

Antioxidant and Hepatoprotective Activities of *Strobilanthes kunthianus* against Carbon Tetrachloride-Induced Hepatotoxicity in Rats

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ABSTRACT

Background: *Strobilanthes kunthianus* Nees T Anders (Neela kurinji) is a shrub in the grasslands of Nilgiris, Western Ghats in India. It is well known for many biological properties including antioxidant. However, there is no *in-vivo* antioxidant and hepatoprotective activities has been carried out previously on *S. kunthianus*. **Objectives:** The present study was aimed to evaluate the antioxidant and hepatoprotective activities of methanolic flower extract of *S. kunthianus* (MFESK) against carbon tetrachloride (CCl₄)-induced hepatotoxicity in experimental rats. **Materials and Methods:** The Wistar rats were divided into six groups comprising six animals to each. Group I was served as normal control and group II as CCl₄ treated. Both these groups were received sodium CMC (0.3%, 5 ml/kg). Groups III, IV and V animals were treated with MFESK at different dose levels (100, 150 and 200 mg/kg). Group VI was treated with standard silymarin (100 mg/kg). All these treatments were given orally for eight consecutive days. On the 8th day of treatment, except the normal group I, all the other group of animals from III to VI were received CCl₄ in liquid paraffin (1:1, 1 ml/kg, i.p., single dose) after 1 h of the vehicle. On the 9th day, the animals were anesthetized and blood was collected from the abdominal artery, then the serum was separated and used for the biochemical estimations. Serum marker enzymes such as ASAT, ALAT, ALP, TGL, CR, TP, TC, TB and albumin were measured using Ecoline kits by using autoanalyzer. Further, blood serum and the supernatant solution of homogenized liver and kidney were used for the estimation of antioxidant parameters such as CAT, SOD and TBARS by spectrophotometrically. **Results:** The administration of CCl₄ caused a significant increase (P<0.001) in the levels of ASAT, ALAT, ALP, TGL, TC, TB and TBARS and decrease in the levels of CR, TP, Albumin, CAT and SOD in serum. A significant (P<0.001 and P<0.01) restoration of these values towards the normal level was observed in all the three tested doses of MFESK. Similar results were observed for CAT, SOD and TBARS in both liver and kidney tissues. These results designated the strong antioxidant and hepatoprotective nature of MFESK. The histopathological investigation of liver and kidney tissues also confirmed the observed activities. **Conclusion:** These findings afford incitement for the development of a novel hepatoprotective herbal drugs.

Key words: *Strobilanthes kunthianus*, Antioxidant, Carbon tetrachloride, Liver disease, Hepatotoxicity, Hepatoprotective.

INTRODUCTION

Liver has an essential role in the regulation of physiological processes and most of the hepatic diseases are usually caused by large amount consumption of alcohol, exposure of toxic chemicals, autoimmune disorders and viral related infections. Carbon tetrachloride (CCl₄), a toxic chemical that can damage liver cells commonly by prompting lipid peroxidation (LPO) and oxidative stress in liver.¹⁻⁵ The mechanism involved in the pathogenesis of hepatic damage prompted by CCl₄ is due to LPO and that is initiated by P450 enzyme system to the highly reactive trichloromethyl radicals.⁶ This Trichloromethyl radicals can react with glutathione (GSH) and protein thiols as well. In addition, CCl₄ also modifies antioxidant enzymes like glutathione peroxidase (GPx), superoxide dismutase (SOD), glutathione-S-transferase (GST), catalase (CAT), glutathione reductase (GR) and

thiobarbituric acid reactive substances (TBARS).⁷ Enriched LPO formed during the liver microsomal metabolism of ethanol may leads to cirrhosis and hepatitis.⁸

According to the World Health Organization (WHO) report⁹, around 90% of the acute hepatitis is mainly due to viruses and now ranked as seventh prominent cause of mortality worldwide. There are five major hepatitis viruses such as type A, B, C, D and E, among these, Hepatitis B and C infections often results to chronic liver disease and its complications like cirrhosis and liver cancer. WHO estimated that globally there are 257 million persons with hepatitis B and every year up to 900000 people succumb to hepatitis B-related deaths. Also estimated that 71 million people are infected with hepatitis C globally, and the infection that kills nearly 400000 people in every year.⁹ The antioxidant activity or the inhibition of the generation of free radicals is important in

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providing protection against hepatic damage. A wide number of natural products that have been reported to possess the hepatoprotective activity by enhancing antioxidant properties.¹⁰

There are over 200 species of *Strobilanthes* nearly all in Asia and over 150 occurs in India, especially in Western Ghats and Nilgiris alone claims more than thirty species. Many of the species flower at longer intervals, usually between six and twelve years and in some even after 35 years. Species of *Strobilanthes* grow wild on the Nilgiris, ranges between 6000 to 7000 feet. The genus *Strobilanthes* is known for its numerous biological activities. *S. crispus* has been used as antidiabetic, anxiolytic, laxative, anti-HIV, antileukemic and hepatoprotective.¹¹⁻¹⁷

Strobilanthes kunthianus Nees T Anders (*S. kunthianus*, Neela kurinji) is a shrub in the grasslands of Western Ghats in India. The Nilgiris, which literally means the blue mountains got its name from the purplish blue flowers of Neela kurinji that blossoms gregariously once in twelve years. *S. kunthianus* is well known for its medicinal properties. It was reported to possess many biological activities including anti-inflammatory and anti-osteoarthritic, analgesic, antioxidant, antibiofilm, enzyme inhibitor, central nervous depressant, anti-giardial, antifungal, antibacterial, antiseptic, antimicrobial, cytotoxicity and protect skin against UV.¹⁸⁻²⁴

Singh *et al.*²⁰ reported *in-vitro* antioxidant activity of *S. kunthianus* flower extract using DPPH and H₂O₂ methods. Other than this preliminary study, there is no report has been found about antioxidant activities of *S. kunthianus*. However, no work has been carried out on hepatoprotective effect of *S. kunthianus*, though the gene has been reported for the treatment of hepatic disorders. Thus the present study was aimed to evaluate *in-vivo* antioxidant and hepatoprotective activities of methanolic flower extract of *S. kunthianus* (MFESK) against CCl₄ induced hepatic damage in rats.

MATERIALS AND METHODS

Instruments and chemicals

Autoanalyser - Merck Microlab 200, manufactured by M/s Vital Scientific N.V., Darmstadt, Netherlands. Centrifuge - Remi centrifuge and R-8c Laboratory centrifuge, Remi Motors Ltd., Mumbai, India. Homogenizer - Elvenjan homogenizer, Remi Motors Ltd., Mumbai. Rotary Evaporator - Rotavapor R-205, Buchi Laboratory Equipments, Flawil, Switzerland. Spectrophotometer - Shimadzu 160-A UV-VIS, Koyoto, Japan. ASAT, ALAT and ALP diagnostic kits were obtained from Span Diagnostic Ltd., Surat, India. Silymarin was obtained from Ranbaxy India Ltd, New Delhi, India. Ibuprofen was procured from Overseas Pharma, Bangalore, India. Commercial animal feed was purchased from Sai Durga Feeds and Food, Bangalore, India. All the chemicals used were of analytical grade.

Extraction

The whole plant of *S. kunthianus* was collected from Thalaikuntha region, near Udhamandalam, Nilgiris district, Tamilnadu, India. The plant was identified and authenticated at Botanical Survey of India, Coimbatore. The separated flowers of *S. kunthianus* (500 g) were dried, grinded into a coarse powder form and extracted with 2.5 l of methanol in a Soxhlet apparatus for 18-20 h. The obtained methanolic flower extract of *S. kunthianus* (MFESK) was concentrated in a rotary evaporator under reduced pressure at 35-40 °C and stored at 4 °C in a refrigerator till further use.

Pharmacological screening

Animals

Wistar albino rats weighing 180-220 g were obtained from the animal house of JSS College of Pharmacy, Udhamandalam, Tamilnadu, India

(JSSSCP/IAEC/PHD/PH.CHEM/02/08-09). All the animals were kept under standard laboratory conditions with 12±1 h light-dark cycle. Animals were provided with standard rat feed and water *ad libitum*.

Acute oral toxicity studies of the MFESK

It was carried out as per OECD-423 guidelines with 2000 mg/kg of MFESK were administered (maximum dose) orally to different group of rats comprising 10 in each group. The animals were observed for body weight changes, clinical signs and mortality daily for a period of 15 days. At the end of the study period, all the animals were subjected to gross necropsy.

In-vivo antioxidant and hepatoprotective activities of MFESK

Preparation of the samples and standard

The suspension of different concentrations of MFESK and standard silymarin (100 mg/ml) were prepared in 0.3% sodium carboxy methyl cellulose (CMC) in distilled water and administered orally to the animals with the help of intragastric catheter.

Procedure

The rats were divided into six groups, each group covering six animals. Group I was used as normal control and group II was classified as CCl₄ treated. Sodium CMC (0.3%, 5 ml/kg) was received in both groups. Animals in groups III, IV and V were treated with MFESK at dose levels of 100, 150 and 200 mg/kg. Group VI animals were treated with 100 mg/kg of silymarin (Standard drug). All these therapies were orally given for 8 days. On the 8th day of treatment, after 1 h of the vehicle, MFESK or standard treatments, all animals except the normal group I were received CCl₄ in liquid paraffin (1:1, 1 ml/kg, i.p., single dose).

On the 9th day, the blood was obtained under anaesthetized conditions from the abdominal artery of animals and kept at 4 °C for 30 min. Serum was isolated by centrifugation and used for biochemical parameter estimation. Serum marker enzymes including ASAT, ALAT, ALP, TGL, CR, TP, TC, TB, and albumin have been determined by autoanalyzer using Ecoline kits. SOD, CAT, and TBARS were obtained by spectrophotometric measurements.²⁵⁻²⁷

Following the collection of blood samples, liver and kidney were excised, rinsed in normal ice-cold saline, followed by cold potassium chloride (0.15 M, pH 7.4), and blotted dry. A 10% w/v homogeneous Elvenjan homogenizer fitted with Teflon plunger was prepared in a potassium chloride buffer (0.15 M) and centrifuged at 2500 rpm at 4 °C for 15 min. The supernatants were used both in liver and kidney to measure SOD, CAT, and TBARS. A portion of the tissues in the liver and kidney were fixed in 10% formalin, cut into 5 µm thick sections and stained using hematoxylin-eosin, and histopathological observations were made.

Estimation of antioxidant parameters

Estimation of CAT

The potassium phosphate buffer (2.25 ml, 65 mM, pH 7.8) and 100 µl of serum or tissue homogeneous or sucrose (0.32 M) were incubated for 30 min at 25 °C. The reaction was started by adding H₂O₂ (0.65 ml, 75 mM). The absorption changes were measured at 240 nm for 2-3 min and dy/dx was determined for 1 min for each assay and the results were expressed as CAT units/mg of tissue.²⁶

Estimation of SOD

The sodium carbonate buffer (2.8 ml, 0.05 mM) and 0.1 ml of serum or homogeneous tissue or sucrose were incubated at 30 °C for 45 min. Then, the absorbance was changed to sample at zero. Adding adrenaline solution (10 µl, 9 mM) then triggered the reaction. The

shift in absorbance during 8-12 min was noted at 480 nm. Throughout the assay the temperature was kept at 30 °C. Similarly, the calibration curve for SOD was prepared by taking 10 unit/ml as standard solution. One unit of SOD inhibits the auto-oxidation of adrenaline by around 50%. The findings have been expressed as SOD activity unit (U)/mg of tissue.²⁸

Estimation of TBARS

Added serum or tissue homogeneous (1 ml), sodium lauryl sulfate (0.2 ml, 8.1%), 20% acetic acid (1.5 ml, pH 3.5), and thiobarbituric acid solution (1.5 ml, 0.8% w/v, pH 7.4). This incubation mixture was made up to 5 ml and then heated for 30 min in boiling water bath. After refrigeration, the red chromogen was collected and centrifuged for 10 min at 4000 rpm. The supernatant was taken and measured its absorbance at 532 nm. As external standard 1,1,3,3-Tetraethoxy propane (TEP) was used. The findings were expressed as nM of wet tissue MDA/mg or ml of serum using the molar chromophore extension coefficient ($1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$). The calibration curve was also prepared for TEP, and the findings were expressed as nM of tissue MDA/mg.²⁹

nM of MDA/mg of tissue or ml of serum =

$$\text{OD} \times \frac{\text{Volume of homogenate} \times 100 \times 10^3}{1.56 \times 10^5 \times \text{Volume of MFESK taken}}$$

Estimation of hepatoprotective parameters

Biochemical estimations in serum were carried out by using Ecoline diagnostic kits.

Assay of ASAT

Reagents 1 and 2 of Ecoline diagnostic kit for ASAT were mixed at the ratio of 4:1 and the temperature was maintained at 30 °C. To 50 µl of the sample, 0.5 ml of the reagent solution was added and mixed. After 1 min, the decrease in absorbance was measured every min for 3 min at 340 nm.

$$\text{Enzyme activity [U/I]} = (\Delta A/\text{min}) \times 2143$$

Where, ΔA is the decrease in absorbance per min.

Assay of ALAT

Reagents 1 and 2 of Ecoline diagnostic kit for ALAT were mixed at the ratio of 4:1 and the temperature was maintained at 30 °C. To 50 µl of the sample, 0.5 ml of the reagent solution was added and mixed. After 1 min, the decrease in absorbance was measured every min for 3 min at 340 nm.

$$\text{Enzyme activity [U/I]} = (\Delta A/\text{min}) \times 2143$$

Where, ΔA is the decrease in absorbance per min.

Assay of ALP

Reagents 1 and 2 of Ecoline diagnostic kit for ALP were mixed at the ratio of 4:1 and the temperature was maintained thermostat at 30 °C. To 20 µl of the sample, 1 ml of the reagent solution was added and mixed. After 1 min, the increase in absorbance was measured every min for 3 min at 405 nm.

$$\text{Enzyme activity [U/I]} = (\Delta A/\text{min})$$

Where, ΔA is the increase in absorbance per min.

Estimation of total bilirubin (TB)

Reagent 1 and 2 of Ecoline diagnostic kit were mixed at the ratio of 4:1.

The mixture, known as diazo solution, was maintained at 30 °C. To 50 µl of the sample, 50 µl of the diazo solution and 250 µl of acceleration solution (mixture of caffeine 5%, sodium benzoate 7.5% and sodium acetate 12.5%) were added and mixed. The reaction mixture was maintained at room temperature. After 1 h, 250 µl of alkaline Fehling's solution II was added and the reaction mixture was incubated at room temperature for 5 min. The absorbance of the sample and standard were measured against the reagent blank, after 5 min at 578 nm.

$$\text{Total Bilirubin (mg \%)} = (A_{\text{sample}} - A_{\text{blank}}) \times 27.78$$

Where, A_{sample} is absorbance of sample and A_{blank} is absorbance of the blank.

Estimation of triglycerides (TGL)

To 0.10 ml of sample or standard triglyceride (supplied by Ecoline diagnostic kit), 1 ml of the reagent was added, mixed well and incubated for 10 min at 37 °C. The absorbance of sample and standard were measured against reagent blank within 60 min at 546 nm.

$$\text{Triglycerides [mg/dl]} = (A_{\text{sample}}/A_{\text{standard}}) \times \text{Concentration of standard}$$

Estimation of total cholesterol (TC)

To 0.10 ml of the sample or standard cholesterol (supplied by Ecoline diagnostic kit), 1 ml of the reagent was added, mixed well and incubated for 5 min at 37 °C and the absorbance of sample and standard were measured at 546 nm within 60 min.

$$\text{Total Cholesterol [mg/dl]} = (A_{\text{sample}}/A_{\text{standard}}) \times \text{Concentration of standard}$$

Estimation of total protein (TP)

Reagents 1 and 2 of Ecoline diagnostic kit for assay of total protein were mixed at the ratio of 4:1 and the temperature was maintained always at 37 °C. To 0.10 ml of sample/standard protein (supplied by Ecoline diagnostic kit), 1 ml of the reagent was added, mixed well and incubated for 1 and 5 min, and the absorbance of the sample and standard were measured within 60 min.

$$\text{Total Protein [g/dl]} = (A_{\text{sample}}/A_{\text{standard}}) \times \text{Concentration of standard}$$

Estimation of creatinine (CR)

Buffer solution and picric acid solution from E-Merck diagnostic kit for creatinine were mixed in the ratio of 1:1 and incubated for about 10 min before use. To 1 ml of this reagent solution, 0.20 ml of sample/standard was added, and mixed well. The absorbance was measured exactly after 1 min and 5 min at 492 nm. The concentration of creatinine was calculated by using the following formula.

Creatinine concentration (mg/dl) =

$$\frac{A_2 - A_1}{A_{St1} - A_{St2}}$$

A_1 and A_2 = absorbance of sample after 1 and 5 min

A_{St1} and A_{St2} = absorbance of standard after 1 and 5 min.

Estimation of albumin

To 1 ml of the reagent solution from E-Merck diagnostic kit for albumin, 100 µl of the sample or standard was added, mixed well and incubated at 37 °C for 10 min. The absorbance of the sample and standard was measured against the reagent blank solution within 60 min. the concentration of albumin was calculated by using the formula.

$$\text{Albumin (mg/dl)} = (A_s/A_{std}) \times \text{Concentration of standard}$$

A_s = Absorbance of sample and A_{std} = Absorbance of standard

Histopathological studies

The liver and kidney were dissected out of all the animals on the 9th day of the experiment, cleansed off their surrounding tissues and washed with normal saline. The materials were initially fixed for 48 h in buffered neutral formalin (10%) and then for 6 h in bovine solution. They have been prepared for implanting paraffin. Blocks of paraffin were made and parts taken using microtome at a thickness of 5 μ m were mounted on microscope slides. Such sections were treated with hematoxylin and alcoholic eosin, then microscopically analyzed to determine histopathological changes.²⁹

Statistical analysis

The significance of the findings of the *in-vivo* analysis was analyzed by one-way ANOVA, followed by Tukey-Kramer multiple comparison tests and $P < 0.05$ was considered statistically significant.

RESULTS

In the acute toxicity studies, MFESK was found to be non-toxic and no mortality was observed up to 15 days when administered independently as a single dose (up to 2000 mg/kg, p.o.) and there were no gross results of necropsy.

The serum levels of hepatic enzymes, such as ASAT, ALAT, ALP and TGL, TC, CR, TB and TBARS used as biochemical parameters for evaluation of early hepatic injury were significantly elevated ($P < 0.001$) and the levels of TP, albumin, CAT and SOD were significantly decreased ($P < 0.001$) in the CCl_4 treated animals. The treatment of MFESK at 100, 150 and 200 mg/kg dose to CCl_4 intoxicated rats significantly inhibited the increased levels ($P < 0.001$ and $P < 0.01$) of ASAT, ALAT, ALP, TGL, TC, TB and TBARS towards normal control when compared to CCl_4 treated animals. There was a significant increase ($P < 0.001$) in the levels of CR, TP and albumin towards the normal control. The high dose at 200 mg/kg also exhibited a significant reversal ($P < 0.001$) of all the changes caused by CCl_4 administration towards the normal control (Table 1). However, the standard silymarin treatment at 100 mg/kg showed better result than MFESK.

The CCl_4 treatment caused a significant decrease ($P < 0.001$) in the levels of antioxidant enzymes CAT, SOD, and a significant increase ($P < 0.001$) in the levels of TBARS in serum, liver and kidney when compared to normal control. The treatment with MFESK at 100 mg/kg to CCl_4 intoxicated rats exhibited a significant reversal ($P < 0.01$) of CAT, SOD and TBARS in serum, liver and kidney towards the normal level when compared to CCl_4 treated. The high dose of MFESK, 200 mg/kg also produced significant changes ($P < 0.001$) in all these biochemical parameters when compared to CCl_4 treated. The standard silymarin at 100 mg/kg also produced similar significant ($P < 0.001$) results, except the TBARS in kidney (Table 2). The MFESK treatment at 200 mg/kg was found to be almost similar to the standard silymarin in reversing most of the biochemical parameters towards the normal control.

Histopathological examination of liver sections showed normal cellular architecture with distinct hepatic cells, sinusoidal spaces and a central vein in normal control group animals (Figure 1a). The liver sections of the rats of CCl_4 treated group showed disarrangement of normal hepatic cells with high degree of damage, characterized by the focal necrosis, bile duct proliferation and centrilobular necrosis (Figure 1b). The sections of the rats treated with MFESK at 100, 150 and 200 mg/kg and intoxicated with CCl_4 exhibited less bile duct proliferation and centrilobular necrosis when compared to the CCl_4 treated group (Figure 1c-e). Dose dependent results were observed and the high dose (200 mg/kg) showed better liver protection. The sections of the rats treated with standard silymarin at 100 mg/kg and intoxicated with CCl_4 showed almost normal architecture of the liver with few centrilobular fatty changes and bile duct proliferation (Figure 1f).

Kidney sections of normal control group of animals showed normal histological appearance (Figure 2a) and CCl_4 treated group exhibited high degree of tubulointerstitial nephritis (Figures 2b). The sections of MFESK treated group of animals at all the three doses and the standard silymarin treatment groups showed normal histological appearance (Figures 2c-f). These findings clearly designate that the liver and kidney tissues, that were damaged by CCl_4 intoxication showed recovery with MFESK and silymarin treatments.

Table 1: Effect of MFESK on biochemical parameters in CCl_4 -induced toxicity in rats.

Treatments	Dose mg/kg	ASAT	ALAT	ALP	TP	TGL	TC	CR	TB	Albumin
Normal control	-	97.17 \pm 3.27	82.00 \pm 2.21	156.50 \pm 2.98	6.67 \pm 0.21	85.83 \pm 2.21	105.30 \pm 3.40	0.70 \pm 0.04	0.60 \pm 0.04	4.32 \pm 0.14
CCl_4 treated	-	313.70 \pm 3.99 ^c	230.80 \pm 3.30 ^c	521.30 \pm 3.24 ^c	2.50 \pm 0.22 ^c	204.20 \pm 3.08 ^c	237.70 \pm 3.28 ^c	4.03 \pm 0.15 ^c	1.75 \pm 0.04 ^c	2.48 \pm 0.12 ^c
Silymarin + CCl_4	100	109.20 \pm 2.82 ^a	93.33 \pm 2.25 ^a	177.80 \pm 3.02 ^a	6.50 \pm 0.22 ^a	107.50 \pm 2.41 ^a	125.50 \pm 3.64 ^a	1.05 \pm 0.08 ^a	0.80 \pm 0.04 ^a	4.02 \pm 0.05 ^a
MFESK + CCl_4	100	177.50 \pm 3.30 ^a	166.30 \pm 3.45 ^a	255.00 \pm 4.02 ^a	5.50 \pm 0.22 ^a	175.70 \pm 2.95 ^a	182.50 \pm 2.96 ^a	2.87 \pm 0.07 ^a	1.45 \pm 0.04 ^a	3.12 \pm 0.06 ^b
	150	152.00 \pm 3.14 ^a	138.50 \pm 2.88 ^a	230.70 \pm 4.21 ^a	6.12 \pm 0.17 ^a	142.00 \pm 2.99 ^a	153.30 \pm 1.94 ^a	1.67 \pm 0.10 ^a	1.22 \pm 0.03 ^a	3.55 \pm 0.15 ^a
	200	126.80 \pm 1.91 ^a	108.70 \pm 2.96 ^a	201.70 \pm 4.89 ^a	6.67 \pm 0.21 ^a	121.50 \pm 2.26 ^a	134.00 \pm 2.60 ^a	1.17 \pm 0.09 ^a	0.83 \pm 0.03 ^a	4.18 \pm 0.09 ^a

Values are given as mean \pm SEM for groups of six animals each; Values are statistically significant at ^b $p < 0.01$, ^a $p < 0.001$ in CCl_4 Vs treated groups & ^c $p < 0.001$ between CCl_4 treated Vs normal control group.

Table 2: Effect of MFESK on SOD, CAT and TBARS in CCl_4 -induced toxicity in rats.

Treatments	Dose (mg/kg)	SOD (Unit/min/mg of tissue)			CAT (IU/min/mg of tissue)			TBARS (n mole of MDA/mg of tissue)		
		Serum	Liver	Kidney	Serum	Liver	Kidney	Serum	Liver	Kidney
Normal control	-	0.36 \pm 0.01	0.38 \pm 0.01	0.29 \pm 0.01	1.35 \pm 0.03	4.12 \pm 0.05	1.26 \pm 0.02	3.49 \pm 0.09	4.48 \pm 0.06	5.64 \pm 0.05
CCl_4 treated	-	0.17 \pm 0.01 ^c	0.24 \pm 0.01 ^c	0.12 \pm 0.00 ^c	0.36 \pm 0.01 ^c	2.27 \pm 0.04 ^c	0.93 \pm 0.02 ^c	4.50 \pm 0.07 ^c	6.77 \pm 0.06 ^c	7.20 \pm 0.04 ^c
Silymarin + CCl_4	100	0.32 \pm 0.01 ^a	0.34 \pm 0.01 ^a	0.27 \pm 0.00 ^a	1.41 \pm 0.01 ^a	3.72 \pm 0.07 ^a	1.12 \pm 0.02 ^a	3.27 \pm 0.06 ^a	4.74 \pm 0.06 ^a	5.80 \pm 0.04 ^a
MFESK + CCl_4	100	0.22 \pm 0.01 ^a	0.28 \pm 0.00 ^b	0.18 \pm 0.00 ^a	1.06 \pm 0.04 ^a	2.85 \pm 0.05 ^a	0.97 \pm 0.02 ^b	4.03 \pm 0.03 ^a	5.78 \pm 0.03 ^a	6.91 \pm 0.03 ^a
	150	0.25 \pm 0.01 ^a	0.31 \pm 0.00 ^a	0.22 \pm 0.00 ^a	1.23 \pm 0.02 ^a	3.30 \pm 0.06 ^a	1.10 \pm 0.02 ^a	3.55 \pm 0.02 ^a	5.13 \pm 0.04 ^a	6.08 \pm 0.02 ^a
	200	0.31 \pm 0.01 ^a	0.33 \pm 0.01 ^a	0.25 \pm 0.01 ^a	1.29 \pm 0.02 ^a	3.58 \pm 0.05 ^a	1.22 \pm 0.01 ^a	3.42 \pm 0.04 ^a	4.89 \pm 0.04 ^a	5.89 \pm 0.02 ^a

Values are given as mean \pm SEM for groups of six animals each; Values are statistically significant at ^b $p < 0.01$, ^a $p < 0.001$ in CCl_4 Vs treated groups & ^c $p < 0.001$ between CCl_4 treated Vs normal control group.

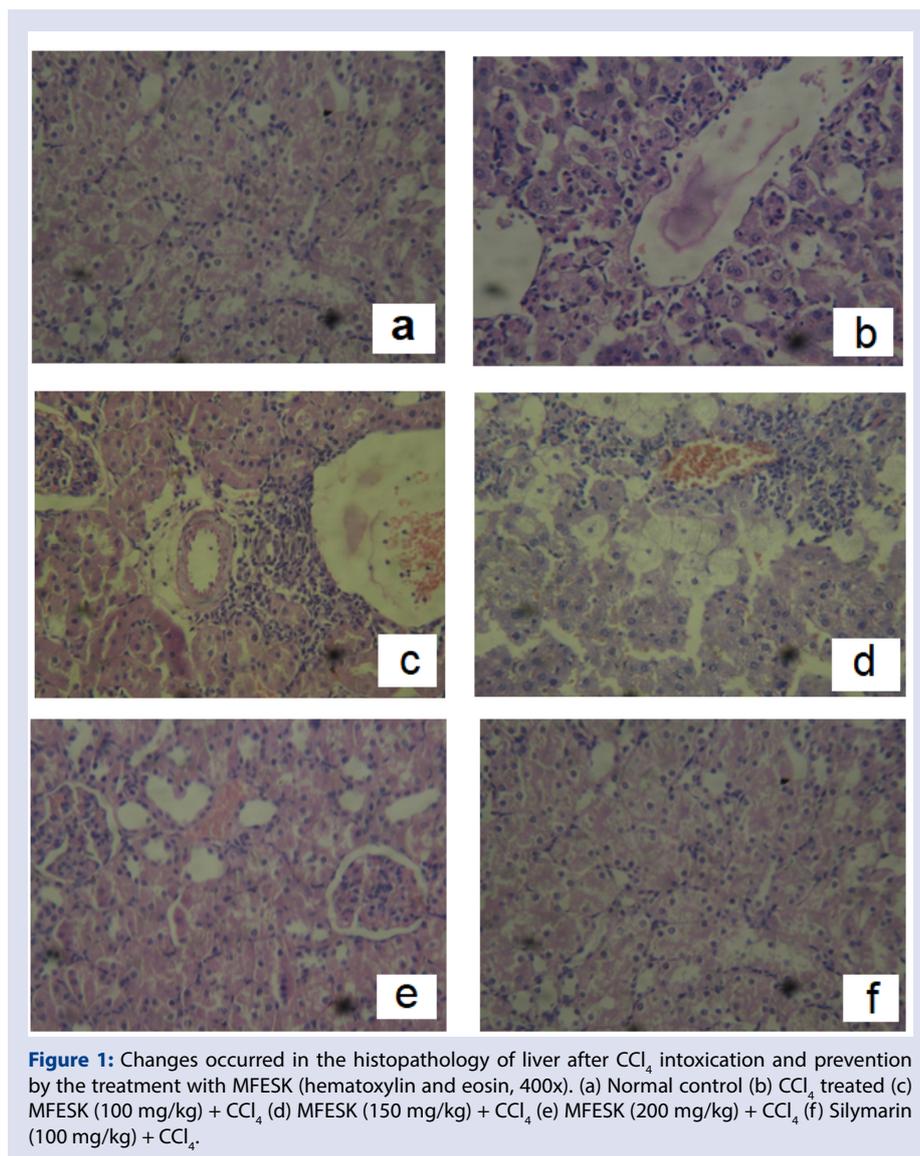


Figure 1: Changes occurred in the histopathology of liver after CCl_4 intoxication and prevention by the treatment with MFESK (hematoxylin and eosin, 400x). (a) Normal control (b) CCl_4 treated (c) MFESK (100 mg/kg) + CCl_4 (d) MFESK (150 mg/kg) + CCl_4 (e) MFESK (200 mg/kg) + CCl_4 (f) Silymarin (100 mg/kg) + CCl_4 .

DISCUSSION

Metabolic processes are primarily controlled by the liver and its disruption contributes to liver injury.³⁰ Due to the lack of a trustworthy liver protective medication in the modern medicine system, a variety of herbal formulations are recommended in Ayurveda for the treatment of liver diseases.³¹ Some studies indicate that natural products are considered safe and effective alternative treatments.

In the present study, CCl_4 mediated hepatotoxicity was selected as experimental model. Since the changes associated with CCl_4 induced liver damage are similar to that of acute viral hepatitis.³² The ability of a hepatoprotective drug to lessen the harmful effects or to preserve the normal hepatic physiological mechanisms, which have been distressed by a hepatotoxin, is the index of its protective effects.³³ Due to its free radical metabolite $\text{CCl}_3\cdot$ which alkylates cellular proteins and other macromolecules with a concurrent attack on polyunsaturated fatty acids in the presence of oxygen, the hepatotoxicity induced by CCl_4 results in lipid peroxides, resulting in hepatic damage.³⁴

Hepatic cells participate in a number of metabolic processes and contain a host of enzymes. ASAT and ALAT are present in tissues at higher concentrations in cytoplasm and ASAT also occurs in mitochondria in particular. In liver injury, the transport mechanism of the hepatocytes is disrupted, resulting in plasma membrane leakage,

resulting in an increased serum enzyme level, and subsequent release of soluble enzymes such as ASAT may occur. The elevated serum activities of ASAT and ALAT suggest cell leakage and loss of functional integrity of cell membranes in the liver.³⁵ CCl_4 administration significantly increased ($P < 0.001$) the serum level of enzymes such as ASAT and ALAT was observed in rats. Oral administration of MFESK at a dose of 200 mg/kg resulted in a significant decrease ($P < 0.001$) in the activity of the above-mentioned enzymes, which can be a consequence of the stabilization of the plasma membrane and the repair of CCl_4 damage to the hepatic tissue.

Serum ALP activity was also elevated during administration of CCl_4 . ALP is usually excreted by the liver through bile. In hepatotoxin liver damage, there is a faulty excretion of bile by the liver which is reflected in their increased serum levels. Hyperbilirubinaemia is a highly sensitive test to substantiate the functional integrity of the liver and the extent of necrosis that increases the binding, conjugation and excretory ability of hepatocytes proportional to the rate of erythrocyte degeneration.³⁶ Suppression of ALP activity in the serum of rats treated with MFESK indicates stabilizing biliary dysfunction of rat liver during chronic injury with CCl_4 .

The rates of TP like albumin would be reduced in hepatotoxic conditions due to defective protein biosynthesis in the liver.³⁷ Intoxication with CCl_4 induces disturbance and disassociation of polyribosomes on

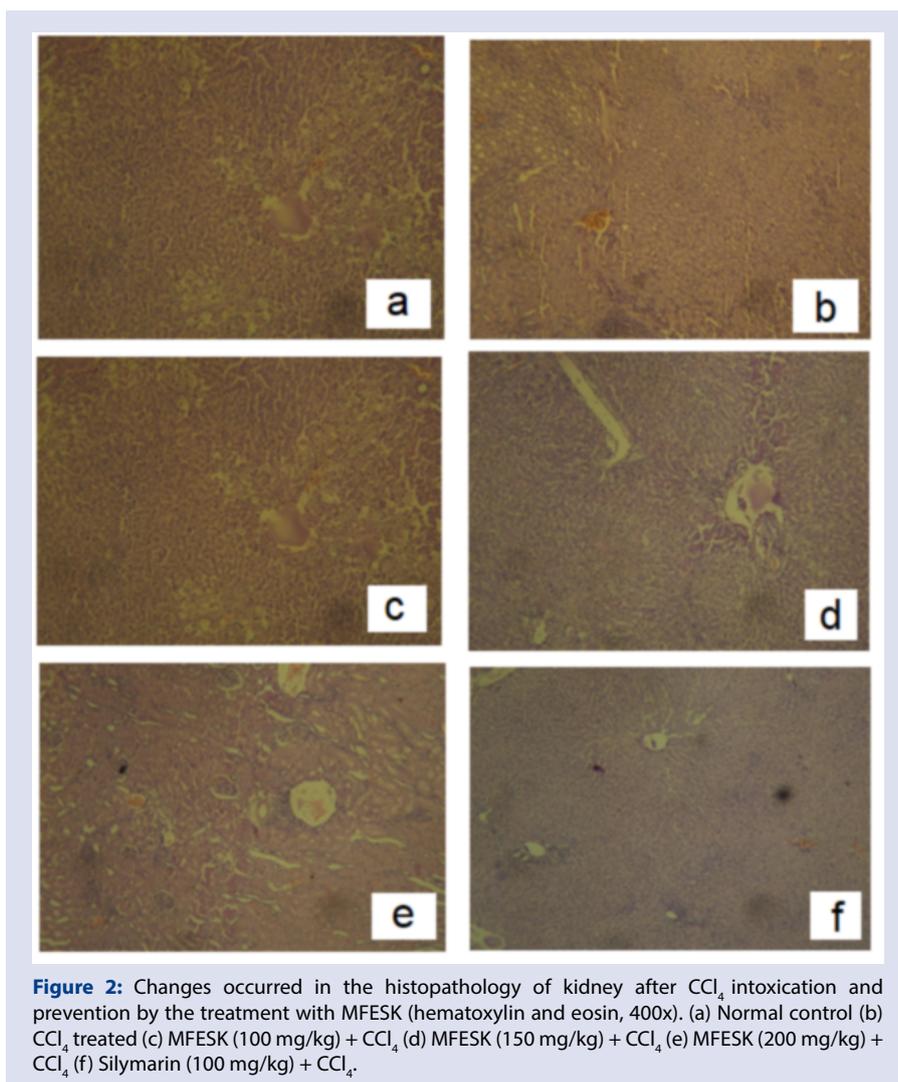


Figure 2: Changes occurred in the histopathology of kidney after CCl_4 intoxication and prevention by the treatment with MFESK (hematoxylin and eosin, 400x). (a) Normal control (b) CCl_4 treated (c) MFESK (100 mg/kg) + CCl_4 (d) MFESK (150 mg/kg) + CCl_4 (e) MFESK (200 mg/kg) + CCl_4 (f) Silymarin (100 mg/kg) + CCl_4 .

endoplasmic reticulum and thereby decreases protein biosynthesis. MFESK pretreatment preserved the synthesis of the proteins by preserving the polyribosomes.

Hepatocellular damage induces a mild hypertriglyceridemia, due to the metabolic modifications induced by the removal of triglycerides from the liver.³⁸ The same is evident in rats caused by the CCl_4 . There was a significant rise ($P < 0.001$) in cholesterol in rats caused by CCl_4 , which may be attributed to the failure of the diseased liver to extract cholesterol from circulation. Pretreatment with MFESK caused a significant dose-dependent reversal ($P < 0.001$ and $P < 0.01$) of the altered levels of TGL and TC toward the normal.

A high serum concentration of TB is an indicator of an increased risk of erythrocyte degeneration.³⁶ Because of the liver damage caused by hepatotoxin, there is a deficient excretion of bile by the liver which is expressed in their elevated serum levels.³⁹ Pretreatment of MFESK at 200 mg/kg significantly decreased ($P < 0.001$) the serum TB level to normal control suggesting improved hepatic capacities.

Hypoalbuminemia is affected by liver diseases and abnormally higher levels of CR indicate the possible breakdown or failure of the kidneys. A significant reversal ($P < 0.001$ and $P < 0.01$) was observed by the MFESK treatment towards the normal when compared to CCl_4 treated animals representing the protection of kidneys by MFESK. It has been recommended that glycogen supports as an energy buffer capable of providing quick and short-range energy.⁴⁰ The elevation of depressed

glycogen stores by MFESK in CCl_4 treated rats may be attributed to either an inhibition of hepatic glucose output improvement in plasma insulin levels or by synthase responsible for the incorporation of glucose moieties into pre-existing glycogen chains.

Inhibition of free radicals or antioxidant activity is playing a major role in protecting against hepatopathy induced by CCl_4 .⁴¹ The human body has an active defensive mechanism to limit and neutralize the free radical damage caused. A group of endogenous antioxidant enzymes such as SOD and CAT are capable of doing this. These enzymes build a compassionate team of defense against ROS in the same way.⁴² In CCl_4 induced hepatotoxicity, the stability between ROS development and these antioxidant fortifications may be lost, resulting in 'oxidant stress' which relaxes the cellular functions that lead to hepatic necrosis through a series of events. The hepatic damage in the rats treated with CCl_4 was usually determined and suggested based on the reduced SOD and CAT activities. The treated MFESK groups (100, 150 and 200 mg/kg) showed significant increase ($P < 0.001$ and $P < 0.01$) in the level of these enzymes, which explains their *in-vivo* antioxidant activity.

The membrane destruction and changes in cellular membrane structure and function were calculated based on the lipid peroxide level. Rise of lipid peroxidation in CCl_4 treated rats liver was observed in the current research. The increase of liver MDA levels suggests enriched lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanisms to prevent the production of unwarranted free

radicals.⁴³ Both of these modifications are significantly reversed by MFESK therapies ($P < 0.001$). Hence, it is possible that the mechanism of hepatoprotection of MFESK may be in line for its antioxidant action.

In the emergence of degenerated nuclei and vacuolated hepatocytes, the CCl_4 infected rats had totally lost its natural liver architecture. Fatty shifts, vacuolization, and hepatocyte necrosis in the centrilobular region were significant. CCl_4 poisoning resulted in severe confessional development of connective tissue and scar enlargement. Minimal disruption of the hepatocyte structure was observed in rat liver tissue intoxicated with CCl_4 and treated with MFESK. CCl_4 's rein parts showed a high degree of tubulointerstitial nephritis compared with normal regulation. Kidney tissue treated with MFESK displayed an almost normal histological appearance suggesting its hepatoprotective function. This findings complimented the findings of biochemical assessments, where a reversal of CCl_4 's effects was observed towards the normal.

Our previous analysis of the constituents present in MFESK included flavonoids, alkaloids, glycosides, phenolics, saponins, steroids, triterpenoids, and tannins.⁴⁴ Therefore, these active constituents could be responsible for such antioxidant and hepatoprotective activities.

CONCLUSION

The MFESK was significantly restored ($P < 0.001$ and $P < 0.01$) all the tested parameter values towards the normal, against CCl_4 induced liver injury in rats. Similar results were observed for CAT, SOD and TBARS in both liver and kidney tissues. These results indicated strong antioxidant and hepatoprotective effect of MFESK. The histopathological examination of liver and kidney tissues also confirmed these activities. These findings provide incitement for the development of a novel hepatoprotective herbal drugs. There is further scope for research to detect the isolated compounds from MFESK that are responsible for this liver protection. There is also a need to establish the possible mechanism of the observed activities.

CONFLICTS OF INTEREST

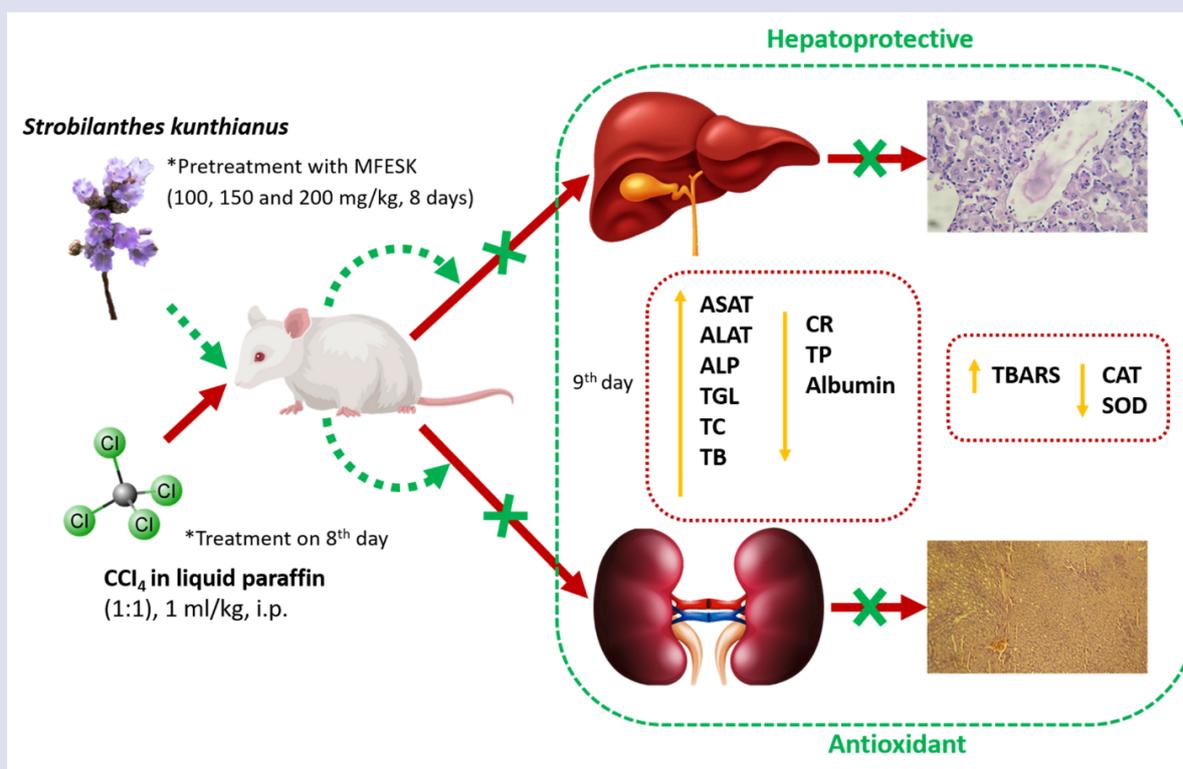
The authors declared that they have no competing interests.

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GRAPHICAL ABSTRACT



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