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THE EFFECT OF HYDROGEL EXTRACT OF KIRINYUH LEAF ON 2ND DEGREE BURN ON WISTAR RAT WITH REGARD TO ANGIOGENESIS AND FIBROBLAST AMOUNT

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ABSTRACT

Burn injuries continue to pose a significant health concern for society, as they can lead to permanent impairment of body functions and appearance, as well as disrupt daily life, including future employment prospects. This research aims to evaluate the potential of kirinyuh leaf hydrogel extract as a topical therapeutic agent in accelerating the healing of second-degree burn wounds in Wistar rats. The parameters assessed include the expression of VEGF, the quantity of neovascularization, and fibroblast count in the burn wounds. This study utilized an experimental design with a Post-Test Only Control Group, administering 5%, 10%, and 15% kirinyuh leaf hydrogel extract to Wistar rats induced with second-degree burns. Data normality was then analyzed using the Shapiro-Wilk test, followed by a One-Way ANOVA test. The results revealed a significant increase in VEGF expression, neovascularization, and fibroblast count when using kirinyuh leaf hydrogel extract compared to the negative control group, with the most significant effect observed at a concentration of 15% ($p<0.05$).

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1. Introduction

Burns are still a concerning health issue for society, which can have a permanent impact on body function and appearance as well as disrupt daily activities.1 According to WHO, the majority of burn cases occur in developing countries, and almost two-thirds of cases occur in Africa and Southeast Asia. Furthermore, according to the American Burn Association, although the majority of burns have a high survival rate of 96.8%, approximately 486,000 people who suffer burns require medical treatment. The most frequent cause of burns is fire, which occurs at 43%, and most often occurs at home, at 73%. The burns prevalence occurring at home frequently happens due to activities in the house, such as cooking, and most often occur in women. In addition, burns are the fifth cause of non-fatal injuries in children. Burns are divided into three degrees, and one of the most frequent complications of burns is bacterial infection, which can result in the prolonged healing of the wound.

Burn treatment is one of the factors that must be considered in accelerating healing and preventing wound complications. Various studies have been carried out to produce drugs for burn wound therapy, one of which is the use of plants. The use of medicinal plants continues to increase in line with the development of the

traditional and modern pharmaceutical industry, considering the ease of obtaining raw materials, relatively low prices, and relatively fewer side effects.

Kirinyuh (Chromolaena Odorata L.) leaves, a type of plant from the Asteraceae family, contain many chemical compounds, including flavonoids, tannins, and saponins. These compounds have antimicrobial and antiseptic properties, which can help in the wound healing process. Flavonoids can inhibit bleeding by increasing the number of platelets in the wound area, thereby promoting wound healing. Tannin acts as an astringent that can cause skin pores to shrink, stop exudates, and stop light bleeding. These activities can cover wounds and prevent bleeding from appearing in wounds. Meanwhile, saponins work as antimicrobials by disrupting the stability of bacterial cell membranes, causing bacterial cell cycles. Traditionally, kirinyuh leaves are used as a medicine for healing wounds, sore throats, cough, headache, and malaria. They have been traditionally used due to their antimicrobial, astringent, antispasmodic, antihypertensive, antipyretic, and anti-inflammatory activities. The essential oil from the leaves contains α-pinene, cadinene, camphor, limonene, β-caryophyllene, and candinol isomers.

According to Ramdani et al. (2018) kirinyuh leaf extract can promote wound closure more quickly than betadine in rabbit models. In line with this, research conducted by Vijayaraghavan et al. (2017) reported that kirinyuh leaves can heal excision wounds in female Wistar rats in four days; faster than betadine. Meanwhile, for burns, Khairul (2016) found that kirinyuh leaf extract with a concentration of 15% could accelerate the healing of grade 2B wounds in Wistar rats compared to distilled water as reflected by the reduction of the wound diameter, redness, and edema as well as the initial formation of scabs and shedding of the scab. Despite these promising findings, mechanistic studies of kirinyuh leaves are still limited.

Hydrogel is a pharmaceutical dosage form that can be used topically and is ideal to cover wounds due to its ability to remove dead tissue. Hydrogel also possesses a cooling effect on the wound area so that it can alleviate pain and provide comfort to the patients. Additionally, the hydrogel can reduce swelling in the wound area and create moist conditions conducive to promoting the healing process. The physical stability test of the ethanol extract gel preparation of kirinyuh leaves has been carried out with variations of carbopol and HPMC, showing stability in accordance with the gel requirements for all observed parameters. Previous studies found that the effective doses of kirinyuh ethanol extract were 2.5%, 5%, 10%, and 15%.

This research was aimed to evaluate the effect of kirinyuh leaf hydrogel extract on second-degree burns in male Wistar rats. Before being administered to the animal model, the kirinyuh leaf hydrogel underwent a stability test. The VEGF expression, number of fibroblasts, and the level of neovascularization were further examined to study the mechanisms of action. By conducting this research, we intended to scientifically prove the activity of the kirinyuh leaf hydrogel extract so that it can be developed into a pharmaceutical dosage form in accordance with BPOM standards aimed at healing burns.

2. Method

2.1 Materials

The materials used in this research were kirinyuh leaves, 96% ethanol (technical grade), CMC-Na (technical grade), glyserin (technical grade), propylene glycol (technical grade), aquadest, formaldehyde, 70% ethanol (technical grade), paraffin, haematoxylin, eosin, and Bioplacenton®.

2.2 Methods

2.2.1 Preparation of Kirinyuh Leaf Hydrogel Extract

One kg of dried kirinyuh leaves were pulverized and then macerated with 96% ethanol for five days with stirring. Extract was filtered, and the powder was re-macerated. The collected extract was evaporated using a rotary evaporator at 70°C to obtain concentrated extract.

Formulation of the hydrogel followed previous research by Sayuti (2015) as presented in Table 1. The ethanol extract of kirinyuh leaves was dissolved in water then heated while stirring. CMC-Na was gradually added to the mixture with continuous stirring to prevent CMC-Na clumping. Next, glycerin, propylene glycol, and water were added and stirred until a homogeneous gel was formed. Once formed, the hydrogel was stored in a dark and cool place for 1 night at a temperature between 10-15°C. Formula 1 was made accordingly without the addition of the extract.

Table 1. Formulation Of Kirinyuh (Chromolaena Odorata L.) Leaf Hydrogel Extract

Ingredient	Formula (g)			
	F1	F2	F3	F4
Kirinyuh leaf ethanol extract	-	1.25	2.5	3.75
CMC-Na	1.25	1.25	1.25	1.25
Gliserin	2.5	2.5	2.5	2.5
Propylene glycol	1.25	1.25	1.25	1.25

2.2.2 Evaluation of Kirinyuh Leaf Hydrogel Extract

The hydrogel formulas were subjected to stability tests using the cycling method. Samples were stored in a climatic chamber, and the temperature was changed between 4 and 40°C every 24 h for 6 days. Several parameters, i.e., organoleptic, pH, homogeneity, viscosity, and spreadability, were tested before and after the cycling test.

2.2.3 Experimental Design

Twenty-five Wistar male rats weighing 150-200 grams, aged 75-90 days, were randomly assigned to five different groups, i.e., gel base (F1), 5% (F2), 10% (F3), and 15% (F3) hydrogel extract, and positive control (PC) groups. Test animals were acclimatized for 7 days in a room with a temperature of 22°C and given sufficient standard food and drink. The lightroom was set with a 12:12-h light-dark cycle.

After seven days, the rat's back area was cleaned and shaved to a size of approximately 2-3 cm. The shaved back was disinfected with 70% alcohol and locally anesthetized using 2% lidocaine. A burn wound was inflicted using a 2 cm x 2 cm iron plate, which had been pre-heated to a temperature of 120°C (measured with an infrared thermometer for 10 seconds). Treatment was given topically to the assigned groups starting from day 8 to day 21. Treatment was administered twice a day.

2.2.4 Histopathological Examination

For histological analysis, the wounded area was harvested on day 22 after the rats were culled. The samples were fixed in 10% neutral buffered formalin and further processed for Hematoxylin and eosin (H&E) staining. Observation was carried out for the following parameters:

1. VEGF expression. Cells with large nuclei and brownish cytoplasm indicated cells that express VEGF. The samples were observed under a microscope in five different fields, and the cells that express VEGF were counted using the immunoreactive score (IRS). The expression results were divided into four categories: 0-1 cells = no protein expression; 2-3 cells = low protein expression; 4-8 cells = medium protein expression; 9-12 cells = high protein expression.
2. Neovascularization. The neovascularization level was determined by counting the number of blood vessels forming on the burnt tissue with a typical size of 7-9 µm. Observations were carried out using a microscope with 100x magnification in 5 fields of view.
3. Fibroblast. Fibroblast cells are elongated with an oval cell nucleus. These cells are distributed along bundles of collagen fibers. The number of fibroblasts was determined under a microscope (100x) in 5 fields of view.

3.3 Data Analysis

The data was analyzed using Shapiro-Wilk to determine the data normality, followed by One-Way ANOVA and Tukey's post hoc to determine significant differences among the groups (p-value was <0.05) in Graphpad Prism (GraphPad Software, Boston, US).

3. Results and Discussion

3.1 Stability of Kirinyuh Leaf Hydrogel Extract

Cycling test is an accelerated stability testing used to obtain information on a product's instabilities. The samples were alternately kept alternately at 4°C and 40°C every 24 h to simulate extreme storage conditions. Observation against organoleptic, pH, homogeneity, viscosity, and spreadability were done before and after the cycling test to assess the stability of the F1-4 formulas.

The organoleptic examination consisted of color, smell, and texture observation. The hydrogel without extract (F1) was clear and transparent, while the formulas containing kirinyuh leaf extract had a dark green color, which was aligned with the extract color. There was no clumping in all formulations. After the cycling test, no changes were observed (Table 2).

Table 2. Organoleptic observation results of kirinyuh leaf hydrogel extract

Formula	Color		Smell		Texture	
	Before	After	Before	After	Before	After
F1	Clear and transparent	Clear and transparent	Typical gel smell	Typical gel smell	No clump	No clump
F2	green	green	Typical extract	Typical extract	No clump	No clump
F3	green	green	Typical extract	Typical extract	No clump	No clump
F4	Dark green	Dark green	Typical extract	Typical extract	No clump	No clump
F1	Clear and transparent	Clear and transparent)	Typical gel smell	Typical gel smell	No clump	No clump

Before the cycling test, the increasing concentration of the extract in the formula resulted in an increased viscosity (Figure 1). In line with this, a previous study reported that the addition of extract into a hydrogel formulation contributed to increased viscosity of the hydrogel due to of viscous nature of the concentrated extract. Following the cycling test, the viscosity of all formulas decreased. The change in viscosity may occur because the container was not airtight, making the hydrogel to absorb water vapor from the surroundings.

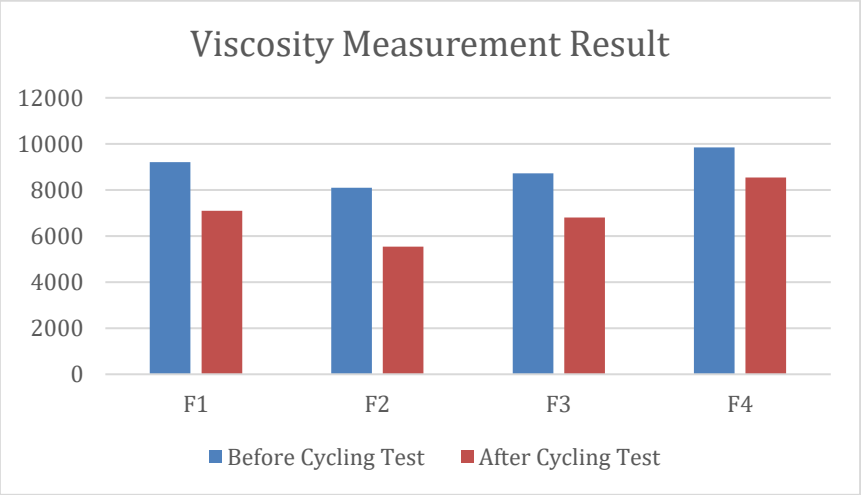


Figure 1. Viscosity of kirinyuh leaf hydrogel extract before and after cycling test

The pH of all formulas before the cycling test was within the normal human skin pH range (pH = 4.5-7; Figure 2). The addition of the extract to the formulation decreased the pH, where the higher the concentration of the extract, the more the pH decreased. This may be plausible because the pH of the extract tended to be acidic, thus affecting the overall pH of the hydrogel. Even though the addition of the extract lowered the pH, the pH values were still within the normal skin pH range. Similarly, the pH of all formulas decreased after the cycling test.

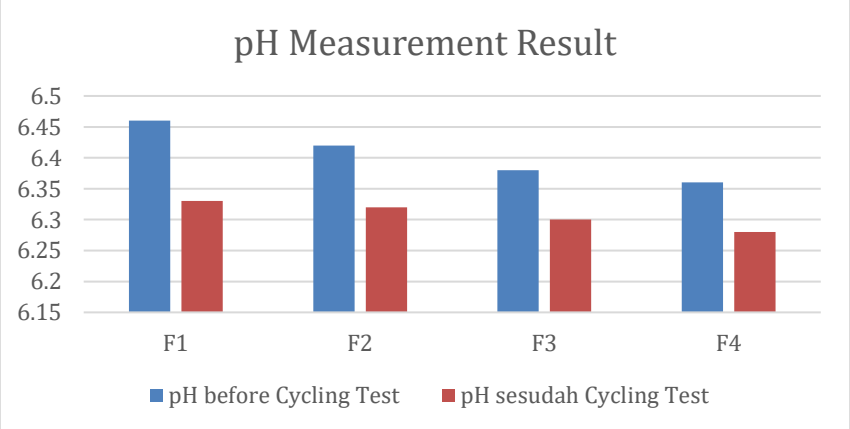
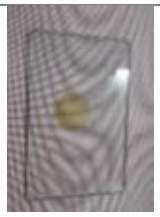


Figure 2. pH measurement of kirinyuh leaf hydrogel extract before and after cycling test

Visual observation found that the formulation of the kirinyuh leaf hydrogel extract (F2-4) and gel base (F1) were homogeneous before and after the cycling test. Homogeneity was reflected in even distribution and the absence of insoluble particles and lumps.

The last parameter observed in the cycling test was spreadability. Before the cycling test, as the amount of extract in the hydrogel increased, the gel spread became more widespread. This trend was also observed after the cycling test. Overall, the gel distribution became wider after the cycling test in all formulations compared to before the test (Table 3). This finding is consistent with the viscosity results, where the viscosity decreased after the cycling test.

Table 3. Spreadability of kirinyuh leaf hydrogel extract before and after cycling test

Formula	**Before Cycling Test**			**After Cycling Test**		
F1						
Weight	0 gram	100 gram	125 gram	0 gram	100 gram	125 gram
diameter	3.5 cm	4.2 cm	4.5 cm	3.9 cm	4.2 cm	4.8 cm
F2						
Weight	0 gram	100 gram	125 gram	0 gram	100 gram	125 gram
diameter	3.5 cm	4 cm	4.4 cm	4.2 cm	4.7 cm	5 cm
F2						
Weight	0 gram	100 gram	125 gram	0 gram	100 gram	125 gram
diameter	4 cm	4.5 cm	4.8 cm	4.4 cm	5 cm	5.3 cm
F2						
Weight	0 gram	100 gram	125 gram	0 gram	100 gram	125 gram
diameter	4.2 cm	4.7 cm	4.9 cm	4.8 cm	5.2 cm	5.5 cm

Taken together, no significant changes occurred on all observed parameters after the cycling, indicating that the formulas used were stable.

3.2 Effect of Kirinyuh Leaf Hydrogel Extract on VEGF Expression

Treatment with kirinyuh leaf hydrogel extract at 5%, 10%, and 15% significantly promoted the expression of VEGF compared to the rat-treated hydrogel base ($p<0.0001$), where at 15%, the extract showed the highest level of VEGF compared to other concentrations (Figure 3). Only F4 presented a similar level of VEGF

expression compared to CP (p=0.68), indicating that the 15% kirinyuh leaf hydrogel extract had similar level of activity compared to Bioplacenton®.

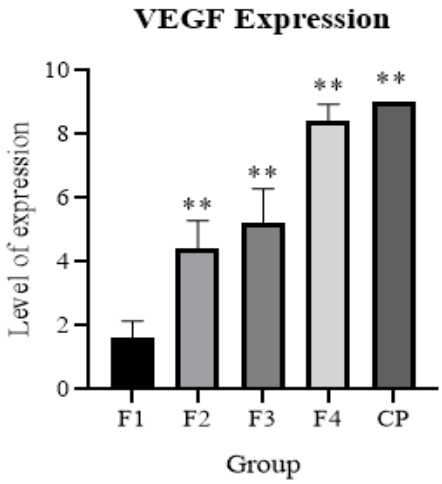


Figure 3. Effect of kirinyuh leaf hydrogel extract on VEGF expression. **indicates p<0.0001 compared to F1

Extract activity that increases VEGF expression was also observed in previous research. Awwaliyah (2020) showed that treatment with kirinyuh leaves extract had an effect on mouse model of burns. This result was also corroborated by Vijayaraghavan et al. (2017), who reported that the extract was able to accelerate the wound healing process four days faster than using betadine.

VEGF is a proangiogenic factor that, at adequate levels, is believed to be able to heal wounds well. This signaling protein is produced by various cells involved in the burn wound process, such as endothelial cells, fibroblasts, smooth muscle cells, platelets, neutrophils and macrophages, stimulating angiogenesis and increasing vascular permability. This eventually repairs the epidermal layer and triggers the formation of granulation tissue, thereby improving the overall appearance of the wound.

Tannins and flavonoids are components of kirinyuh leaves that have been studied and proven to affect the healing process of burn wounds. Tannins are thought to play a role in regulating the transcription and translation of VEGF, which have a re-epithelialization effect and, at the same time, restore angiogenesis and oxygen perfusion. Meanwhile, one of the flavonoids, quercetin, can stimulate the production of VEGF in the angiogenesis process. In addition, the flavonoids in the extract function as anti-inflammatory and inhibit the phospholipase enzyme so that phospholipids in the cell membrane cannot be converted into arachidonic acid. This will inhibit the cyclooxygenase and lipoxygenase pathways, thereby disrupting the synthesis of prostaglandins and leukotrine. Disruption of these inflammatory mediators can reduce vasodilation of blood vessels so that inflammatory cells and redness can be reduced.

3.3 Effect of Kirinyuh Leaf Hydrogel Extract on Neovascularization

Treatment with kirinyuh leaf hydrogel extract increased neovascularization in burn wound area, although significance toward the rat-treated hydrogel base group could only be observed at 15% concentration (p=0.0048). This result suggests that the extract promotes wound healing through neovascularization. However, the activity of 15% kirinyuh leaf hydrogel extract was significantly lower than Bioplacenton® (p<0.0001), suggesting a weaker activity compared to Bioplacenton®. Our results are in line with Mulyani's (2019) research, which showed enhanced wound healing in rabbits after treatment with macerated kirinyuh leaves extract. Similar research has also been conducted by Rahman, demonstrating the effect of kirinyuh leaf extract ointment on the cut wounds healing in laying hens. Based on the time for inflammation to occur, the length of time for the wound to dry, and the length of cut closure, Norma and Oktavina reported that the dose of kirinyuh leaves ointment extract that was found to be the most effective at 15%.

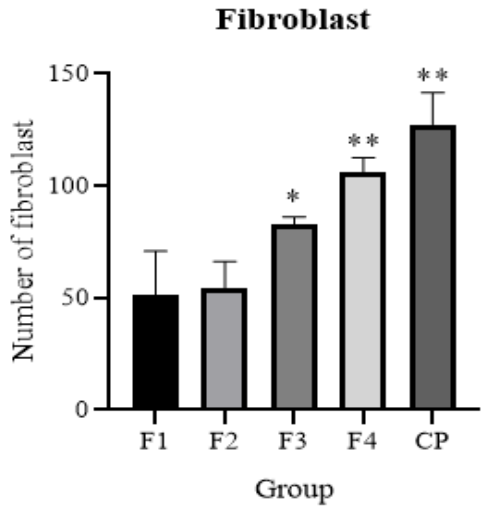


Figure 4. Effect of kirinyuh leaf hydrogel extract on neovascularization. * Indicates $p<0.05$ compared to F1 and **indicates $p<0.0001$ compared to F1

The fibroplasty or proliferation phase is one of the phases of the wound healing process. This phase begins 4 days after the injury occurs and lasts 3-4 weeks or more, depending on the size of the wound. This phase is characterized by the formation of angiogenesis, reepithelialization, and fibroblastia. The proliferation of fibroblast cells originates from mesenchymal cells that have not yet differentiated. The formation of granulation tissue consists of fibroblast and collagen cells. Fibroblasts are useful in producing structural proteins during tissue reconstruction.

Kirinyuh leaves contain compounds such as alkaloids, flavonoids, glycosides, saponins, tannins, and steroids or triterpenoids. These compounds are potential as antibacterials and antivirals. Kirinyuh leaves also contain phenolic compounds, which can protect skin cells.

Saponins and flavonoids in kirinyuh leaves were found to have a role in the fibroplasty phase. The saponin contained in kirinyuh leaves can increase the number of fibroblast cells and also stimulate collagen formation. In addition, saponins increase the ability of fibroblast TGF-β receptors to bind to TGF-β. Meanwhile, flavonoids work by inhibiting bleeding through increasing the number of platelets during wound healing process. When the body experiences bleeding, the platelets will burst, resulting in the thrombokinase, which will then act as an enzyme to activate fibrinogen to form fibrin monomers, which are assisted by Ca2+ and vitamin K found in blood plasma.

Overall, our histological evaluations showcase the concentration-dependent activity of kirinyuh leaf hydrogel extract, where 15% of the extract was found to be the most effective dose. This result is in line with Ramdani et al., which showed that increasing the dose would positively affect would healing process even further. However, having extremely high extract concentration, for instance 20%, could result otherwise. The reason for the activity reduction at high concentrations remains elusive and warrant further study.

4. Conclusion

This study demonstrates the activity of kirinyuh leaf hydrogel extract at 15% in alleviating 2nd degree burns on male Wistar rats, as evidenced by the significant VEGF expression and increased neovascularization and number of fibroblasts. The increase in VEGF expression and neovascularization further indicates the extract's activity in enhancing angiogenesis caused by burns during wound healing. As such, we conclude that kirinyuh leaves extract works by increasing angiogenesis and the number of fibroblasts. The findings in this study further strengthen the evidence for the effectiveness of kirinyuh leaf for burns, showing its potential in making products for burns on the market. Further mechanistic studies should be carried out to elucidate which phytochemicals are responsible for the wound healing activity.

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