

Cassava Leaf Flavonoid Extract on Enhancing the Gingival Epithelium Thickness of Lipopolysaccharide-Induced Rats

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Abstract

This study was aimed to determine the effect of administration of flavonoid extract gel of cassava leaves (*Manihot esculenta*) on gingival epithelial thickness in rat models induced by periodontitis lipopolysaccharide (LPS) of *E. coli*.

This study was an experimental laboratory research on 27 male Wistar rats randomly divided into 9 groups induced by lipopolysaccharide. Groups K11, K12, and K13 were the control groups, and groups P11, P12 and P13 groups were treatment groups with 25% flavonoid extract gel of cassava leaves; while P21, P22, and P23 groups were treatment groups with 50% flavonoid extract gel of cassava leaves. K11, P21, P31 were decapitated on day 3; K12, P12, P22 were decapitated on day 7; K13, P13, and P23 were decapitated on day 14. The measured parameter was the thickness of the epithelium after the lipopolysaccharide was induced. Data analysis used one way ANOVA.

Flavonoid extract of cassava leaf (*Manihot esculenta*) at concentrations of 25% and 50% are not effective in increasing the thickness of the gingival epithelium of the periodontitis rat model, which are decapitated either on day 3; 7th day; or 14th day.

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Introduction

Periodontitis are the group of infections, predominantly caused by colonization of gram-negative, anaerobic pathogens on sub-gingival areas.¹ Lipopolysaccharides are endotoxins that will induce local factors i.e. proinflammatory cytokines such as interleukin-1 α (IL-1 α , IL-1 β), IL-6, tumor necrosis factor- α (TNF- α) and eicosanoids, i.e. prostaglandin (PGE2). Proinflammatory cytokines cause inflammation. The inflammatory response caused by LPS is part of the first immune system to pathogens.²

The process of healing *periodontal wounds* basically includes the process of tissue regeneration and the formation of new attachments.³ Wound healing is a mechanism

whereby the body attempts to restore the integrity of the injured part. It is a summation of processes that follow injury and include coagulation, inflammation, matrix synthesis and deposition followed by angiogenesis, fibroplasia, epithelialization, contraction, remodeling and scar maturation. Wound healing is commonly staged into three phases, namely inflammatory phase, proliferation phase (angiogenesis, epithelialization and fibroplasia) and maturation phase.⁴

Epithelialization is an essential component of wound healing used as a defining parameter of a successful wound closure. A wound cannot be considered healed in the absence of re-epithelialization. The epithelialization process is impaired in all types of chronic wounds.⁵ Epithelialization can be accelerated with the help of medicines from herbal plants, such as cassava leaves.

Cassava leaves are rich in vitamin B, C, Carotene, Calcium and Iron.⁶ Cassava leaves have many health benefits since they have a high level of vitamin C and some organic compounds, such as flavonoids, triterpenoids, tannins, and saponins. Flavonoids are polyphenolic

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compounds that occur ubiquitously in plants having a variety of biological effects both in vitro and in vivo tannins. They have been found to have antimicrobial, antiviral, anti-ulcerogenic, cytotoxic, antineoplastic, mutagenic, antioxidant, antihepatotoxic, antihypertensive, hypolipidemic, antiplatelet and anti-inflammatory activities.⁷ Previous studies have revealed that 25% and 50% concentration of flavonoid extract of cassava leaf can reduce TNF- α levels in rat *periodontitis* models.⁸

Periodontitis treatment can be conducted through surgical and non-surgical methods (chemical drugs), growth factors, bone substitution and stem cells.⁹ Some researchers had prepared and reported a newer drug formulation named as in situ gel, which is able to reside in an oral cavity for a longer period of time.¹⁰ Therefore, the cassava leaves (*Manihot esculenta*) is made into a gel to increase the absorption of flavonoid content in tissues that experience periodontitis and reduce chemical side effects.

The number of experimental researches on the effect of flavonoid extract gel of cassava leaf (*Manihot esculenta*) in accelerating periodontitis wound healing is still small. Therefore, the authors wanted to find out the effect of flavonoid extract gel of cassava leaf (*Manihot esculenta*) at concentrations of 25% and 50% on the thickness of the gingival epithelium in the rat periodontitis models induced by *LPS E.coli*.

Materials and methods

All procedures in this research were approved by the Ethics and Advocacy Committee of Faculty of Dentistry, University of Gadjah Mada (No.00366/KKEP/FKGUGM/ EC/2015). This research is a laboratory experimental research using post-test control group design.

The study was started by making flavonoid extract of cassava leaf (*Manihot esculenta*) at the Laboratory of Chemical Engineering, Politeknik Negeri Malang. The 450 grams of cassava leaves (*Manihot esculenta*) were washed, then cut into small pieces and aerated for 2 days in a room which was not exposed to direct sunlight, and then dried in the oven for 24 hours at 40°C. These processes made the weight of cassava leaves decrease into 238.54 grams. The dried leaves were grinded and then sieved with 80-maze mesh, so that it

could obtain 207.25 grams of fine powder. Furthermore, the cassava leaf powder was macerated with 96% ethanol for 3 days and stirred every 24 hours. Afterwards, the solution was concentrated with a rotary evaporator at a temperature of 50°C and a rotation of 90 rpm to result the extract of cassava leaf (*Manihot esculenta*) with a concentration of 100%, as much as 20 grams. 20 grams of crude extract of cassava leaves were then added to 100 ml of absolute ethanol and then exposed to ultrasound for 10 minutes. After that, it was added with 10 ml of 5% H₃PO₄ and then heated at a temperature of 80°C for 30 minutes, then left for 8 hours. Furthermore, the resulted top layer was taken by vacuum-filtration. The filtrate was extracted with 10 ml petroleum ether (repeated 3 times). The results of the extract were oven-dried at a temperature of 60°C. To reduce the level of ethanol, water was added up to 5 ml. The next stage was the addition of 20 ml of Acetonitrile and sonification for 5 minutes. Then, it was centrifuged at 4000 rpm for 5 minutes. The resulted top layer was taken and dried, so that it became the results of flavonoid extract of cassava leaf. The results of flavonoid extract of cassava leaf were tested using LC-MS/MS to determine the flavonoid levels. The procedure was based on a modification of two different protocols proposed by Docheva *et al.* and Muhammad *et al.*^{11,12}

Gel making was carried out at Pharmaceutics Laboratory, Faculty of Pharmacy, University of Jember. The process of making base gel started with Carbopol, developed in hot water in a mortar, and then stirred until the gel was homogeneous. Triethanolamine (TEA) was then added a little until the mass of the gel was collided. Flavonoid extract of cassava leaf was mixed with tween 20 until it became homogeneous. The extract mixture and tween 20 were then mixed into the gel base and stirred until it was homogeneous. The remaining distilled water was added to the gel in small increments until homogeneous. The procedure of making the gel was based on a modification of protocol proposed by Ahmed *et al.*¹⁰

Treatment of experimental animals was carried out at the Laboratory of Physiology, Faculty of Dentistry, University of Jember. This study used 27 male Wistar rats divided into 3 groups. Group control induced with *LPS E.coli* for 2 weeks, Group (P1) induced with *LPS E.coli*

for 2 week and treated with the topical flavonoid extract gel of cassava leaf at a concentration of 25%. Group (P2) induced with LPS *E.coli* for 2 week and treated with the topical flavonoid extract gel of cassava leaf at a concentration of 50%. Then each group decapitated at day 3, day 7 and day 14 after administration. In the early stage, those Wistar rats were anaesthetized using ketamine at a dose of 0.5 ml/ kg, injected into their quadriceps muscle/triceps muscle of their right rear-foot. The rats were injected with either 10 μ l of saline or LPS *E.coli* (Sigma) (1 mg/ ml) at the gingival sulcus of their first right mandibular molar, in which 5 μ l into lingual part and 5 μ l into buccal part. It was injected every three days for two weeks using a tuberculin syringe with a 30-gauge syringe to trigger periodontitis.¹³

After periodontitis occurred, therapy was given with 25% and 50% flavonoid extract gel which was applied topically in the gingival sulcus region of the mandibular right first molar twice a day for 14 days using the blunted syringe needle. The excess gel on the gingival sulcus then was cleaned with a cotton pellet.¹⁴

Decapitation was conducted on days 3, 7 and 14 after the administration of flavonoid extract gel of Cassava leaf. Decapitation on day 3 was considered as inflammatory phase, and then followed with proliferative phase considered on day 7, then wound remodeling with scar tissue formation as wound healing process on day 14. Tissue processing was processing using hematoxyline staining.

Research data were obtained from observation of histological preparations from each group. Observation of histology preparations used a light microscope (Olympus) assisted with OptiLab at 400x magnification. Examination of epithelial thickness was carried out by measuring the thickness of the epithelium from the stratum basalis to the stratum corneum using raster image software and place of measurement on 1 piece in 3 selected visual fields, and then the results were summed and averaged.

The results of the research data are presented in the average epithelial thickness. To determine the data normality, normality test was applied using Shapirowilk. Then homogeneity test was conducted by using Levene Test. Then, the Two Way Annova parametric test was conducted.

Results

Histological features of epithelial thickness of rat gingival are shown in Figure 1 below.

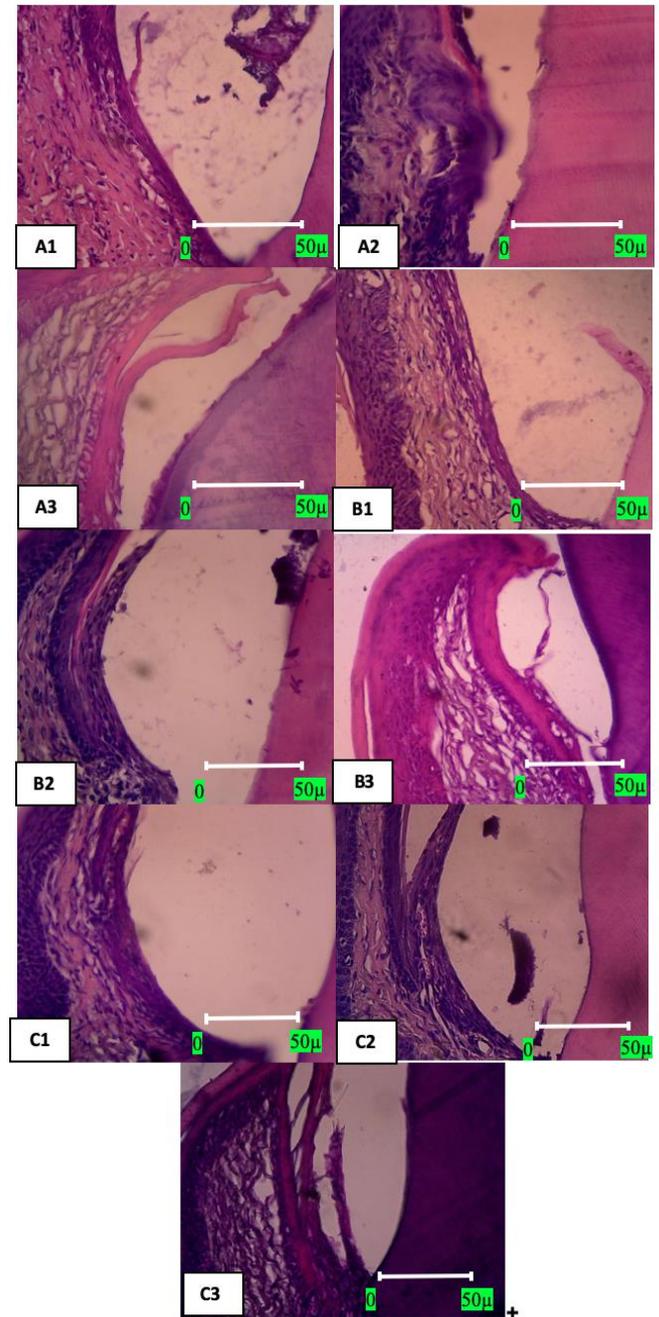


Figure 1. Histological features in rat gingival epithelial tissue. **Control group** day 3 (A1), day 7 (A2) and day 14 (A3). **25% flavonoid extract gel cassava leaf group** day 3 (B1), day 7 (B2) and daya 14 (B3). **50% flavonoid extract gel cassava leaf group** day 3 (C1), day 7 (C2) and daya 14 (C3). magnification 400x.

The results of the calculation of the average gingival epithelial thickness of rats with HE which is staining in each group are shown in Table 1 and Figure 2 below. Table 1 shows the treatment group of 25% flavonoid extract gel decapitated on day 3 has the thinnest gingival epithelial thickness (38.06µm). The treatment group of 50% flavonoid extract gel which was decapitated on day 14 has the thickest average thickest epithelium (100.11µm).

Time of decapitation	Treatment Group ($\bar{x} \pm SD$) (n=3)			P
	Control	EFDS 25%	EFDS 50%	
Day 3	56.79 ± 1.49	38.06 ± 1.45	57.92 ± 1.49	0.258
Day 7	49.70 ± 2.03	48.98 ± 1.44	64.90 ± 1.67	0.488
Day 14	72.58 ± 1.46	59.86 ± 1.58	100.11 ± 1.29	0.036*

Table 1. Mean gingival epithelial thickness

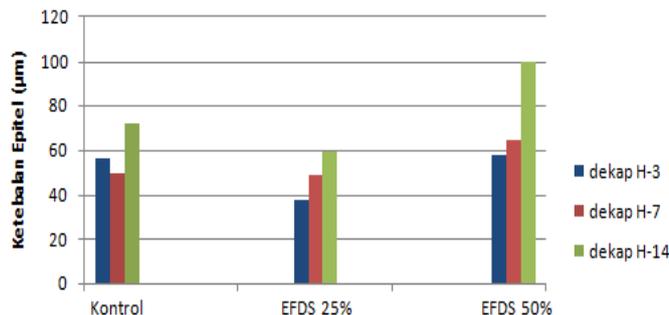


Figure 2. Histogram of the average thickness of rat gingival epithelium in each group.

Notes:

Epithelial thickness unit: µm

N: number of samples

$\bar{x} \pm SD$: Average ± Standard Deviation

Control: No treatment

EFDS 25%: Treatment of 25% flavonoid extract gel of cassava leaf

EFDS 50%: Treatment of 50% flavonoid extract gel of cassava leaf

P: Significance value

* p < 0.05 : Significantly different, followed by LSD test

Figure 2 shows mean of epithelial thickness on the 25% and 50% flavonoid extracts of cassava leaf which showed the epithelial thickness are increased from day 3 until day 14 after decapitation. The data obtained was then tested using Shapiro-Wilk to determine the normality of the data. The results of the normality test showed a significance value P=0.560, so that it can be concluded that the data were normally distributed. Then, the data were tested

for homogeneity using Levene test, which obtained a significance value of 0.987 (p>0.05). From Levene test, it was found that the data were homogeneously distributed. Furthermore, One Way Anova test was conducted to find out whether or not there were significant differences in data (Table 4.1).

One Way Anova test showed that the group decapitated on day 14 was significantly different otherwise the group decapitated on days 3 and 7 was not significantly different. LSD test results shows that there are significant differences on decapitation day 14 between treatment group of 25% extract gel flavonoid of cassava leaf and treatment group of 50% flavonoid extract gel of cassava leaf. However, there were no significant differences between the control group and the treatment group of 25% flavonoid extract of cassava leaf and between the control group and treatment group of 50% flavonoid extract gel of cassava leaf.

Discussion

This research to know the effect of flavonoid extract gel of cassava leaf (*Manihot esculenta*) on gingival epithelial thickness in rat periodontitis models induced with lipopolysaccharide. This study used two concentrations of flavonoid extract of cassava leaf selected based on a preliminary study which revealed that flavonoid extract of cassava leaf with a concentration of 25% and 50% had the ability to reduce TNF-α expression.⁸

The results of this study, epithelial thickness in the group decapitated on day 3 did not show significant differences between another group. There is no differences, on day 3 is the initial phase of the occurrence of mitotic epithelial cells and the migration of epithelial cells to the wound has just begun, so there is not increase in epithelial thickness.⁴ Previous studies showed on day 3, there is still an inflammatory phase. This phase occurred for 3 days, in the inflammatory phase there is an infiltration of acute inflammatory cells into the affected area.¹⁵

Flavonoid of cassava leaf is assumed to play a role in reducing inflammation process. Flavonoid compounds as anti-inflammatory can cause a decrease in the level of proinflammatory cytokines through inhibition of Nuclear Factor Kappa B (NF-κB).¹⁶ The isolated bioactive flavanoid Mesuaferrin-A from *Mesuaferrea L.*

bark ethyl acetate extract acts as a dual inhibitor by inhibiting 5 LOX, COX-2 enzymes and inhibiting carrageenan-induced paw edema.¹⁷ Other flavonoid anti-inflammatory activities are carried out through inhibition of the cyclooxygenase and lipoxygenase cycles, so that migratory inflammatory cells are limited, and clinical signs of inflammation are reduced.¹⁸

Based on the results of the study, epithelial thickness in the group decapitated on day 7 did not show a significant difference. At day 7, there is proliferation phase which includes epithelialization, angiogenesis and fibroplasia. This phase occurred from 4th to 14th day where cellular activity is more dominant.¹⁵

There is no significance different among the group is probably due to mitosis and epithelial cell migration to the periphery of the wound has not healed completely on day 7. Epithelial cells will continuously proliferate and replace cells dead. Epithelial cell proliferation will stop if the tissue has undergone perfect epithelialization.¹⁸ Flavonoid extract gel of cassava leaf is thought to play a role in accelerating the healing process of the tissue. Furthermore, the inflammatory process shorter and the proliferative ability of growth factors is not inhibited.

At day 14th is maturation phase which occurs 8-365 days, where the reorganization process begins, and vascularization has been greatly reduced.⁸ On day 14, the proliferation phase occurred perfectly, so that the increase in epithelial thickness by the epithelialization process stops.¹⁸ Based on the results of the study, epithelial thickness in the control group decapitated on day 14 showed a significant difference. The results of LSD test in the group decapitated on day 14 indicated that the control group did not show a significant difference compared to the treatment group of 25% and 50% flavonoid extract gel of cassava leaf. This occurred because in groups 25% and %0% flavonoid extract gel of cassava leaf there had been maturation where the epithelialization process had been completed.¹⁵ The treatment group of 25% flavonoid extract of cassava leaf showed a significant difference from 50% flavonoid extract gel of cassava leaf group. This was probably due to the less optimal anti-inflammatory power of flavonoid extract gel of cassava leaf. This is supported by previous study which states that 25% concentration is not

effective in inhibiting the expression of cyclooxygenase-2 enzyme.¹⁹

Epithelial thickness in the treatment groups of 25% and 50% flavonoid extract gel showed more thickening from day 3, day 7 to day 14 of decapitation. The effect of anti-inflammatory activity of flavonoid compounds such as flavonoids function to inhibit the release of inflammatory mediators. Flavonoid compounds as anti-inflammatory can causes a decrease in the level of proinflammatory cytokines through the inhibition of Nuclear Factor Kappa B (NF-κB). NF-κB became active because of the stimulus of ROS agents that caused epithelial dysfunction, pathogen exposure, DNA damage and physical stress. NF-κB functions to control the expression of genes encoding proinflammatory cytokines and chemokines such as TNF-α, IL-1β, IL-6 and other proteins. Other flavonoid anti-inflammatory activities are carried out through inhibition of the cyclooxygenase and lipoxygenase cycles, so that inflammatory cells that migrate are limited and clinical signs of inflammation are reduced. Cyclooxygenase is an enzyme that can increase the production of *prostaglandin E2* (PGE2).¹⁶

Prostaglandin E2 (PGE2) is a potent inflammatory mediator to trigger periodontitis by breaking bonds between kappa B (IκB) inhibitors and Nuclear Factor Kappa B (NF-κB). Inhibition NF-κB transcription factors will suppress the production of proinflammatory cytokines, so they will not induce further epithelial cell damage. This has an effect in decreasing epithelial cell damage. Furthermore, the inflammatory reaction will be shorter, and the proliferative ability of growth factors is not inhibited.^{15,16} Kumar⁴ states that if the inflammatory process can occur shorter, tissue healing will be achieved earlier. Migration of epithelial cells starts from the wound edges within a few hours of wounding. A single layer of cells initially forms over the defect, accompanied by a marked increase in epithelial cell mitotic activity around the wound edges. Cells migrating across them attach to the provisional matrix below. When the advancing epithelial cells meet, migration stops, and the basement membrane starts to form.²⁰

Conclusions

Based on the results, it can be concluded there is no different in increasing the thickness of the gingival epithelium of the periodontitis rat

model between flavonoid extract of cassava leaf (*Manihot esculenta*) at concentrations of 25% and 50%.

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Declaration of Interest

All the authors have equal contribution in the manuscript and declare no conflict of interests.

References

1. Partha N, Snigdha P, Laxmidhar M. Formulation development and in vitro evaluation of dental gel containing ethanol extract of tephrosia purpurea linn. *Int J Pharm Pharm Sci* 2016;8(8):132–41.
2. Yiemwattana I, Chaisombon N, Yeesibsarn J, Pangcharoen S. Differential Induction of MAPK Signaling Pathways by Porphyromonas Gingivalis and Escherichia Coli Lipopolysaccharide in Human Monocytes. *Journal of International and Dental Medical Research* 2017;10(2): 202-6.
3. Agustina FE, Sudiana KI, Soetjipto, Rubianto M. Expression of NF- κ B and MMP-7 on Defenses of The Gingival Epithelium Injected LPS Porphyromonas Gingivalis with the Administration of Curcumin. *Journal of International and Dental Medical Research* 2019 12(3): 941-6.
4. Kumar S, Gupta KK, Bhowmick D, Singh A. Concept of Healing in Periodontal Therapy-Part 1. *Journal of Dental and Medical Sciences* 2015;10(11):89-101.
5. Pastar I, Stojadinovic O, Yin NC, et al. Epithelialization in Wound Healing: A Comprehensive Review 2014;3(7):445–64.
6. Biokanesereme, Umar FY, Patrick N, Okechukwu. Anti Inflammatory, Analgesic and Anti-Pyretic Activity Of Cassava Leaves Extract. *Asian Journal Of Pharamceutical And Clinical Research* 2013;6(4):89-92.
7. Rathee P, Chaudhary H, Rathee S, Rathee D, Kumar V, Khli. Mechanism of Action of Flavonoids as Anti-inflammatory Agents: A Review. *J Inflammation & Allergy* 2009;8(3): 229-35.
8. Meilawaty, Z. Effect of Cassava Leaf Flavonoid Extract on TNF- α Expressions in Rat Models Suffering from Periodontitis. *Dental Journal* 2016;49(3):137-142.
9. Banu, S Ramamurthy, J. Role of Animal Models in Periodontal Research-A Review. *Asian Journal Of Pharamceutical And Clinical Research*. 2018;11(7):47-51.
10. Ahmed MG, Choudhari R, Acharya A. Formulation and evaluation of in situ gel of atorvastatin for the treatment of periodontitis. *RGUHS J.Pharm Sci* 2015;5(2): 53-60.
11. Docheva M, Dagnon S, Statkova-AS. Flavonoid content and radical scavenging potential of extracts prepared from tobacco culvitar and waste. *Natural Product Research* 2014;28: 1328-34.
12. Muhammad AA, Pauzi NAS, Arulselvan P, Abas F, Fakurazi S. In vitro wound healing potential and identification of bioactive compounds from Moringa oleifera Lam. *BioMed Research International* 2013: 1-10.
13. Buduneli E, Vardar S, Buduneli N. Effect of Combined Systemic Administration of Low-Dose Doxycycline and Alendronate on Endotoxin-Induced Periodontitis in Rats. *J Periodontol* 2009; 75(11):1516-23.
14. Sato S, Fonseca MJV, Ciampo JOD, Jabor JR, Pedrazzi V. Metronidazole-containing gel for the Treatment of Periodontitis: an in vivo evaluation. *Braz Oral Res* 2008;22(2): 145-50.
15. Abbas AK, Lichtman AH. Cellular and Molecular Immunology. Saunders, USA. 2015:345-55.
16. Nair M, Mahajan S, Reynolds J, Aalinkeel R, Nair H, Schwartz S, Kandaswami, C. The Flavonoid Quercetin Inhibits Proinflammatory Cytokine (*Tumor Necrosis Factor Alpha*) Gene Expression in Normal Peripheral Blood Mononuclear 2006; 13(3):319-28.
17. Chaithanya KK, Gopalakrishnan VK, Hagos Z, Rao DG. Mesuaferrin A, bioactive flavonoid isolated from the Bark of Mesua Ferrea L. Against Phospholipase A2, Cyclooxygenase and Lipoxygenase: an in vitro, in vivo and in silico approach. *Int J Pharm Pharm Sci* 2018;10(2):102–6.
18. Jia L, Han N, Du J, Guo L, Luo S, Liu Y. Pathogenesis of Important Virulence Factor of Porphyromonas Gingivalis Via Toll Like Receptors. *Front Cell Infect Microbiol* 2019;9(262):1-13.
19. Meilawaty, Z. The Effect of *Manihot utilissima* Extracts on COX-2 Expression of Monocytes Induced by LPS E. coli. *Dental Journal*. 2013;46(4): 196-201.
20. Velnar. T, Bailey. T, Smrkolj. V. The Wound Healing Process: an Overview of the Cellular and Molecular Mechanisms. *The Journal of International Medical Research* 2009; 37: 1528-42.