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Hepatoprotective and *in vivo* Antioxidant Effects of *Careya arborea* against Carbon tetrachloride Induced Liver Damage in Rats

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Abstract: The present study was carried out to evaluate the hepatoprotective and antioxidant effect of the Methanol Extract of *Careya arborea* Roxb (MECA) (Family- Myrtaceae) stem bark in Wistar albino rats. The different groups of animals were administered with carbon tetrachloride (CCl_4) (30 % CCl_4, 1 mL Kg^{-1} b. wt. in liquid paraffin 3 doses (i.p.) at 72 h interval). The MECA at the doses of 50, 100 and 200 mg Kg^{-1} and silymarin 25 mg Kg^{-1}were administered to the CCl_4 treated rats. The effect of MECA and silymarin on serum transaminase (GOT, GPT), Alkaline Phosphates (ALP), bilirubin, uric acid and total protein were measured in the rats induced hepatotoxicity by CCl_4. Further, the effects of the extract on Lipid Peroxidation (LPO), enzymatic antioxidant (Superoxide Dismutase (SOD) and Catalase (CAT)), and nonenzymatic antioxidant (Glutathione (GSH), vitamin C and vitamin E) were estimated. The MECA and silymarin produced significant ($p < 0.05$) hepatoprotective effect by decreasing the activity of serum enzymes, bilirubin, uric acid, and lipid peroxidation and significantly ($p < 0.05$) increased the levels of SOD, CAT, GSH, vitamin C, vitamin E and protein in a dose dependent manner. From these results, it was suggested that MECA possess potent hepatoprotective and antioxidant properties.

Key words: *Careya arborea*, hepatoprotective effects, antioxidants, carbon tetrachloride

INTRODUCTION

The experimental intoxication induced by carbon tetrachloride (CCl_4) is widely used for modeling liver injury in rats. Hepatotoxicity is connected with severe impairment of cell protection mechanisms. The location of liver injury Is defined mainly by the biotransformation of CCl_4, which is cytochrome P-450 dependent. Free radicals initiate the process of lipid peroxidation, which is generally caused of inhibition of enzyme activity[1,2]. It is now generally accepted that the hepatotoxicity of CCl_4 is the result of reductive dehalogenation, which is catalyzed by P450, and which forms the highly reactive trichloromethyl free radical. This then readily interacts with molecular oxygen to form the trichloromethyl peroxy radical. Both trichloromethyl and its peroxy radical are capable of binding to proteins or lipids, or of abstracting a hydrogen atom from an unsaturated lipid, initiating lipid peroxidation and liver damage and by doing so playing a significant role in pathogenesis of diseases[3].

Careya arborea Roxb. commonly known as Wild Guava belongs to the family Myrtaceae medium sized deciduous tree, bark dark grey exfoliating in thin strip. Widely available in India, Ceylon, Malay and Peninsula. The plant has been extensively investigated and a number of chemical constituents from the barks, leaves and seeds of the plant have previously reported in a number of instances which includes triterpenoids[4-6] flavonoid[7], cumarin[8,9], saponins[10] and tannins[11].

Stem bark of *Careya arborea* was traditionally used in the treatment of tumors, anthelmintic, bronchitis, epileptic fits, astringents, antidote to snake-venom and skin disease[12], It also used as remedy for diarrhea[13], dysentery with bloody stools and ear pain[14,15]. Antipyretic[5], leech repellant, fish poison and antivenin activities were also reported in literature[16-18]. The aqueous extract of fresh root bark used as fish poison[10]. The tribal peoples of Kolli Hills of Tamilnadu used the stem bark of the plant for the treatment of liver disorders. Pervious report from our laboratory shows invitro antimicrobial and antioxidant activity of

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Careya arborea[19]. Based on the Pervious report, traditional usage and chemical constituents we selected this plant for the study. The purpose of the present study was to evaluate the hepatoprotective and *in vivo* antioxidant activities of Methanol Extract of *Careya Arborea* (MECA).

Plant derived natural products such as flavonoids, terpenoids and steroids etc. have received considerable attention in recent years due to their diverse pharmacological properties including hepatoprotective and antioxidant activity[20,21]. There has been growing interest in the analysis of certain flavonoids, triterpenoids and steroids stimulated by intense research in to their potential benefits to human health. One of their main properties in this regard is their antioxidant activity, which enables them to attenuate the development of tumor and inflammatory disease. Antioxidant plays an important role in inhibiting and scavenging radicals, thus providing protection to humans against infection and degenerative diseases. Realizing the fact, this research was carried out to evaluate the hepatoprotective and antioxidant activity of Methanol Extract of *Careya Arborea* (MECA) against Ccl_4-induced liver damage in rats.

MATERIALS AND METHODS

Plant materials and Extraction: The plant *Careya arborea* (Family: Myrtaceae) stem bark was collected in the month of March 2004 from the Kolli Hills, Tamil Nadu, India. The plant material was taxonomically identified by Botanical Survey of India, Kolkata, India, and the Voucher specimen (No.GMS-3) was retained in our laboratory for the future reference. The dried powder material of the stem bark of *Careya arborea* was extracted with methanol (Yield 7.45 %) in a soxhlet apparatus. The methanol extract was then distilled, evaporated and dried in vacuum. The chemical constituents of the extract were identified by qualitative analysis followed by their confirmation by thin layer chromatography.

Animals: Studies were carried out using male Wistar albino rats (150–180 g). They were obtained from the animal house, Indian Institute of Chemical Biology (IICB), Kolkata, India. The animals were grouped and housed in polyacrylic cages (38 x 23 x 10 cm) with not more than six animals per cage and maintained under standard laboratory conditions (temperature 25 + 2°C) with dark and light cycle (14/10 h). They were allowed free access to standard dry pellet diet (Hindustan Lever, Kolkata, India) and water *ad libitum*. The mice were acclimatized to laboratory condition for 10 days before commencement of experiment. All procedures described were reviewed and approved by the University Animal Ethical Committee.

Drugs and Chemicals: Silymarin was purchased from Micro labs Holar Tamilnadu India, 1-Chloro-2, 4-dinitrobenzene [CDNB], Bovine serum albumin (Sigma chemical St. Louis, MO, USA), Thiobarbituric acid, Nitrobluetetrazolium chloride (NBT) (Loba Chemie, Bombay, India), 5,5'-dithio *bis*-2-nitrobenzoic acid (DTNB), Carbon tetrachloride, (SICCO research laboratory, Bombay). The solvent and / or reagent obtained were used as received.

Toxicity study: For toxicity studies groups of 10 mice were administered (p.o.) with test compounds in the range of doses 100-1600 mg Kg^{-1}. and the mortality rates were observed after 72 h. The LD_{50} was determined using the graphical methods of Litchfield and Wilcoxon[22].

Carbon tetrachloride-induced liver damage in rats: Healthy male albino rats were divided into 6 groups each containing 6 animals. Group 1 Normal (Liquid paraffin 1mL Kg^{-1} body weight, p.o.) Group 2 (Control) received 30% CCl_4 in liquid paraffin (1 mL Kg^{-1} body weight, i.p.). Group 3, 4 and 5 received MECA 50, 100 and 200 mg Kg^{-1}p.o, respectively and Group 6 received standard drug Silymarin (25 mg Kg^{-1}p.o) once in a day and CCl_4 as mentioned above. Treatment duration was 10 days and the dose of CCl_4 was administered after every 72-h (23). Animals were sacrificed 24 h after the last injection. Blood was collected, allowed to clot and serum separated. The liver was dissected out and used for biochemical studies.

The blood was obtained from all animals by puncturing retro-orbital plexus. The blood samples were allowed to clot for 45 min at room temperature. Serum was separated by centrifugation at 2500 rpm at 30°C for 15 min and utilized for the estimation of various biochemical parameters namely Serum glutamyl pyruvate transaminase (SGPT), Serum glutamyl oxalacetic acid transaminase (SGOT)[24], Serum alkaline phosphatase (SALP)[25], serum bilirubin[26], protein content[27] and plasma uric acid[28].

After collection of blood samples the rats were sacrificed and their livers excised, rinsed in ice cold normal saline followed by 0.15 M Tris-HCl (pH 7.4) blotted dry and weighed. A 10 % w/v of homogenate was prepared in 0.15 M Tris-HCl buffer and processed for the estimation of lipid peroxidation[29]. A part of homogenate after precipitating proteins with Trichloroacetic acid (TCA) was

used for estimation of glutathione[30]. Vitamin C[31] and vitamin E[32], with slight modification by Baker and Frank (1951)[33] were also estimated. The rest of the homogenate was centrifuged at 15000 rpm for 15 min at 4° C. The supernatant thus obtained was used for the estimation of SOD[34] and CAT activities[35].

Statistical analysis: Results are reported as means ± S.E.M. ANOVA was used to evaluate differences between groups. If significance was observed between groups, the Student's *t*-test was used to compare the means of specific groups, with $p < 0.05$ considered as significant.

RESULTS

Acute toxicity: The methanol extract of stem bark of *Careya arborea* was found to be non-toxic up to doses of 1.6 g Kg^{-1} and did not cause any death of the animals tested.

Effect of MECA on serum enzymes, bilirubin, uric acid and protein: Alteration in the activities of serum enzymes (GPT, GOT and ALP), bilirubin, uric acid and total protein content in the serum of CCl_4 induced liver damage in rats as evidence from Table1. The level of serum marker enzymes GPT, GOT, ALP, bilirubin and uric acid were found to be significantly increased and protein content significantly decreased in CCl_4-induced liver damage rats when compared with the normal group ($p < 0.01$). Where as treatment with MECA at the dose of 50, 100 and 200 mg Kg^{-1}showed decreased the activity of serum transaminase, ALP, uric acid, bilirubin and increased the protein content in CCl_4-induced liver damage in rats compared to that of control groups ($p < 0.05$). Silymarin (25 mg Kg^{-1}) also significantly decreased the levels of serum enzymes, bilirubin uric acid and increased the protein content in CCl_4 treated groups as compared with the respective control group.

***In Vivo* Lipid peroxidation:** The localization of radical formation resulting in lipid peroxidation, measured as MDA in rat liver homogenate, is shown in Table 2. MDA content in the liver homogenate was increased in CCl_4 control group compared to normal group ($p < 0.001$). MDA level of MECA 50, 100 and 200 mg Kg^{-1}group, were inhibited by 18.76, 47.14 and 92.86 % compared to CCl_4 control ($p < 0.05$). At the same time, the effect of silymarin 25 mg Kg^{-1}on MDA levels in CCl_4 was inhibited by 94.41 %, respectively.

SOD and CAT activity in liver tissues: The effect of MECA on SOD and CAT activities in liver is shown in Table 2. SOD activity in CCl_4 control group was examined to be lower than in normal group ($p < 0.001$). SOD activities in MECA 50, 100 and 200 mg Kg^{-1}groups were observed to be higher than in Ccl_4 control group ($p < 0.05$). SOD activities of MECA 50, 100 and 200 mg Kg^{-1}were improved by 8.36, 29.19 and 81.11 %, respectively. Silymarin 25 mg Kg^{-1}also restored the SOD activity in CCl_4 treated groups. CAT activity of CCl_4 control group was measured to be strikingly lower than in normal group ($p < 0.001$). Liver CAT activities in MECA 50, 100 and 200 mg Kg^{-1}groups were increased by 17.37, 43.86 and 79.97 %, respectively when compared with

Table 1. Effect of methanol extract of *Careya arborea* stem bark (MECA) on serum enzymes (GPT, GOT and ALP), bilirubin, protein and uric acid in CCl_4 induced hepatic damage in rats

Parameters	Normal (liquid paraffin 1mL Kg^{-1}, b.wt)	Control (Ccl_4 1mL Kg^{-1}, b.wt)	Silymarin (25 mg Kg^{-1})+ Ccl_4	MECA (50 mg Kg^{-1}) + Ccl_4	MECA (100 mg Kg^{-1})+ Ccl_4	MECA (200mgKg^{-1})+ CCl_4
SGOT (U/l)	62.52 ± 5.21	178.21 ± 8.92*	64.32 ± 5.72** (98.44)	147.51± 8.53** (26.53)	108.52 ± 7.81** (60.25)	67.41± 6.24** (90.85)
SGPT (U/l)	52.15 ± 4.21	127.51± 9.12*	55.21 ± .4.90** (99.92)	102.15 ± 7.41 (33.64)	80.53 ± 6.42** (62.33)	58.54 ± 5.12** (91.51)
SALP (U/l)	67.42 ± 4.31	118.51± 7.52*	69.14 ± .3.94 (96.63)	102.31± 8.71** (31.69)	88.75± 7.90 (58.24)	72.54 ± 6.84** (89.97)
Bilirubin (mg dL)	0.95 ± .0.32	2.54 ± 0.45*	0.98 ± 0.11** (98.11)	2.12 ± 0.22** (26.41)	1.13 ± 0.12** (57.23)	1.02 ± 0.14** (95.59)
Protein (mg dL)	7.02 ± 0.51	5.45 ± 0.32*	7.01± 0.52** (99.36)	5.93 ± 0.52 (30.57)	6.41± 0.42** (61.14)	6.96 ± 0.31** (96.17)
Uric acid (mg dL)	2.87 ± 0.25	1.46 ± 0.11*	2.92 ± 0.21 (95.13)	2.36 ± 0.21** (56.73)	2.45 ± 0.25** (70.21)	2.72 ± 0.26** 89.36)

The data in the parenthesis indicate percent protection in individual biochemical parameters from their elevated values caused by the CCl_4. The % of protection is calculated as 100 X (values of CCl_4 control -values of sample) / (values of CCl_4 control - values of vehicle control)

Values are mean ± S.E.M. number of rats=6.

Control group compared with normal group * $P < 0.001$

Experimental groups compared with CCl_4 control group * * $P < 0.05$

Table 2. Effect of the methanol extract of *Careya arborea* stem barks (MECA) on lipid peroxidation (LPO) antioxidant enzymes (SOD and CAT) and non enzymatic antioxidant (GSH, vitamin E and vitamin C) in the liver of CCl_4 intoxicated rats.

Parameters	Normal (liquid paraffin 1mL Kg^{-1}, b.wt)	Control (Ccl_4 1mL Kg^{-1}, b.wt)	Silymarin (25 mg Kg^{-1})+ Ccl_4	MECA (50 mg Kg^{-1}) + Ccl_4	MECA (100 mg Kg^{-1})+ Ccl_4	MECA (200mg Kg^{-1}) + Ccl_4
Lipid peroxidation (n mole of MDA/mg protein)	0.88± 0.05	7.01± 0.51*	1.01± 0.05 (96.41)	5.86± 0.41** (18.76)	4.12±0.42 (47.14)	1.32± 0.11** (92.82)
Glutathione content (µg/mg protein)	5.31± 0.49	0.59± 0.07*	5.30± 0.34 (99.78)	1.19± 0.26** (12.71)	3.25± 0.29** (56.35)	4.86± 0.25** (90.46)
Vitamine C (mg/g/wet tissue)	4.07±0.37	2.14±0.24*	3.86±0.35 (89.11)	2.65±0.24** (26.42)	3.32±0.31** (61.13)	3.80±0.25** (89.01)
Vitamine C (mg/g/wet tissue)	1.49±0.13	0.41± 0.12*	1.42±0.14 (93.51)	0.73±0.11** (29.62)	1.04±0.16** (58.33)	1.38±0.12** (89.81)
Superoxide dismutase (U/mg protein)	91.76± 7.35	57.23±4.26*	91.56±7.43 (99.42)	60.12± 5.23** (8.36)	67.31± 6.29 (29.19)	85.24± 7.32 (81.11)
Catalase (U/mg protein)	364.61±20.07	276.92 ±22.07*	363.63±31.25 (98.89)	292.14±33.03** (17.37)	315.37± 9.09** (43.86)	344.41± 29.05** (76.97)

The data in the parenthesis indicate percent protection in individual biochemical parameters from their elevated values caused by the CCl_4. The % of protection is calculated as 100 X (values of CCl_4 control -values of sample) / (values of CCl_4 control - values of vehicle control)
Values are mean±S.E.M. number of rats=6. Control group compared with normal group * $p < 0.001$
Experimental groups compared with CCl_4 control group * * $p < 0.05$

control group ($p < 0.05$). MECA and silymarin completely restored the enzyme activity to the normal level at the respective doses of 200 mg Kg^{-1} and 25 mg Kg^{-1}.

Glutathione, vitamin C and vitamin E levels in liver tissues: The effect of MECA on glutathione content, vitamin C and vitamin E levels in the liver is shown in Table 2. GSH level in normal group was measured to be higher than in CCl_4 control group. GSH level of MECA 50, 100 and 200 mg Kg^{-1}groups were increased by 12.71, 56.35, and 90.46%, respectively as compared to CCl_4 control group ($p < 0.05$). Silymarin almost completely restored the glutathione level in CCl_4 treated groups to the normal level. The levels of Vitamin C in the liver of CCl_4 control group decreased in comparison with the normal group ($p < 0.01$). After administration of MECA at the dose of 50, 100 and 200 mg Kg^{-1}., b.w. increased the levels of vitamin C by 23.62, 58.33 and 89.81%, respectively, as compared to that of the CCl_4 control group ($p < 0.05$). The vitamin E level in CCl_4 control group decreased in comparison with the normal group ($p < 0.01$). Treatment with MECA at the dose of 50, 100 and 200 mg Kg^{-1}., b.w. increased vitamin E levels by 26.42, 61.13 and 89.01%, respectively when compared to that of the CCl_4 control ($p < 0.05$).

DISCUSSION

In the assessment of liver damage by CCl_4 hepatotoxin, the determination of enzyme levels such as SGPT and SGOT is largely used. Necrosis or membrane damage releases the enzyme in to circulation; therefore, it can be measured in serum. High levels of SGOT indicate liver damage, such as that due to viral hepatitis as well as cardiac infarction and muscle injury. SGPT catalyses the conversion of alanine to pyruvate and glutamate, and is released in a similar manner. Therefore, SGPT is more specific to the liver, and is thus a better parameter for detecting liver injury[36]. Our results using the model of CCl_4-induced hepatotoxicity in the rats demonstrated that MECA at the different doses caused significant inhibition of SGPT and SGOT levels. Serum ALP and bilirubin levels on other hand are related to the function of hepatic cell. Increase in serum level of ALP is due to increased synthesis, in presence of increasing biliary pressure[37]. Our results using the model of CCl_4-induced hepatotoxicity in rats demonstrated that MECA at different doses caused significant inhibition of SALP and bilirubin levels. Effective control of bilirubin level and alkaline phosphatase activity points towards an early improvement in the secretory mechanism of the hepatic cell.

Uric acid, the metabolic end product of purine metabolism, has proven to be a selective antioxidant, capable especially of reacting with free radicals and hypochlorous acid[38]. The reduced level of uric acid in hepatotoxicity conditions may be due to the increased utilization of uric acid against increased production of the free radicals, which is a characteristic feature of cancer condition. The reversal of altered uric acid level to near normal in MECA treated rats could be due to strong antioxidant property of MECA, which contributes to its antioxidant potency.

Liver cell injury induced by CCl_4 involves initially the metabolism of CCl_4 to trichloromethyl free radical by the mixed-function oxidase system of the endoplasmic reticulam. It is postulated that secondary mechanisms link CCl_4 metabolism to the widespread disturbances in hepatocyte function. These secondary mechanisms could involve the generation of toxic products arising directly from CCl_4 metabolism or from peroxidative degeneration of membrane lipids[39]. In our study, elevations in the levels of end products of lipid peroxidation in liver of rat treated with CCl_4 were observed. The increase in MDA level in liver suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanisms to prevent formation of excessive free radicals. Treatment with MECA significantly reversed these changes. Hence it may be possible that the mechanism of hepatoprotection of MECA is due to its antioxidant effect.

Biological systems protect themselves against the damaging effects of activated species by several means. These include free radical scavengers and chain reaction terminators; enzymes such as SOD, CAT and GPx system[40]. The SOD dismutates superoxide radicals O_2- into H_2O_2 plus O_2, thus participating, with other antioxidant enzymes, in the enzymatic defense against oxygen toxicity. In this study, SOD plays an important role in the elimination of ROS derived from the peroxidative process of xenobiotics in liver tissues. The observed increase of SOD activity suggests that the MECA have an efficient protective mechanism in response to ROS. And also, these findings indicate that MECA may be associated with decreased oxidative stress and free radical-mediated tissue damage.

CAT is a key component of the antioxidant defense system. Inhibition of these protective mechanisms results in enhanced sensitivity to free radical-induced cellular damage. Excessive production of free radicals may result in alterations in the biological activity of cellular macromolecules. Therefore the reduction in the activity of these enzymes may result in a number of deleterious effects due to the accumulation of superoxide radicals and hydrogen peroxide. Administration of MECA increases the activities of catalase in CCl_4 induced liver damage rats to prevent the accumulation of excessive free radicals and protects the liver from CCl_4 intoxication.

GSH is a naturally occuring substance that is abundant in many living creatures. It is widely known that a deficiency of GSH within living organisms can lead to tissue disorder and injury. For example, liver injury included by consuming alcohol or by taking drugs like acetaminophen, lung injury by smoking and muscle injury by intense physical activity[41], all are known to be correlated with low tissue levels of GSH. From this point of view, exogeneous MECA supplementation might provide a mean of recover reduced GSH levels and to prevent tissue disorders and injuries. The present study, we have demonstrated the effectiveness of MECA by using CCl_4 induced rats, which are known models for both hepatic GSH depletion and injury.

The availability of vitamin C is a determined factor in controlling and potentiating many aspects of host resistance to cancer. The decreased level of vitamin C was found CCl_4 control animals. Vitamin C can protect cell membranes and lipoprotein particles from oxidative damage by regenerating the antioxidant form of vitamin E[42]. Thus, vitamin C and E act synergistically in scavenging a wide variety of ROS. The recoupment of vitamin C to near normal level in drug treated rats was found to be due to the potent antioxidant activity of bark extract which may induce the regeneration of ascorbic acid. Vitamin E is the most significant antioxidant of its kind in animal cells and it can protect against chemical carcinogenesis and tumor growth[43]. Significantly decreased vitamin E levels in CCl_4 control animals might be due to the excessive utilization of this antioxidant for quenching enormous free radicals produced in these conditions. The increased level of vitamin E in drug treated rats reveals the antioxidative nature of the MECA. The extract may scavenge the free radicals and thus maintains the normal level of vitamin E.

It has been reported that *Careya arborea* contain flavonoid and triterpenoid[6,7]. A number of scientific reports indicated certain flavonoids, triterpenoids and steroids have protective effect on liver due to its antioxidant properties[20,21]. Presence of those compounds in MECA may be responsible for the protective effect on CCl_4 induced liver damage in rats.

In conclusion, the results of this study demonstrate that MECA has a potent hepatoprotective action upon carbon tetrachloride-induced hepatic damage in rats. Our results show that the hepatoprotective and antioxidant effects of MECA may be due to its antioxidant and free radical scavenging properties. Further, investigation is underway to determine the exact phytoconstituents that is responsible for its hepatoprotective and antioxidant activity.

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