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Hepatoprotective activity of *Symplocos racemosa* bark on carbon tetrachloride-induced hepatic damage in rats

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ABSTRACT

The present study aims to evaluate the hepatoprotective activity of ethanol extract of *Symplocos racemosa* (EESR) bark on carbon tetrachloride (CCl4)-induced hepatic damage in rats. CCl4 with olive oil (1 : 1) (0.2 ml/kg, *i.p.*) was administered for ten days to induce hepatotoxicity. EESR (200 and 400 mg/kg, *p.o.*) and silymarin (100 mg/kg *p.o.*) were administered concomitantly for fourteen days. The degree of hepatoprotection was measured using serum transaminases (AST and ALT), alkaline phosphatase, bilirubin, albumin, and total protein levels. Metabolic function of the liver was evaluated by thiopentone-induced sleeping time. Antioxidant activity was assessed by measuring liver malondialdehyde, glutathione, catalase, and superoxide dismutase levels. Histopathological changes of liver sample were also observed. Significant hepatotoxicity was induced by CCl4 in experimental animals. EESR treatment showed significant dose-dependent restoration of serum enzymes, bilirubin, albumin, total proteins, and antioxidant levels. Improvements in hepatoprotection and morphological and histopathological changes were also observed in the EESR treated rats. It was therefore concluded that EESR bark is an effective hepatoprotective agent in CCl4-induced hepatic damage, and has potential clinical applications for treatment of liver diseases.

Key words: Carbon tetrachloride, hepatoprotective, *Symplocos racemosa*, silymarin

INTRODUCTION

The liver is a vitally important organ, playing a pivotal role in regulating various physiological processes in the body. It possesses great capacity to detoxicate toxic substances and synthesize useful principles.[1] Liver diseases are mainly caused by toxic chemicals, excess consumption of alcohol, infections, and autoimmune disorders. Most hepatotoxic chemicals damage liver cells mainly by inducing lipid peroxidation and other oxidative damages.[2] Modern medicine knows no effective drugs that stimulate liver functions, offer protection to the liver from damage, or help to regenerate hepatic cells. In the absence of reliable liver protective drugs in modern medicine, there exists a challenge to explore the potential of hepatoprotective activity of plants on the basis of traditional use.[3] Several medicinal plants are already known to play important roles in liver disorders. Others need to be studied for their therapeutic potential. A literature survey revealed the following plants, which have been recently reported as hepatoprotective: *Amaranthus spinosus,*[4] *Saururus chinensis,*[5] *Vernonia amygdalina,*[6] and *Zanthoxylum armatum.*[7]

Symplocos racemosa Roxb (Symplocaceae) is commonly known as "Lodhra" in Sanskrit or "Rodhra." Its bark is useful in bowel complaints such as diarrhea, dysentery, eye diseases, liver complaints, fever, ulcer, scorpion sting, diabetes, and liver disorders.[8] It has been scientifically reported as an antimicrobial,[9] anticancer,[10] anti-inflammatory,[11] antiulcer,[12] and gynaecological disorder.[13] However, to this date, its possible hepatoprotective activity has not been scientifically investigated. Hence, the present study was undertaken to evaluate its possible hepatoprotective

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effects on carbon tetrachloride (CCl_4)-induced hepatic damage in rats.

MATERIALS AND METHODS

Collection of plant material and preparation of extract

Bark of *S. racemosa* was collected from the local area of Pune in the month of November and authenticated at the Agharkar Research Institute, Pune (Auth09-131). The bark was coarsely powdered and defatted with petroleum ether (60-80°C). The marc was subjected to maceration for seven days in ethanol (95%) with daily occasional shaking. This ethanol extract of *S. racemosa* (EESR) was evaporated to dryness under reduced pressure (% yield = 6% w/w).

Phytochemical analysis of ethanol extract of *Symplocos racemosa*

The EESR was analyzed for preliminary phytochemical tests for the presence of carbohydrates, alkaloids, glycosides, sterols, flavonoids, phenolics, and triterpenoids.[14]

HPTLC profile

EESR 10 mg was dissolved in 10 ml of methanol and sample of 5, 10, and 20 µl were applied as 8-mm wide bands, under a continuous flow of nitrogen, using CAMAG LINOMATE V automatic sample applicator. Sample was applied with a 100-µl syringe (Hamilton Bonaduz, Switzerland) at a constant application rate of 0.1 µl/s and the distance between adjacent bands was 15 mm. The plate was developed using the solvent system methanol : benzene : chloroform [4 : 4.5 : 3.5 (v/v)] and scanned by a densitometer (CAMAG) at 580 nm.

Experimental animals

Wistar rats (150-200 g) and albino mice (20-25 g) of either sex were procured from National Institute of Bioscience, Pune, and housed in an environmentally controlled room, maintained at uniform standard laboratory conditions. They were provided with food and water *ad libitum*. The animals were acclimatized for seven days before experiments were performed. The animal studies were approved by Institutional Animal Ethics Committee (SCOP/IAEC/Approval/2009-10/11) of Sinhgad College of Pharmacy, Pune-411041, India.

Acute oral toxicity test

The acute oral toxicity study for EESR was carried out according to OECD guidelines 423.[15] Swiss albino mice were fasted overnight, water also being withheld. The EESR was administered at a dose of 2 000 mg/kg. Animals were observed individually during the first 30 minutes and periodically during 24 hours, with special attention given during the first 4 hours and daily thereafter, for a total 14 days.

Experimental design

Wistar rats were divided into five groups (n = 5). Group I (Normal Control) was served as control and received 2% acacia solution. Groups II (CCl_4 Control) to V were injected daily with a mixture of CCl_4 and olive oil (1 : 1) at a dose of 0.2 ml/kg, *i.p.* for 10 days. Group III (Silymarin) served as the standard group and were administered silymarin (100 mg/kg, *p.o.*). Groups IV (EESR200) and V (EESR400) were treated with 200 and 400 mg/kg, *p.o.* EESR, respectively, for 14 days.[16]

Evaluation of thiopentone-induced sleeping time

Thiopentone sodium (40 mg/kg, *i.p.*) was administered to all groups (I to V) of rats on the last day, and the time between loss of the righting reflex and its recovery was noted.[17] The hepatoprotective activity, expressed as percentage hepatoprotection (H), was calculated using the following equation,

$$H = [1-(T-V)/(C-V)] \times 100$$

Where, T = mean value of treatment group, C = mean value of CCl_4 control group, and V = mean value of normal control group.

Evaluation of biochemical parameters

All the rats were sacrificed on 14th day, 30 minutes after the administration of the last dose of test or standard drug under light ether anesthesia. Blood samples were collected by the retro orbital method, and allowed to stand for 30 minutes. Serum was separated at 2 500 rpm for 10 minutes and biochemical investigations (*viz.,* aspartate transaminase (AST),[18] alanine aminotransferase (ALT),[18] alkaline phosphatase (ALP),[19] bilirubin,[20] albumin,[21] and total proteins[22]) were carried out by spectrophotometric method (UV1800, Shimatzu, Japan) using commercial diagnostic kits (Biolab, India) (UV1800 is model number not wavelength).

Estimation of *in vivo* liver antioxidant property

The liver of each rat was isolated, washed, and perfused with chilled normal saline. Approximately 1 g was minced and homogenized in 10 ml of 0.15 M KCl solution in an ice bath using a tissue homogenizer. The homogenate was centrifuged at 800 g for 10 minutes at 4°C. The supernatant obtained was used for the estimation of malondialdehyde (MDA), glutathione (GSH), catalase (CAT), and superoxide dismutase (SOD) as described below.

Malondialdehyde

0.2 ml of 8.1% sodium dodecyl sulfate, 1.5 ml of 20% acetic acid solution adjusted to pH 3.5 with NaOH, and 1.5 ml of 0.8% aqueous *thiobarbituric acid* (TBA) were added to 0.2 ml of liver homogenate, the volume was made up to 4 ml with distilled water, heated at 95°C for 60 minutes, and cooled. To this, 5 ml of a (15 : 1, v/v) mixture of

n-butanol and pyridine were added, shaken vigorously, and centrifuged at 4 000 rpm for 10 minutes. The absorbance of the organic layer was read at 532 nm. MDA levels were calculated using the standard curve of malondialdehyde and its level expressed in nmol/mg of protein.[23]

Glutathione

GSH was determined by the method of Beutler and Kelly. 0.2 ml of tissue homogenate was mixed with 1.8 ml of Ethylenediaminetetraacetic acid (EDTA) solution. To this, 3.0 ml precipitating reagent (1.67 g of metaphosphoric acid, 0.2 g of EDTA disodium salt, 30 g sodium chloride in 1 000 ml of distilled water) was added, mixed thoroughly, and kept for 5 minutes before centrifugation. To 2.0 ml of the filtrate, 4.0 ml of 0.3-M disodium hydrogen phosphate solution and 1.0 ml of DTNB (5, 5-dithio bis 2-nitro benzoic acid) reagent were added and read at 412 nm. GSH activity was calculated using the standard calibration curve, and expressed as µg/mg of protein.[24]

Catalase

To 100 µl of liver homogenate, 1.9 ml of phosphate buffer (pH 7) was added and absorbance was read at 240 nm. The reading was taken again 1 minute after adding 1 ml of 1 nM Hydrogen peroxide solution to the reaction mixture. One international unit of catalase utilized is that amount that catalyzes the decomposition of 1 nM H_2O_2/min/mg of protein at 37°C. Catalase activity was calculated using the standard calibration curve, and expressed as µg/mg of protein.[25]

Superoxide dismutase

To 100 µl of 10% liver homogenate, 1 ml of sodium carbonate (1.06 g in 100 ml water), 0.4 ml of 24 mM NBT (nitroblutetrazolin), and 0.2 ml of EDTA (37 mg in 100 ml water) was added and the zero minute reading was taken at 560 nm. The reaction was initiated by addition of 0.4 ml of 1 mM hydroxylamine hydrochloride, incubated at 25°C for 5 minutes, and the reduction of NBT was measured at 560 nm. SOD level was calculated using the standard calibration curve, and expressed in µg/mg of protein.[26]

Histopathological examination

A portion of liver tissue in each group was preserved in 10% formaldehyde solution for histopathological studies. Hematoxylin and eosin were used for staining; later, the microscopic slides of the liver cells were photographed.

Statistical analysis

Values were expressed as mean ± SEM. The data were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test.

RESULTS

Preliminary phytochemical screening

Preliminary phytochemical screening showed presence of carbohydrates, alkaloids, glycosides, sterols, flavonoids, phenolics, and triterpenoids constituents in EESR.

HPTLC analysis of ethanol extract of *Symplocos racemosa*

Optimized High Performance Thin Layer Chromatograme (HPTLC) of EESR at 580 nm in Figure 1 showed the presence of total nine components with their Rf value and concentration sequentially as Rf - 0.13 (17.45%), 0.23 (37.46%), 0.42 (1.51%), 0.54 (10.82%), 0.66 (5.97%), 0.73 (3.98%), 0.77 (11.15%), 0.85 (4.73%), and 0.90 (6.93%). Component number 2 at 0.23 Rf showed maximum concentration.

Acute toxicity study

EESR administered at a dose of 2 000 mg/kg did not show any signs or symptoms of toxicity or mortality during the observation period. The starting dose was selected as $1/10^{th}$ and $1/5^{th}$ of 2 000 mg/kg.

Assessment of biochemical parameters

Significant hepatotoxicity was observed after 10 days administration of CCl_4, as indicated by increases in serum AST, ALT, ALP [Figure 2], and bilirubin [Figure 3], although decrease in albumin and total protein levels [Figure 4]. EESR (200 and 400 mg/kg/, *p.o.*) exhibited an ability to counteract the CCl_4-induced hepatotoxicity by significantly decreasing the AST, ALT, ALP, and bilirubin levels, and increases in albumin and total protein levels, compared with CCl_4 control rats. The results of EESR treatment were similar to that of the standard drug silymarin.

Assessment of thiopentone-induced sleeping time

Administration of CCl_4 for 10 days resulted in significant ($P<0.01$) increase in thiopentone-induced sleeping time. Treatment with EESR (200 and 400 mg/kg, *p.o.*) and silymarin (100 mg/kg, *p.o.*) daily for 14 days reduced thiopentone-induced sleeping time compared with CCl_4 control rats [Figure 5].

Assessment of *in vivo* antioxidant property

The administration of CCl_4 for 10 days resulted in increase in liver MDA and decrease in GSH, CAT, and SOD levels compared with normal controls rats. Treatment with EESR (400 mg/kg, *p.o.*) daily for 14 days showed significant decrease in liver MDA and increases in GSH, CAT, and SOD levels, whereas EESR (200 mg/kg, *p.o.*) only increased GSH and CAT levels significantly compared with CCl_4 control rats [Figures 6-9].

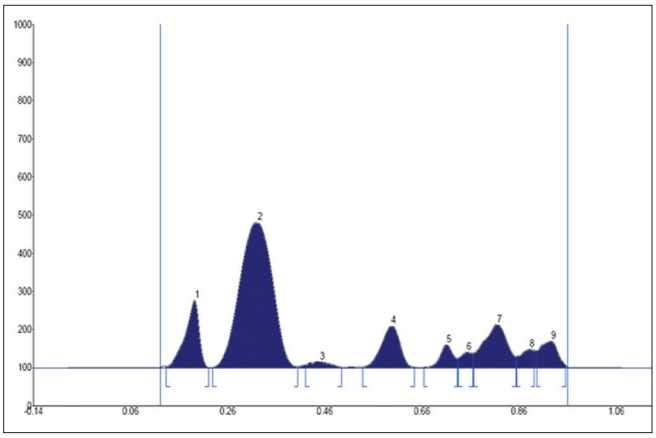

Figure 1: Optimized HPTLC chromatogram of EESR at 580 nm

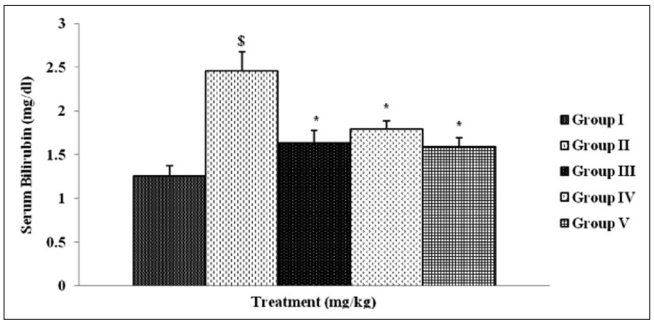

Figure 3: Effect of EESR on serum bilirubin ; Values are expressed as mean ± SEM; *n* = 5 for each group. $*P*<0.001 compared with normal control group and **P*<0.05

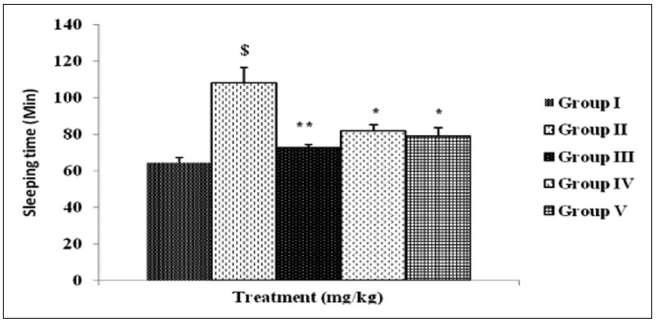

Figure 5: Effect of EESR on sleeping time; Values are expressed as mean ± SEM; *n* = 5 for each group. $*P*<0.001 compared with normal control group and **P*<0.05, ***P*<0.01 compared with CCl4 control group

Assessment of histopathological examination

Liver of the normal control rats were without any pathological changes or abnormalities. CCl_4 control rats showed various degrees of pathological changes, starting from centrilobular necrosis of hepatic cells to central lobular fatty degeneration. Sections of liver taken from the rats treated with standard drug silymarin showed a hepatic architecture similar to that of normal control rats. In contrast, EESR (200 and 400 mg/kg, *p.o.*) attenuated the pathological changes and showed significant protection against CCl_4-induced hepatic damage [Figure 10].

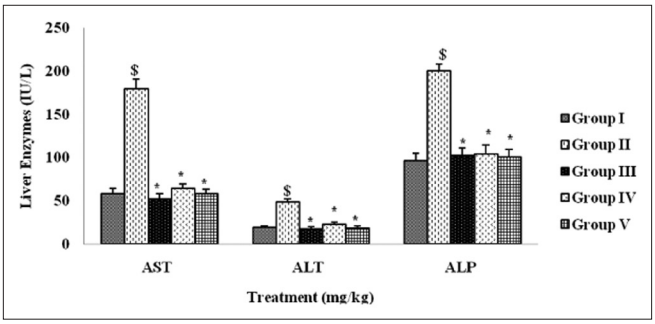

Figure 2: Effect of EESR on liver enzymes; Values are expressed as mean ± SEM; *n* = 5 for each group. $*P*<0.001 compared with normal control group and **P*<0.001 compared with CCl4 control group

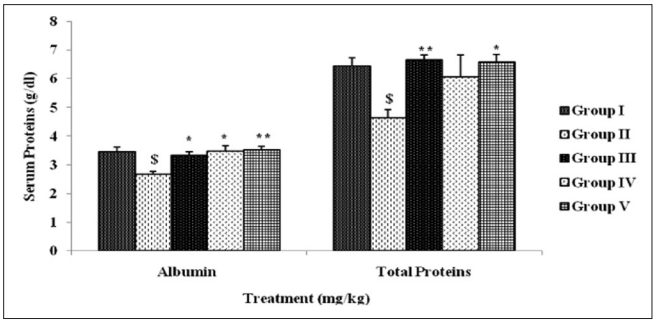

Figure 4: Effect of EESR on serum proteins; Values are expressed as mean ± SEM; *n* = 5 for each group. $*P*<0.001 compared with normal control group and **P*<0.01, ***P*<0.001 compared with CCl4 control group

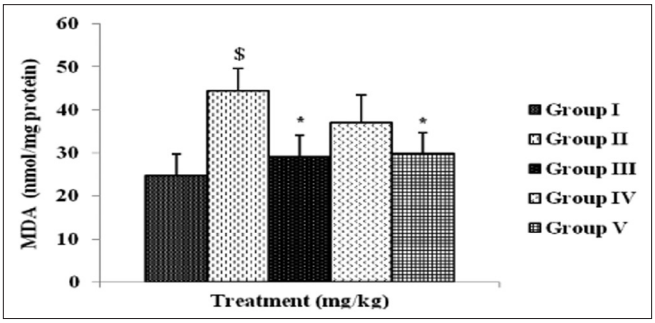

Figure 6: Effect of EESR on serum MDA; Values are expressed as mean ± SEM; *n* = 5 for each group. $*P*<0.05 compared with normal control group and **P*<0.05 compared with CCl4 control group

DISCUSSION

The liver is a versatile organ concerned with regulation of the internal chemical environment. Damage to the liver by a hepatotoxic agent is therefore of grave consequence. In the present study, phytochemical investigation of EESR showed the presence of carbohydrates, alkaloids, glycosides, sterols, flavonoids, phenolics, and triterpenoids. It has been reported that plants possessing flavonoids are responsible for hepatoprotective activity due to their antioxidant property.[27]

CCl_4 is commonly used to induce hepatotoxicity in animal models.[28] Metabolic processes convert CCl_4 into

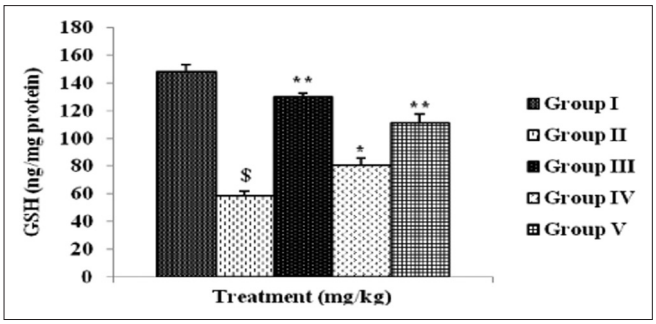

Figure 7: Effect of EESR on serum GSH; Values are expressed as mean ± SEM; n = 5 for each group. $*P*<0.001 compared with normal control group and **P*<0.01, ***P*<0.001 compared with CCl4 control group

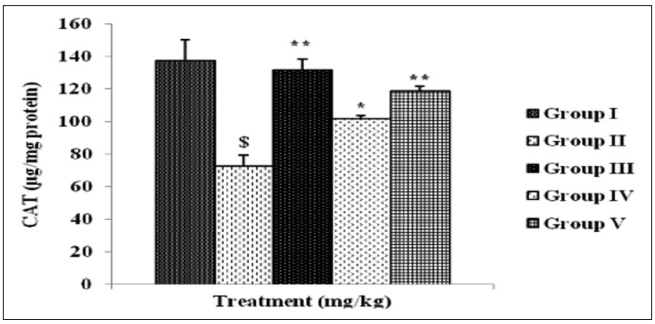

Figure 8: Effect of EESR on serum CAT; Values are expressed as mean ± SEM; *n* = 5 for each group. $*P*<0.01 compared with normal control group and **P*<0.01, ***P*<0.001 compared with CCl4 control group

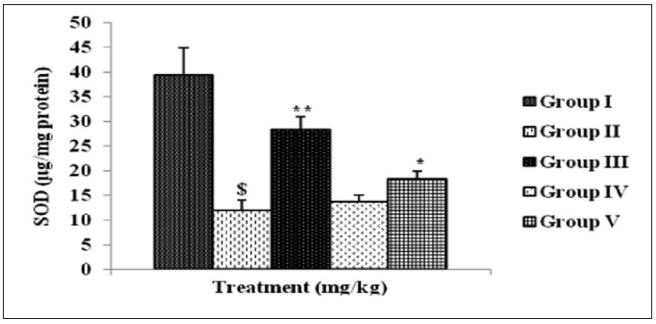

Figure 9: Effect of EESR on serum SOD; Values are expressed as mean ± SEM; *n* = 5 for each group. $*P*<0.05compared with normal control group and **P*<0.05, ***P*<0.01 compared with CCl4 control group

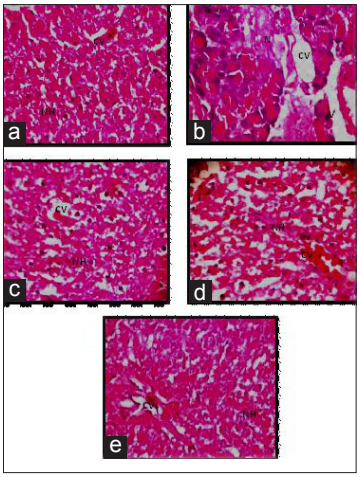

Figure 10: Transverse section of liver hepatic cells of (a) Normal control (b) CCl4 control (c) silymarin (100 mg/kg) (d) EESR (200 mg/kg) (e) EESR (400 mg/kg) (H and E stain, at × 400); CV - Central vein, NH - Normal hepatocyte, N - Necrosis, V- Vacuolization

the trichloromethyl radical (CCl_3-) which interacts with O_2 to yield the highly reactive trichloromethylperoxy radical (CCl_3O_2-). Both radicals are capable of binding to proteins and lipids or abstracting a hydrogen atom from unsaturated lipids, which induces lipid peroxidation and leads to changes in the endoplasmic reticulum, reduction in protein synthesis, and elevation of serum transaminase enzyme levels.[29,30]

Normal liver functions are characterized by balanced activities of the enzymes AST, ALT, and ALP (used as serum marker enzymes), which are found in high concentrations in the cytoplasm of liver cells. In hepatic injury, the lysosomal instability due to CCl_4 leads to leakage of these marker enzymes into the blood stream.[31] In the present study, significant increase in the serum marker enzymes, *viz.,* AST, ALT, and ALP, while decrease in the level of albumin and total protein was observed in CCl_4-treated rats. EESR, like silymarin, significantly reduced the elevated levels of liver enzymes, and increased the levels of albumin and total protein, indicating hepatoprotection. This might have been due to regeneration of hepatocytes with no evidence of inflammatory infiltration.

Drug-induced liver injury (DILI) is a major health problem that challenges not only healthcare professionals, but also the pharmaceutical industry and drug regulatory agencies. In general, the type of liver injury that leads to severe DILI is a predominantly hepatocellular injury; when sufficient to cause hyperbilirubinemia, it is an ominous indicator of the potential for a drug to cause serious liver injury. Serum bilirubin is one of the most sensitive tests employed in the diagnosis of hepatic diseases. It provides useful information about how well the liver is functioning. Bilirubin, a chemical breakdown product of hemoglobin, is conjugated with glucuronic acid in hepatocytes to increase its water solubility. Unconjugated hyperbilirubinemia may be the result of mass inhibition of the conjugation reaction, and consequent release of bilirubin itself from damaged hepatocytes.[32] Serum bilirubin levels decreased significantly in rats treated with EESR (200 and 400 mg/kg,p.o.) and silymarin.

Liver is the primary site for the metabolism of xenobiotics like barbiturates. Hepatic damage requires longer time to inactivate thiopentone, resulting in prolonged loss of righting reflex induced by short acting barbiturates. EESR stimulates liver drug metabolizing enzymes and

considerably shortens the duration of thiopentone-induced sleeping time, which provides indirect evidence for their hepatoprotective activity.[33]

Lipid peroxidation as measured by MDA formation increased in the liver tissue of rats treated with CCl_4.[34] Administration of EESR 400 mg/kg significantly decreased the MDA formation in the liver tissues. These results suggest that EESR interacts with polyester fatty acids and inhibits the enhancement of lipid peroxidation processes leading to MDA formation.[35]

A major defense mechanism involves the antioxidant enzymes, GSH, CAT, and SOD, which convert active oxygen molecules into nontoxic compounds.[36] Decrease in SOD activity is a sensitive index of hepatocellular damage. SOD scavenges the superoxide anion to form hydrogen peroxide, thus diminishing toxic effects caused the free radical.[37] CAT is an enzymatic antioxidant widely distributed in all animal tissues; highest concentrations are found in erythrocytes and liver cells. CAT decomposes hydrogen peroxide and protects the tissues from highly reactive hydroxyl radicals.[38] Therefore, reduction in the concentration of CAT may result in a number of deleterious effects due to the assimilation of superoxide radical and hydrogen peroxide. Glutathione is one of the most abundant tripeptide, non-enzymatic biological antioxidants present in the liver. It removes free radical species such as hydrogen peroxide and superoxide radicals and maintains membrane protein levels.[39] Reduced levels of GSH are associated with enhanced lipid peroxidation in CCl_4-treated rats. CCl_4-treated rats showed significant decreases in GSH, CAT, and SOD levels compared with controls. In contrast, GSH, CAT, and SOD levels were significantly increased in EESR-treated groups, suggesting enhanced antioxidant properties.

Liver sections from normal (control) animals showed the presence of normal hepatic parenchyma; however, after administration of CCl_4, they showed severe degenerative changes, centrilobular necrosis, and fatty infiltration, indicating severe damage to the liver cytoarchitecture.[40] Results of EESR treatment included dose-dependent mild to normal cytoarchitecture, reduced centrilobular necrosis, fatty filtration, and improvement in hepatoprotection and histopathological changes.

Thus, the ethanolic extract of *S. racemosa* bark showed significant hepatoprotective activity. It should be considered for possible clinical applications for the treatment of liver diseases.

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