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Original research article

Topical application of indigo-plant leaves extract enhances healing of skin lesion in an excision wound model in rats

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Abstract
Objectives: This study aims to evaluate the pharmacological role of indigo extract in accelerating the wound healing in a rat model.
Methods: Female Sprague-Dawley rats were anesthetized with ketamine (30 mg/kg, i.p.) and the full thickness of the marked skin was then cut carefully and wounds were left undressed. Indigo extract (5%) in PBS was applied topically twice daily until healing was complete. A control group of rats was treated with povidone-iodide (Betadine®). Rats treated with phosphate buffer saline were used as a negative control group. The rate of wound healing was assessed daily. Histopathological examination of skin sections were qualitatively assessed by independent evaluators. The inflammatory and apoptotic markers were assessed in skin tissue homogenates using ELISA.
Results: Histopathology data showed that applying indigo to skin wounds enhanced the healing process, resulting in a significant decrease in dermal inflammation in comparison to untreated rats. Topical application of indigo significantly increased antioxidant enzyme activities with reduced malondialdehyde (MDA) levels in wound tissues. The levels of matrix metalloproteases-2 and -9 were significantly lower with an accompanied increase in the level of TGF-β1 in skin tissues from rats treated with indigo compared to the control group treated with PBS.
Conclusions: The antioxidant and anti-inflammatory properties of indigo leaf extract accelerate the healing of skin injuries.

Keywords: Excision wound model; Indigo; MMP-2; MMP-9; TGF-β1; Wound healing

Highlights:
- Indigo leaf extract accelerates wound healing.
- Indigo leaf extract reduces the inflammatory and apoptotic markers in injured skin.
- Indigo extract significantly increases antioxidant enzyme activities in injured skin.

Introduction

Cutaneous wound healing is a process by which skin can repair itself after injury, burns, and microbial infection, or after acute outbreaks of chronic skin conditions such as eczema or psoriasis. This process is divided into 4 phases: hemostasis, inflammation, proliferation, and re-modeling. Immediately after injury, the clotting cascade is triggered, and a fibrin clot is formed to start the wound healing (Chodorowska and Roguś-Skorupska, 2004). During the inflammation stage, several chemotactic proteins are secreted to recruit the infiltration of phagocytic cells to engulf any invading pathogens. In addition, several inflammatory mediators such as transforming growth factor-beta 1 (TGF-β1) are released, stimulating the formation of fibroblasts and the growth of epithelial cells.

During the proliferation stage, angiogenesis is activated and fibroblasts start to produce extracellular matrix (ECM) – mainly in the form of collagen – to create a granuloma that protects the damaged tissues. Finally, disorganized ECM is re-modeled by dermal fibroblasts (Shedoeva et al., 2019).

The healing of wounds can be easily interrupted by several factors, some of which could adversely affect the healing process. These factors include infection, contamination, age, diabetes, and treatment with some medications such as corticosteroids, which lead to non-healing chronic wounds (Rodriguez et al., 2008). These factors should be considered, and a suitable protocol for treatment should be followed. The majority of wound healing agents are topical preparations and are used mainly to prevent infection, not to induce the healing process. These medications have several side effects when compared to natural preparations that can be used as an alternative ther-

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apy (Ahmad et al., 2021). Recently, topical treatments have received criticism for an effect known as topical steroid withdrawal (TSW), which refers to symptoms that may appear in patients who apply topical steroids for 2 weeks or longer and then discontinue use. These symptoms include redness, a burning sensation, and itchiness (Hajar et al., 2015).

Herbal therapy has been prescribed for health, healing, and longevity for thousands of years in traditional eastern medicine (Li et al., 2021). Additionally, the use of natural products in alternative medicine has become popular in the form of ointments or herbal-dyed textile products (Shedoeva et al. 2019). Moreover, several recent studies have highlighted the medicinal use of plant extracts such as *Aloe vera, Calendula officinalis, Abrus cantoniensis*, and *Ampelopsis japonica* in treating skin lesions (Lee et al., 2015; Zeng et al., 2016). The widespread belief that Indigo-dyed clothes have positive effects on the skin is what initially motivated us to undertake this study (Ho and Chang, 2002).

Indigo naturalis is prepared from stems and leaves of *Persicaria tinctoria* (Family: Polygonaceae) and processed into a dark blue powder, mass, or granules. Indigo leaf extract was reportedly used in traditional Chinese and Japanese medicine to treat clinical disorders such as ulcerative colitis and psoriasis (Naganuma, 2019). In a multi-center, double-blind, randomized study, patients with active ulcerative colitis were treated with indigo preparation. Interestingly, treated patients showed a significantly higher response compared to patients treated with a placebo, and the rate of mucosal healing was also higher in the treated group (Naganuma et al., 2018). Topical preparation containing indigo showed promising results in the treatment of skin inflammation in psoriasis (Lin et al., 2007, 2014, 2020; Naganuma et al., 2020).

Materials and methods

Ethics statement

Animal procedures and experimental methods were revised and approved on 15/11/2021 by the Ethical Committee of the Faculty of Pharmacy, Delta University, Egypt, FPDU-12/2021.

Plant material and preparation of extract

Dried crushed leaves of *Persicaria tinctoria* (Aiton) Spach, also called *Polygonum tinctorium* Lour (family Polygonaceae) – known as Japanese indigo – were provided by AIZOME (JM Mark Inc.) from their dyeing facility. One-hundred grams of the dried leaves were added to 500 ml of 50% ethanol in water in a round bottom flask and kept in the dark at room temperature for 7 days. The extract was then shaken for 15 min, and filtered through a 0.45 nm membrane filter. The filtered ethanolic extract was evaporated in a water bath at 65 °C until completely dry. A 5% solution of the dry extract was prepared in phosphate-buffered saline (PBS) and stored at room temperature. The chemical composition and characterisation of the plant extract has been previously described by several publications (Choi et al., 2020; Kimura et al., 2015; Lee et al., 2018; Seimandi et al., 2021; Tokuyama-Nakai et al., 2018).

Excision wound model

Female Sprague-Dawley rats (200–220 g) were depilated on their back, and an area of about 500 mm^2 was then marked on the depilated skin with a standard ring. Rats were anesthetized with ketamine (30 mg/kg, i.p.) and the full thickness of the marked skin was then cut carefully and wounds were left undressed. Indigo extract (5%) was applied topically twice daily until complete healing was observed. A control group of rats was treated with povidone-iodide (Betadine®). Rats treated with phosphate buffer saline were used as a negative control group. Changes in the wound area were monitored regularly to record the rate of wound healing. The rate of wound healing was scored daily for wound contraction, epithelization, and formation of a hard scar using a scoring system from 1 to 5, with 5 indicating a superior healing process (Mukherjee et al., 2020).

Histopathology

The cross-sectional full-thickness skin specimens were collected after 10 days of treatment for histopathological examination. Samples were fixed in 10% buffered formalin, processed, blocked with paraffin, then sectioned into 7 μm sections and stained with hematoxylin and eosin. Sections were qualitatively assessed by independent evaluators under a light microscope and observed in respect to fibroblast proliferation, collagen formation, angiogenesis, and epithelialization.

Cytokines and apoptotic protein analysis

For the measurement of cytokines and inflammatory markers in the skin samples, 300 mg of skin was washed with an ice-cold phosphate buffer then homogenized in 3 ml of the same buffer using a polytron homogenizer at 40 °C. Tissue homogenate was centrifuged at 3000 × g for 10 min, and the supernatant was then separated and kept frozen at –80 °C. Commercially available ELISA kits were used to measure matrix metalloproteinase 2 and 9 (MMP-2 & MMP-9), transforming growth factor beta 1 (TGF-β1) (Cloude-clone, USA), nuclear factor-kappa B (NF-κB) (MyBiosource, USA), superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase enzyme, and lipid peroxidase were assessed in the tissue homogenate using the commercially available kit (Sigma-Aldrich).

Statistical analysis

GraphPad Prism version 8 was used to calculate statistical significance between the studied groups using One-Way ANOVA followed by Dunnett's test as a *post hoc* analysis. P values < 0.05 were considered significant as indicated within each figure. All the graphs were prepared in Graphpad prism version 8:
https://www.graphpad.com/scientifc-sofware/prism/.

Results

The application of indigo on skin wounds enhances the healing process

Animals were observed during experiments for any symptoms that might affect their health condition, such as contamination of wounds with bacteria. A rapid wound closure was observed after 6 days in both the indigo and povidone-iodide (Betadine®) treated groups, compared to the Phosphate buffer treated group – with complete closure observed after 2 weeks. The healing score shows a significantly higher rate of healing and epithelization in the indigo and povidone-iodide (Betadine®) treated groups, as compared to the control group (*** P < 0.001) – Fig. 1. Histopathological examination of skin biopsy sections 10 days after skin injury showed healed skin structures associated with marked improvement in collagen formation and neovascularization in rats treated with indigo or povidone-iodide (Betadine®), and a marked decrease in the dermal inflammation compared to rats treated with PBS (Fig. 2). Sections were assessed by 3 independent evaluators.

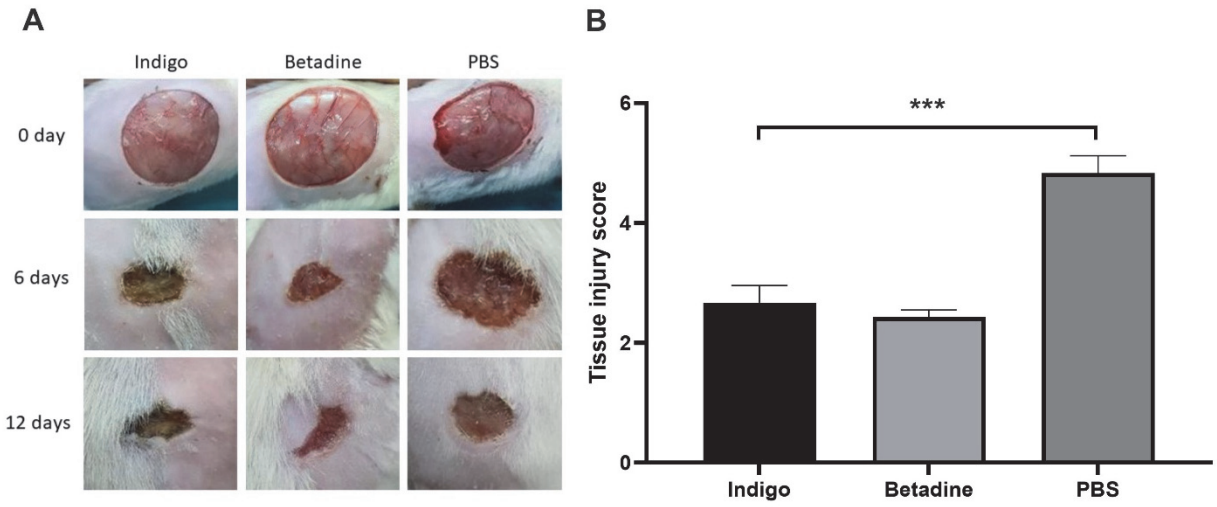
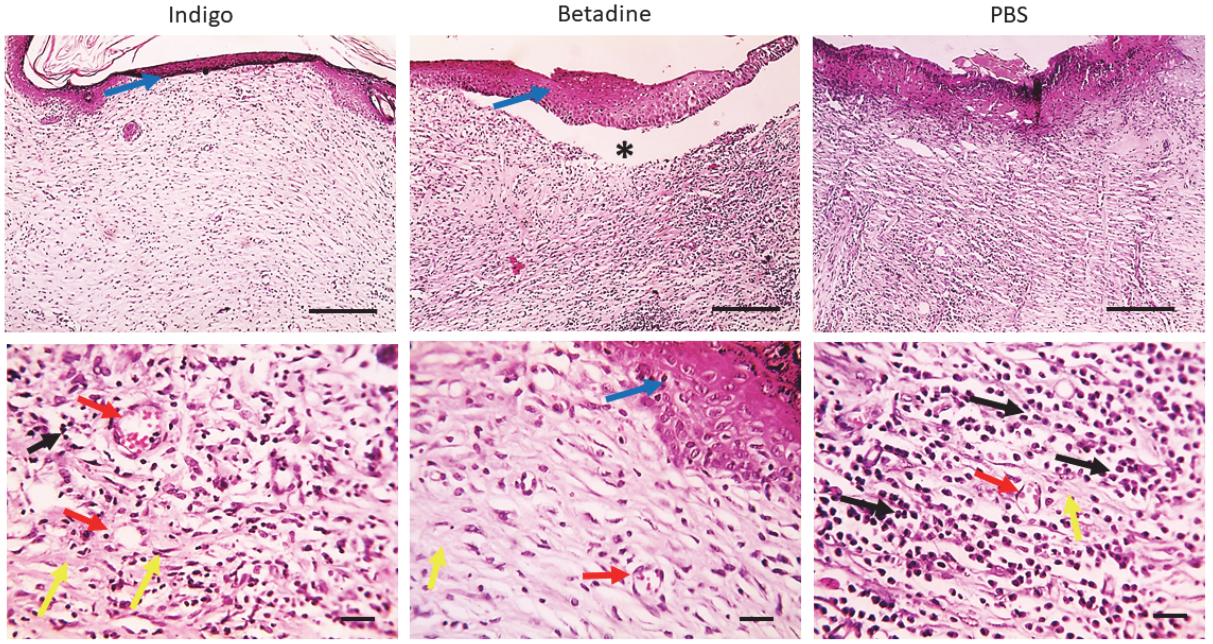


Fig. 1. The application of indigo on skin wounds enhances the healing process. Topical application of indigo extract on the wounded skin improves the healing process and wound closure 6 to 10 days from injury, compared to wounds treated with PBS alone (**A**). Wound healing scoring showed a significant improvement in the skin healing process after treatment with indigo, compared to the PBS treated group (**B**). *** $P < 0.001$. Povidone-iodide (Betadine®) was used as a positive control.



	Re-epithelization	Collagen	Neovascularization	Inflammatory cells
Indigo	++	++	++	+
Betadine	+++	+++	+++	-
PBS	-	+	+	+++

Fig. 2. Skin sections (7 μm) from indigo, povidone-iodide (Betadine®), and PBS treated rats were stained with hematoxylin and eosin stain. Sections were assessed for re-epithelization (blue arrows), dermal inflammation (black arrows), neovascularization (red arrows), collagen deposition (yellow arrows), and separation between epidermis and dermis (*). Sections from rats treated with indigo and povidone-iodide (Betadine®) showed a marked increase in the levels of collagen deposition, neovascularization, and re-epithelization compared to control rats treated with PBS. Inflammatory cells and dermal inflammation were significantly decreased in rats treated with indigo and povidone-iodide (Betadine®), as compared to the control group. Low magnification (upper panel): scale bar = 100 μm and high magnification (lower panel): scale bar = 50 μm.

Topical application of indigo improved antioxidant enzyme activities and blunted MDA levels in wound tissue

Skin injury in our rat model induced severe inflammation associated with a marked increase in oxidative stress-mediated cellular damage. The application of indigo twice daily reduced inflammation and enhanced the healing process. To further assess the role of indigo extract in improving cellular stress and antioxidant defense mechanisms in the skin, the levels of oxidative stress markers were assessed in skin tissues. A significantly lower level of MDA, a potential marker of oxidative damage, was observed in the indigo treated group, compared to the control group treated with PBS (Fig. 3A). Interestingly, high levels of anti-oxidant enzymes activity of catalase, SOD, and Gpx were observed in skin tissues of rats treated with indigo, as compared to the control group (Fig. 3B–D).

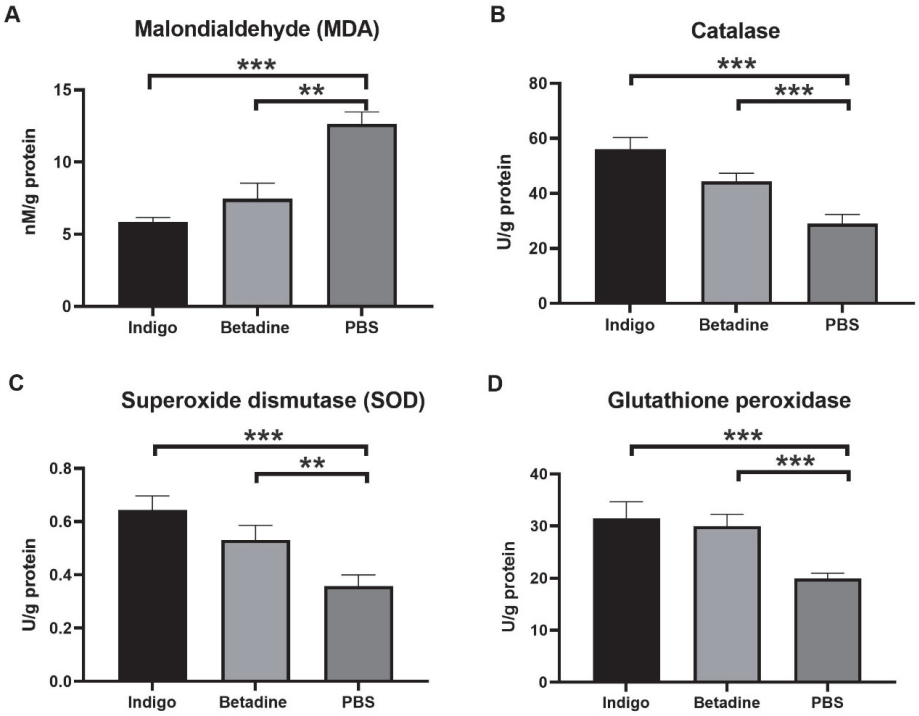


Fig. 3. Evaluation of indigo extract effect on the levels of oxidative stress markers in skin tissues homogenate. Rats treated with indigo extract showed a significantly lower level of MDA compared to the PBS treated group (**A**). In addition, significantly higher levels of catalase, SOD, and Gpx were observed in skin tissue homogenate of rats treated with indigo compared to PBS treated animals (**B–D**). Results are means ± SEM (n = 7). *** P < 0.001, ** P < 0.01. Data were analyzed with One-Way ANOVA, followed by *post hoc* comparisons using the Dunnett test.

Indigo diminshed matrix metalloproteinases, enhanced TGF-β1 levels and thus accelerated tissue repairs

Matrix metalloproteinases and TGF-β1 are key tissue remodeling factors that play a major role in the wound healing process. In our rat model of skin excision, the levels of MMP-2, MMP-9 were significantly lower in skin tissues from rats treated with indigo, as compared to the control group treated with PBS, while the level of TGF-β1 was significantly higher in rats treated with indigo or povidone-iodide (Betadine®), compared to the control group (Fig. 4).

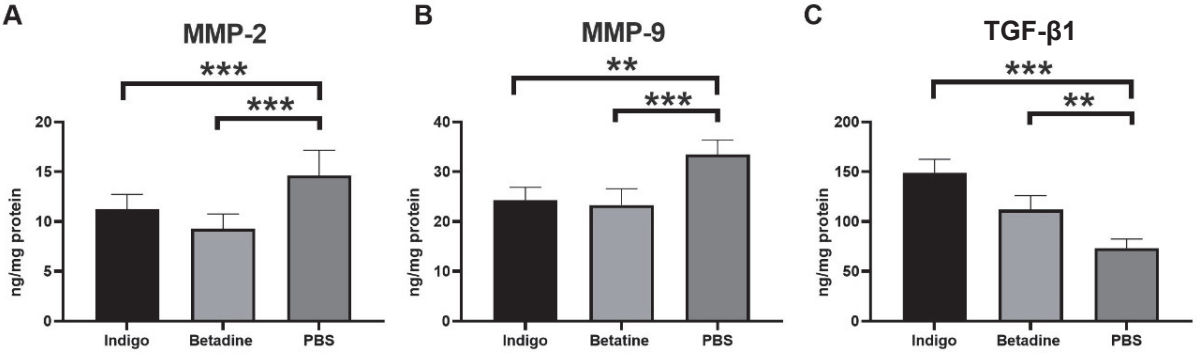


Fig. 4. Application of indigo extract diminshes the levels MMP-2, MMP-9 and TGF-β1, and enhances wound healing. Skin tissues from rats treated with indigo or povidone-iodide (Betadine®) show a significantly lower level of MMP-2, MMP-9, compared to skin tissues from the control group treated with PBS. Tissues from rats treated with indigo or povidone-iodide (Betadine®) have higher levels of TGF-β1 compared to control groups. *** P < 0.001, ** P < 0.01. Data were analyzed with one-way ANOVA, followed by *post hoc* comparisons using the Dunnett test.

Discussion

Skin injuries are of major interest to the public health sector because they affect a large number of people, and consequently reduce the quality of their life. A delay in the healing process or wound contamination with microbes might require antibiotic treatment and extended hospitalization time. Limited information is known about the potential use of topical agents that accelerate wound healing (Rizzi et al., 2010). Natural substances and herbal medicines are important sources of substances that were successfully used as therapeutic agents. Indigo leaf extract was used in traditional Chinese medicine as an agent that might have anti-inflammatory effects and could accelerate wound healing (Wei et al., 2005). Our data demonstrate that the topical application of indigo leaf extract on an open skin injury significantly improves the healing process and accelerates wound closure with marked increase in the levels of collagen formation, angiogenesis, and epithelialization. The anti-inflammatory effect of indigo was previously reported, where it significantly reduced the release of superoxide anions from neutrophils in an *in-vitro* assay (Lin et al., 2009). In addition, indigo was proven to contain a potent anti-inflammatory agent that has successfully been used in treatment of gastric ulcers (Farias-Silva et al., 2007).

In this study, application of indigo extract on skin wounds reduced cellular stress and oxidative damage by lowering the levels of lipid peroxidation and enhancing the antioxidizing activities of catalase, SOD, and Gpx in skin tissue. The presence of high levels of antioxidants and polyphenolic compounds justifies the possible use of indigo as an antioxidant agent. In addition, analysis of indigo extract identified the presence of several bioactive compounds such as indirubin, indigo, and tryptanthrin which might have medicinal uses (Liau et al., 2007). Tryptanthrin is reported to have an anti-inflammatory effect, as it reduces the inflammatory effect by inhibiting cyclooxygenase-2 (Danz et al., 2001). Indirubin is also reported to reduce the levels of inflammation in delayed type hypersensitivity and to inhibit the production of interferon-γ, interleukin-6, and RANTES chemokine (Kunikata et al., 2000; Mak et al., 2004). In this study, significantly high levels of TGF-β1 were observed in skin tissue homogenates taken from rats treated with indigo, as compared to rats treated with the control vehicle. TGF-β1 modulates events of normal wound healing through multiple pathways that influence cell infiltration, proliferation, angiogenesis, extracellular matrix synthesis, and remodeling (Wang et al., 2017). The use of TGF-β1 was described to have a beneficial effect in improving the healing process of poorly vascularized or non-healing wounds in diabetic or elderly patients (Akbik et al., 2014). Tissue matrix homeostasis is an essential process in normal growth, development, and wound healing. Matrix metalloproteinases (MMP-2 and MMP-9) are endopeptidases involved in the initial inflammatory process, and both play a major role in regulating extracellular matrix degradation and deposition that is essential for wound re-epithelialization. The excess protease activity of MMP-2 and MMP-9 can lead to a chronic non-healing wound (Asimakidou et al., 2017). Our results demonstrate that the application of indigo leaf extract reduces the levels of MMP-2 and MMP-9 during the healing of injured skin in rats and enhances wound closure. In a previous work, the use of selective inhibitor of MMP-2 and MMP-9 in a mouse model of skin injury accelerated wound healing by lowering inflammation and enhancing angiogenesis and re-epithelialization of the wound, indicating the important role of the pharmacological targeting of matrix metalloproteinase pathways in wound healing (Gao et al., 2015). In this study, data from histopathology of skin section examination showed a marked reduction in the infiltration of inflammatory cells in skin, associated with the improvement of collagen formation and neovascularization in rats treated with indigo as compared to animals treated with PBS. This was in line with other data showing a significant change in other inflammatory markers such as MMP-2, MMP-9 and TGF-β1.

Interestingly, the use of indigo extract to relieve the inflammation in psoriasis showed promising results, and opened a new avenue to consider natural products as agents that can accelerate wound healing. The use of alternative therapy will help to reduce the use of topical preparations containing steroids and hence minimize their side effects.

Conclusions

Indigo leaf extract has several natural active components with antioxidant and anti-inflammatory effects which play a role in improving and accelerating the healing process of injured skin. These data confirm the use of indigo leaf extract as a topical natural agent to reduce inflammation and enhance wound healing, positioning indigo leaf extract as a viable alternative to topical steroids that may offer an improved quality of life to individuals suffering from skin injury. Further work will thus be arranged to evaluate the anti-inflammatory effect of indigo leaf extract, and to determine the scope of healing agents maintained throughout the dyeing process of textiles in a limited clinical trial.

Author contributions

AS, MS, YA designed and performed the experiments. AS, MS, MM, YA wrote and revised the manuscript. MM provided essential reagents and revised the manuscript.

Ethical aspects and conflict of interests

MM is employed by AIZOME (JM Mark Inc.), YA worked as a consultant at AIZOME (JM Mark Inc.).

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