Research

Eco-Sustainable Production of Bioactive Sialyated-Mucin (SiaMuc) Glycopeptide from Edible Bird's Nest Co-Product

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ABSTRACT

Edible bird's nest (EBN) is the dried salivary secretion of swiftlets during the breeding season. The widely consumed EBN for its highly unique nutrients has contributed to a high demand for international exportation. However, the processing of EBN to remove inedible materials (i.e. sand, dust, feathers) has contributed to several wastes. The disposal of this waste is hazardous to the environment and a waste of value due to abundant sialylated-mucin (SiaMuc) glycoprotein attached in the impurities. This "waste" is the processing co-product of EBN. Therefore, this study aimed to recover SiaMuc-glycoprotein in EBN co-product (EBNco-P) in the form of bioactive SiaMuc-glycopeptide. Through enzymatic hydrolysis, an eco-friendly alternative to transform insoluble SiaMuc-glycoprotein in EBNco-P into soluble bioactive SiaMuc-glycopeptide, valuable nutrients are separable from the impurities. Previously, through our research, this biotechnology has been applied to cleaned EBN. The research finds that the enzymatic hydrolysis of EBN that produces EBN glycopeptide has led to bioconversion into a high-grade product with enhanced nutritional bioavailability and functionality. Similar results are also revealed in the analyses of cleaned-EBN and EBN co-products. Whereby, the result revealed that the optimum hydrolysis period was at 90 min, in which the DH and enhanced solubility indicated a complete breakdown of glycoprotein into bioactive glycopeptide. This result is in line with the physicochemical analysis result. The hydrolysis has significantly decreased ($p≤0.05$) the EBN protein content, while the levels for peptide, glycopeptide, polysaccharides, and sialic acid showed the opposite. This study may contribute to broadening the EBN product development in terms of nutraceutical functionality. With the availability of low-cost EBN co-products, this study is beneficial not only scientifically, but also to the consumers, commercialization, and industrialization.

Key words: Enzymatic hydrolysis, food production waste, glycoprotein, protein hydrolysate, swiftlet's nest

INTRODUCTION

Edible bird's nest (EBN) is the dried salivary secretion of swiftlets during the breeding season. The EBN contains many unique nutrients such as bioactive glycoprotein, sialic acid, and epidermal growth factor, which then makes EBN a precious source that is beneficial in enhancing health. For instance, scientific evidence has confirmed that EBN has health-beneficial functionalities such as anti-hypertensive, boosting immunity, anti-oxidant, anti-aging through promoting cell-proliferation, helps in neurological and memory development, anti-arthritis and even anti-diabetic (Safwani *et al*., 2016; Nurul Nadia *et al*., 2017; Alexandra *et al*., 2018; Ramachandran *et al*., 2018). Therefore, EBN is widely consumed for its highly unique nutrients and thus, contributed to a high demand for international exportation (Hui Yan *et al*., 2021).

For consumption purposes, the harvested swiftlet's nest required a cleaning process due to the involvement of inedible materials trapped within the nest. This includes bird feathers, dust, sand, grass, and more. Generally, the

cleaning process was done by soaking the swiftlet's nest for swelling purposes to ease the removal of inedible materials using tweezers. The swiftlet's nest was then reshaped and dried for market later. The EBN cleaning process of EBN has therefore led to a certain amount of waste. Commonly, this so-called "waste" is disposed of like normal rubbish with the landfill method. However, within this EBN "waste" itself, there is an abundant amount of valuable glycoprotein that remains trapped and tightly attached to the inedible materials (Noor *et al*., 2022). This phenomenon happens naturally when the swiftlets build their nest using saliva which is spit to the wall of the nesting building such as the cave. In short, proper treatment of this "waste" or EBN co-product may contribute to reducing wastage and environmental hazard issues. Hence, this study aimed to recover the EBN from its co-product with the use of enzymatic biotechnology and further investigate its properties.

Previously, through our research, we applied enzymatic biotechnology to the cleaned EBN (Hui Yan *et al*., 2021). The research found that enzymatic hydrolysis of EBN produces soluble EBN glycopeptide. Which, this has led to the bioconversion of EBN into high-grade products with enhanced nutritional bioavailability and functionality. Therefore, it is proposed that this is also applicable to EBNco-P. Through this eco-friendly enzymatic biotechnology, it breaks down the insoluble SiaMuc-glycoprotein in EBNco-P into soluble bioactive SiaMuc-glycopeptide. By using filtration, the separation of valuable nutrients from the inedible impurities can be easily achieved.

In addition to the advantages of applying enzymatic biotechnology, the bioactive SiaMuc-glycopeptide hydrolysate from the EBN co-product is also a lower-cost alternative for all consumers with a higher grade of nutritional functionality. In other words, this research has led to not only a wider industry utilization, it also helps in the development of EBN in terms of nutraceutical functionality yet with a lower cost.

MATERIALS AND METHODS
Materials
Edible bird's nest (EBN) co-product was provided by Mobile Harvester Sdn. Bhd, Selangor, Malaysia. The sample was ground and stored in an air-tight container until further analysis.

Sample preparation
The sample underwent a double-boiling process followed by enzymatic hydrolysis as described elsewhere (Hui Yan *et al*., 2022).

Double-boiling process
The sample (1% wt/vol) was weighed and added to a conical flask. Distilled water (100 mL) was then added to the flask and well-mixed with the sample. In a shaking water bath with boiling temperature, the sample underwent heating for 30 min. The heating process was timed as the temperature of the sample mixture achieved 90 °C and above. The double-boiling process was performed with continuous shaking and stirring. The sample was then cooled down before further process for enzymatic hydrolysis.

Enzymatic hydrolysis and filtration of sample
Hydrolysis was performed in a shaking water bath of 60 °C. Protease was added to the double-boiled sample once the required temperature was achieved. The pH of the sample was maintained at pH 8.0 by using 1.0 M sodium hydroxide. The hydrolysis was carried out for 90 min as described in the previous study. The sample was then brought to a boil for 5 min to deactivate the enzymatic activity.

The sample then underwent a filtration process to remove inedible materials using a cloth strainer, followed by filter paper (Whatman No.1) using a vacuum filter system. The filtrate was then freeze-dried for further analysis.

Recovery yield
Sample filtrate was weighed after freeze-drying, as well as the inedible materials after oven-drying (Noor *et al*., 2018). The recovery yield of the EBN co-product was determined using:

– Equation 1

$$Recovery\ yield\ (\%) = \frac{wt_F}{wt_S} \times 100$$

Whereby,
Wt$_F$ is the weight of the freeze-fried sample after double-boiling and hydrolysis.
Wt$_S$ is the total weight of the input sample before double-boiling and hydrolysis.

Proximate analyses

Proximate analyses were performed according to the method of the Association of Official Analytical Chemistry (AOAC) as described in the report of Latimer (2019). The protein content of the sample was determined using the Kjeldahl method. Sample (0.5 g) was added with catalyst (5.0 g) and concentrated sulphuric acid (12 mL), then heated for digestion. The blue-green solution was then titrated with sodium hydroxide. Distillation was performed with the addition of boric acid. Titration was then carried out by using hydrochloric acid (0.1 M). The % nitrogen was calculated to obtain protein content with a conversion factor of 6.25. The determination of fat content was carried out using the Soxhlet method. The sample (1.0 g) was wrapped in filter paper and placed into a cellulose thimble for the extraction of fat using hexane for 2 h. Ash or mineral content was determined by the dry-ashing method through overnight combustion in a furnace. The differences between the initial and final weight of the sample were used to calculate the mineral content. Moisture content was determined by the oven drying method. The sample was heated at 50-60 °C until no changes in weight were observed. The carbohydrate content was determined using the subtraction method. Which, a total of 100% was used to subtract the % protein content, fat content, mineral content, and moisture content.

Biochemical analyses

Biochemical analyses were carried out according to the previous study (Ling *et al*., 2020).

Soluble protein content

Soluble protein content was determined using the Bradford method. The sample (1.0 mg/mL) was mixed with Coomassie Brilliant Blue Dye G-250, and the absorbance was read at a wavelength of 595 nm. Bovine serum albumin (BSA) was used for standard calibration.

Peptide content

Determination of peptide content was performed using o-phthaldialdehyde (OPA) spectrophotometric assay. Generally, the OPA reagent (300 µL) was mixed with the sample (50 µL) for a 2-min incubation and then read under a spectrophotometer at 340 nm. Leucine was used as standard. The OPA reagent was prepared from a mixture of 25 mL of 100 mM sodium tetra-hydroborate, 20% (w/w) sodium dodecyl sulfate, 40 mg of OPA solution dissolved in 1 mL of methanol, and 100 µL of b-mercaptoethanol, then topped up with distilled water (50 mL).

Polysaccharide content

Phenol-sulphuric acid (PHA) assay was performed to quantify the polysaccharide content of the sample. The sample (1 mL, 1.0 mg/mL) was well-mixed with 5% phenol (0.5 mL) and then added with concentrated sulphuric acid (1.5 mL). The absorbance of the sample was read with a spectrophotometer at 490 nm and glucose was used as standard.

Reducing sugar content

A dinitro salicylic acid (DNS) assay was used to determine the reducing sugar content. The sample was well-mixed with DNS reagent at a ratio of 1:4, heated under a boiling water bath for 10 min then cooled down immediately. Absorbance was read using a spectrophotometer at 540 nm and glucose was used as standard.

Sialic acid content

The Resorcinol method was used to assess the sialic acid content of the sample. The sample was added with resorcinol reagent at a ratio of 1:1, covered with chilled marble, and heated in a boiling water bath for 15 min. An amount of 4 folds of 1-butanol was added, then vigorously mixed and chilled in an ice-water bath for 10 min. Absorbance was read at a wavelength of 580 nm using a spectrophotometer.

Glycoprotein content

Periodic-acid Schiff assay was used to assess the glycoprotein content. Briefly, the sample (2 mL) was incubated with a periodic acid solution (0.2 mL) for 2 h at 37 °C, then further incubated with Schiff fuchsin sulfite solution for 30 min at room temperature. The sample's absorbance was read at a wavelength of 555 nm and horseradish peroxidase was used as standard.

Anti-oxidative activities

Antioxidative activities were determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6sulfonic acid) (ABTS) radical scavenging activity, and ferric reducing

antioxidant power (FRAP) assays as described in previous study (Noor *et al.*, 2022). Ascorbic acid was used as standard in these analyses.

DPPH scavenging activity
      The sample was incubated with DPPH reagent at a ratio of 1:2 for 30 min in a dark environment. The absorbance of the sample was read at 517 nm and the DPPH scavenging activity was calculated using equation:

$$DPPH\ (\%) = \frac{Abs_R - Abs_S}{Abs_R} \times 100$$

— Equation 2

Whereby,
Abs_R is the absorbance of the reagent; Abs_S is the absorbance of the sample.

ABTS radical scavenging activity
      The sample (50 µL) was incubated with ABTS reagent (950 µL) in the dark for 10 min. The sample's absorbance was read at 734 nm., ABTS was prepared with 7 mM ABTS and 2.45 mM of potassium persulfate left in the dark overnight before usage. The ABTS was diluted to an absorbance of 0.70±0.20.

Ferric reducing antioxidant power (FRAP)
      The sample (250 µL) was incubated with FRAP reagent (1.25 mL) in the dark for 30 min, then read under a spectrophotometer at 595 nm. FRAP reagent was prepared with 300 mM sodium acetate buffer, 10 mM of 2,4,6-tris (2-pyridyl)-s-triazine (TPTZ), and 20 mM Ferum Chloride hexahydrate at a ratio of 10:1:1. The FRAP activity was calculated using Equation 3.

$$FRAP\ (\mu gAAE/mg) = \frac{Abs_S}{Abs_{AA}} \times AA \times DF$$

— Equation 3

Whereby,
Abs_S is the absorbance of the sample; Abs_{AA} is the absorbance of ascorbic acid; AA is by concentration of ascorbic acid; DF is the dilution factor of the sample.

Statistical analysis
      All analyses were conducted in triplicates and one-way analysis of variance (ANOVA) was used to determine the statistical differences of all means at a significance of $p \leq 0.05$.

RESULTS AND DISCUSSION
Proximate content of EBN co-product
      Figure 1 shows the proximate result of EBN co-product. Whereby, the protein content has the largest portion, followed by the carbohydrate content, moisture content, and mineral content. Unexpectedly, the nutritional content of EBN co-product is similar to the proximate results of cleaned-EBN reported by many studies (Noor *et al.*, 2018; Wong *et al.*, 2018; Lee *et al.*, 2021; Hui Yan *et al.*, 2022). Whereby, the cleaned EBN was reported with approximately 80-90% glycoprotein made up of 60% of protein and 20-25% of carbohydrate (Hui Yan *et al.*, 2021). In this study, the EBN co-product consists of approximately 62.34% of protein, 19.13% of carbohydrate, 2.51% of mineral content, and 0.80% of fat. Since the study of EBN co-product is a new direction for the edible bird's nest industry, information on this eco-sustainable research is scarce, and only a few were reported. For example, Noor *et al.* (2022) have reported a nutritional content of EBN co-product with protein content (54.29–56.57%), carbohydrate (18.98-20.77%), mineral (2.31–10.70%), and fat (0.43–0.80%). Surprisingly, the fat content of the EBN co-product was revealed to be higher when compared to the cleaned EBN. This may be related to the presence of a bird's feathers that is attached to the EBN co-product. Whereby, the bird feathers contain residual grease (Tesfaye *et al.*, 2017).
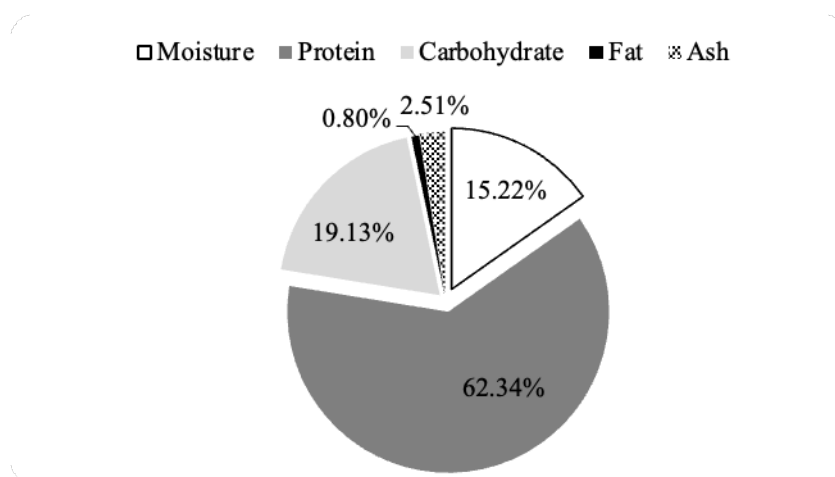
Fig. 1. The proximate analyses of nutritional content for EBN co-product (*n*=3). Note: EBN is meant by an edible bird's nest.

Recovery yield

Figure 2 discloses the recovery yield of bioactive glycopeptide from EBN co-product after enzymatic hydrolysis. It is shown that the EBN co-product has a recovery yield of 61.0±1.0%. Whereby, the inedible materials such as dust, sand, and tiny feathers that are successfully separated from the bioactive EBN glycopeptide is approximately 39.0±1.0%. As shown in Figure 3, the filtrate of EBN hydrolysate recovered from the co-product was freeze-dried for further analysis and storage. Also, Figure 3 (c) displays the inedible materials that are successfully separated from the bioactive EBN SiaMuc-glycopeptide.
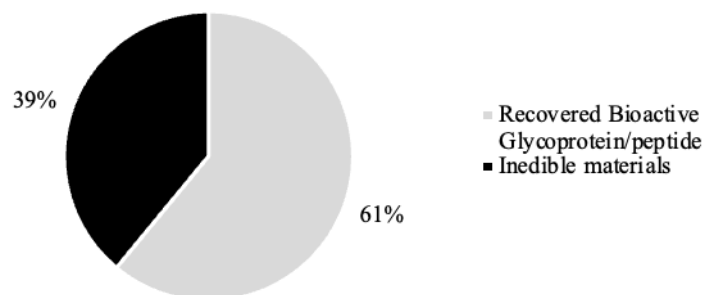

Fig. 2. Recovery yield of bioactive SiaMuc-glycopeptide from EBN co-product. Note: EBN is meant by an edible bird's nest.
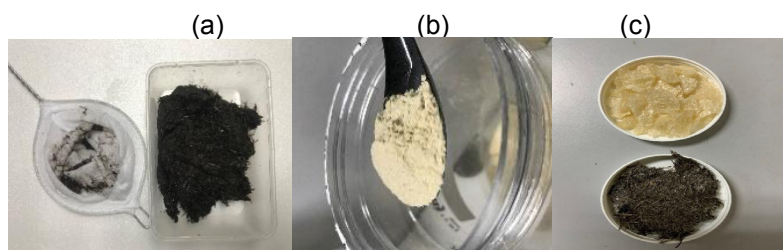

Fig. 3. (a) Raw EBN co-product before enzymatic hydrolysis. (b) Freeze-dried filtrate of the recovered EBN from co-product in the form of bioactive SiaMuc-glycopeptide. (c) Inedible materials separated from the filtration of EBN co-product hydrolysate. Note: EBN is meant by an edible bird's nest.

From our preliminary study previously, the EBN by-product has a high recovery yield of 89-99% with the use of enzymatic hydrolysis (Noor *et al.*, 2022). This finding indicates that the current study has a lower recovery yield in comparison. It is proposed that this is related to the different production batches which may lead to the yield of different recovery rates. As the cleaning process of EBN is done

by man, the conformation of the co-product may be different depending on the skill of the workers during the removal of inedible materials from the EBN (Tan *et al.*, 2018; Tan *et al.*, 2022). In other words, better-skilled workers can remove the inedible materials from EBN more efficiently with less wastage of valuable glycoprotein and vice versa. Also, the origin of the EBN might be another factor affecting the recovery yield of EBN from its co-product. Whereby, EBN from different sources may affect the cleanliness of the harvested EBN. This includes the location, habitat, environmental factors, and even the species of swiftlets itself (Babji *et al.*, 2015; Tan *et al.*, 2018). For instance, a bird's nest from a habitat with a better and/or cleaner environment has less involvement of dirt and other inedible materials. Also, the quality of the harvested bird's nest from different swiftlets affects the recovery yield of the co-product. Whereby, a bird's nest made by white-nest swiftlets (*Aerodramus fuciphagus*) is easier to clean, which produces a lower recovery yield in its co-product. Meanwhile, bird's nest from black-nest swiftlets (*Aerodramus maximus*) has a higher involvement of bird's feathers, grass and weeds, and other inedible materials. Thus, the cleaning process of these black bird's nests may yield a higher recovery of bioactive SiaMuc-glycopeptide of the co-product when enzymatic hydrolysis is applied. In short, the recovery yield of bioactive SiaMuc-glycopeptide from co-product may vary depending on the production skill and many other external factors.

Biochemical content of EBN co-product

Figure 4 shows the biochemical content of the bioactive SiaMuc-glycopeptide recovered from the EBN co-product. The result showed a 72.85±6.44% glycoprotein content, peptide content of 64.40±8.84 %, soluble protein content of 2.31±0.74%, polysaccharide content of 26.12±3.69%, reducing sugar content of 16.76±1.45%, and sialic acid content of 36.23±0.36%. This indicates that bioactive glycopeptide recovered from EBN co-product consists of a high glycoprotein content. It also contains peptide content, polysaccharide content, and sialic acid content. In addition, the bioactive SiaMuc-glycopeptide from the EBN co-product showed the least content of soluble protein. This result is in line with the proximate nutritional content of raw EBN co-product reported in Figure 1.
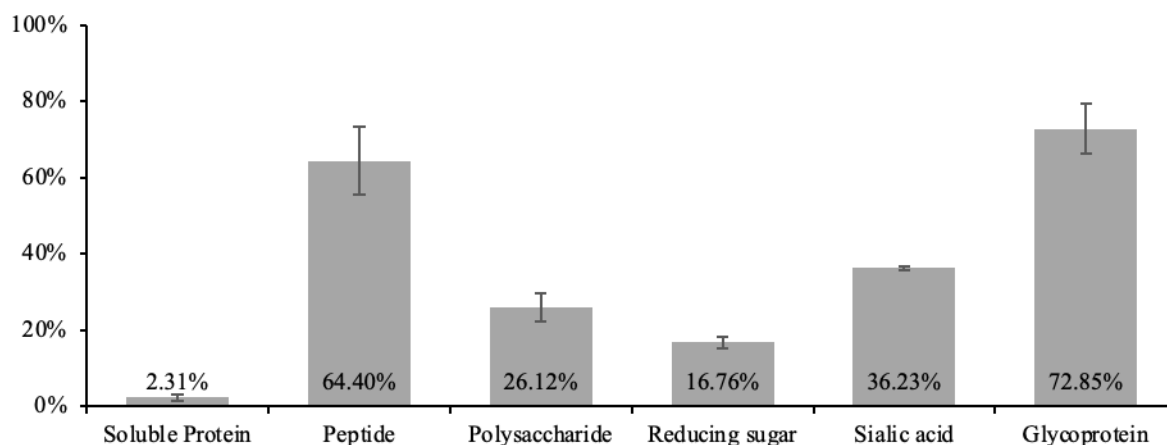
Fig. 4. Biochemical content of soluble protein, peptide content, polysaccharide content, reducing sugar content, sialic acid, and glycoprotein content in EBN co-product. Note: Data is presented in the form mean of *n*=3 and the error bar indicates the standard deviation at a confidence level of 95%.

The application of enzymatic hydrolysis on the EBN co-product breaks down the entrapped EBN glycoprotein into the form of bioactive SiaMuc-glycopeptide and free peptides. Similar studies on cleaned EBN have revealed that enzymatic hydrolysis reduces the molecular weight of EBN and solubilizes the macro-glycoprotein (Nurul Nadia *et al.*, 2017; Ling *et al.*, 2020; Ng *et al.*, 2020). In other words, enzymatic hydrolysis on EBN does not alter the existing nutritional content of the EBN but enhances its bioavailability and bioaccessibility with a simpler structure. This explains the findings in Figure 4, whereby the biochemical content of bioactive hydrolysate from EBN co-product is similar to the proximate nutritional content of its raw form.

In detail, Figure 4 revealed that EBNco-P has a major component of glycoprotein. This is also reported in the study of cleaned-EBN (Daud *et al.*, 2019). It is stated that EBN is made up of 80-90% of glycoprotein. This EBN glycoprotein is composed of 52-60% of protein and/or peptide, and carbohydrate (approximately 20-25%). In this study, EBN co-product is obtained from the cleaning process of EBN to remove inedible materials such as bird feathers. Whereby, these inedible materials contain an abundant amount of EBN tightly attached to the actual waste. As the EBN hydrolysate in this study is recovered from the co-product which is collected from the cleaning process of harvested swiftlet's nest, therefore the content of the recovered EBN is supposed to have a similar nutritional and biochemical content. Thus, this further explains the results in Figure 4. In which, bioactive hydrolysate from the EBN co-

product contains glycoprotein (72.85%) as a major component. From this glycoprotein, the protein and peptide stand for approximately 66% (2.31% & 64.40%), while the glycan-carbohydrate part stands for 26–36%.

Similarly, a previous study on the bioactive EBN hydrolysate produced through enzymatic hydrolysis of cleaned-EBN also reported a biochemical content of glycoprotein 0.98 mg/mL, soluble protein of 0.032 mg/mL, and peptide content of 0.83 mg/ml (Hui Yan *et al.*, 2022). Similarly, the finding in Figure 4 showed a high amount of peptide and a low amount of soluble protein. This then further validifies that enzymatic hydrolysis on EBN co-product hydrolyzed the insoluble EBN glycoprotein into soluble bioactive glycopeptides and free peptides.

At the same time, it is also reported that protease hydrolysis of EBN glycoprotein only influences the protein and peptide content, while the glycan-carbohydrate of EBN remains the same (Hui Yan *et al.*, 2022). This is in line with the current study on EBN hydrolysate recovered from the co-product. In detail, the polysaccharide content was around 26% and the reducing sugar was around 16%. However, it is also shown in Figure 4 that the sialic acid content of EBN hydrolysate from the co-product was approximately 36%. Sialic acid or N-acetylneuraminic acid is the primary carbohydrate component in EBN (Halimi *et al.*, 2014). Which, sialic acid stands for a total of 9% of the total glycan-carbohydrate of EBN. Therefore, the value of 36% of the sialic acid of EBN hydrolysate from the co-product is part of the polysaccharide content. Yet, the value of polysaccharide was lower than the sialic acid content. This is due to the inaccessibility of the PHS assay as the carbohydrate part of the EBN-glycan appeared mostly in the form of oligosaccharides.

In summary, enzymatic hydrolysis in recovering the bioactive EBN hydrolysate from the co-product does not negatively affect the biochemical content of the EBN. A similar study was performed by Noor *et al.* (2018), Ling *et al.* (2020), and Ng *et al.* (2020) in which it is suggested that this is an alternative to reduce wastage, at the same time enhancing nutritional functionality and application of EBN in industry.

Anti-oxidative activities of EBN co-product

Figure 4 shows the anti-oxidative activities of the EBN co-product. The EBNco-P disclosed a DPPH scavenging activity of 34.48%, ABTS value of 52.3%, and FRAP activity of 16.1 ug AAE/mg respectively.
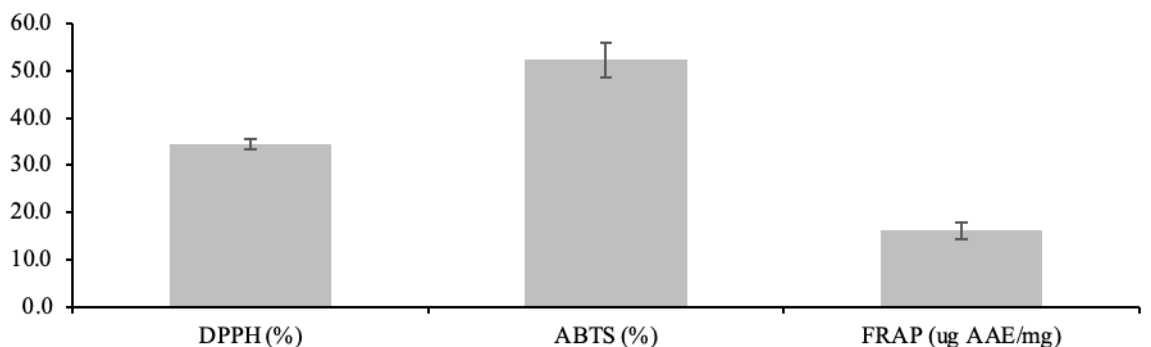
Fig. 5. Anti-oxidative activities of DPPH scavenging activities, ABTS radical scavenging activity, and Ferric reducing antioxidant power (FRAP) of EBN co-product. Note: EBN is meant by edible bird's nest

Our previous study on the antioxidative activities of cleaned EBN reported a DPPH scavenging activity of 25.32% and 28.35-30.93% for the raw EBN and hydrolysate respectively. The study also reported that raw cleaned-EBN has an ABTS radical scavenging activity of 32.66%; and 49-64% of ABTS activity for cleaned-EBN hydrolysate. The FRAP value of raw cleaned-EBN was disclosed at 1.95%; and cleaned-EBN hydrolysate with FRAP value of 6.34-9.73 µgAAE/mg (Hui Yan *et al.*, 2022). In other words, this finding concluded that the anti-oxidative activities of EBN co-product are higher than those in cleaned-EBN. This is in line with the study of Noor *et al.* (2022), in which lower-grade EBN (i.e., EBN co-product) has a higher antioxidative activity. The EBN hydrolysate grades B, C, and D in this study showed DPPH scavenging activities of 21-43% when compared to the cleaned-EBN hydrolysate with a DPPH value of 25-26%. The ABTS value of the low-grade EBN hydrolysate was reported to be 58-90%, while the cleaned-EBN hydrolysate has an ABTS value of 62-79%. Meanwhile, the FRAP value of low-grade EBN hydrolysate was disclosed at a range of 0.47 to 0.53 µmoL/L AAEAC; the cleaned-EBN hydrolysate was disclosed at approximately 0.50 µmoL/L AAEAC. A similar conclusion was also reported in the study by Gan *et al.*, (2020).

The DPPH scavenging activity measures the primary antioxidant activity of the study subject, while the FRAP value evaluates both primary and secondary antioxidative activities (Ling *et al.*, 2020).

It is shown in Figure 4 that the EBN co-product has a higher antioxidative value on ABTS, followed by DPPH, while the FRAP activity of the EBN co-product has the lowest value. This may be related to the shortcomings of the DPPH assay, in which proteins are usually precipitated in the alcoholic reaction medium (Shah & Modi, 2015). In which, EBN co-product has a major nutrient content of protein as reported earlier in Figure 1. Thus, the DPPH value measure is less suitable which then gives a lower value. Whereas, the ABTS assay enables the evaluation of antioxidative activities of both hydrophilic and lipophilic compounds due to the ABTS radical itself being soluble in water and organic solvents. As the bioactive SiaMuc-glycopeptide of the EBN co-product is soluble in water, thus contributes to the high value of ABTS antioxidative activity. Meanwhile, the low FRAP value of the EBN co-product may be related to the FRAP limitation of the redox potential of Fe^{3+} and Fe^{2+}. FRAP value may be low due to the falsely low Fe^{3+} reduction (Wojtunik-Kulesza, 2020).

However, in short, the overall antioxidative activities of EBN co-product were observed to be higher when compared to the cleaned EBN reported in many other studies. This made EBN co-product an alternative food source for antioxidants suitable with a lower cost for all levels of consumers.

CONCLUSION

The study finds that the bioactive sialylated-mucin (SiaMuc) glycopeptide from edible bird's nest (EBN) co-product has physicochemical and nutritional properties similar to and even better than those in cleaned-EBN. Moreover, the eco-sustainable production method of EBN from co-products is a low-cost alternative for all consumers. As reported earlier, EBN co-products have a lower cost of just a fraction (approximately USD 200 per kilogram) of the cleaned EBN with a high price of USD 5000 per kilogram.

ETHICAL STATEMENT
Not applicable.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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