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Antigangrene Activity of Transdermal Patch of Insulin Leaves (*Smallanthus Sonchifolius*) to Diabetic Gangrene on White Rats

(Aktivitas Antigangren Patch Transdermal Daun Insulin (Smallanthus sonchifolius) terhadap Gangrene Diabetik pada Tikus Putih)

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ABSTRACT

Background: Diabetic gangrene is a chronic complication of diabetes mellitus caused by neuropathy, blood vessel disorders, and infection by *Staphylococcus aureus*. *Smallanthus sonchifolius* leaves contain flavonoid as hypoglycemic agents and sesquiterpene lactones as antibacterial. Unfortunately, oral administration of *S. sonchifolius* leaves infusion causes kidney toxicity. **Objective:** The aimed of the study was to determine the effectiveness of the transdermal patch of *S. sonchifolius* leaves ethanol extract on gangrene wound healing with macroscopic parameters and neoangiogenesis of gangrenous wounds in white rats that have been induced by diabetes mellitus. **Material and Methods:** This study used 4 treatment groups: positive control (Bevalex® cream), negative control (patch without *S. sonchifolius* leaves extract), F1 (*S. sonchifolius* leaves patch without enhancer), and F2 (*S. sonchifolius* leaves patch with Tween 60 as enhancer). The dose of *S. sonchifolius* leaves given was 400 mg/kg BW. Alloxan-induced diabetic rat feet were injected with *S. aureus* to form gangrene. Observations were made on the 7th and 14th days. **Results:** Based on the Wagner-Meggit scale on macroscopic observations, administration of a transdermal patch of *S. sonchifolius* leaves accelerates gangrene healing. The statistical results of neoangiogenesis on the 7th and 14th days showed a significant difference ($p \leq 0.05$) between the positive control, F1, and F2 to the negative control. F2 showed the highest angiogenesis on day 7 (114.00 ± 5,00) and 14 (161.00 ± 5.29) compared to all groups. Tween 60 as enhancer increase the number of angiogenesis. Both F1 and F2 did not show a significant difference to the positive control. **Conclusion**: *S. sonchifolius* leaves ethanol extract patch accelerated the diabetic gangrene healing process based on macroscopic and neoangiogenesis observation on the 7th and 14th days. Toxicity examination in white rats are needed before clinical study in human.

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ABSTRAK

Latar belakang: Gangren diabetes merupakan komplikasi kronis dari diabetes mellitus yang disebabkan neuropati, gangguan pembuluh darah, dan infeksi oleh bakteri *Staphylococcus aureus*. Daun *Smallanthus sonchifolius* ini mengandung flavonoid yang dapat menurunkan kadar glukosa darah serta seskuiterpen lakton yang berperan sebagai antibakteri. Akan tetapi, penggunaan per oral infus daun *S. sonchifolius* menyebabkan toksisitas pada ginjal. **Tujuan:** Penelitian ini bertujuan untuk mengetahui aktivitas *patch* transdermal ekstrak etanol daun *S. sonchifolius* dengan *enhancer Tween 60* terhadap pengamatan makroskopis dan neoangiogenesis luka gangren tikus putih. **Bahan dan Metode:** Penelitian ini menggunakan 4 kelompok perlakuan, yaitu kontrol positif (krim Bevalex®), kontrol negatif (*patch* tanpa ekstrak daun *S. sonchifolius*), F1 (*patch* ekstrak etanol daun *S. sonchifolius* tanpa *enhancer*), dan F2 (*patch* ekstrak etanol daun *S. sonchifolius* dengan *enhancer* Tween 60). Dosis daun *S. sonchifolius* yang dipilih sebesar 400 mg/kgBB. Tikus putih dibuat diabetes dengan induksi aloksan, kemudian diinjeksi *S. aureus* pada bagian kaki untuk membentuk gangren. Pengamatan dilakukan pada hari ke-7 dan ke-14. **Hasil:** Berdasarkan skala Wagner-Meggit pada pengamatan makroskopis, pemberian patch transdermal daun *S. sonchifolius* mempercepat penyembuhan gangren. Hasil statistik neoangiogenesis pada hari ke-7 dan ke-14 menunjukkan perbedaan bermakna ($p \leq 0.05$) antara kontrol positif, F1 dan F2 terhadap kontrol negatif. Kelompok F2 menunjukkan angiogenesis tertinggi pada hari ke-7 (114.00 ± 5,000) dan ke-14 (161.00 ± 5,292). Tween 60 sebagai *enhancer* meningkatkan jumlah angiogenesis. Namun terhadap kelompok kontrol positif, F1 dan F2 tidak menunjukkan perbedaan bermakna. **Kesimpulan:** Sediaan *patch* transdermal ekstrak etanol daun *S. sonchifolius* mempercepat penyembuhan gangren berdasar parameter makroskopis dan neoangiogenesis pada hari ke-7 dan ke-14. Pengujian toksisitas pada tikus putih diperlukan sebelum uji klinis pada manusia.

Kata kunci: *Smallanthus sonchifolius, Patch*; Gangren, Neoangiogenesis, Makroskopis

INTRODUCTION

Diabetic gangrene is one of the complications of diabetes and as the result of multiple contributing causes. Gangrene usually occurs due to reduced blood supply and is characterized by necrosis of underlying tissue and skin. Gangrene can be classified as dry or wet gangrene. Dry gangrene is a condition characterized by tissue death, that turns to dark, and dry up because of arterial occlusion. Wet gangrene occurs due to motionless venous or arterial blood flow, leading to bacterial infection. Various factors contribute to diabetic gangrene, but the major cause is neuropathy, lack of glycemic control, and ischemia from peripheral disease arteria (Al Wahbi, 2018). Local edema and thrombosis due to toxins from bacteria (mainly *Staphylococcus sp*. and *Streptococcus sp*.) can cause ischemic necrosis of the lower extremity of the patient with diabetes, mainly the tip of the toe or toes. Pharmacological approaches for gangrene management includes pain management, circulation management, and antibiotics. The bacteria that cause diabetic gangrene are generally found to have a polymicrobial pattern. The most common bacteria were *Pseudomonas aeruginosa* (30.57%), *Klebsiella* (22.29%), *Escherichia coli* (16.56%), *and Staphylococcus aureus* (12.74%)(Aumiller & Dollahite, 2015).

The wound-healing process is usually characterized by four sequential phases. The first phase is hemostasis, a variety of cytokines and growth factors are released into the wound during this stage, mediating the communication and synergizing the activity of different cell players to accomplish the task of healing. The second is the inflammation phase, involving mainly activation of the innate immune system. Neutrophils and monocytes rapidly migrate into the injured skin. Monocytes differentiate into macrophages and support healing. The third is the proliferation phase, reepithelization, and proliferation of keratinocytes occur. The fourth is the remodeling phase, collagen III is replaced by collagen I, which has a higher tensile strength. The number of new blood vessels and the blood flow decline. A mature avascular and acellular environment is formed (Landén et al., 2016).

Insulin or yacon (*Smallanthus sonchifolius*) is a plant originating from the Andes mountains, in Peru. In Indonesia, this plant is widely cultivated in the Wonosobo. Empirically *S. sonchifolius* leaves are used as an antidiabetic drug by boiled or brewed and used two to three times a day to lower blood glucose levels. The active compounds in *S. sonchifolius* leaves are fructooligosaccharides, phenols, chlorogenic acid, and flavonoids which can reduce blood glucose levels (Pahlawan & Oktaria, 2016). Flavonoids have antidiabetic effects through several pathways such as reducing pancreatic cell apoptosis, promoting *S. sonchifolius* secretion, glucose regulation in hepatocytes, reducing inflammation and oxidative stress in skeletal muscle cells and adipose tissue, and increasing glucose uptake in skeletal cells (Triastuti et al., 2020). The flavonoid from *S. sonchifolius* leaves also have an antibacterial effect by inhibiting *Staphylococcus aureus* growth. The mechanism of action is to damage of permeability of the bacterial cell wall and inhibit bacterial motility (Ramonah dkk., 2020). Other compounds, sesquiterpene lactones in *S. sonchifolius* leaves are also shown antihyperglycemic and antimicrobial activity. There are 6 types of sesquiterpene lactones metabolite identified from *S. sonchifolius* leaves and have been tested for antihyperglycemic and wound healing activity. The sesquiterpene lactone enhydrin was suggested responsible for the antihyperglycemic and wound-healing activity of yacon leaves (Herowati dkk., 2018). Unfortunately, oral administration of 2% *S. sonchifolius* leaves infusion at a dose of 100 mg/kg BW for 90 days causes kidney toxicity (Dos Santos et al., 2017). For this reason, we propose alternative delivery by transdermal delivery for *S. sonchifolius* leaves.

A transdermal patch is a dosage form administered through the skin to reach systemic circulation. This transdermal route provides several advantages such as increased patient compliance, maintaining sustained steady-state blood levels, avoidance of gastric acid irritation, and first-pass metabolism in the liver (Ali, 2017; Pastore et al., 2015). Stratum corneum is the outer layer from the skin. This layer of the skin is the most impermeable, forming a laminate of compressed keratin-filled corneocytes attached in a lipophilic matrix. Permeation enhancers are the compounds which promote skin permeability. The nonionic surfactants enhance absorption by inducing fluidization of the stratum corneum lipids (Das & Ahmed, 2017; Pandey, 2014). The aimed of this study was to determine the effectiveness of the transdermal patch of *S. sonchifolius* leaf extract on gangrene wound healing with macroscopic parameters and neoangiogenesis of gangrenous wounds in white rats that have been induced by diabetes mellitus.

MATERIAL AND METHODS

Materials

The materials were ethanol extract of *S. sonchifolius* leaves, HPMC, propylene glycol, Tween 60, and carrageenan. All the chemicals were pharmaceutical grade (obtained from PT. Brataco). Bevalex® cream was used as the positive control (betamethasone valerate 1 mg and neomycin sulfate 5 mg), and glucometer (Easy touch®). The animals used were male white rats (*Rattus norvegicus*) Wistar strain to avoid hormonal change. The certificate for ethical clearance was E/116/UHT.KEPK.03/IX/2021

Methods

Production of *Smallanthus sonchifolius* leaves ethanol extract

The *S. sonchifolius* leaves powder of 1 kg was macerated with 3.5 L of 70% ethanol for 24 hours and then filtered. Remaceration with 2.5 L of 70% ethanol for 24 hours and then concentrated by rotavapor.

Preparation of *Smallanthus sonchifolius* leaves ethanol extract patch

The formula of *S. sonchifolius* leaves ethanol extract patch presented in table 1, made using the solvent evaporation technique in a petri dish. There are two formulas to compare antigangrene effect from transdermal patch with and without enhancer. Formula 1 (F1) contain no enhancer and formula 2 (F2) contain Tween 60 as penetration enhancer. The solution was dried at a temperature of 40°C for 24 hours and then cut to a size of 2 cm x 2 cm. The physical properties of the patch were evaluated by parameters: organoleptic, weight uniformity, thickness, moisture content, folding endurance, aeration, and pH

Table 1. Formulation of *Smallanthus sonchifolius* Leaves Ethanol Extract Patch

Composition	**Control -**	**Formula 1**	**Formula 2**
Ethanol extract of *S. sonchifolius* leaves	-	1.5 g	1.5 g
Tween 60	0.4 g	-	0.4 g
HPMC	0.8 g	0.8 g	0.8 g
Propyleneglycol	4 mL	4 mL	4 mL
Aquadest until	50 mL	50 mL	50 mL

Preparation of *Staphylococcus aureus* bacteria suspension

One sterile wire loop of *S. aureus* stock colonies is inoculated into Mannitol Salt Agar (MSA) sterile, followed by incubation at 35°C–37°C for 24 hours. Then the result of *S. aureus* proliferation was put into a test tube with sterile wire loops containing 2 mL of 0.9% NaCl. The suspension was shaken until a cloudy suspension formed and compared with 0.5 McFarland's turbidity (99.5 mL of 0.36 N H_2SO_4 solution was mixed with 0.5 mL of 1.175% $BaCl_2.2H_2O$ solution in erlenmeyer) (Naimi et al., 2017).

Induction of diabetes in the white rat

Before being given the sample, 48 white rats were adapted for 14 days. White rats were injected intraperitoneally with alloxan 2% dissolved in NaCl 0.9% at the dose of 120 mg/kg. Rats were observed to increase blood sugar levels for three days after the alloxan injection. Blood glucose levels were measured via the tail vein using a glucometer to confirm hyperglycemia. White rats with blood glucose levels above 200 mg/dL or fasting blood glucose above 150 mg/dL were declared diabetic rats (Shah & Khan, 2014).

Induction gangrene in diabetic rats

On the fourth day, 0.1 mL of 1% (w/v) carrageenan was injected subcutaneously into the plantar area of the hind leg to induce inflammation (edema) in diabetic rats. Carrageenan is dissolved in 0.9% saline for no more than 24 hours. After being injected, inflammation characterized by edema was observed at 1 to 2 hours. After the rise of edema, the hind limb is incised by a depth of 0.25 cm, and then 0.2 mL of *Staphylococcus aureus* is injected 1x10 cells/mL of bacteria to trigger gangrene. Observe for gangrene for one weeks (Qosimah et al., 2019).

Treatment with *S. sonchifolius* leaves ethanol extract patch

White rats were divided into two groups that will be observed after treatment by emulgel for 7 and 14 days. Each group was divided into smaller four groups consisting of six rats in each smaller group. Each smaller group will be treated by positive control (C+)(Bevalex®), negative control (C-), Formula 1 (F2), and Formula 2 (F2). Each group received an *S. sonchifolius* leaves patch once a day every morning.

Macroscopic Observation

After diabetic gangrene developed, the hind limbs of rats were observed and evaluated on day 0, day 7, and day 14 visually and characterized by the Wagner-Meggit scale. Macroscopic observed include wound, edema, redness, and red to dark color as a sign of diabetic gangrene (Yadwadkar, 2016). Observations were made on the for 7^{th} and 14^{th} days according inflammation phase, proliferation phase and maturation phase of wound healing.

Angiogenesis observation

After the macroscopically observed, animals were euthanized by anesthesia and cervical dislocation. The tissue of the hind limb of rats was collected for histological analysis, fixed with 10% formalin solution for 24 hours, and then cut into 5 mm thickness. Tissue preparation for histopathology analysis using general coloring Hematoxylin-Eosin to observe neoangiogenesis. Observations were made on samples of the hind limb tissue of white rats on the seventh and fourteenth days after gangrene formation. The tissue observation using a microscope at 400x magnification by the fifth field of view randomly.

Microscopic signs of angiogenesis can be seen clearly in the form of a cavity surrounded by bright red endothelial cells.

Data analysis

Statistical analysis was performed using SPSS 19.0. The normal distribution of data was analyzed using the Kolmogorov-Smirnow test. Parameters were compared among the group by ANOVA followed by the Duncan test. The difference with $p<0.5$ was considered statistically significant.

RESULTS AND DISCUSSION

Physical properties of *Smallanthus sonchifolius* leaves patch

The physical properties of the *S. sonchifolius* leaves patch are presented in table 2. Based on this data, it is known that the patch of *S. sonchifolius* leaves has met the requirements. The appearance of this patch was soft, smooth, flexible, and homogeneous. The thickness and weight had low standard deviations and no differences between formulas. This condition ensures patch is reproducible with a negligible variation. Folding endurance will describe the flexibility of the formed patch preparation. The moisture content varies between 7.21-8.9%, meeting the requirement that moisture content should be 2-10% in transdermal patches**.** The lower moisture content will increase stability, reduce brittleness and protect the patch from microbial contamination (Shabbir et al., 2017).

Table 2. Physical Properties of Patch *Smallanthus sonchifolius* Leaves

Formula	Thickness (mm)	Weight (gram)	Aeration	Folding endurance	Moisture contains (%)
Control -	$0.4083^{a} \pm 0.0144$	$0.3320^{a} \pm 0.0072$	No	>150	$8.14^{a} \pm 0.74$
Formula 1	$0.4167^{a} \pm 0.0287$	$0.3343^{a} \pm 0.0042$	No	>150	$8.07^{a} \pm 0.75$
Formula 2	$0.3667^{a} \pm 0.0287$	$0.3253^{a} \pm 0.0047$	No	>150	$8.49^{a} \pm 0.68$

*Data presented mean ± standard deviation, different superscripts (^{a}) mean statistically difference ($p<0.05$) by Duncan test

Observation of Blood Glucose Levels after alloxan induction

The results of blood glucose levels before and after being induced with alloxan are presented in table 3. Induction by alloxan increased blood sugar levels about four times. Alloxan is a diabetes-inducing agent that is widely used in experiments because it selectively damages pancreatic B cells (El-Desouki et al, 2015). In this experiment, all rats had a significant increase in blood sugar levels ($p<0.05$), indicating pancreatic damage in rats, so experimental animals could be used for the next step.

Table 3. Results of Observation of Glucose Levels Before and After Alloxan Induction

Groups	Blood glucose levels before induction (mg/dL)	Blood glucose levels after induction (mg/dL)
Control +	$84.33^{a} \pm 10.65$	$352.83^{b} \pm 12.25$
Control -	$88.17^{a} \pm 8.49$	$348.33^{b} \pm 12.11$
Formula 1	$80.83^{a} \pm 14.47$	$349.00^{b} \pm 6.81$

Formula 2	$80.50^{a} \pm 10.38$	$347.33^{b} \pm 9.54$

*Data presented mean ± standard deviation, different superscripts (^{a,b}) mean statistically difference ($p<0,05$) by Duncan test

Macroscopic observation

Diabetic gangrene was observed macroscopically on days 0, 7, and 14 and scored according to the Wagner-Meggit classification (Yadwadkar, 2016). The result from macroscopic observation was reported in Figure 1 and Table 4. On day 0, based on the Wagner-Meggit classification, all the rats were categorized at grade 4. The gangrene on the hind limb is characterized by the presence of open wounds, swelling, and discoloration to blackish red. On the seventh day, diabetic gangrene in the positive control and treatment groups (F1 and F2) began to improve. Redness and blackish red were no longer seen, but in the untreatment group (C-) gangrene still exists. On fourteen days, wound, edema, and redness still exist in the untreated group and no sign in another group. The presence of Tween 60 as an enhancer on *S. sonchifolius* leaves transdermal patch increases the healing of diabetic gangrene such as positive control.

Table 4. Macroscopic Observation by Wagner-Meggit Classification

Day	Groups	Macroscopic observation				Wagner Meggit classification
		Wound	Edema	Redness	Blackish red	
0	Control +	√	√	√	√	Grade 4
	Control -	√	√	√	√	Grade 4
	Formula 1	√	√	√	√	Grade 4
	Formula 2	√	√	√	√	Grade 4
7	Control +	√	-	√	-	Grade 1
	Control -	√	√	√	√	Grade 4
	Formula 1	√	√	-	-	Grade 2
	Formula 2	√	-	-	-	Grade 1
14	Control +	-	-	-	-	Grade 0
	Control -	√	√	√	-	Grade 2
	Formula 1	-	-	-	-	Grade 0
	Formula 2	-	-	-	-	Grade 0

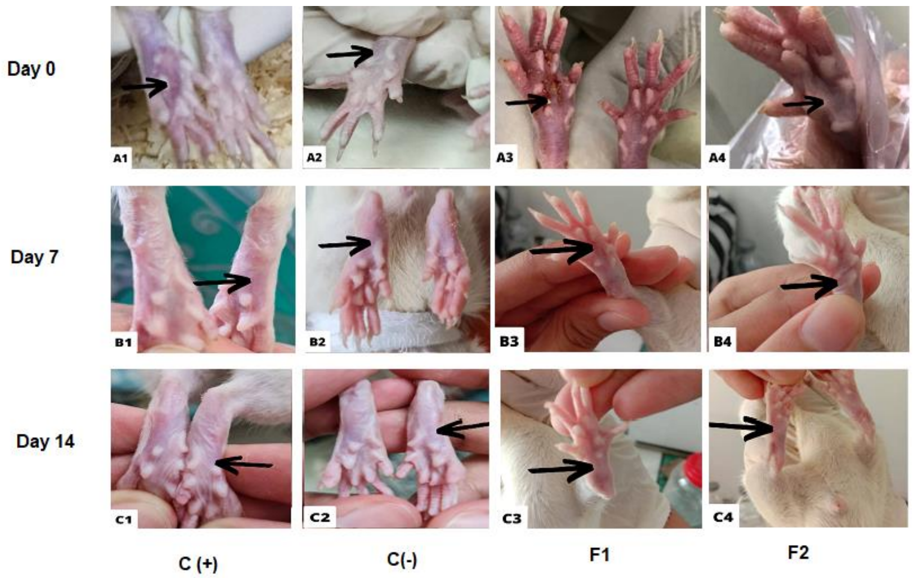

Figure 1. Macroscopic Observations of Gangrene on the Legs of White Rats

Observations of neoangiogenesis

Neoangiogenesis observed in gangrenous wounds in white rats can be seen in Table 5 and Figure 2.

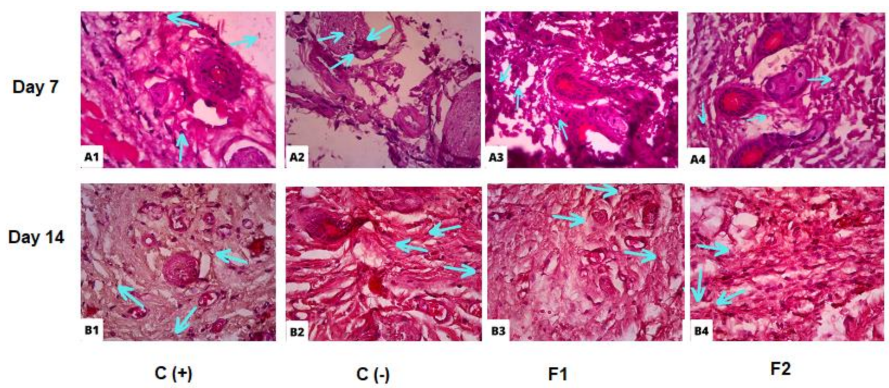

Figure 2. Neoangiogenesis in Gangrene Wounds in White Mice

Table 5. Neoangiogenesis in White Rats

Groups	Neoangiogenesis	
	Day 7	Day 14
Control +	$105.00^b \pm 2.000$	$148.00^b \pm 5.000$
Control -	$40.00^a \pm 6.557$	$79.33^a \pm 8.021$
Formula 1	$109.67^b \pm 5.033$	$154.00^b \pm 8.185$
Formula 2	$114.00^b \pm 5.000$	$161.00^b \pm 5.29$

*Data presented mean ± standard deviation, different superscripts ($^{a, b}$) mean statistically difference ($p<0.05$) by Duncan test

In the observation of the number of angiogenesis, both on the 7th and 14th days, C+, F1, and F2 gave a significantly different in the neoangiogenesis than the negative control group ($p < 0.5$). The F2 treatment group gave the highest mean result on the 7th day. The number of angiogenesis on the 14th day was higher than on the 7th day in all treatment groups. This result is in line with the results of macroscopic examination of gangrene wounds above.

The number of new blood vessel formations in each group was indicated by an increase in the amount of angiogenesis. Angiogenesis is an important process in healing at the site of injury, and in the development of collateral circulation at the site of ischemia. Endothelial cells lining the inner blood vessels are the target cells of angiogenic regulators. Blood vessels are composed of a monolayer of endothelial cells attached to the extracellular matrix and stabilized by pericyte. Endothelial cells can be induced by angiogenic factors to replicate and form new blood vessels in response to physiological and pathological stimulation. When the number of angiogenic factors is produced more than of angiogenic inhibitors, the endothelial cells will be activated, resulting in the formation of new blood vessels (Chronic et al., 2022). The formation of new blood vessels will provide a supply of nutrients to tissue damaged by gangrene and accelerate gangrene wound healing.

The use of Tween 60 as an enhancer has been shown to increase the effectiveness of the *S. sonchifolius* leaves patch. Tween 60 is a partial fatty acid ester that differs in the number of polymerized oxyethylene subunits and the number and type of fatty acid groups. Tween increased transdermal permeation by inducing fluidization of the tight bilayer lipid of the stratum corneum (Saravanakumar et al., 2015).

Based on all data, the administration of *S. sonchifolius* leaves patch affects the healing process of diabetic gangrene wounds. It is predicted that the blood glucose-lowering and antibacterial effects of *S. sonchifolius* leaves simultaneously accelerate wound healing. The ethanol extract of *S. sonchifolius* leaves contains compounds that are efficacious as blood glucose-lowering agents, including protocatechuic, chlorogenic, caffeic acid, and ferulic acid in the leaves which have antidiabetic and antioxidant effects. In addition, *S. sonchifolius* leaves also contains sesquiterpene lactone enhydrin compounds which work by inhibiting the process of glycogenolysis which is the process of breaking down glycogen into glucose in the liver and gluconeogenesis which is a process of glucose synthesis (Dos Santos et al., 2017). Enhydrin had an antibacterial effect which inhibits the development of 2 strains of Methicillin-resistant *S. aureus* (Elawati & Yuanita, 2021).

The limitations of this research are the active compounds that cross the skin membrane have not been identified so it is not known which is compound that have dominan effect, and this research have not carried out toxicity examination.

CONCLUSION

Taken all together, our research showed that patch contained *Smallanthus sonchifolius* leaves ethanol extract can be to accelerate the diabetic gangrene healing process based on macroscopic and neoangiogenesis observation on the 7th and 14th days. Toxicity examination in white rats are needed before clinical study in human in the future.

CONFLICT OF INTEREST

The author declare that there are no conflict of interest

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