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Protective effect of *Euphorbia neriifolia* saponin fraction on CCl₄-induced acute hepatotoxicity

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The present investigation aims at assessing the hepatoprotective effect of saponin fraction isolated from the leaf of *Euphorbia neriifolia* on CCl₄-induced hepatotoxicity on rat. CCl₄ (1.5 mg/kg, i.p) is a potent hepatotoxic agent, which induces peroxidative degeneration of membrane lipids causing hypoperfusion of the membrane. Cytosolic enzymes like SGPT, SGOT and ALP elevates in the blood and hepatic glutathione and SOD decreases. The hepatoprotection of triterpene was compared with silymerin, a well known standard hepatoprotectant. Euphol was isolated from *E. neriifolia* leaf total sapogenin fraction after separation and instrumentation. Pretreatment with total saponin fraction (50, 125 and 175 mg/kg, p.o once a day for 4 days before CCl₄ and continued further for 3 days) attenuated the CCl₄-induced acute increase in serum SGPT, SGOT and ALP activities and considerably reduced the histopathological alterations. Further, saponin fraction reduced thiopentone (4 mg/kg, i.p) induced sleeping time, suggesting the protection of liver metabolizing enzymes. Saponin administration replenished the depleted hepatic GSH and SOD by improving the antioxidant status of the liver. Saponin pretreatment improves bromsulphalein clearance and also increases the cellular viability. These effects substantiate protection of cellular phospholipid from peroxidative damage induced by highly reactive toxic intermediate radicals formed during biotransformation of CCl₄.

Key words: *Euphorbia neriifolia*, triterpenoidal saponin, euphol, β -amyryn, hepatoprotective.

INTRODUCTION

Nature has given us a large number of medicinal plants, some of which are yet to be explored and validated for their medicinal value. The 21st century has seen a paradigm shift towards therapeutic evaluation of herbal products in liver diseases by carefully synergizing the strengths of the traditional medicine with the modern concept of evidence based medicinal evaluation, standard-

dization and randomized placebo controlled clinical trials to support clinical efficacy. Several herbs are known to possess antioxidant properties and may be useful as liver protective agents. Herbs belonging to Euphorbiaceae are reported to have antioxidant proper-ties like triterpenes and flavonoids, both of which are reported to possess hepatoprotective and antioxidant activity (Jyothi et al., 2008a,b)

There are over 1500 species of Euphorbias in the world ranging from annual weeds to trees. *Euphorbia neriifolia* Linn. (Euphorbiaceae) grows widely around the dry, rocky and hilly areas of North, Central and South India. *E. neriifolia* is an herb full of spine, popularly known as 'sehund' or 'thohar' in Hindi. Leaves are thick succulent, 6 - 12 inch long and ovular in shape. In traditional system, leaves are used as aphrodisiac, diuretic, cough and cold treatment, bleeding piles and in ana-rectal fistula (Kirtikar and Basu, 1996). The tribal population of Chattisgarh region uses the milky latex as an ingredient of aphrodisiac

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Abbreviations: ENSF, *Euphorbia neriifolia* leaf sapogenin fraction; LD₅₀, half maximal lethal dose; CCl₄, carbon tetrachloride; SOD, superoxide dismutase; MDA, malondialdehyde; SGOT, serum glutamic oxaloacetic transaminase; SGPT, serum glutamic pyruvic transaminase; ALP, alkaline phosphatase; NAD, nicotinamide adenine dinucleotide; BSP, bromsulphthalein.

mixture. Latex is used to de-root skin warts, earache and in arthritis (Anonymous, 1976). The plant is bitter, laxative, carminative, improves appetite, useful in abdominal troubles, bronchitis, tumors, leucoderma, piles, inflammation, enlargement of spleen, anemia, ulcers, fever and in chronic respiratory troubles. Natives of Chattisgarh use externally boiled 'thohar' milk in castor oil with salt to cure the deep cracks in soles of legs. The milk of 'thohar' is also used commonly like aloe gel in case of burns. 'Thohar' milk can be used successfully for healing of wounds. Application of lukewarm 'thohar' leaves reduces itching pain and swelling in piles (Oudhia, 2003).

E. neriifolia hydroalcoholic extract was found to contain sugar, tannins, flavonoids, alkaloids and triterpenoidal saponin on preliminary phytochemical analysis. Several triterpenoids like glut-5-en-3 β -ol, glut-5(10)-en-1-one, taraxerol and β -amyryn has been isolated from powdered plant, stem and leaves of *E. neriifolia* (Anjaneyulu and Ramachandra, 1965). Antiquorin has been isolated from ethanol extract of fresh root of *E. neriifolia* (Ng, 1990). Neriifolione, a triterpene and a new tetracyclic triterpene named nerifoliene along with euphol, was also isolated from the latex of *E. neriifolia* (Ilyas et al., 1998; Mallavadhani et al., 2004).

E. neriifolia latex showed wound healing activity in guinea pig by increasing epithelization, angiogenesis, tensile strength and DNA content in wounds (Rashik et al., 1996). We have already reported mild central nervous system (CNS) depressant, wound healing and immunomodulatory activity of leaf hydro-alcoholic extract (Bigoniya and Rana, 2005, 2007, 2008a, b). Saponin separated from *E. neriifolia* leaf contains euphol as a major constituent. Total saponin possesses good hemolytic and *in-vitro* antioxidant activity but it is devoid of antibacterial activity of upto 10 mg/ml concentration (Bigoniya and Rana, 2006). The present study was designed to assess the hepatoprotective effect of separated and purified *E. neriifolia* leaf saponin fraction.

MATERIALS AND METHODS

Plant material

E. neriifolia leaves were collected from cultivation field hedge plants of suburban areas of Bhopal (latitude 23.21°, longitude 77.84°, BHOP), Madhya Pradesh, India, in September 2005. The plant was identified with the help of available literature and authenticated by Dr. AP Shrivastava, taxonomist and Principal, P.K.S Government Ayurveda College and Institute, Bhopal, India. A voucher specimen was deposited in the herbarium of the department (No. 1085).

Extraction, isolation and characterization of compound

One kilogram (1 Kg) of dried powder of leaf was extracted with cold ethanol (70%) by maceration for seven days and solvent was removed under reduced pressure. Ethanolic extract gives positive test for reducing sugar, tannins, flavonoids, alkaloids, triterpenoidal saponin and absence of glycoside and fixed oil. The crude ethanolic

extract was resuspended in water and chloroform in HCl (50% v/v) was added to carry out acidic hydrolysis of saponin to isolate sapogenins. Chloroform phase was separated and concentrated under 40°C up to 1/3 of the original volume. Chloroform phase was exhaustively extracted three times with water saturated n-butanol and solvent was removed under reduced pressure. Brown colour dried powder with 2.41% of yield represents the crude sapogenin mixture and was designated as *E. neriifolia* leaf sapogenin fraction (ENSF). It showed positive results for Salkowski and Noller's test.

Total sapogenin was subjected to column chromatography on silica gel using chloroform, solvent gradient of chloroform/ethyl acetate (80:20, 60:40, 40:60 and 20:80), ethyl acetate and methanol. Five fractions were collected and chromatographed on silica gel G plates using CHCl₃ : MeOH (50:50), fractions obtained are: Fraction-1 (mixture), Fraction-2 (ENS-1) which gives Rf value of 0.385, Fraction-3 (ENS-2) which gives Rf value of 0.360, Fraction-4 (no spot) and Fraction-5 (ENS-3) which gives Rf value of 0.314.

Fraction 3 gives positive test for Salkowski and Noller's test and negative result for Libermann Burchard test, indicating presence of triterpene. The residue obtained was carefully crystallized on methanol which gives a solid, white crystal (232 mg), m.p. 116°C. UV δ_{\max} 270 nm; IR (KBr, cm⁻¹): 3400 and 1030 (3- β -OH), 2923 and 2854, 1637 (-C=C-), 1461 and 1376, 925, 862, 802 and 723; ¹H NMR (CDCl₃ 300 MHz): δ 5.43 (1H, t, H-24), 3.29 (1H, m, H-3 β) 1.62 (Me-26), 1.77 (Me-27), 0.74 (Me-18), 0.85 (Me-19), 0.91 (Me-28), 1.16 (Me-29), 1.21 (Me-30), 1.12 (Me-21); ¹³C NMR (CDCl₃ 75 MHz): δ 81.01 (C-3), 135.62 (C-8), 37.26 (C-20), 129.20 (C-24), 20.18 (C-21), 17.52 (C-18), 21.14 (C-19), 18.72 (C-26), 27.32 (C-27), 17.78 (C-28), 26.56 (C-29), 30.14 (C-30); EIMS *m/z* (%): 426 (M⁺), 408 (M⁺-H₂O), 297 0.24% M⁺-H₂O-C₈H₁₅ and other fragments suggesting the fragmentation of side chain 111 (16.42% C₈H₁₅), 97 (25.08% C₇H₁₃), 83 (42.96% C₆H₁₁), 69 (70.93% C₅H₉), 55 (66.15% C₄H₇) and 41 (100% C₃H₅). All the data were compared with published data for Euphol from *Kansui Radix* as reported by Lin et al. (2000).

Experimental animals

Laboratory bred Wistar albino rats of both sexes (150 - 200 g) were maintained under standard laboratory conditions at 22 \pm 2°C, relative humidity 50 \pm 5% and photoperiod (12 h light: 12 h dark), were used for the experiment. Commercial pellet diet (Hindustan Lever, India) and water were provided *ad libitum*. Experimental protocol was approved by the Institutional Animal Ethical Committee (approved body of Committee for the Purpose of Control and Supervision of Experiments on Animals, Chennai, India) of Dr. H.S. Gour University, Sagar, India and care given to the animal was as per the WHO 'guidelines for the care and use of animals in scientific research'.

Determination of LD₅₀

Acute oral toxicity of ENSF was determined according to the guidelines of Organization for Economic Co-operation & Development (OECD) following the Up & Down method (OECD guideline No. 425) and Fixed dose method (OECD guideline No. 420). Based on these methods, a limit test was performed to categorize the toxicity class of the compound and then main test was performed to estimate the exact LD₅₀. The animals (nulliparous and non-pregnant female Wistar albino rats) were fasted overnight with free access to water, weighed and a single dose of the test substance was administered. Animals were observed individually during first 30 min, periodically during 48 h with special attention given during first 4 h (short-term toxicity) and daily, thereafter for

total of 14 days (short-term toxicity). LD₅₀ was found to be greater than 2000 mg/kg, in limit test. The test substance could be classified in the hazard classification as Class 4, 300 mg/kg <LD₅₀ <2000 mg/kg in the globally harmonized system (GSH). LD₅₀ of ENSF was found to be 979.24 mg/kg from main test (Diener et al., 1995). A dose range of 50, 100 and 150 mg/kg was selected for ENSF.

Hepatoprotective activity study

In view of multiplicity and complexity of the liver functions, it is obvious that no single test can establish the disturbances in liver function. Thus, a battery of liver function test was employed for accurate diagnosis, to assess the severity of damage, judge prognosis and evaluate therapy.

The rats were divided into six groups of six animals each. Animals of group I- vehicle control, group II- negative control (Carbon tetrachloride, 1.5 mg/kg, i.p), group III- silymerin (20 mg/kg), group IV, V and VI were administered *E. neriifolia* saponin (ENSF) at a dose of 50, 125 and 175 mg/kg, p.o, respectively. Vehicle used for ENSF was propylene glycol and tween 80 in 4:1 ratio. All the animals of group I, III and IVth to IXth were treated with different doses of vehicle, silymerin and ENSF for four days. On the 4th day, 2 h after drug administration, all animals including group II were treated with CCl₄ in liquid paraffin (1:1) in a dose of 1.5 ml/kg i.p. Drug treatment schedule was repeated for more three days. All the hepatoprotective parameters were assessed on the last day (7th day), 2 h after drug administration.

Thiopentone induced sleeping time

Sleeping induced by short acting barbiturate is significantly prolonged in the event of any hepatic damage and this can be used as a measure of the function of the drug metabolizing enzymes. On the 7th day, a single dose of thiopentone (4 mg/kg, i.p.) was given to the animals and the time between loss of the righting reflex and its recovery was taken as duration of thiopentone induced sleeping time (Singh et al., 2001b).

Effect on serum biochemical parameters

7th day of treatment, all the animals were sacrificed to collect liver and blood samples. Blood samples were centrifuged at 3000 rpm for 5 min, serum was collected and analyzed for estimation of biochemical parameters, that is, alkaline phosphatase (Kind and King, 1980), glutamate pyruvate transaminase and glutamate oxaloacetate transaminases (Reitman and Frankel, 1957), total and direct bilirubin (Jendrassik and Gorf, 1938), total protein (Lowry et al., 1951), albumin (Keyser, 1962), cholesterol (Allain et al., 1974) and triglyceride (McGowan et al., 1983).

Estimation of free radical scavenging ability of liver

Free radical mediated cell injury plays an important role in chemical induced hepatotoxicity. Liver tissues were excised, weighed, homogenized and supernatant was used for estimation of free radical scavenging ability. Glutathione reduces H₂O₂ directly to water or react directly with the free radicals such as O₂²⁻, OH[·], O[·] by a radical transfer process, which yields thiol radicals. This thiol radical presents in glutathione forms a colored complex with DTNB, which is measured colorimetrically at 412 nm (Tietze, 1969). Lipid peroxidative degradation of biomembranes is one of the principal causes of hepatotoxicity of CCl₄ (Shenoy et al., 2001). Thiobarbituric acid reactive substance of (malondialdehyde) lipid peroxidation

was determined following the method of Okhawa et al. (1979). Superoxide dismutase was estimated as per the method of Misra and Fridovich (1979).

Histopathological studies of liver

The isolated liver slices were fixed in Aqua Bouin's fluid and processed for histopathological assessment of liver damage following method of Nanji et al. (2001).

Viability study of liver cells

Loss of cell viability is most often measured as loss of membrane integrity. This event may be primarily due to necrosis or secondarily due to apoptosis. Trypan blue exclusion is a cell viability assay based on the ability of the liver cells to exclude the trypan blue and uptake of the dye by the dead cells due to alteration in the membrane permeability. Viability was measured by trypan blue exclusion test following the method of William et al. (1971).

Bromsulphalein uptake test

It is generally agreed that in the passage of bromsulphalein (BSP) from the plasma to the bile, it undergoes storage, metabolism and excretion by the liver. It is well documented that CCl₄ produces morphological and functional changes in the liver. Bromsulphalein clearance test is the most sensitive and dependable method to assess the physiological status of liver function. The test indicates the excretory function of the liver. The abnormal functional effects produced by CCl₄ are easily demonstrated by the retention of BSP. Liver slices kept in ice cold phosphate buffer (0.2 M) at pH 7.4 were incubated in media (KCl: 10 mM, MgSO₄: 1 mM, NaCl: 1 mM in phosphate buffer) containing 30 µg BSP/ml at 38°C. An aliquot of reaction mixture was analyzed after 10, 20 and 30 min to determine the concentration of BSP in the media at 580 nm (Rajan and Subrahmanyam, 1965).

Statistical analysis

All data are presented as means ± SEM. Experimental data was analyzed using one-way ANOVA followed by student's t-test to compare the difference between the control and treated values. P value <0.05 were considered significant. Graph Pad Prism Version 3.02 was used for statistical calculations.

RESULTS

Thiopentone induced sleeping time

E. neriifolia saponin fraction showed extremely statistical significant (P < 0.001) hepatoprotective (85.23%) activity by reducing the sleeping time to 24.25 ± 1.62 min compared to 65.72 ± 2.43 min of negative control at 175 mg/kg dose as shown in Table 1.

Effect on serum biochemical parameters

ENSF treatment significantly (P < 0.001) decreased SGOT, SGPT and ALP levels of treated animals at all the

Table 1. Hepatoprotective effect of *E. neriifolia* saponin fraction on thiopentone induced sleeping time against CCl₄ induced liver damage on rats.

Treatment (mg/kg, p.o)	Sleeping time (min)	Hepatoprotection (%)
Vehicle control	17.06 ± 1.50	---
CCl ₄ (1.5, i.p)	65.72 ± 2.43	---
Silymerin (20)	20.56 ± 1.08 ^c	94.86
ENSF (50)	45.78 ± 2.32 ^c	40.98
ENSF (125)	32.67 ± 2.90 ^c	67.92
ENSF (175)	24.25 ± 1.62 ^c	85.23

Sleeping time was expressed in mean ± standard error of mean; n = 6. Percentage of hepatoprotection was calculated using the equation: $H = [1 - (T - V / C - V)] \times 100$. Where T is mean value of group treated with test drug, C is mean value of group treated with CCl₄ alone and V is the mean value of control animals. ^cP < 0.001 when compared to negative control (CCl₄ treated) group.

tested doses compared to negative control group (Figure 1). Triglyceride level was also decreased at 125 and 175 mg/kg doses, which is extremely significant (P < 0.001). ENSF significantly (P < 0.001 and 0.05) increased cholesterol level of serum at 125 and 175 mg/kg doses. Saponin treatment did not have any significant effect towards normalization of albumin and direct bilirubin level in treated animals. ENSF significantly decreased serum total bilirubin (P < 0.001) at 175 mg/kg dose (Table 2).

Estimation of liver free radical scavenging ability

CCl₄ intoxication reduced superoxide dismutase enzyme level expressed in unit/mg of protein in liver tissue (Table 3). ENSF treatment showed significant (P < 0.05) enhancement ability of SOD only at 175 mg/kg dose. ENSF at 175 mg/kg dose showed extremely significant (P < 0.001) reduction in lipidperoxidase and elevation of glutathione.

Histopathological parameters

ENSF treatment showed hepatoprotection by reducing the liver weight of CCl₄ intoxicated animals. Liver weight/100 g of body weight for vehicle control, negative control, silymerin and ENSF (175 mg/kg) were 3.29, 5.18, 3.94 and 4.02 g, respectively (Figure 2).

Liver sections of vehicle control animals indicate normal appearance of hepatic parenchyma. CCl₄ hepatotoxin treated animals showed degenerative changes, bile duct hyperplasia, zonal necrosis plus extensive diffuse vacuolar degeneration engorged with blood and microvesicular fatty changes in hepatocytes which indicates completely damaged cytoarchitecture of liver. Silymerin treated animals showed slightly altered hepatic parenchyma with focal necrosis, lobular necrosis, centrolobular

necrosis and sinusoidal dilation with slightly altered hepatic parenchyma. ENSF (175 mg/kg) treatment showed sinusoidal dilation with mild focal coagulative necrosis and fatty vacuolation of hepatic parenchyma. Histopathological observations and photomicrographs of liver slices are shown in Figure 3.

Viability of liver cells

ENSF treatment at all the tested doses showed extremely significant (P < 0.001) hepatoprotection against CCl₄ by increasing the viable liver cell count. Silymerin showed 87.89% of viable cell compared to 61.56% of negative control and 92.99% of vehicle control, respectively, as shown in Table 4. ENSF at 175 mg/kg dose showed 82.93% viable liver cell.

Bromsulphalein uptake test

Liver slices of ENSF treated animals showed extremely statistical significant (P < 0.001) hepatoprotection (54.33%) at 175 mg/kg dose. It showed 75.44 ± 4.12 µg of BSP uptake per g of liver tissue compared to 42.11 ± 2.38 µg of CCl₄ treated group (Figure 4).

DISCUSSION

Cameron and Karunarathe first reported liver injury due to oral administration of carbon tetrachloride (Cameron and Karunarathe, 1936). CCl₄ induced liver damage in experimental animals is a commonly used model for the screening of hepatoprotective drugs. The hepatotoxic effects of CCl₄ are largely due to biotransformation by the cytochrome P-450 system to active metabolite, trichloromethyl radical. Covalent binding of the trichloromethyl radical to cell protein is considered the initial step in a chain of events that eventually leads to lipid peroxidation of the cell membrane and endoplasmic reticulum. Lipid peroxidation in turn gives products like malondialdehyde (MDA) that cause damage to the membrane. The peroxidative products induce hypoperfusion of the membrane and finally cytosolic enzymes appear in the blood (Recknagel et al., 1989).

Intoxicated liver prolongs duration of sleeping time for hexobarbitone, thiopentone, zoxazolamine and pentobarbitone, etc in animals due to damage of hepatic microsomal drug metabolizing enzymes. Thiopentone induced sleeping time in animals with liver intoxication is increased as the enzyme responsible for metabolism of thiopentone is reduced or destroyed (Singh et al., 2001a). Therefore protective effect exhibited by *E. neriifolia* saponin fraction may be due to protection of hepatic drug metabolizing enzymes as evidenced from decrease in thiopentone induced sleeping in treated animals.

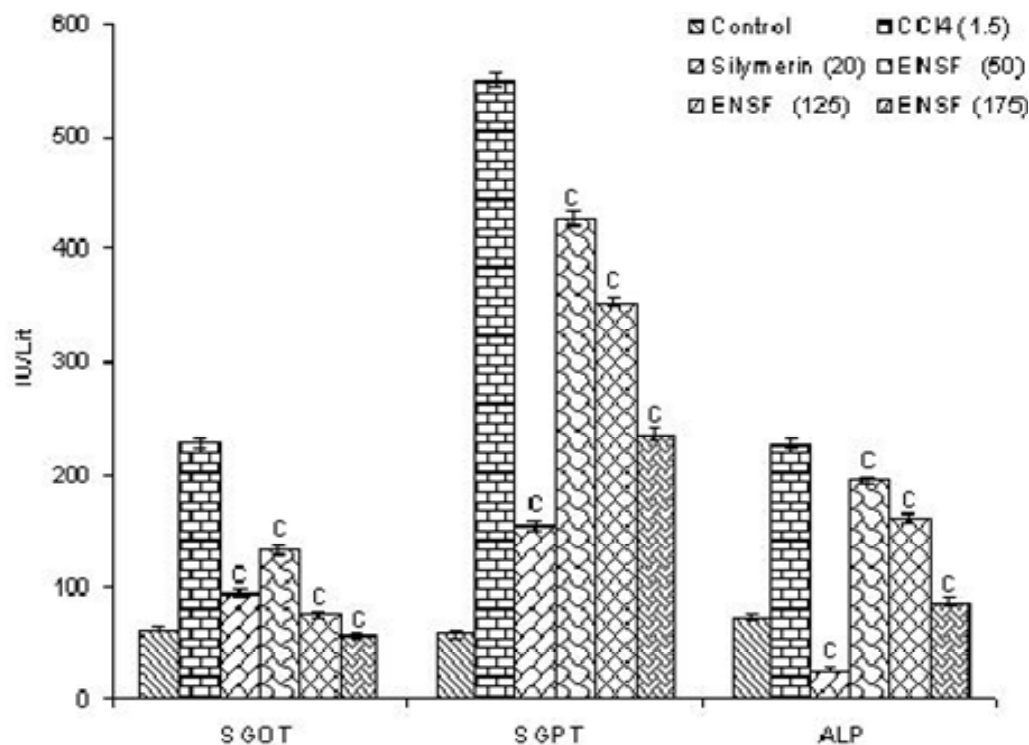


Figure 1. Effect of *E. neriifolia* saponin fraction on serum biochemical parameters of CCl₄ treated rats. n = 6, ^bP < 0.01, and ^cP < 0.001 when compared to negative control (CCl₄ treated) group.

Table 2. Effect of *E. neriifolia* saponin fraction on serum biochemical parameters of CCl₄ treated rats.

Treatment (mg/kg, p.o)	Cholesterol (mg/dl)	Triglyceride (mg/dl)	Protein (g/dl)	Albumin (g/dl)	Bilirubin (mg/dl)	
					Total	Direct
Vehicle control	152.60 ± 3.60	113.50 ± 4.55	8.88 ± 0.34	3.57 ± 0.65	0.75 ± 0.04	0.41 ± 0.02
CCl ₄ (1.5, i.p)	68.92 ± 3.55	220.05 ± 5.72	5.85 ± 0.40	1.52 ± 0.35	8.36 ± 0.79	5.28 ± 0.68
Silymerin (20)	72.57 ± 3.21 ^{ns}	131.54 ± 4.32 ^c	8.46 ± 0.24 ^a	2.58 ± 0.35 ^{ns}	0.55 ± 0.02 ^c	1.15 ± 0.22 ^c
ENSF (50)	78.33 ± 2.90 ^{ns}	205.60 ± 4.22 ^{ns}	5.92 ± 0.33 ^{ns}	1.39 ± 0.12 ^{ns}	4.25 ± 0.46 ^c	5.96 ± 0.57 ^{ns}
ENSF (125)	89.81 ± 3.22 ^a	163.99 ± 3.33 ^c	6.74 ± 0.56 ^{ns}	2.18 ± 0.06 ^{ns}	1.48 ± 0.25 ^c	5.67 ± 0.58 ^{ns}
ENSF (175)	113.87 ± 3.80 ^c	137.43 ± 3.80 ^c	8.13 ± 0.71 ^a	2.63 ± 0.21 ^{ns}	0.98 ± 0.05 ^c	4.51 ± 0.70 ^{ns}

Values are expressed in mean ± standard error of mean; n = 6. ^aP < 0.05, ^cP < 0.001 and ns = not significant when compared to negative control (CCl₄ treated) group.

Table 3. Effect of *E. neriifolia* saponin fraction on free radical scavenging ability of CCl₄ treated rat liver.

Treatment (mg/kg, p.o)	Superoxide dismutase (unit/mg of protein)	Lipid peroxidase (nmol/g of protein)	Glutathione (µg/g of liver)
Vehicle control	5.72 ± 0.87	3.43 ± 0.68	22.26 ± 1.92
CCl ₄ (1.5, i.p)	1.53 ± 0.22	32.97 ± 1.75	7.46 ± 0.67
Silymerin (20)	4.53 ± 0.62 ^a	5.59 ± 0.60 ^c	19.65 ± 1.40 ^c
ENSF (50)	1.95 ± 0.22 ^{ns}	24.25 ± 1.74 ^b	10.56 ± 1.24 ^{ns}
ENSF (125)	3.05 ± 0.45 ^{ns}	20.34 ± 1.09 ^c	13.50 ± 1.35 ^a
ENSF (175)	4.12 ± 0.76 ^a	14.70 ± 1.22 ^c	17.04 ± 1.06 ^c

Values are expressed in mean ± standard error of mean; n = 6. ^aP < 0.05, ^bP < 0.01, ^cP < 0.001 and ns = not significant when compared to negative control (CCl₄ treated) group.

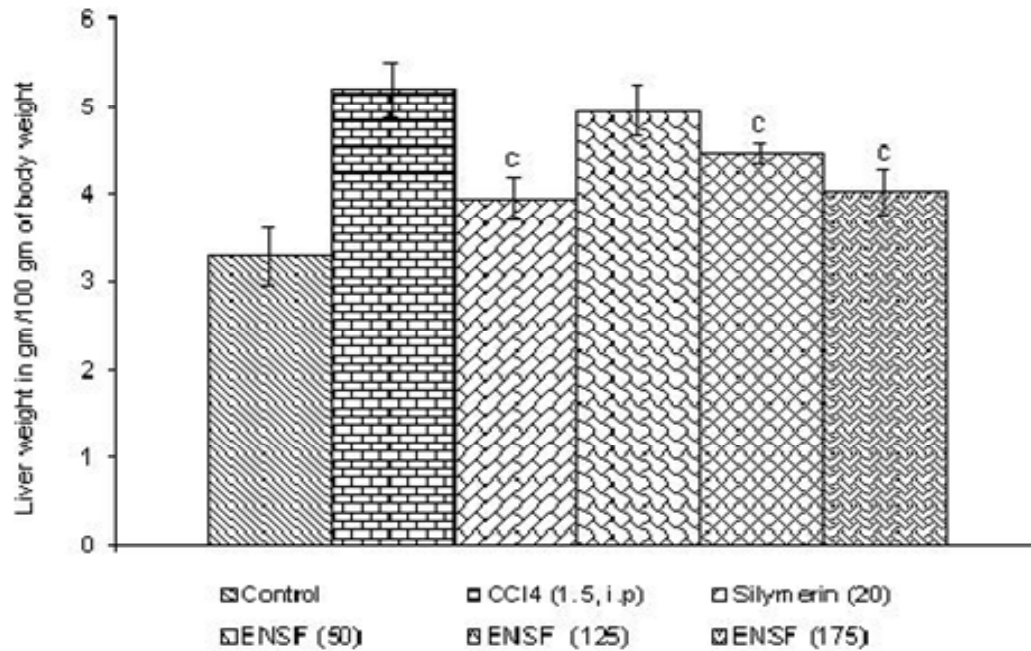


Figure 2. Effect of *E. neriifolia* saponin fraction on relative liver weight of CCl₄ treated rats. n = 6. °P < 0.001 when compared to negative control (CCl₄ treated) group.

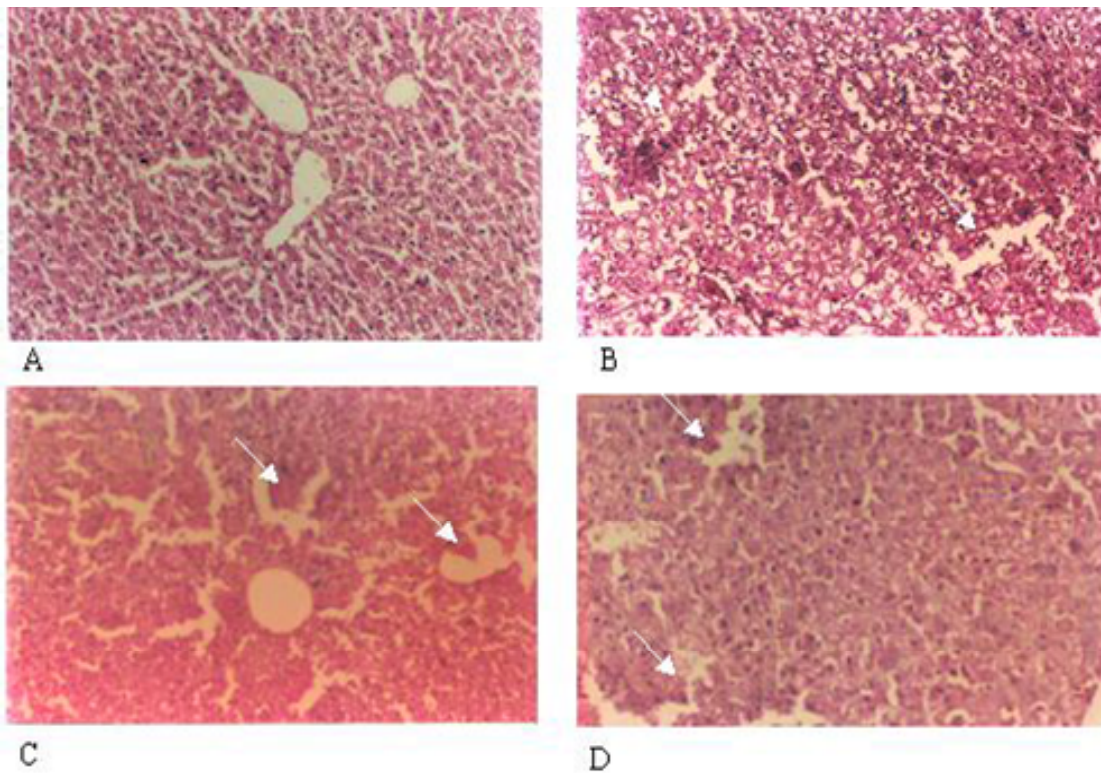


Figure 3. Assessment of CCl₄ induced hepatotoxicity by histopathology in haematoxylin-eosin stained liver sections. A: Section from normal liver tissue. B: Three days after CCl₄ intoxication, liver tissue showing zonal necrosis, extensive diffuse vacuolar degeneration engorged with blood and microvesicular fatty changes in hepatocytes. C: Silymerin treated liver tissue showed focal necrosis, lobular necrosis and sinusoidal dilation with slightly altered hepatic parenchyma. D: ENSF (175 mg/kg) pretreatment showed focal coagulative necrosis, mild fatty vacuolation and dilated sinusoids (10X).

Table 4. Effect of *E. neriifolia* saponin fraction on viability of CCl₄ treated rat liver cells.

Treatment (mg/kg, p.o)	Number of cell counted	Number of viable cell	Percent viable cell
Vehicle control	212.33 ± 4.25	197.45 ± 2.33	92.99
CCl ₄ (1.5, i.p)	205.54 ± 4.78	126.55 ± 2.79	61.56
Silymerin (20)	207.55 ± 4.22	182.43 ± 3.43 ^c	87.89
ENSF (50)	210.17 ± 3.40	132.45 ± 3.67 ^c	63.02
ENSF (125)	207.65 ± 5.82	156.25 ± 3.32 ^c	75.25
ENSF (175)	209.25 ± 4.95	173.55 ± 2.11 ^c	82.93

Values are expressed in mean ± standard error of mean; n = 6. The viability was calculated as percentage from the following equation: % of viable cells = no. of cells excluding dye/total no. of cells counted × 100. ^cP < 0.001 when compared to negative control (CCl₄ treated) group.

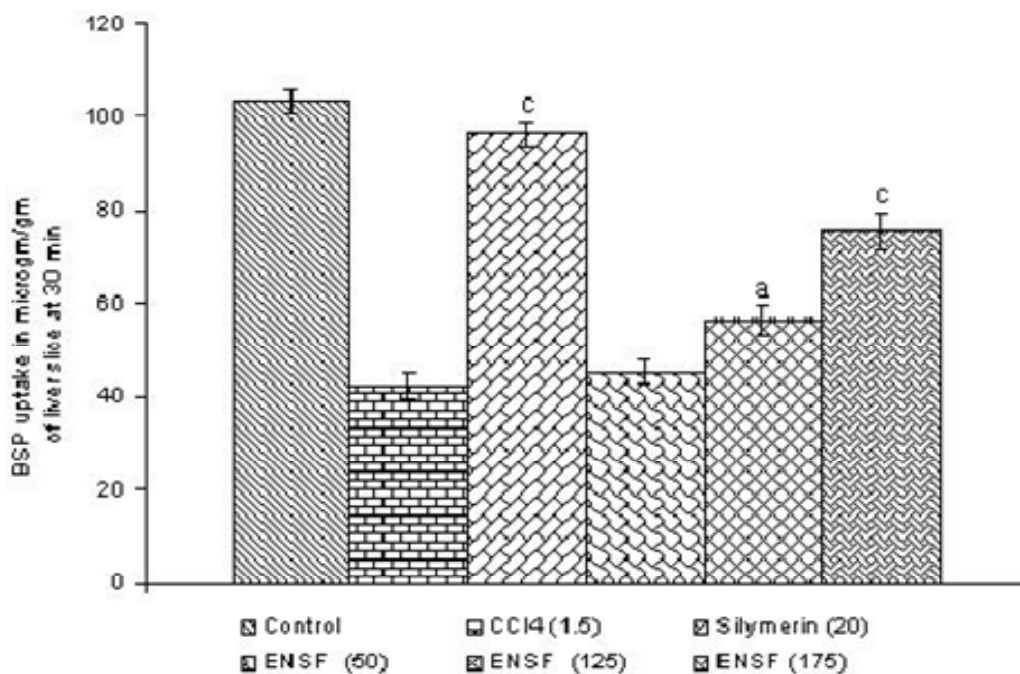


Figure 4. Effect of *E. neriifolia* saponin fraction on BSP uptake of CCl₄ treated rat liver slices. n = 6. ^cP < 0.001 and ns = not significant when compared to negative control (CCl₄ treated) group.

Hepatotoxin CCl₄ get converted into CCl₃O⁻ by liver enzymes and attacks the unsaturated fatty acids of cell membrane in presence of oxygen which consequently gives rise to lipid peroxides which alters the functional integrity of liver mitochondria leading to liver damage. The levels of marker enzymes SGOT, SGPT and ALP are found to be elevated in cytoplasm as well as in blood. Serum triglyceride and bilirubin (total and direct) level elevates, on the other hand serum total protein, albumin and cholesterol level decreases. The tendency of these enzymes to return to a near normal level in ENSF treated rats is a clear manifestation of antihepatotoxic effect of *E. neriifolia*.

Liver toxicants cause disturbances in synthesis and metabolism of triglycerides, cholesterol and lipoproteins,

thus damaging the basic resource for living cells. In CCl₄ toxicity, liver lesion develops within one-two hours, however, late toxic effects may appear after a delay of several hours or two to three days. The increased triglyceride content in the blood is in correlation with the fatty degeneration of the liver (Gergely et al., 1995). The lowering of different serum marker enzyme level is a definite indication of hepatoprotective action of ENSF. ENSF treatment caused a considerable reduction in serum triglyceride content and an increase in cholesterol level may be due to normalization of its synthesis rate.

The lower level of total proteins recorded in the serum of CCl₄ treated rats reveals the severity of hepatopathy. Hypoalbuminemia is most frequent in advanced chronic liver diseases and can be deemed as a useful index of

the severity of cellular dysfunction. ENSF normalizes protein content of CCl₄ intoxicated rats signifying effect on cellular regeneration of macromolecules. Certain drugs like rifampin and probenecid interfere with the net uptake of bilirubin by the liver cell and may produce a mild unconjugated hyperbilirubinemia (Rubin, 1995). Both total and direct bilirubin level rise in disease of hepatocytes, obstruction to biliary excretion into duodenum, hemolysis, in defects of hepatic uptake and conjugation of bilirubin pigment such as in Gilbert's disease. Hyperbilirubinemia results from impaired hepatic uptake of unconjugated bilirubin in liver cell injury (Recknagel et al., 1989). ENSF treatment significantly reduced the elevated serum level of total bilirubin.

Animals of negative control group significantly lost their body weight and showed reduced food consumption as compared to control group. Liver toxicity increases liver weight due to fatty changes along with fall in serum lipids, as in paracetamol intoxication. Bhanwara et al. (2000) reported this as a parameter in ascertaining the hepatoprotective effect of drugs. ENSF treatment showed a significant reversal towards loss of body weight suggesting normalization of CCl₄ toxicity inducing regeneration of hepatocytes.

Highly reactive CCl₃[·] radical induced lipid peroxidation, disturbs Ca²⁺ homeostasis and finally results in cell death. ENSF treatment significantly increased the number of viable hepatocytes, which may be attributed to the inhibition of toxin induced free radical generation and in turn stabilizing the cell membrane. Increase in glutathione activity indicates the restoration of vital molecules such as NAD, cytochrome and glutathione. Restoration of SOD can help in cellular defense mechanism by preventing cell membrane oxidation. ENSF significantly decreased lipid peroxidation, by virtue of antioxidant activity. The saponin fraction preserved the structural integrity of the plasma cellular membrane of the hepatocytes and protected it from breakage by the reactive metabolites produced. ENSF at higher dose have significantly improved the capacity of the damaged liver to take up BSP. This increased uptake of BSP by the liver slices showed that it enhanced capacity to excrete the dye from the blood.

Simultaneous treatment of ENSF with CCl₄ exhibited less damage to the hepatic cells as compared to the rats treated with CCl₄ alone. Hepatocytes showed normal appearance, only some cells showed higher number of vacuoles in the cytoplasm. Although ENSF did not appear to bring a complete reversal of drug-induced injury in the liver but had minimized the effect of CCl₄. The histopathological observations showed a faster regeneration of hepatic cells which seem to suggest the possibility of ENSF being able to condition the hepatic cells to a state of accelerated regeneration, thus decreasing the leakage of SGPT, SGOT and ALP into the circulation (Hewawasam et al., 2003).

The hepatoprotective activity of *E. neriifolia* saponin fraction may be due to the presence of phytoactive compounds which prevents liver plasma membrane alteration

and promotes liver cell repair. The observed protective effect can be attributed to the presence of triterpenoidal saponin containing several triterpenoids like glut-5-en-3 β -ol, taraxerol, β -amyirin and euphol. Plants containing triterpenoidal constituents are reported to have hepatoprotective activity against ethanol, paracetamol, cadmium, ter-butyl hydroperoxide, galactosamine, aflatoxin and carbon tetrachloride. Hepatoprotective effect of ENSF correlates to its all ready reported free radical scavenging and antioxidant activity (Bigoniya and Rana, 2006). Hepatoprotective and antioxidant activity of *Euphorbia antiquorum* containing euphol (Jyothi et al., 2008a), *Euphorbia tirucalli* (Jyothi et al., 2008b) and *Euphorbia nematocypha* (Ito et al., 1990) are reported. Alpha and beta-amyirin had been reported to have hepatoprotective activity against cadmium-induced hepatotoxicity and acetaminophen-induced liver injury, which is predominantly attributed to its antioxidant activity (Oliveira et al., 2005). In conclusion, this study supports the traditional use of *E. neriifolia* leaf as a herbal remedy for jaundice and other liver disorders, suggesting the feasibility of developing herbal formulation and clinical studies.

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