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ORIGINAL ARTICLE

CD68 Expression on Macrophages as Anti-Inflammatory Effect of Tamarillo (*Solanum betaceum* Cav.) Fruit Peel *Ethanol* Extract (Study on Carrageenan-Induced Buccal Mucosa of Rats)

Eksprsi CD68 pada Makrofag sebagai Efek Antiinflamasi Ekstrak *Etanol* Kulit Buah Tamarillo (*Solanum betaceum* Cav.) (Studi pada Mukosa BukalTikus yang Diinduksi Karagenan)

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ABSTRACT

Background

Inflammation is one problem in the oral cavity that patients often complain about. Anti-inflammatory drugs are generally used to treat inflammation but these drugs have side effects therefore currently many anti-inflammatory drugs are developed from natural ingredients, one of which is Tamarillo. The flavonoid of Tamarillo fruit peel can inhibit inflammation. Carrageenan is an irritant that is often used as an indicator of inflammation because it has many advantages. Macrophages are innate immune cells that are important in inflammation. Macrophage cluster of differentiation 68 or CD68 expression can be used to detect inflammation activity. The objective of this study is to detect CD68 expression on macrophages as the anti-inflammatory effect of Tamarillo fruit peel (*Solanum betaceum* Cav.) ethanol extract.

Methods

This study used biological specimens of in vivo experimental research by observing immunohistochemical preparations of 5 sample groups, diclofenac sodium as a positive control, NaCl as a negative control, and Tamarillo fruit peel extract groups at doses of 70, 140, and 280 mg/kg.BW. Statistical analysis was performed using the one-way ANOVA test.

Results

There were significant differences ($p < 0.05$) in CD68 expression between each treatment group on the 24, 48, and 72 hours with the lowest number of expressions on the 72 hours of 280 mg/kg.BW group.

Conclusions

Ethanol extract of Tamarillo (*Solanum betaceum* Cav.) fruit peel has an effect on CD68 expression of macrophage cells in the buccal mucosa of carrageenan-induced rats with the most optimal dose of 280 mg/kg.BW on 72 hours.

Keywords: CD68, Flavonoids, Immunohistochemistry, Inflammation, Macrophage, Tamarillo.

ABSTRAK

Latar Belakang

Inflamasi merupakan salah satu masalah dalam rongga mulut yang sering dikeluhkan pasien. Obat antiinflamasi umumnya digunakan untuk mengobati inflamasi namun memiliki efek samping oleh karena itu, saat ini banyak dikembangkan obat anti infl amasi dari bahan alam salah satunya adalah Tamarilo. Kandungan flavonoid dari kulit buah Tamarillo dapat berperan menghambat inflamasi. Karagenan merupakan iritan yang sering digunakan sebagai indikator inflamasi karena memiliki banyak kelebihan. Makrofag merupakan sel imun *innate* yang berperan penting dalam inflamasi. Untuk mendeteksi makrofag dapat digunakan Cluster of differentiation 68 atau CD68 yang merupakan penanda sitokimia untuk makrofag. Tujuan penelitian ini untuk mengetahui efek anti inflamasi ekstrak etanol kulit buah Tamarillo (*Solanum betaceum* Cav.) terhadap ekspresi CD-68 pada makrofag.

Metode

Penelitian ini menggunakan bahan specimen biologi penelitian eksperimental *in vivo* dengan melakukan pengamatan pada sediaan Imunohistokimia menggunakan antibodi CD68 sebagai marker makrofag pada spesimen mukosa bukal tikus yang telah diberi perlakuan berupa natrium diklofenak sebagai kontrol positif, NaCl sebagai kontrol negatif dan kelompok ekstrak kulit buah Tamarillo dengan dosis 70 mg/kgBB, 140 mg/kgBB dan 280 mg/kgBB. Uji statistic dilakukan dengan menggunakan uji *one way ANOVA*.

Hasil

Terdapat perbedaan bermakna ($p < 0.05$) dari ekspresi CD68 antara tiap kelompok perlakuan pada jam ke-24, 48 dan 72.

Kesimpulan

Ekstrak etanol kulit buah Tamarillo memiliki efek terhadap ekspresi CD68 pada sel makrofag mukosa bukal tikus yang diinduksi karagenan dengan dosis optimal 280 mg/kg.BW pada 72 jam.

Kata Kunci: CD68, Flavonoids, Imunohistokimia, Inflamasi, Makrofag, Tamarillo

INTRODUCTION

Health of the oral cavity is a very important part of body health. However, dental and oral health problems in Indonesia are increasing. One of the problems inside the oral cavity that patients often complain about is inflammation.[1] Inflammation is the body's protective response to adverse disorders such as microbial infections, tissue injuries, and other dangerous conditions. Inflammation is an essential immune response that allows the body to eliminate harmful stimuli and heal damaged tissues.[2] Inflammation is characterized by the main signs such as *rubor, calor, tumor, dolor, and functio laesa*.[3] The inflammatory response involves leukocytes such as neutrophils, lymphocytes, and macrophages. The cells release substances that prevent damage from getting worse produce healing and restore tissue function. Such substances include vasoactive amines, peptides, eicosanoids, pro-inflammatory cytokines, and acute-phase proteins.[4]

Inflammatory treatment is generally to slow down the process of tissue damage that occurs in the inflammatory area.[5] Anti-inflammatory drugs are divided into two, namely non-steroidal anti-inflammatory (AINS) and steroidal anti-inflammatory.[6] However both drugs have adverse side effects for the body, such as impaired kidney function, edema, hypertension, and gastrointestinal bleeding.[7] As for anti-inflammatory steroids or corticosteroids, they can cause changes in metabolism, the functioning of the cardiovascular system, kidneys, endocrine system, and nervous system.[8] Therefore many developed anti-inflammatory drugs from natural ingredients.[9]

Since ancient times the Indonesian people have used plants as medicines. [10] In Indonesia 1,845 species of medicinal plants have been identified.[11] Tamarillo is one of the many plants that contain excellent nutrients and contain natural antioxidants.[12] However, the use of Tamarillo is still limited to the fruit alone while the skin has not been widely used so it becomes waste whereas the skin of tamarillo fruit (*S.betaceum* Cav.) contains anthocyanins, β- carotene, and flavonoids.[13] Flavonoids contained in the ethanolic extract of Tamarillo fruit peel can function as antioxidants that can inhibit inflammation by capturing free radicals that cause tissue damage that initiate the biosynthesis of arachidonic acid and inhibit the cyclooxygenase enzyme thus inhibiting the formation of prostaglandins.[14]

Carrageenan is a large-molecule sulfate polysaccharide used as an inflammatory indicator. The advantage of carrageenan compared to other irritant compounds is that it does not damage tissues leaves marks and gives a more sensitive response to inflammatory drugs.[15]Carrageenan induces cell injury by releasing mediators that initiate inflammation.[16]

Macrophages are *innate* immune cells that play an important role in inflammation. The inflammatory stimulus will activate macrophages thus triggering the release of various inflammatory mediators.[17] One of the inflammatory mediators secreted by macrophages is cytokines. Such cytokines can initiate an inflammatory response.[18] Macrophages are mature cell forms of blood monocytes that migrate to connective tissue. When inflammation occurs, monocytes in the connective tissue become macrophages that are many times more numerous also the macrophages present in the connective tissue become activated. Although inflammation is the body's defense response an excessive amount of macrophages in the body can result in tissue damage. A decrease in the number of macrophages signals a healing process of inflammation.[19]

To detect macrophages can be used *Cluster of Differentiation* 68 or CD68.[20] CD68 is a molecule expressed by macrophages and monocytes.[20-21] CD68 is used as a cytochemical marker for macrophages in histochemical analysis of inflamed tissues.[22] In previous studies it was said that CD68 provides good macrophage detection results in predicting the prognosis of cancer patients.[20]

METHODS

This research was carried out in the OPaDCORE laboratory, Faculty of Dentistry, Trisakti University in December 2022 – January 2023 with the type of research used as an experimental laboratory of biological material stored *in vivo* in the form of specimens of the buccal mucosa of rats. The number of ethical clearances of this study is KET-762/UN2F1/ETIK/PPM.00-02/2021. The research samples used were 30 samples classified into five groups, namely the negative control (NaCl), the positive control (sodium diclofenac), and the Tamarillo fruit peel extract treatment groups which were dosed at 70, 140, and 280 mg/kg BW. The control group serves as a comparison to determine the apparent differences between the treatment group and the control group. The immunohistochemistry staining was done to see the CD68 positive expression on macrophages which were then counted by *ImageJ*.

Immunohistochemistry staining of CD68 as a macrophage marker

Paraffin blocks from treated specimens of the buccal mucosa of rats were cut using microtomes with a thickness of approximately 4 µm. Then deparafinized by introducing the preparation in the xylene solution 2 times for 5 minutes each followed by a stratified alcohol solution (100%, 96%, 70%) for 10 minutes each. *Retrieval* antigens were performed using the HIER method in a microwave oven with a temperature of 90 °C for 15 minutes. Endogenous peroxidase was blocked using 3% H202 incubation in methanol at room temperature and the preparation was rinsed using PBS. Non-specific binding was blocked using a super block blocking buffer for 5 minutes at room temperature. Apply CD68 primary antibody (Elabscience, CD68 Polyclonal Antibody) with a dilution of 1:500 for 30 minutes at room temperature, and then the secondary antibody (ultraTek Anti-Polyvalent &UltraTek HRP), drip chromogen substrate DAB and apply to the preparation for 5 minutes. *Counter*staining was carried out by hematoxylin staining, dehydrating with stratified alcohol (70%,96%,100%), cleaning the preparation with xylene, drip mounting solution, and covering with cover glass.[21-22]

Observation of the number of CD68$^+$ expression on macrophages

CD68 expression was observed by 2 observers under a digital microscope at the 3 highest density viewing fields, with an initial magnification of 10x10, and continued with a magnification of 40x10 to calculate the number of macrophages. CD68$^+$ is characterized by a brown distorted cytoplasm with a rounded shape and a single nucleus on the sub-mucosa area. The number of macrophages was calculated using the *ImageJ* application.[23-24]

RESULTS

CD68$^+$Expression on Macrophages

Observations of CD68$^+$ expression on macrophages were carried out by 2 observers using a digital microscope with an initial magnitude (10x10) to determine the three fields of view that gave the highest CD68 expression. After that, cell calculations with magnification (40x10) were carried out, and CD68 expression was positive in the presence of brown distorted macrophage cytoplasm. Cell counting is performed using the *ImageJ* application to calculate the number of macrophages expressed positively (Figures 1, 2, 3). Table 1, can be seen the average result of the number of macrophages expressing CD68 from each field of view in each study group. The number of macrophages expressing CD68 is categorized into high density when the number is more than or equal to the cut-off value (as much as 11) and low density when the number is less than the cut-off value (<11). The cut-off value is determined based on the median value of the average value of each specimen (Table 1). The number of CD68$^+$ macrophages in each group at 24, 48, and 72 hours were shown in Figures 1, 2, and 3.

Table 1. The number of macrophages expressed CD68⁺ on 24, 48 and 72 hours

	24 hours	Category	48 hours	Category	72 hours	Category
Control (+)	14	High	11,5	High	9	Low
Control (+)	15,5	High	10,5	Low	8	Low
Control (-)	19	High	15	High	11,5	High
Control (-)	16	High	14,5	High	13,5	High
Extract 70 mg/kgBW	11	High	11	High	10	Low
Extract 70 mg/kgBW	14	High	12,5	High	9	Low
Extract 140 mg/kgBW	11	High	9	Low	9	Low
Extract 140 mg/kgBW	13	High	11	High	9,5	Low
Extract 280 mg/kgBW	9	Low	8	Low	6	Low
Extract 280 mg/kgBW	10	Low	9,5	Low	8	Low

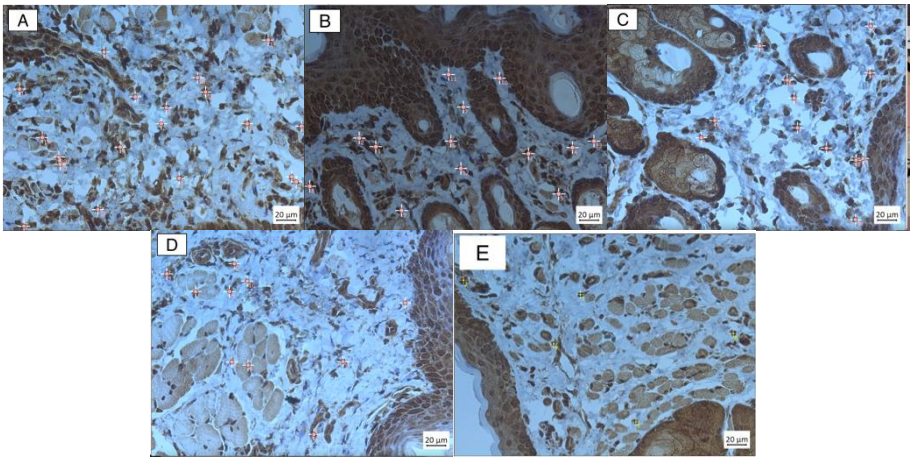

Figure 1. The number of CD68⁺ macrophages in the 24 hours was counted by ImageJ. (A) The negative control group: 19 ; (B) The positive control group: 13 ; (C) The .extract group at a dose of 70 mg/kg.BW: 12; (D) The extract group at a dose of 140 mg/kg.BW: 10 ; (E) The extract group with a dose of 280 mg/kg.BW: 10.

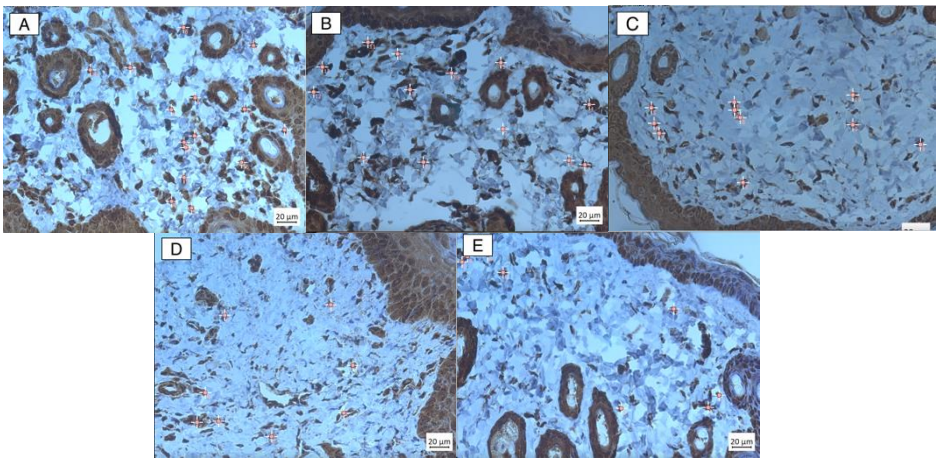

Figure 2. The number of CD68⁺ macrophages in 48-hours was counted by ImageJ. (A) The Negative control group: 14 ; (B) The positive control group: 11 ; (C) The extract group at a dose of 70 mg/kg.BW:10 ; (D) The extract group at a dose of 140 mg/kg.BW: 8 ; (E) The extract group at a dose of 280 mg/kg.BW: 8.

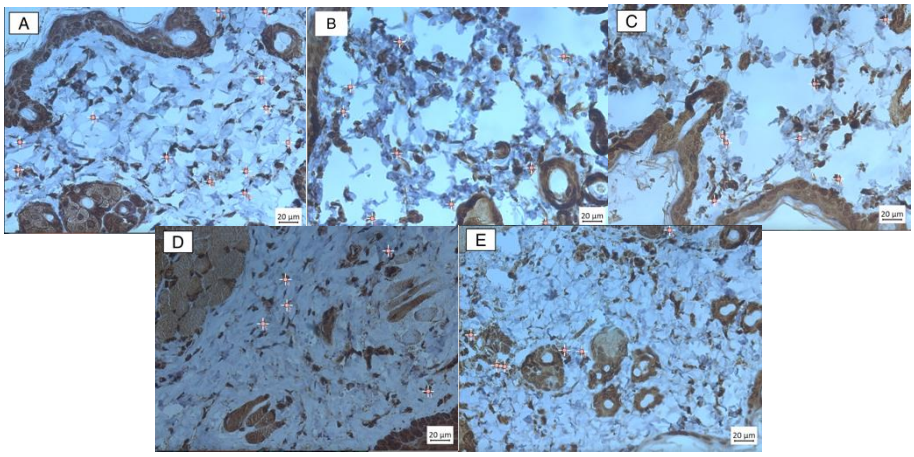

Figure 3. The number of CD68⁺ macrophages in 72 hours was counted by ImageJ. (A) The negative control group: 10 ; (B) The positive control group: 8 ; (C) The extract group at a dose of 70 mg/kg.BW: 7 ; (D) The extract group at a dose of 140 mg/kg.BW: 5 ; (E) The extract group at a dose of 280 mg/kg.BW: 6.

Normality Test Data Analysis

The Normality Test was performed using the *Shapiro-Wilk* test to find out whether the data was normally distributed or not. In Table 2, it can be seen that the results of the Shapiro-Wilk test using *SPSS 26 software* show that all data on observation of preparations 24, 28, and 72 hours are normally distributed, so it is continued with the One-Way ANOVA *test.*

Table 2. Normality Test (Shapiro-Wilk)

Number of CD68⁺	Shapiro-Wilk (Sig.)	Information
24 hours	0.827	Normal Distributed Data
48 hours	0.694	Normal Distributed Data
72 hours	0.571	Normal Distributed Data

One-way ANOVA Test Analysis

The result of the One-way ANOVA using SPSS 26 software is shown in Table 3. It can be seen that the significant value (p-value) on the observation of the 24-hour specimens is 0.026 ($p<0.05$), 48 hours is 0.012 ($p<0.05$) and 72 hours is 0.022 ($p<0.05$). This pointed to a significant effect on each group, and then a Post Hoc test with Tukey was carried out.

Table 3. One-Way ANOVA Test

Treatment		One Way ANOVA		
		Average	*p-value*	Conclusion
24 hours	Positive Control	14.75	0.026	There is an Influence
	Negative Control	17.50		
	Extract 70 mg/kg.BW	12.50		
	Extract 140 mg/kg.BW	12.00		
	Extract 280 mg/kg.BW	9.50		
48 hours	Positive Control	11.00	0.012	There is an Influence
	Negative Control	14.75		
	Extract 70 mg/kg.BW	11.75		
	Extract 140 mg/kg.BW	10.00		
	Extract 280 mg/kg.BW	8.75		
72 hours	Positive Control	8.50	0.022	There is an Influence
	Negative Control	12.50		
	Extract 70 mg/kg.BW	9.50		
	Extract 140 mg/kg.BW	9.25		
	Extract 280 mg/kg.BW	7.00		

Post Hoc (Tukey) Data Analysis

The result of the Post Hoc test conducted using SPSS 26 software is shown in Table 4. There was a significant difference between the number of positive CD68 expressions in the 280 mg/kg.BW extract group and the negative control group, while there was no significant difference between the negative control group and the extract group of 140 mg/kg.BW and 70 mg/kg.BW. There was also a significant difference in the number of CD68 expressions in the positive control group with the negative control group but there was no significant difference between the positive control group and the extract group.

Table 4. Post Hoc Tukey Test at the 24 hour

Group	N	Subset	
		1	2
Negative Control	2		17.50
Positive Control	2	14.75	14.75
Extract 70 mg/kg.BW	2	12.50	12.50
Extract 140 mg/kg.BW	2	12.00	12.00
Extract 280 mg/kg.BW	2	9.50	

At the 48 hours, in the post-hoc Tukey test (Table 5) there was a significant difference in the number of positive CD68 expressions between the 280 mg/kg.BW dose extract and the negative control group but there was no significant difference between the negative control and the 70 mg/kg.BW and 140 mg/kg.BW dose extract groups. There was a significant difference between the positive control with the negative control group but there was no significant difference between the positive control and the extract group.

Table 5. Post Hoc Tukey Test at the 48 hours

Group	N	Subset	
		1	2
Negative control	2		14.75
Positive control	2	11.00	11.00
Extract 70 mg/kg.BW	2	11.75	11.75
Extract 140 mg/kg.BW	2	10.00	
Extract 280 mg/kg.BW	2	8.75	

At the 72 hours (Table 6), there was a significant difference between the negative control and the extract dose group of 280 mg/kg.BW. Meanwhile, in the extract group doses of 70 mg/kg.BW and 140 mg/kg.BW there was no significant difference with the negative control group. In the positive control group, there was a significant difference with the negative control group but no significant difference with the extract group.

Table 6. Post Hoc Tukey Test at the 72 hours

Group	N	Subset	
		1	2
Negative Control	2		12.50
Positive Control	2	8.50	8.50
Extract 70 mg/kg.BW	2	11.75	11.75
Extract 140 mg/kg.BW	2	9.25	9.25
Extract 280 mg/kg.BW	2		14.75

DISCUSSION

This study was conducted at the OPaDCORE Laboratory of Universitas Trisakti by 2 observers who made observations on the immunohistochemistry staining of CD68 antibodies derived from 30 samples of rat buccal mucosal paraffin blocks obtained from biological specimens of experimental research *in vivo*. There were 5 treatment groups on rat buccal mucosal preparations, a positive control group given diclofenac sodium, a negative control group given NaCl, and a treatment group given ethanol extract of Tamarillo fruit peel with doses of 70, 140, and 280 mg/kg.BW which was done before carrageenan injection.

The treatment was done before carrageenan induction to inhibit the formation of arachidonic acid and cyclooxygenase enzymes.[14] Immunohistochemistry staining was performed using CD68 antibodies with a concentration of 1:500 as markers of macrophages.[22] Anti-inflammatory testing was performed by looking at the number of macrophages expressing CD68 by ImageJ apparatus using a digital microscope in 3 viewing fields.[24]

CD68 expression is categorized as high density when the number of positive CD68 expressions is greater than or equal to the cut-off value which was counted as much as 11 and low when the number of positive CD68 expressions is less than the cut-off value. The cut-off value is derived from the median value of the average number of positive CD68 expressions of each preparation.[24]

Based on the results of data analysis with the One-way ANOVA test which were obtained in Table 3, there was a significant difference ($p<0.05$) in the number of CD68 expressions at the 24, 48 and 72 hours so that it can be interpreted that there was a difference in the number of CD68 expressions between the treatment groups at the 24th, 48th and 72nd hours. In Tables 4, 5, and 6, with the *Post Hoc Tukey* test, there was a significant difference ($p<0.05$) between the negative control group and the 280 mg/kg.BW dose extract group while there was no significant difference ($p>0.05$) between the negative control group and the 70 mg/kg.BW and 140 mg/kg.BW dose extract groups.

The positive control group did not have a significant difference ($p>0.05$) with the extract group. So it can be stated that Tamarillo fruit peel ethanol extract has an effect on CD68 expression with the optimal effects at a dose of 280 mg/kg.BW and has an effect that is almost similar to the administration of diclofenac sodium as a positive control.

In Figures 1, 2, and 3, it can be seen that there is a decrease in the number of $CD68^+$ on macrophages that have started at the 24th hour and continued at the 48th hour, and at the 72nd hour CD68 expression is seen to decrease, with the lowest amount of $CD68^+$found in the extract group at a dose of 280 mg/kg.BW. The decrease of CD68 expression is caused by the flavonoid content of Tamarillo fruit peel which can inhibit the inflammatory response as also stated by other studies of Priamsari et al (2006).[14]

This research is in accordance with the results of a study conducted by Siswarni et al (2017) who said that the skin of Tamarillo fruit contains flavonoids that can act as anti-inflammatories.[25] Studies conducted by Priamsari et al also line with this study, in the study it is said that Tamarillo fruit peel ethanol extract has anti-inflammatory effects and the highest effect is obtained at the highest dose.[14] It is following the results of this study (Table 1) that the lowest amount of $CD68^+$ is at the highest extract dose.

Research conducted by Li Novelya and Li Wilvia also supports the results of this study, in their study it was concluded that tamarillo fruit peel ethanol extract is effective in reducing levels of inflammatory biomarkers.[26] In this study, Tamarillo fruit peel extract was considered effective in reducing levels of pro-inflammatory cytokines secreted by macrophages.

Salsabila and Sudiono in their research also said that tamarillo fruit peel extract has anti-inflammatory effects but the most effective dose is a dose of 140 mg/kg.BW.[27] However, there is a difference in the inflammation indicators that were used among the studies, the inflammatory indicators used in that study were IL-6 levels in the blood while in this study CD68 expression of macrophages in tissues was used as an inflammatory indicator.

CONCLUSION

The conclusion obtained from this study is that tamarillo fruit peel ethanol extract has an anti-inflammatory effect characterized by decreased CD68 expression with the most optimal effect on the highest dose of extract, namely, 280 mg / kgBW, and at the 72nd hour. The anti-inflammatory effect of ethanol extract skin of Amarillo fruitis equivalent to the commercial anti-inflammatory drug sodium diclofenac used as a positive control.

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AUTHORS CONTRIBUTION

Jelita FB developed the specimen model, performed simulations, and analyzed data and simulation results. Janti S performed a literature study, designed the method of study, data measurement, and analysis. Both authors designed the project and wrote the paper.

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CONFLICT OF INTEREST

There was no conflict of interest in the making of this study.

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