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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

Abstract

Ethnopharmacological relevance: X.*granatum* J.Koenig (XG) is a popular plant that grows in the mangrove forest Bali. Traditionally, this plant is used mainly for antiinflammation, dysentery, fever, and abdominal problems. Flavonoids, tannins and saponins are active metabolites of this plant, which has antiinflammation, antioxidant and healing effects.

Purpose: The aim of present study to evaluate the effectiveness XG extract seeds cream on burn wound grade IIA in mice

Methods: XG extract was evaluated for antioxidant activity by DPPH method. The two cream formulations were measured for physical quality for organoleptic, homogeneity, and pH. The wound healing activity of XG creams 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 Statistical Package for the Social Science (SPSS, Version 26).

Results: XG extract has a very strong antioxidant activity with an IC50 value of 7,939 ppm. The preparation of XG extract cream has homogeneous and pH of 6,5. The treatment with XGC 15% promote significant increases in wound contraction rate, proliferation of fibroblasts and re-epithelialization with SSD (p>0,05)

Conclusion: These results suggest that XGC 15% is effective in wound healing in mice by increasing wound contraction rate, proliferation of fibroblasts and re-epithelialization

Introduction

Burn injury is defined as tissue injury caused by friction, heat, radiation, light, cold, chemical or electricity. The majority of burn wounds are common household and workplace injuries that caused by heat from hot liquids and solids, or fire. Burn wounds remain one of harmful trauma with significant cost and time consuming management (Shahzad and Ahmed, 2013; Bahramsoltani, Farzaei and Rahimi, 2014; Fan *et al*., 2015; Jeschke *et al*., 2020). The classification of burn wounds based on depth and size. Depending on the size and depth severity, burn wounds are classified into three degree. More than 50% percent of burn wound cases in Iraq, Saudi Arabia, Taiwan and Indonesia were second-degree burn as superficial partial-thickness burns (Chen *et al*., 2014; Lami and Al Naser, 2019; Alajmi, Aldosari and Al-Ghamdi, 2021; Sudarsa *et al*., 2021). Burn wounds as superficial partial-thickness burns (known as 2A burns) are painful, need dressing and wound treatment, but not require surgery (Martin and Falder, 2017; Jeschke *et al*., 2020).

Initial treatments for burn wound using the 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 wound, slows and incomplete re-epithelization, generation of black scars, and hypersensitivity 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, 2012; Oaks and Cindass, 2021).

Treatments goals in burn management are prevention of infection, elimination of nonviable tissue, wound healing, and controlling pain, with minimal side effects (Kowalske, 2011; Panahi, 2012). Eighty percent of populations in Asia and African countries trust on traditional medicine

for primary health-care (Dorai, 2012). To date, the alternative and complementary medicines, such as traditional medicine are less expensive with moderately beneficial to effective without toxicity or low toxicity (Fan *et al*., 2015; Sharifi *et al*., 2021). 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, 2012; Fan *et al*., 2015; Sharifi *et al*., 2021).

Xylocarpus granatum J. Koenig is a one of mangrove species which have secondary metabolites diversity, such as tannin, terpenoid, alkaloid, flavonoid, anthraquinone, and cardiac glycoside (Shi *et al*., 2017; Tomizawa *et al*., 2017; Islam *et al*., 2019; 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. A recent study showed that the lotion with combination *Xylocarpus granatum* fruit and sodium alginate extracts possess potent in wound treatment (Pringgenies *et al*., 2021). The other study showed that *Xylocarpus granatum* have potency strong antioxidant and anti-inflammatory potential (Islam *et al*., 2019). Furthermore, *Xylocarpus granatum*'s seeds have a skin-emolliating properties (Pringgenies *et al*., 2021). Based on this studies, *Xylocarpus granatum*'s seeds have potential as an active compound in burn wound treatment cream. To date, there have been no researches about the *Xylocarpus granatum* for burn wound. Therefore, the potential possessed by this plant, *Xylocarpus granatum* may have to potency as a burn wound therapy. Thus, the aim of this study to determine the effectiveness extract of *Xylocarpus granatum*'s seeds cream on burn wound grade II A in mice. A study on effectivity of extract of *Xylocarpus granatum*'s seeds cream as burn wound grade IIA treatment provides information about utilization *Xylocarpus granatum*'s seeds.

2. Materials and methods

2.1 Plant materials

Xylocarpus granatum J. Koenig collected 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. The seeds were cut into smaller pieces and rinsed with distilled water until clean. Furthermore, the seeds of XG were dried in an oven at a temperature of 40°C 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 40°C. The crude extract was kept in a glass bottle covered with aluminum foil in the refrigerator at 4°C.

2.2. Cream Formulation

The extract was formulated into an oil in water (O/W) based cream preparation (Table 1). 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 was formed. Stop the heating and then the extract was added to the cream mass and stirred until homogeneous. These two cream formulas will then be tested for their physical stability.

Table 1. The composition of cream formulation

Ingredients	Function	XGC 10%	XGC 15%
X. Granatum seed extract	Active ingredient	10%	15%
Liquid paraffin	Emollient, solvent	45%	45%
Cera Alba	Stiffening agent	13,5%	13,5%
TEA	Emulgator	2,7&	2,7&
Methylparaben	Preservative	0,05%	0,05%
Propylparaben	Preservative	0,05%	0,05%
Water	Vehicle	28,7%	23,7%

XGC 10%: treated with 10% concentration of *Xylocarpus granatum* J. Koenig extract cream, and XGC 15%: treated with 15% concentration of *Xylocarpus granatum* J. Koenig extract cream

2.3 Evaluation of Physical Quality of Cream Preparations

2.3.1 Determination of organoleptic properties and homogeneity

The appearance of the cream was determined by its smell, texture, and color (Srirod and Tewtrakul, 2019). Homogeneity testing is done by applying cream on the object glass, observed if there is a phase separation (Rahmawati *et al*., 2018).

2.3.2 Determination of the pH

The pH of the formula was measured using a pH meter that has been calibrated using a standard buffer solution (Srirod and Tewtrakul, 2019).

2.4 Antioxidant activity

The free radical scavenging activity of XG extract was analyzed by DPPH method. Two milliliters of 40 ppm DPPH solution was added to 2 ml the extract solution of each concentration (2 ppm; 4 ppm; 6 ppm; 8 ppm; 10 ppm; 12 ppm), the mixture was vortexed and stored in a dark room at room temperature for 30 minutes. Optical density (OD) 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 following formula:

Scavenging % = $(A_0 - A_1) / A_0 \times 100$ …… (Basma, Zakaria and Latha, 2011)

A_0 = negative control absorbance (without sample)

A_1 = sample absorbance

2.5 Burn Wound Model

Institutional Ethical Committee, University of Surabaya approved all protocols of this animal experiment (approval number: 185/KE/VIII/2021). All mice aged between 3-4 months and weighed between 25-35 g. Mice were anesthetized using ketamine at a dose of 40 mg/kgBW and xylazine at a dose of 5 mg/kgBW (im). 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 4 groups, namely cream base group (CBG) as negative control, silver sulfadiazine (SSD) as positive control, *Xylocarpus granatum* cream 10% (XGC 10%) and *Xylocarpus granatum* cream 15% (XGC 15%) as extract therapy group. Wounds were treated 2 times a day every morning and evening for 14 days.

2.6 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 14. Percentage of wound contraction was measured with following formula (Kalantar *et al*., 2016).

$$\text{Wound contraction (\%)} = \frac{\text{initial wound size} - \text{specific day wound size}}{\text{initial wound size}} x100\%$$

2.7 Histological Analysis

The process of histological preparations by excising 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 a series of alcohol concentration. Tissue samples were sectioned at 3-4μm. Hematoxylin and Eosin (H&E) staining was used to determine 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.

2.8 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).

3. Result

3.1. Preliminary Phytochemical Screening

Phytochemical screening of XG extract using color reaction, obtained secondary metabolites including: flavonoids, saponins and tannins.

3.2. Physical Quality of Cream Preparations

XGC 10% cream and XGC 15% cream formulas appearance a semisolid texture with a reddish-brown color due to the component of XG extract. Cream has good homogeneity, with a pH of 6.5.

3.3 Antioxidant Activity

The results of the antioxidant activity of XG extract obtained an IC_{50} value of 7.939 ppm. This shows that the antioxidant activity of XG extract is very strong. 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 antioxidants (Blois, 1958; Kuspradini *et al*., 2018).

3.4. Determination of wound contraction

Macroscopic observations showed that on days 1 and 2 there was fluid secretion in the wound area for all the 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 reddish color in wound area of the 15% SSD and XGC groups. Granulation tissue to remove on day 9 for the XGC 15% group, replaced by whitish epithelial tissue on day 10. An opposite pattern was seen in negative control, epithelial tissue not occur until day 14 treatment (Table 2). The formation of capillaries stops when the body's physiological needs are sufficient (Li and Wang, 2011).

Table 2. Macroscopical observation of each groups

Macroscopic observation	Day			
	BC	**SSD**	**XGC 10%**	**XGC 15%**
Wound burn	0	0	0	0
Secreting fluid	1-3	1-2	1-3	1-2
Scab	4-6	3-4	4-6	3-4
Granulation tissue	7-14	5-10	7-11	5-9
Epithelial tissue	-	11	12	10

Macroscopic observation of the wound area during 14 days. CBG: negative control group (treated with cream base), SSD: treated with silver sulfadiazine cream, XGC 10%: treated with 10% concentration of *Xylocarpus granatum* J. Koenig extract cream, and XGC 15%: treated with 15% concentration of *Xylocarpus granatum* J. Koenig extract cream

Wound healing was assessed by comparing the wound area and percentage 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 wound area in all group was almost similar. On the 7th day, the effectiveness of XGC 15% in reducing 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 not significantly different from SSD ($P>0.05$), with the percentage of wound contraction increasing.

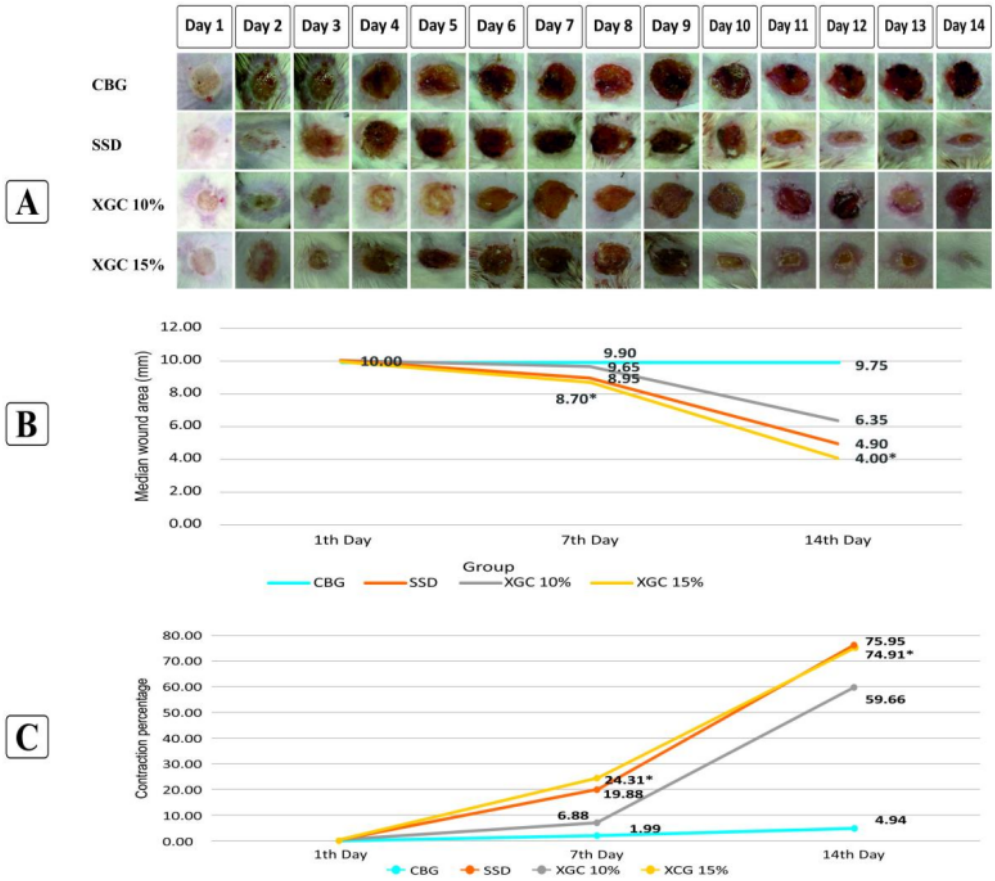

Figure 1. (A) Macroscopic view of burn wound on day 1 to day 14. The wound area was photographed by the same examiner from days 0 to 14 to evaluate the progress of wound closure; (B) Wound diameter in three time intervals between the studied groups, presented in the median value; (C) Contraction percentage 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

3.5. Histological evaluation

The results of post hoc Tukey analysis showed that between XGC 15% and SSD was no significant difference in the score of fibroblasts 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 3) (Figure 2). The score of fibroblasts will decrease at day 14 when the wound ECM has the same tensile strength as the surrounding healthy tissue. Myofibroblast apoptosis will increase when the wound is closed, 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).

Table 3. Statical analysis of wound assessment data at the 14th Day

Wound Assessment	N	Treatment Group	Mean±Standar Deviation	*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**

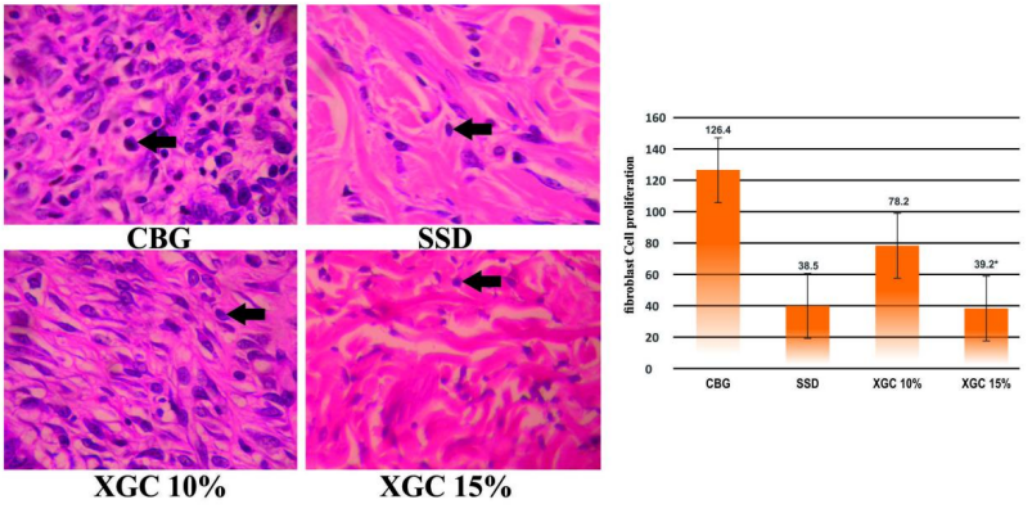

Figure 2. Histological appearance of fibroblasts in H&E stained with H&E at day 14 are shown (A). Fibroblasts will be purple with H&E staining. Migration of fibroblasts was more apparent in the wound margins in all groups. Magnification : 400x. Abbreviations: H&E, hematoxylin and eosin; (*) not significant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

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 that significant difference in epithelial thickness compared with 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 that significant decrease in epithelial tissue compared to other days. The study of Demilew, Adinew and Asrade, (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 that of CBG at day 14 (Figure 3).

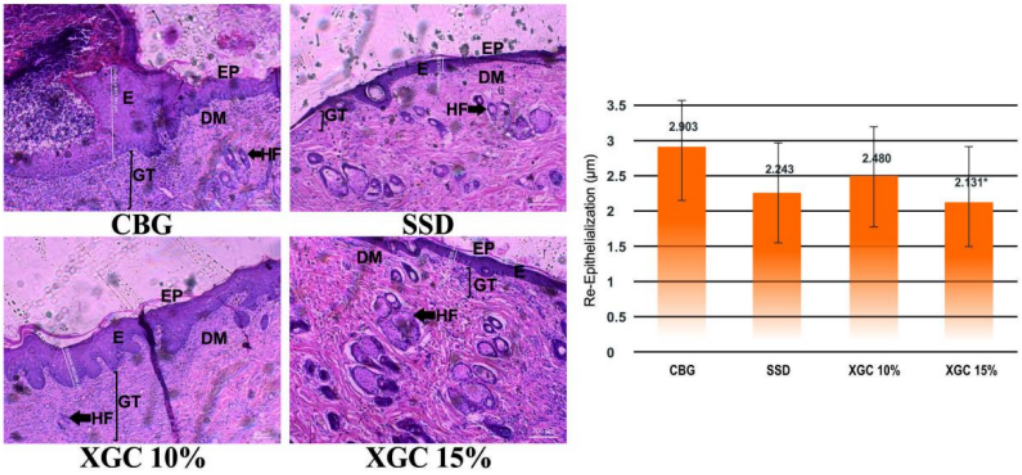

Figure 3. Histological appearance of burn wound stained with H&E at day 14 are shown (A). The XGC 15% group showed the same wound healing as SSD (re-epithelialization and development of granulation tissue). Magnification : 400x. Abbreviations: CBG, Cream base group; SSD, silver sulfadiazine cream; XGC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

Discussion

The preparation of XG extract cream has ideal homogeneity which is 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 the active ingredient. The therapeutic effect cannot be obtained continuously if the active ingredients are not evenly dispersed in the base material (Suena, Antari and Cahyaningsih, 2017). XG extract cream has met the pH criteria for cream preparations, which is in the range of 4-6.5 (Pengon *et al*., 2018; Purwaningsih, Romlah and Choirunnisa, 2020), which is in accordance with the skin pH range of 4.5-6.5 (Mulia, Ramadhan and Krisanti, 2018) so that it is 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, Antari and Cahyaningsih, 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.

In response to tissue injury, the body initiate an inflammatory response. Immune cells travel to the wound area and secrete pro-inflammatory cytokines which are 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, thereby accelerating wound healing (Bhatia *et al*., 2014; Elshamy *et al*., 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, play a notably 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 of vascular endothelial growth factor (VEGF) production, thereby accelerating angiogenesis. H_2O_2 facilitates migration and proliferation of epidermal cells, triggers activation of keratinocyte growth factor (KGF) and receptors for 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, 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 will 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 are significantly able to 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).

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). 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), they 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), able to induce 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 which is important in re-epithelialization (Kim *et al*., 2011).

Tannins can increase the formation of capillaries which are important in the process of angiogenesis. The secretion of FGF, PDGF and TGF-b by platelets can trigger angiogenesis due to hemostatic blockage (Singh, Young and McNaught, 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 activation and growth of endothelial cells, macrophages and blood vessels (Li *et al*., 2011; Singh, Young and McNaught, 2017). Tannins function as astringent agents that cause skin pores to shrink, stop bleeding and exudate so as to prevent bleeding in wounds. Tannins are also known to have antibacterial activity against *Staphylococcus aureus* and *Klebsiella pneumoniae* bacteria which are often found in wounds. The antibacterial mechanism of tannin against *Staphylococcus aureus* and *Klebsiella 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).

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Conclusions

XGC 15% facilitated the wound healing in the 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 that have very strong antioxidant activity and responsible for wound healing. Further studies with purified secondary metabolites are needed to know the complete mechanism of burn wound healing activity using XG extracts.

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