doi.org/10.36721/PJPS.2023.36.4.REG.1169-1176.1

Wound healing activity of ethanol extract of green algae (*Ulva lactuca* L.) gel in mice

Wahyu Widyaningsih*, Vivi Sofia, Sapto Yuliani, Yeni Rahmawati, Rooidatun Nahda, Devia Hanum Kemuning and Afifah Gustin
Faculty of Pharmacy, Universitas Ahmad Dahlan, Yogyakarta, Indonesia

Abstract: *Ulva lactuca* L. contains bioactive substances with anti-inflammatory, antibacterial, and antioxidant properties that aid in healing cut wounds. This research was done to ascertain the histopathological parameter's effects on the wound-healing capacity of gels made from the ethanol extract of *U. lactuca* (EEUL). The 45 mice were equally divided into five groups: The gel-based control (group I), the positive control (group II), the pain control (group III), and the treatment groups (groups IV and VI), which received EEUL gel at concentrations of 5 and 10%, respectively. On the mice's back, a 1 cm-long incision was made. A one-way ANOVA, post hoc least significant difference (LSD), and/or Mann-Whitney test were used to statistically analyze the data. Reduced wound scores, healing times, and wound length were observed (p 0.05). On the other hand, the macrophage score, the number of blood vessels, the thickness of the epithelium, and the fibroblast count increased (p0.05%). Topical application of 5% or 10% EEUL gel has accelerated wound healing by increasing macrophage scores, blood vessel density, epithelial thickness, and fibroblast counts while decreasing wound description scores and wound length.

Keywords: Green algae, ethanol extract gel, wound, macrophage.

INTRODUCTION

A cut refers to a type of wound inflicted by sharp objects that damage the skin tissue (Irfan-Maqsood, 2016). Based on the 2013 National Basic Health Research Report in Indonesia, the prevalence of wounds in the national population is 8.2%. The three most common types of injuries are abrasions/ bruises (70.9%), sprains (27.5%), and cuts (23.2%) (Kemenkes, 2013).

One of the plants used to treat cuts is *Ulva lactuca* L. (green algae), which has phytochemicals such as alkaloids, carbohydrates, proteins and amino acids, as well as sterols, saponins, phenols, starch, quinones, flavonoids, cardiac glycosides, melatonin and chlorophyll (Widyaningsih and Afdaliah, 2020). Because of its flavonoids, tannins, alkaloids, tocopherols and melatonin content, *U. lactuca* is useful as an antioxidant that guards against oxidative stress damage, an antibacterial that stops bacterial growth, and an anti-inflammatory that helps lessen inflammation at wound sites (Ardita *et al*., 2021; Yang *et al*., 2021). Antioxidants diminish inflammation and aid in healing in addition to protecting against oxidative stress-related damage (Comino-Sanz *et al*., 2021). According to Barbalace *et al*., 2019 this characteristic of *U. lactuca* inhibits the signaling system that activates inflammatory enzymes. As an antibacterial, the plant can prevent the synthesis of bacterial nucleic acids, bind to proteins that lyse bacterial cells, reduce adhesion, biofilm formation, and membrane permeability, as well as inhibit the formation of extra cellular enzymes, promote enzyme complexation, and decrease intracellular substrates (Akiyama *et al*., 2001; Shamsudin *et al*., 2022; Tekbas *et al*., 2008; Yan *et al*., 2021).

More drug molecules can collect in the wound area when medications are applied topically compared to when they are administered in other ways (Yunanda and Rinanda, 2017). This has been proven to improve wound healing outcomes. Because the medicine directly penetrates through the underlying skin layer or mucous membrane, it has a localized effect where it is applied. some of the main benefits include the fact that, unlike intravenous therapy, it is not subject to first-pass metabolism, has little to no risk of discomfort, and has little to no risk of various absorption conditions, such as changes in pH level, the activity of drug-metabolizing enzymes, and gastric emptying time (Bhasha *et al*., 2013). Topical preparations like gels can administer medications more effectively than ointments since they are semi-solid and contain a lot of water. Carbopol is frequently used in the formulation of gels to increase the viscosity and pace of drug absorption, hence enhancing wound healing (Pangestu *et al*., 2020).

Blood vessels and macrophage cells can be used to identify the wound healing mechanism. The three primary roles of macrophages are antigen presentation, phagocytosis, and immunomodulation. Recruitment of activated cells to infection sites, microbial detection, phagocytosis, microbial killing and cell proliferation are functional responses in cellular host defense.

**Corresponding author:* e-mail: wahyu.widyaningsih@pharm.uad.ac.id

Additionally, macrophages create bioactive chemicals that are crucial for both innate and adaptive immune responses (Fujiwara and Kobayashi, 2005). The proliferative phase, which starts three days after injury and lasts for around two weeks, is when new blood vessels are formed. It occurs concurrently with all reparative stages and is a critical stage in wound healing (Takeshita *et al*., 1994; Velnar *et al*., 2009). The goal of this work is to develop EEUL gels that satisfy the necessary physical specifications and ascertain their activity and mechanism in wound healing based on variations in the density of macrophages, blood vessels, epithelial thickness, and fibroblasts.

MATERIALS AND METHODS

Collection and identification of Ulva lactuca L.

Green algae (*Ulva lactuca* L.) gathered from Drini Beach in Gunungkidul, Yogyakarta, Indonesia, served as the study's primary source of material. Green algae were collected in the evening when the sea water receded. Green algae were identified at Ahmad Dahlan University's Faculty of Biology with the identification number 269/Lab.Bio/B/XI/2020.

EEUL preparation

Maceration was the extraction technique utilised (Widyaningsih *et al.,* 2016). Before steeping for three days in 1000 ml of 96% ethanol, 500 g of the raw green algae were first ground into a powder. The filtrate 1 was created by passing the macerate output through a Buchner funnel and filter paper. The filtrate was collected after the residual pulp had been macerated twice using the same technique and solvent. The majority of the ethanol was subsequently decreased by rotary evaporation of the filtrate at 40°C. The thickening of the extract was then continued in a water bath until it had a drying shrinkage of less than 10% (Widyaningsih and Afdaliah, 2020).

Gels were made with three formulas containing different EEUL concentrations: 0, 5 and 10%. Methylparaben was dissolved in hot water to create the gels, which were then allowed to cool to room temperature. The carbopol was added gradually to the mortar and then the methylparaben and carbopol dispersion were combined and mixed to achieve homogeneity. Triethanolamine was added to the mixture and swirled until a gel base was created. Finally, EEUL was added little by little to the gel base and mixed thoroughly until homogenous (Widyaningsih *et al*., 2021). Widyaningsih *et al.* (2021) evaluated the physical properties, dispersion and adhesion of 5% and 10% EEUL gels. Based on the results, the pH values are in the range of 4.5-7, the mean spread diameter is 5-7 cm and the pull-off time is less than 4 seconds (Widyaningsih *et al*., 2021). All of which are within their respective normal ranges and thus comply with the requirements for a gel dosage form (Irianto *et al*., 2020; Sayuti, 2015).

Animal model and experimental design

Balb/c mice (Mus musculus), which were employed in this study, were 6-8 week old animals weighing 35-40 gram. For seven days, the test animals were allowed to adapt to a predefined temperature of 18-26°C and 40-70% humidity in the Laboratory of Pharmacy at Ahmad Dahlan University, Yogyakarta. The cages received natural light and were exposed to the 12-hour light/dark cycle. Every morning, 3-4 grammes of AD2 were administered to the mice, and they had unlimited access to water. AD2 is composed of 13.5% water, at least 17% crude proteins, at least 7% crude fat, up to 6% crude fibre, 7% ash max, 0.9 and 1.2% calcium and 0.7-0.9% phosphorus. On the test day (Day 0) a shaved area with a diameter of 4 mm was created on the back of the mice. The mice were locally anaesthetised with Emla ointment, and general anaesthetic Ketamine HCl (50mg/ml) was administered subcutaneously at 100mg/kg BW. Afterwards, a 1 cm-long incision was made on the back skin using a scalpel (Nanda *et al*., 2017). The Ahmad Dahlan University research ethics committee has ethically authorised the care and handling practices for the test animals (No. 012011081).

Based on the given treatments, forty-five male Balb/c mice were equally separated into five groups, each consisting of nine test mice, namely pain control (no EEUL gel application), positive control (Hansaplast wound care ointment), negative control (gel base) and two treatment groups (5% and 10% EEUL gel). Every morning at 09:00 Indonesian Western Time (GMT+7), the EEUL gel was smeared along the wound (1 cm) on the back of the mice in the treatment groups, with the dosage of 500 mg once a day. With the same procedure and at the same time, the gel base was applied to that of the negative control. The wound length was measured using a calliper, and the time required to heal the wound was documented until it was tightly closed. Observations were made on Days 4, 7 and 14. The schematic of the wound healing attribute test is presented in fig. 1.

Histology of macrophages, blood vessels, epithelial thickness and fibroblast counts

The wound healing mechanism was observed from the histology of macrophages, blood vessels, epithelial thickness and the number of fibroblasts of the mouse skin samples on Days 4, 7 and 14. Prior to the mice being put to death via cervical dislocation, skin samples with a diameter of 2.5 cm were first collected. The tissue sample was then processed for histopathology according to the established technique at the Laboratory of Pathology, Faculty of Veterinary Medicine, Gadjah Mada University, which included the addition of haematoxylin-eosin (HE) staining. The histological sample was observed using a microscope connected to Optilap program at 400x magnification. The examined parameters were macrophages (scored based on density and distribution),

blood vessels (observed and counted at 400x magnification), the thickness of the epithelium (measured from the stratum basalis to the stratum corneum) and fibroblasts (counted one by one in each field of view). Each parameter was calculated for each histological sample at six selected fields of view. The number of blood vessels in each field was measured using the Image Raster program.

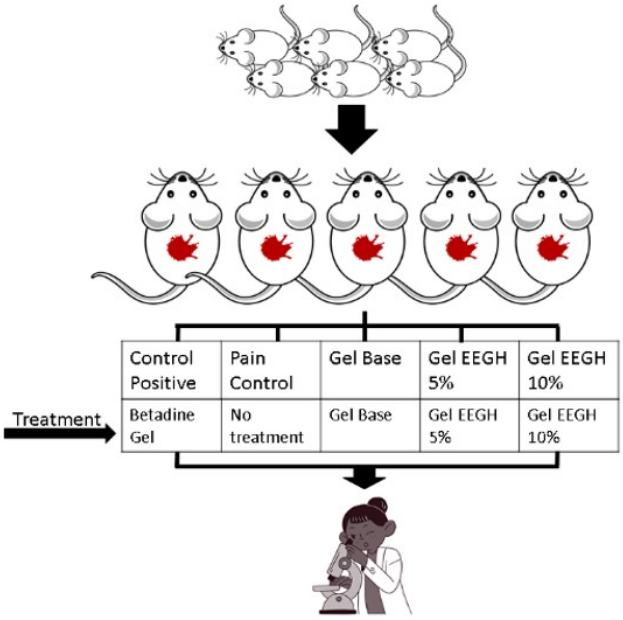

Fig. 1: Schematic of the wound activity test.

STATISTICAL ANALYSIS

The data obtained from the procedures above were processed statistically using the SPSS version 28. A one-way ANOVA with a 95% confidence level and a post-hoc least significant difference (LSD) test to differentiate between the experimental groups were used to statistically analyze the data generated from the aforementioned processes (Wenas *et al*., 2020).

RESULTS

Wound length and description

Fig. 2 displays the macroscopic post-treatment wound conditions of the gel base control and the 5% and 10% EEUL gel groups during the fourteen days of observation.

Table 3 provides the mean length of the incision wounds in each control and treatment group on the day of incision and four, seven and fourteen days after. When compared to the pain control and gel base controls, the 5% and 10% EEUL gels dramatically decreased the mean wound length seven days after the incision was performed. The decline persisted right up until day 14, when the observation was over.

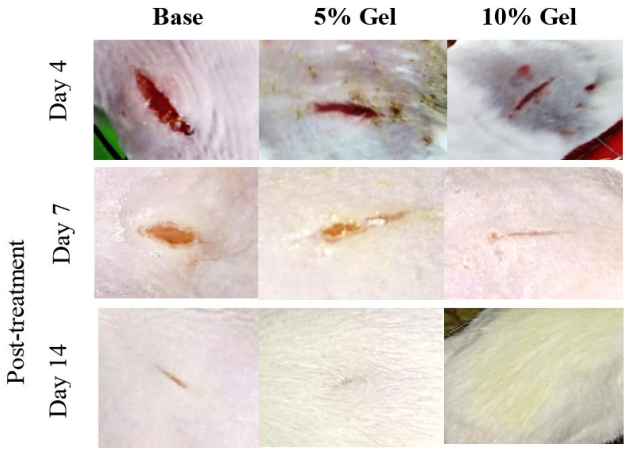

Fig. 2: Incision wound description of the treatment groups on Days 4, 7 and 14

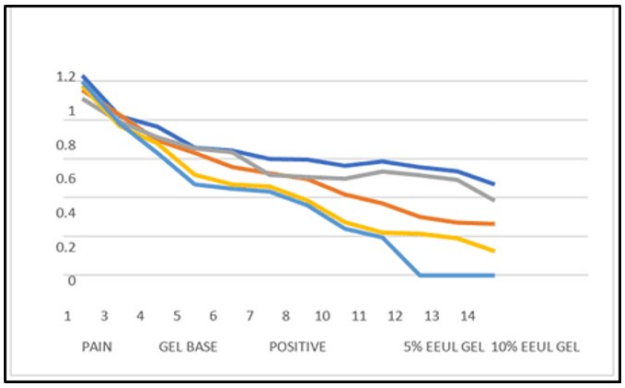

Fig. 3: Correlation between time and wound length in the control and treatment groups

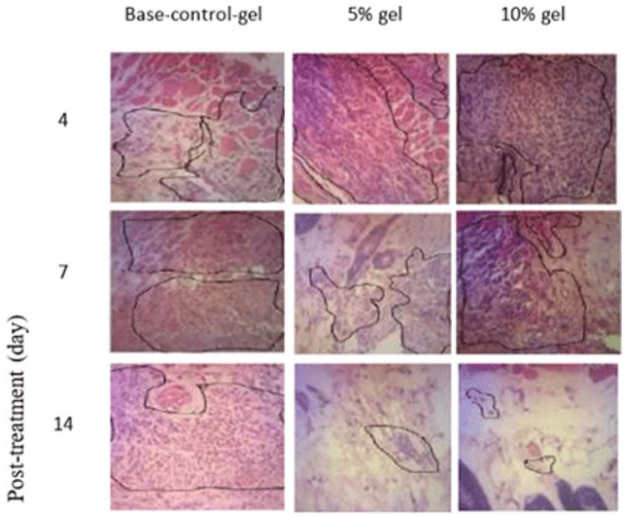

Fig. 4: Histology of the macrophages at the incision site of the gel base control and treatment groups on Days 4, 7 and 14.

Wound healing time

Fig. 3 compares the line plots connecting time with wound length in all control and treatment groups.

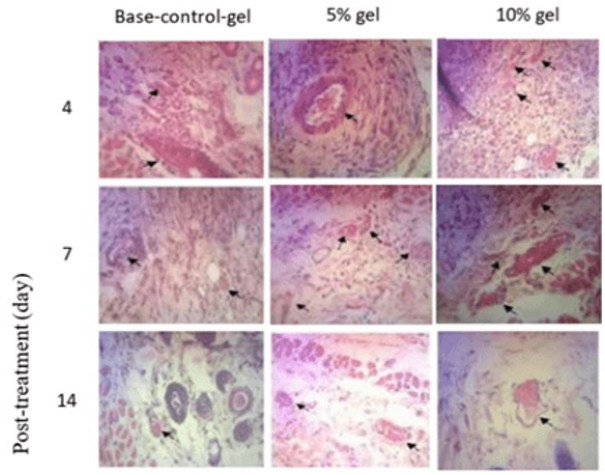

Fig. 5: Histology of the blood vessels at the incision site of the gel base control and treatment groups on Days 4, 7 and 14.

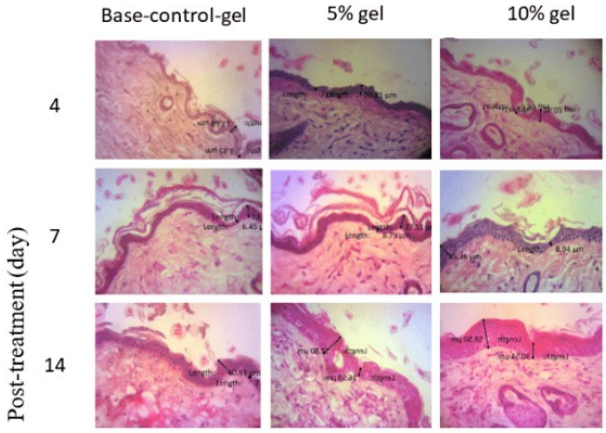

Fig. 6: Histology of the epithelial thickness at the incision site of the gel base control and treatment groups on Days 4, 7 and 14.

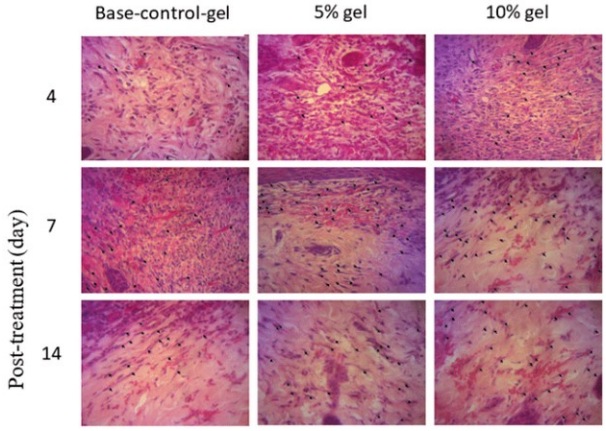

Fig. 7: Histology of fibroblasts at the incision site of the gel base control and treatment groups on Days 4, 7 and 14.

Macropharge scores

Fig. 4 and table 4 show the post-treatment histological description and mean score of macrophages four, seven and fourteen days after the injury.

Number of blood vessels

The post-treatment histology and mean number of blood vessels are displayed in fig. 5 and table 5.

Epithelial thickness

Based on the histology and the average epithelial thickness presented in fig. 6 and table 6, there was an increase in thickness in the proliferative phase.

DISCUSSION

Wound length and description

Incision wounds treated with 5% and 10% EEUL gels had started to close by Days 7 and 14, whereas the wound receiving only the gel base was still wide open and swollen, and the pus was not dried out. In the 5% EEUL gel group, the wound showed no inflammation, but a little pus was still present. However, in the group administered with a higher concentration of EEUL, 10%, a closed wound with no inflammation and no pus was observed on Days 7 and 14. In other words, the incision site was dry and not infected.

Table 2 summarises the wound description scores on the days of observation. Evidently, the topical administrations of EEUL gels at 5% and 10% on Day 7 resulted in significantly different scores from the pain control, gel base control and positive control. Also, as observed on Days 4, 7 and 14 the 10% EEUL gel group had substantially lower wound scores than all the control groups ($p<0.05$).

The topical administrations of 5% and 10% EEUL gels prove effective in healing wounds. It is evident in the wound descriptions: no inflammation, no swelling, no festering wound, dried and tightly closed wound and decreased wound length 14 days after the incision to the back skin. The wound healing effects may be due to the melatonin, flavonoid, chlorophyll and tannin contents in EEUL gels. Flavonoids work as an anti-inflammatory that helps reduce inflammation at wound sites by stimulating the formation of new epithelial cells (cell regeneration) that ends the inflammatory phase (Yunanda and Rinanda, 2017). Melatonin and chlorophyll have antioxidant properties that prevent damage due to oxidative stress at the incision site (Yunanda and Rinanda, 2017). Antioxidants help wound contraction and increase the re-epithelialisation process in the proliferative phase to form new tissue. Tannin is an antibacterial that inhibits bacterial growth in the wound area, preventing infections (Bunganaen *et al*., 2020). In this study, wounds that healed 12 days after the injury without any signs of infection indicate the active role of the compounds in EEUL gel.

Table 1: Formulas of gels containing 5% and 10% ethanol extract of *Ulva lactuca* L.

Ingredients	Gel Base	EEUL Gel 5% (g)	EEUL Gel 10% (g)
EEUL	0	5	10
Carbopol 940	0.1	0.1	0.1
Triethanolamine	2	2	2
Methyl paraben	0.2	0.2	0.2
Aquadest	ad 100 ml	ad 100 ml	ad 100 ml

Table 2: Wound description scores of the control and treatment groups on days 4,7 and 14

Groups	Wound description scores (mean ± SD)		
	Day 4	Day 7	Day 14
Pain control	2.60±0.00	2.40±0.00	1.46±0.23
Gel base control	2.57±0.06	2.56±0.08	1.20±0.00
Positive control	2.40±0.00	2.00±0.00	1.00±0.00
EEUL gel 5%	2.20±0.00	1.53±0.16*#	1.00±0.00*
EEUL gel 10%	1.66±0.17*#	1.10±0,10*#	1.00±0.00*

*Significantly different from the pain control and gel base control
#Signficantly different from the positive control

Table 3: Mean wound length in the control and treatment groups on days 4, 7 and 14

Groups	Wound lengths (mean ± SD, cm)		
	Day 4	Day 7	Day 14
Pain control	0.73±0.04	0.60±0.09	0.35±0.06
Gel base control	0.69±0.06	0.52±0.04	0.30±0.02
Positive control	0.71±0.09	0.51±0.07	0.23±0.02
EEUL gel 5%	0.68±0.05	0.47±0.05*	0.19±0.02*
EEUL gel 10%	0.63±0.07	0.43±0,03*	1.00±0.00*

*Significantly different from the pain control and negative control (gel base)

Table 4: Mean macrophage scores of the control and treatment groups on days 4, 7 and 14

Groups	Macrophage scores (mean ± SD)		
	Day 4	Day 7	Day 14
Pain control	1.33±0.00	3.22±0.19	2.63±0.34
Gel base control	2.11±0.38	2.67±0.34	2.44±0.20
Positive control	2.1±0.173	1.89±0.19	1.76±0.386
EEUL gel 5%	3.67±0.33*	1.89±0.19*	1.00±0.00*
EEUL gel 10%	3.11±0.19*	1.55±0,39*	1.11±0.18*

*Significantly different from the pain control and negative control (gel base)

Table 5: Mean number of blood vessels in the control and treatment groups on Days 4, 7 and 14

Groups	Number of blood vessels (mean ± SD)		
	Day 4	Day 7	Day 14
Pain control	23.00±4.00	31.33±6.51	28.67±6.81
Gel base control	43.00±7,21	79.00±8.54	62.33±9.50
Positive control	73.67±6.50	150.33±11.50	116.33±13.05
EEUL gel 5%	97.00±7.54*#	181.33±11.37*#	142.67±10.02*#
EEUL gel 10%	111.33±8.504*#	241.67±9,712*#	177.00±11.78*#

*Significantly different from the pain control and negative control (gel base)
#Significantly different from the positive control

Table 6: Mean epithelial thickness in the control and treatment groups on Days 4, 7 and 14

Groups	Epithelial thickness (mean ± SD, mm)		
	Day 4	Day 7	Day 14
Pain control	8.68±1.08	15.43±1.62	24.37±2.00
Gel base control	11.41±1.19	16.57±1.43	24.82±1.60
Positive control	13.20±1.22*	18.76±1.65*	26.54±1.76*
EEUL gel 5%	13.92±1.46*	19.67±1.84*	37.07±1.68*#
EEUL gel 10%	15.32±1.37*#	21.09±1.82*#	43.60±2.49*#

Table 7: Mean fibroblast count of the control and treatment groups on Days 4, 7 and 14

Groups	Fibroblast count (mean ± SD, CFU-Fs)		
	Day 4	Day 7	Day 14
Pain control	52±2.00	82±1.00	64.66±1.52
Gel base control	58±2.64	84.66±2.08	68.33±2.51
Positive control	83.66±2.08*	120.33±1.52*	93±2.64*
EEUL gel 5%	97±1.00*#	146.67±2.08*#	118.33±1.52*#
EEUL gel 10%	104±2.00*#	182.66±1.52*#	139±2.00*#

*Significantly different from the pain control and carrier (gel base)
#Signficantly different from the positive control

Macrophage scores

Treating the incision wounds with 5% and 10% EEUL gel started to increase the number of macrophages on Day 4, and a reduction was observed on Days 7 and 14. Throughout the length of the observation, the mean macrophage scores of the 5% and 10% EEUL gel groups were significantly different (sig.<0.005) from the negative control and the pain control. An increase in the number of macrophages indicated the wound-healing activities of the 5% and 10% EEUL gels.

Four days after the incision, macrophage counts rise (see tables 4 and 5) and on the seventh day, a sizable number of new blood vessels can be seen. These findings highlight the crucial function of macrophages in the inflammatory phase (Day 4) and the proliferative phase, which has the largest peak of activity on Day 7, where new blood vessels are formed (Fitrian, 2018; Nofikasari *et al*., 2017).

Number of blood vessels and epithelial thickness

The mean vessel counts in the 5% and 10% EEUL gel groups were significantly different (sig. 0.005) from the pain control, gel base control and positive control after 14 days of observation. These outcomes represented the 5% and 10% EEUL gels developed for the study's wound-healing activities.

The epithelium kept getting thicker right up until the last day of the observation. From Day 4 to Day 14, there was a significantly greater increase in epithelial thickness in the 10% EEUL gel group compared to the pain control, gel base control, and positive control. Similar to the pain control group, a substantial rise was seen in the group receiving 5% EEUL gel between Days 4 and 7 (compared to the pain control and gel base control) and then on Days 14 (compared to the pain control, gel base control, and positive control).

Wound healing process

On day 4, few fibroblasts were generated in each test animal, as evidenced by the histology and the typical number of fibroblasts at the incision site (fig. 7 and table VII). By day 7, though, both the control and treatment groups' numbers had risen. Then, on day 14, it decreased for all groups tracked.

U. lactuca's capacity to hasten wound healing is shown by the thickening epithelium and rising fibroblast count in the groups that received 5% and 10% EEUL gels during the observation period. As a result, the groups receiving 10% EEUL gel, 5% EEUL gel, the positive control, gel base control and then pain control show an increase in epithelial thickness and fibroblast count in the proliferative phase, from highest to lowest. Additionally, substantial variations in the two parameters between the control and treatment groups imply that *U. lactuca*'s metabolite composition synergistically speeds up wound healing. It is feasible because more *U. lactuca* metabolites are present in the gel formulation when greater EEUL concentrations are employed.

Thickening epithelial and increasing number of fibroblasts in the treatment groups may be due to the melatonin content in the EEUL gel. Melatonin can act as an antioxidant by donating its electrons and increasing the ratio of anti-inflammatory cytokines to pro-inflammatory cytokines. These processes speed up the healing of wounds by inhibiting the inflammatory response, causing fibroblasts to produce collagen and inducing angiogenesis

(Fujiwara and Kobayashi, 2005). Within a few hours of the injury, epithelial cells start migrating toward the wound's edge. Later, a single layer of epithelial cells forms over the lesion, and the mitotic activity of the epithelial cells surrounding the wound's borders increases. Cells migrate through the wound and adhere to the matrix. Once the epithelial cells adhere to one another, the migration process is complete and a basement membrane starts to take form (Velnar *et al*., 2009). The proliferative phase, which starts three days after the damage and lasts for roughly two weeks, is when the number of fibroblasts grows. Fibroblast migration and the deposit of newly synthesised extra cellular matrix, which serves as a temporary tissue replacement and contains fibrin and fibronectin, are features of this phase. In this stage of wound healing, there is profuse granulation tissue production on a macroscopic level (Diegelmann and Evans, 2004).

The ratio of M2 polarisation (anti-inflammation) to M1 polarisation (pro-inflammation) is increased by melatonin in macrophages, shortening the inflammatory phase (Liu *et al*., 2020). Both acute and chronic inflammation can be decreased by melatonin and its metabolites (Favero *et al*., 2017) and thereby exert anti-inflammatory effects by blocking transcription factors like activator protein-1 (AP-1), hypoxia-inducible factor (HIF)-1, nuclear factor erythroid 2-related factor 2 (Nrf2) and nuclear factor kappa-B (NF-kB), increasing super oxide dismutase (SOD), catalase (CAT), glutathione per oxidase (GPx), and immune responses triggered by helper T cells while decreasing the expression of COX-2 and inducible nitric oxide synthase (iNOS) and decreasing the production of adhesion molecules and neutrophil infiltration in tissues (Rana, 2018; Tordjman *et al*., 2017). These procedures lessen the production of reactive oxygen species (ROS) and inflammation. Melatonin is a highly intriguing antioxidant to research in this instance, especially for the treatment of inflammation (Reiter *et al*., 2020).

U. lactuca also includes tocopherols, flavonoids, vitamin C, and collagen in addition to the substances mentioned above. Tocopherols are an antioxidant that can hasten the healing of wounds by halting further damage brought on by skin incision wounds (Arief and Widodo, 2018). The development of new blood vessels and blood vessel dilatation are both facilitated by flavonoids (Murti, 2017). Prolyl hydroxylase, which is activated by vitamin C and can stop bleeding caused by blood vessel tears in vascular connective tissue, aids in the formation of collagen (Arief and Widodo, 2018; Pakaya, 2017).

CONCLUSION

Ulva lactuca L's ethanol extract, also known as EEUL (ethanol extract of green algae), can be made into gels that meet the necessary physical requirements. Topical application of EEUL gels in concentrations of 5% and 10% decreases wound description score and length, shortens the time needed for healing, and boosts epithelial thickness and fibroblast count. Both EEUL gels heal incisional wounds by increasing the number of fibroblasts, blood vessels, epithelial thickness and macrophage scores.

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