

## Article

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# Evaluation of wound healing activity of atranorin, a lichen secondary metabolite, on rodents

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**Abstract:** The aim of this study was to investigate the wound healing activity of atranorin cream (Patent requested) on excision wounds. Seventy-two male rats were anesthetized and an excisional wound was performed. Then the rats were randomly assigned into three groups: untreated control group; atranorin 1 (group treated with 1% AT ointment); and atranorin 5 (group treated with 5% AT ointment). Six animals of each group were euthanized 3, 7, 14 or 21 days after surgical procedures and the wounded areas were analyzed and removed. Serial histological sections were obtained and stained by histochemical techniques (Hematoxylin-Eosin-HE and Sirius red) and immunohistochemical techniques. Topical application of atranorin reduced wound areas, induced earlier granulation tissue formation, increased cell proliferation, improved collagenization and modulated the myofibroblasts differentiation when compared to control animals. It is suggested that atranorin modulates the wound healing process. These data suggest that this formulation based on atranorin extracted from *Cladonia kalbii* AHTI may be a new biotechnological product for wound healing clinical applications.

## Introduction

Dermal wound healing is a complex phenomenon characterized by a sequence of independent and overlapped events described as exsudative/inflammatory, proliferative (granulation tissue formation and collagen synthesis) and remodeling phases (Diegelmann & Evans, 2004). The analysis of the kinetic of these biological events in response to novel pharmacological substances is important for the development of efficient therapeutic products capable of stimulating the wound healing (Alborova et al., 2007).

Lichens are symbiotic organisms composed of a fungus and an alga. They produce characteristic secondary metabolites "lichen substances" that seldom occur in other organisms. Lichens and their metabolites have many biological activities such as antimicrobial (Rankovic et al., 2008), antiviral (Huneck, 1999), antioxidant (Behera et al., 2008), wound healing (Nunes et al., 2011), anti-inflammatory (Vijayakumar et al., 2000) and analgesic activity (Maia et al., 2002; Siqueira et al., 2010).

In the Brazilian northeastern area, there is a variation of occurrence of lichen species according to the

shift from the coastal zone (humid), to the inner part of the country, where the climate is semi-arid (an ecosystem known as "caatinga"). In these habitats several lichen species were mentioned to be bioactive (Maia et al., 2002). Atranorin (AT), an important lichen metabolite, has been utilized for medicinal, perfumery, cosmetic as well as ecological applications (Ingólfssdóttir, 2002).

Some studies show biological activities such as antimicrobial (Rankovic et al., 2008), antioxidant (Toledo et al., 2003) and elastase inhibitor (Proksa et al., 1994). In addition, recent studies suggest a possible antinociceptive effect of AT in unspecific tests (Melo et al., 2008) and shows that AT inhibits COX-1 and COX-2 in a dose-dependent manner (Bugni et al., 2009). Additional studies on AT demonstrated that it effectively inhibits the biosynthesis of leukotriene B<sub>4</sub> in bovine polymorphonuclear leukocytes, which could also lead to an anti-inflammatory effect (Kumar & Müller, 1999).

Despite many pharmacological properties involved in the dynamics of the healing process have been related to AT, there is no report regarding to the effects of this lichenic product on in vivo dermal wound healing.

Therefore, the aim of the present study was to investigate the wound healing activity of cream of AT (Patent requested, PI1003289-4; Quintans-Júnior et al., 2012) extracted from *Cladina kalbii* (DES ABB.) Ahti on excision wounds in rats.

## Material and Methods

### *Lichen material and extraction and isolation of atranorin*

*Cladina kalbii* was collected in March 2007 in the Itabaiana County, Sergipe State, northeastern Brazil (10°44'S, 37°23'W). *Cladina kalbii* was identified by M. P. Marcelli (Botanical Institute of São Paulo-SP, Brazil) and herbarium voucher specimens (registry number SP 393235) were prepared and deposited at the Botanical Institute of São Paulo-SP, Brazil. Atranorin was isolated from *Cladina kalbii* as previously described (Melo et al., 2008) and stored at -20 °C.

### *Animal assays*

Male *Wistar* rats (220-260 g) were used for all experiments. Animals were housed under conditions of controlled temperature (25±1 °C) and lighting (lights on: 6 am to 6 pm) and had free access to food and water. All procedures described in the present work were approved by the Animal Research Ethics Committee of the Federal University of Sergipe (CEPA/UFS # 55/07) and were in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication 85-23, revised 1996; <http://www.nap.edu/readingroom/books/labrats/index.html>).

### *Surgical procedures*

Seventy-two rats were anesthetized with thiopental (40 mg/kg, *i.p.*) (Wua et al., 2012) and one full-thickness excisional wound was performed using a 8 mm biopsy punch on the back (in the middle, on the spine, 1 cm under the interscapular area) of each animal (Freitas et al., 2010). The animals were handled in accordance with aseptic principles to avoid exogenous bacterial contamination. Then the rats were randomly assigned into three groups of 24 animals each: CTR (control group or group treated with base ointment-30% lanoline and 70%vaseline); AT1 (group treated with 1% atranorin ointment); and AT5 (group treated with 5% atranorin ointment). All animals were treated immediately after the surgical procedure. A single administration of the ointment (0.1 g, base, with either 1% or 5% atranorin) was daily performed using hands and a spatula till 24 h prior to the euthanasia. Following the daily administration of the ointment, the animals had their wounds covered by healings. Six

animals of each group were euthanized by administration of thiopental (150 mg/mL, *i.p.*) on 3<sup>rd</sup>, 7<sup>th</sup>, 14<sup>th</sup> or 21<sup>st</sup> day after the surgical procedures and the wounded areas were removed and fixed in a 10% formaldehyde solution. The surgical specimens were paraffin-embedded according to the routine laboratorial techniques. Histological sections, serial, of 5 µm thick, were obtained and stained by histochemical and immunohistochemical techniques. Thereafter, the histological analysis of the wounds was carried out as detailed hereafter.

### *Macroscopic analysis and wound contraction*

The clinical features of wounds were monitored regarding to the presence of crust, secretion, necrosis and hypertrophic scars. Craniocaudal and latero-lateral measures of each wound were assessed by using a digital caliper and the final wound areas were obtained through the equation  $A = \pi.R.r/2$ , where A represents the area;  $\pi$ , a mathematical value was used until the 4<sup>th</sup> number after the decimal point, which corresponds to 3.1415; R represents the craniocaudal axis and r corresponds to the latero-lateral axis of the wounds.

### *Histological analysis*

All the histological analyses were performed by two observers blinded to the treatment. Sections stained in HE were analyzed under conventional light microscope to assess the inflammatory response (IR) and epithelization rates (ER). IR was determined by classifying the inflammatory infiltrate according to the predominant leukocyte subset as acute (predominance of polymorphonuclear cells/neutrophils), subacute (balance in the content of polymorphonuclear and mononuclear cells) or chronic (predominance of mononuclear cells), and then graded considering the intensity of the infiltrate as mild/absent (+), moderate (++) or severe (+++), as previously described by Ribeiro et al. (2009). ER were evaluated by measuring the lengths of the epidermal tongues from both wound edges in a microscope with a ruler, and data were expressed as percentage wound closure (distance of migrated keratinocytes from the wound edge/total wound width x 100).

Collagen content was analyzed in the Picrosirius-stained sections, assessed under the ordinary polychromatic and polarized light microscopies. The proportion of tissue stained with picrosirius red content was assessed by descriptive analysis. Collagen fibers were analyzed according to their birefringence pattern (greenish/yellow-greenish or orange/orange-reddish), morphological appearance (wavy or stretched, thin or thick, short or long) and architectural arrangement (reticular, parallel or interlaced).

The quantitative analysis of the content

of collagen fibers deposition (CF) in the healing area was determined by optical density in the image analysis system in different randomly selected fields. The system used consists of a CCD Sony DXC-101 camera, applied to a Olympus CX31 microscope, from which the images were sent to a monitor (Trinitron Sony). By means of a digitizing system (Olympus C-7070 WIDEZOOM) the images were inserted into a computer (Pentium 133 MHz), and processed by a software (ImageTool). A total of ten fields per case were analyzed at a magnification of 100x. The thresholds for collagen fibers were established for each slide, after enhancing the contrast up to a point at which the fibers were easily identified as birefringent (collagen) bands. The area occupied by the fibers was determined by digital densitometric recognition, by adjusting the threshold level of measurement up to the different color densities of the collagen fibers. The area occupied by the fibers was divided by the total area of the field. The results were expressed in percentage of the skin area fraction occupied by the collagen fibers.

#### Assessment of the mean of myofibroblasts for histological field (MF)

Myofibroblasts were detected by using a monoclonal antibody against the  $\alpha$ -smooth muscle actin antigen (clone 1A4; 1:200, 12 h, Dako, Glostrup, Denmark). After washing in PBS, slides were incubated with biotin-labeled antimouse secondary antibodies (Vector Laboratories Inc., Burlingame, CA), then washed in PBS, and incubated with peroxidase-labeled streptavidin (DAKO). The reaction products were visualized by immersing the slides in freshly prepared diaminobenzidine (Dojindo, Kumamoto, Japan). Ten histological sections (40x, 10 ocular, 0.739 mm<sup>2</sup> per field) were randomly selected and the mean of immunostained cells was assessed with an image analysis system Imagelab<sup>®</sup> (Soft-tium, São Paulo, SP, Brasil) as previously described by Ribeiro et al. (2009). Only spindle-shaped and round cells scattered in the connective tissue were regarded as myofibroblasts (Ribeiro et al., 2009).

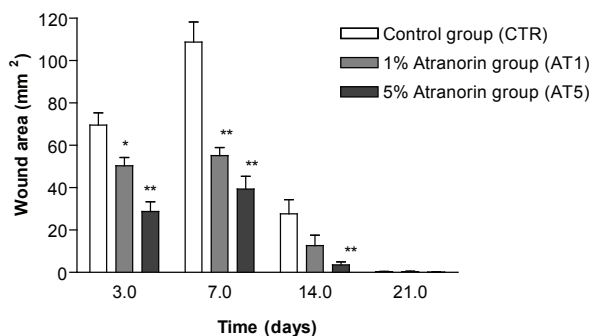
#### Statistical analysis

The histological analysis of the intensity of the inflammatory infiltrate was analyzed by Kruskal-Wallis test, followed by Dunnett's post-test. The epithelization rates and the mean of mast cells were compared among the groups by one-way analysis of variance (ANOVA) followed by Tukey's post test *p* values less than 0.05 were regarded as statistically significant.

## Results

### Analysis of the wound area

Topical application of both formulations based on atranorin decreased significantly the unhealed area on 3<sup>rd</sup> and 7<sup>th</sup> day following the wound, whereas on the 14<sup>th</sup> day later only AT5 provided significant reduction of the wound area (Figure 1). Twenty-one days later there was no difference among the groups (Figure 1).



**Figure 1.** Effect of the atranorin on wound area of rats. Value represents the mean  $\pm$  SEM. \**p*<0.05 and \*\**p*<0.01 when compared to control group. ANOVA followed by Dunnett's test (*n*=6, per group).

### Histological analysis

The intensity of the inflammatory response was severe in absolutely all cases of CTR, AT1 and AT5 at the third day. Besides, the leukocyte infiltrate was predominantly composed of neutrophils, characterizing an acute inflammatory reaction (Figure 2 a/b/c). On 7<sup>th</sup> day, despite the intensity of the inflammatory response had been still severe in all groups, the leukocyte infiltrate was predominantly acute in CTR, subacute in AT1 (rich in both polymorphonuclear and mononuclear inflammatory cells) and chronic (lymphocyte and plasma cell-rich) in AT5 group. In addition, the development of a delicate network of slit capillary vessels forming an immature granulation tissue was observed in both atranorin-treated groups, although in the AT5 it had been observed a conspicuous fibroblastic content in association with the vascular component (Figure 2 d/e/f). At the 14<sup>th</sup> day, the intensity of the inflammatory response was predominantly moderate and the lymphocytes and plasma cells were the most abundant leukocyte infiltrate so that it was classified as nonspecific chronic infiltrate in all groups. A remarkable content of large spindle-shaped cells interpreted as active fibroblasts, mainly in the bottom of the healing area, was also observed, particularly in AT5 group (Figure 2 g/h/i). Moreover, only AT5 group presented the formation of epithelial buddings interpreted as cutaneous appendages rudiments in the epithelial lining (Figure 2i). Finally, at the

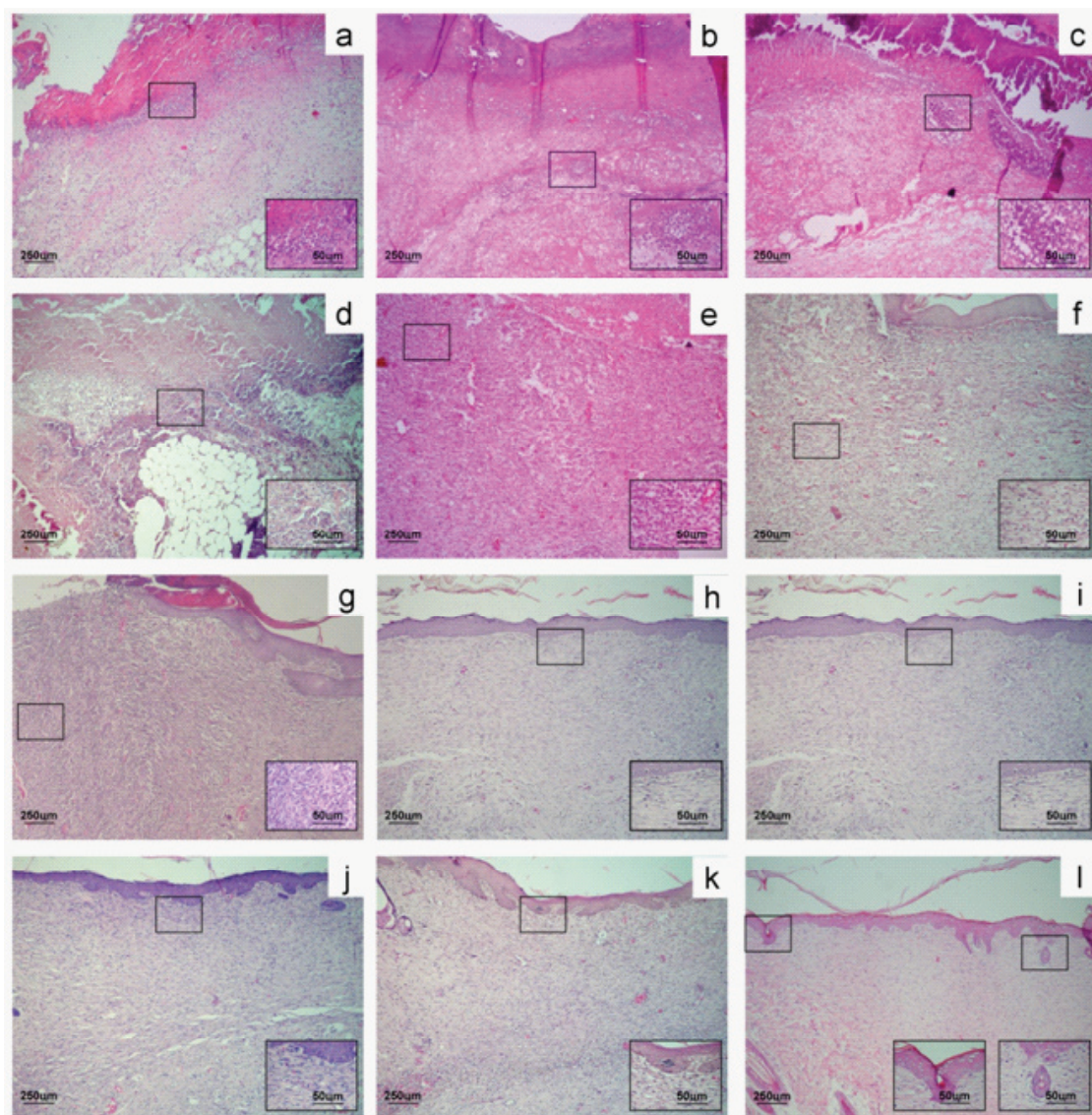


21st day, the inflammatory response was still regarded as moderate in both CTR and AT1 groups, but mild or absent in AT5 one. Plasma cells were the most abundant leukocyte infiltrate found in the connective tissue. Rudiments of cutaneous appendages were frequently found in CTR and AT1 groups, whereas in AT5 well-formed hairy follicles and sebaceous glands were observed within the collagenous scar (Figure 2 j/k/l).

As indicated in the Figure 3, epithelization was inconspicuous until the seventh day after surgery. The epithelization rates were significantly higher in AT1 (94.68%,  $p < 0.05$ ) and AT5 (98.8%,  $p < 0.01$ ) at the 14th day after surgery, but there was no difference among groups on

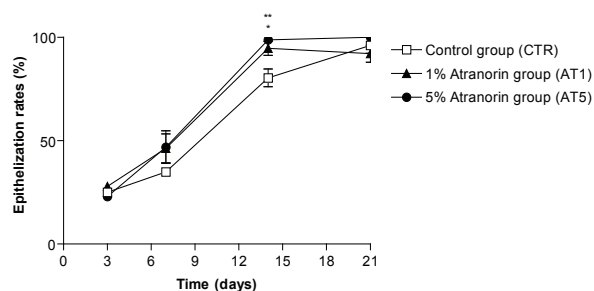
3<sup>rd</sup>, 7<sup>th</sup> and 21<sup>st</sup> day.

Regarding to the collagenization, only scant deposition of thin delicate reticularly arranged fibrils exhibiting greenish and yellow-greenish birefringence (type-III collagen) was observed in all groups three days following surgery. However, in AT1 and AT5 groups, the fibers appeared thicker and longer than in CTR (Figure 4 a/b/c). On the 7<sup>th</sup> day, there was remarkable improvement in the collagen fibers content, with clear reduction of the interfibrillary spaces, in AT1 and AT5 compared with CTR group. However, the newly formed fibrils sustained the yellow-greenish birefringence (type-III collagen molecules) and the reticular arrangement (Figure 4 d/e/f).

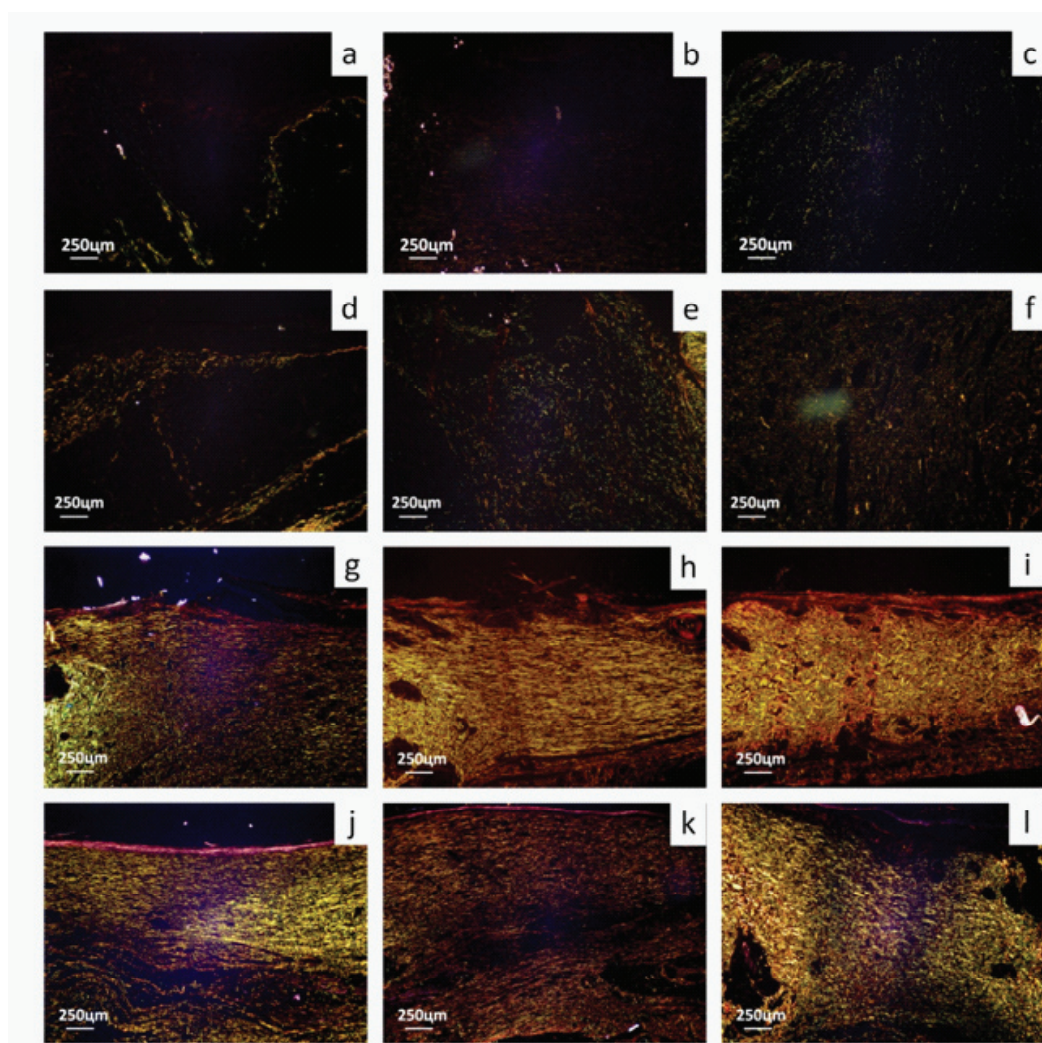


**Figure 2.** Effect of the atranorin on microscopic analysis of wounds in rats. Acute inflammation seen in CTR (d), subacute inflammation seen in AT1 (e) and chronic in AT5 (f) at the 7<sup>th</sup> day. Remarkable content of large spindle-shaped cells interpreted as active fibroblasts, mainly in the bottom of the healing area and formation of epithelial buddings interpreted as cutaneous appendages rudiments in the epithelial lining in AT5 (i). Moderate inflammatory response in CTR and AT1, but mild or absent in AT5 at the 21<sup>st</sup> day (j, k and l).

At the 14<sup>th</sup> day, there was a clear replacement of the delicate type-III collagen fibrils for gross and denser disposed type-I collagen fibers exhibiting orange birefringence. However, the content of collagen fibers was pointedly less conspicuous in CTR than in the other groups. Furthermore, despite AT1 and AT5 groups had presented high levels of collagenization, the pattern of the fibers arrangement observed in AT1 was predominantly parallel whereas in AT5 it was interlaced (Figure 4 g/h/i). Finally, at 21<sup>st</sup> day, CTR group presented intense deposition of gross thick parallel-arranged of both type I and III collagen bundles, less densely deposited in top of scars. The AT5 group showed the same mix of wavy and highly interlaced type I and type III collagen fibers, but the fibers seemed to be thinner and exhibited a dense interlaced arrangement that resembled the normal dermis (Figure 4 j/k/l).



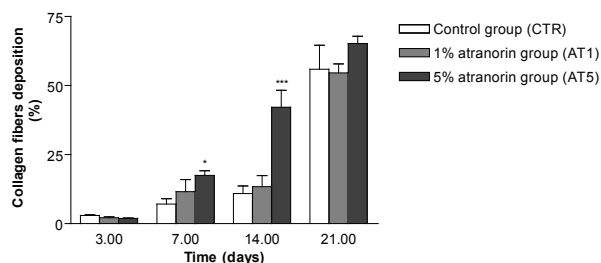
**Figure 3.** Effect of the atranorin in epithelization rates of excision wounds of rats. Value represents the mean±SEM. \* $p < 0.05$  and \*\* $p < 0.01$  when compared to control group. ANOVA followed by Dunnett's test ( $n=6$ , per group).



**Figure 4.** Effect of the atranorin on microscopic analysis of wounds in rats. Remarkable improvement in the collagen fibers, with clear reduction of the interfibrillary spaces, in AT1 and AT5 at the 7<sup>th</sup> day (e and f). High levels of collagenization with pattern of the fibers in parallel arrangement in AT1 (h) and interlaced in AT5 (i). Same mix of wavy and highly interlaced type I and type III collagen fibers, with the fibers less thick and exhibited a dense interlaced arrangement that resembled the normal dermis (l).

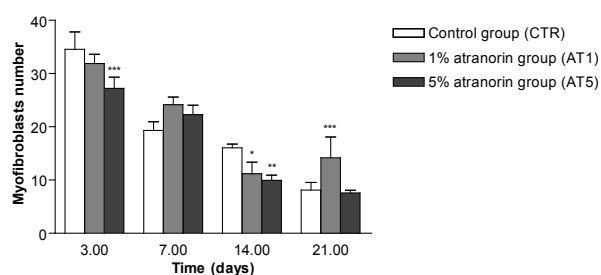


As described in Figure 5, the quantitative analysis of the collagen deposition revealed that AT5, but not AT1, induced significantly more expressive deposition of collagen fibers in 7 ( $p=0.02$ ) and 14 days ( $p=0.000$ ). However, no difference among the groups was observed in 3 ( $p=0.93$ ) and 21 days ( $p=0.64$ ).



**Figure 5.** Effect of the atranorin in collagen fibers deposition of excision wounds of rats. Value represents the mean $\pm$ SEM. \* $p<0.05$ , \*\* $p<0.01$  and \*\*\* $p<0.001$  when compared to control group. ANOVA followed by Dunnett's test ( $n=6$ , per group).

Regarding the MF, A-SM positive myofibroblasts were seen as brown spindle-shaped cells scattered in the connective tissue. On the 3<sup>rd</sup> day, the myofibroblasts content was significantly lower in AT5 than in CTR ( $p<0.001$ ). No significant difference was verified among the groups at the 7<sup>th</sup> day ( $p=0.058$ ). On the 14<sup>th</sup> day, the content of myofibroblasts was significantly higher in CTR than in AT1 and AT5 ( $p<0.05$  and  $p<0.01$ , respectively). However on 21<sup>st</sup> day, the myofibroblasts content was significantly higher in AT1 compared to CTR ( $p<0.001$ ) and AT5 ( $p<0.001$ ) (Figure 6).



**Figure 6.** Effect of the atranorin in myofibroblasts content of excision wounds of rats. Value represents the mean $\pm$ SEM. \* $p<0.05$ , \*\* $p<0.01$  and \*\*\* $p<0.001$  when compared to control group. ANOVA followed by Dunnett's test ( $n=6$ , per group).

## Discussion

The present study demonstrates a novel substance with an important role on wound healing. In this work, we evaluated the skin wound healing process after excision that had been treated with different doses of atranorin, a natural product extracted from *Cladonia kalbii*. Our results showed that atranorin-treated animals presented reduced wound areas, earlier granulation tissue formation, higher

cell proliferation and improved collagenization when compared to control animals.

During the healing process, the inflammatory response should occur rapidly to permit the development of subsequent phases of wound healing. Therefore, despite inflammatory response is absolutely required to provide wound healing, its long-term persistence has been considered one of the most important reasons for the delay of healing process (Clark, 1996). The severe acute inflammatory reaction observed in all the three groups on the 3<sup>rd</sup> day was expected, since soon after injury, as a result of vascular and biochemical changes, a substantial amount of neutrophils migrate into the wound to prevent the invasion and proliferation of microorganisms (Harlan, 1985). However, atranorin administration seems to reduce the content of neutrophils in the wound area of rats treated at the 7<sup>th</sup> day when compared to control rats, facilitating the installation of the chronic inflammatory phase. The positive effect of atranorin on the inflammatory response might be related to the atranorin-induced inhibition of cyclooxygenase (COX-1 and COX-2) activity (Bugni et al., 2009) and blockade of the biosynthesis of leukotriene B4 (Kumar & Müller, 1999), which could lead to an antiinflammatory effect. Atranorin also presented antibacterial and antioxidant properties, which could also contribute to reduce the chemotactic signaling, and consequently the neutrophilic influx into the injured area (Mahmood et al., 2005). Moreover, the fact that the reduction of the neutrophilic infiltrate was more expressive in AT5 than in AT1 group may suggest that the biological effect of this product on the acute inflammatory response is dose-dependent. Notwithstanding, further studies are demanded to ratify this possibility.

The similar inflammatory response among groups on the 14<sup>th</sup> and 21<sup>st</sup> day was expected, since in this periods the latter events of the healing process, such as fibroblastic proliferation and collagen synthesis, had already installed as a result of progressive decrease in the chemotactic stimuli and consequent reduction in the leukocytic infiltrate characteristic of the natural course of the healing process (Boyle et al., 1987).

Epithelization is a process of restoring the epidermis and involves proliferation and migration of keratinocytes (Li et al., 2007). As keratinocytes are supposed to be a source of a variety of cytokines involved in remodeling the collagen fibers deposited at the final stages of the cicatricial repair, the development of epithelial lining is considered a relevant step of wound healing (Balasubramanian & Eckert, 2007). Furthermore, the epithelial bridge is also responsible for the removal of scab by dissolution of its attachments to the underlying connective tissue (Diegelmann & Evans, 2004). In this study, atranorin administration seemed to improve the epithelization during the complete experimental period, although only on the 14<sup>th</sup> day this improvement had

shown to be significant. Surprisingly, previous studies have reported that atranorin presented no effect on the keratinocytes proliferation *in vitro* (Kumar & Müller, 1999).

Nevertheless, it must be remembered that during the formation of the granulation tissue, a wide sort of soluble factors involved in the modulation of keratinocytes proliferation is released into the healing area, including PDGF (Greenhalgh et al., 1990), TGF- $\beta$ 1 (Roberts et al., 1986), EGF (Buckley et al., 1985), and others (Cross & Mustoe, 2003). Therefore, as long as atranorin administration accelerated the granulation tissue formation, the histological findings regarding the epithelization might result of indirect effects associated to such growth factors. Although other still unknown biological processes are likely to be involved in the epithelization observed in this study, the fact that the significant improvement had occurred between 7<sup>th</sup> and 14<sup>th</sup> day seems to reinforce the role played by the granulation tissue-associated growth factors, since this is the period for skin second-intention cicatricial repair in which the most substantial content of such soluble constituents is released into the wound healing area (Werner & Crose, 2003). It must be emphasized that previous studies have already demonstrated that a wide range of natural products can be applied to modulate the kinetics of epithelial cells growth, either down-regulating (Valacchi et al., 2009) or stimulating the keratinocyte proliferation (Nunes et al., 2011).

In normal tissues, collagen fibers provide strength, integrity, and structure; therefore, when tissues are disrupted following an injury, collagen deposition is essential for replacing the lost tissue and restoring anatomical structure and function (Diegelmann & Evans, 2004). A clear improvement in the content of collagen fibers in the atranorin-treated groups was verified in this study. However, the mechanisms responsible for this beneficial effect are unclear. It is possible that the role played by atranorin in the dynamics of collagenization may be related to an upregulation of nitric oxide synthesis. The administration of exogenous nitric oxide has been proved to improve fibroblasts proliferation and collagen synthesis in rodent model (Schaffer et al., 2007). Additionally, some lichen metabolites have been recently implied in the positive modulation of the nitric oxide release in mouse macrophages (Carlos et al., 2009). Thus, we speculate whether atranorin might upregulate the fibroblastic proliferation, deposition of collagen fibers, and wound contraction. However, further investigations are required to clarify this mechanism and prove this theory right.

The deposition of delicate type-III collagen fibrils on 3<sup>rd</sup> and 7<sup>th</sup> day found in this study was expected, since these protein molecules are extensively required to provide spatial orientation to angioblasts migration in order to form the granulation tissue during the very early stages of wound healing (Ramos & Miranda, 2007). Once the granulation

tissue is fully developed, endothelial growth orientation is no longer required, thereby evoking the replacement of the type-III for type-I collagen, a molecule responsible for the tensile strength and mechanical stability of the connective tissue (Rich & Whittaker, 2005). Thus, as long as this collagen substitutive phenomenon was observed in this study, the suitable progression of the healing process was attested.

Although the replacement of a substantial part of the content of type-III collagen for type-I molecules during the healing process is an absolutely expected phenomenon (Rich & Whittaker, 2005), the excessive production of type-I collagen might easily lead to the formation of undesirable hypertrophic scars and keloids (Sandulache et al., 2007; Verhaegen et al., 2009). On the other hand, the moderate content of less thick collagen fibers in AT5 on 21<sup>st</sup> day seems to provide low probability of keloid development. Besides, the pattern of arrangement and balance in the content of both type-I and -III collagen fibers as seen in AT5 may suggest that the remodeling phase of the scar, represented by degradation of the gross connective matrix formed and gradual and progressive deposition of a new depurated matrix rich in both collagen molecules (Junqueira et al., 1983), is highly advanced in comparison to the other groups. These findings could justify the clear resemblance with the normal histological appearance of the dermal collagen. It must also be stressed that since the biological effects of atranorin on fibroplasia dynamics seem to be related to a possible increase in the fibroblast metabolism, both synthesis and degradation of collagen molecules were supposed to be stimulated, as observed in this study. Thus, further investigations are required in order to fully clarify the precise mechanism of the healing modulation pathways provided by this lichenic constituent.

The assessment of CF provided evidences that the 5% atranorin ointment might also improve the collagen synthesis. Despite the over production of collagen may result in hypertrophic scars (Nunes et al., 2011), this pathological phenomenon was not observed in this study. This may suggest that the modulatory effects of the atranorin ointment were limited to the middle-stages of wound healing, not affecting the dynamics of the collagen remodeling phase. In addition, the data obtained after descriptive and quantitative analysis of the collagen content in AT5 seem to be complementary, as long as point to a possible role played by atranorin on the fibroblasts metabolism.

Myofibroblasts are a cell type involved in wound contraction. These cell subsets present a contractile phenotype characterized by a cytoskeleton rich in actin microfilaments, and they can be identified by immunohistochemistry due to their extensive positivity for  $\alpha$ -SMA (alpha smooth muscle actin) (Van Beurden et al., 2005; Ribeiro et al., 2009). Therefore, myofibroblastic

differentiation is supposed to be a crucial event leading to a suitable healing of larger wounds, which have more extensive loss of cells and tissue (Hinz et al., 2007; Ribeiro et al., 2009). In this study, the application of atranorin ointment might have provided fibroblastic transformation into myofibroblasts at the early stages of the wound healing process. Notwithstanding, at 21<sup>st</sup> day, the number of myofibroblasts decreased, most likely due to apoptosis, and scar tissue formed. On the other hand, in AT1, the healing process appeared to be considerably slower, so that the process of myofibroblastic apoptosis apparently had not yet taken place. However further studies are necessary to clarify if this cell phenotype transformation is a direct effect of the atranorin, or a result of the release of differentiating factors by other cells involved in the healing process.

It must be emphasized that several of the  $\alpha$ -SMA positive cells observed in this study were disposed surrounding the newly formed blood vessels, and some of them appeared to be detaching from the capillaries and venules, and they were identified as pericytes (Medrado et al., 2010). The participation of pericytes during wound healing has already been described, and currently they are supposed to work as reserve cells, since their potential to differentiate into osteoblasts, chondrocytes, fibroblasts, leionocytes and lipoblasts has been previously studied (Farrington-Rocket et al., 2004). Despite there is a close resemblance in the phenotype of myofibroblasts and pericytes, their true relationship and association with the healing process is in need of further investigations.

Surprisingly, the myofibroblasts data provide no explanation for the enhanced wound contraction. Despite TGF- $\beta$ -mediated transformation of fibroblasts into myofibroblasts is one of the most important biological phenomenon related to wound contraction, other mechanisms may be involved in this step of the healing process, such as superficial topography of wound. It is well-established that the mechanical strain exerted on collagen fibers orientated in improper angulations, generates deleterious internal forces, which induces the development of piezoelectric current in the wound site (Farahani et al., 2008). On the other hand, the dominance of external forces, to the detriment of such damaging internal ones, in the wounded milieu seems to provide parallel orientation of collagen fibers in relation to the skin surface. This external force dominance minimizes the induction of such piezoelectricity and up-regulates the contractile activity of the previously wounded tissue (Farahani & Kloth, 2008). Therefore, it is possible that this alternative biological process could have been responsible for the wound contraction data observed in this study, but further investigations are necessary in order to clarify this issue.

Taken together, the results presented herein strongly suggest that atranorin (AT) extracted from

*Cladina kalbii* Ahtimodulates the wound healing process in the model of excision wounds. Moreover, we can assert that atranorin was successful in improving certain steps of second-intention wound healing, such as inflammatory profile, epithelization and collagen formation. The precise mechanisms through which AT exerts its action are currently under investigation, but they might be related to the arachidonic acid cascade and/or modulation of pro-inflammatory molecules production.

### Acknowledgements

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### Authors' contributions

RSSB, RLCAJr, AASA and LJQJr designed the research; RSSB, RNPF, JSSQ and ASB contributed with wound healing protocols; RSSB and RNPF performed histological sections stained by HE and Sirius-red; RSSB, JSSQ, JMS, MRVS and VJSF contributed with the immunohistochemical protocols; AASA, LJQJr and LRB contributed essential substance, the atranorin and the atranorin ointment; RSSB, RLCAJr and LJQJr analyzed the data and wrote the paper. All authors read and approved the final manuscript.

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