Anti-inflammatory gel insulin leaf extract (*Tithonia diversifolia)* against lymphocyte cell count of periodontitis wistar rats (*Rattus norvegicus*)

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Abstract

Background: Periodontitis is an inflammatory disease involving dental support tissue caused by pathogenic microorganisms. Periodontitis can result in damage to the periodontal ligaments and alveolar bones, and the formation of periodontal pockets. Periodontitis can cause inflammation; inflammation is one of the first responses of the body's immune system to infection. Inflammation is triggered by the release of chemical mediators from damaged tissues and cell migration. The inflammatory phase is characterized by chronic inflammatory cells namely macrophages, lymphocytes, and plasma cells. Insulin leaves contain flavonoids that can be used as an anti-inflammatory. This study looked at the anti-inflammatory effect of insulin leaf extract gel against a decrease in the number of lymphocyte cells in periodontitis conditions.

Purpose: To know the effective dose of anti-inflammatory gel insulin leaf extract.

Materials and Methods: Periodontitis Wistar rats were given treatment with insulin gel leaf extract (Tithonia diversifolia) with a concentration of 25%, 50%, and 75%, then the lymphocyte cells were counted using a light microscope.

Results: There were significant differences in lymphocyte cell counts in the control group and three treatment groups with concentrations of 25%, 50%, and 75%.

Conclusions: There were differences in the number of lymphocytes in Wistar rats with periodontitis after administration of insulin leaf extract gel with those that were not given insulin leaf extract gel and concentrations of 75% were the most effective dose to reduce the number of lymphocytes in periodontitis.

Keywords: inflammatory, insulin leaf, lymphocytes, periodontitis

Introduction

Periodontal disease is a public health challenge because it can significantly affect oral health, general health and quality of life. In 2010, periodontitis became the sixth most common disease globally with a prevalence of 10.8% and then increased to 25.6% in the period of 2006-2016. Periodontitis in Indonesia have the highest number of cases from the global average with a prevalence of 17% [1]. Periodontitis involves a complex interaction between specific bacterial pathogens, deleterious host immune responses, and environmental factors and is characterized by the progressive destruction of the supporting tissues of the teeth, including the periodontal ligament and alveolar bone. General features of periodontitis include gingival inflammation, clinical attachment loss, radiographic evidence of alveolar bone loss, deep probing, mobility, bleeding on probing, and pathological migration [2].

Periodontitis is caused due to the accumulation of plaque and calculus in the oral cavity. Several other factors, such as systemic and nutritional diseases are also the cause of periodontitis. Periodontitis is dominated by anaerobic Gram-negative bacteria, such as *A. actinomycetemcomitans, P. gingivalis*, *F. nucleatum*, and *P. intermedia*. The bacteria accumulate in dental plaque along with food residues, virulence factors, and metabolites. Dental plaque as the originator of periodontitis can be both supragingival and subgingival plaques [3].

Inflammation occurs due to the presence of pathogenic molecules and molecules associated to tissue damage that responded by innate immune responses and further extended by adaptive immune responses [4]. The mechanism of inflammation involves white blood cells that have change in the area of inflammation as well as inflammatory mediators and signaling molecules released by immune cells. The microvascular response appears right after microbial infection. The inflammation process occurs rapidly, causing vasodilation and increased permeability of blood vessel so that inflammatory mediators enter the inflammatory area and white blood cell infiltration [5]. The inflammatory process occurs with the migration of leukocytes and plasma proteins from blood vessels to inflamed tissue area and then accumulate and are activated, then causing immune responses. Lymph nodes around the inflammation area respond to inflammatory

conditions by increasing migration and activation of lymphocyte [4].

Lymphocytes are white blood cells involved as adaptive and innate immune cells in humans. Lymphocytes are divided into T lymphocyte, B lymphocyte, and natural killer (NK) cells. T lymphocyte cells and B lymphocyte cells are effectors of adaptive immunity, while NK cells are innate immune lymphocytes that do not have recombinant antigen receptors. Lymphocytes have an important role in the body's response and defense system against external antigens such as microbes, tumor cells, atopy, and systemic inflammatory response syndrome (SIRS) [6, 7]. Lymphocytes derived from hematopoietic progenitor cells. Lymphocytes are formed in bone marrow and liver in fetal phase [8].

Plants as herbal medicines are proven to have therapeutic benefits. The development of plant-based medicine has advantages such as natural activity, a high level of safety, and relatively low cost. Modern drugs such as antibiotics have some side effects, and continuous consumption will lead to resistance [9]. In periodontal therapy, antibiotics are the most commonly used drugs. With time, the consumption of antibiotics can cause resistance due to bacteria gradually evading the effects of antibiotics. In addition, antibiotics have some side effects and contraindications in some cases, such as kidney failure, pregnancy, and immunocompromised patients [10].

The bioactive components in fruits and vegetables, such as flavonoids, carotenoids, vitamins, minerals, and fiber, can be beneficial for health and nutritional value for humans [11]. Pharmacological studies of insulin leaves show this plant has functioned as anti-diabetic, anti-malarial, and anti-inflammatory. All parts of the plant, especially the leaves, are used to treat a wide spectrum of ailments ranging from use as a topical application to treat wounds, abscesses, and dermatological conditions and for oral administration for diabetes, malaria, fever, hepatitis, and infectious diseases [12].

Tithonia diversifolia contains several phenolic compounds, such as total phenols, tannins, and flavonoids [13]. The use of flavonoids in the field of medicine has been widely used for e treatment of diabetes mellitus, inflammatory diseases, and allergic diseases [12]. High concentrations of several flavonoid compounds can inhibit the release of arachidonic acid and the secretion of lysosomal enzymes from membranes by blocking the cyclooxygenase and lipoxygenase pathways [11]. This leaf has been studied in human cell lines, microorganisms, and animal models. *T. diversifolia* is a potential source of medicine for various diseases [14]. In this study, insulin leaf extract was used with a concentration of 25%, 50%, and 75%.

This study aims to prove the anti-inflammatory effect of insulin leaf extract on lymphocyte counts in Wistar rats with periodontitis.

Materials and Methods

Experimental animal preparation

In the study, experimental animals were used in the form of Wistar rats (*Rattus norvegicus*) which were divided into 3 treatment groups and 1 control group, with a total of 10 heads each per group. The number of samples is calculated by the Lemeshow formula. Wistar rats were obtained from the Biochemistry Laboratory of the Faculty of Medicine, Universitas Airlangga.

The criteria for Wistar rats used as experimental animals are: 1) Rats are in good health, 2) Rat weight is 150-200 grams, 3) Male Wistar rats, and 4) Wistar rats are 3-4 months old. Wistar rats are divided into 4 main groups. The control group was in the form of Wistar rats induced by *A. actinomycetemcomitans*. The first treatment group was in the form of Wistar rats induced by *A. actinomycetemcomitans* and given insulin leaf extract gel with a concentration of 25%. The second treatment group was in the form of Wistar rats induced by *A. actinomycetemcomitans* and given insulin leaf extract gel with a concentration of 50%. The third treatment group was in the form of Wistar rats induced by *A. actinomycetemcomitans* and given insulin leaf extract gel with a concentration of 75%. Wistar rats were conditioned in cages for 7 days, with adequate nutrition, with the placement of 10 Wistar rats each cage.

Extraction of insulin leaf

Insulin leaf extract is made at UPT Materia Medica, Malang. Making insulin leaf extract using insulin leaf powder as much as 1 kilogram and dissolved in 95% ethanol as much as 2 liters. Insulin leaf powder is then placed into a jar and soaked with 95% ethanol for 3 liters, and incubated for 24 hours. After 24 hours, the jar is shaken with a digital shaker at a speed of 50 rpm. The powder that has been moistened with ethanol is filtered with a cloth filter and accommodated the extract in Erlenmeyer. The rest of the pulp or powder is put back in the jar and a solvent is added until submerged (at least a solvent 5 cm above the surface), in this case 5 liters of solvent is used, then allowed to stand in a shaker for 24 hours. The liquid extract is then evaporated with a rotary evaporator for 13 hours, then re-evaporated with a waterbath for 2 hours.

Production of insulin leaf extract gel preparations

First, 0.2% weighing of CMC- Na is carried out in 0.2 g in 10 ml of warm aquades. After weighing, insulin leaf extract with a concentration of 25% was weighed as much as 2.5 grams and then mixed with a solution of a mixture of aquades and CMC-Na 2%. Insulin leaf extract with a concentration of 50% was weighed as much as 5 grams and then mixed with a solution of a mixture of aquades and CMC-Na 2%. Insulin leaf extract with a concentration of 75% was weighed as much as 7.5 g and then mixed with a solution of a mixture of aquades and CMC-Na 2%. The three solutions of various concentrations are stirred until homogeneous.

Treatment of experimental animals

Wistar rats were adapted for 7 days, the mice were grouped into 4 groups, then induced with *A. actinomycetemcomitans* with a volume of 0.2 ml and a concentration of 10-9 CFU / ml using a micro syringe. In the control group mice and group mice were treated with the administration of insulin leaf extract 25%, 50%, 75% every three times a week for 17 days on the molar teeth of the upper jaw. The control group was given only drinking and standard feed. The treatment group was given a standard feed and insulin leaf extract gel. After being induced with bacteria, the treatment group was given insulin leaf extract gel using micro brush every 3 times a day for 3 days after being induced by *A. actinomycetemcomitans***.** All experimental animals were grouped on the 3rd day of insulin leaf extract, then rat gingival tissue was taken in the upper jaw molar tooth region. Wistar rats were sacrificed by euthanasia or injection of

ketamine 180mg/kg.

Manufacture of paraffin preparations

Before fixation, the gingival sample is pre-washed with physiological saline solution. After tissue fixation for 6-9 hours, the sample is rinsed under running water. Washing is carried out in order to remove the fixation solution from the tissues after the fixation process is completed. Then dehydration of the sample is carried out to draw water in the tissue so that the entire room between cells in the tissue can be filled with paraffin molecules. After dehydration, the first infiltration process of tissues with a duration of no more than 5 minutes is carried out.

Then after the first infiltration, the tissue is dipped in xylene for 2 to 3 minutes, put in 95 % alcohol for 30 minutes, and washed under running water for 1-2 hours. Then the embedding of tissues into paraffin blocks is carried out, by means of which liquid paraffin is poured into a mold formed of 2 metals, which are arranged to form a box on which sheet metal is based. After the liquid paraffin is poured into the mold, the cut of tissue is inserted using tweezers with the direction of the surface of the tissue to be cut facing the base, the top is labeled with a mark. After the paraffin hardens, the metal mold can be removed. The last stage is cutting the paraffin block using a rotary microtome serially with a thickness of 5 µm. During the cutting time, the block temperature is cultivated in a temperature of about 5-10°C.

Manufacture of histological preparations

The slide is dipped in xylol solution for 2 hours 2 times, absolute ethanol for an hour by 2 times, ethanol 95% for 1 hour by 2 times, ethanol 80% for 1 hour. The slide is then washed with water for 10-15 minutes and put in a solution of Mayer's hematoxylin for 15 minutes to color the cell nucleus, after which it is washed under running water to remove excess paint for 20 minutes. Painting is done with lithium carbonate and the slide is inserted into lithium carbonate for 1-2 minutes to obtain a blue core, then washed in running water for 5-10 minutes. Painting with Eosin is done by inserting a slide into the eosin solution for 15 seconds-2 minutes to color the cytoplasm. The next process is dehydration, which is by inserting a slide into 95% ethanol for 2 minutes 2 times and absolute ethanol for 2 minutes 3 times**.** The clearing process is then carried out to remove the remnants of alcohol, namely by inserting it into the xylol solution for 2 minutes for 3 times. Finally, mounting is carried out, namely the preparation is dripped by entellan and covered with a glass cover. The preparation is then carried out examination under a light microscope.

Lymphocyte count readings

All examinations were carried out using a Nikon H600L light microscope equipped with a 300-megapixel DS Fi2 digital camera and Nikon Imaging System image processing software.

Data analysis

The data obtained were then carried out statistical analysis with the Kolmogorov-Smirnov test and homogeneity test. For data analysis, a one-way ANOVA test is used.

Results

Based on the results of the test to calculate the number of lymphocytes in the control group and the treatment group, it was found that the control group had the highest number of lymphocytes compared to the treatment group's concentration of 25%, 50%, and 75% (Table 1). Based on Table 1, it can be concluded that there were significant differences in the number of lymphocyte cells in the control group and three treatment groups with concentrations of 25%, 50%, 75%.

The histological picture of each group also showed that the highest number of lymphocytes was found in the control group, followed by treatment groups with concentrations of 25%, 50%, and 75% (Figures 1, 2, 3, and 4).

Table 1: Mean and standard deviation of lymphocyte counts in the control and treatment groups after administration of insulin leaf extract

Control	$\bar{X}$ ± SD
0%	25,511 ± 3.5413
25%	19,525 ± 2.9989
50%	17,520 ± 3.6273
75%	14,620 ± 3.2034

After obtaining the mean and standard deviation of the control and treatment groups, it continued with the One-Sample Kolmogorov-Smirnov Test. The value Sig (2-tailed) is 0.944, and the value of 0.944 is greater than 0.05 ($p>0.05$), which means that this data is normally distributed.

The result of the Levene statistic test, the Sig value was 0.788, and the value was greater than 0.05 ($p>0.05$), which means that the data is homogeneous. After processing the normality and homogeneity data as a condition for the Oneway ANOVA test, the Oneway ANOVA test obtained Sig 0.00, which means that this data is significant because the Sig value obtained is less than ($p<0.05$).

Based on the results of the Tukey HSD test (Table 2), it can be concluded that there are significant differences in the number of lymphocyte cells in the control group and the three treatment groups with concentrations of 25%, 50%, and 75%.

In the 25% treatment group, there was an insignificant difference from the 50% treatment group. The 50% treatment group had an insignificant difference from the 75% treatment group.

Table 2: Tukey HSD test

Control	Group	Sig.
0%	25%	.005
	50%	.000
	75%	.000
25%	Control	.005
	50%	.597
	75%	.021
50%	Control	.000
	25%	.597
	75%	.237
75%	Control	.000
	25%	.021
	75%	.237

Based on the results above, it can be concluded that there are significant differences in the number of lymphocyte cells in the control group and the three treatment groups with concentrations of 25%, 50%, and 70%.

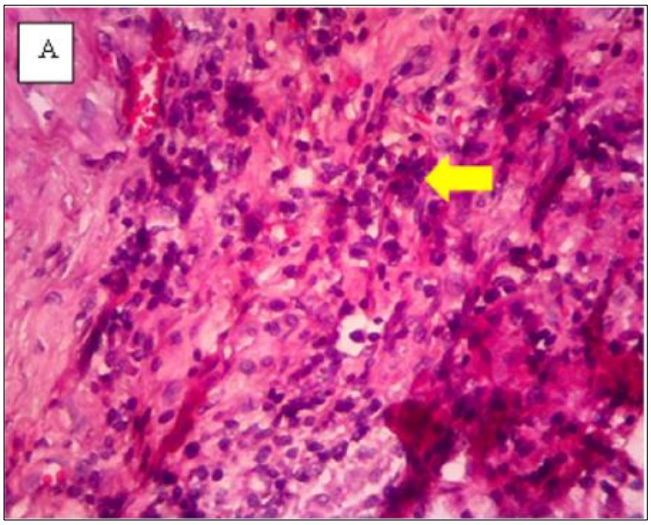

Fig 1: Histological overview of lymphocyte cells (yellow arrow mark) in: A) Control group.

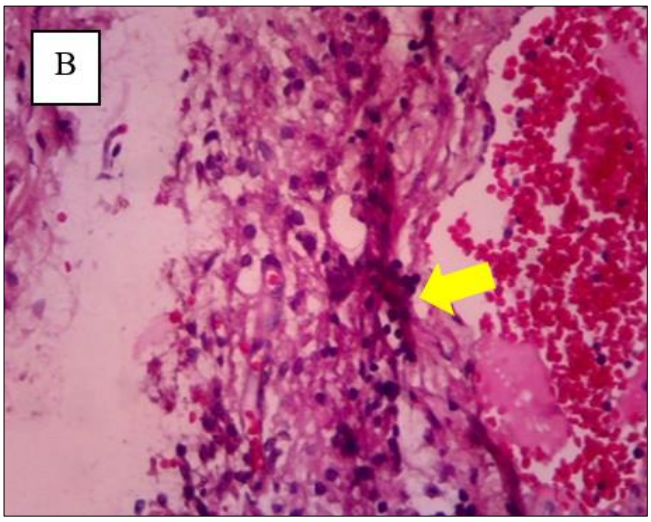

Fig 2: Histological overview of lymphocyte cells (yellow arrow mark) in: B) Extract concentration treatment group 25%

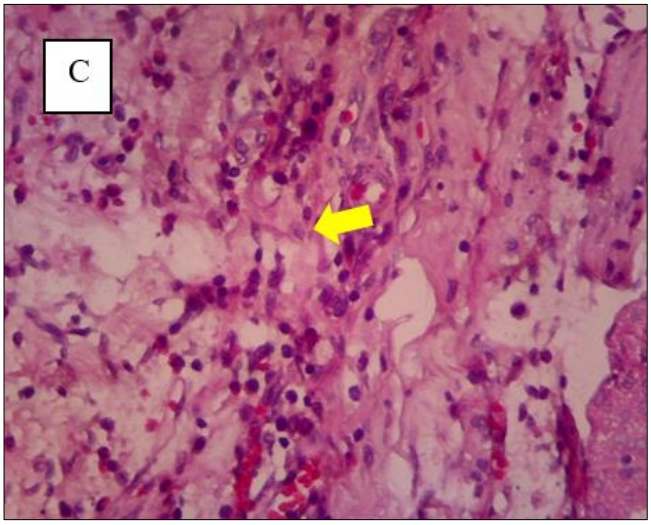

Fig 3: Histological overview of lymphocyte cells (yellow arrow mark) in: C) Extract concentration treatment group 50%

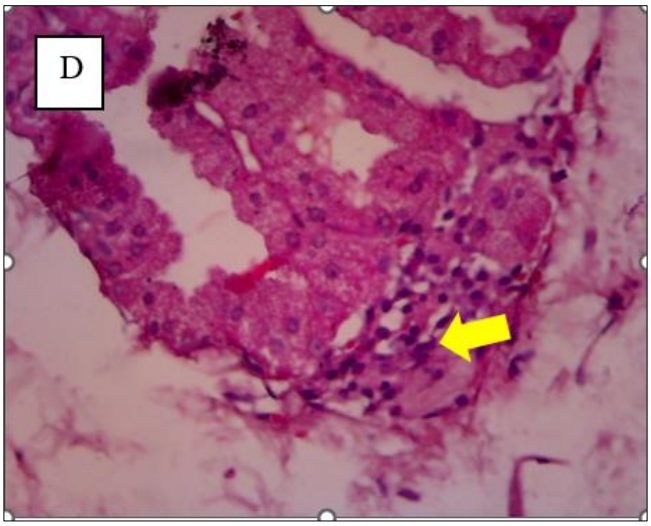

Fig 4: Histological overview of lymphocyte cells (yellow arrow mark) in: D) extract concentration treatment group 75%.

From the figure, the results were obtained that the highest number of lymphocytes was found in the control group, followed by extract groups with concentrations of 25%, 50%, and 75%.

Discussion

The results showed an effect of insulin leaf extract on the number of gingival lymphocyte cells of male Wistar rats that had been induced *A. actinomycetemcomitans*. In the control group, the highest number of lymphocyte cells was obtained because in this group the experimental animals were not given treatment and only as a comparison of the other four treatment groups. Meanwhile, in the 25% treatment group, the highest number of lymphocyte cells was obtained when compared to the other three treatment groups, namely the 50% treatment group, the 75% treatment group.

This is due to the administration of insulin leaf extract gel with a high concentration. This high concentration of insulin leaf extract gel can result in inhibition of cyclooxygenase and lipoxygenase pathways, so that the greater the concentration of insulin leaf extract gel, the higher the content of the active substance and is able to work more effectively. If the COX and LOX pathway are inhibited, the production prostaglandins and leukotrienes will also inhibited [15]. Prostaglandins have an important role in the inflammatory response. In non-inflamed tissues, prostaglandin production is generally very low and in inflammatory tissues the production of prostaglandins increases before the leukocyte migration and immune cell infiltration into the inflamed tissue. Prostaglandins will increase blood flow through vasodilation and increase vascular permeability to inflamed tissues [16, 17]. Thus, decreased levels of prostaglandins will suppress leukocyte migration and immune cell infiltration in the inflammatory areas then the number of lymphocytes will be decreased. The decrease in the number of lymphocytes showed an increase in the immune system in Wistar mice.

In the treatment group, 75% had the least number of lymphocytes when compared to other treatment groups (control group, 25%, and 50%), this is in line with the theory that the higher the concentration of insulin leaf extract, the more active substances contained in the extract and the ability to reduce the number of lymphocyte cells is also higher. Insulin

leaves have a flavonoid content [18]. The mechanism of flavonoids in inhibiting the growth of bacteria, flavonoids can inhibit the attachment and formation of biofilms, porins in cell membranes, membrane permeability, and bacterial pathogenicity. Other studies state that flavonoids can inhibit bacterial growth by releasing transduction energy to the cytoplasmic membrane and inhibiting bacterial motility [19, 20].

Conclusion

Insulin leaf extract gel can lower lymphocyte cell count by acting as an anti-inflammatory. Insulin leaf extract gel can decrease or damage the activity of bacteria, so inflammatory mediators of lymphocyte cells that are damaging to periodontal tissue and caused by the presence of *A. actinomycetemcomitans* can be minimized. From research, it was found that the gel concentration of insulin leaf extract of 75% is the most effective concentration for reducing the number of lymphocyte cells in periodontitis.

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