Journal of Apiculture

2019.34(2) : 125-129
DOI: 10.17519/apiculture.2019.06.34.2.125

Original research article

# *In vitro* Antifungal Activity of 4-Hydroxyderricin and Acetylshikonin against *Ascosphaera apis*

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**Abstract** Honey bees are important pollinators in agriculture, but are threatened by the pathogen *Ascosphaera apis*, which causes chalkbrood. Despite attempts to control this fungus using synthetic fungicides, none of them have been proven to be completely effective. Among 640 natural compounds that we tested, 4-hydroxyderricin (MIC = 3.125, 6.25 mg/L after 24 h and 48 h growth, respectively) exhibited the strongest anti- *Ascosphaera apis* activity, followed by acetylshikonin (MIC = 12.5 mg/L for 24 h and 48 h growth). 4-Hydroxyderricin showed selective growth inhibition of *Ascosphaera apis* and *Rhizopus oryzae* among tested fungus strains. Treatment 4-hydroxyderricin with miconazole revealed a synergistic effect (FICI = 0.65±0.13 at 48 h incubation). These findings suggest that 4-hydroxyderricin, which has antifungal activity against *Ascosphaera apis* but few other fungal species, can effectively control infectious fungal diseases. Combined treatment of bees with 4-hydroxyderricin and miconazole could reduce cytotoxicity and improve the cost effectiveness of treatment.

**Keywords** Honey bee, *Ascosphaera apis*, 4-Hydroxyderricin, Acetylshikonin, Antifungal agent

## INTRODUCTION

Honey bees, *Apis mellifera* L., are the most economically valuable pollinators of many crops, fruits, and wild plants worldwide. Bee populations have been declining dramatically worldwide, and a major contributing factor is infection with the pathogenic fungus *Ascosphaera apis* which cause chalkbrood disease (Aronstein and Murray, 2010; Hedtke *et al*., 2011). Though *Asc*. *apis* does not destroy entire honey bee colonies, the chalkbrood disease can weaken the colony, reduce honey yields, and increase susceptibility to other bee pests and diseases. In addition, this pathogen causes a significant reduction in bee numbers and honey-bee related commercial productivity (Hedtke *et al*., 2011).

Even though the occurrence of chalkbrood disease has been increasing, there are no registered control systems or chemicals available. Burning all potentially contaminated hive material is the only effective means to destroy *Asc*. *apis* (Shin and Kim, 2016) because chemical treatments should be avoided due to the contamination of honey products and honey bees. In the recent years, secondary metabolites from plants have been screened as new drugs because of their potentially lower toxicity

http://journal.bee.or.kr/

Received 16 January 2019; Revised 15 February 2019; Accepted 19 February 2019
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to human and the environments than synthesized chemical compounds (Mourad *et al*., 2005; Kloucek *et al*., 2012; Shin and Kim, 2016).

This study suggested the enhanced antifungal effects of new compound candidates, 4-hydroxyderricin and acetylshikonin, aginst *Asc*. *apis*. Therefore 4-hydroxyderricin and acetylshikonin can be helped to prevent *Asc*. *apis* infection to the honey bees.

## MATERIAL AND METHODS

### *Ascosphaera apis* culture

*Asc*. *apis*, the causal agent of chalkbrood, was obtained and maintained as described previously (Shin and Kim, 2016).

### Strain maintenance

*Aspergillus niger* (KACC 42589), *Aspergillus clavatus* (KACC 40071), *Candida parapsilosis* var. *parapsilosis* (KACC 45480), *Rhizopus oryzae* (KACC 40256), and *Saccharomyces cerevisiae* (KACC 30068) were obtained from KACC (Korea Agricultural Culture Collection, Korea). *Candida albicans* (KCTC 7965, KCTC 7270), *Candida tropicalis* (KCTC 7212), *Candida tropocalis* var. *tropicalis* (KCTC 17762), *Candida glabrata* (KCTC 7219), *F. neoformans* var. *bacillispora* (KCTC 17528), and *Pichia guilliermondii* (KCTC 7211) were purchased from KCTC (Korea Collection for Type Cultures, Korea). *Saccharomyces cerevisiae* (BY4742) was purchased from Life Technologies (Carlsbad, USA). All fungi were maintained on Yeast Extract-Peptone-Dextrose (YPD) medium (Reinaldi *et al*., 2004; Subsomboon, 2012; Shin and Kim, 2016) at 35°C under a normal atmosphere. Growing fungi were monitored regularly for contamination and used in assays.

### *In vitro* evaluation of anti-fungal activity

Anti-fungal efficacy of the natural compounds was determined in vitro by broth microdilution assays, as described previously with slight modifications (Shin and Kim, 2016). Test compounds were dissolved in dimethyl sulfoxide (DMSO) to a 10 mg/mL concentration and then serially 2-fold diluted from 200 μg/mL to 0.2 μg/mL in 100 μL YPD growth medium (TaKaRa, Japan). Then, 100 μL of $OD_{600}=0.1$ *Asc*. *apis* spores were added to 96-well flat-bottomed microtitration plates (SPL, Seoul, Korea). Fungal spore suspensions without compounds were also included as negative controls. Plates were incubated for 24 h or 48 h at 35°C. The same test was repeated three times. Minimum inhibitory concentration (MIC) values were defined as the lowest concentration of the test compound for which no *Asc*. *apis* growth was observed.

### Evaluation of synergistic antifungal activity

To assess the synergistic antifungal activity of acetylshikonin and miconazole or 4-hydroxyderricin and miconazole, broth microdilution assays were performed in a checkerboard fashion (Shin and Kim, 2016). Miconazole was serially 2-fold diluted, and then acetylshikonin or 4-hydroxyderricin was also serially 2-fold diluted vertically and dispensed into 96-well flat-bottomed microtitration plates (SPL, Seoul, Korea) in a total of 100 μL of drug-containing YPD medium per well. Then, 100 μL of $OD_{600}=0.1$ *Asc*. *apis* spores was inoculated into each well of the 96-well flat-bottomed microtitration plates (SPL, Seoul, Korea). Final compounds concentrations ranged from 12.5 μg/mL to 0.1 μg/mL. MIC values of individual drug alone were determined on the same plate. $FIC_i$ equals the MIC of drug *i* in combination divided by the MIC of drug *i* alone. The FICI represents the sum of the FICs of each drug tested. Drug activity was classified as synergistic (FICI, $\leq 0.5$), additive (FIC, 0.5-1), indifference (FIC, 1-4) or antagonistic (FICI, $>4$). Tests were performed at least three times.

### Statistical analysis

The results are presented as the mean values ± SD. In order to determine the reproducibility of the measurements, each antifungal activity assay and cell cytotoxicity assay were carried out in triplicate.

## RESULTS AND DISCUSSION

We screened 640 compounds from NPBANK (http://www.npbank.kr, Korea) using a modified broth microdilution assay and found 32 compounds that inhibited the growth of *Asc*. *apis* (Table 1). 4-hydroxyderricin

(MIC of 3.125 and 6.25 mg/L after 24 h and 48 h treatment, respectively) showed the strongest anti-*Asc*. *apis* activity, followed by acetylshikonin (12.5 mg/L after 24 h and 48 h treatment) (Fig. 1, Table 1).

To determine if 4-hydroxyderricin inhibited the growth of fungi other than *Asc*. *apis*, we investigated the antifungal activity against several fungal species. 4-Hydroxyderricin specifically inhibited the growth of the fungi *Asc*. *apis* and *R*. *oryzae* (MIC of 0.78 mg/L for both 24 h and 48 h treatment) (Table 2). Acetylshikonin had low antifungal activity against *R*. *oryzae* and *S*. *cerevisiae*, but its effect was too weak to classify acetylshikonin as a growth inhibitor of these fungal species (Table 2). Although previously identified anti-microbial compounds were effective when first described, abuse of broad-spectrum anti-microbial compounds has increased the occurrence of antibiotic-resistant infections. Accordingly, there is demand for novel anti-microbial agents with a narrow spectrum of anti-microbial activity (Bax and Green, 2015).

4-Hydroxyderricin is a chalcone and one of the phytochemical components of *Angelica keiskei*. It has various biological activities including anti-inflammatory, anti-diabetic, anti-bacteria, and anti-tumor activities (Ohnogi, *et al*., 2012). 4-hydroxyderricin suppressed the growth of *Staphylococcus aureus* by inhibiting seryl-tRNA synthetase (Battenberg *et al*., 2013). The antifungal activity of this compound might be due to similar mechanisms, but this needs to be confirmed in future studies.

Acetylshikonin is a natural naphthoquinone derivative that is abundant in the roots of traditional medical herbs such as *Lithospermum erythrorhizon* and *Onosma* species. Previous studies have demonstrated that acetylshikonin is significantly cytotoxic toward various types of cancer cells (Cho and Choi, 2015). In addition, acetyl-

**Table 1.** Antifungal activity of hydroxyderricin and acetylshikonin against *Asc*. *apis* compared with miconazole, traditional antifungal drug

| Compound | MIC[a] value (mg/L) | |
|---|---|---|
| | 24 h | 48 h |
| 4-hydroxyderricin | 3.125 | 6.25 |
| Acetylshikonin | 12.5 | 12.5 |
| Miconazole | 1.56 | 1.56 |

[a]MIC = Minimum inhibitory concentration

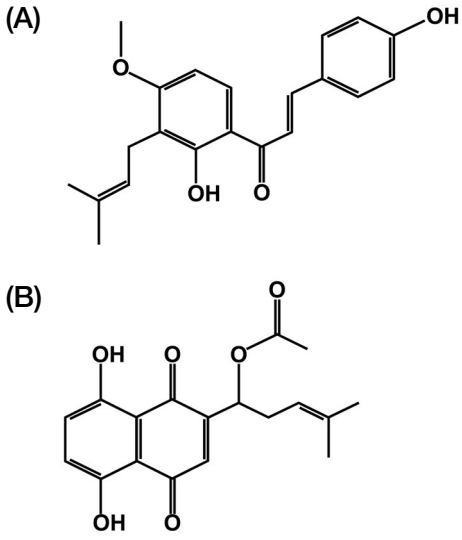


**Fig. 1.** Chemical molecular structure of (A) 4-hydroxyderricin and (B) acetylshikonin.

**Table 2.** Antifungal activity against various fungal strains of 4-hydroxyderricin and Acetylshikonin (MIC values in mg/L)

| Strain | | MIC 4-hydroxyderricin | | Acetylshikonin | |
|---|---|---|---|---|---|
| | | 24 h | 48 h | 24 h | 48 h |
| | *Asc*. *apis* | 3.125 | 6.25 | 12.5 | 12.5 |
| KACC42589 | *Asp*. *niger* | >200 | >200 | >200 | >200 |
| KACC40071 | *Asp*. *clavatus* | >200 | >200 | 50 | 50 |
| KCTC7965 | *C*. *albicans* | >200 | >200 | 25 | 50 |
| KACC45480 | *C*. *parapsilosis* var. *parapsilosis* | >200 | >200 | 50 | 50 |
| KCTC7212 | *C*. *tropicalis* | >200 | >200 | 50 | 50 |
| KCTC17762 | *C*. *tropocalis* var. *tropicalis* | >200 | >200 | 25 | 100 |
| KCTC7219 | *C*. *glabrata* | >200 | >200 | 25 | 50 |
| KCTC17528 | *F*. *neoformans* var. *bacillispora* | >200 | >200 | 12.5 | 50 |
| KCTC7211 | *P*. *guilliermondii* | >200 | >200 | >200 | >200 |
| KACC40256 | *R*. *oryzae* | 0.78 | 0.78 | 25 | 25 |
| KACC30068 | *S*. *cerevisiae* | >200 | >200 | 12.5 | 25 |
| BY4742 | *S*. *cerevisiae* | >200 | >200 | 12.5 | 25 |

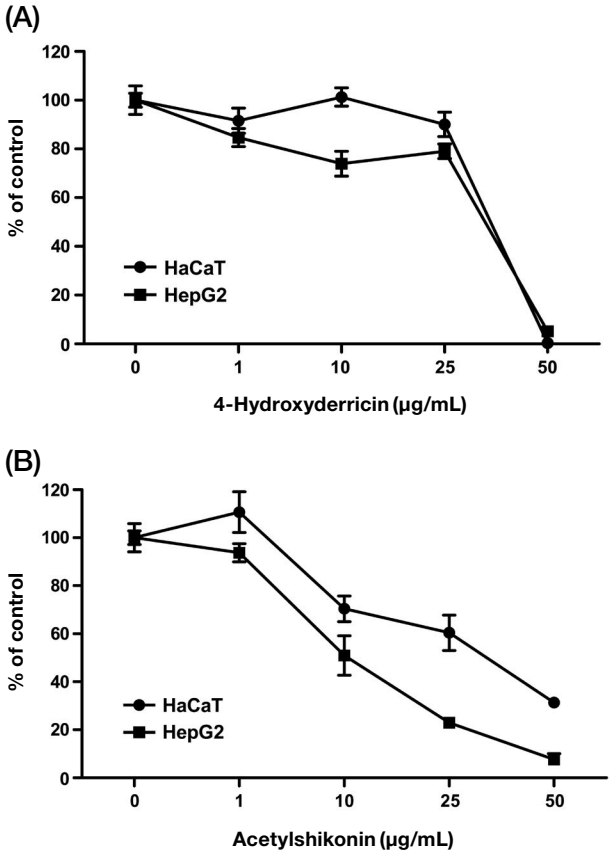


**Fig. 2.** Cytotoxic effects of (A) 4-hydroxyderricin and (B) acetylshikonin against HepG2 or HaCaT cells for 24 h. Data represent mean ± SD of three independent experiments.

shikonin has antifungal activity, but not as high as that of fluconazole or amphotericin B (Sasaki *et al*., 2002). The detailed antifungal mechanism of acetylshikonin has not been studied and requires further elucidation.

We next investigated if 4-hydroxyderricin and acetylshikonin can be cytotoxic to humans. 4-Hydroxyderricin showed very low cytotoxicity toward HepG2 and HaCaT cells, but acetylshikonin showed some cytotoxicity toward HepG2 cells, but not HaCaT cells (Fig. 2).

One of the disadvantages of using natural compounds is the cost of preparation. Combined treatment with natural compounds and miconazole, a well-known synthetic antifungal compound, would increase the cost-effectiveness of 4-hydroxyderricin and reduce the cytotoxicity associated with miconazole usage. We therefore explored that synergy effect of 4-hydroxyderricin or acetylshikonin with micinazole. Acetylshikonin had a synergy effect with miconazole weakly (FICI = 0.83 ± 0.12 and 0.88 ± 0.15 after 24 h and 48 h treatment, respectively), but 4-hydroxyderricin had a more effect with miconazole (FICI = 0.63 ± 0.10 and 0.65 ± 0.13 after 24 h and 48 h treatment, respectively) (Table 3). 4-Hydroxyderricin at 0.78 μg/mL inhibited *Asc*. *apis* growth when combined with 0.78 μg/mL of miconazole (Table 2).

**Table 3.** Effect of combined treatment of 4-hydroxyderricin or acetylshikonin with miconazole against *Asc*. *apis*

| Combination | FICI[a] | |
|---|---|---|
| | 24 h | 48 h |
| 4-Hydroxyderricin + miconazole | 0.63 ± 0.10 | 0.65 ± 0.13 |
| Acetylshikonin + miconazole | 0.83 ± 0.12 | 0.88 ± 0.15 |

[a]FICI values are reported as means ± SD. The experiment was repeated three times with essentially the same results.

4-Hydroxyderricin has antifungal activity against *Asc*. *apis* with less cytotoxicity against human cell lines. The narrow anti-fungal spectrum indicates that it could potentially be used to control honeybee infectious fungal diseases without affecting beneficial fungal species.

## ACKNOWLEDGEMENTS

This work was supported by the IPET (116094-03-1-SB010).

## FOOTNOTES

Financial Disclosure: This research was conducted in the absence of any commercial or financial relationships that could be construed as posing potential conflicts of interest.

## LITERATURE CITED

Aronstein, K. A. and K. D. Murray. 2010. Chalkbrood disease in honey bees. J. Invertebr. Pathol. 103: S20-29.

Battenberg, O. A., Y. Yang, S. H. L. Verhelstb and S. A. Sieber. 2013. Target profiling of 4-Hydroxyderricin in *S. aureus* reveals seryl-tRNA synthetase binding and inhibition by covalent modification. Mol. Biosyst. 9: 343-351.

Bax, R. and G. Samantha. 2015. Antibiotics: the changing regulatory and pharmaceutical industry paradigm. J. Antimicrob. Chemother. 70: 1281-1284.

Cho, S. C. and B. Y. Choi. 2015. Acetylshikonin Inhibits Human

Pancreatic PANC-1 Cancer Cell Proliferation by Suppressing the NF-κB Activity. Biomol. Ther. (Seoul). 23: 428-433.

Hedtke, K., P. M. Jensen, A. B. Jensen and E. Genersch. 2011. Evidence for emerging parasites and pathogens influencing outbreaks of stress-related diseases like chalkbrood. J. Invertebr. Pathol. 108: 167-173.

Kloucek, P., J. Smid, J. Flesar, J. Havlik, D. Titera, V. Rada , O. Drabek and L. Kokoska. 2012. *In vitro* inhibitory activity of essential oil vapors against *Ascosphaera apis*. Nat. Prod. Commun. 7: 253-256.

Mourad, A. K., O. A. Zaghloul, M. B. El Kady, F. M. Nemat and M. E. Morsy. 2005. A novel approach for the management of the chalkbrood disease infesting honeybee *Apis mellifera* L. (Hymenoptera: Apidae) colonies in Egypt. Commun. Agric. Appl. Biol. Sci. 70: 601-611.

Ohnogi, H., Y. Kudo, K. Tahara, K. Sugiyama, T. Enoki, S. Hayami, H. Sagawa, Y. Tanimura, W. Aoi, Y. Naito, I. Kato and T. Yoshikawa. 2012. Six new chalcones from *Angelica keiskei* inducing adiponectin production in 3T3-L1 adipocytes. Biosci. Biotechnol. Biochem. 76: 961-966.

Reinaldi, F. J., M. R. De Giusti and A. M. Alippi. 2004. Inhibition of the growth of *Ascosphaera apis* by *Bacillus* and *Paenibacillus* strains isolated from honey. Rev. Argent. Microbiol. 36: 52-55.

Sasaki, K., H. Abe and F. Yoshizaki. 2002. *In vitro* antifungal activity of naphthoquinone derivatives. Biol. Pharm. Bull. 25: 669-670.

Shin, Y. K and K. Y. Kim. 2016. Macelignan inhibits bee pathogenic fungi *Ascophaera apis* growth through HOG1 pathway. Braz. J. Med. Biol. Res. 49: e5313.

Subsomboon, T. 2012. Culture media for growth of mycelium and for induction of sporulation of *Ascosphaera apis*, the causative agent of chalkbrood disease of honey bee (*Apis mellifera* L.). J. Sci. & Tech. MSU. 32: 51-55.