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RESEARCH ARTICLE

Antimicrobial constituents of *Hypocrea virens*, an endophyte of the mangrove-associate plant *Premna serratifolia* L.

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Abstract: Emergence of multidrug-resistant pathogenic microorganisms has prompted a worldwide search for new antibiotics from various sources. Endophytic fungi from unique habitats are considered as potential sources of novel bioactive compounds. Sri Lankan mangrove ecosystem is such a distinctive and unexploited resource for the discovery of structurally diverse and biologically active metabolites including antimicrobials. Nine endophytic fungi were isolated from the leaves and twigs of *Premna serratifolia* L. from a mangrove habitat in the Negombo lagoon and the antimicrobial activities of their laboratory cultures were evaluated. The most promising antimicrobial activity was exhibited by the endophytic fungus *Hypocrea virens*. Bioassay guided fractionation of the organic extract of this fungus led to the isolation of two known metabolites; the antimicrobial epidithiodioxopiperazine, gliotoxin **(1)**, and the closely related but less active bisdethiobis(methylthio)gliotxin **(2)**. The chemical structures of the two compounds were determined by spectroscopy and confirmed by comparison of mass and nuclear magnetic resonance (NMR) spectral data with the reported values for these molecules. The minimum inhibitory concentration (MIC) values obtained for gliotoxin **(1)** in the current study are 0.13 µg mL^{-1} for *Bacillus subtilis*, 16 µg mL^{-1} for *Staphylococcus aureus*, 32 µg mL^{-1} for Methicillin Resistant *Staphylococcus aureus* (MRSA) and *Escherichia coli*, 64 µg mL^{-1} for *Pseudomonas aeruginosa* and *Candida albicans* fungus. This is the first study to report the isolation of endophytic fungi from *P. serratifolia* and their antimicrobial activities.

Keywords: Bisdethiobis(methylthio)gliotoxin, endophytic, epidithiodioxopiperazine, gliotoxin, secondary metabolites.

INTRODUCTION

The prevalence of antibiotic resistance, specially multidrug resistance among pathogenic bacteria is a serious human health concern (Levy & Marshall, 2004). According to Rice (2008), this resistance has increased in both Gram-positive and Gram-negative bacteria and they effectively escape the effects of current antibiotics. As more and more bacteria develop resistance, it has become crucial that new antibiotics with novel modes of action are introduced to replace the older ones, which have become ineffective. Natural products, specially the ones produced by microbes, are still a promising platform for such discovery (Butler *et al.*, 2014).

Strobel and Daisy (2003) have reported that plants from distinct environmental settings have a high possibility of harbouring endophyitc fungi that produce novel bioactive secondary metabolites. Li *et al.* (2009), Pang *et al.* (2008) and Zhou *et al.* (2014) have stated that endophytic fungi isolated from mangrove plants are a well-established source for structurally diverse and biologically active secondary metabolites. However up to now, the mangrove habitats in Sri Lanka have not been explored for endophytic fungi and their bioactive secondary metabolites. The high biodiversity and unique environment conditions of mangrove habitats such as sharp variation in moisture; temperature and salt concentration; high and low tides of water; anaerobic soil and intense competition among microorganisms; insects and herbivores etc., predestine the mangrove

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flora of Sri Lanka as promising sources for the isolation of endophytic fungi with bioactive properties (Bandaranayake, 2002; Debbab *et al.*, 2013).

In a programme designed to investigate the antimicrobial secondary metabolites of endophytic fungi from distinct environmental settings of Sri Lanka, the isolation and characterisation of helvolic acid from a *Xylaria* sp. endophytic in the endemic orchid *Anoectochilus setaceus* from a rainforest, equisetin from an endophytic *Fusarium* sp. from an arid zone invasive cactus, and solanioic acid, a novel degraded steroid with potent antibacterial activity from an endophytic fungus of *Cyperus rotundus* was reported by us earlier (Ratnaweera *et al.*, 2014; 2015a; 2015b). Our investigations on the antimicrobial constituents of a laboratory culture of the endophytic fungus *Hypocrea virens* inhabiting the mangrove-associate plant *Premna serratifolia* are now reported. This article describes the isolation of the endophytic fungi from *P. serratifolia* and the antimicrobial properties of the organic extracts of their laboratory cultures of which a preliminary account was also presented (Ratnaweera *et al.*, 2013). A bioassay-guided isolation and structure elucidation by nuclear magnetic resonance (NMR), low-resolution mass spectra (LRMS) of gliotoxin and bisdethiobis(methylthio)gliotoxin, and the antimicrobial constituents present in the organic extracts of *Hypocrea virens* are also reported.

METHODS AND MATERIALS

Collection of plant material, isolation of endophytic fungi and investigation of antimicrobial activity

Healthy leaves and twigs of *P. serratifolia* were collected from the Kadol Kale mangrove forest in the Negombo Lagoon Sri Lanka (7°11'41'' – 7°11'54'' N and 79°50'32'' – 79°50'48'' E) in March 2013 and brought to the laboratory in tightly sealed polythene bags under humid conditions. The plant material was identified and confirmed using A Revised Handbook of the Flora of Ceylon (Dassanayake & Fosberg, 1985). For surface sterilisation, the leaves and twigs were first washed thoroughly in running tap water for 10 min, then successively immersed in 70 % ethanol for 1 min, 5.25 % sodium hypochlorite for 3 min and 70 % ethanol for 30 s (Ratnaweera *et al.*, 2014). Finally, the surface sterilised plant parts were washed with sterilised distilled water and allowed to dry in a laminar flow cabinet. Small pieces were cut from these surface sterilised specimens and placed on sterile potato dextrose agar (PDA) dishes. A few drops of sterilised distilled water used for the final washing was also spread on the sterile PDA plates to confirm the sterilisation process by the absence of any fungal growth. The endophytic fungi, which emerged from the tissues were transferred on to new PDA dishes and sequential sub culturing was done until pure cultures were obtained. Each fungus was grown on 5 PDA dishes and after 14 – 21 days, depending on the growth of the fungus. The mycelium together with the medium was cut into small pieces and dipped in 200 mL of ethyl acetate. After leaving for 24 hrs, each ethyl acetate extract was filtered and evaporated under reduced pressure at 40 °C using a rotary evaporator (BUCHI-R-200). The residues obtained (crude ethyl acetate extracts) were tested for antimicrobial activity against Gram-positive *Bacillus subtilis* (UBC 344), *Staphylococcus aureus* (ATCC 43300), Methicillin Resistant *Staphylococcus aureus* (MRSA, ATCC 33591), Gram-negative *Escherichia coli* (UBC 8161), *Pseudomonas aeruginosa* (ATCC 27853) and pathogenic fungus *Candida albicans* (ATCC 90028) at 200 µg per disc using agar disc diffusion method according to the National Committee for Clinical Laboratory Standards (NCCLS, 2003). The bioassay was carried out in triplicate to confirm the activity. polymyxin B (30 µg/disc) for *P. aeruginosa*, *E. coli* and *B. subtilis*, rifamycin (10 µg/disc) for *S. aureus*, MRSA and amphotericin B (20 µg/disc) for *C. albicans* were used as positive controls, while methanol was used as the negative control. The results obtained were statistically analysed by one-way ANOVA using Minitab.

Large scale culturing and extraction of endophytic fungi

The endophytic fungus, which showed the most promising antimicrobial activity was cultured on PDA medium (200 medium sized Petri dishes - 100 × 20 mm) for 15 days at room temperature. At the end of the incubation period, the fungus together with the medium were cut into small pieces and immersed in 1 L of ethyl acetate for 48 hrs and filtered through filter paper (Whatman No.1). This extraction and filtration process was repeated thrice. The filtrates were combined and the organic solvent was evaporated to dryness under reduced pressure at room temperature. The crude extract obtained was weighed and tested for antimicrobial activity at 50 µg per disc using agar disc diffusion method to confirm the presence of antimicrobial activity.

Identification of the endophytic fungi exhibiting antimicrobial activity

First, the colony morphological features of the endophytic fungus was recorded. Then fungal DNA was extracted using a published protocol (Kariyawasam *et al.*, 2012). The extracted DNA was subjected to polymerase chain

reaction (PCR) using universal primers ITS1 and ITS4. Amplified DNA was sequenced and compared with already existing DNA sequences in NCBI GenBank (*http://blast.ncbi.nlm.nih.gov/Blast.cgi*) to identify the fungus. The PCR and DNA sequencing was done commercially.

Isolation and structure elucidation of the bioactive components

To isolate the principal bioactive component(s) from the complex mixture of crude ethyl acetate extract, a series of bioassay guided chromatographic techniques were performed. The initial fractionation of the crude extract (570 mg) by size exclusion column chromatography on Sephadex LH-20 (3 × 115 cm; methanol as the eluent) resulted in seven fractions A-G, based on thin layer chromatographic (TLC) analysis. These fractions were tested for antimicrobial activity against MRSA and *B. subtilis* using a bioautographic TLC assay (Choma & Grzelak, 2011). The most active fraction E (22 mg) was next chromatographed on normal phase silica (2 × 30 cm column) with step-gradient elution (hexane: ethyl acetate 46:54 to ethyl acetate followed by ethyl acetate/methanol mixtures up to 1:1 ratio) to obtain the main active component **1**. A second bioactive fraction D (60 mg) from Sephadex LH-20 chromatography was also chromatographed on normal phase silica (2 × 30 cm column) with step-gradient elution (hexane:ethyl acetate 35:65 to ethyl acetate followed by ethyl acetate/methanol mixture up to 1:1 ratio) to obtain a less bioactive component **2**.

The structure elucidation of the isolated compounds **1** and **2** was done using NMR and mass spectral data. ^{1}H, ^{13}C and 2D NMR spectral datasets in $CDCl_3$ were obtained using a Bruker AVANCE 600-MHz spectrometer with a 5 mm cryoprobe, while the electrospray ionisation mass spectral (ESIMS) data were obtained using Bruker Esquire-LC electrospray spectrometer.

Antimicrobial activity of compounds 1 and 2

The minimum inhibitory concentrations (MICs) of the major bioactive compound **1** was tested for antimicrobial activities against three Gram-positive bacteria, *B. subtilis* (UBC 344), *S. aureus* (ATCC 43300) and MRSA (ATCC 33591), two Gram-negative bacteria, *E. coli* (UBC 8161), *P. aeruginosa* (ATCC 27853) and a pathogenic fungus *C. albicans* (ATCC 90028) using broth micro-dilution method according to NCCLS with modification using Mueller Hinton broth as the medium (NCCLS, 2002). MIC assay was done in triplicate and the mean was taken to calculate the MIC value. The less bioactive compound **2** was checked for bioactivity against the above microorganisms at 200 µg per disc using agar disc diffusion method (NCCLS, 2003). Agar disc diffusion assay was also carried out in triplicate and the mean inhibition zone was taken. The commercial antimicrobial agents polymyxin B, rifamycin and amphotericin were used as positive controls and methanol was used as the negative control.

RESULTS

Isolation and antimicrobial activities of the endophytic fungi, and identification of the active fungal strain

In the current study nine endophytic fungi, seven from the leaves and two from the twigs were isolated from *P. serratifolia.* The antibacterial activities of the crude fungal extracts are given in Table 1. The extract WM-1.6 originating from the twigs showed the most promising antibacterial activities (ANOVA, $p < 0.0001$) being active against Gram-positive *B. subtilis*, *S. aureus*, MRSA and Gram-negative *E. coli*. The extracts WM-1.1 and WM-1.4 showed selective activities against *B. subtilis*, while WM-1.3 and WM-2.3 showed weak inhibitions of *P. aeruginosa*. Although the inhibition zones against *P. aeruginosa* were considerable, the zones were not completely clear of the bacterium, which showed that the extracts did not completely kill the organism at 200 µg per disc. Of the nine extracts, four were inactive against all test organisms while none were active against *C. albicans*.

Fungal mycelium, which led to the extract WM-1.6 was light green and white, and concentric rings were observed in the culture. After 13 – 15 days, it started to secrete a light yellow pigment to the medium. According to DNA sequence data and blast results obtained, this fungus showed 100 % identity to the previously isolated *Hypocrea virens* (Syn. *Trichoderma virens*) species (ex: accession numbers, EF596954.1, JX492998.1, JX174053.1) (*http://www.ncbi.nlm.nih.gov/pubmed*, 17/12/2014). Therefore on the basis of its 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence and 28S ribosomal RNA gene, partial sequence, the WM-1.6 active fungus isolated in the current study was assigned to *H. virens* species.

Isolation and structure elucidation of the active compounds 1 and 2

Size exclusion chromatography of the crude extract (570 mg) followed by silica gel chromatography led to

Table 1: Antimicrobial activities of the crude endophytic fungal extracts isolated from *P. serratifolia* at 200 µg per disc

Fungal endophyte	Plant part used for the isolation	Mean diameter of the inhibition zone (mm) ± SE					
		S. aureus	MRSA	*B. subtilis*	*E. coli*	*P. aeruginosa*	*C. albicans*
WM-1.1	Leaf	-	-	9 ± 0.0	-	-	-
WM-1.2	Leaf	-	-	-	-	-	-
WM-1.3	Twig	-	-	-	-	18 ± 0.2 (weak)	-
WM-1.4	Leaf	-	-	9 ± 0.2	-	-	-
WM-1.5	Leaf	-	-	-	-	-	-
WM-1.6	Twig	22 ± 0.2	22 ± 0.2	20 ± 0.0	12 ± 0.3	-	-
WM-2.1	Leaf	-	-	-	-	-	-
WM-2.2	Leaf	-	-	-	-	-	-
WM-2.3	Leaf	-	-	-	-	15 ± 0.0 (weak)	-
+ Ve		40 ± 0.2	40 ± 0.2	30 ± 0.3	20 ± 0.0	20 ± 0.2	25 ± 0.0
- Ve		-	-	-	-	-	-

+Ve control: polymyxin B (30 µg/disc) for *P. aeruginosa, E. coli* and *B. subtilis*, rifamycin (10 µg/disc) for *S. aureus*, MRSA and amphotericin B (20 µg/disc) for *C. albicans*
- Ve control: Methanol, SE: Standard error

the isolation of 7 mg of the main active compound **1** as a white crystalline compound. This gave a $[M+Na]^+$ ion with *m/z* 349 in the low-resolution electrospray ionisation mass spectrum consistent with a molecular weight of 326 daltons and a molecular formula of $C_{13}H_{14}N_2O_4S_2$. Analysis of ^1H (Figure 1) and ^{13}C NMR data as well as 2D NMR (COSY, HSQC, HMBC, TROESY) spectral data in $CDCl_3$ revealed that the structure of the active compound **1** (Figure 3a) matches that of the known epidi thiodioxopiperazine, gliotoxin (Kaouadji, 1990).

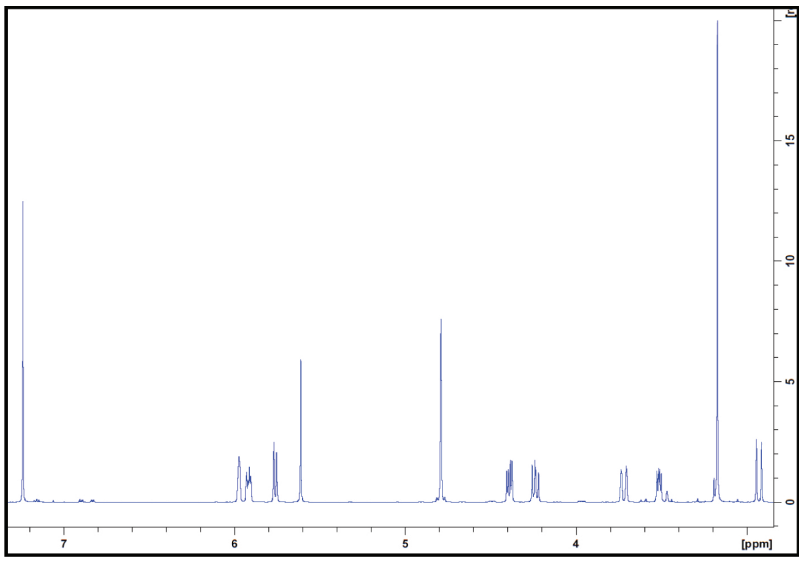

Figure 1: 600 MHz ^1H NMR spectrum of gliotoxin recorded in $CDCl_3$

Silica gel chromatography of the fraction D resulting from size exclusion chromatography led to the isolation of 18 mg of the second bioactive compound **2** as an amorphous solid. This gave a $[M+Na]^+$ ion with *m/z* 379 in its low resolution electrospray ionisation mass spectrum consistent with a molecular weight of 356 daltons and a molecular formula of $C_{15}H_{20}N_2O_4S_2$. Analysis of ^1H, ^{13}C and HSQC NMR spectral data of compound **2** in

$CDCl_3$ revealed that it is closely related to gliotoxin. The only significant differences were the appearance of two additional methyl singlets at 2.22 ppm (12-Me) and 2.24 ppm (11-Me) in the ^1H NMR spectrum (Figure 2) and two additional signals at 13.3 and 14.6 ppm in the ^{13}C NMR spectrum of the compound **2**. These differences could readily be explained by reductive methylation of the disulfur-bridge of gliotoxin resulting in two additional S-methyl groups leading to the known fungal metabolite bisdethiobis(methylthio)gliotoxin (Figure 3b). Comparison of ^1H and ^{13}C NMR data of compound **2** with the reported values of bisdethiobis(methylthio)gliotoxin confirmed this deduction (Lee *et al.*, 2001). A comparison of ^{13}C NMR values obtained in the present study for gliotoxin (**1**) and bisdethiobis(methylthio)gliotoxin (**2**) with already reported data is given in Table 2.

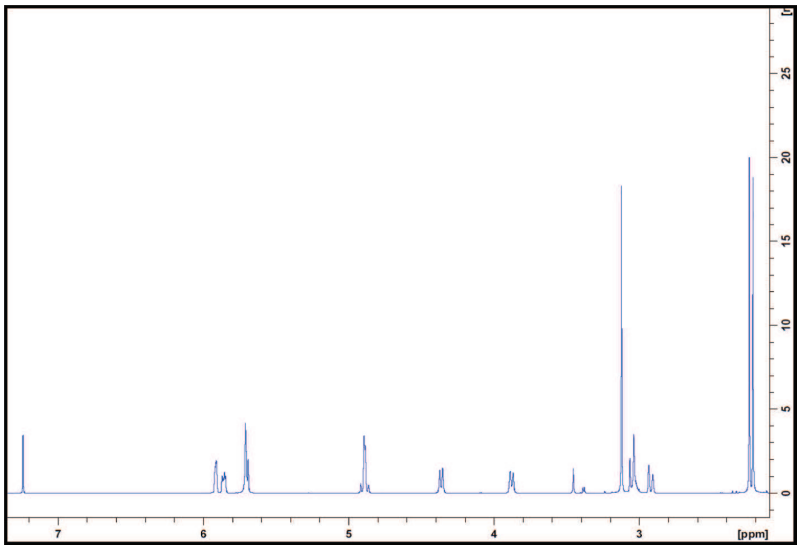

Figure 2: 600 MHz ^1H NMR spectrum of bisdethiobis(methylthio)gliotoxin, recorded in $CDCl_3$

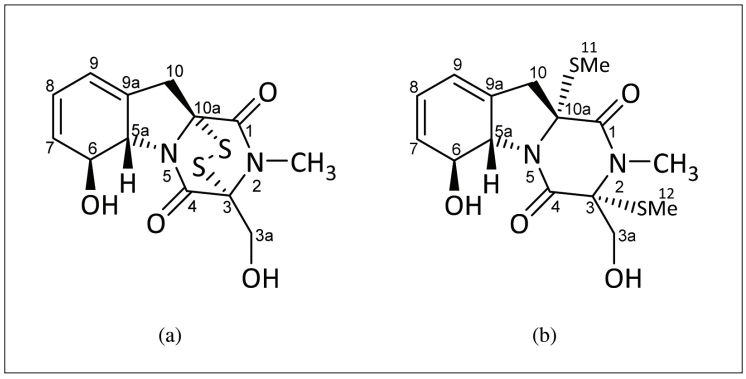

Figure 3: Chemical structures of (a) gliotoxin **1** and (b) bisdethiobis(methylthio) gliotoxin **2**

Antimicrobial activities of gliotoxin and bisdethiobis (methylthio)gliotoxin from *H. virens*

Gliotoxin exhibited antimicrobial activities against the Gram-positive *B. subtilis*, (MIC: 0.13 µg mL^{-1}), *S. aureus* (MIC: 16 µg mL^{-1}) and MRSA (MIC: 32 µg mL^{-1}), Gram-negative *E. coli* (MIC: 32 µg mL^{-1}), *P. aeruginosa* (MIC: 64 µg mL^{-1}) and pathogenic fungus *C. albicans* (MIC: 64 µg mL^{-1}). The MIC for the positive controls, polymixin B was 8 µg mL^{-1} for *B. subtilis*, 4 µg mL^{-1} for *E. coli* and *P. aeruginosa,* while 0.015 µg mL^{-1} for rifamycin against *S. aureus* and MRSA and 0.062 µg mL^{-1}

Table 2: Comparison of ^{13}C NMR data of gliotoxin and bisdethiobis(methylthio)gliotoxin from the present study (in $CDCl_3$) with published data [gliotoxin in $CDCl_3$ and bisdethiobis(methylthio)gliotoxin in DMSO-d_6] (Kaouadji, 1990; Lee *et al*., 2001)

C#	^{13}C δ (ppm) for gliotoxin		C#	^{13}C δ (ppm) for bisdethiobis(methylthio)gliotoxin	
	Present study	Published values		Present study	Published values
1	165.6	166.0	1	165.6	165.2
2-Me	27.1	27.5	2-Me	28.3	28.3
3	76.3	75.6	3	71.4	72.8
3a	60.3	60.7	3a	63.1	63
4	164.9	165.3	4	166.5	166.3
5-N	-	-	5	-	-
5a	69.5	69.7	5a	69.2	69
6	72.8	73.1	6	74.0	73.8
7	129.6	130.1	7	129.6	130.6
8	122.9	123.3	8	122.8	123.5
9	119.9	120.2	9	119.8	119.3
9a	130.3	130.8	9a	131.2	133.1
10	36.1	36.6	10	38.5	38.4
10a	76.7	77.3	10a	71.0	71.5
			11-Me	13.3	12.8
			12-Me	14.6	14.7

for amphotericin against *C. albicans*. Bisdethiobis(meth ylthio)gliotoxin showed 15 ± 0.2 mm zone of inhibition only against *B. subtilis* at 200 µg per disc concentration. The positive control polymixin B (30 µg per disc) gave an inhibition zone of 30 ± 0.2 mm.

DISCUSSION AND CONCLUSION

Although mangrove-derived fungi are well established as a potential source of new bioactive compounds, there have been no reports [other than a preliminary study (Ratnaweera *et al*., 2013)] of endophytic fungi of mangrove and mangrove associated plants of Sri Lanka, and their secondary metabolites. Therefore initiating a programme to investigate the endophytic fungi and their bioactivities from Sri Lankan mangrove habitats becomes a meaningful effort.

This pioneering investigation on the production of antimicrobial constituents by endophytic fungi from the mangrove habitat of Sri Lanka has revealed that the mangrove associated plant *P. serratifolia* harbours several endophytic fungi, which are capable of producing antibacterial substances with selective activities. Our investigation further revealed that the fungus *H. virens,* which showed the most promising antimicrobial activity among the isolated endophytic fungi produces two known epidithiodioxopiperazine antibiotics, gliotoxin and bisdethiobis(methylthio)gliotoxin (Kaouadji, 1990; Lee *et al.*, 2001), making this the first study to identify the antimicrobial constituents of an endophyte isolated from *P. serratifolia*. Gliotoxin was active against both Gram positive and Gram negative bacteria tested and the pathogenic fungus *C. albicans* with varying potencies, and was most active against *B. subtilis* with a MIC of 0.13 µg mL^{-1}. Bisdethiobis(methylthio)gliotoxin was less potent and was active only against *B. subtilis* at 200 µg per disc concentration.

H. virens was first reported from Indiana, USA from a decorticated wood, and it has been found to be the telemorph of *T. virens* according to molecular and morphological characters (Chaverri *et al.*, 2001). *H. virens* has generally been found earlier from soil, decaying wood, on other fungi (such as basidiomycetes) and as opportunistic plant symbionts (Herman *et al.*, 2004; Druzhinina *et al.*, 2011). According to some previous reports *H. virens* and *H. lixii* have been isolated from several mangrove plants, *Rhizophora apiculata, R. mucronata*, *Avicenna officialis* and *A. marina* (Liu *et al.*, 2011; Bhimba *et al.*, 2012). However, to the best of our knowledge this is the first isolation of an endophytic *H. virens* from the plant *P. serratifolia.*

Weindling and Emerson (1936) first isolated gliotoxin as a metabolic product of *Trichoderma lignorum* from soil. The designation of the isolated compound as gliotoxin and structure elucidation ensued in 1941

and 1966, respectively (Weindling, 1941; Beecham *et al.*, 1966). During several years there had been confusion about the nomenclature of the gliotoxin-producing fungus (Webster & Lomas, 1964). However, to date, gliotoxin has been isolated from a variety of fungal microorganisms namely, *Aspergillus fumigatus*, *A. chevalieri*, *A. terrus*, *Gliocladium fimbriatum*, *G. deliquescens*, *T. hamatum*, *T. lignosum*, *H. virens*, *Penicillium obscurum*, *P. cinerascens* and *P. terlikowskii* (Jordan & Cordiner, 1987; Anitha & Murugesan, 2005; Spikes *et al.*, 2008; Liu *et al.*, 2011).

Bisdethiobis(methylthio)gliotoxin was first isolated by Kirby *et al.* (1980) from a *G. deliquescens* species. Later this derivative has been isolated from several other fungal strains such as *Gliocladium flavofuscum*, *A. fumigatus* and an unidentified fungal strain (Waring *et al.*, 1986; Avent *et al.*, 1993; Lee *et al.*, 2001). At the same time some marine-derived fungi, *H. virens* and *Pseudallescheria* sp. isolated from a mangrove plant, *R. apiculata* and a marine brown alga, *Agarum cribrosum* also have afforded bisdethiobis(methylthio)gliotoxin (Li *et al.*, 2006; Liu *et al.*, 2012).

Antimicrobial activities of gliotoxin isolated in the current study also match with the previous reports (Taylor, 1971; Jordan & Cordiner, 1987). According to reported data of Brian and Hemming (1945), gliotoxin has shown MIC values of 3, 10 and 20 μg mL^{-1} against *S. aureus* (NCTC 3750), *B. subtilis* (NCTC 3610) and *E. coli* (NCTC 4144), respectively. Kaouadji (1990) has reported some antifungal activity of gliotoxin during its isolation against *C. albicans* and *C. tropicalis,* while Li *et al.* (2006) has reported 1 μg mL^{-1} activity of gliotoxin against MRSA and multidrug resistant *S. aureus* (MDRSA). It has been reported that the wide range of biological properties, such as antiviral, antibacterial and immunosuppressive activities of gliotoxin is a direct consequence of the reactivity of the epidithio bridge, which is capable of generating reactive oxygen species and engaging in protein conjugation (Waring *et al.*, 1995).

Gliotoxin has attracted considerable attention mainly due to its antibacterial, antifungal and antiviral activities (Jordan & Cordiner, 1987). It has also shown selective toxicity to cells of the haematopoietic system (Mullbacher & Eichner, 1984; Mullbacher *et al.*, 1985). However, their potential to be chemotherapeutic agents has diminished due to the discovery of *in vivo* toxicity. More recent work on this type of toxins has shown that they may be involved in aetiology of fungal diseases as well as design of novel and specific enzyme inhibitors (Waring & Beaver, 1996). Apart from the above, Anitha and Murugesan (2005) have mentioned gliotoxin as the first antibiotic to be used for plant disease control against pathogenic fungi.

According to Li *et al.* (2006), bisdethiobis(methylthio) gliotoxin have shown a 31.2 μg mL^{-1} MIC value against MRSA and MDRSA contradicting the results of this study. However, this compound has not shown any radical scavenging activity against DPPH (Li *et al.*, 2006). The lower potency of bisdethiobis(methylthio)gliotoxin against the tested microorganisms compared to gliotoxin in the current study is in agreement with similar results obtained for anti-angiogenic activities of these two compounds, thus again confirming that the bioactivity is correlated with presence and absence of the disulfide bond in the compounds (Lee *et al.*, 2001).

The investigation of endophytic fungi from terrestrial plants is a relatively new area of research in Sri Lanka. The results of the current investigation together with our recent findings of solanioic acid, a novel degraded steroid with potent antibacterial activity from an endophytic fungus of *C. rotundus* and a nortiterpenoid helvolic acid from an endophytic *Xylaria* sp. from a unique rainforest setting, demonstrate the potential of Sri Lankan plant endophytes for producing novel drug leads (Ratnaweera *et al.*, 2014; 2015b). Thus, it is hoped that this study will encourage further investigations of endophytic fungi of distinctive ecological settings of Sri Lanka for fruitful findings for the welfare of humans.

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