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Anti-inflammatory examination of Tamarillo (*Solanum betaceum* Cav.) fruit peel ethanol extract on VEGF expression (study on carrageenan-induced rat buccal mucosa)

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Abstract

Background: Inflammation is a process of the body's defense function against various stimuli. The ethanol extract of Tamarillo has flavonoid compounds as anti-inflammatory agent.

Aims: to determine the anti-inflammatory effect of Tamarillo (*Solanum betaceum* Cav.) fruit peel ethanol extract on the buccal mucosa of rats induced by carrageenan through VEGF expression.

Study Design: Laboratory experimental *in vivo* study

Place and Duration of Study: This research was done at the OPaD CORE Laboratory, Faculty of Dentistry, Universitas Trisakti at September-December 2022.

Methodology: The research sample was divided into 5 groups which were successively given diclofenac sodium suspension as a positive control, NaCl suspension as a negative control, and the Tamarillo fruit peel ethanol extract suspension with doses of 70, 140, and 280mg/kg.BW as treatment groups. Immunohistochemistry staining was performed and evaluated using microscopic observation by the ImageJ application to see the VEGF expression on the buccal mucosa of the rats. One Way ANOVA test was used to determine differences anti-inflammatory effects among groups.

Results: There was a significant difference of VEGF expression among groups at 72 hours. The highest expression was found at dose of 280 mg/kg.BW on 72 hours of carrageenan induction. Based on the Spearman's Rho test, the power level of VEGF expression on the 72-hour study group correlated very strongly or very high.

Conclusion: The ethanol extract of Tamarillo (*Solanum betaceum* Cav.) fruit peel ethanol extract has the potential as an anti-inflammatory, especially on 72 hours at a dose of 280 mg/kg.BW. This anti-inflammatory potency is equivalent to diclofenac sodium as a positive control.

Keywords: Tamarillo; Inflammation; VEGF Expression

1. Introduction

The normal response to injury is inflammation. Histamin, bradykinin, prostaglandins and serotonin are all released during injury. Capillary walls become more permeable due to the release of these substances which causes vasodilation.1 Proteins and fluids are released from the capillaries once pain receptors are stimulated. Phagocytic cells,

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also known as leukocytes migrate to the site of injury to remove potentially harmful substances. Rubor (redness), dolor (pain), tumor (swelling), calor (heat), and functio laesa (loss of cell function) are signs of excessive phagocytosis. These symptoms cause discomfort to the sufferer and require treatment to relieve it.1,2

NSAIDs (Non-Steroidal Anti-Inflammatory Diseases) class of drugs are commonly used as anti-inflammatory drugs which work by stopping the production of cyclooxygenase (COX) enzymes that convert arachidonic acid to the form of prostaglandins. In addition to their therapeutic effects, non steroidal anti- inflammatory drugs (NSAIDs) have side effects such as the ability to cause stomach or intestinal ulcers, which can lead to anemia. The use of non-steroidal anti-inflammatory drugs (NSAIDs) can cause a number of negative side effects, so looking for natural alternatives that reduce pain and inflammation, especially those derived from plants, can help minimize these negative effects.5

Indonesia is a country in the tropics that has a wealth of biodiversity. This diversity can be utilized, one of which is by utilizing medicinal plants as community herbal medicines. Medicinal plants can be found with various types and properties. However, in fact the existence of medicinal plants has not been optimally explored for their use, one of which is the Tamarillo plant.3

Tamarillo with another name *Solanum betaceum* Cav. is one of the fruits in the Solanacea tribe that grows in subtropical regions. Currently, many farmers cultivate Tamarillo fruit, one of which is in the Karo area, North Sumatra. Based on the data, there was an increase in the yield of Tamarillo planting in Indonesia in 2011 from 482,305 tons to 519,481 tons and continued to increase production up to 545,646 tons in 2013.This shows that Tamarillo production has increased every year. As production increases, the quantity of plant skin waste also increases, especially in North Sumatra. Therefore it is necessary to treat Tamarillo waste which can be utilized, among others, to extract anthocyanins from Tamarillo and to perform antioxidant and anti-inflammatory quercetin tests. Quercetin as flavonoid compound acting as an anti-inflammatory thought to reduce edema as a sign of inflammation through inhibiting inflammatory mediators (serotonin, bradykinin, histamine, and prostaglandins). Flavonoids found in Tamarillo can inhibit cyclooxygenase and lipooxygenase, prevent leukocyte accumulation in areas of inflammation, and reduce inflammation.4 Increasing capillary resistance and maintaining permeability are important functions performed by flavonoids. Consequently, flavonoids are used in diseases such as reduced vascular permeability. Flavonoid as antioxidant compounds will stop the inflammatory response by stopping the cyclooxygenase enzyme from making prostaglandins and capturing free radicals that damage tissue, this will also stop the bio synthesis of arachidonic acid.9

Inflammation testing in the form of carrageenan suspension can be used to ensure anti-inflammatory activity.6 Carrageenan, which comes from the Irish sea moss Chondrus crispus is used as a pathological agent that causes inflammation which is an irritant compound. The carrageenan method is used as an irritant compound which causes cell damage and releases mediators that start the inflammatory process.7 Carrageenan-induced as inflammatory activity test is frequently used in anti- inflammatory activity test. There are a number of benefits in using carrageenan as an inflammatory cytokine; such as does not scar, does not damage the tissue around the injection site, and is more reactive than other irritant compounds to anti-inflammatory drugs.

The process of creating new blood vessels from existing ones is known as angiogenesis. The development of angiogenesis is very important for the wound healing process.5 During this phase, endothelial proliferation continues to form a vascular network that meets all the needs of wound healing cells. Vascular endothelial growth factor (VEGF) is a form of pro-angiogenic glycoprotein to support capillary permeability and endothelial cell proliferation, migration, and survival. Vascular Endothelial Growth Factor is used as an initiator in the body, so that oxygen-starved cells can start increasing blood vessels. This glycoprotein was first described as a protein that can increase endothelial cell proliferation and vascular permeability. It was also found to be a major stimulator of angiogenesis and vasculo-genesis. Vascular Endothelial Growth Factor is triggered by a number of different factors. Tissue or cellular hypoxia is the main agitating factor, followed by various cytokines. There are a number of ways to see the VEGF, one of which is by using the immunohistochemical method. Although circulating VEGF levels are affected and secreted by platelets and leukocytes as part of the normal clotting process, assaying V EGF levels has the advantage of being easier to perform.

2. Material and methods

This experimental laboratory research using samples stored biological material (BBT) paraffin blocks derived from the buccal mucosa of rats from previous studies that experienced carrageenan-induced inflammation with and without administration of ethanol extract of Tamarillo (*Solanum betaceum* Cav) fruit peel prior to induction of inflammation. Tamarillo extract was done by maceration method using 70% ethanol solvent and divided into three doses, namely 70, 140, and 280mg/ kg.BW.

The research consisted of five groups, namely the control negative (0.5% CMC NaCl suspension), positive control (diclofenac sodium suspension in 0.5% CMC at a dose of 7mg/kg.BW) and the treatment group which were successively added to a suspension of Tamarillo (*Solanum betaceum* Cav) fruit peel ethanol extract in0.5% CMC at dose of 70; 140; and 280mg/kg.BW. Immunohistochemical staining was performed to see VEGF expression and the results of VEGF expression were observed microscopically and counted using the ImageJ application.

VEGF expression was observed through immunohistochemistry staining of each sample under a digital microscope with 40x10 magnification in three fields of view. VEGF expression is positive if the endothelial and fibroblast like derived cells in the sub-epithelial area arebrown. VEGF expression was calculated semi-quantitatively using a cell counter in the ImageJ application. VEGF expression was categorized into mild, moderate, and strong. It is said weak category ifthe amount of VEGF expression (1-3), moderate (4-8), severe (9-12) as shown on table 1.

Table 1 VEGF expression category parameters9

Parameters	**Result**	**Score**
Intensity of Expression	Weak	1
Intensity of Expression	Moderate	2
Intensity of Expression	Severe	3
Percentage of positive cells	0-25%	1
Percentage of positive cells	25-50%	2
Percentage of positive cells	50-75%	3
Percentage of positive cells	75-100%	4

The obtained microscopic observation data were analyzed usingunivariate and bivariate analysis. The function of univariate analysis was to identify the percentage of each variable and followed by bivariate analysis to observe the correlation between the dependent and independent variable through the use of Spearman's correlation. Meanwhile, to find out the differences between groups, the One Way ANOVA test was carried out.If a significant difference is found, then proceed with the Post Hoc-Tukey test.

3. Results

The results of the phytochemical tests conducted in this study showed that the Tamarillo (*Solaneum betaceum* Cav) fruit peels ethanol extract positively contained phenolic compounds, flavonoids, tannins, and alkaloid. The results of microscopic observations based on the average amount of VEGFexpression can be seen on figure 1.

Table1 showed the average number of cells or VEGF expression category from each field of view. The score for the VEGF expression category was assessed according to 4 criteria, namely 0 (no VEGF expression), 1 (small/ light amount of VEGF expression), 2 (moderate amount of VEGF expression), and 3 (heavy amount of VEGF expression).

Research Group	Time of Research					
	24 Hours		48 Hours		72 Hours	
	Mean number VEGF expression	VEGF Expression Category	Mean number VEGF expression	VEGF Expression Category	Mean number VEGF expression	VEGF Expression Category
Positive Control	25,6	2	16	1	6,6	1
Positive Control	24	1	-	-	21,3	1
Negative Control	24,3	1	15,3	1	4	1
Negative Control	19,3	1	-	-	12,6	1
Ekstract 70 mg/kg.BW	47,6	2	30,3	2	21,6	1
Ekstract 70 mg/kg.BW	24	2	-	-	18	1
Ekstract140mg/kg.BW	19	1	19,6	1	14	1
Ekstract140mg/kg.BW	24	2	-	-	15	1
Ekstract280mg/kg.BW	17	2	14	1	0	0
Ekstract280mg/kg.BW	17	2	-	-	13,6	1

Figure 1 The average results of VEGF expression and VEGF expressioncategories at 24, 48, and 72 hours

Microscopic observations of VEGF positive expression using Image J application can beseen in Figures 2, 3 and 4.

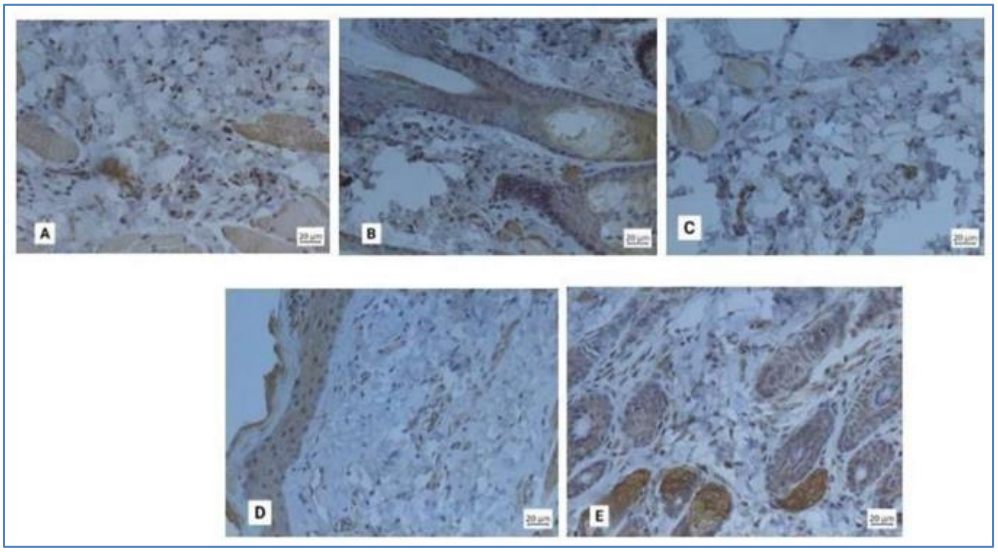

Figure 2 VEGF positive expression at 24 hours using ImageJ application. (A) The positive control group (26 cells); (B) The negative control group (25 cells); (C) The extract group dose of 70 mg/kg.BW (62 cells); (D) The extract group dose of 140 mg/kg.BW (47 cells); (E) The extract group dose of 280mg/kg.BW (46 cells)

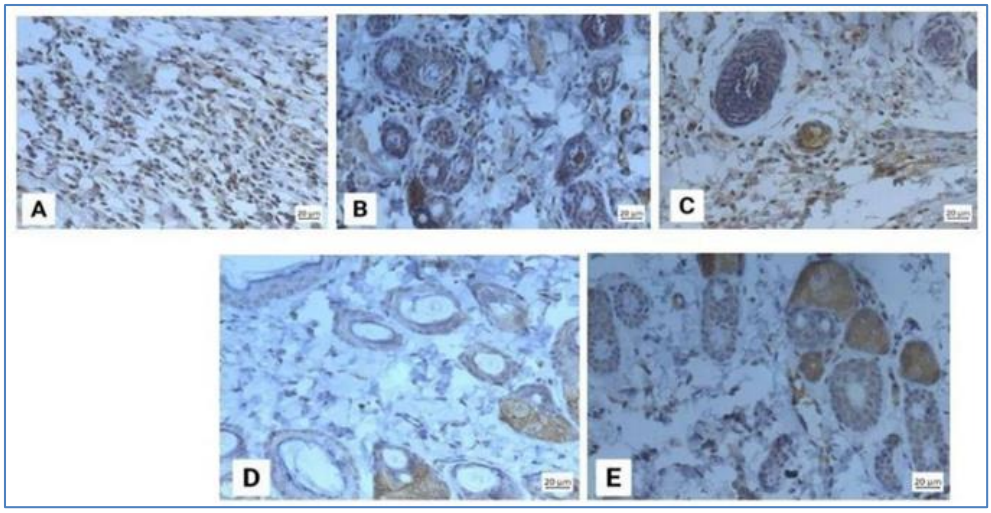

Figure 3 VEGF positive expression at 48 hours using ImageJ application. (A) The positive control group (38 cells); (B) The negative control group (17 cells); (C) The extract group dose of 70 mg/kg.BW (38 cells); (D) The extract group dose of 140 mg/kg.BW (13 cells); (E) The extract group dose of 280mg/kg.BW (11 cells**)**

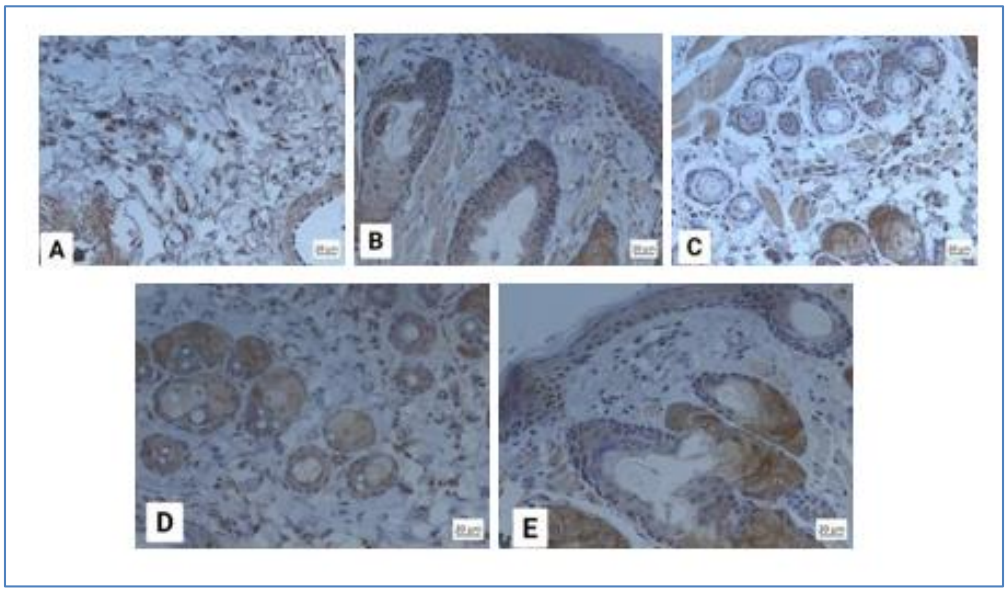

Figure 4 VEGF positive expression at 48 hours using ImageJ application. (A) The positive control group (11 cells); (B) The negative control group (3 cells); (C) The extract group dose of 70 mg/kg.BW (23 cells); (D) The extract group dose of 140 mg/kg.BW (13 cells); (E) The extract group dose of 280mg/kg.BW (1 cells)

The levels of VEGF expression were calculated and followed by comparing the results of measurements in each group. All data was then processed using SPSS version 26 to find out the distribution frequency as well as the relationship between variables. Univariate analysis was performed to find out the mean, minimum, maximum and median value of VEGF expression levels in the control and extract groups. The results of the univariate analysis presented on Table2.

Normality test was done with the result shown on Table3.

Based on the Shapiro-Wilk test on Table3, it showed that all data at 24, 48, and 72 hours of observation are normally distributed and then a homogeneity test was carried out (Table 4).

One Way ANOVA was done with the result showed on Table5.

Table 2 Total VEGF expression levels for each group

Research Groups	Time of research											
	24 hour				48 hour				72 hour			
	Mean ± SD	Minimum Value	Maximum Value	Median	Mean ± SD	Minimum Value	Maximum Value	Median	Mean ± SD	Minimum Value	Maximum Value	Median
Positive Control	25,6±0,57	25,0	26,0	26,0	16±10,14	7,0	27,0	14,0	11,0±4,0	7,0	15,0	11,0
Negative Control	24,3±2,08	22,0	26,0	25,0	15,34±3,78	11,0	18,0	17,0	4,0±1,0	3,0	5,0	4,0
Ekstract 70 mg/kg.BW	47,6±16,92	29,0	62,0	52,0	30,34±7,09	24,0	38,0	29,0	21,67±9,07	12,0	30,0	23,0
Ekstract 140 mg/kg.BW	40,6±19,2	19,0	56,0	47,0	19,67±4,50	15,0	24,0	20,0	14,0±4,35	10,0	19,0	12,0
Ekstract 280 mg/kg.BW	41±8,67	31,0	46,0	46,0	14±10,39	2,0	20,0	20,0	0,0±0,0	0,0	0,0	0,0

Table 3 Normality Test Results (Shapiro Wilk*)*

Number of positive VEGF Expressions	***Shapiro-Wilk (Sig.)***	**Description**
24 hour	0.870	Normally Distributed data
48 hour	0.986	Normally Distributed data
72 hour	0.923	Normally Distributed data

Table 4 Homogeneity test results (Levene's Test)

Number of positive VEGF Expressions	***Shapiro-Wilk (Sig.)***	**Description**
24 hour	0.163	Homogeneous Data
48 hour	0.259	Homogeneous Data
72 hour	0.051	Homogeneous Data

Table 5 One Way ANOVA Test Results

	Treatment		***One Way ANOVA***	
		Average	***p-value***	**Conclusion**
	Positive Control	25.60		
	Negative Control	24.30		
24 hour	Extract 70 mg/kg.BW	47.60	0.147	There is no effect
	Extract 140mg/kg.BW	40.60		
	Extract 280mg/kg.BW	41.00		

48 hour	Positive Control	16.00	0.138	There is no effect
	Negative Control	15.34		
	Extract 70 mg/kg.BW	30.34		
	Extract 140 mg/kg.BW	19.67		
	Extract 280 mg/kg.BW	14.00		
	Positive Control	11.00		
	Negative Control	4.00		
72 hour	Extract 70 mg/kg.BW	21.67	0.002	There is an effect
	Extract 140 mg/kg.BW	14.00		
	Extract 280 mg/kg.BW	0.00		

One Way ANOVA test showed there was no significant differences between VEGF Expression of extract groups on 24 and 48 hours (p=0,147>0.05) and (p=0.138>0.05) respectively (Table5). On the other hand, there was a significant difference on 72 hours (p=0.002<0.05). The statistic analysis continued used Post hoc –Tukey test with the result showed on Table6.

Table 6 Post Hoc –Tukey Result for 72nd hour

Group	**N**	**Subset**		
		1	**2**	**3**
Extract 280 mg/kg.BW	2	0.00		
Positive Control	2	4.00	4.00	
Extract 140 mg/kg.BW	2	11.00	11.00	11.00
Extract 70 mg/kg.BW	2		14.00	14.00
Negative Control	2			21.67

Post-hoc Tukey test showed that at 72 hours, there was a significant difference in the amount of VEGF expression between the 70mg/kg.BW and the negative control group also with the 280mg/kg.BW and the 280mg/kg.BW extract group.

The Spearman's Rho correlation test on VEGF levels between the 24-hour study groups showed the Sig.2-tailed was (0.211>0.05). This meant that there is no significant relationship between the two variables. In addition, a correlation coefficient of 0.433 was also obtained which indicated the level of correlation between the average number of VEGF expression variables while the VEGF expression category only had a sufficient correlation. As for the direction of the relationship, the correlation coefficient is positive, so the relationship between the two variables is uni-direction.

The Spearman's Rho correlation test between the 48 hour study group on VEGF expression levels showed the Sig.2-tailed was (1.00>0.05) which indicates that there is no significant relationship between the mean amount of VEGF expression and the category of VEGF expression.

The correlation between the 72 hour study groups on VEGF expression levels showed the Sig.2-tailed was (0.000<0.05) therefore it can be concluded that there was a significant relationship between the 72 hour study groups on VEGF expression levels. In addition, a correlation coefficient of 1,000 was also obtained, which indicated that the power level of the 72 hour study group had a very strong or very high correlation of VEGF expression levels. As for the direction of the relationship, the correlation coefficient is positive, so the relationship between the two variables is uni-direction.

4. Discussion

Inflammation is a biological response to tissue injury or infection, functioning to maintain the body's homeostasis due to the presence of foreign agents or compounds. Traditional medicine by utilizing natural ingredients has been carried out by Indonesian people, one of which is by using Tamarillo (*Solanum betaceum* Cav) fruit peel which has an antiinflammatory effect. The aim of this study was to determine the antiinflammatory effect of the ethanol extract of Tamarillo (*Solanum betaceum* Cav) fruit peel on the buccal mucosa of rats induced by carrageenan through the expression of VEGF which was observed using immunohistochemical preparations.

Under normal circumstances, VEGF is expressed in varying levels by tissues to control and modulate vascular stabilization to meet tissue demands. Oxygen tension can function as a regulator of VEGF. Exposure to hypoxic conditions will inducerapid expression of VEGF. In contrast, under normal oxygen conditions, VEGF expression decreased and stabilized. The level expression of VEGF depends on the amount of inflammatory cytokines. VEGF is involved in many stages of the angiogenic process, namely stimulating the degradation of the extracellular matrix around endothelial cells, increasing the proliferation and migration of endothelial cells, helping the formation of blood vessel structures. In addition, the expressionlevel of VEGF is also known to increase during wound healing, especially in the granulation phase. [10]

In this study, the results of VEGF expression in the negative control group were lower than those of the treatment and positive control groups. Table3 showed that the ethanol extract of Tamarillo peel fruit increased VEGF expression compared to the negative control. This proves that the ethanol extract of Tamarillo (*Solanum betaceum* Cav) fruit peel can improve the wound healing process by increasing new blood vessels, especially those of the concentration of 280 mg/kg.BW at 72 hours.

The results of this study are also in accordance with the results of a study conducted by other researchers who said that a reduction in inflammation was shown by a suspension of the ethanol extract of Tamarillo fruit peel at doses of 70 mg/kg.BW, 140 mg/kg.BW, and 280 mg/kg.BW, respectively. According to their study, the dose of fruit peel Tamarillo ethanol extract that has the greatest potential to reduce inflammation is also at 280mg/ kg.BW.[11]

Research conducted by other researchers is also in line with this research that the ethanol extract of the Dutch eggplant or Tamarillo peel contains flavonoids that act as anti-inflammatory substances.[12] It is in appropriate with the results of the phytochemicaltests conducted in this study that the ethanol extract of Tamarillo (*Solanum betaceum* Cav) fruit peel has compounds tannins, flavonoids, alkaloids. The anti-inflammatory effect in this study related to the effect of flavonoid content within the extract that increase with the increase of the concentration of extract at the third day (72 hours) of healing process of inflammation in deed the optimum anti-inflammatory effect was at

the concentration of 280 mg/kg.BW at 72 hours which showed the highest EGFR expression. This potency is also showed no significant difference with the anti inflammatory drugs sodium diclofenac used as positive control.

5. Conclusion

The ethanol extract of Tamarillo (*Solanum betaceum* Cav) fruit peel has the potential as an antiinflammatory, especially at 72 hours with a dose of 280 mg/ kg.BW. This antiinflammatory effect is equivalent to sodium diclofenac as an anti-inflammatory commercial drugs used as positive control.

Compliance with ethical standards

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Disclosure of conflict of interest

Authors have declared that no competing interests exist.

Statement of ethical approval

The ethical clearance letter was given by The Ethics Committee of the Faculty of Medicine, University of Indonesia – Cipto Mangunkusumo Hospital with regards of the Protection of human rights and welfare in medical research, No:KET-762/UN2.F1/ETIK/PPM.00.02/2021.

Authors' contributions

This work was carried out in collaboration among all authors. Author JS designed the study, wrote the protocol, and approved the final manuscript. Author PINP managed the experimental laboratory, the statistical analysis and approved the final manuscript. Author PT managed the literature searches and approved the final manuscript. All authors read and approved the final manuscript.

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