https://doi.org/10.48048/tis.2023.6944

Wound Healing Activity of Transdermal Patches of *Carica Papaya*, *Chromolaena Odorata*, and *Averrhoa Bilimbi* Leaves on Incision Wounds of Hyperglycemic Rat

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Received: 4 April 2023, Revised: 29 May 2023, Accepted: 26 June 2023, Published: 25 September 2023

Abstract

Transdermal patches have been used for drug delivery to accelerate the wound healing process with minimum negative effect. This study evaluated the wound healing potential of transdermal patches containing *Carica papaya, Chromolaena odorata*, and *Averrhoa bilimbi* leaves extract on hyperglycemic rat as a diabetic wound model. For this purpose, a total of 40 Wistar rats aged 2 - 3 months were randomly distributed into 10 groups. The first 5 groups (P1, P2, P3, P4 and P5) consisted of normal rats which received normal dressing, TP Dermafix, TP of *C. papaya*, TP of *C. odorata*, and TP *of A. bilimbi*, respectively. The second 5 groups (P6, P7, P8, P9 and P10) were hyperglycemic rats that received normal dressing, TP Dermafix, TP of *C. papaya*, TP of *C. odorata*, and TP *of A. bilimbi*, respectively. Skin incisions were made perpendicular to the spine in the thickest part of the skin with an incision length of 2 cm and a depth of 0.5 cm. Patches were applied to the incisions according to the test group and replaced every 2 days for a period of 13 days. Wound healing activity was determined by evaluating the Clinical Sign of Inflammation **(**CSI) score, wound closure, TGF-β1 concentration, and histology of skin tissue. Data were analyzed using oneway analysis of variance (ANOVA). The results showed that for each parameter observed, the rats intervened with transdermal patches loaded with extract leaves had higher healing potential and different from the control group (normal dressing without intervene) both in normal and hyperglycemic rats. This made us concluded that the TP loaded with *C. papaya, C. odorata,* and *A. bilimbi* extract leaves have potential as wound healing agents and could be considered as alternate to the synthetic transdermal patches.

Keywords: Leaves extract, Wistar rats, Transdermal patches, Wound healing

Introduction

Wound is a state of tissue discontinuity caused by a blunt or sharp object. Everyone has the potential to experience injury while carrying out daily activities both intentionally and unintentionally. Unintentional injury occurs due to accidents while intentional injury occurs as a result of medical action in the form of surgery [1-3]. Wounds cause physiological changes in the body that can cause loss of some or all of the body's functions. One type of wound that is often experienced by the community is an incision wound. Incision wounds occur due to cuts by sharp objects and affect the superficial part of the skin. Improper handling of incisions will delay wound the healing processes and often results into chronic wounds [4]. However, good wound management will prevent such complications or other problems such as chronic wounds, bacterial contamination, and cell death [5,6].

Proper handling of wounds is of great importance during skin healing. must be carried out properly in order to maintain good skin functions. The skin provide protection by acting as a barrier from the environment. Proper wound healing restores skin integrity to normal. Wound healing process is a complex process that involve the repairment of skin tissue damage to be a normal intact skin. This

process causes discomfort due to inflammation reaction. The discomfort was accompanied by pain due to the humoral and cellular elements, released to the nerve, swelling due to accumulation of fluid in the wound area, increased body temperature, the redness of the wound area due to vasodilation of blood vessels, and changes in organ function [7]. Therefore, proper handling of wounds is of a great importance during skin healing in order to maintain good skin function. The skin provide protection by acting as a barrier from environment.

One of the major concerns is diabetic wounds. Diabetic wounds are injuries that occur in diabetic patients which involve disorders of the peripheral and autonomic nerves and abnormalities in blood vessels which was then followed by serious infection [8]. Diabetic patients often experience prolonged wound healing due to hyperglycemia. Wound healing is a complex transitional process in human physiology that involves complex reactions and interactions between cells and mediators. This hyperglycemia will tense affect the body's wound healing. Hyperglycemia will make wound healing more difficult due to a decrease in the ability of blood vessels to contract and relax and hyperglycemia is a fertile environment for the proliferation of pathogenic germs that are anaerobic because the blood plasma of people with hyperglycemia is not well controlled and has high viscosity (viscosity). As a result, blood flow slows down and oxygen supply decreases [9].

The use of medicinal plants as wound healers has been known for generations. Of the 30,000 types of plants, 940 play a role in wound healing, including *Carica papaya (C. papaya)*, *Chromolaena odorata* (*C. odorata*), and *Averrhoa bilimbi* (*A. bilimbi*) [10]. Various studies have proved the presence of wound-healing property in these plants using different application route. The previous results of the GC-MS analysis revealed that *C. papaya, C. odorata*, and *A. bilimbi* extract contained several compounds which process wound healing activities [11]. However, there is no information available on the *in vivo* effect of these plants extract using rat model. Therefore, further studies need to be conducted to validate their wound-healing activity particularly in managing chronic wound in diabetic case.

The development of medicinal plant preparations that are easy to use with optimal, effective, efficient, practical and economical properties needs to be applied to *C. papaya, C. odorata, and A. bilimbi* leaves extract as wound healers in hyperglycemic rats. The use of a transdermal patch is a method that can minimize drug interactions and avoid first-pass effects. However, the patches application produces systemic reactions. Transdermal patches can also be applied to patients who have difficulty in oral administration and their use can be discontinued immediately if side effects occur [10,12,13]. Wound plasters or transdermal patches are known to be an option in healing acute wounds and abrasions [14,15]. The purpose of this study, therefore, was to determine the effectiveness of *Carica papaya*, *Chromolaena odorata* and *Averrhoa bilimbi* leaves extract loaded transdermal patches on wound healing activity in incision wounds of hyperglycemic rats.

Materials and methods

Plant materials

C. papaya, C. odorata and *A. bilimbi* leaves were collected from Lambhuk, Banda Aceh, Indonesia. The leaves were authenticated at the Biology Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Syiah Kuala, Banda Aceh, Indonesia. The leaves were air-dried prior to extraction using ethanol. The extraction process was carried out as described previously by Santi *et al.* [11].

Animal and treatment protocol

This research was conducted at the Faculty of Veterinary Medicine, Universitas Syiah Kuala, Banda Aceh. The experimental animals used were 40 Wistar rats (*Rattus novergicus*), weighed 100 - 200 g and aged 2 - 3 months. This research was approved by the ethical commission of Faculty of Veterinary Medicine, Universitas Syiah Kuala (certificate no 162/KEPH/VIII/2022).

Rats were adapted to the cage condition for 7 days and were fed with growers feed and water ad libitum. The rats were divided into 10 groups with 4 rats in each group. The first 5 groups consist of normal rats (without streptozotocin induction). The normal rats in group P1, P2, P3, P4 and P5 received normal dressing, transdermal patch Dermafix, transdermal patch of *C. papaya* leaf, transdermal patch of *C. odorata* leaf, and transdermal patch of *A. bilimbi* leaves extract, respectively. The second 5 groups were streptozotocin-induced hyperglycemic rat. The hyperglycemic rats in groups P6, P7, P8, P9 and P10 were treated with normal dressing, transdermal patch Dermafix, transdermal *patch of C. papaya* leaf, transdermal patch of *C. odorata* leaf, and transdermal patch of *A. bilimbi* leaves extract, respectively.

The transdermal patches of *C. odorata, A. bilimbi*, and *C. papaya* leaves was prepared according to the procedure of Santi *et al.* [11]. The optimum formulation for the transdermal patch used in this study is

shown in **Table 1**. The thickness, weight, and moisture content of the transdermal patch used were 0.25 mm, 82 mg and 4.39 %, respectively.

Table 1 Transdermal formulations of *C. odorata, A. bilimbi*, and *C. papaya* leaf patches.

Transdermal patch	Formulation		
	Polyvinyl pyrrolidone (mg)	Ethyl cellulose (mg)	Extract (mg)
EECP	200	400	100
EECO	200	400	100
EEAB	200	400	100

Note: EECP = ethanol extract of *Carica papaya*
EECO = ethanol extract of *Chromolaena odorata*
EEAB = ethanol extract of *Averrhoa bilimbi*

Measurement of fasting blood glucose (FBG) levels in rats and streptozotocin (STZ) induction

The FBG of rats in all groups was measured at 0 days after 10 h fasted, but water was provided to ensure that the rats' blood glucose levels were normal. The rats in P6 - P10 group were hyperglycemic induced by single injection of STZ at dose of 30 mg/kg BW intraperitoneally. Then the rats were given 10 % dextrose solution to avoid side effects and the risk of sudden hypoglycemic. FBG was re-checked after 72 h (day 4 post STZ injections). The rats with FBG levels ≥ 126 mg/dL were used for further treatment. For all blood glucose assessment, the blood was drawn from rat tail vein, and blood glucose level was analyzed using Auto check.

Transdermal patch test on incision wounds in Wistar rats

A total of 40 rats were weighed and anesthetized with xylazine 5 mg/kg and ketamine 50 mg/kg by intraperitoneal injection. Dorsal hair was shaved extensively from scapula to flank and sterilized with povidone iodine. Skin incision was made perpendicular to the spine in the thickest part of the skin with an incision length of 2 cm and a depth of 0.5 cm following Özay *et al.* [16]. A transdermal patch was attached to the incision according to the test group and the patch was replaced every 2 days. For the hyperglycemic rat group, patch application was carried out after the FBG level of the rats was ≥ 126 mg/dL.

Wound healing observation

Observation of incision wound healing was carried out for 7 days using Clinical Sign of Inflammation (CSI) parameters (Marini *et al.* 2018). The data obtained was scored according to **Table 2**.

Table 2 CSI Parameters.

Clinical sign of inflammation (CSI)	Score
Redness involving > 50 % of the wound area and/or accompanied by prominent swelling	3
Redness involving < 50 % of the incision area	2
No redness	1

The length of the incision wound was measured on days 3, 7, 9 and 13 using a standard caliper. The percentage of incision wound closure was calculated using the following formula [17]:

$$\frac{A_0 - A_t}{A_0} \times 100\ \%$$

where A_0 is the length of the initial incision and at is the length of the wound at the time of measurement on days 3, 5, 7, 9, 11 and 13.

TGF- β1 analysis

Rat blood samples were taken through the retroorbital plexus (eye vein) as much as 2 - 3 mL on days 7 and 14. Blood serum was obtained through a centrifugation process. Rat TGF-β1 concentration was measured using a specific ELISA kit for rats, Rat Transforming Growth Factor Beta ELISA Kit (Cat. No. BZ-08188861-EB, Bioenzy). The procedure for measuring the concentration of Rat TGF Beta-1 was

carried out following the instructions described in the ELISA kit guide. Rat TGF Beta-1 standard was prepared with a concentration range of 75 to 2,400 ng/L. A 50 µL duplicate of the Rat TGF Beta-1 standard was inserted into the microplate well. After that, 40 µL of the sample was inserted into the well of the microplate and then 10 µL of anti-TGF Beta-1 antibody was added.

This was followed by the addition of, 50 µL of streptavidin-HRP was added to all wells of the microplate (standard and sample), and homogenized by shaking slowly. The microplate was covered with sealer and incubated for 60 min at 37 °C. After incubation, the sealer was removed and washed 5 times with 350 mL/well washing solution and dried using drying paper. After that, 50 µL of substrate A and 50 µL of substrate B were added to each well on the microplate. Next, the microplate was covered with a new sealer and incubated again for 20 min at 37 °C in a dark room. After that, the enzymatic reaction was stopped by using 50 µL of stop solution in each well of the microplate. The absorbance value and concentration of TGF Beta-1 were measured using an ELISA reader at a wavelength of 450 nm (Biolegend, USA).

Histological preparations

On day 14 post wounding day, the rats were terminated by the decapitation method for histological preparations. A thick piece of the rat skin was dissected out from the edges of the wound using a scalpel knife. The skin slices were put in a sample bottle containing 10 % buffered formalin with a ratio of 1 part skin and 9 parts formalin.

Histological preparations were made according to the Kiernan method (1990). In brief, the skin tissue was cut with a thickness ranging from 0.3 - 0.5 mm (trimming) and then placed in a tissue cassette for the dehydration process. The skin tissue dehydration process begins by immersing the samples in 80, 90 and 95 % alcohol for 1 h each and absolute alcohol (3 times), each for 2 h. After the dehydration process, a clearing process was carried out using xylol solution (3 repetitions) for 45 min each, followed by tissue infiltration using paraffin infiltration (3 repetitions) at 60 °C for 30 min each. The next stage was the embedding and blocking process. The tissue block was sliced with a thickness of 5 µm using a microtome and then placed on an object glass. Furthermore, Hematoxylin and Eosin (HE) staining was carried out according to the standard procedures used in the Pathology Laboratory, Faculty of Veterinary Medicine, USK. Finally, the sample were mounted using entellan and covered by a cover glass.

Calculation of fibroblast cells, angiogenesis, collagen thickness and collagen density

The results of HE staining on histopathological preparations were observed on a microscope with a top view application. Observation of fibroblasts was carried out by taking photos of the distribution of fibroblasts using an objective lens with 40× magnifications, and then the number of fibroblasts were counted in the box at 3 fields of view. Measurement of collagen thickness was carried out through histology slide scans using Toup View Software with 40× magnification. Measurements were taken in 3 different areas (left, middle and right) starting from the edge of the wound base and then moving to the dermis. Angiogenesis/vascularization was evaluated by counting the number of capillaries in the form of channels lined with a single layer of endothelium at the bottom of the skin. Collagen density was observed under an Olympus microscope equipped with a digital camera set at a100× magnification.

Scoring parameters for collagen density was measured according to Ummah *et al.* [18] as follows:
0 = No collagen fibers were found in the wound area
+1 = density of collagen fibers in the area of low wounds (less than 10 % part visual field)
+2 = density of collagen fibers in the area of moderate injury (10 - 50 % part visual field)
+3 = Density of collagen fibers in tightly wound areas (50 - 90 % part visual field)
+4 = The density of collagen fibers in the wound area is very tight (90 - 100 % part visual field)

Data analysis

Data on the CSI score, wound closure, TGF-β1 concentration, and skin tissue histology parameters were analyzed using one-way ANOVA with SPSS program for windows ver. 26.0.

Results and discussion

Fasting blood glucose (FBG) levels in rats

All rats used in this study showed normal FBG levels before induced with streptozotocin (H-0) with the lowest FBG levels at P2 (101.0 ± 7.72 mg/dL) and the highest at P8 (130.8 ± 11.2 mg/dL) as presented in **Table 3**. Even though there were differences, all rats in the treatment group were included in

the normal category. Blood glucose levels in male rats before induction were around 75 - 150 mg/dL [19]. The range of normal blood sugar levels in rats is 70 - 140 mg/dL [20].

Induction with streptozotocin in the P6 - P10 group succeeded in increasing the blood glucose levels of all rats (100 %) to reach diabetic levels. This percentage of success is relatively the same as that achieved by Saputra *et al.* [19], which obtained a success percentage of 93.2 %. These results are also consistent with previous reports that blood glucose levels will increase 24 h after induction with streptozotocin. Blood glucose levels 48 and 96 h after induction were 525.0 ± 77.93 and 503.1 ± 90.78 mg/dL, respectively [21]. The category of blood glucose levels can be divided into 4 categories, normal diabetics with glucose levels of 75 - 150 mg/dl, mild diabetics with glucose levels of 150 - 200 mg/dL, moderate diabetics with glucose levels of 200 - 400 mg/dL, and severe diabetics with glucose levels above 400 mg/dL [22]. Based on this category, the P6 - P8 group was included in the severe diabetic category, P9 was included in the mild diabetic category, and P10 was included in the moderate diabetic category.

Table 3 Average blood glucose levels of rats on day 0 and day 4 after induction with Streptozotocin (STZ).

Groups	Blood glucose levels (mg/dl)	
	Day - 0	Day - 4
P1	118.0 ± 2.87	-
P2	101.0 ± 7.72	-
P3	112.0 ± 3.3	-
P4	106.0 ± 6.5	-
P5	103.0 ± 5.12	-
P6	127.0 ± 19.4	474.5 ± 42.4
P7	126.5 ± 23.4	454.0 ± 76.1
P8	130.8 ± 11.2	476.5 ± 48.0
P9	113.5 ± 14.3	165.5 ± 15.8
P10	114.75 ± 5.0	212.5 ± 78.3

Data presented mean ± standard deviation (P1, normal rat + normal dressing; P2, normal rat + transdermal patch Dermafix; P3, normal rat + transdermal patch of *C. papaya* leaf; P4, normal rat + transdermal patch of *C. odorata* leaf; P5, normal rat + transdermal patch of *A. bilimbi* leaf; P6, hyperglycemic rat + normal dressing; P7, hyperglycemic rat + normal dressing + transdermal patch Dermafix; P8, hyperglycemic rat + transdermal patch of *C. papaya* leaf; P9, hyperglycemic rat + transdermal patch of *C. odorata* leaf; P10, hyperglycemic rat + transdermal patch of *A. bilimbi* leaf).

All rats showed FBG levels ≥ 150 mg/dL, thus the rats were classified as hyperglycemic rat and could be proceed for further treatment. Hyperglycemia occurs as a result of streptozotocin's ability to change the DNA of pancreatic ß cells, in the form of DNA alkylation via nitrosourea groups which results in damage to pancreatic ß cells followed by inhibition of insulin synthesis and secretion [23]. Impaired insulin secretion causes all the glucose consumed by the body cannot be processed perfectly, so that glucose levels in the body increase [24]. In addition to the effect of streptozotocin on pancreatic ß-damaged cells, the effect of streptozotocin on pancreatic beta cells also produces a reactive and toxic lipid peroxidation product malondialdehyde (MDA), causing disruption of glucose and fat metabolism [25]. The ability of streptozotocin to damage pancreatic ß cells is facilitated by binding with GLUT-2 so that streptozotocin can enter the cytoplasm of pancreatic β cells, increasing depolarization in mitochondria as a result of Ca2+ ion entry followed by excess energy use resulting in a lack of energy in the cells [24].

Incision wound macroscopic image based on CSI

Effect of transdermal patch loaded with C. *papaya*, *C. odorata*, *A. bilimbi* leaves extract on the wound closure of normal and hyperglycemic rat can be seen on **Figure 1**. The application of transdermal patch at incision wound of rat every 2 days did not show allergic reactions. The clinical sign of

inflammation (CSI) which was directly examined on the wound incision was then scored and the CSI score is presented in **Table 4**.

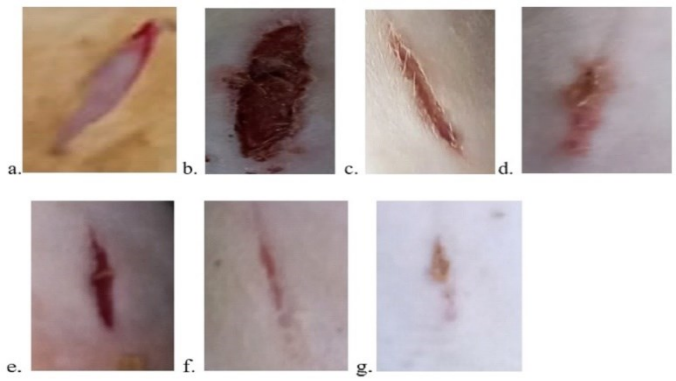

Figure 1 Wound development of normal and hyperglycemic rats treated with transdermal patch. (a) initial wound, (b) normal rat + normal dressing (day 7), (c) normal rat + transdermal patch Dermafix (day 7), (d) normal rat + transdermal patch of leaves extract (day 7), (e) hyperglycemic rat + normal dressing (day 14), (f) hyperglycemic rat + transdermal patch Dermafix (day 14), and (g) hyperglycemic rat + transdermal patch of leaves extract (day 14).

Table 4 Clinical Sign of Inflammation (CSI) scores observed for 7 days after transdermal patch administration.

Groups	CSI score
P1	2.2 ± 0.23^{b}
P2	1.65 ± 0.1^{a}
P3	1.95 ± 0.19^{a}
P4	1.8 ± 0.16^{a}
P5	1.95 ± 0.3^{a}
P6	2.35 ± 0.22^{b}
P7	2.05 ± 0.19^{b}
P8	1.7 ± 0.2^{a}
P9	1.95 ± 0.25^{a}
P10	1.85 ± 0.3^{a}

Data presented mean ± standard deviation. Different superscripts within the same column indicated statistically difference ($p < 0.05$) ((P1, normal rat + normal dressing; P2, normal rat + transdermal patch Dermafix; P3, normal rat + transdermal patch of *C. papaya* leaf; P4, normal rat + transdermal patch of *C. odorata* leaf; P5, normal rat + transdermal patch of *A. bilimbi* leaf; P6, hyperglycemic rat + normal dressing; P7, hyperglycemic rat + normal dressing + transdermal patch Dermafix; P8, hyperglycemic rat + transdermal patch of *C. papaya* leaf; P9, hyperglycemic rat + transdermal patch of *C. odorata* leaf; P10, hyperglycemic rat + transdermal patch of *A. bilimbi* leaf).

The results showed that the CSI score in group P1 and P6 (normal dressing) was significantly higher ($p < 0.05$) compared to others groups except group P7 (hyperglycemic rat that treated with transdermal patch Dermafix). In hyperglycemic rat groups, the application of transdermal patch loaded with *C. papaya*, *C. odorata*, *A. bilimbi* leaves showed significantly lower CSI score ($p < 0.05$). This result indicated that therapy of wound incision with a transdermal patch loaded with *C. papaya* leaves, *C. odorata* leaves, *A. bilimbi* leaves extract had a better wound healing process compared to the group without therapy intervene. The highest CSI score was found in P6, however the healing response to incisions wound in the P6 group continued with the presence of CSI which was considered as a natural body response. This observation was in line with Suryadi [8], who stated that signs of an ongoing inflammatory process are redness (rubor) and swelling (tumor).

The CSI score in this study was different from previously reported on humans [26,27]. Veranita *et al.* [26], reported that there was a relationship between the degree of diabetic foot ulcers and blood glucose levels in people with diabetes mellitus. Lede *et al.* [27], reported that there was an effect of blood sugar levels on the healing time of diabetes mellitus wounds at the Dinoyo Malang Health Center. This difference is probably due to differences in the type of wound and the length of time that hyperglycemia occurs.

Wound closure

Wound closure was measured on days 3, 7 and 13 after incised, and the length was converted to percentages of wound. The results showed that there was a decrease in wounds from day 3 to day 13 as presented in **Table 5**.

Wound healing is characterized by wound closure in very short time. The present results showed that the wound healing marked by a decrease in wound length and an increase in wound closure percentage of the incision wound which was pronounced on day 13 post incised. The process of wound closure in the normal rats and hyperglycemic rats was different in which the wound closure percentage was higher in normal rat than in hyperglycemic rats. Previous studies also showed that the healing processes of non-diabetic and diabetic wounds are different. Özay *et al.* [16], obtained a higher wound healing effect in the nondiabetic group than in the diabetic group on days 7 and 13. The results of our study were consistent with that reported above, wherein incision wound healing took a short time in the group normal rat as compare to hyperglycemic rats (**Table 5**). The supplementation of *C. papaya, C. odorata*, and *A. bilimbi* on the patch which were replaced every 2 days accelerated the healing process. In addition, our study also demonstrated the potential of this plant in wound healing in diabetic wounds even though DM conditions significantly impair wound healing such as dysfunction in the differentiation of extracellular matrix fibroblasts and epidermal cells, as well as poor angiogenesis. The results of this study are in line with Dewiyanti *et al.* [28], who observed that there was a decrease in wound length after administration of Chinese castor sap.

Table 5 Percentage of wound closure in normal and hyperglycemic rats on days 3, 7, and 13.

Groups	Wound closure (%)		
	Day 3	Day 7	Day 13
P1	5.1 ± 2.67^{a}	24.0 ± 1.22^{ab}	48.6 ± 12.61^{a}
P2	20.9 ± 0.96^{de}	51.3 ± 3.38^{cd}	96.0 ± 4.18^{de}
P3	18.5 ± 1.06^{d}	52.9 ± 2.65^{cd}	97.0 ± 4.47^{e}
P4	24.8 ± 4.88^{e}	57.3 ± 4.90^{d}	99.0 ± 2.23^{e}
P5	16.0 ± 1.17^{cd}	46.6 ± 4.99^{c}	94.0 ± 5.47^{cde}
P6	4.6 ± 3.22^{a}	18.9 ± 6.61^{a}	62.0 ± 8.36^{b}
P7	17.5 ± 5.59^{cd}	26.2 ± 6.35^{ab}	66.5 ± 7.82^{b}
P8	9.6 ± 1.19^{ab}	26.0 ± 8.21^{ab}	68.0 ± 5.70^{b}
P9	18.4 ± 7.74^{d}	28.0 ± 10.17^{b}	87.0 ± 5.70^{cd}
P10	1.75 ± 0.41^{bc}	29.4 ± 0.65^{b}	86.0 ± 6.51^{c}

Data presented mean ± standard deviation. Different superscripts within the same column indicated statistically difference ($p < 0.05$) (P1, normal rat + normal dressing; P2, normal rat + transdermal patch Dermafix; P3, normal rat + transdermal patch of *C. papaya* leaf; P4, normal rat + transdermal patch of *C. odorata* leaf; P5, normal rat + transdermal patch of *A. bilimbi* leaf; P6, hyperglycemic rat + normal dressing; P7, hyperglycemic rat + normal dressing + transdermal patch Dermafix; P8, hyperglycemic rat + transdermal patch of *C. papaya* leaf; P9, hyperglycemic rat + transdermal patch of *C. odorata* leaf; P10, hyperglycemic rat + transdermal patch of *A. bilimbi* leaf).

Rat TGF-β concentrations

Transforming growth factor-β plays an important role in wound healing. The results of this study support the results of measuring wound length in rats. TGF-β is a multifunctional protein that controls proliferation, differentiation, and is an important pharmacological agent in accelerating wound healing in rats. TGF-β is regulated and secreted by keratinocytes and macrophages at the start of inflammation for the formation of granulation tissue and regulates myofibroblast differentiation to accelerate wound contraction. Observation of TGF-β levels was chosen as a research parameter because it is considered to

describe the process of wound healing and the formation of scar tissue. The results of rat TGF-β measurements is presented in **Table 6**.

Table 6 TGF-β levels of rat on the 7th and 14th days post incision.

Groups	Rat TGF-β level (ng/L)	
	Day 7	Day 14
P1	148.87 ± 7.52[a]	143.76 ± 6.53[a]
P2	182.51 ± 12.46[a]	184.10 ± 7.10[a]
P3	168.50 ± 4.33[a]	162.51 ± 15.85[a]
P4	188.83 ± 3.79[b]	184.28 ± 13.80[b]
P5	161.46 ± 12.74[a]	149.96 ± 1.56[a]
P6	166.50 ± 1.46[a]	165.71 ± 16.14[a]
P7	158.09 ± 4.01[a]	142.29 ± 7.25[a]
P8	126.57 ± 5.15[a]	123.41 ± 15.80[a]
P9	153.24 ± 7.96[a]	146.64 ± 9.57[a]
P10	129.59 ± 11.99[a]	127.03 ± 3.05[a]

Data presented mean ± standard deviation. Different superscripts within the same column indicated statistically difference ($p < 0.05$) (P1, normal rat + normal dressing; P2, normal rat + transdermal patch Dermafix; P3, normal rat + transdermal patch of *C. papaya* leaf; P4, normal rat + transdermal patch of *C. odorata* leaf; P5, normal rat + transdermal patch of *A. bilimbi* leaf; P6, hyperglycemic rat + normal dressing; P7, hyperglycemic rat + normal dressing + transdermal patch Dermafix; P8, hyperglycemic rat + transdermal patch of *C. papaya* leaf; P9, hyperglycemic rat + transdermal patch of *C. odorata* leaf; P10, hyperglycemic rat + transdermal patch of *A. bilimbi* leaf).

TGF-β is an important cytokine that regulates myofibroblast differentiation and activation, re-epithelialization, and alternative macrophage activation, which are important steps for wound healing. In this study the concentration of TGF-β tended to decrease on H-14 compared to D-7. These results are different from the report of Wulandari *et al.* [29], who reported that there was an increase in TGF-β on H-21 compared to H-5 in acutely injured rats. Oskeritzian [30], stated that an increase in TGF-β causes the activation of fibroblast proliferation which is necessary for collagen synthesis. The accumulated collagen is the cause of scar tissue. The difference in results in this study may be due to differences in the type of wound. It is possible that in the incisions performed in this study, the wound healing process took place faster than in acute wounds. The TGF-β concentration will usually decrease in the early remodeling phase so it is suspected that on the 14th day the remodeling phase has started. This result was also confirmed by the decreasing wound length on day 13 (**Table 5**).

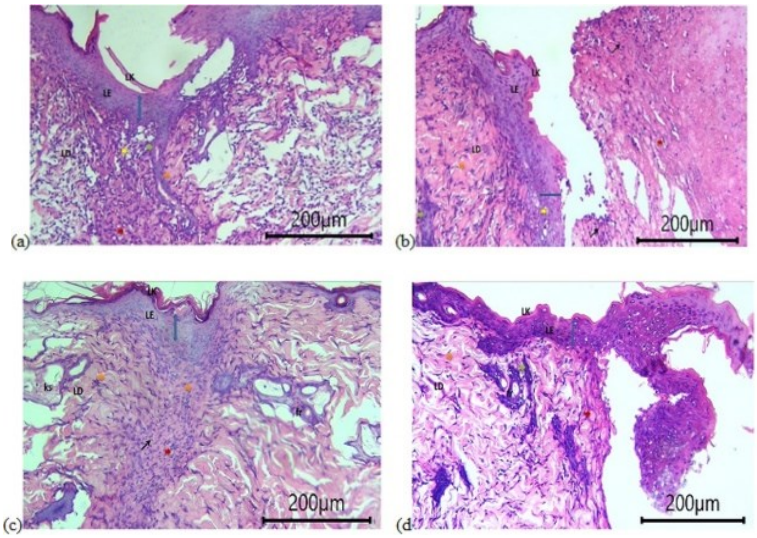

Figure 2 Photomicrographs of rat skin after incision. (a) Day 7 (normal rat), (b) Day 14 (normal rats), (c) Day 7 (normal rat + extract leaves), and (d) 14th day (hyperglycemic rats + extracts leaves). Corneum layer (LK), epidermal layer (LE), dermis layer (LD), sebaceous glands (ks), hair follicles (fr), epithelial areas (blue arrow), collagen (orange arrow), gland ducts (green arrow), angiogenesis (red arrow), inflammatory cell infiltration (black arrow). HE 100×.

Histological parameters of rat skin

Histologically, the inflammatory cells found in this study were generally large fibroblast cells, with irregular branches and round euchromatic nuclei as shown in **Figure 2**.

The densities of collagen and fibroblast cells was considered as an indicator of the granulation tissue in the dermis of the incision wound. **Figure 2** revealed that the administration of a transdermal patch containing *C. papaya, C. odorata*, and *A. bilimbi* leaves showed more obvious collagen density than the control. From the results of the histological tests, it could be seen that treatment with transdermal patch containing the leaves extract resulted in an improvement of incision wound healing process.

On 7th days post incision, it was considered as the proliferative phase of the wound healing process. The proliferative phase begins after the inflammatory phase and fibroblasts are activated in the wound area. Angiogenesis or vascularization is a necessary repair element, by adding chemotactic factors to endothelial cells; angiogenesis can be induced. Angiogenesis is an important factor in the process of wound healing, blood vessels leading to nutrition, oxygenation, cell proliferation and ultimately accelerating wound healing. The growth of blood vessels in the experimental group compared to the control group tends to increase. The 3 leaves can accelerate angiogenesis and the formation of granulation tissue in the wound area. Fibroblasts in response to physiological and pathological stimuli proliferate and can recreate original tissue and repair wounds [31].

Fibroblasts are synthesized as part of the extracellular matrix, such as fibronectin and proteoglycans which provide appropriate cell migration and proliferation. New fibroblasts produce connective tissue elements such as collagen fibers and proteoglycans which promote wound healing [31]. An increase in the number of fibroblasts will result in an increase in collagen which causes the size of the wound to shrink. On day 7, the epidermal layer in rats treated with Dermafix and leaves extracts was thicker and granular tissue full of cells and arteries completely filled the wound as compared to the rats received normal dressing. On the 14th day more collagen, very small layers and re-epithelialization were observed.

As a consequence of skin incision in rats, the inflammation occurs surrounding the incision wound area. Wound healing is a process that occurs after an injury occurs; this process requires interactions between different cell types, structural proteins and growth factors [31]. When an injury occurs, the hemostasis phase immediately takes place, the body will activate both intrinsic and extrinsic coagulation factors and platelet aggregation forms a clot along with fibrin. Platelets secrete cytokines and release growth factors during inflammatory phase which mediate the vasodilation and increase vascular permeability. Inflammatory cells in the inflammatory phase go to the wound area as phagocytic cells to remove foreign particles. These cells include polymorphonuclear (neutrophil cells) and mononuclear (macrophage cells). Macrophage cells stimulate TGF-β and fibroblast cells in the proliferative phase. Fibroblast cells synthesize collagen which functions for the reconstruction of new tissues and maximizes structural strength in the remodeling phase. Inflammatory cells and fibroblast cells play an important role in the wound healing process. Histological observation was carried out to evaluate the number of inflammatory cells and fibroblast cells in in the process of wound healing after therapy intervene [32]. The histological analysis of skin wound healing on the 14th day after treatments are presented in **Table 7**.

Table 7 Histological parameters of skin wound healing on day14 after treatment.

Groups	Fibroblast	Vascularization	Collagen thick	Collagen density
P1	6.2 ± 1.2^{abc}	4.3 ± 1.5^{c}	11.4 ± 1.88^{a}	1.5 ± 1.4^{ab}
P2	11.0 ± 6.5^{e}	2.7 ± 0.6^{ab}	25.2 ± 9.46^{bcd}	3.0 ± 1.4^{bc}
P3	7.0 ± 1.2^{bcd}	2.0 ± 0.0^{ab}	29.1 ± 2.14^{cd}	3.5 ± 0.7^{c}
P4	8.8 ± 2.5^{cde}	2.3 ± 1.2^{ab}	25.0 ± 7.9^{bcd}	3.5 ± 0.7^{c}
P5	6.0 ± 1.0^{abc}	1.3 ± 0.6^{a}	31.5 ± 13.5^{cd}	3.5 ± 0.7^{c}
P6	8.8 ± 0.6^{cde}	3.3 ± 1.2^{bc}	12.1 ± 2.4^{ab}	1.5 ± 0.7^{a}
P7	9.6 ± 1.0^{de}	2.0 ± 1.0^{ab}	14.1 ± 6.2^{ab}	3.5 ± 0.7^{c}
P8	5.2 ± 1.2^{ab}	2.0 ± 1.0^{ab}	33.3 ± 5.7^{d}	2.5 ± 0.7^{abc}
P9	7.8 ± 2.0^{bcd}	2.0 ± 0.0^{ab}	18.9 ± 1.4^{abc}	3.5 ± 0.7^{c}
P10	3.8 ± 2.1^{a}	1.7 ± 0.6^{a}	14.4 ± 7.5^{ab}	3.5 ± 0.7^{c}

Data presented mean ± standard deviation. Different superscripts within the same column indicated statistically difference ($p < 0.05$) (P1, normal rat + normal dressing; P2, normal rat + transdermal patch Dermafix; P3, normal rat + transdermal patch of *C. papaya* leaf; P4, normal rat + transdermal patch of *C. odorata* leaf; P5, normal rat + transdermal patch of *A. bilimbi* leaf; P6, hyperglycemic rat + normal dressing; P7, hyperglycemic rat + normal dressing + transdermal patch Dermafix; P8, hyperglycemic rat + transdermal patch of *C. papaya* leaf; P9, hyperglycemic rat + transdermal patch of *C. odorata* leaf; P10, hyperglycemic rat + transdermal patch of *A. bilimbi* leaf).

Comparison of histological values showed that there was no clear trend was observed in the fibroblast number with the values varied among treatment. The vascularization values were found higher in groups P1 and P6 over others groups. On the other hand, the collagen thickness and collagen density were found lower in groups P1 and P6 as compared to others group. Moreover, collagen density among group received transdermal patch Dermafix and leaves extracts did not differ significantly ($p < 0.05$). The low collagen density indicated that the healing wound process in rats without therapeutic intervene (P1 and P6) were slower than those groups applied with the transdermal patches loaded with leaves extracts. Borges *et al.* [33], stated that in the inflammatory phase, inflammatory cells such as macrophage cells will secrete substances that can trigger the emergence of angioblasts and fibroblasts that function to synthesize collagen that will cover the wound. Administration of a transdermal patch containing *C. papaya, C. odorata*, and *A. bilimbi* leaves showed higher collagen density than the control.

Overall, the results showed that from each parameter observed, the transdermal patch group containing *C. papaya, C. odorata*, and *A. bilimbi* leaves extract had higher healing potential as compared to the group without extract content both in normal rats and hyperglycemic rats. This indicated that *C. papaya, C. odorata,* and *A. bilimbi* have potential as wound healing agents. The wound healing activity of *C. papaya, C. odorata*, and *A. bilimbi* may be due to the contribution of the chemical compound groups contained therein such as flavonoids, phenolics, tannins, saponins, steroids, and triterpenoids [11]. Several mechanisms of this compounds as wound healing agents have been widely reported [34-39].

The results of the GC-MS analysis conducted by Santi *et al.* [11], showed that the ethanol leaves extract of *C. papaya* leaves contained the main compounds such as n-hexadecenoic acid, phytol, methyl linoleate, linolenic acid, oleoyl chloride, and carpaine. Furthermore, the results of the GC-MS analysis of the ethanol extracts of *C. odorata* and *A. bilimbi* leaves showed different compounds compared to the compounds present in the ethanol extract of *C. papaya* leaves, but in general the content of fatty acids and esters such as n-hexadecenoic acid, phytol, linolenic acid were present in all 3 leaf samples. The compounds n-hexadecenoic acid, phytol, methyl linoleate, linolenic acid have been reported to have anti-inflammatory, antioxidant, antimicrobial, antifungal, and wound healing activities [37,40,41].

Hexadecenoic acid is methyl ester belongs to the class of fatty acids with another name methyl hexadecanoate, palmitic acid. This compound is reported to have associated with antioxidant activity and alpha-reductase inhibitors, hypercholesterolemic and hemolytic agents, anti-inflammatory, antioxidant, and antimicrobial [42-44]. Phytol, encapsulates in terpenes and terpenoids, plays an important role in various biological properties as anti-inflammatory which inhibits hyperalgesia, reduces myeloperoxidase (MPO), releases pro-inflammatory cytokines, reduces the production of interleukin (IL)-6, COX-2, significantly downregulates p38MAPK expression, and increases NFκB activity [45]. Linolenic acid has other names, namely 9,12,15-Octadecatrienoic acid; alpha-linolenic acid; all-cis-9,12,15-octadecatrienoic acid. This compound was reported to inhibit prostaglandin synthesis, reduce inflammation, prevent chronic disease, antioxidant, and antifungal [46-49]. Based on the present result, it is evident that the leaves extract of *C. odorata, C. papaya* and *A. bilimbi* infer better wound healing properties.

Conclusions

Transdermal patches containing of *C. odorata, C. papaya* and *A. bilimbi* leaves extract have the therapeutic potency and can accelerate wound healing process in hyperglycemic rat based on on CSI score, wound closure, TGF- β concentration and histological structure of rat skin, and therefore could be considered as alternate to the synthetic transdermal patches.

Acknowledgements

The authors are gratefully acknowledged the Graduate School of Mathematics and Applied Science, Universitas Syiah Kuala, Indonesia for facilitating this research. The acknowledgement also goes to staff of Herbarium Laboratory of the Faculty of Biology Department, Mathematics and Natural Sciences (FMIPA) Indonesia, staff of Animal laboratory, physiology laboratory and Pharmacology laboratory of the faculty of veterinary medicine, Universitas Syiah Kuala, Indonesia who assisted the experiment and analysis.

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