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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 ± 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.