Effect of Glycolic Extract of *Dillenia indica* L. Combined With Microcurrent Stimulation on Experimental Lesions in Wistar Rats

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Abstract: This study evaluated the wound healing activity of a glycolic extract of *Dillenia indica* (GED) prepared from the mature fruits of the plant applied alone or in combination with microcurrent stimulation to skin wounds surgically induced on the back of Wistar rats. Methods. The animals were randomly divided into six groups: (A) negative control group; (B) group receiving microcurrent application (MC; [10 μA/2 mins]); (C) group treated with GED; (D) group treated with an emulsion containing GED; (E) group treated with GED and MC, and (F) group treated with the emulsion containing GED and MC. Tissue samples were obtained 2, 6, and 10 days after injury and underwent structural and morphometric analysis. Results. There were observed differences in wound healing among the various treatments when compared to the control group. The combination of microcurrent plus extract or microcurrent plus emulsion containing GED was advantageous in all of the studied parameters (*P < 0.05*) when compared to the other groups with positive effects seen regarding newly formed tissue, number of fibroblasts, and number of newly formed blood vessels. The morphometric data confirmed the structural findings. Conclusion. Microcurrent application alone or combined with GED exerted significant effects on wound healing in this experimental model. This was probably due to the efficacy of microcurrent application since the extract alone did not significantly accelerate the healing process. *D indica* fruit extract most likely participates in the wound healing process as a result of its anti-inflammatory properties.

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*Dillenia indica* L. (family Dilleniaceae) originated in tropical Asia and has acclimated in Brazil for more than a century. The plant has a thick trunk and a fissured bark surface. The branches have leaves that are concentrated on the terminal region. The flowers are large and white in color and bloom between December and April. The fruits that appear between April and August are large, fleshy, and greenish-yellow in color and contain small, flattened seeds surrounded by a gelatinous substance.  

*D indica* is used as an antipyretic and cardiotonic drug and for the treatment of rheumatoid arthritis and anti-inflammatory processes. Munit et al'
Migliato et al

**KEYPOINTS**
- The objective of this study was to investigate the effects of a glycolic extract of *Dillenia indica* L. fruits and an emulsion containing this extract, either with or without microcurrent stimulation, on the healing of surgically-induced wounds in Wistar rats.

identified the presence of triterpenes and flavonoids in a phytochemical study of the crude extract of *D indica* leaves. Abdille et al.2 observed that the extract of *D indica* fruit contains significant amounts of phenolic compounds with expressive antioxidant activity. Flavonoids, tannins, and other phenolic substances are constituents of plants with antioxidant activity, mainly by acting as radical scavengers of oxygen. The presence of flavonoids in phyotherapeutic agents has been shown to favor the wound healing process in experimental models.10,11

Wound healing is a complex biological process that occurs in response to tissue damage due to trauma or surgical procedures. The wound healing process can be divided into three phases: inflammatory, proliferative, and remodeling. Technological advances facilitated the emergence of a wide variety of wound healing treatments. The application of low amperage electrical stimuli has been shown to modify the healing process in living organisms, especially factors that delay or impair this process.12–16 Several investigators have studied the effects of electrical stimulation using different amplitudes and frequencies and observed modifications in the cellular and tissue responses in experimentally induced wounds.17–19 Stimulation of live cells with low-intensity electrical currents directly affects the membrane potential and is associated with changes in ion gradients across the cell membrane causing an increase in the synthesis of ATP followed by increased protein synthesis.12,20,21

The objective of this study was to investigate the effects of a glycolic extract of *Dillenia indica* L. fruits and an emulsion containing this extract, either with or without microcurrent stimulation, on the healing of surgically-induced wounds in Wistar rats.

**Methods**

Mature fruits of *D indica* L. were collected in February 2009 on the Pinhal Farm in Limeira (São Paulo, Brazil) in the morning. Material was collected from the branches of the same tree for deposition of a voucher specimen, which was deposited by Vinicius Castro Souza, Curator of the Herbarium of the Department of Biological Sciences, ESALQ-USP, Piracicaba Campus (São Paulo) under the number ESA 55549.

**Preparation of plant extracts.** Mature fruits were collected and dried in an oven under circulating air at 45˚C for weight stabilization. The fruits were then ground in a knife mill. The extract was obtained by turbo extraction of 50 g of the sample in 500 mL 70% (w/w) alcohol for 15 minutes followed by filtration in a rotary evaporator under reduced pressure at a maximum temperature of 40˚C until complete elimination of the organic solvent. The turbo extraction technique does not allow the temperature to exceed 40˚C. This procedure was performed because it was considered relatively harmless regarding the extraction of different components. The sample was then lyophilized until all water was removed.

For the physicochemical quality control tests, 10 g of mature fruit was dried at room temperature in the dark. Later, 1.0 g this material was subjected to infrared heat (110˚C) for 1 hour and was weighed afterward. This procedure was performed every hour until the weight did not vary more than 0.25%. Values are expressed as percentage (w/w). The average of determinations was three.22,23

For microbiological analysis of the lyophilized *D indica* extract, total microorganism count, and counts of the pathogens *Salmonella* sp, *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* were determined according to the methods of Migliato et al.24 and Pinto et al.25

**Phytochemical screening.** Preliminary phytochemical analysis of the *D indica* glycolic extract was performed in triplicate according to Simões et al.26 and Harbone.27 A portion of 100 mg of the extract was used for each reaction. The presence of flavonoids was determined using 1% aluminum chloride solution in methanol, concentrated hydrochloric acid, magnesium turnings, and potassium hydroxide solution. Anthraquinones were analyzed by the Borntrager reaction.

**KEYPOINTS**
- Cross-sections of the mid-region of the experimental wound were used to determine tissue repair area, total number of cells (fibroblastic and inflammatory cells), number of newly formed blood vessels, and thickness of the regenerating epithelium.
- Preliminary phytochemical analysis of the glycolic extract of *D indica* fruits showed the presence of flavonoids, saponins, anthraquinones, and tannins; alkaloids were not detected.
tion and saponins by the observation of persistent and abundant foam production. To analyze tannins, the lyophilized extract was dissolved in water and tannins were identified by reaction with 2.5% gelatin, 1% iron salts, and 10% lead acetate. Total alkaloids were assayed using the reagents of Dragendorff, Bouchardat, Mayer, and Bertrand.

Preparation of an emulsion containing the glycolic extract of *Dillenia indica*. The lyophilized *D. indica* extract was solubilized in propyleneglycol:water (1:1) and incorporated into an emulsion containing the following: Butylated hydroxytoluene (BHT [0.05%]), Ethylenediamine tetraacetic acid (EDTA [0.1%]), Lanaxan® (2%), Polawax® (14%), Phenonip® (0.5%), propyleneglycol (3%), *D. indica* glycolic extract (5%), and distilled water qsp (30%).

Animals. Male Wistar rats (*Rattus norvegicus*) weighing 250 g–350 g were housed individually in cages at a constant temperature (23°C ± 2°C). The rats had free access to food and water and were subjected to a 12-hour light/dark cycle. The average weight and behavior of each group at the end of the study did not differ significantly. The animals were anesthetized by intraperitoneal injection of xylazine hydrochloride (20 mg/kg body weight) and ketamine hydrochloride (50 mg/kg). After the position was marked with a dermatographic pen and pachymeter, a 2-cm long and 0.2-cm deep surgical incision was made in the craniocaudal direction. The incision was not sutured. In view of the similar genetic background of the animals and according to the Ethics Committee of University Center, Uniararas (protocol number 809/2006), nine animals were used per group: (A) negative control group receiving sterile saline; (B) group receiving microcurrent application (MC; [10 µA/2 min]); (C) group treated with the glycolic extract of *D. indica* (GED); (D) group treated with the emulsion containing GED; (E) group treated with the GED and MC (10 µA/2 min), and (F) group treated with the emulsion containing GED and MC (10 µA/2 min), according to the protocol of Mendonça et al.16 A transcutaneous electrical stimulator (Physiotonus Microcurrent, Bioset, Rio Claro, São Paulo, Brazil) was used for electrical stimulation. The device was set to microgalvanic-continuous mode with the intensity at 10 µA/2, frequency 0.3 Hz, and was used for 2 minutes. The applications were performed using two metal electrodes with a spherical tip (10 mm) positioned on the wound. The treatments were started 24 hours after surgical intervention and were continued daily for 10 days.

Collection and preparation of wound samples for structural analysis. At days 2, 6, and 10 after the injury, three animals in each group were killed under anesthesia. The total area (approximately 120 mm²–160 mm²) of the wound was removed and submitted for structural and morphometric analysis. Each sample was removed and fixed in 10% formalin in Millonig buffer (pH 7.4) for 24 hours at room temperature. Next, the specimens were washed in buffer and processed for embedding in Paraplast®. Longitudinal sections (7 µm) were stained with hematoxylin & eosin for routine histology and with picrosirius-hematoxylin in order to view collagen fibers. The specimens were examined and documented using a Leica DM 2000 photomicroscope at the Laboratory of Micromorphology, Hermínio Ometto University Center, Uniararas.

Morphometric analysis. Cross-sections of the mid-region of the experimental wound were used for the determination of the following morphometric parameters: tissue repair area (x10³ µm²), total number of cells (fibroblastic and inflammatory cells [n/10³ µm²]), number of newly formed blood vessels (n/10³ µm²), and thickness of the regenerating epithelium (µm). For this purpose, three samples were randomly selected among the sections obtained. All images were captured and digitalized using a Leica DM 2000 photomicroscope. The measurements were made on the digitalized images using the Leica Image Measure™ and Sigma Scan Pro 6.0™ programs. The results were compared by ANOVA and Tukey’s post-hoc test with the level of significance set at 5%. The results were entered into spreadsheets (Biostat for Windows).

Results

Quality assurance is an important factor to be considered in the production of medications, cosmetics, and

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<th>Table 1. Physicochemical analysis of the glycolic extract of <em>Dillenia indica</em> L.</th>
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<td><strong>Parameter</strong></td>
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Structural and morphometric analysis of wound repair. Tissue repair was studied in the different groups by comparing inflammatory (leukocytosis, hemorrhage, and exudate) and proliferative processes (fibroblastic hyperplasia, epithelization, and angiogenesis), and tissue reorganization. Temporal differences in tissue repair were observed among the different treatments.

In the control group (group A), the proliferative phase was observed on day 6 after injury and tissue reorganization was detected on day 10. In the group receiving MC (group B), the size of the tissue repair area and the total number of cells were higher than in the control group on day 6 of treatment. On day 10, the wound area was completely re-epithelialized with the observation of dermis filled with fibrous tissue, reorganized collagen fibers, and compacted fibril elements (Figures 1, 2).

The findings obtained for the group treated with GED (group C) were similar to those observed for the control group in terms of the repair of epidermis and dermis for samples collected on days 6 and 10. A significant increase in the tissue repair area and the total number of cells was observed on days 6 and 10 after experimental injury in the group simultaneously receiving MC and GED (group E) when compared to the other groups. In this group, the dermal wound area was filled with fibrous tissue on day 10 and the collagen fibers were reorganized and compacted, findings indicating tissue repair similar to that observed in animals receiving only MC. No significant difference in the repair of the lining epithelium was observed during application of different treatments (Figures 1, 2).

Figure 1. Photomicrographs of cross-sections of the back skin of Wistar rats obtained on day 10 after experimental injury. A) Control group. B) Group receiving microcurrent application (10 µA/2 min). C) Group treated with the D indica glycolic extract (GED). D) Group treated with the emulsion containing GED. E) Group simultaneously receiving microcurrent application (10 µA/2 min) and the GED. F) Group simultaneously receiving microcurrent application (10 µA/2 min) and the emulsion containing GED. Ep: Epidermis. Arrows: Tissue repair area. The sections were stained with toluidine blue. Bar = 50 µm.
In the group treated with the emulsion containing GED (group D), the tissue repair area and total number of cells on days 6 and 10 after injury were similar to those of the control group. On day 10, the epidermis was completely repaired (Figures 3, 4). A significant increase in tissue repair area and the total number of cells was observed on days 6 and 10 in the group simultaneously receiving MC and the emulsion containing GEC (group F) when compared to group D. However, the collagen fibers of the dermis were poorly compacted.

A significantly larger number of newly formed vessels per tissue area (103 µm²) were observed in groups B, E, and F (Figure 5). Epithelial thickness in the tissue repair area did not differ among groups when compared to control (Figure 6).

Discussion

Phytochemical analysis of the glycolic extract of D indica fruits identified the presence of flavonoids, among other components. These compounds and their derivatives are known to increase vascularization and to delay the process of cell necrosis due to their anti-inflammatory, antifungal, and antioxidant properties.31,32 Studies have shown that most of the antioxidant activity of plant extracts is the result of compounds, such as flavonoids, tannins, isoflavones, flavones, anthocyanins, catechin, and other phenolic compounds.33,34 Flavonoids are especially important in the tissue repair process because of their astringent and antimicrobial properties.6,35 The same properties can be attributed to tannins, which were identified in the present study in the glycolic extract of D indica. According to Jorge Neto et al36 and Bedi and Shenefelt,37 derivatives are known to increase vascularization and to delay the process of cell necrosis due to their anti-inflammatory, antifungal, and antioxidant properties.

The authors identified flavonoids in the C ciliaca (Boiss. & Bal. fruit) extract and suggested that these substances participate in the healing process together with other phytochemical components of the plant.

In the present study, although flavonoids were identified in the D indica extract, no significant effects on the acceleration of tissue repair were observed when the
extract or the emulsion containing the extract was applied alone to skin wounds induced on the back of rats. Although the efficacy of flavonoids in the acceleration of the tissue repair process has been reported by different investigators,\(^{31,32}\) the present results indicate that the presence of these compounds in the \textit{D indica} extract did not have this effect on the healing process.

Analysis of the glycolic extract of \textit{D indica} fruit did not reveal the presence of alkaloids. Similarly, Shome et al\(^3\) characterizing the phytochemical composition of hexane, chloroform, ethanol, and aqueous extracts of \textit{D indica} fruits, demonstrated the presence of triterpenes and the absence of alkaloids in all extracts. Yeshwante et al\(^9\) observed significant anti-inflammatory activity of a methanol extract of \textit{D indica} leaves on edema induced in rats and mice. The authors suggested that this process is the result of the inhibition of prostaglandin biosynthesis, confirming the use of \textit{D indica} in folk medicine. In the present study, application of the \textit{D indica} extract or of the emulsion containing the extract to skin wounds surgically induced in rats did not significantly accelerate the tissue repair process. Positive effects were only observed when microcurrent stimulation was applied simultaneously.

**KEYPOINTS**

- The combination of microcurrent plus extract or microcurrent plus emulsion containing GED was advantageous in all of the studied parameters compared to the other groups regarding newly formed tissue, number of fibroblasts, and number of newly formed blood vessels.
- Although the efficacy of flavonoids in the acceleration of the tissue repair process has been reported by different investigators,\(^{31,32}\) the present results indicate that the presence of these compounds in the \textit{D indica} extract did not have this effect on the healing process. Positive effects were only observed when microcurrent stimulation was applied simultaneously.
observed when microcurrent stimulation was applied simultaneously. These findings suggest that the extract of D indica fruit possesses the same properties as the leaf extract studied by Yeshwante et al., which showed efficacy in treating the effects of inflammatory processes, but did not accelerate wound healing.

Microcurrent application was found to be effective in terms of the parameters analyzed, with positive effects on the area of newly formed tissue, number of fibroblasts and number of newly formed blood vessels, but not on epithelial thickness, when compared to the control group and to the groups receiving only the D indica extract and the emulsion containing the extract. These findings agree with those reported in different studies demonstrating that low-intensity electrical currents stimulate wound healing. Becker reported that electrical currents are present in all biological systems and may promote repair and growth after injury. According to this author, a specific injury stimulus induces another repair stimulus. The author also demonstrated that the membrane electrical potential is altered in injured tissues. The “injury signal” gradually decreases in parallel to the repair process and ceases when the latter is complete. The voltage peaks immediately after injury and gradually decreases as the wound heals, a fact leading to the concept that current flows may be defective in chronic wounds and that the application of electrical currents to wounds may stimulate healing. Biedeback proposed that transmembrane currents open voltage-controlled calcium channels in fibroblasts, inducing ATP resynthesis, activation of protein kinase mechanisms to synthesize new cellular protein, and DNA replication necessary for mitotic cell division. Electrical microcurrent has been used in the treatment of chronic wounds. Lee et al. using a 100 nA current 3 µA in the treatment of chronic wounds and ulcers associated with chronic diseases and found that the application of such currents supposedly provides electrons to tissues and saturated free radicals, facilitating...
tissue repair. Mendonça et al\textsuperscript{16} suggested that microcurrent application to tissue injuries might be used as a coadjuvant to accelerate the healing process. Variations in cell metabolism, as well as fibroblast proliferation, neovascularization, and collagen deposition in the wound area have been observed after microcurrent application.\textsuperscript{16,40} The combined application of a microcurrent and the \textit{D indica} extract or emulsion containing the extract was advantageous in terms of all parameters studied when compared to the control group and to either treatment alone. The simultaneous application of physical and phytotherapeutic agents to wounds has been shown to be effective in both skin repair and reduction of the inflammatory process.

The use of electrical current on transfer of various substances is known in the literature as iontophoresis—a noninvasive technique that ensures the penetration levels of higher concentrations of therapeutic substances when compared to passive diffusion. In iontophoresis, the current is widely used since it generates a unidirectional flow of electrons and constant during the application, triggering the desired therapeutic effects.\textsuperscript{47–49} Maia-Filho et al\textsuperscript{50} investigated the effects of simultaneous application of ultrasound and \textit{Aloe vera} gel on an experimental model of induction of tendinitis in rats and demonstrated that this type of treatment is effective in terms of both skin repair and reduction of the inflammatory process. Also Soares\textsuperscript{51} demonstrated that the combination of antioxidant agents and photodynamic or low-amperage electrical therapy accelerates wound healing in experimental wounds in rats. In addition, the simultaneous application of \textit{Aloe vera} and microcurrent was effective in the treatment of open wounds potentiating wound healing in Wistar rats.\textsuperscript{14} Similar effects were observed in the present study. The combination of the \textit{D indica} glycolic extract or emulsion containing the extract and microcurrent stimulation exerted significant effects on the repair area, total number of cells, and total number of newly formed vessels in the wound area. However, these alterations were not significant when the extract was applied alone. This fact suggests that the positive effects on the acceleration of tissue repair were due to the action of microcurrent stimulation, which has been shown to be effective in accelerating wound healing.\textsuperscript{15,16} However, the \textit{D indica} fruit extract probably participates in the wound healing process as a result of its anti-inflammatory properties.

**Conclusion**

The present results demonstrated that the \textit{D indica} glycolic extract or the emulsion containing the extract was not effective in accelerating the tissue repair process in skin wounds surgically induced in Wistar rats. However, microcurrent application alone or combined with glycolic extract exerted significant effects on wound healing in the experimental model. This finding was probably due to the efficacy of microcurrent stimulation, since this treatment alone accelerated the healing process.

**References**


