RESEARCH COMMUNICATION

Sida rhombifolia ssp. *retusa* Seed Extract Inhibits DEN Induced Murine Hepatic Preneoplasia and Carbon Tetrachloride Hepatotoxicity

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Abstract

   Sida rhombifolia ssp. *retusa* is a well established drug in the Ayurvedic system of medicine used for antirheumatism and antiasthmatism. Inhibitory effects of *S. rhombifolia* ssp. retusa seed extract on DEN induced hepatocellular preneoplastic foci and carbon tetrachloride (CCl_4) induced hepatotoxicity was investigated in rats. Rats received DEN, 1ppm/g b.w. in drinking water for 6 weeks or CCl_4, 0.7 ml/kg i.p. once a week for 4 weeks and seed extract 50 mg, 100 mg/kg b.w. orally prior, during and after exposure to DEN/CCl_4 for 20 or 5 weeks, respectively. Treatment with seed extract significantly inhibited the increase in DEN/CCl4 induced activities of pre-cancerous marker enzymes; gamma-glutamyl transpeptidase, glutathione-S-transferase, hepatotoxicity marker enzymes; glutamate pyruvate transaminase, glutamate oxaloacetate transaminase and alkaline phosphatase as well as lipid peroxidase. Depleted glutathione, protein and albumin levels were restored. Also, histopathological and transmission electron microscopic studies showed prevention of cellular degenerative changes. The chemopreventive and hepatoprotective potentials of seed extract are due to free radical scavenging activity and restoration of cellular structural integrity.

Key Words: *Sida rhombifolia* ssp. retusa seed extract - DEN - hepatic preneoplasia - CCl_4 - hepatotoxicity

Introduction

From time immemorial, for a large number of the world's rural population, medicinal plants are the only source for the prevention and treatment of various pathological diseases. Medicinal plants and their constituents, vegetables, fruits and dietary fibre have been reported to prevent multistage carcinogenesis or may be destroying or blocking DNA damaging mutagens in the cells (Johnson, 1997; Craig,1999).

Sida rhombifolia Linn. ssp. retusa (Linn.) Borss (Malvaceae, syn. Sida retusa) commonly known as "Atibala, Jungli Methi" is an erect annual or perennial branched undershrub about 1.5 m high distributed throughout tropical and subtropical parts of India (Kirtikar and Basu,1933). Phytochemically, it possesses therapeutically active agents viz; alkaloids, phytosterols, ecdysterone, carbohydrates, saponins, cyclopropeniod fatty acids viz; sterculic acid, malvalic acid, linoleic acid, myristic acid, palmitic acid, stearic acid and oleic acid, amino acids, gums and mucilages (Ahmad et al., 1976; Dinan et al., 2001; Khare et al., 2002). This indigenous plant holds a remarkable reputation among the medical practitioners for its anti-rheumatism activity and is an important ingredient in polyherbal formulations. Being a Rasayana drug they are rejuvenating and age-sustaining tonics for promoting vitality and longevity. It is also used for the treatment of asthma and other chest ailments. Earlier studies have showed antipyretic, anti–inflammatory, sedative, hypoglycaemic, antimicrobial, antituberculosis, antimalarial, hepatoprotective, anti-HIV, anti-tumor and cytotoxic activities in this genus. Seeds are aphrodisiac, spermatic tonics, colic, useful in gonorrhoea, cystitis and tensemus (Muanza et al.,1995; Rao and Mishra, 1997; 1998; Khare et al., 2002; Ekramul Islam et al., 2003; Dhalwal et al., 2006).

So far, there is no scientific report on the relationship between antilipid-peroxidative activities and chemopreventive and/or antihepatotoxic property of *Sida rhombifolia* ssp. retusa. Hence, the present investigation is designed to evaluate the role of seed extract in prevention of preneoplastic lesion, oxidative alterations and extent of biotransformation enzyme GST in Diethylnitrosamine (DEN) – initiated rat preneoplasia. Also, its hepatoprotective activity against carbon tetrachloride (CCl_4) - induced liver damage in rats was studied. We report for the first time that chemopreventive and anti-hepatotoxic potentials of *S. rhombifolia* ssp.

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retusa seed extract of is due to free radical scavenging activity and restoration and maintenance of cellular integrity.

Materials and Methods

Plant material and Extraction

Sida rhombifolia ssp. retusa plants along with seeds were authenticated by the National Institute of Science Communication and Information Resources (NISCAIR), New Delhi, India. Seed powder was extracted with 80% methanol for about 36 hr in a soxhlet extractor. The solvent was removed under reduced pressure at 50ºC to give a crude extract. The concentrated extract was dried to give greenish yellow oil (yield 18.8 %). Reversed phase HPLC fingerprint analysis of this methanolic extract was performed on linear gradient system Dionex–Ultimate 3000 HPLC system, USA equipped with Dionex-3000 PDA detector running Chromelon, Dionex software. The major peaks detected at Rt 16 - 35 min. It also revealed the presence of a marker (Rt 20.8 min) could be identified as Ecdysterone of chemotaxonomic significance by comparison with standard Ecdysterone (donated by Dr. Laddha, University Institute of Chemical Technology, Mumbai) and UV spectra (max 245 nm) (see Figure 1).

Animals, carcinogen and chemicals

Male Swiss mice (25-30 g), 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 environmental conditions (Temp. 22±2ºC, relative humidity 45±10%; a 12h light and 12h dark cycles). Commercial pellet diet and water was given ad libitum. Animals received human care and the experiment was carried out following the CPCSEA guidelines and approval obtained by the Institutional Animal Ethics Committee (No. 03/2007) of ACTREC and R. Ruia College (Animal House Registration No. 315/1999/ CPCSEA). All chemicals used were of analytical reagent grade.

Experimental Design

(a) <u>Acute toxicity study</u>: 2 groups of Swiss mice containing 3 mice each were administered orally 200 and 500 mg/kg b.w. seed extracts and observed for toxic effects for 6 hr, 24 hr and then upto 14 days. Control group received only groundnut oil as the vehicle. Body weight and mortality (if any) was recorded.

(b) <u>DEN-induced preneoplasia</u>: Sprague Dawley rats were divided into 5 groups of 6 animals 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 DEN (Sigma Chemicals, USA) 1 ppm/g b.w. in drinking water (5 days/week) over the course of 6 weeks (Parekh and Rao, 2007). Groups 4 and 5: received seed extract (50 mg, 100 mg/kg b.w.) in groundnut oil given orally 5 days/week, pretreatment period for 2 weeks and thereafter were subjected to DEN exposure as in Group 3 followed by simultaneous dosing of extract for 18 weeks. Thereafter drug treatment was discontinued for 2 weeks. At the end
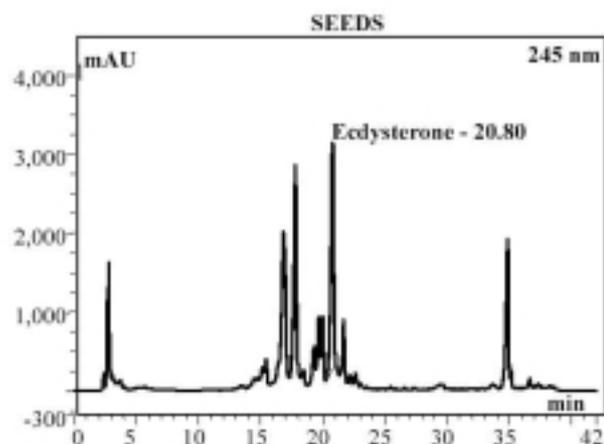
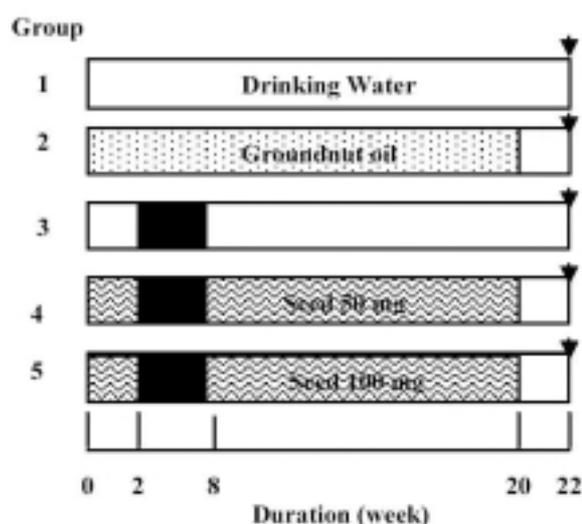**Figure 1. UV Analysis of the Methanolic Seed Extract**

Figure 2. Experimental Design. Black boxes, DEN 1ppm/ gm b.w. in drinking water; Arrows, sacrifice

of 22 weeks the study was terminated.

(c) <u>CCl₄ induced Hepatotoxicity</u>: Wistar rats were divided into 3 groups of 6 animals each. Group 1: Vehicle control received groundnut oil. Group 2: received CCl4 (Qualigens, Mumbai) in groundnut oil 1:1 (v/v), 0.7 ml/ kg b.w. intraperitoneally a single dose once a week for 4 weeks (Katari and Singh, 1997). Group 3: received pretreatment of seed extract (100 mg/kg/day) orally for 1 week. Thereafter CCl_4 was administered as in group 2 followed by simultaneous extract treatment for 4 weeks. At the end of 5th week experiment was terminated.

Body weights, food consumption and mortality (if any) were recorded. Blood was withdrawn through retro–orbital

Table 1. Effects of Seed Extract (SE) on Foci incidence, Body and Liver Weights in DEN treated Rats

Group	No. of Foci	Body wt. (g)	Liver wt. (g)	Relative liver wt. (g)
Control	0	464.3±13.1	9.69±0.14	2.09±0.04
Vehicle	0	441.3±36.7	9.53±0.72	2.14±0.13
DEN	25	400.0±37.8	9.97±0.62	2.50±0.09[a]
DEN+SE (50mg)	14	423.2±26.4	8.98±0.48	2.13±0.09[c]
DEN+SE (100mg)	6	419.8±36.6	8.77±1.11[b]	2.09±0.16[c]

Values are Mean±S.D for 6 animals in each group; [a]P < 0.001 Vs group 2; [b]P < 0.05, [c]P < 0.001 Vs group 3

Table 2. Effect of Seed Extract on Enzymatic Activities in a Model of DEN-induced Hepatic Preneoplasia in Rats

Parameters (Units)	Normal control	Vehicle control	DEN	DEN+SE 50mg	DEN+SE 100mg
SGPT (IU/L)	38.6 ± 6.58	44.1 ± 8.69	98.3 ± 13.8[b]	55.7 ± 6.18[d]	52.7 ± 9.43[d]
SGGT (IU/L)	20.6 ± 3.10	27.5 ± 9.67	55.2 ± 14.2[b]	26.7 ± 8.82[d]	25.0 ± 9.17[d]
STG (mg/dl)	133.9 ± 5.19	140.0 ± 6.24	180.2 ± 39.4[a]	133.2 ± 22.0[c]	132.5 ± 18.8[c]
SALB (g/dl)	4.75 ± 0.38	4.64 ± 0.48	3.37 ± 0.31[b]	3.94 ± 0.26	4.03 ± 0.67[c]
LPO (nM of MDA/g wet liver)	17.4 ± 5.96	21.2 ± 8.81	47.8 ± 10.6[b]	22.5 ± 2.90[d]	18.0 ± 4.72[d]
GSH (μM of GSH/g wet liver)	1.72 ± 0.21	1.88 ± 0.47	1.05 ± 0.14[b]	1.66 ± 0.16[d]	1.74 ± 0.24[d]
GST (nmol of CDNB conjugated/ min/mg protein)					
	548.8 ± 71.0	557.5 ± 15.6	913.5 ± 85.3[b]	542.7 ± 189.8[d]	458.8 ± 108.1[d]
GGT (nmol ofp-nitroanilide released/min/mg protein)					
	0.17 ± 0.08	0.23 ± 0.11	0.35 ± 0.12[a]	0.27 ± 0.26	0.10 ± 0.04c
TPRO (mg /100 mg wet liver)	5.57 ± 0.68	6.53 ± 0.29	4.55 ± 0.72[a]	4.91 ± 1.37	5.66 ± 1.15

Values are Means ± S.D for 6 animals in each group; [a]$P < 0.05$, [b]$P < 0.001$ Vs group 2; [c]$P < 0.05$, [d]$P < 0.001$ Vs group 3

plexus and serum was separated. All the surviving animals were killed and autopsied. Livers were removed, weighed and average number of foci per rat liver was counted. Biochemical parameters; 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 Diagnostic 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 estimation separately 10% homogenate were made in 0.02 M EDTA. GGT (microsomal fraction) (Szazs, 1976), Glutatione-S-transferase (GST, cytosolic fraction) (Habig et al., 1974), Lipid peroxidation (LPO) in liver homogenate (Ohkawa et al., 1979), Reduced glutathione (GSH) (Sedlak and Lindsay, 1968) and total liver proteins (TPRO) (Lowry et al., 1951) 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.

Small blocks of liver from median lobe were fixed in 10% neutral buffered formalin, processed for histopathology by routine methods and Haematoxylin–Eosin (H & E) stained. For EM, pinhead sized blocks of distal end of median lobe of 1-2 mm was fixed in 3% glutaraldehyde in 0.1 M sodium-cacodylate buffer (pH 7.4) for 1 hr at 4°C. The sections were post-stained with

1% osmium tetroxide. Ultrathin sections of (100 nm) were cut on an LKB 2088 Ultramicrotome, mounted on 300 mesh copper grids and stained with uranyl acetate and lead citrate were examined under JEOL JEM–1010 transmission electron microscope operating at 80 kV.

Results

General observation

Acute toxicity study revealed no significant body weight changes, mortality and apparent toxic effects of seed extract signifying its safety profile. Food and water consumption decreased after DEN treatment as evident by sudden reduction in body weight confirming the possible toxic response to hepatocarcinogen treated rats. There was no statistical difference in body and liver weights among the control, DEN and DEN+seed extract groups. However, a significant decrease in relative liver weights ($P<0.001$) was noted in seed extract treated animals in comparison to DEN treated rats alone. Morphometrically, small grayish–white foci and sharp demarcation were easily discernable from the surrounding reddish–brown, non-foci liver parenchyma in DEN treated rats. A significant reduction in foci incidence was observed in seed extract treated rats (Table 1).

Biochemical parameters

The significant increase ($P<0.001$) in the levels of serum biochemical parameters on DEN or CCl_4 exposure and their marked reduction ($P<0.001$) on concomitant seed extract treatment is summarized in Tables 2 and 3. Extent of LPO is measured through malondialdehyde (MDA) activity a pro-oxidant factor which determined the oxidative damage. A significant increase ($P<0.001$) in LPO levels in liver, biotransformation enzyme–cytosolic GST and microsomal GGT as well as marked depletion ($P<0.001$) in GSH activity were observed in DEN or CCl_4 treated animals. However, increase in liver enzymes were significantly reduced ($P<0.001$) on daily administration of seed extract for 22 weeks or 5 weeks period before and during exposure to the toxicants in combination experiment when compared with the DEN or CCl_4 treated group alone. Seed extract treatment markedly enhanced GSH activity ($P<0.001$), protein and albumin levels too were considerably higher preventing their further depletion. Effects of seed extract were almost comparable

Table 3. Effects of Seed Extract on Enzymatic Activity in the Model of CCl$_4$ induced Hepatotoxicity

Parameter	Vehicle control	CCl$_4$	CCl$_4$ + SE
SGPT (IU/L)	73.0 ± 5.94	91.8 ± 6.60[a]	64.2 ± 4.49[b]
SGOT (IU/L)	146.8 ± 5.80	196.6 ± 18.7[a]	161.6 ± 4.83[b]
SALP (IU/L)	231.0 ± 8.34	493.2 ± 44.2[a]	209.0 ± 40.4[b]
SGGT (IU/L)	7.60 ± 2.07	16.80 ± 1.30[a]	5.00 ± 1.58[b]
STBIL (mg/dl)	0.06 ± 0.01	0.34 ± 0.05[a]	0.08 ± 0.01[b]
SCHO (mg/dl)	49.0 ± 4.18	78.6 ± 4.39[a]	49.4 ± 5.68[b]
STG (mg/dl)	42.6 ± 5.59	72.4 ± 3.65[a]	48.8 ± 4.44[b]
STP (g/dl)	6.90 ± 0.82	5.12 ± 0.63[a]	6.40 ± 0.55
LPO (nM of MDA/g wetliver)			
	21.3 ± 0.70	56.4 ± 3.45[a]	31.1 ± 0.80[b]

Values are Mean±S.D for 6 animals in each group; [a]$P < 0.001$ Vs group 2; [b]$P < 0.001$ Vs group 2
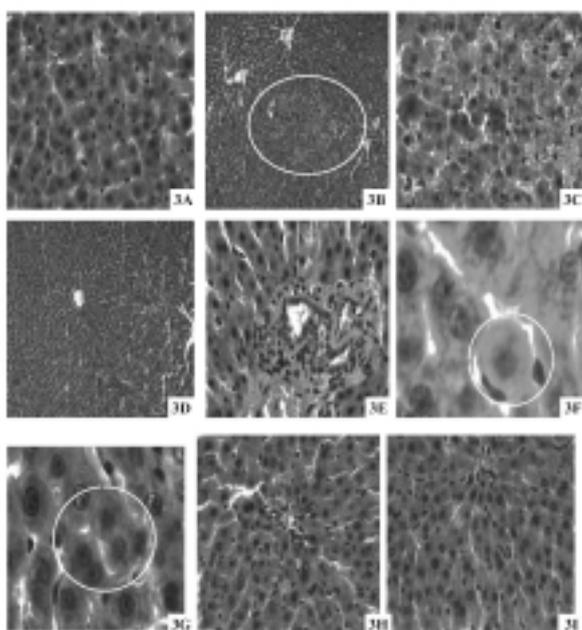
Figure 3. Liver Sections. (A) Vehicle control (x400); (B) DEN (1ppm/gm b.w.) induced Preneoplasia (x100) and at (C) (x400), (D) intense mononuclear cell infiltration (x100) and at (E) (X400), (F,G) single cell enlargement (X400) as well as bi-nucleated nucleus (x1000) ; (H) DEN+50 mg/kg seed extract, (I) DEN+100 mg/kg seed extract showing marked degree of protection with only few preneoplastic cells (x400) (H & E staining)

with normal control group. No statistical difference amongst groups 1, 2 and dose level at 100mg/kg (group 5) was found to exert a maximum protective effect in combating the carcinogenic effects of DEN as compared to the dose 50 mg/kg (group 4) (Table 2).

Histopathology

(a) <u>DEN Vs Seed extract</u>: Livers of DEN treated rats showed greater extent of focal coagulative necrotic changes, presence of bi-nucleated nucleus, focal fatty changes. 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 (Figure 3B-G). Focal infiltration of lymphocytes was observed. So, it scored pathological grade 5 (+++++) severe. Administration of seed extract (100 mg/kg) pre- and post-initiation with DEN resulted in prevention of DEN induced alterations with pathological grade 2 (++) mild degree (Figure 3H and I) and to a lesser extent at dose level 50 mg/kg grade 3 (+++) moderate.

(b) <u>CCl$_4$ Vs Seed extract</u>: Rats treated with CCl$_4$ showed necrosis of hepatic cells, swelling of hepatic cords, infiltration of leucocytes, congestion of capillaries, intense leakage of RBC's and focal mononuclear cell infiltration around the portal blood vessels. But administration of seed extract before and after CCl$_4$, signs of protection to a considerable extent were evident from the marked reduction in swelling of hepatic cords and fatty infiltration of leucocytes and were able to maintain normal architecture of the hepatocytes.

Ultrastructural Pathology

Transmission electron microscopy showed in vehicle control group 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 is occupied by dense mitochondria and fair amount of endoplasmic reticulum (ER). There are more compact and intense intercellular junctional complexes (Figure 4A and B). In DEN treated rats, hepatic cells showed extensive degenerative changes, completely disturbed nuclear envelope and irregularly shaped nucleus with decrease in size. The nucleolus is not well defined. Nucleoplasm is condensed as indicated by dark spots. Enlarged, numerous intensely stained pleomorphic mitochondria surrounded by sparse ER. Swollen or fragmented mitochondria were observed. Cytoplasm showed disturbed or granular rough ER. This state of disorganization was due to fractionation and reduction. Glycogen granules were smaller in size and have become clumped like droplets. 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 4C–G). However, Seed extract (100 mg/kg) treatment before and after DEN showed reappearance of nuclear shape (oval) with prominent nucleoli. Nuclear
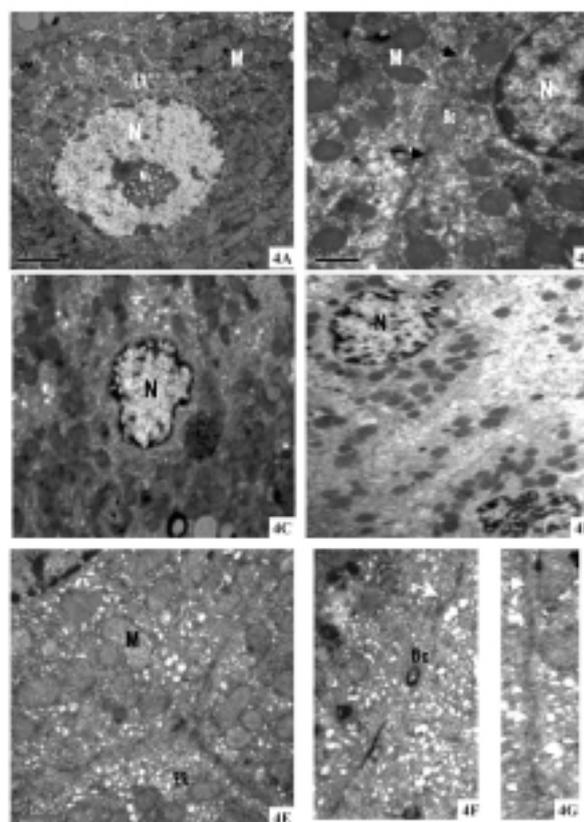

Figure 4. Electron Micrographs of Rat Liver. (A) Vehicle control–hepatic cells showed oval nucleus (N) with nucleolus (NL), dense mitochondria (M), endoplasmic reticulum (ER) (x 8000), (B) bile canaliculus (Bc) with compact intercellular junctional complexes (x 15,000); (C,D) DEN induced Preneoplasia – showing irregularly shaped N, disturbed nuclear envelope (x 4,000), (E) swollen M, sparse ER and (F,G) distended Bc and widened intercellular spaces (gaps, white arrows) (x 15,000)
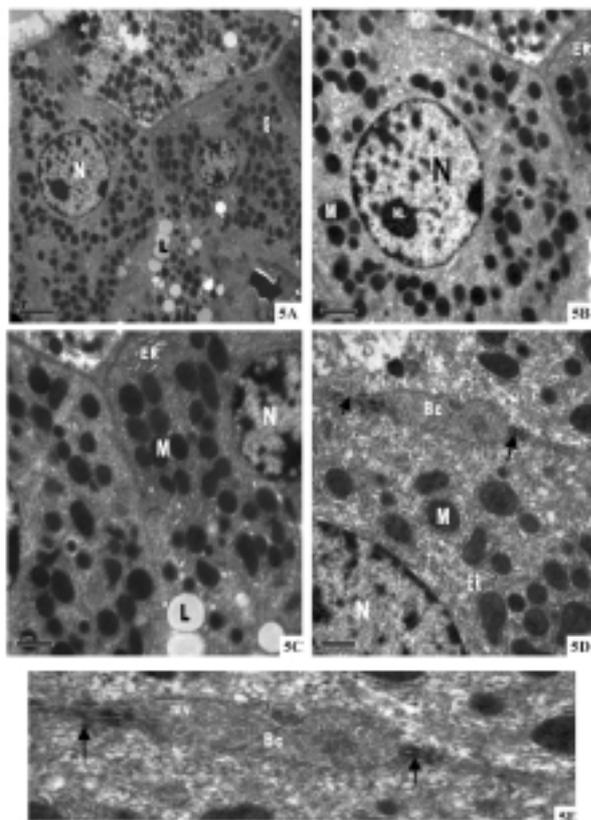
Figure 5. Electron Micrographs of Rat Liver showing Effects of Seed Extract 100 mg/kg on DEN induced Preneoplasia. (A,B,C) Oval N with NL, well-defined nuclear membrane, abundant ER closely packed around M, glycogen granules (G), lipids (L) at x 4, 000, x 8,000, x 12,000, respectively and (D,E) ER, intact Bc filled with microvilli (mv) and Gap junctions at x 15,000. The black arrows indicate intercellular gap junctions

membrane was clear with disappearance of serration. Cytoplasm showed rich distribution of ER, closely packed around the mitochondria. The RER had reappeared, was well organized and abundant. Cytoplasm was occupied by numerous round or oval shaped well defined mitochondria and glycogen granules and lipids. Membranes appeared compact and normal with narrowed intercellular space between adjacent cells (Figure 5A, B and C). Intact tight junctions. The junction is separated at the region of bile canaliculus lined by microvillous projections (Figure 5D and E). Seed extract treatment prevented the DEN induced degenerative changes to almost normal.

Discussion

The results of the present study indicates significant inhibitory effects of seed extract of S. rhombifolia ssp. retusa on the development of preneoplastic foci initiated with DEN and antihepatotoxic activity against CCl4 intoxication in the liver of rats.

Significant increase in serum enzyme activities viz; GPT, GOT, ALP, GGT, TG, CHO, TBIL and the fall in protein and albumin levels caused by DEN or CCl4 have been attributed to the damaged structural integrity of the liver and hepatocellular dysfunction (Sallie et al., 1991). However, treatment with seed extract caused significant

restoration of deficient functioning of these marker enzymes implicating its cytoprotective role by stabilizing action at the membrane level towards normal liver cell function.

Increasing evidence has implicated free radical mechanism in the initiation of carcinogenesis as well as CCl4 acute hepatic injury (Cerutti, 1994; Recknagel and Glende Jr, 1973). CCl_4 is metabolically activated by cytochrome P450 dependent mixed oxidases in endoplasmic reticulum to form a trichloromethyl free radical ($\bullet CCl_3$). Reactive oxygen species (ROS) have damaging effects upon the cells due to peroxidation of unsaturated lipids in biological membranes, interactions with DNA, or attack on enzymes and proteins (Recknagel and Glende Jr, 1973). Cell and tissue destruction leads to more lipid peroxidation because antioxidants are diluted out and transition metal ions that stimulate the peroxidation process are released from the disrupted cells. This increase in lipid conjugated dienes and hydroperoxides was counteracted significantly on treatment with seed extract administration. The cellular antioxidant, reduced GSH is an intracellular thiol rich in tripeptide playing a major role in the protection of cells and tissue structures. GSH content gets markedly depleted leading to LPO. Seed extract treatment enhanced GSH levels and the increase is possibly to control the toxic effects of reactive oxygen compounds. Also GSH either alone or in conjugation with other proteins has been found to protect the cell against LPO. Seed extract possibly due to its free radical scavenging property was capable in augmenting the deficient functioning of impaired enzymes of antioxidant defense system. Recently, Dhalwal et al. (2007) had reported root, leaves, whole plant and stem extracts of this plant for free radical scavenging, reducing power, superoxide anion scavenging, nitric oxide scavenging, and anti-lipid peroxidation activities in vitro.

Another set of precancerous marker enzymes; GGT and GST are known to identify preneoplastic hepatocyte population brought about maximum induction of GGT/ GST activities in DEN treated animals. GST actively participates in the detoxification process either through formation or excretion of GSH adducts and/or of glutathione disulphide (Cameron, 1988; Jeena et al., 1999). Feeding of seed extract before and after DEN treatment prevented increase in these marker enzymes activities.

The findings of biochemical observations are further supplemented by histopathological and ultrastructural analysis which implicates the degenerative changes induced by liver toxicants – DEN or CCl_4 treatment is prevented by administration of the seed extract. It exhibited marked reduction in incidence of preneoplastic lesions. The extract may play a role in maintenance and restoration of the damaged hepatocyte to the near normal hepatocyte. Electron microscopic studies, revealed profound ultrastructural alterations in the liver of rats treated with DEN. The early adverse effects of the hepatocarcinogen indicate manifestations of preneoplastic changes of the liver cell. In this study cytoplasmic degeneration, increased nuclear chromatin, swelling of mitochondria and absence of cell-to-cell contact disturbing the morphological structure were more prominent in DEN

administered animals in comparison to the control animals. However, the significance of these findings lies in the fact that these changes are minimal in animals treated with seed extract pre- and post- administration of DEN. Remarkable changes viz; enhanced cell to cell adhesion among adjacent cells, compact junctional complexes, restoration of morphological architecture and intimate cell contacts with well defined cell boundaries resembling to normal hepatocytes were noticed. This suggests that the seed extract helps in repair of cellular damage and prevents the formation of preneoplastic histopathological changes.

In conclusion, the chemopreventive and antihepatotoxic effects of seed extract are attributed for its suppression of lipid peroxidation, free radical scavenging activity, ability to induce GST and other phase II enzymes involved in carcinogen detoxification and maintenance of structural integrity of the hepatocyte. Therefore, the present study validates the potential usefulness of seeds as a promising hepatoprotectant. Further studies are needed to evaluate the real therapeutic value of this natural extract in terms of isolation and characterization of phytoconstituents.

Acknowledgements

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