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Antioxidant and hepatoprotective activity of *Murraya koenigii* L. against CCl_4-induced hepatotoxicity in rats

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ABSTRACT

Background: Liver disease is one of the global medical problems which cause significant morbidity and mortality. In present study, hepatoprotective and antioxidant property of methanolic extract of *Murraya koenigii* L. (Rutaceae) were investigated in CCl_4 induced hepatotoxicity in rats.

Material and methods: Hepatotoxicity was induced by CCl_4 (2 ml/kg, i.p.) and silymarin was used as a standard drug. Before CCl_4 intoxication, rats were treated with methanolic extract of *Murraya koenigii* L. at a dose of 50, 100 and 200mg/kg once day for 14 days. Treatment with a methanolic extract of *Murraya koenigii* L. showed significant hepatoprotective activity in CCl_4 intoxicated rats which were indicated by reduction in the level of marker of hepatotoxicity. Treatment also showed significantly decreased in hepatic lipid peroxidation and a corresponding increase in tissue antioxidant enzyme.

Result: The results of present study demonstrate the hepatoprotective activity of methanolic extract of *Murraya koenigii* L. against CCl_4-induced hepatotoxicity in rats which might be due to its anti-oxidant activity.

Conclusion: It suggests a potential therapeutic use of *Murraya koenigii* L. in treatment of acute liver diseases and oxidative stress.

Keywords: Liver damage; hepatoprotective effect; antioxidant effect; *Murraya koenigii* L.

INTRODUCTION

The liver is a major organ which plays an important role in synthesis, secretion, metabolism of xenobiotics and elimination and detoxification of drugs. Liver damage characterized by jaundice, cholestasis, liver enlargement, portal hypertension, esophageal varices, ascites, liver encephalopathy and liver failure which may be due to alcohol consumption, infection, malnutrition, anaemia, xenobiotics and medication. It is one of the global medical problems with significant morbidity and mortality (Ansari et al., 2013).

Reactive oxygen species and reactive nitrogen species also known as free radicals are produced in a cell as a result of normal metabolic reaction (Ansari et al., 2012). Mammalian cells contain endogenous antioxidant enzymes, including superoxide dismutase (SOD), glutathione peroxidase and catalase which neutralize excess of free radical and protect the cell from damage. But when level of antioxidant and free radical is imbalance due to endogenous or exogenous cause, it produces oxidative stress where available quantity of antioxidant is insufficient to neutralize free radical (Saeed et al., 2005).

In spite of increased incidence of liver transplantations and death due to liver damage, none of modern medicine available today showed alleviation of liver diseases which further demand investigation for new and effective treatment. A large number of medicinal plants species and their products have been reported as potential therapeutic agents for liver disease (Ansari and Rashid, 2012). *Murraya koenigii* L. (Rutaceae) is a small spreading shrub, about 2.5 meters in height widely used as a spice for its characteristic flavor and aroma. It is reported to possess antibacterial, antidiabetic, antiulcer, antiinflammatory, wound healing, enzyme inhibiting, antioxidant, antidysenteric, anticarcinogenic activity (Vaibhav et al., 2011; Muthumani et al., 2010; Indu and Menon, 2010). Several study suggested that hepatoprotective effects of plant extracts are associated

with their antioxidant plant constituents (Celik et al., 2009).

The present study was designed to evaluate antioxidant and hepatoprotective activity of *Murraya koenigii* L. against CCl_4 induced hepatotoxicity in rats.

MATERIALS AND METHODS

Chemicals

Malondialdehyde (MDA) was obtained from Sigma Chemicals Company, St Louis, MO, USA. Silymarin was obtained from Ranbaxy Laboratories, Delhi, India. CCl_4 was obtained from E-Merck, Mumbai, India. All other reagents used in the experiment were of analytical grade.

Kits

Alanine aminotransferase (ALT), aspartate amino transferase (AST), alkaline phosphatase (ALP), superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH-Px) kits were purchased from Randex Laboratories (San Francisco, CA, USA). Total bilirubin (TB) kits were purchased from Biomerieux, Laboratory of Reagents and Products (Marcy Le´ toile, France). Total antioxidant capacity (TAC) kit was purchased from Biodiagnostic Co. Other chemicals were of the highest purity commercially available.

Preparation of plant extract

The leaf of *Murraya koenigii* L. was collected from Karnataka, India, during the month of September. The 200 g shade-dried powdered of leaf of *Murraya koenigii* L were extracted in 500 ml mehanol for 6 h using soxhlet apparatus. Mixture of extraction was filtered, and the filtrate was evaporated and concentrated in vacuum desiccators until dry yielding 21.8% residue. The extract was suspended in vehicle (0.2% propyleneglycol in saline solution).

Determinations of total phenolic and flavonoid contents

Total phenolic content of methanolic extract of *Murraya koenigii* L. was determined using the Folin– Ciocalteau assay (Ksouri et al., 2009). Total phenolic content was calculated by using the calibration curve obtained from standard solution of gallic acid. Results were expressed in mg/g dry extract, as gallic acid equivalents. Total flavonoid content of methanolic extract of *Murraya koenigii* L. was determined by colorimetric assay (Moreno et al., 2000). Total flavonoid content was calculated by using a calibration curve generated using rutin as standard compound. Results were expressed in mg/g of dry extract, as rutin equivalents.

Animals

Male Wistar rats (100–150 g; 4–6 weeks old) were maintained under controlled conditions of light (12 h/12 h), temperature (26±2 °C) and relative humidity (44–56%) for 1 week before and during the experiments. The animals had free access to standard laboratory feed (Gold Mohur, Hindustan Lever Ltd., Mumbai, India) and water *ad libitum*. For experimental purposes animals were kept fasting overnight but were allowed free access to water. The study was conducted after obtaining ethical committee clearance.

Acute toxicity studies

An acute oral toxicity study was carried out using OECD guidelines-423 for the testing of chemicals. Wistar rats (n=6) of either sex having weight of 180–240 g were selected for the acute toxicity study. The methanolic extract of *Murraya koenigii* L. was administered orally at a dose level of 2000 mg/kg.) to the group of three overnight fasted rats and observed for14 days. If mortality was observed in 2 out of 3 animals, then the dose administered was considered as a toxic dose. If mortality was observed in all animals, then the same dose was repeated again to confirm the toxic dose. If mortality was observed again, the procedure was repeated for lower doses (300, 50 and 5 mg/kg body weight).

Experimental design

All the animals were acclimatized for at least one week prior to experiment and then randomly divided into seven groups (n=6). Treatment was assigned according to the group made below:

Group I (Normal control): 20% Tween 20 solution (v/v) 10 ml/kg p.o.

Group II (Negative control): CCl_4 (2 ml/kg i.p).

Groups III–V: Methanolic extract of *Murraya koenigii* L. (MK), (50, 100 and 200 mg/kg p.o) + CCl_4.

Group VI (Standard): Silymarin (20 mg/kg p.o) + CCl_4.

On the 14th day, the rats were anesthetized using thiopental, and blood was collected from abdominal artery and kept at 37 °C in the incubator for 30 min. Later on, it was cold centrifuged at 2000 rpm for 15 min to isolate the supernatant serum, which was used for the biochemical estimations. The livers from rats were removed, weighted and immediately homogenized (10%, w/v) at 20,000 rpm with homogenizer (IKA T18 basic, IKA-Werke GmbH & Co., Staufen, Germany) in ice chilled 0.9% NaCl solution. The suspension was centrifuged at 2000 rpm at 4 °C for 10 min and the resulting supernatant was used for the following biochemical estimations. Rest of the liver was used to carry out histopathological study.

Assessment of serum biochemical parameters

The activities of serum alkaline phosphatase (ALP), aspartate transaminase (AST), alanine transaminase (ALT), albumin (ALB), total protein (TP) and total bilirubin (TB) were determined at the end of the study. The blood samples were collected from all animals from the retro-orbital venous plexus and serum biochemical parameters were determined according to the manufacturer's instructions.

Assessment of antioxidant enzymes

Liver tissue homogenate was used for the following assay of antioxidant enzyme activity. The activities of GSH-Px, SOD and CAT in the liver were evaluated using commercial kits and a spectrophotometer (UT-1800,

Pgeneral, Beijing, China). The specific enzyme activities of SOD, GSH-Px and CAT were expressed as U/mg protein.

Assessment of tissue biochemistry

MDA, marker of Lipid peroxidation (LPO) was measured by using the TBARS assay in the liver tissue homogenate. Briefly, the homogenate was centrifuged at 3000 rpm for 15 min. The supernatant was transferred to an Eppendorf tube and centrifuged at 12,000 rpm for 30 min. The supernatant was used for the determination of MDA as a lipid peroxidation marker (Nourooz-Zadeh, 1995). The protein concentration of liver homogenate was measured by the method of Lowry et al. (1951) using bovine serum albumin as a standard (Lowry et al., 1951). The results were expressed as the amount of MDA formed per mg of protein.

Total antioxidant capacity (T-AOC) of liver tissue homogenate was estimated by the method of Benzie and Strain (1996) using commercial kits. This kit uses antioxidants in the samples to reduce Fe3+ to Fe2+, which are then chelated with porphyrin to produce a purple complex. The antioxidant activity can then be quantified by measuring the absorbance at 550 nm. The T-AOC of the samples was determined by comparison with a controlled standard and expressed as U/mg protein. The activities of adenosine triphosphatase (ATPase) and glucose-6-phosphatase (G-6-Pase) were also determined in the liver by using the method of Seth and Tangari (1966) and Baginski et al. (1974) respectively.

Histopathology

The liver tissue was collected and fixed in 10% formalin, dehydrated in graduated ethanol (50–100%), cleared in xylene, and embedded in paraffin. 4–5 µm thick Sections were prepared and then stained with hematoxylin-eosin (H–E) dye and examined for histopathological changes under the microscope. The images were taken at 400 x magnification using CARL ZEISS microscope.

Statistical analysis

The obtained raw data in each experimental group were expressed as mean ± standard error of mean (SEM). Groups of data were compared with the analysis of variance (ANOVA) followed by Dunnett's test for multiple comparison of the two treatment groups with the control. Values were considered statistically significant if $p<0.05$.

RESULTS

Acute toxicity studies

The methanolic extract of *Murraya koenigii* L. did not cause any mortality up to 2000 mg/kg, and hence $1/40^{th}$ $1/20^{th}$ and $1/10^{th}$ of the maximum dose administered (i.e., 50, 100, 200 mg/kg, p.o.) were selected for the present study.

Assessment of serum biochemical parameters

The effects of methanolic extract of *Murraya koenigii* L. at three tested dose levels (50, 100 and 200 mg/kg b.w) on serum marker enzymes of hepatotoxicity in CCl_4 induced hepatotoxicity are shown in Table 1. The results of the study revealed a significant elevation of ALT, AST, ALP, and TB in a serum and fall in the serum ALB and TP activities in the CCl_4 intoxicated group compared with the normal controls ($p < 0.01$). The levels of the ALT, AST, ALP, and TB enzymes were markedly decreased in rats treated with methanolic extract of *Murraya koenigii* L compared to the CCl_4 intoxicated group, although this decrease was maximum ($p < 0.01$) in the group receiving methanolic extract of *Murraya koenigii* L. at the highest dose. Also level of ALB and TP was significantly improved in the rats treated with methanolic extract of *Murraya koenigii* L. compared to the CCl_4 intoxicated group.

Table 1. The effects of methanolic extract of *Murraya koenigii* L. at three tested dose levels (50, 100 and 200 mg/kg b.w) on serum marker of hepatotoxicity in CCl_4 induced hepatotoxicity in rats.

Group	ALT (IU/L)	AST (IU/L)	ALP (IU/L)	ALB (g/L)	TP (g/L)	TB (g/L)
Normal control	62.76 ± 12.25	125.45 ± 15.16	280 ± 62	35.87 ± 3.55	70.50 ± 8.45	0.04 ± 0.03
CCl_4 control	825.45 ± 40.34#	580.34 ± 67#	610 ± 98#	22.80 ± 3.21#	58.86 ± 5.54#	0.26 ± 0.07#
MK-I + CCl_4	790.34 ± 38.23*	479.67 ± 56.46*	360 ± 84*	29.50 ± 3.40*	64.45 ± 6.10*	0.11 ± 0.06*
MK-II + CCl_4	435.98 ± 16.45*	336.34 ± 43.51*	330 ± 65*	31.23 ± 3.60*	67.58 ± 7.34*	0.08 ± 0.05*
MK-III + CCl_4	266.12 ± 12.34*	270.47 ± 34.76*	320 ± 58*	32.50 ± 3.72*	68.01 ± 7.25*	0.06 ± 0.05*
Sylimarine+CCl_4	160.45 ± 10.56*	180.89 ± 18.54*	310 ± 55*	33.80 ± 3.80*	69.21 ± 7.50*	0.04 ± 0.04*

Data represented as mean ± SEM. #P< 0.05, compared to normal control group

*P< 0.05, compared to CCl_4 control group, significance by analysis of variance (ANOVA) followed by Dunnett's test for multiple comparison.

Table 2. The effects of methanolic extract of *Murraya koenigii* L. at three tested dose levels (50, 100 and 200 mg/kg b.w) on serum marker enzymes of oxidative stress in CCl_4 induced hepatotoxicity in rats.

Group	GSH (µmol P liberated/ min/mg protein)	SOD (IU/mg protein)	CAT (nmol of H2O2 consumed/min/mg protein)
Normal control	40.56 ± 1.50	5.78 ± 0.10	68.54 ± 1.00
CCl_4 control	14.20 ± 0.56#	2.54 ± 0.07#	23.54 ± 0.95#
MK-I + CCl_4	22.85 ± 0.80*	3.26 ± 0.10*	31.68 ± 1.25*
MK-II + CCl_4	25.00 ± 0.96*	4.00 ± 0.13*	44.22 ± 1.72*
MK-III + CCl_4	27.50 ± 1.01*	4.50 ± 0.15*	52.65 ± 1.65*
Sylimarine + CCl_4	30.87 ± 1.04*	4.88 ± 0.19*	58.50 ± 1.76*

Data represented as mean ± SEM. #P< 0.05, compared to normal control group.
* P< 0.05, compared to CCl_4 control group, significance by analysis of variance (ANOVA) followed by Dunnett's test for multiple comparison.

Table 3. The effects of methanolic extract of *Murraya koenigii* L. at three tested dose levels (50, 100 and 200 mg/kg b.w) on lipid peroxidation and tissue biochemistry in CCl_4 induced hepatotoxicity in rats.

Group	MDA (nmol MDA/mg protein)	T-AOC (U/mg)	Na K-ATPase (µmole of Pi liberated/min /mg protein)	G-6-Pase (µmole Pi/min/ g liver)
Normal control	0.20 ± 0.08	3.00 ± 0.10	1.00 ± 0.10	6.80 ± 0.35
CCl_4 control	0.44 ± 0.12	1.70 ± 0.13	0.60 ± 0.06#	3.50 ± 0.23#
MK-I + CCl_4	0.34 ± 0.07*	2. 24 ± 0.06*	0.85 ± 0.07*	4.60 ± 0.30*
MK-II + CCl_4	0.29 ± 0.06*	2.45 ± 0.08*	0.90 ± 0.08*	5.50 ± 0.33*
MK-III + CCl_4	0.26 ± 0.05*	2.65 ± 0.07*	0.93 ± 0.07*	6.12 ± 0.35*
Sylimrn + CCl_4	0.24 ± 0.04*	2.90 ± 0.09*	0.96 ± 0.09*	6.65± 0.40*

Data represented as mean ± SEM. #P< 0.05, compared to normal control group
* P< 0.05, compared to CCl_4 control group, significance by analysis of variance (ANOVA) followed by Dunnett's test for multiple comparison.

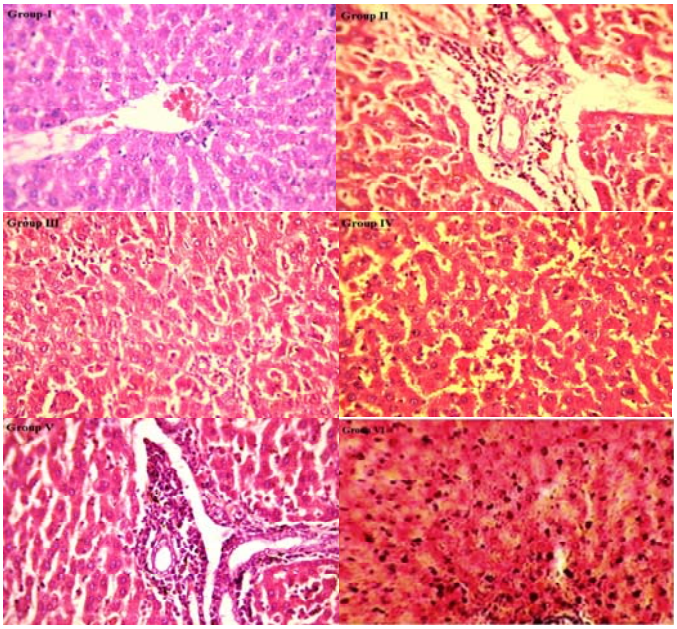

Figure 1. Representative photo micrographs of liver sections from group I to group VI.

The liver section of the control animals (Group I) showed a normal liver architect with central vein surrounded by normal hepatocytes. On the contrary, liver sections from the rats treated with CCl_4 alone (Group II) showed massive changes throughout the lobules, with mononuclear collections around portal triad with sinusoidal dilatation, cellular vacuolization, necrosis, inflammatory infiltrations of the portal triads, and distortion of the central venules. The treatment of animals with MK-I (Group III) revealed lymphocytic collection in sinusoidal space. The treatment of animals with MK-II (Group IV) showed near normal hepatocytes and occasional cell with nuclear condensation where as treatment of animals with MK-III (Group-V) and Sylimarine (Group-VI) showed normal hepatocytes with no damage.

Assessment of antioxidant enzymes

The effects of methanolic extract of *Murraya koenigii* L. at three tested dose levels (50, 100 and 200 mg/kg b.w.) on serum marker enzymes of oxidative stress in CCl_4 induced hepatotoxicity are shown in Table 2. The results of the study revealed a significant decreased in the level of serum GSH, SOD and CAT activities in the CCl4-treated group compared with the normal controls ($p < 0.01$). The levels of the above enzymes were markedly improved in the rats treated with methanolic extract of *Murraya koenigii* L. compared to the CCl_4 intoxicated group, although this improvement was maximum ($p < 0.01$) in the group receiving methanolic extract of *Murraya koenigii* L. at the highest dose.

Assessment of lipid peroxidation and tissue biochemistry

The effects of methanolic extract of *Murraya koenigii* L. at three tested dose levels (50, 100 and 200 mg/kg b.w.) on lipid peroxidation and tissue biochemistry in CCl_4 induced hepatotoxicity in rats are shown in Table 3. The treatment with CCl_4 showed obviously higher MDA levels and decreased in total antioxidant capacity, Na^+ K-ATPase and G-6-Pase in liver tissue than the normal control group ($p < 0.01$). MDA levels in the methanolic extract of *Murraya koenigii* L. and sylimarine treated group were remarkably lower than CCl_4 intoxicated group ($p < 0.01$). In addition, total antioxidant capacity and the level of Na^+ K-ATPase and G-6-Pase in the liver are also significantly improved in methanolic extract of *Murraya koenigii* L. and sylimarine treated group.

Histopathology

The liver lobules of the control animals (Group I) showed a normal liver architect with central vein surrounded by normal hepatocytes. On the contrary, liver sections from the rats treated with CCl_4 alone (Group II) showed massive changes throughout the lobules, with mononuclear collections around portal triad with sinusoidal dilatation, cellular vacuolization, necrosis, inflammatory infiltrations of the portal triads, and distortion of the central venules. The treatment of animals with MK-I (Group III) revealed lymphocytic collection in sinusoidal space. The treatment of animals with MK-II (Group IV) showed near normal hepatocytes and occasional cell with nuclear condensation. Whereas, treatment of animals with MK-III (Group-V) and Sylimarine (Group-VI) showed normal hepatocytes without any damage.

DISCUSSION

The liver is one of the major organs in our body responsible for mtabolism of toxic chemicals and drugs. Thus it is a target organ for all toxic chemicals. The CCl_4

induced hepatotoxicity model is extensively used for the evaluation of antioxidant and hepatopreotective activity of drugs and plant extracts (Amani et al., 2006; Jain et al., 2008). The trichloromethyl free radical (CCl3•) which is an active metabolite of CCl_4, is mainly associated with CCl_4 induced hepatic damage by reacting with sulfhydryl groups of glutathione and protein thiols (Johnston and Kroening, 1998). The covalent binding of these radicals to sulfhydryl-containing proteins in cells initiate a chain of events leading to membrane lipid peroxidation and cell necrosis (Reckengel et al., 1989). In present study, hepatoprotective and antioxidant properties of methanolic extract of *Murraya koenigii* L. (Rutaceae) were investigated in CCl_4 induced hepatotoxicity in rats.

Serum hepatobiliary enzymes such as ALT, AST, ALP, TB, ALB and TP were reported to be altered in liver damage (Drotman and Lawhan, 1978; Wolf, 1999). The present study revealed a significant elevation of ALT, AST, ALP, and TB in a serum and fall in the serum ALB and TP activities in the CCl_4 intoxicated group indicating liver damage induced by CCl_4. Treatment with methanolic extract of *Murraya koenigii* L. showed dose dependent decreased in the levels of the ALT, AST, ALP, TB and increased in the level ALB and TP in the CCl_4 intoxicated group, indicating hepatoprotective activity of methanolic extract of *Murraya koenigii* L.against CCl_4 induced hepatic damage.

Lipid peroxidation is an important marker of oxidative stress. The increase in liver MDA levels induced by CCl_4 suggests enhanced lipid peroxidation, leading to hepatic tissue damage and failure of antioxidant defense mechanisms to prevent formation of excessive free radicals (Souza et al., 1997). The result of present study revealed significance increased in the level of MDA which is a marker of lipid peroxidation and decreased in the level of antioxidant enzymes such as GSH, SOD and CAT in CCL_4 treated group indicating oxidative stress induced in CCl_4 treated group. Treatment with methanolic extract of *Murraya koenigii* L. showed significance decreased in the level of MDA and increase in the level of GSH, SOD and CAT, indication free radical scavenging activity of methanolic extract of *Murraya koenigii* L. in CCl_4 treated group.

In the present study, methanolic extract of *Murraya koenigii* L. restored the altered serum marker of hepatotoxicity, decreased liver lipid peroxidation, and increase level of antioxidant enzymes. Liver histopathology showed that methanolic extract of *Murraya koenigii* L. reduced the incidence of liver lesions induced by CCl_4 in rats.

CONCLUSION

The overall results of the present study indicate hepatoprotective and antioxidant activity in CCl_4 intoxicated rats. Free radical scavenging activity is one of the major antioxidation mechanisms inhibiting the chain reaction of lipid peroxidation induced by CCl_4. Therefore, hepatoprotective activity of methanolic extract of *Murraya koenigii* L. might be attributed to its free radical scavenging and antioxidant activity. However, further study is required to characterize the active ingredient present in the plant.

CONFLICT OF INTEREST

None declared.

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