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Chenopodium ambroisioides in the Repair of Fractures in Rabbits

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ABSTRACT
Chenopodium ambrosioides L. (Amaranthaceae), popularly known as "mastruz" or "erva-de-santa-maria", is a perennial plant found in Brazil and used in folk medicine for the treatment of contusions and fractures. The objective of this study was to evaluate the topical effect of a cataplasm prepared from fresh *C. ambrosioides* leaves on the treatment of fractures experimentally induced in rabbits. Thirty rabbits were divided into three groups (n = 10). After anesthesia, a radius fracture was created and the animals received topical applications of *C. ambrosioides* cataplasm (MZG), diclofenac sodium (DG) and isotonic solution 0.9% NaCl (Control). At 30 days, we evaluated the animal bone regeneration through both qualitative macroscopic analysis of the fracture focus as by determination of serum alkaline phosphatase (ALP). The evolution of bone repair has been verified at 30 and 45 days through the histological analysis. No significant difference in ALP levels was observed between groups. In addition, no allergic reactions or impairment of tissue adjacent to the fracture focus were seen in any of the groups. In contrast, although all groups exhibited similar tissue architecture, the histological analysis revealed greater formation of mature bone tissue in MZG at 30 days, when compared to DG and control group. In addition, higher collagen fiber density was observed in MZG at 45 days. These results indicate *Chenopodium ambrosioides* as a promising therapeutic agent for bone regeneration. The plant may be useful as a raw material for the production of biomaterials for fracture healing, contributing to its validation in ethnomedicine.

Key words: *Chenopodium ambrosioides*, mastruz, fracture healing

INTRODUCTION

More than 6 million fractures occur worldwide each year. Many patients require long-term treatment, with consequent increased costs for them and for the public health system (Giannoudis and Dinopoulos, 2010).

Natural biomaterials, including those derived from medicinal plants used by the population, are of great importance since they are biocompatible and easily applied and stored, in addition to favoring bone growth (Franco *et al*., 2001).

Chenopodium ambrosioides L. (*syn. Dysphania ambrosioides* (L.) Mosyakin and Clemants), Chenopodiaceae, popularly known as "mastruz" or "erva-de-santa-maria") is used by the population in Brazil and Latin America as teas, infusions or syrups for the treatment of inflammatory disorders, leishmanial ulcers (Franca *et al*., 1996) or contusions and fractures (Baptistel *et al*., 2014).

Pharmacological trials suggest that *C. ambrosioides* has action antitumor (Nascimento *et al*., 2006), *in vitro* (Bezerra *et al*., 2006) and *in vivo* leishmanicidal (Patricio *et al*., 2008). Additionally, studies have reported anti-inflammatory and anti-nociceptive activity of this plant species (Ibironke and Ajiboye, 2007; TrivellatoGrassi *et al*., 2013).

Despite its traditional use in contusions and fractures, studies that scientifically validate the therapeutic property of this plant are sparse. In Brazil, the federal government created in 2009 a program that compiled a list of 71 medicinal plant species used by the population as alternatives for the treatment of health disorders. On this list, called the National Register of Plants of Interest to the National Health System (Relação Nacional de Plantas de Interesse do Sistema Único de Saúde-RENISUS), *C. ambrosioides* occupies the 17th position, but without importance for the use in bone fractures.

Preliminary studies conducted by our group showed that the cataplasm prepared from fresh leaves of *Chenopodium ambrosioides* has a potential for repair of soft tissue and bone fractures induced experimentally in rabbits. These findings were obtained by evaluating the inflammatory process, inhibition of edema and radiographic analysis (Pinheiro Neto *et al*., 2005).

Considering the importance of this plant species as a raw material potential for obtaining biomaterials, the aim of this study was to evaluate the effect of the cataplasm of *C. ambrosioides* leaves on bone regeneration fractures in radio rabbits through analysis serum biochemical and histological evaluation.

MATERIALS AND METHODS

Plant material: *Chenopodium ambrosioides* leaves were collected at the time of use in the herbarium of the 'Prof. Dr. Berta Lange de Morretes" Medicinal Plant Garden, UFMA (São Luís- MA, Brazil), in October 2006. A voucher specimen was cataloged, identified and deposited under the registration number 0998.

Preparation of the *Chenopodium ambrosioides* cataplasm: Fresh leaves were washed with distilled water to remove impurities and then dried on filter paper and triturated in a homogenizer. The material obtained was mixed with saline (0.9% NaCl) (3 g of tissue per 1 mL saline) at room temperature. This mixture was called Cataplasm and was directly applied to the fractures.

Experimental animals: Thirty adult male New Zealand rabbits (*Oryctolagus cuniculus*), weighing 3.0±0.5 kg, obtained from the Animal House of the State University of Maranhão (UEMA) were used. The animals were kept at a temperature of 24±1°C and received ration and water *ad libitum*. The animals were acclimated for 10 days and handled under the same conditions. Next, the rabbits were randomly divided into three groups of 10 animals each: Control group, *C. ambrosioides* ("mastruz") group (MZG) and Diclofenac Sodium (DG) group. The protocol was approved by the Ethics Committee on Animal Research of UEMA (Permit No. 0026/2006).

Anesthetic technique and bone defect creation: The animals were anesthetized by intramuscular injection of 5% ketamine hydrochloride (30 mg kg^{-1}) and 2% xylazine hydrochloride (4 mg kg^{-1}). An incision of approximately 3 cm was then made in the skin and subcutaneous tissue in the middle third of the left radius. A complete, simple, transverse diaphyseal fracture (1 cm) was created at this site with an oscillating bone saw, followed by topical application of 10 mL isotonic solution 0.9% NaCl (control), 10 mL of the cataplasm (MZG) or 2 g (gel) diclofenac sodium (DG). The diclofenac sodium was used as anti-inflammatory for positive control. The tissues were closed with simple sutures. During the postoperative period, dressings were applied daily for 10 consecutive days and the sutures were removed after this period (Miranda *et al*., 2005). The animals were euthanized with anesthetics overdose. The evolution of the tissue adjacent to the fracture focus was observed macroscopically throughout the experiment, evaluating features such as color, occurrence of edema, suture dehiscence, presence of a hypertrophic scar and possible allergic reactions.

Biochemical parameters and bone markers: Thirty days after surgery, five animals of each group were anesthetized and blood was collected for biochemical analysis of alkaline phosphatase (ALP). The blood samples were collected into tubes without EDTA and centrifuged at 3000×g for 10 min for the collection of serum. Serum ALP was measured with the Labtest® kit in a semi-automated apparatus-Bioplus® (Bioplus, Barueri, São Paulo).

Histological evaluation: Histological analysis was performed 30 and 45 days after surgery and application of isotonic solution 0.9% NaCl, *C. ambrosioides* cataplasm or sodium diclofenac to monitor the evolution of bone callus formation, considering the neoformation of fibrous, cartilaginous and bone tissues during the healing process. Cross-sections were cut from the fractured bone segments and fixed in 10% buffered formalin for 24 h. After decalcification in 10% nitric acid, the fragments were processed and stained with Hematoxylin and Eosin (HE) and Sirius red (Franco *et al*., 2001). The histological sections were submitted to descriptive qualitative analysis for evaluation of the pattern of bone regeneration.

Statistical analysis: The ALP results are expressed as the Mean±Standard Error of the Mean (SEM) for 05 animals/group and were compared by analysis of variance (ANOVA) followed by the Newman-Keuls post-test, considering $p<0.05$ significant. Statistical analysis was performed using the GraphPad Prism 5.0 program. The histological results are expressed qualitatively.

RESULTS

Qualitative macroscopic analysis of the fracture focus: No alterations were observed in the tissue adjacent to the

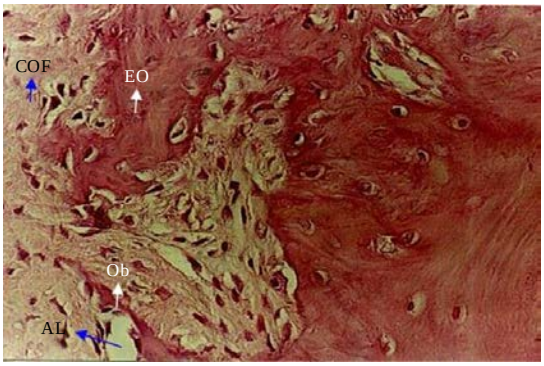

Fig. 1: Fracture site photomicrograph from the control group at 30 days after surgery. AL: Active lacuna, EO: Endochondral ossification, Ob: Osteoblasts, COF: Fibrous bony callus. Hematoxylin and Eosin stain (HE) (400X magnification)

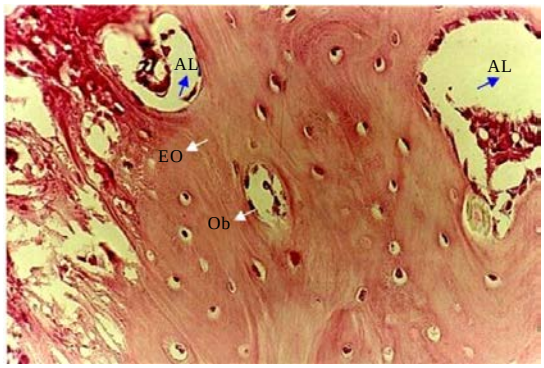

Fig. 2: Fracture site photomicrograph from the diclofenac sodium group at 30 days after surgery. AL: Active lacuna, EO: Endochondral ossification, Ob: Osteoblasts. Hematoxylin and Eosin stain (HE) (400X magnification)

fracture focus in any of the groups studied. In addition, no allergic reactions were seen at the site of application. *Chenopodium ambrosioides* cataplasm was found to be biocompatible in the experimental model used.

Effect of *Chenopodium ambrosioides* cataplasm on serum ALP levels: No significant difference in ALP levels was observed at 30 days. The levels were 104.0±34.0, 103.0±29.0 and 121.0±21.0 UI L^{-1} in the control, DG and MZG, respectively. The reference range of ALP in rabbits reported in the literature is 72.4±29.7 IU L^{-1}.

Histological evaluation of *Chenopodium ambrosioides* cataplasm: Histological analysis of the fracture focus 30 days after the surgical procedure revealed similar bone remodeling in all groups, including the presence of endochondral ossification and osteoblasts. A fibrous bone callus and discrete maturation of lacunae were observed in the control group (Fig. 1). Endochondral ossification was observed in DG during this early stage, with the presence of active lacunae and osteoblasts (Fig. 2). There was an increase in osteoclasts during the process of phagocytosis in MZG, in addition to active lacunae and formation of mature bone (Fig. 3). Histological analysis at 45 days showed endochondral ossification and the presence of osteoblasts in the control group (Fig. 4). Abundant cartilaginous tissue (cartilaginous collar) and large numbers of chondrocytes and osteoblasts were seen in DG (Fig. 5). Fibroblast proliferation and an increase in collagen density were also observed during this phase in the control and DG, accompanied by neovascularization in the former. In contrast, animals of MZG exhibited marked formation of mature bone tissue and a moderate density of collagen when compared to the other groups (Fig. 6).

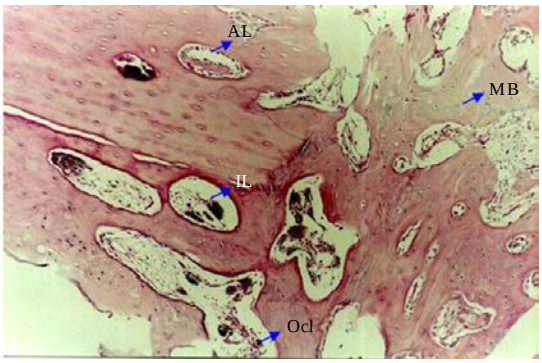

Fig. 3: Fracture site photomicrograph from the *Chenopodium ambrosioides* cataplasm group at 30 days after surgery. AL: Active lacuna, IL: Inactive lacuna, MB: Mature bone, Ocl: Osteoclast. Hematoxylin and Eosin stain (HE) (400X magnification)

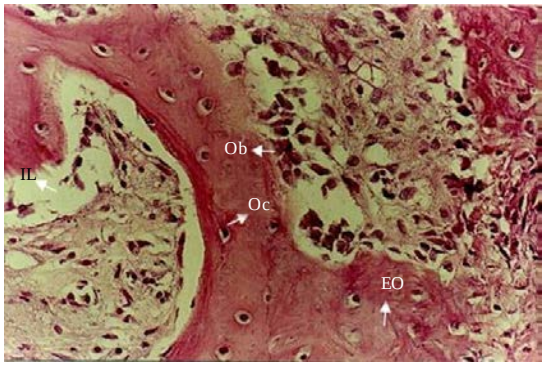

Fig. 4: Fracture site photomicrograph from the control group at 45 days after surgery. IL: Inactive lacuna, EO: Endochondral ossification, Ob: Osteoblasts, Oc: Osteocytes. Hematoxylin and Eosin stain (HE) (400X magnification)

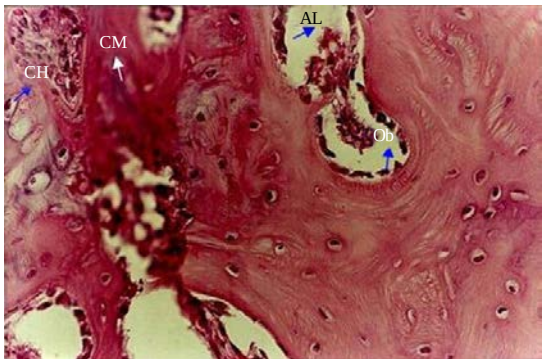

Fig. 5: Fracture site photomicrograph from the diclofenac sodium at 45 days after surgery. AL: Active lacuna, CM: Cartilage Matrix, CH: Hypertrophic chondrocytes, Ob: Osteoblasts, HE: Hematoxylin and Eosin stain (400X magnification)

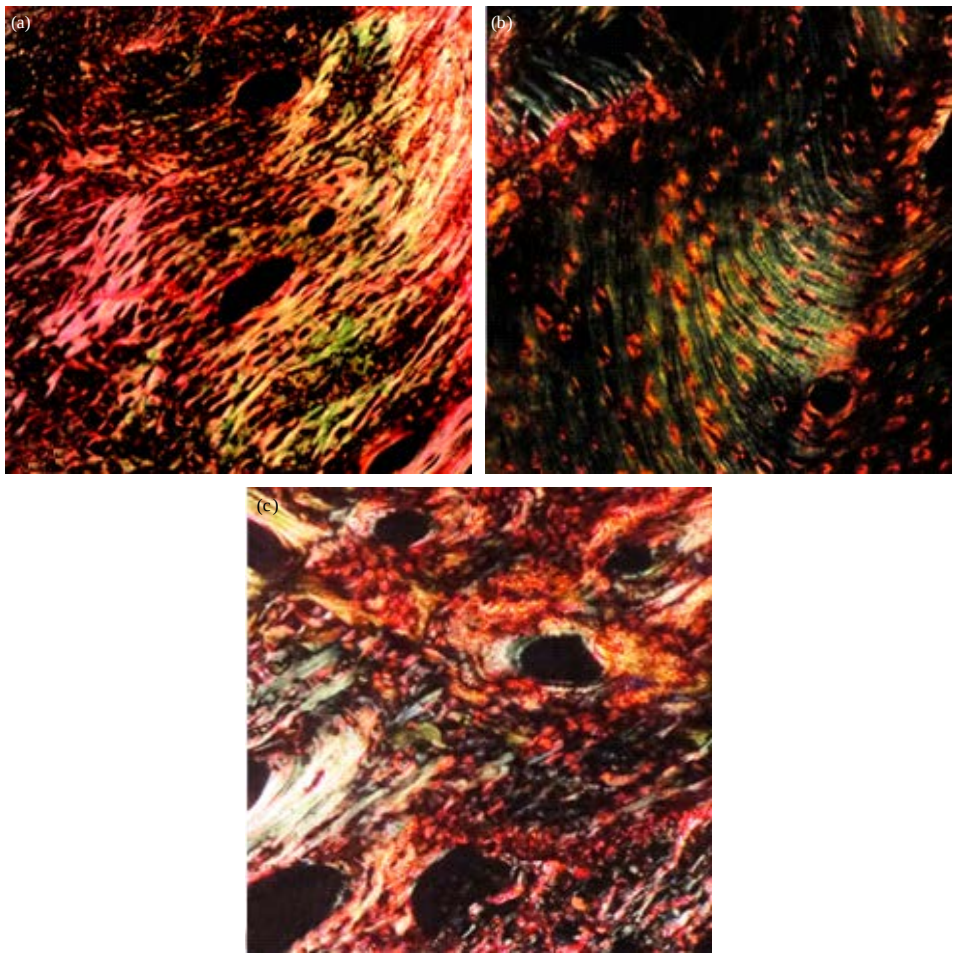

Fig. 6(a-c): Photomicrographs of collagenization of the rabbit radius at 45 days after surgery, (a) Control group, (b) Diclofenac sodium e and (c) *Chenopodium ambrosioides* cataplasma. Sections stained by Sirius red method (400X magnification)

DISCUSSION

The present study shows that *C. ambrosioides* cataplasm acts on the process of bone repair when applied topically to fractures induced in rabbits, suggesting this plant species to be a promising biomaterial since it is biocompatible and easily applied and favors bone growth.

Fractures characterized by substantial bone loss play an important role in clinical-surgical routine in both human and veterinary medicine (Alievi *et al*., 2007). The healing potential

of a bone fracture depends on patient-related variables, location and the treatment employed. These variables must create biological and mechanical conditions that favor the repair process by influencing factors that are determinant for the organization and final process of fracture consolidation (Muller *et al*., 2004).

The main objective of surgical treatment is to fill the fracture with material that can promote osteoinduction and/or osteoconduction and consequently, reparative osteogenesis (Tsonis, 2002). This type of treatment is a constant challenge in orthopedic therapy since the ideal bone implant should not physically modify the tissue and should not induce allergic reactions, in addition to being easily obtained in the amount and shapes necessary to fill the bone defect.

The *C. ambrosioides* cataplasm was found to be applicable and biocompatible since no alterations in adjacent tissue or allergic reactions were observed in the animals studied (qualitative macroscopic analysis) throughout the period of topical treatment of the fracture.

Bone formation involves endochondral ossification and the production of cartilage in the bone tissue which is used secondarily by osteoblasts for the formation of new bone. Bone formation comprises the following steps: Proliferation of chondrocytes, chondrocyte hypertrophy, matrix mineralization, apoptosis, vascular invasion, ossification and remodeling of lamellar bone (Marino and Ziran, 2010).

Histological analysis after 30 days of treatment showed a similar tissue architecture in all groups. However, greater formation of new bone was observed in MZG (Fig. 3) suggesting that the *C. ambrosioides* cataplasm promotes osteogenesis and may serve as a stimulator of bone regeneration (Wong and Rabie, 2006; Pereira-Junior *et al*., 2007; Filho *et al*., 2009; Ishizeki *et al*., 2009).

In addition, a greater density of collagen fibers was observed in MZG at 45 days (Fig. 6). The extracellular matrix which is rich in collagen, contributes to mineralization, angiogenesis and growth factor production and therefore, favors bone regeneration (Roden, 2010).

Within the context of bone remodeling, important markers of real-time bone formation are total ALP and bone ALP (Seibel, 2000; Allen, 2003). In the present study, no difference in ALP activity was observed between MZG and the other groups. This finding might be explained by the fact that this enzyme is not specific for bone neoformation and future studies using osteocalcin and bone ALP are necessary.

The results of this study show that cataplasm of *Chenopodium ambrosioides* leaves induces early bone neoformation, contributing to the treatment of bone repair and opening new perspectives for its use as an alternative, easily obtainable biomaterial.

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