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ORIGINAL ARTICLE

Cytotoxic Action and Proliferation *in vitro* and Analgesic Activity *in vivo* of Resin from *Hymenaea stigonocarpa*

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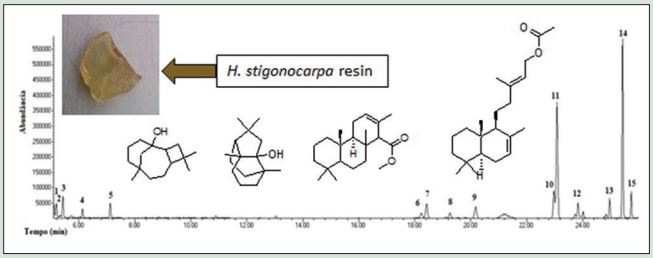
ABSTRACT

Background: *Hymenaea stigonocarpa* Mart. (*Fabaceae*) is one of the plant species commonly found in Brazilian cerrado. It is popularly known as jatobá-do-cerrado. Phytochemical studies of different parts of the plant demonstrated the presence of sesquiterpenes, diterpenes, and oligosaccharides. The species produces an opaque, translucent, pale yellow, or almost white resin rich in labdanóides compounds, widely used in folk medicine, but without proving its pharmacological actions. **Objective:** To analyze the *H. stigonocarpa* resin to confirm its pharmacological actions through specific tests for cicatrization, nociception, and cytotoxicity and to evaluate the chemical composition through the gas chromatography coupled to mass spectrometry (GC-MS). **Materials and Methods:** The cell proliferation/migration capacity was evaluated using the scratch assay method and the cell viability determined by the 3-[4,5-dimethylthiazol-2-yl]–tetrazolium 2,5-diphenyl-bromide cytotoxicity assay. In the pharmacological tests were performed Abdominal Contortions induced by acetic acid and Nociceptive Response evaluated by the Formalin Test. GC-MS was used to trace the profile of the bioactive compounds of the resin. **Results:** It was possible to verify the presence of terpene compounds with the labdanos skeleton. Structures that revealed important pharmacological actions regarding healing, evidenced by the scratch assay test and nociceptive activity demonstrated by the animal tests. No cytotoxic activity was detected at the dosages tested. **Conclusion:** The results obtained confirm the pharmacological action of the species and can be used to elaborate on new therapies, which will increase the therapeutic arsenal available to the population.

Key words: Cicatrizing activities, cytotoxicity, diterpenes, labdanes, nociceptive activities, terpenes

SUMMARY

- In this study to analyze the *Hymenaea stigonocarpa* resin to confirm its pharmacological actions through specific tests for cicatrization, nociception, cytotoxicity and to evaluate the chemical composition. It was possible to verify the presence of terpene compounds with the labdanos skeleton. Structures that revealed important pharmacological actions regarding healing.



Abbreviations Used: ATCC: American Type Culture Collection, GC-MS: Gas Chromatography coupled to Mass Spectrometry, CH_2Cl_2: Dichloromethane, DMEM: Dulbecco's Modified Eagle Medium, DMSO: dimethylsulfoxide, HaCat: Cells, HCl: Hydrochloric acid, MTT: 3-(4,5-dimethylthiazol-2-yl)–tetrazolium 2,5-diphenyl-bromide, KC: Keratinocytes, KOH: Potassium hydroxide, Na_2SO_4: Sodium sulfate, NIH-3T3: fibroblasts cells, PBS: Phosphate-buffered saline.

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INTRODUCTION

Hymenaea stigonocarpa Mart. (*Fabaceae*) is one of the vegetal species commonly found in Brazilian cerrado.[1-3] It is popularly known as jatobá-do-cerrado; it has great importance in the region due to its nutritional value.[4] It belongs to the family Leguminosae (*Fabaceae*), grows to a height of 4–6 meters, and produces fruits that vary between 6 and 18 cm in length and 3–6 cm in diameter.[3]

Phytochemical studies of different parts of the plant have demonstrated the presence of sesquiterpenes, diterpenes, and oligosaccharides.[5,6] The fruits are rich in starch and have high nutritional value. For that reason, it has been used as ingredients in the preparation of cakes, breads, porridge, and biscuits.[4] The bark of the tree and its fruits are widely used in folk medicine. Studies have proven their gastroprotective, antidiarrheal, and cicatrizing effects attributable to the presence of condensed tannins and flavonoids.[2]

H. stigonocarpa also produces an opaque, translucent, pale yellow, or almost white resin.[7] The wound-healing activity of *H. stigonocarpa*, which is attributable to the presence of compounds such as diterpenoid-type epi-labdanoid, has been reported; however, the exact mechanism is still not known.[7,8]

The terpenes of the labdanes class also show beneficial effects against cardiovascular diseases, autoimmune disorders, and Alzheimer's disease.[9] Other studies have confirmed the action of these compounds as tranquilizers, analgesics, antinociceptives, sedatives, anticonvulsants,

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and antidepressants, where the main targets of action are the gabaergic, glutaminergic, dopaminergic, and opioid neurotransmitters.[8,10]

Therefore, the objective of this study was to analyze the *H. stigonocarpa* resin to confirm its pharmacological actions including nociception and healing, cytotoxicity, and chemical composition using gas chromatography/mass spectrometry (GC-MS) techniques.

MATERIALS AND METHODS

Vegetal material

The resin was collected from sutures made on tree trunks of *H. stigonocarpa* Mart. (*Fabaceae*). Samples were harvested from areas of the cerrado biome, located in the city of Montes Claros, in the north of the state of Minas Gerais. A sample was deposited on the form of exsiccate in the Herbarium of the State University of Montes Claros (UNIMONTES) voucher 3318.

Gas chromatography to mass spectrometry

Sample derivation

Resin (20 mg) was used for the hydrolysis reaction, and 5 mL of potassium hydroxide in methanol (0.5 mol L^{-1}) was added, and the mixture was heated to 100°C under reflux for 1 h. Following the esterification reaction, 2 mL of solution hydrochloric acid 36%, methanol (4:1, v/v) was added and heated again at 100°C for 1 h. To extract the methyl esters, 5 mL of distilled water was added to the reaction, and the obtained derivatives were extracted with CH_2Cl_2 (dichloromethane 3 mL × 5 mL), dried over sodium sulfate, filtered, and concentrated on a rotary evaporator. The obtained sample was dissolved in 1 mL CH_2Cl_2 and analyzed in GC-MS.

Gas chromatography to mass spectrometry

The samples were submitted to chromatographic analysis 7890A (Agilent Technologies) coupled to a mass spectrometer (MS 5975C) with a fused silica capillary column DB5-MS (30 m × 0.25 mm × 0.25 μm) using helium (99.9999% of purity) as drag gas with a flow of 1.8 mL min^{-1}. The injector was kept at 220°C, with a flow split ratio of 1:10. The temperature of the column was 160°C for 2 min to 200°C at a rate of 2°C/min. The interface temperature was maintained at 240°C. The system was operated in full scan with electron impact of 70 eV, in the range of 30–600 (m/z). The compounds were identified by the study of their fragmentation data and by comparison of the spectra present in the literature and in the library NIST version 2.0.

Cell lines

NIH-3T3 fibroblasts cells were purchased from the American Type Culture Collection (ATCC CRL-1658, Manassas, Virginia, USA), HaCat cells were acquired from the Bank of Cells of Rio de Janeiro, and human keratinocytes (KCs) were acquired from the Bank of Cells of Healing and Leprosy Laboratory of Ribeirao Preto Medical School of University of Sao Paulo, Ribeirão Preto, São Paulo, Brazil. 3T3 and HaCat were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and KC with Defined KC Medium (GIBCO-Invitrogen Corporation-Grand Island, NY, USA) also supplemented with 10% fetal bovine serum. All were further supplemented with 1% penicillin antibiotic solution (100 U/mL) and antimycotic streptomycin solution (100 mg/mL) and were plated in culture bottles and incubated at 37°C in an incubator under 5% CO_2.

Assessment of cell viability

The toxicity of NIH-3T3 fibroblast, HaCat, and KC cells were determined using the 3-(4,5-dimethylthiazol-2-yl)–tetrazolium 2,5-diphenyl-bromide (MTT) method described by Mohammadi *et al.* (2015) with modifications.[11] The cells were plated in 96-well microplates at a density of 2 × 10^4 cells/mL (volume of 200 μL) per well. The cells were treated with different concentrations of resin: 0.001%, 0.0001%, 00001%, and 0.000001%. The resin was diluted in 0.01% ethanol + 0.09% propylene glycol. The culture medium specific for each cell was used as a positive control and the medium with 50% dimethylsulfoxide (DMSO) was characterized as negative control. The plates were incubated at 37°C in a greenhouse with 5% CO_2 for 24–48 h. Then, 20 μL of the stock solution of MTT (5 mg/mL) in phosphate-buffered saline (PBS) was added to 180 μL of DMEM culture medium (without phenol red). The plates were incubated under the same conditions for 3 h. Then, the whole volume of the cavities was withdrawn, 200 μL of DMSO was added for dissolving the formazan crystals, and the absorbance was read in a spectrophotometer at a wavelength of 570 nm. The experiment was performed in triplicate, and percentage of cytotoxicity was calculated by the following equation:

$$\%\ \text{Cytotoxicity} = \frac{\text{Test absorbance (resin)}}{\text{Positive control absorbance}} \times 100$$

Scratch assay

The cell proliferation/migration capacity was evaluated using the scratch assay method according to Fronza *et al.* modified.[12] The 3T3 cells were seeded in 24-well plates at 3 × 10^4 cells per well. Then, a linear risk was created in the cellular monolayer using a 200 μL plastic tip. The medium was removed and then washed with PBS solution. The cells ware treated with different concentrations of resin 0.001%, 0.0001%, and 0.00001%. The negative control used was DMSO 50% in DMEM and as a positive control culture medium with fetal bovine serum 10%. The solutions prepared were incubated for 24 h under 5% CO_2 at 37°C. The cells were fixed with paraformaldehyde (4%) for 15 min and stained with 4',6-diamino-2-phenyl-indole for 5 min. Four images were obtained from each well, and quantification was done using Image software J 1.46r. The test was realized in triplicate, and the result percentage was calculated by the following equation:

$$\text{Result}\ \% = \frac{\text{Number of migrated cells for the different concentrations of the serum}}{\text{Number of migrated cells for the basic control}} \times 100$$

Pharmacological tests

Animals

Swiss albino adult male mice were used. Each group had six animals with their age ranging from 1 to 2 months and weights ranging from 20 to 35 g. The tests were carried out in the Laboratory of animal experimentation of the UNIMONTES. The animals were fasted for 12 h and deprived of water for 2 h before the tests. Six mice per cage (40 cm × 33 cm × 17 cm) were conditioned in a place exempt from noise, with a temperature of approximately 25°C, humidity 60%, air exhausts, and 12-h artificial light/dark photoperiod.

Model of abdominal contortions induced by acetic acid

The test followed the procedure described by Koster *et al.*[13] The mice were divided into five groups. The negative control group was treated with propylene-glycol vehicle; test group with the resin at 25, 50, and 100 mg/kg; and positive control group with indomethacin 5 mg/kg.

Model of formaldehyde-induced nociceptive response (formalin test)

The procedure used is similar to that described by Hunskaar and Hole with modifications.[14] The animals were divided into six groups. One h before application of the irritant agent, the negative control group was orally treated with the arabic gum carrier in propylene glycol. The test groups were treated orally with the resin at 25, 50, and 100 mg/kg

concentrations, respectively. The positive control group was treated with indomethacin 5 mg/kg. The animals were treated with subcutaneous meperidine 25 mg/kg, 15 min before the application of the irritant agent, characterizing the second positive control group.

Ethics committee

The trials involving the use of albino Swiss mice were submitted to the animal ethics committee and approved under the number CEUA/FIPMOC 06/2014. The procedures that involved the use of human skin fragments to culture KCs were approved by the Ethics Committee of the Hospital das Clinics of the Medical School of Ribeirão Preto, University of São Paulo, under the number of process HCRP 5606/2008.

Statistical analysis

The results of the analgesia analysis were analyzed by statistical test software (SAS statistic) according to one-way ANOVA models with Tukey's variance test to measure the degree of significance ($P < 0.01$). For the analysis of scratch assay and cytotoxicity by the MTT method, applied the statistical tests of variance for multiple comparisons of nonparametric samples (one-way ANOVA and postBonferroni test). Values of $P < 0.05$ were considered to show statistical evidence that there is a difference between the data in question with a 95% confidence interval.

RESULTS AND DISCUSSION

Gas chromatography to mass spectrometry

By means of chromatographic analysis of *H. stigonocarpa* resin, it was possible to detect terpenes such as caryophyllene alcohol (0.92%), ginsenol (1.62%), and some labdanes as shown in Table 1. The chromatogram of total ions obtained by GC-MS of *H. stigonocarpa* resin was evidenced in Figure 1. From this analysis, it was possible to establish the mass spectrum of the *H. stigonocarpa* resin, as well as to identify the characteristic m/z ions and establish the relationship between them. Caryophyllene alcohol is a natural sesquiterpene, known for the anti-inflammatory action, and the ginsenol has wound-healing action, what justifies the results detected in the pharmacological tests.[15,16]

Methyl-8,13-dimethylpodocarp-12-ene-14-carboxylate belongs to the class of terpenes whose pharmacological properties are not yet known.[17] The labdanum is attributed to competitive action on the γ-aminobutyric acid (GABA).[18]

The structures detected in this research work corroborate to that found by other authors who reported that the resin of the species *Hymenaea* is predominantly formed by diterpenes, basing chemically in skeleton of labdanes, abietanes, and pimarane.[7,8]

Assessment of cell viability

Cytotoxicity tests were performed using the colorimetric method, which evaluates the power of toxicity in accordance with the sample treated with the vegetal extracts in study. Cells NIH 3T3 fibroblasts, HaCat, and human KCs were treated with the resin of *H. stigonocarpa* at 0.001%, 0.0001%, 0.00001%, and 0.000001% for 24 and 48 h. By this parameter, it is observed that there is no significant cytotoxicity for the concentrations under study ($P < 0.05$). A fact that can be compiled by Figure 2. The toxic action is available for inhibiting the ability of the cells to convert MTT (salt of yellow color) to formazan (salt of blue color).[19] This conversion occurs via the action of the mitochondrial enzyme tetrazolium-succinate-dehydrogenase in viable cells.[20] Thus, the quantity of MTT-formazan is directly proportional to cellular viability. Cells that are exposed to toxic compounds lose the ability to carry out the conversion.[21] The 3T3 cells are commonly used for toxicity tests. KC and HaCat are KCs (epidermis) and were used with the intention of complementing the results of scratch assay. It is necessary that the compound does not have a toxic effect on the cells of the integumentary system.

Percentage of viability of NIH-3T3 fibroblasts on culture at 24 h and 48 h (A-B), HaCat immortalized KCs on culture at 24 h and 48 h (C-D), and human KCs on culture at 24 h and 48 h (E-F) relative to the control (culture medium – corresponding to 100% viable cells) by MTT colorimetric assay. Medium: culture medium for culturing the cells. V: Ethanol 0.01% + Propylene glycol 0.09%. R1, R2, R3, R4: resin at concentrations of 0.001%, 0.0001%, 0.00001%, and 0.000001%, respectively. One-way ANOVA followed by Bonferroni test ($P < 0.05$).

Scratch assay

The scratch assay is a safe and economically viable tool to test these attributes. This test was developed to demonstrate how vegetable preparations or isolated compounds influence the formation of new cloth.[22,23] The results of this test are described in Figure 3. We found that the resin at concentrations of 0.001%, 0.0001%, and 0.00001% induced 3T3 fibroblast migration over a period of 24 h. The resin, at

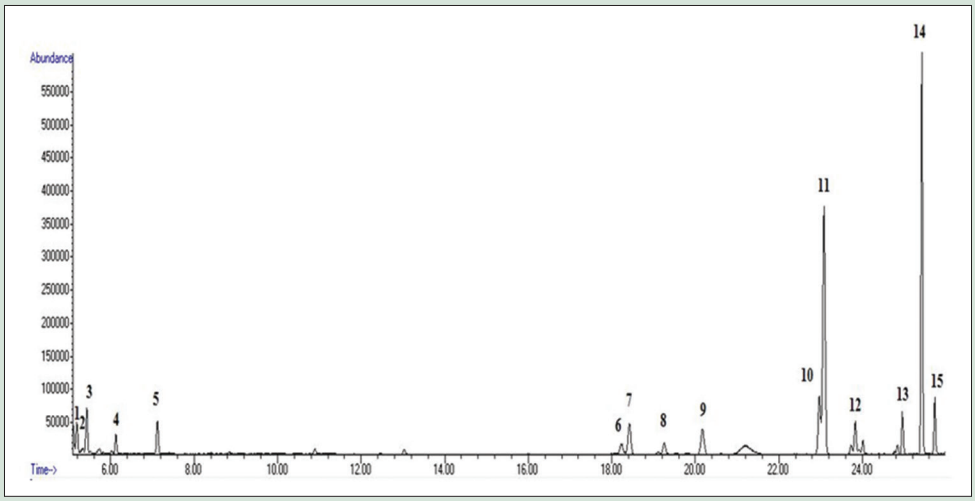
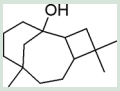
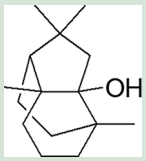


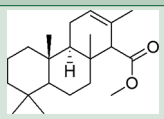
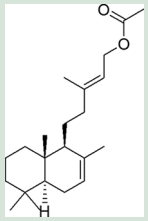
Figure 1: Chromatogram of total ions obtained by gas chromatography coupled to mass spectrometry of *Hymenaea stigonocarpa* resin. Numbered according to table 01

Table 1: Compounds detected in *Hymenaea stigonocarpa* resin by gas chromatography–mass spectrometry analysis

N°	RT	Área (%)	Compounds	MW	SM	SF	CI (m/z)	Reference
1	5,10	0.92	Caryophyllene alcohol	222	$C_{15}H_{26}O$		205 (4), 204 (7), 151 (22), 149 (15), 137 (12), 135 (11), 111 (100), 95 (29), 93 (32), 81 (44), 69 (30), 41 (41)	NIST
2	5,19	1.27	-	-	-	-	221 (6), 204 (5), 193 (23), 161 (11), 137 (10), 125 (100), 123 (10), 81 (11), 55 (11), 41 (14)	-
3	5,4	3.69	Unknown	-	-	-	213 (3), 193 (19), 163 (24), 161 (28), 150 (82), 149 (82), 135 (39), 125 (100), 121 (34), 108 (27), 107 (45), 105 (24), 95 (44), 93 (58), 91 (40), 81 (54), 79 (49), 69 (34), 67 (35), 55 (33), 41 (52)	-
4	6,12	1.62	Ginsenol	222	$C_{15}H_{26}O$		M^+222 (5), 207 (100), 184 (28), 184 (28), 123 (23), 109 (16), 95 (19), 93 (13), 91 (11), 41 (23)	NIST
5	7,1	2.70	Labdano unknown	-	-	-	240 (6), 205 (29), 204 (22), 186 (24), 184 (69), 169 (56), 161 (23), 149 (100), 141 (37), 133 (28), 123 (39), 107 (35), 93 (37), 91 (41), 79 (34)	Chiavari G. *et al.*, 1995
6	18,2	1.77	Labdano unknown	320	-	-	M^+320 (5), 192 (15), 191 (100), 135 (11), 212 (8), 109 (8), 105 (7), 95 (10), 91 (8), 69 (9)	Chiavari, G. *et al.*, 1995; Doménech-Carbó, *et al.*, 2009
7	18,4	4.29	Labdano unknown	320	-	-	M^+320 (2), 192 (14), 191 (100), 135 (10), 121 (7), 109 (7), 107 (7), 105 (5), 95 (8), 70 (8), 55 (6)	Chiavari, G. *et al.*, 1995; Doménech-Carbó, *et al.*, 2009
8	19,2	1.40	-		-	-	229 (3), 192 (17), 191 (100), 135 (14), 121 (9), 107 (6), 105 (6), 95 (9), 69 (10), 55 (9)	Chiavari, G. *et al.*, 1995
9	20,1	3.89	Labdano unknown	320	-	-	M^+320 (13), 305 (22), 196 (37), 192 (11), 191 (81), 177 (15), 149 (20), 135 (20), 124 (26), 123 (30), 122 (85), 121 (41), 109 (100), 107 (46), 105 (25), 95 (49), 91 (33), 69 (39), 55 (38)	Doménech-Carbó, *et al.*, 2009
10	22,9	6.80	Labdano unknown	320	-	-	M^+320 (13), 305 (33), 192 (17), 191 (100), 149 (19), 135 (21), 122 (15), 121 (38), 109 (28), 105 (17), 95 (49), 93 (18), 91 (20), 83 (12), 81 (20), 69 (30), 55 (20)	Chiavari, G. *et al.*, 1995; Doménech-Carbó, *et al.*, 2009
11	23,03	29.25	Labdano unknown	320	-	-	M^+320 (14), 264 (6), 191 (100), 177 (13), 163 (15), 149 (21), 135 (24), 123 (17), 122 (15), 121 (38), 119 (13), 109 (27), 107 (24), 105 (17), 95 (48), 93 (20), 91 (17), 81 (18), 69 (27), 67 (11), 55 (21), 41 (18)	Chiavari, G. *et al.*, 1995; Doménech-Carbó, *et al.*, 2009
12	23,8	3.30	Labdano unknown	320	-	-	M^+320 (12), 305 (23), 196 (25), 192 (11), 191 (82), 149 (16), 136 (18), 123 (38), 122 (100), 121 (27), 109 (100), 107 (40), 105 (22), 95 (46), 81 (36), 69 (36), 55 (31)	Doménech-Carbó, *et al.*, 2009

Contd...

Table 1: Contd...

N°	RT	Área (%)	Compounds	MW	SM	SF	CI (m/z)	Reference
13	24,9	3.39	Methyl-8,13-dimethylpodocarp -12-ene-14-carboxylate	318	$C_{21}H_{34}O_2$		M^+318 (28), 192 (100), 178 (15), 177 (97), 165 (12), 161 (11), 149 (24), 137 (35), 136 (25), 133 (17), 127 (24), 123 (70), 122 (37), 121 (55), 109 (41), 95 (46), 81 (44), 69 (46), 55 (38)	NIST
14	25,4	31.29	Labdano unknown	318	$C_{21}H_{34}O_2$	-	M^+318 (3), 205 (100), 163 (17), 149 (41), 135 (28), 123 (20), 121 (29), 119 (16), 109 (42), 107 (20), 95 (32), 69 (24), 55 (19)	Doménech-Carbó, *et al.*, 2009
15	25,7	4.34	Labd-7,13-dien-15-ol, acetate	332	$C_{22}H_{36}O_2$		204 (100), 189 (11), 161 (17), 147 (14), 135 (51), 124 (19), 114 (20), 109 (99), 95 (20), 93 (23), 81 (67), 41 (19)	NIST

RT: Retention time; MW: Molar weight; SM: Structural molecular; SF: Structural formula; CI: Characteristic ions; NIST: National Institute of Standards and Technology

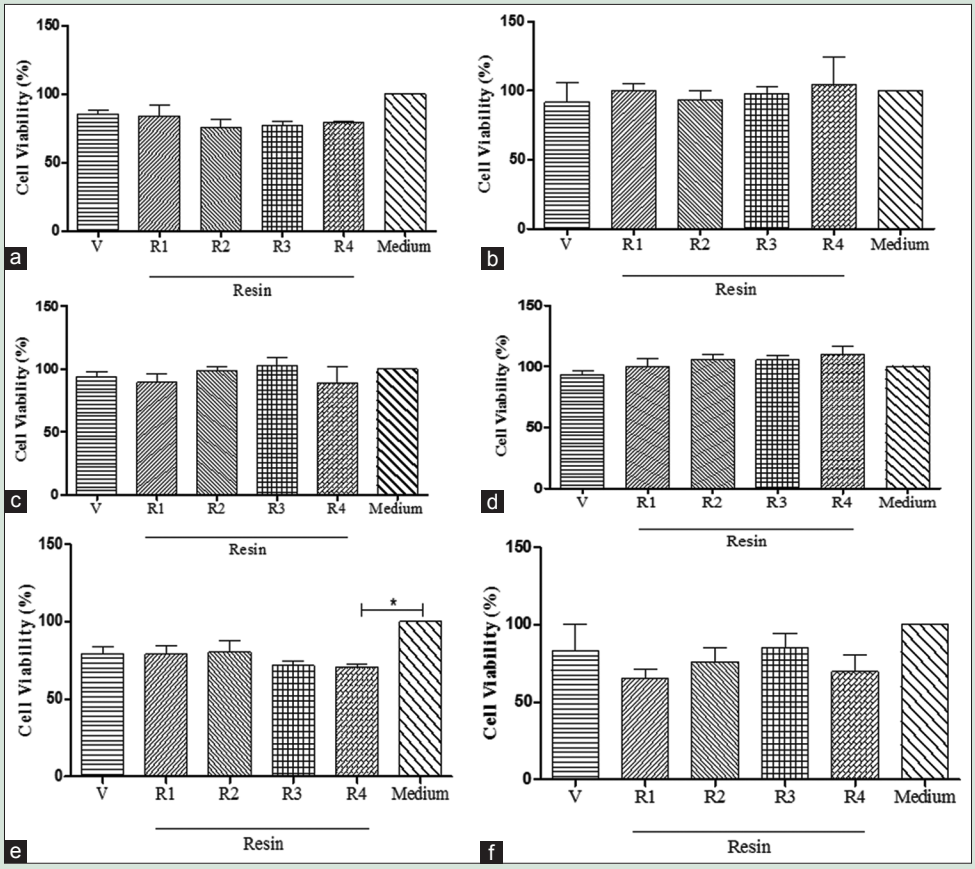


Figure 2: (3-[4,5-dimethylthiazol-2-yl]–tetrazolium 2,5-diphenyl-bromide) colorimetric assay with NIH-3T3 fibroblasts, HaCat, and keratinocytes.Percentage of viability of NIH-3T3 fibroblasts on culture at 24 h and 48h (a and b), HaCat immortalized keratinocytes on culture at 24 h and 48h (c and d) and human keratinocytes (KC) on culture at 24 h and 48h (e and f) relative to the control (culture medium – corresponding to 100% viable cells) by MTT colorimetric assay. Medium: Culture medium for culturing the cells. V: Ethanol 0,01% + Propylene glycol 0,09%. R1, R2, R3, R4: resin at concentrations of 0.001%, 0.0001%, 0.00001% and 0.000001%, respectively. One-way ANOVA followed by Bonferroni test ($P < 0.05$)

doses analyzed, promoted increased cell migration compared to the control and thus ameliorated fibrosis and showed wound-healing activity. Terpenes have excellent healing action. The process of wound reepithelialization consists of estimating the production of matrix

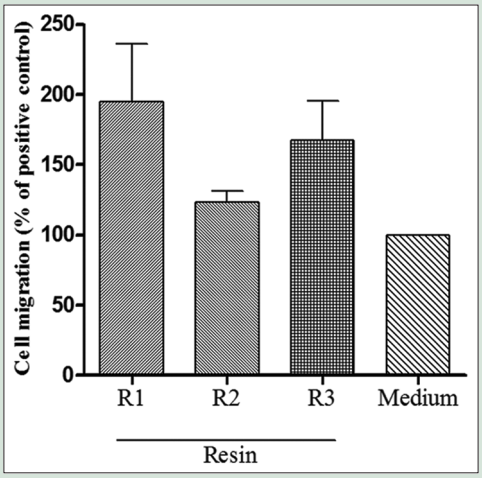


Figure 3: Proliferative/migratory activities of NIH-3T3 fibroblasts in the scratch assay. Effect of preparations from *Hymenaea stigonocarpa* resin on the migratory activities of NIH-3T3 fibroblasts in the scratch assay after 24 h of incubation. Basal is the culture medium supplemented with fetal bovine serum 10%. R1, R2, R3 (Culture Medium with the resins in concentrations 0.001%, 0.0001% and 0.00001%, respectively). The results are expressed in comparison to the basal and analyze the percentage of the number of cells in the wound area. They are represented by the media ±S.E.M of three experiences. One-way ANOVA followed by Bonferroni test ($P < 0.05$)

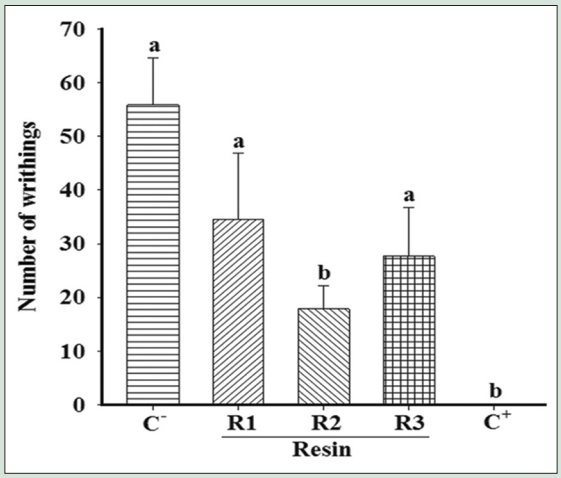


Figure 5: Acetic acid-induced writhing assay. .Effect of resin of *Hymenaea stigonocarpa* on the acetic acid-induced writhing assay. C⁻ (Propylene glycol vehicle), C⁺ (Indometacin 5 mg/kg), R1; R2; R3 (resin at 25, 50 and 100 mg/kg, respectively). Each point represents the average ± S.E.M (n = 6) of the total writhing number in 20 min for the different dosages. The data were analyzed by One-way ANOVA followed by Tukey's test ($P < 0.01$)

proteins in fibroblasts, including fibronectin, collagen, and hyaluronic acid, in addition to the synthesis of collagenases.[12]

Effect of preparations from *H. stigonocarpa* resin on the migratory activities of NIH-3T3 fibroblasts in the scratch assay after 24 h of incubation. Basal is the culture medium supplemented with fetal bovine serum 10%. R1, R2, R3 (culture medium with the resins in concentrations at 0.001%, 0.0001%, and 0.00001%, respectively). The results are expressed in comparison to the basal and analyzed the percentage of the number of cells in the wound area. They are represented by the media ± standard error of the mean (SEM)

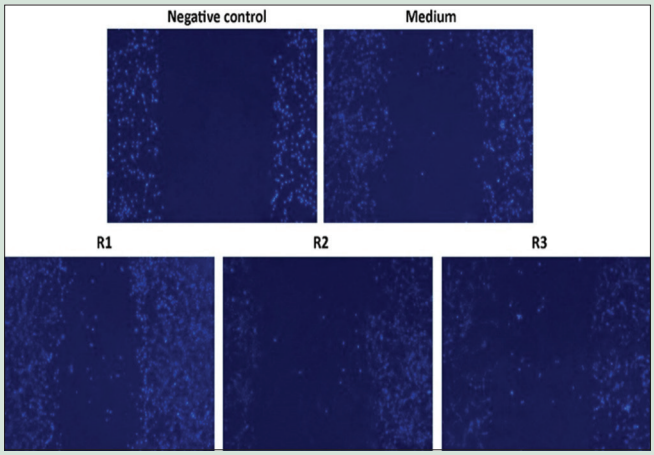


Figure 4: Microscopic fluorescent image for scratch assay .The image quantified by ImageJ Software 1.46r. Evaluates the healing process *in vitro*. A confluent monolayer of NIH-3T3 fibroblasts is used. The results are expressed as a reduction in the width of the denuded region. Basal (medium supplemented with fetal bovine serum 10%). Negative control (basal culture medium with 50% DMSO). R1, R2, R3 (Culture medium with the resins in concentrations 0.001%, 0.0001% and 0.00001%, respectively). Data are expressed compared to the basal

of three experiences. One-way ANOVA followed by Bonferroni test ($P < 0.05$).

Figure 4 shows the process of migration for each analyzed dose along with the positive and negative control. The results are expressed as a reduction in the width of the denuded region, as already proposed by Fronza *et al.*[12]

The image quantified by Image J Software 1.46r evaluates the healing process *in vitro.* A confluent monolayer of NIH-3T3 fibroblasts is used. The results are expressed as a reduction in the width of the denuded region. Basal (medium supplemented with fetal bovine serum 10%). Negative control (basal culture medium with 50% DMSO). R1, R2, R3 (Culture medium with the resins in concentrations 0.001%, 0.0001% and 0.00001%, respectively). Data are expressed compared to the basal.

Abdominal contortions induced by acetic acid

The antinociceptive activity was evaluated using the test of abdominal contortions induced by the intraperitoneal injection of acetic acid. The number of abdominal contortions by mice as a result of the peritoneal irritation was used to compare the antinociceptive activity.[19,24,25] The resin inhibited the number of contortions by 38.21%, 68.06%, and 50.45% at 25, 50, and 100 mg/kg, respectively. Indomethacin which is used as a positive control presented 100% inhibition of the nociceptive effect ($P < 0.01$) [Figure 5]. For better characterization of the antinociceptive action, formalin was injected into the hind paw of the mice.

Effect of resin of *H. stigonocarpa* on the acetic acid-induced writhing assay. C⁻(Propylene glycol vehicle), C⁺ (Indometacin 5 mg/kg), R1; R2; R3 (resin at 25, 50, and 100 mg/kg, respectively). Each point represents the average ± SEM ($n = 6$) of the total writhing number in 20 min for the different dosages. The data were analyzed by one-way ANOVA followed by Tukey's test ($P < 0.01$).

Formalin test

The average time taken by the mice to lick or bite the paw was calculated. For phase 1, the mean time was as follows: vehicle (54.310 s), resin

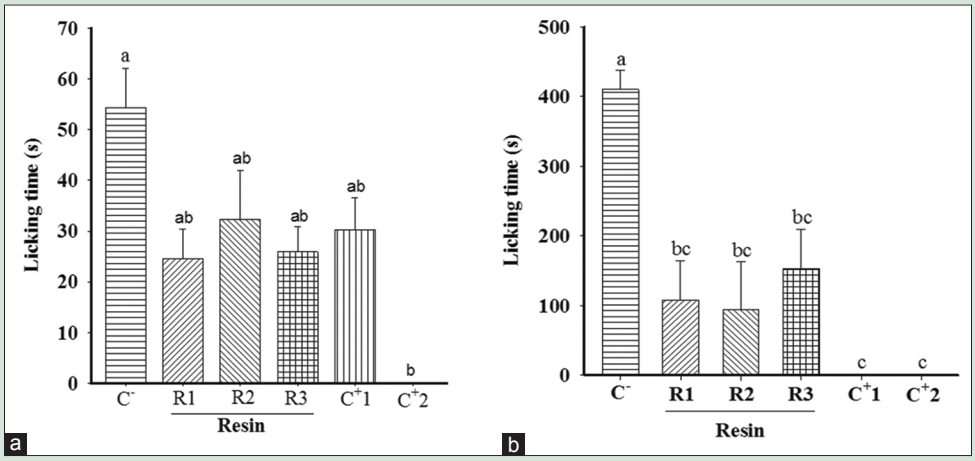


Figure 6: Formalin test in mice for neurogenic phase and inflammatory pain. Effect of resin of *Hymenaea stigonocarpa* in neurogenic phase (0–5 min) (a) and inflammatory pain (15–30 min) (b) of the formalin test in mice. C^- (Arabica gum 4% in propylene glycol vehicle), C^+1 (Indometacin 5 mg/kg), C^+2 (Meperidine 25 mg/kg), R1; R2; R3 (resin at 25, 50 and 100 mg/kg, respectively). The parameter of analysis is established by the licks in the hind paw where the formalin was applied. Values are expressed as a mean ± SEM ($n = 6$) of the pain reaction times in the neurogenic phase (a) and inflammatory pain (b). The data were analyzed by One-way ANOVA followed by Tukey's test ($P < 0.01$)

100 mg/kg (25.892 s), resin 50 mg/kg (32.318 s), resin 25 mg/kg (24.495 s), indomethacin 5 mg/kg (30.293 s), and meperidine (0 s) ($P < 0.01$) as demonstrated in Figure 6a. The resin 50 mg/kg group showed results comparable to those for indomethacin, used as the positive control. This phase (Phase 1) of the experiment is characterized by neurogenic pain (acute pain) that results after formalin injection and lasts for the first 5 min. It can be inferred that the resins at concentrations of 100 and 25 mg/kg showed better action against neurogenic pain, probably by attenuating the chemical stimulation of type C and type Aδ afferent fibers and limiting the release of nitric oxide, substance P, and other excitatory amino acids.[24,26,27] In phase 2 of the experiment, the average time in seconds was as follows: vehicle (410.27 s), resin 100 mg/kg (153.17 s), resin 50 mg/kg (93.72 s), resin 25 mg/kg (107.48 s), indomethacin (0 s), and meperidine (0 s) ($P < 0.01$) as shown in Figure 6b. The resin at a concentration of 50 mg/kg showed better action against the inflammatory pain phase. In phase 2, proinflammatory mediators such as bradykinin, prostaglandins, and serotonin among others are released.[14,19] While meperidine blocked both the phase 1 and phase 2 pathways, indomethacin was only active against the phase 2 pathway.

Effect of resin of *H. stigonocarpa* in neurogenic phase (0–5 min) (A) and inflammatory pain (15–30 min) (B) of the formalin test in mice. C-(Arabica gum 4% in propylene glycol vehicle), C + 1 (Indometacin 5 mg/kg), C + 2 (Meperidine 25 mg/kg), R1; R2; R3 (resin at 25, 50 and 100 mg/kg, respectively). The parameter of analysis is established by the licks in the hind paw where the formalin was applied. Values are expressed as a mean ± SEM ($n = 6$) of the pain reaction times in the neurogenic phase (A) and inflammatory pain (B). The data were analyzed by one-way ANOVA followed by Tukey's test ($P < 0.01$).

CONCLUSION

The results obtained demonstrate pharmacological actions for the use of *H. stigonocarpa* resin; however, additional studies are necessary to complete elucidation of the chemical composition of the species, as well, as clarifying the mechanism of action for the pharmacological activities observed.

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Conflict of interest

There are no conflicts of interest.

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