



**UNIVERSITI PUTRA MALAYSIA**

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

**GHANYA NAJI AL-NAQEEB**

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**By**

**GHANYA NAJI AL-NAQEEB**

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia,  
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By

**GHANYA NAJI AL-NAQEEB**

**August 2009**

**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, antioxidative 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  $\alpha$ -tocopherol content ( $290 \pm$

1.5 mg/100g). HepG2 cells viability was determined using MTT assay and flow cytometry. TQRF and TQ were shown to inhibit HepG2 cells growth with  $IC_{50}$  of 100  $\mu$ g/ml for TQRF and 3.5  $\mu$ g/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  $\mu$ g/ml and TQ at 2  $\mu$ g/ml. The regulatory effects of TQRF at 50 and 80  $\mu$ g/ml and TQ at 2  $\mu$ g/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  $\mu$ g/ml and TQ at 2  $\mu$ g/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  $\mu$ g/ml and TQ at 2  $\mu$ g/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<sup>•</sup>) 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<sup>•</sup> 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.



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**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 metabolisme 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 dioptimumkan 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  $\alpha$  tokoferol ( $290 \pm 1.5$  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  $\mu$ g/ml dan 3.5  $\mu$ g/ml selepas pengeraman selama 72 jam. Analisis dari sitometri pengaliran menunjukkan bahawa pertumbuhan sel HepG2 adalah melebihi 80% apabila sel tersebut dirawat dengan TQRF pada 50  $\mu$ g/ml dan TQ pada 2  $\mu$ g/ml. Kesan rawatan TQRF (pada 50 dan 80  $\mu$ g/ml) dan TQ (pada 2  $\mu$ g/ml) terhadap pengawalan gen yang terlibat dalam metabolisme 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 dirawat dengan TQRF pada 50 dan 80  $\mu$ g/ml dan TQ pada 2  $\mu$ g/ml masing-masing menunjukkan peningkatan pengawalan gen LDLR dengan peningkatan tahap mRNA nya sebanyak 3, 7 dan 2 kali ganda berbanding dengan sel yang tidak dirawat. Sebaliknya, pengawalan gen HMG-CoAR masing-masing direncatkan sebanyak 37, 71 dan 12%, berbanding dengan sel yang tidak dirawat. Selain daripada itu, rawatan TQRF (50 dan 80  $\mu$ g/ml) dan TQ (2 $\mu$ g/ml) juga didapati meningkatkan pengawalan gen Apo A-1 (sebanyak

3, 4 dan 2 kali ganda masing-masing) dan merencatkan pengawalan 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 dirawat oleh TQRF dan TQ adalah lebih rendah daripada plasma tikus dari kumpulan PC. Aktiviti antioksidan plasma tikus telah dinilai melalui ujian pemerangkapan radikal hidroksil ( $\text{OH}\cdot$ ) 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 pengawalan gen-gen yang terlibat dalam metabolisme 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 pengawalan dalam gen LDLR dan gen Apo E tetapi menunjukkan kesan perencatan terhadap pengawalan 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 metabolisme 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 3<sup>rd</sup> 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

**Ph.D.**

---

**PROF. DR. BUJANG KIM HUAT,**

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:

## TABLE OF CONTENTS

	<b>Page</b>
<b>ABSTRACT</b>	<b>ii</b>
<b>ABSTRAK</b>	<b>v</b>
<b>ACKNOWLEDGEMENT</b>	<b>ix</b>
<b>APPROVAL</b>	<b>xii</b>
<b>DECLARATION</b>	<b>xii</b>
<b>LIST OF TABLES</b>	<b>xix</b>
<b>LIST OF FIGURES</b>	<b>xxi</b>
<b>LIST OF ABBREVIATIONS</b>	<b>xxvi</b>
 <b>CHAPTERES</b>	
<b>1 INTRODUCTION</b>	<b>1</b>
<b>2 LITERATURE REVIEW</b>	<b>9</b>
2.1 <i>Nigella sativa</i>	9
2.1.2 Traditional and Therapeutic Uses	11
2.1.2 Viable Nutrients in <i>Nigella sativa</i> Seeds	11
2.1.3 <i>Nigella Sativa</i> Oil Extraction	11
2.1.4 Supercritical Fluid Extraction (SFE)	12
2.1.5 Thymoquinone Isolation and Quantification	12
2.1.6 Hypocholesterolemic Effects of <i>Nigella Sativa</i>	14
2.1.7 Antioxidant properties of <i>Nigella sativa</i>	15
2.2 Risk Factors of Cardiovascular Diseases	16
2.3 Cholesterol and Cardiovascular Diseases	16
2.4 Cholesterol Transportation	17
2.5 Cholesterol Homeostasis and Regulation	18
2.6 Regulation of HMG-COAR	22
2.7 Regulation of LDLR	24
2.8. Regulation of Apolipoproteins B-100	24
2.9. Regulation of Apolipoprotein A-1	25
2.10 Apolipoprotein E	26
2.11 Current Treatment of Hypercholesterolemia	27
2.12 Quantitative Real Time PCR	30
2.12.1 TaqMan Probe Based Detection	21
 <b>3 PREPARATION OF THYMOQUINONE RICH FRACTION FROM NIGELLA SATIVA SEEDS USING SUPERCRITICAL FLUID EXTRACTION</b>	 <b>34</b>
Introduction	34
Materials and Methods	36
<i>Nigella sativa</i> Seed	36
Chemicals	36
Supercritical Fluid Extraction of TQRF	36
Determination of TQ Content	38
Properties of TQRF	40
Statistical Analysis	40
Results	41
Optimization of SFE Parameters	41



Quantification of TQ	42	
Discussion		51
<b>4 REGULATION OF GENES INVOLVED IN CHOLESTEROL METABOLISM IN HEPG2 CELLS BY THYMOQUINONE RICH FRACTION AND THYMOQUINONE</b>		<b>55</b>
Introduction		55
Materials and Methods		56
Materials	56	
Chemicals	56	
Reagents and Chemical Preparation	57	
Culture of HepG2 Cells	59	
Trypan Blue Exclusion Assay		59
Measurement of Cell Viability	60	
Flow Cytometric Analysis	61	
Gene Expression Study	61	
2.2.9 Statistical Analysis		69
Results		70
Cell Viability Assay	70	
Flow Cytometry Analysis	71	
Gene Expression Study	74	
<i>In Vitro</i> Gene Expression of LDLR	86	
<i>In Vitro</i> Gene Expression of Apo A-1 and B100	98	
Discussion		106
<b>5 HYPOCHOLESTEROLEMIC AND ANTIOXIDATIVE EFFECTS OF THYMOQUINONE RICH FRACTION AND THYMOQUINONE <i>IN VIVO</i></b>		<b>117</b>
Introduction		117
Materials and Methods		118
Materials	118	
Chemicals	118	
Animals	119	
Diet Preparation	119	
Preparation of TQRF and TQ Emulsion	120	
Grouping of Animals	121	
Food Intake, Body Weight and Blood Sampling	121	
Tissues Collections	122	
Lipid Profile and Toxicity Analysis	122	
Hydroxyl Radical Scavenging Activity	128	
Gene Expression Study	129	
Statistical Analysis	131	
Results		133
Food Intake	133	
Body Weight	133	
Liver, Kidney, Hearts and Adipose Weights	135	
Lipid Profile Analysis	137	
Toxicity Parameters	144	
Hydroxyl Radical (OH $\cdot$ ) Scavenging Activity	115	
Gene Expression Study	156	

	Discussion	178
<b>6</b>	<b>GENERAL DISCUSSION, CONCLUSION AND FURTHER WORK</b>	189
	6.1 General Discussion	189
	6.2 Conclusion	193
	6.3 Further Work	194
	<b>REFERENCES</b>	196
	<b>APPENDICES</b>	220
	<b>BIODATA OF THE STUDENT</b>	234





## LIST OF TABLES

Table		Page
3.1	The effect of different pressures on <i>Nigella sativa</i> oil yield	41
3.2	The effect of different temperatures on <i>Nigella sativa</i> oil yield	42
3.3	TQ content of different fractions of <i>Nigella sativa</i> oil	46
3.4	Fatty acid composition of TQRF	47
4.1	TaqMan primers and probes specific used <i>in vitro</i> study	68
4.2	Average correlation coefficients and amplification efficiencies of real time PCR.	85
4.3	The effect of HLPDS in the presence or absence of 25OH on LDLR gene in control cells	92
4.4	The effect of HLPDS in the presence or absence of 25OH on HMG-COAR gene in control cells	93
4.5	Regulation of LDLR Gene by TQRF and TQ in the absence of 25OH	94
4.6	Regulation of LDLR Gene by TQRF and TQ in HepG2 cells incubated with 10% HLPDS + 25OH	95
4.7	Regulation of HMG-COAR by TQRF and TQ in HepG2 cells incubated with 10% HLPDS	96
4.8	Regulation of HMG-COAR by TQRF and TQ in HepG2 cells incubated with 10% HLPDS + 25OH	97
4.9	The effect of HLPDS and 25OH on Apo A-1 gene	100
4.10	The effect of HLPDS and 25OH on Apo B100	101
4.11	Regulation of Apo A-1 by TQRF and TQ in the absence of 25OH	102
4.12	Regulation of Apo A-1 by TQRF and TQ with 25OH presence of 25OH	103
4.13	Regulation of Apo B100 by TQRF and TQ in the absence of 25OH	104
4.14	Regulation of Apo B100 by TQRF and TQ in the presence of 25OH	105

5.1	TaqMan Primers and probes specific used <i>in vivo</i> study	132
5.2	Average of food intake by rats at week 8 of treatment	133
5.3	Changes of body weights of rats	135
5.4	Organs weight of experimental rats	163
5.5	Regulation of LDLR mRNA level by TQRF and TQ	163
5.6	Regulation of HMG-COAR mRNA level by TQRF and TQ	167
5.7	Regulation of Apo A-1 gene by TQRF and TQ treatments in vivo	170
5.8	Regulation of Apo E gene by TQRF and TQ	173
5.9	Regulation Effect of TQRF and TQ on expression of Apo B100	176
5.10	Related dose effect of TQRF in comparison with equivalent amount of TQ	188



## LIST OF FIGURES

Figure		Page
2.1(a)	<i>Nigella Sativa</i> Seeds	10
2.(b)	<i>Nigella Sativa</i> Oil	10
2.2	Structure Formula of Thymoquinone	13
2.3	Cellular Cholesterol Homeostasis	18
2.4	The Cholesterol Synthesis	19
2.5	Receptor Mediated Endocytosis Of LDL	20
3.1	HPLC Chromatogram of The TQ Standard	43
3.2	HPLC Chromatogram of The TQRF	44
3.3	Standard Curve of TQ.	45
3.4	HPLC Chromatogram of $\alpha$ -Tocopherol Standard	49
3.5	HPLC Chromatogram of $\alpha$ -Tocopherol Extracted From TQRF	50
4.1 (A)	Cytotoxic Effect of TQRF Against Hepg2 Cells Growth.	70
4.1 (B)	Cytotoxic Effect of TQ Against Hepg2 Cells	71
4.2 (A)	HepG2 Cells At 90% Confluence Before Treatment	71
4.2(B)	HepG2 Cells Treated with 100 $\mu$ g/ml TQRF For 24 H	72
4.2(C)	HepG2 Cells Treated with 4 $\mu$ g/ml TQ For 24 H.	72
4.3 (A)	Flow Cytometric Plots of Untreated HepG2 Cells	73
4.3 (B)	Flow Cytometric Plots of Untreated HepG2 Cells:	74
4.4	Percentage of Cell Population of Flow Cytometric Results For Untreated HepG2 Cells without or with Annexin V And PI. Staining	74
4.5	Flow Cytometric Plots of Annexin V And PI Staining of Treated HepG2 Cells	75
4.6	Percentage of Cell Population of Flow Cytometric Results of Annexin V And PI Staining of HepG2 Cells Treated with TQRF For 24 h.	76
4.7	RNA Extracted from HepG2 Cells	77



4.8	Gel Electrophoresis for Real Time PCR Product of LDLR Gene.	76
4.9	Gel Electrophoresis For Real Time PCR Product of HMG-COAR	76
4.10	Gel Electrophoresis for Real Time PCR Product of Beta Actin	77
4.11	Gel Electrophoresis for Real Time PCR Apo A-1 And Apo B100	77
4.12	Standard Curve of LDLR Gene.	79
4.13	Standard Curves of Beta Actin Gene	80
4.14	Standard Curves of HMG-COAR Gene.	81
4.15	Standard Curves of Apo A-1 Gene.	82
4.16	Standard Curves of Apo B100 Gene.	83
4.17	Real-Time PCR Linearity of Beta Actin and Target Genes: LDLR, HMG-COAR, Apo A-1 Apo B100.	84
4.18	The Absolute Values of The Slope of LDLR	85
4.19	The Absolute Values of The Slope of HMGCOAR	85
4.20	$\Delta C_T$ For LDLR Gene = $\Delta C_T$ For Beta Actin Gene	86
4.21	Raw Data of Amplification Curve For LDLR And Bet-Actin	87
4.22	Amplification Curve for LDLR And Bet Actin	88
4.23	Amplification Curve for HMG-COAR	90
4.24	Induction Effect of HLPDS and Suppression Effect of 25OH on mRNA Level of LDLR and HMG-COAR in HepG2 Cells	91
4.25	Effect of TQRF and TQ Treatment on LDLR mRNA Levels in HepG2 Cells.	93
4.26	Regulation Effect of TQRF And TQ on HMG-COAR mRNA Levels in HepG2 Cells in The Present or Absence of 25OH	95
4.27	Quantitation Curve for Apo A-1 and Bet Actin Genes	96
4.28 (A)	Amplification Curve For Apo B100 And Bet Actin. In Figure A, Cells Were Incubated With HLPDS And In Figure B Cells Were Incubated With HLPDS +25OH	97
4.28 (B)	Amplification Curve for Apo B100 And Bet Actin.	99
4.29	Effect Of HLPDS 25OH On mRNA Level of Apo A-1 and Apo B100 in HepG2 Cells	101

## LIST OF ABBREVIATIONS

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 <sub>T</sub>	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
LDL	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 fecal 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