



**UNIVERSITI PUTRA MALAYSIA**

***ANTI-AGEING PROPERTIES OF EDIBLE BIRD'S NEST ASCERTAINED BY  
IN VITRO AND IN VIVO STUDIES IN ANIMAL MODEL***

***HOU ZHIPING***

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By

**HOU ZHIPING**

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

**August 2015**

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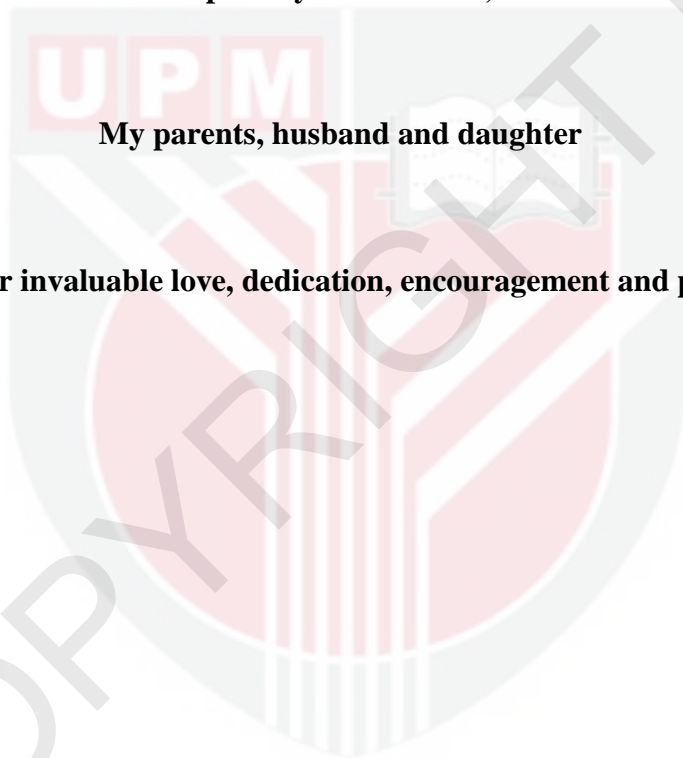
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**Specially dedicated to,**

**My parents, husband and daughter**

**For their invaluable love, dedication, encouragement and patience**



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

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**August 2015**

**Chairman: Maznah binti Ismail, PhD**  
**Faculty : Institute of Bioscience**

According to the World Health Organization (WHO), the world average life expectancy as at 2014 is 66.26 years, having an average of 64.30 years for males and 68.35 years for females. However, the onset of menopause for women, usually around 50 years, has not been postponed along with increasing longevity, but rather brought forward due to the impacts of environment, diet, and lifestyle. Most women spend approximately one-third of their life span in the postmenopausal phase. Hormonal imbalance during menopausal stage has been linked to increased risks of the fatal diseases including Alzheimer's disease, dementia, stroke, diabetes mellitus, and breast cancer. Hormone replacement therapy (HRT) has been used to restore postmenopausal hormonal levels in order to relieve menopausal problems, but its associated side effects including development of cancers and cardiovascular diseases have necessitated the search for other alternatives such as natural food supplementation.

Edible bird's nest (EBN) from the saliva of swiftlet has been esteemed as a precious food tonic by Chinese people since the Tang dynasty (618AD) because it contains rich amount of bioactive compounds such as water - soluble proteins, carbohydrate, iron, inorganic salt, and fiber, and is reported to traditionally possess anti-ageing, anti - cancer, and immunity - enhancing properties. Evidence-based details of its anti-ageing effects including underlying mechanisms are lacking. In this study, EBN was evaluated for its anti - ageing effects and its potential mechanisms were evaluated. First of all, ovariectomized female Sprague - Dawley rats were fed with EBN (6 %, 3 % and 1.5 % in normal pellet) for 12 weeks, then, cognitive function, metabolic indices (serum estrogen, insulin, liver enzyme, kidney function, lipid profile, and antioxidant markers) and hippocampal sirtuin - 1 protein level were in comparison with non - treated ovariectomized rats were observed, and in some instances it showed better results than estrogen therapy. Additionally, EBN produced better transcriptional regulation of hippocampal anti-oxidant genes and an Alzheimer disease isrelated genes, and hepatic insulin signaling genes. Moreover, EBN and its constituents (lactoferrin and ovotransferrin) attenuated H<sub>2</sub>O<sub>2</sub> - induced cytotoxicity, and decreased radical oxygen species through increased scavenging activity, with corresponding transcriptional changes in anti - oxidant and apoptosis - related genes that tended towards neuroprotection.

These data suggested that EBN reduced the risks of neurodegenerative diseases, and may be used as functional ingredient for the prevention of neurodegenerative and metabolism related diseases associated with estrogen deficient ageing.



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

## **SIFAT ANTI-PENUAAN SARANG BURUNG WALIT DITENTUKAN MELALUI KAJIAN *IN VITRO* DAN MODEL HAIWAN *IN VIVO***

Oleh

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Menurut Pertubuhan Kesihatan Sedunia (WHO), purata jangka hayat manusia di dunia pada 2014 adalah 66.26 tahun, di mana lelaki mempunyai purata 64.3 tahun manakala wanita mempunyai 68.35 tahun. Walau bagaimanapun, permulaan menopause bagi wanita, biasanya sekitar 50 tahun, tidak ditangguhkan bersama - sama dengan peningkatan umur, tetapi sebaliknya dipercepatkan oleh kesan persekitaran, diet dan gaya hidup. Kebanyakan wanita menghabiskan kira-kira satu pertiga daripada jangka hayat mereka dalam fasa menopause. Ketidakseimbangan hormon semasa menopause telah dikaitkan dengan peningkatan risiko penyakit - penyakit kronik termasuk penyakit Alzheimer, nyanyuk, strok, kencing manis, dan kanser payudara. Terapi penggantian hormon (HRT) telah digunakan untuk mengubati masalah putus haid, tetapi kesan sampingan seperti kanser dan penyakit kardiovaskular telah mendorong pencarian alternatif lain seperti suplemen makanan semulajadi.

Sarang burung (EBN) daripada air liur burung walit telah digelarkan sebagai tonik makanan yang berharga oleh orang-orang Cina sejak Dinasti Tang (618AD) kerana ia mengandungi jumlah sebatian bioaktif yang kaya seperti protein larut air, karbohidrat, besi, garam bukan organik, dan serat. Ia juga dilaporkan secara tradisi mempunyai anti - penuaan, anti - kanser, dan sifat - sifat meningkatkan imuniti. Maklumat berasaskan bukti saintifik kesan anti - penuaan termasuk mekanisme kefungsiannya adalah amat sedikit. Dalam kajian ini, kesan anti - penuaan daripada EBN telah dikaji dan mekanisme yang berpotensi juga ditentukan. Pertama, tikus Sprague-Dawley betina "yang dibuang ovari" yang diberi makan EBN (1.5 %, 3 %, dan 6% dalam normal pelet) selama 12 minggu menunjukkan fungsi kognitif, indeks metabolik (serum estrogen, insulin, hati fungsi buah pinggang enzymes, profil lipid, dan penanda antioksidan) dan protein sirtuin - 1 pada hippocampus adalah lebih baik berbanding dengan tikus yang dibuang ovari tanpa makanan EBN, dan dalam keadaan tertentu ia menunjukkan keputusan yang lebih baik berbanding dengan terapi estrogen. Di samping itu, EBN menunjukkan pengawalan transkripsi gen yang lebih baik termasuk gen anti-oksida dan gen berkaitan dengan penyakit Alzheimer pada tisu hippocampus, dan juga gen isyarat insulin hepatik. Tambahan pula, EBN dan sebatian aktif (laktoferin, LF dan ovotransferrin, OVF) melemahkan sitotoksiti yang disebabkan oleh H<sub>2</sub>O<sub>2</sub>, dan menurunkan spesies oksigen radikal (ROS) melalui peningkatan aktiviti memerangkap radikal, dengan mengubah transkripsi gen yang berkaitan dengan anti -

oksidasi dan apoptosis, justeru menunjukkan kecenderungan ke arah perlindungan sistem neuron.

Data mencadangkan bahawa EBN mengurangkan risiko penyakit sistem neuron dan boleh digunakan sebagai bahan nutrasutikal untuk mencegah penyakit neuron dan penyakit metabolik yang dikaitkan dengan kekurangan estrogen.





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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy.

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## LIST OF ABBREVIATIONS

AAPH	2,2' - azobis (2 - amidinopropane) dihydrochloride
ABTS	2,2' - azino - bis (3 - ethylbenzothiazoline - 6 - sulphonic acid)
AGE	Advanced glycation end-product
Akt	Protein kinase B
ALP	Alanine transaminase
ALT	Alkaline phosphatase
AO / PI	Acridine orange and propidium iodide
APP	Amyloid Precursor Protein
ATCC	American Type Culture Collection
CA	Cornus ammonis
CASP3	Caspase 3
CAT	Catalase
CNS	Central nervous system
DMEM / F - 12	Minimum essential Eagle's medium, Ham's nutrientmixture
DMSO	Dimethyl sulfoxide
EBN	Edible bird's nest
EGF	Epidermal growth factor
Gapdh	Glyceraldehydes - 3 - phosphate dehydrogenase
Gck	Gluckokinase
GGT	Gamma - glutamyl transferase
GLUT4	Glucose transporter type 4
HDL	High density lipoprotein
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
HOMA - IR	Homeostatic model assessment of insulin resistance
HRT	Hormone replacement therapy
IDE	Insulin - Degrading Enzyme
Ikkb	Inhibitor of kappa light polypeptide gene enhancer in B - cells, kinase beta
Insr	Insulin receptor
Kan(s)	Kanamycin resistance
K <sub>2</sub> S <sub>2</sub> O <sub>8</sub>	Potassium persulphate
KNJC11	Potassium inwardly rectifying channel, subfamily J, member 11
LDL	Low density lipoprotein
LF	Lactoferrin
LPk	Pyruvate kinase-liver isoform
LRP1	Low Density Lipoprotein Receptor - Related Protein1
IRS	Insulin receptor substrate
MAPK	Mitogen-activated protein kinase
MDA	3,4 - methylenedioxyamphetamine
MMP	Mitochondrial membrane potential
MTT	3 - [4,5 - dimethylthiazol - 2 - yl] - 2,5 - diphenyl-tetrazolium bromide
MWM	Morris Water Maze
NaHCO <sub>3</sub>	Sodium biocarbonate
NF - κB	Nuclear factor kappa - light - chain - enhancer of activated B cells
OD	Optical density

OGTT	Oral glucose tolerance test
ORAC	Oxygen radical absorbance capacity
OVF	Ovotransferrin
OVX	Ovariectomy
PARP1	Poly (ADP - ribose) polymerase 1
PI3K	Phosphoinositide - 3 - kinase
PSEN1	Presenilin - 1
PSEN2	Presenilin - 2
p53	Cellular tumor antigen p53
MAPK	Mitogen-activated protein kinase
RA	Retinoic acid
ROS	Radical oxygen species
SIRT - 1	Sirtuin - 1
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances



## CHAPTER 1

### INTRODUCTION

Biological ageing is the process of cumulative changes to molecular and cellular structures that disrupt metabolism with the passage of time, resulting in deterioration and death. Ageing related memory and cognitive decline are increasingly becoming a problem in humans especially due to improvements in healthcare delivery that have given rise to increasing longevity. Life expectancy for women is higher than for men and their menopausal transition is often accompanied with changes. Alteratives in central nervous system (CNS) are often reflected on memory and cognitive function and sensitive with body metabolic status. Over the years, the life span of Malaysian women increased to 76.8 years in 2011 (Malaysia), and the increasing longevity has meant that women live one-third of their lives beyond cessation of endogenous oestrogen production from the ovaries (Hara, et al., 2011). Before menopause, circulating oestrogen level protects women against neurodegenerative diseases, such as stroke, compared to men (Brann, et al., 2007; Mahesh, & Khan, 2007; Scott, et al., 2012; Vadlamudi, & Brann, 2012). Conversely, postmenopausal women have higher morbidity and mortality due to neurodegenerative diseases compared with age-matched men, because of reduced circulating oestrogen level (Appelros, Stegmayr, & Terént, 2009; Persky, Turtzo, & McCullough, 2010). On the other hand, the risk of metabolic diseases, like type 2 diabetes mellitus, increase significantly in women after menopause, and they are the burden of neurodegenerative diseases on the basis of literatures (de la Torre, 2004; Ulas & Cay, 2010).

Oestrogen is secreted and controlled through hypothalamus-pituitary-gonadal (HPG) axis, to regulate biological function. The decline in oestrogen level in menopause is reported to result in memory loss and metabolic perturbation soon after it starts, and sirtuin - 1 protein played important roles in hippocampus dependent memories and synaptic plasticity as reports. Eventually, low levels of oestrogen lead to other secondary metabolic abnormalities including oxidative stress, apoptosis and inflammation that together complicate the ageing-related degenerative diseases through neuropathy, dementia, Alzheimer's disease, cardiovascular disease and diabetes. Also, complications arising from oestrogen - deficit diseases are debilitating and result in a huge expenditure in health care. In addition, these degenerative diseases that are among the most common diseases affecting menopausal women, have been linked with lifestyle especially diet (WHO).

Encouragingly, hormone replacement therapy (HRT) has been shown to prevent neurodegenerative disorders as conventional usage on treating menopausal symptoms. However, the risk of life - threatening complications it induces has limited its use. Data have reported the effects of long term HRT on the risk of breast cancer (Beral, Banks, & Reeves, 2002; Tempfer, et al., 2009) and venous thromboembolism (Jick, Derby, Myers et al., 1996; Vasilakis, & Newton, 1996). Thus, alternatives with better safety profile compared with conventional HRT have received close attention. Herbal medicine has a long history of use and is still widely practiced today, based on the theories, beliefs, and experiences indigenous to different cultures, and used in the maintenance of health as well as in the prevention, improvement or treatment of physical and mental illness (Organisation, 2012).



There is a long history of using traditional medicines in Asian countries. China, one of the early ancient civilisations, has been using edible bird's nest (EBN) since ancient dynasty. EBN is produced by swiftlets from their salivary glue, which is a cementing substance. The nests are considered to have high nutritional and medicinal values, traditionally believed to have everything from anti-ageing and anti-cancer properties to the ability to improve immunity and raise libido. Composition analysis of EBN shows the major composition is protein (62 % ~ 68 %) including glycoproteins (Hamzah, Ibrahim, Jaafar et al., 2013), followed by carbohydrate and mineral salts. Additionally, other bioactives have been reported in EBN including epidermal growth factor (EGF), testosterone (Marcone, 2005), and chondroitin glycosaminoglycans (Matsukawa, Matsumoto, Bukawa et al., 2011). Numerous *in vitro* and *in vivo* researches have shown that administration of EBN was able to boost immunity, improve anti-oxidant ability, promote neutralisation of influenza activity as well as improve osteoporosis. Sadly, however, evidence-based details of other properties of EBN are lacking. Since the practical usage of traditional medicine is not strictly enforced under scientific evidence, herbalism is just recognised as a form of alternative medicine in modern medicine. Despite EBN's long history of medicinal use, there is still a dearth of research and scientific evidence to substantiate the claims of health benefits associated with anti - ageing including its mechanistic basis.

In this study, it was aimed to explore the evidences for the use of EBN as a functional food in preventing and managing ageing-related neurodegenerative diseases in menopause, of which the risk is increased due to loss of protection from oestrogen and related hormones. The brain cells are normally sensitive to the effect of redox system because of their peculiar energetic demands (Gandhi & Abramov, 2012). In brain senescence, radical oxygen species (ROS) starts to accumulate in neurons before clinically evident signs and symptoms of the disease can be detected (Gandhi & Abramov, 2012). When ROS accumulate, oxidative damage is normally prevented by induction of protective factors, like antioxidants. On the other hand, the imbalanced redox status is involved in advanced glycation end-products (AGEs) adjustment pathway, and the depletion of cellular antioxidant mechanisms and the generation of free radicals by AGEs may play a major role in the pathogenesis of neurodegeneration (Kuhla, 2014; Prasad, et al., 2014). Furthermore, AGEs is influenced by glucose and lipid homeostasis, and may play the role in transcriptional and proteomic aspects if the insult is too overwhelming. In such cases, apoptotic mechanisms set in to remove neurons deemed irreparable (Radi, et al., 2014). Loss of neurons through these apoptotic deaths results in severe morphological and functional deficits, which manifest with progressive memory and cognitive decline. Therefore, it was hypothesised that this research will give clues on the effect of EBN in some selected organs and functions that are affected by low estrogenic level such as memory loss, weight increase, insulin deficiency and lipid metabolic abnormalities. Their effects on brain and liver related to anti - oxidation, apoptosis and inflammation were equally considered.

### **Hypotheses of the study were:**

1. EBN and its bioactives (LF and OVF) protect SH-SY5Y cells against H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity and cell oxidative stress;
2. EBN enhances spatial learning and memory in ovariectomized rats;
3. EBN preserves hippocampal SIRT - 1 activity in the menopause model, as the likely basis for enhanced spatial learning and memory;
4. EBN is neuroprotective against oestrogen deficiency - induced damage *via* increasing serum oestrogen level, and decreasing AGEs and oxidative stress;
5. EBN improves metabolic indices like glucose metabolism, lipid profile and antioxidant status in ovariectomized rats.

### **General objective**

To study the anti - ageing effects of EBN and its mechanism through *in vitro* and *in vivo* approaches, including transcriptomic and proteomic analyses.

### **Specific objective**

1. To examine the effects of EBN on ovariectomy - induced memory and cognitive dysfunctions, including transcriptomic and proteomic mechanisms; as well as to obtain sirtuin-1 data on hippocampus and frontal cortex parameters;
2. To determine the effects of EBN on serum AGEs and redox status, and neuro - dysfunction in ovariectomized rats, including mechanistic basis for such effects;
3. To determine the effects of EBN on weight and metabolic indices related to insulin resistance in ovariectomized rats, and mechanistic basis for such effects.
4. To characterize the bioactives (lactoferrin [LF] and ovotransferrin [OVF]) in EBN, and evaluate their anti - oxidant abilities;
5. To determine the neuroprotective potentials of EBN water extract and its constituents, LF and OVF, and related molecular mechanisms involved in H<sub>2</sub>O<sub>2</sub> - induced oxidative stress and apoptosis in SH - SY5Y cells



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