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Hepatoprotective effect of *Carissa carandas* Linn root extract against CCl_4 and paracetamol induced hepatic oxidative stress

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Oral pre-treatment with ethanolic extract of the roots of *C. carandas* (ERCC; 100, 200 and 400 mg/kg, po) showed significant hepatoprotective activity against CCl_4 and paracetamol induced hepatotoxicity by decreasing the activities of serum marker enzymes, bilirubin and lipid peroxidation, and significant increase in the levels of uric acid, glutathione, super oxide dismutase, catalase and protein in a dose dependent manner, which was confirmed by the decrease in the total weight of the liver and histopathological examination. Data also showed that ERCC possessed strong antioxidant activity, which may probably lead to the promising hepatoprotective activities of *C. carandas* root extract. These findings therefore supported the traditional belief on hepatoprotective effect of the roots of *C. carandas*.

Keywords: Antioxidants, *Carissa carandas*, CCl_4, Hepatoprotective activity, Oxidative stress, Paracetamol

Excessive production of reactive oxygen species (ROS) plays an important role in the pathogenesis and progression of various diseases involving different organs[1]. Lipid peroxides produced from unsaturated fatty acids via free radicals cause toxic effects and promote the formation of additional free radicals in a chain reaction. If the *invivo* activity of enzymes or scavengers is not adequate to neutralize these radicals, oxidative stress develops and leads to various diseases such as cancer, cardiovascular diseases, diabetes mellitus, liver diseases, brain dysfunction, or accelerated aging may result[2]. The rationale for the use of antioxidants is well established in prevention and treatment of diseases where oxidative stress plays a major aetiopathological role. Antioxidants may protect the body against ROS toxicity either by preventing the formation of ROS, by the interruption of ROS attack, by scavenging the reactive metabolites or by converting them to less reactive molecules[3]. Therefore the uses of antioxidants, both natural and synthetic are gaining wide importance in the prevention of various diseases.

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Liver is the vital organ responsible for drug metabolism and appears to be a sensitive target site for substances modulating biotransformation[4]. Liver diseases are mainly caused by toxic chemicals, excess consumption of alcohol, infections and autoimmune disorders. Most of the hepatotoxic chemicals damage liver cells mainly by inducing lipid peroxidation and other oxidative stress in liver[5]. Carbon tetrachloride and paracetamol being converted into reactive toxic metabolites by hepatic microsomal cytochrome P-450 in turn cause hepatotoxicity[6]. Therefore, in the present study, the CCl_4 and paracetamol induced acute models have been used to assess hepatoprotective activity.

Various plants have been used effectively as hepatoprotective agents[7]. *Carissa carandas* Linn. (Syn. *Carissa congesta* Wight) is a large dichotomously branched evergreen shrub with short stem and strong thorns in pairs, belonging to the family Apocynaceae. The plant is native and common throughout much of India, Sri Lanka, Java, Malaysia, Myanmar and Pakistan. In traditional system of medicine the plant is used as an anthelmintic, astringent, appetizer and antipyretic, in stomach disorders, rheumatism, disease of the brain, in biliousness and biliary dysfunction[8]. Earlier studies have shown that the extract of the plant possesses cardiotonic, antipyretic and antiviral activity[9-11].

Various cardiac glycosides, triterpenoidal constituents like carissone, carissin and β-sitosterol were reported from different parts of the plant[9,12,13]. It is used by tribal healers of Western Ghats region of Karnataka to treat liver diseases. However, no scientific data are available to validate the folklore claim. Keeping the above information in view, the present study has been designed to evaluate the hepatoprotective potential and *in vivo* antioxidant role of the ethanolic extract of the roots of *C. carandas* in CCl_4/paracetamol induced hepatotoxicity in rats.

Materials and Methods

Plant material and preparation of the extract—The roots of the *C. carandas* were collected from Udupi, Karnataka, during April 2007. It was authenticated by Dr. Gopalakrishna Bhat, Department of Botany, Poorna Prajna College, Udupi, Karnataka, India. A voucher specimen no. 105a is deposited in the herbarium of NGSM Institute of Pharmaceutical Sciences, Mangalore. The collected roots were washed; the bark was peeled off and then dried under shade. The coarse powder of the roots (500 g) was soaked in 1.5 L of 95% ethyl alcohol and extracted in the cold for 4 days with occasional shaking. After 4 days the ethanol layer was decanted off. The process was repeated for 4 times. The solvent from the total extract was filtered by using Whatman No. 1 filter paper, the concentrate was evaporated to dryness under reduced pressure and low temperature (40°C) on a rotary evaporator to give the ethanolic extract (16% w/w yield), which was stored at 4°C until use. Suspension of the extract was prepared in 2 % Tween-80 and used to assess hepatoprotective and antioxidant activities.

Chemicals— All the chemicals and solvents were of analytical grade and were procured from Ranbaxy Fine Chemicals Ltd., Mumbai, India. Carbon tetrachloride (CCl_4) and paracetamol (PCM) were procured from E. Merck (India) Ltd, Mumbai. The standard drug silymarin was obtained as gift sample from Cadila Pharma Ltd, India. Standard kits for SGOT, SGPT, SALP, uric acid and bilirubin were obtained from Span Diagnostics Ltd, India.

Experimental animals—Wistar albino rats of either sex, weighing about 150-180 g were used for experiments. Animals were maintained under standard conditions (12 h light / dark cycle; $25^o \pm 2^o$ C, 45-60% RH) and were fed standard rat feed (Kamadenu Agencies, Bangalore, India) and water *ad libitum*. All the animals were acclimatized to laboratory conditions for a week before commencement of experiment. All experimental protocols were reviewed and approved by the Institutional Animal Ethical Committee (IAEC) prior to the initiation of the experiment and the care of the laboratory animals was taken as per the CPCSEA regulations.

Acute toxicity study—Acute toxicity study of ethanolic extract of the roots of *C. carandas* (ERCC) was determined in Wistar albino rats (150-180 g) according to OECD guidelines No. 425[14]. The animals were fasted overnight and the ethanolic extract was administered orally with a starting dose of 2000 mg/kg, to different groups of animals. Animals were observed continuously for first 3 h and monitored for 14 days for mortality and general behaviour of animals, signs of discomfort and nervous manifestations.

Phytochemical screening—Freshly prepared ethanolic extract of the roots of *C. carandas* (ERCC) was subjected to preliminary phytochemical screening for detection of major chemical constituents[15].

CCl_4 induced hepatotoxicity—Wistar albino rats were randomly divided in to 6 groups of 6 animals each. Group I, the normal control group was administered a single daily dose of normal saline (5 ml/kg, po). Group II, the CCl_4 control group was administered a single daily dose of normal saline (5 ml/kg, po) and CCl_4/olive oil (1:1 v/v, 0.7 ml/kg, ip) on alternate days for 7 days. Group III, the standard group was administered a single daily dose of silymarin (25 mg/kg, po) and CCl_4/olive oil (1:1 v/v, 0.7 ml/kg, ip) on alternate days for 7 days. Group IV, V and VI, the test groups were administered a single daily dose of ERCC (100, 200 and 400 mg/kg, po, respectively) and CCl_4/olive oil (1:1 v/v, 0.7 ml/kg, ip) on alternate days for 7 days[16].

Paracetamol induced hepatotoxicity—Rats were randomly divided in to 6 groups of 6 animals each. All the groups were treated similar to CCl_4 induced hepatotoxicity for 7 days. On fifth day, after the administration of the respective drug treatments, all the animals of groups II, III, IV, V and VI were challenged with paracetamol 2000 mg/kg, po, suspended in sucrose solution (40% w/v)[17].

Assessment of hepatoprotective activity—On the 7th day 2 h after the administration of last dose, the animals were sacrificed by cervical decapitation; blood was withdrawn by intracardiac puncture. Blood was allowed to coagulate for 30 min and serum was

separated by centrifugation at 2500 rpm for 10 min and stored at 4°C until use. The serum was used to estimate serum glutamate oxaloacetate transaminase (SGOT)[18], serum glutamate pyruvate transaminase (SGPT)[18], serum alkaline phosphatase (SALP)[19], uric acid[20], total protein[21] and total bilirubin content[22]. After collection of blood the liver was immediately excised and rinsed in ice cold normal saline, blotted with filter paper and weighed.

Histopathological studies—Portions of the liver from all the experimental groups were fixed in 10% neutral formalin, dehydrated in graded alcohol and then embedded in paraffin. Microtome sections (5 µm thick) were prepared from each liver sample and stained with haematoxylin-eosin (H&E) dye. The sections were examined for the pathological findings of hepatotoxicity[23].

Measurement of antioxidant activity—From all the experimental groups, the portion of the liver was collected and rinsed with 0.15 *M* Tris-HCl (*p*H 7.4). A 10% w/v of liver homogenate was prepared in 0.15 *M* Tris-HCl buffer and processed for the estimation of lipid peroxidation in the form of malondialdehyde (MDA) in liver by measuring the thiobarbituric acid reactive substance (TBARS)[24]. From part of the homogenate, after precipitating proteins with 20% trichloro acetic acid (TCA) containing 1 m*M* EDTA, the supernatant was used for reduced glutathione (GSH) estimation[25]. The rest of the homogenate was centrifuged at 2000 rpm for 10 min at 4°C. The cell free supernatant thus obtained was used for the estimation of super oxide dismutase (SOD)[26] and catalase (CAT) activity[27].

Statistical analysis—The data were expressed as mean±SE, (n = 6). Data were analyzed using One way analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc test (SPSS 10.0 for Windows). Values of $P < 0.05$ were considered statistically significant.

Results

Acute toxicity study—There was no mortality amongst the graded dose groups of animals and they did not show any toxicity or behavioural changes at a dose level of 2000 mg/kg. This finding suggests that the ERCC is safe in or non-toxic to rats and hence doses of 100, 200 and 400 mg/kg, po were selected for the study.

Phytochemical screening—Preliminary phytochemical investigation of the ERCC led to the presence of alkaloids, flavonoids, saponins, cardiac glycosides, triterpenoids, phenolic compounds and tannins.

Effect of ERCC on serum marker enzyme levels—There was a significant elevation in the levels of serum marker enzymes like SGOT, SGPT and SALP content of CCl_4/PCM intoxicated animals. In contrast, pre-treatment with ERCC (100, 200 and 400 mg/kg, po) and silymarin (25 mg/kg, po) exhibited an ability to counteract the hepatotoxicity by decreasing serum marker enzymes in a dose dependent manner (P< 0.05) (Table 1).

Table 1—Effect of ethanol extract of the roots of *C. carandas* (ERCC) on serum enzyme and biochemical parameters in (A) CCl_4 and (B) PCM induced hepatic damage in rats

[Values are mean ± SE from 6 animals in each group]

Group	Dose (mg/kg po)		SGOT (U/L)	SGPT (U/L)	SALP (U/L)	Uric acid (mg/dl)	Total protein (mg/dl)	Total bilirubin (mg/dl)
I Vehicle control	5 ml	A	61.67 ± 2.99	53.85 ± 2.67	66.49 ± 1.48	2.70 ± 0.87	7.06 ± 0.39	0.98 ± 0.19
		B	73.19 ± 2.29	62.86 ± 1.16	66.62 ± 1.66	2.81 ± 0.11	6.98 ± 0.24	1.01 ± 0.19
II CCl_4/PCM Control	0.7 ml	A	174.79 ± 3.56^a	126.64 ± 1.72^a	115.63 ± 1.70^a	1.37 ± 0.45^a	5.38 ± 0.13^a	2.31 ± 0.21^a
		B	179.37 ± 4.60^a	134.13 ± 2.31^a	144.01 ± 2.30^a	1.45 ± 0.25^a	5.26 ± 0.10^a	2.41 ± 0.18^a
III CCl_4/PCM + Silymarin	25	A	64.04 ± 3.4^d	59.49 ± 2.12^d	67.26 ± 1.19^d	2.55 ± 0.15^d	6.99 ± 0.9^d	1.03 ± 0.23^d
		B	82.45 ± 1.64^d	72.26 ± 1.99^d	78.82 ± 2.32^d	2.61 ± 0.10^d	6.73 ± 0.15^d	1.07 ± 0.09^d
IV CCl_4/PCM + ERCC	100	A	143.24 ± 3.69^d	102.29 ± 1.77^d	101.06 ± 2.26^d	1.78 ± 0.60^b	5.68 ± 0.36^b	2.08 ± .42^b
		B	156.86 ± 3.91^d	114.14 ± 2.89^d	130.60 ± 2.13^c	1.90 ± 0.35^b	5.82 ± 0.12^b	2.05 ± 0.15^b
V CCl_4/PCM + ERCC	200	A	103.85 ± 5.27^d	79.48 ± 1.06^d	84.77 ± 3.28^d	2.06 ± 0.55^d	6.31 ± 0.12^d	1.24 ± 0.19^d
		B	110.07 ± 4.68^d	96.93 ± 2.38^d	119.62 ± 2.89^d	2.09 ± 0.14^c	6.31 ± 0.19^d	1.38 ± 0.14^d
VI CCl_4/PCM + ERCC	400	A	71.78 ± 3.11^d	68.95 ± 1.37^d	74.56 ± 2.08^d	2.32 ± 0.91^d	6.63 ± 0.19^d	1.12 ± 0.37^d
		B	93.29 ± 4.98^d	86.12 ± 2.33^d	85.37 ± 2.52^d	2.31 ± 0.23^d	6.49 ± 0.14^d	1.20 ± 0.10^d

One way ANOVA followed by Tukey's multiple comparison post hoc test. ^{a} P< 0.001 when compared with vehicle treated control group, ^{b} P< 0.05, ^{c} P< 0.01, ^{d} P< 0.001 when compared with CCl_4/PCM treated control group.

Effect of ERCC on biochemical parameters—In both the CCl_4 and PCM treated groups, there was a significant increase in total bilirubin and significant reduction in uric acid and total protein content. Whereas, pre-treatment with ERCC (100, 200 and 400 mg/kg, po) caused significant reduction in total bilirubin and significant increase in the activities of uric acid and total protein content dose dependently (Table 1).

Effect of ERCC on antioxidant activity—There was a significant increase in MDA content and reduction in GSH, SOD and CAT activities of both CCl_4/PCM intoxicated animals (Table 2). Pre-treatment with silymarin (25 mg/kg, po) and ERCC (100, 200 and 400 mg/kg, po) significantly ($P< 0.05$) prevented the increase in MDA levels and brought them near to normal level, whereas GSH, SOD and CAT levels were significantly ($P< 0.05$) raised, thus providing protection against CCl_4/paracetamol toxicities.

Effect of ERCC on liver weight—In both CCl_4/PCM intoxicated groups of animals, the weight of the liver was significantly increased, but it was normalized in ERCC and silymarin treated groups of animals (Table 2). A significant reduction ($P< 0.05$) in liver weight supports this finding.

Histopathology—Histopathological studies also provided a supportive evidence for biochemical analysis. The liver sections of the rats treated with CCl_4/PCM intoxicated groups showed hepatic cells with severe toxicity characterized by inflammatory infiltration and necrosis in many areas. Pre-treatment with silymarin and ERCC exhibited significant liver protection against CCl_4/PCM induced liver damage, which is evident by the presence of more or less normal hepatocytes and reduced inflammatory infiltration and necrosis (Fig. 1a-g).

Discussion

Carbon tetrachloride/paracetamol induced hepatic injuries are commonly used models for the screening of hepatoprotective drugs and the extent of hepatic damage is assessed by the level of released cytoplasmic alkaline phosphatase and transaminases in circulation. It is well documented that CCl_4/PCM are biotransformed under the action of microsomal cytochrome P-450 of liver to reactive metabolites[28,29]. These free radicals bind covalently to unsaturated lipid membrane, provoking a sharp increase of lipid peroxides followed by pathological changes such as, elevated levels of serum marker enzymes like SGOT, SGPT and SALP, depletion of GSH, decreased protein synthesis, triglyceride accumulation, increased lipid peroxidation, destruction of Ca^{2+} homeostasis and finally hepatocyte damage[30]. This suggests that, CCl_4/paracetamol induces liver injury by sharing a common property of free radical mechanism.

Hepatocellular necrosis or membrane damage leads to very high levels of serum GOT and GPT released from liver to circulation. Among the two, GPT is a better index of liver injury, since SGPT catalyses the conversion of alanine to pyruvate and glutamate, and is released in a similar manner, thus liver GPT

Table 2—Effect of ethanol extract of the roots of *C. carandas* (ERCC) on lipid peroxidation (LPO), glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) and liver weight in (A) CCl_4 and (B) PCM induced hepatic damage in rats.
[Values are mean ± SE from 6 animals in each group]

Group	Dose (mg/kg, po)		LPO (nM MDA/mg protein)	GSH (µg/mg protein)	SOD (U/mg protein)	CAT (U/mg protein)	Liver weight (Wt/100g bw)
I Vehicle control	5 ml	A	0.98 ± 0.12	5.23 ± 0.11	93.51 ± 1.37	351.45 ± 3.92	3.48 ± 0.12
		B	1.06 ± 0.20	5.34 ± 0.19	92.49 ± 1.94	353.16 ± 4.02	3.54 ± 0.12
II CCl_4/PCM control	0.7 ml	A	6.99 ± 1.93^a	0.75 ± 0.10^a	55.07 ± 1.17^a	262.72 ± 3.02^a	6.38 ± 0.42^a
		B	6.85 ± 0.13^a	0.86 ± 0.13^a	54.48 ± 1.22^a	269.24 ± 2.88^a	6.34 ± 0.15^a
III CCl_4/PCM + Silymarin	25	A	1.10 ± 0.95^d	5.19 ± 0.11^d	89.64 ± 1.70^d	348.30 ± 1.92^d	3.67 ±0.10^d
		B	1.12 ± 0.12^d	5.25 ± 0.15^d	88.31 ± 1.72^d	348.96 ± 4.87^d	3.64 ± 0.10^d
IV CCl_4/PCM + ERCC	100	A	5.69 ± 1.09^d	1.54 ± 0.12^d	63.60 ± 2.16^b	277.81 ± 2.07^c	5.62 ± 0.13^d
		B	6.13 ± 0.21^c	1.45 ± 0.11^c	61.95 ± 1.70^b	283.80 ± 2.03^b	5.74 ± 0.20^c
V CCl_4/PCM + ERCC	200	A	4.01 ± 0.19^d	3.54 ± 0.13^d	68.73 ± 1.74^d	306.06 ± 1.44^d	5.16 ±0.21^d
		B	4.11 ± 0.19^d	3.69 ± 0.23^d	68.08 ± 1.83^d	306.93 ± 3.06^d	5.14 ± 0.18^d
VI CCl_4/PCM + ERCC	400	A	1.77 ± 0.15^d	4.76 ± 0.14^d	80.24 ± 1.29^d	338.27 ± 2.49^d	4.23 ± 0.18^d
		B	2.02 ± 0.31^d	4.81 ± 0.31^d	79.78 ± 1.40^d	337.28 ± 2.36^d	4.17 ± 0.13^d

One way ANOVA followed by Tukey's multiple comparison post hoc test. ^{a} $P< 0.001$ when compared with vehicle treated control group, ^{b} $P< 0.05$, ^{c} $P< 0.01$, ^{d} $P< 0.001$ when compared with CCl_4/PCM treated control group.

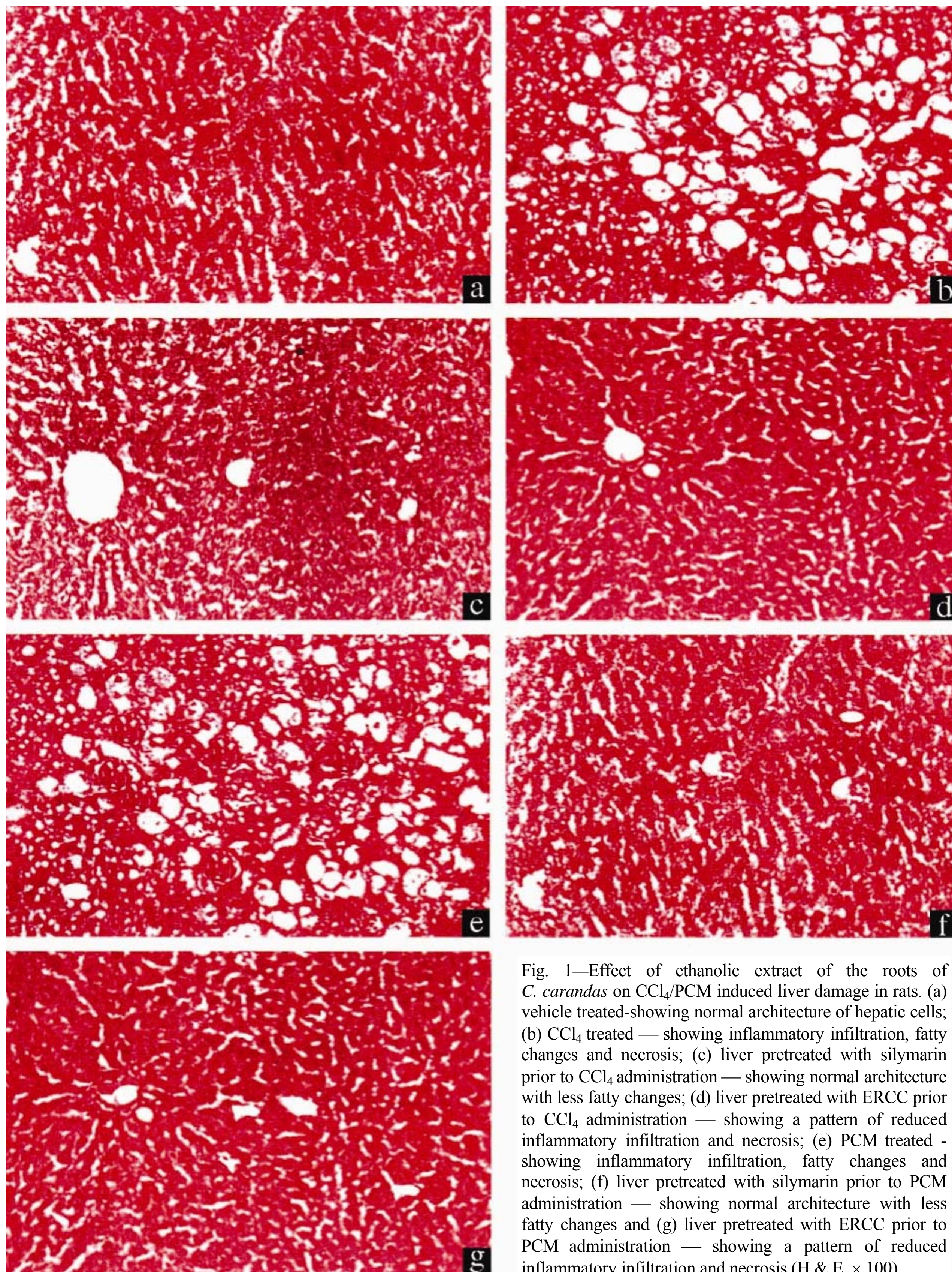

Fig. 1—Effect of ethanolic extract of the roots of *C. carandas* on CCl_4/PCM induced liver damage in rats. (a) vehicle treated-showing normal architecture of hepatic cells; (b) CCl_4 treated — showing inflammatory infiltration, fatty changes and necrosis; (c) liver pretreated with silymarin prior to CCl_4 administration — showing normal architecture with less fatty changes; (d) liver pretreated with ERCC prior to CCl_4 administration — showing a pattern of reduced inflammatory infiltration and necrosis; (e) PCM treated - showing inflammatory infiltration, fatty changes and necrosis; (f) liver pretreated with silymarin prior to PCM administration — showing normal architecture with less fatty changes and (g) liver pretreated with ERCC prior to PCM administration — showing a pattern of reduced inflammatory infiltration and necrosis (H & E, × 100).

represents 90% of total enzyme present in the body[31]. The elevated levels of serum marker enzymes are indicative of cellular leakage and loss of functional integrity of cellular membrane in liver[32]. SALP activities on the other hand are related to functioning of hepatocytes, its increase in serum is due to increased synthesis in the presence of increased biliary pressure[33]. It is well known that toxicants like CCl_4 and PCM produce sufficient injury to hepatic parenchyma cells to cause elevation in serum bilirubin, and in contrast decrease the level of total plasma protein content[34].

In the present study, ethanol extract of the roots of *C. carandas* (ERCC) at a dose of 100, 200 and 400 mg/kg, po caused a significant inhibition in the levels of SGOT and SGPT towards the respective normal range and this is an indication of stabilization of plasma membrane as well as repair of hepatic tissue damage caused by both CCl_4/PCM. On the other hand suppression of elevated SALP activities with concurrent depletion of raised bilirubin level and an increase in the total plasma protein content suggests the stability of biliary dysfunction in rat liver during hepatic injuries with toxicants[35]. These results indicate that ERCC preserved the structural integrity of the hepatocellular membrane and liver cell architecture damaged by CCl_4/PCM which was confirmed by histopathological examination.

CCl_4/PCM caused a significant increase in the liver weight, which is due to blocking of secretion of hepatic triglycerides in to the plasma[36]. The reduced level of uric acid in CCl_4/PCM induced hepatotoxicity is probably due to the increased utilization of uric acid against increased production of free radicals, which is a characteristic feature of cancer and tissue necrosis. The results from the present study suggest that altered uric acid level to nearing normal in ERCC treated animals could be due to strong antioxidant property of the extract.

Lipid peroxidation has been postulated to be the destructive process in liver injury due to CCl_4/paracetamol administration[37,38]. In the present study, an elevation in the levels of MDA in liver of animals treated with both the toxicants was observed. The increase in MDA levels of liver suggest enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defence mechanisms to prevent formation of excessive free radicals. Pre-treatment with ERCC significantly reduced the levels of lipid peroxidation. Hence it may be possible that the mechanism of hepatoprotection by ERCC is due to its antioxidant potential.

Glutathione (GSH) is one of the most abundant naturally occurring tripeptide, non-enzymatic biological antioxidant present in liver[39]. Its functions are concerned with the removal of free radicals such as H_2O_2 and superoxide radicals, maintenance of membrane protein, detoxification of foreign chemicals and biotransformation of drugs[40]. In the present study, the decreased level of GSH has been associated with an enhanced level of lipid peroxidation in CCl_4/PCM intoxicated groups of rats. Pre-treated with ERCC significantly increased the level of glutathione in a dose dependent manner. Thus ERCC may act by inducing the detoxifying enzymes and these enzymes may detoxify the ROS following administration of toxicants.

Serum activities of superoxide dismutase (SOD) and catalase (CAT) are the most sensitive enzymatic index in liver injury caused by ROS and oxidative stress. SOD is one of the most abundant intracellular antioxidant enzymes present in all aerobic cells and it has an antitoxic effect against ROS[41]. CAT is a haemoprotein; it protects the cells from the accumulation of H_2O_2 by dismutating it to form H_2O and O_2[42]. Therefore reduction in the activities of these enzymes may indicate the toxic effects of ROS produced by toxicants. In the present study, it was observed that pre-treatment with ERCC caused a significant rise in hepatic SOD and CAT activities. This suggests that ERCC can reduce ROS that may lessen the oxidative damage to the hepatocytes and improve the activities of the liver antioxidant enzymes, thus protecting the liver from CCl_4/PCM.

Preliminary phytochemical screening of the ethanol extract of the roots of *C. carandas* revealed the presence of alkaloids, flavonoids, saponins, cardiac glycosides, triterpenoids, phenolic compounds and tannins. In the present state of knowledge of the chemical constituents of the extract of this plant, it is not possible to attribute with certainty the hepatoprotective effect to one or several active principles among those detected in the ethanol extract of the roots of *C. carandas*. However, flavonoids[43], triterpenoids[44], saponins[45] and alkaloids[46] are known to possess hepatoprotective activity in animals. It is worthwhile to isolate the bioactive principles which are responsible for these activities, which is in progress in our laboratory.

It can be concluded that the data obtained in the present study suggest that the ethanolic extract of the

roots of *C. carandas* has significant hepatoprotective and antioxidant activities on both CCl_4/PCM induced hepatic damage in rats. These results reveal that the hepatoprotective effect of the ethanolic extract of the roots of *C. carandas* may be due to its ability to block the bioactivation of toxicant and its potent antioxidant activity, and/or by scavenging the free radicals and inhibiting lipid peroxidation.

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