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Development of a Topical Cream from the Ethanolic of *Agave sisalana* Residues with Anti-Inflammatory and Analgesic Properties

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Abstract: Brazil is the largest producer in the world of the species *Agave sisalana*, sisal. The residue of the sisal, which is the result of the extraction of fibers from its leaves, represents 95% of its weight. Considering that sisal leaves have high concentrations of sapogenins and aiming at a future phytotherapeutic, in this study, the alcoholic fraction of sisal, AFS, was developed, and the sapogenins were characterized. In vitro, the cytotoxicity (MTT) and the anti-inflammatory effect of AFS (phagocytosis and hemolysis inhibition) were evaluated. In vivo, the analgesic (formalin test—FT) and anti-inflammatory (paw edema test—PET) activities of AFS, orally, and the cream containing AFS, topical, were analyzed. The results demonstrated that AFS contains hecogenin and tigogenin and is not cytotoxic. In vitro, 0.5, 1, and 2 mg/mL of AFS showed anti-inflammatory activity similar to the positive control (PC). In the FT, the dose of 25 mg/kg did not differ from the PC in the neurogenic phase ($p > 0.05$). In the PET, 25 and 50 mg/kg of AFS differed from the negative control (NC) ($p < 0.05$), and the cream with AFS (5 mg/g) showed activity similar to the PC. The therapeutic activities of AFS probably result from sapogenins. In the future, we expect to develop an anti-inflammatory from the thousands of tons of sisal waste discarded in Brazil.

Keywords: sisal; phytotherapy; sustainability; bioeconomy

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1. Introduction

Inflammatory diseases represent a significant global health issue, leading to growing interest in related research areas. The inflammatory process is the body's response to tissue injuries caused by physical or ischemic lesions, infections, toxin exposure, or other traumas. The symptoms of inflammation are characterized by five pillars: pain, heat, redness, swelling, and loss of function [1]. This process involves cellular changes accompanied by an immune response aimed at repairing damaged tissue [2].

The most common treatments for exaggerated and inappropriate inflammatory response include the administration of non-steroidal agents and/or glucocorticoids [3]. However, their prolonged use is often met with resistance due to associated side effects, such as

renal and physiological homeostasis dysfunction, which can exacerbate pain sensations in patients [4–6]. Therefore, there is a need for the development of new, safe, and effective therapeutic options for the treatment of inflammatory diseases [7].

Recently, there has been a growing interest in alternative therapeutic options, particularly natural bioactive compounds derived from plant extracts [8]. One example is *Agave sisalana* (sisal), which is rich in sapogenins, an important group of secondary plant metabolites that are widely used based on empirical knowledge for treating certain pathologies, such as asthma, tuberculosis, ulcers, cancer, and inflammation, and also help in wound healing and improving memory [7,9–17].

The presence of sapogenins hecogenin and tigogenin has already been found in sisal [15]. However, there are few studies on the therapeutic effects of sisal and its sapogenins in inflammation and induced-pain models for the development of a novel phytotherapeutic [18,19]. Brazil is the world's largest producer of sisal and its hard fibers extracted from the leaves, with fiber extraction yielding 5% of hard fibers from sisal leaves. The remaining residue, consisting of mucilage (solid fraction) and juice (liquid fraction), is typically discarded. Building on a study evaluating the anti-inflammatory activity of hydrolyzed sisal juice [7], this research aims to enhance this activity and develop a future topical phytotherapeutic cream formulation. Specifically, this study focuses on developing the alcoholic fraction of sisal (AFS) to characterize its sapogenins. Additionally, it aims to evaluate in vitro the cytotoxicity (MTT assay) and anti-inflammatory effects (phagocytosis and hemolysis inhibition tests), as well as in vivo analgesic effects (formalin test) and acute and chronic anti-inflammatory effects (paw edema test). Finally, it aims to formulate a cream containing the alcoholic fraction of sisal and evaluate its in vitro acute and chronic anti-inflammatory effects (paw edema test).

This study aims to add economic value to a wasted residue, which will certainly reflect on the social and health aspects of the population that depend exclusively on sisal for its subsistence.

2. Materials and Methods

2.1. Animals

Wistar rats (*Rattus norvegicus*), 10 weeks old, were used in the in vivo experiments. The animals were housed in the vivarium of the Faculty of Arts and Sciences of Assis—UNESP under controlled conditions of temperature (22 ± 2 °C) and light cycle (12 h of light and 12 h of darkness). Food and water were provided. The experimental protocol was approved by the Ethics Committee on the Use of Animals (CEUA), under Process 0022014.

2.2. Human Blood Cells

Peripheral blood samples (4 mL) were collected from ten individuals by venipuncture. The participants, aged 18 to 25 years, of both sexes, were not taking any medication or toxic substances and were selected according to the research criteria. The Informed Consent Form (ICF) was prepared, and the experimental protocol was approved by the Ethics in Research Committee (ERC) under Process 12322010.

2.3. Material of Plant Origin

The raw juice of sisal was obtained in the municipality of Valente, Bahia, Brazil; the species under study was identified as *Agave sisalana* in the Herbarium Assisense of the State University of São Paulo, Assis, São Paulo, where a voucher specimen was deposited under the number 2597.

2.4. Preparation of the Ethanolic Fraction of Agave sisalana (AFS)

The preparation of the AFS was followed as proposed by Barreto et al. 2017, with modifications. The crude juice was centrifuged (Daiki, model 80-2B, model 803, São Paulo, SP, Brazil) at 18 °C and 4000 rpm for 20 min. After this process, the precipitate was separated and dried. Afterwards, 10 g of the precipitate were dissolved in 100 mL of

95% hydrated ethanol (Anidrol, Diadema, SP, Brazil). The solution was stirred for 30 min, filtered, and then 95% of the ethanol was removed by distillation, and the alcoholic fraction of sisal (AFS) was lyophilized (Figure 1).

Figure 1. Preparation of the ethanolic fraction of *Agave sisalana* (AFS).

2.5. Phytochemical Screening by Mass Spectrometry of the AFS

A stock solution of AFS (10 mg/mL, Merck, Darmstadt, Germany) was prepared in methanol and diluted 100-fold in methanol/water (1:1 v/v) with 0.1% formic acid (99%, Exodus, São Paulo, Brazil). The sample was directly injected via syringe pump into an ESI-Orbitrap mass spectrometer (Q Exactive, Thermo Scientific, Bremen, Germany). Spectra were acquired in positive ion mode, with full scan (150–1800 m/z) and MS/MS (50 m/z to above the target ion) using collision energies of 10–40 eV. Instrument settings included a 3500 V spray voltage, 320 °C capillary temperature, 10 psi sheath gas pressure, and 50 V S-Lens RF level. Data were analyzed using Xcalibur software 4.0 (Thermo Scientific, Germany).

2.6. Determination of In Vitro Cytotoxicity of the AFS by the MTT Assay [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide]

The MTT cytotoxicity assay was determined following Kumar [20], with some modifications. For this assay, Peripheral Blood Mononuclear Cells (PBMCs) were inoculated in 96-well microtiter plates and incubated in a culture medium for 24 h at 37 °C, under 5% carbon dioxide (CO_2). These cells were exposed to the three different concentrations of the AFS (0.5, 1 and 2 mg/mL). For the negative control (NC), the AFS was replaced with a physiological solution, and for the positive control (PC), it was replaced with 2% (v/v) Tween® 80. Three incubations points were used, 24, 48, and 72 h.

2.7. Determination of In Vitro Anti-Inflammatory Activity of the AFS

2.7.1. Treatments

The AFS was prepared in distilled water at the following concentrations: 0.5, 1, and 2 mg/mL. For the PC, the AFS was replaced with dexamethasone (0.1 mg/mL), and for the NC, a saline solution was used. In the hemolysis stabilization test, the saline solution was replaced by a 0.18% hyposaline solution (NaCl, Exodus, São Paulo, Brazil) to induce hemolysis.

2.7.2. Phagocytosis

The method described by Fracasso et al. 2023 [11] was used, with few modifications. The percentage of inhibition of phagocytosis (IP) was calculated using Equation (1):

$$\text{IP (\%)} = (E0 - ET)/E0 \times 100 \quad (1)$$

where E0 represents the mean value of the number of cells in the negative control group that phagocytosed the Zymosan particles, and ET represents the mean value of the number of cells of the treated groups that phagocytosed the Zymosan particles.

2.7.3. Induction of Hemolysis by Hypotonic Solution

For the evaluation of the human red blood cell (HRBC) membrane stabilization, the method proposed by Singh et al., 2020 [21] was employed, with some modifications.

The protection against hemolysis was calculated using Equation (2):

$$\text{\% Protection} = (E0 - ET)/E0 \times 100 \quad (2)$$

where E0 represents the mean absorbance value of the negative control group, and ET represents the mean absorbance value in the treated groups.

2.8. Determination of In Vivo Analgesic Activity of the AFS by the Formalin-Induced Method

As described by Demsie et al. [22], this method consists of an intraplantar injection of a 2.5% formalin solution into the left hind paw of Wistar males (n = 6) before treatments.

The inhibition percentage of nociceptive response was calculated using Equation (3):

$$\text{\% Inhibition} = (E0 - ET)/E0 \times 100 \quad (3)$$

where E0 represents the mean value of duration of licking paw observed in the control group, and ET represents the mean value of duration of licking paw observed in the treated groups.

2.9. Determination of In Vivo Anti-Inflammatory Activity of the AFS by the Carrageenan-Induced Paw Edema Method

The carrageenan-induced paw edema method was performed according to Winter et al., 1962 [23]. Male Wistar rats (n = 6), at the beginning of the treatment, were randomly divided into five experimental groups (n = 6/group): negative control, in which the animals were treated with distilled water (5 mL/kg body weight); positive control, in which the animals were treated with dexamethasone (Merck®, Rahway, NJ, USA), administered intraperitoneally at a dose of 5 mg/kg body weight; and AFS/25, AFS/50, and AFS/100, in which the animals were treated with the AFS (25, 50, and 100 mg/kg, respectively).

The evaluation of edema inhibition always took place in the following hours: 1, 2, 4, 6, 24, 48, 76, and 96 h after the first carrageenan administration. The percentage of inhibition was calculated using Equation (4):

$$\text{\% Inhibition} = (E0 - ET)/E0 \times 100 \quad (4)$$

where E0 is the mean value of paw edema observed in the control group, and ET is the mean value of paw edema observed in the treated groups.

After evaluation, the rats of each experimental group were induced to death through the use of an excessive dose of the anesthetic thiopental (Thiopentax®, São Paulo, Brazil).

2.10. Development of the Cream Formulation Containing AFS

The formulation, consisting of two phases, was developed by researchers from the Pharmaceutical Technology Laboratory in Phytoproducts, São Paulo State University (UNESP):

Phase A (Oily Phase): Paramul J wax—10%; Isopropyl myristate—5%; Liquid paraffin—4%; Nipazol—0.05%.

Phase B (Aqueous Phase): Propylene glycol—5%; Nipagin—0.1%; Water—Quantity sufficient to 100 g.

For the preparation of the formulation, phases A and B were heated, and the temperature was monitored with a thermometer to not exceed 85 °C. Once they reached the same temperature and the waxes were completely melted, phase A was poured onto phase B. The mixture was then removed from heat. Subsequently, the mixture was homogenized thoroughly until an emulsion was formed uniformly.

In order to compare the topical anti-inflammatory activity of dexamethasone and AFS, both were incorporated into formulations at the same concentration of 5 mg per gram of cream.

2.11. Determination of the Anti-Inflammatory Activity of the Cream Formulation Containing AFS

In this evaluation, the paw edema test using carrageenan was employed [23]. The experimental protocol followed the same criteria established for oral administration of AFS. However, in this stage, the anti-inflammatory effect of the cream containing AFS was assessed after the application of 50 mg of the formulation topically, at a concentration of 5 mg/g. The cream was rubbed onto the paw for 30 s. The same procedure was conducted with the negative control group treated with the base cream and the positive control group treated with the cream containing dexamethasone, 5 mg/g.

2.12. Statistical Analysis

The data of the in vitro experiments were expressed in terms of mean ± standard deviation. Statistical analysis was performed using BioEstat® (version 5.0) software (Brazil). The figures were made using © 2024 GraphPad Software (Boston, MA, USA). To verify the statistical differences between the groups, a one-way analysis of variance (ANOVA) was performed according to the experimental protocol, followed by Tukey's multiple comparison test. For all analyzes, a p-value of <0.05 was considered significant.

3. Results

3.1. Phytochemical Screening

The AFS HRESI-MS spectrum obtained in positive mode allowed for the detection of 21 peaks (Figure 2A), 18 of which were identified according to the Massbank database (available in http://www.massbank.jp, Acessed on 12 April 2024). The two major peaks were attributed to the sapo-genins, hecogenin and tigogenin (Figure 2B, Table 1).

Table 1. HRESI-MS data of the two sapogenins, hecogenin and tigogenin, present in the AFS.

Compound	Molecular Formula	Calculated [M + H] +	Experimental [M + H] +	Error [PPT]
Tigogenin	$C_{27}H_{45}O_3$	417.33687	417.33573	1.41
Hecogenin	$C_{27}H_{43}O_4$	431.31613	431.31545	0.31

(A)

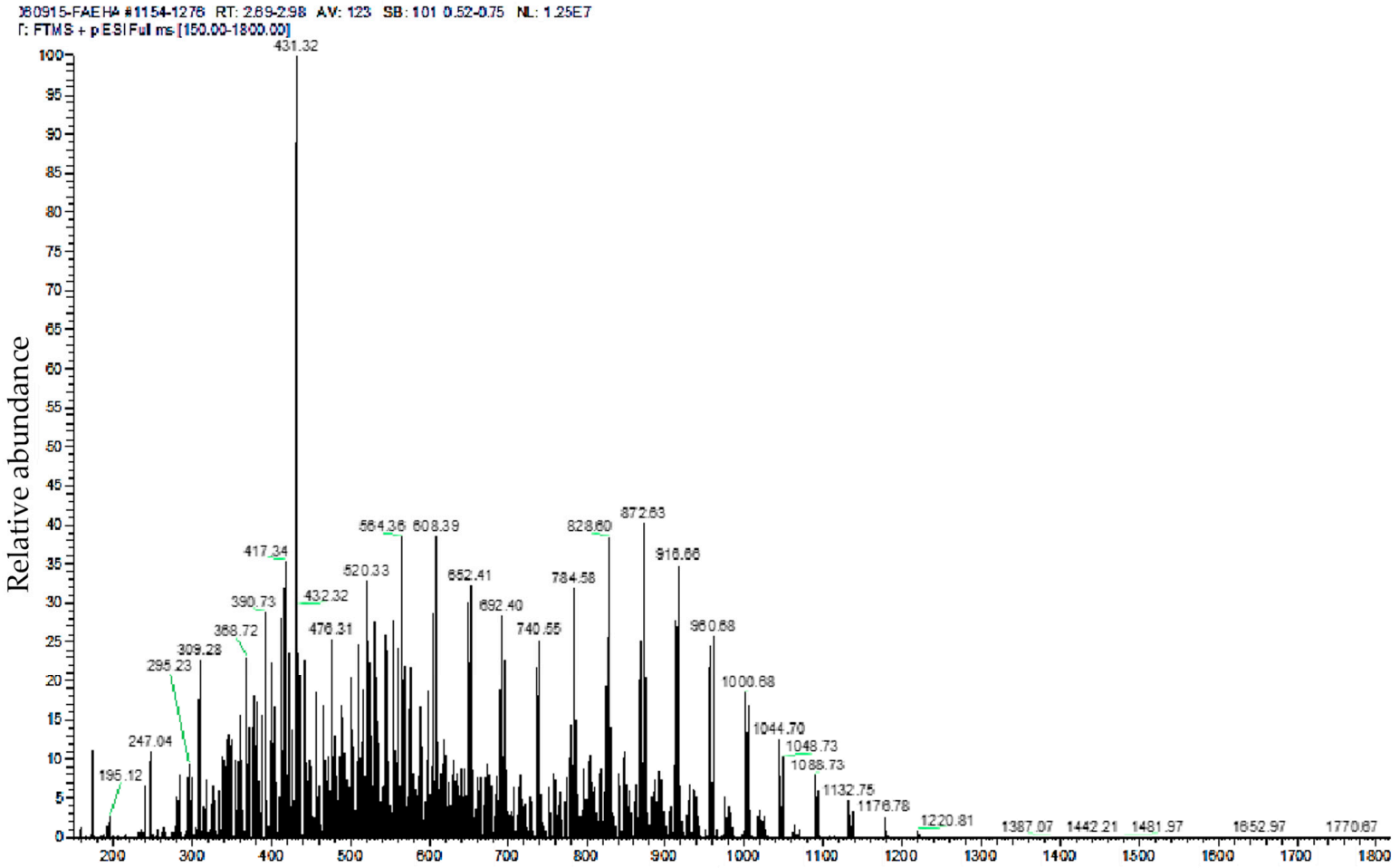

Figure 2. *Cont.*

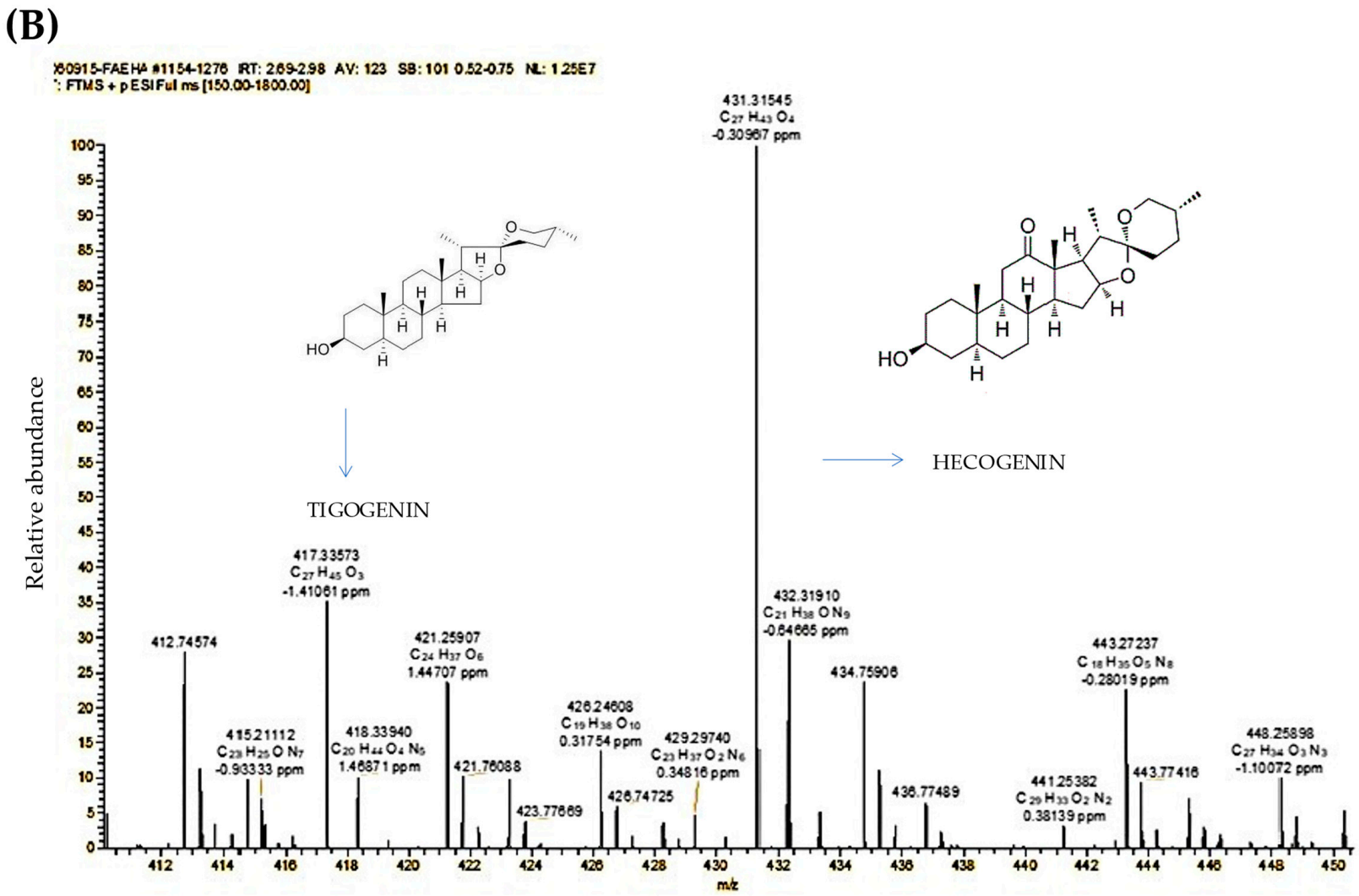

Figure 2. (**A**) Absorption spectrum obtained in the analysis of the AFS by high-resolution mass spectrometry (HRESI-MS). (**B**) Identified peaks belonging to hecogenin and tigogenin.

3.2. Cytotoxicity

The effect of the AFS on PBMCs viability is presented in Figure 3. Only the AFS concentration of 2 mg/mL was able to decrease cell viability below 100% in all incubation points, being more accentuated after 24 h of incubation (70 ± 0.70%) than after 48 and 72 h (cell viability of 89 ± 0.44% and 92 ± 3.48%, respectively), but still above the values attained for the positive control.

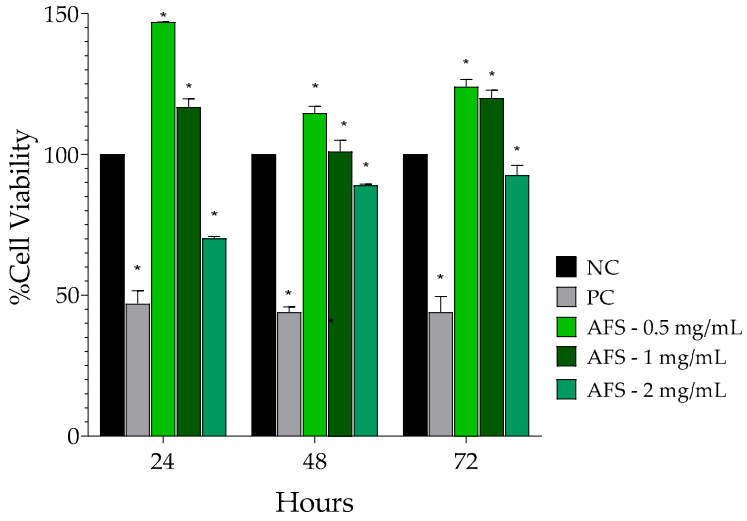

Figure 3. Cell viability after 24, 48, and 72 h of incubation with physiological solution (NC, negative control), Tween® 80 (PC, positive control), and AFS (0.5, 1, and 2 mg/mL) by the MTT method. Results are expressed as mean ± SD. One-way ANOVA followed by Tukey's post hoc test was performed — the asterisk (*) indicates significant differences ($p < 0.05$) compared to the NC.

3.3. Determination of the In Vitro Anti-Inflammatory Activity of the AFS

3.3.1. Phagocytosis

The positive control (dexamethasone) inhibited 45.00 ± 6.22% of phagocytosis, and all three AFS concentrations were able to reach higher values, ranging from 56.00 ± 5.83% (0.5 mg/mL) to 64 ± 1.88% (2 mg/mL), Figure 4.

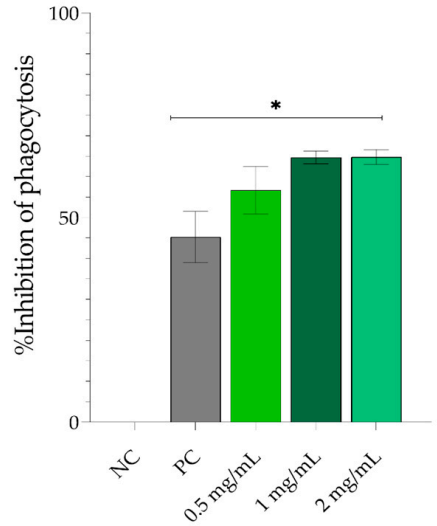

Figure 4. Phagocytosis inhibition after treatment with physiologic solution 0.9% (NC, negative control), dexamethasone (PC, positive control, 0.05 mg/mL), and the AFS (0.5, 1, and 2 mg/mL). Results are expressed as mean ± SD. One-way ANOVA, followed by Tukey's post hoc test, was performed—the asterisk (*) indicates significant differences ($p < 0.05$) compared to the NC.

3.3.2. Membrane Stabilization

Table 2 presents the percentage of protection against erythrocyte membrane hemolysis induced by three AFS concentrations (0.5, 1, and 2 mg/mL). All were able to reach about 98% protection, a value very close to the positive control dexamethasone (99%).

Table 2. Protection against hemolysis (%) induced by each treatment group: dexamethasone (PC, positive control, 0.05 mg/mL) and AFS (0.5, 1, and 2 mg/mL). Results are expressed as mean ± SD. The letters presented after each value indicate whether there were significant differences between the different concentrations ($p < 0.05$). Same letter—no significant difference. Different letter—significant difference. Results are expressed as mean ± SD. One-way ANOVA, followed by Tukey's post hoc test, was performed.

Treatment	% Protection
PC	99.07 ± 0.18 [a]
AFS—0.5 mg/mL	97.53 ± 0.07 [a]
AFS—1 mg/mL	97.88 ± 0.09 [a]
AFS—2 mg/mL	98.47 ± 0.02 [a]

3.4. Analgesic Activity

In neurogenic phase, only the AFS 25 mg/kg and the PC differed significantly when compared to the NC ($p < 0.05$). In the inflammatory phase, all treatments differed significantly when compared to the NC ($p < 0.05$), Figure 5.

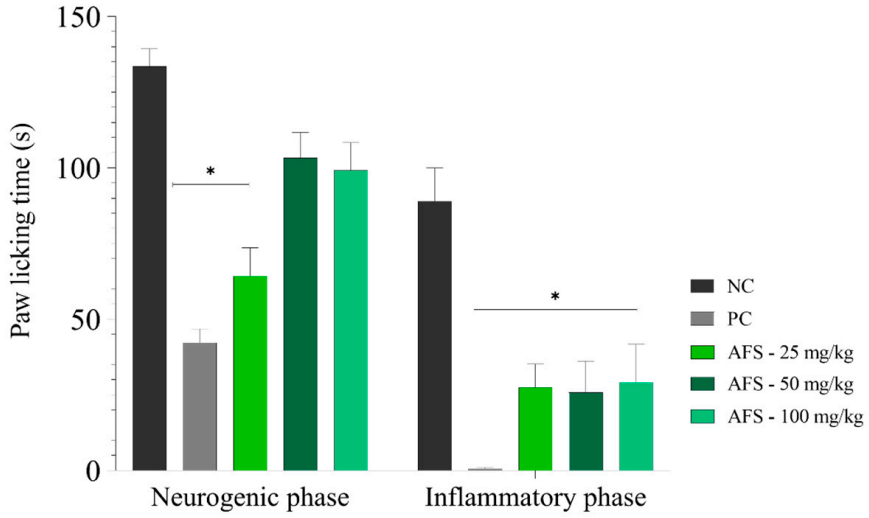

Figure 5. Duration (seconds) of licking paw after gavage administration of saline solution (NC, negative control), Tramal (PC, positive control, 5 mg/kg), and the AFS (25, 50 and 100 mg/kg). Phase I (acute phase-neurogenic)—the first 5 min after intraplantar formalin injection. Phase II (tonic phase–inflammatory)—15 to 30 min after formalin injection. Results are expressed as mean ± SD. One-way ANOVA, followed by Tukey's post hoc test (n = 6), was performed—the asterisk (*) indicates a significant difference ($p < 0.05$) compared to the NC.

In addition, Table 3 presents the percentages of inhibition of the nociceptive response resulting from the treatments in the two analyzed phases. Only in the neurogenic phase did the AFS at 25 mg/kg not differ from the positive control.

Table 3. Pain inhibition (%) in the neurogenic and inflammatory phases induced by gavage administration of Tramal (PC, positive control, 5 mg/kg) and the AFS (25, 50, and 100 mg/kg). The letters presented after each value indicate whether there were significant differences between the different concentrations ($p < 0.05$). Same letter—no significant difference. Different letter—significant difference. Results are expressed as mean ± SD. One-way ANOVA, followed by Tukey's post hoc test, was performed.

Group/Dose	Neurogenic Phase %	Inflammatory Phase %
PC	68.34 ± 8.27 [a]	99.27 ± 0.52% [a]
AFS—25 mg/kg	51.92 ± 1.12% [a]	67.57 ± 3.70% [b]
AFS—50 mg/kg	38.95 ± 13.24% [b]	69.19 ± 2.33% [b]
AFS—100 mg/kg	39.83 ± 16.86% [b]	67.26 ± 3.72% [b]

3.5. Determination of In Vivo Anti-Inflammatory Activity

The mean value of the produced edema was calculated for all groups, both for the acute (Figure 6) and chronic (Figure 7). In the acute anti-inflammatory phase, the PC and the AFS, in the dose of 50 mg/kg, promoted similar inhibition at times of 1, 2, and 4 h. The AFS, 25 and 50 mg/kg, and the PC differed significantly from the NC ($p < 0.05$) at times of 2, 4, and 6 h. The 100 mg/kg AFS differed significantly from the NC ($p < 0.05$) only at a time of 4 h. In the chronic anti-inflammatory phase, the results show that the AFS, 25 and 50 mg/kg, and the PC significantly reduced edema when compared to NC ($p < 0.05$). Even at times of 72 and 96 h, the AFS, 25 and 50 mg/kg, promoted a greater inhibition of edema than PC.

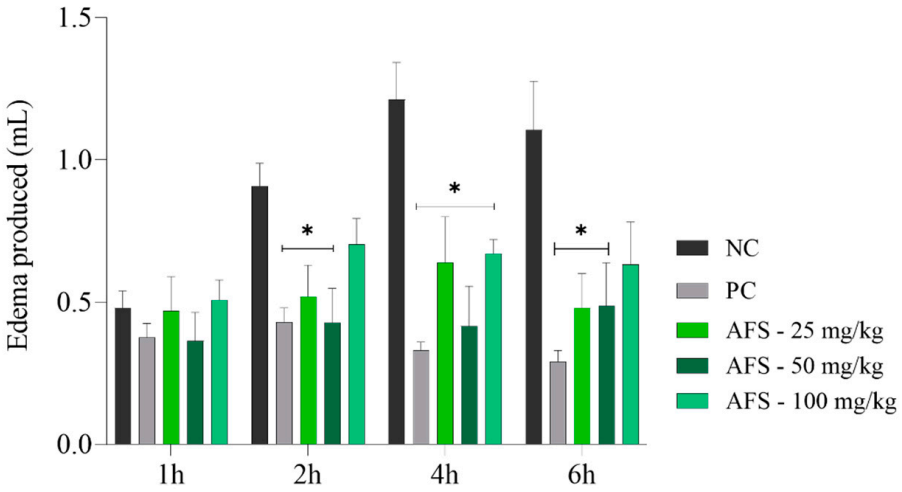

Figure 6. Paw edema (mL) produced in acute inflammatory phase after gavage administration of saline solution (NC, negative control), dexamethasone (PC, positive control, 5 mg/kg) and the AFS (25, 50, and 100 mg/kg). Results are expressed as mean ± SD. One-way ANOVA, followed by Tukey's post hoc test (n = 6), was performed — the asterisk (*) indicates a significant difference ($p < 0.05$) compared to the NC.

In addition, Tables 4 and 5 present the percentages of anti-inflammatory activity resulting from the treatments in the two analyzed phases, acute and chronic. This analysis shows that in both phases of inflammation, the anti-inflammatory activity of AFS at 50 mg/kg and the positive control did not differ from each other at all analyzed time points. However, the AFS at 25 mg/kg exhibited the highest anti-inflammatory activity at 48, 72, and 96 h.

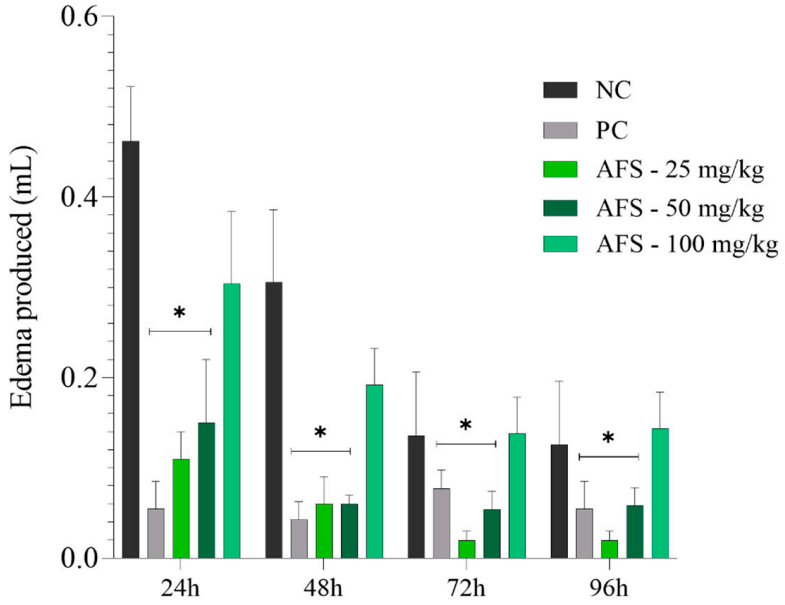

Figure 7. Produced paw edema (mL) for chronic anti-inflammatory activity evaluation after gavage administration of saline solution (NC, negative control), dexamethasone (PC, positive control, 5 mg/kg) and the AFS (25, 50, and 100 mg/kg). Results are expressed as mean ± SD. One-way ANOVA, followed by Tukey's post hoc test (n = 6), was performed—the asterisk (*) indicates a significant difference ($p < 0.05$) compared to the NC.

Table 4. Anti-inflammatory activity in acute inflammatory phase after gavage administration of dexamethasone (PC, positive control, 5 mg/kg) and the AFS (25, 50, and 100 mg/kg). The letters presented after each value indicate whether there were significant differences between the different concentrations ($p < 0.05$). Same letter—no significant difference. Different letter—significant difference. Results are expressed as mean ± SD. One-way ANOVA, followed by Tukey's post hoc test, was performed.

Group/Dose	1 h	2 h	4 h	6 h
PC	21.87 ± 3.02% [a]	52.64 ± 6.79% [a]	72.77 ± 2.28% [a]	73.77 ± 3.95% [a]
AFS—25 mg/kg	2.50 ± 0.88% [b]	49.34 ± 6.79% [a]	63.70 ± 3.66% [a]	62.21 ± 7.24% [a]
AFS—50 mg/kg	28.33 ± 9.8% [a]	59.47 ± 10.80% [a]	74.09 ± 7.49% [a]	51.36 ± 9.15% [a]
AFS—100 mg/kg	5.83 ± 0.71% [b]	22.47 ± 9.51% [c]	44.72 ± 4.18% [b]	42.86 ± 13.56% [b]

Table 5. Anti-inflammatory activity in chronic inflammatory phase after gavage administration of dexamethasone (PC, positive control, 5 mg/kg) and the AFS (25, 50, and 100 mg/kg). The letters presented after each value indicate whether there were significant differences between the different concentrations ($p < 0.05$). Same letter—no significant difference. Different letter—significant difference. Results are expressed as mean ± SD. One-way ANOVA, followed by Tukey's post hoc test, was performed.

Group/Dose	24 h	48 h	72 h	96 h
PC	88.10 ± 6.06% [a]	84.48 ± 5.90% [a]	46.18 ± 6.89% [a]	56.34 ± 9.37% [a]
AFS—25 mg/kg	80.09 ± 4.52% [a]	86.93 ± 5.70% [a]	91.67 ± 1.41% [b]	85.94 ± 4.11% [b]
AFS—50 mg/kg	67.53 ± 5.84% [a]	80.39 ± 3.77% [a]	59.72 ± 3.36% [a]	54.69 ± 2.63% [a]
AFS—100 mg/kg	34.20 ± 7.06% [b]	37.25 ± 3.78% [b]	41,70 ± 5.07% [a]	42.85 ± 3.13% [a]

Figure 8 shows the results obtained with the treatments carried out in the form of a cream, applied topically, to evaluate the anti-inflammatory activity in the acute phase and in the chronic phase of inflammation. The results showed that, when administered topically, at the same concentration, both dexamethasone cream—PC—and AFS cream are

capable of reducing edema in both phases of inflammation and differed significantly when compared to the cream base—NC ($p < 0.05$). However, the AFS cream proved to be more effective in the acute phase and at time times of 24 h, while the dexamethasone cream was more effective at times of 48, 72, and 96 h.

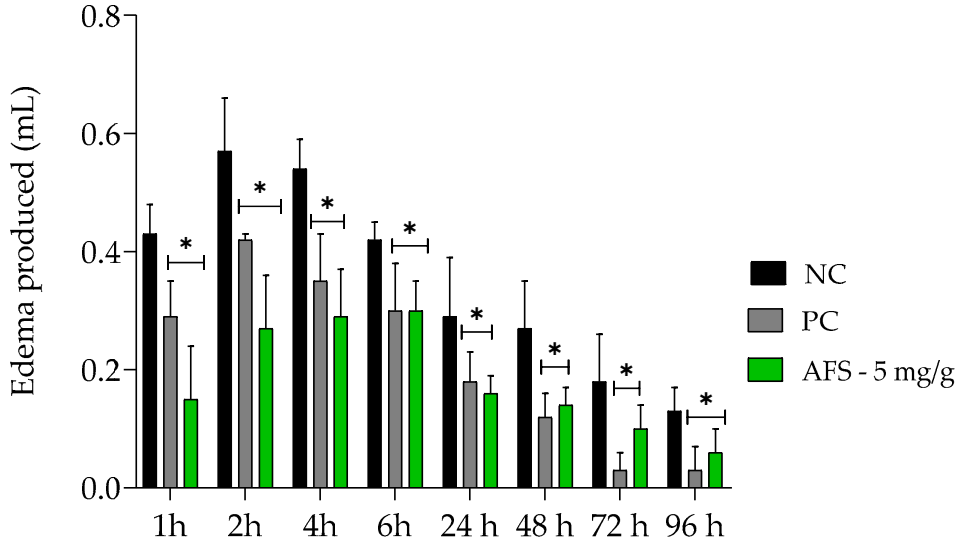

Figure 8. Paw edema inhibition (%) after topical administration of cream base (NC, negative control), dexamethasone cream (PC, positive control, 5 mg/g), and the AFS cream (5 mg/g). Results are expressed as mean ± SD. One-way ANOVA, followed by Tukey's post hoc test (n = 6), was performed — the asterisk (*) indicates a significant difference ($p < 0.05$) compared to the NC.

In addition, Table 6 presents the percentages of anti-inflammatory activity resulting from the treatments with dexamethasone cream (PC, positive control, 5 mg/g) and the AFS cream (5 mg/g) in the two analyzed phases, acute and chronic. This analysis shows that at 1, 2, and 4 h during the acute phase of inflammation, the cream with AFS demonstrated greater anti-inflammatory activity. However, at 72 and 96 h, the cream with dexamethasone exhibited greater anti-inflammatory activity. At other time points, there were no significant differences between the two creams analyzed.

Table 6. Anti-inflammatory activity in acute and chronic inflammatory phases after topical administration of cream base (NC, negative control), dexamethasone cream (PC, positive control, 5 mg/g), and the AFS cream (5 mg/g). The letters presented after each value indicate whether there were significant differences between the different concentrations ($p < 0.05$). Same letter—no significant difference. Different letter—significant difference. Results are expressed as mean ± SD. One-way ANOVA, followed by Tukey's post hoc test, was performed.

Cream	1 h	2 h	4 h	6 h	24 h	48 h	72 h	96 h
PC	33.1 ± 5.8% ^{a}	26.1 ± 3.9% ^{a}	35.6 ± 14.5% ^{a}	28.6 ± 9.5% ^{a}	39.6 ± 7.6% ^{a}	55.6 ± 5.8% ^{a}	83.3 ± 6.1% ^{a}	70.1 ± 7.4% ^{a}
AFS	65.4 ± 8.2% ^{b}	52.1 ± 16.0% ^{b}	45.3 ± 13.2 ^{b}	29.8 ± 7.6% ^{a}	46.3 ± 6.4% ^{a}	49.2 ± 4.1% ^{a}	45.4 ± 6.8% ^{b}	50.6 ± 7.4% ^{b}

4. Discussion

In folk medicine, the *Agave sisalana* leaf is used in the treatment of tick-borne diseases, such as stomach detoxifier and constipation, antimicrobial against pathogen biota of the intestines and stomach, against meningitis and sciatica, skin eruptions, kidney disease, and hepatic affections [9–17]. However, in Brazil, the world's largest producer of sisal, the residue from sisal leaves, which represent 95% of its weight, is discarded [11]. Thus, considering the different therapeutic uses of the *Agave sisalana* species and understanding the importance of reusing the sisal residue that is discarded is important.

Initially, in this study, the presence of sapogenins in the sisal leaves was analyzed. Sapogenins are the lipophilic part of steroidal saponins, also known as aglycones. The other part, the hydrophilic part, contains one or more sugars. Steroidal saponins are one of the main secondary metabolites and exhibit anti-inflammatory, antiviral, and hepatoprotective effects [24,25]. In line with our study, which detected the presence of sapogenins in the AFS using high-resolution mass spectrometry (HRESI-MS) (Figure 1), the literature reports that plants of the *Agave genus* contain steroidal saponins and the sapogenins, tigogenin and hecogenin [11,18]. Moreover, Monterrosas-Brisson et al. [24], Dunder et al. [19], and Costa et al. [7] demonstrated the anti-inflammatory action of steroidal saponins in in vivo models and confirmed the presence of these metabolites in plants of the *Agave genus*. As sapogenins obtained from plant extracts are highly valued due to their numerous biological activities and can exhibit efficacy comparable to synthetic molecules, there is growing industrial interest in developing products containing these molecules [14,24].

The cell viability was evaluated via the MTT assay based on the metabolic activity of the mitochondria. Microsomal enzymes can reduce MTT, break down its substrate, and transform it into insoluble blue–violet formazan crystals. The color intensity of formazan crystals measured by spectrophotometry is proportional to the cell viability [25]. Three AFS concentrations were tested, but even the highest (2 mg/mL) was only able to reduce PMBC cell viability to 70% after 24 h of incubation. *Agave sisalana* extract obtained through acid hydrolysis has already been reported as cytotoxic to cancer cells in the concentrations of 25, 50, and 100 µg/mL by Araldi et al. [11]. Differently, the AFS extract analyzed in this study in healthy cells did not promote MTT test.

Considering that tissue repair comprises three sequential and overlapping healing phases, inflammation, proliferation, and remodeling [26], and that the inflammatory phase involves the formation of clots by platelets and recruitment of phagocytes [27], an uncontrolled process of phagocytosis, rather than repair of the injured tissue, can promote chronic damage [28]. Thus, to analyze the AFS anti-inflammatory activity in vitro, the phagocytosis inhibition assay was performed. The results obtained in this study indicate that the AFS promoted a higher inhibition of phagocytosis than the PC for all the concentrations tested. The literature has no reports on the anti-inflammatory activity of *Agave sisalana* extracts regarding the phagocytosis inhibition assay. Athira and Keerthi [29] observed that *Sigmadocia* extract showed a low level of phagocytosis.

Another in vitro method was used to evaluate the AFS anti-inflammatory activity, the erythrocyte membrane stabilization assay. Anti-inflammatory drugs may act on the lysosomal membrane stabilization, blocking the efflux of enzymes and their by-products and physiological processes responsible for hyperinflammation [21]. Thus, since the erythrocyte and lysosomal membranes are similar [26], a stabilization of the erythrocyte membrane by the AFS indicates that this fraction might also be able to stabilize the lysosomal membrane [23]. In this study, it was demonstrated that the three AFS concentrations tested exerted a potent anti-inflammatory activity, thus promoting lysosome stability in the inflammatory context. Moreover, the human red blood cell stabilization method, which has been used successfully for the evaluation of plant extracts, used this method to analyze the anti-inflammatory activity of a *Solanum paniculatum* L. at concentrations of 15, 30, and 60 mg/mL and observed a protection of the erythrocyte membrane of 25.2%, 30.8%, and 40.5%, respectively, when compared to the negative control group that induced 100% of hemolysis [21]. In addition, for the extract of *Oxalis Corniculata* analyzed in the concentration range of 50–800 µg/mL, a protection of 19.8% to 75.7% was found, compared to the negative control, which promoted 100% hemolysis [24].

In order to relate the sensation of pain to the inflammatory process and analyze in vivo the ability of the AFS to reduce pain sensitivity, the formalin method was used. This model shows two phases of painful sensitivity. The first, beginning immediately after the formalin injection and lasting 5 min, is called the neurogenic phase, in which direct activation of nociceptors occurs locally via formalin. The second phase represents a type of inflammatory pain and involves synaptic transmission reinforced by the spinal cord as well as by the

release of local inflammatory mediators, such as prostaglandins, serotonin, histamine, and bradykinin. The period between the two phases is called a quiescent interval [25].

In the neurogenic phase, only the AFS, 25 mg/kg, and the PC were able to significantly reduce the algic signals' manifestation (51.92% and 68.34% of inhibition, respectively). However, in the inflammatory phase, all doses of the AFS and PC inhibited the nociceptive process significantly, 25 mg/kg being the most effective dose (71.02% of algic signals inhibition). These data point to a possible antinociceptive activity of the AFS and corroborate the results obtained by Dunder et al. 2010 [7]. These authors tested a hydrolyzed *Agave sisalana* extract in an abdominal contortion model and obtained 30.7% of pain inhibition after oral administration of the extract at a dose of 500 mg/kg and 88.7% by intraperitoneal injection at the same dose. Also, Dunder et al. 2013 [6] showed that the hexanic fraction of the hydrolyzed extract of *Agave sisalana* administered at doses of 5, 10, 25, and 50 mg/kg inhibited abdominal contortions when compared to the control group in the proportion of 22, 54, 48, and 30%, respectively. Similar to the results obtained in this study, Dunder's results [6] were not dose-dependent.

The inflammation has two phases, acute and chronic. The acute phase initiates rapidly (in a few seconds or minutes) after the lesion and is characterized by fluid exudation and leukocyte signaling, particularly phagocytes (macrophages and neutrophils). Chronic inflammation is the continuation of the non-termination of the acute phase, in which the presence of other defense cells (lymphocytes) promotes oxidative stress processes, glycation, and consequently causes tissue fibrosis and necrosis [26]. In this study, to evaluate the AFS anti-inflammatory activity in the two inflammatory phases, the carrageenan-induced method was used. In rats, the carrageenan-induced edema presents a biphasic response, with an acute inflammatory peak, which normally occurs in median 4 h after induction, and a second phase, chronic, which extends for 96 h [27,28]. The acute anti-inflammatory activity of the AFS was evaluated 1, 2, 4, and 6 h after carrageenan administration, and the chronic anti-inflammatory activity after 24, 48, 72, and 96 h. The AFS in the doses of 25 and 50 mg/kg, In both evaluated phases of inflammation, it was able to reduce edema. Also, when compared to dexamethasone, an anti-inflammatory drug available in therapeutic, the 25 and 50 mg/kg AFS doses proved to have similar or even higher activity in the chronic inflammatory phase.

The sapogenins' mechanism of action is still unknown, but it is possible to establish a relationship between the analgesic and anti-inflammatory effects of the AFS with the presence of sapogenins in this fraction. Quintans et al. [30] treated mice with a systemic administration of hecogenin acetate in hyperalgesia models by carrageenan and observed a reduction in some cytokines, like the IL1-β, and the expression of IL-10, an anti-inflammatory cytokine. Moreover, Santos et al. [31] demonstrated that hecogenin isolated from *Agave sisalana* reduced the anti-inflammatory activity in vivo. In addition, when analyzing an *Agave americana* extract, Peana et al. [15] identified the sapogenins, hecogenin and tigogenin, and verified that those sapogenins, when administered intraperitoneally, produced a higher antiedematous effect than the commercially available anti-inflammatory drugs indomethacin and dexamethasone, in animal models.

Dunder et al. [18] reported a reduction in paw edema when analyzing the hexanic faction of a hydrolyzed *Agave sisalana* extract in the doses of 10 and 25 mg/kg, being 42% and 58% in the chronic inflammatory phase and 46% and 58% in the acute inflammation phase, respectively. Thus, the results obtained for a dose of 25 mg/kg of the AFS are better, with regard to the chronic inflammation phase.

In addition, Table 6 presents the percentages of anti-inflammatory activity resulting from the treatments with dexamethasone cream (PC, positive control, 5 mg/g) and the AFS cream (5 mg/g) in the two analyzed phases, acute and chronic. Through this analysis, it is evident that at 1, 2, and 4 h during the acute phase of inflammation, the cream containing AFS exhibited greater anti-inflammatory activity. However, at 72 and 96 h, the cream containing dexamethasone demonstrated higher anti-inflammatory activity. No significant differences were observed between the two creams at other time points analyzed.

In the evaluation of the topical cream formulations, the cream containing AFS showed greater anti-inflammatory activity at 1, 2, and 4 h during the acute phase of inflammation, while the dexamethasone cream exhibited greater activity at 72 and 96 h during the chronic phase. In the literature, using a similar methodology, Pashmforosh et al. [32] found that a topical preparation containing 8% *Citrullus colocynthis* significantly reduced paw volume compared to the carrageenan group in a dose-dependent manner. Similarly, Frei et al. [33] observed that topical preparations containing curcumin were significantly more effective against edema than formulations containing diclofenac, as they significantly reduced paw edema 4 h after carrageenan injection.

Thus, the main objective of this experimental work has been achieved. A new topical pharmaceutical formulation containing sisal residue extract was developed. Initially, the absence of cytotoxicity and the anti-inflammatory activity of the extract were demonstrated in vitro. In addition, in vivo analyses confirmed the analgesic activity of the extract, as well as the anti-inflammatory activity of both the extract and the formulation.

5. Conclusions

The gathered results reveal that the AFS presents the steroidal sapogenins, hecogenin and tigogenin, in its composition. The in vitro analyses show the absence of AFS cytotoxicity and an expressive anti-inflammatory activity. The in vivo studies confirmed the AFS anti-inflammatory activity, particularly for the 25 mg/kg dose, and also its analgesic activity. In addition, the topical application of the cream with AFS also showed anti-inflammatory activity, especially in the acute phase of inflammation.

It is thus likely that the AFS' pharmacological activities are a result of the presence of sapogenins in its phytochemical composition. From these results, the performance of complementary studies, phytochemical, pharmacological, and toxicological, will allow for thousands of tons of sisal residue discarded in Brazil to be used in the development of a new medicine that can be used in the treatment of inflammatory diseases and, consequently, promote the social and economic growth of people who depend on sisal for their subsistence.

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