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ANALYSIS OF INTERLEUKIN-6 AND TUMOUR NECROSIS FACTOR-ALPHA EXPRESSION AFTER THERAPY OF ROSELLE FLOWER EXTRACT (HIBISCUS SABDARIFFA L) ON THE INCISION WOUND LIPS WISTAR RAT

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ABSTRACT
Background: *The healing of wounds in the lip region is impeded by the fact that chewing food and speaking can exert pressure and attract the wound. Rosella flowers were chosen because they contain anti-inflammatory flavonoids, anthocyanins, tannins, and saponins, as opposed to chemical medications for wound healing that often induces negative effects.* ***Purpose:*** *This study's objective was to assess the influence of 15% roselle flower extract (Hibiscus sabdariffa L) in a paste formulation on the expression of IL-6 and TNF Alpha during the incision wound healing phase.* ***Method:*** *The method is experimental analytic research with post-test-only control group design. There were two groups: control group and the treatment group. Each group contains 3 Rattus Norvegicus. They were incisions in the upper labial mucosa using blade with a length of 5 mm and a depth 1 mm. Roselle flower extract (Hibiscus sabdariffa L) 15% was applied two times per day for three days in treatment group. After the treatment, the rats were sacrificed to check the expression of IL-6 and TNF Alpha using the Immunohistochemistry method.* ***Result:*** *The results of the analysis of the ANOVA-test show that for TNF Alpha expression it has a significant value (p) of 0.054 or $p > 0.05$, meaning that there is no significant difference. Meanwhile, the IL-6 expression obtained a significant value (p) of 0.009, meaning that there was a significant difference between the control and treatment groups.* ***Conclusion:*** *The study concluded that 15% rosella flower petal extract in a paste reduced IL-6 expression during the inflammatory but not TNF Alpha.*

Keyword : *Roselle flower; Interleukin 6 (IL-6); TNF Alpha, Oral mucosa; Wound healing*
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INTRODUCTION

Vulnus or wound is the tearing of tissue, both as a unit and as tissue components, resulting in the loss of organ function, cell death, bacterial infection, and blood clotting response.[1] The prevalence of injuries in Indonesia in 2018 was 9.2%.[2] The profession of dentistry, particularly the department of oral surgery, must be distinguished from the significance of incision-based surgery (*Vulnus incisivum*)[3], especially labial region.

The wound healing process will respond to cellular and anatomical wound alterations, restoring the integrity of tissue structure and function.[4] The phases of wound healing include the phases of hemostasis, inflammation, proliferation, and remodeling.[5] Failure to heal incision wounds in the oral cavity, one of it is in the lips area, because of regular lip motions, such as eating, drinking, and speaking which exert pressure or tugging on the wound. This hinders the healing process of the wound.

From day 1 to day 3, the wound healing process' inflammatory phase occurs. In the inflammatory phase, cytokines such as Interleukin 6 (IL-6), Interleukin 1 (IL-1), and Tumor necrosis factor alpha (TNFα) are present.[6] Interleukin 6 (IL-6) is a pro-inflammatory cytokine that is released by the body as soon as in response to injury or injury and plays a crucial function in wound healing (so that the wound healing process occurs on time).[7] In contrast, TNFα and IL-6 are pro-inflammatory cytokines that typically work for two to three days before being replaced by other pro-inflammatory cytokines. TNFα is often produced by pro-inflammatory macrophages at the onset of an injury, and it has a role in apoptosis and increasing the permeability of blood vessels.[8] The wound healing process will be hampered if the TNF-α expression in long term, so there will be more than it can interfere with the synthesis of extra cellular matrix proteins (ECM).[9]

The wound healing process can run well if there is a balance between pro-inflammatory and anti-inflammatory. Anti-inflammatory are usually given using chemical drugs such as steroid anti-inflammatory and non-steroidal anti-inflammatory. These chemical

anti-inflammatory drugs can provide adverse side effects, so it is necessary to develop alternative anti-inflammatory drugs from herbal plants. Roselle flower *(Hibiscus sabdarifa L)* consists of flavonoids, anthocyanins, tannins, saponins and organic acids in the form of citric acid.[10] One of the benefits of Roselle flower is their anti-inflammatory properties. Several plants, such as cabbages, sunflowers, carnations, and beets, have anti-inflammatory differences from rosella flower, although rosella flowers have a higher total content of phenol compounds, flavonoid compounds, and anthocyanin compounds.[11] The ethanol extract of rosella flowers decreased COX-2 expression and neutrophils in the oral mucosa of Wistar mice during the wound healing process. The flavonoid content may inhibit COX-2 expression, resulting in lower prostaglandin production and decreased inflammatory response.[12] Rosella extract 15% was able to provide good results in the wound healing process.[13]

Until now research on the effect of rosella flowers on IL-6 dan TNF Alpha expressions in the inflammatory phase has not been carried out, so it is necessary to know the effect of Roselle flower (*Hibiscus sabdarifa L*) 15% on the expression of IL-6 and TNF Alpha in incision wounds of Wistar white rats in the inflammatory stage of the wound healing process, especially on lips mucosa.

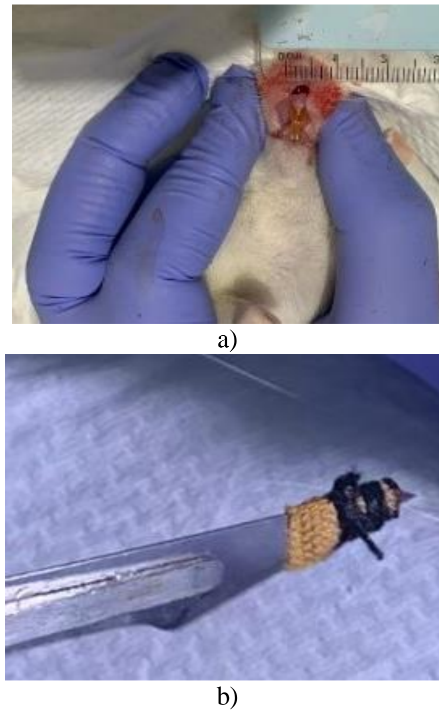
MATERIAL AND METHODS

This research is an in vivo analytical experiment by post-test *only control group design*. Research has received permission from the Health Research Ethics Commission Faculty of Dentistry Universitas Islam Sultan Agung No 326/B.1-KEPK/SA-FKG/XI/2022. Research was conducted on white Wistar rats (*Rattus norvegicus strain Wistar*) male from the Faculty of Animal Husbandry, Diponegoro University. The sample used was six rats which were calculated using the WHO pattern.

Rosella flower extract 15% [13] in paste preparation made by preparing dried roselle flower obtained from Temon District, Kulon Progo Regency, Yogyakarta, as much as 500 grams. The process of drying roselle flower using an oven at 40ºC. Dried roselle flower was crushed using a blender to obtain 330 grams of rosella flower powder. Rosella flower powder was mixed using 70% ethanol solvent and then macerated for 3 days. The rosella flower filtrate was concentrated using *rotary evaporator* at 50ºC temperature, then the remaining solvent was removed using a water bath to obtain a thick rosella flower extract.

Three grams of rosella flower extract mixed with 17 grams of pasta base to get a 15% concentration of rosella flower extract. Rosella flower extract is mixed with a paste base by 2% of carbomer 940, gliserin 30% and Trietanolamin (TEA) 60% then stirred until homogeneous. Preparations that have been put into a container, then labeled and stored in the refrigerator.[13, 14]

They are making incisions using scalpel and blade no 11. The incision wound was made on the upper labial mucosa of the rat with a depth of 1 mm and a wound width of 5 mm. The blade used is modified using plaster as a barrier so that the wound's depth is suitable, and the wound's width is measured using a ruler.



a)

b)

Figure 1. (a) Incision wounds on the upper labial mucosa of rats (b) blade and scalpel modified for rat injury to suit wound depth.

The incision wound in the control group was not given 15% roselle flower extract in a paste preparation, but given aquades steril only. The incisions in the treatment group were given 15% rosella flower extract 37,5 gram in a paste preparation every day at 08.00 and 16.00 for 3 days. The tissue taken is then soaked in the buffer neutral formalin 10% solution, then dehydrated alcohol with graded concentrations for 1.5 hours. They were clearing with xylol I, xylol II, xylol III for 1.5 hours, followed by infiltration process using paraffin I and paraffin II for 2 hours. The paraffin block was cut using a microtome, and the results of the tissue sections were placed on an object glass. Then immunohistochemical staining was performed to see the expression of IL-6 and TNF Alpha. The tissue on the glass object was deparaffinized with xylol for 5 minutes, then rehydrated with 95% and 70% alcohol for 5 minutes then washed with distilled water. The preparations were washed with Phosphate Buffered Saline (PBS) for 2x5 minutes.

The antigen retrieval with Tris EDTA pH 9 at 90º C, then wash with PBS 2x5 minutes. They were performing endogenous protein blocking with 3% H_2O_2 followed by protein blocking with Biocare Background Sniper then performing polyclonal primary antibody incubation and secondary antibody incubation using Universal Link. The preparation was dripped with TrekAvidin-HRP and the DAB chromogen for IL-6 and dripped with streptavidin for 15 minutes, then washed

using PBS for two times within 5 minutes. Furthermore, counterstaining was carried out with hematoxylen mayer then the slide was mounted with entelan. Calculating the average expression of IL-6 and TNF Alpha was obtained from macrophage cells expressing IL-6 and TNF Alpha. A positive result is found when a large cell nucleus with brown cytoplasm is found. A negative result is found a small or large cell nucleus with bluish cytoplasm. The results of the data obtained were analysed using IBM SPSS statistics version 2.

RESULT

The expression of IL-6 and TNF Alpha can be seen from the description of histological preparations that have been stained with immunohistochemistry. When reading the preparation, it was found that the cell's nucleus with brownish cytoplasm indicated macrophage cells with IL-6 and TNF Alpha expression, so it was declared positive.

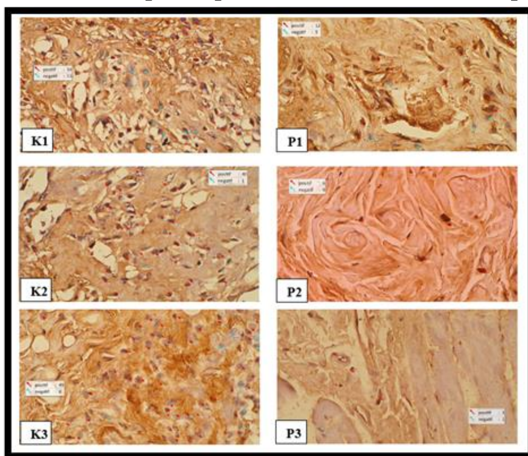


Figure 1. Expression of TNF-α in the upper lip mucosal incision wound tissue of rats in the control group (K) and the treatment group (P) with a microscope magnification of 400 times in the treatment group on day 3. Positive results are marked with red arrows and negative results are colored green

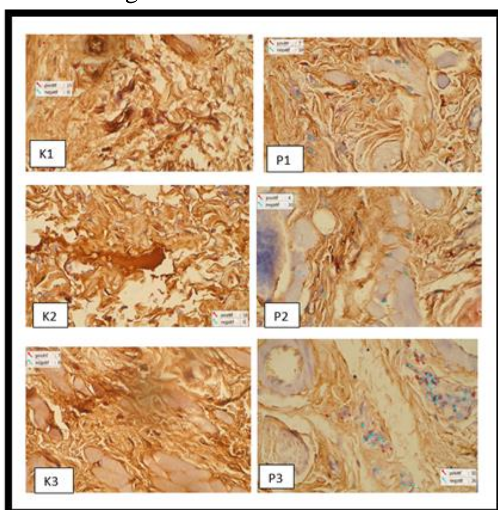


Figure 2. Expression of IL-6 in the upper lip mucosal incision wound tissue of rats in the control group (K) and the treatment group (P) with a microscope magnification of 400 times in the treatment group on day 3. Positive results are marked with brown and negative results are clear blue

The average expression of IL-6 and TNF Alpha is as follows:

No	Group	IL-6 Expression	TNF Alpha Ekspresi
		Mean±Std. Deviasi	Mean : Mean ±Std. Deviasi
1	Treatment	13,67±7,371	40,67±14,64
2	Control	15,33±13,051	136,00±25,00

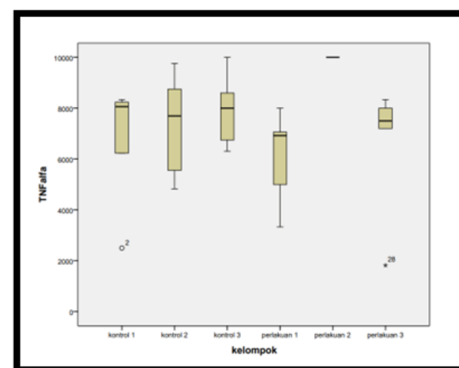
Tabel 1. Average expression of IL-6 and TNF alpha

From the data collected, a statistical analysis of the Anova different test was carried out with the following results:

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
TNF alfa	Between Groups	50254938.400	5	10050987.680	2.567	.054
	Within Groups	93986156.800	24	3916089.867		
	Total	144241095.200	29			
IL-6	Between Groups	87505743.367	5	17501148.673	4.003	.009
	Within Groups	104939768.800	24	4372490.367		
	Total	192445512.167	29			

Tabel 2. Anova different test results Anova different test results



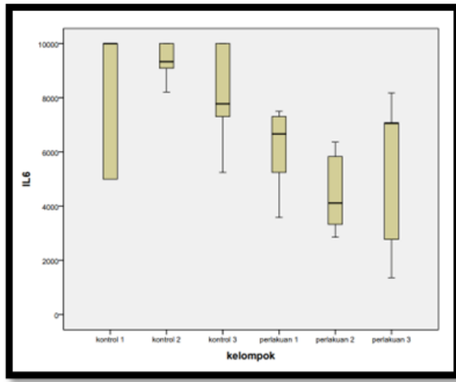


Diagram 1 : Test Result Anova

To analysis which group had the most significant reduction, a post hoc test was carried out. However, because there were homogeneity test values that were not homogeneous, a post hoc test was carried out with Howell's games with the result that there was a significant decrease in the number of IL-6 in the 2nd treatment group compared to the 2nd control group, and there was no significant decrease in TNF Alpha between the control and treatment groups.

DISCUSSION

People know that dried Rosella flower (*Hibiscus sabdariffa Linn*) are often used as tea drink. Rosella has a lot of natural ingredients that are good for health, so it often used as a herbal medicine to treat a wide range of conditions. The flavonoid in the Rosella flower works as an anti-inflammatory and an antibacterial. With the maceration method, the flavonoid content of the ethanol extract of red rosella petals was found to be 2.075.[14-16] Because Rosella flower has a lot of flavonoid compounds, the flower petals can be used as an alternative drug to treat inflammation and bacteria. Flavonoid compounds in Rosella flower work as anti-inflammatory by making pro-inflammatory mediators and stimulating cells that bind during inflammation. These cells are lymphocytes, monocytes, natural killer cells (NK cells), neutrophils, and macrophages.[17]

In the process of wound healing, macrophages create the cytokines TNFα and IL-1. In the acute phase of the healing process, macrophages that have been activated by T lymphocytes, Natural Killer (NK) cells, mast cells, and antigens express TNFα. The release of chemicals and other growth factors by macrophages can aid in the formation of granulation tissue during the wound healing process. TNFα is also essential for promoting fibroblast proliferation, regulating inflammation, and protecting wounds from infection.[18] Expression of TNFα can also detected in monocyte cells, a kind of leukocyte that serves as the first line of defense mechanism. Genetic transcription factors such as nuclear factor (Nf-k) will be activated by monocyte cells. Appropriate expression of TNFα can have a protective and therapeutic impact on tissues, whereas excessive expression of TNFα causes tissue damage. Monocyte cells express TNFα in their cytoplasm and surrounding their brownish membrane. [13]

Based on the results of the ANOVA different test (table 2 and figure 1), it was found that TNF Alpha was not significantly different, with a p-value of 0.054. This could be because the tissue staining failed, leading to false positive results, or because different colors were used to prepare the histological images. This could be because endogenous peroxidase activity wasn't stopped, so the endogenous stopping process didn't happen. Most internal blocking processes use H_2O_2. Background staining can also be lessened by putting tissue sections in a solution with a lot of protein, like serum, before they are stained. The length of the incubation period and the temperature can also cause false positive or false negative results because they change how the tissue looks under microscope. The incubation time for primary antibodies is usually between 20 and 30 minutes. If the temperature is higher than 37ºC, the incubation time can be shortened, which can lead to false positive or false negative results. Incubating primary antibodies at 40ºC needs low concentrations to keep background signalling to a minimum. The drying process between the incubation and washing steps can also cause false negative results.[19] TNFα secreted by macrophages promotes the formation of the extracellular matrix in the injured tissue by inducing the production of proteoglycan and fibronectin by fibroblasts. This meaningless statement is not consistent with the function of TNF Alpha in the healing process.

Meanwhile, in the different test on the expression of IL-6, there is a significant difference with a value of p: 0.009. The content of flavonoids and anthocyanins in Rosella calyx can reduce IL-6 expression because it acts as an anti-inflammatory.[20] The total anthocyanin compounds of Roselle petals using the maceration method showed that Roselle flower contain a total anthocyanin compound of 128.76 mg/100 g.[21] In this study, the weight of Roselle flower extract used was 3 grams with a concentration of 15%. The way flavonoids work is by inhibiting the action of the cyclooxygenase enzyme in the form of Cyclooxygenase-2 (COX-2) which will convert arachidonic acid into prostaglandins and leukotrienes which trigger inflammatory signs, inhibit neutrophil degranulation, inhibit leukocyte accumulation and inhibit histamine release.[22]

As anti-inflammatory agents, anthocyanins suppress the activation of the Nuclear Factor kappa B NFkB) pathway. The pathway regulates the release of multiple cytokines, chemokines, and cell adhesion molecules involved in the inflammatory process, one of which is IL-6. By inhibiting the activation pathway of Nuclear Factor kappa B (NFkB), the synthesis of IL-6 as a proinflammatory cytokine will decrease, resulting in a decreased inflammatory response.[23] In this study, the expression of IL-6 differed significantly, suggesting that Roselle has an anti-inflammatory impact. This study utilized a paste formulation for a number of reasons, including the fact that pastes have a stronger working effect since they adhere longer to the wound site. In

addition, the paste form can shield wounds from sliding or contact with food, chemicals, or other mechanical stimulation.[23, 24] Roselle extract (Hibiscus sabdariffa Linn) 15% in paste preparation has an effect on reducing IL-6 expression in the inflammatory phase applied to the incision wound of the rat's lip mucosa but not on the expression of TNF Alpha.

REFERENCES

1. Kartika RW. Perawatan Luka Kronis dengan Modern Dressing. Cermin Dunia Kedokt. 2015; 42(7): 546-550.
2. Augsornwan D, Surakunprapha P, Pattangtanang P, Pongpagatip S, Jenwitheesuk K, Chowchuen B. Comparison of Wound Dehiscence and Parent's Satisfaction Between Spoon/Syringe Feeding and Breast/Bottle Feeding in Patients with Cleft Lip Repair. J Med Assoc Thai. 2013; 96(Suppl 4): 61-70.
3. Laporan Nasional Riset Kesehatan Dasar. Kementeri Kesehat RI. . 2018, Lembaga Penerbit Badan Penelitian dan Pengembangan Kesehatan, Jakarta.
4. Oktaviani DJ, Widiyastuti S, Maharani DA, Amalia AN, Ishak AM, Zuhrotun A. Review: Bahan Alami Penyembuh Luka. Farmasetika. 2019; 4(3): 45-56.
5. Ramadhani N, Sumiwi SS. Aktivitas Antiinflamasi Berbagai Tanaman Diduga Berasal Dari Flavonoid. Farmaka. 2013; 14(2): 111-123.
6. Poernomo H. Ma'ruf MH. The Effect Of Garlic Extract Gel (Allium Sativum L.) To Macrophage Cell Number Of Guinea Pig (Cavia Porcellus) Gingival Incision Wound Healing. IJKG Interdental J Kedokt Gigi. 2020; 169(2): 36-44.
7. Johnson BZ, Andrew WS, Prêle CM, Fear MW, Wood FM. The Role of IL-6 in Skin Fibrosis and Cutaneous Wound Healing. Biomedicines. 2020; 8(5): 101-18.
8. Ritsu M, Kawakami K, Kanno E, Tanno H, Ishii K, Imai Y, Maruyama R, Tachi M. Critical Role Of Tumor Necrosis Factor-A In The Early Process Of Wound Healing In Skin. Journal of Dermatology & Dermatologic Surgery January. 2017; 21(1): 14-19.
9. Sungkar A, Widyatmoko D, Yarso KY, Wasita B. The Effect Of Duration Of Wound Skin Tissue On MDA,TNF-A, IL-6, Caspase 3, VEGF Levels, And Granulation Tissue Thickness In The White Rat (Rattus Novergicus). Bali Medical Journal (Bali Med J). 2020; 9(3): 918-923.
10. Subaryanti S, Triawan A, Poeloengan M. . Bunga Rosella (Hibiscus sabdariffa L.) Sebagai Antibakteri. Sainstech J Penelit dan Pengkaj Sains dan Teknol. 2020; 23: 78-83.
11. AT Sarwono, DF Suniarti. Roselle Calyx Ethanol Extract Stimulates Oral Mucosal Wound Healing. Int J Appl Pharm. 2017; 9(2): 36-40.
12. Kusumastuti E, Handajani J, Susilowati H. Ekspresi COX-2 dan Jumlah Neutrofil Fase Inflamasi pada Proses Penyembuhan Luka Setelah Pemberian Sistemik Ekstrak Etanolik Rosela (Hibiscus sabdariffa) (studi in vivo pada Tikus Wistar). Maj Kedokt Gigi Indonesia. 2014; 21(1): 13-19.
13. Rambe PS, Putra IB, Yosi A. The Effect Of Roselle Leaf (Hibiscus Sabdariffa L.) Extract Gel On Wound Healing. J Med Life. 2022; 15(10): 1246–1251.
14. Syurgana MU, Febrina L, Ramadhan AM. Formulasi Pasta Gigi dari Limbah Cangkang Telur Bebek. Proceeding of Mulawarman Proceeding of the 6thMulawarman Pharmaceuticals Conferences. Samarinda, 7-8 November 2017.
15. Suzery M, Lestari, S. and Cahyono, B. Penentuan Total Antosianin Dari Kelopak Bunga Rosela (Hibiscus Sabdariffa L) Dengan Metode Maserasi Dan Sokshletasi. Jurnal Sains Dan Matematika. 2010; 18(1): 1-6.
16. Samsuar, Hanifa D. Gel Formulation of Jamblang Leaf Extract (Syzygium cumini L) Skeel and Antioxidant Activity. Oriental Journal Of Chemistry. 2020; 36(5):946-953.
17. Syahrana NA, Akrom and Darmawan, E. Efek Serbuk Bunga Rosela Merah (Hibiscus sabdariffa L.) terhadap Ekspresi IL-10 pada sukarelawan Sehat. Jurnal Farmasi Dan Ilmu Kefarmasian Indonesia, 2017; 4(1): 1-5.
18. Sarbini D, Sargowo D, Rohman MS. Hibiscus Sabdariffa Linn) terhadap NF-κβ, TNF-α dan ICAM-1 pada Human Umbilical Vein Endothelial Cells (HUVECs) Cultured yang dipapar Low Density Lipoprotein (LDL) Teroksidasi. JExp Life Sci feb. 2011; 1(2): 56-110.
19. Dabbs DJ. Diagnostic Immunohistochemistry: Theranostic and Genomic Application: Techniques of Immunohistochemistry: Principle. Pitfalls, and Standarization. Unitede Station of America: Elsevier Saunders 2013: 1-19.
20. Djaeni M, Ariani N, Hidayat R, Utari FD. Ekstraksi Antosianin dari Kelopak Bunga Rosella (Hibiscus sabdariffa L.) Berbantu Ultrasonik: Tinjauan Aktivitas Antioksidan Ultrasonic Aided Anthocyanin Extraction of Hibiscus sabdariffa L. Flower Petal: Antioxidant Activity. Jurnal Aplikasi Teknologi Pangan. 2017; 6(3): 148-151.
21. Stephen S, Jayaraman A, Chapkin RS, Howard M, Mohankumar K, and Shrestha R. Flavonoids: structure–function and mechanisms of action and opportunities for drug development. Toxicol Res. 2021; 37(2): 147-162.
22. Idrus E, Tsary DA, Setiadi DS, Jesslyn, Calfina NE, Halim VA, DF Suniarti, Soedarsono N, Wimardhani YS, Yuniastuti M. Inhibition of Alveolar Bone Destruction by Roselle Extract (Hibiscus Sabdariffa L.). Journal of International Dental and Medical Research. 2020; 13(3): 830-835.
23. Widiastuti IGAA. Ekstrak Pasta Ubi Jalar Ungu (Ipomea batatas L) Mempercepat Angiogenesis Soket Mandibula Pada Penyembuhan Luka Pasca Pencabutan. Journal Caninus Denstistry 2017; 2: 20-30.

24. Nurdianty AV, Apriasari ML, Carabelly AN. The Effect of Topical Toman (Channa micropeltes) Fish Extract on The Number of Neutofil cells in Dibbetes Mellitus Wound Healing. Dentino (Jur Ked Gigi). 2019; 4(2): 110-115.