

Antibacterial Activity of Panduratin A Isolated from *Kaempferia pandurata* against *Porphyromonas gingivalis*

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Abstract Active antibacterial compound against periodontitis-causing bacterium *Porphyromonas gingivalis* was isolated from *Kaempferia pandurata* and identified as panduratin A. Minimum inhibitory concentration (MIC) value of panduratin A was 4 µg/mL, much lower than those of other natural antibacterial agents. Panduratin A also showed antibacterial activity against *Prevotella intermedia* (2 µg/mL), *P. loescheii* (4 µg/mL), and cariogenic *Streptococcus mutans* (4 µg/mL). Damage on cell wall and perturbation of cytoplasmic membrane of panduratin A-treated *P. gingivalis* were visualized through transmission electron microscopy. These results suggest panduratin A, exhibiting strong and preferential antiperiodontal and anticariogenic activities, may be utilized in functional foods for prevention of oral diseases.

Keywords: *Kaempferia pandurata*, panduratin A, *Porphyromonas gingivalis*, antibacterial activity

Introduction

Periodontitis, along with other dental diseases, causes dental plaque in humans. Progression of periodontitis can result in periodontal pocket formation, loosening of teeth, subsequent tooth loss, and oral malodor (1, 2). Recently, several studies have focused on the role of specific bacteria responsible for the initiation and progression of periodontal diseases as well as response to therapy (3). *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Actinobacillus actinomycetemcomitans*, periodontitis-causing bacteria, have been implicated in the advancement of severe adult periodontitis, acute necrotizing ulcerative gingivitis, and localized juvenile periodontitis, respectively (4). These microorganisms are also known to be strongly associated with oral malodor (halitosis); 80 to 90% of all the bad breath originates from oral cavity as well as bacteria generating volatile sulfur compounds (VSC). Persson *et al.* (5) reported that *P. gingivalis* generated 100-200 µmol/L methyl mercaptan and >200 µmol/L hydrogen sulfide in the human heat-inactivated serum.

Extensive efforts have been made to screen effective antibacterial agents from a variety of chemical and biological compounds for incorporation into dental products to mitigate pathogen-mediated diseases. However, such various adverse effects as teeth staining and increased calculus formation have commonly been observed with the currently used chemicals (6). These drawbacks have brought on demands for further research and development of new natural compounds that are safe to use while retaining antimicrobial specificity and efficacy (7).

Kaempferia pandurata is a perennial herb of the Zingiberaceae (ginger family) mainly cultivated in Indonesia, Thailand, and China. The fresh rhizome has been used as a food ingredient and as a folk medicine for

the treatment of colic disorder, aphrodisiac, etc. Several studies have reported various activities of *K. pandurata*, including anti-inflammatory (8), antitumor (9), antidiarrhea (10), antidysentery (11), antifatulence (12), and anti-epidermophytid effects (13). However, few have been reported to date on the antibacterial activity of compounds isolated from *K. pandurata*. This study aimed to isolate and characterize the antibacterial compound from *K. pandurata* against oral pathogens related to periodontitis.

Materials and Methods

Plant material and chemicals *K. pandurata* Roxb. (Zingiberaceae) was collected from the Biofarmaka Research Center of Bogor Agricultural University, Indonesia and was identified by Dr. K. Latifah, Department of Pharmacy. The plant material was shade-dried and ground to powder. A voucher specimen has been deposited at 4°C in the Bioproducts Research Center, Yonsei University, Seoul, Korea.

Extra pure grade solvents were purchased from Duksan Pure Chemicals (Ansan, Korea) and chemical reagents from Sigma Co. (St. Louis, MO, USA), unless otherwise stated.

Test microorganisms and cultures All media for bacterial culture were purchased from Becton Dickinson and Company (Franklin Lakes, NJ, USA). *P. gingivalis* ATCC 53978 was cultured anaerobically in PGB (*Porphyromonas gingivalis* broth) medium [BHI (brain heart infusion) 1.85%, yeast extract 0.5%, cysteine 0.05%, hemin solution 1%, menadione solution 0.1%] in an anaerobic jar with anaerogen (Oxoid Ltd., Hampshire, England). BHI was used for the aerobic cultures of *Streptococcus mutans* ATCC 25175 and *S. sobrinus* ATCC 27351, and anaerobic culture of *Actinobacillus actinomycetemcomitans* ATCC 33384. *Actinomyces viscosus* KCCM 12074 was cultured anaerobically in yeast malt extract, and LBS (*Lactobacillus selection*) broth was

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used for the anaerobic culture of *Lactobacillus acidophilus* KCCM 32820 and *L. casei* KCCM 35465. *Prevotella intermedia* KCTC 3692 and *P. loescheii* KCTC 3691 were cultured in modified chopped meat (MCM) medium (beef extract 1%, 1 N NaOH 2.5%, trypticase 3%, yeast extract 0.5%, K_2HPO_4 0.5%, resazurin solution 0.0001%, cysteine-HCl 0.05%, hemin solution 1%, menadione solution 0.002%) anaerobically.

Determination of minimum inhibitory concentration A series of test tubes, each filled with 2 mL of respective broth, were used to determine the minimum inhibitory concentration (MIC) (14). Sample (2 mL) dissolved in 1% dimethyl sulfoxide (DMSO) was added to the first tube and serially two-fold diluted to give a concentration range of 1,000 to 2 $\mu\text{g/mL}$. The test bacteria, adjusted to 2×10^5 colony forming units (CFU)/mL, were added to each tube. The control was an inoculated growth medium without sample. MIC was determined as the lowest concentration of test compound at which the bacterial growth was completely inhibited after 24 hr incubation at 37°C. Growth inhibition was determined by visually judging the turbidity of the growth media.

Extraction and fractionation Dried rhizomes (1 kg) of *K. pandurata* were ground and extracted twice with 75% methanol (4 L, v/v) for 24 hr at room temperature. The extract was concentrated at 50°C in a vacuum rotary evaporator (Heidolph VV2011, Schwabach, Germany) and lyophilized. The concentrated extract was dissolved in H_2O (1 L) and further partitioned successively with ethyl acetate, *n*-butanol, and water. Each partition was distilled in the vacuum rotary evaporator at 50°C to remove the solvent, followed by dissolving in 1% DMSO for determination of MIC. The ethyl acetate partition, showing the strongest antibacterial activity, was further separated using silica gel column chromatography. The sample was mixed with silica gel (Merck 60, 70-230 mesh, Darmstadt, Germany) and loaded onto a silica gel packed column (5 \times 43 cm) using *n*-hexane : chloroform : ethyl acetate (15:5:1.5, v/v/v) as the eluting solvent. The eluant (10 ml) was collected and divided into four fractions based on the TLC (thin layer chromatography; Merck 60 F₂₅₄) profiles. Fraction II, showing the strongest inhibitory activity against *P. gingivalis*, was further separated using *n*-hexane : ethyl acetate : methanol (18:2:1, v/v/v). The active fraction, Fraction II-B, yielded an active compound, Compound II-B-2 (0.42 g), when eluted with *n*-hexane : chloroform (3:10, v/v). Compound II-B-2 was finally purified using recycling preparative HPLC (Japan Analytical Industry Co., Ltd., Tokyo, Japan).

Instrumentation NMR spectra were recorded on a Bruker Avance-500 spectrometer (Rheinstetten, Germany) at 500 and 125 MHz for 1H and ^{13}C , respectively, in $CDCl_3$ with TMS as an internal standard. Complete proton and carbon assignments were based on 1D (1H , ^{13}C , ^{13}C -DEPT) and 2D (1H - 1H COSY, 1H - ^{13}C HMQC, 1H - ^{13}C HMBC) NMR experiments. Mass spectra (FAB-MS) were measured using JMS-700 (JEOL Ltd., Tokyo, Japan).

Morphology Transmission electron microscopy was

performed for visualization of morphological changes brought about by treatment with panduratin A (15). Bacterial suspension (5 mL) of *P. gingivalis* was centrifuged at $7,000 \times g$ for 5 min and washed three times with 0.1 M potassium phosphate buffer (PPB, pH 7.0). The cells were resuspended in 5 mL PPB. Subsequently, the mixture containing 5 mL cells and equal volume of Compound II-B-2 (4 $\mu\text{g/mL}$) was incubated for 30 min at 37°C, and centrifugation ($10,000 \times g$, 5 min) was performed to obtain the cells. The control and sample-treated cells were fixed with 2% glutaraldehyde and 1% osmium tetroxide at room temperature. After eliminating the remaining glutaraldehyde and osmium tetroxide, dehydration process was conducted using 30, 50, 70, 80, 95, and 100% ethyl alcohol. Each step was performed for 10 min at room temperature. The fixed cells were embedded with araldite, and small blocks of bacteria in the araldite were cut with an ultramicrotome (MT-X, RMC, Tucson, AZ, USA). Ultra-thin sections were cut and observed with a transmission electron microscope (JEM 1010, JEOL Ltd.).

Results and Discussion

Isolation and identification of an active antibacterial compound Because a strong activity against *P. gingivalis* was observed in the ethyl acetate fraction, further separation, monitored by the MICs of each fraction, was performed on the fraction by silica gel column chromatography and recycling preparative HPLC. Compound II-B-2 showed a molecular weight of 406 from the FAB-MS spectrum (m/z , 407, $[M+1]^+$). By comprehensively analyzing the NMR data (data not shown) and referring to the literatures (10, 16), the molecular formula of compound II-B-2 was determined as (2,6-dihydroxy-4-methoxyphenyl)-[3'-methyl-2-(3-methylbut-2''-enyl)-6'-phenylcyclohex-3'-enyl]methanone, or panduratin A (Fig. 1).

Antibacterial activity against *Porphyromonas gingivalis* Table 1 shows MICs of panduratin A against *P. gingivalis* in comparison with some commercial antibacterial agents. Panduratin A exhibited an MIC of 4 $\mu\text{g/mL}$, much lower than those of other natural agents, such as 8 $\mu\text{g/mL}$ of sanguinarine, 125 $\mu\text{g/mL}$ of thymol or green tea extract, and 1000 $\mu\text{g/mL}$ of eucalyptol. It also showed the same MIC as those of the antibiotic chlorhexidine

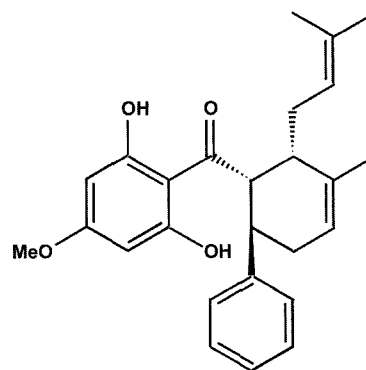


Fig. 1. Molecular structure of panduratin A.

Table 1. Comparison of MIC of panduratin A and commercial antibacterial agents against *Porphyromonas gingivalis*

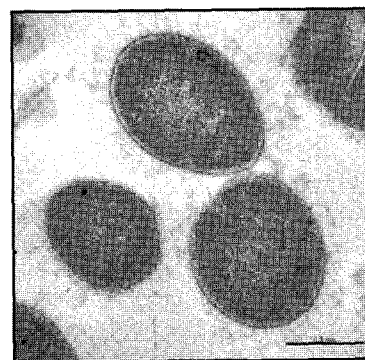
Compounds	MIC ($\mu\text{g/mL}$)
Panduratin A	4
Chlorhexidine	4
Triclosan	4
Sanguinarine	8
Green tea extract	125
Thymol	125
Eucalyptol	1000

and antibacterial chemical triclosan. Chlorhexidine, in a long-term use, has shown adverse side effects, represented by teeth staining (17). Triclosan [5-chloro-2-(2,4-dichlorophenoxy)phenol], showing a broad spectrum against oral pathogens, has been used for the last few decades as a hydrophobic antibacterial agent in a number of products such as toothpaste, detergents, and household commodities. However, triclosan-induced cell death was brought about by apoptosis in human gingival cells (18). Sanguinarine, an alkaloid isolated from the rhizome of *Sanguinaria canadensis*, has been used as a natural agent in a wide range of oral care products such as toothpaste and mouthwash due to its strong antibacterial effectiveness (19). It, however, was reported to be associated with oral leukoplakia, which resulted in a significant reduction in its industrial applications (20). Panduratin A, given that it has been isolated from a widely consumed food material in the tropical countries, may be able to overcome the side effects of these active chemicals.

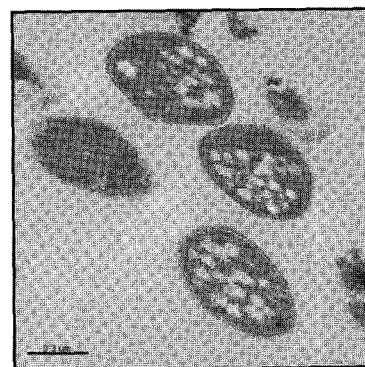
Antibacterial spectrum of panduratin A Besides *P. gingivalis*, panduratin A exhibited preferential antibacterial spectrum against other Gram-positive and -negative oral microorganisms (Table 2). Gram-positive cariogenic bacteria, i.e. *S. mutans* and *S. sobrinus*, were the most sensitive to panduratin A (MIC = 4 $\mu\text{g/mL}$). In contrast, no susceptible activity was observed for *A. viscosus*, *L. acidophilus*, and *L. casei*. Panduratin A also showed strong inhibitory activity against other Gram-negative anaerobic periodontal bacteria, *P. intermedia* (MIC = 2 $\mu\text{g/mL}$) and *P. loescheii* (MIC = 8 $\mu\text{g/mL}$), whereas slight activity against *Actinobacillus actinomycetemcomitans*. These data suggest that

Table 2. MICs of panduratin A against Gram-positive and Gram-negative oral bacteria

Oral Bacteria	Oral Bacteria	MIC ($\mu\text{g/mL}$)
	<i>Streptococcus mutans</i> ATCC 25175	4
	<i>Streptococcus sobrinus</i> ATCC 27351	4
G (+)	<i>Actinomyces viscosus</i> KCCM 12074	500
	<i>Lactobacillus acidophilus</i> KCCM 32820	500
	<i>Lactobacillus casei</i> KCCM 35465	500
	<i>Prevotella intermedia</i> KCTC 3692	2
	<i>Porphyromonas gingivalis</i> ATCC 53978	4
G (-)	<i>Prevotella loescheii</i> KCTC 3691	8
	<i>Actinobacillus actinomycetemcomitans</i> ATCC 33384	500



(a)



(b)

Fig. 2. Transmission electron microscopy of panduratin A-treated *P. gingivalis* (magnification: $\times 40,000$) (A) Control (0.004 % DMSO), (B) 4 $\mu\text{g/mL}$ panduratin A.

panduratin A could be effectively employed as a natural antibacterial agent to inhibit the growth of periodontal and cariogenic bacteria.

Morphology Morphological changes in *P. gingivalis* brought about by panduratin A treatment was observed with TEM. As compared to the intact cell [Fig. 2(a)], significant morphological changes were observed in the cells treated with 4 $\mu\text{g/mL}$ panduratin A [Fig. 2(b)]. Along with the treatment of panduratin A, the cell surface was remarkably disintegrated and the cytoplasmic membrane was detached from the cell wall. Collapse in the cytoplasm can be accompanied by the leakage of intracellular constituents with the loss of bioactive enzymes that are essential for propagations, leading to cell death (21). Further study is needed to elucidate the molecular mechanism of this activity, which may be due to a similar activity as reported with thymol on *P. gingivalis* and *Streptococcus sobrinus* (22). Thymol, as an antibacterial agent, affects the cell membrane, resulting in membrane perforation, leakage of intracellular materials, and collapse of transmembrane potentials. It has been reported to reduce the intracellular ATP level in *S. sobrinus* and inhibit ATP-generating pathways in *P. gingivalis* (23).

Conclusion A strong antibacterial compound against *P. gingivalis*, panduratin A was successfully isolated from the rhizomes of *K. pandurata*. Panduratin A showed equal or

even much stronger antibacterial activity than other commercially used natural agents, strongly suggesting that panduratin A, by inhibiting the growth of *P. gingivalis* and other periodontitic bacteria, could be employed as a new natural antimicrobial agent in oral care products for the prevention of periodontitis.

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