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The protective effect of *Murraya koenigii* leaves against carbon tetra chloride-induced hepatic damage in rats

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PEER REVIEW

Peer reviewer

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Comments

Authors have done a very good piece of work. CCl_4 toxicity in rats is an ideal model that represents toxicity by several agents. The antioxidant and hepatoprotective activity were well supported by liver markers, antioxidant enzymes and histopathological examination. This work offers scientific validation for its use in folk medicine.

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ABSTRACT

Objective: To evaluate the efficacy of methanolic extract of *Murraya koenigii* (MMK) leaves in attenuating the hepatic damage inflicted by carbon tetrachloride (CCl_4), a potent oxidative stress inducer and a model hepatotoxicant.

Methods: Rats were divided into six groups of five each: normal control group, CCl_4 group, CCl_4+silymarin group, CCl_4+MMK group (200 mg/kg body weight), CCl_4+MMK group (300 mg/kg body weight) and CCl_4+MMK group (500 mg/kg body weight). Rats were intraperitoneally injected with 20% CCl_4 in corn oil (1 mL/kg body weight) and MMK was orally administered for 3 weeks. Levels of hepatic markers such as alanine transaminase, aspartate transaminase, alkaline phosphatase and total bilirubin were measured. Activities of superoxide dismutase, catalase and glutathione peroxidase were assayed and malondialdehyde content was measured. For histopathological examination, liver microtome sections were prepared and observed under light microscope.

Results: Oral administration of MMK had significantly reduced the activities of aspartate transaminase, alanine transaminase, alkaline phosphatase and bilirubin content in a dose-dependent manner, which were elevated by CCl_4. However, CCl_4-induced rise in lipid peroxidation and drop in superoxide dismutase, catalase and glutathione peroxidase activities were reversed by MMK administration. Further, the hepatoprotective activity of MMK was supported by histopathological examination of liver microtome sections.

Conclusions: Our biochemical and histological studies demonstrate the potential antioxidant and hepatoprotective activity of MMK and our results scientifically validate the often use of MMK leaves in food preparation and in Ayurvedic medicine in India and neighboring countries.

KEYWORDS

Murraya koenigii, Hepatoprotective, Carbon tetrachloride, Rats, Silymarin

1. Introduction

Humans are exposed to various industrial, occupational and environmental pollutions. Continuous exposure to pollutants such as xenobiotics, pesticides, organic pollutants and consumer products could cause cancers, wounds, inflammation and organ damages[1]. Carbon tetrachloride (CCl_4), a widely used fat soluble industrial solvent, is reported to cause free radicals induced damage to vital tissues such as lungs, kidneys, brain, blood and testis[2-4]. A few studies unequivocally reported CCl_4-induced liver injury in rats[5]. Since the pathological lesions developed in CCl_4 treated animals closely resemble the symptoms of cirrhosis and severe viral hepatitis in humans, it serves as an excellent model to assess the efficacy of hepatoprotection[6]. CCl_4 initially binds with metabolic intermediates and cellular proteins that will alter the cascade of events culminating in cellular necrosis due to membrane lipid peroxidation[5]. Hepatic markers such as alanine transaminase (ALT), aspartate transaminase (AST), bilirubin and reactive oxygen species (ROS) stand implicated in CCl_4 induced hepatic damage[7].

Considering the adverse effects caused by prolonged

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usage of synthetic drugs in treating various ailments, there is a shift in orientation towards natural drugs. Plants and their products have been a rich and valuable source of new molecules that could be well considered as a potential alternative to synthetic drugs. *Murraya koenigii* (*M. koenigii*) is a member of the family, Rutaceae. It is known as Karivepaku in Telugu and curry patta in Hindi, and is extensively used in food preparations in India and several Asian countries[8,9]. In folk and traditional medicine, it is implicated in the treatment of several ailments including traumatic injury, diabetes, jaundice, stomachache and dysentery. The leaves are described to possess antidiabetic[10], antilipidemic[11], antioxidant[12,13], wound healing, immunomodulatory[10,14], and chemomodulatory activities[15].

Previous reports stated that *M. koenigii* leaves contain different phytocompounds including alkaloids, flavanoids, furocoumarins, terpenoids and tannins[16]. A few bioactive compounds such as mahanimbilyl acetate, girinimbilyl acetate and bicyclomahanimbiline have been isolated and reported to possess antimicrobial and antioxidant activity[17,18]. Although CCl_4 model well represents several hepatic disorders, there are scanty studies on the hepatoprotective activity of *M. koenigii* leaves on CCl_4-induced toxicity. Therefore, we aimed to perform this study on Sprague Dawley rats. Our present study offered scientific validation for its usage in food preparations and demonstrated its antioxidant and hepatoprotective efficacy. The protective efficacy of *M. koenigii* was compared with standard drug silymarin, which has been used in clinical practice for the treatment of hepatic diseases[19].

2. Materials and methods

2.1. Chemicals and drugs

CCl_4 (Merck India Ltd.), silymarin (Admac Pharma Ltd., India), diagnostic kits (Lab Care Diagnostics Pvt. Ltd.), solvents *n*-hexane, ethyl acetate, methanol *etc.*, were procured from Hi Media. Silica gel used for column chromatography was purchased from Finar Chemicals, India Pvt. Ltd. Thin layer chromatography plates were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Preparation of methanolic extract and phytochemical analysis

Fresh leaves of *M. koenigii* collected from local market of Tirupati, were shade-dried and made into course powder. This was sequentially extracted with hexane, ethyl acetate, methanol and water in 10 L aspirators by soaking (24 h×3 d) at room temperature. These extracts were evaporated by using rotavapor to obtain respective dry extracts. Phytochemical analysis of these extracts was carried out to find out the nature of compounds present[20]. The obtained crude extracts were tested for their hepatoprotective and antioxidant activities.

2.3. Experimental animals

Adult Sprague Dawley rats (190±10) g and standard normal diet were purchased from National Institute of Nutrition, Hyderabad, India. Rats were housed in polypropylene cages [photoperiod 12 h light/12 h dark cycle, ambient temperature (25±5) °C, humidity 30%-60%]. Animals were allowed to free access of diet and water *ad libitum*. Rats were acclimatized in laboratory condition for 7 d before performing experiments. All procedures involving laboratory animals were in accordance with the Institute's Animal Ethics Committee regulations approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals.

2.4. Induction of hepatotoxicity by CCl_4

Animals were divided into six different groups of five rats each. Animals of Group I served as normal control and received distilled water orally for 14 d and on 7th day olive oil (1 mL/kg body weight) was given intra peritoneally (*i.p*). Group II served as toxic control and received distilled water orally for 14 d and on 7th day CCl_4 and olive oil in 1:1 dilution (1 mL/kg body weight) was administered (*i.p*). Group III was administered with CCl_4 (*i.p.*, on 7th day) and silymarin (25 mg/kg body weight, orally) for 14 d. Groups IV to VI received CCl_4 (*i.p.*, on 7th day), and methanolic extract of *M. koenigii* (MMK) fraction rich in carbazole alkaloids at doses of 200 mg/kg, 300 mg/kg and 500 mg/kg body weight respectively. After 14 d of treatment, rats were anaesthetized and blood was collected from the retro-orbital sinus, and the serum was separated for assessment of different biochemical parameters.

2.5. Assessment of ALP, AST, ALT, total bilirubin and total protein

The activities of hepatic marker enzymes, aspartate transaminase (AST), alanine transaminase (ALT) and protein were assayed by the method of Reitman and Frankel[21]. Serum bilirubin and alkaline phosphatase (ALP) levels were measured by Malloy and Evelyn, and King respectively using assay kits[22,23].

2.6. Assessment of activities of SOD, CAT and GPx

The liver was cleaned with 0.9% ice cold saline, cut into small pieces and homogenised in 0.1 mol/L phosphate buffer (pH 7.4) using a teflon homogenizer. The homogenate was centrifuged at 3 000 *g* for 20 min and the supernatant was used for the estimation of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). SOD activity was measured from the ability of the tissue homogenate to scavenge the superoxide anion generated from the photo-illumination of riboflavin according to the method of Cord and Fridovich[24]. CAT activity was determined from the rate of decomposition of H_2O_2[25]. GPx activity was determined by measuring the decrease in glutathione content after incubating the sample in the presence of H_2O_2 and NaN_3[26].

2.7. Assessment of lipid peroxidation

The liver tissue was collected and homoginized in 0.9% ice cold saline in Teflon homogenizer. The homogenate was centrifuged at 800 g for 10 min and the supernatant was again centrifuged at 12000 *g* for 15 min. The supernatant was used to measure the level of lipid peroxidation which was measured by the thiobarbituric acid method[27].

2.8. Histological studies

Rats were anesthetized, sacrificed and the liver was carefully removed and cut into two pieces. One piece was utilized for histopathology analysis. Liver tissues were fixed in 10% formalin for 24 h, embedded in paraffin, and cut into 6 μm thick sections in a microtome. The sections were stained with haematoxylin-eosin dye and observed under microscope to detect histopathological changes in the liver.

2.9. Statistical analysis

The data are represented as mean±SE. Statistical differences at $P<0.05$ between groups were analyzed by One-way ANOVA followed by Dunnett's multiple comparison tests using SPSS windows version 16.0 software.

3. Results

3.1. Effect of MMK on AST, ALT, ALP, total protein and bilirubin

Phytochemical analysis of different solvent extracts revealed that MMK was rich in alkaloids and tannins. The effect of MMK at three doses (200 mg/kg, 300 mg/kg and 500 mg/kg body weight) on hepatic marker enzymes, total protein and total bilirubin are represented in Table 1. CCl_4 treatment caused substantial raise in levels of AST (4-5 fold), ALT (3-4 fold), ALP (3.0 fold) and serum bilirubin (12.0 fold) which was reversed by administration of MMK in a dose dependent manner.

Table 1

Effect of MMK leaves on hepatic markers in CCl_4 treated rats.

Treatment	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	Bilirubin (mg/dL)	Totalprotein (mg/dL)
Normal control	92.4±2.9	51.9±1.5	109.7±2.6	0.06±0.70	7.65±0.07
CCl_4	383.7±7.6^c	180.3±4.8^c	357.4±3.4^c	0.75±0.06^c	4.16±0.10^c
CCl_4+silymarin	110.2±3.4^c	61.8±1.6^c	138.2±3.4^c	0.05±0.08^{NS}	6.98±0.18^c
CCl_4+MMK 200	148.2±6.8^c	84.5±2.8^c	289.2±3.5^c	0.71±0.07^c	6.56±0.90^c
CCl_4+MMK 300	137.4±5.1^c	71.2±5.4^c	218.3±4.7^c	0.68±0.70^c	6.80±0.30^a
CCl_4+MMK 500	123.8±5.4^a	68.6±1.4^c	162.8±4.2^c	0.05±0.6^{NS}	6.95±0.50^a

Data are expressed as the mean±SE; Comparison is between control group and MMK treated groups. ^{a}: $P<0.05$; ^{b}: $P<0.01$; ^{c}: $P<0.001$; ^{NS}: not significant.

3.2. Effect of MMK on SOD, CAT and GPx

The effects of MMK at three doses (200 mg/kg, 300 mg/kg and 500 mg/kg body weight) on liver antioxidant enzymes in CCl_4 induced hepatic injury are represented in Figures 1, 2 and 3. CCl_4 toxicity caused fall in the activities of SOD, CAT and GPx by 1.4, 1.8 and 3.0 folds respectively. Nevertheless, MMK administration has substantially improved their activities. GPx activity was expressed in terms of glutathione.

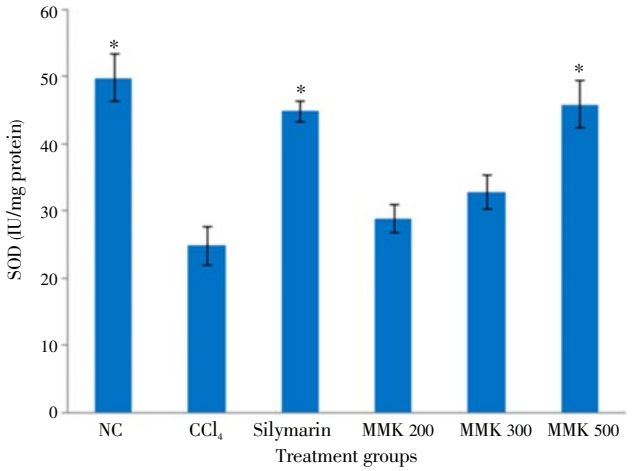

Figure 1. Effect of MMK on SOD activity.
Data are expressed as the mean±SE; * Represents significant difference ($P<0.05$) between CCl_4-treated group and CCl_4+MMK treated groups as well as normal control group.

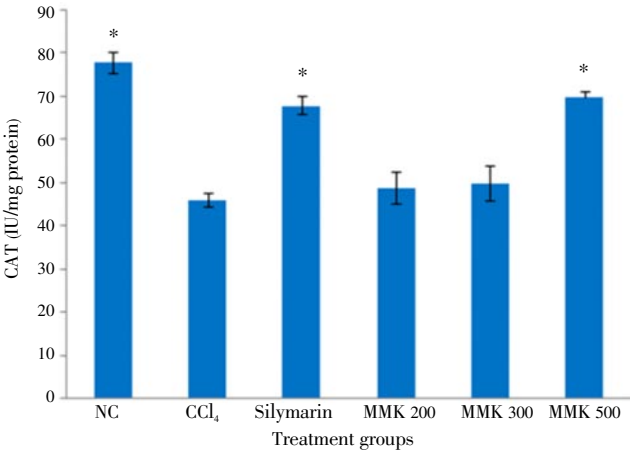

Figure 2. Effect of MMK on CAT activity.
Data are expressed as the mean±SE; * Represents significant difference ($P<0.05$) between CCl_4-treated group and CCl_4+MMK treated groups as well as normal control group.

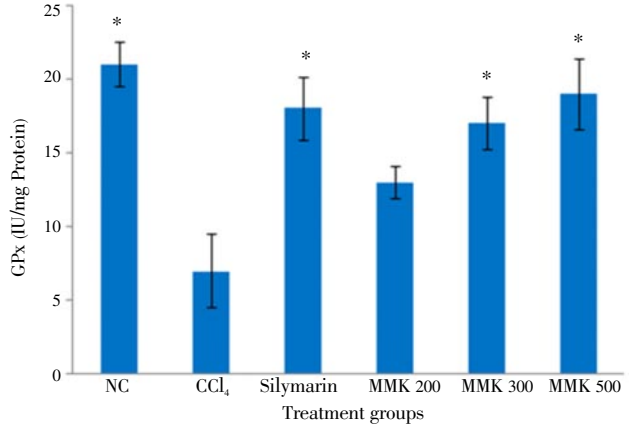

Figure 3. Effect of MMK on GPx activity.
Data are expressed as the mean±SE; *Represents significant difference ($P<0.05$) between CCl_4-treated group and CCl_4+MMK treated groups as well as normal control group.

3.3. Effect of MMK on lipid peroxidation (LPO)

CCl_4 induced hepatic toxicity caused substantial increase (3.3 fold) in malondialdehyde (MDA) levels indicating enhanced ROS generation and consequential LPO. Oral administration of MMK resulted in significant and dose dependent decreases in LPO as evidenced by lowered MDA levels (Figure 4).

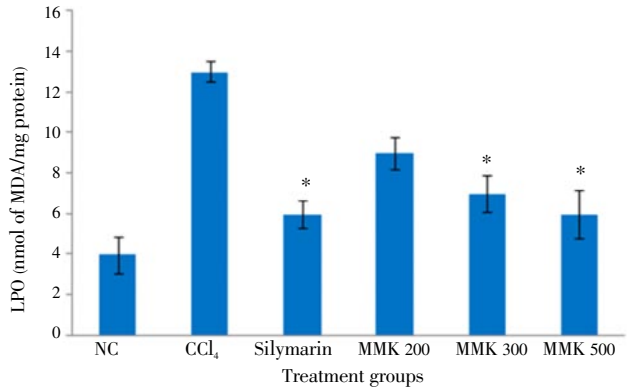

Figure 4. Effect of MMK on LPO (nmol MDA/mg) activity.
Data are expressed as the mean±SE; * Represents significant difference (P<0.05) between CCl_4-treated group and CCl_4+MMK treated groups as well as normal control group.

3.4. Histopathological observations

In order to confirm the hepatoprotective activity of MMK, we further extended our studies to histological examination of liver sections. Microscopic observation of liver sections of normal control rats showed intact central vein surrounded by healthy hepatic cord cells and narrow sinusoidal spaces. CCl_4 treated liver sections showed disruptions in central vein, distended sinusoidal spaces, fatty degeneration, necrosis and vacuole formation. However, the administration of MMK could reduce disruption of central vein, score of fatty degeneration, necrosis and hepatic injury in a dose dependent manner. Histological examination vindicated the hepatoprotective effect of MMK against CCl_4 induced hepatic toxicity (Figure 5).

4. Discussion

Liver is one of the vital organs in the vertebrate body involved in various metabolic reactions, including drug detoxification and excretion of toxic compounds. Continuous exposure to toxic compounds, xenobiotics, drugs and viral infiltrations seriously affect liver functions. Thus, liver diseases remain one of the serious health complications[25]. Liver injury is a classical model for screening the hepatoprotective activity of drugs[26].

CCl_4 is used widely to investigate hepatotoxic effects on various experimental models since CCl_4 induced complications of liver are similar to that of chronic viral hepatitis, necrosis and tissue damages in human and thus preferred as a positive toxic substance in experimental system for various purposes[13].

Earlier works showed that *M. koenigii* contained several important bioactive compounds including alkaloids and polyphenols. The versatile medicinal properties of this plant are attributable to the potent antioxidant and free radical scavenging property of its phytochemicals[27]. In the present study, MMK was assessed for its hepatoprotective and antioxidant activity against CCl_4 induced toxicity

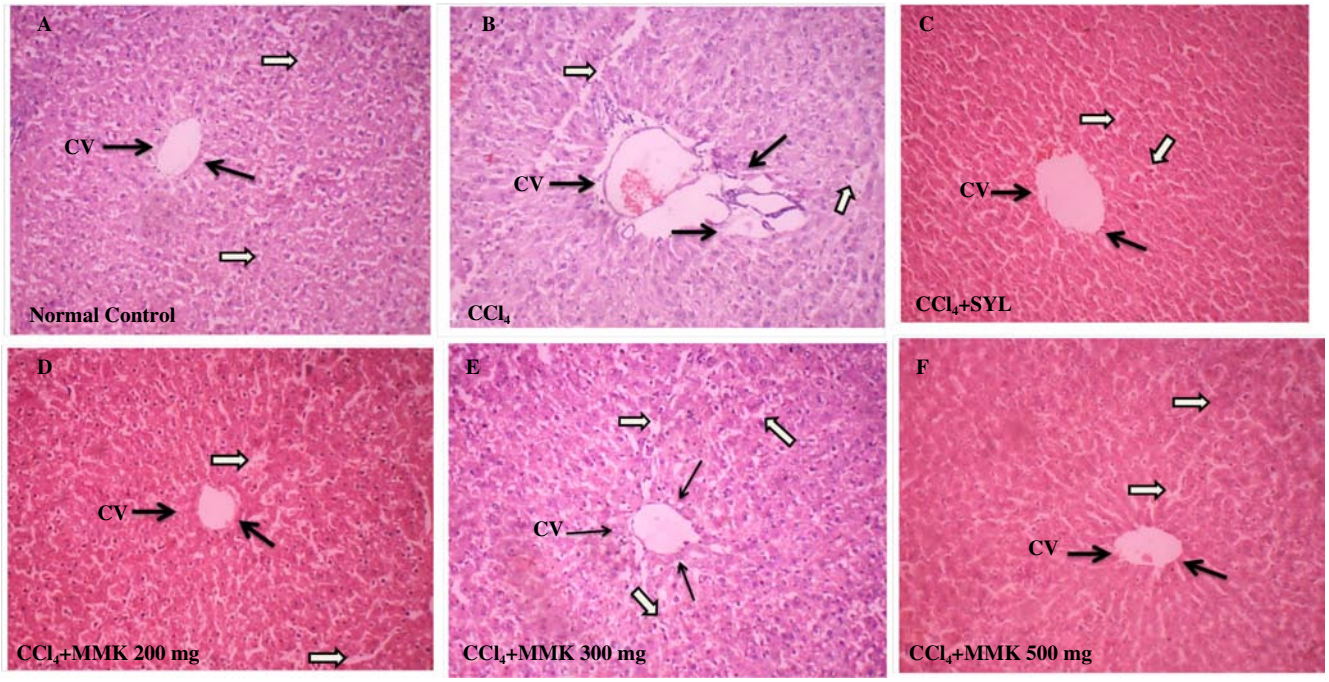

Figure 5. Microphotographs of liver microtome sections.
(A) Liver sections of normal control rats showing intact central vein, narrow sinusoidal spaces and good hepatic cords; (B) CCl_4 treated liver showing disruptions in central vein, widened sinusoidal spaces; (C) Silymarin supplemented liver showing almost normal histological features; (D) MMK extract (200 mg/kg body weight) supplemented liver showing more or less intact central vein but still widened sinusoidal spaces; (E & F) MMK extracts (300 and 500 mg/kg body weight respectively) supplemented liver sections exhibiting near normal hepatic structure.

in rats. A hepatoprotective drug should possess the capability to reduce the adverse effects or to protect the normal physiological functions of liver that have been scattered by a hepatotoxin[28]. CCl_4 gets accumulated in hepatic parenchyma cells and cytochrome P_{450} dependent monooxygenase, converting it as metabolic intermediates, CCl_3 free radicals. These free radicals counteract on poly unsaturated fatty acids in the presence of oxygen to produce LPO contributing to oxidative stress and liver damage[29]. CCl_3 radicals also bind to endoplasmic reticulum resulting in elevated levels of plasma hepatic marker enzymes in experimental rats[30]. Substantially elevated levels of hepatic enzymes AST, ALT and ALP and serum bilirubin are indicative of necrosis and membrane damage of liver.

Oral administration of MMK at different dose levels (200 mg/kg, 300 mg/kg and 500 mg/kg body weight) substantially attenuated the elevated levels of hepatic marker enzymes and bilirubin in CCl_4 treated rats in a dose dependent manner. The maximum activity was shown by 500 mg/kg body weight which was comparable to that of silymarin. Based on the previous reports in our lab on a dose response studies, it was inferred that dosages less than 150 mg/kg body weight did not record any favorable response. Whereas, doses above 300 mg/kg body weight showed identical and comparable result.

In this view, the reduction in the activities of AST, ALT and ALP and bilirubin content by administration of MMK is an indication of the stabilization of plasma membrane as well as a repair of hepatic tissue damage caused by CCl_4. This effect is in concurrence with the commonly established view that serum activities of aminotransferases return to normal with the healing of hepatic parenchyma and regeneration of hepatocytes[7]. ALP is the prototype of these enzymes that reflect the pathological alteration in biliary flow. Thus, the administration of methanolic extract of curry leaves demonstrated the hepatoprotective activity of MMK leaves.

Oxidative stress is the state of disproportion between the level of antioxidant system and production of oxygen-derived species. Increase in ROS such as superoxide radical (O^{2-}), hydroxyl radical (OH^-) and H_2O_2 cause oxidative stress leading to LPO[13]. The body has an effective protection mechanism against free radical induced damage. This is accomplished by various antioxidant enzymes such as SOD, CAT and GPx[31].

SOD, a first line of antioxidant defense enzyme against ROS, plays an important role in scavenging the toxic intermediate of incomplete oxidation. SOD catalyses the dismutation of superoxide radicals to produce H_2O_2 and molecular oxygen, hence diminishing the toxic effects caused by their radicals[32]. Administration of MMK raised the levels of SOD, CAT and GPx in dose dependent manner which were lowered by CCl_4. On the contrary, MMK administration substantially reduced MDA levels indicating reduced LPO. The antioxidant and hepatoprotective activity of the MMK were supported and validated by histopathological examination. Histopathological studies under light microscope showed that CCl_4 treatment caused disruptions in central vein, distensions in sinusoidal spaces and hypertrophy of hepatocytes. However, supplementation of different doses of MMK substantially mitigated CCl_4 induced hepatic alterations in a dose dependent manner.

Our study demonstrates that MMK has potential hepatoprotective and antioxidant efficacy as evidenced by its ability to reverse the CCl_4-induced alterations of hepatic markers, antioxidant enzyme system and hepatic tissue damage. The therapeutic efficacy can be attributed to its carbazole alkaloids, tannins and polyphenols. Our results also scientifically validate the use of MMK leaves in Ayurvedic medicine and in food preparations.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Comments

Background

Liver is one of the vital organs in the vertebrate body involved in various metabolic reactions, including drug detoxification and excretion of toxic compounds. Liver diseases remain one of the serious health problems of the modern world because of changed life styles. Therefore, the search continues to find novel and effective hepatoprotective drugs because of their side effects in place of synthetic drugs.

Research frontiers

In this manuscript, authors have done the protective effects of *M. koenigii* leaves against carbon tetra chloride-induced hepatic damage in rats through evaluation of liver marker enzymes and antioxidant activity.

Related reports

So far, many studies are reported on hepatic damage and necrosis by CCl_4 and paracetamol. The natural compounds isolated from plants and marine source have been used to protect from liver damage.

Innovations and breakthroughs

M. koenigii contains several important bioactive compounds including alkaloids and polyphenols. The versatile medicinal properties of this plant are attributable to the potent antioxidant and free radical scavenging properties of its phytochemicals. In the present work, authors showed potential hepatoprotective and antioxidant role of methanolic extract of *M. koenigii* on CCl_4 induced rats.

Applications

Based on the results done by the authors, MMK can be used

more as a potential nutraceutical than just a flavoring agent to protect liver.

Peer review

Authors have done a very good piece of work. CCl_4 toxicity in rats is an ideal model that represents toxicity by several agents. The antioxidant and hepatoprotective activity were well supported by liver markers, antioxidant enzymes and histopathological examination. This work offers scientific validation for its use in folk medicine.

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