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Research article

Edible *Aerodramus fuciphagus* bird nest for wound healing: In search of the best extraction method to increase sialic acid and its relationship with collagen production

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Abstract

Edible *Aerodramus fuciphagus* bird nest (EBN) has several biological properties, especially wound healing promotion. However, better extraction methods are required to increase the level of sialic acid, which is the major factor determining EBN quality. Furthermore, the relationship between sialic acid and collagen production has not been reported. Therefore, this study evaluated the effect of the source and extraction method on EBN quality to identify the best extraction method to maximize sialic acid in EBN. One-way analysis of variance was used for comparisons of results. In the comparison of EBN types, EBN from houses not only contained higher amounts of sialic acid and protein than those from caves, but the antioxidant activity also seemed to be superior to cave EBN. This study found a new combination of acid and thermal extraction that increased the amount of sialic acid compared to single extraction (mean ± SD = 17.77 ± 1.33 mg/g and 5.41 ± 1.06 mg/g, respectively; $p < 0.05$). The higher amount of sialic acid resulted in a higher amount of collagen production and the mean (± SD) level (1.81±0.21 ×10^4 µg/cell) was significantly ($p < 0.05$) higher than that of the control (1.55 ± 0.18 ×10^4 µg/cell). Furthermore, the extract had a positive effect on L929 fibroblast cell growth and cell migration that are vital factors in wound healing. This straightforward extraction method can be applied for use in large EBN facilities.

Introduction

The wound healing process consists of three steps: inflammatory reaction, proliferation and remodeling, with each step being regulated by multiple cells and cytokines (Ibrahim et al., 2018). Therefore, the development of a wound healing agent mostly focusses on decreasing or increasing those cells and cytokines. The use of a natural healing agent is increasing in many countries especially in Asia, Europe and America because of its biocompatibility and reduced toxicity (Maver et al., 2015). Edible bird nest (EBN) is a natural product produced by swiftlets (*Aerodramus fuciphagus*) who usually generate their nests in caves and custom-built houses (Kew et al., 2014). Most swiftlet habitat is located in Southeast Asia and the South Pacific (Daud et al., 2019). Therefore, these regions represent the largest EBN market. EBN is composed of protein, carbohydrate and dietary elements (Quek et al., 2018b). Consequently, it has several biological activities

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including neuroprotective effects, immunological enhancement, antivirus effects, anti-inflammation, antioxidation and activation of cell proliferation (Zhao et al., 2016; Yida et al., 2015; Ghassem et al., 2017; Haghani et al., 2017; Careena et al., 2018; Lee et al., 2020). Kong et al. (1987) found that EBN extract had an epidermal growth factor-like activity. This activity stimulates collagen production and epithelial tissue proliferation resulting in wound healing promotion (Park et al., 2017). Collagen type I is found at levels greater than 80% in dermis and it is one of the keys in the formation of granulation tissue (Xue and Jackson, 2015). Therefore, an agent that can activate collagen type I promotion has an advantage regarding wound healing acceleration. Because of its health benefits, collagen type I is widely used as a food supplement and active ingredient in cosmetics and traditional medicines (Rodríguez et al., 2018). Nowadays, the price of EBN is continuously increasing due to the high demand and low supply, so that EBN industries are highly competitive. Low EBN quality and counterfeit EBN have started to appear on the market in response to the prospects of higher yield and profit. Accordingly, the quality of EBN is of concern (Dai et al., 2021). Sialic acid is one of the most important indicators of EBN grading (Quek et al., 2018a). EBN that contains a high amount of sialic acid is accepted to have high quality (Chan et al., 2013). Sialic acid is an acid sugar with a nine-carbon backbone and the most prominent sialic acid is N-acetylneuraminic acid (NANA) (Chen and Varki, 2010). Sialic acid is detected at a level of around 9% in EBN (Careena et al., 2018) and is a major contributor to the biological activities of EBN (Anthony and Ravetch, 2010; Bohm et al., 2012; Varki, 2008). The amount of sialic acid depends on the bird species, geography, habitat and extraction methods (Chan et al., 2013). Because of the laws and concessions applying in different countries, competition to be an owner of suitable swiftlet habitat may be limited. Therefore, the development of extraction methods is another valuable option for increasing EBN quality. Finding a straightforward extraction method that can be applied in larger EBN industries is necessary as the appropriate extraction conditions (including the time and solvent) will increase the yield and the amount of sialic acid, resulting in high EBN quality. However, the extraction method affects the structure, physical properties, biological properties and toxicity of the extract, so that those properties should be precisely investigated. Various EBN extraction methods have been developed; however, information is limited for each method in terms of the level of sialic acid. There have been no reports of new combination methods that may be advantageous regarding EBN quality. Furthermore, there has been no evaluation of the relationship between sialic acid and collagen promotion. Accordingly, the objectives of this study were to explore the effects of EBN habitat and extraction methods on the EBN quality with the aim of identifying the best extraction method to maximize sialic acid in EBN. The physical properties, biological properties and cytotoxicity of the extract were also investigated and the relationship was determined between the amount of sialic acid and collagen production.

Materials and Methods

Edible bird nest preparation

The EBN used in this study was created by Thai edible-nest swiftlets (*A. fuciphagus*). EBN collected from four habitats were assessed: deep cave, corner on cave wall, general house and cleaned house, with all sampling sites located in swiftlet habitat in Nakhon Si Thammarat, Thailand (Twin Lotus Co., Ltd; Bangkok, Thailand). The nests (100 g/habitat) were crushed into a powder and then passed through a 500 μm mesh. Powder samples with a particle size of less than 500 μm were extracted using four methods that are described in the following section. The hydrochloric acid (HCl), acetic acid (CH_3COOH), sodium hydroxide (NaOH), pancreatin and other chemicals that were used in this study were of analytical grade (Sigma-Aldrich; St. Louis, MO, USA).

Edible bird nest extraction methods

Thermal extraction

Two different methods of thermal extraction were investigated. A sample (1 g) of each type of EBN powder was immersed in 20 mL of reverse osmosis water. For heat extraction, the sample was boiled for 4 hr at 95°C with shaking at 50 rpm (defined as HeatNest). For autoclave extraction, the sample was autoclaved for 4 hr at 121°C and - 103.42 kPa (defined as AutoclaveNest).

Acid extraction

HCl or CH_3COOH at a concentration of 4 mol/L was used as the solvent in this study. A sample (1 g) of each type of EBN powder was immersed in 20 mL of HCl or CH_3COOH. Then, it was shaken for 4 hr at 25°C and 50 rpm. After that, the pH of the extract was adjusted to pH 7 using NaOH. The EBN samples that were extracted with HCl and CH_3COOH were defined as HCl Nest and CH_3COOH Nest, respectively.

Base extraction

A sample (1 g) of each type of EBN powder was immersed in 20 mL of NaOH at a concentration of 4 mol/L. Then, it was shaken for 4 hr at 25°C and 50 rpm. Finally, the extract was adjusted to pH7 using HCl. This extract was defined as BaseNest.

Enzymatic extraction

Pancreatin solution was prepared at a concentration of 0.5 mg/mL in buffer pH 8. A sample (1 g) of each type of EBN powder was immersed in 20 mL of the pancreatin solution. Then, it was shaken for 4 hr at 25°C and 50 rpm. After that, the enzymatic extraction process was stopped by boiling the extract for 5 min at 95°C. The EBN that was extracted by this method was defined as EnzNest.

All extracts were filtered to remove insoluble substances. The extracts were kept at 4°C until tested.

Experiment part 1: Characteristics and properties of all EBN types extracted using thermal, acid, base, and enzyme

Amount of protein in edible bird nest extracts

The amounts of protein in all EBN types extracted using heat, autoclave, HCl, CH_3COOH, base and enzyme were evaluated in triplicate using a BCA protein assay kit (Thermo Fisher Scientific; Waltham, MA, USA). The absorbance of the protein was measured using an ultraviolet/visible (UV/VIS) spectrometer (PerkinElmer Inc.; Waltham, MA, USA) at 562 nm. Bovine serum albumin was used as a protein standard.

Antioxidant activity of edible bird nest extracts

The modified 2,2-diphennyl-1-picrylhydrazyl (DPPH) radical scavenging assay according to Brand-Williams et al. (1995) was used to measure antioxidant activity in all EBN types extracted using autoclaving, HCl, base and enzyme (triplicate samples). DPPH (0.08 mg/dl) in ethanol was added into a concentration series of the extracts. The mixture was incubated in the dark for 30 min at 37°C. The absorbance of the mixture was measured using a UV/VIS spectrometer (PerkinElmer Inc.; Waltham, MA, USA) at 518 nm. The concentration of the extract that scavenged 50% of DPPH free radicals - was calculated.

Molecular weights of edible bird nest extracts and amino acid analysis

The molecular weights of all EBN types extracted using autoclaving, HCl, base and enzyme were analyzed in triplicate using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The extract was added into sample buffer (1:4). Then, the mixture was incubated at 95°C and was loaded onto the gel. Electrophoresis was performed at 30 mA and 120 V for 90 min. Coomassie Brilliant blue R-250 was used as the stain for band detection. The amino acid analysis of general house EBN extracted using autoclaving and acid (HClNest4h+AutoclaveNestGen) was conducted following an in-house method based on Fischler (1998) and an in-house method based on Mota et al. (2016).

Amount of sialic acid in edible bird nest extracts

The amounts of sialic acid in all EBN types extracted using autoclaving, HCl, base and enzyme were determined in triplicate using a sialic acid (NANA) assay kit (Abcam; Cambridge, MA, USA). The absorbance was measured using a UV/VIS spectrometer (PerkinElmer Inc.; Waltham, MA, USA) at 570 nm.

Experiment part 2 Enhancement of extraction efficiency using autoclaving and HCl extraction method in EBN from general house for sialic acid elevation

The results of the first part of this study presented that the highest amount of sialic acid was detected in EBN obtained from houses compared to other the EBN types. Moreover, HClNest, and AutoclaveNest were the most appropriate extraction method that were used to increase the sialic acid level. Therefore, EBN from general houses extracted using HClNest, and AutoclaveNest were used in the following study.

First, 1 g of the EBN powder from general houses was extracted using autoclave extraction (AutoclaveNestGen). Then, the extract was filtered to separate insoluble substances. After that, HCl at a concentration of 2 mol/L was added to the extract solution (1:1). This concentration was not as harmful as 4 mol/L. The mixture was separated into two parts. The first part was stirred for 0.5 hr (HClNest0.5h + AutoclaveNestGen), while the other part was stirred for 4 hr (HClNest4h+AutoclaveNestGen). Finally, the samples were adjusted to pH7 using NaOH. The amount of sialic acid in the extracts was analyzed using a sialic acid (NANA) assay kit (Abcam, Cambridge, MA, USA) with triplicate samples. Commercial EBN samples from Malaysia (freeze-dried powder and spray-dried powder) that had not been subjected to an extraction process were used as controls.

Cytotoxicity of edible bird nest extracts

The HClNest4h+AutoclaveNestGen and AutoclaveNestGen were investigated. The extracts were dialyzed for 48 hr at 25°C before use in this experiment. The cytotoxicity test was conducted following the ISO 10993-5 guideline (ISO10993-5. Biological Evaluation of Medical Devices—Part 5, 2009) with triplicate samples. For this, L929 mouse fibroblasts (Chinese Academy of Preventive Medical Sciences; Beijing, China) were seeded in 96-well plates (1×10^4 cells/well) and incubated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 1% L-glutamine and 1% antibiotics (penicillin-streptomycin) for 24 hr to allow cell attachment. Then, the medium was replaced with the diluted extract solutions (50 µg/mL, 100 µg/mL, 200 µg/mL or 400 µg/mL) or the control (DMEM and 20 parts per million zinc acetate) and incubated for another 24 hr. The cell viability was determined using the conventional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Cell growth, cell migration and collagen type I production induced by edible bird nest extracts

The same extracts used in the cytotoxicity test were investigated regarding their effect on cell growth and collagen production (triplicate samples). For this, L929 mouse fibroblasts were seeded in 48-well plates (1×10,000 cells/well) and incubated in DMEM containing 10% FBS, 1% L-glutamine, and 1% antibiotics (penicillin-streptomycin) for 24 hr. Then, the medium was replaced with diluted extract solutions (50 µg/mL, 100 µg/mL, 200 µg/mL and 400 µg/mL) or the control (DMEM) and incubated for another 24 or 48 hr. The cell viability was determined using MTT assay. After 72 hr, the amount of soluble collagen type I was evaluated using a Sircol™ collagen assay kit (Biocolor Ltd.; Carrickfergus, County Antrim, UK) at a wavelength of 500 nm, with the calculation based on a bovine collagen type I standard curve. For cell migration, a scratch assay was

tested in triplicate. The L929 mouse fibroblasts were seeded in 24-well plates (8×10^4 cells/well) and incubated in DMEM containing 10% FBS for 48 hr. After formation of a cell monolayer, a linear scratch was generated on the monolayer with a sterile pipette tip. Then, the medium was replaced with diluted HClNest4h+AutoclaveNestGen, AutoclaveNestGen and commercial EBN solutions (400 µg/mL) or the control (DMEM). These were all incubated at 37°C with 5% carbon dioxide. Images were captured at a 200-magnification using a microphotograph (Olympus CK2; -Tokyo, Japan) before and after 48 hr of incubation. The Image J software (1.42q/Java 1.6.0.10; -U.S.National Institutes of Health;Bethesda, -Maryland, USA) was used to evaluate the distance of cell migration.

Statistical analysis

One-way analysis of variance was used for data analysis. Wherever *F*-values were significant ($p < 0.05$) the post-hoc tests were performed using Bonferroni test. The SPSS software (version 22; SPSS Inc.; Chicago IL, USA) was used to calculate all statistics.

Results and Discussion

Experiment part 1 Characteristics and properties of all EBN types extracted using thermal, acid, base, and enzyme

Amount of protein in edible bird nest extracts

The EBN samples were extracted using four methods: thermal extraction (heat and autoclaving), acid extraction (HCl and CH_3COOH), base extraction, and enzymatic extraction. For thermal extraction, all types of EBN were heated or autoclaved. The results showed that the amount of protein in the AutoclaveNest was significantly higher than in the HeatNest (Nests from deep cave 109.65 ± 10.4 mg/g and 11.00 ± 2.97 mg/g, respectively, nests from corner on cave wall 118.52 ± 12.79 mg/g and 15.30 ± 1.06 mg/g, respectively, nests from general house 238.58 ± 18.18 mg/g and 59.06 ± 2.49 mg/g, respectively, and nests from cleaned house 285.59 ± 12.63 mg/g and 46.39 ± 4.41 mg/g, respectively) as shown in Table 1. For acid extraction, HCl and CH_3COOH were used as the solvents. A higher amount of protein was detected in samples of HClNest compared to CH_3COOHNest (nests from deep cave 456.94 ± 17. mg/g and 13.89 ± 5.74 mg/g, respectively, nests from corner on cave wall 540.00 ± 25.54 mg/g and 12.22 ± 7.10 mg/g, respectively, nests from general house 495.11 ± 14.99 mg/g and 13.90 ± 3.78 mg/g, respectively, and nests from cleaned house 518.52 ± 17.70 mg/g and 10.22 ± 6.04 mg/g, respectively). Therefore, autoclaving and HCl extraction were the best representatives of thermal and acid extraction, respectively. Comparing the extraction methods (autoclaving, HCl, base and enzymatic extraction) of all EBN types, the amounts of protein in the extracts from the highest to the lowest were HClNest, BaseNest, AutoclaveNest and EnzNest, respectively. However, there were no significant differences in the amounts of protein in cleaned house EBN extracted using the autoclaving and enzymatic extraction methods. Comparing the EBN types, the amount of protein in house EBN (general house and cleaned house) tended to be higher than for cave EBN (deep cave and corner on cave wall). These results were close to the amounts of EBN protein (48–84%) that were extracted using heat together with gastric fluid digestion (pH 2) by (Wong et al., 2017). In that study, the amount of protein depended on the type and source of EBN (Thailand, Vietnam, Indonesia and Malaysia). (Huda et al., 2008) reported that EBN (Indonesia and Malaysia) protein levels were in the range 24–49% depending on the geography.

Antioxidant activity of edible bird nest extracts

Antioxidant activity is a crucial factor in reducing oxidative stress which is a cause of chronic wounds (Comino-Sanz et al., 2020). Inflammatory reaction in the wound healing process might generate excessive reactive oxygen species leading to prolong inflammation (Eming et al., 2007). Maintaining the balance of those reactive oxygen species in cells avoided abnormal cell growth and disordered immune response (Xu et al., 2020). Consequently, antioxidative agent was effective in wound healing treatment. The antioxidant activity in the current study was presented in terms of the concentration of extract that scavenged 50% of DPPH free radicals (IC_{50}) (Table 2). Comparing among the extraction methods, the IC_{50} values of the AutoclaveNest and HClNest, especially for house EBN extracts, were significantly lower than the IC_{50} values of BaseNest and EnzNest (nests from general house 0.60 ± 0.19 mg/mL, 0.67 ± 0.13 mg/mL, and 1.35 ± 0.27 mg/mL and 1.28 ± 0.32 mg/mL, respectively, and nests from cleaned house 0.84 ± 0.11 mg/mL, 0.54 ± 0.10 mg/mL, and 1.57 ± 0.19 mg/mL and 1.32 ± 0.39 mg/mL, respectively); however, there were no significant differences between AutoclaveNest and HClNest. These results indicated that the antioxidant activity of AutoclaveNest and HClNest was higher than for the others. Comparing the EBN types that were extracted using the same method, the IC_{50} values of EBN

Table 1 Amounts of proteins of edible bird nest extracted using heat, autoclaving, HCl, CH3COOH, base and enzymatic extraction

Method		Amount of proteins (mg/g)			
		Nests from deep cave	Nests from corner on cave wall	Nests from general house	Nests from cleaned house
Thermal extraction	Heat	$11.00\pm2.97^{a,A}$	$15.30\pm1.06^{a,A}$	$59.06\pm2.49^{b,A}$	$46.39\pm4.41^{c,A}$
	Autoclaving	$109.65\pm10.47^{a,B}$	$118.52\pm12.79^{a,B}$	$238.58\pm18.18^{b,B}$	$285.59\pm12.63^{c,B}$
Acid extraction	HCl	$456.94\pm17.35^{a,C}$	$540.00\pm25.54^{b,c,C}$	$495.11\pm14.99^{a,c,C}$	$518.52\pm17.70^{b,c,C}$
	CH_3COOH	$13.89\pm5.74^{a,A}$	$12.22\pm7.10^{a,A}$	$13.90\pm3.78^{a,D}$	$10.22\pm6.04^{a,A}$
Base extraction	Base	$386.35\pm21.17^{a,D}$	$415.50\pm29.58^{a,D}$	$421.04\pm10.30^{a,c,E}$	$455.11\pm12.44^{b,c,D}$
Enzymatic extraction	Enzyme	$50.31\pm4.59^{a,E}$	$42.33\pm5.33^{a,E}$	$106.03\pm22.00^{b,F}$	$263.66\pm27.12^{c,B}$

mean values (± SD) superscripted with different lowercase letters are significantly ($p < 0.05$) different between edible bird nest types within the same extraction method; mean values (± SD) superscripted with different uppercase letters are significantly different ($p < 0.05$) between extraction methods within the same edible bird nest type.

Table 2 Scavenged 50% of DPPH free radical concentration (IC_{50}) of edible bird nest extracted using autoclaving, HCl, base and enzymatic extraction

Method		Scavenged 50% of DPPH free radical concentration (IC_{50}) (mg/mL)			
		Nests from deep cave	Nests from corner on cave wall	Nests from general house	Nests from cleaned house
Thermal extraction	Autoclaving	$0.70\pm0.11^{a,A}$	$0.80\pm0.10^{a,A}$	$0.60\pm0.19^{a,A}$	$0.84\pm0.11^{a,A}$
Acid extraction	HCl	$0.54\pm0.06^{a,A}$	$0.94\pm0.15^{b,A}$	$0.67\pm0.13^{a,A}$	$0.54\pm0.10^{a,A}$
Base extraction	Base	$1.23\pm0.31^{a,B}$	$1.04\pm0.08^{a,A}$	$1.35\pm0.27^{a,B}$	$1.57\pm0.19^{a,B}$
Enzymatic extraction	Enzyme	$0.69\pm0.17^{a,A}$	$0.48\pm0.02^{b,B}$	$1.28\pm0.32^{a,B}$	$1.32\pm0.39^{a,B}$

mean values (± SD) superscripted with different lowercase letters are significantly ($p < 0.05$) different between edible bird nest types within the same extraction method; mean values (± SD) superscripted with different uppercase letters are significantly ($p < 0.05$) different between extraction methods within the same edible bird nest type.

from general house and cleaned house were not significantly different compared to deep cave. However, the EBN from corner on cave wall, particularly using HCl extraction, had showed the significantly highest IC_{50} values compared to the others (0.94 ± 0.15 mg/mL, 0.54 ± 0.06 mg/mL, 0.67 ± 0.13 mg/mL and 0.54 ± 0.10 mg/mL, respectively). Therefore, the antioxidant activity of EBN from the general house and cleaned house samples seemed to be better than for the EBN from the cave samples. The autoclaving and HCl extraction methods were appropriate for house EBN (both general house and cleaned house) extraction, resulting in higher antioxidant activity than for the other methods. These results agreed with (Quek et al., 2018a) who reported the antioxidant activity of house EBN was superior to cave EBN. The sulfhydryl, hydroxyl and carboxyl groups of the amino acids in the EBN might be associated with its antioxidant property (Quek et al., 2018a; Gan et al., 2017).

Molecular weights of edible bird nest extracts and amino acid analysis

The molecular weights of the EBN extracts are shown in Fig. 1. The molecular weights of all EBN types extracted using autoclaving (AutoclaveNest) were higher than 100 kDa. The SDS-PAGE analysis of HClNest and BaseNest showed broad bands with molecular weights ranging from 10 to >250 kDa. However, most of the HClNest molecular weights tended to be higher than 55 kDa, while most of the BaseNest molecular weights were less than 15 kDa. For enzymatic extraction, SDS-PAGE showed clear bands with molecular weights in the range 10–250 kDa. The molecular weight of EnzNest from deep cave samples tended to be lower than for the other EBN types. The current results agreed with (Wong et al., 2017) who reported that the molecular weights of thermal-extracted EBN proteins were over 200 kDa but the molecular weights of the proteins that were dissolved in enzyme were decreased. (Daud et al., 2019) also reported that the molecular weights of water-extracted house EBN varied up to 140 kDa. Most EBN proteins were enzymatically hydrolyzed to small molecular weights (Xian et al., 2010). The important part of the EBN extract was the sialic acid that indicated the EBN quality. (Zhang et al., 2012) indicated that molecular weights of 106 kDa and 128 kDa were associated with the sialic acid content of the EBN protein. The 106 kDa and 128 kDa EBN protein contained 17% and 20% sialic content, respectively, whereas the other parts of the EBN protein had only 1.2% of sialic acid. Both molecular weights may also have had similar protein structures modified by different saccharides. The 106 kDa consisted of 66% protein and 19% total saccharides while the 128 kDa consisted of 60% protein and 24% total saccharides. The current study identified 106 kDA and 128 kDa EBN were found in the molecular weight ranges of AutoclaveNest, HClNest, BaseNest and EnzNest. However, most of the AutoclaveNest and HClNest molecular weights were higher than for BaseNest, and EnzNest. These results implied that it might be easier to detect the sialic acid in the AutoclaveNest and HClNest samples. The highest to lowest amounts of protein in the extracts were for HClNest, BaseNest, AutoclaveNest and EnzNest, respectively. Accordingly, HClNest and AutoclaveNest that contained higher amount of protein and sialic acid had a high potential for EBN extraction. Furthermore, the current results showed that the amino acids in house EBN extract (HClNest4h+AutoclaveNestGen) were aspartic acid (11.25%), glutamic acid (8.20%), arginine (7.28%), lysine (3.73%), histidine

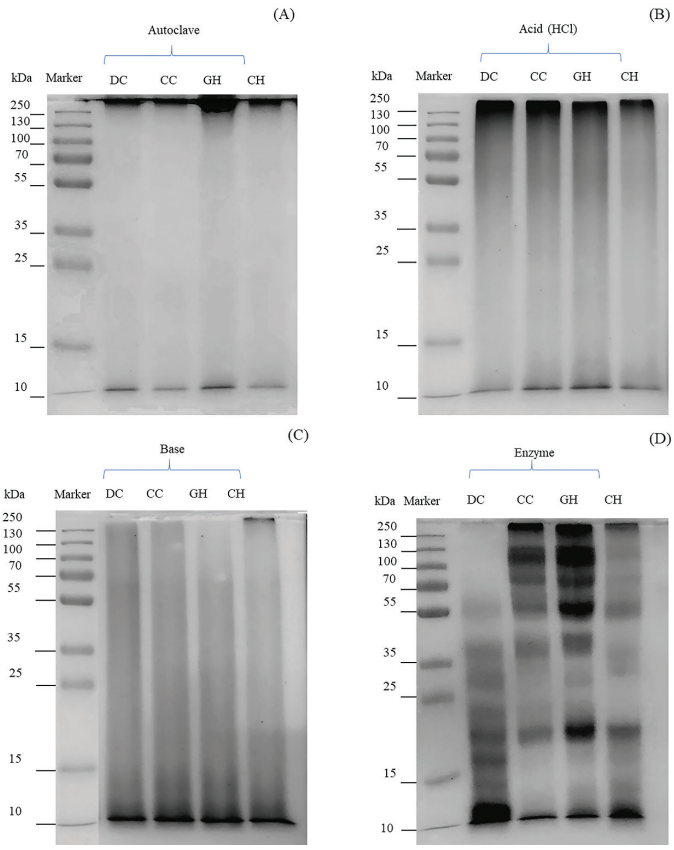

Fig. 1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis and molecular weights of edible bird nests (EBNs) from deep cave (DC), corner on cave wall (CC), general house (GH) and cleaned house (CH): (A) extracted using autoclaving; (B) extracted using HCl; (C) extracted using base; (D) extracted using enzyme

(3.99%), serine (8.38%), threonine (7%), glycine (4.61%), alanine (3.92%), proline (9.20%), valine (7.39%) leucine (7.63%), isoleucine (3.29%), tyrosine (7.40%) and phenylalanine (6.73%). The proteins, especially glutamine and glutamate, were stimulators of collagen synthesis (Karna et al., 2001). Furthermore, amino acid mixtures such as branched-chain amino acids, arginine and glutamine mixtures, and branched-chain amino acids and proline mixtures significantly increased the synthesis rate of skin collagen (Murakami et al., 2012). Consequently, high percentages of glutamic acid, arginine, proline and branched-chain amino acids (valine, leucine and isoleucine) of the EBN extract benefited collagen synthesis.

Amount of sialic acid in edible bird nest extracts

Table 3 shows the amounts of sialic acid of the EBN samples that were extracted using autoclaving, HCl, base and enzymatic extraction. The results showed that the highest amounts of sialic acid were in the HClNest of all the nest types (nests from deep cave 32.92 ± 2.01 mg/g and 1.60 ± 0.17 mg/g, 4.84 ± 0.21 mg/g, 0.21 ± 0.24 mg/g, respectively, nests from corner on cave wall 43.87 ± 1.76 mg/g and 3.20 ± 0.21 mg/g, 8.80 ± 2.24 mg/g, 0.77 ± 0.03 mg/g, respectively, nests from general house 46.71 ± 1.18 mg/g and 5.52 ± 0.33 mg/g, 8.43 ± 0.21 mg/g and 0 mg/g, respectively, and nests from cleaned house 44.85 ± 1.73 mg/g and 11.75 ± 0.19 mg/g, 7.69 ± 0.34 mg/g and 0.87 ± 0.15 mg/g, respectively). For EBN from the corner on cave wall and general houses, sialic acid was detected in descending order in the HClNest, BaseNest, AutoclaveNest and EnzNest samples (nests from corner on cave wall 43.87 ± 1.76 mg/g, 8.80 ± 2.24 mg/g, 3.20 ± 0.21 mg/g and 0.77±0.03 mg/g, respectively, and nests from general house 46.71 ± 1.18 mg/g, 8.43 ± 0.21 mg/g, 5.52 ± 0.33 and 0 mg/g, respectively). However, regarding the amount of sialic acid in EBN from deep caves, there were no significant differences among BaseNest, AutoclaveNest and EnzNest. The two forms of sialic acid in the EBN were the free form of NANA and the conjugated form of NANA (Chan et al., 2018). The conjugated form of NANA was the major form in the EBNs as the NANA is covalently bound to glycan molecules and linked to protein (Chan et al., 2018). For the sialic acid detection, only free NANA was detected in the current study. The results implied that all four extractions methods, especially using HCl, could increase the release of free sialic acid. (Lacomba et al., 2010) and also suggested that HCl or TFA seemed to be the best option for sialic acid hydrolysis. Comparing among nest types, the HClNest for EBN samples from general house, cleaned house and the corner on cave wall had significantly higher amounts of sialic acid than EBN from deep cave samples (46.71 ± 1.18 mg/g, 44.85 ± 1.73 mg/g, 43.87 ± 1.76 mg/g and 32.92±2.01 mg/g, respectively). In addition, the highest amount of sialic acid was detected in general houses EBN samples that had been extracted using HCl. This result agreed with (Careena et al., 2018) who showed that the EBN geography had an effect on the amount of sialic acid. House EBN was also reported to contain a higher amount of sialic acid than cave EBN (Quek et al., 2018a). The degradation of the sialic acid of cave EBN might have been affected by the growth of microorganisms and enzymatic reaction in the cave (Angata and Varki, 2002).

Experiment part 2 Enhancement of extraction efficiency using autoclaving and HCl extraction method in EBN from general house for sialic acid elevation Based on the results of the first part of this study, the sialic acid content in descending order was HClNest, BaseNest, AutoclaveNest and EnzNest, respectively. Therefore, in an attempt to increase the sialic acid level, a combination of methods was tested (HCl and base extraction) regarding the highest amount of sialic acid produced. However, the HCl and base extraction could not be continuously prepared because of the acid-base interaction, so HCl together with autoclave extraction was used to increase the sialic acid level and comparing this extract to autoclave extraction without acid treatment. The autoclave extraction was used for the comparison because it was a non-toxic method. This method not only produced a final product without solvent contamination but would also be harmless in large EBN production facilities. It was also determined in the first part of this study that the highest amount of sialic acid was detected in EBN obtained from houses compared to other the EBN types. Therefore, EBN from general houses was used in the following study.

The results showed that the amounts of sialic acid in HClNest0.5h+AutoclaveNestGen and HClNest4h+AutoclaveNestGen were significantly higher than for the AutoclaveNestGen and commercial EBN (freeze-dried powder and spray-dried powder) with values of 8.84 ± 1.26 mg/g, 17.77 ± 1.33 mg/g and 5.41 ± 1.06 and 0.13 ± 0.05 mg/g, respectively) as shown in Fig. 2. Moreover, the duration of acid extraction had an effect on sialic acid levels. The increase in sialic acid in the HClNest4h+AutoclaveNestGen was significantly greater than that in the HClNest0.5h+AutoclaveNestGen (17.77 ± 1.33 mg/g and 8.84 ± 1.26 mg/g, respectively). In addition, the level was 3.28-fold greater than in the AutoclaveNestGen. Accordingly, the combination of HCl (4 hr) and autoclave extraction was determined as the best method to increase the sialic acid content of the EBN extract. In this study, adding acid to the extraction process might have increased the release of free sialic acid. Therefore, the highest amount of sialic acid was achieved with this method.

Table 3 Amounts of sialic acid of edible bird nest extracted using autoclaving, HCl, base and enzymatic extraction

Method		Sialic acid (mg/g)			
		Nests from deep cave	Nests from corner on cave wall	Nests from general house	Nests from cleaned house
Thermal extraction	Autoclave	$1.60\pm0.17^{a,A,C,D}$	$3.20\pm0.21^{b,A}$	$5.52\pm0.33^{c,A}$	$11.75\pm0.19^{d,A}$
Acid extraction	HCl	$32.92\pm2.01^{a,B}$	$43.87\pm1.76^{b,B}$	$46.71\pm1.18^{b,B}$	$44.85\pm1.73^{b,B}$
Base extraction	Base	$4.84\pm0.21^{a,C}$	$8.80\pm2.24^{b,C}$	$8.43\pm0.21^{b,c,C}$	$7.69\pm0.34^{c,C}$
Enzymatic extraction	Enzyme	$0.21\pm0.24^{a,D}$	$0.77\pm0.03^{b,D}$	$0^{a,D}$	$0.87\pm0.15^{b,D}$

mean values (± SD) superscripted with different lowercase letters are significantly ($p < 0.05$) different between edible bird nest types within the same extraction method; mean values (± SD) superscripted with different uppercase letters are significantly ($p < 0.05$) different between extraction methods within the same edible bird nest type.

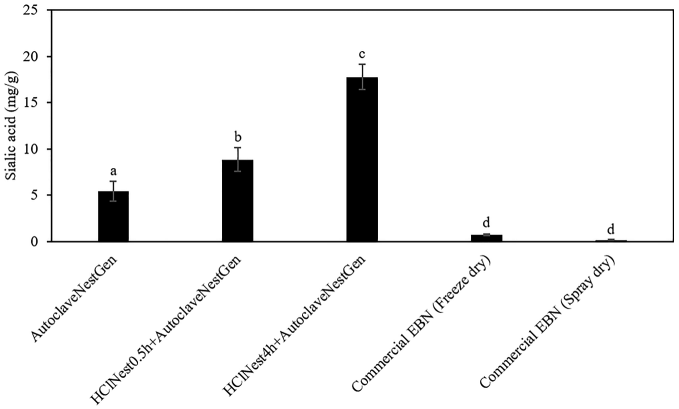

Fig. 2 Amounts of sialic acid in edible bird nests (EBNs) from general house extracted using the following methods: autoclaving and 0.5 hr hydrochloric acid extraction (HClNest0.5h+AutoclaveNestGen), autoclaving and 4 hr hydrochloric acid extraction (HClNest4h+AutoclaveNestGen) and autoclave extraction (AutoclaveNestGen) compared to commercial EBNs, where different superscript letters above bars indicate significant ($p < 0.05$) different between treatments; , error bars indicate ± SD.

The duration of acid incubation also affected the amount of free sialic acid. (van der Ham et al., 2007) found that the conversion of compound sialic acid to free sialic acid using acid hydrolysis was incomplete after 30 min. However, increasing the incubation time for acid hydrolysis from 30 min to 60 min was sufficient to obtain free sialic acid.

Cytotoxicity of edible bird nest extracts

The percentage of cell viability is shown in Fig. 3. The percentages of cell viability of the HClNest4h+AutoclaveNestGen at all concentration (50, 100, 200, and 400 µg/mL) were higher than the AutoclaveNestGen.

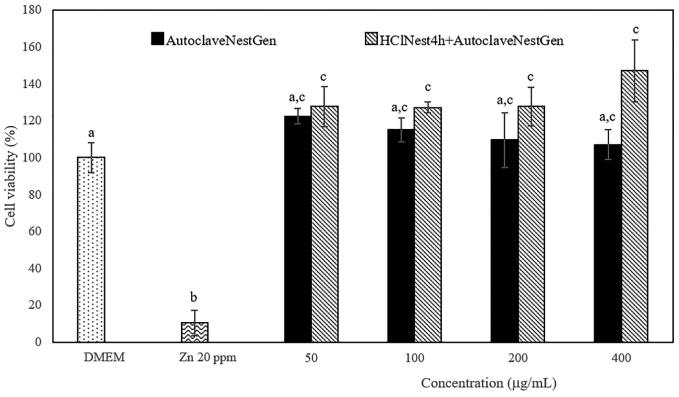

Fig. 3 Percentages of L929 cell viability after culture in various concentration (50 µg/mL, 100 µg/mL, 200 µg/mL and 400 µg/mL) of edible bird nest samples from general house extracted using autoclaving (AutoclaveNestGen) and autoclaving and 4 hr hydrochloric acid extraction (HClNest4h+AutoclaveNestGen) compared to Dulbecco's modified Eagle's medium (DMEM) and zinc acetate (Zn), where different superscript letters above bars indicate significant ($p < 0.05$) different between treatments , ppm = parts per million and error bars indicate ± SD.

They were also significantly higher DMEM (at concentration of 50 µg/mL 127.59±10.90 and 100±8.21, at concentration of 100 µg/mL 127.16±2.94 and 100±8.21, at concentration of 200 µg/mL 127.59±10.63 and 100±8.21, and at concentration of 400 µg/mL 146.99±16.74 and 100±8.21%, respectively; $p < 0.05$). However, all extracts had cell viability more than 80% while Zn (toxic agent) had cell viability less than 80%. Therefore, the HClNest4h+AutoclaveNestGen and the AutoclaveNestGen were not toxic to cells.

Cell growth, cell migration, and collagen type I production induced by edible bird nest extracts

Figs. 4A and 4B show the number of fibroblast cells after culture with various concentrations of the extracts. After 24 hr and 48 hr, the number of cells in the HClNest4h+AutoclaveNestGen treatments (50 µg/mL, 100 µg/mL, 200 µg/mL and 400 µg/mL) were significantly higher than in DMEM (after 24 hr: at a concentration of 50 µg/mL = 2.78 ± 0.24 $\times 10^4$ cells and 2.18 ± 0.18 $\times 10^4$ cells, respectively,

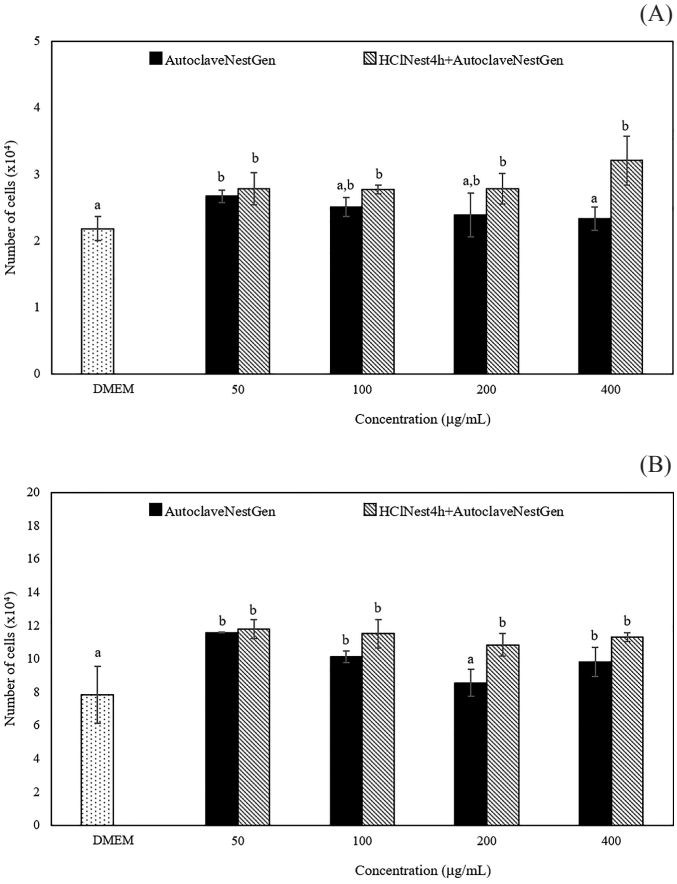

Fig. 4 Number of L929 cells after culture in various concentrations (50 µg/mL, 100 µg/mL, 200 µg/mL and 400 µg/mL) of edible bird's nests from general house extracted using autoclave extraction (AutoclaveNestGen) and autoclave and 4 hr hydrochloric acid extraction (HClNest4h+AutoclaveNestGen) at (A) 24 hr and (B) 48 hr, where different superscript letters above bars indicate significant ($p < 0.05$) different between treatments, error bars indicate ± SD.

at a concentration of 100 µg/mL = 2.77 ± 0.06 ×10^4 cells and 2.18 ± 0.18 ×10^4 cells, respectively, at a concentration of 200 µg/mL = 2.78 ± 0.23 ×10^4 cells and 2.18 ± 0.18 ×10^4 cells, respectively, and at a concentration of 400 µg/mL = 3.20 ± 0.36 ×10^4 cells and 2.18 ± 0.18 ×10^4 cells, respectively; and after 48 hr: at a concentration of 50 µg/mL = 11.80 ± 0.57 ×10^4 cells and 7.84 ± 1.71 ×10^4 cells, respectively, at a concentration of 100 µg/mL = 11.50 ± 0.86 ×10^4cells and 7.84 ± 1.71×10^4 cells, respectively, at a concentration of 200 µg/mL = 10.83 ± 0.67 ×10^4 cells and 7.84 ± 1.71×10^4 cells, respectively, and at a concentration of 400 µg/mL = 11.29 ± 0.26 ×10^4 cells and 7.84 ± 1.71×10^4 cells, respectively). In addition, after 24 hr at a concentration of 400 µg/mL and after 48 hr at a concentration of 200 µg/mL, the number of cells in the HClNest4h+AutoclaveNestGen were also significantly higher than the cells in the AutoclaveNestGen (after 24 hr: at a concentration of 400 µg/mL = 3.20 ± 0.36 ×10^4 cells and 2.33 ± 0.18×10^4 cells, respectively; and after 48 hr: at a concentration of 200 µg/mL = 10.83 ± 0.67 ×10^4 cells and 8.54 ± 0.81×10^4 cells, respectively). Consequently, the HClNest4h+AutoclaveNestGen best elevated sialic acid that promoted fibroblast cell growth.

A scratch assay is an *in vitro* test for a wound healing assay and is performed by creating a gap area for testing cell migration, where the agent that induces a higher percentage of cell migration is referred to as having superior wound healing promotion (Liang et al., 2007). The percentage of cell migration in the HClNest4h+AutoclaveNestGen was significantly higher than the percentage of cell migration in the AutoclaveNestGen, commercial EBN and DMEM (99.07 ± 0.15%, 76.95 ± 0.91%, 70.01 ± 4.81% and 66.50 ± 2.30%, respectively), as shown in Fig. 5. Furthermore, AutoclaveNestGen also had a significantly higher percentage of cell migration than DMEM (76.95 ± 0.91% and 66.50 ± 2.30%, respectively). These results indicated that both EBN extracts (HClNest4h+AutoclaveNestGen and AutoclaveNestGen) accelerated wound healing. The HClNest4h+AutoclaveNestGen containing high sialic acid increased wound healing promotion better than the others.

The collagen concentration is shown in Fig. 6. In the HClNest4h+AutoclaveNestGen, the amounts of collagen in wells treated with 200 µg/mL and 400 µg/mL of the extract treatments were significantly higher than for the 50 µg/mL extract (at a concentration of 200 µg/mL = 1.81 ± 0.21 ×10^4 cells and 1.25 ± 0.09 ×10^4 cells, respectively, and at a concentration of 400 µg/mL = 1.70 ± 0.10 ×10^4 cells and 1.25±0.09 µg/ ×10^4 cells, respectively). In addition, at a concentration of 200 µg/mL, the amount of collagen in the HClNest4h+AutoclaveNestGen was also significantly higher than for the collagen in the AutoclaveNestGen (1.81 ± 0.21 ×10^4 µg/cell and 1.55 ± 0.18 ×10^4 µg/cell, respectively). These results indicated that the extracts (HClNest4h+AutoclaveNestGen) with a high sialic acid content significantly promoted higher collagen type I than the extracts (AutoclaveNestGen) with a low sialic acid content. Because the conjugated form of sialic acid in which NANA is linked to a protein might have been hydrolyzed by the extraction methods applied in the current study, an increase was observed in both the free sialic acid and protein. From the molecular weights results of the EBN extracts and the amino acid analysis, the molecular weights of EBNs that contained sialic acid were easier to detect in HClNest and AutoclaveNest. In addition, HClNest4h+AutoclaveNestGen consisted of amino acid that benefited collagen synthesis, implying that an increase in the free sialic acid was associated with an increase in the protein

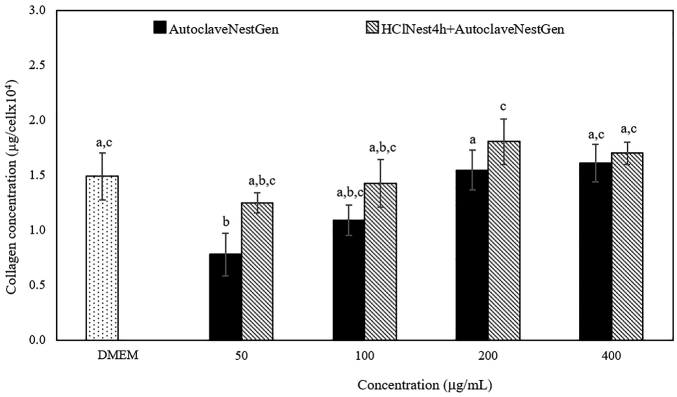

Fig. 6 Collagen concentration of L929 cells after culture in various concentrations (50 µg/mL, 100 µg/mL, 200 µg/mL and 400 µg/mL) of the extracts for 72 hr, different superscript letters above bars indicate significant ($p < 0.05$) different between treatments, and error bars indicate ± SD

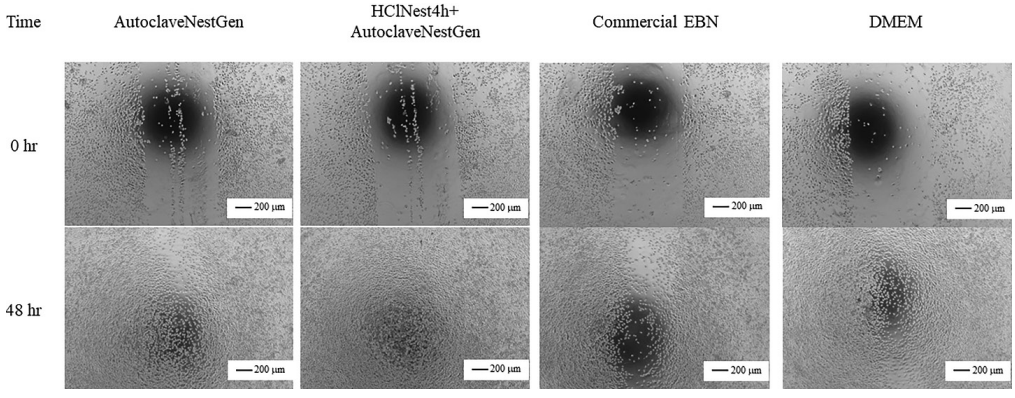

Fig. 5 Migration of L929 cells (scratch assay) before and after culture in edible bird nests (EBNs) samples from general house using autoclave extraction (AutoclaveNestGen), autoclave and 4 hr hydrochloric acid extraction (HClNest4h+AutoclaveNestGen) and commercial EBN for 48 hr, where scale bar = 200 µm-

in the extracted EBN, resulting in cell growth promotion, collagen production and wound healing acceleration (high percentage of cell migration). In general, extraction including thermal extraction might degrade protein; however, it might not affect all protein functions, including cell growth promotion, cell migration and collagen production. (Aramwit et al., 2010) studied the effect of sericin protein on collagen production using m various extraction methods. They reported that autoclave-degraded sericin was the least toxic and could activate the highest collagen production compared to the other extraction methods. In addition, autoclave-degraded sericin promoted cell migration similar to the epidermal growth factor (Aramwit et al., 2013). The current results also showed that both autoclaved EBN extracts (AutoclaveNestGen and HClNest4h+AutoclaveNestGen) increased cell growth, cell migration and collagen more than DMEM (Fig. 4–6). Therefore, some protein in the extracts (AutoclaveNestGen and HClNest4h+AutoclaveNestGen) in the current study might have been degraded but the biological effects were still evident. Sialic acid detection is commonly use in EBN quality testing and could also be used to verify the wound healing activity of EBN. Based on previous research, EBN has an epidermal growth factor-like activity and stimulates thymidine incorporation in quiescent cultures of 3T3 fibroblasts (Kong et al., 1987). (Abidin et al., 2011) showed that 0.5% EBN extract (using heat extraction) could activate cell proliferation in corneal keratocytes. Collagen type I expression in that study also indicated the stability of the keratocytes treated with the EBN extract. The wound healing promotion of EBN was also reported by (Sandi and Musfirah, 2019). In that study, EBN was prepared in the form of EBN ointment (10%, 20% or 30%). The wound lengths of diabetic rats that were treated with a concentration of 30% EBN ointment and povidone-iodine were significantly less than baseline and povidone-iodine treated wound. Similar effects of EBN on wound healing activity were also presented in the results of the study by (Ofiwijayanti et al., 2017).

In conclusion, among the EBN types, the amounts of protein and sialic acid in house EBN were higher than in cave EBN. The antioxidant activity of house EBN also tended to be higher than for cave EBN. The combination of HCl (4 hr) and autoclave extraction was the best method for increasing the content of sialic acid in the EBN extracts. The HClNest4h+AutoclaveNestGen promoted cell proliferation and cell migration. Additionally, the collagen production which is an important factor in wound healing increased to a high concentration in the extracted EBN. The EBN extracts with a high sialic acid content promoted more collagen type I than the EBN extracts with a low sialic acid content. The concentration of 200 µg/mL of the extract was sufficient to increase collagen production. This simple but effective extraction method can be applied in large EBN production facilities; however, the conditions need to be well-controlled.

Conflict of Interest

The authors declare that they have no conflict of interest.

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