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Evaluation of tissue reaction to Aroeira (*Myracrodruon urundeuva*) extracts: a histologic and edemogenic study

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ABSTRACT

Objectives: This study evaluated subcutaneous tissue response to Aroeira (*Myracrodruon urundeuva*) extract employing edemogenic and histological analyses. **Material and methods:** Test groups consisted of aqueous and ethanolic Aroeira extracts and saline (control). For edema quantification, 18 rats received an intravenous injection of Evan's Blue. After 30 min, the extracts and saline were injected on the dorsum of the rats, which were then sacrificed after 3 and 6 h. Readings were performed in a spectrophotometer. For subcutaneous implantation, 30 rats received a polyethylene tube containing the extracts on their dorsum and then they were killed after 7 and 28 days. The samples were processed for histological analysis and evaluated with a light microscope. The inflammatory infiltrate was quantified. **Results:** There were no statistically significant differences between aqueous extract and saline groups in relation to edema quantification in the different periods ($p > 0.05$). Ethanolic solution resulted in more edema independently of the experimental period ($p < 0.05$). Histological analysis showed similar results on the 7-day period for the 3 groups. There was a notable reduction on inflammatory cell number for saline and aqueous extract groups at 28 days. **Conclusion:** The aqueous extract showed biocompatible properties similar to those of saline.

Key words: Inflammation. Edema. Medicinal plants. Anacardiaceae.

INTRODUCTION

Studies on medicinal plants date from the past century, but their use is as ancient as humanity²². According to the World Health Organization (WHO), approximately 3.5 billion people in developing countries believe in the efficiency of herbal medicines and use them regularly⁴, and estimates that 65-80% of this population uses medicinal plants as the only way to treat health problems²³.

Brazil possesses a large biodiversity and has a wealth of traditional knowledge accumulated by local people who have direct access to nature and its

products. Traditional knowledge related to medicinal plants is the basis of folk medicine in Brazil, which is derived from a mixture of Brazilian indigenous cultures and European and African influences from the colonization period⁹.

Myracrodruon urundeuva, popularly known as "Aroeira do sertão", is a Brazilian medicinal plant traditionally used for treating gingival bleeding and gynecological disorders^{11,20}. It is a large tree that grows in Northeastern region of Brazil. Ethanolic extracts from *Myracrodruon urundeuva* stem bark exert anti-inflammatory activity in several experimental models of inflammation, such as

carrageenan-induced paw edema, neutrophil migration to peritoneal cavity and hemorrhagic cystitis induced by cyclophosphamide^{20,21}. Analgesic, antiulcer, hepatoprotective, antidiarrheal and colonic anastomotic wound healing properties have been reported^{5,21}. A chemical study has demonstrated that the ethyl acetate extract has two main fractions. One of them has predominance of substances of a chalcone nature and the other has predominance of catechic tannins²¹. The chalcone demonstrated neuroprotective effects, which are able to reduce oxidative stress and apoptotic injury¹³.

Few studies have addressed the toxic effects of these extracts. Almeida, et al.¹ (2010) found high toxic effects in the ethanolic Aroeira extract. Based on these findings, the aim of this study was to evaluate the immediate and late biological tissue responses to ethanolic and aqueous Aroeira extracts implanted in rat submucosa.

MATERIAL AND METHODS

The study was approved by Ethics committee on animal research of Araçatuba School of Dentistry/UNESP (Process #2007-008938).

Plant extract preparation

The Aroeira (*Myracrodruon urundeuva*) plants were obtained from ecological reserves at Carolina city, Maranhão state, Brazil. The leaves were collected if presented with a healthy aspect, no signs of aging and free of disease and pests. After collection, the plants were washed in water, dried at room temperature and in a heater at 37°C until becoming dry and friable. Subsequently they were pulverized.

The ethanolic and aqueous extracts were prepared according to the methodology described by Tsuchiya, et al.¹⁸ (1994) and Navarro, et al.¹² (1998). For the ethanolic extract, 20 g of leaf powder were mixed to 250 mL of 80% ethanol. The flask was agitated vigorously during 3 min, five times a day, during 12 days. The solution was filtered in paper filters and sterilized by filtration in Millipore membranes (0.22 µm). The aqueous extract was obtained by mixing 20 g of leaf powder with 250 mL of distilled water. The solution was maintained at 100°C for 5 min, 55°C for 1 h and at room temperature for 72 h. Agitation was performed every 24 h. The extract was filtered and sterilized as mentioned before.

Edemogenic test - immediate reaction

Eighteen 4-6 month-old male Wistar rats weighting 250-280 g were divided into 6 groups of 3 animals each according to the experimental period of analysis (3 and 6 h) and treatment with

the ethanolic/aqueous extracts or saline (control). The animals were anesthetized with xylazine (10 mg/kg) and ketamine (25 mg/kg) and received an intravenous injection of 1% Evan's blue (0.2 mL/100 g body weight) (Evan's Blue; Difco Lab, Detroit, Michigan, USA). After 30 min, 0.1 mL of the extracts or saline were injected at the dorsum region, near the tail and using the median line as a reference.

After 3 and 6 h, the animals were sacrificed by anesthetic overdose and a 23 mm of diameter tissue fragment containing a blue halo in the center was removed. The tissue was fixed in 4 mL of formamide for 72 h at 45°C and filtered for spectrophotometric analysis at 630 nm.

Implantation in subcutaneous tissue for histological analysis

Thirty 4-6 month-old male Wistar albino rats weighting 250-280g were divided into six groups of 5 animals each according to the experimental period of analysis (7 and 28 days) and treatment with the ethanolic/aqueous extracts or saline (control).

Sixty polyethylene tubes (1.0 mm internal diameter x 1.6 mm outer diameter x 10 mm length) with a paper cone inside (2 mm short of the cone length) had one side filled with 1 mm of gutta-percha, and were sterilized with nitrous oxide.

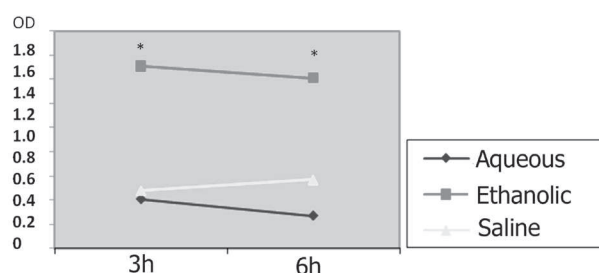


Figure 1- Mean values of edema on both time period and the experimental groups
OD=Optical Density (*P<0.05)

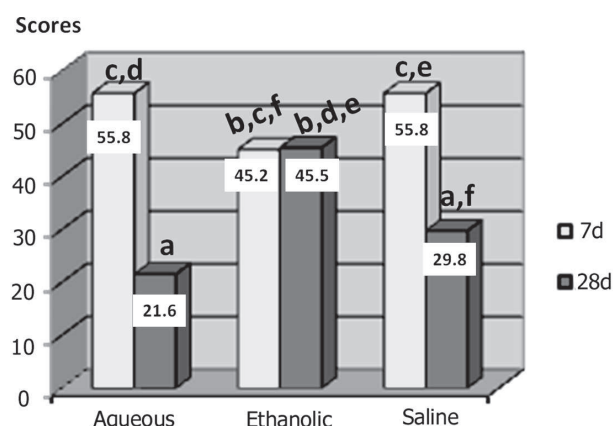


Figure 2- Mean scores of inflammatory cells of the three groups at each time period Different letters=p<0.05. Same letters=p>0.05

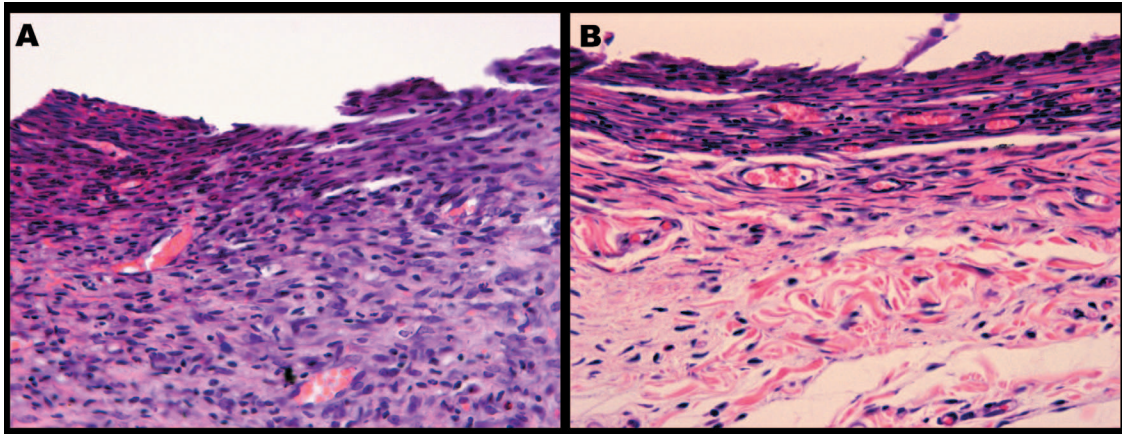


Figure 3- Control group (saline). A – At 7 days, there is an aggressive inflammatory response. The granulation tissue is thick and presents macrophages and lymphocytes. Fibroblasts are near the collagen fibers in a disorganized distribution. Blood vessels are distributed all over the tissue. B - At 28 days, the fibrous tissue present well organized collagen fibers parallel to implant. There are a few inflammatory cells and capillaries. HE – Original magnification=40x

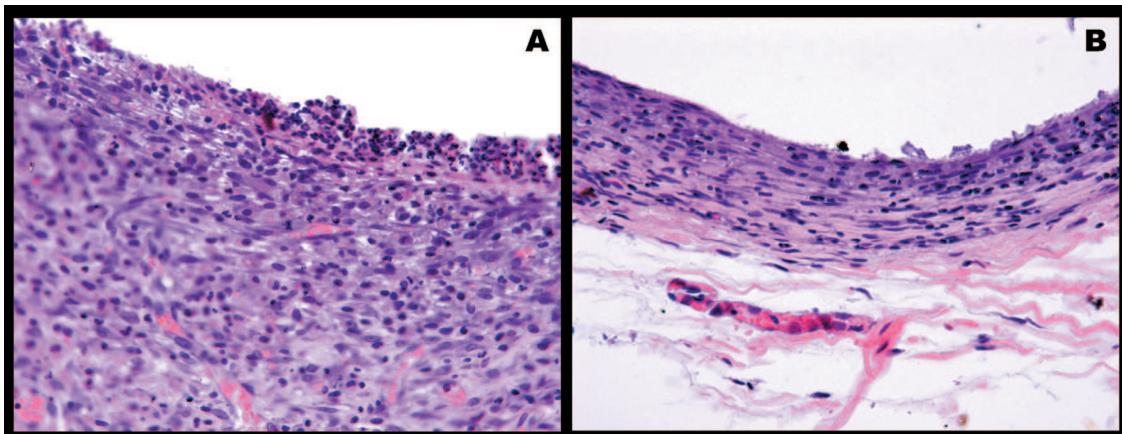


Figure 4- Aroeira's aqueous extract group. A - At 7 days, the granulation tissue is thick and presents inflammatory infiltrate with macrophages and lymphocytes. Fibroblasts appear near the collagen fibers and blood vessels are distributed all over the granulation tissue. B - At 28 days, the connective tissue shows a few fibroblasts, inflammatory cells and blood vessels. Fibroblasts are disposed in line with the collagen fibers. HE – Original magnification 40x

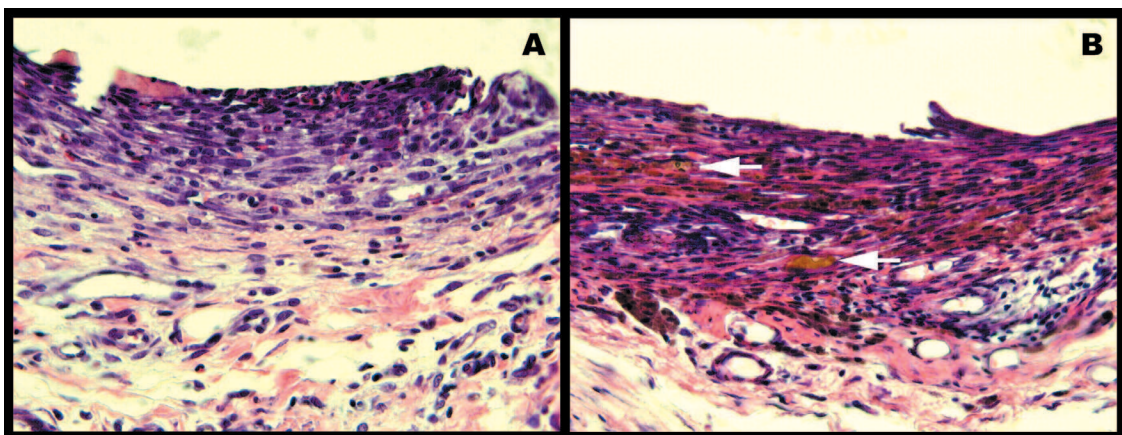


Figure 5- Aroeira's ethanolic extract group. A – At 7 days, the granulation tissue is thick and presents macrophages, lymphocytes and rare neutrophils. Blood vessels are distributed all over the tissue and the fibroblasts are near disorganized collagen fibers at the periphery of the tissue. B - At 28 days, the connective tissue is thin with rare inflammatory cells. Blood vessels are distributed along the granulation tissue. Arrows point a hemorrhagic area pigmented by hemosiderin. Fibroblasts are near collagen fibers at the central and periphery regions of granulation tissue. HE – Original Magnification 40x

The animals received antiseptics with 5% iodine solution and were shaved under anesthesia. The shaved back received an incision in a head to tail orientation with a #15 blade. The skin was reflected creating two pockets: one on the right side and the other on the left side. The tubes were implanted into the spaces created with blunt dissection and the skin closed with a 4/0 silk suture.

Each animal received 2 implants with the same solution totaling 10 implants *per* experimental group. After 7 and 28 days after implantation the animals were killed by anesthetic overdose and the tubes and surrounding tissue were explanted, fixed in 10% formalin and processed and stained with hematoxylin eosin.

Histological analysis

All analyses were performed with a light microscope and original magnification of 40x.

The quantitative analysis of inflammation was performed according to the number of inflammatory cells present and divided in scores^{2,3}.

Absent=absence of inflammatory cells

Low=<25 inflammatory cells

Moderate=25-125 inflammatory cells

Severe=>125 inflammatory cells

Statistical analysis

Data from the edemogenic analysis were analyzed by two-way ANOVA and Tukey's test ($p<0.05$). Data from the histological analysis were analyzed by the Mann-Whitney and Kruskal-Wallis tests. A significance level of 5% was set for all analyses.

RESULTS

Edemogenic analysis

The results for edema quantification showed no statistically significant differences between the experimental periods ($p>0.05$). Among the groups, the aqueous extract and saline presented less edema than the ethanolic extract, which showed significant differences from the other groups in both experimental periods ($p<0.05$) (Figure 1).

Histological analysis

The histological analysis was performed after 7 and 28 days, according ISO 10993-6⁷. The analysis of all groups in relation to the experimental period showed a statistically significant difference between the ethanolic extract and the other groups for the 28-day period ($p<0.05$) (Figure 2).

At 7 days, moderate inflammatory infiltrate was present, probably due to surgical trauma (Figures 3-5). At 28 days, healing was in an advanced stage presenting lower levels of inflammation when compared with 7 days for the three groups (Figures

3-5). These differences were statistically significant ($p<0.05$).

The ethanolic extract group showed persistency of inflammation with large amounts of inflammatory cells up to 28 days, with no statistically significant differences between 7 and 28 days ($p>0.05$) (Figure 5).

DISCUSSION

Approximately 85% of the world's population depends on traditional medical treatments based on herbal medicines, and approximately 25% of the world's pharmaceutical medicines are derived from plants¹⁵.

Myracrodruon urundeuva, called Aroeira in Brazil, was chosen for this study because of its antibacterial properties demonstrated in previous studies⁸. The present study evaluated the inflammatory tissue response to aqueous and ethanolic Aroeira extracts. During the studied period, wound healing was in an advanced stage, presenting diminished levels of inflammation for aqueous and control group. However, the ethanolic extract group demonstrated persistency of inflammation until up to the 28th day. This result may be due to some component released during the preparation of the extract rather than ethanol.

Although clinical studies¹⁰ revealed an absence of significant toxic effects of the plant elixir, Repetto, et al.¹⁶ (1997) showed that the ethanolic Aroeira extract can be considered toxic.

The edemogenic analysis demonstrated slightly reduction of edema by the aqueous Aroeira extracts in relation to the control group, while the ethanolic extract group showed significantly more edema.

The experimental periods for histological analysis were based in previous studies, which showed an unaltered tissue response after 30 days⁶. The use of polyethylene tubes with a paper cone was based on Pullin and Carvalho¹⁴ (1984).

The histological analysis also showed a significantly worse result for the ethanolic extract in relation to persistency of inflammation up to 28 days. There were no signs of adequate wound healing when comparing to other groups. On the other hand, Natural Resources Conservative Service (USA²⁰, 2012) used the same methodology and found better results with ethanolic Araçá extract.

In the present study, the aqueous extract group presented healing evolution similar to that of the saline group and at 28 days was slightly better despite not significant. The moderate inflammatory infiltrate observed at 7 days may be due to surgical trauma rather than a reaction to the extracts¹⁷.

CONCLUSION

In conclusion, the biological response the Aroeira's aqueous extract is biocompatible and similar the response to saline. The ethanolic extract interfered with the wound healing maintaining the inflammation up to advanced periods.

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Authors declare there are no financial/commercial conflicts of interest of this study.

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