

Antifungal Activity of Bee Venom and Sweet Bee Venom against Clinically Isolated *Candida albicans*

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Key Words

anti-fungal activity, bee venom, clinical *Candida albicans*, sweet bee venom

Abstract

Objectives: The purpose of this study was to investigate the antifungal effect of bee venom (BV) and sweet bee venom (SBV) against *Candida albicans* (*C. albicans*) clinical isolates.

Methods: In this study, BV and SBV were examined for antifungal activities against the Korean Collection for Type Cultures (KCTC) strain and 10 clinical isolates of *C. albicans*. The disk diffusion method was used to measure the antifungal activity and minimum inhibitory concentration (MIC) assays were performed by using a broth microdilution method. Also, a killing curve assay was conducted to investigate the kinetics of the anti-fungal action.

Results: BV and SBV showed antifungal activity against 10 clinical isolates of *C. albicans* that were cultured from blood and the vagina by using disk diffusion method. The MIC values obtained for clinical isolates by using the broth microdilution method varied from 62.5 µg/mL to 125 µg/mL for BV and from 15.63 µg/mL to 62.5 µg/mL for SBV. In the killing-curve assay, SBV behaved as amphotericin B, which was used as positive control, did. The antifungal efficacy of SBV was much higher than that of BV.

Conclusion: BV and SBV showed antifungal activity against *C. albicans* clinical strains that were isolated

from blood and the vagina. Especially, SBV might be a candidate for a new antifungal agent against *C. albicans* clinical isolates.

1. Introduction

Yeasts are microorganisms commonly found in nature. They are present in the normal flora (in moist places like the intestinal system, mouth etc.) in the human body. The dimorphic yeast *Candida albicans* (*C. albicans*) is the most prevalent opportunistic pathogen in humans. Candidiasis ranges from superficial and mucosal infections to life-threatening septicaemic mycosis in debilitated patients [1-3], and this fungus is known to be the fourth leading cause of nosocomial infections [4, 5]. Over the last few decades, the incidence of candidal infections due to *C. albicans* has been increasing, paralleling the growing numbers of immune-compromised patients [4-7]. To the patients, disseminated candidiasis is sometimes a serious disease that often results in death [8]. In addition, the fungus causes local infections such as vaginitis and thrush. For medical treatment of fungal infections, amphotericin B has been considered as the drug of choice [9, 10], and the azoles are mainly used in common clinical situations. However, the toxicity of and the resistance to these antifungal drugs are major problems [4, 5]. In the case of amphotericin B, an increased amount of amphotericin B must be administered to patients due to its poor permeability across the membrane [11], which can result in severe side effects, for example, renal damage [12, 13]. To overcome these problems of side-effects, natural products have been considered to be promising antifungal agents with less profound adverse effects. The antifungal activities of several phyto-

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chemicals including polyphenols, phenolics, terpenoids, and alkaloids have been reported [14].

Bee venom (BV) from honey bees (*Apis mellifera* L.) has been utilized for centuries as a pain reliever, anti-coagulant and anti-inflammatory agent for the treatment of chronic diseases, such as arthritis, rheumatism, tendonitis, bursitis, fibrosis and multiple sclerosis [15-18]. In Korea, apitherapy, which uses the stings of live honey bees, has shown therapeutic value for treating piglets, calves and dairy cows with several respiratory diseases [19]. BV, called apitoxin, is a mixture of proteins: melittin (main component 52%), apamin, adolapin, phospholipase A2, hyaluronidase, histamine, dopamine and protease inhibitor [20]. Two major components of BV, melittin and phospholipase A2, are generally thought to play an important role in the induction of the irritation and the allergic reactions associated with bee stings [16]. Also, sweet bee venom (SBV), in which hyper-molecular materials, such as enzymes and histamines that act as allergens in BV pharmacopuncture, have been removed, has been developed [21]. Melittin, a 26 amino acid polypeptide, is known to have anti-bacterial effects [22-24]. However, information on the antifungal efficacies of BV and SBV is not available.

This study was performed to investigate the antifungal activities of BV and SBV against *C. albicans* clinical isolates cultured from blood and the vagina.

2. Materials and Methods

The BV and the SBV used in this study were produced at the Korean Pharmacopuncture Institute (Seoul, Korea). Lyophilized whole BV and SBV were dissolved in distilled water at different concentrations, and were then used in this experiment. To examine the antifungal activities of BV and SBV, *C. albicans* (KCTC 7965) as a standard strain from the Korean Collection for Type Cultures (KCTC) at the Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon, Korea; the 10 *C. albicans* clinical isolates used as test organisms were furnished from the Korean Collection of Medical Fungi (KCMF) at the Konyang Institute of Medical Mycology (KIMM), Daejeon, Korea. Fungal cells were cultured on sabouraud dextrose agar (SDA; BD, Difco, Sparks, MD, USA) plates, and then incubated for 48 hours at 35°C. Amphotericin B (Sigma, St. Louis, MO, USA) and fluconazole (Sigma, St. Louis, MO, USA) were dissolved in 2% dimethyl sulfoxide (DMSO) as a stock solution, and then subsequently diluted.

Disk diffusion was carried out with Mueller-Hinton agar glucose methylene blue (MH-GMB; BD, Difco, Detroit, MI, USA). The inoculum was prepared by using 24-hour plate cultures of *C. albicans* on SDA. The 0.5 McFarland inoculum was swabbed in three directions on the MH-GMB agar plate. A disk (Advantec filter paper No. 26, size 6 mm) was placed on the surface agar, and a 20 µL sample (10 mg/mL each of BV and SBV) was loaded. Amphotericin B (100 µg) and fluconazole (100 µg) were used as a positive control. The plates were incubated for 24 hours at 35°C. The diameters (cm) of the zone of inhibition were measured by using a metric ruler.

A minimum inhibitory concentration (MIC) assay using a

broth microdilution method was performed as described in M27-A2 (CLSI) with modifications [25]. The medium used was Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma Chemical Co., Missouri, USA) with L-glutamine buffered to pH 7.0 by using 0.165 mol/L of morpholino-propanesulfonic acid (MOPS), supplemented with 2% glucose. The strain suspension was prepared in 0.85% saline, with an optical density equivalent to a 0.5 McFarland standard, and was diluted to 1 : 100 in RPMI 1640 medium to obtain a final concentration of $1 - 5 \times 10^4$ colony-forming units per milliliter (CFU/mL). This suspension was inoculated into each well of a microtiter plate previously prepared with BV and SBV at concentrations from 500 µg/mL down to 0.9 µg/mL by using RPMI medium. The 96-well microtiter plate was incubated with agitation for 24 hours at 35°C. The control drug for each strain was amphotericin B and fluconazole diluted in DMSO; the concentrations tested ranged from 50 µg/mL down to 0.09 µg/mL in RPMI 1640 medium. The MIC was defined as the lowest concentration at which the optical density (OD) was reduced to 50% of the OD in the growth control well as measured by using an enzyme-linked immunosorbent assay (ELISA) microplate reader (Biotek).

The kinetics of the antifungal actions of BV and SBV against *C. albicans* cells were evaluated by using killing curve assays. Log-phased fungal cells (2×10^4 CFU/mL) in RPMI 1640 medium were incubated with 125 µg/mL of BV and 62.5 µg/mL of SBV or with 0.1 µg/mL of amphotericin B (at the MIC), which was used as a positive control. The culture was obtained and spread on a SDA agar plate, then, the CFUs were counted after incubation for 24 hours at 35°C [26]. The values represented the averages of measurements conducted in triplicate of three independent assays.

All data were taken in triplicate, and statistical analyses were performed by using the student's *t*-test, with ($P < 0.05$) being considered significant.

3. Results

The antifungal efficacies of BV and SBV against *C. albicans* (KCTC 7965) as a standard strain and 10 *C. albicans* clinical isolates were evaluated by the disk diffusion method with MH-GMB medium, and the results are shown in Table 1 and Fig. 1. The results showed that the diameters of the inhibition zones for *C. albicans* blood isolates and *C. albicans* vaginal isolates were 9 – 12 mm and 10 – 12 mm, respectively, for BV and 12 – 18 mm and 12 – 19 mm, respectively, for SBV. The diameters of the inhibition zones for BV and SBV for the *C. albicans* standard strain were measured as 11 mm and 15 mm, respectively. In the blood isolates and the vaginal isolates, the diameters of the inhibition zones for BV and SBV were not significantly different ($P > 0.05$). However, the inhibition zone diameters of SBV compared with that of BV in clinical isolates were significantly different ($P < 0.05$).

For the clinical isolates, the MIC values, which were obtained by using the broth microdilution method, varied from 62.5 µg/mL to 125 µg/mL for BV and from 15.63 µg/mL to 62.5 µg/mL for SBV. The MIC values for BV and SBV for the *C. albicans* standard strain were 125 µg/mL and

Table 1 Anti-microbial activities of BV and SBV against *Candida albicans* clinical isolates as measured by using disk diffusion assay

Sources	<i>Candida albicans</i>	Diameter of Zone Inhibition (mm)			
		Amphotercin B	Fluconazole	BV	SBV
Blood	KCMF 20025	27	20	11	13
	KCMF 20028	20	14	10	12
	KCMF 20475	20	17	10	18
	KCMF 20485	25	22	12	15
	KCMF 20494	23	15	9	12
Geometric mean		23	17.6	10.4	14
Vagina	KCMF 20030	22	15	10	12
	KCMF 20032	25	15	11	12
	KCMF 20049	25	15	11	13
	KCMF 20054	28	23	11	12
	KCMF 20474	30	20	12	19
Geometric mean		26	17.6	11	13.6
	KCTC 7965*	21	13	11	15

*Standard strain.

BV, bee venom; SBV, sweet bee venom; KCMF, Korean Collection of Medical Fungi; KCTC, Korean Collection for Type Cultures.

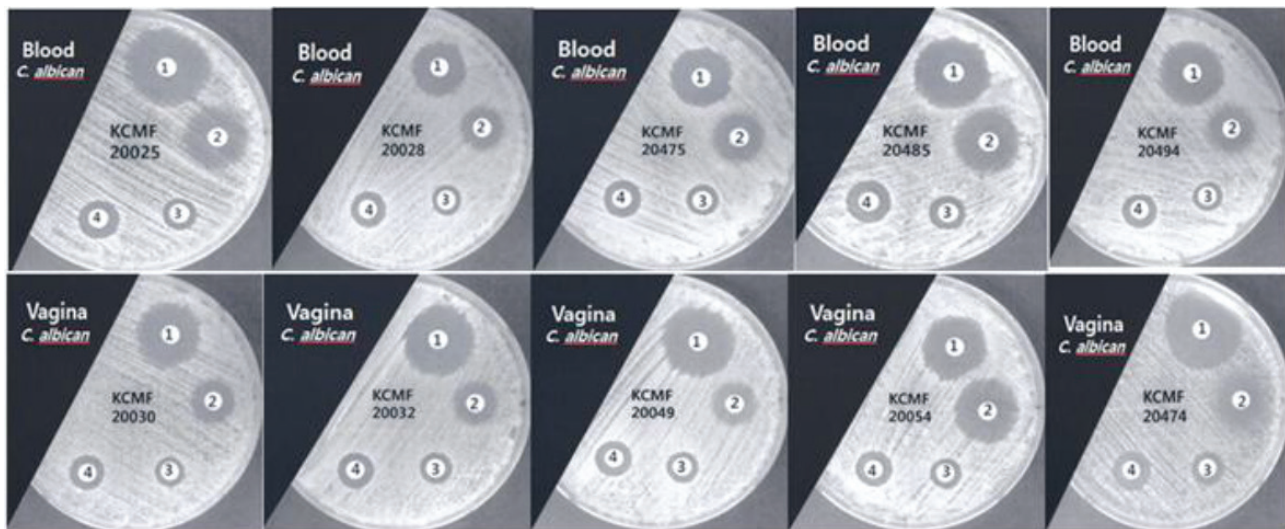


Figure 1 Antifungal activities against 10 *Candida albicans* clinical isolates that were cultured from blood and the vagina as measured by using the disk diffusion method: 1, amphotericin B; 2, fluconazole; 3, bee venom; 4, sweet bee venom.

62.5 µg/mL, respectively. The MIC values for BV and SBV for all clinical isolates were similar to those for the standard strain (Table 2).

A killing-curve assay against *C. albicans* KCTC 7965 cells was conducted to assess the killing potencies of BV and SBV. The results showed that BV and SBV exhibited antifungal activities through fungicidal effects. *C. albicans* cells treated with 50 µg/mL of SBV rapidly decreased in number in a manner similar to the decrease observed with

1 µg/mL of amphotericin B after incubation for 4 hours, however, for 100 µg/mL of BV, the decrease in the number of *C. albicans* was slow (Fig. 2). Thus, the fungicidal effect of SBV was higher than that of BV.

4. Discussion

BV, called apitoxin, is a mixture of proteins: melittin

Table 2 MIC of BV and SBV against 10 *Candida albicans* clinical isolates as measured by using the broth microdilution method

Sources	<i>Candida albicans</i>	Minimal Inhibitory Concentrations ($\mu\text{g/mL}$)			
		Amphotercin B	Fluconazole	BV	SBV
Blood	KCMF 20025	0.195	0.39	125	62.5
	KCMF 20028	0.39	0.39	125	31.25
	KCMF 20475	0.39	0.39	125	15.63
	KCMF 20485	0.09	0.39	125	62.5
	KCMF 20494	0.195	0.39	125	31.5
Geometric mean		0.252	0.39	125	40.63
Vagina	KCMF 20030	0.195	0.39	125	62.5
	KCMF 20032	0.195	0.39	125	31.25
	KCMF 20049	0.195	0.39	62.5	31.25
	KCMF 20054	0.39	0.39	125	31.25
	KCMF 20474	0.39	0.78	125	62.5
Geometric mean		0.273	0.47	112.5	43.75
	KCTC 7965*	0.09	0.39	125	62.5

*Standard strain.

MIC, minimal inhibitory concentrations; BV, bee venom; SBV, sweet bee venom; KCMF, Korean Collection of Medical Fungi; KCTC, Korean Collection for Type Cultures.

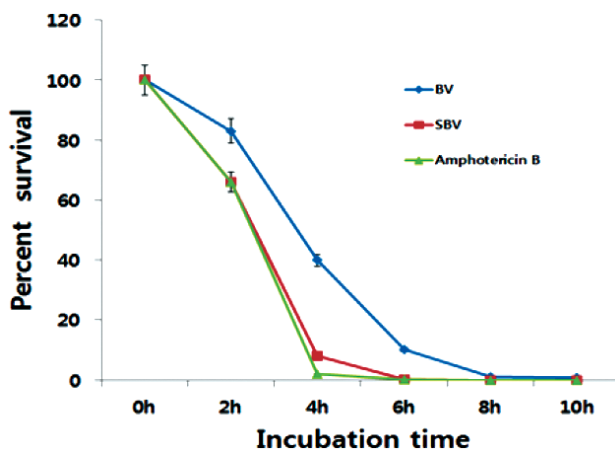


Figure 2 Time-killing plots for BV and SBV against *Candida albicans* KCTC 7965 strain. Fungal cells were incubated with 100 $\mu\text{g/mL}$ of BV, 50 $\mu\text{g/mL}$ of SBV and 1 $\mu\text{g/mL}$ of amphotericin B which was used as a positive control. Viability was determined every 2 hours by using CFUs and was expressed as a percent survival. The error bars represent the SD values for two independent experiments performed in duplicate.

BV, bee venom; SBV, sweet bee venom; KCTC, Korean Collection for Type Cultures; CFUs, colony forming units; SD, standard deviation.

(main component 52%), apamin, adolapin, phospholipase A2, hyaluronidase, histamine, dopamine and protease inhibitor [20]. Two major components of BV, melittin and phospholipase A2, are generally thought to play important roles in the induction of the irritation and the allergic reactions associated with bee stings [16]. Also, SBV which has hyper-molecular materials, such the enzymes and the histamines that act as allergens in BV pharmacopuncture, removed, has been developed [21]. The frequent uses of anti-microbial and antifungal agents such as anti-biotics enable many pathogens to acquire resistances to multiple drugs [27]. The emergence of anti-bacterial-resistant strains of animal pathogens and their potential health risks to humans have captured great attention [28, 29]. Therefore, new anti-bacterial or antifungal agents with fewer adverse effects must be developed for the successful treatment of dermatophytes infections [30].

The anti-bacterial properties of BV as a natural antibacterial agent have been extensively studied, and BV therapy has been suggested for use as an alternative to anti-biotic therapy [31-33]. Strong antibacterial activities of BV against both Gram negative and Gram positive bacteria have been reported [34, 35]. Nakatuji *et al* [36] reported that BV could control the growth of *Staphylococcus aureus*, which plays an important role in the pathogenesis of inflamed lesions in the case of acne vulgaris. Moreover, BV exhibited anti-bacterial activities against skin bacteria such as *Propionibacterium acnes*, *Staphylococcus epidermidis*, and *Streptococcus pyogenes* [37].

BV and SBV anti-candidal activities were observed by using the disk diffusion method and the broth microdilution method, confirming that the compounds have a potential

for use as anti-candidal agents, although their activities are less efficient as compared with amphotericin B and fluconazole, also, in the disk diffusion assay of *C. albicans* isolates from blood and the vagina, the antifungal activities of BV and SBV were similar (Tables 1, 2, Fig. 1). However, Yu *et al* [38] reported that the antifungal activities of BV against *Trichophyton mentagrophytes* and *Trichophyton rubrum* were much stronger than those of fluconazole, one of the commercial antifungal drugs that is currently being used. In the current study, amphotericin B was used as a positive control: amphotericin B is a fungicidal agent that is widely used to treat serious systemic infections [39]. According to the MIC values, the anti-candidal activities of BV and SBV were less potent than those of amphotericin B and fluconazole as a positive control (Table 2). Most anti-biotics show an anti-microbial effect caused by a cidal or a static action. SBV exhibited an effect similar to that of amphotericin B, which is known a cidal agent for fungi; the anti-candidal activities of SBV are due to its killing action, as described by the time killing curve assay with *C. albicans* (Fig. 2).

In this study, the MIC values for *C. albicans* blood and vaginal isolates, as obtained by using the broth microdilution method, varied from 62.5 µg/mL to 125 µg/mL for BV and from 15.63 µg/mL to 62.5 µg/mL for SBV (Table 2). Seoung [39] reported that methanol extracts from *Galla rhois* showed antifungal activity against *C. albicans* isolated from patients with recurrent vaginal candidiasis and the MIC value was 50 mg/mL for *C. albicans*. Also, dichloromethane extract from *Paeonia japonica* showed antifungal activity against *C. albicans*, and the MIC value was 50 mg/mL for *C. albicans* [40]. Thus, the anti-candidal activities of BV and SBV are thought to be much stronger than those of extracts from *Galla rhois* and *Paeonia japonica*. We demonstrated that SBV had very strong antifungal activity, the antifungal activity of SBV being much stronger than that of BV. Moreover, the anti-candidal activities of BV and SBV were much higher than those of phytochemicals, judging by their effective anti-candidal concentration ranges. This study raises the possibility that BV and SBV can be used as an alternative strategy for treating fungal pathogens, which would reduce anti-biotic use. BV and SBV may be candidates for new antifungal agents against *C. albicans* clinical isolates. Although this study, demonstrated that BV and SBV had considerable antifungal activities, further experiments for evaluating the *in vivo* efficacies of BV and SBV for clinical applications are warranted.

5. Conclusion

The antifungal activities of BV and SBV against 10 clinical isolates of *C. albicans* that were cultured from blood and the vagina were determined by using the disk diffusion assay, the broth microdilution assay and the killing-curve assay. BV and SBV showed antifungal activities against those clinical isolates of *C. albicans*, and the antifungal activities of BV and SBV showed similar results on the disk diffusion assay. The MIC values obtained for clinical isolates by using the broth microdilution method varied from 62.5 µg/mL to 125 µg/mL for BV and from 15.63 µg/mL to 62.5 µg/mL

for SBV. In the killing-curve assay, SBV behaved as amphotericin B, which was used as positive control, did. The antifungal efficacy of SBV was much higher than that of BV.

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Conflict of interest

The authors declare that there are no conflict of interest.

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