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HEPATOPROTECTIVE ACTIVITY OF *MURRAYA KOENIGII* AGAINST ETHANOL-INDUCED LIVER TOXICITY MODEL IN EXPERIMENTAL ANIMALS

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ABSTRACT

Murraya koenigii (Linn) Spreng (Indian curry leaf) is a highly valued culinary plant with the aqueous extract of its leaves which are reported to exhibit diverse biological activities. Considering free radical mechanism in hepatotoxicity, dietary antioxidants could provide protection in conditions including alcoholic liver disease. The objective of this study was to investigate its effects on biochemical markers of liver function affected in diseased state. Two high doses of 1 g/kg and 2 g/kg were evaluated for its hepatoprotection on ethanol-induced liver toxicity in Wistar rats. The aqueous extract showed potent antioxidant activity, and at both doses produced a significant increase in reduced glutathione levels with comparable reduction in serum glutamate pyruvate transaminase and alkaline phosphatase levels. Only 1 g/kg dose depleted malondialdehyde levels in comparison to ethanol. Histopathology of liver samples confirmed the above findings. The results suggested significant benefits of nutritive antioxidant compounds in *M. koenigii* contribute to its potential hepatoprotective activity

KEYWORDS

Curry leaves, hepatoprotection, antioxidants, liver functional enzymes

INTRODUCTION

Ethanol abuse is an issue of growing concern, as it is a leading cause of morbidity and mortality throughout the world [1].

Chronic ethanol intake leads to an enhanced microsomal ethanol oxidizing system activity [2], by means of a cytochrome called CYP2E1 in the endoplasmic reticulum of liver cells to form toxic acetaldehyde. This interferes with the levels of cellular antioxidants including reduced glutathione (GSH) leading to mitochondrial damage of hepatocytes that renders susceptibility to free radical-induced injury [3].

The liver function can be enhanced by supplementing with antioxidant nutrients as an alternative therapeutic modality[4].

Murraya koenigii (*Linn*) *Spreng* (Family: Rutaceae), commonly known in India as curry leaf plant, has its characteristic aroma and medicinal properties [5]. The plant is used in Indian system of medicine for its detoxifying action, anti-inflammatory [6] and antihyperglycemic activities [7]. *M. koenigii* leaves mixed with fat separated butterfat is used for the treatment of amoebiasis, diabetes and hepatitis in Ayurveda[8]. Reports mention that fresh crude curry leaf aqueous extract could modulate the levels of drug metabolizing phase-I and phase–II enzymes, antioxidant parameters, lactate dehydrogenase and lipid *per oxidation* changes [9].

The reduced levels of endogenous antioxidants in the free radical-mediated liver injury in ALD and the reported anti-inflammatory action of the herb encouraged us to investigate the aqueous extract of leaves of *M. koenigii* for its hepatoprotective activity on ethanol-induced hepatitis in experimental animals. Its antioxidant potential with influence on lipid *per oxidative* (LPO) property and GSH levels were evaluated. In addition, its effect on modulation of significant serum liver functional markers including serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALKP) and bilirubin were studied with L-ornithine-L-aspartate (LOLA) as a positive control followed by histopathological studies on hepatocytes.

The present study was designed with an objective to investigate whether the antioxidant property of the aqueous extract of *M. koenigii* could be significant for its effects on the liver functional enzymes for claiming its probable hepatoprotective potential.

MATERIALS AND METHODS:

Plant materials

Leaves of *M. koenigii* (Herbarium Sample no. 120401) were obtained from the local market, Mumbai. The sample was suitably authenticated by the Department of Botany, Guru Nanak Khalsa College University of Mumbai, India.

Plant extracts

Maceration technique was used to prepare the aqueous extract by using 500 g of powdered leaves in 1 liter of distilled water for 6-8 h. The slurry was boiled, concentrated, cooled, filtered through a muslin cloth and the filtrate was further concentrated again by boiling. The concentrated slurry was freeze dried to obtain dried extract.

Phytochemical screening

The % yield of extract was calculated and the extract was subjected to a battery of qualitative chemical tests.TLC technique was used to confirm the presence of alkaloids and tannins.

Reagents

Ecoline diagnostic kits manufactured by Merck Ltd Mumbai were used for biochemical estimations. All chemicals and reagents used

were of analytical grade (S.D. Fine Chemicals, India).

Animals and experiment design

Adult Wistar rats of both the sexes M/F weighing 150–200 g were procured from Haffkine Institute, Parel Mumbai. The rats were acclimatized to ICT animal house conditions for about a week.

With the high LD_{50} of more than 5 g/kg body weight of rats as found in the acute toxicity studies, two selected doses namely MK1 and MK2 of 1 g/kg and 2 g/kg of body weight respectively were evaluated for hepatoprotection in rats.

Animals were divided into five groups and each group comprised of six animals. Group I served as a vehicle control while group II received ethanol (1 ml/100 g of animal) as a negative control. Group III and group IV received MK1 with ethanol and MK2 with ethanol respectively. LOLA (Hepamerz) ® 20 mg per rat, (equivalent to 900 mg/day of human dose) with ethanol was administered as a positive control as in group V. (Groups III, IV and V were administered the drugs with simultaneous administration of ethanol after three hours.)

Blood samples were collected and serum was separated on Day 0. The treatment with extract was continued for 21 days with simultaneous administration of 6% v/v ethanol to the last three groups. At the end of the period, blood was collected from rats of all the five groups.

The serum was analyzed for SGPT, bilirubin and ALKP as liver functional tests. Animals were sacrificed at the end of 21 days and livers were collected in normal saline (0.9%) and formal saline (10% formaldehyde solution in normal saline). The livers in normal saline were analyzed for content of GSH and malondialdehyde (MDA). This was followed by histopathology to detect any morphological changes produced in the liver.

All animal experiments were conducted after the approval of Institutional Animal Ethics Committee. (IAEC Approval No: *ICT /PH/IAEC/1204/19*)

Evaluation of antioxidant activity using 1,1-Diphenyl-2-Picryl Hydrazyl (DPPH) assay

The antioxidant activity of aqueous extract in terms of IC_{50} was evaluated at 517 nm at 10-400 µg/ml using DPPH assay [10].

Evaluation of anti-lipid per oxidative property by ex vivo thiobarbituric acid reactive species (TBARS) method

Rat liver was quickly removed from the treated and non-treated animals sacrificed at the end of the experimental period and homogenized in glass teflon homogenizer. The homogenate was quickly placed in ice- cold phosphate buffer saline (PBS). The lipid *per oxidation* was initiated by adding 100 µl of 15 mM ferrous sulfate solution to 3 ml of liver homogenate. After 30 min of incubation at room temperature, 0.1 ml of liver homogenate was taken in a tube containing 0.1 ml sodium dodecyl sulfate (8.1% w/v), 0.75 ml of 20% acetic acid and 0.75 ml of 0.8% thiobarbituric acid aqueous solution and heated on water bath at 95º C for 60 min. The volume was made up to 2.5 ml, to which 2.5 ml of butanol: pyridine (15:1) was added. The reaction mixture was vortexed and centrifuged at 4000 rpm for 10 min. The organic layer was removed and absorbance read at 532 nm in a UV spectrophotometer [11].

The percent inhibition of LPO was calculated by comparing the results of the tests against vehicle control with malondialdehyde bis-diethylacetal as a standard.

Estimation of GSH

The tissue (about 200 mg) from the sacrificed treated and non-treated animals was weighed and homogenized in ice-cold conditions in a tissue homogenizer after adding 5 ml of metaphosphoric acid. The 4% w/v liver homogenate in PBS centrifuged at 3000 rpm for 5 min at 0°- 4° C. For test solution, 0.2 ml of supernatant, 0.8 ml of metaphosphoric acid, 3 ml of buffer and 60 µl of dithiobisnitrobenzoic acid reagent was taken against blank. Quantification was achieved by comparison with standard

curve of known GSH concentration and absorbance read at 412 nm [12].

Estimation of liver functional markers

Estimation of SGPT, bilirubin and alkaline phosphatase on blood serum were carried out using Ecoline diagnostic kits (Merck Ltd, Mumbai).

Histopathological studies on hepatocytes

Liver samples of all the treated and untreated groups in formal saline were studied for its histopathology to see any morphological changes.

STATISTICAL ANALYSES:

All data expressed as mean ± SEM were analyzed using student's *t* test and the *t*-value was calculated. Using the *t* distribution table and the degrees of freedom the statistical significances were determined, against the recorded value at probability levels. The statistical significance of the differences between treated groups and negative control (ethanol) group for each variables were calculated with sample size (n=6) using the table of Fisher and Yates. The results were considered statistically significant if the P values ≤ 0.05

RESULTS

The phytochemistry of extract revealed the presence of alkaloids, glycosides, saponins, tannins and carbohydrates with 0.6% w/w yield. Its considerable DPPH radical scavenging activity in comparison to ascorbic acid must be due to presence of antioxidant phytoconstituents like tannins present in it with an IC_{50} value of 200 µg/ml (Table 1)

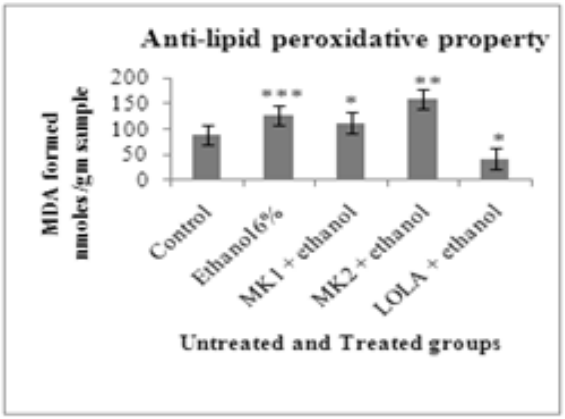
Table 1
Percent inhibition of DPPH radical scavenging activity at various concentrations (10-400 µg/ml) of extract

Concentration (µg/ml)	% DPPH inhibition
10	14.78±2.4
25	22.32±1.4
50	26.45±1.7
100	31.76±2.2
200	49.54±3.1
300	55.67±2.6
400	62.89±2

The percent DPPH inhibition calculated as:
% DPPH inhibitory activity = $A_{(DPPH)} - A_{(DPPH + sample)}$ X 100/ $A_{(DPPH)}$

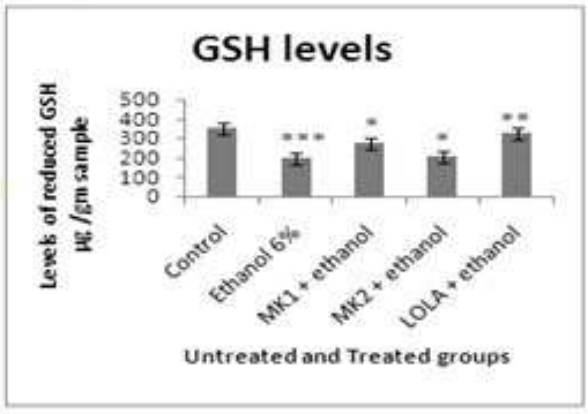
The dose of ethanol chosen was 1 ml of 6% v/v per 100 g body weight of the animals after standardization to produce moderate type of hepatotoxicity. Treatment with MK1 and ethanol exhibited MDA of 113 nmoles/g (P<0.05) while MK2 and ethanol showed higher levels of 159 nmoles/g of MDA (P <0.01) in comparison to ethanol that showed the MDA levels of 128 nmoles/ g (P <0.001). In the case of reduced GSH, ethanol produced a marked decrease of 196.46 µg GSH/g of tissue (P<0.001) while MK1 and MK2 with ethanol showed 273.66 and 204.32 µg GSH/ g of tissue (P <0.05), respectively in comparison to vehicle control (351.33 µg GSH/g of tissue). LOLA, the positive control on the other hand exhibited MDA levels of 42.59 nmoles/g (P <0.05) and 324.84 µg GSH/ g of tissue (P<0.01) (Graphs 1 and 2).

Graph 1
Anti-lipid per oxidative activity



Effects of M. koenigii extracts of MK1, MK2 and LOLA on simultaneous administration with ethanol on the MDA levels in nmoles/g of processed liver tissue sample to assess LPO against vehicle control.
Values represent mean ± SEM (n=6/group).The level of significances are indicated by *at P<0.05, ** at P<0.01 and *** at P<0.001

Graph 2.
Reduced glutathione



Effects of M. koenigii extracts of MK1, MK2 and LOLA on simultaneous administration with ethanol on GSH content in µg/ml of processed liver tissue sample to assess its hepatoprotective potential against vehicle control with * indicating the level of significance at P<0.05

Treatment with MK1 and ethanol produced a significant decrease in SGPT 30.66 U/l (P<0.05) while MK2 with ethanol showed decrease of 31.27 U/l (P<0.05) in comparison to ethanol 41.52 U/l. Concomitant ethanol administration with MK1 and MK2 reduced the levels of ALKP that were 154.57 U/l and 161.38 U/ respectively. LOLA did not produce any significant decrease in SGPT levels (43.8 U/l) but produced a significant decrease in ALKP value of 200.8 U/l (P<0.05).The bilirubin levels of MK1 and MK2 treated animals were almost significantly equivalent to that of vehicle control (2.73 mg/dl) with LOLA exhibiting 2.32 mg/dl of bilirubin levels (P<0.01) (Table 2)

Table 2

Effects on the biochemical parameters on simultaneous administration of MK extract and LOLA individually with ethanol against ethanol control to assess their hepatoprotective Potential

Biochemical parameters	Day	Vehicle control	Ethanol 6% v/v	MK1 + ethanol	MK2 + ethanol	LOLA+ ethanol
SGPT U/l	0	17.24 ± 2.0	29.89± 3.87	21.04 ± 1.35	25.90 ± 2.24	49.0 ± 6.29
	21	16.28 ± 3.21	41.5± 4.64***	30.66± 1.81*	31.27 ± 3.45*	43.8± 2.22**
ALKP U/l	0	115.37± 4.81	127.66 ± 21.18	124.38 ± 7.33	169.72 ± 6.74	232.2 ± 7.21
	21	131.36± 22.30	182.92± 10.27**	154.57± 16.61*	161.38 ± 6.74**	200.8± 13.21*
Bilirubin mg/dl	0	2.44 ± 0.05	2.06 ± 0.05	2.08± 0.06	2.05 ± 0.03	2.61 ± 0.11
	21	2.73 ± 0.35	2.69 ±0.06*	2.8± 0.02*	2.92 ± 0.06**	2.32± 0.03**

† The level of significances are indicated by * at P< 0.05; ** at P< 0.01 and *** at P< 0.001.

The 0 day values were treated as self-control and then the difference between 0 and 21 days were compared with the ethanol group for comparison in the MK1 and MK2 treated groups and negative control group being compared against vehicle control.

Histopathological studies revealed fatty changes in few cells in case of 6% ethanol injury (Fig 3). MK1 with ethanol produced mild to no abnormalities in the liver samples, whereas MK2 produced moderate degree of granular degeneration with sporadic foci of necrosis (+ to ++) and moderate degree of vacuolar degeneration (+ to ++) when administered simultaneously with ethanol. LOLA produced mild degree of cloudy swelling (trace to +).

Histopathological scans

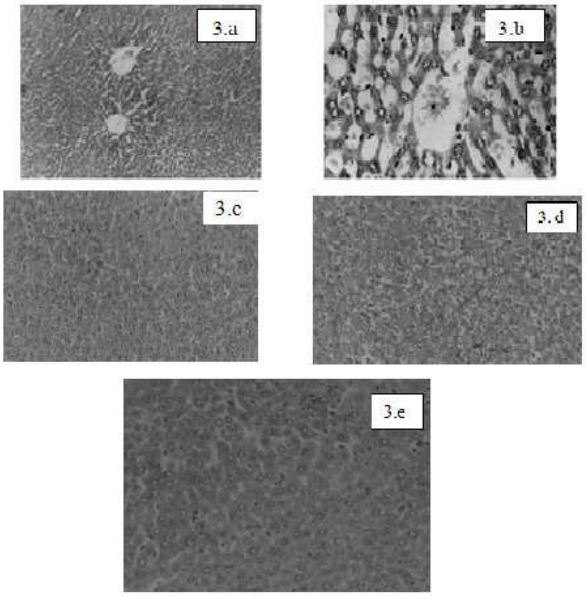


Figure 3

Histopathological scans of hepatocytes in control (3.a) and 6% alcohol treated animals (3.b), MK1 treated (3.c) and MK2 treated groups (3.d) with LOLA treated scans on the lower side (3.e). Scale Bar: 75 µm

DISCUSSIONS

The toxic potential of the chronic ethanol consumption served as an impetus to search for a potential dietary supplement in treatment of ALD. LOLA, a marketed hepatobiliary drug used to treat alcoholic liver damage, fatty liver, hepatitis, cirrhosis and prophylaxis of drug-induced hepatitis served as a positive control [13]
Ethanol-induced oxidative stress was the result of the impaired antioxidant defenses and the production of reactive oxygen species (ROS) by the mitochondrial electron transport chain [14].
The extract produced a good inhibition of DPPH radical (IC_{50}=200 µg/ml) indicating potent antioxidant activity *in vitro*.
One common result of free radical attack was the sequential degradation of cell membranes by lipid *per oxidation*, implicated in pathogenesis of alcohol-induced hepatotoxicity. Ethanol caused increase in the LPO activity due to generation of ROS during its metabolism [15]. MK1 extract with ethanol depleted MDA levels in comparison to ethanol solely. This indicated reduction in the LPO activity offering stability to the cell membrane and inhibiting the cellular necrosis.
Quenching of free radicals was done by conjugation with endogenous GSH and hence its level decreased while scavenging the free radicals. This in turn elevated the MDA levels and reduces the antioxidant defense of the cell [16]. The ability to protect antioxidant defense system was the determinant of an effective antioxidant that provided a therapeutic intervention as hepatoprotectives [17]. Both the doses produced significant increase in the GSH indicating increased antioxidant defense by the cells.
The free radicals also snatched electrons from the polyunsaturated fatty acids of the cell membrane causing necrosis of the cell with the release of SGPT and ALKP in the blood increasing their levels [18]. SGPT served as a fairly specific indicator of liver status and its elevated levels in serum indicated damaged structural integrity of the liver [19]. The extract at both the doses exhibited good protection to alcoholic challenge in terms of SGPT and ALKP levels. Both MK1 and MK2 exhibited comparable reduction in SGPT and ALKP levels less than LOLA.
An important hallmark of ethanolic injury was vacuolar degeneration of the hepatocytes. Diminished oxidation of fats and gross accumulation of fats in the hepatocytes caused ballooning of the cells, followed by degeneration [20]. The extract of *M. koenigii* scavenged the free radicals generated due to ethanol metabolism, inhibited the LPO activity and increased the cellular stability by inhibiting the cellular necrosis as revealed by mild to moderate distortion of hepatic cells.
MK1 exhibited excellent hepatoprotection against the ethanol-induced hepatitis related to the presence of antioxidant phytoconstituents such as tannins, vitamins and dietary nutrients in the extract.
The results obtained related to the previous study wherein the aqueous extract and the crude isolates from *M. koenigii* exhibited excellent hepatoprotective activity on liver carcinoma cell lines Hep G_2 [21]. Amongst another findings, not much major reduction in the serum bilirubin levels were observed in both MK1 and MK2 indicating a poor correlation; which needs to be investigated further.

CONCLUSION

The results suggest that nutritive antioxidant compounds in *M. koenigii* play a pivotal role in the therapeutics of hepatotoxicity by increasing the body's natural antioxidant defenses with depletion in the ethanol-induced oxidative stress and reduction in the elevated levels of liver enzymes.
The present investigation has opened avenues for further research in the development of potent phytomedicine for hepatoprotection from dietary *M. koenigii* leaves.

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