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PROTECTIVE EFFECT OF GUAVA SUPPLEMENTATION IN PERIODONTITIS RATS MODEL

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Abstract

This study aimed to investigate the effect of guava supplementation on acute phase periodontitis rats model. Periodontitis was induced by bacterial-adhered ligature placement around the mandibular incisor tooth. Twenty-four male adult of Sprague Dawley rats were divided into four groups (n=6/group). Sham-operated (C) and positive controls (M=metronidazole 45mg/kg, p.o) were included. The rats were treated with ethanolic guava fruits extract (Gp=Gc=135mg/kg, p.o), 7-days before (Gp) and after induction (Gc) of periodontal inflammation. After 14 days of post-induction treatment, the animals were sacrificed. The clinical parameter was gained through measuring the periodontal pocket depth (PPD) using the Williams probe at mesial and lingual sites of rat's mandibular incisors teeth. Samples representing the periodontal tissue were stored in formalin and prepared for histological processing. Induction by ligature placement caused a significant inflammation on periodontal tissue. Tissue repairment was seen clinically in the M and Gp groups with PPD score of lingual, p=0.012 and 0.046, respectively. Histologically, fibrocollagen regeneration was shown to be significantly different only by M group among the other groups. The result show that pre-treatment with guava extract on Gp group have a positive impact in detaining clinical attachment-loss, better than the post-induction-treatment group at the equal dose ($p<0,05$).

Keywords: Animal model, Guava supplementation, Periodontitis in rats, Protective effect.

1. Introduction

Periodontitis is characterized by inflammation leading to the destruction of the periodontium, triggered by an imbalance between the bacteria infection and host defence. The host defence mechanism is usually influenced by genetic, hormonal, and nutritional factors. Therapy with drugs commonly has a risk of side effects, which possibly cause other diseases such as kidney failure and indigestion. In periodontitis case, it may take a long period and high costs for the treatments. The condition is irreversible so that the preventive approach would be preferable than a curative measure. Tooth loss phenomenon is likely to occur when preventive and conservative treatments in reducing pain are not available [1]. Technological advancement in the medical field does affect the cost of a particular conventional therapy. Successful modern treatment is also usually followed by side effects. Most of the treatments are aimed to reduce the symptoms but do not eliminate the aetiology of the diseases. Plants' active compounds with multi-effects have been promisingly bringing a holistic recovery, particularly in chronic disease cases.

Guava (*Psidium guajava Linn.*), which belongs to the Myrtaceae family, has been traditionally used as herbal medicine for systemic condition and can improve oral hygiene due to its bioactive substances [2]. Guava contains a lot of polyphenol compounds and vitamin C. Almost all parts of the guava have pharmacological activities; however, its leaves are the most frequently used. Its flesh (mesocarp), in the meantime, is more commonly used as supplementary diet food. The extract of guava leaves has been reported to be able to overcome various gastrointestinal disorders such as vomit, diarrhea, inhibition of peristalsis reflex, gastroenteritis, spasmolytic activities, antimicrobial activities, antioxidant, dysentery, flatulence, and other stomach problems. Some of the important active constituents are essential oil, flavonoid, carotenoid, polyphenol compounds, pentacyclic triterpenoid, ester, and aldehydes. The increasing number of *Porphyromonas gingivalis* bacteria indicates a progressive periodontitis status. Consuming guava plays a protective role in periodontitis [3]. In another study, the treatment group consuming 200 g of guava a day, which is equal to having 200 mg of synthetic vitamin C a day, before and during oral hygiene abstention has protective role towards the development of experimental *gingivitis* in comparison with the control group [4].

The abundance of guava leads to its availability and ease of access by the people. Guava is one of natural sources that contains high vitamin C and is easily obtained [5]. However, the use of guava as an alternative of therapy for periodontitis has not been widely studied. Studies evaluating the role of nutrition in periodontitis healing, such guava supplementation was needed, especially related to vitamin C, are still scarce. Investigation of guava effect in this case, cannot be done in humans because of unethical aspects, so a periodontitis model in animals was made. Previous periodontitis model on rats, the induction was performed by injecting bacteria or its parts [6], and some were using ligature only [7-10] for making inflammation to occur. However, in this study, we combined both methods by adding ligature immersed in *P. Gingivalis*. As reported before, periodontal disease induction by ligature placement can cause a significant inflammation of periodontal tissue and alveolar bone loss on rats, which can be observed in 14 days. This study aimed to investigate the effect of guava fruit extract and to characterize the inflammation scale towards periodontitis conditions in rats.

2. Experimental Procedure

The experimental design and protocols were reviewed and approved by the ethics committee in Bandung Institute of Technology, Bandung, West Java-Indonesia (no. 02/KEPHP-ITB/12-2017).

2.1. Plant material and extract preparation.

The medium-ripe red guava (Psidium guajava Linn.) fruits were collected in May 2017 from Cimaung village-Pangalengan, West Java at 7°4′48.6444S 107°33′41.8068″E and 769 m above sea level. The plant was authenticated at the Herbarium Bandungense (No. 4513/I1.CO2.2/PL/2016), The School of Life Science and Technology, Bandung Institute of Technology, West Java, Indonesia.

2.2. Preparation of ethanolic extracts of p. guajava fruits.

The medium-ripe fresh fruits of guava were collected, surface sterilized, chopped, and then macerated in ethanol 96% with ratio 1:10 up to 24 h. The filtrate was evaporated using rotavapor, and the concentrated extract was then stored in air-tight glass bottles at 4°C and later re-dissolved in their respective solvents to the desired concentrations for the various experiments.

2.3. Animals.

The Sprague Dawley rats (170–180 g) were purchased from UPHL IPB, a laboratory animal management unit at Bogor Agricultural University, Indonesia. Two weeks before the experimental period, the animals were housed and adapted at an air-conditioned animal laboratory room (22 ± 3°C) with 12-hour light and dark cycle. The rats were fed with commercial standard rodent pellets and filtered water ad libitum. All of the animals received humane care according to the criteria outlined in the Guide for the Care and the Use of Laboratory Animals 8th edition (2011) prepared by the National Academy of Science and published by the National Institute of Health. The ethics regulations followed national and institutional guidelines for the protection of the animals' welfare during experiments. All of the experimental procedures were performed in the Laboratory of Experimental Animal, School of Pharmacy, Bandung Institute of Technology, Bandung, West Java, Indonesia. Samples' flow diagram is illustrated in Fig. 1. It explains that four animal were excluded in the initial selection because they did not meet the weight subject criteria. In the middle of the study, two animals died. However, this case can be anticipated because the number of animals in the control group has been overstated at the beginning.

2.4. Periodontitis induction.

The *P. Gingivalis*-adhered ligatures were made by immersing the 3/0 non-resorbable sterile silk thread in *P. Gingivalis* (ATCC 33277) suspension. The gingival tissue was separated from the tooth surface to enable inserting ligature into the incisive mandibular sulcus (see Fig. 2(a)) of twenty-four male adult Sprague Dawley rats under general anaesthesia, which was achieved through intraperitoneal injection with a solution of ketamine 10% and xylazine 2% (1:1), 0.1 ml/ 100 g body weight. The periodontitis rats model that had formed was illustrated in Fig. 2(b). The animals were divided into four groups: one group received vehicle (na-

CMC 0,5%) as a negative control (C, *n*=6), one group received Metronidazole as a positive control (M, 45 mg/kg/day p.o, *n*=6), one group received guava extract (135mg/kg/day p.o, *n*=6) started 7-days before induction (Gp, *n*=6), and one group received guava extract with the same dose started 7-days after induction.

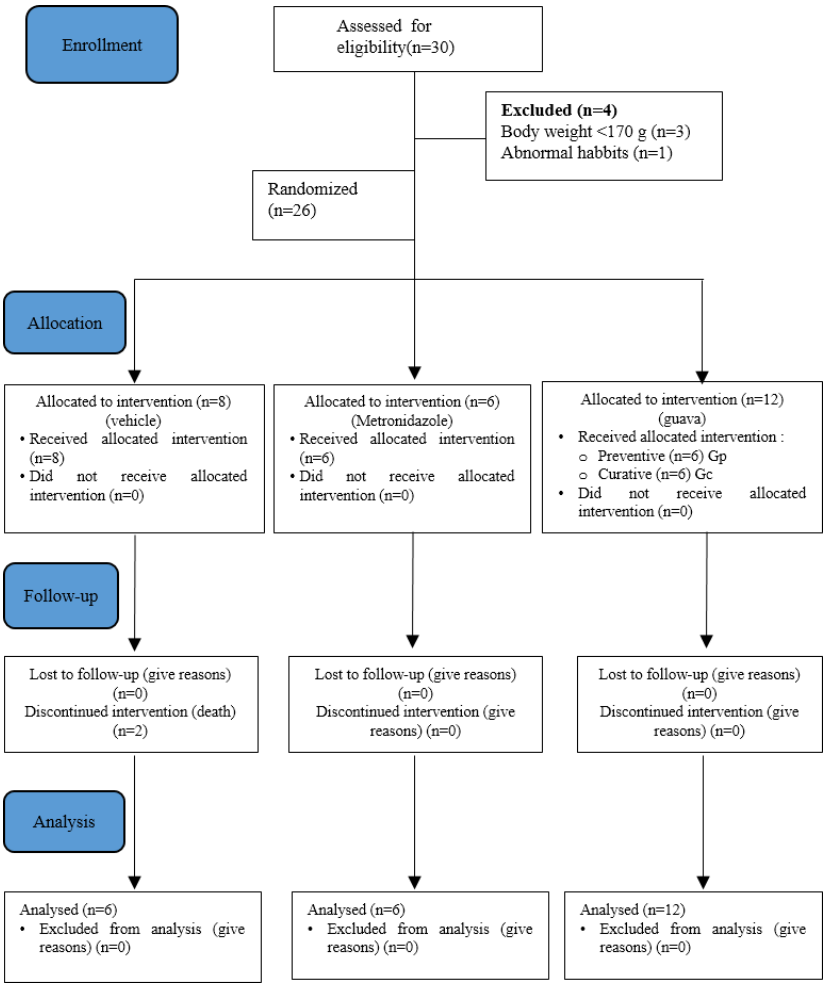

Fig. 1. Samples' flow diagram.

The metronidazole was chosen as positive control based on gold standard therapy for against an anaerobic-bacteria disease including periodontitis [9]. The ethanolic extract of guava (20% water content) was used for the dose of 135 mg/kg/day which is equal to one piece of guava fruit 200 g. In this case, flavonoid content within guava fruit extract is also expected to have antimicrobial activity in addition to its antioxidant activity, because both are needed in periodontitis therapy. The clinical parameter was assessed by measuring the periodontal pocket depth (PPD) using the Michigan O probe with Williams markings at mesial (Fig. 2(c)) and lingual sites (Fig. 2(d)) of rats' incisive mandibular teeth from the margin of gingiva to the bottom of the pocket. On the 14th-day post-induction, the animal has

euthanized dan the PPD were assessed, then the mandibula was taken and stored in formalin for preparing histological analysis [9].

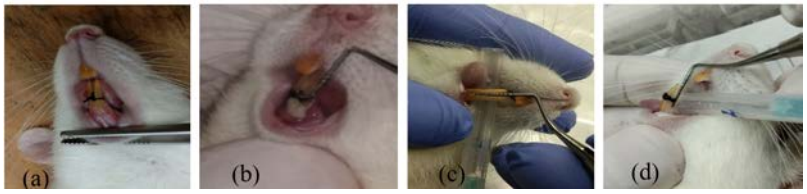

Fig. 2. Illustration of ligature placement on rat's mandibular incisor tooth (a); the periodontitis rats model after induction (b); mesial PPD assessment (c); lingual PPD assessment (d).

2.5. Histological evaluation.

The mandibula was cleaned from muscle and connective tissue and fixed for 72h in 10% neutral buffered formalin. After the fixation completed, the samples were decalcified in a 10% EDTA for 40 days. When decalcification was completed, the periodontal tissue was trimmed longitudinally and dehydrated through successive baths of Isopropyl alcohol (70%, 90%, 95%, and 100%), clarified in xylene, and embedded in paraffin wax. Multiple tissue sections were cut from each paraffin block at 4µm thickness with a rotary microtome. Afterwards, tissue sections were stained with Masson-trichome for fibrocollagen examination under an Olympus BX41 microscope with 400x magnification for assessing the Grade of inflammation histologically. The inflammation grades were categorized based on the fibro-collagen density indicated by the blue colour of Masson's trichome staining as seen as Fig. 3. For the highest density of the fibrocollagen, we gave score 1 on a scale of 3.

2.6. Statistical analysis

The data measurement among the groups was analysed using the Kruskal-Wallis test at a 95% confidence level.

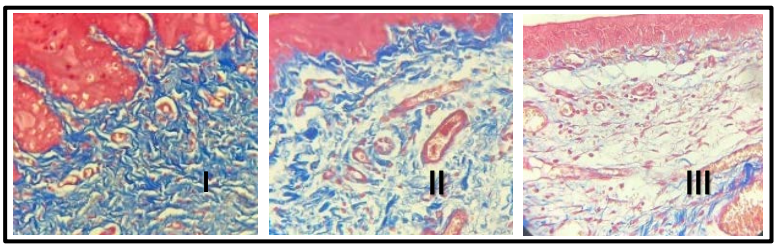

Fig. 3. The scale of inflammation based on fibro-collagen density (blue) through histological observation on the rat's periodontal tissue with a magnification of 400x.

3. Results and Discussion

3.1. Guava fruit selection

The selection of fruit ripeness is a factor to consider in used goal therapy. This is related to chemical changes contained in the guava fruit itself. Mondal reported that

the biochemical activity of guava during ripening were changes, including the enzymes of metabolism. Ascorbate and glutathione contents were found in maximum levels at the turning and ripening stages. Excessive accumulation of ROS due to dysfunction of the ROS scavenger system at later stages of fruit ripening appears to be responsible for the loss of tissue structure as observed in ripe and overripe fruit [10]. Gull's study shows that the flavonoid content of guava decreases following the fruit ripens. Meanwhile, the vitamin C levels increase in step with fruit maturity [11]. The beneficial ingredients needed from guava fruit are at the medium maturity stage to obtain maximum benefits for periodontitis therapy.

3.2. Induction of experimental periodontitis on rats

The induction process of periodontitis involves *P. Gingivalis* bacteria, which is one of the main periodontitis [12]. Binding is applied around the mandibular anterior, which has been previously made on the incisor teeth sulcus. The ligation aims to facilitate the accumulation of plaque bacteria locally, supported by *P. Gingivalis* adhered-silk ligature. The virulence property of *P. Gingivalis* is expected to accelerate the achievement of periodontitis conditions. Artificial wounds in the gingiva sulcus were made to ease the invasion process of *P. Gingivalis*, which manifest gingival inflammation leading to the destruction of the periodontal tissue and alveolar bones of the rats [13-17]. This induction process is conducted while the rats in general anaesthesia to avoid them suddenly waking up, which might lead to induction failure. Seven days after ligation, there were clinically periodontitis symptoms confirmed by the reddish color on the margin of the gingiva, and deepening of the periodontal pocket. This indicated that the induction was successful in achieving periodontitis status in all groups. The *P. Gingivalis* invasion can trigger the release of inflammation mediators such as IL-1 and PGE-2, which can recruit osteoclast and mediate alveolar bone destruction and MMP-8. Consequently, it can ruin the extracurricular matrix from the gingiva, the loss of collagen fiber attachment and periodontal ligament destruction.

3.3. Evaluation of clinical assessment after guava administration

At the end of the experiment, clinical improvement indications leading to the healing were shown by the guava group. This group outperformed the control group in terms of inflammation grade reduction. As seen in Fig. 3, inflammation grade scores were recorded and then transferred to charts and grouped, likewise in the PPD scores. The means and the corresponding standard deviations were computed for variations in both of assessed parameter among the control groups and experimental groups. Profiles of average scores parameter on rats can be seen in Fig. 4. Figure 4 presents that the score improvement in the treatment groups was better than the control group (C). Group M, as a positive control group, showed the lowest score, indicating that the method used was valid as metronidazole as the drug of choice in periodontitis therapy. In clinical practise, Metronidazole acts as an antimicrobial agent, especially for Gram-negative bacteria. One of the purposes of antimicrobial agent uses is to reduce the level of pathogenic micro-organisms and strengthen the groups of beneficial species [9].

A comparison of data was made between the mesial and lingual sites of PPD. Kruskal-Wallis test results of lingual PPD, mesial PPD, and inflammation grade gave p-value = 0.009, 0.018, and 0.032, respectively. This means that the treatment

manifested a significant impact on the experimental groups. The formed periodontal pockets at the lingual side showed a higher score than the mesial side. It is considered being associated with the anatomical position in which the lingual side has potentially enabled for plaque accumulation than the mesial side [16]. If that happens, it will worsen the inflammation and delay the healing process. Guava administration which given as pre-treatment may support a PPD reduction on the rats' lingual site of the mandibular central incisor teeth, in comparison with the control group. The post hoc Dunn's multiple analysis showed that only Gp group whose lingual PPD scale (p-value=0.046) had significant difference statistically compared with Gc groups due to guava extract administration.

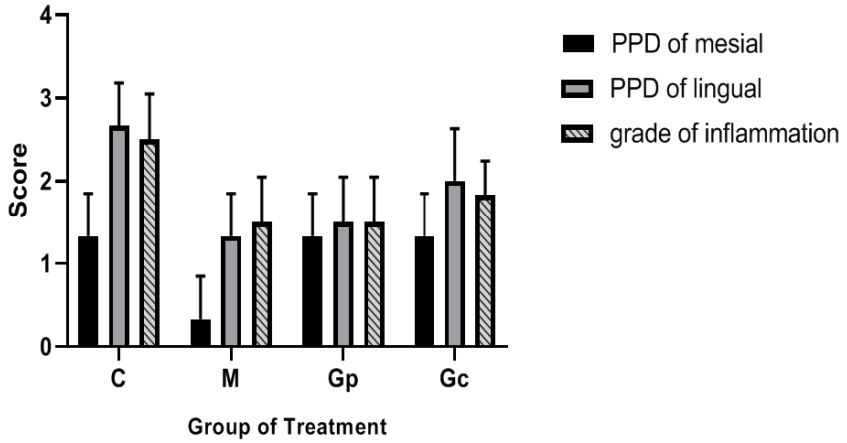

Fig. 4. Clinical sign score of rats after administration of Metronidazole (M) and ethanolic guava fruit extract in preventive (Gp) and curative (Gc) measure.

Reattachment of gingiva and periodontal ligament to their former position occurred due to periodontal ligament regeneration. This regeneration enabled continuity between alveolar bones and cementum as periodontal ligament contains cells that can synthesize and reformation gingiva, periodontal ligament, and alveolar teeth. This fact was also supported by histological data indicating the presence of a healing process in line with the existence of fibrocollagen regeneration, which presented a lower score based on Fig. 3. Fibroblasts may show the end state observed in healthy wound healing. Stage of wound healing indicated by collagen degradation is reduced, which contributes decisively to collagen accumulation in the wound area [15]. Vitamin C contributes as a co-factor in increasing the collagen deposit within the extracurricular matrix [16]. In addition to having high vitamin C, guava is also known to have a variety of flavonoid compounds. The presence of both in guava makes this fruit to have a high antioxidant activity which played a role in scavenging free radicals [17]. This condition facilitates the availability of a competitive substrate for unsaturated lipids in the membrane so that the damaged cell membrane repair is accelerated [10]. A preventive approach is useful in fulfilling the needs of the rats' body of antioxidants as well as in preventing further cell destruction, which in this case is indicated through the decrease of gingival inflammation grade.

Tissue repair also occurred in the control group despite the absence of any treatment. However, the recovery process was most likely to take a longer time compared to that of the rats given the treatment. This phenomenon possibly happened related to the ability of rats, synthesizing their vitamin C naturally in their liver. In this present study, guava supplementation starting one week before the induction in Gp group aimed to provide a pool of vitamin C as prophylactic against infection, by optimizing the reserves of vitamin C which provide in cell and tissue [18]. The integrated vitamin C can play a role as an antioxidant and help to repair the damaged collagen fibers on the gingiva by increasing the fibroblast proliferation and collagen deposition. The regeneration of fibro-collagen tissue will be able to reduce lymphocyte migration at the end of the recovery process. Therefore, rats were still able to regenerate their damaged cells without anti-inflammation medicine. The limitation of this study is the induction site was carried out on the mandibular incisors which had a continuous eruption [19]. This condition renders the ligature attached to not stay long in the sulcus. Thus, cautions must be taken in extrapolating results derived from rat incisors to human dentition.

4. Conclusion

Under the limitation of this study, guava supplementation potentially has a protective role in enhancing wound healing in periodontitis conditions. Different from metronidazole whose mechanisms of action are based on antimicrobial activity, guava tends to follow as the antioxidant agent. It is suggested that the supplementation administration as a preventive approach was able to maintain the intake of vitamin C and other antioxidant needed by the body to regenerate the damaged tissue in a shorter time in comparison with the rats in control (C) group.

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