J.Natn.Sci.Foundation Sri Lanka 2015 43 (2): 119-126
DOI: http://dx.doi.org/10.4038/jnsfsr.v43i2.7939

RESEARCH ARTICLE

Two new antioxidant active polyketides from *Penicillium citrinum*, an endolichenic fungus isolated from *Parmotrema* species in Sri Lanka

K.A.U. Samanthi[1], S. Wickramaarachchi[1], E.M.K. Wijeratne[2] and P.A. Paranagama[1*]

[1] *Department of Chemistry, Faculty of Science, University of Kelaniya, Kelaniya.*

[2] *Southwest Center for Natural Products Research and Commercialization, School of Natural Resources and the Environment, College of Agriculture and Life Sciences, University of Arizona, 250 E. Valencia Road, Tucson, Arizona 85706-6800, USA.*

Revised: 03 November 2014; Accepted: 21 November 2014

Abstract: Bioassay guided fractionation of the ethyl acetate extract of a laboratory culture of *Penicillium citrinum,* an endolichenic fungal strain isolated from a *Parmotrema* species in Sri Lanka, led to the isolation of two new fungal polyketides (1 and 2). The structures of these compounds were determined on the basis of their 1D NMR, 2D NMR and FABMS spectroscopic data. The two compounds (1 and 2) showed radical scavenging activity with IC_{50} values of 159.7 ± 22.3 µg/mL and 68.6 ± 4.3 µg/mL, respectively in DPPH antioxidant assay. The antioxidant activity of compound 2 was comparable to the standard antioxidant compound butylated hydroxytoluene (BHT).

Keywords: Antioxidant activity, biosynthesis, endolichenic fungus, *Penicillium citrinum,* polyketides, secondary metabolites.

INTRODUCTION

Lichens are symbiotic associations of algae or cyanobacteria (photobiont) and filamentous fungi (mycobiont). Endolichenic fungi are microorganisms living in the thalli of lichens that are analogous to the plant endophytic species inhabiting the intercellular spaces of the hosts (Arnold, 2007; Arnold & Lutzoni, 2007). The diversity and prevalence of endolichenic fungi have not been studied extensively, and there are only a few recorded studies on the isolation and identification of endolichenic fungi (Miadikowska *et al*., 2004) and their metabolites (Paranagama *et al*., 2007). The endolichenic fungi in Sri Lanka remain an untapped source of bioactive natural products as their identity and the chemistry of their secondary metabolites have not been studied thoroughly. The first record of endolichenic fungi in Sri Lanka was by a group of researchers from the University of Kelaniya (Kannangara *et al*., 2009). Under the current research programme on the study of new bioactive compounds from endolichenic fungi (Kannangara *et al.,* 2009; Kulasekera *et al.,* 2013; Pary *et al.,* 2013; Samanthi *et al*., 2013 a & b), investigations have been conducted on the endolichenic fungus, *Pennicillium citrinum* isolated from the lichen, *Parmotrema* sp. collected from the Hakgala Botanical Garden, Sri Lanka in December 2010. A literature survey on bioactive secondary metabolites of endolichenic fungi from different geographic locations revealed that its isolation, identification and bioactivity have not been studied extensively. An ethyl acetate (EtOAc) extract of *P. citrinum* cultured on potato dextrose agar (PDA) exhibited a significant antioxidant activity in 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. We report here the bioassay-guided fractionation of ethyl acetate extract of *P. citrinum* leading to the isolation of two new polyketides (1 and 2).

METHODS AND MATERIALS

Isolation of the fungal strain

The lichen host, *Parmotrema* sp., was collected from the Hakgala Botanical Garden situated at an elevation of about 1745 m above mean sea level within the Hakgala Strict Natural Reserve in the Nuwara Eliya District, Sri Lanka. The collected lichen samples were

* Corresponding author (priyani@kln.ac.lk)

kept in sterilized polythene packs and transported to the laboratory at the Department of Chemistry, University of Kelaniya and processed within 24 h.

Fungal isolations were carried out according to the surface sterilization method described by Kannagara *et al.* (2009). Healthy lichen thalli were cleaned in tap water and surface sterilized by consecutive immersion for 10 s in 95 % ethanol, 3 min in 0.5 % sodium hypochloride and 30 s in 75 % ethanol. The thalli were surface dried with sterile filter papers and aseptically cut into small segments. Lichen segments of size 1 × 1 cm were then placed (20 pieces from each of the 5 replicate samples of the lichen species) on 2 % malt extract agar (MEA) supplemented with 0.01 % streptomycin. The plates were sealed with parafilm and incubated up to 14 days at room temperature under ambient light. Fungi grown from each lichen particle were isolated into pure cultures. Slides containing pure cultures were prepared using the sticky tape method (Felgel, 1980) and identified using identification keys (Barron, 1968). The emergent fungi were sub-cultured on 2 % MEA, photographed and deposited as a living voucher in the Department of Chemistry, University of Kelaniya under the accession number US/PA/06.

Molecular identification of the isolated endolichenic fungus

Identification of isolated pure strain of the endolichenic fungus was carried out using a molecular biological protocol by genomic DNA extraction, amplification and sequencing. Promega Wizard® Genomic DNA Purification Kit was used for the extraction of fungal DNA. The fungal strain was identified by analysis of the nuclear internal transcribed spacer (ITS) region of extracted DNA, and selectively amplified by polymerase chain reaction (PCR) using ITS 1 and ITS 4 primers (Arnold & Lutzoni, 2007). Before being subjected to sequencing, the excess nucleotides and remaining primers, enzymes etc., in the PCR product were removed according to a gel purification protocol (Arnold, 2007). First, the desired amplified DNA bands were separated from non specific bands using agarose gel electrophoresis. To isolate the DNA from the gel, Promega Wizard® SV Gel and PCR Clean-Up System was used (Betz & Strader, 2002). The amplified and purified DNA was subjected to DNA sequencing and the obtained sequences were submitted to the GenBank as a nucleotide query and compared with already existing DNA sequences using NCBI BLAST® (*http://www.ncbi.nlm.nih.gov/blast/*).

Extraction and isolation

Penicillium citrinum was cultured on PDA for 2 weeks. The mycelium and the medium were cut into small pieces, extracted with EtOAc (6 × 500 mL) and the solvent was evaporated under reduced pressure, which afforded a dark brown semisolid (5.3 g) that was found to be active in antioxidant assays using DPPH method. A portion (3.6 g) of the EtOAc extract was next subjected to bioassay directed fractionation. It was first partitioned between hexane and 80 % aqueous MeOH and the bioactive aqueous MeOH fraction was diluted to 50 % aqueous MeOH by the addition of water and extracted with $CHCl_3$. The evaporation of solvents under reduced pressure yielded hexane (489.9 mg), $CHCl_3$ (2.9 g) and 50 % aqueous MeOH (44.6 mg) fractions. A portion (2.8 g) of the bioactive $CHCl_3$ fraction was subjected to column chromatography on silica gel (86.0 g) by elution with CH_2Cl_2 followed by increasing amounts of MeOH in CH_2Cl_2, which afforded 67 fractions. These fractions were combined on the basis of their TLC profiles to give seven fractions; F_1 (116.3 mg), F_2 (160.0 mg), F_3 (76.7 mg), F_4 (476.8 mg), F_5 (71.7 mg), F_6 (33.7 mg) and F_7 (175.8 mg). Fraction F_1 was found to contain compounds active in the DPPH antioxidant assay. A portion of (110.0 mg) fraction F_1 was further fractionated by gel permeation chromatography over a column of Sephadex LH-20 (4.0 g) made up in hexane, and eluted with hexane containing increasing amounts of CH_2Cl_2, CH_2Cl_2 only, followed by CH_2Cl_2 containing increasing amounts of MeOH, and finally 100 % MeOH. Twenty two fractions (10 mL each) were collected and combined on the basis of their TLC profiles to obtain two fractions of which the fraction F_{1A} was found to be active in DPPH assay.

A portion (70 mg) of the resulting active fraction F_{1A} was next chromatographed on silica gel to give two active fractions F_{1A-1} (12.3 mg) and F_{1A-2} (20 mg). Final purification of each of these two fractions by normal phase preparative TLC (2 % MeOH in CH_2Cl_2) led to the isolation of two pure compounds, 1 (8 mg) and 2 (12 mg).

Scavenging ability on 2,2-diphenyl-1-picrylhydrazyl radicals (DPPH)

The assay was carried out in a flat bottom 96-well microtiter plate according to the method described by Chatatikun & Chiabchalard (2013) with slight modifications. 160 µL of different concentrations of the sample or standard butylated hydroxytoluene (BHT)

(3.13, 6.25, 12.5, 25, 50, 100 µg/mL) was added to 40 µL of 0.25 mM methanolic DPPH solution in the 96-well plate. All reagents were mixed and incubated for 15 min at room temperature under dark conditions. The absorbance of each well was measured at 517 nm with a Microplate Reader (Biotek, USA). The percentage inhibition was calculated based on the following equation

$$\% \, Inhibition = \frac{A_{control} - A_{sample}}{A_{control}} \text{ x } 100$$

The inhibition rate was calculated and plotted against test concentrations to determine the IC_{50}.

Equipment and thin layer chromatography

1D and 2D NMR spectra of compounds 1 and 2 were recorded in $CDCl_3$ with a Bruker Avance III 400 spectrometer at 400 MHz for ^1H NMR and 100 MHz for ^{13}C NMR using residual $CHCl_3$ as an internal reference. Low-resolution and high-resolution MS were recorded on Shimadzu LCMS-QP8000α and JEOL HX110A spectrometers, respectively. Analytical and preparative thin layer chromatography (TLC) were performed on precoated 0.25 mm thick plates of silica gel 60 F254, sprayed with a solution of anisaldehyde in EtOH followed by heating to visualize the compounds on analytical TLC.

RESULTS

The fungus, US/PA/06 was identified based on the morphological and molecular characteristics. Its colony is fast-growing, velvety and blue-green with a white mycelium edge. Conidiophores arise from the mycelium singly, bearing brush-like sporulating structures, which are characteristic to the genus *Penicillium*. The ITS sequence matched with *P. citrinum* sequences from GenBank with a sequence identity of 99 %. This endolichenic fungus was thus identified as *Penicillium citrinum*.

The antioxidant capacity is widely used as a parameter for medicinal bioactive components in natural extracts. In this study, the total antioxidant activity of the EtOAc extract of *P. citrinum* was determined by DPPH assay and compared with BHT. In the antioxidant assay, the positive control, BHT showed the best results (~68 % at 100 µg/mL). All the test concentrations and the results are presented in Figure 1. The activity increased in a concentration dependant manner compared to BHT. At the concentrations of 100 µg/mL, effect of the crude extract of *P. citrinum* was found to be 35 % for DPPH (Figure 1) when compared with the same concentration of BHT.

The results of the antioxidant assay of EtOAc extract of the solid culture of *P. citrinum* and hexane, $CHCl_3$ and 50 % MeOH fractions obtained after partitioning with the EtOAc extract are shown in Figure 1. The results revealed that the $CHCl_3$ fraction has the highest activity. Bioassay-guided sequential fractionation of the antioxidant active $CHCl_3$ fraction of *P. citrinum* with normal-phase and gel

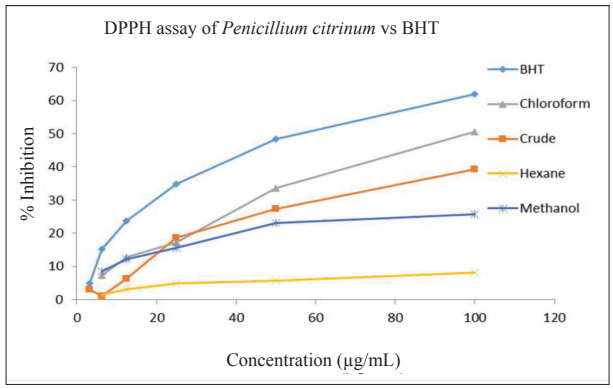


Figure 1: DPPH radical scavenging activity of the crude extract of *P. citrinum* and the resulting hexane, chloroform and methanol fractions compared with the positive control, BHT after 15 minutes of reaction. Each value is expressed as mean ± SD (n = 3)

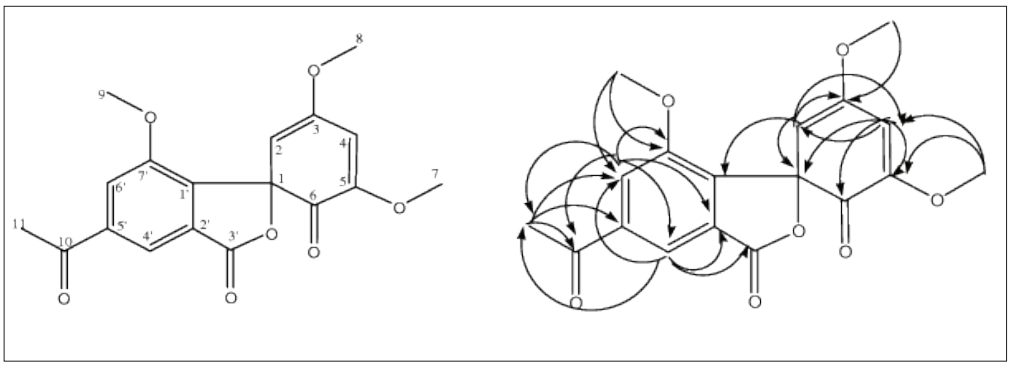


Figure 2: Structure and selected HMBC correlations for compound 1

permeation column chromatography and preparative TLC furnished two pure compounds (1 and 2). Compounds 1 and 2 were isolated as white amorphous solids and were determined to have the molecular formulae $C_{18}H_{16}O_7$ and $C_{18}H_{18}O_7$, respectively using a combination of FABMS and NMR data, which indicated 11 and 10 degrees of unsaturation, respectively.

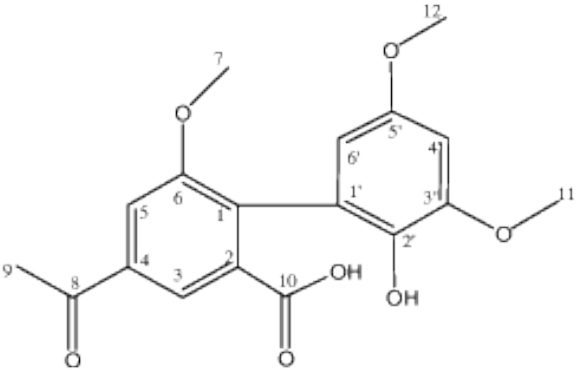
The IR spectrum of compound 1 (Figure 2) showed absorption bands at 1770 and 1681 cm^{-1}, which suggested the presence of lactone and conjugated carbonyl groups, respectively. The UV λ_{max} at 240 nm indicated the presence of carbonyl chromophore. The ^1H NMR spectrum of compound 1, when analyzed with the help of DQF-COSY indicated the presence of two spin systems in addition to four 3H singlets due to three OCH_3 groups (δ_H 3.64, 3.67 and 3.93) and a CH_3 (δ_H 2.42) on a quaternary carbonyl carbon. One of the spin systems consisting of two 1H doublets was shown to be due to the two protons δ_H 7.08 (d, J = 3 Hz) and δ_H 5.75 (d, J = 3 Hz). The two protons, δ_H 6.35 (d) and 6.53 (d) in the ^1H NMR of compound 1 were due to the two protons attached to the aromatic ring system and indicate the second spin system of compound 1 (Table 1).

The ^{13}C NMR spectrum of compound 1 when analyzed with the help of the HSQC spectrum showed the presence of ten aromatic/olefinic carbons of which three were oxygenated (δ_C 169.5, 163.5 and 158.3), four protonated (δ_C 137.2, 105.6, 105.4 and 104.0) and three quaternary carbons (δ_C 152.2, 138.2 and 108.3).

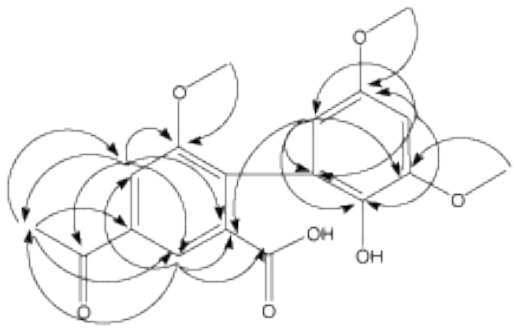
In addition, the ^{13}C NMR spectrum showed the presence of two conjugated ketone carbonyl (δ_C 190.6 and 185.7), one ester carbonyl (δ_C 174.4), three OCH_3 (δ_C 56.8, 56.1 and 52.8), one oxygenated (δ_C 84.0) and a methyl (δ_C 23.3) carbon.

Table 1: ^{13}C (100 MHz, $CDCl_3$) and ^1H (400 MHz, $CDCl_3$) NMR data for compound 1

Position	δ ^{13}C	δ ^1H (multiplicity, nH, J/Hz)	HMBC
1	84.0		
2	137.2	7.08(d, 1H, 3)	1, 3, 4, 8’
3	163.5		
4	104.0	5.75(d, 1H, 3)	1, 2, 5, 6
5	169.5		
6	185.7		
7	56.8	3.64(s, 3H)	4, 5
8	52.8	3.67(s, 3H)	3, 6’
9	56.1	3.93(s, 3H)	6’, 7’
10	190.6		
11	23.3	2.42(s, 3H)	10, 4’, 6’
1’	138.2		
2’	108.3		
3’	174.4		
4’	105.6	6.53(d, 1H, 3)	11, 2’, 3’, 6’
5’	152.2		
6’	105.4	6.35(d, 1H, 3)	10, 11, 4’,3’,7’
7’	158.3		



Partial structure of compound 2



HMBC correlations of compound 2

Figure 3: Structure of compound 2, partial structure of compound 2 and selected HMBC correlations for compound 2

In the HMBC correlations of compound 1 (Figure 2), the proton at δ_H 6.35 showed correlations with C-4' (δ_C 105.6), carbonyl C (δ_C 190.6), C-2' (δ_C 108.3), methyl C of the acetyl group (δ_C 23.2) and C-7' (δ_C 158.3), suggesting that it should be attached to C-6' (δ_C 105.4). HMBC correlations of the signal at δ_H 6.53 with C-2' (δ_C 108.3), C-6' (δ_C 105.4), C-3' (δ_C 174.4) and methyl C of the acetyl group (δ_C 23.3) allowed the assignment of the proton at δ_H 6.53 to H-4'. The olefinic proton at δ_H 7.08 showed HMBC correlations with C-1' (δ_C 138.2), C-4 (δ_C 104.0), C-3 (δ_C 163.5) and C-1 (δ_C 84.0) assigning it to H-2. The other olefinic proton at δ_H 5.75 showed HMBC correlations with C-2 (δ_C 137.2), C-1 (δ_C 84.0), C-5 (δ_C 169.5) and carbonyl carbon at C-6 (δ_C 185.7), assigning it to H-4 and the carbonyl carbon to C-6. The CH_3CO group was placed at C-5' with the help of the HMBC correlations of the signal at H-11 (δ_H 2.42) to C-5' (δ_C 108.3), C-6' (δ_C 105.4), C-4' (δ_C 105.6) and H-4' (δ_H 6.53), and H-6' (δ_H 6.35) to C-11, methyl C of the acetyl group (δ_C 23.2).

With the help of HSQC data the three methoxyl protons, δ_H 3.67, 3.64 and 3.93 were connected to C-9, C-10 and C-11, respectively. The three OCH_3 groups were placed at C-3, C-5 and C-7', as δ_H 3.67 displayed the ^3J HMBC correlation with C-3 (δ_C 163.5), δ_H 3.64 displayed the ^3J HMBC correlation with C-5 (δ_C 169.5) and δ_H 3.93 displayed the ^3J HMBC correlation with C-7' (δ_C 158.3), respectively. The structure of new compound 1 was thus established as 5'-acetyl-3,5,7'-trimethoxy-3'H-spiro [cyclohexa [2,4]diene-1,1'-isobenzofuran]-3',6-dione. Compound 1 showed moderate antioxidant activity with an IC_{50} value of 159.7 ± 22.3 µg/mL (Figure 4).

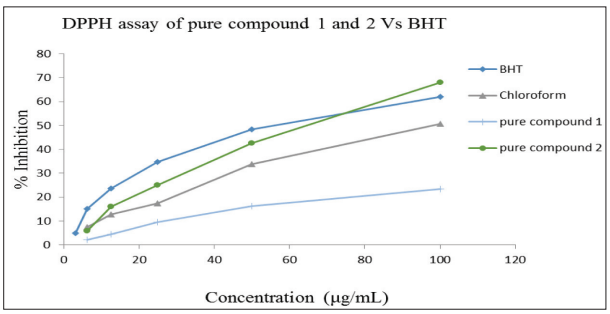


Figure 4: DPPH radical scavenging activity of the chloroform extract of *P. citrinum* and the pure compounds (1 and 2) isolated from the chloroform extract compared with the positive control, BHT after 15 minutes of reaction. Each value is expressed as mean ± SD (n = 3)

Compound 2 analyzed for $C_{18}H_{18}O_7$ by a combination of FABMS and NMR data indicated 10 degrees of unsaturation. The UV λ_{max} at 238 nm indicated the presence of a carbonyl chromophore. The IR spectrum had absorption bands at 3489, 1706 and 1641 cm^{-1}, suggesting the presence of a OH group, carboxylic acid carbonyl and carbonyl functionalities. The ^1H and ^{13}C NMR spectra together with HSQC data of compound 2 (Table 2) showed the presence of an acetyl group attached to the aromatic ring similar to compound 1. The ^1H and ^{13}C NMR data of compound 2 (Table 2) were similar to those of compound 1 and the major difference in NMR data was found to be the absence of an ester carbonyl carbon group at δ_C 174.4, which was assigned to C-2 in compound 1; instead compound 2 showed the presence of a carboxylic acid group (C-10, δ_C 163.9), which exhibited an HMBC correlation to H-3 (δ_H 6.38).

Table 2: ^{13}C (100 MHz, $CDCl_3$) and ^1H (400 MHz, $CDCl_3$) NMR data for compound 2

Position	1 δ ^{13}C	δ ^1H (mult,nH,J/Hz)	HMBC
1	127.7		
2	110.0		
3	110.8	6.38(d,1H,3)	2, 5, 9, 10
4	148.7		
5	103.0	5.99(d,1H,3)	2, 3, 6, 8, 9
6	160.9		
7	55.5	3.23(s,3H)	6
8	199.0		
9	22.5	2.23(s,3H)	3, 4, 5,
10	163.9		
11	52.4	3.58(s,3H)	3'
12	56.3	3.62(s,3H)	5'
1'	131.9		
2'	152.2		
3'	166.3		
4'	109.4	7.14(d,1H,3)	1', 2', 4', 6'
5'	156.4		
6'	114.2	7.51(d,1H, 3)	2, 1', 2', 3'

Further, the ketone carbonyl group (C-6, δ_C 185.7) in compound 1 was absent; instead the ^{13}C NMR spectrum of compound 2 showed oxygenated C-2' (δ_C 152.2). The ^1H NMR spectrum of compound 2 indicated the occurrence of two *meta*-coupled aromatic protons (δ_C 7.51 and 7.14, J = 3 Hz), four 3H singlets of which three were due to OCH_3 groups (δ_C 3.58, 3.62 and 3.23) and the fourth due to a CH_3 group on a quaternary carbon (δ_H 2.42), and two 1H singlets at δ_H 6.38 and 5.99 accounting for 16 out of 18 protons in compound 2. The remaining two protons were suspected to be that of OH groups. The ^{13}C

NMR spectrum assigned with the help of the HSQC data contained signals due to a carbonyl carbon (δ_C 199.0), 12 aromatic/olefinic carbons of which four were oxygenated (δ_C 166.3, 156.4, 152.2 and 160.9) and four protonated (δ_C 103.0, 110.8, 109.5 and 114.2), three methoxyl carbons (δ_C 56.3, 52.4 and 55.5), one methyl carbon (δ_C 22.5), and a carbonyl carbon C of the carboxylic acid (δ_C 163.9). The HMBC spectrum indicated the presence of two partial structures (Figure 2) that contained two aromatic rings with *meta*-coupled protons. The proton at δ_H 6.38 of compound 2 showed HMBC correlations with methyl C of the acetyl group (δ_C 22.5), C-5 (δ_C 103.0), C-2 (δ_C 110.0), and carboxylic acid carbonyl carbon (δ_C 163.9) suggesting that this proton is attached to the aromatic ring at C-3 and the remaining proton (δ_H 5.99) in the aromatic ring should be attached to C-5. The proton at H-5 δ_H 5.99 of compound 2 showed HMBC correlations with C-2 (δ_C 110.0), C-3 (δ_C 110.8), C-6 (δ_C 160.9), C-8 (δ_C 199.0) and C-9 (δ_C 22.5) confirming the presence of the proton (δ_H 5.99) attached to C-5 (δ_C 103.0). These HMBC correlations together with ^{1}H and ^{13}C NMR data established the partial structure B ($C_{10}H_9O_4$) for compound 2 (Figure 4). The cross-peaks between δ_H 7.51 with C-1'(δ_C 131.9), C-3'(δ_C 166.3), C-2' (δ_C 152.2), C-2 (δ_C 110.0); δ_H 7.14 with C-5' (δ_C 156.4), C-2' (δ_C 152.2), C-1' (δ_C 131.9), C-6' (δ_C 114.2); δ_H 3.58 with C-3' (δ_C 166.3); δ_H 3.62 with C-5' (δ_C 156.4) in the HMBC spectrum and the ^{1}H and ^{13}C NMR data for the remainder of the molecule established the presence of the partial structure A ($C_8H_9O_3$) in compound 2 (Figure 3). The proton (δ_H 7.51) showed a correlation with C-2 (δ_C 110.0) indicating that partial structures A and B are connected to each other establishing the structure of compound 2, which contains an additional carboxylic acid group in the aromatic ring A and a hydroxyl group in the aromatic ring B and disappearance of ketone carbonyl group and ester carbonyl group in compound 1 suggesting that the partial structures A and B of compound 2 are linked to each other as shown in Figure 3. The structure of new compound 2 was thus established as 4-acetyl-2'-hydroxy-3',5',6-trimethoxy biphenyl-2-carboxylic acid. Antioxidant assay results of compound 2 showed strong radical scavenging activity in the DPPH assay with an IC_{50} value of 68.6 ± 4.3 µg/mL (Figure 4).

DISCUSSION AND CONCLUSION

Penicillium citrinum is a mycotoxin producing fungus known to produce citrinin, a well-known mycotoxin both nephrotoxic and carcinogenic. Bioactivity of citrinin has been studied extensively and it also possesses antibiotic, bacteriostatic, antifungal and antiprotozoal properties (Abou-Zeid, 2012). There have been several reports on the isolation of bioactive metabolites, and the investigation of the ethyl acetate extracts from the broth and mycelia of *P. citrinum* has led to the isolation of eighteen metabolites (Chen *et al.,* 2011), antioxidant JBIR-124 (Kawahara *et al.,* 2012), cytotoxic penicitrinols C and E, topo IIα-inhibitory tricitrinol B and the cytotoxic dicitrinone B (Khamthong *et al.,* 2012). The isolation of five polyketides including two benzopyranones, one isochroman and two anthraquinone-citrinin derivatives was reported from the sea fan-derived fungus *P. citrinum* (Khamthong *et al.,* 2012). Two benzoquinones and citriquinone A and B have

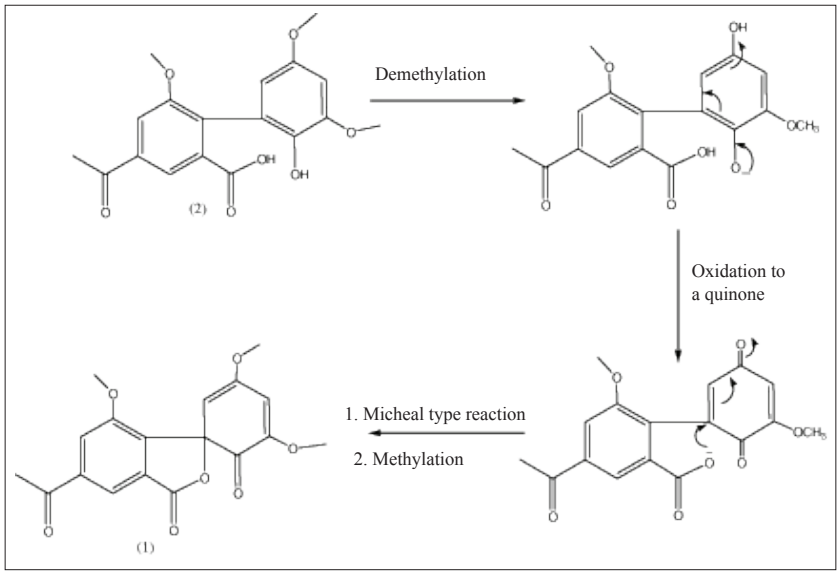


Figure 5: Postulated biosynthetic pathway of compound 1

been reported from the methanol extract of *P. citrinum* isolated from the soil in Sri Lanka. It was reported that citriquinone A exhibited antibacterial activity against *Bacillus* sp. and cell migration inhibitory activity against human cancer cell line HEp 2 (Ranji *et al.,* 2013). In the present study, two new polyketides (1 and 2) have been isolated with strong radical scavenging activity when compared to the control, BHT.

A plausible biosynthetic pathway for the two new polyketides (1 and 2) has been proposed in Figures 5 and 6. Compound 2 contains a similar carbon skeleton as compound 1 except for the absence of a carboxylic acid group attached to C-2. The structural features of compounds 1 and 2 implies that both compounds have the same polyketide origin, and the biosynthetic pathway proposed in Figure 6 suggests that compound 2 is the precursor for compound 1. The proposed biosynthetic pathway given in Figure 5 indicates that compound 2 originated from a heptaketide *via* condensation, reduction, oxidation, carboxylation and other reactions to form the final polyketide product compound 2. Compound 2 is the precursor for compound 1 as compound 2 could undergo oxidation to a quinone to which the carboxylate group can add in a Michael-type reaction as proposed in Figure 6.

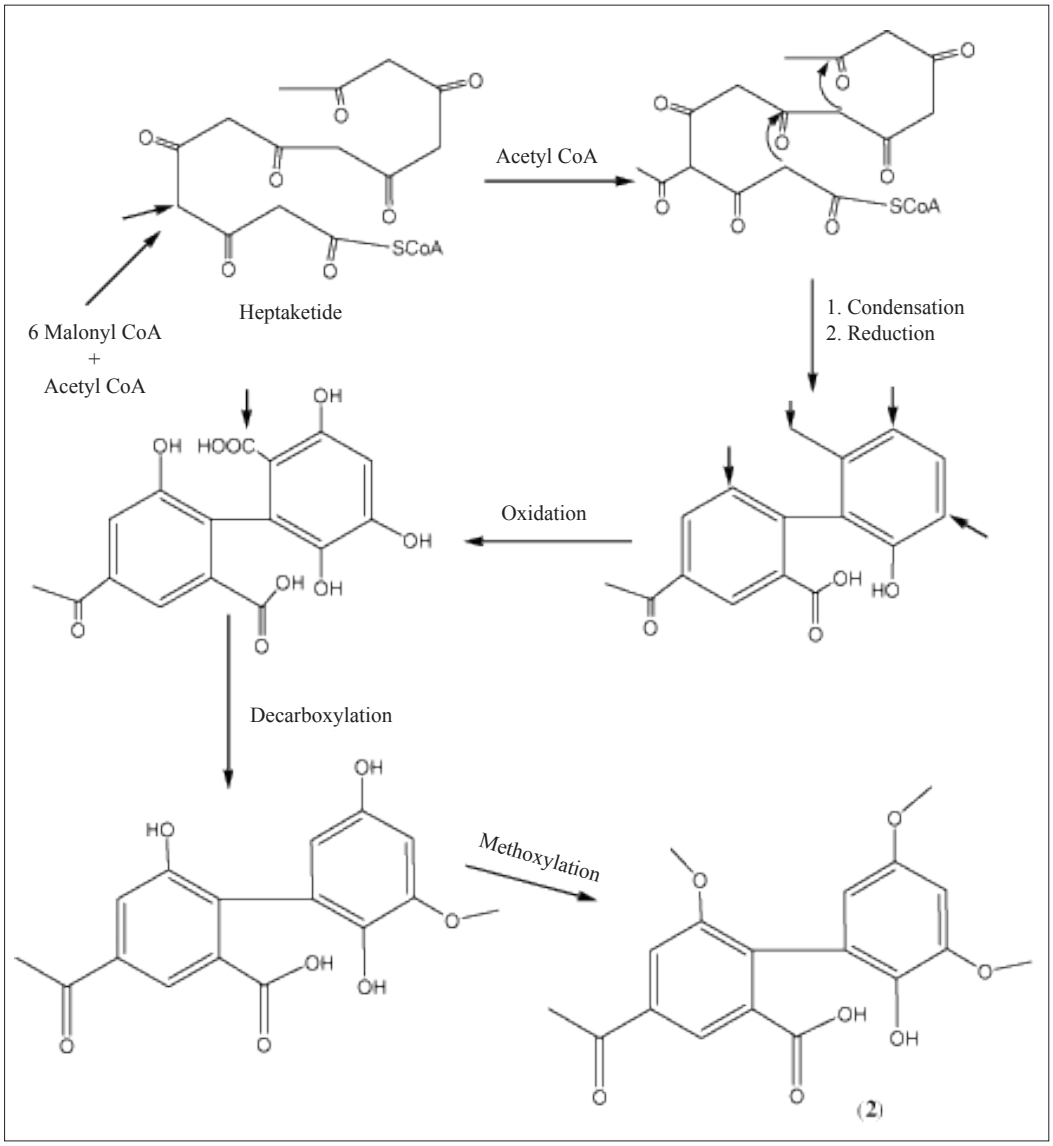


Figure 6: Postulated biosynthetic pathway of compound 2

Acknowledgement

Financial support for this study was provided by the National Research Council of Sri Lanka (grant NRC/08-13). The authors thank Prof. Leslie Gunatilake, Director, Southwest Center for Natural Products Research and Commercialization, University of Arizona, USA for the assistance given to obtain NMR and MS data.

REFERENCES

1. Abou-Zeid A.M. (2012). Review on citrinin: production, effect of some plant extracts and gene involved in its biosynthesis. *Journal of Civil and Environmental Engineering* **2**(113). DOI: http://dx.doi.org/10.4172/2165-784X.1000113
2. Arnold A.E. (2007). Understanding the diversity of foliar endophytic fungi: progress, challenges, and frontiers. *Fungal Biology Reviews* **21**: 51 – 66. DOI: http://dx.doi.org/10.1016/j.fbr.2007.05.003
3. Arnold A.E. & Lutzoni F. (2007). Diversity and host range of foliar fungal endophytes: are tropical leaves biodiversity hotspots? *Ecology* **88**: 541 – 549. DOI: http://dx.doi.org/10.1890/05-1459
4. Barron G.L. (1968). The Genera of Hyphomycetes From Soil, Williams and Wilkins Publishers, Baltimore, USA.
5. Betz N. & Strader T. (2002). Clean-up with Wizard SV for gel and PCR. *Promega* **82**: 1 − 5.
6. Chatatikun M. & Chiabchalard A. (2013). Phytochemical screening and free radical scavenging activities of orange baby carrot and carrot (*Daucus carota* Linn.) root crude extracts. *Journal of Chemical and Pharmaceutical Research* **5**(4): 97 – 102.
7. Chen L., Liu W., Hu X., Huang K., Wu J.L. & Zhang Q.Q. (2011).Citrinin derivatives from the marine-derived fungus *Penicillium citrinum*. *Chemical and Pharmaceutical Bulletin* **59**: 515 – 517.
8. Felgel T.W. (1980). Semi permanent microscope slides of micro fungi using sticky tape technique. *Canadian Journal of Microbiol*ogy **26**: 551 – 553.
9. Kannangara B.T.S.D.P., Rajapaksha R.S.C.G. & Paranagama P.A. (2009). Nature and bioactivities of endolichenic fungi in *Pseudocypellaria* sp*., Parmotrema* sp. and *Usnea* sp. at Hakgala montane forest in Sri Lanka. *Journal of Letters in Applied Microbiology* **48**: 203 – 209. DOI: http://dx.doi.org/10.1111/j.1472-765X.2008.02512.x
10. Kawahara T., Takagi M. & Shin-ya K. (2012). JBIR-137 and JBIR-138, new secondary metabolites from *Aspergillus* sp., fA75. *Journal of Antibiotic* **65**: 45 – 47. DOI: http://dx.doi.org/10.1038/ja.2012.64
11. Khamthong N., Rukachaisirikul V., Phongpaichit S., Preedanon S. & Sakayaroj J. (2012). Bioactive polyketides from the sea fan-derived fungus *Penicillium citrinum. PSU-F51 Tetrahedron* **68**: 8245 – 8250.
12. Kulasekara C.N., Wickramarachchi S. & Paranagama P.A. (2013). Antioxidant and antifungal activities of secondary metabolites of the endolichenic fungus, *Penicillium pinophilum* isolated from the lichen *Pseudocypherllaria* sp., available in Sri Lanka, *Proceedings of the 42nd Annual Sessions, Institute of Chemistry Ceylon*, Adamantane House, Rajagiriya, Sri Lanka, 21 – 22 June, p. 32.
13. Miadikowska J., Arnold A.E., Hofstetter V. & Lutzoni F. (2004). High diversity of cryptic fungi inhabiting healthy lichen thalli in a temperate and tropical forest. *The IAL Book of Abstracts* (eds. T. Randlane & A. Saag), pp. 43. Tartu University Press, Tartu, Estonia.
14. Paranagama P.A., Wijerathne E.M.K., Burns A.M., Marron M.T., Gunathilaka M.K., Arnold A.E. & Gunathilaka A.A.L. (2007). Heptaketides from *Corynespora* sp., inhabiting the Cavern Beard Lichen, *Usnea cavernosa:* first report of metabolites of an endolichenic fungus. *Journal of Natural Products* **70**: 1700 – 1705. DOI: http://dx.doi.org/10.1021/np070466w
15. Pary F., Kulasekera C., Wickramaarchchi S. & Paranagama P. (2013). Isolation and molecular identification of endolichenic fungi inhabiting in the lichen *Pseudocypherllaria* sp., available in Sri Lanka, *Proceedings of the 69th Annual Sessions of Sri Lanka Association for the Advancement of Science*, 2 – 6 December, p. 104.
16. Ranji P.K., Wijeyaratne S.C., Jayawardana K.H. & Gunaherath G.M. (2013). Citriquinones A and B, new benzoquinones from *Penicillium citrinum*. *Natural Products Communications* **8**(10): 1431 –1434.
17. Samanthi U., Wickramaarchchi S. & Paranagama P. (2013a). Isolation of bioactive metabolites in the endolichenic fungus, *Daldinia eschscholzii,* occurring in the lichen, *Parmotrema* sp., in Sri Lanka, *Proceedings of the 69th Annual Sessions of Sri Lanka Association for the Advancement of Science*, 2 – 6 December, p. 161.
18. Samanthi U., Kulasekara C., Adihetti S., Wickramarachchi S. & Paranagama P.A. (2013b). Preliminary investigation of isolation of bioactive secondary metabolites produced by *Penicillium citrinum,* inhabiting the lichen *Parmotrema* sp., available in Haggala montane forest in Sri Lanka, *Proceedings of the 42nd Annual Sessions, Institute of Chemistry Ceylon*, Adamantane House, Rajagiriya, Sri Lanka, 21 – 22 June, pp. 33.