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Evaluation of endophytic fungal fractions of *Andrographis paniculata* (Burm.f.) Wall. Nees leaves for *in vitro* free radical scavenging and hepatoprotective activity

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ABSTRACT

Fungal endophytes are the microbes residing in internal tissues of the plant forming symbiotic, mutualistic, communalistic and trophobiotic relationship. Endophytes from medicinal plants are considered as essential source of secondary metabolites accompanied by interesting biological/pharmacological activities. In this study, an effort was made to isolate, characterize endophytic fungi from leaves of *Andrographis paniculata* and to screen the fungal fractions for *in-vitro* antioxidant and hepatoprotective activity. Two fungal endophytes (APLF-1 and APLF-2) from *Andrographis paniculata* were isolated and fermented to get chloroform (A1C, A2C), ethyl acetate (A1EA, A2EA) and n butanol (A1nB, A2nB) extracts. All the endophytic fractions of APLF-1 and APLF-2 were assayed for free radical scavenging properties against 2, 2-diphenyl-1-picrylhydrazyl (DPPH), hydroxyl radical and reducing power. Fractions of APLF-2 showed good scavenging activity compared to APLF-1 extracts. Further, A2EA and A2nB were evaluated for hepatoprotective activity against CCl_4 induced hepatotoxicity. A2EA (50 mg/kg &100 mg/kg) and A2nB (50 mg/kg &100 mg/kg) reversed the elevated biochemical parameters with respect to CCl_4 treated group ($p<0.001$). The LPO, SOD and CAT levels were also restored by A2EA and A2nB (100 mg/kg p.o). APLF-1 and APLF-2 were studied for rDNA sequencing by PCR technique. The endophytic fungi, APLF-1 and APLF-2 were identified as *Diaporthe sp. A25* and *Preussia sp. PPV3.6* respectively based on their morphology and molecular characterization. The presence of polyvalent secondary metabolites in A2EA and A2nB were confirmed by HPTLC analysis.

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INTRODUCTION

Liver diseases are the most serious ailments leading to mortality throughout the world. The reasons for such diseases are modern food styles, excessive medications, exposure to pollutants, toxic chemicals, surplus intake of alcohol, various infections and autoimmune disorders. (Elberry AA *et al.,* 2010).

Lipid peroxidation and other oxidative parameters damages hepatocytes. No effective drug from synthetic origin is available till today for treating liver

disorders. However, medicinal plants of various traditional systems of medicine and secondary metabolites are found to be effective in regeneration of hepatocytes by preventing lipid peroxidation. But still mortality is recorded due to liver disorders worldwide, thus the availability of drugs become limited and have demand for developing novel potent drugs (Eidi A *et al.,* 2012).

Plant endophytes are group of microorganisms such as bacteria, fungiand actinomycetes which reside in the internal tissues or organs of the plants, showing no marked signs of disease in the medicinal plants (Gunatilaka AL *et al.,* 2006, Schulz B *et al.,* 2002). Endophytes have different types of associations such as symbiotic, mutualistic, trophobiotic and communalistic with the host plant. Endophytes are the chemical synthesizers, useful to their host by producing novel active compounds for applications in agriculture, medicine and in biotechnological industries (Sette LD *et al.,* 2006). Many authors reported different endophytes and their isolated secondary metabolites from various medicinal plants and marine sources which found to exert anticancer (Moore A., 2009, Ding G., 2006), antibacterial (Liu JY *et al.,* 2004, Wang FW *et al.,* 2008) antifungal (YH Ye *et al.,* 2005, Pongcharoen W *et al.,* 2006) antimalarial (Masahiko I *et al.,* 2007, Renee K *et al.,* 2008) and immunomodulatory activities (Zhang AH *et al.,* 2013).

Taxol, an anticancer metabolite was isolated from an endophyte *Taxomyces andreanae* associated with the bark of *Taxus brevifolia,* (Wani MC *et al.,* 1971), and was also produced from *Fusarium redolens,* isolated from Himalayan yew (Garyali *et al.,* 2013). Penicidones A-C were isolated from the cultured *Penicillium* species, an endophytic fungal strain inhabiting in the stem of *Quercus variabilis* (Ge HM *et al.,* 2008). Two metabolites, Cyclo (Pro-Thr) and cyclo (Pro-Tyr) were isolated from the endophytic fungus *Penicillium* species obtained from *Acrostichum aureurm* showing antibacterial activity (Cui HB., 2008). *Streptomyces* sp. LJK109 obtained from *Alpinia galanga* produced 3-methylcarbazole and 1-methoxy-3-methylcarbazole having antifungal activities (Thongchai *et al.,* 2012). KL-4 was isolated from endophytic fungi *Aspergillus* species of seeds of *Gloriosa superba* and showed antimicrobial and anticancer activities (Budhiraja *et al.,* 2013). Crude fractions of endophytic fungi, *Paecilomyces variotti* Bain. isolated from the leaves of *Ocimum sanctum* L. exhibited antioxidant and hepatoprotective activity in CCl_4 induced hepatic damage (Shukla ST *et al.,* 2012).

Andrographis paniculata (Burm.f.) Wall. (Acanthaceae), an annual herbaceous plant, used from centuries in Indian tradition and Chinese herbal medicine. The aerial parts of plant contain the active constituents such as diterpenoids, stigmasterols, flavanoids, andrographolides (Koteswara *et al.,* 2004). It possesses different pharmacological activities such as antimalarial (Dua VK *et al.,* 2004), immunostimulant (Kumar RA *et al.,* 2004), anticancer (Kumar RA *et al.,* 2004), cardiovascular (Khalijah A *et al.,* 2012), hepatoprotective (Soumendra D *et al.,* 2009) activity have all been reported. The researchers also have concluded that the plant is the remedy for treatment for infective hepatitis (Raval *et al.,* 2016). An endophytic bacterium was reported from *Andrographis paniculata* leaves and was screened for antimicrobial activity and antibiotic susceptibility (Arunachalam C *et al.,* 2010). Five fungal endophytes were isolated from various parts of the *Andrographis,* two isolates obtained from the roots were identified as *Aspergillus niger* and *Aspergillus flavus,* other two isolates were obtained from the leaves as *Alternaria* and yeast like fungal species. *Fusarium* sp.was isolated from the internal tissue of the stem of the plant (Gajalakshmi *et al.,* 2012). Also, an endophytic bacterium was also isolated from *Andrographis paniculata* as a plant growth promoter and regulators such as IAA and GA3. (Aziz SA *et al.,* 2013). An endophytic fungus was also isolated from *Andographis paniculata,* for investigation of fungal lipid production as biodiesel precursors (Elfita E *et al.,* 2015).

Data on fungal biodiversity and screening of fungal fractions isolated from *Andrographis paniculata* is limited in the literature and no work was found on hepatoprotective activity of *Andrographis paniculata* associated fungal fraction is reported. Hence, the present investigation aims to isolate, characterize endophytic fungi and to screen the fungal fractions for, free radical scavenging and hepatoprotective activity.

MATERIALS AND METHODS

Plant Material

The leaves of *Andrographis paniculata* were collected from Dharwad district, Karnataka, India. Authentication of the plant was done by Dr. G R Hegde, Karnatak University, Dharwad (India). A specimen is stored in the herbarium, Postgraduate department of Pharmacognosy (SETCPD/Ph. cog/herb/32/12/2015).

Isolation of Endophytes

Andrographis paniculata (Burm.f.) Wall. Nees. leaves were washed thoroughly under tap water to take off any foreign particles adhering to it and were dried. They were then sterilized with 70% ethanol for 1 min, 1% sodium hypochlorite for 30 sec and further cleaned by passing through two

sets of sterile distilled water. After surface sterilization, leaves were cut into small pieces, 1 cm each, the sterile samples were placed on a plate containing potato dextrose agar (PDA) media with 250 µg/mL streptomycin to suppress fungal contamination. The parafilm wrapped petri dishes were incubated at 25 ± 2°C for 7-14 days, till the fungal mycelia started growing on the samples. Fungal colonies were picked randomly from the dilution plates, checked for purity and grouped according to colony morphology (Katoch M *et al.,* 2014). Two pure endophytic fungi namely APLF-1 and APLF-2 (endophytic fungi of *Andrographis paniculata*) leaves were selected for fermentation.

Fermentation and extraction

The Purified isolates of APLF-1 and APLF-2 were inoculated and fermented separately into a 3000 mL Erlenmeyer flask containing 600 mL of potato dextrose broth (Potato infusion from 200 g potatoes+20 g of dextrose, pH 5.1± 0.2, 24 g/L). The flask was incubated at 27°C for 21 days under static culture condition without light. After 21 days of incubation, 500 mL of chloroform was added to the flask, mixed, and left overnight. Chloroform immersed fungus culture was homogenized at 4000 rpm to separate the mycelia from broth for 30 min and filtered by whatman filter paper under vacuum. The filtrate was collected and residual aqueous phase was partitioned twice with equal volumes of chloroform in a separator funnel. Aqueous phase obtained after chloroform extraction was further partitioned three times with equal volumes of ethyl acetate. Aqueous phase obtained after ethyl acetate extracting was further partitioned two times with equal volumes of n-butanol. The chloroform, ethyl acetate and n-butanol fractions of respective endophytic fungi were dried with vacuum rotary evaporator (Superfit Rotavap, PBU-6) and weighed to constitute the crude extract. (Kumar S *et al.,* 2013; Wicklow DT.,1998).

Preliminary phytochemical investigation

The chloroform (A1C), ethyl acetate (A1EA) and n butanol fractions (A1nB) of APLF-1 and chloroform (A2C), ethyl acetate (A2EA) and n butanol fractions (A2nB) of APLF-2 were subjected to preliminary phytochemical investigations following standard procedures (Khandelwal KR., 2002;. Kokate CK *et al.,* 2005).

PHENOTYPIC IDENTIFICATION

Colony Morphology

The colonies grown on the agar slant were studied by the Lacto phenol cotton blue staining. The characteristics were compared with the known organisms from the literature (Astrid Leck., 1999).

MOLECULAR CHARCTERIZATION BY PCR SEQUENTIAL ANALYSIS

Using the genomic DNA extraction kit, genomic DNA was isolated from the given organism (Bhat Biotech Ltd. Bangalore, India) (Junichi K *et al.,* 2015, Mathur SB *et al.,* 2003). Amplification of the 16s rRNA gene was performed using the primers. Forward primer, 5'-TCCGTAGGTGAACCTGCGG-3', Reverse Primer, 5'- TCCTCCGCTTATTGATATGC-3'. PCR was performed with a total volume of 50 µL in a 0.2 mL thin walled PCR tube. The amplification was carried out in a Master cycler® Thermocycler (DNA-AMP Bhat Biotech) using the following program. Initial denaturation was carried out at 95^{o}C for 10 minutes followed by 35 cycles of denaturation at 94^{o}C for a one minute, annealing at 56^{o}C for one minute and extension at 72^{o}C for one minute. Final extension was carried out at 72^{o}C for 10 minutes. The PCR products from ITS gene PCR reactions were purified to remove unincorporated dNTPS and primers before sequencing using GENEASY GEL ELUTION KIT. Both strands of the rDNA region amplified by PCR were sequenced by automated DNA sequence -3037*xl* DNA analyzer from Applied Biosystems using BigDye® Terminator v3.1 cycle sequencing Kit (Applied Biosystems). Sequence data were aligned and dendrograms were generated using Sequence analysis software version 5.2 from applied biosystems. Appropriate software was used to align sequences acquired for plus and minus strands before to phylogenetic analysis. Sequences were compared to the non-redundant NCBI database using BLAST N. The expected value and e values were noted for the most similar sequences. Ten similar neighbors were aligned using CLUSTAL W2. The multiple-alignment file thus obtained was then used to create a Phylogram using the MEGA5 software (Saithou., 1987, Kim., 1993).

HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY OF A2EA AND A2NB

500mg each of A2EA and A2nB were dissolved in 1 mL of ethyl acetate and n-butanol respectively. 3µL and 6µL of the above fractions was applied on a pre-coated silica gel F254 on aluminum plates to a band width of 7 mm using Linomat 5 TLC applicator. The plate was developed in Toluene and Ethyl acetate (1:1). The developed plates were visualized in short UV, long UV and then derivatised with vanillin sulphuric acid and scanned under UV 254nm, 366nm and 620nm. R_f value of the spots and densitometric scan were recorded (Harborne *et al.,* 1998, Wagner *et al.,* 1996).

***In vitro* free radical scavenging activity**

Reaction with DPPH radical

All the fractions of APLF-1 and APLF-2 were used for DPPH free radical scavenging activity of at different concentrations. It was measured from bleaching of the purple colour of 2,2 Diphenyl -1-picryl hydrazyl, where 1 mL solution of different concentration of fraction was added to 1.4 mL of DPPH and kept in dark for 30 min. The absorbance was measured at 517 nm and the percentage inhibition was calculated by using the following equation (Coruh N *et al.,* 2007).

Percentage inhibition (%) = $(A_0 - A_1) / A_0) \times 100$

Where A_0 = Absorbance of control

A_1 =Absorbance of test

The results are expressed in terms of IC_{50} value which is the effective concentration at which the scavenging activity is 50%.

Reaction with hydroxyl radical

Hydroxyl radicals were generated by a Fenton reaction (Fe^{3+}-ascorbate-EDTA-H_2O_2 system), by using the fractions of APLF-1 and APLF-2. The scavenging capacity for the hydroxyl radicals was measured using deoxyribose method (Halliwell B *et al.,* 1987).

The reaction mixture of 2.8 mM of 2-deoxy-2-ribose, 0.1 mM of phosphate buffer (pH 7.4), 20 µM of ferric chloride, 100 µM of EDTA, 500 µM hydrogen peroxide, 100 µM of ascorbic acid and different concentrations from (10-1000 µg/mL) of the test sample was made to a final volume of 1 mL. The mixture was incubated for 1 h at 37 °C. After the incubation, 0.8 mL of the reaction mixture was added to 1.5 mL of 2.8% TCA solution followed by addition of 1 mL of TBA solution (1% in 50 mM sodium hydroxide,) and 2mL of sodium dodecyl sulphate. The mixture was heated for 20 min at 90 °C to develop the colour. After cooling, the absorbance was measured at 532 nm against an appropriate blank solution. All experiments were performed in triplicate. The percentage of inhibition (IC_{50}) was calculated.

Determination of reducing power

The reducing power of the fractions of APLF-1 and APLF-2 were determined by the method described by Oyaizu 1986. 1 mL of different concentrations of endophytic fractions ranging from 10-1000 µg/mL was mixed with 2.5 mL phosphate buffer (0.2 M, pH 6.6,) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated for 20 min. at 50°C. After addition of 2.5 mL 10% trichloroacetic acid, the mixture was centrifuged at 6500 rpm for 10 min. 2.5 mL of the supernatant was mixed with 2.5 mL of distilled water to which 0.5 mL of 0.1% iron (III) chloride and the absorbance was measured at 700 nm using phosphate buffer as blank. These were done in triplicate and the mean values were given in the results. The percentage inhibition was calculated.

Hepatoprotective activity

Based on the results of free radical scavenging activity, A2EA and A2nB were further selected for *in vivo* hepatoprotective activity in CCl_4 induced hepatotoxicity.

Animals

Albino Wister rats (150–200 g) were used and were collected from Venkateshwara Enterprises Bangalore, Karnataka. They were maintained in the animal house of SET's College of Pharmacy, Dharwad for experimental purpose. The animals were maintained under controlled temperature and humidity. All the animals were acclimatized for 7 days before the study. They were randomized into experimental and control groups and housed individually in sanitized polypropylene cages containing sterile paddy husk as bedding. They had free access to standard pellets as basal diet and water *ad libitum*.

Acute toxicity studies

Acute oral toxicity of A2EA and A2nB was determined using Swiss albino mice. The animals were fasted for 12 h prior to the experiment and were administered with single dose of fractions dissolved in 5% gum acacia and observed for mortality up to 48 hour (short term toxicity). Based on the short-term toxicity, the dose of animal was determined as per OECD guideline 420.

Experimental design

Animals were divided into following groups of six animals in each group (n=6)

Group I: Normal Control, rats treated with 0.9% NaCl (2 mL/kg day)

Group II: Rats treated with CCl_4 (2 mL/kg i.p. in olive oil)

Group III: Rats treated with Silymarin (100mg/kg p.o) + CCl_4

Group IV: Rats treated with A2EA (50mg/kg p.o) + CCl_4

Group V: Rats treated with A2EA (100mg/kg p.o) + CCl_4

Group VI: Rats treated with A2nB (50 mg/kg p.o) + CCl_4

Group VII: Rats treated with A2nB (100 mg/kg p.o) + CCl_4

During the treatment, rats were maintained under normal diet and water *ad libitum*. Animals of group

II to VII received CCl_4 (2 mL/kg i.p. in olive oil) subcutaneously, on 2nd and 3rd day after 30 min of administration of the treatment of silymarin and endophytic fractions. On the 6th day, animals were sacrificed, blood was collected by retro-orbital bleeding under mild ether anesthesia, centrifuged (3000 rpm for 15 min) and serum was subjected to biochemical estimations. Liver was dissected out and placed in 10% formalin solution for histopathological study. Liver homogenate was prepared to determine the levels of endogenous enzymes (Suja SR *et al.,* 2004, Qureshi *et al.,* 2007).

Biochemical parameters

Serum was separated and analyzed spectrophotometrically for aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphate (ALP), total and direct bilirubin, total triglyceride (TG), and total protein using diagnostic kits of Swemed diagnostics, Bengaluru, India.

Measurement of enzymatic and non-enzymatic antioxidant parameters

Tissue preparation

Animals were sacrificed and perfused transcardially with ice-cold saline. The whole liver was perfused *in situ* with ice cold saline, dissected out, blotted dry and immediately weighed. A 10% liver homogenate was prepared separately with ice-cold saline-EDTA using Teflon-glass homogenizer (Yamato LSG LH-21, Japan). The homogenate was used for the estimation of proteins and lipid peroxidation. 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 non-enzymatic antioxidants (Lipid peroxidation) and enzymatic antioxidants (Catalase and Superoxide dismutase).

Lipid peroxidation

The concentration of thiobarbituric acid reactive substances (TBARS) in the liver homogenate was estimated using standard procedure. The homogenate was incubated with 15% TCA, 0.375% TBA and 5N HCl at 95°C for 15 min, the mixture was cooled, centrifuged and absorbance of the supernatant measured at 532 nm against appropriate blank. The amount of lipid peroxidation was determined by using the formula €= 1.56 x $105M^{-1}cm^{-1}$ and expressed as TBARS (µ moles) per gram of tissue (Banerjee *et al.,* 2008).

Superoxide dismutase Assay (SOD)

In this assay, 0.5 mL of liver homogenate was taken, and to it, 1 mL of 50 mM sodium carbonate, 0.4 mL of 24µm NBT, and 0.2 mL of 0.1mM EDTA were added. The reaction was started by addition of 0.4 mL of 1mM hydroxylamine hydrochloride. Zero time absorbance was taken at 560 nm followed by recording the absorbance after 5 min at 25º C. The control was run simultaneously without liver homogenate. Units of SOD activity were expressed as the amount of enzyme required to inhibit the reduction of NBT by 50%. The specific activity was expressed in terms of units per mg of proteins (Flohe and Otting., 1984).

Catalase assay (CAT)

Here, 1.95 mL of 10 mM H_2O_2 in 60 mM phosphate buffer (pH=7.0), was added to 0.05 mL of the liver homogenate. The rate of degradation of H_2O_2 was followed at 240 nm/ min. Catalase content in terms of U/mg of protein was estimated from the rate of decomposition of H_2O_2 using the formula

$k=2.303/ \Delta t \times \log (A1/A2)$ s-1

A unit of catalase is defined as the quantity which decomposes 1.0 µmole of H_2O_2 per min at pH=7.0 at 25°C, while H_2O_2 concentration falls from 10.3 to 9.2mM (Claiborne *et al.,* 1985).

Estimation of Protein

Folin phenol reagent method was used to measure total tissue protein content as reported earlier (Lowry OH., 1951).

Histopathological studies

Liver of individual animal were excised, fixed in 10% buffered neutral formalin and fixed in bovine solution. They were further processed for paraffin embedding following standard microtechnique (Galigher AE *et al.,* 1971). Sections of liver stained with alum-haematoxylin and eosin, were observed photomicroscopically for histopathological changes.

Statistical evaluation

The data were expressed as Mean±S.E.M. Statistical comparisons were performed in one-way ANOVA followed by Tukey's t-test using Graph Pad Prism version 5.0, USA.

RESULTS

Two prominent endophytic fungi, namely APLF-1 and APLF-2 were isolated from the leaves of *Andrographis paniculata* (Figure 1 and Figure 2). The percentage yield of fractions of APLF-1 and APLF-2 are illustrated in Table 1.

Preliminary Phytochemical Screening

Preliminary phytochemical investigations revealed the presence of flavonoids, alkaloids, triterpenoid and tannins for APLF-1 and APLF-2 fractions as important secondary metabolites (Table 2).

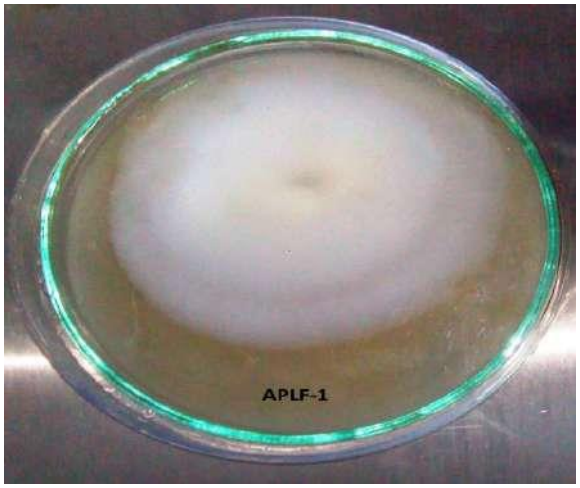

Figure 1: APLF-1 (*Andrographis paniculata* leaf endophyte-1)

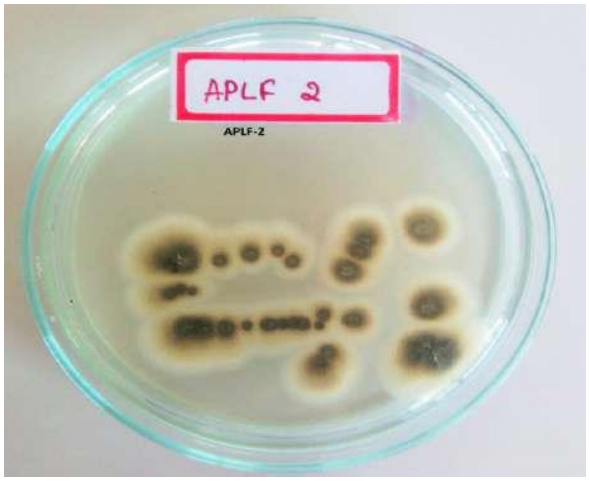

Figure 2: APLF-2 (*Andrographis paniculata* leaf endophyte-2)

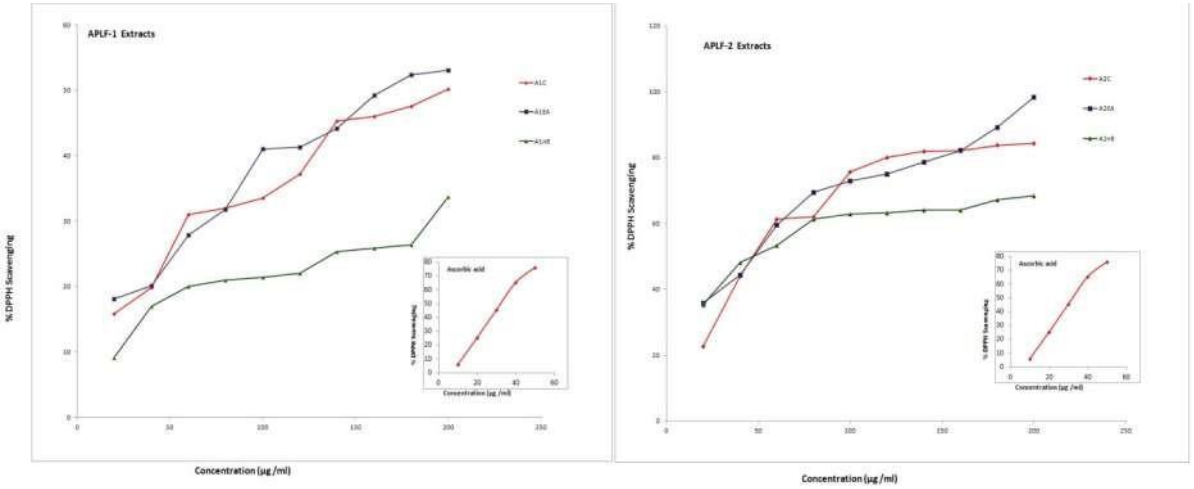

Figure 3: Effect of APLF-1 and APLF-2 fractions on DPPH radical

DPPH assay

The IC50 values for A1C, A1EA and A1nB were found to be 185.4 µg/mL, 168.64 µg/mL and 391.2 µg /mL respectively where as IC50 value for ascorbic acid was found to be 32.53 µg/mL. The IC_{50} values for A2C, A2EA and A2nB extract were found to be 51.68 µg /mL, 43.70 µg /mL and 22.78 µg /mL respectively where as IC_{50} value for ascorbic acid was found to be 32.53 µg/mL (Figure 3). APLF-2 fractions showed significant scavenging activity as compared to APLF-1 extracts.

Hydroxyl Scavenging Assay

The assay showed the abilities of the extract and standard mannitol to inhibit hydroxyl radical-mediated deoxyribose degradation in a Fe^{3+}-EDTA-Mannitol and H_2O_2 reaction mixture. The IC_{50} values for A1C, A1EA and A1nB were found to be 591.06µg/mL, 453.09µg/mL and 458µg/mL respectively. The IC_{50} values for A2C, A2EA and A2nB were found to be 347.61 µg /mL, 144.75 µg /mL and 220.24 µg /mL. APLF-2 fractions were more significant than APLF-1 fractions (Figure 4).

Reducing power Assay

Reducing power of fractions of APLF-1 and APLF-2 (50-450µg/mL) increased with increase in concentration. The A2EA and A2nB showed more effective reductive ability when compared to other extracts. In this assay, Fe (III) reduction is often used as a significant indicator of electron donating activity which is an important mechanism of phenolic antioxidant action by breaking the radical chain by donating a hydrogen atom. The A2EA and A2nB showed highest activity in a dose dependant manner as compared to the other fractions (Figure 5).

HEPATOPROTECTIVE ACTIVITY

Acute toxicity (LD_{50}) studies

Acute toxicity studies were carried out according to OECD guidelines (Up and Down method). No mortility was observed upto 2000 mg/kg body weights were for A2EA and A2nB. Hence, doses of 50 mg/kg and 100 mg/kg body weight were selected to assess the hepatoprotective activity of A2EA and A2nB fractions respectively.

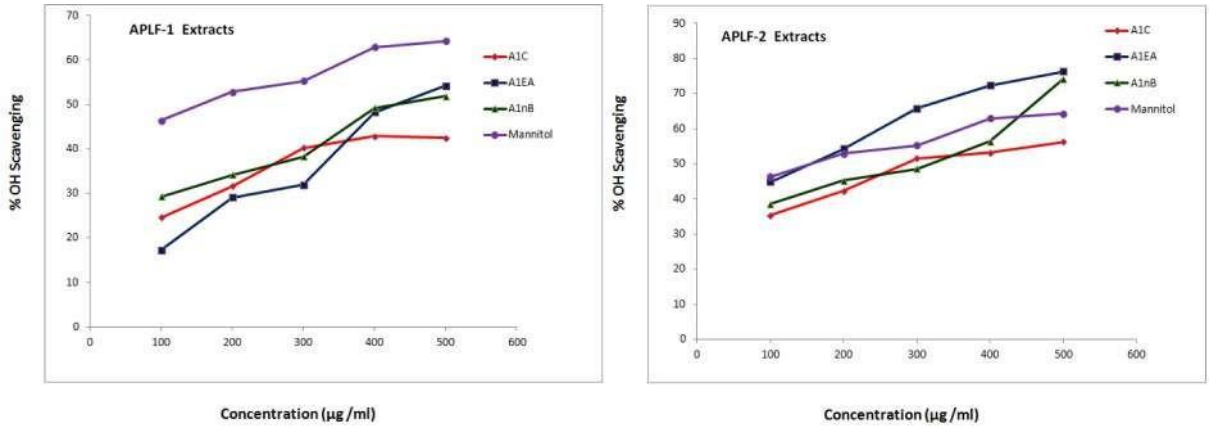

Figure 4: Effect of APLF-1 and APLF-2 fractions on hydroxyl radical

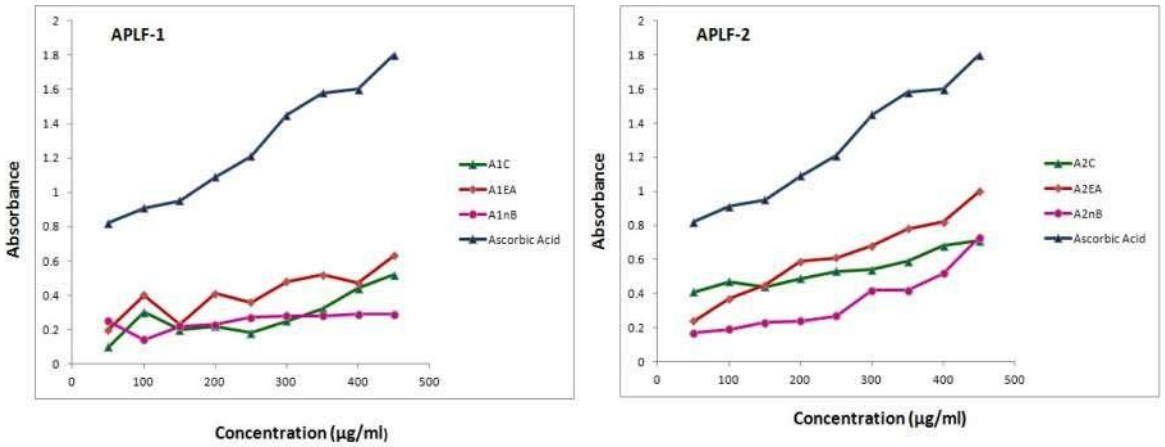

Figure 5: Reducing power assay of APLF-2 APLF-1 and APLF-2 fractions

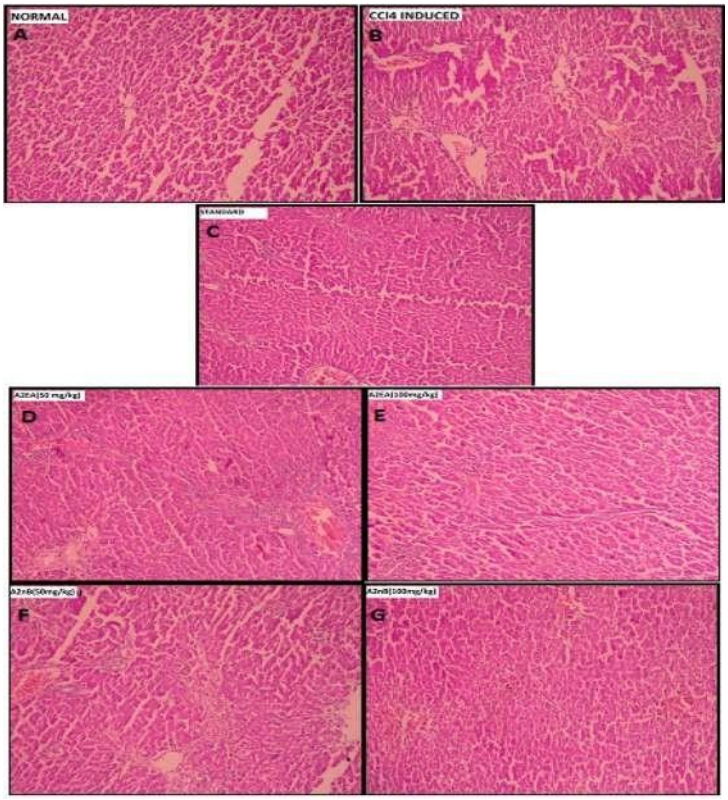

Figure 6: Histopathological Photographs of livers A) Normal rat liver, B) CCl_4 induced rat liver, C) Silymarin treated rat liver, D) A2EA(50mg/kg), E) A2EA(100mg/kg), F) A2nB(50mg/kg)

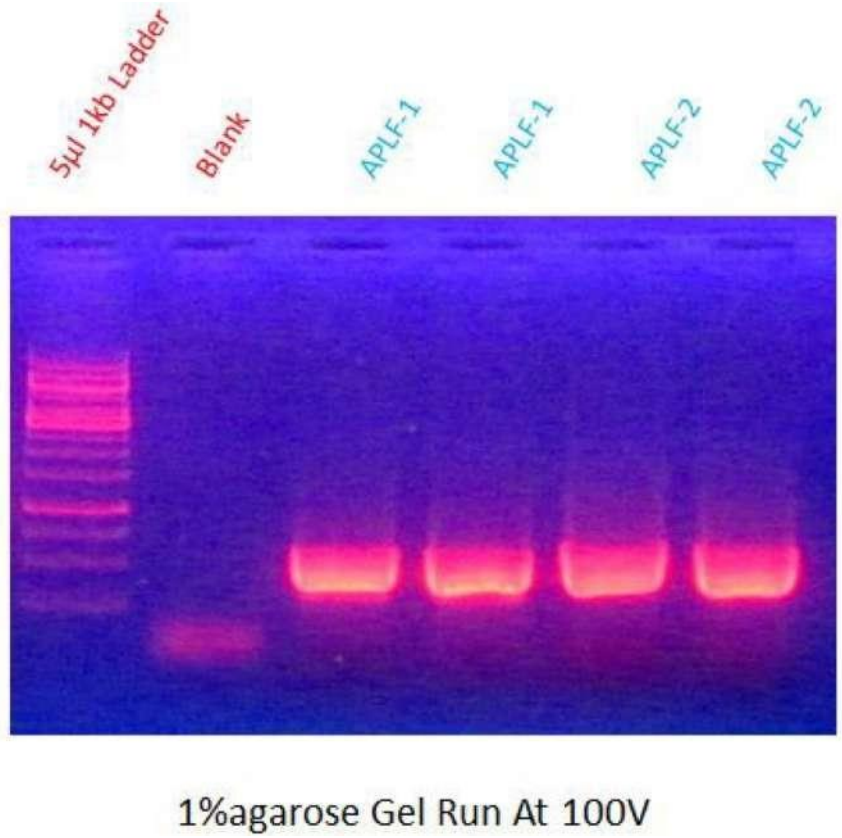

Figure 7: Genomic DNA extracted from APLF-1 and APLF-2 , run on on 0.8% Agarose gel electrophoresis and visualized with propidium Iodide

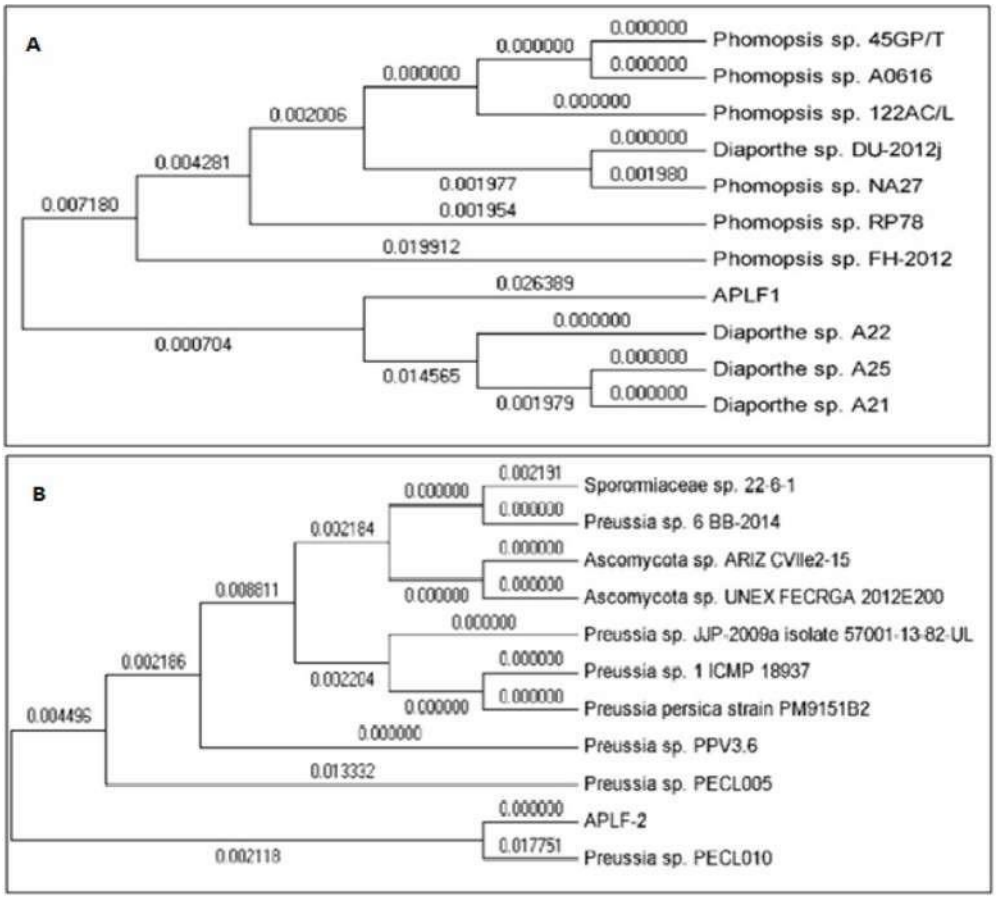

Figure 8: Phylogenetic trees of APLF-1 (A) and APLF-2 (B)

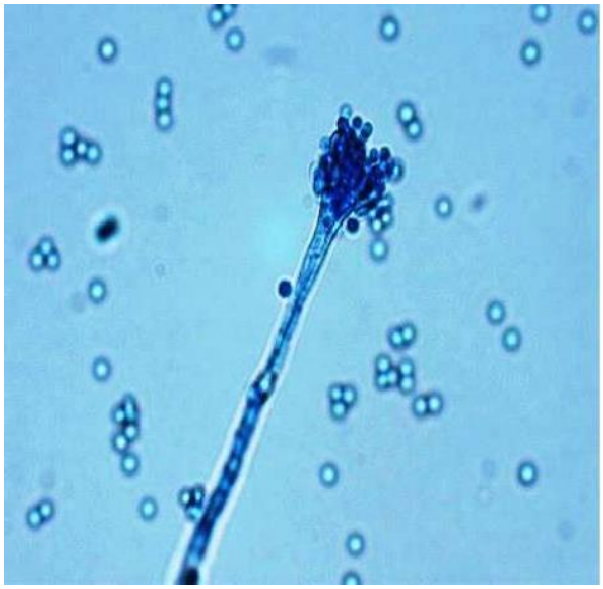

Figure 9: *Diaporthe sp.* A25

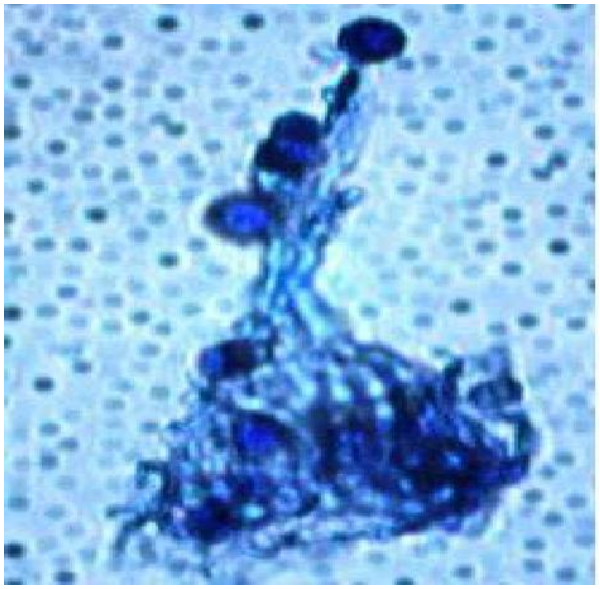

Figure 10: *Preussia sp.* PPV3.6

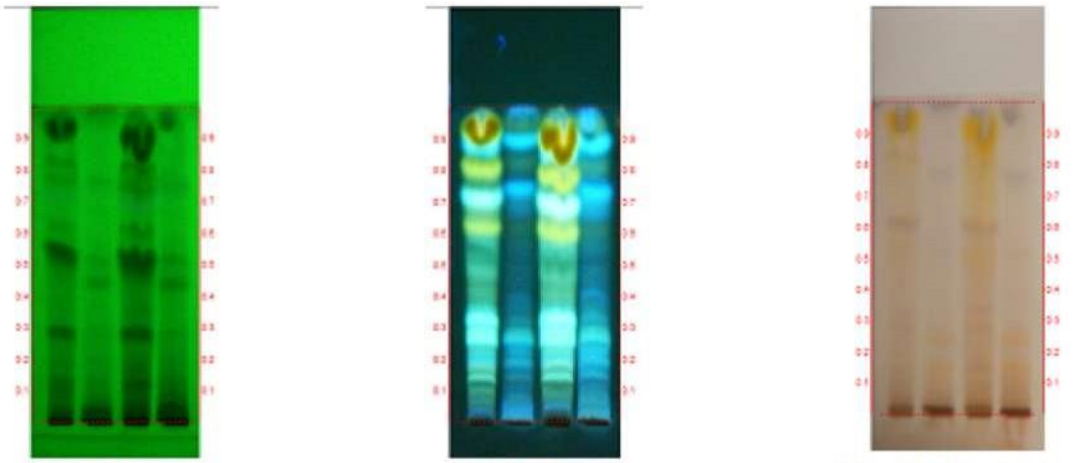

Figure 11: HPTLC of A2EA and A2nB at 254nm, 366nm and post derivatization Track 1- 2EA – 3µl Track 2- 2NB– 6µl Track 3- 2EA – 3µl Track 4- 2NB– 6µl Solvent system – Toluene, Ethyl Acetate (1.0, 1.0)

Effect of APLF-2 on serum biochemical parameters in CCl_4 induced hepatotoxicity in rats

Administration of CCl_4 (2 mL/kg i.p.) significantly increased the levels of ALT, AST, ALP total and direct bilirubin, TG and total protein as compared to normal control. A2EA (50 mg/kg &100 mg/kg) and A2nB (50 mg/kg &100 mg/kg) reversed the elevated biochemical parameters as compared to CCl_4 treated group ($p<0.001$) (Table3).

Effect of A2EA and A2nB on endogenous antioxidant enzymes

Rats treated with CCl_4 group showed decreased SOD ($p<0.0001$) and CAT ($p<0.0001$) levels where as there There was a marked increase in LPO ($p<0.0001$) level in terms of TBARS was observed compared to control. A2EA (100mg/kg) and A2nB (50mg/kg &100 mg/kg) administration showed significantly reversed LPO levels. A2EA (50 &100 mg/kg) and A2nB (100mg/kg) increased the SOD levels showing significance values. A2EA (100mg/kg) and A2nB (100mg/kg) increased the levels of CAT significantly (Table 4).

Histopathology of liver

Histopathological characters of normal liver showed normal hepatic globular structure, inflammation of central vein, portal tract and normal kupffer cells were not seen (Figure 6A). Extensive fatty degeneration, central vein and sinusoidal congestion, spotty necrosis, focal hemorrhage, inflammation and ballooning of hepatocytes were found in CCl_4 treated group (Figure 6B). Silymarin (100 mg/kg) treated group showed the hepatic globular architecture as normal. The central vein and sinusoidal congestion was observed. However, centrilobular degeneration, mild inflammation and

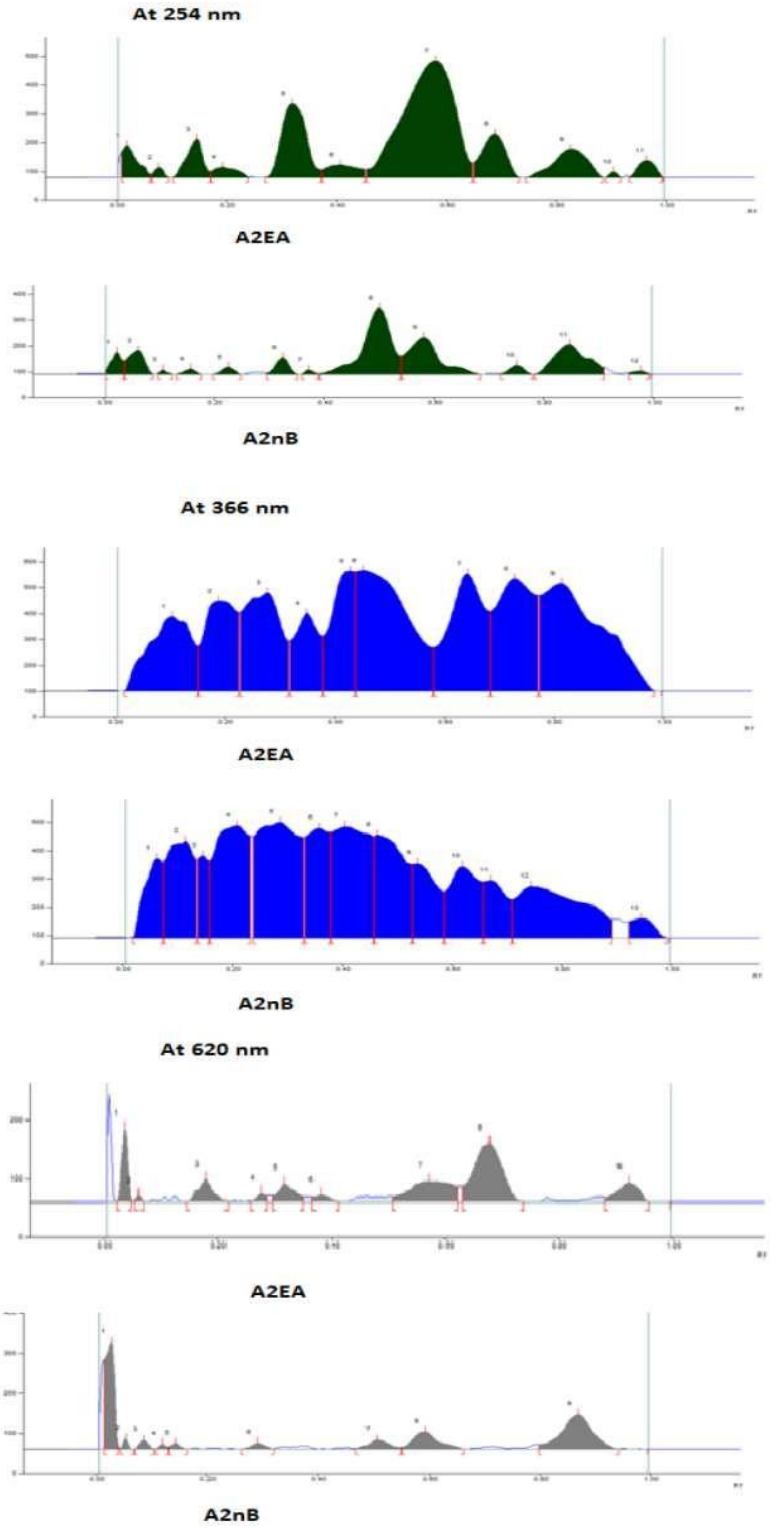

Figure 12: Chromatograms of A2EA and A2nB at 254 nm, 366nm and 620 nm

portal triditis were present. There was regeneration of hepatocytes and no inflammation found (Figure 6C). Livers treated with A2EA (50 mg/kg) showed normal arrangements of hepatocytes around the central vein and sinusoid. Cells around necrosis and fatty vacuoles were regenerated (Figure 6D). Livers treated with A2EA (100 mg/kg) revealed mild congestion of central vein and sinus. Focal haemorrhage and mild inflammation was observed, whereas no degeneration and ballooning of hepatocytes was seen (Fig 6E). Livers treated with A2nB (50 mg/kg) reduced the inflammation of hepatocytes. The injured liver returned to normal by showing reduced degeneration and mild hepatocyte ballooning (Figure 6F). Livers treated with A2nB (100 mg/kg) also showed regeneration of hepatocytes around central vein. Mild inflammation and ballooning of hepatocytes were seen (Figure 6G).

Table 1: Percentage yields of APLF-1 and APLF-2 fractions

Endophyte	Chloroform extract (% yield)	Ethyl acetate (% yield)	N butanol (% yield)
APLF-1	2.24%	0.9%	3.5 %
APLF-2	5.1%	1.66%	5.3 %

Table 2: Preliminary Phytochemical Screening of APLF-1 and APLF-2 extracts

Parameter	A1C	A1EA	A1nB	A2C	A2EA	A2nB
Carbohydrates	-	-	-	-	-	-
Proteins	-	-	-	-	-	-
Flavonoids	-	-	-	+	+	+
Alkaloids	+	+	+	+	+	+
Steroids	+	+	+	+	+	+
Tannins	-	+	+	+	+	-
Test for Andrographolide	+	+	-	-	+	+

Table 3: Effect of A2EA and A2nB on LPO, GSH and CAT levels

Groups	LPO level (µmol/mg/ protein)	SOD level (µmol/mg/ protein)	CAT level (µmol/mg/protein)
Control (2 mL/kg)	13.65±0.78	37.84±0.83	64.70±0.97
CCl_4 treated (2 mL/kg)	30.71±0.66	21.75±0.23	35.68±1.39
Silymarin (25 mg/kg)	15.75±0.80***	28.11±0.97***	51.60±1.04***
A2EA (50mg/kg)	28.00±0.16	28.43±0.60***	36.69±1.02
A2EA 100mg/kg)	21.52±0.95***	31.19±1.29***	45.37±1.08***
A2nB (50mg/kg)	22.31±1.069***	26.99±0.79**	41.49±1.62
A2nB (100mg/kg)	20.14±1.28***	33.60±0.81***	52.37±1.68***

Each value represents Mean ± S.E.M (n06) *p<0.05, ** p<0.01, ***p<0.001 compared to CCl4 treated group. One way ANOVA followed by Tukeys multiple comparison test

PCR sequential analysis of APLF-1 and APLF-2

The sequence of the ITS gene from sample APLF-1 and APLF-2 that of matching sequences from 10 nucleotide sequences were aligned by using the maximum likelihood method based on the Tamura-Nei model (Fig 7). The tree showing highest log likelihood (-952.2880) for APLF-1 and (-798.0617) for APLF-2 is shown. When the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used, otherwise BIONJ method with MCL distance matrix was used. The analysis for APLF-1 and APLF-2 involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 506 positions in the final dataset for APLF-1 and 457 positions for APLF-2 respectively. Evolutionary analyses were conducted in MEGA5. The sequence of the ITS gene for APLF-1 and APLF-2 were compared with existing sequences in the NCBI database using the Blast N programme (Fig 8). Based on these results APLF-1 and APLF-2 were identified as *Diaporthe sp. A25* and *Preussia sp. PPV3.6* respectively. The microscopic view of the identified organisms is shown in Fig 9 & 10.

HPTLC fingerprint

HPTLC fingerprinting of A2EA and A2nB scanned at wavelength 254 nm showed the presence of nine and eleven polyvalent secondary metabolites respectively. The range of R_f values starts from 0.01 to 0.99 in which highest concentration of the metabolite was found to be 30.22 % and 29.05 % with R_f values of 0.45 and 0.72 respectively. However, at 366 nm A2EA and A2nB reveled nine and thirteen secondary metabolites at range of R_f values starting from 0.02 to 0.99 respectively. The highest concentration was found to be 13.12 % with R_f value of 0.44 for A2EA and 10.69 % with R_f value of 0.24 for A2nB respectively After derivatisation at 620nm**,** A2EA and A2nB showed the presence of nine polyvalent secondary metabolites respectively. The range of R_f values starts from 0.02 to 0.94 in which highest concentration of the metabolite was found to be 31.54 % and 52.03% with R_f values of 0.02 and 0.01 respectively (Figure 11 & 12).

Table 4: Effect of A2EA and A2nB on serum biochemical parameters in CCl4 induced hepato-toxicity in rats

Groups	SGPT (mg/dl)	SGOT (mg/dl)	SALP (mg/dl)	Total Bilirubin (mg/dl)	Direct Bilirubin (mg/dl)	Triglyceride (mg/dl)	Total protein
Control (2 mL/kg)	34.64±3.1	131.9±3.6	246.4±2.4	0.25±0.02	0.16±0.018	45.10±1.50	6.45±0.10
CCl_4 treated (2 mL/kg)	183.2±2.2	242.6±2.4	324.3±2.2	0.72±0.01	0.52±0.024	87.87±2.35	3.71±0.089
Silymarin (25 mg/kg)	62.63±2.7***	159.0±3.9***	269.8±2.3***	0.51±0.02***	0.36±0.013***	45.32±1.64***	5.89±0.28***
A2EA (50mg/kg)	139.1±1.3**	172.1±4.6***	280.1±2.0***	0.52±0.02***	0.25±0.017***	71.60±3.35***	4.47±0.090
A2EA 100mg/kg)	93.98±1.4***	120.3±4.0***	252.3±3.6***	0.38±0.019***	0.18±0.010***	55.46±1.06***	5.66±0.20**
A2nB (50mg/kg)	129.9±2.9***	128.3±3.6***	299.0±2.9***	0.57±0.017**	0.44±0.011*	76.93±0.86**	5.06±0.46
A2nB (100mg/kg)	89.03±1.0***	158.5±3.3***	269.9±2.4***	0.34±0.02***	0.35±0.010***	54.73±1.45***	6.68±0.19***

Each value represents Mean ± S.E.M (n06) *p<0.05, ** p<0.01, ***p<0.001 compared to CCl4 treated group. One way ANOVA followed by Tukeys multiple comparison test

DISCUSSION

Endophytic microbes were reported from various plants existing in different ecosystems. All classes of vascular plants and grasses are found to endow with endophytic organisms. They also possess chemical structures and are evolved in biological activities with roles as defensive compounds against competitors/parasites/predators, growth and reproduction Endophytes have the ability to produce a wide range of secondary metabolites with unique structure, synthesized through different metabolic pathways e.g. isoprenoid, polyketide, amino acid derivation (Tan RX *et al.,* 2001). Thus, researchers are provided with numerous leads for compounds with possible development as new drugs for their accessibility and large scale production by the pharmaceutical industry. The diversity of Endophytic fungi is significantly higher in the leaf than stem (Strobel G *et al.,* 2003). This can be assigned to the similarity of endophytes in specific tissues and the availability of nutrients to nourish the plant (Soussi A *et al.,* 2015).

In the present study, two endophytic fungi (APLF-1 and APLF-2) were isolated from the leaves of *Andrographis paniculata*. The phylogenetic or sequential analysis revealed the fungi as *Diaporthe sp. A25 (*APLF-1) and *Preussia sp. PPV3.6* (APLF-2). However, this is the first report on hepatoprotective activity of different fractions of endophytic fungi (APLF-2).

Diaporthe (*Phomopsis*) species are isolated from various hosts and are assigned as plant pathogens, non-pathogenic endophytes or saprobes. *Diaporthe* is one of the most frequently confronted genera of endophytic fungi in various plant hosts (Murali *et al.,* 2006, Botella & Diez., 2011). *Diaporthe* sp was also isolated from a well-known medicinal plant in China, *Cephalotaxus hainanensis* Li which reported the antimicrobial activity (Yang HR *et al.,* 2015). Many reports reveled the presenec of secondary metabolites such as quinoline alkaloids such as quinine, quinidine, cinchonidine, and cinchonine from *Diaporthe* sp. which was isolated from *Cinchona ledgeriana.*(Maehara S *et al.,* 2012). *Diaporthe phaseolorum* (*Phomopsis longicolla*) was isolated from *Trichilia elegans* A. JUSS ssp. Elegans which produced secondary metabolite as 3-nitropropionic acid (Flores AC *et al.,* 2013). *Diaporthe* sp. strains were isolated from the *Camellia sinensis* (tea plant) and rhizomes of *Curcuma longa* respectively. The stereoselective oxidation of (+)-catechin and (−)-epicatechin was biotransformed into 3,4-cis-dihydroflavan derivatives (Agusta & Shibuya., 2005.) whereas conversion of curcumin was biotransformed into a few colorless hydroderivatives (Maehara S *et al.,* 2011).

Preussia is a genus of fungi belonging to family Sporormiaceae. (Lumbsch TH., 2007). This extensive genus has the species that grows on dung or in the soil (Kirk PM et al, 2008). Nine different plants were used to isolate a total of 18 *Preussia* species from Australian dry rainforests. 13 out of 18 *Preussia* sp revealed antimicrobial activity showing the importance of potentially significant bioactive compounds (Mapperson RR *et al.,* 2014). *Preussia* sp was also isolated from twigs of *Juniperus procera* (Cupressaceae) of Taif region, Saudi Arabia, exhibiting antimicriobial activity (Gherbawy *et al.,* 2016).

The important secondary metabolites identified by preliminary phytochemical investigation of APLF-1 and APLF-2 fractions were flavonoids, alkaloids, triterpenoid and tannins. The fractions of APLF-1 and APLF-2 were studied for *in vitro* antioxidant activity by DPPH radical, hydroxyl free radical and

by reducing power. Among all the fractions of APLF 1 and APLF-2, fractions of APLF-2 exhibited good scavenging activity with minimum IC_{50} values and were further set for hepatoprotective studies. The acute toxicity was conducted for as per OECD guidelines which showed that APLF-2 fractions are as non-toxic. Hence, 50mg/kg and 100 mg/kg doses were selected for the *in vivo* studies.

Hepatoprotective activity was carried out for A2EA (50mg/kg and 100mg/kg) and A2nB (50mg/kg and 100mg/kg) in CCl_4 induced hepatotoxicity. The alteration in serum biochemical parameters like ALT, AST and ALP is caused due to CCl_4 as it produce free radicals such as CCl_3^- and CCl_3COO^-, affecting the cellular permeability of hepatocytes leading to elevated levels. Cytochrome P-450 is majorly involved enzyme in the drug metabolism. These radicals covalently get bound to molecules causing lipid degradation of the adipose tissue. Hence, CCl_4 treated rats showed significant hepatic damage, by elevation in the serum AST, ALT, ALP, total and direct bilirubin, TG and total protein levels. High levels of AST and ALT are released from the liver into the blood during necrosis of hepatocytes (Nelson *et al.,* 2005, Emeka, *et al.,* 2011). The AST and ALT levels were slightly decreased to normal after the administration of the fractions of APLF-2 as compared to controls.

In this study, administration of A2EA (50 mg/kg &100 mg/kg) and A2nB (50mg/kg &100 mg/kg) and silymarin showed a decline in AST, ALT and ALP levels. The increased hepatocytic functioning leads to elevated biliary pressure of ALP. Regeneration of hepatocytes can be done due to healing of hepatic parenchyma in serum levels of transaminases.

Antioxidants act by blocking the free radical action as associated in the pathological process of various disorders and aging process (Aruoma OI *et al.,* 2003). Free radicals also are necessary for the body as they regulate the numerous biological processe. Framing cell-signaling is one of the important mechanism that played by free radicals. At the same time they are also harmful to the body. The damage caused by free radicals can be repaired by various enzymes such as SOD, CAT, glutathione, glutathione reductase and peroxidase. Thus, antioxidants play a major role in such defense mechanisms (Bahar E *et al.,* 2013).

The antioxidant effect on DPPH is due to the ability for hydrogen donation. Reaction with DPPH radicals of A1C, A1EA and A1nB showed no significant scavenging activity where as the activity was increased with increasing concentration of A2C, A2EA and A2nB. The reducing power of fractions of APLF-1, APLF-2 and ascorbic acid (50-450µg/mL), increased with increase in concentration. The A2EA showed more effective reduction as compared to other fractions. The reducing power may be due to mono and dihydroxyl substitutions in the aromatic ring, possessing potent hydrogen donating ability.The fractions of APLF-1 and APLF-2 exhibited hydroxyl radical scavenging activity in a dose dependent manner. Molecules inhibiting deoxyribose degradation and chelating the iron ions, prevents complexation with the deoxyribose and imparting them inactive in a Fenton reaction. A2C, A2EA and A2nB showed chelating effect on ferrous ions, as they minimize the concentration of metal in the Fenton reaction compared to EDTA.

Lipid peroxidation elevation is one of major results of CCl_4 induced hepatotoxicity (Brattin & Glende., 1985). In CCl_4 treated group, the TBARS concentration was elevated, which was dose dependent, inhibited by A2EA and A2nB. The data imparted that A2EA and A2nB may exert antioxidant effects by inhibiting the lipid peroxidation. ROS production was increased on administration of CCl_4 as a significant decrease in the endogenous antioxidant enzymes activity such as SOD and CAT. A2EA and A2nB (50 and 100 mg/kg) administration exhibited significant elevation in the SOD and CAT enzymatic activity. Also, the protein expression levels were evaluated by Western blotting analysis. CCl_4 treatment resulted in significant down-regulations of the protein expression levels of SOD and CAT compared with the normal control. A2EA and A2nB (50 and 100 mg/kg) administration significantly increased protein levels of the two antioxidant enzymes in the liver. Our results suggested that A2EA and A2nB (50 and 100 mg/kg) may diminish oxidative stress by augmenting the activity and protein expression of the antioxidant enzymes *in vivo.* Histopathology of normal liver revealed normal hepatic globular architecture, where as in CCl_4 treated group hepatocytes showed extensive fatty degeneration, central vein and sinusoidal congestion, spotty necrosis, focal hemorrhage, inflammation and ballooning of hepatocytes. A2EA (50 mg/kg and 100 mg/kg) showed normal architecture of hepatocytes round the central vein and sinusoid. Cells around necrosis and fatty vacuoles were regenerated, no degeneration and ballooning of hepatocytes was seen. A2nB (50 mg/kg and 100 mg/kg) also showed reduced inflammation and protection against hepatocytes. These fractions returned the injured liver to quite normal by showing reduced degeneration and mild hepatocyte ballooning. These observations showed that A2EA and A2nB (50 and 100 mg/kg) decreased the toxic effects of CCl_4 on hepatic architecture. HPTLC fingerprints profile is an important and powerful procedure for identification of presence of phytoconstituents. It is a liner, precise and accurate method

for identification, authentication and characterization of the medicinal plant extracts. Newer drugs can be formulated by isolating and identifiying the bioactive compounds used for treating various diseases by HPTLC (Elangovan NM *et al.,* 2015). The development of HPTLC fingerprint for A2EA and A2nB shows the direction for the quality control of phytoconstituents from extracts.

Further, investigation is required to characterize the secondary metabolites and quantitative estimation with marker compounds.

CONCLUSION

The ethyl acetate and n butanol endophytic fungal fractions of APLF-2 from leaves of *Andrographis paniculata* exhibited hepatoprotective activity in CCl_4 induced model. The results showed that the antioxidant effects of A2EA and A2nB may be responsible for their hepatoprotective activity. HPTLC fingerprinting analysis exhibited the chemical diversity of A2EA and A2nB of APLF-2. Further investigations are empowered to isolate and identify marker compound(s) responsible for hepatoprotective activity with reference to endophytes.

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