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Effects of Cocoa Pod Husk Extract (*Theobroma Cacao* L.) on Alveolar Bone in Experimental Periodontitis Rats

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Abstract

Periodontitis is a destructive inflammatory disease of the periodontal tissues with a high prevalence in the world. *Phorphyromonas gingivalis* as one of the main bacteria causing periodontitis produces virulence factors that trigger immune cells to produce proinflammatory cytokines that can reduce the number of osteoblasts, thereby triggering alveolar bone resorption. Osteoblast cell differentiation is mediated by a number of growth factors such as cytokines and bone morphogenic proteins (BMPs). SRP mechanical therapy is sometimes not optimal and Metronidazole as an effective antibiotic against periodontitis has been reported to have several adverse effects. Utilization of cocoa pod waste (*Theobroma cacao* L) with its polyphenol content has antibacterial, antioxidant and anti-inflammatory effects. This study aims to determine the effect of ethanol extract gel from cocoa pod husk extract (*Theobroma cacao* L.) on the number of osteoblasts and osteoclasts, as well as the expression of BMP-2 in the alveolar bone of periodontitis-model rats. This laboratory experimental study used 24 rats which were divided into negative control group (CMC-Na gel), positive control group (metronidazole gel), and treatment group (100 mg/mL cocoa pod husk extract gel). 24 Wistar rats were divided into 3 groups which were observed on days 7 and 14. The number of osteoblasts and osteoclasts of alveolar bone were observed by hematoxillin eosin staining and BMP-2 expression by immunohistochemistry staining. One-way ANOVA test showed that there was a significant difference ($p < 0.05$) between groups. The LSD test average of BMP-2 expression in the treatment group on the day 7 and day 14 was higher than the negative control group ($p < 0.05$). The conclusion of the study was that giving 100 mg/mL of cacao pod husk extract gel (*Theobroma cacao* L.) increased the number of osteoblasts and increased the expression of BMP-2 in the alveolar bone of periodontitis rats.

Keywords: Periodontitis, Osteoblasts, Osteoclasts, BMP-2, Cocoa pod husk, *Theobroma cacao*

Introduction

Periodontitis is an inflammatory disease with a prevalence of 57.3 % worldwide [1]. Burden of Disease data shows that periodontitis has the 6th highest prevalence in the world and affects around 743 million people [2]. *Porphyromonas gingivalis* is one of the main causes found in subgingival plaque in periodontitis patients. *P. gingivalis* can produce various virulence factors such as Lipopolysaccharide (LPS), fimbriae and gingipains triggering immune cells to produce pro-inflammatory cytokines such as

interleukin 1-β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and prostaglandin-2 (PGE-2) which can trigger alveolar bone resorption by reducing the number of osteoblasts [3].

Bone resorption is a physiological process important for understanding many major pathologies, and is the most common oral manifestation seen as alveolar bone destruction in periodontitis. Repair of tissue damage requires an understanding of the process of intramembranous bone regeneration from the alveoli after injuries such as tooth extraction, periodontal disease or dental implants [4]. In the process of regenerating periodontal tissue, especially alveolar bone, osteoblast differentiation and proliferation are mediated by various growth factors such as Bone Morphogenetic Protein-2 (BMP-2). BMP-2 plays an important role in bone morphogenesis and repair, promoting differentiation of mesenchymal cells into osteoblasts and inducing new bone formation [5,6]. After the mesenchymal stem cells differentiate into osteoblasts, these cells secrete organic matrix of bone which eventually becomes osteocytes in the bones to form new bone [7]. In periodontitis, if the inflammatory process continues it can inhibit BMPs-induced osteoblastogenesis by suppressing BMPs signaling so that the formation and differentiation of osteoblasts are disrupted which then causes bone resorption [8]. Periodontitis therapy is necessary to prevent more severe bone resorption and accelerate bone repair.

Periodontitis therapy is needed to eliminate periodontitis pathogens so it can prevent more severe bone resorption. Metronidazole is an effective drug for the treatment of periodontitis. However, the use of antibiotics still has drawbacks, such as causing allergy, hematological problems, gastrointestinal problems and resistance if used long term [9]. In order to prevent more bone resorption, natural ingredients are needed that can be used for periodontitis therapy and minimizing the side effects of treatment. Theobroma cacao in cytotoxicity assay showed cocoa pod husk extract increased cell viability and cell proliferation of periodontal ligament fibroblast [10].

Cocoa (*Theobroma cacao* L.) is one of the largest commodity products in Indonesia, as the third largest producer in the world [11]. In general, the seeds of the cocoa pods are taken, while the cocoa pod husk is the biggest waste from the cocoa processing process. Cacao pod husk are very abundant as agricultural waste and have not been utilized properly, even though cocoa pod husk contain active compounds: Flavonoids, saponins, tannins, alkaloids and terpenoids. Flavonoid compounds present in the skin of the cocoa pod have the highest levels compared to the flowers, leaves and cocoa beans [12]. Flavonoids in cocoa pod husk can accelerate the process of osteoblast differentiation into osteocytes in the process of osteogenesis [13]. Based on previous research, cocoa pod extract at a dose of 100 mg/mL worked effectively as an anti-inflammatory in reducing COX-2 expression through inhibiting inflammatory cytokine activity and was effective in inhibiting the growth of *Porphyromonas gingivalis* [14]. The substance contained in cocoa pod husk potential to reduce the degree of inflammation that occurs in periodontitis so that it does not cause further bone damage and can be used as an alternative material for periodontitis therapy. Herbal medicine in the treatment of alveolar bone resorption in periodontitis has begun to be widely used, but the potential of cocoa pod husk in osteogenesis has not been widely studied. Observation on day 7 due to the initiation of new bone formation to synthesize and secrete matrix, and day 14 was the peak of the osteoblasts activity after bone loss. The peak of osteoblast cell activity would lead to increased collagen and matrix deposition by osteoblasts [15]. This study aimed to analyze the number of osteoblasts, osteoclasts and the expression of bone morphogenetic protein-2 in alveolar periodontitis rats at day 7 and 14 after administering the extract gel of cocoa pod husk (*Theobroma cacao* L.).

Materials and methods

Shakerbath *(Memmert WNB7L4, Japan),* rotary evaporator *(YHCHEM RE501,* China), *Ultrasonic bath* (*Elma* S100h, Germany), sentrifuge, decycator, inkubator, spektrofotometer *(Shidmadzu* UV-2600i*, Japan)*, microtome (*Tisuue-Tek*, Japan), W*aterbath* (*Memmert*, Germany), Tissue Prosessor (Tissue-Tek VIP 5Jr AMPSAZ, Japan), Oven (*Memmert* D06058, Japan), microscope (*Olympus* CX21LED, Japan), optilab (Optilab Model Advance Plus, Indonesia), Poly-L-Lysine coated slide (Biogear). antibody sekunder, *P. gingivalis* strain ATCC 33277 suspension, chlorofoam, BMP-2 monoclonal antibody sc-137087 (Santa Cruz Biotechnology, US), enzyme SAHRP (Strep Avidin Horseradish Perosidase), Counterstain Mayer's Hematoxilien, Diaminobenzinidine (DAB) chromogen, metronidazole.

Study design and sample

This research is an experimental laboratory study with the post-test only control group designs. The procedure for the treatment of experimental rats has met the eligibility requirements by the Health Research Ethics Commission of the Faculty of Dentistry, University of Jember with the Number

1641/UN25.8/KEPK/DL/2022. A total of 24 periodontitis rats were divided into a negative control group (CMC-Na gel); positive control group (Metronidazole); and the treatment group (ethanol extract gel of cocoa pods 100 mg/mL). The samples used were 24 male Wistar rats which were divided into 3 groups (negative control group, positive group and treatment group).

Extract preparation

Ripe cocoa pods of forastero type were obtained from the plantations of the Jember Coffee and Cocoa Research Center, East Java, Indonesia. Cocoa pods were cleaned, cut into small pieces, and air-dried. After being baked and blended to a fine powder and weighed with an analytical balance (Ohaus, USA), 5 kg of cocoa pods will produce ± 500 g of cocoa pods powder. The extraction method used is the ultrasonic bath (Elma, German) method which was carried out at the Pharmacy Laboratory of the Universitas Jember. A total of ± 50 g of cocoa pod peel powder plus 300 mL of 70 % ethanol solvent with a ratio of 1:4 (w/v); 1:2 (w/v); 1:1.5 (w/v) into an ultrasonicated glass container for 3×3 min. Every 3 min, the mixture was stirred before being ultrasonicated again. The entire filtrate obtained was then put into a rotary evaporator (B-one, USA), after that it was transferred to a petri dish and put in an oven to be evaporated at 40 °C until a constant weight extract was obtained. Cocoa pod peel extract was made in a gel preparation based on CMC-Na. A total of 48 mL of Aquadest was added with 2 g of CMC-Na and stirred until homogeneous into a 4 % CMC-Na placebo gel. A total of 22.5 g of CMC-Na gel was added to 2.5 g of cocoa pod peel extract, stirred until homogeneous into a 100 mg/mL cocoa pod husk ethanol extract gel [16].

Periodontitis model rats

Wistar rats (*Rattus norvegicus*) were induced 0.05 mL P. gingivalis with a concentration of 2.5×10^9 CFU/mL in the buccal part of the maxillary 1st molar using a tuberculine syringe. P. gingivalis induction was given 3 times a week alternately for 2 weeks until a periodontitis rat model was obtained on the 14th day. Mice are declared periodontitis when there are clinical signs of inflammation in the periodontal tissue in the form of redness, enlargement of the gingival margin, gingival recession and if X-rays are taken, alveolar bone resorption is seen [17].

Treatment procedures for experimental animal

A total of 24 male Wistar rats were divided into 3 groups, negative control group (CMC-Na gel); positive control group (metronidazole gel); and the treatment group (Cocoa pod husk extract 100 mg/mL). Each group observed on the day 7 and 14. The placebo CMC-Na gel and cocoa pod extract gel were applied once a day, while metronidazole was applied once for 7 days and 14 days. Experimental animals were sacrificed by injection of lethal dose of anesthetic using a mixture of 100 mg/kg ketamine and 10 mg/kg xylazine [18]. Tissue was taken on the left maxillary and immediately fixed with 10 % neutral formalin buffer for 24 h, then decalcified with 10 % formic acid. The tissue was processed by the paraffin embedding method and made transversal incisions with a thickness of 5 microns using a microtome and staining with Hematoxylin-eosin and immunohistochemistry.

Measurement of osteoblasts and osteoclasts

Observations were made with a binocular microscope (Olympus CX23) with 40x and 400x magnification on the cervical third, middle third, and apical third of the alveolar bone margin on the mesial side of the tooth extraction socket to observe the number of osteoblasts and osteoclasts. Counting was done with the help of Image J software by 3 observers and the average number of osteoblasts and socket osteoclasts after tooth extraction of Wistar rats was calculated.

Observation of BMP-2 expression

Observed the number of osteoblasts expressing BMP-2 in the defective alveolar bone with 400x magnification on a binocular microscope. Then captured using Optilab viewer 3.0 software, and stored in a file and opened using Quantitative Pathology 0.3.2 software to detect cells that express DAB Immunohistochemical staining. Then counted osteoblast cells that were cuboidal or columnar in shape with adjacent positions, had round nuclei and basophilic cytoplasm with Image J software. The results of counting osteoblast cells expressing BMP-2 were tabulated and averaged.

Statistical analysis

The data obtained were analyzed using SPSS software. Results are presented in terms of mean ± standard deviation (SD), normality test with Saphiro-Wilk, and homogeneity with Levene Test. The data

analyzed using One-Way showed there were differences in group. Furthermore, the data were tested to determine the average differences between the study groups using the Post Hoc Least Significant Difference (LSD) test.

Results and discussion

The results showed that the number of osteoblasts in the cocoa pod extract gel group was higher than the negative control (CMC-Na) and positive control (Metronidazole) groups on the 7th and 14th day of observation and the number of osteoclasts in the cocoa pod extract gel group lower on the 7th and 14th day of observation (**Table 1** and **Figure 1**).

Table 1 The number of osteoblasts and osteoclasts in the alveolar bone of the periodontitis rats.

Groups	Day 7 (x ± SD)		Day 14 (x ± SD)	
	Osteoblast	Osteoclast	Osteoblast	Osteoclast
CMC-Na	52.50 ± 3.87	4.25 ± 2.06	57.25 ± 2.99	3.50 ± 2.52
Metronidazole	56.50 ± 2.08	3.25 ±1.89	62.50 ± 2.65	2.75 ± 0.96
Cocoa pod husk extract	67.00 ± 1.63	3.00 ±1.41	76.00 ± 3.16	2.00 ± 0,82

Table 1 showed that application of cocoa pod husk extract gel increased the number of alveolar bone osteoblast cells in the periodontitis rat model. The results of the LSD test showed a significant difference ($p < 0.05$). The number of osteoclasts in the treatment group with cocoa pod husk extract gel on day-7 showed lower results than the negative control group on day-7 and day-14. However, based on the results of the LSD test, there was no significant difference ($p > 0.05$). The process of bone resorption was lower than alveolar bone formation in male Wistar rats which given cocoa pod husk extract gel. It could be interpreted that the effect of each group in inhibiting osteoclast formation on day 7 and day 14 was the same.

The number of osteoblasts in the cocoa pod husk group showed higher results than the control group **(Figure 1)**. Theses proved that cocoa pod husk contains saponins which can trigger the growth of new cells, as well as reduce the duration of the inflammatory phase. Flavonoids can stimulate the formation of osteoblasts and reduce osteoclast activity by inhibiting IL-6 activity. Flavonoids are able to stimulate the proliferation of osteoblast cells to become osteocytes by increasing the activity of estrogen receptors and increasing growth factors such as TGF-1 so that they can stimulate the proliferation of osteoblast cells. In addition, flavonoids also play a role in preventing cell death and as an anti-inflammatory so that they can accelerate the cell proliferation phase. While the decrease in osteoclasts occurs due to the content of flavonoids which are able to inhibit osteoclast differentiation and bone resorption thereby accelerating the process of bone regeneration [19].

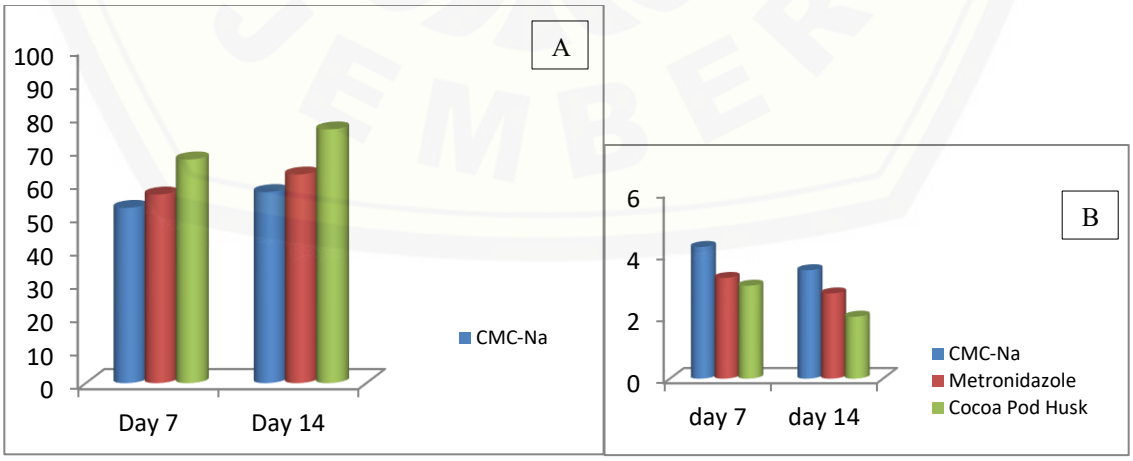

Figure 1 Histograms of osteoblast (A) and osteoclast (B) in alveolar periodontitis rats on day 7 and 14 Polyphenol compounds in cocoa pod husk have useful characteristics in several biological systems.

Cocoa pod husk have properties include antioxidant, anti inflammatory, antiviral, and antimicrobial activities. Polyphenols also showed inhibition osteoclastogenesis in bone loss pathologies [20]. Cacao pod alkaloids act as antioxidants by capturing free radicals in the form of ROS which trigger the kinase pathway to activate NF-κβ. NF-κβ can then translocate to the nucleus and induce transcription of target genes, such as TNF-α, IL-1β, IL-6, and IL-8. So that the content of these alkaloid compounds can suppress inflammation in periodontitis [21].

Table 2 Analysis of the amount of BMP-2 expression in the alveolar bone of the periodontitis rat model.

Groups	Day 7 (x ± SD)	Day 14 (x ± SD)
CMC-Na	18.62 ± 1.53	19.80 ± 2.56
Metronidazole	19.87 ± 0.71	22.87 ± 1.87
Cocoa pod husk extract	23.45 ± 2.18	28.72 ± 2.32

The results of the study with immunohistochemical staining are presented in **Table 2** and **Figure 2**. It shows that the expression of BMP-2 in the Cocoa pod husk extract group showed higher results than the negative control group (CMC-Na gel) and the positive control group (Metronidazole). Bioactive compounds of cocoa pod shell have various biological effects such as antibacterial, antioxidant, anti-inflammatory activity, and directly increase the signaling activity of BMP-2 expression which helps the processes of osteoblast differentiation and alveolar bone formation.

One-way ANOVA test showed that there were differences in the mean expression of BMP-2 in the control negative, control positive and treatment group ($p < 0.05$) (**Table 3**).

Table 3 One-way ANOVA test in each sample group.

	Sum of squares	***df***	***Mean square***	***F***	***Sig.***
Between group	743.977	5	148.795	43.463	0.000
Within groups	61.622	18	3.423		
Total	805.600	23			

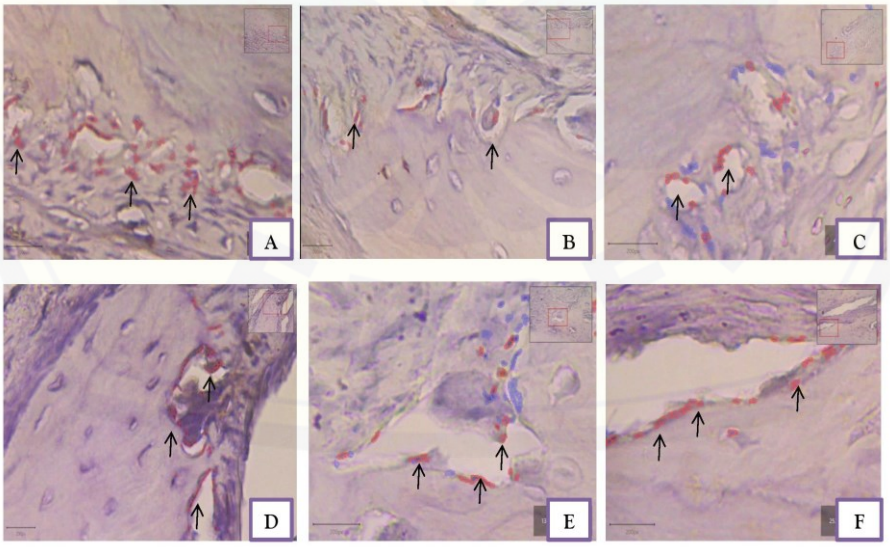

Figure 2 Histopathology of alveolar bone on immunohistochemistry staining (400x). A) the 7th day negative control group by administration of CMC-Na gel; B) the 14th day negative control group with CMC-Na administration; C) positive control group on day 7 with metronidazole gel administration; d) positive control group on day-14 with administration of metronidazole gel; e) the control group was treated on the 7th day by administering cocoa pod extract gel; f) the 14th day treatment group with cocoa pod extract gel (*black arrows* indicate the osteoblast cells expressing BMP-2).

Induction of P. gingivalis bacteria activates the transcription factor Nf-Kβ and proinflammatory cytokines such as IL-1, IL-6, and TNF-α mediated by TLR-4 receptors that bind to LPS, can initiate and cause transcription of these proinflammatory genes. Activation of Nf-Kβ can also inhibit BMP-2 signaling via the Smads 1/5/8 pathway by inhibiting its phosphorylation thereby preventing Smads 1/5/8 from being activated. On the other hand in the nucleus, Nf-Kβ activation inhibits Smads 1/5/8 phosphorylation to join the Smads 4 complex in encouraging transcription of the RunX2 gene. As a result of inhibiting this BMP-2 signal, osteoinductive activity is inhibited and osteoblastogenesis decreases [22].

Table 2 showed that the activity of osteoblast formation and expression of bone morphogenetic protein-2 was highest on the 14th day. In bone regeneration, the period of pre-osteogenic differentiation in the process of osteogenesis begins to increase on the 7th day, and reaches its peak on the 14th day [23]. The immunohistochemical staining presented in **Figure 2** showed that the expression of BMP-2 on day 7 and day 14 had significant differences. According to research, BMP-2 expression began to increase on day 7 and reached its peak on day 14, but on day 21 BMP-2 expression was reported to decrease. This is related to the bone regeneration phase. On the 5th to 11th day, fibrocartilaginous formation occurs where the release of Vascular Endothelial Growth Factor (VEGF) causes angiogenesis and fibrin-rich granulation tissue begins to develop. MSCs begin to be recruited and begin to differentiate mediated by BMP-2 osteoblasts. As a result, osteogenesis begins to occur. On day 11 to day 21, cartilage begins to undergo endochondral ossification. RANKL is expressed, stimulating further differentiation of osteoblasts. As a result, the resulting matrix begins to calcify. Newly formed vessels continue to proliferate allowing further migration and differentiation of BMP-2-mediated MSCs. At the end of this phase, the matrix is calcified but bone has not matured [24].

BMP-2 expression in the CMC-Na gel group was also lower than metronidazole gel as positive control group, and the cocoa pod husk extract group. Metronidazole is one of the antibiotics which is the drug of choice for periodontitis. Metronidazole can stop the etiology of periodontitis by inhibiting P. gingivalis bacteria. The mechanism of action of metronidazole is by inhibiting deoxyribonucleic acid (DNA) gyrase so that it can inhibit DNA synthesis. DNA gyrase is an enzyme present in bacteria that can cause it to open and form a superhelix in DNA. The role of metronidazole can inhibit bacterial DNA replication and cause bacterial death. Bacterial death can prevent excess bacterial colonization so that a more severe infection does not occur so that the inflammatory phase occurs in a shorter time [25].

Expression of BMP-2 in the treatment group showed higher results than the negative control group and the positive control group. It indicated that cocoa pod husk (*Theobroma cacao* L.) extract gel is able to inhibit excessive inflammation and repair bone damage due to periodontitis. Cocoa pod husk (*Theobroma cacao* L.) contains flavonoids, alkaloids, saponins, tannins and terpenoids which have various biological effects such as antibacterial, antioxidant, anti-inflammatory activity and directly increase the signaling activity of BMP-2 expression which helps the processes of osteoblast differentiation and alveolar bone formation. The antibacterial activity of cocoa pod extract gel is found in the content of tannins and terpenoids. Tannins have the ability to inhibit the work of protease enzymes which cause bacterial metabolic processes to be disrupted so that they can cause death in bacteria [26]. Terpenoids compounds are also used as antibacterial agents by damaging the membranes of lipophilic compounds by forming strong polymer bonds and damaging porins, reducing the permeability of the bacterial cell wall so that it can cause bacterial cells to lack nutrition and inhibit bacterial growth or die [27]. Alkaloids compounds contained in cocoa pod husk act as antioxidants by capturing free radicals in the form of ROS which trigger the kinase pathway thereby activating NF-κB. NF-κB can then translocate to the nucleus and induce transcription of target genes, such as TNF-α, IL-1β, IL-6, and IL-8. So that the content of these alkaloids compounds can suppress inflammation in periodontitis [28,29].

In addition, the cocoa pod husk (*Theobroma cacao* L.) ethanol extract gel effectively triggers growth factor, BMP-2, which stimulates bone formation. Flavonoids and saponins in cocoa pod husk increase osteoblast activity by increasing BMP-2 signaling through increasing the Smads pathway involving Smad 1, 5 and 8 [30]. Smad 1, 5, and 8 will then be phosphorylated along with Smads 4 in transcription by activating the Runx2 target gene. This gene acts as a transcription factor in progenitor cells to become osteoblasts. These osteoblast precursors then proliferate and differentiate to form preosteoblasts which will then become mature osteoblasts [31].

Conclusions

Based on the results of the study it was concluded that. Cocoa pod husk can be considered as an alternative to herbal-based therapy. Cocoa pod husk extract gel was effective in increasing alveolar bone regeneration in periodontitis rats model characterized by increased osteoblast numbers and BMP-2 expression.

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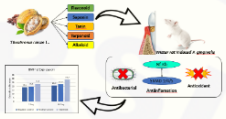

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SECTION

ABSTRACT

Periodontitis is a destructive inflammatory disease of the periodontal

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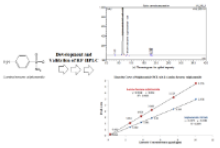

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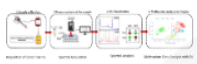

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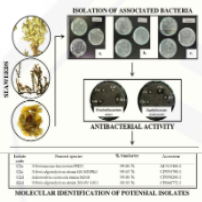

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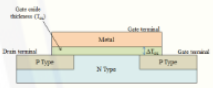

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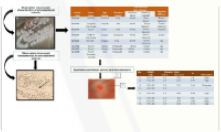

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