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EFFECTS OF LEAF AND SEED ALCOHOLIC EXTRACTS FROM OF *ANNONA CRASSIFLORA* MART. (ANNONACEAE) IN MICE

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ABSTRACT

Annona crassiflora Mart. is a species of the cerrado biome in the family Annonaceae. It is a vegetable with therapeutic potential. However, it should be emphasized that its toxicity when consumed in large doses remains unknown, and clinical studies are strongly suggested for this purpose. The objective of this study was to verify the toxicological effect of leaf and seed extracts of Annona crassiflora Mart. (Annonaceae) in mice. The plant was collected in were collected in cerrado areas in Montes Claros, Minas Gerais, Brazil. Healthy leaves and seeds were selected and dried in a forced air circulating dryer at 40ºC for 72 h. Ethanolic extract (EE) was obtained by maceration of the dried leaves in absolute ethanol. The extracts were filtered and subsequently dehydrated at 40ºC for 48 h. The chromatographic profile was performed using an HPLC system. To evaluate the toxicological effects, 18 Swiss mice, were divided into three groups, composed of three males and three females each as follows: 22 µL PBS (C), 22 µL leaf extract (LE), 22 µL seed extract (SE) that were administered by means of gavage at cumulative doses for four days at the following concentrations: day one, 12.5mg/kg; day two, 25.0mg/kg; day three 50mg/kg and the fourth day, 100mg/kg. To assess the toxicological effects, physical aspects, behavior, and body weight of animals were monitored daily. On the fifth day the mice were euthanized and peripheral blood and target tissues were collected. ALT plasma level was measured. Liver, spleen, heart, and kidney samples were collected, formalin-fisxed, paraffin-embedded, and submited to histopathological analysis. Statistical analysis was performed using Student t tests and ANOVA. The Bonferroni test was applied as post hoc. Valeus of $p < 0.05$ were considered as statistically significant. Our findings showed that *A. crassiflora* Mart. extract is rich in flavonoids and flavones. No clinical signs or symptoms that had suggested toxicity were observed in all animals. However LE and SE animals showed decreased body weight, increased size and wight for liver and spleen organs. Moreover, ALT levels were increased in experimental animals. Histological analysis showed morphological findings of discrete hyperemia in liver and spleen tissues of animals treated with SE. In conclusion, the present study evidenced potential toxic effects of *A.crassiflora* Mart. leaf and seed extract notably in liver and spleen organs. More prospective studies are needed to better understand the cytotoxic effects of A. crassiflora.

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INTRODUCTION

Annona crassiflora Mart., commonly known as panã, araticum-do-cerrado, articum, marolo, pinha-do-cerrado, among others, is a species in the Annonaceae family (LUZIA e JORGE, 2013). In Brazil it is widely distributed in the Cerrado biome, and commonly used in the manufacture of candies, ice creams and liquors. However, the greater relevance of this species is due to its use on traditional medicine (Almeida *et al*., 2008). This species have been target of studies aimed to elucidate its medicinal potential, specially to identify the secondary compounds produced by this species, such as: alkaloids, with antimicrobial and leishmanicidal activity, phenolic with antioxidant and hypoglycemic characteristics (Barbalho *et al*., 2012), and acetogenins that are potent and selective with cytotoxic and antimicrobial properties (Biba *et al*., 2014). The acetogenins also present antimutagenic and antiparasitic (Vilar *et al*., 2008) anti-HIV, antidiabetic, antitumor, wound healing, insecticide and antifungal activities (Chen *et al*., 2012, Gupta *et al*., 2005). A few in vivo studies that evaluated the *A. crassiflora* therapeutic effects, reported abnormal clinical signs in animals treated with the alcoholic extract, such as anxiety and restlessness (ROESLER *et al*., 2007), loss of body weight of approximately 28% as compared to the untreated group (Roesler, 2011). Lambs that received seeds extract for anthelmintic treatment presented diarrhea and severe lesions followed by death, while the control group did not display any similar symptom (Oliveira *et al*., 2011). Toxicological studies are of paramount importance for the development of new drugs and evaluation of potential therapeutic molecules (Parasuraman, 2011). Medicinal plants deserve special attention as they can be therapeutically efficient but toxic to organs and tissues (Chanda, *et al*., 2015). The toxicological studies were incremented in 1920 by the introduction of animals that allow the determination of lethal doses/concentrations and also to evaluate the toxic effects in several levels (from cells to whole body homeostasis) (Parasuraman, 2011). Considering that studies reported abnormal clinical signs *in vivo* and that few studies aimed to investigate the toxicological effect of the *A. crassiflora* extract, it is important to conduct toxicological tests in order to guarantee safety regarding its therapeutic indication. The objective of this study was to verify potential toxicologyeffects of leaf and seed alcoholic extracts of *A. crassiflora* Mart. (Annonaceae) in mice.

MATERIAL AND METHODS

Study area: The research was conducted in Montes Claros City, North of Minas Gerais, Brazil. The area of collection consisted of Cerrado vegetation, with tropical climate characterized by dry summer according to the Koppen classification (Alvares *et al*., 2013). The region is marked by a dry season from May to September and a rainy period in January and February.

Plant material and extract preparation: Healthy-appearing *A. crassiflora* leaves and seeds were selected and dried to constant weight in a forced air circulating drier at 40 º C for 72 h. Dried leaves were ground in a Wiley mill and stored in paper bags, without light. Voucher specimen 1492 was submitted to the Montes Claros Herbarium (HMCMG) of Universidade Estadual de Montes Claros (UNIMONTES). Ethanolic extract (EE) was obtained by maceration of the dried leaves in absolute ethanol, in amber-colored glass containers, kept in darkness for seven days. Extracts were filtered through a gauze funnel and subsequently dehydrated at 40 º C for 48 h, to obtain a residue with constant weight, and stored in paper bags in darkness (Morais-Costa *et al*., 2015; 2016).

Extract characterization: A Waters Alliance 2695 HPLC system composed of a quaternary pump, an auto-sampler, a photodiode array detector (DAD) 2996, and a Waters Empower Pro data handling system (Waters Corporation, Milford, Connecticut, USA) was used for the extract characterization. The analyses were performed on a LiChrospher 100 RP-18 column (250 × 4 mm 5 μm; Merck, Darmstadt, Germany) combined with a LiChrospher 100 RP-18 guard column (4 × 4 mm, 5 μm; Merck) at 40 º C. Water (A) and acetonitrile (B) were used as eluents, both containing 0.1% (v/v) H_3PO_4 at a flow rate of 1.0 mL min^{-1} as follows: 0 min, 95% A and 5% B and 60 min, 5% A, 95% B, followed by 10 min of isocratic elution. Solvents used were of HPLC grade (Merck, Germany) and were degassed by sonication before use. The chromatograms were obtained at 210 nm, and the UV spectra were recorded on-line from 190 to 400 nm. The dried extracts were dissolved in methanol (HPLC-grade), ultrapure water, or hydroethanolic solutions, according to their solubility, to concentrations of 10 mg mL^{-1}. After centrifugation at 8400 × *g* for 10 min, 10 μL of the sample solutions were automatically injected into the apparatus.

In vivo experiments: 18 swiss male mice aged 10 weeks old were used (9 female and 9 male). All animals were healthy with about 28 ± 4 grams of body weight. The animals were obtained from the Biological Sciences Institute (ICB) animal facility of Universidade Federal de Minas Gerais (UFMG). They were kept at the Unimontes animal facility for adaptation period with the following conditions: 22 ± 2ºC, 60 ± 5% relative humidity, 12h light/dark cycles and low sound level <40 dB, with free access to filtered water and balanced food (Purina-Labina®). All procedures were performed in accordance with the principles of animal experimentation approved in the 275/2013 protocol of the Ethics Committee on the use of animals (CEUA) of the Federal University of Minas Gerais, Brazil.

Toxicity analysis: The *A. crassiflora* toxicity test was adapted from Walum (1998) to determine the maximum tolerated dose (MTD) for adult mice. The mice were divided into three groups (n=3 male and n=3 female each). Phosphate Buffered Saline Solution (PBS) was administered to the control group (C) and the leaf extract (LE) and the seed extract (SE) of *A. crassiflora* were administered to the respective groups. Extracts administration was performed by cumulative doses through gavage for four days. On day one, leaf and seed extracts were administered at 12.5 mg/kg body weight, on day two 25.0 mg/kg body weight, on day three and four the extracts were respectively administered at 50.0 mg/kg and 100.0 mg/kg body weight. To evaluate the toxicological effect, clinical and body weight behavior were analyzed daily. After four days of treatment (five day), the blood collection by made by decapitation (Andrade *et al*., 2014), followed by euthanasia with liver, spleen, kidney and heart removal. The biological material was submitted to macroscopic and microscopic evaluation.

Biochemical analysis: In to concerts the functional activity of the liver of animals, blood samples were collected and alanine aminotransferase (ALT) levels were assessed.

Histopathologic Analysis : In order to verify histological alterations, liver, heart, kidney and spleen samples were infiltrated and placed in paraffin using bounding forms. After the paraffin block solidification the samples were submitted to histological sections of 5 µm thickness using a histological microtome (model CUT5062, Slee Technik GmbH, Mainz, Germany). The histological sections were adhered on a glass slide and kept in an oven at 37 ° C for 2h. The slides were submitted to histological staining with hematoxylin and eosin (H&E) and Masson trichrome (TM).

Statistical Analysis: All data were transferred to Graph Pad Prism software. Student's t-test and One-way ANOVA testes were applied. Bonferroni test was applied as post hoc. $p< 0.05$ was considered as statistically significant.

RESULTS AND DISCUSSION

Extract characteristics: The *A. crassiflora* leaves ethanolic extracts evidenced two majority peaks (TR=6.484 and TR=7.704 min) and presented UV spectrum characteristics that suggest presence of flavonoid and flavone compounds, respectively and *A. crassiflora* seeds ethanolic extracts displayed two majority peaks (TR = 15.591 and TR = 47.061). The characteristics presented by the UV spectrum suggest presence of a flavonoid compound (Figures 1A and 1B).

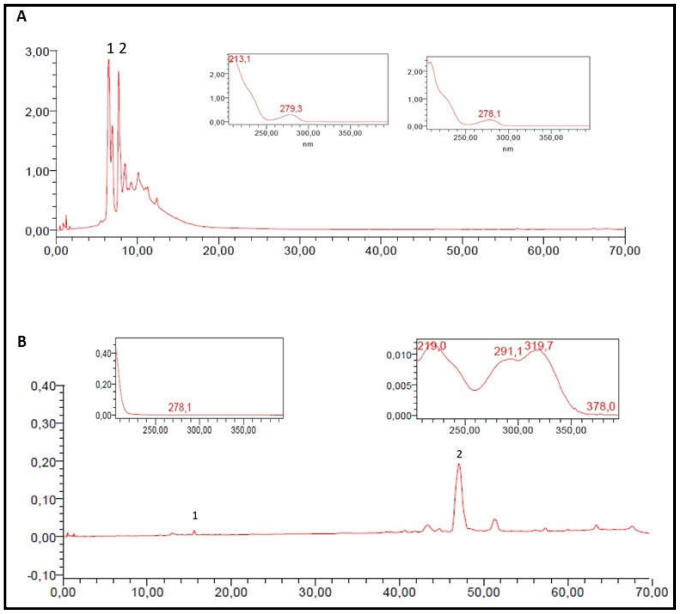

Figure 1. HPLC chromatographic profile of the *A. crassiflora* extracts. (A) Peaks 1 and 2 and their respective retention time represent the flavonoid UV spectrum characteristics of the leaves extracts. (B) Peak 1 indicates flavonoids and peak 2 indicates flavones and their respective retention time are presented for the seeds extracts

Flavonoids and flavones are classified in the same compound subgroups due to their chemical structure (Kozlowska and Szostak-Węgierek, 2014). However, the flavones are widely found in other vegetative organs, such as leaves (Panche *et al*., 2016). Scientific evidence indicate that these substances develop an important beneficial role on the diseases prevention, however, it should be emphasized that their toxicity when consumed in large doses remains unknown, being strongly suggested the conduction of clinical and epidemiological trials for this purpose (Kozlowska and Szostak-Węgierek, 2014). The flavonoids and flavones are described in the literature for their currently use in therapeutic products and are widely consumed in the diet by the population (Niiveldt, *et al*., 2001). However, studies regarding their toxicity are also described in the literature (Wang, *et al*., 2007; Santos, *et al*., 2009; Galati and O'Brien, 2004). A few studies already described potential malefic effects of flavonoids, such as pro-oxidant activity, interaction with enzymes that metabolize drugs, cell cycle regulation and were already described to participate in liver failure, contact dermatitis, and hemolytic anemia, among others (Galati and O'Brien, 2004).

Toxicity test in mice

No clinical signs of toxicity regarding the animals behavior or mortality rate were observed during the four days of extracts administration. However, the mice treated with seeds extract presented decreased body weight (Figure 2) and the blood exam of these animals revealed increased ALT levels for male and female (Figure 3).

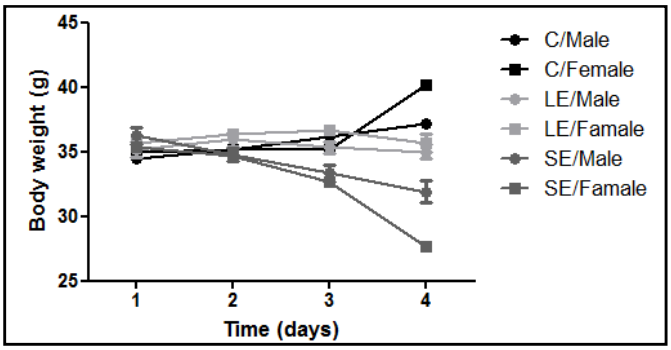

Figure 2. Body weight over the 4-day experimental period for male and female mice treated with *A. crassiflora* seeds and leaves extracts. C: Control; LE: Leaves extract; SE: Seeds extract

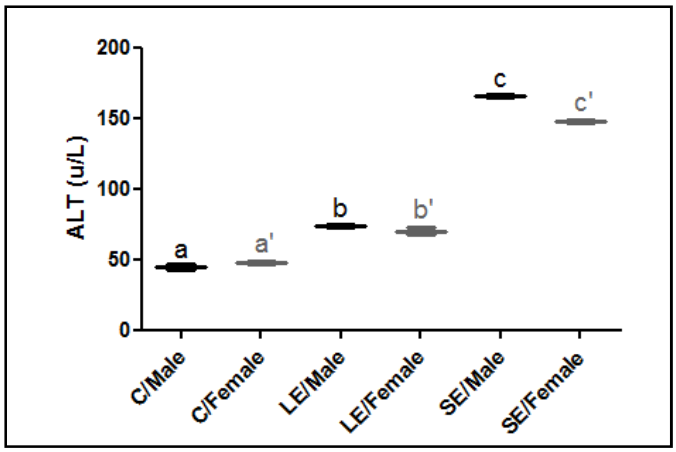

Figure 3. Effects of *A. crassiflora* leaves and seeds extracts on ALT levels. * Reference value for male 65 U/L – 58 U/L and female 26 U/L – 60 U/L. ALT: Alanine Aminotransferase. C: Control; LE: Leaves extract; SE: Seeds extract

The increased ALT levels observed after the extracts administration reflects liver injury as already established in the literature (Amacher, 1998). ALT is a very specific marker for liver injury, which is increased after ischemic or toxic injuries.

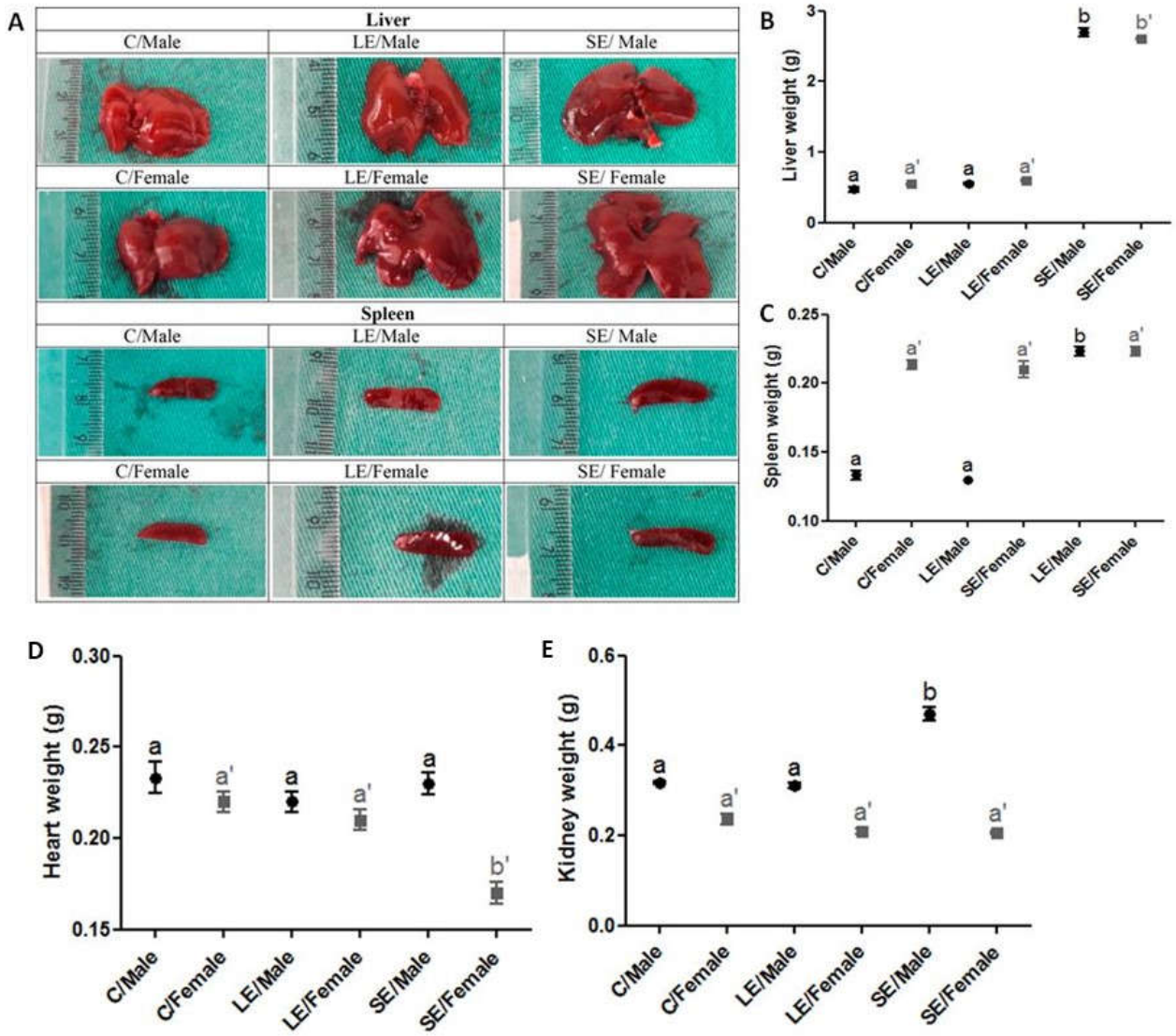

Figure 4. Macroscopic evaluation of the liver and spleen from male and female mice treated with seeds and leaves *A. crassiflora* ethanolic extracts and organs weight. A) Spleen and liver images from male and female mice treated with leaves and seeds extracts and control group. B) Liver weight (g/BW), C) Spleen weight (g/BW), D) Heart weight (g/BW), E) Spleen weight (g/BW). C: Control; LE: Leaves extract; SE: Seeds extract

The literature already reports as guideline the pharmacological history evaluation when the exams report increased ALT levels, as it is common the increase in ALT levels after herbal products or medications consumption (Giannini, *et al*., 2005). Based on the literature, the increased ALT levels observed in the treated animals may correlate with liver injury caused by *A. crassiflora* ethanolic extract, although other liver markers should be used in the clinical practice in order to evaluate more carefully the potential injury. Macroscopically the organs did not present any relevant alterations (Figure 4A), however the liver and spleen of mice treated with the extracts, particularly seeds extract, appear to have undergone a slight increase as compared to the control group, in both males and females (Figure 4B e C). The decreased weight observed in the animals and, increase liver and spleen weight after extracts administration, which might correlate with increase inflammation caused by the extracts consumption. Inflammation is commonly associated to edema, as it induces vasodilatation and also alters protein metabolism (Mercier *et al*., 2002). The organs weight measurements evidenced increased liver and spleen weights in animals treated with both extracts, being more evident in animals treated with seeds extracts.

No weight alterations were observed for the heart and kidneys as compared to control (Figure 4D e E). No morpho-pathological alterations (necrosis, inflammatory infiltration, edema) were observed in the analyzed tissues. However, vascular hyperemia in the kidney and liver of treated animals was observed as compared to control tissues. No differences were observed between male and female mice samples (Figure 5).

Another interesting finding observed in the present study was the vascular hyperemia observed in the liver and kidney of treated animals as compared to control. Vascular hyperemia is the increased blood flow in a specific tissue or organ after a given stimuli (Bliss, 1998), such as oxygen deprivation and metabolites accumulation. Interestingly, Heiss et al. 2003 described that flavonoids exert important vascular effects via increased nitric oxide synthase (NOS) activity that one of the main regulators of vascular response (Heiss, et al., 2003). Once *A. crassiflora* extracts are rich in flavonoids, the vascular hyperemia observed might be associated to this compound.This might be correlated with the pro-inflammatory actions sometimes exerted by flavonoids (Damas, et al., 1985).

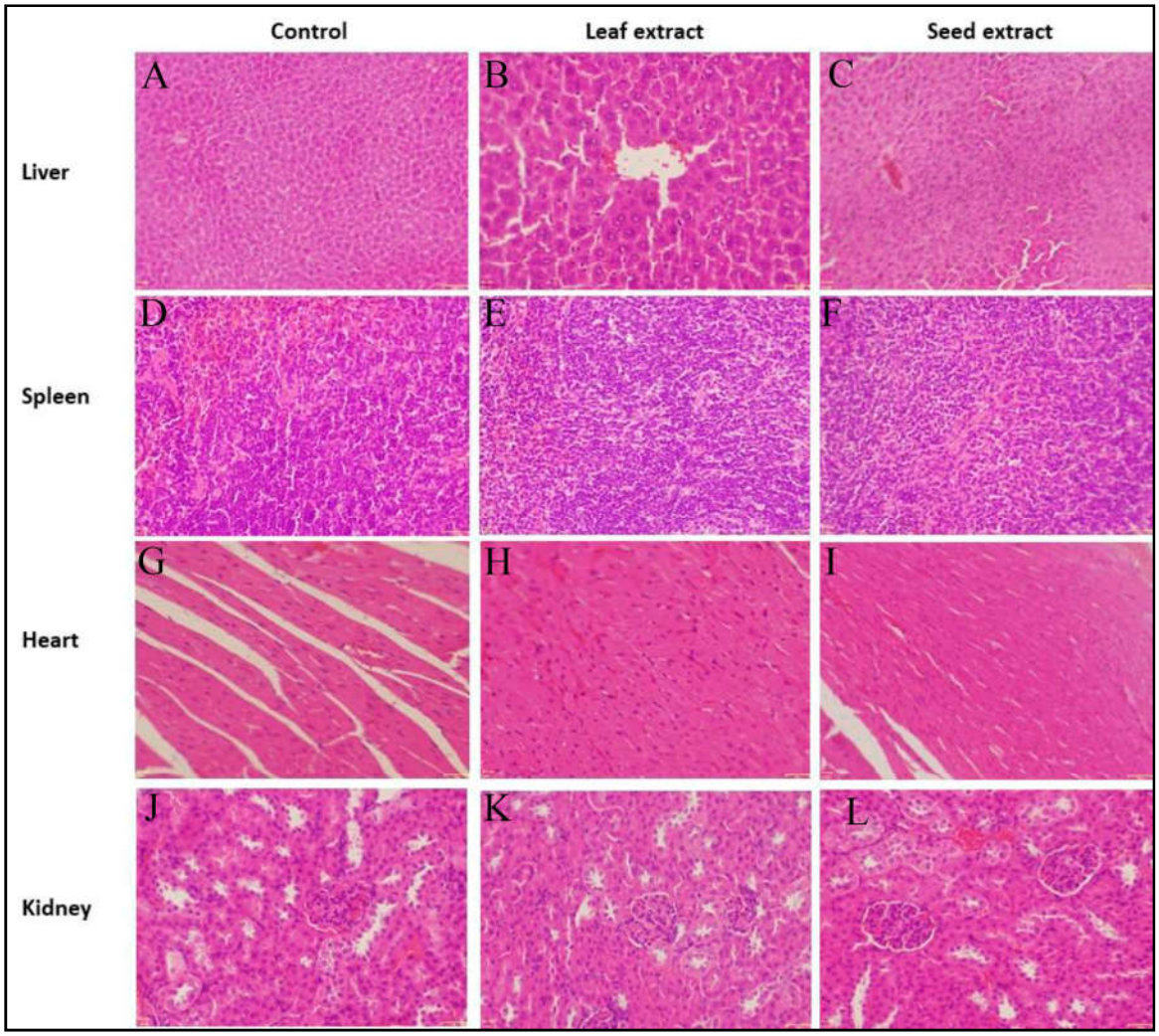

Figure 5. Histopathological fndings from heart, liver, spleen, and kidneys samples from males and females Swiss mice treated with ethanolic extract of leaves and seeds *A. crassiflora* extracts. A, D, E, F, G, H, I e J) absence of morpho-pathological alterations B, C, K e L) presence of vascular hyperemia. Coloration: H&E. Magnification 400X Scale: 100 µm

Conclusion

In summary, the present study indicates that the *A. crassiflora* seeds and leaves ethanolic extracts exert important hemotoxic effects in mice, which might be taken in consideration before the therapeutic use of this species extracts. Future studies aimed to observe the long-term effects of these extracts and more specific toxicity tests are needed. Additionally, analysis capable to isolate the main compounds present in the *A. crassiflora* are needed.

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REFERENCES

Andrade JM, Frade AC, Guimarães JB, Freitas KM, Lopes MT, Guimarães AL, de Paula AM, Coimbra CC, Santos SH. Resveratrol increases brown adipose tissue thermogenesis markers by increasing SIRT1 and energy expenditure and decreasing fat accumulation in adipose tissue of mice fed a standard diet. *Eur J Nutr.* 53(7):1503-10, 2014.

Almeida, S. P.; Agostini-Costa, T. S.; Silva, J. A. Frutas nativas do cerrado: caracterização físico-química e fonte potencial de nutrientes. In: SANO, S. M.; ALMEIDA, S. P.; RIBEIRO, J. F. Cerrado, ecologia e flora Brasília: *Embrapa Informação Tecnológica*, 2008.

Amacher, DE. Serum Transaminase Elevations as Indicators of Hepatic Injury Following the Administration of Drugs. *Regulatory Toxicology and Pharmacology*, 27(2), 119-130, 1998.

Aleksandra Kozłowska, Dorota Szostak-Węgierek. Flavonoids - Food Sources And Health Benefits. *Rocz Panstw Zakl* Hig., 65(2):79-85, 2014

Barbalho SM, Goulart RA, Farinazzi-Machado FMV, De Souza MS, Bueno Guiguer EL, Araujo AC,Groppo M. Annona sp: Plants with multiple applications as alternative medicine -a review. *Curr. Bioact.Comp*., 8:277-286, 2012.

Biba VS, Amily A, Sangeetha S,Remani P. Anticancer, antioxidant and antimicrobial activity of Annonaceae family. *World J.Pharm.Pharm.Sci.* 3:1595-1604, 2014.

Bliss MR. Hyperaemia. J Tissue Viability. 8(4):4-13, 1998.

Chanda S, Parekh J, Vaghasiya Y, Dave R Baravalia Y and Nair R: A Review: Medicinal Plants - From Traditional Use to Toxicity Assessment. *Int J Pharm Sci Res,* 6(7): 2652-70, 2015.

Chen Y, Chen JW, Wang Y, Xu SS, Li X. Six cytotoxic annonaceous acetogenins from *Annona squamosa* seeds. Food Chem. 135:960–6, 2012.

Damas J, Bourdon V, Remacle-Volon G, Lecomte J. Pro-inflammatory flavonoids which are inhibitors of prostaglandin biosynthesis. Prostaglandins Leukot Med. 19(1):11-24, 1985.

Galati G, O'Brien PJ. Potential toxicity of flavonoids and other dietary phenolics: significance for their chemopreventive and anticancer properties. Free Radic Biol Med. 37(3):287-303, 2004.

Giannini, E. G., Testa, R., & Savarino, V. Liver enzyme alteration: a guide for clinicians. CMAJ : Canadian Medical Association Journal, 172(3), 367–379, 2005.

Gupta RK, Kesari AN, Watal G, Murthy PS, Chandra R, Tandon V. Nutritional and hypoglycemic effect of fruit pulp of *Annona squamosa* in normal healthy and alloxan-induced diabetic rabbits. Ann Nutr Metab. 49:407–13, 2005.

Heiss C, Dejam A, Kleinbongard P, Schewe T, Sies H, Kelm M. Vascular Effects of Cocoa Rich in Flavan-3-ols. *JAMA*, 290(8):1030–1031, 2003.

Luzia, D.M.M., Jorge, N. Bioactive substance contents and antioxidant capacity of the lipid fraction of Annona crassiflora Mart. Seeds. *Industrial Crops and Products,* 42:231-235, 2013.

Mercier S, Breuillé D, Mosoni L, Obled C, Patureau Mirand P. Chronic inflammation alters protein metabolism in several organs of adult rats. *J Nutr.* 132(7):1921-8, 2002.

Neide Judith Faria de Oliveira, Eduardo Robson Duarte, Luciana Castro Geraseev, Patrícia Nery Silva Souza, Manoel Ferreira de Souza, João Augusto Maia Mairink, Diogo Gonzaga Jayme, Luiz Alberto do Lago. TOXICIDADE DE Annona crassiflora SOBRE OVINOS EM TESTE DE CAMPO: RELATO DE CASO. Vet. e Zootec. 2011 dez.; 18(4 Supl. 3): IX Congresso Brasileiro Buiatria. 04 a 07 de Outubro de 2011. Goiânia - Goiás, Brasil. P:622-624

Nijveldt RJ[1], van Nood E, van Hoorn DE, Boelens PG, van Norren K, van Leeuwen PA. Flavonoids: a review of probable mechanisms of action and potential applications. *Am J Clin Nutr.* 74(4):418-25, 2001.

Panche, AN, Diwan, AD and Chandra, SR. Flavonoids: an overview. *Journal of Nutritional Science.* 5(e47), 1-15, 2016.

Parasuraman, S. Toxicological screening. *J Pharmacol Pharmacother,* 2(2): 74–79, 2011.

Perry, B.D., Randolph, T.F., McDermott, J.J., Sones, K.R. and Thornton, P.K. Investing in animal health research to alleviate poverty. ILRI (International Livestock Research Institute), Nairobi, Kenya, pp: 148, 2002.

ROESLER, R. et al. Antioxidant activity of Annona crassiflora: characterization of major components by electrospray ionization mass spectrometry. *Food Chemistry,* 104 (3), 1048-1054, 2007.

Setzer RW, Kimmel CA. Use of NOAEL, benchmark dose, and other models for human risk assessment of hormonally active substances. *Pure Appl Chem.* 75:2151–8, 2003.

Stallard N, Whitehead A. Reducing animal numbers in the fixed-dose procedure. *Hum Exp Toxicol.* 14:315–23, 1995.

Vilar, J.B., Ferreira, F.L., Ferri, P.H., Guillo, L.A., Chen, L. Assessment of the mutagenic, antimutangenic and citotoxic ativities of ethanolic extract of araticum (Annona crassiflora Mart. 1841) By micronucleus test in mice. *Brazilian Journal of biology,* 68, 141-147, 2008.

Wang H, Zhao X, Wang Y, Yin S. Potential toxicities of flavonoids. 36(5):640-2, 2007.

Santos, EL. et al. Toxicidade e atividade antioxidante de flavonoides das cascas das raízes de Lonchocarpus filipes. Quím. Nova, 32(9), 2255-2258, 2009 .

Zhengyu Guo, Jorge Francisco González, Julia N. Hernandez, Tom N. McNeilly, Yolanda Corripio-Miyar, David Frew, Tyler Morrison, Peng Yu, and Robert W. Li. Possible mechanisms of host resistance to Haemonchus contortus infection in sheep breeds native to the Canary Islands. Scientific Reports. Pag 1-14, 2016.

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