Original Article

Hepatoprotective And Antioxidant Study Of Ethanolic Extract Of T. Cordifolia Against Cyclophosphamide Induced Liver Injuries

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Abstract

The aim of study is to evaluate the hepatoprotective and antioxidant potential of the ethanolic extract of stem of T. cordifolia against anticancer drug cyclophosphamide. Cyclophosphamide (CPP) is an immunosuppressive agent, generally prescribed in treatment of various types of cancer, autoimmune disorders and also to stabilise the organ transplantations. The major setback of the CPP is that it undergoes extensive hepatic metabolism and transformed into active metabolites 4-hydroxycyclophosphamide, phosphoramide mustard and acrolein. Cyclophosphamide and its produced metabolites altogether alter the hepatocellular membrane permeability by initiating lipid peroxidation and leads hepatic injuries. Present study was conducted to establish the hepatoprotective potential of ethanolic extract of stem of Tinospora cordifolia extract (ETCE) against CPP induced hepatotoxicity. Six groups of Wistar rats (n=6) were constituted. CPP intoxicated animals were treated with ETCE at dose of 250mg/kg, 500mg/kg and 1000mg/kg. Blood samples were collected on 14th day of study and examined for liver function parameters (SGOT, SGPT, ALP and total bilirubin content). A significant ($p<0.001$) fall in the SGOT, SGPT, ALP and total bilirubin content were reported in dose dependent manner of ETCE. Liver homogenates were investigated for antioxidant effect of the ETCE against CPP induced oxidative stress. A significant ($p<0.001$) antioxidant effect at dose of 500mg/kg and 1000mg/kg of ETCE were also reported as decrease in lipid peroxidation along with an increase in GSH, SOD level and catalase activity. Histopathological investigations of liver sections of animals supplemented with ETCE confirmed the restoration of normal hepatic architecture.

Key words: Cancer Chemotherapy, Cyclophosphamide, Tinospora cordifolia, Hepatotoxicity, Oxidative stress, Silymarin.

INTRODUCTION

Cyclophosphamide is nitrogen mustard-alkylating agent. It is widely used in the chemotherapy of cancer and myeloablative therapy of autoimmune disorders. It is also indicated in the organ transplantation to prevent the organ rejection. Cyclophosphamide undergoes extensive hepatic catabolism by Cyp-450 into its active metabolites including 4-hydroxycyclophosphamide, phosphoramide mustard and acrolein [1,2]. Hepatic transformation of 4-hydroxy-cyclophosphmide into phosphoramide mustard, alkylate the cross linking of the purine bases of DNA as to inhibit the DNA, RNA and protein synthesis and causing death of rapid dividing cells.

Direct toxic effect of the cyclophosphamide and its metabolites causes alterations in cell membrane integrity through lipid peroxidation resulting hepatic damage [3,4]. Its metabolites also leads liver tissues injury as sinusoidal obstruction syndrome, characterized as hepatic necrosis, and obstruction in hepatic venous flow [5]. Sinusoidal obstruction syndrome evidenced as sudden onset of the abdominal pain, weight gain and ascites, subsequently reported as jaundice and hepatic dysfunction.

The plant Tinospora cordifolia (Wild.) belonging to the family – Menispermaceae, well-known as "Amrita" or "Guduchi". It is a versatile folk shrub and all parts of the plants such as leaves; stem and roots are used as medicine in Ayurveda. It is also named as Indian bitter and prescribed in the treatment of various types of fevers, management of diabetes, jaundice, urinary problems, gastrointestinal infection (chronic diarrhoea and dysentery), heart disease, leprosy and skin diseases. T. cordifolia widely incorporated in various herbal formulations of Ayurveda. Its stem is used in Ayurvedic formulations for the treatment of jaundice and diarrhoea [6,7]. The stem is bitter, stomachic, diuretic, stimulates bile secretion, enriches the blood and cures jaundice. Recent scientific studies also emphasized its potential to cure of hepatotoxicity induced by antitubercular drugs [8], jaundice [9], cardioprotective [10], leprosy [11], hepatitis [12] and helminthiasis [6]. In the ancient literature of Ayurveda, Ashtang Hridya and Charak Samhita emphasized the use of the Guduchi in the treatment of disease including Javar (fever), Vat Rakta (gout) and Kamala (jaundice) [13,14]. The peoples lived in Mumbai, coastal fishermen and tribes used T. cordifolia in the treatment of fever, jaundice, dysentery and diarrhoea [15].

Our literature survey reveals that till date T. cordifolia were evaluated for hepatoprotective activity against lead nitrate [16] and CCl_4 [17] induced toxicity, but no any attempt has been made to this date to evaluate the hepatoprotective activity against anticancer drug cyclophosphamide induced hepatotoxicity.

Thus, present investigation was designed to assess the hepatoprotective potential of the ethanolic extract of stem of T. cordifolia (stem) against the hepatotoxicity induced by the cyclophosphamide. Investigation includes acute toxicities study of the ethanolic extract of T. Cordifolia; hepatoprotective activity based on the estimation of the serum enzymes including SGPT, SGOT, ALP and total bilirubin content. CPP induced oxidative stress were investigated by estimating the level MDA content, SOD, reduced glutathione and catalase activity of liver tissue homogenates. Histological examination of liver tissue was also conducted to investigate histopathological transformations.

MATERIAL AND METHODS

Chemicals

Cyclophosphamide (Sigma-Aldrich) and Silymarin (Sigma- Aldrich) were used in present study. All other chemicals, solvents and reagents used were of analytical grade.

Collection of plant material

The stems of T. cordifolia were collected from the local region of the Agra, Uttar Pradesh. The specimen of T. cordifolia identified and authenticated (RARI-JHS/1782-28680) by Regional Research Institute, Jhansi. The stems were collected and dried under shade at room temperature and pulverized by mechanical grinder to coarse powder.

Preparation of plant extract

Cold maceration method was preferred for the extraction as to avoid the deterioration of the phytoconstituents. The coarse powdered (500gm) stem of the T. cordifolia successively macerated with petroleum ether (5 lit.) and ethanol (5 lit.). The ethanolic macerated mixture was filtered and the filtrate was subjected to dryness under vacuum at 40^{o}C. The percentage yield was calculated and stored at 4^{o}C for bioactivity and quantitative analysis.

Phytochemical Screening

The ethanolic extract of stem of T. cordifolia (ETCE) was screened for the presence of secondary metabolites such as carbohydrates, alkaloids, glycosides, terpenoids, steroids, tannin, flavonoids and phenolic compounds [18]. The extract was also estimated for the total phenolic and total flavonoids contents.

Estimation of Total Phenolic Content

Total phenolic content in the ETCE estimated with Folin Ciocalteu reagent [19,20]. The Folin-Ciocalteu reagent reduces the polyphenolic compounds and turns the solution blue in colour. The intensity of blue colour represents the amount of total phenolic content in sample solution. 10ml of ethanolic extract of T. cordifolia was prepared with 1ml of the plant extract in distilled water. Further 1.5ml of Folin Ciocalteu's reagent added, and then incubated for 5 min. at room temperature. To this 4ml of Na_2CO_3 (20%w/v) added and makeup the volume up to 25ml with distilled water and stabilised the solution for 30min, at room temperature, then measured the absorbance at 765nm. Calibrated curve of standard Gallic acid was used to estimate the total phenolic content in the plant extract.

Estimation of total flavonoids content

Total flavonoids contents were estimated by a colorimetric assay [21,22]. 1gm of the dried extract were weighed and extracted with 20ml of 60% of ethanol for 30min. filtered in 25ml of the volumetric flask and make the volume up to 15ml with 60% ethanol. 1.5ml of the prepared aliquot of sample / standard solution of rutin and 4.5ml of distilled H_2O pipetted into the 25ml of volumetric flask. 1ml 5% (w/v) $NaNO_2$ solution added and incubated for 6min. To the above mixture 1ml of 10% Al $(NO_3)_3$ solution added and kept a side for 5 min. After incubation of 5min. 10ml of 4% (w/v) NaOH solution added, finally the volume of the mixture solution make up to 25ml with 60% ethanol. Absorbance of the mixture was measured at 510nm against the blank. The mean of three readings was used and the total flavonoids content was expressed in milligram of rutin equivalents/1 g extract. The coefficient of determination was r^2 =0.975.

Pharmacological Studies

Animals

The present studies were carried out on Wistar rats of weighing 210±10g of either sex and procured from PBRI animal house. The animals were housed under standard conditions of humidity, temperature (25±2 ◦C) and light (12 h light/dark). They were fed with standard rat pellet diet and water ad libitum. Animal based experimental studies were conducted as per the ethical guidelines of Institutional Animal ethics Committee (Reg. No. 1824/PO/RC/S/15/CPCSEA).

Acute toxicity study

Acute oral toxicity study of ethanolic extract of stem of T. cordifolia (ETCE) was performed as per the OECD-423 guidelines [23]. The extract was administered orally for four dose levels - 5 mg/kg, 50 mg/kg, 300 mg/kg and 2000 mg/kg and observed for the toxic symptoms, body weight changes and lethality. Results are summarized in table no. 1.

Hepatoprotective activity

The hepatoprotective activity of the ETCE evaluated against the cyclophosphamide induced toxicity [24]. Wister strain of albino rats weighing 200gm were selected and divided into 6 groups (n=6 animals). No drug control animal group were considered since hepatoprotective potential of T. cordifolia previously reported.

Group I: Control treated with distilled water.

Group II: Only Cyclophosphamide 200mg/kg, single dose introduced intraperitoneally on 1st day.

Group III: Cyclophosphamide 200mg/kg, single dose introduced intraperitoneally on 1st day + 250mg/kg of ETCE orally continued for 14 days.

Group IV: Cyclophosphamide 200mg/kg, single dose introduced intraperitoneally on 1st day + 500mg/kg of ETCE orally continued for 14 days.

Group V: Cyclophosphamide 200mg/kg, single dose introduced intraperitoneally on 1st day + 1000mg/kg of ETCE orally continued for 14 days.

Group VI: Cyclophosphamide 200mg/kg, single dose introduced intraperitoneally on 1st day + 100mg/kg of Silymarin (Standard) orally continued for 14 days.

Serum preparation:

After 24 hours of final administration of 14th day of study, the blood was taken from retro-orbital sinus of the experimental animals and placed in Eppendorff Micro tubes to centrifuge at 7000rpm at 4^0C for 15 minutes to obtain clear serum. The resultant serum was transferred in fresh sterilized Eppendorff Micro-centrifuge tubes and estimated for the serum biochemical markers- SGPT, SGOT, ALP and total bilirubin content.

In-vivo antioxidant activity

In the present study the oxidative stress generated by the cyclophosphamide and its numerous metabolites were assessed on the basis of estimation of the level of the lipid peroxidase, superoxide dismutase, glutathione reductase and catalase (Ademola et al., 2016). After 24 hours of final administration of 14th day of study, the animals were sacrificed and their liver tissue were isolated and washed with ice-cold physiological saline and homogenized in 0.1M tris-HCl buffer (pH 7.4) and the aliquots were estimated for the tissue enzymatic antioxidants potential for Lipid peroxidase, Superoxide dismutase (SOD), Glutathione reductase (GSH) and Catalase activity.

Estimation of Superoxide Dismutase (SOD)

The oxidative stress marker superoxide dismutase estimation based on principle of generation of the superoxide radicals by NADH - phenazine methosulfate (PMS) system which cause the reduction of tetrazolium sSGPTs nitro blue tetrazolium (NBT) into blue formazan which further measured spectrophotometrically at 560nm against the blank sample. The SOD in the samples competes for the generated superoxide radical, thereby inhibiting the reaction of tetrazolium reduction. One unit of SOD was calculated and expressed as unit U/mg tissue [25].

Estimation of Lipid peroxidation

Estimation of the lipid peroxidation was conducted to assess the ROS-mediated damage of the hepatocytes cell membranes. Under oxidative stress peroxidation of the polyunsaturated fatty acids produce the malondialdehyde (MDA) as end products. The level of the generated MDA was measured on reaction with thiobarbituric acid (TBARS) in acidic medium at 100^0C as to develop a pink-red colored product which was extracted with butanol: pyridine (15:1) and its absorbance was measured at 520-535nm spectrophotometrically.

Estimation of Reduced Glutathione (GSH)

Glutathione (low molecular weight thiol) in Free State exists in two forms – GSH (reduced form) and GSSH (oxidized form) inside the cells. Normally it exists in the reduced state (GSH) and participates in the metabolic protective functions such as reduction of the hydrogen peroxide, detoxification of the xenobiotics and scavenging of the generated free radicals. Ellman developed a method to estimate the level of GSH **[26]**, based on the ability of the 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB, Ellman reagent) to react with compounds containing sulfhydryl groups, to produce disulfide (GS-TNB) and 2-nitro-5-thiobenzoic acid (TNB). The level of the TNB is quantified

spectrophotometrically by measuring the absorbance of the anion (TNB^{-2}) at 412nm against blank. Absorbance values were compared with a standard curve generated from known GSH.

Estimation of Catalase activity

Catalase is an antioxidant enzyme that splits hydrogen peroxide into water and oxygen. Procedure implicated in the present study is based on colorimetric estimation of splitting out of hydrogen peroxide by catalase; instead hydrogen peroxide could reduce the $K_2Cr_2O_7$ into chromic acetate in the presence of acetic acid. This enzymatic activity of the catalase was measured colorimetrically at 610nm. The catalase activity was measured for the disappearance of hydrogen peroxide and each unit was therefore defined as the amount that degrades 1µmol of hydrogen peroxide per minute.

Histopathological investigation

The liver tissues were collected and preserved in formalin solution (10%) for histopathological examination. These tissues were processed for dehydration in the different grades of alcohol, cleared with toluene, and impregnated in molten paraffin wax. The processed liver tissues finally embedded in fresh molten paraffin wax and allowed to set for histological procedures. Liver sections were prepared for 3µm thick, dried and stained with hematoxylin and 1% aqueous eosin to demonstrate general tissue structure. Stained slides were further dehydrated in various ascending grades of alcohol, cleared with xylene, and mounted in Canada balsam. Liver histological changes were viewed microscopically using 10X objective lenses [26,27].

Statistical Analysis

Results are provided as Mean ± SD (n=6). Results were analyzed statistically using one-way analysis of variance (ANOVA) followed by Bonferroni t-test. $P < 0.05$ was considered as level of significance while comparison between groups.

Results

Cold maceration method with ethanol was used for plant extraction. The percentage yield of ethanolic extract was 3.545%. The phytochemical investigation confirmed the presence of carbohydrates, alkaloids, glycosides, terpenoids, steroids, tannin, flavonoids and phenolic compounds. Quantitative estimation for total phenolic content was found to be 131.60 mg/gm equivalent to Gallic acid and total flavonoids content was found to be 266.20 mg/gm equivalent to Rutin.

Assessment of Acute Toxicity of ETCE

Acute toxicity studies conducted to assess the safety aspect of ETCE. The rats administered with ETCE at dose of 2000mg/kg showed no mortality or any abnormal behaviour changes, during the 72hrs observation for all groups of animals. This complies with the previous results of acute toxicity study of T. cordifolia [28], which confers the safety of the T. cordifolia at the dose of 2000mg/kg of body weight.

Table 1: Acute Toxicity study of ETCE (ethanolic extract of stem of T. cordifolia extract)

Group	Dose (mg/kg)	Rat No.	Day of Death	Body Weight (gm)			No. Death / Tested
				0 Day	7 Day	14 Day	
A	5 mg/kg	R1	--	202.22	204.67	206.81	0/3
	5 mg/kg	R2	--	206.12	208.29	210.93	

	5 mg/kg	R3	--	203.44	205.38	207.81	
B	50 mg/kg	R1	--	203.39	205.11	207.02	0/3
	50 mg/kg	R2	--	203.22	205.55	207.09	
	50 mg/kg	R3	--	201.34	203.76	205.80	
C	300 mg/kg	R1	--	194.32	196.77	198.81	0/3
	300 mg/kg	R2	--	200.32	203.44	205.01	
	300 mg/kg	R3	--	205.47	207.54	208.78	
D	2000 mg/kg	R1	--	200.43	202.29	205.69	0/3
	2000 mg/kg	R2	--	203.29	205.48	208.11	
	2000 mg/kg	R3	--	202.37	204.52	206.92	

Evaluation of hepatoprotective activity of ETCE

The serum biochemical parameters results revealed a remarkable elevation in the level of enzymes of Group II animals treated with cyclophosphamide. This signifies that cyclophosphamide induces intense hepatic injury. Elevation of SGOT and ALP indicated the hepatocytes necrosis with SGPTeration of membrane permeability, causes leakage of these enzymes in the blood circulation. Animals treated with ETCE were observed a significant fall in enzymes level SGOT, SGPT and ALP (table 2) in a dose dependent manner, this indicates T. cordifolia have hepatoprotective potential. The hepatoprotective effect of T. Cordifolia at dose of 500mg/kg fairly near to the effect produces by the standard pure drug Silymarin. The results of serum biochemical liver functional parameters indicate that T. cordifolia at dose of 250mg/kg decrease the elevated level of enzymes up to 50%. This suggests that the 250mg/kg could be considered as a minimum dose for significant hepatoprotective effect of T. cordifolia. At the dose of 1000mg/kg all the enzymes levels were almost restored to the normal.

Bilirubin content signifies the incidence of hepatic necrosis and its accumulation represents functional insufficiency of hepatocytes, biliary obstruction or increase in the haemolysis. Catabolism of haemoglobin yields bilirubin which is conjugated in the liver to di-glucuronide and excreted in the bile. Cyclophosphamide increase the total bilirubin content to 2.11 ± 0.19 (mg/100ml of serum), this suggests an abnormal metabolism of the bilirubin. Treatment with ETCE restores the serum bilirubin level in dose dependent manner and at dose of 500mg/kg serum bilirubin level reached fairly near to normal.

Table 2: Biochemical assessment of hepatoprotective effect of ETCE against cyclophosphamide induced toxicity. ETCE, ethanolic extract of stem of T. cordifolia; CPP, Cyclophosphamide (200mg/kg); SIL, Silymarin (100mg/kg); SGOT, Serum glutamic oxaloacetic transaminase (per min per mg protein); SGPT, Serum glutamic pyruvic transaminase, (per min per mg protein); ALP, alkaline phosphatase (one king Armstrong unit 1 UI-1); Bilirubin, gm/dL. Values are expressed as MEAN±SD at n=6, One-way ANOVA followed by Bonferroni test, ns, p< 0.01, non-significant activity and *P<0.050, **P<0.001 significant activity when compared to the control.

Hepatoprotective effect of T. Cordifolia on Hepatic Serum Biochemical Parameters against Cyclophosphamide induced toxicity								
Group No. / Treatment		**Body wt. (gm)**	**Absolute liver wt. (gm)**	**Relative liver wt. (gm)**	**SGOT**	**SGPT**	**ALP**	**Bilirubin**
I	Vehicle only	214±1.41	5.17±1.17	2.42±0.56	27.4±5.45	25.64±5.46	72.49±7.35	0.3±0.03
II	CPP + Vehicle	211.17±2.32	8.5±1.05	4.02±0.45	113.87±3.28	91.05±3.31	236.83±5.19	2.11±0.19
III	CPP + ETCE (250mg/kg)	210.5±2.43 ^{ns}	5.83±0.98**	2.77±0.45**	59.23±3.31**	48.62±3.31**	135.99±6.2**	0.72±0.2**
IV	CPP + ETCE (500mg/kg)	200.32±5.04**	5.12±0.98**	2.55±0.43**	53.63±9.47**	39.78±3.31**	125.63±4.39**	0.6±0.19**
V	CPP + ETCE (1000mg/kg)	200.31±5.03**	4.9±1.01**	2.44±0.46**	46.56±4.56**	38.01±3.31**	114.81±7**	0.5±0.19**
VI	CPP + SIL (100mg/kg)	203.22±3.01^{ns}	4.5±0.55**	2.21±0.24**	37.13±4.18**	30.04±6.2**	65.25±7.01**	0.29±0.15**

Evaluation of in-vivo antioxidant activity of ETCE

Reactive oxygen species (ROS) are the common by product chemical molecules containing oxygen which are generated on aerobic cellular metabolism. Indeed the hepatic cellular metabolism is also engaged to generate these ROS. Under oxidative stress, excessive free radicals are generated which compromise the hepatic cellular health and contribute into liver cirrhosis by inducing the destruction of the DNA, proteins and lipids peroxidation. Oxidative stress biomarkers are therefore important tools to assess the hepatic cellular health.

Results of oxidative stress studies, shown in table no. 3 revealed that the lipid peroxidation in cyclophosphamide intoxicated liver increases the level of malondialdehyde (MDA) by 3 times, when compared with control group. Supplementation with ETCE decreases the raised levels of MDA in a dose dependent manner. Treatment with ETCE at dose 250mg/kg decreases the lipid peroxidation by 50%. This suggests 250mg/kg could be considered as the minimum dose to inhibit lipid peroxidation.

SOD scavenges superoxide anion and reduce the toxic and deleterious effect of the free radical; enzyme Catalase decomposes H_2O_2 into water and oxygen and protects the tissue from highly reactive hydroxyl radicals; while GSH is the pivotal enzyme for maintaining and regenerating the reduce levels of glutathione in cytoplasm. Cyclophosphamide toxicity decreases the level of the SOD, GSH and CAT below to the normal; this indicates the over accumulation of free radicals. Supplementation with ETCE at dose of 250mg/kg, 500mg/kg and 1000mg/kg restored the SOD, GSH and CAT activity in dose dependent manner, suggesting ability to arrest the generation of reactive oxygen species (ROS) as superoxide anion and reactive hydroxyl radicals; and also restore the level of glutathione. The results of oxidative stress parameters reveals that T. cordifolia at dose of 250mg/kg increase the level of enzymes SOD, GSH and CAT activity up to 50%. This signifies that the 250mg/kg could be considered as a minimum dose of ETCE for antioxidant effect. At higher dose of 1000mg/kg all the enzymes levels were almost restored to the normal.

Table 3 Evaluation of antioxidant activity of ETCE against cyclophosphamide induced oxidative stress. ETCE, ethanolic extract of stem of Tinospora cordifolia extract; CPP, cyclophosphamide (200mg/kg); SIL, Silymarin (100mg/kg); LPO, Lipid peroxidise (nmol MDA/mg tissue); SOD, superoxide dismutase (units/min/mg protein); GSH, Glutathione reductase (1 μM of NADPH/min); CAT, Catalase (μm H_2O_2 consumed/min/mg protein). Values are expressed as MEAN±SD at n=6, One-way ANOVA followed by Bonferroni test, ns, p< 0.01 non-significant activity and *P<0.050, and **P<0.001, significant activity when compared to the control.

Antioxidant activity of T. cordifolia against Cyclophosphamide induced Oxidative Stress					
Group No./ Treatment		**LPO**	**SOD**	**GSH**	**CAT**
I	Vehicle only	15.58±1.85	55.59±6.01	2.41±0.04	38.09±4.89
II	CPP(200mg/kg)+ Vehicle	50.78±2.05	10.57±3.53	0.41±0.02	12.42±0.81
III	CPP + ETCE(250mg/kg)	36.47±1.98**	23.05±2.28**	1.16±0.01**	16.98±1.85 ns
IV	CPP + ETCE(500mg/kg)	22.22±1.39**	29.42±5.13**	1.21±0.01**	22.43±2.86**
V	CPP + ETCE(1000mg/kg)	17.11±1.02**	36.2±2.85**	1.42±0.01**	26.26±4.45**
VI	CPP + SIL	15.69±1.82**	50.16±5.41**	2.09±0.04**	36.65±2.86**

Effect of ETCE on Histopathology

Hepatic histology examined to evaluate the Hepatoprotective effect ETCE against cyclophosphamide induced histopathology.

Group I (Control): Liver sections of animals of control group showed a normal hepatic architecture with radially arranged hepatic cords around the central vein. There were absence of necrosis, and vacuolar degeneration. The hepatic cells were normal with well-preserved cytoplasm and prominent nucleus (Figure 1: PIC-A & PIC-B).

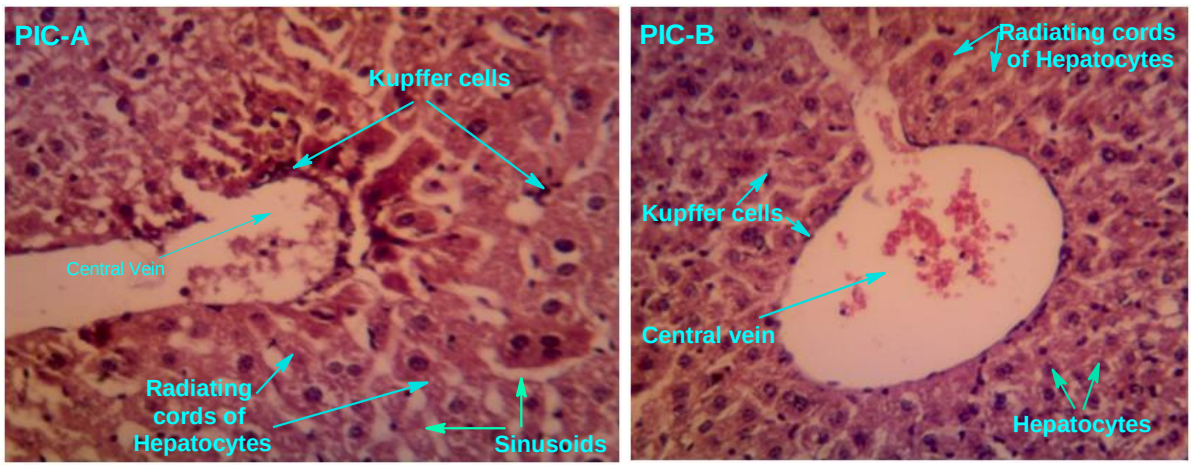

Figure 1 Liver sections (PIC-A & B) of animals received vehicle (distilled water) only.

Group II (Cyclophosphamide 200mg/kg)**:** Cyclophosphamide exposure developed a severe hepatic toxicity evidenced with severe necrosis, hepatic congestion, parenchymal disorganization and inflammation. There were also indication of cholangitis with intense mononuclear infiltration in the portal tract, disruption of hepatic cords and hemorrhagic clots (Figure 2: PIC-C & PIC D).

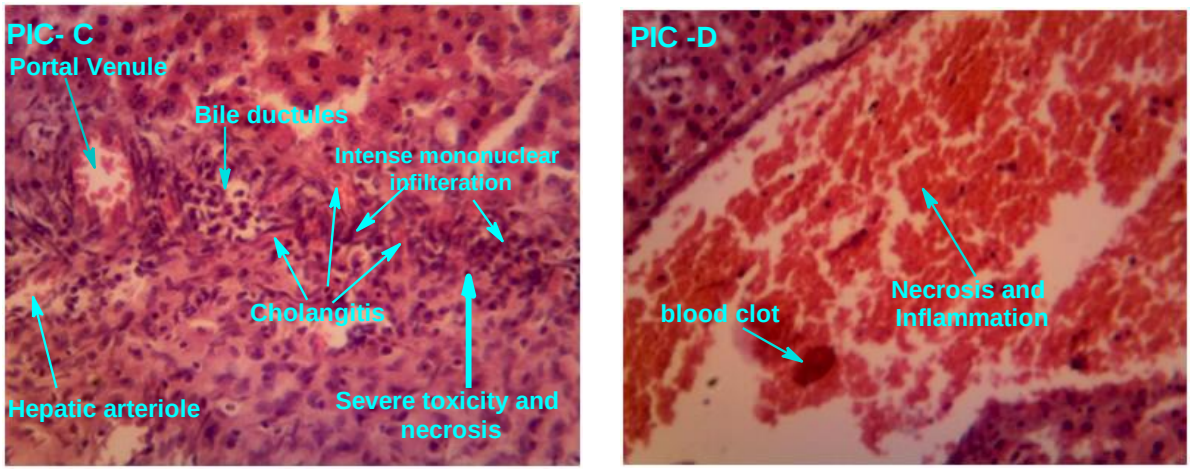

Figure 2 Liver Section (PIC-C & D) of animals received single dose of 200mg/kg of cyclophosphamide .

Group III (ETCE 250mg/kg): Liver histology of animals treated with 250mg/kg of ETCE reveals a some extent of protective effect with initiation of regeneration and restoration of the the normal heaptocytes parenchyma. Hepatic cords are centrilobular arranged but vacuolation and steotic hepatocytes could seen. The adjacent picture of the same group reflects an increase in the normal hepatocytic parenchyma, however some sorts of centrilobular granulocytic infilteration (Figure 3: PIC-E & PIC-F).

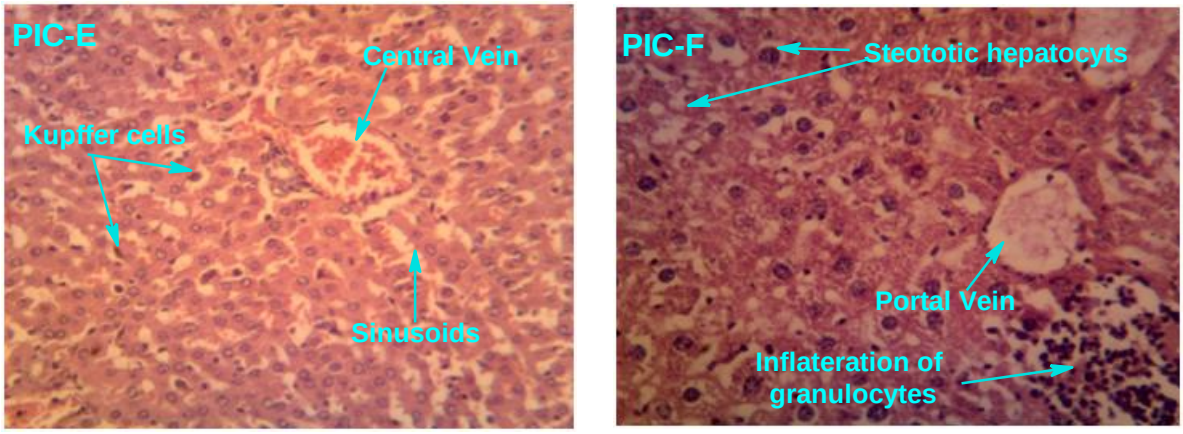

Figure 3 Liver sections (PIC-E & F) of animal treated with 250mg/kg of ETCE

Group IV (ETCE 500mg/kg): Animals supplemented with 500mg/kg of ETCE reported a remarkable regeneration and restoration of hepatic architecture as shown in figure 4 (PIC-G & PIC-H). The pictures also signify the lack of cellular congestion and no any incidence of granulocytic infiltration. Hepatic vacuolarization and steatotic hepatocytes were also not found.

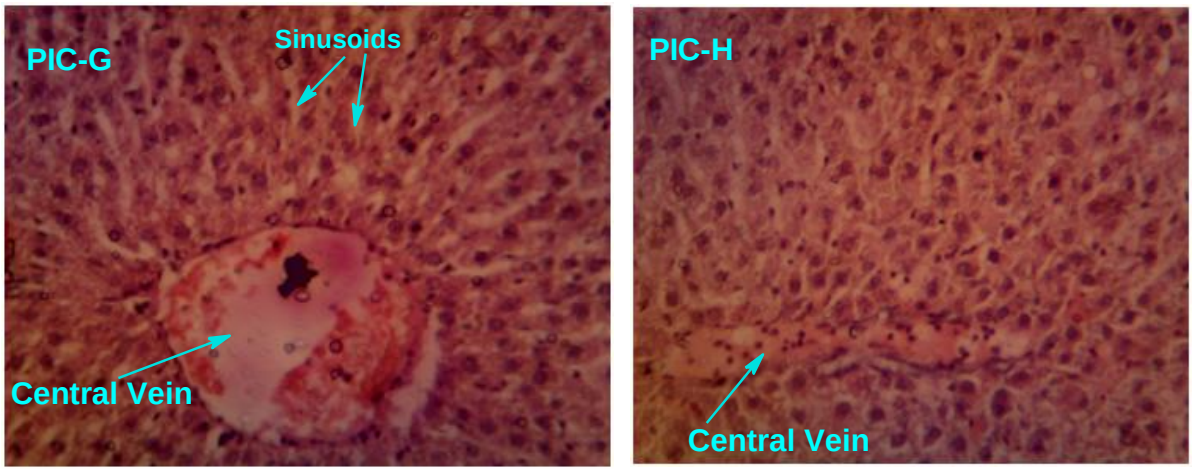

Figure 4 Liver sections (PIC-G&H) of animals treated with 500mg/kg of ETCE

Group V (ETCE 1000mg/kg)**:** Liver sections of Animals treated with 1000mg/kg of ETCE showed a retention of the normal hepatic architecture with regular sinusoids and hepatocytes cords. Degeneration of vacuoles and steototic hepatocytes were also absent. However, a slight granulocytic infilteration at portal traid could observed (Figure 5: PIC-I & PIC-H).

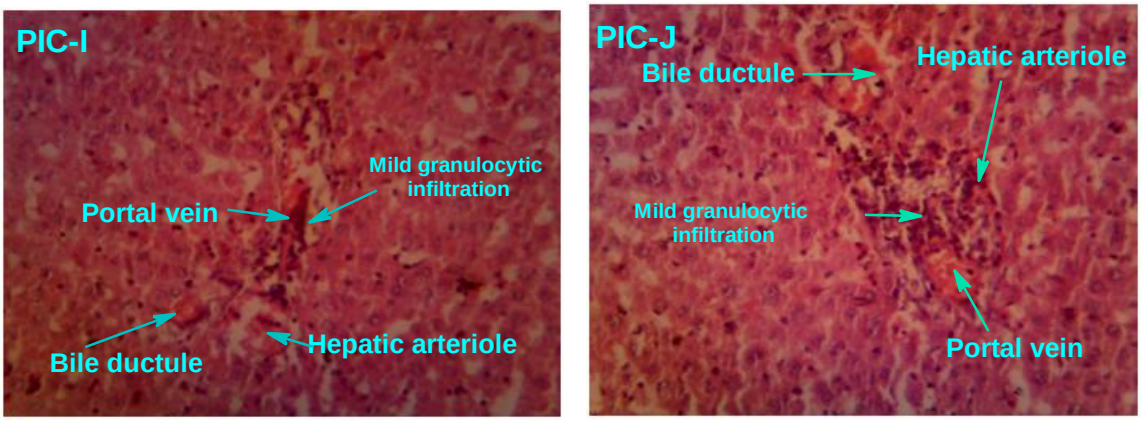

Figure 5 Liver Section (PIC-I & J) of animals treated with 1000mg/kg of ETCE.

Group VI (Silymarin 100mg/kg): Animals treated with silymarin (100mg/kg) showed normal heaptic architecture with radially arrranged centrilobular heaptic cords as shown in figure 6 (PIC-J).

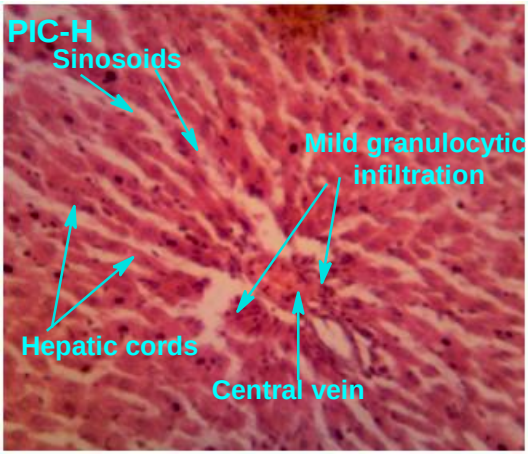

Figure 6 Liver Section (PIC-H) of animals treated with silymarin.

Discussion

Hepatotoxicity is a major side effect of cylophosphamide. Cyclophosphamide and its metabolites initiate the lipid peroxidation (LPO) of the cell membrane and distorted the cell membrane integrity resulting serious consequences reported as hepatic injury with leaching out of the hepato-cellular enzymes such as SGOT, SGPT, ALP and bilirubin into systemic circulation [24] . In our present study cyclophosphamide toxicity reported as serum elevation of the SGOT, SGPT, and ALP. Supplementation with ethanolic extract of stem of T. cordifolia, observed a significant fall in enzymes level SGOT, SGPT and ALP in a dose dependent manner, this confers T. cordifolia have hepatoprotective potential.

Administration of Cyclophosphamide reported for severe hepatic injuries as sinusoidal obstruction syndrome, characterised with hepatic necrosis, obstruction of the hepatic venous flow and jaundice [5]. Raised in the serum level of the bilirubin indicate the prevalence of the jaundice. Serum investigation of the present studies reported an elevation of the total bilirubin content. Treatment with ETCE restores the serum bilirubin level at dose 500mg/kg, which is fairly near to the level of standard drug Silymarin (100mg/kg).

Treatment ETCE at higher dose 1000mg/kg restored and retained the levels of SGOT, SGPT, ALP and bilirubin near to normal. Thus, this could be a positive inference that administration of the ETCE at higher dose 1000mg/kg was not expressing any negative changes in the levels liver functional markers.

Extensive hepatic metabolism by Cyp-450 converts cyclophosphamide into acrolein. Acrolein has high tendency to get bound to block antioxidant nucleophiles such as glutathione (GSH). Depletion of the GSH consequences the generation and excessive accumulation of reactive oxygen species (ROS), reported as oxidative stress [24]. The obtained results of oxidative stress studies revealed that cyclophosphamide increase the level of lipid peroxidation marker MDA along with fall in the level of the SOD and GSH; as well as declination in the CAT activity. Treatment with T. cordifolia extract restored the SOD, GSH and CAT activity, which signify the ability of T. cordifolia to arrest the reactive oxygen species (ROS) as superoxide anion and reactive hydroxyl radicals. This indicates the antioxidant potential of T. cordifolia.

Histopathological investigation reveals that cyclophosphamide exposure developed severe hepatic injuries, which is evidenced in the liver sections as hepatic necrosis, congestion, disorganization of hepatic parenchyma, granulocytic infiltration and inflammation. There is also an indication of cholangitis with intense mononuclear infiltration around the portal tract, disruption of hepatic cords and hemorrhagic clots. Treatment with T. cordifolia extract at the dose of 250mg/kg and 500mg/kg, initiate the regeneration and restoration of the normal hepatic parenchyma with their organized centrilobular arrangement as hepatic cords. Animals treated with higher dose 1000mg/kg of ETCE showed a retention of the normal hepatic architecture, regular arrangement of sinusoids and hepatocytes cords. This supports that T. cordifolia at the higher dose 1000mg/kg restored and retained the normal hepatic histology without inducing any histopathological development. Nevertheless, this expresses a constructive effect of T. cordifolia at higher dose on liver histology.

However, further studies are needed for standardization of T. cordifolia extract, with identification and isolation of the phytoconstituents for its hepatoprotective activity. Indeed a study will be also required to explore the exact mechanism to establish the hepatoprotective potential of T. cordifolia.

Conclusions

The results of present study reveal that CPP exposure elevates the level of serum liver functional enzymes and develop the oxidative stress along with the distortion of hepatic architecture. Supplementation with T. cordifolia of restores the level of serum liver functional enzymes by preventing LPO and reverses the oxidative stress by enhancing the antioxidant defence system. Based on the results of present study T. cordifolia at the dose of 500mg/kg could be consider as a supplement during CPP chemotherapy as to minimise hepatic toxicities.

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Conflict of Interest

The author declares that there is no any conflict of interest.

Author (s) Contribution

The conceptualization and formal analysis of the work was planned and conducted by the PM. The work conducted and completed under the supervision of the SM and SKP. The writing and original drafting of the manuscript was compiled by SKM. The manuscript finally reviewed and edited by the SKP.

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