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Potential Benefit of Flavonoid in Papaya Leave Gel in Neutrophil, Angiogenesis, and Wound size in Rattus Norvegicus

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Abstract: An incision wound is the destruction of several cells in the skin area that has experienced an incision. Several factors influencing wound healing are neutrophils as causative agents of inflammation, angiogenesis, and wound closure. These three factors should undergo physiologically to obtain the optimal healing process. Currently, herbal products are preferred because they have lower side effects than synthetic ones. Papaya leaves contain flavonoids with various functions, including wound closure and anti-inflammatory and antioxidant effects. Topical intervention, especially gel, is good for healing because it can keep the wound moist. This research aimed to determine the effect of the administration of papaya leaf gel on wound closure, neutrophil, and angiogenesis. Thirty white rats were used as the model animals in a randomized experimental study divided into five groups comprising negative control (0,9% NaCl), positive control (10% Povidone-iodine), and three intervention groups treated with gel of Carica papaya leaves at a dosage 10%, 20%, and 30%. All groups were topically administered treatment once a day on the dorsal surface for seven days. Carica papaya leaves contain flavonoids, saponin, tannin, and alkaloids qualitatively and 7,734 mg/kg of the flavonoid quantitatively. There were no significant differences in wound size in all three groups on three days, but Carica papaya leave gel had a superior effect compared to 10% PI and 0,9% NaCl in the wound closure process. There were significant differences in all groups in neutrophil and angiogenesis. Nevertheless, 20% papaya leave gel showed the lowest number of neutrophils and had a superior effect on improving the new blood vessels significantly compared to negative and positive groups.
Keyword: Carica papaya leaves; flavonoid; gel; neutrophil; wound healing

INTRODUCTION

The wound healing process consists of 3 phases. The first is the inflammatory phase, an immediate response to an injury. Primary sensory neurons send signals to the brain to stop bleeding and initiate the inflammatory process. Then slowly, the inflammation subsides, and homeostasis begins. The second process is proliferation, where the purpose of the proliferative phase is to repair damaged tissue and initiate tissue remodeling. Fibroplasia, re-epithelialization, angiogenesis, and peripheral nerve repair are important processes of this phase. Moreover, the last wound healing process is remodeling. The goal of the last phase is to complete the tissue remodeling

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process and restore the integrity of the skin to its original state (Cañedo-Dorantes et al., 2019).

Neutrophils, in normal numbers, play a pivotal role in maintaining the barrier of injured tissue from harmful pathogens. However, the persistent presence of neutrophils can cause excessive inflammation and worsen the wound condition (Phillipson et al., 2019). In the inflammatory phase, the neutrophil response will accumulate immediately after injury. In the subsequent phase, angiogenesis, granulation tissue is formed. Myofibroblasts activated via contraction and signaling molecules, form new tissue remodeling, in which the granulation tissue becomes scar (Ellis et al., 2018). The formation and regeneration of new blood vessels have an important role in distributing nutrients to wounds and transferring immune cells for wound debridement. Regarding this, appropriate intervention is needed, and the wound could heal properly.

Povidone iodine (PI) has good benefits. It is expected to be an effective treatment for acute and chronic wounds in wound healing (Bigliardi et al., 2017), as well as lacerations and deep wounds due to good tissue penetration (Maksym K. Gmur, 2020). PI can suppress pro-inflammatory cytokines, including neutrophils, and also increase the proliferation of endothelial cells at the beginning of the formation of new blood vessels (Wang et al., 2017). However, local cytotoxicity and allergy should still be considered. This drug is contraindicated in people with an allergy to PI or other additives contained in it and hyperthyroidism or thyroid cancer. There was no conclusive evidence for the strong recommendation of Povidone-iodine topically for wound healing in preventing infection (López-Cano et al., 2019). Another intervention in wound care is the use of 0.9% NaCl. Research has shown that NaCl was better than PI in providing comfort and reducing wound size (Gopi, Basava, and Matad, 2017). NaCl or saline can suppress pro-inflammatory activity and increase the proliferation of new blood vessels in the wound (Giri et al., 2021). In contrast, this therapy was as effective as antiseptics (0.1% polyhexanide plus 0.1% betadine) for wound therapy, but additional evidence was needed to strengthen its applicability (Kim et al., 2015).

There is an increasing interest in the continuous production of plant-based drugs to treat various diseases (Aruljothi et al., 2014). Indonesia is in fifth place as the largest producer of papaya (Carica papaya) globally (Tridge, 2019). Papaya plants, especially the leaves, contain more total phenolics and flavonoids than other parts of the papaya tree (Maisarah et al., 2013), which are useful in meeting nutritional and health needs. However, its use in wound healing is still not generally known. The flavonoids in papaya leaves are useful in wound healing (Pang et al., 2017). Papaya leave extract also has strong medicinal effects such as antiviral, anti-inflammatory, antibacterial, antioxidant, to promote cell migration and proliferation (new blood vessels) and, collagen synthesis (Singh et al., 2020), and wound healing (Femilian et al., 2019).

Research on the benefits of papaya leaves on skin wounds, especially its effect on neutrophils and blood vessels, has not been widely studied. In the previous study, using Streptococcus mutans adhesion to neutrophils given papaya leaf infusion was proven to reduce the number of neutrophils (Kurniati, 2021). This is supported by other studies on acute inflammation given papaya leaf methanol extract, which causes suppression of neutrophil counts (Marpaung, Bangun, and Ilyas, 2019). In addition, papaya leaves benefit from the growth of new blood vessels. This has been investigated in the healing process of traumatic buccal ulcers in experimental animals, with results explaining that papaya leaf ethanol extract can significantly increase angiogenesis (Femilian, Agustina, and Subagyo, 2019). Regarding this, papaya leaf extract was made in gel preparation, and neutrophils and new blood vessel growth were observed on dorsal wounds to determine their effect on wound healing, especially in the inflammatory and proliferative phases.

The topical application method of papaya leaves gel to treat the wound as a traditional practice is a good alternative (Aruljothi et al., 2014). Gel preparations are used because they include maintaining oxygen exchange and skin moisture and intensively hydrating the matrix, which promotes wound healing. In addition, it minimizes pain when changing dressings and does not damage newly formed tissue (Rüther et al., 2021). This study aimed to determine the effect of papaya leaf extract gel on the number of neutrophils, new blood vessel growths, and wound size in female rats.

MATERIALS AND METHODS

Study Design

This research was an experimental study using a randomized posttest-only control group design. The study was held in the laboratory of Pharmacology, Brawijaya University, Malang, Indonesia, with the ethical approval letter number 66/EC/KEPK/03/2022. The animal model in this study was 30 rats (Rattus Norvegicus). Some characteristics of the animals were female, healthy, aged 12-16 weeks, and weighing 180-200 grams. These rats were divided into five groups, consisting of the control negative (0,9% NaCl), control positive (10% PI), intervention I (10% gel), intervention II (20% gel), and intervention III (30% gel) group. Before using the rats as an incisional model, the animal underwent acclimatization for seven days, given food and water ad libitum in a 20cm x 30cm x 40cm in size of the cage. On an experimental day, the animals were given all the treatments topically on the wound site once a day.

Rat model of skin incision

Surgical procedures were performed following The Health Research Ethics Committee regulations, Faculty of Medicine, Brawijaya University, Malang, Indonesia. All rats were anesthetized with 0,1 cc ketamine through intramuscular administration, and then they were shaved in the dorsum. The skin incision wound was 2 cm in length and 5 mm in diameter. The incisions were dressed in sterile gauze. The skin incision size was observed and documented on one, three, and seven days for each group.

Carica papaya leave extraction

Carica papaya leaves were harvested from Malang, East Java, Indonesia weighed 5kg. The leaves were subsequently dried in an oven at 50ºC resulting in 1 kg of Carica papaya leaf powder. The subsequent step was extraction with 96% ethanol as a solvent using the maceration method for three days. The rotary evaporator (Janke and Kunkel, RV 05-ST) was used for the evaporation step. The final result of the extraction was a 500gram pasta form of the Carica papaya leaves.

Phytochemical screening

Identifying phytochemical compounds was performed qualitatively and quantitatively. The preliminary screening using Carica papaya leave extract, 3 ml ethanol, 0,5 ml Hydrochloric acid (HCL), magnesium powder (Mg), aquadest and 1 ml butanol, NaCl, dragendorf color reagent, 1% FeCl3, and 1% gelatin. Flavonoid was detected when the formula color changed into yellow (Setyawaty et al., 2020), yellow precipitate, greenish-black precipitate, and foam were the signs of the alkaloid, tannin, and saponin, respectively (Marlinawati et al., 2022). On the other hand, Ultraviolet-Visible (UV-Vis) spectroscopy (Double Beam Spectrophotometer U-2900/2910) was carried out to determine the amount of flavonoid compound (Quercetin equivalent).

This method used 167,6 mg Carica papaya extracts dissolved in 5 ml methanol. The absorbance determined the result with a 430 maximum wavelength.

Composition of Carica papaya leave Gel

The gel comprises water, Carbopol, 0,6% Phenoxyethanol, 7% Propylene glycol, 0,1% Ethylene diamine tetra-acetic acid (EDTA), dan 0,59% Triethanolamine (Jamadar et al., 2017) (Misal et al., 2012) (Thibodeau, 2017) and Carica papaya leave extract (Jamadar et al., 2017).

Table 1. The Papaya Leave Gel Extract Formula

Materials	Amount (gram) Gel Formulation 10%	Gel Formulation 20%	Gel Formulation 30%
Papaya Leaf Extract	2	4	6
Carbopole	0.4	0.4	0.4
Phenoxyethanol	0.12	0.12	0.12
Propylene glycol	1.4	1.4	1.4
EDTA	0.02	0.02	0.02
Triethanolamine	0.118	0.118	0.118
Aquades (add)	16	14	12

(Marlinawati et al., 2022)

Measurement of skin incision size

The camera documented the skin excision of rats for one to three days and measured by image J the wound size. Then the percentage of wound development was calculated with the following formulation.

$$Wound\ size\ (\%) = \frac{Initial\ wound\ area - specific\ day\ wound\ area}{initial\ wound\ area} \times 100$$

(Dev et al., 2019)

Histopathological analysis

Dorsal skin incisions were carried out to observe neutrophils and the new blood vessels on the first, third, and seventh days. A BX35 microscope conducted the observation with 400x magnification and ten fields of view. There were 30 paraffin-embedded specimens stained with Hematoxylin and eosin (HE). In addition, the number of neutrophils and new blood vessels from the angiogenesis process was counted using Image J based on a sample consisting of days 1.3 and 7 from each group that was put together independently.

Statistical analysis

The result of wound closure was analyzed by computer application. Shapiro-Wilk Test was used to check the normality with the statistical significance of p<0,05. Due to unnormal data being detected, non-parametric test, Friedman's 2-way ANOVA, and Kruskal-Wallis test were used, followed by a pairwise test with a significant result on p<0,05.

RESULTS AND DISCUSSION

Flavonoid content in Papaya leaves extract.

Based on a preliminary qualitative analysis using chemical reactions, it was found that papaya leaves contain flavonoids. Where was detected when the formula color changed to yellow (Setyawaty et al., 2020) (Nugroho et al., 2017). Furthermore, tests to determine the total flavonoid compounds quantitatively using Ultraviolet-Visible (UV-Vis) spectroscopy showed the result of 7,734 mg/kg.

Papaya leaves contain phytochemicals that can be useful in wound healing (Femilian, Agustina, and Subagyo, 2019). Papaya leaves contain phytochemical compounds such as flavonoids, alkaloids, and saponins (Ayoola et al., 2010); (Joy Ugo et al., 2019). Flavonoids are secondary metabolites that significantly contribute to wound healing activity (Soib et al., 2020). These components can act as an anti-inflammatory, antimicrobial and antioxidant (Gudimella et al., 2022) which are needed in the wound healing process. Therefore, papaya leaves can be used in herbal-based medicine and as a potential source for drug formulations (Ayoola, Adeyeye, and State, 2010).

Wound area measurement

All 30 Rats, five groups, were enrolled in the skin incision and observed starting from 1 day to 3 days, then analyzed. Five groups of intervention were not significant to wound size (p>0,05). Nevertheless, Carica papaya leaves gel (CPLG) with 10%, 20%, and 30% had a greater effect than PI 10% and NaCl 0,9%.

Table 2. The Wound Size on Related samples Friedman's Two-Way Test Analysis

Intervention	Mean Rank	p-value
The distribution of control positive, negative, and all intervention groups was the same.		0,770
Positive control	2,54	
Negative control	2,92	
CPLG 10%	3,04	
CPLG 20%	3,12	
CPLG 30	3,38	

Description: The significance level is 0,05

Wounds are one of the most common health problems, and the cost of wound care and healing has steadily increased over the past few years (Guo et al., 2021). Effective healing of wounds is very important for wound healing in the skin (Karppinen et al., 2019). The wound healing phase consists of hemostasis, inflammation, proliferation, and remodeling stages. If the wound healing process is hindered from transitioning to the inflammatory phase, it will stimulate pro-inflammatory cytokines, thereby prolonging the inflammatory phase. Wound healing will take longer (Ellis, Lin, and Tartar, 2018).

This study showed that the negative control group with 0.9% NaCl and the positive control group with 10% PI had no significant difference in wound size. However, NaCl had a higher effect than Povidone iodine. The relationship between PI and NaCl was statistically similar to other studies, which showed no significant difference in surgical wound healing after wound care with NaCl and PI (Ancy, 2018). This is also supported by research that states clinically, the infection rate in laparotomy wounds did not increase or decrease with the administration of 5% PI irrigation or with 0.9% saline solution, and there was no significant difference in accelerating wound healing with this type of laceration. or abrasion wound (HG et al., 2018) (Dios et al., 2017).

The results of topical application with CPLG also were not significant; nevertheless, these had a special effect on 10% PI and 0.9% NaCl. Greenish black on the wound area was due to gel color. Another study also revealed that research using animal models given an ethanolic extract of papaya leaves showed faster-wound healing than that of animals given Povidone iodine intervention (Bhar et al., 2013).

Papaya leaves contain flavonoids that have various benefits in wound healing (Karak, 2019). The crude extract and fractionation of papaya leaves showed considerable antioxidant activity, the strongest free radical scavenging effect, and a faster rate of cell migration for 24 hours in wounds (Husin et al., 2019). Papaya leaves significantly affect wound closure, increasing fibroblast cells and forming scar tissue (Ukoba, Adefisan, and Aguwa, 2016). In addition, another beneficial flavonoid content is its antibacterial effect (Shubham et al., 2019). Several bacteria have been shown to significantly affect papaya leaves, such as Escherichia coli, Klebsiella pneumonia, and Staphylococcus aureus (Ajiboye and Olawoyin, 2020; Pertiwi, Hafiz, and Salma, 2019). In this study, there was no infection in the intervention group due to antibacterial activity, and wound closure became faster. This study showed that papaya leaf ethanolic extract gel could accelerate skin wound healing in rats.

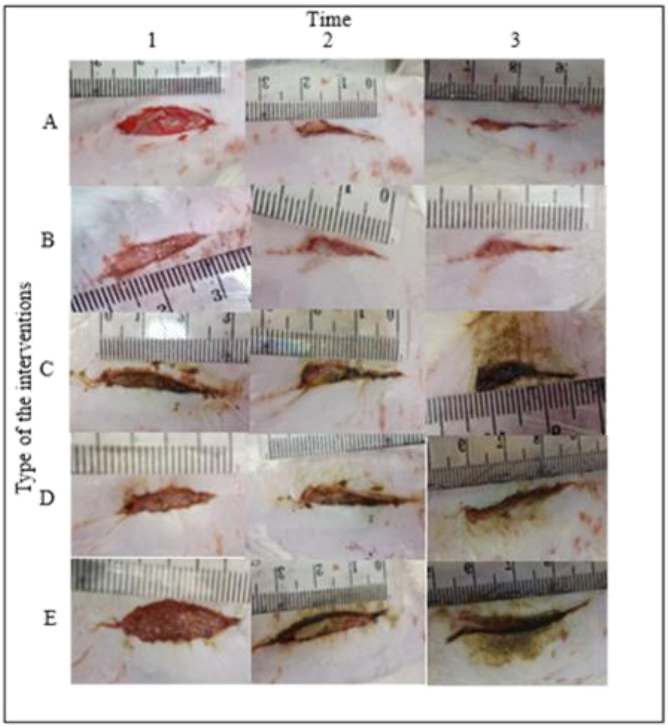

Figure 1. Time: 1= first day; 2= second day; 3= third day.
Type of the interventions: A= NaCl 0,9%; B= Povidone iodine 10%;
C= 10% CPLG; D= 20% CPLG; 30% CPLG.

The number of neutrophils

There was a significant relationship ($p<0.05$) to the number of neutrophils in incised rats among all groups on one, three, and seven days. Sequentially, based on the number of neutrophils from the fewest to the more numerous neutrophils in the median range, there were 20% CPLG, 10% CPLG, 0.9% NaCl, 10% PI, and 30% CPLG. Further tests using pairwise comparisons showed that the 10% and 20% CPLG had a significant difference against the positive control group ($p<0.05$). Compared with the negative control group, 30% CPLG had a significant difference where the gel affects fewer neutrophils. On the other hand, there was a significant difference between papaya leave gel with a concentration of 20% and 30% and 10% and 30 ($p<0.05$). The negative and positive control groups did not differ significantly ($p>0.05$).

Table 3. The Number of Neutrophils on Kruskal Wallis Test Analysis

Intervention group		Mean Rank	p-value
Negative control	Positive control		0,670
	CPLG 10%		0,168
	CPLG 20%		0,66
	CPLG 30%		0,461
Positive control	CPLG 10%		0,071
	CPLG 20%		0,024
	CPLG 30%		0,755
CPLG 10%	CPLG 20%		0,646
	CPLG 30%		0,034
CPLG 20%	CPLG 30%		0,010
Between all groups			0,035
Negative control		17,58	
Positive control		19,75	
CPLG 10%		10,58	
CPLG 20%		8,25	
CPLG 30%		21,33	

Description: The significance level is 0,05

In the early stages of injury, an inflammatory response will appear (Cañedo-Dorantes et al., 2019) as a response to the skin barrier breakdown by recruiting pro-inflammatory cells, one of which is neutrophils. Neutrophils are one of the most abundant and active cells during the wound healing process (Phillipson et al., 2019) by migrating to the wound area via pro-inflammatory signals (Ellis, Lin, and Tartar, 2018). Neutrophil has both advantages and disadvantages for wound healing. These benefits include killing and degrading pathogens at wound sites and cleaning wounds (Wang, 2018). In contrast, excessive levels of proteases in tissues due to highly active neutrophils or persistent neutrophil production can lead to negative effects such as sustained inflammation or collagen formation and re-epithelialization (Wilgus, Roy, and McDaniel, 2013), resulting in chronic wounds.

In this study, the flavonoid compounds of CPLG can significantly regulate the number of neutrophils. The 20% gel intervention had significantly fewer neutrophil counts than the negative and positive control groups (Figure 2). This follows a study that stated that papaya leaves could significantly regulate neutrophils in inflammation conditions by reducing neutrophils (Anjum et al., 2017)(Marpaung, Bangun, and Ilyas, 2019). The regulation of the number of the neutrophils in this study was within the normal number; this was based on the state of the wound, where there were no signs of excessive inflammation, such as rats in good health, no tumor (swelling), and rubor (redness), and loss of function (functio laesa) (Hurlow et al., 2022).

Papaya leaves can regulate the number of neutrophils well, whereas, in a state of excessive inflammation, it reduces the number of neutrophils (Gumay et al., 2020) because it has an anti-inflammatory function (Singh et al., 2020). The antioxidant and anti-inflammatory activities of flavonoids can reduce reactive oxygen species and modulate the pathway of inflammation by inhibiting the pro-inflammatory expression and the expression of inducible nitric oxide synthase (Antunes-Ricardo, Gutierrez-Uribe, and Serna-Saldivar, 2015), reducing ROS, inhibiting the formation of dependent NETs (Nishimura et al., 2013), decreases the release of histamine or prostaglandins from mast cells, inhibits the production of pro-inflammatory cytokines in neutrophils and other immune cells (Maleki et al., 2019) (Freitas et al., 2014).

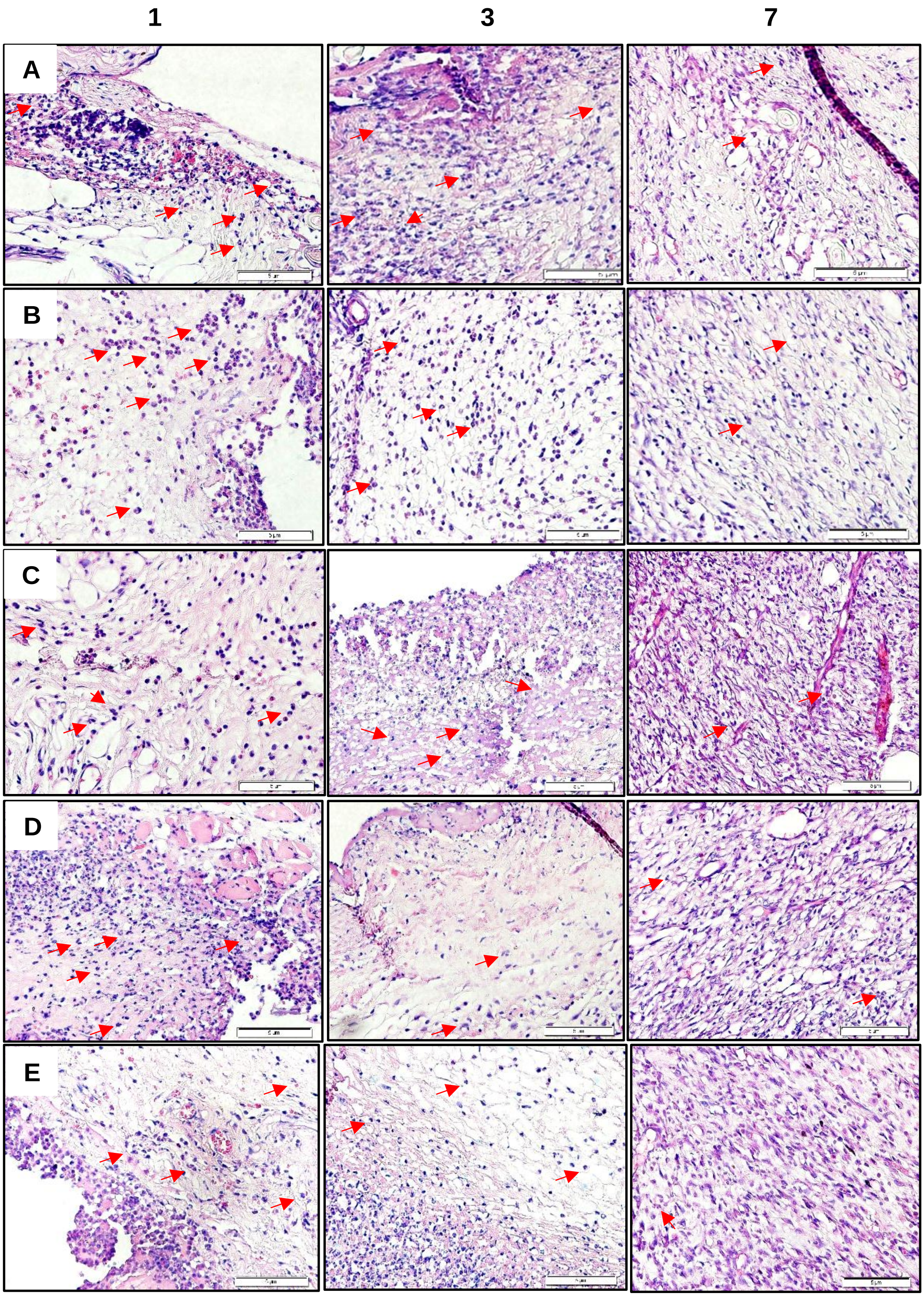

Figure 2. Neutrophil number. Time: 1= first day; 2= second day; 3= seventh day. Interventions: A= NaCl 0,9%; B= Povidone iodine 10%; C= 10% CPLG; D= 20% CPLG; 30% CPLG.

In a wound condition, papaya leaf gel regulates neutrophils in the normal state, resulting in no infection and inflammation, which will accelerate the wound healing process. In contrast, papaya leaves can increase the number of neutrophils in studies using rats induced by *Salmonella typhi* and *Staphylococcus aureus*, in which the increase in neutrophils acts as an anti-inflammatory to inhibit pathogens (Oladunmoye et al., 2007). In conditions that require increased immunity, it also indicates that papaya leaves can increase the number of neutrophils in studies using rats with symptomatic dengue (Razak et al., 2021). Based on this, papaya leave gel can be useful in regulating neutrophils properly based on the host's condition.

The growth of new blood vessels characterizes the angiogenesis process.

There was a significant difference in the intervention given to incised rats from each group on the growth of new blood vessels (p<0.05). In addition, based on the pairwise test, the positive and negative control groups had no significant difference in the growth of new blood vessels (p>0.05). On the contrary, there was a significant difference (p<0.05) between the positive control group and all intervention groups. In contrast, in the negative control group, there was a significant difference (p<0.05) between the 10% and 20% gel intervention. Based on this, the 10%, 20%, and 30% gels have a good effect on the angiogenesis process, especially the growth of new blood, with the best effect being the use of 20% papaya leave gel.

Table 4. The New Blood vessels on Kruskal Wallis Test Analysis

Intervention group		Mean Rank	p-value
Positive control	Negative control		0,600
	CPLG 10%		0,009
	CPLG 20%		0,001
	CPLG 30%		0,019
Negative control	CPLG 10%		0,037
	CPLG 20%		0,004
	CPLG 30%		0,069
CPLG 10%	CPLG 20%		0,441
	CPLG 30%		0,793
CPLG 20%	CPLG 30%		0,301
Between all groups			0,002
Negative control		9,17	
Positive control		6,50	
CPLG 10%		19,75	
CPLG 20%		23,67	
CPLG 30%		18,42	

Description: The significance level is 0,05

Angiogenesis is a physiological process, and the stages of successful angiogenesis are important factors in wound healing. In this study, the newest blood formation occurred on the third day compared to the first and seventh day (p<0,005). This process occurs on the third to fifth day after the injury (Liu, Maloni, and Petrini, 2014). In this phase, the new blood vessels are formed from pre-existing blood vessels by invading the wound clot and incorporating them into the vascular tissue throughout the wound granulation. Influenced by angiogenesis include hypoxia, inflammation, and growth factors (Beyer et al., 2018).

This study resulted in higher new blood vessel formation in gel intervention against negative and positive control groups (Figure 3). To get optimal healing, VEGF, TGF-β, and fibroblast growth factors need to be stimulated (Kumar et al., 2015). Flavonoids can increase the expression and regulation of TGF-β, Smad 2/3, and downregulation of Smad 7 and MMP-2. Increased TGFβ will affect VEGF, triggering angiogenesis (Carvalho et al., 2021) and stimulating new blood vessel formation. Other mechanisms are increased angiogenesis through upregulation of the

Ras/Raf/MEK/ERK pathway, stimulating endothelial cells to form new blood vessels (Carvalho et al., 2021).

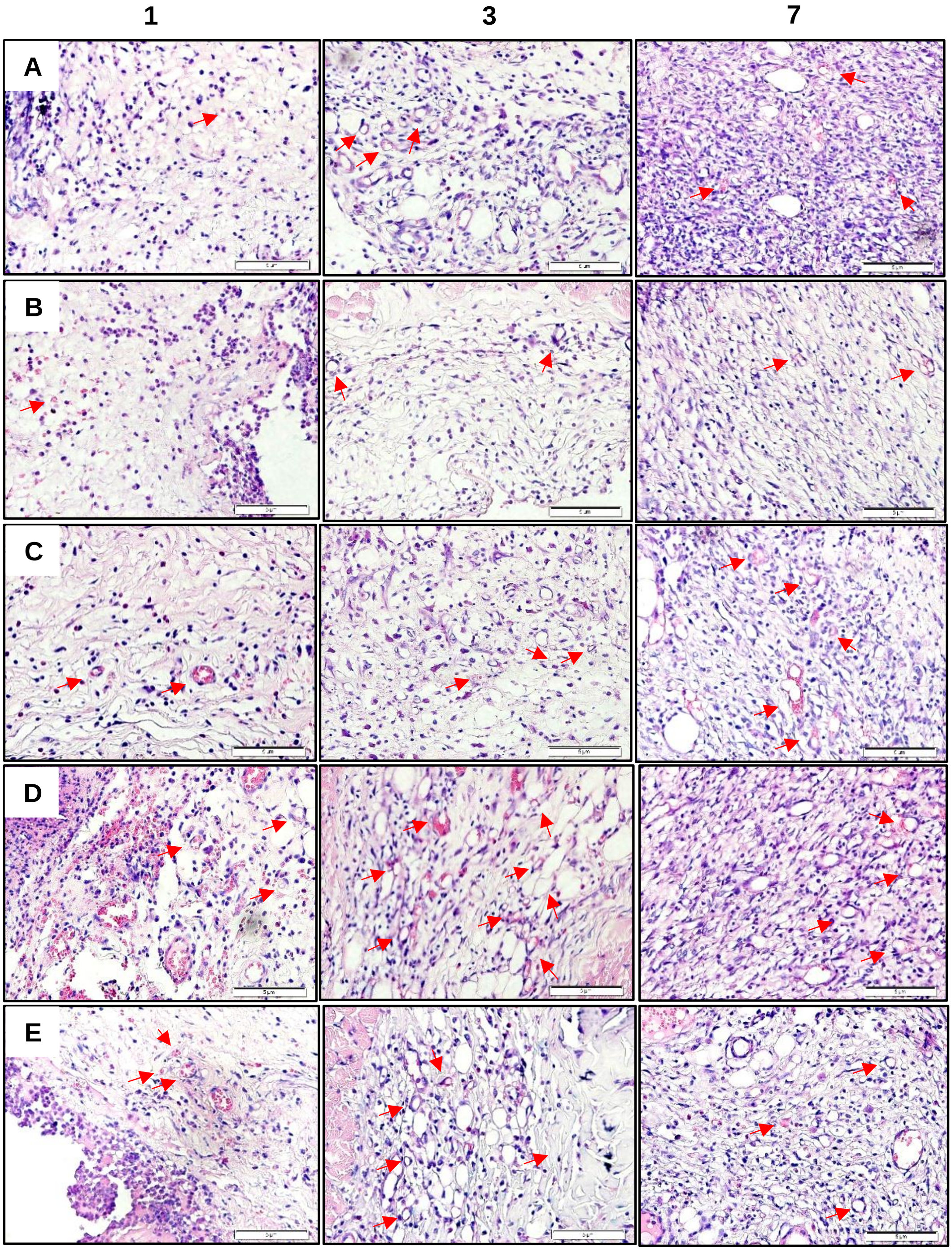

Figure 3. The New Blood Vessels Formation. Time: 1= first day; 2= third day; 3= seventh day. Intervention: A= NaCl 0,9%; B= Povidone iodine 10%; C= 10% CPLG; D= 20% CPLG; 30% CPLG.

Although the limitation of this study is that the study was only carried out until the seventh day, when the wound condition was still not fully healed so it might still be possible to observe the wound area, the number of neutrophils, and the development of blood vessels, but days 1-7 were able to describe the three factors above concerning administration of papaya leaf extract gel.

CONCLUSION

Carica papaya leaves contain phytochemical compounds such as flavonoids. Applying papaya leave gel had benefits in wound healing, especially in the wound size, regulating neutrophils, and forming new blood vessels. Based on this, it could be used to fasten the wound healing process. In addition, further research is recommended to be held to ensure the effectiveness of this gel, especially in clinical research studies.

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CONFLICT OF INTEREST

The research reveals that there is no conflict of interest in this study.

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