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Antifungal and synergistic effects of an ethyl acetate extract of the edible brown seaweed *Eisenia bicyclis* against *Candida* species

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Abstract

With the continuing demand for new solutions in the development of effective and safe candidiasis therapies, we investigated the efficacy of an antifungal agent from the marine brown alga *Eisenia bicyclis*. The methanolic extract of *E. bicyclis* evinced potential antifungal activity against *Candida* species. The ethyl acetate (EtOAc)-soluble extract from *E. bicyclis* demonstrated the strongest antifungal activity against *Candida* species among five solvent-soluble extracts. Indeed, the EtOAc-soluble extract showed minimum inhibitory concentrations (MICs) ranging from 4 to 8 mg/mL. Furthermore, the EtOAc-soluble extract considerably reversed high-level fluconazole resistance of *Candida* species. The MIC values of fluconazole against *Candida* species decreased substantially (from 64 to 4 μg/mL) in combination with the MIC of the EtOAc-soluble extract (4 mg/mL). The fractional inhibitory concentration indices of fluconazole ranged from 0.531 to 0.625 in combination with 4, 2, or 1 mg/mL of the EtOAc-soluble extract against *Candida* isolates, indicating that these combinations exert a marked synergistic effect against *Candida* isolates. These findings imply that compounds derived from *E. bicyclis* can be a potential source of natural antifungal agents against *Candida* species.

Key words: Antifungal activity, *Candida* species, *Eisenia bicyclis*, Synergistic effect

Introduction

Candida species, commonly isolated from clinical material, are a common cause of fungal infections in humans. The most pathogenic *Candida* species are *C. albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, and *C. tropicalis*. Among these, C. *albicans* is generally responsible for 90–100% of mucosal infections and for 50–70% of candidemia episodes. Since Wilkinson first described the association between fungus and vaginal discharge (candidiasis) in 1849, many antifungals such as compounds of polynes and azoles have been used for treatment. Among the drugs used in treating *Candida* infections, fluconazole (an orally active triazole agent) is well established as a first-line management option for both localized and systemic infections. However, antifungal use during the past few years has aided in the development of strains resistant to many

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antifungal antibiotics. Side effects associated with fluconazole include nausea, vomiting, diarrhea, and liver enzyme elevation.

As alternatives with fewer side effects, medicinal plants and marine organisms have been investigated for candidiasis treatment. *Eisenia bicyclis* is a common perennial in the group Phaeophyceae (brown alga), and is generally distributed in the region of Ulleung island in the East Sea of Korea. It has been added to appetizers, casseroles, muffins, pilafs, and soups (Maegawa, 1990; Yoon et al., 2011). The antioxidant activities of *E. bicyclis* phlorotannins, such as eckol (a trimer), phlorofucofuroeckol A (a pentamer), dieckol, and 8,8'-bieckol (hexamers), have been previously described (Okada et al., 2004). Additionally, several medicinal functions of *E. bicyclis* have been reported, including antitumor (Ermakova et al. 2013), anti-Alzheimer's disease (Ahn et al., 2012), antiatherosclerosis (Kang et al., 2006), anti-inflammatory (Jung et al., 2013), anticoagulant (Jeong et al., 2009), antiallergic disease, and anticancer activities (Shibata et al., 2003; Yoon et al., 2013). Phlorotannins have also been known to show potent antimicrobial activity against several microorganisms (Eom et al., 2013). However, very little research has been done on the antifungal activity of *E. bicyclis* against *Candida* species. Here, we demonstrated that *E. bicyclis* methanol (MeOH) extract and its solvent-soluble form have high antifungal effects against *Candida* species, and may act as alternative and therapeutic agents for candidiasis.

Materials and Methods

Plant materials and extraction

In late September 2010, *E. bicyclis* was purchased from Ulleung Trading Co. (Ulleung-Gun, Korea). A voucher specimen was deposited in the author's laboratory. Dried *E. bicyclis* was finely ground and powdered with a food mixer (HMF-1000A; Hanil Electronics, Seoul, Korea). The dried powder was vacuum-packed and kept at –20°C until use. The dried *E. bicyclis* powder (1.0 kg) was extracted with MeOH (10 L × 3) at 70°C for 3 h (3 times), and the solvent was evaporated *in vacuo* with a rotary evaporator (N-1001S-W; Eyela, Tokyo, Japan). The crude MeOH extract of *E. bicyclis* was suspended in 10% MeOH (1.0 L) and partitioned in turn with *n*-hexane (Hexane), dichloromethane (DCM), ethyl acetate (EtOAc), and *n*-butanol (BuOH) in sequence. The concentration of each extract was adjusted to 500 mg/mL by dissolving in dimethyl sulfoxide (DMSO) under sterile conditions and stored at –70°C until used.

Strains and culture conditions

In this study, we used the yeast strain *C. albicans* from the Korean Collection for Type Cultures (KCTC; Daejeon, Korea). *Candida albicans* and *C. glabrata* clinical isolates from a variety of body sites were provided by the Gyeongsang National University Hospital (Jinju, Korea), a member of the National Biobank of Korea. The isolates were maintained at 4°C on Sabouraud Dextrose Agar (Difco, Franklin Lakes, NJ, USA) plates and subcultured for 24 h at 37°C in Sabouraud Dextrose Broth (SDB; Difco) before each experiment to ensure viability. The disk diffusion assay was prepared in Mueller–Hinton agar (MHA; Difco) supplemented with 2% glucose and 0.5 μg/mL of methylene blue, and the broth dilution method was carried out in RPMI-1640 supplemented with 2% glucose (Difco) according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (2004; formerly NCCLS).

Antifungal efficacy was evaluated via disk diffusion assays, as described by the CLSI (2004). In brief, *Candida* species were cultured in SDB at 37°C until cells reached an $OD_{600\ nm}$ of 0.5. One hundred microliters of fungal culture containing approximately 10^4–10^5 CFU/mL was spread on MHA plates. A paper disc (8 mm in diameter) containing 50 mg of each extract was placed on the plates. After incubation for 24 h at 37°C, the diameter of the inhibition zone was measured on fungal culture plates. The experiment was carried out three times and mean values are presented.

Determination of minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs)

The concentrations of *E. bicyclis* MeOH extract and its solvent-soluble extracts were both 500 mg/mL. To determine the minimum inhibitory concentration (MIC) values of the MeOH extract and its solvent-soluble extracts, each extract was diluted with RPMI-1640 supplemented with 2% glucose to obtain a stock solution of 1, 2, and 4 mg/mL. The MICs were at the lowest concentrations of MeOH and solvent-soluble extracts to inhibit the visible growth of microorganisms after aerobic incubation for 48 h using RPMI-1640 supplemented with 2% glucose, modified from the guidelines of CLSI document M27-A3 (CLSI, 2008). MICs of the solvent-soluble extracts were determined by the twofold serial dilution method in 96-well flat-bottomed microtitration plates at a final concentration of 5×10^5 CFU/mL. The microtitration plates were read visually and the MIC was recorded as that of the extracts that exhibited no turbidity. For minimum fungicidal concentration (MBC) testing, an aliquot of inoculum was taken with a MIC test well that did not show turbidity, and was poured onto SDA agar (Difco) plates with *Candida* species. The agar plates were incubated at 37°C until growth was detected in the growth control plates. The MFC value was defined as the lowest concentration required to kill 99.99% or more of the initial inoculum. The MIC and MFC experiments were repeated thrice.

Synergistic effects of ethyl acetate-soluble extract from *E. bicyclis* with fluconazole against *Candida* species

The interaction between the EtOAc-soluble extract from *E. bicyclis* and antibiotic fluconazole (Sigma Chemical Co., St. Louis, MO, USA) against *Candida* species was tested by the checkerboard method (Perea et al., 2002; Weig and Muller, 2001). The synergistic effect was evaluated as a fractional inhibitory concentration (FIC) index. With the checkerboard test, the FIC was calculated as the MIC values of an antibiotic or EtOAc-soluble extract in combination and divided by the MIC of the antibiotic or EtOAc-soluble extract alone. The FIC was then summed to derive the FIC index, which indicated synergy. Index values were determined using the following formulas:

$$FIC_A = MIC_A \text{ in combination}/MIC_A,$$
$$FIC_B = MIC_B \text{ in combination}/MIC_B,$$
$$FIC\ Index = FIC_A + FIC_B$$

where A and B are the MICs of drug A and compound B in the combination, MIC_A and MIC_B are the MICs of drug A and compound B alone, and FIC_A and FIC_B are the FICs of drug A and compound B.

The interaction was defined as synergistic if the FIC index was <1, additive if the FIC index was 1.0, subadditive if the FIC index was between 1.0 and 2.0, indifferent if the FIC index was 2, and antagonistic if the FIC index was >2. Synergy was further subclassified as marked (FIC index ≤ 0.50) or weak (FIC index between 0.50 and 1.0).

Statistical analysis

In all cases, analyses were performed in triplicate and data were averaged. We calculated standard deviation for all measurements. We used Student's t-test ($\alpha = 0.05$) in SPSS 12.0 (SPSS Inc., Chicago, IL, USA) to test differences between MIC values for each individual microorganism.

Results and Discussion

Antifungal activity of the *E. bicyclis* methanol extract and its solvent-soluble extracts against *Candida* species

Fluconazole has been widely used for the treatment of candidiasis. However, it can cause numerous side effects, including nausea, vomiting, and headache, as well as liver damage and altered estrogen (Lilly, 2012). In addition, increasing reports of fluconazole-resistant *C. albicans* strains have been published (Pfaller, 2012). Therefore, a need exists to develop new medicines or alternative therapies for candidiasis.

In an effort to decrease antibiotic use and discover an alternative therapeutic agent for treating *Candida* infections, we tested the MeOH extract and its soluble extracts from *E. bicyclis*, a brown alga. The relative susceptibility of *Candida* species to potential antimicrobial agents was measured by a clear zone of growth inhibition around the disc.

The antifungal activity of the MeOH extract and its solvent-soluble extracts are presented in Table 1. The MeOH extract of *E. bicyclis* exhibited antifungal activity against *Candida* species, suggesting that the extract contained antifungal substances. For *C. albicans*, the clear zones of treatment with the EtOAc-soluble extract had a diameter of 21.0 mm and a concentration of 50 mg per disc. For *C. albicans* isolates, the antifungal activity of the EtOAc-soluble extract was 50 mg per disc (diameter of inhibition: 15–25 mm). For *C. glabrata* isolates, the antifungal activity of the EtOAc-soluble extract was also 50 mg per disc (diameter of inhibition: 12–17 mm). However, the water-soluble form of the MeOH extract did not

Table 1. Antifungal activity of the methanol extract and its solvent-soluble extracts from *Eisenia bicyclis* against *Candida* species

Strains	Zone of inhibition (mm)[a]					
	MeOH[b]	Hexane	DCM	EtOAc	BuOH	H_2O
Candida albicans (KCTC 7122)	20.0	12.0	13.0	21.0	12.0	-[c]
C. albicans isolate 92	22.0	13.0	18.0	25.0	13.0	-
C. albicans isolate 115	22.0	13.0	13.0	15.0	11.0	-
C. albicans isolate 2392	21.0	15.0	16.0	27.0	-	-
C. glabrata isolate 313	20.0	13.0	14.0	17.0	13.0	-
C. glabrata isolate 1808	24.0	12.0	-	12.0	-	-
C. glabrata isolate 2554	22.0	-	-	17.0	-	-
C. glabrata isolate 2672	23.0	13.0	13.0	15.0	12.0	-

[a]The methanol extract and its solvent-soluble extracts from *Eisenia bicyclis* at concentrations of 50 mg/mL was loaded onto a disk (8 mm in diameter).
[b]MeOH, methanolic extract; Hexane, *n*-hexane-soluble extract; DCM, dichloromethane-soluble extract; EtOAc, ethylacetate-soluble extract; BuOH, *n*-butanol-soluble extract; H_2O, water-soluble extract.
[c]–, no detected antifungal activity.

exhibit antifungal properties against all *Candida* species. In Table 1, we show different patterns of antifungal activities for the same species. These differences may be due to the presence of additional mechanisms of resistance. According to Rodloff et al. (2011), different *Candida* species may vary in their susceptibility to antifungals.

Khaled et al. (2012) showed that the EtOAc extract of the brown alga *Padina pavonica* showed significant antifungal activity against *C. glabrata* (diameter of inhibition: 16 mm) and *C. krusei* (diameter of inhibition: 14 mm). The sterile disks were impregnated with different extracts and dried (25 µL/disc). Thus, *E. bicyclis* displays a similar antifungal activity against *Candida* species as other brown seaweeds.

Measurement of MIC and MFC values of *E. bicyclis* extract

To quantitatively evaluate antifungal activity, we investigated the MIC and MFC values of the MeOH extract and its solvent-soluble extracts. The MIC values of five solvent-soluble extracts against *Candida* species varied depending on the polarity of the solvent. Among five solvent-soluble extracts, the EtOAc-soluble extract showed the lowest MIC values against *Candida* species. The EtOAc-soluble extract was able to completely inhibit the growth of *Candida* species at concentrations of 4 and 8 mg/mL. The antifungal activity of the EtOAc-soluble extract against *Candida* species was higher than that of other soluble extracts. Lopes et al. (2013) reported the antifungal activity of the purified phlorotannins extracts from the brown seaweed *Cystoseira nodicaulis* against *C. albicans*. The MIC values of purified phlorotannin extracts of *C. nodicaulis* were 15.6 mg/mL against *C. albicans* (American Type Culture Collection strain 10231) and 62.5 mg/mL against *C. albicans* isolates. In this study, the EtOAc-soluble extract of *E. bicyclis* exhibited increased antifungal activity against *C. albicans* in comparison to that of *C. nodicaulis*. The MFC values of the EtOAc-soluble extract against *Candida* species were 16–32 mg/mL (Table 2). In contrast, the water-soluble extract did not exhibit antifungal activity against *Candida* species. According to Eom et al. (2013), marine-derived polyphenols (phlorotannins) are believed to be the active components of *E. bicyclis*. These are the predominant EtOAc-soluble compounds in brown algae (Choi et al., 2010). Among EtOAc-soluble compounds, polyphenol polymers (eckol, phlorofucofuroeckol A, dieckol, and 8,8′-bieckol) exhibited potent antibacterial activities (Isnansetyo and Kamei, 2009; Nagayama et al., 2002). Differing patterns of antifungal activity were also observed in MIC and MFC values within species. Although the underlying mechanisms of these solvent-soluble extracts were not completely elucidated, evidence exists indicating effects on ergosterol and chitin composition in filamentous fungi, and ergosterol and respiration in yeast (Lopes et al., 2013). Therefore, further research on *E. bicyclis* methanol extract and its solvent-soluble extracts against *Candida* species may provide clues regarding susceptibility to candidiasis.

In addition, we found *Candida* isolates to be highly resistant to the antibiotic fluconazole in this study. Among *Candida* isolates, *C. glabrata* isolates with high-level resistance to fluconazole were detected, with MIC values of 8 mg/mL. Thus, further studies are needed to elucidate the main components of *E. bicyclis* against *Candida* species.

Synergic effects between the ethyl acetate-soluble extract from *E. bicyclis* and the antibiotic fluconazole against *Candida* species

Natural materials such as plant- or marine-derived compounds in combination with traditional medicines can be used as effective approaches for restoring antibiotic activity in treatments against drug-resistant bacteria (Eom et al., 2013). Sharma et al. (2010) reported that pure polyphenol curcumin I from *Curcuma longa* exhibited a marked synergy with ampho-

Table 2. Minimum inhibitory concentrations (MIC) and minimum fungicidal concentrations (MFC) of the methanol extract and its solvent-soluble extracts from *Eisenia bicyclis* against *Candida* species

Strains	MeOH[a]		Hexane		DCM		EtOAc		BuOH		H_2O		fluconazole
	MIC[b]	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	
Candida albicans (KCTC 7122)	32	64	32	64	32	64	8	16	32	64	>32	>32	64 µg/mL
C. albicans isolate 92	16	64	16	64	8	64	4	16	4	32	>32	>32	4 mg/mL
C. albicans isolate 115	16	64	16	64	4	32	4	16	4	32	8	32	512 µg/mL
C. albicans isolate 2392	32	64	32	64	16	64	4	16	8	32	>32	>32	512 µg/mL
C. glabrata isolate 313	>32	>32	>32	>32	>32	>32	8	32	>32	>32	>32	>32	8 mg/mL
C. glabrata isolate 1808	16	64	16	64	16	32	8	16	16	64	>32	>32	8 mg/mL
C. glabrata isolate 2554	>32	>32	>32	>32	>32	>32	4	32	>32	>32	>32	>32	8 mg/mL
C. glabrata isolate 2672	>32	>32	>32	>32	>32	>32	8	32	>32	>32	>32	>32	512 µg/mL

[a]MeOH, methanolic extract; Hexane, *n*-hexane-soluble extract; DCM, dichloromethane-soluble extract; EtOAc, ethyl acetate-soluble extract; BuOH, *n*-butanol-soluble extract; H_2O, water-soluble extract.
[b]MIC and MFC values for the MeOH and its solvent-soluble extracts from *E. bicyclis* are express as mg/mL.

tericin B antibiotics against *C. albicans*. Riccardin C, isolated from the Chinese liverwort *Plagiochasm intermedium* L., also showed synergistic or additive activity when combined with fluconazole against fluconazole-resistant *C. albicans* strains (Xie et al., 2010). Based on these reports, the synergistic effects of marine-derived polyphenol on *Candida* species were assessed in combination with commercial antibiotics to treat candidiasis.

Our results in Table 2 reveal that *Candida* isolates are resistant to fluconazole. Due to their resistance to commonly used antibiotics, a need exists for more effective antifungal agents. Thus, the FIC test for the combination of EtOAc-soluble extract and antibiotics was assessed using the checkerboard test. According to the results displayed in Table 3, the MIC values of fluconazole against *C. albicans* KCTC 7122 decreased from 64 to 4 μg/mL when fluconazole administered in combination with 4 mg/mL of the EtOAc-soluble extract. The MIC values of fluconazole against *Candida* isolates were also greatly diminished when administered in combination with the EtOAc-soluble extract. The FIC indices of antibiotics were between 0.531 and 0.536 in combination with the concentration of the EtOAc-soluble extract (1.0–4.0 mg/mL) against *Candida* isolates, thereby indicating the marked synergistic inhibitory effect of the EtOAc-soluble extract and fluconazole against the growth of *Candida* species. Thus, the results of the checkerboard assay revealed a restoration of antifungal activity against an antibiotic-resistant *Candida* species when used in combination with the EtOAc-soluble extract from *E. bicyclis*.

In conclusion, we evaluated the antifungal activity of the edible marine brown alga *E. bicyclis* against *Candida* species. Since the EtOAc-soluble extract showed the strongest antifungal activity against *Candida* species among five solvent-soluble extracts, the antifungal activity of *E. bicyclis* extracts may correlate with phlorotannins or marine-derived polyphenolic contents. Although the EtOAc-soluble extract from *E. bicyclis* has been shown to have less antifungal activity compared with commercial antibiotics, *E. bicyclis* can be utilized as an effective, safe, and natural source of antifungal agents. The EtOAc-soluble extract in combination with antibiotics is expected to have an additive therapeutic effect for relieving symptoms against *Candida* species. The results of the present investigation will contribute to the development of an alternative phytotherapeutic agent against antibiotic-resistant *Candida* species.

Table 3. Minimum inhibitory concentrations (MIC) and fractional inhibitory concentration (FIC) indices of the ethyl acetate (EtOAc)-soluble extract from *Eisenia bicyclis* at 4.0 and 2.0 mg/mL (I) or at 2.0 and 1.0 mg/mL (II) in combination with fluconazole used for the treatment of candidacies

(I)

Strains	Fluconazole				
	MIC (μg/mL)			FIC index[a]	
	A	B	C	b	c
Candida albicans (KCTC 7122)	64	4	8	0.563	0.625
C. glabrata isolate 1808	512	16	32	0.531	0.563
C. glabrata isolate 2672	512	32	64	0.563	0.625
C. glabrata isolate 2554	512	32	64	0.563	0.625

(II)

Strains	Fluconazole				
	MIC (mg/mL)			FIC index	
	A	B	C	b	c
C. albicans isolate 92	8	0.5	0.5	0.563	0.563
C. albicans isolate 115	8	0.25	0.5	0.531	0.563
C. albicans isolate 2392	8	0.5	1	0.563	0.625
C. glabrata isolate 313	4	0.125	0.25	0.531	0.563

A, without EtOAc-soluble extract from *E. bicyclis*; B to C and b to c, EtOAc-soluble extract from *E. bicyclis* at 4.0 and 2.0 mg/mL (I) or at 2.0 and 1.0 mg/mL (II), respectively. [a] The FIC index indicated synergistic effect as <0.5, marked synergy; 0.5 to <1.0, weak synergy; 1.0, additive; >1.0 to <2.0, subadditive; 2.0, indifferent; >2.0, antagonistic.

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