Effect of thymoquinone on ethanol and high fat diet induced chronic pancreatitis—a dose response study in rats

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A significant increase in serum lipase, amylase, caspase-1 and myeloperoxidase activities, oxidative stress index (OSI), IL-1β and IL-18 was observed in rats receiving ethanol (EtOH) and high fat diet (HFD). Thymoquinone (TQ) supplementation along with EtOH and HFD significantly decreased the levels of serum lipase, amylase, caspase-1, myeloperoxidase, OSI and maintained the antioxidant status when compared to untreated EtOH and HFD fed rats. Among the 4 doses, 100 mg of TQ/kg body weight was found to provide optimum protective effect on pancreas against EtOH and HFD induced abnormal changes. Histological observations added more evidence for the anti-inflammatory effect of TQ.

Keywords: Antioxidants, Ethanol, High fat diet, Inflammation, Pancreatitis, Thymoquinone

Pancreatitis is inflammation of pancreas that occurs when pancreatic enzymes are activated in the gland itself which digest the tissues to cause injury¹. Chronic pancreatitis (CP) is progressive inflammation characterized by irreversible morphological changes and fibrotic replacement of the gland with frequent episodes of abdominal pain. CP affects 5-6% of the world population especially the developing countries like India with the gradual increase in mortality and the major cause reported is the alcohol abuse. Gall stone diseases, hypertriglyceridemia, autoimmunity and excessive production of parathyroid hormone have also been associated with CP that affects the endocrine and exocrine functions of pancreas⁴.

Ethanol (EtOH) has calorific value but when consumed in excess causes serious pathological conditions including fatty liver, cirrhosis, cardiovascular diseases and pancreatitis. EtOH causes secondary malnutrition by impairing the absorption of nutrients in the intestine due to gastro-intestinal complications associated with alcoholism⁶. EtOH is also metabolized in pancreas and the toxic product such as acetaldehyde is oxidized to acetate by xanthine oxidase and aldehyde oxidase and produce harmful reactive oxygen species (ROS). The free radicals are associated with the functional impairment of pancreas due to EtOH toxicity⁷.

High fat diet (HFD) and high triglyceride level were known to aggravate the toxic effects of EtOH⁸. Prooxidant-antioxidant imbalance has been reported in high fat induced abnormalities caused either by active consumption of antioxidants for the clearance of toxic free radicals or excess formation of ROS. ROS are closely associated with tissue injury which triggers the formation of proinflammatory cytokines such as interleukin (IL)-1β and IL-18 to minimize the injury, but can damage even the adjacent normal tissues when produced in excess.

Many allopathic supportive medicines are currently used for the treatment of pancreatitis. But the ever increasing problem of pancreatitis demands the identification of alternative plant based medicines which are less toxic and cost effective in nature. Many natural quinones possess anti-inflammatory property. Thymoquinone (TQ), 2-Isopropyl-5 methyl benzo-1,4- quinone, (Fig. 1) a phytochemical compound present in the seeds of Nigella sativa Lin.

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Fig. 1—Thymoquinone (2-Isopropyl-5 methyl benzo-1, 4-quinone)
(Ranunculaceae) has shown to protect against heart, liver and kidney damage in experimental animals due to its antioxidant nature. In vitro studies show that TQ could kill pancreatic cancer cells by inhibiting histone deacetylases and by affecting genes p53, bax and bcl-2. N. sativa commonly grows in the Middle East, Eastern Europe and Middle Asia is used as food additive and also as natural remedy for many inflammation associated ailments for over thousands of years. The present study is an attempt to evaluate the anti-inflammatory activity of TQ in animals subjected to experimental pancreatitis.

Many experimental models of CP have been reported to evaluate new drugs to be recommended for the treatment of pancreatitis. Among the various models, the combined effect of HFD and EtOH has been shown to accelerate the induction of pancreatitis with reproducible results. In the present study EtOH and HFD induced changes in pancreas and the effect of different doses of TQ on the same have been assessed.

Materials and Methods

Chemicals and reagents—TQ was obtained from Santa Cruz Biotechnology, Inc (Canada). ELISA kits for IL-1β and caspase-1 were purchased from Abcam and ELISA kit for IL-18 was purchased from Invitrogen. All other chemicals and solvents used for the analysis were of analytical grade.

TQ preparation—TQ was suspended in 0.9% saline and mixed thoroughly to get homogenous suspension. The suspension was administrated orally by intragastric intubation.

Experimental protocol—Male albino Wistar rats weighing 175-200 g were maintained on 12:12 h L: D cycle at 22 °C and 50% RH. All animals were individually housed and fed ad libitum standard rat chow obtained from Hanuman Food Products Pvt. Ltd., Bangalore, India during the acclimatization period. Subsequently, rats were randomly assigned into following 7 groups. Groups 1 and 2 were fed ad libitum standard rat chow for 90 days. Groups 3-7 rats were fed with high fat liquid diet according to the report of Tsukamoto et al. and also administered 20% aqueous EtOH equivalent to 8 g/kg/body weight/day for the first 4 weeks and 12 g/kg body weight/day for the remaining days using intragastric tube. Groups 4–7 rats were administrated with 25, 50, 100, 200 mg/kg body weight respectively of TQ suspended in saline for the last 60 days of the experimental period. Group 2 rats were administrated with 200 mg/kg body weight of TQ along with normal standard diet.

Diet composition for experimental animals—Normal diet containing 25% protein, 68.3% carbohydrate, 4.3% fat and required amount of mineral and vitamin mix was obtained from Hanuman Food Products Pvt. Ltd., Bangalore, India. HFD (containing 25% fat) was prepared in the laboratory with required amount of mineral and vitamin mix. Soybean oil was substituted for the supply of essential fatty acids. The energy given by the normal diet is 3.43 Kcal/g and by the HFD along with EtOH is 5.24 Kcal/g. The carbohydrate content in HFD was adjusted isocalorically based on the calories given by EtOH (8 or 12 g/kg body weight/day).

The work protocol was approved by The Animal Care Ethical Committee (XII/VELS/COL/28A/CPCSEA/IAEC/23.09.11). During the experimental period, weight of the animals was recorded weekly along with daily food consumption. After the experimental period of 90 days, rats were fasted overnight and anesthetized by intramuscular injection of ketamine hydrochloride (30 mg/kg body weight) and killed by cervical decapitation. Blood was collected with/without anticoagulant and plasma/serum separated were stored until analyses. In order to rule out the day-to-day variation in results one animal from each group was killed per day.

Tissue homogenate preparation—Immediately after the animal sacrifice, pancreas was removed, washed carefully and homogenized in 0.1 M Tris HCl buffer pH 7.4 and centrifuged at low speed to remove any cell debris. The supernatant was used for the determination of glutathione (GSH), protein, caspase-1, lipid peroxides (TBARS) and antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx).

Biochemical investigations

Determination of serum lipase activity—Lipase (EC: 3.1.1.1) activity in serum was measured by the method of Lowry and Tinsley. The lipolysis reaction was initiated with the addition of serum in 25 ml olive oil/triton X 100 emulsion as substrate. Subsamples of reaction mixture were (0.3 mL) taken at predetermined time intervals and used for the assay of liberated free fatty acids spectrophotometrically at 715 nm. The activity of enzyme was expressed as IU/L.
Determination of serum amylase activity—Amylase (EC: 3.2.1.1) activity was determined by the method of Gomori\(^\text{14}\). The method was based on the activity of enzyme on substrate starch and the measurement of maltose liberated by using lugol’s iodine solution. The enzyme activity was expressed as units/ml.

Determination of myeloperoxidase (MPO) activity—Myeloperoxidase (EC: 1.11.1.7) activity in the pancreas was measured according to the method of Bradley et al\(^\text{15}\). Pre-weighed tissue was homogenized (1:10 w/v) in 0.5% hexadecyltrimethyl ammonium bromide in 50 mM potassium phosphate buffer (pH 6.0) before sonication in an ice bath for 20 sec. Three freeze/thaw cycles were performed followed by sonication (20 sec in icebath). The samples were centrifuged at 17,000 g (5 min, 4 °C) and the enzyme activity was assayed by mixing 0.1 mL of supernatant and 2.9 mL of 10 mM potassium phosphate buffer (pH 6) containing 0.167 g/L o-dianisidine dihydrochloride and 0.0005% hydrogen peroxide (H\(_2\)O\(_2\)). The change in absorbance at 460 nm was measured for 4 min using fluoro spectrophotometer. The enzyme activity was expressed as units/mg protein.

Estimation of lipid peroxides and oxidative stress index (OSI)—The level of lipid peroxides in plasma and pancreas was determined by measuring thiobarbituric acid-reacting substances (TBARS)\(^\text{16}\). The value was expressed as nM/mL plasma and nM/100 mg tissue protein.

The peroxide content was measured in plasma by using FOX 2 method\(^\text{17}\) with minor modifications. The FOX 2 test system is based on oxidation of ferrous ion to ferric ion by various types of peroxides present in the samples, to produce a coloured ferric-xylenol orange complex whose absorbance was measured at 560 nm. Total antioxidant capacity (TAC) was determined by the method of Miller et al\(^\text{18}\). Decolourization of assay mixture containing 2, 2’-azino bis 3-ethyl benzo-thiazoline-6-sulfonate (ABTS) and the sample was monitored by measuring the absorbance at 734 nm and the % inhibition was calculated. The ratio of total peroxides to total antioxidant capacity was calculated as OSI.

Estimation of glutathione and antioxidant enzymes—GSH was determined by the method of Moron et al\(^\text{19}\). Aliquot of plasma or homogenate was mixed with equal volume of ice cold 5% trichloro acetic acid and the precipitated proteins were removed by centrifugation. The supernatant was used for the assay by treating with nitroblue tetrazolium in 0.2 M phosphate buffer, pH 8.0 and measuring at 412 nm. GPx (EC: 1.11.1.9) was assayed by the method of Flohe and Gunzler\(^\text{20}\). The activity of GPx was expressed as nM of GSH oxidized/min/mg protein.

SOD (EC: 1.15.1.1) activity was measured according to method of Kakker et al\(^\text{21}\). The inhibition of reduction of nitroblue tetrazolium to blue coloured formazan in presence of phenazine methosulfate and NADH was measured at 560 nm using n-butanol as blank. The enzyme activity was expressed as units/mg protein. Decomposition of H\(_2\)O\(_2\) in the presence of CAT (EC: 1.11.1.6) was kinetically measured at 240 nm\(^\text{22}\). CAT activity was defined as the amount of enzyme required to decompose 1µM of H\(_2\)O\(_2\)/min. The enzyme activity was expressed as µM of H\(_2\)O\(_2\) consumed/min/mg protein.

Assay of IL-1β—The assay was performed according to manufacturer’s instructions (ab100767). Standards or serum samples were pipetted into IL-1β antibody precoated wells and IL-1β present in a sample is bound to the wells by the immobilized antibody. The wells were washed and biotinylated secondary antibody was added. The unbound biotinylated antibody was washed and added HRP-conjugated streptavidin to the wells. The wells were washed again and TMB substrate solution was added. The intensity of the colour developed was proportional to the amount of IL-1β present in the sample. The stop solution changes the colour from blue to yellow, and the intensity of the colour was measured at 450 nm. The activity of IL-1β was expressed as pg/mL.

Assay of IL-18—The assay was carried out as per the instruction of kit manual (KRC2341). The serum sample and standards were pipetted into antibody immobilized wells. After the incubation, biotinylated secondary antibody was added. After removal of excess secondary antibody, streptavidin-peroxidase was added. Then the substrate solution was added to react with the bound enzyme to produce colour. The intensity of this colour was measured spectrophotometrically at 450 nm. The activity of IL-18 was expressed as ng/L.

Assay of caspase-1—Caspase-1(EC: 3.4.22.36) activity was determined by a colorimetrically assay using serum or pancreatic extract, prepared according to the method of Thornberry\(^\text{23}\), as the enzyme source.
Briefly, the pancreas was homogenized in a lysis buffer (25 mM HEPES [pH 7.5], 1 mM EDTA, 10 µg of aprotinin/ml, 10 µg of leupeptin/ml, 2 mM dithiothreitol) at 5 mL/100 mg of pancreas tissue. Extracts were centrifuged at 15,000 g for 30 min at 4 °C, and the supernatant was centrifuged again at 200,000 g for 1 h at 4 °C. The cytosol was used for caspase-1 activity measurements. The assay in undiluted serum or pancreas extract was performed as per the kit manufacturer instruction. Reactions with enzyme preparation alone, with enzyme mixed with caspase-1 substrate (Ac-YVAD-pNA) or inhibitor (Ac-YVAD-CHO), and with substrate alone were also run as controls. The activity was measured by proteolytic cleavage of Ac-YVAD-pNA for 4 h at 37 °C. The plates were read at 405 nm. A recombinant caspase-1 enzyme was used as a positive control.

**Estimation of protein**—Protein concentration was determined in serum and tissue homogenate by the method of Bradford. The values were expressed as g/dL serum. In tissue homogenate the protein level was used to calculate the enzyme activity.

**Histopathological examination**—For histological examination, the pancreas was excised and rinsed with ice-cold saline (0.9% NaCl) to remove blood and debris of adhering tissues. The tissue was then fixed in 10% formalin for 24 h. The fixative was removed by washing through running tap water, and after dehydration through a graded series of alcohol, the tissue was cleaned in methyl benzoate and embedded in paraffin wax. Sections were cut into 5 µM thickness and stained with hematoxylin and eosin, mounted and observed under light microscope for details.

**Statistical analyses**—Data were analyzed by using a commercially available statistics software package (SPSS for window V.10). The statistical significance of mean values between different groups was determined by applying one way ANOVA with post hoc Bonferroni test and the *P* value < 0.05 was considered as significant.

**Results**

**Effect of TQ on body weight and food consumption**—The weight gain and the average food consumption of control and experimental rats are shown in Table 1. The net weight gain was significantly low (*P* = 0.000) in EtOH and HFD fed rats (group 3) when compared to control rats (group 1). TQ administration along with EtOH and HFD showed improvement in body weight gain. The effect was found to be dose dependent up to 100 mg/kg body weight and 200 mg/kg body weight of TQ received animals did not show further improvement in the weight gain. The average food consumption was also found to be decreased in group 3 rats which received EtOH and HFD alone when compared to group 1 control rats. Co-administration of 100 mg/kg body weight of TQ was found to maintain the level of average food consumption in rats.

**Effect of TQ on serum lipase, amylase and protein**—The activities of serum lipase and amylase, the marker enzymes of pancreatic functions are presented in Table 2. Serum amylase and lipase activities were increased significantly (*P* = 0.000) in EtOH and HFD received animals (group 3) when compared to group 1 rats. The rats (group 4-7) co-administrated with TQ showed decrease in the level of serum amylase and lipase in a dose dependent manner and an optimum decrease was observed in rats received 100 mg/kg body weight of TQ. The serum protein level showed marked decrease in group 3 rats when compared to other groups which received TQ at different doses and the optimum effect was observed with 100 mg/kg body weight of TQ.

**Effect of TQ on MPO, caspase-1 and cytokine activities**—Figures 2-4 show the level of serum IL-1β, IL-18 and caspase-1 respectively in experimental animals. The activities of caspase-1 and MPO in the pancreas (group 1-7) are depicted in Figs 5 and 6. In EtOH and HFD fed rats (group 3) the level of cytokine IL-1 and caspase-1 respectively in experimental animals did not show further improvement in the weight gain. The average food consumption was also found to be decreased in group 3 rats which received EtOH and HFD alone when compared to group 1 control rats. Co-administration of 100 mg/kg body weight of TQ was found to maintain the level of average food consumption in rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Net weight gain (g) (final- initial)</th>
<th>Food consumption (g/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>40±4.6</td>
<td>10.33±1.2</td>
</tr>
<tr>
<td>TQ control</td>
<td>39±4.2**</td>
<td>10.06±1.2</td>
</tr>
<tr>
<td>EtOH+HFD</td>
<td>25±3.3*</td>
<td>7.5±0.9</td>
</tr>
<tr>
<td>EtOH+HFD + TQ</td>
<td>29±3.5**</td>
<td>8.3±0.9</td>
</tr>
<tr>
<td>(25 mg/kg body wt.)</td>
<td>31±4.4*</td>
<td>8.5±0.9</td>
</tr>
<tr>
<td>EtOH+HFD + TQ</td>
<td>34±3.4*</td>
<td>9.5±1.1</td>
</tr>
<tr>
<td>(50 mg/kg body wt.)</td>
<td>35±5.2*</td>
<td>9.6±1.5</td>
</tr>
<tr>
<td>EtOH+HFD + TQ</td>
<td>35±5.2*</td>
<td>9.6±1.5</td>
</tr>
<tr>
<td>(100 mg/kg body wt.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EtOH+HFD + TQ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(200 mg/kg body wt.)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1—Effect of TQ supplementation on weight gain and food consumption in rats

[Values as mean±SD for 6 animals in each group]

Statistical significance was calculated by comparing control vs TQ, control vs EtOH+HFD, EtOH+HFD + TQ vs EtOH+HFD. *P* values: * =0.000, * =0.004, * =0.096, * =0.064, NS = non significant.
(group 4-7) showed a decrease in the level of caspase-1, IL-1β, and IL-18 activities and significant decrease was observed in rats received 100 mg/kg body weight of TQ. The activities of caspase-1 and MPO in pancreas were also significantly \( (P=0.000) \) elevated in group 3 rats and the levels were significantly maintained in rats received 100 and 200 mg/kg body weight of TQ.

**Effect of TQ on oxidative stress**—The levels of TBARS, total peroxide, TAC, and OSI are given in Table 3. Supplementing TQ to EtOH and HFD fed rats significantly decreased the level of TBARS, peroxide concentration and OSI. TAC was found to be decreased in group 3 rats when compared to the control.

![Fig. 2](image1.png)

**Fig. 2**—Changes in the activity of serum IL-1β in EtOH and HFD received rats with/without TQ supplementation. The data were analyzed by oneway ANOVA with post hoc Bonferroni test and values are expressed as mean ± SD for 6 rats in each group. For comparison Group 1 vs Group 3, Group 1 vs Group 2, Group 3 vs Group 4-7 are considered. Statistically significant variations are expressed as \( \*P=0.000 \), NS = non significant.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Lipase (IU/l)</th>
<th>Amylase (IU/l)</th>
<th>Protein (g/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>120.0±14.8</td>
<td>1400±184</td>
<td>5.0±0.57</td>
</tr>
<tr>
<td>TQ control</td>
<td>128.72±17.8(^{NS})</td>
<td>1500±220(^{NS})</td>
<td>4.9±0.73(^{NS})</td>
</tr>
<tr>
<td>EtOH+HFD + TQ (25 mg/kg body wt)</td>
<td>182.70±18.6(^{*})</td>
<td>2300±271(^{*})</td>
<td>4.0±0.51(^{*})</td>
</tr>
<tr>
<td>EtOH+HFD + TQ (50 mg/kg body wt)</td>
<td>140.11±20.7(^{*})</td>
<td>1700±219(^{*})</td>
<td>4.4±0.66(^{NS})</td>
</tr>
<tr>
<td>EtOH+HFD + TQ (100 mg/kg body wt)</td>
<td>122.53±18.3(^{*})</td>
<td>1500±207(^{*})</td>
<td>4.8±0.68(^{NS})</td>
</tr>
<tr>
<td>EtOH+HFD + TQ (200 mg/kg body wt)</td>
<td>120.10±15.4(^{*})</td>
<td>1450±162.4(^{*})</td>
<td>4.9±0.65(^{*})</td>
</tr>
</tbody>
</table>

Statistical significance was calculated by comparing control vs TQ, control vs EtOH+HFD, EtOH+HFD +TQ vs EtOH+HFD. \( P \) values: \( ^{*}=0.000 \), \(^{*}=0.04 \), \(^{*}=0.774 \), \(^{*}=0.006 \) NS = non significant.

![Fig. 3](image2.png)

**Fig. 3**—Changes in the activity of serum IL-18 in EtOH and HFD received rats with/without TQ supplementation. The data were analyzed by oneway ANOVA with post hoc Bonferroni test and values are expressed as mean ± SD for 6 rats in each group. For comparison Group 1 vs Group 3, Group 1 vs Group 2, Group 3 vs Group 4-7 are considered. Statistically significant variations are expressed as \( \*P=0.000 \), NS = non significant.

![Fig. 4](image3.png)

**Fig. 4**—Changes in the activity of serum caspase-1 in EtOH and HFD received rats with/without TQ supplementation. The data were analyzed by oneway ANOVA with post hoc Bonferroni test and values are expressed as mean ± SD for 6 rats in each group. For comparison Group 1 vs Group 3, Group 1 vs Group 2, Group 3 vs Group 4-7 are considered. Statistically significant variations are expressed as \( \*P=0.000 \), NS = non significant.
rats which were co-administered with TQ at different concentration. TBARS concentration in pancreas was found to be decreased significantly by TQ administration at the concentration of 100 and 200 mg/kg body weight of TQ.

Effect of TQ on antioxidants in pancreas—The activities of SOD, CAT and GPx in the pancreas of control and EtOH-HFD received rats with/without TQ administration are presented in Table 4. EtOH and HFD received rats showed low level of enzymatic antioxidants and reduced glutathione in pancreas. TQ co-administration along with EtOH and HFD found to maintain the level of antioxidants in a dose dependent manner with the optimum effect at 100 mg/kg body weight of TQ.

Effect of TQ on the histology of pancreas—The histological changes in the pancreas of control and experimental rats are shown in Fig. 7. The pancreas of control rats showed normal architecture with parenchyma and acinar cells (Fig. 7a). Rats received EtOH and HFD showed significant inflammation,

![Fig. 5—Changes in the activity of pancreas caspase-1 in EtOH and HFD received rats with/without TQ supplementation. The data were analyzed by oneway ANOVA with post hoc Bonferroni test and values are expressed as mean ± SD for 6 rats in each group. For comparison Group 1 vs Group 3, Group 1 vs Group 2, Group 3 vs Group 4-7 are considered. Statistically significant variations are expressed as P=0.000, *=0.01 NS = non significant](image1)

![Fig. 6—Changes in the activity of pancreas MPO in EtOH and HFD received rats with/without TQ supplementation. The data were analyzed by One way ANOVA with post hoc Bonferroni test and values expressed as are mean ± SD for 6 rats in each group. For comparison Group 1 vs Group 3, Group 1 vs Group 2, Group 3 vs Group 4-7 are considered. Statistically significant variations are expressed as *P=0.000, NS = non significant.](image2)

Table 3—Effect of various concentration of TQ on the levels of TBARS, peroxide content, TAC and oxidative stress index in rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Plasma (nM/mL)</th>
<th>Pancreas (nM/100mg protein)</th>
<th>Peroxide (mM/L)</th>
<th>TAC (mM trolox eq./L)</th>
<th>OSI (peroxide/TAC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.163±0.01</td>
<td>3.12±0.46</td>
<td>180.1±20.53</td>
<td>346.66±47.83</td>
<td>0.54±0.05</td>
</tr>
<tr>
<td>TQ control</td>
<td>0.17±0.02 NS</td>
<td>2.98±0.45 NS</td>
<td>192.64±28.70 NS</td>
<td>331.02±42.03 *</td>
<td>0.58±0.07 NS</td>
</tr>
<tr>
<td>EtOH+HFD</td>
<td>0.44±0.04 *</td>
<td>5.09±0.57 *</td>
<td>298.03±44.70 *</td>
<td>224.33±26.42 *</td>
<td>1.32±0.19 *</td>
</tr>
<tr>
<td>EtOH+HFD + TQ (25 mg/kg body wt.)</td>
<td>0.40±0.05 NS</td>
<td>4.72±0.65 NS</td>
<td>260.14±26.53 @</td>
<td>278±41.70 @</td>
<td>1.07±0.13 @</td>
</tr>
<tr>
<td>EtOH+HFD + TQ (50 mg/kg body wt.)</td>
<td>0.32±0.03 *</td>
<td>3.99±0.47 @</td>
<td>244.52±33.49 *</td>
<td>301.72±42.82 *</td>
<td>0.72±0.09 *</td>
</tr>
<tr>
<td>EtOH+HFD + TQ (100 mg/kg body wt.)</td>
<td>0.19±0.02 *</td>
<td>3.59±0.36 *</td>
<td>198.62±25.42 *</td>
<td>328±33.14 *</td>
<td>0.59±0.08 *</td>
</tr>
<tr>
<td>EtOH+HFD + TQ (200 mg/kg body wt.)</td>
<td>0.18±0.02 *</td>
<td>3.42±0.43 *</td>
<td>194±35.80 *</td>
<td>330±40.59 *</td>
<td>0.58±0.08 *</td>
</tr>
</tbody>
</table>

Statistical significance was calculated by comparing control vs TQ, Control vs EtOH+HFD, EtOH+HFD +TQ vs EtOH+HFD. P values: *=0.000, *=0.026, *=0.012, NS = Non significant
mononuclear cell infiltration, fatty changes in and around acinar cell (Fig. 7c and d). TQ co-administration (25, 50 mg/kg body weight) showed mild reduction in fatty changes and inflammation (Fig. 7e and f). There is a significant reduction in fat accumulation, inflammation and hemorrhage in the pancreas of rats received 100 and 200 mg/kg body weight of TQ (Fig. 7g and h).

**Discussion**

Alcohol abuse is a leading cause of death in developing countries like India and excessive alcohol consumption accounts for approximately 40% of all chronic and acute pancreatitis. EtOH is toxic to pancreas that disturbs the metabolic functions and ultimately results in impairment of endocrine and exocrine functions. The present results clearly shows that simultaneous feeding of EtOH and HFD diet to rats resulted in significant decrease in weight gain and also in average food consumption. The HFD formulation contains low carbohydrate and hence reserve carbohydrates and fats are actively utilized as energy source. The alteration produced in pancreas also leads to impaired release of digestive enzymes to intestine which results in malabsorption of nutrients. EtOH at the dose of 7.9 g/kg body weight/day has been shown to reduce weight in rats.25 The decrease in the level of average food consumption might be due to high satiety value of fat diet in group 3 animals which were left without drug treatment.

EtOH is metabolized in liver as well as in pancreas and forms toxic metabolites such as acetaldehyde which can cause tissue injury. This might result in reducing the metabolic functions of liver that synthesize various anabolic proteins. Pancreatic injury may result in reduced supply of proteolytic enzymes for digestion in intestine. This might result in reduced absorption of nutrients and impairment in weight gain. Reduced food consumption has been reported in rats treated with alcohol.26 N. sativa seed extract has been used traditionally for gastrointestinal complications and to promote digestion. The present study also shows the beneficial effect of TQ as a health promoter and the protective action against EtOH and HFD induced weight loss in rats. TQ supplementation was found to improve food consumption as well as the net weight gain showing the modulatory effect on EtOH and HFD induced changes in rats.

Serum amylase and lipase are generally measured to determine the functional efficiency of pancreas. These enzymes are synthesized by pancreatic cells which when subjected to injury release the enzymes to blood circulation to elevate the enzyme levels in serum.27 In this study, EtOH and HFD fed animals showed increased serum level of lipase and amylase due to cell damage in pancreas. The role of TQ as pancreato protective agent is well proved through its effect on reducing the serum lipase and amylase activity in a dose dependent manner. TQ administration is found to reduce the cell damage as well as the release of enzymes into blood circulation. The optimum effect was found in 100 mg/kg body weight of TQ supplemented rats. Many therapeutic agents which protects pancreas from injury has been found in reduce the activity of serum lipase and amylase.

Table 4—Activity of antioxidant enzymes and glutathione in rats administrated with various concentration of TQ

<table>
<thead>
<tr>
<th>Groups</th>
<th>GPx</th>
<th>SOD</th>
<th>CAT</th>
<th>Plasma</th>
<th>Pancreas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(nM of GSH oxidized/min/mg protein)</td>
<td>(units/mg protein)</td>
<td>(µM H2O2 consumed/min/mg protein)</td>
<td>(µg/mg protein)</td>
<td>(mg/g protein)</td>
</tr>
<tr>
<td>Control</td>
<td>1.57 ± 0.22</td>
<td>13.67 ± 1.3</td>
<td>110±15.18</td>
<td>15.13±1.86</td>
<td>13.66±2.04</td>
</tr>
<tr>
<td>TQ control</td>
<td>1.42 ± 0.21</td>
<td>12.95 ± 1.8</td>
<td>105±19.7</td>
<td>14.89±2.03</td>
<td>12.88±1.59</td>
</tr>
<tr>
<td>EtOH+HFD</td>
<td>0.50 ± 0.05</td>
<td>8.53 ± 1.1</td>
<td>68±7.61</td>
<td>10.78±1.55</td>
<td>7.6±1.05</td>
</tr>
<tr>
<td>EtOH+HFD + TQ (25 mg/kg body wt.)</td>
<td>0.67 ± 0.09</td>
<td>9.89 ± 1.33</td>
<td>82.5±12.37</td>
<td>11.01±1.37NS</td>
<td>8.2±0.89NS</td>
</tr>
<tr>
<td>EtOH+HFD + TQ (50 mg/kg body wt.)</td>
<td>0.81±0.09</td>
<td>11.09 ± 1.35</td>
<td>90.87±13.53</td>
<td>12.23±1.30NS</td>
<td>9.78±1.43a</td>
</tr>
<tr>
<td>EtOH+HFD + TQ (100 mg/kg body wt.)</td>
<td>1.08 ± 0.13</td>
<td>12.01 ± 1.80</td>
<td>102±12.95</td>
<td>14.38±1.68#</td>
<td>12.21±1.23</td>
</tr>
<tr>
<td>EtOH+HFD + TQ (200 mg/kg body wt.)</td>
<td>1.11 ± 0.14</td>
<td>12.35 ± 1.82</td>
<td>103.22±14.65</td>
<td>14.50±2.17#</td>
<td>12.78±1.67</td>
</tr>
</tbody>
</table>

Statistical significance was calculated by comparing control vs TQ, control vs EtOH+HFD, EtOH+HFD +TQ vs EtOH+HFD. *P < 0.000, # =0.029, $ =0.025, $ =0.018, %=0.003, NS = Non significant
is traditionally known in Middle Eastern country and referred as “The Blessed Seed” due its powerful healing qualities for many ailments including liver, stomach and intestinal disorders to maintain and to improve overall health. *N. sativa* seeds contain rich amount of TQ and the healing effect of the plant might due to the presence of this quinone.

MPO is an enzyme stored in azurophilic granules of polymorphonuclear neutrophils and macrophages and released into extracellular fluid in the setting of inflammatory process. The observation is that MPO is involved in oxidative stress and inflammation has been a leading factor to study MPO as a possible marker of pancreatitis. EtOH has been shown to cause inflammation in pancreas. The toxic metabolites of ethanol induce injury by means of ROS which promote inflammatory changes in the parenchymal tissues. The results of this study also showed elevated MPO activity in the pancreas of EtOH and HFD received rats. TQ is found to decrease the
activity of MPO in rats fed with EtOH and HFD. TQ has been proved to reduce inflammation associated with diabetes mellitus, arthritis and malignant tumors. This study is an added evidence for the anti-inflammatory activity of TQ by which it protects pancreas from the harmful effects of EtOH and HFD.

Interleukins are inflammatory cytokines, produced in response to cell injury to alleviate the cell damage but when they are produced in excess can even damage the normal cells to promote tissue injury. Interleukins such as IL-1β and IL-18 are known as pro-inflammatory cytokines expressed at the site of chronic inflammation in a variety of diseases including cancer, infectious diseases and myocarditis.

IL-1β and IL-18 are involved in promoting inflammation and also act as markers of tissue inflammation. We could find significant increase in the serum level of IL-1β and IL-18 in EtOH and HFD fed rats and beneficial decrease in TQ co-administered rats. EtOH induced harmful effects are shown to be associated with the formation of pro-inflammatory cytokines which complement the functions of other mediators such as TNF-α and cyclooxygenase.

Alcohol consumers generally prefer fatty diet which all the more increases the complications of alcoholism. Supplementation of TQ to alcohol consumers may be reducing the complications of alcoholism. TQ was found to reduce the systemic concentration of IL-1β and IL-18 showing its anti-inflammatory nature.

IL-1β is closely related to IL-18 by sharing similar 3D structure and common signaling pathways. Binding of IL-18 to its receptors is followed by recruitment of the IL-1β receptor activity kinase. Hence, these two cytokines work hand in hand to promote cell injury. TQ is found to be efficient in reducing the level of these cytokines. However, the detailed study on status of IL-1β and IL-18 receptors in TQ supplemented rats need to be studied.

Caspase-1 is a cysteine protease that proteolytically cleaves other proteins such as the precursor forms of pro-inflammatory cytokines IL-1β and IL-18 into active mature peptides. Caspase-1 itself needs activation by inflammasomes, a protein platform containing different domains such as caspase-1 recruiting domain (CARD) and PYRIN domain. Caspase-1 also triggers a form of cell death with characteristic necrosis and apoptosis that has been called pyroptosis.

In EtOH and HFD fed rats, the caspase-1 activity was found to be elevated significantly both in serum and pancreas showing the role of caspase-1 on the inflammatory changes. TQ was found to reduce the activity of the caspase-1 with the concomitant reduction in the level of IL-1β and IL-18 in a dose dependent manner.

ROS such as superoxide anion, hydroxyl radical and peroxynitrite are involved in promoting inflammation in cells. The deleterious effect of these radicals acts as stimuli for pro-inflammatory cytokines production. EtOH and HFD feeding was found to enhance the free radicals formation which might aggravate the inflammation evidenced by high level of cytokines in blood. TQ was found to minimize the level of TBARS formation and peroxides in serum and pancreas. Among the doses studied, 100 mg/kg body weight of TQ was found to produce optimum free radicals reducing effect. TAC was found to be maintained along with the reduction in oxidative stress index in TQ supplemented rats. This effect shows that TQ ameliorates inflammation probably by reducing the free radical formation in rats fed with EtOH and HFD. Among the promising medicinal plants, N. sativa is a wonder plant with rich traditional background and for its antioxidant content the seeds have been consumed for various ailments in which ROS are involved. TQ has been reported to inhibit the iron dependent microsomal lipid peroxidation in in vitro.

Enzymatic and non enzymatic antioxidants in in vivo, act to counteract the ROS formation and to protect the cells from the harmful effect of free radicals. TQ was found to maintain the level of reduced glutathione and enzymatic antioxidants GPx, SOD and CAT. These enzymes are effective in quenching and clearing the toxic free radicals. GSH a prime antioxidant that can scavenge free radicals and prevent the formation of hydrogen peroxide was found to be maintained significantly in TQ supplemented rats. The decrease in the concentration of pancreatic GSH observed in the present study on EtOH and HFD feeding might be due to its elevated consumption by free radicals. Many pancreatic disorders are associated with depleted GSH level in various organs. Enzymes GPx, CAT and SOD were active in clearing the free radicals and their depletion are also observed in many pathological conditions affecting pancreas. The present results are in accordance with the other reports which show that TQ...
scavenges free radicals and also play an important role in maintaining endogenous antioxidant status in vivo. The present study show that TQ supplementation in EtOH and HFD fed rats markedly reduces the acinar cell damage, inflammation, fatty steatosis and hemorrhage in pancreas. The histopathological study further confirm that TQ co-administration provide optimum protection to the pancreas at the dose 100 mg/kg body weight in rats fed with EtOH and HFD. In conclusion, TQ act as a potent pancreato protective agent by minimizing the release of amylase and lipase from pancreas into the circulation, cytokine production and lipid peroxidation which are associated with EtOH and HFD intake. Among the four doses of TQ studied, 100 mg/kg body weight was found to produce the optimum protective effect in modulating the changes induced by EtOH and HFD. However, further study on the effect of TQ on the regulatory aspects of caspase-1 by means of inflammmasomes is warranted to evaluate the mechanism of action of TQ.

References