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Ameliorative effect of ethanolic extract of roots of *Tetracera akara* (Burm. f.) Merr. on D-galactosamine induced hepatotoxicity in Wistar rats by downregulation of inflammatory mediators like TNFα, COX-2 and iNOS

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Tetracera akara, a climbing shrub locally called Nennalvalli or Pattuvalli, is an ethnomedicinal plant used by Kani tribe of Kerala to treat chronic liver disorders and inflammatory conditions. The present study was aimed to evaluate the hepatoprotective activity of ethanolic extract of roots of *Tetracera akara* root on D-Galactosamine induced hepatotoxicity in Wistar rats. Hepatotoxicity was induced in Wistar rats by intraperitoneal injection of D-GalN (400 mg/kg in saline) in Wistar rats. Ethanolic extract of *T. akara* root (TA ETH) was administered to the experimental rats in varying doses of (50, 150 and 300 mg/kg/day), p. o. for 7 days. The hepatoprotective effect was evaluated by the estimation of biochemical markers of hepatic injury, anti-oxidant status of the liver by estimating hepatic catalase, superoxide dismutase, glutathione and malondialdehyde, gene and protein expression level of inflammatory marker genes and histopathological evaluation of experimental animals. Administration of TA ETH (150 and 300 mg/kg) significantly ($P \leq 0.05$) restored the levels of serum bilirubin, protein and other hepatic enzymes almost comparable to the standard drug Silymarin-treated groups. The levels of antioxidant enzymes like SOD and CAT were elevated and lipid peroxidation was inhibited as evident from the reduced levels of MDA. The gene expression studies by quantitative PCR method showed that TA ETH significantly ($P \leq 0.05$) downregulated pro inflammatory cytokines, inflammatory COX-2 genes and upregulated IL 10 gene levels in D-GalN induced liver tissue, which was further confirmed in protein estimation by ELISA method. The histopathological observations were in correlation with the biochemical findings showing the presence of normal hepatic architecture, which further evidenced the hepatoprotective effect of TA ETH. Ethanolic extract of the root of *T. akara* possesses significant hepatoprotective activity mainly by scavenging reactive free radicals, boosting the endogenous antioxidant system in liver and inhibiting pro-inflammatory mediator like TNF α, COX-2, iNOS and promoting the anti-inflammatory IL 10, thus substantiating the tribal claim.

Keywords: D-GalN, Inflammation, Kani tribe, Kottavalli, Liver disorder, Nennalvalli, Pattuvalli

Liver is the most important organ of metabolism and acute liver injury is generally caused by drug abuse, virus infection, heavy alcohol consumption, etc., which is associated with a high mortality rate due to lack of effective preventions or therapeutic strategies[1]. Among the numerous models of acute liver injury, D-GalN is a highly specific hepatotoxic agent that induces liver injury closely resembling viral hepatitis in its morphological and functional features[2]. It has been commonly used as a mature platform to investigate the mechanisms underlying clinical liver disease and to develop effective hepatoprotective approaches. Toxicity induced by D-GalN is liver specific and does not affect any other organs of the body[3]. D-GalN traps uracil nucleotides by its toxic intermediary metabolites (UDP-galactosamine and UDP-glucosamine) in the liver, thus resulting in the depletion of hepatic uridine triphosphate, UDP-glucose and UDP-galactose, which are essential materials for the biosynthesis of macromolecules such as nucleic acids and proteins[4]. Intense galactosamination results in activation of mast cells to release histamine and loss of activity of the calcium pump which leads to a consequent increase in the intracellular calcium and is considered as one of the major reasons for cell death in D-Gal N induced hepatotoxicity[5]. D-Gal N stimulates Kupffer cells to produce inflammatory factors, such as tumor necrosis factor-α (TNF-α), cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), which causes cell death in various ways including the promotion of oxidative stress and the inflammatory process.

Tetracera akara (Burm. f.) Merr. (Fam.: Dilleniaceae) is a woody climber, locally known as

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'*Pattuvalli*' or 'Nennalvalli', distributed in the Western Ghats region of Kerala and Tamil Nadu. The roots of *T. akara* is used by the Kani tribe of Kerala to cure liver diseases. The medicinal use of *T. akara* was first reported by Dr. Saradamma *et al*.[6] from JNTBGRI in 1987. In Kani tribes, the paste of the fresh root of '*Pattuvalli*' is administered orally in empty stomach to cure chronic liver disorders and inflammatory conditions[6]. The result obtained from our previous studies like comparative *in vitro* antioxidant analysis revealed that *T. akara* ethanolic extract is rich in bioactive phytoconstituents, phenols and flavonoids, and is safe for oral administration in experimental animals[7,8]. Though the antihepatotoxic potential of ethanolic extract of *T. akara* has been demonstrated against paracetamol over dosage in Wistar rats. it still requires more scientific evidence for its hepatoprotection potential in different animal models[9].The protective effect of *T. akara* against D-Gal-induced liver injury has not been reported. In the present study, we have investigated the protective effects of ethanolic extract of roots of *Tetracera akara* against D-galactosamine induced hepatotoxicity in Wistar rats and its potential mechanisms of action.

Materials and Methods

Collection and authentication of plant material

Tetracera akara (Burm. f.) Merr. roots were collected from Kottoor (N 08º 35' 03.8'', E 77º 10' 54.8'' and altitude 585m), Thiruvananthapuram district of Kerala, India, and authenticated by the plant taxonomist of JNTBGRI, Palode. Voucher specimens was deposited at the Institute's Herbarium (TBGT 86868 dated 08/08/2015).

Preparation of ethanolic extract of *T. akara* root

The collected roots were washed in running water, shade dried and powdered. The powder was serially extracted with hexane followed by chloroform and then by 95 % ethanol for 48 h, using a Soxhlet apparatus. The third solvent fraction was then filtered and the filtrate was concentrated under reduced pressure in rotary evaporator, to get the ethanolic fraction with 9.5 % w/w of yield. The dry residue was stored at 4ºC, and at the time of use, was suspended in 0.5% v/v Tween-80. This ethanolic extract was referred to as TA ETH.

Animals

Wistar rats (150-175 g) were obtained from the Institute's Animal House. All the animals were housed in polypropylene cages under standard conditions at temperature 25±2°C, relative humidity 60±10%, room air changes 15±3 times/h and 12 h light-dark cycles, fed commercial rat feed (Lipton India Ltd; Mumbai, India) and boiled water ad libitum. Animals were acclimatized for 1 week before the initiation of an experiment. The study was carried out according to National Institute of Health (NIH) guidelines, after getting the approval of the Institute's Animal Ethics Committee (No. B1/03/2015/EMEP-11).

Commercial kits

Commercial kits for the estimation of aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), γ-glutamyl transferase (GGT), serum bilirubin (SB), triglycerides (TGL), total cholesterol (TC) and total protein (TP) were purchased from Coral Clinical System, Goa, India.

D-galactosamine (D-GalN) induced hepatotoxicity

D-galactosamine induced hepatotoxicity was carried out according to the procedure of Lin *et al.*[10]. Wistar rats were divided into six groups of six animals each. Group I, the normal control group was given a single daily dose of 0.5% Tween-80 for 5 days and injected with a single dose of saline (10 mL/kg, i.p.) on the 6th day. Group II, D-galactosamine control group was given a single daily dose of 0.5% Tween-80 for five days and injected with D-GalN (400 mg/kg in saline, i. p) on the 6th day. Groups III, IV and V, the drug treated groups were administered TA ETH reconstituted in 0.5% Tween-80 at dosages 50, 150 and 300 mg/kg, p.o., respectively. Group VI, the standard group was administered a single daily dose of Silymarin (100 mg/kg, p.o.) for 5 days and on the 6th day dosed with D-GalN as Group II. On the 7th day, 24 h after D-GalN treatment, all the animals were sacrificed by carbon dioxide inhalation and blood samples were collected from the carotid artery for evaluating plasma markers of hepatic injury and liver tissue were collected for evaluating the antioxidant status of liver, gene expression studies and histopathological studies.

Estimation of serum biochemical parameters

The collected blood samples in test tubes without EDTA were allowed to coagulate for 1 h at room temperature (25±2°C). It was centrifuged at 1500 rpm for 15 min at 37°C to separate the serum which was then subjected to the assay of plasma markers of hepatic injury like alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase

(ALP), γ-glutamyl transferase (GGT) and L-lactate dehydrogenase (LDH) were determined. Total serum protein (TP), albumin (ALB), total bilirubin (TBIL) and glucose (GLU) were also estimated using commercial kits purchased from Coral Clinical system, Goa, India.

Estimation of liver tissue parameters

Liver samples of all groups were weighted and homogenized separately using a tissue homogenizer. One portion (10% w/v) was homogenized in 50 mM, pH 7.4 phosphate buffer saline (PBS) which was centrifuged at 6000×g for 15 min at 3°C to remove the cell debris, unbroken cells, nuclei and erythrocytes[11]. The supernatant was used for the estimation of oxidative stress markers in liver like catalase (CAT), superoxide dismutase (SOD), reduced glutathione (GSH) and malondialdehyde (MDA) by comparing with standard curve generated from known amount of standards purchased from Thermo Fisher Scientific, USA.

Estimation of liver catalase

Catalase activity was measured according to the method described by Aebi[12]. Supernatant (0.1 mL) was added to a cuvette containing 1.9 mL of 50 mM phosphate buffer (pH 7.0). Reaction was started by addition of 1.0 mL of freshly prepared 30 mM H_2O_2. The rate of decomposition of H_2O_2 was measured spectrophotometrically by changes in absorbance at 240 nm. Results were expressed as IU of CAT activity/g wet tissue and the absorbance values were compared with a standard curve generated from known amount of catalase.

Estimation of superoxide dismutase

Superoxide radicals react with tetrazolium in the presence of NADH and produce formazan blue. SOD removes the superoxide radicals and inhibits the formation of formazan blue. The intensity of colour is inversely proportional to the activity of the enzyme. The reaction mixture contained 1.2 mL sodium pyrophosphate buffer (pH 8.3, 0.025 mol/L), 0.1 mL phenazine methosulphate (186 mmol/L), 0.3 mL nitroblue tetrazolium (NBT) (300 mmol/L), 0.2 mL NADH (780 mmol/L) and approximately diluted enzyme preparations and water in a total volume of 3 mL. After incubation at 30°C for 90 s, the reaction was terminated by adding 1 mL glacial acetic acid. The reaction was stirred vigorously and shaken with 4 mL n-butanol. The colour intensity of the chromogen in the butanol layer was measured at 560 nm against n-butanol and concentration of SOD were expressed as U/mg protein. The absorbance values were compared with a standard curve generated from known amount of SOD[13].

Estimation of reduced glutathione

Homogenized rat liver samples (10% w/v), 0.2 mL were mixed with 1.8 mL of 1 mM EDTA solution. To this, 3 mL precipitating reagent (1.67 g of met phosphoric acid, 0.2 g of EDTA disodium salt, 30 g sodium chloride in 1 L distilled water) was added, mixed thoroughly and kept for 5 min before centrifugation. To 2 mL of the supernatant, 4.0 mL of 0.3 M disodium hydrogen phosphate solution and 1.0 mL of DTNB [5, 5 dithio-bis (2-nitro benzoic acid)] reagent was added and absorbance was read at 412 nm. The absorbance values were compared with a standard curve generated from known amount of GSH[14].

Estimation of liver malondialdehyde

Malondialdehyde (MDA) in the rat liver was estimated by the modified procedure of Ohkawa *et al*.[15]. Supernatant (1 mL) was mixed with 100 µL of 8.1% sodium dodecyl sulfate (SDS), 600 µL of 20% acetic acid solution and was kept for 2 min at room temperature. Then 600 µL of 0.8% solution of freshly prepared thiobarbituric acid (TBA) was added, heated at 95°C for 60 min in a water bath and cooled with ice cold water at 4°C. A mixture of n-butanol and pyridine (15:1 v/v) were added, shaken vigorously and centrifuged at 10,000 rpm for 5 min. The absorbance of the organic layer was measured against blank at 532 nm. The amount of MDA (thiobarbituric acid reactive substance) was calculated using a molar extinction coefficient 1.56×105 M-1cm-1 and reported as nmoles of MDA/g tissue.

Gene expression studies using Quantitative PCR (qPCR) analysis

Total RNA was extracted from the cells using TRIzol reagent (Sigma-Aldrich, USA) according to the supplier's protocol which was then treated with RNase free DNase I. The purity and the concentration of total RNA was determined using Qubit 3.0 (Life technologies, USA) flurimetric analysis. cDNA was synthesized using Verso cDNA synthesis kit (Thermo Fisher Scientific, USA). The mRNA levels of various genes were evaluated by Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) using a Dynamo flash SYBR Green RT-PCR Kit (Applied

Biosystems, USA) and each sample was assessed in triplicate. qPCR cycling program was carried out according to manufacturer's protocol and the results were analysed using Applied Biosystems step-one data analysis software package. The primer sequence used for the study were shown in Table 1 and relative expression levels of TNFα, COX-2, IL-10 and iNOS were calculated relative to GAPDH (as house-keeping gene) using the comparative cycle threshold method. Here gene expression level of a particular gene of interest in drug-treated groups (toxin control, standard drug treated and TA ETH treated) were compared to that in untreated (normal control) sample. Comparative ΔΔ CT method was followed to study the effect of drug treatment on gene expression and the amount of target, normalized to an endogenous control and relative to a calibrator is given by $2^{-\Delta\Delta\ CT}$ equation[16].

Enzyme linked immune-sorbent assay (ELISA)

The release of cytokines during hepatic injury were estimated using TNFα (Catalog # K1052-100), IL-10 (Catalog #: ab100765) Abcam, ELISA Kits, USA. according to the manufacturer's instructions. The inducible enzyme COX-1 and COX-2 was determined by Rat COX ELISA kit (Code No: 18521 & 27187) from IBL, USA according to manufacturer's procedure.

Histopathological investigations.

Histological examination was performed in three animals, randomly selected from each group/sex. A portion of the internal organ obtained from all the groups was sliced into two pieces of approximately 6 mm^3 sizes and preserved in 10% formalin solution for 24 h. They were subjected to dehydration with acetone of strength 70, 80 and 100%, respectively, each for 1 h. Infiltration and impregnation were done by treatment with paraffin wax, twice each time for 1 h. Paraffin was used to prepare paraffin 'L' moulds. Specimens were cut into sections of 3-7 µm thickness and stained with Haematoxylin and Eosin (H & E). The thin sections of the liver were made in to permanent slides and examined under high resolution microscope with photographic facility and photomicrographs were taken.

Result

D-galactosamine (D- GalN) induced hepatotoxicity study

Estimation of plasma markers of hepatic injury

Treatment of rats with D-GalN induced hepatotoxicity as evident by significant ($P \leq 0.05$) increase in serum AST (282.30±3.42 IU/L), ALT (196.22±2.18 IU/L), ALP (274.01±3.87 IU/L) GGT (24.63±2.15 IU/L), SB (1.50±0.09 mg/dL), TC (204.17±3.08 mg/dL), TGL (241.18±3.75 mg/dL) and decreased levels of TP (3.65±0.62 g/dL) in toxic control group when compared to normal group with AST (74.35±2.26 IU/L), ALT (68.49±2.52 IU/L), ALP (102.63±2.78 IU/L) GGT (6.32±0.93 IU/L), SB (0.49±0.07 mg/dL), TC (88.52±2.34 mg/dL), TGL (106.81±2.81 mg/dL) and TP (6.18±0.62 g/dL) as shown in Table 2. Pre-treatment with TA ETH (50, 150 and 300 mg/kg) caused significant ($P \leq 0.05$) protection against D-GalN intoxicated rats by attenuating AST, ALT, ALP, GGT, SB, TC, TGL and TP elevation in a dose dependent manner. For all the eight biochemical parameters under investigation, TA ETH (300 mg/kg) (AST: 88.58±2.17 IU/L, ALT: 75.16 ± 2.21 IU/L, ALP: 131.25 ± 3.40 IU/L, GGT: 6.89±0.65 IU/L, SB: 0.68±0.04 mg/dL, TC: 82.96±3.31 mg/dL, TGL: 125.20±1.38 mg/dL and TP: 5.80±0.17 g/dL) was found to be more significant than the lower doses studied, which is almost comparable to that of Silymarin (100 mg/kg) (AST: 92.58±2.43 IU/L, ALT: 63.50±2.24 IU/L, ALP: 120.83±2.46 IU/L, GGT: 7.39±0.42 IU/L, SB: 0.61±0.04 mg/dL, TC: 94.72±2.18 mg/dL, TGL: 117.12±2.35 mg/dL and TP: 5.94±0.72 g/dL), the standard drug used in the study.

Evaluation of *in vivo* antioxidant status of liver

The Catalase in liver tissue decreased significantly in D-GalN intoxicated animals (42.28±3.75 U/mg protein) of toxin group when compared to normal control group (154.36±5.47 U/mg protein). The SOD

Table 1 — Primer sequence used in the study

Target Gene	Forward Sequence	Reverse Sequence	Amplified band (base pair)
TNF-α	5′-ACT GAA CTT CGG GGT GAT TG-3′	5′-GCT TGG TGG TTT GCT ACG AC-3′	234
COX-2	5′-TTC AAA TGA GAT TGT GGG AAA ATT GCT-3′	5′-AGA TCA TCT CTG CCT GAG TAT CTT T-3′	210
IL-10	5′-AGA AGG ACC AGC TGG ACA AC-3′	5′-GTC GCA GCT GTA TCC AGA GG-3′	210
iNOS	5′-GAGCCCTACGAGCCGTTGCC-3′	5′-GCGAATGGTCCTGCGGCGTA-3′	195

levels in the toxin group was lowered to 3.45±0.98 U/mg where as it was found to be 12.76±1.03 U/mg in normal control. GSH showed significantly ($P \leq 0.05$) decreased value of 7.86±0.95 µmol/g tissue when compared to normal control. All groups of animals administered with various doses of TA ETH showed an increase in hepatic Catalase, SOD and GSH in a dose dependent manner of which TA ETH at 300 mg/kg showed maximum protection against D-GalN intoxication in animals which is evident from the higher levels of Catalase (132.62±5.18 U/mg protein), SOD (13.72±1.15 U/mg protein) and GSH (50.48±2.87 µmol/g tissue). The MDA levels in toxin control animals (58.20±3.25 µmol/g wet liver) were higher when compared to the normal control (15.44±2.12 µmol/g wet liver) and it was decreased to a normal level in the TA ETH treated groups where the maximum inhibition was obtained in (300 mg/kg) body wt. p.o. of TA ETH treated groups (17.59±1.82 µmol/g wet liver) and it was almost similar to the standard Silymarin (100 mg/kg) treated groups (19.08±2.08 µmol/g wet liver) as shown in Fig. 1.

Gene expression studies using Quantitative PCR (qPCR) analysis

D-GalN significantly altered the synthesis of inflammatory cytokine and inflammatory enzyme as

Table 2 — Effect of TA ETH on hepatic markers of liver injury in D-GalN induced acute liver damage in Wistar rats

	Parameters							
	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	GGT (U/L)	SB (mg/dL)	TC (mg/dL)	TGL (mg/dL)	TP (g/dL)
Normal control	74.35± 2.26	68.49± 2.52	102.63± 2.78	6.32± 0.93	0.49± 0.07	88.52± 2.34	106.81± 2.81	6.18± 0.62
Toxin-D-GalN (400 mg/kg)	282.30± 3.42*	196.22± 2.18*	274.01± 3.87*	24.63± 2.15*	1.50± 0.09*	204.17± 3.08*	241.18± 3.75*	3.65± 0.62*
D-GalN+STD Silymarin (100 mg/kg)	92.58± 2.43**	63.50± 2.24**	120.83± 2.46**	7.39± 0.42**	0.61± 0.04**	94.72± 2.18**	117.12± 2.35**	5.94± 0.72**
D-GalN+TA ETH (50 mg/kg)	238.42± 2.54	167.43± 2.17	229.37± 2.72	19.81± 1.70	1.21± 0.06	189.63± 2.67	196.56± 2.93	3.96± 0.26
D-GalN+TA ETH (150 mg/kg)	137.84± 1.52**	96.33± 2.48**	149.65± 2.53**	10.64± 1.46**	0.76± 0.05**	118.60± 3.67**	135.31± 1.65**	5.01± 0.38**
D-GalN+TA ETH (300 mg/kg)	88.58± 2.17**	75.16± 2.21**	131.25± 3.40**	6.89± 0.65**	0.68± 0.04**	82.96± 3.31**	125.20± 1.38**	5.80± 0.17**

[Values are expressed as mean ± SEM of six values, one way ANOVA followed by Dunnett's multiple comparison test, *$P \leq 0.05$ compared to normal control, **$P \leq 0.05$ compared to D-GalN control]

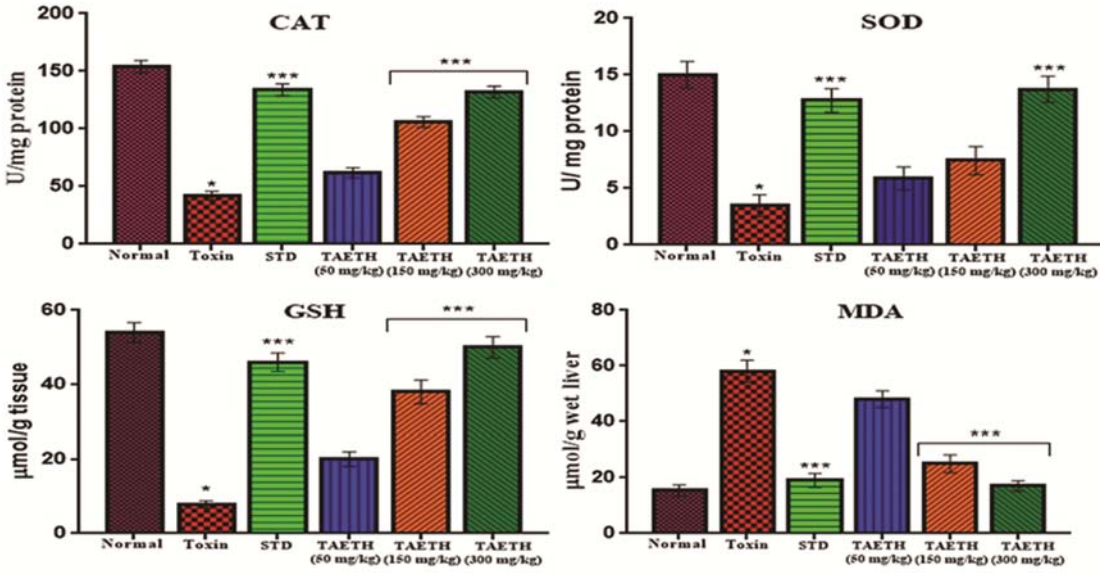

Fig. 1 — Effect of ethanolic extract of roots of *Tetracera akara* (TA ETH) on hepatic CAT, SOD, GSH and MDA of Wistar rats in D-GalN induced hepatotoxicity study. [Values are expressed as mean ± SEM, n=6, one way ANOVA followed by Dunnett's multiple comparison test, * $P \leq 0.05$ compared to normal control, *** $P \leq 0.05$ compared to D-GalN toxin control]

evident from the comparative gene expression study in the experimental animals. The expression level of pro inflammatory cytokine like TNFα was highly elevated, whereas the anti-inflammatory cytokine, IL-10 expression was downregulated in the toxin group when compared with the normal control. The inflammatory enzyme COX-2 and iNOS expression in the toxin control was also elevated when compared to normal, indicating severe inflammation and onset of hepatic damage. The administration of TA ETH significantly ($P \leq 0.05$) downregulated the gene expression pro inflammatory cytokines and inflammatory enzyme, when compared to the toxin group and the maximum protections was offered by TA ETH at (300 mg/kg) as shown in Fig. 2 (A1-A4). The anti-inflammatory IL 10 gene was upregulated in a dose dependent manner in drug treated group. TA ETH at 300 mg/kg body wt. treated animals showed reduced fold change of 0.61±0.16 for TNF-α, 1.98±0.18 for COX-2, 1.14±0.15 for iNOS and 1.75±0.35 for IL 10 indicating lower hepatic damage and inflammatory conditions.

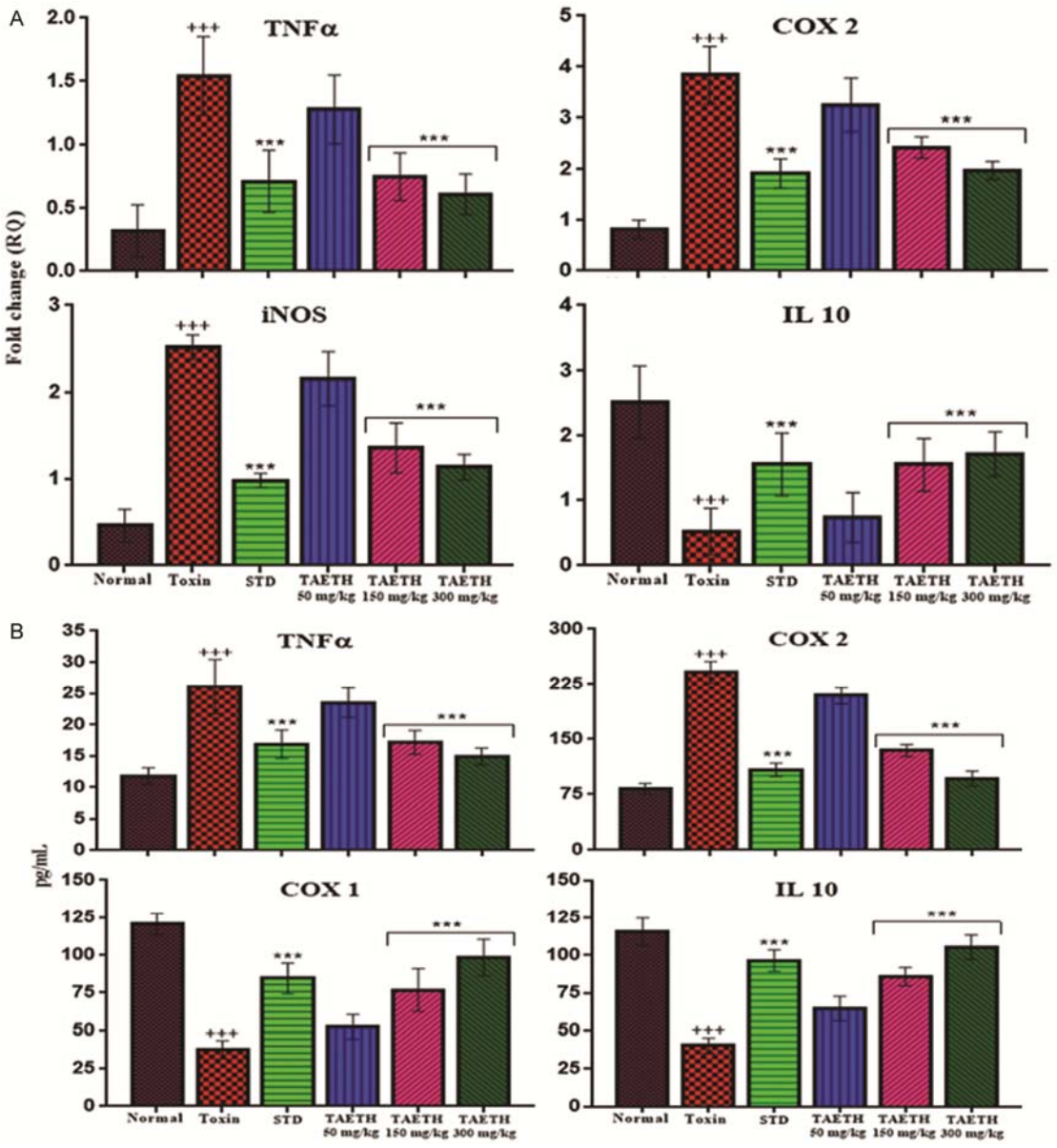

Fig. 2 — (A1, A2, A3 & A4): Effect of ethanolic extract of roots of *Tetracera akara* (TA ETH) on expression of TNFα, COX-2, iNOS, IL-10 genes; and (B1, B2, B3 & B4): Effect of TA ETH on protein level expression of TNFα, COX-2, COX-1, IL-10 in liver tissue of Wistar rats with D-GalN induced acute hepatic damage. [Values are expressed as mean ± SEM, n=6, one way ANOVA followed by Dunnett's multiple comparison test, * $P \leq 0.05$ compared to normal control, *** $P \leq 0.05$ compared to D-GalN toxin control]

Enzyme linked immune-sorbent assay (ELISA).

The results obtained for TNFα, COX 1, COX 2 and IL 10 protein expression in D-GalN induced liver tissues were in coordination with that of the gene expression studies. The results showed that D-GalN induction significantly increased the level of inflammatory COX-2 expression, whereas the production of COX-1 was highly lowered. TA ETH administration reduced the COX-2 protein expression to 96.37±6.15 pg/mL which is comparable to the standard indomethacin treated group (108.46±9.82 pg/mL). When compared with the normal control, the protein levels of proinflammatory cytokine such as TNFα was remarkably increased, whereas the anti-inflammatory IL 10 levels was lowered in D-GalN induced toxin control as shown in Fig. 2 (B1-B4). Oral administration with TA ETH significantly ($P \leq 0.05$) inhibited the cytokine release and elevated the production of IL 10 and maximum inhibition was obtained in 300 mg/kg dose treated group, almost comparable to the standard treated group.

Histopathological investigations

The liver histopathology of control group showed normal hepatic architecture with no evidence of microscopic abnormalities. D-GalN intoxicated animals in toxin group showed enlarged cell, vacuolated cytoplasm and large nuclei with condensed chromatin, ballooning degeneration, the loss of cellular boundaries, nuclear pycnosis, focal hepatic necrosis, Kupffer cell infiltration and congestion of hepatic sinusoids. TA ETH treated group appeared to reduce the Galactosamine toxicity as revealed by the healthy central vein and hepatocytes with clear cytoplasm and nuclei. The histological architecture of liver sections of the rats treated with higher dose of TA ETH showed more or less normal lobular pattern with a mild degree of fatty change, necrosis and lymphocyte infiltration which is almost comparable to the normal control and STD Silymarin groups as shown in Fig. 3.

Discussion

D-galactosamine (D- GalN) induced hepatotoxicity

GalN inhibits the energy metabolism of hepatocytes[17] by blocking enzymes involved in the transport of substrates to the mitochondria and modifies the phospholipid composition of membranes which alters the permeability of the cellular membrane and finally causing enzyme leakage from the cells[18]. Excessive production of free radicals resulting from oxidative stress can damage macromolecules as lipids, decrease levels of hepatic antioxidant enzymes and increased MDA level, a byproduct of lipid peroxidation[19]. Liver damage induced by D-GalN generally reflects as disturbances of hepatocytic metabolism, which lead to characteristic changes in the transport function and activities of serum enzymes and as leakage of enzymes from cells due to altered permeability of membranes[20]. The results obtained from the present study showed a significant increase in the serum levels of AST, ALT, ALP, GGT, SB, TC, TGL and a decrease in the production of TP in toxin control groups which is in accordance with the earlier findings[21]. The levels of these marker enzymes are proportional to the extent of hepatic damage and their estimation can be used for the diagnosis as an indicator of prognosis of the hepatic disease[22]. Hepatocellular necrosis leads to an increase in serum levels of both AST and ALT which are released from the liver into the blood stream. Among the two enzymes, ALT is a better index of liver injury, as liver ALT activity represents 90% of total enzyme activity present in the body. ALP activity when considered, is related to the hepatocyte function. ALP is a membrane bound glycoprotein enzyme reported to be involved in the transport of metabolites across the cell membranes, protein synthesis, synthesis of certain enzymes[23]. An increase in ALP activity is due to elevated synthesis in the presence of increased biliary pressure. The increased SB, TC and TGL in the serum of D-GalN induced rats were also supported by the earlier studies[24,25]. Pretreated with standard drug Silymarin and TA ETH restored the altered serum marker enzymes towards normal in experimental animals. The reduction of AST, ALT, ALP, GGT, SB, TC, TGL and increased TP towards normal values by the administration of TA ETH is an indication of stabilization of plasma membranes as well as repair of damage tissues caused by D-GalN. This effect is in perfect agreement with the fact that serum levels of transaminases return to normal with healing of the liver parenchymal cells and healing of hepatocytes indicating the hepatoprotective activity of *T. akara* against viral hepatitis induced by D-GalN[26].

The activities of antiperoxidative enzymes such as SOD and CAT have been reported to decrease in D-GalN hepatitis[27]. The results obtained showed decreased activities of SOD and CAT activities in

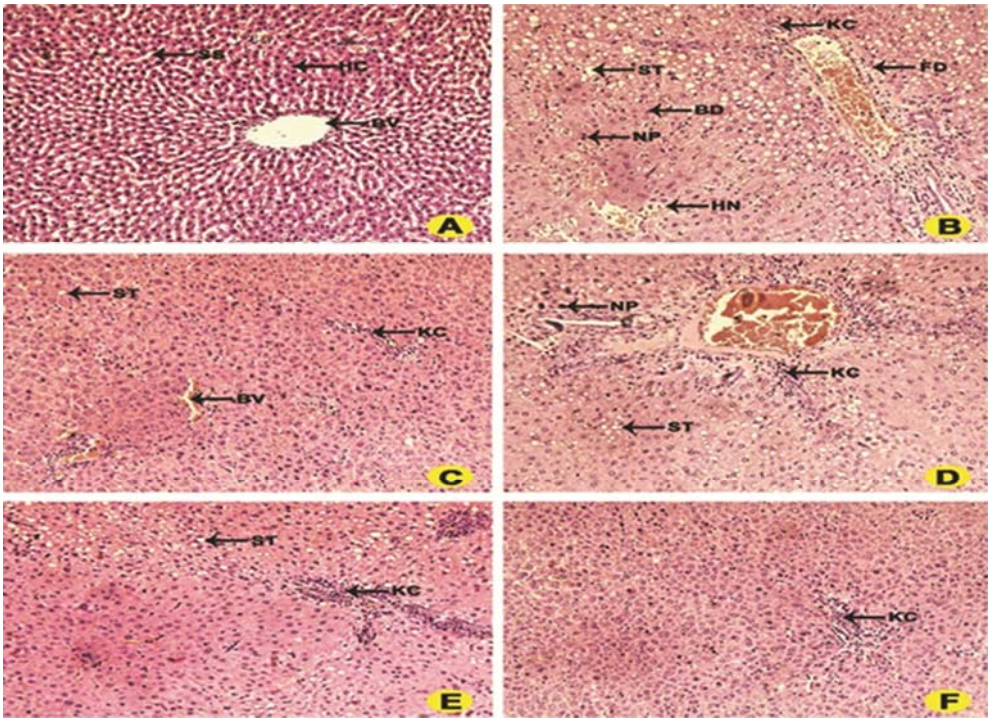

Fig. 3 — Effect of ethanolic extract of roots of *etracera akara* (TA ETH) on the histopathology of D-GalN induced liver damage in Wistar rats (×50, H & E staining). (A) Normal control rat liver histology showing normal hepatic architecture; (B) Toxin control rat liver with vacuolated cytoplasm & large nuclei with condensed chromatin, ballooning degeneration, the loss of cellular boundaries, nuclear pycnosis, focal hepatic necrosis and Kupffer cell infiltration; (C) Standard Silymarin treated group showing almost normal hepatic architecture; (D & E) TA ETH (50 and 150 mg/kg) treated groups showing reduced hepatic damage in a dose depended manner; and (F) TA ETH (300 mg/kg) treated group showing normalized hepatic architecture comparable to the standard. [SS, Sinusoidal space; BV, Blood vessel; HC, Hepatic cells; ST, Steatosis; KC, Kupffer cells; FD, Fatty degradation; BD, Ballooning degeneration; NP, Nuclear pyknosis; and HN, Hepatic necrosis]

toxin group and the decreased activities might be a consequence of irreversible inactivation of enzyme proteins from increased free-radical production. Reduced levels of these enzymes will result in the accumulation of highly reactive free radicals, leading to deleterious effects such as loss of cell membrane integrity and membrane function[28]. Significant increases in the activities of these enzymes were observed in TA ETH administrated animal groups. The antioxidant phytoconstituents in TA ETH scavenges superoxide radicals and hydrogen peroxide radicals, which might decrease the workload of enzymatic antioxidants and reduce the free-radical-mediated inactivation of enzyme proteins, and thereby maintaining the activities of enzymatic antioxidants. The non-enzymatic scavengers, such as GSH, scavenge residual free radicals escaping decomposition by the antioxidant enzymes, acting as the second line of defense[29]. Moreover, enzymatic antioxidants are inactivated by the excessive levels of free radicals and hence the presence of non-enzymatic antioxidants are presumably essential for the removal of these radicals[30]. Glutathione is a major non-protein thiol in living organisms, which play a central role in coordinating the antioxidant defense process in our body. Glutathione reacts directly with ROS and electrophilic metabolites, protects essential thiol group from oxidation and serves as a substrate for several enzymes including GPx[31]. The lowered GSH in D-GalN induced rats represents the increased utilization of GSH as a result of oxidative stress[32]. TA ETH administration improved significantly the GSH level mainly because of inactivation of ROS *via* its radical scavenging effects and retention of enzymic antioxidants in hepatocytes. Excessive production of free radical results in the oxidative stress, which leads to the damage of macromolecules and can induce *in vivo* lipid peroxidation[33]. MDA, a byproduct of lipid peroxidation was found to be elevated in toxin group, indicating membrane destabilization and hepatocytic damage. Pretreatment with TA ETH inhibited lipid peroxidation, suggesting that phytoconstituents in it exerted a stabilizing action on hepatocytic membranes and inhibited MDA formation.

Inflammatory response forms a major contributing factor, leading to liver injury under various stimulations and D-GalN can activate Kupffer cells, leading to the release and accumulation of proinflammatory cytokines[34]. Previous studies have shown that regulation of the inflammatory response is an efficient strategy for the management of acute liver injury[35]. TNF-α has been reported as the most important pro inflammatory cytokine for the progress and development of acute liver injury and in this study, there was a significant difference observed between the toxin group and TA ETH treated group, indicating that the extract attenuated liver injury by downregulation of TNF-α expression. NO is a high reactive molecule synthesized by iNOS from L-arginine and overproduction of NO could suppress the growth of lymphocytes and damaging other normal cells or tissues leading to inflammatory disorder[36]. PGE_2 is also an inflammatory mediator produced by COXs which exists in two forms. COX-1, associated with the production of prostaglandins that activate platelets and protect the stomach and intestinal lining. COX-2 is dramatically upregulated during inflammation and induces inflammatory responses such as fever and vasodilatation by generating PGE_2[37]. In the current study, it was found that the gene and protein level expression of COX-2 was evidently increased in the rats treated with D-GalN indicating severe inflammation and tissue damage, whereas the expression of COX-1 was very much lowered. However, pre-administration of TA ETH markedly decreased COX-2 production compared to D-GalN-stimulated toxin control. In addition, TA ETH pretreatments significantly elevated mRNA as well as protein expression of IL-10 and protein level expression of COX-1 compared to D-GalN-stimulated control.

The histological evidence authenticated the injury caused by D-GalN and the protection offered by TA ETH to hepatocytes. Microscopical examination revealed loss of hepatic architecture with ballooning degeneration, nuclear pycnosis, focal hepatic necrosis, Kupffer cell infiltration and inflammatory collections in the central zone in D-GalN induced rats of toxin group. Prior oral administration with TA ETH extract prevented the histopathological changes in the liver induced by D-GalN in a dose dependent manner. TA ETH (300 mg/kg) treated rats showed normal morphology with more or less normal lobular pattern, necrosis and lymphocyte infiltration without appreciable histological abnormalities, comparable to the standard Silymarin (100 mg/kg) treated animals. The histopathological studies serve as a direct evidence supporting the result of biochemical serum parameters and antioxidant status of D-GalN treated animals. Thus, the bioactive phytoconstituents with antioxidant potential in TA ETH effectively scavenged the free radicals formed during the D-GalN metabolism and reduced the oxidative stress in hepatocytes. The anti-lipid peroxidation effect of TA ETH played a major role in stabilizing the hepatocytic membrane, in turn enhanced the production, action and prevented the leakage of antioxidant enzymes from hepatocyte. It also enhanced the regeneration of hepatocytes, damaged by D-GalN in experimental animals. The protective action of TA ETH against D-GalN toxicity is of clinical importance because there is close resemblance between the multifocal necrosis produced by D-GalN and the lesion of viral hepatitis in humans and the results of this study suggest that *T. akara* could play a major role in the treatment of viral hepatitis.

Conclusion

Resutls of this investigation demonstrated the hepatoprotective potential of ethanolic extract of roots of *Tetracera akara* possibly by scavenging reactive free radicals, boosting the endogenous antioxidant system in liver, inhibiting pro-inflammatory mediators like TNF α, COX-2, iNOS and by promoting anti-inflammatory IL 10. Further research is required to explicate the detailed mechanism of action of phytoconstituents in TA ETH on acute liver damage for exploitation of its broader remedial usage and development of a therapeutic drug with least side effect.

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Conflict of interest

Authors declare no conflict of interests associated with this study.

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