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Research Article

IN VIVO HEPATOPROTECTIVE ACTIVITY OF *CASSIA AURICULATA* POLYMER NANOSPHERES CONTAINING SILYMARIN

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ABSTRACT

Objective: Hepatoprotective activity of herbal drugs has an importance in the treatment of hepatic disorders, but pharmaceutically engineered products are not evolved properly to cure the liver disorders properly based on the demand and also route, target the appropriate site of action.

Methods: In our present work, we have been developed a new formulation that possesses a unique nature and site specificity for targeting the disease state. Here, we examined hepatoprotective activity of *Cassia auriculata* polymer nanospheres containing silymarin against carbon tetrachloride-induced hepatotoxicity in rats using at 50 mg/kg and 100 mg/kg body weight dose levels, and we have been observed that enzyme activities of serum glutamate oxaloacetate transaminase, serum glutamate pyruvate transaminase, alkaline phosphate, total protein, albumin, globulin, total cholesterol, high-density lipoprotein (HDL), glutathione (GSH), and total bilirubin were analyzed.

Results: *C. auriculata* polymer nanospheres and silymarin produced significant ($p<0.001$) hepatoprotective effect by decreasing the activity of serum enzymes, bilirubin, total cholesterol, and increased levels of HDL, total protein, albumin, globulin, and tissue GSH.

Conclusion: From these results, it was concluded that of *C. auriculata* polymer nanospheres would protect the liver cells from carbon tetrachloride from liver diseases.

Keywords: Hepatoprotective activity, Silymarin, *Cassia auriculata*, Nanospheres.

INTRODUCTION

The Greek word, hepar is evolved and it starts as hepato or hepatic in pharmacy or medical terms, which means liver. The liver plays a pivotal role in metabolism, storage, and secretion of enzymes and also stored food[1]. It is referred as great synthetic/chemical factory of the body, which regulates, synthesizes, store, and secret many important enzymes, proteins, nutrients, and it can metabolize the consumed products either form food, chemicals, or pharmaceutical dosage forms, which enter into the liver via systemic circulation and it will eliminate for the body as toxic wastes. The bile produced and secreted form the liver plays very important role in the digestion of food and also fatty acids into cholesterol. The risk of the liver intoxication has recently increased by the higher exposure to environmental toxins, pesticides, and the frequent dosage of pharmaceutical products of a variety of classes[2].

Phytochemical and pharmacological investigation have been done extensively and well established. Phytomedicines show impressive *in-vitro* activity but less *in-vivo* efficacy due to their poor water solubility, lipophilicity, and inappropriate molecular size resulting in poor absorption and hence poor systemic availability. A better understanding of the biopharmaceutics and pharmacokinetics of phytomedicine can also help in designing rational dosage regimens [3].

Nanotechnology is on the threshold of providing a host of new materials and approaches in revolutionizing the medical and pharmaceutical field. Several areas of medical care are already profiting from the advantage of nanotechnology [4].

The use of nanotechnology for treatment, identification, monitoring, and managing biological systems have recently been referred to as nanomedicine. In the herbal formulation research, incorporating the nano-based formulation has a great number of advantages for phytomedicine, including improvement of solubility and bioavailability, safeguard from toxicity, enhancement of pharmacological activity, improvement of stability, increase in tissue macrophages distribution, sustained delivery, and protection from physical and chemical degradation [5].

Aim and objective

Our objective of the study is to investigate the hepatoprotective activity of *Cassia auriculata* polymer nanospheres against carbon tetrachloride induced hepatotoxicity in Wistar rats.

METHODS

Carbon tetrachloride (fine chemicals) and silymarin (Sigma - Aldrich) were used in our study. All other chemicals and reagents used were of analytical grade.

Collection of plant material

The seeds of the plant were collected from the foothill of Tirupati, Andhra Pradesh, in the month of March 2013. The collected plant was identified and authenticated by a botanist. The seeds were shade dried at room temperature for 10 days and coarse powdered and passed through sieve No. 60.

Isolation of mucilage for aqueous extract

The mucoadhesive from *C. auriculata* Linn was prepared by the following method.

Extraction and isolation involves seeds were size reduced to coarse powder. 100 g of this coarse powder was taken in 1000 mL beaker with water of 500 mL and boiled for 45 minutes-1 hr and filter using muslin cloth to obtain gum mucilage. This mucilage was diluted with 500 mL of water to obtain polymerization and finally was mixed with 500 mL of ethanol. The precipitates again dissolved in water, filtered to remove any debris and recrystallized using ethanol. The precipitate obtained after filtration were dried in hot air oven at 45°C for 12 hrs and size reduced to get powdered gum of #80 sieve fraction.

Preparation of nanospheres using *C. auriculata* polymer

C. auriculata polymer nanospheres encapsulated with silymarin, which is a hepatoprotective drug, were prepared by emulsion polymerization method in continuous aqueous phase. *C. auriculata* polymer extracted with ethanol has labeled as CAE, and it was dissolved with dichloromethane. Silymarin was dissolved in 1% solution of Tween 80. The drug solution and polymer solution were emulsified at 15°C using magnetic stirrer for 10 minutes.

This solution was sonicated for 20 minutes at 15°C. The nanoparticles of silymarin were separated by fractional centrifugation using a cooling centrifuge and redispersed in phosphate buffer saline pH 7.2. Six batches of nanospheres were prepared with varied drug polymer ratio and emulsifying agent ratio (Table 1).

***In vitro* release profile of *C. auriculata* polymer nanospheres containing silymarin**

About 100 mg equivalent weight of the silymarin taken in a dialysis tube and placed in 250 mL of phosphate buffer pH 7.4. The medium was stirred using the magnetic stirrer, and the temperature was maintained at 37±0.5°C. Periodically, 1 mL of the samples was withdrawn and diluted to 10 mL using phosphate buffer. After each with drawal, the same quantity of the fresh medium was replaced immediately. Then, the samples were assayed spectrophotometrically, systronics UV spectrophotometer 119 at 288 nm using, medium is blank. The release was compared with marketed silymarin conventional tablets, silybon 140.

Standard graph of silymarin

About 100 mg of silymarin was dissolved with 100 mL of phosphate buffer pH 7.4 to produce the concentration of 1 mg/ml. From that stock solution, 1 mL of the solution was taken, and it was further diluted to produce the concentration of 10 µg/mL. Form that 1-10 mL of the solution was withdrawn and diluted to 10 mL with phosphate buffer in a 10 mL standard flask to produce 1-10 µg/mL concentration. Then, these samples were assayed spectrophotometrically, systronics UV spectrophotometer 119, at 288 nm, using the corresponding medium as blank.

Selection of CAE nanospheres RF1 for hepatoprotective activity

With the about *in vitro* dissolution studies and release pattern of formulations RF1 and RF2. We have been selected RF1 formulation for *in vivo* hepatoprotective activity in Swiss Albino Rats because RF1 was shown better release characteristics than RF2 formulation.

Table 1: Formulation of silymarin nanospheres. CAE: *Cassia auriculata* ethanol extracted polymer

Formulation	Drug	Polymer	Tween 80
RF1	1	1 CAE	1
RF2	2	1 CAE	2

C. auriculata Cassia auriculata

Acute toxicity studies for *C. auriculata* polymer

Acute toxicity studies were performed according to Organisation for Economic Cooperation and Development 423 guidelines. Male Swiss mice weighing 30±5 selected by technique were used. The animals were fasted for 3 hrs with free access to water only; *C. auriculata* polymer was administered orally at a dose of 5 mg/kg initially. Mortality was observed for 2 days. If mortality was observed in two of three animals, then the dose administered was considered as toxic dose. However, it was found to be safe and no mortality was observed, the procedure was repeated for further higher dose such as 50, 100, 200 up to 3500 mg/kg body weight (BW). The animals were observed for toxic symptoms such as behavioral changes, locomotion changes, locomotion convulsion, and mortality for 24 hrs. It was found to be safe as per LD_{50}, and the polymer has allowed for further pharmacological investigations. 1/5 and 1/10 dose of the *C. auriculata* polymer is advisable. It is used as excipient rather than the drug form[6].

Two maximal doses of *C. auriculata* nanospheres of formulation RF1 of 50 and 100 mg/kg BW was calculated and continued further investigations.

Hepatoprotective activity

Animals

- Species: Albino Wistar rats
- Weight/size: Rats, 200±20 g
- Gender: Male
- Number to be used: 6 animals in each group
- Healthy male Wistar rats weighing (200±20) maintained under controlled temperature at 23°C±2°C and a 12 hrs light - 12 hrs dark period will be employed for the experimentation. Animals used for the study were supplied by the Smt. Sarojini Ramulamma College of Pharmacy, Animal House. Animals were housed in large spacious polypropylene cages on clean paddy husk bedding during the experimental period. The study was conducted after obtaining clearance from the Institutional Animal Ethics Committee (Reg. No. 1406/PO/a/12/CPCSEA) of Smt. Sarojini Ramulamma College of Pharmacy, Mahabub Nagar [7].

Number of animals required

Total number of animals required

- Number of animals for acute toxicity=18
- Number of animals in each group=6
- Number of groups=5

Total number of animals required = (6x 5)=30+18=48

Dose

C. auriculata polymer nanospheres containing silymarin, the dose is 50 mg/kg BW and 100 mg/kg BW are taken for two different groups, i.e. Groups IV and V, respectively.

Table 2: *In vivo* hepatoprotective activity of nanospheres containing silymarin with *Cassia auriculata* polymer

Treatment	SGOT/ALT (U/L)	SGPT/AST (U/L)	ALP (U/L)	Total bilirubin (mg/dL)	Cholesterol (mg/dL)	Total protein (gm/dL)	Albuminute (g/dl)	Globulin (mg/dl)	HDL (mg/dl)
Group I control (Tween 80)	123.8±2.82	151.72±1.51	237.46±7.25	1.72±0.19	92.17±2.15	8.71±0.80	5.65±0.29	2.17±0.18	49.16±2.24
Group II CCl_4	342.4±9.4	382.45±5.42	347.48±11.23	2.37±0.17	105.68±1.31	5.20±1.09	3.63±0.30	1.63±0.26	23.83±2.18
Group III Nanospheres RF1+CCl_4	263.27±0.15	210.7±7.34	315.62±1.37***	0.54±0.01	75.41±1.72	6.34±0.18	4.36±0.35	2.56±0.13	35.83±2.08
Group IV Nanospheres RF1+CCl4	164.27±0.27***	164.5±4.63***	257.10±0.72	0.47±0.02	68.41±0.54	5.74±0.63	4.62±0.26	2.35±0.14	29.16±2.12***
Group V Silymarin+CCl_4	307.1±1.27	279.46±1.54	273.46±2.78	0.64±0.03	59.18±1.14	6.48±0.14	5.08±0.35	2.75±0.12	41.5±1.60

The p<0.001, (***p<0.001), *** indicates extremely significant. SGOT: Serum glutamate oxaloacetate transaminase, SGPT: Serum glutamate pyruvate transaminase, ALP: Alkaline phosphate, HDL: High-density lipoprotein

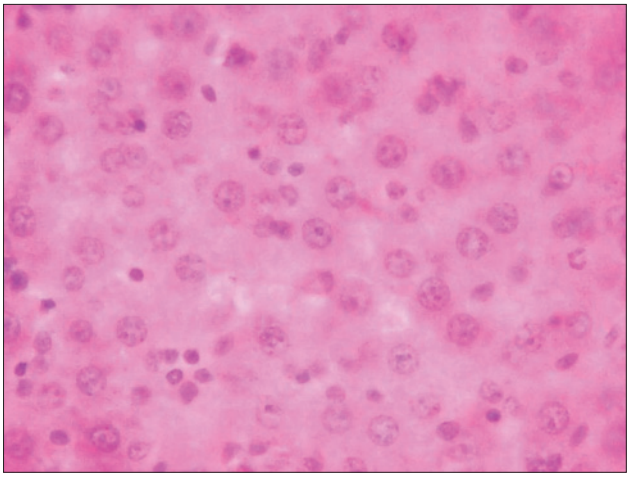

Fig. 1: Group I: Control

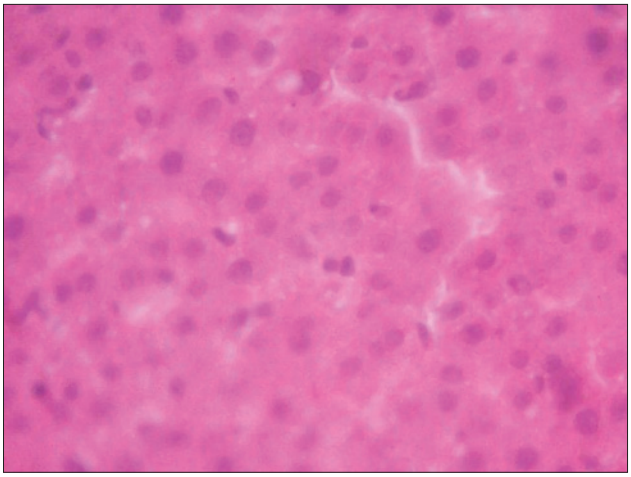

Fig. 4: Group IV: CCl_4 + nanospheres RFI (50 mg/kg body weight)

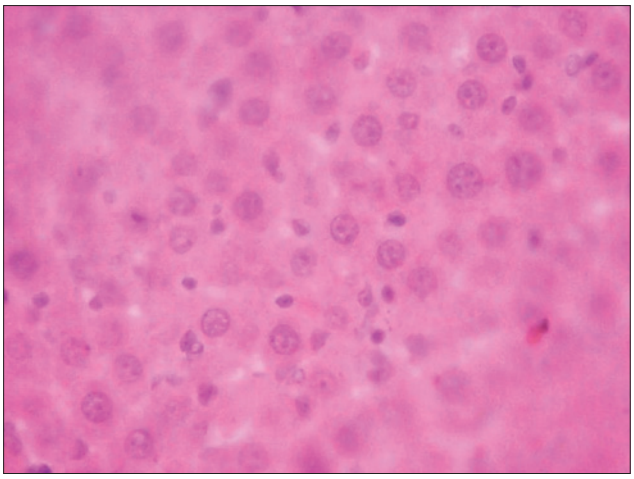

Fig. 2: Group II: Negative control-CCl_4 treated

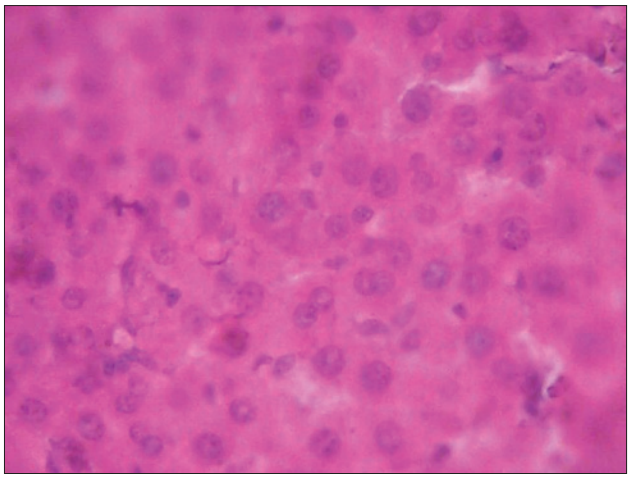

Fig. 5: Group V: CCl_4 + nanospheres RFI (100 mg/kg body weight)

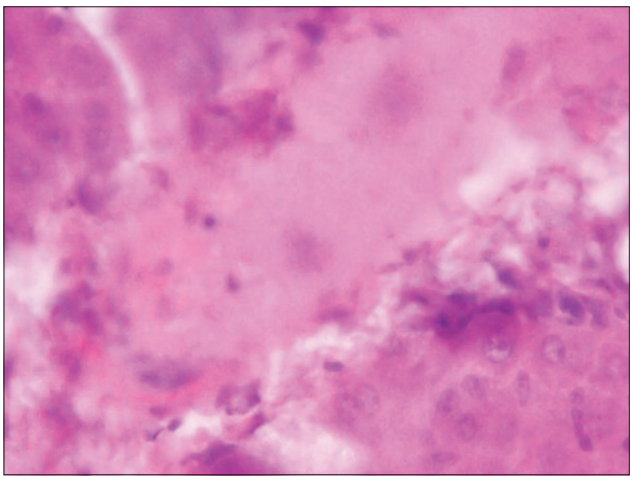

Fig. 3: Group III: Silymarin (50 mg/kg body weight) + CCl_4 treated

Experimental design

Rats were divided into 5 groups, and each group consists of six animals.

Group 1: It was served as control and received vehicle 2% Tween 80 p.o 2 mL/kg BW for 7 days.

Group 2: It was served as negative control and received 2% Tween 80 p.o 2 mL/kg BW for 7 days.

Group 3: It was a standard group, received silymarin 25 mg/kg BW for 7 days p.o.

Groups 4 and 5: These were received *C. auriculata* polymer nanospheres containing silymarin at a dose fo 50 mg/kg BW and 100 mg/kg BW, respectively, for 7 days.

CCl_4 1 ml/kg BW was administered intraperitoneally on 7th day 1 hr later after administration of the vehicle, standard and *C. auriculata* nanospheres, except to Group 1. *C. auriculata* nanospheres containing silymarin drug was administered per oral, and Tween 80-2% solution was used as vehicle. The biochemical parameters were determined after 18 hrs of fasting of the last dose.

Daily records of BW of all groups of animals were maintained during the whole experimental period.

Biochemical studies

After the treatment period, the animals of all groups were anesthetized with anesthetic ether and sacrificed. Blood was collected through the heart puncture.

Non-clinical investigations of a formulation RF1

The collected blood from the heart puncture was allowed to coagulate for 30 minutes, and serum was separated by centrifugation at 2500 rpm[8]. The serum was used to estimate serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase, alkaline phosphate, total protein, albumin, globulin, total bilirubin, total cholesterol, and high-density lipoprotein. The liver was isolated weighed, and morphological changes were observed. Reduced glutathione was estimated using DTNB (0.02% of 5,5'dithio (bis)

nitrobenzoic acid in 1% trisodium citrate) is also called as Ellman's reagent[9]. The yellow developed was immediately measured at 412 nm. The values were calculated using molar extinction coefficient of chromophore (1.36x 104 m^{-1}/cm).

Histopathological study

The liver was transferred to 4% formalin solution for fixation and later on processed for histopathological studies following the standard procedure described by Raghuramulu *et al*. (1983). The microtome sections were cut processed and stained with hematoxylin and eosin (Table 2). The section thus obtained was scanned in Carl-Zeiss microscope (Germany) with photographic facility and photomicrographs were taken. Changes present in the different groups in the cytoarchitecture were noticed [10].

We have taken ×10 and ×40 resolutions of liver were taken for all 5 groups of rats.

Relative organ weight (ROW) analysis

The liver was mopped with filter paper, weighed and the relative weights were calculated and expressed as g/100 g BW (Figures 1-5).

ROW = [absolute organ weight (g)/body weight of rats on sacrifice day (g)] ×100

Bio-statistical analysis

All the results were expressed as mean ± standard error of the mean (Table 4). One way analysis of variance was used for the statistical analysis of data. Dunnett's multiple comparison test was used for determining the significance. A probability value of $p<0.05$ was considered as a significant.

RESULTS AND DISCUSSION

Liver injuries induced by CCl_4 are the best commonly used model for screening of anti-hepatotoxic and/or hepatoprotective activities of drugs. Hepato fibrosis was also investigated extensively. Since the changes associated with CCl_4 induced liver damage is similar to that of acute viral hepatitis, hence CCl_4 mediated hepatotoxicity was chosen as the experimental model. It has been established that CCl_4 is accumulated in hepatic parenchyma cells and metabolically activated by cytochrome p450 - dependent mono-oxygenases to form a trichloromethyl radical (CCl_3). The CCl_3 radical alkylates cellular proteins and other macromolecules with simultaneous attack on polyunsaturated fatty acids, in the presence of oxygen, to produce lipid peroxides, leading to liver damage. Thus, antioxidant or free radical generation inhibition is important in protection against CCl_4 induced liver lesions. Hepatotoxic compounds, such as CCl_4, nearly all of the non-steroidal anti-inflammatory drugs (NSAIDs) have been implicated in causing liver injury, which are commonly used for analgesic and antipyretic activities and often used for the relief of non-specific fever (Radwan, 2000), continue to be important for the palliation of pain[11]. Diclofenac and particularly sulindac are reported to be more commonly associated with hepatotoxicity (Bjorkman, 1998). Several NSAIDs have been withdrawn from clinical use because of associated hepatotoxicity (Rabkin *et al*., 1999). The new more selective COX-2 inhibitors (eg., Celecoxib, rofecoxib, and nimesulide) are also associated with hepatotoxicity (Merlani *et al*., 2001). Hepatotoxicity from NSAIDs can occur at any time after drug administration, but like most adverse drug reactions most commonly occurs within 6-12 weeks of initiation of therapy (Aithal and Day, 1999). There are two main clinical patterns of hepatotoxicity due to NSAIDs (Rabinovitz and Van Thiel, 1992; Aithal and Day, 1999). The first is acute hepatitis with jaundice, fever, nausea, greatly elevated transaminases and sometimes eosinophilia[12]. The alternative pattern is with serological and histological features of chronic active hepatitis. Some of the NSAIDs which cause liver damage are given in the following Table 3. Alcohol is one of the causes of hepatic injury due to the accumulation of reactive oxygen species, which in turn causes lipid peroxidation of cellular membranes and proteins and DNA oxidation (Zhou *et al*., 2002)[13,14].

Table 3: Hepatotoxicants that will affect the liver injury

Serial number	Drug/hepatotoxicant	Use
1	Carbon tetrachloride	Toxicant
2	Acetaminophen	Analgesic, antipyretic (NSAIDs)
3	Diclofenac	Analgesic, antipyretic (NSAIDs)
4	Sulindac	Analgesic, antipyretic (NSAIDs)
5	Celecoxib	COX - 2 inhibitor
6	Rofecoxib	COX - 2 inhibitor
7	Nimesulide	COX - 2 inhibitor

NSAIDs: Non-steroidal anti-inflammatory drugs

The above said hepatotoxicants cause marked elevation in serum enzymes and bilirubin levels. It causes a marked decrease in total protein levels. Silymarin is used as standard hepatoprotective compound since it is reported to have a protective effect on the plasma membrane of hepatocytes [15,16]. CCl_4 has been found to induce extensive liver damage with in a period of 24 hrs following intraperitoneal administration. As a result of this, accumulation of fat in the liver and necrosis in the centrilobular region of the liver occurs. As a consequence, the microsomal enzyme activities are found to decrease and due to lipid peroxidation, the water-soluble enzymes leak into the plasma from the liver. It is shown by the significant decrease in triglycerides and proteins in CCl_4 intoxicated rat hepatocytes.

Treatment with *C. auriculata* nanospheres containing silymarin exhibited significant restoration of the altered biochemical parameters toward normal in CCl_4 intoxicated hepatocytes in rats. The effect of 100 mg/kg body weight *C. auriculata* nanospheres RF1 formulation was found to be better than that of standard silymarin at 25 mg/kg BW. Acute toxicity studies were performed for the *C. auriculata* polymer, and it was found safe for the use in nanospheres. The dose of nanospheres was taken at 50 mg/kg BW and 100 mg/kg BW and standard silymarin at 25 mg/kg BW, respectively. *In vivo* hepatoprotective effect at 50 mg/kg BW and 100 mg/kg BW of *C. auriculata* nanospheres were significant, with the drug availability for desirable clinical response in animals, comparatively with the pure drug silymarin at 25 mg/kg BW, positively supported by the histopathology results.

Table 4: Showing results of CCl_4 induced hepatotoxicity and treated with *Cassia auriculata* nanospheres containing silymarin

Group	Liver weight (g)	GSH (mg%)	Total protein (g/dl)	Relative organ weight (g/100g BW.)
Group I Control	4.23±0.24	1.34±0.02	8.71±0.80	3.30±0.17
Group II Negative CCl_4	5.63±0.16	0.50±0.01	5.20±1.09	3.69±0.42
Group III Standard (25mg/kg BW) + CCl_4	4.25±0.13***	1.23±0.01	8.24±0.20***	3.07±0.29
Group IV Nanospheres (50 mg/kg BW)+ CCl_4	5.13±0.08	0.70±0.01	7.53±0.32	3.41±0.13***
Group V Nanospheres (100 mg/kg BW)+ CCl_4	4.31±0.11	1.10±0.07***	7.23±0.16	3.31±0.24

The $P<0.001$, (***$p<0.001$), *** indicates extremely significant. *C. auriculata Cassia auriculata*, GHS: Glutathione, BW: Body weight

CONCLUSION

In vivo hepatoprotective activity of RF1 formulation of *C. auriculata* nanospheres containing silymarin has shown significant hepatoprotective and inhibition of oxidative stress results, and we have concluded that it should be sent for further pharmaceutical and pharmacological clinical investigations in human subjects.

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