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Mini Review

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Periodontal disease,
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Reactive oxygen species

The bactericidal and antioxidant effects of cacao bean extracts against periodontal pathogen

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Abstract

Cacao beans extract is a polyphenol containing the favorite food such as chocolates. In this mini review, we focused on the bactericidal and antioxidant effects of cacao bean extracts against *Porphyromonas gingivalis*. Cacao bean extracts in this study were used cacao bean polyphenol-rich extract (CBP) and HP cacao extract (PW-VE-R). To determine the bactericidal effects of cacao bean extracts, assessed the improvement effects on periodontal disease using two types of cacao bean extracts against *P. gingivalis*. The bactericidal effect of CBP was stronger than that of PW-VE-R against *P. gingivalis* cells. These extracts were decreased *P. gingivalis* biofilm in a dose-dependent manner. In addition, the scavenging activities against the reactive oxygen species such as superoxide and hydroxyl radical were indicated with cacao bean extract. Electron Spin Resonance analysis revealed that the two types of cacao bean extracts have scavenged the reactive oxygen species such as hydroxyl radicals and superoxide. These results suggested that cacao bean extracts might improvement effects on periodontal tissue destruction.

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Introduction

Periodontal disease is a biofilm-related inflammatory disease in the oral cavity. Oral biofilm is a bacterial community covered a thick matrix composed mainly exopolysaccharides (EPS)[1)]. The EPS matrix can inhibit invade the antibiotics and cause resistant bacterial cells. Therefore, the mainly periodontal treatment is mechanical debridement, such as scaling, root planning, and periodontal surgery. Recently, polyphenols are a useful ingredient for the prevention of several systematic diseases. Cacao bean extract (CBE), a flavonoid, is the seeds of a native Central and South American tree known as *Theobroma cacao*. Cacao bean extract is an effective for arteriosclerosis prevention, CBE reduce cholesterol level and blood pressure[2-4)]. CBE has an antioxidant such as active oxygen and free radical generated in the body in human aging and life-style diseases[5)]. Periodontal disease increases gingival oxidative stress by pro-inflammatory cytokines and nitric stress[6)]. Periodontal disease is also associated with systemic diseases such as cardiovascular disease, diabetes, premature birth, low birth weight infants and

Table 1 Bactericidal effect of the cacao bean extracts against *P. gingivalis*

	concentration (mg/ml)				
	0	0.01	0.1	1	10
BHI	5.3×10^7	−	−	−	−
50% ethanol	2.2×10^7	−	−	−	−
CBP	−	2.63×10^7	2.71×10^6	1.46×10^6	3.3×10^5
PW-VE-R	−	3.03×10^7	2.33×10^7	7.67×10^6	2.07×10^6

aspiration pneumonia[7]. Then, periodontal disease is one of the systemic risk factors. The purpose of this study was to investigate the effects of cacao bean extract against a periodontal pathogen, such as *Porphyromonas gingivalis*. In addition, we determined the scavenging activity of reactive oxygen species with cacao bean extracts (CBE).

1. Bactericidal effect of cacao bean extracts against *P. gingivalis*.

Porphyromonas gingivalis is a Gram-negative, black-pigmented anaerobic rod that is an important periodontal pathogen. *P. gingivalis* colonize the subgingival region, and induce chronic inflammation by the several pathogenic factors such as collagenase, and lipopolysaccharide (LPS)[8,9]. We investigated the bactericidal effect of cacao bean extracts against *P. gingivalis*. Cacao bean extracts in this study were used cacao bean polyphenol-rich extract and HP cacao extract. The cacao bean polyphenol-rich extract (CBP) and HP cacao extract (PW-VE-R) were provided by Meiji Co., Ltd. (Tokyo, Japan). The concentrations of these extracts were adjusted 0.01, 0.1, 1.0, 10 mg/ml by fifty percent concentration of alcohol. *P. gingivalis* ATCC 33277 was grown in brain heart infusion (BHI) broth supplemented with yeast extract (5.0 mg/ml), hemin (5.0 μg/ml), and vitamin K_1 (0.2 μg/ml) at 37°C for 18 hours under anaerobic condition (85% N_2, 10% H_2, 5% CO_2). Bacterial cells were washed and suspended by sterilized phosphate buffered saline (PBS) to an optical density of 1.0 at 550 nm. Ten μL of bacterial suspension (5.3×10^7 colony-forming units (CFU)/ml) was exposed for 60 minutes to 0.01, 0.1, 1.0, 10 mg/ml CBP and PW-VE-R. The same volume of PBS and fifty percent concentration of alcohol were used as controls. After the treatment of CBP and PW-VE-R, *P. gingivalis* cells were carried out by a 10-fold serial dilution and 100 μl of each dilution was spread onto a BHI sheep blood agar plate. The bactericidal effects of cacao bean extracts were determined using the number of CFU after 5 days incubation in an anaerobic condition. Table 1 shows the bactericidal effect of cacao bean extracts against *P. gingivalis*. The effect of CBP and PW-VE-R are more effective than that of controls. The treatment of *P. gingivalis* with 0.01 to 10 mg/ml CBP and PW-VE-R reduced the number of viable cells in a dose-dependent manner. After 0.01, 0.1, 1.0, 10 mg/ml CBP treatment for 60 minutes, the number of *P. gingivalis* deceased by 50.4%, 94.9%, 97.2%, and 99.3% respectively. Otherwise, after 0.01, 0.1, 1.0, 10 mg/ml PW-VE-R treatment for 60 minutes, the number of *P. gingivalis* deceased by 42.8%, 56.6%, 85.5%, and 96.1% respectively. This result showed that the bactericidal effect of CBP was stronger than that of PW-VE-R against *P. gingivalis* cells. A previous study has been reported that CBE inhibited the collagenase activity of *P. gingivalis*. CBE also suppressed nitric oxide and proinflammatory cytokines produced by LPS-stimulated macrophages[10, 11]. CBE may reduce the planktonic bacteria in the oral cavity, and suppress the periodontal pathogenic factors.

2. Influence of cacao bean extracts on *P. gingivalis* biofilm.

We investigated the influence of CBE on *P. gingivalis* biofilm. *P. gingivalis* were grown on 24-well polystyrene plates with the sterilized coverslip (diameter, 12.0 mm; thickness, 0.15 mm) at 37°C for 16.5 hours anaerobically. After non-adherence cells were removed by PBS, fresh BHI broth was transferred into the wells and incubated for a further 24 hours. Bacterial biofilms were treated with 0.01 to 10 mg/ml CBP and PW-VE-R for 1 hour anaerobically. After the incubation, *P. gingivalis* biofilms were stained with SYTO9 and propidium iodide (live/dead staining). The stained biofilm was observed under a fluorescence microscope. Figure 1 shows the fluorescent images of *P. gingivalis* biofilm treated by cacao bean extracts. The numbers of live bacteria was decreased, and the numbers of dead bacteria was increased with the treatment of CBP and PW-VE-R. These extracts were decreased *P. gingivalis* biofilm in a dose-dependent manner. The result suggested that CBE inhibited *P. gingivalis* biofilm

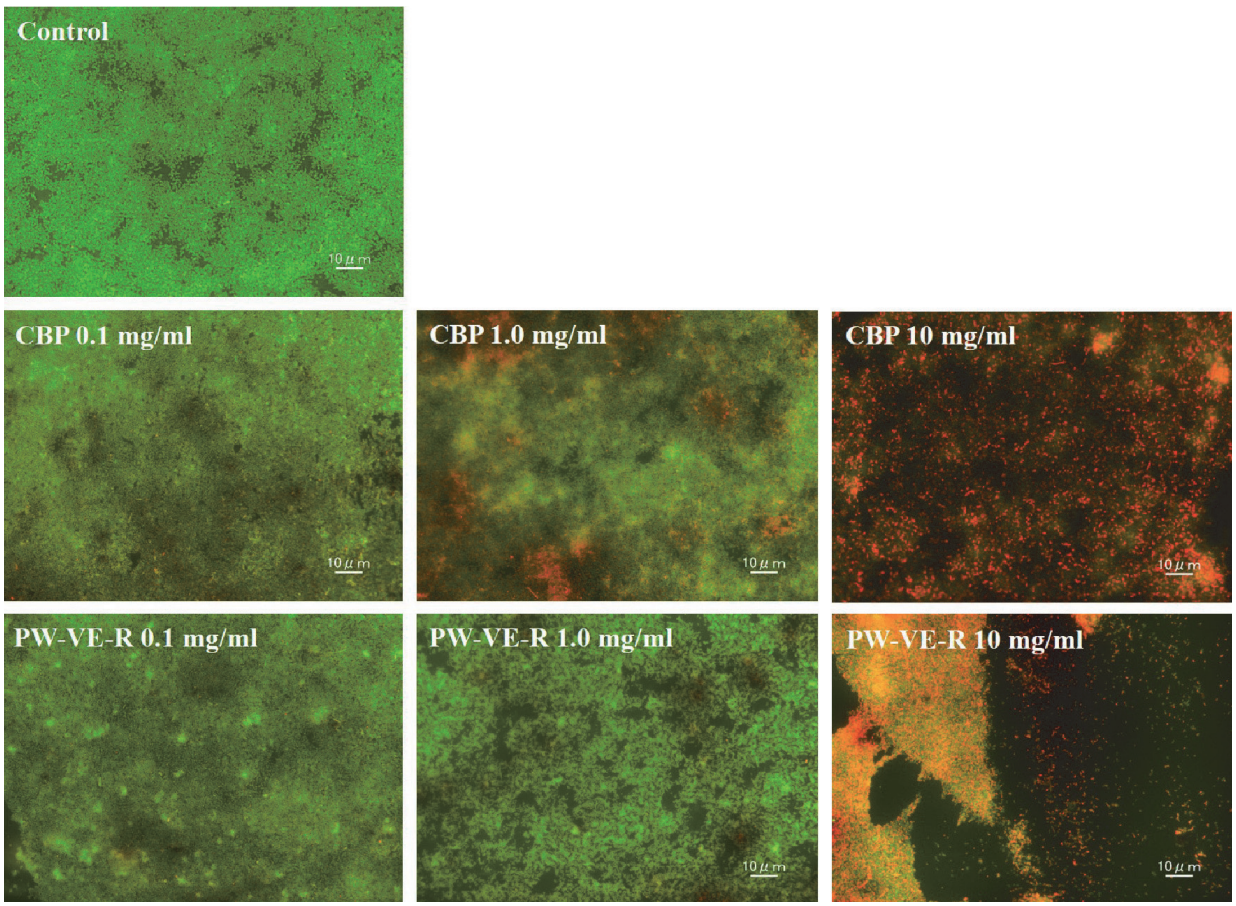

Figure 1. The fluorescent images of *P. gingivalis* biofilm treated by cacao bean extracts. Green staining (viable cells) is due to SYTO9, and red staining (dead cells) is due to propidium iodide in the LIVE/DEAD technique. Scales, 10 μm.

formation.

3. The scavenging activity of reactive oxygen species with cacao bean extracts

Reactive oxygen species (ROS), so-called oxygen radical free radicals, cause deoxyribose damage in the human cells[12]. ROS production is drastically increased from neutrophils and macrophages during the inflammatory diseases[13]. We examined the scavenging activity of ROS (hydroxyl radicals and superoxide) with CBE using Electron Spin Resonance (ESR) spectroscopy. ESR analysis was performed by the previous studies described[14, 15]. ESR analysis revealed that the two types of cacao bean extracts have scavenged the reactive oxygen species such as hydroxyl radicals and superoxide. The hydroxyl radical removal rate stimulated by 0.1 mg, 1.0 mg and 10 mg of CBP were 0%, 55% and 92% respectively. The hydroxyl radical removal rate stimulated by 0.1 mg, 1.0 mg and 10 mg of PW-VE-R were 0% 20% and 89%, respectively. On the other hand, the superoxide removal rate stimulated by 1.0 mg and 10 mg of CBP was 88% and 93%, 1.0 mg and 10 mg of PW-VE-R was 85% and 93%, respectively. ROS is a key factor in the survival of *P. gingivalis* in the inflammatory environment[16]. *P. gingivalis* can change host cellular ROS production to increase its survival[17]. A previous study has been reported that CBE have a great antioxidant activity than other polyphenols[18]. In oral cavity, CBE will contribute the reduction of ROS in inflammatory tissues and maintain the healthy environments.

Discussion

CBE has been used in foods as natural antioxidants and antimicrobials. The free fatty acids contained in cacao have the effect of suppressing the growth of *Escherichia coli* which is the cause of food poisoning. And it also has adhesion inhibition, bactericidal and growth inhibitory effect to a stomach epithelium cell of *Helicobacter pylori*[19, 20]. A previous study has been reported that CBE reduced 20.9% against *Strepto-*

coccus mutans which were used as mouse rinse[21]. CBE can also reduce the dental plaque accumulation in the oral cavity. Inflammation and alveolar bone destruction are characterized in periodontal disease. The future study will be focused on the effects of CBE on alveolar bone resorption by using animal models. CBE may contribute as a therapeutic and preventative ingredient for periodontal disease.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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References

1. Rouabhia M, Chmielewski W. (2012). Diseases associated with oral polymicrobial biofilms. *Open Mycol J*. **6**, 27-32.
2. Osawa K, Miyazaki K, Shimura S, *et al*. (2001). Identification of cariostatic substances in the cacao bean husk: their anti-glucosyltransferase and antibacterial activities. *J Dent Res*. **80**: 2000-2004.
3. Oosawa K, Matsumoto T, Maruyama T, *et al*. (1990). Inhibitony effects of aqueous extract of cacao bean husk of collagenase of *Bacteroides gingivalis*. *Bull Tokyo Dent Coll*. **31**: 125-128.
4. Yasuda A, Natsume M, Sasaki K, *et al*. (2008). Cacao procyanidins reduce plasma cholesterol and increase fecal steroid excretion in rats fed a high-cholesterol diet. *Biofactors*. **33**: 211-223.
5. Toda T. (2011). Oxidative modification and aging of proteins. *Basic aging research*. **35**: 17-22.
6. Chapple IL. (1997). Reactive oxygen species and antioxidants in inflammatory diseases. *J Clin Periodontol*. **24**: 287-296.
7. Cullinan MP, Seymour GJ. (2013). Periodontal disease and systemic illness: will the evidence ever be enough? *Periodontol 2000.* **62**: 271-286.
8. Lamont RJ, Jenkinson HF. (1998). Life below the gum line: pathogenic mechanisms of *Porphyromonas gingivalis*. Microbiology and molecular biology reviews: *MMBR*. **62**: 1244-1263.
9. Lamont RJ, Jenkinson HF. (2000). Subgingival colonization by *Porphyromonas gingivalis*. *Oral Microbiol Immunol*. **15**: 341-349.
10. Ono K, Takahashi T, Kamei M, *et al*. (2003). Effects of an aqueous extract of cocoa on nitric oxide production of macrophages activated by lipopolysaccharide and interferon-gamma. *Nutrition*. **19**: 681-685.
11. Ranneh Y, Ali F, Al-Qubaisi M, *et al*. (2016). The inhibitory activity of cocoa phenolic extract against pro-inflammatory mediators secretion induced by lipopolysaccharide in RAW 264.7 cells. *Springerplus*. **5**: 547.
12. Halliwell B. (2000). Why and how should we measure oxidative DNA damage in nutritional studies? How far have we come? *Am J Clin Nutr*. **72**:1082-1087.
13. Mittal M, Siddiqui MR, Tran K, *et al*. (2014). Reactive oxygen species in inflammation and tissue injury. *Antioxid Redox Signal*. **20**: 1126-1167.
14. Yoshida A, Iwata S, Iizuka J, *et al*. (2014). Blue light from dental resin curing unit causes light-induced vasoconstriction in isolated rat aorta. *OHDM*. **13**: 1147-1151.
15. Yoshida A, Sasaki H, Toyama T, *et al*. (2017). Antimicrobial effect of blue light using *Porphyromonas gingivalis* pigment. *Sci Rep*. **7**: 5225.
16. Henry LG, McKenzie RM, Robles A, *et al*. (2012). Oxidative stress resistance in *Porphyromonas gingivalis*. *Future Microbiol*. **7**: 497-512.
17. Spooner R, Yilmaz O. (2011). The role of reactive-oxygen-species in microbial persistence and inflammation. *Int J Mol Sci*. 1**2**: 334-352.
18. Crozier SJ, Preston AG, Hurst JW, *et al*. (2011). Cacao seeds are a "Super Fruit": A comparative analysis of various fruit powders and products. *Chem Cent J*. **5**: 1-6.
19. Takahashi T, Taguchi H, Yamaguchi H, *et al*. (1999). Antibacterial effects of cacao mass on enterohemorrhagic *Escherichia coli* O157:H7. *Kansenshogaku Zasshi.* **73**: 694-701.
20. Sato S, Taguchi H, Yamaguchi H, *et al*. (1999). The effect of cocoa extract on the growth, adhesion and colonization of *Helicobacter pylori*. *Progr. Med*. **19**: 1208-1213.
21. Srikanth RK, Shashikiran ND, Subba Reddy VV. (2008). Chocolate mouth rinse: Effect on plaque accumulation and mutans streptococci counts when used by children. *J Indian Soc Pedod Prev Dent*. **26**: 67-70.