PROTECTIVE NATURE OF *MURRAYA KOENIGII* LEAVES AGAINST HEPATOSUPPRESSION THROUGH ANTIOXIDANT STATUS IN EXPERIMENTAL RATS

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Summary

The present study deals with the protective nature of methanol extract of *Murraya koenigii* leaves against hepatosuppression through antioxidant status in experimental rats, which has been evaluated in terms of hepatic marker enzymes – AST, ALT (aspartate and alanine transaminases), ALP (alkaline phosphatase), γ-GTP (gamma glutamyl transpeptidase), LDH (lactate dehydrogenase), SDH (Sorbitol dehydrogenase) and total bilirubin, total protein and albumin in serum alongwith the levels of total cholesterol, triglycerides, LPO (lipid peroxidation), SOD (Superoxide dismutase), GSH (reduced glutathione), CAT (catalase) and Vit. C (ascorbic acid) in the liver. Hepatic alterations were significantly ($P \geq 0.001$) induced by CCl_4 (0.2 ml/kg body weight/twice a week, intra peritoneal, with olive oil, 1:1) for 21 days. Simultaneously, the rats were given *M. koenigii* leaves extract, at the doses of 100 and 200 mg/kg body weight/day, orally, which remarkable restored the altered biochemical parameters in a dose dependent manner. However, silymarin showed a significant effect in all the parameters, studied in CCl_4-treated rats. In conclusion, the protective nature of *M. koenigii* leaves extract may be attributed to the combined effect of carbazole alkaloids – Mahanimbine (1), Girinimbine (2), Isomahanimbine (3), murrayazoline (4), Murrayazolidine (5), Mahanine (6) and ascorbic acid, α-tocopherol and mineral (Zn, Cu, Fe) contents. Thus *M. koenigii* leaves extract showing protection in liver, may prove promising as a rich source of free radical quenchers, which have been mediated through hepatocyte membrane stabilizing activity alongwith the reduction of fat metabolism.

Key Words: carbon tetrachloride, fat metabolism, free radical quenching activity, hepatic marker enzymes.

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Introduction

Today, with the extensive use of hepatotoxicants in daily routine life, it has become imperative to safeguard human populations inhabiting poverty against liver cirrhosis because mammalian liver is a highly toxicity sensitive organ and responsible for drug metabolism, mainly detoxifies damaging electrophiles generated during oxidative stress and rich in endogenous antioxidants and related enzymes. The reactive intermediates formed during the metabolism of therapeutic agents, toxins and Carcinogens by *P*-450 enzymes are frequently capable of covelently binding to the tissue macromolecules, which result in tissue damage [1]. Therefore, a need exists for traditionally edible, non-toxic and inexpensive drug for clinical hepatoprotection.

Murraya koenigii (L.) spreng. (Eng.- curry lea tree; Hindi – mitta neem) belongs to the family Rutaceae, and is extensively used for flavouring curries, chutneys, soups, pickles etc. and is commonly reported to be a good source of b-caryophyllene, b-gurjunene, b-elemene and b-phellandrene [2], ascorbic acid, carbazole alkaloids and alpha tocopherols [3], Minerals [4]. The leaves are reported to have hypoglycemic and antidiabetic activities [5] and its carbazole alkaloids have antioxidant activity following the order of ascorbic acid > bismurrayafoline E > euchrestine B, mahanine and alpha- tocopheral > BHT > mahanimbicine and mahanimbine [3] and in comparison of 12-carbazole alkaloids for antioxidant properties suggested that an aryl hydroxyl substituent on carbazole rings plays a role in stabilizing the thermal oxidation and rate of reaction against DPPH radical [6].

According to that views, the present study was to evaluate the protective nature of *Murraya koenigii* leaves against hepatosuppression through antioxidant status in experimental rats.

Materials and Methods

Animals

Colony bread, adult, male albino rats wistar strain, weighing 150 ± 10g, each were used for the present study. The rats were housed in polypropylene cages under controlled conditions of temperature (25±3°C) and light (14L:10D). They were provided with a nutritionally adequate standard laboratory diet (Lipton India, Ltd.) and tap water *ad libitum*.

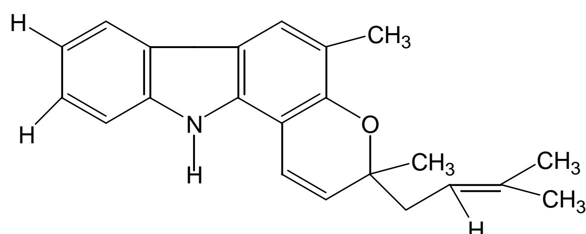
Plant Material and extraction

The leaves of *Murraya koenigii* (L.) Spreng. were collected from the University campus in the month of April 2006. The plant was identified and authenticated from the Deptt. of Botany, University of Rajasthan, Jaipur (Specimen No. RUBL – 19910). The leave have shade dried and Pulverized. The powder was treated with petroleum ether for defatting as well as to remove chlorophyll. The powder was packed in to a soxhlet apparatus and subjected to hot continuos percolation using methanol as solvent. The extract was concentrated under vaccum and dried in a vaccum descicator (yield 8.2% w/w) and for experimentation, the extract was suspended in olive oil with the help of a cyclomixer just before oral administratin.

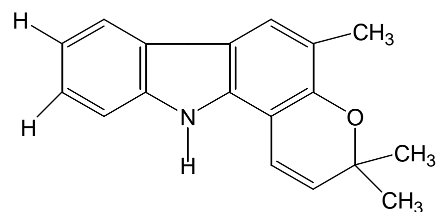
Silymarin was obtained from German Remedies Ltd., Mumbai, for using as a reference standard for experimentation.

Fractionation and isolation of active compounds

The dark brown semisolid mass of methanolic extract of leaves was washed with petroleum ether to remove the fatty portion as well as chlorophyll. The fat and chlorophyll free extract was then fractionated in to chloroform-soluble, acetone-soluble and methanol-soluble parts. The methanolic extract so obtained (30 gms) was subjected to column chromatography. For this purpose, a column filled with si-gel was used (600 gm). The purity of the fractions was checked by qualitative thin-layer chromatography and HPLC using different solvent system. After ascertaining the purity of the compound, it was subjected for detailed spectral analysis (IR, ^{1}H NMR, ^{13}C NMR and MS) to establish the structure. The structures of these 6-bioactive compounds confirmed as carbazole alkaloids- Mahanimbine (1), Girinimbine (2), Isomahanimbine (3), Murrayazoline (4), Murrayazolidine (5), and Mahanine (6), by the spectrometric data.



(1) Mahanimbine



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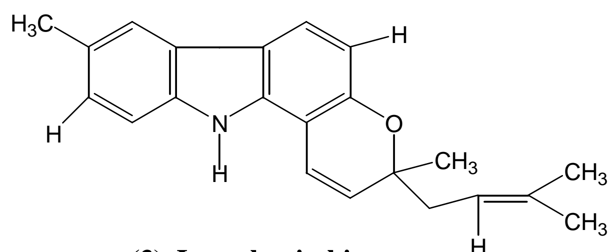
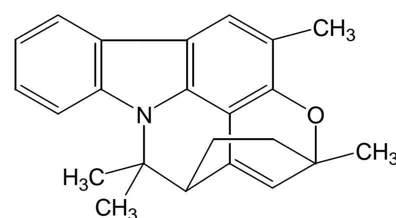
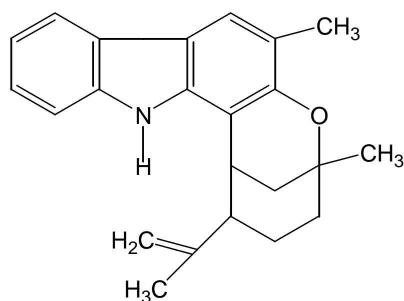


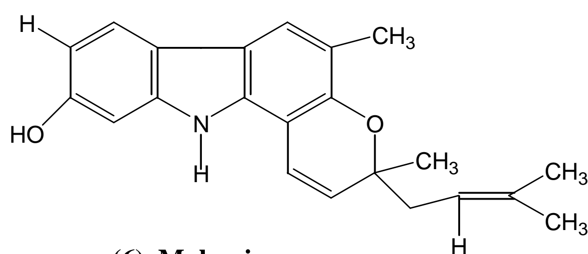
(3) Isomahanimbine



(4) Murrayazoline



(5) Murrayazolidine



(6) Mahanine

Experimental Design

The male albino rats wistar strain were randomly divided in to five groups of 6-animals in each group.

Group I	:	Normal control rats received vehicle only.
Group II	:	Rats treated with carbon tetrachloride (0.2ml/kg b wt/ twice a week, intraperiponeal, with olive oil, 1:1) for 21 days.
Group III	:	Rats treated conjointly with CCl_4 as above and *M. koenigii* leaves extract (100 mg/kg b wt/day) orally.
Group IV	:	Rats treated conjointly with CCl_4 as above and *M. koenigii* leaves extract (200 mg/kg b wt/day) orally.
Group V	:	Rats treated conjointly with CCl_4 as above and silymarin (100 mg/kg b wt / day) orally.

Autopsy

All rats were sacrificed on the day 22nd under ether anesthesia and blood samples were collected by cardiac puncture in sterilized vials. Serum was separated and stored at –20°C untill assayed. The entire liver was perfused immediately with cold physiological saline and thereafter carefully removed, trimmed free of extraneous tissues. After that, the liver was frozen for biochemical analysis.

Serum Biochemistry

Biochemical assay of enzymes, *viz.*- AST, ALT, ALP, γ-GTP, LDH, SDH, total bilirubin, total protein and albumin were performed in serum using kit methods. AST (Batch No. 61105), ALT (Batch No. 60865) and γ-GTP (Batch No. 34004) kits were purchased from Accurex Biomedical Pvt. Ltd.; Mumbai, India. LDH (LOT. No. 6854), SDH (LOT No. 6810), ALP (LOT. No. 7093), Total bilirubin (LOT. No. 6801), Total protein (LOT. No. 6808) and albumin (LOT. No. 6988) kits were purchased from Span Diagnostics Ltd., Surat, India, respectively.

Liver Biochemistry

The quantitative estimation of total cholesterol [7], triglycerides [8], ascorbic acid [9], lipid peroxidation (TBARS) [10], reduced glutathione [11], catalase [12] and superoxide dismutase [13] were performed in liver homogenate.

Statistical analysis

The results obtained in the present study were expressed as the mean ± SEM for each parameter. Results were statistically analyzed by applying Student '*t*' test.

Results

The results of present study indicates that methanol extract of *M. koenigii* leaves possesses hepatoprotective, hypolipidemic and antioxidant activities in a dose dependent manner against CCl_4 induced hepatosuppression.

Administration of CCl_4 caused significant ($P \leq 0.001$) alteration in liver – total cholesterol, triglycerides, LPO, GSH, CAT, SOD, Vit. C contents in comparison to the normal controls. Concurrent administration of CCl_4 and *M. koenigii* leaves extract to rats antagonised the alteration remarkably in total cholesterol, triglycerides, LPO, GSH, CAT, SOD, and Vit. C contents in a dose dependent manner and compared with the reference standard silymarin at 21 days of treatment (Table-1).

A statistically significant ($P \leq 0.001$) alteration in serum – AST, ALT, ALP, γ-GTP, LDH, SDH, total bilirubin, total protein and albumin levels were observed after CCl_4 intoxication in comparison to normal controls. Concurrent administration of CCl_4 and *M. koenigii* leaves extract to rats showed remarkable recoupment in the levels of AST, ALT, ALP, γ-GTP, LDH, SDH, total bilirubin, total protein and albumin levels in a dose dependent manner and compared with the reference standard silymarin at 21 days regimen (Figs. 1-5).

Table-1 : Showing protective nature of *M. koenigii* leaves and silymarin in rats through hepatic antioxidant defense system along with lipid parameters.

Group	Cholesterol (mg/g tissue)	Triglycerides (mg/g tissue)	Lipid peroxidation (TBARS, n mole/mg tissue)	SOD (μ mole/ mg protein)	GSH (n mole GSH/ g tissue)	Catalase (μ mole H_2O_2 consumed/ min/ mg protein)	Ascorbic acid (mg/g tissue)
Normal (Vehicle treated) (Gp.I)	**6.84±0.13**	**9.46±0.17**	**2.23±0.21**	**7.25±0.35**	**3.54±0.28**	**60.15±2.54**	**1.35±0.09**
0.2 ml CCl_4/kg b wt/ twice a week (Gp. II)	**13.25±0.59[a]**	**17.42±0.81[a]**	**7.38±0.47[a]**	**3.65±0.22[a]**	**1.90±0.10[a]**	**35.18±1.24[a]**	**0.72±0.04[a]**
CCl_4+ *M. koenigii* leaves extract (100 mg/kg b wt/day) (Gp. III)	**10.36±0.46[b]**	**13.91±0.85[c]**	**5.43±0.50[c]**	**4.95±0.25[b]**	**2.50±0.17[c]**	**45.80±1.49[a]**	**1.03±0.06[b]**
CCl_4+ *M. koenigii* leaves extract (200 mg/kg b wt/day) (Gp. IV)	**7.76±0.37[a]**	**10.12±0.64[a]**	**3.01±0.39[a]**	**6.22±0.28[a]**	**3.27±0.23[a]**	**56.22±2.02[a]**	**1.24±0.07[a]**
CCl_4+ Silymarin (100 mg/kg b wt/day) (Gp. V)	**7.10±0.33[a]**	**9.72±0.43[a]**	**2.74±0.29[a]**	**6.88±0.23[a]**	**3.33±0.19[a]**	**58.18±1.89[a]**	**1.39±0.05[a]**

Levels of significance :
Data are mean ± SEM (n=6)
a = $P \leq 0.001$ Gp. II compared with control (Gp. I).
a = $P \leq 0.001$; b = $P \leq 0.01$; c = $P \leq 0.05$ Gp. III, IV and V compared with Gp. II.

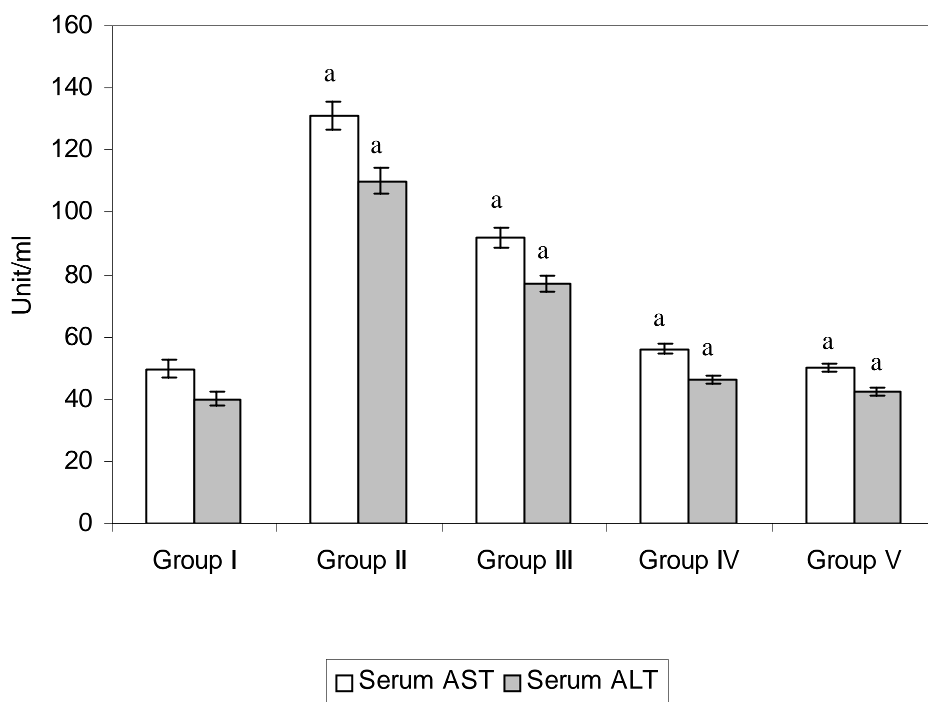


Fig. 1: Protective activity of serum AST and serum ALT in control and experimental rats. Data points with letter notation (a) are statistically significant at a = P ≤ 0.001.

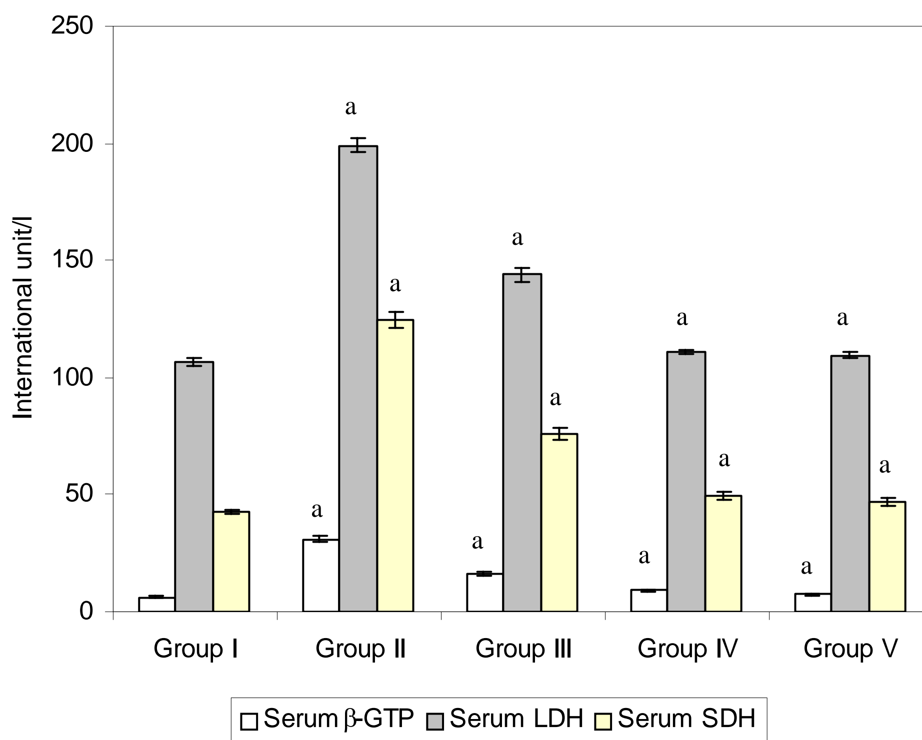


Fig. 2: Protective activity of serum γ-GTP, serum LDH and Serum SDH in control and experimental rats. Data points with letter notation (a) are statistically significant at a = P ≤ 0.001.

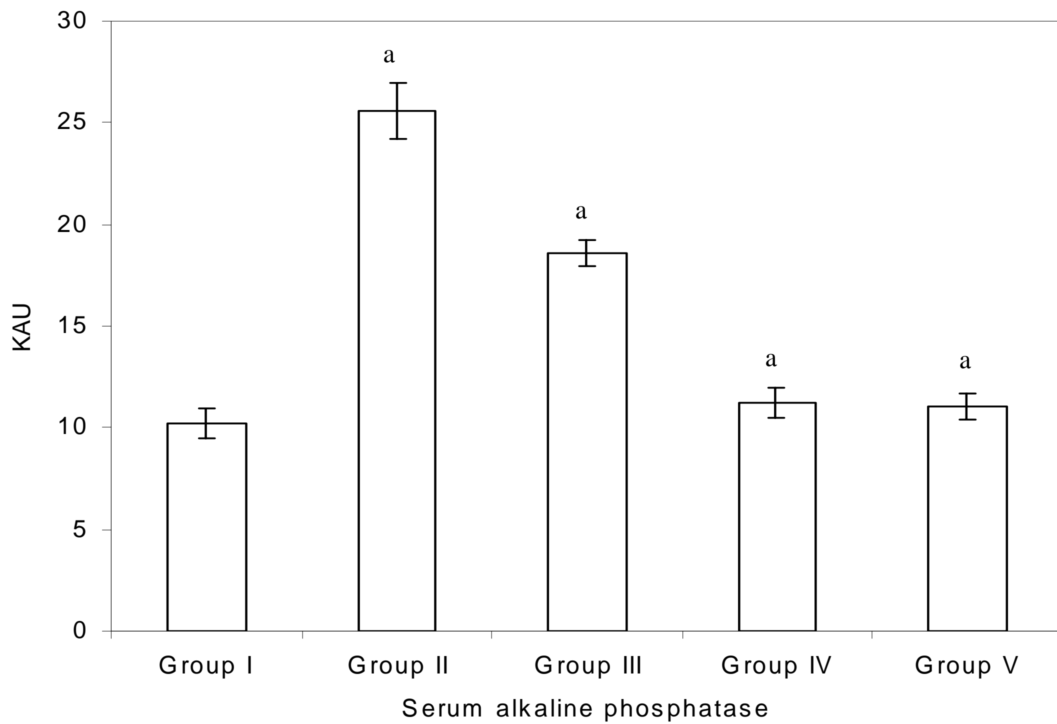


Fig. 3: Protective activity of serum alkaline phosphatase in control and experimental rats. Data points with letter notation (a) are statistically significant at a = P $\leq$ 0.001.

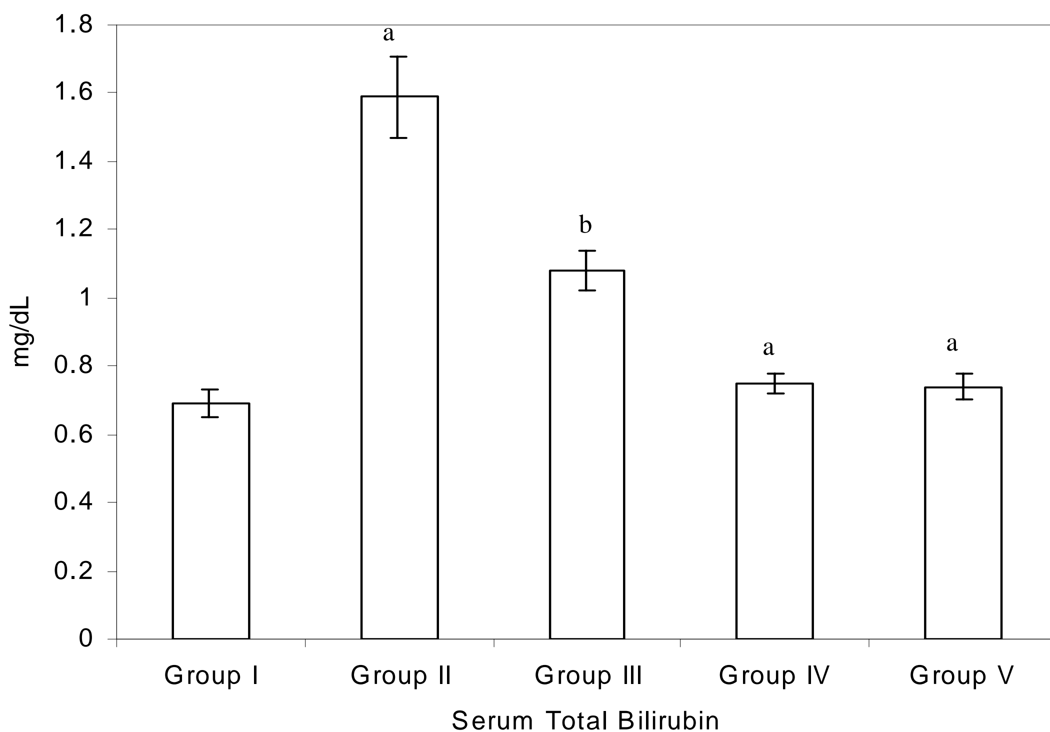


Fig. 4: Protective activity of serum total bilirubin in control and experimental rats. Data points with different letter notations (a,b) are significantly different at a = P $\leq$ 0.001; b = P $\leq$ 0.01.

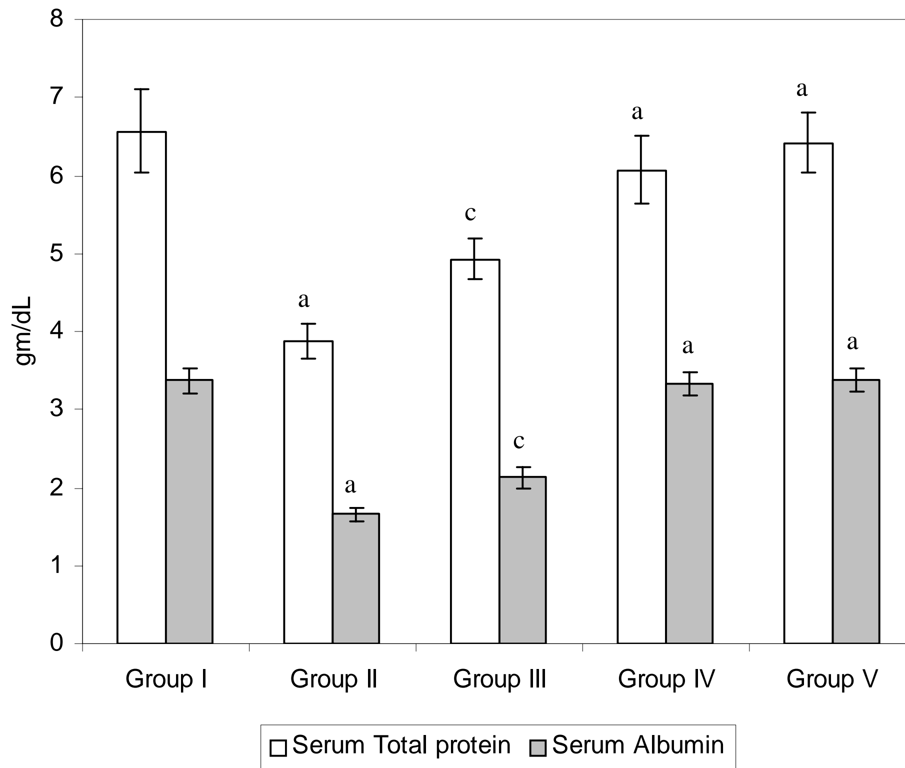


Fig. 5: Protective activity of serum total protein and serum albumin in control and experimental rats. Data points with different letter notations (a,c) are significantly different at a = P $\leq$ 0.001; c = P $\leq$ 0.05.

Discussion

The hepatoprotection ability of *M. koenigii* leaves extract to reduce the injurious effects or to preserve the normal hepatic physiologic mechanisms which have been disturbed by CCl_4 because hepatic cells appear to participate in a variety of enzymatic metabolic activities. Metabolically, CCl_4 is biotransformed by cytochrome *P*-450 dependent monooxygenases to form a trichloromethyl free radical (CCl_3^-) which alkylates cellular proteins (including cytochrome *P*-450) and other macromolecules with a simultaneous attack on polyunsaturated fatty acids in the presence of oxygen to produce lipid peroxidation [14].

LPO is oxidative deterioration of polyunsaturated lipids and it involves ROS and transition metal ions. It is a molecular mechanism of cell injury leading to the generation of peroxides and lipid hydroperoxides, which can decompose to yield a wide range of cytotoxic products, most of which are aldehydes such as malondialdehyde (MDA) and 4-hydroxynonenal. The stimulation of LPO as a consequence of tissue injury can sometimes contribute significantly to a worsening of injury. LPO is a highly destructive process that affects cellular organelles and causes them to lose biochemical functions and/or structural integrity, which may lead to irreparable damage or cell death [15].

In our present investigation, the measurement of lipid peroxidation is a convenient method to monitor oxidative cell damage. Inhibition of LPO has been caused by antioxidant activities of *M. koenigii* leaves extract due to its 6-carbazole alkaloids and probably by its mineral contents because adequate zinc supplementation inhibits LPO and has been described as an antioxidant [15]. Further, inhibition of LPO also observed by silymarin treated group due to its antioxicant and free radical quenching activity through reestablishment of biomembranes of hepatic parenchymal cells.

Rats treated with CCl_4, transaminases activity (AST, ALT), ALP, γ-GTP, LDH and SDH were increased remarkably in plasma by the release of these enzymes from hepatic parenchymal cells, which were indicating a considerable hepatocellular injury [14]. Oral treatment with silymarin and *M. koenigii* leaves extract attenuated these increased enzyme activities produced by CCl_4 and a subsequent recovery towards normalization of these enzymes strongly suggests the possibility of *M. koenigii* extract leaves being able to improve the condition of the hepatocytes so as to cause accelerated regeneration of parenchymal cells, thus protecting against membrane fragility decreasing the leakage of marker enzymes into the circulation stabilization of serum- bilirubin, total protein and albumin levels through the administration of the extract is further a clear indication of the improvement of the functional status of the hepatic cells [14] due to its presence of 6-carbazole alkaloids.

The severe liver toxicity is also increase the peripheral fat metabolism resulting enhance hepatic triglyceride synthesis leads to excess total cholesterol accumulation [16]. The *M. koenigii* leaves extract and silymarin supplementation in our study was potentially effective in reduction of excess total cholesterol accumulation and hepatic triglyceride synthesis.

SOD catalyses the dismutation of the highly reactive superoxide anion to oxygen and hydrogen peroxide. Superoxide anion is the first reduction product of oxygen which is measured in terms of inhibition of generation of O_2 [17]. GSH is a versatile protector and executes its hepatoprotective function through free radical scavenging activity [18].

Catalase catalyses the decomposition of hydrogen peroxide into water and oxygen and its decreased level causes the elevation of LPO. Vit. C is the principal antioxidant in extra cellular fluids and traps peroxyl radicals before they can initiate lipid peroxidation [19]. Therefore, the depleted level of Vit. C, thought to be utilized in the liver. Oral administration of *M. koenigii* leaves extract to rats showed significant elevation in depleted SOD, GSH, catalase and Vit. C levels due to an antioxidant activities of its 6-carbozole alkaloids combinedly and probably by its mineral contents like Zn, Cu, Fe, etc. through scavenging the free radicals generated by CCl_4-intoxication. Further, a significant protection against CCl_4 -induced hepatic antioxidant aberrations was achieved with the silymarin treatment.

Conclusion

It can be concluded that the findings with *M. koenigii* leaves extract, attributed to the combined synergistic effects of its 6-carbazole alkaloids and minerals, which have been shown to possess various biological properties related to antioxidant mechanisms and to improve hepatic system through the body's antioxidant status, which could be achieved by a regular consumption of *M. koenigii* fresh leaves with vegetables.

Further, the comparative evaluation of 6-carbozole alkaloids, as a more effective hepatoprotective agent are in progress and will be reported elsewhere.

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