

# Evaluation of protective effect of *Sapindus mukorossi* saponin fraction on CCl<sub>4</sub>-induced acute hepatotoxicity in rats

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**Aim:** This investigation aimed to assess the hepatoprotective effect of saponin fraction isolated from the fruit pericarp of *Sapindus mukorossi* on carbon tetrachloride (CCl<sub>4</sub>)-induced hepatotoxicity.

**Methods:** Fruit of *S. mukorossi* was collected and authenticated, and dried pericarp powder subjected to extraction with cold ethanol (70%) by maceration followed by isolation of total saponin fraction. Hepatoprotective activity was demonstrated in the CCl<sub>4</sub>-damaged primary monolayer culture. In in vivo studies, pretreatment with total saponin fraction (50,100 and 150 mg/kg per os once a day for 4 days before CCl<sub>4</sub> introduction and continued afterward for 3 days) attenuated the CCl<sub>4</sub>-induced acute increase in serum glutamic pyruvic transaminase, serum glutamic oxaloacetic transaminase, and alkaline phosphatase activities and considerably reduced histopathological alterations. Further, saponin fraction reduced thiopentone-induced (4 mg/kg, intraperitoneal) sleeping time in rats.

**Results:** Saponin fraction pretreatment improves bromsulphalein clearance and also increases cellular viability. Saponin administration replenished depleted hepatic glutathione and superoxide dismutase by improving the antioxidant status of the liver and liver function enzymes. These effects substantiate protection of cellular phospholipids from peroxidative damage induced by highly reactive toxic intermediate radicals formed during biotransformation of CCl<sub>4</sub>.

**Conclusion:** The above findings lead to the conclusion that the saponin fraction of *S. mukorossi* has a protective capability both in vitro on primary hepatocyte cultures and in vivo in a rat model of CCl<sub>4</sub>-mediated liver injury. Hence, we suggest that the inclusion of this *S. mukorossi* fruit pericarp in the management of liver disorders is justified.

**Keywords:** *Sapindus mukorossi*, total saponin content, hepatoprotective

## Introduction

Reactive oxygen species (ROS, such as H<sub>2</sub>O<sub>2</sub>, O<sub>2</sub><sup>2-</sup>, OH<sup>-</sup>) play important physiological functions and can also cause extensive cellular damage. Cells are provided with efficient molecular strategies to strictly control the intracellular ROS level and to maintain the balance between oxidant and antioxidant molecules. Oxidative stress, resulting from an imbalance between the generation of ROS and the antioxidant defense capacity of the cell,<sup>1</sup> affects major cellular components, including lipids, proteins, and DNA. This phenomenon is closely associated with a number of human disorders, including many degenerative diseases such as cardiovascular disease, diabetes, cancer, neurodegenerative disorders,<sup>2,3</sup> and almost all liver pathologies.<sup>4-6</sup> All these conditions appear mostly related to chronic oxidative stress. However, the acute exposure to high levels of ROS seems to be responsible for the development of different

forms of damage during ischemia/reperfusion in the liver.<sup>7,8</sup> Carbon tetrachloride (CCl<sub>4</sub>) is an acute hepatotoxic agent that induces peroxidative degeneration of membrane lipids, causing hypoperfusion of the membrane. Cytosolic enzymes like serum glutamic pyruvic transaminase (SGPT), serum glutamic oxaloacetic transaminase (SGOT) and alkaline phosphatase (ALP) elevate in the blood, and hepatic glutathione and superoxide dismutase (SOD) decrease. Accordingly, effects of antioxidants or free radical scavengers have been widely tested for the prevention and treatment of acute and chronic liver injuries.

A number of medicinal plants are used in traditional medicine for the management of liver disorders. 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 twenty-first century has seen a paradigm shift toward therapeutic evaluation of herbal products in liver diseases, carefully synergizing the strengths of traditional medicine with the modern concept of evidence-based medical evaluation, standardization, 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.<sup>9</sup> Plant saponins are widely distributed amongst plants and have a wide range of biological properties. The more recent investigations and findings on plant saponins have reported many biological activities. In view of these reports, a plant containing saponin was selected to screen for possible hepatoprotective activity.

The genus *Sapindus* belongs to the family Sapindaceae, which has about 2000 species. Most of the species of the *Sapindus* genus are in use for the treatment of several diseases and other commercial purposes. *Sapindus mukorossi*, commonly known as a Reetha, is a deciduous tree. The tree is indigenous to northern and central India and is widely distributed in the Himalayan region, Haryana, Uttar Pradesh, and Chhattisgarh.<sup>10</sup> Traditionally, it is used in the treatment of asthma, snakebite, tooth disorders, piles, dermatological disorders, and hepatic disorders. It is a rich source of potential biological activities.<sup>11–14</sup> A survey of the chemical literature reveals that a great deal of phytochemical work on different parts – fruit, pericarp, seeds, leaves, ripe fruit, roots, and stems – of *S. mukorossi*, *S. saponaria*, *S. trifoliatum*, etc, has been carried out.<sup>15,16</sup> Chemically, the fruit of *S. mukorossi* is valued for the saponins. The present study was designed to assess the hepatoprotective effect of separated and purified *S. mukorossi* saponin fraction.

## Materials and methods

### Chemicals

All chemicals and solvents required for study were obtained from SD chemicals, Mumbai, and HiMedia Laboratories, Mumbai, India. Biochemical estimation kits were procured from Merck India (Mumbai).

### Plant material

An authentic sample of *S. mukorossi* was obtained from authorized herbal supplier Munnalal Dawasas, Hyderabad, India. The plant had been previously identified and authenticated by an expert in the Department of Botany, Osmania University, Hyderabad, India.

### Extraction, isolation, and standardization of saponins fraction

One kilogram of dried fruit powder was extracted with cold ethanol (70%) by maceration for 7 days, and solvent was removed under reduced pressure. Ethanolic extract tests positive for reducing sugar, tannins, flavonoids, alkaloids, and triterpenoidal saponin, and the absence of glycoside and fixed oil. The crude ethanolic extract was resuspended in water, and chloroform in hydrogen chloride (50% v/v) was added to carry out acidic hydrolysis of saponin to isolate sapogenin. The chloroform phase was separated and concentrated under 40°C up to 1/3 of the original volume. The chloroform phase was exhaustively extracted three times with water-saturated *n*-butanol, and solvent was removed under reduced pressure. Brown dried powder with 2.41% of yield represented the crude sapogenin mixture and was designated as *S. mukorossi* saprogenic fraction (SMSF). It showed positive results for Salkowski and Noller's test, indicating the presence of triterpenoids in the saponins fraction.<sup>17</sup>

The isolated saponins fraction was standardized by thin layer chromatography (TLC) profile using precoated silica gel plates as the stationary phase, ethyl acetate:methanol:water (81:11:8), and anisaldehyde-sulphuric acid as the spray reagent. The separation of saponins by TLC revealed the presence of eleven spots in *S. mukorossi*. A compound with an R<sub>f</sub> value of 0.27 confirmed the presence of terpenoid saponins. The TLC profile of this investigation was similar to that reported in the literature. Total saponins fraction was quantified by vanillin-sulphuric acid assay following the procedure of Hiai et al.<sup>18</sup> In brief, 0.5 mL of aqueous solution of the sample, 0.5 mL vanillin solution of 8% (w/v) and then 5.0 mL of sulfuric acid of 72% (w/v) were added and thoroughly mixed in an ice-water bath. The mixture was then warmed in a bath at 60°C for 10 minutes, then cooled in

ice-cold water. Absorbance at 535 nm was recorded against the blank with the reagents using a V-530 UV/VIS spectrophotometer (Jasco, Tokyo, Japan).

### Standard silymarin

Silymarin is a purified extract from milk thistle (*Silybum marianum* (L.) Gaertn), composed of a mixture of four isomeric flavonolignans: silibinin (its main, active component), isosilibinin, silydianin, and silychristin. This extract has been empirically used as a remedy for almost 2000 years, and remains being used as a medicine for many types of acute and chronic liver diseases.<sup>19</sup>

### Experimental animals

Wister albino rats of both sexes, weighing 150–250 g, were acclimatized to the experimental room at 22.2°C, controlled humidity conditions (55%) and 12/12-hour light/dark cycle. They were caged, with a maximum of two animals in one polypropylene cage, were fed with standard food pellets, and water was provided ad libitum supplied by Hindustan Unilever (Mumbai, India). All the studies conducted were approved by the Institutional Animal Ethical Committee of Nizam Institute Pharmacy and Research Institute according to prescribed guidelines of the Indian government's Committee for the Purpose of Control and Supervision on Experiments on Animals.

### Acute oral toxicity studies of SMSF

Acute oral toxicity of SMSF was determined according to the guidelines of the Organisation for Economic Co-operation and Development, following the “up-and-down” method (Guideline 425). Based on the method, a limit test was performed to categorize the toxicity class of the compound and then a main test was performed to estimate the half-maximal lethal dose (LD<sub>50</sub>). The animals were fasted overnight with free access to water, weighed, and a single dose of the test substance was administered. Animals were observed individually for first 30 min, then periodically for 48 hours and daily thereafter for total of 14 days (short-term toxicity). LD<sub>50</sub> was found to be greater than 2000 mg/kg in the limit test. The test substance was able to be classified in the hazard classification as Class 4, 300 mg/kg < LD<sub>50</sub> < 2000 mg/kg, under the Globally Harmonized System. LD<sub>50</sub> of SMSF was found to be 979.24 mg/kg from the main test.<sup>20</sup> Based on this 1/10 of LD<sub>50</sub> value (100 mg/kg body weight), a low dose of 50 mg/kg body weight and a high dose of 150 mg/kg body weight were selected for the study.

### 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. The livers were isolated under aseptic conditions and placed in *N*-2 hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer containing HEPES (0.01 mol/L), NaCl (0.142 mol/L) and KCl (0.0067 mol/L), pH 7.4. The livers were cut into small pieces and then incubated with a second buffer containing HEPES (0.1 mol/L), NaCl (0.0667 mol/L), KCl (0.0067 mol/L) and 0.5% type IV collagenase, pH 7.6, for about 45 min at 37°C in an incubator with constant shaking. Hepatocytes were obtained after filtration and cold centrifugation (4°C, 200 rpm for 2 minutes, three times) and suspended in HEPES buffer. Viability was measured by trypan blue exclusion test following the method of Williams et al.<sup>21</sup>

### 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 CCl<sub>4</sub> produces morphological and functional changes in the liver. The BSP 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 CCl<sub>4</sub> are easily demonstrated by the retention of BSP. On day 7, livers were perfused and part of the liver subjected to the BSP uptake test. Liver slices kept in ice-cold phosphate buffer (0.2 M) at pH 7.4 were incubated in media (KCl 10 mM, MgSO<sub>4</sub> 1 mM, NaCl 1 mM in phosphate buffer) containing 30 mg BSP/mL at 38°C. An aliquot of reaction mixture was analyzed after 10, 20 and 30 minutes to determine the concentration of BSP in the media at 580 nm.<sup>22</sup>

### Isolation of rat hepatocytes

The rat hepatocytes were isolated according to the method of Seglen.<sup>23</sup> The livers were isolated under aseptic conditions and placed in HEPES buffer containing HEPES (0.01 mol/L), NaCl (0.142 mol/L), and KCl (0.0067 mol/L), pH 7.4. The livers were cut into small pieces and then incubated with a second buffer containing HEPES (0.1 mol/L), NaCl (0.0667 mol/L), KCl (0.0067 mol/L), and 0.5% type IV collagenase, pH 7.6, for about 45 minutes at 37°C in an incubator with constant shaking. Hepatocytes were obtained

after filtration and cold centrifugation (4°C, 200 rpm for 2 minutes, three times) and suspended in HEPES buffer. The viability of the hepatocytes was assessed by trypan blue (0.2%) exclusion method.<sup>23</sup>

## Primary cultures of rat hepatocytes

The method of Tingström and Obrink,<sup>24</sup> with slight modifications, was used for the culturing of rat hepatocytes. The freshly isolated viable hepatocytes were suspended in culture medium Roswell Park Memorial Institute 1640 supplemented with calf serum (10%), HEPES, and gentamycin (1 µg/mL). These cells (approximately 1–1.2 × 10<sup>6</sup>/mL) were then seeded into culture bottles and incubated at 37°C in an atmosphere of 5% CO<sub>2</sub> in a carbon dioxide incubator. Upon incubation for 24 hours, the hepatocytes formed a monolayer. The newly formed cells were round, and most appeared as individual cells. These cells were 95%–96% viable, as confirmed by trypan blue exclusion test.

## Hepatoprotective activity study

In view of the multiplicity and complexity of the liver functions, it is obvious that no single test can establish disturbances in liver function. Thus, a battery of liver function tests 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; Tween 80), Group II (negative control; carbon tetrachloride, 1.5 mg/kg, intraperitoneal [IP]), Group III (silymarin; 20 mg/kg body weight), Group IV, V, and VI were administered SMSF at a dose of 50, 100, and 150 mg/kg body weight, per os, respectively, for 4 days. On day 4, 2 hours after drug administration, all animals including Group II were treated with CCl<sub>4</sub> in liquid paraffin (1:1) in a dose of 1.5 mL/kg IP. The drug treatment schedule was repeated for 3 more days. All the hepatoprotective parameters were assessed on day 8, 2 hours after drug administration.

On day 8, all the animals were killed to collect liver and blood samples. Half of the liver was used for estimation of antioxidant enzymes, and the remaining half was subjected to histopathological study. Blood samples were centrifuged at 3000 rpm for 5 minutes, serum was collected and analyzed for estimation of biochemical parameters, ALP,<sup>25</sup> SGPT, SGOT,<sup>26</sup> total and direct bilirubin,<sup>27</sup> total protein,<sup>28</sup> albumin,<sup>29</sup> cholesterol,<sup>30</sup> and triglyceride.<sup>31</sup>

## Thiopentone-induced sleeping time

Sleeping induced by a 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 day 7, a single dose of thiopentone (4 mg/kg, IP) 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.<sup>32</sup>

## 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 H<sub>2</sub>O<sub>2</sub> directly to water or reacts directly with free radicals such as O<sub>2</sub><sup>2-</sup>, OH<sup>-</sup>, and O<sup>-</sup> by a radical transfer process that yields thiol radicals. These thiol radicals present in glutathione form a colored complex with DTNB, which is measured colorimetrically at 412 nm.<sup>33</sup> Lipid peroxidative degradation of biomembranes is one of the principal causes of hepatotoxicity of CCl<sub>4</sub>.<sup>34</sup> Thiobarbituric acid-reactive substance of (malondialdehyde) lipid peroxidation was determined following the method of Okhawa et al.<sup>35</sup> SOD was estimated as per the method of Misra and Fridovich.<sup>36</sup>

## Histopathological studies of liver

The isolated liver slices were fixed in Bouin's fluid and processed for histopathological assessment of liver damage following the method of Nanji et al.<sup>37</sup>

## Statistical analysis

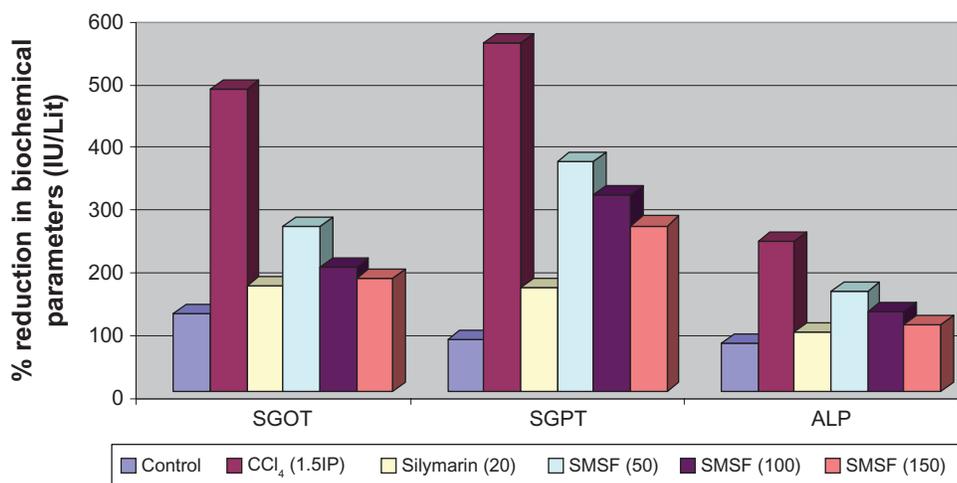
All data are presented as means ± standard error of the mean. Experimental data was analysed using a one-way analysis of variance followed by Student's *t*-test to compare the difference between the control and treated values. *P*-values < 0.05

**Table 1** Hepatoprotective effect of *Sapindus mukorossi* saponin fraction on thiopentone-induced sleeping time against CCl<sub>4</sub>-induced liver damage in rats

Treatment (mg/kg, per os)	Sleeping time (minutes)	Hepatoprotection (%)
Vehicle control	17.06 ± 1.50	–
CCl <sub>4</sub> (1.5, intraperitoneal)	65.72 ± 2.43*	–
Silymarin (20)	20.56 ± 1.08*	94.86
SMSF (50)	45.78 ± 2.32*	40.98
SMSF (100)	32.67 ± 2.90*	67.92
SMSF (150)	24.25 ± 1.62*	86.25

**Notes:** \**P* < 0.001 when compared to negative control (CCl<sub>4</sub> treated) group. Sleeping time expressed as mean ± standard error of mean; n = 6. Percentage of hepatoprotection calculated using the equation: H = (1 – [T – V/C – V]) × 100, where T is the mean value of the group treated with test drug, C is the mean value of the group treated with CCl<sub>4</sub> alone, and V is the mean value of control animals.

**Abbreviations:** CCl<sub>4</sub>, carbon tetrachloride; SMSF, *S. mukorossi* saponin fraction.



**Figure 1** Effect of *Sapindus mukorossi* saponin fraction on serum biochemical parameters of CCl<sub>4</sub>-treated rats (n = 6).

**Notes:**  $P < 0.01$  and  $P < 0.001$  when compared to negative control (CCl<sub>4</sub>-treated) group.

**Abbreviations:** SMSF, *S. mukorossi* saponin fraction; CCl<sub>4</sub>, carbon tetrachloride; IP, intraperitoneal; SGOT, serum glutamic oxaloacetic transaminase; SGPT, serum glutamic pyruvic transaminase; ALP, alkaline phosphatase.

were considered significant. GraphPad Prism version 3.02 (GraphPad Software, San Diego, CA) was used for statistical calculations.

## Results

### Thiopentone-induced sleeping time

SMSF showed extremely statistical significant ( $P < 0.001$ ) hepatoprotective (86.25%) activity by reducing the sleeping time to  $24.25 \pm 1.62$  minutes compared to  $65.72 \pm 2.43$  minutes of negative control at 150 mg/kg dose, as shown in Table 1.

### Effect on serum biochemical parameters

SMSF treatment significantly ( $P < 0.001$ ) decreased SGOT, SGPT, and ALP levels of treated animals at all the tested doses compared to negative control group (Figure 1). Triglyceride level was also decreased at 100 and 150 mg/kg doses; which is extremely significant ( $P < 0.001$ ). SMSF significantly ( $P < 0.001$  and 0.05) increased cholesterol level of serum

at 100 and 150 mg/kg doses respectively. Saponin treatment did not have any significant effect towards normalization of albumin and direct bilirubin level in treated animals. SMSF significantly decreased serum total bilirubin ( $P < 0.001$ ) at 150 mg/kg dose (Table 2).

### Estimation of liver free radical scavenging ability

CCl<sub>4</sub> intoxication reduced the SOD enzyme level, expressed in unit/mg of protein in liver tissues (Table 3). SMSF treatment showed significant ( $P < 0.05$ ) enhancement ability of SOD only at 150 mg/kg dose. SMSF at 150 mg/kg dose showed extremely significant ( $P < 0.001$ ) reduction in lipid peroxidation and elevation of glutathione.

### Histopathological parameters

SMSF treatment showed hepatoprotection by reducing the liver weight of CCl<sub>4</sub>-intoxicated animals. Liver weight/100 g of body weight for vehicle control, negative control,

**Table 2** Effect of *Sapindus mukorossi* saponin fraction on serum biochemical parameters of CCl<sub>4</sub>-treated rats

Treatment (mg/kg, per os)	Cholesterol (mg/dL)	Triglyceride (mg/dL)	Protein (g/dL)	Albumin (g/dL)	Bilirubin (mg/dL)	
					Total	Direct
Vehicle control	$152.60 \pm 3.60$	$113.50 \pm 4.55$	$8.88 \pm 0.34$	$3.57 \pm 0.65$	$0.75 \pm 0.04$	$0.14 \pm 0.02$
CCl <sub>4</sub> (1.5, IP)	$68.92 \pm 3.55$	$220.05 \pm 5.72$	$5.85 \pm 0.40$	$1.52 \pm 0.35$	$8.63 \pm 0.79$	$5.28 \pm 0.68$
Silymarin (20)	$72.57 \pm 3.21^{ns}$	$131.54 \pm 4.32^b$	$8.46 \pm 0.24^a$	$2.58 \pm 0.35^{ns}$	$0.55 \pm 0.02^b$	$1.15 \pm 0.22^b$
SMSF (50)	$78.33 \pm 2.90^{ns}$	$205.60 \pm 4.22^{ns}$	$5.92 \pm 0.33^{ns}$	$1.39 \pm 0.12^{ns}$	$4.25 \pm 0.46^b$	$5.96 \pm 0.57^{ns}$
SMSF (100)	$89.81 \pm 3.22^a$	$163.99 \pm 3.33^b$	$6.74 \pm 0.56^{ns}$	$2.18 \pm 0.06^{ns}$	$1.48 \pm 0.25^b$	$5.67 \pm 0.58^{ns}$
SMSF (150)	$113.87 \pm 3.80^b$	$137.43 \pm 3.80^b$	$8.13 \pm 0.71^a$	$2.63 \pm 0.21^{ns}$	$0.98 \pm 0.05^b$	$4.51 \pm 0.70^{ns}$

**Notes:** Values expressed as mean  $\pm$  standard error of mean (n = 6). <sup>a</sup> $P < 0.05$ ; <sup>b</sup> $P < 0.001$ ; <sup>ns</sup>Not significant when compared to negative control (CCl<sub>4</sub>) group.

**Abbreviations:** CCl<sub>4</sub>, carbon tetrachloride; IP, intraperitoneal; SMSF, *S. mukorossi* saponin fraction.

**Table 3** Effect of *Sapindus mukorossi* saponin fraction on free radical scavenging ability of CCl<sub>4</sub>-treated rat liver

Treatment (mg/kg, per os)	Superoxide dismutase (unit/mg of protein)	Lipid peroxidase (n mol/g of protein)	Glutathione (mg/g of liver)
Vehicle control	5.72 ± 0.87	3.43 ± 0.68	22.26 ± 1.92
CCl <sub>4</sub> (1.5, IP)	1.53 ± 0.22	32.97 ± 1.75	7.46 ± 0.67
Silymarin (20)	4.53 ± 0.62 <sup>a</sup>	5.59 ± 0.60 <sup>c</sup>	19.65 ± 1.40 <sup>c</sup>
SMSF (50)	1.95 ± 0.22 <sup>ns</sup>	24.25 ± 1.74 <sup>b</sup>	10.56 ± 1.24 <sup>ns</sup>
SMSF (100)	3.05 ± 0.45 <sup>ns</sup>	20.34 ± 1.09 <sup>c</sup>	13.50 ± 1.35 <sup>a</sup>
SMSF (150)	4.12 ± 0.76 <sup>a</sup>	14.70 ± 1.22 <sup>c</sup>	17.04 ± 1.06 <sup>c</sup>

**Notes:** Values expressed as mean ± standard error of mean (n = 6). <sup>a</sup>P < 0.05; <sup>b</sup>P < 0.01; <sup>c</sup>P < 0.001; <sup>ns</sup>Not significant when compared to negative control (CCl<sub>4</sub>-treated) group.

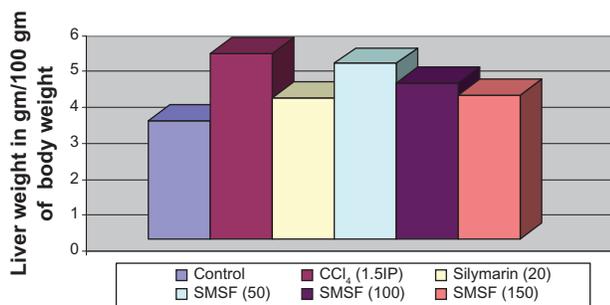
**Abbreviations:** CCl<sub>4</sub>, carbon tetrachloride; IP, intraperitoneal; SMSF, *S. mukorossi* saponin fraction.

silymarin, and SMSF (150 mg/kg) was 3.29, 5.18, 3.94, and 4.02 g, respectively (Figure 2).

Liver section of vehicle control animals indicated normal appearance of hepatic parenchyma. CCl<sub>4</sub> hepatotoxin-treated animals showed degenerative changes, bile duct hyperplasia, zonal necrosis, and extensive diffuse vacuolar degeneration engorged with blood and microvesicular fatty change in hepatocytes, which indicates completely damaged cytoarchitecture of liver. Silymarin-treated animals showed slightly altered hepatic parenchyma with focal necrosis, lobular necrosis, centrolobular necrosis, and sinusoidal dilation with slightly altered hepatic parenchyma. SMSF (150 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.

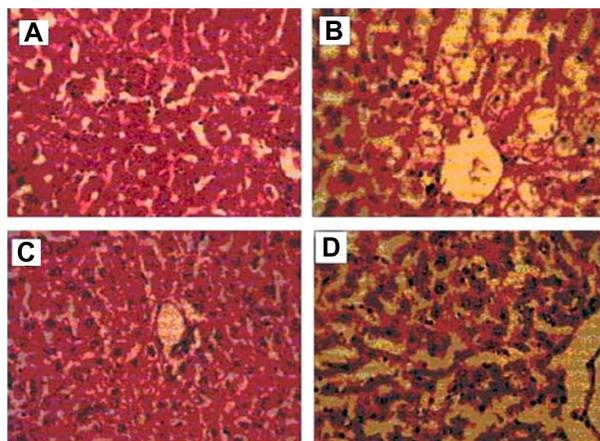
## Viability of liver cells

SMSF treatment at all the tested doses showed extremely significant ( $P < 0.001$ ) hepatoprotection against CCl<sub>4</sub> by

**Figure 2** Effect of *Sapindus mukorossi* saponin fraction on relative liver weight of CCl<sub>4</sub>-treated rats (n = 6).

**Notes:**  $P < 0.001$  when compared to negative control (CCl<sub>4</sub>-treated) group.

**Abbreviations:** CCl<sub>4</sub>, carbon tetrachloride; IP, intraperitoneal; SMSF, *S. mukorossi* saponin fraction.

**Figure 3 (A–D)** Assessment of CCl<sub>4</sub>-induced hepatotoxicity by histopathology in hematoxylin eosin-stained liver section. (A) Section from normal liver tissue; (B) 3 days after CCl<sub>4</sub> intoxicant, liver tissue showing zonal necrosis, extensive diffuse vacuolar degeneration engorged with blood and microvesicular fatty changes in hepatocytes; (C) silymarin-treated liver tissue showed focal necrosis and sinusoidal dilatation with slightly altered hepatic parenchyma; (D) SMSF (150 mg/kg) pretreatment showed focal coagulative necrosis, mild fatty vacuolation and dilated sinusoid (10×).

**Abbreviations:** CCl<sub>4</sub>, carbon tetrachloride; SMSF, *S. mukorossi* saponin fraction.

increasing the viable liver cell count. Silymarin showed 87.93% of viable cell compared to 61.56% of negative control and 92.99% of vehicle control, respectively, as shown in Table 4. SMSF at 150 mg/kg dose showed 82.93% viable liver cell.

## Bromsulphalein uptake test

Liver slices of SMSF-treated animals showed extremely statistical significant ( $P < 0.001$ ) hepatoprotection (54.33%) at 150 mg/kg dose. It showed  $75.44 \pm 4.12$  µg of BSP uptake per gram of liver tissue compared to  $42.11 \pm 2.38$  µg of CCl<sub>4</sub>-treated group (Figure 4).

## Discussion

The hepatotoxic effects of CCl<sub>4</sub> are largely due to biotransformation by the cytochrome P450 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 that cause damage to the membrane. The peroxidative products induce hypoperfusion of the membrane, and finally cytosolic enzymes appear in the blood.<sup>38</sup> 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

**Table 4** Effect of *Sapindus mukorossi* saponin fraction on viability and biochemical parameters of CCl<sub>4</sub>-treated rat liver cells

Treatment (mg/kg, per os)	No of cells counted	No of viable cells (% of viable cells)	ALT (IU/L)	AST (IU/L)
Vehicle control	212.33 ± 4.25	197.45 ± 2.33 (92.99)	18.70 ± 0.46	21.53 ± 0.63
CCl <sub>4</sub> (1.5, IP)	205.54 ± 4.78	126.55 ± 2.79 (61.56)	50.20 ± 0.61	57.50 ± 0.94
Silymarin (20)	207.55 ± 4.22	182.43 ± 3.43 (87.89) <sup>a</sup>	19.46 ± 0.35 <sup>a</sup>	24.10 ± 0.37 <sup>a</sup>
SMSF (50)	210.17 ± 3.40	132.45 ± 3.67 (63.02) <sup>a</sup>	41.33 ± 0.62 <sup>a</sup>	44.53 ± 1.25 <sup>a</sup>
SMSF (100)	207.65 ± 5.82	156.25 ± 3.32 (75.25) <sup>a</sup>	38.50 ± 0.45 <sup>a</sup>	32.80 ± 0.65 <sup>a</sup>
SMSF (150)	209.25 ± 4.95	173.55 ± 2.11 (82.3) <sup>a</sup>	22.23 ± 0.50 <sup>a</sup>	27.33 ± 0.69 <sup>a</sup>

**Notes:** Values expressed as 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. \*P < 0.001, significant reduction compared to hepatotoxin (\*P < 0.05).

**Abbreviations:** ALT, alanine aminotransferase; AST, aspartate aminotransferase; CCl<sub>4</sub>, carbon tetrachloride; IP, intraperitoneal; SMSF, *S. mukorossi* saponin fraction.

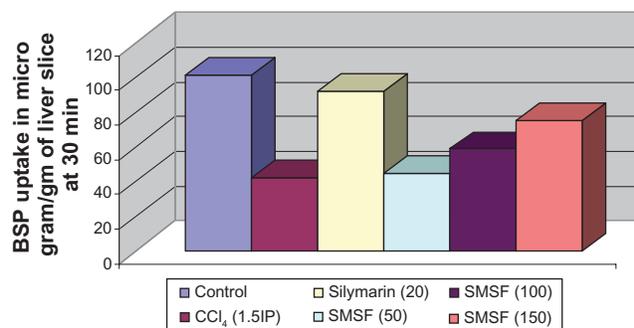
with liver intoxication is increased as the enzyme responsible for metabolism of thiopentone is reduced or destroyed.<sup>39</sup> Therefore, the protective effect exhibited by SMSF may be due to protection of hepatic drug-metabolizing enzymes, as evidenced from the decrease in thiopentone-induced sleeping in treated animals.

Hepatotoxin CCl<sub>4</sub> gets converted into CCl<sub>3</sub>O<sup>-</sup> by liver enzymes and attacks the unsaturated fatty acids of cell membrane in the presence of oxygen, which consequently gives rise to lipid peroxides that alter 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. Liver toxicants cause disturbances in synthesis and metabolism of triglycerides, cholesterol, and lipoproteins, thus damaging the basic resource for living cells. The increased triglyceride content in the blood is in correlation with the fatty degeneration of the liver.<sup>40</sup> Serum triglyceride and bilirubin (total and direct) levels elevate; on the other hand, serum total protein, albumin, and cholesterol levels decrease. In the diseased liver, there are two prime manifestations of liver failure with regard to lipid metabolism. The first of these is the

deposition of triglycerides within the organ itself. This is the basic mechanism of so-called fatty liver, which develops most often as a result of chronic alcoholism. The second prime feature of disordered hepatic lipid metabolism is a diminution in the rate of synthesis of cholesterol. In fact, a decrease below the normal level of serum cholesterol is often found in advanced diffuse liver disease or in severe acute liver disease.

The tendency of liver marker enzymes to return to a near-normal level in SMSF-treated rats is a clear manifestation of the antihepatotoxic effect of *S. mukorossi*. The lowering of different serum marker enzyme levels is a definite indication of the hepatoprotective action of SMSF. SMSF 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 CCl<sub>4</sub>-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. SMSF normalizes the protein content of CCl<sub>4</sub>-intoxicated rats, signifying an 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.<sup>41</sup> Hyperbilirubinemia results from impaired hepatic uptake of unconjugated bilirubin in liver cell injury.<sup>42</sup> SMSF treatment significantly reduced the elevated serum level of total bilirubin. Animals of the negative control group significantly lost body weight and showed reduced food consumption as compared to the control group. Liver toxicity increases liver weight due to fatty changes along with a fall in serum lipids, as in paracetamol intoxication.<sup>43</sup> This is a parameter in ascertaining the hepatoprotective effect of drugs. SMSF treatment showed a significant reversal towards loss of body weight, suggesting normalization of CCl<sub>4</sub> toxicity inducing regeneration of hepatocytes.



**Figure 4** Effect of *Sapindus mukorossi* saponin fraction on BSP uptake of CCl<sub>4</sub>-treated rat liver slice (n = 6).

**Notes:** P < 0.001 and ns = not significant when compared to negative control (CCl<sub>4</sub>-treated) group.

**Abbreviations:** BSP, bromsulphthalein; CCl<sub>4</sub>, carbon tetrachloride; IP, intraperitoneal; SMSF, *S. mukorossi* saponin fraction.

Highly reactive  $\text{CCl}_3\cdot$  radical-induced lipid peroxidation disturbs  $\text{Ca}^{2+}$  homeostasis and finally results in cell death. SMSF 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 mechanisms by preventing cell membrane oxidation. SMSF 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. SMSF at higher dose has 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 SMSF with  $\text{CCl}_4$  exhibited less damage to the hepatic cells, as compared to the rats treated with  $\text{CCl}_4$  alone. Hepatocytes showed normal appearance; only some cells showed a higher number of vacuoles in the cytoplasm. Although SMSF did not appear to bring a complete reversal of  $\text{CCl}_4$ -induced injury in the liver, it minimized the effect of  $\text{CCl}_4$ .

The histopathological observations showed a faster regeneration of hepatic cells, which seems to suggest the possibility of SMSF 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.<sup>44</sup>

The hepatoprotective activity of SMSF may be due to the presence of phytoactive saponin compounds, which prevent liver plasma membrane alteration and promote liver cell repair. The observed protective effect can be attributed to the presence of triterpenoidal saponin, containing several triterpenoids. Plants containing triterpenoidal constituents are reported to have hepatoprotective activity against ethanol, paracetamol, cadmium, tert-Butyl hydroperoxide, galactosamine, aflatoxin, and  $\text{CCl}_4$ . In conclusion, this study supports the traditional use of *S. mukorossi* as a herbal remedy for jaundice and other liver disorders, suggesting the feasibility of developing herbal formulation and clinical studies.

## Disclosure

The authors report no conflicts of interest in this work.

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