Doi: 10.1111/j.1742-7843.2009.00429.x
Journal compilation © 2009 Nordic Pharmacological Society. *Basic & Clinical Pharmacology & Toxicology*, **105**, 243–248

Effects of Embelin on Lipid Peroxidation and Free Radical Scavenging Activity against Liver Damage in Rats

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(Received 17 October 2008; Accepted 2 March 2009)

Abstract: The study aimed to evaluate the hepatic antioxidant capacity of embelin (from *Embelia ribes*) using different antioxidant tests, free radical scavenging activity and lipid peroxidation in albino rats. Carbon tetrachloride (CCl_4) treatment to rats has been more susceptible to peroxidative damage through production of reactive metabolites, namely trichloromethyl-free radicals ($CCl^{\bullet}_3$ and/or $CCl_3OO^{\bullet}$) as measured by thiobarbituric acid reactive species. After the induction of liver damage by CCl_4 intoxication to rats, the concentration of lipid peroxidation was significantly ($P \leq 0.001$) higher in liver and serum, along with concomitant decrease in the levels of antioxidants and cytochrome P450 enzyme in liver as compared to vehicle controls. The activities of marker enzymes – transaminases (AST, ALT), alkaline phosphatase (ALP), γ-glutamyl transpeptidase (GGT), lactate dehydrogenase (LDH) – along with the total bilirubin and total protein levels were altered significantly ($P \leq 0.001$) in the serum of CCl_4-treated rats. When these rats received embelin orally (25 mg/kg) from day 1 to day 15, peroxidative damage was minimal in both liver and serum along with effectively inducing the antioxidant potential in CCl_4-treated rats. The biochemical results were compared with the standard drug silymarin – a combination of flavonolignans of *Silybum marianum* and histology of liver sections. In conclusion, this study suggests that embelin acts as a natural antioxidant against hepatotoxicity induced in rats.

Lipid peroxidation (LPO) is viewed as a complicated biochemical reaction involving free radicals, oxygen, metal ions and a host of other factors in the biological system [1]. Therefore, LPO is the focus of intense activity in relation to its possible involvement in health and disease [2]. Antioxidants and free radical scavengers have been employed to study the mechanism of CCl_4 toxicity as well as to protect liver cells from CCl_4-induced damage by breaking the chain reaction of LPO [3]. Therefore, the search for modern medicine from plants with this property has become a central focus of hepatoprotection today [4].

Embelia ribes (Myrsinaceae) is commonly called 'Vidanga'. It is a large shrub which is found in the hilly parts of India from the central and lower Himalayas down to Sri Lanka and Singapore. Experimentally, *E. ribes* has been reported as a potential antioxidant in diabetic animals [5,6]. Its dried berries (seeds) have a bitter taste and are used in the cure of tumours, ascites, bronchitis, mental diseases, dyspnoea, heart diseases, urinary discharges, snake bites and jaundice in the indigenous system of medicine as well as in Ayurveda [7].

We isolated the main active component embelin (embelic acid) 2,5-dihydroxy-3-undecyl-1,4-benzoquinone from the chloroform extract of *E. ribes* seeds. Furthermore, various pharmacological activities of embelin have been conducted in mammals and cell lines like antitumour, anti-inflammatory and analgesic activity [8], anticancer activity [9], chemopreventive [10] and antioxidant activity by DPPH method [11]. However, no attention has been paid to its hepatoprotective action against peroxidative damage through antioxidant status via LPO reduction in mammals. Hence, the present study aimed to establish the antioxidant potential of embelin using CCl_4-induced peroxidative damage in liver of albino rats.

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Materials and Methods

Animals. Adult, male, Wistar strain, albino rats weighing 150–170 g were used. The animals were housed in standard laboratory conditions and maintained on rat diet (Lipton India Ltd.) and tap water *ad libitum* under a natural light–dark (12 L:12D) cycle.

Plant material. The seeds (dried) of *E. ribes* were provided by an Ayurvedic shop No. 210, Chhoti Chopad market, Jaipur, Rajasthan (India) and authenticated by Professor N.J. Sarana, Department of Botany, University of Rajasthan, Jaipur, India, where a voucher specimen (leaves) has been preserved for future identification (Herbarium Sheet No. – RUBL 19101).

Isolation of embelin. The dried seeds of *E. ribes* were grinded to powder and extracted with petroleum ether for 48 hr at 58–60°. The filtrate obtained was concentrated under reduced pressure and yielded a dark brown semi solid mass. This extract was washed with petroleum ether to remove the fatty portion. The fat-free extract was then extracted with chloroform, where 30 g of crude extract was obtained which was subjected to column chromatography. For this purpose, a column (1.2 m × 5 cm) filled with Si-gel (600 gm) was used. The column was eluted with petroleum ether and benzene (2:3) to afford embelin, which was recrystallized as shiny orange

crystals at a melting point of 144°. The structure was confirmed by ^{1}H NMR and mass spectrometric data [12]. In the experimentation, embelin crystals were dissolved in olive oil for oral dosage given to the rats.

Chemicals. All chemicals and reagents used were of analytical grade and obtained from Sigma Chemicals Company (St. Louis, MO). The kits of SGOT, SGPT (batch no. 61105 and 60805), γ-glutamyl transpeptidase (GGT; batch no. 34004) were purchased from Accurex Biomedical Pvt. Ltd., Mumbai, India and lactate dehydrogenase (LDH; lot no. 6854), alkaline phosphatase (ALP; lot no. 7093), total bilirubin (lot no. 6801) and total protein (lot no. 6808) were purchased from Span Diagnostics Ltd., Surat, India. The standard drug silymarin was purchased from German Remedies Ltd., Mumbai.

Behavioural and toxic effects. Embelin was administered to the test groups in graded doses ranging up to 100 mg/kg body weight/day and the rats were observed for any signs of mortality and behavioural disabilities for 15 days afterwards. Its LD_{50} value was found to be higher than 100 mg/kg body-weight in rats (data not shown). The minimum dose level of embelin viz. 25 mg/kg body weight/day, was used for the further experimentation.

Experimental design. After 7 days of acclimatization, the animals were divided into the following groups of six rats each:

Group I: Vehicle-treated rats were kept on normal diet and served as control.

Group II: Rats were intoxicated with carbon tetrachloride (1 ml/kg body weight once a week, with olive oil, intra-peritoneally, 1:1) for 15 days.

Group III: Rats received oral embelin (25 mg/kg body-weight/day) dissolved in olive oil and CCl_4 as group II, for 15 days.

Group IV: Rats received oral silymarin (25 mg/kg body-weight/day) dissolved in olive oil and CCl_4 as group II, for 15 days.

Biochemical analysis. Twenty-four hours after last dose delivery, all rats of each treated group were autopsied and blood was collected by cardiac puncture. Serum was separated by centrifugation at 1327 ×g at 37° for 20 min. and analysed for AST, ALT (transaminases), ALP, GGT, LDH, total bilirubin and total protein, using diagnostic kits. After the collection of blood, the liver was immediately excised, washed with cold saline, blotted and a part of it was minced and homogenized for superoxide dismutase (SOD) [13], catalase (CAT) [14], reduced glutathione (GSH) [15], glutathione peroxidase (GPx) [16], glutathione-s-transferase (GST) [17], LPO [18] determination. Then, a liver microsomal fraction was prepared [19] and the cytochrome P450 enzyme content in this fraction was measured from a reduced carbon monoxide difference spectrum [20], respectively.

Histology of liver. The liver tissue was dissected out and fixed in 10% formalin, dehydrated in gradual ethanol (50–100%), cleared in xylene and embedded in paraffin wax. Then, it was cut into 5 μm sections and stained with haematoxylin–eosin dye for photomicroscopic observations of hepatocytes of treated groups through light microscopy.

Ethical aspects. The study was approved by the ethical committee of the University Department of Zoology, Jaipur, India. Indian National Science Academy, New Delhi, (INSA, 2000) guidelines were followed for maintenance and use of the experimental animals.

Statistical analysis. Statistical analysis was performed using one-way ANOVA followed by Student's *t*-test. The values are mean ± S.E. for six rats in each group. A single P value ≤ 0.001 was considered significant.

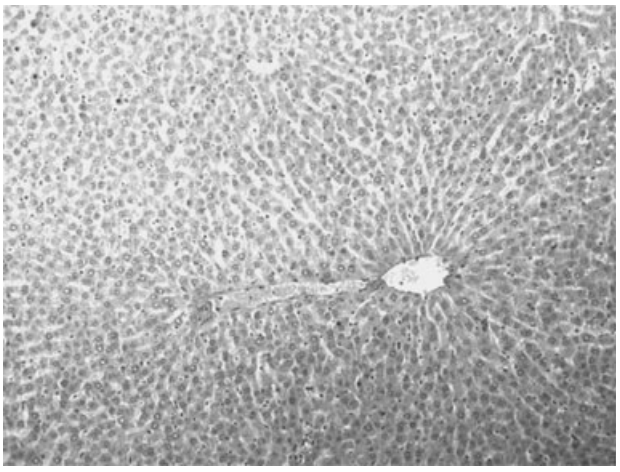

Fig. 1. Photomicrograph of control rat liver section showing well-brought central vein, hepatic cells with preserved cytoplasm and prominent nucleus at haematoxylin and eosin ×100.

Results

The results of biochemical parameters revealed that the administration of CCl_4 to rats caused significant ($P \leq 0.001$) peroxidative damage as evidenced by marker enzymes and antioxidant defence system through liver and serum contents (tables 1 and 2 and figs 1–4).

Rats treated with CCl_4, developed a significant ($P \leq 0.001$) elevation of LPO in both liver and serum contents (tables 1 and 2). In contrast, treatment with embelin (25 mg/kg) and silymarin (25 mg/kg) showed a significant ($P \leq 0.001$) lowering effect on CCl_4-induced elevation of LPO in both liver and serum contents (tables 1 and 2). The depletion of LPO by embelin was statistically similar in nature with the standard drug silymarin.

Table 1 depicts that the activities of hepatic antioxidants such as SOD, CAT, GSH, GPx, GST and enzyme – cytochrome

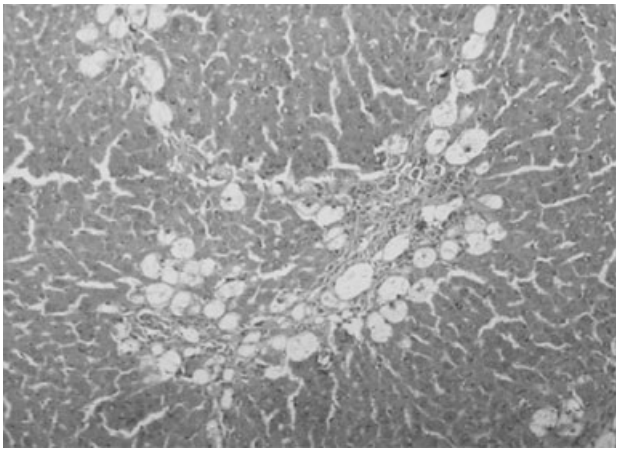

Fig. 2. Photomicrograph of rat liver section with CCl_4 treatment showing marked steatosis of the hepatocytes with ballooning degeneration and distended portal vein, mild periportal fibrosis and necrosis at haematoxylin and eosin ×100.

Table 1.

Effects of embelin and silymarin against oxidative damage in rats through liver parameters.

Treatment design	SOD (μ mole/mg protein)	CAT (μ mole H_2O_2 consumed/min/mg protein)	GSH (n mole/g tissue)	GPx (n mole NADPH consumed/min/mg protein)	GST (n mole CDNB-GSH conjugate formed/min/mg protein)	Cytochrome-*P*-450 (n mole/mg protein)	LPO (n mole MDA/mg protein)
Control (vehicle treated) Group I	12.28 ± 0.44	65.32 ± 2.87	5.22 ± 0.27	15.39 ± 0.26	8.59 ± 0.85	5.22 ± 0.18	1.92 ± 0.08
CCl_4 (1 ml/kg b. wt, ip, once a week with olive oil, 1:1) Group II	5.82 ± 0.16*	36.10 ± 1.98*	2.32 ± 0.13*	8.42 ± 0.19*	3.42 ± 0.26*	2.08 ± 0.14*	5.21 ± 0.19*
CCl_4 + Embelin (25 mg/kg b. wt/day, orally) Group III	10.12 ± 0.22†	56.21 ± 2.32†	4.93 ± 0.32†	12.79 ± 0.38†	6.78 ± 0.42†	4.10 ± 0.12†	3.08 ± 0.10†
CCl_4 + Silymarin (25 mg/kg b. wt/day, orally) Group IV	11.68 ± 0.32†, NS	61.21 ± 2.12†, NS	6.30 ± 0.34†, NS	14.82 ± 0.28†, NS	7.12 ± 0.24†, NS	4.89 ± 0.15†, NS	2.53 ± 0.12†, NS

Levels of significance: Data are mean ± S.E.M. (n = 6).
*P ≤ 0.001 Group II compared to Group I.
†P ≤ 0.001 Group III and IV compared to Group II.
NS, non-significant Group IV compared to Group III.

Fig. 3. Photomicrograph of rat liver section of CCl_4 + Embelin (25 mg/kg b. wt), showing moderately brought central vein, hepatic cells with preserved cytoplasm and prominent nucleus at haematoxylin and eosin ×100.

P450 were declined significantly (P ≤ 0.001) upon CCl_4 administration alone to rats (group II) when compared with group I (vehicle control). Oral administration of embelin as well as silymarin, at the dose of 25 mg/kg, showed a significant (P ≤ 0.001) elevating effect on CCl_4-induced depleted levels of hepatic antioxidants (groups III and IV). The amelioration of hepatic oxidative damage by embelin at the dose of 25 mg/kg was statistically significant and showed equal protection as good as silymarin (25 mg/kg).

After carbon tetrachloride induction to rats, a significant (P ≤ 0.001) elevation in serum enzymatic activities of AST, ALT, ALP, LDH, GGT and total bilirubin along with concurrent decline in totalHH protein levels was shown in group II when compared with vehicle-treated controls (group I). In contrast, treatment with embelin and silymarin showed restoring effect on CCl_4-induced alterations in serum

Fig. 4. Photomicrograph of rat liver section of CCl_4 + Silymarin (25 mg/kg b. wt), showing considerable reduction in necrosis and fatty changes with pyknotic nuclei and cytoplasmic clearing at haematoxylin and eosin ×100.

Table 2.

Effects of embelin and silymarin against oxidative damage in rats through serum parameters.

Treatment design	SGOT (IU/l)	SGPT ((IU/l))	ALP (KAU)	GGT ((IU/l))	LDH (IU/l)	Total bilirubin (mg/100 ml)	Total protein (gm/dl)	LPO (n mole MDA/mg proteins)
Control (vehicle treated) Group I	128.21 ± 2.10	108.32 ± 2.88	21.30 ± 1.45	9.52 ± 0.93	84.15 ± 2.57	0.85 ± 0.06	6.21 ± 0.24	2.45 ± 0.22
CCl_4 (1 ml/kg b. wt, ip, once a week with olive oil, 1 : 1) Group II	206.14 ± 3.87*	187.27 ± 3.09*	35.10 ± 1.59*	28.16 ± 1.42*	142.22 ± 2.85*	1.79 ± 0.10*	3.14 ± 0.17*	8.12 ± 0.61*
CCl_4 + Embelin (25 mg/kg b. wt/day, orally) Group III	141.15 ± 2.42†	129.32 ± 2.89†	25.15 ± 1.18†	17.14 ± 1.17†	108.23 ± 2.33†	1.04 ± 0.09†	5.39 ± 0.19†	4.02 ± 0.29†
CCl_4 + Silymarin (25 mg/kg b. wt/day, orally) Group IV	120.21 ± 2.88†‡	119.18 ± 1.43†, NS	23.85 ± 0.66†, NS	12.30 ± 1.28†, NS	95.10 ± 1.52†‡	0.92 ± 0.08†, NS	6.88 ± 0.23†‡	3.08 ± 0.17†, NS

Levels of significance: Data are mean ± S.E.M. (n = 6).
*P ≤ 0.001 Group II compared to Group I.
†P ≤ 0.001 Group III and IV compared to Group II.
‡P ≤ 0.001; NS = non-significant Group IV compared to Group III.

levels of treated rats in groups III and IV (table 2). The restoration of enzymatic activities by embelin (group III) as well as silymarin (group IV) were significantly (P ≤ 0.001) similar against CCl_4-induced alterations in serum levels of treated groups.

A histopathological study of liver in CCl_4-treated rats showed a lot of vacuoles in the size changing from small to large, mononuclear cell infiltration, picnotic nuclei, the rupturing in the endothelium of some central vein in group II (fig. 2) compared with group I (fig. 1). The rats treated with embelin as well as silymarin at the dose of 25 mg/kg along with CCl_4, showed signs of significant protection against CCl_4 toxicity through considerable extent as evident from formation of normal hepatic cords and absence of necrosis and vacuoles in liver histology in groups III and IV (figs 3 and 4).

Discussion

Peroxidative damage by CCl_4 is the result of reductive dehalogenation, which is catalysed by P450 and forms the highly reactive trichloromethyl-free radical $CCl^{\bullet}_3$. This then readily interacts with molecular oxygen to form the trichloromethyl peroxy radical $CCl_3OO^{\bullet}$. Both radicals are capable of binding to proteins or lipids or of abstracting a hydrogen atom from an unsaturated lipid, which initiates LPO and liver damage and plays a significant role in the pathogenesis of diseases [21].

LPO damages cellular functions by compromising membrane function and by covalent binding to reactive intermediates formed during the CCl_4 intoxication [22]. Gupta *et al*. [23] have suggested that injured hepatic cells produce a substantial amount of hydrogen peroxide and reactive oxygen metabolites that are released into the circulation. Therefore, the increased susceptibility of serum and liver of CCl_4-treated rats could be due to the production of reactive oxygen metabolites during the metabolism of CCl_4. Treatment with embelin offered protection through attenuation of LPO and decreased production of free-radical derivatives, as evident from the decreased levels of serum TBARS. Thus, embelin offered protection to cells against oxidative stress by scavenging free radicals. Silymarin also significantly reduced LPO by its ability to inhibit oxidative damage [24].

The inhibitory effect of CCl_4 on cytochrome P450 level was also compensated by embelin as well as silymarin as it effectively neutralises the free radicals by making more water soluble substances and improving the protein synthesis in mammalian hepatocytes [3,25]. Although, cytochrome P450 is a haemoprotein and a host of enzymes that use iron to oxidise things and metabolise thousands of endogenous and exogenous compounds to dispose of potentially harmful substances by making them more water soluble [26]. Enzyme inhibition generally involves competition with toxicant for enzyme binding sites during toxins removal [27]. CCl_4-induced hepatotoxicity is one of the best documented cases for the participation of CYP2E1 in a toxicologically important

reductive reaction. The reductive reactions of the enzyme could be important for LPO [28].

During hepatic injury, superoxide radicals generate at the site of damage and modulate SOD and CAT, resulting in the loss of activity and accumulation of superoxide radical, which damages liver. Decreased CAT activity is linked to exhaustion of the enzyme as a result of oxidative stress caused by CCl_4. Reduction in hepatic GSH and decreased GPx activity in CCl_4-treated rats also observed in this study indicates the damage of hepatic cells. Administration of embelin as well as silymarin at the dose of 25 mg/kg significantly promoted the SOD and CAT activities and also converted the GSSG (oxidized glutathione) into GSH by the reactivation of hepatic glutathione reductase enzyme, with concomitant reversal of GPx activity to near normal level due to antioxidant potential by detoxifying the endogenous metabolic peroxides after CCl_4 injury in the liver of treated rats. The SOD, CAT and GPx activities with GSH concentration of the embelin-treated groups are in accordance with the report of Raja *et al*. [29]. GST plays a physiological role in initiating the detoxification of potential alkylating agents. However, CCl_4 alters the hepatic GST activity [30]. The GST level was significantly reduced in CCl_4-treated rats and upward reversal was observed after embelin (25 mg/kg) as well as silymarin (25 mg/kg) treatment. This may be attributed to a direct action of embelin on the hepatic GST activation.

The extent of hepatocellular damage by CCl_4 is assessed through most sensitive serum markers employed in the diagnosis in liver diseases [31]. Because of this, a variety of enzymes normally located on the cytosol are released into the blood stream [32]. In the assessment of hepatocellular damage, AST and ALT are widely used [33]. High levels of AST indicate that the liver damage might be due to toxicant induction as well as cardiac infection and muscle injury. Therefore, ALT is more specific to the liver and is thus a better parameter for detecting hepatocellular injury [34]. Increases in the serum level of ALP are due to increased synthesis in presence of increasing biliary pressure [35]. The measurement of GGT has been claimed to be an extremely sensitive test and marker of CCl_4-induced hepatocellular damage [36]. CCl_4 intoxication also has been linked with altered liver metabolism and liver damage, with leakage of cytoplasmic liver enzyme GGT into the blood stream [3]. LDH, a cytosolic enzyme is involved in biochemical regulation reaction of the body tissues and fluids. An elevation of LDH in the serum may indicate a shift towards anaerobiosis resulting in enhanced production of lactic acid, which may be a cause for the convulsions [37].

Oral treatment with embelin and silymarin attenuated these increased enzyme activities produced by CCl_4 and a subsequent recovery towards normalization of these enzymes strongly suggests the possibility of embelin and silymarin being able to improve the condition of hepatocytes so as to cause accelerated regeneration of parenchymal cells, thus protecting against membrane fragility and decreasing the leakage of marker enzymes into the circulation. Stabilization of serum-total bilirubin and total protein levels through the administration of embelin and silymarin to rats is further a clear indication of the improvement of functional status of the hepatic cells [38].

The hepatic antioxidant potential of embelin might be attributed to the presence of hydroxyl group at the ortho position. The *o*-hydroxyl group, because of its resonance property, easily donates e^- to free radicals and effectively neutralises them. Also, the presence of a hydroxyl group in the ortho position increases its antioxidant potential through intermolecular hydrogen bonding involving the –SH group of non-protein thiols and enzymes resulting in the restoration of the antioxidant system against peroxidative damage in liver tissue.

These findings were further confirmed by a comparative histoarchitectural examination of the liver sections from different groups of treated rats. CCl_4-treated rats may potentiate focal hepatocyte damage and degeneration (fig. 2). It is provoked by the increased production of a highly reactive intermediate of CCl_4 like trichloromethyl-free radical, which is normally detoxified by endogenous glutathione and enzymatic antioxidants but in excess it may deplete glutathione stores and the status of other antioxidants, allowing the reactive intermediate to react with and destroy the hepatic cells [39]. Due to embelin (25 mg/kg) treatment in CCl_4-treated rats, all these changes were very much reduced histopathologically. Therefore, the increased levels of GSH and other enzymatic antioxidant activities would be important in the protection against toxicity.

Conclusion

It can be concluded that the probable mechanism by which embelin exerts its protective action against CCl_4-induced hepatocellular metabolic alterations could be by the stimulation of hepatic regeneration through an improved synthesis of proteins, or due to its ability to block the bioactivation of CCl_4 by inhibiting the CYP2E1 activity and/or its accelerated detoxification and the potential to minimize the deleterious effects of free radicals including the peroxy radicals and its antioxidant activity in combination with the inhibition of LPO, thereby embelin can be ranked as a natural antioxidant.

Acknowledgements

The investigation was supported by the University Grant Commission, Regional Centre, Bhopal (M.P.) India. The authors are also thankful to the coordinator, CAS, Head, Department of Zoology, and Head, Department of Chemistry, University of Rajasthan, Jaipur, India for providing necessary facilities.

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