Pharmacognosy

Investigation of wound healing activity of *Lafoensia pacari* (Lythraceae) leaves extract cultivated in Goiás state, Brazil

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Abstract

*Lafoensia pacari* has been used in traditional folk medicine in Brazilian Cerrado to treat wounds. It is important to develop studies that can clarify how the biological activity of *L. pacari* occurs. The aim of this study was to evaluate the healing activity of *L. pacari* leaves extract in an animal model. The extract was prepared from plants cultivated in Goiania-GO, Brazil. The healing activity was assayed using cutaneous wound model in rats, and macroscopic, morphometric and histological analysis of wounds were also conducted. The presence of hydrolysable tannins was detected in thin layer chromatography and in high performance liquid chromatography analysis, may be suggested the presence of ellagitannins. In the evaluation of cutaneous wounds in rats it was possible to observe that the treatment with 10% (w/v) *L. pacari* extract provided a reduction in the time of cutaneous wound healing, with a significant increase in variables involved in healing, such as the number of blood vessels and collagen production. Therefore, this study shows that *L. pacari* wound healing potential may be related to the presence of ellagitannins and corroborates to ethnopharmacological reports regarding this plant.

Key words: bioactivity, Cerrado, medicinal plant, tannin.

Resumo

*Lafoensia pacari* é empregada na medicina tradicional no cerrado Brasileiro para tratar feridas. É importante desenvolver estudos para melhor compreender como ocorre a atividade biológica de *L. pacari*. O objetivo deste estudo foi avaliar a atividade cicatrizante do extrato das folhas de *L. pacari* em modelo animal. O extrato vegetal foi preparado a partir de plantas oriundas de Goiânia-GO, Brasil. Na avaliação da atividade cicatrizante, foi empregado o modelo de feridas cutâneas em ratos. Análise macroscópica, morfométrica e histológica das feridas também foram realizadas. Na cromatografia de camada delgada foi detectada a presença de taninos hidrolisáveis e na cromatografia líquida de alta eficiência, pôde-se sugerir a presença de elagitaninos. Na avaliação das feridas cutâneas em ratos foi possível observar que o tratamento com extrato de *L. pacari* a 10% (m/v) proporcionou uma redução no tempo de cicatrização de feridas cutâneas em ratos, com aumento significativo das variáveis envolvidas na cicatrização, como número de vasos sanguíneos e a produção de colágeno. Portanto, este estudo mostra que o potencial cicatrizante de *L. pacari* pode estar relacionado à presença de elagitaninos, o que corrobora com relatos etnofarmacológicos deste vegetal.

Palavras-chave: bioatividade, Cerrado, planta medicinal, tannin.

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Introduction

Cerrado is the second biggest Brazilian biome, whose area comprises more than 25% of the country area. It represents 33% of national and 5% of global biodiversity (Santos et al. 2010; Brazil 2013) and 44% of its flora is endemic (Klink & Machado 2005). Despite the richness of flora and fauna species, the Brazilian Cerrado is target of devastation and extinction due to agricultural, livestock and extractivism expansion. In this sense, literature reports that over 50% of this biome was devastated (Brazil 2011) and is considered a priority area for conservation due to the high degree of threat of extinction, a worldwide hotspot (Fachim & Guarim 1995; Klink & Machado 2005; Santos et al. 2010).

Among the most representative families of the Cerrado flora is Lythraceae, which comprises 22 genera distributed around the world (Joly 2002; Ribeiro & Dias 2007). In Brazil, Lafoensia genus comprises 6 distinct species, namely Lafoensia pacari A. St.-Hil., Lafoensia densiflora Pohl, Lafoensia glyptocarpa Koehne, Lafoensia replicata Pohl, Lafoensia vandellianna Cham. & Schltdl, Lafoensia nummularifolia A. St.-Hil. (Sano et al. 2008).

Lafoensia pacari is a species widely used by the Cerrado population, being highlighted in the Cerrado Pharmacopoeia (Dias & Laureano 2009), also has potential for landscaping and riparian forest replacement (Camillo et al. 2016). Furthermore, L. pacari is widely distributed in Cerrado, occurring in more than 50% of its area (Ribeiro & Dias 2007). Regarding its morpho-anatomical features, L. pacari is a perennial to semi-deciduous tree, found in Cerrado, Cerradão, riparian forest, dry forest and high-altitude forests. Its canopy has young, reddish, square and sharp terminal branches. The trunk has a diameter of up to 26 cm, with a grayish, scaly rhytidoma presenting sinuous and discontinuous fissures and ridges. Its height may vary from 1 to 10 m (Lorenzi 1998; Carvalho 2003; Camillo et al. 2016). Concerning its phenology, L. pacari flowering at the end of the rainy season, from April to August, fruiting occurs between June and September, with fruit dispersion being more intense in September. Moreover, its leaves fall from July to September and new leaves grow from September to December, with the beginning of the rains (Santos et al. 2009).

Lafoensia pacari is regionally known as “pacari”, “mangava-brava”, “dedal” or “dedaleiro” (Lorenzi 1998) and also as “amarelinho”, “candeia-de-caju”, “pau-tinta”, etc (Camillo et al. 2016). Ethnopharmacological studies describe L. pacari as wound-healer (Vila Verde et al. 2003), being useful for treating ulcer, gastritis, healing and inflammation (Souza 2007; Cabral & Pasa 2009; Jesus et al. 2009).

Literature reports the presence of flavonoids (Santos et al. 2000; Pereira et al. 2018) and tannins (Sampaio et al. 2011; Carneiro et al. 2016; Pereira et al. 2018) in L. pacari leaves. These polyphenols are highly relevant towards the biological activity of plants and have important medical and ecological purposes (Monteiro et al. 2005). Moreover, hydrolysable tannins such as ellagitannins have been reported to occur in Lythraceae (Yoshida et al. 2010).

The healing activity of medicinal plants has shown that these natural agents induce healing and regeneration of lost tissue by multiple mechanisms and is considered a result of the synergistic activity of different secondary metabolites, whose tannins contribute significantly (Logeeswari & Sripathi 2012). Among the species to which wound healing properties can be attributed, there are Stryphnodendron sp. (Lopes et al. 2005; Coelho et al. 2010), Lawsonia inermis L. (Muhammad & Muhammad 2005; Nayak et al. 2007); Schinus terebinthifolius Raddi (Castelo Branco et al. 2006); Tabernaemontana cathaninensis DC. (Janning et al. 2011); Punica granatum L. (Hayouni et al. 2011; Ismail et al. 2012); Ammannia bacifera L. and Blepharis maderaspatensis (L.) B. Heyne ex Roth. (Rajasekaran et al. 2012). Regarding L. pacari, there was a single report using plants extracted in Brazilian Mato Grosso region, in which wound healing effects were detected both in vitro and in vivo (Pereira et al. 2018). In this study, the authors used a species collected from the natural environment, the extract was prepared differently from ours and the experimental model was more complex.

This work aimed to corroborate with studies on the wound healing effects of L. pacari, having as differential the use of a species cultivated in Goiânia, Goiás state, Brazil, using an in vivo model comprising cutaneous wounds in rats.

Materials and Methods

Vegetal material and extract
Lafoensia pacari leaves were collected from adult trees cultivated at the Goiânia Agency of Technical Assistance, Rural extension and Agropecuary Research - EMATER (16°36’19”S, 49°15’48”W, 710 m high) in Goiânia-Goiás, Brazil, in May 2016. The vegetal material was identified by the Professor Maria Teresa Freitas Bara of the
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Lafoensia pacari leaves were cleaned and desiccated at environment temperature. Thereafter, the leaves were milled in knives-mill (Willye-Tecnal) and extracted using acetone/water 50% with ultrasound bath for 30 min at 10% (w/v) drug:solvent ratio. After extraction, the solvent was evaporated using reduced pressure at constant temperature of 35°C. Then, the extract was filtrated and partitioned using analytical grade ethyl acetate (10 × 100 mL). The hydrophilic portion was separated and subjected to lyophilization.

**Lafoensia pacari extract characterization**

In order to characterize L. pacari extract prior lyophilization, solids content and pH were determined according to the 5th edition of Brazilian Pharmacopoeia (Brazil 2010), all assays were conducted in triplicates, and results were expressed as means of three independent experiments. The verification of the presence of hydrolysable tannins was performed using thin layer chromatography (TLC) (Mello & Santos 2017) of the extract prior lyophilization, and high performance liquid chromatography (HPLC) (Okuda et al. 1989) of the lyophilized extract.

TLC was conducted on standard silica sheets (Merck) using 20 µL sampling volume of 1 mg extract in 1 mL methanol. The mobile phase consisted of acetone, toluene and formic acid at proportion of 3:3:1 (v/v/v) respectively. The revelation was performed using vanilin/H₂SO₄ solution, followed by heating or exposure to FeCl₃/HCl at 1%.

HPLC assay was conducted at Waters chromatographer using quaternary pump, e2695 separation module, diode array detector (DAD) 2998, and Empower 2.0 data system. The stationary phase used was a Waters C18 column of 250 x 4.6 mm, injection volume of 10 µL (extract at 1 µg/mL), operational temperature of 25°C, maximum detection at wavelength of 254 nm and mobile phase flux of 1.0 mL/min. The mobile phase consisted of acetonitrile and phosphate buffer 0.01 M (v/v) following a gradient of 8:92 for 20 min, then changed to 18:82 proportion for 15 min, to 50:50 for 10 min, then 80:20 for 3 min and ended at 8:92 proportion, with the total elution time of 50 min.

Animals

20 isogenic female Wistar rats (Rattus norvegicus albinus) were selected for this study. All rats were healthy and aged 60 days old. Moreover, they weighted between 160 to 190 g. All rats were acquired from UFG’s Central Bioterium, and the experiments took place under controlled temperature of 23 ± 2°C, humidity between 50 and 60%, and night/day cycle of 12 h. Water and food were given ad libitum, and all herein described procedures were conducted according to standard animal experimental models, as described by Lopes et al. (2005) and Garros et al. (2006) This experimental protocol was developed in accordance with Brazilian Council for Controlling of Animal Experiments (CONCEA) and were approved by the Research Ethic Council of UFG (number 067/2).

Wound healing study model

All animals were weighted and randomly distributed in 4 treatment groups, each comprising 5 animals. The groups were:

- C1: control group, which was treated with distilled water for 7 days;
- C2: test group, treated with L. pacari extract at 10% in distilled water for 7 days;
- C3: control group, which was treated with distilled water for 14 days;
- C4: test group, treated with L. pacari extract at 10% in distilled water for 14 days.

Macroscopic evaluation

Initially, a surgical procedure was conducted to promote the cutaneous wounds to be assayed in this study. The wound were made in the epilated dorsal-cervical region and using a circular metallic punch of 1 cm diameter. All animals undergone the aforementioned procedures under anesthesia (ketamine 70 mg/kg and xylazine, 10 mg/kg, intramuscular). Immediately after surgery and daily at the same time of each treatment, the wound area was subjected to applications of either 100 µL of distilled water (C1 and C3 groups) or 100 µL L. pacari extract at 10% (C2 and C4 groups). These applications were conducted by dripping with a sterile syringe. During the first two days following surgery, all rats received orally the analgesic tramadol chlorhydrate to minimize pain. All animals were examined daily regarding the general aspect of wounds focusing on episodes of hyperemia, hemorrhage, exudate presence and
other inflammatory or pathologic signals. After 7 days of treatment for C1 and C2 groups, and after 14 days for C3 and C4 groups, the animals were again weighted and euthanized in CO₂ chamber.

Morphometric and histological analysis

The wounds were evaluated at days zero, 7 (C1 and C2 groups) and 14 (C3 and C4 groups) using digital camera with lenses positioned at 27 cm from the wounds. All images were analyzed using Image J 1.3.1 software (NIH, USA). Moreover, the level of area contraction (GC) was calculated taking the original wound area in consideration according to standard protocol (Oliveira et al. 2000).

The histological evaluation was performed using a tissue fragment of the wounds removed after euthanasia of the animals, fixed in buffered formalin, blocked with paraffin, sectioned in microtome and stained with hematoxylin and eosin. At the 7th day, the following variables were evaluated: collagen and fibrin production, hemorrhage, hyperemia and inflammatory infiltrate. At the 14th day of treatment, re-epithelization and epidermal hyperplasia were evaluated. The images were captured using a digital camera and were analyzed using Image J 1.3.1 software (NIH, USA). The variables analyzed were counted in three fields for each processed fragment and for each treatment individual and then the medians of the histological variables evaluated were determined for the groups treated with L. pacari extract when compared to those treated with distilled water (control) (Biondo-Simões et al. 2006).

Statistical analysis

All results were analyzed using Mann-Whitney test, being statistical significance attributed to \( p \leq 0.05 \). Furthermore, all data was treated using GraphPad InStat 3.0 software.

Results and Discussion

Lafoensia pacari extract characterization

Initially, we would like to emphasize that we obtain the vegetal matter in a sustainable way, since it was cultivated in EMATER (Goiânia-GO). This was important because the popular use of L. pacari has been limited due to the peculiarities of its spread during the dry season, when forest fires occur in the Cerrado. In addition, its seeds are particularly susceptible to damage that limits their propagation as they are not protected by any fruit tissue (Camillo et al. 2016). Thus, the cultivation of the species is fundamental.

After processing, L. pacari extract showed solids content of 5.44% ± 0.31 and pH of 4.23 ± 0.02 (prior lyophilization). The acidic pH found on L. pacari extract may be attributed to the extraction of phenolic compounds, which is known to have acidic reaction. Regarding the drying of the extract, it was performed by lyophilization aiming to limit the degradation of thermolabile compounds, such as phenols, which may undergo oxidation (Mello & Santos 2017).

The presence of hydrolysable tannins was showed by TLC, that was revealed using vanillin solution and there were no visible bands. However, upon revelation with iron chloride, blue bands were observed at retention factor of 0.15. The negative results in presence of vanillin-acid solution and positive results upon application of iron chloride allows to suggest this presence (Mello & Santos 2017).

From HPLC analysis, we can state that among the hydrolysable tannins, we have punicalagin. We highlight that in another study of our group (Carneiro et al. 2016), with this same extract and under the same analytical conditions, punicalagin had already been identified through the absorption spectrum, retention time on HPLC and by using spectroscopic methods (ESI-TOF MS, 1D and 2D NMR). So, we refer to the original article that identified it, and in the present work, we only verify this presence by HPLC analysis (retention time and absorption spectrum). HPLC results evidenced the chromatographic profile of L. pacari extract, wherein Figure 1a showed two major peaks at retention times of 14.68 and 17.61 min respectively. By analyzing their absorption spectra (Fig. 1b,c), it was possible to observe three identical maximum absorption bands visible at 214.6, 257.1 and 379.7 nm, which were nonetheless identical to the both peaks visualized in chromatograms. L. pacari extract absorption spectra results were fairly similar to other reports in literature, being observed small variations such as 218, 260 and 379 nm (Machado et al. 2002); 215, 257 and 375 nm (Asres et al. 2001); and 213, 258 and 380 nm (Romani et al. 2012). Furthermore, one of the major features of hydrolysable tannins is the presence of \( \alpha \) or \( \beta \) anomeric hydrogens, which present two peaks under HPLC analysis (Machado et al. 2002). Considering this, the results published by Doig et al. (1990) showed the structural determination of punicalagin anomers from Terminalia oblongata,
which were extracted under similar conditions to the one analyzed in this work. Given the similar findings, we therefore suggest the presence of hydrolysable tannins such as punicalagin in *L. pacari* extract.

Regarding the extraction process herein used, we chose for 50% acetone solution followed by partition with ethyl acetate due to its reported capacity to optimize tannin extraction. In this sense, we can also imply the presence of polar compounds such as flavonoids, phenolic acids and tannin monomers (Mello & Santos 2017), which were conserved post lyophilization and used in experimental models of wound healing.

### Investigation of wound healing activity of *Lafoensia pacari* extract

Results showed that, in the macroscopic evaluation of the wounds treated with *L. pacari* extract was not observed sanguinolent exudate, neither was this found in control group. In the animals treated with *L. pacari* extract, thin scabs covered the wounds after the 2nd day, being thickened over time on the 3rd and 4th day. On the 4th day, the scabs were seemingly united to the tissue and the wound borders started to recede. In the control group there was no visible scab until the 4th treatment day, at which they only became loose on the 6th day. After scabs were united from wound tissue, both wounds reduced gradually in size and in the 14th day, there was no visible wound and partial reconstitution of hair growth. (Fig. 2 - C1, C2, C3 and C4). Therefore, it was possible to observe that *L. pacari* extract at 10% aided in wound recovery time, promoting healing acceleration.

The macroscopic aspect of wound scabs seen in this work were similar to other reports regarding wound healing properties of natural products, wherein the overall aspect of the wound is marked by thick and darkened scabs (Lopes et al. 2005).

In the morphometric evaluation there was no significant difference between wound size in the 7th and 14th day of treatment (Fig. 2). However, there was a qualitative improvement of wound aspect and healing time in the animals treated with *L. pacari* extract at 10%.

The histologic evaluation of wound tissue removed on the 14th day evidenced total re-epithelization in the wound area of all animals. There was moreover no significative difference regarding epidermal hyperplasia.
We remember that healing is a dynamic and gradual process, composed of the following phases: coagulation, inflammation, proliferation, wound contraction and remodeling (Mandelbaum et al. 2003). Coagulation is the immediate onset phase which leads to the release of vasoactive substances and triggering of the coagulation cascade. In the inflammatory phase, in addition to releasing chemical mediators (lymphocytes, macrophages), fibronectin synthesis is also

**Figure 2** – Macroscopic depiction of the wounds in rat dermis. Control groups treated with distilled water on day 0, day 7 (C1) and day 14 (C3). Groups treated with *L. pacari* extract at 10% on day 0, 7th day (C2) and 14th day (C4).

**Table 1** – Medians of histologic variables evaluated at the 7th day in the treated groups (*L. pacari* extract at 10% (w/v) and control group). Mann-Whitney test, and Dunn post-test *p < 0.05.

<table>
<thead>
<tr>
<th>Histologic variables</th>
<th>Control (distilled water)</th>
<th><em>L. pacari</em> extract (10%)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood vessels</td>
<td>1.0</td>
<td>2.5</td>
<td>0.007*</td>
</tr>
<tr>
<td>Fibrin</td>
<td>1.0</td>
<td>1.0</td>
<td>0.67</td>
</tr>
<tr>
<td>Hyperemia</td>
<td>1.0</td>
<td>1.0</td>
<td>0.44</td>
</tr>
<tr>
<td>Hemorrhage</td>
<td>1.0</td>
<td>1.0</td>
<td>0.67</td>
</tr>
<tr>
<td>Inflammatory infiltrate (PMN cells)</td>
<td>1.0</td>
<td>1.0</td>
<td>0.67</td>
</tr>
<tr>
<td>Collagen</td>
<td>1.0</td>
<td>2.0</td>
<td>0.0083*</td>
</tr>
</tbody>
</table>
started. In the proliferation phase, re-epithelization and hyperplasia of the endothelium, fibroplasia for the formation of granulation tissue (with fibronectin, glycosaminoglycans and collagen) and angiogenesis, which leads to greater nutrient supply and tissue oxygenation. The contraction of the wound coincides with the stage of proliferation. The last phase is the long-term remodeling, constituted by extracellular matrix production, wound closure, maturation of the scar tissue and cure (Hardwicke et al. 2008).

In the literature, there is a recent article on wound healing with the use of L. pacari leaves (Pereira et al. 2018), that is carried out more deeply than ours, demonstrating this activity in in vivo and in vitro models. The authors showed that the wound healing activity of L. pacari is seemingly multi-target and involves the inhibition of the proliferative and anti-inflammatory phases, reactive oxygen species scavenging and positive modulation of the remodeling phase. We emphasize that our data corroborate theirs, in the sense of having found some similar results related to an increase in the rate of wound contraction through reepithelialization and also regarding some histological variables such as increased collagen content and number of blood vessels, fibrin and presence of inflammatory infiltrates. However, what we want to highlight most is that we use cultivated species and not collected from the natural environment (as Pereira et al. 2018) and this is the main difference between both studies. Our data allow to suggest that we were able to cultivate the species in order to maintain wound healing activity and we were also able to maintain the biosynthesis of punicalagin, one of the polyphenols also detected by Pereira et al. (2018) and that it was related to play a role in the

**Figure 3** – a-b. Photomicrography of tissue fragments from rat dermis, stained with hematoxylin and eosin after 7 days of treatment. Collagen content (arrows) therein highlighted – a. group treated with distilled water (control); b. group treated with 10% L. pacari extract. Mann-Whitney test, Dunn post-test. * p <0.05.

**Figure 4** – a-b. Photomicrography of tissue fragments from rat dermis, stained with hematoxylin and eosin after 7 days of treatment. Blood vessels (arrows) therein highlighted – a. group treated with distilled water (control); b. group treated with 10% L. pacari extract. Mann-Whitney test, Dunn post-test. * p <0.05.
reported biological effects. These findings are very important because it opens perspectives on the sustainability of the species. We would like to remember that when proposing the cultivation of a medicinal species, one must consider the content of secondary metabolites, which is affected by environmental, genetic and agronomic factors (Sampaio et al. 2011). Thus, it can be said that this work of Pereira et al. (2018) was a tool to monitor the biological activity in relation to the proposed cultivation of *L. pacari*.

Literature states that tannins aid wound healing process as well as accelerate burn healing, hence their capacity to form complexes with proteins, which leads to the formation of a protective layer over wound area, thence allowing the epidermis restructuration (Mello & Santos 2017).

Comparing our findings to that of other tannin-rich plants, similar data are verified as described for *Tabernaemontana catharinensis* extract which presented a healing effect on rat cutaneous wounds with an increase in the number of blood vessels and fibroblasts, while also promoting decrease in wound area (Janning et al. 2011). The hydroalcoholic extract of *Schinus terebinthifolius* delayed the re-epithelization of the wounds in rats, thence presenting a larger wound area than the control group, as well as larger number of mononuclear cells (Castelo Branco et al. 2006). The use of *Stryphnodendron obovatum* and *Stryphnodendron polyphyllum* did not lead to a significant difference in the healing of cutaneous wounds of rats, but ethyl acetate fraction of *S. polyphyllum* (36.16% of tannins) lead to a decreased wound area after 7 days (Lopes et al. 2005). Coelho et al. (2010) demonstrated that ointment with 10% *Stryphnodendron adstringens* extract favored the healing process of cutaneous wounds in rats. Hayouni et al. (2011) found that an ointment with 5% methanolic extract of *Punica granatum* (rich in punicalagin) significantly increased the contraction of the wound, decreased the period of epithelization, increased the levels of collagen, fibrin and hydroxyproline and on the 8th day presented a wound healing of 83.5%. Histological analysis demonstrated the well-formed epidermis, with the presence of hair follicles and absence of inflammatory infiltrate. Ismail et al. (2012) showed that *P. granatum* fruit peels leads to an improvement in epithelialization, resistance to rupture, greater contraction of wounds and increase in hydroxyproline production.

Regarding Lythraceae wound healing properties, the ethanolic extract of the leaves of *Ammannia baccifera*, which contains flavonoids and tannins, induced improvement in the different phases of wound healing process, including collagen synthesis, maturation, wound contraction and epithelization (Rajasekaran et al. 2012). The extract of *Lawsonia inermis* L. leaves provided a reduction in the period of epithelization, high resistance to skin rupture, increase in granulation tissue, reduction of 71% in wound area, increase of collagen and fibroblasts and little inflammatory infiltrate. These benefits were attributed to the chemical compounds present in the extract, amongst them: tannins, gallic acid, mannotol and lawsone (Nayak et al. 2007). Muhammad & Muhammad (2005) suggested that the extract of *Lawsonia inermis* L. leaves may be used in infected burn wounds, since it was able to inhibit the growth of microorganisms that caused pathological infection.

Finally, we highlight that the results found in the experimental conditions described corroborate to the popular use of *L. pacari* leaves in the treatment of wounds. It can also be interpreted as an incentive to expand the cultivation of this medicinal plant, aiming at the sustainability of the plant species and their domestication for therapeutic use.

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**References**


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