Research Journal of Pharmaceutical, Biological and Chemical Sciences

Antioxidant and Hepatoprotective activity of Madhuca longifolia (koenig) bark against CCl_4 - induced hepatic injury in rats: In vitro and In vivo studies

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ABSTRACT

The antioxidant and hepatoprotective effects of 70% ethanolic extract of bark of Madhuca longifolia (koenig) (EEMLK) were studied. The antioxidant property of 70% EEMLK was tested by using reducing power and free radical (hydroxyl and superoxide) scavenging models (invitro); the in-vivo antioxidant activity was assessed by determining the tissue GSH and lipid peroxidation levels. The 70% EEMLK at the doses of 200 and 400 mg/kg and silymarin 100mg/kg were administered to the CCl_4 challenged rats. The effect of 70% EEMLK and silymarin on wet liver weight, liver volume, serum biomarkers like SGOT, SGPT, ALP, direct and total Bilirubin were measured in CCl_4 induced hepatotoxicity in rats. Similarly hepatic tissues were subjected to histopathological observations. The test extract has shown dose dependant antioxidant activity in all the models. The altered biochemical and physical markers by the CCl_4 induced rats brought back to near normal level by the 70% ethanolic extract of MLK in a dose dependent manner.

Keywords: Madhuca longifolia (koenig); Antioxidant; CCl_4; Hepatoprotective

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INTRODUCTION

Liver is an organ of paramount importance as it plays an essential role in maintaining the biological equilibrium of vertebrates. The spectrum of function includes: metabolism and disposition of chemicals (xenobiotics) to which the organ is exposed directly or indirectly; metabolism of lipids, carbohydrates and protein; blood coagulation and immunomodulations [1]. Free radicals and other reactive oxygen species are derived either from normal metabolic process in the human body or from external sources such as exposure to X-rays, Ozone, cigarette smoking, air pollutants and industrial chemicals [2]. The inhibition/quenching of free radicals can serve as facile model for evaluating the activity of hepatoprotective agents. Treatment options for common liver disease such as cirrhosis, fatty liver and chronic hepatitis are problematic. It is therefore imperative to search alternative drugs for treatment of liver disease to replace the currently used drugs of doubtful efficacy and safety.

In India, numerous medicinal plants are evaluated for liver disorders in traditional system of medicine. Some of these plants are evaluated for there hepatoprotective actions against hepatotoxins. The readily available hepatoprotective herbal drugs are not sufficiently active to effectively combat severe liver disorders. Therefore, there is a need to develop satisfactory hepatoprotective drugs.

The present work deals with the study of the bark extract of Madhuca longifolia (koenig) for antioxidant and hepatoprotective activity. The principle chemical constituents of Madhuca longifolia (koenig) consist of bassian β-sitosterol, β-D-glucoside, stigmasterol, β-carotene, oleanolic acid, quercetine, dihydro quericetine, triterpenoids, amyrin acetate, myricetine, palmitic acid, saponin A and B [3][4]. The pharmacological activities reported are inflammations, epilepsy, stimulant, diuretic, refrigerant and anthelmentic dipsia, bronchitis and dermatopathy [5][6][7]. It is also reported that the plant posses analgesic activity [8]. However there is no scientific basis or reports in the modern literature regarding its usefulness as hepatoprotective agent. So, the present work deals with the study of the bark extract of Madhuca longifolia (koenig) for antioxidant and hepatoprotective activity by using CCl_4 -induced hepatic injury in rats.

MATERIALS AND METHODS

Preparation of 70% EEMLK

The bark of plant Madhuca longifolia (koenig) was collected from fields of Harapanahalli, Karnataka in the month of May 2007. It was identified and authenticated by Prof. K.Prabhu, Dept of Pharmacognosy, S.C.S. College of Pharmacy. The bark was shade dried at room temperature and pulverized. The 70% ethanolic extract was prepared by using 70% ethanol in a soxhlet apparatus after de-fatting with petroleum ether. Preliminary phytochemical investigation showed the presence of flavonoid, tannin, saponin, carbohydrade. So 70% EEMLK was selected for the present activity.

Animals

Wister albino rats (150-200g) and mice (18-25 g) of either sex were used for the study, obtained from Venkateshwara Enterprise, Bangalore, Karnataka. After one week of acclimatization the animals were used for further experiments. Approval from the institutional animal Ethical committee (Reg. no.157/1999/CPCSEA) for usage of animal in the experiment was obtained as per the Indian CPCSEA guidelines.

Acute Toxicity studies

The acute toxicity was determined on albino mice by fixed dose method of OECD Guide line no 420 given by CPCSEA. Groups of 6 mice were administered test drug by oral route in the range of 2000-3000 mg/kg and mortality was observed after 24 hr.

Antioxidant activity

Reducing power

The reducing power of 70% EEMLK was determined according to the method of Oyaizu (Oyaizu, 1986)[9]. Different doses of 70% EEMLK were mixed in 1 ml of distilled water so as to get 20μg-100μg concentration. This was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (2.5ml, 1%). The mixture was incubated at 50°C for 20 minutes. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 minutes. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%), and the absorbance (OD) was measured at 700nm. The % reducing power was calculated by using the formula:

$$\% \text{ increase in absorbance} = \frac{\text{Test OD} - \text{control OD}}{\text{Control OD}} \times 100$$

The results are compiled in Table No. 1

Superoxide anion scavenging activity

Measurement of Superoxide anion scavenging activity of 70% EEMLK was done by using the method explained by Nishimiki and modified by Ilhami et al[10]. About 1 ml of nitroblue tetrazolium (NBT) solution (156μM NBT in 100 mM phosphate buffer, pH 7.4), 1 ml NADH solution (468μM in 100 mM phosphate buffer, pH 7.4) and 0.1 ml of sample solution of 70% 70% EEMLK and standard in water was mixed. The reaction was started by adding 100μl of Phenazine methosulphate (PMS) solution (60μM PMS in 100 mM phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25°C for 5 minutes, and the absorbance at 560 nm was measured against blank. The % inhibition of OD was calculated by using the formula:

$$\% \text{ inhibition in absorbance} = \frac{\text{Control OD} - \text{Test OD}}{\text{Control OD}} \times 100$$

The results are complied in the Table No. 1

Hydroxyl radical scavenging activity

Hydroxyl radical generation by phenyl hydrazine has been measured by the 2-deoxyribose degradation, assay of Halliwell and Gutteridge[11]. In 50mM phosphate buffer (pH 7.4), 1 mM deoxyribose, 0.2 mM phenyl hydrazine hydrochloride were prepared. 0.6ml of 1mM deoxyribose and 0.4ml of 70% EEMLK and standard were taken. 0.6 ml phosphate buffer was added to make reaction solution 1.6ml. After 10 min incubation 0.4ml of 0.2 mM phenyl hydrazine was added. Incubation was terminated after 1 hr and 4 hrs and 1 ml each of 2.8% TCA and 1% (w/v) thiobarbituric acid were added to the reaction mixture and heated for 10 mins in a boiling water bath. The tubes were cooled and absorbance was taken at 532 nm. The % reduction in the OD is calculated by using the earlier formula. The results are compiled in Table No. 1.

Hepatoprotective activity

The method of Suja SR et al[12] was used in the study. Healthy Whister albino rats were divided into 5 groups of 6 animals each. Group-I and Group II received distilled water (1ml/ kg) for 5 days. Group III received 100 mg / kg silymarin (standard drug) orally for 5 days. Group IV and Group V received 200 mg/kg and 400 mg /kg 70% EEMLK (orally) respectively for 5 days. Group-I received liquid paraffin (1ml/kg) s.c., on 2nd and 3rd day. Group-II, III, IV and V received CCl_4: liquid paraffin (1:1) at a dose of 2ml/kg s.c. on 2nd and 3rd day, after 30 min of vehicle, 100 mg/kg silymarin, 200 mg/kg and 400 mg/kg of 70% EEMLK administration. On 6th day, blood samples were collected under mild ether anaesthesia and were sacrificed by cervical dislocation and liver

tissue was collected for the estimation of tissue GSH and LPO level and subjected to histopathological studies.

Parameter assess for the hepatic functions

Biochemical studies

The blood was obtained from all animals by puncturing retro –orbital plexus. Collected blood was centrifuged (2000 rpm for 10 mins) to get clear serum and various biochemical studies like SGPT[13], SGOT[14], ALP[15]. Bilirubin (total and direct)[16] were estimated.
Liver weight: The weight of the liver was measured.
Liver volume: The volume of the liver was measured.

In vivo tissue GSH estimation

Tissue Glutathione measurements were performed using a modification of Ellamn procedure[17]. Liver tissue samples were homogenized in ice cold trichloroacetic acid (1gm tissue in 10 ml 10% TCA) in an ultra trux tissue homogenizer. The mixture was centrifuged at 3000 rpm for 10 mins. Then 0.5 ml of supernatant was added to 2ml of (0.3M) disodium hydrogen phosphate solution. A 0.2 ml solution of dithiobisnitrobenzoate (0.4mg/ml in 1% sodium acetate) was added and absorbance was taken at 412 nm.

In vivo lipid peroxidation estimation

The degree of lipid peroxide formation was assayed by monitoring thiobarbituric reactive substance formation [18]. Combine 1.0 ml of biological sample (0.1-2.0 mg of membrane protein or 0.1-2.0 µmol of lipid phosphate) with 2.0 ml of TCA-TBA-HCL and mixed thoroughly. Solution was heated for 15 mins and cooled. Then precipitate was removed by centrifugation at 1000 rpm for 10 mins and absorbance of sample was determined at 535 nm against a blank that contain all the reagents minus lipid.

Histopathology

The liver was dissected out, blotted off blood, washed with saline and stored in 10% formalin and preceded for histopathology to evaluate the details of hepatic architecture in each group microscopically.

Statistical analysis

Results were expressed as mean of # SEM (n=6). Statistical analysis was performed with one way ANOVA followed by Turkey-Kramer multiple comparisons test. P values less than 0.05 was considered to be statistically significant ($p<0.05$).

RESULTS

Acute toxicity

The acute toxicity study showed No animal died even at 2000mg/kg and hence the extract was treated as non-toxic and $1/10^{th}$ and $1/5^{th}$ of the 2000mg/kg was selected for further investigations. Therefore as per the CPCSEA guideline 420 it was thought that 2000 mg/kg was the LD_{50} cut off dose. Therefore $1/10^{th}$ and $1/5^{th}$ dose (200 mg/kg and 400 mg/kg) were selected for all further in vivo studies.

Antioxidant activity

In all models, 70% EEMLK showed dose dependent antioxidant activity (table 1&2). The 70% EEMLK showed 97.62% reducing power, 95.96% hydroxyl radical and 58.54% & 53.59% (in 1hr and 4 hr respectively) super

oxide anion scavenging activities at 100 mcg concentration which are comparable to that of Sodium metabisulphate 25 mcg.

In vivo GSH

There was a marked depletion of GSH level in CCl_4 treated group. Silymarin 100 mg/kg increased tissue GSH by 95.67%. Treatment with 70% ethanolic extract showed a dose dependent increase in the levels of GSH. However, both doses of 70% EEMLK have shown lesser increase GSH level than standard silymarin (Table No 3).

In vivo lipid peroxidation

CCl_4 has enhanced the lipid peroxidation. The treatment with 70% EEMLK has significantly reduced the lipid per-oxidation in a dose dependant manner. Silymarin 100 mg/kg showed 62.64% inhibition, whereas 200 mg/kg of 70% EEMLK showed 63.51% inhibition, which was higher than the standard silymarin (Table No 3).

Table No. 1: In vitro antioxidant effect of 70% EEMLK.

Treatment	Reducing power Mean ± SEM (% increase)	Superoxide anion Scavenging Mean ± SEM (% inhibition)
Control	0.295±0.0023	0.455±0.0033
Standard 25 μg	0.560±0.0017*** (89.83%)	0.033±0.0035*** (92.74%)
70% EEMLK 20 μg	0.377±0.0028*** (27.79)	0.115±0.004*** (74.21)
70% EEMLK40 μg	0.430±0.0028*** (45.76)	0.077±0.002*** (82.73)
70%. EEMLK60 μg	0.489±0.0037*** (65.76)	0.062±0.002*** (86.09)
70% EEMLK80 μg	0.515±0.0003*** (74.57)	0.031±0.001*** (93.04)
70%EEMLK100 μg	0.583±0.0014*** (97.62)	0.018±0.002*** (95.96)

Values are the mean ± S.E.M., n=3, Significance *** P<0.001 compared to control.
STD: Sodium metabisulphate

Hepatoprotective activity

Increased levels of liver weight (4.75gm/100gm), liver volume (5.2 ml/100gm), SGPT (330.51IU/l), SGOT (415.01IU/l), ALP (449.03IU/l), total bilirubin(3.42mg/dl) and direct bilirubin (1.63mg/dl) observed in CCl_4 treated group. The pretreatment with 70% EEMLK (200mg/kg and 400mg/kg p.o.) has brought back the elevated levels of biomarker enzymes of hepatitis in a dose dependant manner (table no.4). Treatment with 400 mg/kg of EEMLK has produced the hepatoprotective activity comparable to that of silymarin 100 mg/kg p.o.

Histopathology

CCl_4 treatment has altered the liver architecture as indicated by the histopathological observations that showing extensive fatty change more around central vein with micro-vesiculisation and fatty change. Liver sinusoids also were congested. However treatment with 70% EEMLK has shown dose dependant improvement in the liver architecture as indicated by the histopathological observations that there was mild inflammation, slight congestion and fatty change to a lesser extent. The reversal of wet liver weight, volume, levels of biochemical markers and histopathological observations reveal that the 70% EEMLK possess hepatoprotective activity against CCl_4 induced hepatotoxicity in albino rats.

Table No. 2: Hydroxyl ion radical scavenging activity of 70% ethanolic extract of the bark of Madhuca longifolia (Koenig):

Group	Absorbance Mean ± SEM (after 1 hr.)	% inhibition	Absorbance Mean ± SEM (after 4 hr.)	% inhibition
Control	0.275±0.002	--	0.306 ± 0.0014	--
Standard 25 μg	0.102±0.0017***	62.90	0.134 ± 0.0023***	56.20
70% EEMLK 10 μg	0.247±0.0038***	10.18	0.268 ± 0.0020***	12.41
70% EEMLK 20 μg	0.211±0.0026***	25.27	0.244 ± 0.0017***	20.26
70% EEMLK40 μg	0.194±0.0026***	29.45	0.210 ± 0.0023***	31.37
70%. EEMLK60 μg	0.165±0.0020***	40.00	0.186 ± 0.0023***	39.21
70% EEMLK80 μg	0.128±0.0011***	53.45	0.163 ± 0.0020***	46.71
70%EEMLK100 μg	0.114±0.0037***	58.54	0.142 ± 0.0017***	53.59

Values are the mean ± S.E.M., n=3, Significance *** $P<0.001$ compared to control.
Std: Sodium metabisulphate

Table No. 3: Effect of 70% EEMLK on tissue GSH and Lipid peroxidation levels in CCl_4 induced hepatotoxicity

Treatment	Tissue GSH level		Tissue lipid peroxidation	
	Mean ± SEM	% Increase	Mean ± SEM	% Inhibition
Negative control (1ml dist. Water p.o.)	0.960±0.03	--	0.232±0.018	--
CCl_4 (positive control) (2ml/kg s.c. + 100 mg/kg s.c.)	0.486±0.025***	--	0.453±0.028	--
CCl_4+ Silymarin (2ml/kg s.c + 100 mg/kg, p.o.)	0.951±0.026***	95.67%	0.170±0.023***	62.64%
CCl_4+70% ethanolic extract (2ml/kg s.c. + 200 mg/kg p.o.)	0.802±0.045***	65.02%	0.221±0.020***	51.21%
CCl_4+70% ethanolic extract (2ml/kg s.c.. + 400 mg/kg p.o.)	0.917±0.052***	88.68%	0.166±0.018***	63.51%

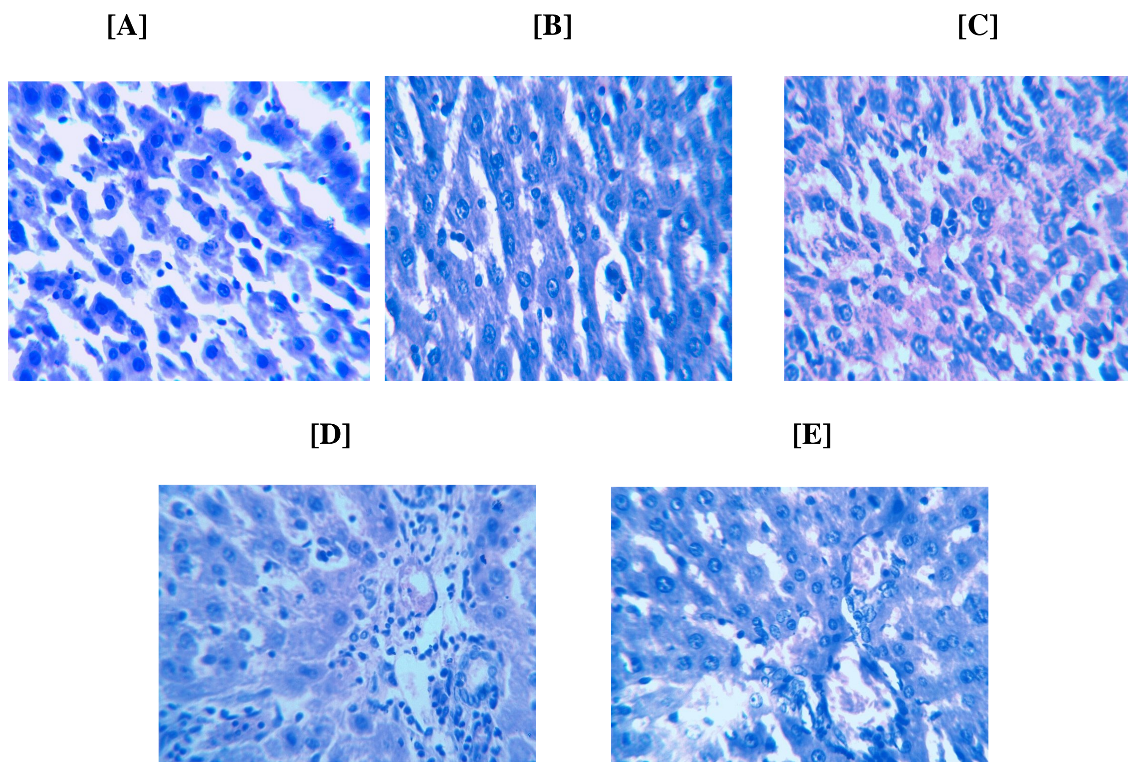
Values are the mean ± S.E.M., n=3, Significance ***P<0.001 compared to CCl4 treatment.

DISCUSSION

The antioxidant activity of 70% EEMLK was assessed on the basis of reducing power, hydroxyl radical, superoxide anion scavenging activity (in vitro antioxidant models) and effect on tissue GSH and lipid peroxidation (in vivo antioxidant model). The 70% EEMLK showed dose dependant reducing power, hydroxyl radical and superoxide anion scavenging activity. Subcutaneous administration of CCl_4 for two days elevated the SGPT, SGOT, ALP, total and direct bilirubin. These findings in +ve control are in conformity with the earlier reports. Pretreatment with 70% ethanolic extract (200 mg/kg and 400 mg/kg p.o.) for 5 days significantly reduced the elevated biochemical markers in a dose dependent manner. Treatment with 400 mg/kg of 70% EEMLK produced the hepatoprotective activity comparable to that of silymarin 100 mg/kg p.o. CCl_4 is metabolized to trichloromethyl $CCl_3^{\bullet}$ radical due to the catalytic activity of CYP 450 $2E_1$ enzyme, which is further converted to trichloromethyl peroxide radical by superoxide anions. This trichloro methyl peroxy radical is the main culprit in causing hepatotoxicity. This particular radical forms a covalent bond with sulphydryl group of membrane GSH, protein thiols and unsaturated fats or lipids. This covalent bonding of free radicals with cellular macro molecules initiates the cascade of reactions leadings to lipid peroxidation [19][20][21]. The lipid peroxidation inturn alter the membrane permeability and initiates chain of reaction leading to tissue damage and necrosis. It was observed that the test extract has shown significant reducing power and superoxide anion scavenging activity. Therefore pretreatment with 70% EEMLK may be preventing the formation of trichloro methyl peroxy radical due to superoxide anion scavenging activity. Thereby tissue GSH levels are not depleted and lipid peroxidation is minimized, this may be the possible mechanism of hepatoprotection offered 70% EEMLK.

However, our studies do not conform whether test extract block CYP 450 $2E_1$ enzyme and thereby inhibit the formation trichloromethyl $CCl_3^{\bullet}$ radical.

Histopathological Studies in CCl_4 induced hepatotoxicity:



[A] Liver architecture of Normal Control (Showing normal central vein and portal vein with no inflammation) [B] Liver architecture of CCl_4 treatment (showing extensive fatty change around central vein and portal vein with sinusoids congestion.[C] Liver architecture of CCl_4 + 100 mg/kg Silymarin treatment (showing mild inflammation and fatty change with no congestion).[D] Liver architecture of CCl_4 + 200 mg/kg of 70% EEMLK (showing mild per portal inflammation and fatty change with mild congestion) [E] Liver architecture of CCl_4 + 400 mg/kg of 70% EEMLK (showing mild congestion and inflammation but central vein was congested).

CONCLUSION

The present study demonstrates that 70% EEMLK possesses antioxidant and hepatoprotective activity. In addition, the hepatoprotective property may be attributed to the antioxidant principles which are present in the plant. Further investigation is going on to isolate, characterize and screen the active principles that possess hepatoprotective property.

ACKNOWLEDGEMENTS

The authors express their gratitude to Sri. Sha. Bra. Chnadramouleshwara Swamiji, President and Sri. T.M. Chandra Shekharaiah, the Manager, for their constant encouragement and the permission to utilize the facilities. We also express thanks to Microlabs for providing the standard drug.

Table No. 4: Effects of 70% EEMLK on Physical parameters and Biochemical markers in CCl_4 induced hepatotoxicity

Treatment	Liver		Biochemical parameters Mean ± SEM				
	Volume (ml/100g)	Weight (g/100g)	SGOT U/L	SGPT U/L	ALP IU/L	Total Bilirubin mg/dl	Direct Bilirubin mg/dl
Negative Control (1ml vehicle)	3.6 ±0.1450	3.09 ±0.1167	147.85 ±2.761	53.89 ±2.100	144.53 ±3.577	0.89 ±0.017	0.18 ±0.018
Positive Control CCl_4 + Liq. Paraffin (1:1) (2 ml/kg s.c.)	5.2 ±0.2509	4.75 ±0.2566	415.01 ±2.418	330.51 ±5.894	449.03 ±15.619	3.42 ±0.19	1.63 ±0.063
CCl_4 + Standard (Silymarin) (2 ml/kg s.c. + 100 mg/kg p.o.)	3.72 ±0.11***	3.55 ±0.16**	165.7 ±3.784***	56.83 ±2.993***	187.2 ±6.813***	1.00 ±0.057***	0.256 ±0.014***
CCl_4 + 70% EEMLK (2 ml/kg s.c. + 200 mg/kg p.o.)	4.1 ±0.15***	4.25 ±0.15***	234.75 ±18.541***	74.97 ±3.540***	272.4 ±3.358***	2.52 ±0.185***	1.21 ±0.076***
CCl_4 + 70% EEMLK (2 ml/kg s.c.+ 400 mg/kg p.o.)	3.56 ±0.23***	3.98 ±0.060***	176.88 ±4.861***	58.53 ±3.114***	197.08 ±4.903***	1.09 ±0.035***	0.33 ±0.021***

Values are the mean ± S.E.M. of six rats/ treatment. Significance **P <0.01 and *** P<0.001, compared to CCl_4 treatment

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