

The Effect of Cocoa Pod Husk (*Theobroma cacao* L.) Extract on Increasing Gingival Fibroblast and Collagen Density in Wistar Rats Induced by *Porphyromonas gingivalis*



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ABSTRACT: Periodontal disease is an inflammatory disease of the oral cavity which characterized by inflammation of the gingiva. *Theobroma cacao* L. is a herbal plant that has antibacterial and anti-inflammatory effects, which is starting to be widely researched as an alternative therapy for inflammation in the oral cavity. The aim of this study was to examined the effect of the ethanol extract gel of cocoa pod husk (*Theobroma cacao* L.) on increasing the number of fibroblasts and the collagen fiber density in the gingiva of rats that have been induced by *Porphyromonas gingivalis*. The sample used 24 male Wistar rats divided into 3 groups. Each group was induced by porphyromonas gingivalis every 3 days for 14 days then anointed with placebo gel (negative control group), metronidazole gel (positive control group), and cocoa pod ethanol extract gel (CPH gel) for 7 and 14 days. Gingival tissue was taken after euthanasia, after which histological preparations were made, and fibroblasts were observed under a microscope and analyzed using quantitative pathology 0.3.2 software, while for collagen density analyzed using Adobe Photoshop CS6 software. The results showed there was a significant difference of the number of fibroblasts between each group ($p < 0.05$). however there was no significant difference between positive control and cocoa pod husk extract at day 14 ($p > 0.05$). The treatment group on day 7 and 14 was higher than the other groups. It was conclude that the ethanol extract of cacao pod husk (*Theobroma cacao* L.) potential to increase the number of fibroblasts and collagen fibers density in gingival tissue of periodontitis rats.

KEYWORDS: gingival fibroblast, collagen fibers, cocoa pod husk, *Theobroma cacao* L

I. INTRODUCTION

Periodontal disease is an inflammatory disease with the highest prevalence in the world. DataWorld Health Organization (WHO) shows that 90% of the world's population suffers from gingivitis and 80% of them are children aged 12 years and under (Zefanya *et al.*, 2021). The main cause of gingivitis is subgingival plaque bacteria which includes gram-negative anaerobes such as *Porphyromonas gingivalis* (Tonetti *et al.*, 2017). *P. gingivalis* always associated with damage to the periodontal tissue, especially gingivitis (Samaranayake, 2012). Bacteria *P. gingivalis* it produces virulence factors such as *gingipain*, *collagenase*, *fibrinolysin*, *phospholipase*, *fimbriae*, *polysaccharide capsule*, and *liposaccharide* which can cause chronic inflammation of the gingiva. (Samaranayake, 2012). This virulence factors trigger immune cells to produce proinflammatory 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 activate tissue fibroblasts, resulting in increased proliferation (Baek *et al.*, 2015).

Fibroblasts play an important role when entering the proliferative phase of the healing process. Under normal circumstances, fibroblast division activity is rarely seen, but when an injury occurs, fibroblasts will immediately migrate to the area and stimulate collagen synthesis gradually so that they are able to heal the wound properly. Fibroblasts will also stimulate macrophages to produce growth factor which aims to synthesize vascularization (Sea *et al.*, 2019). An increase in the number of fibroblasts indicates a healing process. Factors slowing down the healing process are location, wound size, tissue vascularization, and the functional role of the tissue itself (Smith *et al.*, 2019). Collagen is a key component in the phase of wound healing. Display

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of collagen fibrils to the blood will cause platelet aggregation and activation thereby releasing chemotactic factors that start the wound healing process. In the early phase of the wound healing process, the amount of collagen degradation is low, but increases with the maturation of the wound. In the healing process, the main cells involved are fibroblasts and collagen. Fibroblasts are cellular elements that are found in the gingival connective tissue that proliferate and actively synthesize matrix components in the process of wound healing and repair of damaged tissue (Mercandetti, 2002).

In the healing process of gingivitis can be assisted by using chemicals and drugs with herbal ingredients, which can reduce inflammation and cure gingivitis. One of the herbal ingredients that has begun to be widely researched is the cocoa plant (*Theobroma cacao* L.). Based on the results of phytochemical tests, cocoa pod extract contains several active compounds, namely saponins, alkaloids, tannins, flavonoids and triterpenoids (Nugroho *et al.*, 2019). Cocoa is one of the largest commodity products in Indonesia, as the third largest producer in the world. In general, the seeds of the cocoa pods are taken, while the cocoa pod husk is the biggest waste from the cocoa processing process. Cocoa 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* (Rahayu *et al.*, 2023). Based on previous research, cocoa pod extract at a dose of 100 mg/mL worked effectively as an anti-inflammatory in reducing TNF- α , COX-2 and MMP-8 expression through inhibiting inflammatory cytokine activity and was effective in inhibiting the growth of *P. gingivalis* (Rahayu *et al.*, 2020^a; Rahayu *et al.*, 2020^b). This study aimed to analyze the effect of cocoa pod husk (*Theobroma cacao* L.) extract on increasing gingival fibroblast and collagen density in wistar rats induced by *P. gingivalis*.

II. MATERIALS AND METHODS

This research is a laboratory experimental study with a post test only control group design. Ethical approval was obtained from the Health Research Ethics Commission (KEPK) of the Faculty of Dentistry, University of Jember with number 1760/UN25.8/KEPK/DL/2022. Preparation of ethanol extract gel from cocoa pods takes place at Laboratory of Biology Pharmacy, Faculty of Pharmacy, University of Jember, maintenance and care of experimental animals, preparation and rejuvenation of *P. gingivalis* bacterial suspension, tissue preparation with Haematoxylin eosin and Mallory trichrome staining at Laboratory of Microbiology, Faculty of Dentistry, University of Jember

All rats were induced *Porphyromonas gingivalis* (ATCC 33277) once every 3 days for 14 days. After that, the buccal sucus of the maxillary first molar in the left region was given placebo gel once a day for 7 days in the negative control group, metronidazole gel once a day for 7 days in the positive control group. Administration with cocoa pod extract (*Theobroma cacao* L.) (100 mg/ml) once a day for 7 days in the treatment group. Then the upper left jaw of periodontitis rats examined and cut using a scalpel, taken by multiplying 5mm long pieces of tissue in the mesial and distal sulcus of the first molar teeth. The sample is put in buffered formalin for 24 hours so that the tissue is not damaged then decalcified with 10% formic acid. The research data were obtained from observing histological preparations from each group. The number of fibroblasts and collagen density in the gingiva was carried out on day 7 and 14. Observations were made with a binocular microscope with the help of optilab with a magnification of 400X. The sample was observed for the intensity of the pink color arising from HE staining for the observation of fibroblasts which was determined based on the Quantitative Pathology-0.3.2 software while the intensity of the collagen fibers was observed for the blue color. The purplish appearance of Mallory's trichrome coloring was determined based on adobe photoshop CS6 software (Rahayu *et al.*, 2020^b).

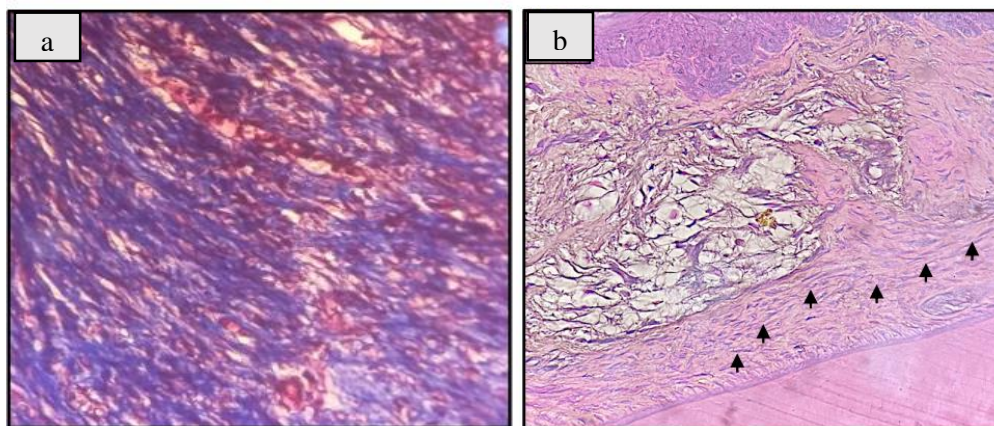


Figure 1. (a) Collagen density at 400x magnification with Trichrome Mallory staining, (b) Fibroblasts at 400x magnification with HE staining.

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The data obtained were then analyzed using the One-Way ANOVA test from SPSS. The sample used was 24 male Wistar rats which were divided into three groups (negative control group, positive control group and treatment group). Each group consisted of 8 rats which were divided into 2 subgroups with each subgroup consisting of 4 male Wistar rats whose data showed the mean and standard deviation in each sample group. The data obtained were tested for normality using the Shapiro-Wilk and homogeneity tests using the Levene Test. Once the data were known to be normally distributed and homogeneous, the data were analyzed using the One-way ANOVA test to determine whether there were significant differences in the number of fibroblasts and collagen density in all groups. The results of the one way ANOVA test showed that there were differences in the average number of fibroblasts and collagen density in the negative control group, the positive control group and the treatment group. Then the data was tested to find out the average difference between study groups using the Post Hoc Least Significant Difference (LSD) test.

III. RESULTS

Based on the results of calculating the average number of fibroblasts, graphs of the average fibroblasts for each group were obtained on day 7 and day 14.

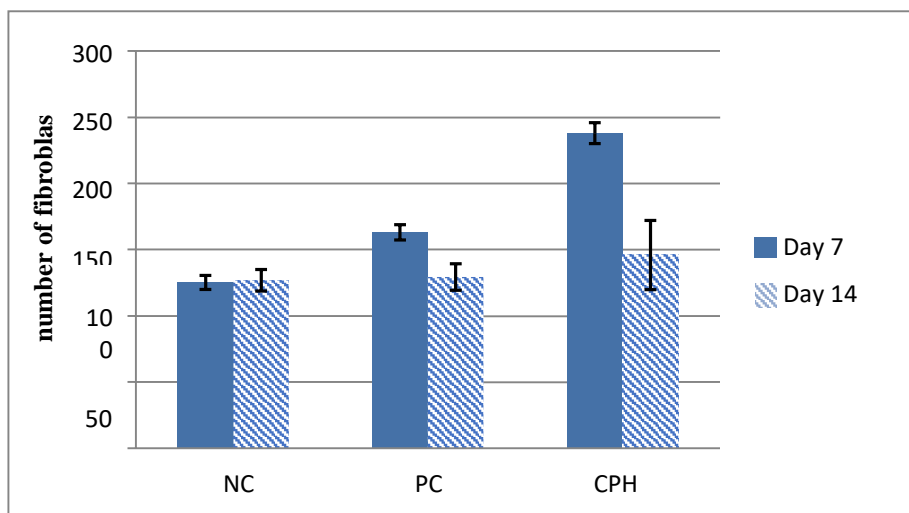


Figure 2. Histogram graph of the average number of fibroblasts in the negative control, positive control and cocoa pod husk groups on day 7 and 14.

The results of calculating the average number of gingival fibroblasts presented in Figure 2 show that the highest number of fibroblasts was in the cocoa pod extract gel group and the lowest number of fibroblasts was in the negative control group. The cocoa pod extract gel group and the positive control group had a higher number of fibroblasts than the negative control group, which means there was an increase in the number of fibroblasts.

Table 1. One-way ANOVA test results on the number of rat gingival fibroblasts

	Sum Of Squares	Df	Mean Square	F	Sig.
Between Groups	36068.517	5	7213.703	44.189	.000
Within Groups	2938.423	18	163.246		
Total	39006.940	23			

The results of the one-way ANOVA test in Table 1 show that the significance value is <0.05 , which is 0.000, which means that there is a significant difference, at least one pair of groups is significantly different. Then the next test, namely Least Significant Difference (LSD) to find out the differences between the two different groups, is presented in Table 2 below.

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Table 2. Results of the Least Significant Difference (LSD) test for the number of gingival fibroblasts in rats

	NC7	PC7	CPH7	NC14	PC14	CPH14
NC7	-	0.006*	0.006*	1.000	0.787	0.250
PC7		-	0.000*	0.009*	0.086	0.433
P7			-	0.000*	0.000*	0.000*
CPH7				-	0.869	0.325
PC14					-	0.913
CPH14						-

Based on the results of the LSD test, a significant value ($p < 0.05$) showed that there was a significant difference between the NC7 group and the PC7 and CPH7 groups; between groups PC7 against CPH7 and NC14; between the CPH7 group and the NC14 group, the PC14 group, the CPH14 group. Whereas in the NC7 group against NC14, PC14 and CPH14; between the PC7 group and the CPH14 group, the CPH14 group; between the NC14 group and the PC14 group, the CPH14 group; between the PC14 group and the CPH14 group there was no significant difference with a significance value ($p > 0.05$). Groups that were not significantly different meant that they had the same number of fibroblasts. This shows that cocoa pod extract gel and metronidazole gel have equal ability to increase the number of fibroblasts.

Based on the results of calculating collagen density, graphs of the average fibroblasts for each group were obtained on the day 7 and 14.

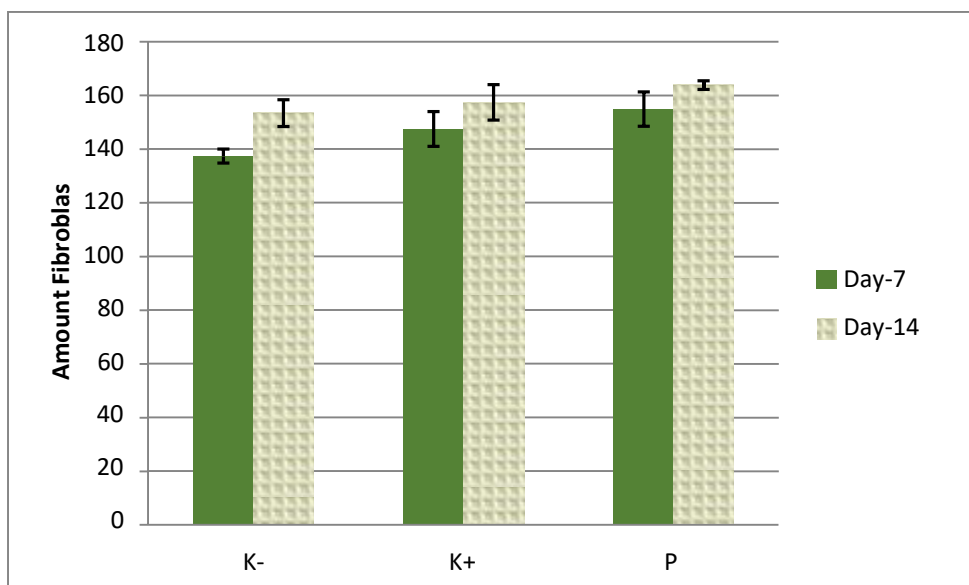


Figure 3. Histogram graph of the average amount of collagen in the negative control, positive control and treatment groups on 7 and 14 day.

Based on Figure 3 the treatment group showed the highest average collagen density when compared to the other groups, both on day 7 (154.9) and day 14 (163.83). In addition, it showed that the highest collagen density was in the cocoa pod extract gel group and the lowest collagen density was in the negative control group. The cocoa pod extract gel group and the positive control group had higher collagen density than the negative control group, which meant that there was an increase in collagen density.

Table 3. One-way ANOVA test results for rat gingival collagen density

	Sum Of Squares	Df	Mean Square	F	Sig.
Between Groups	36068.517	5	7213.703	44.189	.000
Within Groups	2938.423	18	163.246		
Total	39006.940	23			

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The results of the one-way ANOVA test in Table 4 show that the significance value is <0.05 , which is 0.000, which means that there is a significant difference, at least one pair of groups is significantly different. Then the next test, namely Least Significant Difference (LSD) to find out the differences between the two different groups, is presented in Table 4 below.

Table 4. One-way ANOVA test results for rat gingival collagen density

	NC7	PC7	CPH7	NC14	PC14	CPH14
NC7	-	0.006*	0.006*	1.000	0.787	0.250
PC7		-	0.000*	0.009*	0.086	0.433
CPH7			-	0.000*	0.000*	0.000*
NC14				-	0.869	0.325
PC14					-	0.913
CPH14						-

Based on the results of the LSD test, a significant value ($p < 0.05$) showed that there was a significant difference between the NC7 group and the PC7 and CPH7 groups; between groups NC7 against CPH7 and KNC14; between the CPH7 group and the NC14 group, the PC14 group, the CPH14 group. Whereas in the NC7 group against NC14, PC14 and CPH14; between the PC7 group and the PC14 group, the CPH14 group; between the NC14 group and the PC14 group, the CPH14 group; between the PC14 group and the CPH14 group there was no significant difference with a significance value ($p > 0.05$). Groups that were not significantly different meant that they had the same number of fibroblasts. This shows that cocoa pod extract gel and metronidazole gel have equal ability to increase the number of fibroblasts.

IV. DISCUSSION

The results showed that the ethanol extract gel of cocoa pod husk increased the number of fibroblasts in the gingiva of gingivitis rats after *P. gingivalis* induction. This was shown by the average number of fibroblasts in the gingiva of gingivitis rats given cocoa pod ethanol extract gel which was higher than that of gingivitis rats given metronidazole gel and placebo gel. The mean number of fibroblasts in the positive group showed higher results than the negative control group by administering CMC-Na gel. This shows that administration of metronidazole gel can increase the number of fibroblasts, metronidazole gel eliminates *P. gingivalis* by interfering with DNA synthesis and bacterial cell nuclei to prevent colonization or further infection. Thus, inflammation can occur in a short time and be followed by tissue regeneration, one of which is the process of fibroblast proliferation (Atiqah *et al.*, 2021; Cialdai *et al.*, 2022).

The gingivitis rats that were given cocoa pod extract gel had a significantly different number of fibroblasts ($p < 0.05$) from the gingivitis rats that were given placebo gel. This shows that giving cocoa pod extract gel can increase the number of fibroblasts. Cocoa pod skin contains flavonoids, saponins, tannins, alkaloids which can help improve the quality of connective tissue and epithelium in the healing process. In the healing process, compounds are needed that can form collagen by triggering proliferation so that there is an increase in the number of fibroblasts. The content of flavonoids, saponins, tannins, alkaloids has an effect that can play a role in the increase in fibroblasts in the tissue. The anti-inflammatory mechanism of flavonoids is by irreversibly inhibiting the action of *cyclooxygenase* (COX) and lipoxygenase enzymes which results in reduced synthesis of inflammatory mediators such as prostaglandins, especially PGE2, prostacyclin, thromboxane, and leukotrienes which causes the time of the inflammatory process to accelerate which is characterized by increased fibroblast proliferation (Luthfi *et al.*, 2020).

The saponin content of cocoa pods acts as an anti-inflammatory and increases the number of fibroblasts thereby triggering *vascular endothelial growth factor* (VEGF) and increasing the number of macrophages that migrate to the injured area, thereby increasing cytokine production and activating fibroblasts. Saponins inhibit bacterial growth by entering the cell membrane by diffusion. The tannin content in cocoa pod skin has potential as an antioxidant and antibacterial which is useful for stopping bleeding, accelerating inflammation and healing of mucous membranes, and regeneration. new network. These fibroblasts then play a role in the synthesis of collagen which is the main element of the extracellular matrix which is useful for forming strength in scar tissue in wounds (Diller & Tabor, 2022; Rahayu *et al.*, 2023).

The ability of the active substances contained in cocoa pod extract (*Theobroma cacao* L.) to increase the density of collagen is due to the anti-inflammatory effect of flavonoids which consist of catechins, anthocyanins and tannins. *Catechins* and *anthocyanins* in high concentrations act as anti-inflammatory agents by inhibiting the release of arachidonic acid and the release of lysosomal enzymes from the membrane by blocking the cyclooxygenase pathway. The tannins contained in cocoa pod extract

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have the ability as an antibacterial agent, thereby inhibiting bacterial growth and playing a role in fibroblast migration and proliferation. Saponins can increase the permeability of bacterial cell membranes when interacting with bacterial cells resulting in hemolysis of bacterial cells (Schilrreff & Alexiev, 2022).

The increase in average density in the treatment group was also due to the ability of flavonoids, especially quercetin contained in cocoa pod extract (*Theobroma cacao* L.) which could stimulate the induction of *transforming growth factor* (TGF- β). TGF- β has a role in stimulating collagenization and TGF- β has three isoforms namely TGF- β 1, TGF- β 2, TGF- β 3. TGF- β is the main component. TGF- β . This function increases the migration and proliferation of fibroblasts in the area of inflammation. An increase in growth factor secretion can accelerate fibroblast migration and proliferation, furthermore fibroblasts play a very important role in the formation of collagen (Abnaof *et al*, 2014).

V. CONCLUSION

The conclusion obtained from this study was that the ethanol extract gel of cocoa pod (*Theobroma cacao* L.) was effective in increasing the number of fibroblasts and collagen fibers in the gingiva of rats after *P. gingivalis* induction.

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