International Journal of Biomedical and Pharmaceutical Sciences ©2009 Global Science Books

Effect of Andrographolide-Encapsulated Liposomal Formulation on Hepatic Damage and Oxidative Stress

Dilip Kumar Midya • Kartick Chandra Pramanik • Tapan Kumar Chatterjee*

Department of Pharmaceutical Technology, Jadavpur University, Kolkata 700032, West Bengal, India

Corresponding author: * **tkchatterjee81@yahoo.co.in, tkchatterjee_81@rediffmail.com**

ABSTRACT

Andrographolide (diterpinoid lactone) is the major active bitter glycoside obtained from *Andrographis paniculata* and has been found to possess remarkable hepatoprotective activity. We entrapped andrographolide into a liposomal formulation to facilitate the delivery of andrographolide to the liver and enhance the therapeutic efficacy during hepatic damage and stress. Liposomes were prepared with egg lecithin and cholesterol by a freeze drying method using a molar ratio of 4.8:1:1 and the optimal shape and drug content was found to be 44%. A significant increase in liver enzymes and bilirubin were found in CCl_4-treated animals when compared with the control group and this was significantly reversed by liposome- ($P<0.01$) and andrographolide ($P<0.5$)-treated groups. Total protein level decreased in CCl_4-treated animals when compared with control animals which was reversed by liposome- ($P<0.01$) and andrographolide ($P<0.05$)-treated animals. Thiobarbituric acid reactive substances (TBARS) increased significantly whereas antioxidants like glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) levels decreased in the liver homogenates of CCl_4-treated rats when compared with the animal control group. Treatment with andrographolide liposomes (10 mg/kg) successfully attenuated the effects of CCl_4 whereas the effect produced by andrographolide alone was less than that of the liposome-treated group of animals. This may be attributed to their enhanced aqueous solubility as well as physicochemical stability which prolong the activity of andrographolide in the liposomal formulation, which could be safer and more effective. Hepatic damage and oxidative stress in CCl_4-induced rats was significantly protected by the liposomal formulation.

Keywords: antioxidant, CCl_4, hepatoprotective, liver enzymes, liver biopsy

INTRODUCTION

Hepatocellular carcinoma is the fifth most common cancer in the world (Dakshayani *et al.* 2005). The major risk factors of this disease are Hepatitis B and C, viral infection, aflatoxine B_1 toxin and alcohol (Ragaa *et al.* 2004). CCl_4 is a well established hepatotoxin that has been reported to generate free radicals to produce deleterious effects on the cell membrane. These radicals react with biological molecules such as DNA, proteins and phospholipids and eventually destroy the structure of other membranes and tissues (Gomes *et al*. 1995). This hepatotoxin produces dysfunction of rat liver and morphology of the architecture of cells.

Andrographolide is a diterpinoid lactone obtained from *Andrographis paniculata.* Andrographolide is sparingly soluble in water (pers. obs.) and thus would be difficult to administer intravenously or intraperitonially. Liposomes, as slow release reservoirs, endocytose or phagocytose the more vascular liver cells and hence prolong the drug action and cause potentiation (Sinha *et al.* 2000). The outer phospholipid layer of liposomes protects andrographolide against degradation by oxidation, reduction and hydrolysis. To overcome the solubility problem, the present study was designed to evaluate andrographolide-encapsulated liposomal drug delivery for the treatment of liver disorders.

MATERIALS AND METHODS

Chemicals

Phosphatidyl choline (PC), cholesterol, glutaraldehyde, were purchased from Sigma-Aldrich Chemicals (St. Louis, MO). Andrographolide and CCl_4 were purchased from Sigma Chemicals and all other reagents like chloroform, methanol, α-tocopherol, Triton X-100, etc. used in this study were of analytical grade (from Merck Ltd. and Sisco Research Lab).

Animals

Adult male albino Wistar rats (8 weeks), weighing between 180–200 g were used. All animal experiments were approved by the Department animal ethical committee. The animals were allowed water *ad libitum* with normal laboratory pellet diet. Animals were maintained under constant conditions of 12 h light and 12 h dark cycle and at an environmental temperature of 21–23°C.

Preparation of liposomes

Andrographolide was incorporated in the liposomes by a modified thin-film hydration method (Tao *et al.* 2007). The hydrophobic excipients, such as lipids i.e., phosphatidylcholine, cholesterol and andrographolide at a molecular ratio of 4.8: 1: 1) were first dissolved in chloroform and methanol at a 1: 2 ratio and then dried in a rotary evaporator (Hahnvapor HS-2005 V) under an aspirate vacuum and a water bath with temperature maintained at 40°C. The thin film layer formed was flushed with nitrogen gas for 5 min and maintained overnight in a lyophilizer (Freeze Dryer, Instrumentation of India Laboratory) under vacuum to remove traces of chloroform and methanol. The thin film was resuspended in phosphate buffered saline (PBS, pH 7.4) containing polyethylene glycol (PEG) 400 (5% v/v) by rotating the flask at 250 rpm until the lipid film was completely hydrated. Then the liposomes were centrifuged at 28,000 rpm for 60 min to separate the supernatant. Later, the unentrapped andrographolide was removed from the liposome dispersion by centrifuging at 50,000 rpm for 30 min. The supernatant was discarded and the liposome pellets were washed twice with PBS. Liposome particles were then suspended in distilled water containing cryoprotectant (1.7 molar ratios to lipid)

Received: 12 January, 2009. Accepted: 26 April, 2009.

Original Research Paper

and 1 mM EDTA and were freeze-dried in a lyophilizer. The final liposome powders were stored in an air-tight container at 4°C for further experiments.

Particle size analysis

The liposome sample (5–10 µl) was placed in 400-mesh carbon coated copper grid (400 mesh carbon coated copper grid; Sigma-Aldrich, USA) and maintained for 1 min. The excess sample was soaked by blotting paper and 2% uranyal acetate was placed on the grid for 30 sec and again soaked by filter paper and observed under transmission electron microscope (TEM) [Tecnai Spirit (FFI), Japan] at 60 kb. The picture was recorded on a 2/2 CCD camera (Mega view, soft Imaging system, Tecnai Spirit (FFI)) (**Fig. 1**).

Drug encapsulation efficiency

The entrapment efficiency (EE) is defined as the ratio of the amount of the andrographolide encapsulated in the liposome to that of the total andrographolide in the liposome suspension. Briefly, according to Sinha *et al.* (2000), aliquots (0.1 ml each) of liposome suspensions diluted to 1.1 ml by PBS (pH 7.4) immediately after preparation were centrifuged at 1,000 rpm for 10 min to remove any andrographolide particle already released from the liposome. Then, 1 ml of liposome supernatant was precipitated by ultracentrifugation at 50,000 rpm for 30 min. After removing the supernatant by decantation, the precipitate (i.e., liposome pellet) was washed twice with PBS. The liposome pellets were then dissolved in 6 ml of 10% methanolic Triton X-100 and estimated for andrographolide content by HPLC. Andrographolide entrapment in liposomes was done by HPLC. The HPLC method described by Beaulieu *et al.* (2006) was used for the estimation of andrographolide entrapped in MLVs (multilayer vesicles) and ULVs (unilayer vesicles) with slight modifications. The HPLC system (JASCO PU-2089, UV-2075, Column Watres-250 × 4 mm SN-6820 ODS2 5 µm) was operated in a quaternary gradient pump with an intelligent UV/VIS detector. The analysis was performed at 223 nm with a reverse phase column maintained at 25°C (column oven) using a mobile phase of acetonitrile: methanol: water = 55: 30: 15, flow rate 1 ml/min and a sample volume of 20 µl was injected.

Encapsulation efficiency (EE) was calculated from:

Encapsulation efficiency (%) = [Amount of andrographolide in freeze-dried liposome (mg)/Amount of freeze-dried liposome (mg)] X 100

The EE and content were determined in three separately prepared liposome suspensions and were expressed as the mean ± standard deviation.

Animal experiment

The rats were divided into 4 groups each containing 6 animals. Group I rats served as control, Group II rats were injected intraperitoneally (i.p.) with 50% CCl_4 (dissolved in liquid paraffin) at a dose of 2 ml/kg body weight. Group III rats were injected with andrographolide-encapsulated liposomes at a dose of 10 mg/kg bw followed by intraperitonial injection of CCl_4 (2 ml/kg bw). Group IV rats were treated sequentially with andrographolide (4.4 mg/kg bw) and CCl_4 (2 ml/kg bw). The animals had free access to standard pellet diet and water. All the groups were treated for 14 days. CCl_4 was administered i.p. after every 72 h (Manoj and Aqueed 2003). After 24 h of the last administration of CCl_4 with 18 h fasting condition, on the 15th day the animals were sacrificed by cervical dislocation and blood samples, liver tissue were collected from the all animals for evaluation.

Preparation of tissue homogenate

Liver tissues were rinsed with ice-cold normal saline and the surface water was soaked with tissue paper. A small part of liver tissue of different group of animals was preserved in 10% formalin solution and the remaining portion was used to prepare the tissue homogenate.

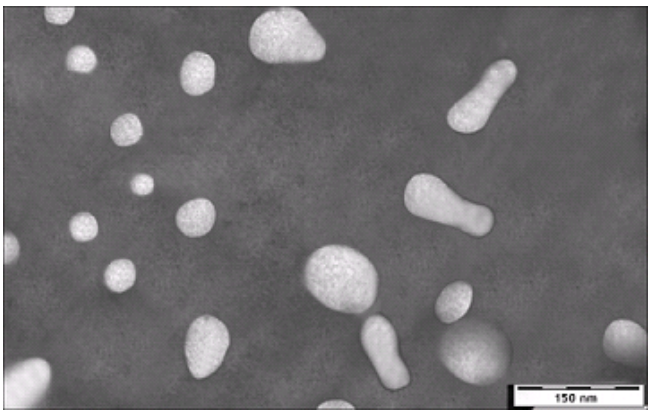

Fig. 1 Liposome retained by 100 kD cutoff membrane filter, washed, stained by 2% urinyl acetate and prepared for TEM. Observed at 60 kBx. 43,000 X magnification.

Post mitochondrial supernatant preparation (PMS)

Liver tissues were homogenized in chilled Tris buffer using a homogenizer. The homogenates were centrifuged at 2000 rpm for 5 min at 4°C to separate the nuclear debris. The supernatant so obtained was centrifuged at 10,000 rpm for 20 min at 4°C to get the post mitochondrial supernatant which was used for the assay of catalase (CAT), reduced glutathione (GSH) and superoxide dismutase (SOD) activities.

Biochemical investigations

Serum glutamate pyruvate transaminases (SGPT), serum glutamate oxaloacetate transaminases (SGOT), alkaline phosphate, total protein, serum urea and bilirubin were determined using an automatic analyzer by use of the Span diagnostic kit (Lab-Care Diagnostics Pvt. Ltd., Kolkata, India). Serum urea was determined by a mono enzyme batholmore test in a spectrophotometer (V-650; Jasco, Japan).

Estimation of lipid peroxidation

Lipid peroxidation in the liver homogenates was estimated calorimetrically according to the method of Nichans and Samuelsson (1968) and Jiang *et al.* (1992) by measuring the thiobarbituric acid reactive substances (TBARS). In brief, 0.1 ml of tissue homogenate (Tris-HCl buffer, pH 7.5) was treated with 2 ml of (in a 1: 1: 1 ratio) TBA-TCA-HCl reagent (thiobarbituric acid 0.37%, 0.25 N HCl and 15% TCA) and placed in a water bath at 95°C for 45 min and cooled at room temp. The absorbance of the clear supernatant was measured against a reference blank at 535 nm.

Assay of catalase (CAT)

CAT activity was determined colorimetrically at 620 nm and expressed as µmoles of H_2O_2 consumed/min/mg protein as described by Sinha (1972). The reaction mixture (1.5 ml) contained 1.0 ml of 0.01 M pH 7.0 phosphate buffer, 0.1 ml of tissue homogenate and 0.4 ml of 2 M H_2O_2. The reaction was stopped by the addition of 2.0 ml of dichromate-acetic acid reagent (5% potassium dichromate and glacial acetic acid were mixed in 1: 3 ratio).

Reduced glutathione (GSH)

Activity of reduced glutathione (GSH) in liver was assayed by the method of Ellman (1959). One ml of supernatant was treated with 0.5 ml of Ellman's reagent and 3.0 ml of phosphate buffer (0.2 M, pH 8.0). The absorbance was read at 412 nm. GSH was expressed as mg/100 g of tissue.

Superoxide dismutase (SOD)

Superoxide dismutase (SOD) activity was assayed by the method of Kono (1978). The assay system consisted of 0.1 mM EDTA, 50

mM sodium carbonate and 96 mM nitro blue tetrazolium (NBT). In the cuvette, 2 ml of the above mixture, 0.05 ml hydroxylamine and 0.05 ml of PMS (phenezine methosulphate) were mixed and the auto-oxidation of hydroxylamine was observed by measuring the absorbance at 560 nm.

Histological examination

Histological observation basically supported the results obtained from serum enzyme assays. Pieces of liver tissue were fixed in 4% formalin (10 ml of 40% formaldehyde made up to 100 ml with normal saline) overnight. Then the tissues were dehydrated with a graded concentration of alcohol, cleared with benzene and embedded with paraffin bath. The paraffin blocks were cut at 5 µm in a rotary microtome (Model: MT-1090A, Optik, City, Country) and the sections were stained with vangeison and mounted in Canada balsam on slides. Thus the slides were prepared for microscopical observation. The liver of CCl_4-intoxicated rats showed massive fatty changes, gross necrosis, and broad infiltration of lymphocytes and Kupffer cells around the central vein and loss of cellular boundaries. We followed an arbitrary scale for determining the degree of liver damage: higher damage = >50% damage, moderate damage = <30% damage, lowest damage = <5-10% damage. The histological pattern of rat livers pretreated with andrographolide-encapsulated liposome and subsequently given CCl_4 showed a more or less normal lobular pattern with the least degree of fatty changes, necrosis and lymphocyte infiltration, which was almost comparable to the normal control group.

Estimation of protein

Protein content was estimated by the Biuret method using bovine serum albumin as the standard at 540 nm.

Statistical analysis

The experimental results were expressed as mean ± SEM. Data were assessed by the method of analysis of ANOVA followed by Dunnett's *t*-test. P<0.05 was considered as statistically significant.

RESULTS

Particle size was analyzed by TEM. Optimum size and shape at a molar ratio of PC: CH 4.8: 1 was obtained (**Fig. 1**). Drug entrapment efficiency was estimated by HPLC. The encapsulation depends upon the lipid molar ratio. At a lipid molar ratio of PC: CH 4.8: 1 drug encapsulation was 44% (**Table 1**), an efficient level compared to the value (46%) reported by Sinha *et al.* (2000).

There was a significant increase in the serum SGPT, SGOT, bilirubin and serum urea levels in CCl_4 treated animals when compared to the control group, and this was significantly decreased in the animals treated with the liposome (P<0.01) and andrographolide (P<0.05). The total protein level was lower in CCl_4 group as compared with normal group whereas protein level significantly increased in liposome and andrographolide treated animals. There was a significant (P<0.05) increase in the serum alkaline phosphate in CCl_4 control group compared with the normal group, which was reduced after administration of andrographolide liposome in the CCl_4-treated group (**Table 2**).

CCl_4 challenge caused a marked increase in lipid peroxidation as evidenced by a significant increase in the TBARS level in both the liver tissues when compared to the control group. The TBARS level of both the andrographolide liposome at 10 mg/kg bw and andrographolide at 4.4 mg/kg bw were significantly (P<0.01, P<0.05, respectively) decreased compared to the CCl_4 treated group. SOD, CAT and reduced glutathione (GSH) levels were lower in the CCl_4-treated group compared to the control group. The levels of SOD, CAT and GSH enzymes in the liver tissue of liposomal formulation- and andrographolide-treated animals were significantly higher (P<0.01, P<0.05, respectively) than the CCl_4-treated group of animals and the level was nearly that of normalcy (**Tables 3, 4**).

A histological study shows a normal liver (**Fig. 2**) and treatment with CCl_4 produced a higher degree of damage to the normal architecture (**Fig. 3**) as characterized by ballooning degeneration, hydrophobic degradation with complete damage. Liposome-treated liver produced the lowest degree of damage (**Fig. 4**) as characterized by a decrease in ballooning degeneration, hydrophobic degradation than andrographolide alone (**Fig. 5**).

Table 1 Estimation of drug encapsulation in liposome formulations.

No. of formulations	Formulation code	Lipid composition	Unit (mol)	Encapsulation efficiency (%)
1	MLV-1	PC:CH:D	1:1:1	12 ± 0.31
2	MLV-2	PC:CH:D	1.5:1:1	13 ± 0.52**
3	MLV-3	PC:CH:D	2:1:1	18 ± 0.41*
4	MLV-3	PC:CH:D	3:1:1	26 ± 0.74*
5	MLV-4	PC:CH:D	4.8:1:1	44 ± 0.63*
6	MLV-1	PC:CH:D	5.5:1:1	43.5 ± 1.21*

PC, phosphotidyl choline; CH, cholesterol; D, drug (andrographolide).
*P<0.01, **P>0.05 vs. MLV-1. Values are expressed as mean ± SEM (n = 4). The results were analyzed by ANOVA followed by Dunnett's test.

DISCUSSION

Today the task of avoiding undesirable drug actions on normal organs and tissues and minimizing side effects of the

Table 2 Effect of andrographolide-encapsulated liposome and andrographolide on serum enzyme (SGPT, SGOT and SALP), bilirubin and total protein in CCl_4-intoxicated rats.

Parameters	Control	CCl_4-treated (2 ml/kg bw)	Liposome-treated (10 mg/kg bw)	Andrographolide (4.4 mg/kg bw)
SGPT (U/L)	51 ± 1.15	7792 ± 780.43	540 ± 24.52 a	1298 ± 211.61 b
SGOT (U/L)	126 ± 2.93	4837 ± 314.51	462 ± 32.23 a	1170 ± 223 b
SALP (U/L)	192 ± 2.96	480 ± 7.82	211 ± 3.47 a	297 ± 4.95 b
TP (mg/dl)	6.4 ± 0.32	4.7 ± 0.24	5.3 ± 0.51 b	4.9 ± 0.32 b
Bilirubin (mg/dl)	0.09 ± 0.01	2.18 ± 0.30	0.9 ± 0.07 a	1.2 ± 0.18 b
Serum urea (mg/dl)	213.34 ± 3.01	1720 ± 40	253.67 ± 62 a	1226.67 ± 162 b

ANOVA followed by Dunnett's test, [a] P<0.01, [b] P<0.05 when compared with treated (CCl_4) group.
SGPT, serum glutamate pyruvate transaminase; SGOT, serum glutamate oxaloacetate pyruvate transaminase; SALP, serum alkaline phosphate

Table 3 Effect of andrographolide encapsulated liposome and andrographolide on anti oxidants and lipid peroxidation in liver.

Parameters	Control	CCl_4-treated (2 ml/kg bw)	Liposome-treated (10 mg/kg bw)	Andrographolide (4.4 mg/kg bw)
Lipid per oxidation (nmole/mg protein)	1.2 ± 0 .1	5.9 ± 0.4	2.1 ± 0.3 a	3.9 ± 0.6 b
Catalase (U^A/mg protein)	187.4 ±12.5	95.6 ± 8.3	152 ± 3.54 a	102 ± 6.32 b
SOD (µ/mg protein)	48 ± 6.3	22.7 ± 5.2	39 ± 4.1 a	32 ± 2.2 b
GSH (mg/100gm tissue)	26.5 ± 3	5.32 ±.89	19.3 ± 2.5 a	12.6 ±1.7 b

ANOVA followed by Dunnett's test, [a] P<0.01, [b] P<0.05 when compared with treated (CCl_4) group.
U^A: µ mole H_2O_2 consumed/min.

Table 4 Effect of andrographolide encapsulated liposome and free drug andrographolide on antioxidants and lipid peroxidation in kidney.

Parameters	Control	CCl_4-treated (2 ml/kg bw)	Liposome-treated (10 mg/kg bw)	Andrographolide (4.4 mg/kg bw)
LOP (nmole/mg protein)	1.68 ± 0.3	4.01 ±0.2	2.12 ± 0.13 a	3.05 ± 0.4 b
Catalase (U^A/mg protein)	152.34 ± 12.5	90.65 ± 7.98	139.43 ± 10.2 a	102.32 ± 9.7 b
SOD (μ/mg protein)	42.79 ± 2.5	23.91 ± 4.2	37.68 ± 4.1 a	29.93 ± 2.1 b
GSH (mg/100 g tissue)	7.97 ± 1.2	5.72 ± 0.9	7.19 ± 0.5 a	6.01 ± 0.43 b

ANOVA followed by Dunnett's test, [a] $P<0.01$, [b] $P<0.05$ when compared with treated (CCl_4) group.
U^A: μmole H_2O_2 consumed/min

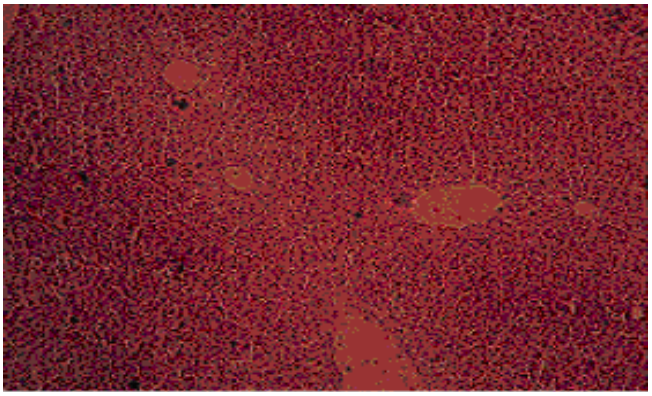

Fig. 2 Animal normal architecture of rat liver (hematoxyine and eosine). 100X magnification.

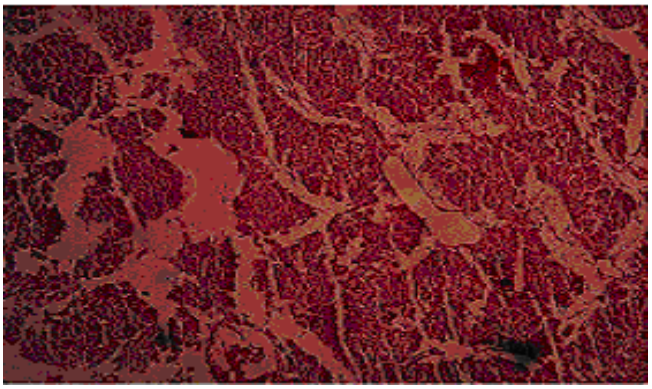

Fig. 3 Liver section of CCl_4 (2 ml/kg)-treated animals shows the liver cells of rat having a higher degree (>50%) of damage, characterized by gross ballooning degeneration, hydrophobic degradation with complete damage of normal architecture of rat liver (hematoxyine and eosine). 100X magnification.

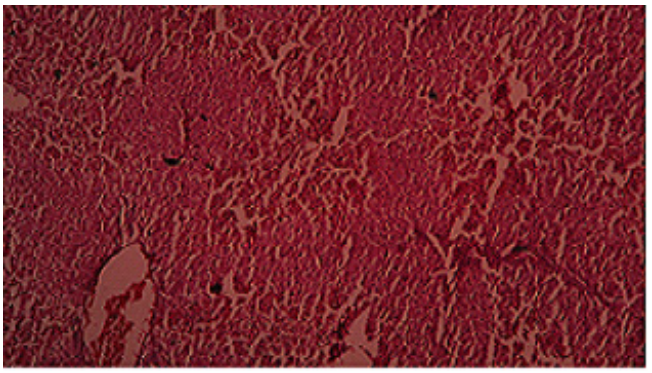

Fig. 4 Liver section of liposome-treated CCl_4 (2 ml/kg)-induced animals shows the liver cells of rat have the lowest degree (<5-10%) of damage, characterized by less ballooning degeneration, hydrophobic degradation with least damage of normal architecture of rat liver (hematoxyine and eosine). 100X magnification.

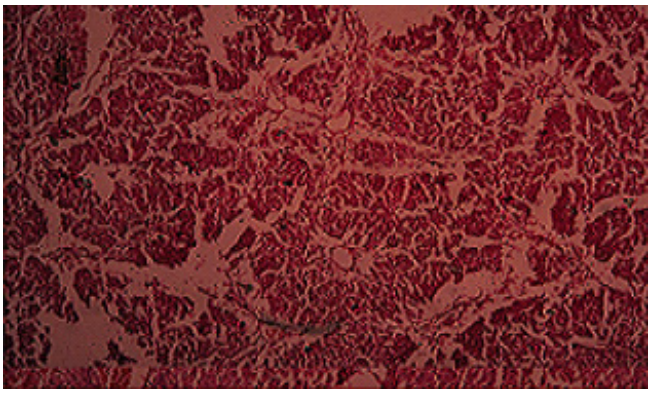

Fig. 5 Liver section of CCl_4 (2 ml/kg)-induced animals treated with andrographolide shows the liver cells of rat have a moderate degree (<30%) of damage, characterized by the ballooning degeneration, hydrophobic degradation with modarate damage of normal architecture of rat liver (hematoxyine and eosine). 100X magnification.

therapy is very important. Thus, screening biologically active compounds is necessary to permitting the choice of drugs with selective action on the appropriate organs or tissues. Liposomes show great potential to effectively deliver drugs to the site of action and of control the release of these drugs at a predetermined rate (Basu 2005). The main objective of this study was to prepare andrographolide liposome for better action against liver cirrhosis induced by CCl_4.

The liver is an important organ actively involved in metabolic functions and is a frequent target of a number of toxicants. Carbon tetrachloride (CCl_4) has been widely used for inducing experimental hepatic damage due to free radical formation during its metabolism by hepatic microsome, leading to lipid peroxidation, and consequently, liver damage. The resulting hepatic injury is characterized by leakage of cellular enzymes into the blood stream and by necrosis and fibrosis. CCl_4 induces liver cell necrosis and apoptosis, and can be used to induce hepatic fibrosis or cirrhosis by repetitive administration (Chang *et al.* 2005; Constandinou 2005). Slight to moderate increases in ALP (1-2 times normal) occurred in liver disorders (Sinha *et al.* 2000). Serum cholesterol is a general indication of the synthetic and general metabolic capacity of the liver (Yim *et al.* 2006). In the present study, CCl_4 induction significantly increased serum ALT, AST, ALP and cholesterol levels, indicating induction of hepatic damage (Yim *et al.* 2006). Andrographolide-encapsulated liposome (10 mg/kg bw) significantly reduces the hepatic cirrhosis by decreasing the level of ALT, AST, ALP, bilirubin and serum urea whereas the free form of andrographolide (4.4 mg/kg bw) does not produce such significant effects.

GSH is a crucial determinant of tissue susceptibility to oxidative damage, and the depletion of hepatic GSH content has been shown to be associated with enhanced toxicity to chemicals, including CCl_4 (Yim *et al.* 2006). Elevated lipid peroxide levels and a decrease in SOD and GSH levels in liver tissue due to CCl_4 toxicity were markedly attenuated by liposomal treatment. This suggested that the maintenance of structural integrity of the hepatocytic cell membrane or regeneration of damaged liver cells by inhibiting lipid peroxidation were the pharmacological activities of andrographolide-encapsulated liposome. These findings were corroborated with histopathological studies. We have followed

an arbitrary scale for determing the degree of liver damage. Thus, andrographolide-encapsulated liposomal formulation can be considered an effective hepatoprotective and good antioxidant (i.e. compared to CCl_4 control). The altered balance of the antioxidant enzymes caused by a decrease in CAT, SOD, and GSH activities may be responsible for the inadequacy of the antioxidant defenses in combating reactive oxygen species-mediated damage.

It can be concluded that increased level of hepatic enzymes and free radicals are associated with decreased antioxidant status in liver of rats treated with CCl_4. Treatment with andrographolide-encapsulated liposome strongly prevent hepatic disorders by scavenging the free radicals generated during toxic CCl_4 administration. Detailed studies should be done in the future to confirm our preliminary data.

ACKNOWLEDGEMENTS

The authors wish to thanks University Grants Commission (UGC), New Delhi for the financial assistance rendered.

REFERENCES

Aragno M, Brignardello E, Tamagno O, Boccuzzi G (1997) Dehydroeppiandrosterone administration prevents the oxidative damage induced by acute hyperglycemia in rats. *Journal of Endocrinol*ogy **155**, 233-240

Basu MK (2005) Liposomal delivery of antileishmanial agent. *The Journal of Applied Research* **5**, 221-236

Beaulieu JM, Robertson J, Doroudchi MM, Durham HD (2006) Protective effect of *Andrographis paniculata* and andrographolide on cyclophosphamide-induced Urothelial Topxicity. *Integrative Cancer Therapy* **5**, 244-251

Camandola S, Aragno M, Cutrin JC, Tamagno E, Danni O, Chiarpotto E, Parola M, Leonarduzzi G, Biasi F, Poli G (1999) Liver AP-1 activation due to carbon tetrachloride is potentiated by 1,2-dibromoethane but is inhibited by alpha-tocopherol or gadolinium chloride. *Free Radical Biology and Medicine* **26**, 1108-1116

Chang ML, Yeh CT, Chang PY, Chen JC (2005) Comparison of murine cirrhosis models induced by hepatotoxin administration and common bile duct ligation. *World Journal of Gastroenterology* **11**, 4167-4172

Constandinou C, Henderson N, Iredale JP (2005) Modeling liver fibrosis in rodents. *Methods of Molecular Medicine* **117**, 237-250

Dakshayani KB, Subramanian P, Manivasagam T, Mohamed Essa M, Manoharan S (2005) Melatonin modulates the oxidant antioxidant imbalance during *N*-nitrosodiethylamine induced hepatocarcinogenosis in rats. *Journal of Pharmacy and Pharmaceutical Sciences* **8**, 316-321

Durk H, Frank H (1984) Carbon tetrachloride metabolism *in vivo* and exhaletion of volatile alkanes: dependence upon oxygen partial pressure. *Toxicology* **30**, 249-257

Ellman GL (1959) Tissue sulfhydryl groups. *Archives of Biochemistry and Biophysics* **82**, 70-77

Gomes EM, Souto PR, Felzenszwalb I (1996) Shark-cartilage containing preparation protects cells against hydrogen peroxide induced damage and mutagenesis. *Mutation Research* **367**, 204-208

Jiang ZY, Hunt JV, Wolff SP (1992) Ferrous ion oxidation in the presence of xylenol orange for detection of lipid hydroperoxide in low-density lipoprotein. *Analytical Biochemistry* **202**, 384-387

Kono Y (1978) Generation of superoxide radical during autoxidation of hydroxylamine and an assay for superoxide dismutase. *Archives of Biochemistry and Biophysics* **186**, 189-195

Manoj B, Aqueed UK (2003) Protective role of *Lawsonia alba* Lam against CCl_4-induced hepatic damage in albino rats. *Indian Journal of Experimental Biology* **41**, 85-87

Mansuy D, Fontecane M, Chottard J (1980) A heme models study of carbon tetrachloride metabolism: mechanism of phosgene and carbon dioxide formation. *Biochemical and Biophysical Research Communications* **95**, 1536-1542

Moskovitz J, Yim MB, Chock PB (2002) Free radicals and disease. *Archives of Biochemistry and Biophysics* **397**, 354-359

Navarro J, Obrador E, Carretero J, Petschen I, Avino J, Perez P, Estrela JM (1999) Changes in glutathione status and the antioxidant system in blood and in cancer cells associate with tumor growth *in vivo*. *Free Radical Biology and Medicine* **26**, 410-418

Niehius WG, Samuelsson D (1968) Formation of malondialdehyde from phospholipid arachidonate during microsomal lipidperoxidation. *European Journal of Biochemistry* **6**, 126-130

Pohl L, Schulick R, George J (1984) Reductive oxygenation mechanism of metabolism of carbon tetrachlorideto phosgene by cytochrome P450. *Molecular Pharmacology* **25**, 318-324

Ragaa EO, Anneleen K, Abdelgadir MYK, Frans JK, Mohammed OI, Ibrahim MEK, Pieter VTV (2004) Population-attributable risk of dietary aflatoxins and hepatitis B virus infection with respect to hepatocellular carcinoma. *Nutrition and Cancer* **48**, 15-21

Recknagel RO (1983) Carbon tetrachloride hepatotoxicity: Status quo and future prospects. *Trends in Pharmacological Sciences* **4**, 129-131

Recknagel RO, Ghoshal AK (1966) Lipoperoxidation as a vector in carbon tetrachloride hepatotoxicity. *Laboratory Investigation* **15**, 132-148

Sinha J, Mukhopadhyay S, Das N, Basu MK (2000) Targeting of liposomal andrographolide to *L. donovani*-infected macrophages *in vivo*. *Drug Delivery* **7**, 209-213

Sinha KA (1972) Colorimetric assay of catalase. *Analytical Biochemistry* **47**, 389-394

Yang T, Cui FD, Choi MK, Lin H, Chung SJ, Shim CK, Kim DD (2007) Liposome formulation of Paclitaxel with enhanced solubility and stability. *Drug Delivery* **14**, 301-308

Yim KY, Lee H, Hong EK, Kim IY, Lee RB, Kim HT, Yi YJ (2006) Hepatoprotective effect of manual acupuncture at acupoint GB34 against CCl_4-induced chronic liver damage in rats. *World Journal of Gastroenterology* **12**, 2245-2249