An Acad Bras Cienc (2022) 94(4): e20210190 DOI 10.1590/0001-3765202220210190
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

HEALTH SCIENCES

Acute toxicity and regenerative dose finding of an extract of *Miconia ferruginata* DC. in a mouse model of Duchenne muscular dystrophy

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Abstract: Duchenne muscular dystrophy (DMD) is a severe disease with no cure caused by a genetic abnormality, promoting progressive muscle degeneration. Corticosteroids are used drugs in treatment associated with adverse effects. The extract of *Miconia ferruginata (Melastomataceae)* (*MF*) has demonstrated potent antioxidant and anti-inflammatory potential *in vitro*. This study used a DMD model *(mdx)* to determine the toxic dose of this plant and found a possible non-toxic dose with therapeutic effects. The *mdx* groups received an intraperitoneal injection of 0 (control group), 50, 100, 200, 300, and 2000 mg kg^{-1} of the aqueous leaf extract following a single-dose acute toxicity protocol and were observed for 14 days. The range of toxicity of the extract and LD_{50} were determined. Histopathological analysis, the quantification of fibrosis, and immunohistochemical analysis of the tissues were performed. The results demonstrated that 2000 mg kg^{-1} was highly toxic, inducing histopathological changes in the tissues evaluated, with 100% mortality in 48 hours. The other doses caused no behavioral changes or signs of toxicity. The *MF* extract led reduction in histopathological changes, fibrosis, and inflammation, a reduction in HSP70 and an increase in MCL-1 proteins. Doses of 50-200 mg kg^{-1} demonstrated regenerative tissue and anti-inflammatory potential.

Key words: *Miconia ferruginata*, Duchenne muscular dystrophy, *mdx* model, acute toxicity, anti-inflammatory, tissue regeneration.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is a progressive genetic disorder with a pattern of recessive inheritance associated with the X chromosome that affects about one in 3,500 live male births (McDonald 2002, Santos et al. 2006, Jarrah et al. 2014). Clinically, DMD is the most serious of human muscular dystrophies and is characterized by progressive, irreversible muscle weakness due to a pathogenic mutation in the dystrophin protein gene, which is related to the motor sarcotubular system (Kueh et al. 2008, Fraysse et al. 2017). Despite advances in knowledge on the disease, there is still no effective treatment and the aim of existing therapies is to reduce disability, prevent complications, prolong mobility, and improve quality of life (Nair et al. 2001, Frezza et al. 2005). Regarding pharmacological treatments, the class of corticosteroids seems to reduce the progression of the disease and improve lung function, but most patients do not tolerate this therapy in the long term due to the many side effects (Campbell & Jacob 2003, Ciafaloni & Moxley 2008).

One of the most efficient ways of studying therapies for DMD is the use of an animal

model of dystrophy – the *mdx* mouse (Seixas et al. 1997, Willmann et al. 2009). Like humans, *mdx* mice have a spontaneous mutation of the X chromosome that leads to dystrophin deficiency (Bulfield et al. 1984, Lefaucheur et al. 1995). Although this model has regeneration cycles with the low deposition of adipose tissue and mild muscle fibrosis, promoting less severe dystrophy, the animals exhibit intense inflammatory infiltrate and myonecrosis, which are characteristic of DMD (Bulfield et al. 1984, Grounds et al. 2008).

The plant *Miconia ferruginata* DC. (Melastomataceae), known locally as *pixirica-do-campo* or *babatenã*, is native of Brazil found to the Brazilian *Cerrado* biome (savanna) and is used in folk medicine as a natural anti-inflammatory to treat allergic skin processes and other inflammatory diseases (Almeida & Bandeira 2010, Goldenberg & Caddah 2015). A natural product research group of the Universidade Federal dos Vales do Jequitinhonha e Mucuri (UFVJM) has evaluated this activity *in vitro* (Barroso 2015, Cruz 2017). Barroso (2015) found that the aqueous extracts of the *M. ferruginata* leaves and stems inhibited the proliferation of lymphocytes, with efficacy 2.5-fold higher compared to the drug dexamethasone and an absence of cell toxicity. They found the presence of flavonols, flavones, phenolic acids and catechins in the extracts and fractions of the leaves and stem, the terpenes (β-caricophylene and α-humulene), caffeoylquinic acid and monocaffeoylquinic in the leaves. In addition, Cunha et al. (2020) found triperpenes (ursolic and oleanoic acids) and flavone (5,6,7-trihydroxy-4'-methoxy flavone) in the leaves.

Chaperone proteins related to cell stress, such as HSP70, and molecules related to cell routes, such as the anti-apoptotic MCL-1, are used in the evaluation of the effects of therapies as well as the monitoring and prognosis of degenerative diseases, such as DMD (De Paepe et al. 2012, Morciano et al. 2016). HSP70 belongs to a large group of proteins known as molecular chaperones, which are over-expressed during acute and chronic episodes of stress and have a cytoprotective function (Slimen et al. 2016). Thus, HSP70 is widely used as a marker of cellular stress levels (Cole & Meyers 2011). MCL-1 is a protein of the Bcl-2 family, which has anti-apoptotic action. This protein is located on the outer membrane of the mitochondria, in the endoplasmic reticulum, and in the cell nucleus. MCL-1 neutralizes the pro-apoptotic function of *Bim* proteins and prevents the activation of death receptors (Wuillème-Toumi et al. 2005).

In an attempt to find more efficient, less toxic therapies for DMD and considering the *in vitro* anti-inflammatory potential of *M. ferruginata*, the aim of this work was to evaluate the *in vivo* toxicity and effect on the inflammatory process of the aqueous leaf extract from *M. ferruginata* in an *mdx* mouse model.

MATERIALS AND METHODS

Collection and identification

M. ferruginata was collected by MSc. Poliana Ribeiro Barroso from the community of Ribeirão de Areia in the city of Diamantina in the northern portion of the state of Minas Gerais, Brazil, on May 19, 2013 and was identified. The species is not on the Official List of Threatened Brazilian Plant Species (ANVISA 2014). The collected material was georeferenced and a voucher was deposited at the Herbário Dendrológico Jeanine Felfili (HDJF) of Universidade Federal dos Vales do Jequitinhonha e Mucuri (UFVJM) under registration number HDJF2405. Dr. Evandro Luiz Mendonça Machado performed the identification. The Ministério do Meio Ambiente issued authorization for the collection of the plant (registration number 64300-13) and the

genetic heritage access was granted by the Conselho de Gestão do Patrimônio Genético (CGEN AACD7CF/2018).

Preparation of extracts

The preparation of the extract was performed at the Laboratório de Química Orgânica e Produtos Naturais of Departamento de Farmácia of Universidade Federal dos Vales do Jequitinhonha e Mucuri (UFVJM). The plant material was dried in a circulating air oven (Biopar® S480AT, São Paulo, SP, Brazil) at about 40°C for 14 days, then ground with the aid of a knife mill (Marconi® MA580, São Paulo, SP, Brazil), and stored in plastic bags in a cool place at a temperature of 20°C protected from light.

The aqueous extract was prepared by infusion (1:10 w/v) using 50 g of the ground material in contact 500 mL of boiling Milli-Q water for 10 minutes. After cooling to room temperature, the infusion was filtered through cotton, frozen in liquid nitrogen, and dried in a freeze dryer (Terroni® LS 3000, São Paulo, SP, Brazil). The extract was transferred to amber bottles and stored in a dry place protected from light.

Animals

The experiments were carried out at the Núcleo de Experimentação Animal, Departamento de Fisioterapia of Universidade Federal dos Vales do Jequitinhonha e Mucuri (UFVJM) and were conducted in accordance with the guidelines of the local Ethics Committee on Animal Use (certificate number: 012/2019) as well as standard guidelines for laboratory animal care (Neves et al. 2013).

Eighteen one-year-old female mice of the C57BL/10ScSn-Dmd^{mdx}/J lineage weighing 20 to 40 g were acquired commercially from Instituto de Ciência e Tecnologia Biomodelos - Instituto FioCruz (Av. Brasil, 4365 - Manguinhos, Rio de Janeiro, Brazil). The animals were housed in plastic boxes under a 12-hour light/dark cycle at 22.0 ± 2.0°C with food and water *ad libitum* throughout the experimental period. The animals were given a seven-day acclimatization period before the onset of the experiment.

Experimental groups

Eighteen non-isogenic, nulliparous, non-pregnant *mdx* females were randomly divided into six experimental groups of three animals each: i) 2000 mg kg^{-1} – single dose intraperitoneally (ip.); ii) 300 mg kg^{-1} – single dose ip.; iii) 200 mg kg^{-1} – single dose ip.; iv) 100 mg kg^{-1} – single dose ip.; v) 50 mg kg^{-1} – single dose ip.; and vi) 0 mg kg^{-1} (distilled water) – single dose ip. (control group). The extract was diluted in distilled water previously filtered through a syringe filter with a 0.22 µm membrane (TPP®, Zollstrasse, Switzerland). The last injection volume was 0.5 mL for all groups.

Acute toxicity of single dose of aqueous extract of *M. ferruginata* and determination of LD50

To determine acute toxicity and LD_{50} of the aqueous extract of *M. ferruginata*, a single dose was administered to the mice in the respective groups following ANVISA Guideline nº 4 2014 (Brasil 2014) and Guideline 423 of the OECD (OECD 2001). For this experimental protocol, the animals had free access to water but were fasted for two hours before the onset of the experiment and for another two hours after the administration of the extract. The observation period after administration of the extract was for 14 days. The results of the acute single-dose toxicity test were evaluated based on the parameters described below.

Hippocratic screening

After the administration of the extract, a single blinded evaluator carefully monitored the animals to detect signs of toxicity after 0.5, 1, 2, 4, 6, 8, 12, and 24 hours, then daily to the 14th day using the Hippocratic screening method proposed by (Malone 1983). A chart was used to record behavior and the results of the clinical examination using a unipolar analog scale ranging from 0 to 4 (absent, rare, little, moderate, and intense) for the following six parameters: i) conscious state (general activity, vocal frenzy, and irritability); ii) central nervous system activity (tremors, convulsions, anesthesia, sedation, ataxia, hypothermia, breathing, and hyperemia); iii) autonomic nervous system activity (lacrimation, cyanosis, ptosis, salivation, and piloerection); iv) activity and coordination of the motor system and muscle tonus (response to touch, response to tail tightening, contortion, position of the posterior train, straightening reflex, body tone, and grip strength); v) reflexes (auricular reflex, corneal reflex, lacrimation, urination, and defecation), and vi) death.

Assessment of weight and water/feed consumption

A semi-analytical scale was used to monitor the gain or reduction in body mass (grams) of the animals (Mars, UX420H, São Paulo, SP, Brazil). Weight was determined before the administration of the extract or water and daily until the end of the 14 days of treatment. Water (in mL) and feed (in grams) consumption were also recorded daily throughout the experimental period.

Determination of LD_{50}

OECD Guideline 423 was used for determination of the LD_{50} (OECD 2001), which is the dose that causes the mortality of 50% of the test population.

Anatomical-histopathological assessment

On the 14th day, a lethal injection of ketamine 300 mg kg^{-1} and xylazine 100 mg kg^{-1} was administered intraperitoneally to euthanize the surviving animals. Immediately after euthanasia, necropsy and macroscopic analysis of the organs were performed. The liver, lung, and tibialis anterior muscle were removed for histological, morphometric, and immunohistochemical analysis. The organs were weighed on a precision semi-analytical scale (Mars, UX420H, São Paulo, SP, Brazil) and submerged in 4% paraformaldehyde for 24 hours. After fixation, the tissues were processed for histopathologic analysis. Three samples per slide were sectioned on a semi-automatic microtome to a thickness of 5 µm, followed by staining with hematoxylin and eosin (HE) and Picrosirius Red. All slides were photomicrographed at 400X magnification with a camera (Axio CAM HRc) coupled to an optical microscope (Kozo, XJS900 series, Nanjing, China) and captured by the ToupView Quick program (ToupTek Corporation, Xihu District, Zhejiang, China).

Morphological analysis

Descriptive morphological analyses were performed under a light microscope (Kozo, XJS900 series, Nanjing, China) in an independent double-blind assay. The qualitative analysis was carried out through of evaluations (magnification: 40X) by two experienced histologists, to investigate reversible (degenerative) and irreversible (necrosis and apoptosis) changes, determine the presence of aberrant cells, and classify the intensity of the inflammatory infiltrate.

Picrosirius Red staining

Morphometry of collagen fiber area in the tissues analyzed was performed on slides stained with Picrosirius Red to evaluate and quantify tissue fibrosis. Histochemical analysis

with Picrosirius Red enables the distinction of collagen fibers deposited in the tissue due to the increased birefringence of the fibers when observed under polarized light (Wegner et al. 2017). Photomicrographs were obtained using a camera (Labomed, LX400p, Fremont, CA, USA) coupled to a polarized microscope, with determination of the histological section avoiding sites with arteries, arterioles, and veins, which are regions rich in collagen. Five randomly selected fields of each slice at 400X magnification (totaling 15 photomicrographs per experimental group) were established, maintaining the same microscope brightness for all images. The photomicrographs were analyzed with the aid of the ImageJ software. The binary analysis procedure (white/black) comprised "Process" > "Binary" > "Make Binary" > "Analyze" > "Set Measurements" > "Selected Area + Area Fraction" > "Analyze" > "Measure". The data on collagen fiber area were expressed as percentage ± standard deviation of the total area.

Immunohistochemical analysis

Sections (5 µm) of paraffin blocks were incubated with affinity-purified mouse monoclonal HSP70 (1:500) and rabbit monoclonal MCL-1 (1:100) using the streptavidin-biotin method, following the manufacturer's instructions (Sigma, Saint Louis, MO, USA). The slides were deparaffinized and the antigens were recovered, followed by incubation for 30 min in 0.3% hydrogen peroxide, rinsing in PBS, and incubation with Protein Block (DAKO X0909, Dako North America, CA, USA). The sections were then rinsed and incubated at 4°C overnight with the monoclonal antibodies diluted in PBS. The tissue sections were then rinsed and exposed to secondary antibody N-Histofine (Nichirei Biosciences, Tsukiji, Tokyo, Japan) and incubated for 30 minutes at room temperature (20°C). The negative control slide did not receive the primary antibody. The antigen-antibody complex was visualized via the chromogen 3,3-diaminobenzidine tetrahydrochloride in the presence of 0.01% hydrogen peroxide. The sections were then counterstained with hematoxylin, dehydrated through a graded series of alcohol, and cleared in xylene prior to cover slipping for the microscopic evaluation and immunopositive cell counts. The slides were photomicrographed with a camera (Axio CAM HRc) coupled to an optical microscope (Kozo, XJS900 series, Nanjing, China) and the photos were captured by the ToupView Help program.

Statistical analysis

The data were expressed as mean ± standard deviation or percentage ± standard deviation and the analyses were performed with the ais of GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA). The Shapiro-Wilk and D'Agostino-Pearson tests were used to determine the normality of the data. Differences between variables with normal distribution were investigated using one-way analysis of variance (ANOVA) and the Bonferroni post hoc test. For variables with asymmetric distribution, the Kruskal-Wallis test was used with Dunn's post hoc test. A p-value <0.05 was considered indicative of statistical significance.

RESULTS

Determination of acute single-dose toxicity

Only the highest dose (2000 mg kg^{-1}) was lethal to all animals in less than 48 hours, demonstrating high toxicity of the extract at this dose. No clinical changes were observed at any of the other doses tested (50, 100, 200, and 300 mg kg^{-1}).

Hippocratic screening

The five behavioral parameters evaluated using the Hippocratic screening method are shown in Figure 1. The most evident behavioral changes were related to the state of consciousness, activity, coordination of the motor system, and reflexes. Significant changes ($p < 0.001$) in these parameters were only observed with the maximum dose tested (2000 mg kg^{-1}). The animals that received this dose initially exhibited greater irritability, agitation, increased vocal frenzy, and disorientation (walking in circles). After about eight hours of observation, the animals became more lethargic and paralyzed, with no response to touch and the absence of corneal and auricular reflexes. This lethargic behavior continued until the death of these animals: one animal died at 12 hours, another at 24 hours, and the third at 48 hours after administration of the extract. No statistically significant changes were found for the other parameters evaluated. Regarding the central nervous system, however, the dose of 2000 mg kg^{-1} caused mild sedation and a reduction in respiratory frequency eight hours after administration.

Body mass assessment

The dose of 2000 mg kg^{-1} of the aqueous extract of *M. ferruginata* affected weight, as the animals did not feed during the brief period of survival, which led to a decreasing body mass curve. In contrast, no change in body mass was found during the 14 days of post-treatment observation with the other doses of the extract (Figure 2).

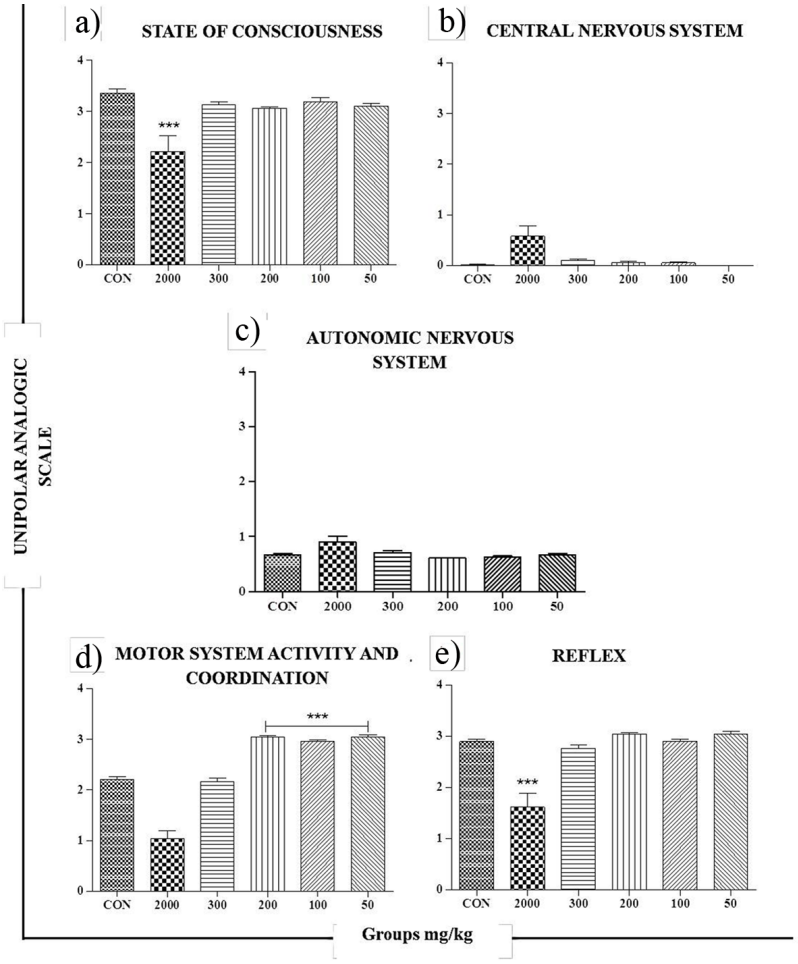

Figure 1. Hippocratic screening for behavioral evaluation of animals submitted to treatment with single dose of aqueous extract from leaves of *M. ferruginata*. CON= control group. a) Estate of consciousness; b) Central nervous system; c) Autonomic nervous system; d) Motor system activity and coordination; e) Reflex. ANOVA with Dunn's post hoc test (50, 100, 200, 300 and 2000 mg kg-1 groups), p <0.001.

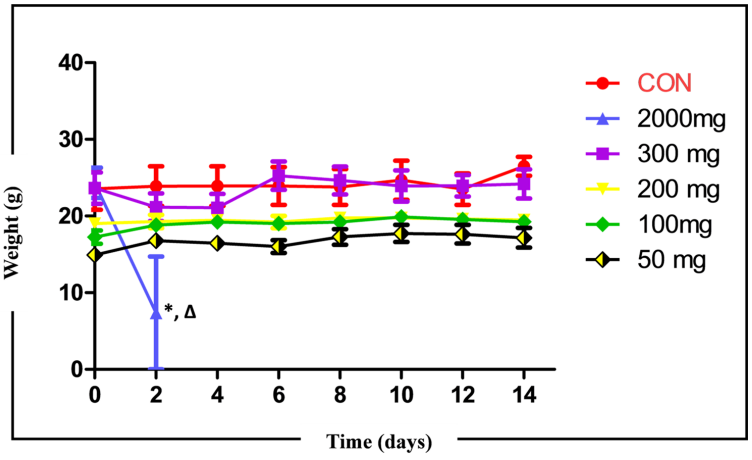

Figure 2. Evaluation of body mass of animals treated with different doses of aqueous extract from leaves of *M. ferruginata*. CON= control group. ANOVA, Dunn's post hoc test, *p <0.001; Δ - death of animals.

Evaluation of water and feed consumption

Water (mL) and feed (grams) consumption reduced significantly in the animals that received the dose of 2000 mg kg^{-1} compared to the control group. No differences in water or feed consumption were found throughout the entire experimental period with the other doses of the extract (Figure 3).

Determination of LD_{50}

Based on the class method recommended by OECD 423 (OECD 2001), the aqueous extract from the leaves of *M. ferruginata* is toxic and the LD_{50} was estimated to be 1000 mg kg^{-1}. The extract was classified in Category 4 of the Globally Harmonized System, with a moderate safety range between > 300 and <2000 mg kg^{-1}.

Morphological analysis (macroscopic and microscopic)

The mean ± standard deviation values for the organ weights of the animals treated with aqueous extracts of the leaves of *M. ferruginata* are listed in Table I. A significant difference in organ weight at the dose 2000 mg kg^{-1} compared to the control group only occurred in the lung ($p < 0.01$).

Macroscopic analysis

Liver

Macroscopically, the liver of animals treated with the aqueous extract from the leaves of *M. ferruginata* at a dose of 2000 mg kg^{-1} was enlarged (hepatomegaly) and exhibited edema as well as a blackish color. Moreover, edema and hepatomegaly, albeit to a milder degree, and a darker color were found in the animals treated with the dose of 300 mg kg^{-1} compared to the control group. In contrast, no changes in size, distension, or color were found with the other doses evaluated (50, 100, and 200 mg kg^{-1}) compared to the control group.

Lung

In the macroscopic analysis, the lungs of animals treated with the aqueous extract from the leaves of *M. ferruginata* at a dose of 2000 mg kg^{-1} exhibited considerable distension, discoloration, severely deformed pleura with several adjacent blood clots, and the collapse of the diaphragm. In the animals treated with the dose of 300 mg kg^{-1}, the lungs exhibited discoloration and pleural deformation but to a milder degree compared to the animals treated with the dose of 2000 mg kg^{-1}. There was also a smaller number of clots adjacent to the pleura and no collapse of the diaphragm. In contrast,

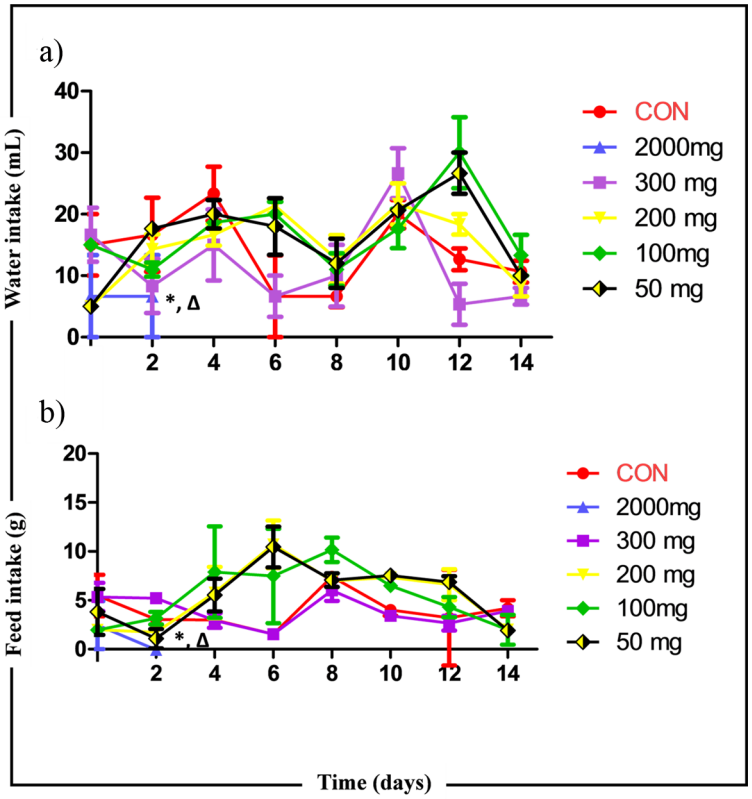

Figure 3. Evaluation of water and feed consumption of animals treated with different doses of aqueous extract from leaves of *M. ferruginata*. a) Water intake (ml); b) Feed intake (g). CON= control group. ANOVA, Dunn's post hoc test (50, 100, 200, 300 and 2000 mg kg-1 groups), *p <0.001; Δ - death of animals.

no macroscopic tissue changes were found in the animals treated with doses of 50, 100, or 200 mg kg^{-1}.

Tibialis anterior muscle

No macroscopic tissue changes were found in the tibialis anterior muscle in the animals treated with different doses of the aqueous extract from the leaves of *M. ferruginata*.

Microscopic analysis

Liver

Figure 4 displays the photomicrographs of the liver after the intraperitoneal administration of different doses of the aqueous extract from the leaves of *M. ferruginata*. In the microscopic analysis, the control group exhibited small lesions, with the presence of inflammatory infiltrate, mild edematous degeneration, few cells in apoptosis, and little necrosis. The animals treated with the dose of 2000 mg kg^{-1} of the aqueous extract from the leaves of *M. ferruginata* exhibited considerable lesions, with an extensive area of hydropic degeneration, numerous fissures around the hepatocytes, and hypertrophy of the Kupffer cells. Most hepatocytes had bi-nucleation or hyperchromic nuclei and cytoplasmic eosinophilia suggestive of a cellular toxicity process. The presence of bright areas in the hepatocytes was suggestive of fatty infiltration. Impressively, the liver of the animals treated with the extract at doses of 300, 200, 100 and 50 mg kg^{-1} exhibited a reduction in cell lesions, with tissue regeneration as well as reductions in edematous degeneration, the number of fissures, hypertrophy of hepatocytes and Kupffer cells, inflammatory infiltrate, and tissue necrosis compared to the control group. Thus, *M. ferruginata* extract exhibited an anti-inflammatory and hepatic regenerative effect in those doses.

Table I. Mean and standard deviation of organ weight of animals treated with different doses of aqueous extract of *M. ferruginata* leaves. Test ANOVA, *post hoc Dunns*, *p<0.01.

Groups	Tissues		
	Lung	Liver	Tibial a. muscle
Control	0,104 ± 0,014	1,130 ± 0,294	0,041 ± 0,005
2000 mg kg^{-1}	0,297 ± 0,168*	0,976 ± 0,100	0,037 ± 0,013
300 mg kg^{-1}	0,053 ± 0,012	1,364 ± 0,326	0,030 ± 0,005
200 mg kg^{-1}	0,152 ± 0,008	1,206 ± 0,212	0,037 ± 0,004
100 mg kg^{-1}	0,100 ± 0,073	1,287 ± 0,044	0,032 ± 0,009
50 mg kg^{-1}	0,127 ± 0,011	1,108 ± 0,129	0,030 ± 0,012

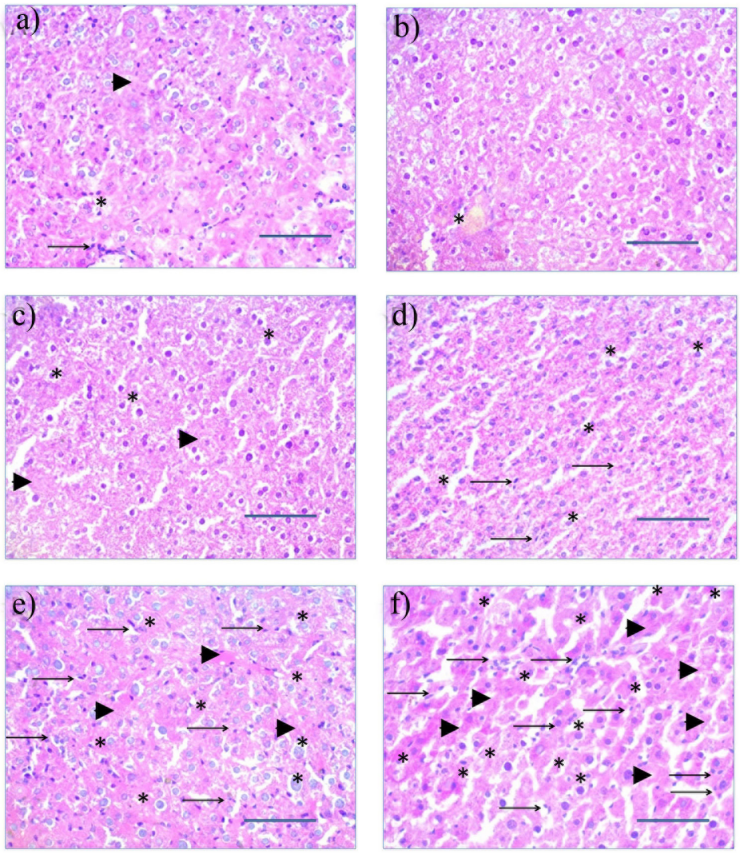

Figure 4. Histological analysis of liver of *mdx* mice treated with different doses of aqueous extract from leaves of *M. ferruginata* (Photomicrography, HE staining). **a)** control group; **b)** 50 mg kg^{-1} group; **c)** 100 mg kg^{-1} group; **d)** 200 mg kg^{-1} group; **e)** 300 mg kg^{-1} group; **f)** 2000 mg kg^{-1} group. Bar: 400 µm. Arrows indicate inflammatory infiltrate. Arrowheads indicate necrosis. Asterisks indicate apoptosis.

Lung

The photomicrographs of the lung after the intraperitoneal administration of different doses of the aqueous extract from the leaves of *M. ferruginata* are displayed in Figure 5. In the microscopic analysis, the control group exhibited mild pulmonary congestion with mild inflammatory infiltrate. As occurred in the liver, the lungs of animals treated with the dose of 2000 mg kg^{-1} of the aqueous extract from the leaves of *M. ferruginata* exhibited severe pulmonary congestion, with dense inflammatory infiltrate and severely degraded pleura and alveoli, with impaired lung function.

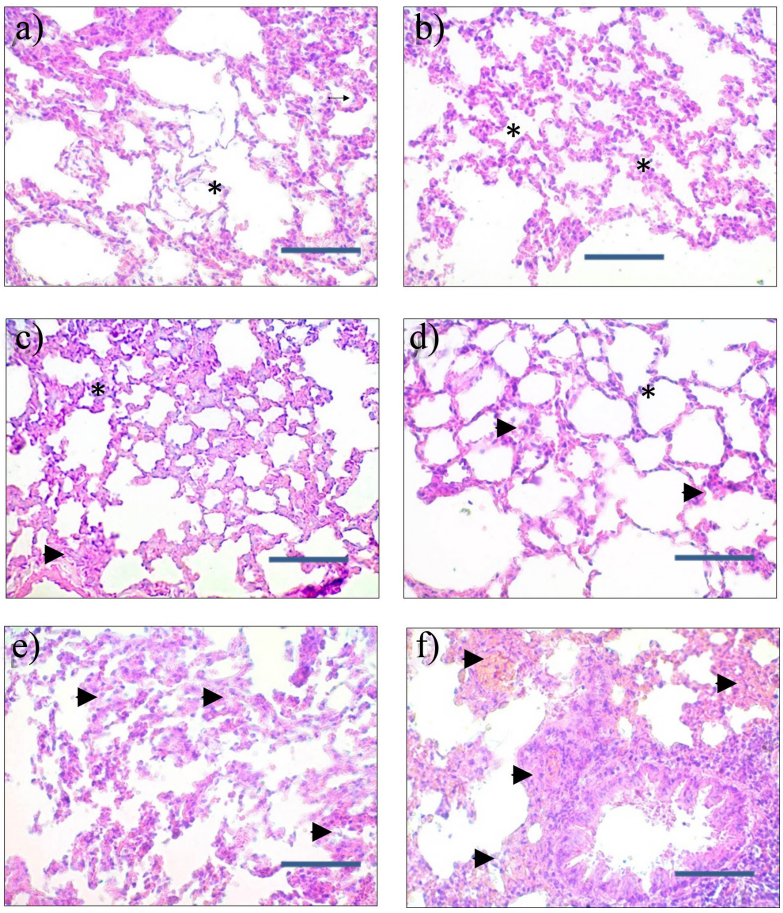

Figure 5. Histological analysis of lungs of *mdx* mice treated with different doses of aqueous extract from leaves of *M. ferruginata* (Photomicrography, HE staining). a) control group; b) 50 mg kg^{-1} group; c) 100 mg kg^{-1} group; d) 200 mg kg^{-1} group; e) 300 mg kg^{-1} group; f) 2000 mg kg^{-1} group. Bar: 400 µm. Arrows indicate inflammatory infiltrate. Arrowheads indicate necrosis. Asterisks indicate apoptosis.

While, at doses of 300, 200, 100 and 50 mg kg^{-1}, an improvement was found in pulmonary and restoration of the pleural congestion, with a reduction in inflammatory infiltrate.

Tibialis anterior muscle

The photomicrographs of the right tibialis anterior muscle after the intraperitoneal administration of different doses of the aqueous extract from the leaves of *M. ferruginata* are displayed in Figure 6. The control group exhibited a moderate level of tissue damage, with inflammatory infiltrate, edematous degeneration, cells in apoptosis, and necrosis. The animals treated with the dose of 2000 mg kg^{-1} exhibited an increase in tissue damage and considerable hydropic degeneration, with numerous fissures and dense inflammatory infiltrate as well as a larger number of necrotic areas and cells in the process of apoptosis. In the animals treated with the dose of 300mg kg^{-1} of the extract, a reduction was found in edematous degeneration, the number of fissures, as well as areas of necrosis and apoptosis compared to the control group and group treated with the dose of 2000 mg kg^{-1}. In the animals treated with the doses of 200, 100 and 50 mg kg^{-1} dose, considerable improvement was found in degeneration characteristic of DMD, with a significant reduction in edematous degeneration and inflammatory infiltrate, few of necrotic areas and of apoptosis. These results suggest that the aqueous extract from the leaves of *M. ferruginata* promotes muscle regeneration and a reduction in the inflammatory process in those doses.

Morphological description and quantification of fibrosis by Picrosirius Red staining

Figure 7 shows the photomicrographs of the muscle tissue stained with Picrosirius Red. The tibialis anterior muscle in the control group exhibited histopathological characteristics typical of dystrophic muscles, with the heterogeneous distribution of collagen fibers between muscle fibers; thick bundles predominated in the perimysium, while thin bundles were found in the endomysium. The aqueous extract from the leaves of *M. ferruginata* led to an increase in the amount of intramuscular collagen fibers, with distribution more uniform and homogeneous, covering larger areas in the muscle.

The morphological analysis of both the liver (Figure 8) and lung (Figure 9) revealed similar histopathological characteristics with the different doses evaluated. At doses of 2000 and 300 mg kg^{-1} of extract, areas were found with similar or increased fibrosis compared to the control group. A moderate to complete reduction in the areas of fibrosis and the morphofunctional maintenance was found at doses of 200, 100 and 50 mg kg^{-1}, with evident tissue regeneration. The animals treated the extract exhibited the complete regression of fibrosis of the tissues, suggesting the potential reversal of tissue fibrosis.

The percentages of the mean area of fibrosis in the different organs are shown in Figure 10. Similar deposition of collagen fibers was found in the different organs of the animals treated with the aqueous extract from the leaves of *M. ferruginata* at doses of 50, 100, and 200 mg kg^{-1}, with a significant reduction ($p < 0.001$) in collagen area deposition. The liver and lung of animals treated with the extract at doses of 300 and 2000 mg kg^{-1} exhibited collagen fiber deposition similar to that in the control group, demonstrating that these doses had no effect on fibrosis. However, a considerable reduction in fibrosis was found in both the liver and lung with doses of 200, 100, and 50 mg kg^{-1}. In contrast, the dose of 2000 mg kg^{-1} led to an increase in collagen deposition for the tibialis anterior muscle, but with no significant difference in comparison to the control group (control: 1.70 ± 2.13; 2000 mg kg^{-1}: 2.50 ± 2.02), whereas a significant reduction in fiber deposition was found in the other groups (control: 1.70 ± 2.13; 300 mg kg^{-1}: 0.92 ± 1.09; 200 mg kg^{-1}: 0.22 ± 0.32; 100 mg kg^{-1}: 0.15 ± 0.53; 50 mg kg^{-1}: 0.11 ± 0.17).

Immunohistochemical analysis of markers of stress and apoptosis (HSP70, MCL-1)

The HSP70 and MCL-1 proteins were immunolocalized in the cytoplasmic region of cells in all tissues analyzed. Regarding the labeling of HSP70, which is related to the cell stress level, an increase in expression was found in the liver (Figure 11), lung (Figure 12), and tibialis anterior muscle (Figure 13) of the animals treated with the dose of 2000 mg kg^{-1} of the extract. MCL-1 was analyzed only in the liver (Figure 14), where a reduction in the expression of this anti-apoptotic protein was found in the animals treated with the dose of 2000 mg kg^{-1} of the extract. Together with the histological and morphometric analyses, the results show the high toxicity of this dose of the aqueous extract from the leaves of *M. ferruginata*. For the animals treated with the other doses (300, 200, 100, and 50 mg kg^{-1}), a reduction in the cell stress marker (HSP70) was found in all tissues and increased expression of the anti-apoptotic protein (MCL -1) was found in the liver compared to the control group, especially at the dose of 50 mg kg^{-1}.

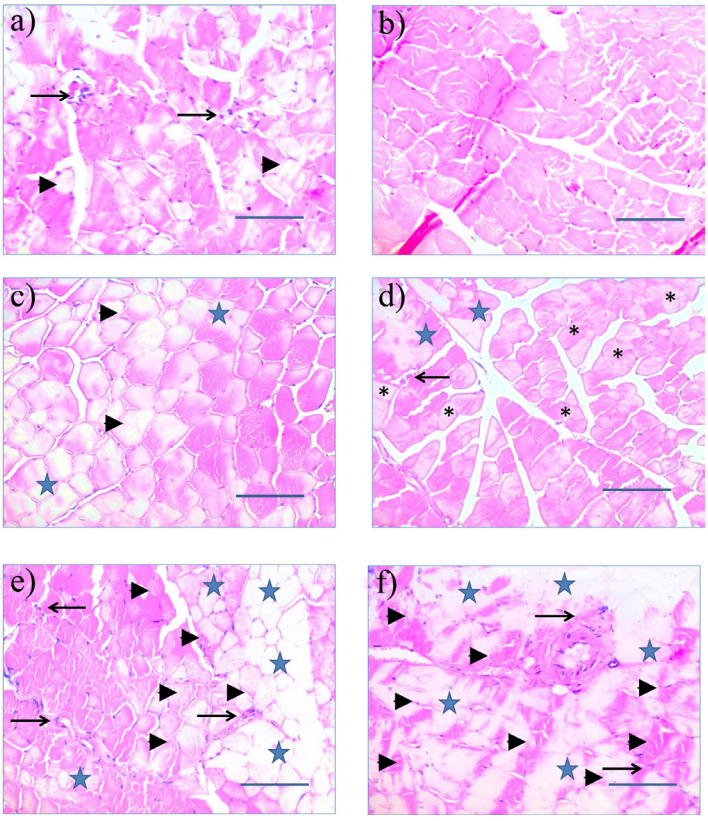

Figure 6. Histological analysis of tibialis anterior muscle (TA) of *mdx* mice treated with different doses of aqueous extract from leaves of *Miconia ferruginata* (Photomicrography, HE staining). **a)** control group; **b)** 50 mg kg^{-1} group; **c)** 100 mg kg^{-1} group; **d)** 200 mg kg^{-1} group; **e)** 300 mg kg^{-1} group; **f)** 2000 mg kg^{-1} group. Bar: 400 µm. Arrows indicate an inflammatory infiltrate. Arrowheads indicate necrosis. Asterisks indicate apoptosis. Stars indicate degenerative areas.

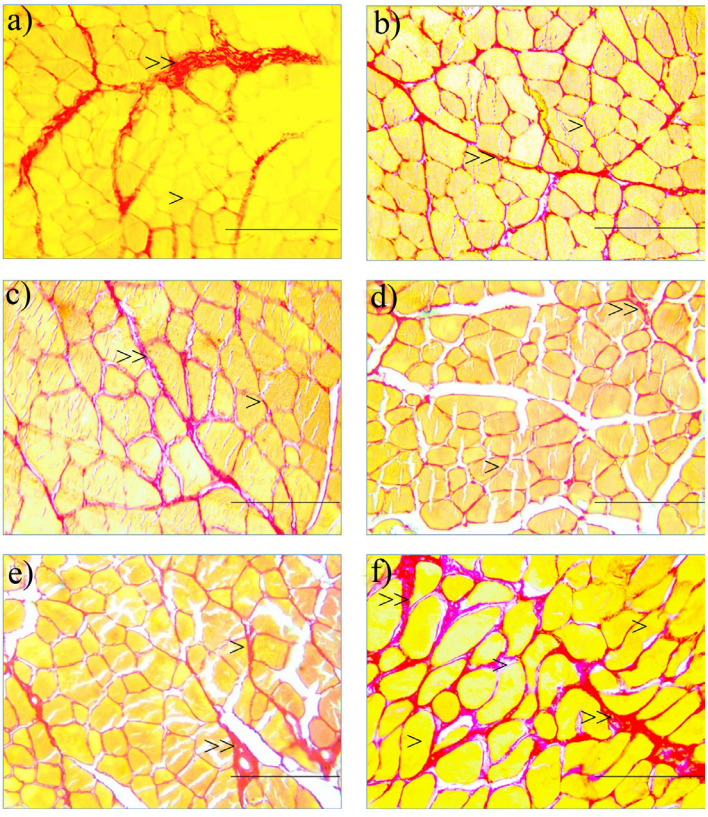

Figure 7. Distribution of collagen fibers in tibialis anterior muscle of *mdx* mice treated with different doses of aqueous extract from leaves of *Miconia ferruginata* (Photomicrography, Picrosirius Red staining). **a)** control group; **b)** 50 mg kg^{-1} group; **c)** 100 mg kg^{-1} group; **d)** 200 mg kg^{-1} group; **e)** 300 mg kg^{-1} group; **f)** 2000 mg kg^{-1} group. Bar: 400 µm. Morphological analysis of deposition of collagen fibers in tibialis anterior muscle of *mdx* mice. Red Picrosirius reaction. >> = Deposition of collagen fibers in perimysium. > = Deposition of collagen fibers in endomysium.

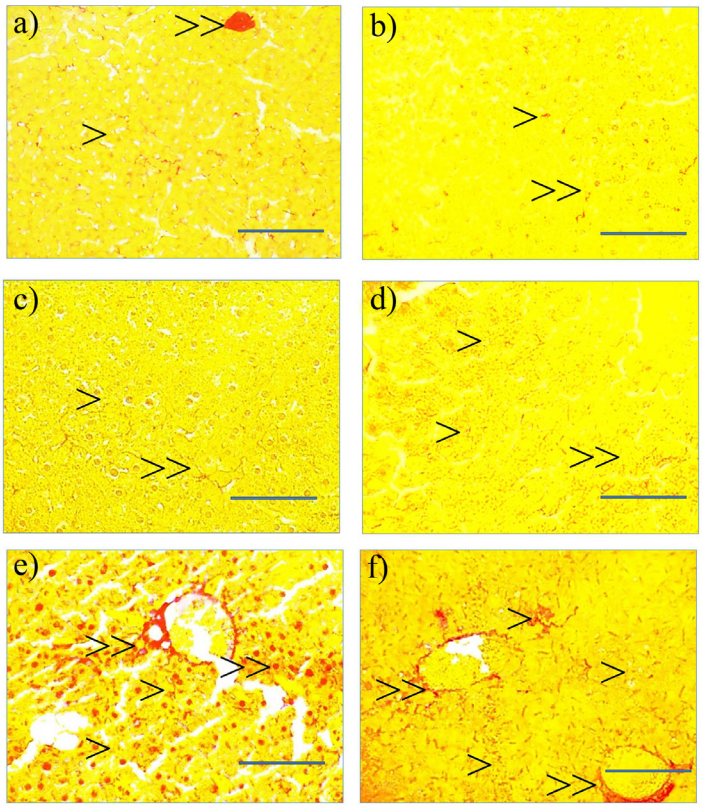

Figure 8. Distribution of collagen fibers in liver of *mdx* mice treated with different doses of aqueous extract from leaves of *Miconia ferruginata* (Photomicrography, Picrosirius Red staining). **a)** control group; **b)** 50 mg kg^{-1} group; **c)** 100 mg kg^{-1} group; **d)** 200 mg kg^{-1} group; **e)** 300 mg kg^{-1} group; **f)** 2000 mg kg^{-1} group. Bar: 400 µm. Morphological analysis of deposition of collagen fibers in tibialis anterior muscle of *mdx* mice. Red Picrosirius reaction. >> = Interlobular deposition of collagen fibers. > = Intralobular deposition of collagen fibers.

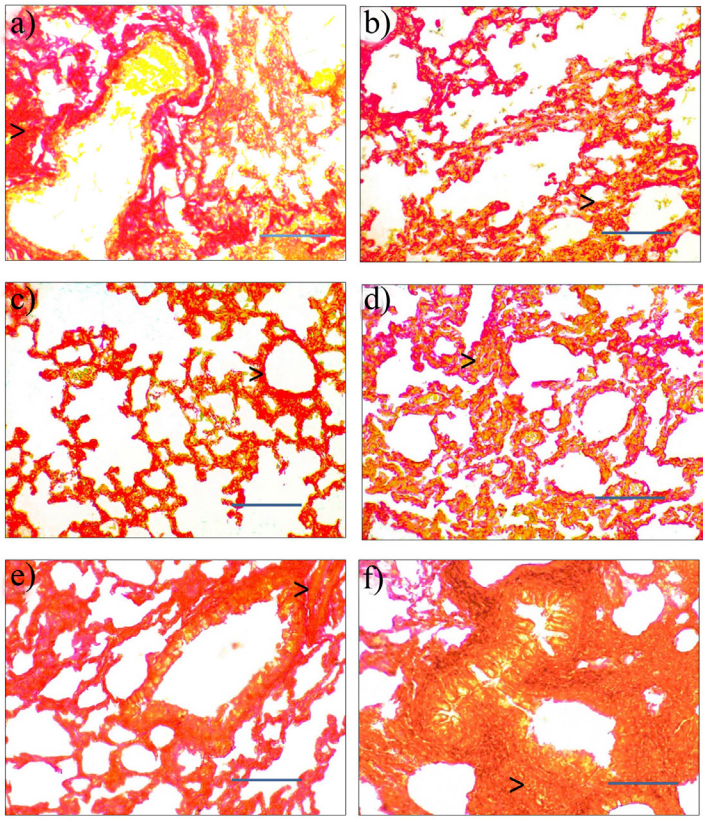

Figure 9. Distribution of collagen fibers in lung of *mdx* mice treated with different doses of aqueous extract from leaves of *Miconia ferruginata* (Photomicrography, Picrosirius Red staining). **a)** control group; **b)** 50 mg kg^{-1} group; **c)** 100 mg kg^{-1} group; **d)** 200 mg kg^{-1} group; **e)** 300 mg kg^{-1} group; **f)** 2000 mg kg^{-1} group. Bar: 400 µm. > = Deposition of collagen fibers in pulmonary parenchyma.

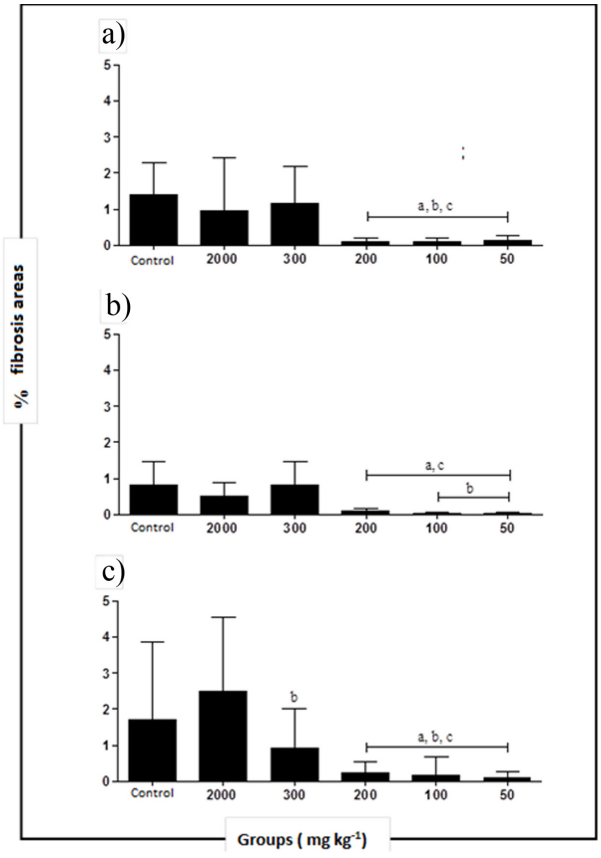

Figure 10. Percentage quantification of area of fibrosis in different organs of *mdx* mice treated with different doses of aqueous extract from leaves of *Miconia ferruginata*. a) Liver tissue; b) Lung tissue; c) Tibialis anterior muscle tissue. ANOVA, Dunn's post hoc test, p <0.001. a: statistically significant difference in comparison to control group; b: statistically significant difference in comparison to 2000 mg kg^{-1} dose group; c: statistically significant difference in comparison to 300 mg kg^{-1} dose group.

DISCUSSION

Popular use and traditional knowledge have been widely used to guide the search for new bioactive compounds for therapeutic purposes. Thus, ethnobotanical surveys are a valuable tool in the selection of plants for bio-prospecting natural products (Basso et al. 2005, Saccaro Jr. 2011, Palma & Palma 2012). According to Almeida & Bandeira (2010), the infusion of *M. ferruginata* leaves has medicinal uses for anti-inflammatory purposes, such as sore throats and dermatitis. With this in mind, we selected the popular form of use for the present study, using the aqueous extract of the leaves obtained through infusion.

The use of medicinal plants requires tests of both efficacy and safety (Talalay & Talalay 2001, Maciel et al. 2002, Berlinck et al. 2017). The acute toxicity test enables determining short-term adverse effects after the administration of a single dose of a drug (OECD 2001). The aqueous extract from the leaves of *M. ferruginata* was toxic at a dose of 2000 mg kg^{-1} and was classified in Category 4 of the Globally Harmonized System (OECD 2001), as all animals died within 48 hours. Toxicity was evident in behavioral and clinical aspects. Besides the loss of body mass, the lack of water and feed consumption are powerful indicators of the systemic toxicity of a compound (Carvalho 2013, Mello 2001, Teo et al. 2002), as observed at the dose of 2000 mg kg^{-1}.

The behavioral reactions after the intraperitoneal administration of this dose of the extract may be explained by a change in the balance of excitatory and inhibitory neurotransmitters (Ibrahem et al. 2011). However, about eight hours after administration of the extract, a severe depressant effect was found, possibly due to the depletion of synaptic vesicles of excitatory neurotransmitters or the prolonged direct stimulation of inhibitory neurotransmitters (Merlo et al. 2011, Brunton et al. 2017). This suggests that the aqueous extract from the leaves of *M. ferruginata* at a dose of 2000 mg kg^{-1} may have some sedative or central nervous system depressant activity. However, specific tests are needed to determine this action.

In contrast, no behavioral or clinical changes were found and there were no deaths with the second highest dose tested (300 mg kg^{-1}). Based on the OECD criteria, the LD_{50} was estimated to be 1000 mg kg^{-1}. This value is acceptable for crude extracts, as $LD_{50} \leq 25$ mg kg^{-1} is considered harmful (Gonçalves et al. 2015). In the present *in*

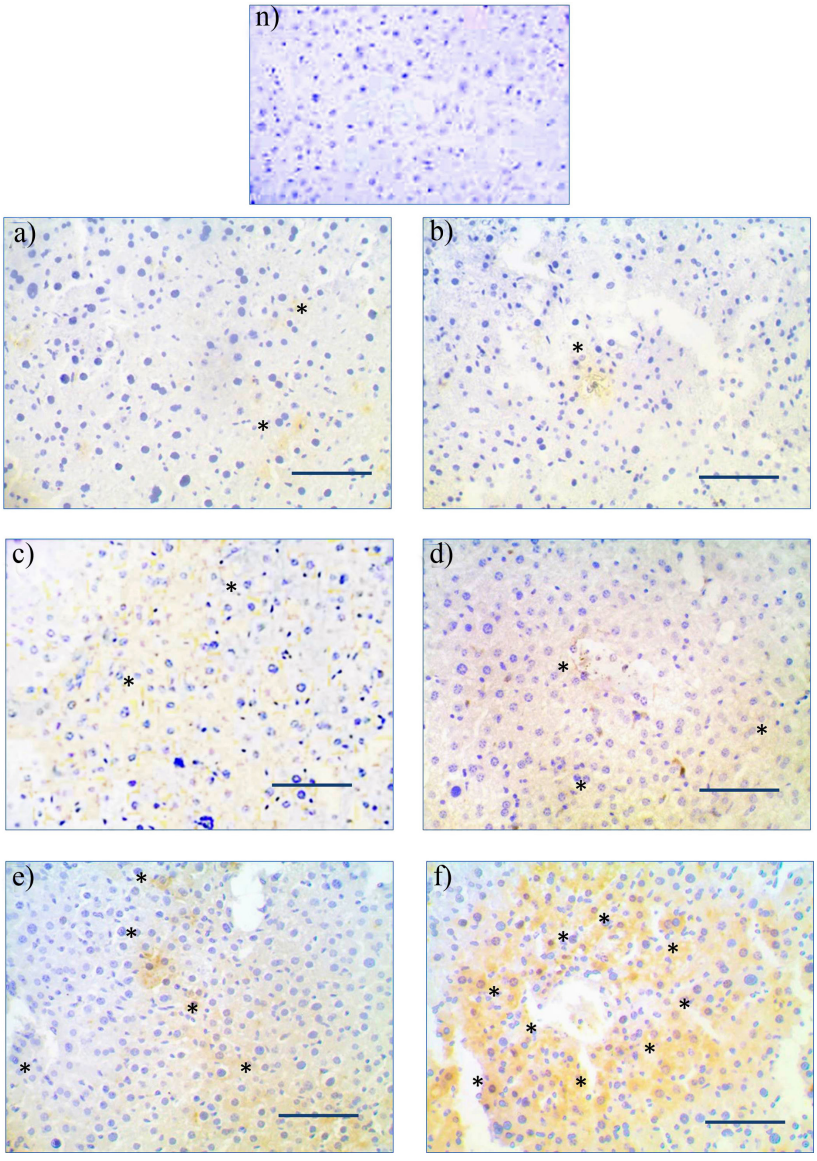

Figure 11. Immunohistochemical analysis of anti-HSP70 in liver tissue of *mdx* mice treated with different doses of aqueous extract from leaves of *Miconia ferruginata* (Photomicrography, 1:500 dilution). **n)** negative control of reaction. **a)** control group; **b)** 50 mg kg^{-1} group; **c)** 100 mg kg^{-1} group; **d)** 200 mg kg^{-1} group; **e)** 300 mg kg^{-1} group; **f)** 2000 mg kg^{-1} group. Bar: 400 µm. Asterisks indicate expression of anti-HSP70 protein.

vivo investigation, doses less than 300 mg kg^{-1} of the extract may have had pharmacological effects, demonstrating the possible regenerative and anti-inflammatory effects of the extract in *mdx* mice.

One of the most important aspects of pre-clinical studies on DMD is the evaluation of mechanisms of the induction and control of the chronic inflammatory response in humans and the experimental *mdx* model (Budel & Claro 2018). The regulation of inflammation is probably a key event in controlling the progression of the disease, for which the main pharmacological therapy comprises the prolonged use of glucocorticoids and immunosuppresses, which have many serious side effects (Ciafaloni & Moxley 2008, Abdel-Hamid & Clemens 2012, De Luca 2012, Wein et al. 2015). In DMD, the most common cell profiles in the inflammatory process are helper T lymphocytes, cytotoxic T lymphocytes, natural killer (NK) cells, eosinophils, and macrophages (Villalta et al. 2009). Thus, plant extracts with anti-inflammatory potential and low toxicity may be an effective, viable,

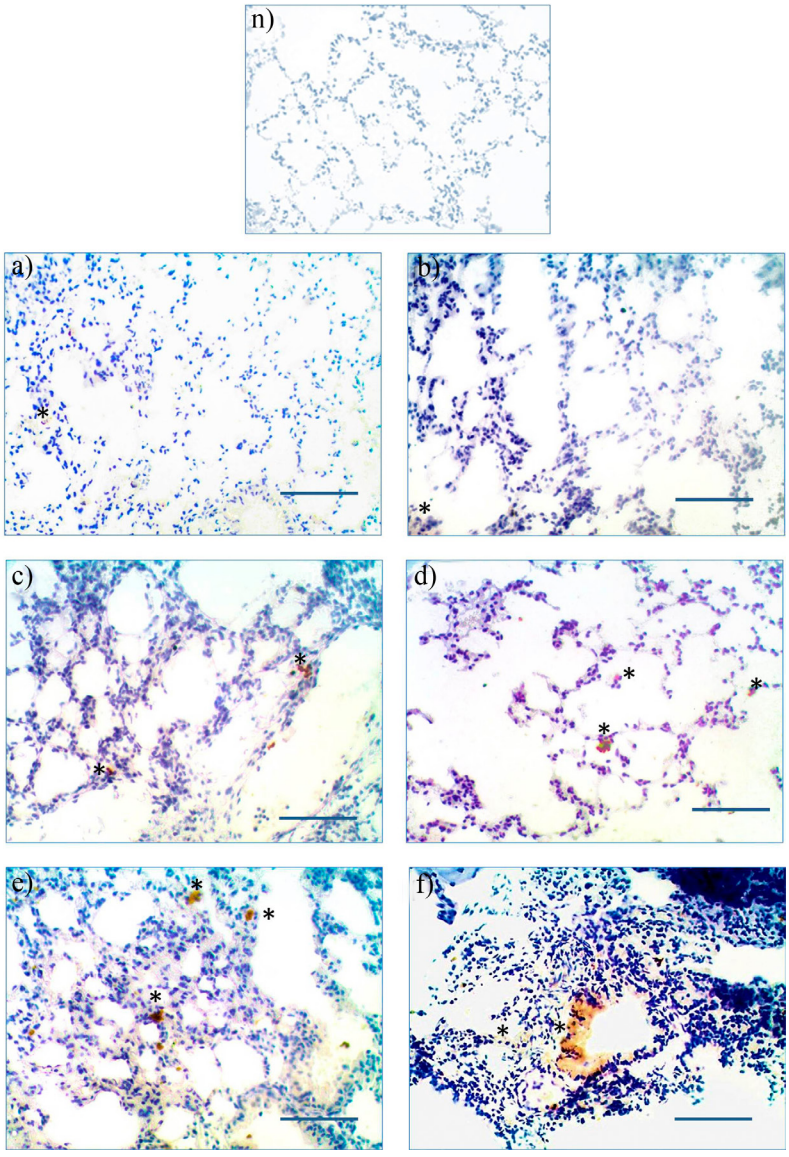

Figure 12. Immunohistochemical analysis of anti-HSP70 in lung tissue of *mdx* mice treated with different doses of aqueous extract from leaves of *Miconia ferruginata* (Photomicrography, 1:500 dilution). n) negative control of reaction; a) control group; b) 50 mg kg^{-1} group; c) 100 mg kg^{-1} group; d) 200 mg kg^{-1} group; e) 300 mg kg^{-1} group; f) 2000 mg kg^{-1} group. Bar: 400 µm. Asterisks indicate expression of anti-HSP70 protein.

low-cost, safe option for individuals with DMD. *In vitro* tests have demonstrated that the extract of *M. ferruginata* promotes reductions in the respiratory burst of neutrophils (Cruz 2017) and the proliferation of stimulated lymphocytes (Barroso 2015), depending on the dose.

The *mdx* mouse has histopathological features typical of pseudo hypertrophic muscular dystrophy. Between four and 12 weeks of age, the animals present muscle regeneration cycles with multiple necrotic foci, dense inflammatory infiltration, and the release of high concentrations of creatine kinase in the plasma (Klyen et al. 2011). Lung tissue exhibits an increase in collagen fibers throughout the lung parenchyma, which is characteristic of fibrosis (Lessa et al. 2015). Likewise, degeneration and regeneration processes, especially in muscle tissue, lead to a fibrotic process that persists throughout the life of the animal. This severely affects the diaphragm and posterior skeletal muscles, such as tibialis and soleus, with the progressive loss of contractile function and muscle weakness, similar to what is found in

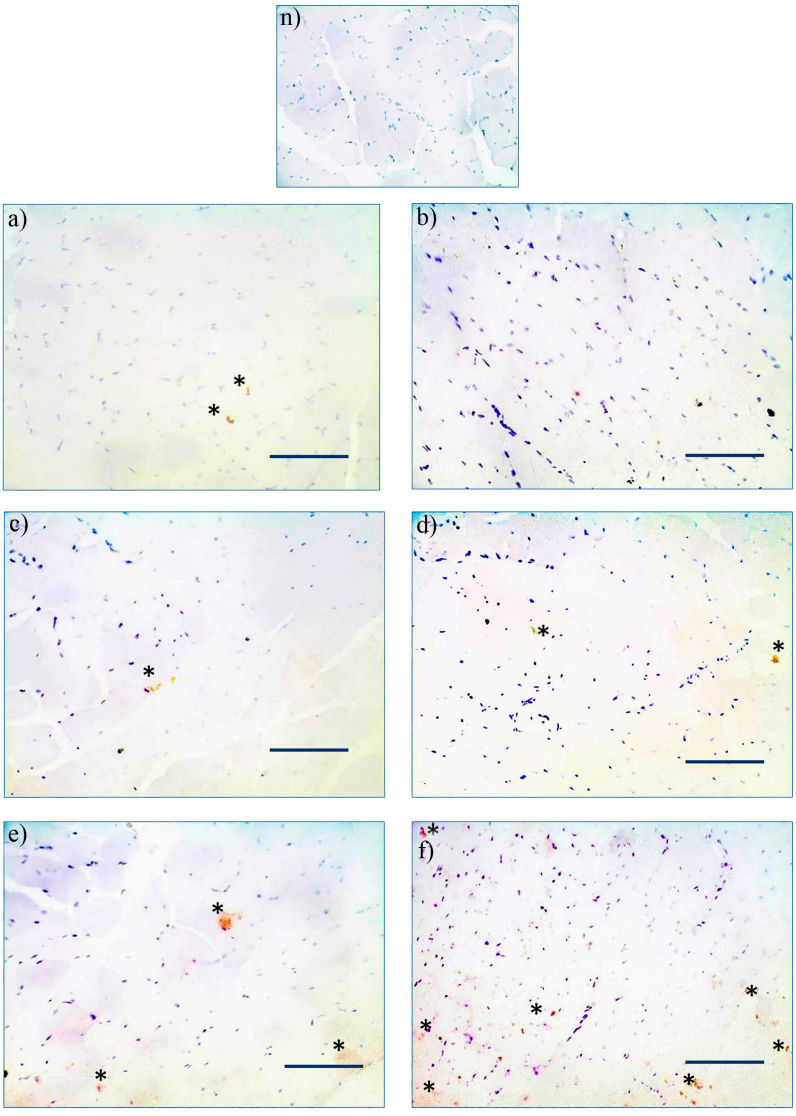

Figure 13. Immunohistochemical analysis of anti-HSP70 in muscle tissue of right tibialis anterior of *mdx* mice treated with different doses of aqueous extract from leaves of *Miconia ferruginata* (Photomicrography, 1:500 dilution). n) control negative of reaction; a) control group; b) 50 mg kg^{-1} group; c) 100 mg kg^{-1} group; d) 200 mg kg^{-1} group; e) 300 mg kg^{-1} group; f) 2000 mg kg^{-1} group. Bar: 400 µm. Asterisks indicate expression of anti-HSP70 protein.

muscular dystrophy in humans (Coirault et al. 2003, Marques et al. 2007).

In the present study, the most severe histopathological changes in all tissues analyzed occurred in the animals treated with the dose of 2000 mg kg^{-1}, such as increased hydropic degeneration with numerous fissures, extensive areas of necrosis, and dense inflammatory infiltrate. These histopathological findings reveal that the aqueous extract of *M. ferruginata* induces cell damage and stress, which is consistent with the systemic toxicity observed in these animals (Upadhyay et al. 2019). The inflammatory infiltrate may have partially contributed to the observed increase in tissue damage (Yonekawa & Harlan 2004). Although the animals treated with the dose of 300 mg kg^{-1} did not exhibit behavioral changes, we found the same histopathological changes in the different tissues as those in the group treated with 2000 mg kg^{-1} but to a lesser degree. Therefore, this dose is also toxic, promoting increased damage and cellular stress compared to the control group.

However, a reduction in histopathological changes was found in the animals treated with

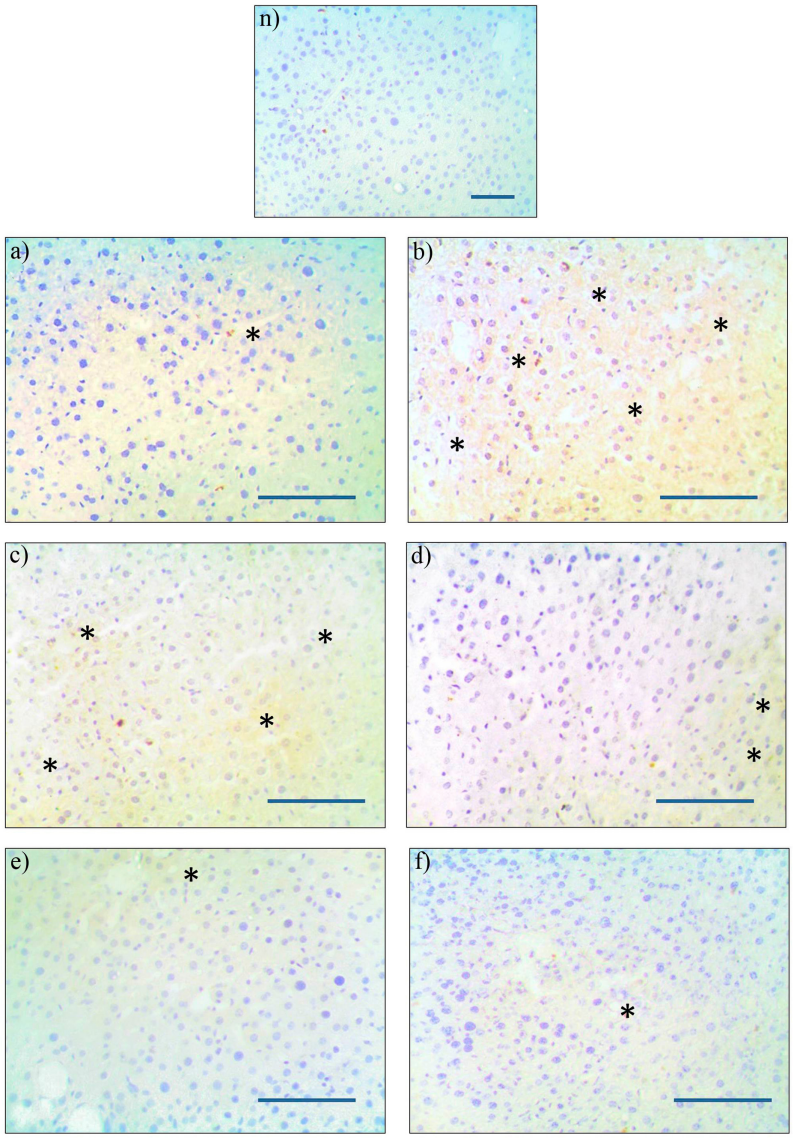

Figure 14. Immunohistochemical analysis of anti-MCL-1 in liver tissue of *mdx* mice treated with different doses of aqueous extract from leaves of *Miconia ferruginata* (Photomicrography, 1:500 dilution). **n)** negative control of the reaction. **a)** control group; **b)** 50 mg kg^{-1} group; **c)** 100 mg kg^{-1} group; **d)** 200 mg kg^{-1} group; **e)** 300 mg kg^{-1} group; **f)** 2000 mg kg^{-1} group. Bar: 400 µm. Asterisks indicate expression of anti-MCL1 protein.

doses lower than 300 mg kg^{-1} of the extract. In the tissues analyzed, reductions were found in edematous degeneration, areas of necrosis, apoptosis, and inflammatory infiltrate compared to the typical changes that occurred in the control group. Moreover, at these doses there was a reversal of the degeneration and necrosis characteristic of DMD, promoting tissue regeneration and a significant reduction in inflammatory infiltrate.

Fibrosis is another typical feature of DMD and involves the deposition of components of the extracellular matrix, especially collagen fibers, in an uncontrolled, excessive manner, leading to a decrease in tissue functioning (Kharraz et al. 2014). Picrosirius Red staining was used to quantify fibrosis, as this histochemical technique highlights collagen networks, specifically when combined with polarized light microscopy (Rittié 2017).

The analysis of the mean percentage quantification of the fibrotic area revealed that doses of 2000 and 300 mg kg^{-1} led to similar collagen deposition in the liver and lung as that found in the control group. Doses of 200, 100, and 50 mg kg^{-1} led to a significant reduction (p

<0.001) in collagen deposition and less tissue fibrosis. For the tibialis anterior muscle, only the dose of 2000 mg kg^{-1} led to an increase in collagen deposition, whereas doses of 300, 200, 100, and 50 mg kg^{-1} promoted a significant reduction in collagen deposition in a dose-dependent manner. A direct association was found between the reduction in the inflammatory process and the reduction is fibrosis in animals treated with the extract of *M. ferruginata,* these doses proved to be promising. The 50 mg kg^{-1} dose is highly promising, as demonstrated by the marked reductions in markers of stress, fibrosis, and local inflammation. This fact may be related to a lower concentration of some toxic secondary metabolite or to a lesser antagonistic effect between metabolites, which expresses an inhibitory effect on inflammatory chemical mediators at this dose. It needs to be further investigated.

The labeling of intracytoplasmic proteins involved in the cytoprotection process has been evaluated when cells are subjected to adverse environmental conditions, such as an increase in temperature, osmotic stress and oxidative stress. (Bukau & Horwich 1998). HSP70 is over-expressed during acute and chronic episodes of stress and has a cytoprotective function (Slimen et al. 2016). Therefore, this protein is widely used as a marker of cellular stress (Cole & Meyers 2011). MCL-1 is a protein of the Bcl-2 protein family that has anti-apoptotic activity and stimulates cell survival. MCL-1 neutralizes the pro-apoptotic function of *Bim* proteins and prevents the activation of death receptors (Wuillème-Toumi et al. 2005). Thus, increased levels of this protein affect the permeability of the mitochondrial membrane and the activation of the extrinsic pathway of apoptosis, contributing to the resistance to apoptosis (Gasparotto et al. 2011). HSP70 in association with co-chaperone HSP40 can modulate the balance of pro-apoptotic and anti-apoptotic proteins, such as Bcl-2 and MCL-1, and affect the balance of apoptosis (Castro et al. 2013).

In line with the histopathological observations, the significant expression of the cell stress marker HSP70 was found in all organs of animals treated with the dose of 2000 mg kg^{-1}. At the dose of 300 mg kg^{-1}, a moderate increase in this marker was found compared to the control group. These data confirm the previous findings of toxicity promoted by the extract at these two doses, as the increase in this protein in the cytoplasm occurs when the cell is undergoing a process of damage or is subjected to stress (Cole & Meyers 2011, Castro et al. 2013). The data suggest that doses of 50, 100, and 200 mg kg^{-1} provide cell protection, as the increase in the anti-apoptotic protein MCL-1 promotes a reduction in apoptosis rates and an increase in cell survival (Wuillème-Toumi et al. 2005). Moreover, these doses demonstrated high repair potential of degeneration and cellular damage and exhibited an anti-inflammatory effect.

CONCLUSIONS

The acute single-dose toxicity test in *mdx* mice demonstrated that the aqueous extract from the leaves of *M. ferruginata* is toxic when administered intraperitoneally at a dose of 2000 mg kg^{-1} and that the LD_{50} is 1000 mg kg^{-1}. Despite not causing behavioral changes in the mice, the dose of 300 mg kg^{-1} promoted moderate histological and morphological changes. However, no toxicity was found when lower doses (200, 100, and 50 mg kg^{-1}) were used, where reduction was observed in necrosis, cell degeneration, and the inflammatory process. These doses were highly promising, as demonstrated by the marked reductions in markers of stress, fibrosis, and local inflammation as well as an increase in the

anti-apoptotic protein in all tissues analyzed, especially the tibialis anterior muscle.

The extract obtained through the infusion of leaves from the plant *M. ferruginata* demonstrated anti-inflammatory potential as well as regenerative and antifibrotic action. The present data demonstrate the promising effects of the use of this extract in the treatment of degenerative diseases aggravated by the inflammatory process, such as Duchenne muscular dystrophy. Further studies are needed to determine the safety and therapeutic effects of the prolonged use of the *M. ferruginata* extract in models, and children.

Acknowledgments

We thank the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil) for the research grant we received during the development of this study. Number: 88882.429298/2019-01 PPGMCF/UFVJM. We are grateful for the use of the Laboratório de Química e Produtos Naturais of Departamento de Farmácia of Universidade Federal dos Vales do Jequitinhonha e Mucuri (UFVJM) for obtaining the plant material and preparing the extract. We also thank Professor Dr. Kinulpe Honorato Sampaio for providing the Laboratório de Histologia of Faculdade de Medicina of Universidade dos Vales do Jequitinhonha e Mucuri (FAMED – UFVJM), where the samples were histologically cut. We also thank Professor Dra. Ana Paula Santos for providing the place where the experiment was carried out Núcleo de Experimentação Animal – Faculdade de Ciências Básicas e da Saúde - NEA / FCBS.

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How to cite

MUNNO PM ET AL. 2022. Acute toxicity and regenerative dose finding of an extract of *Miconia ferruginata* DC. in a mouse model of Duchenne muscular dystrophy. An Acad Bras Cienc 94: e20210190. DOI 10.1590/0001-3765202220210190.

Manuscript received on February 8, 2021; accepted for publication on June 2, 2021

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