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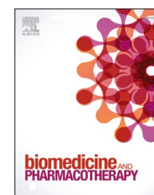

Toxicological and pharmacological effects of pentacyclic triterpenes rich fraction obtained from the leaves of *Mansoa hirsuta*

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A R T I C L E I N F O

Keywords:
Mansoa hirsuta
Acute toxicity
Subchronic toxicity
Inflammation
Analgesia

A B S T R A C T

Mansoa hirsuta is a medicinal plant native to the Brazilian semi-arid region. This approach aimed to investigate the in vitro and in vivo toxicity and anti-inflammatory and analgesic actions of the *M. hirsuta* fraction (MHF). *In vitro* cell viability was assessed in 3T3 cells. *In vivo*, the acute toxicity test, a single dose of the MHF was administered. For the subchronic toxicity test, three doses of were administered for 30 days. Locomotion and motor coordination were assessed using open field and rota-rod. The anti-inflammatory activity was evaluated in carrageenan-induced paw edema and zymosan-induced air-pouch models. Myeloperoxidase (MPO) and total proteins were also measured. The antinociceptive activity MHF was determined using acid acetic-induced abdominal writhing and formalin models. In the cytotoxicity assay, MHF showed no significative impairment of cell viability and in the acute toxicity study, did not cause mortality or signs of toxicity. Repeated exposure to MHF did not cause relevant toxicological changes. The evaluation in the open field test showed that the MHF did not alter the locomotor activity and there was no change in motor coordination and balance of animals. MHF significantly reduced edema, MPO production, the migration of leukocytes and protein leakage. In addition, MHF

Abbreviations: TNF-α, tumor necrosis factor alpha; COX-1, cyclooxygenase-1; NO, nitric oxide; MHF, M. hirsuta fraction; MTT, 3- (4,5-dimethylazol-2-yl) - 2,5-diphenyltetrazolium bromide; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; DSMO, dimethylsulfoxide; PBS, phosphate buffered saline; OECD, Organization for Economic Cooperation and Development; EDTA, ethylenediaminetetraacetic acid; AST, aspartate aminotransferase; ALT, alanine aminotransferase; H&E, hematoxylin and eosin; MPO, myeloperoxidase; HGB, hemoglobin; HCT, hematocrit; PLT, platelets; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red blood cell distribution width; MPV, mean platelet volume; LIN, lymphocytes; MON, monocytes; GRA, granulocytes.

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https://doi.org/10.1016/j.biopha.2021.112478
Received 16 August 2021; Received in revised form 17 November 2021; Accepted 29 November 2021
Available online 3 December 2021
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reduced abdominal writhing and significantly inhibited the first and second stage of the formalin test. The results of this study indicated that MHF has an anti-inflammatory and analgesic potential without causing acute or subchronic toxic effects and it can be a promising natural source to be explored.

1. Introduction

Mansoa is a diversified genus of the family Bignoniaceae, which includes species with a great morphological variety, and in the Brazilian region, it is popularly known as cipó-de-alho [1]. Representatives of this genus are found in both wet and dry tropical forests of Central America, the Amazon, and the Atlantic coastal [2]. In the case of the Brazil, there are records of the occurrence of species restricted to the Caatinga biome: *Mansoa paganuccii*, *Mansoa longicalyx*, *Mansoa ivanii*, and *Mansoa hirsuta* D.C. (www.theplantlist.org) [2,3].

In traditional medicine, *M. hirsuta* leaves have been used to treat diabetes and its stem bark is used for sore throats [4,5]. Some biological activities of this species have been proposed, including antimicrobial [6], antifungal and antitumor activity [4], inhibition of the angiotensin-converting enzyme [7], vasodilatory effect dependent on the endothelium and nitric oxide [8], anti-inflammatory action inhibiting the COX-1 and TNF-α pathways [9,10] and antioxidant properties [7,11].

Results of phytochemical prospecting for this fraction demonstrated the presence of phenols, tannins, steroids, triterpenes, anthocyanins, and flavonoids [11]. Another study showed that the these isolated compounds from MHF were efficient in reducing the lymphoproliferation and production of NO [12]. In view of these results and in order to rationalize the research of phytopharmaceuticals, which seeks more effective and less toxic natural compounds, the fraction was subjected to chromatographic fractionation and ethyl acetate fraction (MHF) was obtained, which is the object of this study.

Investigations about chemical composition found thirteen acid triterpenes in MHF indicates that this fraction is rich in pentacyclic triterpenes derived from oleanolic and ursolic acids [13]. Furthermore, film composed of chitosan and MHF was prepared for wound healing application and showed excellent capacity for stimulating repair and tissue regeneration [13].

Thus, it is vital to continue efforts to study the medicinal properties of this fraction in order to contribute to the search for new therapeutic approaches to human diseases. In addition, to support the safety of the use of this fraction, it is extremely important to carry out toxicity studies in order to identify and evaluate the possible harmful effects since information on its safety profile is not found in the literature. Thus, this study aimed to investigate the in vitro and in vivo toxicity and anti-inflammatory and analgesic actions of fraction obtained from the leaves of *Mansoa hirsuta,* a typical plant in the Brazilian semiarid region.

2. Materials and methods

2.1. Reagents

Non-tumor cells from mouse fibroblasts (NIH/3T3, ATCC CRL-1658), donated by Professor Dr. Hugo Alexandre de Oliveira Rocha from the Department of Biochemistry at the Federal University of Rio Grande do Norte, were acquired from the American Type Culture Collection (Rockville, MD, USA). 3- (4,5-dimethylazol-2-yl) - 2,5-diphenyltetrazolium bromide (MTT) 10% was acquired from Oz Biosciences (San Diego, USA). Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Sigma-Aldrich Co, (Darmstadt, Germany), carrageenan, zymosan and dimethylsulfoxide (DMSO), from Sigma-Aldrich (St. Louis, MO, USA). Thiopental (Tiopentax®) and lidocaine (Xylestesin®) was obtained from Cristália (São Paulo, Brazil).

2.2. Vegetal material

Mansoa hirsuta was collected and described by Teonildes Nunes in Brazil, Bahia, Santo Inácio, Xique-Xique road, towards Gentio do Ouro, before Gameleira do Assuruá (11°19'S, 42°40'W) and a specimen is found deposited in the Herbarium at State University of Feira de Santana (number 59456). Through the partition of the crude ethanolic extract (250 g) of *M. hirsuta* the acetate phase (40 g) was obtained, which was subjected to chromatographic fractionation on a silica gel column resulting in the ethyl acetate fraction (MHF, 15 g). This fractionation was performed by Daniel de Melo Silva (Silva, 2010) at the Natural Resources Research Laboratory (UFAL-AL). This research was registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SISGEN) registration number A350944 in accordance with the Brazilian biodiversity legislation (Federal Law nº 13.123/2015).

2.3. In vitro assays

For the analysis of the cellular viability of the MHF, cultures of non-tumor cells from mouse fibroblasts (3T3) were used, which were maintained in DMEM and supplemented with 10% FBS, in the concentration of 2.5×10^4/mL, in culture bottles of 75 cm^3 , containing 10 mL of medium. Every two days, adherent cells were treated with 0.25% (w/v) trypsin solution in PBS, centrifuged and the pellet was solubilized in fresh culture medium. Cell density was then determined by counting cells in a Neubauer chamber.

2.3.1. Cytotoxicity assay (Lethal concentration for 50% of cells - LC50)

The cytotoxicity of the extracts was established using BALB/c mice splenocytes (5×10^6 cells/well) cultured in 96 plate-well in Dulbecco's Modified Eagle'sMedium (DMEM, Sigma Chemical Co., St. Louis, MO) supplemented with 10% of fetal calf serum (FBS; Cultilab,Campinas, SP, Brazil) and gentamycin (Novafarma,Anápolis, GO, Brazil) at 50 µg/mL. MHF was evaluated in three concentrations (1, 10, and 100 µg/mL), in triplicates. A positive control was made with cells treated with a 1% saponin solution. Cultures were incubated in the presence of 3 H-thymidine (1 µCi/well) during 24 h at 37 ºC and 5% CO2. After this period, the content of the plate was harvested to determine the 3 H-thymidine incorporation using a Beta Radiation Counter (β-matrix 9600, Packard,Meriden, CT). The viability of the cells was determined by 3 H-thymidine incorporation and the cytotoxicity was calculated in relation to untreated cultures.

2.3.2. Cell Viability/MTT Assay (3- (4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide)

Non-tumor cell lines (3T3) were added in 96-well microplates at a concentration of 5.0×10^4/well in supplemented DMEM medium (10% FBS), for maintenance and cell growth, being maintained for 24 h in an oven at 36,5 °C with 3.5% CO_2. Subsequently, the medium was exchanged for DMEM without FBS and after 24 h of incubation (36.5 °C and 5% CO_2), the supernatant was removed and the MHF was added to the wells, in triplicate, at concentrations of 10, 25, 50, 100, 250, 500 µg/mL. As a positive control for cell death, cisplatin diluted in DMEM (10% FBS) was used. After 24, 48 and 72 h of incubation (36.5 °C and 5% CO_2), 10 µL of 10% MTT were added to all wells and after 4 h the supernatant was removed and 100 µL of absolute ethanol was added for solubilization of the crystals formed. Finally, the absorbance was measured in ELISA Epoch (Biotek, Winooski, USA) at 570 nm.

2.3.3. Nitric oxide assay

Peritoneal exudate cells were obtained by washing the peritoneal cavity of mice with cold Hank's balanced salt solution (HBSS; Life Technologies,GIBCO-BRL, Gaithersburg, MD) five days after injection of 3% thioglycollate in saline (1.5 mL per mouse). Peritoneal cells were washed twice with HBSS and re-suspended in RPMI medium (GIBCO-BRL) supplemented with 10% fetal calf serum (Cultilab,Campinas, SP, Brazil), L-glutamine (2 mM), RPMI1640 vitamins solution (1%) (Sigma), sodium pyruvate(1 mM), HEPES (10 mM), 2-mercaptoethanol (50 μM), and gentamycin (50 μg/mL) (Sigma, St. Louis, MO). Cells were plated (2 ×105 cells/well) in 96 well plates. After one hour of incubation at 37 oC, non-adherent cells were removed by washing with complete RPMI. Cultures were then stimulated with 1 μg/mL lipopolysaccharide (LPS from Escherichia coli serotype 0111:B4, Sigma) in combination with 5 ng/mL interferon-γ (IFN-γ;PharMingem, S. Diego, CA) and treated with MHF as described, in a final volume of 200 μL. Cell-free supernatants were collected at 24 h of culture for determination of nitrite concentration using the Griess method, as described previously (Ding et al., 1988).

2.3.4. Lymphoproliferation assay

BALB/c splenocytes suspensions were prepared in RPMI medium (Life Technologies, GIBCO-BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum (Hyclone, Logan, UT), 2 mM of L-glutamine, 0.1% RPMI 1640 vitamins solution (Sigma), 1 mM of sodium pyruvate, 10 mM of Hepes, 50 μM of 2-mercaptoethanol,and 50 μg/mL of gentamycin (Sigma). Splenocytes were cultured in 96-well plates at 4 × 105 cells/well, in 200 μL,in triplicate wells, in the presence of concanavalin A at 2 μg/mL (Con A) alone or various concentrations of MHF, as described. After 48 h, plates were pulsed with 1 μCi of 3 H-thymidine for 12 h, and proliferation was assessed by measurement of 3Hthymidine uptake, as described above. The percentage of proliferation inhibition by the fractions was determined in relation to controls stimulated by mitogen in absence of samples.

2.4. In vivo assays

2.4.1. Animals

Swiss mice (*Mus musculus*) aged 8 weeks (25–45 g) of both sexes (nulliparous and non-pregnant females) were kept in the vivarium of the Health Sciences Center (CCS) at UFRN, under controlled lighting conditions (12 h light/dark cycle) and temperature (23 ± 2 °C) receiving water and food (Presence Nutrição Animal, Paulínia, Brazil) ad libitum. This study was approved by the Ethics Committee for Animal Use (CEUA) from the Federal University of Rio Grande do Norte (088.007/2018 and 087.008/2018). On the days of the experiments, the animals were divided into their respective experimental groups and acclimated for 5 days in polypropylene cages.

2.4.2. Acute toxicity

Male and female mice (n = 6 males and 6 females per group) were divided into a control group and a test group and acclimated for 5 days in polypropylene cages. According to the guideline 423/2002 of the Organization for Economic Cooperation and Development [14], MHF (test group) was administered through orally in a single dose (50 mg/kg). Control groups were treated with DMSO 2% with the same volume and route of administration. All animals (in the test and control groups) were observed during the first 24 h, for immediate toxic signs and daily, during the remaining experimental period, for a total of 14 days, for observation of acute delayed toxic signs. The consumption of water and the body weight of the animals was measured every three days.

Mice (n = 6 per group) were divided into: female control group; male control group; female test group and male test group, which received 300 μL orally of the respective treatments, detailed below. According to the guideline 423/2002 of the Organization for Economic Cooperation and Development [14], MHF (test groups) was administered a single dose (50 mg/kg) and control groups were treated with DMSO 2%. All animals were observed during the first 24 h, for immediate toxic signs and daily, during for a total of 14 days, for observation of acute delayed toxic signs. The consumption of water and the body weight of the animals was measured every three days of the experimental period.

2.4.3. Subchronic toxicity

The mice were randomly divided into four distinct groups (n = 10/group, 5 males and 5 females) and acclimated for 5 days in polypropylene cages. Three different doses of MHF (10, 25 and 50 mg/kg) were administered in the experimental groups while the control group received the vehicle (DMSO 2%). All groups were treated orally in a single daily dose, for 30 consecutive days.

During the entire experimental period, the weight of the animals was monitored every three days, and any signs of toxicity were reported (changes in locomotion, respiratory rate, piloerection, diarrhea, alteration of muscle tone, hypnosis, seizure, hyperexcitability of the nervous system, abdominal contortions and deaths), according guideline 407/2008 and 408/2008 of the Organization for Economic Cooperation and Development [15], with modifications.

The mice were randomly divided into eight distinct groups (n = 5 per group), which received 300 μL orally of the respective treatments, detailed below: female control group (DMSO 2%); male control group (DMSO 2%); female test group (MHF 10, 25 and 50 mg/kg) and male test group (MHF 10, 25, and 50 mg/kg). All groups were treated in a single daily dose, for 30 consecutive days. During the entire experimental period, the weight of the animals was monitored every three days, and any signs of toxicity were reported (changes in locomotion, respiratory rate, piloerection, diarrhea, alteration of muscle tone, hypnosis, seizure, hyperexcitability of the nervous system, abdominal contortions and deaths), according guideline 407/2008 and 408/2008 of the Organization for Economic Cooperation and Development [15], with modifications.

2.4.4. Hematological and biochemical evaluation

After the experimental period (15th day for acute toxicity and 31st for subchronic toxicity), the animals were submitted to general anesthesia with intraperitoneal injection (i.p.) of thiopental, 30–40 mg/kg., associated with lidocaine 2% (10 mg/kg, i.p.) and euthanized by exsanguination by cardiac puncture, according to the Brazilian Society of Animal Science. Still with the animals under anesthesia, blood samples were obtained by cardiac puncture to determine biochemical and hematological parameters. Part of the blood collected was homogenized with the anticoagulant ethylenediaminetetraacetic acid 1% (EDTA) to perform the blood count by the ABX Micros 60-OT automatic analyzer (HORIBA ABX Diagnostics, Jundiaí, Brazil). As for the biochemical analysis, tubes without anticoagulant were used and the blood was centrifuged at 825 rpm for 10 min to obtain the serum. Labmax Plenno automatic biochemical analyzer (Labtest, Lagoa Santa, Brazil) and Labtest® kits were used to determine glucose, total cholesterol, triglycerides, aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine, urea, albumin, and total proteins.

2.4.5. Evaluation of organs and tissues

After collecting blood and the euthanasia of the animals; their liver, stomach, spleen and kidneys were removed, weighed and macroscopically analyzed, and then fixed in 10% buffered formaldehyde. Subsequently they were embedded in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin (H&E) and sent to the Department of Pathology at the Federal University of Rio Grande do Norte for analysis of possible tissue injuries.

2.5. Assessment of central nervous system activity

All behavioral tests were performed between 8:00 am and 12:00 pm

in an silent environment and controlled temperature (25 ± 2 °C).

2.5.1. Open field

The locomotor activity and behavior of the mice were evaluated using the open field test. The apparatus is a box made of wood, covered in formica, black, waterproof, square and containing four compartments measuring 40 cm x 40 cm x 40 cm each. A single dose of MHF (50 mg/kg) was administered through orally to the animals in the test group (n = 8), while those in the control group (n = 8) were treated with DMSO 2%. Each mouse was placed in the center of the open field and the distance covered (in meters) and the immobility time were recorded automatically every 5 min, for 30 min, using a video camera connected to an automated activity monitoring system (Anymaze, Stoelting Co., Wood Dale, IL, USA). After the behavioral evaluation of each mouse, the open field was cleaned with a 5% ethanol solution. The test was carried out in three moments: 1 h, 24 h and 7 days after the administration of MHF (50 mg/kg) or DMSO 2%.

2.5.2. Rota-rod

The evaluation of the motor coordination of the animals was performed through the rota-rod test [16]. The device (AVS Projetos, Riberão Preto, SP, Brazil) consists of a 3 cm diameter bar, with a non-slip surface, raised from the support platform by 22 cm, which rotates clockwise at different speeds, measured in rotations by minute (rpm). Right after the open field test, the same animals were trained for 2 min, left to rest for 1 min and placed on the rotating bar of the device under conditions of acceleration of 10 rpm, for 2 min. The test was carried out in three moments: 1.5 h, 24.5 h, and 7 days after the administration of MHF (50 mg/kg) or DMSO 2% and immediately after the analysis of animal behavior in the open field. At the end, the number of falls and the time the animal remained in the bar were recorded.

2.6. Assessment of anti-inflammatory activity

2.6.1. Carrageenan-induced paw edema model

The carrageenan-induced paw edema model in mice was performed [17] with modifications. The animals received a subplantar injection (50 µL) of lambda-carrageenan 1% (Sigma® Aldrich, USA), except for those in the negative control group that received 50 µL of PBS, pH 7.4. The mice were then treated by orally with 500 µL of saline solution, dexamethasone 2.0 mg/kg or MHF at doses of 50, 20, and 10 mg/kg. Paws were measured at 0 (right after treatment) 1, 2, 3, and 4 h after the administration of carrageenan, with a digital micrometer (Digimess, 100.174BL, Brazil). The paw edema was expressed in millimeters (mm) and calculated as the percentage of the edema. The area under the curve of the time-course (AUC 0–4 h) was also determined using the trapezoidal rule.

The carrageenan-induced paw edema model in mice was performed [17] with modifications. The mice were randomly divided into six distinct groups (n = 5 per group): negative control (PBS), positive control (carrageenan) and test groups (dexamethasone 2.0 mg/kg or MHF at doses of 10, 25, and 50 mg/kg). First, the thickness of the right paws of each animal was measured (baseline) with a digital micrometer (Digimess, 100.174BL, Brazil). Then, the groups received (in the same measured paw) a subplantar injection (50 µL) of lambda-carrageenan 1% (Sigma® Aldrich, USA), except for negative group (received 50 µL of PBS, pH 7.4). Subsequently, the mice were treated by orally with 300 µL of PBS, dexamethasone 2.0 mg/kg or MHF at doses of 10, 25, and 50 mg/kg, according to their respective experimental groups. Paws were measured at 0 (right after treatment) 1, 2, 3, and 4 h after the administration of carrageenan. The paw edema was expressed in millimeters (mm) and calculated as the percentage of the edema. The area under the curve of the time-course ($AUC_{0–4\ h}$) was also determined using the trapezoidal rule.

2.6.1.1. Dosage of the myeloperoxidase enzyme. After performing the paw edema test, the animals were euthanized and their right hind legs were removed and frozen to determine the levels of myeloperoxidase (MPO) [18]. Samples weighting 20–30 mg were perforated with surgical scissors in 1:20 hexadecyltrimethylammonium bromide buffer (p/v), homogenized with a micro homogenizer and subjected to sonification (Schuster L200, Santa Maria, Brazil) for five minutes at environmental temperature. Then, for two days, the samples went through a triple freezing and thawing process in a refrigerated microcentrifuge (Novatécnica NT 805, Piracicaba, Brazil) at 8285 rpm at 4 ºC for ten minutes. Next, 50 µL of the supernatant from each sample was placed in a 96-well microplate (Epoch-Biotek, Winooski, VT, USA), along with 150 µL of the staining reagent (o-dianisidine dihydrochloride, potassium phosphate buffer and 0,05% 1% hydrogen peroxide). The absorbance determined at 450 nm in a microplate reader (Mindray MR-96, Mahwah, USA) through two-point kinetics (0–2 min), where the results were interpolated with a standard curve. A unit of MPO activity was defined as the amount of enzyme that degrades 1 µmol of hydrogen peroxide. The degradation of 1 µmol of hydrogen peroxide was detected as a 1.13×10^{-2} change in absorbance per minute. The results were expressed as units of MPO activity per gram of paw tissue (UMPO/g).

2.6.2. Zymosan-induced air-pouch model

The zymosan-induced air-pouch model was performed [19], with adaptations. All animals received 5 mL of sterile air on the back, subcutaneously, to form the pouch. After 3 days, another 2.5 mL of sterile air was injected in the same location, to reinforce it. Six days after the first application, the animals were treated orally with 300 µL of saline solution, MHF (10, 25, and 50 mg/kg) and dexamethasone (2 mg/kg). Thirty minutes later, 1.0 mL of zymosan solution (1 mg/mL) was injected into the air pouch formed. After 6 h, the animals were euthanized with an overdose of thiopental (120 mg/kg, i.p.) associated with lidocaine 2% (10 mg/g, i.p.). The exudate, in each air pouch, was collected by aspiration with 2 mL of sterile saline solution (0.9 mg/dL) and centrifuged (1500 rpm/10 min/4ºC). The cell *pellet* was diluted with Turk's solution (1:10 v/v) and used for leukocyte counting in a Neubauer chamber, with the aid of a Nikon ECLIPSE E200® microscope (Minato, Tokyo, Japan) with 40x magnification. The results were expressed as number of leukocytes per mL.

The zymosan-induced air-pouch model was performed [19], with adaptations. All animals received 5 mL of sterile air on the back, subcutaneously, to form the pouch. After 3 days, another 2.5 mL of sterile air was injected in the same location, to reinforce it. Six days after the first application, the animals were randomly divided into six distinct groups (n = 5 per group): negative control (PBS), positive control (zymosan) and test groups (dexamethasone 2.0 mg/kg or MHF at doses of 10, 25, and 50 mg/kg). Then, 1.0 mL of zymosan solution (1 mg/mL) was injected into the air pouch formed, except for negative group (received 1 mL of PBS, pH 7.4). Subsequently, the mice were treated by orally with 300 µL of PBS, dexamethasone 2.0 mg/kg or MHF at doses of 10, 25, and 50 mg/kg, according to their respective experimental groups. After 6 h, the animals were euthanized with an overdose of thiopental (120 mg/kg, i.p.) associated with lidocaine 2% (10 mg/g, i.p.). The exudates were harvested by washing with 2 mL of PBS and centrifuged at and centrifuged (1500 rpm/10 min/4ºC). The cell *pellet* was diluted with Turk's solution (1:10 v/v) and used for leukocyte counting in a Neubauer chamber, with the aid of a Nikon ECLIPSE E200® microscope (Minato, Tokyo, Japan) with 40x magnification. The results were expressed as number of leukocytes per mL. The resulting supernatant was used to measure total proteins.

2.6.2.1. Dosage of total proteins. To determine total proteins, supernatants were collected and 10 µL of each sample was added to 96-well plates, followed by the addition of 200 µL of Bradford's reagent. The results were obtained using an ELISA microplate reader (BioTek,

Winooski, VT, USA) at 595 nm and expressed as μg/mL.

2.7. Evaluation of antinociceptive activity

2.7.1. Abdominal writhing test

Male and female mice aged 6–8 weeks (mean weight 30 g) were randomly divided into 5 groups (n = 5): and treated orally with MHF (10, 25, and 50 mg/kg), indomethacin (25 mg/kg) and vehicle (saline solution). After 30 min it was administered via intraperitoneal (i.p.) acetic acid (0.6% v/v), diluted in distilled water. Soon after, the animals were observed and the number of abdominal contortions was counted over a period of 20 min [20].

2.7.2. Formalin test

Male and female mice aged 6–8 weeks (mean weight 30 g) were randomly divided into 5 groups (n = 5) and treated orally with control (saline solution), codeine (7.5 mg/kg), indomethacin (25 mg/kg) and MHF (10, 25, and 50 mg/kg). After 1 h, 20 μL of formalin (2.5% in 0.9% saline) was injected, subplantarly, into their right rear paw. Then, the time (seconds) that the animal spent licking and biting its paw was recorded. This response was measured for 5 min and between 15 and 30 min after the formalin injection [21].

2.8. Statistical analysis

The results were expressed as mean ± standard deviation. The Shapiro-Wilk test was used to observe the normal distribution of variables. One-way analysis of variance (ANOVA) was used to compare means of normal distribution data followed by the Tukey test. Nonparametric data were analyzed using the Kruskal-Wallis and U-Mann-Whitney tests. GraphPad Prism software (San Diego, CA, USA) was used for tabulation and data analysis. Values of * $P < 0.05$ were considered statistically significant.

3. Results

3.1. Cell viability

The assessment of cytotoxicity was tested to choose the MHF concentrations to be used in further assays. The chosen concentrations were those that produced a cytotoxic level lower than 30%. The concentration of 0.01 mg/mL for MHF with 18.62% was determined.

3.2. Cell viability/MTT Assay

In the periods of 24, 48, and 72 h, the fraction showed no impairment of cell viability in any concentration tested (Fig. 1).

3.3. Inhibition of nitric oxide production by fractions of M. hirsuta leaves

Mouse peritoneal exudate macrophages were stimulated with IFN-γ and LPS in the presence or absence of MHF for 24 h. The nitric oxide production was estimated by measuring nitrite using the Griss method. The results were obtained by measuring the values of the stimulated control and cultured in the absence of the fraction. A significant inhibition of nitric oxide production was caused by MHF (57.92%, 0.01 mg/mL).

3.4. Inhibition of lymphoproliferation of M. hirsuta leaf partition fractions

Mouse spleen cells were stimulated by the mitogen concanovalin A in the presence or absence of the fractions for 48 h. Inhibition of lymphoproliferation was determined based on the values of tritiated thymidine incorporation by stimulated cells and the absence of the fraction. To confirm the suppressive effect, the fraction was submitted to

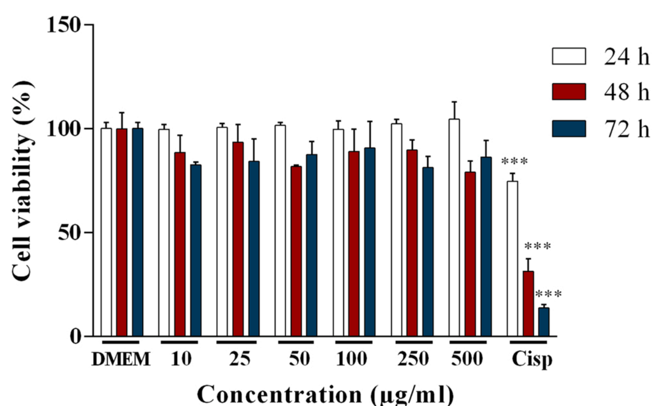

Fig. 1. Cell viability of 3T3 non-tumor cells incubated with MHF at concentrations of 10, 25, 50, 100, 250, and 500 μg/mL in 24, 48, and 72 h. Cisplatin (Cisp) was used as a positive control for cell death and DMEM (10% FBS) was used as a negative control. * $p < 0.0001$ when compared to the negative control.

lymphoproliferation by antigen stimulation and mixed lymphocyte reactions, in which the existence or not of the activity in question could be observed. The results obtained were 99.92% inhibition for MHF.

3.5. Acute and subchronic toxicity

In the acute and subchronic toxicity tests, no animals died and no immediate and delayed acute toxic signs was observed, during the experimental period. In addition, no significant difference was observed in body weight (Fig. 2), water and feed consumption (Table 1), and no behavioral changes.

In addition, the analysis of the relative weights of all organs (Table 2) showed that only the stomach of females treated with MHF at doses of 25 and 50 mg/kg in the subchronic toxicity test was heavier.

The results of the biochemical parameters are shown in Table 3. In the acute toxicity test, there was a decrease in total cholesterol in males who received a dose of 50 mg/kg when compared to the control. In subchronic toxicity, there was an increase in urea in males (10, 25, and 50 mg/kg) and a decrease in creatinine in males and females (25 and 50 mg/kg).

Table 4 represents the hematological parameters of mice treated with control and MHF in the acute and subchronic toxicity test. In the acute exposure to MHF, males showed an increase in platelets and in the amplitude of the red blood cell distribution width (RDW) and a decrease in mean corpuscular volume (MCV). In females treated with MHF, there was a significant increase in erythrocytes. After exposure for 30 days, there was a decrease in hemoglobin and mean corpuscular hemoglobin (MCH) in males treated with 50 mg/kg and a decrease in RDW in males treated with 10 mg/kg. Also, in males, there was a significant decrease in lymphocytes (25 mg/kg), monocytes (10, 25, and 50 mg/kg) and granulocytes (25 and 50 mg/kg). Females, on the other hand, had a decrease in leukocytes and an increase in RDW in the treatment with 25 mg/kg. The other hematological parameters did not show statistically significant results, in both sexes, when compared to the control group.

At necropsy, no significant macroscopic changes were observed in the organs of mice treated with MHF when compared to control groups, both in the acute and subchronic toxicity tests.

Histopathological examination was performed and in the acute (Fig. 3A) and subchronic (Fig. 4) toxicity test, the liver tissue of control and treated male and female mice showed extramedullary hematopoiesis and edematous degeneration/steatosis. In females (25 and 50 mg/kg) mild steatosis was found. Histological analysis of the kidneys (Fig. 3B and Fig. 5) demonstrated mild congestion in all animals analyzed. The histology of the spleen and stomach of the animals

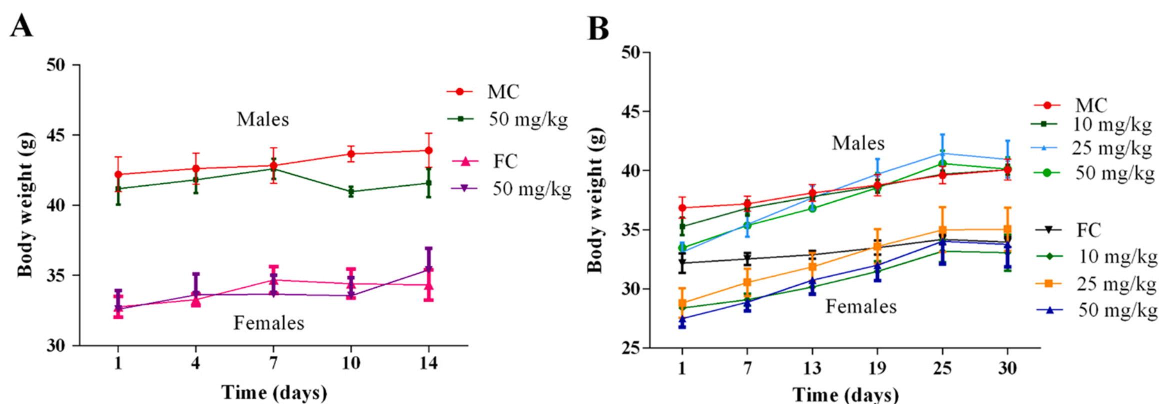

Fig. 2. Effect of the MHF administered orally in mice on their body weight in the acute toxicity test (A) and in the subchronic toxicity test (B). The results represent the mean ± standard deviation. * ** $p < 0.001$, * * $p < 0.01$, and * $p < 0.05$ when comparing the groups treated with MHF and 2% DMSO. MC = male control, FC = female control.

Table 1
Feed intake (g) and water (mL) of the control and treated groups with MHF.

Parameters	Acute toxicity		Subchronic toxicity			
	Control	50 mg/kg	Control	10 mg/kg	25 mg/kg	50 mg/kg
Males						
Feed consumption (g)	6.45 ± 0.41	6.75 ± 0.52	6.20 ± 0.41	8.49 ± 0.77	6.53 ± 0.51	6.16 ± 0.68
Water consumption (mL)Females	8.61 ± 1.46	8.33 ± 1.36	9.66 ± 1.74	12.94 ± 2.25	10.05 ± 2.57	10.27 ± 1.14
Feed consumption (g)	5.65 ± 1.57	5.30 ± 0.86	5.89 ± 0.73	5.58 ± 0.81	5.44 ± 0.67	6.20 ± 1.39
Water consumption (mL)	7.50 ± 1.06	8.33 ± 0.27	10.66 ± 2.41	11.44 ± 0.74	8.11 ± 2.77	9.27 ± 1.52

* $p < 0.05$ when compared to the control group.

Table 2
Weight of relative organs of mice in the control group and treated orally with the MHF in acute and subchronic toxicity.

Parameters	Acute toxicity		Subchronic toxicity			
	Control	50 mg/kg	Control	10 mg/kg	25 mg/kg	50 mg/kg
Males						
Liver (%)	4.81 ± 2.51	4.67 ± 0.54	5.64 ± 0.40	5.26 ± 0.16	5.37 ± 0.47	4.84 ± 0.30
Right kidney (%)	0.79 ± 0.09	0.74 ± 0.14	0.78 ± 0.08	0.90 ± 0.09	0.85 ± 0.16	0.85 ± 0.14
Left kidney (%)	0.80 ± 0.07	0.73 ± 0.17	0.75 ± 0.06	0.87 ± 0.05	0.81 ± 0.14	0.90 ± 0.13
Stomach (%)	1.02 ± 0.12	1.43 ± 0.39	1.24 ± 0.24	1.30 ± 0.39	0.93 ± 0.12	0.98 ± 0.16
Spleen (%)	0.48 ± 0.10	0.39 ± 0.05	0.59 ± 0.12	0.50 ± 0.09	0.53 ± 0.14	0.44 ± 0.07
Females						
Liver (%)	4.34 ± 2.20	5.71 ± 0.75	5.38 ± 0.37	5.28 ± 0.41	5.29 ± 0.81	4.77 ± 0.61
Right kidney (%)	0.60 ± 0.03	0.64 ± 0.08	0.77 ± 0.30	0.73 ± 0.09	0.66 ± 0.15	0.67 ± 0.05
Left kidney (%)	0.61 ± 0.08	0.55 ± 0.07	0.60 ± 0.07	0.64 ± 0.07	0.59 ± 0.10	0.62 ± 0.10
Stomach (%)	1.09 ± 0.49	1.48 ± 0.53	1.73 ± 0.24	1.55 ± 0.24	1.17 ± 0.27 *	1.18 ± 0.31 *
Spleen (%)	0.57 ± 0.09	0.65 ± 0.21	0.53 ± 0.04	0.48 ± 0.14	0.60 ± 0.24	0.49 ± 0.18

The results were expressed as mean ± standard deviation. * $p < 0.05$ when compared to the control group.

showed a structure of normal architecture in all groups.

3.6. Assessment of central nervous system activity

The results of the evaluation in the open field test showed that the acute administration of MHF (50 mg/kg) did not affect the traveled distance (Fig. 6A) nor the mobile time (Fig. 6B), thus not altering the locomotor activity and behavior of the mice, when compared to the control group. There was also no change in motor coordination and balance of animals treated with MHF since no differences were found in time of permanence (Fig. 6C) and number of falls (Fig. 6D) in the rotating drum of the rota-rod test.

3.7. Assessment of anti-inflammatory activity

To evaluate the anti-inflammatory activity of MHF, the carrageenan-induced paw edema model was performed. Carrageenan induced an intense edema formation from the first hour of its application, maintaining this action after 4 h, as can be seen in Fig. 7A. The groups that received carrageenan and were post treated with MHF at doses of 25 and 50 mg/kg, presented the formation of edema significantly decreased, from the second hour of evaluation, when compared to the positive control group (carrageenan). Dexamethasone 2 mg/kg inhibited significantly the second hour analyzed and the group that received PBS, as expected, did not cause significant edema formation since time 0, when compared to the carrageenan group. Taking into account, the progress of the edema reduction within the course of 4 h (ASC $_{0\text{–}4\ h}$), an anti-edematogenic effect could be observed among the different doses of the MHF, indicating that they were able to reduce the edema (Fig. 7B). In addition, MHF was able to significantly decrease the levels of MPO production when compared to the carrageenan group and similarly to dexamethasone ($p > 0.05$), at doses of 25 and 50 mg/kg (Fig. 7C).

Table 3
Biochemical parameters of mice in the control group and treated with the MHF in acute and subchronic toxicity.

Parameters	Acute toxicity		Subchronic toxicity			
	Control	50 mg/kg	Control	10 mg/kg	25 mg/kg	50 mg/kg
Males						
Glucose (mg/dL)	183.28 ± 38.02	182.00 ± 19.81	233.25 ± 43.18	185.20 ± 26.54	206.00 ± 29.23	215.40 ± 23.75
Triglycerides (mg/dL)	93.25 ± 48.12	82.00 ± 34.70	115.75 ± 12.03	105.40 ± 49.69	81.80 ± 19.53	83.20 ± 22.06
Total cholesterol (mg/dL)	126.18 ± 12.28	98.00 ± 9.19 *	107.75 ± 9.91	107.00 ± 12.70	100.40 ± 10.31	93.80 ± 4.38
Total proteins (mg/dL)	5.51 ± 0.24	4.86 ± 0.49	4.84 ± 0.08	5.37 ± 0.16	5.60 ± 0.53	5.30 ± 0.35
Albumin (mg/dL)	2.06 ± 0.11	1.99 ± 0.14	1.86 ± 0.07	1.96 ± 0.14	2.09 ± 0.19	1.95 ± 0.12
AST (U/L)	149.58 ± 39.37	140.40 ± 24.74	164.00 ± 17.45	158.40 ± 25.38	177.80 ± 60.29	148.00 ± 18.54
ALT (U/L)	56.14 ± 8.05	50.00 ± 8.39	54.75 ± 8.01	52.40 ± 6.91	53.40 ± 7.79	58.00 ± 12.36
Urea (mg/dL)	41.75 ± 7.59	59.00 ± 8.86	40.50 ± 8.50	55.60 ± 2.70 *	57.00 ± 10.22 *	53.80 ± 3.19 *
Creatinine (mg/dL)	0.35 ± 0.06	0.29 ± 0.04	0.48 ± 0.22	0.26 ± 0.03 *	0.28 ± 0.03 *	0.31 ± 0.04
Females						
Glucose (mg/dL)	166.20 ± 50.12	192.20 ± 16.51	218.75 ± 29.13	196.20 ± 19.08	198.40 ± 29.57	227.80 ± 19.88
Triglycerides (mg/dL)	55.25 ± 13.30	90.00 ± 8.28	72.00 ± 11.51	99.20 ± 21.98	82.40 ± 10.85	45.20 ± 0.83
Cholesterol (mg/dL)	93.00 ± 9.92	77.20 ± 16.78	81.25 ± 7.80	93.80 ± 4.38	89.80 ± 7.59	85.00 ± 9.13
Total proteins (mg/dL)	5.17 ± 0.16	4.49 ± 0.29	5.30 ± 0.24	5.56 ± 0.33	5.88 ± 0.62	5.67 ± 0.41
Albumin (mg/dL)	2.35 ± 0.15	2.26 ± 0.19	2.32 ± 0.09	2.27 ± 0.17	2.22 ± 0.14	2.30 ± 0.07
AST (U/L)	160.00 ± 92.98	178.00 ± 38.04	191.50 ± 82.72	183.40 ± 76.41	205.00 ± 25.42	184.20 ± 41.43
ALT (U/L)	59.20 ± 14.16	61.00 ± 21.22	58.75 ± 17.21	61.60 ± 9.55	66.60 ± 11.50	88.60 ± 32.54
Urea (mg/dL)	43.50 ± 13.77	47.00 ± 8.00	35.75 ± 7.13	43.00 ± 2.73	41.60 ± 4.56	42.80 ± 4.02
Creatinine (mg/dL)	0.28 ± 0.04	0.24 ± 0.06	0.45 ± 0.17	0.25 ± 0.02 *	0.24 ± 0.05 *	0.30 ± 0.05

The results were expressed as mean ± standard deviation. * $p < 0.05$ when compared to the control group. ALT = Alanine aminotransferase; AST = Aspartate aminotransferase.

Table 4
Hematological parameters of mice in the control group and treated orally with the MHF in acute and subchronic toxicity.

Parameters	Acute toxicity		Subchronic toxicity			
	Control	50 mg/kg	Control	10 mg/kg	25 mg/kg	50 mg/kg
Males						
Leukocytes ($x10^3/mm^3$)	3.24 ± 1.38	3.40 ± 1.29	2.37 ± 0,72	3.35 ± 0.12	3.55 ± 0.49	2.87 ± 1.00
Erythrocytes ($x10^3/mm^3$)	9.13 ± 0.47	9.35 ± 0.60	9.37 ± 1.05	9.45 ± 0.35	9.53 ± 0.64	8.80 ± 0.35
HGB (g/dL)	15.28 ± 2.36	14.50 ± 0.81	16.07 ± 1.60	14.56 ± 1.14	15.24 ± 1.52	13.62 ± 0.70 *
HCT (%)	50.78 ± 5.86	49.70 ± 4.34	52.40 ± 5.87	50.12 ± 3.24	50.24 ± 5.23	45.60 ± 3.47
PLT ($x10^3/mm^3$)	563.60 ± 89.76	775.00 ± 80.01 *	576.00 ± 105.30	611.00 ± 95.67	586.00 ± 131.02	703.60 ± 77.47
MCV (fl)	57.60 ± 5.12	51.40 ± 2.30 *	55.75 ± 1.50	53.00 ± 2.00	52.60 ± 2.07	51.80 ± 3.63
MCH (pg)	16.78 ± 2.70	15.96 ± 1.16	17.20 ± 0.83	16.22 ± 0.68	15.86 ± 0.65	14.80 ± 1.08 *
MCHC (g/dL)	29.32 ± 5.04	30.48 ± 0.57	30.70 ± 0.98	29.96 ± 0.68	29.54 ± 1.08	30.66 ± 1.08
RDW (%)	13.86 ± 0.86	15.42 ± 0.25 *	13.95 ± 0.40	15.56 ± 0.45 *	15.02 ± 0.67	14.44 ± 0.46
MPV	7.30 ± 0.45	7.34 ± 0.27	6.75 ± 0.59	6.24 ± 1.49	7.04 ± 0.81	6.64 ± 0.41
LYM (%)	75.64 ± 4.11	81.50 ± 4.36	80.17 ± 5.54	71.06 ± 2.27	75.58 ± 12.72	71.82 ± 4.14
MON (%)	16.40 ± 4.73	12.94 ± 4.26	11.72 ± 2.93	15.68 ± 1.07	13.46 ± 6.15	16.18 ± 0.78
GRA (%)	7.96 ± 3.00	5.84 ± 0.97	8.35 ± 3.05	14.24 ± 0.99	8.56 ± 7.16	11.92 ± 3.23
LYM ($x10_3/mm^3$)	2.42 ± 1.15	2.42 ± 1.39	7.70 ± 1.57	4.48 ± 4.47	2.00 ± 0.96 *	5.06 ± 4.71
MON	0.46 ± 0.24	0.38 ± 0.13	1.05 ± 0.34	0.34 ± 0.16 *	0.40 ± 0.26 *	0.42 ± 0.21 *
GRA ($x10^3/mm^3$)	0.36 ± 0.11	0.30 ± 0.07	0.90 ± 0.21	0.62 ± 0.13	0.36 ± 0.29 *	0.42 ± 0.13 *
Females						
Leukocytes ($x10^3/mm^3$)	2.78 ± 0.94	4.52 ± 2.08	4.40 ± 1.37	3.72 ± 0.27	2.40 ± 0.25 *	3.08 ± 0.82
Erythrocytes ($x10^3/mm^3$)	8.83 ± 0.29	9.96 ± 0.61 *	9.49 ± 0.89	9.82 ± 0.29	9.63 ± 0.44	9.28 ± 0.55
HGB(g/dL)	15.58 ± 0.74	16.30 ± 0.55	16.07 ± 0.97	14.16 ± 1.17	15.74 ± 0.36	14.30 ± 1.07
HCT(%)	50.00 ± 2.84	55.93 ± 2.70	51.45 ± 4.77	53.02 ± 1.28	51.78 ± 1.55	48.04 ± 2.50
PLT ($x10^3/mm^3$)	649.98 ± 14614	521.40 ± 76.66	583.50 ± 39.48	586.60 ± 2462	610.40 ± 41.74	646.40 ± 4702
MCV (fl)	56.18 ± 2.77	54.60 ± 2.30	54.25 ± 2.21	54.00 ± 2.55	53.80 ± 1.48	51.80 ± 2.77
MCH (pg)	17.08 ± 1.10	15.56 ± 0.80	17.12 ± 0.71	15.02 ± 1.43	15.92 ± 0.19	15.06 ± 0.53
MCHC (g/dL)	30.96 ± 0.94	28.88 ± 0.54	31.62 ± 0.82	27.86 ± 2.42 *	28.34 ± 2.56	29.50 ± 1.42
RDW (%)	14.24 ± 1.01	15.32 ± 0.60	14.10 ± 0.28	15.26 ± 0.43	16.46 ± 0.82 *	15.22 ± 1.09
MPV	7.46 ± 0.55	8.46 ± 1.48	7.48 ± 1.37	6.90 ± 0.14	7.46 ± 0.42	7.64 ± 0.65
LYM (%)	81.52 ± 3.34	80.04 ± 9.97	85.10 ± 4.72	82.20 ± 3.27	69.92 ± 10.04	73.88 ± 7.53
MON (%)	11.50 ± 0.62	10.50 ± 3.63	8.90 ± 1.77	11.10 ± 1.23	13.82 ± 3.58	13.50 ± 4.01
GRA (%)	7.20 ± 3.39	9.32 ± 7.03	6.00 ± 3.62	10.48 ± 2.23	15.54 ± 7.62	10.50 ± 2.74
LYM ($x\ 10^3/mm^3$)	2.40 ± 0.66	2.94 ± 2.36	3.65 ± 1.13	2.96 ± 0.50	1.46 ± 0.28	1.84 ± 0.60
MON	0.40 ± 0.15	0.36 ± 0.18	0.35 ± 0.19	0.26 ± 0.11	0.38 ± 0.19	0.50 ± 0.25
GRA ($x10^3/mm^3$)	0.36 ± 0.27	0.42 ± 0.10	0.40 ± 0.16	0.36 ± 0.08	0.44 ± 0.23	0.46 ± 0.18

The results were expressed as mean ± standard deviation.
* $p < 0.05$ when compared to the control group. HGB, hemoglobin; HCT, hematocrit; PLT, platelets; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, range of distribution of red blood cells; MPV, mean platelet volume; LIN, lymphocytes; MON, monocytes; GRA, granulocytes.

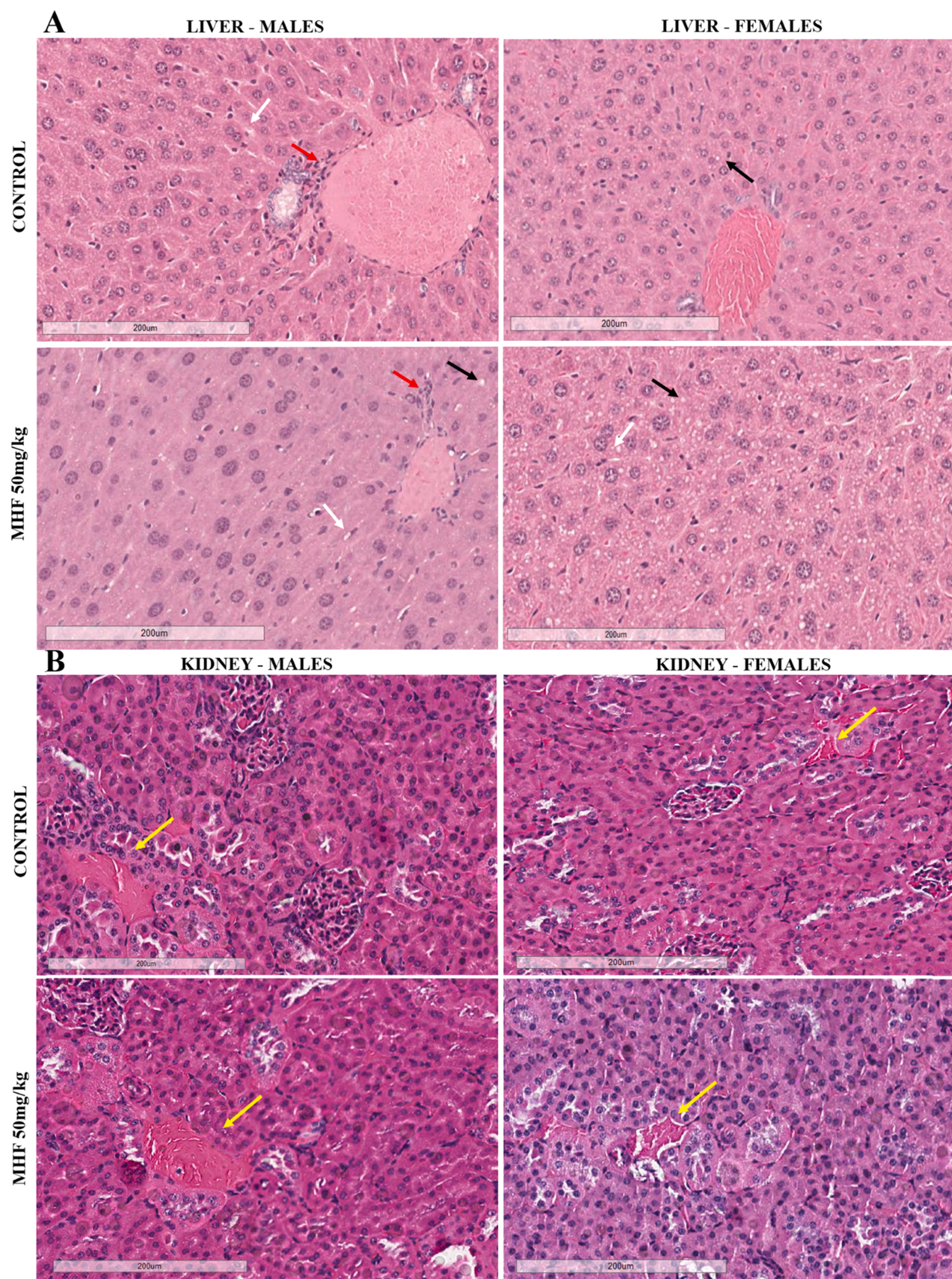

Fig. 3. Histopathological examination of the liver (A) and kidneys (B) obtained from mice treated with DMSO 2% and MHF in the acute (50 mg/kg) toxicity test. The red, white, black and yellow arrows indicate extramedullary hematopoiesis, edematous degeneration, steatosis and mild congestion, respectively. H&E coloring. The bar indicates 200 µm. For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.

In the air pouch model, data revealed that zymosan caused an intense leukocyte migration accompanied by an expressive leakage of proteins into the cavity formed in the animals (Figs. 7D and 7E). In contrast, the groups treated with different doses of MHF (10, 25 and 50 mg/kg), were able to significantly inhibit both leukocyte migration (62%, 73%, and 73%, respectively, see Table 5) and protein leakage caused by the inflammatory, demonstrating the anti-inflammatory activity of the post-treatment of MHF.

3.8. *Evaluation of antinociceptive activity*

As seen in Fig. 8A, oral treatment with MHF at doses of 25 and

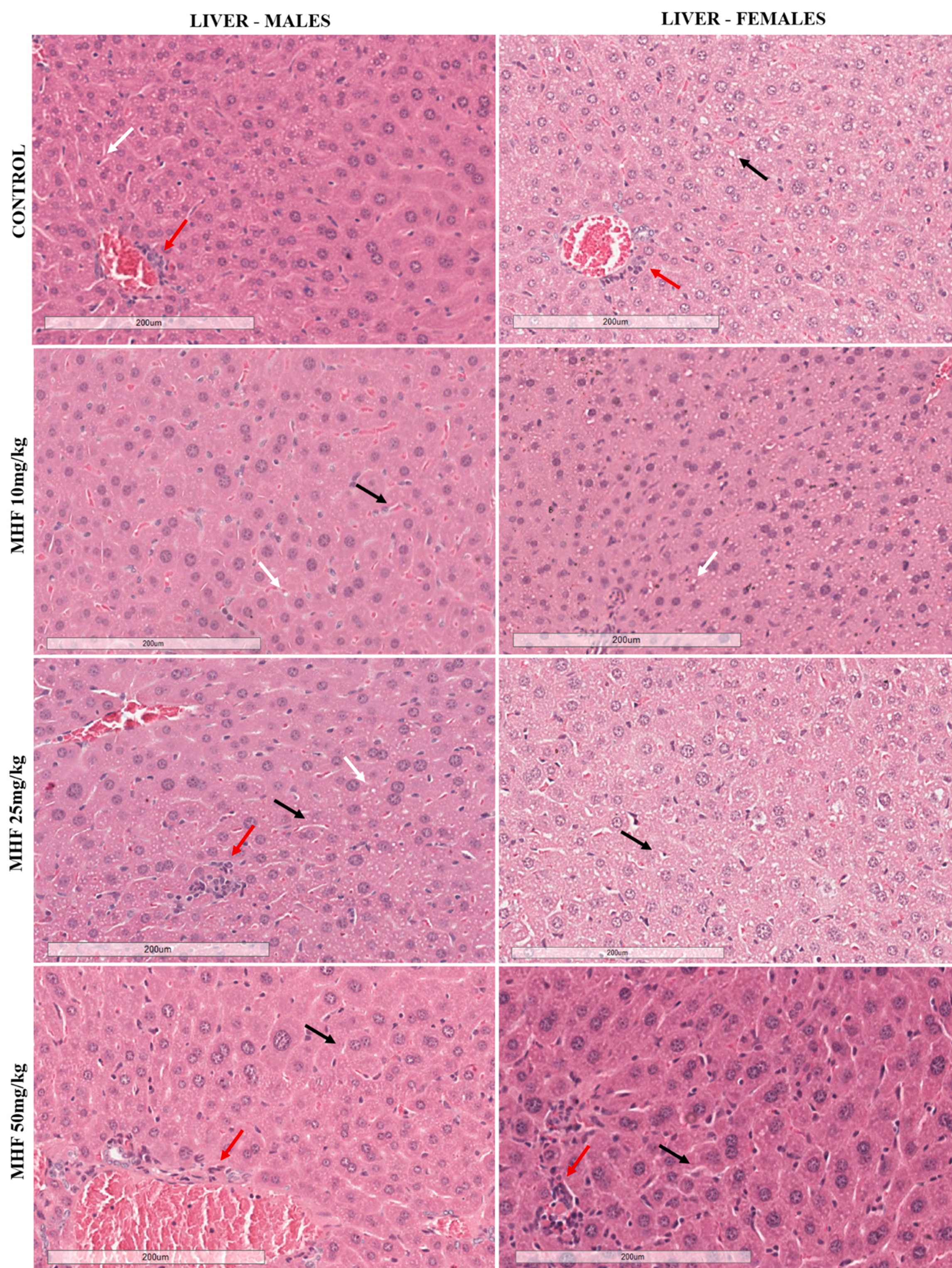

Fig. 4. Histopathological examination of the liver obtained from mice treated with 2% DMSO and MHF in the subchronic (10, 25, and 50 mg/kg) toxicity test. The red, white and black arrows indicate extramedullary hematopoiesis, edematous degeneration and steatosis respectively. H&E coloring. The bar indicates 200 μm. For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.

50 mg/kg significantly reduced abdominal writhing when compared to the control group. Likewise, indomethacin (25 mg/kg) induced significant inhibition of the writhing induced by the acetic acid.

In the formalin test, as seen in Fig. 8B, MHF demonstrated a significant reduction in licking time in the time spent by the mice licking their right paw in the late inflammatory response phase (Phase 2). Indomethacin (25 mg/kg) also caused significant pain inhibition only in the inflammatory phase, whereas morphine (7.5 mg/kg), as expected, inhibited both the neurogenic phase and the inflammatory phase.

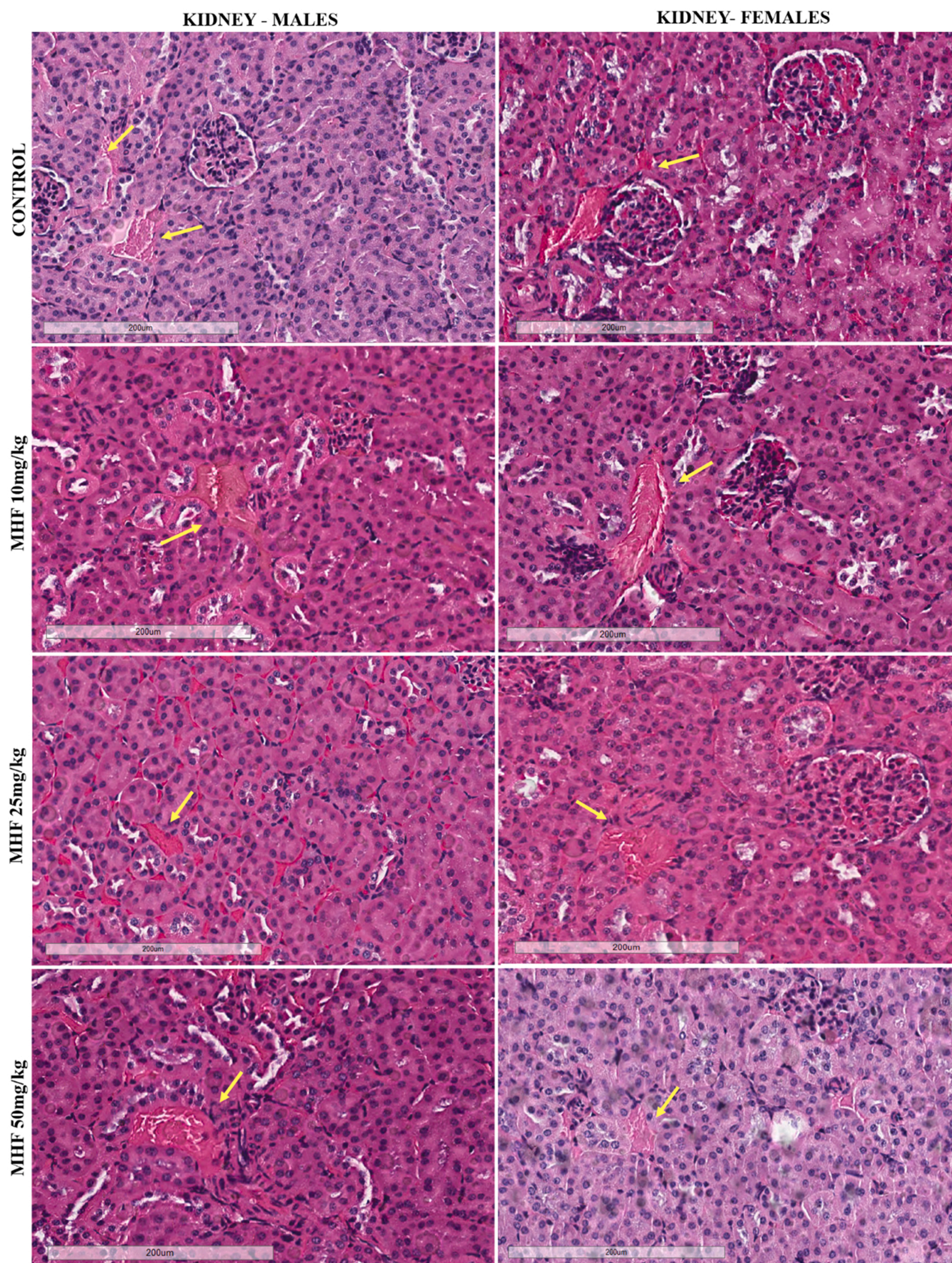

Fig. 5. Histopathological examination of the kidney obtained from mice treated with DMSO 2% and MHF in the subchronic (10, 25, and 50 mg/kg) toxicity test. The yellow arrows indicate mild congestion, respectively. H&E coloring. The bar indicates 200 μm. For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.

4. Discussion

Medicinal plants are potential sources of new pharmacological molecules and are also used as a valuable therapeutic resource for many communities in developing countries. However, they are often used based only on popular knowledge and little is known about their mechanisms of action, effectiveness, and safety. Thus, this study evaluated the toxicological and the pharmacological effects of fraction obtained from the leaves of *Mansoa hirsuta* D.C. since bibliographic research demonstrates that there are no previous studies on possible harmful effects of this important endemic species in the semiarid region of Bahia.

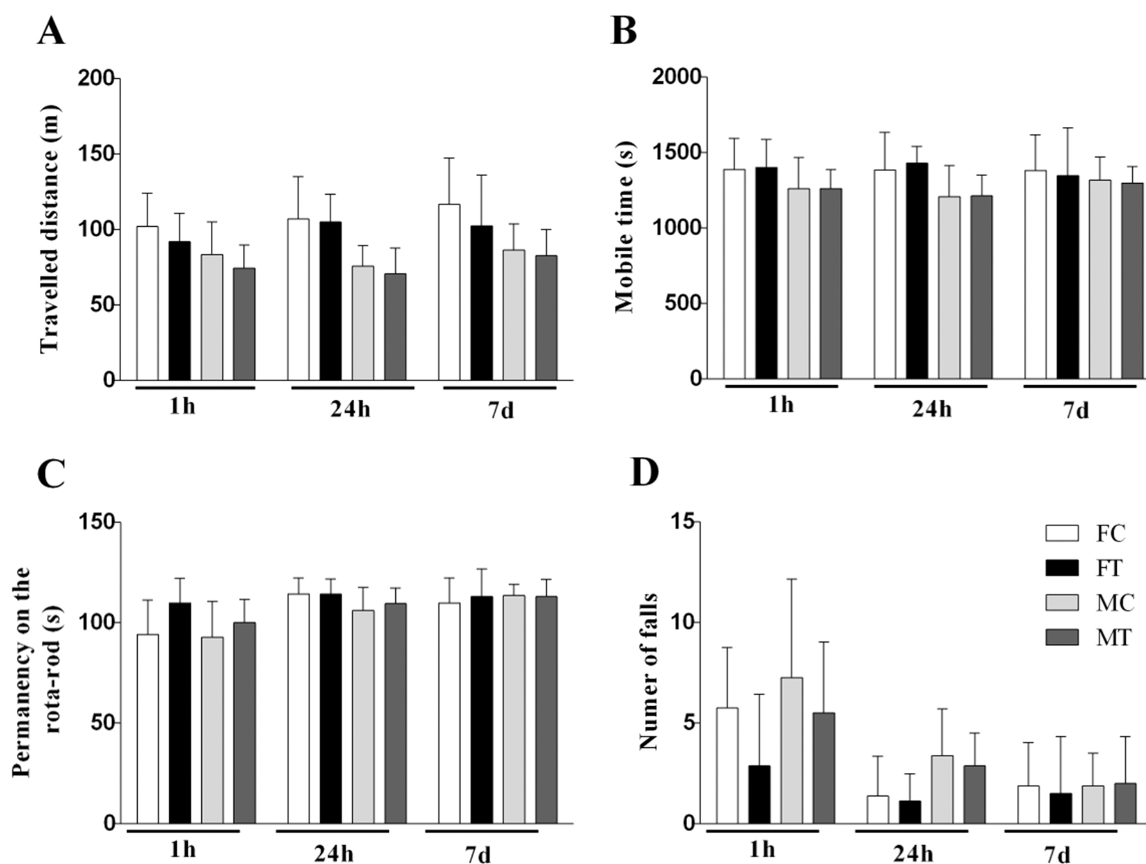

Fig. 6. Effect of the MHF administered orally in mice in the traveled distance (A) and in the mobile time (B) in the open field test and in the sustained time (C) and in the number of falls (D) in the rota-rod test. The mice were pre-treated with vehicle (control group DMSO 2%) and MHF (50 mg/kg) and subsequently placed in the box and rota-rod in three moments: 1 h, 24 h and 7 days. The results represent the mean ± standard deviation (n = 8). * ** $p < 0.001$, * * $p < 0.01$, and * $p < 0.05$ when comparing the groups treated with MHF and 2% DMSO. MC = male control, MT = male test, FC = female control, FT = female test.

As a basis for planning other tests and observing safety in healthy tissues, the cytotoxicity of MHF in 3T3 cells was evaluated by the MTT assay, a method used to analyze cell viability. This assay is based on the metabolic reduction of soluble MTT salt in formazan, measured spectrophotometrically, reflecting the activity of mitochondrial dehydrogenase that directly and proportionately represents the number of viable cells [22,23]. Treating 3T3 cells with MHF for 24, 48 and 72 h did not affect their cell viability. These results indicate that MHF is not toxic for these lines of non-tumor cells. In a previous study, the cytotoxic potential of the MHF against murine V79 culture fibroblasts was verified. The results obtained demonstrated that there was no impairment of cell viability in concentrations up to 100 μg/mL [11]. Furthermore, in this study it was observed that MHF at a concentration of 0.01 mg/mL presented a cytotoxic level of 18.62%. Therefore, considering the results of the in vitro studies, this concentration was used in subsequent tests.

In this study, MHF significantly inhibited nitric oxide production (57.92%, 0.01 mg/mL). NO inhibitor compounds generally have immunomodulatory potential, and in the case of this work it was observed that the same fraction with inhibitory activity of this free radical also has an inhibiting effect on lymphocytes (COSTA, 2004).

In fact, in previous studies, the UPLC-QTOF-MS/MS analysis indicated that MHF is rich in triterpenes that can be derived from ursolic and oleanolic acids [13]. These triterpenes are known for their various pharmacological properties, including anti-inflammatory, analgesic, antineoplastic, antifungal, hepatoprotective, antioxidant, antimicrobial, immunomodulatory, anti-obesity, anti-aging, hypolipidemic and neuroprotective [54,55].

The acute toxicity test represents the first analysis performed in vivo that identifies potential risks of using the tested substance and it also assists in the selection of appropriate doses to be used in further studies [24]. After analyzing information obtained from the acute toxicity test and verifying the absence of toxicity, the possible health risks of repeated exposure are assessed, administering three doses for 30 days. The results of this study demonstrated that the animals did not show clinical signs of toxicity such as mortality and changes in behavior nor decreased appetite, indicating that the acute and subchronic toxicity of MHF has yet not found [25].

Changes in the weight of internal organs are sensitive markers and indicative of substance toxicity [26]. Thus, despite the change in the relative weight of the stomach from the females in the subchronic toxicity test, this finding was not considered toxicologically significant, as it did not occur in both sexes, nor was it accompanied by morphological abnormalities in macroscopic and histopathological investigation.

The biochemical analysis is widely used in toxicity studies, being decisive in the evaluation of damage to organs and their functions [27]. In the results of the biochemical parameters of acute toxicity, there was only a significant decrease in total cholesterol in males. However, this finding was considered to be of no toxicological significance, as no changes in the lipid profile of the treated mice were observed during the subsequent 30 days.

The kidney is highly susceptible to toxic substances, with urea and creatinine being considered important markers of its normal functioning [28]. However, creatinine is a more reliable indicator, as urea levels can be affected by other conditions not related to kidney function, such as diet [28]. In this study, although changes in serum levels of urea and creatinine were found, these changes were considered incidental, therefore not related to the treatment, and not having toxicological

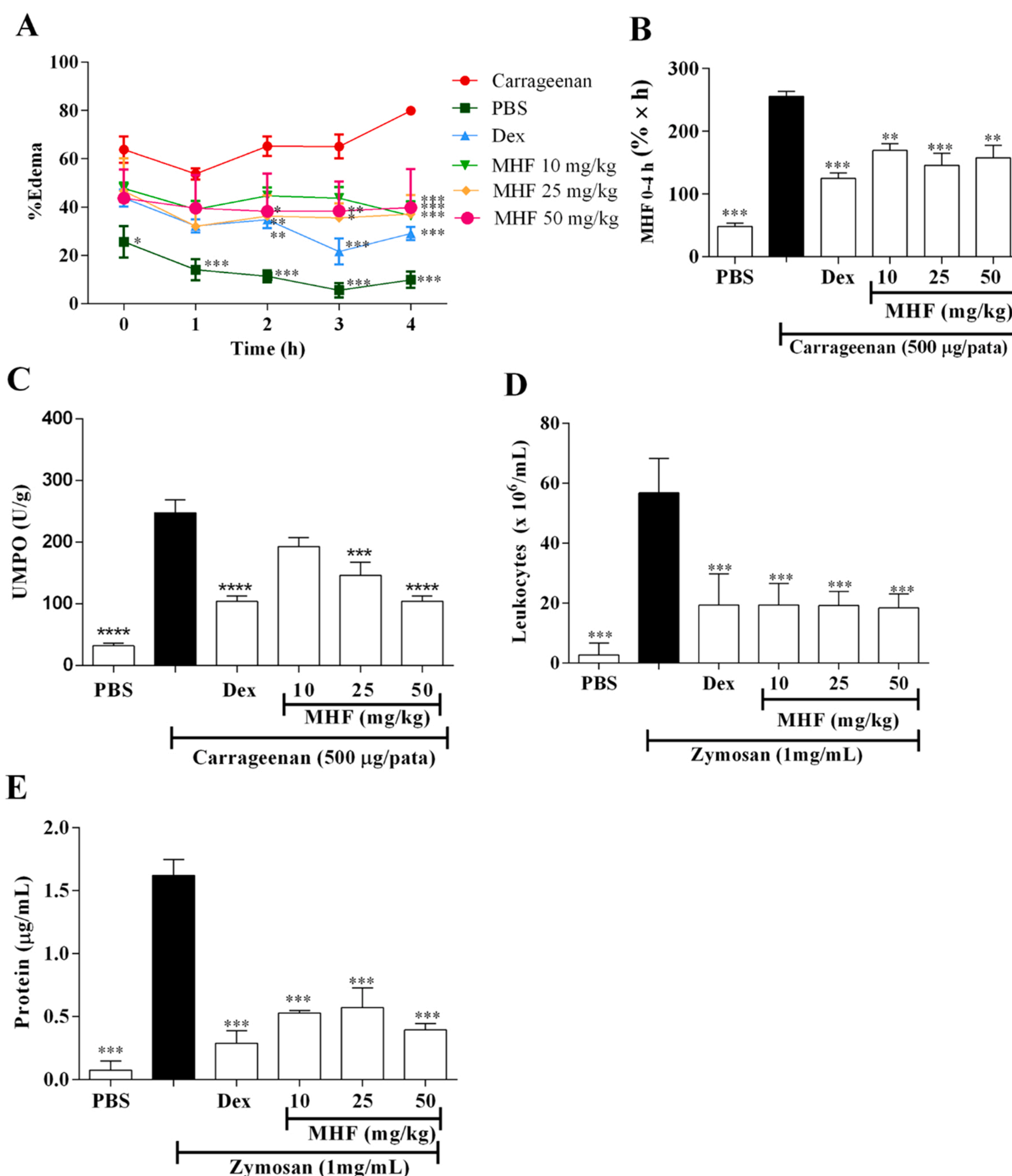

Fig. 7. Effect of post-treatment with MHF in the carrageenan-induced paw edema and zymosan- induced air pouch models. Swiss mice were treated orally PBS, MHF (10, 25, and 50 mg/kg) and dexamethasone (2 mg/kg), and the edema was measured at 0, 1, 2, 3, and 4 h (A and B) after the administration of λ-carrageenan (500 μg/paw). The paw removed after 4 h to determine myeloperoxidase activity (C). For the air pouch model, animals were treated orally with PBS, MHF (10, 25, and 50 mg/kg) and dexamethasone (2 mg/kg), after zymosan (1 mg/mL) administrated into the air pouch. After six hours, the exudate was collected and the total leukocyte count (D) and total proteins were determinated (E). Each horizontal line represents the mean of the values obtained from five animals, and the vertical lines indicate the standard deviations of the mean. * ** * $p < 0.0001$, * ** $p < 0.001$, * * $p < 0.01$, and * $p < 0.05$ compared to the carrageenan and zymosan PBS: phosphate buffered saline (pH 7.4), Dex: dexamethasone.

significance. In addition, there were no significant changes in the levels of total proteins and albumin which reflect protein status and are useful in determining variations in liver and kidney function and the histological analysis of the kidney showed no lesions.

The liver plays a key role in the clearance and transformation of toxic substances, being one of the organs that are the target of toxicity [29]. Serum increases in aminotransferases (AST and ALT) are indicators of liver damage, with ALT being considered the most sensitive and very specific biomarker of hepatotoxicity, as it is primarily located in the liver [30]. In this study, treatment with MHF did not promote significant changes in serum transaminases and the histopathological observation of the liver tissue of animals treated with the *M. hirsuta* fraction did not show any toxic manifestations. These findings may indicate that the MHF at the doses tested did not induce changes in liver function.

The analysis of hematological parameters is very relevant in the evaluation of lesions in the hematopoietic system, one of the most sensitive targets to substance toxicity and an important indicator of the physiological and pathological state in humans and animals [31,32]. Despite the significance found in some parameters, these results were not considered to be a toxic effect because the changes did not occur in

Table 5
Anti-inflammatory activity of the MHF in the zymosan-induced air pouch model.

Groups	(mg/kg)	Cell migration (x10^6/mL)	Inhibition (%)
Zymosan	–	56.750 ± 5.732	–
Dexamethasone	2	18.375 ± 4.695 * **	65
MHF	10	19.375 ± 3.555 * **	62
MHF	25	19.166 ± 2.682 * **	73
MHF	50	18.375 ± 2.313 * **	73

Values expressed as mean ± standard deviation (SD), n = 5, * ** $p < 0.001$, and * * $p < 0.01$, test group compared to the zymosan group.

both sexes, they were not accompanied by correlative findings, and did not show a clear relationship between dose-response.

The behavioral effects of oral treatment with MHF were also investigated through the open field and the rota-rod tests, considered to be classic experimental animal models, well validated to investigate effects on the central nervous system [33]. The open field test is used to investigate exploratory activity and behaviors similar to rodent anxiety and sedation [34], while the rota-rod is used to assess peripheral neuromuscular block and motor coordination [35]. In the present study, the administration of MHF did not impair the locomotor behavior of the animals, indicating that it has no stimulating or sedative effects. In addition, the results obtained in the rota-rod test showed that the MHF, at the doses tested, also did not modify the general activity and motor coordination of the mice, corroborating the absence of sedation.

Considering the promising results of non-toxic effects of MHF obtained in this study, its anti-edematogenic potential in mice was evaluated through a lambda-carrageenan-induced edema, an extensively used polysaccharide that triggers inflammation and it is very useful in the investigation of anti-inflammatory substances [36]. The formation of edema resulting from the production of vasoactive substances, such as histamine, serotonin and bradykinin occurs in the first hours, followed by the release of prostaglandins and the migration of leukocytes to the inflamed site [37,38]. In addition, the intraplantar injection of carrageenan induces hyperalgesia by sensitizing primary afferent nociceptors [39]. The results of the present study revealed that MHF reduced the edema after the third hour of treatment, which suggests that the anti-edematogenic actions of MHF may be due to interference in the activation of nociceptors, inhibition of the enzyme cyclooxygenase (COX) or of one or more intracellular signaling pathways involved in the effects of inflammatory mediators, requiring further investigation to understand the exact mechanism of action.

The MPO enzyme, stored in azurolytic granules of polymorphonuclear neutrophils and macrophages, is characterized by oxidizing halide and pseudohalide ions, producing respective hypohalous acids, presenting important antimicrobial and antiviral activity [40]. However, when released into the extracellular space, it causes tissue damage and is, therefore, detected in a wide range of acute and chronic inflammatory conditions [40,41]. It was observed in this study that the fraction from *M. hirsuta* in the studied doses, resulted in decreased MPO activity, which may be due to the ability to reduce polymorphonuclear migration to the inflamed tissue.

In addition, the anti-inflammatory action of MHF was also evaluated by the air pouch inflammation model using zimosan as an inflammatory agent. Zymosan is a compound derived from the cell wall of the fungus *Saccharomyces cerevisiae* containing mainly β-glucan, mannan and chitin [42,43], capable of activating the alternative pathway of the complement system, inducing secretion of lysosomal enzymes and releasing prostaglandins and leukotrienes [44]. The results showed that the oral administration of MHF in all doses significantly reduced the migration of leukocytes in the air pouch cavity, a key factor for the development of an inflammatory process, as well as the amount of total proteins, corroborating the previous findings of this study, providing scientific evidence for the anti-inflammatory activity of MHF.

The MHF obtained from the leaves of *M. hirsuta* was also evaluated for its analgesic activity using pain models that include the acetic acid-induced abdominal writhing test [45] and the paw-licking test induced by formalin, which represents acute clinical pain due to tissue damage [39]. The abdominal constriction response to intraperitoneal acetic acid injection results from the release of pro-inflammatory cytokines such as the tumor necrosis factor (TNF-α), interleukin 1β and interleukin 8 by resident peritoneal macrophages and mast cells [46], in addition to endogenous substances such as prostaglandins (PGE 2 and PGF 2α), bradykinin, substance P, serotonin, histamine and sympathomimetic amines [47–49]. Central pain stimulation by activation of mitogen-activated protein kinases (MAP), phosphatidylinositol-3-kinase (PI3K) and microglia [50] is also reported. In the present study, MHF reduced abdominal writhing in response to the administration of acetic acid in the highest doses (25 and 50 mg/kg) and may have an analgesic effect due to inhibition of the synthesis of pain mediators, similar to.

non-steroidal anti-inflammatory drugs, such as indomethacin, used as positive control here, are effective in inhibiting prostaglandin

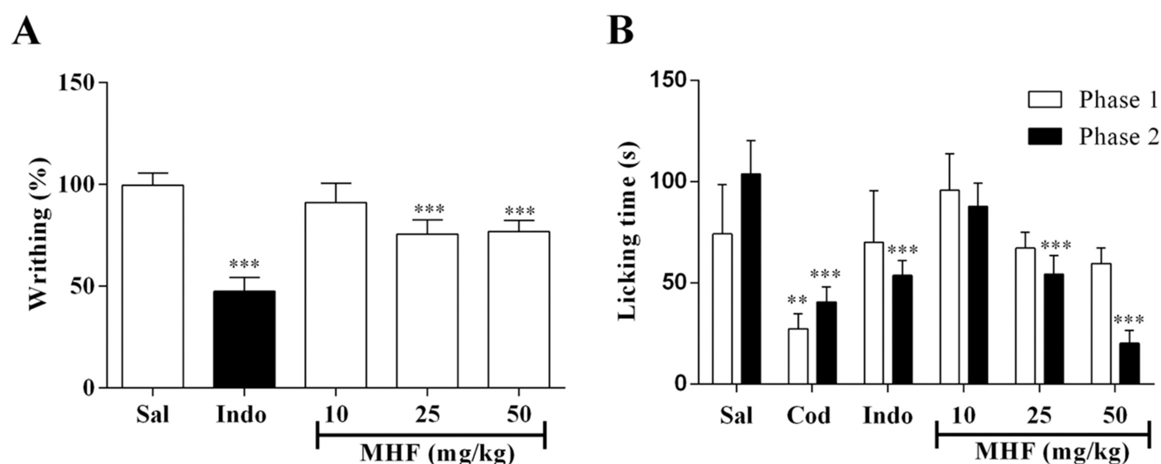

Fig. 8. Analgesic effect of the MHF administered orally in mice in the abdominal writhing test induced by acetic acid and formalin. The mice were pre-treated with vehicle (C, control group Saline solution), indomethacin (Indo, 25 mg/kg) and MHF (10, 25, and 50 mg/kg) 30 min before the intraperitoneal (i.p.) injection of acetic acid. Analgesia was registered by the number of contortions presented by the animal after 20 min of the injection of acetic acid i.p. For the formalin test, the mice were pretreated with vehicle (C, saline control group), codeine (Cod, 7.5 mg/kg), indomethacin (Indo, 25 mg/kg) and MHF (10, 25, and 50 mg/kg) 1 h before the subplantar formalin injection. Analgesia was recorded for the time (seconds) the animal spent licking and biting its paw, measured for 5 min and between 15 and 30 min after the formalin injection. The results represent the mean ± standard deviation (n = 5) * ** $p < 0.001$, * * $p < 0.01$ and * $p < 0.05$ when comparing the groups treated with MHF and indomethacin with the control group.

synthesis.

The paw licking test induced by subcutaneous formalin injection involves two phases of nociceptive responses [39]. The first phase (neurogenic pain) occurs by direct chemical stimulation of nociceptive afferent fibers, mainly C fibers, which can be inhibited by centrally acting analgesics [39,51]. The second phase (inflammatory pain) results from the action of mediators such as prostaglandins, serotonin, histamine, and bradykinin in peripheral tissues, inhibited by non-steroidal anti-inflammatory drugs [52,53]. The results of this study showed that MHF (25 and 50 mg/kg) were effective in reducing pain in second phase, similar to the COX inhibitor positive-control, suggest inhibition of synthesis or release of arachidonic acid and its metabolites, including prostaglandins and leukotrienes. Thus, these data corroborate with previous findings in this work and suggest a possible effect of MHF on neurogenic and anti-inflammatory pain mediated by inhibition of inflammatory mediators, requiring further experiments to elucidate these actions.

5. Conclusion

The excellent results of this study indicated that the fraction obtained from the leaves of *M. hirsuta* did not present significant toxic effects in vitro or when administered orally to mice, but higher doses that produce toxic effects need to be explored. In addition, it has potential benefits in the treatment of inflammation and pain suggesting the possibility of therapeutic applications. However, further studies are still needed to elucidate the mechanisms of action of these pharmacological activities.

CRediT authorship contribution statement

Joquebede Rodrigues Pereira: Investigation, Methodology, Data curation, Formal analysis, Conceptualization, Writing – original draft. **Aldilane Gonçalves da Fonseca:** Investigation, Methodology. **Luzia Leiros de Sena Fernandes**: Investigation, Methodology. **Allanny Alves Furtado:** Investigation, Methodology, Data curation. **Valéria Costa da Silva:** Investigation, Methodology, Data curation. **Valdir Florêncio da Veiga Júnior:** Investigation, Methodology, Data curation. **Antônio Euzébio Goulart Sant'Ana:** Supervision, Investigation, Methodology. **Cláudia Nunes Oliveira:** Investigation, Methodology. **Gerlane Coelho Bernardo Guerra:** Investigation, Methodology. **Matheus de Freitas Fernandes-Pedrosa:** Investigation, Methodology. **Elaine Cristina Gavioli:** Investigation, Methodology. **Ádley Antonini Neves de Lima:** Supervision, Formal analysis, Writing – review & editing. **Daniel de Melo Silva:** Resources, Supervision, Formal analysis, Writing – review & editing. **Telma Maria Araújo Moura Lemos:** Supervision, Data curation, Formal analysis, Conceptualization, Writing – original draft, Writing – review & editing.

Acknowledgments

The authors would like to thank Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for their financial assistance.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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