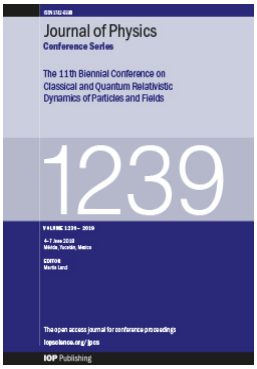

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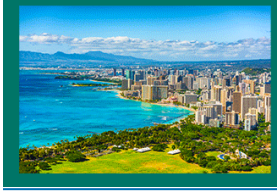

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Rhodomyrtus Tomentosa Extract Activity on Diabetic Wound

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Abstract. *Karamunting* (*Rhodomyrtus tomentosa (Aiton) Hassk*) is a plant that is widely used as a traditional medicine for, among others, wound healing. This study aims to determine the activity of ethanol extract ointment of 70% *karamunting* leaves (*Rhodomyrtus tomentosa (Aiton) Hassk*) and its ability on topical healing of diabetic wounds. A total of 48 rats were divided into 6 treatment groups, namely *karamunting* leaf extract ointment 2.5%, 5%, and 10%, diabetic control, non-diabetic control, and honey. The data obtained were the number of inflammatory cells, the number of fibroblasts, collagen density and epithelial thickness which were observed in days 3, 7, 10 and 14 days after the making of the wound. Based on histological observations, the results showed that the application of ethanol extract ointment with 70% *karamunting* leaf could affect the number of inflammatory cells, fibroblasts, and collagen density as well as significant re-epithelization speed compared to diabetes controls. Of the three concentrations tested, ointment at a concentration of 10% showed comparable results to honey. It can therefore be concluded that the application of ethanol extract ointment of 70% *karamunting* leaves can increase the speed of healing diabetic wounds.

1. Introduction

According to the International of Diabetic Federation (IDF 2015), the global prevalence of people suffering diabetic has increased in 2014 by 8.2% with 387 million people compared to 2013 with 382 million people. The figure is expected to increase to more than 592 million in 2035 (da Rocha Fernandes et al. 2016). The probability of diabetics with diabetic foot wounds is around 15-25%. A research conducted at a hospital in Indonesia discovered that the range of diabetic foot wounds is around 17-32%, with cases of amputation reaching 15-30% (Soewondo et al. 2017)

Wound healing begins with an inflammatory response, whereby inflammatory cells move towards the injured parts and phagocytosis such as bacteria that can cause the infection (Watson 2014). Inflammatory cells consist of neutrophils and macrophages that secrete cytokines and various growth factors (Ebaid et al. 2013). In the next phase, i.e. the proliferation phase, fibroblasts migrate to the wound causing granulation tissue formation, collagen synthesis, and angiogenesis as well as starting to proliferate to produce an extracellular matrix (Velnar, Bailey, and Smrkolj 2009).

Indonesia is a country which has abundant potential for medicinal plants, influencing the society to switch to natural ingredients. The increasing use of traditional medicines, both in quality and quantity, encourages people to use traditional plants as medicine. One of the plants used as a traditional medicine for wound healing is *karamunting* leaves (*Rhodomyrtus tomentosa (Aiton) Hassk*). *Karamunting* is one

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of the plants that must be developed because it has been reported to possess a number of properties, including antidiabetic, anti-diarrheal, and the ability to heal burns (Lim 2016).

The activity of natural ingredients in wound healing is closely related to antioxidant and anti-inflammatory activities, and these two activities are possessed by the *karimunting* plant. The in vitro antioxidant test showed that *karamunting* fruit extract contained 62.09 ± 2.63% RE flavonoids (Wu et al. 2015). Jeong et al (2013) discovered that the methanol extract of *karamunting* leaves showed an anti-inflammatory effect in vivo by inhibiting the production of inflammatory mediators (*nitrite oxide* and prostaglandins) in gastritis and colitis rats. Other studies have shown the activity of *karamunting* leaves as an antibacterial especially in staphylococcus, which is known to be the main cause of infection in diabetic wounds (Saising et al. 2008). Based on this background, this study aims to test the activity of *karamunting* leaves in diabetic wounds

2. Methodology

Ethanol Extraction 70% Leaves Remek Meat

Around 9 kg *karamunting* leaves were cleaned and dried under indirect sunlight. After being dried the plant was then crushed and powdered. The simplicia powder which can be sifted with a 40 mesh sieve was then stored in a clean and tightly closed container. The simplicia powder that was obtained was as much as 750g. They were then extracted by maceration with 70% ethanol for 1 day. During the maceration process, stirring was carried out in the first 6 hours to speed up the dissolving of the components contained in *karamunting* leaves. After 24 hours, they were filtered using filter paper and the dregs were soaked again. The procedure was repeated for 7 days so that the *karamunting* leaves powder could be completely extracted. The maceration results were freed from the solvent by evaporating themusing a vacuum rotary evaporator at a temperature of ± 50°C until a thick extract was obtained. The yield obtained was 18.68% and the water content obtained was 18.86%. The results of the phytochemical screening test are shown in Table 1.

Table 1. Results of Phytochemical Screening Test of *Karamunting* Leaf Extract

No	Filtering	Result	Information
1	Alkaloid	-	Yellow
2	Flavonoid	+	Red
3	Saponin	+	Foam doesn't disappear
4	Tanin	+	Blackish Green
5	Steroid	-	Brown

Test Animals

The research procedure was approved by the Ethics Commission of the Muhammadiyah University Prof. Dr. Hamka (No.02/18.07/012). This study used 48 male white rats. Rats were acclimated in the research room for 14 days in order to adapt to the environment and were given standard feed and drinking ad libitum.

Table 2. Treatment of Test Animals

Day	Group					
	Non-Diabetes	Diabetes	Honey	2.5% concentration	5% concentration	10% concentration
1				Induction of STZ by IP		
5				Blood Sugar Check		
6				Shearing rat		
7				Wound Making		

8-21	Without treatment	Ointment base is applied	Given 2 drops of honey	Topical application of 2.5% extract ointment	Topical application of 5% extract ointment	Topical application of 10% extract ointment
10, 14, 17,21	Tissue collection for histopathological observation					

Induction of Diabetes Wounds

Rats were induced by STZ intraperitoneal, 5 days after STZ induction was carried out on venous blood in the tail. The examination of blood glucose levels using a clinical spectrophotometer. On the 6th day, the rats were shaved in the upper back. On the 7th day, the rats were anesthetized using ketamine at a dose of 40.08 mg/kgBW of rats intramuscularly. The area of the upper back where the wound was created was cleaned with alcohol. On the shaved back, a pattern is made with a diameter of 1 cm, then the skin was pulled and cut using surgical scissors to make an excision wound on the skin below the panniculus carnosus following the pattern (Galehdari et al. 2016). After being injured, the rats were given paracetamol 500mg orally to reduce pain.

Histological Observations

Skin biopsy specimens were taken on day 3, 7, 10, 14 after injury. The tissue taken is the wound tissue to the muscle so that when observing the skin parts are clearly visible. The tissue was taken using a scalpel. Before sampling, the test animals were injected with 230mg/kg i.p ketamine. Afterwards, the tissue was fixed using 10% formalin (Badr 2013). Observation on the number of inflammatory cells, the number of fibroblasts, collagen density and thickness of re-epithelialization were carried out using a light microscope (Olympus) in each group on day 3, 7, 10 and 14 after injury. In observing the number of inflammatory cells and the number of fibroblasts using a magnification of 400x in 10 fields of view, it was observed and calculated using the Image Raster 3.0 application. Meanwhile, observation of collagen density with taking collagen images per 10 fields of view and of the epithelium was carried out in every 5 fields of view in the wound area randomly with a magnification of the object 400x (Ozay et al. 2018). The process of measuring collagen thickness was carried out by observing it using the Photoshop CS 6.0

Data Analysis

The data from the observations were statistically tested in the forms of the number of inflammatory cells, the number of fibroblasts, the density of collagen and the thickness of the re-epithelialization, tested for normality and homogeneity, after which one-way analysis of variance (ANOVA) was carried out, then followed by the Tukey test.

3. Result and Discussion

For diabetic wounds, the healing process occurs slowly due to poor blood circulation, resulting in reduced absorption of nutrients and oxygen (Bhan et al. 2013). In this study, rats were first induced with streptozotocin (STZ) to make them experiencing hyperglycemia. STZ, which is toxic to pancreatic β cells, enters pancreatic β cells through the glucose transporter GLUT 2 (glucose transporter type 2) and inhibits insulin production and causes pancreatic β necrosis (Goud, Dwarakanath, and Chikka Swamy 2015). The dose of STZ used was 40 mg / kgBB for each mouse and the blood sugar was measured on the 5th day after STZ induction.

Wounds in diabetic conditions experience prolonged wound healing due to the inhibition of the wound healing process in each of its phases, which consists of the inflammatory phase, the proliferation phase and the remodeling phase (Koh and Dipietro 2019) (Falanga 2004). The proliferation phase consists of neo-angiogenesis, fibroblast proliferation, collagen synthesis, and re-epithelialization (Dahiru et al. 2016).

The wound healing in the inflammatory phase results showed that the high inflammatory cell was at a concentration of 5% and a concentration of 10%, comparable to that of the non-diabetes group and the

honey group on day 3, where the peak of the inflammatory phase was occurring (Table 3). The peak of the inflammatory phase occurred when a large number of inflammatory cells went to the wound for bacterial phagocytosis. This is significantly different from the diabetes group which had a small number of inflammatory cells ($p<0.05$)

Table 3. Results of the Average Number of Inflammatory Cells

	Total Mean ± SD			
Group	**Day 3**	**Day 7**	**Day 10**	**Day 14**
Non-Diabetes	121,35 ± 15,51[a]	84,52 ± 9,70	63,85 ± 11,65	44,45 ± 10,83
Diabetes	86,85 ± 19,00	116,25 ± 9,63	118,100 ± 16,56	98,02 ± 13,45
Honey	113,62 ± 10,26[ab]	91,67 ± 13,00	70,25 ± 10,95	54,57 ± 7,29
SEDK 2,5%	103,30 ± 12,77[b]	104,600 ± 10,43	86,200 ± 15,59	72,57 ± 9,23
SEDK 5%	108,67 ± 11,08[ab]	100,15 ± 9,10	80,15 ± 12,30	61,87 ± 7,91
SEDK 10%	116,32 ± 13,43[a]	89,82 ± 10,01	66,35 ± 9,78	49,40 ± 10,27

Description: SEDK: karamunting leaf extract ointment. The same superscript letter shows no significant difference $p>0.05$.

In Table 3, on 7th and 10th day, it can be seen that in all groups experienced a decrease in the number of inflammatory cells, but this was different from the diabetes group which experienced an increase and only had a peak of inflammation on day 10. This shows that the diabetes group had a significant difference with other groups ($p<0.05$), therefore there is a delay in the inflammatory phase and a delay in the proliferation phase in diabetic animals. Meanwhile, on day 14, all groups showed a decrease in inflammatory cells as well as the diabetes group.

Table 4 shows the proliferation phase, where fibroblasts were active and would migrate to the wound area, causing granulation tissue formation, angiogenesis and collagen synthesis and starting to proliferate to produce an extracellular matrix (Pastar et al. 2018).

Table 4. Results of Average Number of Fibroblasts

	Total Mean ± SD		
Group	**Day 7**	**Day 10**	**Day 14**
Non-Diabetes	130.73 ± 26.13	169.9 ± 9.71[a]	80.32 ± 9.44
Diabetes	68.03 ± 10.27	85.62 ± 10.69[c]	126.67 ± 14.02
Honey	106.65 ± 13.70	131.22 ± 7.89[b]	86.92 ± 13.44
SEDK 2,5%	90.85 ± 18.14	104.25 ± 12.24 [d]	95.75 ± 11.83
SEDK 5%	92.73 ± 10.15	110.85 ± 10.45[d]	93.55 ± 13.79
SEDK 10%	119.97 ± 19.91	135.85 ± 8.23[b]	81.37 ± 13.13

Description: SEDK: karamunting leaf extract ointment. The same superscript letter shows no significant difference $p>0.05$.

Fibroblasts were cells responsible for collagen synthesis. On day 7 (Table 5), it was seen that on the number of fibroblasts in the non-diabetes group, the administration of 70% ethanol extract of *karamunting* leaf ointment, and honey group were higher than in the diabetes group. The number of fibroblasts in diabetic wounds was significantly lower than in non-diabetic wounds. This means that in diabetic wounds there was a prolonged inflammatory phase, while collagen synthesis was disturbed, angiogenesis was decreased, and the formation of mature granulation tissue was delayed. Data on day 10 demonstrated that the highest increase in the number of fibroblasts was achieved by the non-diabetes group, followed by *karamunting* leaf ointment with a concentration of 10% and honey control. The increase in the mean number of wound fibroblasts in non-diabetic rats with the ethanol extract of *karamunting* leaf with a concentration of 10% was significantly different in the diabetic group and was comparable to the honey control ($p<0.05$). The high number of fibroblasts in the non-diabetes group made the *karamunting* leaf ointment and the 10th day honey group indicated a peak in the proliferation phase. Fibroblasts were responsible for synthesizing collagen, but they also differentiate into myofibroblasts, which help contract the wound edges.

Collagen is a major component of connective tissue which provides strength and flexibility to tissues. Collagen would cover the wounds by forming new tissue bonds. Wounds that were having depth became flush with the wound edges (Tellechea, Ana., Leal, Ermelindo., Veves, Aristidis., Carvalho 2010). Based on observation, the collagen density increased in all groups. Table 5 also shows that on the 10th day there was a peak in the proliferation phase which made collagen synthesis by fibroblasts continue to increase and collagen would accumulate to provide strength and close the wound.

Table 5. Results of Average Collagen Density

Group	Total Mean ± SD		
	Day 7	Day 10	Day 14
Non-Diabetes	97,95±9,55	99,45±8,56[a]	130,58±7,49
Diabetes	66,89±13,51	73,75±8,85[b]	90,96±7,40
Honey	83,29±8,10	90,99±5,35[c]	118,31±10,52
SEDK 2,5%	76,78±8,05	81,18±7,49[d]	101,66±11,41
SEDK 5%	77,7±11,48	84,82±8,09[c,d]	103,26±6,99
SEDK 10%	87,67±8,80	95,07±6,47[a,c]	121,98±7,84

Description: SEDK: karamunting leaf extract ointment. The same superscript letter shows no significant difference p>0.05.

Collagen density in the group that was applied with a 10% concentration of 70% ethanol extract of *karamunting* leaf ointment on day 7 to day 14 increased in comparison to honey control. Collagen density shows that the ethanol extract of 70% *karamunting* leaf played a role in increasing collagen density, resulting in the acceleration of wound healing in diabetes mellitus. The formation of collagen resulted in a tissue repair process in the extracellular matrix which also has an important role in initiating keratinocyte migration in the wound healing process, especially for the re-epithelialization process (Galiano et al. 2004). Re-epithelialization aims to cover the wound surface with an epithelial layer and is based on the differentiation, proliferation and migration of epidermal keratinocytes.

Table 6. Results of Re-Epithelialization Mean

Group	Total Mean ± SD		
	Day 7	Day 10	Day 14
Non-Diabetes	187,50±35,95	197,49±21,75[a]	217,99±34,59
Diabetes	96,63±23,54	123,24±24,54[b]	127,34±14,75
Honey	163,57±26,69	183,64±8,41[c]	192,81±16,19
SEDK 2,5%	137,08±20,64	157,75±19,43[d]	167,68±29,42
SEDK 5%	142,75±16,87	164,74±30,30[c,d]	176,66±21,54
SEDK 10%	173,47±47,10	190,66±30,02[a,c]	207,86±32,75

Description: SEDK: karamunting leaf extract ointment. The same superscript letter shows no significant difference p>0.05.

Table 7 shows that all groups experienced an increase in re-epitheliolysis from day 7 to day 14. *Karamunting* leaf ointment at a concentration of 2.5% experienced an increase in re-epithelialization, which was significantly different from that of diabetes controls. Meanwhile, at a concentration of 10%, there was an increase in epithelialization which was not significantly different from the positive control given by honey. This was because the mechanism possessed by honey and ointment of 70% *karamunting* leaf extract was the same, namely as an antioxidant and anti-inflammatory which worked to increase collagen density and increase re-epithelialization (Goren et al. 2006). Thicker re-epithelialization can close the wound more quickly, resulting in faster wound healing.

The application of ethanol extract of 70% *karamunting* leaf ointment had an activity against diabetic wounds in male white rats. This could be related to secondary metabolite compounds contained in *karamunting* leaf extract that play a role in wound healing, such as flavonoids, saponins and tannins. Flavonoids are powerful antioxidants which play a role in protecting the body against ROS and increase the function of endogenous antioxidants, and increase the levels of antioxidant enzymes in granulation

tissue. Flavonoids can also increase the process of mitogenesis, cell interaction and adhesion of molecules that play a very important role in the proliferation and epithelialization phases of wound healing. In addition, flavonoids can prevent or prolong the onset of cell death, especially excess fibroblasts, along with increased vascularity in wounds, thereby reducing fat peroxidation.

4. Conclusion

Based on the results of the study, it can be concluded that the ethanol extract ointment of 70% *karamunting* leaf with a concentration of 10% had activity on the number of inflammatory cells, fibroblasts, collagen density and thickness of re-epithelialization in diabetic wounds.

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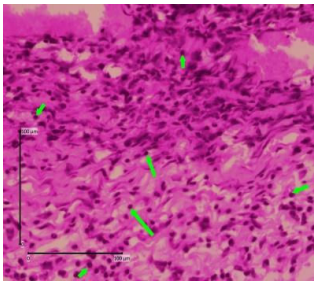
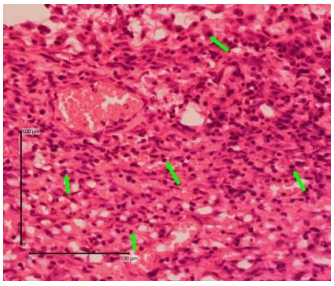
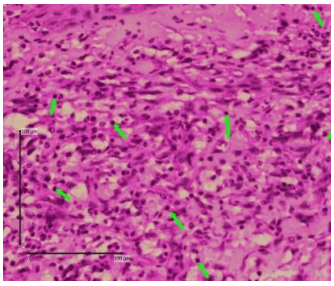
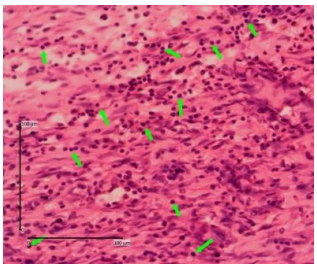
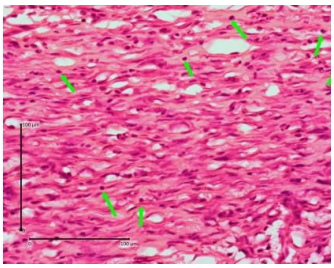
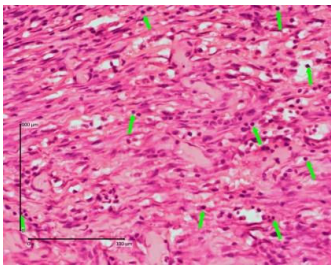
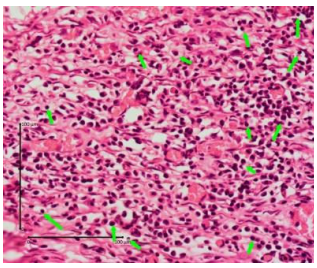
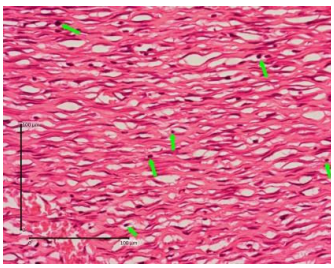
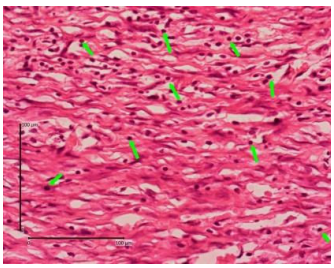
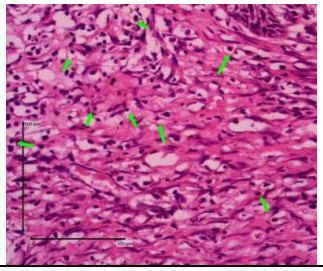
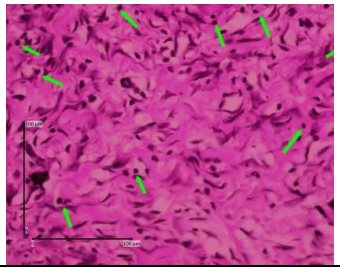
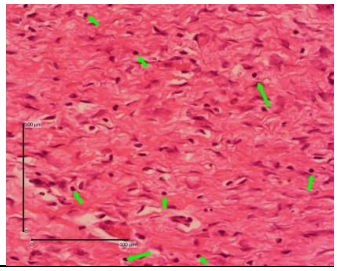
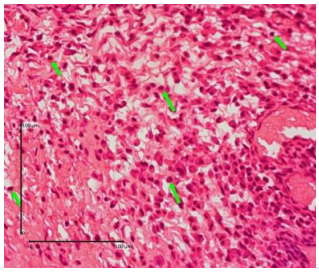
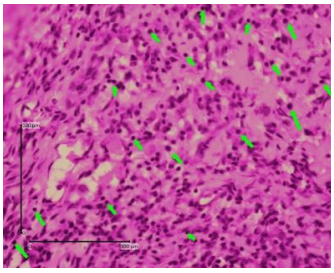
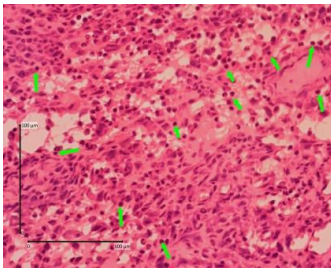
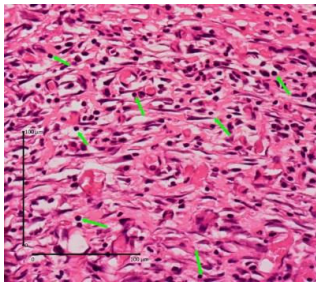
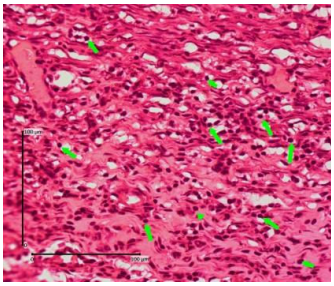
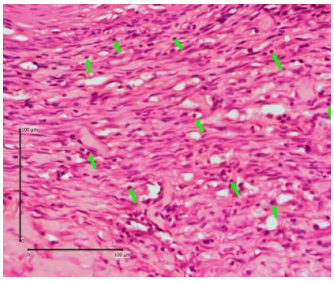
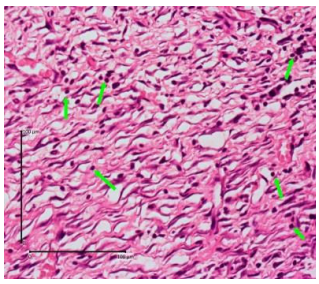
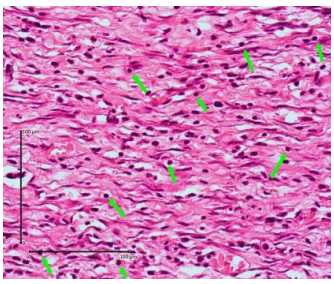
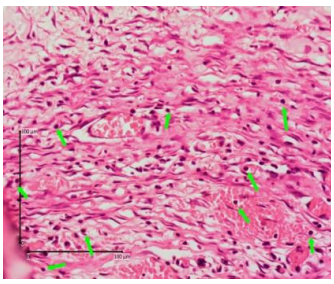
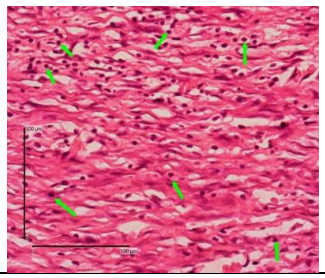
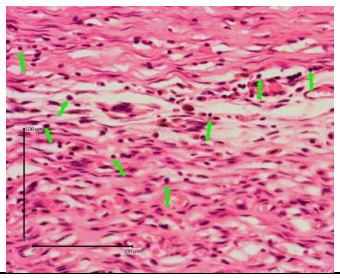
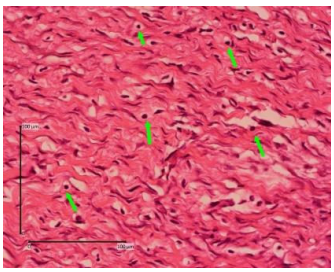
APPENDICES

Appendix 1. Observation Results of Inflammatory Cells

Day	Diabetes	Non-Diabetes	Honey
3			
7			
10			
14			

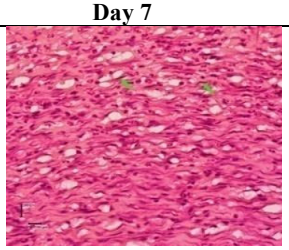
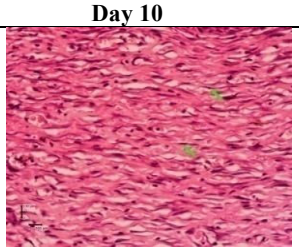
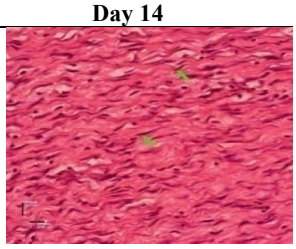
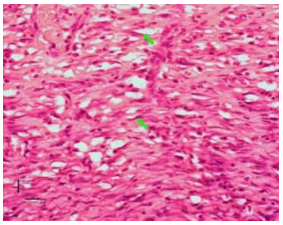
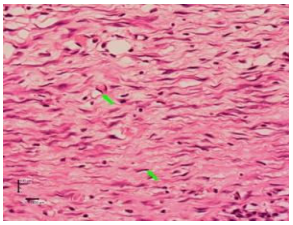
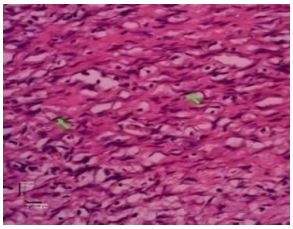
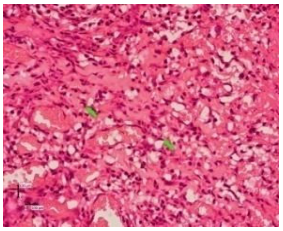
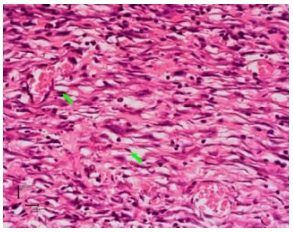
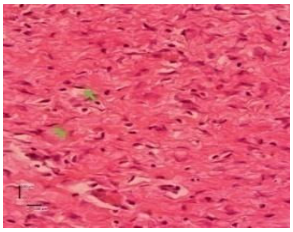
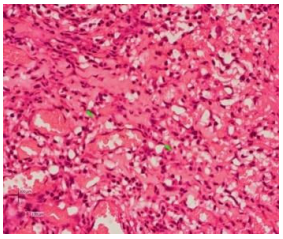
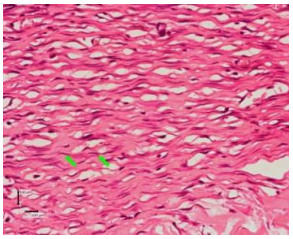
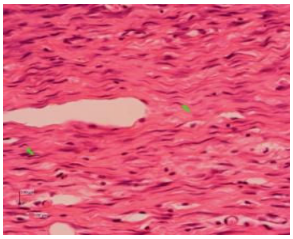
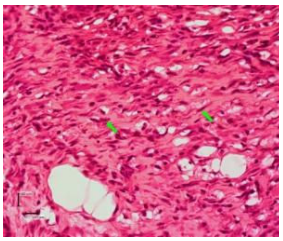
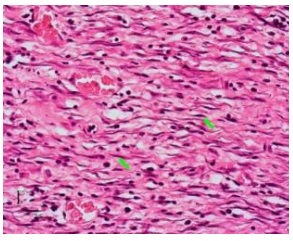
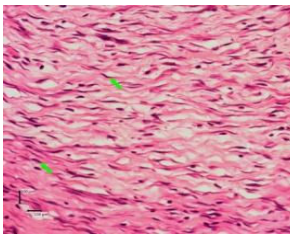
Description: nflammatory cells (green arrow), K = concentration

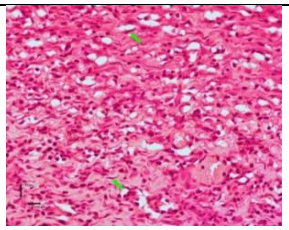
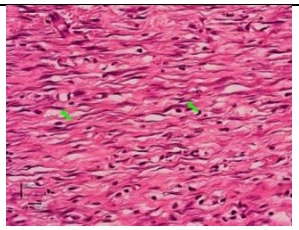
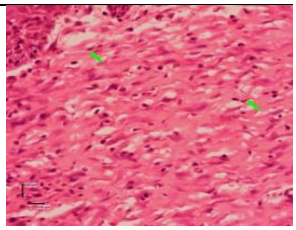
Appendix 2. Observation Results of Inflammatory Cells

Day	K2,5%	K5%	K10%
3			
7			
10			
14			

Description: Inflammatory cells (green arrow), K=concentration

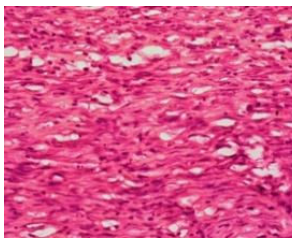
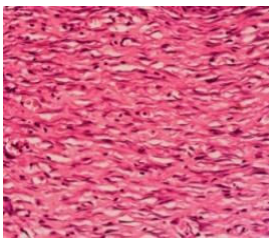
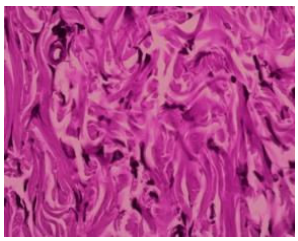
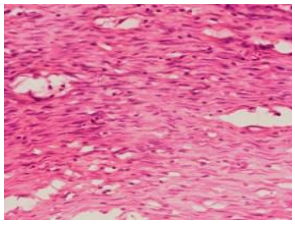
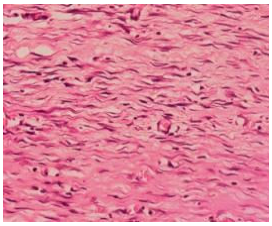
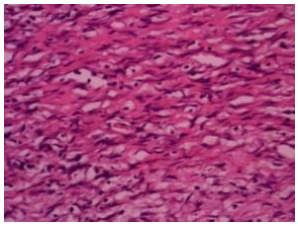
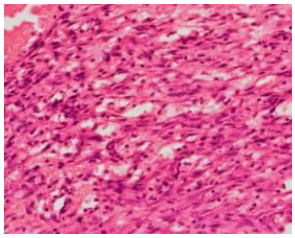
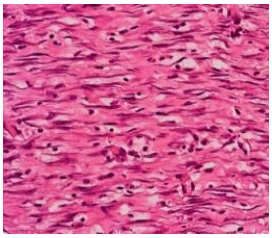
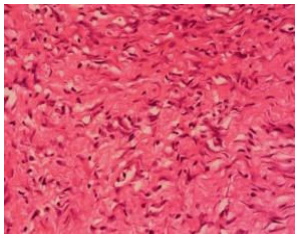
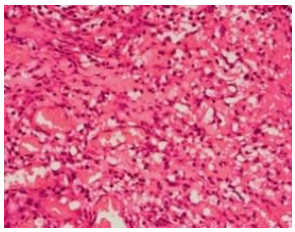
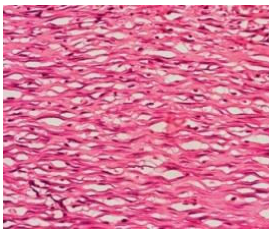
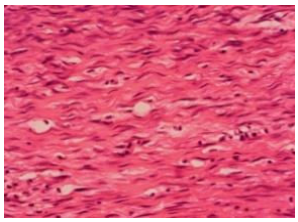
Appendix 3. Results of Fibroblast Observations

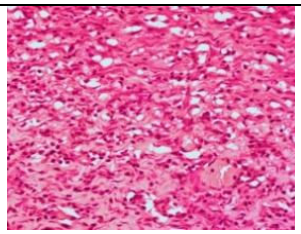
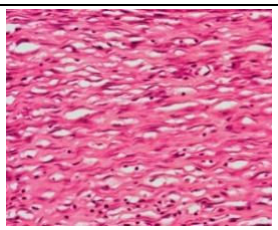
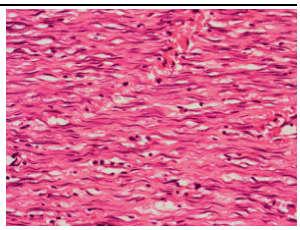
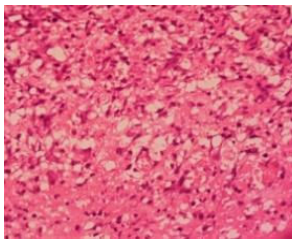
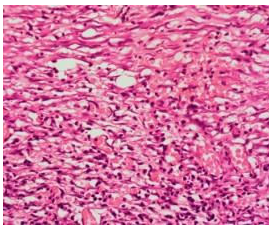
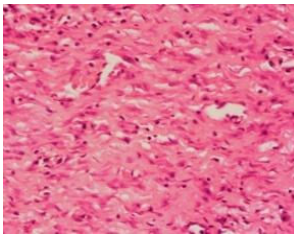
Treatments	Day 7	Day 10	Day 14
Non-Diabetes			
Diabetes			
Honey			
K2,5%			
K5%			

K10%			

Description: Fibroblast cells (green arrow), K=Concentration

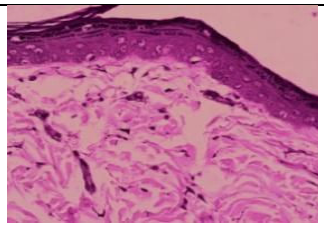
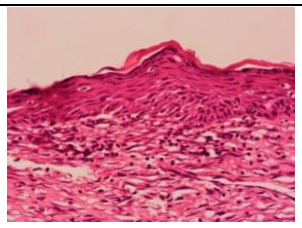
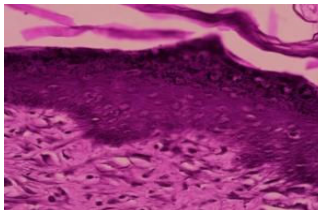
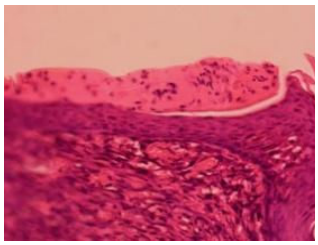
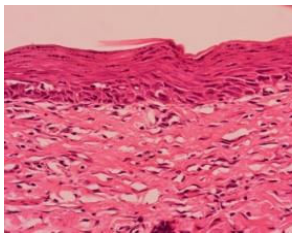
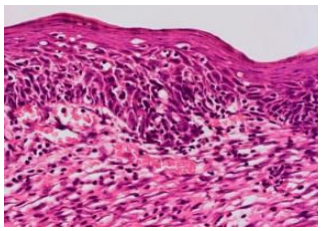
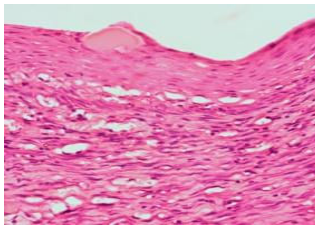
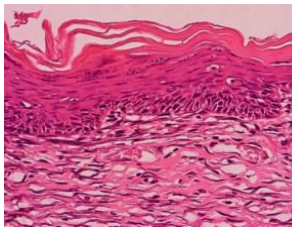
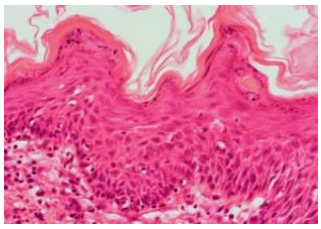
Appendix 4. Results of Collagen Density Histology Observation

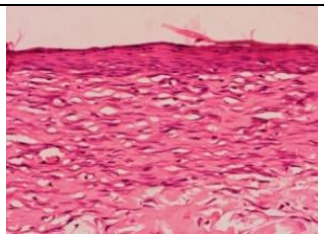
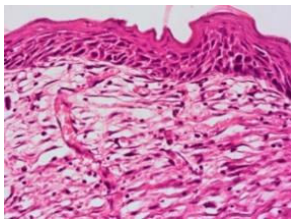
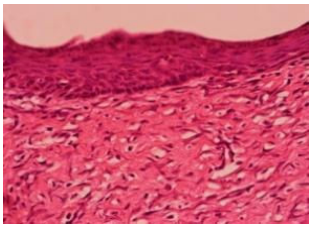
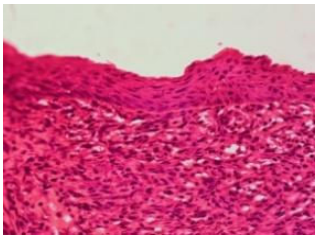
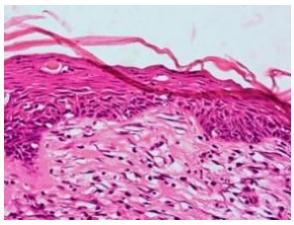
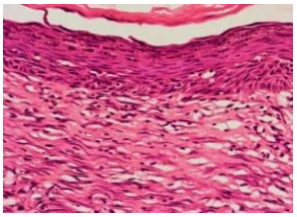
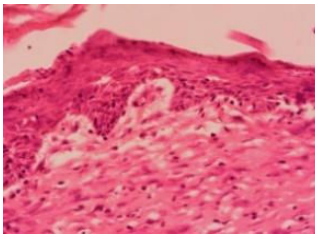
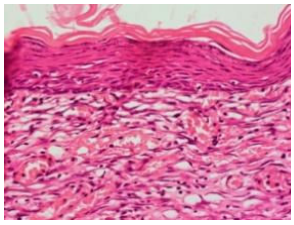
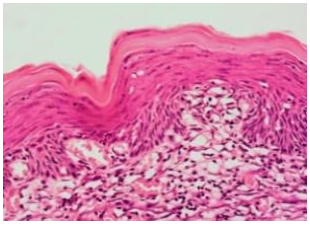
Treatment	Day 7	Day 10	Day 14
Non-Diabetes			
Diabetes			
Honey			
K2,5%			

K5%			
K10%			

Description: Fibroblast cells (green arrow), K=Concentration

Appendix 5. Results of Epithelial Thickness Histology Observation

Treatment	Day 7	Day 10	Day 14
Non-Diabetes			
Diabetes			
Honey			

K2,5%			
K5%			
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