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In Vitro Antifungal Activity of HTI Isolated from Oriental Medicine, *Hyungbangjihwang-tang*

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Hyungbangjihwang-Tang (HT), an Oriental herbal formula, has been known to play a role which helps to recover vigor of human in the Orient. In this study, antifungal substance (HTI) was purified from the ethyl-acetate extracts of HT by using SiO_2 column chromatography and HPLC, and the antifungal effects of HTI and its mode of action were investigated. By using a broth micro-dilution assay, the activity of HTI was evaluated against fungi. HTI showed antifungal activities without hemolytic effect against human erythrocytes. To confirm antifungal activity of HTI, we examined the accumulation of intracellular trehalose as stress response on toxic agents and effect on dimorphic transition in *Candida albicans*. The results demonstrated that HTI induced the accumulation of intracellular trehalose and exerted its antifungal effect by disrupting the mycelial forms. To understand its antifungal mode of action, cell cycle analysis was performed with *C. albicans*, and the results showed HTI arrested the cell cycle at the S phase in yeast. The present study indicates that HTI has considerable antifungal activity, deserving further investigation for clinical applications.

Key words: *Hyungbangjihwang-Tang*, antifungal activity, Oriental medicine, *Candida albicans*

Introduction

In recent years, the rapid increase in microbes that are resistant to conventionally-used antibiotics has been observed [3]. Furthermore, adequate treatment of mycotic infections is difficult since fungi are eukaryotic organisms with a structure and metabolism that are similar to those of eukaryotic hosts. Therefore, there is an inevitable and urgent medical need for indigenous antibiotics with novel antifungal mechanisms.

Chinese herbal formulas evolved through thousands of years of clinical practice. It appears that many of the ancient combination formulas have sound scientific basis through modern pharmacological evaluation. Significant chemical changes occurred during the decoction process of a prescribed herbal formula. Through decoction process, some toxic ingredients were significantly reduced and new active compounds generated due to the chemical interactions among the ingredients [9].

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Hyungbangjihwang-Tang (HT), an oriental herbal formula, has been known to play a role which helps to recover vigor of human in the Orient [8]. HT is a prescription composed of nine oriental medicinal herbs including *Ostericum koreanum*, *Aralia contientalis*, *Ledebouriella seseloides*, *Poria cocos*, *Corns officinalis*, *Rehmannia glutinosa*, *Plantago asiatica*, *Alisma canaliculatum*, and *Schizonepeta tenuifolia* var. *japonica*. It has been used for treatment of various diseases including empyema, rhinitis, and tonsillitis [8]. Though valued biological abilities of HT are known, however, the potential functions of HT as an antifungal agent still remain unknown.

In this study, we purified antifungal substance (HTI) from the ethyl acetate extracts of HT by using silica column chromatography and finally HPLC with Si60 normal-phase column, and reported on the antifungal effects of HTI toward various fungi species and its mode of action regarding antifungal activity against *C. albicans*.

Materials and Methods

Chemicals

The following drugs were obtained from the indicated

commercial sources: YPD broth (Difco, Sparks, MD, USA); fetal bovine serum (FBS) (Cansera International Inc., Rexdale, ON, Canada); amphotericin B, dimethyl sulfoxide (DMSO), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), trehalase, propidium iodide (PI), Triton X-100, 3,5-Dinitrosalicylic acid, RNase A and other chemicals (Sigma Chemical Co., St. Louis, Mo, USA). Stock solution of amphotericin B was prepared in DMSO and stored at −20°C. For all the experiments, final concentration of 2% DMSO was used as solvent carrier.

Extraction and isolation of HTI

An extract of HT was prepared by decocting the dried prescribed nine kinds of oriental medicinal herbs with boiling distilled water (26 g/L). The duration of decoction was about 3 h. After celite filtration water extract was washed with n-hexane and extracted with ethyl acetate. The ethyl acetate extract was evaporated to dryness in vacuo to yield 0.4 g of dark brownish crude oil. This crude material was chromatographed on silica gel column using EtOAc: hexane (from 2:1 to 5:1) as elution solvent with stepwise. The active fraction with 0.4 Rf value on silica TLC (hexane:ethyl acetate=1:2) was concentrated in vacuo to give partially purified material. The purified active fraction was further purified by preparative HPLC equipped with a Lichrosorb Si60 column (10 μm; Merck, Darmstadt, Germany) with PDA detecter at room temperature. The mobile phase consisted of hexane: ethylacetate (1:1) and the flow rate was 1.0 mL/min. The HPLC instrumentation included an CBM-10A system controller (Shimadzu, Kyoto, Japan), a Shimadzu LC-10AT pump, a Shimadzu DGU-14A degasser, an SIL-10A auto sampler, a Shimadzu SPD-10A detector (set at 280 nm), and a computer running Shimadzu software version CLASS-LC10, and active material (HTI) was obtained 33 mg of light browny oil (final yield: 0.12%). HTI was prepared in DMSO and stored at −20°C. For all the experiments, final concentration of 2% DMSO was used as solvent carrier. The ingredients of 26 g HT include 4 g of *Ostericum koreanum,* 4 g of *Aralia contientalis*, 4 g of *Ledebouriella seseloides,* 4 g of *Poria cocos*, 2 g of *Corns officinalis*, 2 g of *Rehmannia glutinosa*, 2 g of *Plantago asiatica*, 2 g of *Alisma canaliculatum,* and 2 g of *Schizonepeta tenuifolia var. japonica* These plant materials were purchased from the department of pharmacy, Daegu Oriental Hospital of Daegu Haany University, Daegu, Korea, and their voucher specimens have been deposited in the same institution [22].

Microorganisms and culture conditions

Saccharomyces cerevisiae (KCTC 7296), *Trichosporon beigelii* (KCTC 7707) and *Aspergillus flavus* (KCTC 1375) were obtained from the Korean Collection for Type Cultures (KCTC) at the Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon, Korea. *Candida albicans* (TIMM 1768) was obtained from the Center for Academic Societies, Osaka, Japan. Fungal cells were cultured in an YPD broth containing yeast extract, peptone, dextrose (50 g/L) with aeration at 28°C.

Determining of antifungal susceptibility

Fungal cells (2×10^4/mL) were inoculated into an YPD broth and dispensed 0.1 mL/well into microtiter plates. MICs were determined by a serial two-fold dilution of test compounds, following a micro-dilution method [2, 13, 16] and MTT assay [15]. After 48 h of incubation at 28 °C, the minimal compound concentration that prevented the growth of a given test organism was determined and was defined as the MIC. Growth was assayed with a microtiter ELISA Reader (Molecular Devices Emax, California, USA) by monitoring absorption at 580 nm. MIC values were determined by three independent assays.

Kinetics of the killing of fungi

The kinetics of the antifungal action of HTI was evaluated with *C. albicans* cells. Log-phased fungal cells (2×10^4 CFU/mL) were incubated with 18 μg/mL of HTI or 10 μg/mL of amphotericin B (at the MIC), which were used as a positive control. The culture was obtained and spread on an YPD agar plate, then the CFUs were counted after incubation for 24 h at 28°C [12, 21]. The values represented the average of measurements conducted in triplicate of three independent assays.

Hemolytic activity against human erythrocytes

The hemolytic effects of HTI were examined by measuring the release of hemoglobin from a 4% suspension of fresh human red blood cells (hRBCs). The hRBCs were washed three times with a phosphate-buffered saline (PBS: 35 mM phosphate buffer/150 mM NaCl, pH 7.4). One-hundred microliters of a hRBC suspension were added to the 96-well microtiter plates, and then 100 mL of the HTI solution in a PBS were mixed into each well. After incubating the

mixtures for 1 h at 37°C, the mixtures were centrifuged at 1,500 rpm for 10 min, and aliquots were transferred to new 96-well microtiter plates. The absorbance of aliquots was measured at 414 nm by using a microtiter ELISA Reader. Hemolytic rates of 0 and 100% were determined in a PBS and 0.1% Triton X-100, respectively [10, 19, 20]. The percentage of hemolysis was calculated by employing the following equation: Percentage hemolysis=[($Abs_{414\ nm}$ in the compound solution−$Abs_{414\ nm}$ in a PBS)/($Abs_{414\ nm}$ in 0.1% Triton X-100−$Abs_{414\ nm}$ in a PBS)]×100.

Determination of intracellular trehalose

One milliliter of *C. albicans* cell suspension (1×10^8 cells), containing 40 mg/mL of HTI, was incubated for 1 h at 28°C. The negative control was incubated without HTI, and a positive control was incubated with 10 μg/mL of amphotericin B. Fungal cells were settled by centrifugation (12,000 rpm for 20 min), and only cells were dried. Intracellular trehalose was extracted from 3 mg (dry weight) of fungal cells with 0.025 mM potassium-phosphate buffer (pH 6.6) and then crude neutral trehalose-containing extractions were extracted by removing the cell debris. The supernatant, containing trehaloses was added 0.05 unit of trehalase. After allowing the enzymatic reaction for 1 h at 37°C, the reaction suspension mixed with water and added 16% DNS reagent (3,5-Dinitrosalicylic acid 1%, NaOH 2%, Sodium potassium tartrate 20%). For reaction of glucose with DNS reagent, the mixture was boiled for 5 min and cooled down. The color formations were measured with OPTIZEN 2120UV spectrophotometer (Mecasys, Daejeon, Korea) at 525 nm. The values represented the average of measurements conducted in triplicate of three independent assays.

The effect of HTI on the dimorphic transition

C. albicans cells were maintained by periodic subculturing in a liquid YPD medium. Cultures of yeast cells (blastoconidia) were maintained in a liquid YPD medium at 37°C. To induce mycelial formation, cultures were directly supplemented with 20% of a FBS. The dimorphic transition in *C. albicans* was investigated from cultures containing 40 μg/mL of HTI, which were incubated for 48 h at 37°C [11, 14]. The dimorphic transition to mycelial forms was detected by phase contrast light microscopy (NIKON, ECLIPSETE300, Tokyo, Japan).

Flow cytometric analysis for a fungal cell cycle

Log-phased cells of *C. albicans* (1×10^9 cells) cultured in an YPD medium, were harvested and treated with 100 μg/mL of HTI. After incubation for 4 h, the cells were washed with a PBS and fixed with 70% ethanol overnight at 4°C. The cells were treated with 200 μg/mL of RNase A and the mixture was left to react for 2 h at 37°C. For DNA staining, 50 μg/mL of PI were added and incubated for 1 h at 4°C in the dark [6]. Flow cytometric analysis was performed by a flow cytometer (Becton Dickinson, San Jose, CA).

Results

***In vitro* antifungal activity**

Previously, HTI was isolated from *Hyungbangjihwang-Tang* by HPLC (Fig. 1 [22]). In this study, the antifungal effects of HTI, on fungi species, were investigated and described in terms of the minimum inhibitory concentration (MIC). These strains, such as *C. albicans* and *T. beigelii*, exist as a commensal of humans and are superficial contaminants that can cause a variety of serious infections in humans. *S. cerevisiae* is a universal yeast strain which has been studied regarding the effects of drugs against yeast strains and *A. flavus* is a filamentous fungus which is one of causes of aspergillosis [4]. In the current study, amphotericin B was used as a positive control; amphotericin B is a fungicidal agent which is widely used to treat serious systemic infections [7].

HTI in MIC values of 9-40 μg/mL showed antifungal activities against fungal strains. HTI exhibited 2-fold less

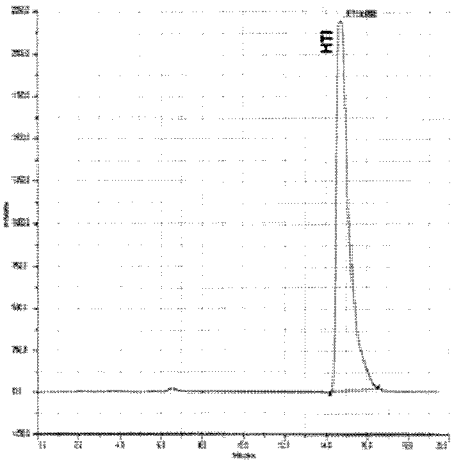

Fig. 1. Silica Si-60 column HPLC chromatogram of the purified HTI isolated from *Hyungbangjihwang-Tang* [22].

Table 1. Antifungal activity of HTI.

Fungi	MIC (μg/mL)	
	HTI	Amphotericin B
C. albicans	18	10
T. beigelii	18	10
S. cerevisiae	9	5
A. flavus	40	20

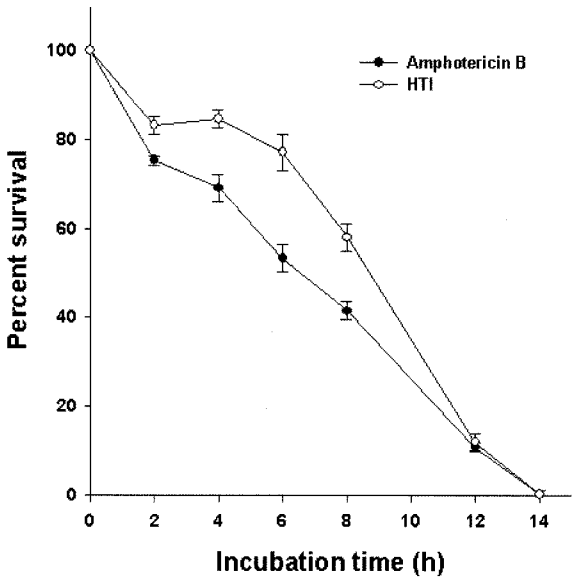

Fig. 2. Time killing plot for *C. albicans* by HTI. Fungal cells were incubated with 18 μg/mL of HTI or 10 μg/mL of amphotericin B used as a positive control. Viability was determined every 2 hours by using colony forming units (CFUs) and expressed as percent of survivals, and the error bars represent the standard deviation (S.D.) values for three independent experiments, performed in triplicate.

potent activities than amphotericin B, showing the MIC values of 5-20 μg/mL on all fungal strains (Table 1).

To assess the killing potency of HTI, we conducted a killing-curve assay against *C. albicans* cells. The results showed that HTI exhibits antifungal activities through fungicidal effects. *C. albicans* cells, at the MIC of HTI, decreased rapidly similar to the decrease in the presence of amphotericin B, after 4 h (Fig. 2).

To estimate the cytotoxicity of HTI against human erythrocytes, hemolytic activity was evaluated by the percentage of hemolysis in 4% suspension of human red blood cells (hRBCs) at various concentrations (from 6.25 to 100 μg/mL) of HTI. HTI exhibited no hemolysis activities at all concentration levels, whereas amphotericin B exhibited potent hemolytic activities at all concentration levels (Table 2).

Table 2. Hemolytic activity of HTI against human erythrocytes.

Compounds	% Hemolysis (μg/mL)				
	100	50	25	12.5	6.25
HTI	0	0	0	0	0
Amphotericin B	100	52	23	8	3

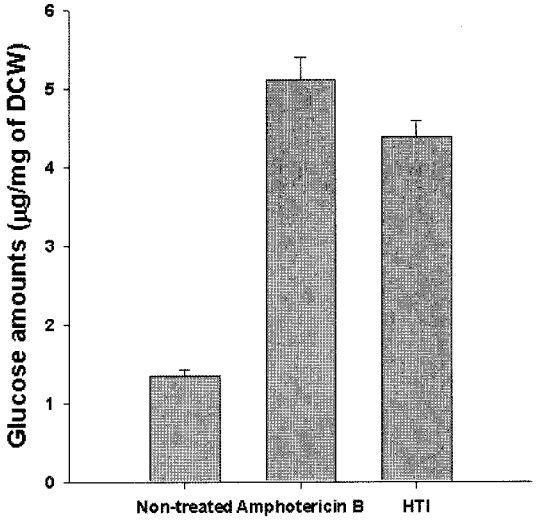

Fig. 3. Trehalose assay after addition of HTI or amphotericin B. Subcultured *C. albicans* cells, with 40 μg/mL of HTI or 10 μg/mL of amphotericin B, were incubated for 1 h at 28°C. The glucose concentrations in the trehalose residue were determined at a wavelength of 525 nm, and the error bars represent the standard deviation (S.D.) values for three independent experiments, performed in triplicate.

Determination of intracellular trehalose

To confirm whether HTI directly functions as a stress factor to fungal cells when HTI exerts antifungal activity, we investigated the amounts of intracellular trehalose on *C. albicans*. *C. albicans* cells were cultured for 1 h at 28°C in the presence of HTI or amphotericin B and the amounts of intracellular trehalose were measured. The results showed that HTI and amphotericin B-treated cells both accumulated more trehalose than the compound-untreated cells. Trehalose amounts, induced by amphotericin B, was measured as being 5.10 μg per fungal dry weight of 1 mg. Trehalose amounts, however, induced by HTI were measured as being 4.38 μg per fungal dry weight of 1 mg. This rate was significantly higher than that induced in the compound-untreated cells, showing trehalose amounts of 1.35 μg (Fig. 3).

The effects of HTI on the dimorphic transition of fungal cells

To investigate the antifungal effects of HTI against *C.*

albicans in the human body, we performed an *in vitro* test of the antifungal effects on the mycelial forms of *C. albicans* cells, which were induced by supplementing with 20% fetal bovine serum. As shown in Fig. 4, the serum-induced mycelia were significantly inhibited from extending and forming in the presence of HTI (Fig. 4C), the mycelia formed normal mycelia in the absence of HTI (Fig. 4B).

The arrest of fungal cell cycle

To understand how the activity of HTI affects cellular physiology, we further investigated the effects on the cell cycle progress of *C. albicans*. The cells were cultured for 4 h at 28°C in the presence or absence [11] of HTI and DNA content was determined via flow cytometry by staining with propidium iodide (PI). PI is a DNA-staining dye that intercalates between the bases of DNA or RNA molecules [23]. As shown in Fig. 5, the percentage of cells

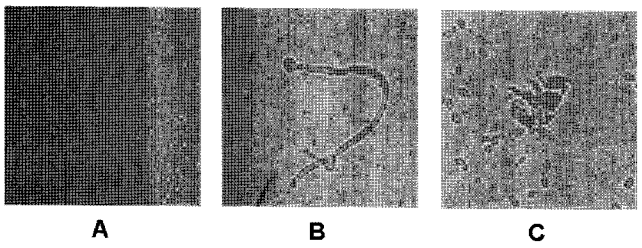

Fig. 4. The effects of the HTI on the dimorphic transition in *C. albicans*. Each culture was incubated with 20% FBS for 48 h in an YPD media with or without 40 μg/mL of the HTI. A, yeast control with no 20% FBS and HTI; B, no treated with HTI; C, treated with 40 μg/mL of HTI in 20% FBS.

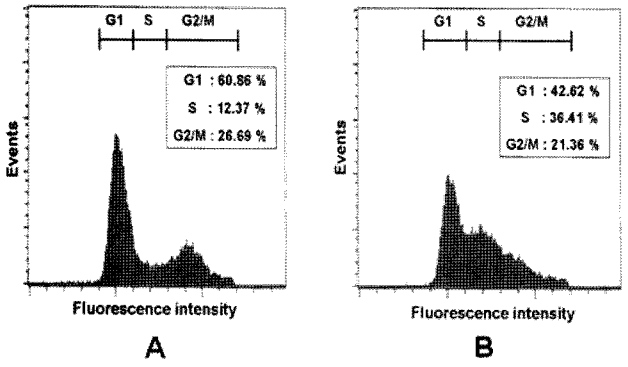

Fig. 5. The effects of HTI on the process of cell cycle of *C. albicans*. *C. albicans* cells were treated with 100 μg/mL of HTI and incubated at 28°C for 4 h under constant shaking. After washing the cells with a PBS, the cells were fixed 70% ethanol (in PBS, v/v) for 12 h, and then, stained with 50 μg/mL of propidium iodide. Cell cycle histogram of *C. albicans*, A, no treated with compound; B, treated with HTI. The control data is quoted from Jung *et al.* (2007).

in the S phase increased by 24%, while that in the G_1 phase significantly decreased by about 18% and that in the G_2/M phase also decreased by about 5% in the presence of HTI.

Discussion

The formation of prescriptions with combination herbal formulas has undergone a considerably long history. Through long clinical practice, ancestors recognized that a recipe composed of two or more drugs proved more advantageous for disease treatment.

Hyungbangjihwang-Tang (HT) has been used for treatment of various diseases including empyema, rhinitis, and tonsillitis. Though valued biological abilities of HT are known, however, the potential functions of HT as an antifungal agent still remain unknown. The present study was aimed to assess the antifungal effects of HTI, antifungal substrate isolated from HT, on various fungal pathogens, and its mode of action for antifungal activity against *C. albicans*.

HTI exhibited less potent antifungal activity against fungal species tested than antifungal activity in MIC values of amphotericin B as a positive control (Table 1). These results demonstrate antifungal activities of HTI against fungi causing human infectious diseases. Most antibiotics show antimicrobial effect by cidal or static action. HTI exhibited similar manner with amphotericin B, which has been known for cidal agent on fungi; antifungal activities of HTI are due to the killing action, as described by time-killing plots with *C. albicans* (Fig. 2). In general, trehalose is a nonreducing disaccharide consisting of two glucose units, which are present in yeast and plant. It has been shown that trehalose can protect proteins and cellular membranes from inactivation or denaturation caused by a variety of stress conditions including desiccation, dehydration, heat, cold, oxidation, and toxic agents [1, 5]. To confirm antifungal activity of HTI, we examined the accumulation of intracellular trehalose as stress response on toxic agents in *C. albicans*. The results showed that HTI and amphotericin B-treated cells both accumulated more trehalose than the compound-untreated cells (Fig. 3). These results demonstrated that intracellular trehalose of HTI-treated cells was accumulated through the stress response caused by antifungal activities of HTI. Thus, it is thought that antifungal activities of HTI induced a stress response in fungal cells. The opportunistic dimorphous pathogen *C. albicans* is of

increasing importance in human medicine, and it can cause deeply-invasive mycoses including candidiasis. The dimorphic transition of *C. albicans* from yeast form to mycelial form, is responsible for pathogenicity with mycelial shapes being predominantly found during host tissue invasion, and mycelial form can be induced by temperature, pH, and serum [17]. HTI also exerts antifungal activity toward serum-induced the mycelial structures of *C. albicans* cells (Fig. 4), suggesting that HTI is a potential extract in the treatment of fungal infectious diseases. Many antimicrobial agents are limited in clinical applications, as they bring about cytolysis of human erythrocytes. HTI shows no hemolytic activity at all tested concentrations, while amphotericin B shows hemolytic activity at all tested concentrations that could be fatal in patients who are treated for fungal infections, via this agent (Table 2). These results confirm the antifungal activities of HTI without cytolytic effect toward human erythrocytes.

The cell cycle is of particular interest as a source of targets for drug development [18]. To elucidate the physiological changes of the fungal cells induced by HTI, we performed flow cytometric analysis for cell cycle. The results showed that HTI arrested the cell cycle at the S phase in *C. albicans* (Fig. 5). This data suggests that HTI induced arrest of cell cycle progress or exerted its killing activity only against replicating cells. In addition, this result probably indicates that antifungal activity of HTI correlates with inhibition of DNA function. Although a detailed experiment on the interaction between HTI and DNA function is needed, the present results lead us to believe that HTI induces the physiological change by impeding DNA function of cells, resulting in the cell death. In conclusion, we demonstrate HTI has considerable antifungal activity, deserving further investigation for clinical applications.

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국문요약

형방지황탕으로부터 분리된 HTI의 항진균활성에 대한 연구

성우상 · 서영배 · 이동건*
경북대학교 자연과학대학 생명과학부

형방지황탕은 한의학에서 중요한 약처방으로 인간의 기력 회복에 약효가 탁월한 것으로 알려져 있다. 본 연구에서는 형방지황탕으로부터 항생 물질(HTI)을 분리하여 항진균활성 및 그 작용기작을 검토하였다. 그 결과 각종 병원성 진균에 대해 항진균 활성을 나타내었으며, 인간 적혈구에 대해서는 세포독성이 나타나지 않은 것으로 확인되었다. 또한 캔디다 알비칸스를 대상으로 스트레스 방어기작과 세포 이형성 유도에 HTI이 미치는 영향을 검토한 결과, 트리할로즈의 증가와 이형성 유도에 영향을 미치는 것으로 나타나 이러한 현상이 항진균활성에 상관관계가 있음을 확인하였으며, 또한 진균의 세포주기에 미치는 영향도 항진균 활성의 요인이 됨을 밝혔다.