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Research Article

The Effect of Red Algae (*Eucheuma cottonii*) Nanoemulgel on Cytokine Il-1 Beta Expression for Traumatic Ulcers Healing

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Abstract

Red algae (*Eucheuma cottonii*) is a type of seaweed (*Rhodophyceae*). *Eucheuma cottonii* contains flavonoids and florotanin as an anti-inflammatory agent. The extracted red algae were formulated into nanoemulgel. This study aimed to determine the effect of red algae nanoemulgel (*Eucheuma cottonii*) in the expression of IL-1β cytokines on the healing of traumatic ulcers. The researcher applied true experimental research with a post-test-only control group design. This research was conducted by giving *Eucheuma cottonii* nanoemulgel concentrations of 20%, 35%, 50%, positive control, and negative control at the labial once a day, followed by evaluation using the IHC method**.** The results revealed that *Eucheuma cottonii* nanoemulgel extract affected the expression of IL-1β cytokines in healing traumatic ulcers by decreasing the amount of IL-1β cytokine expression. The results obtained a p-value of 0.05, which indicates a significant difference in the expression of the number of IL-1β cytokines in traumatic ulcers. This study concluded that the 20% *Eucheuma cottonii* extract nanoemulgel treatment group affected the amount of IL-1β by decreasing the expression of IL-1β cytokines. Nanoemulgel of 20% *Eucheuma cottonii* extract is considered effective as an anti-inflammatory material for traumatic ulcer healing.

Keywords: red algae (*Eucheuma cottonii);* nanoemulgels; cytokine; traumatic ulcer

INTRODUCTION

An ulcer is a pathological state characterized by a loss of epithelial tissue preceded by vesicles or *bulae*, indicating the presence of an imbalance in the oral cavity.[1,2] Ulcers in the oral cavity often occur in women aged 16-25 years and rarely occur at the age of 55 years.[3] The prevalence of traumatic ulcers occurs variably in several countries, in Indonesia 60.5%, Thailand 13.2%, and Malaysia 12.4%.[4]

The etiopathogenesis of the ulcer is still not clearly known, but these lesions can appear due to several factors, such as genetic viral and bacterial infections, food allergies, vitamin deficiencies, stress, and mechanical injuries. Ulcers that appear due to chemical, mechanical, physical, and other pathological injuries are called traumatic ulcers. The initial stage of the appearance of lesions begins with the formation of ulcers and the release of pro-inflammatory cytokines, monocytes T, and lymphocytes in mast cells, as well as plasma cells accumulated in basal cells. In the next stage, polynuclear leukocytes will dominate the central ulcer and the boundary part of the lesion formed by the accumulation of mononuclear cells.[5,6]

There are three stages of healing a traumatic ulcer: inflammation, proliferation, and maturation. The initial stage of the inflammatory phase is

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characterized by the appearance of ulcers on the surface of the mucosa and an increase in pro-inflammatory cytokines and activating immune cells in the area of infection. The increasing number of pro-inflammatory cytokines can cause tissue damage. Hence, the body needs anti-inflammatory cytokines to compensate for the number of pro-inflammatory cytokines.[7] Pro-inflammatory cytokines are produced by macrophages that have been activated and involved in the occurrence of inflammation. P-inflammatory cytokines include IL-1, β IL-6, and TNF-α, which are secreted by neutrophils.[8,9,10] IL-1β is part of IL-1, which also consists of IL-1α. IL-1β is also a powerful pro-inflammatory cytokine and plays a role in the inflammatory process, which will destroy the rest of the extracellular matrix, release proteases, and be stimulated by lymphocytes, macrophages, and monocytes.[9,11] Anti-inflammatory cytokines are needed to balance the production of cytokines that cause inflammation so that inflammation can run well. Anti-inflammatory cytokines produced by the body are IL-10 and TGF-β which function to inhibit the production of pro-inflammatory cytokines (TNF, IL-1, chemokines, and IL-12) and inhibit macrophage function by suppressing the expression of MHC-II and dendritic cells for T-cell activation.[7]

Indonesia is a maritime country that has many biological sources, such as seaweed, that are sourced from the sea. Seaweed is divided into four types, namely *Chlorophyceae* (green algae), *Rhodophyceae* (red algae), *Cynophyceae* (blue algae), and *Phaeophyceae* (brown algae).[12] Seaweed has bioactive components such as sulfate polysaccharides, including laminaran, carrageenan, fucoidan, and ulvan, as well as flavonoids, especially florotanins, carotenoids, especially fucoxanthin, galkaktan sulfta, and ketin.[13] In addition, red algae (*Eucheuma cottonii*) can also produce carrageenan as a polysaccharide that can be used as an antibacterial, anti-inflammatory, and antipyretic.[14] Extract of 20% *Eucheuma cottonii* is more effective in the wound healing process.[15]

Most of these traumatic ulcers are often cured with therapeutic drugs for pain relief; therapeutic drugs for traumatic ulcers are usually available in the topical dosage form. Topical preparations have the disadvantage that particles are larger in size and have slower penetration. Thus, other preparations, such as nanoemulgel,[16] must be developed. Nanoemulgel is an emulgel preparation made with a nanotechnology system and has small particles.[17] The smaller it is, the easier it is to dissolve in liquids to accommodate partitions in the oral mucosa. Therefore, it can be concluded that nanoemulgel preparations have a higher penetration rate into the oral mucosa compared to other preparations, such as gels.[18] For the nanoemulgel formulation, *Eucheuma cottonii* will be washed and ground until smooth and then soaked in an alcohol solution. Then, it was filtered to separate *Eucheuma cottonii* powder from *Eucheuma cottonii* alcohol extract. Islam forbids its followers to consume alcohol, but some studies state that the use of alcohol is permissible. This is allowed if the alcohol is formed naturally through the fermentation process and the alcohol produced by anaerobic fermentation has a content of around 1%-15%. The alcohol is considered *non-halal* (*haram*). However, it is permissible for the industry and production of non-drinking drugs.[19] This study aimed to determine the effect of red algae nanoemulgel (*Eucheuma cottonii*) in the expression of IL-1β cytokines on the healing of traumatic ulcers.

MATERIALS AND METHODS

The type of research carried out was experimental analytical research using post-test-only control group design. This study was conducted by giving *Eucheuma cottonii* nanoemulgel concentrations of 20%, 35%, and 50% positive control using hyaluronic acid gel Gengigel and negative

control without treatment in each group to determine the amount of IL-1β.

The ingredients used in this study include male Wistar rats, red algae (*Eucheuma cottonii),* ethanol, ketamine (Guardian Pharma *Made in Indonesia),* aquadest, *palm oil*, tween 80 (Merck Milipore *Made in* Germany), hematoxyline mayer (Pollar Harmony *Made in* Indonesia), propyllenglikol (*Dow Chemical, Georgia Gulf Corporation Made in* Georgia), carbopol 940 (Sigma Al-Drich *Made in* California), methyl parabn, propyl parabean (Sigma Al-Drich *Made in* California), *neutral* buffer formalin 10%, primary antibody IL-1β (Bio*care Medical Made in California),* secondary antibody IL-1β (Bio*care Medical Made in California),* HCl (*Dow Chemical, Georgia Gulf Corporation Made in* Georgia), xylol, nipasol, aquadest, glycerin, methanol, $FeCl_3$ and nipagin. Meanwhile, the tools used in this research include shakers, scales, stirrers, round burnishers, Bunsen spiritus burners, surgical scissors, microscopes, glass objects, and ultrasonic stirrers.

The first step taken in this study was to make a nanoemulsion of red algae extract, which had been extracted using the maceration method of 250 grams using methanol as much as 1000 mL.[8] The prepared red algae were weighed and washed. Two hundred fifty grams of red algae that had been ground using the maceration method using 1000 mL of methanol solvent for 24 hours was extracted. Mixing of red algae was done manually by stirring the solution every day for 3 days at room temperature until clear. The solution was filtered and evaporated with a rotary evaporator at a temperature of 40-50oC until the extract became thick. The manufacture of nanoemulsions is divided into 3 phases, namely the oil phase, the water phase, and the surfactant phase. The water phase is formed by mixing red algae extract with palm oil and 10 grams of propylene glycol, 0.1 grams of methyl parabean, and 0.02 propyl parabean and mixing using *a* magnetic stirrer at a speed of 1000 rpm for 2 hours. The next stage mixes 3.6 grams of tween 80 as surfactant and aquadest as solvent and ethanol using a homogenizer for 2 hours at a speed of 2000 rpm at a temperature of 30^{o}F to form phased water. After the water phase temperature decreased, it was carried out by entering the oil phase using the titration method using a mechanical stirrer for 6 minutes at 2000 rpm and continued by stirring for 25 min using an ultrasonicator until homogenous.[19]

After making the nanoemulsion by conducting particle analysis tests to prove the size of nanoemulsion particles, it continued with the making of nanoemulgel. Then, nanoemulgel was made by mixing the gel base with the red algae extract nanoemulsion. The gel base was prepared by mixing 0.1 gram of methyl parabean and 0.05 gram of propyl parabean into distilled water (aquadest). After that, mixing 2 grams of carbopol 940 into a measuring cup and continue to insert TEA using a pipette until a gel base is formed. Insert the red algae extract nanoemulsion into the gel base and stir using a *stirrer* at 500 rpm for 15 minutes.[19]

The grouping of samples is carried out by dividing 5 groups as follows: 1) Red algae nanoemulgel treatment group 20%; 2) Red algae nanoemulgel treatment group 35%; 3) Red algae nanoemulgel treatment group 50%; 4) Positive control group with a hyaluronic acid gel; 5) Negative Control group.

Making traumatic ulcers in experimental animals was carried out by anesthetizing the experimental animals using ketamine, which was injected intramuscularly in experimental animals. Experimental animals that are already moving less actively on the labial mucosa of the experimental animal have touched a round burnisher that has been heated for 10 minutes on Bunsen spiritus. Observations were made at 24 hours and 48 hours post-ulcer making.[20] The procedure for applying red algae nanoemulgel was carried out as

much as 1x a day for 3 days after the formation of ulcer.[21]

IHC staining is carried out by making tissue preparate, which is deparaffinated and stained indirectly using *Bioss* brand IL-1 antibodies, and βtissue reading and analysis are carried out using a microscope.[22] This research has received approval from the Health and Medical Research Ethics Commission of the Faculty of Dentistry, Sultan Agung Islamic University, Semarang (No.316/B.1-KEPK/SA-FKG/X/2021). Researchers conducted at the Bionanotechnology Laboratory of Diponegoro University (UNDIP), the Experimental Animal Laboratory of the Faculty of Medicine, Sultan Agung Islamic University (UNISSULA), the Anatomy Pathology Laboratory of the Hospital Islam Sultan Agung (RSISA), and the Laboratory of Anatomical Pathology of Sebelas Maret University (UNS).

RESULT

The results showed IL-1β expressions from labial preparation per group.

Traumatic ulcers are observed at 24 hours and 48 hours post-treatment until an ulcer with a yellowish base is formed. After the ulcer is formed, the smearing of nanoemulgel is carried out in the morning for 3 days. Tissue retrieval in experimental animals is carried out by dislocating the neck and then cutting the oral mucosa of the experimental animal that has formed an ulcer. The ulcer sample that has been obtained is put in formalin for 3 days, and then a paraffin block is performed. After the formation of paraffin blocks, histological preparations and IHC staining are manufactured. Figure 1, 2, 3, 4, and 5 shows the expression of the IL-1β cytokine observed using a microscope with a magnification of 400x and 5 field of view. The average number of IL-1β cytokines obtained in the study is presented in Table 1 below:

Table 1. The average number of cytokines in 3rd days

	Group	Mean ± SD
IL-1β	I	4.87 ± 2.87
	II	11.01 ± 7.53
	III	17.09 ± 6.63
	IV	12.52 ± 5.09
	V	28.49 ± 7.20

Based on Table 1, it was known that the 20% (Group I) *Eucheuma cottonii* extract nanoemulgel treatment group had the least amount of IL-1β cytokine expression. The negative control group (Group V) got the highest number of IL-1β cytokine expressions.

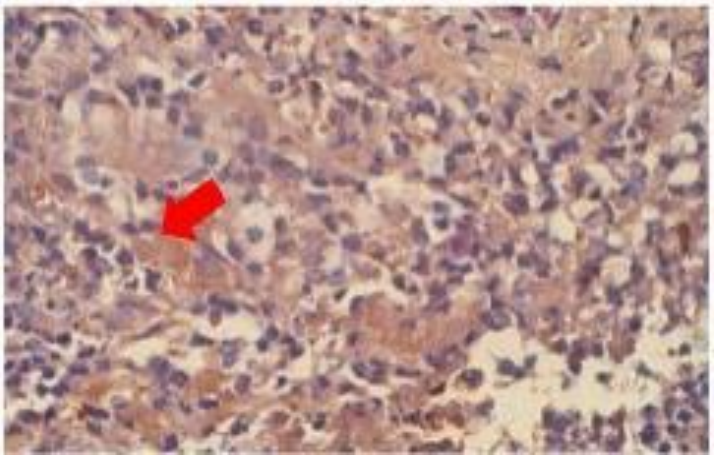

Figure 1. Histologic overview of the 20% *Eucheuma cottonii* extract nanoemulgel treatment group using a microscope magnification 400x. Red arrow: expression of IL-1β cytokines (purple and coccus-shaped).

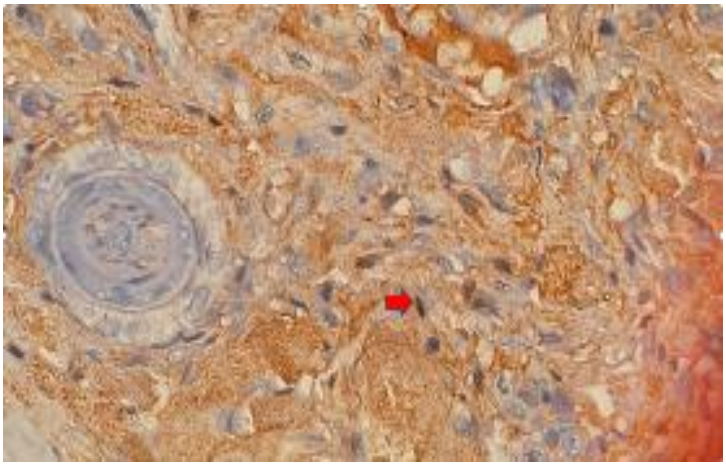

Figure 2. Histologic overview of the *Eucheuma cottonii* extract 35% nanoemulgel treatment group using a microscope magnification 400x.

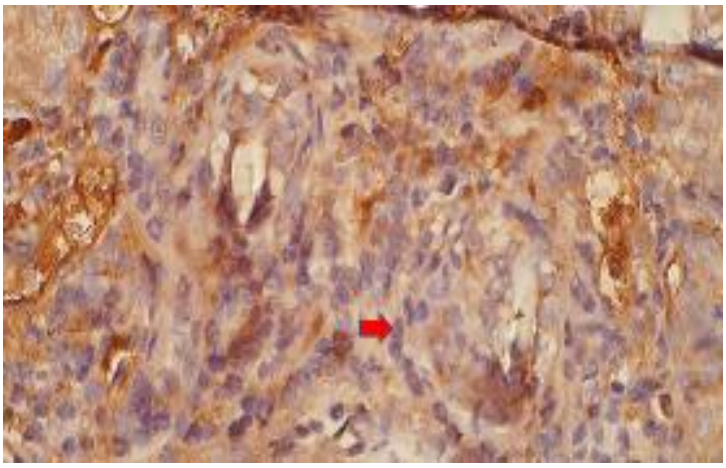

Figure 3. Histologic overview of the *Eucheuma cottonii* extract 50% nanoemulgel treatment group using a microscope magnification 400x

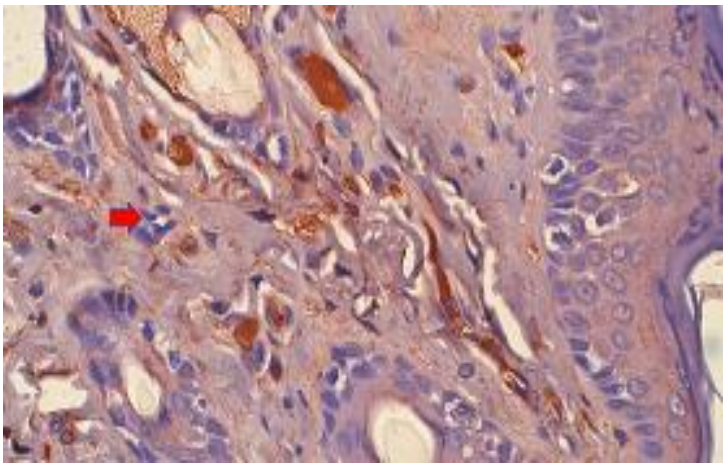

Figure 4. Histologic overview of the hyaluronic acid gel treatment group as positive control using a microscope magnification 400x.

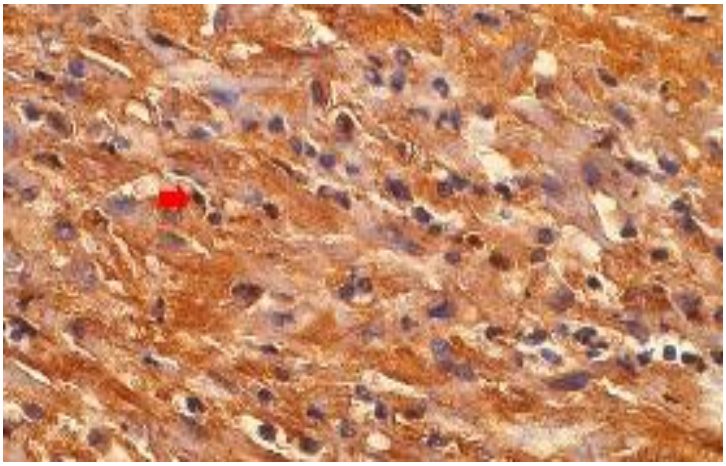

Figure 5. Histologic overview of the without treatment group as negative control using a microscope magnification 400x

Meanwhile, Figure 1, 2, 3, 4, and 5 shows a comparison of the histological picture with a magnification of 400x and shows damaged tissue and healthy tissue.

Table 2. Normality Test Analysis Results (Shapiro-Wilk)

	Group	*Shapiro-Wilk*
IL-1β	I	0.192
	II	0.174
	III	0.932
	IV	0.148
	V	0.404

The normality test can be seen in Table 2, which shows the results that the 20% *Eucheuma cottonii* extract group Nanoemulgel, 35% *Eucheuma cottonii* extract Nanoemulgel, 50% *Eucheuma cottonii extract* Nanoemulgel, the positive control group and the negative group have a p>0.005 value indicating normal data distribution.

The results of the homogeneity test can be seen in Table 3. The homogeneity test results showed p>0.005 (p = 0.417), so the results showed that the study carried out was homogeneous.

Table 3. Homogeneity Test Analysis Results (Levene Test)

	Statistics	*Levene Test*
IL-1β	Based on the average value	0.417

Based on the normality and homogeneity test, homogeneous data were obtained, and all groups were distributed normally. The hypothesis test was carried out using the *One-Way ANOVA* parametric test.

Table 4. Analysis Results of Different Tests in Groups (One Way ANOVA)

	Group	*One Way ANOVA*
IL-1β	I	0.000
	II	
	III	
	IV	
	V	

The results of the *One-Way ANOVA* test can be seen in Table 4, which shows the value of p<0.05 (p=0.000) that there is a significant difference in the expression of the IL-1β cytokine between the study groups. Furthermore, *a Post Hoc LSD (Least Significant Difference)* test was carried out to determine the differences between the 2 experimental groups.

Table 5. Results of Differential Test Analysis between 2 Groups (*Post Hoc* LSD)

IL-1β	I	II	III	IV	V
I		0.128	0.005	0.062	0
II	0.128		0.132	0.701	0
III	0.005	0.132		0.252	0.008
IV	0.062	0.702	0.252		0.001
V	0	0	0.008	0.001	

Post Hoc LSD test results can be seen in Table 5. The results of intergroup treatment tests on the expression value of the amount of cytokine IL-1β were obtained: 1) Between the treatment groups, *Eucheuma cottonii* extract 20% nanoemulgel and *Eucheuma cottonii* extract *50%* nanoemulgel obtained results p = 0.005 (p<0.05), which means that there was a significant difference in the expression value of cytokine IL-1β.; 2) Between the *20% Eucheuma cottonii* extract nanoemulgel treatment group, and the negative control group, a result of p = 0.000 (p<0.05) was obtained, which meant that there was a significant difference in the expression value of the IL-1β cytokine; 3). Between the *35% Eucheuma cottonii* extract nanoemulgel treatment group, and the negative control group, a result of p=0.000 (p<0.05) was obtained, which meant that there was a significant difference in the expression value of the IL-1β cytokine; 4) Between the *50% Eucheuma cottonii* extract nanoemulgel treatment group, and the negative control group, a result of p=0.008 (p<0.05) was obtained, which meant that there was a significant difference in the expression value of the IL-1β cytokine; 5) Between the positive control group and the negative control group, a result of p=0.001 (p<0.05) was obtained, which means that there was a significant difference in the expression value of the cytokine IL-1β.

Table 6. Cumulative Results of Particle Size Analyzer (PSA) Test

PSA Test Cumulative Results	
Z-Avg	15.11 nm
PI	0.257 nm

Table 6 shows that the average size of nanoparticles in nanoemulsions is 15.11 nm with a PI value of 0.257.

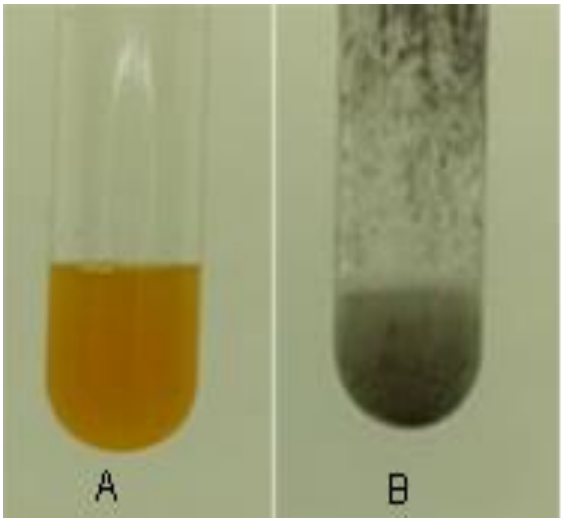

Figure 6. Test results of flavonoids (A) and tannins (B) assay

The results of the assay of flavonoids and tannins (Figure 6) indicated that the preparation positively contained flavonoids (Figure 6.A) and tannins (Figure 6, B).

Figure 7 shows (A) Red algae nanoemulgel treatment group 20%, (B) Red algae nanoemulgel treatment group 35%, (C) Red algae nanoemulgel treatment group 50%, (D) Positive control group with hyaluronic acid gel, (E) Negative Control group. The figure shows the difference between damaged tissue which loses its epithelium which is shown in yellow arrows and healthy tissue which is shown by red arrows.

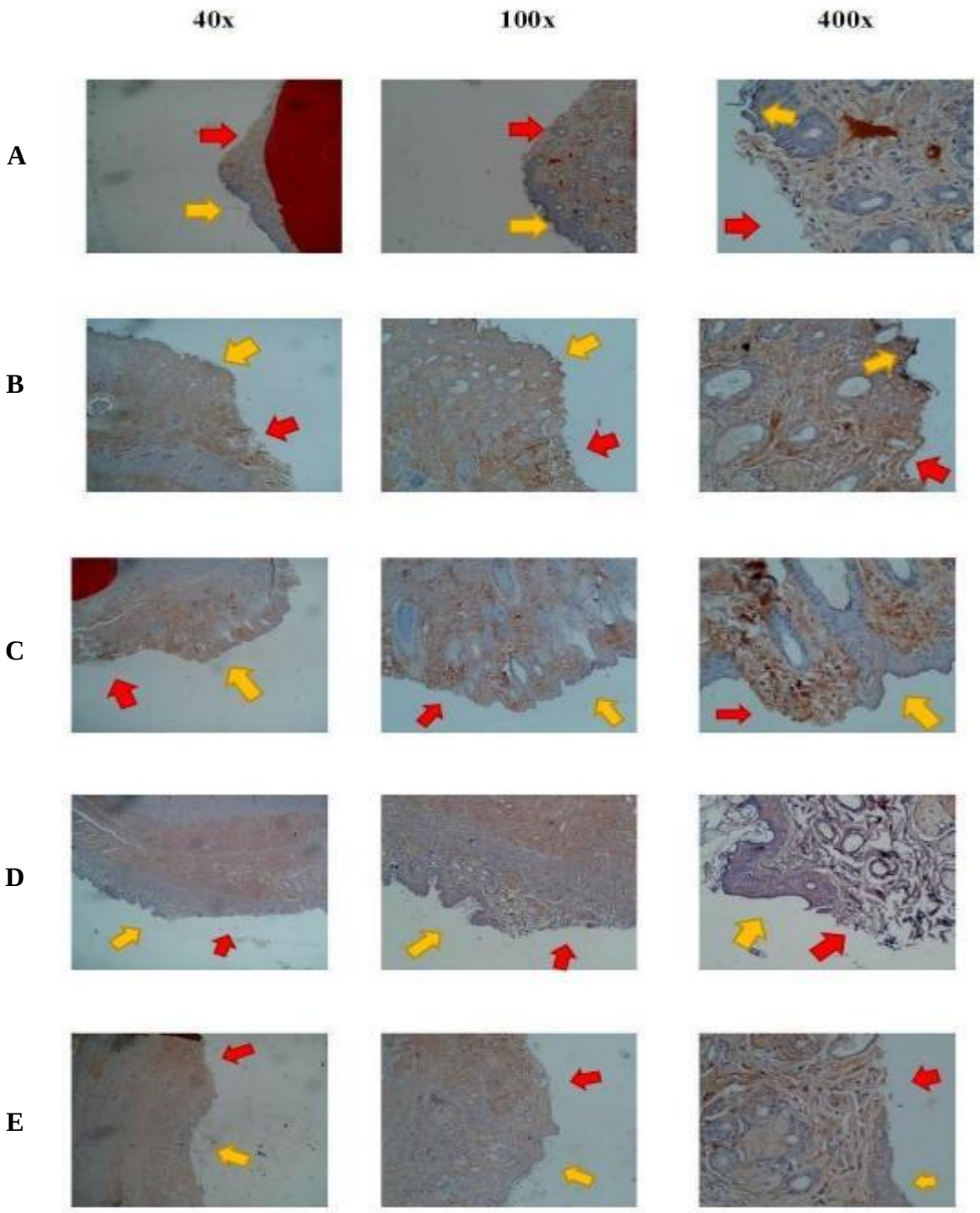

Figure 7. Histological overview of IHC magnification of 40x, 100x, and 400x. The figure shows the difference between damaged tissue, which loses its epithelium shown in yellow arrows, and healthy tissue, which is shown in red arrows. (A) Red algae nanoemulgel treatment group 20%, (B) Red algae nanoemulgel treatment group 35%, (C) Red algae nanoemulgel treatment group 50%, (D) Positive control group with hyaluronic acid gel, (E) Negative Control group.

DISCUSSION

Table 5 shows the use of 20% *Eucheuma cottonii* nanoemulgel, 35% *Eucheuma cottonii* nanoemulgel, and positive control influenced the expression of cytokine IL-1β as an anti-inflammatory by reducing the number of inflammatory cells in the extracellular matrix, allow cell migration and increase proliferation.[23,24] *Eucheuma cottonii* nanoemulgel is more effective at reducing the amount of IL-1β cytokines compared to positive controls by giving hyaluronic acid gel because the use of nanoemulgel preparations in *Eucheuma*

cottonii extract affects the wound healing process. After all, it has nano-sized particles and good adhesive properties, which can help drug penetration in the oral mucosa.[23] In addition, nanoemulgel extract with a concentration of 20% is more effective in the wound healing process compared to nanoemulgel with a higher concentration. According to previous research, the concentration levels of concentration levels of ethanol extract cannot be used as an appropriate reference in the process of wound healing. This is related to the nature of the preparation that affects penetration into the wound in the oral mucosa.[24]

The average size of nanoparticles in nanoemulsions can be seen in Table 6. It showed that the distribution mean of nanoparticles size is 15.11 nm with a PI value of 0.257. Based on research which stated that the poly states that the polydispersity index (PI) value that is close to zero indicates that the uniformity of globule size in the preparation is getting higher.[25] The PSA test obtained a size distribution value based on intensity in nanoemulsions ranging from 5.615 nm to 43.82 nm. This is in line with previous research, which stated that dynamic nenoemulsion as a stable and transparent emulsion has a particle size range of 5-200 nm.[26]

The *one-way ANOVA* test showed a value of P=0.000 (P<0.05), which means that there were differences between the treatment groups of *Eucheuma cottonii* extract nanoemulgel 20%, 35%, 50%, the positive control group (*hyaluronic acid)*, and the negative control group. LSD test results showed that there was a significant difference between the negative control group and the 20% treatment group, and the 20% to 50% treatment group. This is because the 50% preparation sample has a more stable viscosity, which affects the penetration of the nanoemulgel into the oral mucosa. The 20% and 35% treatment groups did not have a significant difference because the viscosity of the preparation which was almost resemblant. Hence, it could affect the penetration properties of the oral mucosa. In addition, the higher viscosity value has better stability.[26]

This study showed that there was an influence of *Eucheuma cottonii* nanoemulgel on the expression of IL-1β cytokines on the healing of traumatic ulcers.

CONCLUSION

The administration of *Eucheuma cottonii* nanoemulgel, which affects the amount of IL-1B cytokine by reducing IL-1B, shows that *Eucheuma cottonii* nanoemulgel acts as an anti-inflammatory. The administration of *Eucheuma cottonii* nanoemulgel on the 4th day shows that *Eucheuma cottonii* nanoemulgel is 20% more effective in reducing IL-1B cytokine expression compared to concentrations of 35% and 50%.

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