



**CHOLESTEROL-LOWERING AND ANTI-ATHEROGENIC PROPERTIES OF
EDIBLE BIRD'S NEST *IN VITRO* AND *IN VIVO***

By

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

September 2022

IB 2022 10

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DEDICATION

In loving memories, Allahyarhamah Prof Dr Maznah binti Ismail. Al-Fatihah.

This dissertation is dedicated to:

My husband,
Wan Nazirul Mubin
For your love, support, and constant patience. From you, I have learned a great deal about sacrifice, discipline, and compromise.

My beloved parents,
**Mohamad Nasir & Fatimah
Wan Azmi & Azidah**
For always being there to support, encourage, and believe in me.

My children,
Wan Zara Sofea & Wan Atif Zikry
For always cheering me up and both of you are the reason for me to keep going chasing my dreams.

My family members,
**Siti Fairuz, Muhammad Faris, Nur Amirah, Aimi Liyana, Wan Azleen
Normaya, Wan Azleen Norliana, Wan Noruddin Rasol, Nur Hanisah,
Wan Nasrul Fikree, Muhd Aqil, Hana Humaira,
Wan Tasneem, Wan Idris**
“Everyone needs a house to live in, but a supportive family is what builds a home” – *Anthony Liccione*

My best friend and research partner,
Ramlah
For all the good times we had together from the beginning till the end of the research project.

My respected supervisors,
Prof Dr Md Zuki, Prof Datin Dr Rozi, Dr. Nor Asma
Thank you for the encouragement and opportunity given to me to pursue my doctoral degree. A great mentor team that keeps me motivated and strives for success.

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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Edible bird's nest (EBN) is a highly nutritious food product with proven cardioprotective effects. However, the fundamental mechanisms involved in preventing atherogenesis remain unknown, particularly those related to cholesterol metabolism and atherosclerotic plaque formation. The present study concentrates on the cholesterol-lowering and anti-atherogenic properties of EBN, using both *in vitro* and *in vivo* models. Two different shapes of EBNS were used in this study, namely half-cup (HC) and stripe-shaped (ST). Major nutrients in EBNS are protein (54-57%), carbohydrates (22-24%), crude fibre (3-20%), calcium (650-740 mg/100g), sodium (500-680 mg/100g), and magnesium (100-130 mg/100g). Both essential and non-essential amino acids were also identified in the EBNS from this study. Full stew (FS) and stew extract (SE) from HC of EBN were selected for the next analyses as it showed high extraction yield (FS: 92.29 ± 2.45%; SE: 12.50 ± 0.89%) and soluble protein concentration (FS: 375.6 ± 0.98 µg/mL; SE: 435.6 ± 2.63 µg/mL) as compared to the ST of EBN ($p < 0.05$). HPLC analysis showed that bioactive sialic acid was detected in both FS (7.91%) and SE (8.47%) of HC EBN, and was confirmed by SDS-PAGE. In this study, a novel protein marker, namely 78-kDa glucose-regulated protein was discovered in FS and SE of HC EBN by using LC-MS/MS. Digested HC EBN (FSh: FS hydrolysate; SEh: SE hydrolysate) possesses high activity as an antioxidant, anti-inflammatory, and inhibitor of HMGCR as compared to unhydrolyzed HC EBN. *In vitro* results demonstrated the potency of FSh and SEh in reducing inflammatory mediator secretion (NO: 42-60%; IL-6: 56-63%; IL-1 β : 34-60%), monocyte migration (24-33%), and macrophage-cholesterol accumulation (40.4-42.9%) with more than 80% of cell viability in RAW 264.7 macrophages and THP-1 monocytes. Further *in vivo* investigation highlighted that dietary intervention with FS or SE of HC EBN at 500 mg/kg b.w/day for 12 weeks significantly improved serum lipid profiles (*i.e.*, reduced TC: 6.8-11.5%, LDL-c: 9.1-20.6%, TG: 42.4-44.2%, and increased HDL-c: 39.5%), atherogenic indices (*i.e.*, reduced CRI-I: 21.2-22.8%, CRI-II: 22.8-35.5%, AI: 19.4-33.4%), hepatosteatosis, stabilization of atherosclerotic plaque

(*i.e.*, reduced I/M ratio: 27.3-43.6%), coagulation status (*i.e.*, increased PT: 30-34%, APTT: 16-19%, and reduced Fb: 40-60%), liver function (*i.e.*, reduced serum ALT: 28-30%, GGT: 26-29%), systemic oxidant-antioxidant balance (*i.e.*, increased serum TAC: 39-48%, SOD: 135-295%, CAT: 27-29%, and reduced serum oxLDL: 20-34%), and inflammatory response (*i.e.*, reduced serum P-selectin: 18-23%, MCP-1: 54-66%, IL-6: 40-47%, IL-1 β : 32-49%) in the hypercholesterolemic rabbits ($p < 0.05$). EBNs supplementation also lowered the levels of hepatic HMGCR (1.3-1.4 fold), hepatic TC (1.6-1.7 fold) and aortic TC (1.9-2.2 fold) ($p < 0.05$). The modulatory effects of EBN on key genes related to cholesterol metabolism in the liver and aorta tissues showed consistent transcript regulation of PPAR γ , LXR α , ABCA1, and LCAT. Gene expression analysis also suggested the potential involvement of the NOS3/NF- κ B pathway in alleviating vascular oxidative stress and inflammation in hypercholesterolemic rabbits. This study provides scientific evidence and proves that consumption of EBN lowers cholesterol levels and could prevent atherogenesis. Therefore, it has a high potential therapeutic target in the prevention of atherosclerosis.

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

CIRI-CIRI PENURUNAN KOLESTEROL DAN ANTI-ATHEROGENIK SARANG BURUNG WALET *IN VITRO* DAN *IN VIVO*

Oleh

NURUL NADIAH BINTI MOHAMAD NASIR

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Sarang burung walet (EBN) ialah produk makanan yang berkhasiat serta dilaporkan mempunyai kesan kardioprotektif. Walaubagaimanapun, mekanisme asas yang terlibat dalam mencegah aterosclerosis masih tidak diketahui, terutamanya yang berkaitan dengan metabolisme kolesterol dan pembentukan plak aterosklerotik. Kajian ini menumpukan kepada ciri-ciri penurunan kolesterol dan anti-atherogenik EBN, menggunakan model *in vitro* dan *in vivo*. Dua bentuk EBN yang berbeza digunakan dalam kajian ini iaitu berbentuk separa cawan (HC) dan berbentuk jalur (ST). Kandungan nutrien utama di dalam kedua-dua bentuk EBN adalah protein (54-57%), karbohidrat (22-24%), serat (3-20%), kalsium (650-740 mg/100g), natrium (500-680 mg/100g), dan magnesium (100-130 mg/100g). Profil asid amino mendapati EBN daripada kajian ini mengandungi kedua-dua asid amino perlu dan tidak perlu. "Full stew" (FS) dan "Stew extract" (SE) daripada EBN HC dipilih untuk analisa selanjutnya kerana ia menunjukkan hasil pengekstrakan (FS: $92.29 \pm 2.45\%$; SE: $12.50 \pm 0.89\%$) dan kandungan protein larut (FS: $375.6 \pm 0.98 \mu\text{g/mL}$; SE: $435.6 \pm 2.63 \mu\text{g/mL}$) yang tinggi berbanding dengan EBN ST ($p < 0.05$). Analisa HPLC menunjukkan bahawa bioaktif asid sialik dikesan dalam FS (7.91%) dan SE (8.47%) daripada EBN HC, serta disahkan melalui kaedah SDS-PAGE. Dalam kajian ini, protein "marker" novel iaitu protein 78-kDa kawalan-glukosa juga telah dikesan dalam FS dan SE daripada EBN HC melalui kaedah LC-MS/MS. EBN HC tercerna (FSh: FS hidrolisat; SEh: SE hidrolisat) mempunyai aktiviti antioksidan, anti-radang dan perencatan HMGR yang tinggi berbanding EBN HC yang tidak terhidrolisis. Hasil kajian *in vitro* mendapati FSh dan SEh berpotensi mengurangkan penghasilan pengantara radang (NO: 42-60%; IL-6: 56-63%; IL-1 β : 34-60%), migrasi monosit (24-33%), dan pengumpulan kolesterol di dalam makrofaj (40.4-42.9%), dengan lebih daripada 80% sel hidup mampu dikekalkan dalam sel-sel makrofaj RAW 264.7 dan monosit THP-1. Kajian *in vivo* selanjutnya memperlihatkan bahawa intervensi pemakanan dengan FS atau SE daripada EBN HC pada tahap 500 mg/kg b.b/hari selama 12 minggu secara

signifikan telah menambahbaik profil serum lipid (iaitu, menurunkan TC: 6.8-11.5%, LDL-c: 9.1-20.6%, TG: 42.4-44.2%, dan meningkatkan HDL-c: 39.5%), indeks aterogenik (iaitu mengurangkan CRI-I: 21.2-22.8%, CRI-II: 22.8-35.5%, AI: 19.4-33.4%), hepatosteatis, penstabilan plak aterosklerotik (iaitu, mengurangkan nisbah I/M: 27.3-43.6%), status pembekuan darah (iaitu, meningkatkan PT: 30-34%, APTT: 16-19%, dan menurunkan Fb: 40-60%), fungsi hati (iaitu, merendahkan serum ALT: 28-30%, GGT: 26-29%), keseimbangan oksidan-antioksidan (iaitu, meningkatkan serum TAC: 39-48%, SOD: 135-295%, CAT: 27-29%, dan menurunkan serum oxLDL: 20-34%), dan tindak balas keradangan (iaitu, mengurangkan serum P-selectin: 18-23%, MCP-1: 54-66%, IL-6: 40-47%, IL-1 β : 32-49%) dalam arnab yang mempunyai hiperkolesterolemia ($p < 0.05$). Pemberian EBN juga menurunkan kandungan HMGCR hepatic (1.3-1.4 kali ganda), serta kolesterol di dalam hati (1.6-1.7 kali ganda) dan aorta (1.9-2.2 kali ganda) arnab tersebut ($p < 0.05$). Kesan modulasi EBN ke atas gen berkaitan metabolisma kolesterol dalam tisu hati dan aorta menunjukkan aturan transkrip yang konsisten pada gen PPAR γ , LXR α , ABCA1, dan LCAT. Analisa ekspresi gen juga mencadangkan potensi pengaktifan hubungan gen NOS3/NF- κ B dalam mengurangkan tekanan oksidatif vaskular dan keradangan dalam arnab yang mempunyai hiperkolesterolemia. Kajian ini memberikan bukti saintifik bahawa pengambilan EBN dapat merendahkan paras kolesterol dan boleh mencegah aterogenesis. Justeru, ia mempunyai sasaran terapeutik yang berpotensi tinggi dalam pencegahan aterosklerosis.

ACKNOWLEDGEMENTS

Praise be to Allah, The Most Merciful, The Most Compassionate, for granting me the opportunity and strength to complete this doctoral program.

I would like to extend my profound sense of gratitude and respect to my esteemed supervisor, Prof. Dr. Md Zuki bin Abu Bakar @ Zakaria, for his sustained commitment, immense knowledge, guidance, motivation, patience, and encouragement throughout my research journey. I am also greatly indebted to my co-supervisors, Prof Datin Dr. Rozi binti Mahmud, and Dr. Nor Asma binti Ab Razak for their sincere guidance, enthusiasm, strong motivation, and intellectual input which enabled me to complete this dissertation work. I could not have imagined having a better advisory team and mentors for my PhD study.

I would like to express my sincere thanks to all staff in the Natural Medicines and Products Research Laboratory (NaturMeds) in the Institute of Bioscience, Universiti Putra Malaysia (IBS, UPM), especially Assoc. Prof. Dr. Ahmad Faizal, Dr. Norsharina, Dr. Chan Kim Wei, Mdm. Zufliha, and Ms. Norhayati for assisting me with the technical aspects in the laboratory. Special thanks to Mdm. Mastura and Mr. Ahmad Termizi for their excellent administrative support for postgraduate students.

Many thanks to all the staff in the Faculty of Veterinary Medicine, UPM especially Dr Abu Bakar Danmaigoro, Dr Mostafa, and Mr. Mohd Jamil for the knowledge sharing and assistance especially during the animal study. Not forgetting to all research scholars, Dr Ooi Der Jiun, Dr Abu Bakar, Dr Ja'afaru, Dr Ramlah, Dr Fiza, Dr Ammar, Abdul Qayyum, Mohd. Adha, Arnisah, Hafiz, Salwana, and others for their moral support and valuable suggestion during my candidature.

I owe a debt of gratitude and respect to my beloved parents, my husband, my children, and other family members, who provided me with distinct character inspiration, consistent support, motivation, and encouragement at every moment of my study. Thank you for all your unconditional love and understanding.

To my late mentor, Allahyarhamah Prof. Dr Maznah binti Ismail who is always in my prayer, thank you for teaching me the importance of education and discipline.

To those who indirectly contributed to this research, your kindness means a lot to me. My utmost appreciation to all these people mentioned above. They have made this PhD journey an interesting, stimulating, and worthwhile learning experience. Thank you very much.

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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LIST OF ABBREVIATIONS

AAPH	2,2'-azobis (2-amidinopropane) dihydrochloride
ABCA1	ATP binding cassette subfamily A member 1
ABTS	2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid
APTT	Activated partial thromboplastin time
Apo	Apolipoprotein
CAT	Catalase
CD36	Cluster of differentiation 36
CETP	Cholesteryl ester transfer protein
CRP	C-reactive protein
CYP7A1	Cytochrome P450 family 7 subfamily A member 1
DPPH	1,1-diphenyl-2-picrylhydrazyl
EBN	Edible bird's nest
eNOS	Endothelial nitric oxide synthase
Fb	Fibrinogen
FS	Full stew of EBN
FSh	Full stew hydrolysate of EBN
GPX	Gluthathione peroxidase
GPX1	Gluthathione peroxidase 1
GSR	Gluthathione-disulfide reductase
IC ₅₀	Half-maximal inhibitory concentration
IDL	Intermediate density lipoprotein
IL-1 β	Interleukin-1 beta
IL-6	Interleukin-6
HCl	Hydrochloric acid

HDL	High-density lipoprotein
HDL-c	High-density lipoprotein cholesterol
HFC	High-fat high-cholesterol
HMG-CoA	3-hydroxy-3methylglutaryl-CoA
HMGCR	HMG-CoA reductase
HOMA-IR	Homeostatic model assessment for insulin resistance
HYL	Hyaluronidase
H ₂ O ₂	Hydrogen peroxide
LCAT	Lecithin-cholesterol acyltransferase
LD ₅₀	Median lethal dose
LOX	Lipoxygenase
LOX-1	Lectin-type oxidized low-density lipoprotein receptor 1
LXR	Liver X receptor
LXR α	Liver X receptor alpha
LDL	Low-density lipoprotein
LDL-c	Low-density lipoprotein cholesterol
LDLR	Low-density lipoprotein receptor
LPS	Lipopolysaccharides
MCP-1	Monocyte chemoattractant protein-1
MMP-2	Matrix metalloproteinase-2
NAFLD	Non-alcoholic fatty liver disease
NaOH	Sodium hydroxide
NF- κ B	Nuclear factor-kappa B
NF κ B1	Nuclear factor kappa B subunit 1
NO	Nitric oxide
NOS3	Nitric oxide synthase 3

O ₂ ^{•-}	Superoxide anion radical
OGTT	Oral glucose tolerance test
ORAC	Oxygen radical absorbance capacity
oxLDL	Oxidized low-density lipoprotein
PPAR γ	Peroxisome proliferator-activated receptor gamma
PARP1	Poly (ADP-ribose) polymerase 1
PT	Prothrombin time
RCT	Reverse cholesterol transport
SE	Stew extract of EBN
SEh	Stew hydrolysate of EBN
SELP	P-selectin
SIRT1	Sirtuin 1
SOD	Superoxide dismutase
SOD1	Superoxide dismutase 1
SOD2	Superoxide dismutase 2
SOD3	Superoxide dismutase 3
SREBP2	Sterol regulatory element-binding protein 2
TEAC	Trolox equivalent antioxidant capacity
TC	Total cholesterol
TG	Triglycerides
TNF- α	Tumour necrosis factor-alpha
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
VCAM-1	Vascular cell adhesion molecule-1
VLDL	Very low-density lipoprotein
WHO	World Health Organization
XO	Xanthine oxidase

CHAPTER 1

INTRODUCTION

1.1 Introduction

Cardiovascular disease (CVD) continues to be the leading cause of death worldwide in both developed and developing countries, accounting for 32% of global mortality in 2019 (WHO, 2021). The number of deaths is expected to increase to about 23 million by 2030 (Virani et al., 2020). More than 80% of deaths from cardiovascular disease (CVD) occur in low- and middle-income countries (LMICs) (Bovet & Paccaud, 2011). As the population ages and unhealthy lifestyles become more common in LMICs, the burden of CVD is predicted to rise significantly. According to the Ministry of Health Malaysia's 2019 Health Facts, CVD mortality was the leading cause of death in government hospitals (21.65%) and the second leading cause of death in private hospitals (23.79%) (MOH, 2019).

Hypercholesterolemia is a significant modifiable risk factor for CVD, and it has been demonstrated that therapies (*i.e.*, pharmacological, nutrition, exercise, and smoking cessation) to reduce plasma cholesterol could reduce cardiovascular risk (Burkhardt, 2015). High plasma cholesterol levels are described as hypercholesterolemia, which is associated with an elevation in low-density lipoprotein (LDL). Hypercholesterolemia can be defined by plasma levels of total and LDL cholesterol (LDL-c) that are higher than the 95th percentile corrected for gender and age in each population (Martinez-Hervas & Ascaso, 2019). Clinicians usually prescribe statins, a 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor to lower the cholesterol level in the patient with hypercholesterolemia.

In hypercholesterolemia, statins work by lowering LDL-c levels by 20% to 50%, reducing triglyceride levels by 10% to 20%, as well as elevating 5% to 10% high-density lipoprotein cholesterol (HDL-c) serum levels (Ramkumar et al., 2016; Odden et al., 2015; Taylor et al., 2013; Huang et al., 2013). Atherogenesis has been reported to be significantly impacted by statins by preventing vascular endothelial dysfunction, intrahepatic vasoconstriction, and inflammation (Kaplan et al., 2019; Trebicka & Schierwagen, 2015; Hognestad et al., 2004). However, a number of adverse effects have been documented in certain individuals, which may impair long-term medication compliance. Most of these side effects are caused by muscle problems and changes in the way the liver works (Chien et al., 2019). Thus, it is important to discover an alternate solution that has cholesterol-lowering properties with fewer adverse effects in alleviating atherosclerosis.

Natural dietary components found in foods that are taken orally and are considered to offer a therapeutic or health advantage are known as nutraceuticals (Souyoul et al., 2018). Curcumin, ginsenosides, red-yeast rice, omega-3 PUFAs, and allicin are examples of active ingredients in natural resources that could help to reduce LDL-c serum levels and prevent atherosclerosis (Momtazi-Borojeni et al., 2019; Moss & Ramji, 2016; Ruscica et al., 2014; Chan et al., 2013a). Edible bird's nest (EBN) is constructed from swiftlet's saliva, mostly from the species of *Aerodramus fuciphagus* (white nest) and *Aerodramus maximus* (black nest), and commonly consumed in a form of soup as traditional delicacy by Chinese community (Chok et al., 2021; Quek et al., 2018). Malaysia is located right at the centre of the swiftlet's habitat and is one of the largest producers in the EBN industry, which contributes about RM (Ringgit Malaysia) 5.2 billion of National Gross Income in 2020 (Daud et al., 2019a; Babji et al., 2015; Rabu & Nazmi, 2015).

Previous studies have shown that EBN has anti-aging, anti-inflammatory, anti-viral, immunomodulatory, antioxidant, and other therapeutic properties (Chua et al., 2021; Hwang et al., 2020; Haghani et al., 2016; Yew et al., 2014; Vimala et al., 2012; Guo et al., 2006). Malaysia exports 600 tonnes of EBN every year (Panyaarvudh, 2018). Each year, China is the largest importer of EBN. Between January and June 2021, China imported roughly 42.3 tonnes of ready-to-drink and processed EBN from Malaysia (Chok et al., 2021). To comprehend the biological activity of EBN as a medication or functional food, it is necessary to study its components. The nutritional analysis showed that EBN consists of high protein (42.0-63.0%), carbohydrate (22.6-27.3%), moisture (7.5-24.3%), ash (2.1-7.4%) and fat (0.14-1.28%) (Lee et al., 2021; Babji et al., 2018; Ma & Liu, 2012). Besides, EBN also contains minerals such as calcium, magnesium, sodium, potassium, iron, and phosphorus (Halimi et al., 2014; Marcone, 2005). The nutritional analysis also shown that EBN rich in amino acids (AAs) constituents with 17% of total essential AAs, which was significantly higher than other protein-rich foods, for instance, egg (4.7 - 7.0%) and milk (1.1%) (Quek et al., 2018). Amino acids are important for human biological functions such as development, metabolism, and immunity and essential AAs are required from the diet as they cannot be synthesised by the human body (He, 2011; Blachier et al., 2013).

Apart from the nutritional composition aforementioned, EBN has been documented to contain sialic acid (5.86-13.6%) which is a major bioactive glycoprotein detected in EBN (Dai et al., 2020; Ling et al., 2020; Quek et al., 2018). Numerous research has shown that sialic acid has a variety of health benefits including promoting neuronal development, preventing hypertension, enhancing the immune response of cancer cells as well as skin whitening (Ling et al., 2022; Khalid et al., 2019; Wang et al., 2018a; Zhou et al., 2019; Wong et al., 2018). Furthermore, the antioxidant and anti-inflammatory of sialic acid play a significant role in ameliorating cardiovascular health by lowering lipid profile and coagulation factors, as well as improving insulin sensitivity (Yida et al., 2015a; Yida et al., 2015b). Hence, sialic acid can be considered a valuable component in EBN with cholesterol-lowering and anti-atherogenic effects.

Despite the effectiveness of EBN in ameliorating cardiovascular events, the underlying mechanisms involved in inhibiting atherogenesis are not fully elucidated, especially on the cholesterol metabolism-related to atherosclerotic plaque formation. Thus, this study aimed to explore the cholesterol-lowering and anti-atherogenic effects of EBN by investigating its roles in cholesterol metabolism and atherosclerotic plaque formation. The effects of EBN on oxidative stress and inflammation related to atherosclerosis were also studied to evaluate its possible antioxidative and anti-inflammatory properties.

1.2 Hypotheses

- i. The physicochemical characteristics and nutritional composition are varied between the different shapes of edible bird's nest (EBN).
- ii. EBN has the ability to inhibit cholesterol synthesis with good antioxidant and anti-inflammatory activities, as well as inhibit early atherogenesis in mouse macrophage (RAW 264.7) and human monocytic (THP-1) cell lines.
- iii. EBN possess anti-atherogenic effects via regulation of biomarkers and genes related to cholesterol metabolism in hypercholesterolemic-induced rabbit model.
- iv. EBN attenuates vascular oxidative stress and inflammation via regulation of biomarkers and genes related to antioxidant and inflammation.

1.3 Objectives

General objective

To determine cholesterol-lowering and anti-atherogenic effects of edible bird's nest in vitro and in vivo

Specific objectives

- i. To determine physicochemical characteristics, nutritional composition, and protein profiling of edible bird's nest (EBN).
- ii. To determine the cholesterol synthesis inhibitory, antioxidant, anti-inflammatory activities as well as the anti-atherogenic potential of EBN using mouse macrophage (RAW 264.7) and human monocytic (THP-1) cell lines.
- iii. To determine the cholesterol-lowering and anti-atherogenic effects of EBN including transcriptional regulation of related genes in hypercholesterolemic-induced rabbit model.
- iv. To determine the effects of EBN on vascular oxidative stress and inflammation including transcriptional regulation of related genes in hypercholesterolemic-induced rabbit model.

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