
**UNIVERSITI PUTRA MALAYSIA**

# *PROXIMATE ANALYSIS AND SAFETY PROFILE OF FARMED EDIBLE BIRD'S NEST IN MALAYSIA AND ITS EFFECT ON CANCER CELLS*

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# PROXIMATE ANALYSIS AND SAFETY PROFILE OF FARMED EDIBLE BIRD'S NEST IN MALAYSIA AND ITS EFFECT ON CANCER CELLS

By

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**November 2018**

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**Faculty : Medicine and Health Sciences**

Edible bird's nest (EBN), a solidified swiftlet's saliva, is the most valuable animal products consumed by human in Asia. EBN was documented to contain high nutritional values and various medicinal properties. Good nutritional profile containing high protein, carbohydrate, fat, and trace elements found in EBN have been well established from previous studies done in different countries in Malaysia, Indonesia and Thailand. However, the previous proximate analysis studies of Malaysia EBN were not representative from all the regions in Malaysia. In recent years, many safety issues which included high nitrate and nitrite contents, presence of heavy metal, fungal infection and cancer cell stimulation *in vitro* were also found to be associated with EBN. Although the EBN are known to be dirtier during haze period as the saliva from the swiftlet are contaminated from air pollution, there has been no prior study to assess the effect on haze towards the quality of EBN. Hence, this study was carried out to determine the proximate analysis, safety profile during normal and haze period, and its effect on cancer cells of farmed EBN in Malaysia.

Seven raw cleaned EBN samples were sourced from 6 different regions in Malaysia. Proximate analysis and were performed by using official AOCA methods according to the Malaysia Standard MS 2509:2012. This study showed a consistency of high protein (53.03%-56.37%) and carbohydrate (27.97%-31.68%) with acceptable level of moisture (10.8-14.04%) and ash (2.22%-3.38%). Besides that, a good safety profile was obtained with low nitrite and nitrate level, undetectable heavy metals and no significant growth for pathogenic microorganism. However, this study had found above tolerance level of mould in EBN. Haze contaminated EBN exhibited no significant differences in terms of nutritional, heavy metal and microorganism profiles. The presence of epidermal growth factor (EGF) like peptides was postulated as the important key of cancer cell stimulation. Chicken EGF Enzyme-linked immunosorbent assay (ELISA) was used for quantification of EGF content. In this study, EGF (30.7 pg/mL and 74.5 pg/mL) were detected in crude EBN 01 (Rompin) and EBN 02 (Sibu), respectively but not in all digested EBN samples

and even after post 10 times concentrated EBN extract. However, both the results were below quantification level.

Microculture tetrazolium assay (MTT) was carried out for the assessment of growth stimulation by different concentration of human EGF (hEGF) in comparison to EBN in 4 different cancer cell lines: MCF-7, Caco-2, HCT116, and A549 for 24, 48, and 72 h. Cancer cell growth was significantly increased after treatment with hEGF. However no significant cell growth was observed after treatment with EBN.

In conclusion, EBN in Malaysia has good consistency of nutritional profile, free from heavy metals, within tolerance level of nitrate and nitrite, and also microorganism profile except mould content. Furthermore, *in vitro* study indicated that EBN is not associated with cancer cell growth stimulation.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

**ANALISIS PROKSIMAT DAN PROFIL KESELAMATAN SARANG BURUNG WALIT MALAYSIA DAN KESAN TERHADAP SEL KANSER**

Oleh

**TAN SIN NEE**

**November 2018**

**Pengerusi : Profesor Madya Christopher Lim Thiam Seong**
**Falkuti : Perubatan dan Sains Kesihatan**

Sarang burung walit (EBN), iaitu air liur burung walit merupakan salah satu produk haiwan yang paling mahal di seluruh dunia. EBN dianggap sebagai bahan makanan mewah dan berprestij yang mengandungi kandungan nutrisi yang tinggi dan pelbagai khasiat perubatan. Pelbagai kajian dari Malaysia, Indonesia dan Thailand telah membuktikan bahawa sarang burung merupakan bahan makanan yang mengandungi kandungan nutrisi yang tinggi termasuklah protein, karbohidrat, lemak dan unsur elemen. Walaubagaimanapun, kajian analisis proksimat EBN Malaysia sebelum ini tidak merangkumi semua kawasan di Malaysia. Sejak kebelakangan ini, pelbagai isu keselamatan telah dikaitkan dengan EBN seperti kandungan nitrit dan nitrat yang tinggi, pencemaran logam, pencemaran kulat atau bakteria serta perangsangan sel kanser dapat dikesan melalui *in vitro*. Selain itu, kelihatan EBN Malaysia adalah lebih kotor semasa musim jerebu disebabkan pencemaran udara, tetapi, tiada kajian sebelum ini yang menilai kesan pencemaran jerebu terhadap kualiti EBN. Oleh demikian, kajian ini adalah bertujuan untuk menentukan analisis proksimat, profil keselamatan dalam udara normal dan semasa pencemaran jerebu, dan kesan EBN Malaysia terhadap sel kanser.

Tujuh EBN mentah yang telah dibersihkan telah dikumpulkan dari 6 kawasan berbeza di Malaysia. Analisa proksimal dijalankan menggunakan kaedah rasmi AOCA mengikut piawai Malaysia (MS) 2509:2012. Keputusan mendapati bahawa EBN Malaysia mengandungi tahap protein dan karbohidrat yang konsisten (53.03% - 56.37% dan 27.97%-31.68%) serta tahap kelembapan dan abu yang boleh diterima (10.8-14.04% dan 2.22%-3.38%). Selain itu, EBN Malaysia juga mempunyai profil keselamatan yang bagus, dengan kandungan nitrit dan nitrat yang rendah, tiada kandungan logam yang dikesan, dan tanpa pembiakan mikroorganisma yang boleh menyebabkan penyakit. Walaubagaimanapun, kajian ini telah menemukan kandungan kulat yang melebihi tahap yang boleh diterima. EBN yang terdedah kepada jerebu juga tidak menunjukkan sebarang perbezaan dari segi nutrisi, kandungan logam, serta profil mikroorganisma. Kandungan peptida merupai faktor pertumbuhan epidermal dianggapkan sebagai factor penting yang berfungsi untuk merangsang sel kanser. ELISA (enzyme-linked

immunosorbent assay) telah digunakan untuk menentukan kuantiti kandungan EGF dalam EBN. Menerusi kajian ini, EGF sebanyak 30.7 pg/mL and 74.5 pg/mL telah dikesan dalam EBN 01 (Rompin) dan EBN 02 (Sibu) yang mentah. Tetapi, EGF tidak dapat dikesan dalam EBN yang telah diproses atau dalam EBN yang telah diproses dan selepas menjalani proses kepekatan 10 kali dari kepekatan asal. Namun begitu, kedua-dua kandungan ini adalah dibawah tahap yang boleh dikuantiti.

Microculture tetrazolium (MTT) digunakan untuk menilai kesan rangsangan terhadap pertumbuhan sel kanser selepas dirawat dengan EBN berbanding kepada EGF manusia (hEGF) yang berlainan kepekatan untuk 4 sel kanser termasuklah MCF-7, Caco-2, HCT116, dan A549 selama 24, 48 dan 72 jam. Pertumbuhan sel kanser meningkat dengan ketara setelah dirawat dengan hEGF. Walaubagaimanapun, tiada pertumbuhan sel kanser ketara yang disaksikan setelah dirawat dengan EBN.

Kesimpulannya, EBN dari Malaysia adalah berkandungan nutrisi yang tinggi, bebas dari logam, dan mempunyai tahap nitrit, nitrat, dan profil mikroorganisma yang boleh diterima. Tambahan lagi, kajian *in vitro* juga menunjukkan bahawa EBN tidak dikaitkan dengan perangsangan sel kanser.

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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 Master of Science. The members of the Supervisory Committee were as follows:

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| AKT | Serine/threonine kinase |
| ANOVA | Analysis of variance |
| AOCA | Association of Official Analytical Chemistry |
| API | Air pollution Index |
| AREG | Amphiregulin |
| ATCC | American Tissue Culture Collection |
| BLAST | Basic Local Alignment Search Tool |
| BTC | Betacellulin |
| CFU | Colony-forming units |
| $CO_2$ | Carbon dioxide |
| DMSO | Dimethylsulfoxide |
| DNA | Deoxyribonucleic acid |
| EBN | Edible bird's nest |
| EDTA | Ethylenediaminetetraacetic acid |
| EGF | Epidermal growth factor |
| EGFR | Epidermal growth factor receptor |
| ELISA | Enzyme-linked immunosorbent assay |
| EPGN | Epigen |
| EREG | Epiregulin |
| ERK | Extracellular signal-regulated kinase |
| FBS | Fetal bovine serum |
| FDA | Food and Drug Authority |
| FIMS | Flow Injection Mercury System |
| GalNAc | N-Acetyl-D-Galactosamine |
| GMP | Good Manufacturing Practice |
| hADSCs | Human adipose-derived stem cells |
| HBEGF | Heparin-binding EGF-like growth factor |
| hEGF | Human epidermal growth factor |
| HFD | High fat diet |
| $H_2O_2$ | Hydrogen peroxide |
| ICP-MS | Inductively coupled plasma-mass spectrometry |
| IG | Immunoglobulin |
| IL | Interleukin |
| ISO | International Organization for Standardization |
| LF | Lactoferrin |
| LPS | Lipopolysaccharide |
| MAPK | Mitogen-activated protein kinase |
| MDCK | Madin-Darby canine kidney |
| MTT | Microculture tetrazolium |
| NANA | N-Acetyl-neuraminic acid |
| NHFs | Normal human fibroblasts |
| NO | Nitrite oxide |
| NS1 | Non-structural protein 1 |
| OA | Osteoarthritis |
| OVF | Ovotransferrin |
| PAGE | Polyacrylamide gel electrophoresis |
| PBS | Phosphate buffered saline |
| PDT | Population doubling time |

| | |
|---|---|
| PPB | Parts-per billion |
| PPM | Parts-per million |
| ROS | Radical oxygen species |
| RPMI | Roswell Park Memorial Institute |
| SD | Standard deviation |
| SDS | Sodium dodecyl sulphate |
| SIRIM | Standards and Industrial Research Institute of Malaysia |
| SPSS | Statistical Package for Social Sciences |
| STAT | Signal transducer and activator of transcription |
| TGF-α | Transforming growth factor-alpha |
| TNF-α | Tumour necrosis factor alpha |
| VEGF | Vascular endothelial growth factor |
| WHO | World Health Organization |

# CHAPTER 1

# INTRODUCTION

## 1.1 Overview

Edible Bird's Nest (EBN), a solidified saliva made from the birds of *Aerodramus* genus family, total of four different species of swiftlets. There are *Aerodramus fuciphagus, Aerodramus maximus, Aerodramus germani* and *Aerodramus unicolor*. EBN has existed many centuries ago and can be traced back as early as the Tang (618– 907 AD) and Sung (960–1279 AD) dynasties whereby they were used as main ingredients in traditional Chinese medicine and Chinese cuisine (Lim and Cranbrook, 2002). EBN was prepared by using double boiler method with additional rock sugar to produce the gastronomic delicacy, well known as "bird's nest soup". Since ancient times, EBN has been also regarded as a luxurious and prestigious food item which contains high nutritional values and various medicinal properties (Oda *et al*., 1998). For the past 500 years, EBN has been traded in Malaysia and it was documented that trading occurred between the Malay Archipelagos and China since the 16$^{th}$ century (Sankaran, 2001).

High nutritional and medicinal values of EBN and the dangers that might be encountered during the nest collection, EBN is one of the world's most valuable animal product which consumed by mankind with average selling price of $ 2500 per kilogram. The Malaysian government recognised the EBN industry has huge economic potential. Malaysia had exported beyond 100 metric tons of EBN with the value of RM 500 million in 2009. EBN trading is expected to contribute more than RM 5.2 billion to the Gross National Income in 2020 (Rabu and Mohd, 2015). Because of the continuous increase demand of the EBN, to avoid overhasvesting practice, it has led to a large decline in cave yields EBN (Sankaran, 2001). In Malaysia, with the present rate of cave EBN hasvesting, cave swiftlet species may be wipe out in 10-15 years time. Malaysia wildlife and National Park Department has reported that drastic decline in nest production as the wild swiftlet population in the Niah Cave has dropped from 1.7 millions in 1935 to 200000 in 1996 and 65000 in 2002 due to uncontrolled and overharvesting (Lim, 2006). The decreased of cave nest production had bloomed the development of EBN farming in many South East Asia countries (Nugroho and Whendrato, 1996). The most famous natural cave swiftlet nesting sites in Malaysia are located in Sarawak and Sabah, and other areas are mainly EBN farming located in Peninsular Malaysia are Sitiawan, Teluk Intan, Kota Bharu, Kuala Terenganu, Bukit Mertajam, Nibong Tebal, Kuantan, Muar, Segamat and many other old townships.

EBN contains high protein, carbohydrate, and trace elements (Marcone, 2005). The proximate analysis of EBN from different countries for example, Malaysia (Huda *et al*., 2008; Norhayati *et al*., 2010; Lee *et al*., 2015), Indonesia (Marcone, 2005) and Thailand (Saengkrajang *et al*., 2013) has been well established. A useful glycoprotein found in EBN is sialic acid, about 7.1% - 9% (Wang *et al*., 2006) which may have benefit on neurological development (Palmano *et al*., 2015). Other than nutritional profile which has been well studied, many researchers have identified some of its impressive medicinal properties include potent anti-inflammatory activity by

suppressing the production of tumour necrosis factor alpha (TNF-α) (Aswir and Wan, 2011), inhibition of influenza virus infection (Haghani *et al*., 2016), alleviation of asthma (Matsukawa *et al*, 2011), chemoprotective properties from cyclophosphamide (Zhao *et al*., 2016), neuroprotective effect (Careena *et al*., 2018), EGF like peptide which promote cell proliferation (Kong *et al*., 1987), and estradiol hormones (Ma and Liu, 2012) which may be beneficial in women health by improving menstrual dysfunctions such as amenorrhea and irregular menstruation (Brendan, 2016).

EGF was found to be present in EBN traced back in 1987 (Kong *et al*., 1987). Human EGF is known to have the ability to stimulate cell growth and proliferation. Total of seven ligands regulation were identified : EGF, transforming growth factor-alpha (TGFA), heparin-binding EGF-like growth factor (HBEGF), betacellulin (BTC), amphiregulin (AREG), epiregulin (EREG), and epigen (EPGN) and four ErbB (HER) family receptor tyrosine kinases: EGFR/ErbB1, ErbB2, ErbB3 and ErbB4 (Wee and Wang, 2017). EGF acts as a mitogenic factor that plays a growth stimulating role in various epidermal and epithelial tissues (Kumar, Abbas, and Aster, 2015). The activation of EGF will lead to the initiation of a cascade of downstream signaling pathways, such as signal transducer and activator of transcription (STAT), mitogen-activated protein kinase (MAPK), and the modulation of calcium channels. Hence, EBN is also deemed to have rejuvenating and anti-aging properties. However, the receptor for EGF (EGFR) has been found highly expressed in various solid tumours and the dysregulation of EGF is also associated with the growth and progression of many cancers. Previous paper had shown that different source and type of EBN stimulates colon cancer cells growth *in vitro* (Aswir and Wan, 2011). This raises an important issue whether the presence of EGF in EBN may stimulate cancer cell growth after consumption.

Besides the presence of EGF and possible of cancer cell stimulation issue, there are many others safety issues arise from Malaysia EBN in recent years for example, high nitrate and nitrite contents (Paydar *et al*., 2013; Quek *et al*., 2015), presence of heavy metal (Chen *et al*., 2014; Lee *et al*., 2015), pathological microorganism and fungal infection (Chen *et al*., 2015). Moreover, Malaysia experiences severe haze episode every years due to open burning from nearby countries. From observation by the expert, the EBN collected from haze affected area with high Air Pollution Index (API) appeared to be dirty and blackish colour. However, there was no previous study has looks into the effect of haze toward the nutritional and safety profile of EBN. Therefore, this paper is to establish the nutritional and safety profile in term of heavy metal, microbiology and nitrate and nitrite contents of EBN, to assess the effect of haze on the quality of EBN, to quantify the EGF in EBN from different regions in Malaysia and to determine the possible growth effect on cancer cells by EGF found in EBN.

## 1.2 Hypothesis

Despite many studies regarding proximate analysis and medicinal properties of EBN but there were still many safety issues arise from EBN like high nitrate and nitrite contents, presence of heavy metal, microorganism and fungal infection. Those safety issues were not throughly investigated before. The EBN issues which arised may potentially harmful to human. Hence, this study was carried out to ensure that EBN is safe for human consumption in term of nitrate and nitrite, heavy metal, microorganism profile and carcinogenic risk.

The null hypotheses in this study were

- EBN has no good nutritional value or safety profile for human consumption.
- There are no significant differences in term of safety and nutritional profile found in EBN collected from different regions in Malaysia.
- There are no significant differences in term of safety and nutritional profile for the haze polluted EBN.
- EBN does not contain EGF.
- EBN does not stimulate human cancer cell growth.

## 1.3 General objective

The general objective of this study was to determine the nutritional, epidermal growth factor, safety and microorganism profile of EBN sourced from various regions in Peninsula and East Malaysia and the potential of cancer cell growth stimulation by EBN *in vitro*.

## 1.4 Specific objective

The specific objectives as follow:

1) To determine the nutritional values and epidermal growth factor level of EBN from different regions in Malaysia
2) To assess the toxicity and safety profile of EBN harvested in normal and haze conditions.
3) To investigate the *in vitro* effect of EBN on various cancer cell lines.

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## BIODATA OF STUDENT

This student was born in Kuala Lumpur on 1987. She had obtained her degree of Doctor of Medicine 2006-2011 from Universiti Putra Malaysia. After that, she served her housemanship in Hospital Teluk Intan and subsequently transferred to Hospital Serdang as Medical officer after 2 years. She took up her Master in Science in Pharmacology & Toxicology under Universiti Putra Malaysia back in 2014. Along the master programme, she had presented a few posters during the conference and managed to publish a paper in Evidence-Based Complementary and Alternative Medicine. While for her career path, she had passed her external paper by Membership of the Royal Colleges of Physicians, London UK in 2016. Currently, she works as a gazetted physician in Hospital Tengku Ampuan Afzan, Kuantan. She is interested in the field of complementary medicine, cancer research and palliative care medicine.

## LIST OF PUBLICATIONS

### Publication

Shobana, C., Sani, D., Tan, S.N., Lim, C.W., Hasan, S., Mohtarrudin, N., Kirby, B., Ideris, A., Basri, H., Stanslas, J., & Christopher, T.S.Lim. (2018). Effect of Edible Bird Nest Extract On Lipopolysaccharide-Induced Impairment of Learning and Memory In Wistar Rats. *Evidence-Based Complementary and Alternative Medicine.* Article ID 9318789. p7.

Tan, S.N., & Christopher, T.S.Lim. (2019). Cryptococcal meningitis in an immunocompetent swiftlet rancher – first case report. Malaysia Journal of Medicine & Health Sciences. 15(1): 82-84.

### Poster

Tan, S.N., Shobana, C., Sani, D., Lim, C.W., Ideris, A., Stanslas, J., Christopher, T.S .Lim. 2014. *Safety Profile and Nutritional Content of Raw Cleaned Edible Bird's Nest.* Poster presentation in 2nd Edible Birdnest Industry Conference 2014, 25th-26th November, Marriott Hotel, Putrajaya. Malaysia. Article published by Department Of Veterinary Services. http://www.dvs.gov.my/dvs/resources/user_1/DVS%20pdf/Aneka%20Haiwan/poster%20papers/14_Tan_UPM.pdf.

Tan, S.N., Shobana, C., Sani, D., Lim, C.W., Ideris, A., Stanslas, J., Christopher, T.S. Lim. 2016. *The potential proliferation of cancer cell by Edible birdnest in Malaysia*. Poster presentation in 3rd Edible Birdnest Industry Conference 2016, 11th-12th October, Marriott Hotel, Putrajaya. Malaysia.

Tan, S.N., Shobana, C., Sani, D., Lim, C.W., Ideris, A., Stanslas, J., Christopher, T.S Lim. 2016. *Quantification of epidermal growth factor in Edible birdnest from different regions in Malaysia*. Poster presentation in 3rd Edible Birdnest Industry Conference 2016, 11th-12th October, Marriott Hotel, Putrajaya. Malaysia.

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