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Antifungal action of chlorogenic acid against pathogenic fungi, mediated by membrane disruption*

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Abstract: Chlorogenic acid is a polyphenol compound, derived from several fruit and plants. The aim of this study was to assess the in vitro antifungal activity of chlorogenic acid and its mode of action. The results indicate that chlorogenic acid exhibits antifungal activities against certain pathogenic fungi in an energy-independent manner, without any hemolytic effect on human erythrocytes. To elucidate the antifungal mode of action of chlorogenic acid, flow cytometry analysis by using $DiBAC_4(3)$ and changes in membrane dynamics using 1,6-diphenyl-1,3,5-hexatriene (DPH) were performed with *Candida albicans*. The results suggest that chlorogenic acid may exert antifungal activity by disrupting the structure of the cell membrane. It is demonstrated that chlorogenic acid is a valid lead compound for the development of bioactive alternatives for treatment of fungal infections.

Keywords: antifungal activity; *Candida albicans*; chlorogenic acid; membrane disruption.

INTRODUCTION

In general, appropriate treatment of fungal infections is difficult since fungi are eukaryotic organisms, the structure and metabolism of which are similar to those of eukaryotic hosts. Furthermore, fungi that are resistant to antifungal drugs have proliferated in recent years [1]. Accordingly, the search for novel antifungal compounds continues to receive special attention.

Several organisms, including plant, marine organisms, and microbes, secrete biologically active compounds in defense against predators and competition from neighbors [2]. In particular, plants produce an enormous array of secondary metabolites, and it is commonly accepted that a significant part of this chemical diversity serves to protect plants against microbial pathogens [3]. Phenolic compounds comprise a family of secondary plant metabolites that are commonly found in plant material such as apples, coffee beans, grapes, pulp, peel, and tea leaves [4–6]. Some of these also seem to serve in defense of plants against invading pathogens such as insects, bacteria, fungi, and viruses. Chlorogenic acid [1,3,4,5-tetrahydroxycyclohexanecarboxylic acid 3-(3,4-dihydroxycinnamate)] is an abundant polyphenolic natural product derived from a variety of plants [7]. Chlorogenic acid displays many of the typical biological properties of polyphenolics in diet, such as antioxidant and anticarcinogenic effects, and

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inhibition of DNA damage [8–10]. Additionally, there have also been reports regarding bioactivity toward microorganisms. Studies have shown that chlorogenic acid displays antimicrobial activity against certain microorganisms treated in combination with other natural compounds [4]. Although these studies demonstrated antimicrobial activity of chlorogenic acid, nevertheless, its antifungal activity and the mode of action, against human pathogenic fungi, still remain unknown.

In this report, we describe an investigation into antifungal activity and the mode of action of chlorogenic acid against certain pathogenic fungi.

MATERIALS AND METHODS

Materials

Chlorogenic acid, amphotericin B, and trehalase were purchased from the Sigma Chemical Co. (St. Louis, Mo, USA). Stock solutions of chlorogenic acid and amphotericin B were prepared in dimethyl sulfoxide (DMSO) and stored at –20 °C. For all the experiments, a final concentration of 1 % DMSO was used as the solvent carrier.

Fungal strains and culture conditions

Trichosporon beigelii (KCTC 7707) and *Malassezia furfur* (KCTC 7744) were obtained from the Korean Collection for Type Cultures (KCTC) of 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 a yeast peptone dextrose (YPD) broth (Difco) with aeration at 28 °C. *M. furfur* was cultured at 32 °C in a modified Bacto yeast extract/malt extract (YM) broth (Difco) and 1 % olive oil.

Determination of antifungal susceptibility

Fungal cells (2×10^4 ml) were inoculated into a YPD or YM broth and 0.1 ml/well were dispensed into microtiter plates. Minimum inhibitory concentrations (MICs) were determined by a serial two-fold dilution of test compound, following a microdilution method and MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide] assay [11]. After 48 h of incubation at either 28 or 32 °C, the minimal concentration of compound to prevent the growth of a given test organism was determined and defined as MIC. Growth was assayed with a microtiter enzyme-linked immunoabsorbent assay (ELISA) reader by monitoring absorption at 580 nm. MIC values were determined by three independent assays.

Effect of chlorogenic acid on the dimorphic transition

C. albicans cells were maintained by periodic subculturing in a liquid YPD medium. The cultures of yeast cells (blastoconidia) were maintained in a liquid YPD medium at 37 °C. To induce mycelial formation, cultures were directly supplemented with a 20 % FBS. The dimorphic transition in *C. albicans* was investigated from cultures containing 40 μg/ml of chlorogenic acid (at half the MIC), incubated for 48 h at 37 °C [12]. The dimorphic transition to mycelial forms was detected by phase contrast light microscopy (NIKON, ECLIPSE TE300, Tokyo, Japan).

Hemolytic activity against human erythrocytes

The hemolytic activity of the compound was evaluated by determining the release of hemoglobin from a 4 % suspension of fresh human erythrocytes at 414 nm with an ELISA plate reader [11]. The hemolysis percentage was calculated by using the following equation: Percentage hemolysis = [($Abs_{414\ nm}$ in

the compound solution – $Abs_{414\ nm}$ in a phosphate-buffered saline (PBS))/($Abs_{414\ nm}$ in 0.1 % Triton X-100 – $Abs_{414\ nm}$ in a PBS)] × 100.

Effects of sodium azide (NaN_3) on antifungal activity

To determine whether the antifungal activity of chlorogenic acid is dependent on the metabolic activity of fungal cells, killing assays were performed in the presence of NaN_3, which blocks mitochondrial respiration. *C. albicans* were seeded on a 96-well microtiter plate at a density of 2×10^4 cells (100 µl per well). Forty micrograms of chlorogenic acid (at half the MIC), with and without 0.002 % NaN_3, a well-known metabolic inhibitor, was added to these fungal cells, to a final concentration [12]. An MTT assay was performed in the same way as that of an antifungal activity test. The results represent the average of measurements taken in triplicate of three independent assays.

Flow cytometric analysis for the plasma membrane potential

For analysis of the membrane integrity after compound treatment, log-phased cells of *C. albicans* (1×10^6 cells) were harvested and resuspended with a 1-ml fresh YPD medium, containing chlorogenic acid (at 3 times the MIC). After incubation for 3 h, the cells were washed with a PBS. To detect depolarization of the cell membrane, 1 ml PBS containing 50 µg of bis-(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4(3)$; Molecular Probes, Eugene, OR, USA), was added and the samples were incubated for 1 h at 4 °C in the dark [13]. Flow cytometric analysis was performed via a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

Measurement of plasma membrane fluorescence intensity

Fluorescence from the plasma membrane of *C. albicans* cells labeled with 1,6-diphenyl-1,3,5-hexatriene (DPH; Molecular Probes) was detected to investigate changes in membrane dynamics. Fungal cells (1×10^6 cells) were treated with compound in the concentration range between 0 and 10 times the MIC value and incubated for 3 h at 28 °C. Labeling and fluorescence measurement were performed, exactly as described [12].

Determining released glucose and trehalose

One milliliter of the *C. albicans* cell suspension (1×10^8 cells), containing 400 µg/ml of chlorogenic acid or 50 µg/ml of amphotericin B (at 5 times the MIC), was incubated for 1 h at 28 °C in a PBS. The fungal cells were settled by centrifugation (12 000 rpm for 20 min). The pellets were dried to calculate their dry weight and supernatants were transferred to a new tube. Released glucose and trehalose-containing supernatants were added to 0.05 units of trehalase. After 1 h of enzymatic reaction at 37 °C, the reaction suspension was mixed with water and 16 % DNS reagent (3,5-dinitrosalicylic acid 1 %, NaOH 2 %, sodium potassium tartrate 20 %) was added. For the reaction of glucose with the DNS reagent, the mixture was boiled for 5 min and cooled. Color formations were measured at 525 nm. The results represent the average of the measurements conducted in triplicate of three independent assays.

RESULTS AND DISCUSSION

Antifungal and hemolytic activities

The antifungal activity of chlorogenic acid on human pathogenic yeast strains have been investigated and described as the MIC. In the current study, amphotericin B, a fungicidal agent widely used in treating serious systemic infections, was used as a positive control [14].

The in vitro antifungal activity of chlorogenic acid against pathogenic yeasts was determined by an MTT assay [11]. The strains, such as *C. albicans*, *T. beigelii*, and *M. furfur*, exist in humans as commensals and are superficial contaminants that can cause a variety of serious infections. All yeast strains are highly susceptible to chlorogenic acid, with MIC values between 40 and 80 μg/ml; however, the antifungal activity of chlorogenic aicd is less potent than that of amphotericin B, with MIC values between 2.5 and 10 μg/ml (Table 1).

Table 1 Antifungal activities of chlorogenic acid against various fungi.

	MIC (μg/ml)		
	C. albicans	*M. furfur*	*T. beigelii*
Chlorogenic acid	80	40	40
Amphotericin B	10	10	2.5

To visualize the antifungal activity of chlorogenic acid against *C. albicans*, the effect of this compound on a dimorphism of *C. albicans* was examined. With respect to *C. albicans*, the alteration from yeast to mycelial form is a crucial factor regarding the pathogenesis [15]. The result shows that the hyphae of *C. albicans* is not only inhibited but also destroyed after treatment of chlorogenic acid (Fig. 1C), whereas the hyphae or pseudohyphae extends normally in the absence of chlorogenic acid (Fig. 1B).

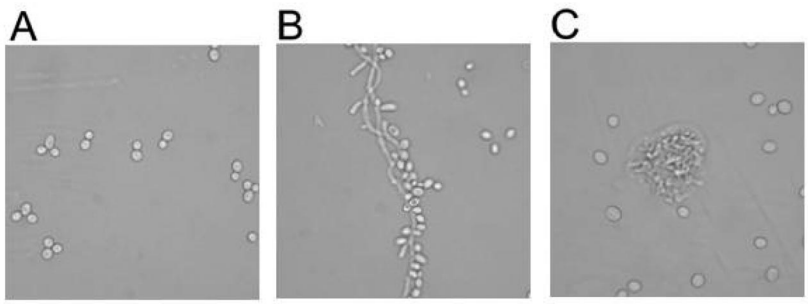

Fig. 1 Effects of chlorogenic acid on the dimorphic transition in *C. albicans*. A, yeast control without a 20 % FBS and chlorogenic acid B, not treated with chlorogenic acid; C, treated with 40 μg/ml of chlorogenic acid in a 20 % FBS.

In order to assess the cytotoxicity of chlorogenic acid against human erythrocytes, hemolytic activity was evaluated by measuring the percentage of hemolysis in 4 % suspension of human red blood cells (hRBCs) at various concentrations of chlorogenic acid. Chlorogenic acid exhibits no hemolytic activity at all tested concentrations, while amphotericin B exhibits potent hemolytic activity at all tested concentrations (Table 2).

Table 2 Hemolytic activities of chloroganic acid against human erythrocytes.

	% hemolysis (μg/ml)					
	80	40	20	10	5	2.5
Chlorogenic acid	0	0	0	0	0	0
Amphotericin B	100	98.6	92.3	86.6	71.2	62.2

Effects of sodium azide on antifungal activity

To investigate the effect of cellular energy consumption toward chlorogenic acid, for membrane-mediated transport pathway, we performed an energy-dependent test on antifungal activity. NaN_3 is a metabolic inhibitor that blocks intracellular ATP synthesis and the ability of ATPase by inhibiting cytochrome oxidase, which further prevents membrane active transport [16]. The viability of the cells is not affected by the presence of 0.002 % NaN_3 (data not shown), but it is exterminated in the presence of chlorogenic acid or amphotericin B, regardless of the presence of NaN_3 (Fig. 2). The current results reveal that the activity of chlorogenic acid is unaffected by NaN_3, suggesting that its effects are mediated by a cellular function, which does not need energy consumption.

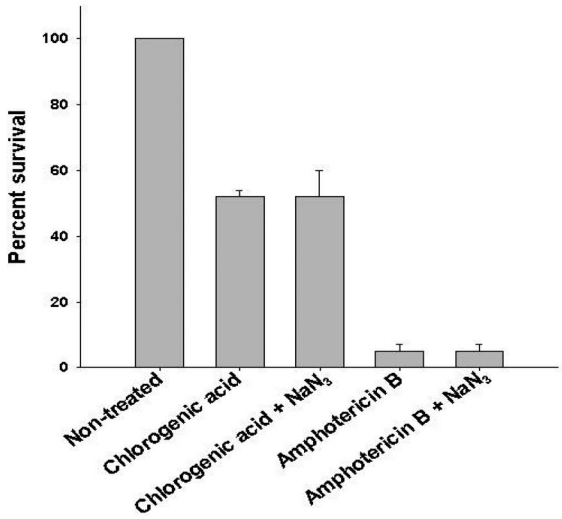

Fig. 2 Effect of NaN_3 on the antifungal activity of chlorogenic acid. Exponential phase *C. albicans* cells were treated with 40 μg/ml of chlorogenic acid or 5 μg/ml of amphotericin B. The cells were incubated 28 °C for 24 h in the presence of 0.002 % NaN_3. The cells that were incubated without NaN_3 were used as positive controls.

Interaction of chlorogenic acid with membranes

In order to provide information about the mode of antifungal action of chlorogenic acid, we selected *C. albicans,* which is both a commensal and opportunistic pathogen of ever-increasing medical importance, as a model organism. This fungus is one of the common causes of life-threatening fungal infections in patients who are immunocompromised due to cancer chemotherapy, organ or bone marrow transplantation, or human immunodeficiency virus infection [17]. To assess whether chlorogenic acid could affect the function of the fungal plasma membrane, its ability to dissipate the membrane potential of *C. albicans* was investigated. *C. albicans* cells were cultured in the presence or absence of chlorogenic acid and the amounts of accumulated $DiBAC_4(3)$ in cells were measured with flow cytometry by staining with $DiBAC_4(3)$. $DiBAC_4(3)$ displays a high voltage sensitivity and can enter depolarized cells where it binds to lipid-rich intracellular components [18]. The result shows that chlorogenic acid-treated *C. albicans* cells pile up $DiBAC_4(3)$ more than that of chlorogenic acid-untreated cells (Fig. 3). It is indicated that chlorogenic acid affects yeast cells by injuring their membranes, thus disrupting membrane potential.

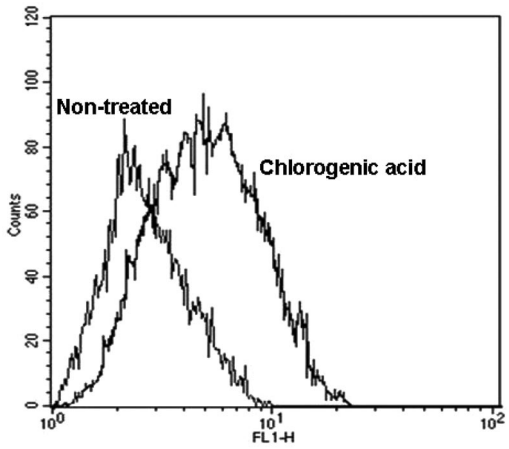

Fig. 3 FACScan analysis of $DiBAC_4(3)$ staining in *C. albicans*. Histograms showed the fluorescence intensity of stained $DiBAC_4(3)$ after *C. albicans* were treated with compound. X-axis (FL1-H) indicates fluorescent intensity for $DiBAC_4(3)$.

To elucidate the membrane fluidity by chlorogenic acid in the plasma membrane of *C. albicans* cells, changes in membrane dynamics were further examined with a fluorescent membrane probe. DPH is a hydrophobic molecule, and this property makes it possible to associate with the hydrocarbon tail region of phospholipids within the cytoplasmic membrane, without disturbing the structure of the lipid bilayer [19]. Assuming the antifungal activity by chlorogenic acid on *C. albicans* is exerted at the level of the plasma membrane, DPH, which interacts with an acyl group of the plasma membrane lipid bilayer, would not be inserted into the membrane. The results show that the DPH fluorescence intensity of chlorogenic acid or amphotericin B-treated cells decreases in a dose-dependent manner and the DPH fluorescent activity in turn reflected the antifungal activity of chlorogenic acid against *C. albicans* cells, by disrupting the plasma membrane (Fig. 4).

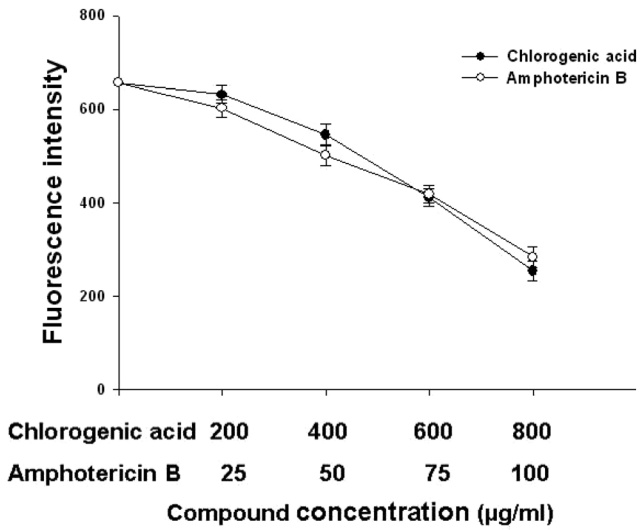

Fig. 4 DPH fluorescence intensity after the addition of chlorogenic acid and amphotericin B. The error bars represent the standard deviation (SD) values for three independent experiments, performed in triplicate.

Fungal cells maintain their membrane potential by establishing multiple ion gradients across the cytoplasmic membrane. The proper maintenance of intracellular components is important to fungal viability. Therefore, *C. albicans* cells were cultured in the presence of chlorogenic acid or amphotericin B, and the amounts of released glucose and trehalose were investigated. Trehalose can protect proteins and biological membranes from inactivation or denaturation caused by a variety of stress conditions, including desiccation, dehydration, heat, cold, oxidation, and toxic agents in yeast [20,21]. The results show that chlorogenic acid or amphotericin B-treated cells both increase more extracellular glucose and trehalose than the compound-untreated cells (Fig. 5). The analysis of glucose and trehalose release, during chlorogenic acid exposure, suggests that it may be one of several intracellular components released during membrane disruption by chlorogenic acid. As for the mechanism by which chlorogenic acid breaks down the membrane permeability barrier, it is possible that chlorogenic acid perturbs the membrane lipid bilayers, causing the leakage of ions and other materials as well as forming pores and dissipating the electrical potential of the membrane.

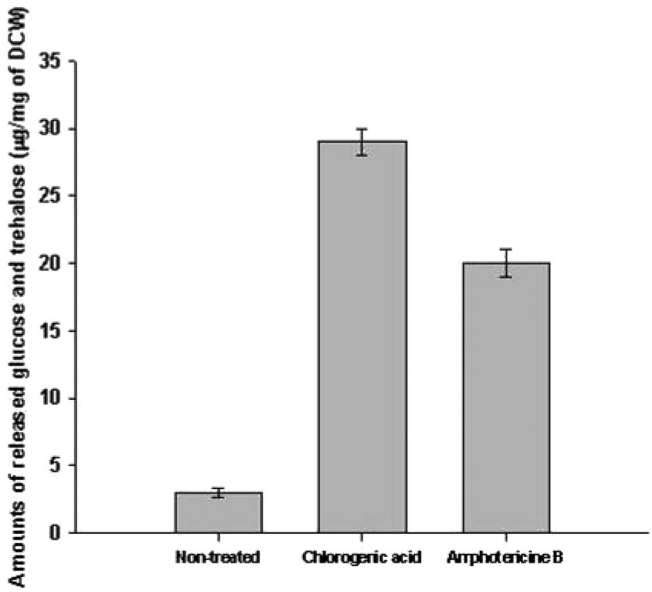

Fig. 5 Concentration of released trehalose and glucose from *C. albicans* by chlorogenic acid and amphotericin B. The error bars represent the SD for three independent experiments, performed in triplicate.

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