Hepatoprotective effect of Asystasia chelonoides var. chelonoides Nees. (Acanthaceae) leaf extracts against CCl_4 induced liver injury in Wistar rats.

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Abstract:The present study was designed to establish the possible preventive and curative hepatoprotective efficacy of ethanolic extract of leaves of *Asystasia chelonoides var. chelonoides*, at different dose levels (50, 150 & 450 mg/kg) on CCl_4 induced liver injury in Wistar rats. Antihepatotoxic potential of the plant extract was assessed by the estimation of 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 (SOD, CAT, GSH and MDA levels) and histopathological evaluation. Oral administration of ethanolic extracts at doses of 150 and 450 mg/kg once daily for 5 days significantly restored serum enzyme levels.The results indicate that rats administered with CCl_4 recorded severe hepatic damage in toxin control when compared to normal group which was evident from the elevated levels of liver marker enzymes and decreased level of total protein. The hepatoprotective effect of the plant was also supported by histopathological evaluation of drug treated animals. In conclusion, the findings of the present study suggest that *A. chelonoides* leaf extract possesses potent prophylactic and hepatoprotective efficacy against CCl_4 rendered liver injury in Wistar rats.

Key words: Antihepatotoxic, *Asystasia chelonoides*, prophylactic.

Introduction

The liver is one of the largest organs in the human body and the chief site for intense metabolic activities and excretion (Ahsan *et al*., 2009). It plays a major role in detoxification and excretion of many endogenous and exogenous compounds; any injury to it or impairment of its functions may lead to many implications on one's health (Subramaniam *et al*., 2015). Hepatic damage is associated with distortion of these metabolic functions. Liver diseases are among the most serious ailment and are mainly caused by toxic chemicals (certain antibiotics, chemotherapeutics, peroxidised oil, aflatoxin, carbon-tetrachloride, chlorinated hydrocarbons, etc.), excess consumption of alcohol, infections and autoimmune disorder. Inspite of development in the medical field with most ultra modern technologies available in the forefront, suitable and remedial measures is still a hope for the treatment of liver complications. Hence specific research methodology is to be put into practice for the development of a proper effective medicine with least side effects for the treatment of liver disease.

Liver plays a vital role in metabolizing carbohydrates, lipids, proteins and detoxifying xenobiotics and drugs. Thus, the liver is proned to injury due to the chronic exposure to drugs, environmental toxicants and other xenobiotics (Maher, 1997). They prove protection against foreign substances through detoxification and elimination processes (Desai *et al*., 2012). Due to increasing incidences of xenobiotic or chemically induced hepatotoxicity,

there is a need for safe protective agents, in lieu of synthetic chemical compounds (Jain *et al*., 2012). Therefore, there is particular interest in developing new drugs from plant sources, to treat liver diseases.

Oxidative stress refers to an imbalance in any cell, tissue or organ between the amount of free radicals and the capabilities of the detoxifying and repair systems. Sustained oxidative damage results only under conditions of oxidative stress when the detoxifying and repair systems are insufficient. Free radical induced damage, when left unrepaired, destroys lipids, proteins, RNA and DNA and can contribute to disease (Sathishsekar, 2005). Oxidative stress has been implicated as a contributing factor to cancer, atherosclerosis, parkinson's disease, schizophrenia, bipolar disorder, emphysema and cataracts. Oxidative stress can cause cellular and tissue of antioxidant levels may lead to free radical caused oxidative stress. Oxidative stress can cause cellular and tissue damages, DNA mutation, Cancer etc. (Tsao, 2004). Besides, in the current world,the human body is significantly exposed to external sources of free radicals. Therefore, the body's antioxidative depends system might not be adequate to prevent oxidative caused damages completely (Simic, 1988). Experimental evidence suggests that free radicals and reactive oxygen species (ROS) can be involved in high number of disorders such as arthritis, connective tissue disorders, diabetis, chronic inflammation, cancer and in the process of ageing

In Ayurveda a number of medicinal preparations have been employed for treating liver disorders and there are no rational drug therapies. The herbal drugs have gained importance and popularity in recent years because of their safety, efficacy and cost effectiveness. (Adewusi and Afolayan, 2010). Inspite of tremendous advances made in allopathic medicine, effective hepatoprotective medicine is still wanting. About 80% of world population relies on folklore medicine for curing ailments related to liver.However, a small number of these medicinal plants as well as formulations used are scientifically evaluated for their activity. In the context of our on going search for new natural substances possessing hepatoprotective efficacy, the present investigation was undertaken by using the leaves of the plant. *Asystasia chelonoides* var. *chelonoides* Nees, belonging to the family Acanthaceae. The hepatoprotective activity of *Asystasia chelonoides* has not been investigated so far. Hence the present investigation was undertaken.

Materials and method

The plant specimen *Asystasia chelonoides* (Acanthaceae) were collected from Western Ghat regions of Thiruvananthapuram and Kollam Districts 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 (150-200gms) were obtained from the institutes Animal House. These animals were housed in polypropylene cages under standard laboratory conditions (temp 24-28ºC, humidity 60-70%). They were fed with commercial rat feed (Lipton India Ltd., Mumbai, India) and boiled water *at 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.

Carbon tetrachloride (CCl_4) induced hepatotoxicity:

Wistar rats were divided into six groups (6 animals/ group). Group I, the normal control group received a single daily dose of 0.5% Tween-80 (1 mL p.o.) on all 6 days and olive oil (2 mL/kg, s.c.) on days 2 and 3. Group II, the Carbon tetrachloride control group, received a single daily dose of 0.5% Tween -80 (1 mL p.o.) on all 6 days and on the second and third day, they were administered s.c., 2 mL/kg of CCl_4: Olive oil (1:1). Groups III, IV and V were administered AC ETH reconstituted in 0.5% Tween-80 at dosages 50, 150, 450 mg/kg, b.w. p. o., respectively for all 6 days and a single dose of CCl_4: Olive oil mixture (2 mL/kg, s.c), on days 2 and 3, 30 min after AC ETH administration. Group VI was administered Silymarin, the standard hepatoprotective drug, at a dose of 100 mg/kg, p.o., on all 6 days and a single dose of CCl_4: olive oil mixture (2 mL/kg, s.c.) on days 2 and 3, 30 min after Silymarin administration. On the 7^{th} day after 24 h starvation all the animals were sacrificed using CO_2 inhalation (Marsillach *et al*., 2009). Blood samples were collected from the carotid artery for evaluating biochemical parameters (estimation of plasma markers of hepatic injury) and liver tissue slices were collected for histopathological studies, antioxidant assays like estimation of Malondialdehyde (MDA), assay of Catalase (CAT), Superoxide dismutase (SOD) and determination of Reduced glutathione (GSH).

Results

Estimation of serum biochemical parameters:

The collected blood samples in test tubes without EDTA were allowed to coagulate for 1h at room temperature. It was centrifuged at 1500 rpm for 15 min at 37ºC 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 3ºC to remove the cell debris, unbroken cells, nuclei and erythrocytes. 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).

Results

Carbon tetrachloride (CCl_4) induced hepatotoxicity study.

The results of the study showed that experimental animals administered with CCl_4 recorded severe hepatic damage in toxin control when compared to normal group which was evident from the elevated levels of liver markers enzyme and decreased level of total protein. All the animals in the toxin group indicated significant ($p \leq 0.05$) increase in serum and decreased level of TP when compared to normal control. In CCl_4 poisoned rats a significant decrease in GSH level was observed compared to normal control group. Pre- and post- treatment with the test extract significantly attenuated CCl_4 induced decrease of GSH content in a dose dependent manner compared to CCl_4 intoxicant toxin group.

Estimation of plasma markers of hepatic injury.

The results indicate that rats administered with CCl_4 recorded severe hepatic damage in toxin control when compared to normal group which was evident from the elevated levels of liver markers enzymes and decreased level of total protein as shown in Table 1. All the animals in the toxin group indicated significant ($p \leq 0.05$) increase in serum AST (244.32 ± 2.81 IU/L), ALT (250.21 ± 2.34 IU/L), ALP (258.12 ± 1.87 IU/L) GGT (19.73 ± 1.12 IU/L), SB (1.21± 0.01 mg/dL), TC (206.12 ± 2.03 mg/dL), TGL (246.13 ± 2.12 mg/dL) and decreased level of TP (2.41 ± 0.16 g/dL) when compared to normal control. Pre-treatment with AC ETH (50, 150 and 450 mg/kg b. w., p. o.) showed significant ($p \leq 0.05$) protection against CCl_4 intoxication in rats in a dose dependent manner by attenuating AST, ALT, ALP, GGT, SB, TC, TGL and TP elevation in dose dependent manner. For all the biochemical parameters studied, AC ETH (450 mg/kg dose) (AST: 80.26 ± 2.24 IU/L, ALT: 92.34 ± 1.68 IU/L, ALP 124.21 ± 2.14 IU/L, GGT: 7.44± 0.38 IU/L, SB: 0.59 ± 0.04 mg/dL, TC: 106.43 ± 2.19 mg/dL, TGL: 127.72 ± 1.38 mg/dL and TP: 5.49 ± 0.12 g/dL) was found to be the most significant dose. The reduction in biochemical parameters exhibited by the higher dose of AC ETH was almost comparable to that of Silymarin (100 mg/kg) (AST: 80.22 ± 0.54 IU/L, ALT: 96.12 ± 1.31IU/L, ALP: 112.32 ± 1.14 IU/L, GGT: 4.71 ± 0.43 IU/L, SB: 0.32 ± 0.03 mg/dL, TC: 98.12 ± 1.02 mg/dL, TGL: 119.12 ± 1.02 mg/dL and TP: 3.32 ± 0.10 g/dL), the positive control used in the study as shown in the table 1.

Table 1: Effect of AC ETH on plasma markers of hepatic injury in CCl_4 intoxicated Wistar rats.

Treatment group	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	70.12±1.32	74.23±1.41	93.12±1.53	3.27±1.32	0.24±0.11	95.23±1.13	116.21±1.28	3.41±0.14
CCl_4 Toxin Control	244.32±2.81*	250.21±2.34*	258.12±1.87*	19.73±1.12*	1.21±0.01*	206.12±2.03*	246.13±2.12*	2.41±0.16*
CCl_4+STD (Silymarin)	80.22±0.54**	96.12±1.31**	112.32±1.14**	4.71±0.43**	0.32±0.03**	98.12±1.02**	119.12±1.02**	3.32±0.10**
CCl_4+AC ETH 50	191.21±1.03	209.13±0.42	214.12±1.03	15.31±1.02	1.21±0.03	172.24±1.41	189.24±1.01	2.02±0.16
CCl_4+AC ETH 150	94.01±1.12**	120.12±0.12**	144.12±2.11**	9.11±0.32**	0.32±0.11**	114.12±1.41**	128.13±0.42**	3.12±0.08**
CCl_4+AC ETH 450	78.12±1.12**	90.12±0.34**	122.12±1.02**	5.21±0.12**	0.34±0.02**	104.21±1.04**	125.51±0.24**	3.24±0.01**

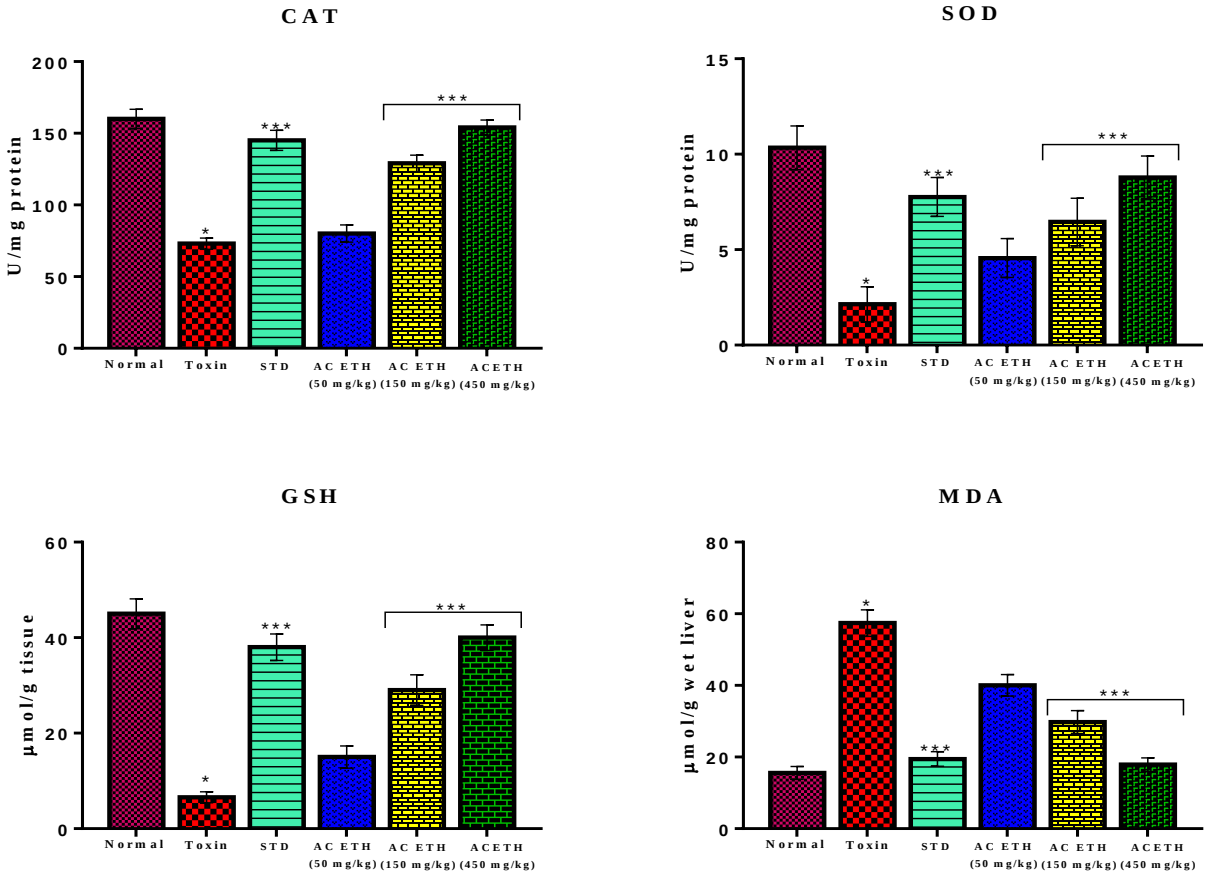
Values are expressed as mean ± SEM of six values, one way ANOVA followed by Dunnett's multiple comparison test, *$p \leq 0.05$ compared to normal control, **$p \leq 0.05$ compared to CCl_4 toxin control.

Evaluation of *in vivo* antioxidant status of liver.

The level of Catalase in liver tissue depleted significantly ($p \leq 0.05$) in CCl_4 intoxicated animals (73.41± 2.63 U/mg protein) of toxin group when compared to normal control group (160.28 ± 4.51 U/mg protein). The SOD levels in the toxin group was lowered to 2.12 ± 0.72 U/mg and GSH showed significantly ($p \leq 0.05$) decreased to 6.54 ± 1.12 µmol/g tissue when compared to normal control. All groups of animals administered 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 showed maximum protection against CCL_4 intoxication in animals which is evident from the higher levels of Catalase (154.62 ± 3.23 U/mg protein), SOD (13.56 ± 1.02 U/mg protein) and GSH (41.73 ± 1.45 µ mol/g tissue). The MDA levels in toxin control animals (57.43 ± 2.40 µ mol/g wet liver) were higher when compared to the

normal control (10.34 ± 1.41 µ mol/g wet liver). The MDA levels were found to be lowered in the AC ETH treated groups and the maximum inhibition of lipid peroxidation was shown by AC ETH (450 mg/kg) treated groups (13.65 ± 1.32µmol/g wet liver) and it is almost comparable to that of the standard Silymarin treated groups (16.34 ± 1.04 µmol/g wet liver) as shown in figure 1.

Fig. 1: Effect of ethanolic fraction of *Asystasia chelonoides*(AC ETH) on hepatic CAT, SOD, GSH and MDA of Wistar rats in CCl_4 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 CCl_4 toxin control.

Histopathological investigations.

Histopathological observations revealed that the CCl_4 treated rats showed extensive cellular injuries, characterized by high hepatocellular degeneration, hydropic changes, macro and micro vesicular steatosis, apoptosis, centrilobular necrosis, ballooning degeneration, fatty deposition and accumulation of inflammatory cells in the toxin group. Silymarin (50 mg/kg) treated group showed almost normalized hepatic architecture with less centrilobular necrosis and fatty deposition. Moderate level of hepatic protection from CCl_4 was achieved in AC ETH (50 and 150 mg/kg) b.w. p.o. treated group. These groups showed restoration of liver structure with minimal cellular necrosis as shown in Fig. 5. Inflammatory changes induced by CCl_4 were remarkably reversed by treatment with AC ETH. Administration of AC ETH (400 mg/kg) to rats almost normalized the effects of CCL_4 intoxication and restored the histological architecture of the liver as shown in figure 2.

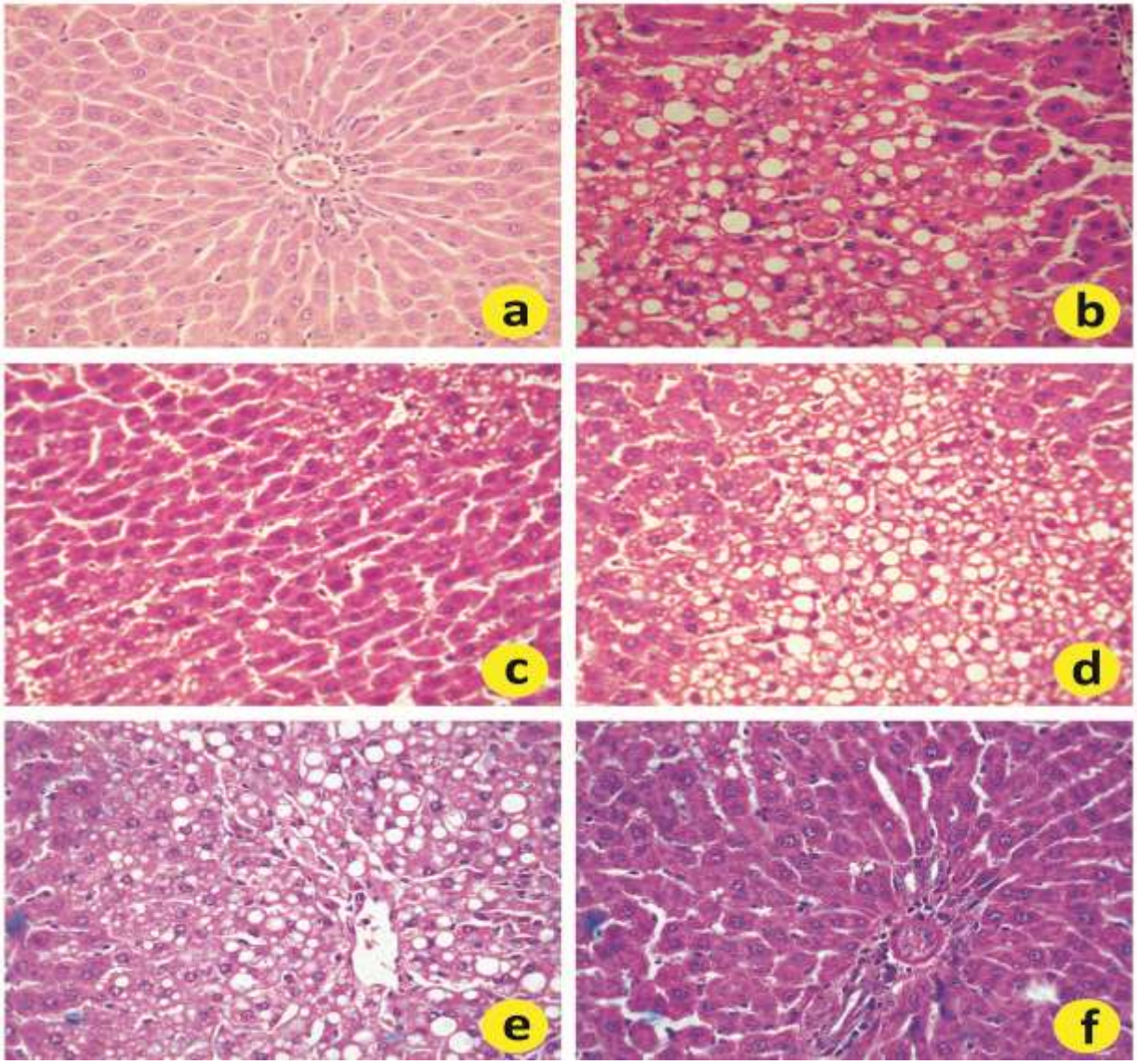


Fig. 2. Effect of ethanolic fraction of *A.chelonoides* (AC ETH) on CCl_4 induced liver damage in Wistar rats (×100, H & E staining). A. Normal control rat liver histology showing normal hepatic architecture. B. Toxin control rat liver with hepatocellular degeneration, steatosis, apoptosis, centrilobular necrosis, ballooning degeneration, fatty deposition and accumulation of inflammatory cells. C. Standard Silymarin treated group showing almost normal hepatic architecture. D, E & F. are AC ETH (50, 150 and 450 mg/kg) treated groups showing reduced hepatic damage in a dose depended manner. (SS-Sinusoidal pace, HC-Hepatic cells, AP-Apoptosis, FA- Fatty accumulation, CN-Centrilobular necrosis, BD-Ballooning degeneration, BV-Blood vessel, SC-Sinusoidal congestion, KC-Kupffer cells, ST-Steatosis, IF-Inflammatory cell accumulation).

Discussion

In the present study, the capability of the extract to protect against CCl_4 induced hepatotoxicity and oxidative stress was investigated. Carbon tetrachloride is a potent hepatotoxin producing centrilobular hepatic necrosis which causes liver injury. The use of many halogenated alkanes such as carbon tetrachloride (CCl_4), chloroform ($CHCl_3$) or iodoform (CHI_3) has been banned or severely restricted because of their distinct toxicity. Yet CCl_4 continues to provide an important service today as a model substance to elucidate the mechanisms of action of hepatotoxic effects and to evaluate the hepatoprotective potential of various herbal extracts or natural compounds (Akindele *et al*., 2010). CCl_4-induced hepatotoxicity model closely resembles human cirrhosis (Lin *et al*., 2002 and Talat, 2008) CCl_4 liver injury depends on a toxic agent that has to be metabolized by the liver NADPH- cytochrome

P450 enzyme system to a highly reactive intermediate. It has been suggested that this toxic intermediate is the trichloromethyl radical (CCl_3) producing maximum damage to liver. (Recknagel, 1983 and Koop, 1992). The free radicals can react with sulfhydryl groups, such as glutathione (GSH) and protein thiols. The covalent binding of trichloromethyl free radicals to cell protein is considered the initial step in a chain of events, which eventually leads to membrane lipid peroxidation and finally cell necrosis (Brattin *et al*., 1985, Recknagel *et al*., 1991 and Waller, 1989). Free radical activation of CCl_4 in mitochondria has also been observed and may contribute significantly to its toxicity. The liver damage was assessed by biochemical studies and histopathological examinations. SGOT and SGPT are well known diagnostic indicators of liver disease. ALT activity is considered to be a sensitive biomarker of hepatotoxicity, as it is primarily localized in liver. In the cases of liver damage with hepatocellular lesions and parenchymal cell necrosis, these marker enzymes are released from the damaged tissues into the blood stream (Drotman *et al*., 1978). Increased levels of SGOT and SGPT in serum of CCl_4 treated animals indicate that the integrity of hepatocytes was abnormal, resulting in the release of intracellular enzymes into the systemic circulation (Eminzade *et al*., 2008). In the present study, pre-treatment with *A.celonoides* extract caused a decrease in the activities of the above enzymes when compared with CCl_4 treatment groups which may be a consequence of the stabilization of plasma membrane as well as repair of hepatic tissue.

Alkaline phosphatase is excreted normally *via*. bile by the liver. Its activity on endothelial cell surfaces is responsible for the conversion of adenosine nucleotides to adenosine, a potent vasodilator and anti- inflammatory mediator that results from injury. So, following the injury, accumulation of Interleukin-6 can lead to production of adenosine by alkaline phosphatase and subsequent protection from injury. This may be the reason for the increment in ALP levels in CCl4 intoxicated rats, which have cell necrosis. The treatment with extracts caused a decrease level of ALP when compared with CCl_4 treatment group, respectively, showing its hepatoprotective potential.

Bilirubin is the breakdown product of heam in red blood cells and hyper bilirubinemia reflects the pathophysiology of liver. It is a most useful clinical indicator of the severity of necrosis and its accumulation is a measure of the binding, conjugation and excretory capacity of liver cells. In liver injury, due to hepatotoxin, there is a defective excretion of bile by the liver which is reflected in their increased levels in serum. Hepatotoxicity is characterized by cirrhotic liver condition which in turn increases the bilirubin release. The result shows that the serum bilirubin levels were elevated in CCl_4 treatment toxin group, which has been reduced in the blood serum of rats treated with plant extracts in a dose dependent manner, indicating its ability to stabilize biliary disfunction of rat liver. This clearly indicates the improvement of the functions of the liver cells and its cytoprotective action which may be due to the inhibitory effect on cytochrome P_{450}. The restoration of serum enzyme levels to normal levels in CCl_4 treated rats after treatment indicates prevention of the leakage of intracellular enzymes by stabilizing the hepatic cell membrane. Restoration of increased hepatic serum enzyme level to normal level reflects the protective effect of the extracts against CCl_4 induced hepatic damage.

Conclusion

In conclusion, the findings of the present study suggest that the extract of *Asystasia chelonoide* leaves possess equally potent prophylactic and curative hepatoprotective efficacy against CCl_4 rendered liver injury in rats.

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Conflicts of interest:

There are no conflicts of interest.

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