RESEARCH COMMUNICATION

Chemopreventive and Hepatoprotective Effects of Embelin on N-Nitrosodiethylamine and Carbon Tetrachloride Induced Preneoplasia and Toxicity in Rat Liver

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Abstract

Embelin, an active constituent isolated from the fruits of *Embelia tsjeriam*–cottam was investigated for its chemopreventive and hepatoprotective effects against N-nitrosodiethylamine (NDEA) induced liver preneoplasia or carbon tetrachloride (CCl_4) induced liver damage. Rats received NDEA, 1ppm/g b.w. in drinking water for 6 weeks or CCl_4, 0.7ml/kg i.p. once a week for 4 weeks and embelin 50mg, 100mg/kg b.w. orally prior, during and after exposure to NDEA/CCl_4 for 20 or 5 weeks, respectively. Embelin treatment significantly prevented NDEA or CCl_4 induced increase in biochemical marker enzymes: glutamate pyruvate transaminase, glutamate oxaloacetate transaminase, alkaline phosphatase, γ-glutamyl transpeptidase, glutathione-S-transferase, lipid peroxidase as well as hypoproteinemia, hypoalbuminuria and glutathione depletion. This was further substantiated by marked decrease in incidence of preneoplastic foci, and inflammatory cells on histopathological and transmission electron microscopic analysis. The present study suggests embelin is a promising chemopreventive and hepatoprotective agent.

Keywords: Embelin - chemoprevention - N-nitrosodiethylamine - hepatoprotective - carbon tetrachloride

Asian Pacific J Cancer Prev, **11**, 1015-1020

Introduction

Numerous epidemiological studies have shown high dietary intake of fruits and vegetables, containing phytochemicals such as carotenoids, antioxidative vitamins, phenolic compounds, flavones, terpenoids, steroids, isothiocyanates, indoles and fibres have been reported for reduction in cancer risk or prevent multistage carcinogenesis (Singletery et al., 2000; Nishino et al., 2005).

Embelin (2,5-dihydroxy-3-undecyl-1,4 - benzoquinone) is a naturally occurring alkyl benzoquinone, the active principle in the fruits of *Embelia tsjeriam*-cottam (syn. *E. robusta*) belonging to family *Myrsinaceae*. Fruits are used extensively in Ayurvedic system of medicine for the treatment of various diseases such as gastrointestinal disorders, dyspepsia, bronchitis, asthma, anaemia and skin diseases (Kirtikar and Basu, 1984; Sharma et al., 2002). Polyherbal formulations containing fruits of *Embelia* have been reported for treatment of various liver diseases like jaundice (Patki et al., 1990; Kothavade et al., 1996). Embelin has been shown to possess antihelminthic, analgesic, anti-inflammatory, antipyretic, wound healing and contraceptive properties (Gupta et al. 1977; Gupta et al., 1991; Kumara et al 2007).

Earlier reports have shown Embelin to possess antiproliferative activity in vitro, regression of fibrosarcoma in rats and chemopreventive activity against DENA induced hepatocarcinogenesis in Wistar rats based on preliminary liver function enzymes. The preneoplastic hepatic foci have been as end point models in assessing the toxic effects of hepatocarcinogens. Preneoplastic lesions have no obvious neoplastic nature, but have a high probability of progressing to a benign or malignant tumor in response to tumor promoters (Bannas ch, 1986; Cameron, 1989). In the present study we evaluated chemopreventive and hepatoprotective effects of Embelin isolated from fruits of *E. tsjeriam*-cottam in N-nitrosodiethylamine (NDEA) induced preneoplastic hepatic foci and cCarbon tetrachloride (CCl_4) induced liver damage in rat model and correlated for the first time biochemical alterations, antioxidant activity with histopathological changes and ultrastructural studies.

Materials and Methods

Isolation of Embelin

The fruits of Embelia tsjeriam-cottam were authenticated

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at National Institute of Science Communication and Information Resources (NISCAIR), New Delhi, India. Powdered fruits were extracted in n-hexane with a Soxhlet extractor for 36 hr. The extract was filtered and concentrated under reduced pressure below 50ºC on a rotary evaporator to give a crude extract. On cooling (0ºC) crude embelin precipitates out which was recrystallized using ice-cold absolute ethanol to give glistening orange crystals of pure embelin (mp. 141-142ºC, mol.wt. 294.39, yield 2 %). Reversed-phase HPLC fingerprint analysis of the major active constituent Embelin was carried out on Dionex-Ultimate 3000 HPLC system, USA equipped with Dionex-3000 PDA detector running Chromelon, Dionex software. The column used for HPLC was Acclaim C18 (25cm X 4.6mm, μm, Make - Dionex, USA) with Solvent A: 0.01 M KH2PO4 : MeOH (90:10)) and Solvent B: MeOH : 0.01 M KH_2PO_4 (90:10) composition in 70 : 30 ratio at a flow rate of 1ml/min, but following injection of the sample 20µl, a low pressure gradient system to 20 : 80 for 40 min was performed and the final mobile phase was maintained 70 : 30 for a further 15min. A single major peak of Embelin (Rt 6.72min) could be identified by comparison with standard Embelin (gifted by Dr. N.D. Grampurohit, Dr. Bhanuben Nanavati College of Pharmacy, Mumbai) and UV spectra (λmax 225nm) are superimposable indicating its purity (Figure 1).

Animals, carcinogen and chemicals

Male Swiss mice (25-30g), Wistar albino rats (180-200 g) (Haffkine Biopharmaceutical Ltd., Mumbai) and Sprague Dawley rats (7 to 8 weeks, ACTREC, Navi Mumbai) were maintained under standard controlled environmental conditions. Commercial pellet diet and water was given ad libitum. The experiment was carried out following the CPCSEA guidelines and approval obtained by the Institutional Animal Ethics Committee (IAEC) of ACTREC and R. Ruia College. NDEA (Sigma Chemicals, USA), CCl_4 (Qualigens, Mumbai) and all other reagents used were of analytical grade.

Experimental Design

(a) Acute toxicity study: 2 groups of Swiss mice containing 3 mice each were given 50 mg and 100 mg/kg of Embelin orally and kept under constant observation for 6 hr, 24 hr, 72 hr and then upto 14 days period. Body weight and mortality (if any) was recorded. Control group received only groundnut oil as the vehicle.

(b) Assessment of NDEA induced Preneoplasia: Sprague Dawley rats were divided into 5 groups containing 6 rats each. The experimental design is shown in Figure 2. Group 1 untreated control - received drinking water. Group 2 vehicle control - received groundnut oil. Group 3 received NDEA 1 ppm/g b.w. in drinking water (5 days/week) over the course of 6 weeks (Parekh and Rao, 2007). Group 4 and 5 received Embelin (50mg, 100mg/kg b.w.) in groundnut oil orally 5 days/week, pretreatment period for 2 weeks and thereafter subjected to NDEA exposure as in Group 3 followed by simultaneous dosing of embelin for 18 weeks. Thereafter drug treatment was discontinued for 2 weeks. At the end of 22nd week experiment was terminated (see Figure 2).

(c) Assessment of CCl4 induced Hepatotoxicity: Wistar rats were divided into 3 groups containing 6 rats each. Group 1: Vehicle treated normal control; Groundnut oil. Group 2: Hepatotoxicant; CCl_4 in groundnut oil 1:1 (v/v), 0.7 ml/kg a single i.p. dose once a week for 4 weeks (Katari and Singh, 1997). Group 3: Treated similarly and Embelin (100mg/kg) was given orally simultaneously. Embelin treatment continued daily for a period of 5 weeks including 1 week pretreatment prior to CCl_4 exposure. At the end of 5th week experiment was terminated.

Body weight and mortality (if any) were recorded. Animals were autopsied; blood and liver tissues were collected. Serum was separated, livers were weighed and the average number of foci per rat liver was counted. Small blocks of liver from median lobe were fixed in 10% neutral buffered formalin, embedded in paraffin blocks and processed for histopathological examination (haematoxylin and eosin staining). For EM, pinhead sized blocks of distal end of median lobe (1-2mm) was fixed in 3% glutaraldehyde in 0.1 M Sodium cacodylate buffer (pH 7.4) for 1 hr at 4ºC and post fixed in 1% osmium tetroxide. Ultrathin sections (100nm) were cut on LKB 2088 ultramicrotome, mounted on 300 mesh copper grids, stained with uranyl acetate and lead citrate and examined under JEOL JEM-1010 transmission electron microscope.

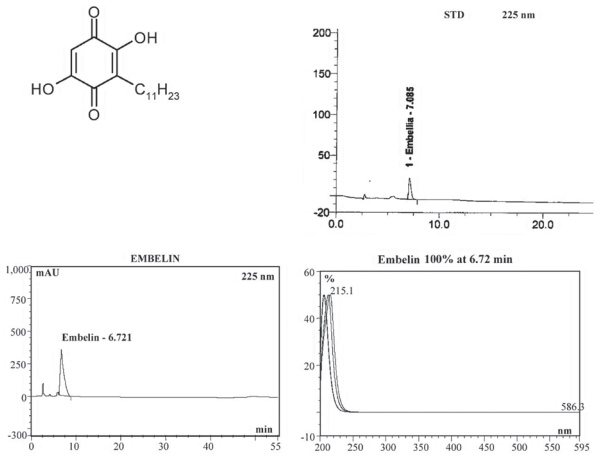

Figure 1. Molecular Structure, HPLC Fingerprint Analysis of the Standard, Embelin and its UV Spectra

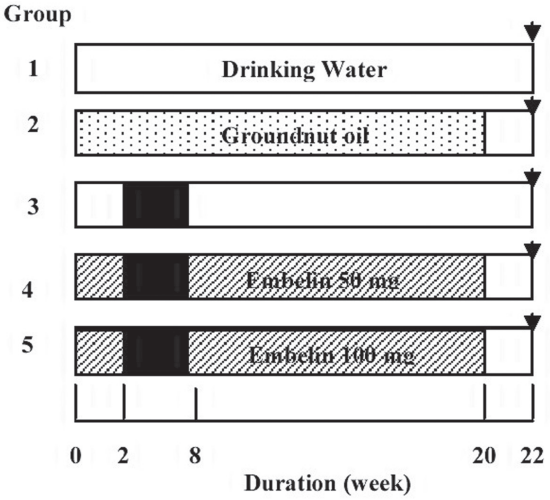

Figure 2. Experimental Design. NDEA 1ppm/gm b.w. in Drinking Water, Embelin, Sacrifice

Biochemical analysis

Serum glutamate pyruvate transaminase (GPT), glutamate oxaloacetate transaminase (GOT), alkaline phosphatase (ALP), γ-glutamyl transpeptidase (GGT), total bilirubin (TBIL), total proteins (TP), albumin (ALB), total cholesterol (CHO) and triglycerides (TG) were determined using Erba Diagnostics kits on XL-300 Fully Automated Random Accesses Chemistry Analyzer, Transasia Bio-medicals Ltd., India. Liver homogenate was prepared in ice-cold 1.15 % KCl. For GSH determination separately 10% homogenate were made in 0.02 M EDTA. Total proteins (Lowry et al., 1951), lipid peroxidation (LPO) in liver homogenates (Ohkawa et al., 1979), Reduced glutathione (GSH) (Sedlak and Lindsay, 1968), GGT activity in microsomal fraction (Szazs, 1976) and Glutathione-S-transferase (GST) in cytosolic fraction (Habig et al., 1974) were determined spectrophotometrically by standard methods. Statistical analysis: One-way Analysis of Variance (ANOVA) followed by the Dunnett t - test for multiple comparisons was applied.

Results

Acute toxicity study

Mice treated with Embelin 50mg and 100mg/kg showed no significant body weight changes, mortality and apparent toxic effects signifying its safety profile.

Body weight, liver weight and hepatic foci profile

There was no significant change in the average body weights of control, NDEA and NDEA+Embelin groups. A significant decrease in liver weights and relative liver weights ($P<0.05$, $P<0.001$) in Embelin treated animals as compared to increased weights in NDEA group alone was noted. Morphologically, small grayish-white foci and sharp demarcation from the surrounding reddish-brown liver were detected in NDEA treated rats. A marked reduction in foci incidence was observed in embelin treated rats (Table 1).

Biochemical profile

The significant increase ($P<0.001$) in serum GPT, GOT, ALP, GGT, TG, CHO, TBIL, liver enzymes viz; LPO, GGT, GST activities on NDEA or CCl_4 exposure and their marked decrease ($P<0.001$) on concomitant embelin treatment is summarized in Tables 2 and 3. With respect to GSH, marked depletion ($P<0.001$) of total soluble sulfhydryl content as well as hypoproteinemia ($P<0.05/P<0.001$) and hypoalbuminuria ($P<0.001$) were observed in DEN or CCl_4 treated animals. Treatment with embelin markedly enhanced GSH activity ($P<0.001$); protein and albumin levels too were considerably higher preventing their further depletion. Effects of embelin were comparable to normal or vehicle group. No statistical difference amongst groups 1-2 and 4-5 were noted.

Histopathological examination

Livers of NDEA treated rats showed greater extent of focal coagulative necrotic changes, presence of binucleated nucleus due to increase in mitotic activity, focal fatty changes, hyperchromasia, single cell enlargement along with change in the nucleus to cytoplasm ratio (1:1 to 1:2) as compared to normal ratio (1:4 to 1:6). These changes indicated preneoplastic changes in comparison to normal hepatocytes. Focal lymphocytic

Table 1. Effects of Embelin on Foci incidence, Body and Liver Weights of NDEA Treated Rats

Treatment (Group)		No. of foci	Body Wt. (g)	Liver wt. (g)	Relative Liver wt. (g)
1	Drinking Water	0	464.33±13.05	9.69±0.14	2.09±0.04
2	Vehicle Control	0	441.33±36.68	9.53±0.72	2.14±0.13
3	NDEA	25	400.00±37.81	9.97±0.62	2.50±0.09[a]
4	NDEA + Embelin (50mg/kg)	5	420.00±34.72	8.75±0.47[b]	2.08±0.07[c]
5	NDEA + Embelin (100mg/kg)	5	434.75±41.80	8.94±0.96[b]	2.06±0.08[c]

Values are Mean ± S.D of 6 animals in each group.; [a] $P < 0.001$ significantly different from group 2, [b] $P < 0.05$, [c] $P < 0.001$ significantly different group 3.

Table 2. Effect of Embelin on Biochemical changes in NDEA induced Hepatic Preneoplasia in rats

Treatment (Group)					
Biochemical Parameters (Units)	1 Normal Control	2 Vehicle Control	3 NDEA	4 NDEA + Embelin 50 mg/kg	5 NDEA + Embelin 100 mg/kg
SGPT (IU/L)	38.61±6.58	44.09±8.69	98.27±13.75[b]	37.84±11.19[d]	37.43±8.57[d]
SGGT (IU/L)	20.60±3.10	27.51±9.67	55.15±14.23[b]	28.47±3.58[d]	32.88±3.86[d]
STG (mg/dl)	133.88±5.19	140.03±6.24	180.20±39.36[a]	134.45±15.87[c]	130.35±22.36[c]
SALB (g/dl)	4.75±0.38	4.64± 0.48	3.37±0.31[b]	3.95 ±0.31	3.93±0.46
LPO (nM of MDA/g wet liver)	17.44±5.96	21.15± 8.81	47.75 ±10.63[b]	14.87±4.35[d]	14.29±4.47[d]
GSH (μM of GSH/g wet liver)	1.72±0.21	1.88±0.47	1.05±0.14[b]	2.11±0.40[d]	1.83±0.34[d]
GST (nmol of CDNB conjugated/ min/mg protein)	548.79±71.00	557.47±15.63	913.49±85.27[b]	487.45±27.65[d]	484.53±49.53[d]
GGT (nmol of p-nitroanilide released/min/mg protein)	0.1 ±0.08	0.23±0.11	0.35±0.12[a]	0.11±0.04[c]	0.11±0.08[c]
TPRO (mg protein/100mg wet liver)	5.57±0.68	6.53±0.29	4.55±0.72[a]	5.83±1.04	5.67±0.99

Values are Mean±S.D of 6 animals in each group.; [a] $P < 0.05$, [b] $P < 0.001$ significantly different group 2. [c] $P < 0.05$, [d] $P < 0.001$ significantly different group 3. NAD No abnormality detected.

Table 3. Effect of Embelin on Biochemical Changes in CCl_4 induced Hepatotoxicity in rats

Treatment (Group)			
Biochemical Parameters (Units)	1. Vehicle Control	2. CCl_4	3. CCl_4 + Embelin
SGPT (IU/L)	73.20± 5.94	91.80±6.60[a]	62.20±2.77 [c]
SGOT (IU/L)	146.80±5.80	196.60±18.70[a]	144.40±13.68 [c]
SALP (IU/L)	231.00±8.34	493.20±44.17[a]	181.20±32.83[c]
SGGT (IU/L)	7.60±2.07	16.80±1.30 [a]	3.80±0.45 [c]
STBIL (mg/dl)	0.06±0.01	0.34±0.05 [a]	0.06±0.02 [c]
SCHO (mg/dl)	49.00±4.18	78.60±4.39 [a]	47.80±5.67 [c]
STG (mg/dl)	42.60±5.59	72.40±3.65 [a]	43.40±4.22 [c]
STP (g/dl)	6.90±0.82	5.12±0.63 [a]	6.90±0.57 [b]
LPO (nM of MDA/g wet liver)	21.29±0.70	56.39±3.45 [a]	25.65±2.66 [c]
GSH (μM of GSH/g wet liver)	0.93±0.08	0.37±0.05 [a]	0.86±0.05 [c]

Values are mean ± SD of 6 animals in each group

infiltration was noticed. Embelin treatment for 20 weeks markedly reduced the preneoplastic changes and adverse effects of NDEA indicating the beneficial effects. CCl_4 treated liver showed intense pathological changes which mainly included swelling of the individual cells as well as hepatic cords, infiltration of leucocytes, congestion of capillaries, intense leakage of RBC's, focal and intense mononuclear cell infiltration. Embelin treatment for 5 weeks markedly reduced the swelling of hepatic cords, inflammatory response and minimized the infiltration of mononuclear cells indicating the effectiveness of the hepatoprotectant drug.

Transmission Electron Microscopy

Liver of vehicle treated group showed a centrally placed oval nucleus with nucleolus (spheroidal) and distinct nuclear membrane characteristic of the hepatocyte. Nucleoplasm was light with fine granules which fills the nuclear space. Cytoplasm was occupied by dense mitochondria and fair amount of endoplasmic reticulum (ER) (Figure 1A). There were more compact and intense intercellular junctional complexes. The junction is separated at the region of bile canaliculus lined by microvillous projections (Figure 1B). In NDEA rats, hepatic cells showed extensive degenerative changes, disturbed nuclear envelope irregularly shaped nucleus with decrease in size and are binucleated. The nucleolus was not well defined showing fragmentation. Nucleoplasm was condensed as indicated by dark spots. The glycogen granules were smaller in size and have become clumped like droplets (Figure 1 C and D). Enlarged, numerous intensely stained pleomorphic mitochondria surrounded by sparse

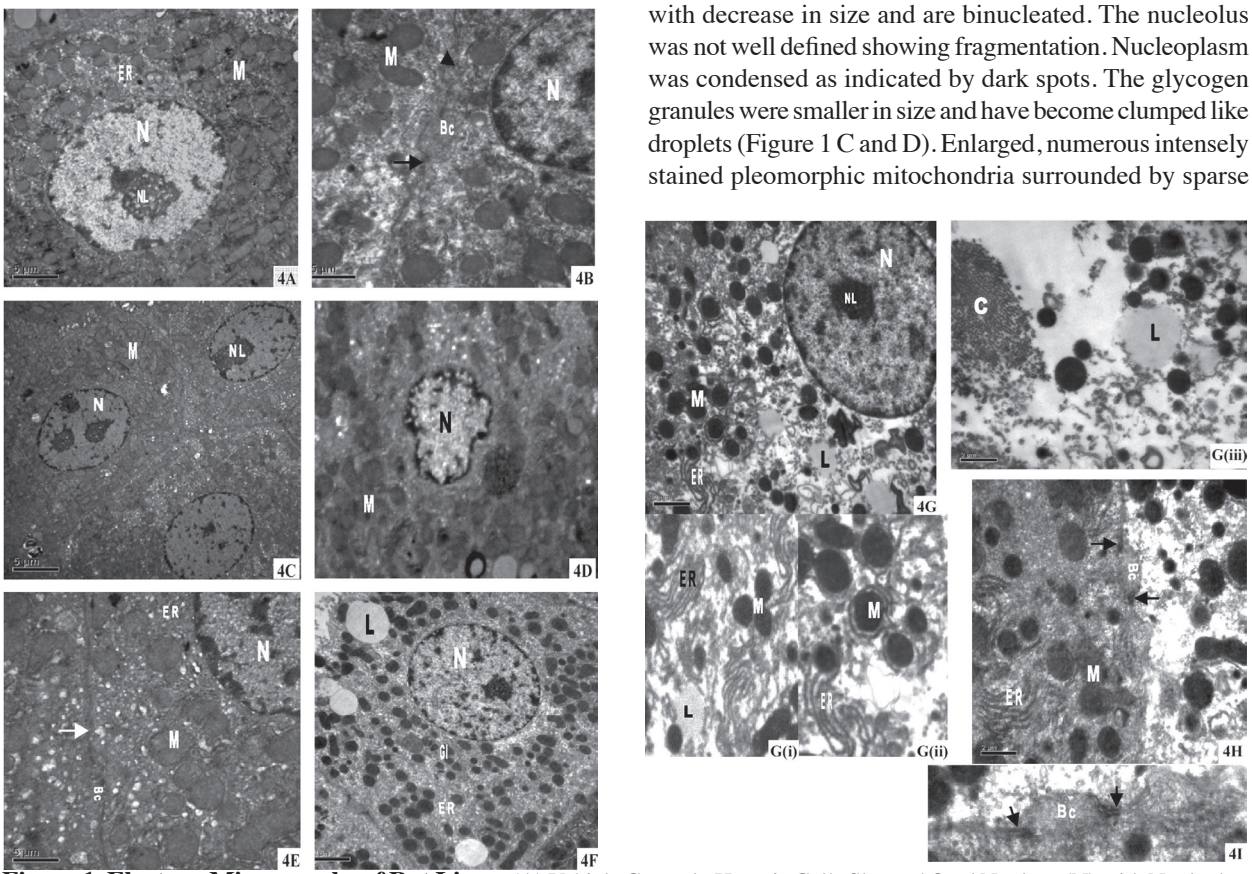

Figure 1. Electron Micrographs of Rat Liver. (A) Vehicle Control - Hepatic Cells Showed Oval Nucleus (N) with Nucleolus (NL), dense Mitochondria (M), Endoplasmic Reticulum (ER) (x8000), (B) Bile Canaiculus (Bc) with Compact Intercellular Junctional Complexes (x15000); (C and D) NDEA Induced Preneoplasia - Showing Irregularly Shaped N, Disturbed Nuclear Envelope, Ill - Defined NL (x4000), (E) Swollen M, Sparse ER, Distended Bc and Widened Intercellular Spaces (Gaps, White Arrow) (X15000) ; (F, G, i and ii) NDEA+Embelin 100mg/kg - Showing Oval N with NL, Well - Defined Nuclear Membrane, Abundant ER Closely Packed around M, Glycogen Granules (Gl), Lipids (L) (x8000), (G iii) Collagen Fibres (C), L (x15000) and (H and I) ER, Intact Bc and Gap Junctions (x15000). The Black Arrows Indicate Intercellular Gap Junctions

ER. A few swollen or fragmented mitochondria were also observed. Cytoplasm showed disturbed or granular rough ER. This state of disorganization is due to fractionation and reduction. Cell membranes were ill defined, with widened intercellular spaces besides complete loss of cell-to-cell contact disturbing the morphological structure. Bile canaliculi are distended (Figure 1E). However, rats treated with NDEA + embelin showed reappearance of nuclear shape (oval) with prominent nucleoli. Nuclear membrane was clear with disappearance of serration. Cytoplasm showed rich distribution of ER, closely packed around the mitochondria. The ER has reappeared, is well organized and abundant. Cytoplasm was occupied by numerous round or oval shaped well defined mitochondria and glycogen granules, collagen fibres and lipids (Figure 1F,G, i, ii and iii). Membranes appeared compact and normal with narrowed intercellular space between adjacent cells, intact tight junctions. Bile canaliculi are filled with microvilli (Figure 1H and I). Embelin treatment prevented the NDEA induced degenerative changes.

Discussion

The results of the present study indicate that embelin exhibited chemopreventive action and possess a potential hepatoprotective activity.

Marked elevations in liver functional markers GPT, GOT, ALP, TG, CHO, TBIL and the fall in protein and albumin levels too reflected the degree of hepatocellular dysfunction caused by NDEA or CCl_4. Significant reduction in these elevated enzyme activities elicited by embelin and enhanced TPRO, ALB levels suggests the protection of structural integrity of hepatocyte cell membrane or stimulatory effects on hepatic regeneration. Also, reflecting the recovery of liver from the toxic effects of NDEA or CCl_4 towards normal liver cell function.

GST and GGT are useful biomarkers of early hepatic focal lesions (Cameron, 1988; Jeena et al., 1999). The precancerous marker enzyme GST is a highly expressed cytoplasmic protein predominant during early carcinogenesis. This higher induction of GST activity in NDEA group was markedly suppressed on combined NDEA and embelin fed rats. A similar effect was observed in GGT activity. GST/GGT are known to identify preneoplastic hepatocyte population and therefore, prevention of NDEA induced increase in GGT/GST activities by simultaneous administration of embelin is a significant finding indicating its chemopreventive role.

The implication of reactive oxygen species (ROS) in carcinogenic nitrosamines and CCl_4 toxic hepatic injury are well documented (Ravid and Korean, 2003; Recknagel and Glende, 1973). The peroxidative breakdown of membrane polyunsaturated fatty acids is involved in many degenerative diseases including cancer. Lipid peroxidation leads to destabilization and disintegration of the cell membranes, altering the activities of membrane-bound enzymes and other proteins, as well as the concomitant release of hydroperoxides and alkoperoxide radicals, which are potentially toxic (Yagi, 1978). Also, the major mechanism involved in CCl_4 hepatotoxicity is LPO by free radical derivatives of CCl_4 viz; $\bullet CCl_3$ (trichloromethyl radical) and $\bullet O2CCl_3$ (trichloromethyl peroxy radical) radicals which trigger cell damage through covalent modification and subsequent proliferative lipid peroxidation (Recknagel and Glende, 1973). Thereby causing damage to hepatic tissue and failure of antioxidant defense mechanisms to prevent the formation of excessive free radicals. Our findings indicate significant increase in Malondialdehyde (MDA), the end of product of LPO was inhibited effectively by embelin is in agreement with earlier reports whereby, naturally occurring quinones like embelin have an O-hydroxyquinone structure are quite reactive to oxidizing reagents and function as H_2O_2 scavengers (Kinoshita et al., 1992; Chitra et al., 1995). Thus, the drug is capable of quenching free radical toxicity. Thiols, particularly glutathione play an important role in trapping reactive carcinogenic species. They protect cells from carcinogenic processes by functioning as an antioxidant and by binding with cellular mutagens. GSH levels were significantly enhanced by embelin against carcinogen-induced GSH depletion which may due to the oxidation by glutathione to oxidized glutathione or to glutathione-quinone conjugate formation. Embelin acts as a potent antioxidant in physiological conditions. In vitro studies have shown it scavenges DPPH radical, inhibits hydroxyl radical induced deoxyribose degradation, LPO and restores impaired mitochondrial Mn-superoxide dismutase (Joshi et al., 2007).

The modifying effects of embelin on NDEA induced preneoplastic foci or CCl4 induced liver damage was further evidenced by histopathological and ultrastructural observations causing prevention of pathological degenerative changes of the NDEA treatment to normalize and exhibiting marked reduction in incidence of focal preneoplastic lesions. Electron microscopically, embelin exhibited significant changes viz; enhanced cell to cell adhesion among adjacent cells, restoration of morphological architecture and intimate cell contacts with well defined cell boundaries resembling to normal hepatocytes were noticed. Therefore, Embelin has proved remarkable in suppressing and /or arresting the degenerative changes, brought about by NDEA.

Earlier reports have shown that Embelin regresses Methylcholanthrene-induced fibrosarcoma in rats (Chitra and Shyamala Devi, 1994), in vitro antiproliferative activity was highlighted by decrease in radioactive (3H) -thymidine uptake (Chitra et al., 1995), chemopreventive activity against DENA induced hepatocarcinogenesis in Wistar rats (Bali and Sreepriya, 2005) and inhibitory effects on the activation of polymorphonuclear leukocytes (Kinoshita et al., 1992). The present *in vivo* results indicate that daily embelin feeding reversed the levels to normal values are in agreement with the above reports. Thereby, establishing the antioxidant potency and chemopreventive potential of embelin.

In conclusion, Embelin effectively prevented formation of preneoplastic foci in rats when given along with the NDEA treatment i.e. during the initiation phase and after the NDEA treatment until the end of the experiment i.e. during the post-initiation stage or CCl_4-induced hepatotoxicity. The preventive effect of embelin was indicated by inhibition of growth and development of

preneoplastic foci. Thus, Embelin seems to be a potential, and promising chemopreventive and hepatoprotective agent.

Acknowledgements

R.P is thankful to Dr. D. S. Suryavanshi, Dr. C. S. Mote (Bombay Veterinary College, Mumbai) for their help in biochemical, histopathological analysis, to Dr. A. D. Ingle for providing the animal house facility, Mr. A. Khole for technical help (ACTREC, Navi Mumbai) and Jaslok Hospital, Mumbai for providing the Electron Microscopy facility.

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