ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 9, Issue 6, 2016

Online - 2455-3891
Print - 0974-2441

Research Article

IN VITRO ANTIOXIDANT AND *IN VIVO* HEPATOPROTECTIVE ACTIVITY OF ETHANOL EXTRACTS FROM THE BARK OF *SHOREA ROBUSTA* (*DIPTEROCARPACEAE*) AGAINST CARBON TETRACHLORIDE-INDUCED LIVER TOXICITY IN RATS

DIPTANU BISWAS[1]*, SHIVRAJ GOUDA T[2], NARAYANASWAMY LACHMANAN GOWRI SHANKAR[3]

[1]Department of Pharmacognosy, Creative Educational Society's College of Pharmacy, Kurnool, Andhra Pradesh, India. [2]Department of Pharmacology, NET Pharmacy College, Raichur, Karnataka, India. [3]Department of Pharmacognosy, Prime College of Pharmacy, Palakkad, Kerala, India. Email: diptanu69@gmail.com

Received: 23 July 2016, Revised and Accepted: 28 July 2016

ABSTRACT

Objective: The study is designed for the evaluation of *in vivo* hepatoprotective and *in vitro* antioxidant activity of ethanol extracts from the bark of *Shorea robusta* (EESR) (*Dipterocarpaceae*) by carbon tetra chloride (CCl_4) induced hepatotoxicity in rats.

Methods: EESR was evaluated for hepatoprotective activity in rats by inducing liver damage by CCl_4. The antioxidant activity of EESR was assayed by various *in vitro* antioxidant methods and activities were compared to standard ascorbic acid.

Results: Ethanol extracts at an oral dose 200 mg/kg and 400 mg/kg exhibited a significant (*p<0.005) protective effects by lowering the level of serum glutamic oxaloacetic transaminase, serum glutamic pyruvic transaminase, alkaline phosphatase, serum bilirubin, total cholesterol, and increasing the level of total proteins as compared to silymarin (50 mg/kg) used as positive control. The extracts exhibit significant antioxidant activity in various *in vitro* antioxidant models.

Conclusion: From these studies, we are concluding that, the EESR have potent hepatoprotective effects and have antioxidant properties, hence can be used as a natural product against liver damage.

Keywords: Antioxidant, Carbon tetrachloride, Hepatoprotective, *Shorea robusta.*

 DOI: http://dx.doi.org/10.22159/ajpcr.2016.v9i6.14271

INTRODUCTION

Shorea robusta is a large such deciduous tree seldom quite leaflets found extensively in North East and Central India [1,2]. Bark is reddish brown-gray in color, smooth and longitudinally a fissured. The plant is traditionally used in dysentery for the plaster of fumigation. It is commonly administered for weak digestion, gonorrhea and as an aphrodisiac [3-5]. Scientifically, the plant has been reported on various activities such as analgesic [6,7], antinociceptive, anti-inflammatory [8,9], oxidative stress [10], wound healing [11,12], and antiulcers [13]. Few novel compounds also isolated from various parts of the plants like a new flavonoids 3,7-dihydroxy-8-methoxy flavones-7-O-α-L rhamnopyranosyl (1→4)-α-L rhanmnoglucoside pyranosyl (1→6) β-glucopyranosyl has been isolated from the seeds of *S. robusta* [14]. Two terpenoids have been isolated from the resin of *S. robusta* namely 3, 25-epoxy-1, 2, 3, 11-tetra-hydroxyurs-12-en-28-oic acid and 3, 25-epoxy 1, 2, 3-tri-hydroxyurs-12-en-28 oic acid [15].

The liver is a glandular organ of the body which plays a key role in the metabolism of carbohydrates, lipids, protein and detoxifying xenobiotics, and drugs. Thus, the liver has a chance to get injured due to its chronic exposure to drugs, environmental toxicants, and xenobiotics [16]. Liver diseases are a global problem and synthetic drugs available for the treatment of liver disorders are believed to have serious adverse effects on the biological system [17]. Numerous medicinal plants and their formulations are used for liver disorders in ethnomedicinal practice as well as the traditional system of medicine in India [18]. The liver is a major organ attacked by reactive oxygen species and primary cells are subjected to oxidative stress-induced liver injury. Antioxidant substances protect the liver from various oxidative damages [19]. Our aim is to find out the hepatoprotective activity of ethanol extracts from the bark of *S. robusta* (EESR) in carbon tetrachloride (CCl4) induced hepatotoxicity in rats. Antioxidant activities of the extracts were also evaluated to test whether the drug has antioxidant potential or not.

METHODS

Plant materials

Barks of *S. robusta* were procured from wild source of the forest of Tripura. The plant material was identified and authentified by Prof. P. Jayaraman, Director plant anatomy center Tambaram, Chennai, with reference number (PARC/2012/1276). The specimen is preserved at herbarium of the institute.

Preparation of the extracts

Coarsely powdered dried bark (500 g) was extracted up to exhaustion with petroleum ether, chloroform and ethanol (70%) using soxhlet apparatus successively. The ethanol extracts thus obtained was dried under reduced pressure yielding 22% which was selected for the study.

Experimental animal

Wister albino rats of either sex were used throughout the experiments and mice was used for acute toxicity studies. After randomization into various groups and before initiation of the experiment, the rats were acclimatized for a period of 7-day-standard environmental conditions such as temperature (26±2°C), relative humidity (45-55%), and 12 hrs. Dark/light cycles were maintained in the quarantine. All the animals were fed with synthetic diet and water was allowed *ad-libitum* under strict hygienic condition. The entire animals were utilized for these studies and used according to the protocol approved by (No. IAEC/CESCOP/2016-07) Institutional Animal Ethics Committee.

Acute toxicity study
The acute toxicity for EESR was determined in albino mice which were maintained under standard conditions. The animals were fasted overnight before the experiment. Acute oral toxicity-acute toxic class method (OCED Guideline no. 423, Annexure-2d) adopted by Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India was followed for toxicity studies. There is no mortality was observed after oral administration of 2000 mg/kg of EESR [20]. Common side effects such as mild diarrhea, weight loss and depression of treated groups were recorded within 7 days of observation [21].

CCl_4 induced hepatotoxicity
In the dose-response experiment, albino rats were randomly assigned into 5 groups consisting of 6 animals in each group. Group I serve as (−ve) control and were received 1 ml 2% acacia solution postoperative (P.O) for 5 days. Group II serves as (+ve) control received distilled for 5 days. Group III received standards silymarin/kg body weight (B.W). for 5 days P.O mixing with 2% acacia solution, Group (IV and V) receiving EESR 200 mg/kg and 400 mg/kg P.O for 5 days by mixing with 2% acacia solution. Group I received olive oil 1 ml/kg B.W, I.P. Group II-V received CCl_4 at a dose 2 ml/kg B.W., by mixing with olive oil (1:1) on the 2nd and 3rd day, after 30 minutes of administration 50 mg/kg silymarin [22], 200 mg/kg and 400 mg/kg of EESR, respectively. Blood samples were collected on the 6th day by retro-orbital plexus for evaluating the various biochemical parameters such as serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), alkaline phosphatase (ALP), serum bilirubin, serum cholesterol, and total protein. They were determined by using biochemical kits of Agape by using Mispa semi auto analyzer of Agape made. The animals were sacrificed; livers were isolated and washed with normal saline. Isolated livers were stored in 40% formalin solution and proceeded to histopathology for evaluation of the detail hepatic architectural changes in each group microscopically [23,24].

Assessments of liver function
Assessment of liver function was done by collecting blood from retro-orbital punctures and centrifuge at 4000 rpms for 15 minutes. Then, after separating the serum various biochemical parameters such as SGOT, SGPT, ALP, total cholesterol, total protein, and total bilirubin [25,26] were evaluated using Mispa semi auto analyzer of Agape.

Histopathology
Histopathological studies were done by isolating the liver to check the cellular damage to the liver cells and hepatic veins. Sections were prepared and stained with hematoxylin and eosin dye for photo microscopic evaluation including cell necrosis, fatty change, ballooning, degeneration, and damage in central vein.

Antioxidant activity
2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity
The free radical-scavenging activities of all samples were being estimated in terms of hydrogen donating or radical scavenging ability. Radical scavenging ability was estimated using the stable radical DPPH. A solution of 0.1 mM DPPH (Himedia, Mumbai) was prepared in ethanol; 1.0 ml of this solution was being added to 3.0 ml of all the extracts solution in water at different concentrations (1-10 µg/ml). 30 minutes later, the absorbance was measured at 517 nm. Ascorbic acid is used as a standard antioxidant. The results were expressed as IC_{50} or inhibitory concentration 50 value, i.e., the concentration of samples exhibited 50% inhibition of DPPH* radicals [27].

Nitric oxide scavenging activity
Nitric oxide scavenging activity of extract was determined using Griess reagent (1% sulfanilamide, 2% phosphoric acid and 1% naphthyl ethylenediamine dihydrochloride). Reaction mixtures containing 3 ml of sodium nitroprusside (10 mmol) in phosphate buffer saline and test extract (1-10 µg/ml) were incubated at 25°C for 150 minutes. Control is also prepared by using 3 ml of sodium nitroprusside in ethanol which is used as solvent to dissolve the extract and allowed for incubation. After incubation, 0.5 ml of *Griess* reagent was added and absorbance was measured at 546 nm using UV-visible spectrophotometer [28]. The IC_{50} value was determined by conventional formula.

Super oxide scavenging activity
Add 100 ml riboflavin (20 µg) solution, 200 ml EDTA (12 mmole) solution, 200 ml methanol and 100 ml nitrobluetetrazolium (0.1 mg) solution in a test tube. The reaction mixture was being diluted up to 3 ml with phosphate buffer (50 mM, pH 7.6). The absorbance of the mixture solution was measured at 560 nm after illumination for 15 minutes, phosphate buffer was used as blank. The optical density (OD) was being taken as control reading. Then to different concentrations of samples (1, 2, 4, 8 and 10 µmg/ml) 100 µmg/ml and add above reaction mixture dilute up to 3 ml with phosphate buffer solution. The absorbance of the solution was measured at 590 nm. L-ascorbic acid was used as a reference standard. Decreased value of absorbance of the reaction mixture indicates an increase in super oxide anion scavenging activity [29].

Reducing power method
Add different doses of sample(s) in 1 ml of ethanol to get 1 µmg, 2 µmg, 4 µmg, 8 µmg, and 10 µmg/ml concentration. A test tube was also made as a control (without any test sample). Add phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 ml, 1%) with the sample. The mixture was incubated at 50°°C for 20 minutes. Portions (2.5 ml) of trichloroacetic acid (10%) added to the mixture and centrifuged at 3000 rpms for 10 minutes. The upper layers of the mixture solution (2.5 ml) have mixed with distilled water (2.5 ml) and 0.1% ferric chloride (0.5 ml). The absorbance (OD) was being measured at 700 nm. Ascorbic acid was used as a reference standard [30]. The results are expressed as IC_{50} or inhibitory concentration 50 value.

RESULTS

Acute toxicity study (LD_{50})
An attempt was made to identify LD_{50} of EESR; acute toxicity study was carried out according to fixed dose method of OECD 423. No mortality was observed at 2000 mg/kg in mice. It was thought that 2000 mg/kg was cut off dose. Therefore, 1/10th and 1/5th (200 mg/kg and 400 mg/kg) dose were taken as effective dose for all further *in vivo* studies.

CCl_4 induced hepatotoxicity
A significant increase (*p<0.05) in biochemical parameters such as serum SGOT, SGPT, ALP, serum bilirubin, and cholesterol level was observed in animals treated with carbon tetrachloride (2 ml/kg i.p.) as compared to normal and total protein level has decreased in toxic group. After treatment of drug extracts, it had significantly increased to normal level to the various biochemical parameters. The results are explained in Table 1. Parameters treatment of EESR (200 mg/kg, 400 mg/kg) and silymarin on 6 days decrease the above parameters significantly (*p<0.05) as compared to CCl_4 treated group. The degree of protection was high with the higher (400 mg/kg) dose of the extracts of *S. robusta.*

Histopathology
Liver histology of normal control animal (Fig. 1) shows normal liver architecture with central veins, cytoplasm and normal polygonal hepatocytes with round nucleolus and glandular cytoplasm. Blood infiltration in central veins, sinusoid space, and Kupffer cell was detected, it was noticed that most of the hepatocytes were normal. The liver with CCl_4 treatment (Fig. 2) shows various degrees of degeneration like ballooning of hepatocytes, infiltration of lymphocytes, and the loss of cellular boundaries with large sinusoidal space. Histopathological examinations of liver section of the rats treated with silymarin and EESR (200 and 400 mg/kg) followed by CCl_4 intoxication resulted in less necrosis and vacuoles formation and less degeneration of

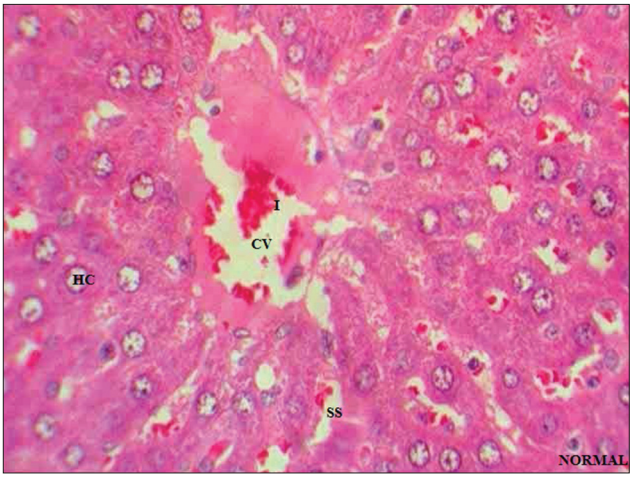

Fig. 1: Normal group treated with (2% acacia) vehicle shows normal hepatocytes cell, with less sindosidial space, hepatic veins shows less dilation but with red blood cell infiltration

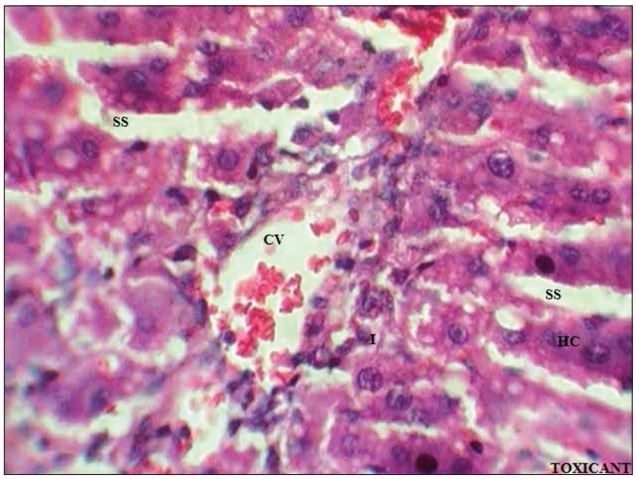

Fig. 2: Carbon tertra chloride at a dose of 2 ml/kg shows the damage in central vein and infiltration of red blood cell, dilation of sinusoidal space in the liver tissues, with ballooning of hepatocytes and damaged nucleus

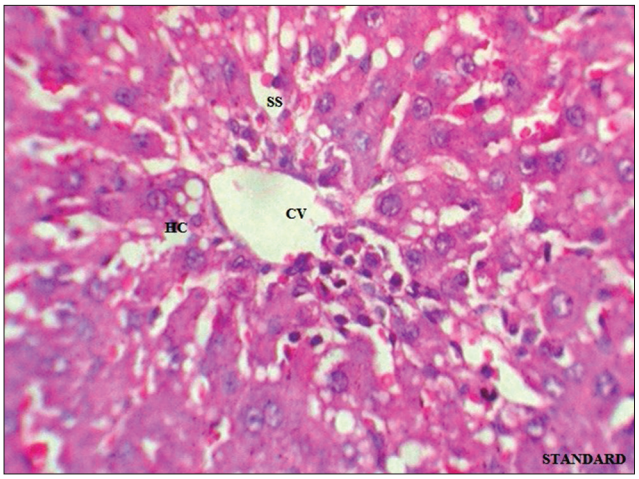

Fig. 3: Standard drug treated at a dose of (silymarin 50 mg/kg) in toxicated by carbon tetrachloride rats shows getting recovering in hepatocytes and reduction of sinusoidal space, and central veins gets normalized with out and infiltration of red blood cell

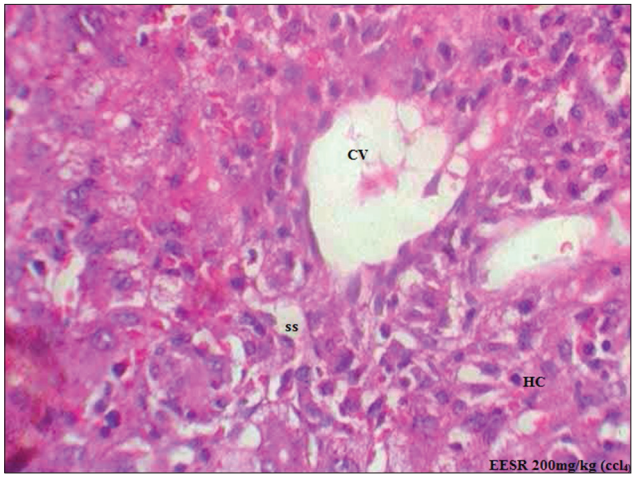

Fig. 4: Drug treated at a dose of (ethanol extracts from the bark of *Shorea robusta* 200 mg/kg) in toxicated by carbon tetrachloride rats shows getting recovering in hepatocytes and regeneration of sinusoidal space, and central veins gets normalized

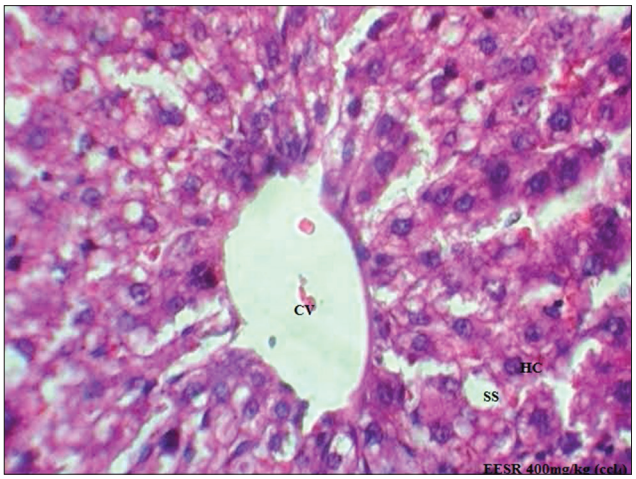

Fig. 5: Drug treated at a dose of (ethanol extracts from the bark of *Shorea robusta* 400 mg/kg) in toxicated by carbon tetrachloride rats shows getting recovering in hepatocytes and reduction of sinusoidal space, and central veins gets normalized with out and infiltration of red blood cell

hepatocytes and less rearrangements (Figs. 3-5). It indicates marked protective activity of EESR at 200 mg/kg and 400 mg/kg in CCl_4 induced hepatotoxicity in rat's liver.

Antioxidant activity

EESR barks possess a concentration relationship of various radical scavenging activities, *viz.,* DPPH radical scavenging activity, nitric oxide scavenging activity, super oxide radical scavenging activity, and radical reducing power activity. Ascorbic acid was used as a standard in DPPH, nitric oxide and super oxide scavenging activity. Rutin was used as the standard for reducing power activity. The results of antioxidant activity were described in Table 2.

DISCUSSION

CCL_4 is a well-known hepatotoxic agents [31], the hepatotoxicity-induced by CCl_4 is due to its metabolites CCl_3 free radicals, a free radical that binds to lipoproteins and leads to peroxidation of lipids of endoplasmic reticulum [32,33]. CCl_4 causes hepatocyte injury that is characterized by centrilobular necrosis that is followed by hepatic fibrosis [34]. Administration of carbon tetrachloride elevated the

Table 1: Effects of ethanol extracts from barks of *S. robusta* on biochemical parameters in rat livers

Groups	Treatment	Parameters					
		SGOT	SGPT	APL	Cholesterol	Total bilirubin	Total protein
Group I	Normal control	140.9±4.417	78.98±4.296	55.45±3.235	119.0±5.235	0.2327±0.0413	5.975±0.2181
Group II	CCl_4	453.3±28.03	243.0±12.71	189.1±7.498	189.1±7.498	2.020±0.217	9.239±0.4360
Group III	Silymarin+CCl_4	136.0±13.15***	156.3±3.268*	125.4±2.474*	125.4±2.474***	0.2733±0.02728**	5.377±0.2397***
Group IV	EESR (200 mg/kg)+CCl_4	182.9±5.377**	188.6±12.20**	102.2±5.560*	169.9±7.757*	0.5833±0.05463*	7.335±0.361**
Group V	EESR (400 mg/kg)+CCl_4	170.5±17.50***	164.1±6.432***	79.81±7.969**	140.1±6.106**	0.4567±0.0445**	7.252±0.3225**

All values are expressed as a Mean±SEM (n=6) One-way ANOVA followed by multiple comparison Tukey test. Symbol represent statically significance ***p<0.001, **p<0.01, *p<0.05 compared with toxic CCl_4 group. SGOT: Serum glutamic oxaloacetic transaminase, SGPT: Serum glutamicpyruvic transaminase. ALP: Alkaline phosphatase, *S. robusta: Shorea robusta*, SEM: Standard error of mean, CCl_4: Carbon tetra chloride

Table 2: Antioxidant activity of EESR

Scavenging methods	Positive control	IC_{50} (µg/ml)	
		EESR	Standard
DPPH scavenging activity	Ascorbic acid	27.82±2.370**	10.63±1.282
Nitric oxide scavenging activity	Ascorbic acid	40.55±1.498*	7.210±0.8577
Super oxide scavenging activity	Ascorbic acid	35.19±2.055**	14.39±0.9484
Reducing power method	Ascorbic acid	37.79±2.839**	15.26±1.994

Vlaues are represents as mean ± SEM as significant compared to blank. Symbol represents *p<0.05, **p<0.001., ***p <0.0001. DPPH: 2,2 diphenyl 1 picrylhydrazyl

serum level of SGOT, SGPT, ALP and bilirubin significantly. A major defiance mechanism involves in the antioxidant including super oxide dismutase, catalase glutathione peroxidase which convert active oxygen molecules into nontoxic compounds. Scavenging of free radicals is one of the major antioxidant mechanism to inhibit the chain reaction to lipid peroxidation [35]. In these studies, CCl_4 induced hepatotoxicity has been done to check the protective effects of ethanol extract from the bark of *S. robusta* in dose-dependent manner at a dose of 200 mg/kg and 400 mg/kg. An attempt also was made to find out the correlation between hepatoprotective activity and antioxidant activity.

The treatment of EESR restored the damaged liver cell and also brought down the elevated levels of SGOT, SGPT, ALP, cholesterol and bilirubin levels related to the hepatic injury. Histology of liver section of normal control animal shows the normal liver architecture of central veins, cytoplasm and normal polygonal hepatocytes with round nucleus and glandular cytoplasm. Blood sinusoid and Kupffer cells were detected normal. CCl_4 treated liver shows various degrees of degeneration like ballooning of hepatocytes, infiltration of lymphocytes, and the loss of cellular boundaries. Histopathological examinations of liver section of the rats treated with EESR (200 mg/kg and 400 mg/kg) and following by CCl_4 intoxication resulted in the absence of necrosis and vacuoles formation and less degeneration of hepatocytes and less rearrangements. It indicates protective activity of EESR at 400 mg/kg and with silymarin-treated groups.

Since the preliminary phytochemical analysis of the extracts has shown the presence of flavonoids and phenol compounds [36], which we have been known for its antioxidant and hepatoprotective activities [37]. The results of various antioxidant activities suggest that ESSR contains a free radical scavenging power which could exert a beneficial action against pathological alteration caused by generated free radical CCl_3 in CCl_4 induced hepatotoxicity in rats.

CONCLUSION

It is concluded that treatment of EESR decrease the CCl_4 induced elevation in biochemical parameters such as SGOT, SGPT, ALP, cholesterol, serum bilirubin. It also significantly increases total protein in the blood of treated animal with test drugs and silymarin. These findings suggest that EESR is effective on bringing the functional improvement of hepatocytes. The healing effects of this extracts were also confirmed by histological observation. The drugs also have antioxidant potential estimated by *in vitro* antioxidant study. Antioxidant may be helpful in the regeneration of hepatic cell from their oxidative damage. Further research is required to find out the correct mechanism for this drug in the treatment of hepatic damage.

ACKNOWLEDGMENT

I would like to thank Principal and Managements of Creative Education Society's College of Pharmacy for providing all the facility to successfully complete my work and my senior colleague Dr. S V Suresh Kumar, HOD, Deprtment of Pharmacognosy for helping me to do the work.

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