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Endophytic *Diaporthe* sp. ED2 Produces a Novel Anti-Candidal Ketone Derivative S

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This study aimed to examine the anti-candidal efficacy of a novel ketone derivative isolated from *Diaporthe* sp. ED2, an endophytic fungus residing in medicinal herb *Orthosiphon stamieus* Benth. The ethyl acetate extract of the fungal culture was separated by open column and reverse phase high-performance liquid chromatography (HPLC). The eluent at retention time 5.64 min in the HPLC system was the only compound that exhibited anti-candidal activity on Kirby-Bauer assay. The structure of the compound was also elucidated by nuclear magnetic resonance and spectroscopy techniques. The purified anti-candidal compound was obtained as a colorless solid and characterized as 3-hydroxy-5-methoxyhex-5-ene-2,4-dione. On broth microdilution assay, the compound also exhibited fungicidal activity on a clinical strain of *Candida albicans* at a minimal inhibitory concentration of 3.1 μg/ml. The killing kinetic analysis also revealed that the compound was fungicidal against *C. albicans* in a concentration- and time-dependent manner. The compound was heat-stable up to 70°C, but its anti-candidal activity was affected at pH 2.

Keywords: *Candida albicans*, *Diaporthe* sp., endophyte, ketone derivative

Introduction

Candida albicans has evolved into an opportunistic pathogen and accounts for the fourth common cause of nosocomial infections in the United States [1]. *C. albicans* is the most common cause of candidiasis, where it colonizes skin, oropharynx, lower respiratory tract, gastrointestinal tract, and genitourinary system [2]. The frequency of candidiasis has increased in the past decade owing to the increased number of immunocompromised patients and gradual rise in azole-resistant strains [3]. Thus, candidiasis has emerged as a serious problem in hospitals worldwide, especially in intensive care units and out-patient clinics [4]. Besides this, the newly isolated clinical strains that show resistance to low-cost antifungal drugs also increase health-care spending.

Endophytes are microorganisms that for all or part of their life cycle invade the tissue of living plants but cause no symptoms of disease to the host [5]. Endophytes, which occupy a unique biotope with a global estimation of up to one million species, are a great choice of bioprospecting to obtain bioactive compounds with novel chemical structure in the study of natural products [6]. Endophytic fungi are a promising source of bioactive and chemically novel compounds with potential application in medical, agricultural, and industrial arenas [7]. The reported natural products from endophytes are highly diverse chemically, and the biological activities exhibited include antibiotic, anticancer, immunosuppressant, antioxidant, anti-diabetic, and anti-insecticidal activities [6].

Diaporthe species (Anamorph *Phomopsis*) are frequently reported as endophytes and saprophytes on a wide range of host plants. They are well known for the production of bioactive compounds that exhibit anti-microtubule, anti-malarial, anti-tubercular, antifungal, herbicidal, algicidal, anti-inflammatory, antimicrobial, and plant growth regulatory

activities [8, 9]. As part of our on-going effort to search for novel bioactive compounds from endophytes, this study aimed to isolate and identify the anti-candidal compound from *Diaporthe* sp. ED2, an endophytic fungus from medicinal herb *Orthosiphon stamieus* Benth. In addition, the anti-candidal activity of the purified compound was also investigated. The effects of temperature, pH level, and shelf life of the purified compound on its anti-candidal activity were also determined.

Materials and Methods

Endophytic Fungus and Storage

The endophytic fungus *Diaporthe* sp. ED2 was previously isolated from *O. stamineus* Benth by Tong *et al*. [10]. The antimicrobial activity was screened and the isolate was deposited at Industrial Biotechnology Research Laboratory, Universiti Sains Malaysia, Penang, Malaysia. The fungal isolate was cultivated on Potato Dextrose Agar (AES) supplemented with powdered host plant materials (5 g/l) and stored at 4°C prior to use. The isolate was subcultured on fresh medium every 4 weeks to ensure its purity and viability.

Culture Medium

Yeast extract sucrose (YES) broth (yeast extract 20 g/l, sucrose 40 g/l, magnesium sulfate 0.5 g/l) supplemented with aqueous extract of *O. stamineus* was used to cultivate *Diaporthe* sp. ED2 in the shake-flask system. The plant extract was prepared by boiling 10 g of dried plant material in 500 ml of distilled water for 30 min. The extract was filtered and mixed with freshly prepared culture medium and autoclaved at 121°C for 15 min.

Fermentation and Extraction

The inoculum was prepared by introducing two mycelial agar plugs into 250 ml Erlenmeyer flasks containing 100 ml of YES medium. The cultures were grown at 30°C in a shaker at 120 rpm. After 20 days of incubation, the fermentative broth and fungal biomass were separated by centrifugation at 5,311 ×*g* (Sigma; Model 4K15). The supernatant was then extracted three times with an equal volume of ethyl acetate (1:1 (v/v)). The upper organic phase was concentrated to dryness, using a rotary evaporator under reduced pressure, to an extract paste.

Separation of the Crude Extract

The ethyl acetate extract was first fractionated by silica gel 60 (Acros, particle size 40–63 µm) column chromatography, using chloroform/methanol at a ratio of 2:3 (v/v) as the mobile phase to yield five fractions. The collection of fractions was performed based on the band color. The 4th fraction, which was yellow in color, was collected and subjected to high-performance liquid chromatography (HPLC) to obtain the purified compound. The reverse phase HPLC used comprised a Waters 717 plus auto-sampler, Waters 1525 Binary HPLC pump, Symmetry C_{18} column (5 µm), and Waters 2489 UV/visible detector coupled with Breeze software. The mobile phase for HPLC analysis was prepared from HPLC-grade organic solvent of methanol and chloroform (7:3 (v/v)). The solvents used were filtered through a Sartorius PTFE membrane filter (47 mm in diameter and 0.45 µm pore size). The HPLC system was operated at room temperature (25 ± 2°C). The equilibration of the system was done under isocratic condition. The mobile phase was run at a flow rate of 1.0 ml/min and a run time of 10 min. The injection volume of the sample was 20 µl and the detection was set at a wavelength of 245 nm. The collection of the separated compound was done at the waste collection based on the retention time.

Identification of Anti-Candidal Compound

The NMR spectra of the pure compound isolated were recorded using a Bruker Avance 500 Spectrometer operated at 500 MHz for ^{1}H NMR, and 125 MHz for ^{13}C NMR. The sample was dissolved in deuterated methanol. All of the experiments were carried out at room temperature (25 ± 2°C). The standard Bruker pulse program was used for the data analysis.

The mass spectroscopy of purified compound was analyzed with an electrospray ionization source operated in positive-ion mode. The positive electrospray ionization conditions included a capillary voltage of 4 V, ion energy of 4 V, desolvation temperature of 120°C, and source temperature of 80°C. The sample was introduced using a syringe pump with a flow rate of 10 ml/min. The Perkin Elmer System 2000 FT-IR spectrometer was used for scanning the infrared spectrum of the compound with potassium bromide pellets. The range of measurement was from 4,000 to 400 cm^{-1}.

Test Isolate

The *C. albicans* strain was previously isolated from a clinical sample taken in Hospital Seberang Jaya, Penang, Malaysia. The yeast was grown on Sabouraud Dextrose Agar plates (Merck) at 37°C. The inoculum suspension was prepared by picking five single colonies from a 24-h-old culture and putting them into 5 ml of sterile physiological saline. The turbidity of the inoculum suspension was adjusted with sterile saline to match the turbidity of 0.5 McFarland standard.

Kirby-Bauer Assay

Using a sterile cotton swab, the test inoculum was streaked onto the surface of a Mueller-Hinton agar plate (Merck) containing 2% dextrose and 0.5 µg/ml methylene blue. A sterile Whatman antibiotic disc impregnated with 25 µg of compound was then placed on the surface of the inoculated medium. Meanwhile, discs containing 25 µg of fluconazole (Sigma) and voriconazole (Sigma) served as reference drugs. The plates were incubated at 37°C for 24 h and the diameter of the clear zone was measured.

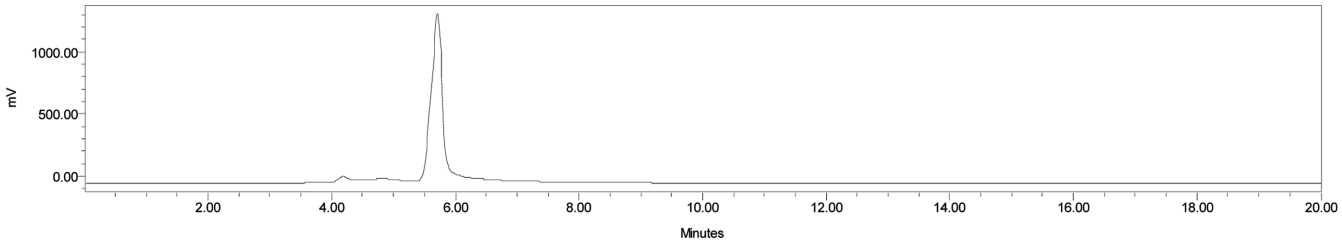

Fig. 1. HPLC chromatogram of the purified anti-candidal compound.

Broth Microdilution Assay

The minimal inhibitory concentration (MIC) of the compound was determined by broth microdilution assay in sterile 96-well microtiter plates as per protocols defined by the Clinical Laboratory Standard Institute [11]. RPMI 1640 medium containing 0.2% dextrose buffered with 0.165 M 3-(*N*-morpholino)propanesulfonic acid to a pH of 7.0 at 25°C was used. Fluconazole and voriconazole were included as reference drugs. The compound was dissolved in 1% dimethylsulfoxide (DMSO) to the concentration of 200 µg/ml. Then, a serial 2-fold dilution of the compound was carried to yield a final concentration range from 100 to 0.78 µg/ml, with final volume of 200 µl in each well. The well containing only 1% DMSO and inoculum was used as the control. The plate was incubated at 37°C for 24 h. After the incubation period, 40 µl of 0.2 mg/ml *p*-iodonitrotetrazolium violet salt (Sigma) dissolved in 99.5% ethanol was added to each well as a growth indicator for *C. albicans*. Wells showing microbial growth were pink colored. To determine the minimal fungicidal concentration (MFC) of the compound, 100 µl of the sample from each well was taken and suitably diluted before spreading on Mueller-Hinton agar plates to judge the viability. The viable cell count method was performed. The MFC was recorded as the lowest concentration of a drug that resulted in 99.9% growth reduction relative to the control.

Killing Kinetic Analysis

First, 100 µl of inoculum suspension (5×10^5 CFU/ml) was inoculated into 10 ml of RPMI 1640 medium. The compound was tested at four concentrations: the MIC susceptibility breakpoint, 2 MIC, MFC, and 2 MFC. The cultures were incubated at 37°C with a rotational speed of 120 rpm. At predetermined time points (0, 4, 8, ….. 40, 44, 48 h), a 100 µl aliquot from each treatment mixture was collected. Viable cell count was performed by inoculating the diluted sample on Sabouraud agar plates. The killing kinetics of the compound was analyzed mathematically relative to the control.

Effects of Temperature and pH on Anti-Candidal Activity

The temperatures employed were 40°C, 50°C, 60°C, 70°C, 80°C, and 90°C, whereas the pH values employed were pH 2, 4, 6, 8, 10, and 12. The pH was adjusted by using 1 M HCl or 1 M NaOH. The compound was subjected to the test condition for 7 days in a glass vial. The shelf life of the compound was also determined at 4°C, room temperature (25 ± 2°C), and 40°C for a period of 3 months. The anti-candidal activity of the compound was tested by Kirby-Bauer assay at a concentration of 25 µg per disc.

Statistical Analysis

The assays were repeated three times in separate tests. The statistical analysis was performed with SPSS ver. 20. Continuous variables were analyzed by ANOVA test, and $p \leq 0.05$ was considered to indicate statistical significance.

Results and Discussion

The ethyl acetate extract was separated into three peaks by reverse phase HPLC, with retention times of 2.43, 5.64, and 7.36 min. The peak with retention time of 7.36 min was the major compound in the fraction by referring to the peak area under the chromatogram. However, the eluent at retention time 5.64 min was the only compound that exhibited anti-candidal activity on Kirby-Bauer assay. Ketone derivatives have been successfully isolated by a few researchers via reverse phase HPLC [12, 13]. The isolation of the compound was evident by the observation of a single peak with retention time 5.64 min on the HPLC chromatogram (Fig. 1).

The purified anti-candidal compound, 3-hydroxy-5-methoxyhex-5-ene-2,4-dione (HMD) (Fig. 2), was obtained as a colorless solid. It gave a $[M + 1]^+$ ion peak at *m/z*

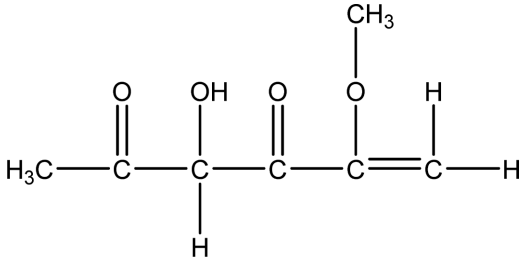

Fig. 2. 3-Hydroxy-5-methoxyhex-5-ene-2,4-dione.

159.0140 (calcd 159.1601) by HRESI, which is consistent with the molecular formula $C_7H_{10}O_4$. The IR spectrum showed absorptions at 3,475 (O-H stretching), 1,715 (C=O stretching), and 1,636 (C=C stretching) cm^{-1}, proposing an unsaturated carbonyl skeleton with hydroxyl group. The ^{1}H-NMR spectrum showed the presence of two single peaks for methyl protons at δ_H 1.77 (s, 3H) and methoxy protons at δ_H 3.94 (s, 3H). The methylene protons at C6 were represented by two singlets at δ_H 5.43 (s, 1H) and δ_H 5.19 (s, 1H), and another singlet at δ_H 5.30 (s, 1H) was assigned to tertiary protons located at C3. The ^{13}C-NMR spectrum showed the presence of seven carbons. Two methyl, one methylene, one methine, and three quaternary carbons were observed by the analysis of DEPT spectra. All protonated carbons of the compound were assigned by the HMQC analysis. The signal at δ_C 90.06 suggested the compound has one hydroxyl (OH) group, and signals at δ_C 116.56 and δ_C 141.66 supported that the compound has a carbon-carbon double bond (C=C). A methoxy carbon at C7 was assigned to δ_C 60.57, and a methyl group at C1 was ascribed to δ_C 17.61. The HMBC spectrum revealed the key ^{1}H-^{13}C connectivity between methoxy protons (δ_H 3.94) with the carbonyl group at C4 (δ_C 181.48). The presence of the C=C double bond at C5 and C6 was deduced on the basis of the correlation of the methylene protons (H6) with C5 at δ_C 141.66. The cross peaks between H3 (δ_H 5.29) with δ_C 173.29 and δ_C 181.48 aided the assignment of C2 at δ_C 173.29 and C4 at δ_C 181.48. The methyl protons (δ_H 1.77) at C1 were correlated with the carbonyl carbon located at C2 (δ_C 173.29), suggesting that the methyl group was attached to the carbonyl group in the molecule. A literature search indicated there were no data reported for this compound. Therefore, it was characterized as 3-hydroxy-5-methoxyhex-5-ene-2,4-dione (HMD), a new ketone derivative isolated from *Diaporthe* sp. ED2. The spectral data of the isolated compound are summarized in Table 1.

Table 1. ^{1}H NMR (500 MHz, CD_3OD), ^{13}C NMR (125 MHz, CD_3OD), and HMBC data for 3-hydroxy-5-methoxyhex-5-ene-2,4-dione.

Position	δ^{13}C	δ^1H	^{1}H-^{13}C HMBC
1	17.61	1.77 (s, 6H)	-
2	173.29	-	H1 H3
3	90.06	5.30 (s, 1H)	-
4	181.48	-	H7 H3
5	141.66	-	H6
6	116.56	5.19 (s, 1H)	-
		5.43 (s, 1H)	
7	60.57	3.94 (s, 3H)	-

A ketone derivative is a compound that is derived from ketone by some chemical and physical processes. Klaiklay *et al*. [14] reported the isolation of a ketone derivative, (2R,3S)-7-ethyl-1,2,3,4-tetrahydro-2,3,8-trihydroxy-6-methoxy-3-methyl-9,10 anthracenedione, from mangrove-derived *Diaporthe* sp. PSU-MA214. Ahmed *et al*. [15] also reported a new natural cyclic-ketone, phomotenone, isolated from endophytic fungus *Diaporthe* sp. A novel antibiotic and cytotoxic compound, 2,2′-dimeric tetrahydroxanthone has been isolated from *Diaporthe longicolla*, an endophyte of endangered mint [16]. Phomoxanthones A and B were also isolated by Isaka *et al*. [17] from *Diaporthe* sp. BCC1323. Phomonaphthalenone A that was isolated from solid culture of *Diaporthe* sp. HCCB04730 also exhibited significant anti-HIV activity [18]. However, the anti-candidal compound isolated from *Diaporthe* sp. ED2 is relatively simple in structure compared with the compounds mentioned above.

A clear inhibition zone with a diameter of 14.7 ± 0.8 mm was observed for HMD on Kirby-Bauer assay (Table 2). The presence of the clear zone signifies the inhibitory activity of the compound on *C. albicans*. On broth microdilution assay, the MIC of the compound was 3.1 μg/ml and the MFC was 12.5 μg/ml. The low MIC indicates the high susceptibility of *C. albicans* to the compound. The anti-candidal activity was concentration dependent, where the MFC was significantly higher than its MIC. A higher concentration of HMD was needed to kill the microbial cells than to inhibit their growth. The inhibitory activity of HMD on *C. albicans* was fungicidal. The anti-candida activity was comparable to voriconazole based on the results of antimicrobial susceptibility tests. Fig. 3 shows the killing kinetic analysis of compound HMD on *C. albicans*. HMD was fungistatic at the concentration of MIC and 2 MIC, as 99.9% reduction in viable cell count, compared with start inoculum, was not observed. The killing kinetic curves for MIC and 2 MIC were generally similar to that of the control, with a lower viable cell count obtained. At the concentration of MFC and 2 MFC, HMD was fungicidal against *C. albicans* in a

Table 2. Anti-candidal activity of 3-hydroxy-5-methoxyhex-5-ene-2,4-dione (HMD) and antifungal drug standards.

Test substance	Antimicrobial activity on *C. albicans*		
	Diameter of clear zone (mm)	MIC (μg/ml)	MFC (μg/ml)
HMD	14.7 ± 0.8	3.1	12.5
Fluconazole	9.4 ± 0.6	25	50
Voriconazole	14.2 ± 0.6	12.5	25

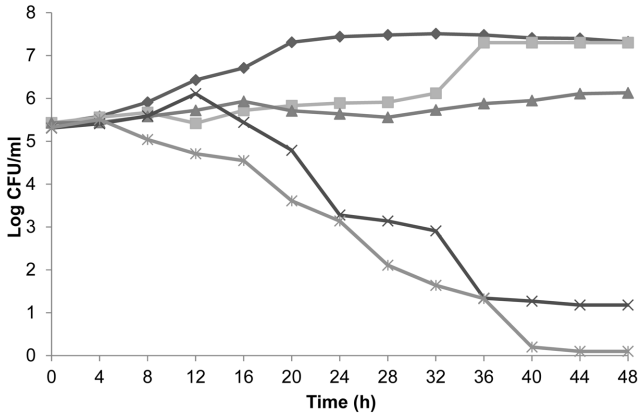

Fig. 3. Killing kinetic analysis of 3-hydroxy-5-methoxyhex-5-ene-2,4-dione on *C. albicans*.
Test concentration: (◆) Control, (■) MIC, (▲) 2 MIC, (×) MFC, (×) 2 MFC.

concentration- and time-dependent manner. A 99.9% reduction in viable cell count relative to the control was obtained after 36 h of exposure to HMD.

The anti-candidal activity of HMD was affected by temperature and pH (Table 3). The compound was not stable at 80°C and 90°C. Temperature has a significant ($p < 0.05$) effect on the activity of the compound and the increment in the temperature decreased the inhibition zone size on *C. albicans*. Heating causes the compounds to vibrate more vigorously, which can lead to the breakage of the chemical bonding and causes the compound to loss its biological activity [19]. No significant difference ($p \geq 0.05$) was detected in the diameter of inhibition zone from 40°C to 70°C, which indicates that the bonding in the compound is still intact. However, at 80°C, the denaturation of the compound occurred, which caused the total loss of anti-candidal activity.

The anti-candidal activity of HMD was reduced by low pH (Table 4). The acidic environment had a more detrimental effect on the stability of the compound than the alkaline environment. The stability of the compound was best at pH 6 and 8, from slightly acidic to slightly basic. The compound is unstable at acidic condition as it can be degraded rapidly owing to the lack of an aromatic ring in its structure. The anti-candidal activity of the compound was retained at 4°C for the entire duration of testing, but a reduction in activity with time was observed (Table 5). The reduction of antimicrobial activity of bioactive metabolites when subjected to different temperature and pH values were reported in other studies [20, 21]. The stability of the compound was significantly different for the three temperatures tested. The duration of storage was found to affect the anti-candidal activity, as HMD can be degraded by the oxidation process, especially at high temperature.

In conclusion, a novel anti-candidal compound was isolated from *Diaporthe* sp. ED2; namely, 3-hydroxy-5-methoxyhex-5-ene-2,4-dione. The compound also exhibited fungicidal activity on pathogenic *C. albicans*. HMD was heat-stable, but its anti-candidal activity was affected by low pH. Further investigations should be done to

Table 3. The effect of temperature on the anti-candidal activity of 3-hydroxy-5-methoxyhex-5-ene-2,4-dione.

Temperature (°C)	40	50	60	70	80	90
Diameter of inhibition zone (mm)	17.2 ± 0.4	17.1 ± 0.8	16.8 ± 1.1	16.7 ± 0.6	-[a]	-

[a]No inhibitory activity.

Table 4. The effect of pH on the anti-candidal activity of 3-hydroxy-5-methoxyhex-5-ene-2,4-dione.

pH	2	4	6	8	10	12
Diameter of inhibition zone (mm)	-[a]	9.8 ± 0.3	17.2 ± 0.8	17.1 ± 0.3	16.1 ± 0.5	16.4 ± 0.9

[a]No inhibitory activity.

Table 5. The shelf life of 3-hydroxy-5-methoxyhex-5-ene-2,4-dione at different storage temperatures.

Storage temperature (°C)	Diameter of inhibition zone after the storage period (mm)					
	20 days	40 days	60 days	80 days	100 days	120 days
4	16.9 ± 0.5	16.8 ± 0.4	17.1 ± 1.1	16.2 ± 0.4	11.4 ± 0.5	11.0 ± 1.3
Room temperature	11.8 ± 0.7	11.4 ± 0.3	-[a]	-	-	-
40	9.2 ± 0.9	-	-	-	-	-

[a]No inhibitory activity.

investigate the toxicity and in vivo anti-candidal efficacy of HMD on animal models.

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