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Activity of Karehau (*Callicarpa longifolia* Lamk.) Leaves Ethanolic Extract as a Wound Healing

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Abstract

Wound is a condition where there is damage to normal tissue structure and function. This tissue damage may be accompanied by infection or not. Wound can be treated with the help of medicines. The Dayak Tunjung community in East Kalimantan has been used karehau leaves as a traditional medicine to treat wounds. The aim of this research was to evaluate the antibacterial and wound healing activity of karehau *(Callicarpa longifolia* Lamk.) leaves ethanolic extract and its gel preparation.This research was divided into several stages, that was an in vitro and in vivo anti-inflammatory activity evaluation, followed by antimicrobial activity evaluation, and lastly the wound healing activity evaluation on rabbit. The results showed that ethanol extract of kerehau leaf (*Callicarpa longifolia* Lamk.) had antibacterial activity with MCI 64 µg / mL in *P. aeruginosa* and *S. Aureus.* Whereas in bacterial infected wounds, there was a decrease in erythema score on day 9 and decreased pus on day 6. Ethanol extract of kerehau leaves gel with concentration of 5%, 10%, 20% had significant difference with negative control in wound healing. This preparation with concentration of 5%, 10%, 20% showed wound healing activity on days 10, 9, 7; respectively. It was concluded that the ethanol extract of kerehau leaves had the antibacterial and wound healing activity.

Keywords: *Callicarpa longifolia* Lamk., Kerehau leaves, antibacterial activity, wound healing activity

INTRODUCTION

Wounds are defined as a missing or damaged part of body tissues that can be caused by sharp or blunt object trauma, temperature changes, chemicals, explosions, electric shock or animal bites [1]. The recovery process that then occurs on the damaged tissue is called the wound healing process. The wound healing process begins since tissue damage occurs, but the healing rate is very slow and allows for microbial infection [2]. In order to avoid infection, the wound need to be treated by stopping the bleeding, local anesthesia, antiseptic administration around the wound, covering the wound with a sterile cloth, and cleaning the wound to remove the foreign object. Prevention of wound infection will affect the wound healing process [3].

One of the plants commonly used by Dayak Tunjung in Borneo Island, Indonesia is kerehau (*Callicarpa longifolia* Lamk.). Empirically, kerehau leaves are used as a medicine to treat swelling, as a mixture in the manufacture of powdery or cold powder to remove acne scars on the face, postpartum treatment to treat perineal lesions (the tear in the normal birth canal), and roots of kerehau used as medicine stomach pain or diarrheal medication [4].

Based on previous research conducted by Setyowati (2010), showed that kerehau plant had the activity as effective antiinflammatory agent with dose of 250 mg/kg bw because kerehau leaves contain secondary metabolite that was alkaloids, flavonoids, tannins and saponins groups [5]. The bacteria that could cause skin infections was the bacteria *Pseudomonas aeruginosa* and *Staphylococus aureus* and also a major bacteria in nosocomial infections [6].

Due to the fact that many products in the form of synthetic were very rare in the form of herbs, this needs to be considered for a new dosage form of the nature of the ingredients [7]. Based on the description above, it encouraged researchers to utilize the leaves of kerehau as an antibacterial and wound healing agent which was formulated in gel preparation form.

MATERIALS AND METHODS

Collection of Plant Material: The plant used in this research was kerehau leaves (*Callicarpa longifolia* Lamk.), obtained in Muara Muntai area, Kutai Kartanegara regency, East Borneo, Indonesia. Based on the results of plant determination and authentication in Mulawarman University, Samarinda, showed that the leaves of kerehau used was *Callicarpa longifolia* Lamk, belonged to the Callicarpa genus and Verbenaceae family.

Preparation of Ethanolic Extract of *Callicarpa longifolia*: As much as 1.2 kg of dried kerehau leaves were macerated using 96% ethanol solvent (1:10). Maserate in the form of liquid extract was then concentrated with a rotary vaporator at a temperature of 40-45°C.

Preparation of Gel Extract of *Callicarpa longifolia*: To increase the effectiveness of using kerehau leaf extract on wound healing, gel preparation was made. This dossage form sometimes called jelly, a semisolid system consists of a suspension made of small inorganic particles or large organic molecules, penetrated by a liquid [8]. The gel preparation was chosen because it was well-dispersed in the skin, had the cold effect, no physiological inhibition of physiological function, ease of washing with water, and suitable for drug release [9].
The gel formula used was as follows:

Table 1

Materials	Function	Concentration (%) b/v			
		NC	KLEG 5%	KLEG 10%	KLEG 20%
Kerehau leaves extract	Active agent	-	5	10	20
Carbopol	*Gelling agent*	2	2	2	2
TEA	pH stabilizer	1	1	1	1
Prophilenglicol	Humectan	10	10	10	10
Glycerin	Humectan	2	2	2	2
DMDM Hydantoin	Preservative	0,6	0,6	0,6	0,6
Aquadest ad	Solvent	100	100	100	100

NC : negative control
KLEG 5% : Kerehau leaves extract gel 5%
KLEG 10% : Kerehau leaves extract gel 10%
KLEG 20% : Kerehau leaves extract gel 20%

All ingredients to be used were weighed according to the weight listed on the formula. The gel preparation begin by mixing the carbopol in aquadest until homogeneous. Then propylenglikol, glycerine and triethanolamine (TEA) were added to form a clear and fluffy gel. To the mixture was added DMDM Hydantoin. After the gel base was formed then kerehau leaf extract was added and mixed until homogeneous. The gel preparation of kerehau leaves extract was stored in a sealed container [10].

Bacteria: Bacteria used in this research were *Pseudomonas aeruginosa* and *Staphylococcus aureus* from Bandung School of Pharmacy. The bacteria were suspended into a liquid media and incubated 18-24 hours at 37°C. The suspension was homogenized and turbidity adjusted to a standard of 0.5 MCF turbidity. The turbidity standard of 0.5 MCF was similar to containing 108 CFU / mL of bacteria, resulting in absorbance of 0.08 to 0.10 at 625 nm wavelengths [11].

Animals: Animal used was New Zealand White strain rabbit (*Oryctolagus cuniculus*) with a weight of 1.5-2.5 kg, 3-4 months old, from Lembang Bandung. Prior to the study, rabbits were acclimatized / adapted in the study room for one week, with the relative temperature and humidity of the cage to be considered. The animals used were healthy animals, ie test animals whose weight did not decrease by more than 10% during the preparation period and visually demonstrated normal behavior.

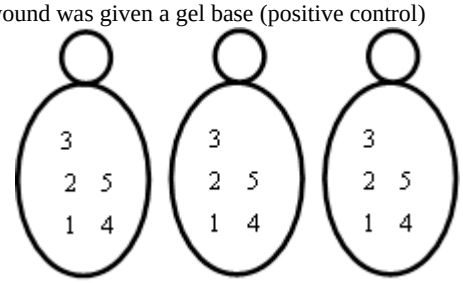
Antibacterial Activity: Evaluation of antibacterial activity using microdilution method was carried out by entering 100 µL Mueller Hinton Broth (MHB) media in the first column as a negative control. Then, a 5 µL bacterial suspension was added to 10 mL Mueller Hinton Broth (MHB) then homogenized with a vortex. A total of 100 µL of the mixture was inserted into the microplate in the second column until the twelfth. In the 12th column, 100 µL of the extract solution was then homogenized. As many as 100 µL transferred to the 11th column. Dilution continues until the 3rd column. Microplate was incubated for at 37 ° C for 18-24 hours. Then it was observed that the clear plate section showed no bacterial growth [12].

Infected wound healing evaluation: The rabbit was adapted for one week before the wound was made. Three male rabbits 2-3 months old and 2-4 kilograms weight shaved and cleaned with alcohol. Furthermore, the back of the rabbit was slashed along 1.5 cm parallel to the vertebrae (vertebrate os) with a wound depth of ± 0.3 cm. To wound the rabbit's backs, a sterile scalpel was used and for the measurement of the length of the wound was used a calipers[13].

Suspension of *Stapylococcus aureus* and *Pseudomonas aeruginosa* were injected subcutaneously by 0.25 mL at each site on the wounded rabbit's back. The observed parameter was erythema after 24 hours and the extract was administrated after 48 hours in the infected area. The ethanol extract of kerehau leaves was applied to 3 locations on the left for *Stapylococcus aureus* bacteria suspension, negative control and positive control; while for right rabbit's back for *Pseudomonas aeruginosa* bacteria suspension, negative and positive control. The injection site was covered with sterile gauze to prevent bacterial contamination. The extract was administrated every day until pus and erythema disappear. The observed parameters was the wound's diameter on the rabbit's back.

Wound healing evaluation: The rabbit was adapted for one week before the cut was made. Three male rabbits 2-3 months old and 2-4 kilograms weight shaved and cleaned with alcohol. Furthermore, on the back of the rabbit slashed along 1.5 cm parallel to the vertebrae (vertebrate os) with a wound depth of ± 0.3 cm. To wound the rabbit's backs a sterile scalpel was used and for the measurement of the length of the wound was used a calipers[13].

The wounded rabbits were treated as follows:
1: The wound was given 20% leaf gel extract
2: The wound was given a gel of kerehau leaf extract 10%
3: The wound was given gel of kerehau leaf extract 5%
4: The wound was given prontosan® gel (standard drug)
5: The wound was given a gel base (positive control)



Picture 1 The wound making on the back of a rabbit with three repetitions.

The gel was applied to the back of the wounded rabbit evenly with three times daily application ± 0.1 g.

The observation was done the day after the rabbit was given treatment to see the speed of wound recovery that had been made, the observation was done for 14 days. Observation of wound healing was done by measuring the length of the wound. Wound healing was characterized by wound drying, scab formation, wound closure and new skin and feathers growth around the wound.

RESULTS AND DISCUSSION

Antibacterial Activity Testing

The antibacterial activity test in this research was using microdilution method. In the microdilution method, if the MCI of extract less than 100 µg / mL, the antimicrobial activity considered to be strong, if MCI 100-500 µg / mL then the antimicrobial activity was medium, if MCI 500-1000 µg / mL then the antimicrobial activity was weak, and if MCI more than 1000 µg / mL the sample was considered inactive [14].

The MCI evaluation of the ethanol extract of kerehau leaves on *P. aeruginosa* and *S. aureus* bacteria used sterile microplate for each bacterium. This was to avoid contamination with the other bacteria in one microplate. The media used was Mueller Hinton Broth (MHB) because this media was a specific media in bacterial isolation, promoting the growth of aerobic bacteria or facultative organisms [10]. The results of the microdilution method showed in Table 2 and Table 3.

From table 2 antibacterial activity test of ethanol extract of kerehau leaves to *P. aeruginosa* bacteria using microdilution method showedthe MCI value at 64 ppm. While tetracycline as a standard drug showed the value of MCI at 16 ppm which means that antibacterial activity of kerehau leaf extract was weaker than the standard antibiotics. As for the KBM values was above 512 ppm which means that kerehau leaves extract had weak antibacterial activity.

Table 2. Antibacterial Activity Evaluation Against *Pseudomonas aeruginosa*

	C (-)	C (+)	1 ppm	2 ppm	4 ppm	8 ppm	16 ppm	32 ppm	64 ppm	128 ppm	256 ppm	512 ppm
A	-	+	+	+	+	+	+	+	-	-	-	-
B	-	+	+	+	+	+	+	+	-	-	-	-
C	-	+	+	+	+	+	+	+	-	-	-	-
D	-	+	+	+	+	+	+	+	-	-	-	-
E	-	+	+	+	+	+	-	-	-	-	-	-
F	-	+	+	+	+	+	-	-	-	-	-	-
G	-	+	+	+	+	+	-	-	-	-	-	-
H	-	+	+	+	+	+	-	-	-	-	-	-

C (-) = negative control C (+) = positive control (-) = clear (no bacterial growth)
(+) = murky (bacterial growth) A – D = kerehau leaves extract E – H = Tetrasiklin

Table 3. Antibacterial Activity Evaluation Against *Staphylococcus aureus*

	C (-)	C (+)	1 ppm	2 ppm	4 ppm	8 ppm	16 ppm	32 ppm	64 ppm	128 ppm	256 ppm	512 ppm
A	-	+	+	+	+	+	+	+	-	-	-	-
B	-	+	+	+	+	+	+	+	-	-	-	-
C	-	+	+	+	+	+	+	+	-	-	-	-
D	-	+	+	+	+	+	+	+	-	-	-	-
E	-	+	+	+	+	+	-	-	-	-	-	-
F	-	+	+	+	+	+	-	-	-	-	-	-
G	-	+	+	+	+	+	-	-	-	-	-	-
H	-	+	+	+	+	+	-	-	-	-	-	-

C (-) = negative control C (+) = positive control (-) = clear (no bacterial growth)
(+) = murky (bacterial growth) A – D = kerehau leaves extract E – H = Tetrasiklin

Table 4. Infected wound's length during treatment

Treatment	1	2	3	4	5	6	7	8	9	10	11	12
***S. aureus* (SA)**	2,00±0,00	2,00±0,00	1,99± 0,01	1,91± 0,06	1,82± 0,04	1,72± 0,05	1,62± 0,06	1,5± 0,04	1,42± 0,05	1,32± 0,04	1,16± 0,11	1,09± 0,13
SA+ Extract	2,00±0,00	2,00±0,00	1,97± 0,02	1,83± 0,02	1,74± 0,00	1,63± 0,03	1,54± 0,00	1,44± 0,02	1,35± 0,01	1,23± 0,02	1,13± 0,03	0,93± 0,05
SA + Tetracycline	2,00±0,00	2,00±0,00	1,90± 0,03	1,79± 0,04	1,70± 0,00	1,61± 0,01	1,5± 0,00	1,40± 0,00	1,30± 0,01	1,23± 0,05	1,05± 0,07	0,75± 0,07
***P. aeruginosa*(PA)**	2,00±0,00	2,00±0,00	1,99± 0,01	1,94± 0,06	1,83± 0,04	1,74± 0,05	1,66± 0,08	1,55± 0,11	1,46± 0,11	1,37± 0,16	1,28± 0,12	1,20± 0,09
PA + Extract	2,00±0,00	2,00±0,00	1,96± 0,03	1,86± 0,03	1,77± 0,05	1,66± 0,03	1,56± 0,03	1,48± 0,05	1,36± 0,02	1,27± 0,05	1,15± 0,04	0,94± 0,17
PA + Tetracycline	2,00± 0,00	2,00± 0,00	1,90± 0,00	1,80± 0,01	1,70± 0,01	1,62± 0,03	1,53± 0,03	1,41± 0,02	1,31± 0,01	1,21± 0,01	1,07± 0,06	0,83± 0,05

From the Table 3, the antibacterial activity test of ethanol extract of leaves to *S. aureus* bacteria using microdilution method showed MCI value at 64 ppm, while the tetracycline as a stamdard drug showed the value of MCI at 16 ppm. This means that the antibacterial activity of kerehau leaf had a weaker antibacterial activity compared to standard antibiotics.

Infected Wound Activity Evaluation

Wound healing activity of kerehau leaf extract was observed everyday until the pus and erythema disappeared. Observed parameters were the wound length in each incision on the rabbit's back. As for microscopic observation of bacteria from pus was performed by taking the pus with sterile lid cotton then scratched on VJA and MCA medium. Observation of wound healing by measuring the length of the wound using the calipers. The wound healing on the animals were marked with loss of erythema and pus. The results of the observation could be seen in the Table 4.

Based on the observation of wound length during the experiment, it could be seen that the tetracycline and extract-administered group showed faster healing time compared to the control group for both *S. aureus* and *P. aeruginosa* bacteria, although not statistically significant.

Wound Healing Activity Evaluation

The observation on the wound healing activity of kerehau leaf extract was performed for 14 days with the administration of the extract and standard drug every morning, noon and afternoon as much as 0,1 gram. The observed parameter of wound healing activity was the length of the wound, measured by using a calipers. The wound was healed marked by scab formation, wound closure, and new skin and hairs growth around the wound. The results could be seen in Table 5.

Table 4 showed that gel administration of kerehau leaf extract with concentrations of 5%, 10% and 20% could heal wounds within 10 days, 9 days and 7 days, respectively. While the standard drug (prontosan® gel) could heal within 6 days, and the negative control (base gel) group was healing within 15 days. The treatment group which showed the fastest effect in wound length reduction was standard drug group (prontosan® gel) in which the active ingredient content was polyhexanide, followed by treatment group with gel extract of 20%, 10% and 5% kerehau leaves extract, and the treatment group which gives effect with the longest wound length reduction is in the negative control group (base gel).

Table 5. Wound's length during treatment

At day-	Wound's Length (cm) ± SD				
	Groups				
	Negative control	Standard drug	KLEG5%	KLEG 10%	KLEG 20%
0	1,50±0,00	1,50±0,00	1,50±0,00	1,50±0,00	1,50±0,00
1	1,50±0,00	1,24±0,01	1,40±0,01	1,38±0,01	1,29±0,01
2	1,50±0,00	1,12±0,02	1,32±0,00	1,27±0,00	1,17±0,03
3	1,46±0,01	0,75±0,08	1,27±0,01	1,16±0,00	0,91±0,01
4	1,42±0,03	0,49±0,02	1,18±0,01	1,08±0,01	0,57±0,05
5	1,38±0,02	0,07±0,12	0,99±0,03	0,80±0,01	0,41±0,09
6	1,35±0,03	0,00±0,00	0,84±0,01	0,68±0,00	0,08±0,13
7	1,31±0,02	0,00±0,00	0,52±0,00	0,35±0,06	0,00±0,00
8	1,27±0,02	0,00±0,00	0,31±0,01	0,08±0,14	0,00±0,00
9	1,10±0,05	0,00±0,00	0,05±0,08	0,00±0,00	0,00±0,00
10	1,03±0,05	0,00±0,00	0,00±0,00	0,00±0,00	0,00±0,00
11	0,92±0,04	0,00±0,00	0,00±0,00	0,00±0,00	0,00±0,00
12	0,75±0,00	0,00±0,00	0,00±0,00	0,00±0,00	0,00±0,00
13	0,63±0,05	0,00±0,00	0,00±0,00	0,00±0,00	0,00±0,00
14	0,31±0,01	0,00±0,00	0,00±0,00	0,00±0,00	0,00±0,00

Table 6. Wound healing percentage during treatment

At day-	Wound Healing Percentage (%) ± SD				
	Group				
	Negative Control	**Positive Control**	**KLEG 5%**	**KLEG 10%**	**KLEG20%**
0	0,00±0,00	0,00±0,00	0,00±0,00	0,00±0,00	0,00±0,00
5	8,22±1,68*	95,11±8,47	34,00±2,00*	46,89±1,02*	72,44±6,58*
6	10,22±2,14*	100,00±0,00	43,78±0,77*	54,44±0,38*	94,89±8,85
7	12,89±1,39*	100,00±0,00	65,56±0,38*	76,67±4,06*	100,00±0,00
8	15,33±1,33*	100,00±0,00	79,11±1,02*	94,44±9,62	100,00±0,00
9	26,67±3,71*	100,00±0,00	96,67±5,77	100,00±0,00	100,00±0,00
10	31,33±3,71*	100,00±0,00	100,0±0,00	100,00±0,00	100,00±0,00

The body naturally has the ability to protect and recover itself. The wound healing mechanism will naturally undergo three phases: the inflammatory phase, the poliferation phase and the maturation phase. Begin with the inflammatory phase occurring immediately after the wound and ending 3-4 days where permeability of the cell membrane occurs so that in this phase there will be inflammation, redness, and pain. But with the help of certain treatments can help to accelerate the process of wound healing [15]. The effectivity of wound healing activity of kerehau leaf extract gel was related to the presence of active compounds contained in the extracts. From the phytochemical screening, it was known that karehau leaves ethanolic extract contained flavonoid, saponin, kuinon, tannin, and steroid/ triterpenoid. The active substance that acts as anti-inflammatory to reduce inflammation in the wound was the flavonoid compound [16]. Flavonoid compounds have antibacterial activity by mechanisms to inhibit bacterial cell wall synthesis [17]. Saponins are also substances that can interact with bacterial cells where bacterial walls become lysis. The presence of these substances as antibacterials can prevent the occurrence of infection in the wound so that wound healing can be accelerated.

From the wound length data, it could be calculated wound healing percentage by the formula:

$$P\% = \frac{po - px}{po} \times 100\%$$

P% : wound healing percentage
po : wound length before treatment (cm)
px : wound length after treatment (cm)

To see whether there was a significant difference of wound healing effect or significant difference from five treatments, statistical test using One Way Anova (Analysis of variant) with 95% confidence level. The results obtained from the anova test were p <0.05 indicating that there was a significant difference in each treatment group. To know the significant difference in each treatment group then continued with LSD (Least Significant Different) test. If the value of one treatment to another treatment> 0.05 then there is no significant difference between the treatment with another while if significant value <0.05 then there is a significant difference between the treatment with other treatment.

Based on the result of LSD (Least significant different) on 5th day, thekerehau leaf extract gel with concentration of 5%, 10%, 20% showed a significant difference (p <0,05) when compared with negative control group (base gel), this could be interpreted that the administration of kerehau leaves extract gel at all concentration hadthe wound healing activity. The percentage of wound healing ofextract based gel at concentration of 5%, 10%, 20% were 34,00%, 46,89% and 72,44%,respectively,showed a significant difference (p <0,05) compared to a positive control group (prontosan® gel) (95.11%).It means that on the 5th day there was a difference of percentage of wound healing between 5%, 10%, 20% concentration of the extract gel with positive control (prontosan® gel).

On the day 6th and 7th, 5%, 10%, 20% gel extract showed a significant difference (p <0.05) when compared with the negative control. Administration of 5% and 10% kerehau leaves extracts showed a significant difference (p <0.05) when compared with standard drug (prontosan® gel), whereas in the gel group of 20% kerehau leaf extract there was no significant difference (p> 0 , 05)

when compared to standard drug (prontosan® gel). So it could be concluded on the 6th and 7th days, the kerehau leaves extract gel with a concentration of 20% had the wound healing activity comparable with the standard drug (prontosan® gel).

On day 8, 5%, 10%, 20% gel extract of kerehau leaves showed a significant difference ($p < 0.05$) when compared with negative control. The 5% extract gel group showed a significant difference ($p < 0.05$) when compared with the standard drug group (prontosan® gel), while the 10% and 20% extract gel were not significantly different (p> 0 , 05) when compared to standard drug (prontosan® gel). So it could be concluded that on the 8th day,10% and 20% extract gel administration had the wound healing activity comparable to standard drug (prontosan® gel).

On the 9th and 10th days, 5%, 10%, 20% extract gel showed significant difference ($p < 0.05$) compared to negative control and no significant difference compared with standard drug. From these results it could be seen that the increase in the concentration of extracts in the preparation was proportional to the percentage of wound healing.

CONCLUSSIONS

Based on this research, it could be concluded that karehau leaves ethanol extract had the activity as antibacterial and wound healing. The best wound healing activity was showed by the karehau extract gel with consentration of 20%.

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