Quercetin ameliorate insulin resistance and up-regulates cellular antioxidants during oleic acid induced hepatic steatosis in HepG2 cells

Satyakumar Vidyashankar a,⁎, R. Sandeep Varma a, Pralhad Sadashiv Patki b

a Cell Biology and Microbiology, Research and Development, The Himalaya Drug Company, Makali, Bangalore 562 123, India
b Medical Services and Clinical Trials, Research and Development, The Himalaya Drug Company, Makali, Bangalore 562 123, India

ARTICLE INFO

Article history:
Received 27 April 2012
Accepted 10 January 2013
Available online 21 January 2013

Keywords:
Oleic acid
HepG2 cells
Non-alcoholic fatty liver disease
Quercetin
Insulin resistance
TNF-alpha
Glutathione

ABSTRACT

Hepatic lipid accumulation and oxidative stress contribute to non-alcoholic fatty liver disease (NAFLD). Thus, we hypothesized that the hypolipidemic and antioxidant activity of quercetin would attenuate events leading to NAFLD. Addition of 2.0 mM oleic acid (OA) into the culture media induced fatty liver condition in HepG2 cells by 24 h. It was marked by significant accumulation of lipid droplets as determined by Oil-Red-O (ORO) based colorimetric assay, increased triacylglycerol (TAG) and increased lipid peroxidation. The inflammatory cytokines TNF-α and IL-8 levels were significantly increased with decreased antioxidant molecules. OA induced insulin resistance which was evident by inhibition of glucose uptake and cell proliferation. Quercetin (10 μM) increased cell proliferation by 3.05 folds with decreased TAG content (45%) and was effective in increasing insulin mediated glucose uptake by 2.65 folds. The intracellular glutathione content was increased by 2.0 folds without substantial increase in GSSG content. Quercetin (10 μM) increased TNF-α and IL-8 by 59.74% and 41.11% respectively and inhibited generation of lipid peroxides by 50.5%. In addition, RT-PCR results confirmed quercetin (10 μM) inhibited TNF-alpha gene expression. Further, superoxide dismutase, catalase and glutathione peroxidase activities were increased by 1.68, 2.19 and 1.71 folds respectively. Albumin and urea content was increased while the alanine aminotransferase (ALAT) activity was significantly decreased by quercetin. Hence, quercetin effectively reversed NAFLD symptoms by decreased triacyl glycerol accumulation, insulin resistance, inflammatory cytokine secretion and increased cellular antioxidants in OA induced hepatic steatosis in HepG2 cells.

© 2013 Elsevier Ltd. All rights reserved.

1. Introduction

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 (Knoebler 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-Yerrer 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, and alcoholic steatohepatitis. 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 (Brown 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). Lipotoxicity has been implicated mainly in the pathogenesis of NAFLD, and free fatty acids appear to be important contributors of lipotoxicity (Feldstein et al., 2004). Elevation of plasma free fatty acid concentration in insulin-sensitive subjects causes a dose dependent decrease in whole body insulin sensitivity, associated with impaired insulin signaling in skeletal muscle (Belfort et al., 2005). Although a causal role for dysregulated FFA metabolism in the development of insulin resistance is well recognized and has been referred to as “lipotoxicity”. Thus, agents with the ability to prevent or attenuate free fatty acids induced lipotoxicity and oxidative stress induced damage represent a promising therapeutic choice for NAFLD.

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). Flavonoids are phenolic phytochemicals that represent essential constituents of the non-energetic part of the human diet. They are thought to promote optimal health, partly via their antioxidant effects in protecting cellular components against reactive oxygen species (ROS) (Boots et al., 2007; Amalia et al., 2007). Plants contain numerous polyphenols, which have been suggested to be the main agents reducting the risks of cardiovascular disease (Arts et al., 2005). In human epidemiological study, vitamin E administration has been documented to have therapeutic effect to counteract liver damage of the non-energetic part of the human diet. Tannin, quercetin and silymarin are phenolic phytochemicals that represent essential constituents of the non-energetic part of the human diet. They are thought to promote optimal health, partly via their antioxidant effects in protecting cellular components against reactive oxygen species (ROS) (Boots et al., 2007; Amalia et al., 2007). Plants contain numerous polyphenols, which have been suggested to be the main agents reducting the risks of cardiovascular disease (Arts et al., 2005). In human epidemiological study, vitamin E administration has been reported to be superior to placebo for the treatment of NASH in human epidemiological study, vitamin E administration has been reported to be superior to placebo for the treatment of NASH in adults without diabetes (Sanyal et al., 2010). In another study, silymarin prevented the palmitate induced lipotoxicity in HepG2 cells (Song et al., 2007).

Quercetin, a polyphenolic flavonoid compound present in large amounts in vegetables, fruits, and tea, exhibits therapeutic potential, including hepatoprotection and the inhibition of liver fibrosis and liver damage (Amalia et al., 2007; Peres et al., 2000; Tieppo et al., 2009). It contains a number of phenolic hydroxyl groups that have strong antioxidant activity (Peres et al., 2000). Recently it has been reported that quercetin ameliorates inflammation and fibrosis in mice with non-alcoholic steatohepatitis (Marcolin et al., 2012). Thus, we hypothesized that hypolipidemic and antioxidant activity of quercetin would attenuate events leading to NAFLD and evaluated along with vitamin E and silymarin using HepG2 cells. HepG2 cells was selected in this study since, they retain and mimic many of the specialized 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).

2. Materials and methods

2.1. Chemicals

Bradford reagent, cytochalasin-B, custom prepared oligonucleotides, cyctochrome-C, DPPH, Dulbecco’s Minimum Essential Medium Eagle (DMEM), Fetal bovine serum (FBS), TRI Reagent®, trypsin, EDTA, glutathione, hydrogen peroxide, insulin, 3-4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), NADPH, oleic acid, quercetin, silymarin, thiobarbituric acid, vitamin-E, xanthine and xanthine oxidase were purchased from Sigma-Alrich (St. Louis, MO, USA). Moloney murine leukemia virus (MMLV) reverse transcriptase, dNTP and Tag DNA polymerase were purchased from MBI (Fermentas Canada Inc., Ontario, Canada). 2-deoxy-3-[H] ß-glucose was purchased from the Department of Atomic Energy, Mumbai, India. All other reagents were of analytical grade.

2.2. 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 (Tarsons) 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.

2.3. Cytotoxicity

HepG2 cells were plated in 96-multiwell culture plates at 1 x 10⁴ cells per well. To study cytotoxicity, 24 h after plating, the medium was discarded and fresh medium containing 0, 10, 20, 30, and 50 µM quercetin was added. After 24 h of incubation cell viability was determined by the MTT assay (Mosmann, 1983).

2.4. Oleic acid induced hepatic steatosis and its inhibition by quercetin

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). The hepatic steatotic inhibitory effect of test compounds and cell proliferation (MTT assay) was carried as described earlier.

2.5. Effect of quercetin on glucose uptake in oleic acid induced hepatic steatosis

The HepG2 cells were rendered steatotic as described earlier and treated with or without quercetin, silymarin and vitamin E along with experimental control for 24 h the cells were then incubated without FBS for 5 h again in the presence or absence of quercetin and experimental controls. The cells were then rinsed with Krebs–Ringer phosphate buffer. The 10 µmol/L 2-deoxy-[H] ß-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).

2.6. Effect of quercetin on lipid peroxidation, inflammatory cytokines, glutathione level in OA induced hepatic steatosis

HepG2 cells were plated in 60 mm culture plates at 7.5 x 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 quercetin and other test compounds 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).

2.7. Biochemical analysis

2.7.1. 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.

2.7.2. 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 isolated nuclei containing 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), sulfuric 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 supernatant to the total DNA.

2.7.3. 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.

2.7.4. TNF-alpha gene expression by RT-PCR

Total RNA was isolated from the cell pellet using TRI reagent®. The isolated RNA was quantitated by spectrophotometer and denaturing agarose gel electrophoresis. Equal concentration of RNA (1 μg) from all the samples was taken for RT experiments. RNA was reverse transcribed using random hexamers/oligo dT primer and MMLV reverse transcriptase (Fermentas, Canada). Further, the PCR amplification was carried out following primer sequences – (TNF-alpha) sense-5’-GTCTATGGCCACAGTCCAACA-3’ antisense-5’-TACCAGGTTTGACTACGC-3’; (GADPH) sense-5’-GACCACGTCCATGCCATCAC-3’ antisense-5’-TCCACACCCCTGTGCTGTAG-3’. The cycling program was carried out with an initial denaturation of 94 °C for 2 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at specific temperature for 30 s and extension at 72 °C for 1 min. The final extension was carried out at 72 °C for 10 min. The PCR products were electrophoresed in 1.5% agarose gels in the presence of ethidium bromide and visualized by ultraviolet fluorescence. The molecular weight of the amplified cDNA was determined by comparison with a standard molecular weight marker (1 Kb ladder). The densitometric analysis was carried out using ImageJ free software (Rashand et al., 2004) for determining relative levels of specific mRNA in comparison with GADPH RNA.

2.7.5. 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.

2.7.6. 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, 1984). Glutathione peroxidase activity was determined by following the enzymatic NADPH oxidation at 340 nm (Flohe and Gunzler, 1984).

2.7.7. Albumin, urea and alanine amino transferase

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

2.8. Statistical analysis

The results are expressed as Mean ± SEM from three independent experiments carried out in triplicates. 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.

3. Results

3.1. Quercetin cytotoxicity and OA induced hepatic steatosis

Quercetin imparted 3.0 – 12.5% cytotoxicity to HepG2 cells at 0–50 μM concentration range when incubated for 24 h as shown in Fig. 1A. The HepG2 cells when treated with 0–2.5 mM concentration of oleic acid for 24 h to induce hepatic steatosis condition and it did not cause cytotoxicity to the cells. While, triacylglycerol content and the recovered Oil-Red-O content was increased significantly by 5.8 folds as shown in Fig. 1B. 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 (photomicrographs not shown). Hence, 2.0 mM oleic acid was used to induce hepatic fatty liver conditions and the effect of quercetin was studied.

3.2. Effect of quercetin 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 quercetin could effectively increase the cell proliferation by 3.05 folds compared to OA treated group. Similarly, silymarin and vitamin E increased the cell proliferation by 2.38 and 2.9 folds respectively as shown in Fig. 2A. The TAG was accumulated as lipid droplets in the OA treated cells and quercetin significantly decreased TAG content by 45%. While, silymarin and vitamin E could reduce the intracellular TAG content by 37.2% and 17.44% respectively when compared to OA group as given in Fig. 2B.
3.3. Effect of quercetin on glucose uptake in OA induced hepatic steatosis

The glucose uptake capacity in OA treated cells was significantly diminished by 67.8% and 77.5% respectively in absence and presence of insulin. Upon addition of quercetin, the specific glucose uptake capacity of cells was significantly increased by 2.02 and 2.65 folds in absence and presence of insulin respectively compared to OA treated cells. While the glucose uptake was increased in silymarin and vitamin E treated cells by 1.77 folds and 1.60 folds in absence of insulin and 1.81 folds and 2.22 folds respectively in presence of insulin compared to OA treated cells as given in Fig. 3.

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

The addition of OA resulted in the 9.45 folds increased lipid peroxidation in HepG2 cells Fig. 4A. Quercetin addition decreased the MDA levels significantly by 50.5% and silymarin and vitamin E inhibited cellular lipid peroxidation by 41.4% and 33% respectively compared to OA treated group. The OA significantly increased the DNA fragmentation by 5.57 folds compared to control as shown Fig. 4B. DNA fragmentation was inhibited by 43% when treated with quercetin and with silymarin and vitamin E, it was inhibited by 22% and 24% respectively compared to OA group.

The GSH level was significantly depleted by 2.49 folds in OA treated HepG2 cells. Addition of quercetin significantly increased the GSH levels by 2.0 folds, whereas silymarin and vitamin E increased GSH content by 1.72 and 1.50 folds respectively compared to OA group. GSSG content was increased by 6.22 folds in OA treated HepG2 cells compared to control. Quercetin significantly brought down the GSSH levels by 2.79 folds compared to OA treated cells as given Table 1. Silymarin and vitamin E brought down the GSSG levels by 2.33 and 2.61 folds respectively.

Fig. 1. (A) Dose dependent effect of quercetin in HepG2 cells. HepG2 cells were incubated with or without quercetin at different concentrations and cell viability was measured by MTT assay. (B) Dose dependent effect of oleic acid (0–2.0 mM) on cytotoxicity and triacylglycerol accumulation in HepG2 cells. Values are Mean ± SEM of three independent experiments carried out in triplicates. *Statistically significant at P < 0.05 compared to control (without quercetin or oleic acid respectively).

Fig. 2. (A) Effect of quercetin on cell proliferation in OA induced hepatic steatosis in HepG2 cells. (B) Effect of quercetin on triacylglycerol accumulation in OA induced hepatic steatosis in HepG2 cells. The cell proliferation and TAG was estimated as described in materials and methods. 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. 3. Effect of quercetin on 2-DG uptake in OA induced hepatic steatosis in HepG2 cells. HepG2 cells were incubated for 24 h with control, quercetin and other experimental compounds. The 2-DG uptake was determined as described in materials and methods. 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.
3.5. Effect of quercetin on inflammatory cytokines in OA induced hepatic steatosis

OA increased TNF-α by 4.65 folds and IL-8 by 5.23 folds respectively compared to control cells (Fig. 5A and B). Whereas, quercetin significantly reverted the increased IL-8 by 41.11% and TNF-α by 59.79% compared to OA group. While, silymarin inhibited IL-8 by 35.87% and TNF-α by 35.11% and likewise, vitamin E inhibited IL-8 by 27.41% and TNF-α by 16.18% compared to OA group. Further gene expression studies by RT-PCR revealed that quercetin inhibited TNF-alpha gene expression by 33% compared to OA group. While the TNF expression was inhibited by 65% and 70% when treated with silymarin and vitamin E respectively (Fig. 6).
activities of catalase, glutathione peroxidise and superoxide dismutase were significantly inhibited by 49.3%, 58.4% and 58% respectively in OA group compared to control cells. Whereas, quercetin could significantly enhance the catalase, glutathione peroxidase and superoxide dismutase activities by 1.68, 2.19 and 1.71 folds respectively compared to OA group. While, silymarin and vitamin E increased catalase, glutathione peroxidase and SOD enzyme activities by 1.26, 1.84 and 1.55 folds and 1.23, 1.52 and 1.61 folds respectively compared to OA treated cells.

3.7. Effect of quercetin 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 3.15 folds compared to control cells. But addition of quercetin, silymarin and vitamin E decreased the ALAT activity by 34%, 24.8% and 18.5% respectively compared to OA treated cells (Table 3). While, amount of urea secreted into the cell supernatant was increased in the quercetin, silymarin and vitamin E treated cells compared to OA treated cells. Similarly, the albumin secretion was significantly increased in quercetin by 2.31 folds and in silymarin and vitamin E it was increased by 1.88 folds compared to OA treated cells.

4. Discussion

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

Table 2

<table>
<thead>
<tr>
<th>Groups</th>
<th>Catalasea</th>
<th>Glutathione peroxidiseb</th>
<th>Superoxide dismutasec</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.75 ± 0.18</td>
<td>21.58 ± 1.35b</td>
<td>35.68 ± 1.56b</td>
</tr>
<tr>
<td>Oleic acid (2 mM)</td>
<td>0.38 ± 0.05&quot;</td>
<td>8.97 ± 0.98&quot;</td>
<td>14.97 ± 1.21&quot;</td>
</tr>
<tr>
<td>Oleic acid (2 mM) + Quercetin (10 mM)</td>
<td>0.64 ± 0.12&quot;</td>
<td>19.68 ± 1.32&quot;</td>
<td>25.64 ± 3.11&quot;</td>
</tr>
<tr>
<td>Oleic acid (2 mM) + Silymarin (0.1 M)</td>
<td>0.48 ± 0.10&quot;</td>
<td>16.58 ± 1.68&quot;</td>
<td>23.28 ± 1.98&quot;</td>
</tr>
<tr>
<td>Oleic acid (2 mM) + Vitamin E(25 mM)</td>
<td>0.47 ± 0.09&quot;</td>
<td>13.67 ± 1.12&quot;</td>
<td>24.15 ± 2.61&quot;</td>
</tr>
</tbody>
</table>

* µmoles of H2O2 decomposed/min/mg protein.
* Statistically significant at P < 0.05 compared to OA group.
** Statistically significant at P < 0.05 compared to control.

Table 3

<table>
<thead>
<tr>
<th>Groups</th>
<th>Urea (µM)</th>
<th>Albumin (mg/mL)</th>
<th>ALAT (Units/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.98 ± 0.23&quot;</td>
<td>0.93 ± 0.12&quot;</td>
<td>3.69 ± 0.54&quot;</td>
</tr>
<tr>
<td>Oleic acid (2 mM)</td>
<td>0.69 ± 0.20&quot;</td>
<td>0.44 ± 0.14&quot;</td>
<td>11.64 ± 0.32&quot;</td>
</tr>
<tr>
<td>Oleic acid (2 mM) + Quercetin (10 mM)</td>
<td>0.84 ± 0.10&quot;</td>
<td>1.02 ± 0.12&quot;</td>
<td>7.68 ± 0.49&quot;</td>
</tr>
<tr>
<td>Oleic acid (2 mM) + Silymarin (0.1 M)</td>
<td>0.81 ± 0.15&quot;</td>
<td>0.83 ± 0.16&quot;</td>
<td>8.75 ± 0.36&quot;</td>
</tr>
<tr>
<td>Oleic acid (2 mM) + Vitamin E(25 mM)</td>
<td>0.83 ± 0.11&quot;</td>
<td>0.82 ± 0.20&quot;</td>
<td>9.48 ± 0.45&quot;</td>
</tr>
</tbody>
</table>

* Statistically significant at P < 0.05 compared to OA group.
** Statistically significant at P < 0.05 compared to control.
studies that suggest a link between lipid peroxidation, cellular pro-oxidant and antioxidant imbalance and obesity-related complications (Feldstein et al., 2004; 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 Bookees, 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 signaling 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, Kupffer cells and hepatic 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 designing therapeutic approaches toward oxidative based liver pathologies (Inoue, 1994). Therapy directed towards countering the oxidative stress might be an effective measure in the management of NAFLD and its complications. The polyphenolic flavonoid compound quercetin (Peres et al., 2000) and others like silimarin (Song et al., 2007) and vitamin E (Sanyal et al., 2010; Barve et al., 2007) have been widely used to treat hepatic disorders and in the present study it is showed that, they are very effective in combating the ROS mediated lipotoxicity induced by oleic acid in HepG2 cells. It is also interesting that the dosage used in this study has a direct clinical implication since, the average circulating plasma vitamin E and quercetin concentration is 26.9 μM and 0.3–7.6 μM respectively in humans (Borel et al., 2007; Radtke et al., 2002) and these in vitro results are more reliable for the reasons that these test compounds have been evaluated at its realistic serum concentrations. The results demonstrated that cells incubated with quercetin 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 quercetin treated steatotic cells without increasing the GSSG levels. It was also showed that DNA fragmentation was decreased by quercetin in steatotic cells. Silymarin and vitamin E were not as effective compared to quercetin in reverting the biochemical parameters, but they too inhibited the lipotoxicity, suggesting the role of quercetin 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 (Kedderis, 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 Cu¹⁰ loaded cells is considered a major intracellular determinant of their susceptibility to cytotoxicity (Vidyashankar and Patki, 2010).

Thus, the potential of quercetin to maintain GSH at reasonably high levels is of importance during oleic acid induced steatosis. Therefore, the ability of quercetin to prevent OA induced GSH depletion reported here is very significant in restoring the cell viability. The GSSG formation was inhibited by quercetin and this may be attributed to the formation of GSH conjugates rather than oxidation to GSSG in OA induced toxic conditions which is indicated by the increased ratio of GSH:GSSG Table 1. Beside this glutathione peroxidase activity was significantly increased and DNA fragmentation was decreased by quercetin during hepatic steatotic toxicity in HepG2 cells. Silimarin could prevent the GSH depletion by 1.72 folds and vitamin E by 1.50 folds. Vitamin E and silymarin prevented the DNA fragmentation which is less compared to quercetin.

Insulin resistance is central to the pathogenesis of NASH (Valenti et al., 2002) and it is shown that quercetin could effectively increase the glucose uptake in steatotic HepG2 cells. Previously, it was reported that stimulation of TNF-α in hepatocytes and adipocytes by FFAs is implicated in the etiology of insulin resistance (Valenti et al., 2002). In agreement with this our results showed that oleic acid triggered TNF-alpha gene expression which is observed with insulin resistance. But quercetin, silymarin and vitamin E inhibited TNF-alpha gene expression by 33%, 65% and 70% respectively compared to OA group. The insulin mediated glucose uptake was significantly higher in HepG2 cells with the addition of quercetin as shown by positive correlation between triacylglycerol content, glucose uptake and decreased lipid peroxidation, TNF-alpha expression and 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 cytochalasin B in the experiments. However an increased level of glucose uptake was observed with the addition of quercetin 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 quercetin to the cells significantly increased the insulin mediated glucose uptake in cells. Insulin mediated glucose uptake mediated by quercetin clearly justified the need for synthesis of new protein relevant to glucose transport. These findings clearly show that quercetin is very effective in restoring the glucose uptake in steatotic cells which was diminished during steatotic conditions with inhibition of TNF-alpha expression. This is the first finding of its kind to show that quercetin is responsible to overcome the insulin resistance during increased FFA levels in experimental studies. Similar to quercetin, silymarin and vitamin E also increased the glucose uptake in HepG2 cells rendered steatotic as observed in our experiment.

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, 1998; Valentí et al., 2002; Wigg et al., 2001). It was shown that, chronic dietary intake of quercetin alleviates hepatic fat accumulation and regulates PPAR-γ, TNF-α and the steatois related gene expression during consumption of western style diet in C57/BL6j mice (Kobori et al., 2011). In similar lines with earlier findings in the present study TNF-α and IL-8 secretion was significantly increased by addition of oleic acid. Addition of quercetin, silymarin and vitamin E significantly decreased the cytokine secretion which is important in amelioration of NAFLD symptoms. Meanwhile expression results showed silymarin and vitamin E are more effective than quercetin to bring down the TNF-α gene expression. The difference observed with respect to TNF-α secretion and vitamin E were more beneficial by decreasing triacyl glycerol accumulation, insulin resistance, inflammatory cytokine secretion and increased cellular antioxidants in OA induced hepatic steatosis in HepG2 cells. Hence quercetin is promising to carry out more experimental and clinical studies to understand the molecular mechanism to overcome NAFLD symptoms.

5. Conflict of interest
N/A

Acknowledgements
Authors thank Dr. Shyam Ramkrishnan, Chief Scientific Officer – R&D, The Himalaya Drug Company, Bangalore, India for his constant support and encouragement during this study.

References


