8. Effect of treatment with hydroethanolic extract of the leaves of *Lafoensia pacari* (HELp, 10, 30, or 100 µg/mL) and dexamethasone (Dexa, 10 µM) on the production of nitrite (NO_2^-) quantified in the supernatant of RAW 264.7 macrophage stimulated with lipopolysaccharide (LPS, 1 µg/mL) at 24 h. NO_2^- concentrations were expressed as Mean ± SEM for two independent experiments (n = 6). One-way ANOVA, followed by the Dunnett's test. $^{***}p < 0.001$ *versus* LPS. $^{+++}$ $p < 0.001$) *versus* Sham.

(Ribeiro et al., 2017). Based on these information and coupled to the fact that its wound healing activity has not been subject of scientific research, we initiated the current study to substantiate its use in the Brazilian ethnomedicine in the treatments of wounds.

We commenced the pharmacological studies on HELp by evaluating its potential cytotoxic effects in CHO-k1 and L929, using alamar blue assay (Nakayama et al., 1997). One of the toxicity test more frequently used for the evaluation of drugs and herbal extracts is tests on cell viability and cytotoxicity. This is because many drugs can cause irreversible damage at the cellular level. The CHO-k1 cell lines are widely used to screen for cytotoxic substances, and to study mechanism(s) leading to cytotoxicity (Adzu et al., 2015; Węsierska-Gądek et al., 2005). Our result showed that HELp did not alter the cellular viability of CHO-k1 and L929 fibroblasts cell lines.

The *in vitro* cytotoxicity result was also substantiated by the lack of *in vivo* acute toxicity, demonstrating HELp high safety margin. Thus, based on these results and other literature findings we conclude that HELp is safe for use in rodents and in humans, since the local population have been using *L. pacari* preparations for ages without any documented reports of its toxicity. Moreover, scientific evidence shows that it possesses cytoprotective and chemopreventive effects in experimental rodents' studies. To the best of our knowledge, there is no available information addressing the safety aspects of *L. pacari* hydroethanolic extracts of its leaves, with the exception of studies on its stem bark extract and specific isolated compounds from the same plant part (Silva Junior et al., 2010).

Tamashiro-Filho (1999) and Tamashiro Filho et al. (2012) demonstrated that the hydroethanolic extract of *L. pacari* stem bark collected in Mato Grosso, Brazil, presented very low toxicity in both acute and subchronic studies in rodents. De Lima et al. (2013) demonstrated that the hydroethanolic extract of the bark of *L. pacari* is devoid of mutagenic activity and moderately genotoxic to bone marrow cells when administered at high doses. According to these authors, at low doses this extract proved to be antimutagenic, antigenotoxic and anticytotoxic.

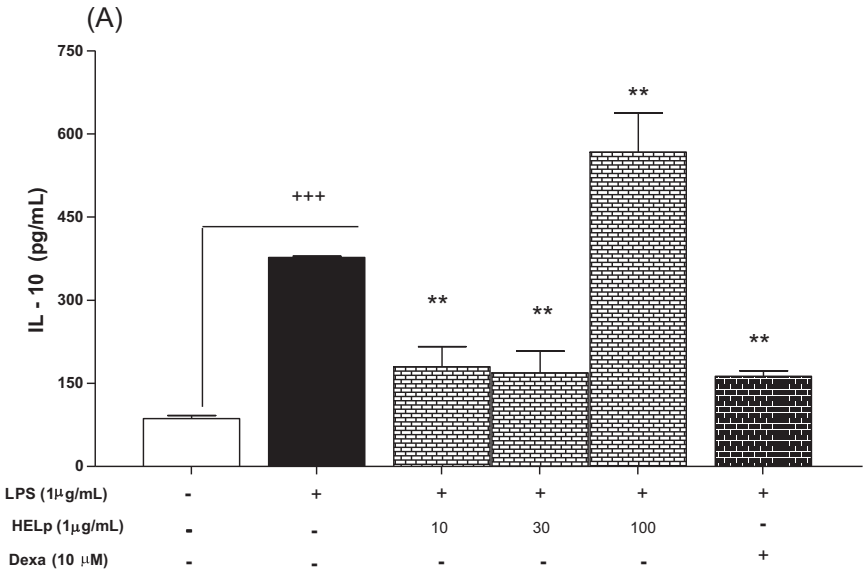
With the purpose of assessing the wound healing activity of HELp, incision and excision wound healing models in rats were employed. Excision model is the most utilized in evaluating substances with potential wound healing effects. It evaluates the evolution of the wound healing process determined with the wound contraction rate and re-epithelialisation of the skin (Shrimanker et al., 2013). Healing of this open defect is brought by reepithelialisation, dermal reconstitution and contraction. The model is useful because it is close to some types of clinical wounds (Gottrup et al., 2000).

In the excision model, HELp demonstrated to be pharmacologically potent as evidenced by increased in the contraction rate and consequently the period of skin reepithelialisation.

In the case of incised wound model, increased tensile strength biomechanics was observed on treatment with HELp at all concentrations tested increased the tensile strength, that suggested good amount of mature collagen deposition (Lopez et al., 1989). The measure of tensile strength is one of the most reliable ways to assess wound healing quality and that its resistance to breakage under tension indicates the quality of tissue repair (Shrimanker et al., 2013). It is therefore plausible to state that HELp wound healing properties might be related to increased collagen synthesis, at least in part.

Histopathological examination further provided additional evidence on the wound healing potential of HELp, as we were able to observe superior effect of the extract compared to the vehicle treated group, particularly in diminishing edema formation in the tissue, increased fibroblast proliferation, collagen deposition and neovascularization. Altogether, these results more evidently demonstrate that HELp predominantly modulates the remodeling phase, in a manner comparable to that of Madecassol®.

In the case of the excision wound, in general, better effects on the rates of contraction were observed at the lower doses of HELp. Although we are unable to explain the exact mechanism responsible for this observation, it may be due to the dynamics of the extract's permeability through the skin layers and subsequently its topical



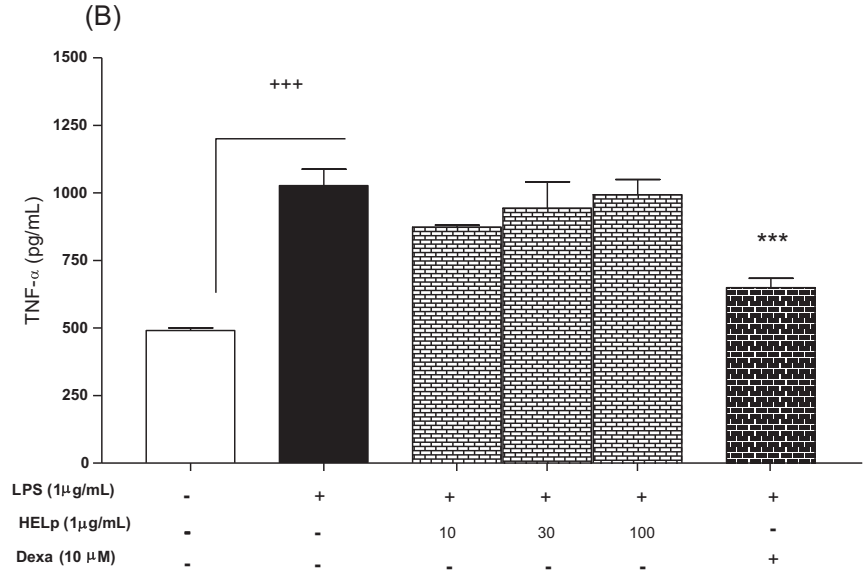


Fig. 9. Effect of pre-treatment with hydroethanolic extract of the leaves of *Lafoensia pacari* (HELp, 10, 30, or 100 μg/mL) and dexamethasone (Dexa, 10 μg/mL) on the concentration of IL-10 (A) and TNF-α (B) by RAW 264.7 cells. Cells were pre-treated with either HELp or Dexa for 1 h, and then incubated with lipopolysaccharides (LPS, 1 μg/mL) for 24 h. The conditioned media were collected, and the concentration of IL-10 and TNF-α were measured in culture medium by a commercial ELISA kit. Results are expressed as mean ± SEM of three independent assays in duplicates (n = 6). Statistical significance was evaluated by one-way analysis of variance, followed by Dunnett's test. $^{+++}$ $p < 0.001$ *versus* basal; *** $p < 0.001$ and ** $p < 0.01$ *versus* LPS.

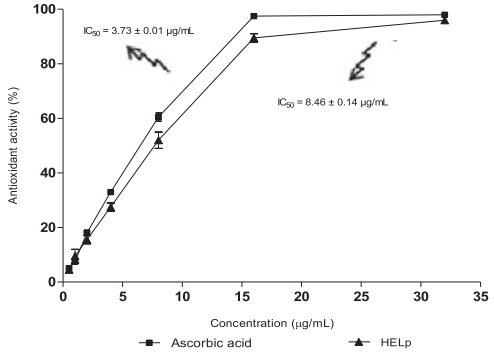


Fig. 10. Inhibitory concentrations 50% (IC_{50}) of free radicals (DPPH) *in vitro* scavenging of the hydroethanolic extract of the leaves of *Lafoensia pacari* (HELp) and of ascorbic acid (reaction time of 30 min). Each value represents mean ± SEM of three independent assays in duplicates (n = 6).

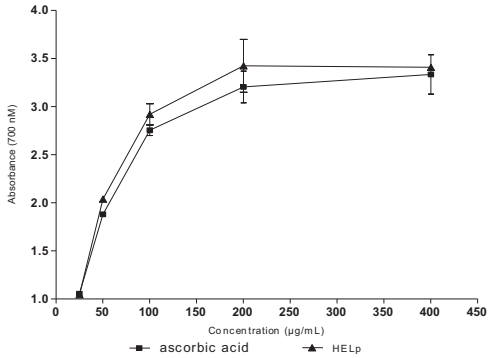


Fig. 11. *In vitro* ferric iron reducing capacities of hydroethanolic extract of the leaves of *Lafoensia pacari* (HELp) and ascorbic acid. Each value represents mean ± SEM of three independent assays in duplicates (n = 6).

absorption, which might have reduced due to many factors (Działo et al., 2016).

In order to assess the effect of HELp on cell proliferation/migration, we performed scratch assay using monolayer of L929 cells. The scratch assay has been widely used to gain insight into potential dermal repair of drugs, by determining the percentage of the enclosed area on 'micro-scratching' (Shrimanker et al., 2013). The fibroblasts are attracted to the wound site to begin the proliferative phase and repair matrix

deposition while keratinocytes are involved in the reepithelialisation phase (Gurtner et al., 2008). HELp at higher dosages increased the proliferation/migration of the fibroblasts in the scratch assay confirming the *in vivo* histopathological findings, a very important step in the healing of wound through increase in the regeneration of the corresponding tissue.

We then investigated the potential mechanism that might be responsible for the proliferative activity demonstrated by HELp in the scratch assay by evaluating ERK 1/2 protein expression, considered the most important factor in cell proliferation (Chen et al., 2005). ERK 1/2 in conjunction with p38 are known to coordinate the dynamic process of wound healing (Sharma et al., 2003).

In fibroblast cultures, HELp caused increased expression of ERK 1/2, comparable to that observed for the PDGF, the gold standard in this assay. This result taken together with others, relating to cell proliferation, supports the hypothesis that the proliferation component might plays important role in the healing activity of the extract.

Many factors influence wound healing by preventing and delaying the process of wound repair, such as immune and inflammatory responses as well as the production of free radicals and microbial infection (Houghton et al., 2005). Bearing these in mind we investigated the anti-inflammatory activities, the free radical scavenging and the potential antibacterial effects of HELp.

Although, the inflammatory phase is essential in the wound healing processes, however, it excess has been associated with impaired wound healing (Eming et al., 2007). Thus, the anti-inflammatory activity of HELp was evaluated in models of peritonitis induced by LPS in mice. HELp reduced leukocyte migration into the peritoneal cavity at the higher doses, with effect comparable to that of dexamethasone.

Some studies have shown that higher NO concentrations, such as occur in the case of continued inflammatory response, is cytostatic/cytotoxic to multiple cell types including endothelial cells, smooth muscle cells, hepatocytes, and fibroblasts (Rizk et al., 2004). In this regard, we sought to analyse NO and cytokines production in LPS-RAW 2647 stimulated cells. Additionally, the release of nitric oxide and production of reactive oxygen species, at excessive levels may leads to cell damage and consequent loss of function (Min et al., 2009). Our result demonstrated that HELp, at the highest concentration, significantly attenuated the LPS-induced elevation of NO, and may thus represent one of its wound healing mechanisms.

In the LPS-stimulated RAW cells, HELp occasioned increased IL-10 levels without affecting TNF-α level. It is likely that the reduction in the migration of the inflammatory cells observed in the *in vivo* peritonitis assay might be due to increased IL-10 levels, as demonstrated *in vitro*. This anti-inflammatory cytokine controls the degree and duration of the inflammatory response either by blocking the expression of genes encoding pro-inflammatory chemokines and cytokines or by increased production of anti-inflammatory molecules (Moore et al., 1993).

In recent years, several studies that confirm the link between harmful effects of ROS in the healing process have been reported (Suntar et al., 2010). The hypothesis is that compounds or substances with considerable antioxidant properties may be useful in promoting wound healing. We therefore evaluated the radical scavenging effects of HELp using DPPH assay and reduction in ferric ion. Our results demonstrate considerable DPPH radical scavenging activity and ferric ion reduction, comparable to ascorbic acid. This result corroborates an earlier report by Solon et al. (2000), who reported a high radical scavenging activity as little as 10 μg/mL of the methanolic extract of *L. pacari*. It is therefore probable that the wound healing activity may be related, at least in part to its antioxidant and free radical scavenging activities (Akkol et al., 2011; Chen et al., 2005).

The avascular wound bed provides an ideal environment for microbial growth, facilitating penetration of pathogens into underlying tissue, with potential for hematogenous dissemination (Soares De Macedo and Santos, 2005). In this case, there is indication for the use of appropriate antibiotics. We thus evaluated if HELp may also be potentially useful in the treatment of infected wounds. Our result demonstrated that HELp was inactive against all the bacterial strains tested, in the *in vitro* antibacterial assay, indicating that in bacteria infected wounds the extract may have to be associated with an antibacterial drug, if there is need. This result however is contrary to a number of studies conducted on different parts of *L. pacari* and using different solvents (Firmo et al., 2016). In many of these studies, *L. pacari* was shown to possess *in vitro* antimicrobial activities against various bacterial and fungal species, although at varying degrees (de Lima et al., 2006; Firmo et al., 2014; Junior et al., 2010; Pereira et al., 2011). We cannot say specifically the exact reasons of this inactivity; however, secondary metabolites content might be highly variable depending on several factors. These includes among others, on the plant phenological stage, on seasonality (harvest time and location) or even to the genetic composition of the plant (variety) which directly influences the levels and composition of compounds responsible for the antibacterial activity (Gouvea et al., 2012; Larissa da Silva et al., 2017; Scognamiglio et al., 2014).

The phytochemical constituents' profile of HELp was investigated using spectrophotometric, fractionation and mass spectrometry analyses to identify relevant secondary metabolites groups and useful chemical markers. Such multiconstituent profile markers are used for quality control of herbal preparations and subsequent validity checks (Adzu et al., 2015). An important finding in this regard is the presence of ellagic acid, punicalagin, kaempferol, quercetin-3-*O*-xylopyranoside and quercetin-3-O-rhamnopyranoside. Some of these bioactive compounds have been previously shown to be active in experimental wound healing studies (Dai and Mumper, 2010; Rice-Evans et al., 1997), while others have been shown to possess considerable free radical scavenging and antioxidant properties. It is therefore conceivable that these components of HELp might be responsible, at least in part for both its *in vitro* and *in vivo* wound healing and antioxidant activities (Dikmen et al., 2011; Bahramsoltani et al., 2014).

5. Conclusion

The present results support the popular use of *L. pacari* leaves in the treatment of cutaneous wounds. Based on the various findings in present study and information in the literature, it is suggested that the wound healing activity observed for HELp may involves different secondary metabolites, emphasizing the potential roles of ellagic acid, punicalagin, punicalin, kaempferol, quercetin-3-*O*-xylopyranoside and quercetin-3-*O*-rhamnopyranoside and that its wound healing activity is multi-targeted. In particular, HELp influenced the proliferative and inflammatory phases that are possibly due to its antioxidant, anti-inflammatory and positive modulation of the remodeling phase of the wound healing processes.

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Conflict of interest

Authors declare no conflict of interest.

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