Fibrogenesis and epithelial coating of skin wounds in rats treated with angico extract (Anadenanthera colubrina var. cebil)[1]

Wagner Soares Pessoa[I], Lígia Reis de Moura Estevão[II], Ricardo Santos Simões[III], Fábio de Souza Mendonça[IV], Luis Evêncio-Luz[V], Liriane Baratella-Evêncio[VI], Rinaldo Florencio-Silva[VII], Fabrício Bezerra de Sá[VIII], Joaquim Evêncio-Neto[IX]

DOI: http://dx.doi.org/10.1590/S0102-865020150050000007

[I]Associate Professor, Histology Division, Department of Morphology, Federal University of Piaui (UFPI), Teresina-PI. Brazil, Conception and design of the study, critical revision.
[II]Fellow PhD degree, Postgraduate Program in Animal Bioscience, Department of Morphology and Animal Physiology, Federal Rural University of Pernambuco (UFRPE), Recife-PE, Brazil. Surgical procedures, acquisition of data.
[III]Fellow PhD degree, Postgraduate Program in Morphology, Department of Morphology and Genetic, Federal University of Sao Paulo (UNIFESP), Brazil. Interpretation of data, manuscript writing.
[IV]Associate Professor, Histology Division, Department of Morphology, UFRPE, Recife-PE, Brazil. Scientific and intellectual content of the study, interpretation of data, critical revision.
[V]Associate Professor, Microbiology Division, Department of Biology, UFPI, Picos-PI. Brazil. Scientific and intellectual content of the study, interpretation of data, critical revision.
[VI]Associate Professor, Histology Division, Department of Histology and Embryology, Federal University of Pernambuco (UFPE), Brazil. Acquisiton and interpretation of data.
[VII]Fellow PhD degree, Postgraduate Program in Morphology, Department of Morphology and Genetics, UNIFESP, Sao Paulo-SP, Brazil. Interpretation of data, manuscript writing.
[VIII]Associate Professor, Anatomy Division, Department of Morphology and Physiology, UFRPE, Recife-PE, Brazil. Conception and design of the study, critical revision.
[IX]Associate Professor, Histology Division, Department of Morphology and Physiology, UFRPE, Recife-PE, Brazil. Conception and design of the study, critical revision.

ABSTRACT

PURPOSE: To evaluate the effects of angico bark extract (*Anadenanthera colubrina* var. *cebil*) in the healing process of the skin of rats.

METHODS: Twenty adult male rats were divided into four groups of five animals each, according to the respective postoperative days, as follow: G4, G7, G14 and G21. Each group received two incisions on skin and subcutaneous tissue in the right and left antimere of the thoracic region, separated by a distance of 2 cm. The right lesion was treated daily with saline and the left with the angico alcoholic extract (5%). At the end of each experimental period, the animals were euthanized and fragments of the wound area with the edges were removed, fixed in 10% formaldehyde solution and processed for paraffin embedding. Histological sections (5 µm of thickness) were stained with hematoxylin and eosin (HE), Gomori trichromic and picrosisirus red for morphological and morphometric analyses. Statistical analysis was done by ANOVA and Tukey-Kramer test ($p<0.05$).

RESULTS: Morphological analysis showed larger fibroblasts and a higher concentration of collagen fibers in skyn wounds treated with the angico extract. Morphometric analysis demonstrated a significant increase in the number of fibroblasts at 7th and collagen in 7th and 14th days ($p<0.01$) in wounds treated with the angico extract.

CONCLUSION: The angico alcoholic extract (*Anadenanthera colubrina* var. *cebil*) induces the acceleration of wound healing in skin wounds of rats.

Key words: Wound Healing. Collagen. Fibroblasts. Phytotherapy. Rats.

Introduction

Medicinal plants have become an important source for the discovery of novel healing agents, which raw extracts from plants and isolated substances have been demonstrated biological activity. Hence, it is important to determine the potential of natural products with biological activity to establish possible harm to normal cells as well as damage to tumor cells, in order to address possible therapeutic uses[1].

Anadenanthera colubrina (Fabaceae-Mimosoideae) is native of South American rain forests, growing at altitudes greater than 400 m. Its distribution is wide, varying in the north from Colombia and northern Brazilian States to the southern State of Paraná[2]. Commonly known as "angico", this is one of the botanical species with medicinal properties most cited by resident populations in an endemic area[3,4].

According to Albuquerque *et al*.[5] the extensive knowledge and reports from *Anadenanthera colubrina* by population that use folk medicine, followed by the fact that it is a native tree with the bark being the most used, its availability is not limited by seasonality. However, there are few scientific data of the reported properties of this medicinal plant by the folk medicine[6]. Lima et al.[7] and Pessoa *et al*.[8] studied the healing activity of alcoholic extract of angico (*Anadenanthera colubrina* (Vell) Brenan) and noted accelerated healing properties in injured tissue, suggesting that this plant present potential medicial properties.

Wound healing is a dynamic process in which the interplay betwee mediators, blood cells and extracelular matrix leads to the regeneration of the inner or outer body surface. After 24–36 h the fibrin clot is infiltrated by macrophages and granulocytes. The following reparative phase comprises the formation of granulation tissue by the sprouting of capillaries and fibroblasts. Subsequently, the granulation tissue is altered by further resorption of the exudate and distinct collagen synthesis into mature scar tissue. This process can last for weeks to months[9]. It should be noted that the stage of remodeling of the extracellular matrix is the period of consolidation, enhancing wound. The deposition, clustering and collagen remodeling are responsible for increased tensile strength and decreased scar size[10,11].

Based on the above considerations, we decided to investigate the effects of alcoholic extract of the bark of *Anadenanthera colubrina* in the healing process of the skin of rats.

Methods

The study protocol was approved by the Animal Ethics Committee of the Federal Rural University of Pernambuco (UFRPE) (process nº 23082.014123/2011).

Twenty adult male rats (*Rattus norvegicus albinus*) weighing approximately 300g were obtained from the Department of Morphology and Physiology of the Rural Federal University of Pernambuco (UFRPE). The animals were housed in individual boxes with commercial chow (Presence®, Purina) and water "ad libitum", maintained at 23-25°C, under a 12 hour light/dark cycle, at the Pharmacy Department of the Federal University of Pernambuco (UFPE) animal colony.

After one week of adaptation, the animals were anesthetized with a combination of xylazine (20 $mg.Kg^{-1}$) and ketamine (100 $mg.Kg^{-1}$) intramuscularly administered. The dorsal fur of the animals was shaved in the thoracic region and antisepsis was performed with topical alcoholic chlorhexidine 0.5%. The area was initially marked with the aid of 1.3 cm diameter cylinder. With a surgical blade and blunt scissors, incisions were made in skin and subcutaneous tissue on the right and left sides of the thoracic region, separated by a distance of 2 cm. The tissue was dissected and removed leaving exposed the adjacent fascia.

Immediately after surgical excision the wounds of the control (Ctrl) group located on the right antimere received daily topical applications of saline (sodium chloride 0.9%), whereas the treated (Treat) group with wounds located in the left antimere received daily topical applications of alcoholic extract of "angico" 5%.

The animals were then divided into four groups according to the time of application of the extract or saline. G4 group - four days of application, G7 group - seven days of application; G14 group - 14 days of application; G21 group - 21 days of application. Euthanasia was performed by deepening of anesthesia.

Preparation of the extract of Anadenanthera colubrina var. cebil

The inner bark and outer bark of Angico (*Anadenanthera colubrina* var. *cebil*) were collected during the morning in November in the city of Pombos, Pernambuco. The plant was identified by a plant taxonomist by comparison with an exemplary deposited in the Sergio Tavares Herbarium of the Forest Science Department of UFRPE (specimen number: 17242).

The extract was prepared from the bark of a tree at the Pharmacy Department of the Pharmacognosy Laboratory, UFPE according to the methodology described by Pessoa *et al*. [8]. An aliquot equivalent to 5g of this extract was diluted in 70% ethanol to yield the hydroalcoholic extract at 5% and was kept in an amber glass container at room temperature until the experiment.

Morphology and morphometry

On 4th, 7th, 14th and 21th days after surgery, the wounds of each group were measured with the aid of a caliper graph (King Tools). To calculate the injured areas the major and minor diameters were observed. From these data the wound area (A) was calculated as A = p.Rr, being R the larger radius and r the smaller radius of the wound.

The calculation of the average degree of contraction (C) was expressed as percentuals, being C = [(A0 – Ai)/Ao].100, where A0 is the initial area of mm the wound (day 0) and Ai is the area of the wound in the 4th, 7th, 14th and 21th days postoperatively.

After macroscopic analysis of the respective groups the animals were anesthetized with isoflurane, administered to animals from each group (G4, G7, G14 and G21). Then, a wound fragment was dissected with a 0.5 cm margin of healthy skin around the lesion, and processed for histological analysis.

Histological analysis

After 24h in 10% formaldehyde, tissue samples were dehydrated in increasing concentrations of ethyl alcohol, diaphanized in xylene, and embeeded in paraffin. In the middle region of the flap, longitudinal sections (5µm) were obtained parallel to the greater axis of the fragments and stained with hematoxylin-eosin (H.E), Gomori trichrome, and picrosirius red for morphological and morphometric analysis.

Morphometric analysis

Five images of each slide was obtained always immediately below the crust, with the aid of a trinocular biological microscope (NIKON 50i) under x400 magnification and adjusted to a capturing image system. Quantification of fibroblasts was performed in the center of the lesion in an area of 0.66 mm^2 (imaging), with the aid of an image analyzer (Imagelab 2000) in a Windows operational system.

Collagen quantification was performed in sections stained with picrosirius red and usig the same equipment used for morphological analysis, but the microscope was adapted to light polarizing filters. In each section, five fields at a magnification of x400 located in the center of the scar were analysed. The percentage of area occupied only by red and yellow collagen fibers (collagen type I) was calculated. Since other types of collagen fibers are present at small fractions, for practical purposes, we considered the sum of collagen I and III as the total collagen scar. The images were analyzed with the program IMAGELAB®.

Statistical analysis

The data was evaluated by ANOVA complemented by Tukey-Kramer test ($p<0.05$). Statistical analysis was performed using Assistat software, version 7.6 beta2.0.

Results

It was not identified signs of infection in the wounds studied. There was no difference in the concentration of neutrophils between Treat and Ctrl lesions; however, it was noted a progressive decrease in neutrophils after the 7th day. Also, data from wound contraction did not differ significantly among the groups (Figure 1).

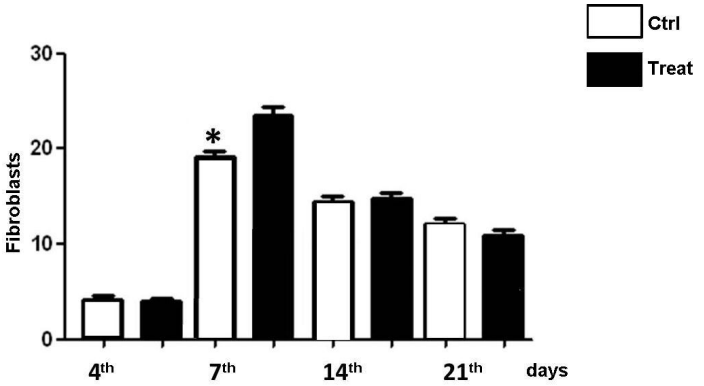

FIGURE 1 - Mean values and standard errors of the number of fibroblasts to the control and treated sides with hydroalcoholic extract of 5% angico for 4, 7, 14 and 21 days. Wound 7treat>wound 7Ctrl. G7> G14 = G21>G4; *$p<0.05$.

Histological analysis showed higher concentration of typical fibroblasts with fusiform appearance, presenting euchromatic nuclei and evident nucleoli at 4th and 7th days in the wounds treated with angico extract, which also showed higher percentage of collagen fibers at 7th and 14th days (Figures 2 to 4). Blood vessels were also noted at higher concentrations in the groups treated with angico extrat at 4th and 7th days, but it decreased in the 21th day. The degree of skin reepithelization between groups was very similar in all perionds, except at 7th day, which a sharper epithelialization was observed in the control group.

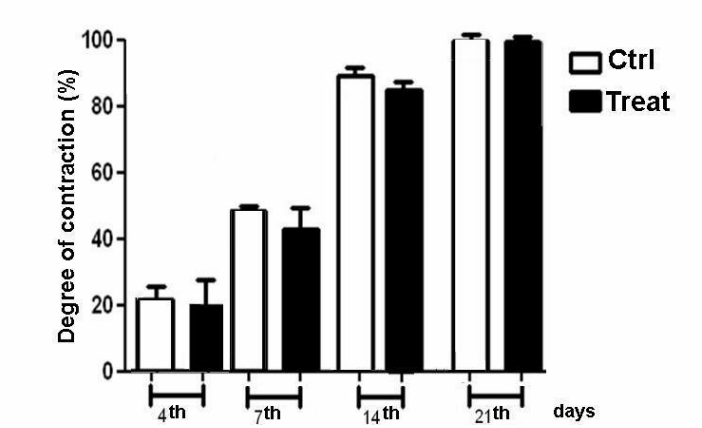

FIGURE 2 - Mean values and standard errors of the percentage of wound contraction control and treated at 4, 7, 14 and 21 postoperative days.

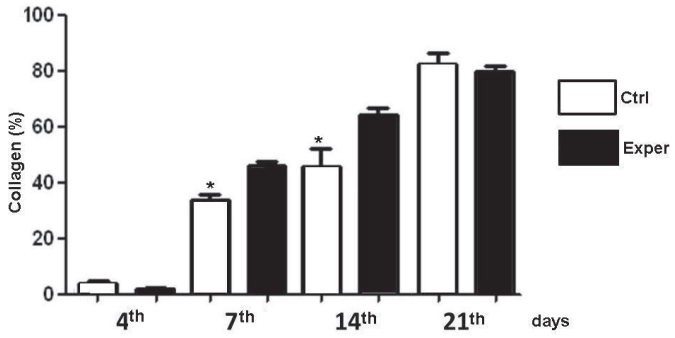

FIGURE 3 - Mean values and standard errors of the percentage of collagen in wound of control and treated at 4, 7, 14 and 21 postoperative days. Wound 7treat > wound 7Ctrl; wound 14Treat > wound 14Ctrl. G21>G14>G4; *p<0.05.

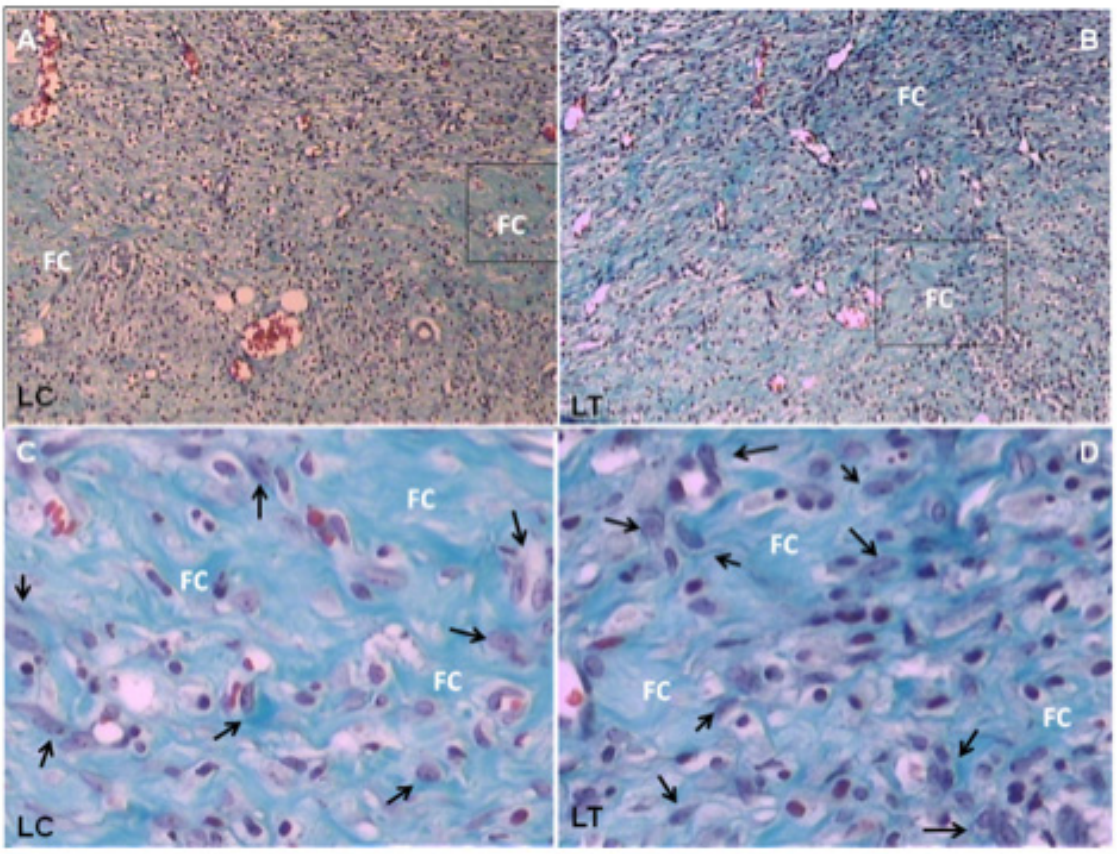

FIGURE 4 - Photomicrographs of skin fragments of rats at the 7th day of postoperative. Control side (LC) and treated side (LT). In **A** and **B** note the formation of collagen fibers (FC). In **C** and **D** (retangle insights in figures A and B) observe collagen fibers (FC) and fibroblasts (arrows). Magnification: **A** and **B** = x115; **C** and **D** = x460. Staining: Mallory trichrome.

Discussion

In this experiment we studied the action of bark extract of the angico on open wounds in rats, since these animals are very resistant to infections. We prefered to study the wound healing by secondary intention, because we also aimed to determine the degree of contraction. In our experiment we did not observe differences in the degree of wound contraction when compared the control with the experimental groups in all periods.

The healing of wounds involves biochemical and physiological phenomena, divided into three sets of events: inflammation, fibroblast phase deposition of extracellular matrix, and matrix remodeling. In such events vasoactive substances, adhesive proteins, growth factors and proteases are released[12].

The clot formation serves to buffer the second degree wounds or wounds coaptar edges, as well as binding to fibronectin, providing a provisional matrix where leukocytes, blood vessels, fibroblasts and keratinocytes can join. Besides the numerous chemical mediators, inflammation depends on the phase of the inflammatory cells such as polymorphonuclear leukocytes, macrophages and lymphocytes[11].

The proliferation phase forming the matrix includes both amorphous and fibrillar, which is important in the formation of granulation tissue. Angiogenesis is essential for the oxygen and nutrient supplies for healing process. The synthesis of collagen by fibroblasts begins around the third or fourth days. As the concentration of collagen fills the wound site, a decrease in the concentration of fibroblasts occurs, marking the end of the stage of initiating repair remodeling phase[11].

In remodeling granulation tissue present more enriched with collagen fibers and begins to acquire the appearance of fibrous mass, a feature of the scar. With the evolution of the process, the emphasis is on collagen deposition and most cells (fibroblasts and endothelial cells) die by apoptosis. The scar remodeling process involves successive stages of synthesis, degradation and orientation of collagen fibrils. Collagen deposition is made initially as randomly oriented to organization of fibronectin and dependent on the nature and direction of the tensional forces applied to the scar[12-14].

Based on the popular experiences angico extract tree is widely used as a healing agent for wounds, with the inner bark, being the most often used part for these purposes[7,15]. We believe that the favorable results of herbal medicines in the healing process are mainly related to flavonoids and tannins[16-18]. According with Paes *et al*.[19] angico (*Anadenanthera colubrina* (Vell) Brenan var. *cebil*) is a plant where tannins are found.

Previous work identified in the inner and outer bark of *Anadenanthera colubrina* var. *cebil* indole alkaloids (oxide of N, N-dimethyltryptamine), sterols (sitosterol palmitate and δ-δ-sitosterol), flavonoids (3,3',4',7,8 - pentahidroflavona), terpenoids (lupenona and lupeol) phenol derivatives (3,4,5-dimethoxidalbergiona, and dalbergiona kuhlmannia) and proanthocyanidins. Proanthocyanidins and other tannins are known to facilitate wound healing. The condensed tannins, known as proanthocyanidins, are known to improve wound healing[16,17,20].

In this atudy, the 4th day evidenced the presence of numerous neutrophils and macrophages and also some fibroblasts in the lesion site, which corroborates the findings of Simoes *et al*.[21], which identified the presence of these cells in the 3th day. The initial lesion and coagulation, platelet mediated to thrombus formation, release factors (serotonin, histamine, bradykinin, prostaglandins, and other neurotransmitters) that promote the migration of leukocytes, macrophages, fibroblasts and mesenchymal cells to the affected area[21].

Angiogenesis is essential for diffusion of oxygen in ischemic or newly formed tissue healing[11,22]. In this experiment a progressive increase in the number of capillaries on days 4, 7 and 14 was observed, confirming the findings of Estevão *et al*. [23], which applied essential oil of aroeira in skin healing in rats.

Sanchez-Neto *et al*.[24] studied the action of papain on skin wounds in rats, and observed more fibroblasts and collagen fibers in the treated groups. Thus our results are similar to the findings of these authors, since at the 7th day postoperatively, the difference in the number of fibroblasts, as well as the percentage of collagen at 7th and14th days were signicant higher in the group treated with angico extract.

The findings of this experiment also corroborates the research done by Simões *et al*.[25], who observed a higher amount of fibroblasts in skin wounds of rats at 7 days postoperatively. In this experiment, after 7 days the skin wounds of Ctrl epithelial lining showed earlier than that of the treated group.

According Majno and Gabbiani[26] inflammatory exudation, neovascularization, cell proliferation, cell migration and reepithelialization ocurrs from the edges of the healing process of open wounds after injury. The Ctrl group showed lesions with more advanced reepithelialization at 14 days postoperatively, while in the treated group this epithelialization did not occur in wounds in two rats. In other animals of treated and GCtrl groups showed epithelium only at the edges of wounds. Simões *et al*.[25] observed that at 21 days lower concentration of fibroblasts and less mature and organized bundles of collagen fibers.

In this experiment the morphometric evaluation did not show significant concentration of fibroblasts between the groups at 21 days PO differences. However, regading the morphology of collagen fibers, the GTreat showed discrete evolution and organization of the collagen fibers, as compared to the GCtrl.

Conclusion

The alcoholic extract of angico bark accelerates the healing process of open wounds in the skin of rats.

References

1. Lima RFL, Alves EP, Rosalen PL, Ruiz ALTG, Teixeira Duarte MC, Góes VFF, Medeiros ACD, Pereira JV, Godoy, GP, Costa EMMB. Antimicrobial and antiproliferative potential of *Anadenanthera*

colubrina (Vell.) Brenan. Evid Based Complement Alternat Med. 2014;2014:802696. doi: 10.1155/2014/802696.
2. Delgobo CL, Gorin PAJ, Jones C, Iacomini M. Gum heteropolysaccharide and free reducing mono and oligosaccharides of *Anadenanthera colubrina*. Phytochemistry. 1998;47(7):1207-14. doi: 10.1016/S0031-9422(97)00776-0.
3. Agra MF, Freitas PF, Barbosa-Filho JM. Synopsis of the plants known as medicinal and poisonous in Northeast of Brazil. Rev Bras Pharmacogn. 2007;17(1):114-40. doi: 10.1590/S0102-695X2007000100021.
4. Santos JS, Marinho RR, Ekundi-Valentim E, Rodrigues L, Yamamoto MH, Teixeira SA, Muscara MN, Costa SK, Thomazzi SM. Beneficial effects of *Anadenanthera colubrina* (Vell.) Brenan extract on the inflammatory and nociceptive responses in rodent models. J Ethnopharmacol. 2013;148(1):218-22. doi: 10.1016/j.jep.2013.04.012.
5. Albuquerque UP, Lucena RFP, Monteiro JM, Florentino ATN, Almeida CFCBR. Evaluating two quantitative ethnobotanical techniques. Ethn Res Appl. 2006;4(1):51-60.
6. Weber CR, Soares CML, Lopes ABD, Silva TS, Nascimento MS, Ximenes ECPA. Anadenanthera colubrina: a therapeutic potential study. Rev Bras Farm. 2011;92(4):235-44.
7. Lima CR, Costa-Silva JH, Lyra MMA, Araújo AV, Arruda VM, Dimech GS, et al. Atividade cicatrizante e estudo toxicológico pré-clínico do fitoterápico sanativo®. Acta Farmacêutica Bonaerense, Buenos Aires, 2006;25(4):544-9.
8. Pessoa WS, Estevão LR, Simões RS, Barros ME, Mendonça Fde S, Baratella-Evêncio L, Evêncio-Neto J. Effects of angico extract (*Anadenanthera colubrina var. cebil*) in cutaneous wound healing in rats. Acta Cir Bras. 2012;27(10):655-70. doi: 10.1590/S0102-86502012001000001.
9. Buţureanu SA, Buţureanu TA. Pathophysiology of adhesions. Chirurgia (Bucur). 2014;109(3):293-8. PMID: 24956331.
10. Boote C, Dooley EP, Gardner SJ, Kamma-Lorger CS, Hayes S, Nielsen K, Hjortdal J, Sorensen T, Terril NJ, Meek KM. Quantification of collagen ultrastructure after penetrating keratoplasty - implications for corneal biomechanics. PLoS One. 2013;8(7):e68166. doi: 10.1371/journal.pone.0068166.
11. Mandelbaum SH, Santis EPD, Mandelbaum MHS. Cicatrização: conceitos atuais e recursos auxiliares - Parte I. An Bras Dermatol 2003;78(4):1-16. doi: 10.1590/S0365-05962003000400002.
12. Balbino CA, Pereira LM, Curi R. Mecanismos envolvidos na cicatrização: uma revisão. Rev Bras Ciênc Farm. 2005;41(1):27-51. doi: 10.1590/S1516-93322005000100004.
13. Oryan A, Shoushtari AH. Histology and ultrastructure of the developing superficial digital flexor tendon in rabbits. Anat Histol Embryol. 2008;37(2):134-40. doi:0.1111/j.1439-0264.2007.00811.x.
14. Verhaegen PD, Schouten HJ, Tigchelaar-Gutter W, van Marle J, van Noorden CJ, Middelkoop E, van Zuijlen PP. Adaptation of the dermal collagen structure of human skin and scar tissue in response to stretch: an experimental study. Wound Repair Regen. 2012;20(5):658-66. doi: 10.1111/j.1524-475X.2012.00827.x.
15. Monteiro JM, Almeida CFCBR, Albuquerque UP, Lucena RFP, Florentino ATN, Oliveira RLC. Use and traditional management of *Anadenanthera colubrine* (Vell.) Brenan in the semi-arid region of northeastern Brazil. J Ethnobiol Ethnomed. 2006;2:6. doi: 10.1186/1746-4269-2-6.
16. Aderounmu AO, Omonisi AE, Akingbasote JA, Makanjuola M, Bejide RA, Orafidiya LO, Adelusola KA. Wound-healing and potential anti-keloidal properties of the latex of Calotropis procera (Aiton) Asclepiadaceae in rabbits. Afr J Tradit Complement Altern Med. 2013;10(3):574-9. PMID: 24146491.
17. Khanna S, Venojarvi M, Roy S, Sharma N, Trikha P, Bagchi D, et al. Dermal wound healing properties of redox-active grape seed proanthocyanidins. Free Radic Biol Med. 2002;33(8):1089-96. doi: 10.1016/S0891-5849(02)00999-1.
18. Peruchi CMS, Silva EB, Andrade RA, Franco SL, Ramalho L. Efecto del propóleos en la cicatrización de lesiones subcutáneas inducidas en el dorso de ratones: estudio histológico. Rev Fac Odontol Univ Chile. 2001;19(2):23-34.
19. Paes JB, Diniz CEF, Marinho IV, Lima CR. Avaliação do potencial tanífero de seis espécies florestais de ocorrência no Semi-Árido brasileiro. Cerne, Lavras 2006;12(3):232-8.
20. Leandro LM, Vargas FS, Barbosa PC, Neves JK, da Silva JA, da Veiga-Junior VF. Chemistry and biological activities of terpenoids from copaiba (*Copaifera* spp.) oleoresins. Molecules. 2012;17(4):3866-89. doi: 10.3390/molecules17043866.
21. Simões MJ, Cabral ACV, Boyaciyan K, Kulay Jr L, Sasso WS. Aspectos ultra-estruturais dos fibroblastos e dos macrófagos durante o processo de reparação da pele de ratos. Rev Paul Med. 1986;104(4):132-5. PMID: 3563265.
22. DiPietro LA. Angiogenesis and scar formation in healing wounds. Curr Opin Rheumatol. 2013;25(1):87-91. doi: 10.1097/BOR.0b013e32835b13b6.
23. Estevão LR, Mendonça FS, Baratella-Evêncio L, Simões RS, Barros ME, Arantes RM, Rachid MA, Evêncio-Neto J. Effects of aroeira (*Schinus terebinthifolius Raddi*) oil on cutaneous wound healing in rats. Acta Cir Bras. 2013;28(3):202-9. doi: 10.1590/S0102-86502013000300008.
24. Sanchez-Neto R, Baroni B, Teves DC, Simões MJ, Novo NF, Juliano Y. Aspectos morfológicos e morfométricos da reparação tecidual de feridas cutâneas de ratos com e sem tratamento com solução de papaína a 2%. Acta Cir Bras. 1993;8(1):18-23.
25. Simões MJ, Uzunian A, Mora AO, Sasso WS. Aspectos ultra-estruturais do processo de reparação da pele de ratos albinos. Rev Paul Med. 1985;103(4):123-6. PMID: 4089424.
26. Gabbiani G, Majno G. Dupuytren's contracture: fibroblast contraction? An ultrastructural study. Am J Pathol. 1972;66(1):131-46. PMID: 5009249.

Correspondence:
Joaquim Evêncio-Neto
Universidade Federal Rural de Pernambuco/DMFA
Rua Manoel de Medeiros, s/n
52171-900 Recife – PE Brasil
Tel.: (55 81)3320-6390
Fax: (55 81)3265-5948
evencio@dmfa.ufrpe.br

Received: Jan 16, 2015
Review: Mar 19, 2015
Accepted: Apr 18, 2015
Conflict of interest: none
Financial sources: FACEPE (Pernambuco Research Foundation), FAPEMIG (Minas Gerais Research Foundation) and CNPq (National Council of Scientific and Technological Development)

[1]Research performed at Department of Morphology and Animal Physiology, Federal Rural University of Pernambuco (UFRPE), Brazil. Part of PhD degree thesis, Postgraduate Program in Animal Bioscience. Tutor: Joaquim Evêncio-Neto.