Chapter 2

Clerodendron glandulosum.Coleb leaf extract ameliorates high fat diet/fatty acid induced lipotoxicity in experimental models of non alcoholic steatohepatitis

INTRODUCTION

Non-alcoholic steatohepatitis is a pathological condition characterized by accumulation of lipids in the liver of non alcoholic individuals and consequent oxidative stress leading to cirrhosis of liver in the long run (Duvnjak *et al.*, 2007). It has received clinical importance only recently after a long lag phase of ignorance mainly due to its asymptotic nature, lack of relevant diagnostic tests and erroneous misinterpretation with hepatitis (Dabhi *et al.*, 2008). Incidence of NASH in USA is 30% of the adult population (Bellentani and Marino, 2009). Number of individuals being diagnosed with NASH even in Asian countries appears to be on the increase (Amarapurkar *et al.*, 2007). Obese individuals are at maximum risk of developing NASH (Duvnjak *et al.*, 2007) and a strong positive correlation exists between insulin resistance (IR) and development of NASH (Chitturi *et al.*, 2002) and, the same has been validated for animals as well (Chidambaram and Venkatraman, 2010).

Despite the increasing understanding of non-alcoholic fatty liver conditions, diagnosis and staging of NASH remain to-date a difficult proposition. The only acceptable mode of diagnosis of NASH that is valid at present is a consideration of medical history along with a liver biopsy. Though liver biopsy alone represents an unequivocal way of staging NASH, it is nevertheless beset with limitations of either over-estimating or under-estimating the degree of disease progression (Ratziu *et al.*, 2005). Due to the many caveats associated with this technique apart from its invasiveness, reliable alternate non-invasive methods need to be pursued for effective diagnosis and staging of NASH. Though many techniques are proposed, none finds clinical acceptance to-date (Erickson, 2009). Diagnosis of NASH usually requires testing for liver biochemistry, as most such cases stand diagnosed subsequent to an

evaluation of abnormal liver function tests and/or ultrasound or computed tomography scans indicating a fatty liver status. Ultrasonography, computed tomography, magnetic resonance imaging, and radionucleotide techniques, are routine techniques employed to characterize hepatic steatosis. Ironically, none of these techniques helps distinguish between simple steatosis and steatohepatitis with progressive fibrosis. NASH being essentially a clinicohistologic entity, histology is of prime importance to confirm the diagnosis (Lewis and Mohanty, 2010).

The level of aminotransferase activity seems typically increased by four times than in alcoholic liver disease with ALT activity being higher than AST. Though bilirubin level remains in the normal range, doubled level of alkaline phosphatase activity appears to be the feature (Charlton, 2004). Detailed history of patients with abnormal liver biochemistry is necessary to exclude the possibilities of excessive alcohol consumption, steatohepatitis inducing pharmacotherapy, surgical procedures and occupational exposure to hepatotoxins, along with a nutritional history (particularly of rapid weight gain or loss), essentially to over-rule clinical conditions associated with steatohepatitis. Some other associated clinical conditions whose exclusion is impossible by simple history include Wilson's disease, viral hepatitis and autoimmune liver disease, thereby requiring serologic/biochemical exclusion. Very often, majority of NASH patients show one or more features of metabolic syndrome such as, increased waist circumference, hypertriglyceridemia, low HDL cholesterol, hypertension and a fasting glucose of 110 mg/dl or higher (Charlton, 2004). It is however not clear as to how far these symptoms find specific association with NASH. A caveat however is that, aminotransferase elevations though used to diagnose NASH, however lack adequate sensitivity to detect patients with NASH and are

entirely nonspecific in predicting liver injury. NASH though characterized by focal areas of fat in the liver can however prove to be irksome due to its inability to distinguish NASH from primary malignancies or metastasis and fine needle aspiration may be required to exclude malignancy. It is also worth noting the possibility of having NASH, especially the chronic progressive from, even in the backdrop of apparently normal values of liver function tests and mild fatty liver. Further complexity in the diagnosis of NASH is its non-obligatory association with obesity, MetS or T2D. Apparently, individuals free of these conditions can, and in fact, do develop NASH. Moreover, not all individuals who are obese or have MetS or T2D develop progressive NASH.

At present, no FDA or EMEA approved drug exists against NASH, as there is no report on any agent with proven benefit. To a certain extent, drugs that reduce insulin resistance such as metformin and thiazolidinediones (rosiglitazone, pioglitazone) seem to be of some promise (Angelico *et al.*, 2007; Tahan *et al.*, 2007) as, few studies on these drugs have shown some improvement in histological manifestations of NASH (Belfort *et al.*, 2006). In general, all drugs that induce weight loss might be beneficial against NASH, in particular when diet and life-style modifications do not work. Both sibutramine and orlistat have shown improvement in some characteristics of NASH such as the sonographically visible degree of liver steatosis and histologically observable degree of steatosis and fibrosis (Harrison *et al.*, 2004; Hussein *et al.*, 2007). Though, some preliminary data tend to suggest efficacy of metformin and glitazones in improving liver histology in patients with non-diabetic NASH, their routine use is not recommendable at present. Some preliminary studies have also tested probiotics and various cytoprotective drugs like ursodesoxycholic

acid (UDCA), antioxidants like vitamin E, anti TNF α agents like pentoxiphylene and anti-fibortic drugs like losartan but with no success in terms of clinical application (Lirussi *et al.*, 2007; Velayudham *et al.*, 2009). There were also some proposals for use of antioxidants and cytoprotective substances like vitamin E, vitamin C, glutathione, betaine, acetylcysteine, S-adenosyl-L-methionine and ursodesoxycholic acid in treating cases of NAFLD and NASH. However, none of these substances showed any promise after a recent Cochrane analysis of validated randomized studies (Lirussi *et al.*, 2007).

Most of the synthetic lipid lowering drugs are efficient in the management of hyperlipidemia and obesity but are of negligible therapeutic value against NASH (Nakamoto *et al.*, 2009). Hence, it is a major challenge for pharmaceutical industry to develop a combination therapy that is effective against NASH in obese and IR individuals. Herbal medicines are becoming increasingly popular and being looked up to for management of hyperlipidemia, obesity and IR, primarily because of their minimal side effects and their multiple modes of action in controlling lipid metabolism. Recently, phytochemical extract of *Eriobotrya japonica* seed (Yoshioka *et al.*, 2010), Olive leaf (Omagari *et al.*, 2010), *Avena sativa L* (Cai *et al.*, 2011), *green tea* (Park *et al.*, 2011), etc. have been shown to be effective in controlling these metabolic disorders including NASH, mainly due to their potent hypolipidemic and antioxidant properties. World Health Organization has also recommended use of herbal medicines in lieu of synthetic counterparts in keeping with their fewer side effects, low cost and multiple therapeutic uses (Calixto, 2000).

Aim: - To evaluate the protective role of *C.glandulosum*.Coleb leaf aqueous extract against experimentally induced non-alcoholic steatohepatitis using *in vitro* and *in vivo* experimental models.

MATERIALS AND METHODS

Plant, preparation of extract and phytochemical analysis

As mentioned in chapter 1

Experimental Animals

Male C57BL/6J mice (6-8 weeks of age) were purchased from National Centre for Laboratory Animal Sciences (NCLAS), National Institute of Nutrition (NIN), Hyderabad, INDIA. They were housed and maintained in clean polypropylene cages and fed with either low fat diet or high fat diet and water *ad libitum*. The experimental protocol was carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India and approved by the animal ethical committee of Department of Zoology, The Maharaja Sayajirao University of Baroda, Vadodara (Approval No.827/ac/04/CPCSEA) .

Experimental Design

A total of 24 mice were randomly allocated into 4 groups of 6 animals each. Group I (CON) consisted of mice fed with low fat diet (regular fat diet). Group II (NASH) was fed with high fat diet (Bose *et al.*, 2008). Group III (NASH+CG1) and Group IV (NASH+CG3) consisted of mice fed with high fat diet containing 1% and 3% (w/w) of CG extract (Table. 1) respectively. All animals were fed with their respective diets for 16 weeks.

At the end of the experimental period, overnight fasted animals were given mild ether anaesthesia and whole blood was collected by retro orbital sinus puncture in EDTA coated vials. Plasma was obtained by cold centrifugation (4°C) of the vials for 10 min at 3000 rpm. Later animals were sacrificed by cervical dislocation and, liver, heart, pancreas, kidney and epididymal fat pad were excised and stored at -80°C (Cryo Scientific Ltd, India) for further evaluations.

Plasma and hepatic lipids: - as mentioned in chapter 1

Plasma markers of hepatic damage

Plasma levels of AST (EC 2.6.1.1) and ALT (EC 2.6.1.2) were measured using commercially available kits (Reckon diagnostics, Baroda, India).

Isolation of hepatic mitochondria

Hepatic tissue of different experimental groups was washed with the isolation medium (0.25 M sucrose, 10 mM Tris-HCl buffer, pH 7.4, 1 mM EDTA and 250 µg BSA/ml) and 10% (w/v) homogenates were prepared using a Potter-Elvehjem type glass-Teflon homogenizer. The nuclei and cell debris sedimented by centrifugation at 1500 rpm for 10 min at 0°C was discarded. Further centrifugation of the supernatant at 10000 rpm for 10 min at 0°C yielded mitochondrial pellet. The same washed by suspending gently in isolation medium was re-sedimented at 8000 rpm for 10 min. The resultant mitochondrial fraction (MF) suspended in isolation medium was then used for biochemical assays (Patel and Katyare, 2006).

Mitochondrial reactive oxygen species and oxidative stress markers

- The assay was as per the method of Mishra *et al.* (2008). Briefly, MF (as prepared above) was first diluted to 0.25% with the isolation buffer to a final volume of 2 ml and divided into two fractions. In one fraction, 40 μL of 1.25 mM 2′-7′-dichlorofluorescin diacetate (DCF-DA; Sigma-Aldrich Ltd) prepared in methanol was added for reactive oxygen species estimation, while only 40 μL of methanol was added to the other fraction that served as a control for tissue auto-fluorescence. All samples were incubated for 15 min in a 37 °C water bath. The fluorescence was determined at 488 nm excitation and 525 nm emission using a spectroflourimeter (Jasco FP-6350). Liver mitochondrial ROS readings were expressed as arbitrary **fluorescence intensity units (FIU at 530 nm).**
- Total thiol (-SH) content was measured using DTNB as the coloring reagent. This reagent reacts with -SH groups to produce a yellow colored complex with a peak absorbance at 412 nm (Ellman, 1959).
- Mitochondrial lipid hydroperoxides (LOOH) was estimated using FOX reagent (250 µM ammonium sulphate, 100 µM xylenol orange, 25 mM H_2SO_4 and 4 mM BHT in 90% (v/v) HPLC-grade methanol) as described by Nourooz-Zadeh et al. (1996). The absorbance was read at 560 nm and LOOH content was determined using the molar absorption coefficient of $4.3\times10^4\ M^{-1}cm^{-1}$.

Measurement of hepatic mitochondrial lipid peroxidation and antioxidants

- Hepatic mitochondrial lipid peroxidation (LPO) was determined by estimating malondialdehyde (MDA) content using thiobarbituric acid (TBA) reactive substances as per the method of Buge and Aust (1975). Commercially available 1, 1, 3, 3-tetraethoxypropane (Sigma-Aldrich Ltd) was used as a standard for calculation of MDA content.

- Reduced glutathione (GSH) content in MF was measured spectrophotometrically using Ellman's reagent (DTNB) as a colouring reagent, as per the method described by Beutler *et al.* (1963). Ten percent homogenate was mixed with precipating reagent and incubated for 5 min at room temperature. It was then centrifuged at 3000 rpm for 15 min, the supernatant was mixed with phosphate solution and DTNB was added.

- Superoxide dismutase (SOD, EC 1.15.1.1) in the MF was assayed by the method of Kakkar *et al.* (1984) involving assay of NADH-PMS-NBT formazan complex. A mixture of phosphate buffer (0.052M), PMS (186 μM), NBT (30μM), NADH (780 nM) and 10% homogenate was incubated for 90sec at 37°C. Acetic acid and n butanol were added, shaken vigorously followed by centrifugation at 2000 rpm for 10 min and read at 560nm.

- Catalase (CAT, EC 1.11.1.6) activity in MF was measured spectrophotometrically at 240 nm by calculating the rate of degradation of hydrogen peroxide (Aebi, 1974). Ten percent homogenate was mixed with H_2O_2 (7.5 mM) and read at 240nm for 3 min at 30 sec interval.

- Glutathione-S-Transferase (EC, 2.5.1.18) activity in MF was assayed by the method of Habig *et al.* 1974. A mixture of sodium phosphate buffer, reduced glutathione (1mM), CDNB (1mM) and 10% PMS in a total volume of 2ml was read at 340nM for 2 min at 30 sec interval. Calculated enzyme activity was expressed as nmol CDNB conjugates formed/min/mg protein using a molar extinction coefficient of $9.6\times10^{3}M^{-1}\ cm^{-1}$.

- Glutathione reductase (GR, EC 1.11.1.9) activity in MF was assayed by the method of Mohandas *et al.* (1984). The enzyme activity was quantitated at 25°C by measuring the disappearance of NADPH at 340 nm, and was calculated as nmol NADPH oxdized/min/mg protein using a molar extinction coefficient of $6.223\times10^{3}M^{-1}\ cm^{-1}$.

- Glutathione peroxidase (GPx, EC 1.11.1.9) activity in MF was assayed by the method of Rotruck *et al.* (1973) based on the reaction between glutathione remaining after the action of GPx and DTNB to form a complex. To a well shaken mixture of phosphate buffer (0.3M), sodium azide (10mM), reduced glutathione (4mM) and 10% homogenate, H_2O_2 (0.2mM) and distilled water were added. It was then incubated for 10 min at 37°C and 10% TCA was added followed by centrifugation at 3000 rpm for 10 min. The supernatant was mixed with phosphate buffer and DTNB was added and read at 412 nm.

Macroscopic and microscopic examination of liver

Fatty liver was initially diagnosed by altered coloration (pink color due to lipid accumulation) and photographed *in situ* and later harvested, rinsed in 0.9 % Nacl, and weighed. Left lobe of the liver was fixed in 4% buffered paraformaldehyde and processed for preparation of paraffin-embedded tissue sections (4 μm) and stained with hematoxylin and eosin (HE) according to the standard protocol; the rest of the tissue was stored at -80°C for further analysis.

Maintenance of HepG2 cells

Human hepatocellular carcinoma cells (HepG2) obtained from National Centre for Cell Sciences, Pune, India, were seeded (1×10^{5}cells/25mm T Flask) and cultured in

Dulbecco's Modified Eagle's Medium (DMEM) from Himedia Pvt Ltd, Mumbai, India, containing 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution (10X) at 37°C with 5% CO_2 (Thermo scientific, forma II water jacketed CO_2 incubator). Cells were subsequently passaged every third day by trypsinization with 0.25 % Trypsin-EDTA (Himedia Pvt Ltd, Mumbai, India) solution.

Qualitative analysis of in vitro NASH

HepG2 cells were cultured in a 96-well microplate (Tarson India, Pvt, Ltd) at 5×10^3 cells/well and treated with 2.0 mM oleic acid (OA) in presence or absence of CG extract (20-200 µg/ml). At the end of treatment period (24 hr), cells were fixed in buffered paraformaldehyde for 10 min and washed with PBS twice. Fifty µl of Oil red O (ORO) solution (1% in isopropanol) was then added to each well and incubated at room temperature for 10 mins. After removing the ORO solution from each well, the cells were washed with PBS until the solution became clear. Wells were dried, mounted in glycerine and examined under a Leica DMIR inverted microscope (Cui *et al.*, 2010) and photographed with a canon Power Shot *S* 72 digital camera.

Quantitative analysis of in vitro NASH

HepG2 cells were maintained as described above and fixed in 4% buffered paraformaldehyde. After washing and drying completely, 100 µl of isopropanol (100%) was added to each well, incubated for 10 min and then transferred to another 96-well plate and was read at 405 nm (Cui *et al.*, 2010) using ELX800 Universal Microplate Reader (Bio-Tek instruments, Inc, Winooski, VT).

Cell viability and cytotoxicity assay

For cell viability assay, HepG2 cells were maintained in 96 well plates as described above for 24 hr. At the end of 24 hr, 10 µl of 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-

Diphenyltetrazolium Bromide (MTT; 5 mg/ml in PBS) was added to wells and the plates were incubated for 4 h at 37°C. This was followed by incubation for 30 min (with constant shaking) after the addition of 150 µl of dimethyl sulphoxide (DMSO). Absorbance was read at 540 nm using ELX800 Universal Microplate Reader (Bio-Tek instruments, Inc, Winooski, VT).

Cytotoxicity was measured as the fraction of lactate dehydrogenase (LDH) released into the medium. HepG2 cells were maintained in 96 well plates for 24 hr as described above. After the collection of supernatants, cells washed with phosphate buffered saline (PBS) were lysed in 1% triton-X-100 in PBS. Cell lysates were collected, vortexed for 15 seconds and centrifuged at 7000 rpm for 5 min. LDH activity was measured in the supernatant and cell lysate by a commercially available kit (Reckon Diagnostics Ltd, Baroda, India).

Measurement of lipid peroxidation levels

HepG2 cells (1x 10^5 cells/well) were maintained in six well plates as described above for 24 hr. At the end of the experimental period, cells were collected from the plate with a cell scraper (Tarson India Pvt Ltd) into a 2 ml centrifuge tube. Lipid peroxidation was measured in the cell suspension as per Buge and Aust (1979) using TBA-TCA-HCL reagent.

Statistical analysis

Data was analysed for statistical significance using one way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test and results were expressed as mean ± S.E.M using Graph Pad Prism version 3.0 for Windows, Graph Pad Software, San Diego California USA.

RESULTS

Plasma and hepatic lipids

As summarized in Table 2, NASH mice recorded significantly ($p<0.05$) higher plasma and hepatic lipids (TG & FFA) compared to CON mice. However, HFD induced increment in plasma and hepatic lipids were significantly ($p<0.05$) attenuated when supplemented with CG extract in HFD fed mice (Figure 1; Table 2).

Plasma markers of hepatic injury

High fat diet fed mice recorded significant ($p<0.05$) increment in activity levels of plasma markers of hepatic damage (AST & ALT). Supplementation of NASH mice with CG resulted in minimal ($p<0.05$) leakage of AST and ALT from hepatic tissue (Figure.1; Table 2).

Hepatic reactive oxygen species measurement by DCFDA

As shown in Figure 3, there was significant ($p<0.05$) elevation in indices of ROS (indicated by increment in DCF florescence) in mitochondria of NASH mice compared to CON. However, ROS generation was significantly ($p<0.05$) minimized by CG supplementation of NASH mice (Figure. 3)

Hepatic markers of oxidative stress

Table 3 shows that, there was a significant ($p<0.05$) increment in mitochondrial levels of hepatic oxidative stress viz. MDA, LOOH and SH in NASH mice. Supplementation of CG extract to NASH mice significantly ($p<0.05$) minimized the increase in these markers of oxidative stress (Figure 2; Table 3).

Hepatic antioxidants

As shown in Table 4, NASH mice recorded significant ($p<0.05$) decrement in hepatic mitochondrial SOD, CAT, GPx, GST and GR activity levels along with decrement in

GSH content compared to CON mice. However, HFD induced depletion in hepatic mitochondrial antioxidants was significantly ($p<0.05$) minimized in NASH+CG mice (Figure 3; Table 4)

Morphological and microscopic evaluation of hepatic tissue

Development of hepatic steatosis in HFD fed mice was prominently visible in the form of pink coloured liver compared to red coloured liver in CON mice. However, a red coloured liver was observed in NASH+CG groups indicating minimal steatosis (Figure 4)

Photomicrographs of liver of NASH mice were characterized by the formation of Mallory's body with hepatocyte ballooning and parenchymatous fat accumulation along with infiltration of inflammatory cells. However, CG treated NASH mice depicted minimal alterations in the normal histoarchitecture of liver (Figure 5).

Qualitative and quantitative evaluation of in vitro NASH

As shown in Figure 6, OA supplementation to HepG2 cells resulted in significant ($p<0.05$) accumulation of lipids in the cytoplasm compared to untreated cells. However, OA induced lipid accumulation was significantly ($p<0.05$) prevented by CG addition. Further, quantitative analysis of ORO staining revealed higher absorbance in the OA treated group than CON and CG exposed cells (Figure. 7A). Oil red O staining of control and treated cells revealed a significantly ($p<0.05$) higher optical density of cells treated with OA as compared to CON and OA+ CG cells (Figure 7A).

Cell viability, cytotoxicity and lipid proxidation in HepG2 cells

OA treatment induced a significant ($p<0.05$) decrement in cell viability and increment in LPO compared to untreated and OA+CG treated cells. Further, an assay of cytotoxicity in terms of LDH activity levels recorded an increment ($p<0.05$) in OA treated cells compared to untreated and OA+CG treated cells (Figure 7B, 7C, 7D).

Table 1: Composition of experimental diets

Ingredients	Control (g/kg)	NASH (g/kg)	NASH+CG1 // NASH+CG3 (g/kg)	(g/kg)
Casein	200	200	200	200
L-Cystine	3	3	3	3
Corn Starch	315	0.0	0.0	0.0
Maltodextrin	35	125	125	125
Sucrose	100	68.8	68.8	68.8
Cellulose	50	50	50	50
Soybean Oil	25	25	25	25
Lard	20	245	245	245
Mineral Mix[1]	10	10	10	10
Di Calcium Phosphate	13	13	13	13
Calcium Carbonate	5.5	5.5	5.5	5.5
Potassium Citrate	16.5	16.5	16.5	16.5
Vitamin Mix[2]	10	10	10	10
Choline chloride	2	2	2	2
Regular chow	195	216.25	206.25	196.25
C.glandulosum.Coleb	00	00	10	30

[1] Mineral mix adds the following components (per g mineral mix): sodium chloride, 259 mg; magnesium oxide, 41.9 mg; magnesium sulfate, 257.6 mg; chromium K sulfate, 1.925 mg; cupric carbonate, 1.05 mg; sodium fluoride, 0.2 mg; potassium iodate, 0.035 mg; ferric citrate, 21 mg; manganous carbonate, 12.25 mg; ammonium molybdate, 0.3 mg; sodium selenite, 0.035 mg; zinc carbonate, 5.6 mg.

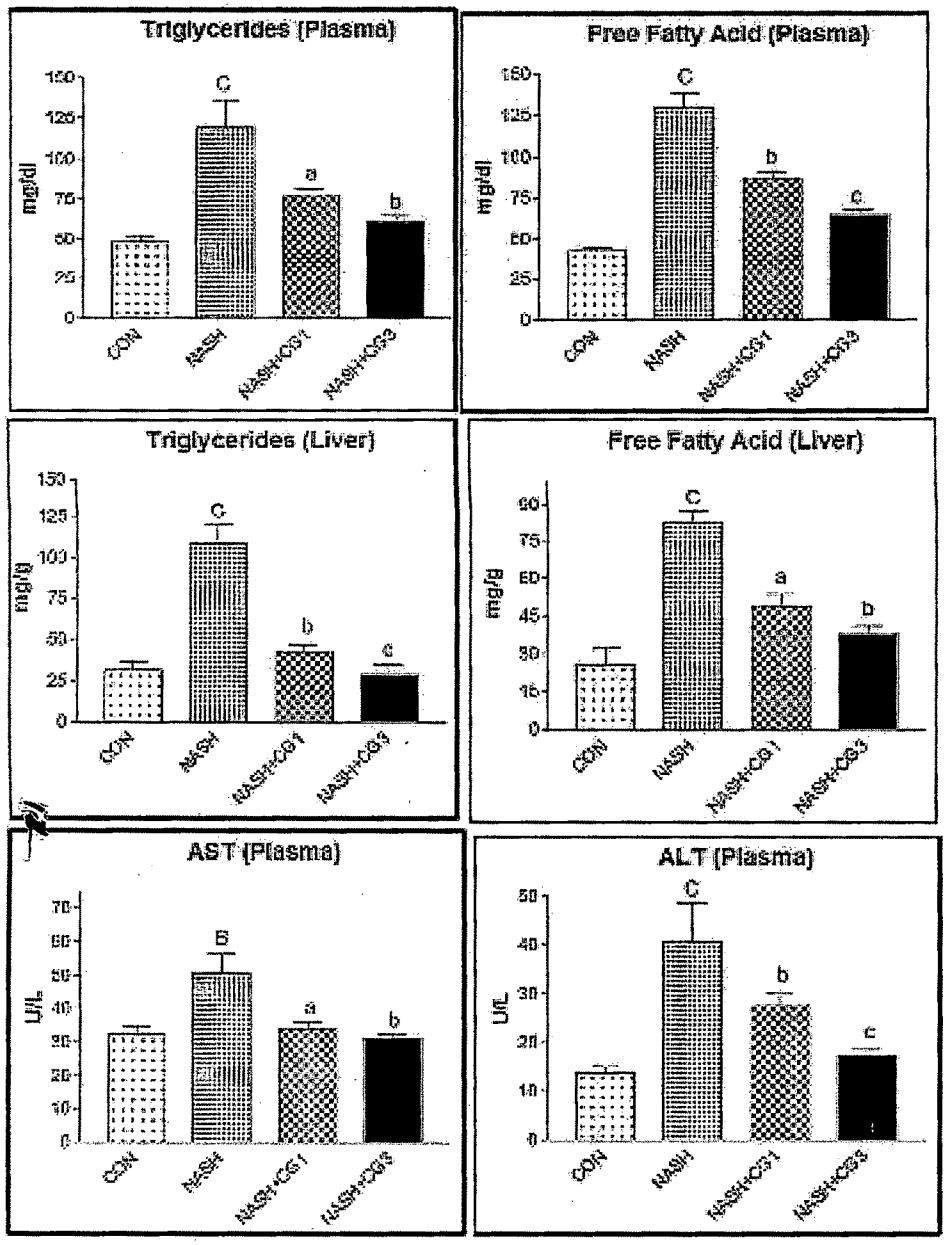
[2] Vitamin mix adds the following components (per g vitamin mix): retinyl acetate, 0.8 mg; cholecalciferol, 1.0 mg; DL-a-tocopheryl acetate, 10.0 mg; menadione sodium bisulfite, 0.05 mg; biotin, 0.02 mg; cyanocobalamin, 1 mg; folic acid, 0.2 mg; nicotinic acid 3 mg; calcium pantothenate, 1.6 mg; pyridoxine-HCl, 0.7 mg; riboflavin, 0.6 mg; thiamin HCl, 0.6 mg.

Table 2: Effect of *C.glandulosum*.Coleb extract on plasma and hepatic lipids and plasma activity levels of AST and ALT.

	CON	NASH	NASH+CG1	NASH+CG3
Plasma				
TG (mg/dl)	49.17±3.41	119.3±17.69^{C}	76.82±5.29^{a}	60.83±3.43^{b}
FAA (mg/dl)	42.75±2.49	130.0±8.99^{C}	86.75±4.19^{b}	64.75±2.72^{c}
AST (U/L)	32.17±2.71	50.83±5.50^{B}	34.00±2.01^{a}	31.00±1.57^{b}
ALT (U/L)	13.83±1.16	40.67±8.20^{C}	27.50±2.60^{b}	17.33±1.25^{c}
Liver				
TG (mg/g)	31.37±5.84	108.9±11.84^{C}	43.07±3.71^{b}	28.79±6.55^{c}
FAA (mg/g)	26.12±5.96	82.55±4.73^{C}	49.12±4.77^{a}	38.03±3.80^{b}

Date expressed as mean±S.E.M for n=6.
$^{A}p<0.05$, $^{B}p< 0.01$, $^{C}p< 0.001$and ^{NS}non-significant Compared to CON.
$^{a}p<0.05$, $^{b}p< 0.01$, $^{c}p< 0.001$ and ^{ns}non-significant compared to NASH.

Figure 1: Effect of *C.glandulosum*.Coleb extract on plasma and hepatic lipids and plasma activity levels of AST and ALT.



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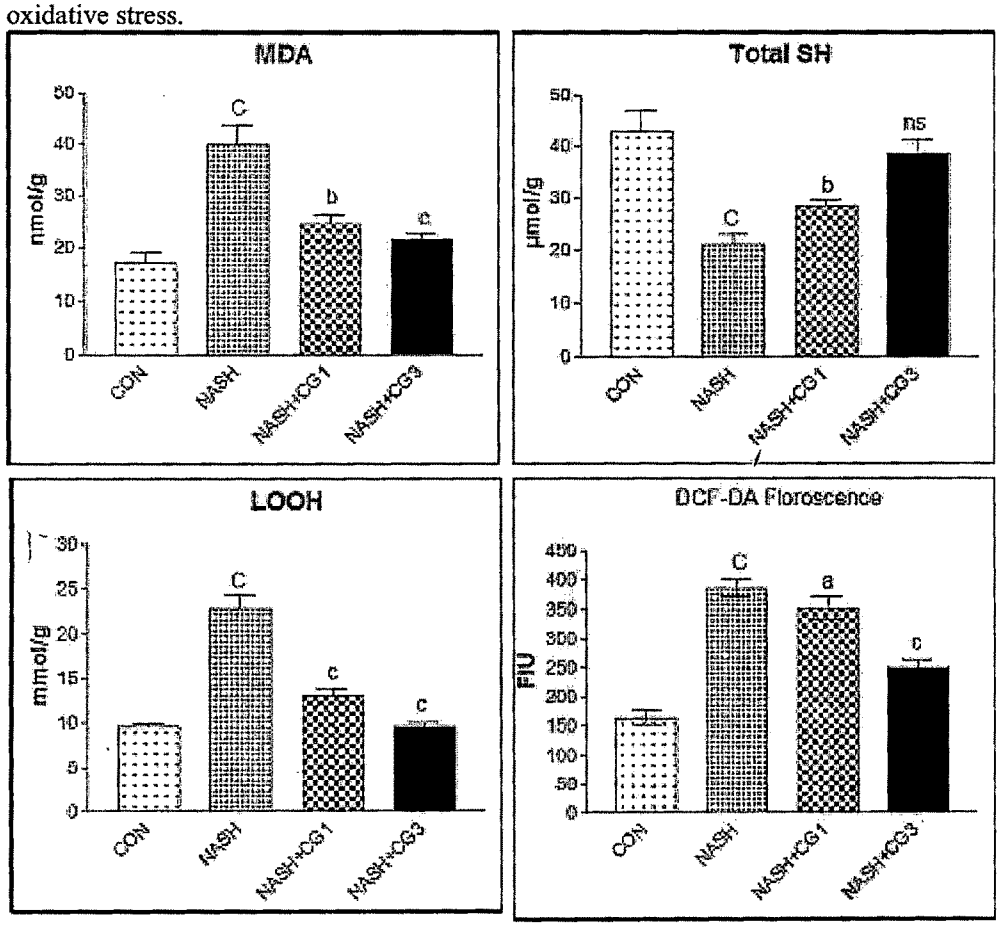
$^{a}p<0.05$, $^{b}p< 0.01$, $^{c}p< 0.001$ and ^{ns}non-significant compared to NASH.

Table 3: Effect of *C.glandulosum*.Coleb extract on markers of hepatic mitochondrial oxidative stress.

	CON	NASH	NASH+CG1	NASH+CG3
MDA (nmol/g)	16.97±2.11	39.70±3.81^{C}	24.50±1.73^{b}	21.50±1.41^{c}
LOOH (mmol/g)	9.55±0.42	22.83±1.45^{C}	13.15±0.62^{c}	9.570±0.45^{c}
Total SH (µmol/g)	42.68±4.02	20.95±2.13^{C}	28.56±1.02^{ns}	38.46±2.75^{b}
DCF DA (FIU)	165.7±11.36	386.2±15.30^{C}	352.3±20.15^{a}	249.0±13.74^{c}

Figure 2: Effect of *C.glandulosum*.Coleb extract on markers of hepatic mitochondrial oxidative stress.



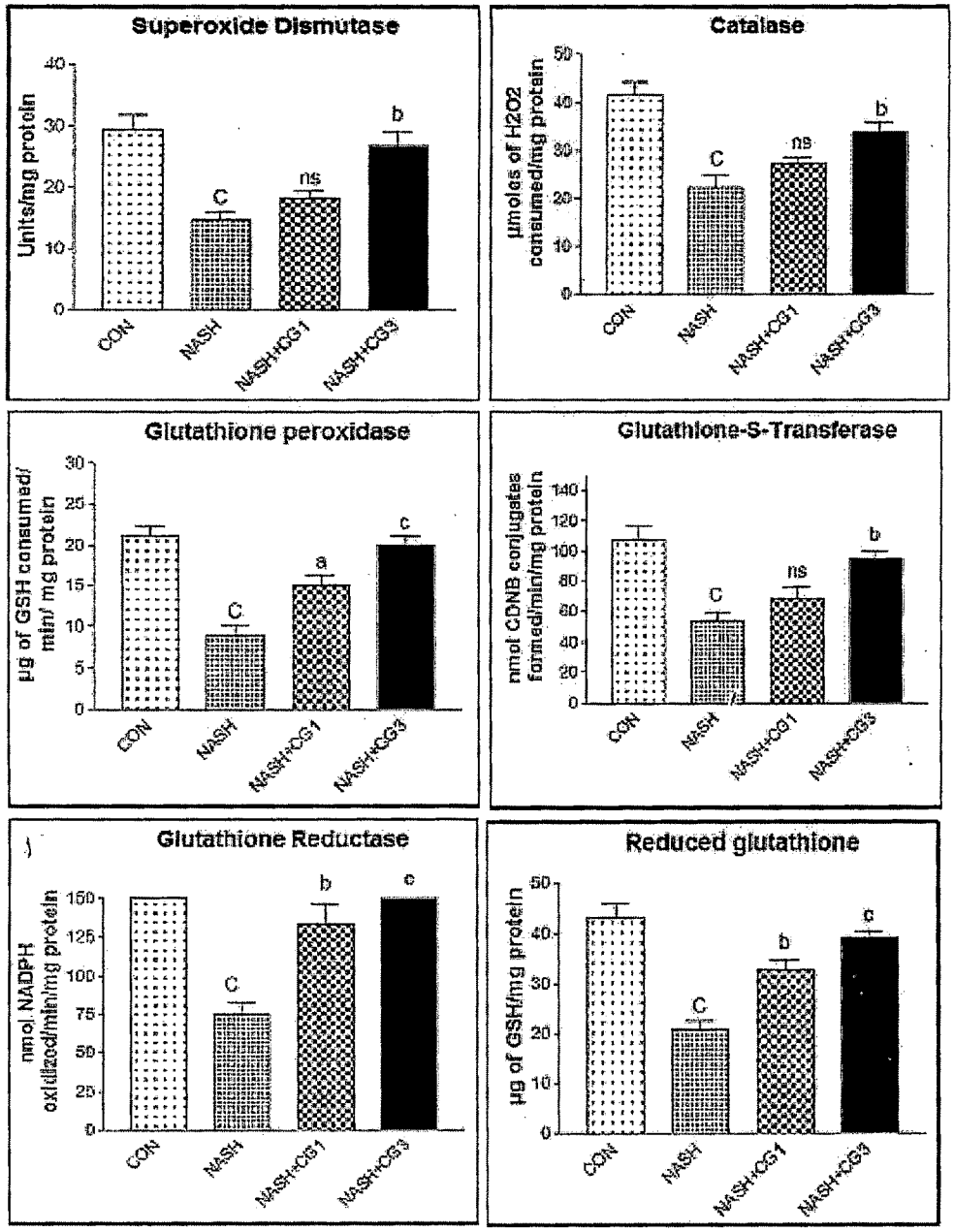
Date expressed as mean±S.E.M for n=6.
$^{A}p<0.05$, $^{B}p< 0.01$, $^{C}p< 0.001$and ^{NS}non-significant Compared to CON.
$^{a}p<0.05$, $^{b}p< 0.01$, $^{c}p< 0.001$ and ^{ns}non-significant compared to NASH.

Table 4: Effect of *C.glandulosum.*Coleb on hepatic mitochondrial enzymatic antioxidants.

	CON	NASH	NASH+CG1	NASH+CG3
SOD (Units/mg protein)	29.40±2.48	14.60±1.50^{C}	18.20±1.24^{ns}	26.80±2.48^{b}
CAT (µmoles of H_2O_2 consumed/mg protein)	41.60±2.67	22.40±2.38^{C}	27.40±1.07^{ns}	34.00±2.00^{b}
GPx (µg of GSH consumed/ min/ mg protein)	21.12±1.21	9.02±0.98^{C}	15.02±1.23^{a}	20.00±1.11^{c}
GST (nmol CDNB conjugates formed/min/mg protein)	107.5±9.33	53.67±5.94^{C}	68.50±7.53^{ns}	94.33±5.58^{b}
GR (nmol NADPH oxidized/min/mg protein)	206.0±15.30	75.17±7.63^{C}	133.8±12.57^{b}	154.0±7.28^{c}
GSH (µg of GSH/mg protein)	43.40±2.57	20.75±1.68^{C}	32.78±1.97^{b}	39.31±1.07^{c}

Date expressed as mean±S.E.M for n=6.
$^{A}p<0.05$, $^{B}p< 0.01$, $^{C}p< 0.001$and ^{NS}non-significant Compared to CON.
$^{a}p<0.05$, $^{b}p< 0.01$, $^{c}p< 0.001$ and ^{ns}non-significant compared to NASH.

Figure 3: Effect of *C.glandulosum*.Coleb on hepatic mitochondrial enzymatic antioxidants.



Date expressed as mean±S.E.M for n=6.
$^{A}p<0.05$, $^{B}p< 0.01$, $^{C}p< 0.001$and ^{NS}non-significant Compared to CON.
$^{a}p<0.05$, $^{b}p< 0.01$, $^{c}p< 0.001$ and ^{ns}non-significant compared to NASH.

Figure 4: Effect of *C.glandulosum*.Coleb extract on morphological changes in the liver.

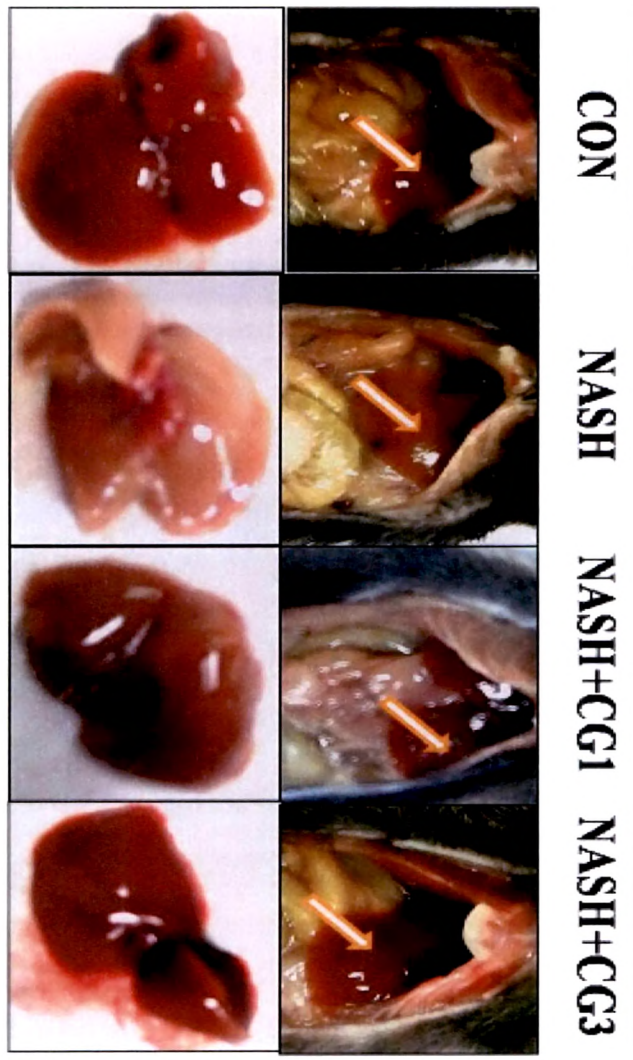


Figure 5: Photomicrographs of liver of (A) control mice showing normal architecture of hepatic tissue, (B) NASH mice showing evidences of parenchymatous lipid accumulation (▲), ballooning hepatocytes (⟶), infiltration of inflammatory cells (★) and Mallory hyaline (→), (C), NASH+ CG1 mice showing only moderate parenchymatous lipid accumulation (▲) and (D) NASH+CG3 showing near normal architecture of hepatic tissue (H X E; 100X).

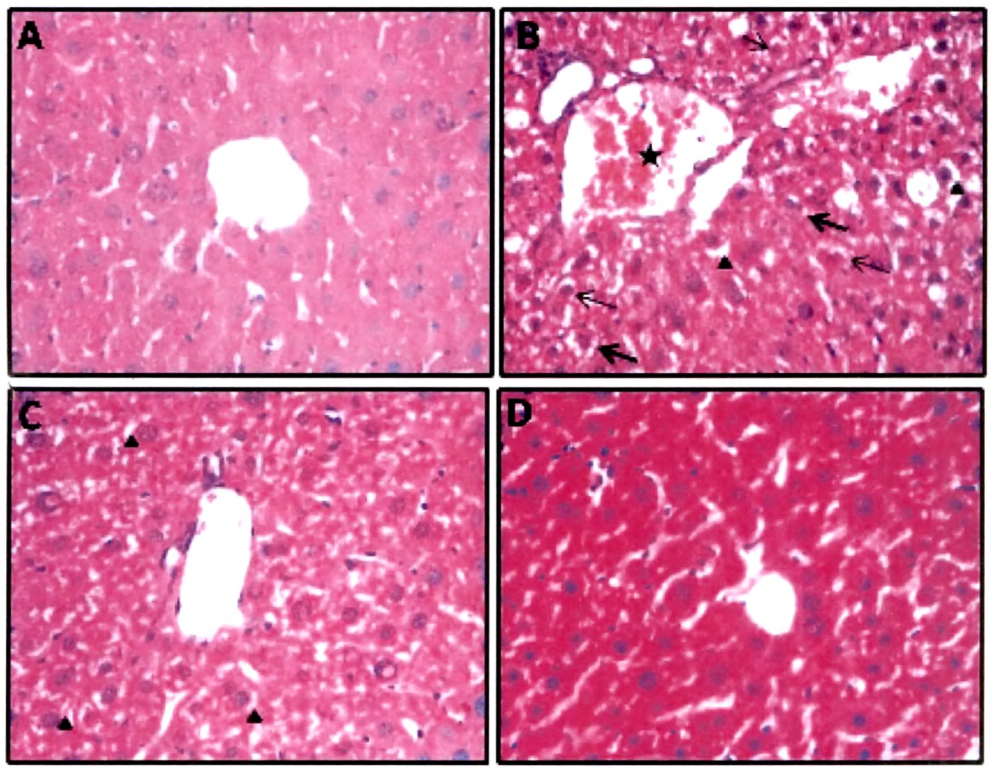


Figure. 6 Photomicrographs showing (A) untreated HepG2 cells, (B) OA (2mM) treated HepG2 cells showing cytoplasmic lipid accumulation, (C) OA (2mM) and CG extract (200µg/ml) treated HepG2 cells showing lesser degree of cytoplasmic lipid accumulation (40 X).

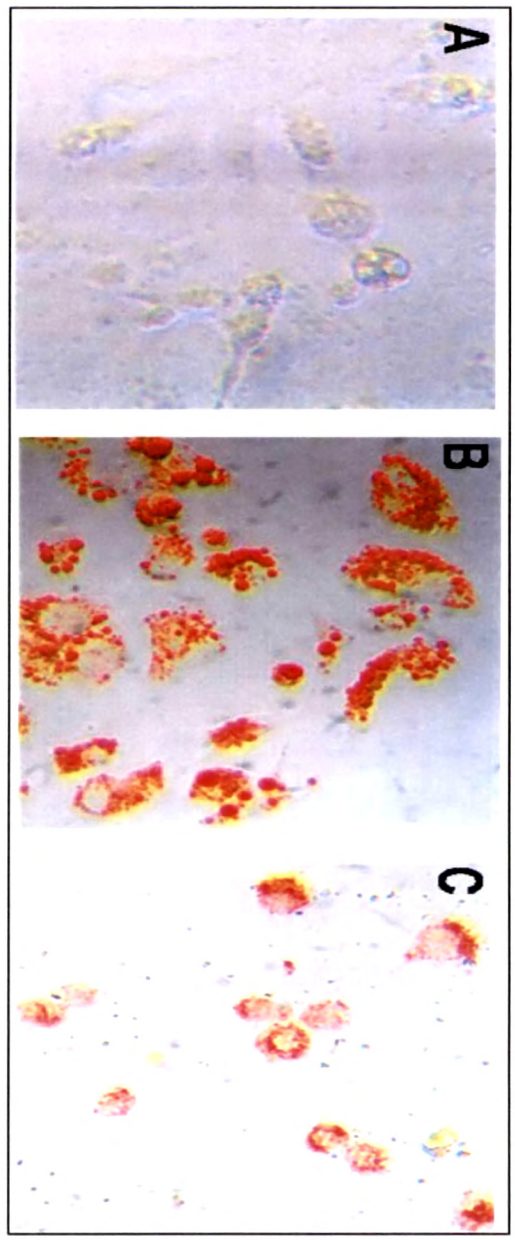
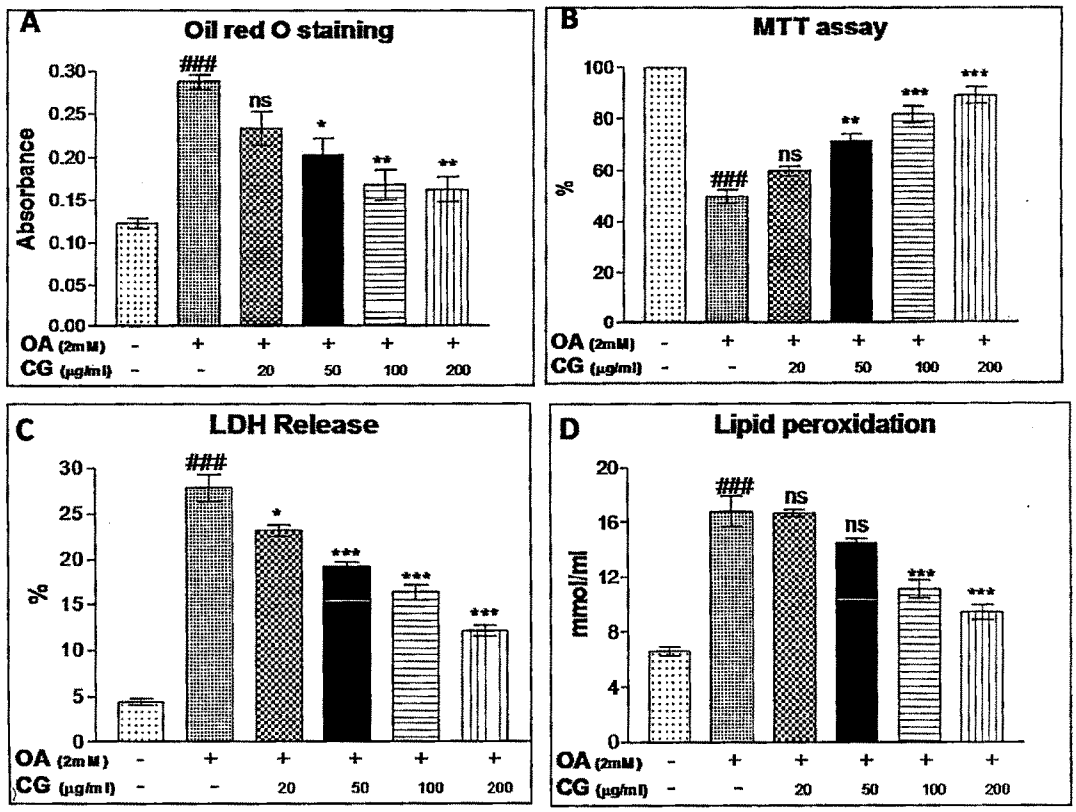


Figure 7: Effect of *C.galndulosum*.Coleb (CG) extract on (A) Oil red o staining, (B) Cell viability, (C) LDH release and (D) lipid peroxidation in OA treated HepG2 cells.



Results are expressed as means ± S.E.M., n = 3.

$^{\#}$ P < 0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 compared with untreated cells, *P < 0.05, **P<0.01 and ***P<0.001 compared OA treated cells NASH and ^{ns} non significant

DISCUSSION

Formulation of a hypothesis on pathogenesis of NASH occurred almost two decades after the first description of the disease in 1980, a hypothesis that found revision immediately after (Day and James, 1998; Day, 2002) and known as a 'two hit' hypothesis. The first hit, marked by TG loading within hepatocytes known as NAFLD is due to overflow of FFA into liver and consequent esterification (Figure 8). Hepatic steatosis marked by high TG accumulation is reflective of excessive inflow of FFA and this, rather than TG *per se*, seems to be the factor responsible for the first hit development of NAFLD (Marchesini *et al.*, 2000; Shiota and Tsuchiya, 2006) and subsequent vulnerability of liver for second hits leading to NASH and/or fibrosis (Figure 8). As already mentioned, insulin resistance of all the causes, is the only metabolic syndrome that appears to depict a consistent association with NASH, which can precipitate hepatic steatosis, lipolysis and hyperinsulinemia. Both lipolysis and hyperinsulinemia lead to higher FFA levels in circulation (adipose tissue lipolysis) and liver (glycolytic synthesis) respectively and, greater hepatic FFA load results in mitochondrial oxidation overload and consequent steatosis due to decreased Apo-B production. This background of hepatic steatosis sets the stage of vulnerability for a second hit which, as it appears now, may represent a set of factors (multi hits) that may involve complex interactions between hepatocytes, stellate cells, adipose cells, Kupffer cells, inflammatory mediators, and reactive oxygen species driving NAFLD state to NASH (Figure 8). Though the cause of progression from NAFLD to NASH/fibrosis remains unclear, animal studies tend to suggest the formation of harmful adducts as by-products of fatty acid oxidation by mitochondria, peroxisomes or microsomes to be the driving force. Fibrosis could be a consequence of hepatic

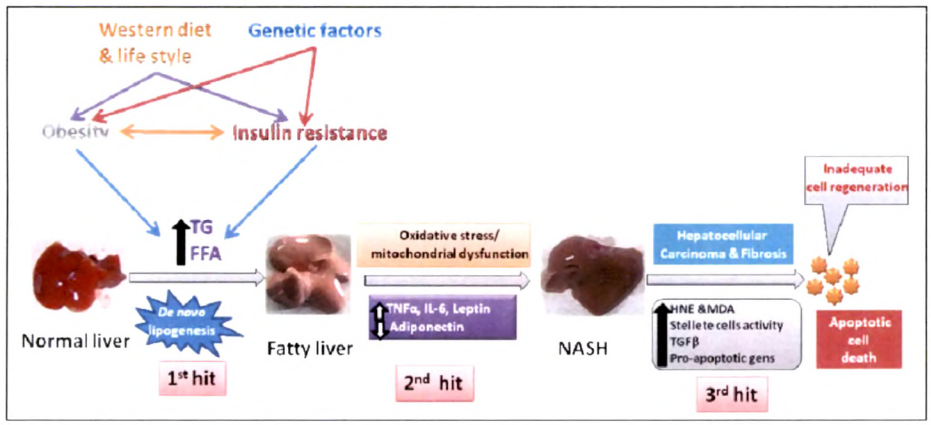
injury inflicted by oxidized by-products (Edmison and McCullough, 2007). Hepatic fibrosis is also a likely effect of increased production of hydroxynonenal (HNE) and malondialdehyde (MDA) by way of lipid peroxidation and oxidative stress, acting through stellate cells and increased production of transforming growth factor-beta (TGF-β) (Browning and Horton, 2004). As mentioned below, an under expression of uncoupling proteins leading to increased generation of reactive oxygen species and Kupffer cell activation might aggravate injury in NASH. Additionally, leptin mediated insulin resistance could also be a factor of significance in fibrogenesis as seen from animal models of NASH (Ikejima *et al.*, 2001; Honda *et al.*, 2002). Role of inflammatory mediators in the progression of NAFLD that could form focus for future development of therapeutics, is also gaining attention. Two of the pro-inflammatory proteins, adiponectin and Tumor necrosis factor-α (TNFα), implicated in the pathogenesis of NAFLD seem to play pivotal roles (Figure 8). Adiponectin, an adipose tissue hormone when lowered, is likely to increase fatty acid oxidation and hepatic gluconeogenesis contributing to increased severity of hepatic inflammation (Yamauchi *et al.*, 2002; Xu *et al.*, 2003; Targher *et al.*, 2006). TNFα, an inflammatory cytokine elaborated by macrophages, adipocytes and hepatocytes has been reportedly elevated in obese patients with insulin resistance and NASH, which can mediate hepatic injury by inhibition of mitochondrial electron transport and release of ROS and promotion of lipid peroxidation (Crespo *et al.*, 2001; Pessayre *et al.*, 2004). Moreover, nuclear factor kappa beta (NF-κB), a proinflammatory transcription factor is also often found elevated in patients with NASH (Lewis and Mohanty, 2010). Recently, inactivation of Kupffer cells, the resident macrophages of the liver that function in both innate and adaptive immunity as active phagocytosing agents and

antigen-presenting cells (via toll-like receptors, among others) to T-cells, has found association with pathogenesis of NASH and impaired hepatic regenerative capacity. Moreover, elimination of Kupffer cells seems to improve NASH, implicating over-activation of Kupffer-cell-mediated immune response to be the underlying cause of liver injury in NAFLD. Increased hepatic lipid load seems to alter the Kupffer cell physiology due to overcrowding of liver sinusoids resulting in prolonged exposure of Kupffer cells to antigens, reduced Kupffer cell outflow, and an attendant sustained inflammatory response. While NAFLD stands histologically defined by hepatic loading of fat, evidence also points to it being a consequence of heightened catabolic events and suboptimal hepatic defenses (Lewis and Mohanty, 2010). Interestingly, patients diagnosed with both NASH and alcoholic liver disease also provide evidences of up regulated expression of the pro-apoptotic gene Bax along with increased caspase activity, marker of cellular apotosis (Ramalho *et al.*, 2006). NASH patients also appear to reveal compromised antioxidant potential as marked by reduced glutathione levels (Vendemiale *et al.*, 2001).

Recently, an additional component representing a 'third hit' stands identified reflecting inadequate hepatocyte proliferation (Figure 8) (Jou *et al.*, 2008). The above publication clearly highlights the competence of healthy liver to undertake compensative replication of mature hepatocytes to replace dead cells and, restore and reconstitute normal hepatic function. Ironically, oxidative stress implicated in NAFLD pathogenesis as a principal feature effectively inhibits the replication of mature hepatocytes and therefore goads the system instead to expansion of the hepatic progenitor cell (oval cell) population (Roskams *et al.*, 2003). The above work highlights the differentiation of these progenitor cells to differentiate into hepatocyte-

like cells and draws a strong correlation between presence of more number of both oval and intermediate hepatocyte-like cells with fibrosis (Roskams *et al.*, 2003). Apparently, as suggested by the above authors, cumulative hepatocyte loss triggers formation and accumulation of progenitor cells and their differentiation towards hepatocytes; an implicated consequence of which is hepatocellular carcinogenesis. In short, the above works reveal a compromised efficacy of hepatocyte regeneration under conditions of chronic liver injury to be the prime mover of the system towards fibrosis/cirrhosis; and therefore, cell death with impaired proliferation of hepatocyte progenitors represents the proposed 'third hit' in NASH pathogenesis.

Figure 8: Pathology of non alcoholic steatohepatitis.



In the present study, HFD feeding to mice resulted in significant increment in plasma and hepatic lipids. However, CG supplementation to HFD fed mice resulted in significant decrement in plasma and hepatic lipids, thus minimizing the impact of "first step". These observations can be attributed CG induced elevation in activity levels of catabolic enzymes (lipoprotein lipase in plasma, liver and adipose tissue), reduced intestinal absorption of dietary lipids and more elimination of the same

through faeces in hyperlipidemic rats (Chapter 1). Again, elevated levels of AST and ALT in NASH mice indicate hepatocyte damage whereas lowered activity levels recorded in NASH+CG group hints at the possible hepatoprotective effect of CG. These results corroborate our previous report on hepatoprotective role of CG against CCL_4 induced hepatotoxic manifestations (Jadeja *et al.*, 2010).

Mitochondrial ROS such as, $^{\cdot}O_2$, H_2O_2, $^{\cdot}OH$ etc has been proposed to play a key role in the progression of NASH. High fat diet leading to infiltration of fat into hepatocytes has been reported to elevate the rate of β-oxidation resulting in excessive production of ROS (Pessayre *et al.*, 2002), which ultimately gives rise to abnormalities in mitochondrial morphology and their dysfunction (Caldwell *et al.*, 1999). Oxidative damage to the mitochondrial membrane has been reported to cause leakage of electrons and their transfer to molecular oxygen that further facilitates the formation of $^{\cdot}O_2$ and H_2O_2 (Pessayre *et al.*, 2002; Fromenty *et al.*, 2004). In the present study, mitochondrial oxidative stress evaluated by DCFDA florescence assay in NASH mice has recorded elevated levels of ROS. However, the same was significantly low in NASH+CG fed mice indicating the protective role of CG in controlling ROS generation and the consequent oxidative damage. These results can be attributed to the free radical scavenging property of CG extract reported by us previously (Jadeja *et al.*, 2009b).

Superoxide radicals are converted to H_2O_2 by SOD and, GPx and CAT further convert H_2O_2 into O_2 and H_2O (Gutteridge, 1995; Thounaojam *et al.*, 2010), thereby preventing H_2O_2 mediated cytotoxicity. Decreased levels of mitochondrial SOD, GPx and CAT in NASH mice indicate a compromised antioxidant milieu and the possible accumulation of $^{\cdot}O2$ and H_2O_2. These radicals are also known to have a synergistic

effect leading to the formation of highly reactive ·HO that could further aggravate mitochondrial oxidative stress in liver. Experimental NASH mice fed with CG, maintained near normal levels of these enzymatic antioxidants indicating minimal mitochondrial damage. This can be attributed to the *in vitro* ·O2, H_2O_2 and ·OH radical scavenging potential of CG extract demonstrated previously (Jadeja *et al.*, 2009b).

GSH is an important non-enzymatic antioxidant and its content in mitochondrial fraction is indicative of the extent and status of oxidative stress in NASH (Malaguarnera *et al.*, 2009). The antioxidant enzymes GPx and GST require GSH as a hydrogen donor to neutralize free radicals (Padmanabhan and Prince, 2006). This process converts more and more GSH into its inactive oxidised form (GSSG), and hence GR activity is crucial in converting GSSG back to GSH (Renugadevi and Prabu, 2009). Compromised activity levels of GPx, GST and GR along with decreased content of GSH observed in NASH mice provide ample testimony to hepatic mitochondrial oxidative stress. Moreover, enzymes such as Glucose-6-Phosphate Dehydrogenase undergo oxidation of their -SH group under conditions of oxidative stress leading to decreased production of NADPH and decreased activity levels of GR (Renugadevi and Prabu, 2009). Observed decrement in total -SH content in the MF of NASH mice can be related with the lowered activity level of GR observed in the present study. Conversely, high -SH content helps improve activity levels of GR, GPx and GST. Significantly higher GSH content recorded in the MF of NASH+CG mice further underscores the protective role of CG against NASH induced hepatic oxidative stress.

Mitochondrial PUFAs are highly susceptible to oxidative damage during NASH and the extent of damage could be assayed by estimating the contents of MDA

and LOOH in MF. Significant increment in MDA and LOOH content recorded in NASH mice in our study is in accordance with published reports (Koruk *et al.*, 2004; Oliveira *et al.*, 2005). However, NASH+CG group recorded minimal increase in MDA and normal LOOH level indicating minimal damage to the mitochondrial membrane.

Diagnosis of NASH individuals is essentially based on a symptomatic elevation of aminotransferases, radiological features of fatty liver and hepatomegaly while, diagnostic establishment is possible in the context of clinical history. A significant feature of NASH associated hepatic TG accumulation seems accountable by a decreased production of Apo-B. Increased output of reactive oxygen species from mitochondria seems to trigger steatohepatitis and fibrosis by three main mechanisms: lipid peroxidation, cytokine induction and induction of fas ligand. Deficiency of enzymes of peroxisomal oxidation that leads to accumulation of significant amount of dicarboxylic acids is apparently another major cause of micro-vesicular steatosis and steatohepatitis; added consequence of deficiency of peroxisomal enzymes is the sustained hyper-activation of perioxisome proliferation activated receptor - α (PPAR- α) regulated genes (Angulo, 2002). Induction of cytokines (TNF-α, TGF-β and IL-8) by reactive oxygen species (ROS) seems triggered by lipid per-oxidation and release of malon-dialdehyde (MDA) and 4-hydroxy noneal (HNE). Moreover, mitochondrial ROS induces expression of fas-ligand on hepatocytes and, interaction between fas ligands of neighbouring hepatocytes may lead to fractional killing (Angulo, 2002) and, MDA and HNE may promote further cell death, Mallory hyaline formation and collagen synthesis (Duvnjak *et al.*, 2007) causing death of hepatocytes. The significantly increased levels

of MDA in NASH mice in the present study can be correlated with the histological observations characterised by the formation of prominent Mallory bodies and evidence of infiltration of immune cells. Lowered MDA content recorded in NASH+CG mice is also marked by no evidence of formation of Mallory bodies or infiltration of inflammatory cells. Briefly, an *in vivo* hepatoprotective role of CG against NASH is clearly indicated from the observations made in the present study.

Human hepatoblastoma cell line (HepG2) has been reported to develop morphological and biochemical transformations due to lipid accumulation when treated with OA. These set of changes are comparable to formation of fatty liver in humans (Okamoto *et al.*, 2002; Janorkar *et al.*, 2009) and hence is an ideal model for studying and quantifying experimentally induced NASH (Cui *et al.*, 2010). It has also been reported that, uptake of OA triggers lipogenesis in HepG2 cells due to up-regulation of enzymatic machinery as reported by Kohjima *et al.*,2009. These anabolic changes induce cytochrome p450E1 expression and subsequent increase in LPO (Sung *et al.*, 2004) leading to cytotoxicity and cell death. In the present study, OA induced NASH in HepG2 cells is confirmed with ORO staining while, low cell viability and high level of LDH released, indicate damage of cell membrane due to fat accumulation. Simultaneous exposure of hepG2 cells to CG and OA *in vitro* shows a dose dependent decrement in lipid accumulation, LPO and LDH release along with increase in cell viability. However, effect of CG on expression of lipogenic enzymes and CYP2E1 needs further investigations.

It can be concluded that, *C.glandulosum*.Coleb extract can prevent high fat diet/fatty acid induced lipotoxicity in *in vivo* and *in vitro* experimental models of NASH by decreasing lipid accumulation and preventing oxidative stress. Reported

antioxidant, free radical scavenging and lipid lowering effects of CG extract could be the possible reasons for its protective role against experimentally induced NASH. Previous report on phytochemical analysis has reported presence of high content of flavonoids, polyphenols and ascorbic acid in CG extract (Chapter 1) and, the same can be held responsible for the recorded protective effect of CG against experimentally induced NASH. Overall, the role of CG extract as a potential herbal therapeutic agent against NASH as indicated in this study warrants a detailed investigation in order to bring this folklore medication into clinical use.

Summary

This study evaluates protective role of CG leaf aqueous extract against high fat diet/fatty acid induced lipotoxicity using HFD fed C57BL/6J mice and OA treated HepG2 cells as experimental models of non alcoholic steatohepatitis (NASH). Plasma lipid profile, merkers of hepatic damage, hepatic mitochondrial reactive oxygen species, markers of oxidative stress and antioxidants and histopathological changes were evaluated in CON; mice fed with low fat diet for 16 weeks, NASH; mice fed with HFD for 16 weeks and NASH+CG1 and NASH+CG3; mice fed with HFD containing 1 and 3% CG extract (w/w) for 16 weeks. Supplementation of NASH mice with CG extract significantly prevented HFD induced elevation in plasma markers of liver damage, plasma and hepatic lipids, mitochondrial oxidative stress and compromised enzymatic and non-enzymatic antioxidant status and histopathological damage to hepatocytes. Furthermore, results from *in vitro* study indicated attenuation of OA induced lipid accumulation in HepG2 cells in presence of CG extract. Moreover, addition of CG extract (20-200µg/ml for 24 hr) to HepG2 cells significantly minimizes lipid peroxidation and cytotoxicity and increases cell viability. These *in vivo* and *in vitro* studies suggest that CG extract has the potential of preventing high fat/fatty acid induced NASH.