Research article

Protective effects of *Asystasia chelonoides* var. *chelonoides* Nees. (Acanthaceae) leaf extracts against Paracetamol-induced hepatotoxicity in Wistar rats

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Abstract: The ethanolic leaf extract of *Asystasia chelonoides* var. *chelonoides* Nees. was evaluated for the hepatoprotective properties against Paracetamol overdose in Wistar rats. Paracetamol administration caused severe hepatic damage in rats as evidenced by the elevated serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and total bilirubin. Hepatoprotective potential of the plant extract was evident from the normalized biochemical parameters of hepatic injury like ALT, AST, ALP, GGT, SB, TGL, TC and TP along with the estimation of antioxidant status of the liver tissue (levels of Catalase, Superoxide dismutase, Glutathione and Malondialdehyde levels). The histopathological studies of liver samples showed recovery from Paracetamol-induced necrosis with almost normalized hepatic architecture in *A. chelonoides* extract-treated animals. The hepatoprotective effects exhibited by plant extract might be mediated through the inhibition of microsomal drug-metabolizing enzymes and in conclusion, the findings of the present study suggest that *A. chelonoides* leaf extract possesses potent hepatoprotective effect against Paracetamol induced hepatic damage in Wistar rats.

Keywords: *Asystasia chelonoides* - Paracetamol - Hepatotoxicity.

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INTRODUCTION

The liver is the key organ of metabolism and, is continuously and variedly exposed to xenobiotics, environmental pollutants, and chemotherapeutic agents (Opoku *et al.* 2007). When this natural protective mechanisms of liver is overpowered by such exposures, it will lead to hepatic injury. Liver diseases are a problem worldwide, and the conventional drugs used in the treatment of liver diseases are sometimes inadequate and can have serious adverse effects. Thus, interest and effort have shifted toward medicinal plants as new sources of hepatoprotective agents (Arhoghro *et al.* 2009). Paracetamol or Acetaminophen (APAP) is a drug of the para-aminophenol group, which is generally considered quite safe at therapeutic doses and is effective as an analgesic to relieve mild to moderate pain, as well as an antipyretic to reduce fever. However, overdosing or chronic use may lead to severe damage to some tissues, especially in the liver. Liver toxicity impairs various normal physiological functions like metabolism; susceptibility of the liver to injury is much higher than any other organ because of its central role in metabolism as well as its ability to concentrate and biotransform xenobiotics (Kumar *et al.* 2015, Bedi *et al.* 2016). The present study was designed to investigate the biological effects of the plant using animal and biochemical methods. Here, we report the hepatoprotective activities of ethanolic extract of *A. chelonoides* in Paracetamol-induced hepatic necrosis in rat models.

MATERIALS AND METHODS

Plant material

The plant specimen [*Asystasia chelonoides* var. *chelonoides* Nees. (Acanthaceae)] were collected from Western Ghats regions of Thiruvananthapuram and Kollam district of Kerala state. Voucher specimens were taxonomically identified and deposited at the herbarium of the Jawaharlal Nehru Tropical Botanical Garden and Research Institute, Palode, India. Care was taken to select healthy plants of *Asystasia chelonoides* (AC). They were thoroughly washed with running water to remove the adherent impurities. The leaves were dried in shade, powdered using mechanical grinder and stored in air-tight glass containers and extracted with ethyl alcohol by Soxhlet's extraction method to obtain ethanolic leaf extract of *Asystasia chelonoides* (AC ETH).

Experimental animals

Male and female Wistar rats (150–200 gms) were obtained from the institutes Animal House. These animals were housed in polypropylene cages under standard laboratory conditions (temp 24–28°C, humidity 60–70%). They were fed with commercial rat feed (Lipton India Ltd., Mumbai, India) and boiled water *ad libitum*. The study was carried out according to National Institute of Health (NIH) guidelines, after getting the approval of the Institute Animal Ethics Committee (IAEC).

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 (APAP) induced hepatotoxicity

Paracetamol induced hepatotoxicity was carried out according to the procedure of Suja *et al.* (2003). Wistar rats were divided into six groups (6 animals / group), Groups I and II were the normal control and Paracetamol intoxicated toxin group respectively. Both of them received a single daily dose of 0.5% Tween-80 (1 ml, p.o.) for all 6 days. Group III, IV and V were administered AC ETH reconstituted in 0.5% Tween-80 at dosages (50, 150 and 450 mg kg⁻¹, p.o.) for all 6 days and Group VI was administered Silymarin, the standard hepatoprotective drug, at a dose of 100 mg kg⁻¹, p.o., for all 6 days. Paracetamol suspension (2.5 g kg⁻¹, p.o.) was administered to Groups II to VI on 5th day, 30 min after plantextract/Silymarin administration. On the 6th day after 24 h starvation, all the animals were sacrificed using CO₂ inhalation. Blood samples were collected from the carotid artery for evaluating the plasma markers of hepatic injury and liver tissue slices were collected for histopathological studies and antioxidant assays like the estimation of Malondialdehyde (MDA), assay of Catalase (CAT), determination of Reduced glutathione (GSH) and superoxide dismutase (SOD).

Statistical analysis

All the data were expressed as mean $\pm$ standard error of the mean (SEM). The significance of difference among the group was assessed using one way ANOVA followed by Dunnett's posttest using GraphPad Prism Version 7.00.

RESULTS

Evaluation of hepatoprotective activity (Estimation of plasma markers of hepatic injury)

Wistar rats treated with over dose of APAP developed liver damage which is indicated by significant ($p \leq 0.05$) increase in serum AST (270.32 ± 4.31 IU L⁻¹), ALT (223.13 ± 3.37 IU L⁻¹), ALP (260.35 ± 3.38 IU L⁻¹), GGT (25.23 ± 2.12 IU L⁻¹), SB (1.43 ± 0.42 mg dL⁻¹), TC (190.44 ± 2.91 mg dL⁻¹), TGL (256.61 ± 3.24 mg dL⁻¹) and decrease in TP (3.53 ± 0.13 g dL⁻¹) in toxin control group compared to normal control. Pre-treatment with AC ETH (50, 150 and 450 mg kg⁻¹ b. w., p. o) caused significant ($p \leq 0.05$) protection against APAP toxicity by attenuating AST, ALT, ALP, GGT, SB, TC, TGL and elevation of TP as shown in dose dependent manner. For all the eight biochemical parameters studied, AC ETH showed potent activity in a dose dependent manner and from among the various doses, AC ETH (400 mg kg⁻¹) (AST: 110.21 ± 2.13 IU L⁻¹; ALT: 71.21 ± 2.34 IU L⁻¹; ALP: 122.21 ± 3.14 IU L⁻¹; GGT: 7.13 ± 0.19 IU/L⁻¹; SB: 0.548 ± 0.02 mg dL⁻¹; TC: 106.42 ± 3.28 mg dL⁻¹; TGL: 119.28 ± 1.43 mg dL⁻¹ and TP: 5.48 ± 0.14 g dL⁻¹) was found to be the significant dose among of *A. chelonoides* studied. The reduction in biochemical parameters exhibited by AC ETH (400 mg kg⁻¹) was almost comparable to that of Silymarin (100 mg kg⁻¹) (AST: 102.76 ± 2.32 IU L⁻¹; ALT: 67.82 ± 0.65 IU L⁻¹; ALP: 118.17 ± 2.121 IU L⁻¹; GGT: 6.22 ± 0.21 IU L⁻¹; SB: 0.52 ± 0.04 mg dL⁻¹; TC: 110.24 ± 2.23 mg dL⁻¹; TGL: 126.21 ± 1.13 mg dL⁻¹ and TP: 5.23 ± 0.14 g dL⁻¹), the standard drug employed in the study as shown in table 1.

Table 1. Effect of AC ETH on plasma markers of hepatic injury in APAP intoxicated Wistar rats. Values are expressed as mean $\pm$ 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 APAP toxin control.

Treatment groups	Parameters							
	AST (IU L ⁻¹)	ALT (IU L ⁻¹)	ALP (IU L ⁻¹)	GGT (IU L ⁻¹)	SB (mg dL ⁻¹)	TC (mg dL ⁻¹)	TGL (mg dL ⁻¹)	TP (g dL ⁻¹)
Normal	82.22 $\pm$ 1.12	60.24 $\pm$ 2.32	93.42 $\pm$ 2.01	5.43 $\pm$ 1.12	0.34 $\pm$ 0.04	96.32 $\pm$ 1.71	122.33 $\pm$ 3.73	6.33 $\pm$ 0.55
Control Toxin	270.32 $\pm$ 4.31*	223.13 $\pm$ 3.37*	260.35 $\pm$ 3.38*	25.23 $\pm$ 2.12*	1.43 $\pm$ 0.42*	190.44 $\pm$ 2.91*	256.61 $\pm$ 3.24*	3.53 $\pm$ 0.13*
Control STD-	102.76 $\pm$ 2.32**	67.82 $\pm$ 0.65**	118.17 $\pm$ 2.21**	6.22 $\pm$ 0.21**	0.52 $\pm$ 0.04**	110.24 $\pm$ 2.23**	126.21 $\pm$ 1.13**	5.23 $\pm$ 0.14**
Silymarin (100 mg kg ⁻¹)								
AC ETH (50 mg kg ⁻¹)	242.23 $\pm$ 2.41	188.43 $\pm$ 3.27	229.48 $\pm$ 2.63	21.24 $\pm$ 2.31	1.34 $\pm$ 0.05	176.22 $\pm$ 2.17	227.46 $\pm$ 2.42	4.03 $\pm$ 0.23
AC ETH (150 mg kg ⁻¹)	138.28 $\pm$ 1.27**	90.47 $\pm$ 2.27**	137.24 $\pm$ 2.46**	12.12 $\pm$ 1.23**	0.61 $\pm$ 0.03**	118.22 $\pm$ 3.41**	135.26 $\pm$ 1.20**	5.01 $\pm$ 0.18**
AC ETH (400 mg kg ⁻¹)	110.21 $\pm$ 2.13**	71.21 $\pm$ 2.34**	122.21 $\pm$ 3.14**	7.13 $\pm$ 0.19**	0.48 $\pm$ 0.02**	106.42 $\pm$ 3.28**	119.28 $\pm$ 1.43**	5.48 $\pm$ 0.14**

Evaluation of in vivo antioxidant status of liver

Figure 1. Effect of ethanolic extract of *Asystasia chelonoides* (AC ETH) on hepatic CAT, SOD, GSH and MDA of Wistar rats in APAP induced hepatotoxicity study. Values are expressed as mean $\pm$ 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 APAP toxin control.

Catalase activity was depleted significantly ($p \leq 0.05$) in APAP intoxicated animals (62.14 ± 3.35 U mg⁻¹ protein) of toxin group when compared to normal control group (157.32 ± 7.43 U mg⁻¹ protein). The SOD levels in the toxin group was also reduced to 4.56 ± 0.62 U mg⁻¹ protein when compared to the normal control group with 17.32 ± 1.25 U mg⁻¹ protein. The level of GSH significantly ($p \leq 0.05$) decreased in APAP intoxicated animals (11.48 ± 1.47 μ mol g⁻¹ tissue) when compared to normal control group (58.26 ± 3.36 μ mol g⁻¹ 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 AC ETH showed an increase in hepatic Catalase, SOD and GSH in a dose-dependent manner. AC ETH at 450 mg kg⁻¹ showed maximum protection against APAP intoxication in animals which is evident from the higher levels of Catalase (146.32 ± 5.74 U mg⁻¹ protein), SOD (14.05 ± 1.08 U mg⁻¹ protein) and GSH (53.46 ± 2.94 μ mol g⁻¹ tissue). The MDA levels in the liver of toxin control animals (45.58 ± 3.42 μ mol g⁻¹ wet liver) were higher when compared to the normal control (13.28 ± 2.06 μ mol g⁻¹ wet liver). The MDA levels were found to be lower in the AC ETH treated groups compared to toxin control groups and the maximum

inhibition was obtained in AC ETH (450 mg kg⁻¹) treated groups (16.23 ± 0.74 nmol g⁻¹ wet liver) and it is almost comparable to that of the standard Silymarin (100 mg kg⁻¹) treated groups (17.22 ± 2.36 μ mol g⁻¹ wet liver) as shown in figure 1.

Histopathological investigations

Histological studies of control animals showed normal hepatic architecture with distinct hepatic cells and sinusoidal space. The liver sections of the toxin control group of animals exhibited disarrangement of normal hepatic cells, intense congestion, hydropic degeneration, pyknosis, centrilobular necrosis, sinusoidal congestion, infiltration of the lymphocytes, Kupffer cells around the central vein, loss of cell boundaries and ballooning degeneration were observed after the intoxication of acetaminophen. The liver histology of Silymarin treated animals showed normal hepatic architecture with few fatty globules and infiltration of Kupffer cells. The liver histology of the animals treated with higher doses of AC 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.

Figure 2. Effect of ethanolic extract of *Asystasia chelonoides* (AC ETH) on the histopathology of APAP induced liver damage in Wistar rats ($\times 50$, H & E staining): **A**, Normal control rat liver histology showing normal hepatic architecture; **B**, Toxin control rat liver showing Centrilobular necrosis, sinusoidal congestion, Kupffer cells infiltration and cellular disintegration; **C**, Standard Silymarin treated group showing almost normal hepatic architecture with mild degree of damage; **D, E & F**, AC ETH (50, 150 and 450 mg kg⁻¹ respectively) treated groups showing reduced hepatic damage in a dose depended manner.

DISCUSSION

Chemical toxins include paracetamol, which is often used as the model substance causing experimental hepatocyte injury in both in-vivo and in-vitro conditions (Remien *et al.* 2014). Frequent use of Paracetamol is considered as a major cause of acute liver failure in many countries. Cytochrome P450 enzymes convert a relatively minor portion of paracetamol to the highly reactive intermediate metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI), which is thought to be responsible for paracetamol-induced hepatic toxicity. Under normal physiological conditions, NAPQI conjugates with glutathione (GSH) and is detoxified. In paracetamol overdose, NAPQI is produced in excess of GSH detoxification capacity, and only part of it can be detoxified by conjugation with GSH. The remaining part of NAPQI subsequently binds to liver proteins and induces oxidative stress, mitochondrial dysfunction, and necrotic cell death. Oxidative stress is recently reported to play a major role in acetaminophen (APAP)-induced hepatotoxicity (Mousah *et al.* 2016).

Paracetamol overdose is also known to be associated with inflammation, marked by an increase in the inflammatory cytokines, tumor necrosis factor- α (TNF- α) and interleukin, as well as the up regulation of nitrogen oxide from serum, macrophages, and hepatocytes (Ghosh *et al.* 2010). Numerous reports are indicating that paracetamol-mediated oxidative stress or hepatotoxicity is attenuated by the use of naturally occurring antioxidants and or free radical scavengers such as vitamins, phenols, terpenoids and flavonoids (Janbaz *et al.* 2004, Ajith *et al.* 2007, Durga *et al.* 2014). Recently, flavonoids have been found to play important roles in non-enzymatic protection against oxidative stress (Elsayed 2006). The free radical scavenging and antioxidant potential of these biomolecules are responsible for many of their beneficial effects and confers a therapeutic potential in diseases such as cardiovascular diseases, gastric or duodenal ulcers, and cancer and hepatic pathologies (Gonzalez-Gallego *et al.* 2007, Bouhali *et al.* 2015).

The liver is highly affected primarily by toxic agents, and hence the liver marker enzymes are very sensitive markers of toxicity and be of great importance in the assessment of hepatic damage. Activities of ALT, AST, ALP, and the level of serum bilirubin are largely used as the most common biochemical markers to evaluate liver injury. Paracetamol -induced hepatic injury is considered as one of the most commonly used models and reliable method for screening of hepatoprotective agents (Remien *et al.* 2014). In the current study, the significant elevation of the enzyme levels, particularly AST, ALT, ALP and bilirubin level in rats treated with paracetamol, are indicative of cellular leakage and loss of functional integrity of the liver cell membrane (Sabiu *et al.* 2014). This is in agreement with previous studies, which reported that overdose of APAP could be toxic to the hepatocytes (Gini *et al.* 2010). The serum levels of transaminases return to normal with the healing of hepatic parenchyma and the regeneration of hepatocytes (Ahmed *et al.* 2001, Pawlikowska-Pawlega *et al.* 2007).

In the present study, paracetamol intoxication also decreased serum TP and Alb, whereas it increased serum bilirubin. The liver is the major source of most of the serum proteins, in which the parenchymal cells are responsible for synthesis of Alb, fibrinogen, and other coagulation factors and most of the α - globulins and β -globulins (Thapa *et al.* 2007). TP reflects the functional status of the liver, because the liver is furnished with machineries for synthesizing serum proteins excluding γ -globulins. Thus, liver damage is characterized by hypoproteinemia and decreased Alb, which can affect the whole physiological status of animals (Iweala *et al.* 2010). Albumin, being the most abundant plasma protein, accounts for 60% of the total serum protein and is incorporated in many physiological processes. Qualitative and quantitative disturbance of protein synthesis is a consequence of impaired hepatic function. The observed decrease in Alb by paracetamol administration in the current study could be a result of a decline in the number of cells responsible for Alb synthesis in the liver through necrosis. The direct interference with the Alb-synthesizing mechanism in the liver as a result of inflammation may also be implicated for decrease in Alb (Jaeschke *et al.* 2003).

Formation of toxic free radicals, such as peroxynitrite, from the reaction of superoxide and nitric oxide, subsequently forming nitrotyrosine adducts inside the mitochondria also induce hepatotoxicity. GSH repletion not only provides surplus cysteine as an energy substrate for the Krebs cycle, it also serves the important role of scavenging for free radicals and peroxynitrite (Jaeschke 2012). Mitochondria, which are critical for cellular respiration and metabolism, suffer damage by the actions of reactive oxygen species and peroxynitrite compounds leading to cessation of ATP synthesis and hepatocytic damage (Yuan & Kaplowitz 2013). Administration of overdose of APAP had shown to develop fulminant centrilobular necrosis in mice and hamsters similar to that observed in humans (Davis *et al.* 1974, Potter *et al.* 1974).

Over dosage of APAP administration caused a complex sequence of events as mentioned above leading to liver injury and the transport function of the hepatocytes gets disturbed, resulting in the leakage of the plasma

membrane (Zimmerman & Maddrey 1995), a variety of enzymes normally located in the cytosol are released into bloodstream. The estimation of these parameters in serum is a useful quantitative marker for the extent and type of hepatocellular damage (Ansari *et al.* 1991). In this study, 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 AC ETH have shown a decrease in the liver enzyme levels in a dose-dependent manner and AC ETH (450 mg kg⁻¹) showed reduced enzyme level similar to the standard hepatoprotective drug, Silymarin (100 mg kg⁻¹) 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 AC ETH against APAP induced liver toxicity. Increased levels of serum bilirubin 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 (Mazer & Perrone 2008). AC 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. Biochemical serum parameters like TC and TGL were also significantly increased in APAP alone treated rats, when compared to normal control. The plant drug also possesses hypolipidemic activity which is evident from the significant reduction in of TC and TGL level. Protein synthesis is the major function of the liver and healthy functioning of liver is required for the synthesis of serum proteins except for the γ -globulins. The decreased Total Protein (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 (Kanchana & Sadiq 2011). It is worth to note that the AC ETH (450 mg kg⁻¹) 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. Equilibrium between free radicals formed due to the increased level of NAPQI (a highly reactive APAP metabolic intermediate) and enzymatic antioxidant enzymes, including superoxide dismutase (SOD) and catalase (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 superoxide dismutase depletion in the pathogenesis of APAP intoxication was supported by various studies (Patel *et al.* 2012). 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. Glutathione 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 hepatic cells is known to play a key role in APAP toxicity. 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 AC ETH effectively enhanced the production of enzymes like CAT and SOD which resulted in the release of oxidative stress by radical scavenging in hepatocytes during APAP metabolism. AC ETH also enhanced the GSH level, which resulted in the effective removal of reactive NAPQI through bile and urine. Malondialdehyde (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 like 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 AC ETH, indicating its ability to break the chain reaction of lipid peroxidation. Maximum inhibition of MDA formation was shown by AC ETH (450 mg kg⁻¹) and the results was comparable to the standard Silymarin (100 mg kg⁻¹) treated group. Antioxidant and anti-lipid peroxidation activity of AC 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.

CONCLUSION

In conclusion, the findings of the present study suggest that the hepatoprotective effect of extract of *Asystasia chelonoides* against paracetamol-induced hepatotoxicity in Wistar rats may be due to the presence of bioactive phytoconstituents. These studies need to be extended for the isolation and characterization of the

active compounds offering protection against various liver ailments.

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