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Research Paper

Antifungal Substances from the Rhizome of *Zingiber ottensii* (Zingiberaceae)

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ABSTRACT: *The rhizome of Zingiber ottensii has been reported to exhibit potent antifungal activities against yeast and dermatophytes. However, the active substances remain unknown. In this study, two antifungal compounds were isolated from the ethanolic rhizome extract of Z. ottensii through bioassay-guided separation. The isolated active substances were zerumbone (**1**) and labda-8(17),12-diene-15,16-dial (**2**) that exhibited anti-yeast activity against Candida albicans and anti-dermatophyte activities against Trhichphyton rubrum, Trichophyton mentagrophytes, and Microsporum gypseum. In addition, the ethanolic rhizome extract of Z. ottensii showed fungicidal activity against C. albicans in the time-kill curve assay. Because of the difference between **1** and **2** in chemical classes, they might have different action modes and enhance their antifungal activities. Consequently, the rhizome of Z. ottensii is a promising medicinal plant for the development of antifungal preparations.*

KEYWORDS: *Zingiber ottensii, antifungal, dermatophytes, zerumbone, labda-8(17),12-diene-15,16-dial*

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I. INTRODUCTION

Dermatomycoses are common fungal skin diseases caused by dermatophytes, yeasts, or mold and are predominant in the tropical and subtropical countries. Most superficial fungal infections are caused by dermatophytes and can usually be treated with topical antifungal agents. [1] Besides, it can be cured by using medicinal plants according to traditional knowledge. [2-3] In the course of our research for the investigation of antifungals from Thai medicinal plants, the rhizomes of many species in the ginger family (Zingiberaceae) exhibited antifungal activities against dermatophytes and yeast. [4] Therefore, the rhizomes of Zingiberaceae plants might be promising sources for the isolation of antifungal agents.

Zingiber ottensii Valeton is a member of the family Zingiberaceae and is widely cultivated in Southeast Asia. The plant has reddish stalk that can grow to 1.5- 2 m tall. The leaves are elliptic, narrowing at the tip. The inflorescence rises directly from the rhizomes to 0.3 m tall, and the flowers protrude from the bracts. The rhizome has purplish inside and pungent smell. This plant has been used as a traditional medicine to relieve bruises, sprains, and strains. [5] The rhizome of *Z. ottensii* has been reported to contain a number of terpenoids. [6] The objective of this study was to investigate antifungal substances from the rhizome of *Z. ottensii* that showed potent antifungal activities in the previous study. [4] The isolated active substances were tested for antifungal activities against yeast and dermatophytes using broth microdilution assay.

II. MATERIALS AND METHODS

2.1 Plant materials

The rhizomes of *Z. ottensii* were collected from Ratchaburi Province, Thailand, in November 2015. The plant was identified by the research officer of the Forest Herbarium Division, and the voucher specimen (Herbarium no. BKF 191214) was then deposited at the Forest Herbarium Division, Forest and Plant Conservation Research Office, Department of National Park, Wildlife and Plant Conservation, Bangkok, Thailand.

2.2 Extraction and Isolation

The dried rhizomes of *Z. ottensii* (38.7 g) were extracted with ethanol (750 mL, 2 times) at room temperature. The extract was concentrated under reduced pressure to yield 1.57 g of dried ethanolic extract. The extract was suspended in distilled water and partitioned successively with *n*-hexane and chloroform to give 0.57 g of an *n*-hexane extract, 0.25 g of a chloroform extract, and 0.63 g of an aqueous extract. The active *n*- hexane extract was subject to Sephadex LH-20 column chromatography and eluted with *n*-hexane-chloroform (2:1 v/v) to yield four major fractions. The most active fraction (fraction 2, 0.11 g) was purified by normal phase HPLC (*n*-hexane-ethyl acetate 95:5, flow rate 1 mL/min, detection UV 254 nm) to obtain compound **1** (17.4 mg, t_R = 5 min) and compound **2** (14.6 mg, t_R = 12 min). The isolated compounds were identified using NMR and MS spectroscopies.

2.3 Assay for Antifungal activity

2.3.1 Microbial strains

Four fungi *Candida albicans* DMST 5815, *Trichophyton mentagrophytes* DMST 19735, *Trichophyton rubrum* DMST 30263, and *Microsporum gypseum* DMST 21146 were obtained from DMST culture collection (Department of Medical Sciences, Thailand) and cultured on Sabouraud dextrose agar (SDA) and potato dextrose agar (PDA) for yeast and dermatophytes, respectively.

2.3.2 Broth microdilution assay

The susceptibility assay of yeast and filamentous fungi was performed using broth microdilution with minor modification according to the method of Clinical and Laboratory Standards Institute (CLSI) documents M27-A3 [7] and M38-A2 [8], respectively. Briefly, the stock suspensions of tested organisms were prepared in 0.85% saline and adjusted for turbidity equivalent to a 0.5 McFarland standard for yeast and a 1.0 McFarland standard for dermatophytes. The suspensions were further diluted 1:500 (yeast) and 1:50 (dermatophytes) in the test medium RPMI-1640 buffered with 0.165 M of MOPS for preparing the test inoculum. The tests were conducted in multiwall microdilution plates in which 50 μL of test medium containing the test sample was 2X (twofold) more concentrated than the final concentration. The microdilution plates were inoculated with 50 μL of test inoculum in each well and incubated at 35°C for 24 h (yeast) and for 96-120 h (dermatophytes). The minimum inhibitory concentrations (MICs) were defined as the lowest concentrations of the test samples that completely inhibited microbial growth.

2.3.3 Time-kill assay

The antifungal time-kill study was carried out by a method previously described. [9] The stock suspension of *C. albicans* was prepared for turbidity equivalent to a 0.5 McFarland standard (approximately 1×10^6 to 5×10^6 CFU/mL) and was then diluted 1: 10 with the test medium to yield a starting inoculum of approximately 1×10^5 CFU/mL. Ten millilitres of inoculum was used to add the desired amount of each test sample. The extract was tested at concentrations equal to MIC and 2X MIC. Ketoconazole at concentrations equal to MIC was used as an antifungal positive control. All solutions were incubated at 35°C with agitation. Aliquots were removed from each test solution for colony count determination at 0, 1, 2, 3, 6, 9, 12, 15, 18, 21, and 24 h. For high and medium inoculum samples, aliquots were diluted and plated onto an SDA plate. When colony counts were expected to be low, 200 μL of the sample was taken directly from the test solution and plated onto an SDA plate without dilution. Plates were incubated at 35°C for 24 to 48 h prior to determination of colony counts.

III. RESULTS

On the guidance of bioassay, the ethanolic rhizome extract of *Z. ottensii* (MIC 50 μg/mL against *C. albicans*) was subjected to liquid-liquid extraction. The *n*-hexane soluble portion (MIC 25 μg/mL) was purified using chromatographic techniques to obtain white crystal of compound **1** and colourless oil of compound **2** as the antifungal substances. Structure elucidation revealed that **1** and **2** were completely identical with zerumbone (**1**) [10] and labda-8(17),12-diene-15,16-dial (**2**) [11,12], respectively. (Figure 1)

(**1**) (**2**)

Figure 1: The structures of isolated compounds

The antifungal activity of the ethanolic extract and the compounds isolated from the rhizome of *Z. ottensii* were tested against *C. albicans*, *T. mentagrophytes*, *T. rubrum*, and *M. gypseum*. The results of the broth microdilution assay are presented in Table 1. Zerumbone (**1**) exhibited antifungal activity with MIC values ranging from 3.13 to 50 µg/mL whilst labda-8(17),12-diene-15,16-dial (**2**) showed with MIC values ranging from 0.39 to 3.13 µg/mL.

Table 1: Antifungal activity of isolated compounds against yeast and dermatophytes

Test Samples	Antifungal Activities (MICs, µg/mL)			
	C. albicans	*T. mentagrophytes*	*T. rubrum*	*M. gypseum*
ethanolic rhizome extract of *Z. ottensii* (ZO)	50	3.13	6.25	3.13
zerumbone (**1**)	50	3.13	12.5	50
labda-8(17),12-diene-15,16-dial (**2**)	3.13	0.39	0.39	0.39
ketoconazole	100	1.56	0.31	1.25

The time-kill results of the ethanolic rhizome extract of *Z. ottensii* (ZO) against the test organism *C. albicans* at test concentrations showed a reduction in the viable colony count greater than 3 $\log_{10}$ CFU/mL whilst ketoconazole showed a slight increase in CFU/mL from the starting inoculum. (Figure 2)

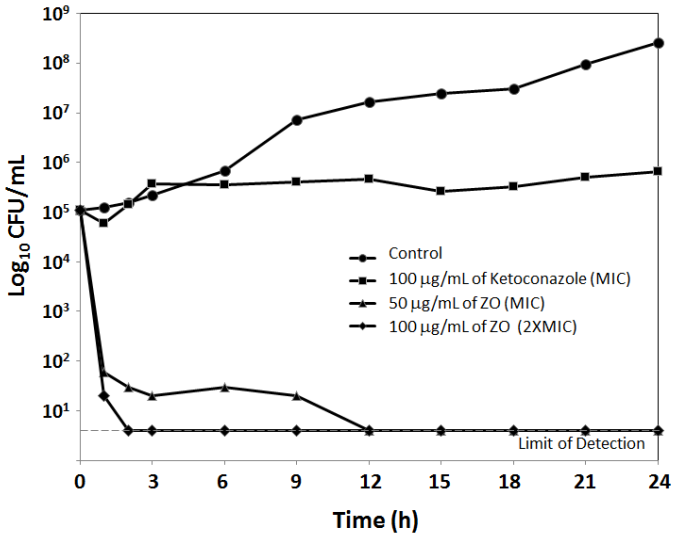

Figure 2: Time-kill curve of the ethanolic rhizome extract of *Z. ottensii* (ZO) against *C. albicans*.

IV. DISCUSSION

Many species of Zingiberaceae plants have been widely used for culinary and medicinal purposes. In traditional medicine, these plants are used to treat various kinds of diseases. For instance, *Curcuma longa* has been used for healing wounds, liver disease, jaundice, and skin disease. [13] *Zingiber cassumunar* is regularly used to treat inflammation, sprains, muscular pain, and wounds. [5, 14] In addition, *Z. ottensii* and *Z. cassumunar* are used with the same indications in Thai folk medicine. [5] Nowadays, essential oils and extracts from many Zingiberaceae species have been reported to possess antifungal and antibacterial activities.[4, 15-16] The fungal and bacterial inhibition properties of certain Zingiberaceae plants support their traditional use for treatment of wound and skin diseases.

In the present study, the ethanolic rhizome extract of *Z. ottensii* showed remarkable antifungal activity against all tested fungi and exhibited fungicidal activity against *C. albicans*, with a reduction of over 3 $\log_{10}$ CFU/mL compared to the starting inoculum (≥99.9% initial inoculum reduction). The rhizome of *Z. ottensii* was shown to possess at least two antifungal substances, comprising zerumbone (**1**) and labda-8(17),12-diene-15,16-dial (**2**). These substances have been isolated from various Zingiberaceae plants. [11-12, 17] Zerumbone (**1**) is a monocyclic sesquiterpene ketone, first isolated from the rhizomes of *Zingiber zerumbet* [17] and also found in several Zingiberaceae species. It has been reported to possess antioxidant, antimicrobial, anti-inflammatory, immunomodulatory, and anticancer activities. [18] In a study by Jyothilakshmi et al. (2017), zerumbone isolated from the rhizome of *Z. zerumbet* has been reported to have antidermatophytic and protease-inhibiting activities

that claim for fungicidal and anti-inflammatory properties.[10] Thus, this is in good agreement with our experimental findings.

Labda-8(17),12-diene-15,16-dial (**2**) is a labdane-type diterpene, first isolated from the seeds of *Alpenia galanga*.[19] This compound can be found in other Zingiberaceae species such as *Alpenia nigra* [20], *Alpinia purpurata* [12], and *Curcuma amada* [11]. It has been reported to exhibit antimicrobial activity towards gram-negative bacteria and delay the growth of *C. albicans* by preventing substrate uptake.[20] In addition, labda-8(17),12-diene-15,16-dial (**2**) was found to possess antifungal activity against dermatophytes and showed fungicidal activity toward *C. albicans*.[11] Therefore, antifungal activity of two substances isolated in our study are in agreement with other previous studies. The result of this study could support antifungal potential of the rhizome of *Z. ottensii* for the treatment of fungal skin infection.

V. CONCLUSION

In summary, the ethanolic rhizome extract of *Z. ottensii* has broad spectrum antifungal activity against cutaneous fungi and acts against *C. albicans* in a fungicidal manner. From the rhizome extracts, two antifungal substances were isolated and identified as zerumbone (**1**) and labda-8(17),12-diene-15,16-dial (**2**), exhibiting antifungal activity with MIC values ranging from 3.13 to 50 μg/mL and 0.39 to 3.13 μg/mL, respectively. This finding indicates that the rhizome of *Z. ottensii* has potential for use to treat fungal skin infections. In addition, zerumbone (**1**) and labda-8(17),12-diene-15,16-dial (**2**) can be used as the marker substances for standardization of the rhizome extracts of *Z. ottensii*.

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