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Antibacterial Polyketide from *Lasiodiplodia theobromae* and *Pyrenula bahiana* on Mangrove Ecosystems in Puttalam Lagoon, Sri Lanka

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Lasiodiplodia theobromae is one of the frequently isolated fast growing endolichenic fungus. This fungus was isolated from the lichen host, *Pyrenula bahiana* collected from the mangrove ecosystems in Puttlam lagoon and its identification was confirmed based on *r*DNA-ITS sequence homology. Secondary metabolites of *L. theobromae* were extracted into ethyl acetate and subjected to antibacterial assay against *Escherichia coli* (ATCC25922), *Staphylococcus aureus* (ATCC25923) and *Bacillus subtilis* (ATCC6051). Crude extract at a concentration of 6.8 µg/mL showed good antibacterial activity against the bacterial strain *S. aureus* compared with the activity of the standard azithromycin at a concentration of 5.0 µg/mL. Active crude extract was partitioned to obtain methanol, hexane and chloroform fractions. Chloroform fraction showed the highest activity to *S. aureus* out of three fractions. This fraction was subjected to bioassay-guided separation on silica gel column chromatography to isolate bioactive pure compounds. The bioactive pure compound was identified as (3*R*)-de-*O*-methyllasiodiplodin using LC-MS, 1D and 2D NMR spectroscopy.

Keywords: Endolichenic fungi, Mangrove systems, Antibacterial activity, *Staphylococcus aureus*, (3*R*)-de-O-methyllasiodiplodin.

INTRODUCTION

Lichen thallus is a unique micro-ecosystem having a symbiotic relationship between a fungus and an alga or a cyanobacterium. In addition to the mycobiont, there are some other fungal species associated with the lichen thalli, which are known as endolichenic fungi (ELF). These ELF are not in a partnership with the symbiotic relationship and are analogous to plant endophytes. Though the exact role of ELF is not clear, numerous studies have suggested that ELF community structure is shaped up by the environment and the host [1]. ELF research community is particularly interested in secondary metabolites produced by these fungi since lichen thallus is a unique yet a challenging environment for ELF. It has been reported that quality, quantity and the type of natural products varied according to the organism's habitats *i.e.* terrestrial, marine and fresh water environments [2]. Natural products based drug development has become an attractive area of research since there are limited options available to treat some infectious diseases such as fever, diarrhea, fatigue, muscle ache and non-infectious diseases such as diabetes, cancer and blood pressure [3].

The first report on such ELF metabolites was on heptaketides isolated from *Corynespora* sp. inhabiting the Cavern beard lichen, *Usnea cavernosa* [4]. Two new heptaketides, corynesporol and 1-hydroxy dehydroherbarin along with herbarin were also isolated from the endolichenic fungal strain, *Corynespora* sp. BA10763 [4]. Since then, a total of 176 compounds has been reported of which 104 compounds identified as new compounds including polyketides, terpenoids, steroids, alkaloids and cyclic peptides. These compounds have shown remarkable antimicrobial, antioxidant and anticancer activities [5].

However, the identity, diversity and chemistry of secondary metabolites of ELF are still not explored sufficiently. Being a biodiversity hotspot, Sri Lanka provides a home for more than 1000 lichen species, and it is estimated that there may be more than 2000 species [6]. They serve as unexplored treasure troves for valuable secondary metabolites.

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Samanthi *et al*. [7] reported two new bioactive polyketides from an ELF, *Curvularia trifolli* obtained from *Usnea* sp. 5-Methoxy-4,8,15-trimethyl-3,7-dioxo-1,3,7,8,9,10,11,12,13, 14,15,15α-dodecahydrocyclododeca[*d*,*e*]-isochromene-15 carboxylic acid displayed > 90 % inhibitory activity at 5 μg/mL on cancer cell lines NCI-H460, MCF-7, SF-268, PC-3M and MIA Pa Ca-2 and 1,14-dihydroxy-6methyl-6,7,8,9,10,10α, 14,14α-octahydro-1*H*-benzo[*f*][1]oxacyclododecin-4(13*H*)-exhibited a strong anti-inflammatory activity. Further, three novel antioxidant polyketides have been obtained from the ELF, *Penicillium citrinum*, from the lichen host *Parmotrema* sp. in Sri Lanka [8,9].

Lasiodiplodia theobromae, has been isolated as a mangrove endophytic fungus from healthy leaves of the marine mangrove, *Acanthus ilicifolius* in the South China sea [10]. This was the first report on isolation of chlorinated preussomerins as natural products and chloropreussomerins A and B have been reported with potent *in vitro* cytotoxicity against A549 and MCF-7 human cancer cell lines, with IC_{50} values ranging from 5.9 to 8.9 μM [10]. Antibacterial activity of chloropreussomerins A and B have been investigated against *Staphylococcus aureus* with MIC values between 1.6 and 13 μg/mL [11].

With these findings, it was presumed that ELF associated with mangrove ecosystems, which are exposed to harsh climatic conditions should be a promising source to discover novel metabolites. Recently, a novel cytotoxic compound (*Z*)-3-{(3-a cetyl-2-hydroxyphenyl)diazenyl}-2,4-dihydroxybenzaldehyde) was isolated from the ELF, *Xylaria psidii* inhabiting *Amandinea medusulina*, a lichen host from the mangrove ecosystem in Puttalam lagoon, Sri Lanka [12].

This study describes the isolation and molecular identification of *Lasiodiplodia theobromae*, an ELF inhabiting the lichen host, *Pyrenula bahiana* collected from mangrove plants in Puttalam lagoon, Sri Lanka. Further, the bioassay guided isolation and identification of the major antibacterial compound, present among other secondary metabolites in *Lasiodiplodia theobromae* is discussed here.

EXPERIMENTAL

Isolation and identification of fungal strains: The lichen host, *Pyrenula bahiana* was collected from the mangrove habitats located in mangrove environments of Puttalam lagoon in Sri Lanka. The isolation of endolichenic fungi (ELF) inhabiting the lichen thalli was carried out according to the surface sterilization method [13]. Out of the various fungal isolates obtained, one of the frequently observed fast growing fungal isolate was selected for further culturing. A pure culture of the fungus (specimen no: KP724987) was obtained by sub-culturing on PDA medium and stored in sterile water at room temperature at the Department of Chemistry, University of Kelaniya. Molecular identification of the fungus was carried out by isolating genomic DNA followed by the polymerase chain reaction (PCR) amplification of *r*DNA-ITS region [13]. Pure PCR products were subjected to bidirectional sequencing and the sequences were manually edited using BioEdit 7.2 [14]. Basic Local Alignment Search Tool (BLASTn) was used to compare the sequence similarities to the available nucleotide sequences in the GenBank and an accession number was obtained.

Preparation of crude EtOAc extract: A seed culture of the isolated fungus was subjected to large scale culturing and the fungal metabolites were extracted into EtOAc [15]. Briefly, after incubation, the mycelia were cut into small pieces along with the PDA medium and transferred into 1 L Erlenmeyer flask. The PDA pieces with fungi were submerged in distilled ethyl acetate (EtOAc) (5 L) and were shaken for 24 h in order to extract the metabolites of *L. theobromae*. The EtOAc extract was filtered under suction using Whatman No.1 filter paper (11 μm) and dried with anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure using rotary evaporator (IKA RV 10, India) at 38 ºC, transferred into a sample vial (5 mL) using CH_2Cl_2 (200 μL) and a stream of N_2 gas was passed to remove the solvent in the extract. The crude extract was kept in the vacuum oven overnight to further confirm the removal of any remaining solvent. Antibacterial activity of the crude extract was determined against *Escherichia coli* (ATCC25922), *Staphylococcus aureus* (ATCC25923) and *Bacillus subtilis* (ATCC6051).

Partitioning of crude EtOAc extract: As crude EtOAc extract showed antibacterial activity, it was subjected to bioassay separation. In brief, a portion (1.31 g) of bioactive crude EtOAc extract was dissolved in 80% methanol in water (150 mL) and then partitioned into hexane. Hexane fraction was separated and the methanolic layer was diluted to 50 % by adding water and again partitioned with chloroform. Total of three fractions *viz*. hexane, chloroform and methanol were obtained separately and then subjected to antibacterial assay against *S. aureus*.

Bioassay directed fractionation of chloroform fraction: A portion of (0.63 g) of chloroform fraction was subjected to silica gel column chromatography as it showed the highest activity among the three studied fractions against *S. aureus.* Sample was dissolved in dichloromethane and introduced onto the column (1.5 cm, 40 cm). A gradient elution system was used from 100% dichloromethane followed by gradually increasing amounts of methanol in dichloromethane and finally 100% methanol was passed through the column. A total of 38 fractions were collected from the column and subjected to TLC analysis with the solvent system 2% (v/v) methanol in dichloromethane. These fractions were combined according to their TLC pattern to obtain five fractions (**A_1-A_5**). Each fraction was subjected to antibacterial assay against *S. aureus* separately.

Preparative thin layer chromatography (PTLC) and isolation of pure compound/s present in A_1 fraction: A PTLC was carried out in order to isolate the major compound/s using 45% hexane in chloroform as the solvent system. The fraction **A_1** was found to be bioactive, therefore, one major compound was isolated and subjected to antibacterial assay against *S. aureus*.

Antibacterial activity: The antibacterial activity was evaluated by the agar well diffusion method [7]. Bacterial inoculum (100 μL) was spread on the nutrient agar plates using a sterile spreader. Four wells with 0.8 cm diameter were cut symmetrically on each spread plate. Standard antibiotic azithromycin (0.5 μg/ mL, 100 μL) was used as positive control. The test

extract was dissolved in DMSO (100 µL) and used as negative control. Prepared plates were incubated at 37 ºC for 24 h and the diameter of inhibitory zone was measured in each well.

Structure elucidation: 1D (^{1}H, ^{13}C and DEPT-135) and 2D (HSQC, HMBC and DQF-COSY) NMR spectra were acquired on a Bruker Avance III 400 spectrometer at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR. ^{1}H spectra were referenced relative to $CDCl_3$ (δ) 7.24 ppm and ^{13}C spectra were referenced relative $CDCl_3$ (δ) 77.0 ppm. Low-resolution and high-resolution MS were recorded on Shimadzu LCMS-QP8000α and JEOL HX110A spectrometer, respectively.

RESULTS AND DISCUSSION

Identification of endolichenic fungal isolate: The fungal isolate was identified as *Lasiodiplodia theobromae*. The *r*DNA-ITS sequence of the fungus showed 100% similarity to the reported *Lasiodiplodia theobromae* sequences (MK860748 - MK860754) in the GenBank. The ITS sequence of the sample was deposited at the GenBank and accession number is KY992569.

Fractionation of bioactive compounds: *in vitro* Antibacterial assay of crude EtOAc extract was performed against three bacterial species, *B. subtilis*, *S. aureus* and *E. coli*. Out of the three tested bacterial species, the most sensitive bacterial strain against the crude extract was *S. aureus* (Table-1) hence further antibacterial assays were limited to *S. aureus* only.

TABLE-1
MEAN DIAMETER OF THE INHIBITION ZONES OF *E. coli*, *S. aureus* and *B. subtilis* BY THE CRUDE EtOAc EXTRACT OF *L. theobromae* IN THE DISC DIFFUSION ASSAY

Bacterial species	Diameter of the inhibition zones (mm)	
	Positive control (5.0 µg/mL)	Crude EtOAc extract (6.8 µg/mL)
Escherichia coli	35 ± 0	–
Staphylococcus aureus	40 ± 0	21 ± 2
Bacillus subtilis	40 ± 0	–

Each value represents the mean of three replicates ± SE (n = 3), dash (–): no inhibition

Partitioning of crude extract based on the polarity of the compounds yielded the hexane (0.10 g), chloroform (0.63 g) and methanol (0.05 g) fractions. The hexane and chloroform fractions were active against *S. aureus* while the methanolic fraction had no activity (Table-2). Herein, the separation and isolation of antibacterial active compounds were done in chloroform fraction only. Since the TLC showed several compounds in chloroform fraction (Fig. 1a), it was subjected to column chromatography. Further separation of chloroform fraction by column chromatography yielded five fractions (**A₁-A₅**) based on their TLC pattern (Fig. 1b). The yields of them were **A₁** = 352.0 mg, **A₂** = 98.1 mg, **A₃** = 65.9 mg, **A₄** = 61.5 mg and **A₅** = 18.8 mg. Antibacterial assay on these five fractions revealed that the fraction **A₁** possesses the highest activity among them (Table-2). In the TLC of **A₁**, a highly UV active major compound along with traces of many other compounds were observed. A preparative TLC on fraction **A₁** furnished the isolation of bioactive pure compound (7.0 mg) (Fig. 1c). The pure compound showed an inhibition zone diameter of 20 ± 2 mm against *S. aureus* (29 ± 2 mm).

TABLE-2
MEAN DIAMETERS OF THE INHIBITION ZONES OF *S. aureus* BY HEXANE, CHLOROFORM AND METHANOL FRACTIONS AND FRACTIONS **A_1-A_5** IN THE DISC DIFFUSION ASSAY

Subsequent fractions of crude EtOAc extract⁺	Diameter of the inhibition zones (mm)	Subsequent fractions of chloroform layer⁺	Diameter of the inhibition zones (mm)
Hexane	17 ± 2	**A_1**	21 ± 0
Chloroform	18 ± 1	**A_2**	13 ± 1
Methanol	–	**A_3**	–
+ve control	38 ± 1	**A_4**	–
		A_5	19 ± 0
		+ve control	36 ± 1

Each value represents the mean of three replicates ± SE (n = 3), dash line (–): no inhibition; ⁺Treatments were tested at 6.8 µg/mL and positive control at 5.0 µg/mL concentrations

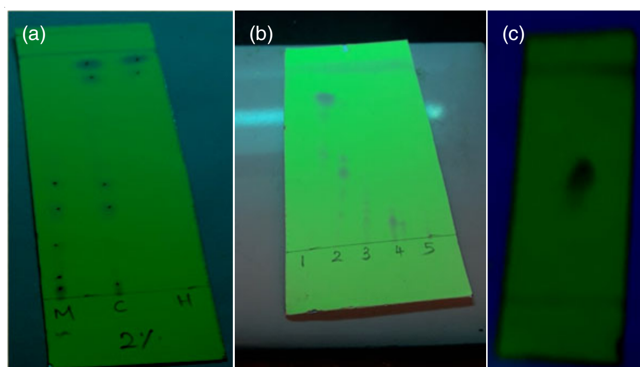

Fig. 1. TLC of (a) hexane, chloroform and methanol fractions (b) fractions **A_1-A_5** (c) pure compound in 2 % MeOH in CH_2Cl_2, under UV light

Structure elucidation of pure compound isolated from *L. theobromae*: Pure compound was isolated as colourless needles having molecular formula $C_{16}H_{22}O_4$, with 7º of unsaturation as confirmed from the data obtained for pseudomolecular ion at 279.19 for M+1 and 277.19 for M-1.

The NMR (^{1}H &^{13}C) data of the pure compound are given in Table-3. The ^{1}H NMR of pure compound indicated the occurrence of one H singlet due to the proton attached to OH group at C-3 or C-5 (δ_H 12.0). It also displayed two aromatic proton attached to *meta*-positions of the aromatic ring δ_H 6.30 (1H, br s) and δ_H 6.22 (1H, br s). DFQ-COSY confirmed that they are coupled to each other, one oxygenated methane, δ_H 5.25 (1H, overlap m), one methyl group 1.30 (3H, d) coupled with oxygenated methane, (δ_H 5.25) show strong HMBC correlations with C-15 (δ_C 75.11), C-14 (δ_C 33.50) and C-13 (δ_C 31.03). Analysis of ^{13}C NMR along with DEPT-135, DQF-COSY, HSQC and HMBC data indicated that this compound contained one methyl group (C-16), one ester carbonyl (C-1), eight methylenes (from C-8/9/10/11/12/13/14/15), six aromatic carbons (C-2/3/4/5/6/7), of which two aromatic methine carbons C-4/6 (δ_C 110.75 and 103.32), two aromatic oxygenated quaternary carbons (C-3/5 at δ_C 165.32 and 160.10). The proton at δ_H 12.0 was assigned to one of the phenolic -OH groups placed at C-3/5 as it shows strong HMBC correlations with C-3 (δ_C 160.10), C-4 (δ_C 101.3), C-5 (165.32), C-6 (δ_C 110.75) and C-7

TABLE-3
^{1}H AND ^{13}C NMR DATA OF THE PURE COMPOUND

δ^{13}C	δ^{1}H	COSY	HMBC
20.09	1.30 3H (d)	1.30, 5.25	21.11, 24.07, 24.66,30.74,31.03, 33.50, 75.11
21.11	1.40 3H (m)		
24.07	1.45 3H (m)		
24.66	1.55 3H (m)	1.55, 2.50	
27.21	1.61 2H (m)		21.11, 24.66, 31.03, 33.50, 75.11
30.74	1.66 3H (m)		
31.03	1.75 1H (m)	1.75, 1.95	20.09, 24.66, 33.50, 75.11
	1.95 1H (m)	1.95, 1.75	171.01
33.50	2.60 1H (m)	2.60, 2.50, 3.40, 2.50, 2.60, 3.40	24.66, 30.74
	3.40 1H (m)	3.40, 2.50, 2.60	24.66, 33.50, 105.44, 110.75, 149.39
75.11	5.25 1H (m)		20.09, 24.66, 31.03, 171.01
101.32	6.30 1H (br s)	6.30, 6.22	
105.44			
110.75	6.22 1H (br s)	6.22, 6.30	31.03, 33.50, 105.44, 110.75, 160.10, 165.32, 171.01
149.39			
160.10			
165.32	12.0 1H (s)		165.32, 160.10, 110.75, 105.44, 101.32
171.01			

(δ_C 105.44) and the absence of HMQC correlations between the proton at δ_H 12.0 and C-1 (δ_C 171.01) and C-2 (δ_C 149.39) suggested this phenolic hydroxyl group attached to C-5. The results revealed that the ester carbon at C1 (δ_C 171.01) show correlations with the protons at δ_H 6.22, 5.25 and 1.95 indicating those protons are attached to C-3, C-15 and C-16. Hence, the spectral data analysis confirmed the structure of the pure compound as (3*R*)-de-O-methyllasiodiplodin (Fig. 2).

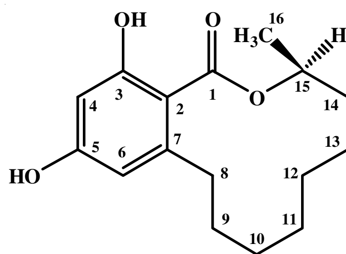

Fig. 2. Struture of (3*R*)-de-*O*-methyllasiodiplodin

Conclusion

Fungal strain isolated from the lichen, *Pyrenula bahiana* was identified as *Lasiodiplodia theobromae*. The structure of the pure compound isolated from crude EtOAc extract of L. theobromae was deduced as (3*R*)-de-O-methyllasiodiplodin, which showed the promising activity against *S. aureus*.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

REFERENCES

1. E. Ren, G. Strobel, J. Sears and M. Park, *Microb. Ecol.*, **60**, 130 (2010); https://doi.org/10.1007/s00248-009-9630-9
2. M.S. Butler, *Nat. Prod. Rep.*, **22**, 162 (2005); https://doi.org/10.1039/b402985m
3. M. Latha, K.M. Ramkumar, L. Pari, P.N. Damodaran, V. Rajeshkannan and T. Suresh, *J. Med. Food*, **9**, 391 (2006); https://doi.org/10.1089/jmf.2006.9.391
4. P.A. Paranagama, E.M. Wijeratne, A.M. Burns, M.T. Marron, M.K. Gunatilaka, A.E. Arnold and A.A. Gunatilaka, *J. Nat. Prod.*, **70**, 1700 (2007); https://doi.org/10.1021/np070466w
5. H. Gao, J. Zou, J. Li and H. Zhao, *Stud. Nat. Prod. Chem.*, **48**, 347 (2016).
6. G. Weerakoon, Fascinating Lichens of Sri Lanka, Dilmah Conservations (2015).
7. K.A.U. Samanthi, S. Wickramarachchi, E.M.K. Wijeratne and P.A. Paranagama, *J. Natl. Sci. Found. Sri Lanka*, **43**, 217 (2015); https://doi.org/10.4038/jnsfsr.v43i3.7950
8. K.A.U. Samanthi, S. Wickramaarachchi, E.M.K. Wijeratne and P.A. Paranagama, *J. Natl. Sci. Found. Sri Lanka*, **43**, 119 (2015); https://doi.org/10.4038/jnsfsr.v43i2.7939
9. S.R. Wickramarachchi, U. Samanthi, K. Wijeratne and P.A. Paranagama, *Int. J. Pharm. Sci. Res.*, **10**, 3412 (2019); https://doi.org/10.13040/IJPSR.0975-8232.10(7).3412-20
10. P.C.Y. Woo, S.K.P. Lau, A.H.Y. Ngan, H. Tse, E.T.K. Tung and K.-Y. Yuen, *J. Clin. Microbiol.*, **46**, 380 (2008); https://doi.org/10.1128/JCM.01137-07
11. R. Chen, M.D. Willcox, K.K. Ho, D. Smyth and N. Kumar, *Biomaterials*, **85**, 142 (2016); https://doi.org/10.1016/j.biomaterials.2016.01.063
12. S. Santhirasegaram, S.R. Wickramarachchi, R.N. Attanayake, G. Weerakoon, S. Samarakoon, K. Wijeratne and P.A. Paranagama, *Nat. Prod. Commun.*, **15**, 1934578X2093301 (2020); https://doi.org/10.1177/1934578X20933017
13. K. Maduranga, R.N. Attanayake, S. Santhirasegaram, G. Weerakoon and P.A. Paranagama, *PLoS One*, **13**, e0200711 (2018); https://doi.org/10.1371/journal.pone.0200711
14. T.A. Hall, *Nucleic Acids Symp. Ser.*, **41**, pp. 95-98 (1999).
15. H.A.K. Maduranga, W.R.H. Weerasinghe, R.N. Attanayake, S. Santhirasegaram, C.D. Shevkar, A.S. Kate, G. Weerakoon, K.A.U. Samanthi, K. Kalia and P.A. Paranagama, *Asian J. Chem.*, **33**, 1425 (2021); https://doi.org/10.14233/ajchem.2021.23229