Effect of Cassave leaf flavonoid extract on TNF-α expressions in rat models suffering from periodontitis

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ABSTRACT

Background: Bacteria playing a role in periodontitis are Gram-negative anaerobic bacteria that can release endotoxin or lipopolysaccharide (LPS). LPS acts as a stimulus to a variety of host cells that can stimulate expression of pro-inflammatory cytokines in periodontal disease, such as IL-1α, IL-1β, and TNF-α. Increased TNF-α then can lead to periodontal tissue destruction. Furthermore, cassava leaves have many health benefits due to flavonoid organic compound contained, known to possess anti-inflammatory activity are used as a medicine. Purpose: This study aimed to determine the effect of cassava leaf flavonoid extract as a basic ingredient of anti-inflammatory gel on TNF-α expression in Wistar rats suffering from periodontitis induced with Escherichia coli (E. coli) LPS.

Method: This study used 24 male Wistar rats. Those rats were divided into six groups. Group 1 consisted of four rats induced with E. coli LPS for 2 weeks, and then decapitation was performed on day 3. Group 2 consisted of four rats induced with E. coli LPS for 2 weeks, and then decapitation was conducted on day 7. Group 3 consisted of four rats induced with E. coli LPS for 2 weeks, treated with the topical cassava leaf flavonoid extract gel (Manihot esculenta) at a concentration of 25%, and then decapitation was performed on day 3. Group 4 consisted of four rats induced with E. coli LPS for 2 weeks, treated with the topical cassava leaf flavonoid extract gel (Manihot esculenta) at a concentration of 25%, and then decapitation was conducted on day 7. Group 5 consisted of four rats induced with E. coli LPS for 2 weeks, treated with the topical cassava leaf flavonoid extract gel (Manihot esculenta) at a concentration of 50%, and then decapitation was performed on day 3. And, group 6 consisted of four rats induced with E. coli LPS for 2 weeks, treated with the topical Cassava leaf flavonoid extract gel (Manihot esculenta) at a concentration of 50%, and then decapitation was performed on day 7. Result: The expression of TNF-α in the control group was more than that in the treatment groups given the Cassava leaf flavonoid extract gel at the concentrations of 25% and 50%. The expression of TNF-α in the treatment groups given the cassava leaf flavonoid extract gel at the concentration of 50% was lower than that in the treatment groups given the cassava leaf flavonoid extract gel at the concentration of 25%. Conclusion: Cassava leaf flavonoid extract gel could be used as an anti-inflammatory gel characterized by a decrease in TNF-α expression in rat models suffering from periodontitis.

Keywords: periodontitis; flavonoids of cassava leaves; TNF-α.

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INTRODUCTION

Periodontal disease is an oral health problem with a fairly high prevalence in all age groups in Indonesia, approximately about 96.58%.

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Bacteria playing a role in periodontitis are Gram-negative anaerobic bacteria, secreting a variety of products including biologically active endotoxin or lipopolysaccharide (LPS).\(^3\) LPS is one of factors triggering periodontal disorders. LPS can stimulate biological activity causing inflammation. The inflammatory response caused by LPS is the first part of the immune system against pathogens. LPS acts as a stimulus to a variety of host cells which will ultimately result in expression of pro-inflammatory cytokines in periodontal disease, such as interleukin (IL)-1\(\alpha\), IL-1\(\beta\), and tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)).\(^5,6\)

In the last few years, many researchers in Indonesia have developed many studies on medicinal plants that are useful as alternative drugs substituting to chemical drugs on markets, including cassava leaves (Manihot esculata). 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 known to have anti-inflammatory activity. Flavonoids are polyphenolic compounds that occur ubiquitously in plants having a variety of biological effects both in vitro and in vivo. They have been found to have antimicrobial, antiviral, anti-ulerogenic, cytotoxic, antineoplastic, mutagenic, antioxidant, antihypertensive, hypolipidemic, antiplatelet anti-inflammatory activities. Flavonoids also have biochemical effects, which inhibit a number of enzymes such as aldose reductase, xanthine oxidase, phosphodiesterase, Cat-2-ATPase, lypoxygenase, cyclooxygenase.\(^7\)

Flavonoids are assumed to suppress TNF-\(\alpha\) expression released during inflammation. Similarly, a research conducted by Peluso shows that flavonoids can reduce TNF-\(\alpha\).\(^8\) Some previous in vitro studies, furthermore, have shown that cassava leaf extract at concentrations of 12.5% and 25% can reduce COX-2 expression, an enzyme that plays a role during inflammation, and also can improve the viability of monocytes exposed by Escherichia coli (E. coli) LPS.\(^9\) For those reasons, this research aimed to determine the effectiveness of the cassava leaf flavonoid extract as a basic ingredient of anti-inflammatory gel on TNF-\(\alpha\) expression in wistar rats suffering from periodontitis induced with E. coli LPS.

### MATERIALS AND METHOD

All procedures in this research were approved by the Ethics and Advocacy Commitee of Faculty of Dentistry, University of Gajah Mada (No. 00 366/ KKEP/ FKG UGM/ EC/ 2015). Cassava leaves were identified at the Indonesian Institute of Sciences in Plant Conservation Center in Purwodadi. Cassava leaves as much as 450 grams was added with 100 ml of absolute ethanol, and then was processed ultrasonically for 10 minutes. It was then added with 10 ml of 5% \(\text{H}_3\text{PO}_4\), heated at a temperature of \(80\degree\) C for 30 minutes, and then settled for 8 hours. The top layer formed was taken, and then vaccum filtration was conducted. The filtrate was extracted with 10 ml of petroleum ether (repeated 3 times). The extract result was roasted at a temperature of \(60\degree\) C. To reduce the amount of ethanol, it was added with water to a volume of 5 ml. 20 ml of acetonitrile was added, and sonication process was conducted for 5 minutes. It was centrifuged at 4000 rpm for 5 minutes. The upper layer formed was taken and dried in order to obtain cassava leaf flavonoid extract. The cassava leaf flavonoid extract then was tested using liquid chromatography-tandem mass spectrometry (LC-MS/MS) to determine the level of flavonoid. The procedure was based on a modification of two different protocols proposed by Docheva et al. and Muhammad et al.\(^10,11\)

The extract was altered into gel at the Laboratory of Pharmaceutical Faculty of Pharmacy, Universitas Jember. The manufacturing process of gel base was started with carbopol developed in hot water in a mortar, and then stirred until homogenous before added with triethanolamine (TEA) in small increments until gel mass was formed. The cassava leaf flavonoid extract was mixed with propylene glycol until homogenous. The mixtures of the extract and propylene glycol were then mixed into the gel base, and stirred until homogenous. The distilled water remained was added to the gel in small increments until homogenous. The procedure of making the gel was based on a modification of protocol proposed by Ahmed et al.\(^12\) Propylene glycol is a solvent that can dissolve a variety of materials, such as corticosteroids, phenol, sulfa drugs, barbiturates, vitamins A and D, alkaloids, and many local anesthetia.\(^13\)

This study used 24 male Wistar rats divided into 6 groups. Group 1 consisted of four rats induced with E. coli LPS for 2 weeks, and then decapitation was performed on day 3. Group 2 consisted of four rats induced with E. coli LPS for 2 weeks, and then decapitation was conducted on day 7. Group 3 consisted of four rats induced with E. coli LPS for 2 weeks, treated with the topical cassava leaf flavonoid extract gel at a concentration of 25%, and then decapitation was performed on day 3. Group 4 consisted of four rats induced with E. coli LPS for 2 weeks, treated with the topical cassava leaf flavonoid extract gel at a concentration of 25%, and then decapitation was conducted on day 7. Group 5 consisted of four rats induced with E. coli LPS for 2 weeks, and then decapitation was performed on day 7. Group 6 consisted of four rats induced with E. coli LPS for 2 weeks, treated with the topical cassava leaf flavonoid extract gel at a concentration of 25%, and then decapitation was conducted on day 7. Group 7 consisted of four rats induced with E. coli LPS for 2 weeks, and then decapitation was performed on day 7.
coli LPS for 2 weeks, treated with the topical cassava leaf flavonoid extract gel at a concentration of 50%, and then decapitation was performed on day 3. Group 6 consisted of four rats induced with E. coli LPS for 2 weeks, treated with the topical cassava leaf flavonoid extract gel at a concentration of 50%, and then decapitation was conducted on day 7.

Afterwards, in the early stage, those Wistar rats were anaesthetized using ketamine (KTM 100) at a dose of 0.5 ml/kg BM, injected into their quadriceps muscle/triceps muscle of their right rear-foot. E. coli LPS (Sigma) at a concentration of 1 mg/ml in PBS was injected at the gingival sulcus of their first right mandibular molar, as much as 5 mL into lingual part and 5 mL into buccal part. It was injected every three days for two weeks using a tuberculin syringe with a 30 gauge syringe to trigger periodontitis. This method was based on the modification of a method proposed by Buduneli et al.15

After the rats suffering from periodontitis, their gingiva enlarged and became reddish. There were also pockets. Radiographically, their alveolar bone declined (Figure 1 and 2). The provision of the cassava leaf flavonoid extract gel with the concentrations of 25% and 50%9 was applied to the gingival sulcus of their first right mandibular first molar using the blunted syringe needle. The gel was applied twice a day for 7 days. This procedure was based on a procedure proposed by Sato et al.16 The excess gel on the gingival sulcus then was cleaned with a cotton pellet.

Decapitation was performed on those rats on days 3 and 7 after the administration of Cassava leaf flavonoid extract gel. Decapitation on day 3 was considered as inflammatory phase. It was then followed with proliferative phase considered as wound healing process on day 7.17 This treatment was performed in Biomedical Laboratory of Faculty of Dentistry, Universitas Jember.

After the decapitation, their lower jaw was taken, and then fixed in a buffered formalin solution. The process of decalcification was conducted using EDTA for ±4 weeks. After their tissues softened, the tissues were excised. The tissues were then cleaned and washed with running water for 60 minutes. The softened and cleaned tissues were soaked in alcohol at concentrations of 70%, 80%, and 90%, as well as absolute alcohol (100%) I, absolute alcohol (100%) II, and absolute alcohol (100%) III to remove the water in the tissues. The tissues were successively soaked at each concentration for 60 minutes. The tissues then were soaked in solutions of xylool I for 2 minutes, xylool II for 2 minutes, and xylool III respectively for 60 minutes. Paraffin infiltration process was gradually carried out in an oven at a temperature of 600°C. The preparations then were put into pure paraffin I, pure paraffin II, and pure paraffin III respectively for 60 minutes. After that, embedding and labeling processes were performed. Cutting then was conducted using a microtome.

Immunohistochemical staining was performed in accordance with a staining procedure was a modification by Schiessl20 and Olsen.21 Deparaffinization of the tissues was carried out using xylol III for 2 minutes, xylol II for 2 minutes, and xylol I for 2 minutes, absolute alcohol III for 2 minutes, absolute alcohol II for 2 minutes, absolute alcohol I for 2 minutes, 90% alcohol for 2 minutes, 80% alcohol for 2 minutes, and 70% alcohol for 2 minutes. Those tissues then were washed three times with PBS each for 5 minutes. The tissues were put in a solution of 0.3% H2O2 in methanol for 20 minutes. Those tissues were washed again with running water for 10 minutes, washed with distilled water for 3-5 minutes, and then washed three times with PBS, each for 5 minutes. They were incubated in antigen retrieval (citrate buffer) using a microwave for 10 minutes, and then cooled at a room temperature for 30 minutes. They were washed three times with PBS, each for 5 minutes.

Ultra V block was applied, and then incubation was conducted for 5 minutes at a room temperature. The tissues were washed three times with PBS, each for 5 minutes. Primary antibody (polyclonal antibody TNF-α) in a ratio 1:100 (10μg antibody : 1cc PBSA) was given, and then

Figure 1. A swollen and reddish rat gingiva.

Figure 2. A rat dental radiography picture (arrows indicating radiolucent area where alveolar bone decreased).
settled for 24 hours at 4°C. They were washed again three times with PBS, each for 5 minutes. Biotinylated goat anti-polyvalent (secondary antibody) was applied, and then incubation was conducted for 5 minutes at a room temperature. They were washed three times with PBS, each for 5 minutes. Streptavidin peroxidase was applied, and then incubation was performed for 5 minutes at a room temperature. They were washed three times with PBS, each for 5 minutes. Incubation then was carried out in DAB chromogen dye (1,3-diamino benzidine), and settled for 10-20 minutes. They were washed again three times with PBS, each for 5 minutes. They then were washed with running water for 10-15 minutes. Counterstain with Mayer’s hematoxylin was performed for 1-5 seconds. Dehydration (as opposed to deparaffinization) was carried out. Mounting then was performed using Canada balsam and then covered with a coverslip.

TNF-α expressions on mesial gingival fibroblasts which cell cytoplasm was brown were observed. Observation was performed under a microscope with a magnification of 400 times. The research data obtained were the mean number of TNF-α expressions calculated per three visual fields.

The data then were tested using a normality test, Shapiro-Wilk test. The results of the Shapiro-Wilk test showed that the data had normal distribution. As a result, a parametric statistical test was performed using one way Anova test to determine differences in TNF-α expression in all groups. LSD test was carried out to compare TNF-α expressions in between the treatment groups.

RESULTS

Microscopically, the color of cells expressing TNF-α was brownish, whereas the color of cells not expressing TNF-α was purplish blue. More details can be seen in Figure 3. The mean and standard deviation of TNF-α expressions in each treatment group can be seen in the following Table 1.

In the control group, the highest mean number of TNF-α expressions on the 7th day was 13.83, whereas in the treatment groups given the Cassava leaf flavonoid extract gel at the concentration of 50% on the 7th day, the lowest mean number was 8.22. Results of the one way Anova test showed that there were significant differences in TNF-α expressions in between the treatment groups.

DISCUSSION

The results of this research showed that the mean number of TNF-α expressions in the control group was higher than that in the groups given the Cassava leaf flavonoid extract gel at the concentrations of 25% and 50% as anti-inflammatory. TNF-α expressions in the control group increased on the 7th day, while TNF-α expressions in the groups given the Cassava leaf flavonoid extract gel at the concentrations of 25% and 50% decreased on the 7th day compared to on the 3rd day. Thus, it can be said that the provision of the Cassava leaf flavonoid extract gel at the concentrations of 25% and 50% could decrease TNF-α expressions.

TNF-α is produced primarily by activated monocytes and macrophages. However, it can also be produced by B cells, T cells, and fibroblasts, playing a role in inflammation.21 TNF-α is also considered as a powerful immune response modulator mediating induction of adhesion molecules and other cytokines, as well as activation of neutrophils. Nevertheless, excessive TNF-α can damage endothelial cells, causing vascular occlusion and improving endothelium permeability.22

Table 1. Mean, standard deviation, and one way Anova test results of TNF-α expressions in the treatment groups based on decapitation time

<table>
<thead>
<tr>
<th>Group</th>
<th>TNF-α expressions</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>( \mu ) ± SD</td>
</tr>
<tr>
<td>On day -3</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>11.33 ± 0.44</td>
</tr>
<tr>
<td>25% GEFDS</td>
<td>9.67 ± 0.28</td>
</tr>
<tr>
<td>50% GEFDS</td>
<td>8.56 ± 0.68</td>
</tr>
<tr>
<td>On day -7</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>12.23 ± 1.05</td>
</tr>
<tr>
<td>25% GEFDS</td>
<td>8.56 ± 0.41</td>
</tr>
<tr>
<td>50% GEFDS</td>
<td>8.17 ± 0.31</td>
</tr>
</tbody>
</table>

Note: 25% GEFDS: Cassava leaf flavonoid extract gel at a concentration of 25%; 50% GEFDS: Cassava leaf flavonoid extract gel at a concentration of 50%; \( \mu \): Mean; SD: Standard Deviation; Sig: Significance of one way Anova test results
Cassava leaves, known as a medicinal plant for mild diseases, can be used as analgesics and anti-inflammatory.

23, 24 Cassava leaves contain some organic compounds, such as flavonoids, saponins, tannins, and triterpenoids. Cassava leaves play a role in suppressing inflammatory process. Flavonoids are potential to suppress the inflammation by blocking the cycle path of cyclooxygenase (COX) and lipoxygenase. As a result, inflammatory cells that migrate are limited, and clinical signs of inflammation are reduced. Inhibition of COX and lipoxygenase pathways also directly causes inhibition of the biosynthesis of eicosanoid. 25

The results of this research, moreover, also showed that the highest number of TNF-α expressions was found in the control group (LPS). LPS is a potent stimulus to secrete TNF since LPS can induce inflammation. Lipopolysaccharide, a product of microorganisms, can involve TLR4 system that can activate NF-κB and generate proteins encoding the protein components of the nonspecific immune responses, including inflammatory cytokines (TNFα, IL-1 and IL-12). 26 In other words, the inflammatory response is triggered by an immune reaction in the cellular level, so other pro-inflammatory cytokines, such as TNF-α, can be improved. 27

Furthermore, the number of TNF-α expressions in the groups given the Cassava leaf flavonoid extract gel at the concentrations of 25% and 50% as anti-inflammatory was less than that in the control group. This is presumably due to anti-inflammatory effects of flavonoids. The anti-inflammatory effects of flavonoids may be due to their action in inhibiting the accumulation of leukocytes at inflammation sites. During inflammation, many endothelial derived mediators and complement factors may lead to adhesion of leukocytes to endothelial wall, as a result, the leukocytes become immobilized and stimulate neutrophil degranulation. 28 Therefore, it can be said that the provision of flavonoids can reduce both the number of immobilized leukocytes and the activation of complements resulting in lower adhesion of leukocytes to the endothelium and can decrease body inflammatory response. 29

In addition, flavonoids play a role as an anti-inflammatory by blocking IkB kinase. Consequently, degradation of IkB preventing activation of NF-κB will not occur. Thus, TNF-α levels will not increase. This condition may be due to quercetin contained. Quercetin flavonoids then can trigger a decrease in TNF-α levels through inhibition of Nuclear Factor Kappa B (NF-κB). NF-κB plays a role in controlling expressions of genes encoding proinflammatory cytokines and chemokines, such as TNF-α, IL-1β, IL-6, and other proteins. 25 It can be concluded that the administration of Cassava leaf flavonoid extract gel as anti-inflammatory can reduce TNF-α expressions in rat models suffering periodontitis.

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REFERENCES


