










Assessment of the Anti-inflammatory Activities of the *Moringa* Leaf Extract in Periodontitis Cases through IL-6 Cytokine Analysis in Wistar (*Rattus novergicus*)

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Abstract

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BACKGROUND: Periodontitis is a chronic inflammatory disease that causes damage to the supporting structures of the teeth, and if left untreated, it can lead to impaired function, appearance, pain, and tooth loss. Periodontitis is caused by bacteria that adhere to and grow on the tooth surface. The "red complex" bacteria consisted of *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*. These bacteria will trigger an inflammatory response in the body. Interleukin-6 (IL-6) is an IL that acts both as a pro-inflammatory cytokine, IL-6 is a stimulator for MMP production. To treat the periodontal disease can be through non-surgical therapy as well as surgical therapy, to maximize therapy, it is accompanied by antimicrobial therapy. However, because of the frequent use of antimicrobials, causing antimicrobial resistance in patients, the use of natural ingredients as additional therapy is very necessary for this study using *Moringa* leaves as a substitute for antimicrobials.

AIM: This study aimed to determine the effectiveness of *Moringa* leaf in influencing the anti-inflammatory cytokine IL-6. The first benefit of this research is to provide scientific information in the field of dentistry regarding the effectiveness of *Moringa* leaves against red-complex bacteria *P. gingivalis* as a cause of chronic periodontitis through anti-inflammatory cytokine analysis.

METHODS: The type of research that will be used is quasi-experimental with a post-test research design with a control group design. This study used a sample consisted of 30 Wistars (*Rattus novergicus*) and was divided into two groups based on periodontal tissue sampling as follows treatment group which was treated with extracts *Moringa* and control group with aquadest irrigation after bacterial induction in the gingival sulcus. Blood samples were taken on days 0, 1, 3, 5, and 7 and centrifuged obtain blood serum and serum cytokine levels (pg/mL) were quantified using a commercial ELISA IL-6 kit.

RESULTS: This study obtained the results that there was a decrease in IL-6 in both groups on the 3rd day of observation (D3) where the treatment group given *Moringa* extract showed a greater decrease in IL-6 levels compared to the control group. There is a significant value in the comparison of IL-6 levels between the two groups with p-value: 0.000 (p < 0.05).

CONCLUSION: *Moringa oleifera* leaf extract showed the anti-inflammatory effect on inflammation induced by the bacterium *P. gingivalis*. *M. oleifera* extract can reduce the production of the pro-inflammatory cytokine IL-6 induced by *P. gingivalis* bacteria in periodontitis.

Introduction

Periodontitis is an inflammation of the supporting tissues of the teeth, caused by microorganisms and can cause progressive damage to the periodontal ligament and alveolar bone, and is accompanied by pocket formation. Periodontitis causes permanent tissue destruction characterized by chronic inflammation, apical migration of the fused epithelium, loss of connective tissue, and loss of alveolar bone. The clinical picture of periodontitis is a change in color to bright red, accompanied by swelling of the gingival margin. Bleeding on probing and probing depth of 4 mm

is due to apical migration of the fused epithelium. There is loss of alveolar bone and tooth mobility [1], [2].

The main cause of the periodontal disease is the presence of microorganisms that colonize the dental plaque. Dental plaque is a structured, soft, and yellow substance that adheres to the tooth surface. The content of dental plaque is various types of microorganisms, especially bacteria, the rest are fungi, protozoa, and viruses. A plaque containing these pathogenic microorganisms plays an important role in causing and exacerbating periodontal infection. An increase in the number of Gram-negative organisms in subgingival plaque such as *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans*, *Tannerella*

forsythia, and *Treponema denticola* initiates periodontal infection [3], [4].

Periodontitis is a multifactorial disease. Many studies have proven that the occurrence of periodontitis involves the presence of dental plaque, individuals who are genetically susceptible to periodontitis, and the presence of one or more risk factors such as stress or depression that can alter immune responses and behaviors related to dental health including oral hygiene. Risk is the chance of developing a specific disease in an individual over a certain period of time. Risk factors are environmental, behavioral, and biological factors that have certain causation with the disease process and can increase the chance of a disease occurring [5], [6].

P. gingivalis is Gram-negative anaerobic bacteria which in normal numbers are a normal microflora that can be found in the oral cavity. *P. gingivalis* is often associated with chronic periodontitis in adults. This bacterium produces a number of unique virulence factors and can be genetically manipulated. The presence of genomic sequences can help understand the biological nature of *P. gingivalis* and how it interacts with the environment, other bacteria, and the host [7].

The ability of *P. gingivalis* to cause periodontitis is determined by its virulence factors. Biofilm formation and bacterial dipeptidyl peptidase IV (DPPIV) contribute to the pathogenic potential of *P. gingivalis*. Furthermore, biofilm formation can increase the virulence of these bacteria by increasing DPPIV activity. *P. gingivalis* is closely associated with chronic periodontitis. Its presence in the periodontal tissues depends on its ability to evade host immunity without inhibiting the overall inflammatory response [7], [8].

These bacteria can be reduced using an appropriate mouthwash; one of the mouthwashes that can be used is chlorhexidine digluconate 0.2%. However, the use of chlorhexidine as an antiseptic turns out to have side effects if used continuously. Side effects that occur are the presence of staining on the teeth, sensation, and an unpleasant taste. Another alternative is needed as a mouthwash raw material with minimal side effects. Alternatives that qualify as antiplaque and antibacterial are herbal ingredients. One of the plants in herbal medicine is *Moringa* leaves [9].

Moringa oleifera is a plant native to Indonesia that can be used as medicine and as an antioxidant. *Moringa* plants grow in the lowlands and highlands. *Moringa* is known to contain more than 90 types of nutrients in the form of essential vitamins, minerals, amino acids, anti-aging, and anti-inflammatory. Data regarding the content of active compounds in *Moringa* leaves are still very rare, some literature states that *Moringa* leaves contain flavonoids, saponins, alkaloids, tannins, and phenols.

Moringa leaves are very rich in nutrients, including calcium, iron, protein, Vitamin A, Vitamin B, and Vitamin C. *Moringa* leaves contain higher iron than other vegetables, which is 17.2 mg/100 g. In addition, *Moringa* leaves also contain a variety of amino acids, including amino acids in the form of aspartic acid, glutamic acid, alanine, valine, leucine, isoleucine, histidine, lysine, arginine, phenylalanine, tryptophan, cysteine, and methionine [10], [11].

The use of *Moringa* plants in dentistry is also widely found and researched for its development. *Moringa* plants can be used as toothpaste, mouthwash, root canal irrigation, wound healing after tooth extraction, medicine for gingivitis, and canker sores and can be used to prevent dental caries with its antibacterial properties. The latest research on the effect of *Moringa* leaves in accelerating the reduction of inflammatory signs of erythema in sterile wounds of guinea pigs (*Cavia porcellus*) stated that the results of the analysis of the ability of *Moringa* leaves were proven to accelerate the wound healing process in this case what was observed was a decrease in signs of inflammatory erythema [12].

The aim of this study is the ability of *Moringa* leaf as an herbal plant that has various substances such as flavonoids, saponins, alkaloids, tannins, and phenols so that it has the potential to be an agent for periodontitis treatment. However, until now, there has been no research on the effect of the active substance *M. oleifera* on periodontitis through anti-inflammatory cytokine analysis.

Materials and Methods

Material

Moringa leaves come from the *Moringa* cultivation garden in the Blora area of the Central Java, Indonesia. *Moringa* leaves are then washed and dried in an oven to produce dry leaves. Dried *Moringa* leaves are then ground and processed by maceration technique to produce thick *Moringa* extract.

Study design

This protocol has been approved by the Health Research Ethics Committee of the Dental and Oral Hospital, Faculty of Dentistry, Hasanuddin University, Ministry of Research, Technology and Higher Education, Indonesia (No.0094/PL.09/KEPK FKG-RSGM UNHAS/2021). This study is an *in vivo* laboratory experimental study using the post-test – only control group design.

Research animals

This study used a sample of male Wistar rats (*Rattus norvegicus*) weighing 200–250 g which were randomly allocated in different groups. Animals were adapted 1 h before testing and were used only once in each experiment. The animal samples were previously acclimatized (adapted) for about 1 week in the animal cage room to familiarize the mice to their new environment and observed general conditions such as weighing and animal health. Wistar rats were placed in cages in groups, light/dark cycle for 12 h, temperature 26–29°C, and humidity 60–70% and were given standard feed and drinking water ad libitum. The cage is in the form of a plastic box with a wire cover measuring 40 cm × 60 cm × 25 cm, covered with rice, and cleaned regularly every 3 times a week to keep the cage dry and healthy. Research sampling was carried out using a simple random sampling technique after meeting the inclusion and exclusion criteria.

The sample consisted of 30 Wistars and was divided into two groups based on periodontal tissue sampling as follows: The control group consisted of 15 Wistars with aquadest irrigation after bacterial induction in the gingival sulcus. The treatment group consisted of 15 Wistars which were treated with extracts *Moringa* and aquadest irrigation after bacterial induction in the gingival sulcus. Blood samples were taken on days 0, 1, 3, 5, and 7 and centrifuged at 5000 g for 10 min at 4°C to obtain blood serum. Serum cytokine levels (pg/mL) were quantified using a commercial ELISA IL-6 kit following the manufacturer's instructions (Elabscience).

P. gingivalis bacteria culture agar media

Preparation of bacterial culture Porphyromonas gingivalis using agar media has several stages. Initially, an agar medium for bacterial culture was made, namely, BHI-A enriched with hemin and Vitamin K. To make 100 ml of BHI-A, 50 l of hemin solution was needed, 10 µl of Vitamin K, 37 g of BHI-A in 100 ml of sterile distilled water, and yeast extract 500 µl. The media is divided into four, and then put into petridish @25 ml and wait until solid. One dose of pure *P. gingivalis* strain ATCC 33277 (F0) was inoculated on each petridish and then incubated for 2 × 24 h.

Bacteria suspension *P. gingivalis*

Before making a suspension of *P. gingivalis* bacteria, 10 ml of liquid media was made, namely, from 0.37 g of BHI-B, 1 l of Vitamin K, 5 l of hemin, and 50 l of yeast extract. Next, the suspension of *P. gingivalis* was made. The liquid media that has been made is divided into two parts @ 5cc. Each liquid medium was given one loop of bacteria from culture on BHI-A agar media. The bacterial suspension obtained was then put in a desiccator and incubated for 2 × 24 h. After incubation,

the concentration of the bacterial suspension was measured to obtain 1.5×10^6 . Furthermore, preparations are made with gram staining to determine the bacteria that are in good condition and not contaminated.

Research methods

Induction of *P. gingivalis* bacteria was carried out by injecting bacterial suspension into the experimental animal's periodontal tissues. After 5 days, periodontitis is expected to occur in the experimental animal's periodontal tissues. Clinical signs of inflammatory periodontitis were observed in the form of gingival enlargement, gingival discoloration to redness, and pus accumulation in some samples. The administration of *Moringa* extract in the treatment group and aquadest irrigation in the control group was carried out after signs of periodontitis inflammation occurred in experimental animals. Blood sampling was carried out on day 0, that is, experimental animals before being given any treatment, day 1, day 3, day 5, and day 7 after both groups were given treatment.

Data analysis

Data processing using IBM SPSS statistics V.25. The homogeneity of the data was tested using Levene's test. The treatment effect test/comparative analysis was carried out using the independent t-test because the data used were two groups of paired data. To analyze the differences between the research groups, one-way analysis of variance was used. The results of the analysis were declared significant or there was a difference if $p < 0.05$.

Results

IL-6 levels in each group were measured for 7 days of observation and the values in each group are

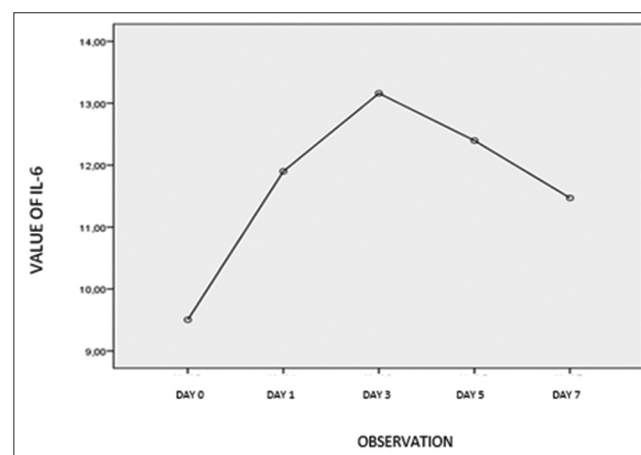


Figure 1: The mean value of interleukin-6 levels in the treatment group on observations. D0, D1, D3, D5, and D7

presented in Figures 1 and 2. The average value of IL-6 levels in both groups was found to be the highest on the 3rd day of observation and the concentration of IL-6 began to decrease on the 5th (D5) to the 7th (D7) day of observation after induction of periodontal tissue with *P. gingivalis* bacteria. The highest mean \pm SD value of IL-6 levels was observed in the control group on the 3rd day of observation and the lowest IL-6 level of mean \pm SD was observed in the treatment group on the 7th day of observation. Based on the t-test by comparing the IL-6 levels of the two groups based on the day of observation, it was found that $p = 0.086$ on D0 and $p = 0.204$ on day 1 where the results were not significant ($p < 0.05$). Comparison of IL-6 levels on day 3 with day 7 in both groups, $p = 0.000$ showed a statistically significant result ($p < 0.05$).

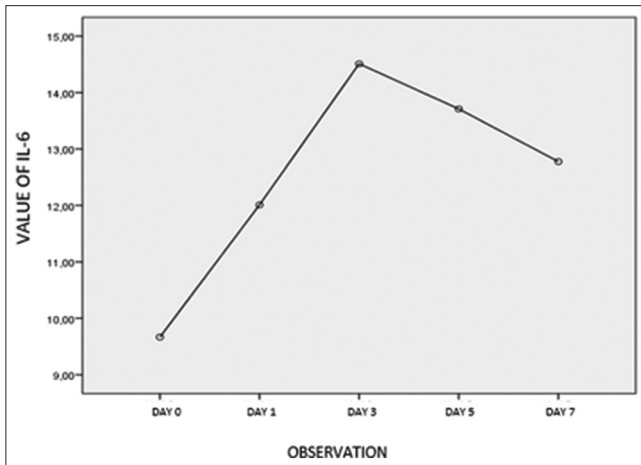


Figure 2: The mean value of interleukin-6 levels in the control group on observations D0, D1, D3, D5, and D7

Figure 3 shows the mean and standard deviation of IL-6 levels in the two groups which shows differences in observations on D0, D1, D3, D5, and D7. This graph shows that the increase in IL-6 concentration in both groups showed the highest increase and starting on the 5th day of observation, there was a decrease in IL-6 levels

in both groups and continued to decrease on D7 where the group given *M. oleifera* extract showed a greater decrease in IL-6 levels faster than the control group. From the graph, it can be seen that the average D3 data in each group was at the highest value, namely, 14.51 ± 0.01 for the control group and 13.16 ± 0.02 for the treatment group. The decrease in IL-6 levels began to be seen at D5 in the control group 13.71 ± 0.07 and 12.39 ± 0.06 in the treatment group and D7 control group 12.77 ± 0.05 and 11.47 ± 0.05 in the treatment group.

Discussion

This study designed the inflammatory reaction by inducing gingival Wistar with *P. gingivalis* bacteria to form periodontitis. The inflammatory response is a non-specific and multi-faceted chemical response of the immune system to various abnormalities or stimuli in the body. These different stimuli trigger a cascade of cytokines and inflammatory mediators leading to vasodilation, vascular leakage, immune cells tissue infiltration and recruitment, and stimulation of mucosal membranes (Owen *et al.*, 2013). These molecular and microscopic tissue changes are visibly manifested as redness, heat, swelling, and pain [13], [14].

Periodontal tissue damage is mainly caused by the interaction of bacterial antigens and inflammatory cells resulting in the production of cytokines. IL-6 is secreted by macrophages in response to inflammation and is involved in leukocyte recruitment and apoptosis and T-cell activation. IL-6 is a pleiotropic cytokine that acts as both anti-inflammatory and pro-inflammatory. IL-6 exhibits anti-inflammatory properties through increased production of tissue inhibitor matrix metalloproteinase and suppression of the pro-inflammatory cytokines IL-1 β and tumour necrosis factor (TNF). IL-6 and its

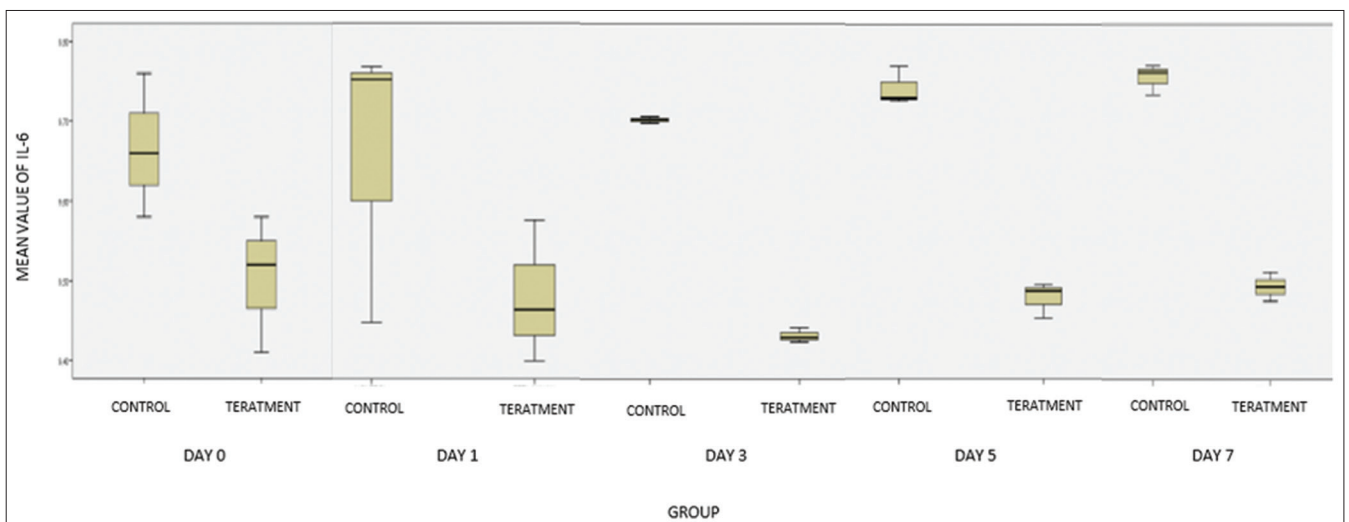


Figure 3: The mean value and standard deviation of interleukin-6 levels in the treatment and control groups

receptors induce bone resorption by increasing nuclear factor K ligand (RANKL) receptor activator or by directly inducing osteoclast formation [13], [15].

In the study of Kou *et al.*, Mengel *et al.*, and Buhlin *et al.* reported that periodontitis has been associated with increased circulating IL-6 levels. This increase appears to be related to the severity of the disease. However, the study of Teles *et al.* showed lower salivary IL-6 levels but there was no significant difference in patients with chronic periodontitis compared with healthy subjects [16]. Pro-inflammatory cytokines, which include TNF- α and IL-6, are classified as the main inflammatory mediators which are produced by monocytes and macrophages in the inflammatory procedure. TNF- α is a key mediator in inflammatory responses, as well as the initiation of apoptosis. It might stimulate the development or expression of IL-6, IL-1 β , PGE2, collagenase, and adhesion molecules eliciting a variety of physiological functions such as septic shock, inflammation, and cytotoxicity. TNF- α and IL-6 are mainly produced by macrophages in response to LPS through NF κ B activation [13], [17].

IL-6 is a pleiotropic cytokine produced in response to tissue damage and infections (Tanaka *et al.*, 2014). After IL-6 is synthesized in a local lesion in the initial stage of inflammation, it moves to the liver through the bloodstream, followed by the rapid induction of an extensive range of acute-phase proteins such as C-reactive protein, serum amyloid A, fibrinogen, haptoglobin, and 1-antichymotrypsin (Heinrich *et al.*, 1990). IL-6 is also involved in the regulation of serum iron with induces hepcidin production, which blocks the action of the iron transporter ferroportin 1 on the gut and, thus, reduces serum iron levels (Nemeth *et al.*, 2004). This means that the IL-6-hepcidin axis is responsible for hypoferremia and anemia associated with chronic inflammation [18], [19].

IL-6 also enhances zinc importer ZIP14 expression on hepatocytes and so induces hypozincemia seen in inflammation (Liuzzi *et al.*, 2005). When IL-6 reaches the bone marrow, it promotes megakaryocyte maturation, thus leading to the release of platelets (Ishibashi *et al.*, 1989). These changes in acute phase protein levels and red blood cell and platelet counts are used for the evaluation of inflammatory severity in routine clinical laboratory examinations [19], [20].

In this study, there were differences in the decrease in IL-6 levels in the two groups; the treatment group was given *Moringa* extract that showed a higher average decrease in IL-6 levels than the control group given aquadest irrigation. This is in line with research conducted by Xu *et al.*, 2019, comparing the anti-inflammatory and antioxidant effects of *M. oleifera* leaves, seeds, and stems which resulted in a decrease in nitric oxide (NO) levels in RAW264.7 macrophage cells as an evaluation of the anti-inflammatory activity [21].

The possible mechanism could be due to inhibition of monocyte infiltration and fibroblast

proliferation. Activated monocytes release a series of pro-inflammatory cytokines, inducing TNF- α which facilitates inflammatory cell infiltration by promoting the adhesion of neutrophils and lymphocytes to the endothelial cell [22]. Hence, it can be construed that the anti-inflammatory effect might be due to active constituents (flavonoids, tannins, rhamnose, xylose, galactose, arabinose, and galacturonic acid) that are present in the aqueous extract of *M. oleifera* leaves. The anti-inflammatory effect of these active constituents has been reported by Mittal *et al.*, 2017 [23].

Moringa leaves contain several chemical compounds in the form of several bioactive compounds, one of which is flavonoids. Flavonoids are polyphenolic compounds produced from secondary metabolism in plants. The main flavonoids in *Moringa*, which include quercetin, kaempferol glucoside, and the flavonoid malonate, exhibit anti-inflammatory activity through inhibition of NO production in LPS-stimulated macrophages (Coppin *et al.*, 2013). Flavonoids are known to have a similar mechanism to non-steroidal anti-inflammatory drugs. Flavonoids can inhibit the activity of mediators of expression of pro-inflammatory mediators other than COX (Izzi *et al.*, 2012). *M. oleifera* can selectively inhibit the production of iNOS and COX-2 and significantly inhibit NO secretion and other inflammatory markers including PGE-2, TNF-, IL-6, and IL-1 β in lipopolysaccharide cells in RAW264.7 cells (Vergara-Jimenez *et al.* 2017 and Kou *et al.*, 2018). Other studies supporting the mechanism *M. oleifera* have been shown an excellent ability to protect against oxidative damage due to its high polyphenols, flavonoids, and flavonols content are very good and non-toxic sources of antioxidants [24], [25], [26].

IL-6, which has been originally defined as a B-cell differentiation factor, is regarded as a multifunctional cytokine that regulates immune responses, hematopoiesis, acute phase response, and inflammation. In the present study, *M. oleifera* leaves extract exhibited significant inhibition of periodontitis-induced IL-6 [27]. These results recommended that *M. oleifera* leaves may possess anti-inflammatory characteristics and help to reduce some inflammatory associated disorders [28]. In this study, the results showed that *M. oleifera* leaf extract can reduce IL-6 levels caused by the activity of active anti-inflammatory compounds owned by this plant such as the main flavonoids in *Moringa* which include quercetin, kaempferol glucoside, and the flavonoid malonate [29], [30], [31].

Conclusions

The present study showed that *M. oleifera* leaf extract exhibited an anti-inflammatory effect

on the inflammation induced by the bacterium *P. gingivalis*. *M. oleifera* extract can reduce the production of the pro-inflammatory cytokine IL-6 induced by *P. gingivalis* bacteria in periodontitis. Further research is needed on the effectiveness of the inhibitory power and dose measurement of the anti-inflammatory activity of the bioactive compounds contained in *M. oleifera* leaf extract so that it can be promoted as an effective treatment for managing inflammatory disorders.

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