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# Hepatoprotective activity of *Phyllanthus niruri* Linn. endophytes

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## Abstract

**Background:** The liver is the vital organ which plays a major role in metabolism with numerous functions in the human beings such as protein synthesis, hormone production, and detoxification. Present research work is focused on hepatoprotective potential of chloroform (PNFC) and ethyl acetate (PNFEA) endophytic fractions from *Phyllanthus niruri* Linn. against CCl<sub>4</sub>-induced hepatotoxicity in albino Wistar rats. To test our hypothesis, both endophytic fungal fractions were tested for vitro antioxidant and in vivo hepatoprotective activity. Serum biochemical parameters like SGOT, SGPT, SALP, cholesterol, bilirubin, and protein were estimated to assess hepatoprotective activity.

**Results:** Group of rats treated with CCl<sub>4</sub> possess marked hepatic damage and oxidative stress which indicates that cellular leakage and loss of functional integrity of cell membrane in liver. PNFC and PNFEA fractions of endophyte from *Phyllanthus niruri* Linn. stem have significantly reduced the elevated levels of biomarkers like SGPT, SGOT, SALP, bilirubin, cholesterol, and total protein in CCl<sub>4</sub>-induced hepatotoxicity in rats. The results obtained confirm hepatoprotective activity of endophytic fractions (PNFC and PNFEA) mediated through the stabilization of plasma membrane, repair of hepatic tissue damage, return of biochemical marker levels to normal, and regeneration of hepatocytes. Histopathological observations revealed improvement in the liver architecture after the treatment of secondary metabolites of endophytic fractions against CCl<sub>4</sub>-induced liver damage. Both fungal endophytes PNFC and PNFEA showed DPPH scavenging activity with IC<sub>50</sub> of 97.79 µg/ml and 108.40 µg/ml, respectively, and possess antioxidant potential. Presence of flavonoids in the both fractions of endophytes may be a possible reason for its antioxidant potential and identified as *Eurotium amstelodami* strain.

**Conclusion:** Both fungal endophytes PNFC and PNFEA possess hepatoprotective potential due to the presence of secondary metabolites of fungi, i.e., *Eurotium amstelodami* strain which support the claim endophytes and act as a potent biomedicine for treatment of various chronic diseases.

**Keywords:** *Phyllan*, Endophytes, Hepatoprotective, Antioxidant, Microbes

## Background

Worldwide, hepatic diseases are the leading cause of death with liver cirrhosis injury [1]. The liver mainly controls vital physiological processes in human beings such as carbohydrate metabolism and storage, fat metabolism, synthesis of bile acid, and detoxification of various drugs in our body [2]. Hazardous chemicals and excess

consumption of alcohol are mainly responsible for liver diseases. Commonly included infections are fatty liver cirrhosis cancer, and various chemicals like tert-butyl hydroperoxide, galactosamine, paracetamol, CCl<sub>4</sub>, acetaminophen, and alcohol, causing potential injury to the liver cells leading to progressive dysfunction. Due to toxic nature of lipid peroxidation (LPO) which is prominent causes liver diseases due to its toxicity [3, 4]. The *Phyllanthus niruri* Linn. has great potential to treat various diseases such as hepatitis, stomach ache, gonorrhoea, asthma, urolithic disease, fever, malaria, vaginitis to

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tuberculosis, antiradicals and DNA cleavage protectors [5, 6]. Endophyte was first coined by De Bary and has become deeply embedded in the literature ever since. Nowadays endophytes defined as microbes which colonize alive internal parts of plant without any hazardous effects over it [7]. Endophytes are novel competent herbal medicine with bioactive natural constituent occupied from endophytes [8]. The aim of this present research work is to determine hepatoprotective activity of chloroform (PNFC) and ethyl acetate (PNFEA) endophytic fractions from *Phyllanthus niruri* Linn. stem against CCl<sub>4</sub>-induced hepatotoxicity.

## Methods

### Isolation of endophytes

The whole plant was cleaned to remove soil with help of running water and then dried. With the help of 4% sodium hypochlorite for 5 min, 70% ethanol for 1 min, and sterile distilled water for 1 min 2–3 times the plant was sterilized. The surface sterilized plant segment were placed on petri dish containing potato dextrose agar supplemented with streptomycin (100 µg/ml) to inhibit bacterial growth and incubated at 23–25 °C. After 7 to 14 days of incubation fungi isolated were purified. Colony morphology was mainly determined by culture purity [9–11].

### Extraction and fermentation

Isolates endophytic fungi were grown on potato dextrose broth plates at 23–25 °C for 7–14 days which are inoculated and fermented separately in potato dextrose broth. After 21 days of incubation at 23–25 °C each fungal culture is filtered and homogenized at 4000 rpm to separate the mycelia from broth. Fractions showing potential in vitro activity were further used for in vivo study [12].

### In vitro steady-state free radical scavenging studies

#### Reaction with DPPH radical

Equal volume of 100 µM DPPH in methanol was added to different concentration of fractions (20–200 µg/ml) in methanol and for 20 min placed in dark after proper mixing. The absorbance at 517 nm was measured using ascorbic acid (ASC) as a standard. Different concentrations (1–5 µg/ml) of ASC were homogeneously mixed with equal volumes of DPPH. Plotting the percentage of DPPH scavenging against ASC concentration gave the standard curve [13].

#### Reducing power assay

The reaction mixture contained 1.0 mL of different concentrations of extracts (2–10 mg/mL), 2.5 mL of 1% potassium ferricyanide, and 2.5 mL of 0.2 mol/L sodium phosphate buffer. The mixture was incubated at 50 °C for 30 min and the reaction was terminated by the

addition of 2.5 mL of 10% trichloroacetic acid, followed by centrifugation at 3000 rpm for 10 min. 2.5 mL of the supernatant was uniformly mixed with 2.5 mL of deionized water and 0.5 mL of 0.1% FeCl<sub>3</sub>. At 700 nm absorbance was measured against blank. Increase in absorbance indicates increased reducing power of the sample [14].

#### Reaction with hydroxyl radical

OH scavenging activity of fraction was determined by degradation of deoxy-D-ribose method. To the reaction mixture having ascorbic acid (0.1 mM) and hydrogen peroxide (2 mM) in different concentrations of the extract/fractions in a volume of 0.3 ml were added to get volume of 3.0 ml. After 30 min of incubation, 1.0 ml of trichloroacetic acid (TCA)-thiobarbituric acid (TBA) reagent (equal volumes of TCA-2.8% and TBA-0.5% in 4 mM NaOH) was added which is followed by boiling the tubes in a water bath for 30 min. At 532 nm, absorbance was recorded once cooling of the tubes and mannitol was used as standard [15].

#### Acute toxicity study

Toxicity of endophytic fractions of *Phyllanthus niruri* Linn in albino Wistar rats will be determined as per reported method. All animals will be given endophytic fractions by oral route, and for next 3 h animals will be observed for mortality and behavioral changes. Animals will be observed for next 48 h for any mortality. LD<sub>50</sub> will be calculated according to the guidelines of OECD.

### Hepatoprotective activity

#### Procedure

Animals were above grouped as shown above and treated for 5 days (Table 1). On the 2nd and 3rd day CCl<sub>4</sub> (2 ml/kg i.p.) in olive oil was administered to all groups other than group I. Silymarin 25 mg/kg p.o. was received as standard drug once in a day and CCl<sub>4</sub> as per above for group III. Whereas, group IV and V were

**Table 1** Experimental design

Sr. No.	Groups	Details of groups
1	Group 1	Normal control rats treated with 0.9% NaCl [2 ml/kg day]
2	Group 2	Rats treated with CCl <sub>4</sub> (2ml/kg i.p. in olive oil)
3	Group 3	Rats treated with Silymarin (25 mg/kg p.o.) + CCl <sub>4</sub>
4	Group 4	Rats treated with fraction PNFC (50mg/kg p.o.) + CCl <sub>4</sub>
4	Group 5	Rats treated with fraction PNFC (100mg/kg p.o.) + CCl <sub>4</sub>
6	Group 6	Rats treated with fraction PNFEA (50mg/kg p.o.) + CCl <sub>4</sub>
7	Group 7	Rats treated with fraction PNFEA (100mg/kg p.o.) + CCl <sub>4</sub>

treated with fractions PNEAF with (50 and 100 mg/kg p.o. each group) and group VI and VII were treated with fractions PNCF with a dose of 50 and 100 mg/kg p.o. each group, respectively. During this period of treatment, the rats were maintained under normal diet and water. On the 6th day, all animals were sacrificed and blood samples were collected by retro-orbital bleeding which was centrifuged for 15 min at 3000 rpm and used for biochemical evaluation. The liver was dissected out and subjected for morphological study such as wet liver weight of each animal. Further histopathological study of liver was performed. Then, the liver homogenate was subjected for in vivo antioxidant estimation [16].

#### Estimation of biochemical parameters

##### Estimation of serum SGPT (IFCC method)

Alanine transaminase (ALT) also known as SGPT located in cytosol of liver cell. At elevated permeability of cell membrane, SGPT was released in blood circulation due to inflammation and break down of liver cells. Hence, determination of ALT is a sign of the extent of liver damage. Pyruvate and L-glutamate was produced after catalysis transfer of amino group from L-alanine to 2-oxoglutarate by SGPT. L-lactate was produced by reaction of pyruvate and NADH. Decrease in absorbance of NADH at 340 nm is indicative of oxidation of NADH equivalent to potency of SGPT action [17].

##### Estimation of SGOT/AST

The reaction of 2-oxoglutarate and L-aspartate was catalyzed by aspartate transaminase which gives product of glutamate and oxaloacetate. In the presence of malate dehydrogenase oxaloacetate react with NADS gives NAD and malate. SGOT activity is directly proportional to decrease in rate of NADH which is mainly calculated photometrically [18].

##### Estimation of serum alkaline phosphatase (SALP)

Serum alkaline phosphatase hydrolyses p-nitrophenyl phosphate into p-nitrophenol and phosphate due to oxidizing agent  $Mg^{++}$ . This reaction is measured as absorbance is proportional to the SALP activity [19].

##### Estimation of bilirubin

Azobilirubin was produced by reaction of bilirubin with sulphanic acid diazotized in acidic medium. Absorbance of azobilirubin gives exact concentration of bilirubin. In an acidic medium, bilirubin directly reacts because of its water solubility. However, surfactant solubilizer bilirubin is solubilized [20].

##### Lipid peroxidation (LPO)

TBARS (thiobarbituric acid reactive substances) in liver homogenate was estimated by using standard protocol.

Homogenate was incubated with 15% TCA, 0.375% TBA, and 5N HCl at 95 against blank. LPO was determined and represented as TBARS (micromoles) per gram of tissue [20].

##### Estimation of cholesterol

At the end of the reaction quinoneimine whose absorbance gives direct concentration of cholesterol [21].

Cholesterol ester ALP  $\rightarrow$  Cholesterol+ fatty acid  
 $Cholesterol + O_2 \xrightarrow{CHOD} Cholest-4-en-3-one + H_2O_2$   
 $2H_2O_2 + 4AAP + phenol \rightarrow POD_4H_2O + Quinoneimine$

##### Estimation of total protein

In alkaline solution, copper ion reacts with peptide bonds of protein to produce blue-violet complex. Five to 6 peptide bonds are complexed with each copper ion. Tartrate was added as stabilizer and auto-reduction of protein concentration is prevented by iodide measured at 546 nm.

#### Identification and characterization of fungal endophyte

##### PCR amplification and DNA extraction

The genomic DNA was isolated from the given organism using genomic DNA extraction Kit (Bhat Biotech)

##### Phenotypic identification

The colonies grown on the plate were studied by the colony characteristics and Lacto phenol cotton blue staining. The significant parameters compared with the standard organisms from the literature.

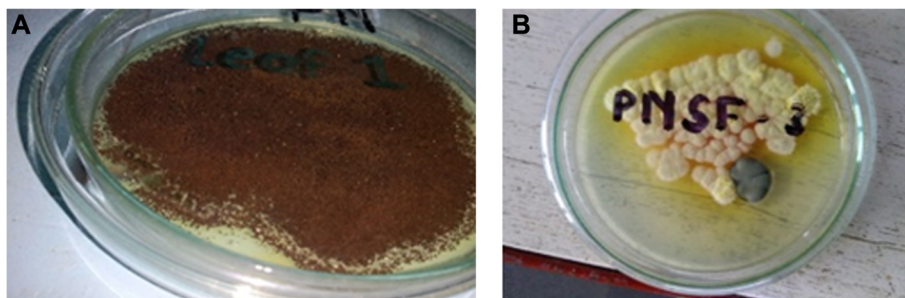
##### PCR amplification and sequencing

The amplification was performed in PCR tube using Master cycler Thermo cycle (DNA mp Bhat Biotech). PCR products were purified from ITS gene with help of Gen easy gel elution kit, Bhat Biotech India Pvt. Ltd. Both strands of the rDNA region amplified by PCR were sequenced by automated DNA sequence -3037x 1 DNA analyzer [22].

## Results

#### Isolation and Identification of endophytes from *Phyllanthus niruri* Linn.

PCR and BLAST analysis studies conforms that two isolated endophytic fungi were isolated from *Phyllanthus niruri* Linn., identified as *Aspergillus niger* strain A6 PNLF (leaf endophyte) and *Eurotium amstelodami* strain CASMB-SEF 9 PNSF (stem endophyte), respectively (Fig. 1). Genomic sequence of both endophytic fungi PNLF and PNSF was identified and by using rDNA sequential analysis and 16sr RNA gene amplification which matches with standard genomic sequence of fungi (Fig. 2). In the



**Fig. 1** Endophytic fungi **a** PNLF and **b** PNSF of *Phyllanthus niruri* Linn.

present investigation, *Eurotium amstelodami* strain *CASMB-SEF 9* was isolated from stem segment of *Phyllanthus niruri* Linn. and was screened for in vitro antioxidant and in vivo hepatoprotective potential (Fig. 3).

**Sequencing of the PCR amplicon of the 16s RNA gene (PNLF)**

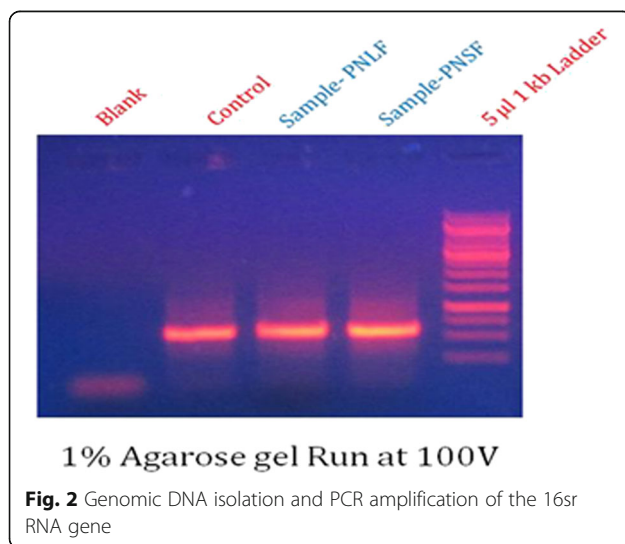
**ITS F**

TTGCTTCGGGTGCGGGTCTTTGGGCCACCTCCCA  
 TCCGTGTCTATTGTACCCTGTTGCTTCGGCGG  
 GCCCGCCGCTTGTGCGCCGCCGGGGGGGCGCC  
 TCTGCCCGCCGGCCCGTCCCCGCCGGAGACC  
 CCAACACGAACACTGTCTGAAAGCGTGCAGTC  
 TGAGTTGATTGAATGCAATCAGTTAAAACCTTCA  
 ACAATGGATCTCTTGGTTCCGGCATCGATGAAGA  
 ACGCAGCGAAATGCGATAACTAATGTGAATTG  
 CAGAATTCAGTGAATCATCGAGTCTTTGAACG  
 CACATTGCGCCCCCTGGTATTCCGGGGGGCATGC  
 CTGTCCGAGCGTCATTGCTGCCCTCAAGCCCCGGC  
 TTGTGTGTTGGGTCGCCGTCCCCCTCTCCGGG  
 GGGACGGGCCGAAAGGCAGCGGCGGCACCCG  
 GTCCGATCCTCGAGCGTATGGGGCTTTGTAC

ATGCTCTGTAGGATTGGCCGGCGCCTGCCGAC  
 GTTTTCCAACCATTTCTTTCCAGGTTGACCTCG  
 GATCAGGTAGGGATACCCGCTGAACTTAAGCA  
 TATCAATAAGCGGAGGAATTCCGAGGGACGCT  
 CCCTTCTAGCAAGCACCAATTCT

**ITS R**

ATACAGGGGGACGCCCTTAGCACCTACAGCTTCC  
 GTAGGGTGAACCTGCGGAAGGATCATTACCGA  
 GTGCGGGTCTTTGGGCCCAACCTCCCATCCGTG  
 TCTATTGTACCCTGTTGCTTCGGCGGGCCCGCCG  
 CTTGTGCGCCGCCGGGGGGGCGCCTCTGCCCC  
 CCGGGCCCGTGCCCGCCGGAGACCCCAACAC-  
 GAACACTGTCTGAAAGCGTGCAGTCTGAGTTGA  
 TTGAATGCAATCAGTTAAAACCTTCAACAATGGA  
 TCTCTTGGTTCCGGCATCGATGAAGAACGCAGCG  
 AAATGCGATAACTAATGTGAATTGCAGAATTC  
 AGTGAATCATCGAGTCTTTGAACGCACATTGC  
 GCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGA  
 GCGTCATTGCTGCCCTCAAGCCCGGCTTGTGTGT  
 TGGGTGCGCGTCCCCCTCTCCGGGGGGGACGGG  
 CCCGAAAGGCAGCGGCGGCACCCGCGTCCGATC  
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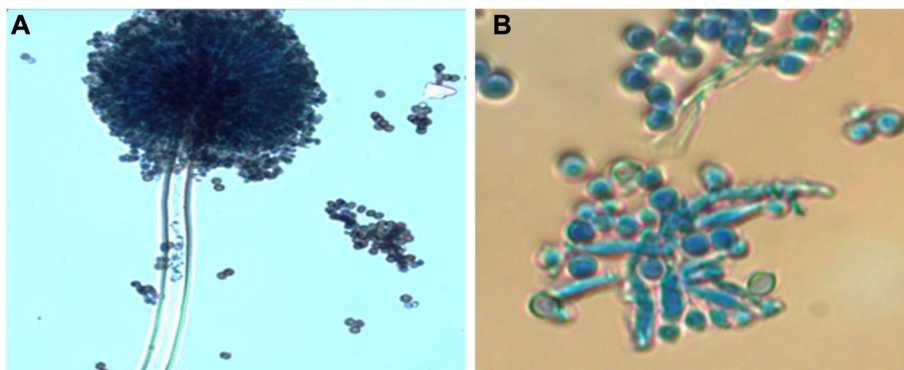


**Sequencing of the PCR amplicon of the 16s RNA gene (PNSF)**

**ITS F**

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 CATCCGTGTCTATCTGTACCCTGTTGCTTCGGCG  
 TGCCACGGCCCGCCGGAGACTAACATTTGAA  
 CGTGTCTGAAGTTTGCAGTCTGAGTTTTTGTAG  
 TTAACAATCGTTAAAACCTTCAACAACGGATCT  
 CTTGGTTCCGGCATCGATGAAGAACGCAGCGA  
 AATGCGATAATTAATGTGAATTGCAGAATTCAGT  
 GAATCATCGAGTCTTTGAACGCACATTGCGCC  
 CCTGGTATTCCGGGGGGCATGCCTGTCCGAG  
 CGTCATTGCTGCCCTCAAGCACGGCTTGTGTG  
 TTGGGCTTCCGTCCCTGGCAACGGGGACGGGC





**Fig. 3** Microscopic view of **a** *Aspergillus niger* A6 and **b** *Eurotium amstelodami* CASMB-SEF 9

CCAAAAGGCAGTGGCGGCACCATGTCTGGTCC  
 TCGAGCGTATGGGGCTTTGTCACCCGCTCCCG  
 TAGGTCCAGCTGGCAGCTAGCCTCGCAACCAA  
 TCTTTTAAACCAGTTGACCTCGGATCAGGTAGG  
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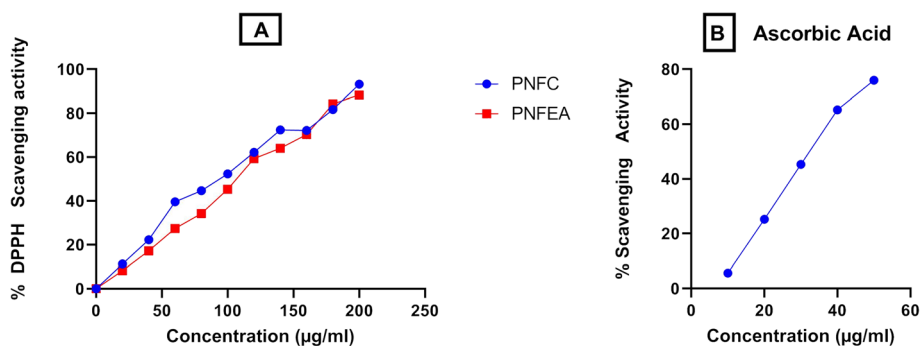
**ITS R**

GAAACCAGCATGGTGGGTGGGGTGCACCGAGG  
 GGGCCACCCCGGAAAGAACCCTCCCGTAGG  
 GGGGACCTGCGGAAGGATCATTACCGAGTG  
 CGGGCCCTCTGGGTCCAACCTCCCATCCGTGTCT  
 ATCTGTACCCTGTTGCTTCGGCGTGGCCACGGCC  
 CGCCGGAGACTAACATTTGAACGCTGTCTGAA  
 GTTTGCAGTCTGAGTTTTTGTAAACAATCG  
 TTAAAACTTTCAACAACGGATCTCTTGGTTCC  
 GGCATCGATGAAGAACGCAGCGAAATGCGATA  
 ATTAATGTGAATTGCAGAATTCAGTGAATCAT  
 CGAGTCTTTGAACGCACATTGCGCCCCCTGGT  
 ATTCCGGGGGGCATGCCTGTCCGAGCGTCATT  
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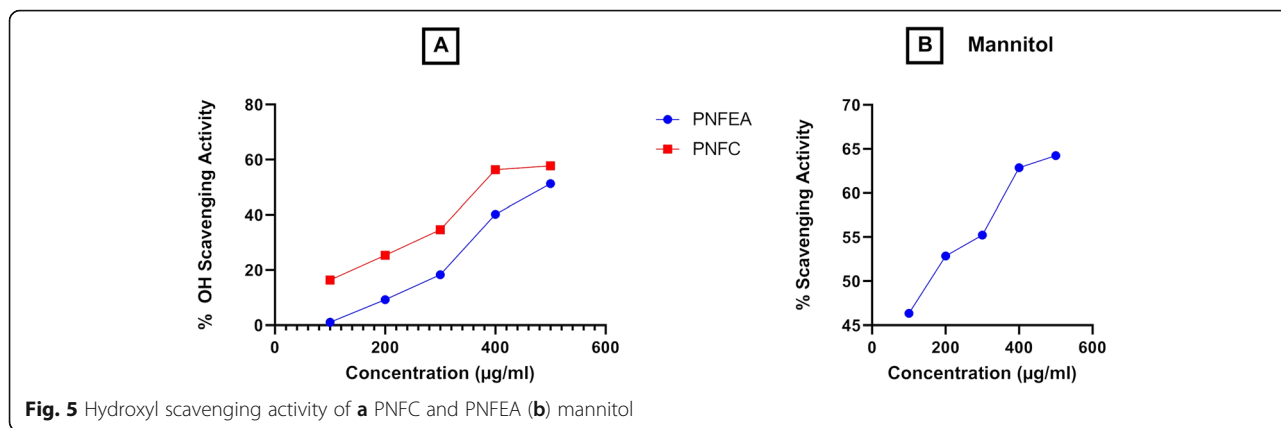
TATGGGGCTTTGTCACCCGCTCCCGTAGGTCC  
 AGCTGGCAGCTAGCCTCGCAACCAATCTTTTT  
 AACCAGGTGACCTCGGATCAGTAGGTCCGTTCC

**In vitro steady-state free radical scavenging studies**

PNFC and PNFEA showed DPPH scavenging activity with IC<sub>50</sub> of 97.79 µg/ml and 108.40 µg/ml, respectively, as compared to standard ascorbic acid having IC<sub>50</sub> of 21.74 µg/ml (Fig. 4). The ability of the PNFC and PNFEA to scavenge the hydroxyl radicals generated through Fenton reaction as measured by the degradation of deoxy-D-ribose yielding malondialdehyde (MDA) was measured as TBARS. Both PNFC and PNFEA have concentration-dependent OH scavenging activity with IC<sub>50</sub> value 433.19 µg/ml and 440 µg/ml, respectively, compared to standard mannitol which showed an IC<sub>50</sub> of 185.5 µg/ml (Fig. 5). Results of scavenging activity conforms concentration-dependent activity of PNFC and PNFEA fractions. As concentration increases percentage absorbance also increased when compared to standard ascorbic acid which is indicative of both fractions possess strong reducing power activity (Fig.6).



**Fig. 4** DPPH radicals scavenging activity of **a** PNFC and PNFE (b) ascorbic acid



**Hepatoprotective activity**

**Acute toxicity studies (LD<sub>50</sub>)**

The endophytic fractions PNFC and PNFEA were subjected to acute toxicity studies as per OECD guidelines (up and down method). *Phyllanthus niruri* Linn. stem did not show any toxicity and behavioral changes, and no mortality was observed in mice up to 2000 mg/kg and hence, doses of 50 mg/kg and 100 mg/kg were selected for hepatoprotective activity.

**Evaluation of hepatoprotective activity**

**Effect of PNFC and PNFEA on biochemical markers in CCl<sub>4</sub>-induced hepatotoxicity**

The administration of CCl<sub>4</sub> leads to significant hepatocellular damage as evident from the increase in serum activities of SGPT (80.14 ± 0.13\*\*\*), SGOT (140 ± 6.73\*\*), SLAP (566.2 ± 0.4\*\*\*), total bilirubin (0.21 ± 0.019\*), direct bilirubin (0.04 ± 0.004\*\*), total cholesterol (79.06 ± 0.82\*\*\*), proteins (6.23 ± 0.07\*\*), and LPO (0.15 ± 0.005\*\*\*) along with decrease in level of CAT (0.11 ± 0.006\*\*\*) and SOD (0.15 ± 0.02\*\*\*) in comparison to normal control group.

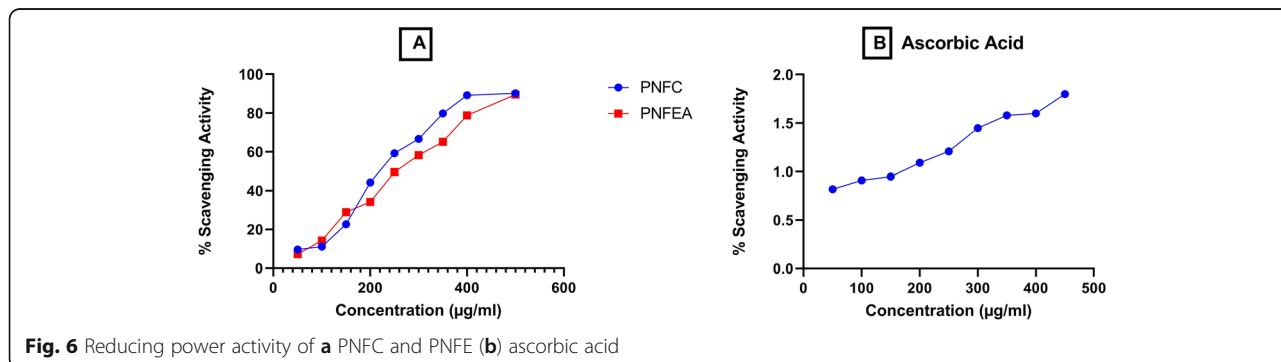
Treatment with PNFC fraction at dose of 100 mg/kg p.o. exhibited significant reduction of induced elevation of serum SGPT (42.66 ± 1.69\*\*\*), SGOT (103 ± 4.82\*\*),

SLAP (434.32 ± 0.5\*\*\*), total bilirubin (0.09 ± 0.011\*), direct bilirubin (0.013 ± 0.002), total cholesterol (69.43 ± 0.70), proteins (5.30 ± 0.07\*\*), and LPO (0.11 ± 0.001\*\*\*) along with increase in level of CAT (0.21 ± 0.006\*\*) and SOD (0.30 ± 0.02\*\*\*) as illustrated in Tables 2 and 3.

Treatment with PNFEA fraction at dose of 100 mg/kg p.o. exhibited significant reduction of induced elevation of serum SGPT (42.51 ± 2.41\*\*\*), SGOT (108 ± 1.83\*), SLAP (409.4 ± 0.68\*\*), total bilirubin (0.07 ± 0.005), direct bilirubin (0.02 ± 0.003), total cholesterol (71.73 ± 0.40), proteins (5.31 ± 0.06\*\*), and LPO (0.12 ± 0.005\*) along with increase in level of CAT (0.25 ± 0.01\*) and SOD (0.32 ± 0.02\*\*) as illustrated in Tables 2 and 3. Treatment with standard silymarin also responded well and reduced hepatotoxicity. However, both fractions PNFC and PNFEA produced hepatoprotective activity in a dose-dependent manner. Comparative study of effect of PNFC and PNFEA on normal control, standard silymarin, and CCl<sub>4</sub> in different serum biochemical parameters is illustrated in Tables 2 and 3.

**Hepatoprotective activity of PNFC and PNFEA**

(A) *In histopathology of normal rat liver* is the liver of a control rat showing normal hepatocytes and normal architecture. (B) *Rat liver histopathology in CCl<sub>4</sub>-treated group* shows focal hemorrhage, inflammation,



**Table 2** Effect of PNFC and PNFEA fractions on SGPT, SGOT, SALP, and total and direct bilirubin in CCl<sub>4</sub>-induced rats

Groups	Dose (mg/kg)	SGPT (mg/dl)	SGOT (mg/dl)	SALP (mg/dl)	Total bilirubin (mg/dl)	Direct bilirubin (mg/dl)
Control	2 ml/kg	39.10 ± 0.27	105 ± 0.67	458.1 ± 0.9	0.045 ± 0.007	0.015 ± 0.002
CCl <sub>4</sub> treated	2 ml/kg	80.14 ± 0.13***	140 ± 6.73**	566.2 ± 0.4***	0.21 ± 0.019*	0.04 ± 0.004**
Silymarin	25 mg/kg	44.44 ± 2.08***	96.5 ± 0.9*	406.2 ± 1.1***	0.13 ± 0.010	0.018 ± 0.003
PNFC	50 mg/kg	50.75 ± 2.51***	111 ± 2.83*	336.7 ± 0.9***	0.12 ± 0.010	0.03 ± 0.002***
PNFC	100 mg/kg	42.66 ± 1.69***	103 ± 4.82**	434.32 ± 0.5***	0.09 ± 0.011*	0.013 ± 0.002
PNFEA	50 mg/kg	53.92 ± 1.35***	114 ± 0.10*	385.3 ± 0.8***	0.14 ± 0.014	0.03 ± 0.002
PNFEA	100 mg/kg	42.51 ± 2.41***	108 ± 1.83*	409.4 ± 0.68**	0.07 ± 0.005	0.02 ± 0.003

The data were expressed as mean ± S.E.M. for six rats in each group. Statistical comparison was performed by one-way ANOVA followed by Turkey's post test \*P < 0.05, \*\*P < 0.01, and \*\*\*P < 0.001 compared to normal control-treated group

ballooning hepatocytes, apoptosis, and degeneration stained with hematoxyline-eosine. (C) *Rat liver histopathology in silymarin (25 mg/kg) treated group* shows no focal hemorrhage stained with hematoxyline-eosine. (D) *Rat liver histopathology in PNFC (50 mg/kg)-treated group* shows no focal hemorrhage and slight ballooning hepatocytes stained with hematoxyline-eosine. (E) *Rat liver histopathology in PNFC (100 mg/kg)-treated group* shows no focal hemorrhage and slight ballooning hepatocytes stained with hematoxyline-eosine. (F) *Rat liver histopathology in PNFEA (50 mg/kg) treated group* shows mild degeneration and reverting to regeneration, stained with hematoxyline-eosine. (G) *Rat liver histopathology in PNFEA (100 mg/kg)-treated group* shows complete regeneration and normal architecture of hepatocytes stained with hematoxyline-eosine (Fig. 7).

**Discussion**

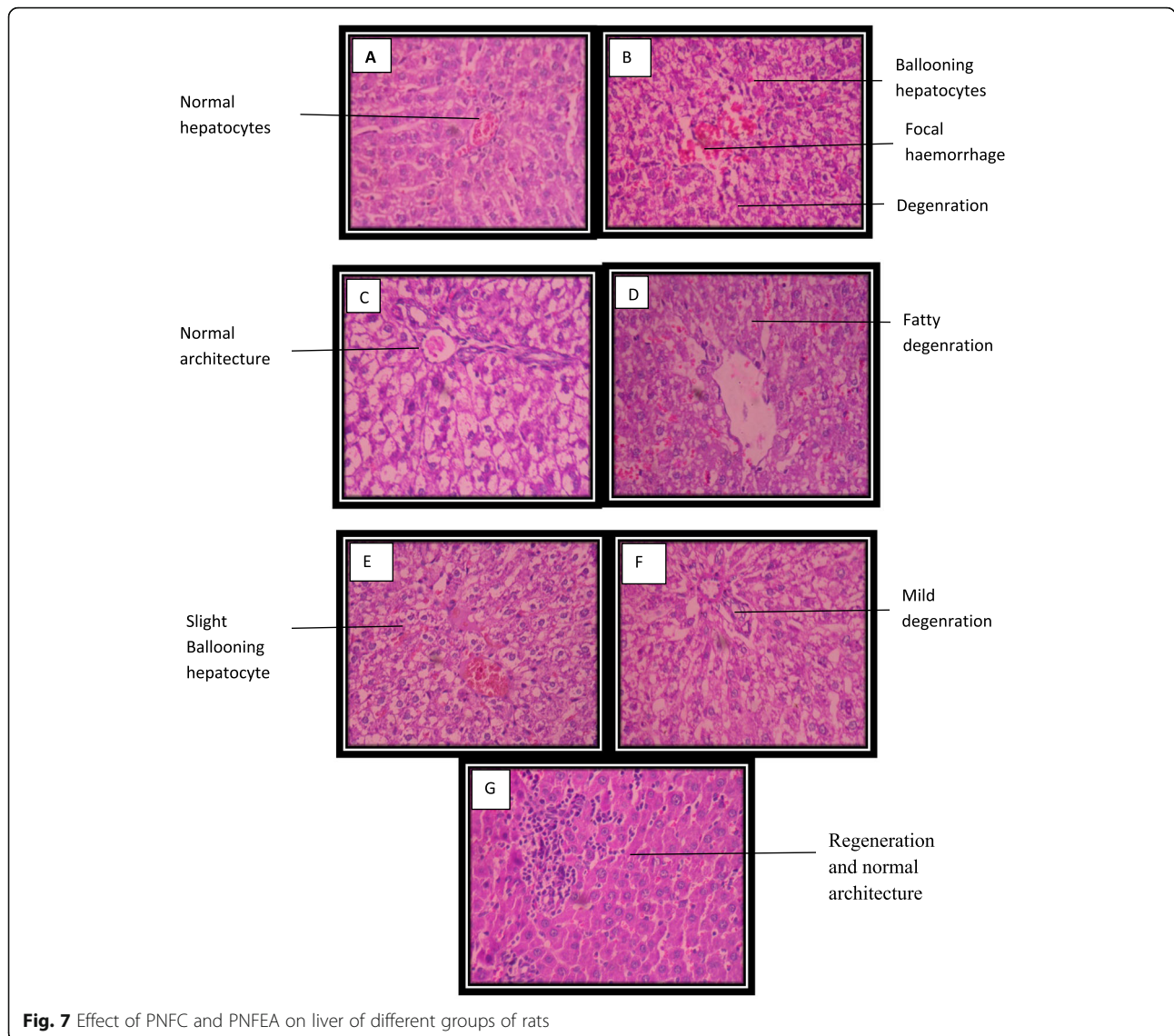
Endophytic fungi *Eurotium amstelodami* was identified and conformed by PCR sequential analysis which is a type of soil borne fungus. These Eurotium species mainly occurs in grain products, poultry feed, bakery products, dried fruits, spices, soil, hypersaline waters, and Dead Sea soil. *E. amstelodami* produced neoechinulin-A and neoechinulin-B as major metabolites [23, 24]. IC<sub>50</sub> is a good measure of antioxidant potential of both endophytic fractions PNFC and PNFEA showed DPPH scavenging

activity with IC<sub>50</sub> of 97.79 µg/ml and 108.40 µg/ml, respectively. Damage to the liver cells by CCl<sub>4</sub> mainly involves metabolism of CCl<sub>4</sub> to trichloromethyl free radical by the mixed function oxidase system of the endoplasmic reticulum. It is postulated that secondary mechanism links CCl<sub>4</sub> metabolism to the wide spread disturbance in hepatocyte function. These secondary mechanisms mainly responsible for the generation of toxic product arising directly from CCl<sub>4</sub> metabolism [25]. In the presence of oxygen, auto-oxidation of the fatty acids occurs in the cytoplasmic membrane which was mediated by free radicals [26]. Functional along with morphological variations in cell membrane occurs due to phospholipids. Furthermore, entry of extracellular Ca<sup>++</sup> ions into cells causes hepatic cell death which produce and release high concentration of SGOT and SGPT in the blood. Total protein, bilirubin, and SALP concentration represents liver function of hepatocyte. Protein level gives a clue regarding hepatoprotective potential as fall in protein level indicates cellular dysfunction in liver diseases and rapid biosynthesis of proteins promote regeneration of hepatocytes which conforms hepatoprotective potential [27]. Bilirubin and SALP levels are indicative of severity of cell death and biliary pressure. Elevated level above normal of total bilirubin produces hepatocellular dysfunction and rapid rise of biliary pressure due to increase in synthesis of SALP [28]. Significant increase (p < 0.01) of total proteins

**Table 3** Effect PNFC and PNFEA of fractions on total cholesterol, total proteins, LPO, CAT, and SOD in CCl<sub>4</sub>-induced rats

Groups	Dose (mg/kg)	Total cholesterol (mg/dl)	Total proteins (mg/dl)	LPO level (µmol/mg protein)	CAT level (µmol/mg protein)	SOD level (µmol/mg protein)
Control	2 ml/kg	69.83 ± 0.30	5.78 ± 0.04	0.09 ± 0.003	0.32 ± 0.008	0.48 ± 0.009
CCl <sub>4</sub> treated	2 ml/kg	79.06 ± 0.82***	6.23 ± 0.07**	0.15 ± 0.005***	0.11 ± 0.006***	0.15 ± 0.02***
Silymarin	25 mg/kg	65.15 ± 0.98	5.46 ± 0.08	0.09 ± 0.0006**	0.18 ± 0.002*	0.28 ± 0.022***
PNFC	50 mg/kg	73.29 ± 0.82	5.36 ± 0.04**	0.11 ± 0.004*	0.15 ± 0.01**	0.26 ± 0.015**
PNFC	100 mg/kg	69.43 ± 0.70	5.30 ± 0.07**	0.10 ± 0.001***	0.21 ± 0.006**	0.30 ± 0.02***
PNFEA	50 mg/kg	73.49 ± 0.98	6.07 ± 0.05*	0.12 ± 0.001*	0.21 ± 0.07***	0.31 ± 0.014**
PNFEA	100 mg/kg	71.73 ± 0.40	5.31 ± 0.06**	0.10 ± 0.005*	0.25 ± 0.01*	0.32 ± 0.02**

The data were expressed as mean ± S.E.M. for six rats in each group. Statistical comparison were performed by one-way ANOVA followed by Turkey's post test \*P < 0.05, \*\*P < 0.01, and \*\*\*P < 0.001 compared to normal control-treated group



**Fig. 7** Effect of PNFC and PNFEA on liver of different groups of rats

observed in  $\text{CCl}_4$ -treated group compared with the normal control rats while group treated with PNFC and PNFEA showed significant decrease ( $p < 0.05$ ;  $p < 0.01$ ), respectively, of total proteins in comparison with the  $\text{CCl}_4$ -treated group. Like standard silymarin-treated group with PNFC and PNFEA, silymarin stimulated the rate of protein biosynthesis in hepatocyte and enhanced the generation and production process of liver cells.  $\text{CCl}_4$ -treated group showed rapid increase of bilirubin and SALP ( $p < 0.01$ ;  $p < 0.001$ ), respectively, when compared to normal control group. These results indicate that  $\text{CCl}_4$  induces hepatobiliary disease and group treated with both endophytic fractions showed decrease ( $p < 0.05$ ;  $p < 0.01$ ) of total bilirubin in comparison with  $\text{CCl}_4$ -treated group which is very much similar to the silymarin-treated group. These finding strongly indicate both endophytic fractions have hypobilirubin activity [29].  $\text{CCl}_4$ -treated group

showed significant increase in total cholesterol level in liver and induce cholesterol deposition which produce adverse effects on the liver tissues and cardiovascular system [24]. Treatment with both endophytic fungi PNFC and PNFEA showed significant decrease in total cholesterol level ( $p < 0.05$ ;  $p < 0.01$ ), respectively, was observed. Results strongly suggest both endophytic fungi strongly enhance capacity of endogenous enzyme to destroy or capture reactive oxygen species (ROS) and prevent the occurrence of certain pathological conditions [30]. After treatment with  $\text{CCl}_4$ , lipid peroxidation has been destructive process in liver injury. Malondialdehyde (MDA) is the end product of lipid peroxidation. Rise of MDA in liver cells which is sing of lipid peroxidation has taken place and incapability of antioxidant defense system to prevent formation of many free radicals and is there for liver tissue damage [31]. Rise in inflammatory signals during the lipid



peroxidation process which are markedly inhibited by both endophytic fractions and silymarin.  $\text{CCl}_4$ -treated group showed significant increase ( $p < 0.001$ ) of MDA level compared to normal control group which is rapidly reduced in rats treated with PNFC and PNFEA ( $p < 0.05$ ;  $p < 0.01$ ), respectively, and silymarin. Hepatoprotective activity is mainly an indication of its protective effects to reduce the injurious and maintain normal hepatic mechanism which is disturbed by hepatotoxins [32]. SOD and CAT are antioxidant enzymes used to remove reactive oxygen species which produces oxidative stress results in liver injury. CAT possess protective potential against the tissue from hydroxyl radicals by converting  $\text{H}_2\text{O}_2$  in to water and oxygen [33]. Reduced potential of CAT and SOD indicate hepatic damage in  $\text{CCl}_4$ -treated group. In present investigation, PNFC and PNFEA significantly increase SOD ( $p < 0.05$ ;  $p < 0.01$ ) and CAT activities ( $p < 0.01$ ;  $p < 0.001$ ), respectively. The restoration of tissue activities of SOD and CAT confirms the protective mechanism of both endophytic fractions. PNFC and PNFEA (100 mg/kg) of *Phyllanthus niruri* Linn. rise in depleted enzyme level induced by  $\text{CCl}_4$  confirms hepatoprotective potential by regeneration of damaged liver cells. Both endophytic fungi are capable of maintaining the activity of hepatic enzymes which are involved in combating reactive oxygen species. The hepatoprotective action of *Phyllanthus niruri* Linn. endophyte was evidenced by the amelioration of biochemical indicators of liver damage and pathological disturbances caused by  $\text{CCl}_4$ .

## Conclusion

From the results, we can conclude that *Phyllanthus niruri* Linn. stem fraction protects the liver from oxidative damage and could be used as an effective protector in  $\text{CCl}_4$ -induced damage. The potent antioxidant and hepatoprotective potential of PNFC and PNFEA may be due to the secondary metabolites of fungi, i.e., *Eurotium stelodami* strain. The present investigation has also opened avenues for further research in pharmacological studies especially endophytes from medicinal plants in development of potent biomedicine for treatment of various chronic diseases.

## Abbreviations

$\text{CCl}_4$ : Carbon tetrachloride; PNFC: Chloroform fraction of *Phyllanthus niruri* L. fungi; PNFEA: Ethyl acetate fraction of *Phyllanthus niruri* L. fungi; SGPT: Serum Glutamate Pyruvate Transaminase; SGOT: Serum Glutamate Oxaloacetate Transaminase; SALP: Serum Alkaline phosphatase; TC: Total cholesterol; LPO: Lipid peroxidation; TBARS: Thiobarbituric acid reactive substances; OECD: Organization for Economic Co-operation and Development; DPPH: 2,2-diphenyl-1-picrylhydrazyl; OH: Hydroxyl; PO: Per Oral; MDA: Malondialdehyde; mg/kg: Milligram/kilogram; PNLF: *Phyllanthus niruri* Linn. leaf fungi; PNSF: *Phyllanthus niruri* Linn. Stem fungi; CPCSEA: Committee for Purpose of Control and Supervision on Experiments on Animals; SEM: Standard error of mean; IAEC: Institutional animal Ethical Committee

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## Plant source

The whole plant of *Phyllanthus niruri* Linn. family (Phyllanthaceae) was collected from Dharwad identified and authenticated by Dr. G.R. Hegde Karnatak University, Dharwad, Karnataka (India), and the voucher specimen no. SETCPD/PH.CO/HERB/05-2016 of herbarium was deposited in the Department of Botany Karnataka University, Dharwad, Karnataka.

## Authors' contributions

RK and SG are the researchers who carried out isolation and all research activities related to endophytes, and SG and DB have major contributors in writing the manuscript. SK and SP are the supervisors who guided in isolation and characterization of endophytes, and PH has major contributions in monitoring pharmacological studies and writing discussion. All authors read and approved the final manuscript.

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## Availability of data and materials

The data sets used and/or analyzed during current research work are available from the corresponding author on reasonable request.

## Declarations

### Ethics approval and consent to participate

All animal studies were approved by the Institutional Animal Ethical Committee (IAEC) of Sonia Educational Trusts College of Pharmacy, Dharwad, Karnataka (reg. No. 112/PO/Re/S/1999/CPCSEA) and conducted under the provisions of the CPCSEA guidelines.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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