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Original Contribution

# *Nigella sativa* thymoquinone-rich fraction greatly improves plasma antioxidant capacity and expression of antioxidant genes in hypercholesterolemic rats

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ABSTRACT

The antioxidant activities of the thymoquinone-rich fraction (TQRF) extracted from *Nigella sativa* and its bioactive compound, thymoquinone (TQ), in rats with induced hypercholesterolemia were investigated. Rats were fed a semipurified diet supplemented with 1% (w/w) cholesterol and were treated with TQRF and TQ at dosages ranging from 0.5 to 1.5 g/kg and 20 to 100 mg/kg body wt, respectively, for 8 weeks. The hydroxyl radical ($OH^{\cdot}$)-scavenging activity of plasma samples collected from experimental rats was measured by electron spin resonance. The GenomeLab Genetic Analysis System was used to study the molecular mechanism that mediates the antioxidative properties of TQRF and TQ. Plasma total cholesterol and low-density-lipoprotein cholesterol levels were significantly decreased in the TQRF- and TQ-treated rats compared to untreated rats. Feeding rats a 1% cholesterol diet for 8 weeks resulted in a significant decrease in plasma antioxidant capacity, as measured by the capacity to scavenge hydroxyl radicals. However, rats treated with TQRF and TQ at various doses showed significant inhibitory activity toward the formation of $OH^{\cdot}$ compared to untreated rats. Upon examination of liver RNA expression levels, treatment with TQRF and TQ caused the up-regulation of the superoxide dismutase 1 (SOD1), catalase, and glutathione peroxidase 2 (GPX) genes compared to untreated rats ($P<0.05$). In support of this, liver antioxidant enzyme levels, including SOD1 and GPX, were also apparently increased in the TQRF- and TQ-treated rats compared to untreated rats ($P<0.05$). In conclusion, TQRF and TQ effectively improved the plasma and liver antioxidant capacity and enhanced the expression of liver antioxidant genes of hypercholesterolemic rats.



Hypercholesterolemia is one of the crucial risk factors for the development of atherosclerosis and subsequent cardiovascular disease [1]. Cholesterol-rich diets are associated with free radical production, followed by oxidative stress and hypercholesterolemia [2,3]. Oxidative stress is, on the other hand, one of the factors that links hypercholesterolemia with atherogenesis [4]. There is evidence that oxidative stress contributes to the development of atherosclerosis in the vascular wall through the formation of reactive oxygen species (ROS) [5,6]. Protection against ROS and the breakdown products of oxidized lipids and proteins is provided by antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPX) [7]. In recent years, studies have been intensively performed on supplementation with natural antioxidant compounds to attenuate oxidative stress-induced pathogenesis of diseases [4]. Dietary intake of antioxidants could inhibit oxidation of low-density-lipoprotein cholesterol (LDLC) and thereby reduce the risk factors for cardiovascular diseases [8–10]. Many studies have reported the antioxidant activity of *Nigella sativa* oil and its active constituent, thymoquinone (TQ), against biologically hazardous ROS [11–14]. According to the findings of El-Saleh et al. [15], oral administration of *N. sativa* oil and TQ at respectively 100 μl/kg and 100 mg/kg body wt for 1 week resulted in significantly increased levels of total antioxidant status in rats. In line with that, Ilhan et al. [16] have reported that *N. sativa* oil elevated the level of GPX in pentylenetetrazol kindling seizures in mice compared to an untreated group. A few studies have also shown that *N. sativa* oil and its active compound TQ are capable of lowering plasma cholesterol levels in animals, probably because of its antioxidant activity [17,18]. However, detailed studies on *N. sativa* oil and TQ on the improvement of plasma antioxidant capacity and antioxidant gene expression are limited. Thus, this study was initiated to investigate the effects of the thymoquinone-rich fraction (TQRF) of *N. sativa* oil and TQ on plasma antioxidant capacity, liver antioxidant enzyme levels, and antioxidant gene expression in rats with induced hypercholesterolemia.

*Abbreviations:* TQ, thymoquinone; TQRF, thymoquinone-rich fraction; ESR, electron spin resonance; TC, total cholesterol; LDLC, low-density-lipoprotein cholesterol; HDLC, high-density-lipoprotein cholesterol; SOD1, superoxide dismutase 1; CAT, catalase; GPX, glutathione peroxidase 2; ALT, alanine aminotransferase; GGT, γ-glutamyltranspeptidase; ROS, reactive oxygen species.

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## Materials and methods

### Chemicals

Cholesterol, Tween 80, triolein, ammonium sulfate, thymoquinone, sucrose, Tris–HCl, phenylmethylsulfonyl fluoride, and EDTA were purchased from Sigma (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (Ottawa, ON, Canada). DMPO (5-dimethyl-1-pyrroline-*N*-oxide) was purchased from Labotech, Ltd (Tokyo, Japan), hydrogen peroxide ($H_2O_2$) was purchased from Bendosen Laboratory Chemicals (Selangor, Malaysia), and ferrous sulfate ($FeSO_4$) was purchased from BDH Chemicals (Poole, England). Total cholesterol (TC), LDLC, and high-density-lipoprotein cholesterol (HDLC) kits were purchased from Randox Laboratories (Crumlin, County Antrim, UK). The RiboPure RNA isolation kit was purchased from Ambion (Austin, TX, USA). The oligo(dT) multiplex primers were supplied by Sigma–Aldrich. The GenomeLab GeXP Start Kit (GenomeLab), ThermoStart Taq DNA polymerase (ThermoScientific), reverse transcription, multiplex PCR, and PCR fragment separation were purchased from Beckman–Coulter (Fullerton, CA, USA).

### Preparation of TQRF

*N. sativa* seeds were cleaned and dried in an oven at 40°C until a constant weight was attained. TQRF was prepared by using a supercritical fluid extractor (SFE) (Thar 1000 F; Thar Technologies, Pittsburgh, PA, USA). One hundred grams of the dried seeds was pulverized for 3 min in a stainless steel grinder (Waring Commercial, Torrington, CT, USA) and placed into a 1-liter stainless steel SFE vessel. After the vessel was tightly sealed, extraction parameters were set at a pressure of 600 bars and a temperature of 40°C. The pressure within the extraction vessel was generated with a constant carbon dioxide flow rate at 30 g/min and regulated by an automated back pressure regulator. The extraction process lasted for 3 h and TQRF was collected from the collection vessel after depressurization to 100 bars. TQRF produced using SFE parameters according to the procedure above is rich in TQ ($2.00 \pm 0.17\%$ TQ w/w) in comparison to TQ content in *N. sativa* oil ($0.57 \pm 0.01\%$ TQ w/w), which was extracted by the conventional Soxhlet procedure.

### Animal study

#### Preparation of TQRF and TQ emulsion

Both TQRF and TQ were administrated to the rats orally in the emulsion form. TQRF at various dosages was slowly added to 20 ml distilled water and 1% Tween 80. Emulsions were prepared at room temperature (25°C) using a laboratory scale homogenizer (Ultra-Turax T25 Basic; IKA-WERKE, Staufen, Germany) at 13,000 rpm for 5 min. TQ emulsion was prepared using triolein as the solvent. Triolein is commonly used to make TQ emulsions because it is hydrophobic and is better able to solubilize bulky lipophilic TQ. This is because TQ has a higher molecular weight than typical liquid hydrocarbon oils and a bulky structure with three branches, which makes solubilization more difficult with a common nonionic surfactant such as an ethoxylated linear alcohol [19]. TQ emulsion was prepared by dissolving a calculated amount of TQ in 1 ml triolein and following the same procedure as for TQRF emulsion. The triolein emulsion was prepared by mixing 1 ml triolein with 20 ml distilled water and Tween 80 at 1% as emulsifier. Rats were fed daily, by gavage in the morning, 2 ml of the freshly prepared emulsion containing the designated dosages of TQRF or TQ.

#### Animals and treatments

Ninety male Sprague–Dawley rats weighing between 150 and 200 g were used in this study. They were purchased from the Faculty of Veterinary Medicine, Universiti Putra Malaysia (Serdang, Selangor, Malaysia). Rats were individually housed in stainless steel cages in a well-ventilated room with a 12/12-h light/dark cycle at an ambient temperature of 25–30 °C. Experiments were carried out according to the guidelines for the use of animals and approved by the Animal Care and Use Committee of the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia. They were fed standard rat chow pellets purchased from As-Sapphire (Selangor, Malaysia), for 2 weeks for adaptation. Nine experimental rat groups were established with 10 rats per group. The groups were as follows: group 1, negative control group (NC) fed a normal prepared diet; group 2, positive cholesterol group (PC) fed a prepared diet supplemented with 1% (w/w) cholesterol; groups 3–5, TQRFL, TQRFM, and TQRFH, fed a cholesterol diet and TQRF emulsion at three doses, 0.5, 1, and 1.5 g/kg body wt, respectively; groups 6–8, TQL, TQM, and TQH, fed a cholesterol diet and TQ emulsion at three doses, 20, 50, and 100 mg/kg body wt, respectively; and Group 9, Triolein group, fed a cholesterol diet and triolein emulsion at 1 g/kg for 8 weeks. Fasting blood samples were collected by cardiac puncture at baseline and after 8 weeks of treatment. At the end of the experiment, all rats were sacrificed and the liver tissues were removed, snap-frozen in liquid nitrogen within 2–5 min of death, and stored at −80 °C for the gene expression study. Analysis of the lipid profile, including the investigation of TC, HDLC, and LDLC, was carried out using a Selectra XL analytical kit (Vita Scientific, Dieren, the Netherlands). The concentrations of alanine aminotransferase (ALT), γ-glutamyltranspeptidase (GGT), urea, and creatinine in plasma collected from the experimental rats were also measured in this experiment using analytical kits (Randox) and by kinetic UV assay using Selectra XL (Vita Scientific).

**Table 1**
Gene name, gene locus, and gene product used in GeXP assays of antioxidant and oxidative stress genes in rat liver

| Gene name | Gene locus | Gene product/description | Function |
|---|---|---|---|
| 18S[a] | BC168964 | 18S | Housekeeping gene |
| GPX | NM_183403 | Glutathione peroxidase 2 | Antioxidant |
| Gapdhs[a] | NM_023964 | Glyceraldehyde-3-phosphate dehydrogenase | Housekeeping gene |
| SOD1 | NM_017050 | Superoxide dismutase 1 | Antioxidant |
| Actb[a] | NM_031144 | β-Actin (mRNA | Housekeeping gene |
| CAT | NM_012520 | Catalase | Antioxidant |
| Knar | | | Internal control |

[a] Gene used for normalization.

#### Plasma antioxidant capacity against hydroxyl radical

After 8 weeks of treatment, plasma antioxidant capacity of experimental rats against hydroxyl radical was measured using an electron spin resonance (ESR) spectrometer (Jeol FA100; Tokyo, Japan). Hydroxyl radical was generated through the Fenton reaction. In brief, the reaction was initiated by mixing 40 μl of 0.4 mM DMPO, 37.5 μl of 0. 2 mM $FeSO_4$, 112.5 μl of 0. 2 mM EDTA, 60 μl of plasma sample, and 150 μl of 1 mM $H_2O_2$. About 200 μl of the mixture was put into a flat cell (200 pl capacity, quartz form) and injected onto the ESR spectrometer. ESR measurements were set as follows: magnetic field $336.450 \pm 5$ mT, microwave power 8 MW, modulation frequency 100 KHz, modulation width 0.1 mT, time constant 0.1 s, amplitude 50, and sweep time 2 min. ESR spectra were measured at room temperature and by using manganese oxide as an internal standard. DMSO was used as standard in this study.

#### Gene expression analysis

##### RNA isolation

RNA was isolated from frozen liver samples using the RiboPure RNA isolation kit (Ambion) according to the manufacturer's instructions.

**Table 2**
Gene name, gene product size, and forward and reverse primer sequences of GeXP assays of antioxidant genes in rat liver

| Gene name | Fragment size | Left sequence with universals | Right sequence with universals |
|---|---|---|---|
| 18S[a] | 188 | AGGTGACACTATAGAATAGCTCCAGGACGGAGTTCATA | GTACGACTCACTATAGGGACAGCAGGTGGAGCTCTGATT |
| Actb[a] | 204 | AGGTGACACTATAGAATAATGTACGTAGCCATCCAGGC | GTACGACTCACTATAGGGAAGGGCAACATAGCACAGCTT |
| SOD1 | 228 | AGGTGACACTATAGAATACTTGCTTTTTGCTCTCCCAG | GTACGACTCACTATAGGGAAAAATGAGGTCCTGCAGTGG |
| Cat | 313 | AGGTGACACTATAGAATAGTGGTTTTCACCGACGAGAT | GTACGACTCACTATAGGGACACGAGGTCCCAGTTACCAT |
| GPX | 221 | AGGTGACACTATAGAATATCAACATCGAGCCTGACATC | GTACGACTCACTATAGGGACAGACTTAGAGCCCCCAGTG |
| Knar[b] | 325 | AGGTGACACTATAGAATAATCATCAGCATTGCATTCGATTCCTGTTTG | GTACGACTCACTATAGGGAATTCCGACTCGTCCAACATC |

[a] Gene used for normalization.
[b] Internal control.

*Primer design*

Primers were designed using GenomeLab eXpress Profiler software. Fragment sizes ranged from 150 to 350 nt with a 7-nt minimum separation size between each PCR product. Genes and primer sequences are listed in Tables 1 and 2. In addition to the three genes of interest, each panel contained an internal control gene (Knar) and three normalization genes (Actb, Gapdhs, and 18S).

*cDNA synthesis and PCR amplification*

The reverse transcription reactions and PCR amplification were performed according to the GenomeLab GeXP Start Kit using the Beckman–Coulter protocol.

*GeXP multiplex data analysis*

The GeXP system was used to separate PCR products based on size by capillary gel electrophoresis and to measure their dye signal strength in arbitrary units of optical fluorescence, defined as the fluorescent signal minus background. PCR product sizes were determined using GenomeLab GeXP software and were compared to the expected PCR product size to identify each transcript. The data were imported into the analysis module of eXpress Profiler software. We chose the housekeeping gene 18S to normalize the results from each gene. The gene expression data were normalized by dividing the peak area of each gene by the peak area of the 18S gene and the fold change of expression of each gene was calculated using the following formula: fold change = normalized data of the gene from treated samples/normalized data of the gene from untreated samples. The data for each gene and technical replicate were averaged and calculated.

*Measurement of hepatic superoxide dismutase and glutathione peroxidase levels*

Liver tissues were homogenized in ice-cold buffer (0.25 M sucrose, 10 mM Tris–HCl, and 0.25 mM phenylmethylsulfonyl fluoride, pH 7.4) and centrifuged at 12,000 *g* for 10 min at 4°C and a portion of the homogenate was measured immediately for SOD and GPX levels using a commercial kit (Randox Laboratories) for Selectra XL (Vita Scientific).

*Statistical analysis*

ANOVA and Duncan grouping were performed using the SPSS Windows program version 14.0 to identify significant differences between samples ($P<0.05$). The Pearson correlation test was conducted to identify correlations between variables.

## Results

*Plasma lipid profile*

As shown in Fig. 1, feeding rats a diet supplemented with 1% cholesterol for 8 weeks resulted in a significant elevation of TC and LDLC levels in the PC and Triolein groups compared to the NC group. Plasma TC and LDLC levels of all groups treated with TQRF and TQ were significantly ($P<0.05$) lower compared to the PC group. On the other hand, no significant changes were observed in plasma HDLC levels in the TQRF and TQ groups compared to the PC group at week 8.

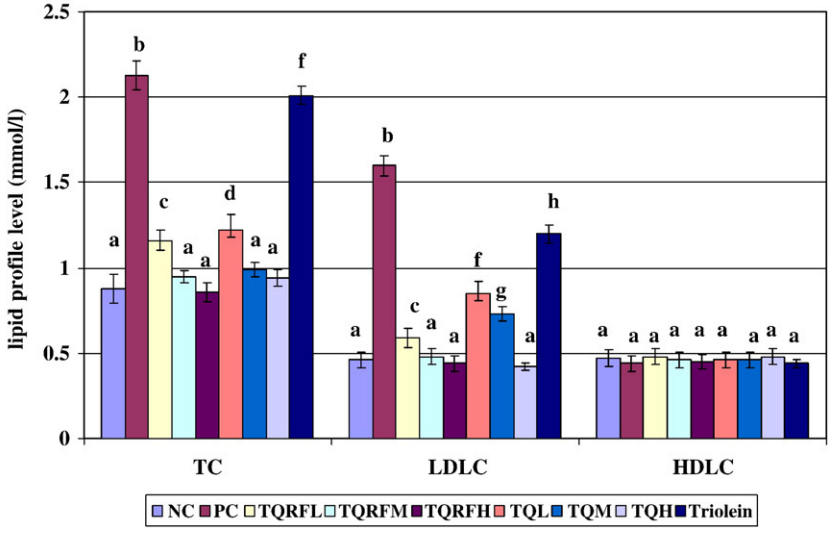


**Fig. 1.** TC, LDLC, and HDLC (mmol/liter) levels in rat plasma after 8 weeks. NC, negative control; PC, cholesterol positive control; TQRFL, group treated with TQRF at 0.5 g/kg; TQRFM, group treated with TQRF at 1 g/kg; TQRFH, group treated with TQRF at 1.5 g/kg; TQL, group treated with TQ at 20 mg/kg; TQM, group treated with TQ at 50 mg/kg; TQH, group treated with TQ at 100 mg/kg; Triolein, group treated with 1 ml/kg triolein for 8 weeks. Each value represents the mean of 10 rats ± SD. Within each parameter different letters indicate significant difference ($P<0.05$).

**Table 3**
ALT, GGT, urea, and creatinine levels in plasma of experimental rats

| Group | ALT (U/L) | GGT (U/L) | Urea (U/L) | Creatinine (U/L) |
|---|---|---|---|---|
| NC | $54.57 \pm 7.61^{a}$ | $4.14 \pm 1.85^{a}$ | $6.26 \pm 1.60^{a}$ | $48.09 \pm 12.97^{a}$ |
| PC | $74.40 \pm 17.12^{b}$ | $9.85 \pm 1.18^{b}$ | $8.45 \pm 0.95^{b}$ | $61.78 \pm 10.45^{a}$ |
| TQRFL | $51.11 \pm 15.35^{a}$ | $4.71 \pm 1.53^{c}$ | $6.31 \pm 1.01^{c}$ | $52.47 \pm 17.12^{a}$ |
| TQRFM | $52.5\ 0 \pm 10.91^{a}$ | $4.85 \pm 1.21^{c}$ | $5.67 \pm 0.86^{c}$ | $49.37 \pm 13.43^{a}$ |
| TQRFH | $52.00 \pm 7.09^{a}$ | $3.57 \pm 0.91^{c}$ | $5.11 \pm 1.20^{c}$ | $48.99 \pm 15.22^{a}$ |
| TQL | $52.71 \pm 6.46^{a}$ | $3.12 \pm 1.15^{c}$ | $4.28 \pm 1.29^{c}$ | $55.50 \pm 11.33^{a}$ |
| TQM | $50.60 \pm 12.50^{a}$ | $4.22 \pm 1.26^{c}$ | $5.34 \pm 0.81^{c}$ | $59.33 \pm 8.23^{a}$ |
| TQH | $50.00 \pm 11.23^{a}$ | $4.57 \pm 0.43^{c}$ | $4.86 \pm 1.89^{c}$ | $58.36 \pm 12.41^{a}$ |
| Triolein | $63.28 \pm 13.14^{a}$ | $7.23 \pm 1.23^{d}$ | $7.25 \pm 0.58^{a}$ | $60.75 \pm 9.65^{a}$ |

NC, negative control; PC, cholesterol positive control; TQRFL, group treated with TQRF at 0.5 g/kg; TQRFM, group treated with TQRF at 1 g/kg; TQRFH, group treated with TQRF at 1.5 g/kg; TQL, group treated with TQ at 20 mg/kg; TQM, group treated with TQ at 50 mg/kg; TQH, group treated with TQ at 100 mg/kg; Triolein, group treated with 1 ml/kg triolein for 8 weeks. Each value represents the mean of 10 rats ± SD. Within a column different letters indicate significant difference ($P<0.05$).

*Plasma ALT, GGT, creatinine, and urea levels*

Table 3 shows the ALT, GGT, creatinine, and urea levels in the plasma of the experimental rats. The results show that feeding rats a diet supplemented with 1% cholesterol resulted in a significant elevation of ALT, GGT, and urea levels in the PC group compared to the NC group. No significant difference was observed in creatinine levels in the PC group compared to the NC group. ALT, GGT, and urea levels were significantly lower in the TQRF- and TQ-treated groups compared to the PC group.

*Plasma antioxidant capacity against hydroxyl radical*

Figs. 2 and 3 show respectively the ESR spectra of DMPO–OH adduct and hydroxyl radical-scavenging activity of plasma samples obtained from experimental rats after 8 weeks of treatment. Hydroxyl radical-scavenging activity of the plasma was calculated through the DMSO standard curve ($y = 0.0093x - 0.1244$; $R^2 = 0.9731$).

The results obtained from this study show that feeding rats a 1% cholesterol diet for 8 weeks (PC) resulted in a significant decrease ($P<0.05$) in plasma antioxidant capacity toward hydroxyl radicals in comparison to the NC group. However, plasma samples obtained from rats fed a cholesterol diet and triolein exhibited a similar anti-peroxide activity compared to NC ($P>0.05$), indicating that a triolein-rich diet is effective in replenishing the antioxidant capacity of the plasma. On the other hand, plasma samples from TQRF-treated groups showed a dose-dependent higher inhibitory activity toward the formation of the DMPO–OH adducts compared to all control groups (PC, NC, and Triolein). This implies that oral administration of TQRF and TQ at the dosages of 0.5–1.5 g/kg and 20–100 mg/kg body wt, respectively, is capable of improving the plasma antioxidant capacity in a murine system. Nevertheless, TQRF groups showed higher $OH^{\cdot}$ scavenging activity compared to TQ-treated groups ($P<0.05$). This indicates that TQRF contains other bioactive compounds in addition to TQ, which might contribute synergistically to the improvement of plasma antioxidant capacity.

*Gene expression study*

Figs. 4A and 4B show the initial electropherogram data from fragment analysis of the gene multiplex assay. The Knar peak at 325 nucleotides served as an internal control for the multiplex reaction. As shown in Figs. 5A, 5B, and 5C, the expression levels of SOD1, CAT, and GPX were significantly lower in hepatic tissue of the PC group (fed a cholesterol diet) compared to the NC group. Treatment with TQRF and TQ at various doses resulted in significant up-regulation of SOD1, GPX, and CAT mRNA levels compared to the PC groups. Furthermore, all TQRF groups showed higher expression levels compared to the TQ groups. In both groups, increases in gene expression were concentration dependent. Among the various doses the expression level was concentration dependent, and a higher expression level was obtained at higher dose compared to the PC group.

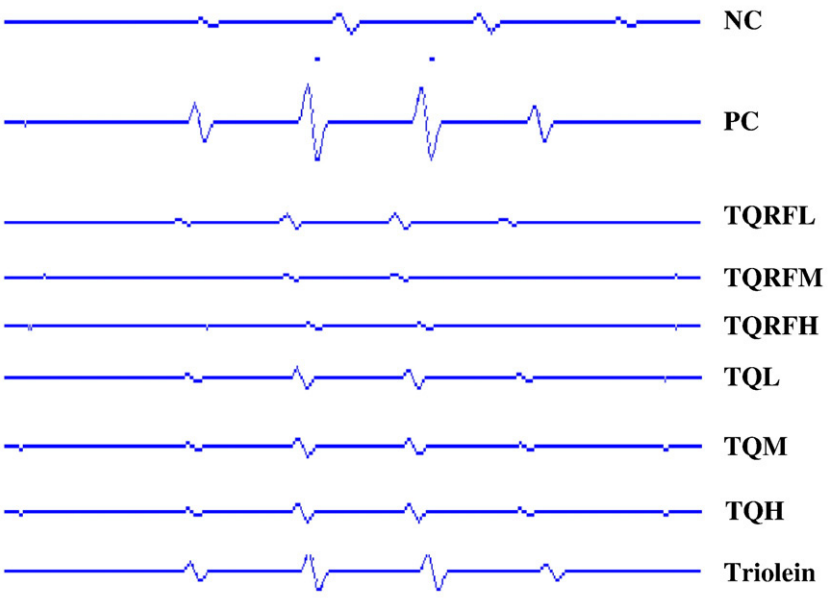


**Fig. 2.** ESR spectra of hydroxyl radical ($OH^{\cdot}$)-scavenging activity of TQ and TQRF in plasma samples collected from experimental rats after 8 weeks of treatment. The reaction $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$ was used as the source for $OH^{\cdot}$ generation. The ESR spectrum of the DMPO–$OH^{\cdot}$ adduct obtained in the reaction mixtures of $H_2O_2$ (1 mM), $FeSO_4$ (0.2 mM), and DMPO (0.4 mM) of various concentrations of DMSO was used as the standard. Various concentrations of DMSO (50, 100, 150, 200 mM) were plotted against the $I_0/I - 1$ value where $I$ is the intensity of the concentration of DMSO, which was calculated from the specific DMSO signal obtained divided by the manganese signal (using the ESR software), and $I_0$ is the intensity of the control sample (DMSO = 0 mM, water was used as a control), which was calculated from the specific control signal obtained divided by manganese signal obtained. Abbreviations are the same as for Fig. 1. Each value represents the mean of six rats ± SD.

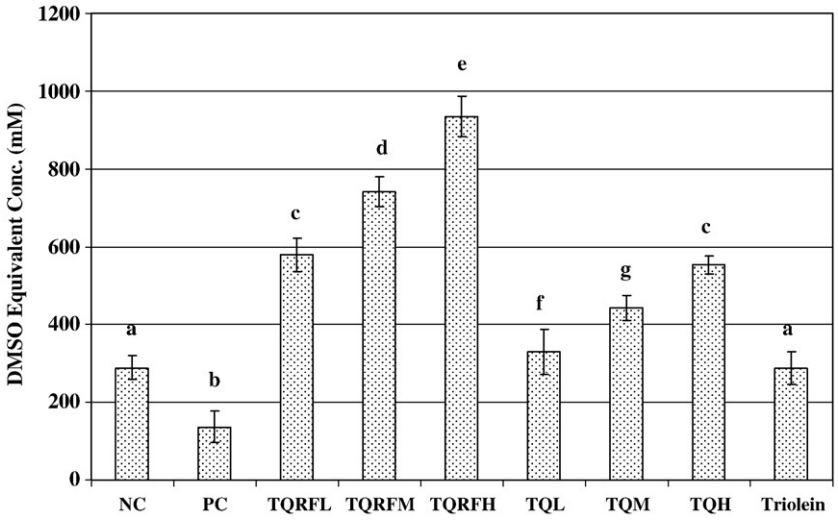


**Fig. 3.** $OH^{\cdot}$-scavenging activity of TQ and TQRF in plasma samples collected from experimental rats after 8 weeks of treatment. Abbreviations are the same as for Fig. 1. Each value represents the mean of three rats ± SD. Different letters indicate significant difference ($P<0.05$).

*Hepatic superoxide dismutase and glutathione peroxidase level*

Table 4 shows that the liver SOD and GPX enzyme levels were significantly decreased in the PC group compared to the NC group after 8 weeks of high-cholesterol diet. However, TQRF and TQ treatments effectively elevated the hepatic SOD and GPX enzyme levels in a dose-dependent manner ($P<0.05$). The TQRF groups showed higher SOD and GPX levels compared to TQ-treated groups. On the other hand, SOD and GPX levels in the Triolein group were similar to those of the NC group ($P>0.05$). This indicates that a triolein-rich diet is capable of replenishing the antioxidant enzymes levels in the rat liver. In summary, the results obtained from this experiment are well correlated and in agreement with the results obtained from the gene expression study in the previous section.

## Discussion

In this study, the lipoprotein profile analysis indicated that the reduction of plasma level cholesterol by TQRF and TQ could be attributed to changes in the LDLC level. The results obtained showed that rats from TQRF- and TQ-treated groups had lower plasma TC and LDL levels compared to the PC rats ($P<0.05$). On the basis of these findings, TQRF and TQ produce a hypocholesterolemic effect by decreasing LDLC levels significantly. The reduction in LDLC levels in the rats is probably due to the effectiveness of TQRF and TQ in regulating genes involved in cholesterol metabolism. More recently we have shown that TQRF and TQ up-regulate the mRNA level of the low-density-lipoprotein receptor and down-regulate 3-hydroxy-3-methylglutaryl-coenzyme A reductase significantly in vivo [20]. In addition, TQRF and TQ were shown to be effective in regulating the apolipoprotein A-1 and apolipoprotein B100 genes, which are linked to cholesterol metabolism in HepG2 cells [21].

The levels of ALT and GGT as well as creatinine and urea in the plasma are key indicators of in vivo hepatocyte damage and renal abnormality, respectively. In this study, a cholesterol-rich diet induced hepatotoxicity and nephrotoxicity in the experimental rats (PC group), with a remarkable elevation in plasma ALT, GGT, and urea levels ($P<0.05$). This finding is in agreement with previous studies that reported the induction of hepatotoxicity and nephrotoxicity in experimental rats by a cholesterol-rich diet [22,23]. Surprisingly, no physical signs of toxicity could be observed in the TQRF- and TQ-treated rats. Plasma ALT and GGT levels in the TQRF- and TQ-treated groups were significantly lower than in the PC group, indicating a possible hepatoprotective effect by TQRF and TQ toward cholesterol-induced hepatocyte damage. According to Mohamed et al. [24], treating rats with *N. sativa* oil reduced serum ALT by 28% and GGT level by 43% compared to control rats. On the other hand, TQ is also reported to be an effective hepatoprotective agent toward *tert*-butylhydroperoxide- and tetrachlorocarbon-induced hepatotoxicity [25,26]. Nagi and Almakki [26] recently reported that oral administration of TQ is effective in increasing the activities of quinone reductase and glutathione transferase, which makes TQ a promising prophylactic agent against chemical carcinogenesis and toxicity.

In addition to lowering the plasma ALT and GGT levels, both TQRF and TQ treatments decreased the plasma urea levels of the experimental rats. This indicates that TQRF and TQ might also serve as excellent nephroprotective agents toward cholesterol-induced renal abnormality. In support of this, some previous studies have reported the nephroprotective effect of *N. sativa* oil and TQ toward kidney damage in rats [27,28].

According to Akihiro et al. [29], feeding rats a high-cholesterol diet triggers excessive production of hydroxyl radicals, which are highly reactive species that can potentially inflict oxidative damage on a wide range of biological molecules. These authors also reported oxidative stress and impairment of in vivo antioxidant capacity. Thus, the current study was initiated to study the radical-scavenging activity of experimental rats' plasma toward Fenton reaction-generated hydroxyl radical. In this study, a cholesterol-rich diet (PC) decreased the plasma antioxidant capacity toward hydroxyl radicals in comparison to NC, indicating a successful oxidative stress induction by cholesterol in the studied murine system.

Treatment with both TQRF and TQ at dosages ranging from 0.5 to 1.5 g/kg and 20 to 100 mg/kg body wt, respectively, improved the plasma antioxidant capacity toward hydroxyl radical in a dose-dependent manner ($P<0.05$). At the highest dosage of TQRF and TQ, the hydroxyl radical-scavenging activity of the plasma samples was elevated by approximately 3- and 1.5-fold, respectively, compared to control plasma samples (NC and Triolein groups). Thymoquinone in TQRF (20 mg TQ/g TQRF) is one of the suggested major bioactive compounds that contribute to antioxidant improvement of rat plasma and its antioxidant properties have been extensively reported [24,30]. This is proven by the strong ($R=0.97$) and positive correlation observed between the TQ content of TQRF and the hydroxyl radical-scavenging activity of the plasma in this study.

Although TQ content in TQRF (20 mg TQ/g TQRF) is lower than the concentration administered in the TQ group (100 mg TQ), plasma samples from the TQRF group exhibited greater antioxidant capacity in comparison to the TQ group ($P<0.05$). This is in agreement with the

findings of Houghton et al. [11], who reported that the fixed oil of the *N. sativa* seed possessed greater antioxidant capacity than TQ. In addition, results from this finding also clearly reveal that TQRF might contain other bioactive compounds, which contribute independently or synergistically with TQ to the improvement of plasma antioxidant capacity.

Oleic acid is one of the major fatty acids (21 to 25%) in *N. sativa* seed oil [31,32]. The hydroxyl radical-scavenging activity results (Fig.

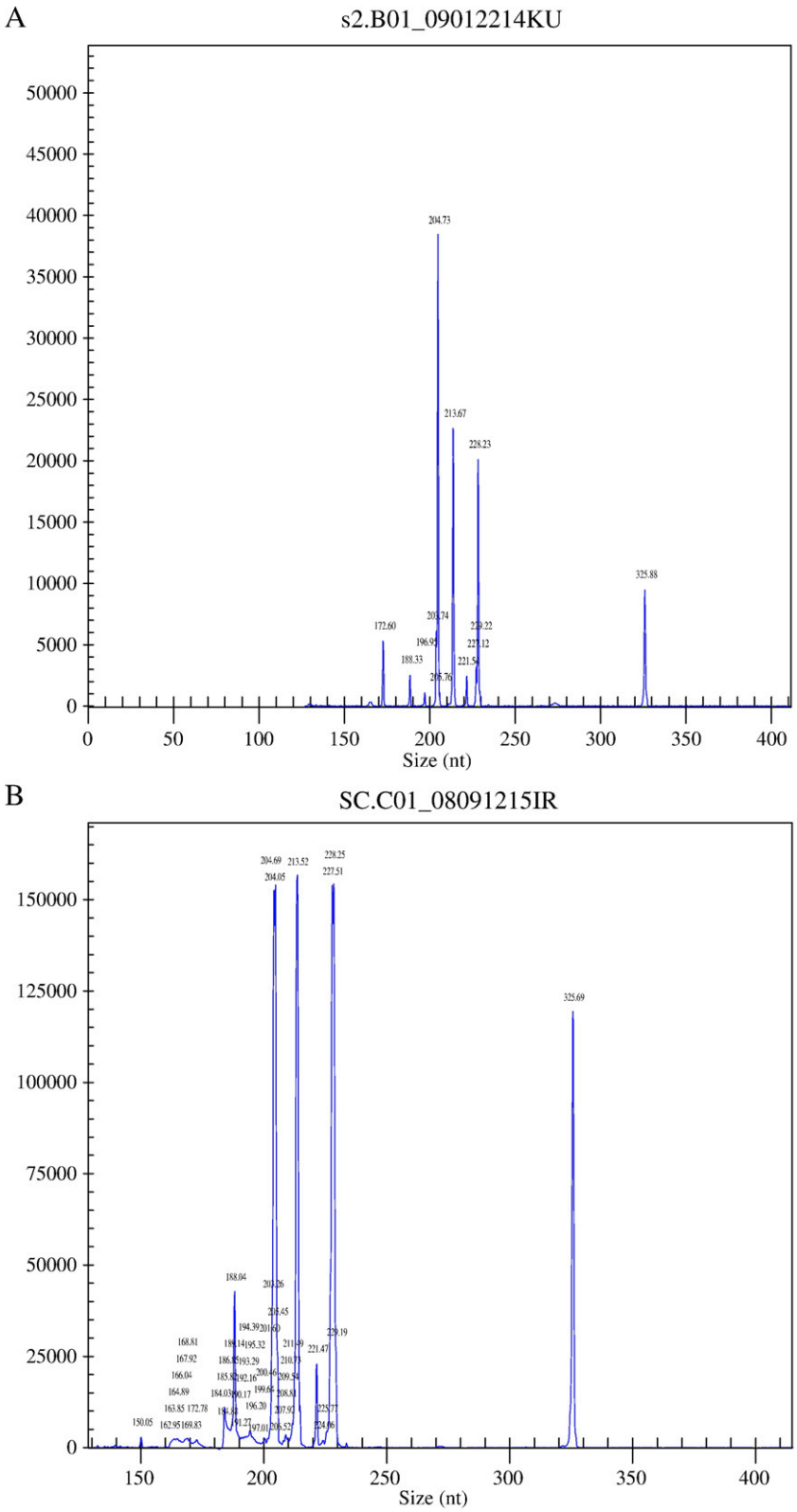


**Fig. 4.** (A) A representative electropherogram from the six multiplex genes expression study obtained from GeXP fragment analysis in the PC group. The Knar peak at 325 nucleotides serves as an internal control for the multiplex GeXP reaction. (B) A representative electropherogram from the six multiplex genes expression study obtained from GeXP fragment analysis in the TQRFH-treated group.

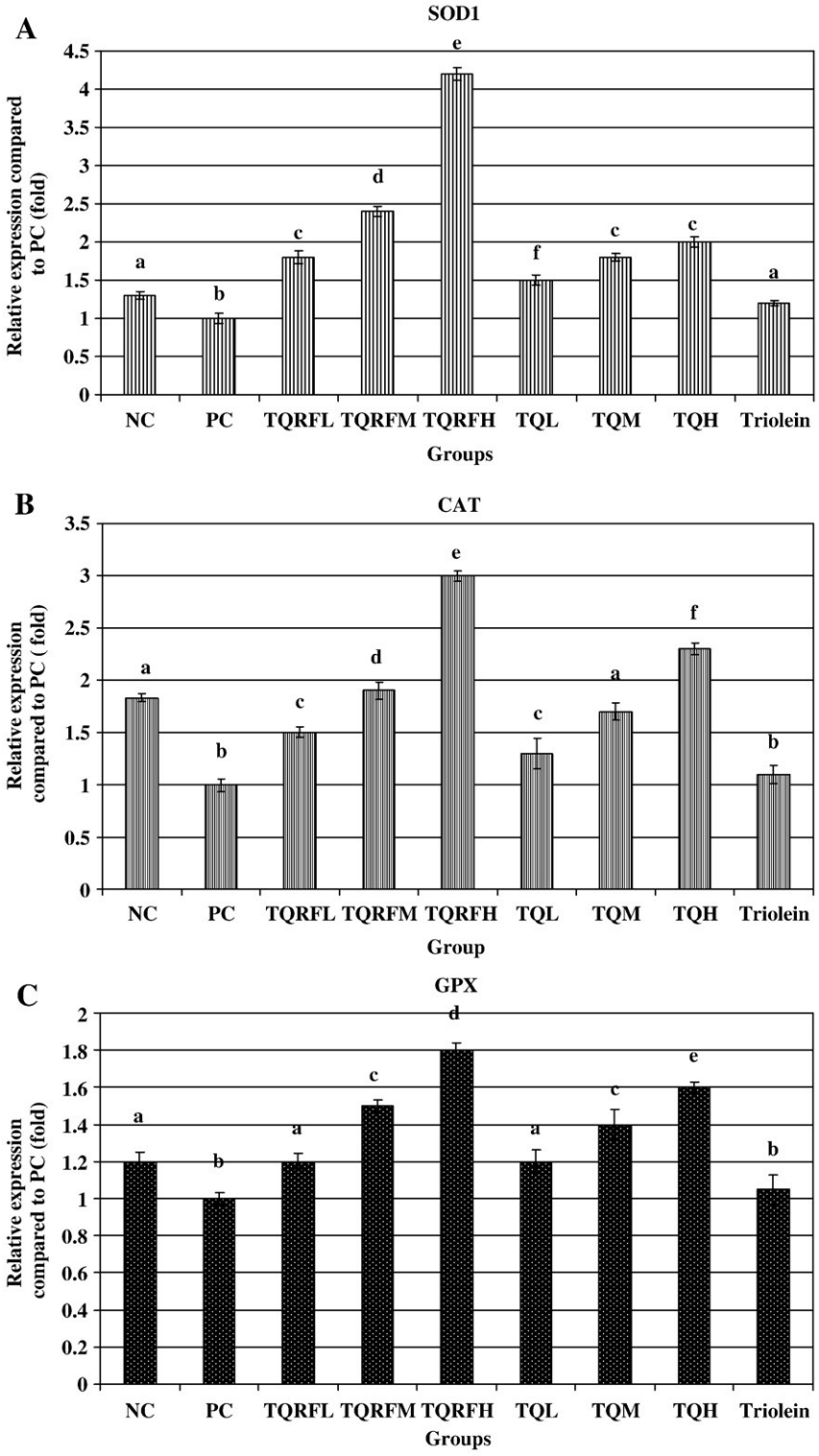


**Fig. 5.** (A) Fold change in mRNA level of the SOD gene in treated rats compared to untreated rats. Data were normalized to the β-actin gene. Abbreviations are the same as for Fig. 1. Each value represents the mean of three rats ± SD. Different letters indicate significant difference compared to the PC group ($P<0.05$). (B) Fold change in mRNA level of the CAT gene in treated rats compared to untreated rats. Data were normalized to the β-actin gene. Each value represents the mean of three rats ± SD. Different letters indicate significant difference compared to the PC group ($P<0.05$). (C) Fold change in mRNA level of the GPX gene in treated rats compared to untreated rats. Data were normalized to the β-actin gene. Each value represents the mean of three rats ± SD. Different letters indicate significant difference compared to the PC group ($P<0.05$).

3) show that plasma samples obtained from rats fed a cholesterol diet and triolein (triglyceride containing three oleic acids) exhibited antiradical activity similar to that of the NC group ($P>0.05$). This elucidated that oleic acid is capable of replenishing the plasma antioxidant capacity and might play an important role in the antioxidant activity of TQRF because oleic acid comprised 21.49% of the TQRF in this study. Other than oleic acid, α-tocopherol (290 ± 1.5 mg/100 g of TQRF) and phytosterols (3.66 g/kg [23]) are also candidate compounds that might contribute to the antioxidant activity of the TQRF.

**Table 4**
SOD and GPX levels in liver of experimental rats

| Group | SOD level (U/ml) | GPX level (U/l) |
|---|---|---|
| NC | $2.75 \pm 0.45^{a}$ | $1783 \pm 126^{a}$ |
| PC | $1.76 \pm 0.21^{b}$ | $1509 \pm 159^{b}$ |
| TQRFL | $4.64 \pm 0.56^{c}$ | $2453 \pm 101^{c}$ |
| TQRFM | $6.47 \pm 0.59^{d}$ | $2759 \pm 123^{d}$ |
| TQRFH | $8.18 \pm 0.85^{e}$ | $3201 \pm 124^{e}$ |
| TQL | $3.98 \pm 0.36^{c}$ | $2149 \pm 46^{f}$ |
| TQM | $4.75 \pm 0.26^{c}$ | $2386 \pm 52^{c}$ |
| TQH | $5.47 \pm 0.59^{g}$ | $2653 \pm 108^{h}$ |
| Triolein | $2.42 \pm 0.52^{a}$ | $1859 \pm 173^{a}$ |

NC, negative control; PC, cholesterol positive control; TQRFL, group treated with TQRF at 0.5 g/kg; TQRFM, group treated with TQRF at 1 g/kg; TQRFH, group treated with TQRF at 1.5 g/kg; TQL, group treated with TQ at 20 mg/kg; TQM, group treated with TQ at 50 mg/kg; TQH, group treated with TQ at 100 mg/kg; Triolein, group treated with 1 ml/kg triolein for 8 weeks. Each value represents the mean of 10 rats ± SD. Within a column different letters indicate significant difference ($P<0.05$).

Free radicals are produced during hypercholesterolemia [33,34]. Living tissues are endowed with innate antioxidant defense mechanisms, including the CAT, SOD1, and GPX enzymes. A reduction in the activity of these enzymes is associated with the accumulation of highly reactive free radicals [35]. Mansour [25] showed that TQ inhibited the nonenzymatic lipid peroxidation of normal mouse liver homogenates induced by $Fe^{3+}$/ascorbate in a dose-dependent manner. His results indicate that TQ is an efficient cytoprotective agent against $CCl_4$-induced hepatotoxicity, possibly through inhibition of the production of oxygen free radicals that cause lipid peroxidation.

Our study showed that feeding rats a cholesterol diet for 8 weeks resulted in a decrease in the mRNA levels of CAT, SOD1, and GPX in untreated rats compared to the normal rats. This may due to production of free radicals by cholesterol feeding that affected the antioxidant defense of the rats. In support of this, our results also demonstrated a significant reduction in the hepatic antioxidant enzymes during experimental hypercholesterolemia, specifically SOD and GPX levels. These data correspond to a number of reports that have shown decreases in SOD and GPX activities with diminution of the respective enzyme mRNA expression [36–38]. Recently, it was reported that the mean activities of CAT, SOD, and GPX in hepatic tissue samples were significantly lower in hypercholesterolemia-induced rats compared to normal rats [39].

Further treatment of rats with TQRF and TQ resulted in a dose-dependent up-regulation of the SOD1, CAT, and GPX genes as well as elevation of hepatic SOD and GPX levels. The up-regulation of the mRNA of SOD, GPX, and CAT in the rats treated with TQRF and TQ could be interpreted as an effort to overcome oxidative stress induced by hypercholesterolemia. Results obtained from gene expression and hepatic antioxidant enzyme studies are in good agreement with the findings on plasma antioxidant capacity in the first part of this study. This suggests that TQRF and TQ enhance the plasma antioxidant capacity of the hypercholesterolemic rats through the up-regulation of the in vivo antioxidant genes (CAT, SOD1, and GPX), which is confirmed by the elevated level of hepatic antioxidant enzymes.

In conclusion, TQRF and TQ are effective as cholesterol-lowering agents with high antioxidant activity. However, clinical trials should be conducted to confirm the hypercholesterolemic antioxidant activities of TQ and TQRF. In addition, subchronic or chronic toxicity assessment should be studied thoroughly before the clinical trial to ensure the safe consumption of TQ and TQRF within the designed dosages.

A limitation of this study is that, because we did not determine the inflammation-associated hypercholesterolemia and oxidative stress, we could not explain the anti-inflammatory properties of TQRF and TQ. Further studies should also be performed to address this effect.

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