Antibacterial, anti-inflammatory, and anti-osteoclastogenic activities of Colocasia antiquorum var. esculenta: Potential applications in preventing and treating periodontal diseases

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This study aimed to investigate the inhibitory effects of Colocasia antiquorum var. esculenta (CA) on Porphyromonas gingivalis (P. gingivalis) growth, inflammation, and osteoclastogenesis. CA was effective in inhibiting the growth of P. gingivalis when applied together with an experimental fluoride varnish. CA also significantly decreased the release of interleukin-6, tumor necrosis factor-α, and nitric oxide from lipopolysaccharide-induced RAW 264.7 cells. No significant differences in viability were noted between the cells treated with CA and the controls. In addition, CA significantly attenuated osteoclast differentiation on bone marrow macrophages. In conclusion, CA inhibited the growth of P. gingivalis and showed anti-inflammatory and anti-osteoclastogenic effects. Therefore, CA may have the potential to act as a novel natural agent for preventing periodontitis.

Keywords: Porphyromonas gingivalis, Colocasia antiquorum var. esculenta, Antibacterial activity, Anti-inflammatory activity, Anti-osteoclastogenic activity

INTRODUCTION

The term “periodontitis” has its etymological origin from the Greek words “peri,” which means around, “odous,” which means tooth, and the suffix “-itis,” which means inflammation in medical terminology; it is biologically defined as an inflammation of the soft tissues caused by bacterial infection in the mouth1-5. Periodontal bacteria are naturally present in the mouth, but their build-up in the form of gingivitis (“gingivae,” which means gums) and progress to periodontitis with bad breath, bleeding, pain, and destruction of the periodontal ligament and alveolar bone ultimately leads to the loosening and potential loss of teeth6.

Of the more than 700 different species of oral bacteria, Porphyromonas gingivalis (P. gingivalis) is one of the predominant gram-negative anaerobic organisms mainly associated with periodontal diseases4,6. When plaque develops on the surface of teeth, a biofilm is formed in the gingival crevice and many anaerobic bacteria, including P. gingivalis, rapidly increase in numbers and lead to inflammation induced by lipopolysaccharides (LPSs), which are constituents of bacterial cell walls5,7. The LPSs of P. gingivalis and Aggregatibacter actinomycetemcomitans are considered key factors in the development of chronic periodontitis8. In the case of P. gingivalis, the LPS stimulates the osteoclastic cells by mediating inflammatory cytokines and inhibits osteoblastic cell differentiation via a host-mediated inflammatory reaction9. LPS stimulates toll-like receptors (TLRs) of macrophages and monocytes to promote the secretion of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-69. Nitric oxide (NO) is an endogenous free radical synthesized from L-arginine by nitric oxide synthase (NOS) in various animal cells and tissues10. Small amounts of NO are important regulators of physical homeostasis, but large amounts of NO have been closely correlated with the pathophysiology of various diseases and inflammation11. Thus, the LPS of P. gingivalis can cause alveolar bone destruction through these inflammatory components, and this process leads to periodontitis12,13.

Recently, we found that a methanol extract of Colocasia antiquorum var. esculenta (CA) inhibited the growth of P. gingivalis. CA is a perennial herb belonging to the family Araceae, and its leaves, stems, and corm in the ground are generally edible. CA has antifungal and antimelanogenic activities, as well as an inhibitory effect on human lanosterol synthase14-16. However, the effect of CA on the prevention, treatment, or improvement of periodontal disease remains unknown. Therefore, we aimed to determine whether CA could suppress inflammatory responses by inhibiting the production of inflammatory cytokines, TNF-α and IL-6, and the production of NO in LPS-induced cells.
and osteoclastogenesis by using tartrate-resistant acid phosphatase (TRAP) staining and a TRAP activity assay. In addition, to investigate the direct effect of CA on the growth of *P. gingivalis*, we evaluated the antibacterial activity of CA against *P. gingivalis*.

**MATERIALS AND METHODS**

**Antibacterial agent**

The plant extract used in this research was obtained from the Korea Plant Extract Bank at the Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea). The plant was collected from Namwon-si, Jeollabuk-do, Korea, in 2007. The plant (120 g) dried in the shade and powdered was added to 1 L of methyl alcohol 99.9% (high-performance liquid chromatography grade) and extracted through 30 cycles (40 kHz, 1,500 W, 15-min ultrasonication, and 120-min standing per cycle) at room temperature by using an ultrasonic extractor (SDN-900H, SD-ULTRASONIC, Seoul, Korea). After filtration and drying under reduced pressure, the methanol extract (5.32 g) was obtained. A stock solution (20 mg/mL) of the extract was prepared in dimethyl sulfoxide (DMSO), and stored at −20°C until use.

**Antibacterial assay**

The antibacterial effects of CA extracts were assessed on *P. gingivalis* (ATCC33277). In all experiments using *P. gingivalis*, the bacteria were cultured under identical conditions as follows: *P. gingivalis* was cultured in the brain heart infusion medium containing 1% hemin, 1% menadione, and 5% sheep blood at 37°C in an anaerobic chamber for 72 h. Thereafter, 90 μL of the cultured *P. gingivalis* at 1.5×10^8 colony-forming unit (CFU)/mL was placed in a 96-well plate and 10 μL of each concentration of CA extract (final concentration of 0, 125, 250, and 500 μg/mL) was added. The concentrations were decided according to the screening concentrations of antibacterial agents (125, 250, and 500 μg/mL) added. The concentrations were decided according to the screening concentrations of antibacterial agents (125, 250, and 500 μg/mL) at Wonkwang University. After 72 h, the absorbance was measured at 600 nm using an enzyme-linked immunosorbent assay (ELISA) reader (Spectra MAX250, Molecular Devices, CA, USA).

**Preparation of the experimental fluoride varnish (FV)**

To serve as a vehicle for CA in the antibacterial activity sustainability test, a FV was prepared as follows: 45 wt% rosin (KR-610, Arakawa Chemical Industries, Osaka, Japan), 50 wt% solvent (ethanol, absolute 99.7% purity, Merck, NJ, USA), and 5 wt% sodium fluoride were mixed using an overhead stirrer (RW20DZM.n, IKA Korea, Seoul, Korea) at 240 rpm for 30 min in a double boiler at 80–100°C on a hot plate (RCH-3, Tokyo Rikakikai, Tokyo, Japan).

**Antibacterial activity sustainability of CA extracts**

To test the antibacterial activity sustainability of CA extracts against *P. gingivalis*, the following samples were prepared: control, FV, and FV+CA (FV [50 μL]+50 mg/mL CA [50 μL]). The control was a polyethylene terephthalate film disc with a diameter of 5 mm. In the FV and FV+CA groups, 5 μL of each material was evenly coated on the surface of the film discs sterilized with ethylene oxide gas and dried on a clean bench under ultraviolet light for 30 min. Four film discs were assigned per group and were placed in Petri dishes (35-mm diameter); then, 2-mL distilled water was added into the Petri dishes. After storing the film discs in a shaking water bath (JSSI-100C, JSR, Gungu, Korea) at 80 rpm and 37°C for 72 h, the discs were removed from the Petri dishes. Thereafter, antibacterial activity sustainability was evaluated using the agar diffusion test. The side of the film disc on which the material was applied was located to face the agar plate inoculated with 1.5×10^7 CFU/mL of *P. gingivalis* suspension. After incubating for 72 h in an anaerobic condition at 37°C, the diameters of the inhibition zones were measured at right angles, and the average diameter was determined as the inhibition zone.

**Anti-inflammatory activity assays in LPS-induced RAW 264.7 cells**

As described previously, all materials for cell culture were purchased from HyClone (UT, USA). The murine macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection (MD, USA) and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 μg/mL streptomycin in a humidified atmosphere of 5% CO_2 at 37°C.

RAW 264.7 cells were grown in a 60-mm dish, at a density of 1×10^6 cells, treated with CA (final concentration of 0, 3, 10, and 30 μg/mL). The concentrations of CA for anti-inflammatory and anti-osteoclastogenic effect were in line with the Korean Chemical Research Institute’s Standard Operating Procedures. After 2 h, the cells were treated with 1 μg/mL LPS (L4130, Sigma-Aldrich, MO, USA) for 1 day. Thereafter, the supernatants were collected and used for quantifying the levels of IL-6 and TNF-α by using ELISA kits (R&D Systems, MN, USA) according to the manufacturer’s instructions. Additionally, NO levels were determined using the Griess reaction. Briefly, 100 μL of the cell culture medium was mixed with 100 μL of Griess reagent (1% w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) N-(1-naphthyl)ethylenediamine dihydrochloride, and then incubated at room temperature for 10 min. Absorbance was then measured at 540 nm by using a microplate reader (VERSA max, Molecular Devices). Fresh culture media were used as blanks in all experiments. Nitrite levels in the samples were determined by comparisons against the standard sodium nitrite curve.

**Cell viability assay**

RAW 264.7 cells were seeded in a 96-well plate at a density of 4×10^3 cells/well. After 24 h, the cells were incubated with CA in the presence of LPS for 1 day. Cell viability was then measured in triplicate by using a Cell Counting Kit-8 (CCK-8, Dojindo Molecular
Receptor activator of nuclear factor kappa-B ligand (RANKL)-induced osteoclastogenesis in bone marrow-derived macrophages (BMMs)

Osteoclasts were differentiated from BMMs as described previously\(^{21,22}\). Isolation of bone marrow cells (BMCs) from 5-week-old male ICR mice (Damool Science, Daejeon, Korea) was carried out in strict accordance with the recommendations in the Standard Protocol for Animal Study of Korea Research Institute of Chemical Technology (KRICT). The protocol (ID No. 7D-M1) was approved by the Institutional Animal Care and Use Committee of KRICT (IACUC-KRICT). Mice were euthanized by cervical dislocation, and the BMCs were obtained by flushing the isolated femurs and tibias with minimum essential medium Eagle-alpha modification (α-MEM) supplemented with antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin). BMCs were cultured for 1 day on a culture dish in α-MEM supplemented with 10% FBS and 10 ng/mL of macrophage colony-stimulating factor (M-CSF). Non-adherent BMCs were plated on a Petri dish and cultured in humidified 5% CO\(_2\) at 37°C for 3 days in the presence of M-CSF (30 ng/mL). After non-adherent cells were washed out, the adherent cells were used as BMMs. When BMMs were cultured with M-CSF (30 ng/mL) and RANKL (10 ng/mL) for 3 days, most of the cells differentiated into TRAP\(^+\)-mononuclear osteoclasts, and TRAP\(^+\)-multinucleated cells (MNCs) generated by the fusion between the mononuclear cells were observed on differentiation day 4\(^{23}\). Therefore, for the complete formation of TRAP\(^+\)-MNCs, BMMs (1×10\(^4\) cells/well in a 96-well plate or 3×10\(^5\) cells/well in a 6-well plate) were cultured with M-CSF, RANKL, and 0, 3, 10, and 30 μg/mL CA for 4 days.

For TRAP staining and activity assay, mature osteoclasts were visualized by staining for TRAP, a biomarker of osteoclast differentiation, as described previously\(^{20}\). Briefly, BMM-derived MNCs were fixed with 3.7% formaldehyde for 5 min, permeabilized with 0.1% Triton X-100 for 5 min, and stained with the Leukocyte Acid Phosphatase Kit 387-A (Sigma-Aldrich). To measure TRAP activity, the permeabilized cells were incubated with the TRAP buffer (100 mM sodium citrate [pH 5.0] and 50 mM sodium tartrate) including 3 mM p-nitrophenyl phosphate (Sigma-Aldrich) at 37°C for 5 min, as described previously\(^{20}\). The reaction mixtures were transferred onto a new plate containing an equal volume of 0.1 N sodium hydroxide, and optical density values were determined at 405 nm by using a Wallac EnVision microplate reader (PerkinElmer, Finland).

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics for Windows, Version 24.0 (IBM, Armonk, NY, USA). Data were analyzed using one-way analysis of variance (ANOVA), with Duncan’s multiple range test (α=0.05) as the post hoc test.

RESULTS

Antibacterial effects of CA and their sustainability

Our study confirmed that CA treatment significantly inhibited the growth of \textit{P. gingivalis} (p<0.05) (Fig. 1A). Specifically, 125 μg/mL and 250 μg/mL CA inhibited the growth of \textit{P. gingivalis} by approximately 20%, while 500 μg/mL CA inhibited it by approximately 40%. The
Fig. 2 Effects of CA on TNF-α (A), IL-6 (B), and NO (C) production in LPS-induced RAW 264.7 cells. The cells were pretreated with different concentrations of CA for 2 h and then exposed to 1 μg/mL LPS for 24 h. The levels of TNF-α, IL-6, and NO in the supernatant were measured at 540 nm by using a microplate reader. (D) The effect of CA on the viability of RAW 264.7 cells was determined using the CCK-8 assay. The (% of control) in the title of the Y-axis of A, B, and C indicates that the data were converted based on the control of RAW 264.7 cells without LPS induction (0 group) as 100%. The (% of control) of D means 0 μg/mL CA group. Different lowercase letters indicate significant differences between groups according to the ANOVA and Duncan’s multiple range test at α=0.05.

Fig. 3 CA inhibits RANKL-induced osteoclastogenesis. BMMs were cultured for 4 days in the presence of M-CSF (30 ng/mL) and RANKL (10 ng/mL) with DMSO (control) that was used to dilute CA. (A) TRAP activity was measured to evaluate the osteoclastogenic activity. Different lowercase letters indicate significant differences between groups according to the ANOVA and Duncan’s multiple range test at α=0.05. (B) TRAP staining was performed to visualize osteoclast differentiation. Stained cells were photographed under a light microscope.
inhibitory effect of 500 μg/mL CA was significantly higher than that of 125 μg/mL and 250 μg/mL CA (p<0.05).

For the sustainability of antibacterial activity, the FV+CA group showed significantly higher antibacterial activity than did the FV group (p<0.05) (Fig. 1B).

Anti-inflammatory activity of CA
The production of the pro-inflammatory cytokines IL-6 and TNF-α as well as that of NO was significantly increased by LPS stimulation of RAW 264.7 cells (p<0.05). In Fig. 2A, CA showed significantly decreased dose-dependent production of TNF-α (p<0.05). IL-6 secretion was significantly inhibited by 3 and 10 μg/mL CA, and was further decreased by 30 μg/mL CA (p<0.05) (Fig. 2B). CA also had a significant inhibitory effect on NO production at 30 μg/mL (p<0.05) (Fig. 2C).

The CCK-8 assay conducted to evaluate the potential cytotoxicity of CA in RAW 264.7 cells revealed that CA exhibited no cytotoxicity regardless of its concentration (p>0.05) (Fig. 2D).

Anti-osteoclastogenic activity of CA
To determine the inhibitory effect of CA on RANKL-induced osteoclast formation, the TRAP activity assay and TRAP staining were carried out. As shown in Fig. 3A, CA attenuated TRAP activity more significantly at 30 μg/mL than at 3 μg/mL and 10 μg/mL (p<0.05). CA inhibited the formation of TRAP+ multinucleated osteoclasts to a greater extent as its concentration increased (Fig. 3B).

DISCUSSION
The antibacterial effect of CA on P. gingivalis and its sustainability were investigated in this study. We also aimed to determine whether CA could suppress osteoclastogenesis by mediating the production of inflammatory cytokines and NO in LPS-induced cells. Therefore, we employed antibacterial assays against P. gingivalis, and measured the production of the pro-inflammatory cytokines IL-6 and TNF-α as well as that of NO, and RANKL-induced anti-osteoclastogenic activity of CA. Our findings showed for the first time that CA has antibacterial, anti-inflammatory, anti-osteoclastogenic activities.

Specifically, CA showed more efficacious antibacterial activity against P. gingivalis at a concentration of 500 μg/mL than at concentrations of 125 or 250 μg/mL (Fig. 1A). With regard to the sustainability of antibacterial activity, CA showed a higher antibacterial effect when mixed with the experimental FV than when the FV was applied alone. FV is clinically very effective in preventing dental caries and has a slight antibacterial activity, CA was combined with the FV. FV can be an effective carrier of an antibacterial agent onto teeth. For how long the antibacterial activity of FV+CA can sustain, further study is necessary. These results highlight the excellent antibacterial effects of CA against P. gingivalis. Moreover, this indicated the possibility that a FV can be used with antibacterial agents to prevent periodontal disease as well as dental caries.

CA also decreased the production of NO and the anti-inflammatory cytokines IL-6 and TNF-α in LPS-activated RAW 264.7 cells without any cytotoxicity. LPS has the ability to induce the expression of various pro-inflammatory cytokines as well as the NO-producing enzyme iNOS in macrophages. The pro-inflammatory cytokines, such as TNF-α, IL-1, and IL-6, stimulated by LPS play critical roles in the inflammatory response that triggers osteogenesis, and their levels are significantly elevated in diseased periodontal sites. TNF-α is an anti-inflammatory cytokine synthesized by activated monocytes, macrophages, and T lymphocytes, and it promotes critical inflammatory responses during periodontal disease. TNF-α also upregulates the production of IL-1β and IL-6. High local levels of TNF-α at diseased sites have a very strong association with active bone resorption. CA could effectively decrease TNF-α expression in a dose-dependent manner. Moreover, CA was effective in inhibiting the production of IL-6 even at concentrations of 3 and 10 μg/mL, but it was more effective at 30 μg/mL and restored the level of IL-6 to that of the negative control before the LPS challenge. IL-6 regulates osteoblast and osteoclast differentiation, and its actions on bones are like that of a “double-edged sword” in that it can promote either bone formation or resorption, depending on the context. NO is another inflammatory mediator that has a paradoxical relationship with bones: although excessive production of NO may be associated with bone loss in some inflammatory conditions, NO also mediates some of the beneficial effects on bone. CA inhibited the production of NO at 30 μg/mL. Thus, the inhibitory effect of CA on TNF-α, IL-6, and NO could also account for its anti-inflammatory effects. More importantly, CA exhibited no cytotoxicity regardless of its concentration, indicating that its anti-inflammatory activity was not caused by cytotoxicity.

Through TRAP staining and activity assay, we demonstrated the RANKL-induced anti-osteoclastogenic activity of CA. Our findings showed that CA suppressed TRAP activity and the RANKL-induced formation of TRAP+ multinucleated osteoclasts. Thus, CA showed an inhibitory effect on osteoclast differentiation. TRAP is considered an established cytochemical marker for recognizing polynucleated and mononucleated osteoclasts. The study of TRAP+ cell formation and activity is a well-known method of determining osteoclast formation and function. The differentiation of osteoclasts by mediating inflammatory cytokines is directly promoted and activated by LPS itself, which can ultimately lead to bone resorption. Therefore, CA could be a potential candidate for the treatment of bone resorption owing to its inhibitory effect on osteoclasts. We have identified the potential of CA in
preventing bone loss caused by periodontal disease by confirming its anti-osteoclastogenic efficacy.

A previous study showed that CA contains various single compounds: \((-\)-)pinosinol and trans-cinnamic acid are single compounds isolated from CA that have antibacterial activity, and \(\beta\)-sitosterol and \(\alpha\)-trans-feruloyltyramine have been reported to have anti-inflammatory activities. The anti-inflammatory and anti-osteoclastogenic effects of CA demonstrated in this study may have resulted from a combination of the bioactivities of various compounds present in CA. Nevertheless, we should determine the safe concentration of CA that can show both effective antibacterial activity as well as anti-inflammatory and anti-osteoclastogenic activities including cell viability. Further study is also warranted to determine the signaling pathway wherein CA plays a role in preventing periodontitis and osteoporosis.

In conclusion, CA directly inhibited the growth of \(P.\) gingivalis and showed antibacterial activity up to 3 days. CA was also effective in inhibiting the production of pro-inflammatory cytokines (TNF-\(\alpha\) and IL-6) and NO, as well as in inhibiting TRAP activity without cytotoxicity. Thus, our findings confirmed that CA could suppress osteoclastogenesis by mediating the production of inflammatory cytokines, such as TNF-\(\alpha\) and IL-6, as well as that of NO. Within the limitations of this study, CA was proven to have the potential to improve periodontitis through a combination of antibacterial, anti-inflammatory, and anti-osteoclastogenic activities.

ACKNOWLEDGMENTS

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea Government (MSIT) (No. 2018R1A2B6002088). The CA extracts used in this study were obtained from the Korea Plant Extract Bank at the Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea). The biological resources used in this research were distributed by KCTC (http://kctc.kribb.re.kr). SHJ and SHK were supported by project grants from Korea Research Institute of Chemical Technology (KK1703-F02, KK1803-F00, and KK1932-20).

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