Antioxidant effect of *Hymenaea courbaril* L (Jatobá) sap on the healing of wounds on mice

Ruth Silva Lima da Costa¹, David Smangoszevski Martins¹, Laan Diego Carvalho Peixoto¹, Bárbara Janaína Paula da Silva², Larissa Barbosa Borges², Emerson Silva Lima², Hector Henrique Ferreira Koolen⁴, Dionatas Ulises de Oliveira Meneguetti¹, Ana Flávia Marçal Pessoa³ and Romeu Paulo Martins Silva¹

¹Post-Graduate Program in Health Sciences on the West Amazon, Federal University of Acre - UFAC, Rio Branco - Ac, Brazil.
²Biological Activity Laboratory, School of Pharmaceutical Sciences, Federal University of Amazonas - UFAM, Manaus - AM, Brazil.
³Department of Surgery, University of São Paulo Medical School (FMUSP), São Paulo, Brazil.
⁴Master’s Program in Biotechnology, Amazonas State University - UEA, Manaus AM, Brazil.

Received 16 January, 2021; Accepted 24 March, 2021

The use of natural products with curative action is a common cultural practice in the Brazilian Amazon, but little explored scientifically. The effect of *Hymenaea courbaril* L sap extract and its contribution to wound healing in mice was evaluated. The antioxidant potential of the extract was studied using the radical 1,1-diphenyl-2-picrilhidrazil and 2,2'-azino-bis (3-ethyl benzothiazoline-6-sulfonic) acid radical, anion superoxide (O2-) radical tests, phenol and flavonoid content, proliferation assay, cell viability and migration. *In vivo* tests were performed on adult Swiss mice, submitted to back injury and treated with a formulation containing 2% sap extract, extracted with ethyl acetate. The animals were treated topically for 14 days, it was observed that the sap extract has antioxidant activity, inhibiting the production of reactive oxygen species, presented potential for the proliferation of fibroblasts and promoted cell migration. Morphometric analysis of wound closure on the 3rd day suggested that animals treated with Jatobá sap had a reduced area of injury when compared with the control group on the 3rd post-injury day for the treated group. On the 14th day, there was no difference between groups. The total closure of the wounds did not occur during the 14-day experiment, but through histological evaluation, it was found that there was re-epithelialization. The results suggest that Jatobá sap extract has the potential to induce the healing of skin wounds due to its antioxidant activity.

**Key words:** *Hymenaea courbaril* L extract, cytotoxicity, antioxidant activity, wound.

**INTRODUCTION**

Wound healing is a complex cellular and biochemical process, necessary to restore structurally defective tissue and involves dynamic interactions between various types of cells, regardless of the causative agent (Grada et al., 2017). This process is also directly related to the general conditions of the organism (De Prado et al., 2018). Currently, the use of antioxidant vitamins (Pessoa et al., 2016), functional foods (Shin et al., 2015), proteins and medicinal plants (Maver et al., 2015) for healing is widely studied.
Among these, the medicinal plants were highlighted, an object of innumerable researches, which have become an effective alternative for treating many pathologies. Such pathologies include skin wounds that affect the quality of life of patients and are considered one of the leading causes of physical disabilities (Oliveira et al., 2019).

Among the many natural riches of Brazil is the *Hymenaea courbaril* L., popularly known as Jatobá, which belongs to the Fabaceae family, Caesalpinioideae subfamily. This tree occurs in all main types of low altitude tropical ecosystems and is well distributed throughout Brazil, occurring in almost all region, presenting uniform distribution in the Amazon (Silva et al., 2019).

Jatobá has different uses, its wood of excellent quality and widely commercialized (Rocha et al., 2019), its fruits are edible with important nutritional properties (Schwartz, 2018), and for centuries, it has been used for culinary purposes. In folk medicine, it has wide therapeutic use with leaves, roots, fruits, resin, sap and bark are used for several types of popular treatments (Silva et al., 2018).

It is popularly used against cough, diarrhea, intestinal colic, lung problems, anemia, and sore throat and for the treatment of kidney problems such as viruses, chronic cystitis, bladder infections. Some species of the genus are also used to deworm and for the treatment of arthritis and inflammatory conditions (Boniface et al., 2017).

Studies carried out with the *H. courbaril* L species reveal the presence of phenolic compounds, such as tannins, flavonoids, essential oils, terpenes and diterpenes. Regarding their biological action, studies carried out with extracts, fractions or compounds isolated from this plant demonstrate antibacterial activity (Aleixo et al., 2015), anti-inflammatory (Brito et al., 2015), antiviral action (Cecílio et al., 2012), antineoplastic and immunosuppressive activity (Suárez and Chávez, 2018; Spera et al., 2019), action antimicrobial (Correa et al., 2020), muscle relaxant properties (Bezerra et al., 2013) and antifungal and antioxidant activity (Menezes et al., 2020). However, the use of the Jatobá sap for healing skin wounds was not yet studied.

Therefore, the objective of this study was to characterize the antioxidant and healing effects on *in vitro* and *in vivo* models present in the Jatobá sap extract and Xylazine were acquired from Ceva (São Paulo, SP-Brazil), Doxorubicin hydrochloride from Eurofarma (SP-Brazil), Isoflurane from BioChimico (Rio de Janeiro, RJ-Brazil), Hank’s Balanced Solution from Vitrocell, (Campinas, SP-Brazil), Paracetamol from SEM (São Paulo, SP-Brazil), and Quercetin from Vetc (São Paulo, SP-Brazil).

The vacuum rotating evaporator was of the Marconi brand (São Paulo, SP-Brazil), the digital camera from Sony Alpha (São Paulo, SP-Brazil), and the microscope was Leica - Microscope (Alemanha - DE).

**Sampling**

The sample of the *H. courbaril* L sap was collected from the Chico Mendes extractive reserve, under Ibama authorization No. 57286-1. Parts of the plant were collected for drying, under the following GPS coordinates - 9°59.50,67°59.2.2ws, identified at the Escola da Floresta and deposited at the herbarium of the Federal University of Acre, with protection number UFACPZ 20025.

**Obtention of the dry extract**

The residue used for preparing the extract was the *H. courbaril* L sap, from which the dry extract was obtained for subsequent analyses. 500 mL of the sap was washed with 500 mL of hexane using a Soxhlet. After extracting until exhaustion, the residue was washed with 500 mL of ethyl acetate. Subsequently, the ethyl acetate was evaporated under vacuum using a rotovap and the residue obtained was stored in a desiccator until use. The extraction yield was of approximately 17.5%.

**Determination of the antioxidant potential**

The extract was submitted to the scanning assay of the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), conducted according to Brand-Williams et al. (1995), with slight modification for the use of microplate of 96 pits. The method was based on the transfer of electrons, which, through the action of an antioxidant (AH) or a radical species, the DPPH, which presents purple coloration and, when reduced, forms the diphenyl-picryl-hydrazine, of yellow coloration, which can monitor the decrease of absorbance.

The determination of the antiradical activity was done through a scanning assay of the 2,2'-azino-bis (3-ethyl benzothiazoline-6-sulfonic acid) radical (ABTS**−**), based on the methodology described by Re et al. (1999). The ABTS radical was formed by adding potassium persulfate, presented in a green coloration. While the antioxidant is mixed with this radical, the ABTS reduces, causing the loss of coloration. The results are expressed in function of the Trolox standard submitted to the same analysis conditions.

The scanning assay of the superoxide anion radical (O$_2^−$) was conducted using the method developed by Ewing and Janero (1995), in which the scanning activity is generated during the metabolism of aerobic organisms, be it by final products of enzymatic reactions or as accidental secondary cellular products of a redox reaction. In this assay, the superoxide anion radical (O$_2^−$) is generated by the Phenazine methosulfate and Nicotinamine Adenine Dinucleotide (PMS-NADH) system, reduced by the oxidation of the Nitro Blue Tetrrozolium.

The dose of total phenols was quantified using the method

**MATERIALS AND METHODS**

**Drugs, reagents, solvents and equipment**

The Galic Acid, Ascorbic Acid, Trypan Blue, Ethyl Acetate, DMSO, DCFH-DA, Methanol, 4% Formaldehyde, Hexane, Paraplast™, 2,2'-azobis amcinopropionate solution, Trolox, Poly-L-Lysine, and Xylol were acquired from Sigma-Aldrich (St. Louis, MO-USA), Ketamine and

*Corresponding author. E-mail: ruttylyma@gmail.com.

Author(s) agree that this article remain permanently open access under the terms of the Creative Commons Attribution License 4.0 International License.
described by Singleton and Rossi (1965). The measurement of total flavonoids was done using the method described by Zhishen et al. (1999), with the objective of evaluating the possible antioxidant potential of the sample.

**Chemical characterization of the extract**

A high-performance liquid chromatography with mass spectrometry system (HPLC-MS), model ifunnel LC-MS 6550 (Agilent Technologies), was used equipped with an electrospray ionization source. The source operated on the negative polarity mode. Substance separation occurred in an HPLC column, model poroshell 120 EC-C18 (dimensions: 2.7 µm of particle, 4.6 mm i.d., column of 50 mm (Agilent Technologies)). Binary water mixtures (A) and acetonitrile (B) were used as the mobile phase. The gradient elution at 35°C was as follows: 0-1 min, 15% B; 1 to 22 min, 15 to 100% (v/v) B; 22-25 min, 100% B at a flow rate of 0.35 mL/min. The temperature of the automatic sampler was maintained at 15°C, and the injection volume was of 15 µL. The ESI origin parameter was as follows: VCap, 3500 V; nozzle voltage, 0 V; fractionator, 100 V; skimmer, 65 V; gas temperature, 280°C; gas flow 14 L/min; nebulizer 45 psi. The MS spectrums and fragmentation (MS/MS) were obtained in the m/z range from 150 to 1000. The MS/MS spectrums were interpreted manually, compared to the previously published data.

**Cellular antioxidant activity**

The evaluation of the cellular antioxidant activity was conducted using the methodology of Wolfe and Liu (2007), based on the detection of the production of intracellular ROS through the use of 2,2'-dichlorofluorescein-diacetate (DCFH-DA) from Sigma-USA.

In this technique, we used cells from the MRC-5 fibroblastic line, sown in the concentration of 6 × 10⁴ cells/pit in 100 µL of growth medium and incubated for 24 h. After the period, the culture medium was removed and the pits were washed with phosphate buffered saline (PBS). Subsequently, we prepared a DCFH-DA solution of 25 µM dissolved in Hank’s buffer (Vitrocell-BRA) and the extract was added to this solution according to the established concentrations. 100 µL of this solution was added to the micropit, incubated for 60 min at 37°C and 5% of CO₂.

Posteriorly, the pits were once again washed with PBS and, shortly after, a solution of 2,2'-azobisamidopropane (AAPH) at 600 µM was prepared, dissolved in Hank’s buffer, which was added to the pits. Subsequently, the micropitates were read with fluorescence, immediately measured at the excitation wavelength of 485 and 520 nm of emission for 60 min in 5-min intervals.

The controls with and without DCFH-DA were prepared and submitted to similar processes. Quercetin (Vetec-BRA) was used as a positive antioxidant activity control.

**Cytotoxicity and cellular proliferation assay**

The cytotoxicity and cellular proliferation assay was conducted using the Alamar Blue method according to Nakayama (1997). The cellular lines (MRC-5), were plated at the concentration of 0.5 × 10⁵ cells per pit (100 µL of DMEM medium with 10% SBF) on micropitates with 96 pits.

After 24 h of incubation and cellular adherence, they were treated with the Jatobá extract at the concentrations of 100, 50, 25, 12.5, 6.25, 3.125, 1.562, and 0.781 µg/mL.

The experiment was conducted in triplicate for each treatment period (24 h). As negative control, we used the DMSO culture medium at 0.01%.

After the treatment period (72 h), we added 10 µL of 0.4% resazurin (1:20 dilution). The incubation period standardized for the cell lines described earlier was of 3 h, the time necessary for the resazurin to metabolize.

The data from the fluorescence reading emitted by the treated cells and the control (without treatment) were acquired and compared. The assay was conducted in triplicate of samples and experiments.

**Growth curve and cellular proliferation**

The assay consisted of the evaluation of the growth curve and cellular proliferation using the Trypan Blue (Sigma-USA) according to the methodology proposed by Freshney (1994). The MRC-5 fibroblasts were plated at the concentration of 3 × 10⁴ cells/mL (DMEM medium, with 10% SBF) in micropitates with 24 pits and maintained in an oven at 37°C and 5% CO₂. After reaching a cellular clonality of 80% of occupied surface, the cells were treated at a non-cytotoxic concentration (50 µg/mL). The proliferation study presented intervals measured at three experimental times: 24, 48, and 72 h of contact with the compounds. After trypsinization and inactivation with the medium and PBS, 90 µL of the cellular suspension was removed and added with 10 µL of Trypan Blue. 10 µL of this solution was transferred to the Neubauer chamber and counted the cells excluding those stained in blue (non-viable cells). As a negative control, the cells were maintained with no treatment, containing only the DMEM culture medium added with 0.1% DMSO.

**Cellular migration assay**

In the experiment for cellular migration, conducted according to the method used by Ascione et al. (2016), MRC-5 fibroblasts were plated at the concentration of 50 × 10⁴ cells/mL (DMEM medium, with 10% SBF) in micropitates with 12 pits, and maintained in an oven at 37°C and 5% of CO₂. After reaching a cellular clonality of 100% occupied surface, a scratch was made at the middle of the pits using a stylus of 100 µL and, subsequently, washed the micropitates with PBS. The cells were then treated with the non-cytotoxic concentrations (12.5, 25, and 50 µg/mL).

The cellular migration study presented intervals measured at three experimental times: 24, 48, and 72 h of contact with the compounds. The micropitates with the treated cells were then analyzed in an optical microscope to evaluate the cellular migration. The registry of the migration was done by capturing the image of the micropitate after photographing with a Sony Alpha NEX digital camera (model – DSC E18-55 24, 4 2MP 3 X optic zoom).

For the negative control, only the DMEM medium was used without treatment, while for the positive control, ascorbic acid (Sigma-USA) was used which induced cell proliferation and Jatobá sap extract at the concentration of 50 µg/mL.

**Preparation of the pharmaceutical formulation**

The Jatobá sap extract, found in pasty consistency was used, homogenized directly in solid vaseline (Rioquimica-BRA), using a mortar and pistil, in the proportion of 2% Jatobá sap extract (p/p). The formulation was identified as Jatobá treatment (JT). Only pure solid vaseline, identified as Vehicle Control (VC), was used as a base.

**Animals**

Male Swiss mice with 70 days of age were obtained from the
The wounded area was immediately photographed (day zero) and subsequently laid on cork strips and fixed in 4% formaldehyde (Sigma-USA) for 8 h at a temperature of 8°C. After the procedure, the animals were submitted to euthanasia using a lethal dose of Xylazine/Ketamine (Ceva-BRA). The skin samples were dehydrated and diaphanized in Xylol (Sigma-USA) and added to Paraplast® (Sigma-USA) for sectioning. Cuts with 7 µm of thickness were obtained using a manual rotative microtome (820 cm × 12 cm) and heated (under light, with ocular protection) to avoid hyperthermia. The animals received a dose of paracetamol analgesic (75 mg/kg of weight) and remained in the individual cages throughout the experiment (Pessoa et al., 2016).

Topical treatment

The animals were divided into two groups: Vehicle Control (VC) group, receiving only the cream vehicle, and Jatobá Control (JC) receiving the cream containing the Jatobá sap extract. The treatments began on day zero, progressing to the 3rd day for the first group and to the 14th day for the second, always at 17 h. 200 µl of cream, measured in a micropipette, were marked on the back of the animals after trichotomy, with the aid of a porous pen, we marked the skin and removed the area surgically using a clamp and small thin tipped scissors. Subsequently, the animals were placed in individuals cages (7.5 × 12 cm) and heated (under light, with ocular protection) to avoid hyperthermia. The animals received a dose of paracetamol analgesic (75 mg/kg of weight) and remained in the individual cages throughout the experiment (Pessoa et al., 2016).

Skin removal, fixation, and tissue processing

To remove the skin, the animals were sedated using a mixture of Xylazine/Ketamine (40 mg/kg of weight). After sedation, the skin was removed using thin tipped surgical scissors and clamps, subsequently laid on cork strips and fixed in 4% formaldehyde (Sigma - USA) for 8 h at a temperature of 8°C. After the procedure, the animals were submitted to euthanasia using a lethal dose of Xylazine/Ketamine.

The skin samples were dehydrated and diaphanized in batteries of ethanol obtained from Sigma-USA (70, 95, and 100%) and Xylool (I and II) (Sigma-USA). Each passage took place with the duration of 1 h and added to Paraplast® (Sigma-USA). Cuts with 7 µm of thickness were obtained using a manual rotative microtome (820 Spencer Microtome) and extended over laminas previously covered with Poly-L - Lysine (Sigma-USA).

Measurement of the wounded area

The wounded area was immediately photographed (day zero) and after 1, 3, 7, 10, 12, and 14 days using a Sony Alpha NEX digital camera (model – DSC E18-55 24.2 MP 3 X optic zoom) at a fixed distance of 20 cm. The wounded area was measured using the Image J program (NIH). To capture the images, the animals were submitted to narcosis with isoflurane (Biochemical-BRA). The results were expressed as a percentage (%) of the original wounded area (Pessoa et al., 2016).

Histological analysis and cell count

The histological analysis was conducted using a Leica microscope after staining the laminas with Hematoxylin/Eosin (H&E). The Leica DMC2990 camera was used for the photomicrographs. The cellularity of the micrographs stained with H&E was measured using the Image J program to measure the amount of the color blue (RGB-Red, Blue, and Green), coloration observed in the nucleus of the cells via histogram.

Statistical analysis

The comparisons between groups were conducted using one-way ANOVA and Kruskal-Wallis for non-parametric tests. The data were analyzed using the GraphPad Prism software. The significance was defined in p<0.05.

RESULTS

In vitro determination of the antioxidant capacity

Table 1 shows that the extract sample extract in ethyl acetate of Jatobá sap presented satisfactory capacity to sequester free radicals, as verified by the DPPH and ABTS tests and scanning assay of the superoxide anion radical, presenting a IC50 µg/mL value similar to the standards used. This demonstrates highly expressive percentages of antioxidant action, considering that the lower the value of IC50 µg/mL (inhibitory concentration) presented by the sample, the higher its antioxidant capacity will be, demanding a lower amount of plant to reduce 50% of the free radicals.

Regarding the doses of phenols and flavonoids in the sample, the results indicate the antioxidant potential of the sample since the percentage found was relatively high when compared with the standards of Gallic acid and Quercetin.

Chemical characterization of the extract

The extract analysis using HPLC-MS demonstrated a complex chemical composition with at least 17 majority substances detected (Figure 1). The fragmentation profile of these substances was identical between distinct spikes, indicating the existence of many isomer phenolic compounds. These compounds were represented by the mixture of (+) and (-)-catequins (spike 1), proanthocyanidin dimers (B1 and B2, spikes 2, 3, 5, 7, and 8), proprapagodin dimers (spikes 4 and 6, tr 2.98 and 3.66 min). In addition to these compounds, most of the sample constitution consists of various isometric versions of proanthocyanidin trimers (spikes 9, 10, 11, and 12). Taxifolin flavonoids (spike 13), two isomeric quercetin di-
Table 1. Evaluation of the antioxidant capacity of the sap of Jatobá extract, extracted with acetate.

| Sample     | DPPH (CI50 µg/ml) | ABTS (CI50 µg/ml) | SOD (CI50 µg/ml) | Phenols | Flavonoids |
|------------|-------------------|-------------------|-----------------|---------|------------|
| Sap acetate| 5.43±0.05         | 1.73±0.05         | 22.56±0.37      | 77.55±3.29 | 13.16±1.64 |
| Standard   | 4.36±0.23         | 3.30±0.18         | 23.96±0.70      | 534.77±22.87 | 65.42±8.87 |
| Trolox     | Trolox            | Gallic Acid       | Gallic Acid     | Quercetin |

Results of the phytochemical triage performed using the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), 2,2′-azino-bis (3-ethyl benzothiazoline-6-sulfonic acid radical (ABTS) tests, superoxide anion radical (O²⁻) scanning assay, dosing of phenols and flavonoids from the sap acetate extract in comparison to the Gallic Acid, Trolox, and Quercetin, at CI₅₀ µg/ml (inhibition mean). The values represent the mean ± standard deviation.

Figure 1. Total ions spectrum of the crude extract sample of the Jatobá sap obtained through the HPLC-MS/MS technique with electrospray ionization in the negative mode.

glycosides (spikes 14 and 15), and two caffeoylquinic acid glycoside isomers (spikes 16 and 17) were also identified when compared with the previously published data (Fracassetti et al., 2013, Kajdžanoska et al., 2010).

Evaluation of the cellular antioxidant activity

The results of the evaluation of the cellular antioxidant activity (Figure 2), demonstrated that the Jatobá sap extract was capable of inhibiting the production of intracellular oxygen reactive species (ROS) in the MRC-5 fibroblastic line when compared with the standard with Quercetin. This suggests its antioxidant activity, demonstrating the potential to neutralize the actions of free radicals, avoiding the occurrence of oxidative stress.

Figure 2. Total ions spectrum of the crude extract sample of the Jatobá sap obtained through the HPLC-MS/MS technique with electrospray ionization in the negative mode.

Evaluation of the cellular viability

In the cell viability assay, performed by the Alamar Blue method (Figure 3), the results demonstrated that at the lowest concentrations (24 and 48 h), the Jatobá sap extract showed an inhibitory effect on the viability of the MRC-5 cells, seeing that cell death did not occur at 100% viability, confirming that the extract is not cytotoxic at the concentrations tested in 24 and 48 h.

Induction of cellular proliferation

The cellular proliferation assay was conducted on MRC-5 cells of the fibroblastic line using the exclusion Trypan Blue method (Figure 4) and is a quantitative cellular proliferation evaluation method. After 72 h of exposure, the Jatobá sap extract induced cellular proliferation, presenting a result near the standard for the ascorbic acid used in the assay, and well above the values found for the negative control, demonstrating that the Jatobá sap extract was capable of inducing the increase in the proliferation of fibroblasts in vitro.
Figure 2. Cellular antioxidant activity. Oxidation induced by the DCFH-DA peroxyl in cells of the MRC-5 fibroblastic line and oxidation inhibition through quercitin and *H. courbaril* (Jatobá) sap extract over 24 h.

Figure 3. Assessment of cell viability after exposure to fibroblast Jatobá sap extract in the concentrations of 50, 25, 12.5, 6.25, 3.125, 1.562 and 0.781 μg/mL, by the Alamar Blue method. Cell lines (MRC-5) were plated at concentrations of 0.5 × 10⁴ cells per well (100 μL of DMEM medium with 10% SBF) in 96-well microplates. The cells were treated with sap extract at concentrations of 50, 25, 12.5, 6.25, 3.125, 1.562 and 0.781 μg/mL. The experiment was carried out in triplicate for each treatment period (24 h). *p<0.001 statistical difference from Jatobá in relation to the control.

Evaluation of the cellular migration

The cellular migration assay was conducted for fibroblasts of the MRC-5 line (Figure 5) using the Jatobá sap extract and ascorbic acid, used as positive control, and resulted in the wound closing in 48 h, presenting confluence of 100% of proliferation for both treatments in 72 h, demonstrating the potential of the sap extract in promoting cellular migration *in vitro*. The *in vitro* results suggest that the Jatobá sap has antioxidant and healing potentials.

Effects of the topical treatment with Jatobá cream on the wound closure on mice

The morphometric analysis of the wound closure on the 3 day post-lesion, inflammatory phase of the healing, suggested that the animals treated with the Jatobá sap cream (JC) presented smaller wound area, as observed along 14 days of experiment by analyzing the images following the healing process (Figure 6A) and cellularity (Figure 7A and B), demonstrated in the histological analysis when compared with the control group (VC).
Figure 4. Evaluation of the growth curve and cellular proliferation of fibroblasts treated with *H. courbaril* (Jatobá) sap acetate extract at 50 μg/mL (Trypan Blue test). *p<0.001, statistical difference of the Jatobá compared to the control; ###p<0.001, statistical difference of the Jatobá compared to ascorbic acid. The fibroblast cells MRC-5 were plated in the concentration of 3×10⁴ de cells/mL (DMEM medium, with 10% SBF) in microplates with 24 pits, maintained in oven at 37°C, and 5% of CO₂. The test presented intervals measured in three experimental times: 24, 48, and 72 h of contact with the Jatobá sap in the concentration of 50 μg/mL. The data was analyzed using the GraphPad Prism® program (One-Way /ANOVA/ Kruskal-Wallis).

Figure 5. Evaluation of the cell migration after exposure to the Jatobá sap extract. Cellular migration assay demonstrating the motility of the fibroblasts treated with 50 μg/mL ascorbic acid and Jatobá sap extract at 50 μg/mL. The MRC-5 fibroblasts were plated in the concentration of 50×10⁴ de cells/mL (DMEM medium, with 10% SBF) in microplates with 12 pits, maintained in oven at 37°C, and 5% of CO₂. The test presented intervals measured in three experimental times: 24, 48, and 72 h of contact with the sap extract. The microplates with the treated cells were then analyzed in an optic microscope for evaluating the cellular motility.
Figure 6. Evolution of the scaring in control animals and Jatobá treatment on days 3rd and 14th days post-lesion. A) Panel presenting the progression of the scaring in control animals and animals treated with Jatobá cream; B) Graphic representing the percentage of the area closure, minimum of 7 animals per group. Treatment with the concentration of 2% Jatobá sap extract. The wounds were photographed over time, and the wounded area was measured using the Image J software program (NIH). The values are presented as mean ± EPM. *p < 0.05, for significant difference compared to the untreated control, as indicated by the one-way ANOVA and post-Kruskal-Wallis test.

Figure 7. Micrograph of the wounded area on the third day post-lesion in control mice without treatment and treated with Jatobá cream at 2%. Histology of the 3rd day post-lesion, by H&E staining. The panel shows the wounded area on the control groups and those treated with Jatobá cream at 2%. Areas visualized with a magnification of 40x (A and B). The values are presented as mean ± SD. (%%) p < 0.01 for a significant difference when compared with the remaining groups, as indicated by the two-way ANOVA and post-Kruskal-Wallis test.
On the 14th day post-lesion, it was still possible to observe the presence of crust and clot on the back of the animals resulted from the healing process, which denoted performing the morphometric analysis (Figure 6A). However, in the histological analysis of the same period, we can observe the re-epithelialization, the presence of dermis cells, such as fibroblasts, and some inflammatory cells, characteristic of this phase, in both groups, demonstrating that the animals that received topical treatment with the Jatobá sap presented a good evolution regarding the healing process (Figure 8A and B).

**DISCUSSION**

The results presented in this work were produced to generate *in vitro* and *in vivo* knowledge on the antioxidant and healing activities of *H. courbaril* L (Jatobá) sap extract. The present study is the first report demonstrating these activities of the Jatobá sap using methodologies that characterize the proliferation process, growth, viability and cellular migration in fibroblasts, and healing analysis in an animal model.

Initially, the ethyl acetate extract of the Jatobá sap was evaluated regarding its capacity for sequestering free radicals through the DPPH, ABTS, and SOD tests, presenting a strong antioxidant activity, with effects comparable to the controls used as positive standard for such activities (Table 1). These results corroborate with a previous study in which the same assays were conducted to evaluate the antioxidant potential of the *H. courbaril* L., but using the hydroethanolic extract from seed, showing its antioxidant potential (Spera et al., 2019).

Understanding the action mechanism of the reduction of DPPH molecules and the main chemical classes present in *H. courbaril* L, it was suggested that the antioxidant action presented may be related to the presence of phenolic hydroxyl compounds available in the plant (Veggi et al., 2014) and to the presence of proanthocyanidin trimers and phenolic compounds such as taxifolin flavonoids identified in the chemical composition of the Jatobá sap extract.

Studies have been conducted with the objective of demonstrating the importance of using substances with antioxidant potential. These studies identified that phenolic compounds are one of the primary groups responsible for this property. Among them are the flavonoids, which can be found in high content in the species *H. courbaril* L (Figueiredo et al., 2016).

The biochemical and pharmacological effects of flavonoids are vast, and among them, the antioxidant, anti-inflammatory, antiplatelet, vasodilator, antimicrobial, and antiallergic activities were highlighted, considering that, the higher the content of phenolic compounds, the higher the antioxidant potential (Batiha, 2020).

Figure 1 shows the significant content of phenolic compounds found in the Jatobá sap extract. This result corroborates with the findings of a similar study in which high contents of phenolic compounds were identified in
the aqueous extract of Malpighia emarginata DC., Platonia insignis Mart., Spondias mombin L., Anacardium occidentale, Psidium guajava, and Tamarindus indica L., performing a positive correlation between the antioxidant activity and the variables of total phenols and total flavonoids. In other words, in as much as the contents increase in the samples, there was a percentage increase of the antioxidant activity. This confirms that the antioxidant activity is directly correlated to the contents of total phenols and flavonoids (Sousa et al., 2011).

This study corroborated with the findings of the present study, given that the strong antioxidant capacity regarding the DPPH, ABTS, and superoxide anion (O₂⁻) radicals can be attributed to the high contents of phenolic compounds.

The phenolic compounds have demonstrated an ideal capacity for removing free radicals due to its chemical structure, donating a hydrogen atom or an electron, presenting antioxidant properties that allow them to act as reducing agents by donating hydrogen, as well as due to their metal chelation potential (Kaurinovic and Vastag, 2019).

In this sense, because of the content of phenols and flavonoids in the sample, the Jatobá sap extract inhibited the production of intracellular reactive oxygen species (ROS) (Figure 2), confirming its cellular antioxidant activity, given that the flavonoids interact with the biomembranes and exercise the function of fluidity modulators. This generates a physical impediment for the diffusion of the ROS and reactive nitrogen species (RNS) causing the decrease of the kinetics of the reactions responsible for the oxidative stress (Barreiros et al., 2006).

In the present study, the sap extract demonstrated the capacity to promote cellular growth and proliferation in MRC-5 cells of fibroblast lines, also presenting no cell death in 50% of viability, as shown in Figures 3 and 4, confirming that the Jatobá sap extract is not cytotoxic in the tested concentrations. This capacity could be related to the content of phenolic compounds present in the sample since the higher the content of phenolic compounds, the higher the antioxidant potential will be.

A study conducted in Gana using 17 plant species with possible antioxidant potential revealed through cellular proliferation assays and in vitro migration that the species Allophylus spicatus, Philenoptera cyanescens, Melanthera scandens, Ocimum gratissimum and Jasminum dichotomum presented healing activity in vitro, with the healing potential attributed to the presence of rutin glycosidic flavonoids in the samples and a quercetin triglycoside in P. cyanescens (Freiesleben et al., 2017).

This result corroborates the cellular migration assay conducted in the present study, as demonstrated in Figure 5 since the H. courbaril L sap extract had the potential to promote cellular migration and proliferation. This mechanism can also be related to the presence of phenols and flavonoids in the sample.

To evaluate the healing potential in vivo, the experimental times of the 3rd and 14th days post-lesion was used to analyze the contraction and re- epithelialization of the wound.

The morphometric analysis of the wound closure on the 3rd day post-lesion suggests that the animals treated with the cream containing Jatobá sap presented significantly smaller wound area and less cellularity (Figure 6A and B). This result is consistent with the findings of a study that evaluated the extract of Zeyheria tuberculosa on healing wounds and presented better results in reducing the diameter of the wound and re-epithelialization in the histological cuts when compared with the control group (Majewska et al., 2011).

The results of this study also corroborate with research that demonstrated that some popularly used medicinal plants could promote tissue repair mechanisms, justifying the potential of therapeutic use in the treatment of wounds, especially regarding the evaluation of its healing potential. This demonstrates the presence of phenolic compounds in the plant composition (Sarmento et al., 2014), obtained by the many extracts and essential oils present, and that the phenolic compounds can be effective for healing wounds (including chronic wounds) and burns (Dzialo et al., 2016).

The present study suggested that the topical treatment with Jatobá sap extract had a tendency for healing a smaller wounded area and, through the analyses, the re-epithelialization and cellularity for both groups could be verified (Figures 7A and B and 8A and B), which demonstrated that the formulation containing Jatobá sap extract contributed to healing. The antioxidant potential of the sample may justify the healing.

According to these results, it is evident that species with the presence of flavonoids contribute to wound healing (Aslam et al., 2018).

Research that aimed to evaluate the effects of total flavonoids of the species Blumea balsamifera L., on surgically induced wounds on the skin of Sprague-Dawley rats, demonstrated significant effects on wound healing of treated animals compared to controls, accordingly with the findings of this study (Pang et al., 2017).

The findings of Güzeli et al. (2019), demonstrated the healing potential in wounds of diabetic Wistar rats, from topical applications of formulations of the species Salvia krenovenburgii Rech. f. (SK) and Salvia euphratica Montbret, Auher & Rech. f. var, showing strong wound healing effects with important antimicrobial and antioxidant activities.

Luteolin (Arachis hypogaea L.), naturally distributed among medicinal plants, vegetables and fruits, structurally rich in flavonoids, was tested to evaluate wound healing in diabetic and non-diabetic Wistar rats, through topical applications of the formulation in the wounds, showing that flavonoids increase the formation of collagen fibrils in the tissues of non-diabetic and diabetic wounds,
since the phenolic compounds present, known as potent antioxidants, contribute to the process of accelerating healing (Ozay et al., 2018).

The phototherapy constituents, such as tannins, terpenoids, diterpenes, sesquiterpenes, phytosterol, phenolic compounds, proteins, flavonoids, saponins, and essential oils, have proven their healing properties, facilitating the healing process by removing the free radicals, increasing the contraction of the affected area and the formation of blood vessels and fibroblasts (Ghosh and Gaba, 2013; Tiago et al., 2020), justifying the results found in this study.

Until the present moment, no clinical or experimental studies have been identified that have evaluated the action of the Jatobá sap extract in the healing process. Therefore, there were no studies with this species to compare the results. Thus, this study is a pioneer in describing this activity for the plant material in question.

The results obtained from the experiments conducted in this work showed, in an unprecedented manner, that the *H. courbaril* L. sap extract presented antioxidant effect, which contributed to the healing of wounds on the back of mice, corroborating the results demonstrated in many ethnomedical studies, and justifying the use of this species in popular medicine.

**Conclusion**

*H. courbaril* L (Jatobá) sap extract has the potential for inducing the scarifying of cutaneous wounds through, among other properties, its antioxidant activity.

**CONFLICT OF INTEREST**

The authors have not declared any conflict of interests.

**REFERENCES**

Aleixo AA, Camaros VN, Herrera KMS, Andrade ACDS, dos Santos M, Miranda VC, Ferreira JMS (2015). Synergistic activity from *Hymenaea courbaril* L. and *Strychnos adstringens* (Mart.) Coville against multidrug-resistant bacteria strains. *Journal of Medicinal Plants Research* 9(26): 741-748. https://doi.org/10.5897/JMPR2014.5502.

Ascone F, Vatasuro A, Caserta S, D’Esposito V, Formisano P, Guido S (2016). Comparison between fibroblast wound healing and cell random migration assays in vitro. *Experimental Cell Research* 347(1):123-132. https://doi.org/10.1016/j.yexcr.2016.07.015.

Aslam MS, Ahmad MS, Riaz H, Raza AS, Hussain S, Qureshi OS, Javed O (2018). Role of Flavonoids as Wound Healing Agent. In Phytochemicals-Source of Antioxidants and Role in Disease Prevention pp. 95-102. https://doi.org/10.5772/intechopen.79179.

Barreiros ALBS, David JM, David JP (2006). Oxidative stress: relationship between generation of reactive species and defense of the organism. *Quimica Nova* 29(1):113-123. https://doi.org/10.1590/S0100-40422006000100021.

Batiha GES, Beshbishy AM, Ikram M, Mulla ZS, El-Hack MEA, Taha AE, Elewa YHA (2020). The pharmacological activity, biochemical properties, and pharmacokinetics of the major natural polyphenolic flavonoid: quercetin. *Foods* 9(3):374. https://doi.org/10.3390%2Ffoods9030374.

Berrera G, Góis R, Brito T, Lima F, Bandeira M, Romero N, Santiago GMP (2013). Phytochemical study guided by the myorelaxant activity of the crude extract, fractions and constituent from stem bark of *Hymenaea courbaril* L. *Journal of Ethnopharmacology* 149(1):62-69. https://doi.org/10.1016/j.jep.2013.05.052.

Boniface PK, Ferreira SB, Kaiser CR (2017). Current state of knowledge on the traditional uses, phytochemistry and pharmacology of the genus *Hymenaea*. *Journal of Ethnopharmacology* 206:193-223. https://doi.org/10.1016/j.jep.2017.05.024.

Brand-Williams W, Cuvelier ME, Berzet CLWT (1995). Use of a free radical method to evaluate antioxidant activity. *LWT-Food Science and Technology* 28(1):25-30. https://doi.org/10.1016/S0023-6438(95)80008-5.

Brito FCR, da Cunha LDC, Gonçalves DO, Olinda TM (2015). Antinflammatory and antioxidutive actions of the ethanol extract of *Hymenaea courbaril* L. in rodents. *Animal Science* 25(3):4-14.

Cecilio AB, de Faria DB, de Carvalho Oliveira P, Caldas S, de Oliveira DA, Sobral MEG, de Almeida VL (2012). Screening of Brazilian medicinal plants for antiviral activity against rotavirus. *Journal of Ethnopharmacology* 141(3):975-981. https://doi.org/10.1016/j.jep.2012.03.031.

Correia MN, Aguirre OER, Palacios JDCA (2020). Antimicrobial activity of *Hymenaea courbaril* L. Fruit. *Asian Journal of Pharmaceutical and Clinical Research* 13(6):200-203. https://doi.org/10.22159/ajpcr.2020.v13i6.37272.

De Prado EML, Rodrigues WD, d’Aleencar T, Guedes RA, Villanova JCO, Severi JA (2018). Therapeutic potential of plants with mucilages in wound healing. Special Topics in Animal Science VII, *Chapter 14*:198-217.

Dzialo M, Mierziak J, Korzun U, Preisner M, Szopa J, Kulma A (2016). The Potential of Plant Phenolics in Prevention and Therapy of Skin Disorders. *International Journal of Molecular Science* 17(2):160. https://doi.org/10.3390/ijms17020160.

Ewing JF, Janero DR (1995). Microplate Superoxide Dismutase Assay Employing a Nonenzymatic Superoxide Generator: Analytical Biochemistry 232(2):243-248. https://doi.org/10.1010/abio.1995.0014.

Figueiredo PA, Spera KD, Gomes AC, Dokkedal AL, Saldanha LL, Ximenes VF, Silva LP, da Silva RMG (2016). Antioxidant activity and chemical characterization of extracts of the genus *Hymenaea*. *Journal of Medicinal Plants Research* 10:330-339. https://doi.org/10.3292/j.rjm.2016.8.06.

Fracassetti D, Costa C, Moulay L, Tomás-Barberán FA (2013). Ellagic acid derivatives, ellagitannins, proanthocyanidins and other phenolics, vitamin C and antioxidant capacity of two powder products from camu-camu fruit (*Myrciaria dubia*). *Food Chemistry* 139(4-5):578-588. https://doi.org/10.1016/j.foodchem.2013.01.121.

Freiesleben SH, Soelberg J, Nyberg NT, Jäger A (2017). Determination of the Wound Healing Potentials of Medicinal Plants Historically Used in Ghana. *Evidence-Based Complementary and Alternative Medicine* 1-6. https://doi.org/10.1155/2017/9480791.

Freshney I (1994). Animal cell culture: introduction to biotechniques edited by SJ Morgan and DC Darling. *Bioessays* 16:218-218.

Ghosh PK, Gaba A (2013). Phyto-Extracts in Wound Healing. *Journal of Pharmacy and Pharmaceutical Sciences* 16(5):760. https://doi.org/10.18433/3831v.

Grada A, Otero-Vinas M, Prieto-Castrillo F, Obagi Z, Falanga V (2017). Research Techniques made simple: analysis of collective cell migration using the wound healing assay. *Journal of Investigative Dermatology* 137(2):e11-e16. https://doi.org/10.1016/j.jid.2016.11.020.

Güzel S, Özay Y, Kumaş M, Uzun C, Özkorkmaz EG, Yildirim Z, Kahrman A (2019). Wound healing properties, antimicrobial and antioxidant activities of *Salvia kronsburgi* Rech. I. and *Salvia eupatoria* Monthb. & Rech. I. var. eupatoria on excision and incision wound models of diabetic rats. *Biomedicine & Pharmacotherapy* 111:1260-1276. https://doi.org/10.1016/j.biopharm.2019.01.038.
