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Original Article

Self microemulsifying formulation of *Lagerstroemia speciosa* against chemically induced hepatotoxicity

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ABSTRACT

Self microemulsifying formulation is an approach used for enhancing the bioavailability of poorly soluble molecules due to their lipidic nature and small particle size. The objective of the present study was to evaluate the hepatoprotective activity of poorly soluble hydroxy- and polyhydroxy-organic phytomolecules rich *Lagerstroemia speciosa* leaves extract in modern formulation i.e. "Self microemulsifying System".

Different doses of SME (Self microemulsifying) formulation of *L. speciosa* leaves extract were evaluated for the hepatoprotective activity against carbon tetrachloride induced liver toxicity in rats. The parameters evaluated were (a) biochemical parameters like serum enzymes: aspartate aminotransferase (AST), serum glutamate pyruvate transaminase (ALT), serum alkaline phosphatase (ALP) and total bilirubin (b) liver antioxidant parameters as estimation of Lipid peroxidation (LPO), catalase (CAT), Superoxide dismutase (SOD) activity and concentration of reduced glutathione (GSH). Oral administration of SME formulation provided the significant protection in marker enzyme of treated group at 100 mg/kg, p.o. as AST ($P < 0.001$), ALT ($P < 0.001$), ALP ($P < 0.001$) and total bilirubin ($P < 0.001$) comparable to the group treated with silymarin. Treatment with SME formulation at the doses of 100 mg/kg, p.o. significantly prevented the rise in levels of LPO significantly ($P < 0.001$). The GSH, SOD and CAT contents had significantly ($P < 0.001$) increased in SME formulation treated groups whereas carbon tetrachloride intoxicated group had shown significant decrease in these parameters compared to control group. Formulation at the dose 100 mg/kg, p.o. has shown maximum protection which was almost comparable to those of the normal control and standard. The histological observations further uphold the results for hepatoprotective activity.

1. Introduction

Lagerstroemia speciosa (L.) Pers. (Lythraceae) reflects their attractive and colorful flower has common names such as queen's flower, queen of flowers, crepe myrtle and pride of India.[1] The main effective chemical constituents previously reported and found in leaves are ellagitannins, ellagic acid, ellagic acid sulfate and four methyl ellagic acid derivatives, including corosolic acid, gallic acid, 4-hydroxybenzoic acid, 3-O-methyl protocatechuic acid, caffeic acid, p-coumaric acid, kaempferol, quercetin and isoquercitrin.[2] Further monomeric and dimeric ellagitannins (flosin A and B, and reginin A, B, C and D) and three new ellagitannins (lagerstannins A, B and C) were isolated and identified from the leaves.[3–5] There is increasing evidence for the hepatoprotective role of hydroxy- and polyhydroxy-organic compounds and particularly from vegetables, fruits and some herbs.[6]

Free radicals are involved in the development of degenerative diseases. They have been implicated in the pathogenesis of liver damage,[7,8] diabetes,[9,10] nephrotoxicity,[11,12] cancer,[13] cardiovascular disorders, neurological disorders, inflammation[14] and in the process of aging.[15] It is well known that a significant increase in steatosis and fibrosis leads to lethal cirrhosis of the liver in humans. Although the pathogenesis of liver fibrosis is not quite clear, there is

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no doubt that reactive oxygen species (ROS) play an important role in pathological changes in the liver.[16] Biological membranes are particularly prone to the ROS effect. Several endogenous protective mechanisms have been evolved to limit ROS and the damage caused by them.[17,18] However, since this protection may not be complete, or when the formation of ROS is excessive, additional protective mechanisms of dietary antioxidants may be of a great importance.[19] Therefore, many natural and synthetic agents possessing antioxidative properties have been proposed to prevent and treat hepatopathies induced by oxidative stress.[20,21]

A large number of studies have shown that the activity of *L. speciosa* is due to presence of corosolic acid, a potent molecule for diabetes treatment.[22,23] However significant amounts of hydroxy- and polyhydroxy-organic compounds and tannins are also present in the plant as discussed above have shown in a recent studies to increase glucose uptake in rat adipocytes, and could be responsible for its effect in lowering the blood glucose levels, antioxidant activity and other various other pharmacological activities. As the standardized extract of *L. speciosa* contains corosolic acid and other hydroxy- and polyhydroxy-organic compounds which have low solubility in water,[22] the *in-vivo* absorption of it may be hampered, which results in low bioavailability.

Self microemulsifying formulation is an approach for enhancing the absorption of poorly soluble phytomolecules due to their lipidic nature and small particle size.[24] Self microemulsifying formulation is mixtures of water insoluble phytomolecule, oil/lipid, surfactant and cosurfactant. After oral administration, they are diluted in aqueous media of gastrointestinal tract (GIT) and form oil-in-water (O/W) microemulsion/nanoemulsion having globule size in the range of 100–500 nm. The energy required for dispersion is provided by gastric motility. The formed microemulsion presents the phytomolecule in a dissolved form which is a premier requirement for poorly water soluble phytomolecule for absorption. Along with this, the specific lipid excipients of self microemulsifying formulation promote the lymphatic transport of phytomolecules results in increase in bioavailability through reduction in first pass metabolism. Another reason of increase in intracellular concentration of phytomolecule is due to reduction in strength of P-glycoprotein efflux system by used lipid and surfactant.[25–29]

The purpose of this study was to investigate the improved hepatoprotective activity of standardized extract of *L. speciosa* leaves in previously optimized formulation of self microemulsifying system against carbon tetrachloride induced acute liver cirrhosis in experimental animals.

2. Material and methods

2.1. Plant collection and identification

The leaves of *L. speciosa* were freshly collected from the road side of Lucknow, Uttar Pradesh. The leaves were identified and authenticated taxonomically by Dr. A.K.S. Rawat, Head of Department, Pharmacognosy and Ethnopharmacology Division, National Botanical Research Institute (NBRI), Lucknow, India. The herbarium, (NBRI/CIF/256/2011), was preserved at department for future reference.

2.2. Materials

Sefsol-218 was kindly provided as gift sample by Nikko chemicals (Tokyo, Japan) and Diethylene glycol monoethyl ether (Transcutol-P) by Gattefosse Corp. (France). Polyoxyl35 castor oil (Cremophor-EL) was obtained as gift samples from BASF Co. (Germany). All other chemicals and reagents used were of analytical grade and procured from Sigma chemicals Co., USA and Qualigens fine chemicals, Mumbai, India.

2.3. Animals

Male albino Wistar rats (200–220 g) were kept in the departmental animal house of National Botanical Research Institute, Lucknow at 27 °C and relative humidity 42–54%, light and dark cycles of 10 and 14 h, respectively, for one week before and during the experiments. Animals were provided with standard rodent pellet diet and the food was with drawn 18–24 h before the experiment though, water was allowed *ad libitum*. All the studies were performed in accordance with the guidelines for the care and use of laboratory animals, as adopted and promulgated by the Institutional Animal Care Committee, CPCSEA, India (Reg. No. 222/2000/CPCSEA). The standard orogastric cannula was used for oral drug administration.[30]

2.4. Preparation of extract

The matured leaves were collected, washed with distilled water to remove dirt and soil, and shade dried up-to 20–25 days. Routine pharmacognostic studies including organoleptic tests, macroscopic and microscopic observations were carried out to confirm the identity of the materials. The dried materials were powdered by grinder and passed through a 10-mesh sieve. The coarsely powdered leaves were defatted by immersing the powder into petroleum ether up-to 12 h by regular shaking. Extraction was done by hot continuous soxhlet apparatus using 50% alcohol at 60 °C for 6 h. After extraction the excess solvent was removed by using a rotary evaporator (Buchi, USA) and then freeze-dried (Freezone® 4.5, Labconco, USA) at high vacuum (133×10^{-3} mBar) and at temperature −40 °C.[31] A net yield of 12.8 gm per 100 gm was obtained. The collected *L. speciosa* leaf extract (LSE) was stored in air-tight glass container for future experiments.

2.5. Preparation of self microemulsifying formulation

Self microemulsifying formulation (1 ml) was prepared by taking specified quantity of oil (Sefsol-218), surfactant (Cremophor-EL) and co-surfactant (Transcutol-P) in a glass vial in a ration of 1:2:2 (% v/v). Then the LSE (10 mg) was added with gentle stirring. The mixture was vortexed and heated at 40 °C on water bath for 15 min. The prepared formulation was stored in tightly closed container at ambient conditions until further use. The characterization of the prepared self microemulsifying formulation was earlier reported by our research group.[32]

2.6. Chemically induced hepatotoxicity

Male albino Wistar rats were divided into five groups, each group had six animals. Group I (control) animals were administered a single daily dose of SME without LSE (1 ml/kg body weight, p.o.). Group II received carbon tetrachloride (1 ml/kg body weight, i.p. 1:1 v/v mixture of CCl_4 and liquid paraffin) alone. Group III and IV received the prepared SME formulation of LSE (equivalent to 50 and 100 mg/kg LSE, p.o.) respectively along with carbon tetrachloride. Group V received silymarin, a known hepatoprotective compound (Sigma Chemicals Company, USA), at a dose of 100 mg/kg, p.o., along with carbon tetrachloride. The SME formulation was given daily while carbon tetrachloride was given for every 72 h for 14 days. Animals were sacrificed 48 h after the last dose of the drug. The liver samples were dissected out and blood was collected.[33]

2.7. Assessment of hepatoprotective activity

The collected blood was allowed to clot and serum was separated at 2500 rpm for 15 min and the biochemical parameters like

serum enzymes: aspartate aminotransferase (AST, U/L), serum glutamate pyruvate transaminase[34] (ALT, U/L), serum alkaline phosphatase[35] (ALP, U/L) and total bilirubin[36] (mg/dL) were assayed using assay kits.

2.8. Assessment of antioxidant parameters

The dissected out liver samples were washed immediately with ice cold saline to remove as much blood as possible. Liver was homogenized (5%) in ice cold 0.9% NaCl with a Potter-Elvenhjem glass homogenizer. The homogenate was centrifuged at 800 rpm for 10 min and the supernatant was again centrifuged at 12,000 rpm for 15 min and the obtained mitochondrial fraction was used for the estimation of lipid peroxidation[37] (LPO), catalase[38] (CAT). Superoxide dismutase (SOD) activity was estimated by the inhibition of nicotinamide adenine dinucleotide (reduced)-phenazine methosulphate-nitrobluetetrazolium reaction system as described by Nishikimi.[39] The concentration of GSH was determined by the method of Anderson.[40]

2.9. Histopathological studies

For histological studies, the liver tissues were fixed with 10% phosphate buffered neutral formalin, dehydrated in graded (50–100%) alcohol and embedded in paraffin. The initial examination was qualitative, with the purpose of determining histopathological lesions in liver tissue. Thin sections (5 M) were cut and stained with routine hematoxylin and eosin (H&E) stain for photo microscopic assessment.[41]

2.10. Statistical analysis

The results were analyzed using one-way analysis of variance followed by Dunnett's test using Graph Pad Prism 5.0 (Graph-Pad Software Inc., San Diego, California, USA). The data are expressed as mean ± S.E.M. The value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of SME formulation on AST, ALT, ALP and total bilirubin

The effect various doses of SME formulation studied on serum marker enzymes and total bilirubin in CCl_4 intoxicated experimental animals. Hepatic injury induced by carbon tetrachloride caused significant changes in marker enzyme as AST by 272.99%, ALT by 395.27%, ALP by 134.85% and total bilirubin by 321.62% compared to control group. The percentage protection in marker enzyme of treated group at 50 mg/kg as AST 52.08 ($P < 0.01$), ALT 53.27 ($P < 0.001$), ALP 44.33 ($P < 0.01$), and total bilirubin 62.82 ($P < 0.001$) compared to carbon tetrachloride treated group. While maximum percentage protection in marker enzyme at the dose of 100 mg/kg and silymarin (100 mg/kg) as AST 68.81 ($P < 0.001$),70.43 ($P < 0.001$), ALT 75.97 ($P < 0.001$), 77.11 ($P < 0.001$), ALP 54.40 ($P < 0.001$) 55.35 ($P < 0.001$) and total bilirubin 73.08 ($P < 0.001$), 73.71 ($P < 0.001$) which is almost comparable to the group treated with silymarin, a potent hepatoprotective drug used as reference standard (Figs. 1 and 2).

3.2. Estimation on LPO, GSH, SOD and CAT in SME formulation treated animals

The results summarized in Figs. 3 and 4 which showed clear significant percentage change in the levels of LPO in CCl_4 intoxicated rats as 253.84 ($P < 0.001$) compared to control group.

$$\% \text{ Change (protection) in activity} = (1-T/C) \times 100$$

where: T = treatment groups (either test groups or toxic group), C = Normal control group or toxic group.

Treatment with SME formulation at the doses of 50 and 100 mg/kg significantly prevented this rise in levels and the percentage protection in LPO were 52.17 ($P < 0.01$) and 68.84 ($P < 0.001$) respectively. The GSH, SOD and CAT contents had significantly increased in SME formulation treated groups whereas carbon tetrachloride intoxicated group had shown significant decrease in these parameters compared to control group. The percentage changed of GSH, SOD and CAT in CCl_4 intoxicated group were 56.63 ($P < 0.001$), 65.00 ($P < 0.001$) and 42.65 ($P < 0.001$) respectively. The percentage protection in GSH as 88.89 ($P < 0.01$), 119.43 ($P < 0.001$) and SOD 113.43 ($P < 0.05$), 157.71 ($P < 0.001$) while in CAT 46.19 ($P < 0.05$), 67.88 ($P < 0.01$) at the doses levels 50 and 100 mg/kg, respectively compared to carbon tetrachloride treated group. In different doses level of SME formulation 100 mg/kg has shown maximum protection which was almost comparable to those of the normal control and silymarin.

3.3. Histopathological observations

The histological observations basically support the results obtained from serum enzyme assays. Histopathology of liver section is well described in Fig. 5 legends.

4. Discussion

In the present investigation, SME formulation was evaluated for hepatoprotective activity using carbon tetrachloride induced hepatotoxicity in experimental rats. The hepatotoxicity induced by CCl_4 is due to its metabolite CCl_3, a free radical that alkylates cellular proteins and other macromolecules with a simultaneous attack on polyunsaturated fatty acids, in the presence of oxygen, to produce lipid peroxides, leading to liver damage.[42] The antioxidant enzymes are therefore the first-line defense against such damage and thus provide protection against the deteriorating outcome.[43] Hepatocellular necrosis leads to elevation of the serum marker enzymes, which are released from the liver into blood.[44]

The present study revealed a significant increase in the activities of AST, ALT, ALP and serum bilirubin levels on exposure to carbon tetrachloride, indicating considerable hepatocellular injury. Administration of SME formulation at different dose levels i.e. 50 and 100 mg/kg, attenuated the increased levels of serum enzymes, produced by carbon tetrachloride and caused a subsequent recovery towards normalization comparable to the control group rats. The effect of SME formulation was further accomplished by the histopathological examination. The SME formulation showed the dose dependent hepatoprotective activity.

In CCl_4 induced hepatotoxicity, the balance between ROS production and these antioxidant defenses may be lost, "oxidative stress" results, which through a series of events deregulates the cellular functions leading to hepatic necrosis. The reduced activities of SOD and catalase indicate hepatic damage in the rats administered with carbon tetrachloride. However, those treated with 50 and 100 mg/kg of SME formulation showed significant increase in the level of these enzymes, which indicate the antioxidant activity of the formulation. Regarding non enzymic antioxidants, GSH is a critical determinant of tissue susceptibility to oxidative damage and the depletion of hepatic GSH has been shown in CCl_4[45] treated group.[46] The increase in hepatic GSH level in the rats treated with 50 and 100 mg/kg of SME formulation may be due to de novo GSH synthesis or GSH regeneration.

The level of lipid peroxide is a measure of membrane damage and alterations in structural and functional cellular membrane.[47] In

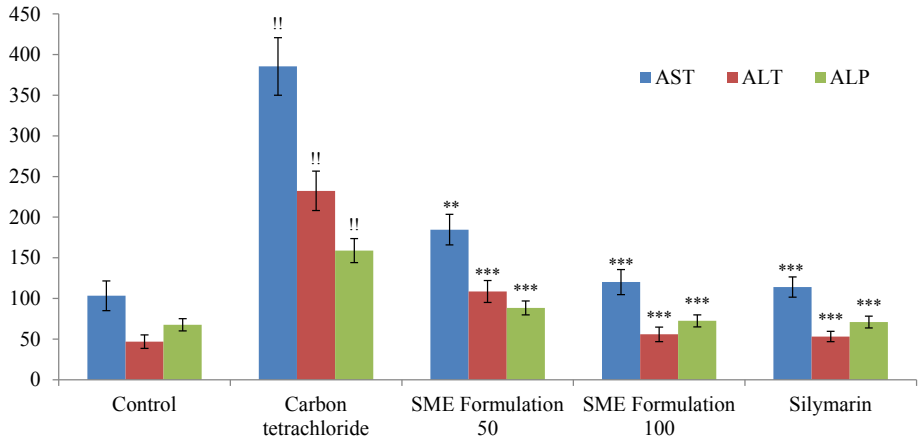

Fig. 1. Effect of SME Formulation on serum AST (U/L), ALT (U/L) and ALP (U/L) against CCl_4 induced liver toxicity in rats. Values are mean ± S.E.M. of 6 rats in each group. P values: ‼ <0.001 compared with respective control group. P values: *<0.05, **<0.01, ***<0.001, compared with the carbon tetrachloride treated group.

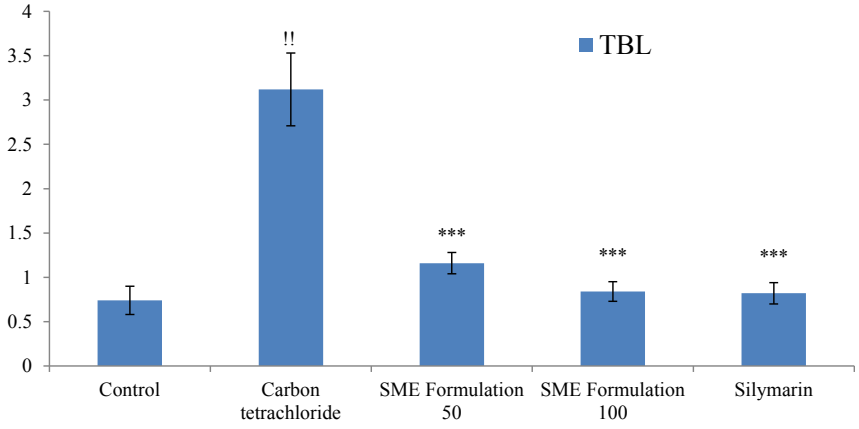

Fig. 2. Effect of SME Formulation on serum Total bilirubin level (mg/dL) against CCl_4 induced liver toxicity in rats. Values are mean ± S.E.M. of 6 rats in each group. P values: ‼<0.001 compared with respective control group. P values: *<0.05, **<0.01, ***<0.001, compared with the carbon tetrachloride treated group.

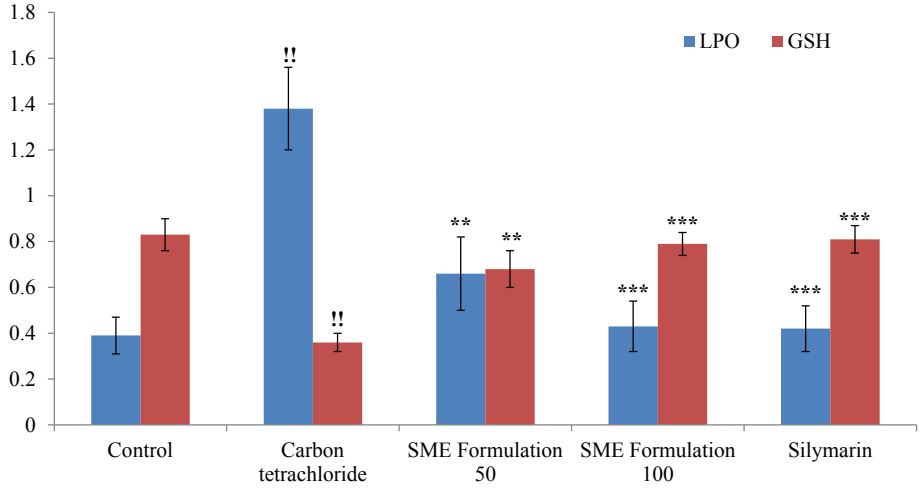

Fig. 3. Effect of SME Formulation on liver LPO (MDA nmol/min/mg of protein) and GSH (nmol/mg of protein) against CCl_4 induced liver toxicity in rats. Values are mean ± S.E.M. of 6 rats in each group. P values: ‼<0.001 compared with respective control group. P values: *<0.05, **<0.01, ***<0.001, compared with the carbon tetrachloride treated group.

the present study, elevation of lipid peroxidation in the liver of rats treated with carbon tetrachloride was observed. The liver microsomal oxidizing systems connected with cytochrome *P*-450 produce reactive metabolites of CCl_4 such as trichloromethyl radical (CCl_3) or trichloroperoxyl radical (CCl_3O_3). These radicals cause lipid peroxidation which produces hepatocellular damage and enhanced production of fibrotic tissue.[48] The increase in LPO level in liver suggests enhanced lipid peroxidation leading to tissue

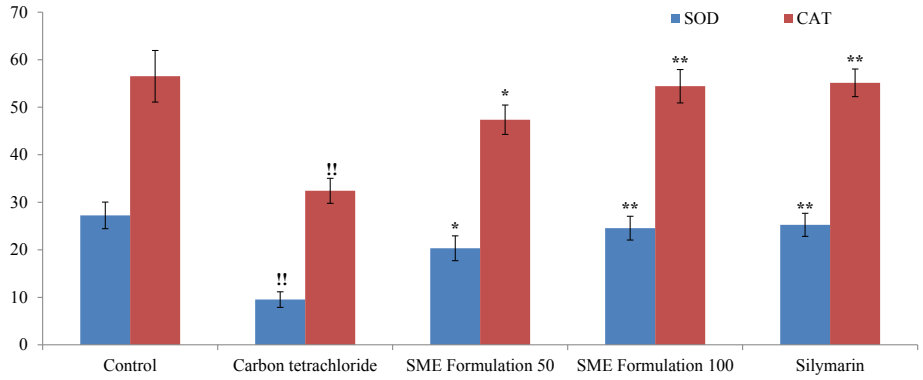

Fig. 4. Effect of SME Formulation on liver SOD (unit/mg of protein) and CAT (units/mg of protein) against CCl_4 induced liver toxicity in rats. Values are mean ± S.E.M. of 6 rats in each group. P values: ‼<0.001 compared with respective control group. P values: *<0.05, **<0.01, ***<0.001, compared with the carbon tetrachloride treated group.

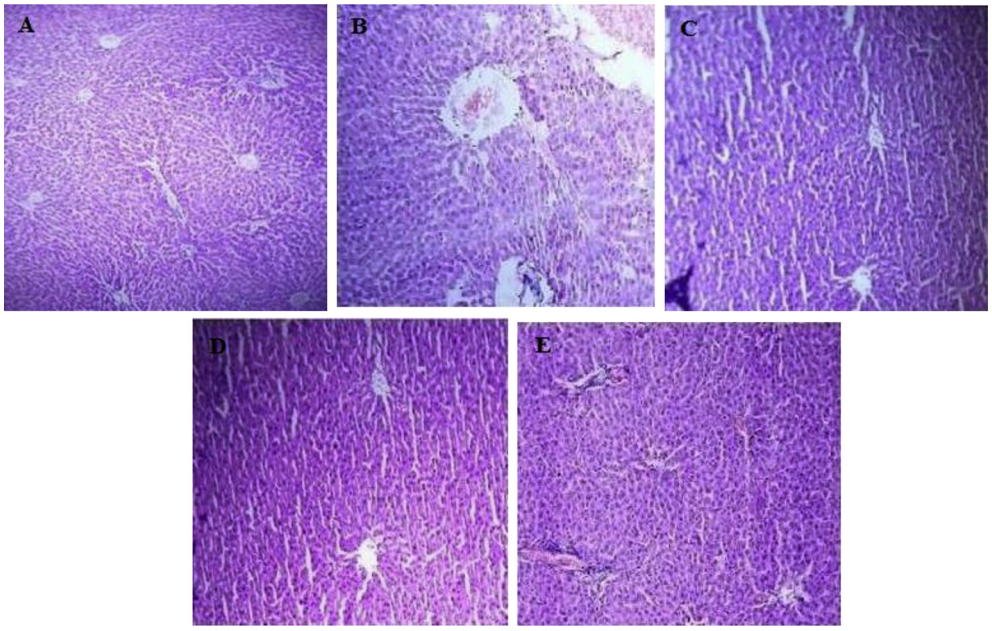

Fig. 5. Effect of SME Formulation on Liver histology against CCl_4 induced liver toxicity in rats. **A**. Section of rat liver treated with vehicle control shows the normal hepatic cells, central vein sinusoid with normal texture. **B**. Section of rat liver treated with CCl_4 shows damaged hepatic cells, central vein, nucleus, endothelium and sinusoids. **C**. Section of rat liver treated with silymarine and CCl_4 shows regeneration of hepatic cells, central vein, nucleus, endothelium and sinusoids. **D**. Section of rat liver treated with SME Formulation (50 mg/kg) and CCl_4 shows regeneration of hepatic cells, central vein, nucleus, endothelium and sinusoids. **E**. section of rat liver treated with SME Formulation (100 mg/kg) and CCl_4 shows regeneration of hepatic cells, central vein, nucleus, endothelium and sinusoids.

damage and failure of antioxidant defense mechanisms to prevent the formation of excessive free radicals.[13] LPO can be prevented at the initial stage by free radical scavengers and antioxidants. Treatment with SME formulation significantly reverses all the changes. Hence, it is possible that the mechanism of hepatoprotective activity of formulation may be due to its antioxidant activity. The protective effect of formulation was also assessed by studying the histopathology of liver tissue. In this study, noticeable changes were observed in the architecture of liver in carbon tetrachloride treated animals.

On preliminary qualitative phytochemical screening, the *L. speciosa* is reported to posses the rich quantity of polyhydroxy-compounds which can function as natural antioxidants in humans and animals, flavonoids, saponins, glycosides, tannins etc. Plant constituents like triterpenoids and flavonoids are well known for their antioxidant and hepatoprotective activity too.[49] As mentioned above SME formulations does contain a variety of compounds with antioxidative and reactive oxygen species scavenging potency, could serve as free radical inhibitors or scavenger or acting possibly as primary antioxidants. The present study shows the enhanced pharmacological activity of the extract due to better bioavailability of these chemical constituents due to self micro-emulsifying system formulation of the extract.

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Conflict of interest statement

We declare that we have no conflict of interest.

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