

SCHINUS TEREBINTHIFOLIUS RADDI (ANACARDIACEAE) IN THE HEALING PROCESS OF GASTRORRAPHY IN RATS

Schinus terebinthifolius raddi (anacardiaceae) no processo de cicatrização de gastrorrafias em ratos

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ABSTRACT – Background - Gastrorraphy, isolated or associated with the use of biological adhesives, was throughout the history of surgery the usual way to promote healing in gastric lesions and the use of herbal medicine has been increasingly more employed. **Aim** - To evaluate the wound healing in the stomach of rats with the use of the hydroalcoholic extract of *Schinus terebinthifolius* Raddi (aroeira). **Methods** - Sixty rats, adult males, were divided into two groups: aroeira group and control group. Each one was subdivided into four subgroups of 15 animals (test groups). Each subdivided subgroup was also subdivided into three subgroups of five rats (deaths periods of 7, 14 and 21 days). All animals underwent the same surgical procedure (injury and stomach suture); animals in the aroeira group received daily dose of 100 mg/kg of hydroalcoholic extract via gavage while the control group received isotonic saline solution. Parameters evaluated were: macroscopic and microscopic changes, test for resistance to insufflation of atmospheric air and test for tensile strength. **Results** - All animals had good healing of the abdominal wall and gastrorraphies without infection and dehiscence. Both groups had adhesions to the gastrorraphies surfaces with neighboring organs. The resistance test by insufflation of atmospheric air and tensile strength showed higher average of pressure on the 7th day and breaking strength in the time periods for the aroeira group. The intensity of chronic inflammation revealed statistically significant differences in the variables fibroblast proliferation and collagen. **Conclusion** - The use of hydroalcoholic extract of *Schinus terebinthifolius* Raddi accelerated the stomach healing in rats

HEADINGS - Rats. Wound healing. Insufflation. Traction.

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DESCRITORES - Ratos. Cicatrização. Insuflação. Tração.

RESUMO – Racional - A gastrorrafia isolada ou associada ao uso de adesivos biológicos constituiu ao longo da história da cirurgia a forma usual de promover a cicatrização nas lesões gástricas e o uso de fitoterápicos tem sido cada vez mais empregado. **Objetivo** - Avaliar a cicatrização de ferida provocada no estômago de ratos com uso do extrato hidroalcoólico de *Schinus terebinthifolius* Raddi. **Métodos** - Foram utilizados 60 ratos, adultos, machos, distribuídos em dois grupos: grupo aroeira e grupo controle, os quais foram subdivididos em três subgrupos de cinco animais conforme o momento das mortes dos animais (7, 14 e 21 dias). Todos os animais foram submetidos ao mesmo procedimento cirúrgico (lesão e refia do estômago) diferindo apenas que os animais do grupo aroeira receberam dose diária de 100 mg/kg do extrato hidroalcoólico via gavagem enquanto o grupo controle recebeu solução salina isotônica. Os parâmetros avaliados foram: alterações macroscópicas e microscópicas, teste de resistência à insuflação de ar atmosférico e teste pela força de tração. **Resultados** - Todos os animais demonstraram boa cicatrização da parede abdominal e das gastrorrafias, sem infecção e deiscência. Ambos os grupos apresentaram aderências à superfície das gastrorrafias com órgãos vizinhos. O teste de resistência por insuflação de ar atmosférico e força de tração mostraram maiores médias de pressão no 7º dia e de força de ruptura nos períodos estudados para o grupo aroeira. A intensidade da inflamação crônica revelou diferença estatisticamente significativa nas variáveis proliferação fobrobrástica e colagenização. **Conclusão** - O uso do extrato hidroalcoólico de *Schinus terebinthifolius* Raddi acelerou a cicatrização do estômago de ratos.

INTRODUCTION

It is important to note that among 250-500 thousand plant species, only about 5% have been phytochemically studied and a smaller percentage evaluated on the biological aspects. Thus, the success of investigations of active principles of plants and animals depends on the degree of multidisciplinary interaction, ie, botany, chemistry and pharmacology⁶.

The aroeira tree (*Schinus terebinthifolius* Raddi) is from Peru with wide geographical distribution in several continents. In Brazil, It is found on the entire coast of the northeast, southeast, south and central-western geographic regions. Known as a toxic plant, many of its properties or its healing effects were attributed to the different polyphenols distributed unevenly in the various plant organs, such as barks, leaves, flowers, fruits and seeds. These polyphenols are substances of great importance to the plant physiology, contributing to its defense mechanism. The phytochemical screening of shells revealed the presence of tannins, phenols and steroids. The popular medicinal use of this plant is based on its anti-inflammatory, astringent and hemostatic actions. Serve these active ingredients as chemical markers for quantification and standardization purposes of this plant, for extracts and herbal products²⁹.

The application of herbal medicines in wound healing of stomach lesions, as well as other organs and tissues, have been extensively evaluated in experimental studies. Medicine at the end of the last millennium and the beginning of the 21st century observed increasing use of plant based drugs in the treatment of several diseases. The investigations were not limited to the development of synthetic drugs, but in the increasing attempts to isolate active principles from plants and animals. In spite of this, there is a major scientific breakthrough involving chemical and pharmacological studies on medicinal plants aimed to obtain new compounds with therapeutic properties. With the development of new spectroscopic techniques, organic chemists have been able to quickly find complex structures of natural constituents, previously difficult to be elucidated⁶.

Several studies have been conducted to evaluate the therapeutic properties of aroeira (*Schinus terebinthifolius* Raddi). Thus, the following pharmacological and ethnobotanical properties are confirmed: anti-diarrheal and anti-inflammatory; astringent, tonic, stimulant and anti-neuralgic; antimicrobial; mutagenic; antioxidant; apoptotic potential and autophagic cell death in DU 145 cells; anti-ulcerogenic; gastritis and dyspepsia; fungicide; healing of tooth extraction in rats; healing of colon, bladder, alba line, skin and stomach. Furthermore, studies on the composition of plant parts, isolated

compounds such as: essential oils, flavonoids, fractions enriched of flavonoids, Shinol (flavonoid) and masticadienoic acid, biflavonoids isolated and triterpenes. Have been studied the morphology and anatomy of the seed, barks and leaves, chemical and microscopic examination of the plant, barks development and fruits development, even clinical drug trials (phase I) of an herbal compound (*Schinus*, *Plectranthus* and *Eucalyptus*)^{1,3,4,5,7,8,9,10,11,15,18,19, 20,21,23,25,26, 28,30,31,32,33,34}.

The present research, that aims to verify the influence of the hydroalcoholic extract of aroeira, through gastric route, in the healing process of gastrorrhaphies in rats, based on morphological and tensiometric analysis.

METHODS

This study was conducted at the Laboratory of Experimental Surgery of Federal University of Maranhão, São Luis, MA, Brazil following Brazilian laws on experimental research and was approved by the Ethics Committee of the Maranhão State University under the protocol 027-2007.

Botanical material and hydroalcoholic extract preparation

The aroeira exsiccate (*Schinus terebinthifolius* Raddi) was obtained from Santa Ana farm and its identity was botanically confirmed. A voucher specimen was deposited at the Atico Seabra Herbarium of Federal University of Maranhão under the registration No. 488. The aroeira in the form of bark suffered crushing and the powder (2400 g) was dissolved in absolute alcohol and distilled water forming 3450 ml crude extract used in the yield and concentration calculation in g/ml.

The crude extract was concentrated in a rotary evaporator under reduced pressure at 55-60°C for solvent total elimination. The material obtained after the concentration showed pasty aspect (596.85 g). For preparing the solution (100 mg/ml) 2.8 g of paste was removed and diluted in 28 ml saline solution, being stored in refrigerator at 10°C. This material (50 ml) was evaluated for sugar content (20.8%), protein (0.966%), lipids (22.93%), ashes (0.5%), moisture (51.87%) and pH (3.9).

Animal manipulation and group distribution

Sixty male Wistar (*Rattus norvegicus albinus*, Rodentia Mammalia) rats aged between 50 to 60 days and weighing on average 141.2 g were used. Animals were randomly assigned during surgery into two groups: aroeira group (AG) and control group (CG), which were subdivided into four subgroups (SG) of 15 rats: insufflation aroeira group (IASG) from 1 to 15, tensile strength aroeira group (TASG) from 16 to 30,

insufflations control group (ICSG) from 31 to 45 and tensile control group (TCSG) from 46 to 60. Each of the subgroups of five animals was killed with seven days interval: 7, 14 and 21.

After seven days of adaptation, surgery was performed with feed withdrawal of six hours before and free access to water. After being weighed, the rats were anaesthetized intramuscularly with 20 mg/kg of 5% ketamine (Vetanarcol®) and 10 mg/kg of 2% xylazine (Kensol®) on the back of the animal's thigh.

Longitudinal laparotomy and stomach exteriorization were performed, in which was carried out longitudinal incision with 1 cm in length and raffia with four separated points of blue wire polypropylene 6-0 (Prolene®, Ethicon). Subsequently, the synthesis of abdominal wall was done and for analgesia dipirone 25 mg/kg/dose was injected intramuscularly every day for five days.

Animals of AG received daily 100 mg/kg/dose of hydroalcoholic extract by gavage and the same volume of 0.9% isotonic saline solution in the control group (CG). Animals were monitored every day and a protocol was used to record all observations, e.g., activity, alertness, body condition, body weight, breathing, coat condition, signs of dehydration, drinking, eating, eyes conditions, face, nose, urine and movement.

Rats were killed with a lethal dose of ketamine and xylazine. They were induced to death on the days previously established (7th, 14th and 21st). Afterwards, the surgical specimen containing the stomach to the pylorus with 4 cm of distal esophagus was removed, not undoing the structures and organs adhered to the gastrorrhaphy in order to not compromising the tests for insufflation and tensile strength. The test for resistance to insufflation of atmospheric air was performed. It consisted in introducing silicone probe n° 6 in the esophagic segment and its fixation with 2-0 cotton threads, connecting the probe to the gauge (Polzin®, Watson Marlow, Berlin, Germany), submerging the piece in water, insufflation with room air at 0.1 ml/s speed until air bubbles release, being recorded the pressure of specimen rupture time (mm Hg).

For the resistance to traction force, surgical specimens were evaluated in universal testing machine (Tiratest 2420, TIRA Maschinenbau GmbH). Immediately before the mechanical test, stomachs were opened by the posterior wall and turned into rectangles (6 x 3 cm), keeping the scar at the centre. Each piece received a 2 N preload with 60 seconds settling time. The speed established for all tests was 5 mm/min and measurements were taken every 0.5 mm. The maximum force (N) in the rupture versus evolution time (days) was determined in each test.

Stomachs were fixed in 10% formalin; slides were stained with haematoxylin and eosin (H&E), Masson's trichrome (MT) and Picrosirius Red (PR) and analyzed

in three fields per double-blind assessment.

Histological evaluation included the following criteria: acute inflammatory infiltrate (polymorphonuclear), edema, vascular congestion and chronic inflammatory infiltrate (mononuclear cells), angiogenesis, fibroblast proliferation and collagen (fibrosis). Scores were adopted to quantify the variables according to intensity (absent, mild discreet, moderate and severe) and qualify collagen (young and mature) ³⁶.

Statistical analysis

Data were evaluated with BioEstat 5.0 (2007) and expressed as mean and standard deviation (SD) in numeric variables by analysis of variance (ANOVA) followed by Dunn test, and when significant difference was applied to the Dunn test. In the classificatory variables (histological) was applied the Mann-Whitney test independent to each observational period (7, 14 and 21 days). The significance level for rejecting the null hypothesis was 5%, ie, it will be considered as statistically significant p value <0.05. Values with p<0.05 were considered statistically significant.

RESULTS

All animals showed good healing of the abdominal wall and stomach, without clinical signs of infection or dehiscence. Adhesions to the surface of gastric sutures occurred nearby organs in both groups, especially the liver, intestine and abdominal wall. According to the Nair score, there were no statistic significant differences with regard to adhesions²⁴.

The test for resistance to atmospheric air insufflation was performed in all rats, stomach rupture occurred at the level of gastrorrhaphy. Means of rupture pressures were higher in AG in 7th day, but there was no significant statistical difference among periods (Figure 1).

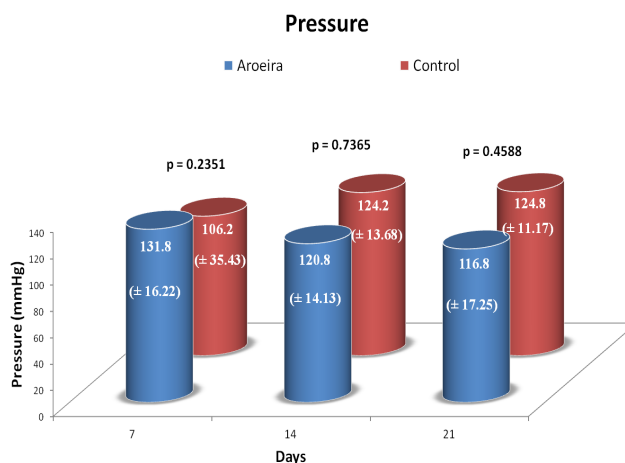


FIGURE 1 - Results of means of pressures obtained by the test for resistance to atmospheric air insufflation

In terms of resistance by tensile strength, mean forces were higher in AG in 7, 14 and 21 days, revealing a statistically significant difference in groups of 7 and 14 days but not on day 21 (Figure 2).

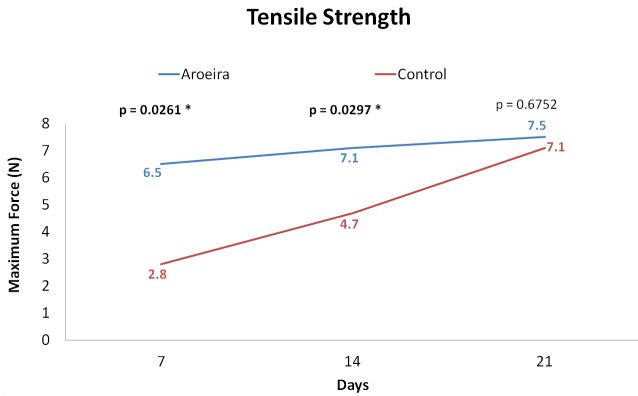


FIGURE 2 -Results of means of the forces obtained by the tensile resistance test.* implies significant difference (P<0,05) between groups

The intensity of acute inflammation and angiogenesis showed discreet in most animals of aroeira and control groups, but there was predominance of rats with moderate and severe intensity in the aroeira group compared to the control group for angiogenesis. Nevertheless, the analysis showed no statistic significant difference in the three periods. The intensity of fibroblast proliferation and collagen was moderate in most rats of both groups. There was statistic significant difference in these variables within 14 days. Considering fibroblast proliferation, such difference was due to the fact that the control group during this period had four rats with severe classification. In relation to collagen, the severe intensity occurred in five control animals in this period, providing statistic significant difference favoring the control group (Figure 3). Photomicrographs of acute and chronic inflammation are shown in Figure 4.

The density of young collagen (type III) was mostly very high (> 75%) within seven days; moderate in 14 days and 21 days (25% to 50%) in both groups, revealing no statistically significant difference in the three periods studied. In relation to mature collagen (type I), the density within seven days showed discrete (<25%) in most rats in both groups; this density was markedly on day 14 (50% to 75%) in the aroeira group and moderate in the control group on day 21, it was predominantly severe in both groups, but very severe intensity in the aroeira group. Thus, there was statistically significant difference in 14 and 21 days, favoring the aroeira group (Figure 5). Photomicrographs of collagen are shown in Figure 6.

Histological variables / p	Dias / Grupos					
	7 Days		14 days		21 days	
	Aroeira	Control	Aroeira	Control	Aroeira	Control
Acute inflammation / p	+ (3) ++ (7)	+ (6) ++ (4)	+ (7) ++ (3)	+ (10)	+ (9) ++ (1)	+ (6) ++ (4)
	0.2568		0.2568		0.2568	
Angiogenesis / p	+ (5) ++ (4) +++ (1)	-(1) + (8) ++ (1)	+ (7) ++ (3)	+ (10)	+ (8) ++ (2)	+ (8) ++ (2)
	0.0821		0.2568		1	
Fibroblast proliferation / p	+ (2) ++ (8)	++ (10)	+ (2) ++ (8)	++ (6) +++ (4)	++ (10)	+ (1) ++ (8) +++ (1)
	0.4497		0.0494*		1	
Collagen formation / p	+ (5) ++ (5)	+ (3) ++ (7)	+ (2) ++ (8)	++ (5) +++ (5)	++ (7) +++ (3)	+ (1) ++ (5) +++ (4)
	0.4497		0.0233*		0.9097	

FIGURE 3 - Histological evaluation of gastrorrhaphy healing parameters in animals treated with aroeira hydroalcoholic extract and control (0.9% saline solution). Mann-Whitney test. Legend: Variables were classified as absent (-), discreet (+), moderate (+ +) or severe (+++), based on the relative intensity of acute inflammation, angiogenesis, fibroblast proliferation and collagen formation by staining HE and TM. The number in parentheses is the number of animals in each classification. * implies significant difference (P<0,05) between groups

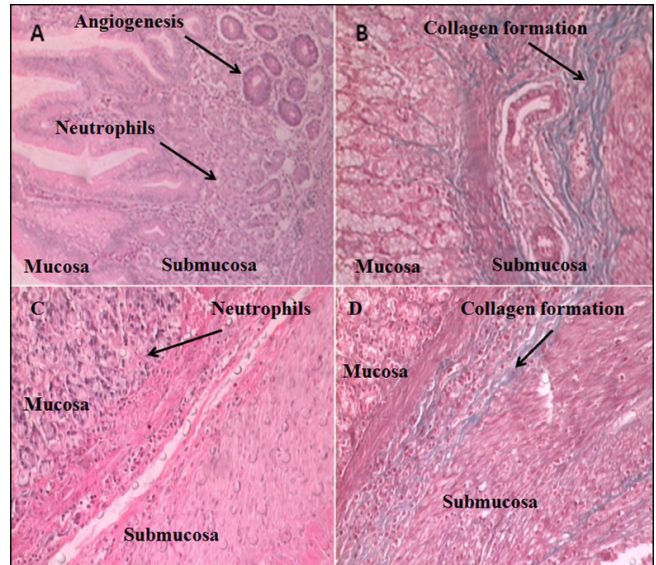


FIGURE 4 - (A) Photomicrograph of intensity of acute inflammation (neutrophils) and angiogenesis with HE staining, 100x magnification. Aroeira Group 7D, submucosa, (B) Photomicrograph intensity of fibroblast proliferation and collagen formation with TM staining, 200x magnification. Aroeira Group 14D, submucosa, (C) Photomicrograph intensity of acute inflammation (neutrophils) and angiogenesis with HE staining, 100x magnification. Control group 7D, submucosa, (D) Photomicrograph intensity of fibroblast proliferation and collagen formation with TM staining, 200x magnification. Control group 14D, submucosa.

Groups	Days/Collagen					
	7th Day		14th Day		21st Day	
	Young	Mature	Young	Mature	Young	Mature
Aroeira	+++ (1)	+ (5)	+(2)	++(2)	+ (1)	+++ (7)
	++++ (9)	++ (3)	++ (4)	+++ (7)	++ (7)	++++ (3)
0.9% Saline Solution	+++ (1)	- (1)	+(2)	-(1)	+ (3)	+ (1)
	++++ (9)	+ (9)	++ (4)	+ (3)	++ (4)	++ (4)
P	1	1	0.8798	0.0009*	0.8501	0.0149*

FIGURE 5 - Histological evaluation of parameters young and mature collagen in healing gastrorrhaphy healing of animals treated with aroeira hydroalcoholic extract and 0.9% saline solution. Mann Whitney Test. Legend: Variables were classified as absent (-), discreet (+) <25%; moderate (++) (25% to 50%); severe (+++) (50% to 75%) or very severe (++++) (> 75%) based on the relative intensity (density) of young and mature collagen by the PR staining. The number in parentheses corresponds to the number of animals in each classification. * implies significant difference (P<0,05) between groups

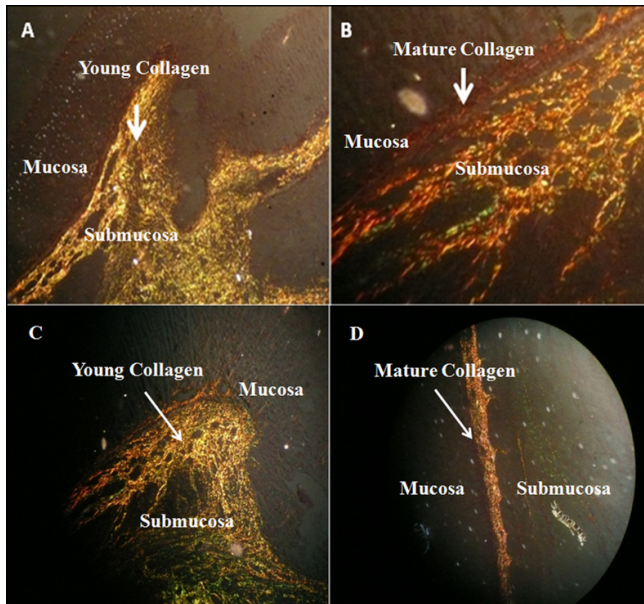


FIGURE 6 - (A) Photomicrograph of young collagen with staining (yellow) in Picosirius Red. Aroeira group, submucosa; (B) Photomicrograph of mature collagen with staining (red) in Picosirius Red. Aroeira Group, submucosa; (C) Photomicrograph of young collagen with staining (yellow) in Picosirius Red. Control group, submucosa; (D) Photomicrograph of mature collagen with staining (red) in Picosirius red. Control group, submucosa

DISCUSSION

To evaluate the aroeira effect on the stomach healing process, it was chosen a rat as experimental animal due to the ease handling and housing, resistance to surgical aggression and infectious processes, also

because this animal is standardized by much research in scarring study, unlike studies using other animals for healing evaluation as guinea pigs, dogs and mice, respectively^{22,27}. Rats used were all male, since hormonal changes of females' estrous cycle could interfere with the stomach's tissue repair mechanism according to other studies. The aroeira was the study target due to their anti-inflammatory and healing actions; however review of the literature showed only one experimental clinical work addressing the oral use of aroeira in the stomach wound healing³¹. In the present study, the chosen aroeira hydroalcoholic extract dose was 100 mg/kg/dose orally, by calculating the DL-50; lower dose than that used in other studies with aroeira (Myracrodruon urundeuva Allemão), 200 mg/kg/dose, from the same family³⁵.

It was evaluated the healing process by resistance to atmospheric air insufflation, tensile strength and morphological and morphometric analyses until day 21 postoperatively, whereas the 7th day in the early phases of the healing process, while the 14 and day 21 was chosen by having healing parameters in intermediate or advanced stages.

The rat's stomach was chosen by having the same morphological characteristics of the human stomach. Preference was given to perform gastrorrhaphy in the body region due to the technical ease. One study evaluated the pattern of healing in incisional wounds on the walls of the stomach and duodenum of rats with polypropylene thread, affirming that the bottom is more extensible than the body portion, antrum and duodenum, requiring more rupture energy^{2,12,14}.

In the immediate postoperative period, the water diet was instituted and the feed was avoided in the first six hours after surgery in order to not favoring possible dehiscence²; on the other hand, the study of Hagio et al.¹⁶ indicated fasten for 48 hours for the animals. The good acceptance of food and maintenance of overall health in daily clinical assessment used to characterize the good evolution of the healing process of gastric sutures, fact corroborated by macroscopic evaluation at necropsy.

In the stomach healing were considered assessments to determine the scar's mechanical resistance to the atmospheric air inflation, tensile strength and the morphological and morphometric study of the tissue, confirming other studies; however one study showed the non-parallelism between the resistance gain of an anastomosis to insufflation with the quantification of hydroxyproline rates¹⁷.

In both groups studied occurred adhesions at the level of gastrorrhaphy, and the structure most frequently involved was the liver, unlike other studies that obtained the greater omentum^{2,13}.

The scar's mechanical strength through the test for resistance to atmospheric air insufflation is one of the main parameters for evaluating the integrity of an anastomosis in the first postoperative days. The

determination of mechanical resistance of a scar can be performed by two techniques: resistance to air or water insufflation and linear tensile strength, when the object of analysis is related to a hollow viscus, such as the gastrointestinal tract, the test for resistance to air insufflation is physiological by reproducing the pressure vectors that normally transmit on the organ wall, being close to the real clinical situation, since the rupture will occur depending on the distension. In this study, both the measure of resistance to air insufflation as the tensile strength were performed in recommended periods corroborating authors that conducted research in similar times; however, it is important to note that the anastomosis resistance in the late period after the 14th day postoperative is best evaluated by linear tensile strength with dynamometers of precision of a single band of tissue, corroborating some authors^{14,17,27}.

The measurement of pressure on air insufflation may be interpreted by the influence of testing in situ and in vitro recording the exact location of gas exhaust in relation to the anastomosis. Most authors recommend the piece removal carefully without breaking the adhesions on it and testing and in vitro, since it allows better identification of the exact location of the gas exhaust, important information for analysis and completion of mechanical strength. In the study, the gas exhaust occurred at the suture line in all animals in evaluated periods, like the observations of some authors. The present study demonstrated that the aroeira group showed higher insufflation pressures on the 7th day and higher average of tensile strength in the periods studied. Importantly, the disruption by insufflation test occurred in specific point of the suture line (weakest point), and may infer that young collagen predominates at this site, while the tensile strength showed linear rupture, ie, dependent on the rupture of both young and mature collagen¹⁷.

The healing process that begins with the inflammatory reaction is a subject of permanent interest in surgery due to the use of sutures, bandages or biological adhesives in order to approximate the tissue; one of the main criteria to be considered is the intensity of tissue reaction induced by the threads, since the excessive inflammatory response may impair the healing process. In this study, a lower acute inflammatory reaction was observed in the aroeira group, confirming the aforementioned information. In this study, the hematoxylin-eosin (HE), Masson (TM) and Picrosirius Red (PR) techniques were used, in which different indicators were classified and quantified according to their presence and intensity. We adopted the classification of the inflammatory process in acute and chronic; neutrophilic infiltrate, interstitial edema and vascular congestion were the indicators of the acute inflammatory process, corresponding to phase I, or inflammatory of the healing process, while the presence of mononuclear infiltrates, fibrosis (collagen

formation), fibroblast proliferation and granulation tissue were indicators of chronic inflammatory process, corresponding to phase II, or proliferative of the healing process.

Picrosirius Red staining was used not only to check the area and density of total collagen, but also to assess the area and density of collagen forming fibers by the differentiation of young and mature fibers, differing from other studies that used only the hematoxylin-eosin and Masson's trichrome staining, which evaluates only the total collagen^{22,24,27,35,36}. In this study was noted that despite the variables fibroblast proliferation and collagen formation favoring the control group, it was noted through PR staining that the collagen formed in this group was predominantly young, whereas in the aroeira group mature collagen has prevailed; fact responsible for the better healing promoted in this group.

Absent or excessive inflammatory response greatly compromises the healing of tissues, as well as studies reporting that as the observation time is extended there is regression of acute inflammatory response and chronic inflammatory response predominates. In the present study, aroeira promoted the healing of several inflammation variables.

CONCLUSION

It is concluded that the use of aroeira hydroalcoholic extract (*Schinus terebinthifolius* Raddi) accelerated the healing process of the stomach regarding the tensiometric and microscopic evaluation.

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