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Antifungal activity of isothiocyanates extracted from horseradish (*Armoracia rusticana*) root against pathogenic dermal fungi

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Abstract To develop natural antifungal agents against pathogenic dermal fungi, the antifungal activity of isothiocyanates (ITCs) extracted from horseradish (*Armoracia rusticana*) root was investigated. A paper disk diffusion assay showed that ITCs inhibited growth of the four pathogenic dermal fungi (*Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Microsporum canis*, and *Epidermophyton floccosum*) at 5000 μg/mL, as well as perfectly inhibited the growth of the fungi at 10,000 μg/mL in a concentration-dependent manner. The minimum inhibitory concentrations of ITCs against *T. rubrum*, *T. mentagrophytes*, *M. canis*, and *E. floccosum* were 200, 200, 100, and 100 μg/mL, respectively. The minimum fungicidal concentrations of ITCs against the four pathogenic dermal fungi were 200 μg/mL. These results strongly suggested that ITCs extracted from horseradish root can be a candidate of natural antifungal agents against pathogenic dermal fungi, even though further study is needed to investigate how to use ITCs in clinical therapy.

Keywords Antifungal activity · Isothiocyanates · Dermatophytes · MIC · MFC

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Introduction

Onychomycosis is a chronic fungal infection of the nails and affects 20% of the world population under 40 years of age [1–3]. Various fungi can cause onychomycosis, the most frequently isolated pathogens are dermatophytes, especially *Trichophyton rubrum* and *Trichophyton mentagrophytes,* which cause approximately 71 and 20% of all case of onychomycosis, respectively. About 90% of all onychomycotic infections of the toenails in North American series arise from dermatophytes, and the infections affect the nail bed causing dystrophy and sometimes leading to complete nail loss [4–6].

The drugs, such as terbinafine tablets, itraconazole capsules, griseofulvin, and ciclopirox nail lacquer, have been proved to have good efficacy and reduced side-effect against dermatophytes, and they are approved by the Food and Drug Administration [7]. Although the effective medicine is available in the therapy of onychomycosis, there are still many unsolved problems, such as toxic side-effects, emergence of resistant strains, and high relapse rate [8–11].

Essential oils and essences of many herbs and spices are known to have antimicrobial activity. Treatment of strawberries [12], celery, and peppers [13] with vapors of methyl jasmonate, which occurs widely in the plant kingdom [14], extends their shelf life by inhibiting microbial growth. Isothiocyanates (ITCs), breakdown products of glucosinolates, are responsible for the pungent flavor of the plants in the Cruciferae family such as horseradish, mustard, broccoli, and wasabi, having the main component of allyl isothiocyanate (AITC). In addition, ITCs were shown to have strong antibacterial [15, 16], and antifungal [17] activities in liquid and vapor phases, such as *Bacillus subtilis*, *Vibrio parahaemolyticus*, *Candida albicans*,

Aspergillus niger, and *Penicillium citrinum*. The antimicrobial activity of ITCs is believed to be due to the inactivation of extracellular enzymes through the cleavage of disulfide bonds [18]. Several mechanisms have been proposed for the antimicrobial activity of ITCs, including modulation of sulfhydryl enzymes, inhibition of ribonucleic acid (RNA) synthesis, partial inhibition of deoxyribonucleic acid (DNA) synthesis, and inhibition of protein synthesis by an action of the ITCs moiety (–N=C=S), [19]. From the above, it may be reasonable to explore the potential activity of ITCs for prevention of dermatophytes. In this study, therefore, the antifungal activity of ITCs extracted from the horseradish (*Armoracia rusticana*) root for development of natural antifungal agents against dermatophytes was investigated.

Materials and methods

Materials

Horseradish (*A. rusticana*) root powder obtained from Biocoats Co., Ltd. (Seoul, Korea) was used to extract ITCs. Allyl isothiocyanate (AITC, Wako, Tokyo, Japan), Phenylethyl isothiocyanate (PITC, Sigma-Aldrich, St. Louis, MO, USA), and 3-Butenyl isothiocyanate (BITC, Sigma-Aldrich) was prepared to measure the concentration in extracted ITCs.

Preparation of ITCs from horseradish root

ITCs were extracted by using a steam distillation method [16]. A mixture of the horseradish root powder (200 g) and distilled water (550 mL) was prepared to extract ITCs. For maximum production of ITCs by an enzyme (β-thioglucoside glucohydrolase) in the horseradish root, the mixture was shaken in a water bath at 40 °C for 2 h. The reacted mixture was distilled and concentrated at 120 °C in an oil bath for 120 min by rotary evaporator (Rotavapor R-200; BUCHI Labortechnik AG, Flawil, Switzerland). Essential oils (ITCs) were extracted from the concentrated solution using centrifugation (Bionova, Model Mega 17R; Hanil Science Industrial, Seoul, Korea) at 5000×*g* for 20 min. ITCs extracted from the horseradish root were analyzed for the identification of the main active components involved in antifungal activities using a 7890A GC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a 5975C mass spectrometer (Agilent Technologies) according to a method by Choi et al. [20]. ITCs were prepared using dissolution in n-hexane (Showa Chemical Industry Co., Tokyo, Japan) at a 1:1 ratio. The hexane fraction was separated using an HP-5 column (30 m × 0.25 mm I.D., 0.25 μm film thickness; Agilent Technologies). The column temperature was maintained at 50 °C for 1 min, and then increased to 250 °C for 3 min. The inlet temperature was 250 °C for 3 min. Helium was used as a carrier gas at a flow rate of 1.0 mL/min. The extraction yield of ITCs was determined using standard concentrations of AITC, PITC, and BITC.

Preparation of fungal inoculum suspension

The four fungal strains were used for performing the antifungal activity assay of ITCs extracted from the horseradish root. *Trichophyton rubrum* ATCC 28188, *Trichophyton mentagrophytes* ATCC 18748, *Microsporum canis* ATCC 36299, and *Epidermophyton floccosum* ATCC 10227 obtained from the Korean Collection for Type Cultures (KCTC, Daejeon, Korea) were pre-cultured in Sabouraud's dextrose broth (SDB, Difco Co., Detroit, USA) for 2 weeks at 28 °C. The inocula were then cultured on a Sabouraud's dextrose agar (SDA, Difco Co.) placed slant at 28 °C for 7 days in order to produce conidia according to a method by Maria et al. [21]. Turbidity of the final conidia was adjusted to 70% transmission at a wavelength of 520 nm using a spectrophotometer, which is corresponding to 1.0×10^6 to 5.0×10^6 CFU/mL.

Paper disk diffusion assay

The antifungal activity of ITCs extracted from the horseradish root was measured using the paper disk diffusion assay. The culture of fungal spores was adjusted to 1.0×10^6 CFU/mL using the sterile culture medium described above, and 100 μL of each fungal culture was spread on a Sabouraud dextrose agar (SDA, Difco Co.) plate with a sterile glass rod. Each 100 μL of ITCs (10,000, 5000, and 2500 mg/L) diluted with sterilized SDB was absorbed into a 8-mm paper disk (Whatman No. 2) placed on the SDA plate and then incubated at 28 °C for 2 weeks.

Minimum inhibitory concentration (MIC) assay

The MICs of ITCs against fungi were determined by the standard M38-A approved by National Committee for Clinical Laboratory Standards (NCCLS, 22) with some modification. The conidia suspensions of the four fungal strains were diluted 1:50 in RPMI 1640 medium (Gibco, Thermo Fisher Scientific, MA, USA) to get 1.0×10^6 CFU/mL. The ITCs (800 μg/mL) extracted from the horseradish root was initially dissolved in ethyl alcohol and then serially diluted in a twofold series with the sterilized RPMI 1640 medium. Each 100 μL of fungus adjusted to 1.0×10^6 CFU/mL was inoculated into microtubes, which had been previously filled with 100 μL medium containing 100 μL of ITCs at different

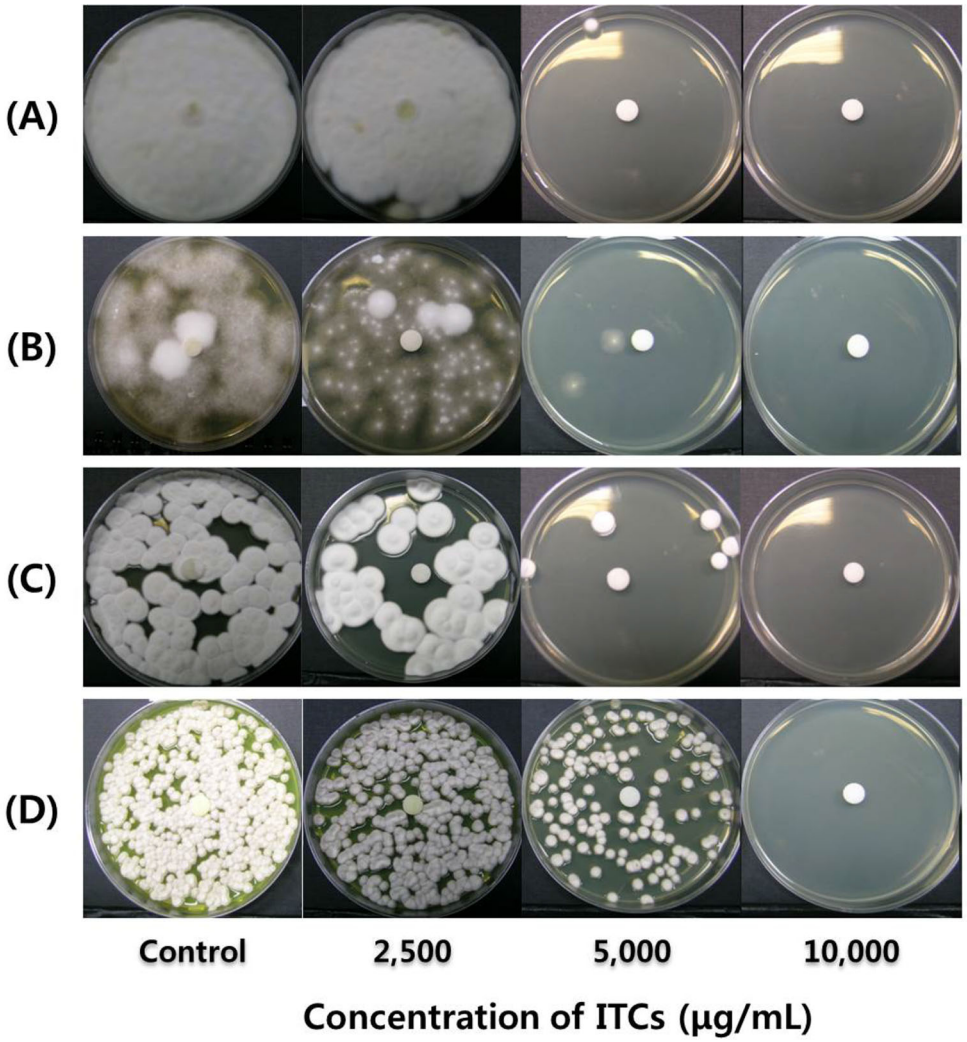

Fig. 1 Antifungal activities of ITCs extracted from the horseradish root against the four fungal strains as analyzed by the paper disk diffusion assay. (A), *Trichophyton rubrum*; (B), *Trichophyton mentagrophytes*; (C), *Microsporum canis*; (D), *Epidermophyton floccosum*

concentrations. The micro-tubes were incubated at 28 °C for 2 weeks. The growth of each fungus was judged by naked eye. MIC was defined as the lowest concentration that shows visible growth. The sterilized RPMI 1640 medium was used as the blank. The negative control was a mixture of 100 μL of the sterilized RPMI 1640 medium and 100 μL of each strain inoculum.

Minimum fungicidal concentration (MFC) assay

The MFCs of ITCs against the four fungal strains were determined according to a method by Bamba et al. [23]. A loopful of each fungal culture without visible growth in the micro-tube was inoculated onto the SDA plate and incubated under the same conditions described above. MFC is defined as the lowest concentration that show no growth on the SDA plates.

Results and discussion

Active components for antifungal activities of ITCs extracted from horseradish root

ITCs extracted from the horseradish root were analyzed using gas chromatograph-mass spectrometer (GC–MS) to identify the main components involved in antibacterial activities (data not shown) as detailed in a previous paper [20]. ITCs contained two major ITC derivatives, 59.995% AITC and 35.819% PITC, and one minor ITC derivative of 1.532% BITC. The concentrations of AITC and PITC in ITCs were calculated using a standard curve. The horseradish extract contained 208,766 μg/mL of AITC and 72,778 μg/mL of PITC [20].

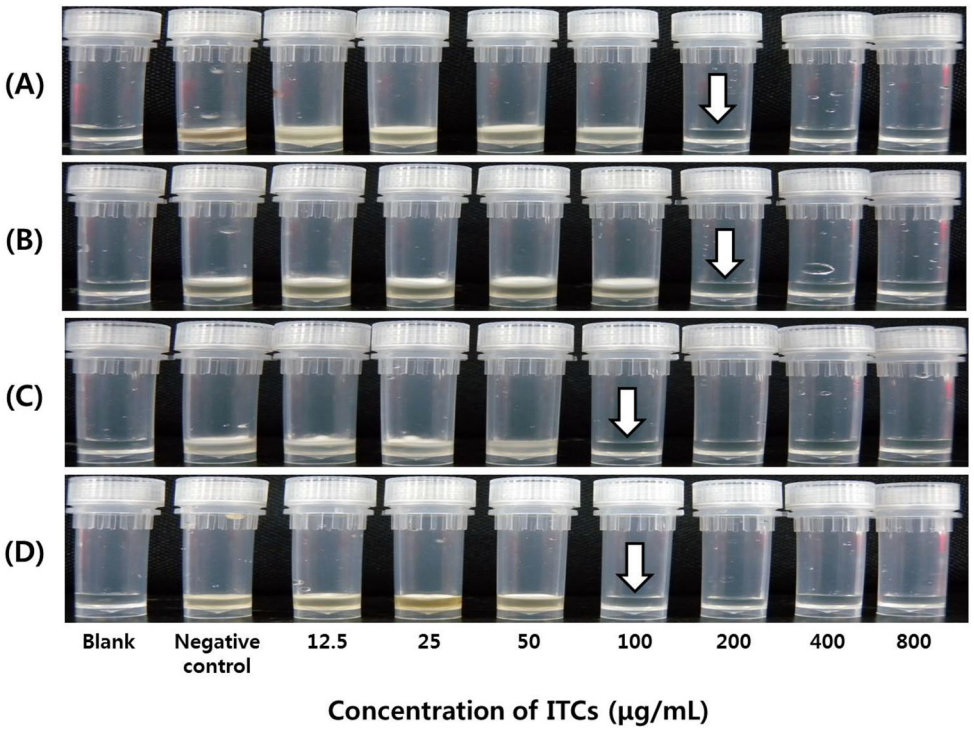

Fig. 2 MIC of ITCs extracted from the horseradish root against the four fungal strains. (A), *Trichophyton rubrum*; (B), *Trichophyton mentagrophytes*; (C), *Microsporum canis*; (D), *Epidermophyton floccosum*

Table 1 MIC of ITCs extracted from horseradish root against four fungal strains

Fungus	Minimum inhibitory concentration (μg/mL)								
	Blank	N.C.	12.5	25	50	100	200	400	800
T. rubrum	–[a]	++[b]	++	+[c]	+	+	–	–	–
T. mentagrophytes	–	++	++	+	+	+	–	–	–
M. canis	–	++	++	+	+	–	–	–	–
E. floccosum	–	++	++	+	+	–	–	–	–

[a] no growth
[b] good growth
[c] growth

Table 2 MFC of ITCs extracted from horseradish root against four fungal strains

Fungus	Minimum fungicidal concentration (μg/mL)								
	Blank	N.C.	12.5	25	50	100	200	400	800
T. rubrum	–[a]	++[b]	++	+[c]	+	+	–	–	–
T. mentagrophytes	–	++	++	+	+	+	–	–	–
M. canis	–	++	++	+	+	+	–	–	–
E. floccosum	–	++	++	+	+	+	–	–	–

[a] no growth
[b] good growth
[c] growth

Antifungal activity of ITCs extracted from horseradish root

The antifungal activities of ITCs extracted from the horseradish root against the four fungal strains as analyzed by the paper disk diffusion assay are shown in Fig. 1. ITCs inhibited growth of the four fungal strains at 5000 μg/mL, and perfectly inhibited their growth at 10,000 μg/mL. Antifungal activities of ITCs against the four pathogenic dermal fungi were concentration dependent. The reason for

the formation of a clear zone all over the agar plate, but not around paper disk, may be the volatile nature of ITCs.

Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

The analysis of MIC and MFC, revealed that the ITCs extracted from the horseradish root possessed potent antifungal activity against all the tested fungi. The MICs of ITCs against *T. rubrum*, *T. mentagrophytes*, *M. canis*, and *E. floccosum* were 200, 200, 100, and 100 μg/mL, respectively (Fig. 2 and Table 1). The MFCs of ITCs against the tested fungi were 200 μg/mL (Table 2).

The appearance of multidrug-resistant fungal strains and high relapse rate of onychomycosis makes it necessary to discover new classes of antifungal agent to solve the problem. For a long time, natural products, either as pure compounds or as extracts, are the focal point in the area of medicine research, and a series of molecules with antifungal activity against different fungal strains have been found, such as crude extracts, essential oils, terpenoids, saponins, phenolic compounds, alkaloids, peptides and proteins [24].

Horseradish (*A. rusticana*) is a perennial plant of the Cruciferae family, both root and leaves were being used as a medicine during the middle ages and the root was used as a condiment on meats in Germany, Scandinavia, and Britain [25]. ITCs are breakdown products of sinigrin, which is the predominate glucosinolate presenting in horseradish, and is proved to be components responsible for the antimicrobial activities [26]. The antimicrobial activity of ITCs is attributed to its amphiphilic structure. Thiocyanate moiety (–N=C=S) is a key factor of the antimicrobial activity of ITCs. ITCs play a role mainly through inducing oxidative stress. [17, 27–29].

This study proved that ITCs have strong antifungal activities against the four pathogenic dermal fungi, with a MIC of 100–200 μg/mL (Table 1) and MFC of 200 μg/mL (Table 2). The antifungal activity of ITCs on *Penicillium notatum* [30], *Alternaria alternate* [31], *Aspergillus flavus* and *Endomyces fibuliger* [17] has been proven. Simultaneously, other essential oils have also been proved to have antifungal activities against dermatophytes. The MICs of onion oil against *T. rubrum, T. mentagrophytes, M. canis*, and *E. floccosum* were 2008 μg/mL [32]. *Melaleuca alternifolia* (tea tree) oil [33], and *Moringa oleifera* Lam oil [34] also have antifungal activities against *Trichophyton* sp. However, we did not compare our data with those results, because the method of measuring the antifungal activity is questionable, such as the initial concentration, exposure time and breakpoints, and is not standardized, although NCCLS methods are widely accepted for determining in vitro antifungal activity. In this study, the MIC (100–200 μg/mL) of ITCs against *T. rubrum, T. mentagrophytes, M. canis*, and *E. floccosum* was lower than those (2008 μg/mL) of onion oil. The MFC (200 μg/mL) of ITCs against the tested fungal strains was lower than MBC (208–2083 μg/mL) of ITCs against 5 dominant bacteria isolated from *Jeotgal*, Korean fermented sea food [16], and MBC (208.3–666.7 μg/mL) of ITCs against antibiotic-resistant bacteria [35]. These results indicate that ITCs are a potential antifungal agent, and the antimicrobial activity of ITCs against fungi is stronger than against bacteria.

From these results, ITCs extracted from horseradish root can be considered a candidate of natural antifungal agents against pathogenic dermal fungi. However, method of application of ITCs to clinical therapy needs further study.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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