Indian Journal of Natural Products and Resources
Vol. 1(4), December 2010, pp. 493-499

Hepatoprotective and antioxidant effect of *Azima tetracantha* Lam. leaves extracts against CCl_4- induced liver injury in rats

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Received 11 January 2010; Accepted 15 April 2010

The chloroform and ethanolic extracts of *Azima tetracantha* Lam. leaves were studied to evaluate the hepatoprotective and antioxidant activities in CCl_4-induced hepatotoxicity in rats. Oral administration of the extracts at doses of 250 and 500 mg/kg once daily for 12 days significantly restored normalization of serum enzyme levels, viz. glutamate oxaloacetate transaminase (SGOT), glutamate pyruvate transaminase (SGPT) and markers viz. total protein, total bilirubin, direct bilirubin and albumin and the results were comparable to the effects of Silymarin. The ethanolic extract at the dose of 500 mg/kg was found to be more potent when compared to chloroform extract at similar dose. The hepatoprotection is also corroborated by histopathology of treated animals. In regard to antioxidant activity, ethanolic extract exhibited a significant effect showing increased levels of enzymatic and non-enzymatic parameters, viz. catalase, GSH, total thiols, and decreased level of malondialdhyde (MDA). The results of this study strongly indicate that *A. tetracantha* leaves have potent hepatoprotective action against CCl_4-induced hepatic damage in rats. The phytochemical screening of the extracts revealed the presence of secondary metabolites like alkaloids, flavonoids, tannins, triterpenoids, steroids, saponins, etc.

Keywords: *Azima tetracantha*, Antioxidant, Carbon tetrachloride, Hepatoprotection.

IPC code; Int. cl.[8] — A61K 36/00, A61P 1/16. A61P 39/06

Introduction

The liver is a vital organ of the body and plays a major role in metabolism, performing a number of functions including glycogen storage, decomposition of red blood cells, plasma protein synthesis, hormone production and detoxification. The liver is necessary for survival; there is currently no way to compensate for the absence of liver function. It produces bile, an alkaline compound which aids in digestion, via emulsification of lipids[1]. The various functions of the liver are carried out by the liver cells, the hepatocytes.

Uncontrolled environmental pollution, poor sanitary conditions, xenobiotics, alcohol intoxications and the indiscriminate use of drugs predispose the liver to a vast array of disorders. However, infection by virus still remains as the major cause of liver disease. Global estimates indicate that there are about 18,000 deaths every year due to liver cirrhosis caused by hepatitis[2]. 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, only a small number of these medicinal plants as well as formulations used are scientifically evaluated for their activity. In the context of our ongoing search for new natural substances possessing hepatoprotective efficacy, the present investigation was undertaken by utilizing the plant *Azima tetracantha* Lam. of Salvadoraceae family.

The plant is known as *Uppimullu* in Kannada and *Kundali* in Ayurvedic medicine. It is a spiny scrambling shrub. Spines are often in whorls of 4; branches 4-angled, leaves are elliptic to oblong, leathery, sharp-tipped at the apex (Plate 1). This plant is reportedly used as antidiarrhoeal, astringent and to relieve cough and given to cattle in rinder pest[3,4]. The root bark is used in muscular rheumatism[5] while the leaf juice is used in tooth and earache[6]. Pharmacological investigations revealed antiulcer, analgesic, anti-inflammatory, diuretic and antimicrobial activities[7-12]. There are few reports on phytochemical composition like presence of dimeric piperdine alkaloids azimine, azcarpaine, carpaine,

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Plate 1 — *Azima tetracantha*

triterpenoids, isorhamnetin 3-rutinoside and novel fatty acids[13-16]. Presence of glucosinolates and neoascorbinogen has also been reported[17].

Materials and Methods

Plant material

The leaves of *A. tetracantha* were collected in April 2007, from the regions around Chitradurga, Chitradurga District, Karnataka, India and authenticated by Prof B B Nandyal, Department of Botany and Biotechnology, SJVP Science College, Harihar, Karnataka. Herbarium specimen was deposited at SJM College of Pharmacy, Chitradurga (No. SJMCP/DP-1001/2006-07).

Preparation of extracts

The collected leaves were shade dried, powdered and extracted successively with petroleum ether (60-80%), chloroform and ethanol in a Soxhlet apparatus, the marc left after extraction was dried and extracted with chloroform water (0.2%) by maceration. All the extracts were concentrated by rotary vacuum evaporator and the residue obtained was dried and weighed. Chloroform (CEAT) and ethanolic extracts (EEAT) were used for the present investigations.

Chemicals

CCl_4 (E-Merck, Mumbai, India), Silymarin tablets (Micro Labs Pvt., Ltd.) and diagnostic reagent kit (ERBA Diagnostics Mannheim Germany and Transasia Bio-Medicals Ltd., Himachal Pradesh, India) were used in the present investigation. All solvents and chemicals used were of analytical grade. Water from Millipore Milli-Q system was used for preparing the solutions and all the solutions were prepared fresh.

Phytochemical analysis

Phytochemical tests were carried out to detect the presence of phytoconstituents, viz. alkaloids, flavonoids, tannins, triterpenoids, saponins, etc[18].

Experimental Models

Acute toxicity study

The Institutional Animal Ethical Committee (Sanction No. SETCP / IAEC / 2008-09 / 0544) approved the pharmacological protocols, according to prescribed guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India[19]. Rats weighing 125-150 g were used for the study. These were divided into groups of 6 animals each. The test extracts were administered orally as a suspension in Tween 80 (1% solution) to the different groups in increasing dose levels of 10, 40, 100, 400, 1000, 2000 and 5000 mg/kg body weight. $1/20^{th}$ and $1/10^{th}$ of maximum tolerated dose i.e. 250 and 500 mg/kg body weight were chosen for the study.

Animals used

Male albino rats (Wistar strain) weighing 150-200 g were used for the experiments. The selected animals were maintained under standard laboratory conditions (temperature 27± 2°C relative humidity 55±10% and 12 h light and dark cycles) and fed standard diet and water *ad libitum.* The animals were adapted to laboratory conditions for 7 days prior to the experiments.

Hepatoprotective activity

Rats were divided into 7 groups, of six animals each. Group I was categorized as normal control while, Group II was the induced control and was administered, a single daily oral dose of vehicle for 12 days. Group III to VI animals were treated with chloroform (CEAT) and ethanolic (EEAT) extracts of *A. tetracantha* at doses of 250 and 500 mg/kg, respectively for 12 days. Group VII animals were

administered with standard Silymarin (100 mg/kg). On 13th day, hepatic injury was induced to all the groups except group I through oral administration of CCl_4 diluted with liquid paraffin oil (1:1) at a dose of 1 ml/kg[20]. 48 h post CCl_4 treatment blood samples were collected from retro-orbital plexus and sacrificed animals. Blood serum was separated and analyzed spectrophotometrically (Semi-auto analyzer: Maygun, MS-500) for SGPT (ALT), SGOT (AST), total bilirubin (TB), direct bilirubin (DB), albumin (ALB) and total protein (TP) using Diagnostic reagent kit (ERBA Diagnostics Mannheim GmbH, Mallaustr, Mannheim/Germany).

Histopathological study

After the collection of blood samples, animals were sacrificed, liver samples were collected, washed with normal saline and were fixed in 10% buffered neutral formalin for 48 h and then washed with water to remove fixative. The tissues were fixed in Bouin's solution for 6 h and processed for microtome sections and examined under light microscope[21].

Antioxidant activity

The liver was excised from the animals, washed with ice-cold saline and blotted dry immediately. A 10% liver homogenate was prepared with ice-cold saline-EDTA using Teflon-glass homogenizer (Remi, Mumbai). Liver homogenate was centrifuged at 10,000 rpm for 10 min and the pellet was discarded. The supernatant was again centrifuged at 20,000 rpm for 1 h at 4°C. The supernatant obtained was used for the estimation of antioxidant parameters, viz. lipid peroxidation (MDA), glutathione (GSH) and total thiols[22,23]. In addition enzymatic antioxidant evaluation was performed by catalase assay[24].

Statistical analysis

The results were expressed as mean ± SE from 6 animals and evaluated using one-way ANOVA followed by Tukey's post-test using Graph pad 4.0, USA. $P<0.01$ was considered as statistically significant.

Results

Preliminary phytochemical screening of the CEAT revealed the presence of triterpenoids and steroids while EEAT fraction showed the presence of steroids, tannins, triterpenoids, saponins, alkaloids and flavonoids.

The effect of oral administration of CEAT, EEAT and reference drug on serum levels of SGPT, SGOT, total proteins, albumin, total and direct bilirubin which serve as reliable markers of hepatotoxicity in normal and CCl_4 treated rats are presented in Table 1. Animals treated with single oral dose of CCl_4 developed significant liver damage in induced control as evident from significant ($P<0.01$) increase in serum activities of SGPT, SGOT, total and direct bilirubin levels (1583.31 IU/l, 256.69 IU/l, 1.34 and 0.81 mg/dl) and decrease in total protein and serum albumin (2.31 g/l and 2.99 g/dl) compared to normal control rats (20.09 IU/l, 42.42 IU/l, 0.56 mg/dl, 0.43 mg/dl, 6.36 g/l and 6.05 g/dl). The elevated levels of serum markers were reduced in the animal groups treated with the plant extracts. Among the two doses of chloroform extract 250 mg showed significant hepatoprotection for only serum albumin. While at 500 mg, in addition to serum albumin, the results were significant for enzyme markers, viz. SGOT, SGPT and for non enzyme markers, viz. direct bilirubin. However, the ethanolic extract at both the

Table 1 — Hepatoprotective effect of *Azima tetracantha* chloroform (CEAT) and ethanol (EEAT) extracts on serum markers of hepatotoxicity in CCl_4-induced hepatotoxic rats

Treatment	Serum SGPT (IU/l) (mg/dl)	Serum SGOT (IU/l)	Total protein (g/l)	Serum total bilirubin (mg/dl)	Serum direct bilirubin (mg/dl)	Serum albumin (g/dl)
Vehicle	20.09 ±2.08	42.42 ±4.17	6.36 ±0.13	0.56 ±0.04	0.43 ±0.04	6.05 ±0.14
CCl_4 (1ml/kg; p.o)	1583.31 ±107.13	256.69 ±24.70	2.31 ±0.18	1.34 ±0.11	0.81 ±0.05	2.99 ±0.23
CCl_4+ CEAT (250 mg/kg)	1567.17 ±081.33	266.20 ±34.81	2.77 ±0.17	1.25 ±0.18	0.70 ±0.06	4.16 ±0.22 *
CCl_4+ CEAT (500 mg/kg)	1126.05±87.07*	215.55 ±11.43*	3.07±0.18	1.23 ±0.18	0.57 ±0.10*	4.59 ±0.25 *
CCl_4+ EEAT (250 mg/kg)	690.70 ±95.87*	193.03 ±12.06*	3.23 ±0.15*	0.73 ±0.03 *	0.48 ±0.07 *	4.86 ±0.14*
CCl_4+ EEAT (500 mg/kg)	452.58 ±98.15*	149.31 ±13.22 *	4.69 ±0.28 *	0.68 ±0.04 *	0.43 ±0.02*	5.24 ±0.22 *
CCl_4+Silymarin (100mg/kg)	370.40 ±76.50*	146.84 ±11.75*	5.08 ±0.27*	0.50 ±0.09 *	0.41 ±0.04 *	5.78 ±0.25 *

Values are mean ± S.E, n=6; ANOVA * $P < 0.01$ vs CCl_4 treated group

doses exhibited significant restoration of serum markers for all the parameters studied in a dose dependent manner indicating its effectiveness. However, the test extract was found to be less potent than the standard drug.

The activities of enzymatic and non-enzymatic antioxidants and results of lipid peroxidation are presented in Table 2. In liver serum GSH, total thiol and catalase levels were considerably reduced (11.47 n moles/mg of protein, 0.09 μmoles/mg of protein and 23.48 U/mg of protein) and MDA level was increased (59.50 n moles/g of tissue) in CCl_4 induced rats compared to normal rats. However, treatment with CEAT and EEAT showed significant protective activities for GSH at both the doses while for total thiols and catalase activity, the CEAT at 500 mg and EEAT at both the concentrations recorded near normal values. In regard to MDA concentration even though the CEAT showed reduction, only the effect of EEAT revealed statistically significant variation when compared to CCl_4 treated animals. It is apparent from the data that the effectiveness of the extracts is in a dose dependent manner. Further, among the two extracts ethanolic extract proved to be more hepatoprotective as evident by its significant hepatoprotective features and comparable to the effect of Silymarin.

The histopathological preparations of various groups are shown in Plate 2 (a to g). Histological profile of control animals showed normal hepatocytes (Plate 2 a). While the animals treated with CCl_4 exhibited intense centrilobular, necrosis, vacuolization and macrovesicular fatty changes (Plate 2 b). Moderate accumulation of fatty lobules was observed in the liver sections of the animals treated with the CEAT and EEAT leaf extracts at 250 mg (Plate 2 c & e). However the liver sections of the animals treated with higher concentration (500 mg) of both the extracts exhibited significant liver protection against CCl_4 as evident by the presence of normal hepatic cords, absence of necrosis and fatty infiltration supporting the protective effect of extract (Plate 2 d & f).

Discussion

The present study provides scientific evidence on the correlative effect of antioxidant and hepatoprotective activities. Elevation of serum markers are a known effect of CCl_4 toxicity and used as biochemical parameters of liver damage[25]. The toxicity produced by CCl_4 is mediated through free radical mechanism. CCl_4 is metabolized by cytochrome P_{450} enzyme and its metabolic products, trichloromethyl free radicals that are highly reactive and induces lipid peroxidation of macromolecules leading to tissue injury[26]. It produces hepatotoxicity by altering liver microsomal membranes in experimental animals[27]. The extent of hepatic damage was assessed by histological evaluation and the level of various biochemical parameters. From the results of the present investigation it was evident that the chloroform and ethanol extracts of *A. tetracantha* were able to reduce the hepatotoxin intoxication induced elevated biochemical parameters. The reduced levels of total proteins and albumin in CCl_4 induced hepatotoxicity is attributed to the initial damage produced and localized in the endoplasmic reticulum which results in the loss of P_{450} leading to its functional failure with a decrease in protein synthesis and accumulation of triglycerides leading to fatty liver[28]. Reduction in the levels of SGOT and SGPT towards the normal value is an indication of

Table 2 — Effect of *Azima tetracantha* chloroform (CEAT) and ethanol (EEAT) extracts on enzymatic and non-enzymatic endogenous antioxidant levels in CCl_4-induced hepatotoxic rats

Treatment	GSH (n moles/mg protein)	Total Thiols (μ moles/mg protein)	MDA (n moles/g of tissue)	Catalase (U/mg of protein)
Normal Control	72.45±4.30	0.69±0.21	10.12±0.53	75.49±4.62
CCl_4 alone (1ml/kg)	11.47±1.13	0.09±0.18	59.50±3.1	23.48±2.56
CCl_4+ CEAT (250 mg/kg)	26.41±1.78*	0.10±0.09	51.37±1.8	31.73±1.04
CCl_4+ CEAT (500 mg/kg)	34.90±2.87*	0.26±0.01*	47.38±1.69	46.26±2.08*
CCl_4+ EEAT (250 mg/kg)	28.78±1.46*	0.18±0.10*	39.18±2.01*	38.29±3.12*
CCl_4+ EEAT (500 mg/kg)	41.28±2.39*	0.47±0.14*	25.68±2.0*	49.16±2.91*
CCl_4+Silymarin (100 mg/kg)	54.18±1.95*	0.58±0.17*	16.29±1.6*	51.27±2.67*

Values are mean ± S.E, n=6; ANOVA * $P < 0.01$ vs CCl_4 treated group

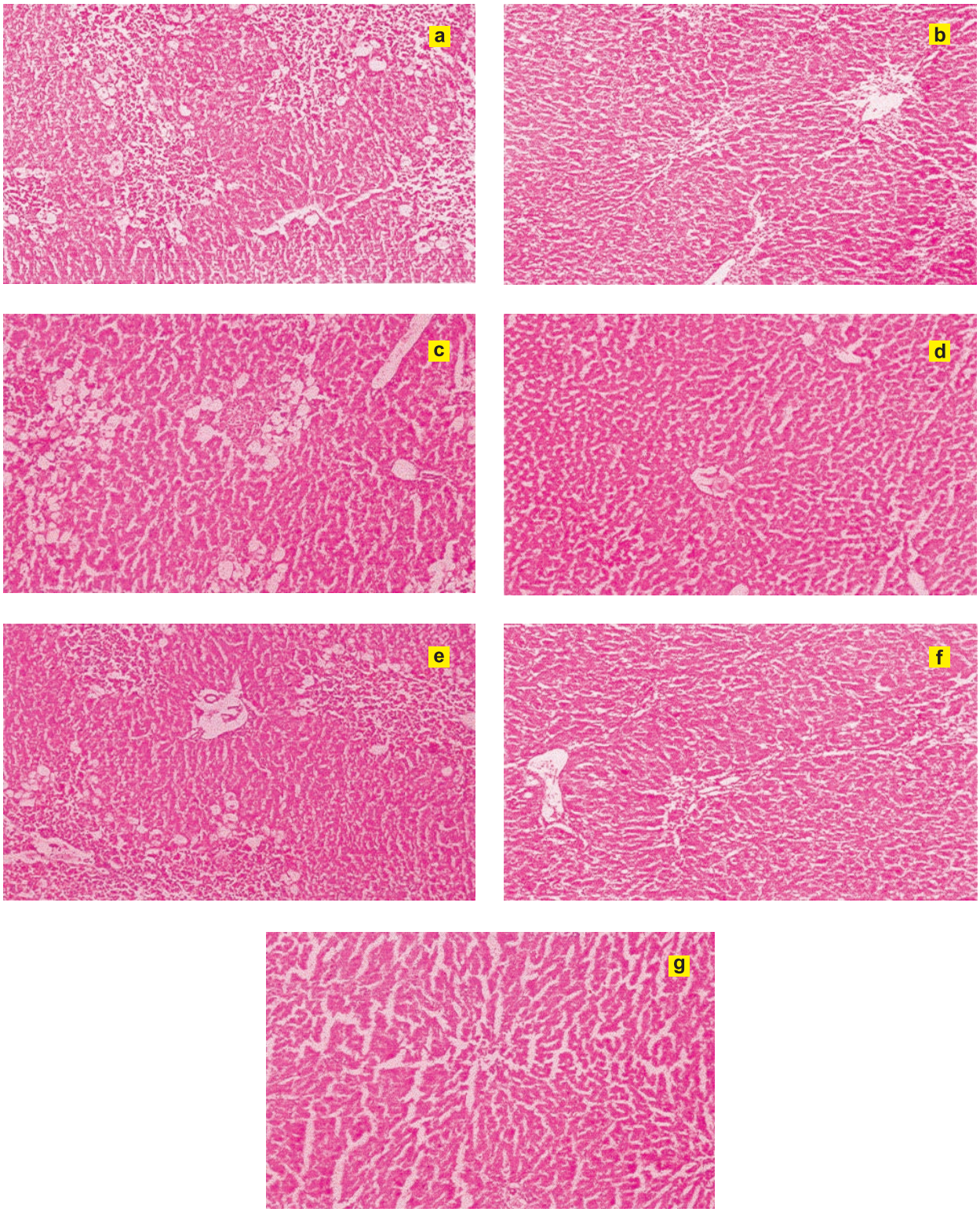

Plate 2 — Histological sections of rat liver: (a) liver sections of normal control rats showing: normal hepatic cells with well preserved cytoplasm; (b) liver sections of CCl_4 treated rats showing: massive fatty changes, necrosis, degeneration and loss of cellular boundaries; (c and e); liver sections of rats treated with CCl_4, CEAT and EEAT extracts (250 mg/kg) showing moderate recovery; (d and f) liver sections of rats treated with CCl_4, CEAT and EEAT extracts (500 mg/kg) showing: hepatic cells well preserved cytoplasm prominent nucleus; (g) liver sections of rats treated with CCl_4 and Silymarin (100 mg/kg) showing: almost normal architecture of liver.

regeneration process. Reduction of raised bilirubin level suggests the stability of the biliary function during injury with CCl_4. The protein and albumin levels were also raised suggesting the stabilization of endoplasmic reticulum leading to protein synthesis. The histopathological observations in CCl_4 rats showed severe necrosis, with disappearance of nuclei, could be due to the oxidative threat caused by trichloromethyl radicals. Ethanolic and Chloroform extracts reversed the histopathological changes. Thus, administration of chloroform and ethanolic extracts of leaves revealed hepatoprotective activity of *A. tetracantha* leaves against the toxic effect of CCl_4, which was also supported by histological studies.

Extensive experimental and epidemiological studies support the involvement of oxidative stress in pathogenesis and progression of many diseases. It is well known that oxygen sometimes becomes toxic and results in the generation of most aggressive agents such as reactive oxygen species (ROS). The high reactivity of ROS can trigger a host of disorders in biological systems. Oxidative stress is an outcome of imbalance between ROS production and antioxidant defenses, which in turn evokes a series of events dysregulating the cellular functions[29]. Endogenous antioxidant enzymes are responsible for preventing and neutralizing the free radical induced damage of tissues.

Catalase (CAT) is an enzymatic antioxidant widely distributed in all animal tissues, and the highest activity is found in the red cells and liver. CAT decomposes hydrogen peroxide and protects the tissues from highly reactive hydroxyl radicals[30]. Therefore reduction in the activity of CAT may result in a number of deleterious effects due to the assimilation of superoxide radical and hydrogen peroxide. Higher dose of both the extracts (500 mg/kg) increases the level of CAT as produced by silymarin. Glutathione is one of the most abundant tripeptide, non-enzymatic biological antioxidant present in the liver. It removes free radical species and maintains membrane protein thiols. Also it is a substrate for glutathione peroxidase[31]. Decreased level of GSH is associated with an enhanced lipid peroxidation in CCl_4 treated rats. Administration of *A. tetracantha* extracts increased the level of GSH in a dose dependent manner. Reduced thiols have long been reported to be essential for recycling of antioxidants like vitamin E and C (Ref. 32). Administration of thiol compounds such as glutathione, cysteine and methionine have been shown to protect against oxidative stress in humans and animals. Treatment with test extracts resulted in increased level of total tissue sulfhydryl (thiol) groups compared to the untreated rats. MDA is the major oxidation product of poly-unsaturated fatty acids and its elevation is an important indicator of lipid peroxidation induced tissue damage due to failure of antioxidant defense mechanisms[33]. Treatment with ethanolic and chloroform extracts significantly reversed it indicating that the phytoconstituents present in these extracts have antioxidant potential.

Conclusion

The reduced lipid peroxidation with simultaneous significant increase in GSH, total thiol and CAT content of liver suggested antioxidant activity of chloroform and ethanolic extracts of *A. tetracantha* leaves and silymarin and thus it can be concluded that possible mechanism of hepatoprotection of leaves may be due to its antioxidant activity. The phytochemical screening of the extracts revealed the presence of triterpenoids and steroids in both the extracts while ethanolic extract in addition has shown tannins, alkaloids and flavonoids. Several phytoconstituents, viz. triterpenes, alkaloids, flavonoids, flavones, glycosides, etc. have been found effective in the hepatoprotection against CCl_4 induced hepatic toxicity[34-36]. Further, the higher efficacy of ethanolic extracts in hepatoprotection could be attributed to the presence of flavonoids in the extract. However, the exact nature of the hepatoprotection exhibited by the plant samples has to be studied.

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