Applications of the hexanic fraction of *Agave sisalana* Perrine ex Engelm (Asparagaceae): control of inflammation and pain screening

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The present study evaluated the anti-inflammatory and analgesic properties of Agave sisalana *Perrine in classic models of inflammation and pain. The hexanic fraction of* A. sisalana *(HFAS) was obtained by acid hydrolysis followed by hexanic reflux. Anti-inflammatory properties were examined in three acute mouse models (xylene ear oedema, hind paw oedema and pleurisy) and a chronic mouse model (granuloma cotton pellet). The antinociceptive potential was evaluated in chemical (acetic-acid) and thermal (tail-flick and hot-plate test) models of pain. When given orally, HFAS (5, 10, 25 and 50 mg/kg) reduced ear oedema ($p < 0.0001$; 52%, 71%, 62% and 42%, respectively). HFAS also reduced hind paw oedema at doses of 10 mg/kg and 25 mg/kg ($p < 0.05$; 42% and 58%, respectively) and pleurisy at doses of 10 mg/kg and 25 mg/kg (41% and 50%, respectively). In a chronic model, HFAS reduced inflammation by 46% and 58% at doses of 10 mg/kg and 25 mg/kg, respectively. Moreover, this fraction showed analgesic properties against the abdominal writhing in an acetic acid model (at doses of 5-25 mg/kg) with inhibitory rates of 24%, 54% and 48%. The HFAS also showed an increased latency time in the hot-plate (23% and 28%) and tail-flick tests (61% and 66%) for the 25 mg/kg and 50 mg/kg doses, respectively. These results suggest that HFAS has anti-inflammatory and analgesic properties.*

Key words: *Agave sisalana* Perrine ex Engelm - hecogenin - tigogenin - anti-inflammatory activity - analgesic activity

Inflammation is an immune response against pathogens, tissue injury and surgical trauma. This response gives rise to the initial cardinal signs of inflammation, which include redness, heat, swelling, pain and loss of function (Serhan & Savill 2005). The study of inflammation is important because of its intimate link to other diseases such as arteriosclerosis, obesity, cancer, chronic obstructive lung disease and asthma (Nathan & Ding 2010). The inflammatory process is a cascade of biochemical events that involves eicosanoids (prostaglandins, leukotrienes and thromboxanes), cytokines and infiltration of leucocytes (neutrophils, macrophages, mast cells and fibroblasts) to the injured area (Basbaum et al. 2009). Neutrophil migration is responsible for tissue damage and the interaction of neutrophils with nerve endings leads to a number of physiological responses during inflammatory reactions, including pain (Fu Sy & Gordon 1997).

The use of non-steroidal anti-inflammatory drugs (NSAIDS) is the most common treatment for inflammation. These drugs inhibit cyclooxygenase enzymes (COX-1 and COX-2) and consequently reduce prostaglandin levels (Vonkerman et al. 2006). However, these compounds provoke undesirable side effects such as peptic ulcer formation, bleeding and perforation of the gastric mucosa (Silverstein et al. 1995). Glucocorticoids, which are alternative drugs for the treatment of chronic inflammation, can induce systemic adverse effects in chronic patients, such as adrenal insufficiency (Barnes 2006). In this context, new drugs to treat inflammation with fewer side effects are necessary.

Several medicinal plants have shown promising results for alleviating pain. Many patients who use such treatments perceive them to be effective (Kanodia et al. 2010). *Agave sisalana* Perrine ex Engelm Asparagaceae is a monocotyledoneous plant that is extensively cultivated around the world, mainly in tropical and semi-arid zones (Nunes-Moreira et al. 1999). In Brazil, *A. sisalana* is broadly cultivated in the state of Bahia (BA), which accounts for 95% of all *A. sisalana* grown in the country (Santos 2006). The crude juice of *A. sisalana* is produced by milling leaves. However, this juice is very fibrous and is therefore not used much. It is rich in saponins, which are necessary for generation of steroidal materials called sapogenins (specifically hecogenin and tigogenin) through acid hydrolysis (Ding et al. 1989). Several studies have shown different activities of extracts derived from the crude juice of *A. sisalana*. The crude juice exhibits bactericidal, antihelmintic and anti-inflammatory

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activity (Santos et al. 2009, Botura et al. 2010, Dunder et al. 2010). In the Dominican Republic, a traditional medicine called *Agave intermixta* Trel is widely used in the treatment of arthritis and infections (Garcia et al. 1999).

Based on other studies of sapogenins obtained from the *Agave* genus in inflammation research, the present study was conducted to investigate the possible anti-inflammatory and antinociceptive effects of the hexanic fraction of *A. sisalana* (HFAS) obtained from the hydrolysed extract.

MATERIALS AND METHODS

Drugs and chemicals - Acetic acid, xylene, methanol (Merck, Brazil), dexamethasone (DEXA) (Hoechst SA, Brazil), ethanol, standard hecogenin, polyethylene glycol-200, morphine hydrochloride, sufentanil, carrageenan and indomethacin (INDO) (Sigma Chemical Co, St. Louis, MO, USA) were used in the present study. Morphine was dissolved in a 0.9% NaCl solution just before use and INDO was prepared in a sodium bicarbonate solution (5%). HFAS was dissolved in a solution of polyethylene glycol-200 (40%, vehicle). All of the reagents used were of high purity.

Plant material - The *A. sisalana* was cultivated at the Riacho do Cedro farm located in the city of Santo Domingo in BA. The botanical identification was performed by Jorge Tamashiro and a voucher specimen was deposited at the herbarium at the State University of Campinas (UNICAMP) (Campinas, state of São Paulo) (voucher 150.429). The leaves were crushed for separation of the juice and for acquisition of the fibre. The *A. sisalana* crude juice was donated by Corona Comércio e Indústria Ltda (Salvador - BA).

Fraction preparation - HFAS was obtained from the *A. sisalana* crude juice, according to the protocol of Peana et al. (1997) with some modifications. To obtain a higher concentration, the *A. sisalana* juice was evaporated at 100ºC until it was reduced to 1/10 of its initial volume. The crude juice (40 g) was subjected to reflux with 300 mL of HCl for 4 h with agitation. After cooling at room temperature, the dark precipitate that formed was filtered, washed with water, neutralized with 10% NaOH and dried. The resulting powder was refluxed with 300 mL of hexane at 60ºC for approximately 60 h. The hexanic solution was filtered and evaporated to provide 1 g (2.5%) of green powder that was rich in steroidal sapogenins. This powder was named HFAS.

Chemical analysis - HFAS was qualitatively analysed by high-performance liquid chromatography (HPLC). HFAS as the standard hecogenin and the mixture of HFAS and the standard hecogenin were solubilised in methanol. The solution (20 µL, 1 mg/mL) was then injected into the HPLC apparatus (Shimadzu-LC 10, Japan) with a column (ACE 5 C 18, 250.0 x 4.6 mm x 5 µm). The mobile phase was methanol (0.6 mL/min for 15 min) for ultraviolet detection (254 nm). The quantitative analysis was performed by standard methods, using a multilevel calibration curve determined by analysis of a pure hecogenin solution at known concentrations (1-10 mg/mL).

Cell culture - The mouse fibroblast cell line BALB/c 3T3 was cultivated in Dulbecco's Modified Eagle's Medium medium supplemented with 10% foetal bovine serum, 1% penicillin (100 U/mL) and streptomycin (100 g/mL) and incubated at 37ºC under 5% CO_2. All of the tests were performed in duplicate.

Animals - All of the experiments were performed on male Unib-WH rats (160 ± 200 g) and male Unib-SW mice (30 ± 5 g) from the Animal House of Multidisciplinary Center for Biological Research/UNICAMP. The animals were fed a diet of regular rodent chow and had free access to tap water. The animals were housed on a 12-h light: 12-h dark cycle at 50% humidity and a temperature of 24 ± 1ºC. All of the experiments were performed in the morning. The experimental protocols were approved by the Animal Use and Care Committee of UNICAMP, protocol 1281-1.

Cell toxicity assay [(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (MTT)] - The cytotoxicity of HFAS was determined using a colorimetric method (Mosmann 1983). This method assesses the ability of the mitochondrial enzyme succinate dehydrogenase in viable cells to convert MTT into formazan. BALB/c 3T3 cells were seeded on 96-well culture plates at a density of 1×10^4 cells/well. The cells were then incubated for 24 h. After this incubation, the cells were treated with increasing concentrations of HFAS (0-500 µM) for 24 h at 37ºC. The culture medium was then replaced by fresh medium containing 0.5 mg/mL MTT and the plates were incubated for 4 h at 37ºC. The medium was then removed and was replaced by 100 µL of ethanol for the solubilisation of formazan. The absorbance was measured using a spectrophotometric microplate reader (ELx 800 BIO-TEK) at a wavelength of λ = 570 nm. The cell viability was expressed as the relative formazan formation in the treated samples relative to the control cells.

Xylene-induced ear swelling in mice - This assay was performed as previously described (Núñez Guillén et al. 1997). The Unib-SW (n = 8) mice were separated into groups. The experimental groups received HFAS [5, 10, 25 and 50 mg/kg orally (p.o.)] and the controls received vehicle (10 mL/kg) and INDO (5 mg/kg). After 1 h of treatment, each animal received 30 µL of xylene on the anterior and posterior surfaces of the left ear lobe. The right ear was considered to be the control. One hour later, the animals were sacrificed by cervical dislocation and circular sections with a diameter of 8 mm were obtained from ears and weighed. The degree of ear swelling was calculated based on the weight of the right ear, which did not undergo xylene application.

Carrageenan-induced rat hind paw oedema - Inflammation in the hind paw was induced as previously described (Winter et al. 1962). Male Unib-WH rats were divided into six groups (n = 5). The groups received HFAS at 5, 10, 25 and 50 mg/kg doses (p.o.) and the controls received vehicle (10 mL/kg) or DEXA (5 mg/kg p.o.). One hour after treatment, 0.2 mL of λ-carrageenan (1%) was administered to the sub-plantar region. The

paw oedema volume was measured using a plethysmometer (UGO Basile, Italy) to determine the oedema at ½, 1 h, 2 h, 3 h and 4 h after λ-carrageenan administration.

Granuloma cotton pellet - The test for cotton pellet-induced granuloma formation was performed as described in a previous study (Swingle & Shideman 1972). Male Unib-WH rats (n = 7) received HFAS (10 and 25 mg/kg, the best doses for acute treatment) or the control treatment (vehicle or DEXA 0.5 mg/kg p.o.) once daily for seven days. A sterilised cotton pellet weighing 20 mg was placed subcutaneously into the back region of rats on the first day. The animals were sacrificed on the eighth day. The pellets were removed with the granuloma tissue and dried at 60ºC. The dry weights were then determined. The percent inhibition was expressed by comparing the mean weight in the test groups with that of the negative control group.

Induction of pleurisy - Groups of UNIB-WH rats were given HFAS p.o. (10 mg/kg and 25 mg/kg) and the controls were given DEXA (2.0 mg/kg) or vehicle (10 mL/kg). One hour after treatment, the animals were slightly anesthetised with isofluorane and 0.2 mL of a sterile solution (consisting of 1% carrageenan suspended in sterile saline) was injected into the right pleural cavity. The animals were killed by CO_2 4 h after the induction of pleurisy. The pleural exudate from each animal was harvested by washing the pleural cavity with 5 mL of sterile phosphate buffered saline solution containing 5 U/mL heparin. The exudates with blood contamination were rejected. The total leucocytes in each sample were counted after Turk staining (2 mL of acetic acid, crystal violet 1%, 1 mL of methanol and 97 mL of water) using a Neubauer counting chamber. The differential cell count in each sample of pleural fluid was determined by haematoxylin and eosin staining, according to a previous study (Mikami & Miyasaka 1983), with some modifications.

Measurement of myeloperoxidase (MPO) levels - Samples of the fluid leakage were collected immediately after the pleural cavity was opened for the MPO assay. The pleural lavage (40 mL) or the standards (MPO from human neutrophils; Sigma M-6908: 0.7-140 mU/mL) were transferred to cuvettes and the reaction was initiated by the addition of 360 mL of assay buffer (0.167 mg/mL of o-dianisidine 2HCl and 0.0005% H_2O_2). The enzymatic reaction was stopped by adding 1% sodium azide after 15 min. Afterwards, the samples were centrifuged (50 *g* for 5 min) and the supernatants were separated. The rate of change in absorbency at 450 nm was determined by using a spectrophotometer (Hitachi U-2001 model 121-0031, Tokyo, Japan). The samples in which the levels of MPO were higher than the upper limit of detection (140 µg/mL) were diluted (2-5-fold) and the concentrations were corrected for the two-five-fold dilution. The results below the lower limit of detection were expressed as 0.7 µg/mL (Fröde-Saleh & Calixto 2000).

Acetic acid-induced writhing - Male Unib-SW (n = 8) mice were used according to a method described previously (Koster et al. 1959). The total number of writhing events following intraperitoneal administration of 0.6% acetic acid was recorded for 15 min, starting 5 min after injection. The animals were pre-treated with HFAS at doses of 5, 10, 25 and 50 mg/kg (p.o.) and the controls were pre-treated with vehicle (10 mL/kg) and INDO (5 mg/kg p.o.) 1 h before acetic acid administration. Antinociceptive activity was expressed as a reduction of the number of abdominal writhes relative to the negative control.

Hot-plate test - The hot-plate test was used to measure the latency of the response as described previously (Eddy & Leimbark 1953) with minor modifications. The temperature of the hot-plate (Ugo Basile, Models-DS 37) was maintained at 56 ± 1ºC. The Unib-SW (n = 8) mice were placed in a glass cylinder with a diameter of 24 cm on a heated surface. The time between the placement and licking of the paws or jumping was recorded as the latency. Control mice were treated with vehicle (vehicle, 10 mL/kg). Morphine was used as a positive control (10 mg/kg subcutaneously) and HFAS was administered (5, 10, 25 and 50 mg/kg p.o.). All of the substances were administered 30 min before the beginning of the experiment. The mice were selected 24 h before the experiment on the basis of their reactivity to the test. This response was defined as the baseline (BL). All of the mice were observed before and 30, 60, 90 and 120 min after drug administration. A latency of 20 s was defined as complete analgesia. The result was expressed as the percentage of the maximal possible effect (MPE), according to the following equation with the following definitions: test line (TL) [value of paw cut-off (CO) during the experiment], BL (response obtained 24 h before the experiment) and CO (maximum latency time of 20 s):

$$\text{MPE (\%)} = \left\{ \frac{\text{TL - BL}}{\text{CO - BL}} \right\} \text{ x } 100$$

Tail flick test - We used the tail-flick test, which is another assay to evaluate the antinociceptive effect, as previously described (Ness & Gebhart 1986). Unib-SW mice received HFAS at doses of 5, 10, 25 and 50 mg/kg or they received the controls vehicle (10 mL/kg) or sufentanil (5 µg/kg intramuscularly). Briefly, the mice were gently held with the tail positioned in the tail-flick apparatus (Ugo Basile, Italy) for radiant heat stimulation (50 ± 5ºC) of the ventral surface of the tail. The intensity of the heat stimulus was adjusted to cause the animal to flick its tail within 2-3 s as the BL for the tail-flick latency. All tail-flick latencies were converted to a percentage of the MPE according to the following equation with the following definitions: TL (value of tail-flick CO during the experiment), BL (response obtained 24 h before the experiment) and CO (maximum latency time of 10 s).

$$\text{MPE (\%)} = \left\{ \frac{\text{TL - BL}}{\text{CO - BL}} \right\} \text{ x } 100$$

Statistical analysis - The results are presented as the mean ± standard deviation (SD). Statistical comparison of the data was performed using the analysis of variance (ANOVA), followed by Dunnett's test. $p < 0.05$ were considered significant. The statistical analysis was performed using Graphpad Prism 5.0 software (GraphPad®, San Diego, CA, USA).

RESULTS

Chemical analysis - According to the HPLC chromatography analyses, we observed the same retention time (RT) for standard hecogenin (RT = 4.34265) (Fig. 1A, B). The other peak in the HFAS is probably tigogenin (RT = 4.9677), because of the literature describes the genus *Agave* as being rich in hecogenin and tigogenin (Peana et al. 1997). As shown in Fig. 1C, a mixture of HFAS and the standard hecogenin was injected into the HPLC. The graph shows the peak of hecogenin in the same RT. The HPLC quantitative analysis in comparison with standard hecogenin revealed that HFAS contains 50% of this compound.

MTT test - Fig. 2 shows the effect of HFAS on the mitochondrial function (reduction of MTT) in BALB/c 3T3 cells. Cells treated with HFAS (250-500 μM) showed a 15% reduction in MTT when compared with the control group, which did not receive HFAS.

Xylene-induced ear swelling in mice - Oral administration of HFAS significantly suppressed xylene-induced ear oedema in mice (Fig. 3). The oedema inhibition produced by HFAS was 52%, 71%, 62% and 42% at doses of 5, 10, 25 and 50 mg/kg, respectively. The reference drug (INDO, 5 mg/kg) exhibited 78% inhibition relative to the negative control (vehicle).

Carrageenan-induced rat hind paw oedema - The results of another acute inflammatory test (carrageenan-induced rat hind paw oedema) are shown in Fig. 4. HFAS (at doses of 10 and 25 mg/kg) exhibited significant inhibition (42% and 58%) of carrageenan-induced rat paw oedema. DEXA produced a 64% reduction in anti-inflammatory activity 4 h after carrageenan injection.

Granuloma cotton pellet - In the chronic model of granuloma (cotton pellet), HFAS inhibited granuloma formation relative to the negative control (vehicle). HFAS (10 and 25 mg/kg) produced 46% and 58% reductions in granuloma weight, respectively. The positive control (DEXA) showed 90% reduction (Fig. 5).

Pleurisy - The values for the estimated amount of cells in the pleural cavity (Table I) show that both of the HFAS doses tested (10 and 25 mg/kg) produced a reduction in total cell infiltration (41% and 50%, respectively). The positive control DEXA produced a 76% reduction in infiltration. HFAS treatment resulted in neutrophil reduction (55% and 62% for the 10 and 25 mg/kg doses, respectively). For the mononuclear cells, a dose of only 25 mg/kg produced a 34% reduction and the positive control DEXA showed a 48% reduction.

Measurement of MPO levels - Fig. 6 shows the results from the MPO pleural lavage obtained from the carrageenan-induced pleurisy in the experimental model. A dose of 25 mg/kg produced a significant decrease in the MPO pleural lavage (43%) and the positive control DEXA (2.5 mg/kg) showed a significant reduction (84%, $p < 0.0001$) relative to the negative control (vehicle).

Acetic acid-induced writhing - The antinociceptive effects were assessed using the acetic acid-induced writhing test. HFAS significantly reduced the number of writhing events induced by acetic acid solution at inhibitory rates of 24%, 54% and 48%. The standard drug

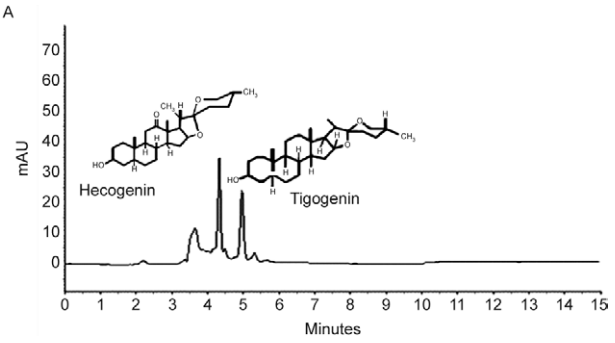

Fig. 1A: high-performance liquid chromatography chromatograms of hexanic fraction of *Agave sisalana* samples at ultraviolet detection (254 nm), hecogenin in the first peak and tigogenin is the second peak. mAU: mass absorbance unit.

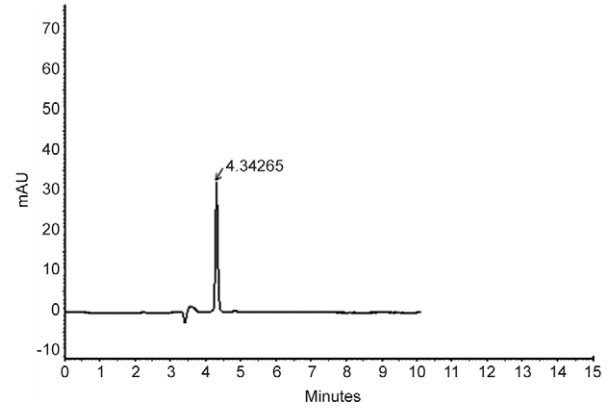

Fig. 1B: high-performance liquid chromatography chromatograms of standard hecogenin in only peak samples at ultraviolet detection (254 nm). mAU: mass absorbance unit.

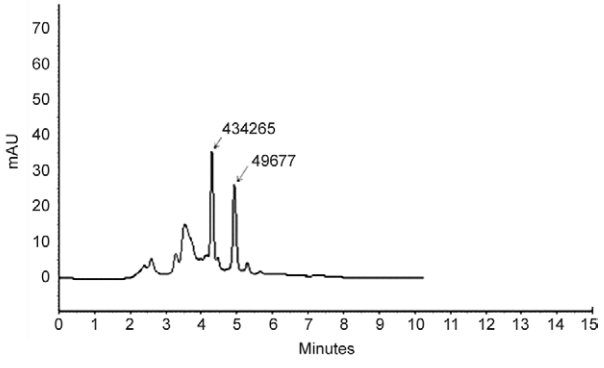

Fig. 1C: high-performance liquid chromatography chromatograms of mixture of hexanic fraction of *Agave sisalana* and standard hecogenin. This figure showed the same peak for hecogenin samples at ultraviolet detection (254 nm). mAU: mass absorbance unit.

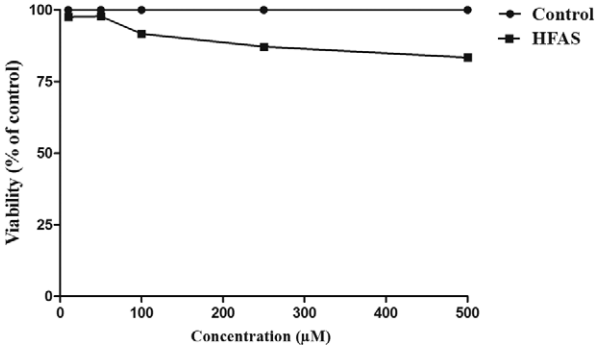

Fig. 2: effects of hexanic fraction of *Agave sisalana* (HFAS) on the cytotoxicity of cells BALB/c 3T3. Cells were grown in a Dulbecco's Modified Eagle's Medium medium with vehicle-control (dimethyl sulfoxide) or 50-500 µM of HFAS for 24 h. The number of viable cells was determined by an (2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide assay.

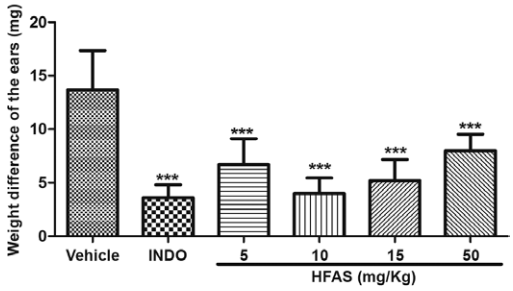

Fig. 3: inhibitory effects of hexanic fraction of *Agave sisalana* (HFAS) at different doses (5, 10, 25 and 50 mg/kg) on the weight of ear oedema in xylene-induced ear inflammation. The control group received vehicle solution and the reference drug was indomethacin (INDO) (5 mg/kg). Asterisks ($p < 0.0001$ values) mean means ± standard deviation compared with vehicle control group (one-way analysis of variance followed by Dunnet's *post hoc* test).

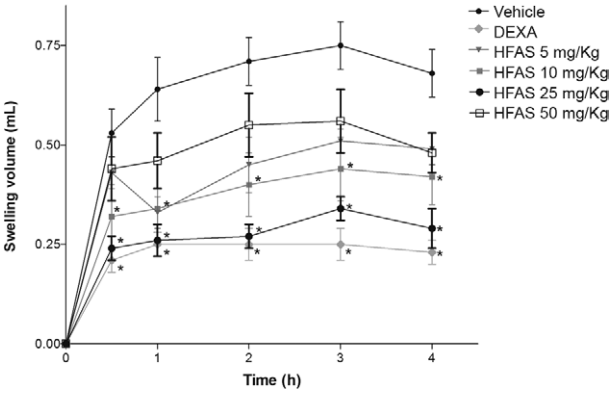

Fig. 4: inhibitory effect induced by hexanic fraction of *Agave sisalana* (HFAS) (5, 10, 25 and 50 mg/kg) on the oedema induced by carrageenan in rats. The control group received vehicle (10 mL/kg) and the reference drug was dexamethasone (DEXA) (2 mg/kg). Asterisk ($p < 0.05$ values) means means ± standard deviation compared with vehicle control group (one-way analysis of variance followed by Dunnet's *post hoc* test).

(INDO) inhibited 45% of writhing. These results are shown in Table II.

Hot-plate test - The results of the hot plate test (Table III) show that HFAS (50 mg/kg) increased the latency time after 90 min (28%) and after 120 min at doses of 10, 25 and 50 mg/kg (20, 23 and 28%, respectively). The standard drug (morphine, 10 mg/kg) showed an increased latency time at 30 min (56%), 60 min (72%) and 90 min (71%).

Tail-flick test - In the tail flick test, HFAS also produced an increased latency time by MPE. Doses of 10, 25 and 50 mg/kg increased the latency time from 60 min on. HFAS showed MPE rates of 42%, 61.5% and 66% for doses of 10, 25 and 50 mg/kg, respectively. Sufentanil, which was applied as the standard drug, increased the latency time of the test (85%). The results are shown in Table IV.

DISCUSSION

Natural products, including plant derivatives, have over the years contributed to the discovery of new therapeutic drugs. Most secondary plant metabolites are known to interfere with or act on several mediators of inflammation (Calixto et al. 2003). In this study, we evaluated the potential of *A. sisalana,* which is rich in steroidal sapogenins, in classic models of inflammation. Chemical HPLC analyses confirmed the presence of hecogenin and tigogenin in HFAS, showing the same RT relative to the standard hecogenin. A quantitative analysis based on the standard hecogenin curve revealed that HFAS contains hecogenin as the major compound.

The MTT cell viability test was used to evaluate the toxicity of HFAS. This method assesses the ability of viable cells to convert MTT into formazan by the mitochondrial enzyme succinate dehydrogenase. Our results clearly showed that HFAS did not induce mortality of BALB/c 3T3 cells at concentrations of 50-500 µM. Furthermore, the MTT test did not reach the 50% inhibitory concentration, which shows that HFAS is not toxic. After HFAS was confirmed to be non-toxic, further analysis of its anti-inflammatory and analgesic activities was performed.

The anti-inflammatory activity of HFAS was evaluated by using a xylene-induced mouse ear oedema model. Xylene acts in the early phase of inflammation

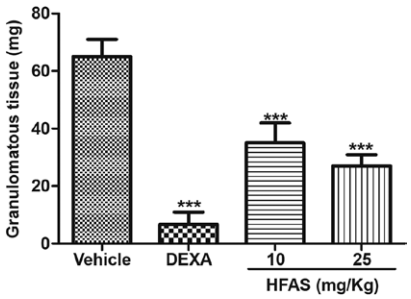

Fig. 5: inhibitory effects of hexanic fraction of *Agave sisalana* (HFAS) (10 and 25 mg /kg) in cotton pellet model. Vehicle dexamethasone (DEXA) (0.5 mg/ kg) was a negative and positive control. Values represent means ± standard deviation. Asterisks mean $p < 0.0001$ (one-way analysis of variance followed by Dunnet's *post hoc* test).

through several events such as vasodilatation and release of histamine, serotonin and substance P. These mediators can induce ear oedema by increasing vascular permeability (Hosseinzadeh et al. 2003). In our study, HFAS showed anti-oedematogenic activity by reducing xylene ear oedema. These results suggest that HFAS can decrease oedema because it contains the steroidal molecules hecogenin and tigogenin. The anti-inflammatory activity was corroborated by another experiment that measured hind paw oedema in a classical model of acute inflammation, which is a good model to examine the anti-inflammatory effects of natural products.

The inflammatory response in the hind paw assay involves two phases. The first phase involves the release of serotonin and histamine and the second phase is mediated by prostaglandin E_2 (PGE_2), which is a product of COX (Torres et al. 2000). Previous studies showed that neutrophil infiltration and activation are involved in the inflammatory responses induced by carrageenan (Godin et al. 2011). HFAS reduced oedema at doses of 10 and 25 mg/kg, which suggests that HFAS may play a key role in decreasing levels of histamine, serotonin and other inflammatory mediators in xylene-induced ear oedema. HFAS maintained the reduction of oedema during the second phase, which involves PGE_2 and leukocyte infiltration. Another study (Dunder et al. 2010) showed that hydrolysed extracts of *A. sisalana* inhibited oedema in the same model, but at a higher dose. In the present study, with a more purified compound that is richer in steroidal sapogenins, more promising results were obtained with lower doses.

Another parameter of inflammation is cellular infiltration. When injected into the pleural cavity, carrageenan (the flogistic agent) causes tissue damage, plasma exudation and protein-rich fluid accumulation followed by an influx of leukocytes (Murai et al. 2003). Therefore, carrageenan-induced acute inflammation has been linked to neutrophil infiltration and the production of neutrophil-derived free radicals (Impellizzeri et al. 2011). The recruitment and activation of neutrophils at the site of inflammation contributes to the release of inflammatory mediators, which increase vascular permeability, maintain inflammation and cause tissue damage (Farias et al. 2012). The present data show that HFAS is able to attenuate leukocyte infiltration into the pleural cavity. HFAS was shown mainly block neutrophil influx rather than influencing mononuclear cells at 4 h after induction with carrageenan. This response may explain the reduction of hind paw oedema. Neutrophils release different inflammatory mediators, such as MPO, which is a member of the peroxidase-COX superfamily that is abundantly expressed in neutrophils and to a lesser extent in monocytes and certain types of macrophages. MPO participates in innate immune defence mechanisms through the formation of microbicidal reactive oxidants and diffusible radical species involved in inflammation (Malle et al. 2007). A biochemical analysis revealed that HFAS reduced the levels of MPO. These data indicate that anti-inflammatory drugs may also exert their effects via inhibition of MPO pathways.

To test the anti-inflammatory activity in a chronic model of inflammation (the cotton pellet granuloma model), the most effective doses of HFAS (10 and 25 mg/kg) were chosen from the acute models of inflammation. The cotton pellet granuloma method is widely employed to assess efficacy against the proliferative phase of inflammation in which tissue degeneration and fibrosis occur (Swingle & Shideman 1972). The evaluated doses of HFAS reduced the proliferative phase of inflamma-

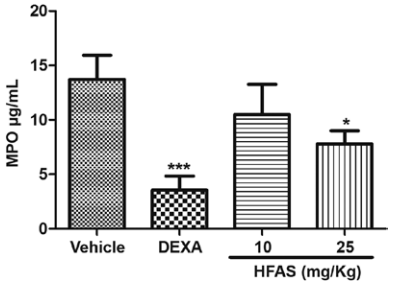

Fig. 6: inhibitory effects of hexanic fraction of *Agave sisalana* (HFAS) (10 and 25 mg/kg) in biochemical quantifications of myeloperoxidase (MPO) in µg/mL. Vehicle and dexamethasone (DEXA) (2.5 mg/kg) was a negative and positive control. Values represent by means ± standard deviation. *: $p < 0.05$; *** $p < 0.0001$ (one-way analysis of variance followed by Dunnet's *post hoc* test).

TABLE I

Effect of hexanic fraction of *Agave sisalana* (HFAS) on carrageenan-induced pleurisy in rats

	Total account		Neutrophils		Mononuclear's	
Groups	Number of cells (10^6)	Inhibiton (%)	Number of cells (10^6)	Inhibiton (%)	Number of cells (10^6)	Inhibiton (%)
Vehicle	-	-	2.86 ± 0.74	-	1.18 ± 0.29	-
DEXA	0.9 ± 0.13[a]	76	0.29 ± 0.07[a]	90	0.61 ± 0.14[a]	48
HFAS 10	2.25 ± 0.48[a]	41	1.29 ± 0.30[a]	55	0.98 ± 0.19	17
HFAS 25	1.89 ± 0.48[a]	50	1.08 ± 0.31[a]	62	0.78 ± 0.16[b]	34

a: $p < 0.001$; b: $p < 0.05$. Data represent means ± standard deviation values from eight-10 rats. DEXA: dexamethasone.

tion, which involves cellular infiltration of leucocytes such as neutrophils and macrophages into the inflammation site, followed by fibroblast infiltration (Swingle & Shideman 1972). The present results show that by reducing the proliferative phase, HFAS plays an important role in leucocyte regulation, mainly in neutrophil cells.

Pain is intimately linked to inflammation because some inflammatory mediators stimulate nociceptors, which results in painful sensations. The present results showed that oral administration of HFAS caused an antinociceptive effect against acute chemical and thermal-induced nociception in mice. Three different analgesic assays were used, including acetic acid-induced writhing and the hot plate and tail flick tests.

In the acetic acid-induced abdominal writhing test (a visceral pain model) arachidonic acid is released via COX. Additionally, prostaglandin biosynthesis plays a role in this nociceptive mechanism (Franzotti et al. 2002). Furthermore, this model releases other mediators such as bradykinin and substance P, as well as cytokines such as interleukin (IL)-1β, tumour necrosis factor alpha and IL-8 (Sekiya et al. 1982). HFAS reduced the number of writhing events. In this test, HFAS was as effective as the standard drug INDO in reducing the amount of abdominal writhing, which indicates its antinociceptive activity.

The hot-plate and tail-flick tests were performed to assess whether HFAS possesses central analgesic properties. The results of the hot-plate test indicate that HFAS (at doses of 25 and 50 mg/kg) significantly increased the latency of the response, which suggests that this compound suppressed the thermal stimulus response.

This latency increase was confirmed by the tail-flick test, in which a thermal stimulus is focused on the tail skin of the animal to activate nociceptors in the superficial layers of the skin. The activation of peripheral nociceptors triggers a complex series of processes at the spinal level and results in the tail flick response. The tail-flick also activates the transient receptor potential vanilloid (TRPV)1 vanilloid receptor. TRPV1 expression in the skin is localized to the terminals of intracutaneous epidermal nerve fibres that convey pain sensations generated by noxious heat (Mandadi & Roufogalis 2008). Activation of TRPV1 receptors induces eicosanoid formation and releases other pro-inflammatory mediators. The eicosanoids, mainly PGE_2, promote a secondary activation of TRPV1 (Dogrul et al. 2007).

HFAS increased the latency time in the tail-flick test, which corroborated the results of the hot-plate test. Some sapogenins have antinociceptive activity. For example, hederagenin and oleanolic acid (derived from *Akebia quinata*) showed analgesic properties in the same models (Choi et al. 2005). Compounds with steroidal char-

TABLE II

Effects of the hexanic fraction of *Agave sisalana* (HFAS) on acetic acid-induced writhing in mice

Group	Writhings	Inbition (%)
Vehicle	47.4 ± 4.3	-
INDO 5 mg/kg	26 ± 2.4[a]	45
HFAS 5 mg/kg	31.8 ± 0.8[b]	32
HFAS 10 mg/kg	21.6 ± 2[a]	54
HFAS 25 mg/kg	24.3 ± 1.8[a]	48
HFAS 50 mg/kg	33 ± 3.3[b]	31

a: $p < 0.0001$; *b*: $p < 0.01$ (one-way analysis of variance followed by Dunnet's *post hoc* test). The control group received vehicle (10 mL/kg) and the reference drug was indomethacin (INDO) (10 mg/kg). Values represent means ± standard deviation.

TABLE III

Analgesic effects of hexanic fraction of *Agave sisalana* (HFAS) in hot-plate test in mice

		Maximal possible effect (%)			
Groups	Dose	30 min	60 min	90 min	120 min
Vehicle	10 mL/kg	2	5.25	4.4	5.2
Morfine	10 mg/kg	56[a,c]	72[a,c]	21	11
HFAS	5 mg/kg	7	11	17	16
HFAS	10 mg/kg	19	27	19	20[b]
HFAS	25 mg/kg	18	15	17	23[a]
HFAS	50 mg/kg	17	23	28[b]	28[a]

a: $p < 0.0001$; *b*: $p < 0.01$; *c*: $p < 0.05$ comparison morphine and HFAS (one-way analysis of variance followed by Dunnet's *post hoc* test). Vehicle and morphine (10 mg/ kg) was a respectively negative and positive control.

TABLE IV

Analgesic effects of hexanic fraction of *Agave sisalana* (HFAS) in tail-flick test in mice

	Maximal possible effect (%)					
Observation time (min)	Vehicle	Sufentanil	HFAS 5 mg	HFAS 10 mg	HFAS 25 mg	HFAS 50 mg
0	-	-	-	-	-	-
15	-2,0	90.6[a,d]	14.1	37.5	18.7	37.2
45	-10.9	95.3[b,d]	9	37.5	42.8[a]	66.2[a]
60	-10.9	95[b,d]	15.7	29.2[a]	61.5[c]	40.7[a]
90	0.8	100[b,d]	13.6	41.8[a]	55.2[a]	27.4[a]
120	-6.5	67.7[c,d]	12.8	29.9	41.7[a]	47.6[a]
180	-7.9	49.1[c,d]	7.3	25.8	34.1[a]	49.3[a]
240	5.3	48.3[c,d]	13.6	6.3	15.9	11.5

a: $p < 0.0001$; *b*: $p < 0.01$: *c*: $p < 0.05$; *d*: $p < 0.05$ comparison sufentanil and HFAS (one-way analysis of variance followed by Dunnet's *post hoc* test). Vehicle and sufentanil as negative and positive control.

acteristics have analgesic activities, but the underlying mechanism is still not well understood. However, Cunha et al. (2008) described an important role of neutrophils in mechanical inflammatory nociception. Furthermore, another study demonstrated that the thermal hyperalgesic response was impaired by blockade of neutrophil recruitment (Lavich et al. 2006). Based on these pre-clinical studies involving inflammation and pain, we believe that the analgesic effects of HFAS involve leucocyte infiltration, mainly neutrophil infiltration.

In summary, a chemical analysis identified two steroidal sapogenins in HFAS (hecogenin and tigogenin) with low toxicity. Results obtained in vivo conclude that this compound is crucial for leucocyte infiltration in acute tests. We also showed that HFAS has anti-oedematogenic properties. In chronic models of inflammation, HFAS showed a slight stronger activity relative to the positive control DEXA. Finally, we showed that HFAS has analgesic properties. Our data provide a scientific basis for new uses of *A. sisalana* in therapeutic studies and new applications for the crude juice. New tests are being performed to identify the mechanisms of action for HFAS in inflammatory and analgesic studies.

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