Interdental Jurnal Kedokteran Gigi
Website: https://e-journal.unmas.ac.id/index.php/interdental
ISSN 1979-9144 (print), ISSN 2685-5208 (online)

Research Article

Moringa (*Moringa oleifera* L.) Leaf Extract Gel Is Able to Reduce TNF-α and IL-6 Post Tooth Extraction in Male Wistar Rats (*Rattus norvegicus*)

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Received date: February 6, 2026 **Accepted date: April 13, 2026** **Published date: April 30, 2026**

KEYWORDS

Moringa leaf extract gel, inflammation, TNF-α, IL-6, tooth extraction

ABSTRACT

Background: Tooth extraction is a procedure that can cause injury to the soft tissue. The inflammatory phase that occurs during wound healing triggers a series of tissue changes mediated by the immune system and cytokines. TNF-α and IL-6 cytokine levels should decrease as the wound heals, but otherwise it will slow wound healing and lead to chronic inflammation. The purpose of this study was to determine the effect of the extract gel of Moringa leaves on the wound healing process

Materials and Methods: This research was conducted on 24 Wistar rats, which were randomly divided into four groups, 70%, 90% Moringa leaf extract gel group 70% and 90%, the control (+) oxyfresh gel, and the control (-) CMC-Na 2%. The left central incisor tooth was extracted, resulting in an injury to the gingival socket, and was given topical treatment every day for 7 days. Blood sampling from the orbital sinus was carried out on the 4th and 7th day after injury, and the levels of TNF-α and IL-6 were measured by ELISA. The data obtained was tested with two-way ANOVA and Post Hoc LSD.

Results: The lowest IL-6 was found in the 90% Moringa leaf extract gel group, with an average of 80.86 ± 1.91, while the lowest TNF-α was found in the 90% day 7 group with 80.56 ± 0.41. There were significant differences between the test groups and the observation time, with $p < 0.05$.

Conclusion: Topical application of moringa oleifera gel was effective in reducing TNF-α and IL-6 levels after tooth extraction in Wistar rats

DOI: 10.46862/interdental.v22i1.13611

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How to cite this article: Dewi NPDC, Jawi IM, Purnawati S. Moringa (Moringa oleifera L.) Leaf Extract Gel Is Able to Reduce TNF-α and IL-6 Post Tooth Extraction in Male Wistar Rats (Rattus norvegicus). Interdental Jurnal Kedokteran Gigi. 2022;22(1):31-39. doi: 10.46862/interdental.v22i1.13611

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INTRODUCTION

In dental practice, tooth extraction is one of the procedures of dental and oral health services, which is often done by dentists in the community because of the pain and the condition of the teeth that can no longer be treated.[1] The results of the 2018 Basic Health Research (Riskesdas) state that the largest proportion of dental problems in Indonesia are damaged/cavities/sick teeth (45.3%). The prevalence of caries or cavities in Bali Province reaches 42.26%. In cases of cavities that were extracted or exfoliated by themselves is around 18.01%. Therefore, the proportion of cavities that are extracted is still relatively high.[2] The prevalence of tooth extraction in dental practice in Indonesia has reached 79.6%.[3]

In the case of teeth that need to be extracted, the extraction must be ideal. Extraction of a tooth or root that is intact without causing pain, with as little trauma as possible to the supporting tissue, so that the wound from the extraction will heal normally and will not cause inflammation.[4]

Post-wound healing extracted tooth is a series of complex processes, and it is a series of events that are interconnected and depend on one another. Handling painful post-extraction who does not inadequate, resulting in interference with the wound healing process. One of the important factors in acute phase wound healing in the first 3 days is TNF-α expression and IL-6. TNF-α and IL-6 are two major inflammatory cytokines that work synergistically to strengthen the inflammatory response and have the effect of accelerating wound healing so that chronic complications do not occur.[5]

To prevent complications after tooth extraction, anti-inflammatory drugs are given, but these anti-inflammatory drugs have adverse side effects, so the use of traditional drugs with natural ingredients becomes a rational treatment.[6]

Herbal medicines are in great demand by society, and their formulations can lead to several new generations of phytomedicines that are more effective than previous formulations. One of them is moringa leaves, which have many therapeutic properties and have been shown to have anti-inflammatory agents. In addition, the seeds have anti-inflammatory effects, antihypertensive effects, and the ability to reduce lipid peroxidation.[7]

Moringa leaf extract has been widely studied, the content of flavonoids in Moringa (Moringa oleifera L.) acts as an anti-inflammatory agent.[8] Anti-inflammatory flavonoids can inhibit the action of arachidonic acid, followed by inhibition of the production of prostaglandins, thromboxane, and leukotrienes as inflammatory mediators, so that the migration of leukocytes to the area of inflammation is reduced.[7]

Based on a series of studies on the pharmacological activity and chemical content of moringa leaf extract (Moringa oleifera L.) that had been carried out before, it was still not known specifically about the ability to heal wounds with parameters of decreasing levels of IL-6 and TNF-α as pro-inflammatory cytokines. The purpose of this study was to prove that moringa leaf extract gel (Moringa oleifera L.) can reduce TNF-α and IL-6 levels in post-tooth extraction in male Wistar rats.

MATERIAL AND METHODS

Ethical clearance tests for test animals were carried out at the Ethics Committee of the Faculty of Medicine, Udayana University, Number B/230/UN14.2.9/PT.01.04/. Extraction, phytochemical testing, and preparation of extract gels were carried out at the Pharmacognosy and Phytochemical Laboratory, Department of Pharmacy, Faculty of Mathematics and Natural Sciences, Udayana University. The wound healing activity test

after tooth extraction was carried out at the Histology Analytical Laboratory, Udayana University. In addition, an analysis of TNF-α levels was also carried out at the Biochemistry Laboratory, Faculty of Medicine, Udayana University. The study was conducted for ± 6 months.

The independent variable in this study is moringa leaf extract gel (Moringa oleifera L.). The dependent variable in this study is the decrease of TNF-α and IL-6.

Tools that is used are glass jars, blenders, mortars, stampers, spatulas, porcelain dishes, animal scales, horn spoons, knives, glassware, analytical balances, filter paper, plastic tubs, wire covers, permanent marker razors, surgical tools, caliper, light microscope, 22 × 10 cm glass cylinder tank, Rotary evaporator (Eyela®), capillary pipette, Plate sealer, Zipper bag. Moringa leaves (Moringa oleifera L), 90% ethanol, The extract gel that consist by mixing CMC-Na with moringa leaf extract. Material for wound healing activity test using IL-6 and TNF-α Rat ELISA Kit, Invitrogen Thermo Fischer, in the form of Standard Solution (9600pg/ml), Pre-coated ELISA Plate, Standard Diluent, Streptavidin-HRP, Stop Solution, Substrate Solution A, Substrate Solution B, Wash Buffer Concentrate (30x), Biotin-Conjugate Anti-Rat IL-6 and TNF-α Antibody.

The research was initiated with the collection and authentication of fresh Moringa oleifera L. leaves. The botanical materials were dehydrated using a controlled drying method to maintain the integrity of bioactive compounds, followed by pulverization into a fine simplicia powder. The Moringa oleifera L. extract was subsequently prepared through [insert extraction method, e.g., maceration], and the resulting extract was subjected to qualitative phytochemical screening to identify secondary metabolites. After that, Moringa oleifera L. was incorporated into a specialized carrier gel system. The formulation was optimized to ensure proper viscosity and stability for topical application on mucosal surfaces. Animal Model and Wound Induction

Male Wistar rats (Rattus norvegicus) were acclimatized to laboratory conditions for seven days. Wound induction was performed via a standardized tooth extraction procedure under general anesthesia. To minimize biological bias, the subjects were stratified into three experimental groups based on uniform criteria for age, body weight, sex, and dietary intake. The experimental groups were administered the respective treatments topically twice daily for a duration of seven days. The extraction of the left mandibular incisor was performed on each experimental subject. General anesthesia was induced via an intramuscular injection of ketamine. The anesthetic agent was administered using a disposable syringe into the triceps muscle of the right hind limb. To initiate the extraction, a semi-circular probe was inserted into the gingival sulcus to disrupt the periodontal attachment. The tooth was subsequently luxated using an artery clamp and elevated or extracted using a small excavator. The entire procedure was executed with minimal force and controlled movement to mitigate iatrogenic trauma to the surrounding tissues.

The experimental subjects were stratified into the following treatment regimens: Negative Control Group: A base gel (vehicle) was applied topically twice daily (morning and evening) from day 1 to day 7. Positive Control Group: A commercial healing gel (Oxyfresh®) was administered twice daily following the same temporal protocol. Treatment Groups (70% and 90% Gel): The formulated moringa leaf extract gels at concentrations of 70% and 90% were applied topically twice daily. For all groups, a systematic euthanasia (decapitation) was conducted for tissue collection. On the 4th and 7th days post-

induction, three rats from each designated group were sacrificed to allow for downstream molecular and histological evaluations.

The wound healing efficacy was evaluated through the quantification of pro-inflammatory cytokines, specifically TNF-α and IL-6. These markers were analyzed using the Enzyme-Linked Immunosorbent Assay (ELISA) technique to assess the inflammatory response. Subjects from each group were euthanized on the 4th and 7th days post-induction.

Data on the percentage of wound healing from the analysis of decreasing IL-6 levels. The obtained IL-6 levels were then tested for distribution with the Shapiro-Wilk test and homogeneity with the Levene test. Data that has been normally distributed and homogeneous is followed by using the one-way ANOVA test and continued with the LSD statistical test. Then, a non-parametric test will also be carried out using the Kruskal-Wallis test, followed by a Mann-Whitney analysis. The treatment group has a p-value <0.05, so it can be stated to be significantly different.

RESULTS AND DISCUSSIONS

The extraction of Moringa oleifera L. leaves was carried out using a cold maceration technique. Fresh leaves (2 kg) were initially desiccated and pulverized to increase the surface area for solvent penetration. A total of 400 g of the resulting powder was subjected to extraction in 90% ethanol for 24 hours under intermittent agitation. Upon completion of the initial incubation, the mixture was filtered, and the recovered residue underwent twofold remaceration to ensure exhaustive extraction of bioactive compounds. The combined filtrates were subsequently concentrated under reduced pressure using a rotary vacuum evaporator at a constant temperature of 68°C. The process was maintained until a dense, crude, viscous extract was yielded for further analysis.Moringa leaf extract (Moringa oleifera L.) was prepared from 402 grams of powder soaked in 90% ethanol. The selected extraction method is the maceration method, which is then evaporated with a rotary evaporator to obtain a thick extract. The viscous extract obtained was 63.48 grams with a total yield value of the moringa leaves (Moringa oleifera L.) extract of 15.79%.

The average value of the water content of moringa leaf extract is 7.23 ± 0.025. According to the Ministry of Health of the Republic of Indonesia (2020), these results have met the requirements set for the water content of the ethanol extract of moringa leaves (Moringa oleifera L.), which is not more than 8.85%.

The results of the phytochemical test showed that the prepared moringa leaf extract (Moringa oleifera L.) contained positive secondary metabolites in the form of flavonoids, triterpenoids, saponins, and tannins.

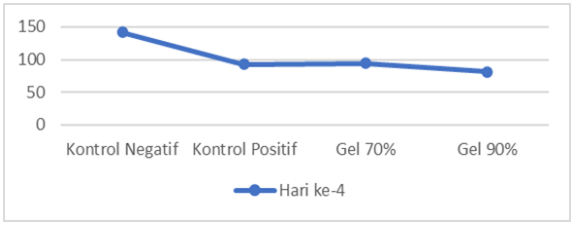

Figure 1. TNF-α levels on day 4 (Description **: Different Meaning)

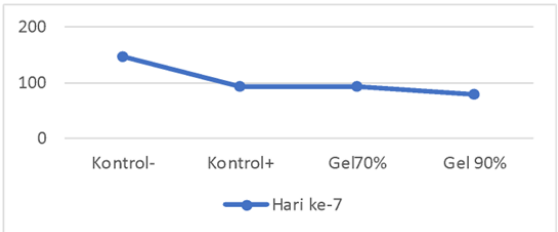

Figure 2. TNF-α levels on day 7 (Description **: Different Meaning)

Figures 1 and 2 show that the TNF-α level of the negative group was higher than that of the other groups. The negative group was the rats that had teeth extracted and applied 2% CMC-NA gel (Placebo). The positive group that was given oxyfresh gel showed a decrease in TNF-α levels compared to the negative group. The 70% and 90% gel test groups showed a decrease in TNF-α levels compared to the negative group. Significant differences in TNF-α levels in the 90% gel test group compared to the negative, positive, and 70% gel groups. Figure 1 shows the relationship between the absorbance value and the concentration/level of TNF-α in the sample. The higher the absorbance value, the higher the concentration/level of TNF-α contained in the tissue sample. The absorbance data is shown in the attachment.

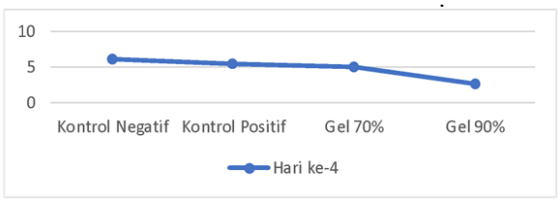

Figure 3. IL-6 levels on day 4 (Description ** : Different Meaning)

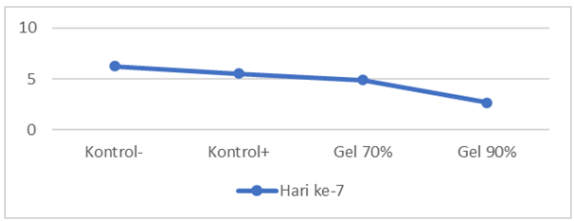

Figure 4. IL-6 levels on day 7 (Description ** : Different Meaning)

Figures 3 and 4 show that the TNF-α level of the negative group was higher than the other groups. The negative group was the rats that had teeth extracted and applied 2% CMC-NA gel (Placebo). The positive group that was given oxyfresh gel showed a decrease in TNF-α levels compared to the negative group. The 70% and 90% gel test groups showed a decrease in TNF-α levels compared to the negative group. Significant differences in TNF-α levels in the 90% gel test group compared to the negative, positive, and 70% gel groups.

Table 1. Percentage of difference in TNF-α and IL-6 levels

Biomarker	Comparison Group	Difference	%
TNF-α	Positive Control vs 70% Gel	1.431	0.07
TNF-α	Positive Control vs 90% Gel	12.134	6.00
IL-6	Positive Control vs 70% Gel	587	0.50
IL-6	Positive Control vs 90% Gel	2800	33.00

Notes:

1. Percentage (%) values indicate relative differences compared with the positive control group.
2. TNF-α = tumor necrosis factor-alpha; IL-6 = interleukin-6.
3. The 90% Moringa oleifera gel group showed greater reductions in both biomarkers than the 70% gel group.

Table 2. Comparison of TNF-α and IL-6 Levels Among Experimental Groups

Group	Day	TNF-α (Mean ± SD)	IL-6 (Mean ± SD)
Positive Control	4	92.82 ± 0.53	5.53 ± 0.02
Positive Control	7	93.94 ± 0.15	5.52 ± 0.02
Negative Control	4	141.59 ± 3.20	6.12 ± 0.88
Negative Control	7	145.53 ± 0.78	6.26 ± 0.05
90% Gel	4	80.86 ± 1.91	2.73 ± 0.06
90% Gel	7	80.56 ± 0.49	2.68 ± 0.49
70% Gel	4	94.25 ± 1.40	4.95 ± 0.03
70% Gel	7	93.89 ± 0.20	4.87 ± 0.22

Notes:

1. Data are presented as mean ± standard deviation (SD).
2. Each subgroup consisted of three samples (n = 3).
3. TNF-α = tumor necrosis factor-alpha; IL-6 = interleukin-6.
4. Two-way ANOVA showed significant differences among treatment groups and observation times ($p < 0.05$).
5. The 90% Moringa oleifera gel group demonstrated the lowest TNF-α and IL-6 levels.

Tables 2 show that there is a difference between the average levels of TNF-α and IL-6 in two or more than 24 samples, with a p value of 0.001 ($p<0.05$). Then the Least Significant Difference (LSD) test was carried out to see whether the average comparison was significant or not between each group.

Table 4. Post Hoc LSD Comparison of TNF-α and IL-6 Levels Between Groups

Comparison	Day	TNF-α Mean Difference	IL-6 Mean Difference	p-value
90% Gel vs Positive Control	4	11.954	2.800	0.001
90% Gel vs Positive Control	7	13.384	2.835	0.001
70% Gel vs Positive Control	4	-1.430	0.597	0.001
70% Gel vs Positive Control	7	0.051	0.651	0.001
Positive Control vs Negative Control	4	-48.777	-0.590	0.001
Positive Control vs Negative Control	7	-51.589	-0.748	0.001
90% Gel vs Negative Control	4	-60.731	-3.390	0.001
90% Gel vs Negative Control	7	-64.973	-3.583	0.001
70% Gel vs Negative Control	4	-47.346	-1.177	0.001
70% Gel vs Negative Control	7	-51.640	-1.399	0.001
70% Gel vs 90% Gel	4	13.385	2.213	0.001
70% Gel vs 90% Gel	7	13.333	2.184	0.001

Notes:

1. LSD = Least Significant Difference post hoc test.
2. Positive values indicate higher biomarker levels in the first group; negative values indicate lower levels.
3. All comparisons were statistically significant ($p < 0.05$).
4. Each subgroup consisted of three samples ($n = 3$).

Table 4 showed significant differences among groups, with p-values < 0.05 for all pair-wise comparisons. Significant differences were observed between the positive control group and the other three groups, namely the negative control group and both extract treatment groups. These findings indicate that administration of the positive control (Oxyfresh gel) significantly reduced TNF-α and IL-6 levels compared with the negative control group ($p < 0.05$).

Administration of *Moringa oleifera* extract gel at both concentrations also resulted in significant reductions in TNF-α and IL-6 levels compared with the negative control group ($p < 0.05$), with effects that were statistically comparable to the positive control group. Among the treatment groups, the 90% gel concentration showed the largest mean differences, indicating that this group had the greatest effect compared with the other groups. The most pronounced reduction in mean IL-6 levels was observed between the 90% gel group and the positive control group, with mean differences of 2.80 on day 4 and 2.83 on day 7. Therefore, it can be concluded that the 90% *Moringa oleifera* gel group was the most effective treatment in reducing TNF-α and IL-6 levels in blood serum after tooth extraction.

Based on the values of TNF-α and IL-6 levels obtained in this study, it was found that the negative control group had the highest average levels of TNF-α and IL-6. The positive group, the 70% gel group, and the 90% gel group experienced an average decrease in TNF-α and IL-6 levels from day 4 to day 7. The gel concentration of 90% had the highest reduction in TNF-α and IL-6 levels.

The difference in mean difference is greatest in the 90% gel with the negative control, which suggests that the 90% gel has the effectiveness of reducing TNF-α levels, and IL-6 was higher than the positive control. Then the difference in mean difference between 70% gel and negative control was not much different from the difference between oxyfresh gel and negative control, so that the giving of 90% and 70% moringa leaf extract gel had effectiveness in reducing TNF-α and IL-6 levels as anti-inflammatory agent candidates, such as oxyfresh gel.

Utilization of Moringa leaves can be used as an anti-inflammatory agent that has low side

effects. Moringa leaves can reduce levels of IL-6 and TNF-α cytokines in male Wistar rats, which contain several chemical compounds in the form of bioactive compounds, one of which is flavonoids. Flavonoids are polyphenolic compounds produced from secondary metabolism in plants.[9] The main flavonoids in moringa leaves, which include quercetin, kaempferol glucoside, and the flavonoid malonate, exhibit anti-inflammatory activity through inhibition of NO production in LPS-stimulated macrophages. NO is involved in the hemostatic-inflammatory and proliferative phases of wound healing by supporting matrix deposition, remodeling, and angiogenesis. NO can also act as an antimicrobial so that it can maintain sterility in the wound area.[10] Flavonoids are known to have a mechanism similar to non-steroidal anti-inflammatory drugs. Flavonoids can inhibit the activity of expression of pro-inflammatory mediators other than COX. Moringa leaves (Moringa oleifera L.) selectively inhibit iNOS and COX-2 production and significantly inhibit the secretion of NO and other inflammatory markers, including PGE-2, TNF-α, IL-6, and IL-1β in lipopolysaccharide cells.[11]

Based on the phytochemical test, it is known that Moringa leaves (Moringa oleifera L.) contain flavonoids, saponins, triterpenoids, and tannins. Several studies have demonstrated the ability of moringa leaf flavonoids to act as wound healers. Flavonoids belonging to the morin, hesperidin, rutin, and chrysin groups are known to be effective in reducing the inflammatory cytokines TNF-α and IL-6 in the process of wound healing. Flavonoids can regulate the cellular activity of cells associated with inflammation, such as mast cells, macrophages, lymphocytes, and neutrophils. For example, some flavonoids inhibit histamine release from mast cells, and others inhibit T-cell proliferation. In addition, certain flavonoids are able to modulate the activity of arachidonic acid (AA) metabolizing enzymes such as phospholipase A2 (PLA2), cyclooxygenase (COX), and lipoxygenase (LOX), and the nitric oxide (NO) producing enzyme, nitric oxide synthase (NOS). Inhibition of this enzyme by flavonoids reduces the production of AA, prostaglandins (PG), leukotrienes (LT), and NO, important mediators of inflammation.

TNF-α and IL-6 are the main pro-inflammatory cytokines that have a significant effect on the site of injury in acute inflammatory responses, such as activation of fibroblasts, endothelium, and leukocytes.[12] TNF-α and IL-6 were produced in the initial wound at relatively high concentrations, indicating that these factors are important for wound healing. However, when TNF-α and IL-6 migrated into the wound, their impact depended on the concentration and duration of exposure. At low levels, TNF-α and IL-6 can promote wound healing by indirectly stimulating inflammation and increasing growth factors produced by macrophages. However, at higher levels of TNF-α and IL-6, especially for a long time, TNF-α has a detrimental effect on healing. It suppresses the synthesis of extracellular matrix proteins (ECM) and inhibits tissue metalloproteinases (TIMPs) while increasing the synthesis of matrix metalloproteinases (MMPs).[13]

In the inflammatory phase, macrophages in the tissue infiltrating neutrophils initiate the pro-inflammatory NF-κB and mitogen-activated protein kinase (MAPK) pathways. Macrophages secrete proinflammatory cytokines, including TNF-α and IL-6, key regulators of the acute inflammatory response. IL-6 has a series of targets

to stimulate Th2 and Th17 differentiation in CD4+ T cells as a cellular immune response.[14] TNF-α and IL-6 cytokine levels should decrease as the wound heals, but otherwise it will slow wound healing and lead to chronic inflammation. The ability of moringa leaf extract gel (Moringa oleifera L.) in reducing IL-6 levels in the 70% and 90% extract test groups is closely related to the content of secondary metabolites contained in moringa leaf extract (Moringa oleifera L.).[15] This is also in accordance with the study by Studha et al.[15] concerning the effect of moringa leaf extract on the gingival wound healing process in Wistar rats, in which moringa leaf extract could reduce IL-1 and IL-6 levels in the wound healing process on day 4 and day 7 of the Wistar rats with a dose of 300 mg/kg BW.

The high levels of TNF-α and IL-6 in the negative control gel CMC-Na 2% are in accordance with the study of Elnar and Ailey[12] that after tooth extraction, the levels of TNF-α and IL-6 will be high in the inflammatory phase if not applied with anti-inflammatory drugs. When compared with positive controls, 90% and 70% gels have effectiveness in reducing TNF-α and IL-6 levels in rat blood serum according to Masoumeh's 2015 study, which was conducted in vitro.[16]

Based on the ability of flavonoids as anti-inflammatory, it is thought to be able to shorten the time of the inflammatory phase in the wound healing process so that chronic inflammation does not occur in the wound healing process after tooth extraction.

CONCLUSION

Based on the results of the research and discussion that have been done, it can be concluded that Moringa leaf extract gel (Moringa oleifera L.) 70% and 90% can reduce TNF-α levels after tooth extraction. Moringa leaf extract gel (Moringa oleifera L.) 70% and 90% can reduce IL-6 levels after tooth extraction.

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