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Bioprospecting of Novel and Bioactive Metabolites from Endophytic Fungi Isolated from Rubber Tree *Ficus elastica* Leaves

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Endophytic fungi are an important component of plant microbiota, and have the excellent capacity for producing a broad variety of bioactive metabolites. These bioactive metabolites not only affect the survival of the host plant, but also provide valuable lead compounds for novel drug discovery. In this study, forty-two endophytic filamentous fungi were isolated from *Ficus elastica* leaves, and further identified as seven individual taxa by ITS-rDNA sequencing. The antimicrobial activity of these endophytic fungi was evaluated against five pathogenic microorganisms. Two strains, Fes1711 (*Penicillium funiculosum*) and Fes1712 (*Trichoderma harzianum*), displayed broad-spectrum bioactivities. Our following study emphasizes the isolation, identification and bioactivity testing of chemical metabolites produced by *T. harzianum* Fes1712. Two new isocoumarin derivatives (**1** and **2**), together with three known compounds (**3**–**5**) were isolated, and their structures were elucidated using NMR and MS. Compounds **1** and **2** exhibited inhibitory activity against *Escherichia coli*. Our findings reveal that endophytic fungi from the rubber tree *F. elastica* leaves exhibit unique characteristics and are potential producers of novel natural bioactive products.

Keywords: Antimicrobial activity, endophytic fungi, *Trichoderma harzianum*, isocoumarin, metabolites, *Ficus elastica*

Introduction

Endophytic fungi are generally regarded as the fungal microorganisms colonizing the internal tissues of healthy plants without causing any apparent negative effects. These fungi are ubiquitously found in most plant species studied so far and exist in various tissues of host plants, such as roots, stems, leaves, flowers, fruits and seeds [1]. Complex interactions exist between endophytic fungi and host plants [2], and many endophytic fungi are considered as beneficial for their hosts in many ways, including promoting host growth and nutrient gain [3], as well as enhancing host resistance to phytopathogens, pests or abiotic stress [4].

In long-term symbioses with their host plants, many endophytes could produce bioactive secondary metabolites to exert positive influence on their hosts [5]. It has been surmised that endophytic fungi and host plants have similar biosynthesis pathways to produce secondary metabolites due to horizontal gene transfer, especially after the discovery of paclitaxel (taxol) in the endophytic fungus *Taxomyces andreanae* [6, 7]. Subbulakshmi *et al*. also proposed that microorganisms associated with host plants rather than plants themselves provided bioactive metabolites with high therapeutic potential [8]. As a characteristic bioresource, endophytic fungi have become an important reservoir to exploit novel bioactive metabolites. A large number of novel secondary metabolites isolated from endophytic fungi have been reported as potential agricultural and/or pharmaceutical candidates for antimicrobial, anticancer, anti-inflammatory, and many more bioactivities [9, 10]. And yet, it is believed that only 5% of the global

fungi have been identified so far, which suggests that a great many new bioactive natural products from endophytic fungi remain to be explored [11].

Over 2000 species of higher plants make latex, though only a few laticiferous plants have been exploited commercially, such as *Hevea brasiliensis* and *Ficus elastica*. *F. elastica*, also known as the Indian rubber tree, is an evergreen plant of tropical Asia that is grown for rubber production [12]. Now, it is grown around the world as an ornamental plant, outside in frost-free climates from the tropics to the Mediterranean and inside in colder climates as a houseplant. However, only a few studies have been focused on the endophytes from *F. elastica*. Solis *et al*. have reported on the diversity patterns of leaf-inhabiting endophytic yeasts of three tropical *Ficus* species collected from botanical garden greenhouses in Germany [13]. Although plenty of research has been conducted on the bioactive natural products synthesized by endophytic fungi from various plants, the bioactive secondary metabolites of endophytic fungi from *F. elastica* have never been investigated. In this study, we selected leaves of *F. elastica* to investigate preliminary characterization of the endophytic fungi. Furthermore, the antimicrobial activities of the identified endophytes were also assayed. Finally, we purified and identified the bioactive constituents present in the highly-active endophyte *Trichoderma harzianum*.

Methods and Materials

Sample Collection and Isolation of Endophytic Fungi

Fresh leaves of *F. elastica* were collected in October 2017, from Liaocheng University Arboretum, Liaocheng, Shandong, China. The samples were placed in disinfected individual plastic bags and transferred to the laboratory. The samples were rinsed with tap water and surface sterilized by immersion in 2% sodium hypochlorite for 3 min, 70% ethanol for 2 min, and washed with sterile water. The leaf pieces (5 × 5 mm) were cut from the processed samples and placed on Petri plates containing potato dextrose agar (PDA). The plates were incubated at 25 °C until hyphae emerged from the cut ends. The hyphal tips were restreaked and subcultured on a new PDA plate. This restreaking process was repeated until pure morphotype colonies were obtained. The strains were sorted into morpho-species according to their appearance (colony color and texture, border type, and radial growth rate) on PDA medium.

Molecular Identification of the Strains

Total DNA was extracted from each strain following the protocol described by Ding *et al*. [14]. The internal transcribed spacer region of rDNA (ITS1-5.8S-ITS2) was amplified with the universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). Details of PCR reaction can be found in Sharma *et al*. [15]. The PCR products were purified and sequenced by Sangon Biotech Co. Ltd. (China). The sequences identified were compared with other sequences in the GenBank database (http://www.ncbi.nlm.nih.gov) by BLAST analysis. Sequence data in this study were deposited in GenBank under the accession numbers MK240330–MK240336.

Fermentation and Extract Preparation

The endophytic fungal strains were cultured using PDA solid medium at 25°C for 5 days. Subsequently, 5 mm diameter plugs with adhering mycelia were added to 250 ml flasks containing 100 ml of potato dextrose broth (PDB) medium. All cultures were grown under shaking condition at 180 rpm and 25°C for 7 days. Each test contained five replicates for each strain.

The fermentation extracts were processed following the protocols established by Ding *et al*. [14]. After cultivation, the mycelia in the fermentation mixture were broken up using a disperser (T18, IKA, Germany), then the fermentation mixture was extracted with an equal volume of ethyl acetate (EtOAc). The extracts were evaporated under reduced pressure and redissolved by MeOH. Sterile PDB medium was extracted using the same procedure. The sterile PDB extract was used as the control in the screening procedure.

Metabolite Fingerprint Analysis

The extracts were analyzed in a HPLC system (Waters Inc., USA), which contained a model 1525 pump, a model 2489 UV detector, and a HPLC column (Pack ODS-A, 250 × 4.6 mm, 5 μm, YMC Co., Ltd., Japan). The gradient increased from 10 to 100% MeOH over 30 min and was retained at 100% for 10 min.

Purification and Identification of Natural Products

Large-scale culture (10 L) and extract preparation of the strain Lcu-Fe1712 were performed in PDB liquid medium using the method mentioned above. 2.7 g of the EtOAc extract was gained and separated by silica gel vacuum liquid chromatography using CH_2Cl_2-MeOH (20:1) to give five fractions (Fractions 1 to 5). Fraction 2 was further separated by Sephadex LH-20 chromatograph eluted with CH_2Cl_2-MeOH (1:1) and then on a semi-preparative HPLC column (Pack ODS-A, 250 × 10 mm, 5 μm, YMC Co., Ltd.) eluted with MeOH-H_2O (80:20, 3 ml/min) to provide compound **4** (5.6 mg, t_R 10.5 min) and compound **5** (20.2 mg, t_R 12.0 min). Fraction 3 was further separated on a Sephadex LH-20 column with MeOH to provide three subfractions (fractions 3-1 to 3-3). Fraction 3-2 was separated by semi-preparative HPLC eluted with MeOH-H_2O (56:36, 3 ml/min), to obtain compound **1** (2.7 mg, t_R 12.5 min), compound **2** (3.2 mg, t_R 15.5 min) and compound **3** (6.2 mg, t_R 23.5 min).

The structures of the compounds were elucidated from extensive MS and NMR. High-resolution electrospray ionization MS (HRESI-MS) spectra were measured on a Micromass EI-4000 Autospec-Ultima-TOF (Micromass communication Inc., UK).

Table 1. Endophytic fungi isolated from *Ficus elastica* as identified by ITS sequences.

Strain number	Closest BLAST match [GenBank accession number]	Query coverage (%)	Identity (%)	No. of bp analyzed	Identification [GenBank accession number]
Fes1714	*Penicillium aeneum [KP016812]*	100	100	516	*Penicillium aeneum*
Fes1701	*Penicillium chrysogenum [JN851002]*	100	99	523	*Penicillium chrysogenum*
Fes1711	*Penicillium funiculosum [GQ337426]*	100	100	517	*Penicillium funiculosum*
Fes1703	*Penicillium variabile [HQ288049]*	100	99	535	*Penicillium variabile*
Fes1707	*Scytalidium lignicola [MH863583]*	99	93	528	*Scytalidium sp.*
Fes1712	*Trichoderma harzianum [KM078037]*	100	100	551	*Trichoderma harzianum*
Fes1702	*Zasmidium anthuriicola [MH863035]*	100	99	315	*Zasmidium anthuriicola*

NMR spectra were recorded on a Varian 500 spectrometer (Varian Medical Systems Inc., USA) using tetramethylsilane as an internal standard, and chemical shifts were recorded as δ values.

Bioactive Assays

Antimicrobial activity of the extracts were evaluated by the well diffusion method. The five microorganisms indicated were the bacteria *Bacillus subtilis* CMCC 63501, *Escherichia coli* CMCC 44102, *Pseudomonas aeruginosa* CMCC 10104, *Staphylococcus aureus* CMCC 26003, and the fungus *Candida albicans* CMCC 98001. The above strains were obtained from the China General Microbiological Culture Collection Center. 100 µl of the microorganism suspensions at a density of 10^6 cells ml^{-1} were seeded onto corresponding medium plates. The crude extracts were dissolved in MeOH to a concentration of 1 mg/ml. The pure compounds were serially diluted in MeOH from 256 µg/ml to 1 µg/ml. 10 µl of the solutions were added to 6.0 mm paper disks, which were placed on the plates. Antibacterial chloramphenicol (0.1 mg/ml) was used as a positive control for bacteria and antifungal fluconazole (0.1 mg/ml) was used for yeast. The assay plates were incubated for 24 h at 37°C, then the diameter of the growth inhibition zone was measured.

Results

Isolation, Identification and Bioactivity of the Endophytic Fungi

A total of 42 endophytic fungi were isolated from healthy leaves of *Ficus elastica*. After being dereplicated by morphological characteristics (Fig. S1), only seven strains were retained and preserved at the Clinical Nutrition

Table 2. Bioactivities of the metabolites from endophytic fungi associated with *Ficus elastica*.

Strain number	Antimicrobial activity: (mm) [a]				
	B. subtilis	*C. albicans*	*E. coil*	*P. aeruginosa*	*S. aureus*
Fes1701	–	9.6 ± 2.0	–	–	10.3 ± 0.7
Fes1702	–	–	–	–	–
Fes1703	–	–	–	–	–
Fes1707	–	–	–	–	–
Fes1711	–	10.4 ± 1.7	16.5 ± 1.6	–	15.9 ± 2.5
Fes1712	–	9.0 ± 1.2	18.5 ± 2.2	9.1 ± 2.2	15.7 ± 1.7
Fes1714	–	–	10.7 ± 3.3	–	9.2 ± 2.5
Chloramphenicol [b]	17.9 ± 1.1	–	26.1 ± 0.8	9.7 ± 1.6	21.0 ± 1.5
Fluconazole [b]	–	20.1 ± 0.8	–	–	–

[a] Antimicrobial activity was estimated by the inhibitory zone (mm) to five indicator microorganisms. The diameter of the inhibition zone: >15 mm is intense (bold), 9–15 mm is medium, <9 mm is low/no activity and not shown. Indicator microorganisms: *B. subtilis*, *Bacillus subtilis* CMCC 63501; *C. albicans*, *Candida albicans* CMCC 98001; *E. coil*, *Escherichia coli* CMCC 44102; *P. aeruginosa*, *Pseudomonas aeruginosa* CMCC 10104; *S. aureus*, *Staphylococcus aureus* CMCC 26003. The crude extracts at concentration of 1 mg/ml were used in the evaluation of antimicrobial activities.

[b] Antibacterial chloramphenicol (0.1 mg/ml) and antifungal fluconazole (0.1 mg/ml) were used as positive controls.

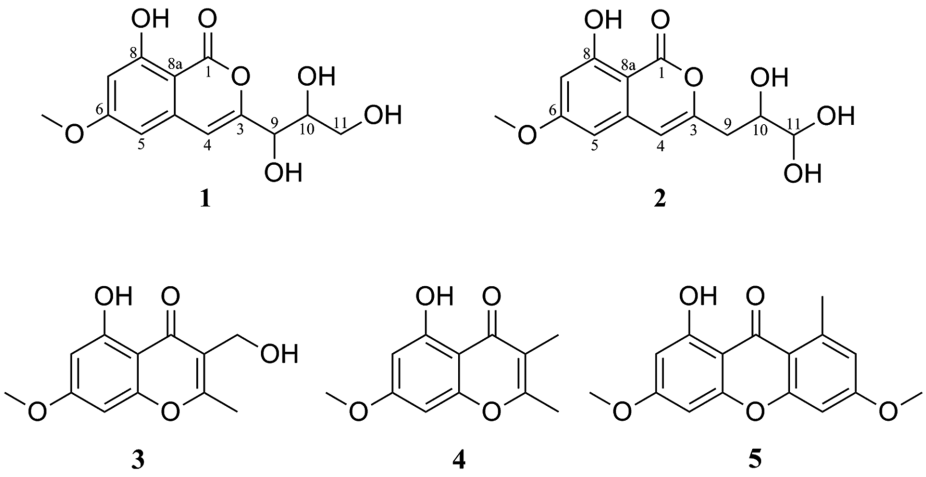

Fig. 1. Chemical structures of isolated compounds **1–5** from *T. harzianum* Fes1712.

Research Center, Liaocheng University, China. These strains were further identified taxonomically based on ITS-rDNA sequences, and assigned to seven individual taxa belonging to the genera *Penicillium*, *Scytalidium*, *Trichoderma*, and *Zasmidium* (Table 1).

The antimicrobial activity of the EtOAc extracts of endophytic fungi isolated from *F. elastica* were evaluated against five pathogenic microorganisms. Results showed that strains Fes1702, Fes1703 and Fes1707 exhibited no inhibitory bioactivity against any of the tested microorganisms, while the other four crude extracts included biologically active compounds against at least one of the tested microorganisms (Table 2). *P. funiculosum* Fes1711 and *T. harzianum* Fes1712 exhibited intense inhibitory activity against both the Gram-negative bacteria *E. coli* and Gram-positive bacterial strain *S. aureus*, while the former also showed stronger inhibitory activity to the fungus *C. albicans* more than any other strain. In addition, only *T. harzianum* Fes1712 exhibited equivalent inhibitory activity to the Gram-positive bacteria *P. aeruginosa* when compared to the positive drug.

Purification, Structural Identification and Bioactivities of the Metabolites from Strain Fes1712

Due to its great antimicrobial activity, the endophyte strain Fes1712 was selected for further chemical investigation using its EtOAc extract. Two new isocoumarin derivatives (**1** and **2**), together with three known compounds (**3–5**) were isolated from the fermentation extract. Structures of these compounds were determined using MS analyses and NMR methods (Fig. 1).

Compound (**1**) was isolated as a white solid powder and had the molecular formula $C_{13}H_{14}O_7$ as determined by the HRESI-MS peak ($[M + Na]^+$ at m/z 305.0624) (Fig. S2). The 1D NMR data (Table 3) showed 13 carbon signals, which were classified by distortionless enhancement by polarization transfer (DEPT) and heteronuclear single quantum coherence (HSQC) spectra as six nonprotonated carbons including four esters or enol ketones and two sp2 hybridized carbons, five methines including two oxygenated and three sp2 hybridized carbons, one oxygenated methylene and one oxygenated methyl (Figs. S3–S6). The

Table 3. ^{1}H and ^{13}C NMR data of Compounds 1 and 2 (500 MHz in $CDCl_3$); δ in p.p.m., *J* in Hz.

Position	Compound (**1**)		Compound (**2**)	
	δ_C	δ_H	δ_C	δ_H
1	165.1(*s*)		165.9 (*s*)	
3	150.8 (*s*)		152.8 (*s*)	
4	108.4 (*d*)	6.55 (*s*)	106.4 (*d*)	6.57 (*s*)
4a	137.7(*s*)		138.8 (*s*)	
5	103.0 (*d*)	6.41 (*s*)	100.9 (*d*)	6.44 (*d*, *J*=2.10)
6	167.0 (*s*)		166.5 (*s*)	
7	101.6 (*d*)	6.54 (*s*)	100.2 (*d*)	6.47 (*s*)
8	163.89 (*s*)		162.5 (*s*)	
8a	100.4(*s*)		99.2 (*s*)	
9	57.7(*d*)	4.66 (*d*, *J*=8.85)	35.3 (*t*)	3.03 (*dd*, *J*=14.90, 3.4) 2.83 (*dd*, *J*=14.90, 9.15)
10	71.2 (*d*)	4.49 (*m*)	72.3 (*d*)	4.38 (*m*)
11	47.2 (*t*)	4.05 (*d*, *J*=11.80) 3.96 (*d*, *J*=11.75)	75.1 (*d*)	5.92 (*d*, *J*=3.4)
6-OCH3	55.8 (*q*)	3.87 (*s*)	55.0 (*q*)	3.89 (*s*)

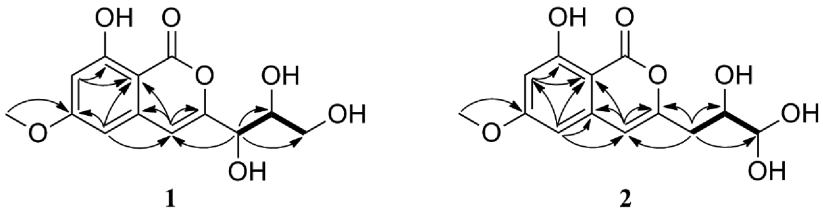

Fig. 2. Key COSY and HMBC correlations of **1** and **2**.

chemical shifts of C-1 – C-8a indicated the presence of an isocoumarin moiety, which was further validated by the heteronuclear multiple bond correlation (HMBC) (Fig. 2). The ^{1}H-^{1}H correlation spectroscopy (COSY) (H-9/H-10/H2-11) and the HMBC from H-9 to C-10/C-11 and from H-11 to C-9, as well as the chemical shifts of C-9 – C-11 (δ_C 57.7/δ_H 4.66, CH-9; δ_C 71.2/δ_H 4.49, CH-10; δ_C 47.2/δ_H 4.05, 3.96, CH_2-11), suggested the existence of a butanetriol residue (Figs. S7–S8). The butanetriol residue was linked with the isocoumarin moiety, as evidenced by the HMBC correlation from H-9 to C-4. The planar structure of **1** was thus established and shown in Fig. 1.

Compound (**2**) was obtained as a white powder with the same molecular formula as that of **1** (Fig. S9). Similarly, the 1D and 2D NMR data of **2** (Table 3, Figs. S10–S15) showed that it shared the same isocoumarin skeleton as **1**. The differences between the observed compounds **1** and **2** were the substitutional positions of hydroxyl groups in the butanetriol residue, shown as the replacement of the 1,2,3-butanetriol group in **1** by a 1,1,2-butanetriol group (δ_C 35.3/δ_H 3.03, 2.83, CH_2-9; δ_C 72.3/δ_H 4.38, CH-10; δ_C 75.1/δ_H 5.92, CH-11) in **2**. However, due to lack of samples, the configurations of **1** and **2** were not determined.

In addition, the other three known compounds (**3**–**5**) were also isolated. By comparison with the published spectroscopic data in the literature (Figs. S16–S21), their structures were identified as 5-hydroxy-3-hydroxymethyl-2-methyl-7-methoxychromone (**3**), 5-hydroxy-2,3-dimethyl-7-methoxychromone (**4**), lichexanthone (**5**), respectively.

The antimicrobial activity of the purified compounds in this study were further investigated (Table 4). Compounds **1** and **2** exhibited growth inhibitory activity against *E. coli* with MIC values of 32 μg/ml. Compound **3** showed the highest antifungal activity against *C. albicans* with MIC values of 128 μg/ml. 128 μg/ml of lichexanthone (**5**) exhibited the broad-spectrum antibacterial activity against *B. subtilis*, *P. aeruginosa* and *S. aureus*.

Discussion

In this study, the diversity characterization and bioactivities of cultivable fungi isolated from fresh leaves of *F. elastica* were investigated. A total of 42 cultivable fungal colonies were isolated and identified as seven individual taxa, that indicated low abundance and diversity of fungal communities in *F. elastica*. In the bioactivities assay, we screened the antimicrobial activity of EtOAc extracts of seven fungal strains, of which two exhibited significant activities.

Three strains of *Penicillium* species in this study exhibited

Table 4. MIC values of the isolated compounds evaluated against the tested microorganisms.

Compound	Strains, MIC (μg/ml)				
	B. subtilis	*C. albicans*	*E. coil*	*P. aeruginosa*	*S. aureus*
1	>256	256	32	128	128
2	>256	256	32	128	128
3	>256	128	256	256	>256
4	>256	256	>256	>256	>256
5	128	>256	256	128	128
Chloramphenicol	16	–	4	64	8
Fluconazole	–	16	–	–	–

Antimicrobial activity was estimated by the inhibitory zone to five indicator microorganisms. The >9 mm diameter of the inhibition zone indicated that the test compound in the corresponding concentration has inhibitory activity. Indicator microorganisms: *B. subtilis*, *Bacillus subtilis* CMCC 63501; *C. albicans*, *Candida albicans* CMCC 98001; *E. coil*, *Escherichia coli* CMCC 44102; *P. aeruginosa*, *Pseudomonas aeruginosa* CMCC 10104; *S. aureus*, *Staphylococcus aureus* CMCC 26003.

moderate antimicrobial activity. The genus *Penicillium* is known to be a significant source of secondary metabolites with various structures and bioactivities [16]. Previous reports on endophytic fungi also have demonstrated that members of genus *Penicillium* could produce a wide array of antimicrobial and antitumor agents [17]. Lin *et al*. found that culture broth of the endophytic fungus *Penicillium* sp. GQ-7 exhibited cytotoxicity in the activity screening [18]. Six new tetramic acid derivatives were further isolated, of which compound penicillenols A_1 showed the strongest inhibitory activity against HL-60 cell line with IC_{50} values of 0.76 μM. Malhadas *et al*. have evaluated the antimicrobial potential of fungal endophytes from *Olea europaea* L. and found that two strains of *Penicillium* species have effective inhibitory activity against Gram-positive and -negative bacteria [19].

Strain Fes1712, identified as *Trichoderma harzianum*, showed broad-spectrum antimicrobial activity in our screening tests. Similar to *Penicillium*, *Trichoderma* species also produce many bioactive secondary metabolites, such as polyketides, terpenoids, alkaloid, butenolides, etc [20]. As such, some novel compounds were isolated from a few endophytic *Trichoderma* species associated with the host plants, and demonstrated a wide range of bioactivities including cytotoxic [21], anti-inflammatory [22], antibacterial [23], and antifungal activities [24, 25]. Pu *et al*. reported that a *T. atroviride* strain isolated from *Camptotheca acuminate* could independently produce camptothecin in the fermentation process, with yields of 197.8 μg/l [26]. In recent bioactive screening of fungal endophytes from *Vinca plants*, Leylaie *et al*. reported cytotoxic and antimicrobial activities of extracts from four *Trichoderma* species [27].

According to the bioactivity results, the chemical constituents extracted from fermentation of *T. harzianum* Fes1712 were investigated farther. In this study, EtOAc was selected as an extract solvent due to its ability to dissolve a broad range of organic compounds. Five polyketide compounds, including two new isocoumarin derivatives (**1** and **2**), two chromone derivatives (**3** and **4**) and lichexanthone (**5**), were isolated and identified.

Isocoumarin and its derivatives are widely distributed in various bioresources and have been shown to possess a series of biological activities due to the combination with different functional residues [28]. Engelmeier *et al*. confirmed that the butyl side-chain attached to C-3 of isocoumarin skeleton was a prerequisite for high antifungal activity [29]. Thongbai *et al*. reported that isocoumarin derivatives linked with chloropropynyl side-chain exhibited pronounced cytotoxic and only moderate antimicrobial activities [30]. However, a sugar moiety linked to the same position of isocoumarins could result in the absence of all biological activity [31]. Previous studies reported that one isocoumarin compound (3,4-dihydro-8-hydroxy-3-methy-lisocoumarin) was found in genus *Trichoderma* fungus, and showed effective antifungal activity [32]. In our study, compounds **1** and **2** were determined as two novel isocoumarin derivatives with a different butanetriol group at C-3, and showed potential inhibitory activity against Gram-negative bacteria.

Both chromone and xanthone compounds have been reported to appear in the fermentation extract of *Trichoderma* species [20]. Jeerapong *et al*. have obtained chromone derivative **3** from the crude extract of soil-derived *T. harzianum* F031, and reported the inhibitory activity of **3** against pathogenic fungus *Colletotrichum gloeosporioides* which could cause anthracnose [33]. In this bioactivity assay, compound **3** also exhibited weak inhibitory activity against fungus *C. albicans*. Compound **4** has been reported as having been isolated from actiniae-derived *Trichoderma* sp. and marine brown alga-endophytic fungus *T. citrinoviride*, respectively [34, 35]. Lichexanthone (**5**) is a well-known metabolite of lichens and is also found in many filamentous fungi species [36, 37]. It has been reported to possess antimicrobial and antitumor activity [38–41]. Compound **5**, as a main product, was isolated from strain Fes1712, and its broad-spectrum antibacterial activity was confirmed by bioactivity tests in this study.

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Conflict of interest

The authors have no financial conflicts of interest to declare.

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