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Investigations into the physicochemical, biochemical and antibacterial properties of Edible Bird's Nest

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ABSTRACT

Edible bird's nest (EBN)islong known as a delicacy and medicine among the Chinese communities all over the world. This work reports thephysicochemical, biochemical and antibacterial properties of EBN obtained from the swiftlet premises in Batu Pahat, Johor, Malaysia. Carbohydrates (46.47%) and proteins (35.8%) were the major constituents, with little fats (1.5%) and no fibre. Amino acid analysis by AccQ.Tag method revealed all eighteen types of amino acids including the eight essential ones. The heavy metal contents were within infant formula specification limits. Sodium, magnesium, potassium and calcium were the major minerals found. Alkaline conditions and heat treatment were the parameters that significantly affected the protein extraction from EBN. Out of the four EBN extracts (Run 1, Run 10, Oda and Goh) prepared; Run 10 had the highest protein content and anti-oxidant power, however, no direct correlations between protein content and the anti-oxidant power could be established. Oda and Goh extracts produced visible bands in the electrophoretic analysis in comparison to the Run 10 and Run 1 extracts. None of the extracts showed any antibacterial activity towards Escherichia coli, Klebsiella pneumoniae, Bacillus subtilis and Staphylococcus aureus.

Keywords: Edible Bird's Nest, Microscopic examination, Proximate analysis, Amino acid analysis and Antibacterial properties.

INTRODUCTION

EBN is only found in the Southeast Asian region of the world. It is considered as a delicacy and traditional medicine in the Chinese communities the entire world over. It is the dried glutinous secretion from the salivary glands of the Southern Asia swiftlets[1]. Scientifically, EBNrefers to the nest of four species of swiftlets, namely *Collocalia fuciphaga, Collocalia germanis,Collocalia maxima* and*Collocalia unicolour*[2]. The saliva is produced by a pair of sublingual glands located beneath the tongue of birds. The nests of the *Collocalia fuciphaga* are amongst the world's most expensive animal products[3]. The price of white EBN being sold by EBN farmers in Thailand has been reported to reach 65,000 Baht (~2170 US$) per kg with its total export valued at around 126 million Baht (~4.2 million US$) per year [4].This has put a lot of pressure on the survival of swiftlets and there has been a decline in EBN cave yields from overharvesting [3]. The continuously increasing demand and the values of EBN in recent years have led to the establishment of EBN farming in many countries [5].

Carbohydrates and glycoproteins are the major components of EBN apart from amino acids, fatty acids and some trace elements such as calcium, sodium, magnesium, zinc, manganese and iron[6]. The composition of EBN makes it esteemed as a nutritional food. Chinese cook nest material in a double boiler with sugar to produce the gastronomic delicacy known as the bird's nest soup[7]. Reportedly, Hong Kong stands as the largest consumer of the EBNs globally, with ethnic Chinese of North America as the second largest consumers[2]. Generally, glycoproteins rich in amino acids, carbohydrates, calcium, sodium and potassium are the main constituents of EBN[8]. Owing to its esteem as a prized food product in the East, EBN is also referred to as the "Caviar of the East"[6]. EBN has been used for millennia as a health supplement in Traditional Chinese Medicine (TCM) as rich source of water soluble proteins, amino acids, carbohydrates, iron, inorganic salts, and fibers etc. Besides, EBN finds profound applications in treating malnutrition, boosting immune system, improving metabolism, enhancing skin complexion and alleviating asthma.

Owing to the fact that the nutrient composition of EBN isdependent upon the country of origin, breeding sites, climate and food intake of birds [8]; several studies have reported the composition of EBNs from certain areas in Thailand, Malaysia and Indonesia [6,8,9]. Some studies have documented the antimicrobial and anti-oxidant properties of certain protein and saliva compounds [10-12]. Glycoproteins from fish epidermal mucus have been found to kill bacteria by forming large pores in their cell membranes [13]. However, till date, very little is known about the antibacterial and anti-oxidant properties of EBN. In view of all these facts, it was thought worthwhile to investigate the physicochemical, biochemical and antibacterial properties of EBN obtained from the swiftlet house in Batu Pahat, Johor, Malaysia. Carbohydrates, proteins, fats and ash were determined by proximate analysis. Amino acids were analyzed by AccQ.Tag method using high performance liquid chromatography (HPLC). The mineral contents were determined by Inductively Coupled Plasma Mass Spectrophotometer (ICPMS) analysis. The anti-oxidant effects were studied by ferric reducing anti-oxidant power (FRAP) assay. Finally, the antibacterial activities towards *Escherichia coli, Klebsiella pneumoniae*, *Bacillus subtilis* and *Staphylococcus aureus*were carried out by disc diffusion method. The results of these findings are presented herein this paper.

EXPERIMENTAL SECTION

Chemicals and Reagents

All the chemicals and reagents were of AR grade and used without further purification. Sulfuric acid, hydrochloric acid, potassium hydroxide, performic acid, hydrogen bromide, lithium hydroxide, sodium hydroxide, bovine serum albumin (BSA),glycerol, folin-ciocalteu phenol reagent and ethanol were purchased from Merck Chemical Company. Ferric chloride, sodium chloride and methanol were purchased from GCE Chemical Laboratory. Glycine, 2,4,6-Tripyridyl-s-Triazine (TPTZ) and glacial acetic acid were procured from Fluka Chemicals. Electrophoresis buffer, acrylamide (electrophoresis grade), bis-acrylamide, tris base, sodium dodecyl sulphate (SDS), tetramethylethylenediamine (TEMED), ammonium persulphate, 2-mercaptoethanol and bromophenol blue were procured from Bio Rad. Other reagents used in this study were Kjeldahl tablets (Lab-scan), petroleum ether (Lab-scan), broad range protein molecular marker (Promega). Mili Q water, AABA (α-Amino-n-butyric Acid)(Perbio), pierce amino acid standard H (Perbio) and AccQ.Fluor derivatization buffer (Waters Corp).Bacterial strains of *Escherichia coli* and *Staphylococcus aureus* were obtained from the Institute of Bioproduct Development, Universiti Teknologi Malaysia while *Klebsiella Pneumoniae* and *Bacillus subtilis*, and nutrient broth were obtained from the Biology Department, Faculty of Science, Universiti Teknologi Malaysia.

Apparatus and Equipments

0.4 mm sieve, dark field and bright field compound microscope (Leica DMLS), digital camera (Power Short S40 Canon), Waters Model 510 HPLC isocratic pump, Waters 717 autosampler and Waters 470 scanning fluorescence detector, millennium chromatography manager, AccQ.Tag column (3.9 x 150 mm) equipped with a Nova-Pak C18 sentry guard column (Waters Corp), hydrolysis bottle, Whatman No.1 filter paper, 0.2 μm cellulose acetate syringe filter, refrigerator, hot water bath (Memmert), pH meter (Hanna), centrifuge (Kubota 2010), centrifuge tubes, freezer Christ Alpha 1-2, conical flasks, hot plate, CWF1100 Carboliter Furnance, Memert drying oven, soxhlet extractor with reflux condenser and distillation flask, PerkinElmer Lamda 25 UV/VIS spectrophotometer, electronic moisture analyzer (Denver Instrument IR-30), Innova 4080 incubator shaker, PE SCIEX Elan 6100 ICPMS, Design Expert 6.0.8 software, mini gel apparatus (bio-Rad Mini Protein III apparatus), power supply (capacity 200V, 500 mA), Hamilton syringe, eppendorf tubes, rotary shaker, micropipettes, beakers, 10 microgram streptomycin (OXOID) and petri dishes.

EBN Sample Preparation
The raw EBN samples were obtained from the swiftlet house in Batu Pahat, Johor, Malaysia. The samples were cleaned by soaking in distilled water for 1 h. Feathers and impurities were manually removed using tweezers and the cleaned nest was dried in oven at 50 °C for 12 h until the moisture content was lower than 15%. The EBN was ground with mortar and pastel and then sieved through a 0.4 mm screen.

Macroscopic Characterization
The features of 50 pieces of raw EBN were examined macroscopically. Raw EBN size was measured by ruler and the shape description was described *via* observation. 5 g of cleaned EBN were soaked in distilled water for 2 h, the swelled EBN dripped to dry *via* 0.4 mm sieve for 10 minutes prior to weighing on the balance. The tests were performed in triplicates.

Microscopic Characterization
The ground EBN was examined directly under a bright field and dark field compound microscope with 40X and 100X magnifications. The photomicrographs of the ground EBN were taken using a digital camera. The cleaned strand and swelled strand of the EBN (after 2 h of soaking) were observed under the microscope by using bright field and dark field modes with low magnification (40X).

Colour Measurement
The ground EBN (0.5 g) was treated separately with 5 ml of H_2SO_4 [98.3 % (v/v)], 2 ml of HCl [38 % (v/v)], 2 ml of KOH [5 % (w/v)] and 2 ml of $FeCl_3$ [5 % (w/v]) for 30 minutes. The colour and physical changes were observed in daylight [14]. The tests were performed in duplicates.

Amino Acid Profiling
AccQ.Tag Method has been used for the identification of amino acids in the EBN. This method involves the use of an amino acid specific derivatizing reagent, i.e. Waters AccQ. Fluor reagent (6-aminoquinolyl-N-hydroxy succinimidyl carbamate). Both primary and secondary amino acids are converted into stable, fluorescent derivatives by the action of this reagent, and itself gets hydrolyzed to 6-aminoquinoline, which is a non-interfering by-product[15]. The AccQ. Fluor reagent reacts rapidly with primary and secondary amino acids to yield highly stable urea that fluoresces strongly at 395 nm. The resulting derivatives are stable at room temperature for up to one week. The Waters AccQ. Tag Chemistry Package includes the Waters Amino Acid Hydrolysate Standard. The standard mixture contains a 2.5 mM concentration of each of the hydrolysate amino acids with the exception of cysteine (Cys) (1.25 mM)[15]. The amino acid profiling was done *via* acidic, alkaline and oxidation pathways as discussed in the following sub-sections.

Acid Hydrolysis
Aspartic acid (Asp), threonine (Thr), serine (Ser), glutamic acid (Glu), proline (Pro), glycine (Gly), alanine (Ala), valine (Val), isoleucine (Ile), leucine (Leu), tyrosine (Tyr), phenylalanine (Phe), histidine (His), lysine (Lys) and arginine (Arg) were determined through acid hydrolysis. 0.3 g of ground EBN was weighed into a hydrolysis bottle and hydrolyzed with 5ml of 6N HCl at 110°C for 24 hin an air force oven. The hydrolysed samples were cooled to room temperature and then filtered through filter paper into 100ml volumetric flasks. 400µl internal standard of 50µmol ml^{-1} of AABA in 0.1M HCl was added and made up to 100ml with distilled water. The aliquotes were filtered through 0.2µm cellulose acetate filter.

For the derivatization process, 10µl of filtered hydrolysed samples or standard were transferred into a 1.5ml glass vial and 70µl of borate buffer solution was added and mixed well. 20µl of AccQ.Fluor Reagent (3mg ml^{-1} in acetonitrile) was added to the mixture and vigorously homogenized using vortex. 10µl of sample and standard were injected into the HPLC system.An AccQ.Tag column with the dimensions of 3.9mm × 150mm was used to separate the amino acids in an aqueous solution of Eluent A (200ml AccQ.Tag mixed with 2L of MiliQ water) and Eluent B (60% acetonitrile) at a constant flow rate of 1ml min^{-1} for 50 minutes. Detection was carried out by a fluorescence detector (λ excitation and λ emission at 250nm). The linear gradient was set as shown in **Table 1**.

Table 1: Different experimental parameters indicating the linear gradient

S. No.	Time (min)	Flow rate (ml min^{-1})	A(%)	B(%)
1	Initial	1.0	100	0
2	0.5	1.0	98	2
3	15	1.0	91	9
4	19	1.0	87	13
5	32	1.0	65	35
6	34	1.0	65	35
7	35	1.0	0	100
8	38	1.0	0	100
9	39	1.0	0	100
10	50	1.0	100	0

Alkaline Hydrolysis

Alkaline hydrolysis was performed to determine tryptophan (Trp). 0.3 g of ground EBN was placed in an evacuated tube and 15ml of fresh 4.3 N lithium hydroxide ($LiOH.H_2O$) was added. The mixture was flushed with nitrogen gas followed by heating in oven at 120 ºC for 16 h. The hydrolysate was transferred into a beaker containing 50 ml of distilled water and 9 ml of 6 N HCl. The pH of sample was adjusted to 4.5 with 0.1 M HCl and filtered through filter paper. The filterate was diluted with distilled water upto 100 ml in volumetric flask.

Standard solutions were prepared by dissolving 0.05 g of trp with 0.1 N HCl in 50 ml volumetric flask. 50 µl of the solution was pipetted out into 10 ml volumetric flask and topped upto the mark with mobile phase. Sample and standard were filtered through 0.2 mm polytetrafluoroethylene microfilter and 10 µl was injected into the HPLC system. A Nova Pack C18 column with the dimensions of 3.9 mm × 150 mm was used to determine tryptophan in a mobile phase of 0.0085 M sodium acetate and methanol (86.7:13.3, v/v) with 4.0 pH at a constant flow rate of 1.5 ml min^{-1}. Detection was carried out by a fluorescence detector (λ excitation at 285nm and λ emission at 345 nm).

Performic Acid Oxidation

Sulphur containing amino acids *viz.*Cys and methionine (Met) were determined through performic acid oxidation. 0.3 g of ground EBN sample was mixed with 2ml chilled performic acid (formic acid: hydrogen peroxide, 9:1, v/v) and then the mixture was stored at 0°C for 16 h. Further, 0.4 ml of HBr were added and the addendum stored for 30 minutes. The sample was then dried in hot water bath to remove the performic acid and proceed with the hydrolysis using 6N HCl hydrolysis as mentioned above.

The standard solutions were prepared by mixing 5ml of 50µmol ml^{-1} of AABA, 10ml of 25µmol ml^{-1}cysteic acid (Cya) and 10ml of 25µmol ml^{-1} Met and made up to final volume of 100ml using 0.1N HCl (solution A). 1.5ml solution A was mixed with 1.5ml amino acid standard H "pierce" (solution B). 160µl of solution B was mixed with 840µl of distilled water. Similar derivatization was performed as in the acid hydrolysis section prior to injection through the HPLC system.

Proximate Analysis

The ground EBN samples were subjected to proximate analysis. Proximate analysis includes the analysis of crude proteins, crude fats, moisture, ash, crude fibre and carbohydrates [18]. Moisture, ash and crude fibre were determined by gravimetric method; fats by soxhlet extraction and gravimetry; proteins by Kjeldahl determination; and carbohydrates by difference. All analyses were carried out in triplicate and the values were then averaged.

Crude Protein Analysis

Crude protein composition was determined by Kjeldahl's method, using 6.25 as a conversion factor [19]. 500 mg sample of EBN was weighed and placed into the disgestion tube. A catalytic amount of $CuSO_4/K_2SO_4$ and 12 ml of sulphuric acid were added into the digestion tube to initiate the digestion process until clear green or blue solution was obtained. The solution was cooled for 10-20 minutes before the addition of 75 ml of distilled water. The

analysis was continued with distillation process through the addition of 25 ml boric acid and 10 drops of bromocresol green as indicator. The cooled digestion tubes were placed in digestion unit. A 50 ml of sodium hydroxide solution was added to samples for 5 minutes in a distillation unit. The distillate was titrated with hydrochloric acid until grey colour was obtained. The crude protein content was calculated using equations (1) and (2).

$$\%\ Nitrogen = \frac{(T-B)\times N\times 14.007}{Sample\ weight\ (mg)} \quad \text{(Eq. 1)}$$

% Crude protein= % Nitrogen x F (Eq. 2)

Where,
T: Sample titration volume (ml)
B: Blank titration volume (ml)
N: Normality of HCl
F: Cude protein factor (6.25)

Crude Fibre Analysis
A sintered glass filter crucible was dried at about 100ºC and then weighed accurately. 1 g of ground EBN was weighed accurately and added into a distillation flask. 0.5 g sodium sulphite, 2 ml decalin and 100 ml neutral detergent solution was added to the EBN containing distillation flask, and the sample mixture refluxed for 60 minutes. The residue left was filtered through the dried crucible with suction, and washed with a little amount of water. 10 ml of amylase solution was allowed to pass through with suction to displace the wash water. The crucible was positioned in a small beaker and more amylase solution added to cover the residue in the crucible. A few dropsof toluene were then added. Finally, the beaker was covered with film or foil and incubated overnight at 37 ºC, and then the sample was filtered under suction. The residue left behind in the crucible was washed with a small amount of water followed by acetone. The washed sample was dried at 100ºC, cooled and reweighed.

The percentage of insoluble dietary fibre in the EBN was calculated according to equation (3):

$$\%NDF = \frac{w2-w1}{w3}\times 100 \quad \text{(Eq. 3)}$$

Where w1= weight of empty crucible (g), w2= weight of crucible with residue (g) and w3=weight of food taken (g)

Crude Fat Analysis
Soxhlet extraction method [18] was used to determine the crude fat content of the ground EBN. The Soxhlet extractor was set up with dried and weighed reflux condenser and distillation flask.2 g of the ground EBN sample was weighed accurately and put into a fat free extraction thimble, plugged lightly with cotton wool. The thimble was then placed in the extractor. Petroleum ether was added until it was siphoned over once. This was followed by further addition of petroleum ether until the barrel of the extractor was half full. Then, the condenser was replaced, ensuring that the joints were tight, and placed on an electric heater. The heating was adjusted such that the solvent boils gently and allows petroleum ether to siphon over EBN at least ten times.

Fat content was calculated as the percentage of the sample taken, given by equation (4).

$$\%Fat = \frac{w2-w1}{w3}\times 100 \quad \text{(Eq. 4)}$$

Where, w1 = weight of empty flask, w2 = weight of flask + fat and w3 = weight of food taken.

Moisture Content Analysis
Electronic moisture analyzer was used to measure the moisture content of ground EBN. 100 mg of the ground EBN was placed in the moisture analyzer at 130°C. The moisture contents (%) measured by the analyzer were recorded.

Ash Content Analysis
4 g of ground EBN was accurately weighed and put into a previously ignited, cooled and weighed crucible. The crucible was transferred to a muffle furnace at about 550°C and was left until a white or light grey ash formed. The crucible was cooled in desiccator and reweighed [18].

The total ash was calculated as the percentage of the original sample as given in equation (5)

$$\%Ash = \frac{weight\ of\ ash}{weight\ of\ original\ food} \times 100 = \frac{w3-w1}{w2-w1} \times 100 \quad \text{(Eq. 5)}$$

Where, w1 = weight of empty crucible,w2 = weight of crucible + food before drying and/or ashing,w3 = weight of crucible + ash.

Carbohydrate Content Analysis
The carbohydrate content of ground EBN was determined by using the difference method [18]. Percentage of the total carbohydrates in the EBN was determined by reducing the sum percentages of moisture, protein, fat, fibre and ash from one hundred as stated in equation (6).This involves obtaining the carbohydrate content by calculation having estimated all the other fractions by proximate analysis.

% Available carbohydrates = 100 – (% moisture + % ash + % fat + % protein) (Eq. 6)

Mineral Analysis
1.0 g of ground EBN was dried in oven at 80 °C for 16 h. The dried EBN was ashed during 24 h in an oven at 400 °C. 0.2 g ash was dissolved with10 ml of 65% nitric acid. After 72 h of digestion in a closed polypropylene tube at 90°C in a heating block, the solution was evaporated to approximately 1 ml and then diluted with ultrapure water until a final volume of 20 ml. Mineral analysis was carried out by ICPMS. Fifteen minerals namely, arsenic, lead, copper, zinc, mercury, cadmium, barium, cobalt, nickel, argentums, lithium, manganese, aluminum, beryllium and titanium were analyzed. Besides, ten other minerals including magnesium, phosphorous, zinc, iron, chromium, calcium, selenium, potassium, ferum, copper and sodium were also analyzed. The analysis was performed in triplicates and the reported values are the averaged ones.

EBN Extraction Methods
Salt Extraction
The salt extraction method was adapted from Marfo and Oke [20]. The proteins were extracted by suspending 0.5 g of the ground EBN samples in 50 ml of deionized water containing 0, 5, 10, 15, 20and 25% (w/v) NaCl concentrations at pH 8. The suspensions were shaken for 3 h at 40°C and at 150 rpm. The resulting slurry was centrifuged at 4000 rpm for 15 min. The supernatants were used for the determination of protein concentration by recording absorbance at 280 nm [11,21]. The effect of NaCl concentration on protein leaching was investigated. The experiment was carried out in duplicate and the average values were recorded.

Alkaline Extraction
The alkaline extraction method was adapted from the method of Ragabet al.[22] and Bilgi and Celik [23]. 0.5 g of the ground EBN samples were added with 50ml deionized water in conical flasks containing 0, 0.050, 0.10, 0.250, 0.50, 0.750, 1.00 and 1.5 M NaOH, respectively. The mixtures were shaken at 150 rpm at 40ºC for 3 h and then centrifuged at 4000 rpm for 15 minutes. The supernatant was used for protein concentration determination at 280 nm. The experiment was carried out in duplicate and the average was recorded.

Heat Extraction
The heat extraction was carried out in accordance with Al-Maamari etal.[24]. 0.5 g ground EBN was suspended in 50 ml of deionized water and incubated at different temperatures*viz.*30ºC, 40ºC, 60ºC, 80ºC and 100ºC at150 rpm. Then, the extract was centrifuged at 4000 rpm and the supernatant was used for protein concentration determination at 280 nm. The experiment was carried out in duplicate and the average values were recorded.

Extraction by Oda et al. Method[25]
The ground EBN (0.5 g) was immersed in 50 ml deionized water overnight and then incubated at 80ºC for 5 h at 150 rpm followed by centrifugation at 4000 rpm. The supernatant was stored at 4°C until used. The protein

concentration was determined by recording absorbance at 280 nm. The experiment was carried out in duplicate and the average values were recorded.

Extraction by Goh et al., Method[26]
The ground EBN (0.5 g) was suspended in 50 ml of deionized water and was allowed to stand for 24 h at 4°C. An aliquot of the extract was then boiled for one hour, followed by centrifugation at 4000 rpm. The supernatant was stored at 4°C until used. The protein concentration was determined by recording absorbance at 280 nm. The experiment was carried out in duplicate and the average values were recorded.

Experimental Design for Protein Extraction
The Box-Behnken experimental design using Design Expert 6.0.8 software was used for designing the experimental work for evaluating the three factors that affected the protein extraction of EBN [27]. Three significant variables were used to study the effect of the independent variables *X*1 [NaOH Concentration(M)], *X*2 [Temperature (°C)] and *X*3 [Duration (h)] at three levels of each factor in the extraction process with seventeen experimental runs. Three conditions (low, middle and high) were used for each variable, *viz.* 0.00, 0.03 and 0.05 M for sodium hydroxide; 30, 55 and 80 °C for temperature; and 1, 3 and 5 h for duration. All experiments were performed in triplicate, using 0.5 g of the ground EBN with agitation at 150 rpm. After extraction, the extracts were centrifuged at 4000 rpm and the supernatant was stored at 4°C until used. **Table 2** shows the design parameters simulated by the software.

Table 2: The simulated design parameters by Design Expert 6.0.8

Run	Factor 1 A: Sodium hydroxide (M)	Factor 2 B: Temperature (ºC)	Factor 3 C: Duration (h)
1	0.05	55	5
2	0.00	30	3
3	0.03	55	3
4	0.03	80	5
5	0.03	55	3
6	0.03	30	1
7	0.03	55	1
8	0.03	30	5
9	0.03	55	3
10	0.05	80	3
11	0.03	55	3
12	0.05	30	3
13	0.00	80	3
14	0.05	55	1
15	0.03	80	1
16	0.03	55	3
17	0.00	55	5

Gel Electrophoresis under Denaturing Conditions
Out of the nineteen protein extracts, only four extracts were further analyzed by SDS PAGE electrophoresis for protein profiling. The chosen extracts are Goh et al. (2000), Oda et al.(1998), Run 1 (0.05 M NaOH, 55 ºC, 5 h) and Run 10 (0.05 M NaOH, 80 ºC, 3 h). These extracts were subjected to 2X dilution factor before loading to the electrophoresis gel.

Run 10 gave the highest protein concentration with yellowish appearance (5.66 mg/ ml). Run 1 gave the highest protein concentration extract (5.09 mg/ ml) with whitish appearance. Goh and Oda were the conventional water extraction methods. The above extracts were selected to compare the developed method and the conventional water extraction method in terms of protein concentration, anti-oxidants, protein profile and antibacterial activity.

Sample Preparation for Electrophoresis
For analysis using simplified electrophoretic methods [21],the 20 µl samples were mixed with 5 µl 5X sample buffer (0.6 ml 1 M Tris-HCl (pH6.8), 5 ml 50% glycerol, 2 ml 10% SDS, 0.5 ml 2-mercaptoethanol and 1 ml 1%bromophenol blue) in an eppendorf tube and heated at 100ºC for 2-10 minutes. Protein solutions were spun down for 1 minute in microfuge.

SDS PAGE

10 µl sample solutions and broad range protein molecular weight markers (225 kDa-10 kDa) were loaded in stacking gel wells using Hamilton syringe. The fitting separating gel was 7.5% acrylamide. The power supply was turned on to 100 V constantly in pre-cooled electrophoresis buffer (25 mM Tris, 192 mM glycine containing 0.1% (w/v) SDS).When the dye had migrated and reached the bottom of the gel, the power supply was turned off. Molecular weight determination was estimated by co-electrophoresis of the molecular weight markers. The sample was then stained by using Coomassie Blue R-250.

Anti-oxidant Analysis

Ferric reducing anti-oxidant power (FRAP) assay was used for anti-oxidant analysis, and was carried out in accordance with Katalinic et al.[28] and Benzie and Strain[29]. Fresh 1 mM aqueous ferrous sulphate solution was prepared and diluted to make a series of solutions of 0.8, 0.6, 0.4, 0.2 and 0.1 mM concentrations. The standard solutions were transferred to cuvettes, and each time one cuvette was positioned in a spectrophotometer (set at 593 nm). The absorbance values were recorded and a graph of absorbance versus concentration was plotted. The chemicals needed in FRAP assay including acetate buffer (300 mM, pH 3.6), dilute HCl (40 mM), TPTZ (2,4,6-tri[2-pyridyl]-s-triazine) (10 mM) and ferric chloride (20 mM) were freshly prepared and used.

FRAP reagent was prepared by mixing 200 ml of acetate buffer, 20 ml of TPTZ solution, 20 ml of $FeCl_3$ solution and 24 ml of distilled water in a plastic bottle. The solution was incubated in a water bath at 37°C for 4 min. First, blank (FRAP reagent only) was transferred to a cuvette and placed in aspectrophotometer set at 593 nm. After 4 min, the blank was zeroed. Then, the sample was prepared by adding 1 ml of the extract with 3 ml of FRAP reagent in a25 ml beaker. The sample was kept in water bath at 37°C for 4 minutes. The samplewas then transferred to cuvette and placed in the spectrophotometer. After 4minutes, the absorbance of the sample was recorded. Similar procedure was repeated for other two replicates. All the steps were repeated for the four extracts, namely, Goh, Oda, Run 1, Run 10 and the control (0.05 M NaOH).

Antibacterial Assays

Disc diffusion method [30] was used for the determination of the antibacterial properties of Oda, Goh, Run 1 and Run 10 EBN extracts against four strains of bacteria; including two gram negative (*Escherichia coli* and *Klebsiella Pneumoniae*)and two gram positive (*Staphylococcus aureus* and *Bacillus subtilis*) strains. The inoculum's density was adjusted with a 0.5 McFarl and turbidity standard before spreading the bacteria on nutrient agar plates. This resulted in approximately 1 to 5×10^6 cells per ml. Antimicrobial susceptibility test discs containing 10 microgram streptomycin were used as positive controls, 0.05 M NaOH as EBN extract control, and methanol as the negative control. A sterile cotton swab was dipped into the nutrient broth and pressed againstthe inside edge of the tube to remove excess liquid. The nutrient agar plate wasstreaked evenly across the entire surface for uniform distribution. The plate was rotated 90 degrees and the streaking procedure was repeated to ensure that the entire surface had been covered in the process. The plate was then allowed to dry at room temperature for 3 to 5 minutes. These steps were repeated for the preparation of *Staphylococcus aureus, Klebsiella Pneumonia* and *Bacillus subtilis.* By using the antibiotics disc dispenser, the discs were gently dispensed on the plates. Small glass dish containing alcohol was used to flame-sterilize the forceps. These flame-sterilized forceps were used to position the discs at least 3 cm from each other and 2 cm from the edge of the plate. Then, the plates were incubated at37 °C for 24 h. After 24 h incubation, the plates were inverted. On the outside of the plate, the diameter of the zone of inhibition was measured. The diameters measured represented the ability of the extracts to inhibit the growth of bacteria.

RESULTS AND DISCUSSION

The EBN samples were cleaned and subjected to marphological examination. The marphological features both microscopically and macroscopically were recorded.

Macroscopic Characterization

The nest cups were half-bowl or u-shaped, and lustrous translucent to opaque as shown in **Fig.1 (a)**. The shape and surface of EBN with fewer feathers and that with more feathers is shown in **Figs. 1 (b)** and **(c)**, respectively. The height of the EBN samples ranged from 3.5-5.0 cm while the length ranged from 7.0-13.0 cm. The diameter of the nest was nearly twice its height, with an average weight of 5.6±1.3 g. From a sample of six nests with an average weight of 5 g, the weights were approximately 52 g after two hours soaking in water at ambient temperature. The 1040% weight increase was due to the absorption of water into the strands. The absorption caused the EBN to swell

two to three times of its original size and made it more resilient. After being stewed for 4 h at 80ºC, the EBN was partially solubilized while the remaining nest remained in its slippery strand form [**Fig. 1 (d)**]. The stewed EBN which absorbed water formed a gelatinous colloidal gel and gave an aluminous smell with a bland taste. The stewed EBN had a pH of 7.5 and thus is considered mildly alkaline.

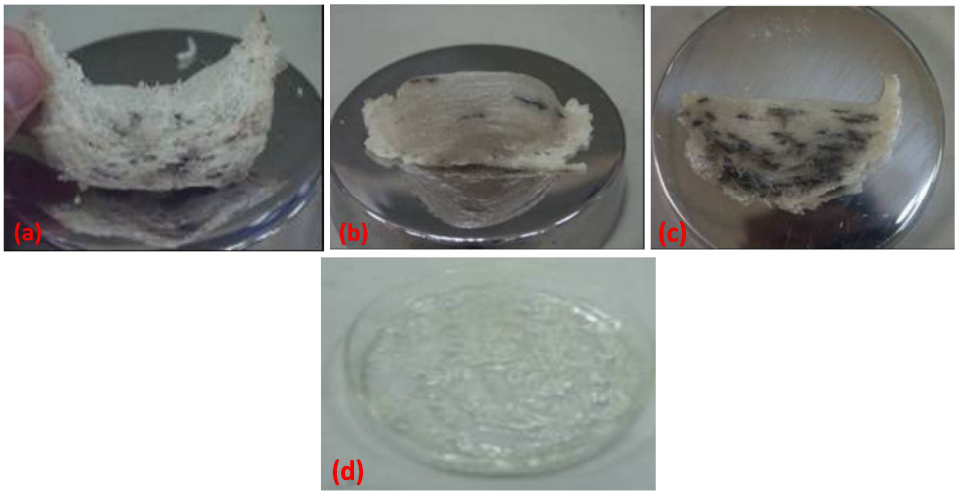

Fig. 1: Macroscopic features of EBN; (a): dorsal view, (b): ventral view with fewer feathers, (c): ventral view with more feathers, and(d) EBN after 4 h stewing

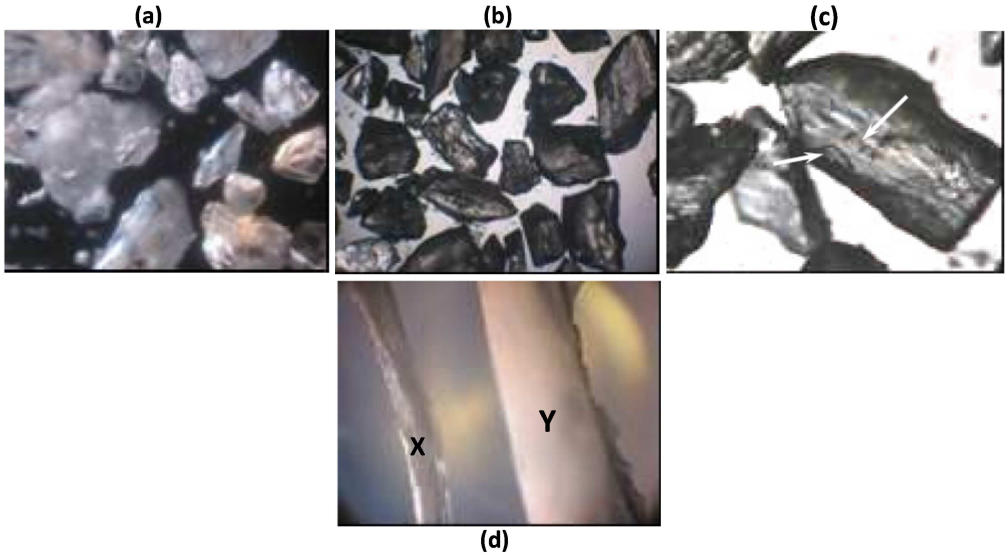

Fig. 2: Microscopic features of EBN; (a): visualization by dark field microscope at 40 X magnification, (b): visualization by bright field microscope at 40 X magnification, (c): air bubbles (indicated by arrows) in the ground EBN explored by bright field microscope at 100 X, and(d) EBN after 4 h stewing, and (d) dry EBN strand (X) and swollen EBN strand (Y)

Microscopic Characterization

The microscopic characterization of EBN revealed the surface or outward physical appearance of the ground EBN. Visualization under a low magnification (40X) dark field microscope [**Fig. 2(a)]** and a bright field microscope [**Fig. 2 (b)]** revealed ground EBN as irregular prisms with lustrous translucent to opaque silver crystalline structures. After about 30 minutes of stewing, air bubbles were found to surround or were captured within the swelled EBN strands**[Fig.2 (c)]**. A comparison between the dry EBN strand and the swollen strand after soaking in water for 2 h and viewed at low magnification (40X) has been presented in [**Fig. 2 (d)]**. It is clear from the figure that the microstructures of both the strands are different. The dry strand (**A**) was smaller and more translucent while as the swollen strand (**B**) was opaque and 2-3 times larger than the dry strand. In addition, the surface of the swollen strand

was smoother and softer than the dry strand. The strand swelling characteristics were used as one of the indications of authenticity of nests in the past. Nests with higher swelling rate were considered more genuine. Even today in the consumers' perspective; swelling rate is absolute towards authenticity.

Colour Analysis

Colour changes were studied to deduce a relation for checking the authenticity of EBN *via* the chemical tests. However, no conclusive results were obtained. The ground EBN showed various colours such as white, yellow, light brown and dark brown on treatment with potassium hydroxide (5%), ferric chloride (5%), hydrochloric acid (38%) and sulphuric acid (98.3%), respectively. Apart from the colour changes, the ground EBN also expanded and softened on treatment with potassium hydroxide (5%), hydrochloric acid (38%) and sulphuric acid (98.3%). However, no similar expansion and softness features were observed with ferric chloride (5%) treatment. Since colour is a highly sensitive property of a substrate, these tests can be used as references for detecting the presence of any fake material added into the EBN.

Proximate Analysis

The protein content in the EBN was found to be 35.80%. Based on the analysis in this study, the protein content of EBN in general is not likely to be as high as 85%; claimed by some commercial EBN websites such as "www.borneodelight.com". EBN investigated in this study had the lowest crude protein content compared to the other reported studies (**Table 3**). The overall mean protein content obtained in this study was however, comparable to that reported by Nurul Huda et al. [31], which ranged from 24-49%. From **Table 3**, it can be seen that the EBN samples from Thailand had the highest protein contents and those from Johor had the lowest. The differential protein contents may be attributed to the negative impacts of the industrial activities to the diversity and the availability of the food supply for the swiftlet populations. Besides, climatic variations are also responsible for the variations in protein content. This is verified by the studies from Mardiastuti and Boedi [32], who documented that the EBN samples collected during rainy seasons had higher protein contents with fewer impurities. Thus, the protein content in EBN is highly dependent on the environmental factors as well as human activities. Importantly, the EBN samples used in this study were obtained directly from swiftlet premises and thus, guaranteed to be 100% pure. Some of the authors have used EBN samples after purchasing from retail shops, which are often subjucted to adulteration and hence, display different protein contents.

Additionally, the protein content of the EBN analyzed in this study was compared with eggs. As per the reports by Brody [33]; an average large egg weighing 50 g provides 6 g or 12% (w/w) of protein. In comparison, 50 g of EBN would provide 17.90 g (35.8%) of EBN's protein, which is 3 times higher than an egg.

Fiber analysis indicated that no fibres were present in the EBN. Fibers would only be detected if some vegetative matter is present in the sample. Thus, the presence of fiber would be a primary indicator that some adulterant of vegetative nature is present in the EBN. Minute fat content (1.30%) was reported in the EBN. The moisture and ash contents were 11.27 and 5.17%, respectively. The carbohydrate content of EBN (46.47%) was 10% higher than the protein content. The possible constituents of the carbohydrate content are thought as D-mannitose, D-galactose, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine and N-acetyl neuraminate [34].

Table 3: Comparison of crude protein composition in EBN sample collected from the swiftlet house in Batu Pahat, Johor with EBN samples from other places

S. No.	EBN Sample	Protein Composition (%±SD)	Reference
1	Johor	35.80±0.12	Present study
2	Kuala Lumpur and Borneo Island	62.50±0.71	4
3	Peninsular Malaysia	58.92±2.53	6
4	Thailand	62.58±2.51	16
5	Thailand	55.32±1.43	17
6	Indonesia	54.68±3.72	17
7	Hong Kong	53.47±0.01	17
8	Vietnam	49.45±1.32	17

Mineral Analysis

The mineral content (magnesium, phosphorous, zinc, iron, chromium, selenium, potassium, copper, calcium and sodium) was analyzed by ICPMS. EBN contained relatively higher amount of sodium (6017 mg/kg), magnesium (344 mg/kg), potassium (138 mg/kg) and calcium (68 mg/kg). Besides, other minerals including phosphorus, iron, chromium and selenium in amounts as 0.037, 4.52, 0.30 and 0.14 mg/kg were also determined. This data confirmed the mineral content as claimed by most of the EBN producers. No mercury and cadmium was detected in the EBN, but trace amounts of arsenic (0.0237 mg/kg), lead (0.0203 mg/kg), copper (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; considers them as heavy metals. However, these heavy metals were within infant formula specification limit as set by the Act. In addition, several other minerals namely, barium (0.0445 mg/kg), cobalt (0.0044 mg/kg) , nickel (0.1172 mg/kg), argentum (0.0020 mg/kg), lithium (0.0119 mg/kg), manganese (0.3468 mg/kg), aluminium (2.5806 mg/kg), titanium (0.0031 mg/kg) and berilium (0.0149 mg/kg) were also detected in the EBN. This finding is contrary to the statement made by the Sarawak Museum Department [1] and Double Swallow Enterprise [35] stating that there is no evidence of any other metals found in the nests. There are speculations that the essential and non-essential minerals present in the EBN are either produced by swiflets themselves or leached from the environment. However, this study indicated that the minerals identified in the nest are produced by the swiflets (naturally occur in the nest) as the EBN has been obtained from the bird's house. There are no evidences that the minerals in the nest have been leached from where the nest was built.

Amino Acid Profiling

The analysis of the amino acid content of a protein involves the breakage of peptide bonds to release the amino acids. Treatment of proteins with 6 N HCl at 110 ºC for 24 h releases most of the amino acids. However, trp, met and cys are unstable to acid digestion in the presence of even a trace amount of oxygen where they would be destroyed at varying degrees[36]. In this study, the standard curves for all the amino acids were linear with correlation coefficients in the range of 0.997-0.999.The amino acid composition of proteins in the EBN samples is presented in **Table 4,** with the sums of 59.86mg/g protein. Ser, Asp, Thr, Tyr and Pro were found to be in significant amounts ranging from 4.15-6.12mg/g protein. This is in good agreement with the findings of Kathan and Weeks[37], Su et al.[17] and Marcone[6], where Ser and Asp have been found abundantly in their respective EBN samples. However, Trp, Ala, Ile, Lys and His were present in the least amounts ranging from 0.80-2.20mg/g protein.

Table 4: Amino acid composition of EBN sample collected from the swiftlet house in Batu Pahat, Johor. Retention factors and profiling methodology for each amino acid are also given

S. No.	Amino Acid	Retention Time (min)	Composition (mg amino acid/g protein)	Profiling Methodology
1	Asp	15.392	4.55	Acid hydrolysis
2	Ser	17.254	6.12	Acid hydrolysis
3	Glu	17.867	4.11	Acid hydrolysis
4	Gly	19.435	2.27	Acid hydrolysis
5	His	20.257	2.20	Acid hydrolysis
6	Arg	23.566	4.06	Acid hydrolysis
7	Thr	23.566	4.47	Acid hydrolysis
8	Ala	25.028	1.42	Acid hydrolysis
9	Pro	27.154	4.15	Acid hydrolysis
11	Val	31.826	3.68	Acid hydrolysis
12	Lys	34.563	1.99	Acid hydrolysis
13	Ile	35.157	1.84	Acid hydrolysis
14	Leu	35.597	4.15	Acid hydrolysis
15	Phe	36.470	3.90	Acid hydrolysis
10	Tyr	30.827	4.37	Acid hydrolysis
16	Cys	13.152	2.00	Performic acid oxidation
17	Met	26.053	3.60	Performic acid oxidation
18	Trp	5.924	0.80	Alkaline hydrolysis

Different sample preparations were performed for the diffrent types of amino acids. There is no single hydrolysis method for the analysis of all residues, and, therefore, hydrolysis is the least controllable part of the whole analysis. Every hydrolysis method aims the quantitative liberation of all amino acids of the subsrate and their quantitative recovery in the hydrolysate. Several factors such as temperature, time, hydrolysing agent and additives affect the completeness of the hydrolysis process. Although, Cys and Met contents in the EBN were detected using the method of acidic hydrolysis, this method is inaccurate in analyzing the sulphur-containing amino acids [36]. These sulphur-containing amino acids could be destroyed to varying extents by HCl at a concentration of 6 N. As a result, the contents of cysteine and methionine would be underestimated. Therefore, performic acid oxidation and hydrolysis were performed to yield the sulphur-containing amino acids in their stable forms. The method involves the conversion of Cysand Met to Cya and methionine sulphone (Meto3), respectively, which is follwed by their quantitation[36].

Acid Hydrolysis

The chromatogram of the separation and identification of amino acids generated by acid hydrolysis is shown in **Fig. 3**. The retention factors and the contents of the detected amino acids in acid hydrolysate are given in **Table 4**. The acid hydrolysate indicates that EBN is rich in several amino acids *viz.* Ser (6.12%), Asp (4.55%), Thr (4.47%), Tyr (4.37%), Leu (4.15%), Pro (4.15%) and Glu (4.11%). The contents of Ala, Ile and Lys are relatively lower with percentages of 1.42%, 1.84% and 1.99%, respectively.

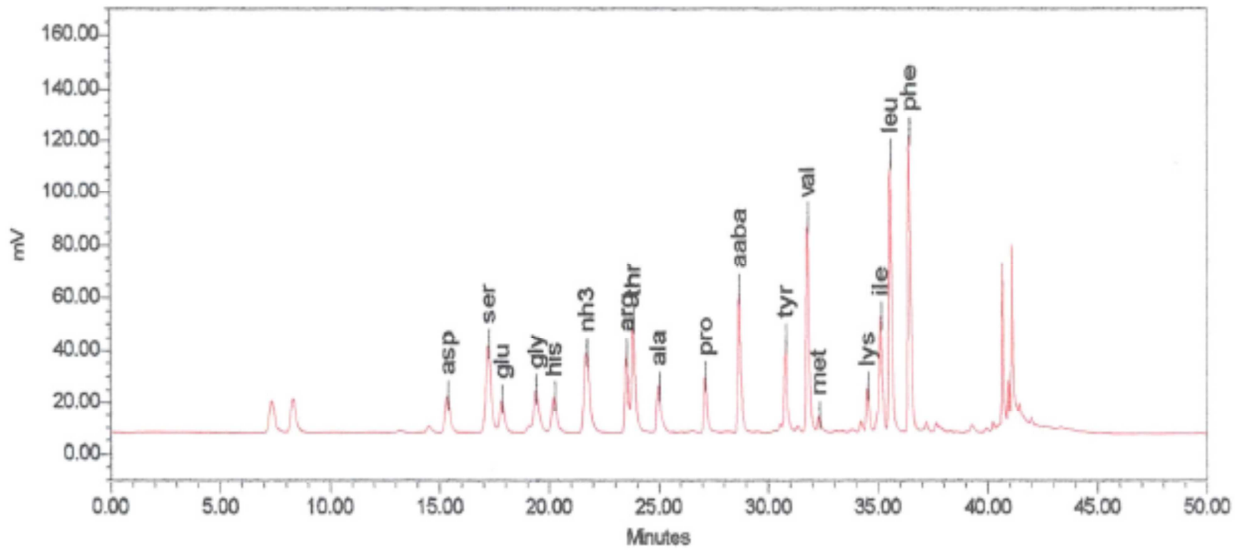

Fig. 3: HPLC chromatogram of the EBN amino acid hydrolysate generated by hydrochloric acid hydrolysis

Alkaline Hydrolysis

Trp is usually determined after digestion under alkaline conditions or after proteolytic digestion of the protein. **Fig. 4** shows the HPLC chromatogram of the EBN following treatment with alkali. It was worthwhile to note that EBN contains only a trace amount of Trp (0.80%) with a retention time of 5.924 minutes **(Table 4)**.

Performic acid oxidation

Cys and Met were converted to Cya and Meto3, respectively *via* performic acid oxidation and then quantitated by HPLC.**Fig. 5** shows the chromatogram of Cya and Meto3 in the EBN following chromatographic separation of performic acid-treated EBN.Following the treatment using performic acid, the EBN was found to contain Cyaand Meto3 as 2.00 and3.60% in terms of their contents with retetion times of 13.152 and 26.053 minutes, respectively (**Table 4**).

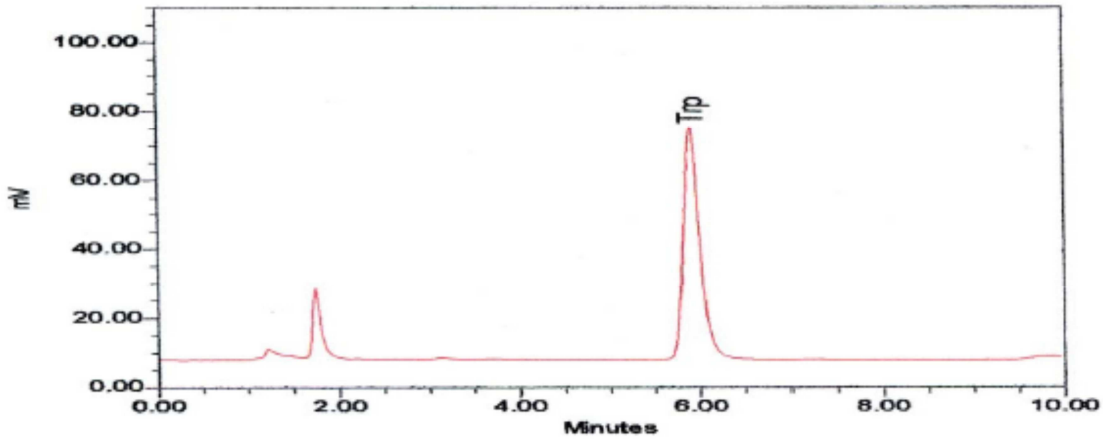

Fig. 4: HPLC chromatogram of the EBN amino acid hydrolysate generated by alkaline sample preparation

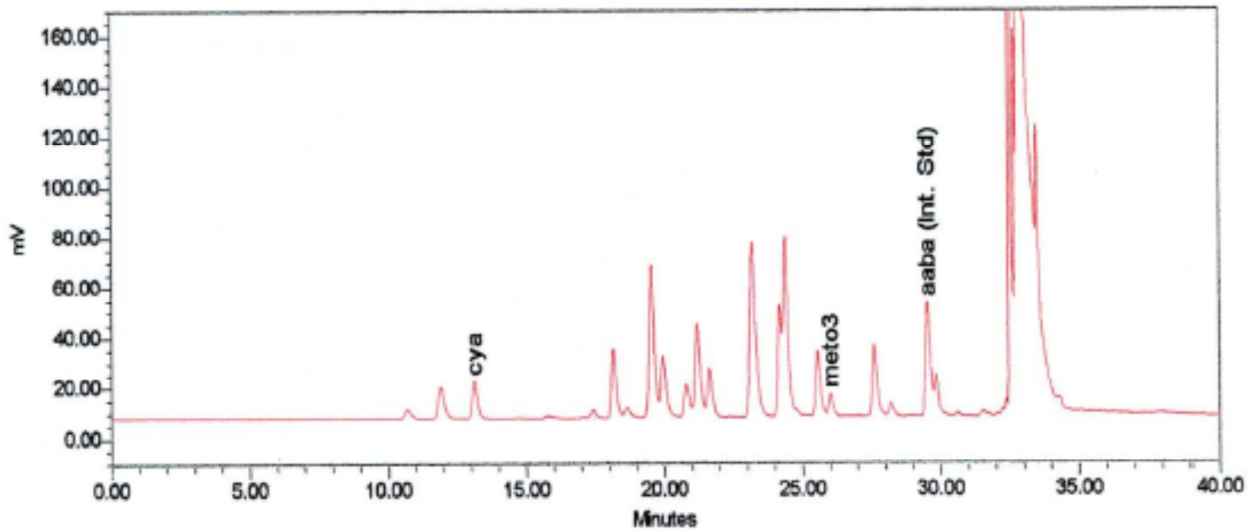

Fig. 5: HPLC chromatogram of the EBN amino acid hydrolysate generated by performic acid sample preparation

Table 5: The five major amino acids in EBN reported by various studies

S. No.	Authors of the study	Units of Quantification	Major amino acids	References
1	Present Study	(w/w %)	Serine (6.12), Aspartic acid (4.54), Threonine (4.47), Tyrosine (4.37) and Leucine (4.15).	
2	Kathan & Weeks (1969)	µmoles/100 mg	Serine (30.4), Proline (25.2), Glutamic acid (21.2), Threonine (21.0) and Aspartic acid (20.6).	37
3	Su et al. (1998)	Nitrogen content (%)	Aspartic acid (0.65), Histidine (0.58), Proline (0.56), Serine (0.46) and Glycine (0.46).	17
4	Marcone (2005)	Molar %	Serine (15.4), Valine (10.7), Isoleucine (10.1), Tyrosine (10.1) and Aspartic acid+arginine (9.5).	6
5	Chua et al. (2014)	mg/g of sample	Aspartic acid (53.86), proline (43.56), threonine (41.01), serine (37.20) and valine (36.43).	38

The amino acid analysis in this paper was compared with the amino acid analysis carried out by Kathan and Weeks[37], Su et al. [17], Marcone[6] and Chua et al. [38]. The comparative results in terms of the relative contents of the major amino acids found in the respective EBN samples are given in **Table 5**. It is clear from the table that all the studies have documented aspartic acid and serine as the major amino acids present in EBN. However, the other major amino acids reported by fellow researchers are different, because it is likely that the samples obtained from different places have different major amino acid contents.

Extraction Studies of EBN

The extraction of EBN for further studies was dependent on its solubility behaviour. Since a large portion of EBN consists of glycoprotein, the efficacy of the extraction was measured through the amount of glycoprotein extracted within the defined solution. Preliminary experiments including the effect of salt concentration, alkaline concentration and temperature; were carried out to determine the factors which influence the extraction of the EBN protein content.

The Box-Behnken experimental design using Design Expert 6.0.8 was used to conduct a simulated experimental design based on the concentration of NaOH, temperature and time duration. Out of the seventeen sets of experiments, two sets of protocols were chosen for protein molecular size profile, and anti-oxidant and antimicrobial activities of the EBN. The first chosen protocol was Run 10 (alkaline concentration: 0.05 M; time: 3 h; temperature: 80°C) which gave the highest yellowish protein concentration extract (5.66 mg/ml). The second protocol was Run 1 (alkaline concentration: 0.05 M; time 5 h; temperature: 55°C), it gave the highest whitish protein concentration extract (5.09 mg/ml). Besides, two additional modified water extraction protocols namely Oda et al.[25] and Goh et al. [26] were chosen.

Salt Extraction

Different NaCl concentrations were used in this study. **Fig.6**showed that the protein concentration extracted into the salt solution decreased as the NaCl concentration increased. Hence, NaCl was not chosen as one of the parameters for further extraction. The decreasing solubility of EBN proteins in the presence of salts may furnish additional information for EBN products preparation. If the ionic strength of the solution is either very high or very low, proteins will tend to precipitate at their isoelectric point. The solubility is also a function of ionic strength and as the ionic strength increases by adding salt, proteins will precipitate or in the context of this study, proteins are more difficult to be extracted into the solution [39]. The lower solubility of the proteins in salt solutions than water indicates that EBN contains more albuminous proteins than globular proteins [20].

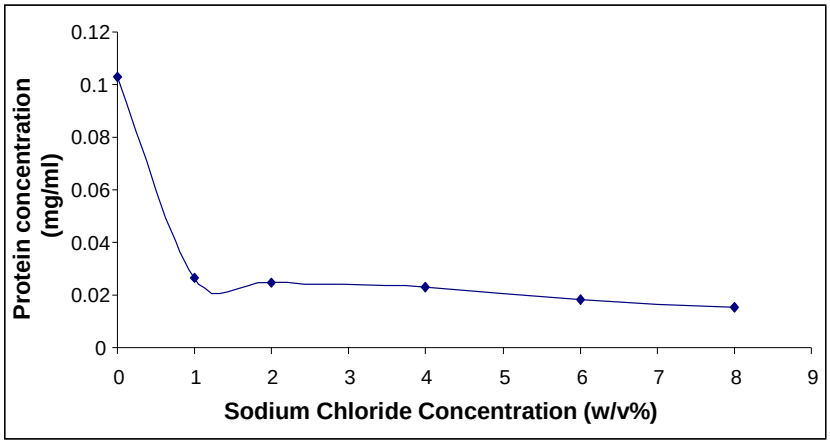

Fig. 6: Protein concentration following salt extraction

Alkaline Extraction

Alkaline extraction was carried out at various NaOH concentrations from 0 to 1.5 M. The effect of NaOH concentration on the extractability of EBN protein is shown in **Fig. 7**. Protein concentration for EBN extract was higher at higher concentrations of NaOH where it reached its maximum limit at 0.5 M NaOH. At higher alkaline concentration, the EBN extract was observed to change to yellowish colour from clear extract. In addition, the

extract discharged a nauseating odour. Higher concentrations of NaOH increased the extractability, therefore, NaOH concentration was selected as one of the parameters for further extraction study.

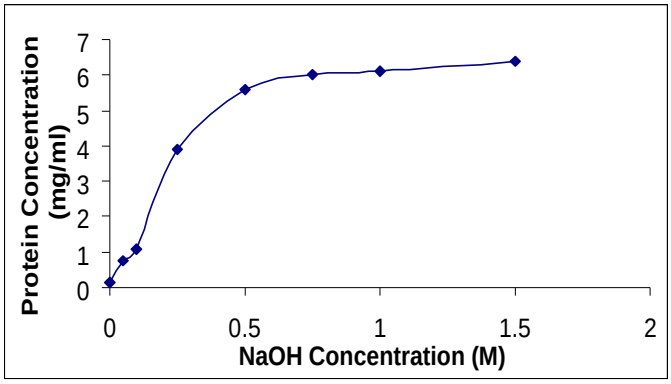

Fig. 7:Protein Concentration through alkaline extraction

Proteins in a biological sample are generally present in their native state. They are often associated with other proteins as well as integrated into membranes or are part of large complexes [39]. By adjusting pH of the extract solution, the EBN extract protein content increased. Proteins are usually soluble in water solutions because of the hydrophilic amino acid side chains on their surfaces that interact with water molecules. Besides, proteins have isoelectric points at which the charges of their amino acid side chains allow them to be soluble in solution at suitable pH. Although the alkalinity increased solubility of the proteins but it is certain that proteolysis will also take place.

Heat Extraction

Fig.8 shows the results of the heat extraction experiments performed between 30°C to 100°C. It was found that at lower temperature (below 60°C), heat had no significant impact on the extraction of proteins from the EBN. At temperatures between 60°C and 80°C, protein concentration of the extract drastically increased but gradually reduced above 80°C. Thus, temperature influences the extraction and solubility of proteins from the EBN. Hence, it was used as one of the parameters for further extraction studies. The interaction between protein surface groups and the surrounding solution is virtually fundamental to physico-chemical separation. Thus, the solubility of the EBN proteins in solution depends upon the properties of its exposed surface groups, pH, solution temperature, and the presence of ionic species in solution (NaCl) which have a biological affinity for the protein. Hence, protein solubility of the EBN is primarily due to interactions between its hydrophilic surface groups and water molecules. The solubility also depends on the solution conditions maximizing surface charge and preventing aggregation, which allows more EBN proteins to elute into the solution [40].

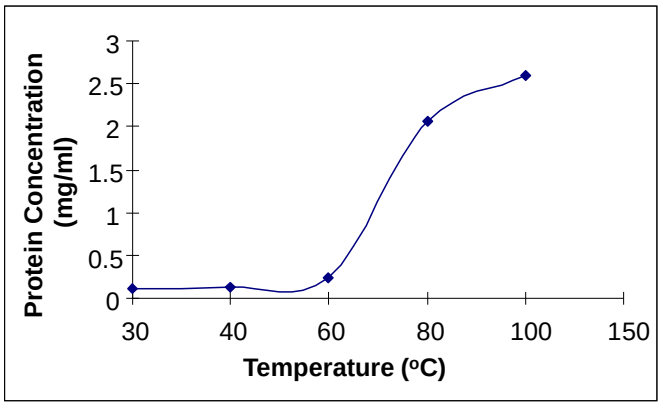

Fig. 8:Protein concentration following heat extraction

As a consequence of these results, alkaline concentration and temperature were chosen because of their significant influence on the extraction of the EBN.

Selection of Extraction Protocol

A series of experiments was conducted based on the experimental design simulated by Design Expert. The protein content extracted from each of the experiments has been summarized in **Table 6**. The protein yield of nineteen extracts (including Oda and Goh) varied from 0.15-5.66 mg/ml. The maximum extraction of protein contents as well as the whitish appearance of the extracts were the desirable factors. From **Table 6**, it is clear that the extracts contained higher protein content at higher temperature and sodium hydroxide concentration. Mailard reaction and protein denaturation are generally the issues to be avoided during the extraction. However, Mailard reaction, which caused yellowish extract was still observed in Run 4, Run 10 and Run 15. High temperature, high relative humidity and alkaline conditions; all promote the Maillard reaction. During the reaction, the condensation of a reducing group of the glycan moieties and an amino group from a protein or amino acid in the EBN forms a polymeric carbonyl-amine product of low solubility. The conjugated double bonds of the products account for the brownish or yellowish coloration [41]. It was observed that Run 10 gave the highest protein concentration with yellowish appearance (5.66 mg/ ml) and Run 1 gave the highest protein concentration extract (5.09 mg/ ml) with whitish appearance. Therefore, the two extracts (Run 1 and Run 10), which yielded highest protein concentrations were selected for protein size, anti-oxidant and antibacterial studies.

Table 6: Protein concentrations and colours obtained by various extraction runs

Run	Protein Concentration (µg/ml)	Extract Colour
1	5088.9	Whitish
2	149	Whitish
3	3277.54	Whitish
4	5154.16	Yellowish
5	3353.8	Whitish
6	437.876	Whitish
7	227.722	Whitish
8	684.634	Whitish
9	3263.43	Whitish
10	5657.68	Yellowish
11	3327.29	Whitish
12	592.118	Whitish
13	3255.63	Whitish
14	1402.53	Whitish
15	4933.53	Yellowish
16	3400.58	Whitish
17	471.799	Whitish
Oda	4079.01	Whitish
Goh	2628.78	Whitish

From **Table 6**, it can be deduced that different methods or different combinations of parameters result in different EBN protein solubilities. Combination of temperature and sodium hydroxide (Run 10 and Run 1) gave higher yields of protein contents as compared to the water and temperature extracts as carried out by previous workers [265,26]. Proteins have net negative charge at alkaline pH, which facilitated the segregation among the protein molecules and increased their solubility in Run 10 and Run 1 extracts [42].

Protein Profile of the EBN Extracts

EBN protein was characterized by SDS PAGE on 7.5% polyacrylamide gel. Extracts of Run 1, Run 10, Oda and Goh were electrophoresed, and the results are shown in **Fig. 9**. It was observed that Run 1 and Run 10 extracts did not show clear bands at the upper portion of the SDSPAGE gel. Besides, Run 10 extract did not have a clear smear and protein bands as the proteins were eluted out of the gel. It is possible that the proteins were hydrolyzed to smaller peptides or amino acids during the extraction process [20]. The Run 1 extract showed a smear effect when run through the gel. This result indicated the overloading effect due to the high concentration of protein sample produced in high temperature conditions, which induced the clogging effect when the denatured protein diffused

into the gel. Hence, a more diluted sample was carried out; however, more diluted form of extract did not produce a clear band in the gel due to for example lower protein concentration. Through the gel, 53 kDa protein was the only observable protein band that remained in the Run 1 extract. Both Oda (Lane 7 and 8) and Goh extracts (Lane 9 and 10) gave identical protein bands; however the Goh extract gave a more clear band as it contains higher protein concentration. Both extracts showed the pattern of the main bands at 135 kDaand 110 kDa, whereas the intermediate bands were at 82 kDa, 66 kDa and 53 kDa.

For Oda and Goh extracts, some faint unclear bands were observed in the molecular weight range from 47-37 kDa as shown in **Fig. 9**(Lanes 7, 8, 9 and 10). A smeared electrophoresis pattern was present in the upper part of profile and the blue band observed at the top of the wells. Thus EBN contains large and complex molecular weight proteins as the higher molecular weight proteins cannot enter the stacking gel [43].From the SDS PAGE analysis of the four extraction methods, it was found that the Oda extract produced better and clearer protein bands compared to the other extraction methods. Run 1 and Run 10 extracts contained higher protein content compared to Oda and Goh extracts. In **Fig.9**, the SDSPAGE smeared and unclear electrophoresis patterns of the Run 1 extract revealed that protein contained in the EBN can be easily extracted using NaOH solution, but the protein band could not be separated by the gel. It is possible that the proteins started to hydrolyze to smaller peptides. Alkaline extraction method at the higher temperature 80°C hydrolyzed the proteins to smaller peptides, which eluted out of the gel; this can be seen in **Fig.9** Run 10 extract. However, this does not necessarily mean that the extracts obtained by this method were not useful. For example, Guo et al.[26], documented that the EBN extract hydrolyzed by Pancreatin F into smaller peptides (lower than 25 kDa) was more propitious for anti-viral effects than the unhydrolyzed proteins in the EBN. The extraction methods in this study showed no 77kDa bands *via* SDS PAGE analysis. Under non-hydrolysing extraction conditions as studied by Oda et al. [25] and Goh et al. [26], the EBN showed several distinct bands as shown in **Fig.9**.The bands were positioned at 135 kDa, 110 kDa, 82 kDa, 66 kDa, 53 kDa and some unclear bands at around 47-37 kDa. Thus, the SDS PAGE analysis, indicated that the extraction method for the extraction of EBN affects the profile of protein bands in the SDS PAGE.Thus, the fresh and unprocessed EBN samples were found to contain more and distinct protein bands, which suggested that the commercial processing must be reducing the amount of intact protein originally present in the fresh nest [26].

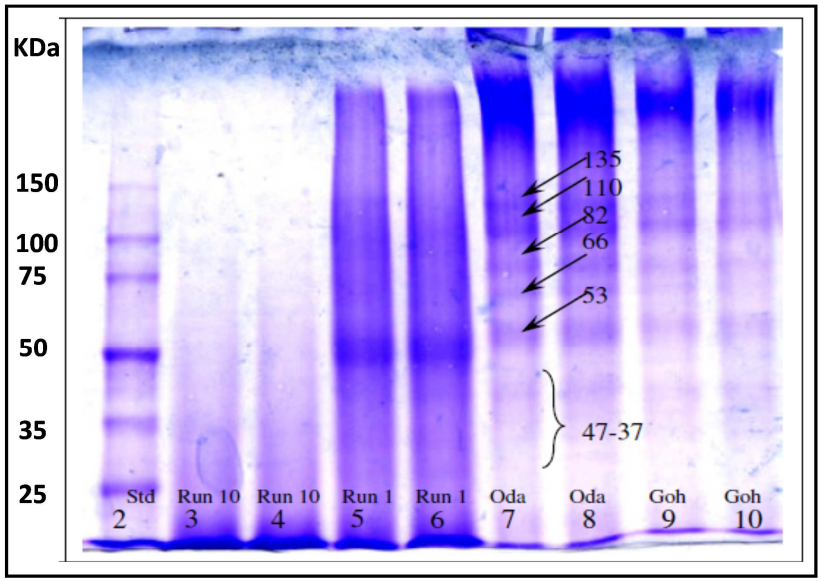

Fig. 9: 2X dilution of the EBN extract solutions. Lane 2: Protein standard, lane3: Run 10, lane 4: Run 10, lane 5: Run 1, lane 6: Run 1, lane 7: Oda extract, lane 8:Oda extract, lane 9: Goh extract, lane 10: Goh extract

Anti-oxidant Analysis

The anti-oxidant capacity of Run 10, Run 1, Oda and Goh extracts by FRAP assay demonstrated the effects of extraction on the anti-oxidant capacity of the EBN. Run 10extract showed the highest anti-oxidant power (1.0981 mM/L), while the lowest anti-oxidant power was shown by Goh extract (0.059 mM/L). Run 1 and Oda extracts had anti-oxidant powers of 0.7696 and 0.0769 mM/L, respectively. Till now, it is not exactly known how the EBN

extracts work as anti-oxidants. May be the anti-oxidant activities are due to the aminoacids present in the EBN as some of the proteins and amino acids detected in the EBN have known anti-oxidant activities. For example, Cys, Met, His, Try and Lys are the amino acids that have been proven for their anti-oxidant activities.A plausible mechanism of anti-oxidant activity may be due to the involvement of hydroxyl and carboxyl groups of the amino acid chains.

The correlation between the protein concentration and the anti-oxidant capacity of the EBN extracts is given in **Fig.10**.This figure depicts no significant correlations between protein concentration and anti-oxidant activity. The higher anti-oxidant capacity was seen in the extracts obtained through alkaline extraction. The hydrolyzed proteinextracts (Run 10 and Run 1) gave higher anti-oxidant capacitiesas compared to the unhydrolyzed protein extracts (Goh and Oda). The hydrolyzed proteins might have exposed more amino acids in the solution and thus gave a higher anti-oxidant power to the extracts.These results are in good agreement with the work of Yida et al. [44] who recently demonstrated that EBN after digestion in gut released antioxidants from their bound matrix and exert ehnanced antioxidant activity. Overall, the anti-oxidant analysis showed that EBN exhibited better anti-oxidant properties after hydrolysis, which may possibly account for some of its functional effects after consumption.

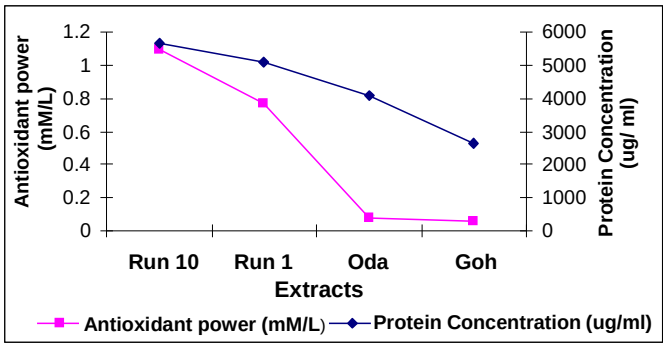

Fig. 10:Correlation between protein concentration and antioxidant capacity of the four selected EBN extracts

Antibacterial Assays

The antibacterial activities of the four extracts, Run 10, Run 1, Oda and Goh were carried out by using two gram positive(*Bacillus subtilis* and *Staphylococcus aureus*), and two gram negative(*Escherichia coli* and *Klebsiella pneumoniae*) bacterial strains. The results of Run 10extract are shown in **Fig. 11 (a-d)**. From the figures it can be seen that no zones of inhibition were observed in the discs, which contained the EBN extracts. Thus, Run 10 is ineffective against the four tested bacterial strains.

The other three extracts showed negative results for antibacterial activities. Thus, it can be concluded that the four EBN extracts had no effects towards the inhibition of *Escherichia coli*, *Klebsiella pneumoniae*, *Bacillus subtilis* and *Staphylococcus aureus*. The lack of antibacterial properties of EBN may be due to the fact that EBN is a proved food material and therefore, might have displayed a similar character towards the tested bacterial strains. However, it may be effective against some other bacterial strains, and therefore, further studies are needed in this direction.

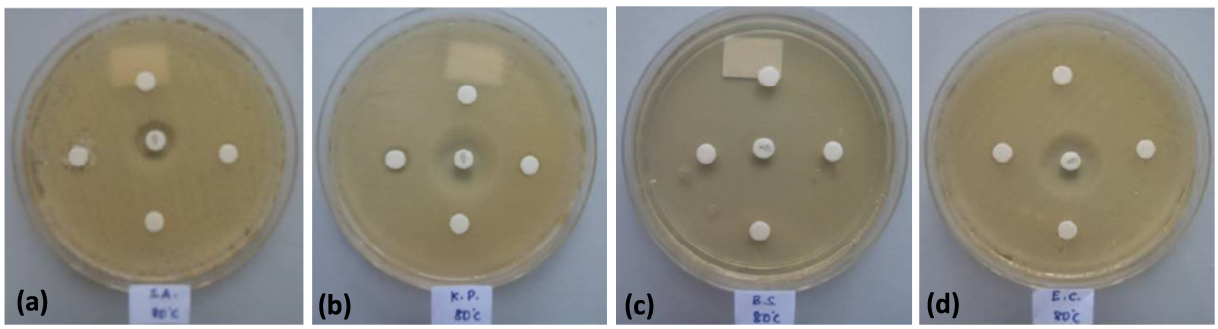

Fig. 11: Petridishes containing bacterial strains and Run 10 extract of the EBN. The bacterial strains are; (a): *Staphylococcus aureus*, (b): *Klebsiella pneumoniae*, (c): *Bacillus subtilis* and(d): *Escherichia coli*

CONCLUSION

The EBN obtained from the swiftlet house in Batu Pahat, Johor, Malaysia was thoroughly characterized by microscopic and macroscopic examinations, and chemical and biochemical analysis. The average weight of EBN was 5.6±1.3 g. The nests with inner filaments were heavier than nests without inner filaments. EBN swelled two to three times of its original size and the weight increased up to 1040%after soaking in water for 2 h. EBN had high protein content (35.8%), minute fat (1.5%) and ash (4.5%)content. No fiber was found in the EBN. The presence of fiber means some vegetative matter is present in the sample. Therefore, the presence of fiber would be a primary indicator that some adulterant of vegetative nature is present in EBN. The carbohydrate content of EBN was 10% higher than the protein content, which contradicts the reports of several researchers who claimed proteins as the major components of EBN. All the eighteen types of amino acids including the eight essential ones were found in EBN. The heavy metal contents were within infant formula specification limits. Sodium, magnesium, potassium and calcium were the major minerals found. Alkaline conditions and heat treatment were the significant parameters that affected the protein extraction from EBN. Run 10 extract had the highest protein content and anti-oxidant power. Protein contents and anti-oxidant powers of Oda and Goh extracts were lower as compared to Run 10 and Run 1, however, the former extracts produced visible bands in the SDS PAGE analysis. Interestingly, none of the extracts showed any antibacterial activity towards *Escherichia coli*, *Klebsiella pneumoniae*, *Bacillus subtilis* and *Staphylococcus aureus.* Conclusively, this study proves EBN from Batu Pahat, Johor, Malaysiaas a rich source of carbohydrates, proteins, amino acids and minerals. However, development of new extraction technologies in which the nutritional values of EBN are preserved are greatly demanding. The discussions and comparisons of the findings in this paper with other research works carried out on EBN samples from other places are quite helpful, and more of such studies will be useful in establishing standard rationales.

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