IJPSR (2019), Volume 10, Issue 7 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 11 November 2018; received in revised form, 07 February 2019; accepted, 08 March 2019; published 01 July 2019

A NEW ANTIOXIDANT ACTIVE COMPOUND FROM THE ENDOLICHENIC FUNGUS, *PENICILLIUM CITRINUM* INHABITING THE LICHEN, *PARMOTREMA* SP.

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Keywords:

Endolichenic fungi, Lichen, *Penicillium citrinum*, Antioxidant activity, Bioactivity

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ABSTRACT: Context: Endolichenic fungi are the microorganisms living inside the thalli of the lichen and found to be a new source of bioactive secondary metabolites. This is an underutilized source of bioactive compounds and has not been studied extensively. Genus, *Parmotrema* is one of the foliose lichens with largely rounded lobes and occurs on rocks of rain forests, sub-montane and montane forests in Sri Lanka. **Aims:** The endolichenic fungi available in Sri Lanka and the chemistry of their secondary metabolites have not been explored thoroughly. This study aims at exploring novel bioactive compounds available in *Penicillium citrinum* inhabiting *Parmotrema sp.* **Methods and Material:** The lichen, *Parmotrema sp.*, was collected from Hakgala Botanical Garden in Sri Lanka and its fungal strains were isolated and identified by Genomic DNA sequencing. Compounds were first extracted to ethyl acetate and subsequently subjected to bioassay-guided fractionation to isolate the bioactive compounds. The DPPH assay was used to determine the antioxidant activity. The active compound/s was separated by column chromatography, and its structure was elucidated by IR, 1D and 2D-NMR, ^{13}C-NMR and MS. **Statistical Analysis Used:** The IC_{50} of the crude extract was calculated using Probit analysis (MINITAB® Release 14.1. Minitab Inc. 2003 Statistical Software). **Results:** A bioactive compound, named PP-PC-03 together with PP-PC-01 and PP-PC-02 was isolated from *P. citrinum*. All three compounds showed antioxidant activity in the DPPH assay with IC_{50} values 159.6 ± 22.3, 120.1 ± 11.7 and 68.6 ± 4.3 µg/mL respectively. **Conclusion:** Based on spectral analysis the structure of PP-PC-03was determined as 10-Ethylidene-2,4,9-trimethoxy-10,10a-dihydro-7,11-dioxa-benzo[b] heptalene-6,12-dione and identified as a new compound. Comparatively PP-PC-02 and PP-PC-03 had moderate antioxidant activities while PP-PC-01 showed strong radical scavenging activity.

INTRODUCTION: 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 without causing deceptive damage to their hosts [1].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.10(7).3412-20

The article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.10(7).3412-20

The diversity and prevalence of endolichenic fungi have not been studied extensively, and there are only a few recorded studies available on isolation and identification of endolichenic fungi [1-4].

The endolichenic fungi found in Sri Lanka and the chemistry of their secondary metabolites have not been explored thoroughly. The prevalence of endolichenic fungi in the lichens in Sri Lanka was published, and 29 endolichenic fungal strains have been isolated from the lichens, *Parmotreama* sp., *Usnea* sp. and *Pseudocyphellaria* sp. from Hakgala montane forest in Sri Lanka [2].

Recent studies have demonstrated that endolichenic fungi can produce structurally diverse bioactive small molecules. The first report of isolation and identification of secondary metabolites from an endolichenic fungus was published by Paranagama, *et al*., 2007 [1]. In that study isolation of two new heptaketides, corynesporol and 1-hydroxydehydro-herbarin along with herbarin have been reported. All compounds were evaluated for their cytotoxicity and ability to inhibit migration of human metastatic breast and prostate cancer cell lines MDA-MB-231 and PC-3M, respectively. Dehydroherbarin inhibited migration of both cell lines at concentrations not toxic to these cell lines [1]. In our continuing search for novel bioactive secondary metabolites from endolichenic fungi inhabit in lichens found in Sri Lanka, we have been particularly interested in the identification of compounds with antioxidant and anticancer activities. Recent reports indicated the isolation of anticancer and antioxidant polyketides from two endolichenic fungi, *Curvularia trifolii* and *Penicillium citrinum* [4, 5].

In the present study, we focused on the endolichenic fungus, *P. citrinum*, inhabit in the lichen, *Parmotrema sp.* collected from Hakgala montane forest in Sri Lanka. It is reported that *P. citrinum* obtained from different sources have produced bioactive secondary metabolites with diverse chemical structures. Isolation of citrinin, phenol A acid, dihydrocitrinone and dihydrocitrinin, together with a cytotoxic dimer, dicitrinin A had been reported from solid phase fermentation of an Australian *P. citrinum* [6]. A thorough literature survey on bioactive secondary metabolites in endolichenic fungi from different geographical locations revealed that isolation of bioactive secondary metabolites in *P. citrinum* had not been studied extensively except for the recent reports published by Samanthi *et al*. (2015a) [4]. In our recent study, bioassay-guided fractionation of ethyl acetate extract of *P. citrinum* isolated from the lichen, *Parmotrema* sp. led to the isolation of two bioactive polyketides [4]. The present study is a continuation of exploring more bioactive compounds available in *P. citrinum*.

SUBJECTS AND METHODS: IR spectra for KBr disks were recorded on a Shimadzu FTIR-8300 spectrometer. NMR spectra were recorded in $CDCl_3$ with a Bruker DRX-400 instrument at 400 MHz for ^1H NMR and 100 MHz for ^{13}C NMR, using residual $CHCl_3$ as an internal standard. The chemical shift values (δ) are given in parts per million (ppm), and the coupling constants are in Hz. Mass spectrum was recorded on a Shimadzu LCMS-8000 QPR and a JEOL HX110A mass spectrometer. Column chromatography was performed using Merck Kieselgel (230-400 mesh) or Sephadex LH 20. Analytical and preparative thin layer chromatography (TLC) was performed on pre-coated 0.25 mm thick plates of silica gel 60 F_{254}.

Isolation and Identification of Fungal Strain: The lichen host, *Parmotrema* sp. was collected from Hakgala Botanical Garden situated in Nuwara Eliya District, Sri Lanka. Fungal isolations were carried out according to the surface sterilization method described by Samanthi *et al.* (2015a) [4]. The fungi grown on PDA were deposited in 10% glycerol in H_2O at -80 ºC at the Department of Chemistry, University of Kelaniya, Sri Lanka under the accession number US/PA/06. Molecular identification of the pure isolated strains of the endolichenic fungi 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 [4].

Preparation of EtOAc Extract of *P. citrinum* and its Antioxidant Activity: A seed culture of *P. citrinum* grown on PDA for two weeks was used for inoculation. Large-Scale culture of *P. citrinum* was inoculated into 56 large PDA plates and incubated for 14 days at room temperature. The crude extract was extracted using EtOAc, and the antioxidant assay was carried out in a flat bottom 96-well microtiter plate, according to the method described by Chatatikun *et al*. (2013) [5] with slight modifications. The inhibition rate was calculated and plotted against the test concentrations to obtain the IC_{50} value. The IC_{50} of the crude extract was calculated using Probit analysis (MINITAB® Release 14.1. Minitab Inc. 2003 Statistical Software).

Isolation of Bioactive Compounds in the EtOAc Extract: A portion (3.6 g) of the crude extract was partitioned with hexane, $CHCl_3$, and 80% methanol

in water as described by Parangama *et al*. (2007a) [1]. The antioxidant activity of each fraction was evaluated using the methods described above.

Bioassay Directed Fractionation of the $CHCl_3$ Fraction: A portion (2.8 g) of the $CHCl_3$ fraction was subjected to column chromatography on silica gel, and the column was eluted with CH_2Cl_2 followed by increasing amounts of MeOH in CH_2Cl_2. These fractions were combined by their TLC profiles. The seven fractions were analyzed using normal phase silica TLC plate using 1% MeOH in CH_2Cl_2 solvent system.

Purification of Antioxidant Active Fraction Using Sephadex LH-20 Column: A portion of the bioactive fraction (110.0 mg) was further fractionated by gel permeation chromatography over a column of Sephadex LH-20 made up in hexane and eluted with hexane containing increasing amounts of CH_2Cl_2, followed by CH_2Cl_2 containing increasing amounts of MeOH, and finally 100% MeOH. Twenty-two fractions (10 mL each) were collected and combined by their TLC profiles to obtain two fractions. Purification of one of the fractions led to the isolation of two bioactive compounds, PP-PC-01 and PP-PC-02 [4] and the other fraction was further purified using silica gel column chromatography.

Isolation of PP-PC-03: A portion of the fraction (475.0 mg) was subjected to column chromatography on silica gel and eluted with CH_2Cl_2 followed by increasing amounts of MeOH in CH_2Cl_2. Six fractions were obtained, and the DPPH assay was carried out to evaluate the antioxidant activity of each fraction. The antioxidant active fraction was purified using preparative TLC with the solvent system of CH_2Cl_2: EtOAc (9: 1) to obtain the pure compound PP-PC-03.

RESULTS:

Fractionation of the EtOAc Extract and their Antioxidant Activities: ITS sequence of the endolichenic fungi isolated from the lichen, *Parmotrema* sp. matches with *P. citrinum* sequences from Gene-Bank with a sequence identity of 99%. Therefore this endolichenic fungus was identified as *P. citrinum* [4]. Antioxidant activity of the EtOAc extract and subsequent fractions was determined by DPPH assay and compared with BHT. The results are presented in **Fig. 1** and **2** [4].

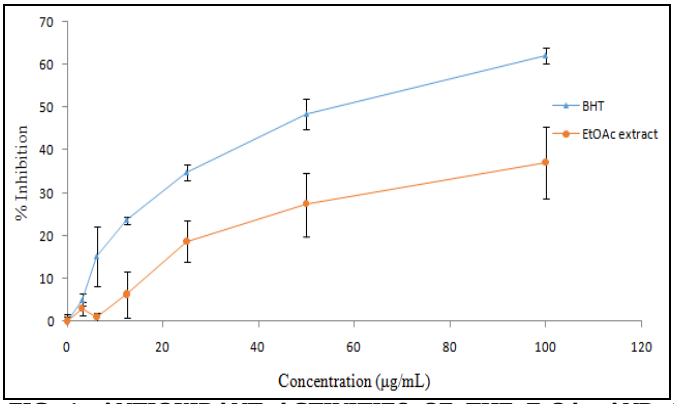


FIG. 1: ANTIOXIDANT ACTIVITIES OF THE EtOAc AND STANDARD SYNTHETIC ANTIOXIDANT, BHT. EACH VALUE IS EXPRESSED AS MEAN ± SD (n=3)

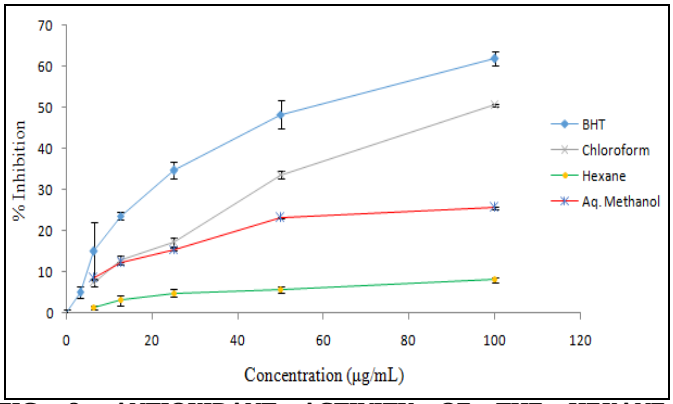
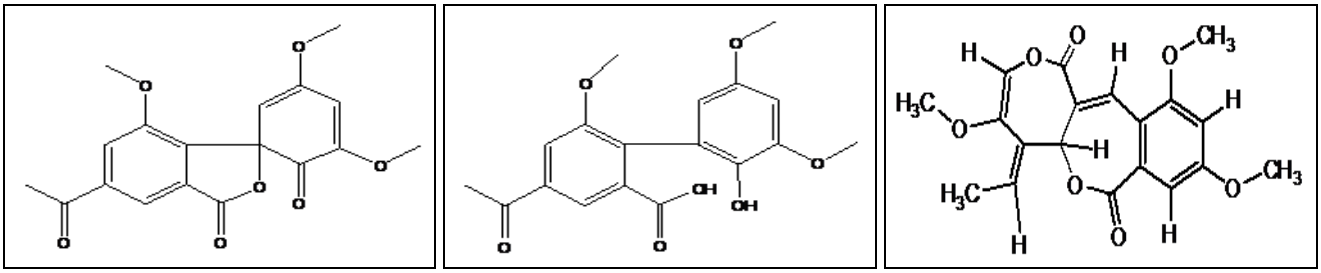


FIG. 2: ANTIOXIDANT ACTIVITY OF THE HEXANE, CHLOROFORM, METHANOL FRACTIONS COMPARED WITH THE POSITIVE CONTROL, BHT AFTER 15 min OF REACTION. EACH VALUES IS EXPRESSES AS MEAN ± SD (n=3)



PP-PC-01 **PP-PC-02** **PP-PC-03**

FIG. 3: DEDUCED STRUCTURES OF PURE COMPOUNDS ISOLATED FROM *P. CITRINUM*

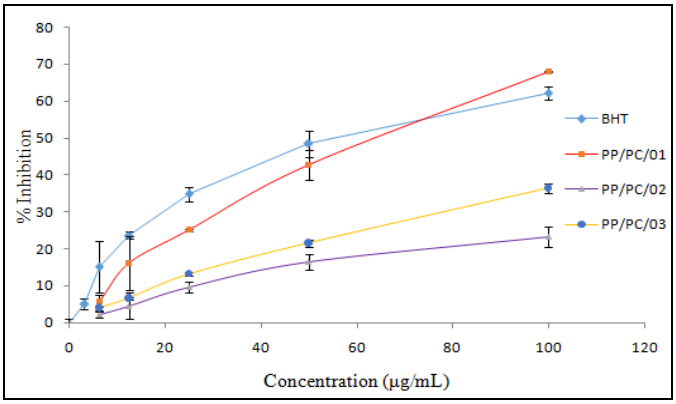


FIG. 4: THE DPPH RADICAL SCAVENGING ACTIVITY OF THE PURE COMPOUNDS (PP-PC-01, PP-PC-02, AND PP-PC-03) ISOLATED FROM THE CHLOROFORM EXTRACT OF *P. CITRINUM* COMPARED WITH THE POSITIVE CONTROL, BHT. EACH VALUE IS EXPRESSED AS MEAN ±SD (n=3)

Further fractionation of $CHCl_3$ fraction which showed the highest activity, furnished a new pure compound PP-PC-03 together with recently reported PP-PC-01 and PP-PC-02 [4] **Fig. 3**. Compound PP-PC-03 was a white amorphous solid with UV activity. This appeared as a blue-white fluorescent compound under long wavelength UV light on a normal phase TLC. PP-PC-03 showed moderate antioxidant activity **Fig. 4**.

Structure Elucidation of PP-PC-03: Structure of PP-PC-03 was fully established by a combination of FABMS and NMR spectroscopy. ^{1}H NMR and ^{13}C NMR data with selected HMBC correlations observed are given in **Table 1**. **Fig. 5** and **6** show the UV-Vis and IR data of PP-PC-03 respectively.

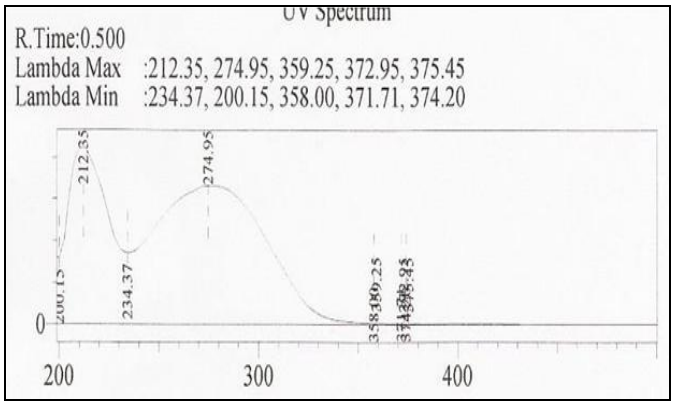


FIG. 5: UV SPECTRUM OF PP-PC-03

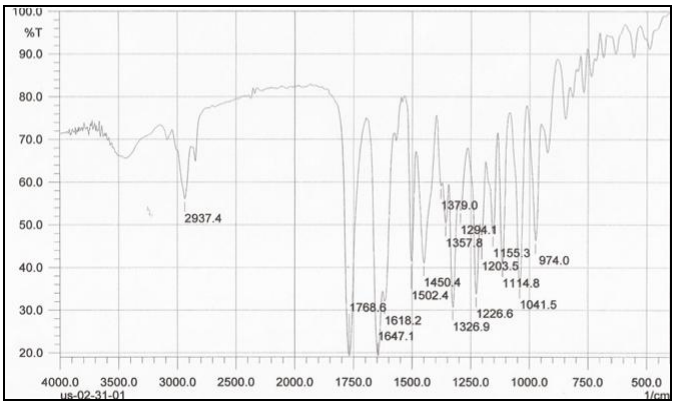


FIG. 6: IR SPECTRUM OF PP-PC-03

TABLE 1: NMR DATA OF PP-PC-03; ^{1}H(400MHz, $CDCl_3$) &^{13}C (100MHz, $CDCl_3$)

Position	δ ^{13}C	δ ^{1}H (mult, nH, J/Hz)	HMBC
1	98.9	6.96(d, 1H, 2)	2,3, 12, 12a
2	163.0		
3	105.1	6.65(d, 1H, 2)	1, 2, 4, 4a
4	154.8		
4a	127.8		
5	152.5	7.39(s, 1H)	4, 5a, 6, 10a
5a	123.5		
6	173.7		
8	134.7	6.54 (s, 1H)	10, 16
9	142.7		
10	155		
10a	73.7	6.44(s, 1H)	4a, 5, 5a, 6
12	170.1		
12a	129.3		
13	55.8	3.86 (s, 3H)	2
14	56.0	3.76 (s, 3H)	4
15	60.6	3.89 (s, 3H)	9
16	118.7	6.55 (q, 1H)	8, 10, 17
17	18.9	1.92 (d, 3H)	8, 9, 10, 16

The ^{1}H NMR spectrum of PP-PC-03 is given in **Fig. 7** and **Fig. 8** is its COSY. **Fig. 9** and **10** are the ^{13}C NMR and HSQC spectra of PP-PC-03. **Fig. 11** is the HMBC spectrum and **Fig. 12** illustrates some selected HMBC correlations of PP-PC-03. **Fig. 17** shows the NOEDIFF correlations of PP-PC-03.

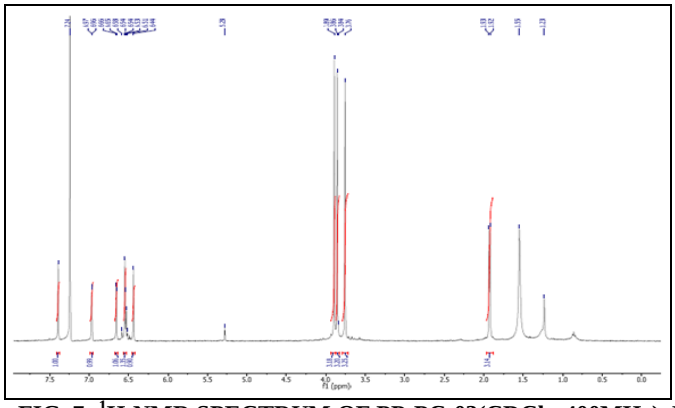


FIG. 7: ^{1}H-NMR SPECTRUM OF PP-PC-03($CDCl_3$, 400MHz)

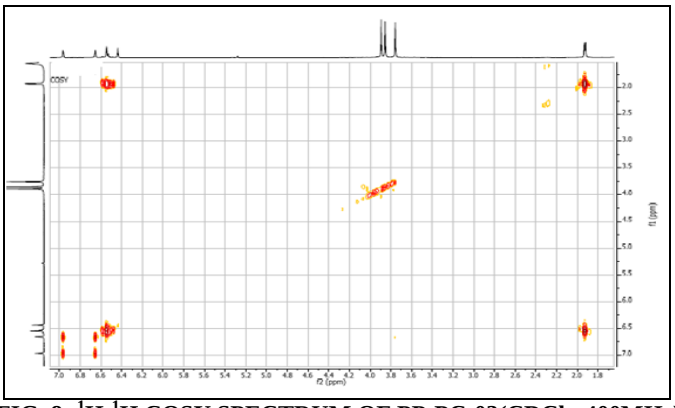


FIG. 8: ^{1}H-^{1}H COSY SPECTRUM OF PP-PC-03($CDCl_3$, 400MHz)

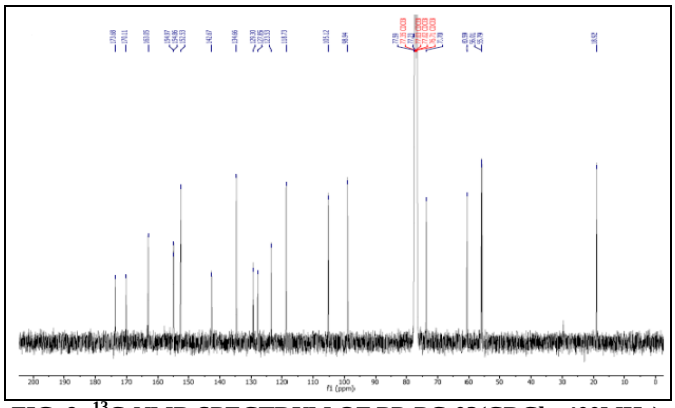


FIG. 9: ^{13}C-NMR SPECTRUM OF PP-PC-03($CDCl_3$, 400MHz)

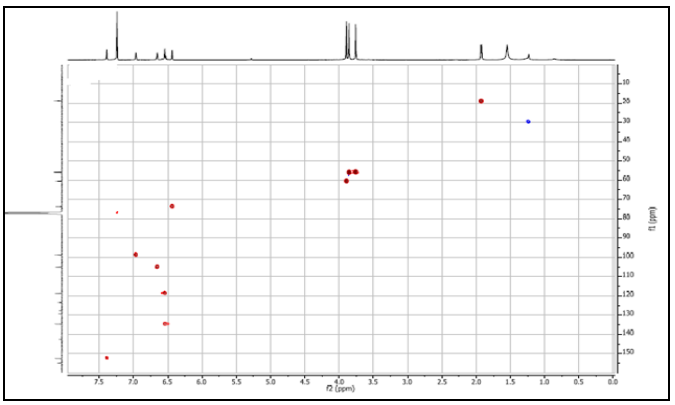


FIG. 10: HSQC SPECTRUM OF PP-PC-03($CDCl_3$, 400MHz)

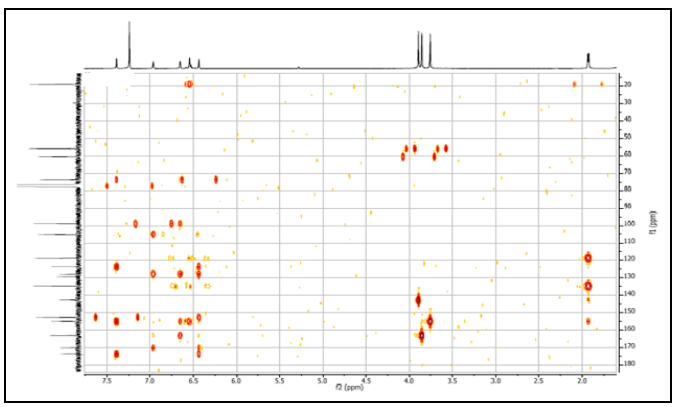


FIG. 11: HMBC SPECTRUM OF PP-PC-03(CDCl3, 400MHz)

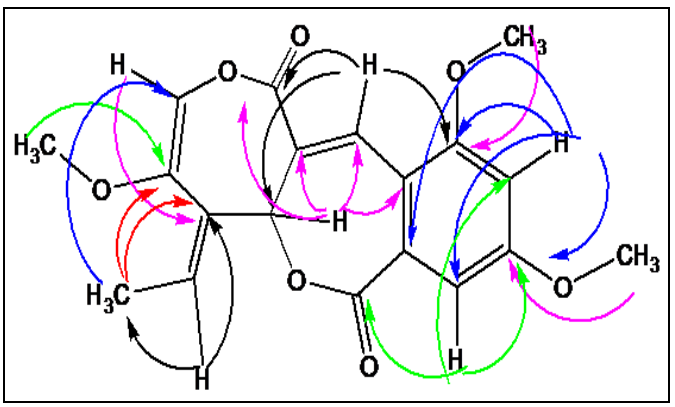


FIG. 12: SELECTED HMBC CORRELATIONS OF PP-PC-03(ARROWS POINTED FROM H TO C) ($CDCl_3$, 400 MHz)

DISCUSSION: Since, the EtOAc extract of *P. citrinum* showed antioxidant properties **Fig. 1**, it was partitioned with hexane, $CHCl_3$ and 80% aqueous MeOH to obtain three fractions and the antioxidant assay revealed that the $CHCl_3$ fraction has the highest activity out of three fractions **Fig. 2**.

The bioactivity of PP-PC-01 and PP-PC-02 have been established [3]. PP-PC-02 showed similar activity to the positive control, BHT whereas the other two compounds, PP-PC-01, and PP-PC-03 showed moderate antioxidant activities with IC_{50} values 159.7 ± 22.3 µg/mL and 120.1 ± 11.7 µg/mL **Fig. 4**.

By a combination of FABMS and NMR spectroscopy, the molecular formula of PP-PC-03 was determined to be $C_{19}H_{18}O_7$ and indicated 11 degrees of unsaturation. UV absorption maxima at 274, 359, 372 and 375 nm **Fig. 5** were indicative of a conjugated chromophore and its IR spectrum **Fig. 6** with absorption bands at 1768, 1647, and 1502 cm^{-1}, PP-PC-03 suggested the presence of α, β-unsaturated lactone carbonyl and olefinic groups.

The ^{1}H NMR spectrum of PP-PC-03 **Fig. 7** analyzed with the help of COSY **Fig. 8** indicated the presence of two spin systems in addition to four 3H singlets due to three OCH_3 groups attached to C-2, C-4, and C-9 (δ_H3.86, 3.76 and 3.89) and a CH_3 (δ_H1.92). The spin system consisting of two 1H doublets was shown to be due to a set of meta-coupled one-proton doublets, δ_H 6.65 (d, J = 2 Hz) and δ_H 6.96 (d, J = 2 Hz). In addition to other signals two olefinic protons, δ_H 6.54 (s) and 6.55 (s) in the ^{1}H NMR of PP-PC-03 were observed and should be due to two protons attached to the dienyl group **Table 1**. The ^{13}C NMR spectrum of PP-PC-03 **Fig. 9** analyzed with the help of the HSQC spectra **Fig. 10** showed the presence of nineteen aromatic/olefinic/ester /methoxy/methyl carbons of which four were oxygenated (δ_C73.7, 142.7, 154.8 and 163.0), five protonated (δ_C98.9, 105.1, 118.7, 134.7 and 152.5) and four deoxygenated quaternary carbons (δ_C123.5, 127.8, 129.3 and 154.9), two esters or lactone carbonyl (δ_C 170.1 and 173.7) and three OCH_3 (δ_C55.8, 56.0 and 60.6) and a methyl (δ_C18.9) carbon.

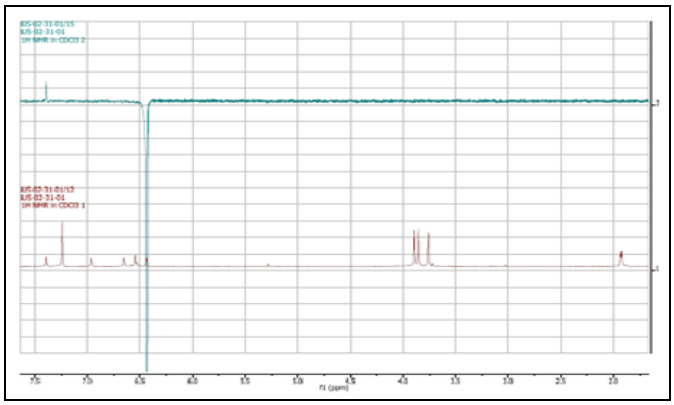


FIG. 13: NOEDIFF SPECTRUM OF PP-PC-03 WHEN IRRADIATE H-8 (δ-6.54)

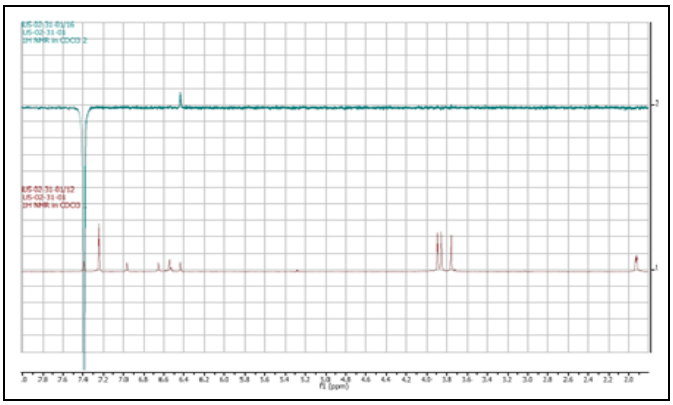


FIG. 14: NOEDIFF SPECTRUM OF PP-PC-03 WHEN IRRADIATE H-4 (δ-7.39)

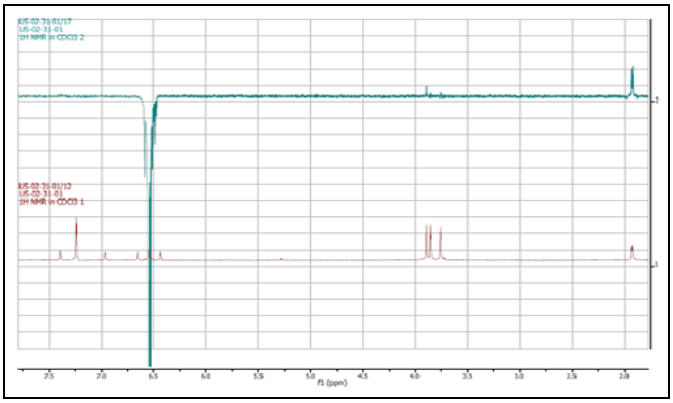


FIG. 15: NOEDIFF SPECTRUM OF PP-PC-03 WHEN IRRADIATE H-12 (δ-6.55)

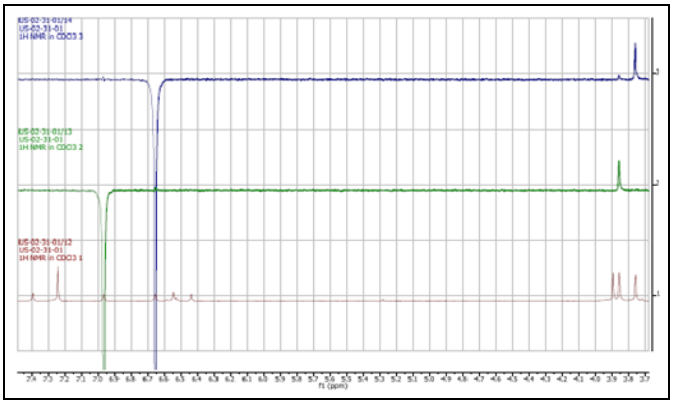


FIG. 16: NOEDIFF SPECTRUM OF PP-PC-03 WHEN IRRADIATE H-1 (δ-6.69) AND H- 6.65 SEPARATELY ($CDCl_3$, 400MHz)

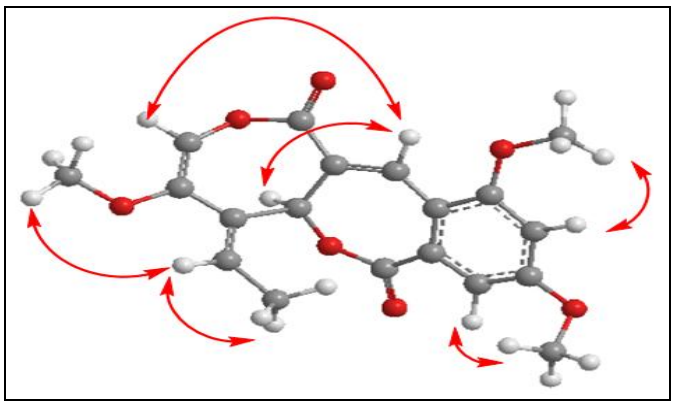
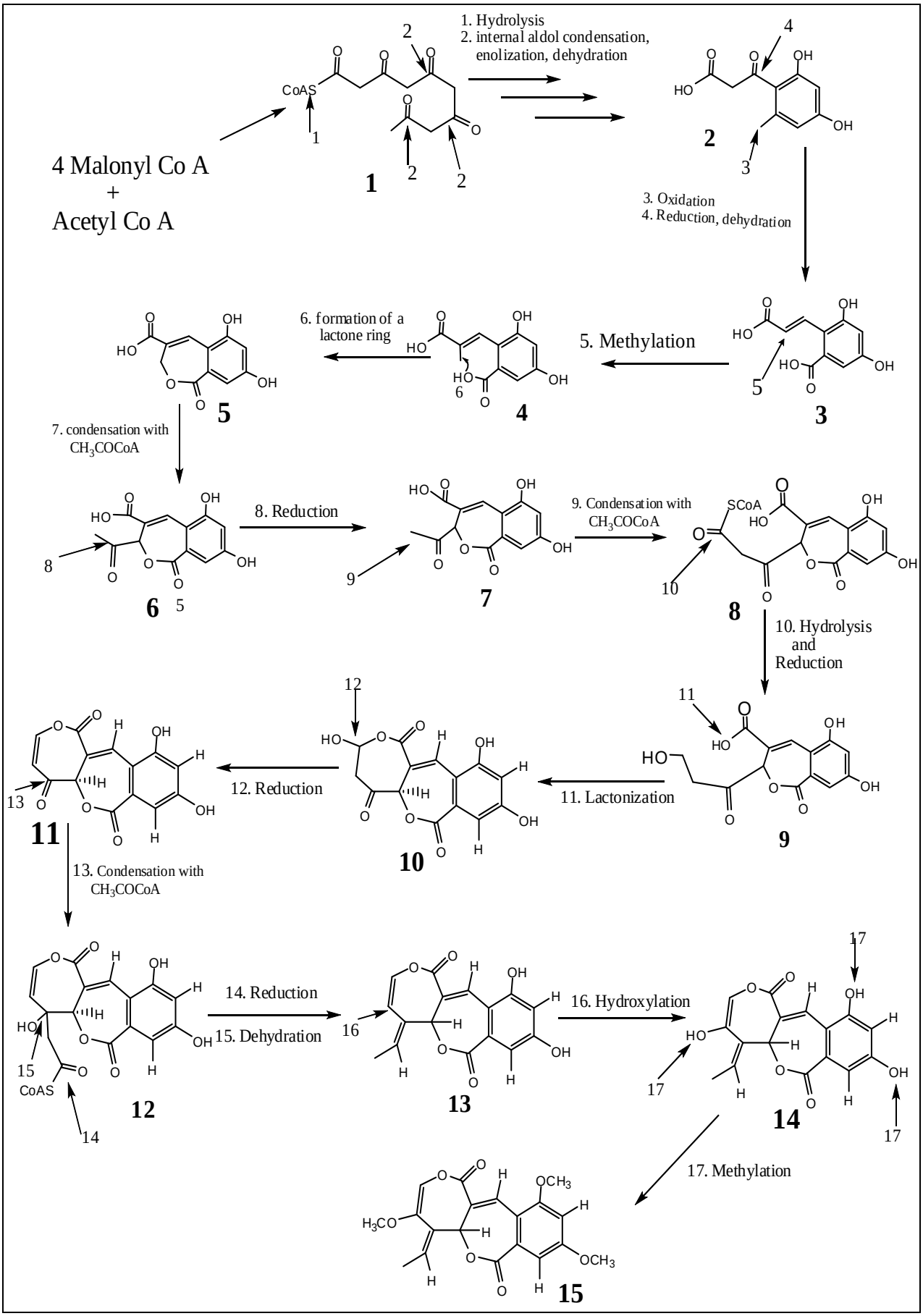


FIG. 17: SELECTED NOE OBSERVED IN THE NOEDIFF EXPERIMENT OF PP-PC-03($CDCl_3$, 400MHz)



SCHEME 1: POSTULATED BIOSYNTHETIC PATHWAY OF PP-PC-03

The cross peaks between δ_H 1.92 with δ_C 118.7 (C-16), δ_C 142.7 (C-9) and δ_C 154.9 (C-10); δ_H 6.44 with δ_C 123.5 (C-5a), δ_C 127.8 (C-4a), δ_C 152.5 (C-5) and δ_C 173.5 (C-6); δ_H 6.54 with δ_C 154.9 (C-10); δ_H 6.55 with δ_C 18.9 (C-17) and δ_C 154.9 (C-10); δ_H 6.96 (H-1) with δ_C 105.1 (C-3), δ_C 127.8(C-4a), δ_C 163 (C-2) and δ_C 170.1 (C-12); δ_H 6.65 (H-3) with δ_C 98.9 (C-1), δ_C 129.3 (C-12a), δ_C 154.8 (C-4) and δ_C 163 (C-2), ; δ_H 7.39 (H-5) with δ_C 173.7 (C-6), δ_C 154.8 (C-4),123.5 (C-5a) and δ_C 73.7 (C-10a) in the HMBC spectrum **Fig. 11** established the connectivity of the two lactone rings present in the proposed structure. In addition to the above low-field signals, the ^{1}H NMR spectrum of PP-PC-03 indicated the presence of a tertiary methyl group at δ_H 1.92 (H-17, d, J=6.1 Hz), two methine protons at δ_H 6.55 (q, J=6.1 Hz) and δ 6.54(s) attached to olefinic carbon, δ_C 118.7 and134.7 respectively.

The ^{13}C NMR spectrum of PP-PC-03 when analyzed with the help of the HSQC spectra, HMBC and COSY spectra PP-PC-03showed that proton at δ_H 1.92 (H-17) is coupled with the proton, δ_H 6.55 (H-16). In the HMBC spectrum, the protons at δ_H 1.92 show correlations with δ_C 118.7 (C-16), 154.9 (C-10), 134.7 (C-8) and 142.7 (C-9) and with the help of HSQC data, two of the three OCH_3 groups were placed at C-2 and C-4 as δ_H 3.86 displayed the ^3J HMBC correlation with C-2 (δ_C 163) and δ_H 3.76 displayed the ^3J HMBC correlation with C-3' (δ_C 163.0). The third OCH_3 group was placed at C-9 as δ_H 3.89 displayed the ^3J HMBC correlation with C-9 (δ_C 142.7). The relative stereochemistry of PP-PC-03 was determined with the help of ^{1}H NMR, COSY and the distinct NOEDIFF enhancement of H-1/H-13, H-3/H-14, H-5/H-10a and H-8, H-16/H-15 and H-17 observed in the NOEDIFF spectra **Fig. 17**. The Irradiation of δ_H 6.54 (H-8) proton led to significant enhancement of H-5 (δ_H 7.39) **Fig. 13**, δ_H 7.39 (H-5) caused enhancement of H-10a (δ_H 6.44) **Fig. 14**, δ_H 6.55 (H-16) caused enhancement of H-17 (δ_H 1.92) and H-15 (δ_H 3.89) **Fig. 15** suggesting that H-8 and H-5; H-5 and H-10a; H-16 and H-17; H16 and H-15 were on the same side of the molecule.

Irradiation of δ_H 6.65 (H-3) and δ_H 6.96 (H-1) during the NOEDIFF experiment caused enhancement of H-14 (δ_H 3.76) and H-13 (δ_H 3.86) respectively suggesting that these two methoxy groups and aromatic protons are near each other **Fig. 16**.

In order to fulfill all the requirement, the unsaturation number of 11 for PP-PC-03 two lactone rings have been proposed at C-4a and C-5a with a side chain, CH_3-CH=C at C-10 have been proposed. By the above data, the structure of PP-PC-03 was thus elucidated as 10-Ethylidene-2,4,9-trimethoxy-10,10a-dihydro-7,11-dioxa-benzo [b]heptalene-6,12-dione **Fig. 3**.

Postulated Biosynthetic Pathways of PP-PC-03: The compound, PP-PC-03 likely to have the same biogenetic origin as the two previously identified polyketides, PP-PC-01 and PP-PC-02, *via* the polyketide pathway and is biosynthesized from acetyl coenzyme A [4]. A plausible biosynthetic pathway for this polyketide, PP-PC-03 is proposed in **Scheme 1**. Formation of the aromatic ring in 2 of **Scheme 1** is proposed due to condensation of tetraketide in 1 of **Scheme 1**. Oxidation of methyl group in 2 produced a carboxylate group in 3, which undergoes methylation and lactonization to yield 4 and 5 respectively. Formation of the second lactone ring is postulated from condensation with acetyl CoA to form 6 and reduction and condensation with a second acetyl CoA molecule to yield 8, which undergoes hydrolysis followed by reduction and lactonization to form 9 and 10. Subsequently, 10 undergoes reduction to form 11, and it condenses with an acetyl CoA to produce 12. After undergoing reduction, dehydration, and hydroxylation 13 changes to 14 which is methylated to yield 15 (PP-PC-03).

CONCLUSION: The structure of PP-PC-03 was determined as 10-Ethylidene-2,4,9-trimethoxy-10, 10a- dihydro- 7, 11-dioxa-benzo[b]heptalene-6, 12-dione and identified as a new compound. PP-PC-03 showed a moderate activity comparative to PP-PC-01 where as PP-PC-03 has similar activity to PP-PC-02.

ACKNOWLEDGEMENT: This work was supported by the National Research Council, Sri Lanka Grant: NRC-08-02.

CONFLICT OF INTEREST: No conflicting interest.

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How to cite this article:
Wickramarachchi SR, Samanthi U, Wijeratne K and Paranagama PA: A new antioxidant active compound from the endolichenic fungus, *Penicillium citrinum* inhabiting the lichen, Parmotrema sp. Int J Pharm Sci & Res 2019; 10(7): 3412-20. doi: 10.13040/IJPSR.0975-8232. 10(7).3412-20.