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Research Article

HEPATOPROTECTIVE AND ANTIOXIDANT POTENTIAL OF *SPHAERANTHUS INDICUS* [*LINN*] ON LIVER DAMAGE IN WISTAR RATS

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ABSTRACT

The hepatoprotective activity and antioxidant potential of the methanol extract of *Sphaeranthus indicus* (MES) was investigated against carbon tetrachloride (CCl_4) induced liver damage in rats. At the dose of 1ml/kg, CCl_4 induced liver damage in rats as manifested by statistically significant increase in serum alanine amino transferase (ALT), aspartate amino transferase (AST) and alkaline phosphatase (ALP). Significant decrease in total protein and antioxidant levels of SOD, CAT and GST were also noted in the liver injured rats. Treatment of rats with two doses (500 mg/kg and 750mg/kg) of MES after CCl_4 dosing statistically restored the serum liver enzyme, total protein and antioxidant levels. Simultaneous treatment with Livomyn (reference drug) also offered significant protection. Histopathological studies supported the biochemical assessment. The results thus suggest that the MES can act as a hepatoprotective and antioxidant agent against CCl_4 induced liver toxicity.

Keywords: Hepatoprotection, *Sphaeranthus indicus*, Antioxidants, Carbon tetrachloride, Rat.

INTRODUCTION

Multitude of physiological roles and biochemical functions of liver makes it an important effector of health and a target in disease. Acute hepatic injury induced by hepatotoxins continues to be one of the major causes of progressive liver disease. A large percentage of these injuries make the organ susceptible to the development of more severe and irreversible conditions such as cirrhosis and hepatocellular carcinoma. Therapeutic intervention of hepatic injury at the early stages of the disease has demonstrated significantly improved outcome in various animal models and human studies. Reactive oxygen species (ROS) generated spontaneously in cells during metabolism is implicated in the aetiology of different degenerative diseases. Chemical-induced liver injury mediated by the formation of reactive species during the microsomal detoxification is hypothesized to drive the pathogenesis. For example during the biotransformation of toxic substance like CCl_4, Cytochrome P450 in the endoplasmic reticulum generate trichloromethyl radical ($\cdot CCl_3$) which eventually leads to necrosis. Cells are protected against such oxidative stress by an interacting network of antioxidant enzymes[1,2]. Exogeneous antioxidants have also been shown promising results in the treatment of fatty liver diseases and biliary cirrhosis, major free radical mediated liver diseases. In addition, induction of anitoxidant ezymes using natural products has opened potential opportunities in the treatment by modulating physiological response. The recent studies showed that the plants such as *Piper longum* and Livergen, a poly herbal formulation are capable of performing the antioxidant and hepatoprotective activity against CCl_4 induced liver damage[3, 4].

Treatment and management of liver disease still pose a significant challenge to the modern medicine due to the lack of rational therapy. In this context a number of medicinal plant preparations whose usage have been in vogue since centuries recommended by the Ayurvedic system of medicine for the treatment of liver disorders hold promise[5,6]. Several reports suggest alternate medical uses of plant preparations due the commonality in all or part of their mechanism of actions. Traditionally, many medicinal plants are currently used in India for the treatment of liver ailments.

Sphaeranthus indicus Linn. (Family- Asteraceae) is a branched herb with purple flowers that grows abundantly in rice field and distributed throughout India. The plant is commonly known in Hindi as Gorakhmundi, an annual spreading herb, distributed throughout the plains of wetlands of India, Sri Lanka and Australia[7]. All parts of the plant have medicinal uses. The whole herb is used in ayurvedic preparations to treat epilepsy, mental disorders[8], piles, hepatitis[7] and have protection against immunosupression. Bioactive fraction of *S. indicus* has been shown to influence both humoral and cell-mediated immunity and protection against cyclophosphamide-induced immunosuppression in mice[10]. Ethanolic extract of the roots of *S. indicus* demonstrated antidiabetic, antihyperlipidemic, and *in vivo* antioxidant properties in streptozotocin- (STZ-) induced type 1 diabetic rats[11]. The anti-inflammatory activities of 7-hydroxy-frullanolide, a small molecule sesquiterpene lactone isolated from the fruit of *S. indicus*, in experimental models of acute and chronic inflammation has also been reported[12]. Current study aimed at investigating the hepatoprotective effects and antioxidant properties of *S. indicus* demonstrate that methanol extracts of *S. indicus* protect against CCl_4-induced liver injury in rats by potentiating the antioxidant enzymes in the liver.

MATERIALS AND METHODS

Chemicals

All chemicals used for the study were analytical grade and obtained from Sisco Research Laboratories (India).

Plant material

The plant was collected from Puliyarai (9°1'1''N, 77°11'37''E), Thirunelveli district of Tamil Nadu during February 2010. The materials were identified and authenticated by the Department of Botany, University of Kerala, Thiruvananthapuram. The plant material was thoroughly washed in water, stems and leaves were separated, finely chopped, shade dried for a week and then powdered with a mechanical grinder and stored in airtight container. The powder obtained was extracted using methanol (99.8 %) in a Soxhlet extractor for 72 hours. The methanol extract was then concentrated using a rotary vacuum evaporator under reduced pressure at 80°C. The methanol extract of *S. indicus* yielded 12.6 % w/w. The crude extract was stored at 4 °C.

Experimental Animals

Male Albino rats of Wistar strain weighing 150-200g were used in the study. They were purchased from Sree Chithira Tirunal Institute of Medical Science and Technology, Thiruvananthapuram. The animals were housed in polypropylene cages maintained in controlled temperature (27±2°c) and light cycle (12h light and 12h dark). Standard Food pellet (Sai Durga, Banglore) and water were provided *ad libitum*. The animals were acclimatized for one week before the experiment. Ethical clearance for handling the animals was obtained from the Institutional Animals Ethical Committee (IAEC-KU-18/09-10-ZOOL-GP (1)) prior to the beginning of the experiment.

Experimental Design

Animals were divided into 5 groups of 4 animals each and treated as follows. Group I served as control and were given the vehicle,

Carboxy methyl cellulose (CMC) at a rate of 1 ml/kg body weight. The group II rats received CCl_4 (1ml /kg body weight) for 10 days. The animals in group III were simultaneously treated with CCl_4 (1ml/kg) and a standard reference drug, Livomyn (100mg/kg body weight) for 10 days. Groups IV and V received CCl_4 (1ml/kg) for first 10 days and followed by administration of MES in doses 500mg/kg and 750 mg/kg respectively for next 10 days. At the end of the experimental period, animals were sacrificed by cervical decapitation. Blood samples were collected by direct heart puncture. Serum was separated by centrifuging at 3000 rpm for 15 minutes and analyzed for various biochemical parameters. Liver tissue was collected and processed for biochemical and histopathological analysis. .

Biochemical estimation

Levels of Aspartate amino transferase (AST), Alanine amino transferase (ALT) and Alkaline phosphate (ALP) assays were carried out using standard reagent kits (Span Diagnostic Ltd Surat. Code No. L G031). The procedures were essentially those described in the literature available with the kits. The total serum protein content was evaluated by using standard procedure of Lowry *et al*[13]. Activities of Superoxide dismutase (SOD)[14], catalase (CAT)[15] and glutathione transferase (GST)[16] were also assessed.

Histopatgological examination of liver

A portion of the liver in each group was fixed in 10% neutral buffered formalin for histopathological studies. Serial sections of 3 µm thickness were made from the fixed liver tissues and studied with haematoxylin and eosin to evaluate the details of hepatic architecture in each group microscopically.

Phytochemical Screening

Successive solvent extraction followed by qualitative examination for phytochemical screening was done. Screening of the phytoconstituents like alkaloids, carbohydrates, phytosterols, phenolics and proteins were done using standard procedures[17] (Table 1).

Statistical Analysis

The values were expressed as mean ± SE. Statistical analysis was performed by one way analysis of variance (ANOVA) followed by Tukey multi comparison test. P values < 0.005 were considered as significant.

Table 1: Preliminary phytochemical screening of Successive Extracts of *Sphaeranthus indicus*

	Alkaloids	Carbohydrates	Phytosterols	Phenolics	Proteins
Pet. Ether	-	-	+	-	-
Benzene	-	-	+	-	-
Chloroform	-	+	+	-	-
Acetone	+	+	-	+	-
Ethanol	-	+	-	+	-
Water	-	+	-	+	-

+ = present **-** = absent

RESULTS AND DISCUSSION

Intoxification of rats with CCl_4 significantly altered the liver biochemical parameters when compared with the normal control rats ($p<0.001$). In CCl_4 intoxicated rats (Group II), there was elevation in the levels of AST, ALT and ALP in comparison with the control group (Group I). However these elevated levels of ALT, AST and ALP were significantly lowered when treated with MES at doses 500 mg/body weight and 750 mg/body weight (Group IV and V, respectively) (Table 2). The liver injured rats that received 100 mg / kg body weight of the drug Livomyn (Group III) also restored the increased levels significantly when compared with the CCl_4 treated group. A significant ($p<0.001$) decrease in protein content was noted in CCl_4 treated rat (Group II). Treatment with *S. indicus* increased the protein level in a dose dependent manner (Groups IV and V).

Table 2: Biochemical activity of MES on serum (ALT, AST, ALP, Total protein) in CCl_4 hepatotoxicated rats

Treatment	Dose ml/mg /kg	ALT U/L	AST U/L	ALP U/L	Total protein mg%
Control	-	41.48±0.525	36.51 ±0.05	35.04±2.23	0.776±0.007
CCl_4	1ml	64.29±5.53*a***	54.81±8.44*a***	118.71±7.52*a***	0.4075±0.006*a***
Livomyn	100	17.53±1.005*a***b***	17.37±0.595*a***b***	49.62±6.89 *a*** b***	1.435±0.0926*b***
S. indicus	500	7.14±3.15 *a***b***	5.16±0.636 *a***b***	4.12±0.975 a** b**	0.997±0.0992*a**b***
S. indicus	750	7.67±3.2 *a***b***	3.932±0.50 *a*** b***	4.35±0.84*a***b***	1.009±0.1116*a**b***

Values are expressed as the mean + SEM for n=4 rats per group. p values: ***a*** indicates significant difference with control and ***b*** indicates significant difference with CCl_4 treated group. *= p<0.05, **=p<0.01

A significant decrease in ($p<0.001$) antioxidant enzymes like CAT, SOD, and GST activity was observed in the liver of CCl_4 administered (Group II) rats when compared to normal (Group I) rats that had received vehicle alone (Table 3). Treatment with the MES appeared to exert a beneficial effect since the activities of these enzymes were significantly higher in the liver of Group III (Livomyn treated), Group IV (MES at 500 mg/body weight) and Group V (MES at 750 mg/body weight) than that of Group II rats. The significant change in the levels of biochemical parameters refers to the effect of plant extract in protecting the liver by restoring the altered levels of hepatospecific parameters.

Table 3: Effect of *S. indicus* on antioxidant levels in CCl_4 hepatotoxicated rats

Treatment	Dose ml /mg/kg	SOD U/mg protein	CAT U/mg protein	GST U/mg protein
Control	-	0.485±0.017	1.1±0.08	0.557±0.009
CCl_4	1 ml	0.2125±0.0115*a***	0.65±0.06	0.2510±0.021*a***
Livomyn	100	0.2205±0.0285 *b***	0.8±0.05 *c**	0.4295±0.0105 *a***b***
S. indicus	500	0.2310±0.046 *b***	0.7±0.04	0.6399±0.0430 *a*** b***
S. indicus	750	0.3048±0.0690 *b**	0.72±0.03 *c**	0.5753±0.040 *a*** b***

Values are expressed as the mean + SEM for n=4 rats per group. p values: ***a*** indicates significant difference with control ***b*** indicates significant difference with CCl_4 treated group and *c* indicates significant difference with Livomyn treated group. *= p<0.05, **=p<0.01

The hepatoprotective effect of the test drug was further confirmed by histopathological examination of the livers of control, CCl_4 treated, CCl_4 plus Livomyn treated and CCl_4 plus test drug extract treated groups. The histopathological pattern of the livers of the rats treated with CCl_4 plus extract showed restitution of lost hepatic architecture and crowded central vein. From the present study it has been confirmed that leaves and stem methanol extract of *S. indicus* has strong protective role in repairing liver damages induced by a well known toxin, carbon tetrachloride. Carbon tetrachloride (CCl_4) is an organic solvent widely used in chemical industry. CCl_4 get accumulated in hepatic parenchyma cells and the bio-transformation of CCl_4 occurs in the endoplasmic reticulum that is mediated by CyP450[18]. CyP450 is inhibited suicidally by the reactive metabolites of CCl_4. CCl_4 is metabolically activated by the Cytochrome P450 dependent mixed oxidase in the endoplasmic reticulum to form trichoromethyl free radical ($\bullet CCl_3$).

This free radical induces an adverse reaction by forming other free radicals after its administration in the early stage between intracellular uptake and transformation into storage types. Many biological substances such as membrane lipids, proteins, and nucleic acids are known to be injured by trichloromethyl radicals[19]. The trichloromethyl radical ($.CCl_3$) is converted into $.CCl_3O_2$ through its reaction with molecular oxygen. Lipid peroxidation is initiated by the interaction of this reactive free radical, $.CCl_3O_2$, with polyunsaturated fatty acids (PUFA) of the membrane lipids[20, 21, 22, 23]. This result in change of structures of the endoplasmic reticulum and other membrane, loss of metabolic enzyme activation, reduction of protein synthesis and loss of glucose 6- phosphatase activation leading to liver injury[24, 25, 26, 27]. When rats were treated with CCl_4 it induced hepatotoxicity by metabolic activation, therefore it selectively causes toxicity in liver cells maintaining semi-normal metabolic function. Necrosis or membrane damage releases the enzyme into circulation and hence it can be measured in the serum[28]. Elevated levels in hepatospecific enzyme principally found in the cytoplasm in rats following the administration of CCl_4 is attributed to the increased release of the enzymes from damaged liver parenchymal cells[29]. These are indicative of cellular leakage and loss of functional integrity of cell membranes in liver cells[30]. The reversal of increased serum enzymes in CCl_4 induced liver damage by the extract could be due to the prevention of the leakage of intracellular enzymes by its membrane stabilizing activity. This is in agreement with the commonly accepted view that serum levels of transaminase reduces considerably with the healing of hepatic parenchyma and the regeneration of hepatocytes[31].CCl_4 might have checked the incorporation of amino acids to synthesize protein and which could be the reason for the decreased protein values. These parameters were brought back to the normal levels in the plant extract treated animals. Hence, *S. indicus* extract proved protection against the injurious effects of CCl_4 that may result from the interference of Cytochrome P450, resulting in the hindrance of the formation of hepatotoxic free radicals. Both Livomyn and the plant extract decreased CCl_4 elevated enzyme levels in tested groups, indicating the protection of structural integrity of hepatocytic cell membranes and regeneration of damaged liver cells.

Antioxidant or free radical generation inhibition is important in protection against CCl_4 induced lesions. This is accomplished by a set of endogenous antioxidant enzymes such as SOD, CAT, and GST. In the present study, the data suggested that high dose of CCl_4 in liver could lead to decreased level of antioxidant enzymes. It is evident that *S. indicus* causes a significant increase in hepatic SOD activity and thus reduces reactive free radical induced oxidative damage to liver. Reduction in the activity of CAT may result in a number of deleterious effects due to the assimilation of super oxide radical and hydrogen peroxide. A higher dose of selected plant extract, 750 mg/kg body weight and a dose of 100mg /body wt. of standard hepatoprotective drug, Livomyn increased the level of CAT and helped the liver to regain the normal health status. GST removes free radical species such as H_2O_2, superoxide radicals and maintains membrane protein thiols. The decreased level of GST is associated with an enhanced lipid peroxidation in CCl_4 treated rats. Administration of *S. indicus* significantly ($p<0.001$) increased the level of GST in a dose dependent manner.

Histology of liver tissues of normal group of animals exhibited hepatic cells with well-defined cytoplasm, prominent nucleus, and well brought out central vein (Fig 1a) whereas that of CCl_4 intoxicated group animal showed total loss of hepatic architecture with crowding of central vein (Fig 1b). Livomyn treated animals exhibited a significant improvement in liver structure. Treatment with MES at a dose of 500 mg /kg body weight showed weak activity in protecting the liver cells from CCl_4 injury and treatment with *S. indicus* at a dose of 750 mg /kg body weight returned the injured liver to quite normal (Fig 1 c & d). Among the two experimental doses of plant extracts, a significant hepatoprotective activity was observed at a dose of 750 mg/kg body weight even though the indicatives of liver repair mechanism between the two doses are not statistically significant.

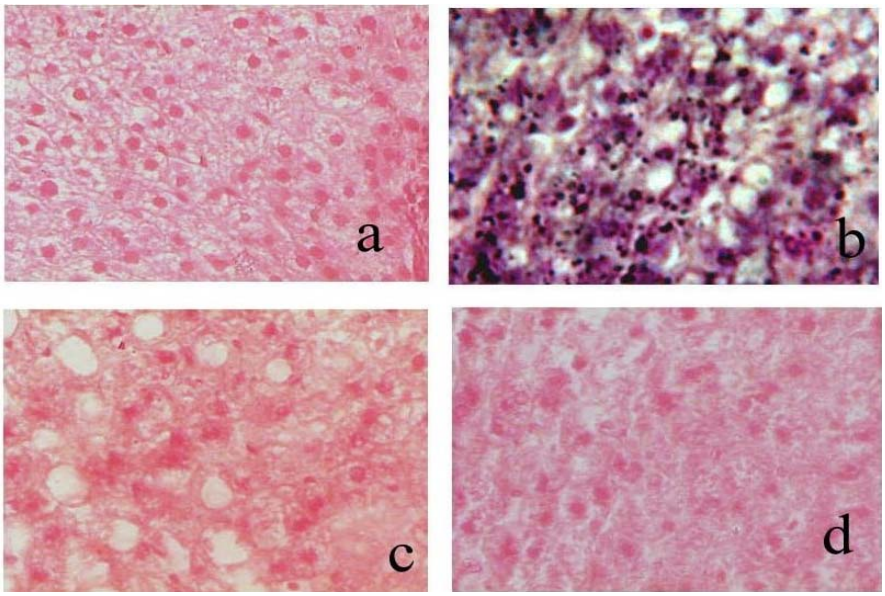

Fig. 1: (a) Liver section of control rat showing hepatic cell with well-defined cytoplasm, prominent nucleus and well brought out central vein; (b) Liver section of CCl_4 intoxicated animal group with total loss of hepatic architecture with crowding of central vein; (c) Liver section of the rat treated with plant extract 500 mg/kg, 40 x, haematoxilin- eosin stain. Liver section shows reduction in hepatic necrosis; (d) Liver section of the rat treated with plant extract 750 mg/kg 40 x, haematoxilin- eosin stain. Liver section shows reduction in hepatic necrosis and the cells are more or less similar to normal

Free radical mediated process has been implicated in pathogenesis of most of the diseases. The protective effect of *S. indicus* on CCl_4 induced hepato toxicity in rats appears to be related to the enhancement of antioxidant enzyme levels in addition to free radical scavenging action. As indicated by qualitative examinations, the alcoholic extracts of the plant contain phenolic compounds. Flavonoids, a polyphenolic derivative could be the major contributory factors in hepatoprotectivity in the present study as reported by others[32, 33]. The present study reveals that a cost effective and clinically highly efficient liver protection drug can be derived from this plant which is now considered as weed in paddy fields of India.

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