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SEÇÃO: ARTIGOS DE PESQUISA

The molecular mechanism underlying in vitro wound healing property of Stryphnodendron astringent (Mart.)

Mecanismo molecular subjacente à propriedade de cicatrização de feridas in vitro do Stryphnodendron adstringens (Mart.)

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Abstract

Aim: to analyze the mechanism of the hydroalcoholic extract of barbatimão in the induction of healing in dermal fibroblast cells (HFF-1), in an in vitro model.

Methods: two concentrations of barbatimão used in commercial ointments were studied here (B1 = 0.49 mg/mL; B2 = 0.99 mg/mL), and their effects were

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evaluated with the following markers at 24 and 72 hours after induction of the in vitro wound: (1) oxidative metabolism [DNA oxidation, lipoperoxidation, protein carbonylation, antioxidant enzymes (superoxide dismutase, SOD; catalase, CAT; glutathione peroxidase, GPX)]; (2) pro-inflammatory cytokines (TNF-, interleukin 6); (3) apoptotic markers [caspase 3 (CASP3), CASP8, and growth factors: keratinocyte growth factor (KGF) and fibroblast growth factor-1 (FGF-1)].

Results: treatment with both concentrations showed a decrease in pro-oxidant, pro-inflammatory, and pro-apoptotic markers, while increasing antioxidant levels and growth factors.

Conclusions: barbatimão acts through a pleiotropic cascade mechanism involving antioxidant, anti-inflammatory, anti-apoptotic, and fibroblast migration/proliferation modulation. Our data could explain the effect and importance of barbatimão extract in wound healing mechanisms.

Keywords: polyphenols, wound healing, antioxidant, anti-inflammatory.

Resumo

Objetivo: analisar o mecanismo do extrato hidroalcoólico de barbatimão na indução da cicatrização em células fibroblastos (HFF-1), em um modelo *in vitro*.

Métodos: foram estudadas duas concentrações de barbatimão utilizadas em pomadas comerciais (B1 = 0,49 mg/mL; B2 = 0,99 mg/mL), e seus efeitos foram avaliados com os seguintes marcadores às 24 e 72 horas após a indução da ferida *in vitro*: (1) metabolismo oxidativo [oxidação do DNA, lipoperoxidação, carbonilação de proteínas, enzimas antioxidantes (superóxido dismutase, SOD; catalase, CAT; glutationa peroxidase, GPX)]; (2) citocinas pró-inflamatórias (TNF-, interleucina 6); (3) marcadores apoptóticos [caspase 3 (CASP3), CASP8, e fatores de crescimento: de queratinocítico (KGF) e de de fibroblastos-1 (FGF-1)].

Resultados: o tratamento com ambas as concentrações mostrou diminuição nos marcadores pró-oxidantes, pró-inflamatórios e pró-apoptóticos, enquanto aumentava os níveis de antioxidantes e fatores de crescimento.

Conclusões: o barbatimão atua por meio de um mecanismo pleiotrópico em cascata que envolve a modulação antioxidante, anti-inflamatória, anti-apoptótica e de migração/proliferação de fibroblastos. Nossos dados poderiam explicar o efeito e a importância do extrato de barbatimão nos mecanismos de cicatrização de feridas.

Palavras-chave: polifenóis, cicatrização de feridas, antioxidante, anti-inflamatório.

Introduction

Brazil offers several medicinal plants with potential wound-healing properties, which includes *Stryphnodendron adstringens* (Mart.) (1–5). This therapeutic plant is commonly referred to as barbatimão (6). Stem barbatimão bark has traditionally been used for wound healing by the Brazilian's Cerrado biome inhabitants. Chemical characterization of barbatimão extracts has revealed high phenolic and tannin contents in their secondary metabolites which include gallic acid, caffeic acid, alkaloids, terpenes, flavonoids, steroids, and several catechins such as epigallocatechin-gallate and robinetinidol (6–9) have also detected rutin, quercetin, and kaempferol molecules in the hydroalcoholic extracts of barbatimão. Besides all these properties, this plant also presents antioxidants, antiulcerogenic, antitumor properties, and antibacterial activity against Gram-positive and Gram-negative bacteria (5,10,11).

Specific commercial products are manufactured using barbatimão extract and prescribed for treating wounds, mainly for healing scars in elderly and/or bedbound patients. Considering the prevalent use of barbatimão as a wound-healing plant, the number of studies on the safety and efficacy of barbatimão has increased recently. Accordingly Pellenz et al. (12)Mart. analyzed the potential *in vitro* toxic effects of barbatimão plant extract on human keratinocytes (HaCAT) and fibroblasts (HFF-1) in commercial cell lines. They found that hydroalcoholic extract of barbatimão at concentrations of 0.49 mg/mL and 0.99 mgCASP/mL, like the pharmacological levels, demonstrated genoprotective activity in these cells. Moreover, decreasing levels of DNA oxidation (oxDNA) were evaluated by quantifying 8-hydroxy-2'-deoxyguanosine reactive oxygen species (ROS), and apoptotic caspase (CASP) (caspase 3 and caspase 8) levels. However, the underlying mechanisms associated with barbatimão wound healing properties remain unclear.

Thus, the present investigation analyzed the barbatimão healing mechanism using an *in vitro* wound healing scratched model in fibroblast cells. Fibroblast cells were chosen due to their crucial role in wound healing. During the proliferative phase, these cells migrate from various sources to the wound site, thereby contributing to granulation tissue formation. This results in the development of a new extracellular matrix, followed by the closure of the wound (13). The analysis performed in this study evaluates the altering effects of barbatimão on oxidative, inflammatory, and growth factor molecules in scratched fibroblasts.

Methods

Chemical characterization of barbatimão hydroalcoholic extract used here was according to Pellenz et al. (12)Mart. using the High-performance liquid chromatography method and standardized to 14.48±0.05 mg/g gallic acid: 8.06±0.02 mg/g caffeic acid, 8.16±0.04 mg/g quercetin, 5.93±0.01 mg/g total catechin, and 4.71±0.01 mg/g rutin. The stimulatory effect of the hydroalcoholic extract of barbatimão was tested using two concentrations (0.49 mg/mL and 0.99 mg/mL) in scratched fibroblast cultures. These concentrations were according to the Brazilian pharmaceutical levels used in phytotherapeutic commercial ointments. Untreated cells were considered the negative control group (C), whereas cell cultures exposed to 0.49 mg/mL and 0.99 mg/mL of barbatimão extract were identified as B1 and B2, respectively.

Initially, the effect of B1 and B2 extracts on cell migration was evaluated in the scratched fibroblast cultures. The scratched cell cultures were analyzed at 0h, 12h, 24h, and 72h for each treatment. The result was expressed as relative cell migration, calculated by dividing the percent change in the scratch area of the barbatimão-treated cells at 12h, 24h, or 72h compared to the scratch area at 0h in each experiment.

Subsequently, the significance of barbatimão was evaluated using an in vitro model by analyzing the modulation of various signaling molecules that contribute to wound healing at 24 h and 72 h in scratched fibroblast cultures. We focused on the following markers: (a) Oxidative markers: studies have shown that mitochondria generate elevated ROS levels in fibroblasts, which are essential regulators of wound healing mechanisms (14). Thus, we analyzed the effects of B1 and B2 on oxDNA, lipoperoxidation, protein carbonylation (PCarb), and on the modulation of primary antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX). (b) Proinflammatory cytokines: tumor necrosis factor-alpha (TNF-) and interleukin 6 (IL-6) are expressed by fibroblasts during wound healing. In chronic wounds, these elevated proinflammatory cytokines inhibit wound healing and contribute to non-closure mechanisms (15-17). Thus, any modulation in TNF- and IL-6 levels by hydroalcoholic extract of barbatimão can be considered a potential underlying mechanism in its wound healing activity. (c) Growth factors: fibroblast growth factor 1 (FGF-1) and keratinocyte growth factor (KGF) were also evaluated in this study. Data from 24 h cell cultures were calculated according to the unit measures of each variable analyzed in this study, while data from 72 h cell cultures was initially standardized to % of the control group, and results are expressed as % mean ± standard deviation of the control group.

Commercial HFF-1 cells were obtained from the American Type Culture Collection via Rio de Janeiro Cell Bank, a non-profit organization. HFF-1 aliquots were obtained with a certificate of analysis indicating that the cells were free of mycoplasma or any other contamination by microorganisms. Cells were cultured in the standardized condition in Dulbecco's modified Eagle medium (DMEM) culture medium supplemented with 15% fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin. Cells were maintained at 37˚C with 5% CO_2 and 95% humidified atmosphere. The culture medium was supplemented with hydroalcoholic extract of barbatimão that was filtered with sterile filter for 'Pes' syringe with pore 0.22uM before use to avoid the presence of microorganisms.

Migration of fibroblast cells in the scratched region of culture at 12h and 24h was evaluated with and without barbatimão supplementation. The effect of barbatimão on fibroblast's wound healing response was determined by scratch wound healing assay as performed in previous studies by Nicolaus et al. (18) with minor modifications. Briefly, the cells were seeded at a density of 1×10^4 cells/well in a 24-well culture plate and incubated overnight. After the incubation, the Dulbecco's modified Eagle medium culture medium was aspirated, and the adherent cell layer was scratched with a sterile 200 µL pipette tip. Further, cell debris was removed by rinsing with Phosphate-Saline Buffer (PBS). The complete medium with and without the hydroalcoholic

extract of barbatimão was then added to each well. The image of the scratched area was captured under bright field microscopy (20×), and the migration was analyzed using Digimizer software (version 5.3.4), which allowed precise manual measurements and automatic object detection with measurements of cell culture characteristics. The wound area was measured using controller software.

The following molecules were quantified by immunoassay using Simple Step ELISA® Quantikine Human Kits (ABCAM; Cambridge, MA, USA): 8-hydroxy-2'-deoxyguanosine (to detect oxDNA), SOD, CAT, GPX, TNF-, IL-6, FGF-1, and KGF. The assay was performed with cell culture supernatant according to the manufacturer's instructions and as previously described by Barbisan et al. (19).

All reagents and working standards were prepared, and the excess microplate strips were removed. Then, 50 µL of assay diluent (RD1W) was added to each well followed by 100 µL of each standard. After that, 100 µL of sample and 1X Control Solution were added to the appropriate cells. Further, 50 µL of appropriate antibody was added and incubated for 3h at room temperature (18-25˚C). The liquid from each well was aspirated and rinsed with 300 µL of Wash Buffer. Further, 100 µL of substrate solution was added to each well and incubated in the dark for 12-15 minutes at room temperature, followed by 100 µL of Stop Reagent to each well. Results were recorded immediately or within one hour (when stored in the dark at 2-8˚C) after the addition of the Stop Reagent. The absorbance of each well was measured on a spectrophotometer at 450 nm.

Considering that oxidative stress can damage cell membranes, thiobarbituric acid reactive substances (thiobarbituric acid reactive substances) were measured to evaluate lipid peroxidation following the protocol described by Jentzsch et al. (20). Carbonylation triggers fragmentation of protein chains and causes oxidation of major kinds of amino acids with excess production of carbonyl compounds. These damages were measured by detecting the formation of carbonyl groups based on the reaction with dinitrophenylhydrazine according to the method described by Morabito et al. (21).

The results of the treatment were statistically compared using GraphPad Prism Software (version 8.4.3). All experiments were performed in triplicates with a minimum of five repetitions for each treatment. The data obtained from the treatments used in this study was used in the in vitro analysis to compare with those obtained from other reported studies (22). Outliers were eliminated, and only upper and lower values of

standard deviation were considered for analysis. Results of the treatment were compared by one-way analysis of variance (ANOVA) followed by the Tukey post hoc test. A statistically significant difference was considered at $p < 0.05$.

Results

The effect of the hydroalcoholic extract of barbatimão on fibroblast migration was evaluated by analyzing the scratched area in the culture. A captured image of the scratch area at 0h is shown in **Figure 1A**, while **Figure 1B** shows fibroblast migration at 12h. **Figure 1C** shows the scratched area at 72h in serum-free control fibroblasts with lower cell concentration than in barbatimão treated cells (0.49 mg/mL) (**Figure 1D**). Hydroalcoholic extracts of barbatimão at both concentrations (0.49 mg/mL and 0.99 mg/mL) revealed an increase in fibroblast migration compared to the migration rate observed in the SF control group (**Figure 1E**).

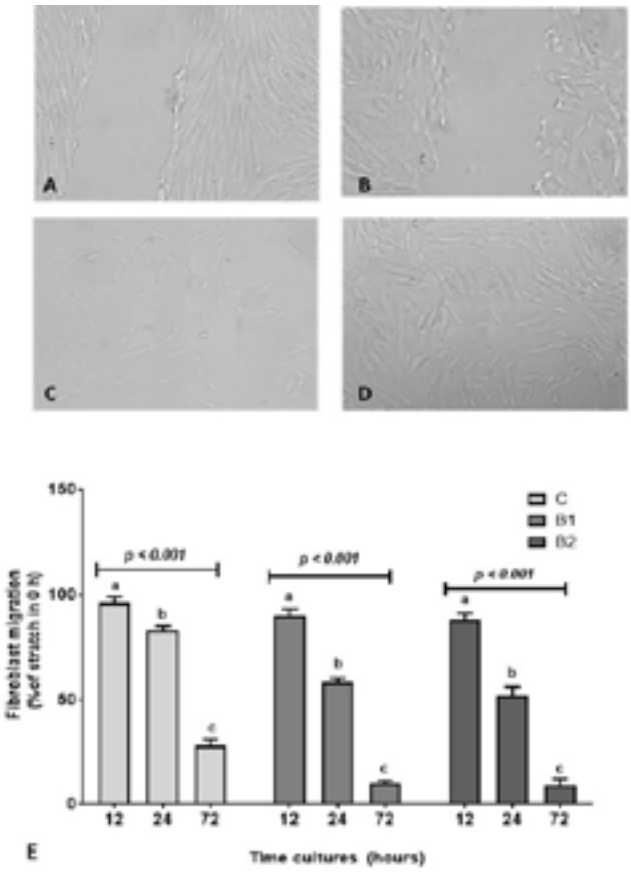

Figure 1. Analysis of fibroblast migration using scratch wound healing assay. Time in hours (post-scratch). (A) Scratched area (S) of fibroblast culture; (B) fibroblast migration at 12 h in S of fibroblast culture; (C) fibroblast migration at 24 h in control untreated culture; (D) fibroblast migration in B1-treated cells at 24 h. (E)fibroblast migration (% of wound healing from time point 0 h). Control (C); barbatimão hydroalcoholic extract at (B1) 0.49 mg/mL and (B2) 0.99 mg/mL. Time period of each treatment to heal the wound was compared by One-Way analysis of variance followed by Tukey post hoc test. Statistically significant differences are denoted by different letters corresponding to each treatment (a, b, c).

The effect of barbatimão on prooxidant and enzymatic antioxidant molecules was also evaluated (**Figure 2**). On comparing different time points within the control group, 72 h post scratch culture showed higher oxDNA (125±3.2 %), lipoperoxidation (112.2±2.8%), and PCarb (118.5±3.4%) compared to their respective measurements in 24 h post scratch culture ($p < 0.001$). On the contrary, significantly lower levels of SOD (91.7±2.3 %), CAT (86.1±4.4%), and GPX (68.9±4.3%) were observed at 72 h compared to their observed values in control cells at 24 h post scratch ($p < 0.001$). On comparing markers of oxidative metabolism, it was revealed that barbatimão treated cells showed a dose-dependent response. Overall, B1 and B2 treatment showed significantly lower levels of oxDNA, lipoperoxidation, and PCarb compared to their respective values in untreated control scratched fibroblasts at a given time point ($p<0.001$). On the contrary, B1 and B2 showed higher antioxidant enzyme levels than those in the control group ($p< 0.001$). Further, it was observed that the effect of barbatimão was dose-dependent on its concentration. Thus, our results suggest that wound healing induced oxidative stress conditions in dermal fibroblasts. However, oxidative stress was attenuated on treating fibroblasts with hydroalcoholic extract of barbatimão.

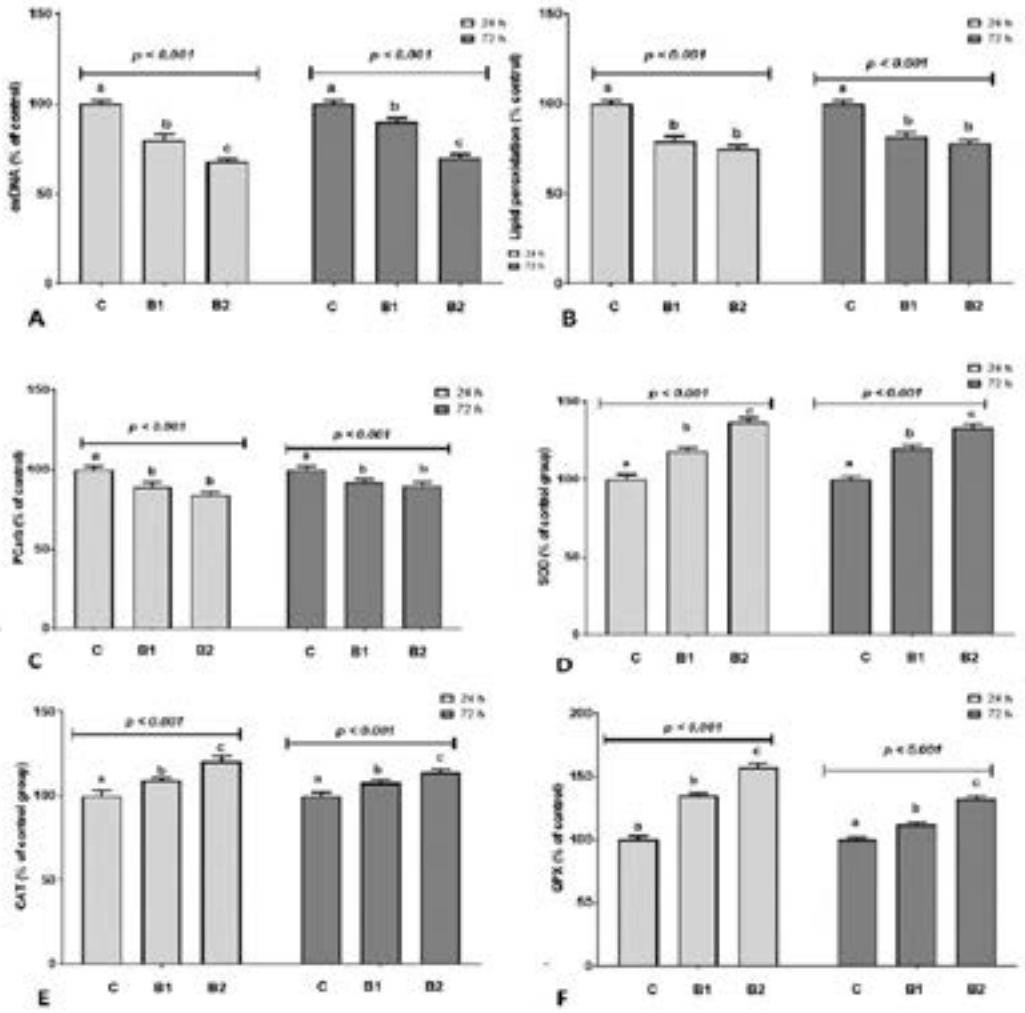

Figure 2. Comparison of prooxidant and antioxidant markers in control cells (C) and barbatimão hydroalcoholic extract treated cells at two different concentrations (B1 = 0.49 mg/mL; B2= 0.99 mg/mL) at 24 h and 72 h post scratch culture. OxDNA was quantified by 8-hydroxy-2-deoxyguanosine assay. Lipoperoxidation was quantified by thiobarbituric acid reactive substances-malondialdehyde (TBARS-MDA) assay. Protein carbonylation (PCarb), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX). Statistical comparison was evaluated by two-way analysis of variance followed by Tukey post hoc test. Statistically Significant differences are denoted by different letters corresponding to each treatment (a, b, c).

Initially, the consequence of scratch on expression levels of proinflammatory cytokines such as TNF- and IL-6 was evaluated in the control group. It was observed that TNF- as well as IL-6 were highly expressed in the control group at 72 h post scratch compared to their expression levels at 24 h post scratch (TNF- = 110.1±2.8%; IL-6 = 118.8±4.7%) ($p < 0.001$). Comparing the cytokine expression in control with the cytokine expression in B1 and B2 treatments (**Figure 3**) revealed that B1/B2 treated cells expressed lower levels of these proinflammatory cytokines at 24 h and 72 h post scratch. On evaluating CASP3 and CASP8 levels, it was revealed that the control group showed higher expression levels of caspase at 72 h compared to caspase expression at 24 h post scratch (CASP3 = 140.0±3.3%; CASP8 = 128.1±4.5%). The expression levels of these apoptotic molecules were significantly lower at 24 h and 72 h post scratch in B1/B2 treated cells compared to the expression levels of the untreated control group at the same time point (Figure 3). Both 24

h and 72 h scratched cell cultures showed that B1 and B2 significantly decreased the expression levels of proinflammatory cytokines (TNF-, IL-6) compared to their expression levels in the control group. Similarly, lower concentration of apoptotic molecules such as CASP3 and CASP8 was also observed at 24 h and 72 h in barbatimão-treated cells. On the other hand, B1 and B2 revealed higher FGF-1 and KGF levels in scratched cell cultures at 24 h and 72 h. The two investigated growth factors, induced by the wound healing mechanism, showed higher levels at 72 h compared to their expression level at 24 h post scratch in the untreated control group (FGF-1 = 138.1±3.4%, KGF = 115.5±2.1%). B1 and B2 induced higher levels of growth factors in a dose-dependent way at 24 h and 72 h compared to the expression pattern of growth factors in the control group.

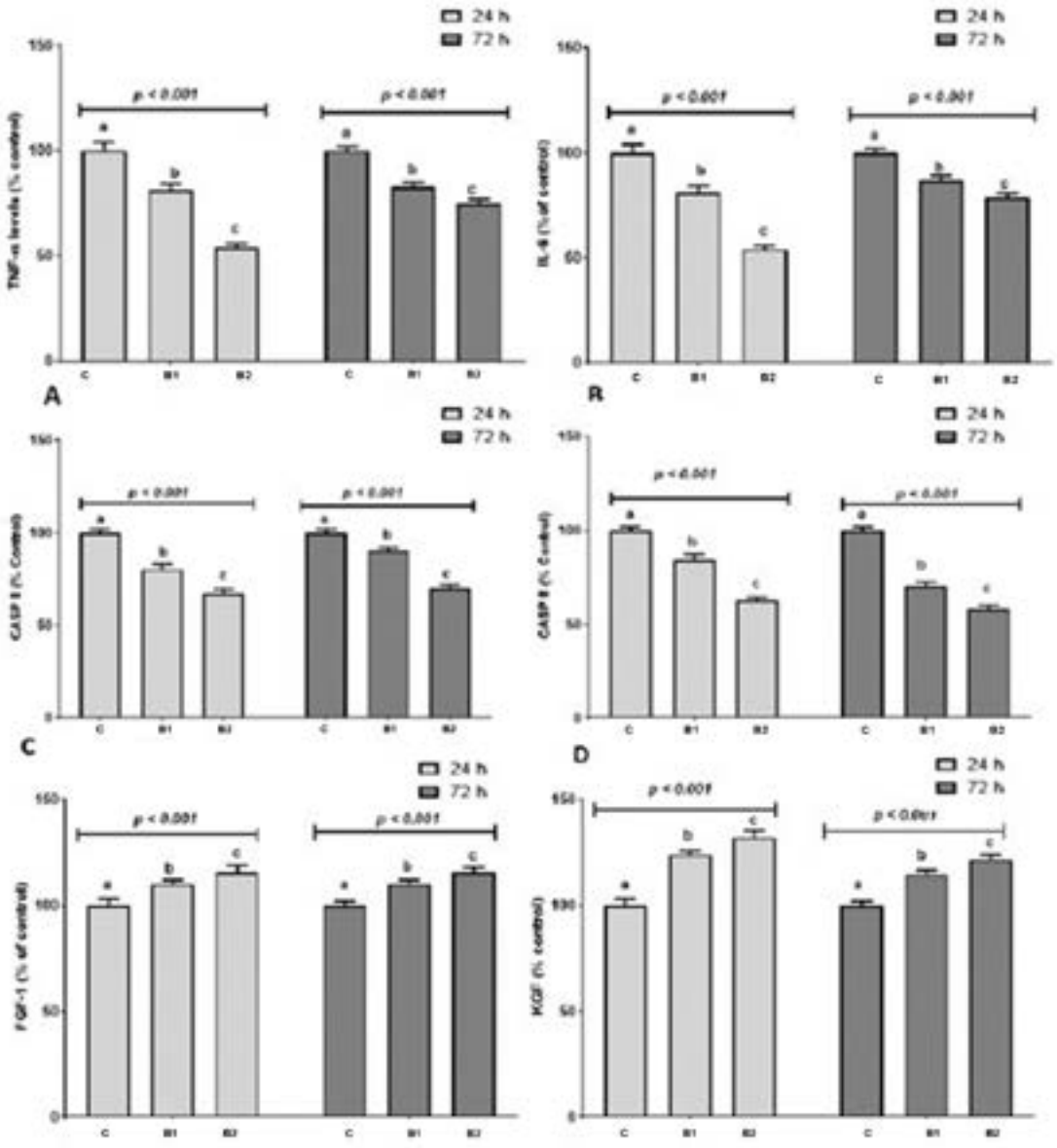

Figure 3. Comparison of proinflammatory cytokines (TNF-, IL-6), apoptotic markers (CASP3, CASP8) and growth factors (FGF-1, KGF-1) in scratched fibroblasts at 24 h and 72 h in untreated control (C) group and barbatimão hydroalcoholic extract treated cells at two different concentrations (B1 = 0.49 mg/mL, B2= 0.99 mg/mL). Statistical comparison was analyzed by two-way analysis of variance followed by Tukey post hoc test. Statistically significant differences are denoted by different letters corresponding to each treatment (a, b, c).

Discussion

The present study described the potential pleiotropic effect of hydroalcoholic extract of barbatimão on *in vitro* dermal fibroblasts using scratch wound healing assay. In the presence of B1 and B2 treatments, scratched fibroblast cultures showed lower levels of prooxidant, apoptotic, and proinflammatory markers while higher levels of antioxidant enzymes and growth factors such as FGF-1 and KGF.

Wound healing is a complex process involving multiple steps and regulatory molecules produced mainly by fibroblasts, keratinocytes, and immune cells. Conventionally, the process is divided into four phases which are connected: (a) Hemostasis phase, (b) Inflammatory phase, (c) Proliferative phase, and (d) Maturation and remodeling phase (23)requiring a coordinated response by immune cells, hematopoietic cells, and resident cells of the skin. We review the classic paradigms of wound healing and evaluate how recent discoveries have enriched our understanding of this process. We evaluate current and experimental approaches to treating cutaneous wounds, with an emphasis on cell-based therapies and skin transplantation.","container-title":"Science","-DOI":"10.1126/science.1253836","ISSN":"0036-8075, 1095-9203","issue":"6212","journalAbbreviation":"Science","language":"en","page":"941-945","source":"-DOI.org (Crossref. Several studies have shown that natural products can be beneficial in wound healing due to their characteristics of being anti-inflammatory, antioxidant, antibacterial, and inducing procollagen synthesis (24).

Such effects can be associated with specific polyphenol molecules that are also found in the chemical matrix of barbatimão. For example, Li et al. (25) described that epigallocatechin--gallate can enhance the therapeutic effects of mesenchymal stem cells in skin wound healing. Moreover, some other studies have associated wound healing with other chemical molecules present in the barbatimão extract (12)Mart. such as gallic acid (26) and caffeic acid (27). Thus, it can be concluded that bioactive molecules present in the hydroalcoholic extract of barbatimão are directly responsible for the significant effects on the scratched fibroblast cells analyzed in this study.

It is believed that the increased expression of proinflammatory cytokines, as well as ROS levels in wound healing, is limited to the immune cells. However, dermal fibroblasts were also found to contribute to elevating the cytokine and ROS levels. As dermal fibroblasts are a group of heterogeneous and distinct cells, they also regulate inflammation by interacting with immune and endothelial cells and participate in cell proliferation (28, 29). In *vitro* studies have proved that scratched fibroblast cell cultures show increased pro-inflammatory and ROS levels (16). These results suggest that mechanical injury, which causes cell necrosis, may be triggering the wound healing response of fibroblasts by modulating inflammatory molecules (29). As a result, it is possible to evaluate the effect of barbatimão based on oxidative, apoptotic, inflammatory, and growth factor molecules using *in vitro* scratched fibroblast model.

In this study, B1 and B2 extracts with different concentrations were able to modulate the oxidative metabolism of scratched fibroblasts by decreasing the expression of prooxidants that contribute to oxDNA, lipoperoxidation, and PCarb while increasing antioxidant enzyme levels (Figure 1). Furthermore, reduction in CASP3 and CASP8 levels revealed attenuation of apoptotic events in the scratched cultures (Figures 2D and 2E). These results can be further elucidated as when a skin wound occurs, a blood clot is immediately formed by aggregation of platelets at the injury site and conversion of fibrinogen to fibrin.

When a wound occurs, the first inflammatory phase is significant as it produces a temporary matrix that recruits local immune cells (neutrophils, macrophages, mastocytes, and natural killer T cells and fibroblasts (30). During this phase in an injured skin tissue (*in vivo*), immune cells from the blood vessels secrete proinflammatory cytokines and increase the expression of ROS. Elevating ROS levels is essential as a protective mechanism against microbial infections. However, ROS production must be highly regulated as high ROS

levels can cause wound healing impairment and fibrosis (29). Moreover, uncontrolled ROS levels can lead to oxidative stress, causing irreversible damage to proteins, lipids, and DNA. Further, DNA damage would promote mutations or trigger apoptosis via caspases (31).

From the obtained results, we infer that barbatimão extract could contribute positively to wound healing due to its antioxidant properties that have been described in previous studies by Baldivia et al. (32) and Pellenz et al. (12)Mart.. Specifically, the survey conducted by Pellenz et al. (12)Mart. also showed that barbatimão hydroalcoholic extract reduced the levels of prooxidant and apoptotic molecules in the intact keratinocytes and dermal fibroblast cells.

With progression in the inflammatory phase, ROS concentration increases causes elevation of antioxidant enzymes to regulate the oxidative stress condition. Evidence have suggested that when high levels of superoxide anion are expressed during the inflammatory phase, expression of the SOD enzyme also increases. The SOD enzyme dismutase superoxide anion to hydrogen peroxide (H2O2) that signals angiogenesis, cell migration, and proliferation events (33). However, high H2O2 concentrations can cause fibrosis, and its regulation is relevant during the proliferative phase of wound healing (34). GPX and CAT are antioxidant enzymes that interact to control oxidative stress and avoid excessive H2O2 accumulation (35). In the present study, barbatimão induced higher levels of antioxidant enzymes (SOD, CAT, and GPX) compared to the expression levels in control scratched fibroblast cells. It should be noted that we are the first to reveal the antioxidant activity of barbatimão through this study. Thus, the antioxidant effect of barbatimão does not depend on the direct reduction of prooxidants. However, it might be due to modulation of the endogenous antioxidant system, which might be responsible for inducing antioxidant properties.The present study also observed lower proinflammatory cytokines in scratched fibroblast cells when treated with B1 or B2 extracts. Polyphenols such as quercetin, present in the barbatimão extract (36, 37), could directly modulate this effect.

Despite the methodological limitations concerning *in vitro* protocols, our results showed that barbatimão can modulate critical molecules that participate in the inflammatory and proliferative phases of wound healing in scratched dermal fibroblasts. These results open the perspective that barbatimão could also act on other skin and immune cells related to wound healing, which further investigations need to confirm.

Notes

This study is part of the result of a doctoral thesis in the Postgraduate Program in Pharmacology at the Federal University of Santa Maria, by one of the authors (NLKP), entitled "Research of mechanisms associated to the healing and regenerative effect of barbatimão (*Stryphnodendron adstringens*) in cellular models *in vitro*".

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Conflicts of interest disclosure

The authors declare no competing interests relevant to the content of this study.

Authors' contributions

All the authors declare to have made substantial contributions to the conception, or design, or acquisition, or analysis, or interpretation of data; and drafting the work or revising it critically for important intellectual content; and to approve the version to be published.

Availability of data and responsibility for the results

All the authors declare to have had full access to the available data and they assume full responsibility for the integrity of these results.

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