Liv.52 up-regulates cellular antioxidants and increase glucose uptake to circumvent oleic acid induced hepatic steatosis in HepG2 cells

Satyakumar Vidyashankar a, *, L.M. Sharath Kumar b, Vandana Barooah a, R. Sandeep Varma a, Krishna S. Nandakumar a, Pralhad Sadashiv Patki c

a Cell Biology and Biochemistry, Research and Development, The Himalaya Drug Company, Makal, Bangalore 562 123, India
b Phytochemistry, Research and Development, The Himalaya Drug Company, Makal, Bangalore 562 123, India
c Medical Services and Clinical Trials, Research and Development, The Himalaya Drug Company, Makal, Bangalore 562 123, India

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A B S T R A C T

HepG2 cells were rendered steatotic by supplementing 2.0 mM oleic acid (OA) in the culture media for 24 h. OA induced hepatic steatosis in HepG2 cells was marked by significant accumulation of lipid droplets as determined by Oil-Red-O (ORO) based colorimeter assay, increased triacylglycerol (TAG) and increased lipid peroxidation. It was also marked by increased inflammatory cytokines TNF-α and IL-8 with decreased enzymic and non-enzymic antioxidant molecules and decreased cell proliferation associated with insulin resistance and DNA fragmentation. Addition of Liv.52 hydro-alcoholic extract (LHAE) 50 µg/mL to the steatotic cells was effective in increasing the insulin mediated glucose uptake by 3.13 folds and increased cell proliferation by 3.81 folds with decreased TAG content (55%) and cytokines. The intracellular glutathione content was increased by 8.9 folds without substantial increase in GSSG content. LHAE decreased TNF-α and IL-8 by 51% and 6.5% folds respectively, lipid peroxidation by 65% and inhibited DNA fragmentation by 69%. The superoxide dismutase, catalase and glutathione peroxidase activities were increased by 88%, 128% and 64% respectively. Albumin and urea content was increased while the alanine aminotransferase (ALAT) activity was significantly decreased by LHAE. Hence, LHAE effectively attenuate molecular perturbations associated with non-alcoholic fatty liver disease (NAFLD) indications in HepG2 cells.

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I n t r o d u c t i o n

Non-alcoholic steatohepatitis (NASH), the inflammatory form of non-alcoholic fatty liver disease (NAFLD) is a chronic disease that occurs in individuals without significant alcohol consumption (Ludwig et al. 1980). Liver biopsy remains the cornerstone for the diagnosis of NASH, with macrovesicular fat infiltration and lobular inflammation being characteristic (Luyckx et al. 2000). Patients with primary NASH typically have the insulin resistance syndrome (Knobler et al. 1999). Initially, the cause of NASH was unknown and there was no defined therapy. More than 2 decades later, this clinical syndrome is better understood, but still there is no Food and Drug Administration – approved therapy (Falck-Ytter et al. 2001). NASH is increasingly recognized as a major cause of cryptogenic cirrhosis and an indication for liver transplantation. The pathogenesis of NASH is complicated, and the prevailing theory is the “two hits” hypothesis proposed by Day and James (1998). The “first hit” is the deposition of liver free fatty acid and triglyceride in hepatocytes (steatosis). The second “hit”, steatosis progresses to NASH and this progress is associated with factors such as oxidative stress, mitochondrial dysfunction, and cytokines capable of inducing inflammation, fibrosis, or necrosis (Day and James 1998). The elevated cytokine interactions with oxidative stress mediators and lipid peroxides have been postulated to play a role in induction of steatohepatitis in both alcoholic and non-alcoholic origin. TNF-α is an important cytokine in the development of many forms of liver injury (Day and James 1998; Valenti et al. 2002; Wigg et al. 2001).

Oxidative stress has been recognized to be mainly involved in the etiology of liver diseases such as hepatocellular carcinoma, viral and alcoholic hepatitis, NASH, alcoholic steatohepatitis etc. It is known that reactive oxygen species [ROS] and reactive nitrogen species play a crucial role in disease induction and progression (Adachi and Ishii 2002). Oxidative stress results from an imbalance between pro-oxidant and antioxidant chemical species that leads to oxidative damage of cellular macromolecules (Browning and Horton 2004). It may be secondary to the release of inflammatory mediators which are the prime mediator of cell injury. ROS including oxygen ions, free radicals and peroxides are the main pro-oxidants in the body. The ROS are generated physiologically during oxidative phosphorylation (Robertson et al. 2001).
Antioxidants from herbal and dietary origin have been well documented to have therapeutic effect to counteract liver damage (Scalbert et al. 2005; Park et al. 2011; Cai et al. 2011). Lipotoxicity has been implicated in the pathogenesis of NAFLD, and free fatty acids appear to be important contributors of lipotoxicity (Feldstein et al. 2004a,b). The agents with the ability to prevent or attenuate free fatty acids induced hepatotoxicity and oxidative stress induced damage represent a promising therapeutic choice for NAFLD.

In this context, Liv.52 a novel herbal formulation is known to play a pivotal role in combating liver disorder due to various infections and damage due to biological and chemical toxicants (Hussein et al. 2005; Mitra et al. 2008; Vidyashankar et al. 2010; Vidyashankar and Patki 2010) in humans, animals as well in vitro models. Liv.52 is rich in phenolic compounds and in particular polyphenols are believed to be at least in part, responsible for such effects (Vidyashankar et al. 2010). The HepG2 cells retain and mimic many of the special functions, which characterize normal human hepatocytes and used extensively to study the phase I, phase II and antioxidant enzymes ensuring that they constitute a good model to study cytoprotective, genotoxic and antigenotoxic effects of compounds in vitro (Mersch-Sundermann et al. 2004). Hence, HepG2 cells were rendered steatotic with oleic acid and Liv.52 hydro-alcohol extract (LHAE) was tested for its possible beneficial effect.

Materials and methods

Chemicals

Ammonium acetate, Bradford reagent, cytochalasin-B, cytochrome-C, DPPH, Dulbecco’s Minimum Essential Medium Eagle (DMEM), fetal bovine serum (FBS), trypsin, EDTA, glutathione, hydrogen peroxide, insulin, MTI, NADPH, oleic acid, thiofibarbituric acid, xanthine and xanthine oxidase were purchased from Sigma–Aldrich (St. Louis, MO, USA). 2-Deoxy-[3H] glucose was purchased from the Department of Atomic Energy, Mumbai, India. Methanol, acetonitrile and water were procured from Merck (LC–MS grade). All other solvents reagents used were of analytical grade.

Composition of Liv.52

The Liv.52 is the approved proprietary medicine by drug regulatory authority Department of AYUSH, Ministry of Health and Family Welfare, Government of India. The Liv.52 formulation contains extracts of the following medicinal plants in definite proportions – Himsra (Capparis spinosa) 65 mg, Kasani (Cichorium intybus) 65 mg, Kakamachi (Solanum nigrum) 32 mg, Arjuna (Terminalia arjuna) 32 mg, Kasamarda (Cassia occidentalis) 16 mg, Binranjasipa (Achillea millefolium) 16 mg, Jhavuka (Tamarix gallica) 16 mg. The good agricultural and collection practices (GACP) were employed for plants used in the formulation. Plants were identified and certified by Botanist and a voucher specimen of each constituent plant has been archived in the herbarium of R&D, The Himalaya Drug Company, Bangalore, India.

Liv.52 hydro-alcoholic extract (LHAE)

Liv.52 granules (100 g) was ground into powder under liquid nitrogen with a mortar and pestle, and extracted with 1000 mL of ethanol–water (70:30 v/v) at RT for 30 min. The mixture was then extracted in a shaking water bath (300 rpm) at 37 °C for 24 h. After cooling to room temperature, the slurries were centrifuged at 1000 rpm for 15 min, and the supernatant was collected. The solution was then evaporated under reduced pressure to obtain the ethanol extract, with the yield being 2.3% of the dry weight of Liv.52. Thus obtained Liv.52 hydroalcoholic extract was used to evaluate the inhibitory effect on OA-induced fatty liver model in vitro.

Liquid chromatography–mass spectrometer analysis

The LC–MS/MS instrument consisted of an HPLC (Shimadzu LC-20AD) coupled with API-2000 mass spectrometer–MS/MS [Applied Biosystem-MDS SCIEX, Canada]. The 20 μL LHAE at the concentration of 2 mg/mL in methanol was injected through SIL-HTC Shimadzu auto sampler for phytochemical screening. The Luna RP- C18 (5 μm, 250 mm × 4.6 mm) (Phenomenex Torrance, CA, USA) column was used for separation and analysis of LHAE. The column oven temperature was maintained at 40 °C throughout the analysis by CTO–10ASVP column oven. The binary mobile phase is the combination of A (10 mM ammonium acetate and 0.1% formic acid in water) and B (acetonitrile). The gradient was varied linearly from 3% to 9% (B) in 0–5 min, 9% to 16% (B) in 5–15 min, 16% to 50% (B) in 15–45 min, 50% to 90% (B) in 45–48 min, and held with 90% (B) at 48–51 min followed by 90% to 3% (B) in 51–60 min delivered at a flow rate of 600 μL/min with split out 200 μL/min to mass spectrometer. An API-2000 mass spectrometer coupled with electron spray ionization (ESI) interface was used to obtain the MS/MS data using Analyst 1.5 version software. The ionization conditions were optimized and the following conditions were adopted – ionization voltage was −4500 V; curtain gas (CUR) 25 psi; focussing potential (FP) −300 V; entrance potential (EP) −2 V; declustering potential (DP) −20 V; ionization source temperature 420 °C; ion source gas 1 (GS1) 55 psi and ion source gas 2 (GS2) 65 psi. Collision energy (CE) for MRM of precursor to product ion was optimized by multiple run through LC until most intense precursor to product ion transition state is obtained. The data was recorded in negative multiple reaction monitoring (MRM) mode. Compounds identified by LC–MS/MS (Fig. 1) were characterized according to Rabaneda et al. (2003) and given in Table 1.

Cell culture

HepG2 cells (hepatocellular carcinoma cell line), obtained from the National Center for Cell Science (NCCS) Pune, India, were maintained in culture in 25 cm² polystyrene flasks (Tarsorns) with DMEM containing 10% FBS, 1% antibiotic–antimycotic solution, and 3.7 g/L sodium bicarbonate under an atmosphere of 5% CO₂ at 37 °C with 95% humidity.

LHAE cytotoxicity

HepG2 cells were plated in 96-multiwell culture plates at 1 × 10⁵ cells per well. To study LHAE cytotoxicity, 24 h after plating, the medium was discarded and fresh medium containing LHAE at various concentrations was added. At different time points, cellular viability was determined by the MTT assay (Mosmann 1983).

Oleic acid induced hepatic steatosis and its inhibition by LHAE

The confluent HepG2 in 96 well culture plate were washed in PBS and added with medium containing 0–2.0 mM oleic acid–bovine serum albumin (OA–BSA) complex (molar ratio 4:1). Then the cells were further incubated for 24 h. The medium with only BSA was selected as the control. The extent of steatosis was quantified by oil-red-O (ORO) based colorimetric assay (Cui et al. 2010) and measuring triacylglycerol content at various time intervals using triglyceride estimation kit (Pointe Scientific, Mumbai, India). To study the hepatic steatosis inhibitory effect of LHAE, the confluent HepG2 cells were added 2.0 mM (OA–BSA) complex
Fig. 1. Compounds detected in Liv.52 hydroalcoholic extract in negative MRM mode. LC–MS/MS conditions as described in the text.
Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Peak retention time (min)</th>
<th>M_0 (relative molar mass)</th>
<th>Precursor ion (m/z)</th>
<th>Product ion (m/z)</th>
<th>DP (V)</th>
<th>CE (V)</th>
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<tbody>
<tr>
<td>Apigenin</td>
<td>44.10</td>
<td>270</td>
<td>269.2</td>
<td>151.1</td>
<td>−20</td>
<td>−35</td>
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<tr>
<td>Ferulic acid</td>
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<td>193.2</td>
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<td>−20</td>
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<tr>
<td>Caffeic acid</td>
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<td>−20</td>
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<td>Gallic acid</td>
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<td>169.2</td>
<td>125.1</td>
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<td>−20</td>
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<td>Comaric acid</td>
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<td>163.2</td>
<td>119.1</td>
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<td>Protocatechuic acid</td>
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<td>154</td>
<td>153.2</td>
<td>109.1</td>
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<td>−20</td>
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<tr>
<td>Hyperoside (Quercetin-3-O-galactoside)</td>
<td>29.50</td>
<td>464</td>
<td>463.2</td>
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<td>−38</td>
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<td>Isoquercetin (Quercetin-3-O-glucoside)</td>
<td>29.80</td>
<td>464</td>
<td>463.2</td>
<td>301.1</td>
<td>−20</td>
<td>−32</td>
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<tr>
<td>Apigenin-7-O-glucoside</td>
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<td>431.2</td>
<td>260.1</td>
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<td>−35</td>
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<tr>
<td>Chlorogenic acid</td>
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<td>353.2</td>
<td>191.1</td>
<td>−20</td>
<td>−20</td>
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<tr>
<td>Quercetin</td>
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<td>301.2</td>
<td>151.1</td>
<td>−20</td>
<td>−35</td>
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<tr>
<td>Luteolin</td>
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<td>286</td>
<td>285.2</td>
<td>151.1</td>
<td>−20</td>
<td>−35</td>
</tr>
</tbody>
</table>

with or without LHAE along with experimental control. The hepatic steatotic inhibitory effect of LHAE was quantified as described earlier.

Effect of LHAE on glucose uptake in oleic acid induced hepatic steatosis

The HepG2 cells were rendered steatotic as described earlier and treated with or without LHAE along with experimental control for 24 h. The cells were then incubated without PBS for 5 h again in the presence or absence of LHAE and experimental controls. The cells were then rinsed with Krebs–Ringer phosphate buffer. The 10 μmol/L 2-deoxy-[3H]glucose (2-DG) (1 μCi/mL) uptake was measured over a 10 min period under conditions in which the uptake was linear. The uptake measurement was made in triplicate. Nonspecific uptake was determined in the presence of 10 μmol/L cytochalasin-B and was subtracted from the total uptake. The uptake of 2-DG was terminated after 10 min by rapidly aspirating off the radioactive incubation medium and washing the cells three times in ice-cold phosphate-buffered saline. The radioactivity associated with the cells was determined by cell lysis in 0.5 N NaOH with neutralization by the addition of 0.5 N HCl, followed by liquid scintillation using Packard (Downers Grove, IL, USA) Top Count-NXT™ liquid scintillation counter. Aliquots from each well were used to determine the protein concentration using Bradford reagent. In experiments in which the effect of insulin was examined, 10−7 M insulin was added to the mixture in KRPH for 20 min before transport studies. Nonspecific uptake and absorption were always <15% of the total uptake (Yonemitsu et al. 2001).

Effect of LHAE on lipid peroxidation, inflammatory cytokines, DNA fragmentation, glutathione levels and antioxidant enzyme activities in OA induced hepatic steatosis

HepG2 cells were plated in 60 mm culture plates at 7.5 × 10⁴ cells per well. Forty hours after plating, the medium was discarded and fresh medium containing 2.0 mM (OA-BSA) complex with or without LHAE along with experimental controls were added. Twenty-four hours later, cell culture medium and cell scrapings were harvested and kept at −80°C for following quantification of several parameters. Cell scrapings were harvested in lysis buffer (25 mM KH₂PO₄, 2 mM MgCl₂, 5 mM KCl, 1 mM EDTA, 1 mM EGTA, 100 μM PMSF, pH 7.5) after rinsing the cells with PBS (pH 7.4).

Biochemical analysis

Lipid peroxidation

The extent of lipid peroxidation was estimated by the levels of malondialdehyde measured using the thiobarbituric acid reactive substances (TBARS) assay at 535 nm (Ohkawa et al. 1979). The results are expressed as nmol/mg of protein using a molar extinction coefficient of 1.56 × 10⁵ M cm⁻¹.

Cytokine assay

The anti-inflammatory cytokines TNF-α and IL-8 was measured using commercially available enzyme linked immunosorbent assay (ELISA) (Krishgen Biosystems, Mumbai, India) following the user guide provided with kit.

DPA assay for DNA fragmentation

The diphenylamine (DPA) reaction was performed by the method of Perandones et al. (1993). Perchloric acid (0.5 M) was added to the cell pellets containing uncult DNA (resuspended in 200 μL of hypotonic lysis buffer) and to the other half of the supernatant containing DNA fragments. Then two volumes of a solution consisting of 0.088 M DPA, 98% (v/v) glacial acetic acid, 1.5% (v/v), sulphuric acid, and a 0.5% (v/v) concentration of 1.6% acetaldehyde solution were added. The samples were stored at 4°C for 48 h. The reaction was quantified spectrophotometrically at 575 nm. The percentage of fragmentation was calculated as the ratio of DNA in the supernatants to the total DNA.

Measurement of nonenzymic antioxidants

Cells were homogenized in trichloroacetic acid (5% w/v), and deproteinized supernatant was used for GSH assay. The glutathione levels from the cell homogenates was determined by the DTNB–GSSG reductase recycling assay as previously described (Anderson 1985) with some modifications. The results are expressed as nmol GSH/mg of protein.

Measurement of enzymic antioxidants

The activity of antioxidant enzymes, namely superoxide dismutase (SOD), catalase and glutathione peroxidase, (GPx) were assayed in 1000 × g supernatants of cell homogenates. Total SOD activity was determined by monitoring the inhibition of the reduction of ferricytochrome C at 550 nm, using the xanthine – xanthine oxidase system as the source of superoxide. One unit of the SOD is defined as the amount of the enzyme required to inhibit 50% of the rate of cytochrome C reduction (Flohe and Otting 1984). Catalase activity was measured by following the rate of H₂O₂ consumption spectrophotometrically at 240 nm and expressed as μmol H₂O₂ oxidized/min/mg protein (Aebi 1974). Glutathione peroxidase activity was determined by following the enzymatic NADPH oxidation at 340 nm (Flohe and Gunzler 1984).

Biochemical and enzyme analysis

Cell supernatants were collected for analysis of albumin, urea nitrogen and alanine transferase and analysed using an automated Nanolab chemistry analyzer (Trivitron Diagnostics Pvt. Ltd., Chennai, India) with commercial assay kits obtained from Diasys.
Diagnosys Systems (Holzheim, Germany) following the manufacturer’s instructions.

Statistical analysis

Results were expressed as mean ± SEM. Statistical significances were determined by one-way ANOVA by employing Tukey Kramer post test using graph pad prism 4 (La Jolla, CA, USA). Results are considered to be significant at \( p < 0.05 \).

Results

LHAE cytotoxicity and OA induced hepatic steatosis

LHAE did not impart cytotoxicity to HepG2 cells at 0–200 \( \mu g/mL \) concentration for 24 h as shown in Fig. 2 (A). The HepG2 cells when treated with 0–2.5 mM concentration of oleic acid for 24 h to induce hepatic steatosis condition did not show cytotoxicity to the cells. While, triacylglycerol content and the recovered Oil-Red-O content was increased significantly by 3.8 folds as shown in Fig. 2(B). Further, microscopic examination revealed HepG2 cells treated with increasing concentration of oleic acid had significant difference in its morphology and the triacylglycerol accumulation as lipid droplets (determined with ORO staining) was prominent with 2.0 mM oleic acid. Hence, 2.0 mM oleic acid was used to induce hepatic fatty liver conditions to study the effect of LHAE in all the experiments.

Effect of LHAE on cell proliferation and TAG accumulation in OA induced hepatic steatosis

The cell proliferation was significantly decreased by 2.0 mM oleic acid to the extent of 55% compared to control cells. The LHAE could effectively increase the cell proliferation by 3.81 folds compared to OA treated group. Similarly, silymarin and vitamin E increased the cell proliferation by 2.61 and 3.24 folds respectively as shown in Fig. 3(A). The photomicrograph of HepG2 cells upon microscopic observation shows protective effect of LHAE and other compounds on morphological features of HepG2 cells treated with OA (Fig. 4). The TAG was accumulated as lipid droplets in the OA treated cells and LHAE significantly decreased TAG content by 55%. While, silymarin and vitamin E could not effectively reduce the intracellular TAG content when compared to LHAE as given in Fig. 3(B).

Effect of LHAE on glucose uptake in OA induced hepatic steatosis

The glucose uptake capacity in OA treated cells was significantly diminished by 64% and 77% respectively in absence and presence of insulin. The addition of LHAE to cells could significantly increase the specific glucose uptake up 2.66 and 3.13 folds in absence and presence of insulin compared to OA treated cells. While the glucose uptake was increased in silymarin and vitamin E treated cells by 43% and 47% in absence of insulin and 42% and 22% respectively in presence of insulin compared to OA treated cells as given in Fig. 5.

Effect of LHAE on lipid peroxidation, DNA fragmentation and glutathione content in OA induced hepatic steatosis

The addition of OA resulted in the 2.4 folds increased lipid peroxidation in HepG2 cells (Fig. 6(A)). LHAE addition decreased the MDA levels significantly by 63% and silymarin and vitamin E inhibited cellular lipid peroxidation by 40% and 50% respectively compared to OA treated group. The OA significantly increased the DNA fragmentation by 3.87 folds compared to control as shown in Fig. 6(B). DNA fragmentation was inhibited by 69% when treated with LHAE and with silymarin and vitamin E, it was inhibited by 83% and 48% respectively compared to OA group.

The GSH level was significantly depleted by 8.0 fold in OA treated HepG2 cells. Addition of LHAE significantly increased the GSH levels.
Fig. 4. Photomicrograph of OA induced hepatic steatosis in HepG2 treated with LHAE and other compounds, (a) Control, (b) Oleic acid (2 mM), (c) OA (2 mM) + LHAE (50 μg/mL), (d) OA (2 mM) + silymarin (0.1 M) and (e) OA (2 mM) + vitamin E (25 μM). The cells were stained with 5% Giemsa and morphological changes were recorded using Leica Microscope (Germany) at 10× magnification.

Table 2

<table>
<thead>
<tr>
<th>Groups</th>
<th>GSH (nmol/mg protein)</th>
<th>GSSG (nmol GSH equiv./mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>90.45 ± 0.21</td>
<td>2.58 ± 0.35</td>
</tr>
<tr>
<td>Oleic acid (2 mM)</td>
<td>11.28 ± 0.15</td>
<td>18.97 ± 0.98</td>
</tr>
<tr>
<td>Oleic acid (2 mM) + LHAE (50 μg/mL)</td>
<td>100.64 ± 0.12</td>
<td>4.68 ± 1.32</td>
</tr>
<tr>
<td>Oleic acid (2 mM) + silymarin (0.1 M)</td>
<td>70.89 ± 0.19</td>
<td>7.58 ± 1.68</td>
</tr>
<tr>
<td>Oleic acid (2 mM) + vitamin E (25 μM)</td>
<td>82.58 ± 0.21</td>
<td>5.68 ± 1.12</td>
</tr>
</tbody>
</table>

HepG2 cells pre-treated with OA were incubated with LHAE (50 μg/mL), silymarin (0.1 M) and vitamin E (25 μM) and GSH and GSSG were measured as described in materials and methods section. Values are mean ± SEM of three independent experiments carried out in triplicates.

* Statistically significant at p < 0.05 compared to OA group.

** Statistically significant at p < 0.05 compared to control.

by 8.9 fold, whereas silymarin and vitamin E increased GSH content by 6.28 and 7.32 folds respectively compared to OA group. GSSG content was increased by 7.35 folds in OA treated HepG2 cells compared to control. LHAE significantly brought down the GSSH levels by 4.05 fold compared to OA treated cells as given in Table 2. Sily-

Fig. 5. Effect of LHAE on 2-DG uptake in OA induced hepatic steatosis in HepG2 cells. HepG2 cells were incubated for 24 h with control, LHAE and other experimental compounds. The 2-DG uptake was determined as described in materials and methods section. Values are mean ± SEM of three independent experiments carried out in triplicates. * Statistically significant at p < 0.05 compared to control. ** Statistically significant at p < 0.05 compared to OA group.

Fig. 6. (A) Effect of LHAE on lipid peroxidation in OA induced hepatic steatosis in HepG2 cells. (B) Effect of LHAE on DNA fragmentation in OA induced hepatic steatosis in HepG2 cells. HepG2 cells pre-treated with OA were incubated with LHAE (50 μg/mL), silymarin (0.1 M) and vitamin E (25 μM) lipid peroxides and DNA fragmentation was measured as described in materials and methods section. Values are mean ± SEM of three independent experiments carried out in triplicates. * Statistically significant at p < 0.05 compared to control. ** Statistically significant at p < 0.05 compared to OA group.

Effect of LHAE on inflammatory cytokines in OA induced hepatic steatosis

OA increased IL-8 by 21 folds and TNF-α by 3.33 folds respectively compared to control cells (Fig. 7(A) and (B)). LHAE significantly reverted the increased IL-8 by 6.35 folds and TNF-α by 51% compared to OA group. While, silymarin inhibited IL-8 by 1.83
folds and TNF-α by 60% and likewise, vitamin E inhibited IL-8 by 2.53 folds and TNF-α by 57% compared to OA group.

**Effect of LHAE on antioxidant enzymes in OA induced hepatic steatosis**

The effect of LHAE in OA treated cells on antioxidant enzymes was evaluated and the results are given in Table 3. The activities of catalase, glutathione peroxidase and superoxide dismutase were significantly inhibited by 38%, 29% and 22% respectively in OA group compared to control cells. Whereas, LHAE could significantly enhance the catalase, glutathione peroxidase and superoxide dismutase activities by 2.28, 1.63 and 1.87 folds respectively compared to OA group. While, silymarin did not significantly increase catalase and glutathione peroxidase activity but vitamin E could enhance the enzyme activities by 68% and 19% respectively compared to OA treated cells. The superoxide dismutase activity was significantly increased in silymarin and vitamin E treated cells by 75% and 27% respectively compared to OA treated cells.

**Effect of LHAE on biochemical markers in OA induced hepatic steatosis**

Oleic acid addition to HepG2 cells led to significant decrease in urea and albumin secretion whereas, ALAT activity was increased by 99% compared to control cells. But addition of LHAE, silymarin and vitamin E decreased the ALAT activity by 41%, 9.5% and 32% respectively compared to OA treated cells (Table 4). While, amount of urea secreted into the cell supernatant was increased in the LHAE, silymarin and vitamin E treated cells compared to OA treated cells. Similarly, the albumin secretion was significantly increased in LHAE by 2.11 folds and in silymarin and vitamin E it was increased by 1.64 folds compared to OA treated cells.

**Discussion**

HepG2 cell retain biochemical and morphological properties characteristic of hepatocyte and has proved useful for studying hepatocyte/liver injury and metabolism. Hepatic steatosis results from increased fatty acid influx to the hepatocytes and reduced lipid oxidation and decreased VLDL excretion (Cui et al. 2010). In this study, HepG2 cells were supplemented with pathophysiologic levels of oleic acid to mimic the influx of excess FFAs into hepatocytes, giving rise to hepatic steatosis. Our data demonstrate that exposure of HepG2 to pathophysiologically relevant concentrations of FFA (Oleic acid) results in increased TAG content, lipid peroxidation associated perturbations and with decreased insulin mediated glucose uptake ability and proliferation of HepG2 cells which is in agreement with several studies that suggest a link between lipid peroxidation, cellular pro-oxidant and antioxidant imbalance and obesity-related complications (Feldstein et al. 2004a,b; Barve et al. 2007; Kugelmas et al. 2003).

Increased fatty acid influx into the HepG2 cells may have resulted in the generation of reactive oxygen species (ROS) by the accumulation of free fatty acids in mitochondria due to saturation of mitochondrial β-oxidation and excess H2O2 production during peroxisomal β-oxidation (Abdul-Ghani et al. 2008). Thus, oxidative stress, resulting from an imbalance between pro-oxidant and antioxidant balance resulted in the generation of ROS and affects major cellular components including lipids, proteins and DNA. ROS play important physiological functions and can also cause extensive cellular damage (Hoffman and Bookes 2009). But cells are provided with efficient molecular strategies to strictly control the intracellular ROS level and to maintain the balance between pro-oxidant and antioxidant molecules (Hoffman and Bookes 2009; Sies 1985). In this context recent evidence shows that reactive oxygen species (ROS) mediated cellular signalling contribute to the development of viral, alcoholic and non alcoholic liver diseases. ROS can also be considered as molecular second messenger within the cell as they can be generated during triggering of particular cellular responses by cytokines, hormones, growth factors and other soluble mediators such as extracellular ATP (Lander 1997).

It is observed in the experiments that oleic acid induced oxidative stress during steatosis in HepG2 cells resulted in the increase of free radicals production together with a decrease in antioxidant defence system which is in agreement with the earlier studies (Cui et al. 2010). The normal liver is provided with very efficient enzymatic and non enzymatic antioxidant systems. In particular, Kupfer cells and biliary stellate cells are potentially more exposed to ROS molecules and it has been well documented that hepatic antioxidant systems are significantly decreased in several chronic liver diseases. The involvement of the classical intracellular ROS scavengers such as superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT) is of fundamental importance in setting up therapeutic approaches towards oxidative based liver pathologies (Inoue 1994). Therapy directed towards counteracting the oxidative stress might be an effective measure in the management of NAFLD and its complications. The herbal composition like Liv.52 rich in natural antioxidants (Vidyashankar et al. 2010; Vidyashankar and Patki 2010) and other health beneficial compounds like silymarin (Song et al. 2007) and vitamin E (Sanyal et al. 2010; Barve et al. 2007) have been widely used to treat hepatic disorders and might be very effective in combating the ROS mediated lipotoxicity induced by oleic acid in HepG2 cells.
The results demonstrated that cells incubated with LHAE could effectively inhibit the TAG accumulation and promoted cell proliferation, while the SOD, GPX and CAT activities were significantly increased. The lipid peroxidation was significantly decreased and the depleted GSH levels were replenished in LHAE treated steatotic cells without increasing the GSSG levels. It was also shown that DNA fragmentation was decreased by LHAE in steatotic cells. Silymarin and vitamin E were not as effective compared to LHAE in reverting the biochemical parameters, but they too inhibited the lipotoxicity, suggesting the role of LHAE and other antioxidant molecules in combating the ROS mediated molecular perturbation.

It is known that, GSH plays an important role in hepatocyte defence against ROS, free radicals and electrophilic metabolites (Redderis 1996; Castell et al. 1997). Hence, severe GSH depletion leaves cells more vulnerable to oxidative damage by radicals and increases protein thiolation or oxidation of SH groups that may lead to alterations in cellular calcium homeostasis (Castell et al. 1997). A sustained increase in cytosolic calcium levels results in activation of enzymes (phospholipases, non-lysosomal proteases, endonucleases) and cytoskeletal damage, which ultimately causes cell death (Castell et al. 1997). The decrease of GSH levels has indeed been suggested as one of the primary mechanisms of ROS induced toxicity in liver cells (Jewell et al. 1986; Buc-Calderon et al. 1991; Martin et al. 2001) which is generally followed by an increase in the intracellular levels of calcium (Bellomo et al. 1982; Nicotera et al. 1988; Buc-Calderon et al. 1991). Earlier we have shown that, cytotoxicity induced by tertiary butyl hydroperoxide in HepG2 cells has been shown to be proportional to the depletion of GSH (Vidyashankar et al. 2010) and also low GSH levels in Cu2+ loaded cells is considered a major intracellular determinant of their susceptibility to cytotoxicity (Vidyashankar and Patki 2010).

Thus, the potential of LHAE to maintain GSH at reasonably high levels is of importance during oleic acid induced steatosis. Therefore, the ability of LHAE to prevent OA induced GSH depletion by 8.9 folds is very significant in restoring the cell viability. The GSSG formation was inhibited by LHAE and this may be attributed to the formation of GSH conjugates rather than oxidation to GSSG in OA induced toxic conditions. Beside this glutathione peroxidase activity was significantly increased and DNA fragmentation was decreased by LHAE during hepatic steatotic toxicity in HepG2 cells. Silymarin could prevent the GSH depletion by 6.28 folds and vitamin E by 7.32 folds. Vitamin E and silymarin prevented the DNA fragmentation which is less compared to LHAE.

Insulin resistance is central to the pathogenesis of NASH (Valenti et al. 2002) and it is shown that LHAE could effectively increase the glucose uptake in steatotic HepG2 cells. Previously, it was reported that stimulation of TNF-α in hepatocytes and adipocytes by FFA is implicated in the aetiology of insulin resistance (Valenti et al. 2002). In this study, insulin mediated glucose uptake was significantly higher in HepG2 cells with the addition of LHAE as shown by positive correlation between glucose uptake and decreased lipid peroxidation, cytokine levels and increased cell proliferation in culture conditions. This increased glucose uptake observed was not due to non specific glucose transportation since it was ruled out by inclusion of cyclochalin B in the experiments. However an increased level of glucose uptake was observed with the addition of LHAE in the steatotic rendered HepG2 cells. While, the insulin mediated glucose uptake was not increased in cells rendered steatotic with oleic acid whereas, addition of LHAE to the cells significantly increased the insulin mediated glucose uptake in cells. Insulin mediated glucose uptake mediated by LHAE clearly justified the need for synthesis of new protein relevant to glucose transport. These findings clearly show that LHAE is very effective in restoring the glucose uptake in steatotic cells which was diminished during steatotic conditions. The synergistic effect of processed phytochemical compounds present in the LHAE is responsible for this activity since most of the ingredients and its compounds in the composition are known to possess anti diabetic activity in experimental and clinical studies (Ganesh et al. 2009). Silymarin and vitamin E also increased the glucose uptake in HepG2 cells rendered steatotic but it was not statistically significant compared to LHAE.

The vast majority of cytokine abnormalities were observed in animal models of alcoholic liver disease are also observed in animal models of NASH (Tilg and Diehl 2000). Increased supply of FFAs to the liver may play a major role in the development of hepatic inflammation and result in secretion of cytokines (Day and James 2007)
1998; Valentì et al. 2002; Wigg et al. 2001). In the present study, higher levels of cytokines was significantly brought down by addition of LHAE, silymarin and vitamin E and established that restoring cytokine to normal levels has a beneficial role in amelioration of NAFLD symptoms as reported earlier. It was also shown earlier that, Liv.52 regulated ethanol induced PPAR-γ and TNF-α expression in HepG2 cells (Mitra et al. 2008).

The albumin and urea content in the culture media treated with LHAE suggests the positive correlation with cell proliferation. The decrease in the alanine amino transferase (ALT) activity in the cell supernatant treated with LHAE gives additional evidence towards restoring normal biochemical parameters in OA induced steatotic liver cells (Sorbi et al. 1999). Silymarin and vitamin E were effective in increasing the albumin and urea content in the cell supernatant by increased secretion. In similar lines the alanine amino transferase activity was decreased in cell supernatant treated with LHAE, silymarin and vitamin E which decreased the ALT activity in HepG2 cells rendered steatotic.

In conclusion, compounds present in LHAE could effectively reverse the molecular perturbations underlying NAFLD symptoms suggesting its importance to ameliorate OA induced hepatic steatosis in HepG2 cells. Hence treatment with LHAE could be a new perspective to carry out more experimental and clinical studies to understand the molecular mechanism to overcome NAFLD symptoms.

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References


