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Antimicrobial Effect of Furaneol Against Human Pathogenic Bacteria and Fungi

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Abstract Furaneol, a key aroma compound found in strawberry, pineapple, and processed foodstuffs, has been known to possess various biological activities on animal models. In this study, the antimicrobial effects of furaneol against human pathogenic microorganisms were investigated. The results indicated that furaneol displayed a broad spectrum of antimicrobial activities against Gram-positive and Gram-negative bacteria and fungi without hemolytic activity on human erythrocyte cells. To confirm the antifungal activity of furaneol, we examined the accumulation of intracellular trehalose as a stress response marker on toxic agents and its effect on dimorphic transition of *Candida albicans*. The results demonstrated that furaneol induced significant accumulation of intracellular trehalose and exerted its antifungal effect by disrupting serum-induced mycelial forms. These results suggest that furaneol could be a therapeutic agent having a broad spectrum of antimicrobial activity on human pathogenic microorganisms.

Key words: Furaneol, 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone, antimicrobial activity, strawberry

The need for efficient antimicrobial agents increases with the emergence of pathogens resistant to current therapies. Among different approaches to find novel, safe, and effective antimicrobial agents, the discovery and use of naturally occurring antimicrobial compounds are attracting an increasing amount of attention [19, 22, 23]. This is due to the fact that, unlike many currently used antimicrobial compounds, natural compounds show little tendency to elicit resistance [2].

In general, plants have developed an arsenal of chemicals to survive attacks by microbial invasion [9]. These include both physical barriers as well as chemical ones, such as the presence or accumulation of antimicrobial metabolites. These metabolites are either preformed in the plant (prohibitins) or induced after infection (phytoalexins) [6]. Phytoalexins are a group of low-molecular-weight antibiotics produced by higher plants in response to infection by relatively avirulent microorganisms. At least 64 different chemicals so designated have been isolated from 75 or more plant species representing 20 families of plants, wherein they appear to be associated with resistance to infection. A given host plant may produce several different phytoalexins in response to various microorganisms or other (chemical, physical) elicitors [10].

Furaneol (2,5-dimethyl-4-hydroxy-3(2*H*)-furanone), a main aroma compound found in strawberry [24], pineapple [21], and various processed foods [4, 12, 28], is formed in the Maillard reaction of amino acids and sugars. Specifically, high levels of furaneol are present in strawberry (up to 55 mg/kg strawberry fruit Fomular Weight) [15]. Furaneol has been shown to possess antioxidative activity that exhibits an anticataract effect on spontaneous cataract in the rat [25] and inhibition of mouse forestomach neoplasia [14]. Although furaneol has been known to possess many biological effects on animal models, the effect of this compound on human pathogenic bacteria and fungi have not been reported.

In this study, we investigated the antimicrobial activities of furaneol against various pathogenic bacterial and fungal strains, and suggest its potential as a therapeutic agent to treat human infectious diseases caused by microorganisms.

Materials and Methods

Materials

Furaneol, propionic acid, and amphotericin B were purchased from Sigma Chemical Co. (St. Louis, U.S.A.). Stock solutions of furaneol and propionic acid were prepared in sterile distilled water, and that of amphotericin B was prepared in dimethyl sulfoxide (DMSO). They were stored at −20°C.

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Bacterial and Fungal Strains

Escherichia coli (KCTC 1682), *Escherichia coli* O-157 (ATCC 43895), *Proteus vulgaris* (KCTC 2433), *Staphylococcus aureus* (KCTC 1621), *Staphylococcus epidermidis* (KCTC 1917), *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), Daejon, Korea. *Candida albicans* (TIMM 1768) was obtained from the Center for Academic Societies, Osaka, Japan.

Assay of Antibacterial Activity

Bacterial cells were grown to the midlog phase in an LB [bactotryptone: yeast extract: NaCl, 10 g: 5 g: 10 g per liter (pH 7.0)] medium, and seeded in the wells of a 96-well microtiter plate in LB medium at a density of 2×10^6 cells (100 μl per well). The bacterial cells were added to 10 μl each of the serially diluted solutions of the compounds (from 2.5 to 80 μg/ml), and incubated for 12 h at 37°C. The negative control was incubated without furaneol. The minimum inhibitory concentration (MIC) was determined by measuring the turbidity of each well at 620 nm by using a microtitrater ELISA reader (Molecular Devices Emax, California, U.S.A.). The MICs were the average of triplicate measurements in three independent assays.

Assay of Antifungal Activity

The fungal strains were grown at 28°C in YPD (Yeast extract: Peptone: Dextrose, 10 g: 20 g: 20 g per liter) medium. The fungal cells were seeded in the wells of a 96-well microtiter plate in YPD medium at a density of 2×10^3 cells (100 μl per well). These fungal cells were added to 10 μl each of the serially-diluted solutions of the compounds (from 2.5 to 80 μg/ml), and incubated for 24 h at 28°C. The negative control was incubated without furaneol. Five μl of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) solution [5 mg/ml MTT in phosphate-buffered saline (PBS), pH 7.4] was added to each well, and the plates were incubated for 3 h at 37°C. Thirty μl of a 20% (w/v) SDS solution containing 0.02 M HCl was then added, and the plates were incubated for 16 h at 37°C to dissolve the formazan crystals that were formed [18, 26]. The absorbance of each well was measured at 580 nm by using a microtiter ELISA reader (Molecular Devices Emax, California, U.S.A.). The MICs were the average of triplicate measurements in three independent assays.

Assay of Hemolytic Activity

The hemolytic effect of furaneol was measured against 4% a human red blood cells (RBCs) suspension. Human RBCs were washed three times with phosphate-buffered saline (PBS: 35 mM phosphate buffer/150 mM NaCl, pH 7.4). One-hundred μl of the RBCs suspension were added to 96-well microtiter plates, and then 100 ml of the serially-diluted solution in PBS was mixed into each well. After incubation of 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 PBS and 0.1% Triton X-100, respectively [18, 19]. The percentage of hemolysis was calculated using the following equation:

$$\text{Percentage hemolysis}=[(\text{Abs}_{414\,nm} \text{ in the compound solution} - \text{Abs}_{414\,nm} \text{ in PBS})/(\text{Abs}_{414\,nm} \text{ in 0.1\% Triton X-100} - \text{Abs}_{414\,nm} \text{ in PBS})]\times100.$$

Determination of Intracellular Trehalose

One ml of *C. albicans* cell suspension (1×10^8 cells), containing 2 mg/ml of furaneol, was incubated for 1 h at 28°C. The negative control was also run without furaneol, and a positive control was incubated with 50 μg 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 the cell debris were removed by centrifugation (12,000 rpm for 20 min). The supernatant, containing trehaloses, was mixed with 0.05 unit of trehalase (Sigma, T8778). After allowing the enzymatic reaction to proceed for 1 h at 37°C, the reaction suspension was mixed with water, and ml of 16% DNS reagent (3,5-dinitrosalicylic acid 1%, NaOH 2%, sodium potassium tartrate 20%) was added. For the reaction of glucose with DNS reagent, the mixture was boiled for 5 min and cooled down. The color formed was measured at 525 nm. The results were the average of triplicate measurements in three independent assays.

Effect of Furaneol on the Dimorphic Transition

Cultures of cells (blastoconidia) were maintained by periodic subculturing in liquid YPD medium at 28°C. To induce mycelial formation, cultures were supplemented directly with 20% fetal bovine serum (FBS). The dimorphic transition of *C. albicans* was investigated with cultures containing 40 μg of furaneol, incubated for 48 h at 37°C, and was

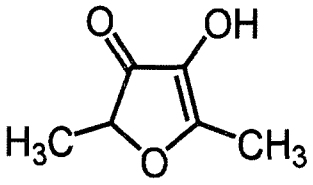

Fig. 1. Chemical structure of furaneol (2,5-dimethyl-4-hydroxy-3(2*H*)- furanone).

Table 1. Antibacterial activity of furaneol against various bacteria.

	MIC (μg/ml)				
	Gram-positive bacteria		Gram-negative bacteria		
	S. aureus	*S. epidermidis*	*E. coli*	*E. coli* O-157	*P. vulgaris*
Furaneol	20–40	20	20	20	20
Propionic acid	20	20	10–20	10	10

The bacterial strains were grown in LB medium at 37°C. The bacterial cells were seeded in the wells of a 96-well microtiter plate of LB medium at a density of 2×10^6 cells (100 μl per well). The serially diluted compound solutions were added to each well, and the cell suspension was incubated for 12 h at 37°C. The turbidity of each well was measured at 620 nm by using a microtiter ELISA reader.

detected by phase contrast light microscopy (NIKON, ECLIPSETE300, Tokyo, Japan).

Results and Discussion

The antibacterial activity of furaneol (Fig. 1) is expressed as minimum inhibitory concentration (MIC). The result indicated that furaneol showed MIC values of 20–40 μg/ml against Gram-positive and Gram-negative bacterial strains and was little less potent than propionic acid with MIC value of 10–20 μg/ml (Table 1). Propionic acid, a positive control for the antibacterial assay in this study, is widely used as a food preservative, because of its broad spectrum of antimicrobial activity against Gram-positive and Gram-negative bacteria [5].

The antifungal activities (MIC) of furaneol against human pathogenic fungal strains were also determined by MTT assay [18, 26], and the result indicated that furaneol showed potent antifungal activity at the MIC values of 5–20 μg/ml against three fungal strains, and this compound was less potent than amphotericin B having the MIC values of 2.5–5 μg/ml (Table 2). Amphotericin B, used as a positive control, is an antifungal agent widely used to treat serious systemic infections. However, the use of this compound is limited, because of high toxicity such as hemolytic effect against human erythrocyte cells [1, 11].

Additionally, furaneol also showed antifungal effect against a filamentous fungus, *A. flavus*, which is one of the causes of aspergillosis [7]. Mycelial growth inhibition of *A. flavus* by furaneol was detected by the agar hole assay. As shown in Fig. 2, the mycelial growth of *A. flavus* was significantly inhibited at the circle center treated with furaneol on YPD agarose plate.

Table 2. Antifungal activity of furaneol against various fungi.

	MIC (μg/ml)		
	C. albicans	*T. beigelii*	*S. cerevisiae*
Furaneol	5–10	10–20	10
Amphotericin B	2.5–5	5	5

The fungal strains were grown in YPD medium at 28°C. The fungal cells were seeded in the wells of a 96-well microtiter plate of YPD medium at a density of 2×10^3 cells (100 μl per well). The serially diluted compound solutions were added to each well, and the cell suspension was incubated for 24 h at 28°C. Five μl of MTT was added to each well, and the plates were incubated for 3 h at 37°C. The absorbance of each well was measured at 580 nm by using a microtiter ELISA reader.

To assess the cytotoxicity of furaneol against mammalian cells, its hemolytic effect against human erythrocyte cells was evaluated by percentage of hemolysis of a 4% human RBCs suspension at various concentrations (from 2.5 to 80 μg/ml) of furaneol. The result indicated that furaneol showed no hemolytic activity at all the concentrations tested, whereas amphotericin B exhibited a potent hemolytic activity at all the concentrations tested and propionic acid exhibited a low hemolytic activity at a high concentration (Table 3). These results demonstrated that furaneol has a broad spectrum of antimicrobial activities against Gram-positive and Gram-negative bacteria and fungi without hemolytic activity against human erythrocyte cells.

To confirm whether furaneol functions directly as a stress to fungal cells when exerting antifungal activity, the amount of intracellular trehalose in *C. albicans* was measured

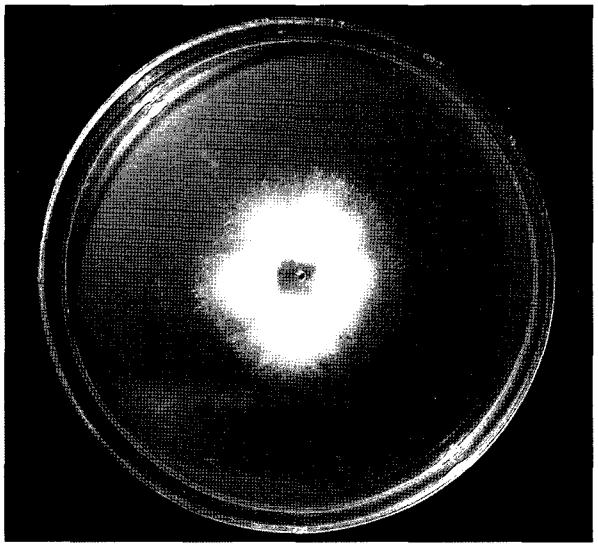

Fig. 2. Agar hole assay for the antifungal effect of furaneol against filamentous fungus, *Aspergillus flavus*.
Furaneol (1 mg/100 μl) was placed at the center of an *A. flavus* mycelium disk on the YPD agar plate. The plate was incubated for 3 days at 28°C.

Table 3. Hemolytic activity against human erythrocyte cells.

	% Hemolysis (μg/ml)					
	80	40	20	10	5	2.5
Furaneol	0	0	0	0	0	0
Propionic acid	5.2	0	0	0	0	0
Amphotericin B	100	98.6	92.3	86.6	71.2	62.2

The hemolytic effect of furaneol was evaluated by determining the hemoglobin release of 4% fresh suspensions of human RBCs at 414 nm. The percentage of hemolysis was calculated by using the following equation:

percentage hemolysis=[($Abs_{414\,nm}$ in the compound solution $-Abs_{414\,nm}$ in PBS)/($Abs_{414\,nm}$ in 0.1% Triton X-100 $-Abs_{414\,nm}$ in PBS)]×100.

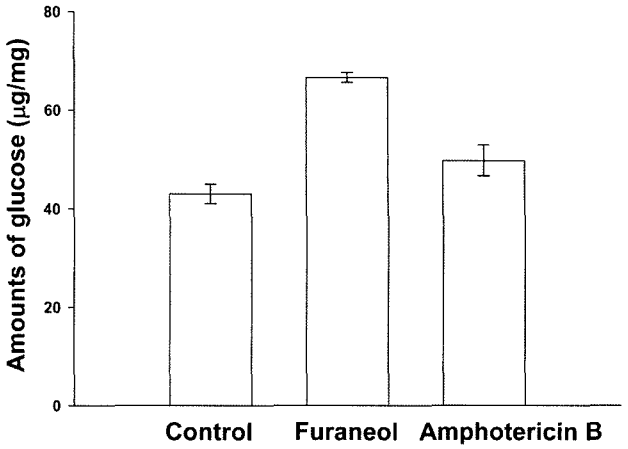

Fig. 3. Trehalose assay after addition of furaneol or amphotericin B.

Subcultured *Candida albicans* cells, with 500 μg of furaneol or 50 μg of amphotericin B, were incubated for 2 h at 28°C. Compound-treated cells were washed with phosphate-buffered saline (pH 7.4) and dried under vacuum. To determine glucose in the trehalose residue, samples were submerged in boiling water and 10 μl (0.05 units) of trehalase was added. After incubation, DNS reagent was added, and the mixture was boiled for 15 min. The glucose concentrations were spectrophotometrically determined at 525 nm.

in the presence or absence of furaneol and amphotericin B. 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 [3, 8]. In the present study, the result showed that the accumulation of intracellular trehalose in furaneol or amphotericin B-treated cells was more active than that of non-treated cells, and that of furaneol-treated cells was slightly more than that of amphotericin B-treated cells (Fig. 3). This result demonstrates that intracellular trehalose of the furaneol-treated cells accumulated through the stress response caused by the antifungal activity of furaneol. Thus, it is highly likely that the antifungal activity of furaneol induced a stress response in fungal cells.

The deeply invasive mycoses such as candidiasis have frequently been emerging through opportunistic infections with the advent of organ transplantation, cancer chemotherapy, or human immunodeficiency virus infection [12]. The ability of some fungi to undergo a morphological transition between unicellular forms and mycelial structures may be considered to be a simple model of cellular development [16]. *C. albicans* is the prototypic dimorphic yeast and an opportunistic pathogen, causing a variety of superficial and deep-seated mycoses. Specifically, dimorphic transition of *C. albicans* plays a crucial role in pathogenesis, with mycelial shapes being predominantly found during host tissue invasion [20]. To induce filamentation and investigate the effect of furaneol on the dimorphic transition of *C. albicans*, this transition was examined in cultures containing 40 μg of furaneol for 48 h at 37°C with 20% fetal bovine serum. As shown in Fig. 4, furaneol destroyed the mycelial forms by exerting its antifungal activity, indicating that furaneol could be applied for treating infection of *C. albicans* in the human body.

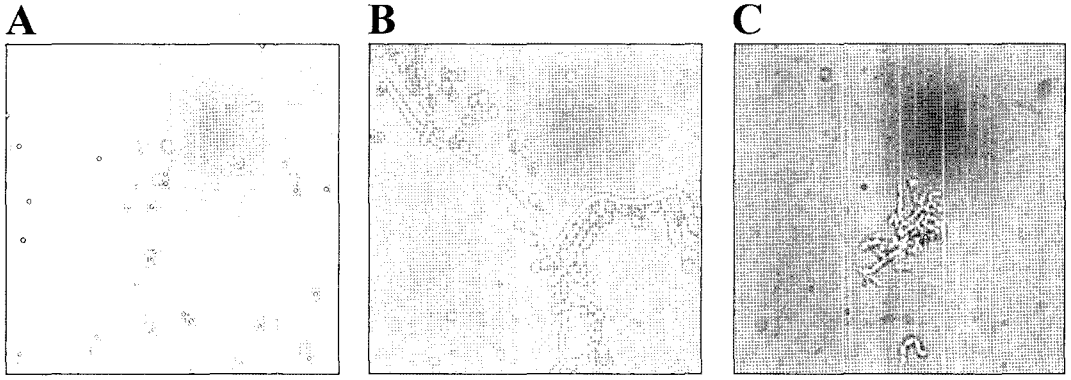

Fig. 4. The effect of furaneol on the dimorphic transition of *Candida albicans*.

A. Yeast control with no FBS and furaneol. **B**. Cells with only 20% FBS. **C**. Cells with 20% FBS and 40 μg of furaneol.

In conclusion, furaneol, a key aroma compound found in strawberry, pineapple, and processed foodstuffs, showed antimicrobial activities against human pathogenic Gram-positive and Gram-negative bacteria and fungi, without hemolytic activity on human erythrocyte cells. We suggest that furaneol is an excellent candidate as a therapeutic agent for human disease caused by pathogenic microorganisms.

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