

EVALUATION OF ANTIOXIDANT, ANTIMICROBIAL AND WOUND HEALING ACTIVITY OF *POIKILOSPERMUM SUAVEOLENS*

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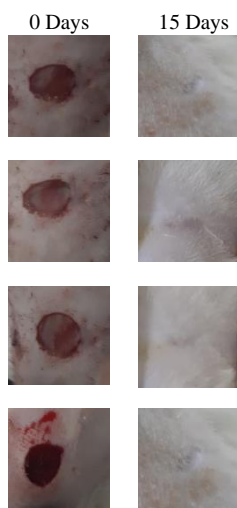
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Graphical abstract



Abstract

Poikilospermum suaveolens is a plant could grows in the limestone area in Maros, Indonesia. The leaves are generally widely used as traditional medicine for treatment of wounds by local communities. This study aims to examine the wound healing potential of *P. suaveolens* leaves extract as well as scientific clarify of the plant. The plant leaves were extracted by maceration. The measurement of antioxidant activity by the DPPH technique (*1,1 diphenil-2-pikrilhidrazil*) spectrophotometrically. The antimicrobial activity was evaluated by formation of the inhibition zone. The wound healing activity was performed the percentage of injury closure in rats skin injury. The antioxidant activity showed that 70% ethanol and ethyl acetate extracts were not significantly different, however, highly distinct to the positive control (*Butylated Hidroxyanisole*). The antimicrobial activity of extract showed 70% ethanol and ethyl acetate extracts were not significantly against *Staphylococcus aureus* and *Candida albicans*, but significantly against *Pseudomonas aeruginosa*. The evaluation of *P. suaveolens* extract on the rate of wound closure activity quickly, i.e., within 15 days the injury on the test animal was recovered. This study concludes that the *P. suaveolens* plant extracts are potential as a sources of active substances for wound healing.

Keywords: Antimicrobial, Antioxidant, wound healing activity, *Poikilospermum suaveolens*

Abstrak

Poikilospermum suaveolens adalah sejenis tumbuhan yang boleh tumbuh di Kawasan batu kapur di Maros, Indonesia. Daun ini biasanya digunakan secara meluas sebagai ubat tradisional untuk rawatan luka oleh masyarakat tempatan. Kajian ini bertujuan untuk mengkaji potensi penyembuhan luka dari ekstrak *P. suaveolens* serta penjelasan santifik tumbuhan. Daun *P. suaveolens* diekstrak dengan maserasi. Ukuran aktiviti antioksidan mengikut teknik DPPH (*1,1 diphenil-2-pikrilhidrazil*) secara spektrofotometrik. Aktiviti antimikrob telah dinilai oleh pembentukan zon perencatan. Aktiviti penyembuhan luka telah dilakukan peratusan penutupan kecederaan pada kulit tikus. Aktiviti antioksidan menunjukkan bahawa 70% ekstrak etanol dan etil asetat tidak jauh berbeza, bagaimanapun, sangat berbeza dengan kawalan positif (*Butylated Hidroxyanisole*). Aktiviti antimicrobe pada 70% ekstrak etanol dan etil asetat tidak berbeza secure significant terhadap *Staphylococcus aureus* dan *Candida albicans*,

bagaimanapun, bervariasi terhadap *Pseudomonas aeruginosa*. Penilaian ekstrak *P. suaveolens* pada kadar aktiviti penutupan luka dengan cepat, iaitu dalam masa 15 hari kecederaan pada haiwan ujian telah pulih. Kajian ini menunjukkan bahawa ekstrak tumbuhan *P. suaveolens* berpotensi sebagai sumber bahan aktif untuk penyembuhan luka.

Kata kunci: Antimikrob, Antioksidan, Aktiviti penyembuhan luka, *Poikilospermum suaveolens*

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1.0 INTRODUCTION

The wound healing process is quite complex in that occur the reunites of the anatomical structure of the body's tissues. As a biological process which normally occurs in human body, the four phases respective distinct, namely hemostatis, inflammation, proliferation, and maturation [1]. Wound healing successfully occurs when all the four phases manifested in the right order and time. There are several factors could interfere with one or more phases of this process, cause the wound healing failure. According to Sanchez et al. (2018) [2], the factors affecting wound healing process is oxidative stress, aging, and poor health, such as diabetes infection, smoking, excessive alcohol intake, and impaired nutritional status.

An important phase regulator in the wound healing process is the Reactive Oxygen Species. The low ROS concentrations necessary to regulate the signal transmission for cell survival as well as against invasive microorganisms [2], [3]. In contrast, high concentrations of ROS actually increase oxidative stress, causing wound healing slowly. The uncontrolled excessive oxidative stress leads to prolongation and breakdown of the inflammatory process. Therefore, the elimination of ROS could occur an accelerated healing process, especially in cases of chronic wounds [3].

The healing process is also affected by microbial infection in the wound area. There are several studies have reported that bacteria invade many cases of chronic wounds such as *Staphylococcus*, *Streptococcus*, *Corynebacterium*, and *Pseudomonas* [4]. Microbial infection causes delayed in the wound recovery process through the formation of persistent inflammatory mediators, continuous neutrophil activation, reduction of fibroblasts, and competition with host cells for nutrients and oxygen. There are certain pathogenic bacteria tend to protect themselves through the biofilm formation mechanism as a defense mechanism [5]. *Pseudomonas aeruginosa* is one of the pathogenic bacteria plays main role in inhibiting the chronic wound healing process through the mechanism of biofilm-producing [6]. Besides *P. aeruginosa*, *Staphylococcus* is also known to contribute in bacterial wound infections. The utilization of extracts or plant-derived compounds in the treatment and management of

wounds is well documented. As reported by Farahpour and Habibi (2012) [7], some traditional medicinal plant derived from plants which known the beneficial to injury healing. *Poikilospermum suaveolens* is one of the tradisional medicinal plants used as wound healing, known as Indonesian local name, Mentawan.

P. suaveolens is an epiphytic plant (liana), growing in areas an altitude ranges from 500-600 m above sea level, humid places, tropical rain forests, monsoon forests, rivers area. Native distribution were found in various countries such as Malaysia, Philippines, India, Kalimantan, Vietnam, Thailand, and Indonesia [8]

Ethnobotany and ethnopharmacology studies reveal that various communities uses this plant as an ingredient for traditional medicine such as the treatment of muscle pain and aches caused by flatulence [9], asthma and cough [10], diarrhea and gastritis [11], menstruation, numbness and cervical cancer [12], eye pain, fever, malaria, and itching [13], syphilis [14], breast cancer [15], and scabies [16]. [13], syphilis [14], breast cancer [15], and scabies [16]. This plant grows adaptable to limestone areas in the Maros region, South Sulawesi, Indonesia. Local communities are widely used the plants leaves in a traditional medicine for wound healing

Scientifically, the compounds component in these plant that show effects in the wound healing process are not yet known. The purpose of this study was carried out to support the scientific validation on utilizing empirical medicine treatment this plant by community.

2.0 METHODOLOGY

2.1 Plant Materials

P. suaveolens leaves were collected from native habitats in the Karst Maros Region of South Sulawesi. The samples were identified by Indonesian Institute of Sciences (LIPI), Indonesia. The leaves were washed thoroughly, then drained and weighed 1000 g of wet weight. Furthermore, it were chopped into small pieces and dried in an oven at 40°C (\pm 10% moisture content). The dry sample was weighed and blended to making powder (60 mesh) [17].

2.2 Extraction Procedure

The extraction process was carried out using the maceration method [18]. The samples were mashed into powder weighed 150 grams, then macerated with 70% ethanol and ethylacetate as much as 482 mL for 24 hours. Furthermore, the filtrate was concentrated using vacuum rotary evaporator, to find extracts. The ethanol and ethylacetate extracts were labeled ET and EA, respectively.

2.3 Determination of Phenolic Content

The total phenol content of extract was measured using the *Folin-Ciocalteu* reagent method. The extract was diluted by weighing the sample approximately 0.05 grams, distilled water (H₂O) was added. An aliquote of 20 µl sample was transferred into each test tube, then was added 1.58 ml of H₂O and 100 µl of *Folin-Ciocalteu* reagent, let it for 7 mins. After this, 300 µl of sodium carbonate (Na₂CO₃) was added, plates were stored for 30 mins in a dark room, the absorbance was recorded using a spectrophotometer at 765 nm wavelength. The experiment was performed thrice replication. The total phenolic compounds of the extract were expressed as milligrams in gallic acid equivalent per gram of the dry weight extract (mg GAE/g). The content of phenolic compounds in sample extract was calculated by the formula:

$$T = \frac{C \cdot V}{M} \quad (1)$$

Where:

T : Total phenolic content (mg/g)

C: The gallic acid concentration (mg/ml)

V : The volume of the leaf extract (ml)

M : The weight of the leaf extract (mg)

2.4 Antioxidant Activity

The determination of the antioxidant activity of ethanol and ethylacetate extract was carried out by transferred each of 77 µl of ethyl acetate and ethanol extract into a test tube, then aliquots 3 ml of DPPH solution was added, homogenizing, and incubated for 30 mins in a dark room. The antioxidant activity was performed by a spectrophotometer at 517 nm of wavelength.

The chemical substances standard, BHA (*Butylated Hidroxyanisole*) approximately 0.025 g diluted in 1 ml of metanol, whereas 1 ml of methanol was used as a blank. The antioxidant activity was performed in the same prosedure as desribed previously. All experiment were carried out in thrice replication. The free radical scavenging activity of extracts were calculated by the formula as follow [19]:

$$\text{DPPH radical (\%)} = \frac{A_{\text{control}} - A_{\text{Sample}}}{A_{\text{Control}}} \times 100 \quad (2)$$

Where A_{control} is the absorbance of control and A_{sample} is the absorbance of extracts sample.

2.5 Antimicrobial Activity

The antimicrobes activity of plant extracts were evaluated using the agar well diffusion methods. Each of Nutrient agar (NA) and Potato dextrose agar (PDA) plates were inoculated *Pseudomonas aeruginosa* and *Candida albicans* by spreading 0.1 ml of 10⁶ cfu/ml tested microbes culture with sterile swab cotton, respectively. Well with 6 mm diameter were cut on the plates surface by using a sterile cork borer. Fifty microliter of 50 mg/ml of each ethanol 70% extract (EA) and ethylacetate extract (EA) was placed into the well. The wells were filled with DMSO 10% and chloramphenicol 100ug/ml were used as a negative and positive control, respectively. All plates were incubated at 30°C for 48 h. All experiment were carried out thrice replication.

2.6 Wound Healing Evaluation

Healthy male albino wistar rats weighing (200-250 g) were selected for the in vivo study. Test animals were given feed and water ad libitum, then acclimatized for 7 days at the UNM Biology Laboratory before the treatment was applied. The rats were anesthetized with ketamine subcutaneously at a dose of 120 mg/Kg. The study was approved by the ethics committee for animal experiment, Veterinary department, faculty of medicine, Hasanuddin University.

The sample ointment are prepared according to the formula as follows: the basic ingredients of the base gel composition consist of 5% Carbopol, 10% Glycerin, 5% propylene glycol, 0.5% triethanolamine, and 69.5% water. All ingredients of the ointment base were mixed and heated gently in a waterbath (50°C) with stirring until homogenous, and then mixed until cooled. After that, the formula was homogenized using a homogenizer for 10 minutes at 1500 rpm. For preparing extract ointment formula, the *P.suaveolens* extract was taken and mixed with the base gel to find the 10% and 15% of final extract concentration. Subsequently, the dorso-costal area of each rat was shaved and the excision wound was performed using a 6 mm sharp dermal biopsy punch. The animals were divided randomly into four groups, each consisting of 6 animal. Group 1 was treated with 0.5 g ointment base and it used as a negative control; group 2 was treated with 5% povidone iodine as a positive control; Groups 3 and 4 were treated with an ointment formula containing active ingredients of EA and ET.

The treatment was applied immediately after the skin of the test animals are injured, treatment was carried out in every 24 hours for 15 days. All animal wounds were subjected to the same treatment throughout the experiment. The wound size area was measured after giving injury treatment (day 0) and wound measurements were carried out on days 3, 5, 9, 13, and 15. The percentage of wound closure was calculated using the following formula [20].

Wound closure (%) =

$$\frac{\text{initial wound size} - \text{specific day wound size}}{\text{Initial wound size}} \times 100 \quad (3)$$

2.7 Histology Analysis

On the scar tissue, a biopsy was performed from each group of mice on the 15th day. The skin was then washed with physiological NaCl and placed in 10% formalin for 48 hours. The tissue was dehydrated with the solutions of 70%, 80%, and 95%, namely absolute ethanol, xylol, and blocked paraffin fat, respectively. The skin tissue was cut with a 5 µm thick microtome, then placed on a slide, stained using hematoxylin-eosin and microscopically was observed.

2.8 Data Analysis

Data were analysed by ANOVA ($\alpha = 0.05$), while the Tukey's further test was performed to determine the significantly between the treatments. This significance was based on the p value of <0.05 .

3.0 RESULTS AND DISCUSSION

3.1 Extract Rendament Yield

The plant leaves samples were extracted by maceration method using 2 types of solvents, namely 70% ethanol and ethylacetate. The results showed that the 70% ethanol solvent was recovered the extract yield of 21.69%, while the ethylacetate solvent is 5.93%. These results show that the extraction by 70% ethanol was yielded more than ethylacetate. This finding reveal that the ethanol more suitable solvent for improved yield extract. It may be influenced by the polarity of extract component and solvent in sample.

3.2 The Determination of The Total Phenolic Content

The total phenolic content of ethanol and ethyl acetate extracts showed that the total phenolic content in the ethanol extract (1.54 mg GAE / g) was higher than ethylacetate (0.86 mg GAE/g) (Figure 1).

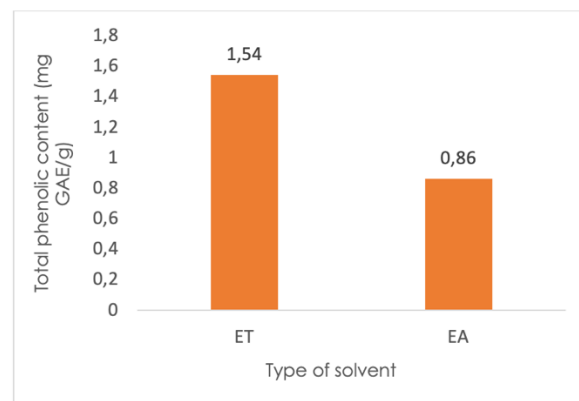


Figure 1 Total of phenolic content of 70% ethanol extract and ethylacetate *P. suaveolens*

Phenol is one of the secondary metabolite with the potential as antioxidant activity. This is due to the presence of hydroxyl group in phenol compounds. The hydroxyl group acts as a contributor to the hydrogen atom to bind with radical compounds, namely through the electron transfer to inhibit the oxidation process. Phenolic compounds are well known as antitumor, antibiotic, antiviral and antioxidant activities [21].

3.3 Antioxidant Activity

The determination of antioxidant activity was carried out using the DPPH method. This is a fast, simple and inexpensive technique for measuring the antioxidant capacity of food or plant extract compounds. Also, it involves the use of free radicals, 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) which is widely used to test the ability of compounds that act as free radical scavengers or hydrogen donors [22].

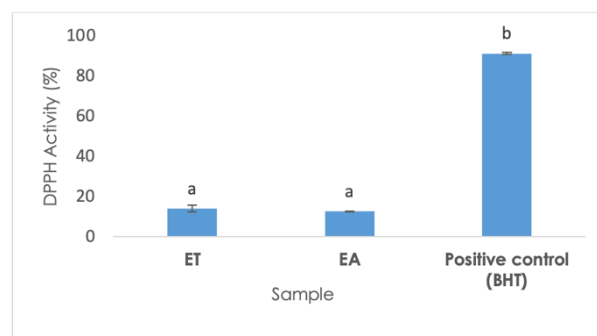


Figure 2 Percentage of DPPH activity

The antioxidant activity of ET and EA extracts showed that the percentage of free radical scavenging activity of the two types of extracts, namely ET (13.98%) and EA (12.62%) was not significantly. However, it was not significantly when compared to BHA as a positive control (91,16). %

(Figure 2). The high percentage of ET and EA treatments were compared to the negative control occurred due to the activity of metabolites content in the extracts. Phenol as a secondary metabolites found in the plant extracts play an important role in reducing ROS levels, so that free radicals can be eliminated. Phenolic compounds are reported exhibit as antioxidant activity in both in vivo and in vitro. It is assumed that the antioxidant capacity of phenolics increases with the increase in the amount of free hydroxyl and side chain conjugation of the aromatic ring [23].

The low antioxidant activity of 70% ethanol and ethyl acetate extracts compared to BHA was due to the low concentration and purity of the active compounds in the extracts. BHA is a synthetic compound for antioxidant purposes. According to Seliha *et al.* (2018) [24] that BHA is a monophenol antioxidant could soluble in fat or oil. BHA has an important mechanism in scavenging free radicals in cells through reduces ROS values. This mechanism affects proteins as a target molecules in cells. However, the presence of antioxidant activity of both extracts indicated their antioxidant potential compared to the negative control.

Phenolic compounds are contains one or more hydroxyl groups, which bind to electronegative (O, N) atoms in the peptides and proteins by hydrogen bonding. The OH-groups present in phenolic compounds partially dissociate into the negatively charged ions under favorable physiological conditions [25]. These negatively charged OH groups readily form ionic bonds with positively charged amino acid residues (such as lysine, arginine). When polyphenols formed multiple hydrogen and ionic bonds with the binding or catalytic site of a protein, the structural and functional flexibility of the targeted protein was reduced. Similar to the secondary metabolite situation and reactive functional groups forming covalent bonds with proteins. The modulation of transcription factors by phenolics, indirectly influence gene regulation. Therefore, these compounds carry out their biological activity [26].

3.4 Antimicrobial Activity

The activity of the extract as an antimicrobial was indicated by the presence of a clear zone, or the diameter of the inhibition area that was formed around the well. The diameter of the inhibition zone of each extract was shown in Table 1.

The antimicrobial activity of extracts were indicated by the formation of an inhibitory zone of all test microbes. The ET extract with a concentration of 15% showed the highest antimicrobial activity against *S. aureus* and *C. albicans*. While the EA extract with a concentration of 15% showed the highest antimicrobial activity against *P. aeruginosa*. These results indicate that both extracts were not significantly inhibit the microbial growth of *S. aureus* and *C. albicans*, but significant effect on inhibit

growth of *P. aeruginosa* bacteria, especially the ethylacetate extract at the 10% and 15% concentrations.

Table 1 Diameter of the inhibition zone of ET and EA samples against *S. aureus*, *P. aeruginosa*, and *C. albicans*

Sample	Percentage	Diameter of the inhibition zone (mm)		
		<i>S. aureus</i>	<i>P. aeruginosa</i>	<i>C. albicans</i>
ET	5%	8,72 ± 0,47 ^a	8,00 ± 0,00 ^a	8,00 ± 0,00 ^a
	10%	8,98 ± 0,40 ^a	8,00 ± 0,00 ^a	8,69 ± 1,19 ^a
	15%	8,98 ± 0,37 ^a	8,00 ± 0,00 ^a	9,04 ± 1,80 ^a
EA	5%	8,00 ± 0,00 ^a	8,14 ± 0,24 ^a	8,00 ± 0,00 ^a
	10%	8,00 ± 0,00 ^a	9,34 ± 0,18 ^b	8,00 ± 0,00 ^a
	15%	8,00 ± 0,00 ^a	9,98 ± 0,20 ^b	8,00 ± 0,00 ^a
Negative control		8,00 ± 0,00 ^a	8,00 ± 0,00 ^a	8,00 ± 0,00 ^a
Positive control		14,99±1,92 ^b	16,28±0,98 ^c	22,61±0,21 ^b

The same letter shows the results that are "not significantly different" based on Tukey's test with a confidence level of $\alpha = 0.05$

According to Compean & Ynalvez (2014) [27] that the varying of different component of plant extract may cause the antimicrobials differences activity due to diverse secondary metabolite compounds.

3.5 Wound Healing Activity

Wound excision was performed to evaluate the healing activity of the treatment group (ET and EA) and the control group. The wound closure activity of the plant extract was shown in Figure 3, 4 and 5.

Wound healing is a normal biological process that occurs in four precise and highly programmed phases [28]. The mechanism of this process is a complex repairing, in which an inflammatory response occurs leads to the formation of ROS, a detrimental effects. In addition, infection with *S. aureus* and *P. aeruginosa*, delays the inflammatory phase and disrupts the normal clotting system, thereby affect the slowing angiogenesis. The wound healing effect is enhanced through the role of plant extracts which is attributed to its antioxidant and antimicrobial properties. A positive role are show between the free radical scavenging action and the wound healing process [29]. In this study, ET and EA extracts of *P. suaveolens* were shown the effect of the antioxidant and antibacterial activity in vitro.

The experimental results reveal on the 3rd day of treatment show a significant in both the treatment group and the control group. However, the 5th, 9th, 13th, and 15th days show no significant. The average percentage of wound closure was not significantly in the treatment and control groups. However, ET and EA treatment has a good effect towards the wound healing process

On the 15th day of the experimental period the percentage of wound healing reached 100%, this result was show the higher compared the positive and negative controls. The highest percentage of wound healing on days 3 to 15 is 10% EA

concentration. As reported by Firdous & Sautya (2018) [30], plants as a source of wound healing potential which to be developed as new drugs for humans. Medicinal plants have been reported used as wound healing potential through angiogenesis, nf-kb activation, pro-inflammatory cytokine support, increased expression of inducible nitric oxide synthase (inoS), collagen alpha 1 type 1, and antioxidant activity.

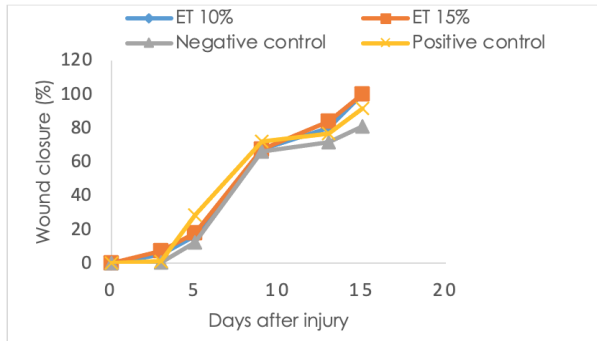


Figure 3 Wound closure of ET Treatment on days 3, 5, 9, 13, and 15

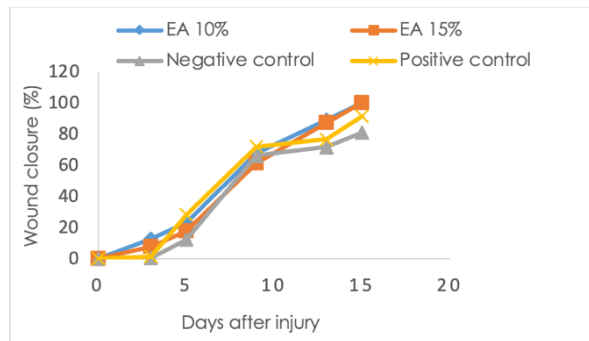


Figure 4 Wound closure of EA treatment on days 3, 5, 9, 13, and 15

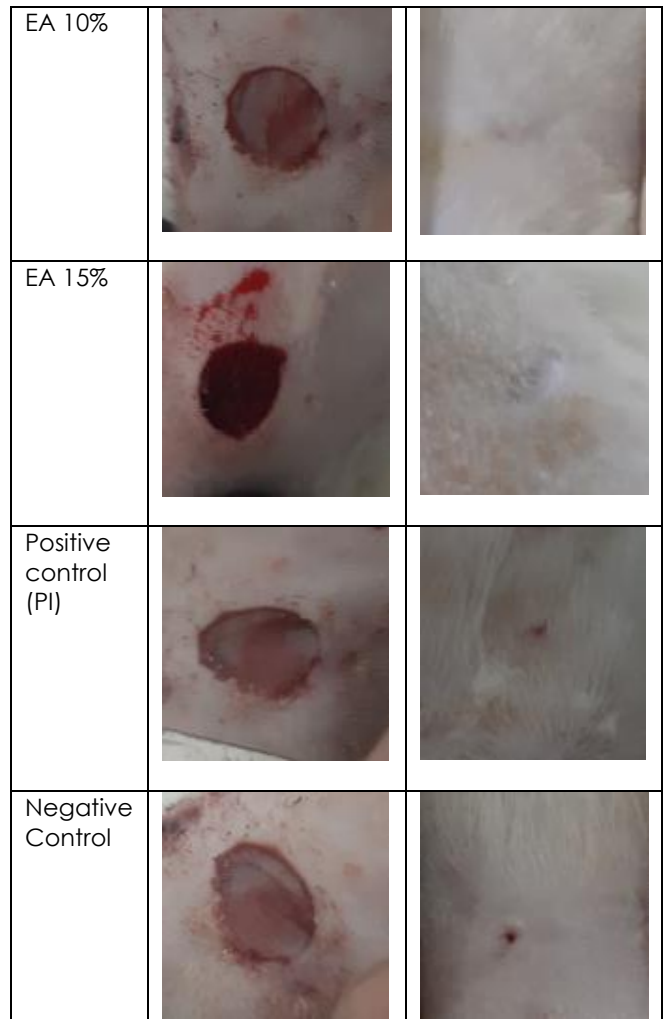


Figure 5 Morphology wound closure activity of *P. suaveolens* extract at days 0 and 15

Sample	0 Days	15 Days
ET 10%		
ET 15%		

The rate of wound healing in the ET and EA treatments was higher compared with the control group. It is caused by the active compounds in extract such as flavonoids, tannins, triterpenoids, and saponins. The study was reported by Bouassida *et al.*, (2017) [31] concluded that the presence of tannins, polyphenols, and flavonoids in *Urtica dioica* extract synergistically enhances angiogenesis. It was also reported that polyphenols regulate in vivo angiogenesis and epithelial cell proliferation [32]. This results are supported by a study conducted by Hartati *et al.* (2018) [20] that the rate of wound healing quickly in the animal wound treated leaf extract of *C. kujete*. The extract was reported contain active compounds, namely alkaloids, flavonoids, tannins, and saponins. In addition, these compounds are reported to inhibit bacterial growth, anti-inflammatory and antioxidant. According to Dunnill *et al.* (2017) [33] that the normal physiology of the wound healing process depends on low levels of reactive oxygen species (ROS) and oxidative stress.

3.6 Histological Analysis

The histological analysis of the wound healing of rat skin on the 15th day, shown in Figure 6. The results showed that treatment with 10% ET extract and 15% ET extract caused skin regeneration, while the 10% EA and 15% EA samples showed necrosis on positive and negative controls.

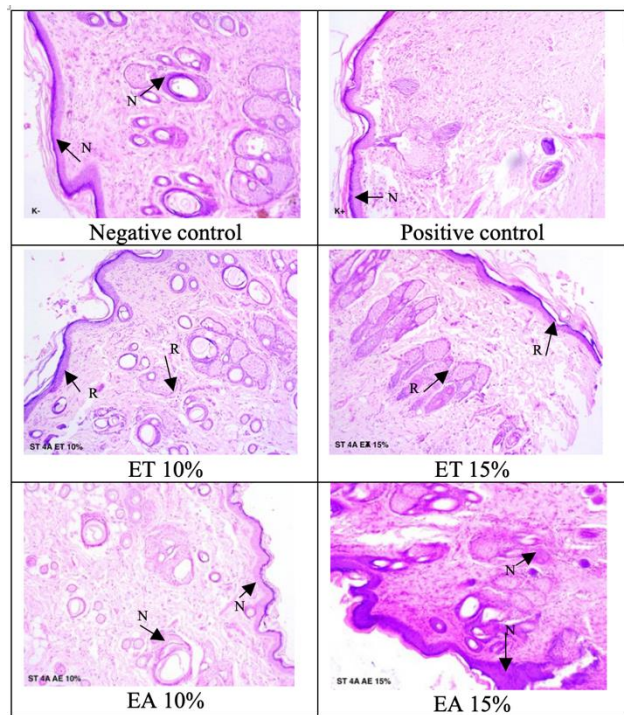


Figure 6 Representative images of histological sections of the six experimental groups in day 15, Necrotic (N), Regeneration (R), magnification x100

Skin regeneration of wound healing process based on histological analysis was observed in the 10% and 15% ET treatment groups (Figure 6). These results indicate the occurrence of regeneration of epidermal cells and hair follicles, inflammatory cells, moderate amounts of macrophages and hyaline. The process of epidermal regeneration is influenced by the production of keratinocytes which actively interact with fibroblasts, endothelial cells and immune cells in the wound [34].

4.0 CONCLUSION

P. suaveolens leaves 70% ethanol extract were recovered show higher phenolic content than the ethyl acetate isolate. Both extracts were able to inhibit the growth of *S. aureus*, *P. aeruginosa*, and *C. albicans*. The antioxidant and antimicrobial activity of the extract could have implications for wound healing in test animals. Treatment by application of the plant

extract showed the ability to heal wound towards test animals within 15 days

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