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The Inhibitory Activity of Citral against *Malassezia furfur*

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Abstract: The lipophilic yeast *Malassezia furfur*, is a member of the cutaneous commensal microbiota and is associated with several chronic diseases such as dandruff, pityriasis versicolor, folliculitis, and seborrheic dermatitis, that are often difficult to treat with current therapies. The development of alternatively effective antifungal therapies is therefore of paramount importance. In this study, we investigated the treatment effect of citral on *M. furfur*. The minimal inhibitory concentration of citral for *M. furfur* was 200 μg/mL, and the minimal fungicidal concentration was 300 μg/mL. Citral significantly increased the proportion of yeast cells to mycelial forms 2.6-fold. Phosphatidylserine externalization, DNA fragmentation, and metacaspase activation supported a citral-induced apoptosis in *M. furfur*. Moreover, citral at sub-minimum inhibitory concentrations reduced the invasion of *M. furfur* in HaCaT keratinocytes. Finally, we demonstrated that citral inhibited IL-6 and TLR-2 expression and enhanced HBD-2 and TSLP expression in *M. furfur*-infected HaCaT keratinocytes. These results showed that citral has antifungal activity at high concentrations and can decrease the infection of *M. furfur* by modulating the keratinocyte immune responses at low concentrations. Our results suggest that citral is a potential candidate for topical therapeutic application for *M. furfur*-associated human skin diseases.

Keywords: *Malassezia furfur*; citral; yeast apoptosis; human keratinocyte; immunomodulation

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1. Introduction

The lipophilic yeast *Malassezia furfur* is a member of the cutaneous commensal microbiota of human skin, and is found particularly in areas rich in sebaceous gland content. *M. furfur* infection can result in several chronic superficial dermatitis such as dandruff, pityriasis versicolor, seborrheic dermatitis, folliculitis, and atopic dermatitis. *M. furfur* is a dimorphic fungus that can alter its morphology from a unicellular yeast form to a mycelial form. *M. furfur* primarily shows a yeast form in normal conditions and standard cultures. However, it transforms into a mycelial form under some stimulations which play a predominant role in the pathogenesis of some diseases, such as pityriasis versicolor [1–5].

Several azoles, such as ketoconazole (KTZ) in solutions, creams, gels, and shampoo forms are used in routine clinical treatment of *M. furfur*. However, KTZ demonstrates toxicity to mammalian cells and may cause urticarial [6]. *M. furfur*-related diseases are often refractory to therapy and require extended use of antifungal and anti-inflammatory medications, which may lead to drug resistance [7–9]. Therefore, finding a safe, effective, and side-effect-free treatment is required.

Plants and their derivatives are known sources of a variety of biologically active components. They have great potential due to their low cost, low toxicity, and safety. Previous studies have shown that extracts or purified ingredients from *Trigonella foenum-graecum*, *Asparagus racemosus*, *Hypericumper foratum*, *Dittrichia viscosa*, and *Vitis vinifera* had

treatment effects on *Malassezia* spp. infections [10–14]. *Cymbopogon citratus*, a herb and perennial tropical grass commonly known as lemon grass, is widely used in mid-tropical countries such as Southeast Asia, South America, and Africa as food seasoning and perfume material and as herbal medicines for its analgesic and anti-inflammatory properties [15]. Citral (3,7-dimethyl-2,6-octadienal) is the major constituent of *Cymbopogon citratus* and is classified as a "generally recognized as safe" (GRAS) substance and is used in food, perfume, and cosmetics and as a pharmaceutical component because of its lemon-like flavor. Citral is a mixture of two isomeric acyclic aldehydes, geranial (*trans*-citral, citral A) and neral (*cis*-citral, citral B). Several studies have demonstrated that citral possesses antifungal, antimicrobial, and anti-inflammatory activities [16,17]. The antifungal activity exerted by citral has been demonstrated in varied conditions. Recently, it has been shown that citral can destroy the integrity of the cell membrane. Citral could also exert its antifungal effect by inhibiting ergosterol biosynthesis and mycelial growth. Citral inhibits fungal growth by damaging oxidative phosphorylation and cell membranes through massive ROS accumulation [12,16]. Citral could be added to many finished products. The maximum acceptable concentrations of citral in finished products are reviewed in the literature. For example, the maximum acceptable concentration of citral in hand-cream products is about 0.15%; in products applied to the hair with some hand contact is about 0.2%; and in products with body and hand exposure but which are primarily rinsed-off is about 1.2% [18].

Keratinocyte are a primary cell type in the epidermis that forms an essential barrier against invading microorganisms. Keratinocytes are also involved in innate immune defense mechanisms. Previous studies have shown that keratinocytes induces the production of pro-inflammatory cytokines by co-culturing with *Malassezia* yeasts [19,20]. Keratinocytes also produce different antimicrobial peptides, such as the β defensins family, contributing to host defense against microorganisms.

Cymbopogon citratus possesses various pharmacological activities, but little is known about the immunomodulating effects on keratinocytes. Although several studies have demonstrated that citral possesses antifungal, antimicrobial, and anti-inflammatory activities [16,17], no studies have investigated its antifungal activity and action mechanism against *M. furfur*. Citral's yeast apoptotic process has been reported in common disease-causing yeasts such as *Candida albican* [3,21–23], but not in *Malassezia* spp. Therefore, this study aimed to investigate the inhibitory activity of citral against *M. furfur* and its immunomodulatory effect on keratinocytes in vitro.

2. Materials and Methods

2.1. Materials

Citral (Sigma-Aldrich, St. Louis, MO, USA) with 96% purity was dissolved in dimethyl sulfoxide (DMSO) to prepare the stock solution just before performing the assays. The antifungal drug KTZ (Sigma-Aldrich) stock solution was prepared using DMSO as the solvent and stored at −20 °C. The final concentration of DMSO in every assay was 1%.

2.2. Microorganism and Cultivation

M. furfur BCRC 22,243 was purchased from the Bioresource Collection and Research Center (BCRC; Taiwan). To prepare budding yeast suspensions, the strain was grown in modified Dixon (mDixon) medium (3.6% malt extract, 2.0% desiccated ox bile, 0.6% peptone, 1.0% tween 40, and 0.2% oleic acid, pH 6.0) and incubated for three days at 30 °C. For the human keratinocyte invasion experiment, *M. furfur* was cultured on agar plates of modified Leeming and Notman (mLNA) agar (1% peptone, 1% glucose, 0.2% yeast extract, 0.8% desiccated ox bile, 1% glycerol, 0.05% glycerol monostearate, 0.5% tween 60, 2% olive oil, and 1.5% agar, pH 6.0) for three days at 30 °C [24].

2.3. Antifungal Susceptibility Tests

The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of citral to *M. furfur* were determined using the microdilution broth method. Serial

two-fold dilutions of citral were made in 24-well microtiter plates to obtain concentrations of 1.0 to 1000 µg/mL. *M. furfur* was suspended in mDixon medium to the final density of 5×10^5 CFU/mL. Each well was inoculated with 0.5 mL of the inoculum suspension and incubated at 30 °C for 48 h. KTZ was utilized as the control drug. The MIC was defined as the lowest drug concentration that would inhibit the visible growth of a microorganism after incubation. MFC was determined as the lowest drug concentration that killed >99.9% of the initial inoculum. To determine MFC, 50 µL from each well showing no growth was spread on the mDixon plates and incubated at 30 °C for 72 h [25].

In the time–killing assay, yeasts were treated with different concentrations of citral. After incubation at various time points, the cells were plated out on mDixon plates for viable counts.

2.4. Morphological Analysis

M. furfur cells were stained with lactophenol cotton blue (LPCB) staining after being treated with different concentrations of citral for 48 h at 30 °C. The proportions of yeast to mycelial conversion of *M. furfur* treated with various concentrations of citral were determined using a light microscopy at 1000× magnification [7].

2.5. Analysis of Apoptosis Markers

2.5.1. Phosphatidylserine Externalization

For early-stage apoptotic marker analysis, phosphatidylserine externalization was determined using the Annexin V-FITC apoptosis detection kit (BD Pharmingen, San Jose, CA, USA). *M. furfur* yeast cells (1×10^6 CFU/mL) were harvested by centrifugation, and washed and digested with lysing enzyme (20 mg/mL) and lyticase (50 U/µL) in 0.1 M potassium phosphate buffer (PPB; pH 6.0) containing 1 M sorbitol for 2 h at 30 °C. Protoplasts of *M. furfur* were incubated with citral or H_2O_2 for 2 h at 30 °C, washed and resuspended in annexin binding buffer. Cells were incubated with 5 µL/mL of annexin V-FITC and propidium iodide (PI) for 20 min. Flow cytometry was performed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) [26].

2.5.2. TUNEL Assay

DNA fragmentation was analyzed by the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay using APO-DIRECT™ kit (BD Pharmingen) to observe late stage of apoptosis. Cells (1×10^6 CFU/mL) were treated with citral for 24 h at 30 °C, then fixed and protoplasted, labeled with DNA labeling solution, and stained with PI. Cells were then analyzed with a FACSCalibur flow cytometer [27].

2.5.3. Metacaspase Activation

Activated metacaspases in *M. furfur* were measured using the CaspACE FITC-VAD-FMK In Situ Marker (Promega, Madison WI). Cells (1×10^6 CFU/mL) were treated with citral or H_2O_2 for 3 h at 30 °C. The cells were washed in PBS, suspended in a staining solution containing 10 µM FITC-VAD-FMK, incubated for 20 min at room temperature in the dark, and analyzed with a FACSCalibur flow cytometer [28].

2.6. Cell Culture

A human keratinocyte (HaCaT) cell line was cultured as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 µg/mL streptomycin at 37 °C with 5% CO_2. The medium was changed every 2 days.

2.7. MTT Assay

The cytotoxic effect of citral on HaCaT cells was determined using MTT assay. HaCaT cells (5×10^5 cells/mL) were treated with 25–800 µg/mL citral at 37 °C for 48 and 72 h. The medium was removed and the cells were washed once with PBS; DMEM containing

0.5 mg/mL MTT was added, and the cells were incubated at 37 °C for 4 h. The absorbance was measured at 570 nm. The data are expressed as the percentage of viable cells compared to the 1% DMSO-treated control [2].

2.8. Treatment of HaCaT Cells with M. furfur in the Presence or Absence of Citral

HaCaT cells were infected with *M. furfur* at a ratio of 1:20 or 1:30 (HaCaT cells:yeasts), and treated with or without citral for 24 and 48 h. After treatment, cells were washed with PBS and stained with May–Grunwald and Giemsa stain, then examined under a light microscope. The percentage invasiveness was determined by counting the HaCaT cells that the *M. furfur* yeast had penetrated.

The infected cells were treated with or without DMEM containing 0.25 µg/mL KTZ for 4 h at 37 °C. After this period, cells were scraped and diluted in PBS and plated on mLNA plates. The plates were then incubated for 72 h at 30 °C, and colonies were counted (M1 cfu/mL). Yeast cells treated with KTZ were also counted (M0 cfu/mL). The adhesion percentage was then calculated as % adhesion = (M0 − M1/M0) *·100 [29].

2.9. ELISA Analysis

For measuring the secreted IL-6, IL-10, TNF-α, TGF-β, and TSLP, cell culture supernatants were collected and tested using the human cytokine ELISA kit (Invitrogen, CA, USA; TSLP from eBioscience) according to the manufacturer's instruction.

2.10. RNA Extraction and RT-PCR Analysis

Total RNAs were isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and RNeasy Mini Kit (Qiagen, Germantown, MD, USA) from the HaCaT cells treated and not treated with *M. furfur*, then cDNA was synthesized with the random hexamer primers using the RevertAid H Minus First Stand cDNA Synthesis Kit (Thermo Scientific, Madison, WI, USA). Real-time RT-PCR (ABI Step One real-time PCR system, Foster City, CA, USA) was used to analyze the mRNA expression of the target genes with the Maxima SYBR Green qPCR master mix kit (Fermentas, Waltham, MA, USA) according to the manufacturer's protocol. Table 1 summarizes the primer sets information and reaction conditions.

Table 1. Primer sets for real-time RT-PCR.

Gene	Sense and Anti-Sense Sequence	Size (bp)
TLR2	5′-TGTCTTGTGACCGCAATGGT-3′ 5′-TGTTGGACAGGTCAAGGCTTT-3′	101
TSLP	5′-TAGCAATCGGCCACATTGCC-3′ 5′-CTGAGTTTCCGAATAGCCTG-3′	145
HBD-2	5′-ATCAGCCATGAGGGTCTTGT-3′ 5′-GAGACCACAGGTGCCAATTT-3′	172
GAPDH	5′-TGAACGGGAAGCTCACTGG-3′ 5′-TCCACCACCCTGTTGCTGTA-3	307

TLR2, Toll-like receptor 2 [2]; TSLP, thymic stromal lymphopoietin [30]; HBD-2, human beta-defensin 2 [31]; GAPDH, glyceraldehyde-3-phosphate dehydrogenase [32].

2.11. Statistical Analysis

Each experiment was performed at least in triplicate. Results were analyzed for statistical significance by unpaired Student's t-test using SigmaPlot v14. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Antifungal Activity of Citral against M. furfur

The MIC and MFC of citral against *M. furfur* were analyzed in triplicate using the microdilution method to determine the fungicidal activities of the citral. KTZ was used as a positive control. The MIC and MFC of citral were 200 µg/mL and 300 µg/mL, and

KTZ were 0.13 μg/mL and 0.25 μg/mL, respectively (Table 2). The MIC and MFC of citral against *M. furfur* were higher than those of known antifungal agents such as KTZ. The time–killing curves showed that the fungicidal activity of citral against *M. furfur* depended on its concentration (Figure 1). Citrate exhibited rapid killing during the first 2–4 h of the assay above MFC. Citral treatment also significantly increased the proportions of yeast cells to mycelial forms, by approximately 2.6-fold (Figure 2).

Table 2. The antifungal activity of citral and ketoconazole (KTZ) against *M. furfur*.

	MIC (μg/mL)	MFC (μg/mL)
Citral	200 ± 15	300 ± 25
Ketoconazole	0.13 ± 0.05	0.25 ± 0.09

All results are the means of three determinations.

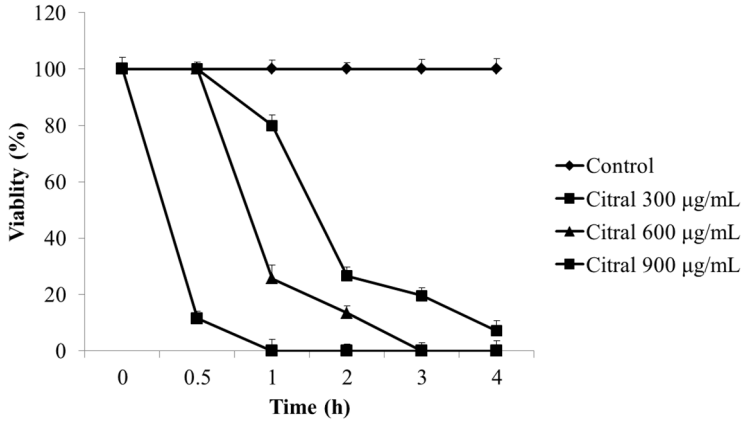


Figure 1. Time–killing curves of the citral against *Malassezia furfur*. *M. furfur* was treated with different concentrations of citral in the mDixon medium. After 1, 2, 3, and 4 h, the yeasts were spread on the mDixon plates and incubated for 72 h at 30 °C. The colony-forming units were counted and compared to control plates.

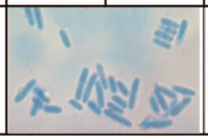
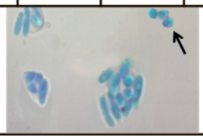
	Medium	1% DMSO	Citral (μg/mL)			
			75	100	150	200
Yeast	12%	13%	19.5%	23%	25.5%	31%
						

Figure 2. Citral increased the proportions of yeast cells to mycelial forms. *M. furfur* was treated with different concentrations of citral for 48 h at 30 °C. Yeast cells were stained with the LPCB method and examined at 1000× magnification using a light microscope.

3.2. Citral Induced Apoptosis in M. furfur

3.2.1. Phosphatidylserine Externalization

In combination with membrane-impermeable dye, PI, Annexin V-FITC stain, which binds to phosphatidylserine with high affinity in the presence of Ca^{2+}, was used to determine the citral-induced apoptosis in *M. furfur*. As shown in Figure 3, citral-treated *M. furfur* cells showed similar phosphatidylserine externalizations to H_2O_2-treated *M. furfur* cells in which H_2O_2 was an inducer of apoptosis in the yeast cells.

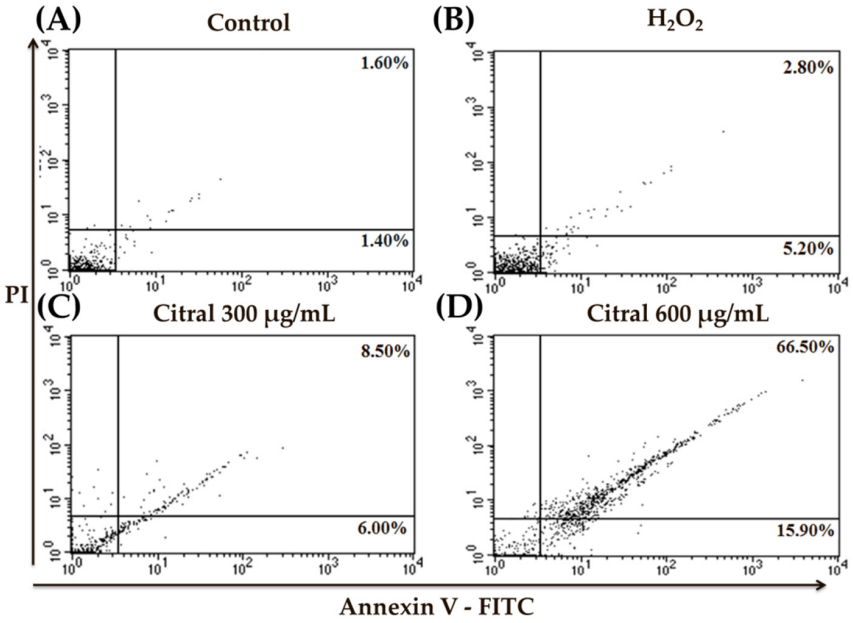


Figure 3. Effect of citral on externalization of phosphatidylserine at the cytoplasmic membrane. Phosphatidylserine externalizations were determined by Annexin V-FITC and PI staining in citral-or H_2O_2-treated *M. furfur* cells using a flow cytometer. (**A**) Control, (**B**) 10 mM H_2O_2 as positive control, (**C**) 300 µg/mL citral, and (**D**) 600 µg/mL citral.

3.2.2. DNA Fragmentation

DNA fragmentation is one of the late apoptotic phenotypes. To investigate whether citral induces the late stage of apoptosis in *M. furfur*, we evaluated DNA fragmentation using TUNEL assay and flow cytometry. TUNEL assay is a standard method for detecting apoptotic DNA cleavage in individual nuclei by labeling the fluorescent dUTP at the 3′-OH ends of DNA. We found that *M. furfur* cells exposed to citral and H_2O_2 showed increased fluorescence intensity compared to untreated cells (Figure 4).

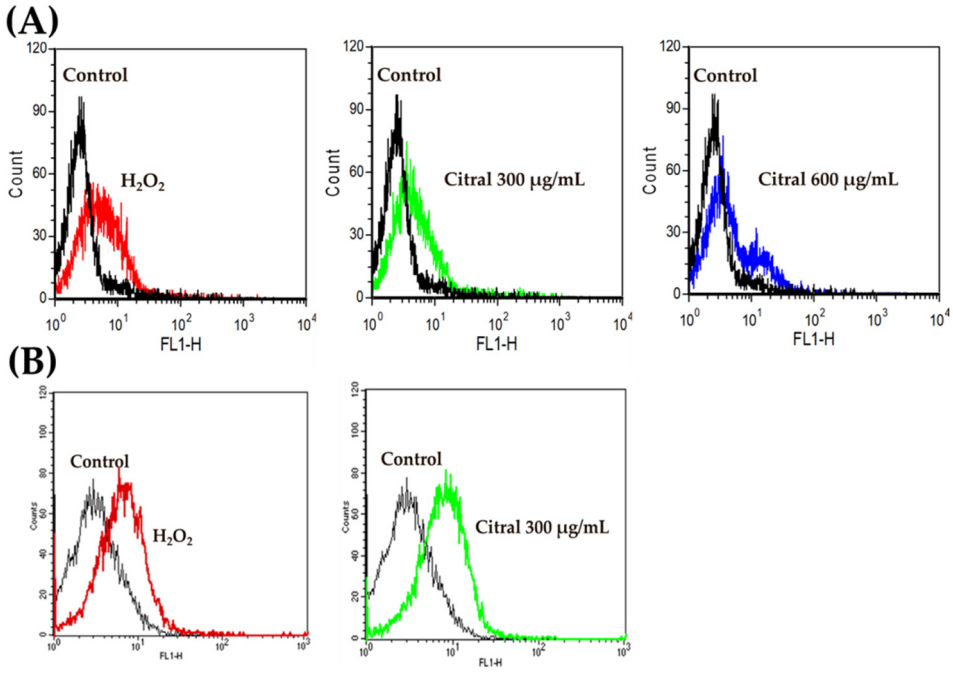


Figure 4. Effect of citral-induced DNA fragmentation in *M. furfur* cells. Cells were treated with citral or H_2O_2 for (**A**) 3 h and (**B**) 24 h. Cells were stained with TUNEL staining and analyzed using flow cytometry.

3.2.3. Metacaspase Activation

The activation of metacaspase plays a vital role in apoptosis. Citral-treated *M. furfur* cells were incubated with FITC-labeled VAD-FMK to monitor the metacaspase activation. The FITC-labeled caspase inhibitor VAD-FMK, a cell-permeable fluorescent maker, binds specifically to the active center of metazoan caspases in apoptotic cell. As shown in Figure 5, cells treated with citral showed increased fluorescence intensity, consistent with the positive control cells treated with H_2O_2. These results suggest that citral induces the biochemical feature of apoptosis in *M. furfur* cells, including membrane depolarization, DNA fragmentation, and metacaspase activation.

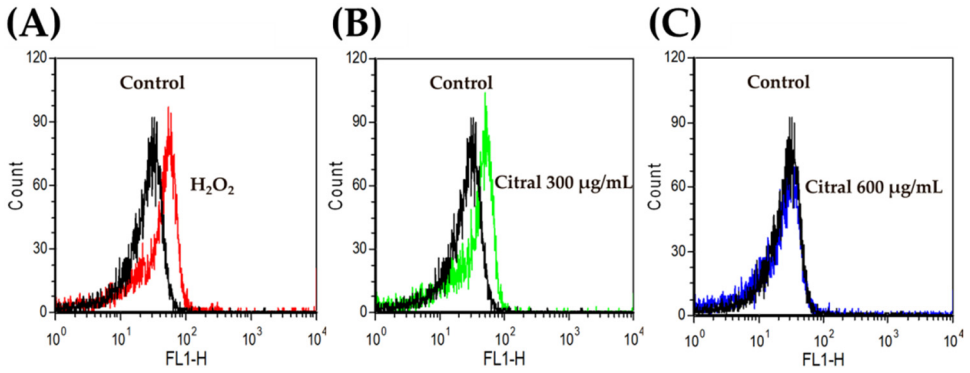


Figure 5. Effect of citral on the activity of metacaspase in *M. furfur* cells. Cells were stained with FITC-VAD-FMK, and analyzed using a flow cytometer. (**A**) 10 mM H_2O_2, (**B**) 300 μg/mL citral, and (**C**) 600 μg/mL citral.

3.3. Citral Can Decrease the Adhesiveness and Invasiveness of M. furfur to Human Keratinocytes at Sub-MIC

3.3.1. The Cytotoxic Effect of Citral on HaCaT Cells

For determine the cytotoxic effects in HaCaT cells, cell viabilities were measured using an MTT assay. After treatment with different concentrations of citral, the viability percentages of HaCaT cells were >98% (Figure 6). These results demonstrate that citral has no cytotoxic effect in HaCaT cells at the antifungal concentrations.

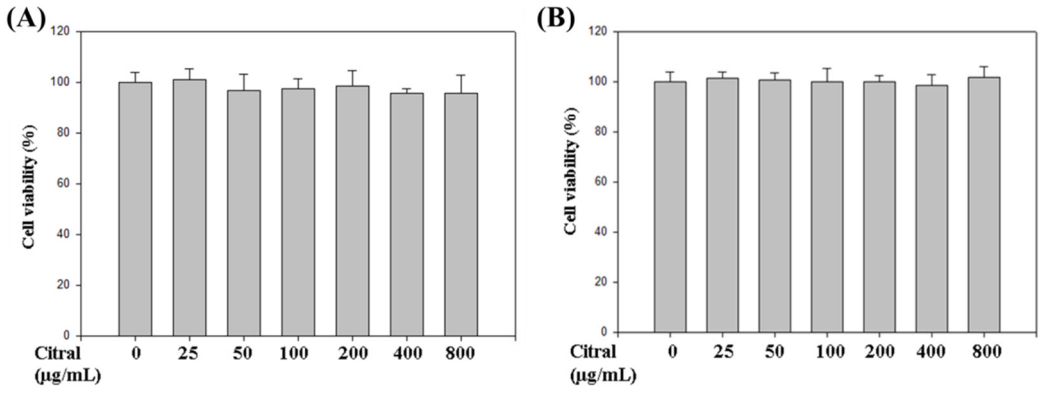


Figure 6. Cell viability of HaCaT cells to citral. HaCaT cells were treated with different concentrations of citral for (**A**) 48 h and (**B**) 72 h. An MTT assay was used to determine the cell viabilities.

3.3.2. The Inhibitory Effect of Citral on *M. furfur* Adhesion to HaCaT Cells

For determining whether citral could interfere with *M. furfur* invasion, the HaCaT cells were treated with different concentrations of citral and infected with *M. furfur* (1:20 or 1:30, cells:yeasts) for 24 h. We can found that at low concentrations (25, 50 μg/mL), citral can inhibit the adhesion of *M. furfur* to HaCaT cells and the inhibition was more apparent at higher concentrations (100, 200 μg/mL) compared with the control (Figure 7).

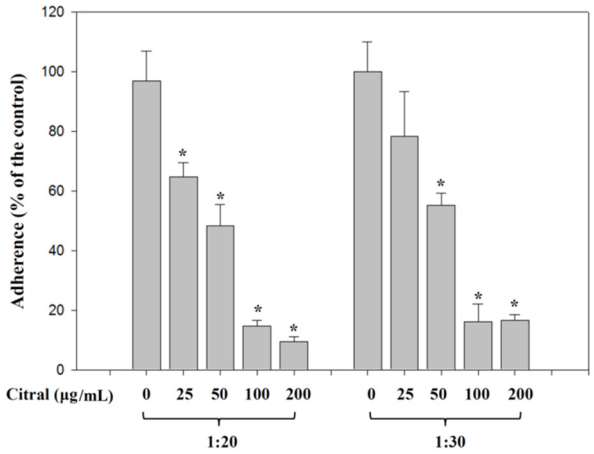


Figure 7. The effect of citral against *M. furfur* adherence on HaCaT cells. HaCaT cells were cultured with citral and *M. furfur* at a ratio of 1:20 or 1:30 (HaCaT cells:yeasts). After 24 h, the infected cells were treated with or without DMEM containing 0.25 μg/mL ktz for 4 h. The adhesion percentage was then calculated as % adhesion = (M0 − M1/M0) *·100. (M1,CFU of treated with various citral concentrations; M0, CFU of treated with ktz). Adherence differences were determined using the unpaired Student's *t*-test (* $p < 0.05$).

3.3.3. The Inhibitory Effect of Citral on *M. furfur* Invasiveness into HaCaT Cells

The invasion of *M. furfur* to HaCaT cells was monitored using May−Grunwald and Giemsa staining. Some HaCaT cells showed yeast engulfment in the negative-control group and low-concentration citral-treatment groups (Figure 8A). Cell invasiveness of 15% and 30% were determined at HaCaT cells to yeasts ratios of 1:20 and 1:30 (Figure 8B). Like the adhesion, citral reduced the invasion of *M. furfur* to HaCaT cells (Figure 8C).

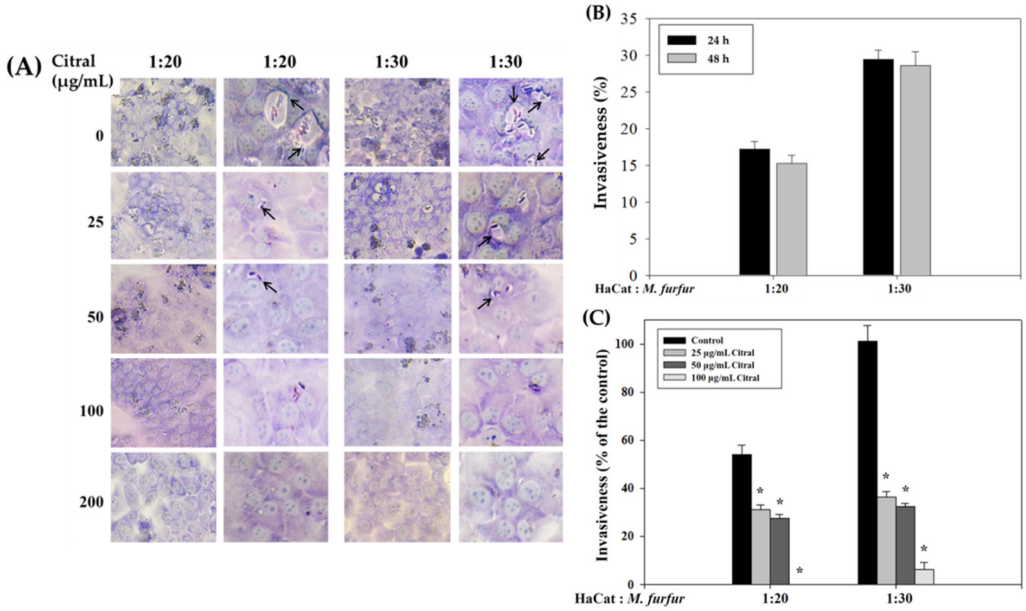


Figure 8. The effect of citral against *M. furfur* invasion on HaCaT cells. HaCaT cells treated with citral and *M. furfur* at a ratio of 1:20 and 1:30 (HaCaT cells:yeasts) for 24 and 48 h. (**A**) HaCaT cells were stained with the May–Grunwald and Giemsa method after incubation with yeast. Some cells show yeast engulfment. (**B**,**C**) Yeasts were taken up into HaCaT cells after being treated different concentrations of citral for 24 and 48 h. The percentage of invasiveness was determined by counting the HaCaT cells that the *M. furfur* yeast had penetrated. The negative control group (cells:yeasts ratio of 1:30) was set as 100%. Differences in adherence were determined using the unpaired Student's *t*-test (* $p < 0.05$).

3.4. Citral Can Modulate the Immune Response of HUMAN keratinocytes and Interfere with M. furfur Infection

3.4.1. TLR2, HBD-2, and TSLP Gene Expression

We next examined the effect of citral on the mRNA expression of TLR2, HBD-2, and TSLP by real-time RT-PCR in the *M. furfur*-infected HaCaT cells. HaCaT cells were treated with *M. furfur* (cells:yeasts of 1:30) at different citral sub-MICs. The TLR2 mRNA expression was 30-fold higher in *M. furfur*-treated HaCaT cells at 8 h treatment. In contrast, when cells were cotreated with *M. furfur* and citral, the increased TLR2 mRNA expression was significantly downregulated by citral (Figure 9A), and the TLR2 mRNA expression downregulations were also found after 24 h treatment (Figure 9B). Moreover, both citral and *M. furfur* induced HBD-2 production, with the strongest effect occurring in *M. furfur*-infected cells after 24 h treatment (Figure 10). Finally, we tested the effect of citral on TLSP mRNA expression. The TSLP mRNA expression increased in the *M. furfur*-infected cells, and citral had a more apparent effect on TSLP transcript after 24 h treatment (Figure 11). These results demonstrate that citral efficiently inhibits the *M. furfur*-induced TLR2 mRNA expression and enhances HBD-2 and TSLP mRNA expression in HaCaT cells.

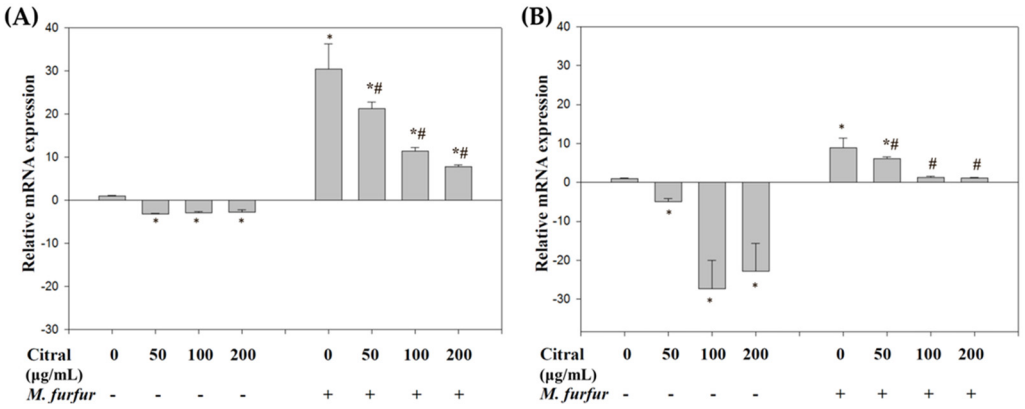


Figure 9. The gene expression of TLR2 in citral against *M. furfur*-infected HaCaT cells. HaCaT cells were cotreated with citral and *M. furfur* at a cells to yeasts ratio of 1:30 for (**A**) 8 h and (**B**) 24 h. Cells were scrap-harvested and quantitatively analyzed using real-time RT-PCR to determine the TLR2 mRNA expression. All values are expressed as mean ± SD. Differences in the expression of TLR2 were determined using unpaired Student's *t*-test (* $p < 0.05$). (* means comparison with non-treatment control, # means comparison with *M. furfur*-infected cells.).

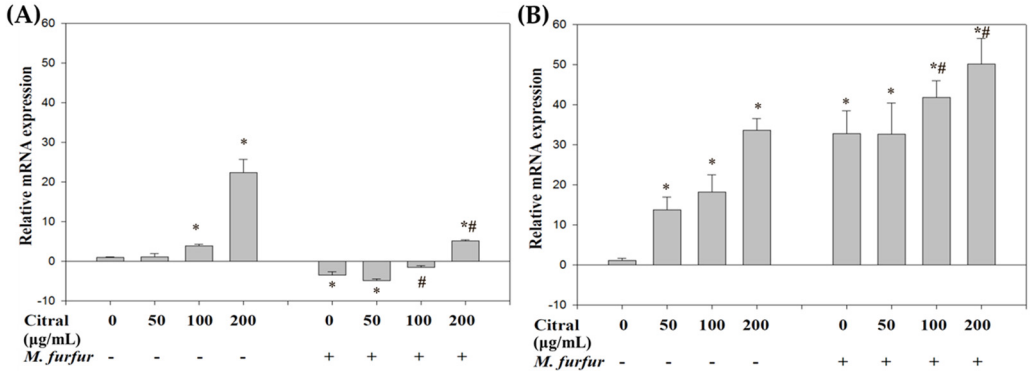


Figure 10. The gene expression of HBD-2 in citral against *M. furfur*-infected HaCaT cells. HaCaT cells were cotreated with citral and *M. furfur* at cells to yeasts ratio of 1:30 for (**A**) 8 h and (**B**) 24 h. Cells were scrap-harvested and quantitatively analyzed using real-time RT-PCR to determine the HBD-2 mRNA expression. All values are expressed as mean ± SD. Differences in the expression of HBD-2 were determined using unpaired Student's *t*-test (* $p < 0.05$). (* means comparison with non-treatment control, # means comparison with *M. furfur*-infected cells.).

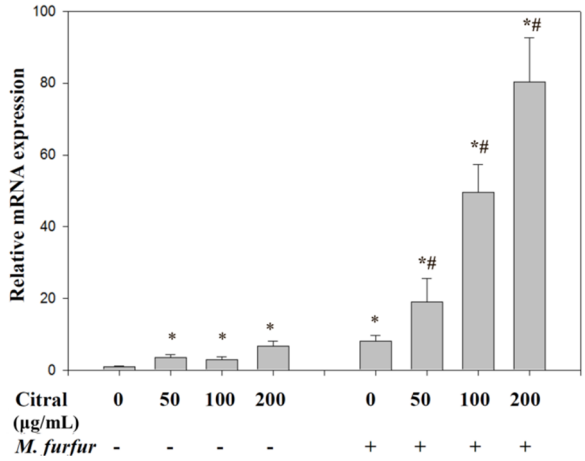


Figure 11. The gene expression of TSLP in citral against *M. furfur*-infected HaCaT cells. HaCaT cells were cotreated with citral and *M. furfur* at cells to yeasts ratio of 1:30 for 24 h. Cells were scrap-harvested and quantitatively analyzed using real-time RT-PCR to determine TSLP mRNA expression. All values are expressed as mean ± SD. Differences in TSLP expression were determined using unpaired Student's *t*-test (* $p < 0.05$). (* means comparison with non-treatment control, # means comparison with *M. furfur*-infected cells.).

3.4.2. Cytokine Expression Assay

To determine how citral interferes with *M. furfur*, we examined the expressions of proinflammatory cytokines IL-6 and TNF-α, and anti-inflammatory cytokines IL-10 and TGF-β using ELISA. IL-6 levels increased in *M. furfur*-infected HaCaT cells after 24 h of treatment. In contrast, a significant decrease in IL-6 level was observed when the cells were treated with citral (Figure 12). The results clearly demonstrated that citral inhibits the proinflammtory cytokine IL-6 expression induced by *M. furfur* in HaCaT cells. However, expression differences in TNF-α, IL-10, and TGF-β were not detectable (data not shown).

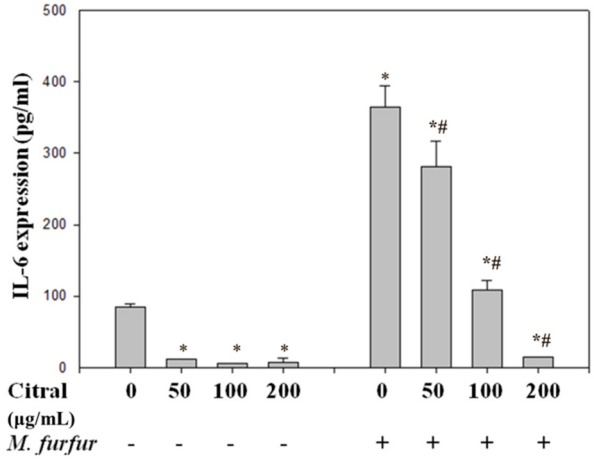


Figure 12. IL-6 expression in citral against *M. furfur*-infected HaCaT cells. The secretion of IL-6 induced by the *M. furfur*-infected HaCaT cells was measured using ELISA. Cell supernatants were collected at 24 h after infection with *M. furfur* in the presence or absence of citral. The negative control was the non-treated cells. The results are means of triplicate experiments. All values are expressed as mean ± SD. Differences in IL-6 expression were determined using unpaired Student's *t*-test (* $p < 0.05$). (* means comparison with non-treatment control, # means comparison with *M. furfur*-infected cells.).

4. Discussion

Malassezia spp. yeasts are dimorphic fungi and normal fungal skin flora in humans and other warm-blooded animals. They can cause skin-related diseases through different

mechanisms. Killing bacteria or bacterial inhibiting was the core concept of antibiotic development and an essential milestone in medicinal chemistry. Destroying critical components during bacterial growth is an efficient antibacterial strategy. Although clinical studies have confirmed the azoles such as ketoconazole (KTZ) effectively treat *Malassezia*-related skin diseases, the long-term safety of antifungal drugs remains unknown [7]. Toxicity, low efficacy, and drug resistance of the antifungal drugs have limited their clinical usage. Besides, they are costly when an extended treatment time is necessary and many patients leave the therapy before being cured. One way to prevent antibiotic resistance of pathogenic species is to find and use new compounds [3,21].

Both mycelial and yeast forms have been found in several diseases believed to be caused by *M. furfur*. Our study showed that citral has lower antifungal activity below sub-MIC but can block the yeast to mycelia transformation of *M. furfur*, although the mechanism and effect remained unclear.

In the early phases of apoptosis, phosphatidylserine is translocated from the inner leaflet of the plasma membrane bilayers to the outer leaflet. Phosphatidylserine exposure generally precedes DNA fragmentation and nuclear condensation. A putative caspase (metacaspase) has been shown to be involved in apoptosis. Our results suggested that citral at lower concentrations induces apoptosis in *M. furfur* yeast cells and necrosis at higher concentrations. The yeast apoptosis process has been found in common clinical yeast such as *C. albican*, but remains unclear in *M. furfur* [7,32–34].

In the current experiment, we chose the HaCaT cell line to examine the effect of citral on *M. furfur*-infected cells. At first, *M. furfur* cultured in the mDixon medium barely infected the HaCaT cells. *Malassezia* spp. has cell walls with a very thick multi-layered structure, and the lipid compositions alter when cultivated in the different medium. The lipid layer of *Malassezia* spp. plays an essential role in modulating proinflammatory cytokine production by keratinocytes [35]. Therefore, we changed the mDixon medium to mLNA medium with higher lipid content to cultivate *M. furfur* for cell infection experiments and found that the infection rate of mLNA-cultured *M. furfur* in HaCaT cells increased significantly compared to that of mDixon-cultured *M. furfur*.

Toll-like receptors (TLRs) are crucial players in the innate immune responses to microbial invaders, TLRs and β-defensins are crucial elements in the innate immune response against bacteria, fungi, and viruses affecting the human skin and TLR2 is one of the main receptors on the cell surface that recognizes fungus [2]. Our study demonstrated that citral downregulates TLR2 gene expression in *M. furfur*-infected keratinocytes. In addition, citral also increased the expression of HBD-2 antimicrobial peptides in HaCaT cells. The antimicrobial peptides directly bind to the cell membranes of microorganisms and alter their permeability resulting in a bacteriostatic effect. Citral may stimulate HaCaT cells to secrete antimicrobial peptides, which inhibit *M. furfur* growth. The binding of HBD-2 may also prevent the binding of *M. furfur* to cells due to steric structural barriers.

In addition to recognizing foreign pathogens, TLR2 also initiates host innate immunity and affects subsequent adaptive immune responses. In recent studies, *Malassezia* stimulates human keratinocytes to produce cytokines and inflammatory molecules [20,36]. We found that citral inhibits IL-6 production in *M. furfur*-infected HaCaT cells. A previous study has shown that citral has an anti-inflammatory ability on lipopolysaccharide (LPS)-induced RAW 264.7 mouse macrophages and can inhibit the inflammatory cytokines molecules such as IL-6 and IL-10 [37]. Because the TLR2 precedes IL-6 and IL-8 pathways, it is reasonable to speculate that citral inhibits the TLR2 expression in HaCaT cells, resulting in a decrease in IL-6 secretion. Further research is required.

5. Conclusions

In conclusion, citral has antifungal activity at high concentrations and can block morphogenesis of *M. furfur* at low concentrations. Citral decreases the infection of *M. furfur* through modulation of the keratinocyte immune response. Citral modulates the *M. furfur* infection in HaCaT cells by inducing the secretion of HBD-2 antimicrobial peptides. In

addition, citral inhibits TLR2 expression and cell recognition of *M. furfur*. Citral also affects the expression of *M. furfur* surface adhesion factor, reducing the ability of *M. furfur* to adhere and aggregate. Our findings suggest that citral is a potential drug component to be formulated in therapeutics for *M. furfur*-associated human skin diseases.

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