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Antioxidant Activity and Hepatoprotective Potential of *Artemisia aucheri* in Rat

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Abstract

Background/Objectives: Medicinal plants play an important role in human health. A large number of plants have claimed for hepatoprotective potential. The aim of this study was to assay the hepatoprotective potential of hydro alcoholic extract of *Artemisia aucheri* on Carbon tetrachloride induced hepatotoxicity in rats. **Materials and methods:** Hydro alcoholic extract of *Artemisia aucheri* at dose of 150, 300 and 600 mg/kg administered on rats for 28 days. The hepatoprotective effect was assessed by serum biochemical tests such as alaine aminotransferase, aspartate aminotransferase, alkaline phosphatase, total bilirubin, total protein and albumin. Antioxidant enzymes (superoxide dismutase and catalase), malondialdehyde, F2α-isoprostanes and reduced glutathione were determined along with histopathological studies on liver tissue. **Results:** Serum levels of total protein, albumin, superoxide dismutase, catalase activities and reduced glutathione were decreased in carbon tetrachloride (toxin) group. However alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, total bilirubin, malondialdehyde, F2α-isoprostanes were increased. In treatment groups all biochemical parameters restored toward normal by use of hydroalcoholic extract of *A. aucheri*. **Conclusion:** Hydro alcoholic extract of *Artemisia aucheri* displayed protective potential against carbon tetrachloride induced hepatotoxicity which demonstrated by biochemical tests in tissue homogenate and serum. Also histopathological studies in liver tissue.

Keywords: Alanin Amino Transferase, Antioxidant Activity, Aransferase Artemisia Aucheri, Carbon Tetrachloride, F2α-isoprostanes, Hepatoprotective

1. Introduction

Liver is the largest body organ which regulates a wide variety of biochemical reactions, biosynthesis, hormones regulation and catabolism of biological compounds[1]. Hepatitis is one of the most liver-related diseases with high mortality and morbidity in developing countries despite of much progress in hepatology field. There are some major risk factors for hepatitis such as alcoholism, virus-induced hepatitis, using liver toxic medicines specially antibiotics, acetaminophen and carbon tetrachloride[2]. Today, in new medicine, medicinal plants are highly interested for treatment of liver diseases due to; 1) the absence of protective drugs for treat of hepatic disorders, 2) a small number of reliable hepatoprotective drugs from plant sources are available in practice[3]. Thus, people show more interest in using medicinal plants with antioxidant activity for treat of hepatic disease.

Reactive Oxygen Species (ROS) are highly reactive molecules that are generated during normal metabolic process and cause an imbalance of pro-oxidants and antioxidants in the organism can cause oxidative stress and result in different disease such as liver, heart and brain tissue damage and cell death[4].

Antioxidants such as ascorbic acid, alpha-tocopherol, Superoxide Dismutase (SOD), catalase (CAT) and peroxidases can reduce pathological conditions and oxidative stress[4].

Antioxidants are found in medicinal plants, vegetables, fruits, grain cereals and nuts. It has been shown

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that some types of plants may lower risk against some disease (e.g. cancer) which act in neutralizing free radicals[5]. The medicinal values of plants assumed more important because they contain minerals, primary metabolites and diverse array of secondary metabolites with antioxidant potential and therapeutic effects. So, they are now preferred to the synthetic ones because of safety concerns[6,7]. Recently, widespread screening of plants for possible antioxidant properties and the isolation of antioxidants from natural origin was carried out for hepatoprotective potential[1,8].

Artemisia genus belongs to the Compositae family. These herbs grow in temperate climates usually in dry or semiarid habitats (e.g. Iran) and have wide range of traditional use in different diseases (e.g. malaria, hepatitis, cancer, diabet, stomach ulcer[9,10]).

Artemisia aucheri possesses anti-inflammatory, anti-fungi, anti-bacteria, anti-parasite and anti-viral, antifebrile and anti-cough properties. Many species have been used since ancient times as folk remedies for some treatment purposes such as hepatitis. A number of Iranian Artemisia species such as *A. aucheri* contain different flavonoids compound such as quercetin and retinoid and some other phytochemical including monoterpenes and sesquiterpene with potent antioxidant potential[9,10]. To date, no study have been carried out on the effects of *A. aucheri* on hepatotoxicity that induced by carbon tetrachloride (CCl_4), therefore, the main purpose of present study was to investigate the effect of hydroalcoholic extract of *A. aucheri* (HEAA) on CCl_4-induced hepatotoxicity in male wistar rats. CCl_4 was used to induce hepatotoxicity in a rat model.

2. Materials and Methods

2.1 Collection of Plant Materials and Preparation of Extract

A. aucheri was collected on the August and September 2011, province of Fars, Iran. The plant was identified by the taxonomist and voucher specimen has been deposited in clinical biochemistry department at the herbarium No. MPRC 025. The aerial parts of plant was shade dried at 25–30 °C. Hydro-alcoholic (Ethanol 70% V/V) extract of the dried material was prepared by Soxhlet method for 4 h[11]. The extract was collected and dried by rotary evaporator (Hyedolph, type: Heizbad Hei-VAP, Germany) and were kept in a refrigerator.

2.2 Animals

Thirty-six male wistar rats weighting 180–200g during the study under condition of controlled temperature (25±2 °C), illumination (12 h light cycle starting at 06:00 am) were fed on laboratory chow diet and tap water freely.

2.3 Experimental Design

LD50 of *A. aucheri* was determined in a primarily study by a so called 'staircase method'. Five doses were then chosen for the determination of oral LD50 in five groups of rats (3 in each group). The animals were observed for first 2 hours and then at 6th and 24th hour for any toxic symptoms. After 24 hours, the number of dead rats was counted and LD50 was estimated[12].

The rats were divided into six groups of six each as shown below.

Group I – Normal Control (distilled water 1ml/kg, p.o).

Group II – CCl_4 (toxin) group (1 ml/kg diluted with olive oil, i.p.).

Group III – HEAA Control (300mg/kg, p.o).

Group IV – treatment 1: CCl_4 (1 ml/kg, i.p.)+HEAA (150mg/kg, p.o).

Group V – treatment 2: CCl_4 (1 ml/kg, i.p.)+HEAA (300mg/kg, p.o).

GroupVI–treatment 3: CCl_4 (1 ml/kg, i.p.)+HEAA (600mg/kg, p.o).

All groups except normal and toxin groups were treated with HEAA once daily for the period of 28 days. After the administration of the last dose, rats were fasted for 12 hr. animals then sacrificed under ether anesthesia. Blood was collected by heart puncture for biochemical parameters assay. A small portion of liver tissue from each animal was fixed in formalin solution for histopathological study.

2.4 Biochemical and Histopathological Studies

Activity of AST, ALT, ALP in serum and total protein, albumin and total billirubin levels were determined by commercial kits for rats using an automated analyzer (RA1000-USA).

2.4.1 Catalase Activity Assay Protocol

For determination of catalase activity Cayman's catalase assay kit utilized[13,14].

2.4.2 SOD Activity Assay Protocol

The method of Misra and Fridovish was used for the determination of SOD activity[15].

2.4.3 MDA and F2α-isoprostanes Assays

These assays are based on the reaction of a chromogenic reagent. In order to assessment of liver tissue F2α-isoprostanes. Tissue samples were manually homogenate by a homogenizer (ULTRA-TURRAX-model: T18B, made in Germany).

2.4.3.1 Preparation of Liver Tissue Homogenate

One ml of homogenization buffer (0.1 M phosphate buffer, pH 7.4 containing 1mM EDTA and 0.005% BHT) per 100mg of tissue added. Then liver tissue samples homogenized. After homogenization, samples centrifuged at 8000rpm for ten minutes and supernatant transferred to a clean tube.

2.4.3.2 Liver Tissue F2α-isoprostane Assessment

Then for measurement of F2α-isoprostanes (IsoP) a Cayman's EIA kit was used[16,17].

2.4.3.3 Liver Tissue MDA Assessment

Malondialdehyde (MDA) level of liver tissue homogenate was determined by the method first described by Hoyland[18].

2.4.3.4 Preparation of Liver Homogenate

Liver tissues were homogenized with 10 times (w/v) ice – cold 0.1 M phosphate buffer pH 7.4. Then aliquot homogenates from rats liver were used to determine MDA.

2.4.3.5 Preparation of MDA Reagent

A - Make 100 ml Hcl 0.25 M
B - Weigh 0.375gr TBA and 15gr TCA
C - Mix A and B to each other

2.4.3.6 Assay Protocol

500µl of tissue supernatant was added to 2 ml of MDA reagent, and allowed this mixture to heat in a boiling water bath for 15 minutes. The tube containing this mixture was centrifuged at 1700rpm for 15 minutes. After cooling at room temperature, a pink colored chromophore was formed. The absorbance of the chromophore was measured at 532 nm. The concentration of MDA was expressed as micromole of MDA/gm liver tissue.

2.4.4 GSH Assay Protocol

Glutathione (GSH, g-glutamylcysteinylglycine), was measured by Ellman's reagent, 5,5'-dithio-bis-(2-nitrobenzoic acid) as also known as DTNB by a modification of the method first described by Tietze[19].

2.4.4.1 Assay Protocol

1. To measurement of GSH, it made 10mM DTNB stock solution by dissolving 40mg DTNB in 10ml DMSO.
2. Diluted the stock solution 100 fold with 0.1M Tris-HCl pH 7.5 to make 0.1mM DTNB working solution.
3. Aliquot 950µl of 0.1mM DTNB work solution to each 1.5ml centrifuge tube.
4. Add 50µl each tissue homogenate sample and mix by brief vortexing.
5. Set a blank by adding 50µl of 0.1M Tris-HCl pH 7.5 to 950µl of 0.1mM DTNB work solution.
6. Incubated 2 minutes at room temperature.
7. Measured the absorbance of the test sample with a spectrophotometer against blank at 412nm.
8. Calculated the concentration of free sulfhydryls in the sample from the molar extinction coefficient of NTB (14.15 mM-1 cm-1) as follow:

$$\text{mM free sulfhydryls} = \frac{\text{Absorbance}}{(\text{path length} \times 14.15) \times 20}$$

2.4.5 Histopathological Study

For histopathological studies, the liver tissue was dissected and the tissue samples fixed in formalin solution (10% V/V) and embed in paraffin with 4 mm thick. The slides were stained with hematoxylin and eosin for light microscopic examination.

2.5 Statistical Analysis

Values are expressed in Mean ± S.E.M for six animals in each group. P-value was calculated using ANOVA followed by tukey test for multiple comparisons using IBM SPSS statistics ver.21. Values of $P < 0.05$ were considered significant.

3. Results

In oral acute toxicity test did not show any mortalities in the surviving rats even at the highest dose 5000 mg/kg body weight after 3 days. Therefore, LD50 values of HEAA were calculated more than 5000 mg/kg in male wistar rat follow an oral gavages. Intra peritoneal injection of CCl_4 increased serum levels of ALT, AST, ALP, T-Bill and tissue MDA and 8-isprostane in all groups except negative control.

HEAA administration in highest concentration (600mg/kg) has been significantly restored mentioned parameters toward normal ($p<0.05$) (Table 1 and 2). CCl_4 (toxin) group displayed significant decrease ($p<0.05$) in serum SOD and CAT activities (Table 1).

Simultaneous administration of CCl_4 and HEAA at concentration of 600 mg/kg significantly increased level of SOD (5.19±0.1U/mg protein), CAT (65.15±1.9 U/mg protein), TP (5.04±0.16gm/dl) and Alb (50.16±1.36gm/l) in serum respectively ($p<0.05$), (Table 1 and 2).

Administration of HEAA at highest concentration was caused a significant decrease in AST (148±2.5IU/L), ALT (212±1.19IU/L), ALP (618±5.4 IU/L) activities and T-Bill (0.57±0.01 mg/dl) and MDA (20.8±1.1nmol/ml), 8-isprostane (4.02±0.52 pg/ml) and GSH (0.39±0.09 mm free sulfhydryls/ml) levels in treatment groups compared to toxin group (Table 1 and 2). There was no significant difference between plant extract and negative control.

3.1 Histopathological Studies

Liver architecture in negative control was normal pattern with central vein and sinusoids (Figure 1). However in CCl_4 group, focal necrosis with fatty cells, congestion, centrilobular swelling and hepatocyte vacuolation was reported (Figure 2).

In treat 3 with highest dose of HEAA extracts, the most of pathological changes in toxic group were normalized when compare to negative control (Figure 3).

4. Discussion

Oxidative stress is characterized by an imbalance between increased exposure to free radicals, principally derived from oxygen, and antioxidant defenses, comprised of both small molecular weight antioxidants, such as glutathione, and antioxidant enzymes, such as superoxide dismutase and catalase.

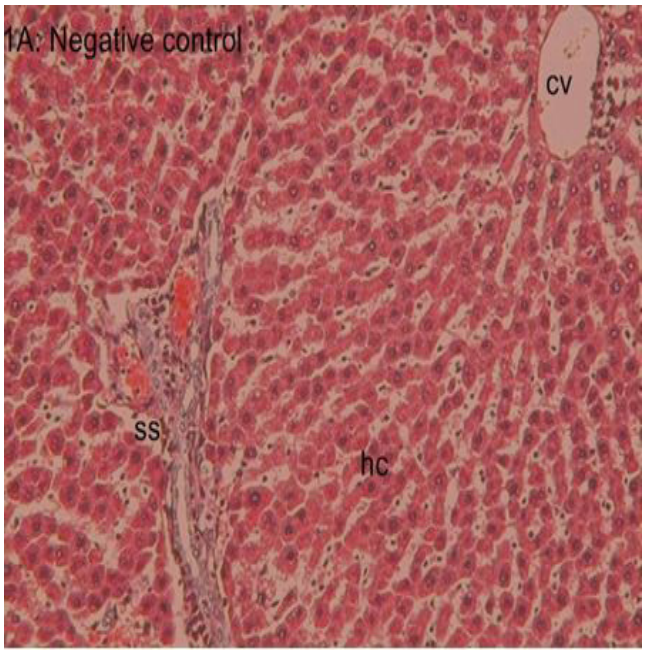

Figure 1. Normal liver tissue was shown by histopathological study in negative control group, (HE, ×50) cv: central vein; hc: hepatocyte; ss: sinusoidal space; vc: vacuole.

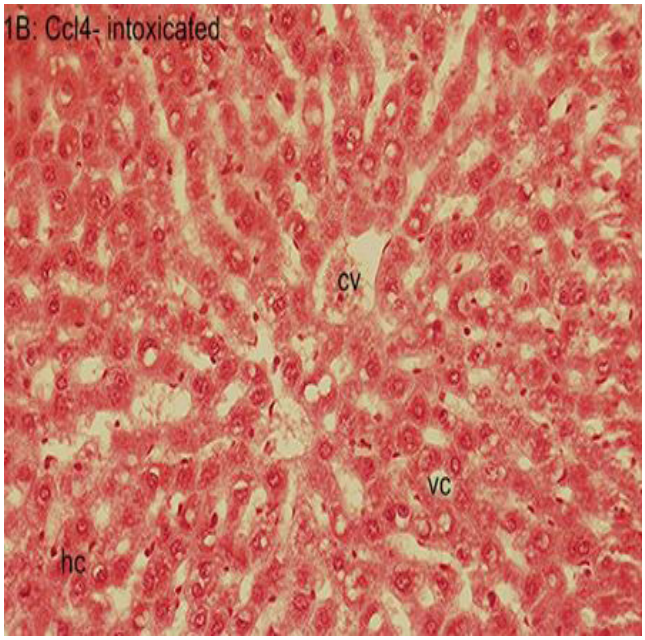

Figure 2. Hepatocyte necrosis and vacuolization of periportal vein are observed in CCl4- intoxicated liver and congestion of the sinusoids and central vein were reported (HE, ×50); cv: central vein; hc: hepatocyte; ss: sinusoidal space; vc: vacuole.

Table 1. Effects of HEAA on ALT, AST, ALP, TP, Alb and T-Bill in CCl4-induced hepatotoxicity in male rats

Group	BIOCHEMICAL PARAMETER					
	AST(IU/L)	ALT(IU/L)	ALP(IU/L)	TP(gm/dl)	Alb(gm/dl)	TBill(mg/dl)
I	†126.6±10.92	75.0±12.41	533.8±19.67	9.02±0.22	3.81±0.55	0.85±0.04
II	†384.6±89.77	†435.8±67.7	†1045.3±65.42	†6.96±0.17	3.33±0.15	1.07±0.01
III	121.5±3.91	60.16±7.89	523.1±56.74	8.90±0.38	3.46±0.15	0.86±0.01
IV	406.5±55.98	331.0±51.31	1058.8±180	7.80±0.28	3.53±0.25	1.05±0.07
V	445.7±74.82	304.0±49.72	1012.0±104.80	7.87±0.31	3.55±0.29	1.05±0.02
VI	†148.4±14.01	†235.0±44.08	†618.2±36.17	†8.26±0.11	3.74±0.05	†0.89±0.01

Values are expressed as Mean±S.E.M; n = 6 in each group; †significant at p<0.05 compare to toxin group. Group I; Negative control. Group II; CCl4 (toxin) group. Group III; HEAA control. Group IV; treatment 1. Group V; treatment 2. Group VI; treatment 3. HEAA: hydroalcoholic extract of A. aucheri. ALP: alkaline phosphatase. AST: aspartate aminotransferase. ALT: alanine aminotransferase. Tp: total protein. Alb: albumin. T-Bill: total billirubin.

Table 2. Effects of HEAA on serum SOD, CAT and liver tissue GSH, MDA and IsoP in CCl4-induced hepatotoxicity

Group	BIOCHEMICAL PARAMETER				
	SOD(U/mg)	CAT(U/mg)	GSH(mM free sulfhydryls/ml)	MDA(µmol/ml)	IsoP(pg/ml)
I	6 ±0.1	1.2±70.05	0.5±0.11	3.51±0.15	2.02±0.3
II	0.1±23.2†	1.8±43.21†	†0.1±0.008	†8.82±0.25	†10.5±2.2
III	0.2±5.2	2.3±69.28	0.4±0.14	2.21±0.2	3.31±0.25
IV	0.5±3.28	1.4.12 ±05	0.23±0.03	5.5±0.85	7.52±0.36
V	0.4±4.6	1.3±47.50	0.22±0.05	6.75±0.58	6.52±0.66
VI	0.1±5.19†	1.9.08 ± 65 †	†0.39±0.09	†4.1±0.22	†4.02±0.52

Values are expressed as Mean±S.E.M; n = 6 in each group; †significant at p<0.05 compare to toxin group. Group I; Negative control. Group II; CCl4 (toxin) group. Group III; HEAA control. Group IV; treatment 1. Group V; treatment 2. Group VI; treatment 3. HEAA: hydroalcoholic extract of Artemisia aucheri. MDA: malondialdehyde. GSH: g-glutamylcysteinylglycine. CAT: catalase. SOD: superoxide dismutase. Isop: 8-isneoprostane.

Free radicals can be generated endogenously or derived from exogenous sources such as CCl_4. Free radicals cause direct damage to critical biomolecules including DNA, lipids, and proteins. Oxidative stress is now recognized to be a prominent feature of many acute and chronic diseases including cancer, cardiovascular disease and etc[20].

Lipid peroxidation is a well-established mechanism of cellular injury in both plants and animals, and is used as an indicator of oxidative stress in cells and tissues.

Polyunsaturated fatty acid peroxides generate malondialdehyde (MDA) and 8-isoprostanes upon decomposition. Measurement of malondialdehyde and isoprostanes has been used as an indicator of lipid peroxidation[20].

Exposure to high concentrations of carbon tetrachloride can affect the central nervous system, degenerate the liver, kidney disease and cancer[21].

Lipid-solubility of CCl_4 allows it to cross membranes and to be distributed to all organs. The cytochromes P450

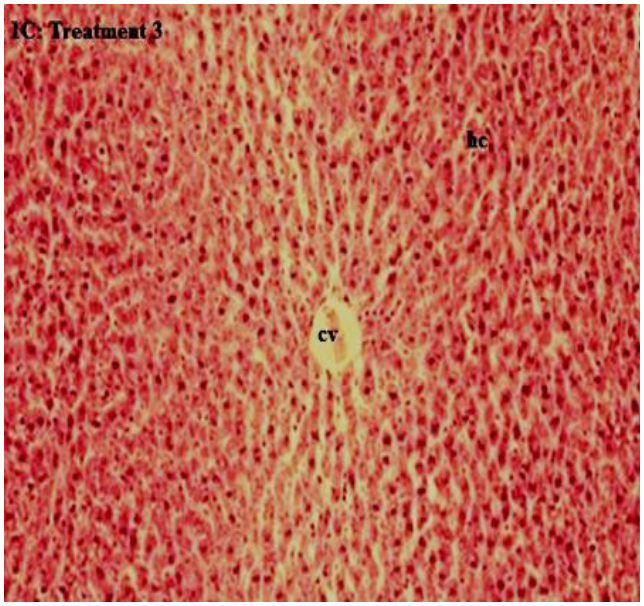

Figure 3. In treatment group with highest dose (600mg/ml), minimal congestion, vacuolization and tissue injury on hepatocytes were demonstrated (HE, ×50); cv: central vein; hc: hepatocyte; ss: sinusoidal space; vc: vacuole.

(CYP) metabolize CCl_4 to trichloromethyl radicals that can cause lipid peroxidation and cell injuries. Several Antioxidants are intimately involved in the prevention of cellular damage by scavenging free radicals. Therefore prevents CCl_4-induced lipid peroxidation process[22].

Hepatic enzymes following CCl_4 exposure either alone or in combination with HEAA indicated a significant change in the peroxidative process. Animal cells contain endogenous antioxidant enzymes; including superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) which are repelled the harmful effects of oxidative stress[23].

Superoxide, the one–electron reduced form of molecular oxygen, is a precursor of other ROS such as hydrogen peroxide, hydroxyl radical, and singlet oxygen that have the potential of reacting with biological macromolecules and thereby inducing tissue damages[24]. So Super Oxide Dismutase (SOD) is an antioxidant enzyme which protects the cell and tissues from injuries it cause decreased superoxide radicals via conversion to hydrogen peroxide. Superoxide scavenging property of plant extract might be due to the existence of secondary metabolites such as total phenol and flavonoid compounds[25,26]. In the present study, CCl_4 intra peritoneal injection (i.p) made significant damage on liver tissue, which is evidenced by significant changes in enzymes activities such as AST, ALT and ALP. Levels of SOD and CAT enzymes were decreased by CCl_4, thus transforming the tissue to the peroxidative damage.

Increased in AST, ALT, ALP, MDA and 8-isoprostanes accompanied by a significant reduction in SOD and CAT in serum and GSH in liver tissue homogenate. Assessment of these markers revealed the liver damage was occurred. Administration of the HEAA (600 mg/kg) extract caused decrease in CCl_4–induced oxidative stress that indicating its protective role in liver tissue. It was supported by significant decrease toward normal range in serum levels of AST, ALT, ALP, total protein, albumin and total billirobin, also increase in serum enzyme activities of SOD and CAT.

Normalization of the enzymes activity follows administration of HEAA supporting the antioxidant and hepatoprotective role of plant extracts in present study. Malondialdehyde and isoprostanes are markers of lipid peroxidation. The defence of antioxidant enzymes with free radicals results in the depletion of the antioxidants and formation of lipid peroxidation that evident in elevation of MDA and 8-isoprostane levels. CCl_4 significantly increased serum MDA and 8-isoprostane levels into the normal control group ($p<0.05$). HEAA (600 mg/kg) significantly diminished CCl_4-elevated MDA and 8-isoprostanes content ($p<0.05$).

HEAA at dose (600mg/kg) produced protective effects in liver against CCl_4 toxicity. Co-administration of HEAA and CCl_4 preserved hepatic architecture and decrease the fibrosis, congestion, hepatocyte swelling, hepatocyte vacuolation and fatty changes.

Similar to present study, hepato-protective and antioxidant potential of artemisia due to free radicals scavenger property was reported in literature[10,27].

According to results of some study, quercetin, flavonoids and sesquiterpenes with antioxidant activity neutralize free radicals in different disorders such as liver diseases and also inhibits lipid peroxidation[28]. Parallel to recent study antioxidant activity and lipid peroxidation inhibition of HEAA was reported by researchers[29,30]. The hepatoprotective potential of HEAA is may be due to the presence of major phytochemical compounds.

5. Conclusion

Administration of HEAA in CCl_4-induced hepatotoxicity in male wistar rats had hepatoprotective potential.

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