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Hepatoprotective properties of phenolic acids from *Thespesia populnea* Soland ex. Correa

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Abstract

The alcohol extract of *Thespesia populnea* Soland ex. Correa, a traditional medicinal plant, was shown to have antihepatotoxicity against carbon tetrachloride (CCl_4) toxicity in rats. Follow up studies were carried out to isolate the active principle or fraction and evaluate its efficacy against various toxic chemicals-induced liver damages and hepatitis B virus. An active ethyl acetate fraction was isolated from the alcohol extract of *T. populnea* by activity guided solvent fractionation. The fraction was further separated into two major components, using column chromatography. The fast moving component on thin layer chromatogram (TLC) with an Rf value of 0.8 was found to be the active component which showed positive reaction to phenolic acids. HPLC analysis showed 3 closely related compounds in the phenolic acid fraction (PF). The hepatoprotective activity in rats of the PF against CCl_4, acetaminophen and thioacetamide was evaluated by measuring levels of serum marker enzymes for liver damage, serum proteins, bilirubin, glucose, antioxidant enzymes and lipid peroxides. Histological studies were also carried out for obtaining supportive data. The activity of the PF was found to be dose dependent and, interestingly, the activity at 2 mg/kg against CCl_4 toxicity in rats was comparable to that of 100 mg/kg silymarin, a standard herbal drug. PF was found to be effective both in pre and post treatment to CCl_4 challenged rats. PF protected rats from acetaminophen and thioacetamide induced hepatotoxicity also. Further, PF showed antihepatitis B viral activity in HepG2.2.15 cell line. Thus, PF is promising for the development of a medicine for liver diseases.

Key words: *Thespesia populnea*, Hepatoprotection, Antihepatitis B viral activity, Phenolic acids, Carbon tetrachloride, Acetaminophen, Thioacetamide

Introduction

Liver diseases are among the most life threatening diseases. Major causes of liver diseases include infection with hepatitis viruses and exposure to toxic chemicals. The hepatitis B virus (HBV) is a major global health problem and the world's ninth leading cause of death (Xing *et al*., 2008). Although several antiviral drugs have been approved for the treatment of hepatitis B viral infection, including interferon-alpha and nucleoside analogues, unresolved significant issues remain with current drugs, such as moderate efficacy, dose-dependent side-effects and drug resistance (Perrillo, 2005). Satisfactory treatment to severe liver diseases is not available in the conventional medicine. Herbal medicines play a vital role in the management of liver diseases. Numerous medicinal

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plants and their formulations are used for liver disorders in ethnomedical practices as well as in traditional systems of medicine in India (Subramoniam *et al*., 1998).

Thespesia populnea Soland ex. Correa, a plant of the Malvaceae family, is used in folk-medicine in India for the treatment of liver diseases. The decoction of the bark is used by Ayurvedic physicians for the treatment of skin and liver diseases (Varier, 1994). The bark and flower of *T. populnea* possess pharmacological properties such as hepatoprotection, antioxidant activity, anti-inflammation, memory enhancement property and hypocholesterolemic activity (Yuvaraj and Subramoniam, 2009; Shirwaikar *et al*., 1995; Ilavarasan *et al*., 2003; Vasudevan *et al*., 2007; Vasudevan and Parle, 2006 and Sathyanarayana *et al*., 2004).

In our preliminary studies, remarkable hepatoprotective activity of this plant (stem bark) powder (water suspension, 500 mg/kg) and ethanol extract (60 mg/kg) against carbon tetrachloride-induced liver damage in Wistar rats was observed (Yuvaraj and Subramoniam, 2009). The present study was aimed at isolation, identification and characterisation of the active principle(s) or fraction and evaluation of its efficacy against hepatotoxic chemicals (CCl_4, acetaminophen and thioacetamide) in rats and determination of *in vitro* antihepatitis B viral activity. Further, the post treatment (curative) effect of the herbal drug on CCl_4 toxicity was also evaluated.

Materials and Methods

Chemicals

Carbon tetrachloride, thioacetamide and acetaminophen were purchased from Merck (Darmstat, Germany). The solvents and chemicals used were of HPLC and analytical grade, respectively.

Animals

Male albino rats of Wistar strain weighting 190 ± 10 g were used for the study. They were housed in polypropylene cages under standard conditions (23 ± 2°C, humidity 60-70%, 12 h light/dark cycles) and fed with standard pellet diet (Sai Durga Feeds and Foods, Bangalore, India) and water ad-libitum. The protocol was approved by Institutional Animal Ethics Committee, constituted as per the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals).

Plant materials, preparation of extract and fractions

T. populnea stem bark was collected from Thodupuzha, Kerala, India in the month of March. The specimens were identified and voucher specimens were deposited in the Herbarium (TTP-876) of Nagarjuna Herbal Concentrates Ltd, Kerala, India. The dried and coarsely powdered stem bark of *T. populnea* was macerated with ethanol for 4 h by continuous stirring at room temperature and then filtered and evaporated the filtrate to dryness under reduced pressure. The yield of ethanol extract was 23% of the stem barks powder. The dried ethanol extract was then suspended in distilled water (1:50, w/v) and fractionated through successive extractions (twice each) with chloroform (1:1, v:v), ethyl acetate (1:1, v:v), n-butanol (1:1, v:v)) saturated with water. Each fraction, except, water fraction was concentrated to dryness under reduced pressure and below 45°C on a rotary vacuum evaporator. The water fraction was freeze dried in a lyophilizer. The yields of chloroform, ethyl acetate, butanol and water fractions of ethanol extract were 28, 14, 26 and 21 percentages, respectively.

Thin layer chromatography (TLC) of ethyl acetate fraction

Ethyl acetate fraction which showed activity was subjected to silica gel (NP-Silica Gel 60 F_{254} plates, Merck, Germany) TLC using solvent system: hexane-ethyl acetate-acetic acid (5:5:0.1, v:v:v). The developed plates were allowed to air dry and chromatograms were observed under visible and UV light.

Fractionation of ethyl acetate fraction and isolation of phenolic acid fraction

In order to isolate the components indicated on TLC analysis, the ethyl acetate fraction was subjected to silica gel 60-120 mesh size (Merck, Mumbai) column chromatography (column 15 X 400 mm, 20 g silica gel and 1g sample) and eluted with hexane-ethyl acetate (100:0), (90:10), (80:20), (70:30), (60:40), (50:50), (40:60), (30:70), (20:80) and (10:90), successively; each fraction was extracted in a volume of 10 ml of the solvent mixture. Eluents were combined according to TLC (NP-Silica Gel 60 F_{254} plates) behaviour using solvent system: hexane-ethyl acetate-acetic acid (5:5:0.1, v:v:v). The developed plates were allowed to air dry and chromatograms were observed under visible and UV light (k = 254 nm). Each fraction was tested for hepatoprotective activity against CCl_4 toxicity in rats.

Chemical analysis of active component/fraction

In the column chromatography, fraction 25-50 showed a single band on TLC with an Rf value of 0.8. This component was found to be active. This was subjected to chemical analysis. The plates were sprayed with Folin - Ciocalteu's phenol reagent and fumigated ammonia vapour, the plate developed dark blue colour band (Harborne, 1973). The yield of active component was 53% of ethyl acetate fraction and 1.7 percentages of plant stem bark powder. This active component was used for further studies. The phytochemical identification was carried out by the methods of Trease and Evans (1983) and Harborne (1973).

High performance liquid chromatographic (HPLC) analysis of phenolic fraction

The active component, isolated by column chromatography was subjected to HPLC analysis. In order to determine the

concentration of active column fractions (25-50), following HPLC conditions were used: column: Merck make Lichrospher NP- Si 60 column 150 × 4 mm (5 micron); wavelength: 289 nm; flow rate: 1 ml/min; mobile phase: Hexane-Iso propylalcohol-Orthophosphoric acid [90:10:0.10]; sample was injected with Rheodyne sample injector with 20 µl loop at 20 µl in Shimadzu HPLC system comparing of LC-8 A pump, SPD 10 A.

Experimental procedure/ protocol

Evaluation of different fractions of T. populnea on CCl_4-induced liver damage in rats

Animals were randomly divided into nine different groups, each of six rats. The first group served as the control. Groups 2-9 animals were orally administered with CCl_4 (1:1 in liquid paraffin, 2 ml/kg) on third day of the experiment. Groups 3, 4, 5, 6, 7 and 8 animals were given chloroform fraction (16 mg/kg), ethyl acetate fraction (4, 8 and 16 mg/kg), n-butanol fraction (15 mg/kg) and water fraction (12 mg/kg) of alcohol extract of *T. populnea*, respectively for 4 days (dosage equal to yield of respective fractions from 60 mg/kg ethanol extract). Group 9 animals were given silymarin (100 mg/kg), a well known hepatoprotective herbal drug for 4 days. All treatments were done orally and the animals were sacrificed under mild anesthesia after 48 h of CCl_4 administration. The blood samples were allowed to clot and serum was separated. Liver was dissected out and weighed and a portion was used for histopathological studies.

Identification of hepato-protective component of the ethyl acetate fraction of T. populnea

To test the 2 major components of ethyl acetate fraction for hepatoprotective activity, animals were randomly divided into four groups, each group contain six rats. Group I (vehicle control) animals received liquid paraffin and 5% Tween 80 (0.5 ml/animal), Group II (CCl_4 control) animals received CCl_4 and 5% Tween 80 (0.5 ml/animal); Group III animals received CCl_4 and four daily doses of the component I (TLC - band A., Rf: 0.8) 2 mg/kg in 5% Tween 80 and Group IV animals received CCl_4 and four daily doses of the component II (TLC- band B), 2 mg/kg, in 5% Tween 80. CCl_4 (2 ml/kg of 1:1 mixture in liquid paraffin) was administered on day 3. All animals were sacrificed 48 h after CCl_4 administration.

Dose response study of phenolic acids fraction

For dose response study of active component, 36 rats were equally divided into six groups: Group I (vehicle control), Group II (CCl_4 control), Groups III, VI, V and VI, where the animals received CCl_4 and four different doses of the active component at 0.5, 1, 2 and 4 mg/kg, respectively. Details of treatments are as given above.

Post-treatment (curative study) with phenolic acids fraction

For curative study, 24 rats were divided into four groups of 6 rats each. Group I (vehicle control); Group II (CCl_4 control), Groups III animals received CCl_4 and three daily doses of the phenolic acid fractyion (2 mg/kg) and Group IV animals received CCl_4 and silymarin (100 mg/kg). Groups II–IV animals were administered orally with a single dose of CCl_4 (2 ml/kg of 1:1 mixture in liquid paraffin) on day 1. Groups III and IV received the active component and silymarin respectively on day 2, 3 and 4. All animals were sacrificed 96 h after CCl_4 administration (Tao *et al*., 2008).

Acetaminophen-induced liver damage

Rats were divided into control, acetaminophen and test (active component + acetaminophen) groups, each comprising of six animals. Acetaminophen was suspended in 40% (w/v) aqueous sucrose solution and administered orally at a dose of 3 g/kg (Porchezhian and Ansari, 2005). Normal control rats received single daily dose of 5% Tween 80 in water (5 ml/kg, p.o.) for 4 days and a single dose of 40% sucrose solution (1 ml per rat, p.o.) on day 3. Acetaminophen control group received single daily dose of 5% Tween 80 solution for 4 days and single dose of acetaminophen suspension on day 3. Test groups received daily doses of the active component (2 mg/kg) in 5% Tween 80 (1ml per rat), for 4 days and a single dose of acetaminophen on day 3. The animals were sacrificed 48 h after acetaminophen administration by mild ether anaesthesia. From all the three groups, blood and liver samples were collected for biochemical and histological studies.

Thioacetamide induced hepatotoxicity

Rats were divided into three groups, consisting of six animals in each group. Group I was normal control, Group II was thioacetamide control, both received 5% Tween 80 for four days. Groups III received the active component at an oral dose of 2 mg/kg in 5% Tween 80 (1ml per rat) for four days. Groups II and III received a single dose of thioacetamide (200 mg/kg; i.p.) on the third day. The animals were sacrificed 48 h after the thioacetamide injection by mild ether anaesthesia. From all the three groups, blood and liver samples were collected for biochemical and histological studies.

Determination of anti-HBV activity in HepG2.2.15 cells

HepG2.2.15 cells (human hepatoma cell line HepG2 stably transfected with HBV genome) were cultured and maintained in minimal essential medium (MEM), supplemented with 0.25% fetal calf serum (FCS), 100 IU/ml penicillin, 100 mg/ml streptomycin, and 200 mg/ml G418 at 37° C in a humidified incubator with 5% CO_2. The cells were incubated at a density of 5 X 10^4/ml per well in 96-well culture plates. The cells were grown with various concentrations of drugs for 9 days with changes of drug-containing medium every day. The culture medium was harvested. An aliquot of the culture medium (5 ml) was used for estimation of HBsAg (hepatis B surface antigen).

Cell viability assay using XTT (Sodium-2,3-bis-[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide)

The XTT assay was performed according to Pettit *et al*. (2005) with some modifications. The sodium salt of XTT (Sigma) was dissolved in saline to give a concentration of 1 mg/ml. Menadione (Sigma) was dissolved in acetone to give a concentration of 1 mmol/l. The XTT/Menadione reagent was prepared freshly prior to each assay and contained 12.5 parts XTT/1 part Menadione. For cell culture preparation, 200 ìl of overnight cells, adjusted to 5 x 10^5 cells/ml in MEM, was added to 96-well flat bottom plate. Following a 17 h treatment of the 96-well plate cells with different concentrations of *T. populnea* phenolic acid fraction (PF), in each experiment, different concentrations of PF (40, 80, 120, 160, 200, 240 and 280 mg/ml) were tested in three separate wells and the cytotoxicity curve was constructed from at least three different experiments; the plates were gently shaken in a shaker incubator and 100 ìl from each well was transferred to a new plate. Then, to each well 25 ìl of XTT/Menadione was added and the plates were gently shaken. After an incubation period of 1 h at 37 °C, the plates were again gently shaken and the absorbance at 490 nm was measured using a microplate reader (Universal Microplate Reader, ELx 800 UV).

Biochemical analysis

Commercial kits from SPAN Diagnostics Ltd, Surat, India and Agappe Diagnostics Pvt. Ltd, Kochi, India were used to measure the hepatic marker enzymes, serum bilirubin, glucose, total protein and albumin. Lipid peroxide levels in serum were estimated using thiobarbituric acid method (Ohkawa *et al*., 1979). GSH levels were determined by the method of Moron *et al*. (1979) and the amount of GSH was expressed as n mole / mg protein.

Assay of antioxidant enzymes

The activities of catalase (Takahara *et al*., 1960), super oxide dismutase (Kakkar *et al*., 1984), glutathione peroxidase (Rotruck *et al*., 1973) and glutathione-S-transferase (Habig *et al*., 1974) were determined in liver homogenate.

Histopathological evaluation

Immediately after the sacrifice, the liver of each rat was removed and an approximately half part was placed in 10% buffered formalin to fix the tissue for microscopic examination. The paraffin sections were prepared in an automatic tissue processor and sliced into 5 µm thick sections in a rotary microtome and then stained with haematoxylin and eosin and mounted with Canada balsam. The histopathological examination of slides was performed under Carl Zeiss photomicroscope and photographed. Images were captured using a Carl Zeiss photomicroscope at original magnification of 400 x.

Statistical analysis

The values are expressed as the Mean ± SD and the significance between different groups were determined by one-way-analysis of variance (ANOVA) coupled with Duncan's Multiple Range Test (DMRT), taking $p < 0.05$ as significant. The percentage of protection was calculated by considering enzymes and bilirubin level difference between CCl_4 control and normal rats as 100 %.

Results

Effect of different fractions of alcohol extract on liver damage

Rats treated with CCl_4 developed liver damage as observed from elevated serum levels of marker enzymes (AST, ALT, ALP) for liver damage and bilirubin levels (Table 1). Activity of GGT in serum was also increased in CCl_4-intoxicated rats. Out of the four fractions of alcohol extract of *T. populnea*, ethyl acetate fraction showed maximum protection against CCl_4-induced alterations in the serum enzyme levels and bilirubin. Chloroform, n-butanol and water fractions have shown some levels of activity (Table 1). The ethyl acetate fraction at 8 mg/kg body weight showed maximum hepatoprotection. The activity of 8 mg/kg body weight ethyl acetate fraction was similar to that of 100 mg/kg body weight silymarin. The histopathological studies of the liver showed ballooning, centrilobular necrosis, inflammatory cells and swelling of hepatocytes in CCl_4-treated rats in comparison with normal control rats. Administration of ethyl acetate fraction of the plant exhibited a significant recovery of the histological alterations. The ethyl acetate fraction at a dose of 8 mg/kg showed almost complete normalization of the histological changes (Figure 3).

Separation and characterisation of phenolic acids fraction

The ethyl acetate fraction was resolved in to two major components on TLC, component I and component II (Figure 1). These components were separated in sufficient quantities by column chromatography.

The component/ fraction which showed a Rf. value of 0.8 on TLC showed activity. Pretreatment with this component (2 mg/kg body weight) provided remarkable hepatoprotection. Serum marker enzymes for liver damage, ALT and AST were reverted to near normal. The other component did not show sufficient hepatoprotective activity (Table 2). The most active component was separated and used for further studies.

Phytochemical analysis indicates that the active component is phenolic acids. However, the component is not a single chemical entity. It showed 3 peaks in HPLC (Figure 2).

Dose dependent effect of phenolic acids fraction (PF)

The dose dependent responses of the hepatoprotective component of *T. populnea* are given in Table 3. Pretreatment

of rats with 0.5, 1, 2 and 4 mg/kg body weight, p. o., of the active component exhibited a dose dependent reduction in the CCl_4-induced increase in the levels of ALT, AST, ALP and bilirubin. Optimum level of protection was observed at 2 mg/kg level. At this dose, the hepatoprotective efficacy of 2 mg/kg of the component was almost equal to that of 100 mg/kg standard herbal drug silymarin (Table 3).

Curative effects of PF

In the post-treatment (curative-treatment) experiments, rats administered with CCl_4 (CCl_4 control), showed an elevated level of serum AST, ALT GGT, ALP, bilirubin, total protein, albumin, glucose and lipid peroxide. Treatment with the active component (2 mg/kg) 24 h after CCl_4 treatment (for 3 days) cured the rat liver from CCl_4-induced hepatotoxicity. Standard herbal drug, silymarin (100 mg/kg) also showed similar curative effect towards CCl_4 intoxication (Table 4). Rats treated with the active component (2 mg/kg) and silymarin (100 mg/kg) after the establishment of some level of toxic injury showed recovery of hepatic damage. This fact was confirmed by histological observation also (Figure 4).

Acetaminophen-induced hepatotoxicity

Administration of acetaminophen (3 g/kg, p.o.) induced a marked increase in the serum hepatic enzyme levels, ALT, AST, ALP, GGT, bilirubin (total and direct), total cholesterol, triglycerides, glucose, TBARS and urea whereas total protein and albumin levels were decreased compared to normal controls indicating liver damage. Pretreatment of the rats with the active component (2 mg/kg) prior to acetaminophen administration caused a reduction in the values of ALT, AST, ALP, GGT, bilirubin (total and direct), total cholesterol, triglycerides, glucose, TBARS, urea and also almost restored the decreased levels of total protein and albumin (Table 5). The liver antioxidant enzymes (catalase, SOD, GPx, GST) and GSH levels were decreased and lipid peroxidation levels were increased in the acetaminophen administered animals. Pretreatment of the rats with PF (2 mg/kg) increased the activities of tissue antioxidant enzymes (catalase, SOD, GPx, and GST) and GSH whereas lipid peroxidation levels were decreased towards their normal control values (Table 6).

Histopathological examination of liver tissue also confirmed the hepatoprotective activity of the active component against acetaminophen toxicity in rats. The histoarchitecture of acetaminophen treated liver sections showed fatty degeneration of hepatocytes with centrilobular necrosis. Treatment with the active component (2 mg/kg) resulted in normalization of these defects in histo architecture (photomicrograph not shown).

Thioacetamide-induced hepatotoxicity

Activities of marker enzymes for liver damage (ALT, AST and ALP, GGT), bilirubin (total and direct), total cholesterol, triglycerides, glucose, TBARS and urea were markedly elevated, whereas total protein and albumin levels were decreased in the group of rats given thioacetamide (200 mg/kg i.p.). The pretreatment with the active component (2 mg/kg) exhibited prevention of thioacetamide induced enhancement in the levels of all the biochemical parameters studied and increased serum total protein and albumin levels, resulting in restoration towards their normal control values (Table 7). The liver tissue parameters catalase, SOD, GPx, GST and GSH were decreased whereas TBARS levels were increased when the animals were challenged with thioacetamide (200 mg/kg i.p.). Treatment with the active component (2 mg/kg) restored these changes almost to the levels of their normal control values (Table 8).

Histopathological examination of liver tissue also confirmed the hepatoprotective activity of the active component against thioacetamide toxicity in rats. The histoarchitecture of thioacetamide treated liver sections showed the appearance of centrilobular necrosis with tiny vacuoles, lymphocyte infiltration and fat accumulation. Treatment with the active component (2 mg/kg) resulted in normalization of these defects in the histo-architecture (photomicrograph not shown).

Anti-HBV activity of *T. populnea* in HepG2.2.15 cells

Treatment of HepG2.2.15 cells with *T. populnea* at various concentrations for 9 days resulted in significant reduction of HBsAg secretion in dose-dependent manner. After 6 days treatment of *T. populnea* at concentration of 240 µg/ml and 280 µg/ml had significantly inhibited HBsAg secretion at 67%. The continuation of treatment for 8^{th} and 9^{th} days, still significantly reduced HBsAg secretion; 240 µg/ml of *T. populnea* on 8^{th} day inhibited HBsAg secretion (93%) and on 9^{th} day 240 µg/ml PF showed remarkable (97.3%) reduction of HBsAg secretion. These data demonstrated that the inhibition of HBV gene expression by PF was efficient and persistent (Figure 5).

Cell viability assay using XTT

The growth of the HepG2.2.15 cells in the presence of various concentrations of *T. populnea* (PF) was examined. The results from the XTT test showed that there was significant difference of cell viability between PF treated groups whose concentration was 240 µg/ml and control group. PF inhibited the growth of HepG2.2.15 cells at 240 µg/ml and above (Figure 6).

Table 1: Effect of different fractions of ethanol extract of *T. populnea* (bark) on serum biochemical parameters in carbon tetrachloride induced hepatotoxicity in rats

Groups	ALT (IU/L)	AST (IU/L)	ALP (KA unit)	GGT (IU/L)	Total bilirubin (mg/dl)
Normal control	54.2 1.8[a]	95.4 ± 3.7[a]	67.8 ± 2.4[a]	2.4 ± 0.3[a]	0.54 ± 0.02[a]
CCl_4 Control	245.1 ± 17.5[b]	278.9 ± 13.7[b]	130.4 ± 5.2[b]	6.9 ± 0.1[b]	1.60 0.24[b]
Chloroform fraction (16 mg/kg)	131.3 ± 8.2[c] (60)	139.9 ± 6.1[c] (76)	85.5 ± 5.2[c] (72)	3.8 ± 0.3[c](68)	0.69 ± 0.10[c] (86)
Ethyl acetate fraction (4 mg/kg)	104.1 ± 9.4[d](74)	132.7 ± 9.4[c](80)	84.7 ± 4.7[c](73)	3.6 ± 0.1[c](73)	0.70 ± 0.09[c](85)
Ethyl acetate fraction (8 mg/kg)	70.7 ± 2.6[e] (91)	112.7 ± 7.7[d] (91)	78.4 ± 2.2[d] (83)	2.7 ± 0.2[a](93)	0.63 ± 0.06[ac] (92)
Ethyl acetate fraction (16 mg/kg)	86.1 ± 4.9[f](83)	123.0 ± 7.2[e](85)	81.4 ± 4.6[cd](78)	3.1 ± 0.3[d](84)	0.65 ± 0.05[ac](90)
n-Butanol fraction (15 mg/kg)	141.8 ± 9.3[g] (54)	156.0 ± 10.1[f] (67)	96.9 ± 2.3[e] (53)	4.5 ± 0.3[e](54)	0.82 ± 0.05[d] (74)
Water fraction (12 mg/kg)	198.5 ± 6.5[h] (24)	184.4 ± 4.9[g] (52)	107.9 ± 9.0[f] (36)	5.1 0.4[f](41)	0.94 ± 0.04[e] (62)
Silymarin (100 mg/kg)	63.5 ± 5.4[e](95)	102.9 ± 9.5[a](96)	69.5 ± 3.5[a](97)	2.5 ± 0.3[a](98)	0.56 ± 0.10[a](98)

CCl_4, Carbon tetrachloride. Values are Mean ± SD, n= 6 rats in each group. [a-h] In each column, means with different superscript letter differ significantly at $p < 0.05$ (DMRT). Values in parentheses indicate percentage of protection. The dose was selected in each case based on % yield of each fraction. ALT - Alanine aminotransferase; AST - Aspartate aminotransferase; ALP - Alkaline phosphatise; GGT - g-glutamyl transferase

Table 2: Effect of the two components (separated by column chromatography) of ethyl acetate fraction of *T. populnea* (alcohol extract) on serum transaminases in CCl_4 -induced liver damage in rats

Groups	ALT (IU/L)	AST (IU/L)
Normal control	45.5 ± 2.4[a]	76.0 ± 2.8[a]
CCl_4 treated	226.1 ± 5.2[b]	246.3 4.7[b]
Component I [band A] (2 mg/kg)	53.9 ± 4.7[c] (95)	84.6 ± 3.0[c] (95)
Component II [band B] and very minor bands (2 mg/kg)	211.5 ± 5.0[d] (8)	236.7 ± 9.2[d] (6)

Values are Mean ± SD; n= 6 rats in each group. [a-d] In each column, means with different superscript letter differ significantly at $p<0.05$ (DMRT). Values in parentheses indicate percentage of protection. (In TLC, ethyl acetate fraction showed two major bands: one with Rf value of 0.8 (band A); this was separated by column chromatography; the remaining matter in the ethyl acetate fraction was taken as band B for activity determination). CCl_4 - Carbon tetrachloride; TP - Thespesia populnea; ALT - Alanine aminotransferase; AST - Aspartate aminotransferase

Table 3: Effect of different doses of *T. populnea* (phenolic acid fraction) on serum ALT, AST, ALP, and bilirubin in carbon tetrachloride induced hepatotoxicity in rats

Groups	ALT (IU/L)	AST (IU/L)	ALP (KA unit)	Total bilirubin (mg/dl)	Direct bilirubin (mg/dl)
Normal control	49.2 ± 4.9^{a}	87.3 ± 5.5^{a}	70.9 ± 1.9^{a}	0.51 ± 0.05^{a}	0.21 ± 0.04^{a}
CCl_4 control	269.2 ± 1.1^{b}	234.4 ± 0.5^{b}	142.6 ± 1.3^{b}	0.81 ± 0.07^{b}	0.55 ± 0.03^{b}
Active component (0.5 mg/kg) + CCl_4	159.1 ± 4.5^{c} (50)	143.0 ± 3.5^{c} (62)	88.2 ± 5.4^{c} (76)	0.65 ± 0.06^{c} (53)	0.36 ± 0.03^{c} (57)
Active component (1 mg/kg) + CCl_4	72.2 ± 5.8^{d} (90)	118.1 ± 5.0^{d} (79)	79.6 ± 2.5^{d} (88)	0.58 ± 0.12^{c} (78)	0.29 ± 0.03^{d} (76)
Active component (2 mg/kg) + CCl_4	52.3 ± 3.2^{a} (99)	92.7 ± 3.2^{e} (96)	73.9 ± 1.1^{a} (96)	0.53 ± 0.05^{a} (94)	0.24 ± 0.10^{ad} (90)
Active component (4 mg/kg) + CCl_4	50.4 ± 4.1^{a} (99)	89.4 ± 3.3^{ae} (99)	71.9 ± 1.1^{a} (99)	0.52 ± 0.01^{a} (97)	0.23 ± 0.04^{ad} (93)
Silymarin (100 mg/kg) + CCl_4	53.8 ± 3.2^{a} (97)	94.1 ± 3.1^{e} (94)	75.8 ± 1.8^{a} (93)	0.54 ± 0.06^{a} (91)	0.25 ± 0.11^{ad} (88)

CCl_4 - Carbon tetrachloride. Values are Mean ± SD, n= 6 rats in each group. $^{a-e}$ In each column, means with different superscript letter differ significantly at $p<0.05$ (DMRT). Values in parentheses indicate percentage of protection. ALT - Alanine aminotransferase; AST - Aspartate aminotransferase; ALP - Alkaline phosphatase

Table 4: Effect of *T. populnea* (phenolic acid fraction) post treatment (curative study) on serum biochemical parameters in carbon tetrachloride induced hepatotoxicity in rats

Parameters	Normal control	CCl_4 control	TP active component (2 mg/kg)	Silymarin (100 mg/kg)
ALT (IU/L)	47.0 ± 2.9^{a}	177.6 ± 4.2^{b}	49.8 ± 1.9^{ac}	50.3 ± 3.1^{ac}
AST (IU/L)	97.6 ± 1.0^{a}	183.3 ± 4.7^{b}	99.3 ± 2.0^{a}	99.6 ± 2.3^{a}
ALP (KA unit)	69.7 ± 2.1^{a}	131.9 ± 3.3^{b}	71.8 ± 1.6^{a}	70.64 ± 1.9^{a}
GGT (IU/L)	2.35 ± 0.17^{a}	5.09 ± 0.41^{b}	2.39 ± 0.29^{a}	2.93 ± 0.30^{a}
Total bilirubin (mg/dl)	0.54 ± 0.05^{a}	1.03 ± 0.12^{b}	0.58 ± 0.08^{a}	0.56 ± 0.03^{a}
Direct bilirubin (mg/dl)	0.20 ± 0.02^{a}	0.93 ± 0.03^{b}	0.22 ± 0.03^{a}	0.21 ± 0.03^{a}
Total protein (g/dl)	6.54 ± 0.52^{a}	4.37 ± 0.30^{b}	6.50 ± 0.25^{a}	5.52 ± 0.41^{a}
Albumin (g/dl)	4.70 ± 0.11^{a}	3.00 ± 0.09^{b}	4.69 ± 0.06^{a}	4.13 ± 0.09^{a}
Glucose (mg/dl)	76.45 ± 3.60^{a}	89.02 ± 6.02^{b}	77.51 ± 4.13^{a}	76.64 ± 5.02^{a}
TBARS (nano moles MDA/ml)	1.68 ± 0.17^{a}	3.96 ± 0.35^{b}	1.72 ± 0.35^{a}	1.66 ± 0.23^{a}

CCl_4 - Carbon tetrachloride, TP - *Thespesia populnea*. Values are Mean ± SD for 6 rats in each group. $^{a-b}$ In each row, means with different superscript letter differ significantly at $p < 0.05$ (DMRT). ALT - Alanine aminotransferase; AST - Aspartate aminotransferase; ALP - Alkaline phosphatise; GGT - g-glutamyl transferase; TBARS - Thiobarbituric acid reactive substances; MDA- malondialdehyde

Table 5: Effect of phenolic acid fraction on acetaminophen induced hepatotoxicity in rats

Parameters	Normal control	Acetaminophen (3 g/kg)	TP active component (2 mg/kg) + Acetaminophen (3 g/kg)
ALT (IU/L)	55.3 ± 1.9^{a}	244.2 ± 18.3^{b}	60.17 ± 5.00^{a}
AST (IU/L)	92.9 ± 3.0^{a}	243.5 ± 7.5^{b}	102.37 ± 5.10^{c}
ALP (KA unit)	74.2 ± 2.9^{a}	161.4 ± 5.3^{b}	78.84 ± 4.94^{c}
GGT (IU/L)	2.2 ± 0.1^{a}	6.86 ± 0.31^{b}	2.48 ± 0.14^{a}
Total bilirubin (mg/dl)	0.58 ± 0.04^{a}	1.89 ± 0.12^{b}	0.64 ± 0.07^{ac}
Direct bilirubin (mg/dl)	0.20 ± 0.08^{a}	$1.06 \pm .08^{b}$	0.26 ± 0.02^{a}
Total protein (g/dl)	7.06 ± 0.48^{a}	4.45 ± 0.25^{b}	6.72 ± 0.46^{a}
Albumin (g/dl)	5.31 ± 0.11^{a}	3.48 ± 0.11^{b}	5.10 ± 0.17^{c}
Cholesterol (mg/dl)	85.99 ± 4.22^{a}	144.79 ± 4.83^{b}	89.21 ± 2.50^{a}
Triglycerides (mg/dl)	65.70 ± 2.49^{a}	91.72 ± 2.45^{b}	70.74 ± 3.86^{c}
Glucose (mg/dl)	73.56 ± 6.78^{a}	93.94 ± 5.39^{b}	76.10 ± 1.91^{a}
TBARS (nanomoles/MDA/ml)	1.77 ± 0.23^{a}	5.54 ± 0.18^{b}	2.20 ± 0.14^{c}
Urea (mg/dl)	26.07 ± 1.08^{a}	31.35 ± 2.44^{b}	26.04 ± 2.71^{a}

TP - *Thespesia populnea*. Values are Mean ± SD for 6 rats in each group. $^{a\text{-}c}$ In each rows, means with different superscript letter differ significantly at $p < 0.05$ (DMRT).

Table 6: Effect of *Thespesia populnea* (phenolic acid fraction) on acetaminophen induced hepatotoxicity in rats

Parameters	Normal control	Acetaminophen (3 g/kg)	TP active component (2 mg/kg) + Acetaminophen (3 g/kg)
Catalase (nanomoles of H_2O_2 decomposed/min/mg protein)	70.24 ± 3.57^{a}	44.52 ± 1.26^{b}	66.95 ± 2.03^{c}
SOD (Unit /mg protein)*	6.72 ± 0.50^{a}	2.58 ± 0.53^{b}	6.47 ± 0.16^{a}
TBARS (nanomoles malon dialdehyde/mg protein)	1.60 ± 0.25^{a}	7.90 ± 0.44^{b}	2.20 ± 0.28^{c}
GSH (nanomoles/g wet tissue)	5.87 ± 0.12^{a}	2.28 ± 0.18^{b}	5.65 ± 0.12^{c}
GPx (nano mole glutathione oxidized/min/mg protein)	7.18 ± 0.51^{a}	3.59 ± 0.93^{b}	6.54 ± 0.64^{c}
GST (µ moles of CDNB conjugate formed/min/mg protein)	12.28 ± 0.83^{a}	5.05 ± 0.25^{b}	11.32 ± 0.24^{c}
Cholesterol (mg/100 g wet tissue)	340.66 ± 7.57^{a}	520 ± 32.65^{b}	352.5 ± 8.38^{a}
TG (mg/100 g wet tissue)	294.61 ± 4.55^{a}	363.38 ± 3.90^{b}	296.21 ± 4.07^{a}

TP - *Thespesia populnea*. Values are Mean ± SD for 6 rats in each group. $^{a\text{-}c}$ In each rows, means with different superscript letter differ significantly at $p<0.05$ (DMRT).

*One unit of enzyme activity was taken as the enzyme reaction, which gave 50% inhibition of nitroblue tetrazolium reduction in one min.

Table 7: Effect of *T. populnea* (phenolic acid fraction) on thioacetamide induced hepatic damage in rats

Parameters	Normal control	Thioacetamide (200 mg/kg)	TP active component (2 mg/kg) + Thioacetamide
ALT (IU/L)	52.04 ± 0.81[a]	280.66 ± 14.16[b]	58.73 ± 5.44[ac]
AST (IU/L)	90.59 ± 2.65[a]	255.08 ± 3.51[b]	96.84 ± 4.24[c]
ALP (KA unit)	65.02 ± 1.56[a]	167.57 ± 3.62[b]	69.73 ± 1.48[c]
GGT (IU/L)	2.31 ± 0.11[a]	6.71 ± 0.30[b]	2.66 ± 0.11[c]
Total bilirubin (mg/dl)	0.54 ± 0.03[a]	1.82 ± 0.12[b]	0.62 ± 0.05[ac]
Direct bilirubin (mg/dl)	0.20 ± 0.08[a]	1.09 ± 0.02[b]	0.25 ± 0.01[ac]
Total protein (g/dl)	6.82 ± 0.47[a]	4.40 ± 0.19[b]	6.30 ± 0.25[c]
Albumin (g/dl)	5.13 ± 0.11[a]	3.41 ± 0.05[b]	4.96 ± 0.19[c]
Total cholesterol (mg/dl)	90.70 ± 2.97[a]	145.22 ± 2.27[b]	96.15 ± 3.09[c]
Triglycerides (mg/dl)	61.63 ± 1.65[a]	92.61 ± 1.11[b]	68.27 ± 2.14[c]
Glucose (mg/dl)	75.99 ± 6.05[a]	94.95 ± 4.37[b]	80.27 ± 1.83[c]
TBARS (nanomoles malondiald ehyde/ml)	1.81 ± 0.11[a]	5.16 ± 0.28[b]	2.11 ± 0.13[c]
Urea (mg/dl)	24.73 ± 0.26[a]	30.46 ± 0.99[b]	25.96 ± 0.60[c]

TP - *Thespesia populnea*. Values are Mean ± SD for 6 rats in each group. [a-d] In each rows, means with different superscript letter differ significantly at $p < 0.05$ (DMRT). Values in parentheses indicate percentage of protection.

Table 8: Effect of *T. populnea* (phenolic acid fraction) on thioacetamide induced hepatic damage in rats

Parameters	Normal control	Thioacetamide (200 mg/kg)	Phenolic acid fraction (2 mg/kg) + Thioacetamide
Catalase (nanomoles of H_2O_2 decomposed/minute/mg protein)	68.17 ± 1.81[a]	43.06 ± 1.17[b]	66.11 ± 1.54[c]
SOD (Unit / /mg protein)*	6.75 ± 0.37[a]	2.63 ± 0.62[b]	6.05 ± 0.23[c]
TBARS (nanomoles malon dialdehyde/mg protein)	1.49 ± 0.16[a]	8.15 ± 0.44[b]	1.94 ± 0.25[c]
GSH (nanomoles/g wet tissue)	5.62 ± 0.06[a]	2.22 ± 0.09[b]	5.45 ± 0.12[c]
GPx (nano mole glutathione oxidized/min/mg protein)	7.53 ± 0.78[a]	2.73 ± 0.78[b]	7.01 ± 1.06[ac]
GST (µ moles of CDNB conjugate formed/min/mg protein)	12.93 ± 0.75[a]	4.69 ± 0.17[b]	11.90 ± 0.70[c]
Cholesterol (mg/100 g wet tissue)	309.69 ± 6.88[a]	466.66 ± 10.49[b]	316.96 ± 3.78[c]
Triglycerides (mg/100 g wet tissue)	274.41 ± 4.55[a]	361.27 ± 2.10[b]	281.48 ± 3.24[c]

Values are Mean ± SD for 6 rats in each group. [a-c] In each rows, means with different superscript letter differ significantly at $p < 0.05$ (DMRT).

* One unit of enzyme activity was taken as the enzyme reaction, which gave 50% inhibition of nitroblue tetrazolium reduction in one minute.

Figure 1: Silica gel TLC separation of ethyl acetate fraction (Solvent system: hexane:ethyl acetate:acetic acid (5:5:0.1, v:v:v). A - Component I; B - Component II

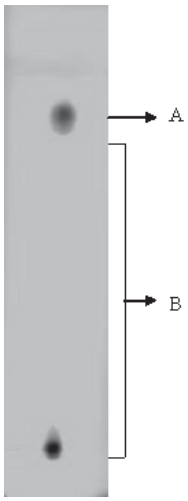


Figure 2: HPLC profile of phenolic acids fraction from *Thespesia populnea*. Three peaks are seen (retention time: 2.649, 3.158 and 3.806 min at 289 nm).

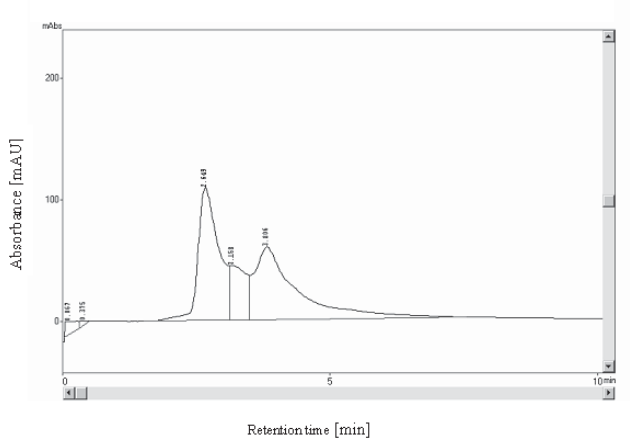
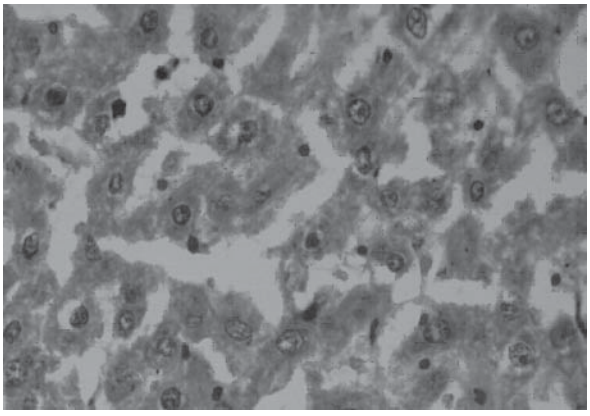
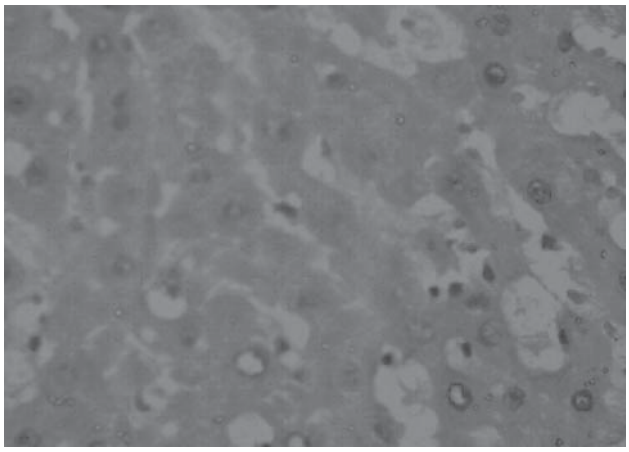


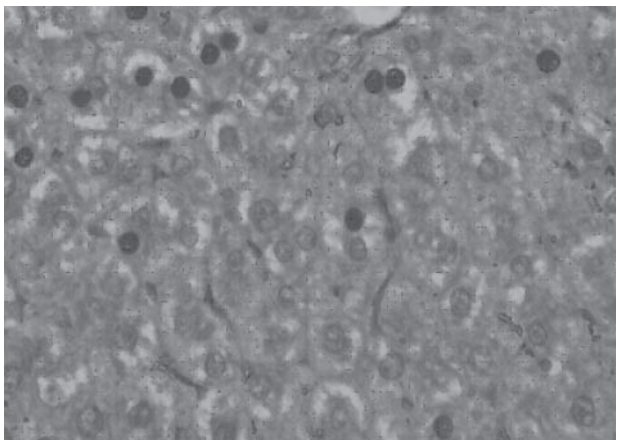
Figure 3: Effect of *T. populnea* (ethyl acetate fraction) on liver histopathology in CCl_4 toxicity in rats (protective study)



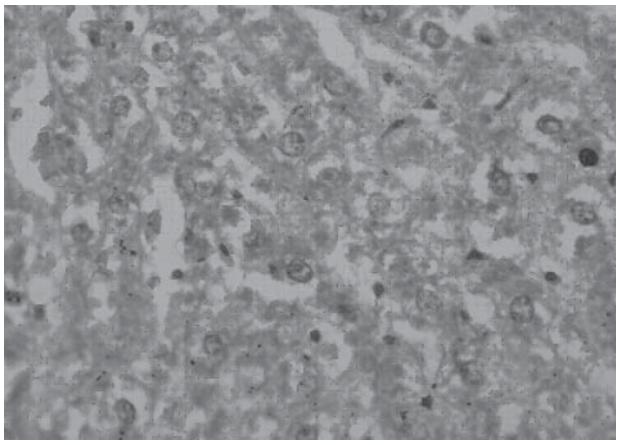
A. Control rat liver (H&E x 400)
Normal architecture of liver



B. CCl_4 treated rat liver (H&E x 400)
Ballooning, centrilobular necrosis and inflammation of cell

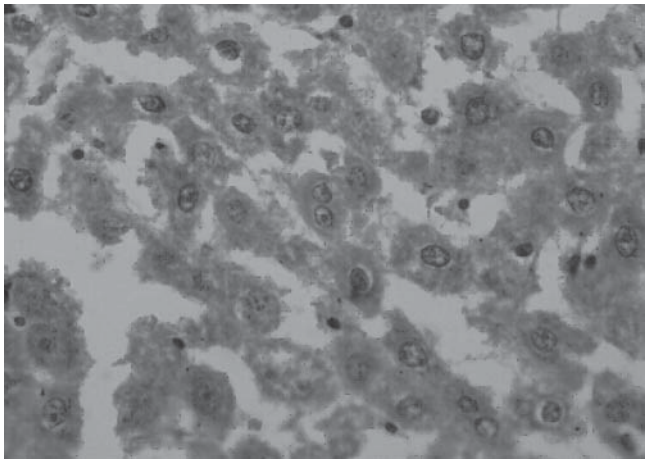


C. Ethyl acetate fraction (8 mg/kg) + CCl_4 treated rat liver (H&E x 400) Normal hepatocytes and mild inflammation

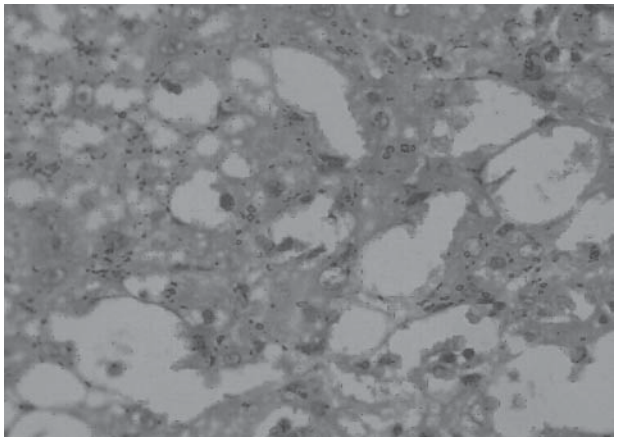


D. Silymarin (100 mg/kg) + CCl_4 treated rat liver (H&E x 400) Mild cellular damage and feathery degeneration of hepatocytes

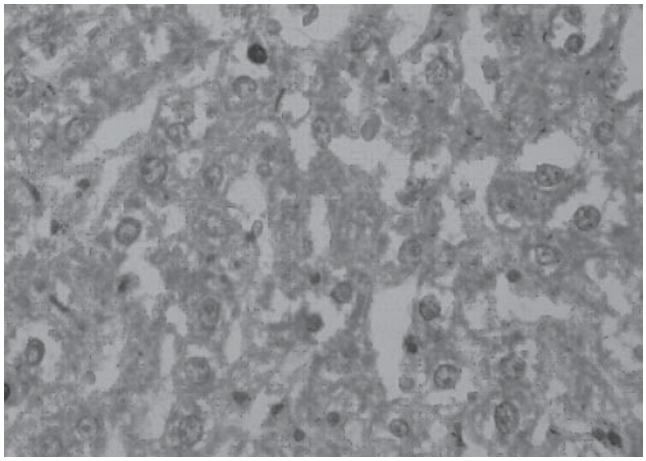
Figure 4: Effect of *T. populnea* (phenolic acids fraction) post treatment on liver histology in CCl_4 toxicity in rats (curative study)



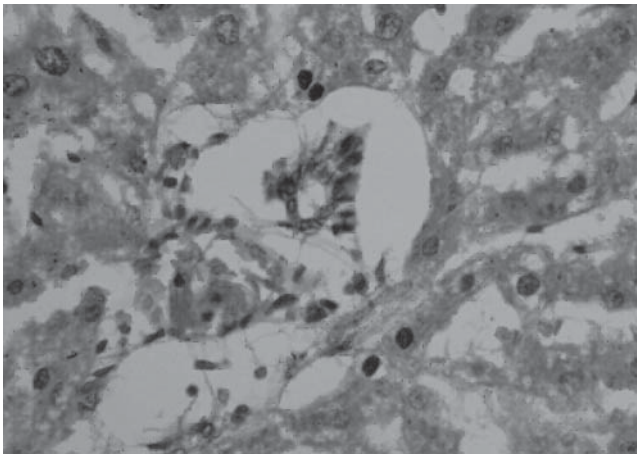
A. Control rat liver (H&E x 400)
Normal architecture of liver



B. CCl_4 treated rat liver (H&E x 400)
Microvascular fatty changes, focal necrosis and pericular inflammatory



C. Te phenolic acid fraction (2 mg/kg) + CCl_4 treated rat liver (H&E x 400)
Normal hepatocytes and sinusoidal dilatation



D. Silymarin (100 mg/kg) + CCl_4 treated rat liver (H&E x 400)
Microvascular fatty changes

Figure 5 : Anti-HBV activity of T. populnea in HepG2.2.15 cells

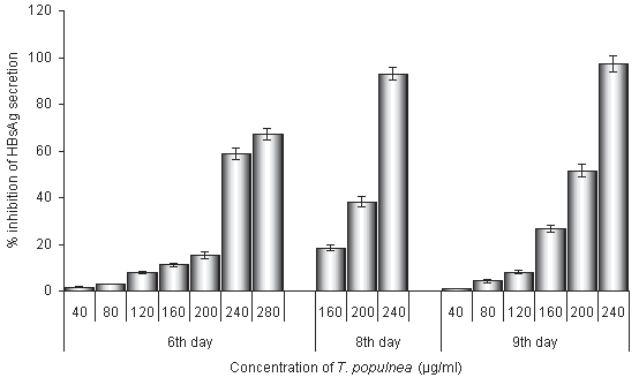
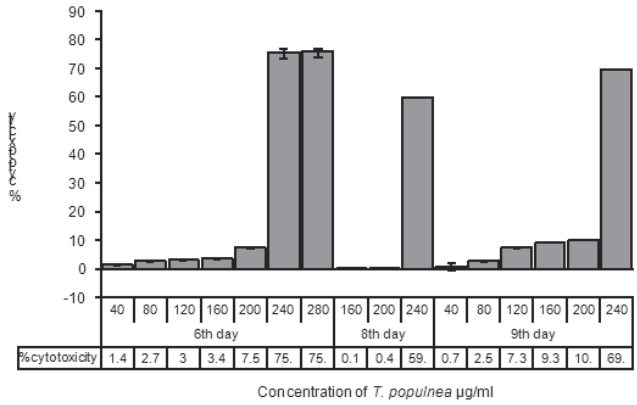


Figure 6: Cytotoxicity in HepG2.2.15 cell line



Discussion

A highly active hepatoprotective phenolic acids fraction (PF) has been isolated for the first time from *T. populnea* stem bark. Structures of these phenolic compounds remain to be elucidated. It is not known whether or not all of the 3 compounds present in PF are active. It is of interest to note

that the hepatoprotective efficacy of 2 mg/kg PF against CCl_4 toxicity was comparable to that of 100 mg/kg silymarin, a standard currently used hepatoprotective herbal drug from Silybam marianum.

Although, studies in the recent past have revealed hepatoprotective activity in many medicinal plants, none of them showed activity at a very low dose of 2 mg/kg (Ko *et al*., 1998; Subramoniam *et al*., 1998; Subramoniam and Pushpangadan 1999; Achliya *et al*., 2004; Asha *et al*., 2004; Porchezhiana and Ansari 2005 and Youvaraj *et al*., 2007). In this context, it is of interest to note that the phenolic acids fraction at a low dose (2 mg/kg) provided protection against all the three hepatotoxins tested. CCl_4 is an industrial toxin which induces liver damage mainly by its highly reactive pro-oxidant breakdown products formed in the liver (Castro *et al*., 1974; Noguchi *et al*., 1982; Packer *et al*., 1978 and Recknagel *et al*. 1989). Acetaminophen (paracetamol) is a safe and effective anti-pyretic and analgesic drug when used at therapeutic levels and an over dose of the drug can induce severe hepatotoxicity (Amar and Schiff, 2007). Thioacetamide was originally used as a fungicide to protect against decay of oranges (Childs, 1946). Thioacetamide toxicity is due to the formation of thioacetamide-5-oxide which is responsible for the change in cell permeability, increased intracellular concentration of calcium and inhibition of mitochondrial function (Neal and Halpert, 1982 and Bruck *et al*., 2002). Thus, PF may act against a wide variety of hepatotoxic compounds. However, the mechanism of action remains to be elucidated.

One of the mechanisms could be the antioxidant activity of the herbal drug. Several studies have reported that specific polyphenols are able to scavenge superoxide and hydroxyl radicals, reduce lipid peroxides and inhibit lipid per oxidation (Ko *et al*. 1998). The antihepatotoxic effect reported in the present study might possibly, partly, due to the potent antioxidant activity of the molecules as seen from the *in vivo* antioxidant activities (increase in the activities of antioxidant enzymes and decrease in lipid peroxides in the herbal drug treated animals in the present study). Further, the phenolic acids isolated from the plant showed promising *in vitro* antioxidant activities also (unpublished observations of the authors). The influence of these active phenolic acid components on the levels of proinflammatory cytokines is being investigated in this laboratory.

HepG2.2.15 cell contains multiple copies of the HBV genome, which are stably integrated into the host cell genome (Sells *et al*., 1988) and is widely used as a useful *in vitro* model for evaluation of novel anti-HBV drugs. So in our experiment, we chose the HepG2.2.15 cell line as *in vitro* cellular model. Inhibition of HBsAg secretion may be achieved at the transcription, translation, or post-translational level. Although the replication and life cycle of HBV is complex, most known antiviral agents with activity against HBV act at the polymerase or reverse transcriptase (Lee *et al*., 1989; Price *et al*., 1989 and Doong *et al*., 1991).

PF was cytotoxic to HepG2.2.15 cell line in XXT assay at 240 µg/ml level and above. At the same time, in normal macrophages and thymus cells PF did not show cytotoxicity effects up to 500 µg/ml in trypan blue exclusion method (data not shown). Transformed cells could be more sensitive to PF.

Since the active phenolic compounds have curative property also in the post-treatment experiments against CCl_4 toxicity, this herbal drug could prove to be a valuable drug against liver diseases induced by toxic chemicals. The herbal drug may have stimulatory effect on regeneration of liver cells. This remains to be investigated.

T. populnea is a small tree which may take a few years on cultivation to yield reasonably matured stem bark for medicinal purposes. However, this plant can be grown for large scale production of the plant material for drug development. Seasonal and ecotype variation in the medicinal quality of the bark, if any, remain to be studied. Attempts can also be made for the production of the active principles containing biomass of this plant by tissue culture techniques. Elucidation of the structure of the active phenolic acids may pave the way for the production of the active principle by chemical synthesis.

At any rate, the present study opened up a new vista for the likely development of a successful drug for, at least, certain hepatic diseases. In this context, in should be noted that for most of the severe liver diseases the conventional medicine is devoid of a satisfactory treatment. Herbal drugs and herbal combination drugs are used for the treatment of liver diseases. Development of improved herbal drug with regard to safety and efficacy is the need of the hour. The present study is a significant step towards that direction.

Conclusion

The study reports for the first time that the hepatoprotective principles in *T. populnea* (bark) is phenolic acids. The active principles possess highly potent hepatoprotective activity against toxic chemicals induced liver damages at low doses. Further, the *in vitro* study suggests the antihepatitis B activity of the phenolic acids. It is very promising for the development of safe and effective medicine for liver diseases.

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