Research Article

Antihepatotoxic effect of the ethanolic fraction of roots of *Tetracera akara* (Burm. f.) Merr., on Acetaminophen-induced hepatic damage in Wistar rats

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ABSTRACT

Objective: The objective of the study was to evaluate the hepatoprotective activity of *Tetracera akara* root ethanolic fraction on paracetamol-induced hepatotoxicity. **Materials and Methods:** Hepatotoxicity was induced in Wistar rats by the oral administration of paracetamol (2.5 g/kg/day for 6 days). The ethanolic fraction of *T. akara* root 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 of the extracts was evaluated by the assay of biochemical markers of hepatic injury (total bilirubin, serum protein, alanine aminotransaminase, aspartate aminotransaminase, and alkaline phosphatase activities), antioxidant status of the liver by estimating (hepatic catalase [CAT], superoxide dismutase [SOD], glutathione, and malondialdehyde), and histopathological evaluation of the liver. **Results:** In *T. akara* root ethanolic fraction administered animals, the toxic effect of paracetamol was controlled significantly by restoration of the levels of serum bilirubin, protein, and hepatic enzymes as compared to the normal and the standard drug silymarin-treated groups. Oral administration of plant drug elevated the levels of antioxidant enzymes such as SOD, CAT in liver and inhibited lipid peroxidation as evident from the reduced levels of MDA. The histopathological observations were in correlation with the biochemical findings. The animals treated with the extracts showed the presence of normal hepatic cords, the absence of necrosis and fatty infiltration, which further evidenced the hepatoprotective activity. **Conclusion:** Ethanolic fraction of the root of *T. akara* possesses significant hepatoprotective activity, thus substantiating the tribal claim.

KEY WORDS: Kani tribe, Paracetamol, Pattuvalli, *Tetracera akara*

INTRODUCTION

Tetracera akara (Burm. f.) Merr. locally known as "Pattuvalli," belongs to the family Dilleniaceae. It is a woody climber distributed in the Western Ghats region of Kerala and Tamil Nadu and the roots of which is used by the Kani tribe of Kerala to cure liver diseases. The medicinal use of *T. akara* was first reported by S. Rajasekharan and his team from JNTBGRI in 1987, during ethnobiological studies carried out with the help of a tribal healer, Mallan Kani residing in the Chonnampara tribal settlement of Thiruvananthapuram district. According to Mallan Kani, the paste of the fresh root of "Pattuvalli" is administered orally in an empty stomach to cure chronic liver disorders and inflammatory conditions.[1] However, no scientific studies have been reported so far regarding the hepatoprotective property of this plant until the date, and it has become imperative to scientifically validate the tribal claim. The result obtained from the previous studies like comparative *in vitro* antioxidant studies revealed that *T. akara* ethanolic fraction possessed potent antioxidant potential[2] and is safe for oral administration in experimental animals as reported earlier.[3]

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MATERIALS AND METHODS

Collection and Authentication of Plant Material

T. akara (Burm. f.) Merr. roots were collected from Kottoor (N 08º 35' 03.8", E 77º 10' 54.8", and altitude 585 m), Thiruvananthapuram district of Kerala, India, and authenticated by the plant taxonomist of JNTBGRI, Palode. Voucher specimens were

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deposited at the Institute's Herbarium (TBGT 86868 dated 08/08/2015).

Preparation of Ethanolic Fraction 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 4°C, and at the time of use, was suspended in 0.5% v/v Tween-80. This ethanolic fraction was referred to as TA ETH.

Animals Used

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 guidelines, after getting the approval of the Institute's Animal Ethics Committee (No. B-l6/02/2015/EMEP.15).

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.

Paracetamol (Acetaminophen [APAP]) Induced Hepatotoxicity

Paracetamol-induced hepatotoxicity was carried out according to the standardized procedure.[4] Wistar rats were divided into six groups (6 animals/group), Groups I and II were the normal control and paracetamol intoxicated group, respectively, and both received a single daily dose of 0.5% Tween-80 (1 mL, p. o.) for all 6 days. Groups IV-VI were administered TA ETH reconstituted in 0.5% Tween-80 at dosages (50, 150, and 300 mg/kg, p. o.) for all 6 days and Group III was administered silymarin, the standard hepatoprotective drug, at a dose of 100 mg/kg, p.o., for all 6 days. Paracetamol suspension (2.5 g/kg, p.o.) was administered to Groups II-VI on the 5th day, 30 min after plant extract/silymarin administration. On the 6th day after 24 h of starvation, all the animals were sacrificed using CO_2 inhalation. Blood samples were collected from the carotid artery and allowed to coagulate for 1 h at room temperature and later centrifuged at 1500 rpm for 15 min at 37°C to separate the serum. The serum was then used for evaluating the above- mentioned biochemical parameters (estimation of plasma markers of hepatic injury). Liver samples collected in phosphate buffered saline were subjected to assays such as estimation of malondialdehyde (MDA), assay of catalase (CAT), determination of reduced glutathione (GSH), and superoxide dismutase (SOD) to reveal the antioxidant status of liver and liver slices preserved in 10% neutral buffered formaldehyde solution were subjected to histopathological examination.

Histopathological Investigations

Liver slices preserved in buffered formalin 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" molds. Specimens were cut into sections of 3–7 µm thickness and stained with hematoxylin and eosin. The thin sections of the liver were made into permanent slides and examined under high-resolution microscope with photographic facility and photomicrographs were taken.

Statistical Analysis

All the data were expressed as a mean ± standard error of the mean. The significance of difference among the group was assessed using one-way analysis of variance (ANOVA) followed by Dunnett's posttest using GraphPad Prism version 7.00. $P \leq 0.05$ was considered statistically significant.

RESULTS

Estimation of Plasma Markers of Hepatic Injury

Wistar rats administered with overdose of APAP developed liver damage which is indicated by significant ($P \leq 0.05$) increase in serum AST (272.62 ± 5.61 IU/L), ALT (225.16 ± 4.67IU/L), ALP (262.65 ± 4.48 IU/L), GGT (27.43 ± 2.28 IU/L), SB (1.86 ± 0.32 mg/dL), TC (192.84 ± 3.90 mg/dL), and TGL (258.73 ± 3.37* mg/dL) and decrease in TP (3.56 ± 0.16 g/dL) in toxin control group compared to normal control. Pre-treatment with TA ETH (50, 150, and 300 mg/kg b. w., p. o) caused significant ($P \leq 0.05$) protection against APAP toxicity by attenuating AST, ALT, ALP, GGT, SB, TC, and TGL and elevation of TP as shown in Table 1. For all the eight biochemical parameters studied, TA ETH showed potent activity in a dose-dependent manner and from among the various doses, TA ETH (300 mg/kg) (AST: 110.62 ± 2.36 IU/L; ALT: 71.93 ± 2.68 IU/L, ALP: 122.42 ± 3.22 IU/L, GGT 7.38 ± 0.16 IU/L, SB: 0.57 ± 0.04 mg/dL, TC: 106.76 ± 3.60 mg/dL, TGL: 119.66 ± 1.82 mg/dL, and TP: 5.96 ± 0.12 g/dL) was found

Table 1: Effect of TA ETH on plasma markers of hepatic injury in APAP-intoxicated Wistar rats

Treatment groups	Parameters							
	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	GGT (IU/L)	SB (mg/dL)	TC (mg/dL)	TGL (mg/dL)	TP (g/dL)
Normal control	80.42±1.18	61.38±2.71	93.84±2.08	6.76±1.37	0.36±0.05	95.62±1.63	122.56±3.72	6.52±0.45
Toxin –APAP (2.5 mg/kg)	272.62±5.61*	225.16±4.67*	262.65±4.48*	27.43±2.28*	1.86±0.32*	192.84±3.90*	258.73±3.37*	3.56±0.16*
APAP+STD silymarin (100 mg/kg)	102.73±2.14**	67.52±0.82**	118.32±2.18**	6.92±0.17**	0.52±0.08**	110.78±2.52**	126.14±1.26**	5.75±0.16**
APAP+TA ETH (50 mg/kg)	242.45±2.16	188.56±3.70	229.75±2.78	21.36±2.42	1.65±0.08	176.74±2.38	227.58±2.34	4.08±0.17
APAP+TA ETH (150 mg/kg)	138.34±1.18**	90.52±2.34**	137.38±2.72**	12.25±1.48**	0.63±0.05**	118.12±3.62**	135.65±1.60**	5.13±0.27**
APAP+TA ETH (300 mg/kg)	110.62±2.36**	71.93±2.68**	122.42±3.22**	7.38±0.16**	0.57±0.04**	106.76±3.60**	119.66±1.82**	5.96±0.12**

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 APAP toxin control, CAT: Catalase, AST: Aspartate transaminase, ALT: Alanine transaminase, ALP: Alkaline phosphatase, GGT: γ-glutamyl transferase, SB: Serum bilirubin, TGL: Triglycerides, TC: Total cholesterol, TP: Total protein, APAP: Acetaminophen, SEM: Standard error of the mean

to be the significant dose among of *T. akara* studied. The reduction in biochemical parameters exhibited by TA ETH (300 mg/kg) was almost comparable to that of silymarin (100 mg/kg) (AST: 102.73 ± 2.14 IU/L, ALT: 67.52 ± 0.82 IU/L, ALP: 118.32 ± 2.18 IU/L, GGT 6.92 ± 0.17 IU/L, SB: 0.52 ± 0.08 mg/dL, TC: 110.78 ± 2.52 mg/dL, TGL: 126.14 ± 1.26 mg/dL, and TP: 5.75 ± 0.16 g/dL), the standard drug used in the study.

Evaluation of *in Vivo* Antioxidant Status of Liver

CAT activity was depleted significantly ($P \leq 0.05$) in APAP intoxicated animals (75.35 ± 4.04 U/mg protein) of toxin group when compared to normal control group (168.42 ± 8.23 U/mg protein). The SOD levels in the toxin group were also reduced to 4.56 ± 0.62 U/mg proteins when compared to the normal control group with 17.32 ± 1.25 U/mg proteins. The level of GSH significantly ($P \leq 0.05$) decreased in APAP intoxicated animals (11.48 ± 1.47 µmol/g tissue) when compared to normal control group (58.26 ± 3.36 µmol/g tissue) and all the drug doses showed significantly ($P \leq 0.05$) increased the GSH values. In all the animals treated with various doses of TA ETH showed an increase in hepatic CAT, SOD, and GSH in a dose-dependent manner. TA ETH at 300 mg/kg showed maximum protection against APAP intoxication in animals which is evident from the higher levels of CAT (146.32 ± 5.74 U/mg protein), SOD (14.05 ± 1.08 U/mg protein), and GSH (53.46 ± 2.94 µmol/g tissue). The MDA levels in the liver of toxin control animals (45.58 ± 3.42 µmol/g wet liver) were higher when compared to the normal control (13.28 ± 2.06 µmol/g wet liver). The MDA levels were found to be lower in the TA ETH treated groups compared to toxin control groups, and the maximum inhibition was obtained in TA ETH (300 mg/kg) treated groups (16.23 ± 0.74 nmol/g wet liver) and it is almost comparable to that of the standard silymarin (100 mg/kg) treated groups (17.22 ± 2.36 µmol/g wet liver) as shown in Figure 1.

Histopathological Investigations

Histological examination of control animals showed normal hepatic architecture with distinct hepatic cells (HC) and sinusoidal space. The liver sections of toxin control group of animals exhibited disarrangement of normal HC, intense congestion, hydropic degeneration, pyknosis, centrilobular necrosis (CN), sinusoidal congestion (SC), infiltration of the lymphocytes, Kupffer cells (KF) around the central vein, loss of cell boundaries, and ballooning degeneration were observed after the intoxication of paracetamol. The liver histology of silymarin-treated animals showed normal hepatic architecture with few fatty globules and infiltration of KF. The liver histology of the animals treated with higher doses of TA ETH showed normal hepatic cords and absence of

severe congestion, pyknosis, and occasional necrosis and the normal cellular architecture was retained as compared to those of the control rats as shown in Figure 2.

DISCUSSION

Hepatic injury and subsequent hepatic failure due to both intentional and non-intentional overdose of paracetamol (APAP) have affected patients for decades and involve the cornerstone metabolic pathways which take place in the microsomes of hepatocytes. Overdosage of APAP leads to liver injury and disturbance in the transport function of the hepatocytes, resulting in the leakage of plasma,[5] and a variety of enzymes normally located in the cytosol are released into bloodstream. In this study, the paracetamol-induced hepatic damage is evident from the significant increase in the level of serum marker enzymes, namely AST, ALT, ALP, and GGT in APAP administered groups as compared to the normal control. The animal group treated with TA ETH has shown a decrease in the liver enzyme levels in a dose-dependent manner, and TA ETH (300 mg/kg) showed reduced enzyme level similar to the standard hepatoprotective drug, silymarin (100 mg/kg) administered group. The lowering of enzyme levels in the extracts administered group may be attributed to the hepatoprotective activity offered by the bioactive phytoconstituents in TA ETH against APAP-induced liver toxicity. Increased levels of SB in APAP administered toxin group may be due to the excessive destruction of hepatocytes, blockage of the biliary tract, mass inhibition of the conjugation reaction of hepatic enzymes or the release of unconjugated bilirubin from damaged and dead hepatocytes.[6] TA ETH administered groups showed significantly lower bilirubin levels compared to the toxin group which reveals the ability of the plant extract to bring the liver function into normal mainly by promoting conjugation reaction and maintaining cellular integrity of hepatocytes.

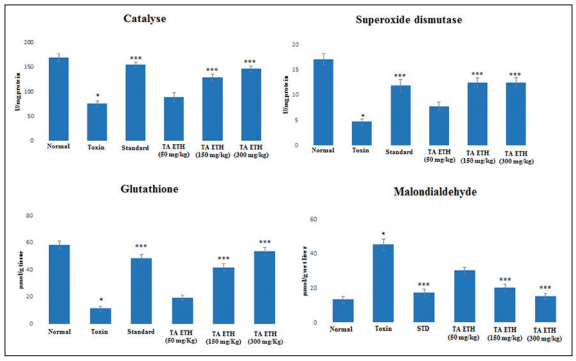

Figure 1: Effect of the ethanolic extract of roots of *Tetracera akara* (TA ETH) on hepatic catalase, superoxide dismutase, glutathione, and malondialdehyde of Wistar rats in acetaminophen (APAP)-induced hepatotoxicity study. Values are expressed as a mean ± standard error of the mean, $n = 6$, one-way ANOVA followed by Dunnett's multiple comparison test, $^{*}P \leq 0.05$ compared to normal control, $^{***}P \leq 0.05$ compared to APAP toxin control

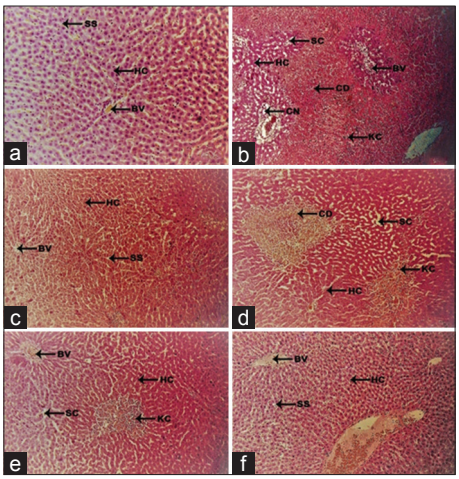

Figure 2: Effect of the ethanolic extract of roots of *Tetracera akara* (TA ETH) on the histopathology of acetaminophen-induced liver damage in Wistar rats (×50, H and E staining). (a) Normal control rat liver histology showing normal hepatic architecture. (b) Toxin control rat liver showing centrilobular necrosis (CN), sinusoidal congestion (SC), Kupffer cells (KF) infiltration, and cellular disintegration (CD). (c) Standard silymarin-treated group showing almost normal hepatic architecture with mild degree of damage. d-f are TA ETH (50, 150, and 300 mg/kg) treated groups showing reduced hepatic damage in a dose-depended manner SS - sinusoidal space, HC - hepatic cells, and BV - blood vessel

Biochemical serum parameters such as TC and TGL were also significantly increased in APAP alone treated rats, when compared to normal control. Elevated levels of TC and TGL indicate impaired fat metabolism due to hepatocytic damage. Administration of *T. akara* ethanolic fraction significantly decreased serum lipid profile in paracetamol intoxicated rats, from which it can be hypothesized that the administration of TA ETH helped to restore the fat metabolism. The plant drug also possesses hypolipidemic activity which is evident from the significant reduction in of TC and TGL levels. Protein synthesis is the major function of the liver, and healthy functioning of the liver is required for the synthesis of serum proteins except for the γ-globulins. The decreased TP in the toxin control group may be due to decreased protein metabolism and lipid peroxidation in the liver and is a feature of chronic hepatic damage.[7] It is worth to note that the TA ETH (300 mg/kg) effectively enhanced the protein metabolism and reduced the hepatic damage when compared to the toxin control group.

Both enzymatic and non-enzymatic antioxidant system is essential for cellular response to deal with oxidative stress under physiological condition.

Therefore, antioxidant enzymes such as CAT, SOD, and non-enzymatic electron receptors such as GSH are affected and used as indexes to evaluate the level of oxidative stress. The equilibrium between free radicals formed due to the increased level of NAPQI (a highly reactive APAP metabolic intermediate) and enzymatic antioxidant enzymes, including SOD and CAT, are crucial and important for preventing hepatic damage by oxidative stress. Superoxide production is generated from various sources such as auto-oxidation of leukoflavins and the products of mitochondrial respiration. The role of SOD depletion in the pathogenesis of APAP intoxication was supported by various studies.[8] High amounts of superoxide radicals inhibit CAT, another important antioxidant enzyme which decomposes hydrogen peroxide and protects tissue from reactive hydroxyl radicals is widely distributed in all animal tissues.[9] GSH is an important non-enzymatic antioxidant that protects the liver against APAP-induced damage by forming conjugates with the harmful NAPQI and removing it from the hepatocytes. The depletion of cellular GSH level in the HC is known to play a key role in APAP toxicity.[10] Results obtained in the present investigation showed a decreased level of CAT, SOD, and GSH indicating an oxidative stress in the APAP alone treated group.

Administration of TA ETH effectively enhanced the production of enzymes such as CAT and SOD which resulted in the release of oxidative stress by radical scavenging in hepatocytes during APAP metabolism. TA ETH also enhanced the GSH level, which resulted in the effective removal of reactive NAPQI through bile and urine. MDA is one of the end-products of polyunsaturated fatty acid peroxidation and is a good indicator of the degree of lipid peroxidation, which can be related to APAP-induced hepatic damage. The decreased level of antioxidant enzymes such as CAT and SOD results in an increased lipid peroxidation rate indicated by high levels of MDA. In the present study, a significant increase in the MDA level observed in liver homogenate of APAP-intoxicated rats was reduced by treatment with the TA ETH, indicating its ability to break the chain reaction of lipid peroxidation. Maximum inhibition of MDA formation was shown by TA ETH (300 mg/kg), and the results were comparable to the standard silymarin (100 mg/kg) treated group. Antioxidant and anti-lipid peroxidation activity of TA ETH is one of the main reasons behind the hepatocytic membrane stabilization, which helped to maintain normal serum enzyme levels and antioxidant status of the liver.

The results obtained from the biochemical analysis of serum and liver homogenate can be correlated with the histopathological analysis of liver samples from APAP-intoxicated animals. The hepatic histopathology of toxin group exhibited loss of cell boundaries, ballooning degeneration, CN, SC, and infiltration of KF around the central vein which are the characteristic features of paracetamol-induced toxicity. Treatment with TA ETH effectively inhibited lipid peroxidation and prevented the loss of cell boundaries and ballooning degeneration in a dose-dependent manner. The liver histology of silymarin (100 mg/kg)-treated animals showed normal hepatic architecture with few fatty globules and infiltration of KF. The liver histology of the animals treated with higher doses of TA ETH (300 mg/kg) showed normal hepatic cords, and absence of occasional necrosis as the plant drug effectively removed the free radicals and NAPQI before causing cellular damage during APAP metabolism. The probable mechanism by which *T. akara* exerts its protective action against paracetamol-induced hepatocellular metabolic alterations could be by the stimulation of hepatic regeneration through an improved synthesis of protein, free radical scavenging activity, anti-lipid peroxidative effect and accelerated detoxification, and excretion of NAPQI from the hepatocytes.

CONCLUSION

Thus, it can be concluded that the hepatoprotective effect of an ethanolic fraction of roots of *T. akara* may be due to the presence of bioactive phenolic compounds and flavonoids as evident from the previous study. These studies need to be extended on the isolation and characterization of the active compounds, which are responsible for this bioactivity. This investigation, however, substantiates the tribal claim on *T. akara* and provides evidence to initiate detailed exploration on the medicinal property of plant offering protection against various liver ailments.

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