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Research Article

ANTIOXIDANT AND CURATIVE EFFECT OF *LEUCAS ASPERA* METHANOLIC EXTRACT AGAINST CARBONTETRA CHLORIDE INDUCED ACUTE LIVER INJURY IN RATS

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ABSTRACT

Objective: Aim of the present study was to investigate the curative effect of the methanolic extract of Leucas aspera(MELA) against carbontetra chloride(CCl4) induced acute liver injury in rats.

Methods: Toxic control, MELA and standard drug *Silymarin* treated rats were received a single dose of CCl_4 (150 µl/100 g, 1:1 in ground nut oil). In post-treatment groups, rats were treated with MELA at doses of 100 and 200mg/kg and Silymarin at a dose of (50mg/kg), 2,24 and 48 hrs after CCl_4 administration.

Results: Rats treated with the extract after the establishment of CCl_4 induced liver damage showed significant ($p^*\leq0.05$) protection of liver as evidenced from normal AST, ALT, ALP and lipid peroxide levels. Hepatic glutathione levels were significantly ($P^*\leq0.05$) increased by the treatment with the extracts. Histopathological changes induced by CCl_4 were also significantly($p^*\leq0.05$) reduced by the extract treatment in curative groups. The antioxidant status of the animals were also assessed by measuring the activity of GSH, Catalase, SOD, GST and GPx. The extent of lipid peroxidation was also measured. Phytochemical screening of this plant revealed the presence of sitosterol, flavonoids and alkaloids which could be responsible for the possible hepatprotective action.

Conclusion: This study demonstrates the antioxidant and curative effect of MELA and thus scientifically proves the use of entire plant in traditional medicine for hepatic disorders.

Keywords: Leucas aspera, Silymarin, Carbon tetrachloride, Hepatotoxicity, Lipid peroxidation

INTRODUCTION

Herbal medicines have become more popular in the treatment of many diseases due to popular belief that green medicine is safe, easily available and with lesser side effects. It is believed that crude extract from medicinal plants are more biologically active than isolated compounds due to their synergistic effects [1]. Many folk remedies from plant origin are evaluated for its possible antioxidant and hepatopotective effects against different chemical-induced liver damage in experimental animals.CCl_4–induced hepatotoxicity model is frequently used for the investigation of hepatoprotective effects of drugs and plant extracts [2,3].

Leucas aspera belonging to the family Lameaceae is used as an insecticide and indicated in traditional medicine for coughs, colds, painful swellings and chronic skin eruptions [4]. The compounds isolated from the plant includes long chain aliphatic compounds, triterpene-levolactone, sterols-sitosterol, campesterol, stigmasterol and a novel phenolic compound [5-8].

Most of these compounds prevent peroxidative damage to liver microsomes and hepatocytes [9].The main objective of the study is to evaluate the antioxidant and curative effect of *L.aspera* to verify tribal or folk claims in using this herb against liver disorders. In the present investigation the antihepatotoxic activity of methanolic extract of *L.aspera* against CCl_4 induced acute liver injury in curative model was conducted.

MATERIALS AND METHODS

Chemicals

Carbon tetrachloride was purchased from Merck, Mumbai. Silymarin was purchased from sigma chemical Co., St. Louis, MO, USA. 5,5'-dithiobis-(2-nitrobenzoic acid),1-chloro-2,4-dinitrobenzene were purchased from Sisco Research laboratories, Mumbai, India. Assay kits for serum marker enzymes AST, ALT, ALP were purchased from span diagnostics Ltd., Surat, India. All other chemicals were of analytical grade.

Collection of plant material and preparation of extracts

Leucas aspera was collected from kottayam, kerala. The whole plant was washed thoroughly and dried at room temperature in shade and authenticated. A voucher specimen (**SBSBRL.04**) is maintained in the institute. A 100gm of dried powder of the whole plant of *L. aspera* was soxhlet extracted with 500ml of methanol for 48 hr. The step was repeated with a new set of dried powder and solvent until the required quantity was achieved. The extract was concentrated in a rotary evaporator and yield recorded was 4.6%. It was suspended in 5% Tween80 and stored in refrigerator till further use.

Preliminary phytochemical studies

Various phytochemical investigation studies shows the presence of alkaloids, sterols, triterpenoids, flavonoids and saponins in MELA.

Invivo antioxidant studies

Animals and diets

Male wistar rats (150-200g) were used for the study. The animals were housed in well ventilated cages and given standard rat chow (Sai Feeds, Bangalore, India) and water adlibitum. The animals were maintained at a controlled condition of temperature of 26-28^{0}C with a 12 h light: 12 h dark cycle. Animal studies were followed according to Institute Animal Ethics Committee (IEAC) regulations approved by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) (Reg. No. B2442009/5) and conducted humanely.

Acute oral toxicity

Acute oral toxicity of MELA was determined using wistar albino rats. The overnight fasted rats were weighed and selected. Methanolic extract was dosed in a stepwise procedure, with the initial dose being selected as the dose expected to produce some signs of toxicity and were observed for a period of two weeks. LD_{50} values of the methanolic extract was (1000mg/kg,po), hence 100mg/kg was considered as effective for the hepatoprotective activity. Selected doses of MELA were 100mg/kg and 200mg/kg.

Experimental design

In the post-treatment, animals were divided into five groups with six rats in each group. Group1 was normal control.Group11 to V animals were administered orally with a single dose of CCl_4 (150µl/100g of 1:1 mixture in ground nut oil) on day 1. Groups 111 to V received *Silymarin* (50mg/kg) and MELA at doses of 100 and 200mg/kg respectively 2, 24 & 48 hrs after CCl_4 administraion.

Biological assay

Serum enzyme analysis

Blood was collected from the neck blood vessels under mild ether anaesthesia and kept for 30min at 4^0C.Serum was separated by centrifugation at 2500rpm at 4^0C for 15min. The liver was removed rapidly and cut into separate portions for hepatic glutathione, antioxidant enzymes, lipid peroxide estimation and histopathological studies. AST (E.C 2.6.1.1), ALT (E.C 2.6.1.2) and ALP (E.C 3.1.3.1) levels were assayed by using span diagnostic kits by kinetic method using semi autoanalyzer (RMS, India). Antioxidants and lipid peroxidation levels were measured photometrically.

Tissue analysis

Liver was excised, washed thoroughly in ice-cold saline to remove the blood stain. Ten percent of liver homogenate was prepared in 0.1 M tris HCl buffer (pH-7.4). The homogenate was centrifuged at 3000 rpm for 20 min at 4^oC and the supernatant was used for the estimation of Superoxide Dismutase (SOD), catalase (CAT), glutathione peroxidise (GPx), glutathione –S-transferase (GST), reduced glutathione (GSH) and thiobarbituric acid reactive substances (TBARS).

Reduced glutathione was determined according to Moron etal [10] method using Ellmann's reagent. The procedure is based on the reduction of Ellmann's reagent by SH groups to form 5,5´-dithiobis(2-nitrobenzoicacid),the intensity of yellow colour developed was measured spectrophotometrically at 412 nm using a spectrophotometer. Results were expressed as nmol GSH/mg protein. SOD(E.C 1.15.1.1) was assayed by following the method of Kakkar et al [11]. Activity of SOD was expressed as units/mg protein. The CAT (E.C 1.11.1.6) activity was determined from the rate of decomposition of H_2O_2 according to the method of Cohen etal [12]. A 0.3 ml of supernatant was added to 3ml of 0.067 M phosphate buffer (pH 7.0) and 0.75ml of H_2O_2. The rate of decomposition of H_2O_2 was measured spectrophotometrically at 240nm.Activity of catalase was expressed as U/mg of protein. GPx (E.C 1.11.1.9) activity was determined by measuring the decrease in GSH content after incubating the sample in the presence of H_2O_2 and NaN_3. Activity of GPx was determined by the method of Rotruck etal [13] and was expressed as µmoles of GSH oxidized/min/mg protein. GST(E.C 2.5.1.18) activity was determined from the rate of increase in conjugate formation between reduced glutathione and CDNB by Beutler [14].

Lipid peroxidation levels in liver homogenate was estimated by Nichan's and Samuelson method [15].In brief, the amount of malondialdehyde(MDA) was measured in terms of thiobarbituric acid reactive substances(TBARS),which is undertaken as an index of lipid peroxidation using 1'1'3'3'tetramethoxypropane as standard [16]. Results were expressed as nmol MDA/mg protein for TBARS. Protein content in the tissue was determined using bovine serum albumin (BSA) as standard [17].

Histopathological studies

Small pieces of liver, fixed in 10% buffered formalin were processed for embedding in paraffin [18]. Sections of 5-6µm were cut and stained with hematoxylin and eosin and examined for histopathological changes under the microscope (Motic AE 21, Germany). The micrographs were taken using Moticam 1000 camera at original magnification of 100X.

Liver sections were graded numerically to assess the degree of biological features in acute hepatic injury. Centrilobular necrosis, characterised by the damage of several liver cells around the central vein, fatty infiltration, prominent ballooning etc. and bridging hepatic necrosis, a form of confluent necrosis of liver cells linking central veins to portal tracts or portal tracts to one another were prominent in histological findings [19].

A combined score of centrilobular necrosis & bridging hepatic necrosis was given a maximum value of 6. Descriptive modifiers such as mild, moderate & severe was appilied to activity and staging. The parameters were graded from score 0 to 6, with 0 indicating no abnormality, 1 to 2 indicating mild injury, 3 to 4 indicating moderate injury and 5 to 6 with severe liver injury.

Statistical analysis

Results were expressed as mean ± S.D and all statistical comparisons were made by means of one way ANOVA test followed by Tukey's post hoc analysis and P values less than or equal to 0.05 were considered significant

RESULTS

Serum enzyme analysis

In the post-treatment groups, rats administered with CCl_4 showed an elevated level of serum AST, ALT, ALP and lipid peroxides. Treatment with the extract of *L.aspera* at doses of 100 and 200mg/kg significantly ($p \leq 0.05$) protected the rats from CCl_4 induced hepatotoxicity. Standard drug *Silymarin* also showed a remarkable protection towards CCl_4 administration. Treatment with 100mg and 200mg/kg of MELA and *Silymarin* (50mg/kg) exhibited a protection of 96.1%, 98.8% and 97.1% on AST levels; 96.5%, 98.5% and 96.9% in ALT levels and 94.5%, 96.9% and 95% ALP levels respectively (Fig 1).

Tissue analysis

Estimation of reduced (GSH), GST, GPx, SOD and Catalase

In the post–treatment groups, rats administered with CCl_4 alone were found significantly ($p^* \leq 0.05$) lowered the hepatic GSH, GST, GPx, SOD and Catalase levels. Various doses of MELA and *Silymarin* treated rats completely prevented the lowering of tissue antioxidant levels (Table 1).

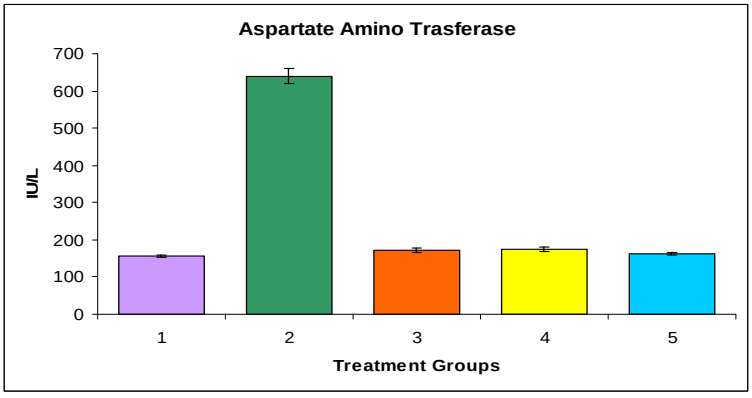


Fig. 1a: Aspartate aminotransferase levels

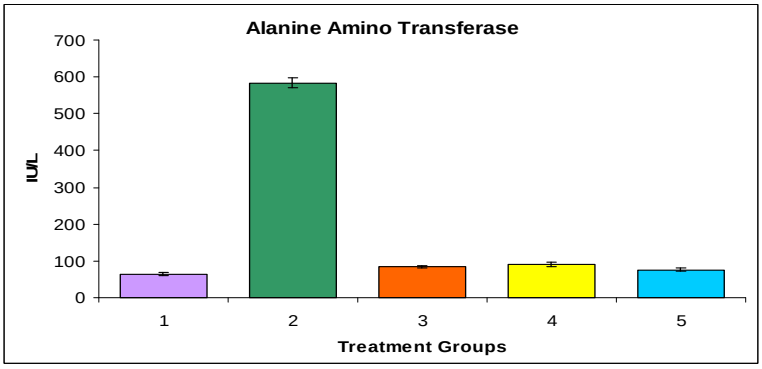


Fig. 1b: Alanine aminotransferase levels

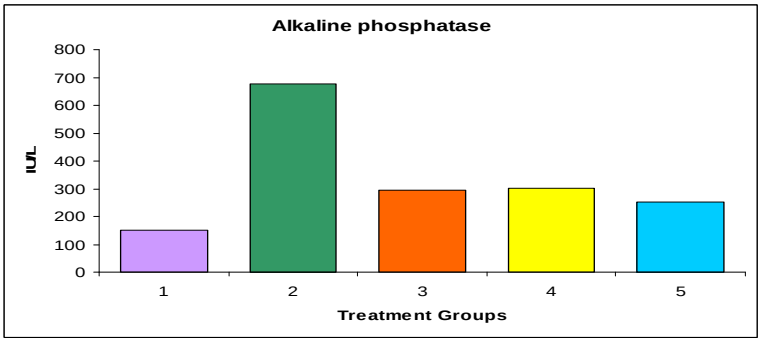


Fig. 1c: Alkaline phosphatase level

Fig. 1: Effects of MELA and Silymarin treatment on serum marker enzyme levels

1. Normal control 2. CCl_4 Control (150 µl/100g 3. Silymarin (50mg/kg) +CCl_4 4. MELA (100mg/kg) +CCl_4 5. MELA (200mg/kg) +CCl_4.

Estimation of lipid peroxides

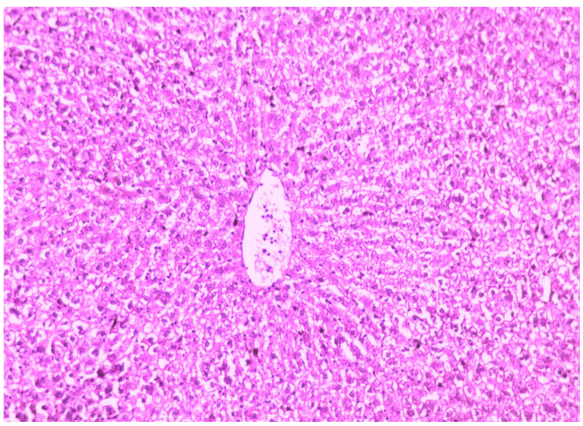
A significant increase ($p^*\leq 0.05$) in tissue lipid peroxide level (TBARS) was observed in CCl_4 alone treated rats. However CCl_4 induced elevation of TBARS concentration were lowered significantly ($p^*\leq 0,05$) by the post-treatment of the rats with the extract. In MELA treated rats, TBARS levels were remarkably reduced which was comparable to Silymarin (Table 1).

Table 1: Effect of MELA and Silymarin on TBARS and antioxidant enzyme levels

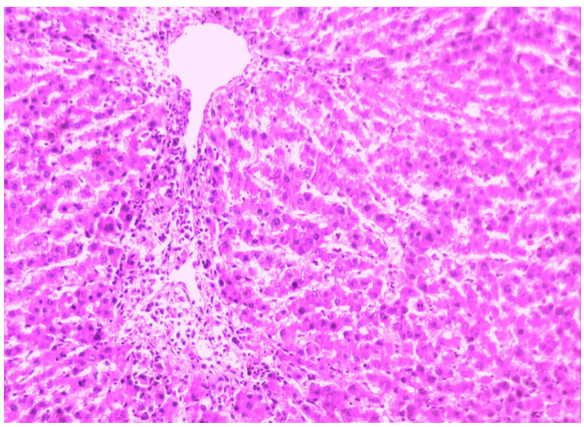
Treatment groups	TBARS	GSH	SOD	CAT	GPx	GST
Normal control	2.01±1.61	2.33± 0.29	166.2 ±10.1	33.26 ±3.9	39.23 ±2.01	0.49 ±0.03
CCl_4Control (150 µl/100g)	15.95 ±2.12#	0.70 ±0.05#	92.51±8.5#	20.1± 2.85#	17.42± 3.05#	0.17 ±0.02#
Silymarin (50mg/kg+CCl_4)	2.82± 2.3*	2.22 ±0.28*	135.1 ±8.2*	27.72 ±3.2*	31.19 ±3.31*	0.35 ±0.02*
L.aspera (100mg/kg+CCl_4)	3.14 ±1.89*	2.02± 0.21*	125.5 ±7.9*	24.26 ±3.1*	29.51 ±2.82*	0.31 ±0.03*
L.aspera (200mg/kg+CCl_4)	2.33 ±1.82*	2.29± 0.30*	160.9 ±9.3*	31.31 ±3.4*	36.59 ±2.21*	0.46 ±0.02*

Values are mean ± S.D., n = 6 #P*≤ 0.05 Vs normal control ; P*≤ 0.05 Vs CCl_4 control.

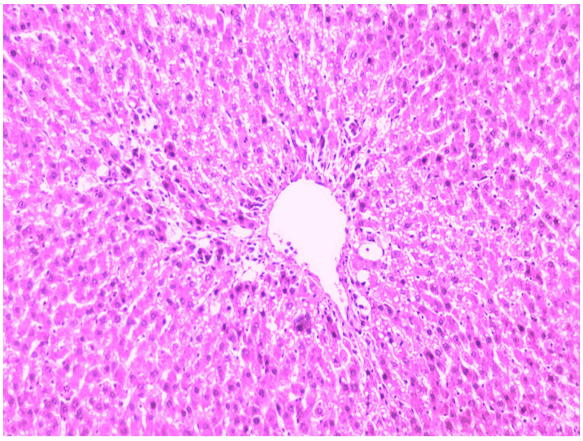
TBARS: n moles of MDA/gm tissue; GSH: nmole/mg protein; SOD: Units/min/mg protein; CAT: µ moles of H_2O_2 consumed/min/mg protein; GPX: Moles of GSH oxidized/min/mg protein; GST: µ molesof CDNB-GSH conjugate formed/min/mg protein.



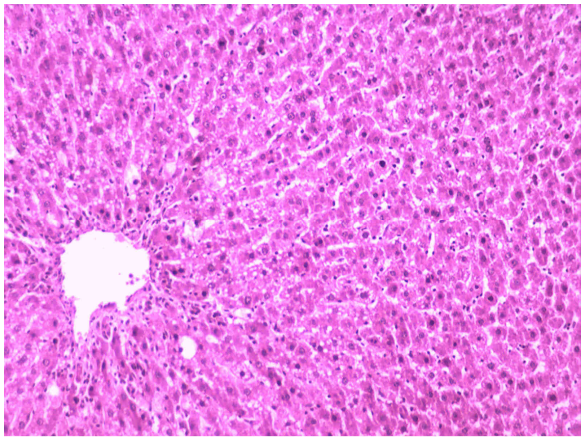
(A)Normal control



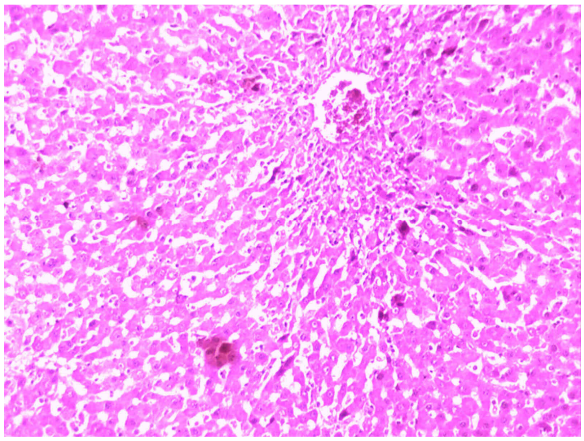
(B) CCl_4 control



(C) CCl_4+Silymarin(50mg/kg)



(D) CCl_4+MELA(100mg/kg)



(E) CCl_4+MELA(200mg/kg).

Fig. 2: Histopathological changes in the liver of control and treated animals (H&E x100)

Histopathological analysis

In rats treated with carbontetra chloride, the normal architecture of liver (figure 2B) was completely lost with the appearance of centrilobular necrosis and bridging hepatic necrosis scoring 5.4±0.4 (mean ±S.D ; n=3) (fig 2). The post-treatment animals administered with plant extract at 100 and 200 mg/kg and Silymarin (50mg/kg) showed a significant ($p^*\leq 0.05$) protection from CCl_4 induced liver damage as evident from hepatic architectural pattern with mild to moderate hepatitis with scores 3.3 ± 0.4; 2.7±0.65; 3.2±0.6 (mean ± S.D ; n=3; $p^*\leq 0.05$) respectively (figure 2 C -2E).

DISCUSSION

CCl_4 has been widely used in animal models to investigate chemical toxin–induced liver damage. The most remarkable pathological characteristics of CCl_4 -induced hepatotoxicity are fatty liver, cirrhosis and necrosis, which have been thought to result from the formation of reactive intermediates such as trichloromethyl free radicals (CCl_3^*) metabolized by the mixed function Cytochrome p_{450} in the endoplasmic reticulum [20]. Usually the extent of hepatic damage is assessed by the increased levels of cytoplasmic enzymes(AST, ALT and ALP), thus leads to leakage of large quantities of enzymes into circulation. This was associated by massive centrilobular necrosis, ballooning degeneration and cellular infiltration of the liver [21].

Aminotranferases are present in high concentration in liver, an important class of enzymes linking carbohydrate and aminoacid metabolism. Alanine aminotransferase and aspartate aminotransferase are well known diagnostic indicators of liver disease. Alkaline phosphatase is a membrane bound enzyme and its elevations in plasma indicate membrane distruption in the organ. Alkaline phosphatases, although not a liver specific enzyme, liver is the major source of this enzyme.Measurement of these enzyme levels has proved to be powerful tools in the assessment of hepatotoxicity [22].The results obtained clearly depict the extent of damage created by CCl_4 to the hepatic tissue.

Administration of plant extract significally prevented CCl_4 induced elevation of AST, ALT and ALP indicating the hepatoprotective activity of this herb. In a dose response study, it was found that the extract at a dose of 200mg/kg could completely protect the liver from toxic injury.*L.aspera* extract at a dose of 100mg/kg was comparable to standard control drug, *Silymarin* which also showed better protection towards CCl_4 induced liver damage. The methanolic extract significantly reduced the elevation of lipid peroxide levels which was comparable to *Silymarin*. This extract also prevented the decrease of hepatic GSH, GST, GPx, SOD and Catalase in CCl_4 induced rats and retained the levels to standard control rats, indicating the antioxidant activity of the extract. Antioxidant enzymes represent one protection against oxidative damage [23].

SOD converted O_2 into H_2O_2 and catalase metabolize H_2O_2 to non-toxic products. The GSH antioxidant system plays a fundamental role in cellular defense against reactive free radicals and other oxidant species. This system consists of GSH and an array of functionally related enzymes, of which GPx and GST work together with GSH in the decomposition of H_2O_2 and other organic hydroperoxides. Hepatic GSH, GST, GPx, SOD, Catalase and TBARS formation were completely restored to normal values by the treatment with the extract and *Silymarin*. Antioxidant property is claimed to be one of the mechanisms of hepatoprotective drugs.

The histological changes induced by CCl_4 treatment as evidenced by centrilobular necrosis and bridging hepatic necrosis and its protection to normalcy by the treatment with the plant extract was indicative of the hepatoprotection of the extract.Histopathological

evaluation showed negligible damage to a few hepatocytes present in the close vicinity of central vein in extract treated rats and the improvement of histological scores proved the efficacy of the extract as an antihepatotoxic agent.

Phytochemical screening and antioxidant activity of different extracts of *Leucas aspera* was studied by Das et al [24]. The results revealed the presence of significant amount of alkaloids, glycosides,tannins and flavonoids in ethanol extract. The protective activity of *Leucas aspera* in carbon tetrachloride(CCl_4) induced acute hepatotoxicity in rats were studied by K Mangathayaru et al [25]; Shirish.S. Pingala [26] and Radhika & Brindha [27]. The result of serum biochemical parameters, level of hepatic lipid peroxides, hepatic glutathione, antioxidant enzymes and histopathological studies in the post-treatment groups support the highly potent hepatoprotective and antioxidant activity of MELA and also supports the tribal/folk use of *L.aspera* for the treatment of hepatic disorders. The identified class of compounds in single or in combination with other components present in the extract might be responsible for the hepatoprotection in the curative groups.

CONCLUSION

The present study validates the hepatoprotective and antioxidant potential of l.aspera in curative model.Histological studies supported the data. From this it is concluded that L.aspera could be used for the development of phytomedicines against hepatic disorders. The hepatoprotective nature could be attributed to the presrnce of β-sitosterol,alkaloids and flavonoids in it.

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