Stricto Ensu Editora

EFFECT OF THE ETHANOLIC EXTRACT OF *Dysphania ambrosioides* (MASTRUZ) ON BONE REPAIR IN *Rattus norvegicus*: A STUDY BY HISTOLOGICAL AND BIOCHEMICAL ANALYSIS, AND RAMAN SPECTROSCOPY

Esmeralda Maria Lustosa Barros[1], Pedro Vítor Lopes-Costa[2], Waldilleny Ribeiro de Araújo Moura[3], Lianna Martha Soares Mendes[4], Valmir Oliveira Silvino[3], Rayane Carvalho de Moura[5], Talvany Luís de Barros[6], Ana Karolinne da Silva Brito[7], João Victor Barbosa Moura[8] and Cleânio da Luz-Lima[9]

1. Sciences and Health Program, Health Sciences Center, Federal University of Piauí, Teresina, PI, Brazil.
2. Department of Gynecology, Health Sciences Center, Federal University of Piauí, Teresina, PI, Brazil.
3. Departament of Biophysics and Physiology, Health Sciences Center, Federal University of Piauí, Teresina, PI, Brazil.
4. Department of Specialized Medicine, Health Sciences Center, Federal University of Piauí, Teresina, PI, Brazil.
5. Department of Nutrition, Health Sciences Center, Federal University of Piauí, Teresina, PI, Brazil.
6. Integral Diferencial College (FACID/Devry),Teresina, PI, Brazil.
7. Medicinal Plants Research Center, Federal University of Piauí, Teresina, PI, Brazil.
8. Science and Technology Center, Federal University of Cariri, Juazeiro do Norte, CE, Brazil.
9. Department of Physics, Natural Sciences Center, Federal University of Piauí, Teresina, PI, Brazil.

ABSTRACT

Due to the photochemical composition and possible biological effects of *Dysphania Ambrosioide* (mastruz), we investigated the effect of the ethanolic extract of the mastruz leaf (EEM) on the bone repair of rats. Female Wistar rats (250-300 g) were subjected to bone defect in the tibia and divided into the following groups: EEM-t: Topical filling of the lesion with EEM (5mg/kg) (n = 12), EEM-o: oral treatment with EEM (5 mg/kg) (n = 12), Positive control group - PCG: filling with Bio oss® (n = 12), and Negative control group - NCG: filling with blood clot and saline (n = 12). Each group was subdivided into two subgroups: 15 and 30 days of treatment (n = 6 / treatment time). After the experiment, analysis of serum calcium (SCA) and alkaline phosphatase (APH) was performed. The tibiae were sent for histological analysis and Raman spectroscopy. After 15 days of treatment, there were no significant differences in the APH levels of EEM when compared to PCG and NCG. SCA levels were significantly lower ($p < 0.05$) in EEM-o compared to NCG. When treated for 30 days, EEM-o

and EEM-t showed significantly lower APH ($p < 0.05$) than PCG, with no difference in relation to NCG, while their SCA levels did not differ in relation to NCG. The tibias treated with EEM-t showed similar results after 15 and 30 days, with mild inflammatory infiltration, edema, and granulation tissue. In EEM-o for 15 days, there was no inflammation and organized bone formation, while after 30 days, the analysis showed a total absence of inflammation, while Raman analysis showed complete bone healing. EEM-o at both treatment times of treatment produced improvement in parameters related to bone repair in rats.
Keywords: Dysphania Ambrosioides, Raman spectroscopy, Bone defect and Inflammation.

1. INTRODUCTION

The bone has a protein structure strengthened by calcium phosphate and possesses a great regenerative capacity. The entire formation process occurs through biological integration of biochemical, cellular, and hormonal processes, which are facilitated by a state of deposition, reabsorption, and bone remodeling (RAMOSHEBI et al., 2002).

It is estimated that, in the United States, 500,000 bone grafting procedures are carried out every year due to accidents or diseases which lead to loss of bone tissue (BOSTROM; SEIGERMAN, 2005). Bone graft is considered to be the gold-standard procedure regarding bone injury treatment, as it is widely accepted and efficient, although it is not perfect. Some of its disadvantages are; the difficult acceptance by the patients (as it is necessary to remove bone from another area), the volume and the limited shape of the donor areas, and the defect generated in the graft extraction and the postoperative, which usually presents more complications than in the receiving area (SOOST et al., 2001). For this reason, there is a constant concern in the medical and dental areas for bone repair procedures, as well as materials and techniques used for their optimization (PEREIRA et al., 2011).

Many plants are popularly used for nutritional or therapeutic purposes, as well as in the development of new drugs for the treatment of degenerative diseases, such as osteoarthritis. Studies show that several natural products have the ability to enhance the bone repair process (KUMAR et al., 2007; ALMEIDA et al., 2010; BLOKHUIS; ARTS, 2011; MARÃO et al., 2011; SANTOS et al., 2014; GARCÍA-GARETA et al., 2015). The mastruz plant is an annual or perennial shrub species with a strong aromatic odor and is widely distributed around the world. It is considered by the World Health Organization as the most used species among traditional remedies in folk medicine. Its use is widespread empirically in the treatment of wounds and inflammations of the skin through the use of compresses, bandages and/or ointments. It also serves to treat bruises and fractures (LORENZI, 2008; BARACUHY et al., 2016).

The mastruz has in its composition a high concentration of monoterpenes, such as ascaridol, one of the most abundant compounds. It is found in ethanolic extracts of mastruz leaves and has anti-nociceptive, sedative, and anti-inflammatory effects (DEMBITSKY et al., 2008; OLAJIDE et al., 1998; IBIRONKE; AJIBOYE, 2007; TRIVELLATO et al., 2013). In addition, the mastruz leaves are rich in phenolic derivatives and have different classes of secondary metabolites, such as terpenes and flavonoids (ASSAIDI et al., 2019; REYES-BECERRIL et al., 2019).

Considering that orthopedic trauma causes many sequelae in patients, it is necessary to investigate the therapeutic use of mastruz in the anti-inflammatory and osteoconductive action in bone regeneration in experimental conditions, as it may have clinical application in humans. Thus, due to the nature and photochemical composition of mastruz and its biological effects, we evaluated the anti-inflammatory action and osteoconductive capacity of mastruz in bone repair in Rattus norvegicus Wister. The tibiae were sent for histological analysis and Raman spectroscopy for assess inflammatory processes and the phosphate group formed. Raman spectroscopy has been usad as for studying bone repair and to monitor the progression of some pathologies through qualitative and quantitative analyses (SOUZA et al., 2012; MAIA FILHO et al. 2014).

2. MATERIAL AND METHODS

2.1 OBTAINING PLANT MATERIAL

The leaves of mastruz were collected at the Nucleus of Aromatic and Medicinal Plants (Nuplam), Federal University of Piauí (UFPI), in Teresina, PI, Brazil. An exsiccata is deposited at the Herbarium Graziela Barroso of UFPI and registered under number 31.653.

The leaves were sanitized and then dried naturally at room temperature in an airy place in the shade for 10 days. Next, they were grinded using an industrial blender (METVISA). 75 g of greenish colored powder was obtained, and 400 ml of absolute ethyl alcohol was added to extract the chemical compounds. The mixture was manually stirred for five minutes for 7 days. Afterwards, the concentrated ethanolic extract was placed on a rotary evaporator (IKA RV-10) under reduced pressure and controlled temperature (50-55 °C). The extract underwent a lyophilization process at 50 to 20 ºC.

2.2 ANIMALS AND EXPERIMENTAL PROCEDURES

In this study, the ethical principles of animal experimentation were applied in accordance with the guidelines of the National Council for Animal Experimentation Control, following the Normative Resolution No. 37 of February 15, 2018, approved by the Animal Use Ethics Commission (CEAU / UFPI), registration no. 520/18.

The experiment was carried out at the Biophysics and Physiology Laboratory of Federal University of Piauí (UFPI) using 48 female Wisters (Rattus norvegicus), aged 60 days and weighing between 250 to 300 g, clinically healthy. They were born in the vivarium center at Federal University of Piauí (UFPI) and kept in hygienic environments, in polypropylene cages since birth, fed with a standard diet of the vivarium ration (Labina TM) and water 'ad libitum' (at will) with light and dark cycle 12 h, at room temperature between 20 to 24º. The specimens were randomly distributed in 4 groups with 12 animals, where each group was subdivided into 2 subgroups according to the euthanasia period (15 and 30 days after surgery).

The animals were weighed and underwent dissociative anesthesia through ketamine hydrochloride (50 mg/mL) (Dopalen®, Sespo Indústrias e Comércio Ltda, Paulínia, SP, Brazil) and Xylazine (20 mg/mL) (Anasedan®, Sespo Indústrias e Comércio Ltda, Paulínia, SP, Brazil) in a 1:1 ratio (0.1 ml - 100 g) (MASSONE, 2003). Next, the skin was shaved and asepsis was performed with topical povidone-iodine solution (Ceras Johnson® Ltda, Manaus, AM, Brazil). A 20 mm long linear incision was made in the craniocaudal direction using a scalpel 15, followed by the divulsion of the skin, muscle, and periosteum for the exposure of the medial bone surface, with preservation of the periosteum. Using a spherical carbide drill No. 08 mounted on a surgical micromotor with irrigation with 0.9% saline, the bone was defected in 2 mm in diameter in the metaphysis region of the right tibia until reaching the medullary canal. The procedures ended with a simple suture with the needle thread of Nylon nº 0.4.

For antimicrobial therapy, all animals received a single dose (0.02 ml/100 g) of broad-spectrum Pentabiotic (Fort Dodge®) post-surgical, deep intramuscularly. For analgesic therapy in the postoperative period, 0.03 ml/100 g of Sodium Dipyrone was orally administered every 12 h during the first two days of the study. The groups were divided into: topical filling of the lesion with EEM (5mg/kg) (EEM-t); oral treatment with EEM (5mg/kg) (EEM-o); Positive control group (PCG) filled with Bio oss® and Negative control group (NCG) filled with blood clot and saline.

The animals were euthanized 15 and 30 days post-surgery, in accordance with appropriate ethical principles (CONASEMS, 2018). The animals received 10 mg/kg lidocaine anesthetic peritoneal followed by a barbiturate (100 mg/kg sodium pentobarbital via intraperitoneal Tiopentax®, Cristália Produtos Químicos Farmacêuticos, Itapira, SP, Brazil).

2.3 BIOCHEMICAL ANALYSIS (ALKALINE PHOSPHATASE AND SERUM CALCIUM)

For the blood drawing, the animals were fasted for 8 h and underwent dissociative anesthesia ketamine hydrochloride (50 mg/mL) (Dopalen®, Sespo Indústrias e Comércio Ltda, Paulínia, SP, Brazil) and xylazine (20 mg/mL) (Anasedan®, Sespo Indústrias e Comércio Ltda, Paulínia, SP, Brazil) (MASSONE, 2019). Next, the animals were cardiac punctured and 3-5 ml of blood was collected, which was stored in tubes (Vacuete Serum Sep Clot Activator Capacity 5 ml) and centrifuged at 2500 rpm for 10 minutes (Excelsa II bench Model 206 BL). The serum concentrations of alkaline phosphatase and calcium were measured by the colorimetric method using the Labtest Kit (Minas Gerais, Brazil).

2.4 HISTOLOGICAL ANALYSIS

The tibiae with bone defects were surgically disjointed and kept in liquid nitrogen at -196º C until Raman tests were performed. Afterward, they were fixed in 10% buffered formaldehyde for at least 48 h and decalcified in 20% formic acid solution. The bone parts were sent to the routine laboratory process for histological analysis.

The slides were analyzed using an Olympus optical microscope at a 5X objective. A Samsung SDC-415 camera and an Image Capture software, attached to the Nikon Eclipse E200 trinocular microscope, were used to capture the images (up to 100x). Two sections per slide representing the entire lesion area were selected. For each area of the lesion, a region adjacent to the defect with similar dimensions was chosen on the same slide, representing normal bone (100% new bone formation). The descriptive histological analysis was based on the presence of inflammatory infiltrate, necrosis, fibrosis formation, bone neoformation (immature and lamellar bone tissue), and collagen fibers.

2.5 STATISTICAL ANALYSIS

The entire statistical analysis was performed using the software Graph pad Prism version 5.0, software (GraphPad Software Inc., La Jolla, CA, USA). One-Way ANOVA with Tukey post hoc analysis of variance was used. The significance level of 95% ($p < 0.5$) was established for multiple comparisons between the treated groups and the control groups.

2.6 RAMAN SPECTROSCOPY ANALYSIS

Raman spectroscopy analysis was performed using a Senterra Bruker micro-Raman spectrometer. A laser with $\lambda = 785$ nm was used as a source of excitation. The spectrometer slot was adjusted to a resolution of 4 cm^{-1}. An optical microscope (Olympus BX-50) with an Olympus MPlan 10x/0.25NA objective was used to focus the sample surface and obtain images, as well as Raman spectra. The spectra were obtained in the spectral region at 500 to 1300 cm^{-1}, with 3 accumulations of 20 s. Then the tibiae with the bone defects were fixed in 10% buffered formaldehyde for 48 h. After that period, they were placed in a 25% formalin decalcified solution and sent for routine histological procedures. Paraffin blocks were included where each piece underwent a longitudinal cut in a rotating microtome (thickness of 5 µm), resulting in semi-closed cuts of the region of interest. They were stained with hematoxylin and eosin. The slides were evaluated by light microscopy.

3. RESULTS AND DISCUSSION

Raman analysis were carried out in two different regions (Figure 1) in which the inset in the figure has a superior image showing the injury caused. The circular and square points outside and inside the lesion indicate where Raman analysis were performed.

The analysis performed outside the lesion aimed to show the characteristic spectrum of the bone (peak at ~ 967cm^{-1}, peak this of the phosphate group) and compare it with the spectra obtained within the lesion in order to verify bone regeneration (Figure 1). When there is the regeneration, this band must be observed, and its corresponding characteristics, intensity and area, in comparison with the reference (spectrum obtained outside the lesion) shows how fast this regeneration is occurring (SOUZA et al., 2012).

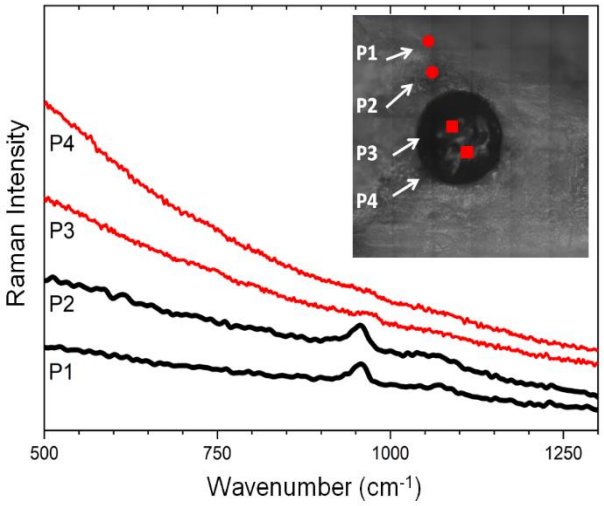

Figure 1. Raman spectra obtained inside and outside the lesion. In the inset, we have the indication of the corresponding points where the spectra were obtained: points P1 and P2 outside, and P3 and P4 inside the lesion.

Figure 2 shows the spectra of each group. The spectra observed in figure 2A at 15 days and in figure 2B at 30 days after the lesion in NCG. In the spectrum of points P1, we have the characteristic peak of bone formation (967 cm^{-1}), which is not observed in the spectrum of point P2 (spectra in the lesion) with the same intensity. Thus, there was no complete bone formation at 15 days, whereas for the 30-day sample there was a low-intensity peak, evidencing the beginning of this formation.

The PCG spectra shown in the figures 2C and 2D show the peak of bone formation in the lesion area (P2). The P2 peak is less intense (Figure 2C) than that of the P2 (Figure 2D), which is the same order of intensity as the peak obtained outside the lesion, these results corroborates with the images in the inset of this figure. The image of the lesion is completely closed in figure 2D.

The EEM-t spectra (Figures 2E and 2F) show in points P2 that this treatment resulted in low bone regeneration. The peak only occurred at 30 days after the surgery (967 cm^{-1}), but of very low intensity. Inset images show that the lesion was poorly covered at 15 days and partially covered at 30 days. However, there was no bone formation in this coverage, indicated by the non-appearance of the peak at 967 cm^{-1} in the Raman spectrum.

The EEM-o spectra at 15 and 30 days are shown in figures 2G and 2H, respectively. When comparing to the EEM-t, the peak has half the intensity outside the lesion (P1) at 15 days and, the same intensity inside and outside the lesion (P2) at 30 days of treatment (peak at 967cm^{-1}). Thus, EEM-o presented a result than that of the EEM-t and compatible with that of the positive control group.

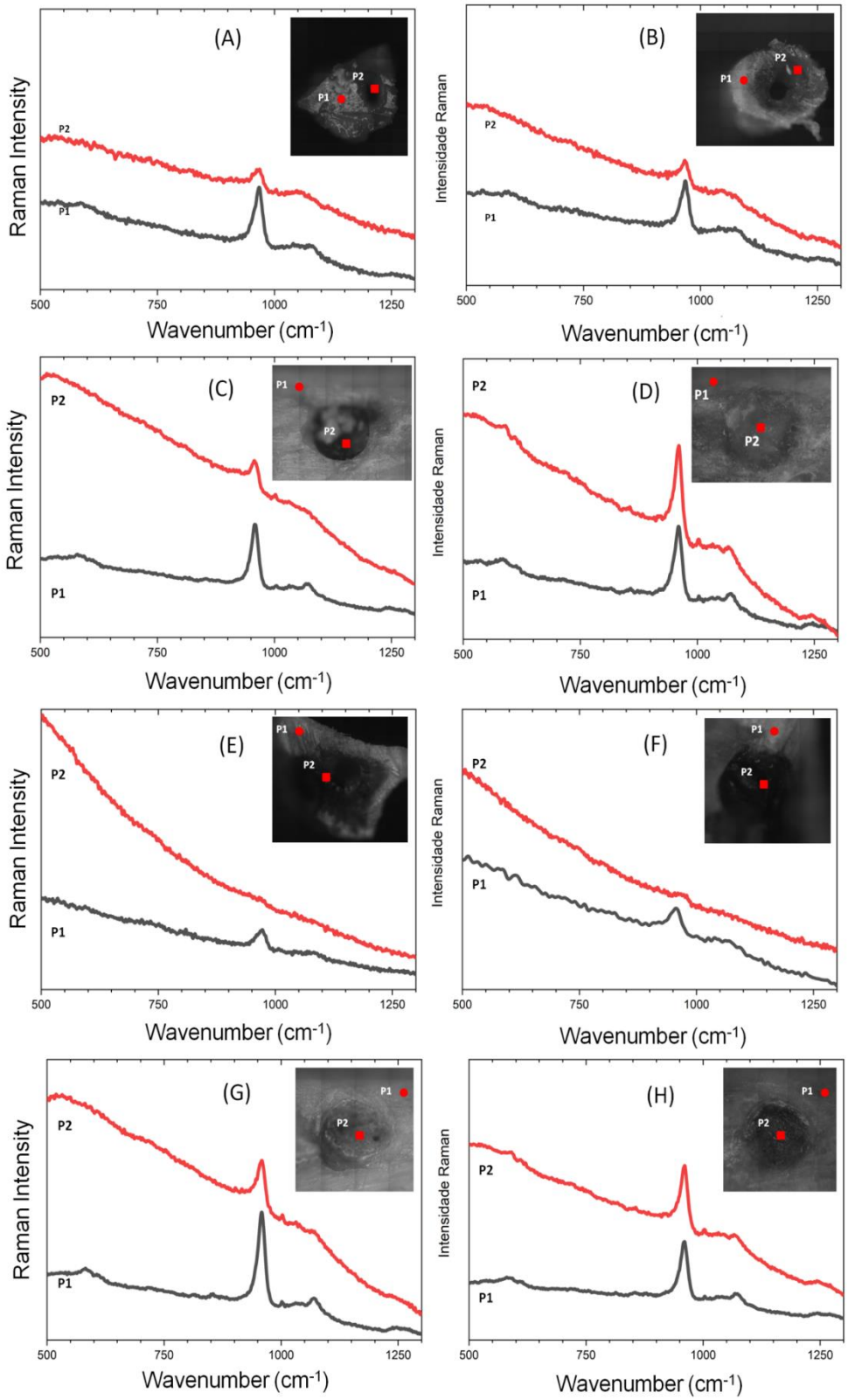

Figure 2. Representation of the bone regeneration intensity. Negative Control groups, 15 days (A), 30 days (B); Positive Control groups: 15 days (C), 30 days (D); EEM-t groups, 15 days (E), 30 days (F); EEM-o groups: 15 days (G), 30 days (H).

For quantify the regeneration if the each sample, the intensity and area of the peaks at 967 cm^{-1} outside and inside the lesion were identified for 15 and 30 days, these were normalized by dividing the intensities and areas of the peaks within the lesion by the respective intensities and areas peak of the analyzes outside the lesion, the result is shown in figure 3. These results show that the samples, EEM-t and NCG, obtained low bone regeneration, maximum regeneration in 30 days of 30% and 40% respectively. The samples EEM-o and PCG showed high regenerations, reaching complete regeneration in 30 days.

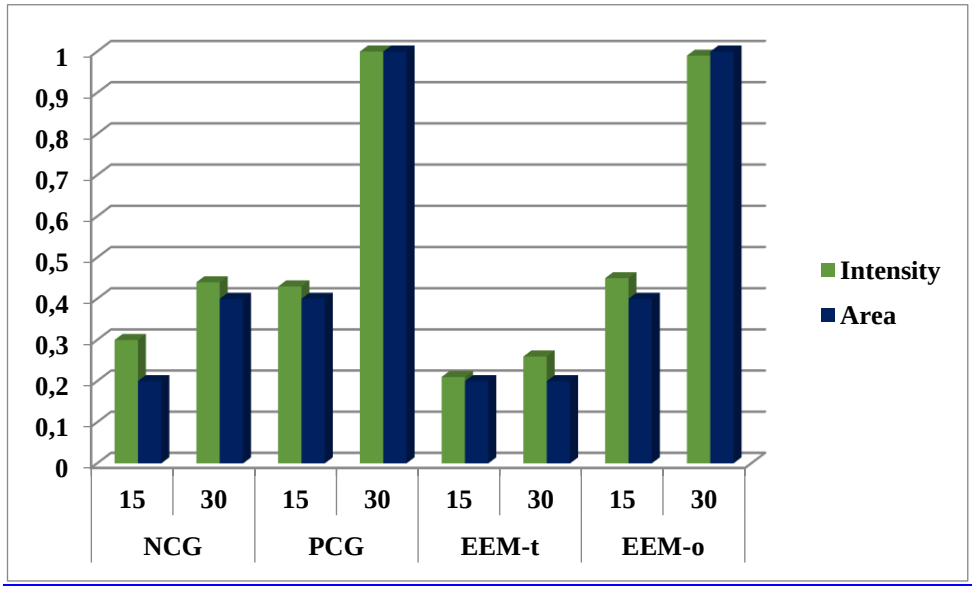

Figure 3. Areas of the peaks within the lesion.

Used to analyze the progress of treatment and/or diagnosis of bone problems, Alkaline Phosphatase Level - APL test show that: After 15 days of treatment, there were no differences in the levels of alkaline phosphatase in the EEM-o group when compared to the PCG (Bio oss®) and NCG (Saline Solution, blood cells) and serum calcium levels were lower in the EMS-o group compared to the saline and blood cells ($p < 0.05$) (Figure 4).When treated for 30 days, EEM-o and EEM-t groups showed significantly lower levels of alkaline phosphatase ($p < 0.05$) than PCG, with no difference in relation to NCG, while their serum calcium levels did not differ in relation to NCG (Figure 5). Regarding the results of the Photomicrography, it was observed that, after 15 days, postoperatively with the EEM-t lead to a mild to moderate inflammatory infiltrate, although there was a good bone healing in this group (Figure 6A). The EEM-o group showed no inflammation and excellent bone healing (Figure 6B). The tibiae of animals treated with synthetic bone showed no inflammation or effective healing (Figure 6C), whereas the NCG group presented necrosis with moderate inflammation (Figure 6D).

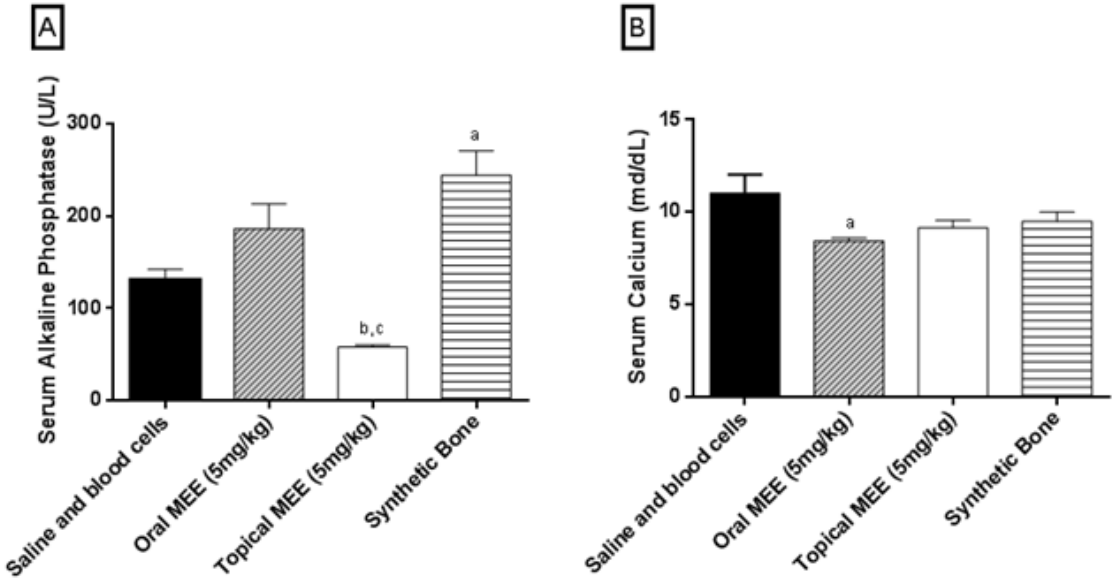

Figure 4. Serum alkaline phosphatase (U/L) and serum calcium (mg/dL) after 15 days of bone defect in the tibia of Wistar rats. The animals were treated orally and topically with ethanol extract of mastruz (EEM) 5mg/kg, saline solution with blood cells, and synthetic bone (Bio oss®). [a]p <0.05 in relation to the saline group and blood cells, [b]p <0.05 in relation to the synthetic bone group, [c]p <0.05 in relation to the EEM-o.

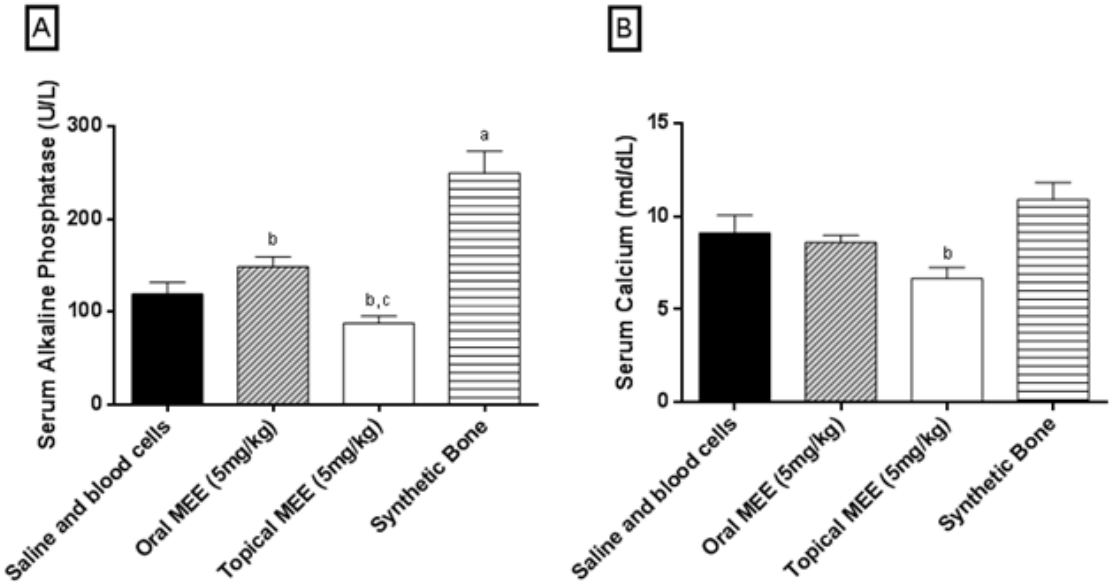

Figure 5. Serum alkaline phosphatase (U/L) and serum calcium (mg/dL) after 30 days of bone defect in the tibia of Wistar rats. The animals were treated orally and topically with ethanol extract of matruz (EEM) 5 mg/kg, saline solution with blood cells, and synthetic bone (Bio oss®). [a]p <0.05 in relation to the saline group and blood cells, [b]p <0.05 in relation to the synthetic bone group, [c]p <0.05 in relation to the EEM-o. (One-way ANOVA / Tukey).

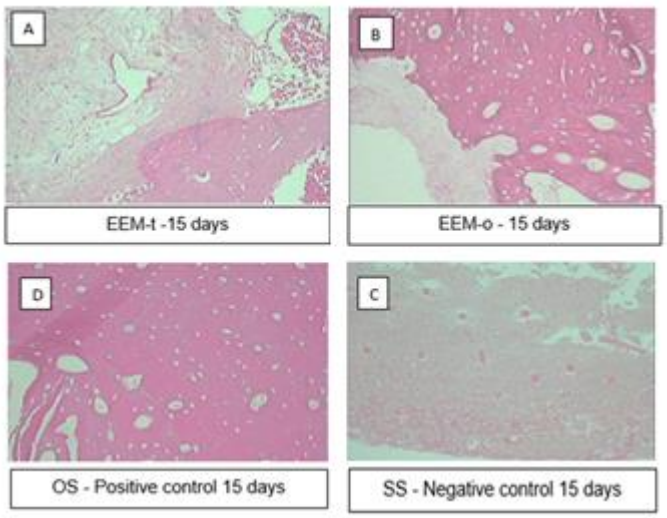

Figure 6. Photomicrograph of the bone repair in the experimental groups at 15 days after the surgery. (A) Treated with topical matrix. Increase 40x H.E. (B) Treated with oral mast. Increased 100x H.E. (C) Treated with Bio Bone®. Magnification 100x H.E. (D) Without treatment. Necrosis and moderate inflammation.

After 30 days, it was observed that the tibial lesions of animals treated with EEM-t (Figure 7A) also showed a discrete inflammatory infiltration with good bone healing, no change in their histological characteristics. The use of EEM-o (Figure 7B) resulted in the total absence of inflammation, and the area where the bone defect was performed was even difficult to be identified due to its similarity with the intact bone. Regarding the positive control group (Figure 7C) and negative control group (Figure 7D), there was no inflammation with good healing, and mild inflammation with the immature healing area, respectively.

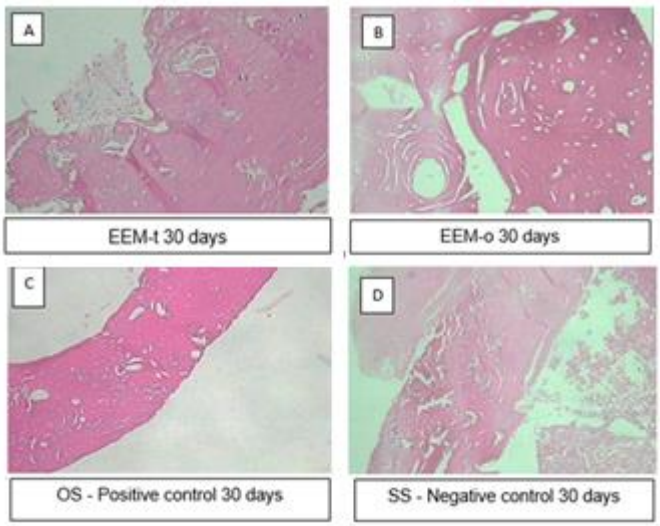

Figure 7. Photomicrograph of the bone repair in the experimental groups at 30 days after the surgery. (A) Treated with topical matrix. Increase 40x H.E. (B) Treated with oral mast. Increased 100x H.E. (C) Treated with Bio Bone®. Magnification 100x H.E. (D) Without treatment. Increase 40x H.E). Necrosis with moderate inflammation

The measurement of the serum concentration of alkaline phosphatase had a pre-clinical purpose of assessing rates of auxiliary bone training. That's because the isoenzymes of bone-specific alkaline phosphatase are located in the plasma membrane of the osteoblast. Thus, a large amount of isoenzymes can be secreted when the mechanical membrane is active (FREEMONT, 1998; VIEIRA, 2007).

Topical treatment with EEM did not alter the serum levels of alkaline phosphatase when compared to the positive control group. These results contrast with those reported by Pereira et al. (2018), in which they observed that, after 30 and 60 days of the fracture, an increase in the serum levels of alkaline phosphatase in animals that received the C. ambrosioides graft. They revealed that this medicinal plant can stimulate alkaline phosphatase activity during early fracture healing in a time-dependent manner, which suggests the occurrence of osteogenesis.

However, our data suggest that topical treatment with EEM for 30 days did not affect serum levels of alkaline phosphatase. This corroborates with Soares et al. (2015), who verified that, after 30 days of treatment with hydroalcoholic extract of the mast (50 mg/kg), there was no difference in the alkaline phosphatase levels in rats.

The serum calcium dosage was used in order to evaluate the behavior of Ca^{2+} metabolism ion in the organisms when using EEM. For calcium to maintain its homeostasis state in the body, it is necessary to involve the processes of absorption, excretion, secretion, and storage in the bone (CASHMAN, 2002).

Although mastruz leaves have high calcium content (541 mg/100g) (ALMEIDA et al., 2010), serum calcium levels were lower in animals that had topical administration with EEM than those in the synthetic bone group after 30 days of treatment.

It is important to note that serum calcium circulates in two main forms: ionized calcium (which has a biological action) and protein-bound and complexed calcium. Taking this into consideration, and a series of clinical circumstances, including the bone regeneration process, the determination of total calcium alone cannot be considered a determining factor to determine functional calcemia (VIEIRA, 2007).

The hydroalcoholic extract of mastruz plays a key role in bone metabolism, since it is capable of altering blood proteins and enzymes, which prevents bone loss and the replacement of bone marrow cells with adipocytes (SOARES et al. 2015).

Although the route of administration is different, the histological results of the present study are similar to those found by Pinheiro Neto et al. (2015), They carried out the treatment using *Chenopodium ambrosioides L*. poultice in rabbits with bone fractures and observed its

biocompatibility, with a marked formation of mature bone tissue and a moderate density of collagen, as well as an increase in osteoclasts during the phagocytosis process.

The bone marrow of animals treated with EEM cells has more native cells and fewer adipocytes. This suggests a possible EEM mechanism in activating osteogenesis and suppressing adipogenesis, which would imply a greater number of osteoblasts and, consequently, greater bone formation (SOARES et al., 2015).

In accordance with the results of our study, Pereira et al. (2010) found that the hydroalcoholic extract of mastruz leaves at a dose of 5 mg/kg did not induce toxic changes, suggesting that it is a safe therapeutic dose.

4. CONCLUSION

The oral treatment with ethanol extract of mastruz leaves at a concentration of 5 mg/kg had an excellent anti-inflammatory response that possibly benefited bone neoformation, besides not causing signs of toxicity, behavioral changes or deaths. We still have the mechanisms involved in obtaining and administration of its use as alternative biomaterials that are easy to obtain. This result prospects new, broader studies and will allow a better understanding of the mechanisms involved and the use of mastruz as an alternative and accessible biomaterials.

5. ACKNOWLEDGMENTS

C. Luz-Lima, Ph.D, acknowledges the support from the MCTI/CNPQ/Universal (Grants#) and the PQ – (Grants#426995/2018-0). The other authors thank CNPq and CAPES for the financial support.

6. REFERENCES

ALMEIDA, J.M.; SANTOS, M.R.; GARCIA, L.A.; THEODORO, L.H.; GARCIA, V.G. Estudo histomorfométrico do processo cicatrização óssea em defeitos cirúrgicos de tamanho crítico em calvárias de ratos preenchidos com polímero de mamona. **Rev Odontol**, v. 31, p. 22–27, 2010.

ASSAIDI, A.; DIB, I.; TITS, M.; ANGENOT, L.; BELLAHCEN, S.; BOUANANI, N.; et al Chenopodium ambrosioides induces an endothelium-dependent relaxation of rat isolated aorta. **J Integr Med**, v. 17, n. 2, p, 115–124, 2019.

BARACUHY, J.G.V.; FURTADO, D.A.; FRANCISCO, P.R.M.; LIMA, J.L.S.; PEREIRA, J.P.G. **Plantas medicinais de uso comum no Nordeste do Brasil**, 2ª ed. Campina Grande: UFCG/ CEDAC, 2016.

BOSTROM, M.P.G.; SEIGERMAN, D.A. The Clinical Use of Allografts, Demineralized Bone Matrices, Synthetic Bone Graft Substitutes and Osteoinductive Growth Factors: A Survey Study, **HSS J**, v. 1, p. 9–18, 2005.

BLOKHUIS, T.J.; ARTS, J.J.C. Bioactive and osteoinductive bone graft substitutes: Definitions, facts and myths. **Injury**, v. 42, p. s26–s29, 2011.

CASHMAN, K.D. Calcium intake, calcium bioavailability and bone health. **Br J Nutr,** v. 87, n. suppl 2, p. 169–177, 2002.

CONASEMS. Conselho Nacional de Secrecretarias Municipais de Saúde. **Política Nacional de Medicina e Práticas Complementares PMNPC**, 2018. Disponivel em: <www.conasems.org.br>. Acessado em: 25/06/2018.

DEMBITSKY, V.; SHKROB, I.; HANUS, L.O. Ascaridole and related peroxides from the genus Chenopodium. **Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub,** v. 152, n. 2, p. 209–215, 2008.

FREEMONT, A.J. (I) bone. **Curr Orthop,** v. 12, n. 3, p. 181–192, 1998.

GARCÍA-GARETA, E.; COATHUP, M.J.; BLUNN, G.W. Osteoinduction of bone grafting materials for bone repair and regeneration. **Bone,** v. 81, p. 112–121, 2015.

IBIRONKE, G.F.; AJIBOYE, K.I. Studies on the Anti-Inflammatory and Analgesic Properties of Chenopodium Ambrosioides Leaf Extract in Rats. **Int J Pharmacol**, v. 3, n. 1, p. 111–115, 2007.

KUMAR, R.; MISHRA, A.; DUBEY, N.; TRIPATHI, Y. Evaluation of Chenopodium ambrosioides oil as a potential source of antifungal, antiaflatoxigenic and antioxidant activity. **Int J Food Microbiol**, v. 115, n. 2, p. 159–164, 2007.

LORENZI, H.; MATOS, F.J.A. **Plantas medicinais no Brasil: nativas e exóticas**, 2ª ed. Nova Edessa: Instituto Plantarum, 2008.

MAIA FILHO, A.L.M.; AMARAL, F.P.M.; MARTIN, A.A. Evaluation of inorganic and organic bone components after application of an apatite-coated Al2O3 implants as scaffolds for bone repair. **Braz Arch Biol Technol,** v.57, n.3, p.334-339, 2014.

MARÃO, H.F.; PANZARINI, S.R.; GUSTALDI, F.P.S.; PEREIRA, C.C.S.A.; GARCIA JÚNIOR, I.R. Regeneração óssea guiada com o uso de matriz óssea bovina desmineralizada em defeitos ósseos experimentais em cães. **Rev Bras Cir Cranio Maxilofac**, v. 14, n. 2, p. 86–90, 2011.

MASSONE, F. **Anestesiologia veterinária: Farmacologia e técnicas**, Rio de Janeiro: Guanabara Koogan, 2019.

OLAJIDE, O.A.; OLADIRAN, O.O.; AWE, S.O.; MAKINDE, J.M. Pharmacological evaluation

ofHoslundia opposita extract in rodents. **Phyther Res,** v. 12, n, 5, p. 364–366, 1998.

PEREIRA, N.S.L.; SOUZA, R.B.; SOARES, L.C.; SANTOS, I.M.S.P.; ARAÚJO, K.S. Regeneração óssea guiada utilizando membrana reabsorvível fixada com etilcianoacrilato. **Rev Bras Odontol**, v. 69, n. 1, p. 39–42, 2011.

PEREIRA, W.S.; SILVA, G.P.; VIGLIANO, M. V.; LEAL, N.R.F.; PINTO, F.A.; FERNANDES, D.C.; et al. Anti-arthritic properties of crude extract from Chenopodium ambrosioides L. leaves. **J Pharm Pharmacol,** v. 70, n. 8, p. 1078–1091, 2018.

PEREIRA, W.S.; RIBEIRO, B.P.; SOUSA, A.I.P.; SERRA, I.C.P.B.; MATTAR, N.S.; FORTES, T.S.; et al. Evaluation of the subchronic toxicity of oral treatment with Chenopodium ambrosioides in mice. **J Ethnopharmacol,** v. 127, n. 3, p. 602–605, 2010.

PINHEIRO NETO, V.F.; RIBEIRO, R.M.; MORAIS, C.S.; CAMPOS, M.B.; VIEIRA, D.A.; GUERRA, P.C.; et al. Chenopodium ambrosioides as a bone graft substitute in rabbits radius fracture. **BMC Complement Altern Med**, v. 17, p. e350, 2017.

RAMOSHEBI, L.N.; MATSABA, T.N.; TEARE, J.; RENTON, L.; PATTON, J.; RIPAMONTI, U. Tissue engineering: TGF-[beta] superfamily members and delivery systems in bone regeneration. **Expert Rev Mol Med**, v. 4, n. 20, p. 1-11, 2002.

REYES-BECERRIL, M.; ANGULO, C.; SANCHEZ, V.; VÁZQUEZ-MARTÍNEZ, J.; LÓPEZ, M.G. Antioxidant, intestinal immune status and anti-inflammatory potential of Chenopodium ambrosioides L. in fish: In vitro and in vivo studies. **Fish Shellfish Immunol,** v. 86, p. 420–428, 2019.

SANTOS, J.A.A.; SOUSA, M.F.A.M.; SILVA, E.L.V.; AGUIAR JÚNIOR, F.C.A. Avaliação histomorfométrica do efeito do extrato aquoso de urucum (norbixina) no processo de cicatrização de feridas cutâneas em ratos. **Rev Bras Plantas Med**, v. 16, n. 3, p. 637–643, 2014.

SOARES, C.D.; CARVALHO, M.G.F.; CARVALHO, R.A.; TRINDADE, S.R.P.; RÊGO, A.C.M.;ARAÚJO-FILHO, I.; et al. Chenopodium ambrosioides L. extract prevents bone loss, **Acta Cir Bras,** v. 30, n. 12, p. 812–818, 2015.

SOOST, F.; KOCH, S.; STOLL, C.; AMTHAUER, H.; GROßE-SIESTRUP, C.; ZORN, P. Validation of bone conversion in osteoconductive and osteoinductive bone substitutes. **Cell Tissue Bank**, v. 2, n. 2, p. 77–86, 2001.

SOUZA, R.A.; et al. Influence of creatine supplementation on bone quality in the ovariectomized rat model: an FT-Raman spectroscopy study. **Lasers In Medical Science**, v. 27, n. 2, p. 487-495, 2012.

TRIVELLATO, G.L.; MALHEIROS, A.; MEYRE-SILVA, C.; BUSS, Z.S; MONGUILHOTT, E.D.; FRÖDE, T.S.; et al. From popular use to pharmacological validation: A study of the anti-inflammatory, anti-nociceptive and healing effects of Chenopodium ambrosioides extract. **J Ethnopharmacol**, v. 145, n. 1, p. 127–138, 2013.

VIEIRA, J.G.H. Diagnóstico laboratorial e monitoramento das doenças osteometabólicas. **J Bras Patol e Med Lab,** v. 43, n. 2, p. 75-82, 2007.