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생물소재를 이용한 황색포도상구균의 바이오필름 억제 연구

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Biomaterials Inhibiting Biofilm Formation of *Staphylococcus aureus*

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요 약: 바이오필름은 부유 미생물이 피부 표면에 부착되어 형성된 미생물 집락이며 형질적 · 생화학적 특성에서 부유 상태와는 차이가 있다. 바이오필름으로 증식하는 미생물은 부유 상태의 미생물 보다 숙주의 방어나 항생제에 대한 저항성이 훨씬 높기 때문에 바이오필름이 형성되면 감염 상태를 치료하기 위해 더욱 많은 항생제를 사용해야 한다. 따라서 감염된 세균의 내성을 피하면서 치료를 하기 위해서는 단순히 세균을 사멸하는 것이 아니라 표적을 달리하는 새로운 전략이 필요하다. 이번 연구에서는 아토피 등 염증성 피부 질환의 원인균의 *S. aureus*의 바이오필름을 억제하는 기작에 초점을 맞추어 연구를 진행하였다. 이 연구의 목적은 *S. aureus*의 바이오필름의 형성을 억제하여 피부질환을 조절할 수 있는 후보 물질을 찾는 데에 있다. 슬라이드글라스를 human placental 콜라겐으로 코팅하고 시험 물질과 함께 배양하여 억제된 바이오필름의 양을 crystal violet 염색법으로 측정하여 정량적으로 측정하였다. 이 실험에서는 표준균인 *S. aureus* ATCC 6538 strain이 사용되었다. 실험 결과 편백다당체가 바이오필름의 형성을 강하게 억제하였으며 녹차다당체와 황촉규근은 오히려 바이오필름의 형성을 촉진하였다. 자일리톨은 1 %의 낮은 농도에서는 바이오필름을 촉진하나 그 이상의 높은 농도인 3 %와 5 %에서는 억제하여 농도 의존적인 결과를 보였다.

Abstract: Biofilms are surface-attached microbial communities with phenotypic and biochemical properties distinct from free-living planktonic cells. Biofilm bacteria show much greater resistance than planktonic counterparts and much higher concentration of biocide is needed to treat biofilms compared to the dosage used for planktonic bacteria. As a result, alternative strategies or more effective agents exhibiting activity against biofilm-producing micro-organisms are of great interest. Therefore, we turned our attention to control of biofilm of *S. aureus*. The aims of this research are to investigate substances which inhibit the formation of biofilm by *S. aureus* and to suggest effective materials for controlling skin problems. We coated slide glasses with human placental collagen and the coverslip was incubated with test materials and bacteria. The coverslip was stained with crystal violet and we measured optical density of each sample. The biofilm inhibitory activity was calculated by crystal violet staining degrees. In this study, *S. aureus* ATCC 6538 was used as test organism. Our results show that both water soluble and insoluble *Hinoki cypress* polysaccharide strongly inhibited biofilm formation. Whereas, green tea and sunset hibiscus root extract promoted biofilm. Xylitol showed a concentration dependent effect; high concentration (3 % and 5 %) of xylitol reduced biofilm while promoted biofilm formation at a concentration of 1 %. These results support that *Hinoki cypress* polysaccharide and xylitol have ability to suppress biofilm formation.

Keywords: *Staphylococcus aureus, biofilm, biomaterials, Hinoki cypress, xylitol*

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1. Introduction

It was reported that *Staphyloccus aureus* (*S. aureus*) and its biofilm are related to human skin problems. For instance, eczema skin lesions of atopic dermatitis are usually colonized with this bacteria[1,2].

Chamaecyparis obtusa polysaccharide (Japanese cypress, *Hinoki cypressis*) is a species of cypress native to central Japan. A recent study of Jung et al. [3] showed that Hinoki cypress have antioxidative and antimicrobial activity of skin flora.

Hibiscus manihot L. (Sunset Hibiscus) known also as a hibiscus, this obscure Chinese species is a tropical perennial, half shrub that can reach 6 feet in height. Its root extract is used to manufacture Korean traditional paper (Hanji). Polysaccharides from *Hibiscus sabdariffa* flowers known as stimulating proliferation and differentiation of human keratinocytes[4].

A previous study showed that xylitol has a clear inhibitory effect on the formation of the experimental biofilms[5]. The study concludes that it is not only efficient on the acid production of cariogenic bacteria, but also on the formation of a multispecies biofilm. It confirms the relevance of the use of this polyol for the other bacterial skin infections as well as oral diseases caused by lactic acid bacteria.

Decursin, a chemical structural derivative of decursinol, is one of the major coumarins of korean traditional herbal medicine, Cham-Dang-Gui. Cham-Dang-Gui (Korean Angelica, the dried root of *A. gigas Nakai*), has been widely used in traditional Korean folk medicine not only for the treatment of anemia, but also as a sedative, an anodyne, or a tonic agent. It is also known that this herbal medicine ensures healthy pregnancies and easy deliveries, and that coumarins such as decursin and decursinol angelate are the major constituents of this plant[6].

Panduratin A, isolated from *Kaempferia pandurata* Roxb, is a natural chalcone compound with a molecular weight of 407. Panduratin A possesses some biological properties, such as anti-inflammatory, anticancer, and anti-oxidant effect. The bactericidal activity of panduratin A against planktonic *Porphyromonas gingivalis* cells also has been reported[7].

Green tea has been reported ato potentially have anti-oxidative, anti-aging, and anti-microbial proterties. The antibacterial activity of green tea extracts against cariogenic streptococci and other harmful mouth flora has been reported[8].

In this study, we measured biofilm inhibitory activity of these natural compounds using crystal violet staining degrees. The adsorption of the crystal violet was used as an indication of the amount of biofilm.

2. Materials and Methods

2.1. Antimicrobial Activity

S. aureus (ATCC 6538) was used for the antimicrobial tests. The minimal inhibitory concentrations (MICs) were determined by the microbroth dilution method in tryptic soy broth. The MIC was taken as the lowest concentration at which observable growth was inhibited. The average values were calculated from the results of two samples in each experiment.

2.2. Collagen Coating

Human placental collagen type IV (Sigma Chemical Co.) was dissolved in 0.2 % acetic acid at a concentration of 2 mg/mL for 1 h. The collagen solution was then filtered through a 5 μm syringe filter. The above filtrate was filtered again through 0.45 μm cellulose acetate filter apparatus. This filtrate was stored as stock solution at 4 °C until needed.

2.3. Observation of Biofilm Formation

A plastic coverslip coated with type IV collagen was put in each well of a 4-well tissue culture plate with 0.5 ml of human plasma (Cosmobio, Japan) and 0.5 mL of TSB medium. *S. aureus*, which had been incubated at 37 ℃ for 24 h in TSB medium, was inoculated into the medium (1×10^8 cfu/mL). After incubation for 6 days, each coverslip was washed three times with sterile water and stained with 0.4 % crystal violet.

2.4. Image Analysis

Slides stained with crystal violet were pictured with

Table 1. MIC and Test Concentrations

No.	Name	MIC (*S. aureus*)	Test concentration
N	Negative control	-	-
P	Positive control	-	-
1	*Hinoki cypress* (soluble)	〉0.4 %	0.10 %
2	*Hinoki cypress* (insoluble)	〉0.2 %	0.10 %
3	*Sunset hibiscus* root.	〉0.8 %	0.80 %
4	Xylitol 1 %	〉10 %	1 %
5	Xylitol 5 %	〉10 %	5 %
6	Xylitol 3 %	〉10 %	3 %
7	Decursin	〉0.2 %	0.05 %
8	Green tea	0.40 %	0.10 %
9	Panduratin	25 ppm	10 ppm

(n = 2)

Table 2. Representative Results of Biofilm Assay

No.	Name	Mean Density[a] (OD_{590})	Biofilm formation ratio (%)	Biofilm inhibition ratio (%)
N	Negative control	0 ± 3.92	0	100
P	Positive control	56.33 ± 0.37	100	0
1	*Hinoki cypress* (soluble)	33.02 ± 40.0	58.61	41.39
2	*Hinoki cypress* (insoluble)	30.19 ± 5.35	53.59	46.41
3	*Sunset hibiscus* root.	70.65 ± 1.41	125.42	-25.42
4	Xylitol 1 %	69.26 ± 1.30	122.95	-22.95
5	Xylitol 5 %	42.72 ± 0.52	75.84	24.16
6	Xylitol 3 %	48.64 ± 5.21	86.34	13.66
7	Decursin	70.21 ± 2.74	95.64	4.36
8	Green tea	122.7 ± 5.66	119.96	-19.96
9	Panduratin	95.88 ± 3.93	93.74	6.26

[a] Results are expressed as means ± standard deviation (n = 2)

digital camera, saved as a jpg file and converted to 24 bit bmp file. The optical density (OD) per unit area was calculated using gauge (ver. 3.0, Fujifilm) and OD_{590} of each sample was measured. The biofilm inhibitory activity was calculated against positive control (0 %) and negative control (100 %).

3. Results and Discussion

To find a biofilm inhibition agent that has a mechanism different from that of a conventional antimicrobial, we screened for inhibitors of biofilms of *S. aureus*.

The biofilm inhibitory activities of *Hinoki cypress*, sunset hibiscus, xylitol, decursin, green tea and panduratin within subminimum inhibitory concentrations (sub-MICs) were determined using crystal violet straining method. MIC and test concentration are presented in Table 1.

Table 2 shows a graph of mean density, biofilm formation rate and biofilm inhibition rate. As shown in Table 2 and Figure 1, The effect of xylitol is concentration dependent; high concentration of xylitol (3 ~ 5 %) reduced biofilm while promoted biofilm formation at a concentration of 1 %. Both water soluble and in-

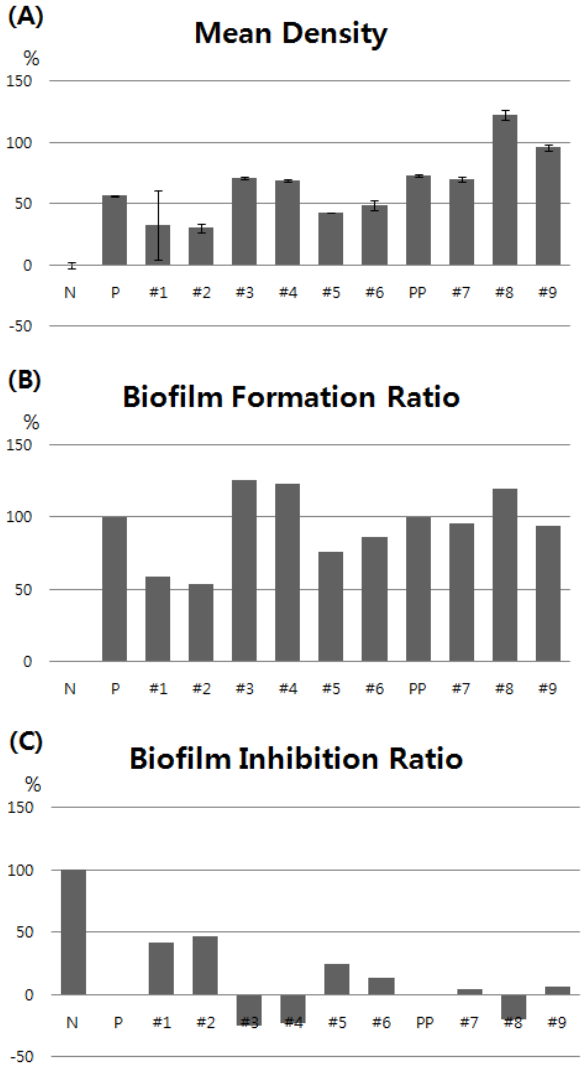

Figure 1. Biofilm formation of *S. aureus* on test biomaterials, stained with 0.1 % crystal violet. Mean density of OD_{590} per unit area ± standard error (n = 2) (A) and biofilm formation rate and biofilm inhibition rate of *S. aureus* by several materials; (B) and (C) respectively.

soluble 0.1 % *Hinoki cypress* polysaccharide strongly inhibited biofilm formation, however, Green tea (0.1 %) and sunset hibiscus (Hibiscusmanihot) root extract (0.8 %) promoted biofilm formation. However, the mechanisms of biofilm inhibition and biofilm formation activity of each biomaterials have not been clearly elucidated.

Until today, antibiotics are commonly used to treat atopy dermititis but these mechanisms need a high dosage and the bacteria can weaken or kill normal flora. Our results support that Hinoki cypress polysaccharide and xylitol have potential for inhibiting biofilm formation and controlling skin diseases like eczema and atopic dermatitis due to their inhibitory effects on biofilm formation by *S. aureus*. Additional investigations need to be performed in order to confirm the safety of effective concentrations for human consumption.

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