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연구보문

The Antidermatophytic Potential of the Marine Isolate of *Aspergillus* sp. Collected from South Coast of Korea

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ABSTRACT: This study was carried out to assess the antidermatophytic potential of the ethyl acetate (EtOAc) extract of the marine isolate of *Aspergillus* sp.. The fungus was isolated by serial dilution, and was identified *Aspergillus* sp.. The EtOAc extract of the fungus was examined to evaluate the antidermatophytic efficacy against the fungal pathogens infecting human skin using the disc diffusion and MIC (minimum inhibitory concentration) determination methods. The EtOAc extract (5 μl disc^{-1}) was considered to have the antidermatophytic activity based on the inhibition percentage of the mycelial growth of the fungi tested such as *Trichophyton mentagrophytes* KCTC 6085, *Microsporum canis* KCTC 6591, *Microsporum canis* KCTC 6348, *Trichophyton rubrum* KCTC 6352, *Microsporum canis* KCTC 6349 and *Trichophyton mentagrophytes* KCTC 6316. The percentage of the inhibition ranged from 54% to 81, and the MIC obtained was 62.5, 62.5, 250, 125, 125, and 125 μg ml^{-1}, respectively. The extract had a strong detrimental effect on the spore germination of the tested skin infectious pathogens. These findings strongly support the role of the ethyl acetate extract as a potential antidermatophytic agent.

Key Words: marine fungus, *Aspergillus* sp., dermatophytes, minimum inhibitory concentration, spore germination

INTRODUCTION

Dermatophytoses are the most common forms of fungal infections found in most countries, affecting skin, hair and nails. They are caused by keratinophytic fungi. Treatment demands the use of the antifungal agents such as griseofulvin and amphotericin B[1]. However, the high cost of this kind of treatment, especially in developing countries the long period of therapy and the emergence of the resistant strains to the antifungal agents should hinder the eradication of these diseases[2,3].

The lack of some new antifungal agents, the apparent increase of the infections by the dermatophytes and the emergence of the resistant to the antifungal therapy lead to the identification of new antifungal agents with potential interest in the treatment of serious fungal infections. As one of the sources for the development of new drugs, natural products are of particular significance[4].

Recently, the abuse of the antibiotics resulted in the development of the drug resistance of some fungal pathogens. This is why the fungal infections are the higher prevalence of risk factors for the patients in hospital[5]. Accordingly, there is an urgent need to search for the more potent antifungal agents. Owing to the limitation of the sources of terrestrial organisms like plants and microbes, most of which have been well investigated chemically and biologically, scientists have begun to extend their attention to those living in oceans since the early 1960s. Antimicrobial substances from natural sources like microorganisms have been investigated to achieve higher levels of human safety standards[6]. Moreover, the whole population of marine microorganisms is being thought to possess a strikingly bigger biodiversity than those of animal and plant kingdoms. Therefore, marine microbes are currently receiving much more attention highlighting

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that some marine microbial products have become potential for their different bioactivities[7,8]. The screening of such natural marine products should offer potential resources since their use is widespread with much of the research population relying on them.

The aim of this study was to investigate the antidermatophytic spectrum of the ethyl acetate extract of the marine based fungus *Aspergillus* sp. against human skin infectious pathogens.

MATERIALS AND METHODS

Isolation of the fungal isolate

The fungus *Aspergillus* sp. was isolated by serial dilution from the blackish woody substrate collected from the upper floating surface of the sea water of Haeundae South Coast, Pusan, Republic of Korea in July 2006[9].

Culture media

The germination and the growth of isolated fungus was maintained on potato dextrose agar (PDA) medium containing per liter 17 g enzymatic digest of casein, 3 g enzymatic digest of soybean meal, 5 g NaCl, 2.5 g dipotassium phosphate and 2.5 g dextrose without supplementing the sea water.

Test microorganisms

The human skin infectious fungal pathogens used in this study were *Trichophyton mentagrophytes* KCTC 6085, *Microsporum canis* KCTC 6591, *Microsporum canis* KCTC 6348, *Trichophyton rubrum* KCTC 6352, *Microsporum canis* KCTC 6349 and *Trichophyton mentagrophytes* KCTC 6316, which were obtained from Korean Agricultural Culture Collection (KACC), Suwon, Republic of Korea. All the strains were maintained on SA (Sabourauds agar, Difco) at 4℃.

Preparation of the EtOAc extract of *Aspergillus* sp.

The fresh mycelium plugs of *Aspergillus* sp. grown on PDA medium at 24 ± 1℃ for 5-6 days were transferred separately into 3000 ml Erlenmeyer flasks containing 1500 ml potato dextrose broth (PDB) medium. After 12 days of incubation at 24 ± 1℃, 12 liter culture liquid was extracted with EtOAc followed by incubation for 2 - 3 days at 24 ± 1℃ and 150 rev min^{-1} on a rotary shaker. Evaporation of the solvent from the extract *in vacuo* gave a residue, which was subjected to the antidermatophytic potential.

Preparation of the spore suspension and test sample

The spore suspensions of *T. mentagrophytes* KCTC 6085, *M. canis* KCTC 6591, *M. canis* KCTC 6348, *T. rubrum* KCTC 6352, *M. canis* KCTC 6349, and *T. mentagrophytes* KCTC 6316 were obtained from their respective 10 day old cultures, mixed with sterile distilled water to obtain a homogenous spore suspension of 1×10^8 spore ml^{-1}. The ethyl acetate extract of *Aspergillus* sp. was dissolved in the same solvent used for extraction to prepare the stock solution with its known weight, which was further diluted to prepare test sample, where the final concentration of the solvent was 0.5% (v/v).

Disc diffusion assay

Petri dishes (9 cm diameter) containing 20 ml of PDA medium were used for the antifungal activity assay, performed in solid media by the disc diffusion method[10]. Sterile Whatman paper discs of 6 mm diameter were impregnated with 5 μl $disc^{-1}$ EtOAc extract of *Aspergillus* sp. and placed in the agar plates equidistantly. A disc of fungal inoculum (6 mm in diameter) was removed from a previous culture of all the fungal strains tested and placed upside down in the center of the petri dishes. The plates were incubated at 28℃ for 5 - 7 days. The inhibition of the growth of each fungal strain was calculated as the percentage of inhibition of radial growth relative to the control along with antifungal effect on fungal mycelium. The plates were used in three replicates for each treatment.

The growth inhibition of treatment against control was calculated by percentage, using the following formula:

Inhibition ratio (%) = {1- mycelium growth of treatment (mm) / mycelium growth of control (mm)} × 100

Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentrations (MICs) of EtOAc extract were determined by two-fold dilution method against *T. mentagrophytes* KCTC 6085, *M. canis* KCTC 6591, *M. canis* KCTC 6348, *T. rubrum* KCTC 6352, *M. canis* KCTC 6349 and *T. mentagrophytes*

KCTC 6316[11]. Ethyl acetate extract was dissolved in the same solvent according to its known weight. This solutions was serially diluted with ethyl acetate and was added to Sabourauds broth (SB) to achieve final concentrations of 31.25, 62.5, 125, 250, 500, 1000 μg ml^{-1}, respectively. A 10 μl spore suspension of each test strain was inoculated in the test tubes in Sabourauds broth (SB) medium and incubated for 2 - 7 days at 28℃. The control tubes containing SB medium were inoculated only with fungal suspension. The minimum concentrations at which no visible growth was observed were defined as the MICs, which were expressed in μg ml^{-1}.

Spore germination assay

Six concentrations of EtOAc extract of *Aspergillus* sp. (50, 100, 200, 300, 400 and 500 μg ml^{-1}) were tested for spore germination against the skin infectious pathogens[12]. Aliquots of 10 µl fixed with lactophenol-cotton blue, mixed with fungal spore obtained of each test fungi, were placed on both chambers of hemocytometer by carefully touching the edges of cover slip with the pipette tip and allow capillary action to fill the counting chambers and observed under the microscope for spore germination. About 200 spores were counted and percent spore germination was calculated. All experiments were conducted in three replicates.

Statistical analysis

Statistical analysis was performed by using Student's t-test. Values are given as mean ± of standard deviation.

RESULTS AND DISCUSSION

The fungus isolated from the south coast of Korea was identified to be *Aspergillus* sp. through comparing its morphology with the documented description based on its microscopic features[13-15]. The microscopic structures are unique to certain species and constitute the key features for species identification together with the surface color of the colony. The microscopic features of *Aspergillus* sp. were found identical to the morphology with the documented description such as the hyphae were found septate and hyaline. The conidiophores originated from the basal foot cell located on the supporting hyphae and terminate in a vesicle at the apex (Fig. 1). Vesicles are the typical formation for the genus *Aspergillus*, whereas the morphology and color of the conidiophore vary from one species to another. Covering the surface of the vesicle entirely ("radiate" head) or partially only at the upper surface ("columnar" head) were the flask-shaped phialides which were uniseriated and attached to the vesicle directly. Over the phialides, the round conidia (2-5 µm in diameter) forming radial chains were found in newly identified *Aspergillus* sp. as the unique feature of genus *Aspergillus*.

The *in vitro* antifungal activity of EtOAc extract of *Aspergillus* sp. against the skin infectious fungal pathogens was qualitatively assessed by the mycelial growth inhibition diameter, MIC determination and the spore germination assay. As shown in Table 1, a total of six fungal pathogens were tested that infect human skin. The EtOAc extract exhibited promising

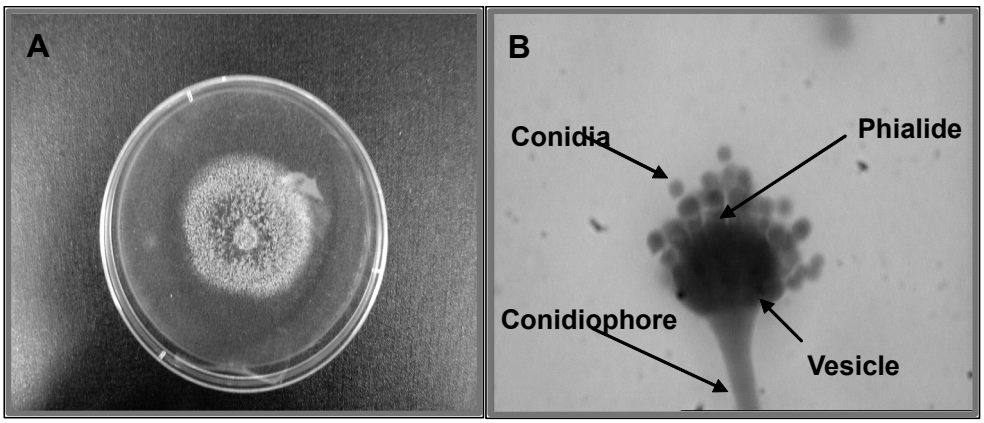

Fig. 1. Morphology of new marine isolate of *Aspergillus* sp..
A: Potato dextrose agar, 25°C, 7 days; B: Radiate conidial heads (dissecting microscope, bar = 100 μm).

antidermatophytic effect against all the strains at the concentration of 5 μl disc^{-1}. In particular, the EtOAc extract exhibited the antidermatophytic effect against *T. mentagrophytes* KCTC 6085, *M. canis* KCTC 6591, *M. canis* KCTC 6348, *T. rubrum* KCTC 6352, *M. canis* KCTC 6349 and *T. mentagrophytes* KCTC 6316 with their respective fungal mycelial growth inhibition percentage of 81.03, 80.90, 53.93, 77.10, 80.93 and 66.26%. The EtOAc extract exerted comparatively moderate antidermatophytic effect against *M. canis* KCTC 6348.

Minimum inhibitory concentrations (MICs) of the EtOAc extract of *Aspergillus* sp. were found greatly low against the tested skin infectious pathogens (Table 2). As control, only ethyl acetate did not affect the growth of tested strains at the concentration used in this study. The minimum inhibitory concentrations defined as the lowest concentrations of the EtOAc extract that resulted in complete inhibition of visible growth against *T. mentagrophytes* KCTC 6085, *M. canis* KCTC 6591, *M. canis* KCTC 6348, T. rubrum KCTC 6352, *M. canis* KCTC 6349 and *T. mentagrophytes* KCTC 6316 were found as 62.5, 62.5, 250, 125, 125 and 125 μg ml^{-1}, respectively.

The results obtained for EtOAc extract of *Aspergillus* sp. from the spore germination assay of each of the test dermatophytes are shown in Fig. 2. A separate control run simultaneously in the presence of ethyl acetate (0.5% v/v) did not inhibit spore germination of any of the pathogens tested. There was a significant inhibition of fungal spore germination by different concentrations of EtOAc extract. EtOAc extract at the concentration ranging from 400 to 500 μg ml^{-1} exhibited 100% fungal spore germination inhibition against all the pathogens. Also, the ethyl acetate extract at the concentration of 500 μg ml^{-1} revealed 80% spore germination inhibition of *M. canis* KCTC 6348.

Secondary metabolites produced by microorganisms can be used potentially for the treatment of many human fungal diseases. Earlier studies on the analysis of antifungal effect of various crude extracts showed that they had varying degree of antidermatophytic

Table 1. The antidermatophytic effect of the EtOAc extract (5 μl disc^{-1}) of the marine isolate of *Aspergillus* sp.

Microorganism	EtOAc extract of *Aspergillus* sp.	
	Mycelial growth (mm)	Mycelia growth inhibition (%)
Control	45.0 ± 0.0	0.0 ± 0.0
Microsporum canis KCTC 6591	5.10 ± 0.26	80.90 ± 0.65
Microsporum canis KCTC 6348	12.16 ± 0.76	53.93 ± 0.11
Microsporum canis KCTC 6349	5.03 ± 0.15	80.93 ± 0.05
Trichophyton mentagrophytes KCTC 6316	8.96 ± 0.25	66.26 ± 0.64
Trichophyton mentagrophytes KCTC 6085	5.03 ± 0.15	81.03 ± 0.05
Trichophyton rubrum KCTC 6352	6.03 ± 0.25	77.10 ± 0.36

*Values are represented as the mean ±S.D. of three experiments.

Table 2. Minimum inhibitory concentration (MIC) of the EtOAc extract of the marine isolate of *Aspergillus* sp. against the tested dermatophytes

Microorganism	EtOAc extract of *Aspergillus* sp.[a]
	MIC[b]
Microsporum canis KCTC 6591	62.5
Microsporum canis KCTC 6348	250
Microsporum canis KCTC 6349	125
Trichophyton mentagrophytes KCTC 6316	125
Trichophyton mentagrophytes KCTC 6085	62.5
Trichophyton rubrum KCTC 6352	125

[a]EtOAc: ethyl acetate; [b]MIC: minimum inhibitory concentration (values in μg ml^{-1}).

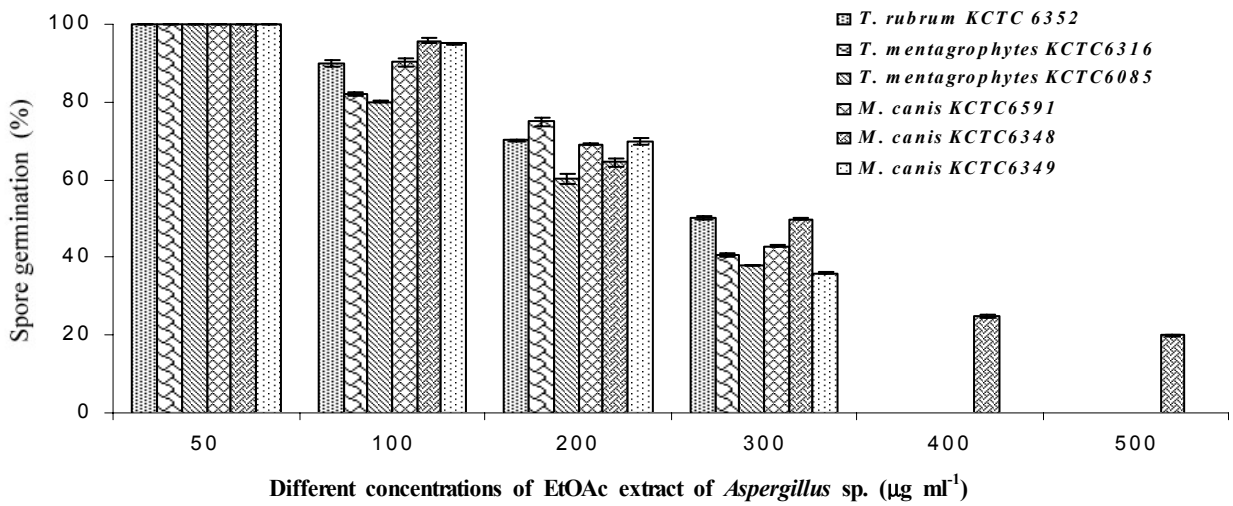

Fig. 2. The effect of the different concentrations (μg ml^{-1}) of the ethyl acetate (EtOAc) extract of the marine isolate of *Aspergillus* sp. on spore germination of the tested skin infectious pathogens.
***Values are represented as the mean ±S.D. of three experiments.**

effect against different skin infectious microorganisms[16]. Our study revealed comparatively better results of the antidermatophytic effect of EtOAc extract of *Aspergillus* sp. against the tested human pathogens. These results suggest the availability of various crude extracts from the different origins for trials in controlling such human infectious fungal pathogens. These findings are strongly supported by the work of some of the researchers as they also observed that ethyl acetate (EtOAc) extract of a fungal strain under the number Z16 isolated from the spermary of *Argyrosomus argentatus* was found strongly inhibitory to the growth of the human pathogenic fungi *Candida albicans, Aspergillus niger* and *Trichophyton rubrum*[17]. These pathogens are responsible for serious human pathogenic disorders in various parts of the world and, although control measures are available, they are of limited effectiveness[1]. As a result, work on alternative approaches to control such pathogens is important.

Thus, based on the overall results, it can be estimated that EtOAc extract of *Aspergillus* sp. has strong antidermatophytic effect and can be used as a natural therapy in clinical approaches to inhibit such skin infectious dermatophytes causing severe fungal infections in human beings such as tinea capitis, tinea pedis, tinea corporis and onychomycosis.

In conclusion, the primary significance of this study is the observation that EtOAc extract could effectively inhibit the growth of dermatophytes, fungal infections that have caused patients to seek medical attention. To our knowledge, this is the first study to apply EtOAc extract of the marine isolate of *Aspergillus* sp. successfully to human fungal pathogens. Thus we anticipate developing and identifying novel bioactive molecules present in EtOAc extract of *Aspergillus* sp. responsible for this antidermatophytic effect. We also believe that such antidermatophytic effects of EtOAc extract on human infectious fungal pathogens will contribute in medical mycology as the supplement to cure human pathogenic fungi in future as the fast and reliable alternative.

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