NUTRITIONAL ANALYSIS, METABOLOMICS AND PROTEOMICS OF EDIBLE BIRD NEST FROM DIFFERENT GEORAPHICAL REGIONS OF MALAYSIA

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By

DANISH ADLI BIN ZULKIFLI

Thesis Submission to the School of Graduate Studies Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Master of Science

December 2018

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DEDICATION

I would like to dedicate this thesis to my parents
who through tough and thin was right there beside me

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

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December 2018

Chairman: Rozaihan Binti Mansor, PhD
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Edible bird's nest (EBN) is made of the salivary secretions from the swiftlet species *Aerodramus fuciphagus*. It is well-known in traditional Chinese medicine as well as among Asian countries due to their beneficial effects on health. Previous studies have shown that consumption of EBN provides various health benefits such as promoting epidermal growth, inhibiting infection caused by influenza viruses and others. Nowadays EBN is prepared and incorporated into many food products including drinks, food additives and cosmetics. Despite the continuous demand on EBN, strong justification on the nutritional and medicinal benefits of the farmed and commercialised EBN is still lacking. This study compared the chemical, amino acid, mineral constituents and the metabolites and protein profiles using proteomic and metabolomic approaches between the commercialised EBN bought from a reputable drug store and farmed EBN collected from the Central, Southern, East Coast and East of Malaysia. The chemical composition of the EBN was determined according to the Food Analysis Laboratory Manual with slight modifications. The elemental analysis was done in accordance of the official AOAC method. The detection of amino acid was done with High Performance Liquid Chromatography (HPLC) with Pico-Tag Amino Acid Analysis Column. ^{1}H-Nuclear Magnetic Resonance (NMR) spectroscopy was then used to detect the metabolites in the EBNs while Chemomx NMR Suite 8.2 was used to analyse the detected metabolites. The analysis of the EBN's proteome was done through the use of two-dimensional gel electrophoresis (2-DGE) followed by matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF/TOF MS). The results showed that the farmed EBN was mainly comprised of protein followed by carbohydrate while the commercial EBN had similar protein and carbohydrate composition. The total amount of essential amino acid in commercial EBN was higher (237.9 mg/g protein) compared to the farmed EBN (156.81 –

236.6 mg amino acid/ g protein) with valine the highest found in both the farmed and commercialised EBN. For the elemental analysis, both farmed and commercialised EBN were found to have a good amount of calcium and potassium content. There were 26 metabolites identified and phenylalanine; an essential amino acid was found significantly in abundance in all farmed EBN but not in commercialised EBN. In addition, choline; a vitamin-like essential nutrient and acetic acid were also found in large amount in all farmed EBN while acetone and 3-hydroxyisovaleric acid were found high in commercialised EBN. A number of metabolites from chemical classes such as organic acids and amino acids were identified from the use of NMR spectroscopy. The proteins detected through the proteomic process includes complement C1q, alpha-(1,3)-fucosyltranferase 6, glutamine-rich protein and tropomyosin alpha-1 chain. The complement C1q is known to involve in the clearance of apoptotic cells and pathophysiology of the central nervous system while alpha-(1,3)-fucosyltranferase 6 involved in the protein glycosylation pathways. Tropomyosin alpha-1 chain involved in the regulation of the straited muscle contraction. The overall findings suggested that the nutritional analysis, metabolites profiling and the proteomic analysis can give a better understanding about Malaysian EBN, benchmarking procedure and quality determination of EBN in the future

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

ANALISIS NUTRITISI, PROTEOMIK DAN METABOLOMIK SARANG BURUNG WALET DARIPADA KAWASAN SEKITAR MALAYSIA

Oleh

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Sarang burung walet (EBN) terbuat dari rembesan air liur dari spesies burung walet *Aerodramus fuciphagus*. Ia terkenal dalam perubatan tradisional Cina dan juga di kalangan negara-negara Asia yang lain kerana kesan baiknya terhadap kesihatan. Kajian terdahulu menunjukkan bahawa pemakanan EBN memberi pelbagai manfaat terhadap kesihatan seperti menggalakkan pertumbuhan epidermis, menghalang jangkitan yang disebabkan oleh virus influenza dan lain-lain. Pada masa kini EBN disediakan dan dimasukkan ke dalam banyak produk makanan termasuk minuman, bahan tambahan makanan dan juga kosmetik. Walaupun tedapat permintaan berterusan terhadap EBN, justifikasi yang kuat mengenai manfaat pemakanan dan ubat-ubatan dari EBN yang diusahakan dan dikomersialkan masih kurang. Kajian ini membandingkan bahan kimia, asid amino, juzuk mineral dan profil metabolit dan protein menggunakan pendekatan proteomik dan metabolomik antara EBN komersial yang terdapat dari kedai ubat yang bereputasi dan EBN ternakan yang dikumpul dari kawasan Tengah, Selatan, Pantai Timur dan Timur Malaysia. Komposisi kimia EBN ditentukan berdasarkan Manual Laboratori Analisis Makanan dengan sedikit pengubahsuaian. Analisis unsur dilakukan mengikut kaedah AOAC rasmi. Pengesanan asid amino dilakukan dengan kromatografi cecair prestasi tinggi (HPLC) dengan Kolum Analisis Amino Acid Pico-Tag. ^{1}H spektrofotometri resonans magnet nukleus (RMN) kemudiannya digunakan untuk mengesan metabolit dalam EBN manakala Chemomx NMR Suite 8.2 digunakan untuk menganalisis metabolit yang dikesan. Analisa proteinom EBN dilakukan melalui penggunaan elektroforesis gel dua dimensi (2-DGE) diikuti dengan masa pengionan laser bantuan matriks masa penerbangan (MALDI-TOF / TOF MS). Keputusan menunjukkan bahawa EBN ternakan mempunyai komposisi protein yang lebih tinggi daripada karbohidrat manakala EBN yang dikomersial mempunyai protein dan komposisi karbohidrat yang serupa. Pengumpulan jumlah asid amino penting dalam EBN yang dikomersialkan adalah lebih tinggi (protein 237.9 mg / g) berbanding EBN (156.81 - 236.6 mg / g) yang diternak dengan valina adalah asid amino tertinggi dalam kedua-dua EBN yang diternak dan dikomersilkan. Untuk analisis unsur, kedua-dua farmasi dan komersil EBN didapati

mempunyai kandungan kalsium dan potassium yang baik. Terdapat 26 metabolit yang dikenal pasti dan fenilalanina; Asid amino penting didapati dengan banyaknya dalam kesemua EBN ternakan tetapi tidak dalam EBN yang dikomersialkan. Di samping itu, kolina; asid nutrien penting dan asid asetik juga didapati dalam jumlah besar di kesemua EBN ternakan manakala aseton dan asid 3-hidroksiisovalerik didapati tinggi dalam EBN yang dikomersialkan. Sejumlah metabolit dari kelas kimia seperti asid organik dan asid amino dikenal pasti daripada penggunaan spektroskopi NMR. Protein yang dikesan melalui proses proteomik termasuk pelengkap komplemen C1q, alfa-(1,3)-fucosiltranferase 6, protein tinggi glutamina and rantai alfa-1 tropomiosin. Komplemen C1q diketahui melibatkan apoptosis sel dan patofisiologi sistem saraf pusat manakala alfa-(1,3)-fucosiltranferase 6 yang terlibat dalam laluan glikosilasi protein. Rantai alfa-1 tropomiosin yang terlibat dalam pengawalan pengetatan otot yang berlainan. Penemuan keseluruhan mencadangkan bahawa analisis pemakanan, profil metabolit dan analisis proteomik dapat memberikan pemahaman yang lebih baik mengenai EBN Malaysia, kaedah untuk menanda aras dan menentukan kualiti EBN pada masa akan datang.

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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 are follows:

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LIST OF ABBREVIATIONS

°C	Degree celcius
%	Percentage
±	Plus-Minus
µL	Microliters
ANOVA	Analysis of Variance
BSA	Bovine Serum Albumin
cm	Centimeters
DNA	Deoxyribonucleic acid
EBN	Edible Bird's Nest
EGF	Epidermal Growth Factor
g	Grams
GC	Gas chromatography
GLU	Glutamic acid
h	Hour
H_2SO_4	Sulphuric acid
kDa	Kilodalton
L	Liters
LC	Liquid chromatography
LSD	Fisher Least Significant Difference
M	Mole
mg	Miligrams
mins	minutes
mL	Mililiter
NaOH	Sodium hydroxide
NMR	Nuclear Magnetic Resonance
TNF-α	Tumor necrosis factor alpha
TYR	Tyrosine
w/v	Weight per volume
w/w	Weight per weight

CHAPTER 1

INTRODUCTION

1.1 Background

Swiftlets are small insectivorous birds which can be found throughout South East Asia and the South Pacific region. They are roughly similar but are not closely related to swallows (*Hirundo rustica*) and sparrows (*Passer domesticus*) (C. Lim, 2006). Only a few out of the many swiftlet species during the breeding season uses the salivary secretion to produce edible bird's nest (Gausset, 2004) which compose mainly of mucin-like glycoproteins. Most species use saliva as an agent to bind leaf, moss or feathers into nests however swiftlets build their nest wholly based on their mucilaginous secretion which upon drying, forms a hard cement in a shape of cup (Sankaran, 2001). They are usually found in a large colony at their nesting and hunting sites which are in deep, dark caves. Most swiftlet species are able to use echolocation to navigate and hunt in the caves. (Chua & Zukefli, 2016)

The nests are thought to have first been consumed by Vietnamese which can be found along the coasts and islands (Thorburn, 2015). Harvesting the nests is a dangerous occupation as it requires the harvester to climb up to 60m to reach the walls and roofs of the caves. Overharvesting that occurred in many locations has contributed to some extinction issues on some swiftlet populations (Gausset, 2004; Thorburn, 2015). The history of swiftlet farming in Malaysia started as a cottage-style operation in natural caves (Gausset, 2004). As the industry expanded and the demand for BEN increased, collection of EBN from caves was not enough. Thus, to maintain the supply of the EBN, they made a simulated habitat of the swiftlet natural cave environment. The birds frequently breed colonially in old buildings as such colony can be observed in Penang, Malaysia between 1976 to 1977 (Langham, 1979). The swiftlet industry has grown drastically since then and Malaysia currently is the third largest EBN producer after Thailand and Indonesia (Ain & Jumri, 2014). The bird nest is occasionally refered to as 'The Caviar of the East' and 'Gold from the Sky' is coveted as one of the most expensive animal by products. Harvesting the EBN is considered a well-paid industry in many of the South East Asia countries.

Naturally, EBNs are in dull or dirty white to dull yellowish in colour but rarely, the red EBNs are seen in caves and swiftlet houses which can fetch a higher price than the white EBNs in the market (But et al., 2013). It was once believed that the red colour attains from the bird's blood of exhausted swiftlets hurrying to build their nests before laying eggs (But et al., 2013; Thorburn, 2015) but the truth is, environmental factors such as nitrifying bacteria in the nest cement is reacting to ammonia vapours from the swiftlet guano droppings causing the nest to turn red colour.

However, the EBN's quality is determined based of multiple factors such as size, type, colour, shape and origin. Due to all this factors, the uncertainty of the EBN quality has

caused the overall value of the Malaysian EBN to be unstable (Looi & Omar, 2016). In accordance to the expansion of the swiftlet industry, it is common to see non-genuine EBN being sold in the market. EBN adulteration has been an issue as EBN suppliers introduced various materials into the EBN to increase the net weight. Karaya gum, red seaweed, pork skin and also egg white were among the several common adulterants added to the EBN (Wu et al., 2007). These have been added to increase its weight before sale to obtain higher profits and were extremely difficult to detect its incorporation due to the similarity in texture, colour and appearance to the actual nest (Yang et al., 2014).

EBN is known to have many medicinal benefits and this is reflected in their nutritional content. It was thought to have medicinal properties as it was used as traditional Chinese medicine dated back to the Tang dynasty (618 AD). Current studies have confirmed that EBN has haemagglutination inhibiting properties against influenza viruses, proliferate cell, improve bone strength and dermal thickenss, reduce tumor protection, treating erectile dysfunction and oestroporosis (Chua et al., 2013; Matsukawa et al., 2011). Past reports from Marcone (2005) and Saengkrajang et al., (2012) have revealed that EBN is comprised of mostly protein and carbohydrate however although the protein content between these two researches is similar the carbohydrate and also the ash content of the EBNs were different. This has led to a conclusion that there are minor differences between EBN from different parts and areas of the world due to the swiftlet's local environment.

Further research found that main proteins found in the EBN is made out of glycoproteins which are proteins attached to a carbohydrate strand while the main composition of the carbohydrate of the EBN was sialic acid. This has led the researchers to believe that the nutritional and medicinal benefits of the EBN were from these macro molecules and tend to focus the research on these part of the EBN (Shim et al., 2017). Although proteomic of the EBN was thought to be key to unlock the secrets to the health benefits of the EBN, some researchers shift the focus to the metabolites of the EBN to add an alternative perspective to this food product. This has led to over 78 metabolites to be detected and screened to compare the metabolites present with other regions (Chua et al., 2014). The minor differences in the metabolites of the EBN from different regions was also concluded to be because of the food source of the swiftlets which came from the local environment.

In contrast to the rapid growth of the EBN demand and value, there are still limited research on the nutritional and the medicinal properties of the EBN especially since these properties tend to vary due to the harvesting location and time. (Norhayati et al., 2010).

1.2 Problem statement and justification

EBN is a highly priced food product and it is expected to improve significantly in the near future in Malaysia with trends of demand and value increasing every year. However, in contrast to the rising demand and value, the scientific investigation on the nutritional as well as the medicinal properties of Malaysian EBN is still limited compared to neighboring countries such as Thailand. The Malaysian EBN is not well characterized and there are limited number of metabolite and proteins found in the Malaysian EBN. Currently there is no set benchmark for the authentication or to determine the quality of the EBN. This research intends to thoroughly investigate the nutritional values of the EBN collected from different regions of original Malaysian EBN through the nutritional, proteomics and metabolomics study of the EBN and also comparing them to the processed and commercialised EBN to further add and improve the library of determined metabolite and proteins. With the improved understanding of the EBN, the method to benchmark and determine quality of the EBN could be determined by future research.

1.3 Objectives and aims

Objectives

1. To analyse and compare the nutritional content of different types of EBN
2. To profile and compare metabolites present in different types of EBN
3. To identify protein/peptides from different types of EBN

Hypothesis

1. The nutritional content, proteomic and metabolite content of the farmed EBN and commercialised EBN is different.
2. There are differences in the nutritional content, proteomic and metabolite content between farmed EBN

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