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## RESEARCH ARTICLE

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# The Effect of Extract of Red Pomegranate (*Punica granatum Linn*) on The Number of Macrophages of Ulser Traumatic of Rat Model

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## ABSTRACT

Therapy for traumatic ulcers using steroid causes serious side effects. Red pomegranate (*Punica granatum Linn*) can be used as alternative therapy which contains bioactive elements to reduce the number of macrophages in traumatic ulcers. The aimed of this study was to determine the potential of red pomegranate extract to reduce the number of macrophages in the rat model of traumatic ulcers. The 24 model rats were experimented within 3 groups including 8 animals each group (negative control using CMC-Na placebo, positive control using triamcinolone acetonide 0.1%, and treatment group using red pomegranate extract gel). Each group was divided into two (4 animals with a 3-day test and 4 animals with a 5-day test). Thermal-induced trauma was given to the labial mucosa of rats and then observing the number of macrophages histologically with 400x microscope magnification. The mean macrophages counted on the 3-day-tested groups of red pomegranate extract gel therapy, triamcinolone acetonide 0.1% therapy, and CMC-Na placebo therapy were 9.1, 16.5, and 22.3. While on the 5-day-tested groups, the mean of macrophages counted were 27.2, 23.6, and 28.9. In conclusion, red pomegranate extract had a significant effect in decreasing the number of macrophages in the inflammatory reaction of traumatic ulcer.

**Keywords:** red pomegranate; traumatic ulcer; macrophage; anti-inflammatory

## INTRODUCTION

Traumatic ulcers on the mucosa are one of the abnormalities in the oral cavity that can be caused by temperature trauma, physical trauma, chemical trauma, or electrical trauma<sup>(1)</sup>. The oral cavity has conditions that support the lesion of traumatic ulcers healing process by preventing the infection within 7 to 10 days. However, these lesions cause pain and discomfort that can reduce appetite so that the wound healing process is disrupted<sup>(2)</sup>. Inflammation in traumatic ulcers is the initial phase of wound healing that begins with the infiltration of neutrophils into the wound area which is then replaced by macrophages on the 3<sup>rd</sup> day or 72 hours after the trauma happened. Monocytes need approximately 48 hours to mature themselves and become macrophages, thus the number of macrophages is at its peak on the 5<sup>th</sup> day after the trauma happened<sup>(3,4)</sup>. Macrophages in the wound healing process get the job to clean apoptotic cells and phagocytize bacteria that enter the tissue as the body's mechanism to prevent further infection<sup>(5)</sup>. Inflammation in the wound healing process should be limited, if it occurs continuously it can cause abnormal wound healing process and become pathological inflammation. The longer it takes for macrophages to phagocytize infectious agents, the more pro-inflammatory cytokines will occur. The overproduction of pro-inflammatory cytokines can lead to ongoing inflammation and tissue injury leading to excessive pain or tenderness<sup>(6)</sup>.

The commonly done treatment for traumatic ulcers is by eliminating the local cause and applying topical medications like corticosteroids to reduce the inflammation. One of the most effective corticosteroids in the treatment of traumatic ulcers is triamcinolone acetonide 0.1%<sup>(7)</sup>. In several research cases, the usage of corticosteroid anti-inflammatory drugs can cause side effects such as a burning sensation and irritation, so many treatments are developed from natural ingredients such as red pomegranate (*Punica granatum Linn*) which contains bioactive ingredients that act as anti-inflammatory, antibacterial, antimicrobial, and antioxidant agents.

Pomegranate (*Punica granatum Linn*) contains polyphenol compounds that work as antioxidants in preventing several diseases. The antioxidant and anti-inflammatory properties of pomegranate are assumed to be caused by ellagic acid in free or bound forms, vitamin C, and flavonoids<sup>(8)</sup>. Pomegranate extract is also assumed to be more effective than the administration of only one active ingredient since it constitutes a combination of several active substances that form a synergistic, mutually formulating formula<sup>(9)</sup>. This study aimed to determine the potential of pomegranate extract to reduce the number of macrophages in the rat model of traumatic ulcers.

## METHODS

The study constituted experimental laboratory research using pretest and posttest control group design and involving the use of animal subjects received ethical approval from the Faculty of Dentistry, University of Jember (No.1050/UN25.8/KEPK/DL/2020). Before the procedure of making traumatic ulcers was performed, ketamine hydrochloride was injected into the thigh muscles of the subjects. A 15-40 mg/kg dose of general anesthesia was administered before a traumatic ulcer being created using a 2.5 mm diameter flame-shaped amalgam stopper tip that had been heated in a bunsen burner flame for  $\pm 15$  seconds before being applied to the lower lip mucosa of the subjects for one second. Observation of the traumatic ulcers formed was conducted 24 hours after the ulcer was made. By then, the ulcers were measured by placing a straight periodontal probe (UNC-15) on the vertical and horizontal sides as well as one of the diagonal sides to equalize the sizes of each ulcer<sup>(10,11)</sup>.

The 24 rats with traumatic ulcers were divided into six groups, namely; 2 treatment groups (3-day test and 5-day test), 2 positive control groups (3-day test and 5-day test), and 2 negative control groups (3-day test and 5-day tests). Each group contained four subjects which were isolated before the application using cotton buds of the designated material, namely; red pomegranate extract gel at 75% concentrations for the treatment groups, the application of triamcinolone acetonide 0.1% for the positive control groups, and the application of CMC-Na placebo gel for the negative control groups. Each ingredient was topically applied using a micro brush twice a day (at intervals of no more than 12 hours)<sup>(7)</sup>.

On the 4<sup>th</sup> day, the 12 rats of the 3-day test groups were then decapitated and then the labial mucosal tissue of the mandible was taken. While the other 12 rats of the 5-day test groups were decapitated on the 6<sup>th</sup> day and the labial mucosal tissue of the mandible was taken by then. The tissue of the traumatic ulcer area that had been taken was then soaked in formalin for 24 hours and then continued with making the microscope slide sets of the traumatic ulcer tissues using hematoxylin-eosin staining. After the slide sets were ready for use, then the counting of macrophages was done by 3 random fields of view in each microscope slide set by 400x magnification.

## RESULTS

Observation of macrophage cells using a light microscope with 400x magnification (Figure 1 and Figure 2) so that macrophages can be seen and has an oval or kidney-shaped nucleus located eccentrically<sup>(12)</sup>.

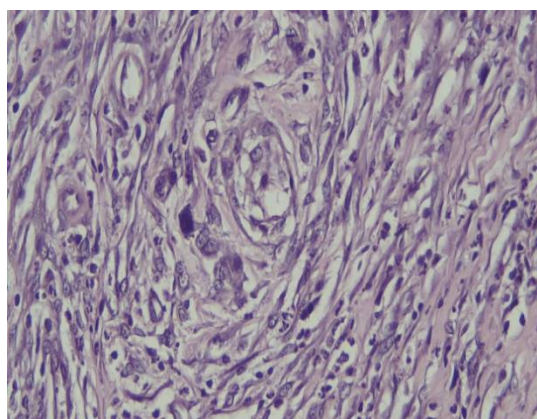


Figure 1. Histopathological appearance of the labial mucosa of 3-day-tested rat model of traumatic ulcer with CMC-Na placebo gel therapy using *Hematoxylin-Eosin* staining (400x magnification, transverse section)

The average value of the number of macrophages, as shown in Table 1, in the red pomegranate extract group was 9.1 on the 3<sup>rd</sup> and 17.1 on the 5<sup>th</sup> day. The average value of the number of macrophages in the triamcinolone acetonide group is 16.5 on the 3<sup>rd</sup> day and 23.6 on the 5<sup>th</sup> day. The mean value of the number of macrophages in the group CMC-Na placebo was 22.3 on the 3<sup>rd</sup> day and 28.9 on the 5<sup>th</sup> day. Data analysis was carried out using the Shapiro-Wilk normality test with the number of samples used is less than 50 samples. Data is said to be normally distributed if the value  $p > 0.05$ . The results obtained from the normality test macrophages

show all data distributed normally. The data is declared normal then Levene's test of homogeneity of variance was performed. From the results of Levene's test homogeneity test, the data the number of macrophages has a significance value of ( $p > 0.05$ ). The results show that data from the calculation of the number of macrophages have homogeneous variance or data comes from a population with the same variance. From the result calculation, it is found that all data is homogeneous, then the parametric test is carried out Anova with a 95% confidence level. In the ANOVA parametric test, as shown in table 2, it was found that there was a significant difference meaningful in the day group with  $p$ -value = 0.000 ( $p < 0.05$ ).

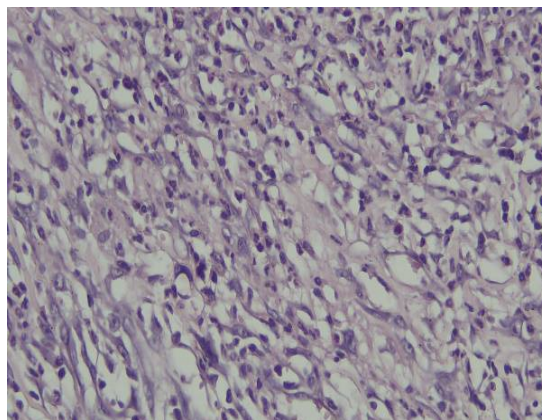


Figure 2. Histopathological appearance of the labial mucosa of 3-day-tested rat model of traumatic ulcer with the pomegranate extract gel therapy using *Hematoxylin-Eosin* staining (400x magnification, transverse section).

Table 1. The mean and standard deviation (SD) of the number of macrophage cells in each experimental group

Groups	3-day test (mean±SD)	5-day test (mean±SD)
Negative control	22.3±6.7	28.9±8.9
Positive control	16.5±3.7	23.6±4.6
Treatment	9.1±2.7	17.1 ±5.2

Table 2. The mean and standard deviation (SD) of the number of macrophage cells in each experimental group

ANOVA		
	F	p-value
Between groups	9.226	0.000

Table 3. The summary of the LSD test results of each experimental group (p-value)

Groups	NC3	NC5	PC3	PC5	T3	T5
NC3	-	0.054	0.087	0.695	0.01*	0.117
NC5		-	0.001*	0.113	0.00*	0.002*
PC3			-	0.001*	0.033*	0.871
PC5				-	0.00*	0.056
T3					-	0.023*

Note:

- NC3: 3-day-tested negative control group (CMC-Na placebo gel therapy)
- NC5: 5-day-tested negative control group (CMC-Na placebo gel therapy)
- PC3: 3-day-tested positive control group (*triamcinolone acetonide* 0.1% gel therapy)
- PC5: 5-day-tested positive control group (*triamcinolone acetonide* 0.1% gel therapy)
- T3: 3-day-tested treatment group (pomegranate extract gel therapy)
- T5: 5-day-tested treatment group (pomegranate extract gel therapy)

The data that has been obtained is next analyzed by using a follow-up test, namely the Post Hoc LSD, as shown in table 3, test to find out the group of days and which treatment shows the different meaning. In the LSD Post Hoc Test, the results obtained are that there is a difference significant with  $p < 0.05$  between several groups, namely: a) CMC-Na placebo 5-day test group with triamcinolone acetonide 3-day test group with a score of 0.001; b) CMC-Na placebo 3-day test group with CMC-Na placebo 5-day group with a score of 0.001; c) the red

pomegranate extract gel 3-day test group with all CMC-Na placebo groups and all triamcinolone acetonide groups; d) The red pomegranate extract gel 5-day test group with the CMC-Na placebo 3-day test group, the triamcinolone acetonide 5-day test group, and the red pomegranate extract gel 3-day test group.

The result showed that the red pomegranate extract (*Punica granatum* Linn.) has the fewer number of macrophages than the number of macrophages in the triamcinolone acetonide group and the CMC-Na placebo group.

## DISCUSSION

Based on the results of the study, the average number of macrophage cells in the negative control group, positive control group, and treatment group on day 3 was smaller than on day 5 in the same group. This is because theoretically the infiltration of monocyte cells (the origin of macrophages) into the injured area only starts after 3 days of injury. The results of the study on day 3 also showed that the treatment group had a smaller average number of macrophages than the negative control group and positive control group on day 3. These results prove that red pomegranate extract can inhibit the migration of macrophage cells to the wound area since the beginning of macrophage infiltration. This is confirmed by the content of ellagic acid in red pomegranate which has anti-inflammatory activity. Ellagic acid can reduce the inflammatory process through inhibition of Nf- $\kappa$ B activation so that there is a decrease in the synthesis of IL-1 which plays a role in the migration activity of monocytes and macrophages<sup>(13)</sup>.

In the 5-day test of the negative control group, the positive control group, and the treatment group showed a higher number of macrophages compared to the 3-day test in the same group. This is following the theory that macrophages are most abundant in the tissue on day 5 because after monocytes migrate to the tissue, it takes 48-72 hours to differentiate into macrophages in the area of injury. The results of the study on the 5<sup>th</sup> day showed that the treatment group had a lower mean number of macrophages than the negative control group and the positive control group. This can be caused by the bioactive ingredients of red pomegranate extract which can inhibit macrophage migration<sup>(4)</sup>. Besides ellagic acid, red pomegranate extract also contains flavonoids and vitamin C which work as antioxidants that can neutralize free radicals. Flavonoids have a hydroxyl group attached to the aromatic ring carbon so that they can ward off free radicals resulting from thermal reactions by donating one hydrogen atom to stabilize free radicals. Meanwhile, vitamin C works by donating electrons to prevent other compounds from being oxidized and attracting superoxide anions, hydroxyl radicals, and lipid hydroperoxides. Catching these free radicals will help ellagic acid in inhibiting Nf- $\kappa$ B activation through weak oxidation pressure molecules so that stimulation of Nf- $\kappa$ B activation will be disrupted<sup>(14,15,16)</sup>.

Overall, the number of macrophages in the treatment group showed a decrease compared to the negative control group and the positive control group, proving that red pomegranate extract had anti-inflammatory and antioxidant effects. The decrease in the number of macrophages due to the effect of red pomegranate extract can help the wound healing process but the decrease that occurs needs to be considered so as not to exceed the minimum limit.

Based on the results of this study, it was found that the application of red pomegranate extract gel can reduce the number of macrophages which are assumed to be caused by the active compounds contained in red pomegranate which act as anti-inflammatory, antibacterial, and antioxidant. The decrease in the number of macrophages in the trauma area produced by pomegranate extract will cause a decrease in the release of proinflammatory cytokines such as TNF- $\alpha$ , IL-6, and IL-8. The decrease in the release of TNF- will prevent the inflammatory response, namely an increase in temperature in the injured and systemic areas caused by the capture of PGE2 which can affect the regulation of body temperature located in the hypothalamus. In addition, a decrease in IL-6 will reduce the hyperalgesia response in unactivated glial cells in the CNS so that the pain caused by the inflammatory response will decrease. By decreasing the inflammatory response caused by the traumatic ulcer, the ulcer tends not to cause discomfort to the patient so that the nutritional needs of the patient will be maintained to optimize the wound healing process.

## CONCLUSION

The application of red pomegranate extract gel can reduce the number of macrophages which are assumed to be caused by the active compounds contained in red pomegranate which act as anti-inflammatory.

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