UNIVERSITI PUTRA MALAYSIA

EXTRACTION, GENE REGULATION AND HYPOCHOLESTEROLEMIC EFFECTS, AND ANTIOXIDATIVE ACTIVITY OF THYMOQUINONE RICH FRACTION AND THYMOQUINONE FROM *NIGELLA SATIVA*

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By

GHANYA NAJI AL-NAQEEB

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Chairman: Professor. Maznah Ismail, PhD

Faculty : Medicine and Health Sciences

Nigella sativa and its active constituent thymoquinone (TQ) have been used for various health benefits such as antitumor, antidiabetic, antihypertensive, antioxidatative and antibacterial. The present study involves thymoquinone rich fraction (TQRF) prepared from *Nigella sativa* seeds using supercritical fluid extraction (SFE) and commercial available thymoquinone (TQ) to investigate their regulatory effects on genes involved in cholesterol metabolism *in vitro* using Human liver cancer cells (HepG2 cells) and *in vivo* using Sprague-Dawley rats. In the present study, SFE extraction procedures with different parameters of pressure at 400 and 600 bar and temperature at 40, 60 and 80°C were optimized to prepare TQRF. The yield of the oil content was highest (37%) at a high temperature of 80°C and a high pressure of 600 bars. The TQ content in different fractions of oil was the highest (2%) at a low temperature of 40°C and a high pressure of 600 bars compared to other used parameters. The major fatty acids in TQRF were oleic and linolenic which exist as unsaturated fatty acids. Palmitic, stearic and myristic acids were the main saturated fatty acids. TQRF was rich in α-tocopherol content (290 ±

1.5 mg/100g). HepG2 cells viability was determined using MTT assay and flow cytrometry. TQRF and TQ were shown to inhibit HepG2 cells growth with IC_{50} of 100 µg/ml for TQRF and 3.5 µg/ml for TQ after 72 h incubation. Flow cytometry analysis showed that the cells viability was more than 80% when HepG2 cells were treated with TQRF at 50 µg/ml and TQ at 2 µg/ml. The regulatory effects of TQRF at 50 and 80 µg/ml and TQ at 2 µg/ml on genes involved in cholesterol metabolism were studied in HepG2 cell using real time polymerase chain reaction. These genes included low density lipoprotein receptor (LDLR), 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-COAR), apolipoproteins (Apo) including Apo A-1 and Apo B100. When cells were treated with TQRF at 50 and 80 µg/ml and TQ at 2 µg/ml mRNA level of LDLR gene was up-regulated by 3, 7 and 2 fold respectively, compared to untreated cells. On the other hand, mRNA level of HMG-COAR was down-regulated by 37, 71 and 12% respectively, compared to untreated cells. The mRNA level of Apo A-1 gene was up-regulated by 3, 4 and 2 fold and Apo B100 was suppressed by 60, 70 and 49% respectively, when cells were treated with TQRF at 50 and 80 µg/ml and TQ at 2 µg/ml compared to untreated cells. TQRF at dose of 0.5, 1 and 1.5 g/kg and TQ at 20, 50 and 100 mg/kg body weight in emulsion form were administrated orally to the rats fed diet supplemented with 1% cholesterol for 8 weeks. There was a significant reduction in plasma total cholesterol levels (TC) and low density lipoprotein cholesterol (LDLC) in both TQRF and TQ at different doses compared to cholesterol control rats (PC). The concentration of the alanine aminotransferase (ALT), gamma glutamyltranspeptidase (GGT), urea and creatinine of plasma collected from experimental rats were also measured in this experiment to test any toxic effect of TQRF and TQ on liver and kidney of rats. ALT, GGT and urea levels were

significantly lower in TQRF and TQ treated groups compared to PC group. The antioxidant activity of TQRF and TQ treatment as hydroxyl radical (OH·) scavenging activity in plasma samples collected from experimental rats was also carried out using electron spin resonance (ESR). The findings showed significantly higher of OH· scavenging activity in TQRF and TQ treated rats at different doses compared to untreated rats. The regulatory effect of TQRF and TQ treatment on hepatic genes involved in cholesterol metabolism including LDLR, HMG-COAR, Apo A-1, Apo B100 and Apo E genes were also investigated *in vivo*. Treating rats with TQRF at dose of 0.5, 1 and 1.5 g/kg and TQ at 20, 50 and 100 mg/kg body weight showed up-regulation of LDLR and Apo E genes whereas both treatments in the same doses showed down-regulation of HMG-COAR and Apo B100 genes compared to untreated rats. In conclusion, the present study suggests that TQRF and TQ generated a hypocholesterolemic effect through regulation of key genes involved in cholesterol metabolism. Both *in vitro* and *in vivo* results demonstrate potential value of TQRF and TQ as a novel cholesterol lowering and antioxidant candidate.

Abstrak tesis dikemukakan kepada Senat Universiti Putra Malaysia bagi memenuhi keperluan Ijazah Doktor Falsafah

PENGESTRAKAN,PENGAWALATURAN GEN-GEN, KESAN HIPOKOLESTEROLEMIK DAN AKTIVITI ANTIPENGOKSIDAAN FRAKSI KAYA TIMOKUINON DAN TIMOKUINON DARIPADA *NIGELLA SATIVA*.

Oleh

GHANYA NAJI AL-NAQEEB

Ogos 2009

Pengerusi: Profesor. Maznah Ismail, PhD

Fakulti : Perubatan dan Sains Kesihatan

Nigella sativa dan sebatian aktifnya, timokuinon (TQ) telah digunakan secara meluas dalam bidang perubatan dan kesihatan sebagai agen antitumor, antidiabetik, antihipertensi, antioksidatif dan anti-bakteria. Kajian ini bertujuan untuk mengkaji kesan rawatan fraksi kaya timokuinon (TQRF) (disediakan dari biji benih *Nigella sativa* dengan menggunakan kaedah pengekstrakan bendalir superkritikal, SFE) dan timokuinon (TQ) komersial terhadap pengawalaturan gen yang terlibat dalam metabolisma kolesterol, dengan menggunakan sel kanser hati manusia (sel HepG2) secara *in vitro* dan tikus Sprague-Dawley secara *in vivo*. Dalam kajian ini, tekanan pengekstrakan SFE yang berjulat dari 400 hingga 600 bars bersama-sama dengan suhu pengekstrakan pada 40, 60 dan 80°C telah dioptimakan untuk menyediakan TQRF. Kebolehdapatan minyak yang tertinggi (37%) didapati apabila pengekstrakan SFE dijalankan pada suhu 80°C bersama-sama dengan tekanan

tinggi iaitu pada 600 bars. Kandungan TQ yang tertinggi (2%) pula didapati pada minyak SFE yang diekstrak pada suhu rendah iaitu 40°C bersama-sama dengan tekanan pengekstrakan 600 bars. Kandungan asid lemak utama dalam TQRF adalah oleik dan linoleik yang hadir sebagai asid lemak tak tepu. Manakala asid palmitik, stearik dan miristik pula merupakan asid lemak tepu yang utama dalam TQRF. Fraksi kaya timokuinon didapati kaya dengan α tokoferol (290 ± 1.5 mg/100g). Pertumbuhan sel HepG2 telah dikaji dengan menggunakan kaedah asai MTT dan sitometri pengaliran. TQRF dan TQ telah menunjukkan kesan perencatan terhadap pertumbuhan sel HepG2 dengan bacaan IC_{50} masing-masing pada 100 µg/ml dan 3.5 µg/ml selepas pengeraman selama 72 jam. Analisis dari sitometri pengaliran menunjukkan bahawa pertumbuhan sel HepG2 adalah melebihi 80% apabila sel tersebut dirawati dengan TQRF pada 50 µg/ml dan TQ pada 2 µg/ml. Kesan rawatan TQRF (pada 50 dan 80 µg/ml) dan TQ (pada 2 µg/ml) terhadap pengawalaturan gen yang terlibat dalam metabolisma kolesterol dalam sel HepG2 telah dikaji dengan menggunakan kaedah tindakbalas berantai polimerase masa nyata. Gen – gen yang terlibat adalah termasuk reseptor lipoprotein berketumpatan rendah (LDLR), reduktase 3-hidroksi-3-metilglutaril-koenzim A (HMG-CoAR), apolipoprotein (Apo) iaitu Apo A-1, Apo B100 dan Apo E. Keputusan menunjukkan bahawa sel yang dirawati dengan TQRF pada 50 dan 80 µg/ml dan TQ pada 2 µg/ml masing-masing menunjukkan peningkatan pengawalaturan gen LDLR dengan peningkatan tahap mRNAnya sebanyak 3, 7 dan 2 kali ganda berbanding dengan sel yang tidak dirawati. Sebaliknya, pengawalaturan gen HMG-COAR masing-masing direncatkan sebanyak 37, 71 dan 12%, berbanding dengan sel yang tidak dirawati. Selain daripada itu, rawatan TQRF (50 dan 80 µg/ml) dan TQ (2µg/ml) juga didapati meningkatkan pengawalaturan gen Apo A-1 (sebanyak

3, 4 dan 2 kali ganda masing-masing) dan merencatkan pengawalaturan gen Apo B100 (sebanyak 60, 70 dan 49% masing-masing). TQRF pada dos 0.5, 1 dan 1.5 g/kg dan TQ pada 20, 50 dan 100 mg/kg berat badan dalam bentuk emulsi telah diberikan secara oral kepada tikus yang telah diberi makanan yang mengandungi 1% kolesterol tambahan selama 8 minggu. Rawatan TQRF dan TQ pada kepekatan yang berbeza-beza didapati menurunkan tahap kolesterol jumlah plasma (TC) dan kolesterol lipoprotein berketumpatan rendah (LDLC) dalam tikus secara signifikan jika dibanding dengan tahap kolesterol tikus kawalan (PC). Kepekatan alanina aminotransferase (ALT), gama glutamiltranspeptidase (GGT), uria dan kreatinin dalam plasma tikus telah diukur dalam ujikaji ini bagi mengenalpasti ketoksikan TQRF dan TQ terhadap hati dan buah pinggang tikus. Keputusan menunjukkan bahawa tahap ALT, GGT dan uria dalam plasma tikus yang dirawati oleh TQRF dan TQ adalah lebih rendah daripada plasma tikus dari kumpulan PC. Aktiviti antioksida plasma tikus telah dinilai melalui ujian pemerangkapan radikal hiroksil ($OH^{\cdot}$) yang diukur dengan spektrometer resonan pemusingan elektron (ESR). Keputusan ESR menunjukkan bahawa rawatan TQRF dan TQ pada kepekatan yang berbeza-beza didapati meningkatkan aktiviti pemerangkapan radikal hidroksil dalam plasma tikus secara signifikan jika dibanding dengan tikus kawalan. Kesan rawatan TQRF dan TQ terhadap pengawalaturan gen-gen yang terlibat dalam metabolisma kolesterol (LDLR, HMG-COAR, gen Apo A-1, Apo B100 dan Apo E) turut dikaji dalam model *in vivo*. Keputusan menunjukkan bahawa rawatan TQRF pada kadar dos 0.5, 1 dan 1.5 g/kg dan TQ pada kadar 20, 50 dan 100 mg/kg berat badan menunjukkan peningkatan pengawalaturan dalam gen LDLR dan gen Apo E tetapi menunjukkan kesan perencatan terhadap pengawalaturan gen HMG – CoAR dan gen Apo B100 dalam model tikus. Kesimpulannya, kajian ini

mencadangkan bahawa TQRF dan TQ menunjukkan kesan hipokolesterolemik melalui proses pengawalaturan gen – gen penting yang terlibat dalam metabolisma kolesterol. Hasil dari ujikaji secara *in vitro* dan *in vivo* mengesyorkan bahawa TQRF dan TQ mempunyai potensi yang tinggi sebagai agen penurunan paras kolesterol dalam badan dan sebagai antipengoksida.

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I certify that an Examination Committee met on 3rd August/ 2009 to conduct the final examination of **Ghanya Naji Al-Naqeeb** on her of Doctor of Philosophy thesis entitled "**Extraction, Regulation of Genes Involved in Cholesterol Metabolism, Hypocholesterolemic Effect and Antioxidative Activity of Thymoquinone Rich Fraction and Thymoquinone from *Nigella Sativa*"** in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

Zaitun Yassin, Ph.D.
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Asmah Rahmat, Ph.D.
Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

Rokiah Mohd Yusof, Ph.D.
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

Musalmah Mazlan, Ph.D.
Professor
Faculty of Medicine
Universiti Kebangsaan Malaysia
External Examiner

PROF. DR. BUJANG KIM HUAT,

Ph.D.

Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

This thesis submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee are as followed:

Maznah Ismail, PhD
Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Rozita Rosli, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

Zeenathul Allaudin PhD
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

HASANAH MOHD. GHAZALI, PhD
Professor and Dean,
School of Graduate Studies
Universiti Putra Malaysia

Date: 16 October 2009

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

GHANYA NAJI AL-NAQEEb

Date:

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25OH	25 Hydroxycholesterol
ALT	Alanine aminotransferase
ANOVA	One way ANOVA
Apo A-1	Apolipoprotein A-1
Apo B100	Apolipoprotein B100
Apo E	Apolipoprotein E
bp	Base pair
cDNA	Complementary deoxyribonucleic acid
CHD	Coronary heart disease
C_T	Threshold cycle
CVD	Cardiovascular diseases
DEPC	Diethylpyrocabonate treated distilled water
DMEM	Dulbecco's minimum essential medium
DMSO	Dimethyl sulfoide
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
ESR	Electron spin resonance
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GGT	Gamma glutamyltranspeptidase
HDLC	High density lipoprotein cholesterol
HLPDS	Human lipoprotein deficient serum
HMG-COAR	3-Hydroxy-3-methylglutaryl COA reductase
HPLC	High performance liquid chromatography
h	Hour
ILDL	Intermediate density lipoproteins
LDLC	Low density lipoprotein cholesterol

LDLR	Low-density lipoprotein receptor
min	Minute
MMLV	Moloney Murine Leukemia Virus
mRNA	messenger RNA
MTT	3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide
NC	Negative control, reference group
OH·	Hydroxyl radical
PBS	Phosphate buffered saline
PC	Positive Cholesterol Control Group
PCR	Polymerase chain reaction
PI	Propidium iodide
Real time PCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
RSA	Radical scavenging activity
sec	Seconds
SFE	Supercritical fluid extraction
SREBP	Sterol regulatory element binding protein
TBE	Tris/borate/EDTA Electrophoresis Buffer
TC	Total cholesterol
TG	Triglycerides
TQ	Thymoquinone
TQRF	Thymoquinone rich fraction
VLDL	Very low density lipoproteins
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

Cardiovascular diseases (CVD) are considered to be the frequent cause of most deaths in the world, and the current trend is expected to persist till the year 2020 with over 25 million deaths annually (Murray and Lopez, 1997). An estimated 17.5 million people died from cardiovascular disease in 2005, representing 30 % of all global deaths. Of these deaths, 7.6 million were due to coronary heart disease (CHD) (WHO, 2005). In the United States, 300,000 individuals each year suffer sudden CVD (Rosamond *et al*., 2007) 80% of which are of atherosclerotic origin (Wang 2005). CVD was accounted for between 23% to 26% of deaths in Malaysian government hospitals from 1994 to 2001 (Zambahari, 2004).

Generally, the risk of CVD is correlated with elevated plasma levels of total cholesterol (TC) and low density lipoprotein cholesterol (LDLC) as well as low level of high density lipoprotein cholesterol (HDLC) (Hironori *et al*., 2005). Apolipoproteins are also used in risk assessment for cardiovascular disease. The plasma levels of apolipoprotein B 100 (Apo B100), apolipoprotein A-I (Apo A-I) and apolipoprotein E (Apo E) have been reported to be even more discriminatory in determining the risk of developing CHD than the cholesterol concentration (Kaptein *et al*., 1992).

Despite the fact that cholesterol is essential in the body, its level should be maintained within normal range to preserve cell functions. The regulation of

cholesterol synthesis and uptake from plasma in humans is dependent on coordinated changes in the level of mRNAs of key genes that are known to regulate cholesterol synthesis and cholesterol uptake (Horton *et al*., 2002). Genes including low-density lipoprotein receptor (LDLR), 3-hydroxy-3-methylglutaryl COA reductase (HMG-COAR) and apolipoproteins such as Apo B100, Apo E and Apo A-1 are related with CVD and atherosclerosis.

It has been established that increased LDLC is a risk factor for atherosclerosis and the underlying cause of CHD and stroke (Yilin *et al*., 2007). Low-density lipoprotein receptor (LDLR) is a key regulator of human plasma LDLC homeostasis (Brown and Goldstein, 1986). LDLR expressed on the surface of hepatocytes captures LDLC and internalizes it into the cells, leading to a decrease in circulation LDLC. Thus the expression level of LDLR directly influences the level of plasma LDLC (Brown and Goldstein, 1997). Up regulation of liver LDLR gene is effective in treating hypercholesterolemia (Kong *et al*., 2004) and is thus of primary importance in controlling the plasma cholesterol and further suggests a reduced risk of cardiovascular disease.

Two-third of the body's cholesterol is synthesized in the liver with 3-hydroxy-3-methylglutaryl coenzyme A (HMG-COA) reductase as the rate-limiting enzyme of the mevalonate pathway for cholesterol biosynthesis (Goldstein and Brown, 1990). Earlier studies showed that dietary cholesterol synthesis inhibits cholesterol synthesis, suggesting that cholesterol synthesis is a regulated process. Decrease cholesterol synthesis in response to cholesterol feeding seems to be the major compensatory mechanism to prevent an increase in whole body cholesterol levels

(Peter *et al*., 1996), whereas the increase of facial bile acid and neutral steroid excretion play secondary role in hypocholesterolemic and normal individuals (Cai and Carr, 1999). Up-regulation of LDLR and down-regulation of HMG-COAR are key mechanisms to control elevated plasma LDLC (Marc *et al*., 2004). The regulatory mechanisms that control expression of apolipoproteins genes are important since they represent the main structural components of the LDL and HDL particles, and their improper concentrations lead to cardiovascular disorders (Goldstein and Brown, 1997).

The liver is the central organ in the cholesterol metabolism and plays an important role in whole body cholesterol homeostasis because it is an essential organ in the removal of excess cholesterol from the blood circulation for excretion into the bile (Spady *et al*., 1999). The liver regulates its intra hepatic cholesterol homeostasis, by maintaining an appropriate balance between the regulatory free cholesterol and the more inert cholesterol ester pool (Menno *et al*., 2005). The human hepatoma cell line HepG2 has been used as a suitable model for studying the cholesterol regulation and lipoprotein synthesis and metabolism (Kaptein *et al*., 1992).

Among the most common pharmacological drugs currently used as lipid lowering agents in the clinical practices are statins. They act through inhibition of cholesterol synthesis and subsequent increase in hepatic LDLR expression (Kreisberg and Oberman, 2003). Statins effectively lower the plasma concentration of LDLC and reduce mortality and morbidity from CHD (Larosa *et al*., 1999). However, some patients are unable to tolerate statin treatments due to musculoskeletal symptoms and other side effects (Ballantyne *et al*., 2003; Rader, 2001). High rate of side effects