Effectivity Test of Loquat (*Eriobotrya japonica* (Thunb.) Lindl.) Leaves Extract on the Incision Wound Healing in Mice (*Mus musculus* L.)

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Keywords: *Eriobotrya japonica* (Thunb.) Lindl., Incision Wound, Mice, Wound Healing.

Abstract: The wound healing process is divided into four phases; 1) coagulation 2) inflammatory 3) proliferation 4) remodelling. Loquat (*Eriobotrya japonica* (Thunb.) Lindl.) leaf contains triterpenoid, flavonoid, alkaloid, and tannin as anti-inflammation, antibacterial, anti-allergic that can accelerate the wound healing process. The aims of this research were to find out the effectiveness of ethanol extract of loquat leaves and to examine the histological appearance of wound healing process on mice's skin in day 14th. This research used 25 male mice (*Mus musculus* L.) that were divided into five different treatment groups. The treatment groups were treated with two control groups; povidone-iodine (K+) and without treatment (K-) and loquat leaves ethanol extract with three concentration of 15% (PI), 30% (PII), and 45% (PIII). Incision wound was made into 1 cm length, the ointments were applied onto the wound and observed for twice a day in 14 days. Histological preparation was made to calculate epithelial thickness, fibroblasts, and lymphocytes. The data were analyzed statistically using SPSS. The result of this research showed significant difference of average time span of wound healing of each group's, K-, K+, PI, PII, and PIII was subsequently 8 days, 9.2 days, 7.6 days, 8.4 days, and 9.4 days, with $p=0.048$ ($p<0.05$). Histological observations showed the average of epithelial thickness, fibroblasts, and lymphocytes in the treatment group PI is higher than the other treatment groups. In conclusion, loquat leaves ethanol extract with concentration 15% (PI) was the most effective to accelerate the wound healing process.

1 INTRODUCTION

A wound is defined as damage or disturbance that occurs in the structure and function of normal anatomy tissue caused by a defense, chemical or trauma. Incision wound is a wound that can be healed properly if complications do not occur. The criteria for an incision wound are new, sudden and fast to healed (Perdanakusuma, 2007). Wound healing is a natural process that occurs after an injury, predictable progression of steps used by the body to resolve impaired tissue integrity (Szycher and Lee, 1992).

Wound healing is a complex process that requires a series of steps, some of the steps are granulation, collagen maturation, and scar formation. (Zheng and Qin, 2007) which divided into four phases: 1) coagulation and hemostatis; 2) inflammation; 3) proliferation; 4) remodeling of a wound (Velnar *et al.,* 2009).

The hemostatic phase will begin the healing process where the skin slashed then blood clots containing fibrin and blood cells fill a narrow space at the edge of the incision. Followed by an inflammatory process, which starts 24 hours after the incision occurs (Ross and Pawlina, 2011). The inflammatory phase is the phase of the formation of immunity such as leukocytes which plays an important role for the initial inflammatory response after the occurrence of wounds that can prevent the entry of microorganisms to avoid acute injuries (Robson *et al.,* 2001). Proliferative phase is characterized by the formation of granulation tissue within the wound bed, composed of new capillary network, fibroblast, and macrophages in a loose arrangement of supporting structure (Prasettyono, 2009).

Indonesia has many plants that have various benefits, one of them as medicine. The use of plants as traditional medicine is widely used as an

Sabri, E. and Zahra, R.
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DOI: 10.5220/0010138100002775
In *Proceedings of the 1st International MIPAnet Conference on Science and Mathematics (IMC-SciMath 2019)*, pages 162-170
ISBN: 978-989-758-556-2

alternative for healing because it is easy to obtain, use and safer in terms of side effects.

The loquat (*Eriobotrya japonica* (Thunb.) Lindl.) leaves have been used as a component in Chinese medicine. Loquat is grown well in Kabanjahe, Kabupaten Karo, North Sumatera, Indonesia, has potential genetic compares with another loquat. The fruit is large, has a very sweet taste, and becomes multifunction plants. The leaves and the seeds are containing bioactive compounds that are commonly used for Karo traditional medicine to cure diabetic disease and for expectorant to relieve cough (Nurwahyuni *et al.*, 2017).

Loquat leaves contain various triterpenes, sesquiterpenes, flavonoids, tannins, and megastigmane glycosides and these compounds have been reported as anti-inflammation, anti-viral, antioxidant, anti-mutagenic, and anti-tumor (Banno *et al.,* 2005), anti-bacteria and anti-allergic (Tan *et al.,* 2017). In a study conducted by Zhang et al. (2015) founded 19 secondary metabolite contents in loquat leaves for various bioactivities, such as anti-inflammatory, antioxidant, and anti-cancer properties. Mong these 19 ingredients, there are 15 components, most of which are classified as triterpenoids and flavonoids that show antiinflammatory activity.

Various pharmacological and biology reports showed that the medicinal value of plants lies in bioactive phytochemical constituents that produce definite physiological action on the human body. The herbal extracts promote fast wound healing than control and non medicated group in different in vivo studies, the process is promoted by several herbal extracts, which are composed of active agents like triterpenes, alkaloids, flavonoids, tannins, saponins, anthraquinones, and other biomolecules (Thakur *et al.,* 2011). According to the research of Kimura et al. (2008), Lee et al. (2006) and Liu et al. (2008) that asiaticoside, a triterpenoid component can accelerate the wound healing process in mice by increasing antioxidant activity, collagen synthesis, and angiogenesis. Two triterpenes compounds, asiaticosides and madessicosides, showed better result wound healing pattern in a view of histological examination (Wu *et al.*, 2012).

From the various research data above, it shows that the loquat leaves have the potential to heal the wounds due to the content of secondary metabolites which have anti-inflammatory, antibacterial, antiallergic and antioxidant effects that an accelerate wound healing. In this case, a study was conducted to test whether ethanol extracts of loquat leaves can accelerate wound healing in mice *(Mus musculus).*

2 MATERIALS AND METHODS

2.1 Grouping and Dosing of Animals

This study was taken in Laboratory Animal Structure and Physiology, Faculty of Mathematics and Natural Science, Sumatera Utara University. Twenty-five male mice (*Mus musculus* L.) were used for the study. Mice weighed 25-30 grams and aged 8-10 weeks. Mice were housed in cages and provided with *adlibitum* pellet and water. The study protocol was approved by the Ethics committee of the Faculty of Mathematics and Natural Science, Sumatera Utara University, AREC. Mice were divided into five groups. The treatment groups were treated with two control groups; povidone-iodine (K+) and without treatment (K-) and loquat leaves ethanol extract with three concentration of 15% (PI), 30% (PII), and 45% (PIII).

2.2 Plant Extraction

Loquat leaves were dried for 3 three weeks under the shade. The dried leaves were grinded to coarse powder. The powder was macerated with 70% ethanol as a solvent in 6 hours with occasional shaking and stirring, then soaked for 18 hours. The extract was then filtered. The maceration process is repeated once again. The filtrate was then combined and evaporated in a rotary evaporator at 40°C and concentrated in water bath until a thick extract is obtained (Kemenkes RI, 2013).

2.3 Ointment Formulation

Simple ointment of the loquat leaves ethanol extract with vaseline was prepared following the formula (Table 1) based on Kusumawardhani *et al.* (2015):

$$L = \frac{a}{b}\ x\ 100\ \% \quad (1)$$

L : Ointment concentration (%)
a : Loquat leaves ethanol extract (gram)
b : Ointment (50 gram)

Table 1: Ointment formulation.

Ingredients	Ointment (gram)		
	15%	30%	45%
Vaseline	42,5	35	27,5
Ethanol extract of loquat leaves	7,5	15	22,5

2.4 Incision Wound Model

After acclimatization in a week, mice were anesthetized in the same manner to reducing the pain. The dorsal of each mouse was then shaved and a 1 cm long incision was made through the skin. The scalpel was marked on the tip for the depth of the wound. Mice were treated under grouping dosing section and the ointment formulation as described. Treatments were started from day 1st until day 14th. Observed in twice a day, at 08.00 AM and 05.00 PM.

2.5 Histological Assessment

The skin samples were taken in day 14th. Histological assessment using paraffin methods and stained by Hematoxylin-Eosin stain (Suntoro, 1983).

2.6 Data Parameters

Data parameters were divided into macroscopically and microscopically. Macroscopical data was the average time span of wound healing in each group. Microscopical data was average of epithelial thickness, fibroblasts, and lymphocytes on day 14th in skin mice. Microscopical examination used OptiLab Microscope Camera with magnification 100x for epithelial thickness and 400x for calculating fibroblast and lymphocyte cells. The data were analyzed using SPSS version 22.0.

3 RESULTS

3.1 The Average Time Span of Wound Healing

The effect of ethanol extract of loquat leaves on wound healing day was marked by the wound having healed. Data on the average time span of wound healing day of mice can be seen in Table 2.

Table 2: Average time span of wound healing.

Groups	Average time span ± SD
K-	$8,0^{ab} \pm 0,70$
K+	$9,2^{b} \pm 0,83$
PI	$7,4^{a} \pm 1,14$
PII	$8,4^{ab} \pm 1,14$
PIII	$9,4^{b} \pm 1,14$

Data were tested with One Way ANOVA and obtained significant results of 0.028 ($p < 0.05$), followed by Duncan test and the results showed that the treatment group of ethanol extract of leaves of 15% concentration (PI) was the most potent treatment on wound healing seen from average time span of wound healing in mice.

Wounds treated with a concentration of 15% (PI) provide a faster healing effect when compared to other treatments and treatment with a concentration of 45% (PIII) provides the longest healing effect of all treatments. Normal wound healing can take place naturally without assistance such as healing that occurs in negative control treatments (K-), but herb extracts such as at a concentration of 15% (PI) loquat leaves will help in accelerating wound healing because it contains secondary metabolites that have an effect anti-inflammatory and antibacterial properties such as terpenoids and alkaloids.

Accordance to Rahman et al. (2013) said that the speed of the wound healing process can be influenced by the compound of secondary metabolites such as terpenoid, alkaloid, and tannin which have the function to improve the repair and strengthen the skin cells and stimulate the growth of connective tissue. Krishnaiah et al. (2009) said that triterpenoids are components that have an active role in wound healing. Triterpenoid help strengthens the skin structure, increase the concentration of antioxidants and restore inflammatory or inflamed tissue by increasing blood supply to the wound area and accelerate the process of wound healing.

Loquat leaves have secondary metabolite compounds such as triterpenes, flavonoids, tannins, which have an affect as an antiinflammatory, antiviral, antioxidant (Banno *et al.,* 2005), antibacteria, antiallergic (Tan *et al.,* 2017). Triterpene compounds in *Centella asiatica,* mainly including two glycosides are considered to facilitate burn wound healing via activating growth factors such as TGF-β that favors fibroblast proliferation and could elevate collagen synthesis. The compounds can accelerate the time span of the wound healing process (Wu *et al.*, 2012).

The hemostasis/inflammation phase of acute wound healing reflects the time recruit the many celullar elements in several days to weeks that are activated during early repair and the absence of mechanical strength (Robson *et al.* 2001). So that, PI has a potential effect on wound healing to the average time span of wound healing.

Macroscopical examination of wound area of each group *(Mus musculus* L.*)* in the day 1st, day 7th, day 14th can be seen in Figure 1.

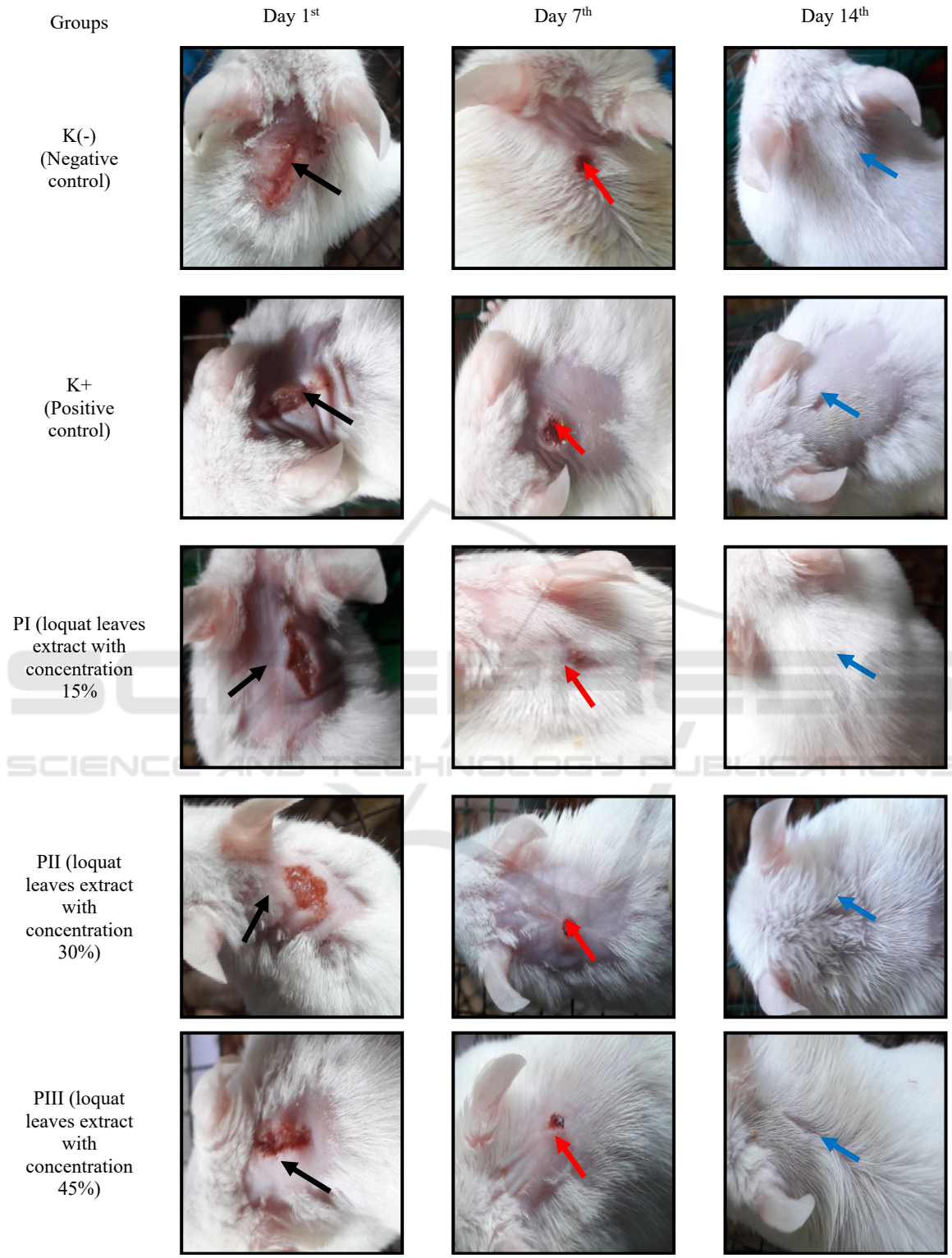

Figure 1 : macroscopical examination of wound area of each group of treatment.(➡) day 1st , wounds red and swollen, (➡) day 7th, wounds shrink and dry, (➡) day 14th, wounds healed.

3.2 The Average Number of Fibroblast Cells

Histological observations of the fibroblast cells on the histological preparations of the mice skin with a magnification of 400 x. Data on the average number of fibroblast cells in each group on the day 14th can be seen in Table 3.

Table 3: The average number of fibroblast cells.

Groups	Fibroblasts ± SD
K(-)	$6,42^{a} \pm 4,36$
K(+)	$12,26^{b} \pm 2,93$
PI	$19,16^{c} \pm 5,83$
PII	$15,42^{bc} \pm 3,85$
PIII	$14,74^{bc} \pm 3,55$

Based on Table 3, it can be seen that the highest average number of fibroblasts on the day 14th was in the treatment group, a concentration of 15% (PI) with 19.16, while the lowest was in the negative control treatment group (K-) with 6.42.

Based on the statistical test, data was obtained significant results 0.002 ($p < 0.05$). Followed by the Duncan test and the result is that the treatment group concentration 15% (PI) is the group that the most potential on wound healing seen from the number of fibroblasts.

The effect of ethanol extract of loquat leaves on the number of fibroblasts is shown from the lower extract concentration, the higher the number of fibroblasts. The use of ethanol extracts of loquat leaves might effect the addition of nutrients derived from loquat leaves in the wound area which can optimize wound healing by increasing the number of fibroblasts.

Alkaloid compound can accelerate soft tissue repair. Reyes *et al.*, (1993) presented evidence that alkaloid compound, taspine, extracted from *Croton lechleri* by its chemotactic process toward fibroblasts that migrate into the wound from local tissues and increasing extracellular matrix synthesis due to their increased number.

Two glycoside triterpene compounds were able to enhance collagen type I and type III synthesis mainly through activating skin fibroblasts (Wu *et al.*, 2012). Collagen fibers type III is synthesized first in the proliferation phase by fibroblasts that are stimulated by growth factors TGF-β from fibroblast cells and macrophages itself (Sabirin *et al.,* 2013).

The large amount of connective tissue in the wound area could help accelerate the wound contraction. So, the wound side will be contracted and caused the wound area to be smaller (Prasetyo *et al.,* 2010).

Fibroblasts synthesize and release glycosaminoglycans and proteoglycans, which are also important components of the extracelullar matrix of granulation tissue. Simultaneously, vascular generation (angiogenesis) occurs with the use of the maturing matrix. The acute wound fibroblast density reaches a maximum between 7 and 14 days after injury (Robson *et al.,* 2001).

Fibroblast is one indicator that healing process occurred faster. Increasing of fibroblast as a result of lymphokines induced which secreted by $CD4^{+}$ lymphocytes, which has a role as healing promotor to cellular immune response. Depletion of $CD4^{+}$ lymphocytes can decrease skin tension, angiogenesis and extracellular matrix component (Prasettyono, 2009).

3.3 The Average Number of Lymphocyte Cells

Histological observations of the lymphocyte cells on the histological preparations of the mice skin with a magnification of 400 x. Data on the average number of lymphocyte cells in each group on the day 14th can be seen in Table 4.

Table 4: The average number of lymphocyte cells.

Groups	Lymphocyte ± SD
K(-)	$5,10^{a} \pm 2,96$
K(+)	$4,70^{a} \pm 2,04$
PI	$18,08^{c} \pm 3,97$
PII	$11,22^{b} \pm 3,63$
PIII	$13,38^{b} \pm 4,98$

Based on Table 4, it can be seen that the highest average number of lymphocytes on the day 14th was in the treatment group, a concentration of 15% (PI) with 18.08, while the lowest was in the positive control treatment group (K+) with 4.70.

Based on the statistical test, data was obtained significant results 0.000 ($p < 0.05$). Followed by the Duncan test and the result is that the treatment group concentration 15% (PI) is the group that the most potential on wound healing seen from the number of lymphocytes.

The effect of ethanol extract of loquat leaves on the number of lymphocytes is shown from the lower extract concentration, the higher the number of lymphocytes. Secondary metabolite compounds in loquat leaves might able to accelerate the inflammatory phase as seen from the high number of lymphocytes in the treatment group of loquat leaves

extract which can inhibit the growth and kill microorganisms in the wound area.

Loquat fruit and leaves have high concentration of Vitamin A. Vitamin A can play a role in accelerating the inflammatory phase to the proliferation phase by increasing monocytes, lymphocytes and macrophages to the wound area which will eliminate bacteria from the wound area and produce growth factors needed for proliferation of fibroblast cells and angiogenesis (Kumar *et al.,* 2014; Negara *et al*., 2014).

Lymphocytes were present within the wound at one day, increased to peak numbers between days 8 and 14 post-wounding and remained present. Lymphocytes are an important regulator of fibroblast activity both directly and indirectly through the macrophages during wound healing. Martin and Muir, 1990).

On the day 7th, inflammatory cells and macrophages begin to migrate together with fibroblast cells into the wound tissue. Fibroblasts will proliferate with the help of growth factors, especially transforming growth factor -β (TGF-β) and basic fibroblast growth factor (bFGF) which are secreted by platelets and macrophages. Macrophages will experience a reduction in the number as a result of tissue repair which process will be followed by fibroblast, endothelial cells and (Sabirin *et al.,* 2013).

On proliferation phase, $CD4^+$ lymphocytes induce keratinocytes to release IL-1 in the wound area. Keratinocytes have a potential role on epithelization, proliferation, and maturation of epidermis. IL-1 that has been released by keratinocytes induces endothelial cells to form angiogenesis and fibroblast to form extracellular matrix (Prakoso and Kurniasih, 2018).

The histological figure of fibroblast and lymphocytes of mice skin on day 14^{th} with 400x magnification can be seen in Figure 2 below:

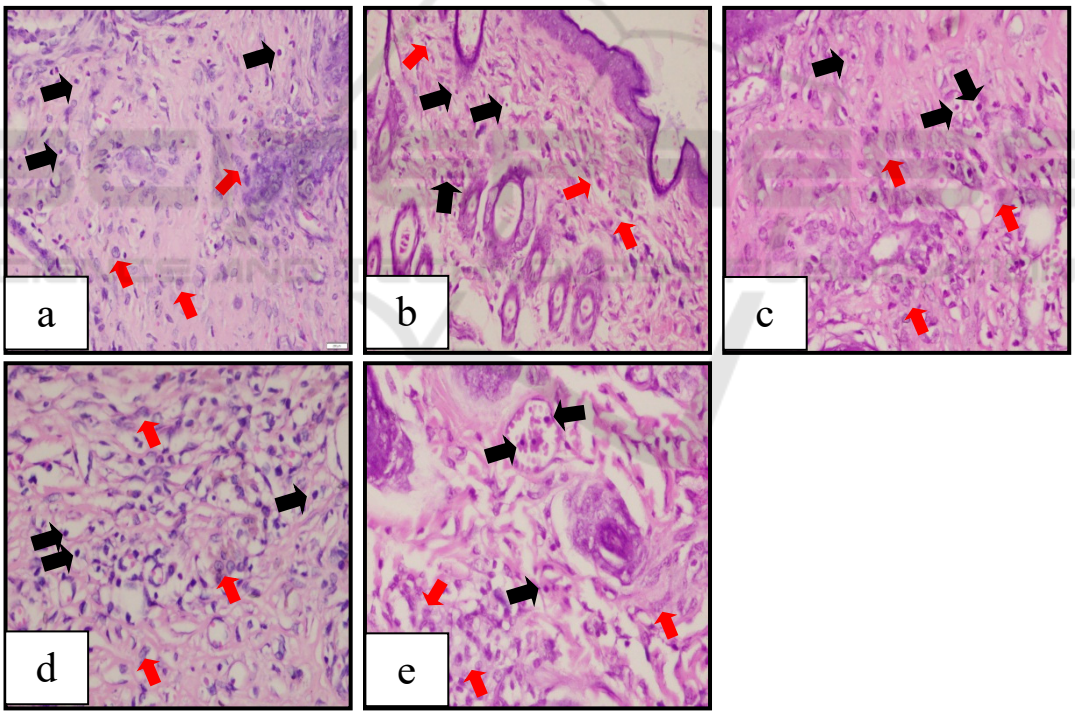

Figure 2: Microscopical examination of fibroblasts and lymphocytes.
(a) negative control (K-); (b) positive control (K+); (c) loquat leaves extract with concentration 15% (PI);
(d) loquat leaves extract with concentration 30% (PII); (e) loquat leaves extract with concentration 45% (PIII), (➡) fibroblas (➡) limfosit.

According to Balqis *et al.,* (2014) that on the 14^{th} day of the histological slide, infiltration of inflammation cells was still visible and collagen fibers have spread. In normal tissue, fibroblast cells are rarely found. After the injury, fibroblast will actively migrate to the wound area, will proliferate collagen which plays a role in new tissue formation until the skin returns to normal.

3.4 The Average Number of Epithelial Thickness

Histological observations of the epithelial thickness on the histological preparations of the mice skin with a magnification of 100x. Observation of epithelial thickness is measured from the *stratum corneum* layer to the *stratum basale.* Data on the average epithelial thickness in each group on the day 14th can be seen in Table 5.

Table 5: The average number of epithelial thickness.

Groups	Ephitelial thickness (µm) ± SD
K-	$88,18^{a} \pm 27,71$
K+	$110,60^{ab} \pm 41,99$
PI	$171,61^{c} \pm 35,62$
PII	$166,12^{c} \pm 40,85$
PIII	$156,88^{bc} \pm 48,59$

Based on Table 5, it can be seen that the highest average number of epithelial thickness on the day 14th was in the treatment group, a concentration of 15% (PI) with 171,61 µm, while the lowest was in the positive control treatment group (K+) with 88,18 µm.

Based on the statistical test, data was obtained significant result 0.010 ($p < 0.05$). Followed by the Duncan test and the result is that the treatment group concentration 15% (PI) is the group that the most potential on wound healing seen from the number of epithelial thickness.

Group PI, PII and PIII has shown the highest number. The epithelial thickness can indicate the faster process of reepithelization, so that can accelerate the wound healing. The faster process in the treatment group might be affected by loquat leaves extract. Loquat leaves contain tannin, vitamin A, Pratiwi *et al.* (2015) suggested that vitamin A, C, E, tannins. and saponins in clove flower bud extract can help the process of reepithelization by increasing the differentiation of epithelial cells.

Loquat contain secondary metabolites such as alkaloids which have antibacterial and antioxidant effects that are high enough to maintain skin integrity (Kumar *et al.,* 2014), and enough to play a role in the wound healing process by increasing collagen formation, differentiation of epithelial cells and increasing immunity (Negara *et al*., 2014). The antioxidant effect can thicken the epithelial layer. Substitution epithelial tissue occurs on the surface of epithelial cells that continue to experience cell death (Yohana, 2015).

Microscopical examination of epithelial thickness with 100x magnification can be seen in Figure 3.

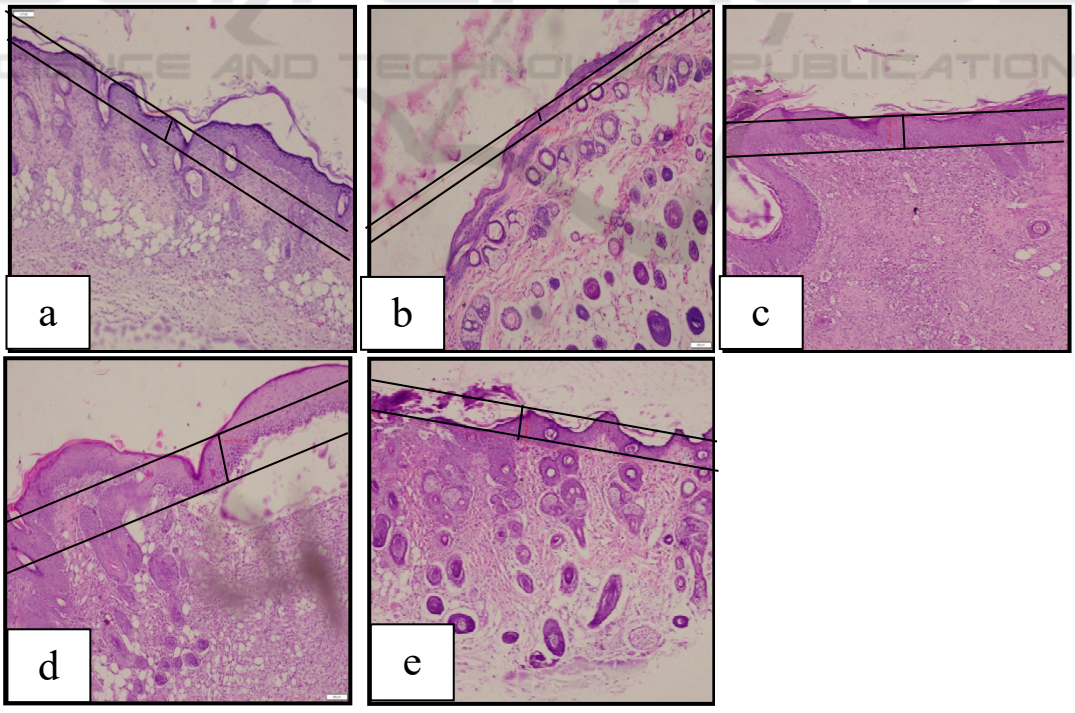

Figure 3: Microscopical examination of ephitelial thickness.
(a) negative control (K-); (b) positive control (K+); (c) loquat leaves extract with concentration 15% (PI); (d) loquat leaves extract with concentration 30% (PII); (e) loquat leaves extract with concentration 45% (PIII), (➡)fibroblas (➡) limfosit.

4 CONCLUSIONS

The average time span of wound healing for each group K-, K +, PI, PII, and PIII were subsequently 8 days, 9.2 days, 7.6 days, 8.4 days, and 9.4 days. Wounds treated with 15% concentration of loquat leaf ethanol extract (PI) showed the fastest healing effect while the 45% concentration of loquat leaf ethanol extract (PIII) showed the longest healing effect. The average number of epithelial thickness, fibroblasts, and lymphocytes in the treatment group of loquat leaf ethanol extract with a concentration 15% (PI) is higher than the other treatment groups with the significant results of statistical analysis.

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