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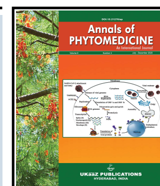

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Hepatoprotective activity and constituents of *Nigrospora* sp. CMH2_13: An endophytic fungus isolated from leaves of *Phyllanthus amarus* Schum. and Thonn.

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Abstract

Endophytes are microorganisms that dwell within plant tissues by having a symbiotic association, one of the sources of secondary metabolites in plants. In this study, an attempt was made to isolate, characterize endophytic fungi, from leaves of *Phyllanthus amarus* Schum. and Thonn., and to screen the fungal fractions for hepatoprotective activity, followed by isolation of secondary metabolites from the endophytic fraction. An endophytic fungus, PALF-2 was identified as *Nigrospora* sp. CMH2 by molecular characterization. PALF-2 was fermented to get ethyl acetate (P2EA) and n butanol (P2nB) fractions. P2EA and P2nB were evaluated for hepatoprotective activity against paracetamol-induced hepatotoxicity. Based on the activity, P2EA was subjected to column chromatography and analyzed by spectroscopic methods. P2EA and P2nB at doses of 50 and 100 mg/kg reversed the elevated biochemical parameters as compared to CCl_4 treated groups. P2EA and P2nB restored endogenous enzyme levels. Chromatographic studies of P2EA led to the isolation of hypophyllanthin, phyllanthin, quercetin-3, 4-di-O-glucoside, and kaempferol-3-O-rutinoside, the structures were confirmed by spectroscopic analysis. LC-MS studies of the oil confirmed the presence of seven constituents. This is the first report on the isolation of these constituents from the endophytic fungus of *P. amarus*. Thus, it can be concluded that, *Nigrospora* sp. CMH2_13 could be a good commercial source for the production of hepatoprotective and antioxidant constituents.

1. Introduction

Microbial community has the capability to colonize plants which assist them in healthy growth of the plant. Depending on the plant environment, plants get colonized by pathogens and nonpathogenic endophytes, as they occupy part of their life cycle inner surface of the plants (Brader *et al.*, 2017). Medicinal plants are known to nurture potent endophytic micro-organisms, due to the presence of secondary metabolites. Thus, they constitute a fascinating source of natural metabolites. Reports exhibited that several medicinal plants get colonized by endophytes. These are the micro-organisms that reside in tissues of living plants and act to improve their growth (Pedra *et al.*, 2018). Bioactive compounds obtained from the endophytes are valuable in biotechnological applications, inoculation of either these microbes or constituents helps in improving plant health. Endophytes have an important role in abiotic stress management and can withstand during drought conditions (Barkodia *et al.*, 2018).

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Phyllanthus is the important group of plants in India. *Phyllanthus amarus* Schum. and Thonn. is being distributed as a weed in waste and cultivated lands (Meena *et al.*, 2018). *P. amarus*, historically has been used by people, because of its rich medicinal values. *P. amarus* (family Euphorbiaceae) is a plant traditionally used in the treatment of viral hepatitis in India (Kiemer *et al.,* 2003). *P. amarus* contains hypophyllanthin, phyllanthin, niranthin, kaempferol, niranthin, nirtetralin, isobubbialine, epibubbialine, securinine, nor-securinine, dihydrosecurinine, geraniin, corilagin, 1,6-digalloylglucopyranoside rutin, quercetin 3-O glucopyranoside, amarulone, phyltetralin, demethylenedioxy-astragalin, *etc.*, chemical constituents in its different parts (Yoganarasimhan, 1996; Gupta and Vaghela, 2019). *P. amarus* are rich in secondary metabolites and are the key factors for various pharmacological activities such as antidiabetic, antitumor, immunomodulation, antifungal, antibacterial and anti-inflammation activities and antioxidant effects (Meena *et al.*, 2018).

Reports revealed the presence of triterpenoids including cycloeucalenyl acetate, ergosta-5,7,22-trien-3-ol acetate, macdougallin, 17-(1,5-Dimethylhexyl)-6- hydroxy-5-methylestr-9-en-3-yl acetate, steroids such as stigmasterol and β-sitosterol in the hexane fraction, 6,7-epoxypregn-4-ene-9,11,18-triol-3,20-dione,11,18-diacetate, bufalin, olean-13(18)-ene, methyl ursolate, barringenol R1 and 7,8-epoxylanostan-11-ol,3-acetoxy in the

methanol fraction (Zubair *et al.*, 2017). One endophytic fungus, namely; *Acinetobacter* sp., and one endophytic bacteria namely; *Bacillus* sp. were isolated from leaves of *P. amarus* and identified by 16s rRNA sequencing (Joe *et al.*, 2016). Thirteen fungal species of endophytic fungi were isolated and reported from stems and roots of *P. amarus* (Kandasamy *et al.*, 2017). The other endophytic fungi isolated from *P. amarus* showed antibacterial activity against gram (+) and gram (–) bacteria (Sowparthani and Kathiravan, 2011). Literature survey revealed, no work has been reported associated with *in vitro* antioxidant, *in vivo* hepatoprotective activity and isolation of secondary metabolites from endophytic fungi of *P. amarus* leaves. So, a venture was made for the study.

2. Materials and Methods

2.1 Materials

All the chemicals and reagents (analytical grade) were purchased from Sigma Aldrich (Dorset, UK) and Hi Media for extraction and chromatographic studies. Melting points of all the isolated compounds were determined by Shital-digital programmable melting point apparatus. IR spectra were recorded using Bruker FT-IR. Proton and ^{13}C NMR spectra for isolated compounds were obtained using a Bruker Avance 500 NMR spectrophotometer. Chemical shifts are reported in δ (ppm). A mass spectrum was recorded using XEVO G2XS QTOF mass spectrophotometer by studying m/z.

2.2 Isolation of endophytic fungi

Healthy stems and leaves of *Phyllanthus amarus* Schum. and Thonn. were collected from the surroundings of Dharwad, Karnataka, India. The plant was identified and authenticated by a Taxonomist, Department of Botany, Government Science College, Dharwad, Karnataka, India and a voucher specimen (SETCPD/Ph.cog/herb/86/12/2017) was deposited at the herbarium of the Postgraduate Department of Pharmacognosy and Phytochemistry, SET's College of Pharmacy, Dharwad, India. Briefly, plant materials were washed thoroughly in sterile water to remove adhered foreign material, surface disinfected by soaking in 70% ethanol for 3 min and 4% sodium hypochlorite (NaOCl) for 1 min, rinsed in sterile demineralized water and stored aseptically. The washings were tested to ascertain proper surface sterilization. Small pieces of inner tissues (1 cm x 1 cm) were permeated on potato dextrose agar (PDA), supplemented with antibiotic streptomycin (125 µg/ml) in petri plates and incubated at 27°C ± 2°C until fungal growth was initiated. The purity of fungal colonies was checked randomly and grouped according to colony morphology. Purified strains were stored on PDA slants at 4°C until further use (Sowparthani and Kathiravan, 2011; Katoch *et al.*, 2014; Kandavel and Sekar, 2015; Puri *et al.*, 2018).

2.3 Molecular characterization of endophytic fungi by PCR sequential analysis

Isolated fungal endophytes were identified using microscopic and molecular techniques. Using the genomic DNA fractionation kit (Bhat Biotech Ltd. Bangalore, India), genomic DNA was isolated from the given organism (Shukla *et al.*, 2012). Phylogenetic analysis of endophytes was performed by the acquisition of ITS1-5.8S-ITS4 ribosomal gene sequencing. Internal transcribed spacer (ITS) region of the fungi was amplified using the forward primer ITS1 5'-TCCGTAGGTGAACCTGCGG-3' and reverse primer ITS4 5'-TCCTCCGCTTATTGATATGC-3' using polymerase chain reaction (PCR) as per the previously described method.

2.4 Fermentation and preparation of endophytic fractions

The purified fungal isolate was inoculated and fermented separately into a 3L Erlenmeyer flask containing 600 ml of potato dextrose broth (PDB), incubated at 25°C - 27°C for 21 days. After incubation, 500 ml of chloroform was added to flask and left overnight. Further, homogenized at 4000 rpm for 30 min to separate the mycelia from broth and filtered by Whatman filter paper under vacuum. Aqueous phase obtained after chloroform fraction was further partitioned three times with equal volumes of ethyl acetate, followed by two times partition with equal volumes of n-butanol. All the fractions were dried using rotary flash evaporator (SuperfitRotavap, PBU-6) and weighed (Kandavel and Sekar, 2015; Puri *et al.*, 2018).

2.5 Hepatoprotective activity of endophytic fractions

Ethyl acetate (P2EA) and n-butanol fractions (P2nB) were screened for *in vivo* liver protective activity in CCl_4 induced hepatotoxicity.

2.6 Animals

Albino Wister rats weighing 180-200 gm were used and procured from Aditya biosys Pvt. Ltd. Tumukuru, Karnataka, India and were housed in polypropylene cages containing sterile paddy husk as bedding under controlled conditions of temperature 23 ± 2°C, controlled humidity of 60-70% and 12 h light and dark cycles in a registered animal house (Reg No.112/1999/CPCSEA, dated 19-05-1999). They were acclimatized for 7 days before the study. They had free access to standard nutritionally balanced pellets diet (VRK nutritional solutions, Sangli, India) and water *ad libitum*. Ethical clearance for animal studies was obtained from the Institutional Animal Ethical Committee and standard operating procedures and protocols were followed as per CPCSEA.

2.7 Acute toxicity studies

Swiss albino mice were used to determine the acute toxicity of P2EA and P2nB. The animals were fasted for 12 h prior to the experiment were administered with a single dose (2000 mg/kg) of fractions dissolved in 5% gum acacia and observed for mortality up to 48 h (short term toxicity). Based on the short-term toxicity, the dose of the next animal was determined as per OECD guideline 420.

2.8 Assessment of hepatoprotective activity

Animals were divided randomly into six groups (n= 6 animals per group)

Group I: (Normal control): Rats received distilled water (2 ml/kg, body weight, p.o) for the whole period of the experiment (5 days).

Group II: (Negative control): Rats administered with CCl_4 in olive oil v/v intraperitoneally (2 ml/kg body weight) on 2nd and 4th day.

Group III: (Positive control): Rats administered with CCl_4 in olive oil v/v intraperitoneally and treatment of Silymarin (100 mg/kg p.o.)

Group IV: (P2EA treated): Rats administered with CCl_4 in olive oil v/v intraperitoneally and treatment of P2EA (50 mg/kg p.o.)

Group V: (P2EA treated): Rats administered with CCl_4 in olive oil v/v intraperitoneally and treatment of P2EA (100 mg/kg, p.o.)

Group VI: (P2nB treated): Rats administered with CCl_4 in olive oil v/v intraperitoneally and treatment of P2nB (50 mg/kg, p.o.)

Group VII: (P2nB treated): Rats administered with CCl_4 in olive oil v/v intraperitoneal and treatment of P2nB (100 mg/kg, p.o.)

Treatment with respective drugs was continued for a period of five days as reported earlier (Shukla *et al*., 2012; Puri *et al*., 2018).

2.9 Serum biochemical analysis

The tail-snip method was used to collect the blood from all the experimental rats for biochemical estimations such as serum glutamic pyruvic transaminase (SGPT), Serum glutamic oxaloacetic transaminase (SGOT), Serum alkaline phosphatase (SALP), total bilirubin, direct bilirubin, triglycerides and total protein. Parameters in blood serum were estimated using semi-autoanalyser (Swemed Biomedicals Pvt. Ltd. ARTOS, India), following the manufacturer's protocol of standard kits (Swemed diagnostics, India).

2.10 Measurement of enzymatic and non-enzymatic antioxidant activity

Animals were euthanized and the whole liver was perfused *in situ* with ice cold saline, dissected out, blotted dry and immediately weighed. Liver homogenate (10%) was prepared separately with ice cold saline EDTA using Teflon-glass homogenizer (Yamato LSG LH-21, Japan) used for the estimation of proteins and lipid peroxidation. The liver homogenate was centrifuged at 10,000 rpm for 10 min and the pellet discarded. The supernatant was again centrifuged at 20,000 rpm for 1 h at 4°C. Both the liver supernatants obtained were used for the estimation of nonenzymatic antioxidant (Lipid peroxidation) (Banerjee *et al*., 2008) and enzymatic antioxidants (Superoxide oxide dismutase and Catalase), following established methods reported (Flohe, 1984; Claiborne, 1985).

2.11 Histopathological evaluation

Livers were removed after the animal's euthanization and a portion of each tissue was fixed at 10% buffered neutral formalin and embedded in paraffin. Using microtome, solid sections of 5 µm thickness were taken and stained with hematoxylin-eosin (H & E) (Galigher and Kozloff, 1971). Histopathological changes were studied using Olympus Magnus microscope camera taking the help of a veterinarian pathologist.

2.12 Statistical evaluation

The data were expressed as Mean ± standard error of means for each group. One analysis of variance (ANOVA), followed by Tukey's multiple comparison tests was used to calculate the statistical difference using Graph pad prism version 7.0 USA software. Values considered $p<0.05$ were considered statistically significant.

2.13 Isolation of secondary metabolites from ethyl acetate fraction of *Nigrospora* sp. CMH2_13

20 g of the ethyl acetate fraction (P2EA) was mixed with 20 g of silica gel (60-120) mesh, using ethyl acetate as the solvent. The drug adsorbed silica was then loaded on top of the glass column which was previously packed with 200 g of silica gel (120) mesh using petroleum ether by wet packing method. The column was eluted first with 100% petroleum ether followed by Petroleum ether: chloroform graded mixtures (95:5, 90:10, 80:20, 70:30, 60:40, 50:50) then with 100% chloroform followed by chloroform: ethyl acetate graded mixture (95:5, 90:10, 80:20, 70:30, 60:40, 50:50) then with 100% ethyl acetate, followed by ethyl acetate: MeOH graded mixture (95:5, 90:10, 80:20, 70:30, 60:40, 50:50) then with 100% MeOH. Each test tube contained 10 ml of elutes which were concentrated and monitored by TLC (Silica Gel GF 254, visualization under UV (254 and 366 nm). A total of 23 fractions were eluted out of which five fractions were collected in their pure form as a single spot.

Elutions were carried out with graded mixtures of petroleum ether: chloroform (50:50) resulted in a single spot as orange oil and monitored by gas chromatography, designated as PAE-1. Elutes obtained with chloroform: ethyl acetate (90:10) solvent was pooled together and concentrated to get a single spot as yellow crystals. A single spot was confirmed by TLC studies with mobile phase toluene: ethyl acetate (5:5) and detected in UV at 254 nm, designated as PAE-2. Elutes obtained with chloroform: ethyl acetate (80:20) solvent resulted in a yellow crystallized compound which was rechromatographed and designated as PAE-3. Elutes obtained with ethyl acetate (100%) solvent were pooled together and concentrated to get a single spot as a brown powder. A single spot was confirmed by TLC studies with mobile phase toluene: Ethyl acetate (3:7), detected in UV at 254 nm, designated as PAE-4. Elutes obtained with ethyl acetate: MeOH (95:5) solvent was pooled together and concentrated to get a single spot as a brown powder. A single spot was confirmed by TLC studies with mobile phase toluene: ethyl acetate (3:7) and detected in UV at 254 nm, designated as PAE-5.

2.14 Rechromatography of PAE-3

1 gm of the fraction was adsorbed with 1 gm of Silica gel for column chromatography (240-400 mesh). 20 g of Silica gel for column chromatography (240-400 mesh) with petroleum ether. Then it was eluted with the mobile phase to collect fractions and was concentrated. Each fraction was evaluated by TLC. Fractions showing the same number of spots and R_f values were pooled, concentrated and evaporated to dryness. The elutions were carried out with flow rate of 10 drops per min with 100% petroleum ether followed by petroleum ether: Chloroform graded mixtures (50:50), then with 100% chloroform followed by chloroform: ethyl acetate graded mixture (99:1, 98:2, 96:4, 94:6, 92:8, 90:10) then finally by 100% ethyl acetate. All the elutions were monitored by TLC (Silica gel GF 254; visualization by UV 254 nm, 366 nm). Elutes obtained with 99:1, 98:2, 96:4, 94:6, 92:8 of chloroform: ethyl acetate resulted in a single prominent spot on TLC. A single spot was confirmed with mobile phase toluene: ethyl acetate (45:55) and detected in UV at 254 nm designated as PAE-3.

3. Results

3.1 Identification of endophytic fungus

Microscopic and molecular identification of the fungus confirmed that it belongs to the genus *Nigrospora*. The homology analysis based on phylogenetic tree revealed the best matching with *Nigrospora* sp. CMH2_13 (KF 227831.1).

3.2 PCR sequential analysis of PALF-2

The sequence of the ITS gene from PALF-2 and that of matching sequences from 10 nucleotide sequences were aligned by using the

maximum likelihood method based on the Hasegawa Kishino Yano model. The tree shows the highest log likelihood (-601.8862). The sequence of the ITS gene for PALF-2 was compared with the existing sequence in the NCBI database using BLAST N programme. Based on these results, PALF-2 was identified as *Nigrospora* sp. CMH2_13 (Figure 1).

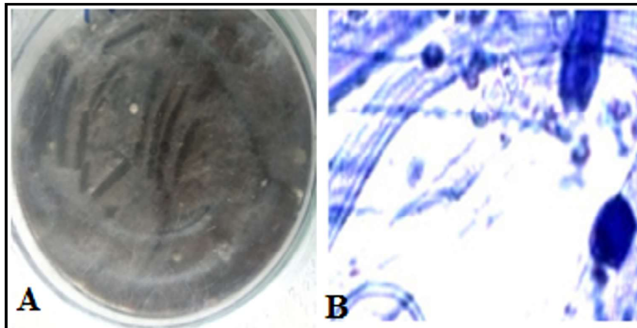

Figure 1: Colony morphology (A) and microphotograph of lactophenol cotton blue staining of colonies of *Nigrosporasp*.CMH2_13 observed on agar slant (B).

3.3 Hepatoprotective activity of P2EA and P2nB

3.3.1 Acute toxicity (LD_{50}) studies

Acute toxicity studies were performed following OECD guidelines (up and down method). No mortality was observed at 2000 mg/kg body weight after P2EA and P2nB administration. To assess the hepatoprotective activity of P2EA and P2nB, doses of 50 mg/kg and 100 mg/kg body weight were selected, respectively.

3.3.2 Effect of P2EA and P2nB on serum biochemical parameters

Wister rats showed a significant elevation in the levels of serum ALT, AST, ALP, total and direct bilirubin, TG and total protein, following intoxication with CCl_4, (2 ml/kg i.p.) as compared to normal control. A CCl_4 stimulated an increase in the AST levels of rats was significantly reduced following treatment of animals with P2EA and P2nB (50 mg/kg and 100 mg/kg) and reference drug silymarin (200 mg/kg p.o.) ($^{***}p$<0.001). Serum ALT, ALP, total and direct bilirubin, TG and total protein levels also showed a significant reduction, following treatment with different concentrations of P2EA and P2nB (Table 1).

3.3.3 Effect of P2EA and P2nB on endogenous antioxidant enzymes

Rats treated with CCl_4 showed decreased SOD and CAT levels and a marked increase in LPO level in terms of TBARS as compared to control. P2EA and P2nB (100 mg/kg) decreased the elevated LPO level significantly ($^{***}p$<0.001). P2EA (50 and 100 mg/kg) and PA2nB (100 mg/kg) significantly increased the SOD and CAT levels ($^{***}p$<0.001). The results are shown in Figure 2.

3.4 Histopathological studies

Histopathology of the liver treated with CCl_4 showed significant induction of hyperplasia of the bile duct, interlobular fibrosis, vacuolation of hepatocytes and infiltration of lymphocytes. Treatment with P2EA and P2nB (50 and 100 mg/kg) showed significant improvements in CCl_4 induced injuries by reducing inflammatory responses, bile duct hyperplasia and interlobular necrosis followed by regeneration of hepatocytes (Figure 3).

3.5 Secondary metabolites from *Nigrospora* sp. CMH2_13

3.5.1 LC-MS analysis of oil (PAE-1)

LC-MS analysis of PAE-1 (orange oil) showed the presence of 7 components namely 1-dococene (a), hexadecanoic ethyl ester (b), ethyl oleate (c), oleic acid (d), *cis* vacceric acid (e), 1,2, benzene dicarboxylic acid dis octyl ester (f), oxalic acid allyl hexadecyl ester (g) (Figure 4). Structural confirmation of these components was done by comparing the data with the literature library.

3.5.2 Quercetin-3, 4'-di-o-glucoside (PAE-2)

Quercetin-3,4'-di-O-glucoside was obtained as yellow crystals, MP:228°C–230°C: R_f : 0.54 ($CHCl_3$:EA, 90:10); IR (KBR) cm^{-1}; 3390.86 (OH), 2939.52 (CH str. of CH_3), 1406.18 (CH deformation in CH_3), 1211.30 (C-O-C);^{1}H NMR (DMSO D_6): (500 MHZ, δ ppm) : 12.91 (s, 2H, C_{31}, C_{32}-H), 10.16 (s, 2H, C_{33}, C_{34}-H), 7.08 (s,1H,C_{30}-H), 6.98 (s, 1H, C_{12}-H), 6.56-6.51 (m, 3H, C_{15}, C_{16}, C_{23}-H), 6.21-6.15 (m, 2H, C_7, C_9-H), 3.33 (s, 4H, C_{35}, C_{36}, C_{37}, C_{39}-H), 3.59 (s, 4H, C_{40}, C_{41}, C_{42}, C_{43}, C_{44}-H), 3.41 (s, 5H, C_4, C_{38}, C_{43}-H), 2.51 (s, 6H, C_{19}, C_{20}, C_{21}, C_{26}, C_{27}, C_{28}-H), 2.14 (s, 6H, C_2, C_3, C_{18}, C_{22}, C_{25}, C_{29}-H).^{13}C NMR ($DMSOD_6$): (100 MHZ, δ ppm): 168.97, 162.56, 161.33, 157.39, 150.78, 140.57, 116.73, 110.52, 108.61, 108.11, 107.26, 107.08, 105.74, 105.68, 104.94, 103.84, 97.38, 96.92, 77.80, 74.05, 73.16, 71.68, 61.49, 56.60, 56.56, 20.64, 20.38. Mass spectra: ESIMS: found: 625.15 (M^+-1), calculated: 626.52 (M^+).

Table 1: Effect of P2EA and P2nB on serum biochemical parameters in CCl_4 induced hepatotoxicity in rats

Groups	Dose	SGPT (mg/dl)	SGOT (mg/dl)	SALP (mg/dl)	Total bilirubin (mg/dl)	Direct bilirubin (mg/dl)	Triglycerides (mg/dl)	Total protein (mg/dl)
Control	(2 ml/kg)	83.07 ± 0.39	148.1 ± 0.43	242.5 ± 0.37	0.35 ± 0.01	0.16 ± 0.01	58.06 ± 0.46	5.37 ± 0.07
CCl_4 treated	(2 ml/kg)	216.7 ± 0.59***	271.1 ± 0.87	323.8 ± 0.53	1.03 ± 0.01	0.62 ± 0.07	105.01 ± 1.31	9.47 ± 0.08
Silymarin	(25 mg/kg)	125.3 ± 0.26***	186.1 ± 0.51***	276.2 ± 0.77***	0.73 ± 0.01***	0.43 ± 0.02***	73.76 ± 0.47***	4.48 ± 0.07***
P2EA	(50 mg/kg)	211.5 ± 1.43***	225.6 ± 0.57***	304.9 ± 0.90***	0.89 ± 0.06***	0.43 ± 0.01***	98.13 ± 0.93*	8.35 ± 0.06***
P2EA	(100 mg/kg)	187.5 ± 0.47***	216.5 ± 0.61***	286.4 ± 0.81***	0.94 ± 0.04**	0.35 ± 0.01***	96.91 ± 1.46**	8.207 ± 0.02***
P2 nB	(50 mg/kg)	203.5 ± 1.5***	266.6 ± 0.75*	313.9 ± 0.71*	0.82 ± 0.07***	0.47 ± 0.05**	80.02 ± 0.55***	8.768 ± 0.13*
P2 nB	(100 mg/kg)	179.0 ± 1.05***	256.3 ± 0.69***	283.5 ± 0.70***	0.77 ± 0.01***	0.33 ± 0.01***	72.39 ± 0.63***	8.227 ± 0.02***

Each value represents Mean ± S.E.M (n=6) *p<0.05, **p<0.01, ***p<0.001 compared to CCl_4 treated group. One-way ANOVA followed by Tukey's multiple comparison tests.

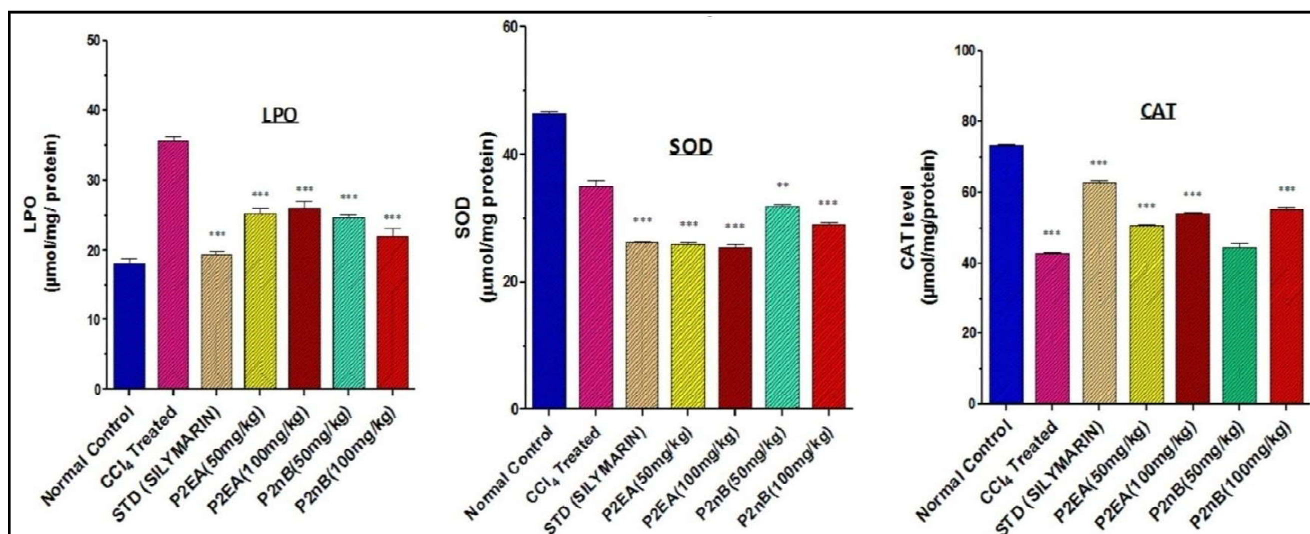

Figure 2: Effect of P2EA and P2nB on endogenous antioxidant levels in CCl4 treated hepatotoxic rats

Each value represents Mean ± SEM (n=6) **p*<0.05, ***p*<0.01, ****p*<0.001 compared to CCl_4 treated group. One-way ANOVA followed by *Tukey's* multiple comparison tests.

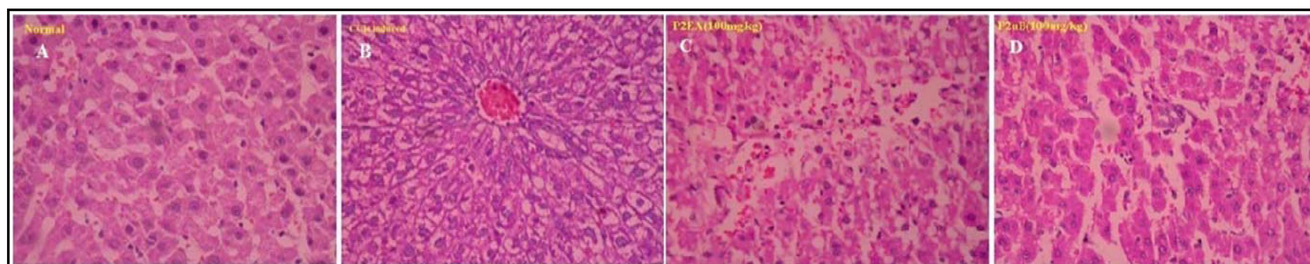

Figure 3: Microscopic evaluation of P2EA and P2nB on CCl4 induced acute hepatotoxicity (200 X stained with Haematoxylin and Oesin)

(A) Normal rats (B) CCl_4 treated rats showing massive fatty accumulation, centrilobular necrosis, ballooning degeneration, infiltrating lymphocytes and loss of cell boundaries. (C) P2EA (100 mg/kg + CCl_4 showing normal lobular pattern with mild fatty degeneration and reduction in necrosis (D) P2nB (100 mg/kg + CCl_4 showing normal lobular pattern with mild fatty degeneration and reduction in necrosis.

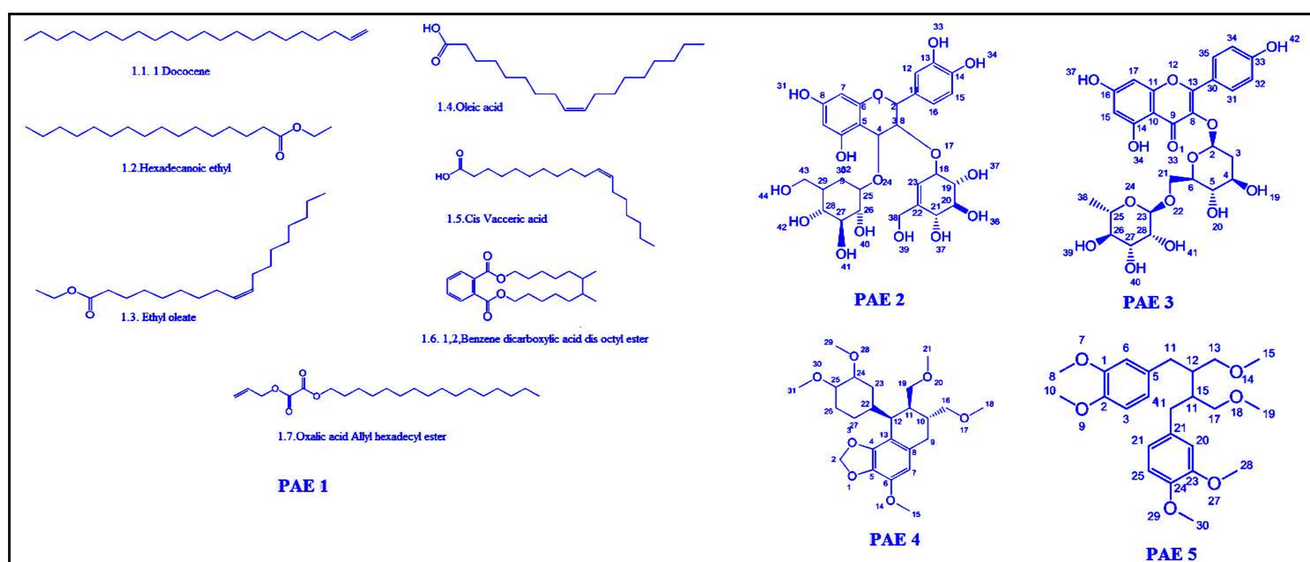

Figure 4: Secondary metabolites from ethyl acetate fraction of endophytic *Nigrospora* sp. CMH2_13

PAE 1- An orange oil containing seven constituents, PAE 2- quercetin-3,4'-di-O-glucoside; PAE 3- kempferol-3-O-rutinoside; PAE 4- hypophyllanthin; PAE 5- phyllanthin.

3.5.3 Kaempferol-3-O-rutinoside (PAE-3)

Kaempferol-3-O-rutinoside was obtained as yellow crystals, MP:205°C-210°C; R_f:0.86 ($CHCl_3$:EA, 80:20); IR (KBR) cm^{-1}: 3369.64 (br. OH), 2933.73 (CH str. of CH_3), 2865.36 (CH str. of CH_2), 1651.07 (C=C str. of Aromatic ring), 1606.10 (C=C str. of Aromatic ring), 404.18 (CH deformation in CH_3);^{1}H NMR (DMSO D_6): (500 MHZ, δ ppm); 12.91 (br, s, 1H, C_5-OH), 10.16 (br, s, 1H-C_7-OH), 6.98 (s, 2H, C_3, C_5-H), 6.51 (t, 1H, C-6), 6.21 (d, 1H C_8-H); 6.16 (d, 1H C_1-H), 3.31 (s, 1H C_2-H), 3.383 (s, 1H, C_3-H), 3.50 (s, 1H, C_4-H), 3.41 (s, 1H, C_5-H), 3.93 (d, 1H, C_{6a} -H), 3.59 (s, 1H C_{6b}-H); 3.94 (d, 1H C_1-H), 3.41 (s, 1H C_2-H), 3.93 (s, 1H C_3-H), 2.51 (m, 1H C_4-H), 3.93 (s, 1H C_5-H), 1.12 (s, 2H C_6-H); ^{13}C NMR (DMSOD_6): (100 MHZ, δ ppm); 184.46, 169.33, 162.49, 159.01, 152.93, 150.72, 140.70, 117.22, 110.22, 108.11, 105.73, 103.85, 100.88, 97.28, 96.97, 76.28, 74.46, 71.18, 70.10, 61.95, 61.50, 20.62; Mass spectra: ESI-MS: found: 595.14 (M^++1), calculated: 594.52 (M^+).

3.5.4 Hypophyllanthin (PAE-4)

Hypophyllanthin was obtained as brown powder, MP:128°C-130°C; Rf: 0.29 (EA, 100%); IR (KBR) cm^{-1}; 2918 (CH str. of CH_3), 2850.79 (CH str. of CH_2), 1656.85 (C=C str. of Aromatic ring), 1440.83 (CH deformation in CH_3); ^{1}H NMR (DMSO D_6): (500 MH_Z, δ ppm): 7.998 (d, 1H, CH, C-7), 7.536 (d, 1H, CH, C-23), 6.997 (d, 1H, CH, C-26), 6.53 (d, 1H, CH, C-27), 5.838-5.844 (d, 2H, OCH_2, C-16), 4.030-4.848 (m, 1H, CH, C-12), 3.816 (s, 3H, OCH_3, C-15), 3.802 (s, 3H, OCH_3, C-18), 3.673 (s, 3H, OCH_3, C-21), 3.417 (s, 3H, OCH_3, C-29), 3.406 (s, 3H, OCH_3, C-31), 3.313-3.505 (m, 2H, CH_2, C-9), 3.025-3.187 (m, 2H, CH_2, C-19), 2.501-2.515 (m, 2H, Ar-CH_2, C-7), 1.548 (s, 2H, CH_2, C-2),1.096-1.063 (m, 1H, CH, C-11),^{13}C NMR (DMSOD_6): (100 MH_Z); 40.48, 54.92, 71.61, 31.76, 73.90, 39.48, 130.55, 103.08, 136.50, 133.28, 167.40, 118.77, 136.47, 109.36, 147.92, 168.75, 119.02, 124.11, 58.67,57.4, 56.48, 54.92, 55.23, 98.78; Mass spectra: ESI-MS: 432.18 (M+2H).

3.5.5 Phyllanthin (PAE-5)

Phyllanthin was obtained as brown powder, MP: 96°C- 97°C; R_f:0.24 (EA:MeOH, 95:5); IR (KBR) cm^{-1}: 2914.44 (Ar-C-H), 1465.90 (C-O-C), 1276.88 (C-O-C); ^{1}H NMR (DMSO D_6): (500 MH_Z); 7.05-6.96 (m, 3H, C_{22}, C_{25}, C_{26}-H), 6.62-6.57 (m, 3H, C_3, C_6, C_4-H), 4.68-3.70 (m, 4H, C_{11}, C_{13}, C_{17}, C_{20}-H), 1.34-1.18 (m, 18H, C_8, C_{10}, C_{15}, C_{19}, C_{28}, C_{30}-H), 0.81-0.71 (m, 2H, C_{12}, C_{16}-H); ^{13}C NMR (DMSOD_6) (100 MH_Z); 148.78, 147.84, 133.28, 124.11, 118.77, 109.31, 72.96, 58.67, 56.48, 55.23, 39.51; Mass spectra; ESI-MS: Mass calculated as 418.53; Mass found: 417.6 (M^{-1}).

4. Discussion

Endophytes are any of the micro-organisms that populate within the plants without causing any immediate negative effects and found in all types of vascular plants (Hodkinson *et al*., 2019). The major role of endophytes is plant growth promotion, plant fitness nitrogen fixation, phosphate solubilization, and protection against pathogens and abiotic stress (Loiret *et al*., 2004; Taechowisan *et al*., 2012; Shahzad *et al*., 2018; Ikram *et al*., 2018). They are synergistic to their host and some of them are useful to the plant by producing chemically diversified secondary metabolites that prevent the host from being attacked by phytopathogens (Garyali *et al*., 2013). The biological/pharmacological activities of endophytic fractions and or their metabolites are also well documented (Kumar *et al*., 2013; Kusari *et al*., 2009; Eyberger *et al*., 2006). Ethyl acetate extract of endophytic fungus *Achaetomium* sp., depicted hepatoprotective activity in CCl_4 exposed HepG2 cells (Uma Anitha and Mythili, 2017). In continuation of our work in search of antioxidant and liver protective compounds from endophytic fungi, an endophytic fungus (PALF-2) was isolated from the leaves of *P. amarus* leaves. Molecular techniques followed in the current study identified the fungus as *Nigrospora* sp. CMH2_13. *Nigrospora sphaerica* is an airborne filamentous fungus in the phylum Ascomycota. It is found in soil, air, and plants as a leaf pathogen. It can occur as an endophyte where it produces antiviral and antifungal secondary metabolites (Webster, 1952; Zhang *et al*., 2009). Authors have also reported four antifungal compounds from *Nigrospora* sp. LLGLM003, an endophytic fungus from the roots of *Moringa oleifera* Lam (Zhao *et al*., 2012). Oxidative damage is an etiological factor in chronic human diseases. The liver is one of the major organs attacked by ROS by damaging hepatic cells. Hepatotoxicity of CCl_4 is well documented in both animals and humans and is used extensively for evaluating the liver protective activity of synthetic and natural products (Ingawale *et al*., 2014; Dong *et al*., 2016). In the CCl_4 treated group, the levels of ALT, AST, ALP, direct bilirubin, total bilirubin, triglycerides and protein were elevated as compared to normal.

Cytochrome P-450 in endoplasmic reticulum activates CCl_4 to trichloromethyl radicals (CCl_3*). The covalent binding of the CCl_3* radical initiates the inhibition of lipoprotein secretion causing steatosis, whereas reaction with oxygen, forms trichloromethyl radicals initiating lipid peroxidation (Meharie *et al*., 2020). It also generates structural changes in the endoplasmic reticulum, mitochondria and protein synthesis reduction causing liver disorders (Ahsan *et al*., 2009). Treatment with P2EA, P2nB (50 mg/kg and 100 mg/kg) and silymarin treated rats restored the elevated biochemical parameters when compared to CCl_4 treated group rats which give increased protection of the liver

Lipid peroxidation elevation was also observed in CCl_4 induced hepatotoxicity which is mainly attributed to increase malondialdehyde (MDA), a lipid peroxidation product in the liver of rats. Enhanced lipid peroxidation was observed in CCl_4 induced group by elevation in TBARS level leading to damaging of tissue and antioxidant defence mechanism failure and finally cell death. Treatment with P2EA and P2nB restored the increased lipid peroxidation levels caused by CCl_4 which might be due anti TBARS action (Parmar *et al*., 2010). Administration of P2EA and P2nB (50 and 100 mg/kg) showed a remarkable increase in the SOD and CAT levels (Maheswari *et al*., 2008). This may be due to the presence of antioxidant compounds in P2EA and P2nB. P2EA and P2nB (50 and 100 mg/kg) administration improved significantly protein expressions of the SOD and CAT enzymes in the liver. P2EA and P2nB (50 and 100 mg/kg) may have reduced the oxidative stress by

increasing the activity and protein expression of the antioxidant enzymes *in vivo.* Treatment with P2EA and P2nB recovered the injured liver to normal and normal hepatic cell arrangement was seen as compared to CCl_4. From these findings, it can be inferred that P2EA and P2nB significantly protected the liver from the toxic effects of CCl_4 in rats. Finally, the hepatoprotective effect of *Nigrospora* sp. CMH2_13 can be attributed to the presence of secondary metabolites in P2EA and P2nB. Presence of phenols, flavonoids and tannins were observed in endophytic fungal extracts. Earlier studies on flavonoids reported the isolation of 2, 4-dihydroxy-6-methoxy-3 5-dimethylchalcone from by an endophytic fungus *Ceriporia lacerate* isolated from *Cleistocalyx operculatus* (Wang *et al*., 2013). In our study, chromatographic and spectroscopic techniques led to the isolation of oil followed by flavonoids and lignans (Elsawy *et al*., 2019; Ezzat *et al*., 2020). LC-MS analysis of oil revealed the presence of seven known metabolites. Isolated compounds were identified as quercetin 3, 4'di-O-glucoside, kempferol-3-O-rutinoside, hypophyllanthin and phyllanthin. Hence, it is speculated that hepatoprotective activity of P2EA and P2nB may be due to the synergistic effect of these metabolites which act through inhibition of oxidative stress, pro-inflammatory response and metabolizing CYP enzymes in the liver.

5. Conclusion

The endophytic fungus, *Nigrospora* sp. has never been reported capable of producing phyllanthin, hypophyllanthin and antioxidant compounds. The present study is the first to isolate, characterize and identify phyllanthin and hypophyllanthin producing *Nigrospora* sp. from *Phyllanthus amarus* which may be valuable for basic research and industrial applications.

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Conflict of interest

The authors declare that there are no conflicts of interest in the course of conducting the research. All the authors had final decision regarding the manuscript and decision to submit the findings for publication.

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