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Antimicrobial activities of mycoleptodiscin B isolated from endophytic fungus *Mycoleptodiscus* sp. of *Calamus thwaitesii* Becc.

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ABSTRACT

Endophytic fungi are a promising source of novel biologically active compounds including antimicrobials. Plant endophytic fungi of Sri Lanka, an island with exceptionally high biodiversity and endemism, are a vastly untapped resource. Therefore this study was initiated with the objective of examining the antimicrobial producing potential of the endophytic fungi of *Calamus thwaitesii* Becc. from Sri Lanka. This examination resulted in the isolation of 21 fungi with 7 of them exhibiting antimicrobial properties. Further investigation of the *Mycoleptodiscus* sp. isolated from the leaves, which showed the best activity among them, resulted in the isolation of the known alkaloid mycoleptodiscin B and its structure was elucidated and confirmed by mass and nuclear magnetic resonance spectral data. Mycoleptodiscin B showed promising antimicrobial activity against *Bacillus subtilis* (MIC 0.5 µg mL^{-1}) and *Staphylococcus aureus* (MIC 1 µg mL^{-1}), and was less potent against methicillin resistant *Staphylococcus aureus* (MRSA, MIC 32 µg mL^{-1}) and the pathogenic fungus *Candida albicans* (MIC 64 µg mL^{-1}). This is the first study to report the isolation, identification and antimicrobial properties of endophytic fungi of *C. thwaitesii* and the antimicrobial activities of the alkaloid mycoleptodiscin B.

INTRODUCTION

Fungal endophytes colonize healthy tissues of host plants with no manifestations of disease symptoms and are an outstanding source of biologically active compounds with potential medicinal and agricultural applications (Aly *et al.*, 2011). They are purportedly found in all plants occupying tropical and temperate climates to extreme arctic, alpine and xeric environments and their numbers are estimated to reach into millions of species (Zhang *et al.*, 2006). Thus far only a very small percentage of endophytic fungi have been investigated for their secondary metabolite producing capacity and biological activities (Strobel and Daisy, 2003; Radic and Strukelj, 2012). Further perusal of the biosynthetic abilities of endophytic fungi, especially those occupying unique ecological niches, may lead to new molecular scaffolds useful as drug leads and result in more effective utilization of this resource.

Sri Lanka, a small island nation with a variety of climatic conditions exhibits high biodiversity with a remarkably high level of endemism among its flora (Myers *et al.*, 2000; Mittermeier, 2005) that may harbor endophytes with distinctive biosynthetic abilities. In the backdrop that effective and innovative antibiotics are needed to combat drug resistant pathogenic bacteria, exploring the antibacterial producing capacity of such endophytes becomes meaningful. So far, only a few Sri Lankan plants have been systematically investigated for the production of antibacterial substances by their fungal endophytes. A recently initiated program to investigate the antimicrobial producing potential of endophytic fungi from distinct ecological settings of Sri Lanka has shown inspiring results justifying the continued research on this resource (Ratnaweera *et al.*, 2014; Ratnaweera *et al.*, 2015a & b).

Calamus thwaitesii Becc. is a rattan, belonging to the family Palmae (Arecaceae) distributed in the Western Ghats of India and Sri Lanka (Sreekumar and Renuka, 2006). Due to over-

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exploitation for furniture industry *C. thwaitesii* is rapidly decreasing and in Sri Lanka it is listed as a vulnerable species, facing a high risk of extinction in the wild (MOE, 2012). The fungal endophytes of this plant or their bioactive metabolites have not been investigated previously. Herein we describe the isolation and identification of the endophytic fungi of *C. thwaitesii* and the antimicrobial activities of their laboratory cultures. The isolation, structure elucidation and antimicrobial activities of mycoleptodiscin B, a known alkaloid, from the endophyte *Mycoleptodiscus* sp. with the most promising activity are also described.

MATERIALS AND METHODS

Isolation of the endophytic fungi from *C. thwaitesii*

Healthy specimens of *C. thwaitesii* were collected from the village of Udugampola, in the Gampaha District, Sri Lanka in December 2013 and the identity of the plant was confirmed and authenticated by comparing with voucher specimens in the National Herbarium, Royal Botanical Gardens, Peradeniya, Sri Lanka. A voucher specimen (No. UOC/PTS/RD-2014) of the plant used in the current investigation was deposited in the Department of Plant Sciences, University of Colombo, Sri Lanka.

Collected leaves and stems of *C. thwaitesii* were surface sterilized according to the protocol described in (Radji *et al.*, 2011), within 5 hrs. of collection, and sterilized plant parts were dried, cut into small segments and placed on dilute malt yeast agar (dMYA) (HIMEDIA) dishes enriched with antibiotic (Chloramphenicol 50 mg/L) under aseptic conditions and incubated at room temperature for several days. The endophytic fungi emerging from the edges of the plant segments were repeatedly sub-cultured on antibiotic absent sterile potato dextrose agar (PDA) dishes until pure cultures were obtained.

Identification of the endophytic fungi

The isolated endophytic fungi were initially identified through colony morphological features and by microscopic (OLYMPUS-CX21FS1) examination of hyphae and reproductive structures using slide cultures. The identities were confirmed by using molecular biological techniques. For this, first the fungal DNA was extracted from the mycelia using a published protocol (Kariyawasam *et al.*, 2012). The target ITS region including the 5.8S gene, was amplified by polymerase chain reaction (PCR) using universal primers ITS 1 and ITS 4 under the conditions, initial denaturation of 5 min. at 94 ^{o}C, followed by 35 cycles of 30 sec. at 94 ^{o}C, 1 min at 55 ^{o}C and 2 min at 72 ^{o}C, with a final extension of 7 min. at 72 ^{o}C (Diaz *et al.*, 2012).

Amplified DNA was sequenced commercially and was analyzed by BLAST [National Center for Biotechnology Information (NCBI)] and accession numbers were obtained for the gene sequences. The fungal voucher specimens were preserved on PDA slants at 4 ^{o}C at the Pathology Laboratory, Department of Plant Sciences, University of Colombo, Sri Lanka.

Fermentation, extraction and antimicrobial activity

Each isolated endophytic fungus was grown separately on six PDA dishes and incubated for 2-5 weeks. When the fungi reached the sporulation stage, each fungus together with the culture medium was cut into small pieces and immersed in 200 mL of ethyl acetate (EtOAc) for 24 h, the resulting extracts were filtered and the filtrates were evaporated to dryness under reduced pressure (BUCHI-R-200 rotary evaporator). The resulting fungal extracts were tested, in triplicate against four pathogenic bacteria, *Staphylococcus aureus* (ATCC 25923), *Bacillus cereus* (ATCC 11778), *Pseudomonas aeruginosa* (ATCC 9027) and *Escherichia coli* (ATCC 35218) at 300 and 50 µg/disc concentrations using standard agar disc diffusion assay. [positive control - Gentamycin (20 µg/disc); negative control – methanol]. After incubation overnight, the mean diameter of the inhibition zones were recorded. The anti-fungal bio assays of crude fungal extracts were performed using the disc diffusion method against *Fusarium oxysporum, Rigidoporus microporus, Colletotrichum gloeosporioides, and Aspergillus niger* at 300 µg/disc. (Positive control - a 1:1 A mixture of Ketoconazole and Itraconazole (10 µg/disc)) The growth inhibitions were visually examined by comparing with the positive control.

Fungal strain (RDWW-02) which showed the best antimicrobial activity was grown in large scale, on sterile PDA in 400 Petri dishes (size, 100 × 120 mm) and was incubated for 14 days at 28 ^{o}C. Next, this large scale fungal culture was extracted thrice with EtOAc (3 x 2.5 L). The combined extracts were filtered and were concentrated *in vacuo*. This EtOAc extract was tested for antimicrobial activity against the two Gram-positive bacteria, *Staphylococcus aureus* and *Bacillus cereus* at 50 µg/disc concentration using the agar disc diffusion method, to confirm the activity before proceed to the next step.

Isolation of Mycoleptodiscin B

The EtOAc extract (400 mg) of the fungal strain RDWW-02 was first subjected to solvent/solvent partitioning between hexane and MeOH/H_2O, 8:2 (500 mL each) and after the separation of the hexane layer, the polarity of the aqueous layer was increased to MeOH/H_2O, 6:4 by the addition of H_2O and extracted with $CHCl_3$ (500 mL). The $CHCl_3$ layer was separated and the aqueous layer was concentrated at reduced pressure and was partitioned between H_2O and EtOAc. Next the $CHCl_3$ soluble fraction (130 mg) which retained the antimicrobial activity was further purified using normal phase silica column chromatography (2 × 25 cm) using gradient elution (starting with hexane, then mixtures of hexane/EtOAc to EtOAc). The resulting active fraction (58 mg) was again purified by normal phase silica column chromatography (1.5 × 35 cm) with gradient elution (hexane/EtOAc, 4:1 to hexane/EtOAc, 1:9). The resulting active fraction was next subjected to size-exclusion chromatography (Sephadex LH 20; 1.5 × 60 cm with methanol) and finally purified by C_{18} reversed-phase HPLC using a CSC-Inertisil 150A/ODS2, 5 µm 25 x 0.94 cm column with 4:1 MeCN/H_2O as eluent with a flow rate of 2 mL min^{-1} to yield mycoleptodiscin B.

Structure elucidation of Mycoleptodiscin B

The structure elucidation of mycoleptodiscin B, was done using nuclear magnetic resonance (NMR) and mass spectral data. ^{1}H, ^{13}C and 2D NMR spectral data sets in DMSO-d_6 were obtained using a Bruker AV-600 spectrometer with a 5 mm CPTCI cryoprobe, while low and high resolution ESI-QIT-MS were recorded on a Bruker-Hewlett Packard 1100 Esquire–LC system mass spectrometer.

Antimicrobial activity of Mycoleptodiscin B

Mycoleptodiscin B was assayed for antimicrobial activity against three Gram-positive bacteria, *B. subtilis* (UBC 344), *S. aureus* (ATCC 43300) and methicillin resistant *S. aureus* (MRSA, ATCC 33591), two Gram-negative bacteria, *E. coli* (UBC 8161), *P. aeruginosa* (ATCC 27853) and the pathogenic fungus *C. albicans* (ATCC 90028). The minimum inhibitory concentrations (MICs) were determined using broth micro-dilution method according to National Committee for Clinical Laboratory Standards with modification using Mueller Hinton broth as the medium (NCCLS, 2002). The MIC end point was taken as the lowest concentration with more than 90 % growth inhibition. Optical density of the microbial growth was determined (at 600 nm) using a DTX 880 (Beckman Coulter Inc.) plate reader. The commercial antimicrobial agents polymyxin B for *B. subtilis*, *E. coli* and *P. aeruginosa*, rifamycin for *S. aureus* and MRSA and amphotericin for *C. albicans* were used as positive controls (Concentration series used: 2.0-0.004 µg mL^{-1}).

RESULTS AND DISCUSSION

Isolation and identification of the endophytic fungi

A total of 21 morphologically distinct endophytic fungi, 12 from leaves and nine from stems were isolated. The identities, accession numbers and incubation periods are given in Table 1. The endophytic fungi isolated belonged to 13 genera with each genus *Colletotrichum*, *Curvularia*, *Phomopsis* and *Aspergillus* represented by four, three, three, and two species respectively and the rest of the genera present as a single species. These fungi belong to three Ascomycota classes. The *Aspergillus* species belong to Eurotiomycetes, while *Curvularia*, *Lasiodiplodia*, *Dendryphiella*, *Setosphaeria*, *Bipolaris*, *Macrophomina* species belong to Dothideomycetes and *Mycoleptodiscus*, *Phomopsis*, *Diaporthe*, *Calonectria*, *Fusarium*, *Colletotrichum* species belong to Sordariomycetes class. Most of these genera including *Colletotrichum*, *Curvularia*, *Phomopsis*, *Aspergillus* and *Fusarium* are common as plant pathogenic fungi though non-pathogenic endophytic forms of these also exist (Strange and Scott, 2005; Yeasmin and Shamsi, 2013). Some reports have stated that endophytes remain latent, with symptomless nature inside the host plant only until the environmental conditions are favourable for the fungus to turn into aggressive saprophytes or opportunistic pathogens (Sieber, 2007; Rodriguez and Redman, 2008). The fungi in the current study were isolated from a healthy, symptomless plant after thorough surface sterilization, which confirm the isolated fungi were in the endophytic form during the isolation period.

Antimicrobial activity of the crude endophytic fungal extracts

The mean diameter (± Standard error (SE)) of the inhibition zones of the active crude fungal extracts in the antimicrobial bioassays are shown in Table 2. None of the extracts were active against the two Gram negative bacteria tested, while seven fungal extracts inhibited the growth of at least one Gram positive bacterial strains at 300 µg/disc. As suggested by (Hugo, 1998), this may be due to the rigid and complex cell wall structures in Gram negative bacteria. Among them RDWW-12 and RDWW-16 were only active against *S. aureus* and other five extracts were active against both *S. aureus* and *B. cereus*. Only RDWW-02, *Mycoleptodiscus* sp., showed antibacterial activity against Gram positive bacteria at 50 µg/disc concentration,

Table 1: Identities of isolated endophytic fungi with their origin, incubation period, and accession numbers.

Endophytic fungal strain	Isolated plant section	Incubation period	Identity of the endophytic fungus	Accession number
RDWW-01	leaves	18 days	*Calonectria pteridis*	KR 092388
RDWW-02	leaves	14 days	*Mycoleptodiscus* sp.	KP 119836
RDWW-03	stem	7 days	*Colletotrichum gigasporum*	KR 092389
RDWW-04	leaves	14 days	*Bipolaris* sp.	KR 092390
RDWW-05	leaves	7 days	*Aspergillus fumigatus*	KR 092391
RDWW-06	stem	7 days	*Curvularia geniculata*	KT 150263
RDWW-07	leaves	10 days	*Lasiodiplodia theobromae*	KR 092392
RDWW-08	stem	18 days	*Colletotrichum horii*	KT 150264
RDWW-09	stem	18 days	*Curvularia* sp.	KT 150265
RDWW-10	stem	21 days	*Diaporthe* sp.	KT 150266
RDWW-11	stem	7 days	*Colletotrichum gloeosporioides*	KT 150267
RDWW-12	stem	18 days	*Dendriphiella* sp.	KT 150268
RDWW-14	leaves	14 days	*Phomopsis* sp.	KT 150270
RDWW-15	leaves	21 days	*Aspergillus terreus*	KT 150271
RDWW-16	leaves	7 days	*Macrophomina phaseolina*	KT 150272
RDWW-17	stem	7 days	*Colletotrichum* sp.	KT 150273
RDWW-18	leaves	7 days	*Curvularia* sp.	KT 150274
RDWW-19	leaves	18 days	*Fusarium solani*	KT 150275
RDWW-21	leaves	18 days	*Phomopsis* sp.	KT 150277
RDWW-23	leaves	7 days	*Phomopsis* sp.	KT 150279
RDWW-24	stem	7 days	*Setosphaeria rostrata*	KT 150280

Table 2: Antimicrobial activity of the crude extracts of endophytic fungi of the plant *C. thwaitesii*.

Endophytic fungal strain	Antibacterial activity Mean diameter of the inhibition zone (mm) ± SE				Antifungal activity 300 µg/disc	
	S. aureus		*B. cereus*			
	300 µg/disc	50 µg/disc	300 µg/disc	50 µg/disc	*F. oxysporum*	*R. microporus*
RDWW-01	07.0 ±0.2	-	09.0 ±0.3	-	-	-
RDWW-02	22.4 ±1.3	13.2 ±0.5	16.3 ±0.6	10.1 ±0.2	+	+
RDWW-07	10.3 ±0.1	-	12.2 ±0.5	-	-	-
RDWW-12	12.1 ±0.3	-	-	-	-	-
RDWW-14	13.0 ±0.6	-	09.0 ±0.4	-	-	-
RDWW-15	09.4 ±0.2	-	09.6 ±0.4	-	+	+
RDWW-16	11.9 ±0.4	-	-	-	-	-
+Ve control	23.1 ±1.0	21.7 ±0.9	20.6 ±0.8	22.3 ±0.8	+++	+++
-Ve control	-	-	-	-	-	-

+ growth inhibition of the fungus

thus this was selected for the isolation of bioactive metabolites. The genus *Mycoleptodiscus* is known as a plant-associated fungus and has previously been isolated from *Desmotes incomparabilis* (Rutaceae) in Panama and from the medicinal plant *Tinospora crispa* (Menispermaceae) in Thailand as endophytes (Ortega *et al.*, 2013; Siriwach *et al.*, 2012).

Only two crude extracts, RDWW-02 and RDWW-15, out of 21, exhibited antifungal activity against *F. oxysporum*, *R. microporus* (Table 2). None of the extracts were active against *C. gloeosporioides* and *A. niger*. The two extracts that showed antifungal activity also showed activity against Gram positive bacteria. Although antifungal activities of plant endophytic fungi have been reported (Fisher, 1984; Liu *et al.*, 2001), antifungal activities of plant endophytic fungi are not as common as antibacterial activities (Hugo, 1998).

Isolation and structure elucidation of the bioactive metabolite of the *Mycoleptodiscus* sp.

The bioassay guided fractionation of the EtOAc extract of RDWW-02 led to the isolation of the active compound (2 mg) as a reddish orange amorphous solid eluting at a retention time of 16 minutes under the HPLC conditions used. This compound gave a $[M+Na]^+$ ion in the HRESIMS at m/z 390.2023 appropriate for the molecular formula of $C_{23}H_{29}NO_3$. Analysis of ^1H and ^{13}C NMR data as well as 2D NMR (COSY, HSQC, HMBC, tROESY) spectral data in DMSO-d_6 revealed that the structure of the active compound matches that of the known alkaloid mycoleptodiscin B (Fig. 1). A comparison of ^{13}C values obtained for mycoleptodiscin B in the present study and published data are given in Table 3 (Ortega *et al.*, 2013). The ^1H NMR (DMSO-d_6) values obtained in the present study and their assignments are: δ 12.4 (NH, s), δ 7.13(H-2, s), δ 5.56 (H-14, s), δ 4.14 (OH, s), δ 2.87 (H-3_{ax}, d, 16.4 Hz), δ 2.52 (H-3_{eq}, d, 16.4 Hz), δ 1.84 (H-12_{ax}, dt, 12.8, 4.0 Hz), δ 1.72 (H-12_{eq}, td, 12.8, 5.6 Hz), δ 1.69 (H-10, dd, 12.5, 2.1 Hz), δ 1.57 (H_2-11, m), δ 1.51 (H-6_{ax}, m), δ 1.40 (H_2-7, m), δ 1.30 (H-8_{eq}, m), δ 1.25 (H-6_{eq}, m), δ 1.22 (H_3-18, s), δ 1.09 (H_3-21, s), δ 1.05 (H-8_{ax}, dt, 13.6, 3.5 Hz), δ 0.87 (H_3-19, s), δ 0.84 (H_3-20, s).

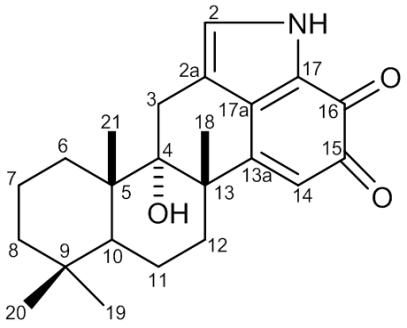

Fig. 1: Chemical structure of the alkaloid, mycoleptodiscin B.

Antimicrobial activity of mycoleptodiscin B

Mycoleptodiscin B showed strong selective antibacterial activities against Gram-positive *B. subtilits* (UBC 344), and *S. aureus* (ATCC 43300) with MIC values of 0.5 and 1 µg mL^{-1} respectively (Table 4). It is less active against MRSA (ATCC 33591) and the pathogenic fungus *C. albicans* (ATCC 90028) and inactive against the Gram negative bacteria *E. coli* (UBC 8161) and *P. aeruginosa* (ATCC 27853) (Table 4). This is the first report of the antimicrobial activities of mycoleptodiscin B.

Mycoleptodiscin B, together with mycoleptodiscin A, has previously been isolated from the *Mycoleptodiscus* species endophytic in *D. incomparabilis* collected in Panama. In that study mycoleptodiscin B is reported to possess moderate cytotoxic effects against four cancer cell lines (H460, A2058, H522-T1 and PC-3) with IC_{50} values ranging from 0.60 to 0.78 µM (Ortega *et al.*, 2013). The present study is the first to report the antimicrobial activities of the alkaloid Mycoleptodiscin B. Additionally, a new chromone derivative, mycoleptione has been isolated from the *Mycoletodiscus* species endophytic in *T. crispa* (Siriwach *et al.*, 2012).

The stem sap of *C. thwaitesii* is reported to be used as an antifertility drug (Bhandary *et al.*, 1995). However, there are no reports of any antibacterial activity attributed to this plant. Harboring endophytes which are capable of producing potent antimicrobial substances such as mycoleptodiscin B may be a valuable adaptation of hosts, which are unable to biosynthesize antibacterial substances on their own, to thwart potentially damaging microbial attacks.

Table 3: Comparison of ^{13}C NMR data of mycoleptodiscin B from the present study (in DMSO-d_6) with published data (in CD_3OD) (Ortega *et al*., 2013).

C#	^{13}C δ (ppm) for mycoleptodiscin B		C#	^{13}C δ (ppm) for mycoleptodiscin B	
	Present study	Published values		Present study	Published values
2	128.5	130.1	13	43.6	45.4
2a	119.8	121.6	13a	164.3	166.2
3	24.5	25.8	14	114.6	116.4
4	80.2	82.5	15	166.2	167.7
5	42.7	44.5	16	185.1	187.1
6	31.6	33.3	17	124.6	126.0
7	17.9	19.4	17a	130.2	132.6
8	41.1	42.7	18	24.5	25.5
9	32.8	34.2	19	33.5	34.1
10	45.2	47.3	20	21.5	22.1
11	17.7	19.3	21	17.6	18.6
12	30.8	32.6			

Table 4: MIC values obtained for mycoleptodiscin B and the positive controls.

	MIC values (µg mL^{-1})					
	B. subtilis	***S. aureus***	**MRSA**	***E. coli***	***P. aeruginosa***	***C. albicans***
Mycoleptodiscin B	**0.5**	**1**	**32**	**>64**	**>64**	**64**
Polymixin B	8	-	-	4	4	-
Rifamycin	-	0.015	0.015	-	-	-
Amphotericin	-	-	-	-	-	0.062

CONCLUSION

This study revealed that *Calamus thwaitesii* harbors many fungi capable of producing antimicrobial substances active especially against Gram positive bacteria, and thus is a potentially valuable resource for the isolation of novel antimicrobial compounds. Bioassay guided fractionation of the organic extract of *Mycoleptodiscus* sp. led to the isolation of the alkaloid mycoleptodiscin B, with potent and selective antimicrobial activity.

CONFLICT OF INTEREST STATEMENT

Authors declare that they have no conflict of interest

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