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CARDIOPROTECTIVE EFFECTS AND NUTRIGENOMIC STUDY OF EDIBLE BIRD'S NEST IN VITRO AND IN VIVO

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By

ZHANG YIDA

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

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Chairman : Maznah Ismail, PhD
Institute : Bioscience

Cardiovascular disease (CVD) is one of the major cause of morbidity and mortality globally, which is contributed by multiple risk factors including hyperlipidemia, insulin resistance, hypercoagulation, inflammation and oxidative stress. Current therapies have several limitations and are not able to tackle all metabolic perturbations related to CVD. Alternative therapies like edible birds nest (EBN) are therefore receiving closer attention. EBN has been used for thousands of years to improve wellbeing in Asia but there is lack of scientific evidence to back up its use. The present work focused on the cardioprotective effects of edible bird's nest and the nutrigenomic basis for such effect, *in vitro* and *in vivo*. *In vitro* antioxidant potentials of EBN extracts were determined by 2,2′-azinobis- (3-ethylbenzothiazoline-6- sulfonic acid) (ABTS) and oxygen radical absorbance capacity (ORAC) assays, while their effects against oxidative stress were tested in HepG2 cell line. The results showed that EBN extracts possessed potent antioxidant potentials especially after simulated gastrointestinal digestion; at 1000μg/mL, there was up to 50% radical scavenging without signs of toxicity. This was followed by *in vivo* testing of the EBN using a high fat diet induced hyperlipidemic Sprague Dawley rat model, in which high fat diet (HFD)+2.5%EBN, HFD+20%EBN, HFD+10mg/kg/day simvastatin, HFD alone and normal pellet were fed to different rat groups for 12 weeks. The results showed that the EBN groups improved HFD-induced hyperlipidemia (total cholesterol = 6.04 mmol/L (19.14%), triglyceride = 0.54 mmol/L (55.37%), and low density lipoprotein = 4.52 mmol/L (9.24%) for HFD+2.5%EBN, and 4.17 mmol/l (44.18%), 0.44 mmol/L (63.64%), 2.98 mmol/L (40.16%) for HFD+20%EBN, respectively), similar to simvastatin (4.99 mmol/L (33.20%), 0.63 mmol/L (47.93%) and 3.6 mmol/L (27.71%), respectively) in comparison with the HFD alone group (7.47 mmol/L, 1.21 mmol/L and 4.98 mmol/L, respectively). EBN also lowered the risk of HFD-induced insulin resistance unlike simvastatin which increased such risk (homeostatic model of insulin resistance was 1.63±0.71, 2.83±0.79 and 2.46±0.22 for HFD+20%EBN, HFD+simvastatin and HFD alone, respectively). EBN also improved HFD-induced inflammation, oxidative stress and coagulation as evidenced by attenuation of HFD-induced alterations of Tumor necrosis factor-alpha (TNF-α), Interleukin-6 (IL-6), C-reactive protein (CRP), ABTS, Thiobarbituric acid reactive substances (TBARS), Activated partial thromboplastin time (APTT), Prothrombin time (PT), Bleeding time (BT), Platelet count (PC),

Oxidized low density lipoprotein (OxLDL), platelet aggregation, leptin, adiponectin and Nitric oxide synthase 3 (NOS3). Furthermore, HFD-induced transcriptional changes were attenuated by EBN; HFD+20% EBN group showed upregulation of the insulin receptor substrate 2 (IRS2), phosphatidyl inositol-3-kinase (PI3K), glucokinase (GCK), gluthatione reductase (Gsr), superoxide dismutase (SOD) and gluthatione peroxidase (Gpx) genes, and downregulation of the inhibitor of nuclear factor kappa-B kinase subunit beta (IKBKB), mitogen-activated protein kinase 1 (MAPK1), Chemokine (C-C motif) ligand 2 (Ccl2), C-reactive protein (CRP), nuclear factor kappa beta1 (Nfkb1), tumor necrosis factor (TNF), von willibrand factor (vWF) and plasminogen activator inhibitor-1 (PAI-1) genes which were the possible basis for the improved insulin resistance, inflammation, oxidative stress and coagulation. EBN was also found to be predominantly protein (57%) of EBN, and sialic acid was the major protein constituent (11%) of EBN. Sialic acid was therefore tested to determine if it was a major contributor to the effects of EBN, with results showing that it contributed significantly as evidenced by similar effects it produced in comparison with the EBN treatment. The findings thus far suggests that EBN and sialic acid may be good candidates for cardioprotection through regulation of hyperlipidemia, insulin resistance, inflammation, oxidative stress and hypercoagulation which are all linked with cardiovascular disease.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

KAJIAN KESAN PERLINDUNGAN KARDIOVASKULAR DAN NUTRIGENOMIKS SARANG BURUNG WALIT *IN VITRO* DAN *IN VIVO*

Oleh

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Penyakit kardiovaskular (CVD) merupakan salah satu punca utama morbiditi dan kematian di seluruh dunia yang disumbangkan oleh pelbagai faktor risiko termasuk hiperlipidemia, rintangan insulin, pembekuan lampau, keradangan dan tekanan oksidatif. Terapi semasa masih tidak dapat menangani semua gangguan metabolik yang berkaitan dengan penyakit kardiovaskular dan ini menimbulkan batasan dalam bidang perubatan. Sehingga kini, pengambilan sarang burung walit sebagai terapi alternatif untuk menangani penyakit kardiovaskular semakin mendapat perhatian. Pemakanan sarang burung walit telah diamalkan sejak beribu-ribu tahun dahulu untuk meningkatkan tahap kesihatan di Asia, namun bukti saintifik terhadap kesan sarang burung walit kepada kesihatan didapati terhad. Oleh yang demikian, kajian ini berfokus kepada kesan perlindungan kardiovaskular dan ciri-ciri nutrigenomik sarang burung walit secara *in vitro* dan *in vivo*. Aktiviti anti-pengoksidaan ekstrak sarang burung walit ditentukan melalui ujian ABTS dan ORAC secara *in vitro*. Di samping itu, aktiviti ekstrak sarang burung walit terhadap tekanan oksidatif dikaji dengan menggunakan sel HepG2. Hasil kajian menunjukkan bahawa ekstrak sarang burung walit berpontensi tinggi sebagai bahan antioksidan terutamanya selepas simulasi pencernaan gastrousus. Pada kepekatan 1000 µg/mL, ekstrak sarang burung walit mampu menghapuskan 50% radikal bebas tanpa memberi kesan ketoksikan. Ini diikuti dengan kajian ekstrak sarang burung walit secara *in vivo* dengan menggunakan tikus 'Sprague Dawley' yang diaruh diet tinggi lemak selama 12 minggu, di mana pembahagian kumpulan tikus adalah seperti berikut: diet tinggi lemak + 2.5% ekstrak sarang burung walit, diet tinggi lemak + 20% ekstrak sarang burung walit, diet tinggi lemak + 10 mg/kg/hari simvastatin, diet tinggi lemak + kawalan, pelet normal. Hasil kajian menunjukkan bahawa ekstrak sarang burung walit dan simvastatin menambahbaik semua kumpulan tikus yang diberi diet tinggi lemak berbanding dengan kumpulan kawalan (kumpulan 2.5% ekstrak sarang burung walit: jumlah kolesterol = 6.04 mmol/L (19.14%), trigliserida = 0.54 mmol/L (55.37)%, lipoprotein ketumpatan rendah = 4.52 mmol/L (9.24%); kumpulan 20% ekstrak sarang burung: jumlah kolesterol = 4.17 mmol/L (44.18%), trigliserida = 0.44 mmol/L (63.64%), lipoprotein ketumpatan rendah = 2.98 mmol/L (40.16%); kumpulan simvastatin: jumlah kolesterol = 4.99 mmol/L (33.20%), trigliserida = 0.63 mmol/L (47.93%), lipoprotein ketumpatan rendah = 3.6 mmol/L (27.71%); kumpulan kawalan: jumlah kolesterol = 7.47 mmol/L, trigliserida = 1.21 mmol/L, lipoprotein ketumpatan rendah = 4.98 mmol/L). Sarang burung walit juga mengurangkan risiko kerintangan insulin disebabkan oleh diet tinggi lemak dan simvastatin mempunyai

kesan yang sebaliknya (kerintangan insulin untuk kumpulan diet tinggi lemak + 20% ekstrak sarang burung walit, kumpulan diet tinggi lemak + simvastatin dan kumpulan diet tinggi lemak kawalan adalah 1.63 ± 0.71, 2.83 ± 0.79 dan 2.46 ± 0.22 masing-masing). Ekstrak sarang burung walit menambahbaik keradangan, tekanan oksidatif dan pembekuan darah yang disebabkan oleh diet tinggi lemak dan ini boleh dibuktikan dengan hasil kajian menglibatkan TNF-α, IL-6, CRP, ABTS, TBARS, APTT, PT, BT, PC, OxLDL, pengagregatan platelet, leptin, adiponektin dan NOS3. Sebagai tambahan, ekstrak sarang burung walit telah dibuktikan berjaya mengurangkan perubahan transkripsi gen yang disebabkan oleh diet tinggi lemak. Kumpulan diet tinggi lemak + 20% ekstrak sarang burung walit didapati meningkatkan regulasi gen IRS2, PI3K, GCK, Gsr, SOD, GPX1 sementara mengurangkan regulasi gen IKBKB, MAPK1, Ccl2, CRP, Nfkb1, TNF, vWF dan PAI-1. Ini mungkin merupakan asas kepada penambahbaikan rintangan insulin, keradangan, tekanan oksidatif, dan pembekuan darah. Protein merupakan unsur dominan dalam sarang burung walit (57% daripada sarang burung walit) dan asid sialik merupakan konstituen protein utama (11% daripada sarang burung walit). Dengan itu, asid sialik turut dikaji untuk memastikan sumbangannya dalam ekstrak sarang burung walit yang digunakan. Hasil kajian membuktikan bahawa rawatan asid sialik memberi kesan yang sama berbanding dengan rawatan ekstrak sarang burung walit. Penemuan daripada kajian ini mencadangkan bahawa sarang burung walit dan asid sialik adalah sumber yang bagus untuk perlindungan kardio melalui pengawalan hiperlipidemia, rintangan insulin, keradangan, tekanan oksidatif dan hiperkoagulasi yang mana berkait dengan penyakit kardiovaskular.

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I certify that a Thesis Examination Committee has met on 23 March 2016 to conduct the final examination of Zhang Yida on his thesis entitled "Cardioprotective Effects and Nutrigenomic Study of Edible Bird's Nest *In Vitro* and *In Vivo*" 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 degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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AAPH	2,2′-azobis (2-amidinopropane) dihydrochloride
ABTS	2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
Adipoq	Adiponectin
ALP	Alkaline phosphatase
ALT	Alanine transaminase
AO-PI	Acridine orange–propidium iodide
ApoB	Apolipoprotein B
APTT	Activated partial thromboplastin time
AST	Aspartate transaminase
BT	Bleeding time
CAT	Catalase
Ccl2	Pyruvate kinase
CHD	Coronary heart disease
CRP	C-reactive protein
CVD	Cardiovascular disease
CYP7A1	Cholesterol 7 alpha-hydroxylase
DCFH-DA	Dichloro-dihydro-fluorescein diacetate
EBN	Edible bird’s nest
EBNH	High dose edible bird’s nest
EBNL	Low dose edible bird’s nest
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
Gck	Glucokinase
Gpx	Glutathione peroxidase
Gsr	Glutathione reductase
H2O2	Hydrogen peroxide
HDL	High-density lipoprotein
HFD	High fat diet
HMGCR	HMG-CoA reductase
HOMA-IR	Homeostatic model assessment of insulin resistance
IDL	Intermediate-density lipoprotein

Ikbkb	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta
IKK-β	Inhibitor of nuclear factor kappa-B kinase subunit beta
IL-1	Interleukin-1
IL-6	Interleukin-6
Insr	Insulin receptor
IRS	Insulin receptor substrate
$K_2S_2O_8$	Potassium persulphate
KCNJ11	Potassium inwardly rectifying channel, subfamily J, member 11
LDH	lactate dehydrogenase
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LXR	Liver X receptor
MAPK	Mitogen-activated protein kinase
MCP1	Monocyte chemotactic protein 1
M-CSF	Monocyte-macrophage colony-stimulating factor
MDA	Malondialdehyde
mTOR	Mammalian target of rapamycin
MTT	(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide)
$NaHCO_3$	Sodium bicarbonate
Neu5Ac	N-acetylneuraminic acid
Neu5Gc	N-glycolylneuraminic acid
NF-κB	Nuclear transcription factor kappa-B
NOS3	Nitric oxide synthase 3
OGTT	Oral glucose tolerance test
ORAC	Oxygen radical absorbance capacity
OxLDL	Oxidized low density lipoprotein
PAI-1	Plasminogen activator inhibitor-1
PC	Platelet count
PCSK9	Proprotein convertase subtilisin/kexin type 9
PDGF	Platelet derived growth factor
PEP	Phosphoenolpyruvate
PG	Prostaglandins

PI3K	Phosphoinositide-3-kinase
Pk	Pyruvate kinase
Prkcz	Protein kinase C, zeta
PT	Prothrombin time
RBC	Red blood cell
ROS	Reactive oxygen species
SA	Sialic acid
SAH	High dose sialic acid
SAL	Low dose sialic acid
SIM	Simvastatin
SOD1	Superoxide dismutase 1
SOD2	Superoxide dismutase 2
SREBP	Sterol regulatory element-binding proteins
TBARS	Thiobarbituric acid reactive substances
TC	Total cholesterol
TG	Triglyceride
TGFβ	Transforming growth factor β
TNF-α	Tumor necrosis factor-alpha
VLDL	Very low-density lipoprotein
vWF	Von Willebrand factor
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

Morbidity and mortality associated with cardiovascular diseases (CVD) is severe and significant. CVDs cause nearly 30% of all death worldwide. It accounts for more than 17 million deaths globally every year. By 2030, the figure is estimated to grow to 23.6 million. Around 80% of CVDs burden can be found in the low and middle income countries [WHO, 2015]. The fact that these countries already have a huge burden of communicable diseases, the growing burden of CVDs and other non-communicable diseases may be too much to bear.

Atherosclerosis has been reported to be central to most CVD morbidity and mortality, and is promoted by hypercholesterolemia, insulin resistance, coagulation, inflammation and oxidative stress [Libby et al., 2002; Van Gaal et al., 2006; Pawlak et al., 2003; Chuang et al., 2007]. There are many therapeutic options available for managing CVDs, and most are targeted at the factors that promote CVD. Limited successes have been recorded despite wide availability and use of these drugs, and in fact the burden of CVDs is expected to grow in the near future if no urgent action is taken. Insights into the pathogenesis of CVDs and other chronic diseases have indicated that diets play more significant role than was previously anticipated [Estruch et al., 2013; O'Keefe and Cordain, 2004; Joint WHO/FAO Expert Consultation, 2003]. Based on the contribution of diet to the development of CVDs and side effects associated with currently used drugs, there are increasing efforts to also look at diets that can prevent and manage CVDs [Shikany and White Jr, 2000; Amine et al., 2002; Mccullough et al., 2000].

Studies have indicated that the dietary intervention can improve CVD through diverse mechanisms [Erdman, 2000; Stephens et al., 2010; Imam et al., 2014] including anti-inflammation, antioxidation, and amelioration of hyperlipidemia and other metabolic problems. Whole foods have been shown to mediate some of these effects [Imam et al., 2014; He et al., 1995; Lithander and Mahmud, 2015], although in some cases, individual bioactives are better at producing such effects [Ryan et al., 2007; Hu et al., 2015]. Irrespective of the form (whole foods, extracts or bioactive fractions), however, the outcomes are promising. In this regard, oats have been used to manage CVD through lowering cholesterol levels [He et al., 1995], while lupin and germinated brown rice have been shown to regulate oxidative stress, lipid metabolism and insulin sensitivity [Lithander and Mahmud, 2015; Belski et al., 2011; Imam et al., 2014]. There is increasing awareness of the synergistic effects of foods to address the burden of diseases like CVD, which have multiple underlying perturbations, which has prompted the search for foods that have multiple bioactive compounds that are able to regulate several perturbations at once.

Edible bird’s nest is used traditionally by the Chinese for its health promoting properties. Its many years of use have not recorded any major safety concerns, prompting its continuous and wide spread use. Scientific evidence supporting the use of EBN for medicinal purposes is very scarce, and information on the bioactives responsible for its functional effects is sadly lacking. Additionally, there are limited *in vitro* tests that have indicated that EBN may have antioxidant and anti-inflammatory properties [Aswir and Wan Nazaimoon, 2011; Kim et al., 2012]. Thus, based on the antioxidant and anti-inflammatory effects of EBN, it was hypothesized that it may be beneficial in managing CVD, since both effects could independently improve CVD. Hence, this study was aimed to investigate the functional effects of EBN on selected parameters of CVD. In this study, the antioxidant potentials of EBN *in vitro*, and its effects on lipid metabolism, insulin resistance, inflammation, oxidative stress and anticoagulation were determined including underlying transcriptional regulation, which have all been associated with CVD, in a continuing study using an animal model of hyperlipidemia. The regulation of these different parameters by EBN indicated that it is a good candidate for use in the management of CVD among Malaysians, where EBN is produced extensively. Additionally, Malaysia is one of the largest exporters of EBN and as such this benefit can be extended to the international community. In view of the increasing drive to use safer alternatives for the management of chronic diseases like CVD, the results from this study could have profound implications on the health of majority of individuals since EBN is consumed by majority of people in Asia, where CVD is the leading cause of death. By extension, the use of EBN by people around the world could also impact positively on global mortality due to CVD, which remains the highest cause of morbidity and mortality all over the world. Similarly, in view of the possible contributory effects of the major constituent of EBN, sialic acid, to the functional effects of EBN, it was hypothesized that sialic acid, if it is able to produce similar effects to EBN, could also be used in isolation as a nutraceutical for individuals that prefer to use supplements instead of foods. Overall, the results could impact on the health of majority of people, because the metabolic disturbances targeted in the study underlie the major causes of death globally, especially CVD.

General objective:

To study the cardioprotective effects of edible bird's nest and the nutrigenomic basis for such effects, *in vitro* and *in vivo*.

Specific objectives:

1. To determine the antioxidant activities of EBN extracts and its cytotoxicity *in vitro* using HepG2 cell line.
2. To determine the effects of EBN and sialic acid on lipid metabolism and insulin resistance in high fat diet-induced hypercholesterolemic Sprague Dawley rat model, including the underlying nutrigenomic basis for understanding mechanistic action.
3. To determine the antioxidant and anti-inflammatory effects of EBN and sialic acid in high fat diet-induced hypercholesterolemic Sprague Dawley rat model, including the underlying nutrigenomic basis for understanding mechanistic action.
4. To determine the anti-coagulation effects of EBN and sialic acid in high fat diet-induced hypercholesterolemic rat model, including the underlying nutrigenomic basis for understanding mechanistic action.

Hypotheses of the study were:

1. EBN has high antioxidant potential and is non-toxic to human cell.
2. EBN and sialic acid have lipid-regulating properties.
3. EBN and sialic acid can improve CVD risk factors such as insulin resistance, hypercoagulation, oxidative stress and inflammation.

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