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# Effect of Cocoa Pod Husk Proanthocyanidins Extract on Cyclooxigenase-2 (COX-2) Expression in Gingival Crevicular Fluid of Periodontitis Rats

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**ABSTRACT**

Inflammation of periodontal tissue due to Porphyromonas gingivalis which invades the gingival tissue due to the activity of virulence factors. This causes the host immune system changes and increases COX-2 production in periodontal tissue so resulting excessive tissue destruction. The aim of this study was to analyze the potency of proanthocyanidins extract of cacao pod husk to inhibit COX-2 expression in rat gingival sulcus fluid. 3 groups in this study, namelynegative control, positive control, and treatment. In all groups, on the 0 day, gingival sulcus fluid was taken, then the rats were induced by Porphyromonas gingivalis once every 3 days for 2 weeks; then in the negative control group a placebo gel was applied and the treatment group was applied proanthocyanidins extract gel of cacao pod husk extract every day for 7, 14, and 28 days and serial gingival crevicular fluid collection was carried out on days 7, 14, and 28. The expression of COX-2 in the gingival crevicular fluid was observed using the ELISA method. The statistical test used was Anova. There were a significant difference in COX-2 expressions ($p < 0.05$). There was a decrease in the expression of COX-2 in rat gingival sulcus fluid induced by Porphyromonas gingivalis and given 10% cacao pod husk extract. Bioactive compounds in cocoa pod husk extract potential to reduce COX-2 expression in periodontitis rats.

**KEYWORDS:** COX-2, cocoa pod husk, proanthocyanidins, periodontitis

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## INTRODUCTION

Cocoa pod husk only used as animal feed and if they are not used properly, they will increase theamount of waste from the cacao pod, even though from a medical perspective, the cacao pod husk has many ingredients that can be isolated and utilized.[1] One of the largest ingredients is polyphenols in the form of catechins (37%), anthocyanins (4%), and proanthocyanidins (58%).[2] The polyphenol of cacao pod extract, can be used as an immunomodulatory, anti-cancer, antioxidant, antibacterial, and anti-inflammatory agent.[3] Proanthocyanidin, the largest polyphenol type of cacao pod extract, can be used as a natural alternative for healing inflammation, such as periodontal disease.[4] The damage that occurs inperiodontal tissue is caused by various virulence factors of P. gingivalis such as lipopolysacharide, capsule, fimbriae, and gingipains. [5] P. gingivalis is a species that is closely related to the formation process of chronic periodontitis, 85% in periodontal tissue that is inflamed. [6]

Virulence factors possessed by P. gingivalis bacteria can cause inflammation by releasing proinflammatory cytokines, *interleukin* (IL-1, IL-6), and *tumor necrosis factor* (TNF- $\alpha$).[7] These proinflammatory cytokines activate transcription of the c*yclooxigenase* (COX-2) gene. [8]. Proinflammatory cytokines can cause inflammation of the periodontal tissue through the arachidonic acid metabolic pathway. [9] Cyclooxygenase is an enzyme that has an important role in the metabolic process of arachidonic acid to produce inflammatory mediators, especially COX-2. COX-2 is one of the enzymes responsible for the synthesis of inflammatory mediators, *thromboxane A2* (TXA2) and *prostaglandin E2* (PGE2). COX-2 is not expressed continuously, its expression increases when it receives inflammatory stimuli such as *lipopolysaccharides*, hormones, *growth factors* and proinflammatory cytokines, which will cause an increase in inflammatory mediators. Continuous increase of inflammatory mediators can cause

tissue damage. [10]

COX-2 will be secreted in the gingival crevicular fluid in periodontal inflammation. The resulting ofsecretions can be used as biomarkers in determining the diagnosis and severity of periodontal disease. Detection of the presence ofthe COX-2 enzyme is easiest by taking gingival crevicular fluid, in patients with periodontitis the amount of gingival crevicular fluid will increase. [11] The advantages using gingival crevicular fluid as a method of analyzing periodontal inflammation are due to easy, inexpensive, and non-invasive method.[12]

## MATERIALS AND METHODS

Tools and materials used in this research among others were analytical scales, oven, measuring flask, erlenmeyer, rotary evaporator, waterbath shaker, falcon tube, test tube, ose, centrifuge, spectrophotometer, stopwatch agar desicator, 1.5 ml eppendorf tube, refrigerator for storingsamples at minus 60 C, vortex, yellow tip, micropipette, channel 8 micropipette, 96-well plate, COX-2 ELISA kit, and ELISA reader. Cocoa pod extract, CMC-Na, acetone, sterile distilled water, P. gingivalis ATCC 33277 0.05 ml with a concentration of 2x109 CFU/ml, paper point number 15, cotton roll, and Phosphate Buffered Saline (PBS).[13]

### Animal preparation

The treatment procedure for experimental animals has received approval (ethical clearance)by the Health Research Ethics Commission of Gajah Mada Faculty of Dentistry, no. 0019/KKEP/FKG-UGM/EC/2019. The experimental animals were adapted first for 7 days in a closed cage and given standard food and drink. This aims to obtain uniformity prior to conducting research to control experimental animals.

### Extraction proantocyanidins of cocoa pod husk

The cocoa pod husk (Theobroma cacao L.) was cut and dried in the sun to dry, then shaved and blended to get a fine powder. Powdered cocoa pod husk were freed from fat using n-hexane solvent by maceration with a ratio of powder to solvent (1: 5) three times. The fat-free sample was dried. Powder cocoa pod 100 gram was put into an erlenmeyer containing 700 ml of 70% acetone solution and 300 ml of distilled water, then stirred until homogeneous and put in a waterbath shaker at 50°C for 20 minutes. The extract solution was separated from the supernatant by centrifugationat 2000 rpm for 10 minutes. The extract solution was then put into a rotary evaporator, after whichit was transferred to a petri dish and put in the oven. The petri dish was removed from the oven,then the thick part that is at the bottom of the petri dish was removed and placed in a beaker.[14]

### Chromatography analysize used HPLCmethod

Proanthocyanidins analyze was performed using the column HPLC method which was operated at25°C. The compounds were detected at 200 and 400 nm wavelengths. The mobile phase of HPLC consisted of 2% (v/v) acetic acid in water (eluent A) and 0.5% acetic acid in water andacetonitrile (50:50, v/v; eluent B). The mass spectrophotometric analysis of the extract was carried out by a mass spectrometer in negative ionization mode. The nebulizer pressure was set to 45 psi and the drying gas flow rate was 5 l/min. The flow rates and temperature of the casing gas were 11 l/min and 350°C, respectively. The mass ranges from 50 m/z to 2000. Chromatographic separation was performed on an ODS C18 analytical column (4.6 × 250 mm), using the Agilent 1290 Infinity HPLC system (Agilent Technologies, USA). About 0.3 ml/min of eluent was introduced into the mass detector. Selecting ion monitoring (SIM) was used to select molecular ion isomers from the procyanidins group in the proanthocyanidinextract of the cacao peel for quantification. The Agilent Mass Hunter Workstation was used for data acquisition and processing.[1]

### Preparation for Proanthocyanidins of cacao pod husk extract gel

96 ml distilled water was measured with a volumetric flask and poured into the mortar. Then

4 grams CMC-Na were measured by analytical scales and putted into a mortar containing distilled water. Let stand 10-15 minutes, stirring ituntil expands and forms a yellow gel. The mixture of CMC-Na and distilled water that has becomegel was weighed as much as 45 grams and 100% proanthocyanidin of cocoa pod husk extract as much as 5 grams, then it was putted in the mortar and mix until homogeneous to get a gel of proanthocyanidins of cocoa pod extract with 10% concentration.[16]

### Preparation of P. gingivalis suspention

After making the culture media, one ose of P. gingivalis with 33277 ATCC pure was inoculated in each petridish, then incubated for 2x24 hours.[17] The suspension was made by taking 1 ose P. gingivalis bacteria from the culture preparation and it dissolving in 1 cc of saline/PZ solution in the tube. The reaction after that was homogenized by centrifuge and measured at a concentration of 2x109 CFU/ml according to the Mc Farland 0.5 standard using a spectrophotometer.[13]

### Applied of P.gingivalis and proanthocyanidins

The negative control, positive control, and treatment groups were injected with 0.05 ml of P. gingivalis ATCC 33277 at a concentration of 2x109 CFU/ml in the distobuccal and distopalatalof gingival sulcus of maxillary first molar. Theinjection was repeated once every 3 days for 2weeks.[17] after obtaining the periodontitis ratmodel, then taking the rat gingival sulcus fluid onday 0 for the negative control and treatmentgroup. Applying placebo gel for the negativecontrol group, metronidazole for the positive control group and proanthocyanidin extract cacao pod 10% for treatment group, daily for28 days. Gingival crevicular

fluid was take on days 7, 14, and 28. Placebo gel and proanthocyanidin extract gel of cocoa pod husk 10% was applied to the distobuccal and distopalatalgingival sulcus area of the maxillary first molar using a plastic filling instrument.[18]

**Measurement of COX-2 expression**

Teeth were cleaned with cotton to control saliva, then gingival crevicular fluid (GCF) samples were taken with paper point number 15 for 30 seconds. The paper point was positionedhorizontally in the area of the gingival groove inthe distobuccal part of the maxillary first molar. Taking GCF should be done carefully so did notmake a injury to the gingival groove area which in turn will cause contamination. Paper points were inserted into 0.5 mL eppendorf tube and stored at -20°C, until the ELISA test was performed. Before the ELISA test, the eppendorf tube was added with 50 µL 0.02 M of Phosphate Buffer Solutions (pH 7.0-7.2), followed by 2000- 3000 RPM centrifugation at room temperature 18-25°C for 20 minutes, after that the ELISA test was carried out with the COX-2 ELISA kit. Then the test results are read using an ELISA reader with a wavelength of 450 nm for a maximum of 30 minutes after giving the stop solution.[19]

**Statistical analysis**

The data obtained were analyzed using the Statistical Package for the Social Sciences (SPSS). *Kolmogorov-Smirnov* test used to test for normality and *Levene's* test for homogeneity. *One-way analysis of variance* (ANOVA) would be carried out to compare the TNF-a expressionbetween each treatment group, followed by *LeastSignificant Different* (LSD). Groups differences were significant if the p value was < 0.05.

**RESULTS AND DISCUSSION**

The expression of COX-2 was presented in **Table 1**. There were significant difference of COX-2 expression on 0, 7, 14 and 28 days (p= 0.000). On 0 day, the most expression was negative control group (71.740±3.56), proantocyanidins group higher than positive control group (56.577±1.070); on the 7 day, the most expression of COX-2 was negative control group (113.393±12.527), positive control group higher than proantocyanidinsgroup (97.897±6.931); on the 14 day, the most expression of COX-2 was negative control group (194.094±6.592), positive control group higher than proantocyanidins group (78.397±2.134); and on the 28 day, the most expression of COX-2 was negative control group (55.924±1,706), positive control group higher than proantocyanidins group (54.081±2,644).

**Table 1. The COX-2 expression in the rat GCF (U/l) and Anova test.**

| Groups | COX-2 expression (Mean ± SD) | | | | p |
|---|---|---|---|---|---|
| | 0 day | 7 days | 14 days | 28 days | |
| NC | 71.740 ±3.56 | 113.393±12.527 | 194.094±6.592 | 55.924 ±1,706 | *0.000 |
| PC | 52.666 ±0.00 | 97.897 ± 6.931 | 78.397 ±2.134 | 54.081 ±2,644 | |
| PA | 56.577 ±1.070 | 92.811 ± 2.662 | 74.019 ±4.594 | ±2,175 | |

*Significant difference p<0.000

NC: negative control (CMC-Na gel)

PC: positive control (metronidazole gel)

PA: Proanthocyanidins cocoa pod husk extract gel

The difference of COX-2 expression between group was presented in Table 2 . On the 0 day, there were difference between negative and positive control group (0.04); on the 7 day, there were difference between negative control and proanthocyanidins group (0.02); between negative control and proanthocyanidins group (0.00), also between positive control and proanthocyanidins group (0.00); on the14 day, there were difference between negative and positive control group (0.00), also between negative control and proanthocyanidins group (0.00)

**Table 2. The result of different test of The COX-2 expression by LSD test (p).**

| | 0 day | | | 7 days | | | 14 days | | | 28 days | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Groups | NC | PC | PA | NC | PC | PA | NC | PC | PA | NC | PC | PA |
| NC | | *0,04 | 0,10 | | 0,09 | *0,02 | | *0,00 | *0,00 | | 0,83 | 0,72 |
| PC | | | 0,66 | | | 0,57 | | | 0,62 | | | 0,87 |
| PA | | | | | | | | | | | | |

The damage that occurs in periodontal tissues iscaused by various virulence factors from P. gingivalis such as lipopolysacharides, capsules, fimbriae, and gingipains.[5] The presence of lipopolysaccharides will stimulate the formation of inflammatory mediators through the cyclooxygenase (COX) pathway.[9] COX is an enzyme that useful for

accelerating the synthesisof prostaglandins from arachidonic acid. Arachidonic acid is an unsaturated fatty acidfound in the phospholipid bilayer.[20]. The inflammatory stimulus causes the activation of phospholipase A2 which causes the release of arachidonic acid from the cell membrane to the cytosol. Arachidonic acid metabolism throughthe cyclooxygenase (COX) pathway will produce prostaglandin E2 (PGE2) and thromboxane A2 (TXA2). [21]

Prostaglandin E2 causes increased vasodilation and endothelial permeability resulting in increased infiltration of inflammatory cell. PGE2 is the most type prostaglandin in the pathogenesis of periodontitis.[22] Increased proinflammatory cytokines (IL-1 and TNF-α) andPGE2 can stimulate bone resorption by increasing osteoclast formation. Proinflammatorycytokines and PGE2 will also inhibit the formation of osteoprotegerin (OPG) which functions to inhibit osteoclast formation, resultingin increased osteoclast formation and bone resorption.[23]

The results of this research showed the treatment group with 10% proanthocyanidin extract gel had the lowest TNF-α and COX-2 expression. This was caused by the proanthocyanidins in the cocoa pod husk extract having anti-inflammatory andantibacterial properties.[24] Proanthocyanidin activity in inhibiting COX-2 is probably by inhibiting the activity of proinflammatory cytokines. This was supported by severalprevious studies showing that proanthocyanidin can inhibit the growth of P. gingivalis and reduce COX-2 expression through inhibition of inflammatory cytokines activity.[25]

According to research conducted by La, proanthocyanidin can inhibit the invasion of P. gingivalis and inhibit the activity of virulence factors such as gingipain. Gingipains functions to activate proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β ) and interleukin-6 (IL-6) which can trigger inflammation. Based on research by Lee et al. (2017), proanthocyanidin from grape seeds can also decrease the expression of IL-1β, IL-6 and TNF-α.[19] Proanthocyanidin from cranberries can decrease the expression of IL-8 and CCL-5 from the oral epithelial cells induced by P. gingivalis.[5]

Previous study showed that there was an increase in COX-2 mRNA expression after induced by IL-1β for nine hours and decreased significantly after proanthocyanidins administration. The study also showed that metronidazole gel which was used as a positive control could reduce COX-2 expression and there was a significant difference in the average COX-2 expression on days 7, 14, and 28. The decrease was due to metronidazole gel are bactericidagainst anaerobic gram-negative bacteria such as P. gingivalis by interfering for bacterial DNA synthesis.

The decreased of IL-1 and TNF-α expression will decrease COX-2 expression due to the signal inhibition of IL-1 and TNF-α for therelease of phospholipids from the cell membrane, whereas COX-2 expression in the negative control group is higher. The amount of mRNA and gingival COX-2 protein in subjects with chronic periodontitis was higher than in healthy ones.[28] This is reinforced by the results of Mesa et al. study that COX-2 expression in patients with gingivitis or periodontitis was higher than in healthy gingiva.[23]

Research conducted by Mori et al. demonstrated that P. gingivalis gingipain can induce COX-2, wherein COX-2 mRNA levels are greatly increased after 2 hours and can still be detected at 6 hours and 12 hours after exposure by gingipain, lipopolysaccharides, capsules, and fimbriae. P. gingivalis can regulate inflammatory.[24] Tamura et al. showed that cells induced by IL-1$\beta$ increased theexpression of mRNA and protein COX-2 and PGE2.

The changes of COX-2 expression based on time, in the proanthocyanidins group COX-2 increased on day 7 and decreased slowly until day 28. Meanwhile, COX-2 levels in the negative control group continued to increase until day 14 and decreased drastically until day

28. The enhance of COX-2 expression was probably due to the negative control group still experiencing an inflammatory process, where COX-2 is an important enzyme for the synthesisof inflammatory mediator precursors. Based on the research of Paulasilva et al., it was shown that in the periodontal tissue of rats induced by P. gingivalis, there was fluctuation in COX-2 expression, where the highest expression of COX-2 occurred on day 14.[26]

## CONCLUSIONS

The proanthocyanidins contained in the cocoa pod husk extract (Theobroma cacao L.) potential to reduce COX-2 expression in periodontitis rats. Based on the results of the study, the highest COX-2 expression was in the negative control group, and there was a significant difference in the average COX-2 expression in the negative control group on days 0, 7, 14 and 28.

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