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Anti Inflammatory and Wound Healing Potential of Liverwort Extract and Collagen-Transdermal Patch in Diabetic Rats

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ABSTRACT

Patients with diabetes often experience impaired wound healing, which is associated with intense inflammation. Liverwort extract is rich in secondary metabolites with anti-inflammatory properties, while collagen is crucial for skin regeneration and wound healing. This study investigated the effect of a transdermal patch containing liverwort extract and collagen derived from tilapia bones on inflammation and wound healing in diabetic rats. A completely randomized design was used, consisting of eight groups in total: a normal control group (C) on a standard diet, a negative control group (NC) injected with alloxan at a dose of 120 mg/kg body weight, a positive control group (PC) injected with alloxan and treated with a transdermal patch containing an antibiotic, and five groups injected with alloxan and treated with transdermal patches containing different ratios of liverwort extract and collagen for 14 days. An analysis of variance was used for all data with 5% significance levels ($p<0.05$). The results showed that alloxan significantly increased blood sugar levels, confirming that the rats were in a diabetic condition. Wound healing was compared among the non-diabetic group, the diabetic group, and the diabetic-treated groups, with significant differences observed. The percentage of white blood cells observed in peripheral blood smear examinations was significantly lower in the diabetic-treated groups compared to the diabetic group, indicating substantial improvement. An increased number of white blood cells is associated with greater inflammation. The topical application of the liverwort and collagen transdermal patch reduced this effect. These findings indicate that the liverwort and collagen transdermal patch has the potential for reducing inflammation and promoting wound healing.

Keywords: Diabetes, collagen, liverwort, transdermal patch, wound healing

Introduction

Diabetes mellitus (DM) has become a major global public health concern, including in Indonesia. According to the International Diabetes Federation (IDF), in 2021, Indonesia ranked fifth globally in diabetes prevalence, with 19.5 million cases, and is projected to rise to 28.6 million by 2045 (Ministry of Health, 2024). DM is a metabolic disorder caused by the inability or lack to produce insulin or can be due to malfunctioning insulin receptors, which prevents cells from receiving glucose for metabolism (Antar et al., 2023). This condition is known as the "mother of all diseases" due to the numerous problems that can arise if left untreated including diabetic ulcers caused by macroangiopathy leading to vascular insufficiency, inflammation, and neuropathy.

Diabetic ulcers increase the risk of amputation 10-20 times compared to people without diabetes (IDF, 2017). These ulcers are primarily caused by infections resulting from high blood glucose levels, which promote bacterial growth and immune system deficiencies that prolong wound inflammation. Initially, the pro-inflammatory response is characterized by low cytokine levels in the wound and increased levels of neutral endopeptidase, an enzyme that degrades

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various substances in the skin. In the next stage, reduced stimulation of keratinocytes, fibroblasts, and endothelial cells further impairs healing. However, in diabetic condition, this process is delayed and can lead to the pro-inflammatory development of chronic wounds. The chronic wound induces an inflammatory response that is characterized by the accumulation of leukocytes (macrophages, neutrophils, basophils, and T cells) due to overexpression of the complement system (Worsley et al., 2023). This response produces free radicals through non-enzymatic glycation, glucose oxidation, and increased lipid peroxidation, leading to oxidative stress that damages enzymes, weakens tissues, and contributes to insulin resistance (Asmat, 2016).

Chronic wounds in diabetic patients show significant challenges, impeding recovery also increasing healthcare costs and the risk of severe complications, such as infections and amputations (Frykberg & Banks, 2015). Due to the limitations of conventional diabetic ulcer treatments—including issues like antibiotic resistance and hypersensitivity—interest has grown in alternative treatments. Natural treatments, commonly used in complementary and alternative medicine, are promising options for managing chronic wounds in diabetic patients. In Indonesia, traditional medicine remains widely used; according to Kemenkes RI (2018), 59.12% of households use traditional remedies, with usage increasing from 63% to 71% between 2016 and 2019. This trend reflects the public perception that natural ingredients are safer, more accessible, and affordable. Lau and Sunarti (2018) further observed that the belief in their low side effects drives a preference for natural remedies over modern treatments.

One promising natural resource is bryophyta, particularly liverworts (Marchantia), which are rich of secondary metabolites like terpenoids and flavonoids and have a long history of use in traditional medicine across various countries (Ludwiczuk & Asakawa, 2019). Indonesia, with the second highest biodiversity globally, is well-suited for such resources due to its tropical climate, vast forests, and high rainfall. Home to approximately 30,000 plant species—about 10% of the world's flora—Indonesia is rich in bryophytes, including liverworts, which thrive in humid environments and can grow in a wide range of habitats. Liverwort extracts have shown antibacterial, anticonvulsant, antipyretic, and antiseptic properties (Motti et al., 2023), and a study by Purnamasari (2013), has highlighted the anti-inflammatory effects of liverwort ethanol extracts. However, research on liverwort's potential in diabetes treatment as an anti-inflammatory remains limited, particularly within Indonesia.

Fish scales, skin, and bones are increasingly recognized for their potential in collagen production. Tilapia (*Oreochromis niloticus*) bones, in particular, are known as a valuable collagen source that plays an essential role in skin regeneration and wound healing. A study by Holmes et al. (2013) has shown that collagen can be highly effective in treating diabetic ulcers. Collagen assists in wound repair by kickstarting clotting through platelet activation, drawing immune cells to release cytokines, and encouraging cell migration. Additionally, collagen fragments promote fibroblast activity and stimulate the production of growth factors, which are crucial for new tissue formation and blood vessel development. Finally, during wound remodeling, collagen helps balance new matrix production and breakdown, enhancing the wound's strength and resilience (Mathew-Steiner et al., 2021). In Indonesia, tilapia is a major aquaculture product, with most fish processed as fillets, leaving around 50-70% as by-products like bones, skin, and scales (Muralidharan et al., 2013). Without sustainable use, these leftovers can lead to environmental concerns such as methane emissions, a greenhouse gas contributing to global warming. However, repurposing these by-products for collagen extraction presents an eco-friendly opportunity (Liu & Huang, 2016).

A direct method of drug delivery is the use of transdermal patches, which release medication through the skin for systemic effects at a controlled rate. Transdermal patches offer several advantages, such as ease of use, reduced dosing frequency, stable bioavailability, fewer side effects, and rapid metabolism, enabling drugs to enter systemic circulation effectively (Nurul et al., 2016). These patches contain adhesives with active compounds designed to deliver medication in precise doses to deeper skin layers, reaching peripheral blood vessels to treat wounds topically.

The skin as a delivery route offers benefits, including a short drug half-life, reduced bioavailability issues compared to oral delivery, avoidance of gastrointestinal irritation, and improved therapeutic efficacy. Additionally, transdermal delivery bypasses hepatic metabolism, enhancing drug availability while lowering the risk of systemic side effects due to minimized plasma concentrations. It also allows steady drug release at the application site, reduces fluctuations in plasma drug levels, and avoids injection. Furthermore, transdermal delivery prevents sudden drug pulses in the bloodstream, which can lead to side effects (Hanistya & Samlan, 2021).

Therefore, in this study, a transdermal patch of combination liverwort extract and tilapia collagen was developed to accelerate the healing of diabetic ulcers. The effectiveness of this transdermal patch was evaluated by observing wound appearance and the number of leukocyte, particularly neutrophil, from peripheral blood smears. The number of leukocyte or white blood cell can be used as a key indicator of inflammatory activity within the wound. Elevated white blood cell levels indicate ongoing inflammation and are associated to the pathogenesis of insulin resistance (Santoso et al., 2018). We expect that the combination of liverwort extract and collagen in transdermal patch can facilitate the direct release of active substances and accelerate the healing process.

Material and Methods

Research Materials and Tools

Research Tools

The tools used in this study include a blender, rotary evaporator, freeze dryer, hotplate, magnetic stirrer, analytical balance, centrifuge, microscope, vortex, glucometer, oven, plastic buckets, beaker glass, evaporation flask, measuring cup, stirring rod, glass funnel, filter paper, scissors, tweezers, petri dish, glass vial, micropipette, glass tube, micropipette, glass tube, microcentrifuge, micropipette, eppendorf tube, sterile cotton swab, and ruler.

Research materials

Liverwort (*Marchantia emarginata) was* obtained from a local vendor in West Java and tilapia (*Oreochromis niloticus*) bones were obtained from a fish fillet manufacturer. Tilapia (*Oreochromis niloticus*) obtained from a fish fillet producer, rats as test animals, distilled water, 96% ethanol, CH_3COOH, NaOH, NaCl, alloxan, glucostick, Whatman 42, polymers PVP and HPMC, plasticizers PEG 400 and Propylene glycol, Amoxicillin and plaster.

Extraction procedure

Liverwort extraction process

Extraction was carried out on liverwort samples. Samples in fresh form were cleaned with water. Drying was done at room temperature and protected from direct sunlight. The dried samples were then blended, so that the samples were obtained in a dry powder state. Furthermore, liverwort is extracted using ethanol by maceration method until fully submerged 3 times 24 hours and shaken using a shaker. Furthermore, the mixture was filtered with a vacuum filter using Whatman 42. The filtrate obtained was centrifuged at 4000 rpm and obtained supernatant in this process. The supernatant was put in an extraction flask and evaporated using a rotary evaporator. The result of evaporation is a thick liquid and obtained ethanol extract of liverwort (Nuriman, 2013).

Tilapia bone extraction process

The collagen extraction process refers to a modification of the method of Ata et al. (2016) Samples were immersed in 0.1 M NaOH solution with a ratio of 1:10 (b/v) at room temperature (32 ± 2°C) 3 times 24 hours to remove non-collagen protein and fat (*degreasing*) and every day the NaOH solution was replaced with a new solution. Fish bones were washed using distilled water until the pH was neutral. The collagen extraction process was carried out by immersing the bones,

skin, and scales respectively in a CH_3COOH 0.75 M solution as much as 1:10 (b/v) at room temperature for 3 times 24 hours. The extraction results were filtered using a calico cloth to separate the residue and supernatant. The supernatant was precipitated by adding 0.9 M NaCl (salting out process) for 24 hours. The precipitate was centrifuged at 8000 rpm for 30 minutes then the collagen was dried using a freeze dryer. This process will produce the dried extract of Tilapia bones.

Phytochemical analysis

Phytochemical screening is one way to identify the content of secondary metabolite compounds in a plant (Luhurningtyas et al., 2020). Qualitative tests were conducted to identify phytochemical compounds including alkaloids, phenols, flavonoids, saponins, triterpenoids, and sterols. Meanwhile, quantitative tests include the determination of total phenols, total flavonoids, and antioxidant activity (Prayoga et al., 2019).

Phytochemical analysis was performed using the following test methods: Lieberman-Burchard and Salkowski test for terpenoid, NH_3 test for flavonoid, Test with NH_3 0,05N and H_2SO_4 2N and Mayer reagent for an alkaloid test. The purpose of this analysis is to identify the primary and secondary metabolites of liverwort extract.

Preparation of transdermal patches

Transdermal patch formulation refers to the modification of Julianti et al. (2024). Formulation using solvent evaporation method. HPMC and PVP polymers were dissolved in distilled water at 100˚C and stirred, then plasticizers in the form of PEG 400 and propylene glycol added, liverwort extract, and tilapia bone collagen and stirred with a magnetic stirrer for 15-20 minutes. The formed mass was molded and baked at ± 40˚C until dry.

Table 1. Formulation of transdermal patches with liverwort and collagen ratios

No	Active Ingredients	PVP	HPMC	Propylene glycol	PEG 400	Ethanol	Aquadest
1	(Liverwort: Collagen) 1:0	0,5 gr	1 gr	0,5mL	0,5mL	5 mL	45mL
2	(Liverwort: Collagen) 1:2	0,5 gr	1 gr	0,5mL	0,5mL	5 mL	45mL
3	(Liverwort: Collagen) 1:1	0,5 gr	1 gr	0,5mL	0,5mL	5 mL	45mL
4	(Liverwort: Collagen) 2:1	0,5 gr	1 gr	0,5mL	0,5mL	5 mL	40mL
5	(Liverwort: Collagen) 0:1	0,5 gr	1 gr	0,5mL	0,5mL	5 mL	50mL
6	Antibiotics 0,05 gr	0,5 gr	1 gr	0,5mL	0,5mL	5 mL	50mL
7	-	0,5 gr	1 gr	0,5mL	0,5mL	5 mL	50mL

Treatment of rats

The research was conducted in accordance with ethical clearance guidelines and received ethical exemption (No.093/LE.003/VII/04/2024). Rats were acclimatized for a week. Rats were placed in cages with adequate air circulation and rats could easily eat and drink by ad libitum. Rats are injected with alloxan to make them diabetic. Alloxan is a classical diabetogenic chemical that exerts selective cytotoxic influences on pancreatic β-cells, resulting in the destruction of β-cells and type 1 diabetes (Yin et al., 2018). Blood glucose levels of rats were measured using BloodChem Analyser before and after alloxan injection with a single dose of 120 mg/kg BW intraperitoneally for 7 days (Suarsana et al., 2021). According to Rahman (2014), normal rat blood

sugar levels are 50-135 mg/dl and are considered hyperglycemic or diabetic if the rat blood sugar level is >135 mg/dl. Rat fur was shaved about ± 4 cm around the skin area to be wounded. Rats are wounded with a wound diameter of ± 1 cm and a wound depth of ± 0.5 cm or up to the subcutaneous area (Wijonarko et al., 2016). Wounds in the diabetic rat group were treated using transdermal patches for 14 days, while the normal rat group was not treated. Observations were conducted in 3 phases, namely *Pre* (day-0), *Mid* (day-9), and *Post* (day-14) of the treatment.

Research design

This type of research is experimental analytic which uses a case-control research design. Grouping of Wistar male rats (*Rattus norvegicus*) was carried out using the formula Federer's. There were 8 treatment groups including 3 control groups and 5 treatment groups with differences in the ratio of liverwort extract and collagen. The number of samples for each treatment group is 4 rats/group, with the following details:

C : Normal group without treatment,
NC : Negative control group, diabetic rats, with transdermal patch base administration
PC : Positive control group, diabetic rats with transdermal patch administration of antibiotics Amoxicillin
P1 : Treatment group, diabetic rats with transdermal patches of liverwort and collagen 1:0,
P2 :treatment group, diabetic rats with transdermal patches of liverwort and collagen 1:2,
P3 :treatment group, diabetic rats with transdermal patches of liverwort and collagen 1:1,
P4 :treatment group, diabetic rats with transdermal patches of liverwort and collagen 2:1,
P5 :treatment group, diabetic rats with transdermal patches of liverwort and collagen 0:1.

Diff Quick staining of Blood Smears

Peripheral blood smear is an examination with microscopy techniques to see the morphology of blood cells and even other components that can provide considerable and meaningful information about a person's hematologic condition. Peripheral blood smear preparations are used to help assess the morphology of various types of blood cells such as erythrocytes, leukocytes, and platelets, and count the number and type of leukocytes (Ardina & Rosalinda, 2018). To facilitate the observation of cells and their components in peripheral blood smears with precision, it is necessary to perform a staining technique. Good staining produces a clear picture of the color contrast of the nucleus and cells (Susilowati et al., 2022). Diff Quick staining can be an option to produce good and fast staining. In Diff Quick staining, the preparation is soaked in methanol 1 dip, stained in eosin for as much as 8 dips then dry, stained with methylene blue for as much as 8 dips then rinsed with running water and dry. Cover with cover glass and observe under a microscope the shape of the cell, the color contrast of the nucleus, and the background (Dila et al., 2023).

Leukocyte cell count calculation

Leukocytes are one of the cells that have a major role in the body's defense system or immune system (Subaiyah et al., 2018). In laboratory examinations, leukocyte count examination is a routine examination to assess the condition of the immune system and inflammation levels in the body. An increase in leukocyte count is usually associated with the presence of infection, inflammation, and tissue necrosis (Canzoneri, 2009). The measurement of leukocytes, especially neutrophils, is necessary when inflammation occurs. Calculation of leukocyte area and neutrophil cell counts were performed on five fields of view under 400x microscope observation.

Data analysis

The histopathological features of the pancreas and the wound closure process were analyzed descriptively by comparing between the negative control (diabetic rats) and the treatment groups. Data represented in numerical form is the average result of 4 replicates. One-way ANOVA parametric test was used as long as the data was homogeneous and normally distributed, which was then followed by Tukey's post-hoc test. If the assumptions of normal distribution and homogeneity of data were not met, the Kruskal-Wallis non-parametric test was used followed by the Mann-Whitney U test. A p-value of <0.05 with a 95% confidence interval means there is a significant difference between the data tested. The results of the statistical test was analyzed by using SPSS.

Results and Discussion

Extraction result

The extraction process was done by maceration method using ethanol solvent to attract non-polar compounds from liverwort including terpenoids and flavonoids as anti-inflammatory compounds. The extract was then concentrated using a rotary evaporator, yielding a thick liquid extract. The extraction 31,61 grams of liverwort extract, with a yield of 3,04 (Table 2.).

Table 2. Liverwort extraction result

Plants Name	**Weight Symplasia (g)**	**Extract Weight (g)**	**Percentage of yield (%)**
Liverwort (*Marchantia emarginata*)	1.038,61 g	31,61 g	3,04%.

The yield of collagen was obtained based on the wet weight. The yield represents the percentage of collagen powder obtained relative to the initial weight of the raw materials (bones, skins, scales) in a wet state (Alves et al., 2017). Yield is a crucial indicator to show how much of the raw material can be used, and provide as an essential parameter for assessing the economic value and effectiveness of a product (Suptijah et al., 2018). In this study, the extraction process produced 35.2 grams of collagen powder from tilapia bones, resulting in a yield of 4.48%.

Table 3. Tilapia bone extraction result

Plants Name	**Weight Symplasia (g)**	**Extract Weight (g)**	**Percentage of yield (%)**
Tilapia bones (*Oreochromis niloticus*)	785,8 g	35,2 g	4,48%

Phytochemical analysis

The results of phytochemical using various methods confirmed the presence of active compounds, including terpenoid-steroids and flavonoids. Terpenoids play a role in multiple mechanisms involved in inflammation triggered by various etiological factors, as research has demonstrated their therapeutic potential in managing inflammatory conditions (Prakash, 2017). Meanwhile, flavonoid exhibit anti-inflammatories properties by inhibiting cyclooxygenase and lipooxygenase enzymes, offering the potency for the treatment of inflammation and allergy symptoms (Hidayati et al., 2008). With these bioactive compounds, liverwort extract holds significant potential as an anti-inflammatory compounds.

Table 4. Phytochemical Analysis of Liverwort Extract

Compound Group	Reagent/Test	Result
Terpenoid-steroid	Liebermann-Burchard Test	+ (purple-red color formed)
	Salkowski Test	+ (red ring formed)
Flavonoid	Tested with NH_3	+ (yellow color formed)
Alkaloid	Tested using NH_3 0.05 N and H_2SO_4 2N and reagent Mayer	- (no brown precipitate formed)

Note: (+) positive reaction; (-) negative reaction.

pH test

The test was conducted to determine the pH of the patch surface that shows in Table 5. Value of pH was determined by placing pH paper on the patch surface, then calculating the average value and calculating the standard deviation (Wardani & Saryanti, 2021). All formulations exhibit pH values within the acceptable skin-friendly range of 5–6.5, ensuring minimal risk of skin irritation (Hermanto et al., 2019). The pH test is essential when the product applied on the skin. An excessively low (acidic) pH can cause skin irritation, while a high (alkaline) pH may lead to dryness and scaling in the surface of skin (Wardani & Saryanti, 2021).

Table 5. pH results of different patch formulations

Preparation		pH Result	Average ± SEM
Formulation	Replication		
NC	1	5	5.33 ± 0.47
	2	6	
	3	5	
PC	1	5	5.33 ± 0.47
	2	5	
	3	6	
P1	1	6	6.00 ±0.00
	2	6	
	3	6	
P2	1	6	5.67 ± 0.47
	2	5	
	3	6	
P3	1	5	5.33 ± 0.47
	2	5	
	3	6	
P4	1	5	5.00 ± 0.00
	2	5	
	3	5	
P5	1	5	5.33 ± 0.47
	2	6	
	3	5	

Blood glucose level

Alloxan is a compound that has diabetogenic properties and is toxic, especially to pancreatic beta cells and when given to experimental animals, namely rats, it will cause the rats to become diabetic. The mechanism of action of alloxan that causes damage to pancreatic beta cells is to enter the pancreatic beta cells first and then be absorbed by pancreatic beta cells (Prameswari & Widjanarko, 2014). Damage to pancreatic beta cells will result in necrosis, this is due to the empty space in the islets of Langerhans and black cell nuclei due to cell nucleus condensation (Jorns et al., 1997). The pathological effects of alloxan occur through glucokinase inhibition and the formation of the ROS (reactive oxidative species) cycle in pancreatic beta cells, resulting in a decrease in the quantity of insulin production, an increase in blood glucose levels, and resulting in type 1 diabetes (Ighodaro et al., 2017).

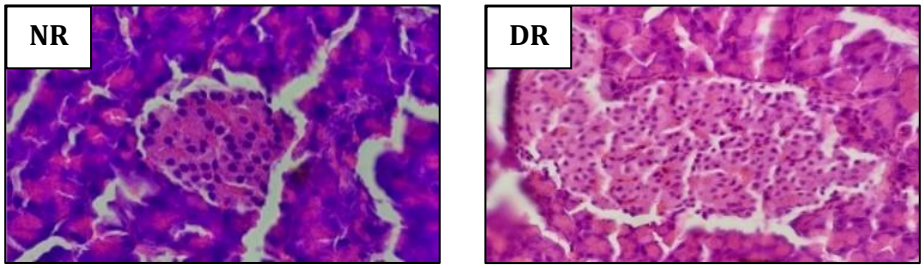

Figure 1. Representative histopathology of rat pancreas. NR: a group of normal, non-diabetic rats, and DR: a group of diabetic rats injected with alloxan 120mg/kgBB. Observations were performed under a microscope with 400X magnification

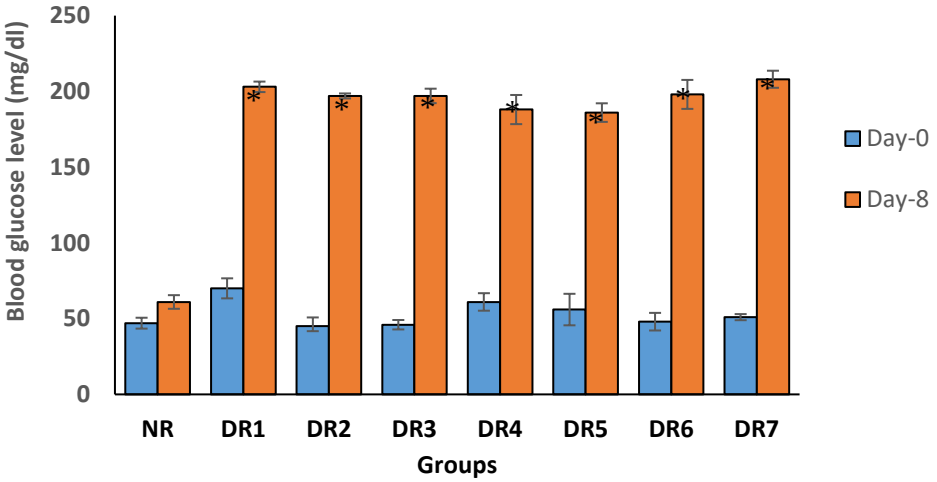

Figure 2. Bblood glucose level (mg/dl) of rats in each group on day 0 (before alloxan injection), day 8 (after alloxan injection). Description: NR: a group of normal, non-diabetic rats, and DR: a group of diabetic rats injected with alloxan 120mg/kgBB. DR groups will then be grouped according to the treatment listed in the research method. Star (*) represent statistically significant differences between NR and DR1-7groups at each time point (p-value < 0.05).

Measurement of blood glucose levels of rats was conducted on day 0 (before alloxan injection) and day 8 (after alloxan injection). The fasting blood glucose levels of rats ranged from 46-70 mg/dL, below 135 mg/dL, indicating that the rats were in normal condition. On the 8th day, the group of rats that had been injected with alloxan had increased blood glucose levels, ranging from 186-208 mg/dL. One-way ANOVA test analysis of the data before alloxan injection showed a significance value of 0.054, greater than 0.05, so it can be concluded that there is no significant

difference on day 0 among all treatment groups. Rats are considered in diabetic condition if the blood glucose level is ≥ 135 mg/dL or in hyperglycemia condition (Rahman, 2014).

Wound development

Diabetic ulcers are often indicated by infections, with pus formation observed as early as the second day following the injury (pre-treatment). As shown in Figure 3, the wound's appearance and surface area changed gradually across different stages: on day 10 (pre-treatment), day 15 (mid-treatment), and day 21 (post-treatment). A notable improvement was first seen on day 15, five days after applying a transdermal patch enriched with additional of active ingredients. In groups PC, P2, and P3, the wounds showed signs of drying, whereas in groups C, P1, P4, and P5, the wounds remained red. By day 21, the wound in group P3 had fully closed, with the redness almost entirely reduced, while the other treatment groups displayed partial closure with lingering redness. In contrast, the wound in group NC had not fully closed by day 15 and continued to exude pus, although some closure was observed by day 21, although with persistent pus discharge. The presence of pus indicates bacterial infection, as bacteria release leukocidin, which breaks down neutrophils and leaves the wound inflamed and filled with pus (Ekawati et al., 2018). Diabetic ulcers typically extend the inflammatory phase due to elevated blood glucose levels, which impair circulation and slow the healing process (Dasari et al., 2021).

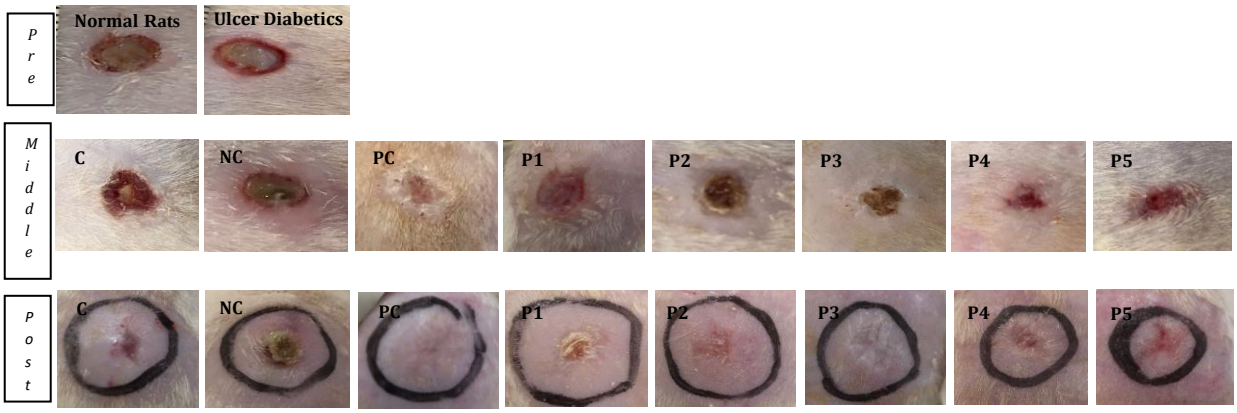

Figure 3. Wound Development in Diabetic Rats. This figure shows the progression of wound healing in diabetic rats across three time points: pre-treatment (day 0), mid-treatment (day 9), and post-treatment (day 14). Images compare normal and diabetic ulcer wounds at the pre-treatment stage. Treatment groups include a control (C), a negative control (NC), a positive control (PC), and five patch formulations (P1 to P5) containing varying ratios of liverwort and collagen

Figure 3. indicated that the combination of transdermal patches from liverwort and collagen from tilapia bone can potentially serve as an anti-inflammatory compound. The inflammatory response is the body's way of responding to a wound. Inflammation is characterized by rubor (redness), tumor (swelling), calor (warmth), and dolor (tenderness) (Gurtner, 2007). The goal of inflammation is to kill bacteria in the wound. The inflammatory phase is critical in wound healing, as effective recovery depends on the initial recruitment of inflammatory cells and the release of various mediators during this stage. Inflammatory mediators will trigger vasodilation, increased vascular permeability and activation of leukocyte cells that will circulate to the site of the inflammatory agent to remove the causative agent. The vasodilation process allows inflammatory cells such as neutrophils, monocytes, macrophages and lymphocytes to arrive at the injured tissue. Neutrophils are the first inflammatory cells to arrive, peaking at 24 hours. They phagocytose bacteria, clearing microbes and other cellular debris. In addition, polymorphonuclear leukocytes (PMNs) release reactive oxygen species that amplify this killing process (Rodrigues et al., 2019).

Leukocyte and neutrophil

Leukocyte is often measured to quantify the degree of inflammation. Leukocyte increases during inflammation by migrating to the site of infection or injury. Neutrophils are a type of white blood cell (leukocytes) that act as your immune system's first line of defense. Neutrophils have been found to play a crucial role in chronic inflammation. They are consistently drawn to areas of persistent inflammation, where they help sustain the process by releasing serine proteases, forming neutrophil extracellular traps (NETs), and activating other immune cells (Herrero-Cervera et al., 2022).

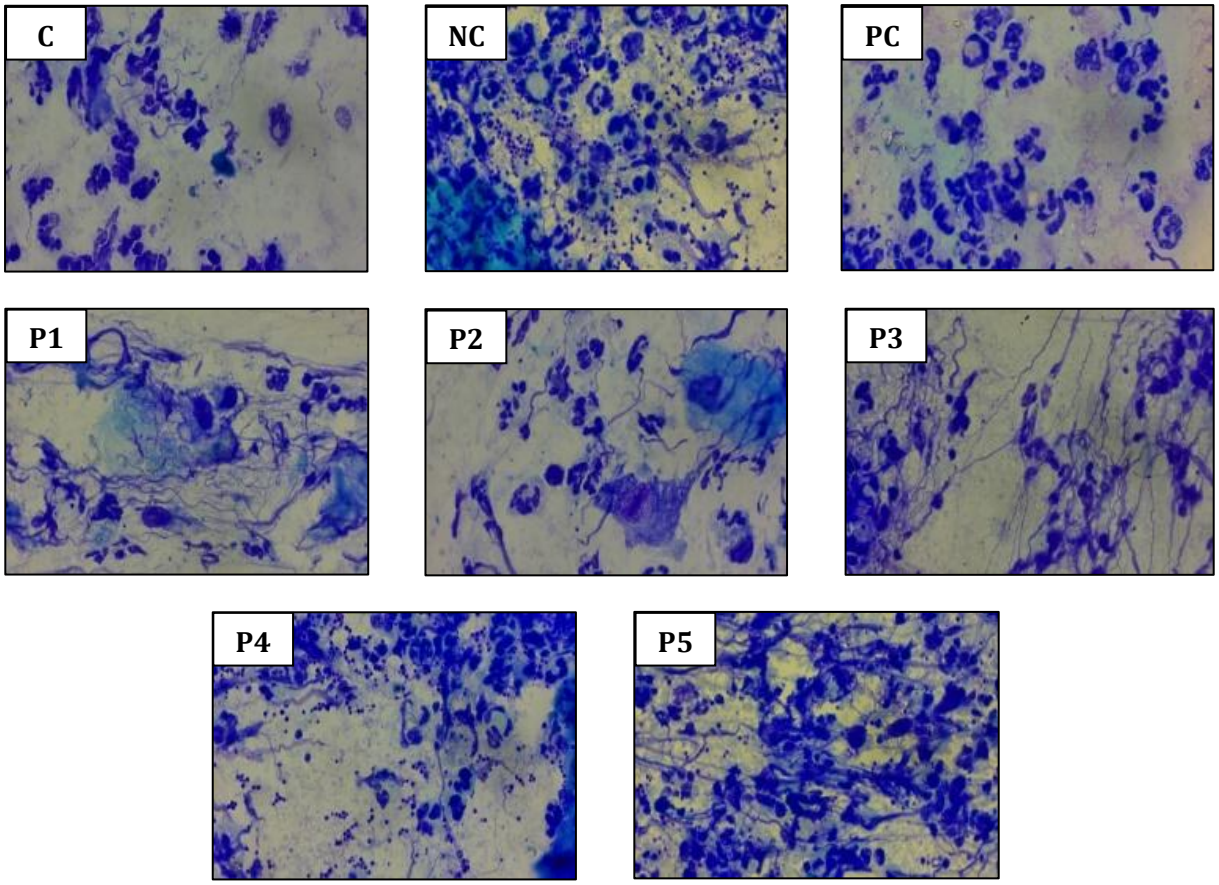

Figure 4. Histological images of leukocytes in blood smears under a microscope 400X. The images display stained leukocyte areas for the control group (C), negative control (NC), positive control (PC), and five patch formulations (P1-P5).

The Figure 5. shows that among the various transdermal patch formulations tested, P3 (a combination of liverwort extract and tilapia bone collagen) demonstrated the most effective anti-inflammatory properties, significantly reducing leukocyte areas from pre-treatment to post-treatment. Similar to positive control group, P3 showed the reversed effect compared to control and negative control groups. Other formulations, such as P1, P2, P4, and P5, showed moderate reductions in leukocyte areas but were not as effective as P3. This highlights its potential to reduce inflammation indicated by reducing leukocyte area.

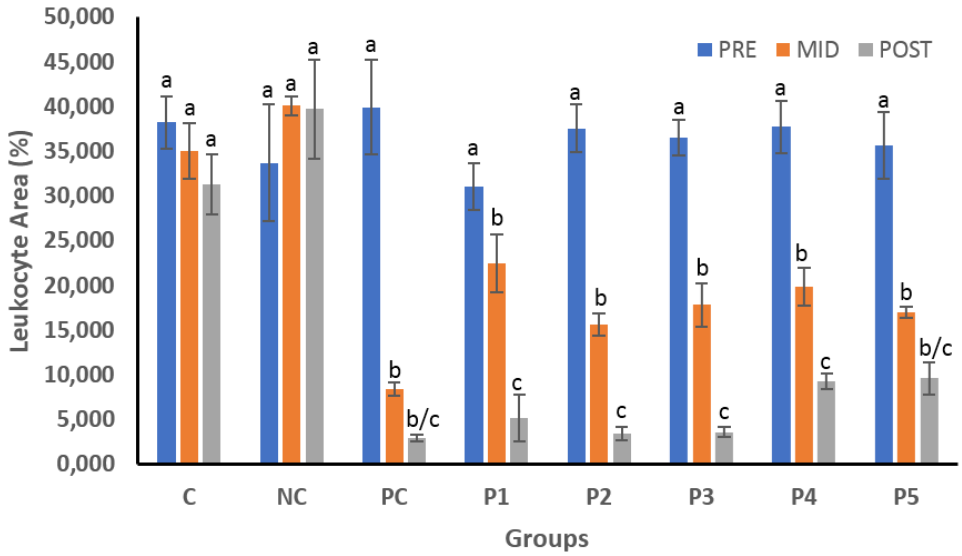

Figure 5. The effect of transdermal patch treatment on the percentage of leukocyte area over three time points: Pre-treatment (PRE), Mid-treatment (MID), and Post-treatment (POST). The groups compared include Control (C), Negative Control (NC), Positive Control (PC), and five different patch formulations (P1–P5). Different letters (a, b, c) represent statistically significant differences between groups at each time point (p-value < 0.05).

Table 5. showed that the NC group exhibited the highest number of neutrophils at all time points, indicating a robust inflammatory response, likely due to a diabetic condition. In contrast, the PC group, which received antibiotic transdermal, showed a moderate number of neutrophils that suggest some level of efficacy.

Table 5. The number of neutrophil cells from various treatment groups which measured at three time points: Pre-treatment (PRE), Mid-treatment (MID), and Post-treatment (POST). The groups included: control (C), negative control (NC), positive control (PC), and five different transdermal patch formulations (P1-P5). Values are presented as mean ± standard error mean (SEM). Different letters indicate statistically significant differences between groups at the each time point

	PRE	MID	POST
C	41,4±4,49[a]	37,9±6,65[a]	22,1±1,97[ab]
NC	65,4±11,8[a]	40,5±6,11[a]	23,6±4,99[a]
PC	61,8±9,7[a]	12,3±1,76[b]	6,8±1,26[c]
P1	45,1±9,1[a]	33,5±0,28[a]	7,4±1,63[c]
P2	58,3±17,1[a]	38,8±5,39[a]	7,1±1,29[c]
P3	43,4±17,3[a]	13,8±1,05[b]	5,3±0,32[b]
P4	68,9±2,29[a]	17,1±3,07[b]	15,1±2,55[b]
P5	66,1±5,53[a]	14,3±1,25[b]	14±3,33

Among the transdermal patch formulations, P1, P2, P3, P4, and P5 demonstrated varying degrees of effectiveness. Patch formulation at P3 with 1:1 ratio exhibited significant improvement in number of neutrophil, particularly in the MID and POST stages, suggesting that this formulation may be particularly effective in enhancing the healing process compared to the other formulations. Meanwhile, P4 also showed promising results, although they did not reach the levels

observed in P3. These findings contribute to understanding the potential of specific transdermal patch formulations in managing inflammation and enhancing wound healing, particularly in diabetic models. This interpretation aligns with existing literature that emphasizes the role of inflammatory markers in wound healing and the significance of potential treatments for effective recovery.

Conclusion

The results demonstrated that the treatment increased the wound healing process and significantly decreased the number of leukocytes, particularly neutrophils, indicating anti-inflammatory effects and improved healing processes in diabetic wounds. The most effective transdermal patch formulation for assisting the wound healing process is the combination of liverwort extract and tilapia bone collagen at a 1:1 ratio (P3). These findings suggest that liverwort and tilapia bone collagen transdermal patches have the potential as wound care treatment for diabetic ulcers. Further research is needed to investigate the detail histological changes in skin tissues including epithelial thickness, collagen density, and the number of fibroblast.

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