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The Effect of *Xylocarpus granatum* J. Koenig Seed Extract Cream on the Number of Fibroblast and Re-Epithelialization in IIA Degree Burn Wound Healing

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ABSTRACT

Xylocarpus granatum J. Koenig (XG) is a popular plant that grows in the mangrove forest of Bali. Flavonoids, saponins, phenolics and tannins are active metabolites of this plant, which have antiinflammation, antioxidant and wound healing effects. IL-1β, IL-6 and TNF-α are pro-inflammatory cytokines involved in the pathophysiology of the wound. Flavonoids trigger increased wound contraction and reduced the period of re-epithelialization. Re-epithelialization plays a role in wound closure. Saponins increase fibroblast migration and proliferation as indicated by the amount of high cell density. Fibroblasts support granulation tissue synthesis, ECM production, and collagen synthesis, thereby accelerating wound healing. Tannins have antibacterial activity and increase the angiogenesis. Phenolics can stimulate wound contraction, migration of fibroblasts, and re-epithelialization processes in wound healing. The present study aims to evaluate the effectiveness of XG extract seeds cream on burn wound grade IIA in mice. XG extract was evaluated for antioxidant activity using the DPPH method. The wound healing activity of XG creams (10% and 15% concentration) were evaluated from wound contraction, histological analysis of fibroblast proliferation and re-epithelialization in mice. Statistical analyses were performed with post hoc Tukey using analysis of variance and were analyzed using SPSS, Version 26. XG extract has a very strong antioxidant activity (IC_{50}=7.939ppm). The treatment with XGC 15% promotes significant increases in wound contraction rate (74.91%), proliferation of fibroblasts (p value = 0.521) and re-epithelialization (p value = 0.521) with SSD. These results suggest that XGC 15% is effective in wound healing in mice by increasing the wound contraction rate, proliferation of fibroblasts and re-epithelialization.

Key words: antiinflammation, antioxidant, cream, wound healing, *X granatum* extract

INTRODUCTION

Burn injury is defined as tissue injury caused by friction, heat, radiation, light, cold, chemical or electricity. Most burn wounds are common household and workplace injuries caused by heat from hot liquids and solids or fire. Burn wounds remain one harmful trauma with significant cost and time-consuming management (Shahzad and Ahmed, 2013; Bahramsoltani, Farzaei and Rahimi, 2014; Fan *et al.*, 2015a; Jeschke *et al.*, 2020a).

Table I. The formulation of *X. granatum* cream

Ingredients	Function	XGC 10%	XGC 15%
***X. Granatum* seed extract**	Active ingredient	10.00%	15.00%
Liquid paraffin	Emollient, solvent	45.00%	45.00%
Cera Alba	Stiffening agent	13.50%	13.50%
TEA	Emulsifier	2.70%	2.70%
Methylparaben	Preservative	0.05%	0.05%
Propylparaben	Preservative	0.05%	0.05%
Water	Vehicle	28.70%	23.70%

XGC 10%: treated with 10% concentration of *X. granatum* extract cream; XGC 15%: treated with 15% concentration of *X. granatum* extract cream

The classification of burn wounds is based on depth and size. Depending on the size and depth severity, burn wounds are classified into three degrees. More than 50% percent of burn wound cases in Iraq, Saudi Arabia, Taiwan and Indonesia were IIA degree burns as superficial partial-thickness burns (Chen *et al.*, 2014; Lami and Al Naser, 2019; Alajmi, Aldosari and Al-Ghamdi, 2021; Sudarsa *et al.*, 2021). Superficial partial-thickness burns (known as 2A burns) are painful, need dressing and wound treatment, but don't require surgery (Martin and Falder, 2017; Jeschke *et al.*, 2020b).

Superficial partial-thickness burns are treated with topical antiphlogistic and antibiotic ointments such as silver sulfadiazine. However, its use should be restricted for long therapy. Silver sulfadiazine has limitations, including limited penetration to the depth of the wound, slow and incomplete re-epithelization, generation of black scars, and hypersensitivities such as skin discoloration, multiforme, pruritis, rash, and Stevens-Johnson syndrome. The most common side effects of silver sulfadiazine is hematologic effects, including aplastic anemia, agranulocytosis, hemolytic anemia, and leukopenia (Panahi, 2012a; Oaks and Cindass, 2021).

Treatment goals in burn management are prevention of infection, elimination of nonviable tissue, wound healing, and controlling pain with minimal side effects (Kowalske, 2011; Panahi, 2012b). Eighty percent of populations in Asia and African countries trust traditional medicine for primary health care (Dorai, 2012). To date, alternative and complementary medicines, such as traditional medicine, are less expensive and moderately beneficial to effective without toxicity or low toxicity (Fan *et al.*, 2015b; Sharifi *et al.*, 2021a). Several biological activities of plants have been identified to possess potent wound-healing or burn-wound-healing through anti-inflammatory, anti-infectious, and antioxidant activity (Panahi, 2012b; Fan *et al.*, 2015b; Sharifi *et al.*, 2021b).

X granatum J. Koenig is a mangrove species with secondary metabolites diversity, such as tannin, terpenoid, alkaloid, saponin, flavonoid, anthraquinone, and cardiac glycoside (Shi *et al.*, 2017; Tomizawa *et al.*, 2017; Islam *et al.*, 2019a; Polyium, 2020). These plants are distributed in tropical and subtropical coastal, especially in Indonesia. Traditionally, this plant is used for inflammation, dysentery, fever, and abdominal problems. Flavonoids trigger increased wound contraction and reduce the period of re-epithelialization, saponins can increase fibroblast migration and proliferation, and tannins have antibacterial activity and increase angiogenesis. The presence of secondary metabolites in X. *granatum* means this plant has the potential to shorten the healing time of superficial partial-thickness burns (IIA degree) (Alizadeh *et al.*, 2020; Pringgenies *et al.*, 2021).

A recent study showed that the lotion with the combination of *X granatum* fruit and sodium alginate extracts is potent in wound treatment (Pringgenies *et al.*, 2021). The other study showed that *X granatum* have potency strong antioxidant and anti-inflammatory potential (Islam *et al.*, 2019b). Furthermore, *X granatum*'s seeds have skin-emolliating properties (Pringgenies *et al.*, 2021). Most secondary metabolites, such as flavonoids and terpenoids are highly polar and consequently poorly absorbed in lipids, as there is difficulty in crossing highly lipid-soluble biological membranes, leading to poor bioavailability (Sawant and Yadav, 2020). Based on these studies, *X granatum*'s seeds have potential as an active compound in burn wound treatment cream.

To date, there has been no research about using *X granatum* for burn wounds. Therefore, the potential possessed by this plant, *X granatum* may

have potency as a burn wound therapy. The selection of cream preparations is based on the cream vehicle, an important role in the drug delivery mechanism. Cream preparations will increase the thermodynamic activity of the formulation, thereby promoting drug transport through epithelial surfaces such as skin. The cream is easy to apply and stable enough; therefore, it doesn't need frequent application and causes minimal irritation to the skin (Brown *et al*., 2018; Simões *et al*., 2018). Thus, this study aims to determine the effectiveness of *X granatum*'s seeds extract cream on burn wound grade IIA in mice. A study on the effectiveness of *X granatum*'s seeds extract cream as burn wound grade IIA treatment provides information about utilization *X granatum*'s seeds. *X granatum* extract as the main ingredient formulated into the cream can be facilitated into wound healing in male mice models.

MATERIALS AND METHODS

Plant materials

J. Koenig collected *X granatum* from Pemogan Village, Bali, Indonesia. The sample was determined at The Indonesian Institute of Sciences (LIPI) Plant Conservation Center, Eka Karya Bedugul Botanical Gardens, Bali (B.217/IPH.7/AP/III/2017). The seeds were cut into smaller pieces and rinsed with distilled water until clean. Furthermore, the XG seeds were dried in an oven at a temperature of 40ºC for 5 days, then ground into powder. A total of 100 g of XG seed powder was extracted with 1.500 L of ethanol 80% by maceration for 5 days at room temperature, filtrated, and evaporated at 40ºC. The crude extract was kept in a glass bottle covered with aluminum foil in the refrigerator at 4ºC. The crude extract rendement was calculated using the following formula (Susilowati and Purwati, 2021):

$$\text{Rendement (\%)} = \frac{\text{weight extract after extraction}}{\text{weight simplicial before extraction}} x\ 100$$

Phytochemical screening

A total of 0.5 g of XG extract was dissolved in 50 mL of 70% ethanol. This diluted extract was qualitatively tested for the presence of alkaloids, flavonoids, saponins, phenols, tannins, and terpenoids using standard procedures (Manandhar, Bajgain and Neupane, 2021; Siregar, Sinaga and Silalahi, 2022).

Determination of Ash Content

The ash content of the XG extract was examined using the method as described in a previous report (Huang *et al.*, 2020). The ash content was calculated using the following formula:

$$\text{Ash Content (\%)} = \frac{\text{the weight of the ash residue}}{\text{the weight of the sample}} x\ 100$$

Determination of Moisture Content

The moisture content of the XG extract was examined using the method as described in a previous study (Sadiq Abdulrahman *et al.*, 2020a). The moisture content was calculated using the following formula:

$$\text{Moisture (\%)} = \frac{\text{loss in weight after drying}}{\text{initial samples weight}} x\ 100$$

Determination of Total Phenol Content (TPC)

The total phenol content was evaluated using the Folin-Ciocalteu colourimetric method based on oxidation-reduction reaction. Gallic acid (10, 20, 40, 60, 80, 100 mg/L) was used as a standard solution to obtain a calibration curve. The procedure followed a previous study (Phuyal *et al.*, 2020). The total phenol was measured using the spectrophotometer at a wavelength of 760 nm. TPC was expressed as gallic acid equivalent per 100 g extract (g GAE/100 g).

Determination of Total Flavonoid Content (TFC)

The total flavonoid content was calculated with minor modifications using the method from a previous study (Josipovic *et al*., 2016; Nayaka *et al*., 2020). The total flavonoids were measured using the spectrophotometer at a wavelength of 510 nm with a linearity range of the calibration curve from 0.01 to 0.08 mg/ml. Quercetin (40-100 mg/mL) was used as a standard solution. The total flavonoid content was expressed as quercetin equivalent per 100 g extract (g QE/100 g)

Cream Formulation

The extract was formulated into an oil in water (O/W) based cream preparation (Table I). There are two formulas with each extract concentrations of 10% and 15%. The water-soluble component and preservative (propylparaben) were dissolved in the aqueous phase and heated to a temperature of 70-75^{0}C in a water bath. The emulsifier, preservative (methylparaben), and oil soluble components were dissolved in the oil phase and heated to 70-75^{0}C. After dissolving, the water phase was added to the oil phase, stirring continuously until homogenous and a creamy mass formed. The heating was then stopped, and the extract was added to the cream mass and

stirred until homogeneous. These two cream formulas will then be tested for their physical stability.

Antioxidant activity

The free radical scavenging activity of XG extract was analyzed using the DPPH method. Two milliliters of 40 ppm DPPH solution were added to 2 ml of the extract solution of each concentration (2 ppm; 4 ppm; 6 ppm; 8 ppm; 10 ppm; 12 ppm), and the mixture was vortexed and stored in a dark room at room temperature for 30 minutes. Optical density (OD) was measured at 517 nm (UV/Vis spectrophotometer). The IC50 was calculated using a calibration curve in the linear range by plotting the corresponding scavenging effect vs. the extract concentration. The radical scavenging activity was measured with the following formula:

$$\text{Scavenging \%} = \frac{(A_0 - A_1)}{A_0} x\ 100 \ldots \text{(basma } et\ al.\text{, 2011)}$$

A_0 = negative control absorbance (without sample)
A_1 = sample absorbance

Burn Wound Model

The Institutional Ethical Committee, University of Surabaya approved all protocols of this animal experiment (approval number: 185/KE/VIII/2021). All mice (*Mus musculus L*) were aged between 3-4 months and weighed between 25-35 g. The mice were anesthetized using ketamine at a dose of 40 mg/kgBW and xylazine at a dose of 5 mg/kgBW (im). The hairs on the dorsal of mice were removed 3-5 cm. The process of IIA degree burns, an iron with a diameter of 1 cm is heated in boiling water at 100ºC for 3 minutes and affixed to the dorsal of the mice for 10 seconds (Kalantar *et al.*, 2016; Oryan *et al.*, 2018). The 24 male mice were randomly and equally divided into four groups: the cream base group (CBG) as negative control, silver sulfadiazine (SSD) as positive control, *X granatum* cream 10% (XGC 10%) and *X granatum* cream 15% (XGC 15%) as the extract therapy group. The wounds were treated twice a day every morning and evening for 14 days.

Wound Healing Measurement

Wound contraction and macroscopic view were the two factors examined for burn wound healing observation. The wound area was measured, and photographs of the wound area were taken on day 0 until day 14. The percentage of wound contraction was measured using the following formula (Kalantar *et al.*, 2016).

$$\text{Wound contraction (\%)} = \frac{A - B}{A} x\ 100\%$$

A : initial wound size; B: specific day wound size

Histological Analysis

The process of histological preparations excised 1 cm of skin in the wound area. Tissue samples were immersed in 10% neutral buffered formalin for 24 hours for histological analysis. Tissue were embedded in paraffin wax after tissue samples were degraded in a series of alcohol concentration. Tissue samples were sectioned at 3-4μm. Hematoxylin and Eosin (H&E) staining was used to determine the microstructure of the skin tissue, including granulation tissue, score of fibroblasts, and re-epithelialization. Wound tissue was seen with an OLYMPUS XC10 series photomicroscope equipped with Olyvia software (Viewer for Imaging Applications) with a magnification of 400 times per field of view.

Statistical Analysis

Quantitative data are presented as mean or median ± standard deviation. Statistical analyses were performed with post hoc Tukey using analysis of variance and were analyzed using Statistical Package for the Social Science (SPSS, Version 26).

RESULT AND DISCUSSION

Characterization Extract

Phytochemical screening of XG extract using color reaction, obtained secondary metabolites, including flavonoids, saponins, phenolic and tannins (Supplementary A). The crude extract rendement of XG was obtained at 13.751±0.001%. The percentage of rendement was used to determine the weight of simplicia compared to the weight of the crude extract (Susilowati and Purwati, 2021). The XG extract ash content was 5.569±0.014%. Another report revealed that the ash content of the *X granatum* seed extract obtained 2.06% and the *X moluccensis* seed extract obtained 9.77% (Patil, 2019). High ash content indicates the percentage of inorganic mineral elements present in the extracts (Sadiq Abdulrahman *et al.*, 2020a). The XG extract moisture content was 2.052±0.002%. Meanwhile, the moisture content of the *X granatum* extract obtained in the Sindhudurg district of India was 9.36% (Patil, 2019). The low moisture content indicates that the XG extract will not be susceptible to enzyme activities and microbial growth (Sadiq Abdulrahman *et al.*, 2020b).

Table II. Macroscopical observation of each groups

Wound healing process	Macroscopic observation for each group (days)							
	CBG		SSD		XGC 10%		XGC 15%	
	Initiation	Duration ($\bar{x}$±SD)	Initiation	Duration ($\bar{x}$±SD)	Initiation	Duration ($\bar{x}$±SD)	Initiation	Duration ($\bar{x}$±SD)
Wound burn	0	0	0	0	0	0	0	0
Secreting fluid	1	1.92±0.20	1	0.92±0.20	1	1.75±0.42	1	0.83±0.26
Scab	4	1.92±0.20	3	0.92±0.20	4	1.83±0.26	3	0.83±0.26
Granulation tissue formation	7	6.83±0.26	5	4.75±0.42	7	3.83±0.26	5	3.75±0.42
Epithelial tissue formation	-	-	11	*	12	*	10	*

CBG: negative control group (treated with cream base); SSD: treated with silver sulfadiazine cream; XGC 10%: treated with 10% concentration of *X. granatum* extract cream; and XGC 15%: treated with 15% concentration of *X. granatum* extract cream. (*) Epithelial tissue usually completely formed more than 14 days, but this study only observed macroscopic healing process until 14 days.

TPC and TFC in analysed XG extracts

The total flavonoid content of the XG extract was 11.686±0.004 g QE/100 g. Research conducted by Saeed shows a correlation between effective capacity of scavenging for superoxide radical of extract with total flavonoid content; therefore, suggesting its antioxidant potential (Saeed, *et al*., 2012). The total phenolic content of the XG extract was 4.684±0.035 g GAE/100 g. The antioxidant activity of phenolic compounds depends on the degree of hydroxylation of the aromatic ring and the most active from three to six hydroxyl groups (Merghem and Dahamna, 2020).

Physical Quality of Cream Preparations

XGC 10% cream and XGC 15% cream formulas appear as a semisolid texture with a reddish-brown color due to the XG extract component. The cream has good homogeneity, with a pH of 6.5. The preparation of the XG extract cream has ideal homogeneity, indicated by no separation of the water and oil phases (Al-Busaid *et al.*, 2020). Homogeneity is important to ensure that each part of the preparation contains the same amount of active ingredients. The therapeutic effect cannot be obtained continuously if the active ingredients are not evenly dispersed in the base material (Suena, *et al.*, 2017). The XG extract cream has met the pH criteria for cream preparations, in the range of 4-6.5 (Pengon *et al.*, 2018; Purwaningsih, *et al.*, 2020). This is in accordance with the skin pH range of 4.5-6.5 (Mulia *et al.*, 2018), so it's not expected to cause irritation if the pH of the preparation is too acidic or causes the skin to become dry if the pH of the preparation is too alkaline (Suena *et al.*, 2017). XG extract cream is considered to have good physical quality, where the homogeneity and pH of the preparation are safe for use on the skin.

Antioxidant Activity

The results of the antioxidant activity of the XG extract obtained an IC_{50} value of 7.939 ppm. This shows that the antioxidant activity of the XG extract is very strong. The sample, which had IC_{50} values lower than 50 ppm, was a very strong antioxidant, 50-100 ppm was a strong antioxidant, 101-150 ppm was a medium antioxidant and greater than 150 ppm was a weak antioxidant (Blois, 1958; Kuspradini *et al.*, 2018).

Determination of Wound Contraction

Macroscopic observations showed that on days 1 and 2, there was fluid secretion in the wound area for all groups. The scab started to form in the SSD and XGC 15% groups on day 3 but were delayed in negative controls and XGC 10% (day 4). Granulation tissue was formed on day 5, which was marked by a reddish color in wound area of the 15% SSD and XGC groups. Granulation tissue to remove on day 9 for the XGC 15% group was replaced by whitish epithelial tissue on day 10. An opposite pattern was seen in the negative control, where epithelial tissue did not occur until day 14 treatment (Table II). The proliferative phase begins once homeostasis is achieved, and the inflammatory response is balanced. This phase includes the process of angiogenesis, granulation tissue formation, wound retraction, collagen deposition, and epithelialization (Singh, Young and McNaught, 2017). The formation of capillaries stops when the body's physiological needs are sufficient (Li and Wang, 2011).

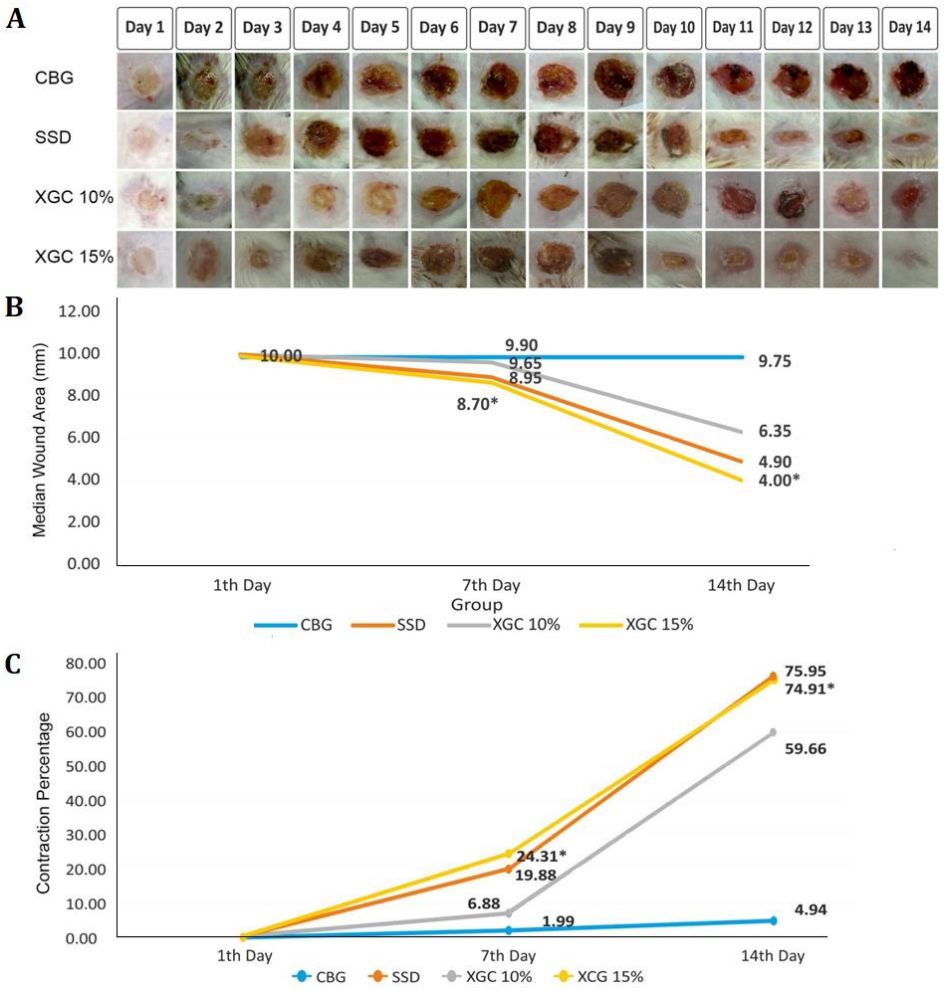

Figure 1. (A) Macroscopic view of burn wound on day 1 to day 14. The wound area was photographed by the same examiner from days 0 to 14 to evaluate the progress of wound closure; (B) Wound diameter in three time intervals between the studied groups, presented in the median value; (C) Contraction percentage in three time intervals between the studied groups. (*) not significant (p>0,05) compared with positive control group using Kruskal Wallis with post-hoc Mann-Whitney

Wound healing was assessed by comparing the wound area and percentage of wound contraction on days 1, 7 and 14. The decrease in wound area was linear with the increase in the percentage of wound contraction (Figure 1). On day 1, the median of the wound area in all groups was almost similar. On the 7th day, the effectiveness of XGC 15% in reducing the wound area was not significantly different from SSD, which was offset by an increase in the percentage of wound contraction. On the 14th day, the wound area on XGC 15% decreased significantly (P<0.05) compared to CBG and was not significantly different from SSD (P>0.05), with the percentage of wound contraction increasing. The higher percentage of wound contraction of the XGC 15% may be due to its dose-dependent antibacterial activity or induction of macrophage cell proliferation (Demilew, *et al.,* 2018)

Histological Evaluation

The results of post hoc Tukey analysis showed no significant difference in the score of fibroblasts between XGC 15% and SSD, with p value = 0,521 (P> 0.005). The mean value of fibroblasts in XGC 15% (39,23±0,46) was lower than CBG (126.40±1.32) (Table III) (Figure 2).

Table III. Statistical analysis of wound assessment data at the 14th Day

Wound Assessment	N	Treatment Group	($\bar{x}$±SD)	*p*-value
Wound diameter	6	Negative control group	-0.610±0.096	0.001
	6	Positive control group	0.702±0.032	0.128*
	6	Intervention group 1	0.555±0.038	0.001
	6	Intervention group 2	0.775±0.014*	0.128*
Epithelialization tissue's	6	Negative control group	2.903±0.085	0.001
	6	Positive control group	2.243±0.115	0.351*
	6	Intervention group 1	2.480±0.074	0.001
	6	Intervention group 2	2.131±0.164*	0.351*
Fibroblast	6	Negative control group	126.40±1.32	0.001
	6	Positive control group	38.50±0.59	0.521*
	6	Intervention group 1	78.20±1.02	0.001
	6	Intervention group 2	39.23±0.46*	0.521*

*not significant (p>0,05) compared with positive control group

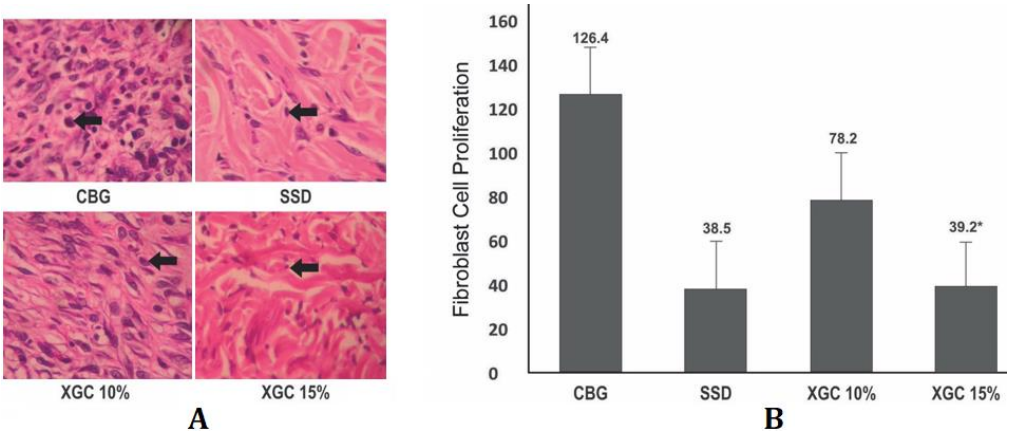

Figure 2. (A) Histological appearance of fibroblasts in H&E stained with H&E at day 14 are shown. Fibroblasts will be purple with H&E staining. Migration of fibroblasts was more appearance in the wound margins in all groups. (B) The mean value of fibroblast cell proliferation. Magnification: 400x. Abbreviations: H&E, hematoxylin and eosin; (*) not significant (p>0,05) compared with positive control group

The score of fibroblasts decreased on day 14 when the wound ECM had the same tensile strength as the surrounding healthy tissue. Myofibroblast apoptosis will increase when the wound is closed, and apoptosis triggers granulation tissue to develop into scar tissue (Bainbridge, 2013). Fibroblasts are important in wound healing, especially at the cell migration stage, so that the wound is closed (Eming, Martin and Tomic-Canic, 2014).

Histologically, XGC 15% had significant effectiveness with SSD in the re-epithelialization (P>0,05), with mean SSD values (2,243±0,115) and XGC 15% (2,131±0,164). The XGC 10% showed significant difference in epithelial thickness compared with the 15% XGC group. The epithelial tissue in the CBG was the thickest compared to the other groups, with a mean value of 2,903+0.085. (Hammad *et al.*, 2011) showed that the epithelial tissue was thickest in the initial phase of the wound (day 0) and decreased gradually (day 7, 14, until day 21). Day 14 of his observations showed significant decrease in epithelial tissue compared to other days. The study of (Demilew *et al.,* 2018) showed the re-epithelialization period was achieved on day 13; (in the extract therapy group, whereas in the positive control, the epithelialization period was achieved on day 15). These studies support the results of this study, where the epithelial tissue on XGC 15% was thinner than CBG on day 14 (Figure 3).

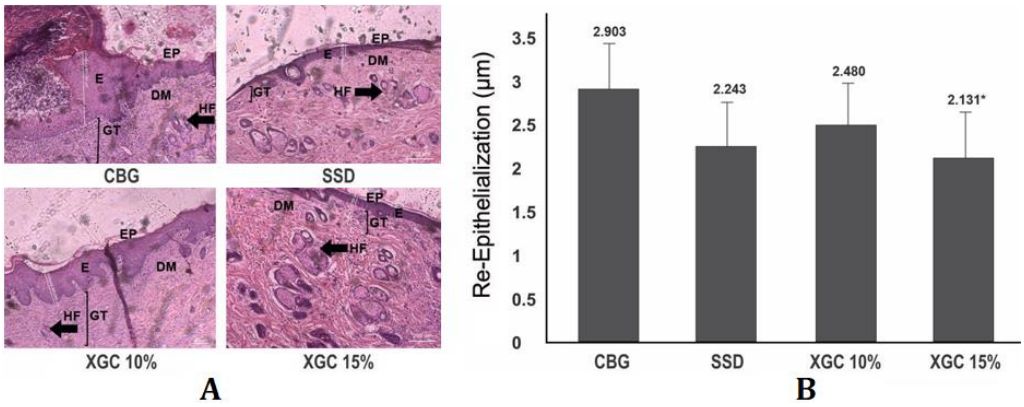

Figure 3. (A) Histological appearance of burn wound stained with H&E. The XGC 15% group showed the same wound healing as SSD (re-epithelialization and development of granulation tissue). (B) The mean value of re-epithelialization at day 14. Magnification : 400x. Abbreviations: CBG, Cream base group; SSD, silver sulfadiazine cream; XGC 10%: *X. granatum* extract cream 10% concentration; XGC, 15%: *X. granatum* extract cream 15% concentration; EP, epidermis; DM, Dermis; E, Epithelialization; GT, granulation tissue; HF, hair follicle; (*) not significant (p>0,05) compared with positive control group.

In response to tissue injury, the body initiates an inflammatory response. Immune cells travel to the wound area and secrete pro-inflammatory cytokines, characterized by increased levels of IL-1β (Interleukin-1β), IL-6 (Interleukin-6) and TNF-α (Tumor Necrosis Factor-α) (Ibrahim *et al.*, 2018; Elshamy *et al.*, 2020). These pro-inflammatory cytokines can induce the synthesis of MMP in inflammatory cells and fibroblasts. High levels of proteases and cytokines can trigger the secretion of proteases that can slow wound closure and damage tissues (Muhammad *et al.*, 2016). Flavonoid compounds have anti-inflammatory activity by lowering levels of IL- 1β and TNF-α, ther (Bhatia *et al.*, 2014; Elshamy *et al.*, (2014; 2020).

The Free radicals (Reactive Oxygen Species) are mostly produced by neutrophils and macrophages when injured (Kim *et al.*, 2011; Kurahashi and Fujii, 2015). ROS is normally produced in low levels and plays as cellular signaling in response to stimuli. ROS are also involved in the re-epithelialization process. Several studies have shown that moderate levels of H_2O_2 regulate the vascular endothelial growth factor (VEGF) production, thereby accelerating angiogenesis. H_2O_2 facilitates the migration and proliferation of epidermal cells, triggers activation of keratinocyte growth factor (KGF) and receptors for the epidermal growth factor (EGF), induces the production of TGFα (EGF member) in fibroblasts and protects the body from bacterial infections (Kurahashi and Fujii, 2015; Ibrahim *et al.*, 2018). However, in the early stages of injury, ROS (e.g., peroxin nitrite and super peroxide) are produced in excessive amounts, increasing tissue damage and inhibiting angiogenesis due to increased microvascular permeability (Bhatia *et al.*, 2014; Kurahashi and Fujii, 2015). High levels of ROS can also trigger the activation of several transcription factors, including activator protein 1 (AP-1), nuclear factor kappa B (NF-kB), mitogen-activated protein kinase (MAPK) pathways, and nuclear factor erythroid-derived 2-like 2 (Nrf2) (Cruz, 2020). XG extract has an IC_{50} value of 7.939 ppm and is categorized as a very strong antioxidant. Flavonoids in XG extract play as antioxidants that bind to ROS to form inactive compounds (Bhatia *et al.*, 2014; Kurahashi and Fujii, 2015; Kalantar *et al.*, 2016). Flavonoids can reduce lipid oxidation by increasing vascularity and preventing cell necrosis (Begashaw *et al.*, 2017). Flavonoids can significantly heal wounds by increasing wound contraction, increasing collagen deposition, and reducing the period of epithelialization (Avula *et al.*, 2013). In addition, flavonoids also have antibacterial and astringent activity (Muhammad *et al.*, 2016; Nagar *et al.*, 2016).

In this study, topical administration of XGC 15% showed an increase in the rate of wound contraction and a decrease in the period of re-epithelialization. Saponins can increase fibroblast migration and proliferation as indicated by the number of new blood vessels in the wound area and

high cell density (Begashaw *et al.*, 2017). Fibroblasts play a role in granulation tissue synthesis, collagen synthesis, and ECM production (Singh, Young and McNaught, 2017). Fibroblasts appear early in the proliferative phase (24-48 hours) and produce matrix metalloproteinases (MMPs) to degrade fibrin (Bainbridge, 2013). In wound tissue, fibroblasts will differentiate into myofibroblasts, which then produce extracellular matrix (ECM) to replace MMPs. This process occurs simultaneously with the secretion of transforming growth factor (TGF-β) and fibroblast growth factor (b-FGF), inducing matrix deposition and hyaluronic acid secretion, thereby accelerating wound healing.

The components of ECM include glycoproteins, collagen I–IV, XVIII, thrombospondins, proteoglycans, hyaluronic acid (HA) and heparan sulfate, glycosaminoglycans (GAGs), and laminin. Extracellular matrix triggers angiogenesis, generation-tissue, and epithelization (Kim *et al.*, 2011; Li and Wang, 2011; Bainbridge, 2013; Addis *et al.*, 2020; Pringgenies *et al.*, 2021). In healing wounds, saponins work as anti-inflammatory, antioxidant and antibacterial. Fruticesaponin B is known to have a very high anti-inflammatory activity. Saponins can trigger epidermal cell proliferation and increase the rate of keratin cell migration, important in re-epithelialization (Kim *et al.*, 2011).

Tannins can increase the formation of capillaries, which are important in the angiogenesis process. The secretion of FGF, PDGF and TGF-b by platelets can trigger angiogenesis due to hemostatic blockage (Singh *et al.,* 2017). In the formation of granulation tissue, tannins play a role in increasing wound contraction and the number of fibroblasts. TGF-b and PDGF trigger fibroblasts to migrate to the wound area. In the remolding phase, tannins trigger the cicatrisation process by forming scar tissue. The cellular mechanism of tannins occurs through up-regulation of immunohistochemistry, transcription, and translation of VEGFA. VEGF triggers angiogenesis through the activation and growth of endothelial cells, macrophages and blood vessels (Li *et al.*, 2011; Singh *et al.,* 2017). Tannins function as astringent agents that cause skin pores to shrink, stop bleeding and exudate to prevent bleeding in wounds. Tannins are also known to have antibacterial activity against *S aureus* and *K. pneumoniae* bacteria, often found in wounds. The antibacterial mechanism of tannin against *S. aureus* and *K. pneumonia* is to destroy the bacterial cell wall (Li *et al.*, 2011; Fetse *et al.*, 2014; Muhammad *et al.*, 2016; Su *et al.*, 2017; Demilew, Adinew and Asrade, 2018). Phenolic compounds can stimulate wound contraction, migration of fibroblasts, cell proliferation, COL-1, VEGF, FGF, TGFβ and PDGF expression, and re-epithelialization processes in wound healing. Phenolic compounds also act as antioxidants, anti-inflammatory and antimicrobial activity (Juneja *et al.*, 2020; Momtaz *et al.*, 2020; Melguizo-rodríguez *et al.*, 2021)

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CONCLUSIONS

X granatum extract as the main ingredient formulated into the cream can facilitate wound healing in male mice models. Topical administration of XGC 15% increased the rate of wound contraction in the treated group. A histological analysis, XGC 15% had significant effectiveness with SSD to scores of fibroblast proliferation, granulation tissue, and re-epithelialization. Flavonoids are secondary metabolites of XG extract with very strong antioxidant activity and are responsible for wound healing. However, this study is focused on establishing the role of XG seed extract in wound healing by increasing wound contraction rate, the proliferation of fibroblasts and re-epithelialization. Further research is needed to evaluate wound healing activity using fractionated extracts of XG.

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