Chapter 2

Sida rhomboidea.Roxb extract alleviates pathophysiological alterations and mitochondrial oxidative stress associated with non-alcoholic steatohepatitis (NASH): Studies on *in vivo* and *in vitro* models.

The present study aims to evaluate the possible protective/ therapeutic potential of an aqueous extract of *Sida rhomboidea*. Roxb (SR) against NASH in C57BL6/J mice *in vivo* and against oleic acid induced lipid accumulation and cytotoxicity in HepG2 cells *in vitro*.

Published as: - **Thounaojam MC**, Jadeja RN, Dandekar DS, Devkar RV, Ramachandran AV. (2010). *Sida rhomboidea*.Roxb extract alleviates pathophysiological changes in experimental *in vivo* and *in vitro* models of high fat diet/fatty acid induced non alcoholic steatohepatitis. *Experimental and Toxicologic Pathology*. DOI:10.1016/j.etp.2010.02.010.

INTRODUCTION

The term non-alcoholic fatty liver disease (NAFLD) indicates a pathophysiological state of fatty liver that develops in patients without any history of alcohol abuse. Prevalence of NAFLD is common in developed nations (20 to 40%) and is the most prevalent form of chronic liver disease (Browning *et al.*, 2004). This condition causes structural and physiological alterations in liver due to fat accumulation (steatosis). Conditions of steatosis exceeding 5–10% of body weight and advanced fibrosis leading to cirrhosis and hepatocellular carcinoma are termed as non- alcoholic steatohepatitis (NASH). Ludwig *et al.* (1980) introduced the term and, fibrosis, cirrhosis and hepatocellular carcinoma usually mark the progression of NAFLD to NASH (Caldwell, 2004). However, only 10-20% of the patients suffering from NAFLD may develop and manifest physiological changes associated with NASH. NASH is more prevalent in obese and diabetic individuals (Clark, 2002; Chitturi, 2004) and the prevalence in the western world is approximately 2-6%. In the United States, NASH is considered to be the 3rd most common liver disease after hepatitis C and alcoholic fatty liver (Patel and Lee, 2001).

High fat diet leads to elevation in plasma and hepatic lipids that tilts the metabolic balance towards anabolism. This imbalance results in net fat accumulation in hepatocytes, a step referred to as the "first hit" in relation to the onset of NASH. Subsequently, it results in intracellular oxidative stress, inflammation and gross injuries to hepatocytes leading to fibrosis due to the greater generation of reactive oxygen species (ROS), because of exaggerated fat accumulation and mitochondrial fatty acid oxidation. Cumulatively, these events are referred to as the "second hit" for induction of NASH

(Duvnjak *et al.*, 2007). Asymptotic and unexplained increase in the activity levels of plasma aspartate transaminase (AST) and alanine transaminase (ALT) are often correlated with the onset of NASH (Angulo, 2002). Non-invasive radiological imaging techniques such as ultrasonography, computed tomography scan and magnetic resonance imaging are not capable of distinguishing between fatty liver and NASH. This makes the entire process of diagnostics and therapy of NASH a difficult proposition difficult and, biopsy alone serves as the comprehensive option for its confirmation (Dabhi *et al.*, 2008).

Although exercise and diet improvement may reduce the overall magnitude of this disease, development of novel dietary supplements or drugs that can successfully prevent the onset of NASH is desired (Nakamoto *et al.*, 2009). Recently, consumption of natural antioxidants and hepatoprotective plant products has gained popularity mainly due to their cost effectiveness and minimal side effects (Verma and Singh, 2008). In this context, a recent study has demonstrated effectiveness of a herbal drug with multi-therapeutic potential against NASH (Haddad *et al.*, 2009).

The present study therefore aims to evaluate the possible protective/ therapeutic potential of an aqueous extract of *Sida rhomboidea.* Roxb (SR) against NASH in C57BL6/J mice *in vivo* and against oleic acid induced lipid accumulation and cytotoxicity in HepG2 cells *in vitro.*

MATERIALS AND METHODS

Plant and preparation of extract: 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

Eighteen animals randomly allocated to three groups of six animals each served the experimental purpose. While group I (CON) received LFD, groups II (NASH) and III (NASH+SR1) received HFD or HFD+SR respectively for 16 weeks (Bose *et al.*, 2008). At the end of the experimental period, blood collected in EDTA coated vials by retro orbital sinus puncture from overnight fasted animals under mild ether anaesthesia was cold centrifuged (4°C) for 10 min at 1500 rpm to obtain plasma. Later, liver, heart, pancreas, kidney and epididymal fat pad excised from animals subjected to cervical dislocation under mild anaesthesia were 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).

Measurement of mitochondrial reactive oxygen species generation by DCFDA

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 ROS 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).

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). 10% 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).

10% homogenate was mixed with H2O2 (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 and Jakoby (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. A mixture of phosphate buffer (0.3M), sodium azide (10mM), reduced glutathione (4mM) and 10% homogenate was mixed well and 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 mm) 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 (1×10^5cells/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 SR 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. 50 µl of Oil red O (ORO) solution (1% in isopropenol) 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 isopropenol (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 significant increment in the levels of plasma and hepatic lipids compared to CON mice. However, HFD induced increase in plasma and hepatic lipids showed attenuation on supplementation with SR extract (Table.2 & Figure.1).

Plasma markers of hepatic damage

NASH mice recorded significant increment in liver weight and activity levels of plasma markers of hepatic damage (AST & ALT). Supplementation with SR extract resulted in minimal leakage of AST and ALT from hepatic tissue (Table. 2 & Figure.1).

Hepatic mitochondrial antioxidants

As shown in Table 3, NASH mice recorded significant decrement in hepatic mitochondrial SOD, CAT, GPx, GST and GR along with decrement in GSH content compared to CON mice. However, HFD induced depletion in hepatic mitochondrial antioxidants was significantly minimal in NASH+SR3 mice (Table. 3 & Figure.2)

Hepatic reactive oxygen species and lipid peroxidation

As shown in Figure. 3, there was significant elevation in the indices of ROS (indicated by increment in DCF florescence) and MDA in mitochondria of NASH mice compared to CON. However, ROS generation and lipid peroxidation were significantly minimized by SR supplementation to NASH mice (Table.4 & Figure. 3)

Morphological and microscopic evaluation of hepatic tissue

Development of hepatic steatosis in HFD mice was evident by the pink coloured appearance of liver compared to the red coloured liver of CON mice. Prevention of hepatic steatosis in NASH+SR3 group was evident by the more reddish appearance of liver compared to NASH mice (Figure. 4). Photomicrographs of sections of liver of NASH mice were characterized by the formation of Mallory body with hepatocyte ballooning and parenchymatous fat accumulation along with infiltration of inflammatory

cells. However, NASH+SR3 mice depicted minimal alterations from the normal histoarchitecture of liver (Figure. 5).

Qualitative and quantitative evaluation of in vitro NASH

As shown in Figure. 6, OA addition in the culture medium of Hep G2 cells resulted in significant accumulation of lipids in the cytoplasm compared to untreated cells. SR supplementation prevented OA induced lipid accumulation to a significant extent. Moreover, quantitative analysis of ORO staining revealed higher absorbance in the OA treated cells compared to CON and SR supplemented groups (Figure.7).

Cell viability, cytotoxicity and lipid peroxidation in OA treated HepG2 cells

Data on cell viability, cytotoxicity and levels of LPO of control and treated HepG2 cells appear in Figure.7. There was significant decrement in cell viability along with higher cytotoxicity and lipid peroxidation in OA treated HepG2 cells compared to untreated cells. Changes in cell viability and cytotoxicity appeared minimal with co-presence of SR along with OA (Figure. 7).

Table. 1 Composition of experimental diets

Ingredients	Control (g/kg)	NASH (g/kg)	NASH+SR1 (g/kg)
Casein	200	200	200
L-Cystine	3	3	3
Corn Starch	315	0.0	0.0
Maltodextrin	35	125	125
Sucrose	100	68.8	68.8
Cellulose	50	50	50
Soybean Oil	25	25	25
Lard	20	245	245
Mineral Mix[1]	10	10	10
Di Calcium Phosphate	13	13	13
Calcium Carbonate	5.5	5.5	5.5
Potassium Citrate	16.5	16.5	16.5
Vitamin Mix[2]	10	10	10
Choline chloride	2	2	2
Regular chow	195	216.25	206.25
S.rhomboidea.Roxb	00	00	10

[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 *S.rhomboidea.*Roxb extract on plasma and hepatic lipids and plasma activity levels of AST and ALT.

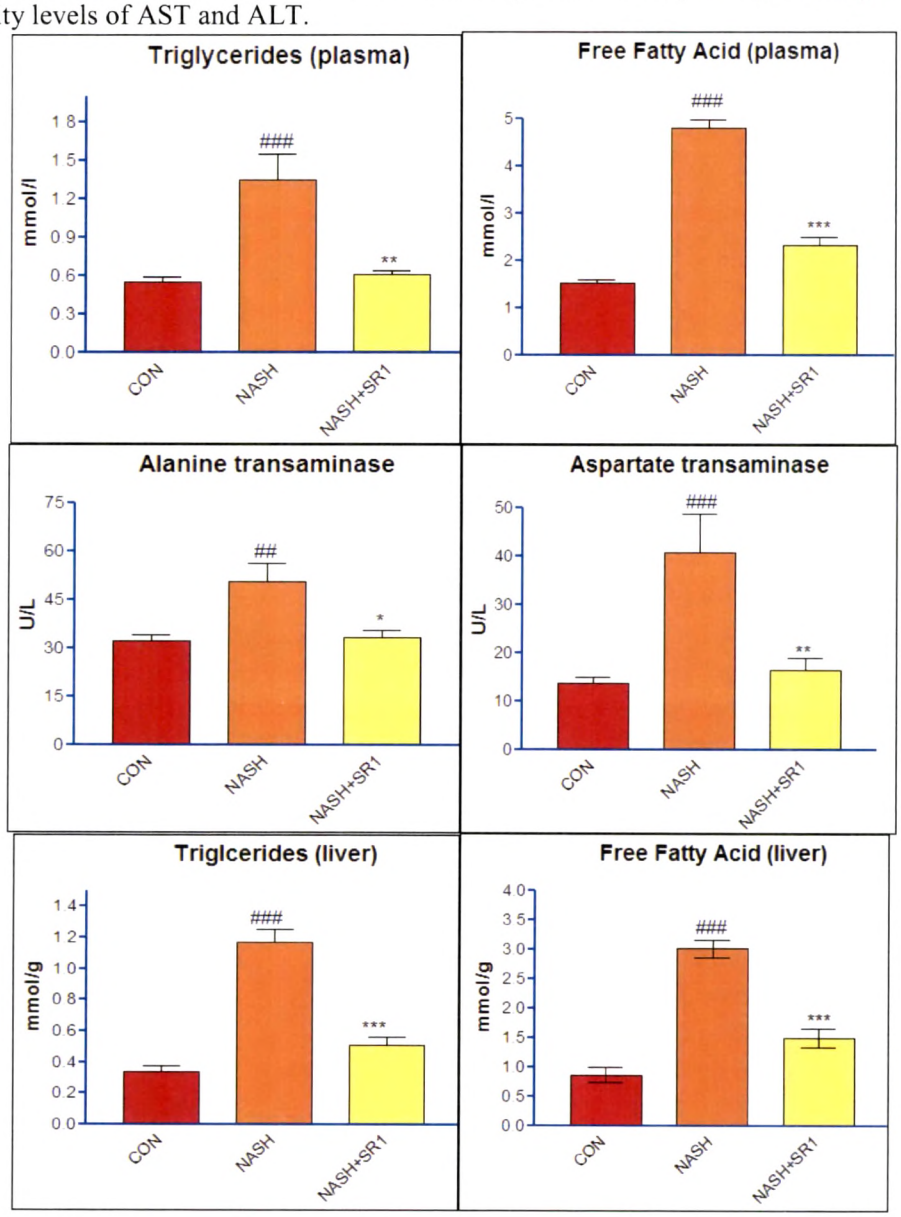
	CON	NASH	NASH+SR1
Plasma			
Triglycerides (mmol/l)	0.55±0.04	1.35±0.20$^{###}$	0.61±0.03**
Free fatty acids (mmol/l)	1.54±0.07	4.81±0.17$^{###}$	2.34±0.16***
ALT (U/L)	32.17±2.71	50.83±5.50$^{##}$	33.50±2.26*
AST (U/L)	13.83±1.16	40.67±8.20$^{###}$	16.50±2.60**
Liver			
Triglycerides (mmol/g)	0.34±0.04	1.17±0.08$^{###}$	0.51±0.05***
Free fatty acids (mmol/g)	0.87±0.13	3.02±.15$^{###}$	1.50±0.16***

Table. 3 Effect of *S.rhomboidea.*Roxb extract on hepatic mitochondrial antioxidants.

	CON	NASH	NASH+SR1
SOD (Units/mg protein)	29.40±2.482	14.60±1.503$^{###}$	24.05±1.02**
CAT (µmoles of H_2O_2 consumed/mg protein)	41.60±2.676	22.40±2.379	39.99±2.69***
GPx (µg of GSH consumed/ min/ mg protein)	21.12±1.21	9.02±0.98$^{###}$	18.09±1.23**
GST (nmol CDNB conjugates formed/min/mg protein)	107.5±9.330	53.67±5.942$^{###}$	92.09±5.98***
GR (nmol NADPH oxidized/min/mg protein)	206.0±15.30	75.17±7.639$^{###}$	162.09±8.01***
GSH (µg of GSH/mg protein)	43.40±2.571	20.75±1.677$^{###}$	34.02±1.29***

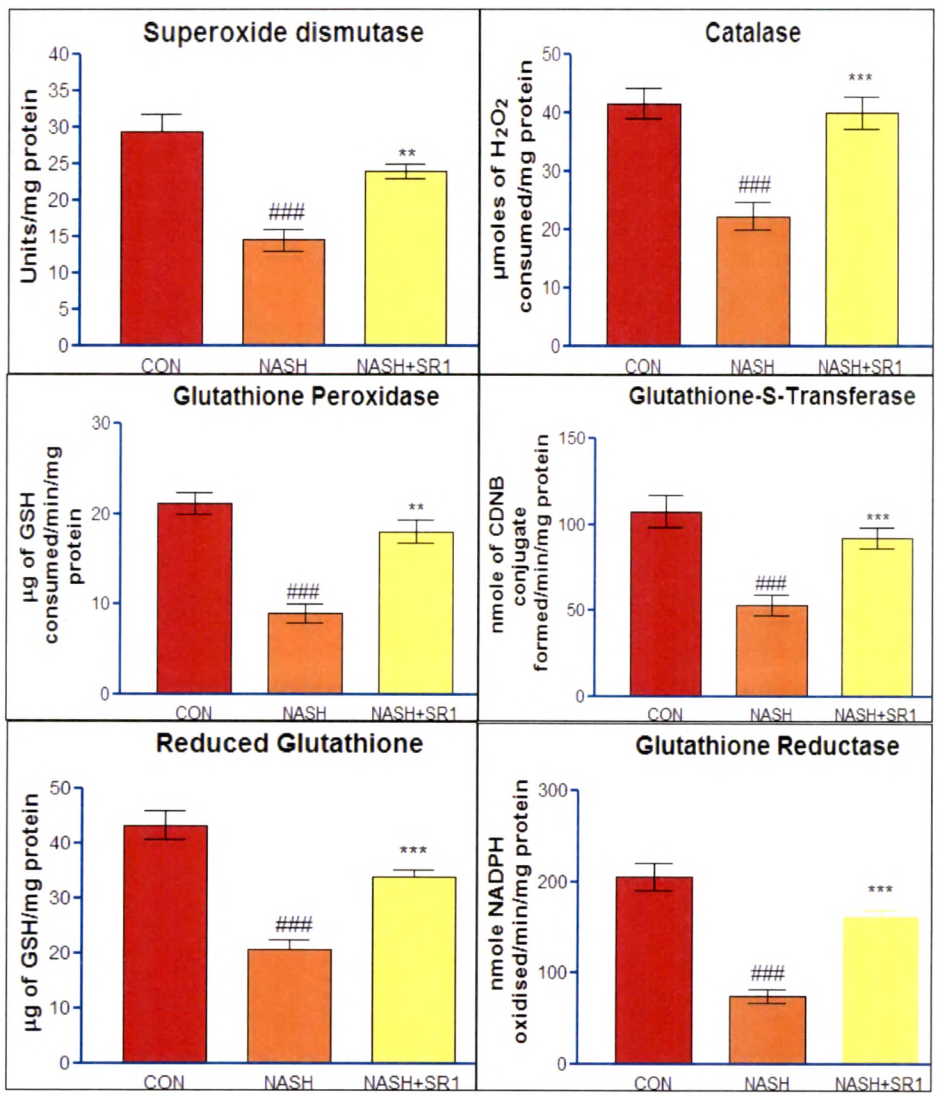
Results are expressed as means ± S.E.M., n = 6. Where, $^{#}$ P < 0.05, $^{##}$p<0.01 and $^{###}$p<0.001 compared with CON, *P < 0.05, **p<0.01 and ***p<0.001 compared with NASH and ^{ns} non significant.

Figure.1 Effect of *S.rhomboidea.*Roxb extract on plasma and hepatic lipids and plasma activity levels of AST and ALT.



Results are expressed as means ± S.E.M., n = 6. Where, $^{\#}P < 0.05$, $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ compared with CON, $^{*}P < 0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ compared with NASH and ^{ns} non significant.

Figure.2 Effect of *S.rhomboidea.*Roxb extract on hepatic mitochondrial antioxidants.

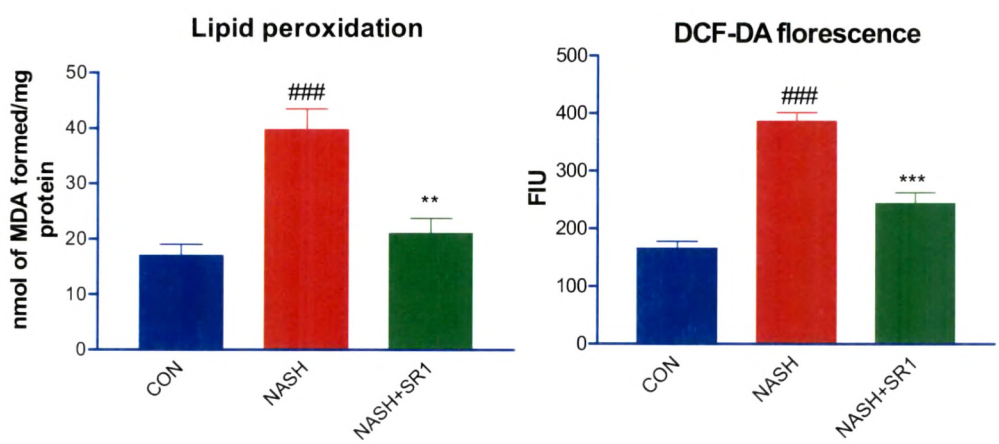


Results are expressed as means ± S.E.M., n = 6. Where, $^{\#}$ P < 0.05, $^{\#\#}$p<0.01 and $^{\#\#\#}$p<0.001 compared with CON, *P < 0.05, **p<0.01 and ***p<0.001 compared with NASH and ^{ns} non significant.

Table.4 Effect of *S.rhomboidea.*Roxb extract on hepatic mitochondrial (A) lipid peroxidation and (2) reactive oxygen species formation.

	CON	NASH	NASH+SR1
Lipid peroxidation (nmol of MDA formed/mg protein)	16.97±2.11	39.70±3.80$^{###}$	21.00±2.72**
Reactive oxygen species formation (Florescence intensity units)	165.70±11.63	386.20±15.30$^{###}$	243.70±18.59***

Figure. 3 Effect of *S.rhomboidea.*Roxb extract on hepatic mitochondrial (A) reactive oxygen formation and (2) MDA formation.



Results are expressed as means ± S.E.M., n = 6.
$^{\#}$ P < 0.05 compared with CON, *P < 0.05 compared with NASH and ^{ns} non significant

Figure. 4 Morphological evaluation of fatty liver in NASH mice. (A) liver of control mice showing red coloured appearance, (B) Fat laden liver of NASH mice showing pale coloured appearance and (C) liver of NASH+SR1 mice showing red coloured appearance of liver that is similar to the liver of control mice.

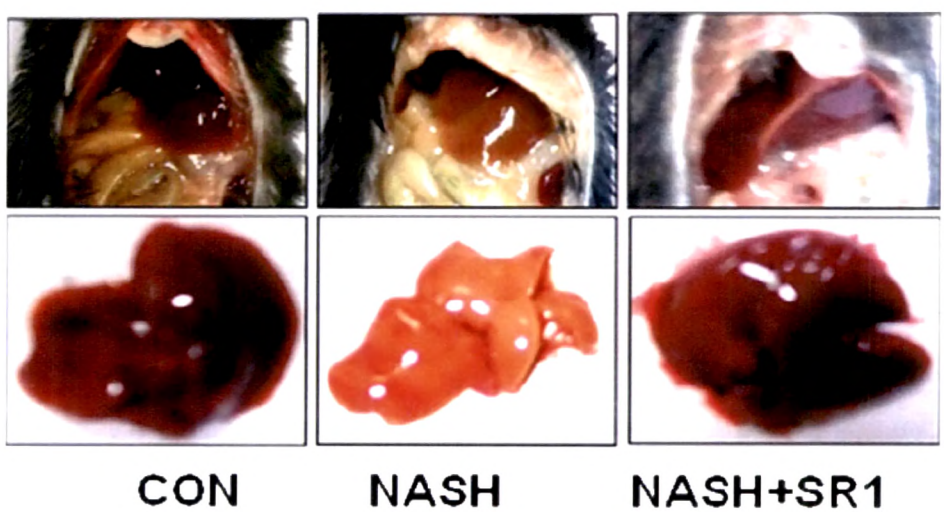


Figure.5 Photomicrographs of liver of (A) control mice showing normal architecture of hepatic tissue showing normal hepatocytes (black arrow), (B) NASH mice showing evidences of parenchymatous lipid accumulation and ballooning hepatocytes (blue arrow), infiltration of inflammatory cells (red arrow) and Mallory hyaline (green arrrow) and (C) NASH+SR1 showing only moderate alterations in architecture with only few scattered hepatocytes showing parenchymatous fat accumulation (blue arrow) with majority of normal hepatocytes (black arrow) (H X E; 100X).

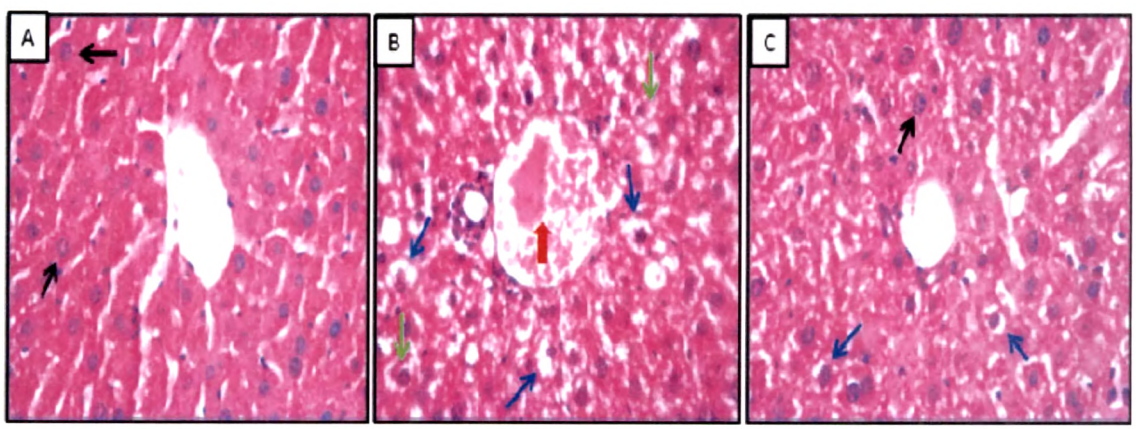


Figure. 6 Photomicrographs showing (A) untreated HepG2 cells, (B) oleic acid (2mM) treated HepG2 cells showing cytoplasmic lipid accumulation, (C) oleic acid (2mM) and *S.rhomboidea*.Roxb extract (200µg/ml) treated HepG2 cells showing lesser degree of cytoplasmic lipid accumulation (40 X).

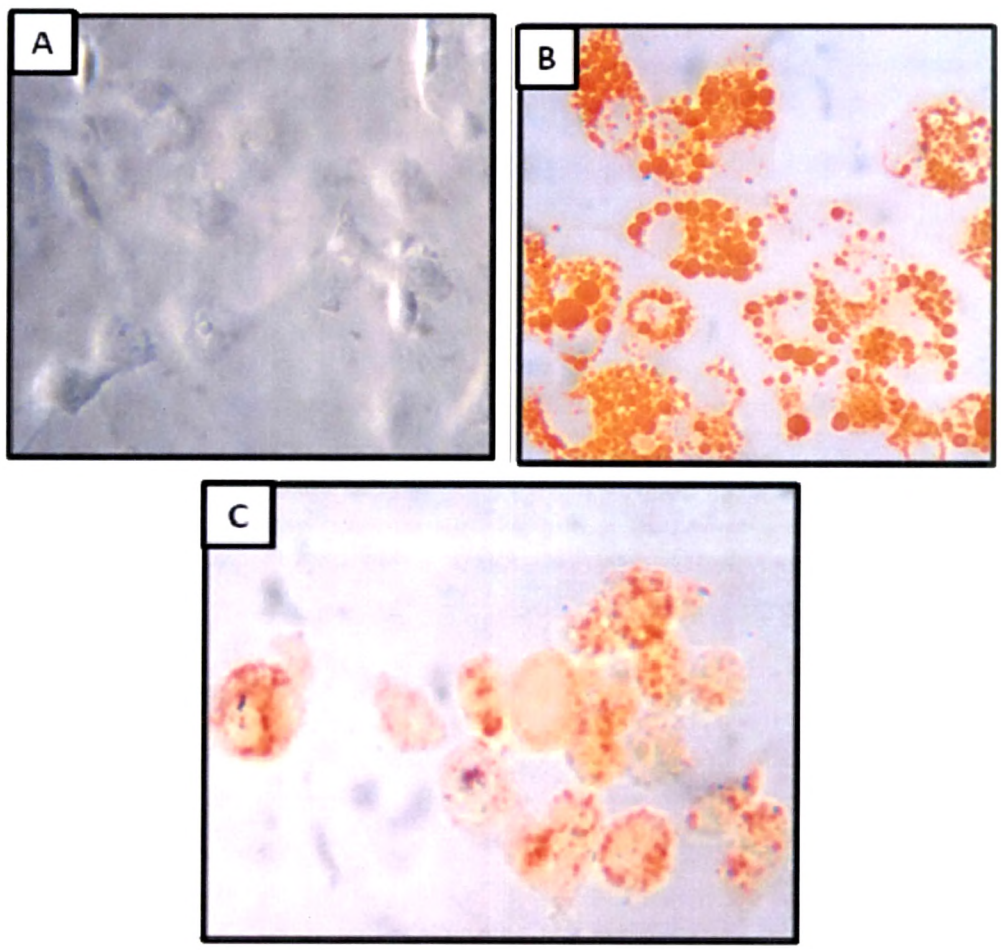
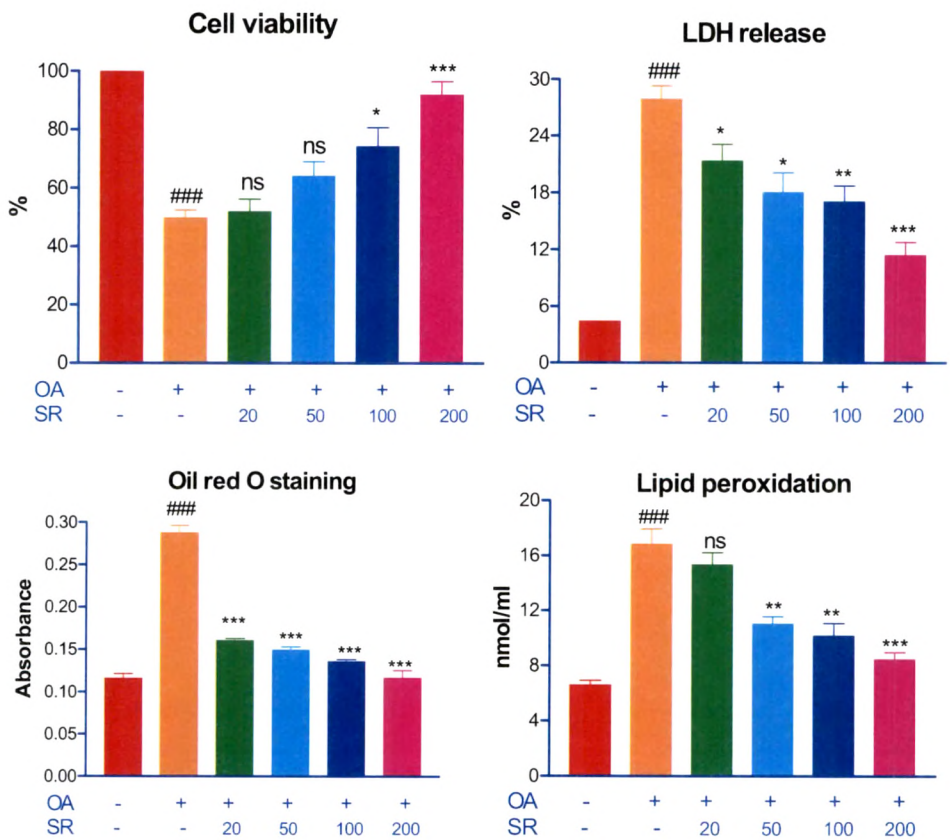


Figure. 7 Effect of *S.rhomboidea*.Roxb (SR) extract on (A) Cell viabillity, (B) LDH release, (C) Oil red O staining and (D) lipid peroxidation in oleic acid treated HepG2 cells.



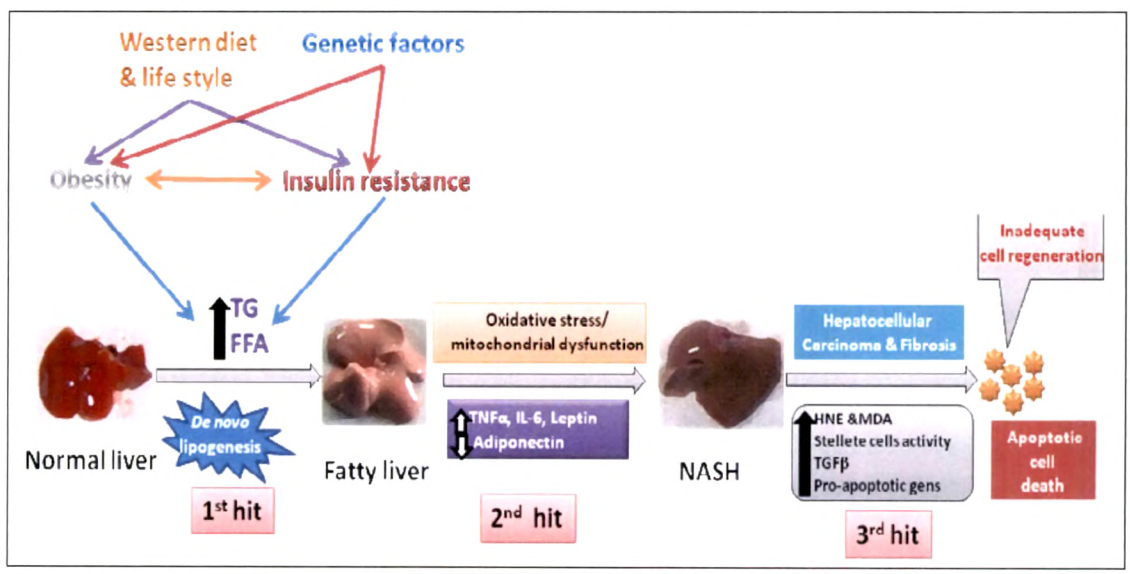
Results are expressed as means ± S.E.M., n = 3.
$^{\#}$ $P < 0.05$ compared with untreated, $^{*}P < 0.05$ compared with oleic acid (OA) and ^{ns} non significant

DISCUSSION

Lipid content in liver is influenced by several physiological processes such as, import of free fatty acids (FFAs) from the adipose tissue, *de novo* FFA synthesis in hepatocytes, beta-oxidation of FFAs, esterification of FFAs into triglycerides and export of triglycerides as VLDL. Hepatic steatosisis is a consequence of imbalance of these processes in favour of excessive TG accumulation (Tiniakos *et al.*, 2010). The present study on HFD induced experimental NASH in C57BL/6J mice records elevated plasma levels of ALT and AST and plasma and hepatic TG and FFA. These observations tend to suggest probable hepatic dysfuntioning due to heavy lipid loading. Simultaneous supplementation with SR extract seems to offset almost completely the diet induced hepatic lipid loading and hepatocyte damage. This becomes evident by the recorded significant resistance to the diet induced increase in TG and FFA and plasma levels of AST and ALT. Apparently, SR seems to be hepato-protective against diet induced NASH and is validated by our earlier reports of lipid lowering effects of SR (Chapter 1).

Increased hepatic lipid load, leading to augmented mitochondrial fatty acid oxidation and generation of ROS seems to contribute to the onset of "second hit" in NASH as, oxidative stress plays a very crucial role in the pathogenesis and progression of NASH. Free radicals such as superoxide anion radical (O2), hydroxyl radical (OH•−), and hydrogen peroxide (H_2O_2) are generated by biochemical redox reactions during normal cell metabolism (Figure.8).

Figure.8 Pathology of non alcoholic steatohepatitis



The deleterious effects of these ROS are kept under check by a delicate balance between the rate of their production and the rate of their elimination by endogenous antioxidant defence systems. But under conditions of toxic insult, overproduction of ROS burdens the cellular antioxidant defence system that results in a compromised oxidative status eventually culminating in cellular oxidative damage to all major groups of biomolecules (DNA, protein, lipids and small cellular molecules; Halliwell, 1999). Mammalian cells have evolved a variety of antioxidant enzymes to counteract the ROS generated during normal cell metabolism and/or various pathophysiological processes. The cellular antioxidant defence systems include enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidise (GPx) and non-enzymatic

antioxidants such as glutathione (GSH), vitamins C and E, playing important role in scavenging oxidants and preventing cell injury (Ames *et al.*, 1999; Halliwell, 1994). Glutathione is the major cellular anti-oxidant and under conditions of oxidative stress, it acts either as an electron donor that neutralizes hydrogen peroxide and lipoperoxides or, as a direct free radical scavenger. SOD is a manganese-containing enzyme in mitochondria that catalyze the dismutation of superoxide anions into H_2O_2 and O_2 (Cao *et al.*, 2003). As H_2O_2 is still harmful to cells, catalase and GPx further catalyze the decomposition of H_2O_2 to water. GSH-Px metabolizes H_2O_2 and hydroperoxide to non-toxic products and terminates the chain reaction of lipid peroxidation by removing lipid hydroperoxides from the cell membrane. Overproduction of ROS contributes to a series of pathological events characterized by reduction of the activities of antioxidant enzymes, lipid peroxidation, and induction of mitochondrial permeability transition, Ca2+ overload, necrosis and apoptosis (Ren *et al.*, 2010). Thus, the coordinated actions of various cellular antioxidants in mammalian cells are critical for effectively detoxifying ROS. Higher concentrations of free radicals lead to lipid peroxidation (LPO) because of undesirable unsaturated fatty acid oxidation of membrane phospholipids. Lipid peroxidation is autocatalytic, progressive and most often irreversible. It represents the most distinct negative phenomenon in free radical activity in a two-phase process. In the first phase, hydrogen from the double-bonded unsaturated fats is eliminated and alkyl radicals and other primary products of LPO are formed (conjugated dienes, peroxide radical and lipid hydroperoxides). In the second phase, these unstable primary products give rise to secondary products such as short-chained carbohydrates, aldehydes and

ketones as well as, malondialdehyde (MDA) as the ultimate product of lipid peroxidation. Paralleling these, there is formation of free radicals, which can reactivate these reactions leading to recurrence of cyclic lipid peroxidation (Rajadurai and Prince, 2006). The results obtained from the present study clearly demonstrate the efficacy of SR extract in containing NASH associated increase in mitochondrial ROS load, which is correlatable with the earlier reported potent free radical scavenging potential of SR (Thounaojam *et al.*, 2010b). Compromised endogenous anti-oxidant status along with increased ROS generation can account for heightened mitochondrial oxidative stress and, as such, NASH animals in the present study reveal significantly depleted hepatic mitochondrial levels of both non-enzymatic and enzymatic antioxidants. Interestingly, co-supplementation with SR extract prevents the decrement in endogenous mitochondrial antioxidants caused due to HFD induced NASH (Hsu and Yen, 2007; Kelly *et al.*, 2009). Precipitous fall in NASH induced mitochondrial non-enzymatic and enzymatic antioxidants, and its prevention by concurrent supplementation with SR extract, is in concurrence with our earlier reported antioxidant potential of SR extract (Thouanaojam *et al.*, 2010b).

Hepatic steatosis caused due to fat overload in experimentally induced NASH (Matteoni *et al.*, 1999) is marked by histopathological lesions such as hepatocyte ballooning, lobular inflammation and Mallory's hyalinization (Brunt, 2007).

Fat accumulation and increased ROS generation lead to lipid peroxidation and damage of mitochondrial membrane. Further, higher MDA generated due to elevated LPO induces the formation of neo-antigens by interacting with hepatic proteins resulting in inflammation and as such, the present histopathological observations are marked by

significantly higher score of infiltration by inflammatory cells, as also reported by others (Bose *et al.*, 2008; Haddad *et al.*, 2009). Favourable effect of SR extract in preventing lipid accumulation, ROS formation and LPO, is also well reflected in the histologically observed minimal score of inflammation. However, a potent role of SR in combating inflammation appears feasible in the light of the reported anti-inflammatory role of SR (Venkatesh *et al.*, 1999). Swelling and/ or enlargement of hepatocytes seen in the present study in NASH mice is a characteristic form of degeneration visible in the form of hepatocyte ballooning, as also reported in cases of human NASH (Heilbronn *et al.*, 2004). Mallory's hyalinization, also known as Mallory's body represents histoarchitectural aberrations of hepatocytes caused due to cross-linking of cytokeratin, a feature reported in NASH (Albano *et al.*, 2005). Both, hepatocyte ballooning and Mallory's bodies clearly visible in liver sections of NASH mice, are however not observable in SR supplemented mice, suggesting the protective effect of principles in SR extract against NASH associated histopathological alterations.

Human hepatocarcenoma cells (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 (Janorkar *et al.*, 2009; Okamoto *et al.*, 2002) and hence is an ideal model for studying and quantifying experimentally induced NASH (Cui *et al.*, 2010). *In vitro* studies on HepG2 cells undertaken essentially to draw confirmatory evidence in favour of the *in vivo* observations on NASH has revealed significant lipid accumulation in OA treated HepG2 cells. Lactate dehydrogenase (LDH) is an oxidoreductase that catalyzes the reaction of

transforming pyruvate into lactate (under anaerobic conditions), and vice versa, transforming lactate to pyruvate under aerobic conditions. Within the cells, it is located in cytoplasm but under conditions of oxidative stress, this enzyme leaches out of cells due to loss of cell membrane integrity in the course of lipid peroxidation. Cytotoxicity of OA on HepG2 cells is characterised by augmented LDH release and lipid peroxidation along with reduced cell viability. However, presence of SR extract along with OA significantly prevented lipid accumulation and LDH release, lipid peroxidation and cytotoxicity as assessed by cell viability test. These results are indicative of the protective role of SR extract against OA induced lipid accumulation and cytotoxicity.

It can be concluded from the present study that, supplementation with SR extract is potentially capable of preventing NASH induced biochemical and pathophysiological changes in the liver of HFD fed C57BL/6J mice. This conclusion is aptly supported by the observed containment of hepatic mitochondrial oxidative stress, and LPO and, maintenance of enzymatic and non-enzymatic antioxidants in NASH+SR3 mice. *In vitro* studies with HepG2 cells further validate the *in vivo* results and suggest a therapeutic role for SR extract in prevention and/ or treatment of NASH.

Summary

The present study was aimed to evaluate protective role of *Sida rhomboidea*.Roxb (SR) extract against high fat diet/fatty acid induced pathophysiological alterations in experimental model of non alcoholic steatohepatitis (NASH). Effect of SR extract on plasma levels of markers of hepatic damage, plasma and hepatic lipids, mitochondrial oxidative stress, status of enzymatic and non-enzymatic antioxidants and histopathological changes in liver tissue were evaluated in high fat diet fed C57BL/6J mice. Also, the effect of SR supplementation on lipid accumulation, lipid peroxidation, cytotoxicity and cell viability were evaluated in oleic acid treated HepG2 cells. Supplementation of NASH mice with SR extract prevented high fat diet induced elevation in plasma marker enzymes of liver damage, plasma and hepatic lipids, mitochondrial oxidative stress and compromised enzymatic and non-enzymatic antioxidant status. Further, addition of SR extract to *in vitro* HepG2 cells minimized oleic acid induced lipid accumulation, higher lipid peroxidation, cytotoxicity and reduced cell viability. These *in vivo* and *in vitro* studies suggest that SR extract has the potential of preventing high fat/fatty acid induced NASH mainly due to its hypolipidemic and antioxidant activities.

Schematic summary