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Letter

Hinokitiol and pyrrolidone carboxylate zinc or corn oligosaccharides: A Synergistic approach to combating scalp microorganisms in seborrheic dermatitis

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ABSTRACT — Seborrheic dermatitis (SD) is a prevalent condition that results in dandruff, itching, and discomfort, and affect approximately 3–10% of the general population. Proliferation of the genus *Malassezia*, a microorganism inhabiting the scalp, is considered a contributing factor. Despite reducing the *Malassezia* population, other diseases, including SD, may still develop due to an increase in *Staphylococcus aureus*, which is associated with atopic dermatitis (AD) and SD, or a decrease in *Staphylococcus epidermidis*, which produces glycerol (moisturizer) and inhibits *S. aureus* growth. Therefore, we investigated the concentrations of anti-microbial reagents (pyrrolidone carboxylate-zinc [PCA-Zn] and Hinokitiol) and malt oligosaccharides (MT: corn-derived oligosaccharide mainly containing maltotetraose) that inhibited or promoted the growth of three types of scalp microorganisms. Individually, 0.50–1.00 mM PCA-Zn or 0.05–0.20 mM Hinokitiol displayed a marked growth-inhibitory effect on *Malassezia furfur* without a decline in *S. epidermidis* or an increase in *S. aureus*. Conversely, 0.02% MT individually exerted a growth effect on *S. epidermidis* but not on *M. furfur*. We then examined the effects of a mixture of the above-mentioned reagents on scalp-resident microorganisms. Our results indicated that 0.10 mM or 0.20 mM Hinokitiol combined with 0.02% MT markedly inhibited *M. furfur* growth and were the most effective at increasing *S. epidermidis* or decreasing *S. aureus*, compared to the single or combined effects of other reagents. Overall, our study provides valuable information on Hinokitiol and oligosaccharides concentrations in mixtures for use in shampoo-type cosmetics, and quasi-drugs, to prevent and treat SD.

Key words: Hinokitiol, Oligosaccharides, Pyrrolidone carboxylate-zinc, Seborrheic dermatitis, Malassezia, Staphylococcus

INTRODUCTION

Seborrheic dermatitis (SD) of the scalp causes dandruff and hair loss, which have detrimental effects on a patient's confidence, and also cause discomfort through itching and inflammation (Lin *et al*., 2021). SD affects 3–10% of the general population and is linked to *Malassezia* proliferation (Dall'oglio *et al*., 2022). Despite being a chronic illness prone to relapse after treatment, azole antifungals are recommended as first-line therapy for SD (Leong *et al*., 2021; Filatov *et al*., 2023). However, long-term use of these drugs can lead to reduced susceptibility in the genus

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Malassezia, resulting in diminished efficacy (Leong *et al*., 2021; Filatov *et al*., 2023). The decreased sensitivity to azole antifungals is attributed to induced ABC transporter expression, which enhances drug excretion, rendering one-third of patients with SD clinically unresponsive to treatment (Samarei *et al*., 2017). Therefore, identifying compounds with potent anti-microbial activity is crucial.

Among the various available compounds, the pyrrolidone carboxylic acid (PCA), a natural moisturizing factor [NMF]), combined with zinc (Zn) is considered effective for treating SD symptoms. PCA-Zn exhibits antibacterial effects from Zn ions and moisturizing effects from PCA (Jarrousse *et al*., 2007; Takino *et al*., 2012; Abebe *et al*., 2020). Application of a specially formulated gel cream (piroctone olamine, antifungal; biosaccharide gum-2, antifungal; stearyl glycyrrhetinate, anti-inflammatory; Zn l-pyrrolidone carboxylate, antiseborrheic) significantly reduces *M. furfur* and *Staphylococcus aureus* proliferation in SD patients (Micali *et al*., 2021). These findings suggest the effectiveness of PCA-Zn; however, further research is needed to clarify its individual effects, effective concentrations, and impact on *Staphylococcus epidermidis*.

Hinokitiol, found in some topical products, demonstrates anti-microbial activity against *Malassezia furfur* (Yoshimasa *et al*., 1988; Aoshima *et al*., 2009), *S. epidermidis* (Aoshima *et al*., 2009), and *S. aureus* (Okabe, 1993; Morita *et al*., 2007; Le *et al*., 2023) at various concentrations. Its anti-microbial action is attributed to inducing reactive oxygen species (ROS) production and respiratory dysfunction in iron-containing enzymes through its metal-chelating ability (Murakami *et al*., 2005; Hachlafi *et al*., 2021).

While evaluating compounds that reduce *S. epidermidis* and *Malassezia*, it is crucial to consider their combined effects and to explore compounds that supplement the moisturizing function of *S. epidermidis* or increase bacterial diversity. Malt oligosaccharides (MT), which are primarily composed of maltotetraose (corn-derived oligosaccharides), serve as moisturizing agents. The solid content of MT is 70%, with 35% maltotetraose and the remaining 35% maltotriose, maltose, and glucose, which are considered useful carbon sources contributing to microorganism growth. Although *S. aureus* has been reported to grow in response to treatment with dextrin and glucose (Choueiry *et al*., 2022), the effects of maltotetraose on the growth of *S. epidermidis* and *S. aureus* remain unclear.

In this study, we investigated the optimal concentrations of existing anti-microbial agents (PCA-Zn and Hinokitiol) samples (MT) considered useful, which exhibit growth-supporting or inhibitory effects against three types of scalp microorganisms (*M. furfur, S. aureus, and S. epidermidis*). Furthermore, the individual and combined effects of these mixtures were evaluated.

MATERIALS AND METHODS

Samples

PCA-Zn was purchased from AJINOMOTO Co., Inc., Hinokitiol was from TAKASAGO INTERNATIONAL CORPORATION, and Leogard-MT (MT) was from Lion Specialty Chemicals Co., Ltd. All other chemicals were of reagent grade and were purchased from commercial sources.

Sample preparation

Hinokitiol (5 mM) was prepared by adding 1 mL of sterile water to 0.8 mg of the compound, and incubating at 60°C for 15 min. After dissolution by vortexing, the mixture was allowed to sit at 4°C for 30 min and then vortexed for an additional 15 min. PCA-Zn was dissolved in sterile water at a concentration of 100 mM. MT was diluted with sterile water and adjusted to 20% (containing 7.0 g of maltotetraose and 7.0 g of maltotriose, maltose, and glucose combined). Each sample was diluted with sterile water and used for the microorganism growth tests.

Test strains

Three distinct types of scalp microorganisms were used in this study: *S. aureus* NBRC12732, *S. epidermidis* NBRC12993, and *M. furfur* NBRC0656. Specimens were supplied by the Biological Resource Center of the National Institute of Technology and Evaluation (NBRC).

Culture medium

S. aureus and *S. epidermidis* were cultured aerobically at 37°C in NBRC802 medium, which comprises 1% hypopeptone, 0.2% yeast extract, and 0.1% magnesium sulfate heptahydrate. *M. furfur* was cultured aerobically at the same temperature in YM medium, comprising 1% glucose, 0.5% peptone, 0.3% yeast extract, and 0.3% malt extract, with olive oil added at a final concentration of 1.0%.

Microorganism growth test

To quantify the growth-supporting or inhibitory effects of each sample on the microorganisms, a modified broth microdilution method was used. Each test strain was cultured in liquid medium, and the turbidity of the liquid medium in an L-shaped tube was evaluated using a Miniphoto 518R (Taitec Co., Ltd.) to measure the OD600. The test strain in each agar medium was added to 5.0 mL

of liquid medium and incubated at 37°C for more than 10 hr (OD600 = 0.8–1.0) in a Bioshaker®V·BR-36 (Taitec Co. Ltd.). The microorganism culture was then diluted (OD600 = 0.3–0.5), and after shaking the culture at 37°C for 1 hr, the diluted culture (OD600 = 0.08–0.13) was further diluted 100-fold and used as the microorganism solution. Next, 200 µL of culture medium (comprising 180 µL of each microorganism solution, 20 µL of the sample, or sterile water) was added to each well of a 96-well plate.The plate was then incubated at 37°C for 24 hr in a Maximizer M BR-022UP (Taitec Co., Ltd.). Turbidity of the culture medium was measured at 0, 5, 10, and 24 hr) using an Infinite 200Pro MPlex (Tecan Japan Co., Ltd.) and growth was assessed by calculating the mean and standard deviation.

Statistical analysis

Reagent efficacy was formally evaluated using a one-way analysis of variance, followed by Dunnett's test to identify significant differences between the turbidity of samples treated with varying concentrations of the reagents and the turbidity of the control (water) at the same time for each microorganism. If a significant difference was detected using Dunnett's test, the percentage increase or decrease in turbidity of samples, treated with single or combined reagents, was calculated as a percentage ± standard deviation, with the average turbidity of the control set at 100% after 24 hr. One-way analysis of variance was conducted, followed by Tukey's test, to assess the combined effectiveness against each microorganism. The significance level was set at 5%. Statistical analyses were conducted using EZR software (Kanda, 2013).

RESULTS

Single-reagent effect on scalp-resident microorganisms

To investigate the optimal concentration that exhibits growth-supporting or inhibiting effects on microorganisms, a single-reagent test was conducted on scalp-resident microorganisms. After 24 hr, 0.10 mM PCA-Zn had no effect on *S. aureus* and *M. furfur* compared with that of the control, whereas it significantly increased the growth of *S. epidermidis* (Fig. 1). Further, 0.50 mM PCA Zn had no effect on *S. aureus* but significantly increased the growth of *S. epidermidis* and markedly inhibited the growth of *M. furfur*. PCA-Zn at 1.00 mM had no effect on *S. epidermidis* but showed a significant decline in *S. aureus* and a marked growth-inhibitory effect on *M. furfur*. These results indicate that 0.50 and 1.00 mM PCA-Zn are effective compounds that exhibit a marked growth-inhibitory effect on *M. furfur* without promoting *S. aureus* growth.

After exposure to 0.10–0.20 mM Hinokitiol for 24 hr, no observable effects were observed in the *S. epidermidis* group when compared to the control. However, significant growth inhibition was observed in *M. furfur*, and a significant decrease was observed in *S. aureus* (Fig. 2). Moreover, using 0.05 mM Hinokitiol produced similar results (data not shown). Additionally, 1.00 mM Hinokitiol demonstrated a pronounced inhibitory effect on all three microorganisms (data not shown). Thus, 0.05–0.20 mM Hinokitiol effectively inhibited the growth of *S. aureus* and *M. furfur*, with no observable effect on *S. epidermidis*.

Subsequently, we used MT, a hydrolyzed cornstarch, to determine its effects on the growth of *S. epidermidis* (Fig. 3). After 24 hr, 0.02% MT exhibited significant growth of *S. epidermidis* alone compared with that of

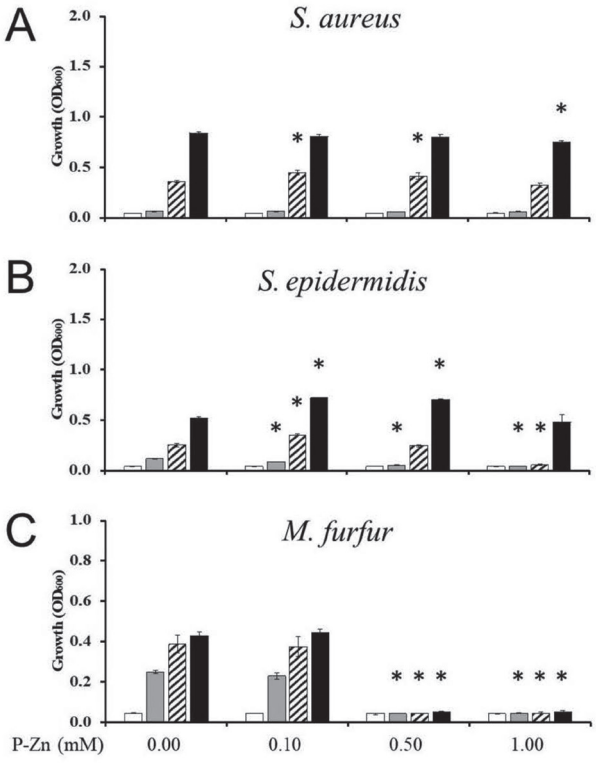

Fig. 1. Effect of PCA-Zn. After culturing each microorganism for 0, 5, 10, and 24 hr, the difference between the control (H_2O) and PCA-Zn (P-Zn: 0.10, 0.50, 1.00 mM) was assessed simultaneously. ANOVA Dunnett, n = 4, *P < 0.05

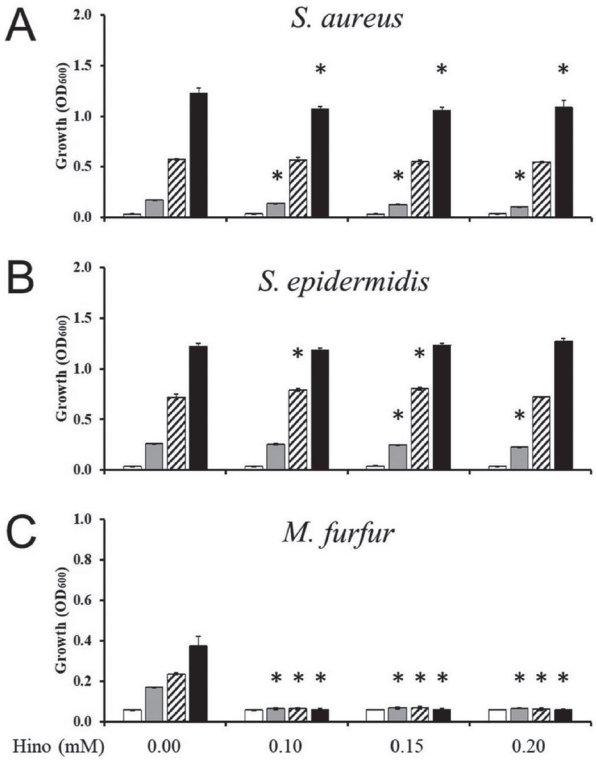

Fig. 2. Effect of Hinokitiol. After culturing each microorganism for 0–24 hr, the difference between the control and Hinokitiol (Hino: 0.10, 0.15, and 0.20 mM) was assessed simultaneously. ANOVA Dunnett, n = 4, *P < 0.05

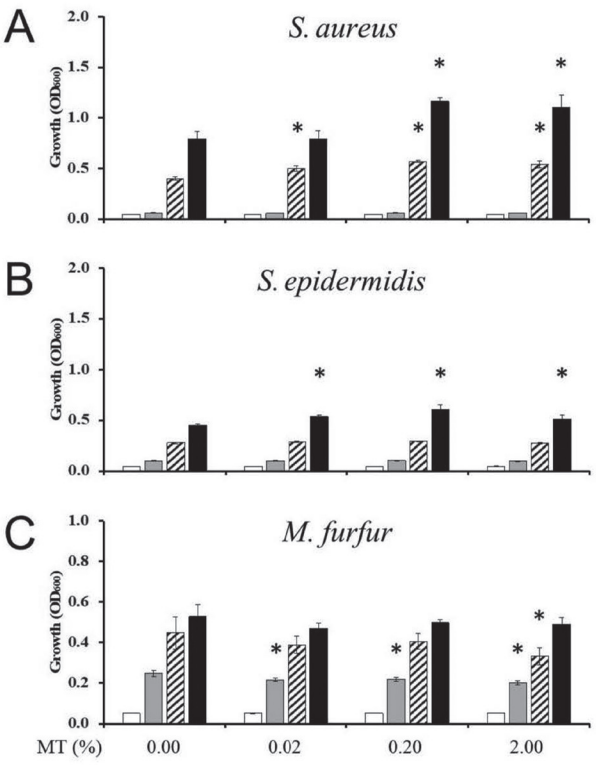

Fig. 3. Effect of MT. After culturing each microorganism for 0–24 hr, the difference between the control and MT (0.02, 0.20, and 2.00%) was assessed simultaneously. ANOVA Dunnett, n = 4, *P < 0.05

the control. Moreover, 0.20% and 2.00% MT significantly increased the growth of both *S. epidermidis* and *S. aureus*, without causing any growth in *M. furfur*. These results indicate that 0.02% MT is effective at promoting the growth of *S. epidermidis* without stimulating the growth of *S. aureus* or *M. furfur*.

Combined effect of two reagents on scalp resident microorganisms

After assessing the individual effects of two reagents (mixture of 0.5 mM PCA-Zn/0.2 mM Hinokitiol) on microorganisms, the results revealed a notable inhibitory impact on the growth of the three types of microorganisms when compared to the control group (data not shown). Subsequently, experiments were conducted using varying concentrations of PCA-Zn and Hinokitiol, (0.01 mM to 0.20 mM). The mixture of 0.05 mM PCA-Zn/0.20 mM Hinokitiol showed no significant difference in the growth of *S. epidermidis* compared to the control after 24 hr. However, it exhibited a pronounced inhibitory effect on *M. furfur* and significantly decreased *S. aureus* growth (Fig. 4).

In the combination of 0.10–1.00 mM PCA-Zn and 0.02–0.20% MT, the mixture of 0.50 mM PCA-Zn/0.02% MT or 0.50–1.00 mM PCA-Zn/0.20% MT had no effect on *S. aureus* after 24 hr compared to the control; *M. furfur* was markedly inhibited while *S. epidermidis* growth was increased (Fig. 5). The mixture of 1.00 mM PCA-Zn/0.02% MT after 24 hr had no effect on *S. epidermidis* compared to the control; *M. furfur* exhibited marked growth inhibition while *S. aureus* showed a significant decline (Fig. 5).

The interaction between 0.10–0.20 mM Hinokitiol and 0.02–0.20% MT resulted in varying effects on the growth of *S. aureus*, *M. furfur*, and *S. epidermidis* (Fig. 6). Specifically, the mixture of 0.10 mM Hinokitiol/0.02% MT after 24 hr showed no impact on *S. aureus* compared to the control, but exhibited a marked inhibitory effect on

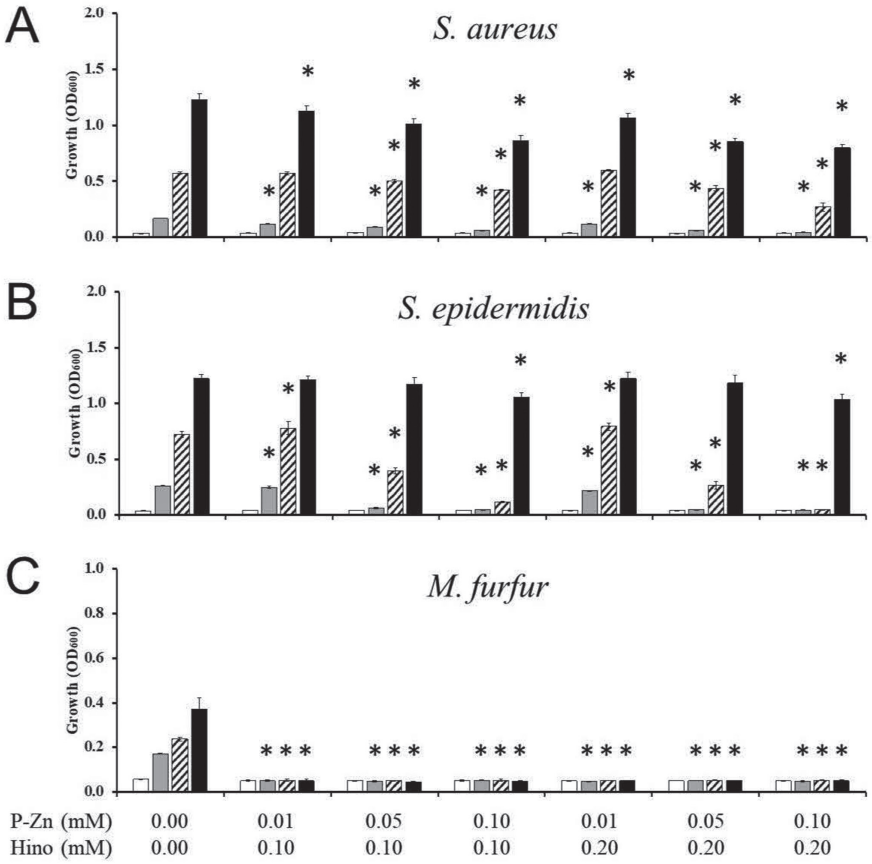

Fig. 4. Effect of combination of PCA-Zn and Hinokitiol. After culturing each microorganism for 0–24 hr, the difference between the control and two mixtures (0.01–0.10 mM P-Zn and 0.1–0.2 mM Hino) was assessed simultaneously. ANOVA Dunnett, n = 4, *P < 0.05

M. furfur and a significant increase in *S. epidermidis*. Conversely, the mixture of 0.20 mM Hinokitiol and 0.02% MT after 24 hr had no impact on *S. epidermidis* compared to the control, but significantly inhibited *M. furfur* growth decreased *S. aureus* population. Additionally, the mixture of 0.15 mM Hinokitiol and 0.2% MT mixture demonstrated a marked inhibitory effect on *M. furfur* growth and significant reduced *S. aureus* population, while demonstrating a tendency for *S. epidermidis* to grow.

DISCUSSION

Table 1 presents the concentrations of the reagents and their combinations that were effective against the microorganisms when used alone or in combination after 24 hr (Fig. 1–6). PCA-Zn at 1.00 mM effectively decreased *S. aureus* and inhibited *M. furfur* growth (Table 1). Additionally, 0.50 mM PCA-Zn effectively increased *S. epidermidis* growth and inhibited *M. furfur*. Zinc acts as a cofactor for many enzymes, including superoxide dismutase (SOD), an ROS-scavenging enzyme (Mohd Yusof *et al*., 2019). However, an increase in the Zn concentration inhibits growth because of the interaction (damage) with the microorganism membrane and an increase in ROS (Abebe *et al*., 2020). Zn is reported to exhibit different effects depending on its solubility, concentration, time of action, and properties of microorganisms (Abebe *et al*., 2020). These data suggest that PCA-Zn may exert different effects depending on the Zn concentration and properties of the microorganisms.

The results presented in Table 1 indicate that 0.20 mM Hinokitiol effectively reduced the growth of *S. aureus* and inhibited the growth of *M. furfur*. Hinokitiol exhibits

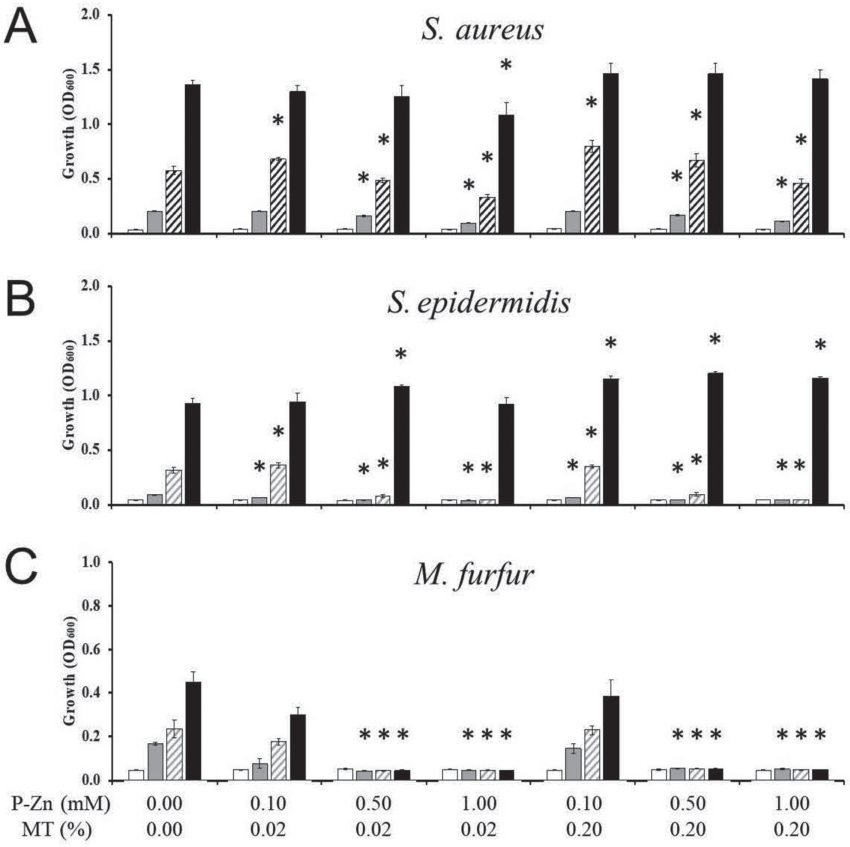

Fig. 5. Effect of combination of PCA-Zn and MT. After culturing each microorganism for 0–24 hr, the difference between the control and two mixtures (0.10–1.00 mM P-Zn and 0.02–0.20% MT) was assessed simultaneously. ANOVA Dunnett, n = 4, *P < 0.05

anti-microbial and antioxidant properties (Murakami *et al*., 2005; Koufaki *et al*., 2010; Hachlafi *et al*., 2021). Its anti-microbial activity has been attributed to its ability to induce ROS production (Murakami *et al*., 2005; Hachlafi *et al*., 2021). Additionally, Hinokitiol forms complexes with metal ions providing it with antioxidant properties (Koufaki *et al*., 2010). These findings suggest that the efficacy of Hinokitiol against different microorganisms varies with its concentration.

The effectiveness of MT (0.02%) in enhancing the growth of *S. epidermidis* is shown in Table 1. The growth of *S. aureus* on dextrin and glucose has been previously documented (Choueiry *et al*., 2022). High glucose treatment has been observed to impede bacterial development (Luo *et al*., 2020). MT is composed primarily of maltotetraose and glucose. Thus, monosaccharides and oligosaccharides present in MT were hypothesized to exert distinct effects on each microorganism, depending on their concentration.

A combination of 0.05 mM PCA-Zn and 0.20 mM Hinokitiol or 0.20 mM Hinokitiol and 0.02% MT effectively at reduced the presence of *S. aureus* by 30.1% or 42.2%, respectively, while inhibiting the growth of *M. furfur* (Table 1). These mixtures demonstrated a synergistic effect in reducing *S. aureus*, achieving a greater reduction compared to Hinokitiol or PCA-Zn alone (both 11.2% reduction). Zn ions are known to react with Hinokitiol, facilitating its entry into cells and exerting antiviral effects (Krenn *et al*., 2009). This synergistic effect may be attributed to the interaction of the Zn ions in PCA-Zn with Hinokitiol, which facilitates its entry into the cell. This phenomenon may be attributed to the optimal concentrations of Zn ions in PCA-Zn and Hinokitiol. Oligosaccharides derived from pectin have also been reported

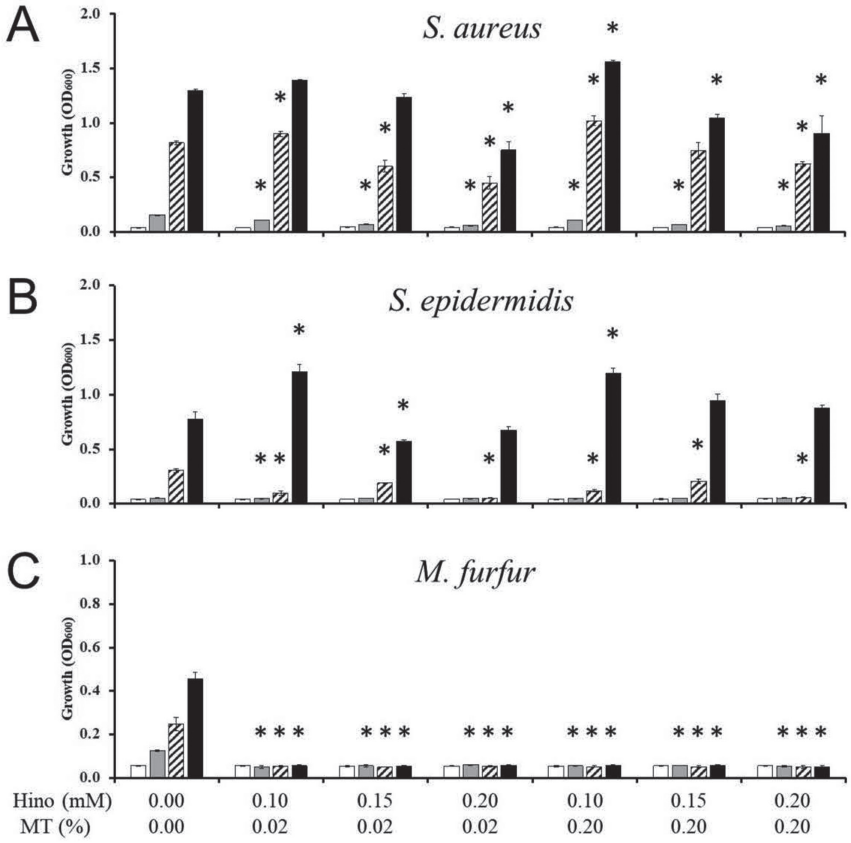

Fig. 6. Effect of combination of Hinokitiol and MT. After culturing each microorganism for 0–24 hr, the difference between the control and two mixtures (0.1–0.2 mM Hino and 0.02–0.20% MT) was assessed simultaneously. ANOVA Dunnett, n = 4, *P < 0.05

to inhibit the growth of *S. aureus* (Hasui and Nishiyama, 2014). A similar component, maltotetraose (a type of oligosaccharide), may have contributed to the significant reduction of *S. aureus* in the mixture of 0.20 mM Hinokitiol and 0.02% MT. This combination was found to be more effective against *S. aureus* than the mixture of 0.05 mM PCA-Zn and 0.20 mM Hinokitiol (Hasui and Nishiyama 2014).

The mixture of 0.10 mM Hinokitiol and 0.02% MT was more effective at increasing *S. epidermidis* (55.8% increase) and inhibiting the growth of *M. furfur* (Table 1). This mixture had a significant synergistic growth effect on *S. epidermidis*, greater than that of 0.02% MT or 0.50 mM PCA-Zn alone (18.7% or 35.6% increase). The significant increase in *S. epidermidis* may be attributed to the overlapping effects of maltotetraose or glucose on *S. epidermidis* and the antioxidant effects of Hinokitiol (Koufaki *et al*., 2010; Choueiry *et al*., 2022). Further studies are needed to understand why the combination of 0.02% MT with 0.1 mM or 0.2 mM Hinokitiol shows different synergistic effects against *S. aureus* or *S. epidermidis*, respectively.

The outcomes of treatment with the mixture of 1.00 mM or 0.50 mM PCA-Zn and 0.02% MT, resulted in a 20.3% reduction in *S. aureus* or a 30.3% increase in *S. epidermidis*, similar to the outcomes of the single treatments with 1.00 mM PCA-Zn or 0.50 mM PCA-Zn, which achieved a 11.2% reduction or 35.6% increase, respectively (Table 1). These results indicate that the combination of PCA-Zn and MT did not exhibit an additive effect or an interaction with MT.

The use of anti-microbial agents can reduce resident microorganisms, potentially compromising barrier function and allowing harmful microorganisms to prolifer-

Table 1. Effects of each reagent singly or in combination on microorganisms.

Reagents	Concentration	*S. aureus*	*S. epidermidis*	*M. furfur*
PCA-Zn	1.00 mM	↓ (11.2 ± 1.6%) $^{\alpha,\beta}$	±	MI
PCA-Zn	0.50 mM	±	↑↑ (35.6 ± 0.9%) $^{\alpha,\beta}$	MI
Hinokitiol	0.20 mM	↓ (11.2 ± 5.4%) $^{\gamma,\delta}$	±	MI
MT	0.02%	±	↑ (18.7 ± 4.2%) $^{\beta,\gamma,\delta}$	±
PCA-Zn Hinokitiol	0.05 mM 0.20 mM	↓↓ (30.1±1.9%) $^{\alpha,\gamma,\varepsilon}$	±	MI
Hinokitiol MT	0.20 mM 0.02%	↓↓ (42.2 ± 6.0%) $^{\beta,\delta,\varepsilon,\zeta}$	±	MI
Hinokitiol MT	0.10 mM 0.02%	±	↑↑↑ (55.8 ± 9.1%) $^{\alpha,\gamma,\varepsilon}$	MI
PCA-Zn MT	1.00 mM 0.02%	↓ (20.3 ± 8.5%) $^{\zeta}$	±	MI
PCA-Zn MT	0.50 mM 0.02%	±	↑↑ (30.3 ± 1.6%) $^{\delta,\varepsilon}$	MI

Single or combined effects showed a significant decrease or increase compared to the control for each microorganism after 24 hr. ANOVA Dunnett, n = 4, $P < 0.05$. No significant difference (±), marked growth inhibition (MI), significant increase (↑) or decrease (↓) by 10–25%, significant increase (↑↑) or decrease (↓↓) by 30–45%, and significant increase (↑↑↑) by 50–65%. For each microorganism, significant differences are indicated between the same symbols (α, β, γ, δ, ε, and ζ). ANOVA Tukey, n = 4, $P < 0.05$.

ate. To maintain barrier integrity and prevent and treat diseases, it is crucial to suppress disease-causing microorganisms while preserving beneficial ones. PCA-Zn and Hinokitiol have shown to be useful in this study, and the combined effect of 0.10 or 0.20 mM Hinokitiol and 0.02% MT was found to be even more effective than that of either agent alone. This treatment was thus considered effective against SD-related diseases.

M. furfur is proposed to play a role in exacerbating atopic dermatitis (AD) (Tajima, 2005). Management of AD involves decreasing the levels of *S. aureus* and increasing the levels of *S. epidermidis*, which promote hydration. Specifically, a combination of 0.10 or 0.20 mM Hinokitiol with 0.02% MT may serve as a useful ingredient for managing both SD and AD.

Conflict of interest---- This study was conducted in collaboration with Beauty Hi-tech Innovation Co., Ltd. and received a grant.

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