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Antioxidant potencies in enzymatic hydrolysates from cup and corner sections of edible bird's nest

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Abstract

Edible Bird's Nest (EBN) has many health-promoting properties, including antioxidants. Although the antioxidant properties of the EBN hydrolysates have been reported in the previous literature, little study has been done on the antioxidant potencies of the enzymatic hydrolysates within different sections of EBN. Thus, this study investigated the antioxidant potencies of the enzymatic hydrolysates from both the cup and corner sections of EBN. Therefore, using different enzymatic hydrolysis durations (1, 2, and 3 hr), this study examined the antioxidant potencies of enzymatic hydrolysates from cup and corner sections of EBN. The antioxidant potencies of alcalase-treated EBN hydrolysates were evaluated using *in-vitro* chemical assays (DPPH, FRAP, and ABTS). The hydrolysates from the corner section of EBN consistently exhibited significantly higher (p<0.05) DPPH, FRAP, and ABTS values compared to the hydrolysates from the cup section of EBN at all hydrolysis times, except FRAP assay at the 2-hr hydrolysis time. Meanwhile, the antioxidant values obtained from this study suggest that, despite the variations in hydrolysis time, certain data points exhibited comparable antioxidant potencies between cup and corner samples in FRAP and ABTS assays. It is also noteworthy that the ABTS assay demonstrated higher sensitivity in detecting antioxidant activity in the hydrolysates of EBN compared to DPPH and FRAP. In conclusion, the hydrolysates from the corner section of EBN showed stronger antioxidant potencies when treated with alcalase compared to the cup hydrolysates. These results open way for further investigation into the factors influencing the antioxidant activity of the hydrolysates from the cup and corner sections of EBN.

1. Introduction

Edible bird's nest (EBN) also known as "Caviar of the East", is a dried glutinous secretion from the salivary glands of certain species of swiftlets (genus *Aerodramus*) such as *Apodidae, Collocalia, Aerodramus Maximus* (Black-nest swiftlet), and *Aerodramus Fuciphagus* (White-nest swiftlet) (Marcone, 2005; Benjakul and Chantakun, 2022). These small birds are notable for their ability to build up the EBN triennially (three times a year) in deep and dark caves or cave-like environments (bird house) which mainly can be found throughout Southeast Asia and the South Pacific (Thorburn, 2015; Looi and Omar, 2016). Malaysia is the world's third-largest producer of EBN, accounting for around 9% of the global supply in 2006, behind Indonesia (60%) and Thailand (20%) (Looi and Omar, 2016). EBN is well-known worldwide due to its richness of nutrients.

Several studies have found that EBN is abundant in nutrients such as carbohydrates, high-valued glycoproteins as well as calcium, sodium, amino acids, and potassium that promote beneficial effects for the human body (Gawade, 2021). Research has proven that EBN's glycoprotein exhibits antioxidant properties (Fan *et al.*, 2022; Loh *et al.*, 2022). The antioxidant qualities of EBN are ascribed to the presence of multiple bioactive constituents, such as sialic acid, fatty acids, minerals, vitamins, lactoferrin, and glucosamine (Loh *et al.*, 2022). The presence of these bioactive substances in the mucin of EBN, a type of extensively glycosylated big glycoprotein found in saliva, can be hydrolysed to yield

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glycoprotein hydrolysates (Yuan *et al.*, 2023). The hydrolysis produced small bioactive peptides, which improved the biological function and activity of the EBN. The functional properties exhibited by EBN hydrolysates can be altered and influenced by the degree of hydrolysis, consequently determining the antioxidant potencies of EBN hydrolysates (Gan *et al.*, 2020). Commonly, the antioxidant potencies of EBN hydrolysates have been determined by using *in-vitro* chemical techniques such as DPPH, FRAP, and ABTS assays (Chantakun and Benjakul, 2022; Lee *et al.*, 2023).

According to previous studies, most of the EBN antioxidant-related research conducted used the whole EBN (Ramachandran *et al.*, 2017; Quek *et al.*, 2018). Nevertheless, very little investigation focuses specifically on the antioxidant activities of different sections of EBN. The corner section of EBN requires a longer hydrolysis time to hydrolyse the structure. Hence, in this study, the antioxidant potencies of hydrolysates from different sections of EBN were investigated through *in-vitro* chemical assays (DPPH, FRAP, and ABTS).

2. Materials and methods

2.1 Preparation of edible bird's nest sample

Raw-uncleaned EBN samples (*n*=6) (Figure 1) were cleaned by an EBN-producing company (Nanyang Dreams International Trading Sdn. Bhd.). The raw-cleaned EBN samples were separated into two different sections (cup and corner).

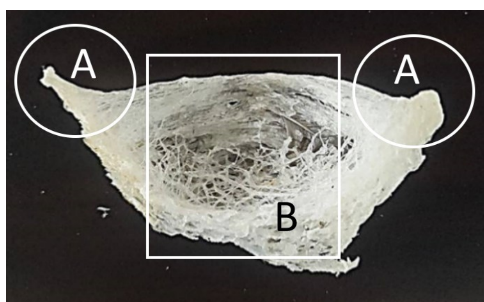

Figure 1. A) Corner section, and B) Cup section of raw-uncleaned EBN.

The composite samples of both corner and cup sections were individually processed into coarse granules using a grinder (Nima®, Japan) and subsequently sieved for 5 min utilising a sieve shaker (Endercott's Minor 200 -2647, England) with standard vibration mode. The samples were selected at aperture size <355 µm. The coarsely grounded EBN samples were kept in airtight containers and labelled and stored at room temperature in dark conditions for further usage.

2.2 Preparation of edible bird's nest protein hydrolysate solutions

The EBN coarse granules (corner and cup) were soaked in distilled water at a ratio of 1:20 at a chilled temperature overnight respectively to soften the cement. Then, the mixture was boiled for 45 mins in a 100°C water bath (LabTech LSB-015S, Daihan LabTech Co., Ltd., Korea) and cooled down to 65°C. The mixture was homogenised at 10,000 rpm using Ultrasonic homogeniser (WiseTis® HG-15A, Daihan Scientific Co., Ltd., Korea). After homogenising the mixture, the sample was hydrolysed using Alcalase in the optimum pH condition suggested by the manufacturer (pH 8.6 – 8.71) at 55°C in the water bath (LabTech, Daihan LabTech Co., Ltd., Korea). The enzyme (0.5% (v/v)) was added to the homogenised mixture, and the hydrolysis was carried out for 1, 2, and 3 hr. The resulting hydrolysates were heated at a temperature of above 90°C with boiling water for 15 mins to inactivate the enzymes. Then, the hydrolysates were centrifuged at 3,800 rpm for 30 mins using a centrifugal machine (Kubota 2420, Japan). The supernatant was separated and transferred into a 15 mL centrifuge tube and then wrapped using aluminium foil prior to storage in a freezer (3°C) (Nadia *et al.*, 2017)

2.3 DPPH assay

Radical scavenging activity was conducted based on the method developed by Ahmad *et al.* (2005) with slight modifications. Accurately, 200 µL of tested sample solution was added to 4.0 mL of 50 μM DPPH solution in buffered methanol. This buffered methanol was prepared by mixing the stock solution of 60 mL methanol and 40 mL (0.1M, pH 5.5) acetic acid buffer, respectively (Sharma and Bhat, 2009). Then, the mixture of sample and DPPH was vortexed and allowed to react in the dark conditions for 30 mins at room temperature. The samples were prepared in triplicate and the absorbance reading at wavelength, λmax = 517 nm was measured spectrophotometrically (PG Instruments T80+ UV-Vis's spectrophotometer). Ascorbic acid (Sigma-Aldrich, Germany) was used as a standard in comparison with the sample hydrolysates. A series of standards ranging from 0.00 – 0.57 mM standard calibration curves were established. The difference in absorbance between a test sample and the control (DPPH) is expressed as % radical scavenging activity (Ravisangkar *et al.*, 2014).

$$\%\ DPPH\ Scavenging\ Assay = \left[1 - \left(\frac{Absorbance\ sample}{Absorbance\ control}\right)\right] \times 100$$

2.4 FRAP assay

The antioxidant potencies of the extract based on ferric reducing power have been determined according to

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the method described by Siti Azima *et al.* (2014) with some modifications. The stock solutions included 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ solution. The working solution was freshly prepared by mixing the acetate buffer, TPTZ solution, and $FeCl_3 \cdot 6H_2O$ solution in a 10:1:1 ratio and then was incubated at 37°C for 10 mins prior to analysis. Samples (0.1 mL) were allowed to react with 2.9 mL of the FRAP solution for 30 mins in the dark condition. The readings were measured spectrophotometrically at λmax = 593 nm. The linear standard calibration curve ranging from 0 – 1.0 mM Trolox was established. The final results were expressed in μM TEAC/g of fresh extract weight sample (Siti Azima *et al.*, 2014).

2.5 ABTS$^+$ assay

The assay is a modified method of Ali *et al.* (2019). The stock solutions (7 mM ABTS solution and 2.45 mM potassium persulfate ($K_2S_2O_8$)) were prepared. The working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react in the incubator for 12-16 hr at room temperature in the dark. The resulting solution was diluted with distilled water to obtain an absorbance of 0.706±0.001 units at 734 nm using the UV-vis spectrophotometer. The sample (1 mL) was added to 3.5 mL of the diluted ABTS solution. The mixture was shaken vigorously for 30 sec and left in the dark for 10 min at room temperature. The absorbance of the resultant solution was measured at 734 nm using the spectrophotometer. A standard curve was prepared by reacting 1 mL of Trolox (0 – 2.0 mM) with 3.5 mL of diluted ABTS$^+$ solution. The degree of ABTS radical-scavenging activity of the hydrolysates was expressed as % radical scavenging activity (Najafian *et al.*, 2013; Ali *et al.*, 2019).

$$\%\,ABTS\ Radical\ Scavenging\ Activity = \left[1 - \left(\frac{Absorbance\ sample}{Absorbance\ control}\right)\right] \times 100$$

2.6 Statistical analysis

All experiments were performed in triplicate. Statistical analyses were performed using Statistical Analysis System 3.8, SAS® Studio software. The Analysis of Variance (ANOVA) procedure, specifically Duncan's Multiple Range Test at 5% significance level was used to compare significant differences among the mean of the antioxidant values within the hydrolysis time for each sample. A T-test of Least Significant Difference (LSD) was calculated to compare differences between antioxidant values between the hydrolysates from different sections of EBN (cup and corner). Values were expressed as means ± standard deviation (SD).

3. Results

3.1 DPPH radical scavenging activities of the hydrolysates of edible bird's nest

Figure 2 shows the percentage of DPPH radical scavenging activity for the hydrolysates from both cup and corner sections of EBN. All the tested samples exhibited moderate scavenging activity against DPPH. The range values for hydrolysates at three different hydrolysis time is between 28.15 – 32.67% (cup), and 46.64 – 58.59% (corner). The highest DPPH values were recorded for bother sections at 1 hr of hydrolysis time, and these differences were found to be statistically significant (p<0.05).

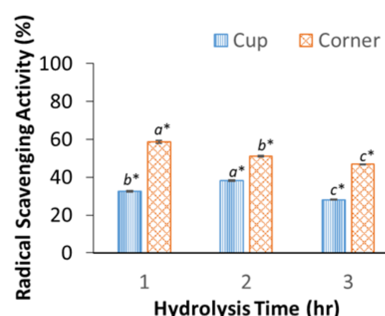

Figure 2. The percentages of DPPH radical scavenging activities for the hydrolysates from the cup and corner sections of EBN at various hydrolysis times (1, 2, and 3-hr). Data presented are mean± SD of triplicate samples. Bars with different alphabets are statistically significantly different (p<0.05) between the hydrolysates of both cup and corner EBN at various hydrolysis times (1, 2, and 3-hr). The asterisk (*) denotes a statistically significant difference between the hydrolysates of both cup and corner EBN within the same hydrolysis time, with a significance level set at p<0.05.

Nonetheless, the DPPH values for both hydrolysates decreased over the hydrolysis time. The lowest DPPH values for corner and cup EBN hydrolysate were observed at 3-hr hydrolysis time, which was 46.64% and 28.15%, respectively, with a significant difference (p<0.05). Despite this decline, the antioxidant potencies of both cup and corner EBN hydrolysates were comparatively low when compared to the standard of ascorbic acid.

3.2 FRAP assay reducing power of hydrolysates of edible bird's nest

Figure 3 presents all EBN hydrolysates with different hydrolysis times that had a certain degree of electron donation potency. The range of antioxidant values of cup samples at three different hydrolysis times is between 25.61 - 37.29 μM TEAC/g while 39.35 – 48.61 μM TEAC/g for corner hydrolysate samples. The corner EBN hydrolysate after 1 hr hydrolysis showed

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significantly higher ($p<0.05$) reducing power (48.61 μM TEAC/g) compared to cup EBN hydrolysate (37.29 μM TEAC/g).

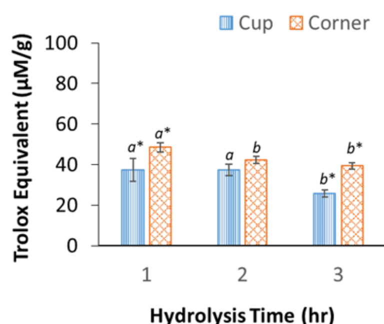

Figure 3. Reducing power of cup and corner EBN within 1, 2, and 3 hr of hydrolysis time. Data presented are mean± SD of triplicate samples. Bars with different alphabets are statistically significantly different ($p<0.05$) between the hydrolysates of both cup and corner EBN at various hydrolysis times (1, 2, and 3-hr). The asterisk (*) denotes a statistically significant difference between the hydrolysates of both cup and corner EBN within the same hydrolysis time, with a significance level set at $p<0.05$.

However, the FRAP values for both hydrolysates have the same pattern as DPPH analysis which was decreased over the hydrolysis time. The lowest FRAP value for corner and cup EBN hydrolysate was at 3-hr hydrolysis time, which was 25.61 μM TEAC/g and 39.35 μM TEAC/g, respectively with significant differences ($p<0.05$).

3.3 ABTS scavenging activities of hydrolysates of edible bird's nest

According to Figure 4, all of the tested hydrolysate samples exhibited relatively high scavenging activity against ABTS. At three different hydrolysis times, the range ABTS value of cup EBN hydrolysate is between 98.70 – 99.63% whereas for the corner samples is 99.24 – 99.81%. Corner EBN hydrolysate demonstrated the highest ABTS value (99.81%) compared to the value for cup EBN hydrolysate (99.63%) with significant differences ($p<0.05$). Both are recorded at 3-h of hydrolysis time. At 1-hr hydrolysis time, both samples still exhibited relatively high scavenging activity which is 99.24% for corner EBN hydrolysate and 98.70% for cup EBN hydrolysate with significant differences ($p<0.05$). Then, the antioxidant potencies of the cup and corner EBN hydrolysates were in the range of a standard of ascorbic acid.

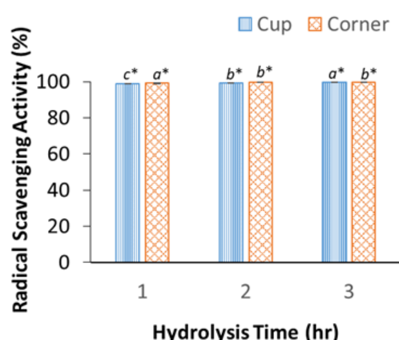

Figure 4. Percentage of ABTS radical scavenging activity of cup and corner EBN within 1, 2, and 3 hr of hydrolysis time. Data presented are mean± SD of triplicate samples. Bars with different alphabets are statistically significantly different ($p<0.05$) between the hydrolysates of both cup and corner EBN at various hydrolysis times (1, 2, and 3-hr). The asterisk (*) denotes a statistically significant difference between the hydrolysates of both cup and corner EBN within the same hydrolysis time, with a significance level set at $p<0.05$.

4. Discussion

Based on the results, the ABTS assay demonstrated the highest percentage of scavenging activity compared to DPPH and FRAP assays. Nonetheless, this finding cannot be compared to that of the DPPH and FRAP assays as different mechanisms were used in each assay (Gan *et al.*, 2020).

The ABTS assay is a great method for measuring the antioxidant activity of hydrogen donor compounds (scavengers of aqueous phase radicals) as well as of chain-breaking antioxidants (scavengers of lipid peroxyl radicals) (Loganayaki *et al.*, 2013). The ABTS radical is relatively stable and is easily reduced by antioxidants. Due to the high free radical captured by the activity of ABTS, antioxidant compounds present in the hydrolysates are probably assumed to be hydrophilic (Klompong *et al.*, 2007; Binsan *et al.*, 2008). Generally, free radicals might interact with hydrolysates of EBN that contain peptides or proteins, which were hydrogen donors, changing them into more stable products, thereby breaking the radical chain reaction (Khantaphant and Benjakul, 2008). On top of that, the hydrogel properties of EBN arise from the presence of hydrophilic polysaccharides in the glycoprotein polymer, which intensifies the interaction with ABTS radicals (Shim *et al.*, 2016).

In comparison with the ABTS assays, the DPPH assay is another method commonly used for determining antioxidant activity. DPPH assay, however, is more suitable for plant extracts. Even so, by minor modification of the chemical utilised in the assay, the antioxidant findings for the animal protein hydrolysate can still be produced. Since protein is a polar substance and is very stable in non-polar solvents, buffered methanol is employed in the assessment of the antioxidant activity of the DPPH assay (Sharma and Bhat, 2009; Lee *et al.*, 2020). In this assay, a molecule or antioxidant with weak hydrogen bonding will interact with a stable free radical DPPH, hence the radical is

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scavenged, which is visualised by turning its colour from purple to yellow, and the absorbance is reduced at maximum absorbance, 517 nm (Nagarajan *et al.*, 2017).

Besides that, the FRAP assay also demonstrated a positive value from the analysis of antioxidant potencies of the EBN hydrolysates. This assay evaluates the reducing ability of the antioxidant to react with the ferric tripyridyl triazine (Fe^{3+} - TPTZ) complex. When the antioxidant donates a hydrogen atom to a ferric complex, it breaks the radical chain reaction and blue-colour ferrous formed at maximum wavelength, λ_{max}=593 nm (Guo *et al.*, 2003).

The antioxidant value of EBN is known to be associated with the biopeptide, amino acid, and sialic acid content in the EBN compound. The bioactive peptides act as the natural antioxidants in the EBN and amino acids function to enhance antioxidative activities (Tan *et al.*, 2021). It is supported by another study in which peptides play a major role in the antioxidant action of proteins (Shahi *et al.*, 2020). According to the results obtained in this study, corner EBN hydrolysates demonstrated a significantly higher (p<0.05) antioxidant value compared to cup EBN hydrolysates even though the antioxidant potencies were evaluated with different antioxidant assays. Thus, it can be assumed that corner EBN hydrolysate may likely contain a higher number of those compounds compared to cup EBN hydrolysates since it demonstrates higher antioxidant potencies.

However, the outcome of this study is contrary to a previous study (Mohamad Nasir *et al.*, 2021). A previous study found that the crude protein content and amino acid profile in cup EBN (59.96±0.09%) was higher than in the corner EBN (54.70±0.16%). In addition, the study also revealed that the full stew (FS) method (without filtration)yielded significantly higher extraction yield (p<0.05) of soluble protein concentration (cup EBN: 92.29±2.45%; corner EBN: 79.35±0.91%) compared to the stew extraction (SE) method (filtered using muslin cloth) (cup EBN: 12.50±0.89%; corner EBN: 10.99±0.11%) (Mohamad Nasir *et al.*, 2021). Previous studies demonstrated that the cup EBNs have relatively higher soluble protein concentration when compared to the corner EBN in both extraction methods.

The contradictory result could perhaps be due to the differences in the hydrolysate preparation techniques used, particularly the difference between enzymatic hydrolysis and water extraction in the former case. The traditional water extraction technique has recovered an inadequate yield of water-soluble protein, meanwhile, protein solubilisation by using enzymes could improve the cleavage of peptide bonds in EBN proteins into bioactive glycopeptides (Bourgougnon, 2014; Mohamad Nasir *et al.*, 2021). Moreover, higher protein content in the cup section may not necessarily generate higher antioxidant potencies, it depends on the proteolysis reaction and production of bioactive peptides (López-García *et al.*, 2022). Previous research indicated that soy protein isolate hydrolysate exhibited several-fold higher antioxidant potency compared with soy protein isolate (De Castro and Sato, 2014).

In spite of the fact that limited recent data on different sections of EBN hydrolysate, there is little information regarding the impact of enzymatic hydrolysis duration on the antioxidant potencies of EBN hydrolysates. Hence, this study was conducted on various hydrolysis times (1, 2, and 3 hr) treated with *Alcalase* enzyme for different sections of EBN. Theoretically, the corner section is harder compared to the cup section of EBN (Mohamad Ibrahim *et al.*, 2021), hence, it was expected that the cup section of EBN may have a higher degree of hydrolysis, Moreover, the degree of hydrolysis will usually increase by increasing the hydrolysis time. The chain length of the peptides became shorter, and the molecular weight distribution decreased, due to the breakdown of the peptide bonds. Thus, the amount of free amino acids increased and subsequently induced the antioxidant potencies (Shahi *et al.*, 2020). Antioxidant peptides produced from food such as EBN not only decrease the generation of reactive oxygen species but may also activate natural antioxidant defence systems in cellular and animal models (Zhu *et al.*, 2022).

However, the antioxidant values of DPPH and FRAP assays demonstrated that the antioxidant values of the cup and corner EBN hydrolysate demonstrated fluctuations and significant downward trends over the hydrolysis time (p<0.05). The finding was similar to the results obtained in the previous research works (Corrêa *et al.*, 2011; Xia *et al.*, 2012; Mahdavi-Yekta *et al.*, 2019). These antioxidant peptides may undergo further degradation, and breaking down the bioactive sequences responsible for antioxidant activity, the primary determinants of their antioxidant activity might be the distinct chemical structures of peptides. The hydrolysis process of precursor protein structures may also have an impact on the antioxidant properties of peptides (Zou *et al.*, 2016). In regards, this occurs because, at a certain point, the proteins or peptides are further broken down into inactive free amino acids, whereby these free amino acids are no longer functional as antioxidants (Samaranayaka and Li Chan, 2011). Other research also supports that further hydrolysis will lead to the formation of shorter peptides (tri- and dipeptides) and free amino acids (Sbroggio *et al.*, 2016). Hence, the antioxidant potency of peptides can be reduced or completely lost after conversion to free amino acids (Tironi and Añón,

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2010).

5. Conclusion

In this study, it can be concluded that corner EBN enzymatic hydrolysate possesses significantly higher antioxidant potencies compared to EBN enzymatic hydrolysate from the cup section ($p<0.05$). These findings highlight the potential differences in antioxidant properties between the cup and corner sections of EBN and provide valuable insights for understanding their health-enhancing effects. Therefore, these findings could be valuable for the EBN industry in formulating functional food. However, there are a few limitations during the sample preparation for both sections of EBN samples. Since EBN has hydrogel properties, the extraction of EBN hydrolysate was not fully hydrolysed even after 3 hr of hydrolysis. Thus, more work to be done to determine the maximum antioxidant potencies in EBN hydrolysates. As a recommendation, EBN samples may be exposed to the longer enzymatic hydrolysis time in future studies.

Conflict of interest

The authors declare no conflict of interests.

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