

Phytochemical screening and wound healing effect of *Alocasia longiloba* Miq. petiole ethanolic extract and its fractions in excision wound rat model

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ABSTRACT

Alocasia longiloba Miq., or 'keladi candik' is traditionally used for wound healing in Malaysia. Preliminary studies have been performed to determine the dose range of *A. longiloba* for potential wound healing. Therefore, this study continues to determine the phytochemical screening of fractions and their ability in accelerating wound healing. Ethanol crude extract of *A. longiloba* and its fractions of hexane, chloroform, and ethyl acetate was prepared and used for phytochemical screening and wound healing formulation. Seven groups of Sprague-Dawley rats (n = 3) were experimentally wounded in the dorsal area for 12 days. Group I was left untreated, Group II was dressed in petroleum jelly as the vehicle group, Group III was treated with Solcoseryl jelly 10% as the positive drug, and the rest of the groups were dressed with respective formulations at 6% concentration. The wound area was photographed and measured, and the histological examinations of granulation tissues were examined. The phytochemical screening of chloroform fraction shows an abundance of alkaloids, flavonoids, and tannins, which are considered responsible for wound healing activity. The chloroform

fraction proved more effective, showing a significant percentage of wound contraction ($p < 0.05$) compared to the negative and vehicle control groups. Moreover, histological observation of wounds treated with chloroform fraction of *A. longiloba* showed an organized epithelial layer, fewer blood vessels, and dense collagen fibres comparable with normal rat skin.

Keywords: *Alocasia longiloba* Miq., petiole extract, excision, wound, Sprague-Dawley rat, histology

INTRODUCTION

Wound healing is replacing dead tissue with newly produced tissue to restore skin integrity, which involves three phases, inflammation, proliferation, and remodelling (Tottoli et al., 2020; Zhang et al., 2020). The inflammatory phase begins immediately after injury to stop blood loss and fight pathogenic infections through the action of macrophages and neutrophils (Moghadamtousi et al., 2015; Minutti et al., 2017). The second phase is the proliferation phase, where the focus is on covering the wound area through the epithelization and the formation of granulation tissues (Reinke & Sorg, 2012; Pastar et al., 2014). The final phase is maturation or tissue remodelling. In this stage, extracellular matrix components alter during the maturation period, such as forming type I collagen from immature type III collagen, which is more stable (Campelo et al., 2018). Besides, the number of cells that had been used in wound healing but are no longer needed, such as fibroblasts, blood vessels, and inflammatory cells, decreases due to apoptosis or unknown cell death mechanisms in this phase (Gonzalez et al., 2016).

Medicinal plants or herbs have been used to speed up wound healing since ancient civilizations (Vitale et al., 2022). Researchers today are more interested in finding novel natural therapeutic compounds from medicinal plants since they are secure, accessible, and free of adverse effects, including in the healing of wounds (Safna et al., 2020; Monika et al., 2022). *Alocasia longiloba* Miq., known as 'keladi candik' by the Malay people in Malaysia, is a plant from the *Araceae* family (Nur-Izzati et al., 2021). The species is indigenous to Australia, Asia, and Southeast Asia. They can be found across tropical and subtropical woods, with the types of wet lowlands changing between different geographic areas and nations (Arbain et al., 2022). The Malays in Malaysia have traditionally employed *A. longiloba* to treat external wounds where the outer bark of the stem of *A. longiloba* is peeled and wrapped over the wound to speed healing. According to studies, *A. longiloba* has a greater total phenolic and flavonoid content and good antioxidant scavenging activity on 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) tests. Our previous studies have proven the effectiveness of *A. longiloba* in accelerating wound healing. In the study, we used different solvent extracts (95% ethanol, 50% ethanol, and hexane) with different concentrations (0.5, 3, and 6%) to determine which solvent extract and concentration were most effective on wound healing. From the study, we found that 6% of 95% ethanol

extract provided the highest healing properties compared to other solvent extracts and concentrations (Che Hamzah et al., 2019). To the best of our knowledge, no study has been conducted to determine the effect of the active fraction of *A. longiloba* ethanol extract on wound healing. The importance of studying the effects of different fractions of crude plant extract is that they have been documented to show varying levels of biological activity (Adewumi et al., 2020). In the present study, *A. longiloba* ethanol crude extract was fractionated and used to determine the phytochemical screening, fractions' ability on wound closure percentage, and histological features associated with wound healing.

MATERIALS AND METHODS

Plant Material

The plant material (*A. longiloba*) was collected from Kg. Sering, Kota Bahru, Kelantan, Malaysia in December 2019. The plant material has been identified and verified by Dr Zulhazman Hamzah at the Faculty of Earth science, Universiti Malaysia Kelantan, Malaysia. The vouchers were deposited at the herbarium of Universiti Malaysia Kelantan, Faculty of Earth Science, with voucher number UMK00288. The separated petiole samples were carefully rinsed using tap water to remove dust particles and air-dried in an oven at 40°C for seven days. After that, an electrical blender was used to grind the dry petioles into powder.

Animals

This wound healing study obtained ethical approval from Animal Ethics Committee (approval project code: USM/IACUC/2018/ (115) (958)). Male Sprague-Dawley rats (180 – 200 g), approximately 8 – 12 weeks old were used in this study. The animals were maintained in individual cages in a well-ventilated and temperature-controlled animal room (22 ± 2°C) and provided with a rodent chow diet and water ad libitum prior to the experimental period. The animals were divided into seven groups of three rats each as follows:

1. No treatment (NT)
2. 100% petroleum jelly (PJ)
3. 10% Solcoseryl gel (SG)
4. 6% ethanol extract (EE)
5. 6% hexane fraction (HF)
6. 6% chloroform fraction (CF)
7. 6% ethyl acetate fraction (EAF)

Extraction and Fractionation

Dry powdered samples of *A. longiloba* (60 g) were extracted using a Soxhlet extractor with ethanol (300 ml) at 60 – 65°C. Then the solvent was evaporated to obtain semisolid extract (26.11 g) by a rotary evaporator. Semisolid ethanol extract (20 g) was then suspended in water (1:20) before being extracted by different solvents with increasing polarities using a separatory funnel to give hexane (12.43 g), chloroform (0.23 g), ethyl acetate (0.11 g), and residual ethanol fractions (4.4 g). The extraction method for each solvent was repeated three times and combined.

Formulation of the Extract and Fraction

The extract of *A. longiloba* was prepared in 100% pure petroleum jelly with a concentration of 6% (w/w) (6 g of extract/ 94 g of 100% pure petroleum jelly). The formulation was chosen based on the prior preliminary study (Che Hamzah et al., 2019). The specified amount of the extract/fraction was added to the melting base and continuously swirled until a homogeneous dispersion was attained (Umeh et al., 2014; Imran et al., 2015).

Phytochemical Screening Analysis

1 g of ethanol extract (EE), hexane fraction (HF), chloroform fraction (CF), and ethyl acetate fraction (EAF) was completely dissolved in 100 mL of Dimethyl Sulfoxide (DMSO). The obtained stock solution used for phytochemical screening is as follows:

Alkaloids

Wagner Test | 1 ml of stock solution was taken in a test tube. Then the stock solution was added with 1 ml of dilute HCl (10%) and Wagner's reagent and shaken well. The formation of a reddish-brown precipitate showed the presence of alkaloids (Rufai et al., 2016).

Saponins

Form Test | 0.5 gm of the extract was shaken with 2 ml of water. The formation of foam persists for ten minutes indicating the presence of saponins (Tiwari et al., 2011).

Flavonoids

Naoh Test | The stock solution (1 mL) was taken in a test tube and added 1 ml of dilute NaOH (10%) solution. An intense yellow colour appeared in the test tube and it became colourless when the addition of 1 ml of dilute acid (10% HCl) indicated the presence of flavonoids (Hossain et al., 2013).

Tannins

Braymer Test | 10% alcoholic ferric chloride was added to 2 – 3 ml of stock solution (1:1). Dark blue or greenish grey colouration of the solution indicated the presence of tannins (Sasidharan et al., 2011).

Steroids and Terpenoids

Salkowski Test | 1 mL stock solution was treated with 1 mL of chloroform and 1 mL concentrated sulfuric acid, slowly until double phase formation. The appearance of a dish-brown colour in the middle layer was indicative of a steroidal ring (María et al., 2018).

Wound Healing Studies

Excision Wound Model

Intraperitoneal (IP) injections of ketamine (90 mg/kg) and xylazine (5 mg/kg) were used to anaesthetize the rats. Three full-thickness excision wounds were produced using sterile biopsy punches of 6 and 3 mm in diameter and thickness, respectively. The ointments were carefully administered once daily from day 0 to day 12 to cover the injured area after 24 hours of wound creation. The negative control group was left untreated. The vehicle control group was topically applied with 20 mg of 100% petroleum jelly, and the positive control group with 20 mg of 10% Solcoseryl jelly. Treatment groups were topically applied to each rat with 20 mg of 6% ethanol extract and its fractions (hexane, chloroform, and ethyl acetate) of *A. longiloba*.

Macroscopic Evaluation of Wound

The wound excision was monitored by measuring the wound contraction percentage on days 3, 6, and 12 and photographed on the same days for all experimental rat groups. Transparent tracing paper and a 1 mm² graph sheet were used to measure the wound diameter (mm) (Anima et al., 2019). The wound contraction percentage was determined using the following formula:

$$\text{Wound contraction (\%)} = [A \text{ Day } 0 - A \text{ Day } X] / A \text{ Day } 0 \times 100$$

Where Day X = Day 3; Day 6; Day 12 and A = diameter of the wound

Microscopic Evaluation of Wound

After macroscopic examination, the rats were euthanized on Day 12 with 100% carbon dioxide (CO₂) inhalation. Then, 6 mm granulation tissues were removed and fixed in 10% formalin buffer for histological analysis. Fixed granulation tissues were processed, embedded, and sectioned before being stained with haematoxylin and eosin (H&E) and Masson's trichrome (MT) staining. A microscope with an image analyser (Olympus BX-41) was used to visualize the histological features of wound healing (Loh et al., 2018).

Statistical Analysis

The results are shown as mean standard deviation (SD). One-way analysis of variance (ANOVA) was used to analyze the data, followed by the Tukey test. Statistical significance was considered when the comparison with untreated and vehicle-treated groups gave a value of $p < 0.05$.

RESULTS

Phytochemical Screening

Phytochemical screening was conducted to evaluate the qualitative chemical composition of plant extract by utilizing precipitation and colouring to identify the principal natural chemical groups including alkaloids, tannin, flavonoids, terpenoids, tannin, steroids, and phenols (Naik et al., 2014). In this study, phytochemical screening of *A. longiloba* petiole extract/ fractions, revealing the extract contains alkaloids, flavonoids, steroids/ triterpenoids, tannin, and saponin as shown in Table 1. All the extracts resulted positive to the Wagner test observing an orange-reddish-brown precipitate showed the presence of alkaloids. Clear solutions were discovered in the ethanol extract and chloroform fraction, indicating an abundance of flavonoid, however only moderate flavonoid was detected in the hexane and ethyl acetate fractions. A perfect steroidal ring was discovered in the ethanol extract, indicating an abundance of steroids/ triterpenoids. Meanwhile, a moderate amount of dish-brown colour was detected in the intermediate layer of the hexane and chloroform fractions, while a small amount was detected in the ethyl acetate fraction. An abundance of tannin was detected in the ethanol extract and the chloroform fraction, while the hexane and ethyl acetate fractions had only a moderate amount and a very low amount of tannin, respectively. Finally, poor saponin was found in all *A. longiloba* extracts and fractions.

Table 1 Phytochemical constituents of *A. longiloba* extract/fraction

Biochemicals	Extract/fractions			Ethyl acetate
	Ethanol	Hexane	Chloroform	
Alkaloids	+++	+++	+++	+++
Flavonoids	+++	++	+++	++
Saponins	+	+	+	+
Steroids	+++	+	++	++
Tannins	+++	++	+++	+

Key: (-) Absence, (+) Poor, (++) Moderate, (+++) Abundant

Wound Contraction Rate

Topical application of *A. longiloba* ethanol extract/ fraction ointments in the excision wound model has significantly increased ($p<0.05$) the rate of wound contraction compared to the negative and vehicle control groups and is comparable to the contraction value of the positive control group (Figure 1). The wound closure on day 3 shows a significant difference for the positive control (20.21 ± 2.45), ethanol extract (20.82 ± 2.41), chloroform fraction (21.93 ± 1.37), and ethyl acetate fraction (18.84 ± 2.06) treated groups compared to the negative (10.44 ± 2.64) and vehicle (12.35 ± 2.02) control groups. However, the hexane fraction (17.49 ± 2.22) shows a significant difference compared to the negative group only. On day 6, wound closure of chloroform fraction (71.80 ± 1.77) showed significantly higher than the positive control (63.67 ± 2.37), hexane fraction (62.42 ± 1.72), and ethyl acetate fraction (62.39 ± 1.08) treated groups but not for ethanol extract (66.05 ± 2.19). At the same time, wound closure of all extract/fraction treated groups showed significant differences compared to the negative (35.63 ± 2.45) and vehicle (38.55 ± 2.91) control groups. On day 12, wound closure of positive control (86.71 ± 0.55), ethanol extract (87.68 ± 2.09), and chloroform fraction (90.66 ± 2.82) were significantly higher than hexane fraction (78.83 ± 2.40) and ethyl acetate fraction (79.20 ± 0.36) respectively. Moreover, all treated groups showed significantly higher wound closure than the negative (65.23 ± 0.97) and vehicle (67.36 ± 1.20) control groups. The highest percentage of wound closure on day 12 was in the chloroform fraction group, followed by the ethanol extract group, and the lowest was in the negative control group.

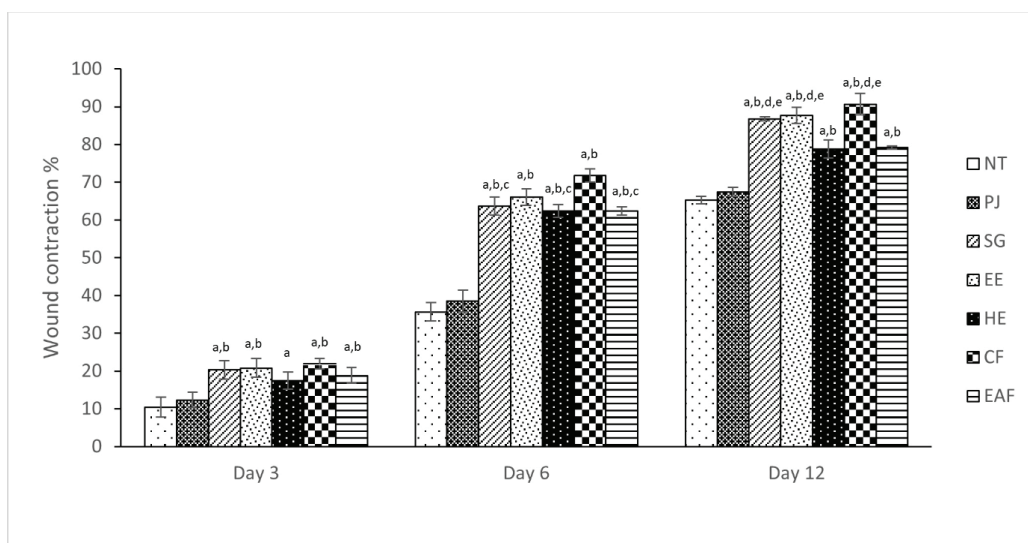


Figure 1 Bar charts represent the wound contraction percentage in *A. longiloba* extract/fractions and control groups on days 3, 6, and 12. The results were expressed as mean \pm S.E.M., $n = 3$ per group. ^a $p < 0.05$ compared with NT; ^b $p < 0.05$ compared with PJ; ^c $p < 0.05$ compared with CF; ^d $p < 0.05$ compared with HF and ^e $p < 0.05$ compared with EAF.

Macroscopic Evaluation of Wound

The macroscopic images of excision wound healing on days 0, 3, 6, and 12 treated with 6% of *A. longiloba* extract/fractions and the control groups were presented in Figure 2. On day 0, the wound showed redness due to blood covering the wound area after wound creation for all groups. On day 3, the images showed that scabs began to cover the wounds of all groups, but complete scab formation could be seen in the chloroform fraction treated group. On the 6th day, scabs appeared to disappear for the groups treated with 10% Solcoseryl jelly, ethanol extract, chloroform fraction, and ethyl acetate fraction, causing the wound area to shrink and reveal pink granulation tissue. Meanwhile, no changes in scabs were found for the negative, vehicle, and hexane fraction groups. On the 12th day, the scabs completely dropped in 10% Solcoseryl jelly, ethanol extract, chloroform fraction, and ethyl acetate fraction. However, there are still scabs that have not fallen on the negative, vehicle, and hexane fraction groups, which causes a delay in wound healing. As we can see in the group treated with ethyl acetate fraction, although the scabs disappeared completely, the size of the granulation tissues was still wide, showing a slight contraction of the wound.

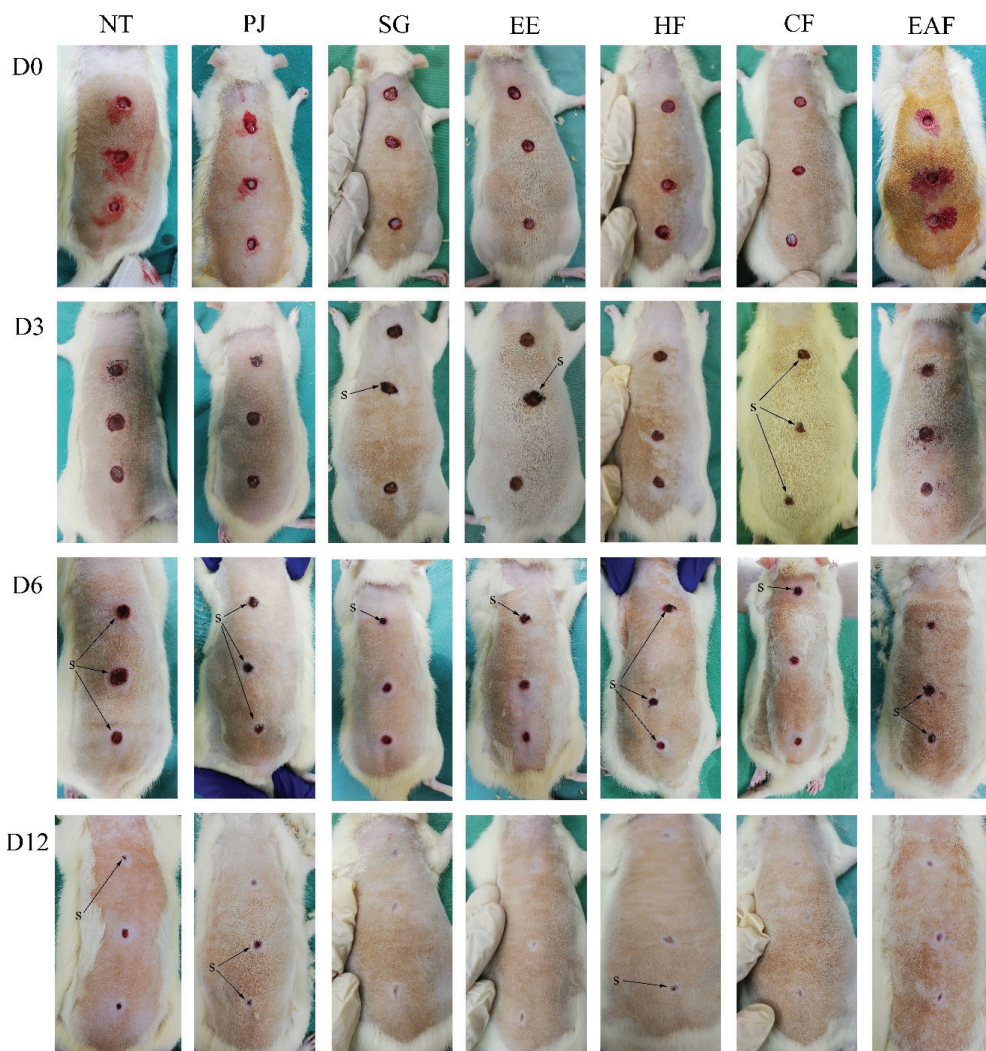


Figure 2 Macroscopic images of excision wound healing on days 0 (D0), 3 (D3), 6 (D6), and 12 (D12) in Sprague-Dawley rats treated with 6% of *A. longiloba* extract/ fractions and the control groups. Abbreviations: scab (s).

Microscopic Evaluation of Wound

Microscopic evaluation of wound healing for all treated groups on day 12 is shown in Figure 3. The histological analysis of all treated groups was compared with the histology of normal skin to get a better view of its wound healing quality (Figure 3). The results show that ethanol extract, chloroform fraction, and 10% Solcoseryl treated groups had well-defined epidermal and dermal layers, fibroblasts, discrete vascularisation, and organized collagen deposition comparable to the normal rat skin. In contrast, hexane

and ethyl acetate fraction groups revealed complete epidermal and dermal formation, the presence of fibroblasts, and organized collagen. However, they still had intense angiogenesis indicating an incomplete wound healing process as in the negative and vehicle control groups. In addition, the images also show inflammatory cells in the negative control group and the vehicle, which proves the incomplete healing of this control treatment group compared to the other groups.

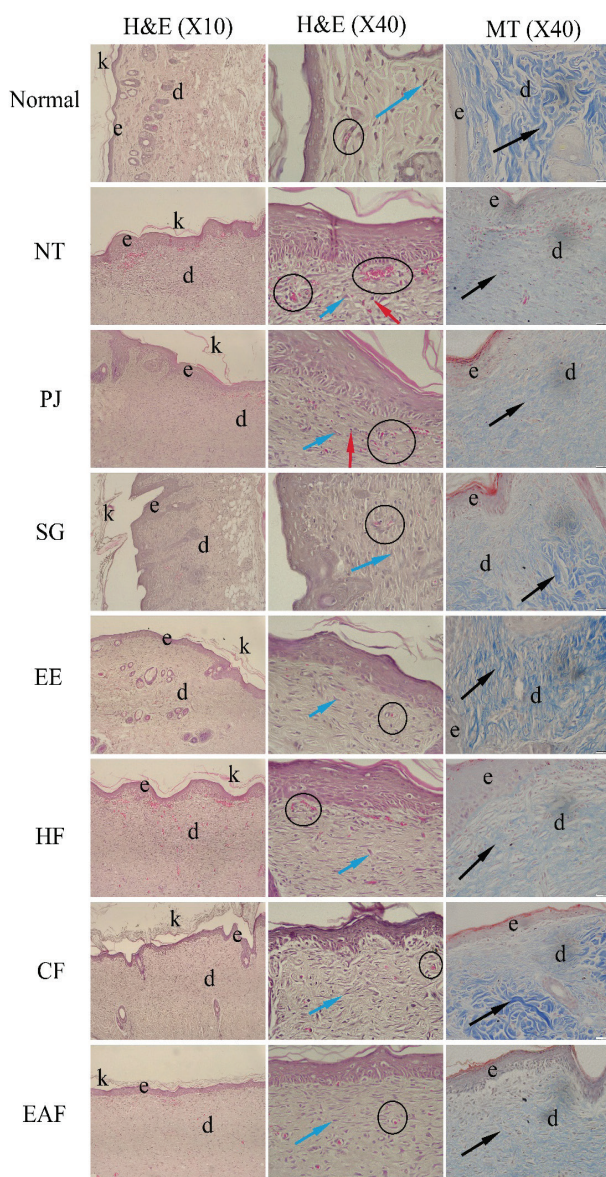


Figure 3 Light microscopic images of epithelization (e: epidermis, d: dermis, k: keratin), fibroblasts proliferation (blue arrows), inflammatory cells (red arrow), blood vessel (circle), and collagen organization on day 12 for all treated groups stained with H&E (Bar scale: 100 μ m and 50 μ m) and Masson's Trichrome (Bar scale = 50 μ m).

DISCUSSION

Nowadays, people use modern medication to treat wounds. However, a problem has arisen due to multi-resistant organisms and a decline in newer antibiotics. As a result, new formulations using various ways, such as ancient therapeutic procedures using traditional medicine, must be developed to overcome this problem (Dorai, 2012). *A. longiloba* is a traditional medicine used by Malays in Malaysia to treat wounds, but the clinical evidence is still unclear. They used *A. longiloba* directly by wrapping the wound area with the outer layer of the petiole. They believed that the water from petiole had properties to heal the wound. Now, extracting technology is used to obtain the maximum extraction yield or increase the quality of the extraction product by using solvent (Geow et al., 2021). There are many types of solvents available for extraction but the choice of solvent depends on the type of plant, the part of the plant to be extracted, the nature of the bioactive compounds, and the availability of solvent. Water is the most popular solvent used for extraction because it is cheap and capable of extracting various polar compounds. However, water has the disadvantage that it promotes the growth of bacteria and mould that can change the phytochemical properties of the extract and requires a large amount of heat to concentrate the extract. Alcohol is also another popular polar solvent used in extraction where it can extract polar secondary metabolites, is self-preserving and little heat is needed to concentrate the extract (Abubakar & Haque 2020). Alcoholic solvents such as ethanol are suitable for polyphenol extraction, non-toxic, and safe for human consumption (Do et al., 2014). Our previous study also proved that 95% ethanol extract of *A. longiloba* has the highest healing properties compared to 50% ethanol and hexane extract (Che Hamzah et al., 2019). Therefore, in this study, the effects of the ethanolic extract of *A. longiloba* petiole and its fractions were observed on full-thickness excision wounds using an experimental rat model. The importance of studying the effect of different fractions on wound healing is that we can identify the bioactive compounds responsible for healing from the fraction because different fractions have different bioactive compounds regarding the solvent used during fractionation (Adewumi et al., 2020).

Phytochemicals are biologically active compounds derived from plants which are also known as secondary metabolites of plants (Sharif et al., 2018). Its function in plants is to give colour, aroma, and taste as well as protection from infections and predators (Martinez et al., 2017). The use of this phytochemical can provide beneficial health effects to the human being. Suggestions from the research show that its antioxidant and anti-inflammatory activity is effective in treating various diseases (Süntar et al., 2020). Generally, polyphenols, alkaloids, flavonoids, steroids, saponins, tannins, and terpenoids are the main phytochemicals that medicinal plants contain (Rex et al., 2018). Alkaloids, terpenoids, flavonoids, and saponins have been discovered to have antioxidant, antibacterial, antiviral, anti-gout, anti-inflammatory, and anti-arthritis properties. They are also beneficial in the prevention and treatment of numerous illnesses, including cancer (Abdulhafiz et al., 2020). In addition, alkaloids, tannins,

and flavonoids are some of the phytochemicals that promote the wound-healing process by fighting against infection and accelerating the healing of wounds (Rex et al., 2018). Meanwhile, tannins enhance wound healing mechanisms by enhancing the regeneration and organization of new tissues (Kothari & Jain, 2017). In this study, we found that the chloroform fraction has an abundance of alkaloids, flavonoids and tannins than the hexane and ethyl acetate fractions. Alkaloids, flavonoids, and tannins are some phytochemicals that promote wound healing by preventing infection and hastening the healing of wounds (Rex et al., 2018). Therefore, this could be the reason for the wound-healing activity in the chloroform fraction compared to other fractions.

The main objective of wound healing is to heal or restore the damaged tissue to its original state in a short time and with minimal pain and discomfort to the patient. Restoration of damaged tissue to its original condition is marked by wound contraction or the process of shrinkage in the injured area (Begashaw et al., 2017). From this study, the ethanolic petiole extract of *A. longiloba* and its fractions demonstrated a significant wound contraction percentage compared to the control-treated group. The results showed that topical application of chloroform fraction gave the highest wound contraction percentage on day 12. This result is similar to the study by Olugbuyiro et al. (2010) where in both infected and non-infected groups, the chloroform fraction of *Flabellaria paniculata* leaf extract revealed considerable wound healing activities compared to the aqueous fraction (. It has been suggested that the presence of tannins and other astringents in this plant contributes to the wound-healing ability of the chloroform fraction. Macroscopic evaluation of this study also proved that chloroform fraction gives the highest wound healing effect. As we can see on day 3, the scab formation occurred entirely in the chloroform fraction treated group compared to the other groups indicating rapid fluid secretion from inflammatory cells. The findings indicated that chloroform fraction possesses anti-inflammatory properties that can reduce wound inflammation and hasten the healing process (Ahmad et al., 2021). The delay in the inflammatory process causes a delay in the wound healing process, as in the case of negative and vehicle groups.

The histological analysis of normal tissue is shown to have a thin epidermal layer, a dermis layer rich in appendages such as hair follicles and sebaceous glands, and dense collagen fibres with H&E and trichrome staining (Figure 3). In this study, complete epithelization can be observed in *A. longiloba* petiole extract/fractions and positive treated groups, comparable with normal skin. This suggests that *A. longiloba* petiole extracts positively affect epithelization in wound healing. Compared to the negative and vehicle control groups showed partial epithelization. During the proliferation phase, the amount of blood vessels is highest in granulation tissue to improve circulation to provide oxygen, and essential nutrients for the healing process include re-epithelialization. According to Häkkinen et al. (2011), granulation tissue serves as a framework for connective tissue healing. As the granulation tissue matures into mature scar tissue, some new vessels will experience regression, causing the number of vessels to normalize and return to the level of normal skin as shown in the ethanol extract, chloroform fraction, and

positive groups (Johnson & Wilgus, 2014; DiPietro, 2016). However, the negative, vehicle, hexane fraction, and ethyl acetate fraction groups showed a high number of blood vessels appearance due to neovascularisation, indicating an incomplete proliferation phase. This finding is consistent with a previous study by Cheng et al. (2018) in which untreated type 1 diabetic wound showed the highest number of new blood vessels on day seven. However, the number of blood vessels decreased on Day 14 and increasingly decreased by plasma treatment.

In addition, the presence of inflammatory cells in the negative and vehicle control groups may indicate incomplete healing because inflammatory cells are critical for the release of soluble growth factors and cytokines, which is crucial for controlling fibroblast proliferation, chemotaxis, migration, and gene expression required for the formation of granulation tissue (Häkkinen et al., 2011). Moreover, the ethanol extract, chloroform fraction, and a positive group showed compact collagen fibres in a parallel configuration comparable to normal tissue. This is due to the formation of muscle type I collagen from type III collagen produced in the proliferative phase (Rebolla et al., 2013). In contrast to the negative, vehicle, hexane, and ethyl acetate fraction groups with irregularly arranged and loosely packed collagen fibres. These results are supported by the study by Abdul Latif et al. (2015) where histological analysis of wounds treated with 3% of *Alocasia denudata* stem juice showed a large number of fibroblast proliferation and collagen synthesis resulting in increased wound tensile strength and better wound healing effects. Collagen is the primary structural protein of the extracellular matrix produced by fibroblasts, which is crucial for epithelization, tensile resistance, and structural support (Nejjari et al., 2019). The high content of collagen fibres in the granulation tissue of ethanol extract and chloroform fraction indicates the effectiveness of *A. longiloba* petiole extract. The lower collagen content in the granulation tissues of the negative and vehicle groups may be due to the longer duration of the inflammatory phase, during which time collagen breakdown is more pronounced than collagen synthesis (Asumang et al., 2021). Thus, the results obtained from the study support the usefulness of *A. longiloba* in folk medicine for the treatment of wound healing among Malay communities in Malaysia.

CONCLUSION

In conclusion, this study showed that the application of ethanol extract of *A. longiloba* and its fraction improves the healing activity. Phytochemical examination of the chloroform fraction of *A. longiloba* showed an abundance of alkaloids, flavonoids and tannins, which are responsible for its wound-healing activity. The 6% chloroform fraction of *A. longiloba* shows remarkable wound healing activity compared with other fractions in improving wound contraction, epithelialization, collagen synthesis, and maturation. Therefore, the 6% chloroform fraction of *A. longiloba* has the potential to be developed as an effective natural wound-healing agent.

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