MORPHOLOGICAL CHANGES IN LIVER CELLS OF HYPERCHOLESTEROLAEMIC-INDUCED RATS AND HEPG2 CELL LINES ASSOCIATED WITH EDIBLE BIRD'S NEST SUPPLEMENTATION

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia in the Fulfilment of Requirements for the degree of Doctor of Philosophy

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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February 2020

Chairman : Assoc. Prof. Intan Shameha binti Abdul Razak, DVM, PhD
Faculty : Veterinary Medicine

Edible bird's nest (EBN) is an animal-based natural product that has high interest in Chinese committee. Since in the Tang Dynasty (618-907 CE), it had been consumed frequently by the royal families for well-being purposes. This polymerized swiftlet's salivary secretion is mainly composed of protein that presence in the form of glycoprotein. It has been anecdotally claimed to have broad medicinal benefits, including metabolic stimulant. Nonetheless, the effect of EBN supplementation on the cholesterol metabolism with scientific evidence is poorly elucidated. Therefore, we hypothesised the EBN supplementation is able to improve cholesterol metabolism in HepG2 cell lines and hypercholesterolaemic-induced rats. This study focuses on evaluating the effect of EBN supplementation on the cholesterol metabolism via assessing the relevant genes expression, quantifying hepatic cholesterol concentration, measuring blood lipid profiling, localizing important structure and receptor in cholesterol metabolism, and assessing hepatic and extra-hepatic microscopic changes in the HepG2 and hypercholesterolaemic-induced rats. Cellular toxicity assessment of the edible bird's nest extract (EBNE) at different concentrations (0.2, 0.5, 0.8, 1.0 and 1.5 mg/mL) was done and revealed the cell viability remains as high as 71% even at the highest concentration (1.5 mg/mL) after 24 hours incubation. Thus, in the subsequent assay the Hep-G2 was supplemented with EBNE at three different concentrations (0.5, 1.0 and 1.5 mg/mL) in high lipid media [exogenous lipid (1:500) and cholesterol (1:250)] for 24 hours. Besides that, there were three control groups including baseline control (BC) that cultured in AMEM media only, negative control (NC) in high lipid media, and positive control (PC) in high lipid media with Simvastatin (4.60 µg/mL) as an anti-cholesterol drug. Quantification of gene expression for both low-density lipoprotein receptor (LDLR) and acyl Coenzyme A: cholesterol acyltransferase 2 (ACAT2) were significantly up regulated in a dose-dependent manner. However, 3-hydroxyl-3-metylglutaryl coenzyme A reductase (HMGCR) was observed significantly to down-regulate this enzyme at the highest dose of EBNE (1.5 mg/mL). Quantitatively, distribution of immunofluorescence intensity against LDLR protein and lipid droplets (LDs) was increased as the EBNE concentration increased. Total cholesterol storage was showing the amount of cholesterol stored in the LDs increased as the concentration of EBNE increased. In

the 12 weeks *in-vivo* study, a total of 30 male Sprague Dawley rats aged 10-week old were randomly assigned into five different groups (n=6); BC (normal rat diet), NC [hypercholesterolaemia induced with high-fat diet (HFD) and Triton-X 100 (150 mg/kg SQ q42d)], PC (hypercholesterolaemia with Simvastatin 10 mg/kg PO SID), EBNE [hypercholesterolaemia with EBNE (6.5 mg/kg PO SID)] and EBNS [hypercholesterolaemia with EBNS (843.2 mg/kg PO SID)]. Post-treatment blood lipid profiling was showing the EBNS group significantly reduced the triglycerides (TAG), total cholesterol (TC), low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL), compared to the NC group and but only TAG and LDL significantly reduced up to BC group. Meanwhile, the EBNE group showed significant reduction for TC and LDL only compared to NC but not reduced up to the BC group. Statistically, both EBNS and EBNE group were showing significant elevation of high-density lipoprotein (HDL) compared to the NC and BC group but not as high as in the PC group. Nonetheless, the HDL of EBNS group had two-fold significant increment compared to EBNE group. Remarkably, cardiogenic indices that predicting cardiac diseases and atherosclerosis occurrence showed a significant protective effect in the EBNS group only, which comparable with PC's indices. The hepatic cholesterol concentrations in the EBNS and EBNE were significantly reduced as compared to the NC. Gene expression revealed EBNS significantly down-regulated HMGCR and proprotein convertase subtilisin/kexin 9 (PCSK9) which stimulating and maintaining the upregulation of LDLR via cleavage of sterol regulatory element-binding protein 2 (SREBP2). Concomitantly, EBNS up-regulated also the cytochrome P450 family 7 subfamily A member 1 (CYP7a1) for bile production. On the other hand, EBNE was observed to up-regulate the expression of LDLR via cleavage of SREBP2 only. Grossly, liver of rat fed on EBNS appeared mild yellowish discolouration, meanwhile the EBNE group was observed moderate yellowish discolouration. Histological examination of the liver revealed mild hepatic steatosis in the EBNS group and mild non-alcoholic steatohepatitis (NASH) in EBNE group. This finding was consistent with the ultrastructure finding (TEM), whereby the mitochondria of EBNS were demonstrated enlarged with intact mitochondrial membrane, meanwhile the mitochondria in the EBNE group showed swollen with loss of cristae and translucent matrix indicating liver injury. The hepatic tissue sections were also immunologically-labelled with fluorescence against the LDLR and demonstrated high intensity expression of LDLR in the EBNS (5.65 ± 0.12), which statistically equivalent to the PC (5.53 ± 0.17) group. Meanwhile, EBNE (3.54 ± 0.04) group appeared lower LDLR distribution compared to EBNS group, but ameliorated than NC (1.81 ± 0.06). Quantitative SEM revealed the cranial thoracic aorta of NC and EBNE were significantly occluded the aortic lumen up to 31% and 30%, respectively; compared to the BC (0%). Meanwhile, EBNS documented insignificant aortic lumen occlusion (<1%). These outcomes were consistent with degree of immunoreactivity in the CD40 immunoperoxidase assay, in which detecting atherosclerosis biomarker. Renal tissue demonstrated absence of glomerulonephritis in the EBNS group, but prominently observed in the EBNE with thickening of the glomerular basement membrane. These findings were in line with detection of the renal inflammasome (NLRP3) in the tubules of EBNE group which covered up to 61% of the tissue section, compared to the EBNS which less than 17% immunoreactivity. Concurrently, inflammatory cells in the renal interstitial space was showing mild and moderate infiltration in respective group. As a conclusion, EBNE shows significant cholesterol improvement in the *in-vitro* study not in the *in-vivo* model. Nonetheless, in the hypercholesterolaemic-induced rats, EBNS was

profoundly exhibited a significant effect in improving cholesterol metabolism and slowing progression of non-alcoholic fatty liver disease (NAFLD), atherosclerosis and chronic kidney disease (CKD).

Abstrak tesis yang dikemukan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PERUBAHAN MORFOLOGIKAL PADA SEL HATI TIKUS TERINDUKSI-HIPERKOLESTEROLEMIA DAN TISU KULTUR HEPG2 YANG DITAMBAHAN DENGAN SARANG BURUNG WALET

Oleh

MOHD AKMAL BIN MOHD NOOR

Februari 2020

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Sarang burung walet (EBN) merupakan produk semulajadi berasaskan haiwan yang mempunyai nilai tersendiri dalam kalangan masyarakat Cina. Sejak zaman pemerintahan Dinasti Tang (618-907 SM), ia menjadi makanan kesihatan alternatif yang lazim bagi golongan bangsawan diraja. Produk ini merupakan cecair liur burung walit terpolimer yang terdiri daripada protein sebagai kandungan utama dan wujud di dalam bentuk. Secara tradisionalnya, sarang burung walit dipercayai mempunyai kebaikan perubatan seperti agen penggalak metabolisme. Namun demikian, kesan pengambilan EBN terhadap metabolisme kolesterol masih kurang dibincangkan secara saintifik. Oleh itu, kajian ini menghipotesiskan pengambilan EBN mampu meningkatkan metabolisme kolesterol di dalam pengkulturan HepG2 dan tikus terinduksi hiperkolesterolemia. Penyelidikan ini memfokuskan kesan pengambilan EBN terhadap metabolisme kolesterol melalui penilaian pengekspresian gen-gen berkaitan, pengkuantitian kepekatan kolesterol di dalam hati, analisis profil lemak darah, pengesanan struktur dan reseptor penting di dalam metabolisme kolesterol, dan pemeriksaan perubahan mikroskopik hepatik dan ekstra-hepatik melalui penggunaan tisu kultur HepG2 dan tikus terinduksi hiperkolesterolaemia. Penilaian toksisiti selular EBNE (0.2, 0.5, 0.8, 1.0 dan 1.5 mg/mL) telah menujukkan sel mampu untuk terus hidup pada kadar 71%, walaupun ditambah pada dos EBNE yang tertinggi (1.5 mg/mL) selama 24 jam. Lanjutan daripada itu, tisu kultur HepG2 telah ditambah dengan EBNE (0.5, 1.0 dan 1.5 mg/mL) di dalam media tinggi lemak [lemak eksogenus (1:500) dan kolesterol (1:250)] selama 24 jam. Selain daripada itu, terdapat tiga kumpulan kawalan yang terdiri daripada kawalan asas (BC) yang dikultur di dalam larutan AMEM sahaja, kawalan negative (NC) di dalam media tinggi lemak dan kawalan positif (PC) di dalam media tinggi lemak bersama Simvastatin (4.60 µg/mL) sebagai anti-kolesterol. Kuantifikasi pengekspresian gen bagi kedua-dua LDLR dan ACAT2 telah menujukkan kesan positif yang berkadar terus dengan peningkatan dos. Walau bagaimanapun, gen HMGCR telah menunjukkan perencatan yang signifikan hanya pada dos EBNE yang tinggi (1.5 mg/mL). Secara kuantitatif, taburan penandaan imunofluoresen terhadap LDLR protein dan titisan lemak (LDs) semakin meningkat apabila dos EBNE meningkat. Jumlah kolesterol tersimpan di dalam HepG2 turut menunjukkan peningkatan apabila dos EBNE semakin meningkat.

Di dalam 12 minggu kajian in-vivo, sejumlah 30 ekor tikus Sprague Dawley yang berusia 10 minggu telah diagihkan secara rawak kepada lima kumpulan (n=6) yang terdiri daripada BC (diet tikus biasa), NC [tikus hiperkolesterolemia diaruh melalui pemberian makanan diet tinggi lemak (HFD) dan Triton-X 100 (150 mg/kg SQ q42d)], PC (tikus hiperkolesterolemia dengan Simvastatin 10 mg/kg PO SID), EBNE [tikus hiperkolesterolemia dengan EBNE (6.5 mg/kg PO SID)] dan EBNS [tikus hiperkolesterolemia dengan EBNS (843.2 mg/kg)]. Profil lemak darah bagi kumpulan EBNS selepas eksperimen telah menunjukkan penurunan trigliserida (TAG), jumlah kolesterol (TC), lipoprotein ketumpatan rendah (LDL) dan lipoprotein ketumpatan sangat rendah (VLDL) yang signifikan berbanding kumpulan NC, tetapi hanya TAG dan LDL sahaja yang menunjukkan penurunan signifikan yang setara dengan kumpulan BC. Manakala, kumpulan EBNE hanya mencatatkan penurunan TC and LDL yang signifikan berbanding kumpulan NC, tetapi tidak setara dengan kumpulan BC. Secara statistik, kedua-dua kumpulan EBNE dan EBNS telah menunjukan peningkatan signifikan bagi lipoprotein ketumpatan tinggi (HDL) berbanding kumpulan BC dan NC, tetapi tidak setinggi yang direkodkan dalam kumpulan PC. Secara perbandingan, bacaan HDL di dalam kumpulan EBNS adalah dua kali lebih tinggi berbanding kumpulan EBNE. Berdasarkan data ini, hanya kumpulan EBNS telah menunjukkan bacaan perlindungan indeks jangkaan penyakit jantung dan aterosklerosis yang signifikan berbanding kumpulan-kumpulan lain dan setara dengan indeks yang dicatatkan dalam kumpulan PC. Selain itu, jumlah kepekatan kolesterol hepatic di dalam kumpulan EBNE dan EBNS telah merekodkan penurunan yang signifikan berbanding kumpulan NC. Ekspresi gen hati di dalam kumpulan EBNS menunjukkan perencatan dwi-gen HMGCR dan PCSK9 yang signifikan dan menyumbang kepada ransangan dan pengekalan peningkatan gen LDLR melalui pengaktifan gen SREBP2. Pada masa yang sama, kumpulan EBNS telah mencatatkan peningkatan ekspresi gen CYP7a1 yang penting di dalam penghasilan jus hempedu. Manakala, EBNE telah merekodkan peningkatan ekspresi gen LDLR melalui pengaktifan tunggal gen SREBP2 sahaja. Secara kasar, hati kumpulan EBNS menunjukkan perubahan warna yang sedikit kekuningan, berbanding hati kumpulan EBNE yang kelihatan sederhana kekuningan. Pemeriksaan histologi mendapati hati EBNS dan EBNE masing-masing adalah steatosis hepatik sederhana dan steatohepatitis bukan-alkoholik (NASH) sederhana. Dapatan ini adalah selari dengan penemuan ultra-struktur di dalam TEM, yang mana mitokondria hati kumpulan EBNS kelihatan membesar dengan struktur membran yang masih utuh, berbanding mitokondria kumpulan EBNE yang membengkak dengan kehilangan kristae dan matriks yang kosong. Apabila LDLR protein pada tisu hati ditanda dengan imunofluoresen, kumpulan EBNS (5.65 ± 0.12) menunjukkan taburan protein yang banyak berbanding kumpulan EBNE, dan ini adalah setara dengan penemuan di kumpulan PC (5.53 ± 0.17). Walau bagaimanapun, tisu hati EBNE (3.54 ± 0.04) menunjukkan taburan yang lebih banyak berbanding kumpulan NC (1.81 ± 0.06). Kuantitatif SEM pada aorta torasik kranial, menunjukkan lumen aortik kumpulan NC dan EBNE adalah tersumbat sehingga 31% dan 30% berbanding kumpulan BC (0%). Manakala, kumpulan EBNS menunjukkan peratusan lumen aortik tersumbat kurang daripada 1% dan setara dengan kumpulan PC. Kuputusan ini adalah selari dengan dapatan di dalam imunoperoksidase bio-penandaan aterosklerosis (CD40). Tisu ginjal pula menunjukkan EBNS mampu mencegah glomerulonefritis, berbanding EBNE yang menunjukkan penebalan membran dasar glomerular atau glomerulonefritis. Penemuan ini adalah selari dengan pengesanan inflamasom (NLRP3) pada tubul renal kumpulan EBNE yang meliputi 61% potongan tisu, berbanding kumpulan EBNS yang

menunjukkan imunoreaktiviti pada kurang daripada 17%. Pada masa yang sama, masing-masing telah menujukkan kehadiran sel inflamatori di dalam ruang interstitial renal pada kadar yang sedikit dan serdahana bagi kedua-dua kumpulan tersebut. Secara keseluruhannya, EBNE telah menunjukkan peningkatan metabolisme kolesterol di dalam tisu kultur HepG2 tetapi tidak pada tikus terinduksi hiperkolesterolemia. Manakala, EBNS telah menunjukkan kesan yang signifikan pada peningkatan metabolisme kolesterol dan memperlahankan pembentukan penyakit hati berlemak bukan-alkoholik (NAFLD), aterosklerosis dan penyakit ginjal kronik (CKD).

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I certify that a Thesis Examination Committee has met on 24 February 2020 to conduct the final examination of Mohd Akmal bin Mohd Noor on his thesis entitled "Morphological Changes in Liver Cells of Hypercholesterolaemic-Induced Rats and HepG2 Cell Lines Associated with Edible Bird's Nest Supplementation" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The committee recommends that the student be awarded the Doctor of Philosophy.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

6-OHDA	Neurotoxin 6-Hydroxydopamine
ACAT	Acyl Coenzyme A: Cholesterol Acyltransferase
ACE	Angiotensin I Converting Enzyme
ACP	Aggrecan Core Protein
ACS	Apoptosis-associated Speck-like Protein Containing A Caspase Activation and Recruitment Domain
AGEs	Advanced Glycation End-Products
AKI	Acute Kidney Injury
AMEM	Advanced Minimum Essential Media
APOA5	Apolipoprotein-A5
ApoB-100	Apolipoprotein B-100
APP	Amyloid Precursor Protein
APTT	Activated Partial Thromboplastin Time
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
BC	Baseline Control
bHLH-Zip	Basic Helix-Loop-Helix Leucine Zipper
CAT	Catalase
CCL2	Chemokine (C-C Motif) Ligand 2
CD40	Cluster of Differentiation 40
CD40L	Cluster of Differentiation 40 Ligand
CE	Cholesteryl Ester
CETP	Cholesteryl Ester Transport Protein
CKD	Chronic Kidney Disease
CLS	Crown-like Structure
CMS	Cardiometabolic Syndrome
CNS	Central Nervous System
Co-A	Coenzyme-A
COL	Collagen
Co-Q	Coenzyme-Q
COX-2	Cyclooxygenase-2
CRP	C-Reactive Protein
CSPCP	Cartilage-Specific Proteoglycan Core Protein
CVD	Cardiovascular Disease
DAMP	Damage-Associated Molecular Pattern
DM	Diabetes Mellitus
DN	Diabetic Nephropathy
E_2	Estradiol
EAA	Essential Amino Acid
EBN	Edible's Bird Nest
EBNE	Edible's Bird Nest Extract
EBNS	Edible's Birds Nest Soup
ECM	Extracellular Matrix
EGF-A	Epidermal Growth Factor-A
eLDs	End Lipid Droplets
ESRD	End Stage Renal Disease
ETC	Electron Transport Chain
$FADH_2$	Flavin Adenine Dinucleotide

FATP1	Fatty Acid Transport Protein 1
FC	Free Cholesterol
FFA	Free Fatty Acid
Fox03a	Forkhead Box 03a
FPE	Fermented Fine Needle Extract
FSH	Follicular Stimulating Hormone
GAG	Glycosaminoglycan
GALNT2	Polypeptide N-Acetylgalactosaminyltransferase 2
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GBM	Glomerular Basement Membrane
GCK	Glucokinase
GC-MS	Gas Chromatography-Mass Spectrometry
GEnCs	Glomerular Endothelial Cells
GI	Gastrointestinal
GIT	Gastrointestinal Tract
GN	Glomerulonephritis
Gpx	Glutathione Peroxidase
GRF	Glomerular Rate Filtration
Gsr	Glutathione-Disulphide Reductase
HA	Hemagglutinin
HCD	High-Cholesterol Diet
HDL	High-Density Lipoprotein
HFD	High-Fat Diet
HMGCR	3-Hydroxy-3-Methylglutaryl-CoA Reductase
HNF-1α	Hepatic Nuclear Factor 1 Alpha
HOMA-IR	Homeostatic Model Assessment of Insulin Resistance
HPLC	High-Performance Liquid Chromatography
HRT	Hormone Replacement Therapy
HSPs	Heat-Shock Proteins
IAV	Influenza A Virus
IFN-γ	Interferon-Gamma
IHC	Intrahepatocellular Concentration
IHC	Immunohistochemistry
IL	Interleukin
IL-18Rβ	Interleukin-18 Receptor Beta
IL-1R	Interleukin 1 Receptor
IL-1β	Interleukin 1 Beta
iLDs	Initial Lipid Droplets
iNOS	Inducible Nitric Oxide Synthase
INSR	Insulin Receptor Precursor
IRS2	Insulin Receptor Substrate 2
JAK2	Janus-Activated Kinase 2
KCNJ11	Potassium Voltage-Gated Channel Subfamily J Member 11
LAL	Lysosomal Acid Lipase
LC3-II	Autophagosome Membrane Protein
LC-MS/MS	Liquid Chromatography-Mass Spectrometry/Mass Spectrometry
LDL	Low-Density Lipoprotein
LDLR	Low-Density Lipoprotein Receptor
LDs	Lipid Droplets
LF	Lactoferrin

LH	Luteinizing Hormone
LIPC	Lipase C
LOX-1	Oxidised Low-Density Lipoprotein Receptor 1
LPL	Lipoprotein Lipase
MCH-I	Major Histocompatibility Complex Class I
MCH-II	Major Histocompatibility Complex Class II
MCP-1	Monocyte Chemoattractant Protein 1
MDA	Malondialdehyde
MetS	Metabolic Syndrome
MGN	Membranous Glomerulonephritis
MLXIPL	MLX Interacting Protein Like
MMPs	Matrix Metalloproteinases
MPGN	Membranoproliferative Glomerulonephritis
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NA	Neuraminidase
NADH	Nicotinamide Adenine Dinucleotide
NAFLD	Non-Alcoholic Fatty Liver Disease
NASH	Non-Alcoholic Steatohepatitis
NC	Negative Control
nCEH	Neutral Cholesteryl Ester Hydrolase
NF-κβ	Nuclear Factor Kappa Beta
NLRP3	NOD-like receptor family, pyrin containing domain 3
NO	Nitric Oxide
NOD	Nucleotide-binding Oligomerization Domain
OA	Osteoarthritis
OVF	Ovotransferrin
Ox-LDL	Oxidised Low-Density Lipoprotein
PC	Positive Control
PCSK9	Proprotein Convertase Subtilisin/Kexin Type 9
PD	Parkinson's Disease
PE	Pine Needle Extract
PI3K	Phosphoinositide-3-Kinase
PK	Pyruvate Kinase
PON3	Serum Paraoxonase/Lactonase 3
PRR	Pattern Recognition Receptor
PSEN	Presenilin
PT	Prothrombin Time
ROS	Reactive Oxygen Species
S1P	Site-1 Protease
S2P	Site-2 Protease
SCAP	SREBP Cleavage-Activating Protein
SEM	Scanning Electron Microscopy
SOD	Superoxide Dismutase
SRA	Scavenger Receptor A
SRE	Sterol Regulatory Element
SREBP	Sterol Regulatory Element-Binding Protein
TAG	Triglycerides
TCM	Traditional Chinese Medicine
TECs	Tubular Epithelium Cells
TEM	Transmission Electron Microscopy

TGF-β1	Transforming Growth Factor Beta 1
TLR	Toll-liked Receptor
TNF-α	Tumour Necrosis Factor Alpha
TRAIL	Tumour Necrosis Factor-related Apoptosis Inducing Legend
TRX	Triton X-100
VLDL	Very Low-Density Lipoprotein
VRF	Vanillin Rich Fraction
vRNP	Viral Ribonucleoprotein Complex
VSMCs	Vascular Smooth Muscle Cells

CHAPTER 1

INTRODUCTION

The edible bird's nest (EBN) is an animal-derived natural product that serves as the Chinese delicacy for thousands of years. The history of this natural product was documented back in the Tang Dynasty (618-907 CE) and frequently consumed by the Chinese royal family due to their extensive medicinal properties and well-being effects (Marcone, 2005). In worldwide, there are two type of EBN that are available in the market including house-EBN and cave-EBN (Looi and Rahman, 2016). In general, EBN is mainly composed by conjugated glycoprotein such as sialic acid, glucosamine and galactosamine (Ma and Liu, 2012b). Most of the protein is highly solubilized amino acids. Thus, most of the extraction methods in recent studies were adopting water-based extraction, which is a traditional way in which the Chinese community prepared the EBN soup (Marcone, 2005; Utomo *et al.*, 2014; Chua *et al.*, 2014a and Zulkefli *et al.*, 2017). Besides that, it also contained minerals, vitamins and some hormones (Yu-Qin et al., 2000; Ma and Liu, 2012a). This product is derived from the secretion of swiftlet's sublingual salivary glands that is produced all year round, but predominantly between Septembers to December during the breeding season. Both sexes are involved in the nest building but mainly built by the male swiftlets (Looi and Omar, 2016). Microscopically, the EBN composed of polymerized salivary secretion with varies degree of the feather and plumage composition that will support the nestling and the mother up to 40 days (Langham, 1980; Ma and Liu, 2012b). In general, swiftlets (*Aerodramus fuciphagus*) is an aerial insectivore that mainly inhabits in limestone cave situated more than 1300 meters above the sea level to avoid interspecies competition (Lim *et al.*, 2002). This amazing bird is anatomically different from other common swift, as they have shorter metatarsal bones and digits together with underdeveloped (smaller and thinner) caudoproximal of pelvic muscles bundle (biceps femoris, semitendinosus, semimembranosus and gastrocnemius), resulting inability of the swiftlets to perch, walk and stand (Zuki *et al.*, 2012).

In recent years, extensive preclinical studies including *in-vitro* and *in-vivo* experiments were discovered several potential medicinal effects of the EBN. Based on those studies, EBN able to improve cognitive function, insulin resistance, regulate coagulation pathway, serves as therapeutic and prophylactic agents including anti-hypertensive, antioxidant, anti-inflammatory, antiviral, natural hormonal supplement and chondroprotective agent. In the ancient Traditional Medicine (TCM) books, EBN had been claimed to improve metabolism. Therefore, we were postulating the EBN might have a potential effect in regulating cholesterol metabolism as well. Cholesterol metabolism is a well-established biochemical pathway in the body and alteration of this metabolism can lead to the development of cardiovascular disease (CVD) (Charlton-Menys and Durrington, 2007). A major risk factor of CVD is the atherogenic dyslipidaemia which characterized with the elevation of triglycerides (TAG) and low-density lipoprotein (LDL), and reduction of high-density lipoprotein (HDL) in the blood (Miller *et al.*, 2011). When there is a dysregulation of lipoprotein metabolism, particularly in LDL, which commonly known as bad cholesterol, it can increase the risk of an individual to succumb with CVD (Chong *et al.*, 2011). As this LDL is readily infiltrated to the sub-endothelial space of the arteries wall due to light

molecular weight, these particles are rapidly phagocytized by arterial wall macrophages in the tunica intima, which eventually transforms into the foam cells (Zilversmit, 1979; Myasoedova *et al.*, 2017). Prolong accumulation of foam cells on the arterial wall will trigger an inflammatory reaction that will lead to the formation of a solidified substance, known as a plaque (Laderis-Lopes *et al.*, 2015). As the arterial plaque is growing, important atherosclerosis biomarker, which is CD40 will start to express at any stage of the development (Leivens *et al.*, 2009; Lusis, 2000).

Formation of the plaques will result in high blood pressure and eventually, multiple diseases can be observed; such as stroke, acute pulmonary, liver failure or renal failure (WHO, 2014). As the dyslipidaemia advanced, the hepatic manifestation of the metabolic disease will be triggered to progress with the maturation of key cholesterol metabolism genes; including 3-hydroxy-3-methyglutaryl-CoA reductase (HMGCR) and sterol regulatory element-binding protein 2 (SREBP2) (Mi *et al.*, 2012). These two genes will induce accumulation of free cholesterol that contributed to the development of non-alcoholic fatty liver diseases (NAFLD), despite accumulation of other lipid components (TAG and cholesteryl ester) (Polimeni *et al.*, 2015; Ioannou *et al.*, 2017); which can be ranged from simple hepatic steatosis to hepatocellular carcinoma (Tacer and Rozman, 2011; Polimeni *et al.*, 2015). NAFLD is characterized with an elevation of lipid load in the hepatic parenchyma which causing histological and physiological alteration that can lead to a diseased liver (Jayakumar *et al.*, 2011; Tacer and Rozman, 2011; Polimeni *et al.*, 2015; Tsutsumi *et al.*, 2017). As the liver is undergoing pathological changes, an abundance of damaged cellular components that accompanied with high blood cholesterol remain to circulate in the bloodstream. All these components will be served as damage-associated molecular pattern (DAMP) which will activate the NLRP3 inflammasome in the kidney and leading to the wide range of glomerulonephritis (GN) and tubular nephritis (Hutton *et al.*, 2016; Anders and Schaefer, 2014; Fioretta and Mauer, 2007; Chun *et al.*, 2016)

Initiated with alteration of lipid metabolism, or particularly dysregulation of cholesterol metabolism, the clinical manifestation of lesions in the several internal organs is widely described in the previous pre-clinical studies and series of case reports. Therefore, the significance of cholesterol metabolism in diseases development currently is the main highlight in the biomedical research, which aims to improve cholesterol metabolism. In line with the claim that has been anecdotally documented in the TCM books, undiscovered and novel potential anti-cholesterol properties of the EBN is incompletely described. Thus, the present study was conducted to evaluate the effect of the EBN(s) in the *in-vitro* (HepG2 cell lines) and *in-vivo* (hypercholesterolaemic-induced rat) setting, particularly elucidating the biochemical regulation in the liver, and histological alteration in the cranial thoracic aorta, liver and kidney, which can be observed upon supplementation of EBN.

1.1 Problem Statement

In line with the emerging of metabolic diseases or collectively known as metabolic syndrome (MetS); the occurrence of this problem was withdrawing interest among the medical practitioner worldwide; as it was causing among the highest mortality rate in

the human population for the past few decades (Polimeni *et al.*, 2015). Incidence of this syndrome is highly associated with prolonging consumption of high-calories diet, sedentary physical activities and metabolic gene polymorphism (Chan, Barrett and Watts, 2014). Association of MetS with metabolism impairment was epidemiologically explained, and one of the approaches to solve this problem is consumption of chemically derived medications to alter such abnormalities (Charlton-Menys and Durrington, 2007). As the flourishing of holistic approaches in this century, discovering of potential natural product was withdrawing researchers' attention, and it was eccentrically studied from the ethnic's origin, beliefs and religion (Calixto, 2000).

One of the natural products that has been extensively studied in the previous five years, is the edible bird's nest (EBN). In the early Traditional Chinese Medicine (TCM) books such as "Shen Non Ben Cao Jing" (1695), "Ben Cao Cong Xin" (1759) and "Ben Cao Qiu Zhen" (1769), they have been claimed that the EBN able to maintain general health wellness, including several medicinal properties such as antioxidant, antiviral, immune booster and metabolism stimulant (Haghani *et al.*, 2016). Nonetheless, for the past few decades, there were insufficient scientific evidence to prove such claims (Ma and Liu, 2012). In the experimental setting, EBN was supplemented in two forms, either EBN extract (EBNE) and/or EBN soup (EBNS), depended on the study design. EBNE was predominantly tested in the *in-vitro* studies, as the EBNS will be causing mortality in the cell culture. Meanwhile, in *in-vivo* studies, both forms of EBNs can be supplemented via oral-gavage for hypothetical testing purposes. According to TCM books, the EBN was traditionally claimed to stimulate and boost body metabolism. However, the terminology of 'metabolism' itself was not clearly stated, and which metabolism the EBN able to trigger upon the consumption? Thus, the definition of metabolism was literally elaborated to get a better explanation. Metabolism is a sum-up sequential chemical reactions in the living organism, that is crucial for life-sustaining; which initially processed at the cellular level and mediated by all the nutritional metabolites including carbohydrates, protein, lipid, minerals and vitamins (Matthews *et al.*, 2013). As the component of the metabolism is well-known and broadly explained above, the current study was postulating the EBN might have the specific effect on the sub-type of lipid component which cholesterol, that plays a major risk in modulating metabolic syndrome; particularly hypercholesterolaemia. Finally, the effect of EBN on cholesterol metabolism is poorly been described, and this research will be the first and novel study to describe it.

1.2 Hypothesis

Supplementation of the edible bird's nest (EBN) can up-regulate cholesterol metabolism in the HepG2 cell lines and hypercholesterolaemic-induced rats, which consequently ameliorates blood lipid profiling, hepatic lipid concentration and histological changes of liver, kidney and cranial thoracic aorta.

1.3 General Objectives

The main objective of this study is to evaluate the effect of edible bird's nest (EBN) supplementation on the cholesterol metabolism in HepG2 cell lines and hypercholesterolaemic-induced rats.

1.3.1 Specific Objectives

1. To quantify relevant gene expression on cholesterol metabolism in in the HepG2 cell lines and hypercholesterolaemic-induced rats supplemented with EBNs.
2. To localize hepatic distribution of low-density lipoprotein receptor (LDLR) and lipid droplets (LDs) in the HepG2 and hypercholesterolaemic-induced rats supplemented with EBNs.
3. To determine hepatic lipid concentration in the HepG2 cell lines and hypercholesterolaemic-induced rats supplemented with EBNs.
4. To assess blood lipid profiling in hypercholesterolaemic-induced rats supplemented with EBNs.
5. To evaluate histological and ultrastructure changes in the liver, kidney and cranial thoracic aorta of hypercholesterolaemic-induced rats supplemented with EBNs.

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