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Original Article

THE INCREASING OF VEGF EXPRESSION AND RE-EPITHELIALIZATION ON DERMAL WOUND HEALING PROCESS AFTER TREATMENT OF BANANA PEEL EXTRACT (*MUSA ACUMINATA* COLLA)

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ABSTRACT

Objective: The objective of the study was to determine the efficacy of topical administration of an alcoholic bark extract of *Musa acuminata* Colla on cutaneous wound healing including expression of VEGF and re-epithelization on dermal in rats.

Methods: Model was performed to evaluate the excision model of wound healing activity. Full-thickness excision wounds were made on the back of rat and *Musa acuminata* Colla extract was topically administered (doses 25%, 50% & 75%). The formation of granulation tissue was observed on day 4, 8, 12 and 16 (post-wound) to indicate VEGF and collagen. The extract increased cellular re-epithelialisation and collagen synthesis at the wound site, shown by increase in VEGF, and total collagen content of granulation tissues. The rate of contraction in wounds was determined by tracing the wound surface onto a transparent graph paper and measuring the surface area by planimetry.

Results: From the observation in EBP, all doses were found to have wound healing activity in wound contraction and period of epithelialization. The increasing of VEGF expression was found greater in the extract treated group than control group. The tensile strength of extract treated group was increased significantly.

Conclusion: The results indicated that *Musa acuminata* Colla peel extracts has significant wound healing activity.

Keywords: Dermal wound healing, *Musa acuminata* Colla, VEGF, re-epithelialization.

INTRODUCTION

Wound healing consists of an orderly progression of events that establish the integrity of the damaged tissue. The process of wound healing is essential to prevent the invasion of damaged tissue by pathogens and to partially or completely reform the damaged tissue [1]. This complex cascade of event starts from the moment of injury and can continue for varying periods of time depending on the extent of wounding. The process can be broadly categorized into three stages; inflammatory phase (consisting the establishment of homeostasis and inflammation); proliferate phase (consisting of granulation, contraction and epithelialisation) and finally the remodelling phase which ultimately determines the strength and appearance of the healed tissue [2, 3].

The process of wound healing is promoted by several natural products [4], plant products, which are composed of active metabolites such as triterpenes, alkaloids, and flavonoids [5]. These agents usually influence one or more phases of the healing process. The earlier reports shows the wound healing properties of two tropical plants, *Centella asiatica* L [6] and *Terminalia chebula* Retz [7] on dermal wound healing in rats.

Bananas are one of the most popular fruits on the world market. It is well known that fruits contain various antioxidants, such as vitamin C, vitamin E, and b carotene [8]. Antioxidant activity of a fruit may be due to its content of compound such as flavonoids. Macheix et al (2000) has been reported that banana contained interesting different types of antioxidants, such as phenol and tannin [9]. Further, Atzingen et al (2011) conducted an experimental study in which unripe banana peel gel was used at different concentrations (2%, 4% and 10% gel) in the treatment of surgical wounds in rats, and reported wound contraction rates similar to animals that were treated with 4% gel for 21 days [10]. Gel from unripe *Musa sapientum* L peel at 4% concentration applied to surgical wounds in rats resulted in increased number of polymorphonuclear cells on day 7, and reduced wound contraction and vascular proliferation, as well as increasing concentration of collagen fibers on day 21 [11]. The previous findings also reported that the catechin of banana pulp has antioxidant activity [12].

Then, this study was designed to identify the active metabolites of *Musa acuminata* Colla and examine their antioxidant activity. This study was taken up to investigate the efficacy of topical application of *Musa acuminata* Colla by biophysical parameter, immunohistochemistry, and histological approaches in the process of wound healing.

MATERIALS AND METHODS

Materials

Musa acuminata Colla obtained from Lumajang, Indonesia. The plant was identified, confirmed and authenticated by Balai Konservasi Tumbuhan Kebun Raya Purwodadi, Pasuruan, Indonesia. Catechin was obtained from E. Merck, Darmstadt, Germany, rat polyclonal antibody of VEGF was from Sigma Chemical Co., St. Louis, USA. All of other reagents were analytical grade from E. Merck, Darmstadt, Germany.

Preparation of alcoholic extract of banana peel (EBP)

The bananas were ripe. The peels were separated manually, then cut into small pieces (±2 mm), dried and powdered. The dried of banana peel (50 g) was extracted with 250 ml 70% ethanol at room temperature for 5 days. The extract was then concentrated using a rotary evaporator. The crude extract weighted and then kept in dark glass bottle in a freezer dryer for further use.

Animal

Healthy adult male Wistar rats with weight 150 to 200 g were obtained from the Laboratory Animal Gadjah Mada University. The animals were maintenance under 12:12 h day and light schedule with temperature between 22 to 24^{o}C. The animals were placed in large spacious hygienic cages during the experimental period. The animals were given food and water regularly. All procedures were carried out according to the stipulations of the Institutional Animal Care and Use Committee (IACUC) Airlangga University, Indonesia.

Excision wound model

A total of 64 animals were divided into four groups (control and extract treated 3 groups). Each group containing sixteen animals were anaesthetized. The rats were depilated on the back and cutaneous circular wound of 1.5 cm diameter were inflicted on the pre-shaved sterile dorsal surface of the animal by cutting. Each animal were given single wound. The wound was left undressed to the open environment. Then the treatment was started in the following manner: Control (Carbophol-400) group, 25% EBP-gel group, 50% EBP-gel group, and 75% EBP-gel group. EBP was topically administrated once a day after cleaning with surgical cotton wool for a period of 16 days. The formation of granulation tissue was observed and blood samples were taken on 4, 8, 12 and 16 days post-wounding for used analyses. The assessment of area wound healing used semitransparent paper and calculation was done using graph paper. Determination of angiogenesis, VEGF, used imunohistochemistry method by staining wound tissue. And determination of collagen used histopathological method by staining studies wound tissue.

Wound assessment

The rate of contraction of wounds was determined by tracing the wound surface using a transparent graph paper and measuring the surface area by planimetry. The period of epithelialization was measured of wound on day 4, 8, 12, and 16. And wound tissues was cut at least 5 µm and stained with Haematoxylin Eosin (HE). The sections were examined under light microscope and photomicrographs were taken.

VEGF assay

Ex-vivo studies involved immunohistochemical analysis for VEGF expression in skin tissue wound animal. The intensity of VEGF positivity was assessed semiquantitative analysis using the Quick Score Method [13], which takes into account intensity and distribution of VEGF containing cell. The intensity of VEGF was scored as follow:

- Negative (no staining of any nuclei at high magnification) = score 0
- Weak (only visible at high magnification) = score 1
- Moderate (readily visible at low magnification) = score 2
- Strong (strikingly positive at low magnification) = score 3

The number of stained cells was scored as follow: 0% = score 0, 1-25% = score 1, 26-50% = score, 51-75% = score 3 and 76-100% = score 4. The intensity score added by the stained cells score gave a Quick score in the range of 0-7 (0 = negative; 1-5 = moderately positive; 6-7 = strongly positive).

Masson's Trichrome

Sections were dewaxed and rehydrated conventionally, placed in Weigert's haematoxylin stain for 1 h, rinsed under lukewarm water for 5 min, immersed in Masson solution for 15 min, and rinsed in deionized water before placing in phosphomolybdic acid for 10 min. Subsequently, sections were immersed in 2% aniline blue for 15 min, rinsed in 1% acetic acid, 95% ethanol, and absolute ethanol in turn, immersed in xylene for 10 min, and mounted with resin. Collagen fibers were stained blue, cytoplasm and erythrocyte were stained red, and nuclei were stained bluish brown.

Statistical analysis

The results were expressed as mean ± standard error mean (SEM). The statistical significance was assessed using one way analysis of variance ANOVA and non-parametric Mann–Whitney *U*-test. $P < 0.05$ was considered significant. The statistical analyses were performed using SPSS statistical version 20.0 software package (SPSS® Inc., USA).

RESULT

The rate of contraction wound in control and extract treated group are depicted in Figs. 1 and 2. The wounds in extract treated group were found to contract much faster than control group. The epithelialization period of the treated wounds showed a significant decrease in 50% EBP-gel (1.32 ± 0.30) and 75% EBP-gel (0.85 ± 0.36) than control group (2.06 ± 0.07) on day 12 after wounded (Table. 1). Fig 1 illustrated the comparison of tensile strength of tissues for control and extract treated group.

Table 1: Effect re-epithelization of EBP on area wound in each group of rats for 16 days

Group	Wound Contraction (cm²)			
	4	8	12	16
Control	4.12 ± 0.20	3.19 ± 0.32	2.06 ± 0.07	0.69 ± 0.36
25% EBP-gel	3.51 ± 0.49	3.00 ± 0.47	2.02 ± 0.20	0.49 ± 0.26
50% EBP-gel	3.06 ± 0.37	2.84 ± 0.28	1.32 ± 0.30	0.15 ± 0.04
75% EBP-gel	3.39 ± 0.41	2.81 ± 0.23	0.85 ± 0.36	0.03 ± 0.02

Value are expressed as mean ± SEM; n = 4, One Way ANOVA followed Tukey *t*-Test F-values denotes significant at $^{*}P<0.05$.

The epithelialization period of the extract treated group showed a significant decrease on day 16 (Fig. 1D). Fig. 1D compares the tensile strength of tissues from the control and experimental incision wounds. Observations on day 16 at dose 75% showed that the wound become close and started to grow hair on parts of the wound.

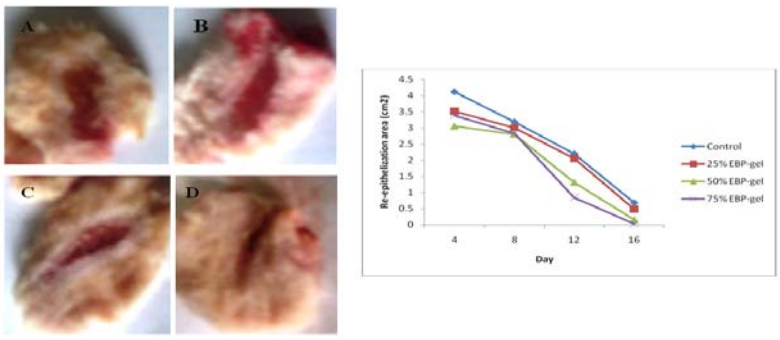

Fig. 1: Administration EBP gel topically accelerated wound healing in rats. Full-thickness excision wounds about 1.5 cm in diameter were created on the back male rats. Different concentration of EBP gel and gel base was administered topically once daily for 16 consecutive days starting at wounding. The wounds were completely closed in 75% EBP gel group (D), while in control group (A), 25% EBP-gel group (B), and 50% EBP-gel group (C) were still some open wounds of varied extent

Immunohistochemical analysis expression of VEGF, a marker of vascular endothelial cell, showed that there was a statistically significant increase in the number of new capillary sprouts in the three EBP gel treatment groups compared with the control groups. Expression of VEGF in 50% EBP gel was 4.37 ± 1.11 and 75% EBP-gel was 6.56 ± 0.51 compared with control group was 1.81 ± 0.62 on day 12 (fig. 2).

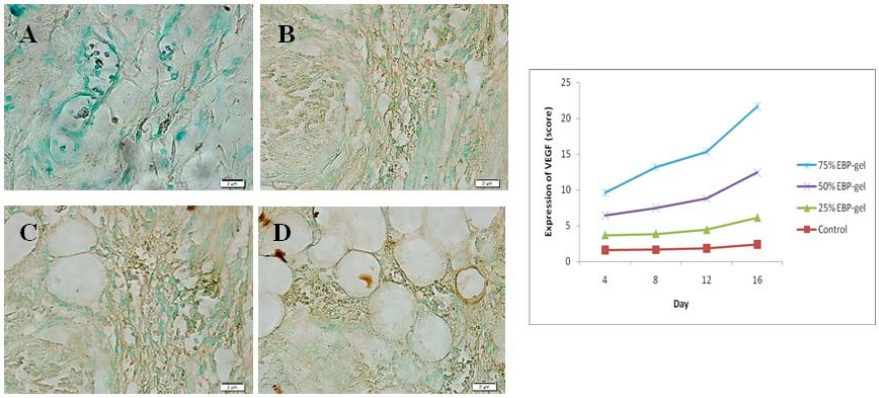

Fig. 2: Expression of VEGF related antigen in wound tissue on day 16 after wounding in each group. Immunostaining of VEGF related antigen, a marker of vascular endothelial cell, in control group (A), 25% EBP gel group (B), 50% EBP gel group (C) and 75% EBP gel group (D). The number of new capillary sprouts was counted under 400x visual at least five visual fields of wounds were counted per slide. Statistical analysis showed that there was a significant increase in new capillary sprouts in two EBP gel treatment groups compared with control groups on day 12 and day 14. Expression of VEGF was showed in 50% EBP gel (6.31 ± 2.05) and 75% EBP-gel (9.25 ± 2.24) groups compared with control group (2.37 ± 1.03) on day 16

Masson trichrome staining and histological evaluation showed correlation between macroscopic and microscopic appearances of wounds. Masson trichrome staining showed that wounds developed vascular granulation tissue tended to be populated with inflammatory cells and fibroblasts, and partially formed newly formed epithelium at the edge of the wounds in all of the three groups at day 12 after wounding. At day 12, there was thicker and more highly organized collagen fiber deposition, populated with less inflammatory cells and more fibroblasts, in wounds treated with EBP, especially in gel 75% EBP group.

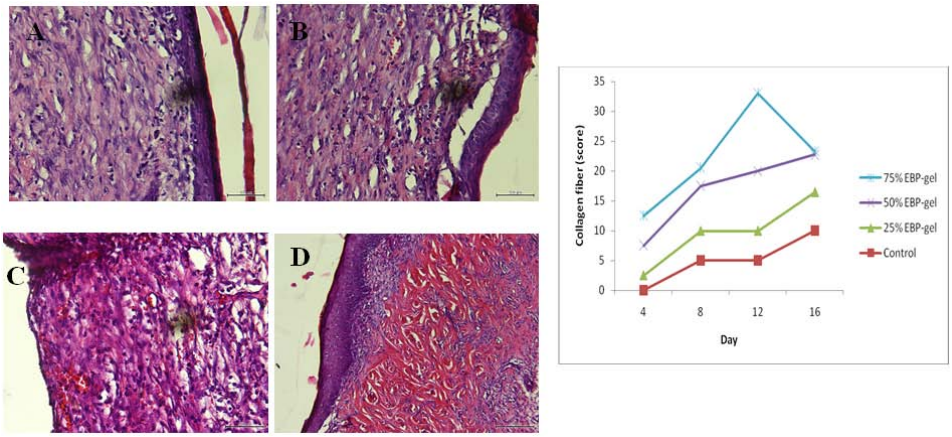

Fig. 3: Masson trichrome was staining of the wounds on day 14 after wounding in each group. Masson trichrome stained tissues from control group (A), 25% EBP-gel group (B), 50% EBP-gel group (C), and 25% EBP-gel group (D) on day 12. The staining results showed that wounds developed vascular granulation tissue, tended to be populated with inflammatory cells and fibroblasts, and partially formed newly formed epithelium at the edge of the wounds in all of the two groups on day 8 after wounding, while on day 12, there were thicker and more highly organized collagen fibers deposition in wounds treated with EBP-gel, especially in 75% EBP-gel group

DISSCUSSION

The clinical treatment of skin loss due to severe and massive burns or wounds continues to be a major problem in surgical procedures. A therapeutic agent selected for the treatment of wounds should ideally improve one or more phases of healing without producing deleterious side effects. The present investigation describes some unique features with respect to the therapeutic effect of bark extract from a tropical evergreen plant *Musa acuminata* Colla on rat dermal wound healing. Plant products are potential agents for wound healing and largely preferred because of their widespread availability, non-toxicity, absence of unwanted side effects and their effectiveness as crude preparations. Several study has been reported that *Centella asiatica* L and *Terminalia chebula* Retz were effective in wound healing in rats [6, 7]. Collagen fibers treated with the plant flavonoid compound, catechin, have been found to be stable [14]. These findings prompted us to further investigate other tropical plants, which had reported medicinal values, for wound healing. Accordingly, systematic in vivo studies of *Musa acuminata* Colla in wound healing activity were carried out.

The processes involved in wound healing are epithelialization, contraction with connective tissue deposition. The involvement of each phase varies over a spectrum dependent largely on the type, location, and milieu factors influencing the wound. Epithelialization is the process where keratinocytes migrate from the lower skin layers and divide. Contraction is the process where the wound contracts, narrowing or closing the wound. The movement of fibroblast in the wound area facilitates matrix formation and collagen is laid down over and throughout the amorphous material.

The processes of wound repair were also controlled by a wide variety of different growth factors and cytokines, such as VEGF [15], fibroblast growth factor, epidermal growth factor, and so on. Their expression dynamics showed characteristic temporal and spatial regulation, and changes in the expression pattern of growth factors were associated with impaired wound healing.

VEGF is released in large amounts from degranulating platelets, and it is present in wound fluid, particularly early after injury. The expression of VEGF related antigen, a marker of vascular endothelial cell, a marker of proliferation, in the wounds showed that there was a increase in the number of new capillary sprouts in the three EBP gel groups at varied extent, especially in 75% EBP gel groups, compared with that in control group on day 12 and day 16 after wounding.

At day 12, Masson trichrome staining showed there were thicker and more highly organized collagen fiber deposition, populated with less inflammatory cells and more fibroblasts, in wounds treated with EBP gel, especially in 75% EBP gel group. However, the granulation tissue was thin, dominated by inflammatory cells, and collagen fiber alignment was disorganized in wounds of control group. The effects of topically applied EBP gel were dose dependent, especially 75% EBP gel per day, had much higher percentage wound closure, re-epithelialization area, new capillary sprouts compared with rats receiving 50% EBP-gel per day. Topically administered drugs are effective in faster wound contraction due to the larger availability at the wound site. In our study, the rate of wound contraction in treated rats was significantly higher. Furthermore, the period of epithelialization was shorter in the treated wounds. These results further support the effectiveness of *Musa acuminata* Colla wound healing. Deposition of newly synthesized collagens at the wound site increases the collagen concentration per unit area and hence the tissue tensile strength.

CONCLUSSION

Topical administration of *Musa acuminata* Colla extract improved the different phases of wound healing through VEGF expression, collagen synthesis and re-epithelialization.

CONFLICT OF INTERESTS

Declared None

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