Research Article

Hepatoprotective Effect of *Eugenia singampattiana* Bedd Leaf Extract on Carbon Tetrachloride Induced Jaundice

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Accepted on: 03-04-2013; **Finalized on:** 30-06-2013.

ABSTRACT

The aim of the study is to investigate the hepatoprotective activity of ethanol extract of leaf of Eugenia singampattiana in CCl_4 induced hepatoprotective rats. Administration of hepatotoxins (CCl_4) showed significant elevation of serum GOT, GPT, ALP, ACP, LDH, bilirubin, conjugated, unconjugated and lipid peroxidation. Treatment with E. singampattiana (100 and 200 mg/kg) significantly reduced the above mentioned parameters. The plant extract also enhanced the antioxidant activity. The ethanol extract of E. singampattiana have significant effect on the CCl_4 induced hepatotoxicity animal models.

Keywords: Bilirubin, CCl_4, Hepatotoxicity, MDA.

INTRODUCTION

Liver plays major role in detoxification and excretion of many endogenous and exogenous compounds, any injury to it or impairment of its functions may lead to many implications on one's health. Liver disease is still a worldwide health problem. Unfortunately, conventional or synthetic drugs used in the treatment of liver diseases are inadequate and sometimes can have serious side effects, whereas; herbs play a role in the management of various liver diseases. Many fold remedies from plant origin have been long used for the treatment of liver diseases. This is one of the reasons for many people in the world over including those in developed countries turning complementary and alternative medicine. Many traditional remedies employ herbal drugs for the treatment of liver ailments.[1, 2]

Carbon tetrachloride (CCl_4) is one of the most commonly used hepatotoxins in the experimental study of liver diseases. The hepatotoxic effect of CCl_4 is largely due to its active metabolite, trichloromethyl radical.[3] The administration of CCl_4 in rats enhances hepatic protein oxidation and results in the accumulation of CCl_4 oxidized proteins in the liver.[4] The present study was conducted to evaluate the hepatoprotective effect of the ethanol extract of leaves of Eugenia singampattiana on carbon tetrachloride induced liver damage in experimental rats.

E. singampattiana Bedd belong to the family Myrtaceae. It is commonly known as "Kattukorandi" by Kanikkar tribals of Agasthiarmalai, Biosphere Reserve, Western Ghats, Tamil Nadu, India. The paste prepared from the leaf of E. singampattiana is given to treat asthma and giddiness. Paste prepared from equal quantity of leaves and flowers is consumed by Kanikkar tribals to cure body pain and throat pain. Paste prepared from equal quantity of leaves, flowers and tender fruits are consumed by the Kanikkars to relief from leg sores and rheumatism. Paste prepared from equal quantity of stems, leaves and flowers is consumed with palm sugar to get relief from gastric complaints.[5] E. singampattiana leaf extracts were reported for the biological activities such as antitumor, anti diabetic, anti hyperlipidaemic and in vitro antioxidant activity.[6-8] Since there are no particular reports on hepatoprotective activity of leaves of the plant, it was considered worthwhile to evaluate the leaves for hepatoprotective activity.

MATERIALS AND METHODS

Plant Material

The leaves of Eugenia singampattiana Bedd were freshly collected from the well grown healthy plants inhabiting the natural forests of Karaiyar, Agasthiarmalai Biosphere Reserve, Western Ghats, Tamil Nadu. The plant were identified and authenticated in Botanical Survey of India, Southern Circle, Coimbatore, Tamil Nadu, India. A voucher specimen was deposited in Ethnopharmacology Unit, Research Department of Botany, V.O.Chidambaram College, Tuticorin, Tamil Nadu.

Preparation of plant extract for phytochemical screening and hepatoprotective studies

The aerial part of the plant was dried under shade and then powdered with a mechanical grinder to obtain a coarse powder, which was then subjected to extraction in a Soxhlet apparatus using ethanol. The extract was subjected to qualitative test for the identification of various phytochemical constituents as per standard procedures.[9-11] The ethanol extracts was concentrated in a rotary evaporator. The concentrated ethanol extract were used for hepatoprotective studies.

Animals

Normal healthy male Wistar albino rats (180-240g) were used for the present investigation. Animals were housed under standard environmental conditions at room temperature (25±2°C) and light and dark (12:12h). Rats

were fed with standard pellet diet (Goldmohur brand, MS Hindustan lever Ltd., Mumbai, India) and water ad libitum.

Acute Toxicity Studies

Acute oral toxicity study was performed as per OECD-423 guidelines (acute toxic class method), albino rats (n=6) of either sex selected by random sampling were used for acute toxicity study.[12] The animals were kept fasting for overnight and provided only with water, after which the extracts were administered orally at 5mg/kg body weight by gastric intubations and observed for 14 days. If mortality was observed in two out of three animals, then the dose administered was assigned as toxic dose. If mortality was observed in one animal, then the same dose was repeated again to confirm the toxic dose. If mortality was not observed, the procedure was repeated for higher doses such as 50, 100 and 2000 mg/kg body weight.

Experimental Design

In the investigation, a total of 25 rats (20 CCl_4 hepatic toxicity induced rats and 5 normal rats) were taken and divided into five groups of 5 rats each.

Group I: Rats received normal saline was served as a normal control.

Group II: CCl_4 hepatic toxicity induced control: Rats received 2.5ml/kg body weight of CCl_4 for 14 days.

Group III: Liver injured rats received ethanol extract of leaf of E. singampattiana at the dose of 100mg/kg body weight for 14 days.

Group IV: Liver injured rats received ethanol extract of leaf of E. singampattiana at the dose of 200mg/kg body weight for 14 days.

Group V: Liver injured rats received standard drug silymarin at the dose of 100mg/kg body weight for 14 days.

Biochemical Analysis

The animals were sacrificed at the end of experimental period of 7 days by decapitation. Blood was collected, sera separated by centrifugation at 3000g for 10 minutes. Serum protein[13] and serum albumins was determined quantitatively by colorimetric method using bromocresol green. The total protein minus the albumin gives the globulin. Serum glutamate pyruvate transaminase (SGPT) and serum glutamate oxaloacetate transaminase (SGOT) was measured spectrophotometrically by using the method of Reitman and Frankel.[14] Serum alkaline phosphatase (ALP) and serum acid phosphatase (ACP) measured by the method of King and Armstrong.[15] Lactate dehydrogenase (LDH) was determined by the method of Mercer.[16] Total bilirubin and conjugated bilirubin were determined as described by Balistrei and Shaw.[17] The unconjugated bilirubin concentrations were calculated as the difference between total and conjugated bilirubin concentrations. Liver homogenates (10%W/V) were prepared in ice cold 10mM tris buffer (pH7.4). Quantitative estimation of MDA formation was done by determining the concentration of thiobarbituric acid reactive substances (TBARS) in plasma by the method of Satoh.[18] Enzymatic antioxidants, superoxide dismutase (SOD)[19] and glutathione peroxidase (GPx)[20] were also assayed in erythrocytes.

Statistical Analysis

The data were expressed as the mean ± S.E.M. The difference among the means has been analyzed by one-way ANOVA. $p<0.01$ and $p<0.01$ were considered as statistical significance using SPSS Software.

RESULTS AND DISCUSSION

Liver is the largest organ and it is the target for toxicity because of its role in clearing and metabolizing chemicals through the process called detoxification.[21] Drug induced liver disorders occurred frequently can be life threatening and mimic all forms of liver diseases.[22] CCl_4 is one of the most commonly used hepatotoxin. CCl_4 produces an experimental damage that histological resembles viral hepatitis. Toxicity begins with the change in endoplasmic reticulum which results in the loss of metabolic enzymes located in the intracellular structures.[23] The toxic metabolic, CCl_3 radical is produced and further reacts with oxygen to give trichloro-methyl peroxy radical. Cytochrome P450 is the enzyme responsible for this conversion. Both the radicals can bind covalently to the macromolecules and induce peroxidative degradation of the membrane lipid of endoplasmic reticulum rich in polyunsaturated fatty acids.[23] This leads to the formation of lipid peroxidases followed by pathological changes such as depression of protein synthesis, elevation levels of serum marker enzymes such as SGOT, SGPT, ALP, ACP and LDH depletion of GSH-Px and SOD and increase in lipid peroxidation.

The ethanol extract of leaf of E. singampattiana subjected for phytochemical screening showed the presence of alkaloids, coumarin, glycosides, flavonoids, saponins, terpenoids, steroids, phenols, tannins and xanthoproteins. The ethanol extract did not show any sign and symptoms of toxicity and mortality upto 200 mg/kg dose. The effect of ethanol extract of E. singampattiana on serum transaminases, alkaline phosphatase, acid phosphatase and lactate dehydrogenase in CCl_4 intoxicated rats are summarized in Table 1. There was a significant ($p<0.01$) increase in serum GOT, GPT, ALP, ACP and LDH levels in CCl_4 intoxicated group (Group II) compared to the normal control group (Group I). Ethanol extract of E. singampattiana at a dose of 100 and 200 mg/kg orally administered significantly decreased the elevated serum marker enzymes. The total protein and albumin levels were significantly ($p<0.05$) decreased to 5.12 g/dl and 3.22 g/dl in CCl_4 intoxicated rats from the levels of 7.78 g/dl and 4.05 g/dl respectively in normal rats (Table 2).

Table 1: Effect of ethanol extract of Eugenia singampattiana leaves on the enzyme activity of serum GOT, GPT, ALP, ACP and LDH on the CCl_4 induced liver injured albino rats

Treatment group	Hepatic Marker Enzyme				
	GOT(U/L)	GPT(U/L)	ALP (U/L)	ACP (U/L)	LDH(U/L)
Group – I	36.34 ± 2.87	41.56 ± 4.78	165.67 ± 7.39	143.89 ± 5.89	94.56 ± 4.90
Group – II	116.45 ± 6.45**	136.54 ± 5.91**	221.34 ± 11.98**	245.67 ± 10.67**	134.45 ± 4.38*
Group – III	69.43 ± 3.23*	89.54 ± 3.45*	189.45 ± 6.12*	199.56 ± 7.54**	121.77 ±7.83^{NS}
Group – IV	54.33 ± 2.36^{aa}	49.48 ±3.14^{aa}	178.35 ± 5.76^{aa}	152.39 ± 4.78^{aa}	98.34 ± 3.26^{aa}
Group – V	35.99 ± 1.49^{aa}	43.21 ± 2.81^{aa}	173.56 ± 6.56^{aa}	148.79 ± 5.66^{aa}	92.56 ± 3.65^{a}

Each value is SEM ± 5 individual observations; * p < 0.05 ; **p<0.01 compared with normal control vs. liver injured rats and drug treated rats; a p < 0.05; aa p<0.01 compared with liver injured rats vs. drug treated rats; NS- Not Significant.

Table 2: Effect of ethanol extract of Eugenia singampattiana leaves on the concentration of total bilirubin, conjugated bilirubin and un conjugated bilirubin in the serum of CCl_4 induced liver injured albino rats

Treatment group	Parameters		
	Total Bilirubin (µmol/L)	Conjugated (µmol/L)	Un conjugated (µmol/L)
Group – I	0.88 ± 0.02	0.20 ± 0.01	0.68 ± 0.03
Group – II	5.78 ± 1.89**	1.46 ± 0.02*	4.32 ± 1.23*
Group – III	2.99 ± 0.37*	1.07 ± 0.04*	1.92 ± 0.12^{NS}
Group – IV	1.08 ± 0.48^{aa}	0.32 ± 0.03^{a}	0.76 ± 0.06^{aa}
Group – V	1.05 ± 0.80^{aa}	0.22 ± 0.03^{a}	0.83 ±0.05^{aa}

Each value is SEM ± 5 individual observations; * p < 0.05; ** p<0.01 compared with normal control vs. liver injured rats and drug treated rats; a p < 0.05; aa p<0.01 compared with liver injured rats vs. drug treated rats; NS- Not Significant.

Administration of ethanol extracts of E. singampattiana leaf reversed the altered protein and albumin to almost normal level.

In the present study, it was observed that, the rats treated with CCl_4 resulted in significant hepatic damage as shown by the elevated levels of serum markers. These changes in the marker levels will reflect in hepatic structural integrity. The rise in the SGOT is usually accompanied by an elevation in the SGPT which play a vital role in the conversion of amino acids to keto acids.[24] Ethanol extract of E. singampattiana leaf at doses of 100 mg/kg and 200 mg/kg significantly attenuated the levels of the serum markers. The normalization of serum markers by ethanol extract of E. singampattiana suggests that they are able to condition the hepatocytes so as to protect the membrane integrity against CCl_4 induced leakage of marker enzymes into the circulation. The above changes can be considered as an expression of the functional improvements of hepatocytes.

Alkaline phosphatase concentration is related to the functioning of hepatocytes, high level of alkaline phosphatase in the blood serum is related to the increased synthesis of its by cells lining bile canaliculi usually in response to cholestasis and increased biliary pressure25. Increased level was obtained after CCl_4 administration and it was brought to near normal level by E. singampattiana treatment.

Lactate dehydrogenase is localized in the cytoplasm of cells and thus is extruded into the serum when cells are damaged or necrotic. The measurement of total lactate dehydrogenase can be useful when only a specific organ, such as the liver is known to be involved. Lactate dehydrogenase is increased in acute necrosis of the liver. Lactate dehydrogenase is a sensitive intracellular enzyme which increase in serum is also an indication of liver cell damage.[26]

Protein metabolism is a major project of liver and a healthy functioning liver is required for the synthesis of the serum proteins. Hypoproteinemia is a feature of liver damage due to significant fall in protein synthesis. Albumin is decreased in chronic liver disease. Hypoproteinemia was observed after CCl_4 ingestion but the trend turns towards normal after E. sigampattiana treatment.

The effect of ethanol extract of E. singampattiana on total, conjugated and unconjugated bilirubin is shown in Table 3. A significant elevation of total, conjugated and unconjugated bilirubin in the serum of CCl_4 intoxicated group (Group II) were observed when compared to normal control (Group I). The ethanol extract of E. singampattiana at the dose of 100 and 200 mg/kg reduced the levels of total, conjugated and unconjugated bilirubin.

Bilirubin is a yellow pigment produced when haeme is catabolized. Hepatocytes render bilirubin water soluble and therefore easily excretable by conjugating it with glucuronic acid prior to secreting it into bile by active transport. Hyperbilirubinemia may result from the production of more bilirubin than the liver can process, damage to the liver impairing its ability to excrete normal amount of bilirubin or obstruction of excretory ducts of the liver.[27] Serum bilirubin is considered as one of the

true test of liver functions since it reflects the ability of liver to take up and process bilirubin into bile. Elevated levels may indicate several illnesses. High levels of total bilirubin in CCl_4 treated rats may be due to CCl_4 toxicity. This may have resulted in hyperbilirubinemia. The significant reduction in the level of total bilirubin in the serum of E. singamapttiana leaf extract treated rats suggested the hepatoprotective potential of leaf extract against CCl_4 intoxication.

Table 4 showed the levels of plasma MDA and erythrocyte GSH-Px and SOD. CCl_4 treated rats had an elevated level of MDA and decreased the level of GSH-Px and SOD as compared to normal control rats. Rats treated with ethanol extract of E. singampattiana at the doses of 100 and 200 mg/kg significantly ($p<0.01$) decreased the elevated lipid peroxidation levels and restored the altered glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD).

Table 3: Effect of ethanol extract of Eugenia singampattiana leaves on the concentration of Total Protein, Albumin, Globulin in the serum of CCl_4 liver injured albino rats

Treatment group	Parameters			
	T. Protein (mg/dl)	Albumin (g/dl)	Globulin(g/dl)	A/G Ratio
Group – I	7.78 ± 0.2	4.05 ± 0.3	3.73 ± 0.2	1.08:1
Group – II	5.12 ± 0.4*	3.22 ± 0.4*	1.90 ± 0.1*	1.7 :1
Group – III	7.01 ± 0.2	4.26 ± 0.6	2.75 ± 0.5	1.25:1
Group – IV	7.48 ± 0.1[a]	4.09 ± 0.4[a]	3.39 ± 0.4[a]	1:18:1
Group – V	7.46 ± 0.3[a]	4.31 ± 0.4[a]	3.15 ± 0.6[a]	1.22:1

Each value is SEM ± 5 individual observations; * $p < 0.05$; **$p<0.01$ compared with normal control vs. liver injured rats and drug treated rats; a $p < 0.05$; aa $p<0.01$ compared with liver injured rats vs. drug treated rats; NS- Not Significant.

Table 4: Effect of ethanol extract of Eugenia singampattiana leaves on plasma MDA and erythrocyte GSH-Px and SOD levels in the serum of CCl4 induced liver injured albino rats

Treatment group	Parameters		
	MDA (nmol/ml)	GSH-Px (U/g Hb)	SOD (U/gHb)
Group – I	3.15 ± 0.51	58.67 ± 4.86	2654.87 ± 288.67
Group – II	8.56 ± 0.76*	23.58 ± 2.61*	1245.54 ± 298.44**
Group - III	3.66 ± 0.46[a]	55.45 ± 1.78[a]	2438.48 ± 178.36[aa]
Group – IV	4.01 ± 0.56	49.16 ± 2.69*	2344.90 ± 266.78*
Group – V	3.56 ± 0.39[a]	54.754 ± 3.57[a]	2113.67 ± 278.44[aa]

Each value is SEM ± 5 individual observations; * $p < 0.05$; **$p<0.01$ compared with normal control vs. liver injured rats and drug treated rats; a $p < 0.05$; aa $p<0.01$ compared with liver injured rats vs. drug treated rats; NS- Not Significant.

Lipid peroxidation has been postulated to destructive process of liver injury due to CCl_4 administration. In the present study, the elevations in the levels of end products of lipid peroxidation in the liver of rat treated with CCl_4 were observed. The increase in malondialdehyde (MDA) levels 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 ethanol extract of E. singampattiana significantly reversed these changes. Hence, it may be possible that the mechanism of hepatoprotection by ethanol extract of E. singampattiana is due to its antioxidant effect.

Glutathione peroxidase (GSH-Px) is a seleno enzyme two third of which is present in the cytosol and one third in the mitochondria. It protects cells from damage due to free radicals like hydrogen and lipid peroxides.[28] It catalyses the reaction of hydroperoxidases with reduced glutathione to form glutathione disulphide and the reduction product of hydroperoxide. In the present study, decline in activity of glutathione peroxidase has been associated with oxidative stress elicited of E. singampattiana leaves significantly increased the level of glutathione peroxidase in a dose dependent manner.

Superoxide dismutase (SOD), a metallo protein is the most sensitive enzyme in the enzymatic antioxidant defense system. It scavenges the superoxide anion to form hydroxide peroxide and oxygen, hence diminishing the toxic effect caused by this radical.[29] In the present study, it was observed that, the ethanol extract of E. singampattiana leaves significantly increased SOD activity in CCl_4 intoxicated rats thereby diminished CCl_4 induced oxidative damage.

CONCLUSION

In conclusion, the results of this study demonstrate that, the ethanol extract of E. singampattiana leaves have a potent hepatoprotective action against CCl_4 induced hepatic damage in rats. It's mode in affording the hepatoprotective activity against CCl_4 induced liver damage may be due to cell membrane stabilization, hepatic cells regeneration and enhancement of antioxidant enzymes. The hepatoprotective and antioxidant potential of leaf extract could have been brought about by various phytochemical principles i.e.,

flavonoids, alkaloids, phenolics and tannins present in E. singampattiana leaf. So results of this study demonstrated that the E. singampattiana has significant protection on CCl_4 induced hepatotoxicity.

Acknowledgement: Thanks to Dr. Sampathraj, Honorary Advisor, Samsun Clinical Research Laboratory, Tirupur for their assistance in animal studies. The last two authors are thankful to University Grants Commission – New Delhi, for their financial support (Ref. No: 39-429/2010(SR) dated 7th JAN 2011).

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Source of Support: Nil, **Conflict of Interest:** None.