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In vitro antimicrobial and anticancer potential of hinokitiol against oral pathogens and oral cancer cell lines

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Abstract

Hinokitiol is a natural component isolated from *Chamacyparis taiwanensis*. It has antimicrobial activity, and has been used in oral care products. The minimal inhibitory concentration (MIC) and minimal microbicid MRSA, *Aggregatibacter actinomycetemcomitans*, determined by the agar and broth dilution m paradoxical inhibition phenomenon (PIP) wa *mutans*. The PIP can be described as microbia and low concentrations of a compound, betw PIP was confirmed using a kinetic microplate and inhibition zone methods. The PIP was also observed in MRSA. The low autolysin activity somehow correlated to the PIP positive. The cell diameter was increased in all the pathogens, and the transition was inhibited in *C. albicans* following hinokitiol treatment. Hinokitiol is also a potential anticancer drug. The 200 μM of hinokitiol has significant antimicrobial and cytotoxic activities against oral pathogens and oral squamous cell carcinoma cell lines, respectively, and lower cytotoxic effects for normal human oral keratinocytes, indicating that hinokitiol displays a high potential for safe and effective applications in oral health care.

Keywords

Autolysin; Hinokitiol; Minimal inhibitory concentrations; Minimal microbicidal concentration; Oral squamous cell carcinoma; Paradoxical inhibition phenomenon (PIP)

1. Introduction

The timber of the Taiwan cypress tree *Chamacyparis taiwanensis* is resistant to insects, fungi, and bacteria, making it one of the most resilient building materials used since ancient times. Hinokitiol is a chemical compound of aromatic tropolone and a component of essential oils first isolated from the heart wood of *C. taiwanensis*. Early clinical uses included treatments for lung gangrene, tuberculous fistula, pulmonary tuberculosis, and decubitus ulcers.

Hinokitiol shows antibacterial, antifungal, antiviral, and insecticidal activities (Inamori et al., 2000, Arima et al., 2003, Krenn et al., 2009). Certain health care products contain hinokitiol, such as safe preservatives for toothpaste, cosmetics, hair tonics, oral care gel, eyelid cleanser, hair restorers, skin lotions, and body soap, because of its multi-biological activities (Higashi et al., 2009, Gilbard et al., 2010, Nagao and Sata, 2011). However, few studies have been conducted on hinokitiol antimicrobial mechanisms. *Staphylococcus* aureus that was isolated from atopic dermatitis patients was inhibited by 50 μg/mL hinokitiol (Arima et al. 2003). Exposure to 500 μg/mL hinokitiol for 30 min did not cause morphological changes in *Escherichia coli* IFO 3301 and *S. aureus* IFO 12732 cells; however, cellular respiration, nucleic acid synthesis, and protein synthesis were reduced (Morita et al.
Candida albicans strains was 5–10 μg/mL, hinc
expression but did not inhibit ATP synthesis
also been shown to inhibit influenza virus re
mansoni (Miyamoto et al., 1998, Chisty et al., 2

The oral cavity is a warm, humid, and hypoxi
pathogens flourish. Caries, periodontal disease, and candidiasis are common oral diseases that often result from imbalances between the normal oral microflora and host immunity. MRSA is a facultative-anaerobic *Staphylococcus* sp. for which the colonization and infection is 77.8% among oral cancer patients following surgery (Miyake et al. 2007). *Aggregatibacter actinomycetemcomitans* is a spherical or rod-shaped bacterium that colonizes the periodontal pockets of oral cavities and damages tooth-supporting tissues. *Streptococcus* mutans is a facultative-anaerobic *Streptococcus* sp. that is common in the human oral cavity and significantly contributes to dental caries. The fungal pathogen *C. albicans* causes oral candidiasis in immune deficient humans, such as cancer, transplants, and AIDS patients.

Hinokitiol has also been shown to cause apoptosis and cell cycle arrest in murine embryonal carcinoma cells (Tanaka et al. 1999). Treatments with 5 μg/mL hinokitiol for 24 and 48 h produced cytotoxic effects in a murine P388 lymphocytic leukemia cell line, and 50 μM hinokitiol blocked androgen receptor signal transduction, inhibiting the growth of the prostate cancer cell line LNCaP (Liu and Yamauchi 2006). Hinokitiol was also shown to induce caspase-3-mediated apoptosis through a time- and dosage-dependent suppression of DNA synthesis in murine F9 teratocarcinoma cells (Ido et al. 1999). However, no developmental toxicity or carcinogenic effects have been observed for hinokitiol (Ema et al., 2004, Imai et al., 2006). Thus, Hinokitiol has demonstrated specific antimicrobial and anticancer properties. The purpose of our study was to examine the antimicrobial and cytotoxic properties of hinokitiol for clinical applications for oral disease pathogens and oral squamous cell carcinoma (OSCC). We examined the antimicrobial and cytotoxic properties of hinokitiol on oral disease pathogens and OSCC. We verified hinokitiol MIC and MMC in MRSA, *A. actinomycetemcomitans*, *S. mutans*, and *C. albicans*, and found hinokitiol the PIP in MRSA, *A. actinomycetemcomitans*, and *S. mutans*. The hinokitiol PIP is associated with bacterial autolysin activity. The microbes in the study were swollen, and *C. albicans* transition was inhibited after hinokitiol treatment. Hinokitiol has significant antimicrobial and cytotoxic activities against oral pathogens and OSCC cell lines, respectively, and lower cytotoxic effects for normal human oral keratinocytes (OK).

2. Materials and methods

2.1. Microorganisms culture

Methicillin-resistant *S. aureus* (MRSA, ATCC number: 33591), *A. actinomycetemcomitans* (ATCC number: 33384), *S. mutans* (ATCC number: 25
study. The MRSA and *S. mutans* were cultured
actinomycetemcomitans was cultured in brain h
cultured in a yeast extract peptone dextrose n
loop transfer from frozen tube slants of 3 mL
actinomycetemcomitans, and *S. mutans* were cul
30 °C for 24 h. Bacteria from these cultures w
medium and incubated overnight. Selected colonies were transferred to the appropriate liquid medium, and incubated 4–6 h to achieve log phase growth. The optical density at 600 nm (OD600) of each culture was adjusted to 1.0 using fresh broth to give a standard inoculum of 10^6 cfu/mL, which was verified by counting colony numbers on agar media following a 10-fold series dilution. Stock cultures were maintained at –80 °C in growth broth containing 15% sterile glycerol.

2.2. Minimum inhibition concentration and minimum microbicidal concentration determinations Agar dilution method

concentrations of hinokitiol between 5 μM and 200 μM, and 0.2% DMSO (0 μM hinokitiol, control) were prepared in a 12-well plate format (2 mL/well). Chlorhexidine (CHX, Sigma–Aldrich), a popular antimicrobial agent in mouthwashes, was used as a parallel control in our study. Bacterial cultures were prepared on a solid medium using 2 μL of 10^6 cfu/mL, and were incubated in the dark at 37 °C overnight (*C. albicans*, yeast form in 30 °C, and hyphal form in 37 °C). The solid medium containing the least concentration of hinokitiol and CHX, which showed no visible growth, represented the MIC. MIC is the lowest concentration of an antimicrobial that inhibits the visible growth of a microorganism after overnight incubation. The MIC plates were swabbed with a sterile inoculating loop that was then used to inoculate a 6 cm nutrient broth agar plate, which was hinokitiol free, and the 6 cm plates were then incubated for 24 h. The left and right side of the agar plate were used for the agar dilution method MMC and broth dilution method MMC, respectively. The least concentration at which no growth was observed represented the MMC. MMC is the lowest concentration of antibiotic required to kill a particular microorganism.

2.3. Broth dilution method

The broth dilution method was modified from Mohapatra's methods (Mohapatra et al. 2011). Cell suspensions were prepared in 2 mL of liquid medium with various hinokitiol and CHX concentrations in 15 mL culture tubes by inoculation with 2 μl of 10^6 cfu/mL from each glycerol stock. The cultures were incubated at 30 or 37 °C at 250 rpm for 24 h, and OD600 for 10-fold dilutions of each culture. Tubes showing no visible turbidity represented the MIC, and subsequently, were inoculated on the right side of the sterile 6 cm nutrient agar plates without hinokitiol and incubated for 24 h. The least concentration, at which no growth was observed, represented the MMC.

2.4. Kinetic microplate method

The kinetic microplate test was conducted in
previously described method (Holowachuk et
concentrations of hinokitiol in 1 mL of liquic
inoculated with 1 μL of 10^6 cfu/mL from the g
96-well plates. Unused wells were filled with
evaporation. The 24-h kinetic analysis of the culture growth was performed at the species-appropriate incubation temperature (30 or 37 °C). The kinetic analysis included a 5-s shaking step before each of the time point measurements of OD600 that were recorded at 30 min intervals and analyzed using VersaMax™ and Softmax® Pro (version 5.4.1) software, respectively.

2.5. Inhibition zone

diameter filter paper discs, before air drying overnight. Molten 1.5% agar broth was equilibrated in a 50 °C water bath for 30 min following autoclave sterilization. Cultures were inoculated with 10^6 cfu/mL of glycerol stock by swirling before being poured into plates. The dried filters placed on the surface of the solidified agar, and the cultures were incubated for 24 h at 30 or 37 °C. The diameter of the inhibition zone was recorded and photographed.

2.6. *C. albicans* transitional assay

C. albicans were treated with 0.2% DMSO, 25, 50, 100, and 200 μM hinokitiol in colony morphological tests, hyphal morphological tests, and germ tube formation ability tests.

I. Colony morphological test

C. albicans were cultured on YPD agar containing 4% FBS, 80 mg/mL uridine, and different dosages of hinokitiol in 37 °C for 3 d.

II. Hyphal morphological test

C. albicans were cultured on bacto agar containing 4% FCS, 80 mg/L uridine, 20 mg/L arginine, 20 mg/L histidine, and different dosages of hinokitiol in 37 °C for 7 d.

III. Germ tube formation ability test

C. albicans were cultured in YPD agar with different dosages of hinokitiol in 30 °C for 1 d, and then inoculated in YPD broth contained 80 mg/L uridine, and incubated in 37 °C for 3 h. The germ tubes were observed and photographed by light microscopy.

2.7. Scanning electron microscope

The test microorganisms were treated with 200 μM hinokitiol, and adsorbed on a glass slide. The cells were fixed using 4% glutaraldehyde [illegible] ethanol washes, and dried with tertiary-butyl [illegible] palladium, and the colonies were observed us[illegible] electron microscope (SEM).

2.8. Autolysin activity

Determinations of the ability of MRSA, *A. actinomycetemcomitans*, and *S. mutans* to produce autolysin were performed using a modified solid medium assay (Fontana et al. 1990). Autolysin activity was defined as the ability to lyse heat-killed bacteria. MRSA, *A. actinomycetemcomitans*, and *S. mutans* were heat-killed by autoclaving and used as substrates. The heat-killed bacteria were added to molten BHI or TSB agar, and OD600 was adjusted to 0.5. Following solidification, 10 μL of the test pathogen were spotted onto the agar surface, and the plates were incubated at 30 or 37 °C for 24–48 h. Bacteria that showed a clear lysis zone on heat-killed bacteria plates were deemed autolysin producers.

University Hospital). The OSCC cell lines HSC3, SAS, and SCC4 were kindly gifted by Dr. M.-C. Kao (China Medical University). These cell lines were cultured, and cell viability after 24 h hinokitiol treatment was used for the MTT test as described (Shieh et al., 2010, Shih et al., 2012).

2.10. Statistical analysis

All the assays were duplicate to quadruplicate. The Mann–Whitney test was used for analysis. Data were shown as the mean ± standard error. Differences between the variants were considered significant when $p < 0.05$.

3. Results

3.1. MIC and MMC of hinokitiol determined by agar/broth dilution methods

The agar dilution method was used to determine hinokitiol MIC of MRSA, *A. actinomycetemcomitans*, *S. mutans*, and *C. albicans*. The hinokitiol MIC values for MRSA, *A. actinomycetemcomitans*, *S. mutans*, *C. albicans* yeast form, and hyphal form were 110, 90, 70, 60, and 90 μM, respectively. However, the antibacterial activity of hinokitiol exhibited a paradoxical inhibition phenomenon (PIP) for *A. actinomycetemcomitans*, and *S. mutans* bacteria. The PIP can be described as microbial growth occurring in the presence of both high and low concentrations of an antimicrobial compound, between which microbial growth is inhibited. No *A. actinomycetemcomitans* growth was observed in the presence of 10–30 μM hinokitiol, but growth was observed in the presence of 40–80 μM hinokitiol. Similarly, no *S. mutans* growth was observed in the presence of 10–40 μM hinokitiol, but growth was observed in the presence of 50–70 μM hinokitiol. In a parallel control experiment, the CHX MIC for MRSA, *A. actinomycetemcomitans*, *S. mutans*, and *C. albica*
respectively. No PIP effect was observed with
(Fig. 1A).

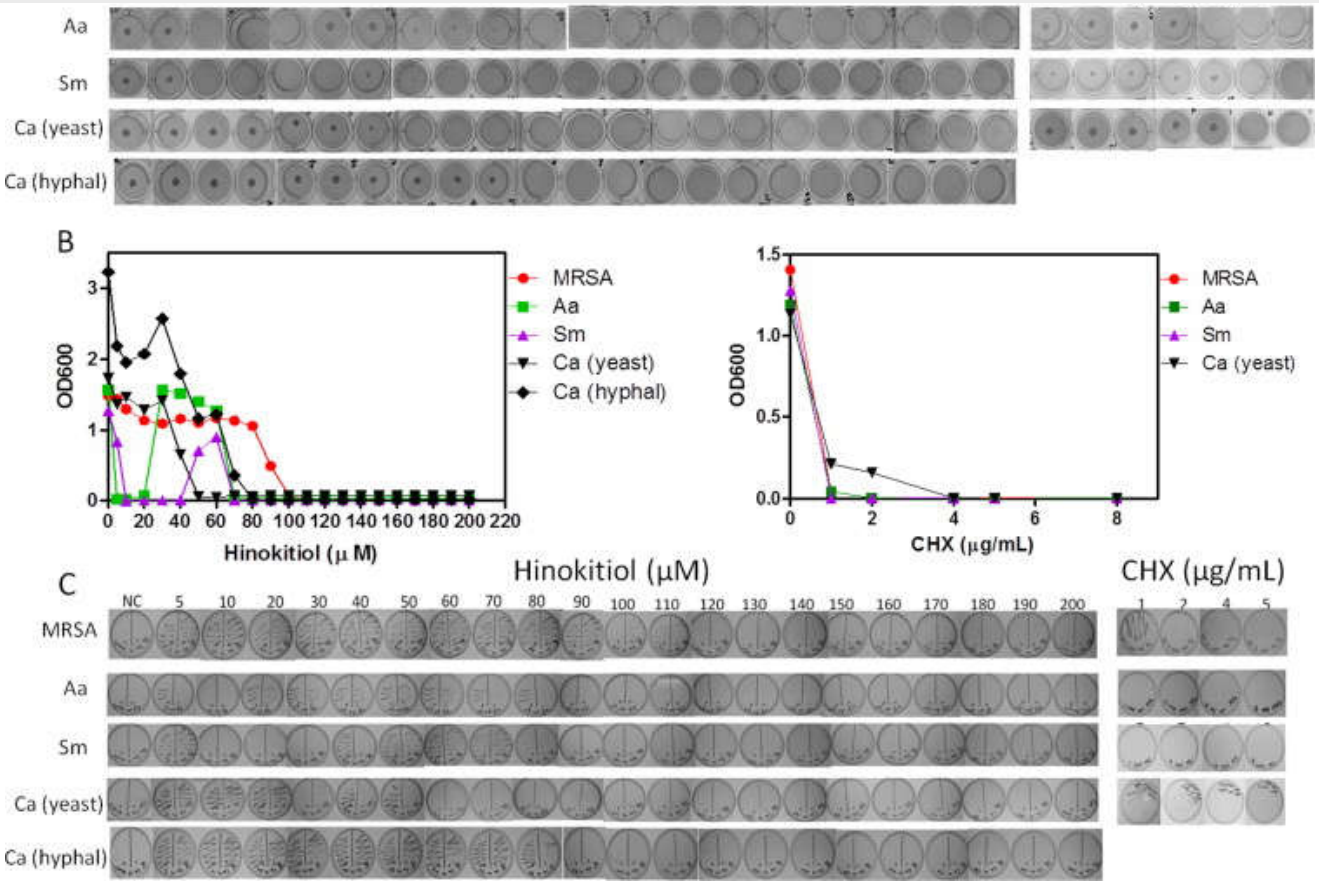

Fig. 1. Agar/broth dilution methods determination of the hinokitiol MIC/MMC for MRSA, *A. actinomycetemcomitans* (Aa), *S. mutans* (Sm), and *C. albicans* (Ca). Hinokitiol, from 0 to 200 μM, and CHX, from 0 to 8 μg/mL. (A) Agar dilution method, MIC. (B) Broth dilution method, MIC. (C) Agar/broth dilution methods MMC. The agar dilution method is represented on the left side of the plate, and the broth dilution meth
NC indicates the negative control (no microo
dilution method.

The broth dilution method was used to verify
dilution method, and the MIC of hinokitiol for MRSA, *A. actinomycetemcomitans*, *S. mutans*, *C. albicans* yeast form, and hyphal form cells were 100, 70, 70, 40, and 80 μM, respectively. The PIP was also observed for *A. actinomycetemcomitans*, and *S. mutans*. The *A. actinomycetemcomitans* culture medium was clear for the 5–20 μM hinokitiol samples, but the culture was turbid in the 30–60 μM. The *S. mutans* culture medium was clear for the 10–40 μM hinokitiol samples, but the culture was turbid in the 50–60 μM. Using the broth dilution method, the CHX MIC for MRSA, *A. actinomycetemcomitans*, *S. mutans*, and *C. albicans* yeast form were <1, <1, <1, and 4 μg/mL, respectively (Fig. 1B).

are represented on the right side. The MMC values for hinokitiol of MRSA, *A. actinomycetemcomitans*, *S. mutans*, *C. albicans*, yeast form, and hyphal form, which were determined using the agar dilution method, were 130, 90, 80, 60, and 130 μM. However, *A. actinomycetemcomitans* and *S. mutans* growth were not observed in 10 μM, or 10–30 μM hinokitiol samples, respectively. The MMC values for hinokitiol of MRSA, *A. actinomycetemcomitans*, *S. mutans*, *C. albicans*, yeast form, and hyphal form that were determined using the broth dilution method were 130, 70, 80, 50, and 90 μM. *A. actinomycetemcomitans* and *S. mutans* growth were not observed in the 10 μM, and 10–40 μM hinokitiol samples, respectively. The CHX MMC that was determined using the broth dilution method was 2, <1, <1, and 4 μg/mL for MRSA, *A. actinomycetemcomitans*, *S. mutans*, and *C. albicans*, respectively (Fig. 1C).

3.2. The MRSA PIP of hinokitiol was detected by the kinetic growth curve and inhibition zone test

In the kinetic analysis, the value under the curve was the highest detected value of OD600 during 24 h in each sample. The result of kinetic analysis showed that the MIC and PIP were consistent in the broth dilution method in *A. actinomycetemcomitans*, and *C. albicans*, but different in MRSA and *S. mutans*. *S. mutans* growth was inhibited in the presence of 10–60 μM hinokitiol, significant growth occurred in the presence of 70–130 μM hinokitiol, and growth was again inhibited in the presence of 140 μM hinokitiol (Fig. 2A). An OD600 greater than 0.02 was defined as the starting point of the log phase of microbial growth. The OD600 was 0.685 for the 20 μM hinokitiol samples but was 1.131 for the 30 μM hinokitiol samples in MRSA, indicating that growth was more extensive at a higher hinokitiol concentration. The inhibition of microbial growth in the 0 and 80 μM hinokitiol samples is shown in Fig. 2B. The MRSA, *A. actinomycetemcomitans*, *S. mutans*, and *C. albicans* log phases were 4, 6.5, 6, and 8 h in duration, respectively. Although the PIP of M
dilution methods based on growth at 24 h, it
B). Plotting the area under the growth curve (
linear curve that was used to quantify the ant
The results of agar/broth dilution and kinetic
Antimicrobials are usually regarded as bacter
MIC. The data indicated that hinokitiol is an

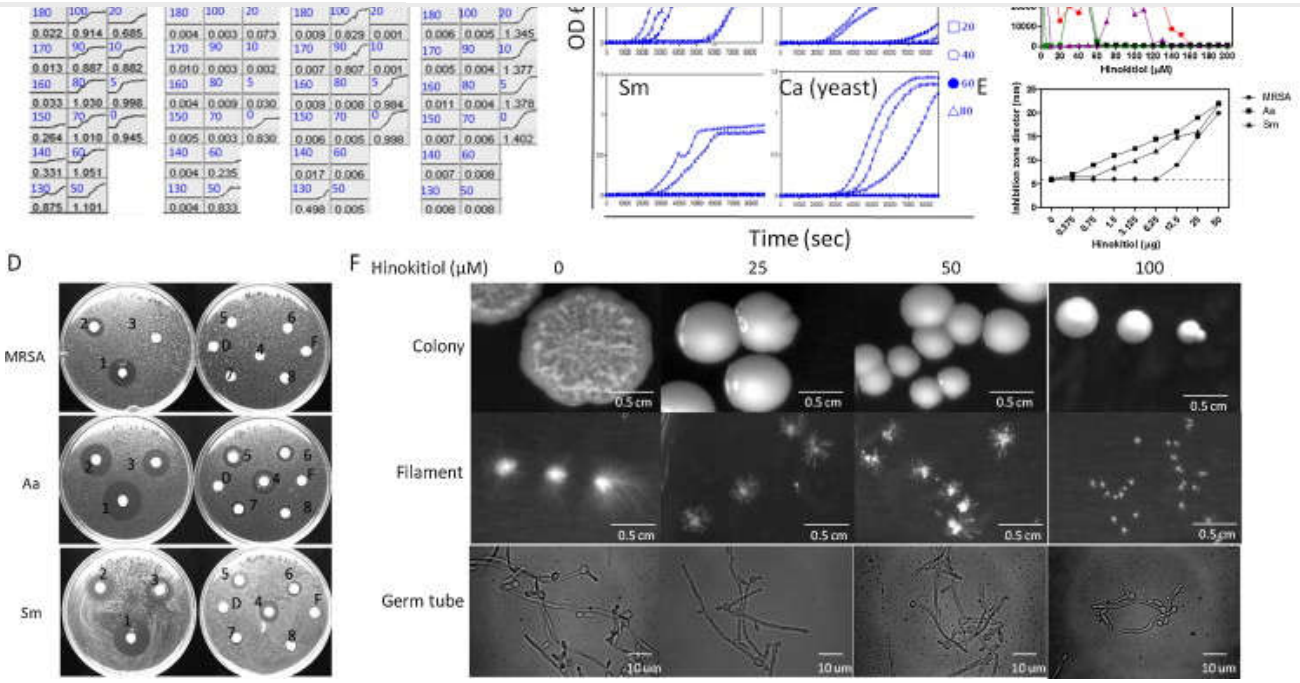

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Fig. 2. MIC, MMC, and PIP of hinokitiol determined by kinetic microplate and inhibition zone method. (A) Growth curve analysis of MRSA, *A. actinomycetemcomitans*, *S. mutans*, and *C. albicans* in the presence of hinokitiol (0–200 μM) using the kinetic microplate method. The value above the curve represents hinokitiol concentration, and the value below the curve represents the maximum OD600 during the 24 h period. (B) Growth in the presence of hinokitiol, 0–80 μM. (C) Plots of area under the growth curve (AUGC) versus hinokitiol concentration yielded diagram of curves. (D) Hinokitiol inhibition zone tests. Drops of DMSO containing 50.0, 25.0, 12.5, 6.25, 3.13, 1.56, 0.781–0.325 μg hinokitiol were absorbed on the filter paper discs labeled 1–8, respectively. Discs D and F contained 6 μL DMSO and no reagent (control). (E) The diameter of inhibition zone
plotted for each microorganism. The dotted l
disc (6 mm). (F) *C. albicans* transitional test in
panel is colony, the middle panel is filament,

Table 1. Hinokitiol and CHX MIC/MMC.

Hinokitiol (μM)	CHX (μg/mL)

	(MIC/MMC)	(MIC/MMC)	method (MIC)		(MIC/MMC)
	Agar dilution method (MIC/MMC)	**Broth dilution method (MIC/MMC)**	**Kinetic microplate method (MIC)**	**Agar dilution method (MIC)**	**Broth dilution method (MIC/MMC)**
MRSA	110/130	100/130	160	5	<1/2
Aa	90/90	70/70	70	4	<1/<1
	PIP: 10–30/10	PIP:5–20/10	PIP: 5–20		
Sm	80/80	70/80	140	5	<1/<1
	PIP: 10–40/10–30	PIP: 10–40/10–40	PIP: 10–60		
Ca (yeast)	60/60	40/50	50	5	4/4
Ca (hyphal)	90/130	80/90	–	–	–

MIC, MMC, and PIP data for hinokitiol and CHX treatments of MRSA, *A. actinomycetemcomitans* (Aa), *S. mutans* (Sm), and *C. albicans* (Ca) in 3 independent experiments using the agar dilution method, the broth dilution method, and the kinetic microplate method.

The PIP of hinokitiol that was observed for *A. actinomycetemcomitans* and *S. mutans* bacteria was also demonstrated in the results of the inhibition zone test. In the analysis of MRSA, the inhibition zone of the 50 μg hinokitiol disc w
growth that was visible in the outermost area
indistinct for the 12.5 μg disc, and no clearly
hinokitiol disc for the MRSA. The 25 and 50
zone for *A. actinomycetemcomitans*, and *S. muta*
discs showed a distinct area of *A. actinomycete*
proximal to the disc and a distal region in wh
the inhibition zone was dose-dependent for the 0.38–1.5 μg hinokitiol discs regarding *A. actinomycetemcomitans*, and *S. mutans* growth (Fig. 2D). The diameters of the inhibition zones are shown in Fig. 2E. There were no significant differences between the data collected at 24 and 48 h. In the *C. albicans* transitional test, wrinkled colonies were observed in the control, and smooth colonies were observed in 25, 50, and 100 μM hinokitiol. The colony numbers, size, filaments density, and germ-tube lengths were decreased by hinokitiol in a dose dependent manner. No visible colony, filament, or germ tube was observed in 200 μM of hinokitiol. The data demonstrated that hinokitiol could block the yeast-to-hyphal transition and germ tube formation in *C. albicans* (Fig. 2F).

25, 50, 100, and 200 μM hinokitiol for 24 h (Fig. 3A). SEM analysis showed that morphological changes occurred in MRSA, *A. actinomycetemcomitans*, *S. mutans*, and *C. albicans* cells following treatment with 200 μM hinokitiol for 24 h, with all species exhibiting increases in cell diameter. In addition, the SEM images show that *C. albicans* wrinkled and ruptured following hinokitiol treatment (Fig. 3B). The changes in cell diameter that were measured for MRSA, *A. actinomycetemcomitans*, *S. mutans*, and *C. albicans* before and after hinokitiol treatment were 0.64 ± 0.024 to 0.86 ± 0.040 μm, $p = 0.011$; 1.00 ± 0.084 μm to 1.60 ± 0.114, $p = 0.016$; 0.79 ± 0.010 to 1.00 ± 0.032 μm, $p = 0.0097$; and 3.04 ± 0.160 to 3.78 ± 0.017 μm, $p = 0.0358$, respectively (Fig. 3C). The pH of the culture media did not significantly decrease following the addition of 200 μM hinokitiol. The pH of the TSB medium (MRSA and *S. mutans*) before and after addition of hinokitiol was 7.42–7.41, respectively. The pH of the BHI medium (Aa) was 7.06–7.05, respectively, and the pH of the YPD medium (Ca) was 6.21–6.19, respectively.

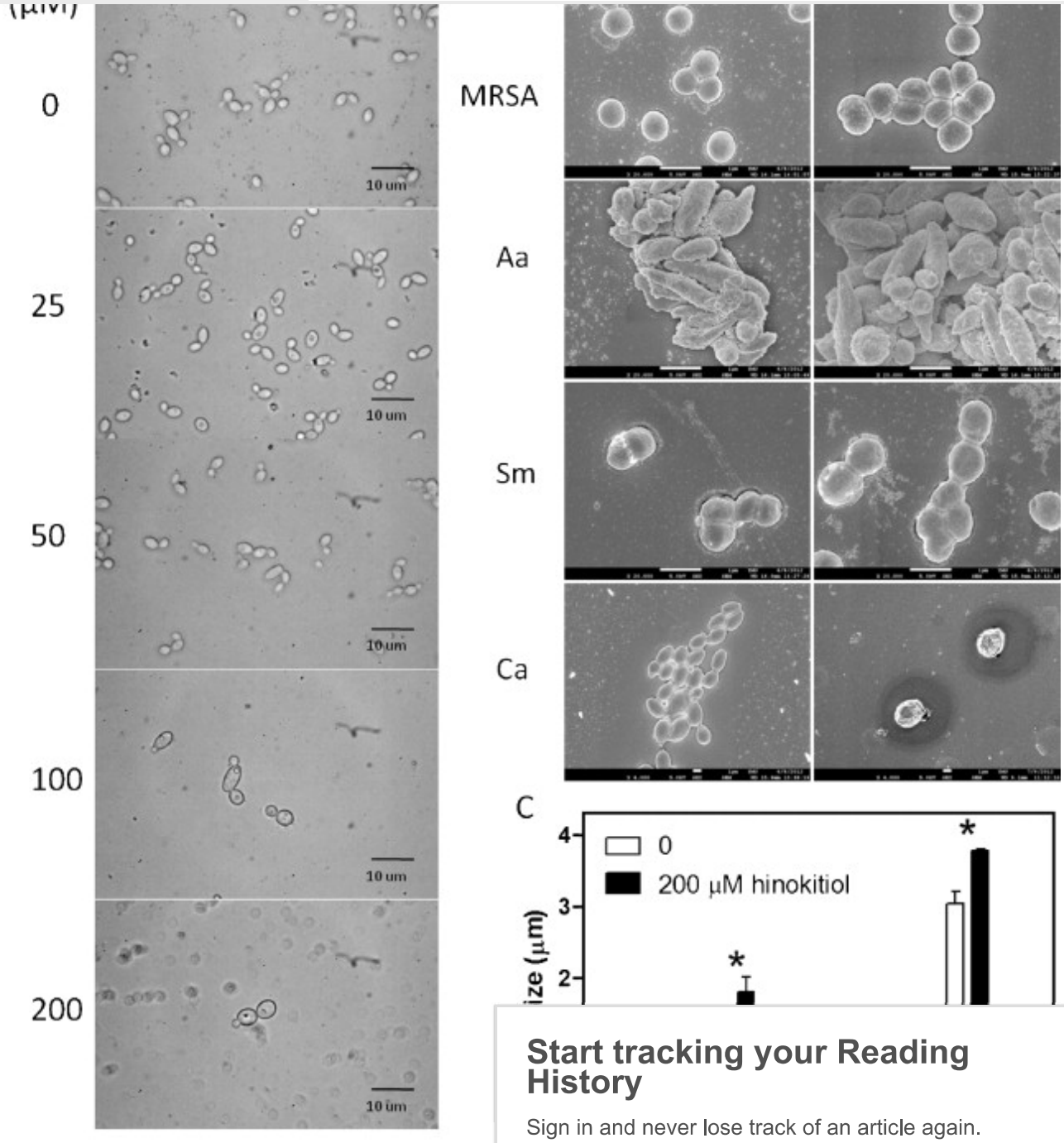

Fig. 3. Morphological examination using scanning electron microscopy. (A) The morphology of untreated cells is represented in the left panel, and cell morphology following treatment with 200 μM hinokitiol is shown in the right panel. The photographs of MRSA, *A. actinomycetemcomitans*, and *S. mutans* were taken at 20,000× magnification, and those of *C. albicans* were taken at 4000× magnification. (B) The diameter of the cells treated with and without the 200 μM hinokitiol treatment was analyzed using the Mann–Whitney test. The asterisk indicates $p < 0.05$, and the double asterisk indicates $p < 0.01$.

of agar plates tested (heat-killed MRSA in TSB agar, heat-killed *A. actinomycetemcomitans* in BHI agar, and heat-killed *S. mutans* in TSB agar). The MRSA bacteria displayed high autolysin activity in all three agar types. We considered that the high autolysin activity observed for the MRSA bacteria may have been the result of higher cell density, compared with that of the *A. actinomycetemcomitans*, and *S. mutans*, cells, because the MRSA replication rate was relatively higher. However, inoculation with one-eighth and one-half the volume of the original MRSA inoculum did not decrease the autolysin radius (data not showed). There were no significant differences observed between the data collected at 24 and 48 h (Fig. 4).

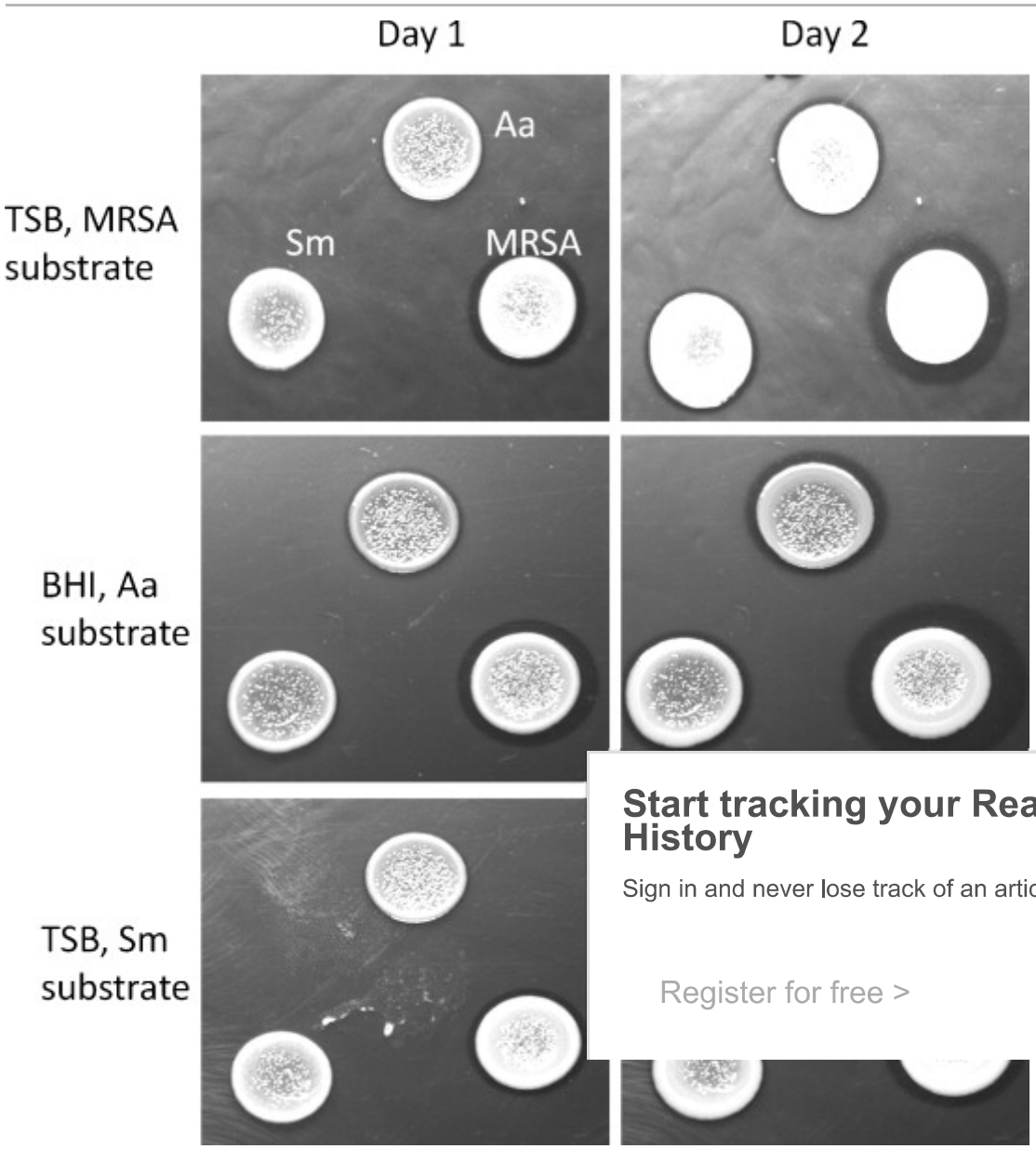

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Fig. 4. Autolysin activity tests of MRSA, *A. actinomycetemcomitans*, and *S. mutans*. The panels represent results on day 1 (left) and day 2 (right). Heat-killed MRSA, *A. actinomycetemcomitans*, and *S. mutans* cells were used as the substrates. The MRSA was inoculated on the lower-right

3.5. OSCC were more sensitive to hinokitiol than OK

The results of the MTT assay analysis of cell viability for the OSCC cell lines HSC3, SAS, and SCC4 showed that treatment with 200 μM hinokitiol resulted in growth rates of 15%, 45%, and 30%, respectively. However, the OK cell viability was 65% following hinokitiol treatment, indicating that the effect of hinokitiol was significantly greater in the cancer cells. In addition, although the CHX control treatment displayed a greater cytotoxic in OK cells than in the OSCC cell lines (Fig. 5A and B).

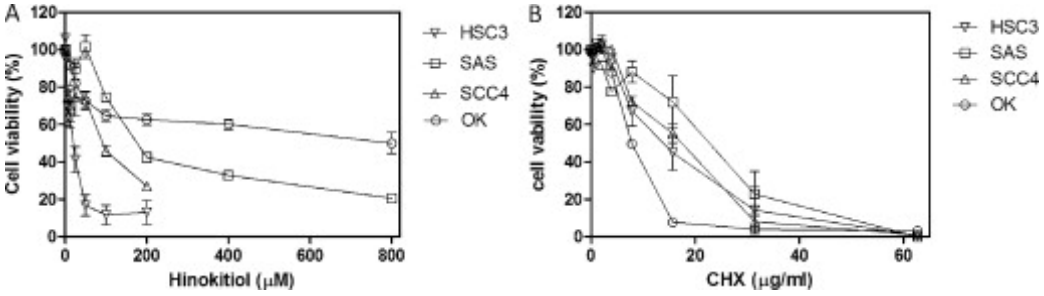

Fig. 5. Cytotoxicity for hinokitiol and CHX treatments of OK and OSCC cell lines, HSC3, SAS, and SCC4. (A) hinokitiol, MTT test, (B) CHX, MTT test.

4. Discussion

The MIC and MMC of hinokitiol obtained fo
different methods that were used for the eval
the result of different incubation conditions,
liquid culturing methods, culture volume, th
methods, and aeration. For example, the brot
15 mL tube but the kinetic growth curve anal
recording in 96-well plates. Shaking of culture increases dissolved oxygen and increases the growth of aerobic microorganisms. However, although facultative anaerobes grow well under such conditions, growth rates may be suboptimal, compared with anaerobic conditions.

Numerous antibiotics were recommended at higher dosages for patients with serious infections. That can cause a theoretical risk if the antibiotic is PIP positive. We investigated hinokitiol MIC, MMC, and PIP dosage in oral pathogens for this reason. All 3 methods demonstrated a significant PIP for both *A. actinomycetemcomitans* and *S. mutans* and the MIC and MMC values were similar for each. However, although MRSA did not exhibit the PIP in

was not observed using the agar/broth dilution methods. The values of the CHX MIC and MMC for MRSA, *A. actinomycetemcomitans*, *S. mutans*, and *C. albicans* were similar to those reported in previous studies (Holbrook and Kippax, 1979, Meurman et al., 1989, Sheng et al., 2009), which validates our results.

The PIP has been reported for penicillin, caspofungin, and hinokitiol; however, the mechanism of the PIP for these antimicrobials is unknown (Eagle, 1948, Arima et al., 2003, Stevens et al., 2004). One explanation is that a high-concentration antimicrobial agent might be self-antagonising the receptor, such as penicillin. An antimicrobial agent could possibly precipitate out of the solution so that the antibacterial activity is either not seen or the colorimeter is detecting crystals of the antimicrobial. The possibility of hinokitiol precipitates has been excluded because the OD600 of BHI containing 5–200 μM hinokitiol without *A. actinomycetemcomitans* did not significantly increase (OD600 = 0.000–0.002) and no precipitates were observed after centrifuging at 3000 rpm for 5 min in our study (data not showed). The PIP was obvious in the gram-positive (MRSA and *S. mutans*) and gram-negative bacteria (*A. actinomycetemcomitans*). This suggests that the PIP of hinokitiol was not associated with gram-stain classification.

Autolysins are naturally produced by peptidoglycan-containing bacteria. Peptidoglycan hydrolytic autolysins are critical for separating daughter cells after cell division (Zoll et al. 2010). Excessive autolysin degrades the peptidoglycan matrix and causes the cell to burst because of osmotic pressure (Blackman et al. 1998). Fine-tuned spatial and temporal control of autolysins could maintain the peptidoglycan network and prevent suicidal cell death (Rice and Bayles 2003). Autolytic defects often lead to antibiotic tolerance (Mitchell and Tuomanen 2002). *Enterococcus faecalis* treated with penicillin displayed the PIP, and the PIP was associated with autolysin activity (Fontana et al. 1990). The autolysin activity observed in our study was highest for MRSA and lowest for *A. actinomyc* relationship between autolysin activity and th that of the previous penicillin study (Fontana

Hinokitiol reduced *E. coli* IFO 3301 and *S. au* synthesis, and protein synthesis (Morita et al. 1 (CYR1), and RAS1, which function in the re *efg1* and *cph1* were regulated by RAS signal transmission, and they might be inhibited by hinokitiol to block *C. albicans* hyphal growth transition (Brown and Gow 1999). However, whether hinokitiol treatment blocks *C. albicans* hyphal from formation because of *efg1* and *cph1* gene down-regulation requires further investigation in the future.

The PIP of hinokitiol might have implications for long-term antimicrobial treatments for oral disease prevention because high doses of hinokitiol are progressively diluted by saliva over time, resulting in a subsequent, second antimicrobial treatment at the lower concentration of

CHX is commonly used in mouthwash to reduce dental plaque and oral bacteria, but it is harmful at high concentrations. In our cytotoxicity tests, the OK cells were more sensitive to CHX than the OSCC cell lines. However, the OK cells were less sensitive to hinokitiol, compared with the significant reductions in the viability in all the OSCC cell lines that were observed following hinokitiol treatment, according to the results of the MTT assays. This suggests a high degree of safety for potential uses of hinokitiol for oral disease prevention and therapy. In addition, as an anticancer agent that demonstrates specific inhibition of cancer cell growth and low cytotoxicity in normal cells, the combination of the anti-cancer and the anti-bacterial properties of hinokitiol indicate that it may be useful for the treatment of oral cancer patients following surgery. Hinokitiol has been used in oral health care products, such as toothpaste, mouth wash, and oral care gel; however, hinokitiol must still be analyzed carefully in greater detail before clinical application in oral disease prevention and therapy.

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