Quercetin and β-sitosterol prevent high fat diet induced dyslipidemia and hepatotoxicity in Swiss albino mice

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High fat diet group showed a significant rise in serum and hepatic total cholesterol, triglyceride and atherogenic index which are major biomarkers of dyslipidemia and cardiovascular risk. The liver function markers, lipid peroxidation and proinflammatory cytokine levels were elevated in high fat diet group whereas antioxidant levels significantly reduced. These findings manifest hepatic damage which was further confirmed by histological findings. Quercetin and β-sitosterol though structurally different yet both ameliorate the sickening changes in different mechanism. The current investigation is perhaps the first report of the mechanistic role of two polyphenols over dyslipidemia and subsequent hepatotoxicity.

Keywords: β-sitosterol, Dyslipidemia, Hepatotoxicity, Quercetin, Steatosis

High fat diet consumption over a long period of time causes chronic dyslipidemia and steatosis1,2. Though cholesterol is an essential component of the cells, its excess has serious implications on organism health. Cholesterol levels are tightly regulated by a balance between dietary uptake, endogenous synthesis and its excretion3. Dyslipidemia is defined as the disorder of lipoprotein metabolism which is generally manifested by elevated plasma levels of total cholesterol (TC), low density lipoprotein cholesterol (LDL-C) and triglyceride concentration (TG) and a decrease in plasma high density lipoprotein cholesterol level (HDLC). The above dyslipidemic condition can contribute to cardiovascular and cerebrovascular diseases like atherosclerosis, hypertension, stroke etc.4,5. Liver plays an essential role in regulating plasma lipid level through LDL-C clearance and HDL-C recruitment6. As a result of excess lipoprotein and TG burden liver gets damaged which causes hepatotoxicity. This is better known as non-alcoholic fatty liver disease (NAFLD) which leads to form non-alcoholic steatohepatitis (NASH). If it is left untreated cirrhosis develops7. The liver injury, in NAFLD is frequently accompanied with obesity8.

The conventional treatment of dyslipidemia is mainly by statin group of drugs, bile acid sequestrants or resins, nicotinic acid or fibrin acid derivatives. But all of them possess adverse effects9-13. Particularly, the long term use of statin in event reduction in the context of metabolic disorder has some questionable role. Therefore a natural product or a phytochemical with hypolipidemic effect is the prime choice against dyslipidemia. Among the phytochemicals, phenolic compounds are widely distributed in fruits and vegetables and show the best medicinal properties. Polyphenols can donate electron to the free radicals and generate relatively non reactive reduced forms, thus, acting as chain-breaking antioxidants14. Quercetin (3, 3′,4′,5,7-pentahydroxyflavone) belongs to polyphenolic flavonoid compounds almost ubiquitous in plants and plant food sources (Fig. 1a). It is a strong antioxidant compound acting as free-radical terminators. Presence of central C-ring along with high number of hydroxyl groups and conjugated π orbitals makes the compound a strong reducing agent15. It scavenges the excess free radicals formed during overt metabolism in the cells. Quercetin (Q), due to its high reducing power effectively neutralized the reactive oxygen species (ROS) and maintained the in vivo antioxidant status. Therefore, all consequent ROS mediated events like systemic inflammation and subsequent organ damage are efficiently inhibited by Q16.

β-sitosterol (BS) on the other hand is a primary plant sterol, containing cyclopentano perhydro phenanthrene ring as in cholesterol (Fig. 1b). Therefore it is likely that it competes for cholesterol
absorption in the lower intestine and in turn reduces the levels of cholesterol in the blood\textsuperscript{17}. There might be other reasons in favour of the prevention of dyslipidemia and liver toxicity by $\beta$S. Therefore this study has been undertaken to figure out what are the pleiotropic roles of $\beta$S other than competition at the intestinal bed.

The main objective of this article is to identify how effectively these two structurally diverse compounds can prevent dyslipidemia, hepatic injury and inflammation. Swiss albino mouse was chosen to develop the experimental model to promulgate the objectives. The results show their effectiveness in preventing the rise in lipid profile, inflammatory cytokines, liver function markers, hepatic lipid peroxidation (LPO) level apart from histopathological findings. The study also promise a future note that they can be further compared or combined to reinforce the improvement of metabolic disorder.

Materials and Methods

Chemicals and animals—Quercetin, $\beta$-sitosterol, cholesterol, glutathione reductase, thiobarbituric acid (TBA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Other reagents were procured from Merck (Darmstadt, Germany). All reagents were of highest analytical grade. Male Swiss albino mice were purchased from Bengal Chemical and Pharmaceuticals Ltd. (Kolkata, India).

Animal treatment—The experiments were carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India. Animal room was maintained at 24±2 °C, 50±10% RH and a 12:12 h L:D cycle. The mice were allowed to access food and water \textit{ad libitum}. After initial 1 week of acclimatization they were divided into following 4 groups of 8 each. All the animals were given standard laboratory diet.

Gr. I: Control (C): Standard laboratory diet, Gr. II: high fat diet (HFD): 2% cholesterol along with the standard diet, Gr. III high fat diet + Quercetin (HQ): 2% cholesterol and Quercetin (100 mg/kg body weight) with the diet, and Gr. IV: high fat diet + $\beta$-sitosterol (H$\beta$): 2% cholesterol and $\beta$-sitosterol (100 mg/kg body weight) with the diet.

The dosage was selected from dose-response curve.

Blood and tissue collection—After 8 weeks of treatment animals were sacrificed by decapitation after application of anaesthesia. Blood was collected and serum was isolated for the estimation of lipid profile, liver function enzymes and ELISA for TNF-$\alpha$ and IL-6. Liver was taken out and stored properly for different biochemical and histological experiments.

Lipid profile—The hepatic lipids were extracted using the procedure developed by Folch et al\textsuperscript{18}. Total cholesterol and HDL-C were measured by enzymatic method using kit from Randox Laboratories Ltd. (Antrim, United Kingdom) according to the manufacturer’s protocol. Triglyceride in serum was measured using kit from Merck (Darmstadt, Germany). The LDL-C was determined directly by semi-autoanalyzer (Merck, Darmstadt, Germany) using kit from Labkit, Chemelex, S.A. (Canovelles, Barcelona, Spain) according to the manufacturer’s protocol.

Determination of in vivo antioxidant profile—Liver tissue homogenate (10%) was prepared by sonication using lysis buffer (pH 7.4). The homogenate was then centrifuged at 6000 $g$ at 4 °C for 15 min and supernatant was collected for experiments. The protein content was determined from this liver tissue homogenate by Lowry method\textsuperscript{19}. Using this supernatant superoxide dismutase (SOD)$^{20}$, catalase$^{21}$ and glutathione content$^{22}$ were assayed.

Histological study of liver tissue—For histology, a small portion of liver tissue was cleaned, processed and embedded in paraffin wax. Paraffin blocks were cut 5 µm in thickness, processed and stained with hematoxylin and eosin for histopathological evaluation of liver$^{23}$. The stained slide for each group was observed using light microscope (Olympus 207444, Tokyo, Japan) at 100x magnification. The photomicrograph was taken using the Camera, Canon Power Shot S70.

Estimation of liver function enzymes and hepatic lipid peroxidation—Three main liver enzymes, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were estimated using enzymatic
 kit of Span diagnostic Ltd. (Surat, India) according to the manufacturer’s protocol. Hepatic lipid peroxidation (LPO) was assayed according to Ohkawa et al.24.

Enzyme-linked immunosorbant assay (ELISA) for TNF-α and IL-6—The levels of serum tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) were measured using a sandwich ELISA Kit purchased from Endogen Inc. (Rockford, IL, USA).

Statistical analysis—Statistical analysis was performed using one-way analysis of variance (ANOVA) using Origin software version 8.0. Results were expressed as mean ± SE for 8 mice in each group. P values <0.05 were considered significant.

Results

Serum lipid profile—There was about 65% and 70% increase in TC and TG respectively in HFD group (Gr.II) in comparison to control (Table 1). The LDL concentration had a rise by 80% in Gr.II. The atherogenic index also significantly increased in HFD group in comparison to control (P<0.05). By Q and βS treatment the levels of the above parameters significantly decreased in comparison to HFD group (P<0.05). There was about 30 and 34% reduction of TC and TG level respectively by Q treatment in comparison to HFD. LDL level and atherogenic index was also reduced by 22 and 49% respectively. On the other hand βS treatment reduced TC and TG by 19 and 31% respectively. LDL concentration and atherogenic index was reduced by 33 and 40% respectively. Therefore, both the phytochemicals prevented the serum TC and TG and atherogenic index. This lipid profile markers and atherogenic index are major predictor of the CVD risk.

Hepatic cholesterol and triglyceride content—The HFD group showed about 83 and 53% rise in hepatic cholesterol and triglyceride respectively (Fig. 2). With Q and βS treatment their value decreased significantly (P<0.05). By Q treatment cholesterol and triglyceride value decreased by 32 and 21% respectively in comparison to HFD group. βS treatment reduced their value by 41 and 27% respectively.

Hepatic antioxidants—Hepatic antioxidant profile is an overall index of organism’s metabolic status. In vivo oxidative environment manifests several physiological anomalies if remains untreated. In a metabolically active organ like liver, this stands out to be a major concern if the endogenous protectors are exhausted or impaired. There was about two fold decrease in both SOD and catalase activity in HFD group (Table 2). GSH content reduced by 47% in HFD group. Contrarily, when Q was administered along with HFD, SOD and catalase activities were enhanced by over 100 and 50% respectively. In βS treatment, the cases were over 66 and 36% respectively in comparison to HFD group.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (a)</th>
<th>HFD (b)</th>
<th>HQ (c)</th>
<th>Hβ (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mg/dL)</td>
<td>84.25 ± 2.90</td>
<td>138.75 ± 3.40</td>
<td>98.25 ± 3.15</td>
<td>112.15 ± 2.85</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>68.80 ± 2.50</td>
<td>116.35 ± 2.80</td>
<td>76.75 ± 3.65</td>
<td>80.25 ± 3.10</td>
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<tr>
<td>LDL-C (mg/dL)</td>
<td>42.55 ± 1.80</td>
<td>76.40 ± 2.35</td>
<td>52.25 ± 4.10</td>
<td>51.20 ± 3.00</td>
</tr>
<tr>
<td>Atherogenic index*</td>
<td>0.75 ± 0.03</td>
<td>1.23 ± 0.01</td>
<td>0.63 ± 0.10</td>
<td>0.75 ± 0.07</td>
</tr>
</tbody>
</table>

**Table 1—Serum lipid profile**

| Values are mean ± SE from 8 mice in each group |

* Atherogenic index = (TC – HDL)/HDL.

Significance was compared between a/b, b/c and b/d (P<0.05) and for TG significance of difference was compared between e/f, f/g and f/h (P<0.05). There is significant difference between a/b, b/c and b/d (P<0.05) and e/f, f/g and f/h (P<0.05).

Fig. 2—Effect of Quercetin and β-sitosterol on hepatic lipid profile. For hepatic TC significance of difference was compared between a/b, b/c and b/d (P<0.05) and for TG significance of difference was compared between e/f, f/g and f/h (P<0.05). There is significant difference between a/b, b/c and b/d (P<0.05) and e/f, f/g and f/h (P<0.05).
Table 2—In vivo antioxidant profile
[Values are mean ± SE from 8 mice in each group]

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (a)</th>
<th>HFD (b)</th>
<th>HQ (c)</th>
<th>Hβ (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gr. I</td>
<td>Gr. II</td>
<td>Gr. III</td>
<td>Gr. IV</td>
</tr>
<tr>
<td>SOD *</td>
<td>2.00 ± 0.30</td>
<td>0.90 ± 0.14</td>
<td>1.92 ± 0.22</td>
<td>1.50 ± 0.10</td>
</tr>
<tr>
<td>Catalase **</td>
<td>42.25 ± 4.50</td>
<td>25.30 ± 2.75</td>
<td>38.10 ± 2.25</td>
<td>28.60 ± 3.20</td>
</tr>
<tr>
<td>GSH ***</td>
<td>1.12 ± 0.13</td>
<td>0.60 ± 0.09</td>
<td>1.07 ± 0.08</td>
<td>0.82 ± 0.07</td>
</tr>
</tbody>
</table>

* Unit/mg of protein/minute  ** μ moles of H₂O₂ decomposed/mg of protein/minute  *** μmoles/mg of protein
Significance was compared between a/b, b/c and b/d (P<0.05)

Liver histology—There was obvious damage found in HFD. The tissues were found disintegrated. There were distinct signs of degenerative changes in the hepatic morphology (Fig. 3). All these indicated the hepatic damage after high cholesterol and triglyceride accumulation and subsequent generation of free radicals due to overt metabolism. The Q and βS treatment however, kept the liver morphology normal. Both the photomicrographs represent a near normal morphology in comparison to HFD.

Liver function enzymes and LPO level—Table 3 shows hepatic enzyme activities among the different groups. The liver function markers reflect overall hepatic homeostasis. Thus, any toxic conditions are immediately predicted by these markers. The lipid peroxidation proves fatal if remains unabated. Contingent upon any toxic insult to liver, the proxidants injure the membrane by oxidizing the membrane lipids, which in turn leads to the leakage of liver enzymes. The AST, ALT and ALP activities in HFD group results were up by about two fold in comparison to control group (Table 3). This reflects the obvious hepatic injury. In support of this membrane damage was prominent since the TBARS level remained higher by 89%. These findings were further confirmed by hepatic histology findings. Both Q and βS treatment restored the hepatic physiology with significant values (P<0.05). As a consequence of liver injury there was about 90% increase in peroxidation level in HFD group in comparison to control. Q and βS treatment reduced TBARS level by 40 and 34% by respectively in comparison to HFD.

ELISA of TNF-α and IL-6—Hepatic insults always accompany the upsurge of pro-inflammatory cytokines. Their levels increase during hepatic inflammation and damage. In HFD group the TNF-α and IL-6 concentrations increased by 34 and 40% respectively in comparison to control (Fig. 4). Q treatment significantly reduced the levels of the above two cytokines (P<0.05). Perhaps, the scavenging of oxidative elements triggers by Q did initiate the inflammatory cascade. βS treatment also reduced their levels, however, the IL-6 reduction was not significant. βS due to its chemical structure, is not an efficient scavenger of oxidative molecules.

Discussion

High fat diet consumption for a long period of time results in some serious physiological consequences. In the present communication the efficacy of two major phytochemicals that prevented serious anomalies induced by high cholesterol diet for chronic
Table 3—Status of Liver function enzymes and LPO
[Values are mean ± SE from 8 mice in each group]

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (a) Gr. I</th>
<th>HFD (b) Gr. II</th>
<th>HQ (c) Gr. III</th>
<th>Hβ (d) Gr. IV</th>
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</thead>
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<tr>
<td>AST (IU/l)</td>
<td>17.15 ± 2.10</td>
<td>41.45 ± 4.80</td>
<td>25.70 ± 2.70</td>
<td>23.35 ± 3.55</td>
</tr>
<tr>
<td>ALT (IU/l)</td>
<td>28.20 ± 2.40</td>
<td>52.40 ± 3.50</td>
<td>34.75 ± 2.85</td>
<td>37.25 ± 3.65</td>
</tr>
<tr>
<td>ALP (KA units)</td>
<td>26.65 ± 3.20</td>
<td>46.30 ± 2.90</td>
<td>31.50 ± 4.60</td>
<td>34.25 ± 2.55</td>
</tr>
<tr>
<td>LPO *</td>
<td>3.14 ± 0.42</td>
<td>5.95 ± 1.0</td>
<td>3.55 ± 0.83</td>
<td>3.79 ± 0.92</td>
</tr>
</tbody>
</table>

* Nanomoles of TBARS/mg of protein. Significance was compared between a/b, b/c and b/d (P<0.05).

Fig. 4—ELISA of TNF-α and IL-6. For hepatic TC significance of difference was compared between a/b, b/c and b/d (P<0.05) and for TG significance of difference was compared between e/f, f/g and f/h (P<0.05). There is significant difference between a/b, b/c and b/d (P<0.05) and e/f, f/g (P<0.05).

term is presented. The pathophysiological states are dyslipidemia, a major source of cardiovascular risk and subsequent steatosis. To execute the experimental objective, serum lipid profile, endogenous antioxidants, liver function markers, LPO, inflammatory cytokines and liver histopathological studies were performed.

From the present results, HFD feeding for 8 weeks led to significant rise in serum TC, TG and LDL-C (Table 1) and made the mice dyslipidemic. The dyslipidemic state reflects the cardiovascular risk as shown by a steep increase in the atherogenic index (Table 1). The chronic cholesterol diet might have facilitated the infiltration of TG into the liver. Hepatic TC and TG in the HFD group increased. This metabolic situation in lipid burden led to oxygen toxicity and hence excess reactive oxygen species (ROS) production in the mitochondria. The surplus ROS generation exhausted the endogenous antioxidants. Due to such condition a significant (P<0.05) reduction in SOD, catalase and GSH in high fat group compared to control (Table 2) was observed. In addition there were degenerative changes in the liver as found from the histological picture (Fig. 3).

The ROS activates key pro-inflammatory cytokines and thus trigger hepatic inflammation creating a state of low grade inflammation. There were significant rise in TNF-α and IL-6 in HFD group in comparison to control (Fig. 3). Thus, excess lipid infiltration, ROS and inflammatory cytokines trigger a condition of liver toxicity. As a consequence the liver function markers (AST, ALT and ALP) showed significant leakage in the serum and indicated the membrane damage of the hepatic cells. The later is further confirmed by a steep rise in LPO level (Table 3). This stage if remains unattended develop steatosis or NAFLD that further complicates the hepatic physiology.

At the outset of high fat diet mediated dyslipidemia and liver toxicity, a significant improvement in biochemical and histopathological parameters by both βS and Q treatment was observed. In case of βS, it might be explained by competitive inhibition in absorption and transport of cholesterol since it shares the similar chemical structure with cholesterol. It also regulates the cholesterol synthesis by regulating hydroxy methyl glutaryl CoA reductase (HMG CoA reductase) and Sterol regulatory element binding protein-2 (SREBP-2) (unpublished data).

On the other hand Q, due to its high reducing power effectively neutralized the ROS and maintained the in vivo antioxidant status. Therefore, all consequent ROS mediated events like hepatic inflammation and subsequent liver damage were efficiently inhibited by Q (Fig. 5). The histological picture thus shows better tissue integrity with no such damage as found in the high fat group. Recently it has been shown that Q can alleviate the inflammation developed after short term HFD treatment in Swiss albino mice.
The HFD with high cholesterol definitely generated an inflammatory state in the mice. The inflammatory state was down regulated by the actions of the βS and Q. A strong anti-inflammatory effect of these phytocompounds was observed since the pro-inflammatory cytokines, TNF-α and IL-6 were reduced in serum after their treatments. Further, the Q and βS both can modulate a number of signalling pathways. The high cholesterol triggered an elevated metabolic state in the mitochondrial system and more generation of ROS followed. This ROS is responsible for a low grade inflammation and elevation of TNF-α and IL-6 level which are responsible for the vicious cycle of impaired electron flow and more generation of ROS causing liver steatosis. Moringa oleifera leaf extract (MoLE) successfully prevented development of liver injury and steatosis like events in mice. In a recent study the leaf extract of the plant also found to prevent oxidative stress induced DNA damage by its potent antioxidative power. In fact the βS and Q are the two important constituents of MoLE. Therefore, the roles of the phytocompounds were reinstated in the present study. Another interesting issue of this study is that βS and Q, two structurally dissimilar phytocompounds inhibited the cardiovascular risk and inflammatory development in the liver through their pleiotropic functions. The present set of experiments however have the limitation to defend the molecular mechanism that how the cholesterol regulatory role is manifested by quercetin. On the other hand how the anti-inflammatory role was manifested by βS, has to be investigated further. In any case, the present findings warrant the development of functional food including these components against cardiovascular diseases and liver toxicity even after chronic high fat diet.

Conclusion

The present results show that chronic high fat diet intake induces dyslipidemia and hepatic toxicity and two plant phenolics though structurally not similar prevent the generation of dyslipidemia and hepatic damage. Quercetin being a powerful free radical scavenger ameliorates the damage and βS mainly inhibit the damage by interfering with the lipid absorption and metabolism. The further investigation will be to address whether these two have any synergistic or additive role when applied in combination.

Acknowledgement

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References