

# In Vivo Wound Healing Activity of Ethyl Acetate and *n*-Butanol Fraction of *Ampelociscus rubiginosa* Lauterb. Tuberos Root in Incisional Wound Model Rats

Khoerul Anwar<sup>1\*</sup>, Muhammad Rafi'i<sup>1</sup>, Nurul Faridah<sup>1</sup>, Nurlely<sup>1</sup>, Liling Triyasmono<sup>1</sup>, Sudarsono<sup>2</sup>, and Agung Endro Nugroho<sup>3</sup>

<sup>1</sup> Program Studi Farmasi, Universitas Lambung Mangkurat, Banjarbaru, Indonesia

<sup>2</sup> Bagian Biologi Farmasi, Fakultas Farmasi, Universitas Gadjah Mada, Yogyakarta, Indonesia

<sup>3</sup> Bagian Farmakologi dan Farmasi Klinik, Fakultas Farmasi, Universitas Gadjah Mada, Yogyakarta, Indonesia

**Abstract.** Previous research showed the wound healing activity of ethanol extract of tuberous root of *Ampelociscus rubiginosa* Lauterb. on incision model rat. This study aims to determine the activity of ethyl acetate (EAF) and *n*-butanol fraction (NBF) of ethanol extract of *A. rubiginosa* tuberous root in gel dosage form as wound healing agents. The experimental animals used were rats with incision wound models. Three concentrations of EAF and NBF (0.75%, 1.00%, and 1.25%) were given topically as gel preparations. Bioplacenton was used as a reference. On the 16th days, the length of wound area, tensile strength, and histopathology of skin tissue were determined. The results showed that percent wound contraction was observed significantly greater in EAF and NBF for all concentrations groups ( $p < 0.05$ ). Tensile strength has varied results, but in general both fractions have higher tensile strength value than negative control groups. Histopathological observations showed improvement in re-epithelialization, neocapillarization, and fibroblast. In conclusion, this study suggested that both ethyl acetate and *n*-butanol fraction of tuberous root of *A. rubiginosa* have potential benefit as wound healing agents with comparable activity.

**Keywords:** Wound healing, *Ampelocissus rubiginosa* Lauterb., Ethyl acetate and *n*-butanol fraction, Incision wound

## 1. Introduction

Wounds are damage to the skin tissue that is often experienced by most of the world's population. Wound prevalence in Indonesia is relatively high. The three most common types of injuries experienced by the Indonesian population were abrasions/ bruises (70.9%), dislocations (27.5%), and laceration (23.2%) [1]. Damaged skin tissue due to injury triggers a natural healing process to restore tissue integrity and skin function as the outer protector of the body [2]. The wound healing process can be supported by using synthetic or traditional drugs that contain compounds anti-inflammatory, antibacterial and or analgesic [3]. Medicinal plants have great potential in improving wound healing, such as eliminating swelling, pain, and scars on wounds [4]. More than 70% of pharmaceutical products for wound healing are plant-based products,

while 20% are based on minerals and the rest are based on animal products [5].

Tawas ut (*Ampelocissus rubiginosa* Lauterb.) is a shrub from the family of Vitaceae, has great potential as a traditional medicine to heal wounds. This plant has been empirically used by residents of Central Kalimantan as medicines for various diseases, including as a wound medicine. The results of phytochemical screening showed that *A. rubiginosa* tuberous root contained alkaloids, flavonoids, tannins, and saponins [6,7]. Previous studies have shown that ethanol extract of *A. rubiginosa* tuberous root has an incision wound healing activity, by accelerating re-epithelialization, neocapillarization, and increasing collagen density in histopathological observations [7]. This extract also has antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* [8].

The compound in tuberous root of *A. rubiginosa* which is thought to potentially accelerate the wound healing

\*Corresponding author: endrance@gmail.com

process is a relatively polar compound. Therefore, fractionation is carried out to take compounds based on their polarity level. Semi polar ethyl acetate tends to attract both polar and nonpolar active compounds [9]. The *n*-butanol solvent is a semi-polar solvent, but is more polar than ethyl acetate, capable of attracting organic compounds such as phenols and flavonoids [10]. This study aims to determine the healing activity of incision wounds from ethyl acetate and *n*-butanol fractions from ethanol extract of *A. rubiginosa* tuberous root. The parameters observed from this study were wound contraction, as well as based on tensile strength, and histopathology study.

## 2. Methodology

### 2.1 Plant material

The tuberous root of *A. rubiginosa* was obtained from the traditional market in Palangkaraya, Central Kalimantan. This plant has been identified in the Basic Laboratory of FMIPA ULM Banjarbaru (No. 017e / LB. LABDASAR / I / 2017). The plant material that have been dried are then powdered for further testing.

### 2.2 Extraction and fractionation

The powdered material of *A. rubiginosa* tuberous root (1466.4 grams) were macerated using ethanol 70% (ratio 1: 2.5 parts of the solvent). Extraction was carried out for 24 hours while stirring occasionally. Macerate then filtered with a flannel cloth and collected in an Erlenmeyer flask. The residue left behind is macerated 2 times. All filtrate evaporated with rotary evaporator (temperature 65°C) and concentrated using waterbath [11].

The thick extract was first suspended with distilled water (ratio of 1: 2 extract and distilled water), then put into a separating funnel and added with ethyl acetate solvent (ratio of 1: 5). The separating funnel is then shaken out and allowed to stand until 2 layers are formed. Ethyl acetate layer was separated and collected. Water layer obtained is then fractionated again with *n*-butanol solvent. The obtained *n*-butanol layer was collected. Ethyl acetate (EAF) and *n*-butanol fraction (NBF) of ethanol extract of *A. rubiginosa* tuberous root evaporated using a rotary evaporator and then thickened on the waterbath.

### 2.3 Preparation of formulation

EAF and NBF gel preparations were made, each in three concentration 0.75%, 1.00%, and 1.25%. The marketed formulation, Bioplacenton Gel (PT. Kalbe Farma, Indonesia) was took as a reference for comparison [7].

### 2.4 Experimental protocol

Male Wistar rats were selected for this study (aged 2-3 months with a body weight of 200-260 grams). The food used is laboratory standards feed (Comfeed, Indonesia) and *ad libitum* drinking water. This research has received ethical clearance from the Health Research Ethics Commission FK ULM Banjarmasin (No. 335/KEP-FK UNLAM /EC/V/2017).

Five animals were taken in each group for this experiment. The group I was referred as control group, given only gel base while Group II, III and IV denoted as treatment groups were received topically EAF gel (0.75%, 1.00%, and 1.25% concentration respectively). The Group V, VI, and VII were received 0.75%, 1.00%, and 1.25% NBF respectively. The Group VIII received Bioplacenton (Kalbe Farma, Indonesia) and served as reference group. All of treatment giving twice daily. Healing property was assessed in terms of physical parameters and histopathological study.

### 2.5 Incision wound creation

The hair around the back of animal was shaved and 70% alcohol is applied to the skin as an antiseptic. Rat was anesthetized first using ether before incision. Wounds were made 4 cm long with a depth of  $\pm 2$  mm [12]. The blood that comes out during the wound was cleaned using 0.9% NaCl until the bleeding stops. Clean wounds were sewn at a distance of  $\pm 1$  cm.

### 2.6 Physical properties of wounds

#### 2.6.1 Wound contraction

Wounds were observed and the wound length was measured on days 4, 8, 12, and 16 after injury. The observations made were measuring the average length of wound for each group, and calculating the percentage of wound contraction [13]:

$$\text{Percentage} = \frac{\text{wound length day 0} - \text{wound length day } n}{\text{wound length day 0}} \times 100\% \quad (1)$$

$n$  = days of measurement (days of 4, 8, 12, 16, and 20<sup>th</sup>)

#### 2.6.2 Tensile strength

After the mouse sacrificed at the end of the treatment, the skin on the wound area is taken 2 x 3 cm. The skin was stretched with tensiometer [12]. The initial load given starts from 500 g, which is added in a 20-second interval [14]. Tensile strength can be calculated by the formula [15]:

$$\text{Tensile strength} = \frac{\text{weight loaded (g)}}{\text{wound area(mm}^2\text{)}} \quad (2)$$

resulted in 6.55% thick ethyl acetate fraction and *n*-butanol fraction with a yield percentage of 39.78%.

## 2.7 Histopathological study

Wound tissue specimens from the control group, EAF, NBF, and reference were fixed first in a 10% formalin buffer. The skin tissue was printed on melted paraffin [13]. Paraffin block was cut with a thickness of 5  $\mu\text{m}$  and stained with HE (hematoxylin-eosin). Parameters observed were epidermal layer (re-epithelialization), neocapillary formation and fibroblast cells in the wound [15-17].

## 2.8 Data analysis

All pharmacological data were presented as Mean $\pm$ SEM for five rats. Data were analyzed by One-Way ANOVA to see a significant difference in the controlled variables with a 95% confidence level ( $p = 0.05$ ).

## 3 Result and Discussion

### 3.1 Extraction and fractionation

The coarse powder of *A. rubiginosa* (1,466.4 grams) was extracted by maceration method using 70% ethanol solvent because it easily penetrates into the plant cell membrane, making it easy to attract the active substances contained in the sample [18]. The thick extract obtained from the maceration process was 452.83 grams with a yield of 30.88%. Secondary metabolite compounds in ethanol extract of *A. rubiginosa* tuberous root still too complex. So it is necessary to separate the compounds through the fractionation process. Fractionation of extracts was done by partitioning method. The partition is the addition of two solvents which are not mixed with the polarity that increases into the extract [19]. The use of solvents with different levels of polarity can affect the types of compounds extracted [20]. Forty grams ethanol extract of *A. rubiginosa* tuberous root was fractionated with partition method,

### 3.2 Phytochemical screening

Phytochemical screening was carried out to identify the content of active compounds from plant samples by adding certain reagents. Based on the results of phytochemical screening tests, NBF was found to be positive for alkaloids, flavonoids, tannins and saponins; while NBF gave same result except negative saponins (Table 1).

Table 1. Phytochemical screening of EBF and NBF

Extract	Alkaloids	Flavonoids	Tannins	Saponins
EBF	+	+	+	-
NBF	+	+	+	+

### 3.3 Wound contraction

Rat skin incision wound closure began to be appear on day 4. The percentage of wound closure showed a difference from each treatment in the process of wound closure in rats (Table 2). Wound healing in the negative control group still occurs because a healthy body has a natural ability to protect and restore itself [21]. A visual observation shows that the condition of the wound that is initially in moist conditions is seen to dry immediately after the scab formation. Scab that forms above the skin surface forms homeostasis and prevents contamination of wounds by microorganisms [22]. Observations on the 3<sup>rd</sup> day after injury, scab formed in the EAF, NBF, and positive control groups, while the negative control of scab formed on the 6<sup>th</sup> day. Scab formation shows that the wound healing process enters the early stage of proliferation phase where there is formation of granulation tissue in the wound (fibroblasts and inflammatory cells) [23]. The speed of scab formation from each treatment group indicates the speed of wound healing [22]. The mean percentage of wound length closure of test animals on the 16<sup>th</sup> day showed a significant difference between the groups given gel containing EAF, NBF, and reference gel compared to control group ( $p < 0.05$ ).

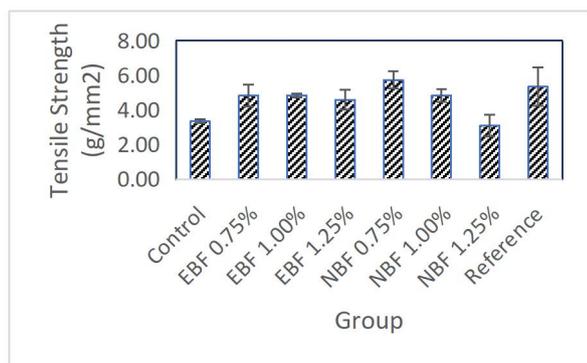
Table 2. Effect of treatment on wound contraction on different days

Group	Wound contraction (%) (Mean±SEM)			
	Day 4	Day 8	Day 12	Day 16
Control	0,50 ± 0,50	26,50 ± 1,69	71,60 ± 1,91	91,50 ± 2,57
EBF 0.75%	19,0±2,57*	69,0±2,03*	91,50±3,59*	100±0,00*
EBF 1.00%	9,50±2,42*	70,0±2,34*	92,00±5,56*	100±0,00*
EBF 1.25%	15,00±2,62*	72,0±3,48*	94,50±1,66*	100±0,00*
NBF 0.75%	1,00 ± 0,61	69,50 ± 3,65*	91,50 ± 3,02*	98,50±1,00*
NBF 1.00%	1,50 ± 0,61	76,50 ± 2,03*	90,00 ± 2,23*	99,50±0,50*
NBF 1.25%	1,00 ± 0,61	50,00 ± 3,79*	89,00 ± 1,87*	99,50±0,50*
Reference	9,50±7,04*	81,5±3,41*	95,5±0,93*	100±0,00*

n=5 rats per group, value represents Mean±SEM, \*p<0.05 when treated group compared with control group

### 3.4 Tensile strength

Tensile strength was used to see wound healing process based on the resistance of skin tissue to the maximum load given. Large tensile strength values indicate the number of fibroblasts and collagen synthesis in injured skin tissue (Figure 1). All groups gave significantly different tensile strength results compared to controls (p<0.05) except in the NBF group 1.25%. Increased tensile strength in the incision wound shows that there is an increase in the number of fibroblasts and the rate of collagen synthesis [24]. Collagen fibers are a factor that can affect the tensile strength of a wound [25]. The breakdown of collagen fibers will reduce the ability of the skin to withstand the pulling load, so the tensile strength decreases [26]. The greater the amount of collagen, the greater the value of tensile strength in the skin tissue [27-29].



\* p<0.05 when treated group compared with control group

Fig. 1. Effect of treatment on tensile strength on rats

### 3.5 Histopathological study

The results obtained from histopathological observation of skin tissue showed an improvement in re-epithelialization, neocapillarization and fibroblast formation at all doses of the EAF, NBF, and reference groups compared to controls (Figure 2). Improvement of skin structure (epithelialization) in the treatment group (EAF and NBF) was better compared to the control group. Re-epithelialization is a stage of wound repair that includes mobilization, migration, mitosis, and epithelial cell differentiation. These stages will restore lost skin integrity [17]. Re-epithelialization begins several hours after injuries. Epidermal cells from the wound will proliferate from the inner edge of the wound and eventually form a barrier that covers the surface of the wound so as to prevent the entry of microorganisms [30]. Increased epithelial strength occurs significantly in the third to sixth weeks after injury [31].

Neocapillarization is the formation of new blood vessels in the form of shoots that are formed from blood vessels and will develop into new branching in wound tissue. Neocapillarization will be anastomosed to each other and form a dense blood circulation network in the wound tissue [32]. Blood vessels have an important role in tissue repair to provide nutritional intake for tissues that are regenerating. Blood vessels also deliver inflammatory cells formed in the bone marrow to approach the injured tissue until the inflammatory cell emigrates [33]. The number of neocapillary formed began to decrease in the EAF 1.0%, NBF 1.25%, and reference group. This shows that wound healing enters the early maturation phase, where the role of the capillary in providing nutrients for regeneration of cells during the healing period of the wound has begun to decrease. In the EAF 0.75%, EAF 1.25%, NBF 0.75%, NBF 1.0%, and control groups there are still many inflammatory cells and still need nutritional intake which results in long-lasting wound healing.

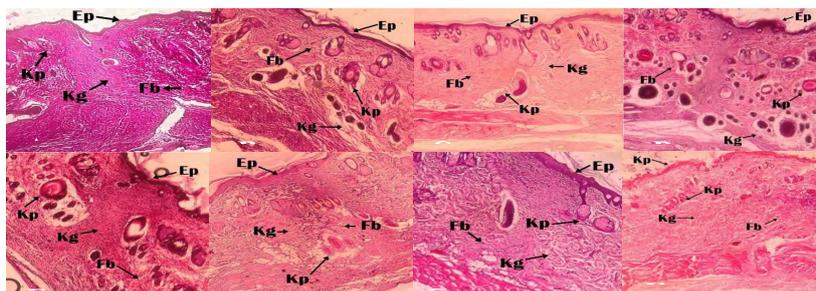


Fig. 2. Histopathology of rat skin after post wounding with different treatment.

A: Control; B: EAF gel (0.75% w/w); C: EAF gel (1.0% w/w); D: EAF gel (1.25% w/w); E: NBF gel (0.75% w/w); F: NBF gel (1.0% w/w); G: NBF gel (1.25% w/w); H: Reference gel. (Ep = re-epithelization, Kg = collagen, Fb = fibroblast dan Kp = neocapillarization).

Fibroblasts play a role in the wound healing process at the proliferation stage. Fibroblast density in NBF 1.25% and the reference group showed that wound contraction was faster. This is because the more connective tissue in the wound, the greater the contraction strength of the wound so that the side of the wound will be attracted and cause the wound to become smaller. More fibroblasts in the treatment group caused epithelialization to occur faster than the control group. Increased fibroblast cells due to chronic inflammation when monocytes enter the network and differentiate into macrophage cells will phagocytose damaged tissue including polymorphonuclear PMN cells that have died and will produce Transforming Growth Factor- $\beta$  (TGF- $\beta$ ) which helps fibroblast proliferation which then digests agents including bacteria in the vacuole [34].

This study showed that the administration of ethyl acetate and n-butanol fraction of *A. rubiginosa* tuberous root showed wound healing activities for incisions. Phytochemical content in EAF (alkaloids, flavonoids, tannins, and saponins) and NBF (alkaloids, flavonoids and tannins) play a role in this activity. Alkaloids have the ability as antibacterial by disrupting the constituent components of peptidoglycan in bacterial cells, so the cell wall layer is not formed completely and causes bacterial cell death [35]. In addition to antibacterial effects, flavonoids have anti-inflammatory activity by stimulating cells such as macrophages to produce growth factors and cytokines such as Epidermal Growth Factor (EGF), Transforming Growth Factor- $\beta$  (TGF- $\beta$ ), interleukin-1 (IL-1), interleukin -4 (IL-4), and interleukin-8 (IL-8) [36]. Tannin compounds also play a role in the healing process of wounds because they are useful as astringents [31]. Astringent is a fastening material that has the power to contract and shrink the skin tissue, so that bleeding in the wound can stop quickly, and the wound dries faster [37]. Saponins will inhibit the production of excessive wound tissue and act as a cleanser and antiseptic which functions to kill or prevent the growth of microorganisms that arise in the wound so that the wound does not have a severe infection [38-39].

From histopathological study it can be concluded that the healing phase of NBF 1.25% and reference group has entered the remodeling phase because the number of blood vessels is reduced and collagen maturation has occurred. The control group just started to enter the proliferation phase because there have been many new blood vessels formed but epithelial tissue has not yet formed. While in the EAF group (0.75%, 1%, and 1.25%), NBF 0.75%, and NBF 1% healing phase has reached the final phase of proliferation and will enter the remodeling phase because the number of blood vessels has been reduced and the tissue epithelium has begun to form.

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