Sys Rev Pharm 2020;11(10):884-889
A multifaceted review journal in the field of pharmacy

The Effect of Gel *Abelmoschus Manihot* (L.) Medik Leaf Extract on Second Degree Burn Wound Healing Process

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ABSTRACT

Background: World Health Organization (WHO) estimated around 265000 of death is caused by burn injury. *Abelmoschus manihot* (L.) Medik consists of active compound such as hibifolin, stigmasterol, γ-sitosterol, myricetin, cannabistrin, myricetin 3-O-beta-D-glucopyranoside, 2,4-Dihydroxy benzoic zcid, asam maleat, quercetin, guanosine, adenosine.
Material and methods: Thirty male rats (*Rattus norvegicus*) weighting 200g were divided into 5 experimental groups (T0, T1, T2, T3 and T4). The skin sample was observed by using histopathology preparation and blood sample was also observed.
Results: Density of collagen on T0 showed significant difference to T1 and T2 ($p<0,05$), but P0 didn't show significant difference to T3 and T4 ($p> 0,05$). Arrangement of collagen on T0 showed significant difference to T1, T2 and T4 ($p< 0,05$), but no significant difference to T3 ($p> 0,05$). Based on statistical analysis fibroblast showed that P0 has no significant difference to T3 ($p >0,05$) but has significant difference to T1, T2, T4 ($p<0,05$).
Conclusion: The skin evaluation that consists of density and arrangement of collagen and total of fibroblast shows that T3 is the best concentration of extract *Abelmoschus manihot* (L.) Medik leaf that can be used as a topical therapy for burn injury.

Keywords: Collagen, ethanol 96%, fibroblast, Gedi Merah leaf, leukocyte, second degree

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INTRODUCTION

Thermal trauma is one of the most common problems faced in the emergency room. It may cause multiple organ injury distant from the burned area; therefore, morbidity and mortality is increased in thermal trauma patients [1] In addition to direct tissue damage, inflammatory reactions and infection as major complications [2].

For survivors, the most persisting problem is scarring, so the process of wound healing and the final outcome of this process is under investigation with the hope of decreasing the problems related to scar. Burn wound healing is a complex process including inflammation, granulation, and remodeling of the tissue [3].

Knowledge of the physiology of the normal wound healing trajectory through the phases of hemostasis, inflammation, granulation and maturation provides a framework for understanding the basic principles of wound healing. Through this understanding, the healthcare professional can develop the skills required to care for a wound and the patient can be helped with the complex task of tissue repair [4].

During the years, specific herbals have been developed and used to facilitate and accelerate the process of healing and tissue regeneration. There are some studies that explain alternative therapies in burn management [5,6,7]. For example moist exposed burn ointment which is an herbal remedy for burn treatment was developed and used in China [8]. Saffron (*Crocus sativus*) extract was used for healing of second-degree burn wounds in rats [9]. There are other studies supporting the effect of herbal remedies in treatment of burn wounds [10, 11, 12, 13]

Abelmoschus manihot (L.) Medik consists of active compound such as hibifolin, stigmasterol, γ-sitosterol, myricetin, cannabistrin, myricetin 3-O-beta-D-glucopyranoside, 2,4-dihydroxy benzoic acid, maleate acid, quercetin, guanosine, adenosine [14]. Prior research showed that *Abelmoschus manihot* (L.) Medik has activity as antibacteria, antivirus, antiinflammation dan antioxidan, and also used as a chronic bronchitis and dental illness [15].

MATERIAL AND METHODS

Ethical approval

This research used a standard procedure that has been certified by the ethical board of Faculty of Veterinary Medicine, Universitas Airlangga, with certification number 269/HRECC.FDOM/X/2018.

Ethanol extract of *Abelmoschus manihot* (L.) Medik leaf

Abelmoschus manihot (L.) Medik leaf (AMML) were dried at room temperature and were powdered using a grinder. Aqueous ethanol (70%) was gradually added to the powdered flowers, and the mixture was kept at room temperature without exposure to direct light for 72 h. After filtration, the solution was concentrated in a rotary evaporator under reduced pressure. The temperature of the rotary evaporator was from 40 to 50°C.

Preparation of gel formula

Extract used in this study were gel form with concentration of 6.25, 12.5, and 25% and were made with the following composition respectively.

R/	Extract of AMML		6.25%
	CMC		5%
	Glycerin		10%
	Propilene glycol		5%
	Nipagin		0.2%
	Aquadest (dH_2O)	ad	15g
R/	Extract of AMML	12,5%	
	CMC		5%

	Glycerin		10%
	Propylene glycol		5%
	Nipagin		0.2%
	Aquadest (dH_2O)	ad	15g
	#		
R/	Extract of AMML		25%
	CMC		5%
	Glycerin		10%
	Propylene glycol		5%
	Nipagin		0.2%
	Aquadest (dH_2O)	ad	15g

Animals and experimental procedure

Thirty male Wistar rats (*Rattus norvegicus*) with a mean weight of 150 to 200g were randomly divided into 5 experimental groups (T0, T1, T2, T3 and T4) all with an equal number of animals.

All animals except T0 were anesthetized via intramuscular injection of combination Ketamine and Xylazine (100 mg/kg bw and 5 mg/kg bw), and a 3×3cm area of dorsal hair was shaved (Fig.1). A second degree burn injury were made by applying the electrode to the skin purposely with 85±5°C temperature for 5 seconds [16] (Fig. 2)

All animals were kept under standard laboratory conditions and were provided with identical food and water during the study period. After recovery, rats were individually housed in separate cages. The cages were cleaned daily and kept free from infectious agents. In T0, T1, T2 and T3, the animal were treated with 1% silver sulfadiazine, AMML extract with concentration 6.25%, 12.5% and 25% respectively. The gel were applied to the wound twice daily for 2 weeks. Each of the animals was kept in a separate cage to prevent licking of the applied ointment by other rats, so we did not use any cover or dressing over the wounds after applying the gel.

After 14 days of treatment, all animals were sacrificed. The excised skin stored in 10% neutral buffered formalin solution was dehydrated in ascending alcohol series, cleared in xylene, and embedded in paraffin. Paraffin sections (3 µm thick) were cut, deparaffinized, and stained with hematoxylin and eosin (HE). All sections were double blindly examined and photographed under a light microscope (Olympus-CX41RF, Olympus Corporation, Tokyo, Japan) by an experienced histologist. Hematoxylin and eosin staining were provided for evaluation of collagen density, collagen arrangement and the number of fibroblasts.

Microscopic observation was done to examine the density and arrangement of collagen by using the method of Shakya [17] and Nasiri [18]. The skin sample slides were observed by using microscope with the magnification 100x for density of collagen and 400x for the arrangement of collagen. The examination method for the number of fibroblasts was using the method of Fuadi [19] with modification.

Blood sample were also collected after 14 days of treatment. The blood was collected in venoject tube with anticoagulant *Ethylene Diamine Tetraacetic Acid* (EDTA). Total leukocyte and differential counting of leukocyte were examined by using hematology analyzer. The differential counting of leukocyte consisted of eosinophils, basophils, neutrophils, lymphocytes, and monocytes.

Statistical analysis

Statistically, all data are expressed as mean ± standard deviation. The data of collagen density and arrangement scoring were analyzed using *Kruskal-Wallis* and continued with *Mann-Whitney.* The number of fibroblast data and white blood examination and were analyzed using *Analysis of Varians* (ANOVA) and continued with Duncan. Statements of statistical significance are based on $p<0.05$. These analyses were carried out using SPSS statistical analysis system (Release 10.0, SPSS. Inc)

RESULTS

Density and arrangement of collagen

Collagen density on T0 showed significant difference with T1 and T2 ($p< 0.05$) but has no significant difference with T3 and T4 ($p> 0.05$). The density of collagen on T1, T2, T3 and T4 showed no significant difference ($p> 0.05$). Mean of collagen density ± SD on T0, T1, T2, T3 and T4 were 1.00 ± 0.00, 2.50 ± 0.58, 2.25 ± 0.50, 1.25 ± 0.50 and 1.5 ± 0.58 respectively (Table 1).

The similar result were found in the arragement of collagen. T0 showed significant difference with T1, T2 and T4 ($p< 0.05$), but no significant difference with T3 ($p> 0.05$). There were no significant difference treatment T1, T2, T3 and T4 on arrangement of collagen showed no ($p>0.05$). Mean of arrangement of collagen on T0, T1, T2, T3 and T4 respectively were 1.00 ± 0.00, 3.00 ± 0.82, 3.35 ± 0.50, 1.75 ± 0.96 dan 2.25 ± 0.25 (Table 1).

Table 1. The Density and Arrangement of Collagen on Each Treatment

Treatment	Mean ± SD	
	Density of collagen	Arrangement of collagen
T0	1.00^{a} ± 0.00	1.00^{a} ± 0.00
T1	2.50^{b} ± 0.58	3.35^{b} ± 0.50
T2	2.25^{b} ± 0.50	3.00^{b} ± 0.82
T3	1.25^{ab} ± 0.50	1.75^{ab} ± 0.96
T4	1.5^{ab} ± 0.58	2.25^{b} ± 0.25

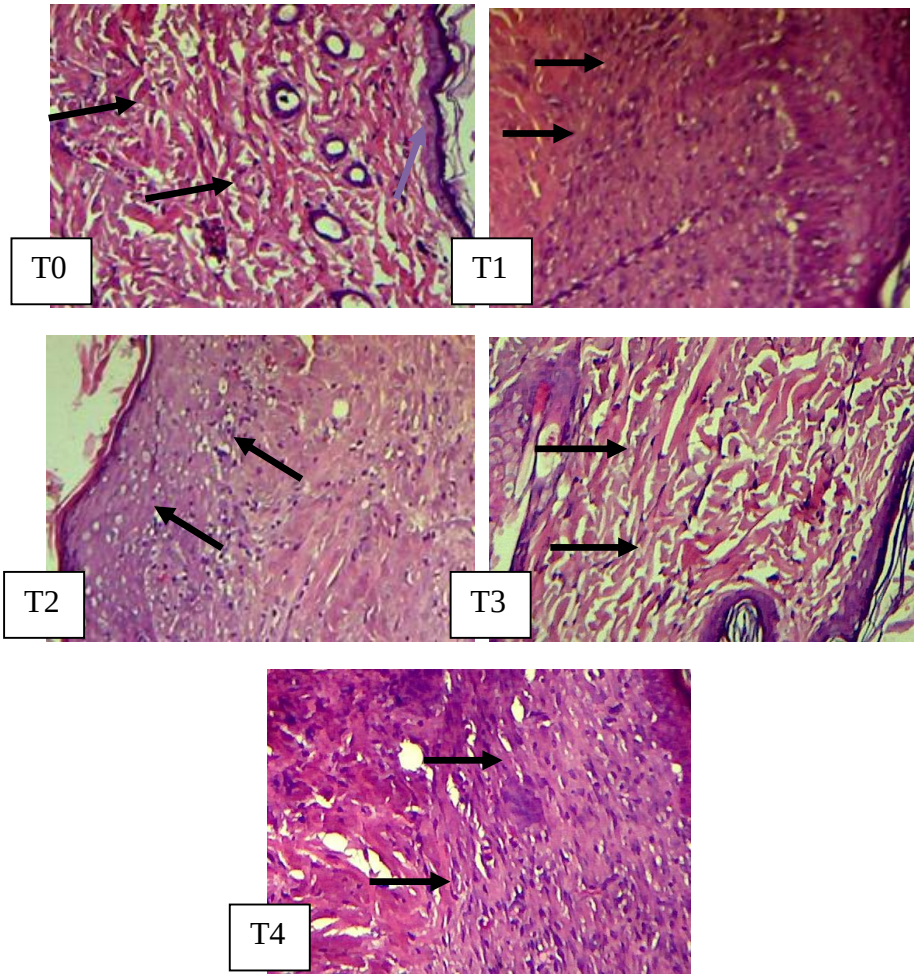
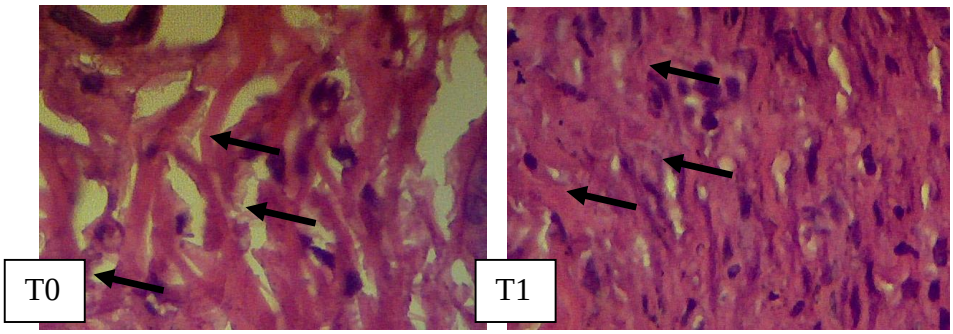


Figure 1: Histopathological appearance of skin collagen density on day 15 post burn with no treatment (T0) treated with SSD 1% (T1) treated with 6.25% AAMML (T2) treated with 12.5% AMML (T3) treated with 25% AMML (T4) (HE, 400x)

T0 : Normal collagen density
T1 : Showed a moderate amount of collagen in the dermis and less dense of overall collagen bundle
T2 : Showed a less amount of collagen in the dermis and less dense of overall collagen bundle.
T3 : Showed large amount of mature collagen and very dense of the whole collagen bundle.
T4 : Showed large amount of collagen in the dermis layer and dense of the collagen bundle.



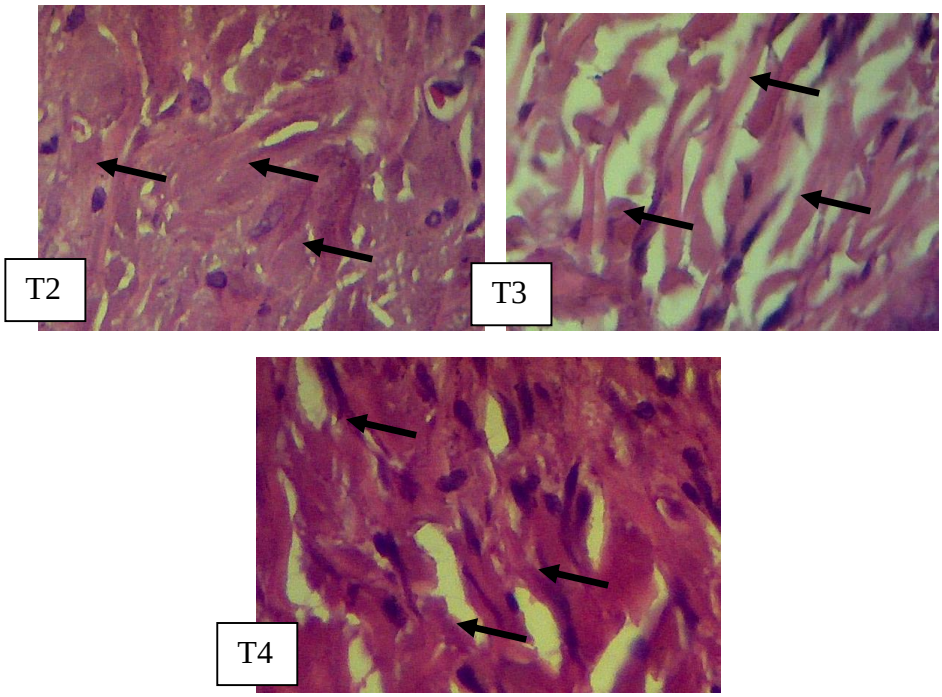


Figure 2: Histopathological appearance of skin collagen arrangement on day 15 post burn with no treatment (T0) treated with SSD 1% (T1) treated with 6.25% AMML (T2) treated with 12.5% AMML (T3) treated with 25% AMML (T4) (HE, 400x)

T0 : Collagen fibers showed clear boundaries and very good collagen arrangement
T1 : Collagen fibers showed unclear and thinner boundaries and well-oriented collagen fibers.
T2 : Collagen fibers showed clear and thick boundaries but poorly oriented collagen fibers.
T3 : Collagen fibers showed clear and thick boundaries and very well-oriented collagen fibers.
T4 : Collagen fibers showed clear boundaries and well-oriented collagen fibers

Total of fibroblast

Statistical analysis number of fibroblasts showed that T0 showed no significant difference to T3 ($p > 0.05$) and has significant difference to T1, T2, T4 ($P < 0.05$). Mean of total of fibroblast ± standard deviation on T0, T1, T2, T3, and T4 respectively were 28.72 ± 9.118; 59.96 ± 16.095; 72,92 ± 19.539; 30.76 ± 9.205, and 71.52 ± 7.026. The lowest mean number of fibroblasts was on T0 (Table 2).

Table 2. Mean and Standart Deviation Number of Fibroblast on Each Treatment

Treatment	Fibroblast (Mean ± Standard Deviation)
T0	$28.72^{a} \pm 9.118$
T1	$59.96^{b} \pm 16.095$
T2	$72.92^{b} \pm 19.539$
T3	$30.76^{a} \pm 9.205$
T4	$71.52^{b} \pm 7.026$

Total and differential count of leukocyte

Based on statistical analysis total and differential count of leukocyte on day 8 and 15 showed no significant difference ($p > 0.05$). However, eosinophils, basophils, neutrophils (T1 and T2), lymphocytes (T0 and T1) and monocytes tend to decrease on day 15 compared to 8 (Table 3)

Table 3. The Total and Differential Counting of Leukocyte on Each Treatment on Day 8 and15

Day	Parameter	T0	T1	T2	T3	T4
8	Total leukocytes	$13.60^{a} \pm 2.74$	$11.70^{a} \pm 6.27$	$11.77^{a} \pm 2.81$	$9.10^{a} \pm 2.21$	$9.70^{a} \pm 2.95$
	Eosinophils	$0.14^{a} \pm 0.03$	$0.12^{a} \pm 0.06$	$0.05^{a} \pm 0.08$	$0.07^{a} \pm 0.06$	$0.16^{a} \pm 0.09$
	Basophils	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.04^{a} \pm 0.07$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$
	Netrophils	$1.87^{a} \pm 0.71$	$2.09^{a} \pm 1.11$	$2.41^{a} \pm 0.47$	$1.73^{a} \pm 0.65$	$1.46^{a} \pm 0.71$
	Lymphocytes	$11.02^{a} \pm 3.18$	$8.66^{a} \pm 4.95$	$8.11^{a} \pm 1.68$	$6.67^{a} \pm 2.25$	$7.66^{a} \pm 2.00$
	Monocytes	$0.57^{a} \pm 0.50$	$0.83^{a} \pm 0.62$	$1.15^{a} \pm 0.65$	$0.63^{a} \pm 0.09$	$0.42^{a} \pm 0.38$

15	Total leukocytes	$10.37^{a} \pm 1.08$	$10.77^{a} \pm 3.70$	$11.90^{a} \pm 1.78$	$12.07^{a} \pm 5.90$	$11.93^{a} \pm 1.36$
	Eosinophils	$0.07^{a} \pm 0.06$	$0.09^{a} \pm 0.11$	$0.08^{a} \pm 0.07$	$0.06^{a} \pm 0.05$	$0.08^{a} \pm 0.07$
	Basophils	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$
	Netrophils	$2.24^{a} \pm 0.58$	$1.79^{a} \pm 0.53$	$2.24^{a} \pm 0.57$	$2.16^{a} \pm 0.08$	$1.71^{a} \pm 0.94$
	Lymphocytes	$7.85^{a} \pm 1.12$	$8.32^{a} \pm 4.15$	$8.96^{a} \pm 1.78$	$9.36^{a} \pm 5.32$	$9.31^{a} \pm 1.65$
	Monocytes	$0.21^{a} \pm 0.02$	$0.56^{a} \pm 0.39$	$0.63^{a} \pm 0.15$	$0.49^{a} \pm 0.55$	$0.83^{a} \pm 0.63$

DISCUSION

In this study, the efficacy of gel containing *Abelmoschus manihot* (L.) Medik leaf extract was evaluated for treating second degree skin burn wounds in rats. The results of this study showed that gel containing 12.5 and 25% AMML extract facilitated the healing of the burned skin. Topical gel containing 12.5% AMML extract were more effective in inducing wound healing than creams containing 1% SSD.

On day 15 after the burn injury, the wound size was significantly smaller in rats treated with gel containing 25% AMML extract than in rats treated with the other agents. It has been extensively studied about the benefits of AMML including antioxidant, analgesics, an antibacterial activity [20]. The identification of chemical compounds reported the presence of flavonoid in AMML extracted with ethanol solvent. The level of flavonoid content of AMML extracted with 96% ethanol was 41.56%. Flavonoid compounds have various important functions for health, among others, in reducing the risk of cardiovascular disease, blood pressure, atherosclerosis, and as an antioxidant [21].

Our study showed that gel containing 12.5% AMML extract were more effective than creams containing 1% SSD for treating burn wounds. Treatment with gel containing 12.5% AMML extract accelerated the time for wound healing than for wounds treated with the other agents.

Collagen density and arrangement (organization) was also higher in wounds treated with gel containing 12.5% AMML extract than in wounds treated with the other agents, Otherwise the number of fibroblasts decreased dramatically compared to other treatment except T0. Preliminary chemical analysis of the AMML extract showed that it contained high concentrations of myricetin, maleic acid, benzoic acid, quercetine, guanosine and adenosine [22]. Previous studies have reported that AMML extract had antiinflammation [23], antiviral [24], accelerate wound healing [25] and cerebral ischemic reperfusion activity [26].

Wound healing is a multiphase process characterized by wound contraction, granulation, epithelialization, and collagenation. Wound healing involves 3 phases, that is, inflammation, proliferation, and remodeling [27]. Proliferation is followed by epithelialization, angiogenesis, and collagen formation. Fibroblasts, collagen, edema, and new blood vessels are formed and undergo maturation in the remodeling phase, resulting in the formation of scar tissue. Collagen is the main protein that contributes to wound strength [28, 29]. The barrier function of the skin is disrupted after thermal injuries, which may result in the development of infections in the wounded area. Infection complicates burn wounds and delays their healing. Therefore, wound dressing should be performed appropriately to prevent the entry of environmental microorganisms [30].

We observed that the healing of wounds treated with gel containing 12.5% AMML extract was faster than that of wounds treated with the other agents. This may be because of the beneficial effects of AMML extract on wound healing parameters such as revascularization, fibroplasias, wound contraction, and collagen synthesis. In addition, this beneficial effect of AMML extract may be associated with its antibacterial, anti-inflammatory, and antioxidant properties [31]. These properties of AMML extract probably augment together and promote wound healing compared with the antibacterial effects of standard 1% SSD.

Creams containing SSD are commonly used for treating burn injuries because of the antibacterial property of SSD. Although creams containing SSD are recommended as the standard treatment for treating burn wounds, the use of SSD may increase the duration of hospitalization [30]. Because gel containing AMML extract promoted faster wound healing than creams containing 1% SSD, the use of these creams may result in a shorter hospital stay of patients with burn injuries. The treatment of wounds with gel containing 12.5% AMML extract resulted in better wound healing process than the treatment of wounds with the other treatment. On day 15 of the study, for wounds treated with cream containing 1% SSD, gel with 6.75% and 25% AMML extract, respectively, indicating that gel containing 12.5% AMML extract had the best effect on wound healing.

CONCLUSION

Administration of 12.5% of *Albelmoschus manihot* (L.) Medik leaf extract increase total number of fibroblast, increased collagen density, and improve collagen arrangement in the healing process of grade IIB skin burns.We are intending to determine the main ingredients and substances in *Albelmoschus manihot* (L.) Medik which may contribute to its wound healing effects and formulize a biomaterial for treatment of second degree burn wounds.

ACKNOWLEGMENT

The authors are highly thankful to Dean and Colleague of the Faculty of Veterinary Medicine, Universitas Airlangga for their cooperation and provision of research facilities.

FUNDING SOURCES

This research did not receive any specific grant from funding agencies in the public, or not-for-profit sectors.

CONFLICT OF INTEREST

There are no editorial or financial conflicts of interest.

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