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Hepatoprotective Potential of Hydroalcoholic Extract of *Santalum album* Linn. Leaves

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ABSTRACT

The present study was designed to evaluate the hepatoprotective activity of leaves of *Santalum album* in experimentally induced liver injury by carbon tetrachloride and paracetamol. The levels of serum marker enzymes, bilirubin, total protein and antioxidant status were determined by measuring lipid peroxidation, glutathione, superoxide dismutase and catalase activity. Total wet weight and histopathological study of isolated liver was also carried out. The oral pre-treatment with hydroalcoholic extract of the leaves of *S. album* (200 and 400 mg/kg) showed significant hepatoprotective activity against CCl_4 and paracetamol induced hepatotoxicity by decreasing the activities of serum marker enzymes, bilirubin and lipid peroxidation, and significant increase in the levels of glutathione, superoxide dismutase, catalase and protein in a dose dependent manner, which was confirmed by the decrease in the total weight of the liver and histopathological examinations. Data also revealed that the extract possessed strong antioxidant activity, which might leads to the promising hepatoprotective activity.

Keywords: Carbon tetrachloride, Hepatoprotective activity, Paracetamol, *Santalum album*.

INTRODUCTION

Excessive production of reactive oxygen species (ROS) plays an important role in the pathogenesis and progression of various diseases involving different organs. [1] Lipid peroxides produced from unsaturated fatty acids via free radicals cause toxic effects and promote the formation of additional free radicals in a chain reaction. If the *in-vivo* activity of enzymes or scavengers is not adequate to neutralize these radicals, oxidative stress develops and leads to various diseases such as cancer, diabetes mellitus, liver diseases, brain dysfunction, or accelerated aging may result. [2] The rationale for the use of antioxidants is well established in prevention and treatment of diseases where oxidative stress plays a major etiopathological role. Antioxidants may protect the body against ROS toxicity either by preventing the formation of ROS, by the interruption of ROS attack, by scavenging the reactive metabolites or by converting them to less reactive molecules. [3] Therefore the uses of antioxidants, both natural and synthetic are gaining wide importance in the prevention of various diseases.

Liver is the vital organ responsible for drug metabolism and appears to be a sensitive target site for substances modulating biotransformation. [4] Liver diseases are mainly caused by toxic chemicals, excess consumption of alcohol, infections and autoimmune disorders. Most of the hepatotoxic chemicals damage liver cells mainly by inducing lipid peroxidation and other oxidative stress in liver. [5] Carbon tetrachloride and paracetamol being converted into reactive toxic metabolites by hepatic microsomal cytochrome P-450 causes hepatotoxicity. [6] Among the many diseases that can affect the liver, the most common is 'viral hepatitis' and it can also caused by drugs, bacteria, mushrooms, parasites like amoebas or giardiasis. About 20,000 deaths found every year due to liver disorders. [7] Conventional or synthetic drugs used in the treatment of liver diseases are sometimes inadequate and can have serious adverse effects. [8]

Medicinal plants have been recognized and are extremely valued all over the world as a prosperous source of bioactive for the prevention and treatment of ailments. Various plants have been used effectively as hepatoprotective agents. [9] Some of the hepatoprotective plants as well as formulations used in traditional medicines have been pharmacologically evaluated for their efficacy. However, still more numbers of plants are needed to be screened for their hepatoprotective potentials.

One such plant *Santalum album* Linn (Family: Santalaceae) is locally available has been used in traditional system of medicine. The wood, root, bark and leaves of the plant used

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for the treatment of the liver disease like jaundice by the tribal healers. [10] This plant is very beneficial for treating gastric irritability, jaundice, dysentery, tension and confusion. Previous reports have proved that sandal wood and root bark possessed abortifacient, hepatoprotective, urinary antiseptic, stomachic, anti-viral and anti-herpetic activities. [11] The leaves of this plant are known to possess array of flavonoids. [12-13] However, there is paucity of scientific data for the hepatoprotective activities of leaves of the plant. On the basis of use of leaves in Ayurveda for the treatment of jaundice and the presence of various bioactive phytoconstituents like flavonoids, in the present study an attempt is made to evaluate the hepatoprotective activity of the plant of *S. album* Linn leaves.

MATERIALS AND METHODS

Plant material

The fresh leaves of *Santalum album* Linn used for the present studies were collected from Mangalore in April 2013 and authenticated by Mr. Dinesh Nayak, Advisor (Green belt), Mangalore SEZ Limited.

Preparation of plant extracts

The collected leaves material were cleaned to remove the adhered dust particles and were then shade dried. The dried plant materials were coarsely powdered, weighed and stored in an air tight container till use. The coarse powder was packed into Soxhlet column and extracted with 70% ethanol for about 48 h. The solvent was evaporated using rotary flash evaporator to get syrupy consistency. Then the dried extract was stored in airtight container in refrigerator below 8°C until used.

Phytochemical screening

Freshly prepared hydroalcoholic extract of the leaves of *S. album* (HASA) was subjected to preliminary phytochemical screening for the detection of major chemical constituents. [14]

Experimental animals

Healthy Wistar albino rats of either sex weighing 150-200 g were used. Animals used in the study were procured from registered breeder. The animal care and handling was carried out according to CPCSEA guidelines. Animals were acclimatized to the animal quarantine for one week prior to the experiment under controlled conditions of temperature (27 ± 2^{o}C) and were housed in sterile polypropylene cages containing paddy husk as bedding material with maximum of six animals in each cage. The rats were fed on standard food pellets and water *ad libitum*. The studies conducted were approved by the Institutional Animal Ethical Committee, Srinivas College of Pharmacy, Mangalore, Karnataka (Approval No.: SCP/CPCSEA/P15/F150/2012).

Acute toxicity study

Acute toxicity study of hydroalcoholic extract of the leaves of *S. album* (HASA) was determined in Wistar albino rats according to OECD guidelines No. 425. [15] The animals were fasted overnight and the extract was administered orally with a starting dose of 2000 mg/kg, to different animals. Animals were observed continuously for first 3 h and monitored for 14 days for mortality and general behaviour of animals, signs of discomfort and nervous manifestations.

CCl_4 -induced hepatotoxicity

Wistar albino rats were randomly divided in to 5 groups of 6 animals each. Group I, the normal control group was administered a single daily dose of 1% Tween 80 (1 ml/kg, p.o). Group II, the toxic (CCl_4) control group was administered a single daily dose of 1% Tween 80 (1 ml/kg, p.o) and CCl_4/olive oil (1:1 v/v, 0.7 ml/kg, i.p) on alternate days for 7 days. Group III, the standard group was administered a single daily dose of silymarin (100 mg/kg, p.o) and CCl_4/olive oil (1:1 v/v, 0.7 ml/kg, i.p) on alternate days for 7 days. Group IV and V, the test groups were administered a single daily dose of HASA (200 and 400 mg/kg, p.o, respectively) and CCl_4/olive oil (1:1 v/v, 0.7 ml/kg, i.p) on alternate days for 7 days. [16]

Paracetamol induced hepatotoxicity

The animals were divided in to 5 groups of 6 animals each. Group I and II (Normal and toxic) was administered a single daily dose of 1% Tween 80 (1 ml/kg, p.o). Group III, (standard) was administered a single daily dose of silymarin (100 mg/kg, p.o). Group IV and V, the test groups were administered a single daily dose of HASA (200 and 400 mg/kg, p.o, respectively) for 7 days. On 5th day, after the administration of the respective drug treatments, all the animals of groups II, III, IV and V were challenged with paracetamol 2 g/kg, p.o, suspended in sucrose solution (40% w/v). [17]

Assessment of liver function

On the seventh day 2 hour after the administration of last dose, the animals were sacrificed and blood was withdrawn by intracardiac puncture in both the models. Blood was allowed to clot at room temperature for 30 min, subjected to centrifugation (3000 rpm for 15 min). The serum was used to estimate serum glutamate oxaloacetate transaminase (SGOT), [18] serum glutamate pyruvate transaminase (SGPT), [19] serum alkaline phosphatase (SALP), total protein (TP) [20] and bilirubin content (total (TB) and direct (DB)). [21] The liver was immediately isolated and washed with normal saline, blotted with filter paper and weighed. One portion of liver was subjected to histopathological examination and other portion was subsequently subjected to estimation of endogenous antioxidant parameters like lipid peroxidation (LPO), [22] super oxide dismutase (SOD), glutathione (GSH) [23] and catalase (CAT). [24]

Statistical analysis

The data were expressed as mean ± SE, (n = 6). Data were analyzed using One way analysis of variance (ANOVA) followed by Dunnett's't' test. Values of $p<0.05$ were considered statistically significant.

RESULTS

Phytochemical screening

Preliminary phytochemical investigation of the HASA reveals the presence of flavonoids, carbohydrates, steroids, saponins, glycosides and tannins.

Acute toxicity study

Acute toxicity studies were carried out according to OECD guidelines. No mortality was observed at 2000 mg/kg body weight. Therefore 1/10th and 1/5th doses were taken as low and high effective dose for the evaluation of hepatoprotective activity.

Effect of HASA on serum biochemical parameters

Animals challenged with CCl_4/paracetamol (Toxic control group) developed significant liver injury as evident from a significant elevation in the biochemical markers like SGPT, SGOT, SALP, TP, total bilirubin and direct bilirubin when compared with normal control group. Treatment of animals with HASA at the dose of 200 mg/kg and 400 mg/kg, p.o

markedly prevented the CCl_4/paracetamol induced elevation of serum SGPT, SGOT, SALP, bilirubin (total and direct) and increased the level of total protein. Hepatoprotective potency of the test extract at the dose 400 mg/kg was found closer to that of standard (Table 1).

Effect of HASA on endogenous antioxidant enzymes

It was observed that animals treated with CCl_4/paracetamol developed a hepatic damage, as increase in LPO and decrease in GSH, CAT and SOD when compared to normal control (Table 2). Animals treated with standard (silymarin) showed extremely significant (P<0.001) increase in GSH, CAT, SOD and decrease in LPO. Treatment with HASA (200 and 400 mg/kg, p.o) significantly prevented the increase in LPO levels and brought them near to normal level, whereas GSH, SOD and CAT levels were significantly raised, thus providing protection against CCl_4/paracetamol toxicities.

Effect of HASA on liver weight

The liver weight was increased in CCl_4/paracetamol treated animals when compared to normal control. The animals which treated with HASA 200 and 400 mg/kg showed significant (P<0.001) decrease in liver weight (Table 2).

Histopathology

Histopathological studies also provided a supportive confirmation for biochemical analysis. Both the CCl_4/paracetamol intoxicated groups of animals showed inflammation, centrilobular degeneration and necrosis. Treatment with HASA (200 and 400 mg/kg) showed reduced inflammation, centrilobular and bridging necrosis. Liver section of these groups revealed normal hepatocytes with significant reduction in areas of necrosis when compared to toxic group. These changes showed protective effect of the drug against hepatic damage induced by CCl_4/paracetamol (Fig.1).

DISCUSSION

Carbon tetrachloride/paracetamol induced hepatic injuries are commonly used models for the screening of hepatoprotective drugs and the extent of hepatic damage is assessed by the level of released cytoplasmic alkaline phosphatase and transaminases in circulation. It is well documented that CCl_4/PCM are biotransformed under the action of microsomal cytochrome P-450 of liver to reactive metabolites. [25] These metabolites attributed to damage structural integrity of liver and raise the levels of SGPT, SGOT, ALP and bilirubin. Further depletion of GSH, decreased protein synthesis, triglycerides accumulation, and increased lipid peroxidation results in hepatocyte damage. [26] The hepatotoxic effects of CCl_4 are largely due to its active metabolite, trichloromethyl radical ($CCl_3^{\bullet}$) in presence of cytochrome P450 which in turn disrupts the structure and function of lipid and protein macromolecules in the membrane of the cell organelles. Due to liver injury, the transport function of hepatocytes get disturbed, resulting in the leakage of plasma membrane, thereby causing an increased enzyme level in serum. While the wet liver weight and liver volume was found to be increased, this may be due to enlargement of liver cells, accumulation of fluids and fatty changes in the liver. CCl_4 has important role in reduction of intracellular antioxidant reduced glutathione (GSH), increased LPO, membrane damage, decreased protein synthesis and alteration of hepatic enzymes level.

The present study revealed a significant alteration in physical and biochemical parameter after exposure to the CCl_4. The plant extract HASA 200 and 400 mg/kg showed dose dependent decrease in the elevated serum biomarkers (SGPT, SGOT, SALP and bilirubin) and endogenous enzymes (GSH, SOD, CAT), it also been observed that increase in total protein levels which were comparable to the standard and significant reduction in the level of lipid peroxidation.

Paracetamol is metabolized primarily in the liver and eliminated by sulfate and glucuronide conjugation, and excreted by kidney.

Table 1: Effect of HASA on biochemical parameters in (A) CCl_4 and (B) paracetamol induced liver damage in rats

Groups	Treatment		ALP (U/l)	SGOT (U/l)	SGPT (U/l)	TB (mg/dl)	DB (mg/dl)	T.P (g/dl)
Normal control	1% Tween 80	A	136.3±3.53	86.66±3.04	59.61±1.38	0.945±0.012	0.24±0.009	8.07±0.06
		B	143.3±3.569	74.31±1.759	65.74±1.57	1.01±0.02	0.21±0.01	7.98±0.06761
Toxic control	CCl_4 0.7 ml/kg, i.p	A	359.7±7.06[a]	177.4±7.56[a]	135.6±2.25[a]	2.878±0.018[a]	0.54±0.009[a]	4.73±0.12[a]
	Paracetamol 2 g/kg, p.o	B	307.5±10.35[a]	181.2±9.159[a]	147.6±4.48[a]	2.44±0.06[a]	0.52±0.05[a]	5.00±0.13[a]
Standard	Silymarin	A	178.4±5.84***	98.32±2.06***	76.74±2.46***	1.120±0.01***	0.27±0.008***	7.70±0.07***
	100 mg/kg, p.o	B	161.8±3.62***	104.4±3.481***	87.10±2.84***	1.12±0.01***	0.24±0.006***	7.17±0.11***
Low dose	HASA	A	302.6±4.24***	152.8±1.91**	107.8±1.99***	2.817±0.005**	0.50±0.006**	6.68±0.067***
	200 mg/kg, p.o	B	261.5±7.502**	141.2±4.140**	123.3±4.20***	2.12±0.03*	0.44±0.03**	6.53±0.050***
High dose	HASA	A	252.6±4.26***	107.5±3.07***	97.60±2.93***	2.808±0.006**	0.49±0.005**	7.28±0.073***
	400 mg/kg, p.o	B	225.6±10.60***	118.8±6.98***	100.8±2.815***	1.95±0.15**	0.428±0.02**	6.95±0.07***

One way ANOVA followed by Dunnett's't' test. All the values are Mean±SEM, n=6. [a]p< 0.001 when compared with vehicle treated control group. ns, p>0.05, *p<0.05, **p<0.01, ***p<0.001 when compared with toxic control.

Table 2: Effect of HASA on lipid peroxidation (LPO), superoxide dismutase (SOD), glutathione (GSH), catalase (CAT) and liver weight in (A) CCl_4 and (B) paracetamol induced hepatic damage in rats

Groups	Treatment		LPO (Abs at 535 nm)	SOD (Abs at 560 nm)	GSH (Abs at 412nm)	CAT (Abs at 620 nm)	Liver weight
Normal control	1% Tween 80	A	0.06±0.009	0.83±0.009	0.63±0.009	0.52±0.013	3.53 ±0.06
		B	0.07±0.006	0.88±0.019	0.67±0.01	0.55±0.013	3.48±0.055
Toxic control	CCl_4 0.7 ml/kg, i.p	A	0.35±0.007[a]	0.18±0.008[a]	0.25±0.009[a]	0.25±0.013[a]	4.95±0.07[a]
	Paracetamo2 g/kg, p.o	B	0.33±0.013[a]	0.20±0.02[a]	0.33±0.02[a]	0.28±0.009[a]	5.06±0.07[a]
Standard	Silymarin	A	0.15±0.009***	0.65±0.02***	0.54±0.011***	0.42±0.015***	3.72±0.05***
	100 mg/kg, p.o	B	0.13±0.008***	0.71±0.02***	0.56±0.01***	0.45±0.015***	3.84±0.06***
Low dose	HASA	A	0.25±0.007***	0.24±0.007*	0.30±0.013**	0.31±0.008**	4.19±0.036***
	200 mg/kg, p.o	B	0.24±0.012***	0.30±0.01**	0.39±0.007*	0.34±0.012*	4.133±0.05 ***
High dose	HASA	A	0.23±0.008***	0.26±0.006**	0.47±0.010***	0.32±0.011**	3.928±0.054***
	400 mg/kg, p.o	B	0.20±0.009***	0.31±0.018**	0.41±0.011**	0.36±0.019**	3.90±0.05***

One way ANOVA followed by Dunnett's't' test. All the values are Mean±SEM , n=6. [a]p< 0.001 when compared with vehicle treated control group. ns, p>0.05, *p<0.05, **p<0.01, ***p<0.001 when compared with toxic control.

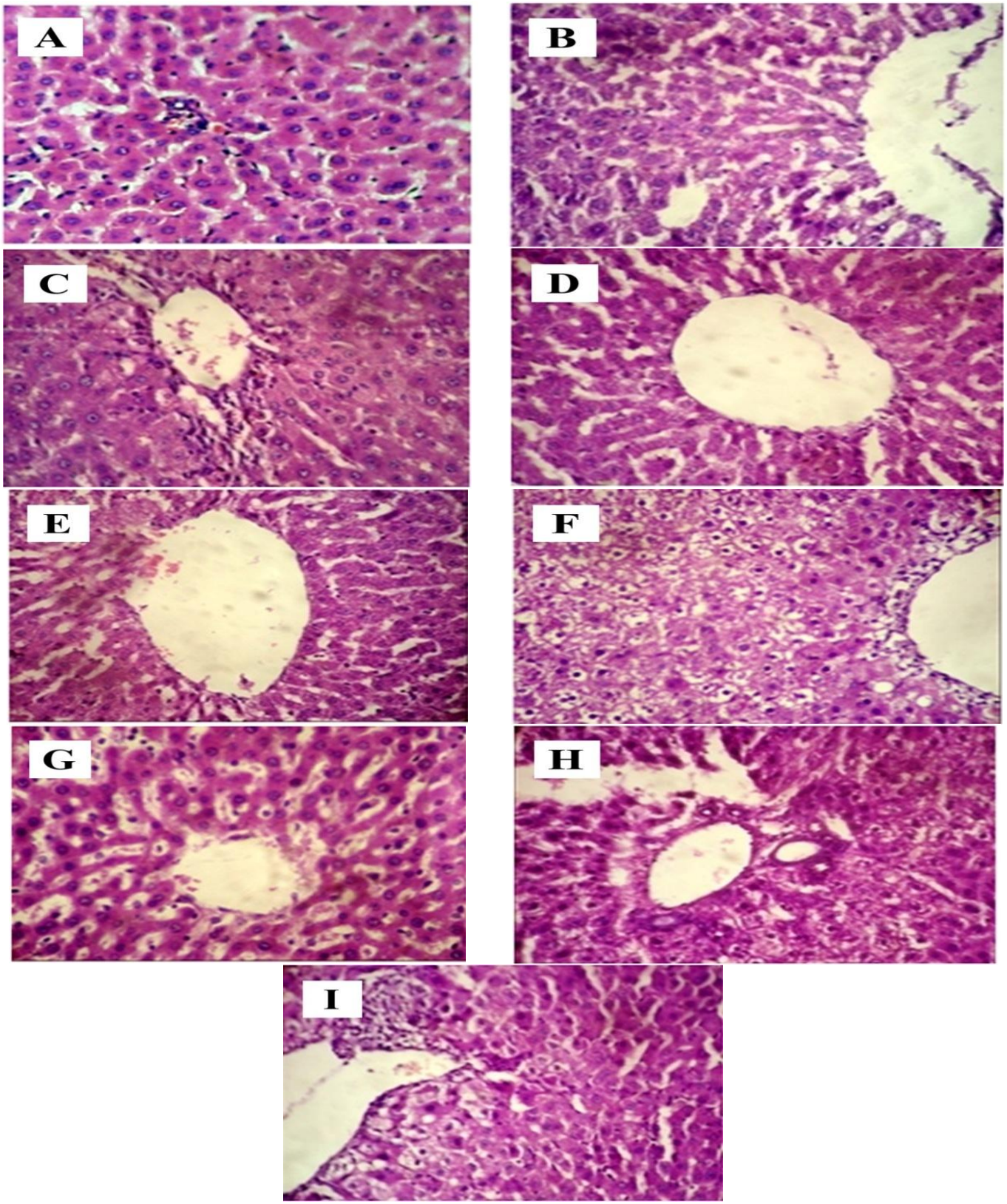

Fig. 1: Effect of Silymarin and HASA on liver histology in CCl_4 /Paracetamol induced liver toxicity
A: Liver of normal rat; **B**: Liver of CCl_4 induced rat; **C**: Liver treated with silymarin prior to CCl_4 administration; **D**: Liver treated with HASA 200 mg/kg prior to CCl_4 administration; **E**: Liver treated with HASA 400 mg/kg prior to CCl_4 administration. **F**: Liver of paracetamol induced rat; **G:** Liver treated with silymarin prior to paracetamol administration; **H:** Liver treated with HASA 200 mg/kg prior to paracetamol administration; **I:** Liver treated with HASA 400 mg/kg prior to paracetamol administration.

Moreover, paracetamol hepatotoxicity has been attributed to the formation of toxic metabolites, when paracetamol is activated by hepatic cytochrome P-450 to a highly reactive metabolite N-acetyl-p-benzoquinoneimine (NAPQI). Toxic metabolites (N-acetyl-p-benzoquineimine) can alkylate and oxidize intracellular GSH, which results in liver GSH depletion subsequently leads to increased lipid peroxidation by abstracting hydrogen from a polyunsaturated fatty acid and ultimately liver damages due to higher doses of paracetamol. [27]

Elevated levels of serum biomarkers are indicative of cellular leakage and loss of functional integrity of hepatic cell membranes implying hepatocellular damage. Lipid peroxidation has been suggested being the destructive process in liver injury due to CCl_4/paracetamol administration. The present study revealed a significant alteration in physical and biochemical parameter after exposure to the paracetamol. The plant extract HASA-200 and HASA-400 showed dose dependent decrease in the elevated serum biomarkers (SGPT, SGOT, ALP and bilirubin) and endogenous enzymes (GSH, SOD, CAT), it also been observed that increase in total proteins levels which were comparable to the standard and significant reduction in the levels of lipid peroxidation. Hence it might be possible that the mechanism of hepatoprotection by hydroalcoholic extract is due to its antioxidant potential.

Protective effect in CCl_4 and paracetamol induced liver toxicity was also proved by decrease in relative organ weights in animals treated with both HASA-200 and HASA-400. Histopathological studies of liver, treated with CCl_4/paracetamol alone revealed affected the architecture of liver parenchyma with damaged hepatocytes. Treatment with HASA (200 and 400 mg/kg) revealed the significant improvement in architecture of liver parenchyma and regenerating hepatocytes indicating hepatoprotection.

The hydroalcoholic extract prepared was subjected to phytochemical tests and the outcome of these tests revealed the presence of carbohydrate, glycosides, flavonoids, tannins, steroids and saponins.

The number of investigators has reported that flavonoids, saponins and other phenolic compounds [28, 29] are known to possess hepatoprotective activity in animals. It is therefore to speculate that the phytoconstituents present in this plant extracts might responsible for the observed hepatoprotective activity.

It can be concluded from the above data that the hepatoprotective activity was found to be more significant in high dose (HASA-400 mg/kg) compared to low dose (HASA-200 mg/kg) in both the animal models. The treatment with hydroalcoholic extract of leaves of *S. album* could able to restore the organ (liver) weight at considerable range, which were elevated in hepatotoxic animals, hence proving organ protective activity. The hepatoprotective potential of extract in both the experimental models might be due to the presence of flavonoids, saponins and other polyphenolic compounds which are attributed for the antioxidant activity.

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