EDIBLE BIRDS NEST - CHEMICAL COMPOSITION AND POTENTIAL HEALTH EFFICACY AND RISKS

EDITED BY: Karl Tsim, Ting Hun Lee, Geng Li and Gallant Chan
PUBLISHED IN: Frontiers in Pharmacology

frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88974-491-6
DOI 10.3389/978-2-88974-491-6

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EDIBLE BIRDS NEST - CHEMICAL COMPOSITION AND POTENTIAL HEALTH EFFICACY AND RISKS

Topic Editors:
Karl Tsim, Hong Kong University of Science and Technology, Hong Kong, SAR China
Ting Hun Lee, University of Technology Malaysia, Malaysia
Geng Li, Other, China
Gallant Chan, Hong Kong University of Science and Technology, Hong Kong, SAR China

Citation: Tsim, K., Lee, T. H., Li, G., Chan, G., eds. (2022). Edible Birds Nest - Chemical Composition and Potential Health Efficacy and Risks. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88974-491-6

Table of Contents

frontiers in Pharmacology

EDITORIAL
published: 28 January 2022
doi: 10.3389/fphar.2021.819461

Editorial: Edible Bird's Nest—Chemical Composition and Potential Health Efficacy and Risks

Geng Li[1]*, *Ting Hun Lee*[2], *Gallant Chan*[3] and *Karl Wah Keung Tsim*[3]

[1]Laboratory Animal Center, School of Chinese Pharmaceutical Science, Guangzhou University of Chinese Medicine, Guangzhou, China, [2]School of Chemical and Energy Engineering, Faculty of Engineering, Universiti Teknologi Malaysia, Johor, Malaysia, [3]Division of Life Science and Center for Chinese Medicine, The Hong Kong University of Science and Technology, Hong Kong, China

Keywords: edible bird's nest, chemical composition, potential health efficacy, risk, anti-aging effect

Editorial on the Research Topic

Edible Bird's Nest—Chemical Composition and Potential Health Efficacy and Risks

Edible bird's nest (EBN) is a popular delicacy in the Asian Pacific region: this is a salivary secretion from several *Aerodramus* spp. swiftlets, mainly *Aerodramus fuciphagus*, which originated in Southeast Asian countries, including Indonesia, Malaysia, Thailand, and Vietnam. The historical record of EBN for human consumption can be tracked back to about 1,500 years ago in China. Today, the consumption of EBN as food or as a medicinal product is very common in Asian populations. According to ancient literature, EBN is able to alleviate respiratory health conditions, such as asthma, and is believed to have an impact in skin physiology. Despite the potential functional roles and popularity of this ethnomedicine in Asian cultures, the pharmacological research on EBN is still very limited. These claims of beneficial effects are questionable due to the lack of strong supporting experimental evidence. On the other hand, the production of EBN today is totally depending on swiftlets. The natural habitat of swiftlets is varied according to their surrounding environment, which therefore greatly affects the final product of EBN. Foreign contaminants not related to bioactive proteins or peptides secreted from the swiftlet are frequently identified. The chemical variation, as well as possible contaminants, of EBN is inevitable, leading to possible risks for the consumers. Nevertheless, the scientific community should provide experimental evidence in identifying the chemical composition, pharmacological property, and diversity of contaminants of EBN.

A total of 10 articles involved in the understanding of EBN in terms of chemical composition and functional properties are included in this research topic, including six original articles and four reviews. Seventy-two authors form Malaysia and China participated in this research topic, which reflected that EBN attracted the attention of many scholars.

In the chemical analysis of EBN, Yeo *et al.*, in this collection, have summarized the possible contaminants in EBN, which included adulterants, chemicals, and microbials, as well as the current methods in measuring the authenticity and chemical composition. Moreover, the possible sources of contaminants have been proposed to be derived from swiftlet farms, processing, storage, and transportation of EBNs. The design and management of swiftlet houses as well as EBN post-processing could greatly affect the EBN color. The review also provided the variation of chemical composition, as well as beneficial effects, of EBN, which could depend on various factors, such as geographical location, harvesting place, harvesting season, and processing procedure. The authors have proposed that standardized EBN processing methods are required to better preserve the bioactive effects. Besides this, the health concerns of EBN consumption, as caused by adulterant, chemical, and microbial contaminations, should be addressed by strict adherence to standardized guidelines. Several studies demonstrated that the plausible authentication methods could be

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Edited and reviewed by:
Javier Echeverria, University of Santiago, Chile

***Correspondence:**
Geng Li
lg@gzucm.edu.cn

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: *21 November 2021*
Accepted: *25 November 2021*
Published: *28 January 2022*

Citation:
Li G, Lee TH, Chan G and Tsim KWK (2022) Editorial: Edible Bird's Nest—Chemical Composition and Potential Health Efficacy and Risks. Front. Pharmacol. 12:819461. doi: 10.3389/fphar.2021.819461

translated into industrial settings but would require further development in terms of cost-effectiveness and equipment availability.

In the pharmacological analysis of EBN, Chua *et al.* have provided evidence of EBN in suppressing replicated virus within host cells, which therefore led to reduced viral replication, endosomal viral trafficking, intracellular viral autophagy, secretion of pro-inflammatory cytokines, reorganization of the actin cytoskeleton, and increase in lysosomal degradation of viral materials. In addition, the authors have discussed and proposed the potential application of EBN in clinical treatments of influenza A and coronavirus.

Lee *et al.* have reviewed 124 articles from the Web of Science, which consisted of 119 original research articles and five review papers. They reviewed the possible roles of EBN in anti-aging effects, inhibition of influenza virus infection, alternative treatments in athletes and cancer patients, corneal wound healing, stimulation of human adipose-derived stem cells, potentiation of mitogenic responses, epidermal growth factor-like activities, enhancement of bone strength and dermal thickness, eye care, and neuroprotective and antioxidant effects.

On the other hand, Lim *et al.* have provided experimental evidence to support the role of EBN in preventing kidney injury, as induced by gentamicin, in a rat model, suggesting the reno-protective properties of EBN. They found that pre-treatment with 125, 250, and 500 mg/kg EBN has significantly resulted in lesser scores than the model group. However, the protocol that they used in serum renal profiles as a measurement of kidney function will not be a suitable method to assess renal function in such a short duration of an experiment like ours. Thus, they proposed that future similar trials should have a minimum of 14 days post-intervention to have a better assessment of renal recovery.

Rashed *et al.* have found that the cell viability results showed that there are no cytotoxicity effects in all neuroblastoma and epithelial cell lines when exposed to sialic acid at a concentration of 60 μg/ml or below. The Tukey *post-hoc* test also revealed that the number of active mitochondria in SH-SY5Y is significantly higher when induced with sialic acid compared with the control. The number of active mitochondria could be increased by 195% after the treatment of sialic acid, a major active ingredient of EBN, in cultured SH-SY5Y cell lines. Thus, the authors have proposed the possible use of EBN in treating Alzheimer's disease.

Fan *et al.* have found the protective effect of EBN in dextran sulfate sodium-mediated colitis in mice: this event could be mediated mainly by restoring the balance of T helper 17/regulatory T cells. Moreover, the authors have found that EBN not only restored β-cell function and insulin signaling by attenuation of oxidative stress-mediated chronic inflammation in type 2 diabetic mice but also enhanced the sperm concentration, sperm motility, as well as the levels of FSH and LH. The characterization of EBN through liquid chromatography–mass spectrometry has identified testosterone as one of the peaks. Besides this, amino acids, estradiol, and sialic acid were among the major peaks being identified.

In terms of the cosmetic function of EBN, Lai *et al.* have revealed that the effects of enzymatic digested EBN showed robust skin moisturizing effects as demonstrated in both *in vitro* and *ex vivo* models. In cultured keratinocytes, the expressions of S100-fused type proteins contributing to skin barrier function in the stratum corneum, *e.g.*, filaggrin and filaggrin-2, were determined in both mRNA and protein levels, which were markedly induced when treated with digested EBN extract. These lines of evidence therefore suggested the water moisturizing effect of EBN in skin function.

In summary, this research topic has a collection of articles on the chemical and pharmacological analyses of EBN. The critical parameters in standardizing EBN during production have been given. The pharmacological functions of EBN includes reno-protection, skin moisturizing, mitochondrial protection, relieving oxidative stress and inflammation, improving type 2 diabetes, and enhancing male reproduction. This comprehensive description of EBN should stimulate the investigation and application of EBN.

AUTHOR CONTRIBUTIONS

GL, TL, and GC wrote this article. KT reviewed this article.

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frontiers in Pharmacology

REVIEW
published: 23 March 2021
doi: 10.3389/fphar.2021.631136

Potential Residual Contaminants in Edible Bird's Nest

Bee-Hui Yeo[1], *Teck-Kim Tang*[1], *Shew-Fung Wong*[2], *Chin-Ping Tan*[3], *Yong Wang*[4], *Ling-Zhi Cheong*[5] *and Oi-Ming Lai*[1,6]*

[1]Institute of Bioscience, Universiti Putra Malaysia, Serdang, Malaysia, [2]School of Medicine, International Medical University, Centre for Environmental and Population Health, Institute for Research, Development and Innovation (IRDI), International Medical University, Kuala Lumpur, Malaysia, [3]International Joint Laboratory on Plant Oils Processing and Safety (POPS) JNU-UPM, Department of Food Technology, Faculty of Food Science and Technology, Universiti Putra Malaysia, Serdang, Malaysia, [4]International Joint Laboratory on Plant Oils Processing and Safety (POPS) JNU-UPM, Department of Food Science and Engineering, College of Science and Engineering, Jinan University, Guangzhou, China, [5]Department of Food Science and Engineering, College of Food and Pharmaceutical Sciences, Ningbo University, Ningbo, China, [6]Department of Bioprocess Technology, Faculty of Biotechnology & Biomolecular Sciences, Universiti Putra Malaysia, Serdang, Malaysia

Edible bird's nest (EBN) is recognized as a nourishing food among Chinese people. The efficacy of EBN was stated in the records of traditional Chinese medicine and its activities have been reported in many researches. Malaysia is the second largest exporter of EBNs in the world, after Indonesia. For many years, EBN trade to China was not regulated until August 2011, when a safety alert was triggered for the consumption of EBNs. China banned the import of EBNs from Malaysia and Indonesia due to high level of nitrite. Since then, the Malaysia government has formulated Malaysia Standards for swiftlet farming (MS 2273:2012), edible bird's nest processing plant design and management (MS 2333:2010), and edible bird's nest product quality (MS 2334:2011) to enable the industry to meet the specified standards for the export to China. On the other hand, Indonesia's EBN industry formulated a standard operating procedure (SOP) for exportation to China. Both countries can export EBNs to China by complying with the standards and SOPs. EBN contaminants may include but not limited to nitrite, heavy metals, excessive minerals, fungi, bacteria, and mites. The possible source of contaminants may come from the swiftlet farms and the swiftlets or introduced during processing, storage, and transportation of EBNs, or adulterants. Swiftlet house design and management, and EBN processing affect the bird's nest color. Degradation of its optical quality has an impact on the selling price, and color changes are tied together with nitrite level. In this review, the current and future prospects of EBNs in Malaysia and Indonesia in terms of their quality, and the research on the contaminants and their effects on EBN color changes are discussed.

Keywords: edible bird's nest, nitrite level, heavy metal, color changes, bacteria, fungi

OPEN ACCESS

Edited by:
Geng Li,
Guangzhou, China

Reviewed by:
Yang Liu,
Peking University Third Hospital, China
Xu-Dong Zhou,
Hunan University of Chinese Medicine, China
Karl Tsim,
Hong Kong University of Science and Technology, Hong Kong

***Correspondence:**
Oi-Ming Lai
omlai@upm.edu.my

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: *19 November 2020*
Accepted: *04 February 2021*
Published: *23 March 2021*

Citation:
Yeo B-H, Tang T-K, Wong S-F, Tan C-P, Wang Y, Cheong L-Z and Lai O-M (2021) Potential Residual Contaminants in Edible Bird's Nest. Front. Pharmacol. 12:631136. doi: 10.3389/fphar.2021.631136

INTRODUCTION

Bird's nest is built by swiftlet using secretions from its salivary glands under the tongue. Nesting can be used as a shelter for canaries to breed and roost. There are more than 24 species of insectivorous, echolocation swiftlets all over the world (Hamzah et al., 2013). However, only two species of swiftlets are responsible for producing commercially valuable edible bird's nest (EBN) currently. They are species of the *Apodidae*, *Collocalia*, i.e., *Aerodramus fuciphagus* (white nest swiftlets) and

Aerodramus maximus (black nest swiftlets). EBNs are nests made from the regurgitated saliva. During reproduction, mucin glycoproteins are secreted in saliva from a pair of unique sublingual glands beneath the swiftlet's tongue, repeatedly interwoven together to build the half bowl shaped, palm size, white nests (Shim and Lee, 2018). When fresh, the nested cement (glutinous secretion/mucin glycoprotein) is soft and viscous, but gradually dries and hardens when exposed to air (Lim and Cranbrook, 2002). EBNs from *A. fuciphagus* are favored by processors and traders because they are predominantly hardened nest-like cement with traces of feathers and impurities (Seow et al., 2016), while bird's nest from others with more feathers and impurities. EBNs are mainly produced in Southeast Asia.

Swiftlets may come from the swiftlet's house/cave in the morning and forage in the wild like on grass, rice fields, hills, rivers or waters, valleys and trees. Swiftlets do not and never live in any place other than nests, so as to avoid direct interaction or contact with other birds or poultry on land. After looking for food, usually in the afternoon or evening (before dark), the swiftlets return to their own nests. They only return to swiftlet house/cave to rest and lay eggs. Some swiftlets are kept in swiftlet house designed to resemble cave conditions. Swiftlets cannot be cultivated because there is no substitute for food and swiftlets are very dependent on their nature environment. If disturbed or undernourished, swiftlets may not return again to their own nests (Badan Karantina Pertanian, 2013). The diet of swiftlets are mainly insects. The swiftlets perform all their activity in flight, including mating and eating, because swiftlets cannot perch. The EBN in the farmhouse (called the house EBN) is a nest built in the man-made swiftlet house that mimics the cave structure. These swiftlets are attracted by the recorded swiftlet sounds and build nests in the swiftlet house. On the other hand, the cave EBN is the edible bird's nest in the cave, built by the swiftlet from the natural nesting activities (Quek et al., 2015).

For centuries, EBN is well-known in the Chinese community for its nutritional and medicinal values, is an important ethnomedicinal commodity. The first consumption of EBN dated back to the Tang Dynasty (618–907 AD), when it was the supreme delicacy and was sent to the court of the Chinese emperor. Since then, the medicinal value of EBN has been well documented, and it was later recognized as one of the four major supplements in the late Ming (1405–1433 AD) and early Qing (AD 1644–1911 AD) Dynasties (Chye et al., 2017). EBN is believed to have nourishing effects, such as strengthening the immune system, treating malnutrition, improving metabolism, enhancing skin complexion, relieving asthma, helping to clear sputum, reducing coughs, nourishing children, improving libido, enhancing kidney function, recovery from diseases and surgery rehabilitation, and improving concentration (Tong et al., 2020).

The beneficial properties of EBN have also been proven through modern science and technology, revealing its nutritional values and pharmacological activities, including 1) body maintenance and enhancement of the immune system (Marcone, 2005; Ma and Liu, 2012); 2) stimulation of cell growth (Kong et al., 1987); 3) anti-inflammatory effect (Vimala et al., 2012); 4) protection from joint degeneration and chondro-protection against osteoarthritis (Matsukawa et al., 2011; Chua et al., 2013); 5) enhancement of antioxidant capacity-antioxidative effect (Hu et al., 2016); 6) anti-influenza or as anti-viral agent (Guo et al., 2006; Haghani et al., 2016; Hu et al., 2016); 7) skin whitening, anti-aging, anti-inflammatory and wound healing (Zeng and Lai, 2019; Hwang et al., 2020); 8) promotion of corneal wound healing (eye caring) (Zainal Abidin et al., 2011); 9) improvement of stem cell proliferation (Roh et al., 2012); 10) learning and memory functions of multi-generational mice - (Haghani et al., 2016; Careena et al., 2018; Xie et al., 2018; Khalid et al., 2019; Mahaq et al., 2020); 11) neuroprotection in Alzheimer's or Parkinson's disease (Hou et al., 2017; Yew et al., 2018); 12) anti-obesity effects (Yida et al., 2015); 13) prevention of cardio-metabolic and diabetic diseases (Hou et al., 2015); 14) anti-hypertensive effect (Ramachandran et al., 2018); and 15) amelioration of the detrimental effects of lead acetate (LA) toxicity in the uterus (Albishtue et al., 2019). Therefore, EBN has proved its nutritional and therapeutic values.

Raw uncleaned (RUC) EBNs are EBNs harvested from caves and ranches (swiftlet house) which may include sorting, drying, grading, trimming, weighing and packing without any cleaning process and raw-cleaned (RC) EBNs are EBNs that have undergone cleaning processes such as sorting, soaking, picking of feathers and impurities, molding, drying, grading and packing (Department of Standards of Malaysia, 2010). The RC EBN products can be classified as bird's nest cup (largely maintains its original shape), bird's nest strip, bird's nest coner/head (hard ends on both sides of original RUC EBN), bird's nest crumble (fragments and net of EBN that were collected after processing and packed together), and bird's nest cake/biscuit (in which EBN fragments or strips or combination are packed into a mold of any shape) (Xing et al., 2012). Traditionally, RC EBN purchased from shop is cooked by double boiling method. The bird's nest, rock sugar and water are placed in an inner pot, and the inner pot is immersed in an outer pot at about 100°C. The stewed product is the bird's nest soup.

The World Health Organization reports that an estimated 600 million people (approximately one tenth) of the world get sick after eating contaminated food each year, and 420,000 people die each year, resulting in 33 million healthy life years. The WHO also emphasized that nutrition and food safety are inseparable. Unsafe food can lead to a vicious circle of disease and malnutrition, especially affecting infants, young children, the elderly and the sick (WHO, 2020a). EBN is nutritious as listed above, but what about the safe consumption of EBN? This review summarizes previous studies on potential residual contaminants in EBNs, including 1) issues and regulations regarding EBN; 1) nitrite and nitrate content in EBN; 2) the color of EBN; 3) bacteria, fungi and mites in EBN; 4) allergens in EBN; and 5) heavy metals and excessive mineral contents in EBNs and including different stages of EBN, i.e., RUC EBN, RC EBN and EBN after treatment.

ISSUE AND REGULATIONS REGARDING EBN

China is the largest importer of raw clean (RC) EBN products, accounting for 82% of global trade (Zhang et al., 2020). The bird's nest industry in Malaysia and Indonesia suffered a severe blow in 2011 when China banned exporters' EBNs due to high concentrations of nitrate, lead and arsenic in certain products. The price of raw uncleaned EBNs plummeted by 50% (Ramalingam, 2014). In 2014, bird's nest products could be exported to China again. EBN products exported from Malaysia and Indonesia need to be cleaned in accordance with standard operating procedures to reach the EBN safety level required by China. The industry still suffers huge economic losses, and the price of bird's nest remained stable after 2016 (Chan et al., 2018).

Subsequent to the ban, Malaysia and Indonesia had a few bilateral discussions with the Chinese Authorities and on the April 24, 2012, a Memorandum of understanding (MOU) on the Protocol of Inspection, Quarantine and Hygiene Requirements for the Importation of Bird Nest Products from Indonesia into China was sealed and signed (Badan Karantina Pertanian, 2018); the protocol was sealed and signed between Malaysia and Indonesia on September 19, 2012 (Rohaizan, 2017). Every RC EBN exported to China must comply with this protocol to ensure food safety.

In order to meet the requirements listed in the protocol, Indonesia stipulates that every EBN exported to China should have a traceability system, free from avian influenza, and its nitrite content should be less than 30 ppm (Yusuf et al., 2020). Here is the guidelines provided by Indonesia which can be obtained from the website under Ministry of Agriculture Indonesia government - https://karantina.pertanian.go.id/media.php?module=home: 1) "Pedoman Persyaratan dan Tindakan Karantina Hewan Terhadap Sarang Walet dari Wilayah Negara Republik Indonesia ke Republik Rakyat Cina"- Guidelines for Animal Quarantine Requirements and Measures on EBN, including the traceability system; 2) "Pedoman Pemantauan Karantina Terhadap Pengeluaran Sarang Walet dari Wilayah Negara Republik Indonesia ke Republik Rakyat Tiongkok" - Quarantine and monitoring guidelines for the export of bird's nest from the territory of the Republic of Indonesia to the People's Republic of China; 3) "Pedoman Pemanasan Sarang Walet untuk Pengeluaran ke Negara Republik Rakyat Tiongkok" – Guidelines for Heat Treatment of EBN for Expenditure to the People's Republic of China, to ensure free from avian influenza; 4) "Pedoman Pemeriksaan Kandungan Nitrit Sarang Walet untuk Pengeluaran ke Negara Republik Rakyat Tiongkok" - Guidelines for Examination of EBN Nest Nitrite Content for Exportation to the People's Republic of China, to make sure the nitrite content is less than 30 ppm.

For Malaysia, the requirement listed in the protocol to China also included requirement of traceability system, free from avian influenza and the nitrite content <30 ppm. The quality of RC EBNs from Malaysia for export to China is set by Department of Standard Malaysia as Malaysia Standard. These Malaysia standards include: 1) MS 2333:2010 Good Manufacturing Practice (GMP) For Processing Raw-Unclean and Raw-Clean Edible-Birdnest (EBN); 2) MS2334:2011 Edible- Birdnest (EBN)- Specification; 3) MS 2612:2015 Raw-Unclean Edible Birdnest (EBN)- House nest- Specification; and 4) MS 2509:2012 (P) Test method for Edible-birdnest (EBN)- Determination for nitrite (NO_2^-) and nitrate (NO_3^-) content. It is worth noting that, besides the above standards, the Malaysia standards also provides standards for farming EBN's swiftlet, i.e., MS 2273:2012 Good Animal Husbandry Practice - Edible-nest Swiftlet ranching and its premises. MS 2273: 2012 and MS 2333: 2010 are the two important references for EBN industry, inside the standards including a few guidelines to reduce the potential contamination in EBN. Where MS 2273:2012 has showed a few guidelines in ranching practices of edible-nest swiftlet, including the ranch design and maintenance; hygiene of premises; and sign of illness of swifltet. On the other hand MS 2333: 2010 has showed guidelines in designing the processing premises to avoid cross-contamination; control procedure of operation; premise and personal hygiene; and hygiene control system (control contaminant). Those guidelines can reduce the heavy metal, nitrite and microorganisms content in EBNs. Malaysia standard can be purchased from the Department of Standards Malaysia (http://www.standardsmalaysia.gov.my) or SIRIM Berhad (http://sirim.my). Standards and Industrial Research Institute of Malaysia (SIRIM) and Department of Veterinary Service Malaysia (DVS) have published a guideline which is freely available: SIRIM/DVS 2:2014 Requirements for Traceability of Raw Edible- Birdnest (EBN). The Food Safety and Quality Division (FSQD), Ministry of Health (MOH) is the Competent Authority that establishes food safety over the EBN products supply chain in order to ensure that the edible bird's nest products produced will be safe for human consumption by complying to the Food Act 1983 and Food Regulations 1985, Food Hygiene Regulations 2009 as well as the importing countries' requirements. FSQD has published a few reference documents: 1) Compliance Listing and Verification Protocol for Export of Raw Clean EBN To China; 2) Standard Operating Procedure (SOP) on the Control of the Safety of Raw EBN Along the Food Supply Chain; 3) SOP on the Control of Nitrite Level in EBN; 4) SOP for Monitoring of Raw Clean EBN; and 5) SOP for Issuance of Health Certificate for the Export of Raw Clean EBN To China. These regulations are implemented and enforced by the Department of Veterinary Services of the Ministry of Agriculture and Food Safety and Quality Division of the Ministry of Health, Malaysia. For meeting the export requirements, an important step is that the EBN products must be processed with heat treatment; the core temperature of the products shall be lower than 70°C and retained at least 3.5 s to effectively kill avian influenza virus.

As an importing country, the Chinese authorities (Chinese Academy of Inspection and Quarantine, CAIQ) have issued two documents on the production of RC EBNs. They are 1) CAIQ-RZ-2015001 Bird's Nest Product Processing Enterprise, Hygienic Technical Specifications; and 2) CAIQ-RZ-2015002 Bird's Nest Product Certification Implementation Rules. These two documents can be obtained from the website http://ebn.caiq.

TABLE 1 | Malaysia and Indonesia Standards RC EBNs for export to China.

Category	Parameters	Tolerance level		
		Malaysia	Indonesia	China
Physical	Feather and dirt contamination	N/A	Looks clear of hair and visual debris from the naked eyes at distance of 20–30 cm	N/A
	Metal and wood contamination	N/A	Nil from the naked eyes at distance of 20–30 cm	N/A
Microbiology	Total Plate Count	≤2.5 × 10^6 cfu/g	≤1 × 10^6 cfu/g	≤1 × 10^6 cfu/g
	Coliforms	≤1,100 MPN/g	≤100 cfu/g	≤100 cfu/g
	Escherichia coli	≤100 MPN/g	≤10 cfu/g	N/A
	Salmonella sp.	Nil	Nil	Nil
	Staphylococcus aureus	≤100 MPN/g	≤100 cfu/g	≤100 cfu/g
	Yeast and mold	≤10 cfu/g	N/A	≤10 cfu/g
Residue	Nitrite	≤30 ppm	≤30 ppm	≤30 ppm
	Hydrogen peroxide	Nil	N/A	N/A
	Sulfur dioxide	N/A	N/A	Nil
Heavy metal	Lead (Pb)	≤2 ppm	N/A	≤2 ppm
	Arsenic (As)	≤1 ppm	N/A	≤1 ppm
	Mercury (Hg)	≤0.05 ppm	N/A	≤0.05 ppm
	Cadmium (Cd)	≤1 ppm	N/A	≤1 ppm
Excessive mineral	Copper (Cu)	≤1 ppm	N/A	N/A
	Iron (Fe)	≤0.3 ppm	N/A	N/A

org.cn/. The content of CAIQ-RZ-2015001 includes the guidelines for RUC EBNs to RC EBNs processing controls and processing premises while the content of CAIQ-RZ-2015002 includes the guidelines on requirements for the procedures and management of bird's nest product certification activities carried out by certification agencies and this includes the standard of RC EBNs. **Table 1** shows the quality of RC EBNs (house nest only) from Malaysia and Indonesia for the export to China. The tolerance levels of different parameters associated with RC EBNs are obtained from MS 2334:2011 for Malaysia Standard; Pedoman Persyaratan dan Tindakan Karantina Hewan Terhadap Sarang Walet dari Wilayah Negara Republik Indonesia ke Republik Rakyat Cina for Indonesia Standard; and CAIQ- RZ-20052 for China Standard. The methods for testing for the parameters were not mentioned in detail for all the parameters. Only heavy metals were mentioned in MS 2334:2011 where AOAC Atomic Absorption Spectrophotometer (AAS) method was used; and nitrite method mentioned in Indonesia Standard uses spectrophotometry or high performance liquid chromatography (HPLC).

NITRATE AND NITRITE CONTENT IN EBN

In August 2011, the Chinese government banned EBN products imported from overseas because of the high levels of nitrite (NO_2) detected in these edible bird's nest products. The highest content of nitrite reached 11,000 ppm (cave EBN). According to the report of the Chinese government in Zhejiang Province, the discovery of nitrite pollution in 2011 has aroused public concerns about the safety of EBN consumption. It also aroused the public's suspicion whether these edible EBNs are really "edible" (Paydar et al., 2013; Quek et al., 2015; Chan et al., 2018).

Nitrate (NO_3) consists of one nitrogen atom and three oxygen atoms; while nitrite (NO_2) consists of one nitrogen atom and two oxygen atoms. Nitrite and nitrate are natural chemicals in our food and water. Nitrate is relatively inert and stable, it is unlikely to change and cause harm. Nitrite may become: nitric oxide, which is good for the body; or nitrosamines, which may be harmful. Nearly all manufacturers add nitrite to the meat to protect them, where nitrite is used as a food additive and a preservative, however, the usage is under a strict regulation. In meat, nitrite is converted to nitric oxide. This will react with the protein in the meat, change its color and preserve the meat (Gunnars, 2020).

The daily intake of nitrite acceptable to the World Health Organization is 0–3.7 mg/kg body weight per day or 222 mg/day for 60 kg adults. In the body, nitrite can be converted into nitric oxide (signaling molecule) that can cause blood vessels to dilate and lower blood pressure. When nitrite and amino acids coexist, carcinogenic compounds called nitrosamines are formed during high-temperature cooking (Gunnars, 2020). In order to obtain optimal cardiovascular health and consider potential negative health risks of nitrate and nitrite intake in the diet, foods containing nitrate and nitrite should be guided by reasonable diet (Bedale et al., 2016) and the daily intake limit must not be exceeded.

Table 2 shows the nitrite data from relevant literatures. The data include nitrite from raw uncleaned (RUC), raw cleaned (RC, after processing), house nest, cave nest, different color EBNs and from different parts of EBNs. The table shows that most of the nitrite in white RC house EBNs is less than 30 ppm but not the RC cave nests. The cave nest has a higher nitrite concentration than the house nest. Even after processing, the data shows that the nitrite level of most cave nests is still higher than the allowable level (30 ppm). After processing, the nitrite level of RC EBN is significantly lower than that of RUC EBN, but it is worth paying attention to how effective the processing is for different parts of

TABLE 2 | Nitrite and nitrate levels in different colors of EBNs.

NO	References	Color of EBNs (visual observation description/measurement)	Nitrite (ppm)	Nitrate (ppm)	Source of sample
1	Chan (2013); Chan et al. (2013)	Red Yellow White	600 (median) 510 (median) 100 (median)		RC: 48 randomly purchased cubilose from Hong Kong market. 25 White EBN, 6 Yellow EBN, and 17 Red EBN. All of these EBN samples were imported from Indonesia, Malaysia, Thailand and Vietnam
2	Hamzah et al. (2013)		28.4 (RUC) 0.4 (RC) 10.2 (RUC) 0.2 (RC) 8.5 (RUC) 0.5 (RC)	349.3 (RUC) 1.2 (RC)	Cave nest from Langkawi, Malaysia Java, Indonesia Balikpapan, Indonesia
3	Paydar et al. (2013)	White Brown Red	7.9, 12.9, 22 47.44, 212.9 65, 39.2	20.4, 23.7, 87.5 12,168.2, 2128.6 30,016.7, 30,016.7	RC: Three house-EBNs, Malaysia RC: Two brown cave nests, Southeast Asia RC: Two red cave nests, Southeast Asia
5	Quek et al. (2015)	Off white to ivory L* value: 50.7 ± 1.7 a* value: 2.1 ± 0.2 B* value: 15.3 ± 0.8	5.7 ± 6.7	98.2 ± 33.7	RC EBN: Four house nests as from Segamat, Johor; Kapar, Selangor; Nibong Tebal, Penang and Sarikei, Sarawak. Cleaned in lab
		Light brown L* value: 37.2 ± 0.8 a* value: 3.0 ± 0.4 B* value: 13.6 ± 0.5	843.8 ± 460.9	36,999.4 ± 38,738.7	RC EBN: Four cave nests as from Gua Gomantong, Sabah; Gua Niah, Sarawak (2 samples); and Gua Subis, Sarawak. Cleaned in lab
6	Susilo et al. (2016)	White	93.12 ± 4.4		RUC EBN : Kalimantan Selatan, Indonesia
7	Quek et al. (2018)	L* value: 50.43 ± 1.84 a* value: 2.08 ± 0.22 B* value: 15.83 ± 1.27	31.63 ± 54.99	133.43 ± 79.22	RUC: Five house nests, four from Peninsular Malaysia, one from East Malaysia. All are *A. fuciphagus* nests
		L* value: 42.19 ± 7.45 a* value: 4.09 ± 1.73 B* value: 17.30 ± 5.47	702 ± 473	31,992 ± 29,569	RUC: Six cave nests, All from East Malaysia. Two samples are *A. fuciphagus* nests and four samples are *A. maximus* nests
8	Sirenden et al. (2019)	White	7.8 (body of EBN) 4.8 (nest of EBN) 17.4 (head of EBN)		RC EBN: PT. Waleta Asia Jaya. Bahan baku dari Kecamatan Sepaku, Kabupaten Penajam Paser Utara, Kalimantan Timur
9	Yusuf et al. (2020)		32.4, 66.5 and 47.9 7.6, 4.8, and 23.6 164.9, 48.8, 136.8		RUC EBN: Wajo Regency, South Sulawesi, Indonesia RUC EBN: Pare-pare Regency, South Sulawesi, Indonesia RUC EBN: Pinrang regency, South Sulawesi, Indonesia
10	Tan et al. (2020)	Whitish	10.1 ± 0.4 10.4 ± 0.2 18.4 ± 0.4 15.8 ± 0.1 11.0 ± 0.2 10.3 ± 0.1 11.4 ± 0.2	24.9 ± 0.5 39.4 ± 1.0 52.6 ± 0.9 47.0 ± 0.6 41.5 ± 0.5 31.1 ± 0.5 35.9 ± 0.1	RC: Alor Setar, Malaysia RC: Sibu, Sarawak RC: Rompin, Pahang, Malaysia RC: Kuala Selangor, Selangor RC: Johor Bahru, Johor RC: Jerantut, Pahang, Malaysia RC: Port Klang, Selangor, Malaysia

EBN. Sirenden et al. (2019) showed that the nitrite in different parts of the same EBN is different after processing and suggested that the thickness of each part of the bird's nest and also the area of the contact surface during processing affects the decrease in nitrite levels.

The results also showed that the nitrate concentration of both house nests and cave nests is always much higher than that of nitrite. Quek et al. (2015) suggested that this situation is due to the stability of nitrate, and nitrate may also be produced by the oxidation of nitrite. Chan et al. (2013) showed that the nitrite content of EBN has a huge range, from non-detectable to 6,430 ppm. **Table 2** shows the median concentration of each color of EBNs reported in the study.

Although the nitrite and nitrate in the cave EBNs are significantly higher than the house EBNs on average, it can be seen from the **Table 2** that the readings between the samples are widely distributed and the standard deviation value is high, especially in the cave EBNs. These nitrite and nitrate changes may be attributed to different cave and swiftlet farm environments, such as humidity, pH and climate; age of EBN when harvesting (harvesting time); contamination during harvest and the cleaning processes of the collected EBNs may all cause the

different concentrations of nitrate and nitrate levels (Paydar et al., 2013; Tan et al., 2020). The good management of the swiftlet houses, such as the frequent removal of swiftlet guano while the guano of the cave is left in the cave and not cleaned may contribute to lower nitrite level in the house nests. The guano can produce nitrite through fermentation (to be discussed later). In addition, good ventilation design of the swiftlet houses also helps to reduce the bacterial anaerobic fermentation process, thereby reducing the concentration of nitrite (Quek et al., 2015; Tan et al., 2020). Therefore, the management and design of the swiftlet houses results in a relatively low concentration of nitrite and nitrate in the house nests compared to the cave nests.

Generally, in any swiftlet house or cave, the presence of nitrite and nitrate is a natural phenomenon. Paydar et al. (2013) hypothesized that the sources of nitrite and nitrate can be obtained from ammonia through anaerobic fermentation by the bacteria. Nitrite is produced by the nest itself and is also absorbed by the swiftlet nesting environment, especially from the floor where organic matter is decomposed. Quek et al. (2015) agreed with Paydar et al. (2013) that the fermentation process of bird droppings and natural environmental resources such as atmosphere, water and soil have caused the infiltration of nitrite and nitrate in bird's nests.

Chan and his team researched the source of nitrite contamination. In order to find the source of nitrite, they collected swiftlet droppings and water samples from EBN production sites in Malaysia and Indonesia. The results showed that they contained a lot of nitrate instead of nitrite. They also performed proteomic analysis of EBN protein extracts by mass spectrometry. The analysis identified microbial nitrate reductase, which converts nitrate in EBN to nitrite. In EBN, the nitrate/nitrite metabolism process may occur. Under the enzymatic conversion of nitrate reductase, a large amount of nitrate that may originate from swiftlet is triggered to form nitrite. A specific nitrate reductase inhibitor, when added to the EBN under development, can successfully eliminate the nitrate reducing activity found in EBN, thereby reducing the final content of nitrite in EBN. They have successfully proved this possibility in their published study. Therefore, the nitrite on EBN may be the result of environmental pollution and nitrate and microbial nitrate reductase (Chan 2013; Chan et al., 2013; Chan et al., 2018). Swiftlet dropping/guano is source of nitrite content in EBNs, MS 2273:2012 has suggested that farmers should apply Effective Microorganism (EM) on the guano and mine the guano frequently and not allow it to build up.

The nitrite content in bird's nests should be strictly controlled to ensure that bird's nests can be eaten safely. According to Malaysia Standard MS2334:2011, raw cleaned edible bird's nests (RC EBN) which after undergone cleaning processes should not contain more than 30 ppm of nitrite. A proper cleaning process can reduce the nitrite and nitrate concentrations in EBNs (Chan et al., 2013; Hamzah et al., 2013). There are only limited studies (Quek et al., 2018; Tan et al., 2020) comparing the levels of nitrite and nitrate in house/farmed EBNs from different regions. Most studies compared between houses and cave/wild EBNs. In addition, the comparative study of nitrite and nitrate of different RC EBN products (such as cup-shaped EBN, instant cook, etc.); and how different treatment methods affect the content of nitrite have yet to be reported.

THE COLOR OF EBN

When customers choose EBNs, color is an important attribute and indicator of food quality and food acceptability. The color of house EBN is usually white, off-white, light yellow, brown, golden yellow and orange red. Majority of the house EBNs are off white and light yellow. On the other hand, the color of cave EBNs is usually white but with "red head," red, orange-red and brown. Majority of cave nests are red and orange in color. In fact, all EBNs start from white. After 2–3 months, the EBN color changes from white to yellow. After about 6 months, when RUC EBN is still in ranches, the EBNs finally turn red (Quek et al., 2015). Unlike cave EBNs, not all house EBNs will eventually turn red after a long period of time.

According to ancient traditional Chinese medicine, white EBN is mainly used to treat coughs and other respiratory diseases, and only red EBNs can be used to treat children with dysentery i.e., blood in the stool (Chan et al., 2013). The supply of red EBNs or cave EBNs is very limited. Higher demand than the supply leads to higher price of red EBNs than white EBNs. The high nutritional and medicinal values and the higher price of the red EBNs lead to issues of adulteration (Ma and Liu, 2012). Marcone (2005) mentioned that on occasion white nests have been treated with red pigments which are either partially or wholly water-soluble; and But et al. (2013) reported that white nest was fumigated with "bird soil" under hot and humid condition. After treatment of the white EBNs, falsified appearance as red EBN will be sold as higher grade and athigher price.

The bird's nest is initially white, but the color may remain white or change to other colors during harvest. Different colors of EBN are initially secreted through the beaks of the same species of swiftlet, so they are not different due to structural coloration (Shim and Lee, 2018). The causes of color changes has been a puzzle for centuries. The following shows the process and reasons for the color change of bird's nest from the previous studies. But et al. (2013) showed that white EBNs could turn yellowish/red/darker red by vapors from bird soil. The white EBNs turn yellowish when "washed bird soil" was used for fumigating the EBNs. The white EBNs turn red (lighter) when "untreated bird soil "was used for fumigating the EBNs. The darker red EBNs are observed, when the white EBNs were fumigated with "heated bird soil" (bird soil placed in oven for 5 h) and "nitrite-enriched bird soil" ("wash bird soil" mixed with sodium nitrite). The study has also shown that the vapors produced by sodium nitrite ($NaNO_2$) under acidic conditions (2% HCl) can turn white EBNs into red, while the vapors produced by $NaNO_2$ in distilled water cannot (But et al., 2013). Thus, swiftlet excrement can be a source for changing the color of raw uncleaned bird nests.

Paydar et al. (2013) showed two important findings; i.e., 1) no hemoglobin was detected in red "blood" nests; and 2) nitrite and nitrate in EBN affect EBN's color. No hemoglobin was detected in red "blood" nest or cave nest and this answers to an old folklore which asserted that the red EBNs are swiftlet blood mixed with

saliva when exhausted swiftlets hurried to finish their nests before laying eggs. Paydar et al. (2013) also found that when white EBNs were exposed to vapor from $NaNO_2$ in 2% HCl, or bird soil turn to brown/red colors, where nitrite and nitrate contents also increased significantly. This results agree with the comparison between house (white) nests and cave (red) nests, where cave nests (red/brown) contained higher intensities of C-N and N-O bonds compared to house nests. However, Payder et al. found that nitrite and nitrate are not the only factor that affect the reddening process, because some brown EBNs nitrite and nitrate are higher than red EBNs; and white EBNs soaked into the $NaNO_2$ in 2% HCl and exposed to vapor from bird soil nitrite enriched bird soil turned to yellowish and not turned to red (Paydar et al., 2013). Chan (2013)'s finding is consistent with Paydar et al. (2013)'s finding, where white and yellow EBNs turned into red after 10–20 days of incubation with 1M potassium nitrate. On the other hand, no significant color change occurred for the samples without nitrate sources. A strong positive correlation between nitrite with redness (a^* color component) and negative correlation with lightness (L^* color component) were found with Pearson correlation (Quek et al., 2015).

Shim and Lee (2018) reported that the white nests turned to red/orange color because of the vapor of reactive nitrogen species reacting with the mucin glycoprotein tyrosine. This reaction is named as nitration of tyrosine. The reactive nitrogen species used in the study is nitrous acid (HNO). In the bird house or cave, after aerobic bacteria (nitrifying bacteria) decompose bird soil, they produce nitrous acid and nitric acid vapor. Shim and Lee showed that the nitration of the tyrosine residues gave 3-nitrotyrosine residue in the glycoprotein (3-NTyr) and caused the red color. Tyrosine has been proposed as one of the markers for differentiating between house and cave EBNs, as house EBN has higher tyrosine compare with cave EBN (Seow et al., 2016). This is due to the fact that tyrosine in white EBN is not nitrated.

Wong Z. C. F. et al. (2018) suggested that EBN has potential metallic binding sites, where they found that white EBN was changed to red with the addition of Fe^{3+}; and changed to blue when Cu^{2+} was added. They proposed that transition metals induce color change in EBNs, and binding of Fe ion to acidic mammalian chitinase-like (AMCase-like) protein directs the origin of red color in EBNs. They proposed that $NaNO_2$ can be an oxidant in EBN, through the oxidization of iron ions process, oxygen is bound to Fe and leads to the color change in EBNs where increased in Fe-O bond intensity was found (Wong ZCF. et al., 2018).

Gan and his team in 2016 and 2017 have conducted a series of studies regarding the relationship between rehydration/drying and color change in EBNs. EBN samples were subjected to 1) convection hot air drying at 40, 50, 70, 80, and 90°C, air circulation at 4.6 m/s; 2) intermittent with infrared coupled with ultraviolet C (UVC) at 25 and 40°C, intermittent $\alpha = 0.2$, 0.33, 0.67 (tempering periods of 240, 120, and 60 min) and $\alpha = 1$; and 3) heat pump drying intermittent ($\alpha = 0.2$, 0.33, 0.67, and 1.0) with air velocity at 28.6 and 40.6°C, during "on": high air velocity at 5.4 ± 0.5 m/s and during "off" low air velocity of 1.0 ± 0.5 m/s. The lowest total color change happened at intermittent $\alpha = 0.2$ air velocity at 28.6°C; and the highest color change was reported at 90°C hot air drying. Overall, results demonstrated that air velocity or infrared and intermittent UVC contributed to lower color change. Gan and his team suggested that during the tempering period, in the low-temperature dehumidified air in the drying chamber, the moisture from the center of the material will be redistributed to the surface, which is likely to prevent dehydration of the surface and has a significant effect on reducing color changes because of the rate of Maillard reaction which is reduced and non-enzymatic browning. On the other hand, hot air drying method contains high levels of oxygen, which stimulates the enzymatic browning reaction (Gan et al., 2016, 2017).

Zhang et al. (2020) successfully showed that low-energy X-ray can inactivate foodborne pathogen, but the irradiation also affects the color of EBNs. Study showed that the when irradiation dose increases, the yellowness value increases while the lightness decreases respectively. Studies above showed that color changes in EBNs occur when in the swiftlet houses and during the processing from RUC to RC. Based on studies by Paydar et al. (2013), Chan (2013), Shim and Lee (2018) and Wong Z. C. F. et al. (2018), one can conclude that nitrite and nitrate play an important role in EBN color changes. Bird soil could be the most likely sources of the nitrite and nitrate. But the source is not limited to nitrite and nitrate as the metal like Fe ion and high temperature during the drying process can also affect the color changes in EBNs. Here, the studies focused mainly on the color change from white to darker color like yellow, brown and red nests. How about from yellow or brown nests to white nests? Recently, consumers prefer house nests that are white or ivory in color but not yellow or brown nests. To the best of our knowledge, there is no study that focus on the EBN color changes from darker to lighter shades. Semicarbazide (SEM), a potentially harmful agent, was detected in instant bottled bird's nests, cup-shaped EBN and bird nest cakse. The hypothesized sources of SEM were from edible gum and hypochlorite used for bleaching (Xing et al., 2012). Bleaching is a step that can make the color of EBN become lighter, but until recently there are still no detailed study on the effect of bleaching to the EBNs.

BACTERIAL, FUNGI AND MITES IN EBNS

Bacteria associated with EBNs may cause food-borne diseases if ingested. Bacteria enter the body through contaminated food or water. Food-borne pathogens can cause severe diarrhea or debilitating infections such as meningitis (WHO, 2020a). As shown in the **Table 1**, in the EBN study, several microorganisms were of concerns, namely coliforms, *Escherichia coli* (*E. coli), Salmonella, Staphylococcus aureus*, yeast and mold. Coliform is the "indicative organism" of food microbiology. Coliforms are Gram-negative, rod-shaped, non-spore forming bacteria, such as *Citrobacter*, *Enterobacter, Escherichia,* and *Klebsiella* species (Martin et al., 2016).

Salmonella sp. and *E. coli* are the most common food-borne pathogens, affecting millions of people every year, and can be fatal (WHO, 2020a). *Enterobacter* sp. are another coliform that cause nosocomial infections. *Staphylococcus aureus* cause serious threat

to human health globally as Staphylococcal food poisoning. Elimination of *Staphylococcus aureus* is very important for food industry, but is very challenging (Fetsch and Johler, 2018). Fungi are the other most resilient spoilage microorganisms. Fungi can overcome the food safety control strategy adopted by the food industry. Fungi can multiply in foods with extremely limited water supply and have extremely high heat resistance as they can survive and thrive in commercial sterilized foods (Snyder and Worobo, 2018). Fungi can appear as yeast, mold or a combination of the two forms. In addition to the controlled microorganisms that affect the quality of EBNs, there are another organisms that might affect the quality of the EBNs such as mites. Mites have been identified as pathogens and are the most common source of allergens that cause respiratory allergies and anaphylaxis. After ingesting heated or unheated mite-contaminated food, systemic anaphylaxis can occur in those who have been sensitized (Sanchez-Borges et al., 1997).

Table 3 shows that mites, fungi and bacteria have been successfully isolated and identified in previous studies while **Table 4** shows the concentration of mites, fungi and bacteria that have been reported. Kew et al. (2014) revealed the presence of mites, fungi, bacteria and feathers on both RUC and RC EBNs through structural analysis of EBNs under a scanning electron microscope. Mite egg-shells and fecal particles as well as body parts of other arthropods were found on the RUC EBNs. Bacterial (streptococci, cocci and rod-shaped) and fungal structures (yeast, hyphae and fungal spores) were detected on the surface of RUC EBNs. The mites, bacteria and fungi were also detected on the surface of RC EBNs.

Sien et al. (2013) successfully isolated 500 bacterial isolates (11 types, **Table 3**) from the swiftlet feces collected from a farmhouse in Sarawak, Malaysia. The 16srRNA analysis showed that 96% of the isolates were identified as Gram-positive bacteria, and the remaining 4% of the isolates were Gram-negative bacteria. *Staphylococcus sp*. was the most prevalent bacteria found in feces.

E. coli, S. aureus (Sani et al., 2015) and *Enterobacter* (Wong ZCF. et al., 2018) were detected in the RC bird nests. No *Salmonella* was detected by both studies including RUC EBNs. Mold (Sani et al., 2015) and fungi (soil and environmental fungi) (Wong S. F. et al., 2018) were detected in RC EBNs also.

Sani et al. (2015) reported the concentration of total plate count (TPC), coliforms, *E. coli*, *S. aureus*, and yeast and mold before and after irradiation. Before the radiation, RC EBNs readings exceeded the permittable limit set by Standard Malaysia. Similarly, as reported by Chen et al. (2015), all EBNs had fungal CFU that exceeded the limit (100 CFU/g). However, when RUC EBNs and RC EBNs were compared, there were no significant difference found between the total number of bacteria and fungi in both the samples but they did show significant difference in types of isolates (Chen et al., 2015; Wong S. F. et al., 2018).

Wong S. F. et al. (2018) reported that *Enterobacter, Exiguobacterium, Brevibacillus, Caryphanon,* and *Solibacillus* species were found exclusively in the commercial EBNs. RUC and RC EBNs were purchased separately and therefore, the raw materials before processing into RC EBNs might differ from the RUC EBNs. *Bacillus* species were found in all type of samples, including bird's nest feces, RUC EBN, RC EBN and double boiled samples. *Bacillus cereus* can cause foodborne illness through the production of distinct toxins which lead to diarrhea and emetic syndrome (Griffiths and Schraft, 2017). In addition, the spores of *Bacillus cereus* are highly resistance to processing and harsh conditions, and can germinate to vegetative cells under any favorable conditions (Lv et al., 2019). Therefore, it is recommended that attention should be paid to the existence and quantity of *Bacillus* in the EBN industry.

Plant fungi were only detected in RUC EBN samples, but not RC EBNs and also boiled samples. *Aspergillus* sp. and *Penicillum* sp. are the common fungi isolated from the unboiled and boiled raw (RUC) and commercial (RC) EBNs (Chen et al., 2015; Sani et al., 2015). *Aspergillus* sp. and *Penicillum* sp. are environmental fungi and can be easily isolated from spoiled food. They produce mycotoxins that are harmful to human health (Greeff-Laubscher et al., 2020). This is not only for the safe consumption of EBN products, but also may pose risk of exposure to farmers and workers who deal with or process the raw uncleaned EBNs.

Kew et al. (2015) observed that there were no live mites detected in RC EBNs. This is consistent with the structural analysis, where the mites observed at RC EBNs were partially embedded in the nests. Kew et al. (2015) have successfully isolated and identified thirty types of mites. They suggested that these isolates probably are feather mites, house dust and storage mites, mesostigmatid mites, prostigmatid mites, astigmatid mites and oribatid mites.

Previous studies (Kew et al., 2014; Chen et al., 2015; Wong et al., 2018a) suggested that the bacteria, fungi and mites on the EBNs may be originated from the fauna and detritus found in the cave, swiftlet houses and the surrounding as well as the insects ingested by the swiftlets. Remnants of insects in their mouths and possibly the microbes and mites on their body are incorporated into the nests when the swiftlets build their nests with their saliva. Bacteria, fungi and mites in the houses and/or surrounding the ranches can directly contaminate the EBNs during harvest or contaminate the swiftlets, or inhabit the feathers and skin of swiftlets which indirectly contaminate the EBNs. Fungi and microbes can be introduced during EBN processing, storing and transporting.

From **Tables 3**, **4**, we can observe that double boiling can significantly reduce types and number of the bacteria. However, this method may not be effective in removing heat-resistant bacteria such as *Bacillus sp*. and *Brevibacillus* sp. In addition, double boiling of EBNs was not effective in reducing the types and number of fungi, and this suggested fungi also possessed heat-resistant properties. Gamma radiation was reported to reduce the number of yeast and molds. Other studies have shown that microwave sterilization can reduce the number of *Salmonella* and *E. coli* in EBN drink (Than et al., 2018); and heat sterilization can remove yeast and mold, coliform*, E. coli*, *Salmonella,* and *S. aereus* effectively with no detectable growth in EBN beverages (Lam, 2018). Low-energy X-ray irradiation (350–400 Gy) can decreased *E. coli* O157:H7 and *S. Typhimurium* in dry EBNs from 6.35 to 5.84 log CFU/g, respectively, to undetectable level (Zhang et al., 2020).

TABLE 3 | Bacteria, fungi and mites associated with EBNs.

No	References	Type of samples	Microbes	Microbes After treatment
1	Wong et al. (2018a)	Raw uncleaned (house nest)	Bacteria (isolates) *Acinetobacter sp.,Brevibacterium sp., Bacillus subtilis, Bacillus shackletonii, Bacillus sp., Bacillus megaterium, Bacillus pumilus, Bacillus flexus, Bacillus circulans, Bacillus cereus, Bacillus aryabhattai, Deinccoccus sp., Enterococcus faecalis, Enterococcus sp. , Listeria fleischmannii, Microbacterium sp. ,Paenibacillus sp., Paenibacillus sp. 23-13, Paenibacillus agglomerans, Paenibacillus alvei, Staphylococcus nepalensis, Staphylococcus Kloosi, Staphylococcus sp., Staphylococcus sciuri, Staphylococcus sp. Y3 Virgibacillus halophilus*	Double boiling *Bacillus subtilis, Bacillus sp.,*
		Raw cleaned (commercial EBNs)	*Bacteria (isolates) Acinetobacter sp., Acinetobacter radioresistens, Acinetobacter calcoaceticus, Brevibacillus sp. Brevibacterium sp., Bacillus sp., Bacillus badius, Bacillus cereus, Bacillus flexus, Bacillus lichniformis, Caryphanon sp., Deinococcus sp. Enterobacter cloacae, Enterobacter hormaechei Exiguobacterium sp., Solibacillus silvestris Staphylococcus sp., Staphylococcus pasteuri, Staphyloccus saprophyticus, Staphylococcus sciuri. Sporosarcina saromensis*	Double boiling *Brevibacillus sp., Brevibacillus agri, Bacillus sp.*
2	Kew et al. (2015)	Raw and commercial nests	Mites (Isolates) *Eustathia cultrifer*, *Pteroherpus garrulacis*, *Pterodectes amaurochalinus*, *Laminalloptes* sp., *Berlesella alata, Neochauliacia* sp., *Suidasia* sp., *Austroglycyphagus* sp., *Aleuroglyphus ovatus, Dermanyssus sp., Cheyletus sp.,* Tarsonemid, cunaxid mites*, Collocalidectes sp., Streetacarus sp., Hemisarcoptes sp* and unidentified oribatid mites	N/A
3	Sien et al. (2013)	Swiftlet feces in swiftlet farm houses	Bacteria (Isolates) *Bacillus sp., Dermacoccus sp. 103, Enterococcus harae strain ss33b, Escherichia coli, Leucobacter iarius strain 40, Lysinibacillys sp. B4, Paenibacillus sp. Gh-134, Proteus sp., Pseudomonas aeruginosa strain 123, Sporasarcina sp., Staphylococcus sp.*	N/A
4	Sani et al. (2015)	Raw cleaned EBN	Mold (Isolates) *Aspergillus* spp. and *Penicillium* spp.	
5	Chen et al. (2015)	Raw uncleaned (house nest)	Fungi (Isolates) Soil Fungi: *Blastobotrys* sp., *Lichtheimia* sp., *Nigrospora* sp., *Paecilomyces* sp., *Perenniporia* sp., *Phialosimplex* sp. *Syncephalatrum* sp., *Sagenomella* sp., *Stephanoascus* sp. *Talaromyces* sp, Plant Fungi: *Coprinellus* sp., *Fomitopsis* sp., *Lasiodiplodia* sp., *Lenzites* sp., *Letendraea* sp., *Polyporales* sp., *Rigidoporus* sp. Environmental Fungi: *Aspergillus* sp., *Candida* sp., *Cladosporium* sp., *Neurospora* sp., *Penicillum* sp., *Eurotium* sp.	Double boiling Soil Fungi: *Phialosimplex* sp. Plant Fungi: -Environmental Fungi: *Aspergillus* sp., *Candida* sp., *Cladosporium* sp., *Neurospora* sp., *Penicillum* sp., *Eurotium* sp.
		Raw cleaned (commercial EBNs)	Fungi (Isolates) Soil Fungi: *Chrysosporium* sp., *Nigrospora* sp., *Sagenomella* sp., *Sebanicales* sp. Plant Fungi: -Environmental Fungi: *Aspergillus* sp., *Candida* sp., *Cladosporium* sp., *Neurospora* sp., *Penicillum* sp.	

ALLERGENS IN EBNS

Food allergy is defined as hypersensitivity reaction towards a food. Food allergic reactions can be classified as immediate (IgE mediated reaction) and delayed (generally non-IgE-mediated reaction) (Kemp et al., 2010). Anaphylaxis is a severe systemic hypersensitivity reaction with rapid onset; characterized by life-threatening airway, breathing, and/or circulatory problems; and usually associated with skin and mucosal changes (Reber et al., 2017). EBN can be a potential source of life-threatening food allergy to those who are sensitized to its components or contaminants. The National University Hospital of Singapore reported that among children, 0–15 years old, the most common food allergen is bird's nest soup, which surpasses other clear food allergens, such as milk, eggs, peanuts and crustaceans (Goh et al., 1999). The allergic symptoms after consumption of bird's nest soup were typical of a type I hypersensitivity reaction (angioedema, wheezing, urticaria, and abdominal cramps), and no deaths were reported.

Goh and his team continued to study allergens in EBNs, and they found that IgE-mediated hypersensitivity occurs after consumption of bird nest soup. Investigations revealed that 66 kDa protein is the main putative allergen responsible for this reaction. The protein is homology with ovoinhibitor,

TABLE 4 | Bacteria, fungi and mites contents in EBNs.

No	References	Type of samples			Enumeration method	Source of samples
		Raw uncleaned EBN	Raw cleaned EBN	After treatment/ Others		
1	Tan et al. (2020)		ND *E. coli, S. Aureus* and *Salmonella* Total Plate Count: $2.3*10^5$–$25*10^5$ cfu/g Coliform ND- 43 cfu/g Mould <10–140 cfu/g Yeast <10–10 cfu/g		Australian Standard- *Escherichia coli, Samonella spp*., Coliform, and total plate count. Official AOAC method –*Staphylococcus aureus* Bacteriological Analytical Manual of Food and Drug Authority- mould and yeast	RC: Seven RUC house nest samples from different regions in Malaysia then cleaned in lab. Seven regions include: Alor Setar, Kedah; Sibu, Sarawak; Rompin, Pahang; Kuala Selangor; Johor Bahru; Jerantut, Pahang; and Port Klang, Selangor
2	Wong et al. (2018a)	$6.0*10^2$–$1.02*10^5$ CFU/0g		*Double boiling* 0–$2.4*10^2$ CFU/g	Total Plate Count	RUC: Five Malaysia house nest from Kuala Sanglang, Pantai Remis, Kluang, Kajang and Kota Bharu
			$4.0*10^1$–$1.5*10^5$ CFU/g	*Double boiling* 0–$2.4*10^2$ CFU/g		RC: Six commercial sample purchase from five different Chinese traditional medicine shops from Malaysia and one from Medan, Indonesia
3	Sien et al. (2013)			Swiftlet feces 6.03–9.22 log10 CFU/g	Total Plate Count	Swiftlet feces from swiftlet houses located in ten places, including Kota Samarahan, Saratok, Semarang, Betong, Sarikei, Sibu, Sepinang, Maludam, Kuching and Miri in Sarawak
4	Sani et al. (2015)		7.64–7.66 log CFU/g	*Gamma Irradiation 20 kGy* <2 log CFU/g	Total Plate Count	RC: Raw cleaned samples from Pahang and Terengganu
			5.61–5.95 log CFU/g	Gamma irradiation 5 kGy <2–4.64 log CFU/g	Plate count -agar Brilliance Coliform-Coliforms	
			2.47–2.67 log CFU/g	Gamma irradiation 1 kGy <2 log CFU/g	Plate count agar Brilliance *E. coli*-*E. coli*	
			4.55–4.66 log CFU/g	Gamma irradiation 5 kGy <2 log CFU/g	Plate count – agar Rabbit Plasma Fibrinogen - *Staphylococcus aureus*	
			4.8–5.10 log CFU/g	Gamma irradiation 5 kGy <3 log CFU/g	Plate count-agar Dichloran rose Bengal chloramphenicol- Yeast and molds	
			Not detected	N/A	Plate count- agar xylose lysine deoxycholate agar and brilliant green agar- *Salmonella spp*.	
5	Chen et al. (2015)	40–18,080 CFU/g	40–2,640 CFU/g	N/A	Plate count- Sabouraud Detrose Agar- Fungi	RUC and RC: Same sample batch with Wong et al. (2018a) exclude sample from Medan
6	Kew et al. (2015)	Live 0–66.4 mites/g. Dead 15.9–2,613 mites/g. Total 18–2,613 mites/g.	Live 0 mites/g. Dead 0–88 mites/g. Total 0–88 mites/g.	N/A	Stereomicroscope- Mite	RUC and RC: Same sample batch with Wong et al. (2018a) exclude sample from Medan.

Kazal-type serine protease inhibitor, that is mainly found in chicken egg white (Goh et al., 2000; Goh et al., 2001; Ou et al., 2001). One band with a molecular weight of approximately 77 kDA protein found from white and red "blood" nests, this protein that similar to those of the ovotransferrin protein in eggs to the highly allergenic ovotransferrin protein in eggs. This protein can be partially responsible for the allergic reactions (Marcone, 2005).

The source of allergens in EBNs has not been determined, because of the 39 cases of allergic reactions, 14 of them had eaten edible bird's nest before, without any allergic reactions (Goh et al., 2000). Those who develop allergic reactions after consuming bird's nest soup may be sensitized to the bird nest components or other associated contaminants. Kew et al. (2015) suggested that the possible sources of allergens found in the EBNs may originate from the saliva or feathers of the swiftlets, the insects ingested by the swiftlets, the microorganisms and arthropods (mites) associated or inhabit with the nests or swiftlet, the cleaning processes of the raw nests, the adulterants added to the raw cleaned EBNs and the contaminants introduced, and the infestation of arthropods or other organisms during the storage of the nests. Most of the possible allergens listed above can be removed by proper cleaning process and management after cleaning. However, the heat-resistant bacteria, fungi and mite allergens can be causes of concern.

HEAVY METALS AND EXCESSIVE MINERAL CONTENTS IN EBNS

This section summarizes the studies on heavy metals (lead, arsenic, mercury and cadmium) and excessive minerals (iron and copper) in EBNs, and the content of these elements is subjected to the maximum limit of RC EBN in Malaysian standards (**Table 1**). Metals and metalloids (a combination of metal and non-metal elements) found in foods can be beneficial or harmful when within or exceed the recommended dose (FDA, 2020c). Pb, As, Hg, and Cd are heavy metals that have no established health benefits and are harmful to human (FDA, 2020a). Fe and Cu are beneficial metals to human when taken in optimum dosage and not exceeded. It is discussed here as excessive mineral (as listed in Malaysia Standard).

Lead may be a cumulative toxic substance that affects multiple body systems. Long-term exposure to low concentrations for an extended time may also be dangerous. It is toxic to humans, especially harmful to vulnerable people such as babies, young children, pregnant women and their fetus, and people with chronic diseases. Lead within the body is distributed to the brain, liver, kidneys and bones. It is stored in teeth and bones and accumulates over time. High levels of lead exposure can seriously endanger the health and development of kids, especially the brain and nervous system. It affects learning abilities, causes behavioral difficulties and lowers IQ. Important sources of environmental pollution to lead pollution include mining, smelting, manufacturing and recycling activities, continued used of leaded paints, leaded gasoline and leaded aviation fuel in certain countries/regions. Lead is detected in food due to its presence in the environment and enters our food supply through the following methods: 1) lead within the soil can settle or be absorbed by plants, fruits or vegetables or plant-based dietary supplements; 2) lead in food cannot be removed completely by washing or other food processing steps; 3) The animals we eat may also ingest and absorb lead in plants or water, and pass it on to us; 4) lead may inadvertently enter through the manufacturing process (for example, lead-containing pipes can contaminate water employed in food production); 5) lead in some pottery and other food containers may leach out and enter into food when food is prepared, served or stored (FDA, 2020b; WHO, 2020b).

Arsenic (As) is omnipresent components in environment and humans are exposed to arsenic through air, ground water and food sources. Significant wellsprings of arsenic defilement could be either through geological or anthropogenic activities (Mohammed Abdul et al., 2015). Arsenic in the environment is normally low, except for volcanic emissions, mining, breaking, coal-terminated force plants, arsenic-treated wood, and arsenic-containing pesticide contamination in certain zones. Arsenic enters the food supply through soil, water or air. Arsenic in food can cause life-threatening health problems (FDA, 2020a). Arsenic influences practically all cellular processes and organ functions in our body, like integumentary, cardiovascular and reproductive systems. In addition, arsenic is able to cause epigenetic modifications and tumorigenesis, subsequently causing cancer (Mohammed Abdul et al., 2015).

Mercury can be classified into three main groups: metallic mercury (Hg^0), inorganic mercury (Hg^{2+}), and organic mercury (methyl mercury: CH_3Hg^+). Minamata disease is a typical example of the mercury pollution-related health damage (Sakamoto et al., 2018). Mercury has been widely used in many measuring instruments, electric appliances, agricultural chemicals and mildew-proofing agents. Human exposure to mercury occurs through leakage from the instruments and appliances and land run-off of this chemical. Environment may be polluted by mercury through discharge from industrial activities (anthropogenic) or derived from volcanoes (natural) (Clarkson, 1997; Sakamoto et al., 2018). The health effects of mercury include but are not limited to behavioral and cognitive changes; prenatal exposure to methylmercury (MeHg) is associated with low birth weight, delayed neurodevelopment, and growth and development of children; and increased risk of psychiatric symptoms (Clarkson, 1997; Ha et al., 2017).

Cadmium (Cd) is a toxic heavy metal and is a non-essential trace metal that has strong teratogenic and mutagenic effects in living organisms. General population exposure to cadmium is through occupational exposure (example working at metal refinery industry), diet (the main source), breathing, smoking, or drinking water (Fatima et al., 2019; Geng and Wang, 2019). Local residents near an active lead-zinc mine and copper smelter acquired the Cd via dietary intakes of rice and vegetables (Du et al., 2020). The impact of cadmium on human health includes damage to the lungs, glucose impairment which leads to diabetes, restriction of fetal growth, impaired hormone synthesis, kidney diseases (renal failure, renal anemia, etc.), anaemia, osteoporosis, cardiovascular diseases and cancer (Nordberg et al., 2018; Fatima et al., 2019; Geng and Wang, 2019; Kumar and Sharma, 2019).

Copper within the atmosphere is produced by natural processes and human activities. Natural processes include volcanic eruption, windblown dust and microbial activities while human activities include industrial activities such as

TABLE 5 | Heavy metals and excessive mineral contents in EBNs.

NO	References	Sample	Element concentration (ppm)						Source of sample
			Lead, Pb	Arsenic, As	Mercury, Hg	Cadmium, Cd	Copper, Cu	Iron, Fe	
1	Tan et al. (2020)	House nest	<0.02	<0.01	<0.01	<0.01			RC: Seven RUC house nest samples from different regions in Malaysia then cleaned in lab
2	Quek et al. (2018)	House nest	0.164 ± 0.089	0.069 ± 0.014	0.081 ± 0.007	0.003 ± 0.001	5.22 ± 0.45	21.58 ± 10.56	RUC: Five house nests, four from Peninsular Malaysia, one from East Malaysia. All is *A. fuciphagus* nest
		Cave nest	0.190 ± 0.086	0.067 ± 0.007	0.073 ± 0.005	0.005 ± 0.002	5.13 ± 0.75	23.01 ± 9.05	RUC: Six cave nests, All from East Malaysia. Two samples are *A. fuciphagus* nest and Four samples are *A. maximus* nest
		Peninsular Malaysia	0.184 ± 0.089	0.075 ± 0.007	0.083 ± 0.006	0.003 ± 0.001	5.33 ± 0.44	18.67 ± 9.74	RUC: Four house nests. All is *A. fuciphagus* nest
		East Malaysia	0.174 ± 0.088	0.065 ± 0.010	0.073 ± 0.005	0.005 ± 0.002	5.08 ± 0.67	24.47 ± 9.12	RUC: One house nest and six cave nests. Three samples are *A. fuciphagus* nest and Four samples are *A. maximus* nest
3	Salim et al. (2018)	House nest Cave nest		<0.05 0.1–0.3					Received from Ministry of Health, Malaysia
4	Chen et al. (2014)	RUC: House nest	<0.592	0.000055–0.034.45	0.000060–0.070.180 *1 sample >0.05 ppm	0.060–1.870	<0.500	1.309,725–1.3496320	Same sample batch with Wong et al. (2018a) exclude sample from Medan
		RC: Commercial	<0.067	<0.01	Nd- 0.0144		0.19113–0.53883	>0.3 <5.790,405 *All >0.3 ppm	

acid drainage from mining operation. Human exposure to copper is through daily intake, mainly from food like seafood and dry fruits, and water (Latorre et al., 2019). Copper is an important micronutrient in the human body, and it contains about 100 mg of copper in the human body. Copper is involved in a variety of biological processes including antioxidant defense, neuropeptide synthesis and immune function (Bost et al., 2016). However, excess or deficit of copper is linked to several human disorders like diabetes, cardiovascular diseases and cancer. Wilson disease (copper excess) and Menkes disease (copper deficiency) are the two major genetic disorders that have been widely studied in terms of copper homeostasis (Latorre et al., 2019).

Maintaining iron homeostasis in human body is important. Deficiency or over exposure to iron has noticeable effects on human health. Iron is an essential nutrient for human as it participates in a wide variety of metabolic processes, including supporting oxygen binding and transport (e.g., hemoglobin); electron transport (e.g., heme-containing enzymes), oxidative metabolism (e.g., NADH dehydrogenase), cellular proliferation and DNA synthesis. Iron deficiency always manifests as anemia, which results in reduced immunity, reduced work ability and impaired mental function. Maintaining excessive iron in the human body can lead to neurodegenerative diseases, liver damage, endocrine disease, cardiac dysfunction, and increased risk of liver cancer (Abbaspour et al., 2014; Wessling-Resnick, 2017). Therefore, maintaining the homeostasis state of copper and iron in the human body is vital.

Table 5 shows the concentration of heavy metals and excessive minerals detected in EBNs. Studies found that 1) lead, arsenic and cadmium are always below the regulatory limits even for the concentration in RUC EBNs; 2) most of the RUC EBNs had higher mercury and copper levels than the limits; 3) however mercury and copper concentrations in RC EBNs were below the regulatory limits; and 4) all samples had higher iron concentration than the permissible limit of 0.3 ppm. There are very limited studies on heavy metals and minerals in EBNs. From the classification, latent Dirichlet allocation model suggested that arsenic and mercury contributed most significantly ($p < 0.05$) to the geographical origin differentiation (between Peninsular Malaysia and East Malaysia (Quek et al., 2018). Quek et al. (2018) suggested that the difference is due to location of swiftlet houses

and caves, where the Peninsular Malaysia is concentrated with many industrial activities. These activities emit toxic metal pollutants that contaminate the environment and ecosystem, including forages for the swiftlets. Swiftlets are aerial insectivores that usually forage for insects around their habitats. Another reason they suggested is due to indoor environments of the swiftlet houses, for example paints used for painting the swiftlet houses could be the source of mercury contamination, pesticides used to control the bacteria and pests in or around the swiftlet houses can also be sources for arsenic and mercury contamination. Research on heavy metals and minerals is very limited, especially on RC EBNs. There is no research reporting on the source of mercury, copper or iron contamination, or whether current cleaning processes are able to remove these contaminants effectively.

RECOMMENDATIONS AND CONCLUSION

This critical review has made an attempt to discuss the most important findings in the understanding of potential residual contaminants in EBNs. A number of knowledge gaps have been identified in each of the potential residual contaminants, including nitrite and nitrate contents; bacteria, fungi and mites; heavy metals; and other contaminants in EBNs and their effects on EBN color changes and allergenicity.

Some of the identified gaps that need to be investigated include:

1. Comparative study of potential residual contaminants in different RC EBN products, such as cup-shaped EBN, instant cook, etc.;
2. Comparative study of different processing (including cleaning, drying and sterilization) methods (from RUC EBN to RC EBN) in removing and mitigating the contaminants;
3. Study on the effect of bleaching as during the cleaning process;
4. Study on the factors which influencing color changes during the drying process.

There also needs to be a close working relationship between researchers and EBN industry players to fill the gap of knowledge. In addition, the policy makers can formulate reasonable and effective policies based on science and "real" industry conditions. The heat-resistant bacteria or fungi and allergens should be included in the RC EBN standards formulated as well. In a nutshell, there is an urgent need for more investigation to gain insights into potential residual contamination of EBNs.

AUTHOR CONTRIBUTIONS

Conceptualization, B-HY and O-ML; Data curation, T-KT and B-HY; Funding acquisition, O-ML and B-HY, Project administration, YW and L-ZC, B-HY, and O-ML; Supervision, O-ML, S-FW, and C-PT; Drafting the article, B-HY and O-ML; Writing- review; editing, O-ML, S-FW, YW, L-ZC, and B-HY; All authors have read and agreed to the published version of the manuscript.

FUNDING

The authors acknowledge the support given by Department Veterinary Service, Malaysia and CAIQTEST Innovation Malaysia Service Center. This study was supported by National Industry-UPM Edible Birdnest Processing Research Group Scheme (Project Code 997) in Universiti Putra Malaysia.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 30 March 2021
doi: 10.3389/fphar.2021.631402

Edible Bird's Nest Supplementation Improves Male Reproductive Parameters of Sprague Dawley Rat

Farah Hanan Fathihah Jaffar[1], *Khairul Osman*[2], *Chua Kien Hui*[1], *Aini Farzana Zulkefli*[1] *and Siti Fatimah Ibrahim*[1]*

[1]Department of Physiology, Faculty of Medicine, Universiti Kebangsaan Malaysia (UKM), Cheras, Kuala Lumpur, Malaysia, [2]Forensic Science Program, Faculty of Health Sciences, Universiti Kebangsaan Malaysia (UKM), Bangi, Selangor, Malaysia

Edible bird's nest (EBN) is reported to have a positive *in vitro* proliferative effect and contain male reproductive hormones. Spermatogonia cells proliferate during spermatogenesis under male reproductive hormones stimulation that include testosterone, follicle-stimulating hormone (FSH), and luteinizing hormone (LH). Characterization of EBN through liquid chromatography-mass spectrometry (LCMS) has found testosterone as a base peak. Six types of amino acids, estradiol and sialic acid were among the major peaks that have been characterized. Based on the presence of these reproductive components, this study evaluated different doses of EBN on sperm parameters and male reproductive hormones of Sprague Dawley rats. Sixteen Sprague Dawley rats at the age of eight weeks were randomly and equally divided into four groups, which are Control, 10 mg/kg BW/d 50 mg/kg BW/d, and 250 mg/kg BW/d EBN group. The rats were fed with EBN enriched pellet daily and water ad-libitum. Rats were sacrificed and the organ was weighed for organ coefficients after eight weeks of treatment. Sperm concentration, percentage of sperm motility, and sperm viability were evaluated. Meanwhile, ELISA method was used to measure testosterone, FSH, and LH. Findings showed that there were no significant differences in organ coefficient between groups. Supplementation of 250 mg/kg BW/d EBN demonstrated a significant increase in sperm concentration, percentage of sperm motility as well as FSH and LH level compared to 10 mg/kg BW/d group. There was a dose-dependent increase in testosterone level but was not significant between groups. Based on these findings, EBN is concluded to have crucial effects on male reproductive parameters.

Keywords: edible bird nest, sperm concentration, sperm motility, testosterone, follicle stimulating hormone (FSH), leutinizing hormone (LH)

OPEN ACCESS

Edited by:
Ting Hun Lee,
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Reviewed by:
Eddie Ti Tjih Tan,
MARA University of Technology, Malaysia
Mark Hiew,
Putra Malaysia University, Malaysia

***Correspondence:**
Siti Fatimah Ibrahim
timi@ukm.edu.my

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: *20 November 2020*
Accepted: *22 February 2021*
Published: *30 March 2021*

Citation:
Jaffar FHF, Osman K, Hui CK, Zulkefli AF and Ibrahim SF (2021) Edible Bird's Nest Supplementation Improves Male Reproductive Parameters of Sprague Dawley Rat. Front. Pharmacol. 12:631402. doi: 10.3389/fphar.2021.631402

INTRODUCTION

Edible bird's nest (EBN) has been a delicacy in Chinese traditional medicine since the 16th century (Medway, 1969). The nest was partly built by the male swiftlet from the specialized salivary glands secretion of *Aerodramus fuciphagus* during the breeding season (Medway, 1962) and is found dominantly in South East Asia (Phach and Voisin, 2007; Aowphol et al., 2008; Babji et al., 2015; Manchi, 2015).

The major nutritional composition of EBN was reported to consist of protein followed by carbohydrate, fat, ash, and some moisture (Marcone, 2005; Hamzah et al., 2013; Saengkrajang et al.,

2013). Besides, EBN contains high sodium and calcium content despite the presence of other mineral elements, which include magnesium, potassium, phosphorus, and iron. Essential amino acids like histidine, isoleucine, leucine, lysine, phenylalanine, methionine, threonine, and valine have also been detected and mentioned by various researches (Marcone, 2005; Saengkrajang et al., 2013). All these nutritional contents might contribute to a remarkable therapeutic potential of EBN.

The therapeutic potential of EBN has been scientifically proven including anti-osteoporosis effect (Matsukawa et al., 2011), osteoarthritis treatment (Chua et al., 2013), and the ability to enhance the immune system (Zhao et al., 2016). Moreover, EBN supplementation also has been proven to improve metabolic diseases (Yida et al., 2015), accelerate wound healing (Abidin et al., 2011), improves the length and weight of uterine and vaginal in ovariectomized rats (Zhiping et al., 2015) as well as improves the cognitive function of ovariectomized rats (Zhiping et al., 2015).

Despite the vast scientific exploration of EBN therapeutic potential, evaluation of its effect on male reproductive system is still lacking. Evaluation of EBN supplementation of the male reproductive system is important for two main reasons. First, due to its proven positive proliferative effect in human adipose-derived stem cells (hADSCs) and normal human fibroblasts (NHFs) (Roh et al., 2012). This positive effect may also promote the proliferation of spermatogonia during the spermatogenesis event in the testis.

Secondly, Ma and Liu (2012) had discovered the presence of male reproductive hormones, which include testosterone, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) in EBN (Ma and Liu, 2012). Another study was done by Ma et al. (2012) also demonstrated that EBN supplementation in castrated male rats had a significant increase in serum testosterone and LH. This finding suggested that EBN can become a potential erectile dysfunction treatment (Ma et al., 2012). To date, this is the only study that evaluates the association of EBN with the male reproductive system.

Based on this characteristic, EBN is postulated to have a favorable effect on the male reproductive system. Since there is a limited number of EBN studies related to male reproduction, it is imperative to evaluate the effect of EBN supplementation on male reproductive parameters.

MATERIALS AND METHODS

EBN Source and Extraction Method

The EBN was obtained from an identified swiftlet's house at Bera, State of Pahang, Peninsular Malaysia. The identified swiftlet's house is referring to the house that has been well maintained, good sanitation condition for high-quality EBN production, and swiftlet population is at minimum 2000 birds habitat in the house.

A single batch of EBN Extract was prepared from the collected EBN and used for the whole project. The whole extraction procedures are briefly described as follows:The EBN was cleaned from its impurities and dried at 50°C for 24 h before grounded and filtered with a 600 μm pore size mesh. The grounded EBN was then kept in distilled water (20 g/L water) at 25°C for 12 h before heated at 95°C for 120 min. The supernatant was filtered using Grade 1 Whatman filter paper and the filtrate was freeze-dried later. The freeze-dried EBN extract was stored at 4°C until use.

Orbitrap Liquid Chromatography-Mass Spectrometry Analysis of EBN Extract

This analysis was done to characterize the compounds presence in the EBN extract. Approximately 2 mg of EBN extract was dissolved in 1 ml of HPLC grade methanol. The solution was sonicated for 15 min and filtered through a membrane filter with pore size 0.22 μm before analysis. About 10 μL of the sample was injected into the AccelaTM UHPLC System (Thermo Scientific, San Jose, United States) equipped with quaternary pump, a build degasser, a PDA detector and an auto-sampler. Separation of the EBN sample was carried out by using A Luna Kinetex RP C18 column (2.6 μm, 2.1 mm I.D. x 150 mm) at a flow rate of 200 μL/min over 40 min by using acetonitrile: 0.1% formic acid in water as eluent. The step gradient and isocratic solvent composition was depicted in **Supplementary Table S1**.

The sample was analyzed by LTQ mass spectrometer (Thermo Scientific, San Jose, United States) with m/z spectrum ranged from 50 to 1000 was recorded in negative ionization mode. The setting of electrospray ionization modes was as follows: source accelerating voltage, 4.0 kV; capillary temperature, 350°C; sheath gas flow, 40 arb and auxiliary gas, 20 arb.

A reference standard of 18 types of amino acid (AAS18, analytical standard) was obtained from Sigma-Aldrich and used in this analysis. This reference standard was separated and analyzed under the same setting explained above.

Animals

A total of 16 (N = 16) Sprague Dawley male rats at the age of 8 weeks with an initial average weight of 250 ± 50 g were acquired from Universiti Kebangsaan Malaysia (UKM) Laboratory Animal Research Unit (LARU). They were divided randomly into four groups with four rats (n = 4) in each group. The first group was the Control group which was only fed with a standard food pellet. The other three groups received 10 mg/kg BW/d, 50 mg/kg BW/d and 250 mg/kg BW/d EBN extract respectively. The doses were supplied daily for eight consecutive weeks. The lowest EBN dose applied in this setting was based on the study done by Ma et al. (2012).

The rats were housed individually in ventilated cage (IVC), clean water was supplied *ad libitum* and kept at 12-h light: dark cycle in a room. Ambient temperature was maintained at 22 ± 5°C throughout the experiment. After eight weeks of treatment, rats were sacrificed by anesthetizing intraperitoneally with an overdose mixture of ketamine (3.34 mg/kg, Ilium, USA), xylazine (3.34 mg/kg, Ilium, United States) and Zoletil-50 (1.66 mg/kg, Virbac). The death of the animal was confirmed by the loss of righting reflex, cessation of heartbeat, loss of tail pinch reflex, loss of a pedal withdrawal reflex in the forelimbs and hindlimbs and lack of corneal reflex.

All animal procedures were approved by The National University of Malaysia (UKM) Animal Ethics Committee with

the approval number FISIO/PP/2018/SITIFATIMAH/28-MAR./908-MAR.-2018-DEC.-2020.

EBN Supplementation

EBN supplementation to the animals was done by natural feeding. This was done by enriching the food pellet with EBN extract. Enriching was done by mixing the dried EBN extract powder in a 100 g ground rats' food pellet. The mixing weight was standardized at 100 g to ensure it is well-mixed. Following this, 100 ml of sterile tap water was added to mix it further. It was shaped into a pellet, and dried overnight at 60°C.

For every EBN doses, rats' food pellet was enriched with 2 mg/g (10 mg/kg BW/d), 10 mg/g (50 mg/kg BW/d) and 50 mg/g (250 mg/kg BW/d) respectively. To supply daily doses to the animals, the following formula was applied:

$$\frac{\text{Daily dose}\,(\text{mg/kg}) \times \text{Weight of an animal}\,(\text{kg})}{\text{Concentration of EBN enriched food pellet}\,(\text{mg/g})} = \text{Daily amount of EBN enriched food pellet}\,(\text{g}).$$

The amount of EBN enriched food pellet that needs to be given daily to achieve daily doses was provided first for each animal to finish. The supply of normal food pellets was then given after all the EBN enriched food pellets were taken by the animals.

Serum Sampling

Blood was drawn immediately from retro-orbital sinus following sacrifice. Blood was collected in BD Vacutainer SST II Advance Plus Blood Collection Tube (BD, United States) and left undisturbed for at least 30 min at room temperature. The collection tubes were then transferred into the centrifuge and serum was separated by centrifugation at 1500 *g* for 10 min at 4°C. Serum yields were aliquoted and stored at −80°C until analysis.

Evaluation of Reproductive Organ Coefficient

Both sides of the testes and epididymis as well as seminal vesicle were carefully dissected and cleaned from surrounding adipose tissue before accurately weighed. The organ coefficient of each dissected reproductive organ was expressed according to the equation: the wet weight of organ (g)/body weight (g) x 100 (Feng et al., 2015).

Epididymal Sperm Collection

Cauda epididymis was dissected out and minced in 2 ml of pre-warmed PBS (ThermoFisher Scientific, United States). Epididymal sperm were collected following 40-min incubation at 37°C to allow sperm to swim out of the epididymal tubules.

Evaluation of Sperm Parameters

Sperm Concentration

A drop of 10 µl sperm suspension was placed on Makler Chamber (Sefi Medical Instruments Ltd. Haifa, Israel). Sperm concentration was counted as an average of five rows under 10x magnification of bright field microscope (Olympus CH-2, Japan).

Sperm Motility

Sperm motility was determined according to the World Health Organization (WHO) (WHO, 2010) recommendations. The sperm motility was categorized into three categories which are A: progressive motile sperm, B: non-progressive motile sperm and C: immotile sperm. A drop of 10 µl sperm suspension was placed on a microscope slide and cover slipped. A total of 200 cells were counted in duplicate under 40x magnification bright field microscope (Olympus CH-2, Japan). The sperm motility was reported as the percentage of total motile sperm (A + B/total counted sperm) x 100.

Sperm Viability

Evaluation of sperm viability was done by using the hypo-osmotic swelling test (HOST). The sperm suspension was mixed with a hypo-osmotic swelling solution in a ratio of 1:10. The hypo-osmotic swelling solution was prepared by adding 0.735 sodium citrate dehydrate (Sigma Aldrich, Germany) and 1.351 g D-fructose (Sigma Aldrich, Germany) in 100 mL distilled water. The mixture was incubated at 37°C for 30 min. About 10 µl from the mixture was placed on the microscope slide, smeared and let dry at room temperature.

In order to enhance the visibility of the sperm under bright field microscope visualization, the smear was stained with Diff Quick staining where the slides were dipped in Diff Quick Fix, Diff Quick 1 and Diff Quick II for 5 min respectively. Following this, the slides were rinsed and room dry. The viable sperm were then counted under 40 × magnification out of 200 sperm cells. Counting was done in duplicate.

Determination of Testosterone, FSH and LH by ELISA

Serum testosterone was measured by competitive enzyme-linked immunosorbent assay (ELISA, Elabscience, Wuhan, China). On the other hand, serum FSH and LH were measured by sandwich ELISA (Elabscience, Wuhan, China). The intra-assay and inter-assay variability (CV) for all the ELISA kit was less than 10%.

All the assay procedures were done according to the kit instruction. Briefly, 50 µl standard and serum were pipetted to the 96 well testosterone ELISA plate in duplicate. Immediately, 50 µl biotinylated antibody was added into the well, sealed and incubated for 45 min at 37°C.

For FSH and LH hormone analysis, 100 µl standard and serum were pipetted to the 96 well of respective ELISA plate in duplicate. The plate was sealed and incubated for 90 min at 37°C and all the liquid was removed before immediately adding 100 µL respective biotinylated antibodies into the well. This was further followed up by incubation for 1 h at 37°C.

Following incubation with respective biotinylated antibody, all the solution was removed from the well before being washed three times. About 100 µl horseradish peroxidase (HRP) conjugated Avidin was added exclusively into each well. Further incubation was done for another 30 min at 37°C before being washed five times. Color development was done by adding 90 µl Substrate Reagent, covered from light and further incubated for another

15 min. The reaction was stopped by adding 50 μL Stop solution and the absorbance was measured at 450 nm.

Testosterone, FSH and LH serum levels were interpolated from 8 points standard curve plotted according to a 4-parameter logistic (4 PL) by using MyAssays.com.

Statistical Analysis

Statistical analysis was performed using SPSS version 22.0 software (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) followed by Dunnet and Tukey's post-hoc analysis was conducted owing to the normal distribution and variance homogeneity of the data. A p-value < 0.05 was considered statistically significant.

RESULTS

Characterization of the Compound in EBN Extract

Orbitrap LCMS analysis of EBN extract showed 20 major peaks (**Supplementary Figure S1**). From the chromatogram, it was found that peak number 3 showed a relative abundance of 100%. Characterization of this peak revealed that it is most likely to represent testosterone hormone. Other reproductive hormones that has been identified are estradiol and progesterone. Sialic acid is also among the compound that has been characterized. Besides, at least six types of amino acids were present as well as vitamin K and ionic compounds of calcium and magnesium iodide. Characterization of all probable compound from the LCMS data is depicted in **Supplementary Table S2**.

Organ Coefficient

Findings showed that there was no statistically significant difference in organ coefficient between all the groups (**Supplementary Table S3**).

Sperm Concentration, Motility and Viability

Sperm concentration showed a gradual increase from 10 mg/kg BW/d EBN to 250 mg/kg BW/d EBN group (**Supplementary Figure S2A**). However, there was no significant difference in sperm concentration between the Control, 10 mg/kg BW/d EBN and 50 mg/kg BW/d EBN group. On the other hand, the sperm concentration in the 250 mg/kg BW/d EBN group showed a significant increase compared to Control and 10 mg/kg BW/d EBN group with $p < 0.05$.

For the percentage of total motile sperm, there was also no significant difference between the Control, 10 mg/kg BW/d EBN and 50 mg/kg BW/d EBN group. However, the percentage of total motile sperm in the 250 mg/kg BW/d EBN group showed significant increased compared to the 10 mg/kg BW/d EBN group ($p < 0.05$) (**Supplementary Figure S2B**).

On the other hand, the 50 mg/kg BW/d EBN group showed significant difference compared to the Control group with $p < 0.05$ in the percentage of viable sperm. There was no significant difference in the percentage of viable sperm between the Control group, 10 mg/kg BW/d EBN and 250 mg/kg BW/d EBN group (**Supplementary Figure S2C**).

Testosterone, FSH and LH Hormone Analysis

Testosterone, FSH and LH level in serum of Sprague Dawley rats showed a similar trend of gradual increase with increasing EBN dose. However, testosterone levels showed no significant difference between the groups (**Supplementary Figure S3A**). On the other hand, the 250 mg/kg BW/d EBN group showed a significant increase of FSH (**Supplementary Figure S3B**) and LH (**Supplementary Figure S3C**) compared to the 10 mg/kg BW/d EBN group where $p < 0.05$.

DISCUSSION

Traditionally, the consumption of EBN is claimed to increase libido. Libido in men usually is affected by a multitude of factors but it is closely regulated by the testosterone hormone (Allan et al., 2008; Corona et al., 2014; Rizk et al., 2017). Thus, this traditional claim of EBN on libido may be due to the presence of testosterone hormone in the EBN and its effect on promoting the testosterone release.

In this study, characterization of the major peak in Orbitrap LCMS analysis of EBN extract has identified the presence of several reproductive hormones. This includes testosterone, progesterone, and estradiol. However, the presence of FSH and LH were not detectable among the major peak. Testosterone, FSH, and LH are the well-known male reproductive hormones that play a vital role in controlling the complex process of spermatogenesis (Silber, 2018). Therefore, this study further evaluated the effect of EBN supplementation on these three hormones through serum hormonal analysis. It was found that there was a dose-dependent increase of testosterone, FSH, and LH which might suggest a direct correlation to the hormonal promoting effect of EBN.

Estradiol, another prominent reproductive hormone is also detected in our EBN extract. This is a predominant form of estrogen which is important for the female reproductive system. On the same note, estradiol also plays a critical role in a male sexual function where it is essential for modulating libido, erectile function, and spermatogenesis (Schulster et al., 2016). However, the testosterone and estradiol (T/E) ratio needs to be maintained above the cut-off point of 10 (Pavlovich et al., 2001). An increment of estradiol level which leads the T/E ratio to fall below the lower limit may cause decreased semen parameters (Raman and Schlegel, 2002).

Unfortunately, this study did not measure the serum estradiol level following EBN supplementation to clarify the T/E ratio. The evaluation of sperm parameters, however, reflects that EBN supplementation enhances spermatogenesis. This study found that there was a trend of gradual increase in sperm concentration and percentage of motile sperm with the increase of EBN dose. These positive findings in sperm concentration and motility reflected that major reproductive hormones were enhanced to the level that preserves and improves the spermatogenesis following EBN supplementation.

Despite the hormonal factors, the increment of sperm concentration might be related to the positive proliferative effect of EBN (Aswir and Wan Nazaimoon, 2011; Roh et al.,

2012) that is highly associated with the presence of sialic acid (Aswir and Wan Nazaimoon, 2011). Since sialic acid is characterized as one of the major peaks, therefore, sialic acid might act synergistically with other factor presence in EBN to promote spermatogonia proliferation during spermatogenesis in a way yet to be understood.

EBN supplementation also supports the sperm maturation process by significantly increased the percentage of sperm motility and viability. Supplementation of 50 mg/kg BW/d EBN showed a significant increase in sperm viability compared to the Control group. On the other hand, 250 mg/kg BW/d EBN demonstrated a slight decrease in the percentage of viability without a statistically significant difference compared to 50 mg/kg BW/d EBN. A discrepancy in the result of sperm viability may probably due to the different membrane sensitivity of the epididymal sperm (Hall et al., 1991; Lagarrigue et al., 2020). The process of harvesting sperm from the cauda epididymis might be mixed up with the adjacent caput epididymis. Subsequently, this led to a mixture of different sperm maturity with immature sperm membranes and therefore demonstrated a different sensitivity toward the viability test (HOST). There is a high possibility that the percentage of sperm viability may show a different result if ejaculated sperm is used for evaluation.

Reduction in organ weight is the most prominent sign of testicular toxicity (Fallahzadeh et al., 2017; Nowrouzi et al., 2019). All EBN doses applied in this study, however, demonstrated no obvious testicular toxicity as the organ coefficient of the testes, epididymis, and seminal vesicle showed no significant difference between the groups. Therefore, overall study findings prove that EBN has a positive effect on the male reproductive parameters.

However, future study is needed to evaluate the protective and/or curative effect of EBN with various factors affecting male fertility. Furthermore, male infertility is often a multi-dimensional problem (Muhammad et al., 2015). Thus, the suitability of EBN supplementation in various factors affecting male fertility needs to be further evaluated. At this moment, EBN holds multiple factors which include reproductive hormones and the proliferative effect that help to improve male reproductive parameters. It is well known that EBN also has antioxidant properties (Yida et al., 2014; Zulkifli et al., 2019) that might benefit from oxidative stress associated with male infertility in the future. Therefore, EBN could be a good candidate to fit with the multi-dimensional problem of male infertility.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the National University of Malaysia (UKM) Animal Ethics Committee with the approval number FISIO/PP/2018/SITIFATIMAH/28-MAR./908-MAR.-2018-DEC.-2020.

AUTHOR CONTRIBUTIONS

SI and CH conceived and conceptualized the experimental design. FJ and AZ performed the experiments and data collection. KO performed the Orbitrap LCMS analysis. FJ, KO, SI, and CH interpreted the data and critical analysis. FJ wrote the manuscript while SI and CH provided revisions to the scientific content of the manuscript. All authors read and approved the final manuscript.

FUNDING

This study was financially supported by FRGS/1/2019/SKK06/UKM/02/3 and FF-2018-193 research grants.

ACKNOWLEDGMENTS

We are grateful to the staff of the Department of Physiology for their assistance during the animal scarification day for sample collection. We also would like to express our appreciation to Gylcofood Sdn Bhd for providing the EBN material throughout the study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.631402/full#supplementary-material.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 12 April 2021
doi: 10.3389/fphar.2021.633303

The Potential Use of Sialic Acid From Edible Bird's Nest to Attenuate Mitochondrial Dysfunction by *In Vitro* Study

Aswir Abd Rashed[1]*, *Hafandi Ahmad*[2], *Siti Khadijah Abdul Khalid*[1] and *Devi-Nair Gunasegavan Rathi*[1]

[1]Nutrition, Metabolism and Cardiovascular Research Centre, Institute for Medical Research, National Institutes of Health, Ministry of Health, Shah Alam, Malaysia, [2]Departments of Veterinary Preclinical Sciences, Faculty of Veterinary Medicine, Universiti Putra Malaysia, Serdang, Malaysia

OPEN ACCESS

Edited by:
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Guangzhou University of Chinese Medicine, China

***Correspondence:**
Aswir Abd Rashed
aswir@moh.gov.my

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: 25 November 2020
Accepted: 22 February 2021
Published: 12 April 2021

Citation:
Rashed AA, Ahmad H, Abdul Khalid SK and Rathi D-NG (2021) The Potential Use of Sialic Acid From Edible Bird's Nest to Attenuate Mitochondrial Dysfunction by In Vitro Study. Front. Pharmacol. 12:633303. doi: 10.3389/fphar.2021.633303

Edible bird's nest (EBN) is one of the expensive functional foods in herbal medicine. One of the major glyconutrients in EBN is sialic acid, which has a beneficial effect on neurological and intellectual capability in mammals. The aims of this research were to study the effects of sialic acid from EBN on cell viability and to determine its effect on mitochondria membrane potential (MtMP) in Caco-2, SK-N-MC, SH-SY5Y, and PC-12 cell lines. Fourteen samples of raw EBN were collected from four different states in Malaysia. The confluency of the epithelial monolayers measurement of the tight junction for all the cell lines was determined using transepithelial electrical resistance (TEER), and the sialic acid uptake study in cell lines was determined by using ultra-high performance liquid chromatography (UHPLC). The MTT assay was conducted for cell viability study. The MtMP in cell lines was determined using the Mito Probe JC-1 Assay by flow cytometer analysis. We have recorded a statistically significant difference between the uptake of sialic acid from EBN and the standard solution. A higher amount of sialic acid was absorbed by the cells from extract of EBN compared to the standard solution. The amounts of sialic acid uptake in Caco-2, SK-N-MC, SH-SY5Y, and PC-12 cell lines were (0.019 ± 0.001), (0.034 ± 0.006), (0.021 ± 0.002), and (0.025 ± 0.000) μmol/L, respectively. The MTT results indicated that the concentration of sialic acid increased the cell viability and showed no cytotoxicity effects on cell lines when they were exposed to the sialic acid extract and sialic acid standard at all the tested concentrations. The number of active mitochondria was found to be significantly higher in SH-SY5Y cell lines with a 195% increase when treated with sialic acid from EBN. Although many researchers around the globe use SH-SY5Y and SK-N-MC for Alzheimer's disease (AD) study, based on our finding, SH-SY5Y was found to be the most suitable cell line for AD study by *in vitro* works where it has a known relationship with mitochondrial dysfunction.

Keywords: edible bird's nest, sialic acid, cell lines, in vitro, mitochondrial dysfunction, SH-SY5Y

INTRODUCTION

Human body is composed of many vital organs, where one of the largest and most complex organs is the brain (Jawabri and Sharma, 2020). Under normal circumstances, brain aging among healthy adults is known to undergo various changes, in terms of structural, chemical, functional, and neuronal alterations. Several of these changes are indicated *via* decrease in brain size, as a whole, as well as declining neurotransmitter systems (Marsman et al., 2013). Despite the fact that all healthy adults are subjected to these changes, it is important to highlight that age-related neurodegenerative disorders are not inclusive as a part of regular healthy aging conditions. These are mainly referred to Alzheimer's disease (AD) and other forms of dementia (Dobrowolski, 2014).

Alzheimer's disease or a related form of dementia is estimated to affect approximately 44 million people worldwide (Duthey, 2013). To date, elucidation to demonstrate the mechanism involved in AD pathogenesis is yet to be reported. Amyloid cascade reaction is the most widely recognized action mechanism among the numerous hypotheses that are proposed and available. This reaction puts forward the role of neurotoxic β-amyloid proteins that are deposited within the brain. The presence of these proteins instigates pathological changes that include amyloid plaques aggregation and intracellular neurofibrillary tangles (Murphy et al, 2010). Apart from the explained hypothesis above, there are also evident studies that suggest interrelatedness of mitochondrial damage and AD (Wang et al., 2020). The idea behind this is that the presence of healthy mitochondria is vital for neuron-based activity and also as a protection mechanism to minimize possible oxidative damage (Wang et al., 2020). As such, damaged mitochondria are believed to interfere with these essential roles.

Mitochondria are essential and of high importance for several roles, with their main function channeled toward energy production. The synthesis of high energy molecules in the form of adenosine-5′-triphosphate (ATP) is associated with the presence of mitochondria. The mechanism involved here includes conversion of metabolites energy to reduced nicotinamide adenine dinucleotide (NADH) followed by electron transfer from NADH while protons are also pumped across inner membrane to intermembrane space. This process creates transmembrane potential that utilizes ATP synthase for reflow of protons across inner membrane, and finally this energy is the driving factor of adenosine diphosphate (ADP) phosphorylation to ATP (Nicholls, 2010; Rich and Maréchal, 2010; Divakaruni and Brand, 2011; Bonora et al., 2012). Three factors were proposed as the possible reason that could lead to mitochondrial dysfunction, which include inability to provide required substrates, insufficient mitochondria, and failure of electron transport and ATP synthesis machinery (Nicolson, 2014).

Sialic acid presence is deemed essential for brain development and participates within ganglioside with specific role to enhance learning capability as well as memory (Tram et al., 1997; Wainscot, 2004). Cognitive ability in mammals is related to the variations observed with brain sialic acid concentration. In young mammals, concentrations of ganglioside- and protein-bound sialic acid were enhanced upon exogenous supplementation (Oliveros et al., 2018). Moreover, the exogenous sialic acid is localized to the synapse and the movement of positive neurotransmitters, transmitter release, and altering existing synaptic morphology are influenced by the sialic acid supplementation (Morgan and Winick, 1979). Dietary sialic acid has a role in brain development. Previous study done by Sprenger and Duncan has shown a significant rise in the sialic acid concentration in brain gangliosides and glycoprotein *via* oral administration of sialic acid during an initial postnatal period in the rodents (Sprenger and Duncan, 2012). Another study found that a decline in exogenous sialic acid concentration in brain leads to irreversibly decreased cognitive function but supplementary sialic acid will improve the learning process (Tram et al., 1997). Thus, nutritional interference research is important to evaluate the benefits in digestion and absorption system associated with neurodevelopment function. This will allow detailed analyses of cognitive function and behavior at numerous stages of development and show the association between dietary sialic acid supplementation and cognitive function development (Wang, 2012).

The sialic acid can be found in edible bird's nest (EBN) and it is one of the eight glyconutrients which has helped to increase cell tissue repair (Kong et al., 1987), promotes cell division and cell proliferation (Aswir and Wan Nazaimon, 2010). EBN has also been reported to be effective in the treatment of neurodegenerative disorders located in the hippocampal and cortical neurons in the brain such as AD and Parkinson's disease (PD) (Yew et al., 2014; Zhiping et al., 2015), and was able to improve the physiological human health (Guo et al., 2006; Matsukawa et al., 2011). However, extensive research is required to verify the effective levels and safety issues of EBN before it can be marketed more progressively worldwide and consumed by human. This is important because EBN is considered very precious, and its high consumption might not necessarily benefit the body.

In view that there is restricted access to human brain tissue in neuron-based disorders, most researches were channeled toward application of *in vitro* cell line. One of the most common cell lines used is the neuroblastoma (SH-SY5Y) cells. These cells were applied as a prototype for Aβ cytotoxicity in AD (Xie et al., 2010). Another cell line that could also be used is the human induced pluripotent stem cells (iPS) isolated from familial AD (FAD) patient. The iPS cell line is differentiated cells and is hence suitable to be evaluated for presenilin mutation effects (Penney et al., 2020). In addition to the two cell lines, immortal rat hippocampal (IRH) cell lines obtained from embryonic rat hippocampus were also deemed vital as the hippocampal neurons are accountable for both cognitive and memory ability (Eves et al., 1992). Furthermore, previous study by Gilbert and Ross mentioned that this cell line is more beneficial pertaining to its malignant and lack of cell lineage specificity nature, comparatively to tumor cells (Gilbert and Ross, 2009).

As mentioned above, the progression of β-amyloid may result in loss of memory function; thus, the objective of our study was to focus on the effect of sialic acid on mitochondrial dysfunction by using several types of cell lines.

MATERIALS AND METHODS

EBN and Sialic Acid Extract Source

A total of 14 raw unprocessed EBN samples were collected from four states of Peninsular Malaysia representing each region. The samples were collected during the breeding season of edible nest swiftlet within April to August 2016, manually cleaned, and finely grounded using a grinder. The sialic acid was extracted from raw EBN samples at SIRIM Berhad, Malaysia, using the high performance liquid chromatography (Siti Khadijah et al., 2019).

Cell Lines and Culture Conditions

Four different types of cell lines were used in this study: the colorectal adenocarcinoma (Caco-2/ATCC cat. no. HTB-37); the neuroepithelioma (SK-N-MC/ATCC cat. no. HTB-10); the neuroblastoma (SH-SY5Y/ATCC cat. no. CRL-2266); and the pheochromocytoma (PC-12/ATCC cat. no. CRL-1721). The entire cell lines were purchased from American Type Culture Collection (ATCC, United States). Each of the cell lines was seeded and maintained in 25 cm^2 culture flasks (Constar, Cambridge, MA) until use.

The PC-12 and SH-SY5Y cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; GIBCO New York, United States) supplemented with 20 and 15% v/v fetal bovine serum (FBS), while SK-N-MC cells were grown in Eagle's Minimum Essential Medium (EMEM; GIBCO New York, United States) supplemented with 20% v/v fatal bovine serum. The entire medium contained 1% v/v nonessential amino acid (GIBCO New York, United States), 1% antibiotic (penicillin–streptomycin) (GIBCO New York, United States), and 1% v/v L-glutamine (GIBCO New York, United States). Only PC-12 cell was added with 15% horse serum (GIBCO New York, United States). All the cells were maintained in the same conditions, at 37°C in an incubator with 5% carbon dioxide, 95% humidity, and air atmosphere. The medium was replaced every 2–3 days. The cells were maintained until they reached 80% confluency.

Transepithelial Resistance Values Measurement

The cells will reach the maximal levels of differentiation after several days in incubation. To confirm this process, transepithelial resistance values (TEER) can be monitored using EVOM2™ because fully differentiated polarized cells have tight junctions with a TEER of >200 Ω*cm^2 (MacCallum et al., 2005). The EVOM2™ measures cell monolayer health and cellular confluence *via* qualitative and quantitative measurement, respectively. Before measurement, STX2 electrodes (World Precision Instrument, New Haven, CT, United States) were equilibrated and sterilized according to the manufacturer's recommendations. Two hundred microliters of culture medium was added to the upper compartment of the cell culture system. The ohmic resistance of a blank (culture insert without cells) was measured in parallel. The blank value was subtracted from the total resistance of the sample, in order to obtain sample resistance. The final unit area resistance (Ω*cm^2) was calculated by multiplying the sample resistance by the effective area of the membrane (0.33 cm^2 for 24-well millicell insert plates).

Cell Viability

The MTT (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is commonly used to measure cell viability and proliferation (Ahmad et al., 2006). Adherent cells were dissociated from their substrate by trypsinization or scraping. Then, the cells were centrifuged at 830 × g for 5 min. The supernatants were discarded, and the cell pellet was resuspended in DMEM at a density of 20,000 cells/cm^2 into a 96-well plate. The cells were then incubated overnight in an incubator with 5% carbon dioxide (CO_2), 95% air atmosphere, and 95% relative humidity at 37°C. The next day, the medium was changed to medium-free FBS following exposure to (or treatment with) sialic acid at the required concentration (20, 40, 60, 80, and 100 μg/ml), and the cells were incubated for 24 h. MTT reagent at a concentration of 5 mg/ml and a volume of 10 μL was added to each well and incubated for 3–4 h until the purple precipitate was visible. Later, the medium was aspirated from the wells as completely as possible without disturbing the formazan crystals and cells on the plastic surface. Then, 100 μL of dimethyl sulfoxide (DMSO) was added to each well, followed by agitation on a plate shaker for 5 min, and finally, the optical density was read at 570 nm. The number of surviving cells is directly proportional to the level of the formazan product created (Van de Loosdrecht et al., 1994).

The absorbance value for the blanks should be 0.00 ± 0.1 optical density (O.D) units. The absorbance range for untreated cells should typically be between 0.75 and 1.25 O.D. units. Selection of a cell number (i.e., providing values between the range of 0.75 and 1.25) allows for the measurement of both stimulation and inhibition of cell proliferation. If the absorbance values of the experimental samples are higher than the control, this indicates an increase in cell proliferation. Alternatively, if the absorbance rates of the experimental samples are lower than the control, this indicates a reduction in the rate of cell proliferation or a reduction in overall cell viability.

Sialic Acid Uptake Studies in Caco-2, SK-N-MC, SH-SY5Y, and PC-12 Cell Lines

For the purpose of sialic acid uptake studies, the cells were grown on transwell plates for 21 days. The culture medium was removed before adding 2 ml of uptake buffer (140 mM NaCl, 5 mM KCl, 1 mM NaH_2PO_4, 10 mM Mes, 0.5 mM of $MgCl_2$, and 1.0 mM $CaCl_2$ at pH 6.0) and incubated at 37°C for 2 min. Later, 1 ppm (~3 μmol/L) of sialic acid extract was added to each well and incubated for 15 min at 37°C. Then, the buffer was aspirated, and cells were rinsed with cold buffer for three times before adding

1 ml of 200 mM NaOH to solubilize the cells and they were left overnight at 4°C. After an overnight incubation at 4°C, the cells were subsequently removed; the levels of sialic acid in buffer and cells were determined by using UHPLC. This procedure follows the method of Derakhshandeh and his colleagues with slight modification (Derakhshandeh et al., 2011).

Mitochondrial Membrane Potential

This procedure was performed using the Mito Probe JC-1 Assay Kit for flow cytometry (Life Technology, United States). The kit contains 30 µg powdered dye, DMSO, carbonyl cyanide 3-chlorophenylhhydrazone (CCCP), and 10× phosphate-buffered saline (PBS). The cell lines were fixed with 150 µL of sialic acid extract and standards for 24 h prior to staining the cells for flow cytometer measurement. In brief, all reagents were equilibrated at room temperature before beginning the experiment. Two hundred micromolar of JC-1 powder stock solution was prepared by dissolving the vial with 230 µL of the DMSO. Then, 1×10^6 cells/mL of cell lines were collected by scrapping the cells and centrifuged in warm PBS before being incubated at 37°C for 5 min. To provide the positive control, 1 µL of 50 mM of CCCP (50 µM in final concentration) was added to the tube and incubated for 5 min. After the incubation, 10 µL of JC-1 stock solution (2 µM in final concentration) was added to all tubes and incubated for another 30 min at 37°C. Later, all cells were washed using the warm PBS and centrifuged to obtain the cell pellet. Then, the cells were resuspended by adding 500 µL of PBS to each tube. All tubes were analyzed using a flow cytometer machine (BD FACSAria™) with 488 nm excitation using emission for Alexa Fluor® 488 dye and fluorescence microscopy. The JC-1 dye detection was used with bandpass filters centered around 529 nm (green fluorescence) and bandpass filters centered around 595 nm (orange fluorescence). The logarithmic signal amplification was used with the typical green–orange electronic signal compensation near 4% and orange–green signal compensation around 10% (Cossarizza and Salvioli, 2001). The depolarization of mitochondria in cells is indicated by decreasing ratio of fluorescence intensity (red JC aggregates/green JC monomer).

Statistical Analysis

All data were analyzed using the SPSS software version 16 (IBM Software, Inc., New York, United States). The viability of the cell lines induced with sialic acid was analyzed using the one-way analysis of variance (ANOVA) with post hoc Tukey's test to show significant difference between groups. $p < 0.05$ was considered statistically significant.

RESULTS

Transepithelial Resistance Values Measurement

The determination of cell cultures confluency stage for entire cell lines using EVOM2™ was shown in **Figure 1**, **2**. Caco-2 and SK-N-MC cell lines reached their 50% confluency at day 12 with 289.91 ± 137.89 $\Omega^{*}cm^2$ and 292.71 ± 80.61 $\Omega^{*}cm^2$, respectively, while SH-SY5Y and PC-12 cell lines reached their 50% confluency at day 15 with 305.91 ± 80.61 $\Omega^{*}cm^2$ and 280 ± 127.98 $\Omega^{*}cm^2$, respectively. The Pearson Rank correlation analysis was performed to determine the relationship between the days of culture and TEER reading. Based on results in **Figure 2**, a strong positive correlation between culture duration and all cell lines was recorded ($p < 0.01$).

Cell Viability

Table 1 shows the effect of sialic acid extract and standard at 20, 40, 60, 80, and 100 µg/ml concentrations on cell viability in serum-free medium, which represents the conditions for the sialic acid uptake study. The cell viability results showed that there are no cytotoxicity effects in all neuroblastoma and epithelial cell lines when exposed to sialic acid at the concentration of 60 µg/ml or below. All cell lines showed significant differences in cell viability ($p < 0.05$).

The percentage of cell viability in epithelial and neuroblastoma cell lines was significantly higher when they were induced with sialic acid extract compared to sialic acid standard. Above the concentration of 60 µg/ml, all cell lines showed negative effect of sialic acid on cell viability.

Sialic Acid Uptake Studies in Caco-2, SK-N-MC, SH-SY5Y, and PC-12 Cell Lines

Table 2 shows the concentration of sialic acid that has been absorbed by the entire cell lines after selected concentration of sialic acid was added to the cells. For cell monolayer, all cell lines showed significant differences between the extraction and control ($p < 0.05$) and between the extraction and standard extraction ($p < 0.05$), respectively. However, only Caco-2 cell lines showed a significant difference between the control and standard ($p < 0.05$). For basal solution, the mean absolute difference (MD) of sialic acid uptake was significant between the control and the extraction in all cell lines. The MD of sialic acid uptake was also significant between the extraction and the standard. In contrast, only SH-SY5Y cell line has recorded the differences in sialic acid uptake between the control and the standard.

Mitochondrial Membrane Potential

Table 3 shows the percentage of active mitochondria once being treated with sialic acid in cell lines using flow cytometry analysis. **Figures 3–6** present the excitation peak of entire cell exposed to JC-1 dye using 488 nm wavelength. One-way ANOVA showed that there was a significant difference in numbers of active mitochondria between all groups of treatment ($p < 0.05$). The Tukey post hoc test revealed that the number of active mitochondria in SH-SY5Y is significantly higher when induced with sialic acid compared with control ($p = 0.000$). However, even without treatment with sialic acid, all other cell lines have a higher number of active mitochondria at the start of the experiment.

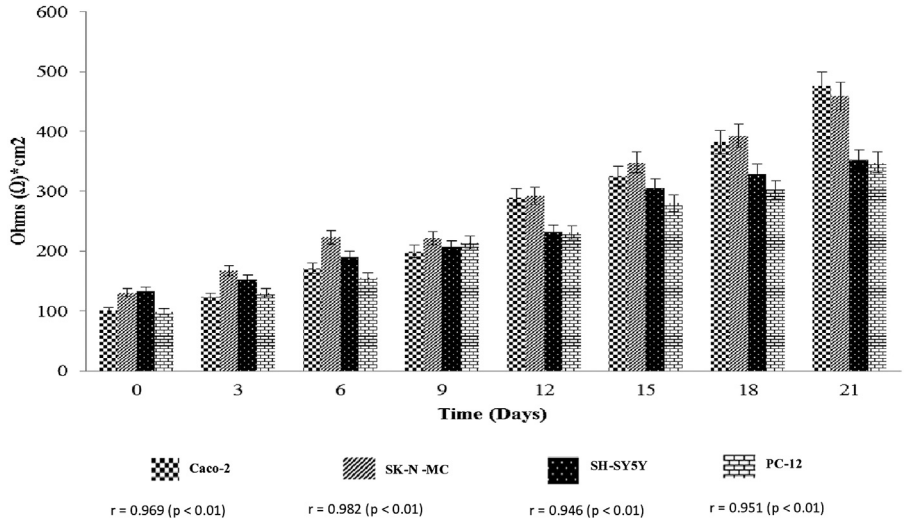

FIGURE 1 | Transepithelial electrical resistance assay (TEER) (Ohms*cm^2) measurement of cell lines. Values are expressed as mean ± SEM of triplicate experiments.

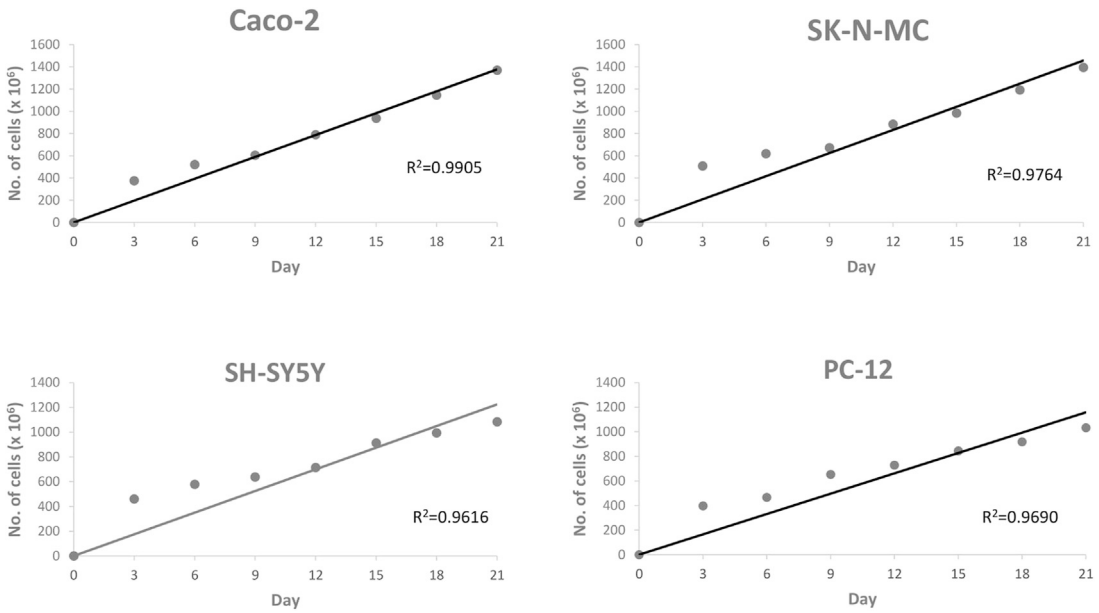

FIGURE 2 | Scattered plot shows the correlation between cell lines and day of culture. Values are given as 1 × 10^6 (mean ± SEM) for three independent biological determinations.

DISCUSSION

In studies that involve cell line usage, it is important to control possible variations especially in terms of standardizing optimal duration after seeding as this ensures generation of differentiated cultures with excellent uniformity. Furthermore, development of tight junction in these cells is highly associated with the culture duration (Srinivasan et al., 2015). As an example, Caco-2 cells are derived from colorectal adenocarcinoma cells and spontaneously undergo differentiation between 0 and 21 days. It is important to also note that the Caco-2 cells are nonidentical to normal duodenal enterocytes (Mahraoui et al., 1994; Sharp et al., 2002; Mariadason et al., 2000). Based on reported literature, fully differentiated polarized Caco-2 cells have tight junctions with a TEER value of >200 Ω*cm^2, and as such the differentiation process is confirmed by careful monitoring of the cell's polarization process (Eves et al., 1992). On the other hand, fully differentiated neuroblastoma cells (SH-SY5Y) express

TABLE 1 | Effects of sialic acid from EBN on cell lines in 1×10^6 cells/ml.

Treatment concentration (μg/ml)	Cell viability (%)							
	Sialic acid extract				Sialic acid standard			
	Caco-2	SK-N-MC	SH-SY5Y	PC-12	Caco-2	SK-N-MC	SH-SY5Y	PC-12
0	8.9 ± 4.48^{c}	9.27 ± 0.61^{d}	9.3 ± 1.57^{d}	9.6 ± 1.76^{d}	9.6 ± 1.99^{b}	9.0 ± 4.69^{d}	9.5 ± 6.22^{c}	9.5 ± 2.50^{c}
20	79.13 ± 3.01^{b}	71.6 ± 4.38^{b}	61.63 ± 1.76^{b}	52.0 ± 8.58^{c}	65.93 ± 3.62^{a}	65.57 ± 2.48^{b}	54.0 ± 1.82^{b}	39.53 ± 4.89^{b}
40	77.67 ± 5.52^{b}	77.33 ± 3.48^{b}	64.57 ± 2.55^{b}	63.93 ± 3.62^{b}	63.9 ± 5.87^{a}	73.03 ± 0.81^{b}	54.0 ± 3.01^{b}	57.5 ± 6.54^{a}
60	90.2 ± 5.46^{a}	86.57 ± 4.85^{a}	81.93 ± 8.65^{a}	72.53 ± 2.67^{a}	78.23 ± 3.21^{a}	82.27 ± 0.91^{a}	65.77 ± 1.94^{a}	63.57 ± 4.25^{a}
80	74.47 ± 4.77^{b}	83.77 ± 4.56^{a}	53.3 ± 1.28^{c}	63.33 ± 4.95^{b}	69.07 ± 9.81^{a}	79.43 ± 3.17^{a}	51.0 ± 3.76^{b}	61.37 ± 7.90^{a}
100	70.6 ± 2.65^{b}	64.77 ± 5.46^{c}	49.17 ± 5.06^{c}	62.00 ± 2.91^{b}	64.67 ± 12.40^{a}	70.77 ± 6.76^{b}	43.46 ± 8.51^{b}	53.37 ± 7.03^{a}

Cells were treated with sialic acid extract and sialic acid standard for 24 h in serum-free medium. The optical density was determined by spectrophotometer at 570 nm. Values are expressed as mean ± SEM of triplicate experiment. $^{a, b, c, d}$ *Means without common letters in their superscript are significantly different (*$p < 0.05$*).*

various markers of mature neurons. These are inclusive of growth-associated protein (GAP-43), neuronal nuclei (NeuN), synaptophysin (SYN), synaptic vesicle protein II (SV2), neuron specific enolase (NSE), and microtubule associated protein (MAP) (Murillo et al., 2017). At the same time, the fully differentiated cells also indicate that they lack expression of glial markers such as glial fibrillary acidic protein (GFAP) (Liu et al., 2009).

TEER measurement is subject to certain level of variations, especially among distinct groups in different studies. These variations observed are possibly contributed by a number of factors. The identified factors include difference in actual junction tightness, temperature, cells handling technique during measurements, and potential difference in measuring equipment (e.g., chopstick or cup electrodes, impedance measurements). In addition, it should also be highlighted that translating TEER into a functional estimate of tightness is tough. This is due to the fact that composition of the tight junction complexes and the size of the compound of interest are the underlying factors that influence the endothelial monolayer tightness (Srinivasan et al., 2015; Helms et al., 2016). In our study, we found that the human epithelial cell lines (Caco-2) and human brain barrier cell lines (SK-N-MC, SH-SY5Y and PC-12) reached more than 300 $\Omega^{*}cm^2$ at 21 days. In comparison to previous reported review by Helms and colleagues, our finding is considered higher than the reported standard range of TEER values for blood–brain barrier cell culture, which is around 40–200 $\Omega^{*}cm^2$ (Helms et al., 2016).

In comparison to *in vivo* studies, *in vitro* cytotoxicity and cell viability assays were shown to be more advantageous in terms of speed, lower cost, and automation potential. Due to these reasons, studies conducted with human cells are deemed more appropriate than *in vivo* research (Chrzanowska et al., 1990). MTT assay is a colorimetric assay that assesses the cell metabolic activity. Before any dietary components are investigated for their effects on iron uptake in Caco-2 cells, it is prudent to evaluate cell viability under incubation conditions and the reduction of tetrazolium salts, as part of the MTT assay, which is now recognized as a safe and accurate test for cell viability (Yew et al., 2014). NADPH and NADH that are synthesized by dehydrogenase enzymes in metabolically active cells are responsible for this conversion (Xu et al., 2015). It was found that the cell viability of the Caco-2, SK-N-MC, PC-12, and SH-SY5Y cell lines were upregulated alongside the steady increase in the concentration of sialic acid in the media. At 1×10^6 cell lines, sialic acid extract and sialic acid standard did not show any cytotoxicity effects on cell viability up to a concentration of 60 μg/ml. However, above 80 μg/ml concentration, there was a reduction in cell viability for all the cell lines. This result is similar to our previous published finding on cell proliferation where it was concluded that stimulation with different concentration of sialic acid from EBN and sialic acid standard into cells will give rise to a dose dependent increase in cell viability (Aswir and Wan Nazaimon, 2011).

The cell viability of Caco-2 and SH-SY5Y cell lines showed remarkable difference when treated with sialic acid extract compared to the standard sialic acid. This discrepancy could be due to the variation in extraction process. Besides, there are many different types of standard sialic acids commercially available and containing a mixture of sialic acids found in humans and animals. The extraction of standard sialic acids also varies from sulfuric acid, phosphoric acid, acetic acid, trifluoroacetic acid (TFA), and HCl. None of the sialic acid on the market is obtained from bird's nests. Since bird's nests naturally contain higher bioactive compounds including sialic acid, it may be one of the reasons why sialic acid obtained from bird's nest has a slightly higher absorption rate than the commercially available sialic acid. In addition, the increase in cell viability with higher concentration might be influenced by the mitogenic properties in sialic acid extract from EBN that promoted the cell growth as manifested by previous studies which showed the enhanced cell division in rabbit corneal keratocytes using EBN (Zainal Abidin et al., 2011; Yew et al., 2014). Although sialic acid standard also showed increase in cell viability, this effect was observed to be slightly lower than the sialic acid extract. This could be due to low activities associated with the varied process and preparing the treatment as previously mentioned (Yew et al., 2014). Moreover, the quantity of absorbance signal created is dependent on numerous parameters including the concentration of MTT reagent, the time of incubation period, the numbers of viable cells, and also their metabolic activities (Riss et al., 2016).

In general, sialic acid rarely exists free in nature and is usually available as component of oligosaccharide chains of mucins, glycoproteins, and glycolipids. In terms of its position, sialic acid usually occupies terminal, nonreducing positions that are highly exposed and functionally essential. These commonly refer to nonreducing positions of oligosaccharide chains of complex

TABLE 2 | Sialic acid uptake studies by Caco-2, SK-N-MC, SH-SY5Y, and PC-12 cell lines.

Treatment	Sialic acid uptake							
	Basal solution (μmol/L)				Cell monolayer (μmol/L)			
	Caco-2	SK-N-MC	SH-SY5Y	PC-12	Caco-2	SK-N-MC	SH-SY5Y	PC-12
Control	0.003 ± 0.002^{b}	0.003 ± 0.003^{b}	0.004 ± 0.001^{c}	0.003 ± 0.000^{b}	0.003 ± 0.001^{c}	0.003 ± 0.005^{b}	0.003 ± 0.08^{b}	0.002 ± 0.000^{b}
Extraction	0.020 ± 0.001^{a}	0.055 ± 0.006^{a}	0.043 ± 0.002^{a}	0.043 ± 0.000^{a}	0.019 ± 0.001^{a}	0.034 ± 0.006^{a}	0.021 ± 0.002^{a}	0.025 ± 0.000^{a}
Standard	0.006 ± 0.002^{b}	0.004 ± 0.006^{b}	0.027 ± 0.003^{b}	0.003 ± 0.000^{b}	0.012 ± 0.001^{b}	0.004 ± 0.001^{b}	0.009 ± 0.003^{b}	0.003 ± 0.000^{b}

Values are expressed as mean ± SEM in triplicate experiment. $^{a, b, c}$ Means without common letters in their superscript are significantly different ($p < 0.05$).

carbohydrates on both outer and inner membrane surfaces, mainly to galactose, N-acetylgalactosamine, and other sialic acid moieties. The highest concentration of sialic acid is present in mammalian central nervous system, where majority of it is found in gangliosides (65%), followed by glycoproteins (32%), while the remaining exists as free sialic acid (Brunngraber et al., 1972).

To date, there are limited findings on digestion and mechanisms involved among sialic acid compounds. It was reported that rat intestinal walls are highly permeable to free sialic acids. In addition, it was highlighted that sialidases of bacterial origin could possibly cleave sialic acid residues from milk oligosaccharides in colon; however it was not evident if sialic acid is capable of absorption across colonic mucosa (Wang and Brand-Miller, 2003). Hence, a cell model was established in order to understand and evaluate the mechanisms involved in sialic acid transport across cellular barriers. One relevant cell model is the Caco-2 epithelial cells that plays its role in studying transport from gastrointestinal (GI) lumen into the blood (Wilson et al., 1990), where sialic acid is taken orally. In this study, we determine the concentration of sialic acid that has been absorbed by the cell using UHPLC instead of radiolabeled isotope. A monolayer of Caco-2 cells was applied as the model uptake prototype to demonstrate sialic acid uptake across the GI epithelium. The same model was applied for sialic acid uptake in the brain through a monolayer of SK-N-MC, SH-SY5Y, and PC-12 cell lines.

Based on literature, it was found that the normal range of total sialic acid (TSA) level found within serum or plasma falls in the range of 1.58–2.22 mmol/L. From this, only about 0.5–3.0 μmol/L corresponds to free form of SA (Sillanaukee et al., 1999). Thus, we used 3 μmol/L of sialic acid extract to mimic the normal range of free sialic acid in the human body. Our study has recorded sialic acid uptake by the cell lines. A number of transport mechanisms across cytoplasmic membrane have evolved in response to sialic acid transport in bacteria. Tripartite ATP-independent periplasmic (TRAP), ATP binding cassette (ABC), major facilitator superfamily (MFS), and sodium solute symporter (SSS) transporter families are among the common identified mechanisms (Vimr and Troy, 1985; Allen et al., 2005; Post et al., 2005; Severi et al., 2010; North et al., 2017; Wahlgren et al., 2018). Based on our finding, there is high possibility of the presence of sialic acid transporter in monolayer cell lines that helps in the transportation of sialic acid extract across the membrane to the basal chamber. From the original concentration of 3 μmol/L, we found that high concentration of sialic acid was recorded in neuroblastoma (SK-N-MC, SH-SY5Y, and PC-12) cell lines compared to epithelial (Caco-2) cell lines. Although we did not perform a study on the sialic acid transporter, this finding, however, shed some lights and indirectly gave insights for the reason behind high sialic acid concentration in brain compared to the other parts of the body. The previous study done by Bardor and his colleagues (Bardor et al., 2005) which reported that human neuroblastoma cell lines could be incorporated with sialic acid with efficiency comparable with Caco-2 cells also supports our findings. Thus, this could suggest that the mechanism of sialic acid uptake study can also occur in other human cells but with varying degrees.

Eukaryotic cells contain several types of organelles, which may include nucleus, mitochondria, chloroplasts, the endoplasmic reticulum, the Golgi apparatus, and lysosomes. Each of these organelles performs a specific function critical to the cell's survival. Since cell lines were also obtained from the eukaryotic cells, they also have the same organelles. Mitochondria study gained attention in 1970s, when research was focused on studying energy conservation mechanism as well as ATP synthesis. At the same time, chemiosmotic theory of oxidative phosphorylation was also established, which led to conferment of Nobel Prize in Chemistry awarded to Peter Mitchell in year 1978 (Brand et al., 2013). Any part of the body can be affected by mitochondrial diseases such as the cells of the brain, nerves, muscles, kidneys, heart, liver, eyes, ears, and pancreas. Mitochondrial dysfunction arises upon failure of the mitochondria in ATP synthesis due to certain underlying conditions or diseases. These diseases can lead to secondary mitochondrial dysfunction, including but not limited to Alzheimer's disease, muscular dystrophy, diabetes, and cancer (Brand et al., 2013).

TABLE 3 | The numbers of mitochondria in flow cytometer analysis for Caco-2, PC-12, SH-SY5Y, and SK-N-MC cell lines.

Types of cell lines	Numbers of mitochondria (%)		
	Control	Sialic acid standard	Sialic acid extract
Caco-2	96.04 ± 0.03^{a}	98.10 ± 0.94^{a}	80.40 ± 0.55^{b}
PC-12	95.86 ± 1.15^{a}	92.98 ± 1.00^{a}	91.96 ± 0.65^{a}
SH-SY5Y	48.34 ± 1.22^{b}	96.39 ± 0.61^{a}	90.56 ± 0.85^{a}
SK-N-MC	93.76 ± 0.87^{a}	93.51 ± 0.70^{a}	94.80 ± 0.30^{a}

Values are expressed in mean ± SEM for three independent experiments. $^{a, b}$ Means without common letters in their superscript are significantly different ($p < 0.05$).

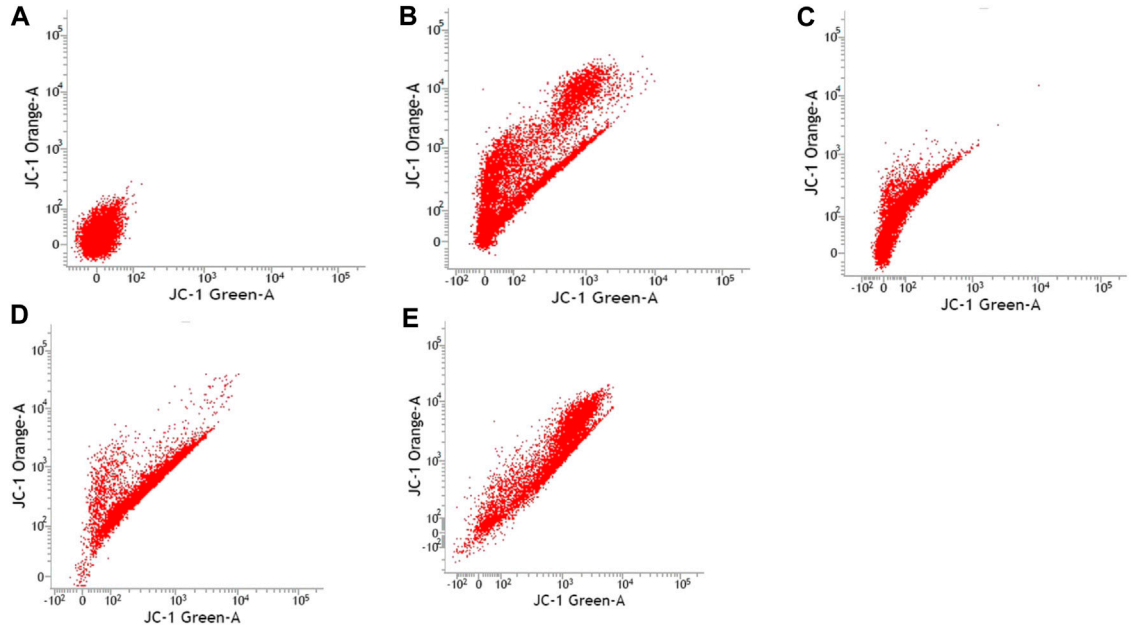

FIGURE 3 | Caco-2 cell lines with or without treatment with sialic acid using JC-1 Mito Probe by BD FACSAria™: **(A)** untreated cell; **(B)** control without CCP; **(C)** control with CCP; **(D)** treatment with standard sialic acid; **(E)** treatment with extraction of sialic acid.

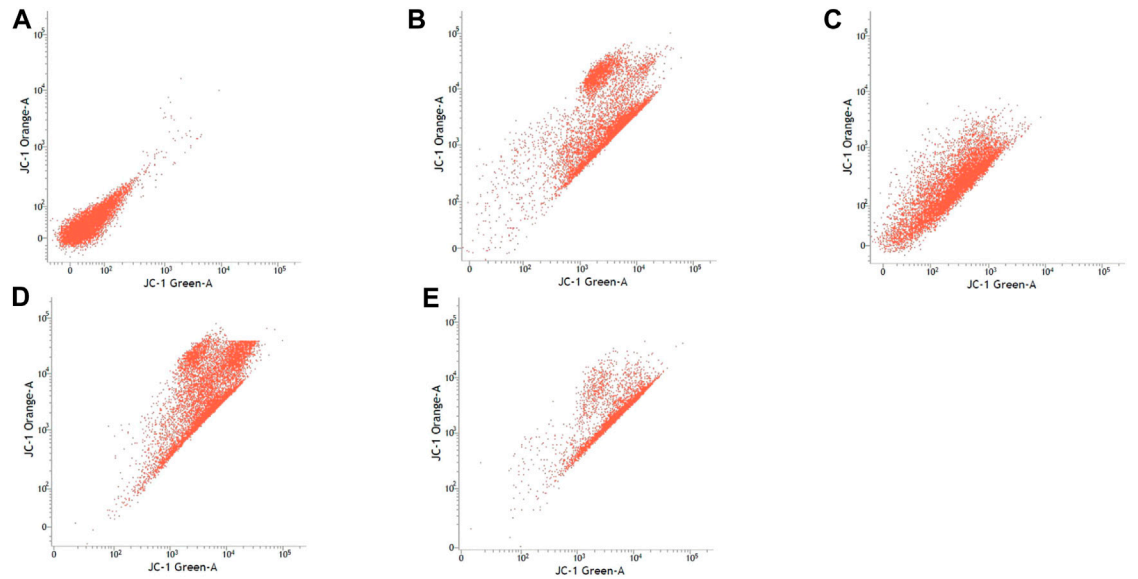

FIGURE 4 | SK-N-MC cell lines with or without treatment with sialic acid using JC-1 Mito Probe by BD FACSAria™: **(A)** untreated cell; **(B)** control without CCP; **(C)** control with CCP; **(D)** treatment with standard sialic acid; **(E)** treatment with extraction of sialic acid.

In this study, the JC-1 was used because it is more specific for measuring changes in mitochondria membrane potential (MtMP) (De Biasi et al., 2015). It is also consistent in response to the depolarization of the mitochondria compared to other cationic dyes such as Rhodamine-123 and 3,3′ dihexyloxacarbocyanine iodide (DiOC6) which are toxic to the mammalian cells (Shapiro, 1994). The carbonyl cyanide 3-chlorophenylhhydrazone (CCCP) was used as a control to confirm that JC-1 dye responses to membrane potential fluctuations and also determines compensation percentage that is necessary in order to quantify 488-excited J-aggregates accurately (Perelman et al., 2012; Sivandzade et al., 2019). Throughout oxidative phosphorylation, essential components that are required in energy storage are the MtMP derived by

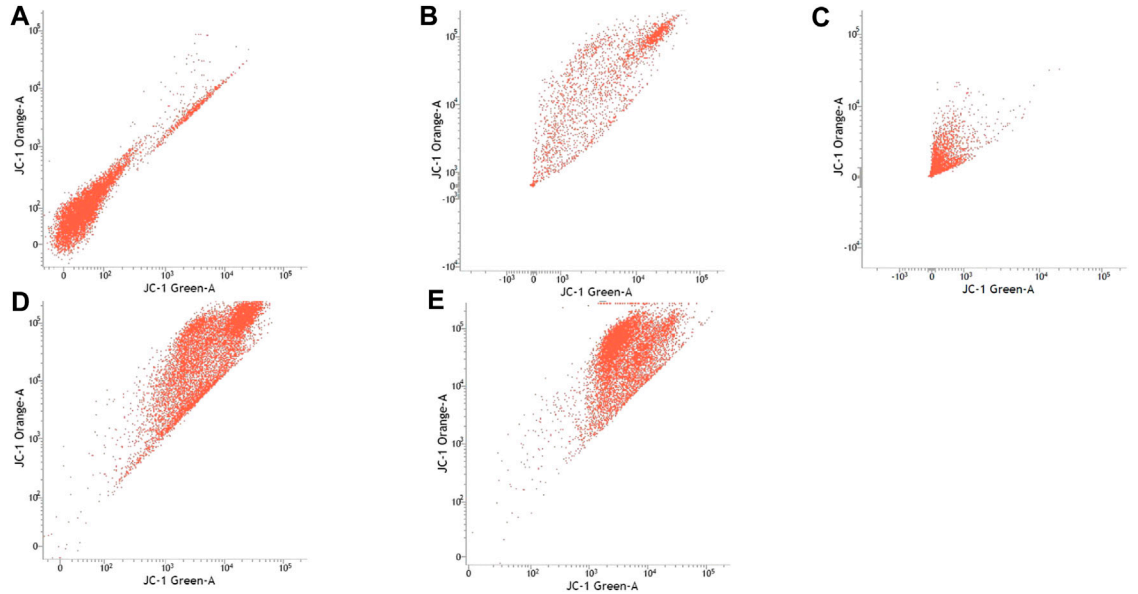

FIGURE 5 | SH-SY5Y cell lines with or without treatment with sialic acid using JC-1 Mito Probe by BD FACSAria™: **(A)** untreated cell; **(B)** control without CCP; **(C)** control with CCP; **(D)** treatment with standard sialic acid; **(E)** treatment with extraction of sialic acid.

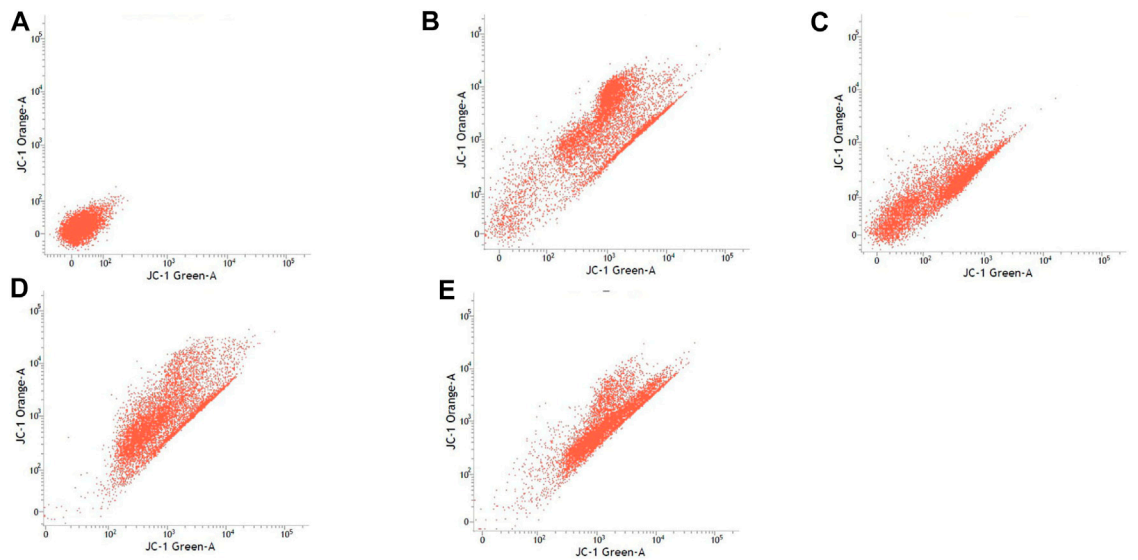

FIGURE 6 | PC-12 cell lines with or without treatment with sialic acid using JC-1 Mito Probe by BD FACSAria™: **(A)** untreated cell; **(B)** control without CCP; **(C)** control with CCP; **(D)** treatment with standard sialic acid; **(E)** treatment with extraction of sialic acid.

proton pumps, specifically called Complexes I, III, and IV (Zorova et al., 2018). Based on our findings, Caco-2, PC-12, and SK-N-MC cell lines revealed a high number of active mitochondria compared to SH-SY5Y cell lines in control group (**Table 3**). The sialic acid extract exhibited less numbers of active mitochondria in Caco-2 cells compared to sialic acid standard in respond to the sialic acid stimulus. The impact developed and was upregulated almost 100% when sialic acid was added to the cells compared to the control.

Previous study showed that the mitochondrial functionality can be restored by natural product such as herbal medicine which helps in preserving the dopaminergic neurons in Parkinson disease (Kim et al., 2004), and in this study the sialic acid caused the depolarization of the mitochondria cells. The percentage of mitochondria was slightly increased when sialic acid standard was added to the media containing Caco-2 cell lines compared with the control. However, the

percentage was reduced significantly when sialic acid extract was added to the media. Although these findings were interesting, the root cause of this phenomenon is unknown. Conversely, in the SK-N-MC cell line, the percentage of mitochondria was slightly higher in sialic acid extract (94.80%) compared to sialic acid standard (93.51%). This is because the SK-N-MC is a human brain cell and according to Schauer (Schauer, 1982), neural cell in membrane contains 20 times more sialic acid than other types of membrane and clearly indicated that sialic acid is of utmost importance in neuronal development. The results were similar to the study done by Rosernberg (Rosenberg, 1995), where ganglioside in nervous tissue is composed of sialic acid as glycosphingolipids that are available in cerebral cortex of human brain at high concentration. Intriguingly, sialic acid extract and standard were found to be able to increase the number of active mitochondria significantly in SH-SY5Y cells compared with the control. With SH-SY5Y cells originally derived from a metastatic bone tumor biopsy, the number of active mitochondria in them seem to be able to effectively change when sialic acid was added to the media. Although there are many types of cell lines available to represent AD study *in vitro*, SH-SY5Y cell line is more pronounced if the study is focusing on mitochondrial dysfunction. Apart from its suitability for AD, it can also be applicable for other studies focused on mitochondrial dysfunction in other diseases.

CONCLUSION

Our finding has indicated that MtMP measurement could be used to study mitochondrial dysfunction by *in vitro* technique. The increase observed in mitochondrial membrane potential in entire cell lines when subjected to sialic acid treatment indicated presence of healthy cells. This is vital to study on MtMP measurement for its effect on mitochondrial dysfunction. The sialic acid uptake also was noticed to occur with varying degrees in cell lines. Based on our findings among all the tested cells, SH-SY5Y was found to be the most suitable cell line especially for studies that will be focused on the expression of active mitochondria.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

All authors were involved in conceptualization, literature review, editing tables, writing, reviewing, editing, revision, and final editing. All authors have read and agreed to the published version of the manuscript.

FUNDING

The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article. This project was funded by COE Swiftlet. However, for the publication fees, the payment will be made by the National Institutes of Health (Malaysia).

ACKNOWLEDGMENTS

The authors wish to thank the Director General of Health, Malaysia, and the Director of Institute for Medical Research (IMR), for giving permission to publish this article. They would also like to express their gratitude to all the experts within the MOH, Malaysia, who participated in this workshop for their tremendous support.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 13 April 2021
doi: 10.3389/fphar.2021.632169

Effect of Hydrolyzed Bird's Nest on β-Cell Function and Insulin Signaling in Type 2 Diabetic Mice

Ker Woon Choy[1], Zuhaida Md Zain[2], Dharmani Devi Murugan[2], Nelli Giribabu[3], Nor Hisam Zamakshshari[4], Yang Mooi Lim[5] and Mohd Rais Mustafa[2,4]*

[1]Department of Anatomy, Faculty of Medicine, Universiti Teknologi MARA, Selangor, Malaysia, [2]Department of Pharmacology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia, [3]Department of Physiology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia, [4]Centre for Natural Product Research and Drug Discovery (CENAR), Wellness Research Cluster, University of Malaya, Kuala Lumpur, Malaysia, [5]Department of Pre-Clinical Sciences, Centre for Cancer Research, Faculty of Medicine and Health Sciences, University Tunku Abdul Rahman, Selangor, Malaysia

OPEN ACCESS

Edited by:
Geng Li,
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Reviewed by:
Xiaoping Lai,
Guangzhou University of Chinese Medicine, China
Jieru Lin,
Hospital Authority, Hong Kong

***Correspondence:**
Dharmani Devi Murugan
dharmani79@um.edu.my

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: *22 November 2020*
Accepted: *22 February 2021*
Published: *13 April 2021*

Citation:
Choy KW, Zain ZM, Murugan DD, Giribabu N, Zamakshshari NH, Lim YM and Mustafa MR (2021) Effect of Hydrolyzed Bird's Nest on β-Cell Function and Insulin Signaling in Type 2 Diabetic Mice. Front. Pharmacol. 12:632169. doi: 10.3389/fphar.2021.632169

Type 2 diabetes mellitus is characterized by both resistance to the action of insulin and defects in insulin secretion. Bird's nest, which is derived from the saliva of swiftlets are well known to possess multiple health benefits dating back to Imperial China. However, it's effect on diabetes mellitus and influence on the actions of insulin action remains to be investigated. In the present study, the effect of standardized aqueous extract of hydrolyzed edible bird nest (HBN) on metabolic characteristics and insulin signaling pathway in pancreas, liver and skeletal muscle of *db/db*, a type 2 diabetic mice model was investigated. Male *db/db* diabetic and its euglycemic control, C57BL/6J mice were administered HBN (75 and 150 mg/kg) or glibenclamide (1 mg/kg) orally for 28 days. Metabolic parameters were evaluated by measuring fasting blood glucose, serum insulin and oral glucose tolerance test (OGTT). Insulin signaling and activation of inflammatory pathways in liver, adipose, pancreas and muscle tissue were evaluated by Western blotting and immunohistochemistry. Pro-inflammatory cytokines were measured in the serum at the end of the treatment. The results showed that *db/db* mice treated with HBN significantly reversed the elevated fasting blood glucose, serum insulin, serum pro-inflammatory cytokines levels and the impaired OGTT without affecting the body weight of the mice in all groups. Furthermore, HBN treatment significantly ameliorated pathological changes and increased the protein expression of insulin, and glucose transporters in the pancreatic islets (GLUT-2), liver and skeletal muscle (GLUT-4). Likewise, the Western blots analysis denotes improved insulin signaling and antioxidant enzyme, decreased reactive oxygen species producing enzymes and inflammatory molecules in the liver and adipose tissues of HBN treated diabetic mice. These results suggest that HBN improves β-cell function and insulin signaling by attenuation of oxidative stress mediated chronic inflammation in the type 2 diabetic mice.

Keywords: hydrolyzed bird nest, type 2 diabetes mellitus, insulin signaling, oxidative stress, inflammation

INTRODUCTION

Diabetes mellitus is a chronic metabolic disorder of endocrine system which can be categorized into two types: type 1 diabetes (insulin dependent diabetes mellitus) and type 2 diabetes (T2DM, non-insulin dependent diabetes mellitus). T2DM account for 90% of the clinical cases and is the most prevalent form of diabetes (Domingueti et al., 2016). In 2019, approximately 463 million adults (20–79 years) are living with diabetes and this figure is expected to rise to 700 million by 2045 (International Diabetes Federation, 2019). The characteristics of the T2DM are peripheral insulin resistance, insulin insensitivity, decreased β-cell function, and impaired regulation of hepatic glucose production which ultimately leads to pancreatic β-cell failure (Halim and Halim, 2019). The development of T2DM is positively linked with chronic low levels of inflammation (Lontchi-Yimagou et al., 2013).

Insulin, the main hormone that is capable of reducing the blood glucose level, interacts with its receptor to activate an intrinsic tyrosine protein kinase, which autophosphorylates the receptor as well as Insulin Receptor Substrates (IRS). The IRS then activates a cascade of serine-protein kinases which includes Akt (protein kinase B). Akt is a major branch point with numerous downstream substrates leading to a variety of physiological functions including the regulation of energy homeostasis (Beale, 2013). Besides the liver and pancreas, adipose tissue also regulates metabolism by regulating insulin sensitivity in target tissues by several ways 1) storing fat as triglyceride and releasing it as fatty acids and glycerol as needed; and 2) releasing a variety of hormones, collectively known as adipokines (Beale, 2013).

Currently, drugs such as metformin, glibenclamide and thiazolidinediones are mainly used for clinical management of diabetes (Ahren et al., 2017). However, prolonged use of these drugs are associated with long-term side effects such as liver and kidney damage (Prescrire International, 2014). Therefore, there is an on-going need to develop anti-diabetic agents with better efficacy and less adverse effects.

Recently, natural products have gained attention in clinical medicine as a potential treatment in management of type 2 diabetes mellitus due to their improved efficacy and less side effects (Li et al., 2017). Edible bird's nest is an Asian traditional food supplement known for its nutritional value and is believed to enhance energy levels, prevent aging and improve overall health (Vimala et al., 2012). It's also been proven in several studies as good source of antioxidants, is anti-inflammatory, and desirable bone-strengthening effects (Matsukawa et al., 2011; Vimala et al., 2012; Yew et al., 2014; Yida et al., 2014). Furthermore, Yida et al., showed that edible bird's nest prevents high-fat diet induced insulin resistance in rats (Yida et al., 2015). Recently, we have demonstrated the vascular protective effect of hydrolyzed bird's nest in hyperglycaemic condition (Murugan et al., 2020). However, not much is known on the effect of edible bird nest in type 2 diabetes. Therefore, in the present study, the anti-diabetic effects and mechanism of hydrolyzed bird's nest (HBN) is investigated through measurement of general and metabolic parameters, protein expression and morphology changes in liver, adipose, pancreas and kidney to provide further a basis for the use of HBN as a potential nutraceutical supplement in type 2 diabetes mellitus.

MATERIALS AND METHODS

Preparation of HBN and Chemical Profiling of HBN

The HBN was prepared and kindly provided by Professor Lim Yang Mooi from University Tunku Abdul Rahman, Malaysia. A voucher specimen was deposited in the Nature Inspired UM Natural Products Library, University Malaya (voucher number UMCNA1801). Briefly, the raw edible birds nest from Aerodramus fuciphagus (white nest) from swiftlet houses was cleaned and made to powder. The raw cleaned EBN powder was then suspended in distilled deionized water with the ratio of 0.2% (w/v) for 24 h at 4°C. The mixture was then heated at 80°C in the distilled water for an hour. The extracts were allowed to cool and further centrifuged at 2,700 *g* to collect the supernatant for subsequent lyophilization. The dried extract was stored at −20°C until further analysis. Chemical profiling of HBN was carried out using LCMS-QTOF. Solvents system in LCMS consist of 0.1% formic acid in water (A) and acetonitrile (solvent B) were used with the following gradient: starting with 100% B and reduction to 50% B at 18 min, and finally 5% B from 18 to 30 min. The system controller was stopped at 20 min. The solvent's flow rate was 0.8 ml/min. Samples (10 µl) were injected in to a C18 reversed-phase column (150 × 4.6 mm i.d, 3.0 µm particle size). Mass spectrometric detection was performed with a quadrupole-TOF-MS operated in the positive mode. Information dependent acquisition was conducted using a TOF-MS survey scan 100–1,100 Da (100 ms) and up to 10 dependent TOF MS scans 100–1,100 Da (100 ms) with Collision Energy (CE) of 45 V with Collision Energy Spread (CES) of ± 30 V. The identification of the peaks was conducted with Metlin database.

Animal Preparation

Healthy *db/db* and C57BL/6J male mice (8 weeks old) were purchased from Jackson Laboratory and Monash University (Sunway Campus, Malaysia), respectively. The mice were allowed 2 weeks for acclimatization to the housing facility. All the experimental procedures were approved by the University of Malaya Animal Care and Ethics Committee (Ethics Reference No: 2015–180709) and accredited by Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). Animal study was carried out in strict accordance with the established institutional guidelines and the NIH guidelines on the use of experimental animals. The animals were housed in individual ventilated cages with temperature (24 ± 1°C), lighting condition (12 h light/dark cycles), room pressure (10 Pa) and humidity (50–70%) with free access to standard chow (Specialty Feeds Pty Ltd., Glen Forrest, Australia) and tap water ad libitum.

Experimental Design and Study Protocol

Animals were divided into five groups with six mice in each group as follows:

Group 1: C57BL/6J mice-nondiabetic control mice (received distilled water) *via* oral gavage for 28 days
Group 2: *db/db* mice-control type 2 diabetic mice (received distilled water) *via* oral gavage for 28 days
Group 3: *db/db* mice receiving 75 mg/kg HBN *via* oral gavage for 28 days
Group 4: *db/db* mice receiving 150 mg/kg HBN *via* oral gavage for 28 days
Group 5: *db/db* mice receiving 1 mg/kg glibenclamide (Glib) *via* oral gavage for 28 days (Murugan et al., 2020).

HBN and anti-diabetic agent, glibenclamide (positive control) was dissolved in distilled water. Body weight was measured weekly by using a digital weight scale. Fasting blood glucose (FBG) with blood pricked from tail vein was measured weekly by using a digital glucometer (Accu-Check, Roche Diagnostics). At the end of 28 days, the animals were sacrificed by carbon dioxide (CO_2) inhalation and blood were collected immediately by cardiac puncture. Liver, pancreas and skeletal muscle were collected for investigating the morphological changes and immunohistochemistry. Some part of the liver and epididymal fat were collected for Western blot.

Oral Glucose Tolerance Test

Oral Glucose Tolerance Test was performed on overnight fasted animals 2 days before sacrificing the animals at the end of the HBN treatment period. Briefly, glucose load of 3 g/kg of glucose was given orally and blood glucose levels were checked by tail pricking at 0 min (prior to glucose load), 30, 60, 90, and 120 min after loading of glucose (Kunasegaran et al., 2017).

Determination of Serum Insulin

Blood samples were allowed to clot for 30 min at room temperature, centrifuged at 2,000 rpm for 10 min at 4°C to obtain serum and stored at −80°C immediately until further use. Serum insulin levels were determined using enzyme-linked immunosorbent assay (ELISA) kit (Mercodia Mice Insulin ELISA Kit's instructions' (i-DNA Biotechnology) according to the manufacturer's protocol. In brief, insulin in the sample reacts with peroxidase-conjugated anti-insulin antibodies in the microtiter wells. The samples were washed to remove the unbound enzyme-labeled antibody. The bound conjugate was detected by reaction with 3, 30, 5, 50-tetramethylbenzidine, then stopped with acid which gave a colorimetric endpoint. Optical density was measured by using a micro-plate reader (Tecan, Mannedorf, Switzerland) at wavelength of 450 nm.

Measurement of Serum Inflammatory IL-6 and TNF-α Levels

The volume/unit of IL6 and TNF-α protein complex were measured in the blood serum by using ELISA kits (Biosource International Inc., Camarillo, CA) following manufacturer's guidelines. IL-6 and TNF-α were determined from a standard curve and their levels were expressed in pg/ml.

Histology and Immunohistochemistry

Liver, pancreas and skeletal muscle were harvested and immediately fixed in 10% buffered formalin overnight, then embedded in paraffin and manually cut into 5 μm thick sections by using a microtome (Histo-line laboratories, ARM-3600, Viabrembo, Milan, Italy). Sections were then dewaxed in two changes of xylene, hydrated in two changes of 100% of ethanol, followed by 95 and 80% of ethanol and finally rinsed with H_2O. Sections were then stained with hematoxylin and eosin (H and E). The stained sections were dehydrated with 80% ethanol followed by 95% ethanol, placed in two changes of 100% of ethanol and cleansed with two changes of xylene. Histopathological changes were viewed by using a phase contrast microscope (Nikon H600L, Nikon DS camera control Unit DS-U2, Version 4.4, Tokyo, Japan), with an attached photograph machine (Nikon H600L).

For immunochemistry, the sections were incubated with 0.01 M citrate buffer, pH 6.0 for 10 min at 100°C and then 3% H_2O_2 in phosphate buffered saline (PBS) to neutralize the endogenous peroxidase. Blocking for non-specific binding was done with normal serum (Santa Cruz Biotechnology, Santa Cruz, CA, United States). Following that, sections were incubating with primary polyclonal antibodies for insulin (sc-9168), GLUT-4 (sc-53566) and GLUT-2 (sc-9117) from Santa Cruz Biotechnology, at a dilution of 1:500 in 5% normal serum for 1 h at room temperature. The sections were then rinsed three times with PBS before incubation with biotinylated secondary antibody for 30 min at room temperature, exposed to avidin and biotinylated HRP complex in PBS for another 30 min (ImmunoCruzTM ABC Staining System). 3,30-Diaminobenzidine (DAB) were used to visualize antibody binding site that produced dark-brown precipitate. Sections were counterstained with hematoxylin-eosin (H and E) for nuclear staining.

Western Blotting

Protein samples obtained from the liver and epididymal fat were lyzed in ice-cold 1X RIPA buffer consists of EGTA 1 mM, EDTA 1 mM, NaF 1 mM, leupeptin 1 μg/ml, aprotinin 5 μg/ml, PMSF 100 μg/ml, sodium orthovanadate 1 mM, and β-glycerolphosphate 2 mg/ml. The lysates were centrifuged, and the supernatant was used for Western blotting. Protein concentrations were quantified using standard Lowry assay protocol by (Bio-Rad Laboratories, Hercules, CA, United States). Twenty micrograms of protein samples were electrophoresed at 100 V through 7.5 or 10% SDS-polyacrylamide gels based on the size of target proteins and transferred to an Immobilon-P polyvinylidene difluoride membrane (Millipore, Billerica, MA, United States). The membranes were incubated with 3% bovine serum albumin (BSA) in 0.05% Tween 20 PBS with gentle shaking to block non-specific binding. The membranes were then incubated with primary antibodies against insulin receptor β (1:1,000, Cell Signaling), p-IRS1 (1:1,000, Cell Signaling), IRS1 (1:1,000, Cell Signaling), p-PI3K (1:1,000, Cell Signaling), p-AKT (1:1,000, Cell Signaling), AKT (1:1,000, Cell Signaling), NFκB (1:1,000, Cell

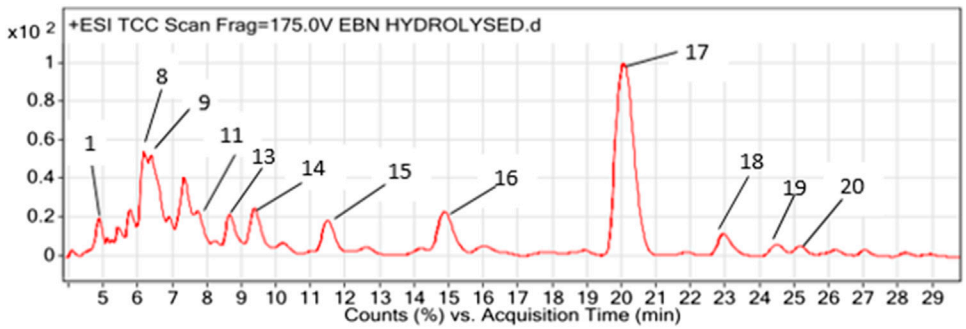

FIGURE 1 | LCMS chromatogram profiling of hydrolyzed bird's nest (HBN).

TABLE 1 | Metabolites detected from hydrolyzed birds nest (HBN) using LCMSQTOF.

No	Retention time (min)	Ion	Mass	Molecular formula	Tentative identification
1	4.885	$[M + H]^+$	293.1120	$C_{11}H_{19}NO_8$	N-Acetylmuramic acid
2	4.905	$[M + H]^+$	309.1069	$C_{11}H_{19}NO_9$	N-Acetylneuraminic acid
3	4.906	$[M + H]^+$	291.0964	$C_{11}H_{17}NO_8$	N-Acetyl-2,7-anhydro-alpha-neuraminic acid
4	4.927	$[M + H]^+$	203.0812	$C_9H_9N_5O$	3-Phenyl-5-ureido-1,2,4-triazol
5	5.317	$[M + H]^+$	216.111	$C_9H_{16}N_2O_4$	Threonyl-proline
6	5.456	$[M + H]^+$	115.0641	$C_5H_9NO_2$	D-Proline
7	5.897	$[M + H]^+$	129.1519	$C_8H_{19}N$	Octylamine
8	6.182	$[M + H]^+$	408.2143	$C_{22}H_{22}N_2O_2$	Cascarillin
9	6.419	$[M + H]^+$	230.1633	$C_{11}H_{22}N_2O_3$	L-Leucyl-L-Valine
10	7.378	$[M + H]^+$	354.2169	$C_{19}H_{26}N_6O$	Roscovitine
11	7.789	$[M + H]^+$	225.137	$C_{12}H_{19}NO_3$	Terbutaline
12	8.475	$[M + H]^+$	132.0427	$C_5H_8O_4$	2-Acetolactic acid
13	8.657	$[M+2H]^{+2}$	618.410	$C_{32}H_{54}N_6O_6$	Unknown 1
14	9.336	$[M + H]^+$	385.2578	$C_{19}H_{35}N_3O_5$	Actinonin
15	11.439	$[M+2H]^{+2}$	580.391	$C_{40}H_{52}O_3$	(3S,4R,3′R)-4-hydroxyalloxanthin
16	14.863	$[M + H]^+$	169.1472	$C_{10}H_{19}NO$	Lupinine
17	20.028	$[M + H]^+$	729.6106	$C_{42}H_{83}NO_8$	N-(octadecanoyl)-1-beta-glucosyl-sphinganine
18	22.913	$[M + H]^+$	462.3184	$C_{28}H_{46}O_3S$	Vitamin D3
19	24.681	$[M + H]^+$	427.3047	$C_{22}H_{41}N_3O_5$	Unknown 2
20	25.57	$[M + H]^+$	311.2099	$C_{17}H_{29}NO_4$	Unknown 3

Signaling), NOX4 (1:1,000, Cell Signaling), SOD-1 (1:1,000, Cell Signaling) and GAPDH (1:10,000, Abcam) overnight at 4°C. Following that, the membranes were washed three times with TBS-T and incubated with horseradish peroxidase-conjugated secondary antibodies (DakoCytomation, Carpinteria, CA, United States) at a dilution of 1:10,000 for 1 h. Then, enhanced chemiluminescence detection system (ECL reagents, Millipore Corporation, Billerica, MA) was added onto the membrane exposed on X-ray films and developed by SRX-101 (Konica, Wayne, NJ) for visualization. Quantity One software (Bio-Rad) was used for densitometry analysis. The respective protein expression levels were calculated as ratio of each target band/appropriate internal control and expressed over normal control.

Statistical Analysis

All results are presented as mean ± standard error of mean (SEM) for the number of rats (n) in each group. Data were analyzed for statistical significance using Student's t-test for unpaired observations and, for comparison of more than two groups, one-way ANOVA followed by Bonferroni's multiple comparison test was performed using the statistical software GraphPad Prism version 4 (GraphPad Software Inc., San Diego, CA, United States); p value of less than 0.05 was considered to indicate statistically significant difference.

RESULTS

Chemical Profiling of HBN

Figure 1 shows a LCMS- QTOF chromatogram of HBN. A total of 20 metabolites were identified. Based on the chemical MS library and comparison to the literatures, 17 metabolites (**Table 1**) were tentatively identified including the bio-marker compounds, sialic acid. Three types of sialic acid were determined in HBN. They are N-Acetylmuramic acid, N-Acetylneuraminic acid and N-Acetyl-2,7-anhydro-alpha-neuraminic acid. The

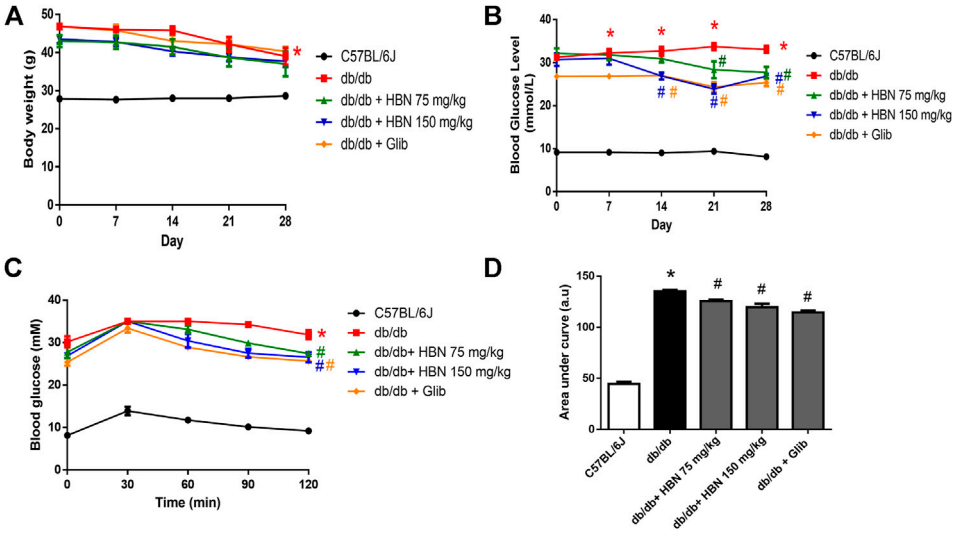

FIGURE 2 | Effect of HBN on body weight, fasting blood glucose and glucose tolerance. **(A)** Weekly body weight and from day 0 to day 28, **(B)** weekly fasting blood glucose **(C)** oral glucose tolerance test (OGTT) and **(D)** area under the curve for OGTT following treatment with HBN (75 and 150 mg/kg) and glibenclamide (1 mg/kg) in *db/db* mice. Results are means ± SEM of six experiments. *$p < 0.05$ compared to C57BL/6J mice, #$p < 0.05$ compared to *db/db* mice.

HBN used for this study contained 1.26 μg sialic acid/mg of dried weight (the major active compound in HBN) and it was standardized to contain the same amount of sialic acid for every batch prepared. Other metabolites found were vitamin D and 3-Phenyl-5-ureido-1,2,4-triazol. As EBN contain high amount of protein, a few amino acids and peptides such as threonine, proline, leucine and valine were also detected in the sample. Secondary metabolites such as octylamine, cascarillin, roscovitine, terbutaline, lupinine and N-(octadecanoyl)-1-beta-glucosyl-sphinganine were also found in HBN sample.

The Effect of HBN on Body Weight

The body weight of *db/db* mice were significantly higher than the non-diabetic mice at day 0 (46.8 ± 0.75 vs. 27.8 ± 0.65 g) and remained high until end of treatment at day 28 (39.00 ± 2.19 vs. 28.7 ± 0.61 g). There were no significant differences in the body weights for all *db/db* groups at day 0. The body weight of all *db/db* mice decreased slightly from day 14 onwards. There was no significant difference in body weight between vehicle, HBN or glibencamide treated *db/db* mice at day 28 (**Figure 2A**).

The Effect of HBN on Fasting Blood Glucose and Glucose Tolerance

The diabetic groups demonstrated a significantly elevated fasting blood glucose (>20 mM) compared to the non-diabetic control (<10 mM) on day 0 and remained high throughout the 28 days. The *db/db* mice treated with HBN 150 mg/kg demonstrated a significant decrease from day 14th while HBN 75 mg/kg and glibenclamide treated groups started showing a significant decrease from day 21. There was about 20% reduction in blood glucose level at the end of treatment at day 28 for all treated groups compared to *db/db* mice (**Figure 2B**).

The diabetic groups demonstrated a significantly increased in glucose tolerance compared to the non-diabetic control. Treatment with HBN (75 and 150 mg/kg) and glibenclamide decreased significantly the glucose tolerance by about 7–10% in *db/db* mice (**Figures 2C,D**). The HBN (150 mg/kg) and glibenclamide treatment showed significant decrease in glucose tolerance starting 60 min after glucose administration and continue to decrease at 90 and 120 min while the treatment with HBN (75 mg/kg) showed significant decrease in glucose tolerance starting 90 min after glucose administration till the end point at 120 min.

The Effect of HBN on Insulin Expression in Islets of Langerhans and Serum Insulin

Insulin expression was significantly reduced in pancreatic islets of *db/db* mice compared to control group. In diabetic rats treated with HBN (75 and 150 mg/kg), increased insulin expression was observed. Higher insulin expression was observed with 150 mg/kg HBN treatment. Treatment with glibenclamide also significantly increased pancreatic insulin content (**Figure 3A**). Serum insulin were markedly elevated in *db/db* mice indicating hyperinsulinemia. Supplementation of HBN (75 and 150 mg/kg) and glibenclamide (1 mg/kg) in the *db/db* mice significantly decreased the elevated serum insulin (**Figure 3B**).

The Effect of HBN Treatment on Pro-inflammatory Cytokines

Pro-inflammatory cytokines, IL-6 and TNF-α were increased by two folds in the serum of *db/db* mice. Treatment with HBN (150 mg/kg) and glibenclamide significantly reduced the

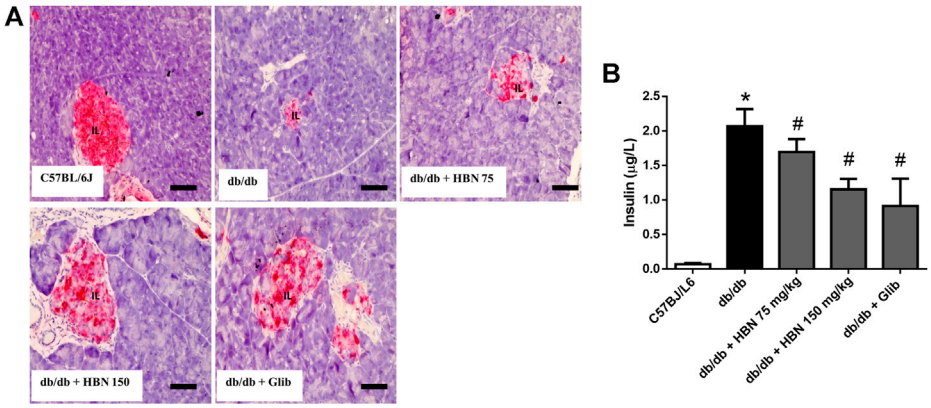

FIGURE 3 | Effects of HBN (75 and 150 mg/kg) and glibenclamide (1 mg/kg) treatment on insulin content. **(A)** Representative immunoperoxidase images showing insulin distribution in the pancreatic islets. The sites of distribution are indicated by dark brown staining. Scale bar = 50 µm. Magnification ×40 **(B)** Serum insulin level (µg/L). Values are expressed as mean ± S.E.M of six experiments. *$p < 0.05$ compared to C57BL/6J mice, #$p < 0.05$ compared to *db/db* mice.

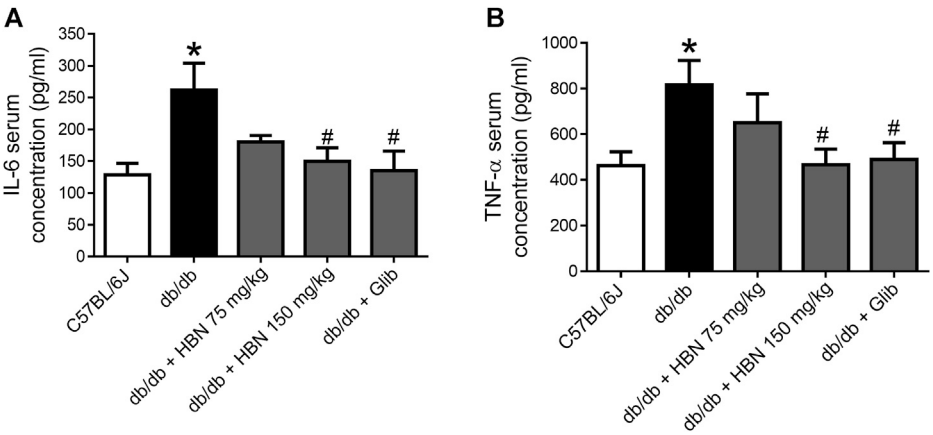

FIGURE 4 | Effects of HBN on serum pro-inflammatory cytokines. The level of pro-inflammatory cytokines of **(A)** IL-6 and **(B)** TNF-α in blood serum in C57BJ/L6 mice and *db/db* mice after treatment with HBN (75 and 150 mg/kg) and glibenclamide (1 mg/kg). Results are mean ± SEM of six experiments. *$p < 0.05$ compared to C57BL/6J mice, #$p < 0.05$ compared to *db/db* mice.

production of pro-inflammatory cytokines of IL-6 and TNF-α. Although not significant, treatment with 75 mg/kg HBN also demonstrated a decrease in serum IL-6 and TNF-α levels in *db/db* mice (**Figures 4A,B**).

The Effect of HBN on Histopathological Changes of Pancreas and Liver

Islet of Langerhans appeared small and degranulated in the diabetic group compared to control group. The acinar cells were swollen, and small vacuoles were observed in almost all acinar cells. Interlobular ducts were lined with flattened epithelium. However, in diabetic rats treated with HBN extracts or glibenclamide, the islets were bigger and less necrotic (**Supplementary Figure S1A**). The liver from normal control mice showed normal histological structure, regular distinct hepatocytes with sinusoidal spaces arranged radially around the central vein. The diabetic group showed some fatty changes with necrosis and necrobiosis in the hepatocytes. However, diabetic mice treated with HBN (75 and 150 mg/kg) and glibenclamide showed decreased fatty changes with lower necrosis (**Supplementary Figure S1B**).

Effect of HBN on Insulin Signaling, Inflammatory and Oxidative Stress Proteins in Liver and Adipose Tissues

In liver (**Figure 5**) and adipose tissue (**Figure 6**), the protein expression of insulin receptors (IRβ) and the downstream proteins of insulin signaling, p-IRS1, PI3K and p-Akt was downregulated in the *db/db* mice compared to non-diabetic mice. Treatment of HBN (150 mg/kg) and glibenclamide in the diabetic mice upregulated the IRβ, p-IRS1, PI3K and p-Akt in both liver and adipose. Treatment with HBN

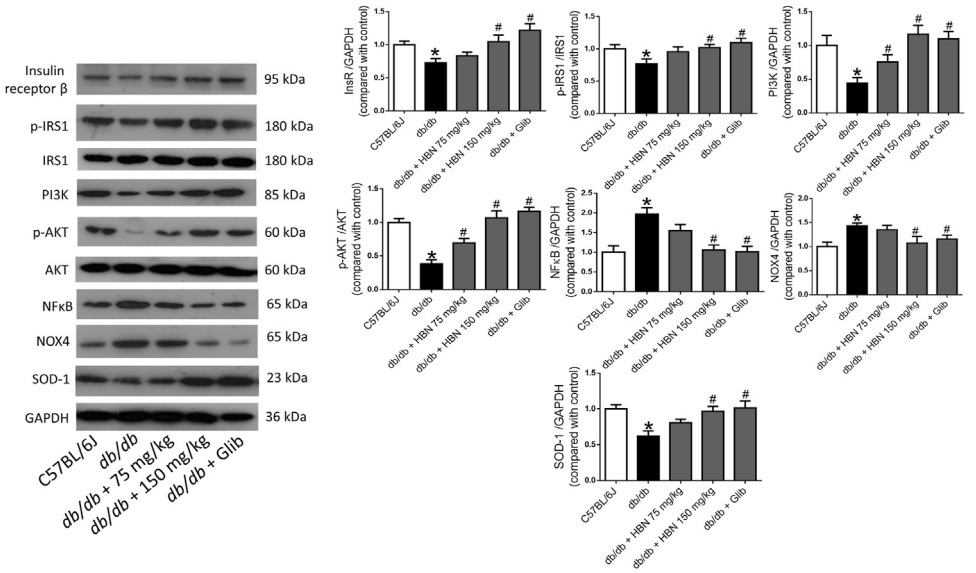

FIGURE 5 | Effects of HBN on hepatic insulin signaling, inflammation and oxidative stress. Western blot and quantitative data showing the protein expression in liver of C57BJ/L6 mice and *db/db* mice after treatment with HBN (75 and 150 mg/kg) and glibenclamide (1 mg/kg). Results are mean ± SEM of six experiments. *$p < 0.05$ compared to C57BL/6J mice, #$p < 0.05$ compared to *db/db* mice.

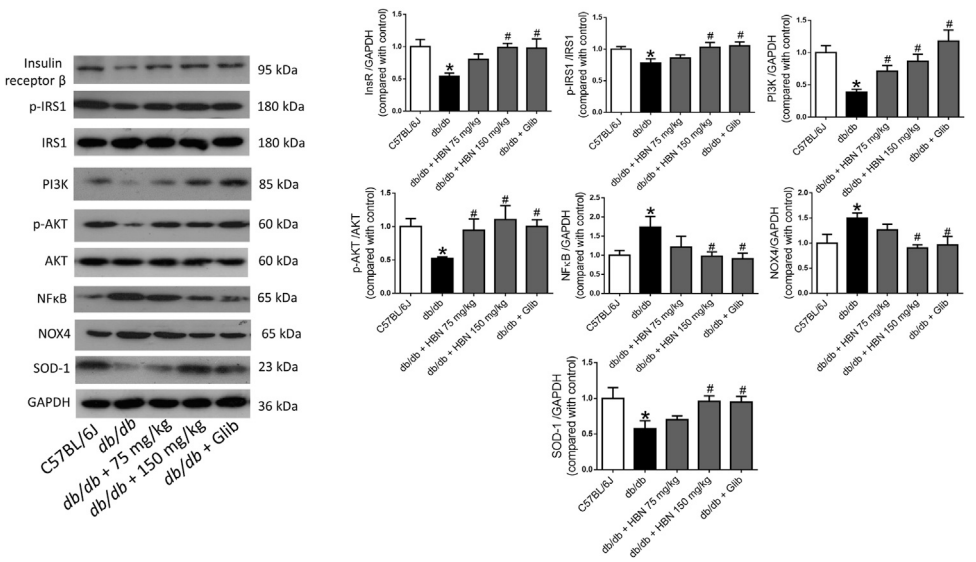

FIGURE 6 | Effects of HBN on adipose insulin signaling, inflammation and oxidative stress. Western blot and quantitative data showing the protein expression in adipose tissue of C57BJ/L6 mice and *db/db* mice after treatment with HBN (75 and 150 mg/kg) and glibenclamide (1 mg/kg). Results are mean ± SEM of six experiments. *$p < 0.05$ compared to C57BL/6J mice, #$p < 0.05$ compared to *db/db* mice.

(75 mg/kg) in liver and adipose tissue significantly upregulated PI3K and p-Akt. Although this dose demonstrated a slight upregulation of IRβ and p-IRS1, it was not significant.

In contrast, the inflammatory protein NFkB were upregulated by 1-fold in both the liver (**Figure 5**) and adipose tissue (**Figure 6**) of the *db/db* group. The treatment with HBN (75 and 150 mg/kg) and glibenclamide reversed the upregulation of NFkB. Similarly, the reactive oxygen species (ROS) marker, NOX4 protein was upregulated in the liver and adipose tissue of the diabetic group and this upregulation was reversed following treatment with HBN (75 and 150 mg/kg) and glibenclamide. In contrast, the antioxidant protein SOD-1 was downregulated in both liver (**Figure 5**) and adipose tissue (**Figure 6**) of the diabetic group. Treatment with HBN (75 and 150 mg/kg) and glibenclamide in the diabetic animals upregulated the decreased protein to control level.

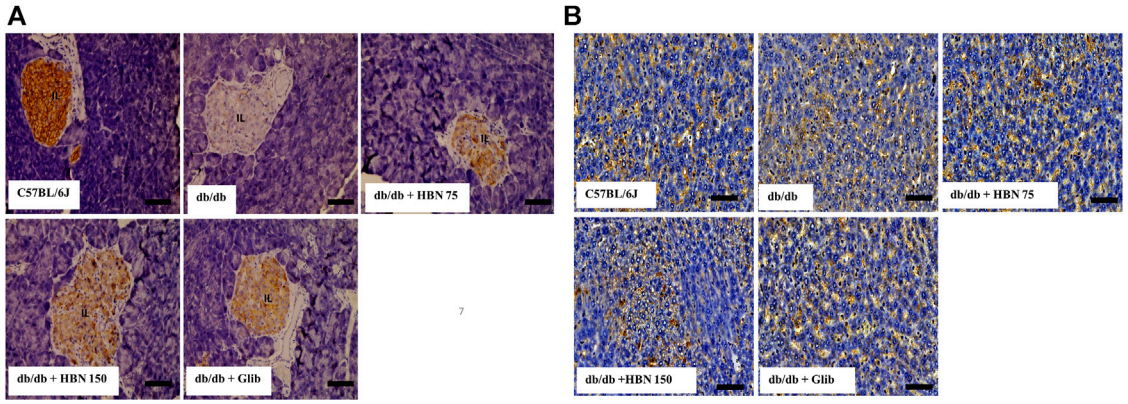

FIGURE 7 | Effects of HBN on glucose transporter 2 (GLUT-2) level. Representative immunoperoxidase images showing GLUT-2 distribution in the pancreas **(A)** and liver **(B)**. The sites of distribution are indicated by dark brown staining. Scale bar = 100 μm. Magnification ×40.

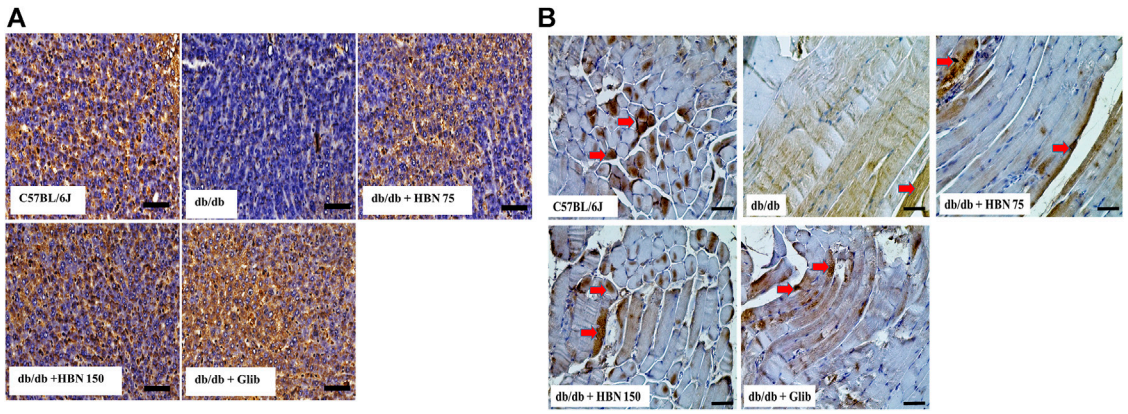

FIGURE 8 | Effects of HBN on glucose transporter 4 (GLUT-4) level. Representative immunoperoxidase images showing GLUT-4 distribution in the liver **(A)** and skeletal muscle **(B)**. The sites of distribution are indicated by dark brown staining. Scale bar = 100 μm. Magnification ×40.

Effect of HBN on GLUT-2 Expression in the Pancreas and Liver

GLUT-2 expression was highest in the pancreatic islets (**Figure 7A**) and liver (**Figure 7B**) of non-diabetic group and significantly lower in the pancreatic islets and liver of the diabetic group. Treatment of diabetic mice with HBN (75 and 150 mg/kg) and glibenclamide resulted in higher GLUT-2 expression in the pancreatic islets and liver compared to untreated diabetic group.

Effect of HBN on GLUT-4 Expression in the Liver and Skeletal Muscle

Immunostaining showed lower levels of GLUT-4 protein expression in the liver (**Figure 8A**) and skeletal muscle (**Figure 8B**) of *db/db* group compared to the C57BL/6J group. Treatment of diabetic mice with HBN (75 and 150 mg/kg) and glibenclamide showed significantly higher GLUT-4 expression in the liver and skeletal muscle.

DISCUSSION

The present study demonstrated that oral treatment with HBN exerted anti-diabetic effect and improved glucose tolerance in type 2 diabetic mice by protecting the pancreatic β-cells and improving insulin signaling in the liver and adipose tissue. The anti-diabetic effects of HBN is partially attributed to inhibition of chronic inflammation and oxidative stress.

The major compositions of edible bird nest (EBN) reported are carbohydrate (25.62–31.40%) and protein (60–66%) (Zainab et al., 2013). Ma and Liu (2012) reported that the carbohydrates in EBN consist mostly of sialic acid (9.0%), galactosamine (7.2%), glucosamine (5.3%), galactose (16.9%) and fucose (0.7%). Studies have demonstrated high amount of sialic acid contributed to brain development and learning and memory enhancing ability (Wang and Brand-Miller, 2003; Careena et al., 2018). Similarly, our earlier work demonstrated that improvement by HBN in endothelial dysfunction due to high glucose was comparable to the effects of sialic acid *in vitro*

(Murugan et al., 2020). Three types of sialic silic acid were determined in HBN. They are N-Acetylmuramic acid, N-Acetylneuraminic acid and N-Acetyl-2,7-anhydro-alpha-neuraminic acid. Normally the sialic acid exist in conjugated form such as oligosaccharides and glycoprotien. However, after hydrolysis of EBN, the neuraminic acid or acetylmuramic acid will be released (Chan et al., 2013).

The *db/db* mice model is one of the most frequently used models to emulate type 2 diabetes in humans. *db/db* mouse lacks leptin receptor which makes them susceptible to obesity, insulin resistance, and type 2 diabetic mellitus (Burke et al., 2017). Our result showed that treatment with HBN and antidiabetic agent, glibencamide did not have significant effect on the body weight of diabetic mice. However, treatment with HBN in diabetic mice significantly decreased the fasting blood glucose starting from day 7 of treatment for the higher dose (150 mg/kg) and day 14 for the lower dose (75 mg/kg). Both doses also improved glucose tolerance further supporting the anti-diabetic effect of HBN. According to Zulkifli et al. 2019, hydrolysis of EBN lead in improving the functional properties of EBN and result in the increasing of antioxidant and anti-hyperglycemic activities which is agreeable with the present study which used hydrolyzed edible bird's nest.

Insulin is produced in pancreatic β-cells and is packaged into membrane-bound secretory granules which is released upon stimulation of β-cells by glucose or other secretagogues (Do and Thorn, 2015). We have demonstrated that supplementation with HBN in diabetic mice preserved pancreatic functions by restoring the histopathological changes in the pancreas as observed by the near normal pancreatic islets and β-cell numbers. Furthermore, HBN treatment reduced hyperinsulineamia in the diabetic group in an attempt to normalize the plasma insulin level.

Persistent hyperglycaemia in diabetes mellitus increases the production of reactive oxygen species (ROS) as well as suppressing antioxidant defense mechanisms which ultimately contribute to oxidative stress (Jha et al., 2018). Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) is a multicomponent enzyme complex that produces reactive oxygen species (ROS) (Yong et al., 2013). Among the NOX isoforms, NOX4 is associated with diabetes where it dysregulates stress signaling, fibrosis, and insulin sensitivity in hepatocytes and Kupffer cells of liver (Bettaieb et al., 2015) and adipocytes (Den Hartigh et al., 2017). In addition, a decrease in potent antioxidants such as superoxide dismutase (SOD), catalase (CAT) and the glutathione (GSH) enzyme family during hyperglycaemic condition will cause an increase in ROS, which eventually contributes to oxidation-induced liver and adipose tissue damages (Mohamed et al., 2016; Maslov et al., 2019). The present results showed that the level of NOX4 was elevated while SOD-1 was reduced in liver and adipose tissue of *db/db* mice, which were all reversed with the supplementation of 150 mg/kg HBN.

In addition, increased level of inflammation is noted in the *db/db* mice as evidenced by increased level of NFκB protein expression in the liver and adipose tissue and increased serum cytokines, IL-6 and TNF-α. Translocation of NFκB into the nucleus increases the expression of inflammatory cytokines such as TNF-α and IL-1β (Liu et al., 2017). The marked reduction of serum TNF-α and IL-6 following 150 mg/kg HBN supplementation reduces inflammation and help to protect the liver, pancreas and adipose tissue from the hyperglycaemic insults. Similar anti-inflammatory and antioxidant effects of edible bird's nest have been demonstrated in high fat diet induced obese rats (Yida et al., 2015) and following exposure to elevated glucose levels *in vitro* to human SH-SY5Y cells (Yew et al., 2014; Hou et al., 2015) and in isolated *db/db* aorta (Murugan et al., 2020).

Chronic inflammation play an important role in the pathogenesis of insulin resistance, the hallmark of type 2 diabetes mellitus (Rehman and Akash, 2016). Activation of TNF-α triggers insulin resistance by increasing uptake of glucose in visceral and subcutaneous adipocytes (Fernandez-Veledo et al., 2009) and through phosphorylation of serine 307 in IRS-1 (Aguirre et al., 2000). It has been reported that IL-1β amplified systemic inflammation and impaired insulin signaling leading to insulin resistant (Boni-Schnetzler and Donath, 2013) and organ dysfunction (Grant and Dixit, 2013) in peripheral tissues and macrophages during type 2 diabetes mellitus. Both TNF-α and IL-1β downregulation insulin receptor β in diabetic insulin-resistant hepatocytes and adipocytes (Jager et al., 2007; Alipourfard et al., 2019).

Phosphorylated IRS proteins activates PI3K/Akt signaling pathway demonstrating its role in the metabolic action of insulin (Huang et al., 2018). In adipose tissue, insulin-Akt signaling promotes utilization of glucose, gluconeogenesis and lipid biosynthesis. In liver, it has been reported that PI3K/Akt signaling pathway attenuates production of hepatic glucose and glycogenolysis, increases synthesis of glycogen and fatty acids for storage and subsequent utilization by other tissues (Huang et al., 2018). Similarly, the current study showed that insulin signaling proteins such as insulin receptor β, phosphorylation of IRS, PI3K and phosphorylation of Akt in liver and adipose tissues were significantly downregulated in *db/db* mice, suggesting that the insulin signaling was impaired in type 2 diabetic animal. Treatment with 150 mg/kg HBN in *db/db* mice improved these metabolic changes associated with dysregulation of insulin signaling.

It has been reported that level of Akt and mobility of glucose transporter in the vesicles/membrane is closely related (Kuai et al., 2016). Akt catalyses the AS160 substrate protein phosphorylation which triggers GLUT glucose transporters translocation from cytoplasmic vesicles onto the cell membrane surface which facilitate the insulin-dependent transport of glucose into the cell (Kuai et al., 2016). GLUT-2 and GLUT-4, the isoforms of glucose transporters have essential roles in mediating disposal of body glucose. GLUT-2, unique among the facilitative hexose transporters due to its low affinity and high capacity for glucose, is expressed primarily in cells involved in glucose sensing, such as hepatocytes and pancreatic β cells. When plasma glucose concentrations are elevated, which are typical of the postprandial state, GLUT-2 responds with rapid, continual net uptake of glucose for insulin secretion (Kuai et al., 2016).

Meanwhile, the majority of peripheral glucose uptake into adipose tissue and skeletal muscle is achieved through similar signal transduction pathways and is mediated by the insulin responsive GLUT-4 facilitative glucose transporter (Stringer et al., 2015). GLUT-4 disruption in adipose or muscle tissue will cause insulin resistance and thus promote higher risk for diabetes (Abel et al., 2001). In the present study, GLUT-2 and GLUT-4 were decreased in the diabetic mice, and treatment with HBN increased the expression of both glucose transporters. The increase in GLUT-2 level with HBN treatment was more prominent in the pancreas than in the liver. On the other hand, the expression of GLUT-4 level was similarly increased in the liver and skeletal muscle of the HBN-treated diabetic groups. This finding suggest that increased in GLUT-2 receptors subsequently promote uptake of glucose into the β-cells to stimulate insulin secretion by the pancreas while GLUT-4 promotes uptake of glucose to tissues.

In conclusion, the present findings suggest that oral supplementation of HBN exhibited anti-diabetic effect in type 2 diabetic *db/db* mice as evidenced by the improvement of β-cell function and insulin sensitivity *via* the insulin-PI3K/Akt signaling pathway. This is potentially achieved through reduction in oxidative stress and chronic inflammation. HBN may be useful as a nutraceutical supplement in improving glucose control in type 2 diabetes mellitus.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the University of Malaya Animal Care and Ethics Committee (Ethics Reference No: 2015–180709) and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

AUTHOR CONTRIBUTIONS

KWC, DDM, and MRM participated in designing the study. KWC, ZZ, and NG performed the in-vivo experiments and analyzed the data. YML prepared the HBN and NZ performed the chemical profiling. KWC and ZZ prepared the first manuscript draft. DDM and MRM participated in editing and preparation of final manuscript draft. All authors read and approved the final manuscript.

FUNDING

This study was funded by Government Agency grant GA001-2017.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.632169/full#supplementary-material.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

REVIEW
published: 19 April 2021
doi: 10.3389/fphar.2021.626233

Edible Bird's Nest: The Functional Values of the Prized Animal-Based Bioproduct From Southeast Asia–A Review

Ting Hun Lee[1,2], Waseem A. Wani[1], Chia Hau Lee[1], Kian Kai Cheng[2], Sheikh Shreaz[3], Syieluing Wong[1], Norfadilah Hamdan[1] and Nurul Alia Azmi[1]*

[1]School of Chemical and Energy Engineering, Faculty of Engineering, Universiti Teknologi Malaysia, Johor, Malaysia, [2]Innovation Centre in Agritechnology for Advanced Bioprocessing, Universiti Teknologi Malaysia, Pagoh Research Center, Johor Darul Takzim, Malaysia, [3]Oral Microbiology General Facility Laboratory, Faculty of Dentistry, Health Sciences Center, Kuwait University, Safat, Kuwait

Edible Bird's Nest (EBN) is the most prized health delicacy among the Chinese population in the world. Although some scientific characterization and its bioactivities have been studied and researched, no lights have been shed on its actual composition or mechanism. The aim of this review paper is to address the advances of EBN as a therapeutic animal bioproduct, challenges and future perspectives of research involving EBN. The methodology of this review primarily involved a thorough search from the literature undertaken on Web of Science (WoS) using the keyword "edible bird nest". Other information were obtained from the field/market in Malaysia, one of the largest EBN-producing countries. This article collects and describes the publications related to EBN and its therapeutic with diverse functional values. EBN extracts display anti-aging effects, inhibition of influenza virus infection, alternative traditional medicine in athletes and cancer patients, corneal wound healing effects, stimulation of proliferation of human adipose-derived stem cells, potentiate of mitogenic response, epidermal growth factor-like activities, enhancement of bone strength and dermal thickness, eye care, neuroprotective and antioxidant effects. In-depth literature study based on scientific findings were carried out on EBN and its properties. More importantly, the future direction of EBN in research and development as health-promoting ingredients in food and the potential treatment of certain diseases have been outlined.

Keywords: edible bird's nest, bioproduct, functional values, anticancer, anti-aging, antioxidant

OPEN ACCESS

Edited by:
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Imma Pagano, University of Salerno, Italy

****Correspondence:***
Ting Hun Lee
leetinghun@utm.my

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: *05 November 2020*
Accepted: *06 April 2021*
Published: *19 April 2021*

Citation:
Lee TH, Wani WA, Lee CH, Cheng KK, Shreaz S, Wong S, Hamdan N and Azmi NA (2021) Edible Bird's Nest: The Functional Values of the Prized Animal-Based Bioproduct From Southeast Asia–A Review. Front. Pharmacol. 12:626233. doi: 10.3389/fphar.2021.626233

INTRODUCTION

Edible Bird's Nest (EBN) is a secretion created by swiftlets. *Erodramus* (echolocating swiftlets) and *Collocalia* (non-echolocating swiftlets) are among the two genera of swiftlets known to produce valuable EBN (Ma and Liu, 2012). Swiftlets are insectivorous birds, predominantly inhabited in South East Asia (SEA) and southern part of China (Aswir and Wan, 2010). The world's largest producer of EBN is Indonesia, which has the largest colony of swiftlets currently, followed by Malaysia. (Hobbs, 2004). Saliva secreted from the pair of sublingual glands of swiftlets are the principal material used in the construction of the EBN. The sublingual glands of swiftlets increase in

weight (2.5–160 mg) and reach their maximum secretory activity during nesting and breeding season (Jamalluddin et al., 2019). The male birds make nests by using their secretion to bind with some feathers and vegetation. The resulting material is shaped into nests with simultaneous attachment to the walls of the caves when is habituated in cave environment. In man-made premises, they are attached to the wooden linter (Lee et al., 2017). The nests are graded based on the dry mass, size, color, impurity and amount of feathers via physical appearance.

EBN has been the delicacy food in Traditional Chinese Medicine (TCM) since the Tang Dynasty (618–907 A.D.) (Marcone, 2005). EBN is cooked using double boiling method with rock sugar to make the Chinese cuisine, namely the bird's nest soup (Hobbs, 2004). It was reported that Hong Kong is the largest importer of EBN globally, followed by the Chinese community from North America. EBN may be regarded as the most expensive animal by-product in the world, costing USD 2,000–10,000 per kilogram for its high nutritional and medicinal therapeutic values (Babji et al., 2015). The key component of EBN are glycoprotein, calcium, sodium, potassium and carbohydrate (Quek et al., 2018a). Owing to its esteem as a prized bioproduct in the East of the globe, EBN is also named as the "Caviar of the East" (Marcone, 2005). EBN has also been used as a health tonic in TCM due to its being a multipurpose general health rejuvenation tonic and social symbolic status delicacy during banquet (Ghassem et al., 2017). TCM claimed that EBN can treat malnutrition, improve metabolism rate, boost immune system and rejuvenate the skin complexion (Bashir et al., 2017). Moreover, in the modern research, EBN also exhibits some interesting therapeutic effects, such as anticancer, anti-aging, phlegm-dissolving, cough-suppressing, anti-tuberculosis, voice-improving, curing general debility and asthenia, and hastening recovery from illness and surgery (Daud et al., 2019a). There is a great amount of research taking place on the investigation of the hidden nutritive and pharmacological properties of EBN. Some of the reviews were focused on the authentication and identification methods of EBN, its bioactive components and food values (Lin et al., 2006; Wong, 2013; Lee et al., 2018). However, none of the reviewers have discussed the latest challenges facing by the researchers in EBN research field, such as the important substrates that contribute to the medicinal properties in EBN. Therefore, it is worthwhile to review the advances in the research involving EBN as a functional food from animal-based bioproducts and to discuss or address the challenges and future research perspectives of EBN. This manuscript will be served as a reference for the EBN researchers.

METHODOLOGY

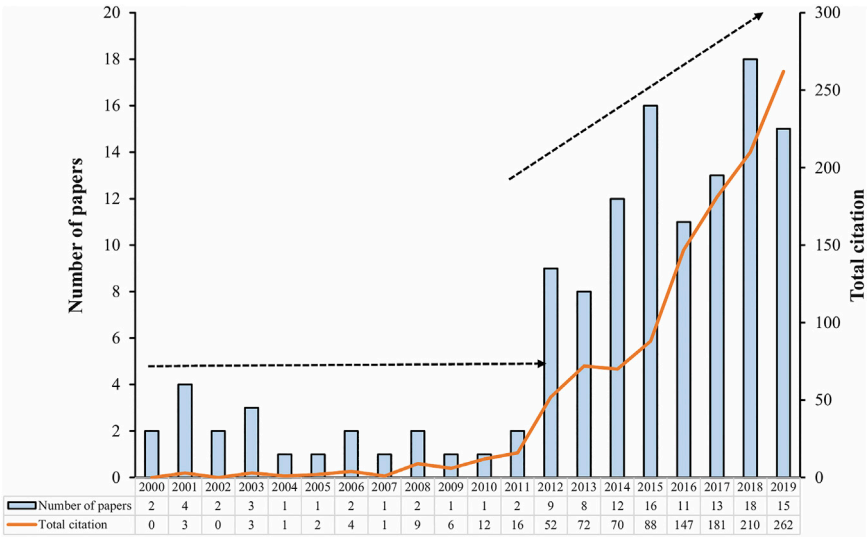
Published data from 2000 to 2019 were retrieved from the Web of Science (WoS), in August 2020, by using the following search string: TS = [(edible bird* nest*)]. Only publications in English language that report the functional values of EBN were included for subsequent analysis. The duplicate results were removed from the search. Current EBN trend observed in the Malaysia industry is also included in this review.

THE TREND OF EDIBLE BIRD'S NEST PUBLICATIONS

A thorough search of the literature on WoS indicated that approximately 170 research publications consisting of various types of documents appeared in this topic. Out of 170 articles, only 124 publications were considered in this review which consists 119 original research articles and five review papers. The available publications discussed several aspects related to collection, extraction, purification, authentication, nutritive values, medicinal significance, and other important facts of EBN. According to the search and summarized in **Figure 1**, the publications on this topic remained low in 2000–2011. However, in 2012–2019, there is an increase in annual publications, with irregular trend. A noticeable and dramatic increase in publications numbers on this topic occurred since 2012. An increase on the number of citations has revealed the significant attention on the EBN work to the global scientific community.

OVERVIEW OF EDIBLE BIRD'S NESTS

EBN is the hardened secretion produced by several species of swiftlets originally inhabiting in the limestone caves. EBN weighs at least 1–2 folds of the swiftlet's body weight and can accommodate only the adult bird and nestlings. The swiftlets take around 35 days to complete the construction of the nest (Marcone, 2005). White nests (**Figure 2A**) are almost entirely made from saliva (Sims, 2008), while black nests (**Figure 2B**) comprised about 45–55% feathers and small dried leaves (Zulkifli et al., 2019). The white nests are mostly produced in the bird premises and only a little amount is found in the caves, whereas the black nests are only harvested in caves. Some slightly or entirely dull orange-red to brownish red nests called *Xueyan* or *Xueyanwo* in Chinese are occasionally found in caves and swiftlet houses. *Xueyan* is a Chinese word with the meaning "blood nest" or the blood-coloured nest which arise from the resemblance in the color of the blood. Red nests or blood nests (**Figure 2C**) are supposed to have higher health benefits and thus, fetch a higher price than white nests in the market (But et al., 2013). The EBN names deserved some special attention also. The first step is to identify them with color, for example, white nest, black nest and also red nest. White and black nests are explained above but the most interesting is the red or blood nests. Blood nest story was invented by the Hong Kong people where it is made to believe that the swiftlets will secrete blood (the best essence) when there is no more saliva to be used to build the nest. This makes the best quality nest (Lee et al., 2017). However, some researchers have suggested that red color might be due to the absorption of the minerals from the wall where the nest was attached (Wong et al., 2018a; Shim and Lee, 2018). Due to the higher price and hence better profit, some of the EBN processors decided to fake the blood nest with all kinds of dreadful methods. This has resulted in the "sodium nitrite crisis issues" that happened in 2011. China government has banned the import of EBN which caused multimillion dollar losses in Malaysia, after detecting a high



	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019
Number of papers	2	4	2	3	1	1	2	1	2	1	1	2	9	8	12	16	11	13	18	15
Total citation	0	3	0	3	1	2	4	1	9	6	12	16	52	72	70	88	147	181	210	262

FIGURE 1 | A pictorial depiction of the steadily growing interest in the research on EBN from 2000 to 2019.

FIGURE 2 | An overview of white **(A)**, black **(B)** and red **(C)** EBNs.

content of sodium nitrite in some of the EBN. Subsequently, the Malaysian government has taken the initiative to standardize the EBN names based on the harvested location: the cave and house nest (Lee et al., 2017). It is categorized into only two major types based on the location where the nest is harvested. They totally did not acknowledge the red nest existence simply because there were too many ambiguous points to categorize them. After the ban was lifted in 2014, the content of sodium nitrite was controlled at 30 ppm which followed the Malaysian Food Regulation 1985 and Malaysia Standard MS 2334:2011 (Quek et al., 2015). Till today, Malaysia remains as one of the highest exporters of EBN to China.

TRADITIONAL VALUE AND COMPOSITION OF EDIBLE BIRD'S NEST

EBN was once portrayed as a symbol of social status in ancient Chinese society (Jamalluddin et al., 2019) due to its rarity and high price. TCM prescribed EBN as the remedy for consumptive illnesses, *tuberculosis*, alleviating asthma, dry coughs, haemoptysis, asthenia, improving voice, difficulty in breathing, general weakness of bronchial ailment and relieving gastric troubles (Ghassem et al., 2017). Besides, EBN is traditionally believed to raise libido, fortify the immune system, promote growth, improve concentration, increase energy and metabolism, and regulate circulation (Bashir et al., 2017). Although the efficacy of EBN extracts in maintaining youthfulness and increasing physical strength have yet to be tested, but there is scientific evidence on EBN supplementation indicating that it could improve skin texture and alleviate the aging processes (Wong, 2013; Hwang et al., 2020). Based on these studies, EBN consumption may promote the human health.

Protein is the major component in EBN which are commonly used for constructing the cells and tissues and consequently driving to other metabolic functions. Based on the previous studies, the average protein content in EBN is ranging from 50 to 55% of the dried weight (Wong et al., 2018c). In addition to the protein contents of EBN, carbohydrates form another major

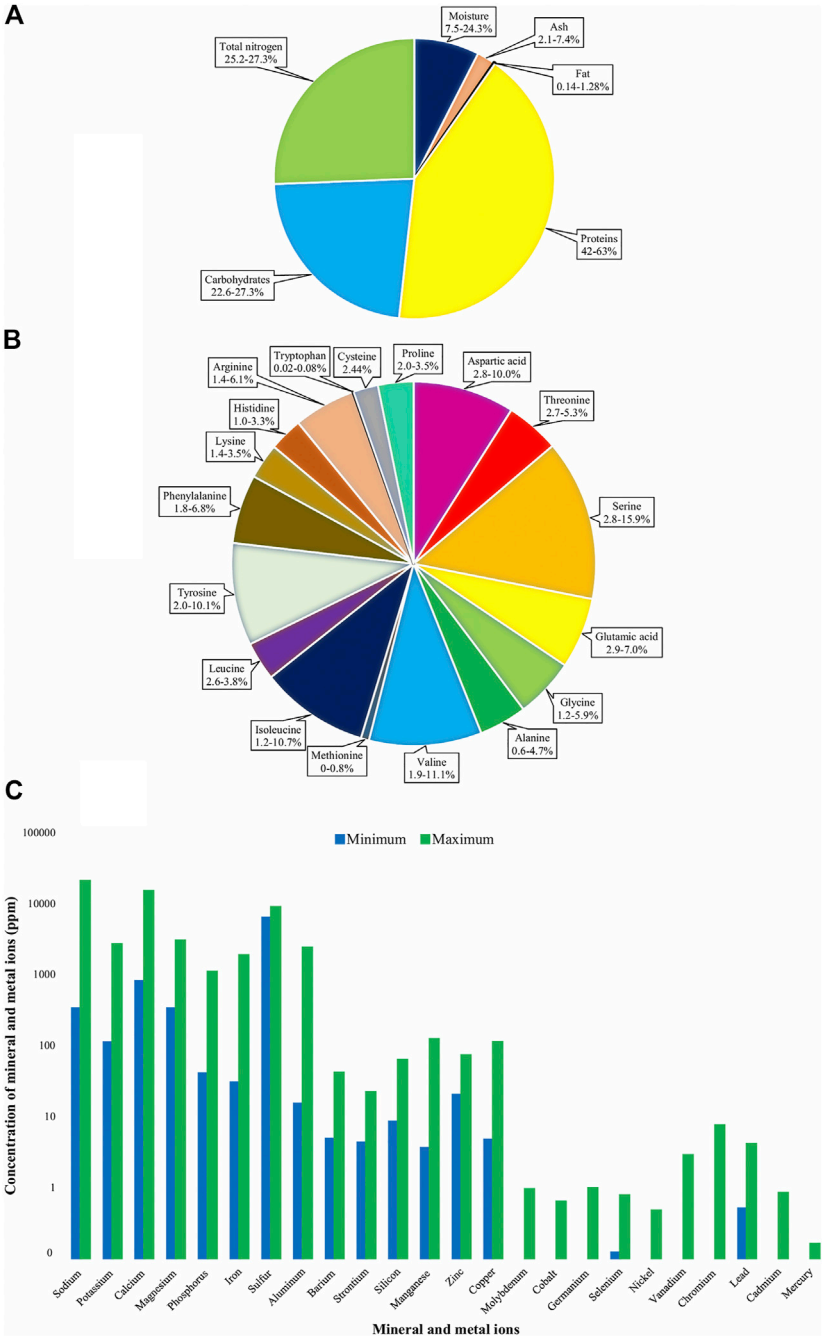


FIGURE 3 | Total composition **(A)**, amino acid **(B)**, mineral and metal ions **(C)** in EBN (Marcone, 2005; Ma and Liu, 2012).

portion of its composition (**Figure 3A**) (Babji et al., 2018). The main carbohydrates present in EBN is sialic acid. Sialic acid facilitates development of gangliosides structure in the brain (Wang and Brand-Miller, 2003). Interestingly, ingestion of it can enhance and improve the neurological and intellectual for infants. Some other main and major ingredients in EBN are the essential trace elements such as calcium, phosphorus, iron, sodium, potassium, iodine and essential amino acids (Hun et al., 2015).

Based on these contents, EBN serves as a highly nutritious and health restorative food suitable for consumption by all age groups and genders. The modern analysis of its composition has been reported by many researchers as displayed in **Figure 3**. Out of the twenty types of amino acids desired by human, eighteen types of amino acids are detected in EBN. These include nine essential amino acids (phenylalanine, valine, threonine, histidine, tryptophan, isoleucine, methionine, lysine and leucine) required by human body for the growth and reparation of the

FIGURE 4 | Some of the famous EBN market products based on foods labeled, outlook and, food products. **(A)** Bird's nest soup, **(B)** Bird's nest instant energy drink, **(C)** Vietnam bird's nest powder, **(D)** Brand new concept EBN powder, **(E)** Bird's nest drink, **(F)** Bird's nest pudding recipe, **(G)** Instant Malaysian cubilose nourishing tonic and **(H)** Bird's nest granules for supplements. The image is adapted from (Yen, 2015).

tissue (Azmi et al., 2021). Out of nine essential amino acids, two of them, namely lysine and tryptophan, are not present in most plant protein. Hence, EBN could provide a complete amino acid for the vegetarians since it is categorized as vegan as it is not meat or animal blood.

Based on the content reported by various researchers, there were some differences in amino acid contents (**Figure 3B**). The actual causes of these differences are not known. However, these variance could be due to the EBN samples that were obtained from different places (Quek et al., 2018b). Also, the samples obtained could have been processed and adulterated (Huang et al., 2018). This is due to the fact that researchers could not standardize the EBN processing and cleaning method. Most of the time, samples were just obtained from sponsors or retailers but not knowing the actual process that had been carried out that make the variants.

The minerals and metal ions content (**Figure 3C**) in the EBN were either produced by the swiftlets (who built EBN) or leached from the environment. The content ranges are fairly wide as the samples were from various places and types. The excess mineral present in the food will cause negative effects and jeopardize human health, especially the heavy metal (Lead, Copper, Zinc, Mercury and Cadmium) when entering the human complex body through either inhalation, ingestion, and dermal contact. As described previously, some of these trace minerals and metal ions such as Lead, Mercury, Arsenic and Cadmium could have long term side effects in humans leading to various type of disease even at the small dose of ingestion or exposure (Zheng et al., 2020). Some of the heavy metal content in EBN showed in **Figure 3C** have alarming excess contents set by the majority food legislations (0–1 ppm). It is suggested that the heavy metal content limit should be enforced as this product is popular among children and more seriously, among pregnant ladies (Lee et al., 2017).

Traditionally, the benefit of EBN consumption in elderly include strengthening of lung and kidney, improving of the spleen, enhancing appetite and phlegm clearances. EBN helps to improve immunity in children, and strengthens the function of the kidney and lung in men (Quek et al., 2018a). Based on EBN's content, in summary, EBN may be termed as a complete food enriched with a huge diversity of proteins, lipids, amino acids, carbohydrates, minerals and vitamins. Some of the essential amino acids, sialic acid, and other key constituents of EBN might have great health benefits in terms of general health especially on lung strengthening, improve skin health and anti-aging (Wang et al., 2019). Some of the recent developed EBN based products are shown in **Figure 4**. Till now, there has been little or none of the research on its functional and medicinal properties of EBN. It is further elaborated and discussed in the following section.

PRE-CLINICAL ANALYSIS AND THERAPEUTIC EFFECTS OF EDIBLE BIRD'S NEST

The effects of EBN extract have been summarized in **Table 1** with details.

Table 1 Summary of pre-clinical studies on the therapeutic effects of EBN extract.

Antiviral Effects

Viruses are micro infectious agents which can only replicate in living cells of organisms that act as a host. Viruses can infect all living organisms that include plants, animals, bacteria and archaea (Koonin et al., 2006). Most of the viral infections have been reported as leading to lysis of the cell by changing the structure of cell membrane that result to the apoptosis of the host cells (Haghani et al., 2016). The most prevalence disorders due to viral infections are Influenza, Chickenpox, Cold Sores, Avian Influenza, Severe Acute Respiratory Syndrome (SARS) and Acquired Immunodeficiency Syndrome (AIDS).

Flu or influenza is a common viral infection that attacks the human population in the world. People who get infected by the

TABLE 1 | Summary of studied effects using EBN extract.

Pharmacological activities	Sample preparation	Model	Dosage	Control group	Results	Proposed mechanism and suggested acting compound	References
Antiviral effects	Water extract (enzyme extraction)	Madin-darby canine kidney cells (MDCK)	4 mg/ml	Non-hydrolyzed EBN and untreated cells/mice	EBN after being hydrolyzed with pancreatin F, EBN showed potent antiviral properties in MDCK cells and prevented the virus' hemagglutinin surface protein from binding to erythrocytes	The bioactive compounds (sialic acid or thymol derivatives) in EBN showed the potential effect toward the antiviral properties by inhibiting the viral genes (NA and NS1) Suggested acting compound: Sialic acid or thymol derivatives	Haghani et al. (2016)
Anticancer effects	Water extract	Human colonic adenocarcinoma cell (Caco-2)	5 ppm	Untreated cells	Two commercial EBN samples showed 84 and 115% cell proliferation, respectively. The unprocessed EBN samples collected from 4 zones, east coast (91%), north (35%), and south (47%) also showed the potent in cell proliferation activity	It is suggested that some of the constituents of EBN must be imparting it with potential to kill rapidly dividing cancer cells	Aswir and Wan (2010)
	Acid extract	Macrophage cells (RAW)	5 ppm	Untreated cells	EBN decreases the production of anti-inflammatory TNF-α in RAW cells	The acting compound not suggested nor tested	
Human adipose-derived stem cells proliferation	Enzyme extraction	Human adipose-derived stem cells (hADSCs)	2000 ppm	Cells were cultures in control medium (DMEM +15% FBS)	EBN extract was strongly found to promote the proliferation of hADSCs mediated by the production of interleukin 6 (IL-6) and vascular endothelial growth factor (VEGF)	The production of IL-6 and VEGF was triggered by the activation of activator protein 1 (AP-1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). The EBN extract induced production of IL-6 and VEGF was inhibited by PD98059 [a p44/42 mitogen-activated protein kinase (MAPK) inhibitor], SB203580 (a p38 MAPK inhibitor) and ammonium pyrrolidinedithiocarbamate (PDTC; an NF-κB inhibitor). Thus, EBN extract-induced proliferation of hADSCs occurred primarily through amplified expression of IL-6 and VEGF genes, which was mediated by the activation of NF-κB and AP-1 through p44/42 MAPK and p38 MAPK.	Roh et al. (2012)
Epidermal growth factor-like activity	Water extract	Primary mouse embryonic fibroblast cell (3T3 fibroblasts)	0.016–2 ng/ml	Glucosamine	EBN stimulated thymidine incorporation in 3T3 fibroblasts cell culture, and this study proved the presence of EGF in EBN.	EGF are known to stimulate DNA synthesis. This study proved DNA synthesis occurred by detecting the present of thymidine	Dawson et al. (2005), Albishtue et al. (2018)

(Continued on following page)

TABLE 1 | (*Continued*) Summary of studied effects using EBN extract.

Pharmacological activities	Sample preparation	Model	Dosage	Control group	Results	Proposed mechanism and suggested acting compound	References
Enhancement of bone strength	Enzyme extraction	Female sprague-dawley rats	100 mg/kg	Fed an AIN93G-based normal diet	The femur of ovariectomized rats that treated with EBN extract showed the increment of calcium level and bone strength ability. Dermal thickness also increased and serum estradiol concentration was not affected by the administration of EBN extract	Estrogen production causes rapid bone loss during first decade after menopause. It was suggested that EBN extract effectively improve bone strength and at the same time regulates the serum estradiol concentration	Matsukawa et al. (2011), Chua et al. (2013), Hou et al. (2019)
	Hot-water extraction	Human articular chondroytes cell (HACs)	0.05–1.00%	Untreated cells	EBN extract increased HACs proliferation, reduced the catabolic genes expression and production of prostaglandin E2 (PGE2). In anabolic activity analysis, type II collagen, aggrecan and SOX-9 gene expression and total sulfated glycosaminoglycan production were increased	EBN extract able to down-regulate matrix metalloproteinases (MMP), cytokines and other catabolic mediator expression that can reduce destruction of cartilage and the degenerative progression of osteoarthritic cartilage	
Eye care effects	Water extract	Rabbit corneal keratocytes cell	0.05 and 1%	Untreated cells	Low concentration of EBN synergistically induced cell proliferation, especially in serum-containing medium. The corneal keratocytes reserved their phenotypes with the addition of EBN, which was confirmed by both phase contrast micrographs and gene expression analysis	EBN induce corneal cell proliferation and also capable to maintain their phenotypes and functionality by synthesizing stromal constituents in maintaining corneal cells This confirmed by increased functional gene expression of collagen type 1, ALDH and lumican which are important corneal keratocytes cell proliferation The acting compound not suggested nor tested	Abidin et al. (2011), Khalid et al. (2019)

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TABLE 1 | (*Continued*) Summary of studied effects using EBN extract.

Pharmacological activities	Sample preparation	Model	Dosage	Control group	Results	Proposed mechanism and suggested acting compound	References
Neuroprotective effects	Water extraction	Human neuroblastoma cell SH-SY5Y	100 μg/ml for crude extract and 200 μg/ml for water extract	Untreated cells	EBN treatment reduces the level of 6-hydroxydopamine-induced apoptotic changes in SH-SY5Y cells that was revealed by morphological and nuclear staining observations	EBN extract more potent in improving reactive oxygen species (ROS) build up, early apoptotic membrane phosphatidylserine externalization and the inhibition of caspase-3 cleavage. This report clearly indicated that EBN extracts might induce neuroprotective effects against 6-hydroxydopamine-induced degeneration of dopaminergic neurons via inhibition of apoptosis	Yew et al. (2014), Hou et al. (2015), Careena et al. (2018)
	Water extract	Human neuroblastoma cell (SH-SY5Y)	1,000 μg/ml	Untreated cells	EBN and its content (lactoferrin and ovotransferrin) attenuated H_2O_2-induced cytotoxicity and decreased radical oxygen species through increased scavenging activity	This report indicated that EBN acts as a neuroprotective (SH-SY5Y human neuroblastoma cell) agent against H_2O_2-induced cytotoxicity and cell oxidative stress Suggested acting compound: Lactoferrin and ovotransferrin	
Antioxidant effects	Water extract	Human neuroblastoma cell (SH-SY5Y)	1,000 μg/ml	Untreated cells	EBN demonstrated protective effects against hydrogen peroxide-induced toxicity and cell oxidative stress on SH-SY5Y cells. Lactoferrin and ovotransferrin also possess antioxidant capacities on SH-SY5Y cells	EBN and its ingredients diminished hydrogen peroxide-induced cytotoxicity, and decreased ROS through increased scavenging activity. Lactoferrin and ovotransferrin in EBN could be contributing toward overall functional properties of EBN Suggested acting compound: Lactoferrin and ovotransferrin	Yida et al. (2014), Hou et al. (2015), Daud et al. (2019b)
Erectile dysfunction	Water extract (enzyme extraction)	Castrated male wistar rats	1 mg/kg/day 3 and 9 mg/kg/day	Un-castrated rats	Castrated rat treated with 9 mg/kg/day of EBN extract exhibited significant higher testosterone and luteinizing hormone level. The penis index was observed to be significantly higher	Authors speculated with the increased dosage of EBN extract (9 mg/kg/day) that contributed to the sexual functions Suggested acting compound: Testoterone but no evidence of EBN extract it contains	Ma et al. (2012)

influenza virus may be having varies symptoms like sore throat, muscle pains, running nose, headache, coughing, tired feeling and sometimes may come together with a high fever. Previous study on EBN has shown its potential to treat influenza virus infection in Madin-Darby Canine Kidney Epithelial (MDCK) cells. It also prevents human erythrocytes from undergoing hemagglutination by influenza A viruses (Haghani et al., 2016). Besides, after hydrolyzation with Pancreatin F, EBN extract also has reported the inhibition of the infection in a host range-depended manner with the human, porcine, and avian influenza viruses (Guo et al., 2006). The bioactive compounds such as sialic acid and thymol derivative have given EBN the potential to inhibit the virus. However, *Collocalia* mucoid or EBN contained a substrate for influenza virus sialidase Saengkrajang et al. (2013), whereby the inhibition can be disrupted by neuraminidase to some extent. Thus, EBN does not protect against influenza viruses sialidase. On the other hand, there was a report that suggested that the potential of EBN extract as an antivirus agent may be attributed to other inhibitory substances in the EBN that may be work together in a complex and bring the antiviral function in EBN. For instance, there was a previous study showed that N-acetylneuraminic acid may play a role in regulating the antiviral activity in EBN (Saengkrajang et al., 2013).

Interestingly, EBN displays no side effects to the MDCK cells and erythrocytes even at a high concentration of 4 mg/ml. Thus, EBN extract which has undergone Pancreatin F treatment and have a smaller molecular size is highly potential to be used in

antiviral treatment due to its effectiveness and safety properties (Guo et al., 2006). Further study was carried out by Yagi et al. (2008) where they presented the N-glycosylation profile of EBN. The authors illustrated a tri-antennary N-glycan with the alpha 2,3-N-acetylneuraminic acid residues as a core component of the EBN. The authors further suggest that the sialylated high-antennary N-glycans are the core components that regulate the inhibition of influenza viral infection.

Overall, there are limited studies that demonstrated the antiviral properties of EBN. Thus, more analyses are needed to investigate the EBN antiviral activity toward the other pathogenic viruses. Although some of the antiviral activity in EBN have been attributed to the presence of N-acetylneuraminic acid and sialylated high-antennary N-glycans, it is important to ascertain other active ingredients in EBN that could possess antiviral properties. It is also vital to establish the active ingredients mechanisms of EBN that showed antiviral effects.

Anticancer Effects

Cancer is one of the most common and lethal diseases after cardiovascular diseases (Zhao et al., 2016). It is a major public health havoc all over the world. Therefore, anticancer agents have always been of great interest (Ali et al., 2011; Saleem et al., 2013). Aswir and Wan (2010) documented the effects of EBN on the progression of epithelial colorectal adenocarcinoma cells (Caco-2 cells) in human. The EBN samples used two commercial brands and four unprocessed samples taken from the Department of Wildlife and National Parks, Kuala Lumpur. Analysis was done using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to determine the anticancer properties of the EBN samples. The authors observed that only 84 and 115% cells proliferated upon treatment using EBN samples from the two commercial brands. Nevertheless, the assay using unprocessed EBN samples from North, South and East Coast zones, resulted in 35, 47, and 91% of cell proliferation, respectively. It was reported the variations in the proliferation percentages of Caco-2 cells is subject to the type and source of EBN used (Aswir and Wan, 2010). These preliminary studies suggested that some of the constituents of EBN must be imparting into human body or cancer cells with potential to kill rapidly dividing cancer cells. However, the exact nature and the fate of the components of EBN responsible for the anticancer effects were not detected.

Complementary and Alternative Medicine (CAM) is a branch of medical and health care systems that include treatments and medications that are not regarded to be part of modern medical practices (Cassileth and Deng, 2004). CAM usage is quite popular among cancer patients. In Singapore, patients from western and eastern cultures who seek for cancer treatment were introduced to CAM practices either by taking TCM health supplements, traditional Indian medicine (Ayurvedic) or traditional Malay medicine (Jamu). Lim et al. (2006) documented EBN used in CAM for pediatric oncology patients in Singapore. The main therapeutic ingredients of CAM are alimentary changes, herbal supplement or tea and EBN. The authors suggested that CAM has a broad impact on every aspect of the healthcare system including pediatric oncology. In a similar fashion, Shih et al. (2009) documented the usage of EBN in CAM for adult cancer patients in Singapore. About 403 adult cancer patients who are taking medication at the Ambulatory Treatment Unit of National *Cancer* Center Singapore have answered a survey form. Based on the questionnaire analyses, 46% claimed taking EBN in their CAM and TCM. As part of the treatment. 54% of the respondents reported the use of EBN during CAM treatment to their oncologist and surprisingly, about 66.4% of the oncologists agreed with the application. Effectiveness from the combination of EBN and CAM to treat cancer is benefited by more than half of the patients. This report shows the advantages of EBN as alternative medicine in improving health of cancer patients.

The studies involving the anticancer evaluation of EBN extracts is yet to be carried out and tested on all range of cancer cells. Based on the literature found, most of the study were very preliminary and it is crucial for the EBN to be screened over a range of cancer cell lines so that a strong justification on its anticancer potential can be documented. Further investigations are needed to elucidate the exact role of different EBN constituents toward cancer cells.

Human Adipose-Derived Stem Cells Proliferation

Stem cells are basic cells which are undifferentiated with a potential to differentiate into many different types of cells. Adipose Stem Cells (ASCs) are generally ubiquitous in all white adipose tissue. The pluripotent ASCs may find differentiation into other types of the mesenchymal cells, such as adipocytes, osteoblasts, chondrocytes and myocytes (Zuk et al., 2001; Zuk et al., 2002). Due to the mesodermal origin of adipose cells, their differentiation into neural tissue of ectodermal origin is unlikely (Tholpady et al., 2006). However, *in vitro* anti-oxidant activity of adipose cells revealed a bipolar morphology which is indistinguishable to neuronal cells (Boone et al., 2000). Stem cells are largely important in the regeneration or repair of aberrant or damaged tissues. ASCs are regarded as the most potent among the mesenchymal stem cells due to its ample confirmations of their pluripotency, multiplying capability and minimum donor morbidity (Ogawa, 2006). Besides, ASCs presence as highly potential agents in regenerative medicine as their cells can be collected in a huge volume with minimum donor-site morbidity. In the last decade, several studies have pointed to the use of ASCs in clinical applications in future. Roh et al. (2012) documented the induction of proliferation of Human Adipose-Derived Stem Cells (hADSCs) by EBN extract. EBN extract was revealed to stimulate the hADSCs cell proliferation *via* the production of vascular endothelial growth factor (VEGF) and Interleukin 6 (IL-6). The production of VEGF and IL-6 was triggered by the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activator protein 1 (AP-1). Interestingly, EBN extract also induced the production of VEGF and IL-6. The EBN extract induced production of VEGF and IL-6 was inhibited by PD98059 [a p44/42 mitogen-activated protein kinase (MAPK) inhibitor], SB203580 (a p38 MAPK inhibitor) and ammonium pyrrolidinedithiocarbamate (PDTC; an NF-κB inhibitor), but not SP600125 [c-Jun

N-terminal kinase (JNK) inhibitor]. Similarly, EBN extract-induced proliferation of hADSCs was also limited by PD98059, SB203580, and PDTC but not SP600125. Thus, EBN extract that promoted hADSCs proliferation mainly occurred by amplified IL-6 and VEGF genes that was mediated by the regulation of activation of NF-κB and AP-1 through p44/42 MAPK and p38 MAPK.

In a nutshell, this report highlighted the potential of EBN extract for the improvement of the self-renewal of hADSCs through the enhancement of growth and multiply capability, which suggested that EBN extract may function as an external element to improve self-renewal by increasing the proliferative capacity of hADSCs. EBN extract affected the proliferation of typical healthy human cells only with no remarkable effects on modified cell lines or mutant cells, which indicated the cell specific effects of EBN extract toward normal cells. However, the details into which the active components of EBN were responsible for these effects still remain to be explored. Therefore, the details of specific component or a group of components of EBN that are responsible for these effects warrant further investigation.

Epidermal Growth Factor-Like Activity

EGF promotes cell growth, dividing and proliferation by joining to the binding site at the epidermal growth factor receptor (EGFR). The size of Human EGF protein is 6,045 Da and it comprises three intramolecular disulfide bonds that linked together with 53 amino acid residues (Harris et al., 2003). EGF binds to the surface of EGFR with high affinity and activates the ligand-induced dimerization (Dawson et al., 2005). The binding will promote the activation of intrinsic protein-tyrosine kinase activity of the receptor. The activation of tyrosine kinase activity induces a signal transduction cascade in several biochemical changes within the cell such as the elevated of intracellular calcium levels, up regulated of the protein synthesis and glycolysis process, and increased of the expression level of the EGFR targeted gene. This leads to DNA synthesis and cell proliferation (Albishtue et al., 2018). Kong et al. (1987) was the first to demonstrate that there is a particular component in the EBN extract that has the EGF-like activity. The EGF-like substance was semi-purified from aqueous extract of the EBN using a Bio-Gel P-10. Following semi-purification, the EGF-like activity of EBN was identified using a series of biochemical analyses such as protein assays that include gel electrophoresis and competitive binding assays. Preliminary study using a specific radioreceptor assay showed the semi-purified EGF-like activity of EBN could generate a competitive binding curve that is parallel to the standard curve. Besides, the EGF-like component present in the EBN extract also could stimulate DNA synthesis by inducing the thymidine inclusion in the quiescent culture of the embryonic fibroblasts (3T3 fibroblasts). Analysis using heat treatment, trypsin digestion and mEGF (EGF isolated from mouse) antibody to investigate the simulation of DNA synthesis in human fibroblasts, the semi-purified EGF-like activity of EBN shown the ability to alter the stimulation of thymidine incorporation with the fibroblasts culture by restricted the trypsin digestion and eventually destroyed the its activity. Consistent results were recorded where the activity of mEGF and EGF-like activity derived from EBN were suppressed when treated with mEGF antibody. This result indicated that the nest EGF shared many similarities with EGF isolated from mouse or shrew in terms of its physical properties.

This section summarised the possible reason of EGF-like substance in the EBN that may contribute to its rejuvenating properties. However, there is a need to find out the possible substance, and characterize its structure through *in vitro* and *in vivo* studies, both alone and in EBN as a formulation.

Enhancement of Bone Strength

Bones are rigid structures inside human body that form part of the skeleton system. They are vital to the support system and protect various important organs in the body. Additionally, bones play important roles in the production of white and red blood cells, minerals storage and involve in regulation of body movements and locomotion. Matsukawa et al. (2011) reported the increase of bone strength and dermal thickness of ovariectomized rats after daily consumption of EBN extract. It was reported that the oral consumption of EBN extract improved bone strength of ovariectomized rats due to the increasing of calcium level in the femur of the rats. More importantly, it was also observed the enhancement of dermal thickness following the EBN extract administration. Interestingly, EBN extract did not alter serum estradiol concentration level after EBN extract consumption. Since the ovarian production of estrogen will decline and this will be the major cause of rapid bone loss after menopause Gruber et al. (1984), Hou et al. (2019), EBN extract consumption can be an alternative and effective way to increase bone mass and at the same time slow skin aging in postmenopausal women.

Osteoarthritis (OA) is an established degenerative disorder caused by the deterioration of joints that includes the articular cartilage and subchondral bone. It is a painful disorder of the joints often causing stiffness and loss of ability. It is assumed that EBN extract poses some active ingredients that may minimized the occurrence of OA and contribute to the regeneration of cartilage (Wong et al., 2018b). In addition, Chua et al. (2013) documented the effects of EBN treatments toward the human articular chondrocytes (HACs) isolated from the knee joint of OA patients. They used hot-water extraction technique to obtain the EBN extract and reported that the supplementation of EBN extract results in increased HACs proliferation. In addition, EBN supplementation down-regulated the expression level of catabolic genes such as matrix metalloproteinases (MMP1 and MMP3), Interleukin 1, 6, and 8 (IL-1, IL-6, and IL-8), cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) in cultured HACs. In addition, the production of PGE2 was significantly reduced in HACs. However, in anabolic activity analysis, total sulfated glycosaminoglycan production was increased and the expression level of targeted gens such as Aggrecan, type II collagen and SOX-9 were also elevated.

This research report clearly indicated that EBN extract has *in vitro* chondro-protection effect on HACs. However, it needs to be seen which components of EBN and how they demonstrate such effects. Therefore, EBN may be have a great potential as a replacement nutrient and supplement for osteoarthritis.

Eye Care Effects

Eyes are the main sensory organs of the human body that react to light and give sight. The rod and cone cells in the retina are important conscious sense organs, which can enhance vision and conscious light perception. Our human eye is known to distinguish approximately 10 million colors (Land and Fernald, 1992). The transparent cornea covers three main organs which are pupil, iris, and the anterior chamber. Three distinct cell layers make up the cornea, including the stroma, epithelium and endothelium. Each individual cornea layer has its specialized visual functions. The cell layers also act as protective barriers from external environment (Lu et al., 2001). Approximately 90% of the corneal volume is comprised of the corneal stroma. It has a highly organized extracellular matrix (ECM) and consist of relatively low keratocyte density (West-Mays and Dwivedi, 2006). Keratocytes derived from the corneal layers are mesenchymal-derived cells which directly regulate in the synthesis and secretion of the ECM components (He and Bazan, 2008). Generally, the cornea is damaged by light injuries including localized burns, scraping or abrasions, and some extensive injuries in terms of surface or depth (Bizrah et al., 2019).

There are a few researches performed on the development of medicinal eye care product from EBN. Abidin et al. (2011) studied the effects of EBN on cultured animal corneal keratocytes through the *in vitro* study, including the isolation of corneal keratocytes with MTT assay in serum contained media (SCM) and serum free media (SFM), morphological observation for detection of phenotypes changes of keratocytes, and determination for gene expression of lumican, collagen type 1 and aldehyde dehydrogenase cells through Reverse Transcription Polymerase Chain Reaction (RT-PCR). Two significant results from Abidin et al. (2011) were reported for better recovery and tissue repair of eye. One significant result was the supplementary effect of EBN from 0.05 to 0.1% showed the highest cell proliferation and the capability to retain phenotypes of corneal keratocytes had been proved by the gene expression and phase-contrast micrographs (Abidin et al., 2011). Another study from Khalid et al. (2019) also clearly indicated that cell proliferation, especially in SCM, was synergistically induced by low EBN concentration. From these literatures, EBN showed great potential to enhance the cell repair from damage through higher cell proliferation rate and proper functioning maintenance in the wound healing of corneal tissues. To develop EBN-based eye drops products before *in vivo* application, the *in vitro* test can be a critical first step in the beginning. However, efforts are needed to see if there are any adverse reactions of EBN on other cell types in the vicinity of corneal keratocytes. Besides, it needs to be seen which of the EBN ingredients is responsible for the activity.

Neuroprotective Effects

Neurodegeneration refers to the progressive loss of the structure or function of neurons. Several neurodegenerative diseases such as Parkinson's (PD), Alzheimer's (AD) and Huntington's (HD), occur as eventual results of neurodegenerative processes. PD is an age-related progressive neurodegeneration. It is projected that the prevalence of PD will exceed nine million globally for people who aged more than 50 years old by the end of 2030 (Szatmari et al., 2019). Factors including depletion of dopaminergic neuronal in the substantia nigra and depletion of dopamine in the striatum are the hallmark pathology of PD (Yew et al., 2018). Przedborski (2005) revealed that the abnormal synthesis of α-synuclein (one protein type of presynaptic neuronal) had contributed to the neurodegenerative diseases. The degeneration of motor functions occurs with dopamine depletion and the patients often show clinical symptoms including slow responsiveness, rigidity, and tremor (Snyder and Adler, 2007).

For the past few years, works on EBN and neuroprotective effects have been studied by a number of scientist. For instance, the examination of neuroprotective effect on Human Neuroblastoma SH-SY5Y (HNS) cells using EBN extracts has been reported by Yew et al. (2014). The study showed that the pancreatin-digested EBN extract was inhibited the cell death of HNS cells up to 75 μg/ml while the maximum non-toxic dose was double (150 μg/ml) for EBN water extract. Nuclear staining and morphological observation indicated that the application of EBN can decrease apoptotic changes induced by 6-hydroxydopamine (6HD) in the HNS cells. Interestingly, cell viability significantly improved with digested EBN extract as compared to the EBN water extract. Nevertheless, EBN water extract showed great roles in the cleavage inhibition of caspase-3, regulate the early apoptotic effect on the phosphatidylserine externalization membrane and neuron recovery with reactive oxygen species build-up. Another similar study also clearly showed that enzyme extraction from EBN might possess neuroprotective effects through the apoptosis inhibition against 6HD-induced degeneration of dopaminergic neurons (Careena et al., 2018). EBN can therefore serve as a viable nutraceutical alternative for the protection against oxidative stress-related neurodegenerative diseases. A different study conducted by Hou et al. (2015) demonstrated the effect of EBN on the toxicity depletion of hydrogen peroxide (H_2O_2) on HNS cells. It was observed that lactoferrin and ovotransferrin within EBN attenuated (H_2O_2)-induced toxicity and cytotoxicity. The contents from EBN further decreased ROS with the enhancement of scavenging process which corresponds to a later work done by (Careena et al., 2018) where they found that EBN supplementation inhibited the production of oxidative markers ROS and TBARS in a Wister rat model of LPS-induced neuroinflammation. These reports indicated that EBN may act as a neuroprotective agent against cell oxidative stress and H_2O_2-induced cytotoxicity.

Although many researches have been reported on neuroprotective effects of EBN, the current scientific reports have not been able to demonstrate which of the specific EBN components or combination thereof has neuroprotective effects. Hence, the research efforts on EBN are needed to conclude this point in the near future.

Antioxidant Effects

The human body has several anti-oxidant mechanisms that counteract oxidative stress from normal metabolic activity (Wang et al., 2019). The food contains the antioxidants components which are able to fight against the cell-disruptive effects. These antioxidants supplied function either individually

or in combination with the endogenous systems. The implications of antioxidants with diet have been shown to be beneficial to human health, but their absence may trigger a number of diseases due to uncontrolled oxidative stress. Numerous vegetables and fruits have been shown to have antioxidant properties against certain cancers and other diseases. Thus, people who regularly rely on fruits and vegetables that are rich in anti-oxidants have lesser frequencies of free radical-induced diseases (Babji et al., 2018). Antioxidants have been the subject of great attention in the present scenario on because of their potential for fighting oxidative stress-related diseases.

EBN has long been first reported to contain antioxidants (Ghassem et al., 2017). As such, the effect of its antioxidants after oral administration is not fully known. EBN's anti-oxidant properties are attributed to the pool of bioactive compounds such as amino acids, sialic acid, triacylglycerol, vitamins, lactoferrin, fatty acids, minerals, and glucosamine (Liu et al., 2012; Zainab et al., 2013; Lee et al., 2020). The anti-oxidative effect of EBN showed the presence of two main constituents, namely ovotransferrin and lactoferrin (Hou et al., 2015). The authors also reported their protective effects against H_2O_2-induced toxicity on HNS cells. Furthermore, transcriptional changes in anti-oxidant related genes were brought about by lactoferrin, ovotransferrin and EBN were in linked with the neuroprotection (Hou et al., 2015). Yida et al. (2014) documented the *in vitro* bio-accessibility and antioxidant properties of water extracts of EBN using Oxygen Radical Absorbance Capacity (ORAC) assays and 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay. It was observed that there were low antioxidant activity (about 1% at 1,000 μg/ml) on the undigested EBN water extract for both ABTS and ORAC assays. On the contrary, the digested EBN samples using pepsin, pancreatin and bile extract at similar concentrations showed improved antioxidant activities around 38 and 50% for ABTS and ORAC assays respectively. Besides, the EBN extracts showed non-toxicity toward human hepatocellular carcinoma (HEPG2) cells and protected HEPG2 cells from H_2O_2-induced toxicity. In short, enhancement of antioxidant activities of EBN after digestion highlighted some of its functional effects after consumption. However, further analysis such as *in vivo* studies are needed further characterize the significance of EBN from clinical perspective.

Miscellaneous Effects

There are many therapeutic claims about EBN with no scientific proof which have been handed down from generation to generation. These include claims to treat *tuberculosis*, dry coughs, asthma, gastric trouble and stomach ulcer (Jamalluddin et al., 2019). Among the Chinese community, it is also renowned for its contribution to the fine porcelain complexion of Chinese beauties. In addition, it is also a normal practice to consume EBN among the Chinese mothers-to-be as a health supplement for both mother and child to have a pair of strong lung and fine complexion (Babji et al., 2018). These legendary claims on the EBN need further research so that the EBN can be developed into traditional medicine with scientific proofs, or at least, a scientifically credited functional food or health supplement. Erectile dysfunction is one of the main male sexual disorders characterized by tenacious inability to keep penile erection enough for pleasing sexual acts (Ma et al., 2012). This disorder mainly occurs due to a continuous spectrum of clinical factors such as difficulties, stress and physical illness in relationship (Corona and Maggi, 2010). Although drugs such as phosphodiesterase-5 inhibitors and testosterone supplication could help to overcome this disorder, the results of these treatments are not always desirable (Tsertsvadze et al., 2009). Ma et al. (2012) studied the effects of EBN on the sexual functioning of castrated rats and found that the seminal vesicle indices and prostate along with the hormone expression of endothelial nitric oxide synthase increased potently in the mouse groups that were treated with EBN. This result indicated that EBN has a potential to be an ideal active ingredient for the development of drugs in treating erectile dysfunction.

CURRENT CHALLENGES AND FUTURE PERSPECTIVES

Despite the scarcity of science on the therapeutic effects of EBN in the past, several scientific papers have appeared on this subject especially for the past decades. Some of the research papers which have documented and summarized these effects include claim of antiviral, anticancer, proliferation effects on stem cells, epidermal growth factor-like activity, bone strength enhancement, eye care, neuroprotective antioxidant and other health-related effects of EBN. Research needs to be addressed so that the fundamental issues, including the molecular and biochemistry pathway of EBN to alleviate asthma, facilitate renal function, improve complexion, stamina and vitality bone health, could be fully understood. The specific components contributing to a specific function need to be identified. Besides, the correlations between dosages and activities of EBN need to be worked out also. Therefore, it would be a great breakthrough to discover the fundamental mechanisms by which the EBN component exerts both its biological effects *in vivo* and *in vitro* studies. Additionally, specific biological functions to specific components of EBN studies and then their isolation and purification component would be valuable. The conclusions and solutions would provide better use of EBN.

From the current literature updates, it can be concluded that EBNs collected from different sources and locations have their differences in composition. Therefore, it would be beneficial to standardize EBN composition and establish a standard operating procedure (SOP) to ensure that a stable and consistent outcome could be obtained. Further investigation focusing on the methodology reported including the complexity and variety of the location sources is needed to justify the variation that exists. If a sample is collected from the market, dealer or a retail shop, it is to be considered as processed since the probability of adulteration is high. One of the most common adulteration is bleaching so that the bird feathers cannot be seen. Others include addition of fortified materials to gain weight such as egg white, jelly, seaweed or even pork skin (Ma et al., 2019). These will

definitely deviate the contents of EBN and hence affect the results of experiments.

EBN has long been used as a traditional remedy for some illness but has never been used as a medicine to cure or treat the sickness. This is simply due to the lack of study on the drug development and effective dose of this unique animal-based bioproduct. To the best of our knowledge, there is still no fractionation and isolation of single component work reported for EBN material leading to no specific component to demonstrate its therapeutic. There were only works done for *in vitro* and *in vivo* testing using the whole EBN extract with no further characterization on its single compound. Hence, due to insufficient scientific findings and reports, EBN could only regarded as food or at most remedy food.

The rise of allergic issues related to the consumption of EBN have been reported in several health cases. Allergic issues like skin rash, nasal obstruction and facial swelling have been reported in Japan after 5 min of consumption of EBN-contained dessert. The condition of allergic reactions can be in different degree of severity and some severe cases might cause death (Goh et al., 2000). One similar case was reported by National University of Singapore which documented that EBN caused food-induced anaphylaxis among the kids. The anaphylaxis occurrence might be due to the presence of putative allergens and abnormal regulation of Immunoglobulin E mediated process (Goh et al., 2000). Therefore, it becomes critical to identify whether a person is allergic or prone to allergy toward EBN protein by undergoing a skin prick test before consumption. These studies have set the precedence of EBN being an allergen. As the report comes from the highly reputable National University of Singapore, it is of great concern. However, as the test samples were obtained from the market or in other terms, it could have been adulterated by the bird's premises handler or producer along the way in order to increase profit. The terms "egg white like" protein provides a good clue on this as the EBN processor normally will add egg white on the surface of EBN to provide the good looking luster on EBN to fetch a higher price (Guo et al., 2018). A better understanding and awareness of the consumer market norms and practices would ensure a good sample is applied for research to arrive at a more accurate conclusion.

There are issues arising in relation to the prescription of EBN to cancer patients. Generally, EGF receptors (which had been discussed in *Epidermal Growth Factor-Like Activity*) are highly expressed in several tumors cells such as non-small-cell lung, breast, colon, ovarian, renal, head and neck cancers (Herbst and Shin, 2002). Therefore, it may be assumed that EBN consumption might stimulate tumor progression and resist chemotherapy/radiation treatment in tumor cells. However, EBN also promotes healthy cell growth as explained earlier. *Cancer* patients should not avoid EBN as if it is a taboo merely based on EGF findings alone as EBN has been found to have apoptosis on cancer cells (Albishtue et al., 2018). Nevertheless, these concepts should be researched further to maximize the cancer prevention or treatments.

CONCLUSION

The discussion in this article proves EBN could be a source of vital health-promoting ingredients with the reported content of amino acids, proteins, carbohydrates, fatty acids and minerals. The discovery of bioactivities in EBN are still in fetal stage and very much unexplored. Overall, the biological effects of EBN are still little explored as the available studies are very much preliminary and have been carried out on limited targets without any emphasis on *in vivo* studies. Thus, more exploration on the evaluation of bioactivities of EBN are needed to narrow the knowledge gap in EBN research studies. Like of the finding of subject or new material, the primary material should be standardized. The extract based on active ingredients or a standardized operating procedure should be filed. Despite some metabolite profiling studies, there are little information regarding the correlation of the specific active compound of EBN with specific medicinal effect. Furthermore, there is a lack of optimization studies related on the fractionation isolation and purification of active components that attributed to the bioactivities process in EBN. The present era demands further proteomic and genomic research to analyze EBN and its components for humanity's welfare comprehensively. Finally, research should be encouraged to explore the biological and medicinal properties of EBN. There is a great need to study the correlations between the components and the functions of EBN so that some new and exciting compositions may be discovered. EBN, its extracts and products hold much for future development as possible food and medicinal-based products.

AUTHOR CONTRIBUTIONS

THL, SW, and NH edited and reviewed the manuscript. KKC and SS conceived and designed the study. WW composed the first draft of the manuscript. CHL and NAA wrote some contents of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Center of Excellence: Swiftlets Malaysia and Ministry of Science, Technology and Innovation Malaysia (MOSTI) (grant numbers 6371400–10301(H6), 6371400–10301(Q6)).

ACKNOWLEDGMENTS

The authors express their sincere gratitude to the internal reviewers, LSK and LTK for their unbiased opinion, valuable suggestion and devoted dedication in making this paper a success.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 21 April 2021
doi: 10.3389/fphar.2021.632602

Edible Bird's Nest Ameliorates Dextran Sulfate Sodium-Induced Ulcerative Colitis in C57BL/6J Mice by Restoring the Th17/Treg Cell Balance

Yaohua Fan[1], Yanqun Fan[2], Kunfeng Liu[3], Piyanuch Lonan[3], Feng Liao[3], Yuhang Huo[3], Xiaohua Zhong[2], Yueliang Liang[4], Yaxin Wang[2], Shaozhen Hou[5], Xiaoping Lai[6], Geng Li[3]* and Weihong Kuang[7]**

[1]Traditional Chinese Medicine Innovation Research Center, Shenzhen Hospital of Integrated Traditional Chinese and Western Medicine, Guangzhou University of Chinese Medicine, Shenzhen, China, [2]Xiamen Yan Palace Seelong Food Co., Ltd., Xiamen, China, [3]Laboratory Animal Center, Guangzhou University of Chinese Medicine, Guangzhou, China, [4]Guangzhou Tongkang Pharmaceutical Co., Ltd., Guangzhou, China, [5]School of Pharmaceutical Sciences, Guangzhou University of Chinese Medicine, Guangzhou, China, [6]Guangdong Provincial Key Laboratory of New Drug Development and Research of Chinese Medicine, Mathematical Engineering Academy of Chinese Medicine, Guangzhou University of Chinese Medicine, Guangzhou, China, [7]Guangdong Key Laboratory for Research and Development of Natural Drugs, Key Laboratory of Research and Development of New Medical Materials of Guangdong Medical University, School of Pharmacy, Guangdong Medical University, Dongguan, China

Ulcerative colitis (UC) is a type of inflammatory bowel disease (IBD) with a complex aetiology that commonly recurs. Most drugs for UC treatment interfere with metabolism and immune responses, often causing some serious adverse reactions. Therefore, the development of alternative treatments, including nutritional supplements and probiotics, have been one of the main areas of current research due to fewer side effect. As both a Chinese medicine and a food, edible bird's nest (EBN) has high nutritional value. Modern pharmacological studies have shown that it has anti-inflammatory, immunoregulatory, antiviral and neuroprotective effects. In this study, UC was induced with dextran sulfate sodium (DSS) to investigate the protective effect of EBN on colitis mice and the related mechanism. The body weight, faecal morphology and faecal occult blood results of mice were recorded every day from the beginning of the modelling period. After the end of the experiment, the length of the colon was measured, and the colon was collected for histopathological detection, inflammatory factor detection and immunohistochemical detection. Mouse spleens were dissected for flow cytometry. The results showed that in mice with colitis, EBN improved symptoms of colitis, reduced colonic injury, and inhibited the increases in the levels of the pro-inflammatory cytokines IL-1β and TNF-α. The T helper 17 (Th17)/regulatory T (Treg) cell balance was restored by decreasing the expression of IL-17A and IL-6 in intestinal tissues, increasing the expression of TGF-β, and decreasing the number of Th17 cells in each EBN dose group. These findings suggest that EBN has a protective effect on DSS-mediated colitis in mice, mainly by restoring the Th17/Treg cell balance.

Keywords: ulcerative colitis, edible bird's nest, immune regulation, Th17 cells, treg cells

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Edited by:
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****Correspondence:***
Weihong Kuang
kuangwh@gdmu.edu.cn
Xiaoping Lai
Lxp88@gzucm.edu.cn
Geng Li
lg@gzucm.edu.cn

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: *23 November 2020*
Accepted: *26 February 2021*
Published: *21 April 2021*

Citation:
Fan Y, Fan Y, Liu K, Lonan P, Liao F, Huo Y, Zhong X, Liang Y, Wang Y, Hou S, Lai X, Li G and Kuang W (2021) Edible Bird's Nest Ameliorates Dextran Sulfate Sodium-Induced Ulcerative Colitis in C57BL/6J Mice by Restoring the Th17/Treg Cell Balance. Front. Pharmacol. 12:632602. doi: 10.3389/fphar.2021.632602

INTRODUCTION

Ulcerative colitis (UC) is a major form of inflammatory bowel disease (IBD) characterized by recurring and diffuse inflammation in the rectal and colonic mucosa. The incidence and prevalence rates of UC in North America and northern Europe are higher than those in Asia, although there is an increasing incidence in Asia (Ordás et al., 2012; Feuerstein et al., 2019). As an important public health problem and a global disease, UC tends to affect young people, which could increase the risk of colorectal cancer (Feuerstein et al., 2019). Risk factors for the development of UC are related to disruption of the intestinal mucosal barriers, which can be caused by infections, medications or familial genetics. Chronic and relapsing immune responses and clinical symptoms seriously reduce the quality of life of patients with UC. As a result of its complicated pathogenesis, UC has not yet been cured, and the goal of therapy in UC is to first induce clinical remission and then achieve steroid-free maintenance of remission (Feuerstein et al., 2019). In addition, due to its structure and long-term treatment, sulfasalazine (SASP), one of common used for UC, could cause some serious adverse reactions such as infertility, nephrotoxicity and hepatotoxicity (Linares et al., 2011). Therefore, there is an urgent need to develop novel therapeutic approaches for UC patient treatment.

Dysregulation of immune responses is considered an important factor in the development of UC. Recent research has shown that the immune response and inflammatory pathway of UC are driven by dynamic and complex interactions of cells and cytokines (Tatiya-Aphiradee et al., 2018). Moreover, T helper (Th) 17 cells and regulatory T (Treg) cells play crucial roles in UC pathogenesis by regulating, suppressing and maintaining inflammation (Tatiya-Aphiradee et al., 2018). Additionally, the homoeostatic balance between Th17 cells and Treg cells is disturbed in the mucosa of patients with UC (Hartog et al., 2015). Moreover, the balance between Th17 cells and Treg cells has a significant role in the T cell-mediated immune response in the intestine, and this balance is a novel and potential therapeutic target in UC (Bettelli et al., 2006; Xu et al., 2007; Lee et al., 2018).

Edible bird's nest (EBN; known as Yanwo in Chinese), which comes from swiftlets belonging to the family *Apodidae* and the genus *Aerodramus*, has multiple nutritional and pharmaceutical benefits, such as pro-conception effects, neuroprotective effects and bone loss improvement (Matsukawa et al., 2011; Careena et al., 2018; Albishtue et al., 2019; Hou et al., 2019). Additionally, some studies have shown that EBN with antiviral activities can regulate the expression of some immune cytokines involved in influenza A virus infection, such as tumour necrosis factor α (TNF-α), interferon (IFN)-γ, interleukin (IL)-1β, IL-6, IL-10 and chemokine (C-C motif) ligand (CCL) 2 (Haghani et al., 2016; Haghani et al., 2017). Several pro-inflammatory cytokines (e.g., IL-1β, IL-6 and TNF-α) that can induce Th17 cell polarization have key roles in the pathogenesis of UC (Acosta-Rodriguez et al., 2007; Gálvez, 2014; Lee et al., 2018). In addition, IL-10 is an anti-inflammatory cytokine that is involved in Treg cell differentiation, leading to aberrant Treg cell function and increased susceptibility to UC (Boden and Snapper, 2008; Achour et al., 2017).

However, the protective effect of EBN in the context of UC is still unclear. Based on the theories mentioned above, we hypothesized that EBN could ameliorate the severity of UC by regulating pro-inflammatory and anti-inflammatory cytokines to maintain the Th17/Treg cell balance. In this study, we investigated the effect and mechanism of EBN in a dextran sulfate sodium (DSS)-induced UC model, which could provide a reference for drug development for UC therapy.

MATERIALS AND METHODS

Animals and groupings

Male C57BL/6J mice (6°weeks old) were obtained from the animal laboratory of Guangzhou University of Chinese Medicine (production license No. SCXK [YUE]-2008–0034). All the experimental procedures were approved by the International Institute for Translational Chinese Medicine Animal Care and Use Committee, Guangzhou University of Chinese Medicine. Animals were housed in a well-ventilated room maintained at a temperature of 22–26°C with 12°h light/dark cycles and 40–70% humidity. Then, animals were randomly divided into six groups, each consisting of six animals, including the control group, model group (DSS, Yeasen biotech Co., Ltd., Shanghai, China), sulfasalazine (SASP, Shanghai Zhongxi Sunve Pharmaceutical Co., Ltd., Shanghai, China) group, low-dose EBN group (EBNL, 0.3 g/kg), moderate-dose EBN group (EBNM, 0.7 g/kg) and high-dose EBN group (EBNH, 1.3 g/kg). UC was induced in mice by administering 5°ml 2% DSS daily for 7°days; the animals in the control group were administered distilled water only. Each time point of drug administration is shown in **Figure 1A**. Body weight was measured once a week from the first week to the sixth week. Additionally, the body weight, faecal morphology and faecal occult blood of mice were recorded every day after the modelling period. At 53°days, the mice were sacrificed by cervical dislocation, and then colon specimens (from the caecum to the anal terminus the colon) and spleen specimens were collected.

Preparation of Edible Bird's Nest Extracts

EBN was provided by XIAMEN YAN PALACE SEELONG FOOD Co., Ltd (XIAMEN YAN PALACE SEELONG FOOD Co., Ltd., Xiamen, China) and was identified as the saliva of *Apodidae Aerodramus fuciphagus*. A voucher specimen was dried at 4°C and stored in an airtight container in the Laboratory Animal Center, Guangzhou University of Chinese Medicine. Briefly, the ground EBN was immersed in distilled water for an hour and boiled at 100°C for 30 min. The extracts were stored at 4°C for further use.

Protein Identification of Edible Bird's Nest Extracts by High Performance Liquid Chromatography/Electrospray Ionization

Characterization of sialic acid in EBN extracts was detected by HPLC (Easy-nLC1000). Chromatographic separation was achieved on a 3 μm Thermo scientific EASY column C18 column (75 μm × 100 mm), with column temperature

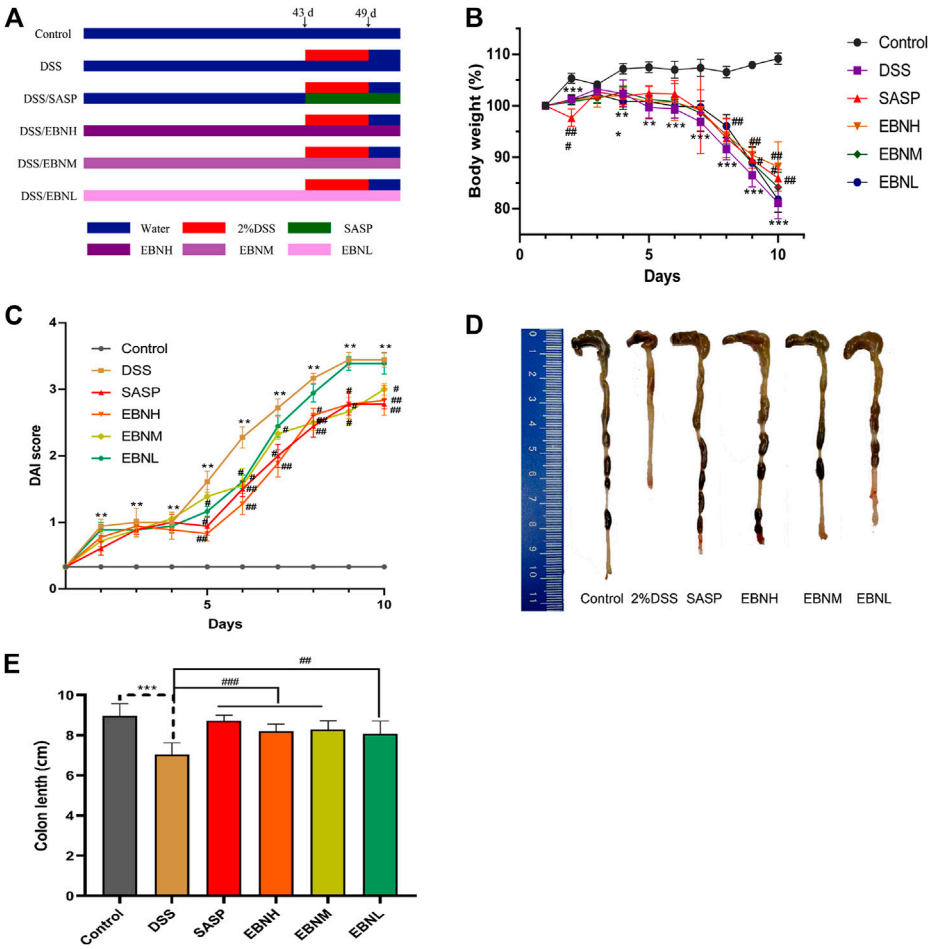

FIGURE 1 | Effects of EBN pretreatment on the clinical symptoms of DSS-induced colitis in C57BL/6J mice. **(A)** Schematic showing the experimental dosing design. **(B)** Effect of EBN on body weight during the modeling period of colitis induced by DSS. **(C)** Effect of EBN on the disease activity index (DAI) of colitis induced by DSS. **(D,E)** Effect of EBN on colon length in mice with colitis induced by DSS. $^{**}P < 0.05$ and $^{***}P < 0.001$ compared with the control group; $\#P < 0.05$, $\#\#P < 0.01$ and $\#\#\#P < 0.001$ compared to the DSS group ($\bar{x} \pm S$, $n = 6$).

maintained at 25°C. The mobile phases consisted of 0.1% formic acid solution (A) and acetonitrile formate in aqueous solution (B) using a gradient elution. The flow rate was 250 nL/min. The mass spectrometer was operated in electrospray ionization (ESI). The full scan setting parameters are as follows: ion polarity: positive ion; parent ion scanning range, 300–1800 m/z; first order mass resolution, 70,000 at m/z 200; Secondary mass resolution, 17,500 at m/z 200; ionspray voltage, 2 kV; capillary temperature, 250°C; collision energy, 27%HCD. The sialic acid was identified by the retention time and quantified by comparison of integrated peak areas with a known amount of sialic acid-labelled C18 standard sample.

Evaluation of Ulcerative Colitis Severity Based on Disease Activity Index Scores

The disease activity index (DAI) has been widely used to assess the severity of UC in animal models in light of the study of Wirtz et al. (2017), Zhu et al. (2019). Briefly, DAI scores are calculated as the mean of the total scores of all parameters including weight loss, stool consistency and the degree of intestinal bleeding. The degree of intestinal bleeding was also assessed by a qualitative detection kit for faecal occult blood (Leagene Biotechnology Co., Ltd., Beijing, China). The criteria of the scoring system are shown in **Table 1**.

Morphological and Histological Evaluation

Colonic tissues were embedded in paraffin and stained with haematoxylin and eosin (HE). Then, we assessed the

TABLE 1 | Scoring system for calculating DAI scores.

Score	Weight loss	Stool consistency	Blood
0	None	Normal	Negative haemoccult
1	1–5%	Soft but still formed	1+
2	6–10%	Soft	2+
3	11–18%	Very soft; wet	3+
4	>18%	Watery diarrhoea	Blood traces in stool visible

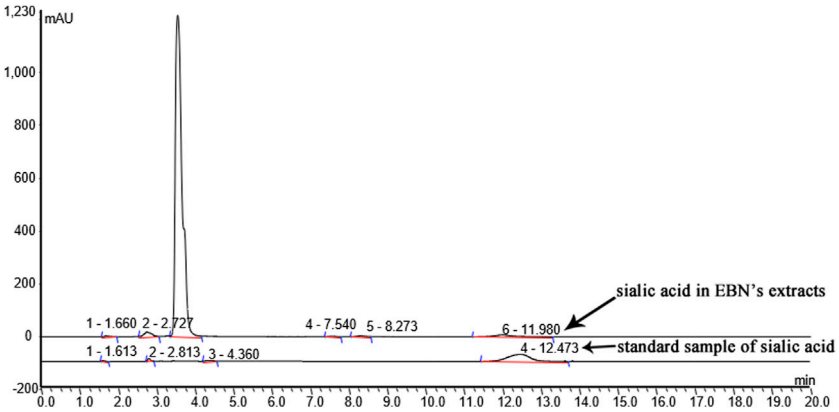

FIGURE 2 | Characterization of EBN extracts.

inflammation-associated histological changes in the colon based on lamina propria inflammatory cell infiltration, and the morphology of the intestinal epithelium and glandular and goblet cells.

Myeloperoxidase Activity Assay

Radioimmunoprecipitation assay (RIPA) lysis buffer (BIOSYNTHESIS BIOTECHNOLOGY Co., Ltd., Beijing, China) was used to lyse tissues, and 10 mg tissue was obtained for each sample. Following washing with cold PBS, the tissues were resuspended in 9 volumes of PBS (included in the myeloperoxidase Activity Assay Kit) and then centrifuged at 4°C and 2,500 r/min for 10 min. The supernatant was collected and transferred to a clean tube, which was then placed on ice. Myeloperoxidase (MPO) activity was assayed using the myeloperoxidase Activity Assay Kit (Abcam Co., Ltd., Cambridge, United States) with measurement of the absorbance of the sample at 460 nm using a microplate reader. The specific MPO activity in the colon was measured as units/g (U/g) protein.

Cytokine Analysis by Enzyme-Linked Immunosorbent Assay

Colonic tissues were soaked in 5 volumes of 1X PBS containing 1% phenylmethanesulfonyl fluoride (PMSF). Then, the tissues were placed on a tissue grinder and ground at 10 Hz for 1 min. Next, the specimens were lysed at 4°C for 30 min and then centrifuged at 4°C and 12,000 r/min for 10 min. The supernatant was collected and transferred to a clean tube. Subsequently, we measured the levels of IL-1β, TNF-α, IL-10, IL-17, IL-6 and TGF-β in the colonic tissues using ELISA kits according to the manufacturer's instructions (MultiSciences Biotech Co., Ltd., Hangzhou, China).

Preparation of Splenic Single-Cell Suspensions

Spleens were placed in 6 mm sterile petri dishes and mechanically dissociated. Following filtration through a 100 mesh cell filter, we obtained splenic cell suspensions. Then, the suspensions were centrifuged at 4°C and 1,500 r/min for 5 min to obtain the cell pellets. The pellets were resuspended in 2 ml PBS and then centrifuged at 4°C and 1,500 r/min for 5 min; the supernatant was discarded. Subsequently, RPMI 1640 medium containing 10% PBS was used to resuspend the pellets, which were then counted, and the cell concentration was adjusted to 1×10^6/ml.

Measurement of the Proportions of $CD4^+IL\text{-}17A^+$ Th17 Cells and $CD25^+Foxp3^+$ Treg Cells by Flow Cytometry

Th17 and Treg cells were isolated from mouse spleen tissues as cell suspensions and evaluated by flow cytometry. The Th17 cells were positively sorted by CD4 and IL-17A. We used FACS analyses and gated on live $CD4^{high}$ cells and $IL\text{-}17A^{high}$ cells. $CD4^{high}/IL\text{-}17A^{high}$ double-positive cells were used to determine the percentages of Th17 cells. The Treg cells were positively sorted by CD25 and Foxp3. We used FACS analyses and gated on live CD25-high cells and Foxp3-high cells. CD25-high/Foxp3-high double-positive cells were used to determine the percentages of Treg cells.

Measurement of the Expression of IL-17A and Foxp3 in Colonic Tissues by Immunohistochemistry

Paraffin sections of colonic tissues were dewaxed; soaked in xylene I, II and III for 15 min each; and hydrated with ethanol (anhydrous ethanol I, anhydrous ethanol II, 85% ethanol and 75% ethanol for 5 min each) and distilled water. After performing antigen repair and blocking endogenous peroxidase activity, the sections were blocked in goat serum at room temperature for 30 min. Then, the sections were washed three times in PBS for 5 min each time and blocked with 3% bovine serum albumin. After blocking, the sections were incubated with anti-IL-17A and anti-Foxp3 (eBioscience Co., Ltd., CA, United States) antibodies overnight at 4°C. The sections were washed 3 times in PBS and incubated with secondary antibodies (EARTH Co., Ltd., CA,

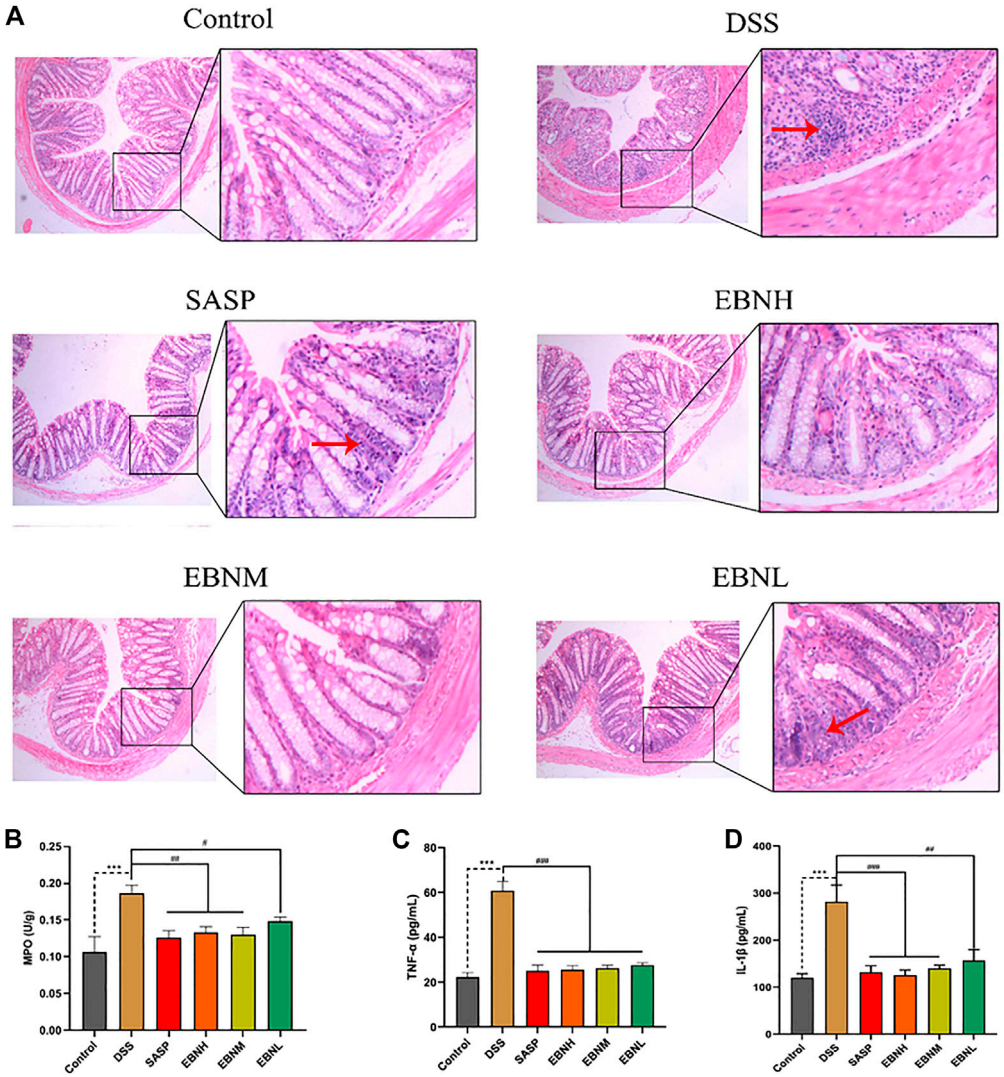

FIGURE 3 | Effects of EBN pretreatment on inflammation in DSS-induced UC. **(A)** Effect of EBN on colonic histomorphology in mice with colitis induced by DSS (50×). **(B–D)** Effects of EBN on MPO activity and TNF-α and IL-1β levels in colonic tissues of mice with colitis induced by DSS. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the control group, $\#P < 0.05$ and $\#\#P < 0.01$ compared to the DSS group ($\bar{x} \pm S$, $n = 6$).

United States) in an incubator at room temperature for 50 min. The tissue sections were incubated with a diaminobenzidine substrate solution (BOSTER Co., Ltd., Wuhan, China).

Measurement of the Expression of IL-17A and Foxp3 by Western Blotting

Colonic tissues were lysed in RIPA buffer (Sigma-Aldrich) containing a PMSF protease inhibitor. The specimens were placed in a Tissue Lyser at 70 Hz for 60 s. The homogenate was lysed on ice for 30 min and centrifuged at 12,000 r/min for 10 min at 4°C. Protein concentrations were determined using a Bradford protein assay kit (Beyotime Co., Ltd., Shanghai, China). The samples were boiled for 5 min in loading buffer. The proteins were transferred to nitrocellulose membranes with 0.45 μm-diameter pores. The membranes were blocked with 5% non-fat dry milk for 1 h at room temperature and then incubated with primary antibodies, including anti-IL-17A and anti-Foxp3 (eBioscience Co., Ltd., CA, United States). After incubation overnight, the membranes were washed 3 times for 10 min each time in PBST and incubated with a secondary antibody (Cell Signaling Technology) for 1 h at room temperature. The protein bands were visualized with Immobilon Western HRP Substrate (Millipore Co., Ltd., MA, United States). Densitometric analyses of the bands were performed using ImageJ software. All data are representative of at least 3 independent experiments.

Statistical Analysis

Results were analyzed with SPSS 22.0 software and are expressed as the mean ± standard deviation. Mauchly's test of sphericity was used to analyse repeated measurement data. If the repeated measurement data did not conform to Mauchly's test of

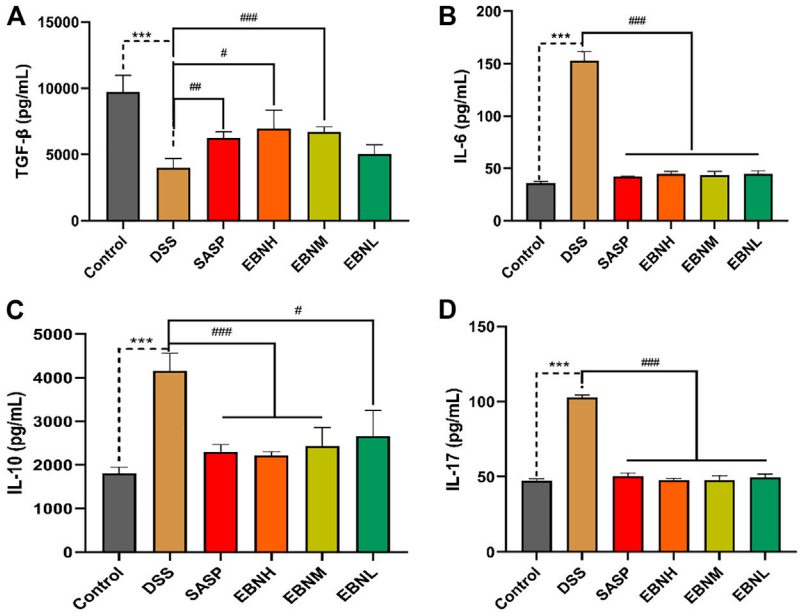

FIGURE 4 | Effects of EBN pretreatment on the expression of Th17 and Treg cell-associated cytokines in colonic tissues. **(A–D)** Effects of EBN on the expression of TGF-β, IL-6, IL-10 and IL-17 in colonic tissues. ***$P < 0.001$ compared with the control group; #$P < 0.05$, ##$P < 0.01$ and ###$P < 0.001$ compared to the DSS group ($\bar{x} \pm S$, $n = 6$).

sphericity ($p < 0.05$), the multivariate test Roy's maximum root was used to identify statistically significant differences ($p < 0.05$). Data considered to conform to a normal distribution (p-value of the Shapiro-Wilk test > 0.05) were analysed by one-way analysis of variance (ANOVA). Data that did not conform to a normal distribution were analysed by nonparametric tests and are shown as quartiles (M [P25–75]). $P < 0.05$, $P < 0.01$ and $P < 0.001$ were considered significant. GraphPad Prism 8.0 software was used to draw images.

RESULTS

Characterization of Edible Bird's Nest Extracts

To identify the sialic acid in EBN extracts, we analyzed the EBN extracts using HPLC/ESI. The polypeptides of EBN were isolated and purified by HPLC. Then ESI formed multi-charge ions, which can be used to detect polypeptide molecules in a small M/Z range, the result was shown in **Figure 2**.

Effects of Edible Bird's Nest Pretreatment on the Clinical Symptoms of Dextran Sulfate Sodium-Induced Colitis in C57BL/6J Mice

First, we evaluated the efficacy of EBN pretreatment in alleviating the clinical symptoms of UC. All mice exhibited normal activity, diet, stool consistency and weight gain before modelling. In comparison with those in the control group, the mice in the model group showed not only observably decreased body weight (**Figure 1B**) and colon length (**Figures 1D,E**) but also observably increased DAI scores (**Figure 1C**, **Supplementary Tables S1–S6**). As shown in **Figure 1C**, the DAI scores of the SASP group and EBN groups began to show relief of disease symptoms in mice on the 5th day after modelling, and the DAI scores of these groups were significantly lower than those of the model group. In addition, the macroscopic morphology of colonic tissues showed colon oedema, a reduced caecal volume and an enteric cavity with bloody stools. Nevertheless, the mice pretreated with EBN exhibited reversed colonic shortening and relieved colonic oedema. These results suggest that EBN can alleviate the clinical symptoms of UC and could be a latent drug for UC.

Effects of Edible Bird's Nest Pretreatment on Inflammation in Dextran Sulfate Sodium-Induced Ulcerative Colitis

To evaluate the extent of damage and inflammation in colonic tissues, histological analysis was performed. As shown in **Figure 3A**, HE staining of tissues from the control group showed an intact colonic structure with aligned crypts and glands and no inflammatory cell infiltration. Consistent with previous studies, the colonic tissues of the model group showed extensive inflammatory cell infiltration in the submucosa, a large area of exfoliated intestinal epithelium, jumbled arrangement of the glands, reduced goblet cell numbers and oedema in the submucosa (Wirtz et al., 2017; Zhu et al., 2019). In contrast, the mice pretreated with EBN showed a low level of inflammation and a more complete structure of the submucosa.

In addition, we detected MPO activity and cytokine levels in colonic tissues, which can reflect the severity of inflammation. As shown in **Figures 3A**,**B** higher level of MPO activity was found in

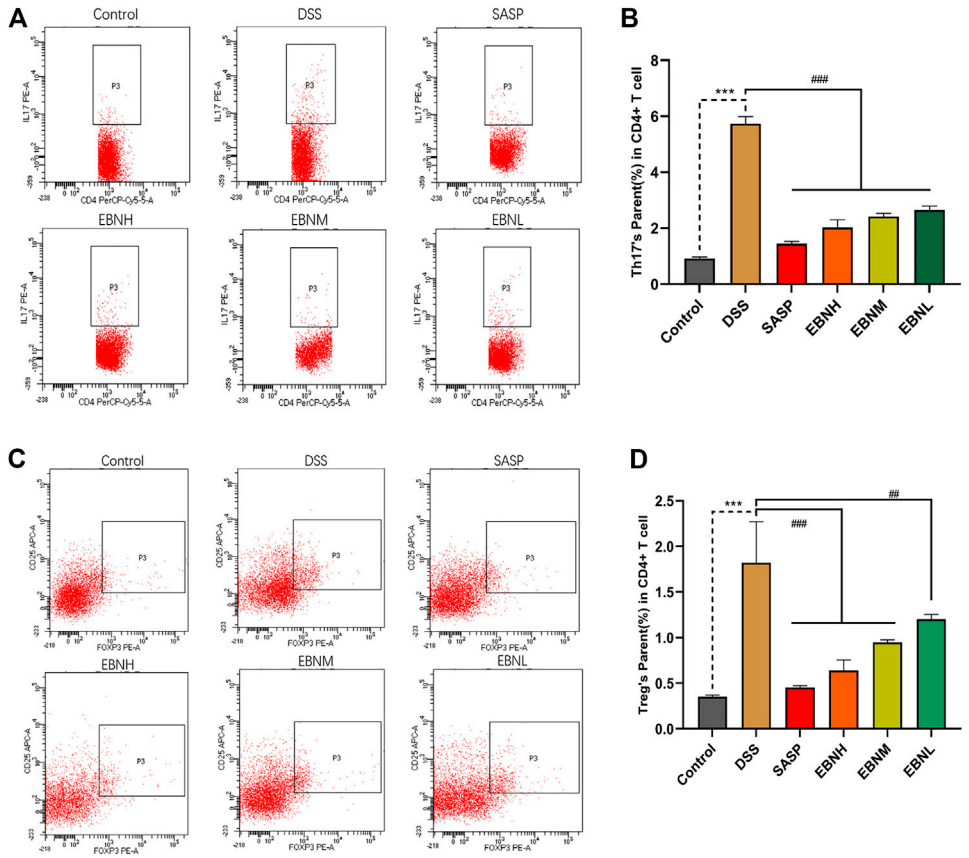

FIGURE 5 | Effects of EBN pretreatment on the proportions of Th17 cells and Treg cells in the $CD4^+$ T cell compartment of the spleen. **(A,B)** Effect of EBN on the proportion of $CD4^+IL\text{-}17A^+$ Th17 cells in the spleen of mice with colitis. **(C,D)** Effect of EBN on the proportion of $CD25^+Foxp3^+$ Treg cells in the spleen of mice with colitis. $^{***}P < 0.001$ compared with the control group; $\#P < 0.05$, $\#\#P < 0.01$ and $\#\#\#P < 0.001$ compared to the DSS group ($\bar{x} \pm S$, $n = 3$).

the model group than in the control group. However, this activity was observably reduced by treatment with EBN or SASP. On the other hand, the EBN-pretreated groups showed reductions in the expression of TNF-α and IL-6 compared with the model group (**Figures 3C,D**). These results also demonstrated that EBN inhibited the inflammation induced by DSS by reducing the levels of pro-inflammatory cytokines.

Effects of Edible Bird's Nest Pretreatment on the Expression of Th17 and Treg Cell-Associated Cytokines in Colonic Tissues

The effects of EBN on DDS-induced Th17 and Treg cell-associated cytokines were next explored. As shown in **Figure 4**, the level of TGF-β secreted by Treg cells was decreased in the colon of DSS-induced C57BL/6J mice compared to that of control group mice, while the levels of IL-6 and IL-17 were upregulated. In contrast, in comparison with those in the model group, the mice pretreated with EBN showed lower levels of IL-6 and IL-17, as well as a higher level of TGF-β. These results suggest that EBN ameliorates the inflammatory response induced by DSS by regulating the secretion of Th17 and Treg cell-associated cytokines.

Effects of Edible Bird's Nest Pretreatment on the Balance of Th17/Treg Cells

To explore the effect of EBN on the immune system in DSS-induced UC, the proportions of Th17 cells and Treg cells in the $CD4^+$ T cell compartment of the spleen were measured by flow cytometry. As shown in **Figure 5**, treatment with DSS increased the proportions of Th17 cells and Treg cells. However, the proportions of Th17 cells and Treg cells were both restored in all EBN pretreatment groups.

We further explored the effects of EBN pretreatment on the expression of Th17 and Treg cell-associated proteins in colonic tissues. Immunohistochemistry (IHC) and western blot results showed that the expression of IL-17A and Foxp3 in the model group was significantly higher than that in the control group (**Figure 6**). However, in comparison with modelling alone, EBN pretreatment dramatically inhibited the overexpression of IL-17A and Foxp3 induced by DSS (**Figure 6**). These results indicated that the counts of Th17 cells and Treg cells in the colon could be restored to a normal balance, which was consistent with the

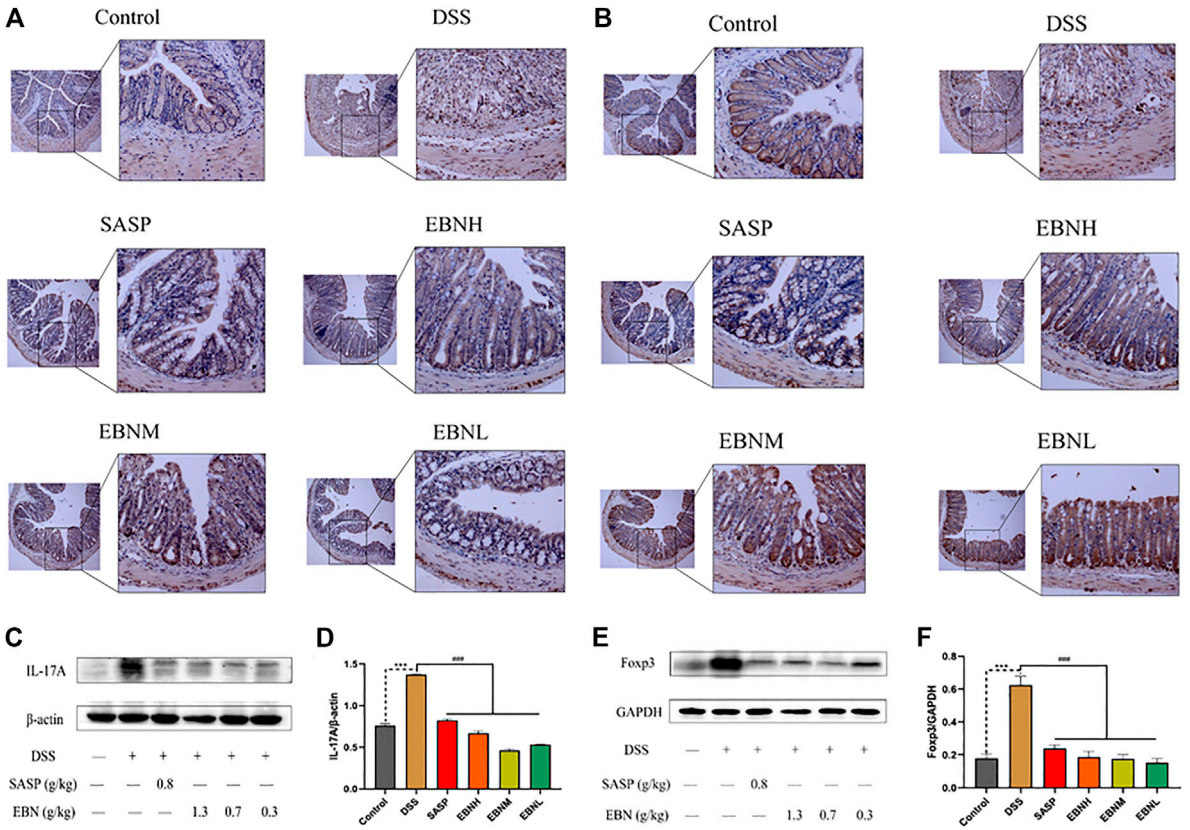

FIGURE 6 | Effects of EBN pretreatment on the expression of Th17 and Treg cell-associated proteins in colonic tissues. **(A,B)** Effects of EBN on the expression of IL-17A and Foxp3 in colonic tissues detected by IHC (100×). **(C,D)** Effect of EBN on IL-17A expression in colonic tissues of mice with colitis. **(E,F)** Effect of EBN on Foxp3 expression in colonic tissues of mice with colitis. ***$P < 0.001$ compared with the control group; #$P < 0.05$, ##$P < 0.01$ and ###$P < 0.001$ compared to the DSS group ($\bar{x} \pm S$, $n = 6$).

results for the spleen. These findings suggest that EBN can help the immune system reverse the overreaction in UC by regulating the balance of Th17/Treg cells.

DISCUSSION

EBN, a natural product with value as both a medicine and a food, has been used to prevent and treat diseases in the Chinese population since the Yuan dynasty (Fan. et al., 2020). Recent studies have found that EBN can potentiate mitogenic responses, increase the lifespan, protect neurons, promote bone mineralization, increase bone density, inhibit influenza virus, enhance the antioxidant capacity, alleviate inflammation, promote B cell proliferation and activation and reduce intestinal immune injury (Roh et al., 2012; Yew et al., 2014; Hu et al., 2016; Zhao et al., 2016; Quek et al., 2018; Hou et al., 2019). Moreover, EBN restores the immune system in the context of infection by influencing viruses through regulation of pro-inflammatory cytokines, such as IL-6 and TNF-α, and cytokines with regulatory properties, such as IL-10 and CCL2, which are also involved in the progression of UC (Haghani et al., 2016; Lee et al., 2018). Hence, we thoroughly examined the effects of EBN on DSS-induced UC and the associated mechanism in C57BL/6J mice.

Consistent with previous studies, DSS-induced C57BL/6J mice showed a series of symptoms of UC, such as weight loss, diarrhoea, haematochezia, abbreviated colon length, relatively high DAI scores and inflammatory cell infiltration in colonic tissues (**Figures 1, 3**) (Wirtz et al., 2017; Cao et al., 2018; Yan et al., 2018).

MPO is a recognized biomarker of tissue inflammation and can catalyse the production of potent reactive oxygen species (ROS). Additionally, MPO activity perpetuates inflammation and contributes to host tissue injury in patients with IBD (Chami et al., 2018). In experimental colitis, disease severity is often positively correlated with the levels of MPO activity and pro-inflammatory cytokines, such as TNF-α and IL-1β (Kim et al., 2012). Our study showed that EBN pretreatment significantly reduced MPO activity and the expression of TNF-α and IL-1β in the colon in a DSS-induced UC model. These results indicated that EBN pretreatment could resist inflammation and protect colonic tissues by reducing the infiltration of neutrophils and levels of pro-inflammatory cytokines.

Three unique components including a thick luminal mucin layer, a single epithelial cell barrier, and secretion of anti-inflammatory factors can protect the gut mucosal lamina propria from luminal resident microbiota (Ueno et al., 2018). In particular, pro-inflammatory and anti-inflammatory cytokines are crucial for the development of T cells

(Ueno et al., 2018). Moreover, the imbalance between Th17 cells and Treg cells substantially contributes to the intestinal immune disturbance and subsequent tissue injury in UC (Xu et al., 2017). Moreover, IL-6 can induce the proliferation of Th17 cells, which can secrete pro-inflammatory cytokines, such as IL-17 and TNF-α, to play pathogenic roles in intestinal inflammation (Kimura and Kishimoto, 2010; Lee et al., 2018). Our results indicated that EBN reduced the infiltration of neutrophils and levels of pro-inflammatory cytokines by inhibiting the proliferation of Th17 cells.

However, several studies have shown opposite results. A clinical study showed a relatively high level of Th17 cells and relatively low level of Treg cells in UC patients (Gong et al., 2016). In addition, relatively high levels of $CD4^+$ Treg cells and IL-10 but little TGF-β has been observed in inflamed sites in the gastric mucosa (Kindlund et al., 2017; Ma et al., 2018). Consistent with previous studies, the proportion of Treg cells in the $CD4^+$ T cell compartment of the spleen was increased by DSS, which could lead to a higher level of IL-10 to limit inflammation. Treg cell levels were increased in the spleen and colon as a negative feedback regulation mechanism of the body in response to a sharp rise in the Th17 cell proportion (Kindlund et al., 2017). Because of the immunomodulatory activity of EBN, the mice in the EBN groups with a state of mild inflammation could inhibit the development of Treg cells by reducing the negative feedback regulation in response to the low level of Th17 cells. Therefore, we inferred that the primary targets of EBN, which could ameliorate DSS-induced UC and restore the Th17/Treg cell balance, are Th17 cells.

CONCLUSION

Our results have shown for the first time that in mice with UC, EBN improves symptoms of UC, reduces colonic injury, and inhibits the increases in the levels of the pro-inflammatory cytokines IL-1β and TNF-α. The Th17/Treg cell balance was restored by decreasing the expression of IL-17A and IL-6 in intestinal tissues and decreasing the proportion of Th17 cells in each EBN dose group. Additionally, our results suggest that EBN has a protective effect on DSS-mediated UC mice, mainly by inhibiting the development and secretory function of Th17 cells. Also, the different efficacy among EBNL group, EBNM group and EBNH group were not statistically significant. Thus, we suggested that the optimal concentration of EBN to treat UC mice is 0.3 g/kg. However, pathological changes in UC include damage to the intestinal epithelium and changes in intestinal mucosal permeability and tight junction proteins. Therefore, whether EBN also protects the intestinal barrier to ameliorate UC by improving intestinal permeability and tight junction protein expression remains unclear and deserves further study. In conclusion, these results provide further evidence for the use of EBN as a treatment for the prevention of UC.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the International Institute for Translational Chinese Medicine Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

WK and GL designed the research. YF (1st author), KL, PL and FL performed the study. YF (2nd author) and YK analyzed the data. YF (1st author) and GL wrote the manuscript. SH and XL revised the manuscript. YL, XZ and YW provided technical support. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the projects of the National Natural Science Foundation of China [81173498], the Guangdong Provincial Science and Technology Planning Project [2017A050506045], Dapeng New District Industrial Development Fund Support Project [KY20180107] and Special Innovation Project of Guangdong Provincial Education Department [2018KTSCX036].

ACKNOWLEDGMENTS

Special thanks to Xiamen Yan Palace Seelong Food Co., Ltd. (Xiamen, China) for providing raw EBN.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.632602/full#supplementary-material.

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Conflict of Interest: Authors YF (2nd author), XZ and YW were employed by the company Xiamen Yan Palace Seelong Food Co., Ltd. Author YL was employed by the company Guangzhou Tongkang pharmaceutical Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
published: 07 May 2021
doi: 10.3389/fphar.2021.633292

The Anti-Viral and Anti-Inflammatory Properties of Edible Bird's Nest in Influenza and Coronavirus Infections: From Pre-Clinical to Potential Clinical Application

Kien Hui Chua[1], *Isa Naina Mohamed*[2], *Mohd Heikal Mohd Yunus*[1], *Norefrina Shafinaz Md Nor*[3], *Khidhir Kamil*[1], *Azizah Ugusman*[1] *and Jaya Kumar*[1]*

[1]Department of Physiology, Faculty of Medicine, Universiti Kebangsaan Malaysia, Cheras, Malaysia, [2]Department of Pharmacology, Faculty of Medicine, Universiti Kebangsaan Malaysia, Cheras, Malaysia, [3]Department of Biological Sciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, Bangi, Malaysia

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The Fourth Clinical Medical College of Guangzhou University of Chinese Medicine, China

***Correspondence:**
Jaya Kumar
jayakumar@ukm.edu.my

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: 25 November 2020
Accepted: 26 April 2021
Published: 07 May 2021

Citation:
Chua KH, Mohamed IN, Mohd Yunus MH, Shafinaz Md Nor N, Kamil K, Ugusman A and Kumar J (2021) The Anti-Viral and Anti-Inflammatory Properties of Edible Bird's Nest in Influenza and Coronavirus Infections: From Pre-Clinical to Potential Clinical Application. Front. Pharmacol. 12:633292. doi: 10.3389/fphar.2021.633292

Edible bird's nest (BN) is a Chinese traditional medicine with innumerable health benefits, including anti-viral, anti-inflammatory, neuroprotective, and immunomodulatory effects. A small number of studies have reported the anti-viral effects of EBN against influenza infections using *in vitro* and *in vivo* models, highlighting the importance of sialic acid and thymol derivatives in their therapeutic effects. At present, studies have reported that EBN suppresses the replicated virus from exiting the host cells, reduces the viral replication, endosomal trafficking of the virus, intracellular viral autophagy process, secretion of pro-inflammatory cytokines, reorient the actin cytoskeleton of the infected cells, and increase the lysosomal degradation of viral materials. In other models of disease, EBN attenuates oxidative stress-induced cellular apoptosis, enhances proliferation and activation of B-cells and their antibody secretion. Given the sum of its therapeutic actions, EBN appears to be a candidate that is worth further exploring for its protective effects against diseases transmitted through air droplets. At present, anti-viral drugs are employed as the first-line defense against respiratory viral infections, unless vaccines are available for the specific pathogens. In patients with severe symptoms due to exacerbated cytokine secretion, anti-inflammatory agents are applied. Treatment efficacy varies across the patients, and in times of a pandemic like COVID-19, many of the drugs are still at the experimental stage. In this review, we present a comprehensive overview of anti-viral and anti-inflammatory effects of EBN, chemical constituents from various EBN preparation techniques, and drugs currently used to treat influenza and novel coronavirus infections. We also aim to review the pathogenesis of influenza A and coronavirus, and the potential of EBN in their clinical application. We also describe the current literature in human consumption of EBN, known allergenic or contaminant presence, and the focus of future direction on how these can be addressed to further improve EBN for potential clinical application.

Keywords: edible bird's nest, anti-viral, anti-infammatory, influenza, coronavirus, COVID-19, cytokine, bird nest

INTRODUCTION

Edible bird's nest (EBN) is a nest produced from the salivary secretion of swiftlets such as *Aerodramus sp.* and *Callocalia sp.*, which are commonly found in the South-East Asian regions. To date, a myriad of studies reported various therapeutic potentials of EBN, including anti-aging (Hwang et al., 2020), anti-inflammatory (Vimala et al., 2012), anti-viral (Guo et al., 2006), immunomodulatory (Haghani et al., 2016), anti-oxidant (Yew et al., 2014), and so forth. Given its broad health benefits, EBN is a highly sought-after medicinal food in Asia (Abidin et al., 2011; Looi and Omar, 2016).

Recent reports on global mortality caused by seasonal influenza indicate up to 290,000 to 650,000 deaths associated with respiratory illnesses alone (Iuliano et al., 2018), and the Global Burden of Disease Study (GBD) attributed 99,000 to 200,000 annual deaths from lower respiratory tract infections directly to influenza (GBD 2017 Influenza Collaborators, 2019). As of October 2020, a novel coronavirus, otherwise known as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has infected more than 50 million and killed no less than one million worldwide (Worldometer, 2020).

Through antigenic shift and drift, influenza viruses learn to evade inherent immune responses (Kim et al., 2018), hence continuous reformulation of immunization and vaccine persists (Principi et al., 2019). Moreover, suboptimal vaccine-induced immune responses in vulnerable populations like the elderly and young children also present a major strain on the management of influenza infections (Sano et al., 2017). The primary drug of choice for influenza, anti-viral medications are dogged by treatment-resistant viral strains (Hayden et al., 2018; Omoto et al., 2018), expensive treatment cost (Yang, 2019), and adverse effects (Witcher et al., 2019), despite their proven benefits. Whereas for SARS-CoV-2, at present, the efficacy of vaccines or anti-viral medications is plagued by fast mutating nature of the virus.

Over the past decades, there has been a growing interest in the use of natural products for their medicinal values (Kamil et al., 2018; Hamid et al., 2020; Kamil et al., 2020; Prom-In et al., 2020). EBN, is an emerging food product with known anti-viral effects, especially against Influenza A virus (IAV) (Guo et al., 2006; Haghani et al., 2016; Haghani et al., 2017), the most common cause of the global pandemic. EBN significantly reduced viral titer, viral-induced hemagglutination (Haghani et al., 2017), virus-binding activity (Guo et al., 2006), and virus-induced endocytosis and autophagosomes (Haghani et al., 2017). In addition to anti-viral, EBN also demonstrated anti-inflammatory (Vimala et al., 2012), anti-oxidant (Yew et al., 2014), and immunomodulatory properties (Haghani et al., 2016), indicating its potential as an all-around prophylactic or therapeutic option for influenza, SARS-CoV-2 and its associated complications, such as cytokine storm. In this review, we described the pathogenesis of IAV and SARS-CoV-2 infections, current anti-viral medications in clinical practice for both infections, chemical compositions, and potential benefits of EBN as an anti-viral, anti-inflammatory, anti-oxidant, and immunomodulatory agent in the treatment of IAV and SARS-CoV-2 infections.

INFLUENZA A

The IAV is an enveloped virus with segmented negative sense, single-stranded RNA materials. The primary glycoprotein antigen on the virion surface, hemagglutinin (HA), and neuraminidase (NA) are the target of inherent immune response and many anti-viral medications (McAuley et al., 2019). To date, 18 types of H antigen, and 11 N antigens have been identified (Tong et al., 2013). Throughout history, IAV is known to cause various pandemics, such as the H1N1 virus pandemic in 1918 (Saunders-Hastings and Krewski, 2016), H2N2 in 1957, H3N2 in 1968, and another H1N1 virus pandemic in 2009 (Guan et al., 2010).

IAV, through HA on the surface of the virion, binds to the terminal sialic acid residues on mucins (Ma et al., 2017), which is released by the actions of NA cleaving off the terminal sialic acid residues (Yang et al., 2014), leading to endocytosis of IAV into the host cell past the mucosal layer. Once in, HA undergoes conformational changes to expose fusion peptide to promote viral endosomal membrane fusion, and IAV core undergoes acidification *via* protein entry through the M2 ion channels, allowing vRNPs to be released into the cytoplasm (Padilla-Quirarte et al., 2019). The IAV genome is transcribed and translated to synthesize HA, NA, M2 ion channel, matrix protein (M1), nuclear export protein (NEP), polymerases (PB1, PB2, PA), nucleoprotein (NP), PB1-F2, PA-X, and non-structural protein 1 (NS1). The synthesized viral particles attach to the host cell membrane due to the interaction between HA and sialic acids and released by the catalytic actions of NA on terminal sialic acid residues (Krammer, 2019).

The major types of sialic acid present in the terminal side of the glycans of mammalian and avian glycoproteins and glycolipids are N-acetylneuraminic acid (Neu5Ac; mostly humans) and N-glycolylneuraminic acid (Neu5Gc) (For review Long et al., 2019). HA from human-adapted viruses is known to bind to α2-6-linked sialic acid, whereas HA from avian influenza viruses binds to α2-3-linked sialic acid (Rogers and Paulson, 1983). The X-ray crystallographic and glycan microarray binding studies revealed a receptor binding site of HA from human-adapted viruses contain a bulkier cis conformation adopted by α2-6-linked sialic acid, compared to the HA of avian influenza viruses with thin and straight trans conformation by the α2-3-linked sialic acid (Shi et al., 2014; Lipsitch et al., 2016). Studies also have reported both α2-3 and α2-6 sialic acid linkages in the human lung and bronchus (Walther et al., 2013), α2-6 linkages in the respiratory tracts of ferrets and pigs (Nelli et al., 2010; Jia et al., 2014), and higher expression of α2-3 sialic acid linkages in non-human primates and mice (Gagneux et al., 2003; Ning et al., 2009). Other features of glycans also determine the interaction between virus and host, such as the presence of other sugar moieties or functional groups, length of sialic acid presenting glycans (Long et al., 2019), and second binding site in addition to a usual catalytic sialic acid binding site of NA, such as the hemadsorption (Hd) site (Uhlendorff et al., 2009). More recent findings suggest the binding to the secondary site may occur prior to the binding to the primary site where the enzymatic cleavage occurs (Durrant et al., 2020).

TABLE 1 | Anti-Viral medications for the treatment of Influenza A virus.

Chemical name	Trade name	Mechanism of action	Infectious agent	Resistance reported	Side effects	Efficacy
Baloxavir marboxil	Xofluza	Selective inhibition of cap-dependent endonuclease activity of the influenza virus polymerase acidic (PA) protein Heo (2018); Takashita et al. (2019); Noshi et al. (2018); Omoto et al. (2018)	Influenza A, B	I38X amino acid substitutions in the influenza PA protein Hayden et al. (2018); Gubareva et al. (2019); Takashita et al. (2019).	The most common; diarrhea. Other reported; bronchitis, nausea, sinusitis, increase in AST and ALT, and headache Hayden et al. (2018); Ison et al. (2020); Shionogi and Co. Ltd (2018).	Oral capsule. Influenza A or B in otherwise healthy adults and children (aged ≥12 years). Influenza A or B in adults and children (aged ≥12 years) with high risk of influenza complications. Hayden et al. (2018); Ison et al. (2020).
Oseltamivir	Tamiflu	Specific inhibitors of enzyme neuraminidase, which promotes virus spread in the respiratory tract and virus release from the infected cells Gubareva et al. (2000); Ohuchi et al. (2006); Hama (2016).	Influenza A, B	i) Mutations within or close to the HA receptor-binding site gene reduces successful virus budding to cellular receptors, which reduces dependence on NA function Gubareva et al. (2000). ii) Substitutions of amino acid at the conserved residues in the active site of NA enzyme -mutation H274Y, R292K and N294S Gubareva et al. (2000); Wang et al. (2002).	Diarrhea, bronchitis, nausea and sinusitis Hayden et al. (2018); Ison et al. (2020); Gubareva et al. (2000); Hama (2016), insomnia, vertigo Moscona (2005); Hama (2016).	Oral capsule and suspension, Influenza A or B in uncomplicated acute illness patients aged >1 year Ward et al. (2005); Samson et al. (2013).
Zanamivir	Relenza	Specific inhibitors enzyme neuraminidase. Gubareva et al. (2000); Ohuchi et al. (2006); Hama (2016).	Influenza A, B	D151 A/E/G/V mutations in inf A viruses Sheu et al. (2008). E119G/D/A substitutions in influenza B viruses Jackson et al. (2005).	Headache, dizziness nausea, diarrhea vomiting sinusitis, bronchitis, cough Moscona (2005); Samson et al. (2013).	Oral inhalation. Influenza A or B treatment, approved for patients older than 7 years Samson et al. (2013).
Peramivir	Rapivab	Specific inhibitors enzyme neuraminidase. Gubareva et al. (2000); Ohuchi et al. (2006); Hama (2016).	Influenza A, B	H274Y mutation in influenza A viruses, Renaud et al. (2010); Scott (2018). V94I and R152K: mutation in influenza B viruses, from a pediatric patient Yoshida et al. (2011).	Diarrhea (mild to moderate), nausea, and vomiting, cough, and pyrexia Komeda et al. (2015); Scott (2018).	Parenteral. Influenza A or B treatment, approved for patients of all ages Samson et al. (2013). Uncomplicated influenza in children from the age of 2 years and adults Scott (2018).
Favipravir	Avigan	Inhibits RNA polymerase activity by preventing RNA elongation Furuta et al. (2013)	Influenza A, B	V43I in PB1 in influenza A viruses Cheung et al. (2014). K229R in PB1 subunit of influenza A virus Goldhill et al. (2018).		

ANTI-VIRAL MEDICATIONS AGAINST IAV

Vaccination is the primary mode of prevention against influenza. Though, most of the vaccines are not 100% effective as the influenza viruses are constantly evolving (Hurt, 2014). Hence, anti-viral medications are in continuous development given their importance in the management of influenza infections, particularly during the initial phases of a pandemic when vaccines are still in the making. **Table 1** shows a comprehensive overview of various anti-virals used to treat IAV infection.

The anti-viral baloxavir marboxil (XofluzaTM) is a cap-dependent endonuclease inhibitor that was developed to treat IAV or B infection (Shionogi and Co. Ltd, 2018). Following Japan in 2018, baloxavir is approved in many other countries, including the United States in 2019 (Takashita et al., 2019). The mechanism of action of baloxavir is dissimilar to neuraminidase inhibitors, where it suppresses the proliferation of virus through inhibition of mRNA synthesis initiation (Koszalka et al., 2017; Noshi et al., 2018). Administration of a single dose of baloxavir within the 48 h of symptom onset rapidly improved the symptoms, with fewer influenza-related complications. Moreover, a single dose of baloxavir also reduced viral replication in high-risk adult and adolescent outpatients with uncomplicated influenza (Portsmouth et al., 2017; Ison et al., 2020). Children older than 12 years of age and adults suffering from IAV or B generally well-tolerated a single dose of baloxavir (Heo, 2018). Otherwise, the most commonly associated adverse effects with baloxavir are diarrhea, nausea, bronchitis, and sinusitis (incidence of ≤3%) (Shirley, 2020), and increase in AST, ALT, and headache (incidence <1%) (Shionogi and Co. Ltd, 2018).

Oseltamivir (TamifluTM) is a specific inhibitor of IAV that has proven clinically effective in adults and children (as young as 1 year) for the chemoprophylaxis and treatment of IAV and B

infections (Ward et al., 2005). Oseltamivir is available in oral form (75 mg twice daily used by a large population of patients) (McNicholl and McNicholl, 2001) and intravenously for those unable to tolerate oral dosing (Kamali and Holodniy, 2013). Oral administration of Oseltamivir (75 mg twice daily) significantly reduced the severity and duration of symptoms (McNicholl and McNicholl, 2001). Oseltamivir is relatively well tolerated, with the most commonly associated side effects such as abdominal pain, vomiting, and transient nausea in 5–10 percent of patients studied (Moscona, 2005). Others have also reported Oseltamivir-related serious adverse effects, such as sudden deaths and accidental deaths due to abnormal behaviors (Hama, 2016). Resistance to the drug has also been reported in the past, among patients who have been exposed to the drug previously, and also without any prior exposure (Kamali and Holodniy, 2013).

Zanamivir (Relenza™) is the first neuraminidase inhibitor to be developed, with a high affinity for the neuraminidase binding site (McKimm-Breschkin, 2013). Zanamivir has poor oral bioavailability, hence administered with an inhaler device (Diskhaler) (Diggory et al., 2001). Therefore, the inspiration flow determines the amount of drugs reaching the respiratory tract, which is a limitation, especially for intubated patients (Ison, 2010). For patients older than seven years of age, Zanamivir (10 mg) is inhaled twice daily for 5 days, whereas prophylaxis is given to patients older than 5 years of age once daily for 10–28 days (Samson et al., 2013). Zanamivir reduces the period of symptom alleviation by 10–24% (0.5–1.25 days) (McNicholl and McNicholl, 2001).

Peramivir (Rapivab®) is a novel cyclopentane neuraminidase inhibitor with potent and selective inhibitory action against IAV and B virus' NA, with similar or more potent *in vitro* inhibitory effects against IAV and B, than zanamivir or oseltamivir (Fage et al., 2017). In 2014, the Food and Drug Administration approved the use of peramivir for the treatment of acute uncomplicated influenza in patients 18 years and older (Alame et al., 2016). Due to its poor oral bioavailability, peramivir is offered only as an intravenous formulation, and its inhibitory activity against influenza is slightly lower than oseltamivir and zanamivir (Fage et al., 2017). Pediatric and adult patients with uncomplicated influenza generally well tolerate a single dose of peramivir. The common adverse reactions reported are nausea (2.4% of patients) and reduced neutrophil counts (3.2%) (BioCryst, 2018). In children (frequency 1 to <10%), the most frequently associated adverse reactions are rashes at injection sites, tympanic membrane hyperemia, pyrexia, pruritus, and psychomotor hyperactivity (BioCryst, 2018).

SEVERE ACUTE RESPIRATORY SYNDROME-RELATED CORONAVIRUS

Coronavirus Disease 2019 (COVID-19) caused by the severe acute respiratory syndrome coronavirus (SARS-CoV-2) has infected more than 50 million, and killed at least 1.2 million worldwide, as of November 2020 (Worldometer, 2020). The Timeline Outbreak by SARs-COV-2 viruses began when severe cases of pneumonia were reported in Wuhan City of Hubei Province in China. Investigations into etiological agents led to the Hunan seafood wet market in Wuhan, China where the sample tested from this wet market was found to be positive for SARS-CoV-2, which was initially known as 2019-nCoV before being renamed as SARS-CoV-2 (Ralph et al., 2020; Wu et al., 2020).

Coronavirus is made of the Membrane (M), Envelope (E), Nucleocapsid (N), and spike protein (S), and two large polyproteins (Yoshimoto, 2020). Among them, the ones that enabled the coronavirus to infect and replicate in humans are S and N proteins. The S protein of SARS-CoV-2 has a receptor-binding domain (RBD) similar to SARS-CoV, which binds to the same receptor Angiotensin-converting Enzyme 2 (ACE2) in humans, but with 10-20-fold higher affinity (Wrapp et al., 2020; Zhang and Holmes, 2020), though MERS-CoV expresses a different receptor, dipeptidyl peptidase 4 or DPP4 (Wang et al., 2013). The binding of the S protein to ACE2 enables the entry of SARS-CoVs into the human cell (Letko et al., 2020), whereas N protein is vital for replication and assembly (V'kovski et al., 2020).

ANTI-VIRAL AGENTS AGAINST SARS-COV-2

Remdesivir (RDV) (sold under the trade name Veklury) gained prominence as it is the first anti-viral drug to be approved by the US FDA to be used as a treatment option for SARS-CoV-2. Initially, RDV was developed as a general anti-viral drug for hepatitis C and Ebola, but results were not encouraging. RDV acts by inhibiting the coronavirus's RNA synthesis through delayed chain termination. RDV was touted as a potential anti-viral therapy option for SARS-CoV-2 early in the pandemic. Trials were quickly initiated. Intravenous RDV was found to have been numerically better but not statistically significant in mortality, clinical improvement, and time taken to clear off the virus (Wang X. et al., 2020). Grein et al. in another trial reported that 68% (36 of 53 patients) of patients showed improvement with regards to oxygen support on day 18, and 84% had significant clinical improvement by day 28 in severe Covid-19 cases (Grein et al., 2020). In both trials, adverse effects were mostly well tolerated with common adverse events reported as constipation, increased total bilirubin, anemia, diarrhea, rash, and hepatic enzymes. Severe adverse events include septic shock, acute kidney injury, and multiple organ dysfunction.

The anti-viral favipiravir (FPV) (sold under the trade name Avigan and Fabiflu) is developed by Toyama Chemical (Japan), has been sold in Japan for the treatment of influenza since 2014. It was one of the early candidates recognized for the pharmacotherapy of SARS-CoV-2 and was used in Wuhan as a treatment option. Since then, a few trials have been initiated in various countries with varying degrees of success. FPV competitively inhibits RNA-dependent RNA polymerase in the virus (Furuta et al., 2013). FPV profoundly improved the latency to relief for cough and pyrexia in patients with COVID-19 (Chen C. et al., 2020). FPV also improved clinical recovery in Day 7 in moderately severe COVID-19 patients as well as decreased auxiliary oxygen therapy and decreased the incidence of

TABLE 2 | Antiviral medications for the treatment of SARS-CoV-2.

Chemical name	Trade name	Mechanism of action	Infectious agent	Resistance reported	Adverse effects	Efficacy
Rem-desivir	Veklury	Inhibits the RNA synthesis by virus through delayed chain termination for all three coronaviruses' RdRp.	SARS-CoV-2	In a review, the authors concluded that there is a high genetic barrier for remdesivir to develop resistance as well as decreased fitness and pathogenicity in remdesivir-resistant mutants, which further encourage the therapeutic potential of remdesivir in the treatment of newly emerging COVID-19 Ko et al. (2020).	The most commonly reported were hypoalbuminemia, anemia, thrombocytopenia, constipation, hypokalemia, and elevated total bilirubin Wang X. et al. (2020).	Intravenous RDV had a numerically better improvement, but did not profoundly reduce the time taken for clinical improvement, mortality, or time for virus clearance in serious COVID-19 cases in comparison to placebo Wang Y. et al. (2020).
					Increase in hepatic enzymes, rash, renal impairment, hypotension, diarrhea. *Generally, AE were more commonly associated in patients receiving invasive ventilation Grein et al. (2020).	Over a median follow-up of 18 days after the first dose of RDV, 36 of 53 patients (68%) showed an improvement in the category of oxygen support, whereas 8 of 53 patients (15%) worsened Grein et al. (2020).
					The most common SAE were septic shock, acute kidney injury, hypotension, and multiple-organ-dysfunction syndrome Grein et al. (2020).	By 28 days of follow-up (RDV group), there were 84% cumulative incidence of clinical improvements in severe cases of Covid-19 Grein et al. (2020).
Favi-piravir	Avigan	Competitively inhibits the RNA-dependent RNA polymerase Furuta et al. (2013).	SARS-CoV-2	None	The most frequently observed FPV-associated AE was raised serum uric acid Chen C. et al. (2020).	Favipiravir profoundly attenuated the latency to relief for pyrexia and cough in COVID-19 patients. Based on post-hoc analysis, Favipiravir effectively improved clinical recovery rate at Day 7 in moderate COVID-19 patients compared to Arbidol. Similar effect was diminished in severe/critical COVID-19 cases.
					The rate of adverse events in patients receiving favipiravir was significantly lower (11.4% vs. control 55.6%; $p < 0.01$) Khamis et al. (2021).	Based on a post-hoc analysis, in moderate COVID-19 patients, Favipiravir was associated with: -Reduced noninvasive mechanical ventilation rate or auxiliary oxygen therapy with marginal significance ($p = 0.0541$). - Profoundly reduced *de novo* incidences of dyspnea Chen L. et al. (2020). In China, an open-label non-randomized trial of COVID-19 80 patients reported a profound decrease in the time to SARS-CoV-2 viral clearance in patients treated with favipiravir compared with historical controls treated with lopinavir/ritonavir The FPV arm also showed profound improvement in chest imaging compared with the control arm, with an improvement rate of 91.43 vs. 62.22% ($p = 0.004$) Khamis et al. (2021).
Lopinavir	Kaletra -Fixed-dose combination with ritonavir (LPV/r) - Ritonavir helps to stabilize lopinavir	Inhibits the protease activity of coronavirus.	SARS-CoV-2	None	Gastrointestinal adverse events were more common in the lopinavir–ritonavir group Cao et al. (2020).	One study showed the positive effects of LPV/r therapy: Four COVID-19 patients were given antiviral treatment including LPV/r. Following treatment, three of them showed marked improvement in pneumonia-associated symptoms. Two patients were confirmed to be COVID-19 negative and discharged, and

(Continued on following page)

TABLE 2 | (*Continued*) Antiviral medications for the treatment of SARS-CoV-2.

Chemical name	Trade name	Mechanism of action	Infectious agent	Resistance reported	Adverse effects	Efficacy
						one of whom was negative for the virus at the first test Wang Z. et al. (2020). A combination treatment with adjuvant drugs and LPV/r has better therapeutic effects in reducing the body temperature and restoring normal physiological mechanisms with no evident toxic and side effects Ye XT. et al. (2020). The efficacy of lopinavir/ritonavir treatment was reported from a single case report from the index patient treated in Korea, whose viral titers diminished following treatment Lim et al. (2020).
	*Combination of (LPV/r) has been proposed for the treatment of coronaviruses because of its potential effect on viral replication at the cellular level.					An *in vitro* study, using Vero cells: The severity of cytopathic effects was lesser in lopinavir/ritonavir-treated cells, and viral load was profoundly decreased in this group compared to the control ($p < 0.001$). Lopinavir/ritonavir significantly inhibited SARS-CoV-2 *in vitro* at its usual plasma concentration Kang et al. (2020). Lopinavir–ritonavir co-administration did not profoundly accelerate clinical improvement, decrease mortality, or reduce throat viral RNA detectability in serious Covid-19 cases. Lopinavir-ritonavir treatment recorded lower 28-days mortality (19 vs. 25%), however without any significant between-group differences Cao et al. (2020). A retrospective study enrolling 134 novel coronavirus pneumonia (NCP) patients reported no significant difference in improvement of symptoms or reduction in viral loads between control ($n = 48$), LPV/r-treated group ($n = 52$), and Abidol-treated group ($n = 34$) Chen L. et al. (2020). *(article in Chinese language)
Ritonavir	Norvir	Inhibit protease Inhibits the metabolizing enzyme CYP450 3A and therefore increases the half-life of lopinavir (other antiretroviral drugs which it is co-administered with) Chandwani and Shuter (2008).	SARS-CoV-2	None. Resistance reported for HIV: The valine at position 82 (Val 82) in the active site of the human immunodeficiency virus (HIV) protease mutates in response to therapy with the protease inhibitor ritonavir.	Same as the lopinavir	Same as the lopinavir

dyspnea (Chen L. et al., 2020). Another trial by Cai et al. (2020) reported a significant decrease in the time SARS-CoV-2 viral clearance and improvement in chest imaging in comparison to ritonavir or lopinavir (Cai et al., 2020). FPV is a well-tolerated drug with the most commonly reported adverse event of elevated serum level of uric acid and had a better safety profile compared to lopinavir or ritonavir (Cai et al., 2020; Chen C. et al., 2020).

Ritonavir (LPV/r) and Lopinavir is a fixed-dose anti-viral combination used for the pharmacotherapy and prevention of HIV/AIDS. LPV/r acts by inhibiting the protease activity of viruses. LPV/r was also touted early in the epidemic as an anti-viral treatment for SARS-CoV-2. A preclinical *in vitro* study by Kang et al. reported lower viral load in LPV/r treated group and concluded profound inhibition of SARS-CoV-2 at plasma concentration (Kang et al., 2020). Many trials have since concluded on its efficacy. Wang et al. reported significant alleviation of pneumonia-associated symptoms (Wang Y. et al., 2020). Cao et al. reported overall lower 28-days mortality (19 vs 25%) on patients receiving LPV/r but it was not statistically significant (Cao et al., 2020). Similarly, Chen C. et al., 2020, in a retrospective study reported no profound difference between LPV/r treated and control groups in symptom improvement or reduction in viral loads (Chen L. et al., 2020). Most studies reported that LPV/r anti-viral is a well-tolerated drug with gastrointestinal adverse effects as the most commonly reported adverse effects. **Table 2** shows a comprehensive overview of various anti-viral medications for SARS-CoV-2.

ANTI-VIRAL EFFECTS OF EBN

The anti-viral properties of EBN were tested using *in vitro* and *in vivo* models and compared across the efficacy of standard anti-viral medications such as oseltamivir and amantadine (Haghani et al., 2017) (**Table 3**). In anti-viral cell culture studies, EBN did not produce any cytotoxic effects up to 50 mg/ml (Nuradji et al., 2018), whereas some reported CC50 of EBN extracts at a lower concentration range 27.2–32 mg/ml (Haghani et al., 2017). For anti-viral *in vivo* studies, concentration in the range of 5–2000 mg/kg was tested in 8–10 weeks old female mice *via* oral gavage and was shown to be free of toxicity and mortality at the end of 14 days of the treatment period (Haghani et al., 2016). The EBN concentration range of 12.5 mg/ml (Haghani et al., 2017), 0.5–4,000 μg/ml (Guo et al., 2006), and 15–35 mg/ml (Nuradji et al., 2018) was employed in most anti-viral investigations against IAV strains of H1N1, H3N2, and H5N1, respectively.

Most of the *in vitro* anti-viral studies of EBN used the water extraction method to prepare the EBN test sample. Usually, EBN is dried at a temperature higher than ambient for few hours to one day before grinding and filtering EBN with wire mesh to separate the feather and impurities. The ground EBN was then soaked in water or water-based buffer before heated to release the water-soluble parts of the EBN. Pancreatin enzyme was commonly used to simulate the gastrointestinal digestion process to break down the larger protein components of EBN. For *in vivo* model, EBN extract without the pancreatin enzyme is preferred in the experiment since the animal gut could do the job (Haghani et al., 2016). As the anti-viral effect of the EBN depends on the amount, concentration, and bioavailability of the functional groups; the location of EBN harvest, seasoning effect, and extraction methods can affect the study findings. **Table 4** shows an overview of the analysis of EBN composition in various studies.

For its anti-viral effect, EBN water extracts significantly reduced hemagglutination activity of IAV (H1N1, H3N2, and H5N1) in a dose-dependent manner (Guo et al., 2006; Haghani et al., 2017; Nuradji et al., 2018), with increasing efficacy when the treatment duration is prolonged (Haghani et al., 2017). The efficacy of EBN with enzymatic treatment (pancreatin F), and without enzymatic treatment was the focus of many *in vitro* investigations. EBN without enzymatic treatment reduced extracellular NA copy number, NS1 expression, and at the same time also increased intracellular NS1 expression. EBN with enzymatic treatment increased intracellular NA copy number, increased extracellular NS1 expression, and increased intracellular M2 expression, suggesting the effects of EBN with enzymatic treatment (Pancreatic F) are more inclined to inhibiting the release of the viral materials (Haghani et al., 2016). EBN (from house nest) with and without enzymatic treatment reduced virus titer, with a percentage of protection 42.47 ± 8 and 45.42 ± 8.4, respectively (Haghani et al., 2017). In line with these findings, Guo et al. (2006) reported significant inhibitory effects of EBN with pancreatic enzyme digestion on hemagglutination activity of IAV, whereas little effects when treated with EBN without the pancreatic enzymes. The findings indicate that EBN extracts with smaller proteins (10–25 kDa) when treated with the pancreatic enzyme produced stronger inhibitory effects compared to the larger proteins (more than 50 kDa) expressed in the EBN without enzymatic treatment. The same researchers also reported that EBN extracts (4 mg/ml) did not inhibit the neuraminidase activity of influenza, and therefore, the EBN extracts act on viral hemagglutinin but not viral neuraminidase (Guo et al., 2006). In addition to reducing viral load, EBN also normalized the cellular shapes and reoriented the actin filaments, and reduced the densities of actin filaments caused by the IAV infection. EBN also increased the density and the number of lysosomes in both infected and non-infected cells (Haghani et al., 2017) (**Figure 1**).

Treatment with another enzyme, neuraminidase significantly affected the inhibitory effects of EBN (Guo et al., 2006), suggesting the important role of glycosidic linkages in the anti-viral properties of EBN. Lectin blotting assay revealed Neu5Ac2-3Gal linkage as the major molecular species of sialic acid in the EBN extract along with O-acetylated Neu5Ac (Guo et al., 2006). Based on the location of collection (house vs cave), EBN extracts differ chemically, where EBN collected from the cave (Gua Madai) was reported to contain Neu2, 4,7,8,9 Ac6, which has more O-acetylated branches (Haghani et al., 2016). O-acetylation of N-acetyl residues confers higher resistance of sialic acids to the actions of sialidases from various infectious agents (Corfield et al., 1981; Haverkamp et al., 1982). EBN from Gua Madai recorded the highest percentage of protection against

TABLE 3 | Antiviral effects of EBN.

References	Cell Lines and Virus strain	Origin of EBN and dosage	Experimental design	Analysis	Summary of findings
Guo et al. (2006)	A/Shizuoka/450/05 (H3N2) and A/Shizuoka/451/05 (H3N2) strains.	Natural cave EBN.	Sample 1 (S1) was collected from a bird's nest from a natural cave.	Hemagglutination inhibition (HAI) assay.	EBN extracts treated with pancreatic enzyme (both S1 and S2) had significant HAI activities. S1 EBN extract inhibited strain A/Aichi/2/68 stronger than S2 and treatment with neuraminidase reduced their effects.
	Madin-Darby canine kidney (MDCK) cells and Human lung carcinoma	House-cultured EBN.	Sample 2 (S2) was collected from house-cultured bird's nest.	Sialidase inhibition assay.	Virus binding activity of S1 EBN was greater than S2, and treatment with neuraminidase markedly reduced binding activity of EBN extract S1.
	A549 cells.			Neutralization assay through lactate dehydrogenase (LDH) level.	EBN extract had no effects on the neuraminidase activity of influenza viruses, and remained side-effects free in all experiments.
				Western blot (detected the binding activity of the viruses to the glycoprotein from the EBN extracts against anti-H3N2).	Human influenza virus strain A/Aichi/2/68 (H3N2) bound to sialyl 2–3 and 2–6 galactose linkages in salylglycoproteins and sialylglycolipids.
				Fluorometric HPLC analysis for sialic acid and lectin blotting method to determine sialic acid linkages.	Neu5Ac2-3Gal linkage was the major molecular species of sialic acid in the EBN extract.
Haghani et al., (2016)	*In vitro*	House nest from teluk Intan,	1) House nest EBN with no enzymatic treatment.	*In vitro*	*In vitro*
	Influenza A virus, strain A/PR/8/34 (H1N1).	Perak, Malaysia.	2) House nest EBN with Pancreatin F-treatment.	RNA changes of viral genes (NS1, NA, NP, and M2)	Antiviral effects:
	Madin Darby canine kidney (MDCK) cell line (viral propagation).	Cave nest from Gua Madai caves, Lahad Datu, Sabah, Malaysia.	3) Cave nest EBN with no enzymatic treatment.	Cytokines (TNFα, IL6, IFNβ, IL27, and CCL2)	EBN was found to reduce the copy number of intracellular NA gene and extracellular NS1 gene. However, EBN increased M2 gene and had no effect on NP gene.
	In vivo	*In vivo*	4) Cave nest EBN with neuraminidase-treatment.	NFκB activation protein (NFAP)	Immunomodulatory effects:
	A/Peurto Rico/8/1934 (H1N1).	100 mg/kg (1 ml/100g body weight using oral gavage).	*In vitro*	*In vivo*	EBN can regulate the excessive innate inflammatory reaction by significantly reducing CCL2, IL-6, and IFN-β expression while significantly increasing the IL-27 and TNF-α expression.
			Infected MDCK cell lines were treated with EBNs and commercial antiviral drugs (Oseltamivir phosphate and Amantadine hydrochloride) for 48 h.	Viral load in lungs	EBN profoundly increased NFκB activation protein (NFAP).
			In vivo Mice were challenged with intranasal 10^5 TCID50 of A/Peurto Rico/8/1934 (H1N1). The mice were treated with EBN or oseltamivir phosphate (OSE) 4 h before viral challenge.	Blood cytokine levels (TNFα, IFNγ, IL1β, IL2, IL4, IL6, IL10, IL12 (p70), and IL15).	*In vivo* Pretreatment of EBN and treatment of OSE both reduced NA copy number in lung. EBN significantly decrease IFN-γ and increased IL-4, IL6, IL-12, and TNF-α by day 3. EBN reduced IL-10, and IL-12 by day 7.
				MTT assay for EBN cytotoxicity.	

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TABLE 3 | (*Continued*) Antiviral effects of EBN.

References	Cell Lines and Virus strain	Origin of EBN and dosage	Experimental design	Analysis	Summary of findings
Haghani et al. (2017)	Infleunza A virus strain A/ Puerto Rico/8/1934 (H1N1).	House nest EBN from teluk Intan, Perak, Malaysia.	1) House nest EBN with no enzymatic treatment.		EBN CC50 ranged from 27.5 to 32 mg/ml, while IC50 ranged 2.5–4.9 mg/ml, amantadine: CC50: 4,523.4 mg/ml, oseltamivir: CC50: 14,373.2 mg/ml.
	Madin Darby Canine Kidney (MDCK) cell line.	Cave nest EBN from Gua Madai caves, Lahad Datu, Sabah, Malaysia.	2) House nest EBN with Pancreatin F-treatment.	Hemagglutination assay.	EVB decreased the influenza virus hemagglutination activity in a dose dependent manner.
			3) Cave nest EBN with no enzymatic treatment.	Endosomal trafficking through immunoblotting of Rab5, RhoA and LC3 translocation and immunofluorescent of actin cytoskeleton and lysosomal bodies.	EBNs could significantly ($p < 0.05$) decreased the expression of Rab5 protein, in par with oseltamivir. Amantadine had no effect on Rab5 protein in infected and normal cells.
			4) Cave nest EBN with neuraminidase-treatment.		EBNs markedly ($p < 0.05$) reduced the RhoA as similar to Amantadine and oseltamivir. EBNs failed to reduce LC3 protein.
			Infected MDCK cell lines were treated with EBNs to measure EBN cytotoxicity and its effects on autophagy process and endosomal trafficking of influenza virus.		EBNs normalized the cellular shapes and reoriented the actin filaments, and reduced densities of actin filaments. At the same time, EBN increased the density and the number of lysosomes in both infected and non-infected cells.
Nuradji et al. (2018)	H5N1 HPAI virus clade 3.2.3 A/DK/BNY/F.2014P1 in chicken embryonic eggs.	Thirteen samples of EBN extract from 12 swiftlet houses from Indonesia were used.	Thirteen samples of EBN extract were used.	Cytotoxic effects of EBN extract:	Up to 50 mg/ml of EBN did not generate cytotoxicity in cell culture. CC50 of EBN extract was detected at more than 50 mg/ml. EBN concentrations begin to show HI activity at 12 μg/ml.
	Vero cell (African Green Monkey Kidney Cells).		Monolayer Vero cells was exposed with different concentrations of EBN extract for 96 h.	Cell shrinkage, blebbing membranes, ballooning cell, chromatin condensation, and cytoplasmic vacuolation. Hemagglutination-inhibition test. Viral titer.	EBN with low concentration (25 mg/ml) showed a high HI titer compared with EBN of higher concentration (35 mg/ml).

TABLE 4 | Analysis of EBN composition.

References	Source of EBN	Preparation of EBN	Methods of analysis	Results
Guo et al. (2006)	Indonesia,	**Grinding**	Fluorometric determination of 5-N-acetyl-neuraminic acid (Neu5Ac) and 5-N-glycolylneuraminic acid (Neu5Gc) through high-performance liquid chromatography (HPLC) method.	S1 has Neu5Ac as the major molecular species of sialic acid.
	Sample 1 (S1)- natural cave	Nests were dried at 70°C for 16 h, ground and sifted to remove the foreign substances and feathers.		S2 has O-acetyl sialic acid species.
	Sample 2 (S2)–house -cultured	**Heating** Nests were placed in 5°C distilled water for 16 h and heated at 60°C for 30–60 min. **Enzymatic treatment** 1) Samples were exposed to pancreatin F (final concentration of 0.5 mg/ml) at 45°C for 4 h at pH 8.5–9.0, and then heated at 90°C for 5 min for enzyme inactivation. 2) Samples were exposed to neuraminidase (final concentration of 0.5 mg/ml) at 45°C for 2 h at pH 8.5–9.0 and then heated at 90°C for 5 min for enzyme inactivation. **Filtration and Storage** The extracts were filtered and freeze-dried at -80 °C after 48 h, and then stored at -80°C for further usage.		The sialyl-glycoconjugates of S1 have Neu5Ac2-3Gal linkages and S2 have an O-acetylated Neu5Ac. The sialyl glycoconjugates with Neu5Ac2-3Gal linkages could inhibit influenza virus infection.
Yagi et al. (2008)	Indonesia	The EBN extract were prepared based on Guo et al. (2006).	HPLC mapping method, MALDI-MS/MS sequencing, gas chromatography– electron impact methylation analysis.	The findings of tri-antennary N-glycan as a major component suggested that the sialylated N-glycans of EBN may contribute to the antiviral effects of EBN.
Halimi et al. (2014)	EBN from Pahang and terengganu by Nest Excel	**Storage**	Nutritional composition of moisture, protein, carbohydrate, ash and fat content.	Protein was the major components in EBN samples (58.55%, Pahang; 55.48%, terengganu) followed by carbohydrate, moisture, ash and fat.
		EBN samples were stored in air-tight container at room temperature.	Amino acid composition	Glutamic acid (9.61%), aspartic acid (6.34%), lysine (5.44%) and leucine (5.30%) were the major amino acids found in the EBN samples.
			Total Solubility	The total solubility of EBN increased with boiling time; 4 h gave the most soluble form.
Hou et al. (2015a)	Sabah and Sarawak, Malaysia	**Grinding** Nests were dried in an oven at 50 °C for three days and finely ground. **Ultrasonication and Centrifugation** Samples were dissolved into 1000 ml of dd H2O, and sonicated in an ice followed by centrifugation. **Filtration and Storage** The supernatants were desalted and condensed in a dialysis bag with a 3500-cut-off molecular weight. The dialyzed protein was stored at –20°C until use.	ELISA for lactoferrin (LF) and ovotransferrin (OVF).	The concentrations of LF and OVF -House white EBN (Sabah): LF: 4.68 ± 0.57 μg/mg, OVF: 10.23 ± 0.72 μg/mg. Cave white EBN (Sabah): LF: 4.27 ± 0.49 μg/m, OVF: 10.63 ± 0.90 μg/mg. Cave black EBN (Sabah): LF: 2.80 ± 0.21 μg/mg; OVF: 1.31 ± 0.07 μg/mg; Cave red EBN (Sabah): LF: 3.43 ± 0.07 μg/mg; OVF: 3.13 ± 0.58 μg/mg; Cave red EBN (Sarawak): LF: 3.13 ± 0.07 μg/mg; OVF: 8.40 ± 0.56 μg/mg.
Haghani et al. (2016)	Teluk Intan, Perak, Malaysia.	The EBN extract were prepared based on Guo et al. (2006).	H-NMR spectra for flavonoid metabolites and sialic acid composition.	EBN from teluk Intan and Gua Madai have different derivatives of N-glucoloylneuraminic acid and N-acetyl-neuraminic groups.

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TABLE 4 | (*Continued*) Analysis of EBN composition.

References	Source of EBN	Preparation of EBN	Methods of analysis	Results
	Gua Madai caves, Lahad Datu, Sabah, Malaysia.			Both EBNs have four sialic acid derivatives; N- glycol neuraminic acid, N-acetyl neuraminic acid, 5-N-acetyl-8, 9-di-O-acetylneuraminic acid and 5-Nacetyl-9-O-acetylneuraminic acid. EBN from Gua Madai consisted of Neu2,4,7,8,9 Ac6 (more O-acetylated branches). Only EBN from Gua Madai contained thymol-β-D glucopyranoside indicating antiviral activity against Herpes Simplex Virus and antibacterial property.
Zhao et al. (2016)	Nha trang, Khanh Hoa, Vietnam	**Grinding and Filtration** Nests were dried at 50°C for 24 h and grinded. The ground nests were kept in PBS at 30°C for 24 h. The suspension then was fully ground using a tissue grinder. Then it was centrifuged for 10 min at 3,000× *g*. The supernatant was collected and filtrate through to 0.2 μm filter.	Protein concentration by BCA Protein Assay. Amino acid distribution (mg/g) was determined by L-8900 automatic amino acid analyzer.	The concentration of the extract's protein was 0.75 mg/ml with a size of 31 KD.
Haghani et al. (2017)	Teluk Intan, Perak, Malaysia Gua Madai caves, Lahad Datu, Sabah, Malaysia	The EBN extract were prepared based on Guo et al. (2006).	Fluorometric HPLC	EBN from Gua Madai contained 6.7 mg/g of Neu5Ac. EBN from teluk Intan contained 3.2 mg/gr of Neu5Ac
Ghassem et al. (2017)	N/A	**Grinding**	DPPH, FRAP, ABTS and ORAC assay for antioxidant activity.	Various fractionated EBN samples (extract, crude hydrolysates, purified peptides) showed different antioxidant activities with purified peptide (<3 kDa) being the strongest antioxidant.
		Nest were pounded in a mortar and suspended in 1:5 (vol/vol) distilled deionized water and eluted for 24 h at 4°C.	LC-ESI-TOF MS/MS for peptide sequence identification.	Following peptide sequencing of <3 kDa fraction, two pentapeptides, PFHPY and LLGDP were discovered to have potent ORAC values. The peptide identified from the extract had amino acid sequences between 5 and 7 amino acids in length and the molecular weight between 514.29 and 954.52 Da. Most of them showed ORAC values > 5 μM of TE μM^{-1} peptide, indicating their antioxidative properties.
		Heating	MTT assay for *in vitro* cytotoxicity (cell viability with MRC-5 cell, oxidative rescue with HepG2 cell line).	PFHPY and LLGDP were not toxic to cells and showed protection against H_2O_2-induced oxidative damage in HepG2 cancer cells.
		The extract was boiled for and then proceed with enzymatic treatment. **Enzymatic treatment** The EBN samples were treated with pepsin at 37 °C for 2 h with continuous shaking. The pH of the mixture was then adjusted to 8 and digested with trypsin for 2 h at 37 °C. The mixture was then boiled in water bath for 15 min. **Centrifugation** The mixture was centrifuged and the supernatants was collected, lyophilized as crude hydrolysate. **Ultrafiltration** The crude hydrolysate was then filtered using ultrafiltration cut-offs membranes producing 3	Gastrointestinal proteases effects on antioxidant activity.	PFHPY and LLGDP maintained their potent antioxidant activity following exposure to gastrointestinal proteases.

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TABLE 4 | (*Continued*) Analysis of EBN composition.

References	Source of EBN	Preparation of EBN	Methods of analysis	Results
		fractions with molecular weight of >30, 10–3, <3 kDa. **Storage** The samples were lyophilized stored at –80°C until further analysis.		
Noor et al. (2018)	EBN Grade A, B, C and D supplied by Mobile Harvesters Malaysia Sdn. Bhd	EBN of various grades were crushed and ground using home blender, then stored at room temperature.	Nutritional composition of moisture, protein, carbohydrate, ash and fat content.	Grade A EBN has highest protein and fat content (60.59 and 1.19%) followed by grade B, C and D. Grade C EBN has the highest carbohydrate content while Grade D EBN has the highest ash content.
	* A = Cleaned EBN, B = Semi-cleaned EBN, C = Washed Residue EBN, D = Unwashed EBN		Amino acid composition	All grade of EBN samples contained all essential amino acids in which valine, leucine, and threonine presented in high amount. Serine was the highest in four grades followed by aspartic acids. The least amino acids found in all EBN grades was methionine.
			Percentage recovery from hydrolysis	Grade A EBN demonstrated highest recovery of hydrolysate following hydrolysis (96–99%) followed by grade D, C and B.
Daud et al. (2019)	EBN supplied by Mobile Harvesters Malaysia Sdn. Bhd	N/A	Protein, carbohydrate and reducing sugar analysis	EBN samples have up to 60% of protein content, followed by carbohydrate (25%). About 6% of reducing sugar obtained from the total carbohydrate.
			FTIR spectroscopy for glycan	Glycan can be extracted following alkaline hydrolysis that remove significant protein amount.
			In vitro digestion	Consumption of glycan from EBN can resist stomach digestion in which it can pass into gut environment to act as prebiotic component.
Nuradji et al. (2018)	Kalimantan Island and Java Island, Indonesia	The EBN extract were prepared based on Guo et al. (2006) with some modifications on heating where aquabides (150 mg/ml) was used instead of distilled water.	Sialic acid content was analyzed by spectrophotometry.	The sialic acid content in EBN was 10.14% (w/w). EBN from Kalimantan island had higher sialic acid than EBN from Java island.
Babji et al. (2018)	Commercial EBN products from Pahang, Malaysia by Nest Excel Resources Sdn. Bhd.	**Heating** EBN was soaked in a water with ratio 1:100 (w/v) and incubated for 16 h at 4°C, and followed by boiling for 30 min at 100°C and cooled to room temperature before pH adjustment. **Gamma irradiation** Irradiation of EBN powder using cobalt-60 irradiator (220 Gammacell® Excel) at a rate of 2.17 kGy.h-1, at doses of 0.0, 1.0, 2.0, 5.0, 7.5, 10.0, 20.0 and 30.0 kGy. **Enzymatic hydrolysis** 1% enzyme alcalase was added to substrate, and followed by hydrolysis for 4 h and boiling of the hydrolysates for 5 min to inactivate the enzyme. **Centrifugation**	Amino acid profiling. Microbiological analysis and antioxidant and angiotensin converting enzyme (ACE) inhibitory assay. LC-TSI-TOF mass spectrometry for identification of peptides.	Microbial analysis showed insignificant microbial activity probably due to gamma irradiation. Hydrolysates of EBN showed higher anti-oxidant ACE inhibitory activities. Significant level of aromatic amino acid composition (histidine, phenylalanine, and tyrosine) and essential free amino acid such as valine and threonine in hydrolysate compared to the raw EBN. Mass spectrometry identified aromatic amino acid (His, Phe, and tyr) composition, valine (Val), serine (Ser), proline (Pro), threonine (Thr) and phenylalanine (Phe).

(Continued on following page)

TABLE 4 | (*Continued*) Analysis of EBN composition.

References	Source of EBN	Preparation of EBN	Methods of analysis	Results
		The samples were centrifuged at 4,000 rpm, 4°C for 10 min. **Filtration and storage** The supernatants were filtered using Whatman No.1 and freeze-dried prior to storage for further analysis. **Ultrafiltration** The high and low molecular weight fractions of EBN protein hydrolysates were separated by ultrafilter membranes.		
Zulkifli et al. (2019)	Raw cleaned EBN obtained from terengganu supplied by Mobile Harvesters Malaysia Sdn. Bhd.	**Grinding** Raw EBN was ground to powder and soaked in distilled water for 16 h at a ratio of 1:100 at 4°C. **Heating** The samples were boiled through double boiling method at 100°C for 30 min, and cooled to room temperature before pH adjustment. **Enzymatic hydrolysis** 1% alcalase (6 µL/100 ml), 1% papain (6 mg/100 ml) and 30% of papaya juice (30 g/100 ml) were added to EBN samples and was carried out for 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 h at 60°C. The enzymatic activities were stopped by boiling in water for 5 min. **Filtration and storage** The samples were filtered using Whatman #4 filter paper, and frozen at -20°C for 48 h and then freeze-dried for further analysis. Boiled EBN without enzymatic treatment was freeze-dried and used as a control.	Bradford assay to assess the soluble protein content. DNS (3,5-dinitrosalyzed acid) method to determine reducing sugar in EBN hydrolysate. APPH assay for anti-oxidant activity. α-glucosidase inhibition to measure anti-hyperglycemic activity.	Degree of hydrolysis was highest through addition of alcalase, especially at 0.5 and 1.5 h compared to others. Increase in hydrolysis time increased the solubility of EBN hydrolysate proteins, except for alcalase (1–3 h; not significant), and papaya juice (increase from 1 to 2 h, but decrease from 2 to 3 h). Protein solubility of boiled EBN was lesser than hydrolyzed EBN. Hydrolyzed EBN contained higher reducing sugar concentration compared to boiled or unhydrolyzed EBN. Increase in hydrolysis increased the anti-oxidant activity of EBN hydrolyzed by alcalase, however no such changes in EBN hydrolyzed with papain and papaya juice. EBN hydrolyzed with papaya juice showed moderate inhibitory activity against α-glucosidase activity whereas boiled and EBN hydrolyzed with alcalase and papain did not inhibit α-glucosidase activity.
Hun Lee et al. (2020)	Johor, Malaysia	**Heating** The feather of EBN samples were removed and dried in oven at 40 °C for 5 h to produce a constant weight. **Grinding**	Protein and amino acids analysi by SDS-PAGE and Waters AccQ Fluor Reagent kit (Waters). HPLC-QTOF/MS for metabolites analysis. Fatty acids and triglyceride analysis by NMR spectroscopy. DPPH, ABTS and catalase assay for antioxidantive, paraoxanase and anti-tyrosinase activities.	EBN contained mostly of proteins (62–63%) and carbohydrates (25.62–27.26%). Protein profile analysis showed 15 protein bands (16, 19, 21, 25, 27, 30, 37, 42, 49, 58, 66, 82, 97.4, 116 and 173 kD). EBN samples identified serine, aspartic acid, phenylalanine and tyrosine as the most abundant amino acids and the least amino acids of tryptophan and methionine.

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TABLE 4 | (*Continued*) Analysis of EBN composition.

References	Source of EBN	Preparation of EBN	Methods of analysis	Results
		Dried EBN samples were blended and grounded into fine powder, then filtered through 1 mm steel filter. **Storage** Samples were stored at room temperature in air-tight container prior to analysis.		EBN samples were rich in poly-unsaturated fatty acids. The total amount unsaturated fatty acids accounted for 73.17% of overall fatty acids. EBN samples demonstrated strong free radical scavenging activity and paraoxonase activity with moderate anti-tyrosinase activity.
Amiza et al. (2019)	Gong Badak, terengganu.	**Cleaning** Raw uncleaned EBN was cleaned by soaking with water and rinsed frequently to remove contaminants and feathers. Feathers were removed manually by using tweezers and the samples were kept in container and stored at -20 °C. **Heating** Freeze EBN samples were homogenized with distilled water at 10,000 rpm for 2 min. Then, the samples were boiled for 30 min in water bath and let cooled. **Enzymatic hydrolysis** The homogenized EBN were treated with Alcalase using water bath shaker at a specified temperature and time. The treatment was inactivated at 85°C for 20 min. **Storage** The samples then were freeze-dried, and the resulting lyophilized samples were crushed into powder. The powder samples were kept in airtight container.	Degree of hydrolysis by optimizing temperature, pH, concentration, hydrolysis time. Amino acid composition	The degree of hydrolysis (37.89%) was highest when EBN samples were enzymatically treated with 2% Alcalase, at 65°C, with a pH of 9.5 for 60 min. Protein was the major nutritional component in dry raw and lyophilized EBN (70.55 and 67.63%). Ash and fat content were lower (except carbohydrate) in lyophilized EBN samples (hydrolysate) than raw EBN. Sixteen types of amino acids were found in EBN hydrolysate. Valine was the highest essential amino acids in the samples while serine was for non-essential amino acids.
Ling et al. (2020)	Commercial EBN products from Mobile Harvester (M) Sdn Bhd, Selangor, Malaysia	**Grinding** EBN clean and co-products were ground using a stainless-steel Waring blender for 2 min and kept in storage for further analysis. **Enzymatic hydrolysis** 3g of clean and EBN co-products were soaked in 100 ml distilled water and maintained for 16 h at 4 ± 1°C. This was followed by boiling at 90 ± 1°C for 30 min and cooled to 60 ± 1°C. Protease from Bacillus licheniformis was added to the solution and boiled for 5 min. **Centrifugation**	Resorcinol method to determine N-acetylneuraminic acid level. Bradford protein assay to determine soluble protein content level. Fourier transform infra-red (FTIR) spectroscopy to identify functional groups in EBN. DPPH and FRAP assay to determine EBN's antioxidant properties.	Recovery glycopeptides yield of clean EBN was 89.09 ± 0.01% and EBN co-products was 47.64 ± 0.26%. The hydrolysate of clean EBN had the highest level of N-acetylneuraminic acid compared to the rest. Hydrolysate of EBN contained significantly higher level of soluble protein compared to RAW EBN, and FTIR spectra revealed carbohydrate and protein as the main components of EBN. Clean EBN showed profoundly higher free radical scavenging activity (DPPH) and reducing power assay activity (FRAP) compared to EBN co-products.

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TABLE 4 | (*Continued*) Analysis of EBN composition.

References	Source of EBN	Preparation of EBN	Methods of analysis	Results
		The samples were centrifuged at 2610 X g for 10 min at 4 ± 1°C. Supernatants were freeze dried and their recovery were calculated. **Storage** The samples were stored in airtight container at room temperature.		
Gan et al. (2020)	Processed clean EBN supplied by Mobile Harvesters Malaysia Sdn. Bhd.	**Grinding**	Physicochemical analysis composed of water activity test, color analysis, viscosity test, degree of hydrolysis, s oluble protein content, and amino acid profiling.	The yield of EBN was highest in samples with freeze drying, followed by oven and spray drying.
		EBN were grounded using a cutting mill then passed through a 0.5 mm sieve.	Functional group identification *via* FTIR spectroscopy.	Oven drying demonstrated undesirable water activity, darker color and inferior bioactivities compared to spray and freeze drying.
		Enzymatic hydrolysis	DPPH, FRAP, ABTS assay for antioxidant activity.	Freeze drying method appeared to be the most desirable technique to achieve good EBN physicochemical properties and bioactivities.
		Grounded EBN samples were soaked in distilled water at room temperature. This was followed by boiling at 90°C for 30 min. Protease from Bacillus licheniformis was added to the solution and incubated in water bath.	Antihypertensive activity analysis.	Freeze-dried EBN samples showed the strongest free radical scavenging activity, ferric reducing antioxidant power but not ABTS scavenging activity.
		Drying (oven, spray or freeze drying) The samples were dried with either oven, spray or freeze drying.		No significant antihypertensive activity was found between different dried EBN.
Hui Yan et al. (2021)	Clean EBN samples from Mobile Harvester (M) Sdn Bhd, Selangor, Malaysia	**Grinding**	Nutritional composition of EBN (protein, moisture, fat, ash and carbohydrate).	The majority of nutrional component of clean raw EBN consist of protein, followed by carbohydrate, moisture, fat and ash.
		EBN samples were crushed and homogenized to granules	Degree of hydrolysis and solubility	Increasing hydrolysis period will result in decreasing molecular weight of SiaMuc-glycoprotein (except at 60–90 min).
		Double boiling	SiaMuc-glycoprotein, protein and peptide content	Alcalase hydrolysis produce high recovery of bioactive SiaMuc-glycopeptide.
		EBN samples were made to swell by soaking in distilled water and stored overnight under 4°C. Then, the samples were double-boiled for 30 min.	Protein molecular weight profile	Alcalase hydrolysis produce high macro fractions of SiaMuc-glycoprotein thus increase the solubility and bioavailability of nutrients in EBN.
		Enzymatic hydrolysis EBN samples were pre-heated in 60°C shaking water bath then, 1% alcalase was added while maintaining the pH level. Enzymatic treatment was investigated in four timepoints.		
		Storage The samples were freeze-dried for storage.		

TABLE 5 | Anti-inflammatory effects of EBN.

References	Cell lines	Origin of EBN and dosage	Experimental design	Analysis	Summary of findings
Aswir and Wan Nazaimoon (2011)	Human colonic adenocarcinoma cell line (Caco-2 cells) and mouse leukemic monocyte macrophage cell line (RAW 264.7)	Two processed, commercial brands (Y1 and X1) and 4 unprocessed samples obtained from 3 zones [North (Zua1 and Zub1), South (ZS1) and East Coast (ZP1)] of Peninsular Malaysia.	EBN treatment was grouped based the brand and origin of the samples, and the working concentration of the EBN used was 5 ppm.	Proliferative effect of EBN was assessed using Caco-3 cells.	Significant cell proliferation ($p < 0.05$) was noticed in cells treated with 5ppm of unprocessed or commercial EBN. The highest proliferation was seen in treatment with EBN (Brand X1) at 215.07 ± 4.74%.
				Anti-inflammatory effect of EBN was assessed using RAW 264.7 cells.	Sialic acid (0, 2, 4, 6, 8 and 10) caused a dose dependently increased the proliferation of Caco-3 cells. At 24h, 2% of sialic acid significantly increased cell proliferation by 50% ($p = 0.027$), and 10% of sialic acid increased proliferation by more than 100% ($p = 0.009$). Brand Y1 reduced the percentage of TNF-α production to 43% (430 pg/ml), EBN from East Zone and South Zone reduced TNF-α production to 31% (310 pg/ml) and 24% (240 pg/ml), respectively. GlcNA was the most effective in reducing TNF-α production at 2ppm.
Vimala et al. (2012)	Mouse macrophage cell line RAW 264.7.	Two processed brands of EBN (Brand X and Y) and eight unprocessed raw (ECZ1, ECZ2, NZ1, NZ2, NZ3, SZ1, SZ2 and SZ3) white EBN from three zones in Peninsular Malaysia; two from East Coast, three from North and South zones	The treatment groups were categorized based on the types of EBN. RAW cells were then treated with EBN at 1, 10 and 100 ppm for 2 h and cultured with LPS from Escherichia coli serotype 0111: B4 (0.1 mg/ml) for 24 h at 37°C.	Nitrite (NO) production TNF-α concentration. Cell viability by MTS assay.	All EBN extracts inhibited TNF-α and NO production in LPS-stimulated RAW 264.7 macrophages. ECZ1 and ECZ3 showed cell viability over 80% by the MTS assay.
Yew et al. (2014)	SH-SY5Y cells.	Crude and water extract of EBN from Perak, Malaysia.	S1 (crude) and S2 (Water extract) of EBN used throughout the study.	Cell viability by MTT assay.	Pre-treatment with EBN extracts and co-incubation with 6-OHDA generally did not significantly improve the viability of cells. Pre-treatment with S1 or S2 prior to 6-OHDA protected SH-SY5Y cells by decreasing cell death, nuclear apoptotic changes in the culture.
			SH-SY5Y cells were exposed to 6-ODHA to induce cytotoxicity and treated with S1 and S2 EBNs.	Morphological examination.	High dose S2 treatment instead of S1 significantly reduced ROS level. The level of ROS was maintained at baseline when SH-SY5Y cells were treated with EBN extracts alone.
				Intracellular ROS level. Apoptosis analysis, mitochondrial membrane potential assessment, caspase-3 detection.	S1 increased apoptotic event in untreated cells and did not reverse the 6-OHDA induced early apoptosis. However, S2 reduced the early apoptosis events and attenuated 6-

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TABLE 5 | (*Continued*) Anti-inflammatory effects of EBN.

References	Cell lines	Origin of EBN and dosage	Experimental design	Analysis	Summary of findings
					OHDA induced caspase-3 activation. No significant difference in MMPs between the EBN and 6-OHDA groups.
Yida et al. (2015)	Sprague dawley rats (10-week-old, 230–280 g).	EBN from terengganu, Malaysia.	The rats were fed high fat-diet (HFD) containing	Thiobarbituric acid reactive species (TBARS).	Both EBN treatments significantly improved percentage of scavenging activity and reduced liver MDA level compared to HFD group.
			4.5% cholesterol and 0.5% cholic acid with or without treatment using Simvastatin or EBN (2.5, 20%) for 12 weeks, except the normal group.	Serum total antioxidant.	Both EBN treatments significantly reduced HFD-induced increase in CRP, IL-6 and TNF-α and reduced serum inflammatory markers CRP and TNF-α but had little effects on serum total antioxidant status.
				Serum C-reactive protein (CRP), IL-6 and TNF-α.	EBN increased hepatic antioxidant genes suppressed by HFD, such as superoxide dismutase [SOD] 1 and 2, glutathione reductase (GSR), and glutathione peroxidase
				Gene expression study.	(GPx), and reduced HFD-induced increase in hepatic inflammatory genes CRP, TNF-α, CCL2, and NFκβ.
Hou et al. (2015b)	SH-SY5Y human neuroblastoma cell line.	EBN from Sabah and Sarawak, Malaysia.	SH-SY5Y cells were treated with varying doses (depends on the assay) of EBN, lactoferrin (LF) and ovotransferrin (OF), respectively.	ABTS radical cation scavenging activity.	EBN (1000 μg/ml) produced the greatest scavenging effect on ABTS followed by LF and OF.
				Oxygen Radical Absorbance Capacity (ORAC) Assay.	EBN showed more than 80% viability on SH-SY5Y cells with 1000 μg/ml EBN producing the greatest effects.
				MTT assay.	Cell appeared normal in EBN, LF, and OF treated cells while H_2O_2 caused early apoptosis.
				Acridine orange and propidium iodide (AO/PI) staining (Cell apoptosis).	Pretreatment with LF, OVF, and EBN for 24 h recovered the SOD activity compared to H_2O_2 treated cells where SOD decreased. EBN reduced intracellular H_2O_2.
				Superoxide dismutase (SOD) and ROS ELISA assays.	EBN upregulated the expression of SOD1, SOD2, and PARP1 genes even in the presence of H_2O_2.
				Real-time Polymerase Chain Reaction.	No significant differences in SOD mRNA levels between EBN, LF, and OVF.
Zhao et al. (2016)	BALB/c mice (female, 18–22 g, 6–8 weeks old).	EBN from Nha trang, Khanh Hoa, Vietnam.	Treatment groups:	*In vitro*	All doses of EBN significantly increased the proliferation of lymphocytes and splenocytes. All EBN extracts improved proliferation of splenocytes.

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TABLE 5 | (*Continued*) Anti-inflammatory effects of EBN.

References	Cell lines	Origin of EBN and dosage	Experimental design	Analysis	Summary of findings
			In vitro	Cell proliferation	Different EBN extracts concentrations markedly elevated percentages of CD19+/CD25+, CD19+/CD71+, CD19+/CD69 + cells compared to control.
			Splenocyte and B lymphocytes collected from BALB/c mice and cultured.	Immunoglobulins detection	EBN extracts promote activation of B-cells in the early, middle, and later periods.
			In vivo	*In vivo*	EBN elevated IgA, IgE, IgG3, and IgM levels, but not IgG1, IgG2a, and IgG2b compared to the control group.
			Control, cyclophosphamide (CY)-treated animals on Day 27.	IgA concentration in intestinal lavage and cD3+/cD19 + lymphocytes among Peyer's patch cells.	All EBN doses significantly increased intestinal secretion of sIgA level compared to CY-treated group, with mid dose showing the greatest effects.
			EBN (low dose (EBNL, 0.42 g/kg), medium dose (EBNM, 0.83 g/kg), and high dose (EBNH, 1.66 g/kg). EBN was given for 28 days, and on day 27 the mice were given CY to induce immunosuppression followed by removal of their small intestine after 24 h.		All doses of EBNE significantly reversed the CY-induced drop in $CD3^+$ T- and CD-19+ B-cells in Peyer's Patch cells with mid dose showing the greatest effects.
Ghassem et al. (2017)	HepG2 (human hepatocarcinoma cell line)		Different cell lines were treated varying concentration of purified peptides from EBN extract.	Antioxidant assay.	EBN showed effective radical-scavenging and promising antioxidant properties.
	MRC-5 (human embryonic fibroblast cell line)			Cytotoxicity assay.	Two novel pentapeptides Pro-Phe-His-Pro-tyr and Leu-Leu-Gly-Asp-Pro, corresponding to f134–138 and f164–168 of cytochrome b of *A. fuciphagus* indicated the highest ORAC values indicating increased scavenging activity.
				ORAC assay.	Scavenging activity increased following hydrolysis (digestive enzymes) and ultra-fractionation due to more cleavage and increased number of low molecular weight peptides. The antioxidant potency of PFHPY and LLGDP peptides was not affected by *in vitro* incubation with gastrointestinal enzymes. No cytotoxicity effect of purified peptides at concentration was observed. Pretreatment with purified peptides enhanced the HepG2 cell viability.

IAV compared to EBN collected from House Nest. Fluorometric High-Performance Liquid Chromatography revealed EBN from Gua Madai contained 6.7 mg/g of Neu5Ac, whereas EBN from House Nest contained 3.2 mg/g of Neu5Ac (Haghani et al., 2017). Both research groups suggest that the higher amount of sialic acid and the diversity of its derivatives increase the anti-viral effects of EBN.

CYTOKINE STORM IN INFLUENZA AND COVID-19 INFECTIONS

Cytokine storm (CS) or aberrant pro-inflammatory responses is a known complication in severe influenza (Woo et al., 2010; Ishikawa, 2012; Kalaiyarasu et al., 2016) and COVID-19 cases (Chan et al., 2020; Wang Z. et al., 2020; Zhao et al., 2020). Infected cells undergoing apoptosis and necrosis trigger acute inflammatory response recruiting leukocytes and plasma cells to the site of infection (Knipe and Howley, 2013). Hence, increasing the production of pro-inflammatory cytokines and chemokines (La Gruta et al., 2007; Shinya et al., 2012) *via* transcription factors such as Nf-κB, AP-1, interferon response factors 2 and 7 (Thompson et al., 2011).

In severe influenza cases, increase in transcription activities of NF-κB and IRF 3/7 promote anti-viral pro-inflammatory responses in the lungs, including excessive levels of interferon, tumor necrosis factor (TNF-α), interleukin (Beigel et al., 2005) (IL-1 and IL-6) (Tisoncik et al., 2012), IL-8 (de Jong et al., 2006), and chemokines such as MCP-1, RANTES, causing severe influenza-induced immunopathy (Berri et al., 2014; Chiang et al., 2014) Increased activities of NF-κB also escalate the expression of IκB (an inhibitor of NF-κB) and anti-inflammatory cytokines to attenuate the NF-κB-induced pro-inflammatory responses. Early responses to IAV infections indicate a rise in the levels of RANTES, MCP-1, IL-8, IFN-α, IFN-β, and IFN-κ (Le Goffic et al., 2007; Xagorari and Chlichlia, 2008), whereas late responses have recorded a surge in IL-1α/β, IL-6, TNF-α, IL18, IFN type I, MCP-1, MIP-1α, MIP-1β, RANTES, MCP-3, MIP-3α, and IP-10, especially secreted by macrophages residing in the lower respiratory tract (Jewell et al., 2010). Such changes in cytokine waves tip the balance between pro-inflammatory and anti-inflammatory responses, along with viral virulence causes respiratory epithelial injuries such as acute lung injury or even ARDS (Shimizu, 2019) reducing oxygen saturation level leading to mortality (For review: Gu et al., 2019).

In COVID-19 cases, an increase in IL-6 level was most frequently associated with the severity of the disease (Chen C. et al., 2020; Gao et al., 2020; Ruan et al., 2020) and other cytokines and chemokines, including IL-1B, IL-7, IL-8, IL-9, IL-10, FGF, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1A, MIP1-β, PDGF, TNF-α, and VEGF (Huang et al., 2020) were also documented. At the early stage of SARS-CoV infection, there is a delay in the release of chemokines, cytokines, hence a low level of IFN. At the same time, the abundant release of pro-inflammatory cytokines such as TNF, IL-1β, IL-6, and chemokines such as CCL-2, CCL03, CCL-5 causes excessive infiltration of inflammatory cells into the lungs (Law et al., 2005; Lau et al., 2013). Pre-clinical findings revealed an early delay in the release of IFN-α/β induced generation of more monocyte chemoattractants such as CCL-2, CCL-7, and CCL-12 by macrophages causing their further accumulation, and production of pro-inflammatory cytokines, including IL-1β, IL-6, TNF-α, and inducible nitric oxide synthase. Cumulatively, IFN-α/β and IFN-γ cause apoptosis of T cells (Channappanavar et al., 2016), airway epithelial cells, alveolar epithelial cells, and endothelial cells (Shimizu, 2019) damaging pulmonary vasculature, leading to vascular leakage and alveolar edema, causing hypoxia (Channappanavar et al., 2016; For review: Ye Q. et al., 2020). Endothelial injuries also cause spillover of cytokines and chemokines into the blood circulation causing multi-organ failure (Tisoncik et al., 2012). In line with this, findings from non-human primates revealed that SARS-CoV-infection-related death is more likely due to excessive immune response rather than virus titer (Smits et al., 2010).

THE USE OF CORTICOSTEROIDS IN INFLUENZA AND COVID-19 INFECTIONS

The severity of- and mortality due to influenza and COVID-19 infections are determined by the host resistance and virulent factors, where severe infections usually trigger hyperactive host resistance, hence causing adverse symptoms (Liu et al., 2020), such as acute respiratory distress syndrome (ARDS), eventually leading to mortality. Influenza patients with ARDS are managed through corticosteroid therapy for their anti-inflammatory actions. Over the years, a myriad of systematic and meta-analyses have consistently reported that routine corticosteroid treatment in influenza patients may not be appropriate. Many reported a significant association between corticosteroid treatment and higher mortality (Zhang et al., 2015; Ni et al., 2019; Lansbury et al., 2020; Zhou et al., 2020), whereas some have reported that low-to-moderate dose of corticosteroids may reduce mortality (Li et al., 2017). Some have claimed that early and high-dose corticosteroid use is associated with hospital mortality (Lansbury et al., 2020; Sheu et al., 2020; Tsai et al., 2020). In addition to high mortality, corticosteroid treatment also correlated to secondary infections (Yang et al., 2015; Lansbury et al., 2019; Ni et al., 2019; Zhou et al., 2020) due to prolonged hospital stay (Yang et al., 2015; Ni et al., 2019).

Systematic review and meta-analyses of randomized clinical trials revealed that corticosteroids reduced the mortality in critically ill COVID-19 patients (WHO Rapid Evidence Appraisal for COVID-19 Therapies (REACT) Working Group et al., 2020; Ma et al., 2021; van Paassen et al., 2020), especially patients with compromised lung capacity (Bartoletti et al., 2021), however with delayed viral clearance and increased secondary infections (van Paassen et al., 2020). Some have also reported increased short-term mortality following corticosteroid use in COVID-19 patients with ARDS (Liu et al., 2020), whereas some found no significant association between corticosteroid use and mortality (Shuto et al., 2020; Albani et al., 2021). In line with these, some have warned against the use of corticosteroids in non-critically ill COVID-19 patients due to longer hospitalization,

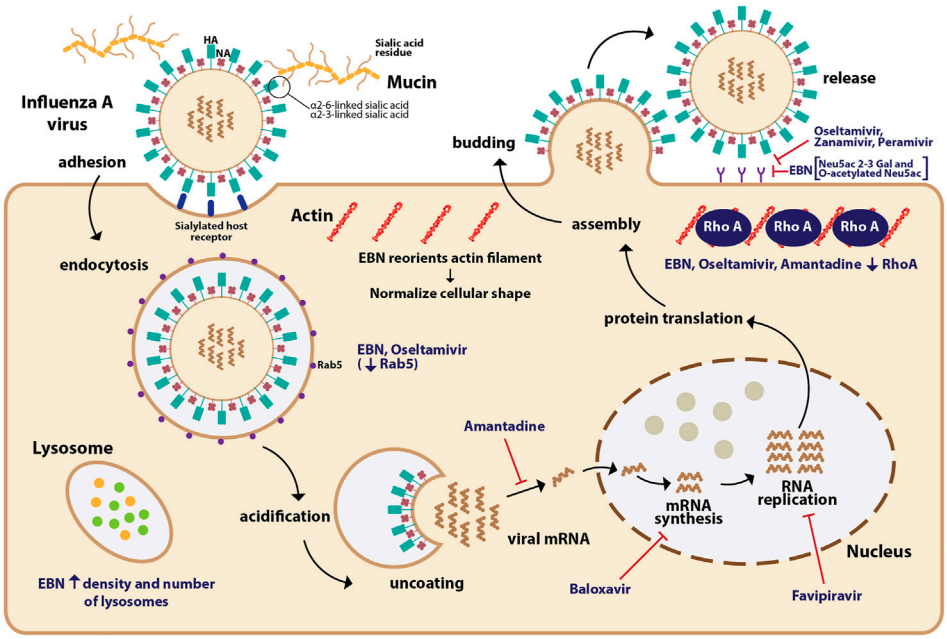

FIGURE 1 | The mechanism of action of EBN and anti-viral drugs on influenza A virus (IAV). The IAV binds to the terminal sialic acid residues on mucins, particularly through α2-6 and α2-3 linkages leading to endocytosis of IAV into the host cell. The IAV core undergoes acidification allowing viral RNPs to be released into the cytoplasm. The IAV genetic material is transcribed and translated to produce viral materials, which once assembled, bud out from the infected cell. Anti-viral drugs such as baloxavir inhibit viral mRNA synthesis, favipiravir inhibits RNA replication, amantadine inhibits the release of viral mRNA, and neuraminidase inhibitors (oseltamivir, zanamivir, and peramivir) inhibit the release of the IAV. EBN increases the density and number of lysosomes in infected cells. Similar to oseltamivir, EBN reduces the expression of Rab5, which facilitates the endocytosis of IAV. EBN reorients the actin filament and normalizes the cellular shape of infected cells. Similar to oseltamivir and amantadine, EBN also reduces the expression of RhoA, a protein involved in actin filament polymerization. EBN, through its sialic linkages such as Neu5Ac 2-3 Gal, and O-acetylated Neu5Ac may prevent the release of IAV.

longer duration of viral shedding, and progression of non-severe to severe cases (Shuto et al., 2020; Yuan et al., 2020). Due to many limitations such as study designs, sample size, age factors, viral strain, and pharmacogenomics discrepancy, at present, it is not empirically possible to conclude the efficacy of corticosteroid use in COVID-19 or influenza patients.

ANTI-INFLAMMATORY EFFECTS OF EBN

EBN (House nest from Teluk Intan), when tested for its anti-viral effects using an *in vivo* model, significantly increased the expression of IFN-γ, IL-1β, IL-2, IL-6, and TNF-α on day 1 following infection, significantly decreased the level of IFN-γ, and upregulated IL-4, IL-6, IL-12, and TNF-α on day 3 post-infection, and reduced IL-10 and IL-12 on day 7. The profound increase in the pro-inflammatory cytokines seen during the early stage of infection is similar to numerous anti-viral medications, including the anti-viral drug oseltamivir used in the same study. On post-infection day 5 and 6, the level of IL-6 remained high in the oseltamivir treated groups, unlike the EBN treated groups where the levels of pro-inflammatory cytokines were well regulated. In the same study, EBN (House nest from Teluk Intan) treated with pancreatic F enzyme significantly decreased the levels of IL-6 and chemokine (C-C motif) ligand 2 (CCL2) *in vitro* (Haghani et al., 2016). In line with these findings, studies using other disease models also reported EBN-induced inhibition of nitric oxide and TNF-α production in LPS-stimulated macrophages (Aswir and Wan Nazaimoon, 2011; Vimala et al., 2012), improvement in the proliferation of lymphocytes and splenocytes (Zhao et al., 2016), free radical scavenging activities and reduced pro-inflammatory markers such as CRP, TNF-α, CCL2, NF-κB (Yida et al., 2015) (**Figure 2**). **Table 5** shows the anti-inflammatory effects of EBN on various models.

The discrepancy in the therapeutic potential of EBN of various sources and preparation techniques should be taken into consideration. For an instance, some researchers have reported increased viability of cells following incubation with EBN (Zhao et al., 2016; Haghani et al., 2017) with increasing concentration of sialic acid (Aswir and Wan Nazaimoon, 2011). Whereas, some reported no improvement in viability of cells following co-incubation of EBN (water extract) with 6-hydroxydopamine (Yew et al., 2014). However, when incubated with crude EBN, 20% of viability was seen (Yew et al., 2014). Moreover, treatment with high-dose crude EBN significantly promoted intracellular production of ROS (twice than 6OH treated level). However, high-dose water extracts of EBN significantly reduced the ROS level (Yew et al., 2014). In mice, all EBN dosages employed in a study induced six division cycles of CD-19 stained (B cells). EBN (0.19, 0.38, and 0.75 mg/ml) only completed one division of CD-3 stained (T cells) cells, indicating that the immunomodulatory

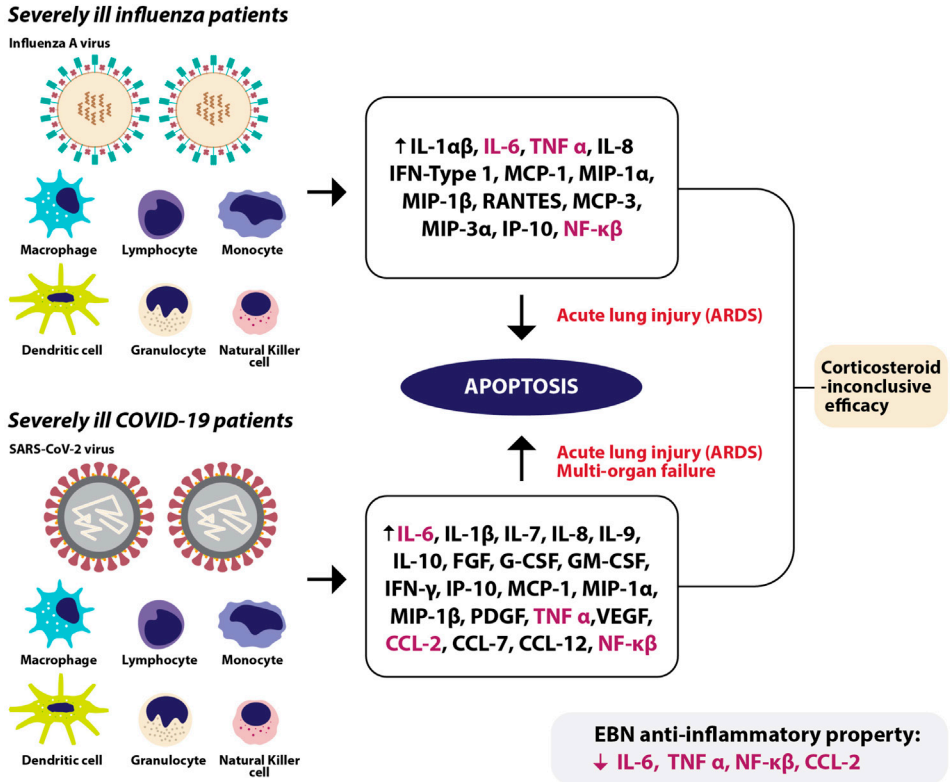

FIGURE 2 | In severely ill influenza patients, immune cells release various cytokines and chemokines leading to acute lung injury, or also known as acute respiratory distress syndrome (ARDS) due to apoptosis. In severely ill COVID-19 patients, various immune cells release a myriad of chemokines and cytokines causing apoptosis, leading to ARDS and spillover of cytokines and chemokines into the blood circulation causing multi-organ failure. Among the escalated cytokines and chemokines in severe influenza and COVID-19 infections, EBN was shown to reduce IL-6, TNF-α, CCL-2, and NF-κβ at the pre-clinical level.

property of EBN could be more directly inclined to B cell responses (Zhao et al., 2016).

BIOACTIVE INGREDIENTS IN EBN

EBN mainly consists of protein (almost 60%), carbohydrate (25%) (Daud et al., 2019), moisture, fat, and ash (Amiza et al., 2019; Hun Lee et al., 2020; Hui Yan et al., 2021). At present, the potential bioactive ingredients in EBN with anti-viral and immunomodulatory properties are glycoprotein or glycopeptides. Sialylated N-glycan was identified as the main component of EBN contributing to its anti-viral property (Yagi et al., 2008). Fluorometric analysis revealed Neu5Ac 2-3 Gal linkages in the cave and O-acetylated Neu5Ac in house-cultured EBN (Guo et al., 2006). Further analysis identified derivatives of sialic acid such as N-glycol neuraminic acid, N-acetyl neuraminic acid, 5-N-acetyl-8,9-O-acetylneuraminic acid, and others such as thymol-β-glycopyranoside in EBN (Haghani et al., 2016). The importance of sialic acid in human nutrition is well known (Ghosh, 2020). Hydrolysis of EBN produced the highest amount of soluble protein compared to other forms of EBN. The hydrolysates of EBN contained high level of N-acetylneuraminic acid with profound anti-oxidant effects (Gan et al., 2020). The degree of hydrolysis was the highest when EBN was enzymatically treated with alcalase (Amiza et al., 2019; Zulkifli et al., 2019; Hui Yan et al., 2021), recovering a high level of bioactive Siamuc-glycopeptide (Hui Yan et al., 2021), and amino acids including valine, serine, aspartic acid, phenylalanine, tyrosine, histidine, threonine, and leucine (Babji et al., 2018; Noor et al., 2018; Amiza et al., 2019; Hun Lee et al., 2020). In line with this, the anti-oxidant activity of enzymatically treated EBN was significantly higher, especially alcalase compared to just boiled EBN (Babji et al., 2018; Zulkifli et al., 2019). Other glycoprotein and protein such as ovotransferrin, and lactoferrin also identified in EBN, especially higher in house and cave white EBN compared to cave red and black EBN (Hou et al., 2015a). Ovotransferrin is known for its anti-bacterial, anti-viral, and anti-inflammatory properties (Giansanti et al., 2016). Similarly, lactoferrin from milk also been well explored for its anti-viral and anti-bacterial activities (Giansanti et al., 2015).

Most of the studies investigating the potential medicinal values of EBN used water extract (Haghani et al., 2016; Haghani et al., 2017), as bioactive compounds in EBN are water-soluble. Water extraction of EBN results in a mixture of glycopeptides (Guo et al., 2006; Yagi et al., 2008; Ling et al., 2020; Hui Yan et al., 2021) which should be isolated or purified through a binding affinity technique utilizing specifically targeted ligands anchored to the

solid phase such as a polyester membrane or cellulose-based surface. The purified or isolated bioactive ingredients can then be released by elution buffer with an appropriate pH. In a large-scale setting, it would be more practical to carry out molecular size range separation (Babji et al., 2018) prior to the purification or isolation process, to reduce the complexity of the extract and slow down the purification step. By fractioning the EBN extract based on their molecular size, the purification step can be further simplified. This should be followed by the identification of bioactive compounds in the fractions. House harvesting of EBN results in various grades of EBN (Grade A-D) (Noor et al., 2018). The fragments and crushed pieces of EBN are of low value for conventional EBN processing. Nonetheless, this EBN might be more applicable as raw material for EBN extract preparation, which involves grinding the EBN into powder, and followed by soaking in water to increase their surface area for reaction with water or enzymes. Subsequently, the crude extract should be subjected to fragmentation based on molecular size and followed by purification. Purified peptides (3 < kDa) showed the strongest anti-oxidant activity compared to extract and crude hydrolysate (Ghassem et al., 2017). Techniques such as freeze-drying removes water while preserving the functional groups of the bioactive compounds. In line with this, freeze-drying has been reported to result in a high yield of EBN compared to oven and spray drying (Gan et al., 2020). The glycopeptides in EBN are easily denatured by the heat drying method, hence the water content should be evaporated under low temperature to preserve the bioactive compounds. Upon usage, the compounds can be reconstituted easily with water to regain their functional property.

EBN STUDIES IN HUMANS: WHAT WE KNOW SO FAR

EBN is an expensive delicacy for the Chinese and sometimes is considered as the "Caviar of the East" (Marcone, 2005). EBN is exclusively popular as part of the traditional Chinese medicine to treat respiratory ailments, moistening the lungs, heart tonics, and stomach nourishments (Zhao et al., 2016). Owing to its documented effects in the old Chinese medical textbooks, the Chinese frequently consumed EBN as a supplement for health benefits (Zhao et al., 2016). Numerous authentic EBN products, particularly in a form of extract or essence, were sold over the counter where most of them were imported exclusively from the Southeast Asian regions (Dai et al., 2020). To date, no findings have directly related the health benefits of EBN in humans. The closest to human testing were the *in vitro* setups involving cultured human cell line such as keratinocytes (Kim et al., 2012; Hwang et al., 2020), SH-SY5Y cells (Hou et al., 2015b), lung carcinoma cells (Guo et al., 2006), colonic adenocarcinoma cells (Aswir and Wan Nazaimoon, 2011), chondrocytes (Chua et al., 2013), and hepatocarcinoma cells (Ghassem et al., 2017). *In vivo* reports were derived from pre-clinical animal models (Yida et al., 2015; Zhao et al., 2016). Hence, the potential benefits of EBN in humans are yet to be proven empirically. In line with human consumption, EBN was reported to cause allergic reactions in pediatric patients due to avian allergen homologous to ovoinhibitor precursor in chicken (66-kd protein) and potentially by other additives or contaminants (Goh et al., 2000; Goh et al., 2001). Compared to fresh EBN extract, commercially prepared EBN showed an undetectable amount of allergen in immunoblot probably due to boiling of the EBN, however still able to trigger an allergic reaction, indicating that the allergen is not heat sensitive. Periodate treatment relinquished the Ig-E binding ability of the allergen suggesting that allergenic epitope could be due to carbohydrate moiety which is known to be resistant to cooking and boiling (Goh et al., 2001). Contaminants such as fungus also been reported to present in EBN, especially plant and soil fungi in raw EBNs and environmental fungi in commercial and boiled EBNs (Chen et al., 2015).

EBN AS A POTENTIAL ANTI-VIRAL AND ANTI-INFLAMMATORY THERAPIES: FUTURE DIRECTION

The anti-viral efficacy of EBN was compared across standard anti-viral medications such as oseltamivir and amantadine, and similar to those drugs EBN also reduced protein involved in viral trafficking such as Rab5 (not amantadine) and protein involved in actin filament polymerization, RhoA. EBN also inhibited the HA activity of the virus (Guo et al., 2006; Haghani et al., 2017; Nuradji et al., 2018). The anti-viral drugs currently being used to treat IAV infection are baloxavir marboxil (inhibits viral mRNA synthesis) (Shionogi and Co. Ltd, 2018), oseltamivir, zanamivir, and peramivir (selective neuraminidase inhibitors) (Gubareva et al., 2000; Ohuchi et al., 2006; Hama, 2015), and favipravir (inhibits RNA polymerase activity) (Furuta et al., 2013). Apart from the neuraminidase inhibitors, it is yet to be explored if a combined therapy of EBN and other classes of anti-viral medications could achieve a synergistic outcome. EBN also attenuated the surge in pro-inflammatory cytokines and chemokines such as TNF-α, CCL-2, NF-κβ, NO, IL-6, and increased IFN-γ (Aswir and Wan Nazaimoon, 2011; Vimala et al., 2012; Haghani et al., 2016), which are dysregulated in severe forms of IAV (Xagorari and Chlichlia, 2008; Tisoncik et al., 2012), and SARS-CoV infections (Gao et al., 2020; Huang et al., 2020). Moreover, EBN also ameliorated apoptosis (Yew et al., 2014), and normalized the cellular shape of IAV-infected cells (Haghani et al., 2017), which may reduce the collateral damage to the host cells in severe infections. Furthermore, EBN also exhibited anti-bacterial property (Hun et al., 2015), and improved B cell activity (Zhao et al., 2016), which may provide additional protection against opportunistic bacterial infections in cases of immunosuppressive use in severely ill patients. Nonetheless, to date, the efficacy of EBN as anti-inflammatory agent is yet to be compared empirically with clinically used anti-inflammatory medications. The allergenic effect of EBN also should be taken into account (Goh et al., 2001), considering the already known high risk of cytokine surge in severe IAV and COVID-19 patients. Techniques such as ultrafiltration tend to isolate larger glycoproteins, with higher allergenic potential, and periodate treatment relinquished the

allergenic activity of EBN (Goh et al., 2001). It is imperative that future investigations should further explore how these techniques affect the bioactive yield of EBN, and their health-promoting effects in living organisms. The presence of contaminants such as fungus (Chen et al., 2015), and residual contaminants (Yeo et al., 2021) should be reflected in pre-clinical settings prior to clinical application. Gamma irradiation reduced the microbial activity of EBN to an undetectable level (Babji et al., 2018), whether this equally affects the efficacy of EBN in various *in vivo* disease models should be investigated further.

CONCLUSION

The anti-viral and anti-inflammatory property of EBN is promising at the pre-clinical level. Nevertheless, future studies should consider the EBN's potential allergenic and contaminant factors in their study designs prior to its clinical application.

AUTHOR CONTRIBUTIONS

KC, NS, MM, IM, and JK performed the literature search and drafted the manuscript. AU, KK, KC, and JK reviewed and finalized the manuscript.

FUNDING

This study was supported by the GUP-2018-055. Fund provided by the National University of Malaysia (UKM).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 20 July 2021
doi: 10.3389/fphar.2021.685982

Edible Bird's Nest, an Asian Health Food Supplement, Possesses Moisturizing Effect by Regulating Expression of Filaggrin in Skin Keratinocyte

*Queenie Wing Sze Lai[1,2], Maggie Sui Sui Guo[1,2], Kevin Qiyun Wu[1,2], Zhitao Liao[3], Dongshi Guan[4], Tina Tingxia Dong[1,2], Penger Tong[3] and Karl Wah Keung Tsim[1,2]**

[1]Shenzhen Research Institute, The Hong Kong University of Science and Technology, Shenzhen, China, [2]Division of Life Science and Center for Chinese Medicine R and D, The Hong Kong University of Science and Technology, Hong Kong, China, [3]Department of Physics, The Hong Kong University of Science and Technology, Hong Kong, China, [4]State Key Laboratory of Nonlinear Mechanics, Institute of Mechanics, Chinese Academy of Sciences, Beijing, China

OPEN ACCESS

Edited by:
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***Correspondence:**
Karl Wah Keung Tsim
botsim@ust.hk

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: *26 March 2021*
Accepted: *03 June 2021*
Published: *20 July 2021*

Citation:
Lai QWS, Guo MSS, Wu KQ, Liao Z, Guan D, Dong TT, Tong P and Tsim KWK (2021) Edible Bird's Nest, an Asian Health Food Supplement, Possesses Moisturizing Effect by Regulating Expression of Filaggrin in Skin Keratinocyte. Front. Pharmacol. 12:685982. doi: 10.3389/fphar.2021.685982

Edible bird's nest (EBN) has been consumed as a Chinese delicacy for hundreds of years; the functions of which have been proposed to prevent lung disease, strengthen immune response, and restore skin youthfulness. To support the skin function of EBN, the water extract and the enzymatic digest of EBN with enriched digested peptides were tested in cultured keratinocyte, HaCaT cell line. The effects of EBN extract and digest in inducing proteins crucial for skin moisturizing were determined in both *in vitro* and *ex vivo* models. In cultured keratinocytes, the expressions of S100-fused type proteins contributing to skin barrier function in the stratum corneum, e.g. filaggrin and filaggrin-2, were determined in both mRNA and protein levels, which were markedly induced in the treatment of EBN extract or digest. The EBN-induced gene transcriptions of filaggrin and filaggrin-2 were mediated by activation of p38 MAPK pathway and various transcription factors, e.g. GATA3, PPARα, PPARβ, and PPARγ: these transcriptional factors were markedly activated by the digested products of EBN, as compared to the extract, in cultured keratinocytes. By using atomic force microscopy (AFM), the EBN-treated keratinocyte was shown to have more liquid-like morphology, as compared to a control cell. The EBN digest showed better induction on these moisturizing effects as compared to the extract. These lines of evidence therefore suggested the water moisturizing effect of EBN in skin function.

Keywords: edible bird's nest, sialic acid, moisturizing, filaggrin, filaggrin-2, atomic force microscopy

INTRODUCTION

Edible bird's nest (EBN; Yan Wo), the solidified saliva secreted by the swiftlets, *Aerodramus fuciphagus*, has been consumed in Asia for several hundred years as a delicacy. Today, the majority of EBN is produced by domesticated swiftlets in specialized houses from Indonesia, Malaysia, Vietnam, and Thailand. According to historical literatures in Chinese medicine, EBN was recorded to prevent lung diseases, to reduce aging and to improve complexion (Wong, 2013). In line with the historical

records, several lines of evidence have suggested the functions of EBN in improving immune response (Zhao et al., 2016), encouraging cell regeneration (Roh et al., 2012), promoting anti-oxidation (Ghassem et al., 2017) and facilitating skin whitening (Chan et al., 2015).

EBN contains a rich source of protein, having over 50% by dry weight, which provides a rich source of glycoproteins and amino acids for human consumption (Halimi et al., 2014; Wong et al., 2018b). Typically, the consumption of EBN is by extensive cooking; but this process may degrade the active ingredients for its nutritious values, such as those small peptides specific for skin regenerating functions. EBN exists as a complex macromolecular form that is, not easily transformed into a dietary supplement or as a skin-care product. Therefore, we have developed methods to maximize the extracting efficiency of protein/peptide in EBN, as well as the soluble sialic acids (Wong et al., 2018a). Having the developed method for EBN extraction, the extractable protein/peptide was significantly increased by 70–90%.

In the skin's surface, the epidermal layer stratum corneum retains water by those hygroscopic agents, named as natural moisturizing factors (NMFs), which are presented in corneocytes and orderly arranged intercellular lipids forming a barrier against trans-epidermal water loss (TEWL) and maintaining water balance of skin. Over 70% of NMFs are synthesized and derived from filaggrin. Filaggrin (filament aggregating protein) is a filament-associated protein that binds to keratin fibers in epithelial cells to prevent TEWL (Sandilands et al., 2009; Vestergaard and Deleuran, 2014). 10–12 filaggrin units are post-translationally hydrolyzed from a large profilaggrin precursor protein during terminal differentiation of epidermal cells (Sandilands et al., 2007). In humans, profilaggrin is encoded by the filaggrin gene, which is part of the S100 fused-type protein family within the epidermal differentiation complex on chromosome 1q21. Besides filaggrin, filaggrin-2, a closely related member of filaggrin family, also contributes to the formation of NMFs (Hoste et al., 2011). Both filaggrin and filaggrin-2 are protein markers of late differentiation of the epidermal cells, localizing in keratohyalin granules at stratum granulosum. Under the proteolysis process, filaggrin and filaggrin-2 are dephosphorylated, deiminated, and finally proteolyzed into NMFs at the stratum corneum (Sandilands et al., 2009; Hsu et al., 2011). Major NMFs formed from filaggrin are histidine, urocanic acid, and pyrrolidone carboxylic acid (Sandilands et al., 2009). In line with this hypothesis, the loss-of-function mutation of filaggrin is the major genetic risk factor of atopic dermatitis; while the reduced expression of filaggrin-2 is closely correlated to psoriatic lesions (Kypriotou et al., 2012). Both proteins are controlling epithelial homeostasis and protecting the skin barrier (Sandilands et al., 2009).

EBN is consumed as soup, or can be applied as external skin-care products, to improve complexion *via* its moisturizing, whitening, and anti-oxidation effects. Despite the recognized moisturizing effect of EBN in skin, the mechanistic action of EBN as a moisturizer has not been illustrated. The moisturizing effect of EBN has been proposed to be originated from polar interactions between sialic acids in EBN and water molecules (Ng, 1991); however, the detailed experimental data has not been shown. Here, we are attempting to demonstrate the moisturizing effect of EBN, both in water extract and enzymatic digested products, by revealing the expressions of filaggrin and filaggrin-2 by real-time PCR analysis, western blot analysis, and immunofluorescence analysis in EBN-treated cultured keratinocytes. Besides, the EBN-induced signaling cascade in activating expression of filaggrin was illustrated here. The moisturizing effect of EBN was further demonstrated in skin *ex vivo* culture, as well as the measurement of cell moisturization of EBN-treated keratinocytes by atomic force microscopy (AFM).

MATERIALS AND METHODS

Preparation of EBN Extract and Digestion

White EBN, originated from Malaysia house production site, was purchased in Hong Kong market: the sample was of the standard "cup" grade. The sample was stored at room temperature. The raw EBN material was first weighed and soaked in 1:100 (w/v) double deionized (DDI) water overnight for expansion. On the following day, the expanded EBN was rinsed with DDI water three times to remove water-soluble inorganics. The softened EBN was boiled at 98 ± 2°C with 1:30 (w/v) DDI water for 8 h under constant stirring. The stewed EBN was filtered, and the filtrate was collected for lyophilization. The lyophilized filtrate powder was considered as EBN extract. The dried EBN extract was weighed and digested with 1: 100 (w/v) simulated gastric fluid (SGF; without enzyme; containing 0.07 M hydrochloric acid and 0.1 M sodium chloride, catalog number: 01651) and 7.6% (w/w) pepsin (from porcine gastric mucosa lyophilized powder, ≥ 2,500 units/mg protein, catalog number: P7012) (Sigma-Aldrich, St Louis, United States) for 48 h at 37°C for complete digestion. After 48 h, the digestion was terminated by neutralizing nine parts of digested solution with one part of 0.7 M sodium hydroxide solution. Finally, lyophilization was performed to prepare the EBN digest.

HPLC Analysis

HPLC-UV chromatographic separation of EBN digest was performed on an Agilent HPLC 1200 series system (Agilent, Waldbronn, Germany) equipped with a diode array detector (DAD). The EBN digest was reconstituted in DDI water and separated by Superdex 75 gel filtration column (catalog number: GE17-5174-01) (GE Healthcare, Little Chalfont, United Kingdom). UV absorbance was measured at wavelength 214 and 280 nm for peptide and protein detection, respectively. Samples were eluted with phosphate-buffered saline (PBS) mobile phase (containing 0.14 M NaCl, 2.68 mM KCl, 0.01 M Na_2HPO_4, and 1.76 mM KH_2PO_4) at a flow of 0.4 mL/min. Protein markers consisting of α-melanocyte-stimulating hormone (1.7 kDa) (Sigma-Aldrich, catalog number: M4135-1 MG), aprotonin (6.5 kDa), ribonuclease A (13.7 kDa), carbonic anhydrase (29 kDa), ovalbumin (43 kDa), conalbumin (75 kDa), and blue dextran (> 2,000 kDa) (GE Healthcare, catalog number: 28-4038-41) were employed for size estimation.

Animals and Cell Culture

C57BL/6 mice were supplied by Animal and Plant Care Facility in The Hong Kong University of Science and Technology (HKUST). All experiments were performed according to the guidelines of Department of Health, The Government of Hong Kong SAR. The experimental procedures had been reviewed and approved by the Animal Ethics Committee of the university (Reference No.: (20–104) in DH/HT&A/8/2/2 Pt.2). Housing was maintained at a constant temperature and humidity, under a fixed 12-h light/dark cycle and free access to food and water. Human epidermal keratinocytes, HaCaT cells (AddexBio, San Diego, United States, catalog number: T0020001), were cultured in Dulbecco's modified Eagle's medium (DMEM) (catalog number: 1280017), or custom made DMEM without calcium chloride, phenol red, nor sodium bicarbonate (catalog number: 31600034), supplemented with 10% (v/v) fetal bovine serum (FBS) (catalog number: 10270-106) and 1% (v/v) penicillin/streptomycin (10,000 U and 10,000 μg/mL) (catalog number: 15140-122) in a humidified atmosphere with 5% CO_2 at 37°C. All culture reagents were purchased from Thermo Fisher Scientific (Waltham, United States).

DNA Transfection

The DNA construct pFLG2-eGFP was composed of the vector pEGFP-N1 (Addgene, Watertown, United States, catalog number: 6085-1) containing a filaggrin-2 promoter, which drives the transcription of a red-shifted variant of wild-type green fluorescence protein reporter. Cultured HaCaT keratinocytes were transfected using jetPRIME reagent (Polyplus Transfection, New York, United States). In short, a transfection mix containing jetPRIME buffer (catalog number: 712-60), DNA construct and jetPRIME reagent (catalog number: 114-15) were added to cell culture and incubated overnight, followed by transferring transfected cells to a 96-well clear-bottom black plate. Seeded at 1×10^5 cells/mL. Cells were treated with or without EBN samples, including extract and digest. $CaCl_2$ (Sigma-Aldrich, catalog number: 31307-500G) at 0.16 mM was used as a positive control. Samples were examined by Nikon fluorescence microscope. Green fluorescent protein (GFP) quantification was performed in a FlexStation® 3 Benchtop Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, United States). Fluorescence signals were measured under excitation/emission wavelength 488/509 nm. GFP activities were normalized to total proteins using Bradford assay (Bio-Rad Laboratories, Hercules, United States, catalog number: 5000006), and the values were expressed as percent increase of transfected untreated control.

Real-Time PCR Analysis

Through real-time PCR analysis, the amounts of filaggrin and filaggrin-2, GATA3, PPARα, PPARβ, PPARγ, CASP14, and GAPDH mRNAs were quantified. HaCaT keratinocytes were grown in a 12-well culture plate in a concentration of 1.5×10^5 cells/mL. EBN samples, including extract and digest, were applied. $CaCl_2$ (Sigma-Aldrich, catalog number: 31307-500G) at 0.16 mM (positive control of filaggrin and filaggrin-2), IL-4 (Thermo Fisher Scientific, catalog number: A42601) at 100 ng/mL (positive control of GATA3), linoleic acid (Sigma-Aldrich, catalog number: 62230-5ML-F) at 30 mM (positive control of PPARs), and 1α, 25-dihydroxycholecalciferol (vitamin D_3) (Sigma-Aldrich, catalog number: C9758-5G) at 10^{-5} M (positive control of CASP14) were applied. Total RNA was extracted after 24 h of EBN application, using RNAzol® RT RNA isolation reagent (Molecular Research Center, Cincinnati, United States, catalog number: RN190). The RNA quality and amount were determined by NanoDrop™ (Thermo Fisher Scientific) measurements at A260/A280 and A260/A230. The extracted total RNA sample was normalized to 2 μg and any contaminating genomic DNA was removed with DNase I (New England Biolabs, Hitchin, United Kingdom, catalog number: M0303S), according to the manufacturer's protocol. 500 ng RNA sample was reverse transcribed to cDNA using PrimeScript™ RT Reagent Kit (TaKaRa, Kusatsu, Japan, catalog number: RR036A), according to the manufacturer's protocol. Relative gene quantification was performed by LightCycler® 480 Real-Time PCR System (Roche, Basel, Switzerland). The sequences of specific primers for filaggrin and filaggrin-2, GATA3, PPARα, PPARβ, PPARγ, CASP14, and GAPDH were shown as followed: sense 5′-GCT GAA GGA ACT TCT GGA AAA GG-3′ and antisense 5′- GTT GTG GTC TAT ATC CAA GTG ATC-3′ for filaggrin; sense 5′-CTG TGG TCA TTC ATG GAG TGG-3′ and antisense 5′-CCC TAG AAG GGC TAA TGT GTG A-3′ for filaggrin-2; sense 5′-GCG GGC TCT ATC ACA AAA TGA-3′ and antisense 5′-GCC TTC GCT TGG GCT TAA T-3′ for GATA3; sense 5′-GCA CTG GAA CTG GAT GAC AG-3′ and antisense 5′-TTT AGA AGG CCA GGA CGA TCT-3′ for PPARα; sense 5′-CAG AAG AAG AAC CGC AAC A-3′ and antisense 5′-CGC CAT ACT TGA GAA GGG T-3′ for PPARβ; sense 5′-CAG GAA AGA CAA CAG ACA AAT CA-3′ and antisense 5′-GGG GTG ATG TGT TTG AAC TTG-3′ for PPARγ; sense 5′-ATA TGA TAT GTC AGG TGC CCG-3′ and antisense 5′-CTT TGG TGA CAC ACA GTA TTA G-3′ for CASP14; sense 5′-ACA ACT TTG GTA TCG TGG AAG G-3′ and antisense 5′-GCC ATC ACG CCA CAG TTT C-3′ for GAPDH. Amplification was performed for 45 cycles. Each cycle consisted of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 20 s. The mRNA levels were determined by calculating $2^{-\Delta\Delta Ct}$ values.

Western Blot Analysis

The relative amount of filaggrin and filaggrin-2, GATA3, PPARα, PPARβ, PPARγ, and CASP14 proteins, regulated under the treatment of EBN samples, were quantified with specific antibodies. HaCaT keratinocytes were grown in a 6-well culture plate in concentration of 3×10^5 cells/mL. After EBN treatment, the cells were lysed with low salt lysis buffer containing 150 mM NaCl [Affymetrix USB (part of Thermo Fisher Scientific), catalog number: 21618-5KG], 10 mM HEPES (Sigma-Aldrich, catalog number: H4034-100G), 1 mM EDTA (GoldBio, St Louis, United States, catalog number: E-210-500), 1 mM EGTA (Sigma-Aldrich, catalog number: E4378-100G), 1% NP-40 (Sigma-Aldrich, catalog number: NP40S-100ML), 0.01% SDS (Sigma-Aldrich, catalog number: 62862-1KG), 0.1 M Tris-HCl (pH 7.6) [Affymetrix USB (part of Thermo Fisher Scientific),

catalog number: 75825-5KG] and protease inhibitors [1:1,000 aprotonin (Sigma-Aldrich, catalog number: A1153-25MG), 1:1,000 leupeptin (Sigma-Aldrich, catalog number: L2884-25MG), 1:200 benzamidine (Sigma-Aldrich, catalog number: 434760-5G), 1:1,000 pepstatin A (Sigma-Aldrich, catalog number: P5318-25MG)]. The protein concentration was measured by Bradford assay (Bio-Rad Laboratories). In the investigation of the p38-MAPK and GATA-3 phosphorylation, HaCaT keratinocytes were grown in a 12-well culture plate in concentration of 3×10^5 cells/mL and stimulated with EBN samples at different time points with or without 2-h pre-incubation of p38 inhibitor SB203580 (Cell Signaling Technology, Danvers, United States, catalog number: 5633). D-sorbitol (S6021-500G) at 400 mM was applied as a positive control in p38 phosphorylation. The cells were lysed, and the lysates were dissolved in lysis buffer containing 0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol [ACROS Organics (part of Thermo Fisher Scientific), catalog number: AC158922500], 2% 2-mercaptoethanol (Sigma-Aldrich, catalog number: M6250-100ML) and 0.02% bromophenol blue (Sigma-Aldrich, catalog number: 32712-5G) and denatured at 95°C for 5 min, three times. Reduced samples were normalized to 40 μg per lane and separated in 10% sodium dodecyl sulphate (SDS)-polyacrylamide gels. SDS-PAGE was run at 60–85 V and then transferred to nitrocellulose membranes. The membrane was blocked with 5% reduced fat milk or 5% BSA for 1 h at room temperature. After blocking, the membrane was incubated with primary antibodies overnight at 4°C. Primary antibodies used: mouse and rabbit anti-filaggrin antibody at 1:100 (Santa Cruz Biotechnology, Dallas, United States, catalog number: sc-66192 and sc-30229), rabbit anti-filaggrin-2 antibody at 1:1,000 (Bethyl Laboratories, Montgomery, United States, catalog number: A305-861A-M), rabbit anti-p38 MAPK antibody at 1:1,000 (Cell Signaling Technology, Danvers, United States, catalog number: 8690S), rabbit anti-phospho-p38 MAPK (Thr180/Tyr182) antibody at 1:1,000 (Cell Signaling Technology, catalog number: 4511S), rabbit anti-GATA3 antibody at 1:1,000 (Cell Signaling Technology, catalog number: 5852S), rabbit anti-phospho-GATA3 (Ser308) antibody at 1:1,000 (Thermo Fisher Scientific, catalog number: MA5-32144), rabbit anti-PPARα + PPARβ antibody at 1:2,000 (Abcam Ltd., Cambridge, United Kingdom, catalog number: ab1788865), rabbit anti-PPARγ antibody at 1:1,000 (Cell Signaling Technology, catalog number: 2435S), rabbit anti-CASP14 antibody at 1:1,000 (Cell Signaling Technology, catalog number: 8519S), and mouse anti-α-tubulin antibody at 1:10,000 (Sigma-Aldrich, catalog number: 3873S). The membranes were followed by secondary antibodies incubation for 2 h at room temperature, where HRP-conjugated antibodies at 1:2,000 (Zymed, South San Francisco, United States, catalog number: Rb7074S and Ms7076S) were used. Non-specific binding of protein was reduced with 0.1% Tween-20 TBST (pH 7.6) (anatrance, Maumee, United States, catalog number: T1003-500ML). The enhanced chemiluminescence (ECL) western blotting detection kit (Thermo Fisher Scientific, peroxide solution, catalog number: 1859701, luminol enhancer solution, catalog number: 1859698) was used. The protein amount was compared with the band intensities, measured under ChemiDoc Imaging System (Bio-Rad Laboratories).

Frozen *ex vivo* Mouse Skin Section

The dorsal skin was shaved and collected from C57BL/6 mice (2-months old) after being sacrificed by cervical dislocation. The isolated skin was rinsed with PBS and cultured in DMEM supplemented with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin (10,000 U and 10,000 μg/mL) in a humidified atmosphere with 5% CO_2 at 37°C. After the treatment, skin was rolled and embedded in optimal cutting temperature (OCT) compound (Thermo Fisher Scientific, catalog number: 6769006) in a tissue mold, followed by an overnight freezing under −80°C. Skin was then cut into 10 μm thick sections at −20°C using CryoStar™ NX70 Crystat (Thermo Fisher Scientific). The sections were melted onto slides, fixed with 4% paraformaldehyde (Sigma-Aldrich, catalog number: P6148-500G) for 30 min at room temperature and preserved at −80°C for following experiments.

Hematoxylin and Eosin Staining

After sectioning, *ex vivo* mouse dorsal skin sections were fixed with 4% paraformaldehyde for 30 min at room temperature. The fixed skin sections were then stained with the hematoxylin and eosin (H and E) staining kit (catalog number: ab245880) purchased from Abcam, Ltd. Sections were first washed with PBS twice for 5 min and dried, before the incubation in hematoxylin solution for 5 min. Hematoxylin-stained sections were washed with DDI water twice. Incubation with bluing reagent was performed for 10–15 s, sections were then washed with DDI water twice followed by absolute ethanol. Incubation with eosin solution was subsequently performed for 3 s, and dehydration with absolute ethanol were conducted. Sample was then mounted with dibutyl phthalate in xylene (DPX) reagent (Sigma-Aldrich, catalog number: 06522-100ML) for microscopic evaluation. Samples were examined by a Zeiss Axio Vert. A1 inverted phase microscope (Zeiss, Jena, Germany) with a 10X objective.

Immunofluorescent Staining

HaCaT keratinocytes were seeded on sterile coverslip (Marienfeld Superior, Lauda-Königshofen, Germany) in 35-mm culture plates at 5×10^4 cells/mL. Cultured keratinocytes and *ex vivo* mouse dorsal skin sections were fixed with 4% paraformaldehyde for 30 min at room temperature, followed by blocking with 5% BSA in PBS with or without permeabilization by 0.1% triton for 2 h at room temperature. After blocking, the cell or tissue sample was incubated with primary antibodies, anti-FLG at 1:50 (Santa Cruz Biotechnology), anti-GATA3 at 1:100 or anti-PPARγ at 1:100, overnight at 4°C. The samples were followed by secondary antibodies incubation for 2 h at room temperature under darkness, where Alexa 488 (donkey anti-mouse IgG, catalog number: ab150105) and 647 (donkey anti-rabbit IgG, catalog number: ab150075)-conjugated antibodies at 1:200 (Abcam Ltd.) were used. Samples were mounted with ProLong Gold Antifade Mountant with DAPI (Thermo Fisher Scientific, catalog number: P36931). Samples were then examined by a Leica SP8 Confocal Microscope (Leica Microsystems, Wetzlar, Germany) with a 63X oil immersion objective.

Atomic Force Microscopy

The mechanical properties of HaCaT keratinocytes were characterized by atomic force microscope (AFM) (MFP-3D, Asylum Research, Santa Barbara, United States) supplemented with a colloidal probe. The colloidal probe was prepared by gluing a glass sphere (~ 12 μm in diameter) onto the free end of a tipless cantilever (CSC38, tipless, MikroMasch) with a typical spring constant $k \sim 0.09N/m$ (Shen et al., 2017). The exact spring constant of the modified cantilever was calibrated by thermal power spectral density method (Shen et al., 2017). Before each AFM experiment, the probe surface was coated with a thin layer of poly (L-lysine)-graft-poly (ethylene glycol) (PLL-g-PEG) (SuSoS AG, Dübendorf, Switzerland) to avoid adhesion between the probe and cell surface. Cultured keratinocytes for AFM measurement were seeded onto a sterile coverslip (Marienfeld Superior) in 35-mm culture plates at 5×10^4 cells/mL and cultured in DMEM supplemented with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin (10,000 U and 10,000 μg/mL). After the application of EBN samples, including extract and digest, for 24 h, cells were rinsed with sterile PBS twice and then transferred into an AFM closed fluid chamber, where ambient buffer environment was provided. The AFM measurement for EBN-treated cells was performed in PBS solution at 37°C in a humidified atmosphere of 5% CO_2. AFM measurements were also performed in hypertonic and hypotonic PBS solutions to characterize different cell properties in various osmotic conditions, serving as negative and positive controls. The hypotonic and hypertonic PBS solutions were prepared based on Ayee et al. (2018), with osmolarity of 227 and 366 mOsm/kg, respectively, measured by Advanced Model 3320 Micro-Osmometer (Advanced Instruments, Norwood, United States).

Statistical Analysis

Data for control and drug-treated groups were compared using one-way ANOVA with Dunnett post-hoc statistical testing provided in GraphPad Prism 8.3.0. Results were calculated from at least 3 independent determinations, performed in triplicates and expressed as percent increase or fold change of control in mean ± SEM. Statistical significance was indicated by * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. Statistical comparison in **Figure 8B** (lower panel) was made by two tailed *t*-test, * $p < 0.05$, and *** $p < 0.001$, compared to indicated group pre-treated with SB203580.

RESULTS

The Extract and Digest of EBN Induce Expressions of Filaggrin and Filaggrin-2

The HPLC chromatograms of EBN extract and its enzymatic digested product were illustrated in **Figure 1**, which were obtained by injections of 10 μL of 10 mg/mL EBN extract, or 100 μL 10 mg/mL EBN digest, or 10 μL 1 mg/mL protein markers. The UV absorbance of protein/peptide was measured at wavelength 280 nm. The peak eluted at 20 min was the only peak observed from EBN extract, suggesting that EBN was formed in a large complex protein with an oversized molecular weight at least >2,000 kDa. After 48 h of digestion by simulated gastric fluid with pepsin, the digested product was subjected to HPLC analysis. In the EBN digest, numerous peaks were identified in the chromatogram, and the peak at 20 min of undigested EBN complex was markedly decreased, i.e. digestion to smaller protein/peptide (**Figure 1**). The chromatogram of protein markers suggested that most of the digested EBN was migrated at small molecular weight, i.e. ~1.7 kDa. By estimation, the protein digestion reached over 90% of completion. This fingerprint of digested EBN could serve as a quality control of the product.

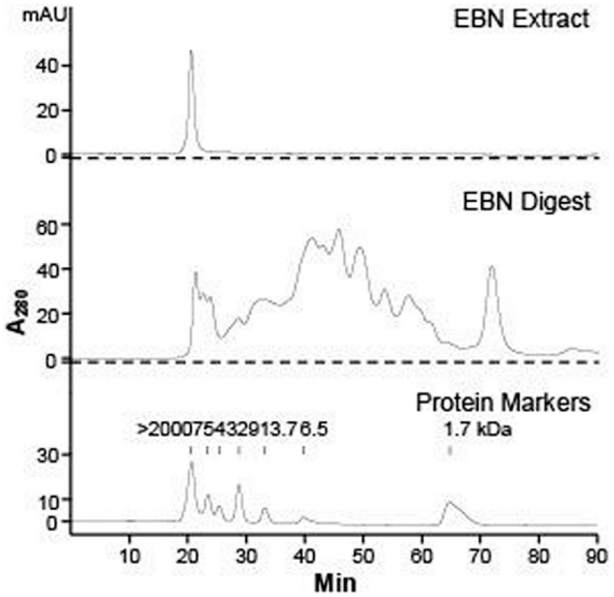

FIGURE 1 | HPLC chromatogram of EBN extract and digest. Gel chromatographic separations of Malaysia white house EBN extract (10 mg/mL, injection volume = 10 μL) and digest (10 mg/mL, injection volume = 100 μL) under UV wavelength of 280 nm are shown. The molecular weight of protein markers of α-melanocyte-stimulating hormone (1.7 kDa), aprotonin (6.5 kDa), ribonuclease A (13.7 kDa), carbonic anhydrase (29 kDa), ovalbumin (43 kDa), albumin (75 kDa), and blue dextran (> 2000 kDa) are indicated.

To assess the ability of EBN in promoting the moisturizing effect of skin keratinocytes, the extract and digest of EBN were tested for regulation of mRNAs encoding filaggrin and filaggrin-2, two specific proteins playing critical role in skin water maintenance. **Figure 2A** shows the location of primers flanking the two genes. The EBN digest, instead of EBN extract, showed robust induction of filaggrin and filaggrin-2 expressions in dose-dependent manners: the maximal induction was at over 2.5-fold as compared to the control under high calcium condition (1.8 mM) (**Figure 2B** upper panel). This induction was also revealed in low calcium culture medium, where EBN digest again showed more significant induction, peaked at 1.5 to 2.2-fold of control (**Figure 2B** lower panel). The mRNA inductions of filaggrin and filaggrin-2 were higher under culture condition with higher Ca^{2+} concentration. In both scenarios, the EBN

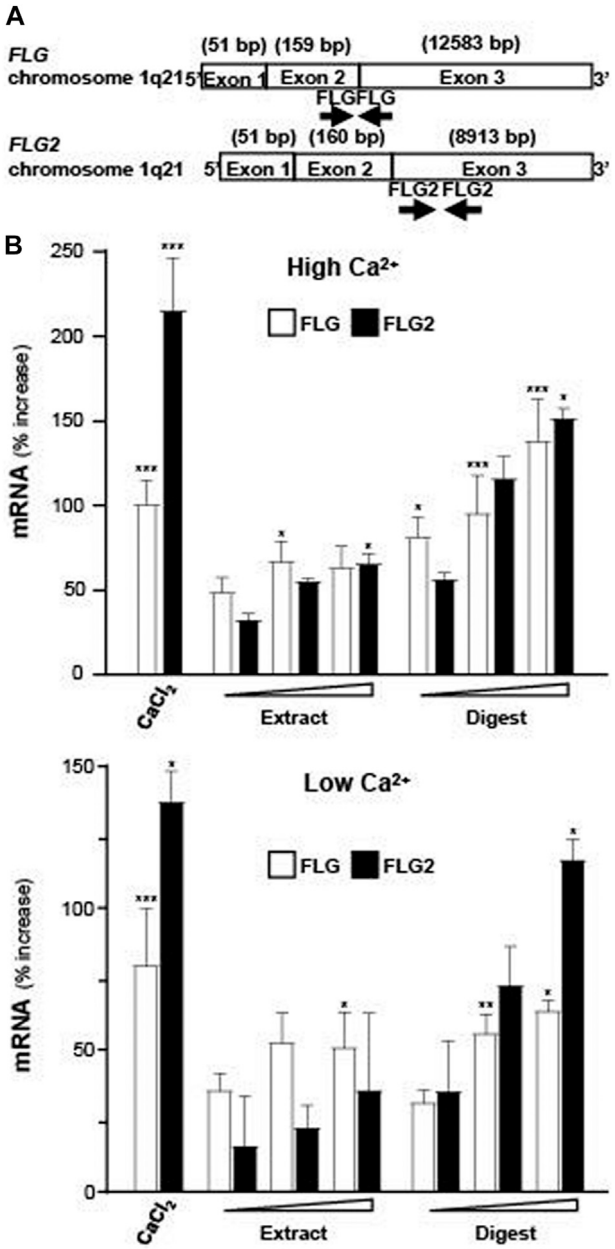

FIGURE 2 | Expressions of filaggrin and filaggrin-2 are evaluated by real-time PCR. **(A)** The schematic diagrams of primers flanking the exons of human filaggrin (FLG) and filaggrin-2 (FLG2) genes on chromosome 1q21 are indicated. **(B)** Filaggrin and filaggrin-2 mRNA levels in cultured differentiated (upper panel; 1.8 mM Ca^{2+}) and undifferentiated (lower panel; low Ca^{2+}) keratinocytes after 24-h treatments of EBN extract and digest, in doses of 1, 10, 100 μg/mL, as indicated. $CaCl_2$ at 0.16 mM was adopted as a positive control. Values are expressed as the percentage of increase in relative to normalized basal expression set at 0, in mean ± SEM, $n = 4$. Statistically significant results are marked with * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ against the control.

digest showed much better efficacy in inducing the mRNA expression than that of EBN extract, suggesting the digestion of EBN could generate effective peptides and/or small chemicals. External applied Ca^{2+} was adopted as a positive control (Li et al., 1995).

The promoters of filaggrin-2, tagged downstream with an eGFP construct (pFLG2-eGFP), was used here for illustration of activated gene transcription. In pFLG2-eGFP transfected keratinocytes, the fluorescent signal, representing the promoter activity, was induced by applications of EBN extract and EBN digest (**Figure 3A**). The quantitation showed robust activity, at least 50% increase of pFLG2-eGFP fluorescence, triggered by EBN digest, similar to the positive control of Ca^{2+} application: the extract of EBN showed insignificant effect (**Figure 3B**). Filaggrin is a non-membrane-bound protein localized in keratohyalin granules (Presland et al., 2001). Thus, the amount of filaggrin protein was revealed by immunochemical staining in cultured keratinocyte. The treatments of EBN extract and digest in keratinocyte induced the overall cellular staining of filaggrin, which again showed better effect in the scenario of EBN digest (**Figures 3C,D**).

The protein expressions of filaggrin and filaggrin-2, induced by EBN, were determined by western blotting. Profilaggrin is dephosphorylated, deiminated and proteolyzed into isomeric repeat intermediates. The bands at ~350 kDa referred to profilaggrin, showing the multimeric complex (**Figure 4A**). The total filaggrin expression was summed for the band intensities corresponding to trimeric (~90 kDa), dimeric (~60 kDa), and monomeric (~25 kDa) forms in the blot (**Figure 4A**). In parallel, the band of ~238 kDa was corresponding to flaggrin-2 (**Figure 4A**). By quantitation of the band intensities, the digest of EBN showed induction of protein expressions, both in filaggrin (**Figure 4B**) and filaggrin-2 (**Figure 4C**), significantly, which were in dose-dependent manners. EBN extract showed the protein induction at low magnitude than that of the digest, which was in line to the results of mRNA and promoter assays.

Signaling of EBN-Induced Expression of Filaggrin and Filaggrin-2

MAPK signaling pathways have been shown to mediate the functions of keratinocyte differentiation and skin barrier (Meng et al., 2018). The activation of p38 resulted from the phosphorylation of Thr-Gly-Tyr (TGY) motif in kinase domain (Cargnello and Roux, 2011). To identify whether EBN in regulating the expressions of filaggrin and filaggrin-2 *via* p38-MAPK signaling, the p38-MAPK phosphorylation, activated by EBN extract and digest, was performed. The treatment of EBN extract and digest increased the p-p38 MAPK/p38 MAPK ratio by 2–3 folds after 60 min (**Figures 5A,C,D**). Furthermore, the pre-incubation of p38 MAPK inhibiter SB203580 significantly suppressed the activation of p38-MAPK, as triggered by EBN extract and/or digest (**Figures 5A,C,D**), showing the specificity of EBN-activated p38-MAPK pathway. D-sorbitol served as a positive control in activating p38 phosphorylation (Clerk et al., 1998) (**Figures 5A,B**).

GATA3, a key transcriptional factor in regulating the expressions of filaggrin and filaggrin-2, was hypothesized here to be involved as an upstream target of the EBN-mediated gene

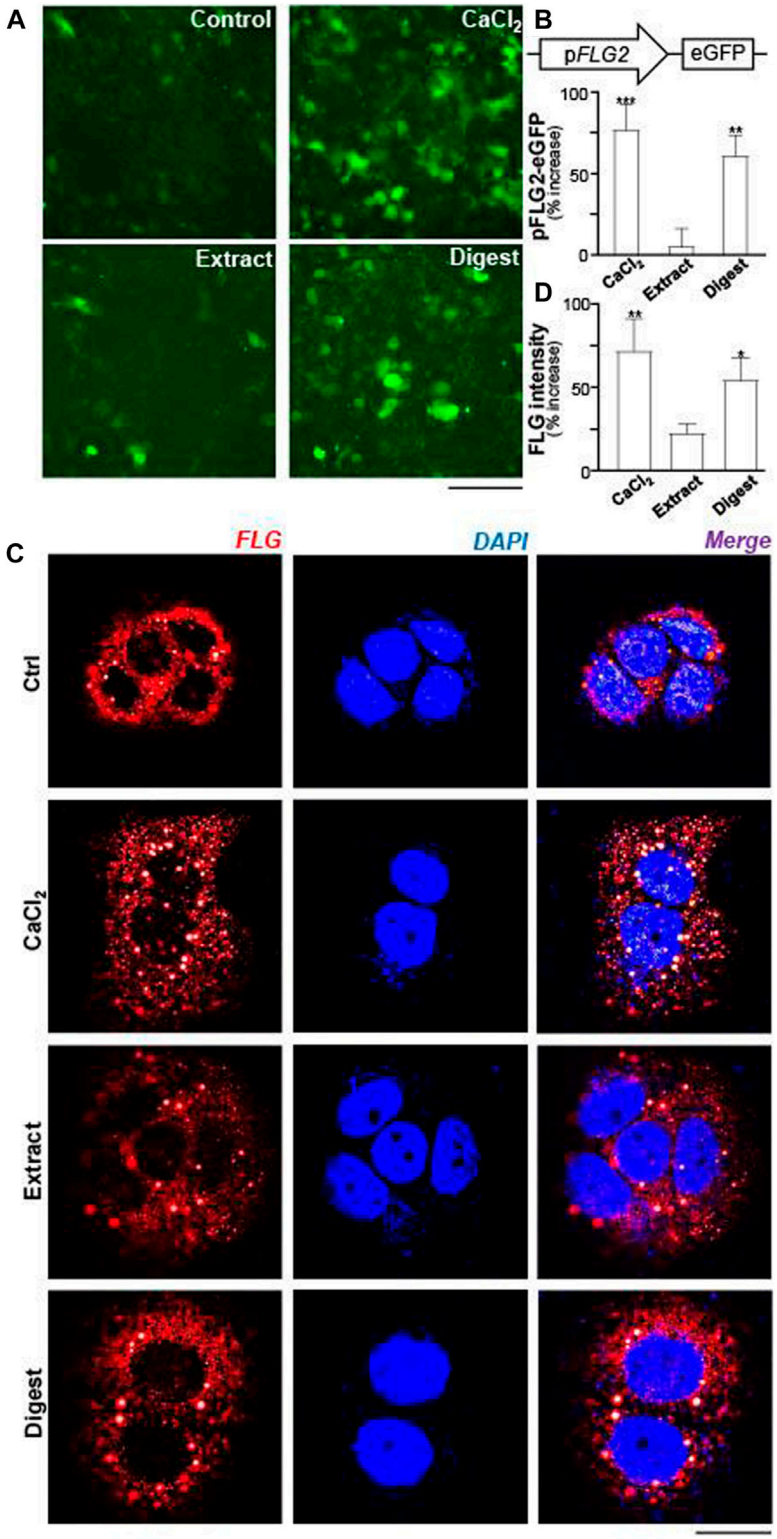

FIGURE 3 | EBN induces the activity of pFLG2-eGFP and filaggrin expression. **(A)** The fluorescence images of pFLG2-eGFP transfected keratinocytes after 24-h treatments of $CaCl_2$ (0.16 mM, a positive control), EBN extract (100 μg/mL), and EBN digest (100 μg/mL) are shown, $n = 3$. Bar = 100 μm. The pFLG2-eGFP plasmid was composed of FLG2 promoter tagged with EGFP reporter. **(B)** The quantifications from **(A)** are expressed as the percentage of increase in fluorescence intensity measured under excitation/emission wavelength 488/509 nm to normalized basal activity set at 0 in mean ± SEM, $n = 6$. **(C)** Cytosolic expression of FLG protein in HaCaT keratinocytes after 24-h treatments of EBN extract (100 μg/mL) and EBN digest (100 μg/mL) are shown, in representative confocal images. $CaCl_2$ at 0.16 mM was adopted as a positive control. $n = 3$. Bar = 20 μm. **(D)** The quantifications from **(C)** are expressed as the percentage increase in filaggrin fluorescence intensity to normalized basal activity set at 0 in mean ± SEM, $n = 3$. The filaggrin fluorescence intensity of each cell was normalized to total cell area for quantification. Statistically significant results are marked with * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ against the control.

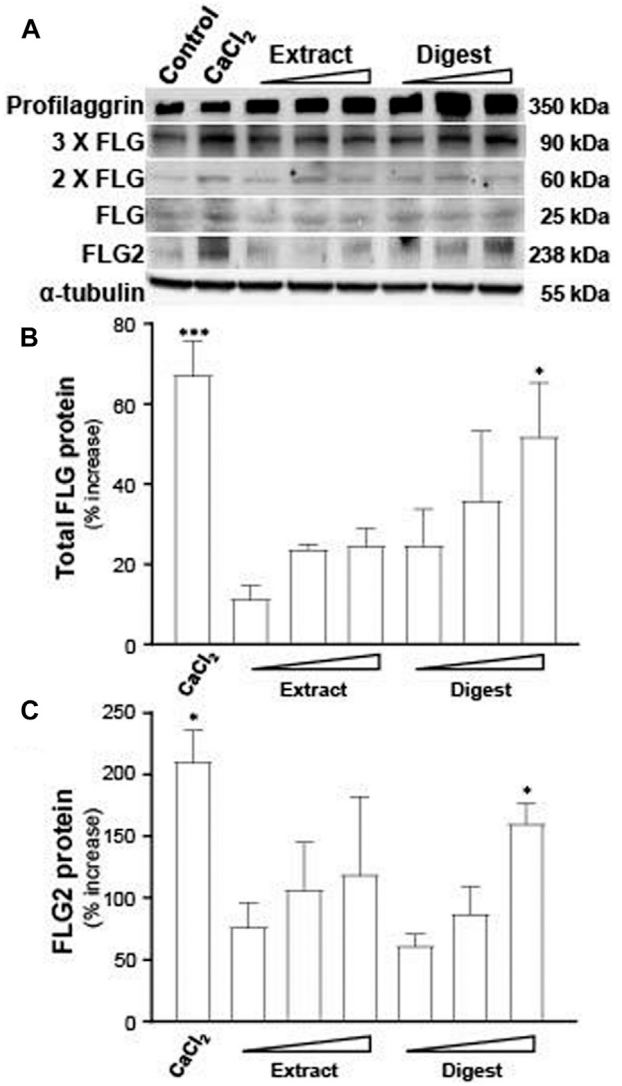

FIGURE 4 | EBN induces the protein levels of filaggrin and filaggrin-2. **(A)** The protein levels of filaggrin (FLG) and filaggrin-2 (FLG2) in cultured differentiated keratinocytes, after 24-h treatments of EBN extract and digest, were determined in doses of 1, 10, 100 μg/mL, as indicated. $CaCl_2$ at 0.16 mM was adopted as a positive control. **(B)** The total filaggrin (summation of the isomeric intermediates) and **(C)** filaggrin-2 protein levels, relative to α-tubulin protein, are expressed as the percentage increase to normalized basal expression set at 0 in mean ± SEM, $n = 3$. Statistically significant results are marked with * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ against the control.

expression in cultured keratinocytes. To validate the role of GATA3, RT-PCR was employed to quantify the expression of GATA3 mRNA under the treatment of EBN. The treatment of EBN digest increased the mRNA level by ~50% in a dose-dependent manner, better than that of EBN extract (**Figure 6**). IL-4 served as a positive control in activating GATA3 transcription (Onderdijk et al., 2015).

Peroxisome proliferator activated receptors (PPARs) are ligand-activated nuclear transcription factors expressed in human skin keratinocytes, and the reported subtypes were PPARα, PPARβ, and PPARγ (Sertznig et al., 2008). The three PPAR variants are known to induce filaggrin expression in both *in vitro* and *in vivo* models (Harding and Rawlings, 2000). To evaluate whether PPARs participated as an upstream target in the EBN-mediated regulation of filaggrin and filaggrin-2 in cultured keratinocytes, the mRNA levels of PPARα, PPARβ, and PPARγ were determined. Linoleic acid (C18:2), a positive control (Kliewer et al., 1994), activated the PPAR variants by 50–150% of increase (**Figure 6**). In cultured keratinocytes treated with EBN, the amounts of PPARα, PPARβ, and PPARγ mRNAs were increased in dose-dependent manners (**Figure 6**). The inducing response was more robust in a scenario of PPARβ under the challenges of EBN digest. In all scenarios, the extract of EBN showed much less induction as that of EBN digest.

Caspase 14 (CASP14) is a Ca^{2+}-dependent cysteine protease involved in the cell senescence of epidermal keratinocytes. CASP14 plays roles in proteolysis and deimination of profilaggrin and filaggrin-2 proteins, leading to keratinocyte differentiation and cornified envelope formation (Hoste et al., 2011; Masse et al., 2014). To assess the ability of EBN in upregulating CASP14 expression, RT-PCR assay was performed. 1α, 25-dihydroxycholecalciferol (vitamin D_3) was adopted as a positive control (Lippens et al., 2004). EBN extract and digest increased the expression levels of CASP14 in a dose-dependent manner: the EBN digest showed better response (**Figure 6**). Thus, EBN could be able to induce CASP14 expression involving in the proteolysis of filaggrin and filaggrin-2 to form NMFs during late differentiation of keratinocyte.

Besides mRNA, the protein expressions of GATA3 (~48 kDa), PPARα (~53 kDa), PPARβ (~50 kDa), PPARγ (~53 kDa), and CASP14 (~28 kDa) were determined (**Figure 7A**). α-Tubulin served as a loading control. In all scenarios, the treatments of EBN extract and digest in cultured keratinocytes were able to significantly induce the protein expressions, in dose-dependent manners (**Figures 7B–F**). The robust activation, triggered by EBN digest, was revealed in all cases (**Figure 7**). EBN extract showed protein induction as well but at a lower magnitude to that of EBN digest.

The levels of those transcriptional factors are not crucial regulators to activate the expressions of flaggrin and/or flaggrin-2 in skin cell. Instead, the activation of transcriptional factors by phosphorylation or dimerization is the key in regulating the gene transcription. For example, the phosphorylation of *p*-GATA3 by ~3-fold in keratinocytes was triggered by IL-4, a positive control, in a transient manner: the IL-4 induced phosphorylation was blocked by a p-38 MAPK inhibitor, i.e., SB203580 (**Figures 8A,B**). The digest of EBN activated the phosphorylation of GATA3 in a time-dependent manner to a maximal increase of ~2.5-fold (**Figures 8A,B**). Similar to the situation of IL-4, the phosphorylation was blocked by applied SB203580.

In EBN-treated keratinocyte, the translocation of *p*-GATA3 was identified by immunochemical staining. The application of EBN extract and digest induced the nucleus localization of *p*-GATA3, as revealed by specific staining at the nucleus (**Figure 8C**). In addition, the nuclear translocation of GATA3 was inhibited by the p-38 MAPK

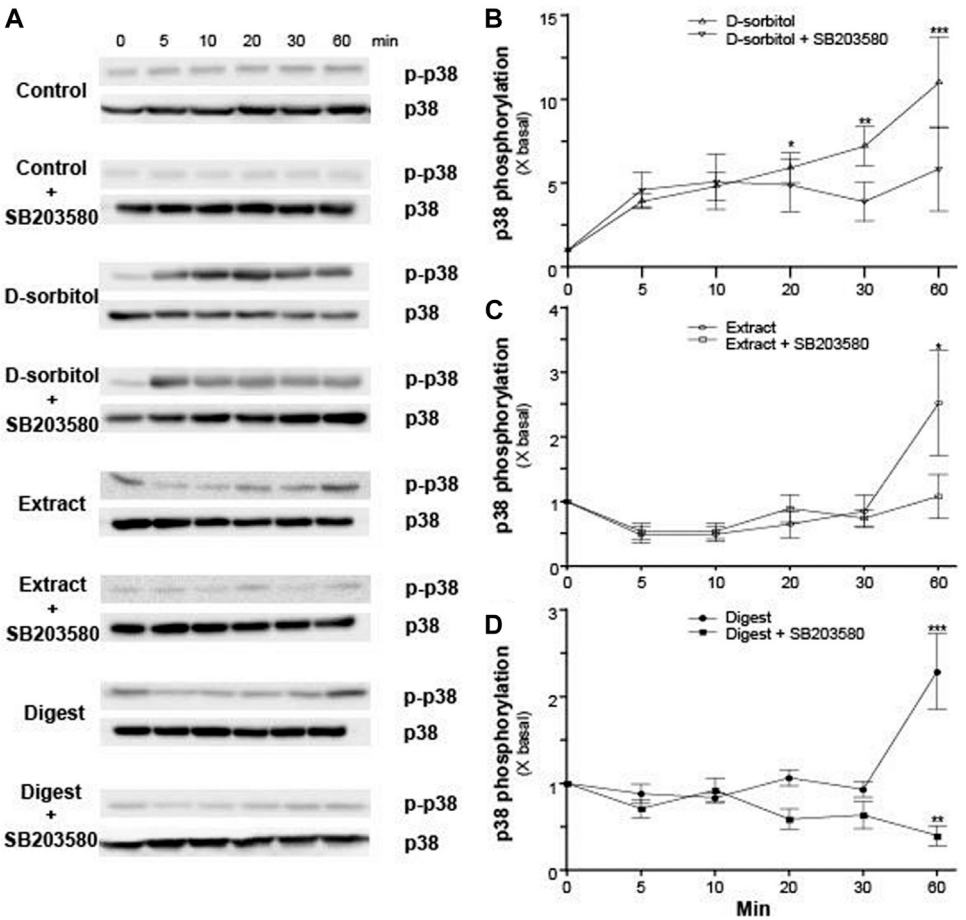

FIGURE 5 | The p38 MAPK inhibitor blocks EBN-induced p38-MAPK phosphorylation. **(A)** After 16-h serum starvation, cultured differentiated keratinocytes were pre-incubated with or without SB203580 (a p38 kinase inhibitor; 10 μM) for 2 h, followed by different times of applied D-sorbitol (a positive control, 400 mM), EBN extract or digest (both at 100 μg/mL). Total p38-MAPK (p38-MAPK) and phosphorylated p38-MAPK (p-p38 MAPK) (both at ~38 kDa) were evaluated by western blot assays. The quantifications of p-p38 MAPK/p38 MAPK expression levels after different time of applied **(B)** D-sorbitol, **(C)** EBN extract, and **(D)** EBN digest with or without SB203580 pretreatment are shown. Values are expressed in mean ± SEM, $n = 3$. Statistically significant results are marked with * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ against the control.

inhibitor (SB203580) (**Figures 8C,D**). In parallel, the translocation of *p*-GATA3 from cytosol to nucleus was further confirmed by staining with total GATA3 specific antibody, which had recognized the nuclear localization of the protein after IL-4 and EBN treatments (**Figure 9A**). In addition, the nucleus translocation of another transcriptional factor, PPARγ, was illustrated. Application of EBN extract/digest and linoleic acid (a positive control) in keratinocyte induced the accumulation of PPARγ in the nucleus (**Figure 9B**). By quantification of percentage of GATA3 and PPARγ intensities in nuclear to cytosol, the digest of EBN showed more significant activation and translocation of the transcription factors (**Figure 9C**).

EBN Induces Filaggrin in Skin and Cell Moisturization in Keratinocyte

The functional role of EBN in regulating filaggrin and filaggrin-2 was further demonstrated in mouse skin *ex vivo*. After the treatment with EBN, the skin section was stained with filaggrin. The amount of staining was robustly increased in the epidermal level of skin, after treatment with EBN extract and digest, as well as the control of external applied Ca^{2+} (**Figure 10A**). All treatments did not increase the epidermal thickness (**Figure 10B**). Similar to the cell culture study, the effect of EBN digest showed more robust induction of filaggrin expression, i.e. moisturizing effect, as compared to EBN extract.

With a multiscale relaxation model, the viscoelastic properties of cultured keratinocytes could be measured by AFM (**Figures 11A,B**) and described with relaxation modulus:

$$E(t) = E_1 e^{-\frac{t}{\tau_1}} + E_2\left(1 + \frac{t}{\tau_2}\right)^{-\alpha} + E_\infty$$

The relaxation modulus $E(t)$ consists of three parts, namely a short-time exponential decay followed by a long-time power law decay, and a persistent modulus that does not vanish at an infinite time. In the above equation, E_1, E_2, E_∞ are the moduli of

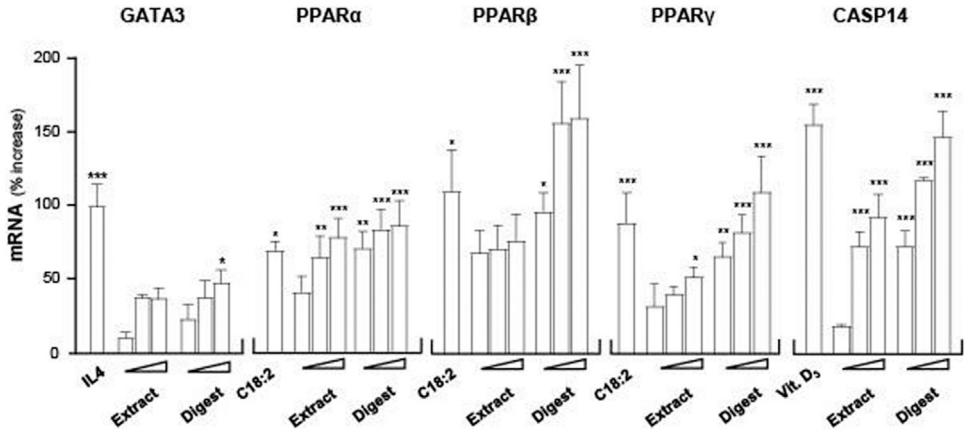

FIGURE 6 | EBN induces mRNA expressions of targeted genes corresponding to moisturize skin. The mRNA levels of GATA3, PPARα, PPARβ, PPARγ, and CASP14 in cultured differentiated keratinocytes after 24-h treatments of EBN extract and digest were determined in doses of 1, 10, 100 μg/mL (as indicated). IL-4 at 100 ng/mL, linoleic acid (C18:2) at 30 μM and cholecalciferol (vitamin D_3) at 10^{-5} M were adopted as positive controls in GATA3, PPARs, and CASP14 expressions. Values are expressed as the percentage of increase in mRNA amount relative to normalized basal expression set at 0 in mean ± SEM, $n = 4$. Statistically significant results are marked with * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ against the control.

exponential component, power-law component, and persistent component, respectively; τ_1, τ_2 are the scales for exponential decay and power-law decay; α is the power-law exponent. The initial modulus $E_0 = E_1 + E_2 + E_\infty$ is the measured modulus at time $t = 0$, which is considered as a measure of apparent rigidity of whole cell when indenting at high speed. A typical measurement of normalized relaxation modulus $E(t)/E_0$ is given (**Figure 11C**).

In this relaxation model, the four parameters: initial modulus E_0, exponential modulus component E_1, exponential relaxation time τ_1 and power-law exponent α are jointly correlating with the moisturization states of the cells. The reasons could be shown below. Firstly, the short-time exponential relaxation represents the reorganization of biopolymers in cytosol. When the cell absorbs water due to osmotic stress or moisturization, the cell becomes softer, and therefore the measured modulus E_0, E_1 become smaller (Moeendarbary et al., 2013; Liao et al., 2018). In addition, the exponential relaxation time is $\tau_1 \sim b^2/D$ (b is the mesh size of cytoskeleton network, and D is the diffusion coefficient of biopolymers within the mesh). Having higher water content in the cell, biomolecules diffuse faster as they are diluted, leading to an increase of diffusion coefficient and subsequent decrease of τ_1 (Miermont et al., 2013; Moeendarbary et al., 2013). Secondly, the power-law relaxation α is relating to the structure changes of the cytoskeleton, which characterizes the fluidity of the cytoskeleton network within a range from 0 to 1 (Hecht et al., 2015). $\alpha = 0$ corresponds to a purely elastic solid; since it does not relax with time. $\alpha = 1$ corresponds to a purely viscous Newtonian fluid (Hecht et al., 2015). The increase of α indicates more liquid-like behavior of the cell skeleton, i.e., moisturization of the cell. Thus, the correlated changes of four parameters provide the state of moisturization in skin keratinocyte.

The parameters, τ_1, α, E_0, and E_1, were measured in cultured keratinocytes after treatment of EBN extract and digest. These parameters were compared to values measured for keratinocytes under hypotonic and hypertonic conditions. For keratinocytes under hypotonic condition, simultaneous decreases of τ_1, E_0, and E_1, as well as increase of α, were identified, which clearly showed the moisturization of cells. In contrast, the hypertonic condition caused opposite changes of these parameters implying dehydration of cells (**Figure 11D**). The correlated statistically significant changes of the four parameters in EBN-treated keratinocytes indicated that EBN extract and digest could promote the moisturization of cells, similar to cell behavior under hypotonic environment (**Figure 11D**). The 24-h treatment of EBN digest caused better changes of α and E_1 values than that of EBN extract, which suggested that the cell skeleton became more fluid-like and more water was retained in the cytosol. Thus, the analysis by AFM further validated the moisturizing effect of EBN digest in cultured keratinocytes from the perspective of cell mechanics.

DISCUSSION

In Southeast Asian countries, EBN has been known for its ability to prevent lung diseases, strengthen the immune system, and improve complexion. Epidermal growth factor (EGF) has been found in EBN, which has proposed to correspond the proliferation effect of EBN in epidermal tissues (Kong et al., 1987). In addition, N-acetylneuraminic acid, contained in EBN, possessed a skin-whitening function (Chan et al., 2015), and additionally EBN was shown to reduce water loss, wrinkle area, and dermal thickness of skin (Terazawa and Shimoda, 2020). Although there are many claims on the skin-promoting effects and medicinal values of EBN, there has been very little scientific evidence showing the functions, and/or the active ingredients, of EBN in moisturizing skin. Here, we have provided different evidence to reveal the signaling pathway of EBN extract and

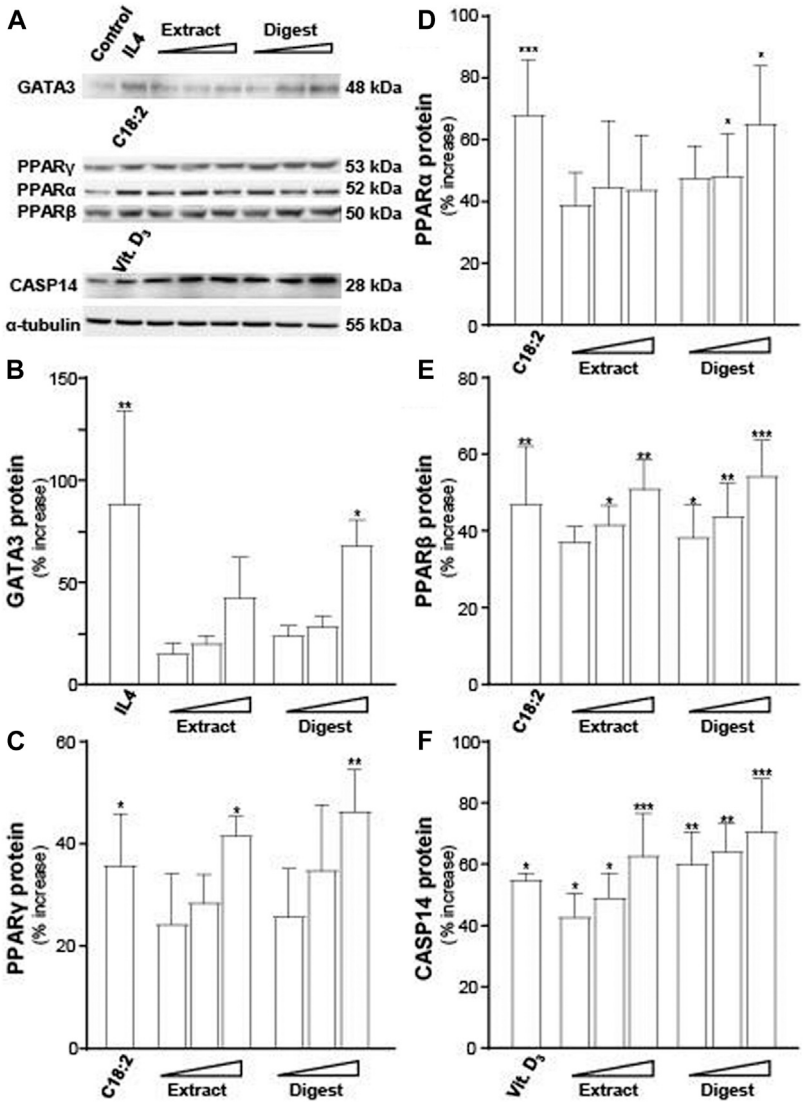

FIGURE 7 | EBN induces the expressions of targeted proteins corresponding to moisturize skin. **(A)** Western blot membranes showing GATA3, PPARs, and CASP14 protein expressions after 24-h treatments of EBN extract and digest were assessed in doses of 1, 10, 100 μg/mL (as indicated). IL-4 at 100 ng/mL, linoleic acid (C18:2) at 30 μM and cholecalciferol (vitamin D_3) at 10^{-5} M were adopted as positive controls in GATA3, PPARs, and CASP14 expressions. The quantification of **(B)** GATA3, **(C)** PPARγ, **(D)** PPARα, **(E)** PPARβ, and **(F)** CASP14 are shown. The expression levels, in relative to α-tubulin protein, are expressed as the percentage of increase to normalized basal expression set at 0 in mean ± SEM, $n = 3$. Statistically significant results are marked with * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ against the control.

digest in regulating the expressions of filaggrin and filaggrin-2, two important skin barrier proteins of the SFTP family playing roles in water balance of skin surface. The EBN-mediated regulation of filaggrin and filaggrin-2 is demonstrated to be triggered by p38-MAPK signaling pathway and various transcriptional factors, e.g. GATA3, PPARα, PPARβ, and PPARγ.

The epidermis composes of four layers: stratum basale, stratum spinosum, stratum granulosum, and stratum corneum from bottom to top. Keratinocytes are proliferative in the basal layer and then keratinized, or differentiated, from the spinous to cornified layers; whereas the Ca^{2+} concentration increases as it moves up the layers (Bikle, 2018). The increase in extracellular Ca^{2+} concentration facilitates the exocytosis of lipid-containing lamellar bodies and the proteolysis of filaggrin into NMFs, which function as retaining water and maintaining elasticity in stratum corneum. The enzymatic digestion of EBN was devised to enhance the protein extraction rate, as well as to increase the release of sialic acid from EBN (Wong et al., 2018a). In the enzymatic digestion, pepsin in simulated gastric fluid was used to mimic the gastric digestion environment in the human body and to maximize the digestion of the large proteins contained in EBN. As shown here, the digested product of EBN significantly increased filaggrin and filaggrin-2 expressions at mRNA and protein levels, dose-dependently. This suggests that the

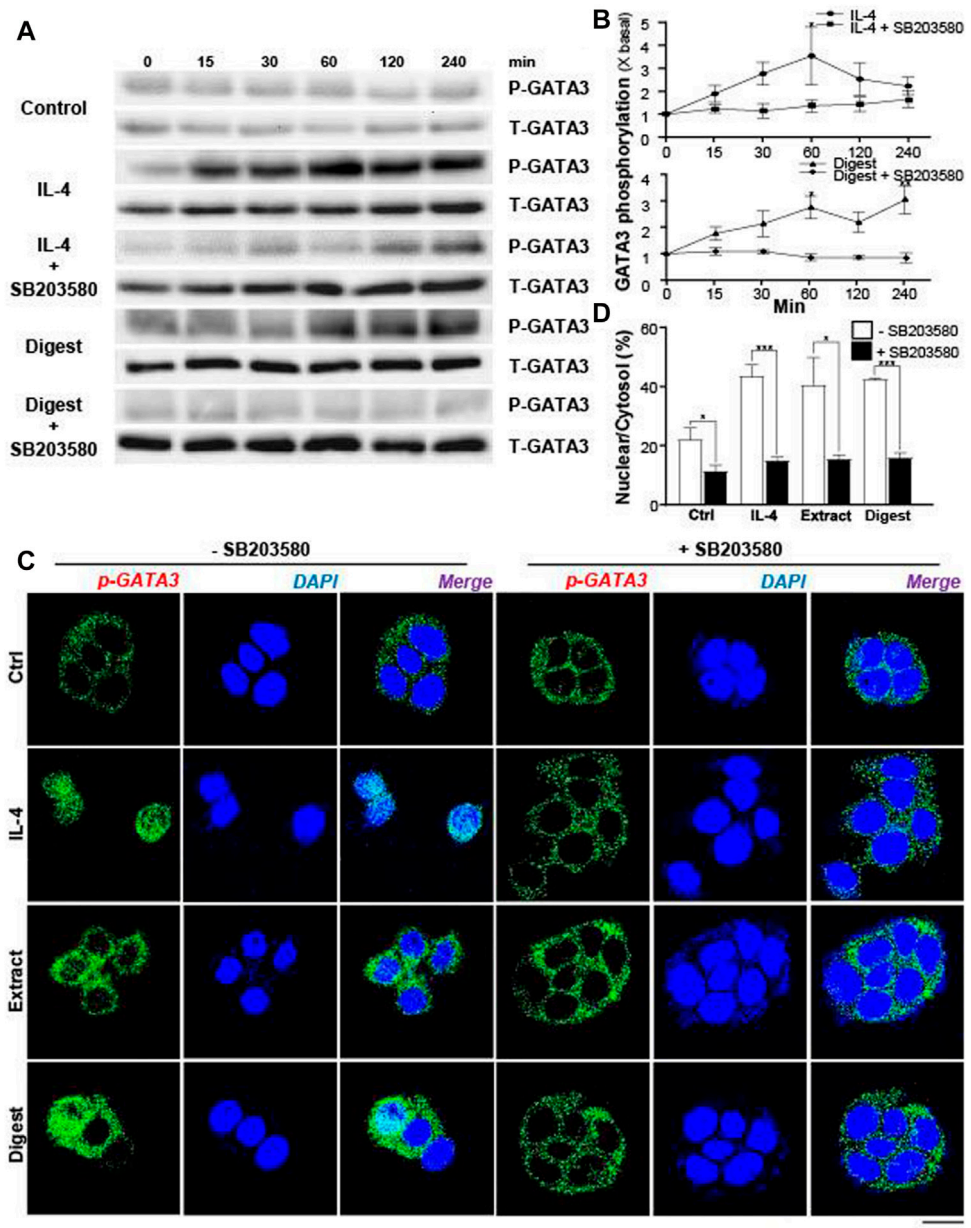

FIGURE 8 | EBN induces phosphorylation of GATA3 and its nuclear translocation. **(A)** After 16-h serum starvation, cultured differentiated keratinocytes were pre-incubated with or without SB203580 (10 μM) for 2 h, followed by different time of applied IL-4 (a positive control, 100 ng/mL) or EBN digest (100 μg/mL). Total GATA3 (T-GATA3) and phosphorylated GATA3 (P-GATA3) (both at ~48 kDa) were evaluated by western blot assays. **(B)** The quantification of the increased fold of P-GATA3/T-GATA3 expression levels from **(A)** after different time of applied IL-4 and EBN digest, with or without SB203580 pre-treatment, are shown. Values are expressed in mean ± SEM, $n = 3$. Statistically significant results are marked with * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ against the control. **(C)** After 16-h serum starvation, cultured differentiated keratinocytes were pre-incubated with or without SB203580 (10 μM) for 2 h, followed by 240-min treatment of IL-4 (100 ng/mL, a positive control), EBN extract (100 μg/mL) and EBN digest (100 μg/mL). Phosphorylated GATA3 protein localization in keratinocytes was determined by immunofluorescence assays. $n = 3$. Bar = 20 μm. **(D)** The quantification of the percentage of nuclear to cytosol level of P-GATA3 intensities under different treatments from **(C)** are shown. Values are expressed in mean ± SEM, $n = 3$. Statistically significant results are marked with * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ against the control or as indicated.

enzymatic digestion can release small functional peptides from EBN that specifically increase the gene expression. Additionally, EBN digest showed better efficiency, as compared to EBN extract, in increasing the protein expressions. Niacinamide, vitamin B3, is a well-known barrier repair vehicle widely used as a moisturizer and body wash ingredient (Del Rosso, 2011), as well as a known PPAR agonist in enhancing filaggrin biosynthesis (Harding and Rawlings, 2000). According to Nisbet et al. (2019), 0.1% niacinamide induced a nearly 3-fold change of filaggrin mRNA level in HaCaT keratinocytes. Thus, the moisturizing effect of digested EBN in protecting skin barrier is comparable to that of niacinamide.

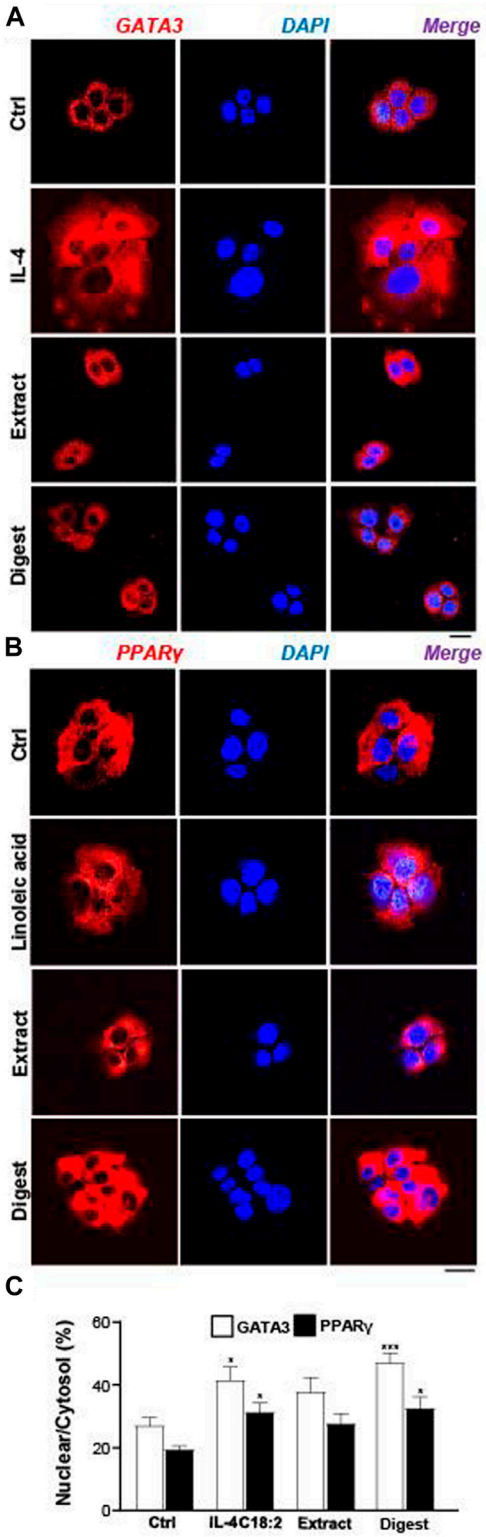

FIGURE 9 | EBN induces nucleus localizations of GATA3 and PPARγ proteins. The immunofluorescent staining of **(A)** GATA3 or **(B)** PPARγ in cultured differentiated keratinocytes after 24-h treatments of EBN extract (100 μg/mL) and EBN digest (100 μg/mL) are shown. IL-4 at 100 ng/mL and linoleic acid (C18:2) at 30 μM were adopted as positive controls in GATA3 and PPARγ expression. Bar = 20 μm. **(C)** The quantification of the percentage of nuclear GATA3 to cytoplasmic GATA3, or PPARγ, are shown. Values are expressed in mean ± SEM, $n = 3$. Statistically significant results are marked with * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ against the control.

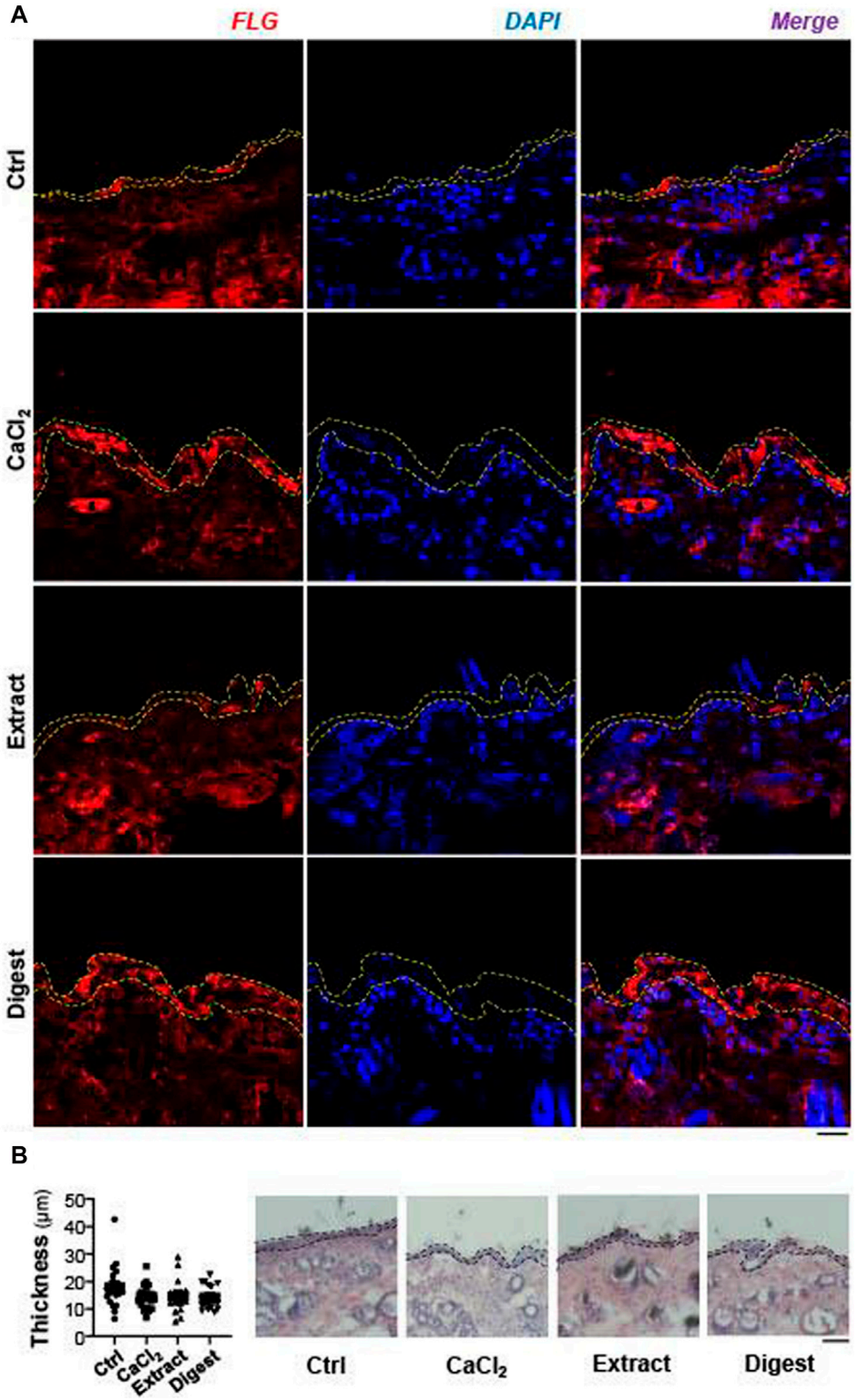

FIGURE 10 | EBN induces filaggrin in *ex vivo* mouse skin. **(A)** The expression of filaggrin (FLG) protein in 2-month-old mouse dorsal skin *ex vivo* after 24-h treatments of EBN extract (100 μg/mL) and EBN digest (100 μg/mL) were assessed by immunofluorescent staining. $CaCl_2$ at 0.16 mM was adopted as a positive control, $n = 3$. Bar = 20 μm. Yellow dash lines indicate the fluorescent area of filaggrin. **(B)** The epidermal thickness of *ex vivo* mouse dorsal skin after 24-h treatments of $CaCl_2$ (0.16 mM), EBN extract (100 μg/mL) and EBN digest (100 μg/mL) were assessed by H and E staining. Black dash lines include epidermis layer (right panel). The quantification of five randomly selected distance points were assessed for the epidermal thickness (left panel). $n = 3$. Bar = 100 μm.

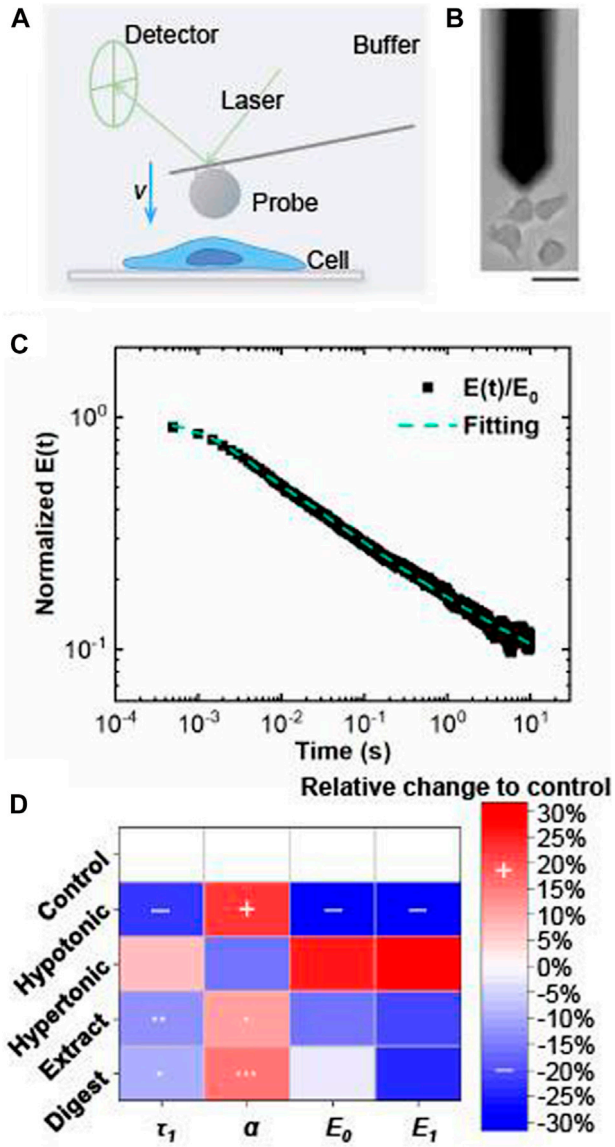

FIGURE 11 | Viscoelastic properties of EBN-treated keratinocyte evaluated by atomic force microscopy. **(A)** Atomic force microscopy (AFM) setup for mechanical measurements of single cell (side view). **(B)** Bright field image of AFM measurement (top view). Bar = 35 μm. **(C)** A sample curve of normalized relaxation modulus $E(t)/E_0$ as the function of time. Green dash line is fitting to the measured relaxation data, showing a short-time exponential decay followed by a long-time power-law decay. **(D)** Relative changes of E_0, E_1, τ_1, and α to the control group under different treatments in cultured keratinocyte, as in **Figure 2**. Simultaneous decrease of E_0, E_1, τ_1, and increase of α show the moisturization of cells; while increase of E_0, E_1, τ_1, and decrease of α show the dehydration of cells. The parameter map indicates that EBN-treated keratinocyte (100 μg/mL extract n = 30, 100 μg/mL digest n = 31) becomes moisturized, similar to cells under hypotonic condition (227 mOsm/kg, n = 6) and opposite to hypertonic one (366 mOsm/kg, n = 10). Statistically significant results are marked with * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ against the control.

In addition to transcriptional regulation, we suspect that EBN could function in regulating the post-translation modification of filaggrin and filaggrin-2. The proteolysis and deimination of profilaggrin and filaggrin-2 are promoted by proteases caspase-14 (CASP14) (Hoste et al., 2011), protein arginine deiminase 1 (PADI1) and 3 (PADI3) (Nachat et al., 2005), and calpain 1 (Sandilands et al., 2009; Hsu et al., 2011). At least as shown here, EBN triggered the expression of CSAP14, which could account for part of the moisturizing effect. The moisturizing factors are essential for stratum corneum hydration, barrier homeostasis, desquamation, and plasticity (Robinson et al., 2010), and therefore the epidermal hydration can be reserved. Loss-of-function mutation in filaggrin gene could reduce the level of NMFs (Kezic et al., 2008), and thereafter the skin could not maintain its hydration level and experience greater water loss. CASP14 is a cysteinyl aspartate specific proteinase confined in cornified epithelium playing a role in epidermal differentiation and NMF formation (Hoste et al., 2011; Masse et al., 2014). Two classes of widely used skin moisturizing ingredients, Cer-2 and Cer-6 ceramides, have been reported to stimulate CASP14 gene expression (Jiang et al., 2013). Having the EBN-induced CASP14 expression here, we suggest that EBN has the potential in developing into a moisturizer ingredient in cosmetics application.

Apart from the downstream pathway of filaggrin and filaggrin-2, the upstream signaling pathways regulating the moisturizing proteins are also important. The inhibition of p38-MAPK signaling pathway has been illustrated to abolish the expression of filaggrin in human epidermal keratinocytes (Nagosa et al., 2017). As demonstrated here with HaCaT keratinocytes, EBN significantly activated the phosphorylation of p38, and therefore the expressions of filaggrin and filaggrin-2 were mediated *via* the p38-MAPK signaling. The over expression of GATA3 in regulating the expressions of filaggrin and filaggrin-2 in human keratinocytes has been demonstrated (Zeitvogel et al., 2017). Here in keratinocytes, EBN significantly increased GATA3 expression, as well as the nuclear translocation of phosphorylated GATA3: these events promoted the expressions of filaggrin and filaggrin-2. Besides, the activation of PPAR expression and its nuclear translocation could also lead to an increased expression of filaggrin (Harding and Rawlings, 2000). The three PPAR isotypes: PPARα, PPARβ, and PPARγ, are all expressed in human skin, playing roles in skin homeostasis (Michalik and Wahli, 2007). EBN, both extract and digest, significantly increased the mRNA and protein levels of PPARs.

Biochemical assays, including western blot and real-time PCR, require an extraction process and disrupt cellular structures, which cannot provide information of EBN in physical appearance of keratinocyte. Thus, we have employed AFM, serving as a novel and effective technique, to reveal the moisturization state of keratinocyte under the treatment of EBN. In our AFM assays, the relaxation modulus $E(t)$ obtained from the mechanical measurements describes how cells behave under mechanical perturbation, which is closely related to the moisturization state of living cells by E_0, E_1, τ_1, and α. By comparing the mechanics between EBN-treated cells and mechanical-induced moisturized or dehydrated cells, we can identify the moisturizing effect of EBN mechanically. The biochemical assay and mechanical characterization are in parallel to confirm the moisturization effects of EBN.

The developed digestion on EBN greatly releases free sialic acids and small functional peptides that could specifically increase the skin moisturizing effect. In our preliminary study, N-acetylneuraminic acid, the most abundant sialic acid contained in EBN, significantly induced the expression of filaggrin in cultured keratinocytes. Besides, the digested peptides of EBN have been fractionated by molecular sizing on a HPLC, and the fractions were probed for testing in inducing filaggrin and filaggrin-2. The fraction containing small peptides of molecular weight <1.7 kDa showed robust induction in expressions of filaggrin and filaggrin-2 (**Supplementary Figure 1**). These lines of evidence support the superior efficacy of EBN digest in moisturizing skin. Generally speaking, the large polypeptides provide limited therapeutic values due to their low membrane permeability, rapid degradation, and native allergen reactivity (Lindgren et al., 2000; Aguilar-Toalá et al., 2019). Recent report has shown that the food-derived peptides could exert better biological functions, as compared to their native proteins (Aguilar-Toalá et al., 2019). In the application of cosmetics products, low molecular weight peptides possess the advantages of higher bioavailability, deeper skin penetration, lower toxicity, and hypo-allergenicity (Aguilar-Toalá et al., 2019). On the other hand, most of the active compounds in current intradermally-injected or topical moisturizer formulations are synthetic chemicals, phytochemicals, metabolites, minerals, or vitamins: these agents may cause side effects, such as skin allergic reactions including irritation or inflammation. In a case reported by Lupton and Alster, (2000), continuous intra-dermal injections of hyaluronic acid gel led to bruising, erythema, and acute red nodules in treatment areas. In contrast, EBN-derived short peptides possess the advantages of lower toxicity and hypo-allergenicity. Furthermore, our preliminary results show that the digested EBN could ameliorate TNF-α induced dermatitis in keratinocytes (data not shown), which supports a greater potential of EBN in developing into anti-inflammatory and moisturizing skincare products.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Ethics Committee of HKUST.

AUTHOR CONTRIBUTIONS

KT conceived and designed the research. QL conducted most experiments. MG and KW participated in mouse dorsal skin section and immunofluorescence staining experiments. ZL performed AFM experiments, analyzed the experimental data, prepared the figures and revised the paragraphs. DG assisted the AFM data analysis. PT supported the AFM experiment. TD and KW provided the reagents and guidance. KT and QL wrote the paper.

FUNDING

This work is supported by The Key-Area Research and Development Program of Guangdong Province (2020B1111110006); Special project of Foshan University of science and technology in 2019 (FSUST19-SRI10); GBA Institute of Collaborate Innovation (GICI-022); Special project of Foshan University of science and technology in 2019 (FSUST19-SRI10); Shenzhen Science and Technology Innovation Committee (ZDSYS201707281432317; JCYJ20170413173747440; JCYJ20180306174903174), Zhongshan Municipal Bureau of Science and Technology (ZSST20SC03); Guangzhou Science and Technology Committee Research Grant (GZSTI16SC02; GZSTI17SC02); Hong Kong RGC Theme-based Research Scheme (T13-605/18-W); Hong Kong Innovation Technology Fund (UIM/340, UIM/385, ITS/500/18FP; TCPD/17–9); TUYF19SC02, PD18SC01 and HMRF18SC06; HMRF20SC07; AFD20SC01; GRF grant: 16300920.

ACKNOWLEDGMENTS

The authors appreciate Gallant Chan for his preliminary data and early paper draft, Zack Wong for his guidance, Sybil Xia for assistance in confocal microscopy, Bella Bai for assistance in immunohistochemistry staining, and Gary Yuen for assistance in nuclear and cytoplasmic protein fractionation.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.685982/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 29 September 2021
doi: 10.3389/fphar.2021.726005

Edible Bird Nest Protects the Kidney From Gentamicin Induced Acute Tubular Necrosis

Christopher T S. Lim[1], Norhafizah M.[2], D. Sani[1], S. N. Tan[1], C. W. Lim[1], Brian P. Kirby[3], A. Ideris[4] and J. Stanslas[1]*

[1]Department of Medicine, Faculty of Medicine and Health Sciences, UPM Serdang, Serdang, Malaysia, [2]Department of Pathology, Faculty of Medicine and Health Sciences, UPM Serdang, Serdang, Malaysia, [3]School of Pharmacy and Biomolecular Sciences, RCSI University of Medicine and Health Sciences, Dublin, Ireland, [4]Department of Veterinary Clinical Studies, Faculty of Veterinary Medicine, UPM Serdang, Serdang, Malaysia

Every year, there are about 13.3 million cases of acute kidney injury (AKI). Although AKI is a preventable and treatable disease, if left untreated, it has high risk of multiple organ failure and progression to end stage kidney disease. Acute tubular necrosis (ATN) has been recognised as one of the major causes of AKI. Till to date, there is no effective supplement or medication in treating or reversing AKI. Most of the treatment strategies involve preventative measure to minimise the occurrence of AKI or to reverse the cause of AKI. Hence one of the primary area of research interests is to explore the potential treatment for AKI. Edible bird nests (EBN) are edible food produce by the swiftlet's saliva, which is rich in sialic acids. Sialic acids are monosaccharides that play a vital role in maintaining the integrity and proper function of the human organs, including kidneys. EBN also contains epidermal growth factor, which is widely believed to have rejuvenation and tissue repairing properties. We initiate this study to study the potential reno-protective effect of edible bird's nests by studying the Wistar rat model of gentamicin-induced AKI. Besides renal profiles, renal histology was also semiquantitatively assessed. In our study, pre-treatment with EBN prevented and ameliorated the gentamicin-induced AKI. To a lesser extent, post-treatment with EBN also protected the kidney from the toxic effect of gentamicin. Our findings are highly indicative that EBN possesses reno-protective properties.

Keywords: edible bird nest, renal protective, sialic acid, epidermal growth factor, medicine

OPEN ACCESS

Edited by:
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Reviewed by:
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****Correspondence:***
Christopher T S. Lim
christopher@upm.edu.my
drchrislim@gmail.com

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: *16 June 2021*
Accepted: *15 September 2021*
Published: *29 September 2021*

Citation:
Lim CTS, M. N, Sani D, Tan SN, Lim CW, Kirby BP, Ideris A and Stanslas J (2021) Edible Bird Nest Protects the Kidney From Gentamicin Induced Acute Tubular Necrosis. Front. Pharmacol. 12:726005. doi: 10.3389/fphar.2021.726005

INTRODUCTION

Based on Kidney disease Improving Global Outcome (KDIGO) guidelines, AKI can be defined by either increase in serum creatinine by > 0.3mg/dl, increase in serum creatinine by > 1.5 times baseline, or a reduction in urine volume <0.5 ml/kg/h for 6 h (Khwaja, 2012). It can be further subdivided into Acute Kidney Injury Network (AKIN) Stages based on changes in serum creatinine

Abbreviations: AKI, acute kidney injury; ATN, acute tubular necrosis; CKD, chronic kidney disease; EBN, edible bird's nest; ESRF, end stage renal failure; NAC, N-acetylcysteine; GTN, gentamicin

level (Bagshaw et al., 2008). AKI, regardless of its stages, carries significant morbidity and mortality risks.

The causes of AKI can be divided into three broad categories: prerenal (caused by decreased blood perfusion to the kidneys), intrinsic renal (caused by a process within the glomeruli and tubules), and postrenal (caused by disruption of drainage of urine). Intrinsic renal causes are also relevant sources of AKI with acute tubular necrosis (ATN) as the most common type of AKI encounter in the patients that are hospitalised. The cause is usually an ischaemic insult or post exposure to nephrotoxic agent.

Edible bird nest (EBN), produced from the saliva of the swiftlets, is consumed worldwide and is often regarded as a medicinal food (Marcone, 2005). EBN comprises many exceedingly nutritious substances like glycoproteins, carbohydrates, minerals and both essential and non-essential amino acids. It is also abundant in hormones, vitamins and fatty acids. According to our previous works, the highest composition contained inside the EBN is protein. Carbohydrates were the next highest ingredient found in the EBN. There is also evidence that there is the presence of sialic acid and epidermal growth factors in the EBN (Tan et al., 2020). Other growth factors contained inside EBN, such as vascular endothelial growth factor (VEGF) and cytokines such as interleukin-6 (IL-6), play a vital role in cellular communication. As intercellular mediators, these molecules play an essential role in regulating survival, growth, differentiation, and functions of cells (Roh et al., 2012).

Although consumption of EBN is believed to have beneficial effect to the kidney for many centuries, there are no previous trials that study its effect on preservation of the kidney function. The beneficial effect is believed to be mediated through down regulation of the inflammatory pathway, reparation of the renal tissues via the presence of epidermal growth factor and maintenance of tissue integrity via sialic acid.

Therefore, this present study was initiated to evaluate the reno-protective effect of EBN using a Wister rat model of gentamicin-induced acute tubular necrosis (ATN).

MATERIALS AND METHODS

Raw cleaned EBN samples were sourced from a swiftlet ranch located on a piece of agriculture land (2°52′51.4″N 103°16′33.1″E) in Pahang, Malaysia. The raw, cleaned EBN was then immerse in reverse osmosis water for 1 hour until it turned soft. Impurities from the nests were then carefully removed by using tweezers under the aid of a magnifying glass. After the dirt was removed, the EBN was then left to dry under the fan in room temperature. Cleaned nests were then sent to a local accredited lab for proximate analysis, toxicology and bacteriology analyses to ensure the quality of the product are met as per the established standard (Department of Standards of Malaysia, 2011). The cleaned EBN was then cooked according to the Chinese technique of double-boiled cooking method for 30 min until it has a gelatinous texture.

Renal Injury Model

This study aims to evaluate the renoprotective effect of using EBN in pre-treatment and post-treatment phase of GTN induced ATN model ATN will be induced in rats by gentamicin. The method used is as described by Moreira (Moreira et al., 2014). For the pre-treatment phase, the rats will be fed EBN (at various doses) orally 7 days before the induction of ATN via injection of gentamicin. In contrast, for the post-treatment arm, rats will be fed EBN (at various doses) immediately after treatment with gentamicin. The control groups for both experiments are rats with tubular injuries but without the pre or post-treatment with EBN. The studied parameters in our study were renal profiles, serum osmolarity and renal histopathology. Renal histopathological slides were read, in a blinded manner, by an experienced histopathologist who would then record the renal pathological changes of ATN as according to Endothelial, Glomerular, Tubular, Interstitial (ETGI) scoring system (Khalid et al., 2016). Blood was collected before and after treatment for measurement of the renal profiles.

Animal Handling and Housing

We used ninety-six female, 12–14 weeks old Wistar rats in this study. The rats were sourced from Takrif Bistari Enterprise, Selangor, Malaysia. They weighed in the 250–300 g range. All of the rats were held in groups in plastic cages for 10 days for them to adapt to the research laboratory setting. The research laboratory has a preset ambient temperature of 25 ± 2°C with alternating 12 h dark and light cycle. The rats were nourished with a constant pellet formula rodent diet recommended for rats (Specialty Feeds, Pte Limited, Western Australia). In contrast, the water supply was provided at an *ad libitum* basis. Our animal research was carried out in conformity with the standards of laboratory animal care as dictated by our local institution Animal Care Use Committee (approval number UPM/IACUC/AUP-R064/2014).

Experimental Animal

Ninety-six Wistar rats were randomly separated into six groups, with each group containing eight rats.

A total of forty-eight rats were used in the six groups of pre-treatment arm: Group I: normal control; Group II: Gentamicin - injected rats without pre-treatment with EBN as negative control; Group III-V: Gentamicin - injected rats which have been pre-treated with different doses of EBN (125, 250 and 500 mg/kg respectively); Group VI: Gentamicin -injected rats which have been pre-treated with N-acetylcysteine as the positive control.

A total of forty-eight rats were used in the six groups of post-treatment arm: Group I: normal control; Group II: Gentamicin-injected rats without EBN given as post-treatment to act negative control; Group III-V: Gentamicin - injected rats which were post-treated with different doses of EBNs (125, 250 and 500 mg/kg respectively); Group VI: Gentamicin -injected rats subsequently receiving N-acetylcysteine (60 mg/kg) as the positive control group.

EBN and Gentamicin Treatment

EBNs were diluted accordingly to achieve the desired dose concentration 125, 250 and 500 mg/kg correspondingly. The various doses were designed to be equivalent to a full, half and quarter dose oral consumption of EBN by human (5 g of cleaned EBN for a 60 kg human body weight). EBNs at different doses is fed orally to the rats (groups III to V) for 7 days before the

administration of the intraperitoneal (IP) injection at a concentration of 100 mg/kg Gentamicin (GTN) (Garasent, Duopharma (M) Sdn Bhd, Malaysia). The GTN is dissolved in the normal saline. N-acetylcysteine was used at positive control and fed orally to the rats in group 6 before the IP GTN injection. For post-treatment phase, IP injection of 100 mg/kg GTN (Garasent, Duopharma (M) Sdn Bhd, Malaysia) was administered to the rats before EBNs at a graded does of 125, 250, and 500 mg/kg were fed orally to the rats for a further 7 days. Similarly, N-acetylcysteine was used as a positive control, and this was given orally to the group 6 rats post-GTN injection. The dose of the vehicle is 10 ml/Kg.

Sample Collection

At the end of the research, all the rats were sacrificed via the administration of carbon dioxide gas. The abdomens were carefully dissected, and the kidneys were harvested. The kidneys were processed using routine histological techniques. The renal tissues were then stained with hematoxylin and eosin. The blood samples are taken via cardiac puncture with 2.5 ml of specimen collected in fluoride blood tubes. The blood was analysed using Olympus © Chemistry Analysers using the ISE modules. Samples are stored at Pharmacotherapeutics Laboratory, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia for posterity purpose.

Statistical Analysis

Results in our study were analysed using SPSS version 25 and expressed as mean ± standard deviation (SD). The data generated were analysed with Anova one-way analysis of variance while post hoc analysis was done using Bonferroni method. A p-value less than 0.05 is considered statistically significant in our study.

RESULTS

Renal Histopathology

The control kidneys which were treated with normal saline showed normal histopathological findings (**Figure 1**). The negative control kidneys, which were given gentamicin and normal saline, showed the classical picture of severe ATN damages, which is the hallmark feature of intrinsic AKI damages. These changes include markedly dilated renal tubules with flattening of the epithelium, diffuse denudation of the renal tubules, tubular atrophy and intratubular cast formation (**Figure 2**). Similar severe ATN features were found in the positive control kidneys (**Figure 3**).

For the pre-treated group with different dose of EBNs, the 125 mg/kg group has near-normal renal tubules with no discernible ATN changes (**Figure 4**). The pre-treated group, which received 250 mg/kg and 500 mg/kg of EBNs, has mild ATN changes (**Figures 5**, **6**). As for post-treated group, all different doses of EBN (125, 250, and 500 mg/kg) have resulted in moderate ATN changes (**Figures 4–6**).

The changes were scored using the EGTI system, as shown in **Tables 1**, **2** (Khalid et al., 2016). A higher score denotes worse renal histopathological damage. Pre-treatment with 125, 250, and 500 mg/kg have signifcantly lesser scores than GTN group ($p < 0.05$). The post-treatment groups 125, 250, and 500 mg/kg scores were lesser than GTN group but higher than pre-treatment groups.

Serum Renal Profiles

Pre-Treatment With EBN

Serum renal profiles were taken for both pre and post-treatment groups. Serum creatinine was significantly higher in the pre-treated 125, 250, and 500 mg/kg groups as compared to the GTN group. Serum urea was only significantly higher in the pre-treated 500 mg/kg group as compare to GTN cohort. Serum sodium and potassium were higher in the pre-treated group, which received 250 and 500 mg/kg of EBNs (**Figures 7–10**).

Post Treatment With EBN

As for post treatment results, serum sodium, urea and creatinine were signficantly higher in the 250 and 500 mg/kg groups as compare to the GTN group. Serum urea levels did not show any significant changes. Serum potassium and chloride were significant increased in 500 mg/kg post treatment groups as compare to GTN (**Figures 11–14**).

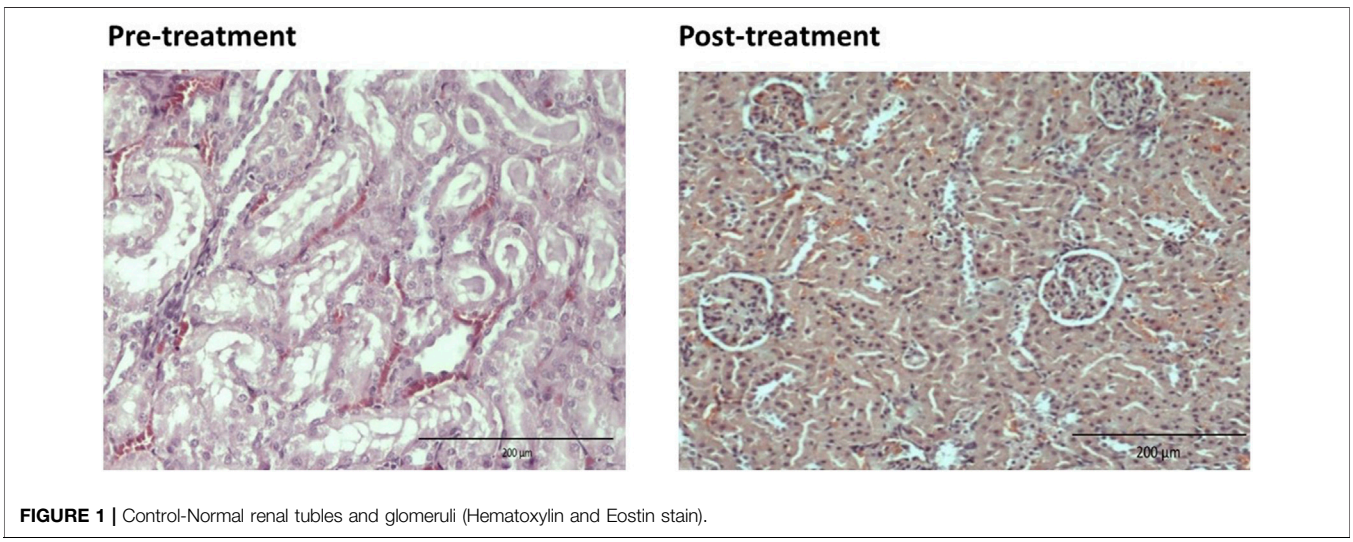


FIGURE 1 | Control-Normal renal tubles and glomeruli (Hematoxylin and Eostin stain).

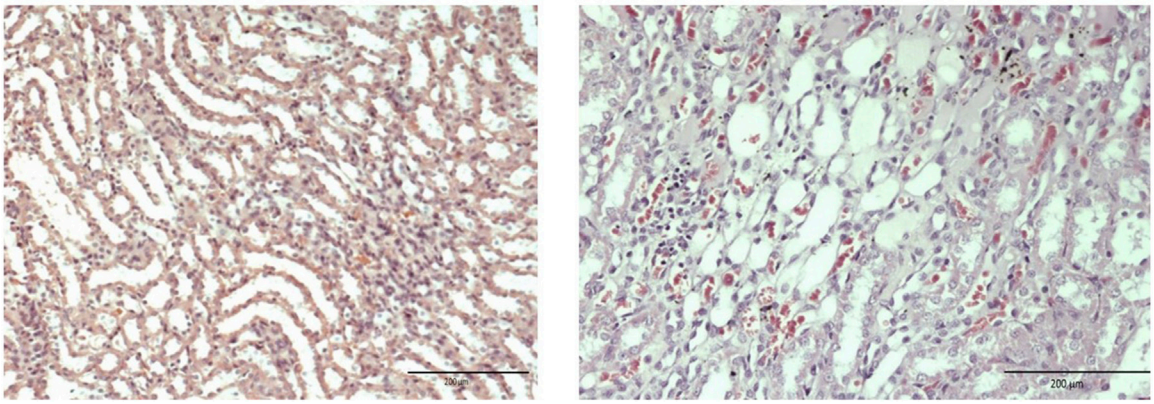


FIGURE 2 | Negative contol (GTN and saline) Severe ATN changes in the tubles.

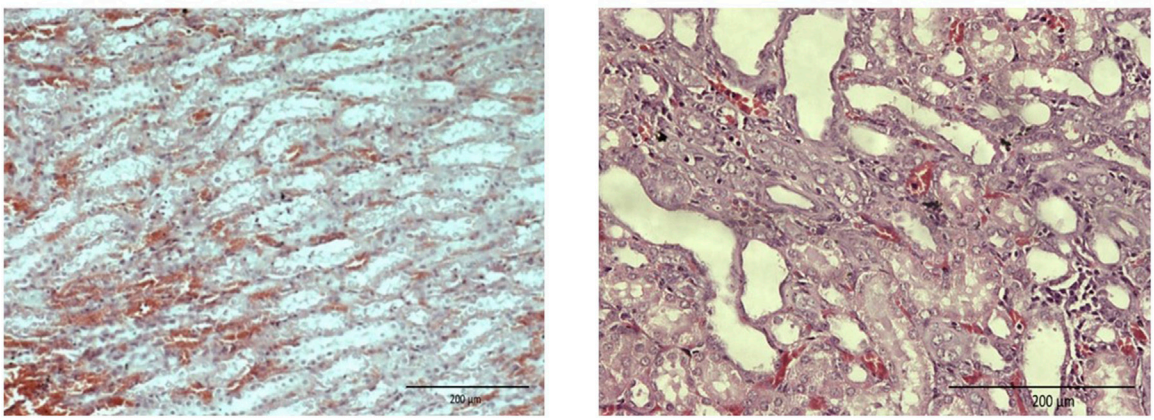


FIGURE 3 | Postive control (GTN and NAC) Severe ATN changes.

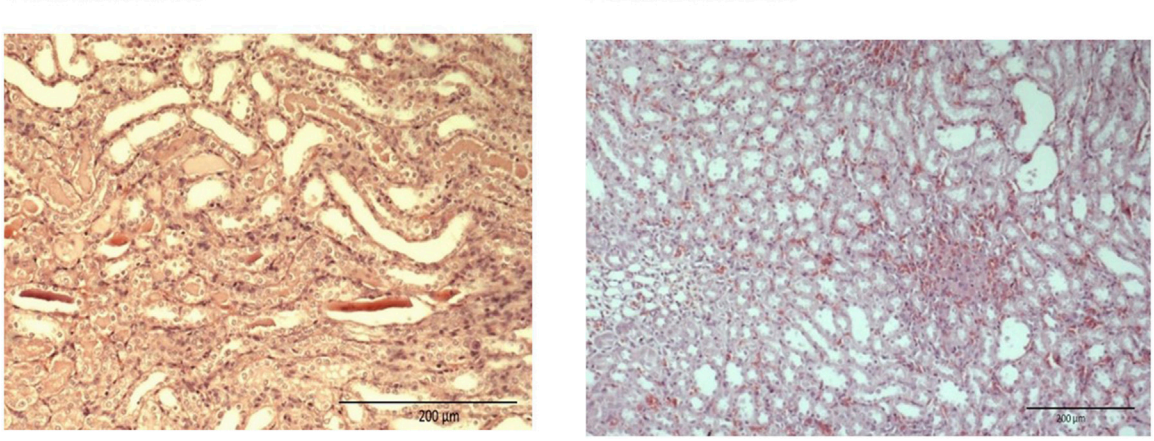


FIGURE 4 | Pre-treatment with EBN 125mg/kg and GTN-relatively normal renal tubles.

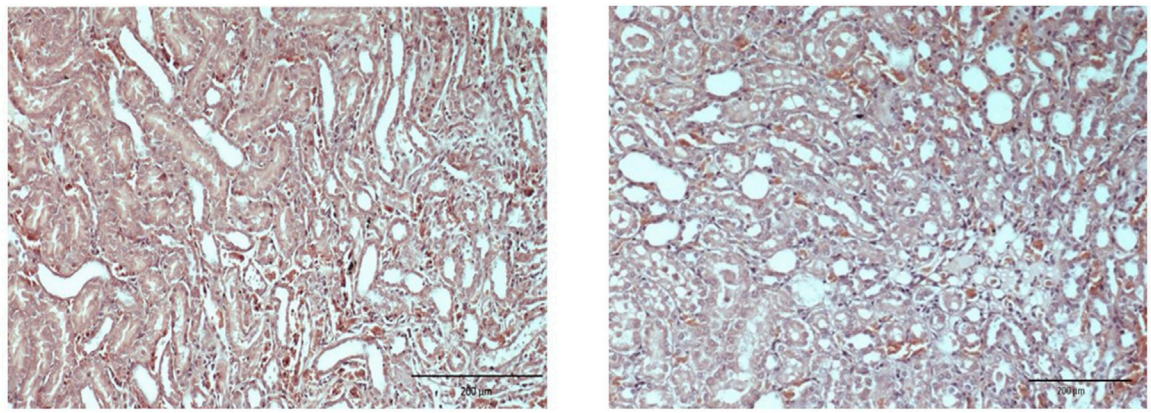


FIGURE 5 | Pre-treatment with EBN 250mg/kg and GTN-mild ATN.

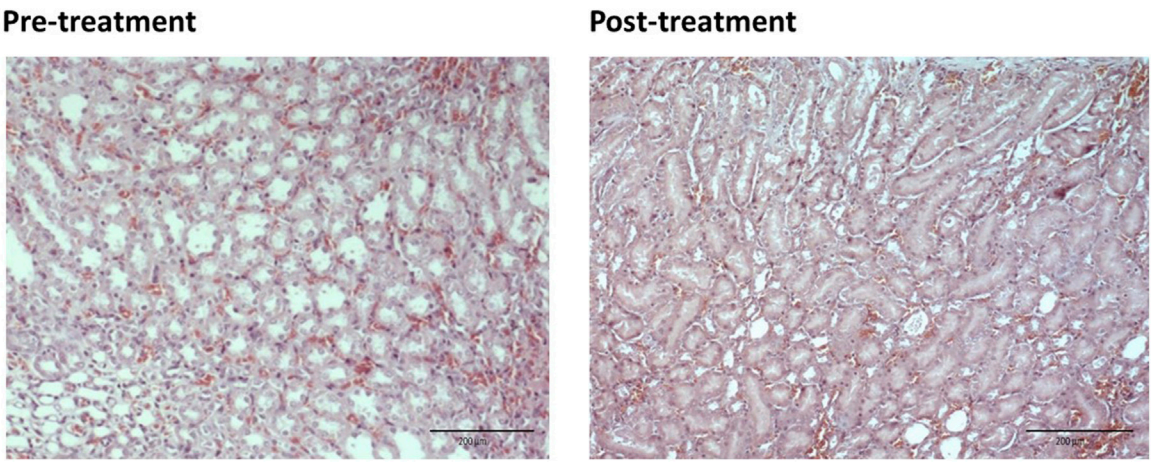


FIGURE 6 | Pre-treatment with EBN 500mg/kg and GTN-mild ATN.

TABLE 1 | The EFTI scoring system for ATN.

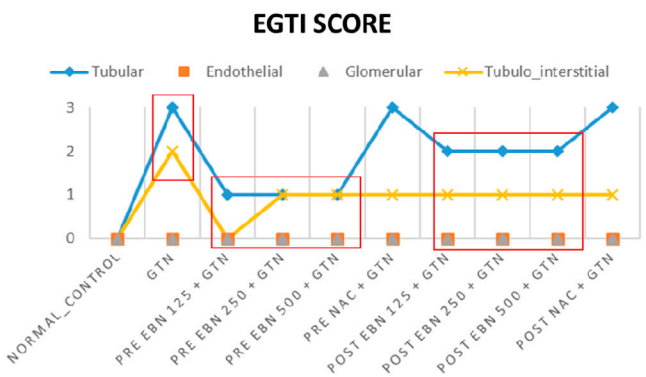
Group	Tubular	Endothelial	Glomerular	Tubulo/Interstitial	P value
CONTROL					
Normal Control	0	0	0	0	
GTN	3+	0	0	2+	
PRE GROUP					
EBN 125 + GTN*	1+	0	0	0	0.025
EBN 250 + GTN*	1+	0	0	1+	0.038
EBN 500 + GTN*	1+	0	0	1+	0.038
NAC + GTN	3+	0	0	1+	NA
POST GROUP					
EBN 125 + GTN	2+	0	0	1+	0.205
EBN 250 + GTN	2+	0	0	1+	0.205
EBN 500 + GTN	2+	0	0	1+	0.205
NAC + GTN	3+	0	0	1+	NA

EFTI: Endothelial, Glomerular, Tubular, Interstitial (ETGI) scoring system.
GTN, Gentamicin; NAC, N-acetylcysteine.
*A bivariate analysis were done to determine the significant different of Pre and Post EBN concentration with GTN (*p < 0.05).*

TABLE 2 | Plots of EGTI scores.

Group	Tubular	Endothelial	Glomerular	Tubulo/Interstitial	P value
CONTROL					
Normal Control	0	0	0	0	
GTN	3+	0	0	2+	
PRE GROUP					
EBN 125 + GTN*	1+	0	0	0	0.222
EBN 250 + GTN*	1+	0	0	1+	0.038
EBN 500 + GTN*	1+	0	0	1+	0.038
NAC + GTN	3+	0	0	1+	NA
POST GROUP					
EBN 125 + GTN#	2+	0	0	1+	0.205
EBN 250 + GTN#	2+	0	0	1+	0.205
EBN 500 + GTN#	2+	0	0	1+	0.205
NAC + GTN	3+	0	0	1+	NA

EGTI : Endothelial, Glomerular, Tubular, Interstitial (EGTI) scoring system GTN : Gentamicin, NAC : N-acetylcysteine A bivariate analysis were done to determine the significant different of Pre and Post EBN concentration with GTN ($p < 0.05$) *#compare mean analysis



Line graph showing the EGTI score. There is significant lower EGTI scoring achieved in the groups received pre-treatment as compared to positive and negative controls treated with GTN

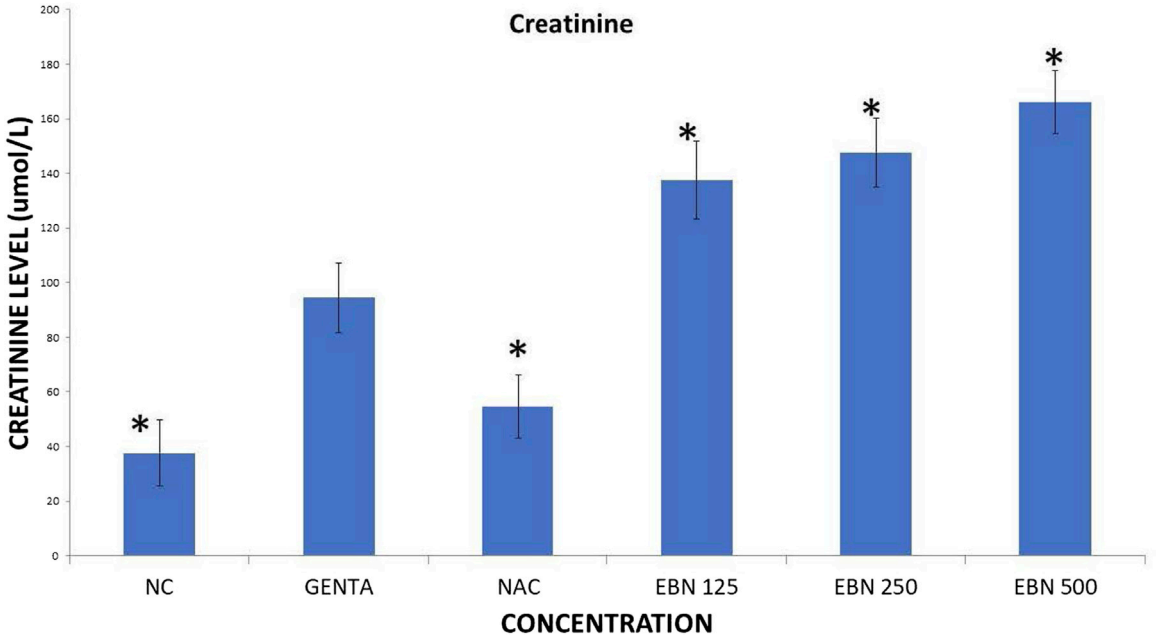


FIGURE 7 | Changes of serum creatinine chnages vs. different pre-treatment group (*$p < 0.05$ compare to gentamicin group).

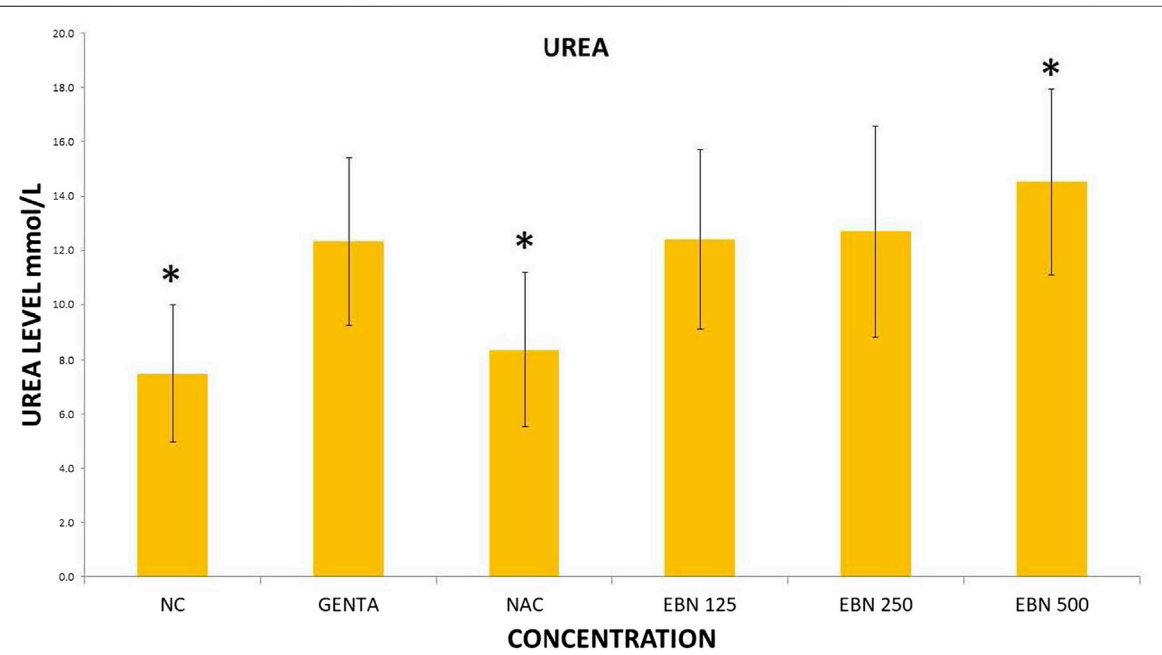


FIGURE 8 | Changes of serum urea changes vs. different pre-treatment group (*$p < 0.05$ compare to gentamicin group).

Chemical Analysis of EBN

By using HPLC, the sialic acid content of the EBN used in this study was 5.47%. In term of proximal analysis, the order of composition, from highest to the lowest is protein (55.5 ± 2.5%), carbohydrate (28.6 ± 6%), moisture (12.1 ± 1.6%), ash (2.8 ± 0.1%) and lipid (0.1%). In term of safety profile, there was acceptable limit of nitrate (35.9 ± 0.1 mg/kg) and nitrite level (11.4 ± 0.2 mg/kg). There were no trace of arsenic, mercury, lead or cadmium inside the EBN. AS for microbiology profile, there were no *E Coli, S. Aureus,*

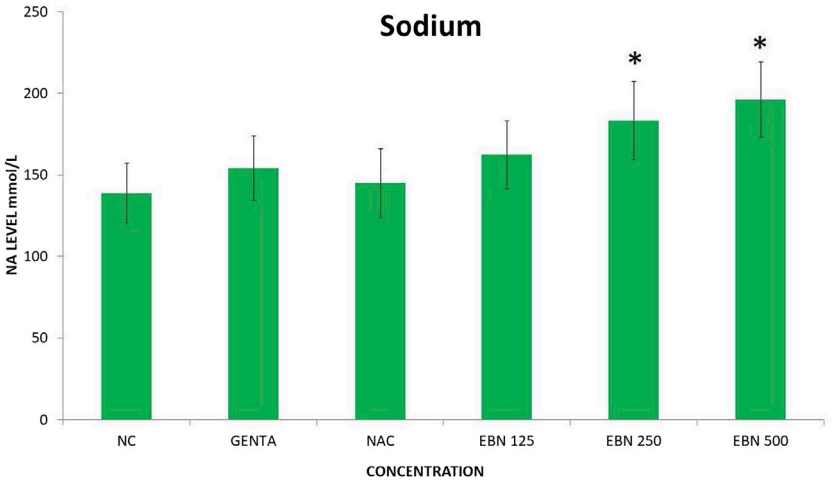


FIGURE 9 | Changes of serum sodium vs. different pre-treatment group (*p < 0.05 compare to gentamicin group).

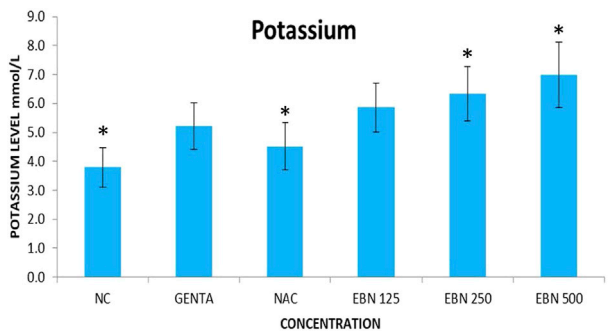


FIGURE 10 | Changes of serum potassium vs. different pre-treatment group (*p < 0.05 compare to gentamicin group).

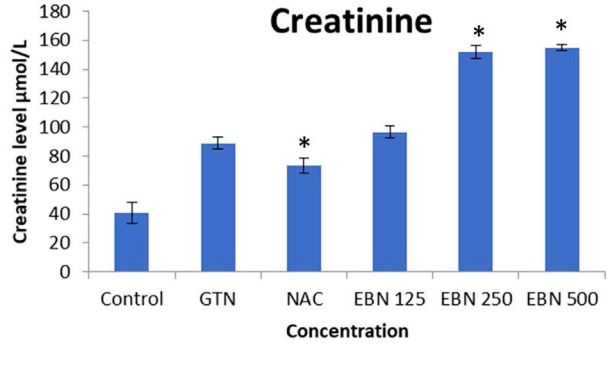


FIGURE 11 | Changes of serum creatinine vs different post-treatment group (*p < 0.05 compare to gentamicin group).

Salmonella, Coliform or mould detected. The full chemical analysis of the EBN used in our study can be obtained in our earlier works that had been published (Careena et al., 2018; Tan et al., 2020).

DISCUSSION

AKI is a sudden episode of loss of renal function that happens within a few hours or a few days. AKI increases the long-term risk of developing chronic kidney disease (CKD) or end-stage kidney disease (ESKD) (Hsu and Hsu, 2016). Moreover, the presence of AKI has been associated with an increase in short-term and long-term morbidity and mortality (Samuels et al., 2011). Aetiologies of AKI are usually divided into prerenal, renal and postrenal causes. Among hospitalised patients in medical wards, AKI usually comes from the prerenal aetiologies (volume depletion or low cardiac output) or acute tubular necrosis (ATN) (nephrotoxin, ischaemia or sepsis). Acute tubular necrosis (ATN) occurs when there is death of tubular epithelial cells that lined the renal tubules of the kidneys. It can only be accurately diagnosed via a renal biopsy.

One of the vital functions of the human kidney is the regulation of water and excretion of nitrogenous waste product generated by the body. Although the measurements of elevated blood urea and creatinine level traditionally serve as the surrogate marker of decreased renal function, they are not accurate per see. An acute rise in serum urea and creatinine can be attributed by hypercatabolic state, excessive protein intake, AKI, gastrointestinal bleeding or dehydration. In clinical practice and in any experimental animal model, a precise diagnosis of AKI will require the presence of ATN confirmed histologically *via* a renal biopsy rather than relying on traditional blood test or biomarkers (Kudose et al., 2018). In the clinical management of AKI, a combination of serum creatinine and histopathological analysis can accurately tell us about the state of AKI. The cytokines and other specific blood and urinary biomarkers (apart from serum creatinine) remain controversial, and most importantly, most of these markers compare their performance with regards to serum creatinine. In a systemic review carried out by our us, we have found that traditional markers like urea and creatinine still have significant roles in clinical management of AKI and CKD compare to emerging biomarkers (Bidin et al., 2019).

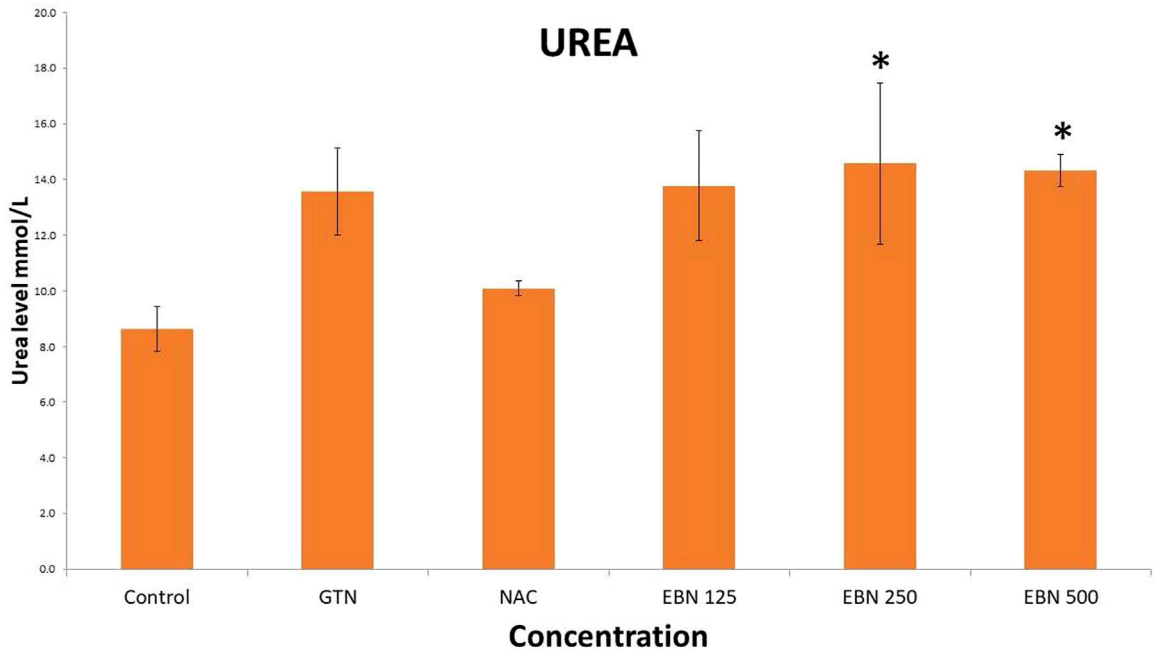


FIGURE 12 | Changes of serum urea chnages vs different post-treatment group (*$p < 0.05$ compare to gentamicin group).

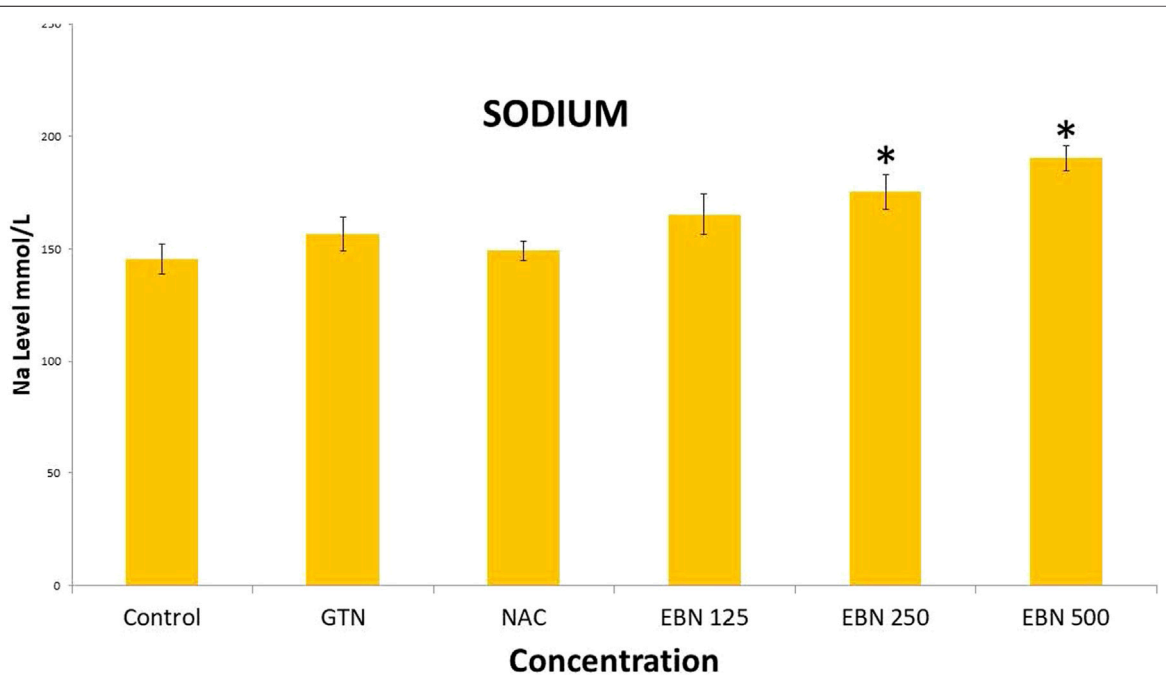


FIGURE 13 | Changes of serum sodium vs different post-treatment group (*$p < 0.05$ compare to gentamicin group).

In our study, renal injury is induced by the usage of GTN, which causes direct nephrotoxicity. GTN is known to cause direct tubular damage through a combination of necrosis of tubular epithelial cells, predominantly in proximal segment and alteration of function of main cellular components involved in transport and conservation of water and solutes (Randjelovic et al., 2017). The degree and severity of ATN in the kidneys correlate with the recovery of renal function in the long term. The more severe the ATN, the higher chances that the patient will end up with chronic kidney disease.

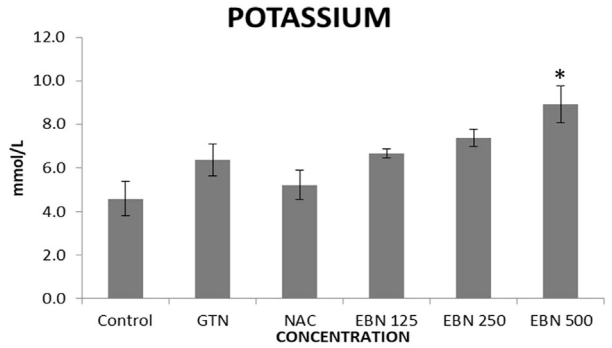


FIGURE 14 | Changes of serum potassium vs different post-treatment group (*$p < 0.05$ compare to gentamicin group).

Our renal histological examination demonstrated that pre-treatment with EBN protects the kidneys from the nephrotoxic effect of GTN, whereby the GTN mediated toxicity was either negated or severely attenuated. For those who had received post-treatment EBN, the renal tubules were similarly protected, albeit not as effective as pre-treatment arms, as modest ATN changes from GTN was observed.

While we were expected to see a corresponding improvement in renal profiles, serum urea and creatinine level were noted to be progressively increased in the pre and post EBN treatment rats despite improvement observed in the renal tissues examination. Of note, the serum urea measurements were disproportionately high as compared to the serum creatinine levels, a finding that suggests the presence of moderate to severe dehydration (define as the ratio of urea/creatinine >1: 20) (Tariq et al., 2009). To confirm the presence of dehydration, we have proceeded to analyse the serum osmolarities of the post-treated samples which showed a trend of increasing osmolarity from 315.9 ± 23.6 mOsm/kg from the group that received 125 mg EBN to the highest of 424 ± 13.4 mOsm/kg for the group that received 500 mg EBN, therefore confirming that the elevated serum urea and contribution were likely to be attributed by dehydration.

As recovery of ATN begins as early as hours after the insults, we designed this study hoping the be able to observe the trend of improving creatinine after 7 days. Unfortunately, we did not see such a trend from the blood test. Animals that have ATN are known to lose the water concentrating ability with polyuria as a norm. Full recovery or the kidney tissues from ATN is expected in 14–60 days after the initial insult. Nancy et al. advocated a full renal recovery will require an average of 42 days (Gary et al., 1976). Hence in retrospective, our protocol that used serum renal profiles as a measurement of kidney function will not be a suitable method to assess renal function in such a short duration of an experiment like ours. We strongly proposed that future similar trials should have a minimum of 14 days post intervention to have a better assessment of renal recovery.

Although EBN is widely believed to have protective effect towards the kidney for many centuries, ours is the first that successfully documented the reno-protective effect of EBN. This reno-protective effect is believed by our research team to be effected through the presence of epidermal growth factor which repair the damaged tissues, maintenance of renal tissue integrity via the sialic acid and lastly, down regulation of the pro-inflammatory pathway.

We postulated that the reno-protective effect of EBN could be attributed to the presence of sialic acid and epidermal growth factors. As our group has reported previously, our EBN has a good amount of sialic acid (Careena et al., 2018). Sialic acid is essential for maintaining renal function. Appropriate sialylation of the glomerular filter is vital for proper maturation and maintenance of the visceral part of the kidney filtration apparatus. Sialylation, through oral sialic acid supplementation therapy, has gained new interest as a potential new therapeutic strategy to cure or delay glomerulopathies such as focal segmental glomerular sclerosis (Niculovic et al., 2019). Apart from preserving the glomerular cells, sialic acid also plays a vital role in maintaining the integrity of renal tubular cells (Sgambati et al., 2011).

Reside inside kidney tissues is a variety of immune cells such as dentritic cells, macrophages, regulatory T cells, which play an important role in the maintenance of tissue homeostasis. Once activated by external trigger such as LPS, these cells produce proinflammatory cytokines that can initiate and propagate the kidney disease which can culminate in irreversible renal scarring (Andrade-Oliveira et al., 2019). EBN can inhibit the generation of proinflammatory cytokines (TNF-α, IL-1β, and IL-6) and oxidative markers. Pre-treatment of sialic acid significantly lessened the LPS-induced detrimental effects on the renal haemodynamics, renal reactive oxygen species production and the LPS-activated TLR4/gp91/Caspase3 mediated apoptosis signalling (Hsu et al., 2016).

EBNs are also believed to contain epidermal growth factors (EGFs) (Tan et al., 2020). EGFs are small mitogenic proteins involved in several mechanisms, such as healthy cell growth and differentiation. Recently EGFs have been found to regulate sodium transport and development of hypertension. EGF receptor activation has been shown to ameliorate renal damage sustained in experimental AKI through the repairing process that involves the regeneration of the renal tubular cells (Melenhorst et al., 2008; Rayego-Mateos et al., 2018).

The limitation of our study is the short duration of the animal study and the failure to keep the animal's adequate hydration during the experiment. However, we have an excellent and comprehensive histological assessment of the renal tubules, which has provided a comprehensive and real time information about renal tubular structure changes during the experiment. Despite the shortcomings, in this pilot trial, we have demonstrated that pre and post treatment with EBN protects the gentamicin treated kidneys from developing ATN. We hope that this preliminary finding can lead to further in depth study of the possible mechanisms of reno-protective properties of EBN.

CONCLUSION

This study revealed that pre-treatment of EBN is a more effective means of preventing GTN induced ATN as compared to post-treatment with EBN. We believe that the renal protective effect could be due to the presence of sialic acid, which is needed to

maintain a proper kidney function. Similarly, EGF could have assisted in the regeneration of the renal tubules post-ATN. Further studies are needed to assess whether external supplementation of EBNs can render reno-protection from other models of AKI or chronic kidney disease.

SAMPLE STORAGE

Samples are stored at Pharmacotherapeutics Laboratory, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia for posterity purpose.; Ethics approval.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Ethical Committee (UPM/IACUC/AUP-R064/2014).

AUTHOR CONTRIBUTIONS

CL, BK, JS, and AI contributed to conception and design of the study. CL, SNT, and DS organized the database. CL, NM, and LCW performed the statistical analysis. CL wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

FUNDING

This research is made possible by a research grant provided by the Centre of Excellence (CoE) Swiflets, Faculty of Veterinary Medicine, Universiti Putra Malaysia (Ref. No: 6471400-10301-H5)

ACKNOWLEDGMENTS

We wish to acknowledge the guidance and support provided by the Centre of Excellence (CoE) Swiflets, Faculty of Veterinary Medicine and Pharmacotherapeutics Laboratory, Universiti Putra Malaysia. The authors also thank Careena Shobana for her assistance in carrying out the animal studies and data collection.

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frontiers in Pharmacology

REVIEW
published: 01 October 2021
doi: 10.3389/fphar.2021.746656

Edible Bird's Nest: Recent Updates and Industry Insights Based On Laboratory Findings

*Kian Chung Chok[1], Ming Guan Ng[1], Khuen Yen Ng[2], Rhun Yian Koh[3], Yee Lian Tiong[4] and Soi Moi Chye[3]**

[1]School of Health Science, International Medical University, Kuala Lumpur, Malaysia, [2]School of Pharmacy, Monash University Malaysia, Selangor, Malaysia, [3]Division of Biomedical Science and Biotechnology, School of Health Science, International Medical University, Kuala Lumpur, Malaysia, [4]School of Postgraduate, International Medical University, Kuala Lumpur, Malaysia

Edible bird's nest (EBN) is a traditional Chinese delicacy made of the saliva of swiftlets found in Southeast Asia. With increasing demands for EBN, quality control of EBN products is important for safe consumption. The processing steps are particularly important for efficient extraction of bioactive compounds. Geographical location, collection place, and harvesting season contribute to differences in nutritional contents in EBN. Concerns regarding presence of adulterant, chemical, and microbial contaminants in EBN as well as authentication and chemical composition measuring methods are discussed in this review. Recent discoveries of beneficial health functions of EBN in antimicrobial and antiviral actions, immunomodulation, cancer prevention and treatment, tissue regeneration, cardiometabolic maintenance, antioxidant action and neuroprotection are also reviewed. Our review provides an update on the recent research on EBN.

Keywords: edible bird's nest, industry insights, laboratory findings, saliva of swiftlets, bioactive compounds, nutritional contents

OPEN ACCESS

Edited by:
Ting Hun Lee,
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Reviewed by:
Aline Priscilla Gomes Da Silva,
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Kennedy Honey Erlwanger,
University of the Witwatersrand, South Africa

****Correspondence:***
Soi Moi Chye
chye_soimoi@imu.edu.my

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: *24 July 2021*
Accepted: *20 September 2021*
Published: *01 October 2021*

Citation:
Chok KC, Ng MG, Ng KY, Koh RY, Tiong YL and Chye SM (2021) Edible Bird's Nest: Recent Updates and Industry Insights Based On Laboratory Findings. Front. Pharmacol. 12:746656. doi: 10.3389/fphar.2021.746656

INTRODUCTION

Consumption of edible bird's nest could be traced back to the 7th century from China, in which EBN was classified as a high-grade health food, tonic, medicine, and a symbol of wealth (Lau and Melville, 1994). Traditional Chinese medicine states that EBN confers health benefits such as moisturizing the lung, resolving phlegm, alleviating cough, and recuperating from diseases (Lim and Earl of Cranbrook, 2002; Hobbs, 2004). EBN is usually referred to the nests of *Aerodramus fuciphagus* (*A. fuciphagus*) (white-nest swiftlet) and *Aerodramus maximus* (*A. maximum*) (black-nest swiftlet) harvested for consumption in Southeast Asia (Hao et al., 2015). The growth and reproduction of swiftlets require appropriate environmental conditions such as humidity of about 80–90%, temperature between 26 and 35°C and sufficient food sources. Hence, EBN-producing swiftlets are mainly found in Southeast Asian countries such as Malaysia, Thailand, Indonesia, Myanmar, Vietnam, The Philippines, Cambodia, and the southern region of China. However, the quality, composition, and nutritional values in EBN could be different based on the swiftlet species, geographical as well as environmental conditions (Dai et al., 2020). Increasing demands for EBN has produced economic value from US$1000.00 to US$10,000.00 per kilogram depending on its grade, shape, species, and origin. Indonesia is the largest bird's nest producer in Southeast Asia, exporting around 2,000 tons/year, followed by Malaysia at 600 tons/year, and Thailand at 400 tons/year (Panyaarvudh, 2018). China is the largest EBN importer every year. From January to June 2021, China imported approximately 128.3 tons, 42.3 tons, and 0.1 tons from Indonesia, Malaysia, and

Thailand respectively, in the form of ready-to-drink beverages and processed EBN (Development Report of Imported Bird's Nest in 2021, 2021). Indonesia exported about US$998 million worth of EBN in 2015 and the value surged up to US$3.64 billion in 2019 (Badan Pusat Statistika, 2020). EBN production in Myanmar is comparatively lower as the industry has just begun a decade ago by building the first EBN house in Bokpyin, southern Myanmar (Big business in bird's nest in Myanmar seaside town, 2017). In China, there are 3 well-known places with swiftlets inhabitant: Huaiji County, Guangdong province, which produces 150 kg/year; EBN collection is prohibited on Dazhou Island, Hainan because swiftlets are listed as endangered species; EBN produced from Jianshui County, Yunnan is only for the consumption by local residents (Geographical distribution of swiftlet inhabitant, 2020). Due to increasing demands for EBN, quality control should be in place to ensure safe consumption of EBN. Studies that investigated the nutritional value, adulterant content, nitrite (NO_2^-), nitrate (NO_3^-), and microbial contamination in EBN have increased in recent decade (Lee et al., 2021). Our review will discuss on EBN extraction methods, nutritional values, health benefits, and the risk of EBN consumption by including the latest findings about EBN. There is an existing review about EBN which includes publications up to 2019 indexed in Web of Science (Lee et al., 2021). Our review aims to provide an update by including those publications which were not discussed in previous reviews and discuss on additional topics including industrial EBN processing based on laboratory findings. This is a narrative review which includes latest EBN publications up to April 2021. Published data were retrieved from PubMed, Semantic Scholar, Meta, X-MOL, and Google Scholar, using search string: edible bird's nest.

CHEMICAL COMPOSITIONS OF EDIBLE BIRD'S NEST

The quality of EBN is based on various factors such as species of swiftlets, habitat (man-made house or natural cave), harvesting season (rainy season or drought season), and geographical location (Malaysia, Thailand, Indonesia, Myanmar, and China). Quality of EBN is usually screened and maintained at the stage of industrial preparation. Despite the challenges and limitations, numerous methods were applied to ensure authenticity, nutritional values, and safety for EBN consumption, ranging from empirical measures to molecular biology-based techniques.

Factors Affecting Chemical Compositions of Edible Bird's Nest

Nutritional and authenticity studies show that EBN acquired from different sources, such as swiftlet premises, natural caves, and retail stores contain significantly varied chemical compositions. The *A. fuciphagus* EBN from swiftlet premise has higher antioxidant activities and sialic acid content whereas the *A. maximus* EBN from the cave has more mineral composition of calcium and magnesium. Total amino acids in *A. fuciphagus* EBN was found to be 23% higher than *A. maximus* EBN (Quek et al., 2018a). A simple gel electrophoretic method could be employed to differentiate house and cave EBN by generating a fingerprint profile of EBN. The cave EBN produces 10 bands with 2 strong bands at 30 and 35 kDa, house EBN produces 9 bands with 2 strong bands at 120 and 140 kDa (Hun et al., 2016). These results show different protein contents in EBN from different origins and hence there is a need to further investigate this difference in protein composition. Cave EBN was also reported to have 3 times more calcium content than house EBN. It is believed that the moist limestone cave wall provides abundance Ca^{2+} to seep into the cave EBN whereas the house EBN is built on timber strips that have no Ca^{2+} to leach out. (Shim and Lee, 2020). In a field study, cave EBN collected from small islands in Indonesia and Malaysia contain higher NO_3^- and NO_2^- levels when compared to house EBN. It is probably due to the symbiotic relationship between plants and nitrogen-fixing bacteria providing space (i.e., root nodules) for the growth of nitrogen-fixing bacteria in exchange for NO_3^- as nutrients. It is speculated that nitrogen-fixing bacteria reaches the mouth of swiftlets when the swiftlets prey on insects or directly from plants or soil (Schultze and Kondorosi, 1998; Chan et al., 2013). The risk of NO_3^- and NO_2^- contamination will be discussed later in this review.

Nutritional compositions in EBN are known to be affected by the harvesting season. Swiftlets feed on insects from *Hymenoptera* (winged ants, fig wasps and bees), *Coleoptera* (small beetles), *Homoptera* (leafhoppers) and *Ephemenoptera* (mayflies) species. The food sources for swiftlets are more abundant during the rainy season. EBN protein content ranged from 60.3 to 63.6 g/100 g for samples from North Peninsular Malaysia and 57.9–61.2 g/100 g from the East Coast Peninsular Malaysia with the highest protein level found in samples collected during December to March. For EBN samples obtained from South Peninsular Malaysia, protein content ranged from 61.8 to 65.2 g/100 g and samples harvested from August to November had the highest protein content. However, the mineral contents found in EBN samples from all different zones are not affected by the season (Norhayati et al., 2010).

Farmed EBN along the seashores of Thailand were reported to be rich in sulphur-containing amino acids, calcium, and magnesium (Saengkrajang et al., 2013). The use of Gas Chromatography-Mass Spectrometry (GC-MS) and Liquid chromatography-Mass Spectrometry (LC-MS) together with orthogonal projection to latent square discriminant analysis (OPLS-DA) could identify the origins of EBNs accurately based on the fatty acid content in the Malaysian, Indonesian, and Thailand EBNs. Generally, the levels of fatty acids and fatty acid amides are highest in Malaysian EBNs followed by Indonesian and then Thai EBNs. Moreover, the Malaysian and Indonesian EBNs could be differentiated with distinct fatty acids profiles in which the fatty acids of Malaysian and Indonesian EBNs are separated along the PC1 axis in the loading plot. (Chua et al., 2014). Thailand has a huge land mass cultivating rice whereas Malaysia and Indonesia have large palm oil plantations (Seeboonruang, 2012; Meijaard et al., 2020). Swiftlets usually feed

on insects nearby their habitats, the swiftlets in Thailand feed on insects with a rice-based diet, resulting in low fatty acids profile in Thai EBN. In contrast, the swiftlets in Malaysia and Indonesia feed on insects with a diet rich in palm oil, resulting in high fatty acids content in their EBNs (Lourie and Tompkins, 2000; Zhou et al., 2003; Tres et al., 2013). In the same study by Chua et al., the cave EBN contains higher level of ergosterol, a component of fungi and heptadecasphinganine, antifungal agent when compared to house EBN indicating higher amount of fungi in cave EBN and production of heptadecasphinganine by the swiftlets (Chua et al., 2014).

EBN is seen to be rich in protein, essential amino acids, essential trace elements, and essential sugars for human biological functions. Pure EBN collected from swiftlet premises in Batu Pahat, Malaysia contains carbohydrate (46.47%) (w/w), protein (35.80%) and a very minute fat content (1.30%). EBN contains relatively high amount of sodium (6017 mg/kg), magnesium (344 mg/kg), potassium (138 mg/kg) and calcium (68 mg/kg). Other minerals such as phosphorus, iron (Fe), chromium, and selenium are 0.037, 4.52, 0.30 and 0.14 mg/kg respectively and trace amounts of heavy metals arsenic (As) (0.0237 mg/kg), lead (Pb) (0.0203 mg/kg), copper (Cu) (0.6783 mg/kg) and zinc (1.2542 mg/kg) were found in the EBN. Malaysia Food Act 1983 (Act 281), Part VII (Incidental Constituent), Regulation 38 consider them as heavy metals. However, these heavy metals were within the infant formula specification limit as set by the Act. No mercury (Hg) and cadmium (Cd) were detected in the EBN. Fibre would only be detected in EBN if adulterant containing vegetative matter was added into EBN (Lee et al., 2015). Proximate nutritional compositions were compared between *A. fuciphagus* EBN from Pahang and Terengganu, Malaysia (Halimi et al., 2014).

Systematic analysis of nutritional composition of EBN collected from different regions in Indonesia was recently conducted. EBN collected from West Sumatra, South Sumatra, West Java, West Kalimantan, Central Sulawesi, and Southeast Sulawesi were analysed. The proximate nutritional compositions are protein (53.09–56.25%), carbohydrate (19.57–23.04%), moisture (17.08–21.50%), ash (5.44–6.25%), and fat (0.07–0.57%). EBN collected from different locations in Indonesia contain 18 types of amino acids, including 10 essential amino acids and 8 non-essential amino acids, ranging between 16.15 and 20.88%. NO_2^- contents were found to be ranging from 3.11–18.28 ppm (average 8.40 ppm) (Elfita et al., 2020).

EBN from Myanmar was found to contain high protein (53.5–59.3%), sodium (0.17%), potassium (237.1 ppm), calcium (0.71%), magnesium (361.3 ppm), and zinc (12.3 ppm). The NO_2^- and NO_3^- levels in EBN were estimated by measuring NO_2^- and NO_3^- contents in filtrate water after soaking with EBN for 4 h. The NO_2^- and NO_3^- levels in filtrate water are 0.27 and 4.0 ppm respectively (Phyu Win et al., 2020).

Authentication of Edible Bird's Nest

In the past, EBN was considered 'Caviar of the East' and was expensive (from US$1000.00 to US$10,000.00 per kilogram).There have been incidents of adulterants added into EBN during the processing to increase the final weight of EBN product. The addition of adulterants possesses risks to the health of EBN consumers as well as economical loss to the consumers when faulty goods were purchased (EBN products containing adulterants). Common adulterants such as tremella fungus (*Tremella fuciformisis*), jelly, fish swimming bladder, egg white, pork skin, karaya gum (*Sterculia urens*), and red seaweed could cause unwanted health effects and allergy if consumed unnoticeably (Thomas et al., 2019; Yeo et al., 2021). The relevant government departments and companies are responsible to authenticate and prevent consumers from consuming EBN products containing health-threatening adulterants.

Gel electrophoretic protein fingerprint profiling could be done to differentiate EBN from white fungus, jelly, fish swimming bladder and egg white. Cave EBN (30 and 35 kDa), house EBN (120 and 140 kDa), white fungus (22, 35, and 75 kDa), egg white (35 and 75 kDa), fish swimming bladder (streaking bands), and jelly (no band). The amino acid fingerprint profile of cave EBN, house EBN, white fungus, egg white, fish swimming bladder, and jelly are all distinct from each other and could be identified by HPLC (Hun et al., 2016). Proximate nutritional analysis shows that addition of adulterants, such as karaya gum, red seaweed, and tremella fungus, could account for 2–10% of the final weight of EBN and reduce the crude protein content of EBN up to 6.2% (Marcone, 2005). Authentic EBN contains more than 3 mg epidermal growth factor (EGF) in 1 g of protein as detected using a simple immunoblotting assay (Yang et al., 2014). Guo et al. established a TaqMan-based real-time polymerase chain reaction (PCR) assay to specifically detect EBN components and 4 common adulterants: white fungus, agar, pork skin and egg white. Five sets of primers and probes were designed for these five components. The assays were specific and reproducible even after the samples have undergone vigorous, tedious, and complicated processing. The relative detection sensitivities were 0.5% EBN, 0.001% white fungus, 0.5% agar, 0.001% fried pork skin and 1% egg white (Guo et al., 2014).

Metabolite profiling refers to holistic analysis of the molecular information of food which has recently gained attention in the food industry (Xu et al., 2006). The use of mass spectrometry coupled with chromatography resulted in improved data quality and sensitivity (Chua et al., 2014). The mass spectrometry method was further improved using GC-MS and LC-MS together with the chemometrics model, orthogonal projection to latent square discriminant analysis (OPLS-DA). These methods could classify the EBNs based on production site, colours, and countries. OPLS-DA has the advantage of being able to predict the identity of unknown samples. GC-MS coupled with OPLS-DA was discovered to be a better EBN authentication method for quality control of EBN compared to LC-MS, of which detection of the metabolite could be affected due to EBN processing (Chua et al., 2014). GC-MS was used to detect the oligosaccharides content in EBN, especially the N-Acetylneuraminic acid (NANA). By using environmental scanning electron microscopy, the micrographs of authentic raw EBN samples display the crater-like structure of irregularly shaped three-dimensional networks, measuring

from less than one, up to several microns. However, the fake EBN samples made from jelly fungus, agar, and pork skin display granule- or cudgel-like structures rather than network structures. In another study, well-established gas chromatography with flame-ionisation detection (GC-FID) was employed to analyse and quantify amino acids in EBN whereas optimised liquid chromatography with tandem mass spectrometry (LC-MS-MS) was applied for monosaccharide analysis. The data generated were combined with Hotelling T2 range plot to identify EBN and non-EBN. Hotelling T2 range plot is a chemometrics plot that reduces the amino acids and monosaccharides into a T2 value. This plot simplifies data analysis process and, more importantly, combines multiple variables to create a unique fingerprint for the sample (Chua et al., 2015). Its application is often found in the quality control of products (Eppe and De Pauw, 2009). This method has since then discovered EBN to contain a group of glycoproteins that are not affected by the EBN's colouration, country of origin, and/or the processing method of the food item (Chua et al., 2015).

Samples damaging during authentication of EBN is the major concern in the authentication process. Contamination or loss of chemical composition could result in false reporting if samples were not handled with care. Thermogravitiy (TG) and differential thermogravity (DTG) techniques require 5–10 mg of EBN samples for rapid authentication of EBN by comparing the TG and DTG curves produced from EBN and adulterated EBN. EBN was heated in the crucible from 25°C to 1000°C and 800°C for unadulterated and adulterated samples to generate the TG and DTG curves. TG is presented in the curve of mass percentage versus temperature whereas the first derivative DTG curve gave the rate of change of mass loss percentage versus temperature. Hence, adulterated EBN (e.g. glucose, sucrose, hydrolysed marine collagen, and monosodium glutamate) has distinctive TG and DTG curves which could be identified when compared to TG and DTG curves of pure EBN (Shim et al., 2017). Shi et al. (2017) designed a rapid and non-destructive method to characterise the distribution map of carbohydrates, proteins, and sialic acid in EBN using hyper-spectral imaging. The chemometrics and spectral signals of EBN were used to build calibration models which then were tested by extracting and predicting spectra of each pixel in EBN hyperspectral images. The distribution map of carbohydrates, proteins, and sialic acid in EBN were characterised with unevenly distributed carbohydrate and protein contents based on the swiftlets diet consumption: Insects and small fish consumption increased protein content whereas seaweed consumption increased carbohydrates content. However, sialic acid is evenly distributed in the entire EBN (Shi et al., 2017). Future studies on these methods to identify adulterated EBN and their potential application in industry EBN preparation is promising to be ventured.

Fourier Transform Infrared (FTIR) spectrum of the raw unprocessed EBN was identical to that of the spectra of processed EBN samples. The spectra of adulterated EBN samples were different from that of processed EBN. Fingerprint region of the spectra of pure edible bird and adulterated edible bird nest samples were different at $<1700\ cm^{-1}$. The NH group was absent in all adulterants except in pork skin and egg white. Interestingly, only pork skin has ester C=O stretch bond and C=CH group which were not present in EBN. Hence, this technique could be used for testing the authenticity of the EBN samples as well as detection of porcine products (Hamzah et al., 2013). Apart from that, indirect enzyme-linked immunosorbent assays (ELISAs) was developed to detect porcine gelatine adulteration using anti-peptide polyclonal antibodies. Three indirect ELISAs were developed for porcine species-specific amino acid sequences of the collagen (I) α2 chain (PAB1 and PAB2) and the collagen (I) α1 chain (PAB3), which had limits of detection of 0.12, 0.10 and 0.11 μg/g respectively (Tukiran et al., 2015).

Environmental factors, such as locations, harvesting places, and harvesting seasons could affect the quality of EBN and a standardised guideline is required for quality control of EBN products. Differences in nutritional, adulterants, and contaminants detected could be due to different EBN handling, beginning from collection, processing, delivery, and storage. The EBN industry in Malaysia and Indonesia suffered a severe blow in 2011 when China banned exportation of EBNs due to high concentrations of NO_3^-, Pb and As in certain products. Various regulations were rolled out in response to the ban (Yeo et al., 2021). The improved approaches for authentication and classification of EBN could benefit the whole industry by having a more economical, rapid, and streamlined industrial preparation of EBN products, as well as providing better guarantee of the quality of EBN products to meet the consumers requirements.

BENEFICIAL HEALTH FUNCTIONS OF EDIBLE BIRD'S NEST

EBN has been a traditional, healthy food delicacy since the Tang Dynasty, however scientific research has been lacking over the last century. EBN-related publications remained scarce between 2000 and 2011, but the number of publications has increased significantly since 2012–2019. Discussions about the functions of EBN can be found in this review article by Lee et al. (2021) while our review will be focusing on recent discoveries of bioactive components in EBN and the functions of EBN that have never been discussed before.

Antiviral, Immunomodulatory, and Antimicrobial Actions

EBN is known to exert antiviral bioactivity against influenza A virus (IAV) (Biddle and Belyavin, 1963). In another study carried out decades later, it was found that the non-pancreatin treated EBN extracts can bind to influenza viruses, but this antiviral effect was not seen in haemagglutination inhibition assay or the neutralization assay of influenza virus infected Madin-Darby Canine Kidney (MDCK) cells. The proteins or peptides which had the best anti-influenza effect were found to be sialylglycoproteins of size 10–25 kDa in the pancreatin treated EBN extract. Although proteins above 50 kDa (including a major allergen of 66 kDa) in the non-pancreatin treated EBN extract could bind to influenza A virus, neutralization against the virus

was limited, indicating small molecular weight proteins of EBN extract was more favourable for its anti-viral effect. Furthermore, the EBN extract after pancreatin treatment did not trigger detrimental effects, such as hemolysis and cytolysis of erythrocytes and MDCK cells even at a higher concentration (4 mg/ml) (Guo et al., 2006). In summary, EBN extract treated with pancreatin is a more effective and safer antiviral agent when compared to non-pancreatin treated EBN extract.

Sialic acid (SA) and its derivatives found in EBN are highlighted by Guo *et al.*, in which they proposed that these compounds potentially contribute to the anti-influenza effects of EBN (Guo et al., 2006). Indeed, sialic acid is the most studied antiviral compound in EBN. One of the identified sialic acid is 5-N-acetylneuraminic acid (Neu5Ac) (Ogura, 2011). Interestingly, the Neu5Ac content in EBN is correlated with its potency in antiviral activity. The concentration of Neu5Ac varies among EBN samples collected at different geographic locations in Malaysia. EBN from Gua Madai contained 6.7 mg/g of Neu5Ac, which is higher than EBN collected from Teluk Intan which contained 3.2 mg/g of Neu5Ac (Haghani et al., 2017). SA residues could interact with the neuraminidase, and prevent this enzyme from cleaving the SA receptor present on the host cell surface hence halting the viral spread and release (Bianco et al., 2001; Haghani et al., 2016; Benton et al., 2017). Treatment with EBN for 24 h after the MDCK cells were inoculated with IAV for 1 h had the highest antiviral activity and percentage of protection against IAV when compared to EBN pre-treatment (MDCK cells treated with EBN for 1 h before IAV inoculation) and co-treatment (MDCK cells treated with EBN and IAV at the same time for 1 h) groups. It is speculated that the antiviral action of EBNs might be related to the viral release process from the cell membrane rather than viral attachment to the host cell. In the same study, EBN reduced early endosomal trafficking of the IAV by downregulation Rab5 and RhoA GTPase proteins. EBN also inhibited the IAV life cycle by regulation of autophagy with decreased expression of LC3-II and increased lysosomal degradation (Haghani et al., 2017). Besides that, EBN also reduced viral proliferation as effective as oseltamivir phosphate (a commercial antiviral drug) as shown by its effect in suppressing the viral enzyme, neuraminidase in IAV-infected BALB/c mice. Concomitantly, EBN showed high immunomodulatory effects against IAV by significantly increasing the levels of interferon-gamma (IFN-γ), tumour necrosis factor-alpha (TNFα), nuclear factor kappa B (NF-κB), interleukin-2 (IL-2), some pro-inflammatory cytokines (IL-1β and IL-6) and other regulatory cytokines (IL-4, IL-10, IL-12, IL-27 and CCL-2) depending on the stage of infection (Haghani et al., 2016).

At the same time, EBN also possesses immune-enhancing properties by modulating the immune system. However, there are only a limited number of experiments which have studied the effects of EBN on immune regulation. Zhao *et al.* studied the immunomodulatory effects of EBN in immunocompromised BALB/c mice treated by cyclophosphamide. Results showed that EBN stimulated proliferation and activation of B cells. Enhanced antibody secretion from B cells and better protection of the B cells from damage were detected in experimental mice, resulting in reduced intestinal immune damage (Zhao et al., 2016). Furthermore, EBN was also proven to enhance T lymphocytes transformation and increase serum immunoglobulin M (IgM) in the mouse model (Zhang et al., 1994). In addition, Cao *et al.* also stated that humoral immune, cellular immune and nonspecific immune were strengthened and regulated through EBN-induced improvements on the spleen and thymus index together with the phagocytosis rate and phagocytosis index of the peritoneal macrophages (Cao, 2012). Last but not least, EBN could also potentiate the proliferation of human peripheral blood monocytes (Ng et al., 1986).

EBN has also shown the potential in curing ulcerative colitis. An *in vivo* study was done by administering 5 ml 2% dextran sulfate sodium (DSS) via oral route daily for 7 days to induce ulcerative colitis in the male C57BL/6J mouse model. EBN was introduced in three concentrations (0.3 g/kg, 0.7 g/kg and 1.3 g/kg). Histologically, EBN-treated mice had a lower inflammation activity and a more complete structure of the submucosa, in comparison to the positive control group (without EBN supplementation) which showed excessive inflammatory cell infiltration, exfoliated epithelium, disrupted glandular arrangement and reduction in number of goblet cells in the submucosa. EBN reduced severe inflammatory reaction by lowering myeloperoxidase (MPO) activity and production of TNF-α and IL-6. In addition, reduced level of IL-17 and increased level of TGF-β were also determined in the EBN-treated groups. The author suggested that EBN ameliorated the inflammation by restoring the balance of Th17 cells and Treg cells, which was seen to be increased in the positive control group. This was further proved by the immunohistochemistry (IHC) and western blots, in which the IL-17A and Foxp3 genes were overexpressed in the positive control group whereas the overexpression of these two genes was inhibited by EBN. Concisely, EBN was proven to alleviate DSS-induced inflammation through immunomodulation by restoring the balance of Th17 and Treg cells and their associated cytokines (Fan et al., 2021).

Type of solvent used for extraction may have an impact on the antimicrobial activity of EBN. A metabolite, thymol-beta-D glucopyranoside was detected in the EBN. This metabolite has been previously reported to be an effective agent against food-borne bacteria (i.e. *Staphylococcus aureus (S. aureus), Escherichia coli (E. coli), Salmonella typhimurium, Shigella flexneri* etc.) (Liang et al., 2007; Chua et al., 2014). Hence, it was speculated that presence of this metabolite would confer antimicrobial activity in EBN. EBN extracted with methanol showed antibacterial activities for *S. aureus* (100 mg/L), *Candida albican (C. albican)* (100 mg/L), *E. coli* (1,000 mg/L), and *Aspergillus niger (A. niger)* (>3,000 mg/L), but the EBN extract derived by soaking extraction in ethyl acetate showed no antibacterial effects. Results of the solvent extraction methods showed that the EBN extracts with ethyl acetate were more effective than using methanol with *C. albican* (20 mg/L) and *A. niger* (20 mg/L) while the solvent extraction method with methanol showed slight effects with *S. aureus* (20–100 mg/L) and *E. coli* (>100 mg/L). In another study, the antibacterial activities of EBN extracted with 0.05 M alkaline with heating were tested on

two Gram-positive (*Bacillus subtilis* and *S. aureus*) and two Gram-negative (*E. coli* and *Klebsiella pneumoniae*) bacterial strains but no antibacterial effects were observed in those tests (Saengkrajang et al., 2011). It is speculated that the extraction method could significantly affect the antibacterial effects of EBN. More studies should be done to identify the optimal extraction method of EBN to retain antimicrobial functions.

In short, EBN has shown its potential in enhancing the immune system in both *in vitro* and *in vivo* models. Nevertheless, more investigations are warranted to understand the mechanisms of immuno-stimulation and antibacterial effects exhibited by EBN.

Cancer Prevention and Treatment

EGF were detected in the crude EBN collected from Rompin (30.7 pg/ml) and Sibu (74.5 pg/ml) from Malaysia using ELISA quantification kit. Tan et al. postulated that the presence of EGF in EBN can stimulate cancer cell growth. Four cancer cell lines (MCF-7 human breast adenocarcinoma cells, A549 human alveolar adenocarcinoma cells, Caco-2 human epithelial colorectal adenocarcinoma cells, and HCT116 human colorectal carcinoma cells) with EGF^{+} phenotype were selected to test for cell proliferative effects of EGF content in EBN using MTT assay. However, results showed no significant growth was observed in all 4 cell lines treated with EBN. It was thought that the EGF content in EBN could be too low to induce cancer growth (Tan et al., 2020). In another study, the cell viability of Caco-2 cell treated with two commercial EBNs were 84 and 115% respectively, while Caco-2 cells treated with unprocessed EBN from the East Coast, North and South Zones of Peninsular Malaysia were 91, 35 and 47% respectively. RAW 264.7 cell is a macrophage cell line that is usually used to study TNF-α expressions in macrophage. The EBNs collected from South and East Coast Zones of peninsular Malaysia as well as one of the commercial EBN, significantly reduced TNF-α production in RAW cells, where it was reduced to 24%, 32%, and 43% respectively. Geographical location and sources of the EBN are the factors resulting in the observed discrepancies in cancer cell proliferation rate. In the same study, commercial EBN products induced Caco-2 colorectal cancer cell growth. It is speculated that the adulterant in commercial EBN products stimulated Caco-2 cell growth, future studies are required to find out the substance responsible for promoting cancer cell growth in adulterants with introduction of better authentication procedure to detect such adulterants (Aswir and Wan Nazaimoon, 2011).

Growth Factors and Tissue Regeneration

There is evidence that EBN possesses wound-healing effects. Hwang et al. studied the wound healing effects of EBN on HaCaTs cells and normal human dermal fibroblasts (NHDFs) which had been irradiated by ultraviolet B (UVB). Results showed that treatment with EBN significantly improved wound healing effects in both HaCaTs and NHDF cells. In fibroblasts, healing rate of the cells treated with 10 μg/ml increased by 39.6% after 24 h as compared to 0 h timepoint. Healing rate is faster in EBN treated cells as compared to fibroblastic self-healing (17% without any treatment) and 10 μg/ml allantoin treated group (28.2%). Hyaluronic acid, also known as hyaluronan, is a straight-chain carbohydrate extracellular matrix polymer that is a key component for the wound healing process (Aya and Stern, 2014). 10 μg/ml of EBN also significantly increased the production of hyaluronan in HaCaTs cells by 109.1%. In the study, EBN was also shown to confer anti-inflammatory effects as EBN reduced the expressions of two inflammatory cytokines, TARC/CCL17 by 89.7% and MDC/CCL22 by 46.1% in TNF-α/IFN-γ-stimulated HaCaTs cells. The author suggested that rapid healing effect induced by EBN was mediated through enhanced production of hyaluronan and the downregulation of MMP-1 and upregulation of procollagen type I expression in UVB-irradiated NHDF cells. In summary, these results indicate that EBNs have the potential to ameliorate UVB-induced skin photo ageing and TNF-α/IFN-γ-stimulated inflammation as well as wound injuries, resulting in faster healing rate (Hwang et al., 2020).

EBN also improves healing in the cornea. It was shown that rabbit corneal tissues treated with 0.05% EBN (with or without the addition of serum) resulted in increased expressions of collagen type I, aldehyde dehydrogenase (ALDH) and lumican, as compared to those treated with either serum-containing medium or serum-free medium (Zainal Abidin et al., 2011).

An *in vivo* study done by Albishtue et al. to study the effects of EBN on female reproductive system using adult female Sprague Dawley rats showed that oral EBN treatment at 60 and 120 mg/kg per day for 9 weeks augmented proliferation of uterine cells, including luminal epithelium, glandular epithelium, and stromal cells, resulting in better uterine and reproductive functions. Besides that, the endometrial receptivity and the number of implantation sites were also increased by EBN treatment. EBN also enhanced production of reproductive hormones, estrogen, progesterone, and prolactin, as well as expression of steroid receptors, progesterone and estrogen receptors. Upregulation of vascular endothelial growth factor (VEGF), EGF, EGF receptor, and proliferating cell nuclear antigen (PCNA) were found in the endometrial tissue. In short, EBN showed its potential to improve embryo implantation and also increased successful pregnancy rates (Albishtue et al., 2019).

Recently, it was reported that the action of Neu5Ac is not only limited to antiviral functions, but it also exhibits skin whitening and bone maintenance effects. In a skin whitening test, pepsin-digested EBN showed stronger inhibition of melanogenesis in cultured murine B16 skin cells and enzymatic activity of tyrosinase, as compared to that of undigested EBN. In addition, the pepsin-digested EBN also exhibited stronger osteogenic activity in cultured MG-63 osteoblasts cells. Neu5Ac in EBN was originally in conjugated form and was released from the conjugated form by treating EBN with pepsin in simulated gastric fluid at pH 2 condition for 48 h. This study indicated that prolonged EBN extraction in a gastric-like condition could achieve the full beneficial functions of EBN. Wong et al. tailored the method of Neu5Ac extraction from EBN (Wong et al., 2018c).

Cardiometabolic Maintenance

EBN also has an impact on metabolism. Western blots showed that diabetic *db*/*db* mice treated with 75 and 150 mg/kg of EBN orally had elevated insulin levels when compared to mice treated with distilled water only. Meanwhile, insulin signalling receptor (IRβ) and downstream proteins (p-IRS1, PI3K and p-Akt) were also upregulated in mice treated with 75 and 150 mg/kg of EBN. Treatment with 75 and 150 mg/kg of raw EBN also decreased expression of pro-inflammatory cytokines, IL-6 and TNF-α, and also inflammatory protein, NF-κB in these mice. Moreover, at these doses, oxidative stress was ameliorated, as shown by downregulation of NADPH oxidase 4 (NOX4) protein, a reactive oxygen species (ROS) marker, and upregulation of superoxide dismutase-1 (SOD-1) protein, an antioxidant protein. In summary, EBN improves β-cell function and insulin signalling by attenuation of oxidative stress-mediated chronic inflammation in type 2 diabetic mice (Choy et al., 2021).

In another study, EBN demonstrated its potential role in preserving endothelial function by reducing oxidative stress in both cultured cells and mouse models. In the *ex vivo* study using male C57BL/6J mice, aortic ring assay indicated that impaired aortic relaxation in high glucose-fed mice was reversed with treatment of raw EBN (15 and 30 μg/ml). Human umbilical vein endothelial cells (HUVECs) were also utilized to examine the effects of EBN on high glucose-induced ROS formation. Results of lucigenin-enhanced chemiluminescence assay showed that treatment with 30 μg/ml of raw EBN significantly diminished intercellular ROS level and vascular superoxide anion production in HUVECs. EBN treatment at 30 μg/ml in HUVECs or 150 mg/kg in *db*/*db* mice reversed the high glucose-induced depletion of nitric oxide (NO). Western blot analysis also showed that EBN treatment at 30 μg/ml in HUVECs or 150 mg/kg in *db*/*db* mice could significantly reduce the level of NADPH oxidase 2 (NOX-2) and nitrotyrosine proteins while increase SOD-1 and p-eNOS protein levels (Murugan et al., 2020).

Antioxidation and Neuroprotection

EBN has antioxidant effects which could serve as a novel alternative therapy for oxidative stress-mediated neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) (Tai et al., 2017). The antioxidant capacity of EBN is higher in extracts obtained via alkaline extraction, it is speculated that the hydrolysed proteins release amino acids such as Cys, Met, His, Try and Lys with antioxidant properties (Lee et al., 2015). Simulated human gastro-intestinal digestion study demonstrated that the antioxidant components of EBN are released after digestion in the human gut. The digested EBN was shown to enhance antioxidative activities and protected HepG2 human liver cells from hydrogen peroxide (H_2O_2)-induced cytotoxicity (Yida et al., 2014).

Pathogenesis of PD involves oxidative stress-induced death of the midbrain dopaminergic neurons. EBN reversed ROS and nitric oxide (NO) build-up, reduced lipid peroxidation, inhibited caspase-3 cleavage, and attenuated apoptotic cell death in an *in vitro* PD model, neurotoxin 6-hydroxydopamine (6-OHDA)-treated SH-SY5Y cells. EBN improved motor function and balancing in 6-OHDA-treated C57BL/6J mice *in vivo* PD model. Inhibition of microglia activation and enhancement of antioxidant enzyme activity was shown in EBN-treated mice (Yew et al., 2014, 2018). Neurotrophic properties of EBN were demonstrated by increased cell proliferation and migration in a neural stem cell model, embryonic mouse neuroectodermal cells (NE-4C) (Yew et al., 2019). In another study, lipopolysaccharide (LPS) elicited cognitive impairment in rats by significantly increasing the escape latency while decreasing the number of entries in the probe trial, which were coupled with increased production of proinflammatory cytokines (TNF-α, IL-1β, and IL-6) and oxidative markers (ROS and TBARS) in the hippocampus. Treatment with EBN (125 mg/kg, 250 mg/kg, and 500 mg/kg; p.o.) effectively reversed effects of LPS on escape latency and probe trial. In addition, these treatments also inhibited the LPS-induced upregulation of pro-inflammatory cytokines and oxidative markers (Careena et al., 2018).

The effects of EBN on spatial learning and memory were also examined. The results showed that EBN supplementation had a dose-dependent improvement on cognition as evidenced by significant shorter escape latency. EBN-induced improvement of spatial learning and memory was also seen in the newly born offspring mice, which was fed on maternal milk of mother mice exposed to EBN supplementation. At the molecular level, EBN also exhibited its effect on attenuating neuro-inflammation and neuro-oxidative stress through increasing expression of SOD and decreasing levels of malondialdehyde (MDA) in the newly born offspring mice fed on maternal milk of mothers exposed to EBN supplementation. Evidence showed that EBN-induced enhancement of cognitive activity was due to elevation of SIRT1 expression in the pyramidal layer and dentate gyrus of the hippocampus. Besides that, EBN exhibited neuro-protective effect through anti-apoptotic mechanism in which the caspase-3 cleavage and early apoptotic membrane phosphatidylserine externalization were inhibited. The antioxidant effect of EBN through decreasing ROS level and increasing the expression of SOD gene in hippocampal neurons (SH-SY5Y neuroblastoma cells) was another neuroprotective mechanism (Ismail et al., 2021). In a recent study, zeroth generation CJ57BL/6 mice gave birth to first and second generations of offspring after 6 weeks of EBN supplementation (10 mg/kg). Both generations of offspring showed improvement in Y-maze cognitive performance at 6 weeks of age. Brain samples of the offspring mice demonstrated upregulation of GNE, ST8SiaIV, SLC17A5, and BDNF mRNA, and increased densities of synaptic vesicles in the presynaptic terminal (Mahaq et al., 2020).

Interestingly, EBN has also shown to have antioxidant effect in fly model. *Drosophila melanogaster* was cultured in four different groups, in which they were fed by food medium supplemented with 0 g/kg (control), 1 g/kg, 3 g/kg and 9 g/kg of EBN. Results of ferric reducing antioxidant power (FRAP) showed that the EBN-treated groups had higher total antioxidant activity in a dose-dependent manner as compared to control which had low total antioxidant activity. EBN enhanced the antioxidant capacity by increasing the SOD and catalase activities (CAT) whereas the MDA level was decreased. In a nutshell, EBN could improve

ageing problems by reducing oxidative stress, hence increasing the lifespan of *Drosophila melanogaster* (Hu et al., 2016).

CHEMICAL AND BIOLOGICAL CONTAMINANTS IN EDIBLE BIRD'S NEST

Based on the Memorandum of understanding (MOU) on the Protocol of Inspection, Quarantine and Hygiene Requirements for importation of bird nest products from Indonesia and Malaysia into China, sealed and signed by Malaysia and Indonesia with China, the contaminants were assessed from the aspects of physical, microbiological, residual, heavy metals and excessive minerals, parameters and tolerance levels (Yeo et al., 2021). Bacteria, fungi, and mites are commonly reported to be found in EBN. Microbial growth in EBN might be attributed to the environment of EBN collection, such as high humidity levels and lower temperature or microbial infestation during EBN storage. The microorganisms could also be originated from saliva or feathers of swiftlets or the nest itself (Kew et al., 2014; Yeo et al., 2021). Additionally, Wong *et al.* revealed that both raw and commercial EBNs contain diverse types of bacteria, including *Staphylococcus sp.*, *Bacillus sp.* and *Acinetobacter sp.* Although the double-boiling treatment could effectively kill most of the bacteria, heat-resistant species like *Bacillus sp.* and *Brevibacillus sp.* were still isolated after treatment (Wong S. F. et al., 2018). Consumption of *Bacillus cereus* (*B. cereus*)-contaminated food can lead to diarrheal and emetic syndrome, resulting from production of *B. cereus* toxins (Griffiths and Schraft, 2017). A promising non-thermal processing method for food preservation by polychromatic low-energy X-ray with a high linear energy transfer (LET) effect results in a high relative biological effect (RBE). Low-energy X-ray with cut-off energy of 150 KeV was applied to inactivate two of the most prevalent foodborne pathogens in dry EBN. X-ray irradiation at 350 and 400 Gy decreased *E. coli* O157:H7 and *S. Typhimurium* from 6.35 ± 0.56 and 5.84 ± 0.67 log CFU/g, respectively, to undetectable levels. Based on dose distribution in 10 stacked pieces of EBN, two-sided irradiation could effectively inactivate pathogens uniformly (Zhang et al., 2020).

Environmental fungi from soil and plants are generally detected in both raw and commercial EBN. Although most of the fungi were removed after boiling of EBN up to 100°C for 3 h, the environmental fungal genera of *Aspergillus sp.* and *Penicillum sp.*, can still be isolated in both EBN samples. It was speculated that these fungi were introduced from the EBN processing facility environment after the boiling process because these are two of the most frequently isolated environmental fungal genera. However, it is also possible that these fungal genera are thermoresistant or thermotolerant (Chen et al., 2015). *Aspergillus sp.* and *Penicillum sp.* are commonly known as food-spoilage fungi and can produce different mycotoxins (e.g. aflatoxins and ochratoxin A), which could cause various diseases in human as well as opportunistic infections in immunocompromised individuals (Greeff-Laubscher et al., 2020). Future studies are warranted for complete elimination and contamination management of these two fungal genera. Moreover, *Cladosporium* sp. and *Eurotium* sp. are detected in raw EBN samples, as these two genera of fungi are known to cause respiratory infections, swiftlet ranchers require personal protective equipment to prevent inhalation of these hazardous microbes (Chen et al., 2015). Mites are known as a source of allergens that could cause anaphylaxis (Sanchez-Borges et al., 1997). Mites, their faeces and eggs, and feather strands were observed on the surface of both raw and commercial EBN through EBN surface structural analysis under the electron microscope (SEM). These contaminants remained on the surface of EBN even after the washing and processing procedures were done (Kew et al., 2014; Tai et al., 2020). EBN is one of the causes of food-related anaphylaxis among children (Goh et al., 1999) and the pathophysiology is suggested to be related to immunoglobulin E (IgE)-mediated hypersensitivity caused by the protein existing in several isoforms seen at 66 kDa. N-terminal sequence of the major putative allergen (66 kD) showing homology to a domain of an ovoinhibitor precursor in chicken (Goh et al., 2001).

Insects consume plants inhabited by nitrogen-fixing bacteria and crops fertilized with nitrogen-based fertilizers. When swiftlets consume these insects, this results in accumulation of nitrites and nitrates in the swiftlets. As the nest is made of salivary secretion of swiftlets and embedded with droppings of swiftlets, high concentrations of NO_3^- and nitrogen-fixing bacteria are detected. The nitrogen-fixing bacteria converts NO_3^- into NO_2^- giving rise to redding of EBN by NO_3^- reductase. Although up to 98% of NO_2^- could be removed after commercial EBN processing, NO_2^- and NO_3^- is still found in high concentrations in EBN, exceeding the acceptable tolerance level which is ≤30 ppm (But et al., 2013; Quek et al., 2018a; Yeo et al., 2021). In fact, the daily intake limit of NO_2^- as advised by the World Health Organization (WHO) is between 0 and 3.7 mg/kg body weight (Yeo et al., 2021). To clear the traditional belief of red EBN being more precious, Paydar et al. (2013) discovered that the red colour in EBN is contributed by NO_3^- and NO_2^- instead of the presence of haemoglobin. NO_3^- is relatively stable but NO_2^- is active and can react with coexisting amino acids to form a carcinogenic compound called nitrosamine (Yeo et al., 2021). Semicarbazide, which originates from bleaching process used to remove impurities, was another carcinogenic compound found in EBN (Xing et al., 2012). A safer method to prevent red colouration of EBN is by using sodium tungstate, an inhibitor of NO_3^- reductase that suppresses formation of NO_2^- in cubilose (Chan et al., 2013). Nonetheless, a recent study reported that the acidic mammalian chitinase-like protein found in EBN contributes to the red colour as a noticeable increase in Fe-O bonding intensity after the colour change in EBN (Wong et al., 2018b).

Heavy metal and mineral contaminations in EBN were also reported in recent studies. Studies showed that in raw EBN, levels of Hg and Cu were higher than the permissible limits whereas the levels of Pb, As and Cd were below the limits (Chen et al., 2014; Quek et al., 2018b). A trace amount of these elements was also found in commercial EBN (Tan et al., 2020). As a matter of fact, the maximum permissible levels of each element is as follows; 1) Hg ≤0.05 ppm; 2) Cu ≤1 ppm; 3) Pb ≤2 ppm; 4) As ≤1 ppm; and 5) Cd ≤1 ppm. In addition Fe level was found to be higher than

the regulatory limit (0.3 ppm) in both raw and commercial EBN (Yeo et al., 2021). All these heavy metals, if consumed in excessive amounts could react with proteins or enzymes in the human body, causing chronic heavy metal poisoning syndrome (Dai et al., 2020). For example, Hg intoxication could cause severe behavioural and cognitive changes as well as delayed development of growth and neural system in children (Ha et al., 2017). Other than that, Cd is known to be a mutagenic compound and Cd consumption could result in cancer development (Fatima et al., 2019). An excess amount of Cu is also associated with certain human disorders, such as cardiovascular diseases, neurotoxicity and hepatic disease (But et al., 2013; Paydar et al., 2013).

In short, EBN consumption undoubtedly has its safety issues due to the potential residual contaminations including microorganisms (bacteria, fungi, and mites), heavy metals (Hg, Pb, As and Cd), minerals (Fe and Cu) as well as NO_2^- and NO_3^- contents. On top of that, adulterants used in EBN production and semicarbazide used in the bleaching process could also pose hazardous effects to human health. Therefore, the quality and authentication of EBN should be strictly regulated to avoid consumers from eating inauthentic EBN and thus the risk of EBN consumption could be reduced to a greaer extent.

EDIBLE BIRD'S NEST PROCESSING

The study of EBN has recently gained attention from researchers in the past decade as recent laboratory studies of EBN demonstrated many optimisation steps for the production of better EBN products. We summarise the optimisation steps based on laboratory findings that could be useful references for setting up the industrial EBN processing with the aims to improve the safety, taste, and preserve the health benefits of EBN products (**Table 1**).

Laboratory and Traditional Industrial Edible Bird's Nest Processing

After collection from the swiftlet premises or caves, EBN is processed following these general procedures: cleaning, drying, grinding, soaking/elution (specific for elution water extraction (eHMG)), heating, acid/alkaline treatment (specific for acid/alkaline extraction), enzymatic treatment (specific for enzymatic extraction), filter, centrifugation, freeze-drying/lyophilisation, storage. Slight changes for processing steps were made as reported in different groups for the consideration of targeted nutrients, convenience, cost-effectiveness, and instruments availability. These processing steps are critical for preservation of nutritional values of EBN (Yew et al., 2014; Gan et al., 2017b; Hong et al., 2020; Tong et al., 2020).

Firstly, the collected EBN is soaked in ultrapure water until softened to loosen the protein strands and cleaned by washing in water or ultrapure water, followed by manual removal of dirt, feathers, and egg shells using forceps (Yew et al., 2014). The dust and lighter impurities floating in the water and EBN could be easily picked with forceps as the EBN expands in size after soaking in water (Hong et al., 2020). Then, the cleaned EBN is dried with or without controlled air circulation, heat, and humidity. EBN could be dried at a faster rate with higher air circulation speed and temperature but this intervention is associated with reduced sialic acid and antioxidant retention in EBN (Gan et al., 2017a; 2017b). The dried EBN, around 10–12% moisture then undergoes grounding with pestle and mortar manually or electric blending based on preferences as no study was conducted for the comparison between manual and automatic grounding process (Gan et al., 2017a; Yew et al., 2019). The grounded EBN is sieved through a wire mesh (0.4–1.0 mm) to further separate the feathers and other impurities (Zainal Abidin et al., 2011). The next step is extraction of chemical compositions of EBN by extraction method of HMG, heat extraction, enzymatic extraction, acid extraction, alkaline extraction, and eHMG.

Grounded EBN is soaked in cold distilled water 2.5% (w/v) or deionized water at 0.2% (w/v) at 4°C, and usually left for overnight but some studies have also left it for 16–48 h (Guo et al., 2006; Yew et al., 2014; Tong et al., 2020). After that, heating of EBN solution is carried out in the range of 40°C to 121°C for 15 min to 4 h (Amiza et al., 2019a; Tong et al., 2020). If it was acidic extraction, the EBN mixture is heated at 80°C with 2% (v/v) of 0.4 M sulfuric acid for 4 h subsequently allowed to cool down and centrifuged at 2,716 g (5,000 rpm) for 15 min. The pH of supernatant collected is neutralized to pH 7.0. White precipitation formed is removed through centrifugation with 2,716 g (5,000 rpm) for 15 min at 4°C. The supernatant is then collected and kept at 4°C for further analysis (Tong et al., 2020). However, if it was an alkaline extraction, 1 g of each raw sample is immersed in 30 ml of 0.1–0.4 M NaOH solution for 48 h. Then, the aliquot of each extract is immersed in the water bath at 65°C for 2 h. The extracted solutions are centrifuged and eventually the supernatant is obtained. After the extraction process, the suspensions are centrifuged at 18,000 rpm for 20 min, and the supernatants are thoroughly dialysed against distilled water (Hun et al., 2016). For enzymatic treatment, enzymes such as pancreatin, alcalase (Khushairay et al., 2014), protease from *Bacillus licheniformis* (Ling et al., 2020), and neuraminidase from *C. perfringens* (Guo et al., 2006) is used. In continuation of the heating, the enzyme is usually added to the EBN mixture at a warm temperature between 38°C-60°C, at pH 8.5–9.0 to facilitate partial protein denaturation. It is then followed by heating at 90°C for 5 min for enzyme deactivation. The EBN mixture is then filtered using filter paper, filtrate is collected and freeze-dried to obtain EBN powder (Guo et al., 2006; Yew et al., 2014). Freeze-drying process aims to produce dehydrated EBN powder while retaining maximum nutrient and bioactive compounds (Bhatta et al., 2020). Lastly, the freeze-dried EBN powder is stored at 4°C or −80°C freezers for future use. In industrial setting, freeze-dried EBN could be made into cosmetics, tablets, ready-to-drink beverages, tonics, spices and baking powder among others (Lee et al., 2021). In the industrial processing of whole raw EBN, it is started with sorting and grading of EBN based on the difficulty to clean the EBN. The cleaning step is important as EBN with more feathers and impurities are graded lower and sold at a lower price. After

sorting, EBN will be soaked in clean water, so that dust and impurities will float on the water. Feathers that remained on the EBN will be hand-picked with forceps or tweezers. This is a labour-intensive process that could be time-consuming. After that, EBN is placed on a mold and left to dry by heating cabinet and fan blowing. This process could lead to nutrients loss. The dried EBNs in cup shape is sorted and graded again based on their colour, cleanliness, and shape for packing (Hong et al., 2020).

Industrial Edible Bird's Nest Processing Optimised With Laboratory Findings

As mentioned earlier, EBN is required to be free from avian influenza. The MOU on the Protocol of Inspection, Quarantine and Hygiene Requirements demands EBN products from Indonesia and Malaysia to be free from avian influenza to be imported into China (Badan Karantina Indonesia, 2014; Rohaizan, 2017). Only EBN obtained from farms registered with the Department of Veterinary Services, Malaysia is authenticated. The Chinese government has imposed stringent procedures for quality assurance by tracing the entire supply chain from EBN harvesting to exporting to China (Thorburn, 2015). Additionally, EBN processing facilities must be inspected and approved by Certification and Accreditation Administration of the People's Republic of China (CNCA) (Minister Department, 2012). According to the information provided by the CAIQ official website http://ebn.caiq.org.cn/overseasRegist, Indonesia has 23 EBN processing facilities and 436 swiftlet houses approved to be exported to China; Malaysia has 41 EBN processing facilities, but there is no information about the registered swiftlet houses; Thailand has 2 EBN processing facilities and 21 registered cave EBN sources (Development Report of Imported Bird's Nest in 2021, 2021).

Department of Veterinary Services of the Ministry of Agriculture and Food Safety and Quality Division, Ministry of Health, Malaysia, implements and enforces that EBN products must be processed with heat treatment, with core temperature of the products shall be higher than 70°C and retained for at least 3.5 s to effectively kill the avian influenza virus (Yeo et al., 2021). Apart from that, environmental fungi in raw EBN should be removed before consumption. Heating EBN to ≥100°C for at least 3 h could remove the environmental fungi in EBN (Chen et al., 2015). The heating temperature and time possess critical effects on extraction of bioactive compounds from EBN as well as retention its nutritional values. Excessive heat treatment is known to cause alteration in protein native structure resulting in unfolding of protein and altered protein surface exposure (Teodorowicz et al., 2017). As the proteins denature, bioactive properties are lost and this would affect its nutritional value.

Furthermore, differential scanning calorimetry have often detected loosely bound water (dehydrates from EBN below 110°C) and tightly bound water (dehydrates from EBN between 100°C and 200°C) in EBN samples. In fact, it implies the wide use of H_2O_2, as a bactericide and bleaching agent in bird nest cleaning industry to make the EBN more whitish, so that it could be graded as high-grade EBN. Some of the H_2O_2 may be tightly hydrogen-bond in coils of the glycoprotein chains, just like the tightly bound water, and may remain trapped in the EBN on drying typically below 60°C, and this could present as a food hazard. It may be necessary to heat dry the cleaned EBN at above 100°C to dislodge and decompose H_2O_2, or to reduce it in the moist EBN with permitted reducing agents like ascorbic acid, or by enzymatic decomposition with catalase, before drying (Shim et al., 2017). This study further supports the importance of heat treatment for EBN. Future studies focusing on the optimum heating condition for pathogenic microorganisms removal and nutrients retention should be carried out. The heating step should be introduced into industrial EBN products processing, especially for those ready-to-drink beverages to prevent potential avian influenza virus infection and food poisoning.

Mites and fungal spores are contaminants widely found in EBN and more mite faecal pellets and eggshells were found in raw EBN compared to commercially processed EBN (Kew et al., 2014). Certain mites are heat-resistant and can survive the cooking process to elicit allergic reactions in consumers (Yan et al., 2008). Bromelain, an enzyme derived from pineapple showed promising results in killing mites and fungi found in raw EBN (Tai et al., 2020). Using bromelain to remove microbial contaminants could be a safer choice than bleaching agents like H_2O_2 and semicarbazide. A study showed that semicarbazide detected in commercial EBN products originated from bleaching of EBN. As semicarbazide is carcinogenic, use of semicarbazide to bleach EBN should be strictly prohibited (Xing et al., 2012). Moreover, the packaging of EBN products should avoid using azodicarbonamide-containing packaging as it could produce semicarbazide due to thermal decomposition (Stadler et al., 2004). Most importantly, adulterants should not be added to EBN products and storage should be kept clean from potential contaminants for safe consumption of EBN. Hypothetically, simultaneous applications of both heat and enzymatic EBN extraction could provide additional advantages after optimisation.

Apart from using heat and enzymatic treatments to remove contaminants, these steps could improve bioavailability of nutrients in EBN. The extracted product compositions are unique for each extraction method due to their physicochemical properties (Tong et al., 2020). Protein functionalities based on its physical and chemical properties including size, shape, amino acid composition, sequence, net charge and distribution, hydrophobicity/hydrophilic ratio, secondary, tertiary, and quaternary structures, molecular flexibility/rigidity, and ability to interact with other components. However, extensive denaturation of proteins often results in insolubilisation, which can consequently impair functional properties that are dependent on solubility. Ideally, partial denaturation of protein often improves digestibility and biological availability of essential amino acids (Damodaran and Parkin, 2017). Enzymatic protein hydrolysis is commonly used to modify the nutritional, physicochemical, functional, digestibility, sensory, and bioactive properties of the protein, as well as to reduce allergenic and anti-nutritional compounds (Tavano, 2013).

A widely studied enzyme, pancreatin is a mixture of digestive enzymes that is secreted from the pancreas with proteolytic,

amylolytic and lipolytic activities. Treatment with pancreatin significantly increases the protein's solubility, degree of hydrolysis and bioactive peptides concentration of the mixture (Khushairay et al., 2014). For example, pancreatin-treated EBN has higher efficiency compared to non-pancreatin treated EBN in reducing cell death in 6-OHDA-challenged SH-SY5Y cells. However, water extract exhibited higher efficacy in ameliorating ROS build up, early apoptotic membrane phosphatidylserine externalization as well as inhibition of caspase-3 cleavage (Yew et al., 2014). In *in vitro assay,* EBN treated with pancreatin had superior anti-viral effect and showed no side effects, rendering the pancreatin treated EBN a safer and more effective antiviral agent as compared to EBN extracted with water (Guo et al., 2006).

The choice of EBN extraction should be made based on desired biomedical application because bioactive compounds could be best extracted by a particular extraction method. Alcalase is an enzyme that has broad specificity and has been employed to modify the functional properties of a range of proteins. It is used mainly to cleave the carboxyl side of the hydrophobic amino acid (Adler-Nissen and Olsen, 1979). It is speculated that combined applications of heat treatment and enzymatic protein hydrolysis may help to maximise bioactive components functionality, digestibility, and bioactivity. When compared to a variety of heating modalities and heating plus alcalase treatment: slow cook with or without alcalase treatment (85–96°C, 60–120 min), autoclave with or without alcalase (121°C, 15 min), and raw (without heating and alcalase treatment), EBN heated at 100°C for 60 and 30 min followed by alcalase treatment gave highest degree of hydrolysis, 22.59 and 20.42% respectively. The degree of hydrolysis is positively correlated with solubility where high solubility of normal boiled, enzymatically hydrolysed EBN possess the potential of EBN being used for food formulation, nutraceutical, and cosmetic products (Amiza et al., 2019a). On top of that, alcalase treatment also helped in removal of impurities (for example ash content), and improves the aesthetics of commercially available EBN (Noor et al., 2018).

Neu5Ac in EBN is known for its antiviral, skin whitening, and bone maintenance functions. The EBN treated with pepsin in simulated gastric fluid at pH 2 for 48 h showed higher potency for the functions as mentioned than those EBN samples without extraction process under such condition. Hence, prolonged treatment of EBN in gastric-like conditions could retain better Neu5Ac bioactivity (Wong et al., 2018c). The application of enzymes in EBN preparation have shown promising results and it is anticipated that these procedures should be incorporated into industrial EBN processing. Treating EBN with enzymes for elimination of contaminants, extraction of bioactive compounds, and allergen deactivation could provide consumers with better and safer EBN products.

The pH value during EBN extraction is one of the important factors to retain maximum bioactivity. Both acid and alkaline extraction methods including hydrochloric acid (Quek et al., 2018a) and sodium hydroxide (Hun et al., 2016) were studied. Extracting bioactive compounds from EBN using acid was shown to be inefficient when compared to acidic enzymatic extraction. Hence, supplementing enzymatic extraction (e.g. pepsin) with addition of acids facilitates protein denaturation and the EBN extract will be neutralized to achieve a pH of 7.0 with alkaline to halt the enzyme activity (Tong et al., 2020). In contrast, Amiza et al. (2019b) demonstrated that the optimum conditions for EBN hydrolysis is at pH of 9.46, Alcalase to substrate concentration of 2%, hydrolysis time of 179.55 min, and 64.99°C, can achieve 37.9% degree of hydrolysis. Alcalase require pH 7.5–9.5 and neutralized with acids after the extraction (Amiza et al., 2019b).

The fatty acids content of EBN is relatively understudied but recently Lee et al. reported that EBN contains 48.43% of poly-unsaturated fatty acids (PUFA), 25.35% of saturated fatty acids (SFA), and 24.74% mono-unsaturated fats (MUFA). These fatty acids explain the antioxidative activity of EBN. Briefly, after HMG was done, the triglyceride fraction from EBN was extracted with acetone water mixture (ratio of 75:25) at room temperature for 2 days. The aqueous solution was then obtained after solvent evaporation under a vacuum that was successively partitioned with ethyl acetate (Hun et al., 2020). One of the important factors for EBN nutrients retention is the pH value during acid extraction (Hun et al., 2020).

Recent studies have reported new extraction methods with high efficiency in extracting the highest amount of nutrients from EBN. The EBN eHMG extraction was first demonstrated by Oda et al. and remained a non-disclosure procedure to extract EBN (Oda et al., 1998). Recently, eHMG was found to be the more effective than acid extraction, enzyme extraction, and HMG in terms of EBN extraction, with the highest number of water-soluble (193) metabolites and extra sialic acids extracted (Tong et al., 2020). A recent study showed that the EBN processed with dynamic high-pressure micro-fluidisation (DHPM) at various pressure (range from 20–200 MPa) displayed a remarkable increase in protein solubility. DHPM treatment significantly improved the solubility accompanied by changes in the structural properties of EBN insoluble proteins, which may be caused by intense shear, turbulence force, and cavitation effect of DHPM treatment. Confirmational changes such as the increase in α-helix and β-turn, reduced particle size and de-aggregation of large proteins in EBN water-insoluble fraction. Hence, optimized DHPM could be an alternative method to traditional stew to release the bioactive compound from EBN (Fan et al., 2020). Mass eHMG and DHPM EBN processing could be developed and tested in the future.

Based on the common practice of current whole EBN processing, whole EBN is dried on a mold after soaking and cleaning, skipping extraction as shown in **Figure 1** (Hong et al., 2020). The drying process after cleaning of EBN was discovered to be critical for sialic acids and antioxidants retention, in turn providing benefit to the quality and grade of EBN. Higher drying temperature causes degradation of sialic acids and antioxidants in EBN, exhibiting first-order kinetics. EBN dried at 25°C had 83.9 and 96.6% retention of sialic acids and antioxidants, 78.7 and 91.5% at 40°C, 42.5 and 38.7% at 70°C respectively. Longer drying time is required for drying at lower temperature to achieve similar level of dryness in the EBN as the processing of EBN would be slowed down but the retention of nutritional value is significantly better. When

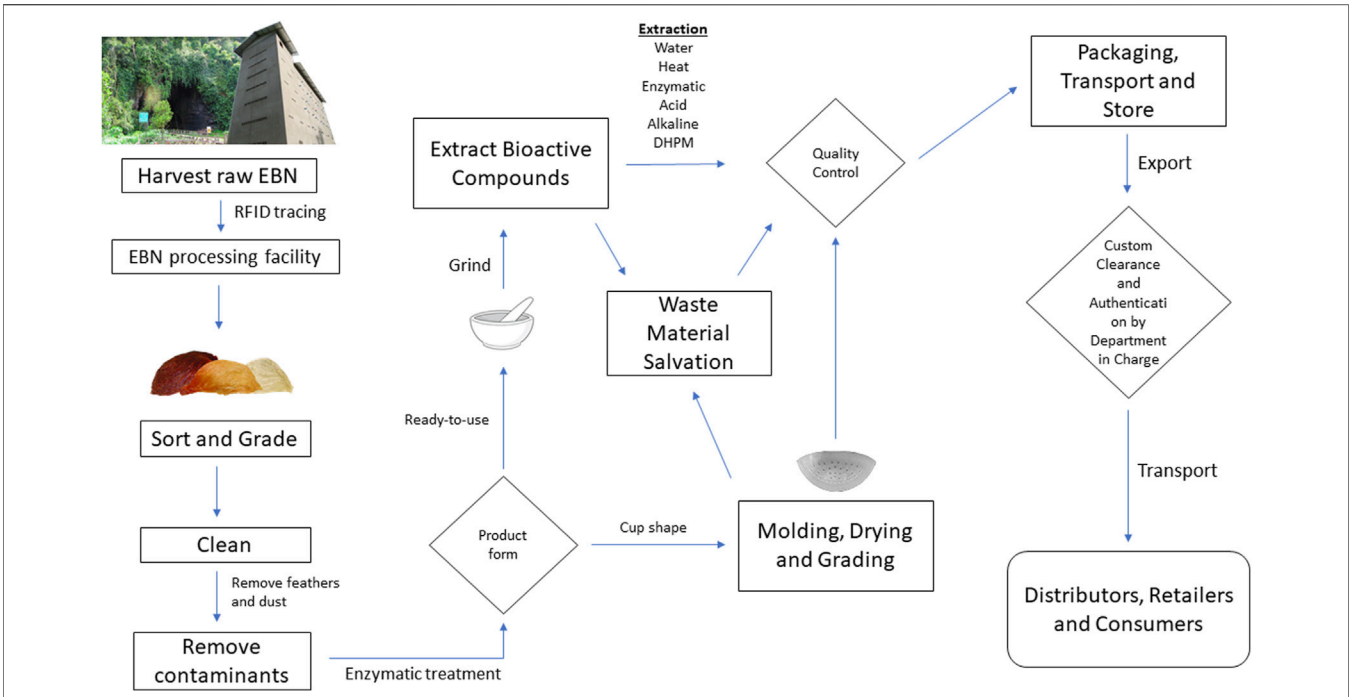

FIGURE 1 | Schematic flowchart of EBN industry.

TABLE 1 | Summary of EBN extraction studies.

Authors	Extraction methods	Nutrients	Bioactive components	Location	Functions
Yew et al. (2014)	Water extraction	-	-	Malaysia: Perak swiftlet premises	Increase antioxidant properties, Inhibit early apoptotic membrane phosphatidylserine externalization, Inhibition of caspase-3 cleavage
	Pancreatin enzymatic extraction	-	-	-	Improves cell viability of 6-OHDA-challenged SH-SY5Y cell
Yew et al. (2018)	Water extraction and Pancreatin enzymatic extraction	-	-	Malaysia: Perak swiftlet premises	Improves motor function and balancing in PD mice, Prevents 6-OHDA-induced loss of dopaminergic neuron in substantia nigra of PD mice, Improves antioxidant in PD mice, Reduces microglia activation in PD mice, Reduces NO production and lipid peroxidation in PD cell
Yew et al. (2019)	Water extraction and Pancreatin enzymatic extraction	-	-	Malaysia: Perak swiftlet premises	Promoting proliferation and migration of the NSC model, embryonic mouse neuroectodermal cells (NE-4C)
	Water extraction	-	Repulsive guidance molecule domain family member B, Protein lin-9, and hyaluronan mediated motility receptor	-	Promote neurite extension, axonal growth, cell proliferation and migration

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TABLE 1 | (*Continued*) Summary of EBN extraction studies.

Authors	Extraction methods	Nutrients	Bioactive components	Location	Functions
Tong et al. (2020)	eHMG	193 metabolites	-	Malaysia: Johor swiftlet premises	-
	pHMG	42 metabolites	-	-	-
	Sulfuric acid extraction	6 metabolites	-	-	-
	Pancreatin enzymatic extraction	1 metabolite	-	-	-
Zainal Abidin et al. (2011)	eHMG	-	Collagen Type I		Major structural collagen of the cornea
			aldehyde dehydrogenase (ALDH)		Oxidation of a wide variety of endogenous and exogenous aldehydes to their corresponding acids
			Lumican		Normal cornea morphogenesis
Khushairay et al. (2014)	Heat and Pancreatin enzymatic extraction	Protein solubility to 163.9 mg/g, Degree Hydrolysis: 86.5% and 109.5 mg/g peptides	-	-	-
	Heat and Alcalase enzymatic extraction	Protein solubility of 86.7 mg/g, Degree Hydrolysis 82.7% and 104.1 mg/g peptides	-	-	-
Ling et al. (2020)	Protease from *Bacillus licheniformis*	Soluble protein: 375.1 ± 4.7 g/kg	Sialic acids: 126.1 ± 4.0 g/kg		Increase antioxidant activity
Guo et al. (2006)	Neuraminidase from Clostridium perfringens	-	*N*-acetylneuraminic acid	Indonesia: cave and swiftlet premises	Neutralize the infection of MDCK cells with influenza viruses, Inhibit hemagglutination of influenza viruses to erythrocytes
Tai et al. (2020)	Bromelain extraction		Bromelain from pineapple		Removal of mites
Amiza et al., 2019a	Heat extraction	Protein Hydrolysis: 5.84–14.54%	-	-	-
	Heat and Alcalase enzymatic extraction	Protein Hydrolysis: 12.16–22.59%	-	-	-
Hun et al. (2020)	Modified water extraction with ethyl acetate, n-butanol, and acetone-water mixture	54 metabolites	Triglycerides	Malaysia: Johor	Improve antioxidant antivities
Fan et al. (2020)	Dynamic high pressure microfluidization (DHPM)	Protein: 69.56 ± 1.74%, Carbohydrate: 17.56 ± 0.45%, Sialic acid: 10.77 ± 0.42%	-	-	-
Wong et al. (2018c)	Pepsin and simulated gastric fluid at pH 2	-	N-acetylneuraminic acid	Indonesia: swiflet premises, Malaysia: swiftlet premises, Thailand: cave, Vietnam: cave	Inhibition of melanogenesis, Stronger enzymatic activity of tyrosinase, Stronger osteogenic activity
Haghani et al. (2016)	Heat extraction, heat and enzymatic extraction	-	Sialic acids and thymol derivatives	Teluk Intan, Perak, Malaysia: swiftlet premises, Gua Madai, Lahad Datu, Malaysia: cave	Heat extraction stronger effects than heat + pancreatin enzymatic extraction: Decrease NS1 and NA gene copies of IAV
Haghani et al. (2017)	Heat extraction, heat and enzymatic extraction	-	a2,3-N-acetylneuraminic acids (sialic acids): Gua Madai = 6.7 mg/g, Teluk Intan = 3.2 mg/g	Teluk Intan, Perak, Malaysia: swiftlet premises, Gua Madai, Lahad Datu, Malaysia: cave	Reduce Rab5 activity, Inhibit autophagy, Increase lysosomal degradation
Fan et al. (2021)	Heat extraction	-	-	-	Decrease expression of IL-1β, TNF-α, IL-17A, and IL-6, Increase expression of TGF-β, Restore Th17/Treg cell balance in intestine of C57BL/6J Mice
Saengkrajang et al. (2011)	Methanol extraction	-	-	-	Inhibits growth of *S. aureus, C. albican, E. coli, A. niger*
	Ethyl acetate extraction	-	-	-	

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TABLE 1 | (*Continued*) Summary of EBN extraction studies.

Authors	Extraction methods	Nutrients	Bioactive components	Location	Functions
					Inhibits growth of *C. albican and A. niger*
Tan et al. (2020)	Heat, pepsin, and pancreatin extraction	protein: 53.03–56.37% and carbohydrate: 27.97–31.68%	EGF	Malaysia: Alor Setar, Kedah. Sibu, Sarawak. Rompin, Pahang. Kuala Selangor. Johor Bahru. Jerantut, Pahang. Port Klang	Insignificant changes of cell viability in MCF-7, A549, Caco-2, HCT116 cells
Aswir and Wan Nazaimoon, (2011)	Heat and hydrochloric acid extraction	-	Sialic acids	North, South, and East coast of Peninsular Malaysia	Increase Caco-2 cell proliferation and reduce TNF-α production
Hwang et al. (2020)	Water extraction	-	-	A: Java city, B: Sumatra/Banka cities	Increase antioxidant properties, Decrease TNF-α, IFN-γ, and matrix metalloproteinase-1 expression, Increase expression of hyaluronan, Increase procollagen type I synthesis
Albishtue et al. (2019)	Heat extraction	-	-	-	Improve embryo implantation and pregnancy rates in Sprague Dawley rats, Increase productions of hormones and hormone receptors, Increase antioxidant properties
Choy et al. (2021)	Heat extraction	17 metabolites	Sialic acids	-	Increase expressions of insulin and insulin receptors, Decrease inflammatory cytokines expression, Increase antioxidant properties in *db/db* diabetic mice
Murugan et al. (2020)	Heat extraction	-	Sialic acids: 1.26 μg/mg	-	Preserve endothelial functions in glucose-treated C57BL/6J mice, Increase antioxidant properties in *db/db* diabetic mice
Lee et al. (2015)	Heat and alkaline extraction Sodium Chloride extraction Hydrochloric extraction	Proteins:35.8% ± 0.12	-	Batu Pahat, Johor, Malaysia	Increase antioxidant properties and no antibacterial function
Yida et al. (2014)	Heat extraction Heat, pepsin, pancreatin, and bile extracts extraction (pH 8.9 -> 2 -> 8.9)	-	-	-	Protect HepG2 cells from hydrogen peroxide-induced toxicity by increasing antioxidant activity
Careena et al. (2018)	Heat, pepsin, and pancreatin extraction at pH 2	-	Sialic acids	Malaysia: East, southern, northern, western coast of Peninsular Malaysia, heavily polluted industrial area, and East Malaysia	Improve brain functions by Increasing antioxidant properties and decreasing proinflammatory cytokines in hippocampus in LPS-induced Wistar rats
Mahaq et al. (2020)	Heat extraction	-	Sialic acids	Malaysia: North and South Peninsular Malaysia, Sabah	Improve spatial recognition memory in next two generations offsprings of CJ57BL/6 mice: Upregulation of GNE, 4ST8SialV, SLC17A5, and BDNF mRNA. Increase densities of synaptic vesicles in the presynaptic terminal
Hu et al. (2016)	Water extraction	-	Sialic acids: N-acetyl neuraminic acid 5.4%	Vietnam: Nha Trang	Increase lifespan, Decrease mortality rate and increase survival rate against

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TABLE 1 | (*Continued*) Summary of EBN extraction studies.

Authors	Extraction methods	Nutrients	Bioactive components	Location	Functions
					heat-stress test, Increase antioxidant properties
Quek et al., 2018	Heat extraction	-	Sialic acids: 13.6 g/100 g	Peninsular Malaysia: Segamat, Johor. Kapar, Selangor. Nibong Tebal, Penang. Klang, Selangor	Increase antioxidant properties
			Sialic acids: 9.1 g/100 g	East Malaysia: Sarikei, Sarawak. Gomantong Cave, Sabah. Baram, Sarawak. Niah Cave, Sarawak. Subis Cave, Sarawak	
Norhayati et al. (2010)	Hydrochloric acid extraction	Proteins: 61.5 ± 0.6 g/100 g, Calcium: 553.1 ± 19.5 mg/100 g, Sodium: 187.9 ± 10.4 mg/100 g, Magnesium: 92.9 ± 2.0 mg/100 g, Potassium: 6.3 ± 0.4 mg/100 g	Sialic acids: 0.7–1.5%	-	-
Halimi et al. (2014)	Heat and hydrochloric acid extraction	Proteins: 58.55%, Carbohydrate: 22.28%, Fat: 0.67%	-	Pahang, Malaysia	-
		Proteins: 55.48%, Carbohydrate: 25.79%, Fat: 0.29%	-	Terengganu, Malaysia	-
Chua et al. (2014)	Chloroform + methanol (1:1) extraction with sonication	43 metabolites	-	Malaysia, Indonesia, and Thailand	-
	Methanol extraction with sonication	35 metabolites			
Saengkrajang et al. (2013)	Hydrochloric acid and chloroform + methanol (1:1) extraction	Proteins: 61.0–66.9%, Essential amino acids: 15.9–31.6 mg/g, Carbohydrates: 25.4–31.4%	-	Thailand: Trat province, Phetchaburi province, Nakhon Si Thammarat, Satun, and Narathiwat provinces	-
Elfita et al. (2020)	Heat and hydrochloric acid extraction	Proteins: 53.09–56.25%, Carbohydrate: 19.57–23.04%, Fat: 0.07–0.57%	-	Indonesia: West Sumatra, South Sumatra, West Java, West Kalimantan, Central Sulawesi, and Southeast Sulawesi	-
Phyu Win et al. (2020)	Heat extraction	Proteins: 53.5–59.3 %	-	Myanmar	-

Table legend: "-" indicates "not studied". Glucosamine (UDP-N-acetyl)-2-epimerase/N-acetylmannosamine kinase (GNE), ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase (ST8SiaIV), solute carrier family 17 member 5 (SLC17A5), and brain derived neurotrophic factor (BDNF).

compared to convective hot air drying, heat pump drying at a temperature lower than 40°C, with forced air in the initial stage of drying and intermittent forced air supply in the later stage could efficiently retain colour and sialic acid in EBN. Traditional EBN drying step could be improved as suggested by Gan et al. to preserve the highest possible amount of nutrients in EBN. Temperature as low as 25°C could be used to best preserve the colour, antioxidants, and sialic acids in EBN although it will take a longer time to dry and therefore affects the turnaround time for the industrial preparation process. In summary, heat pump drying at a temperature lower than 40°C, with forced-air in the initial stage of drying and intermittent forced air supply in the later stage could efficiently retain colour, antioxidants, and sialic acids in EBN (Gan et al., 2017a; 2017b).

Surprisingly, around 50% of glycopeptide can be recovered from the waste material of EBN, particularly N-acetylneuraminic acid, which is 229% higher than EBN hydrolysate after enzymatic extraction. The antioxidant activities found in waste material after enzymatic extraction was higher than the EBN extract, suggesting a significant bioactive portion of EBN was wasted during the extraction process. The cleaning procedure is a time-consuming process that includes immersion, swelling, separation of impurities, sterilization, molding, drying and secondary sterilization. The cost of processed clean EBN was approximately 2200–2500

USD kg^{-1} and unprocessed EBN was 1200–1500 USD kg^{-1} in 2017, and this value is expected to increase in the future. The salvation step should be included in enzymatic extraction procedure to prevent economical and nutritional value loss (Ling et al., 2020).

CONCLUSION

This review compiled recent investigations of EBN and provided insight into various topics related to EBN products. Increasing studies have proven beneficial health effects of EBN consumption but factors, such as geographical location, harvesting place, harvesting season, and processing influences the nutritional contents of EBN. Hence, standardised EBN processing methods are required to preserve the bioactive effects discussed in this review. The health concerns of EBN consumption caused by adulterants, chemical and microbial contamination should be addressed by strict adherence to guidelines. Several studies demonstrated plausible authentication methods could be translated into industrial settings, but cost-effectiveness and equipment availability required further analysis and comparison. Numerous *in vivo* animal studies showed promising health-improving functions of EBN with discoveries of bioactive compounds. Future studies to fill the knowledge gap of EBN in promoting human health is promising.

AUTHOR CONTRIBUTIONS

KCC and MGN wrote the manuscript, and YLT, KYN and RYK critically reviewed the manuscript. SMC formulated the entire concept and reviewed the manuscript.

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