Amino acid composition, antioxidant activity and mineral content of *Achatina fulica* snail slimes and edible bird's nest

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Abstract. This study was performed to investigate whether the snail slimes which also well-known for their anti-aging properties, can be served as an alternative for edible bird's nest (EBN) in our daily lives. The amino acid composition of samples was evaluated using a reversed-phase High Performance Liquid Chromatography. The four major amino acids detected in snail slimes were aspartic acid (3.80 µmol/mL), glutamic acid (2.87 µmol/mL), alanine (2.23 µmol/mL) and serine (1.80 µmol/mL), while for EBN, the top four amino acids were proline (5.31 µmol/mL), serine (5.27 µmol/mL), aspartic acid (4.78 µmol/mL) and threonine (3.97 µmol/mL). In overall, EBN possessed significantly ($p < 0.05$) higher total amino acid content (42.63 µmol/mL) than the snail slimes (23.59 µmol/mL). Besides, the EBN was found to have significantly higher ($p < 0.05$) DPPH free radical scavenging activity (25.12 %) as compared to the snail slimes (16.02 %). The four major elements identified in snail slimes and EBN were sodium, calcium, potassium and magnesium. Snail slimes contained higher levels of minerals compared to EBN. In conclusion, the snail slimes can potentially be served as an alternative of EBN to those people suffered from mineral deficiencies.

1 Introduction

The snails are belonging to phylum Mollusca (the second biggest phylum within animal kingdom) together with slugs, squids, octopus, oysters and cuttlefish [1]. They are members of class Gastropoda and the land snails play several important roles in our mother earth. The land snails are responsible for nutrient recycling in forest ecosystem and serve as prey for some of the small mammals, birds, amphibians, reptiles and others [2]. There are a lot of different types of land snails in the world, such as the garden snails *Helix aspersa*, the edible snails *Helix pomatia*, those giant African land snails (*Achatina fulica*, *Achatina achatina*, *Archachatina marginata*) and others [3]. However, among all of them, *A. fulica* is the only one that being interested in this study. *Achatina fulica* is a member of Achatinidae family and it is originated from East Africa but nowadays it can be found in other places such as China, India, Taiwan, West Indies and United States as well [4]. The body surfaces of the snails are covered by mucus, a viscous-elastic fluid which is secreted by the snails themselves. This

snail mucus or slime has gained public's interest in recent years because of its unbelievable health and medical benefits. The snail slimes are known to exhibit anti-aging and antibacterial properties [5].

Swiftlets (from Apodidae family) are basically kind of birds which are similar to sparrows and swallows, but they are considered not closely related to one another [6]. Currently, there is a total of twenty-four species of swiftlets being recorded in the world and the five common species which can be found within Malaysia are *Aerodramus fuciphagus*, *Aerodramus maximus*, *Hydrochous gigas*, the white belly swifts *Collocalia esculent* and the Asian palm swifts *Cypsiurus balasiensis* [7]. However, most of the edible bird's nests (EBN) that have been commercially exploited nowadays are made from *A. fuciphagus* (white-nest swiftlets) and *A. maximus* (black-nest swiftlets) [8]. EBN (also called "yan wo" in Mandarin and "sarang burung wallet" in Malay language) is a nest which is composed of the salivary secretions from the sublingual salivary glands of swiftlets [9]. These salivary secretions have soft and sticky properties when they are fresh, and gradually become dried and hardened after they expose to the air [10]. The white nests consist of approximately 70 – 80% of salivary secretions and 20 – 30% of impurities (feathers or droppings), while the black nests contain about 85 – 90% of feathers and only 10 – 15% of edible parts [7]. The white nests are preferred and thus sold at a higher price than the black nests in the market.

EBN are widely known for their multiple health benefits and have been used as traditional Chinese medicine for long time. The EBN extracts are found to be able to treat the erectile dysfunction, enhance bone strength and the dermal thickness, inhibit the infection of influenza virus and act as alternative chondro-protective agent when treating the osteoarthritis [11-13]. However, not everyone is able to afford them due to their high market prices. Since the snail slimes have emerged as a new trend currently and also well-known for their health benefits, it yields a question, whether the snail mucus can be served as an alternative for EBN in our daily routines. Furthermore, the snail slimes could be a more sustainable and cheaper source of anti-aging food supplement compared to edible bird's nest. There are close similarities between snail slimes and edible bird's nest such as snail *A. fulica* mucous secretion constituted abundant water-soluble protein, collagen, amino acid and mineral content. The abundant protein, collagen and minerals found in snail mucus suggested that Malaysian snail slimes might contain biological activities and nutritional advantages as those found in the edible bird's nest valuable for commercialisation. The specific objectives of this study are to determine and compare the amino acid profile, antioxidant activity and mineral content of snail slimes from *Achatina fulica* and EBN from *Aerodramus* spp.

2 Materials and Methods

2.1 Sample preparation

A total of 180 wild Giant African snails (*Achatina fulica*) with length of 3 to 8 cm were caught from Kampar, Perak, in which 60 snails were responsible for 1 replicate. Besides, the house-farmed edible bird's nests of swiftlets (*Aerodramus* spp.) were obtained from the local supplier at Sitiawan, Perak.

2.1.1 Snail slimes

The outer shell and body of the snails were cleaned with running water to remove all the dirt. The cleaned snails were then treated with electric shock to stimulate the mucus secretion. An electric current with 5 to 10 volt was applied to the snails for approximately 30 to 60 s [14]. The secreted slimes were collected and 2 volumes of the deionized water were then

incorporated to the slimes to dilute them [15]. Next, the diluted slime solutions were centrifuged at 6,400 rpm and at 8°C using refrigerated Centrifuge 5430 R (Eppendorf, Germany) for 15 min. The supernatant was collected and subjected to UV sterilization for 15 min to eliminate the germs. The sterilized supernatants were frozen overnight and freeze-dried with ScanVac CoolSafe freeze dryer (Labogene, Denmark). Finally, the dried slime solids were converted into powders and stored inside the refrigerator until further analysis.

2.1.2 Edible bird's nests

The unprocessed house-farmed EBN were initially soaked in the clean water and followed by subsequent cleaning process. After that, the impurities such as feathers and dirt were removed manually with forceps [8]. The cleaned EBN were then frozen overnight and subjected to ScanVac CoolSafe freeze dryer (Labogene, Denmark). Lastly, the dried EBN were ground into powder form and kept inside the refrigerator for subsequent analysis.

2.2 Amino acid analysis

2.2.1 Protein hydrolysis

Initially, 10 mg of sample was weighed in the boiling tube and approximately 2 mL of 6 N HCl was added to it. The mixture was purged with nitrogen gas [16]. Later, it was hydrolyzed at 110°C for 24h with vacuum condition. After that, about 1 mL of borate buffer was added to the hydrolyzate to attain a concentration of 10 mg /mL buffer and this sample solution was filtered through polyethersulfone (PES) syringe filter. The instrumental analysis for amino acid profile of samples was done following the methods developed by Agilent Technologies with some modifications [17].

Firstly, about 5 µL of the sample or standard was mixed well with 25 µL of borate buffer and 5 µL of *ortho*-phthalaldehyde reagent, followed by 4 µL of 9-fluorenylmethyl chloroformate reagent. Approximately 320 µL of deionized water was inserted and the mixture was mixed well. After derivatization, the mixture was filtered through 0.2 µm polytetrafluoroethylene (PTFE) membrane filter and 10 µL of sample mixture was injected into the HPLC column.

2.2.2 HPLC Analysis

Agilent 1100 HPLC system (Agilent Technologies, USA) was employed to perform the amino acid analysis. The chromatographic separation was performed by using Agilent ZORBAX Eclipse Plus C18 column (Agilent Technologies, USA) with 4.6 mm × 250 mm and particle size of 5 µm. The column temperature and flow rate of mobile phase were set at room temperature and 1.5 mL/min respectively. HPLC gradient run system was using 40 mM Na_2HPO_4 at pH 7.8 as mobile phase A and mobile phase B with acetonitrile (ACN): methanol (MeOH): water in 45:45:10, v/v/v, respectively. The protocol of flow gradient was pre-set as shown as Table 1. The standard curves of peak area against concentration were plotted for various amino acid standards. Lastly, the amino acid profile of sample was determined based on the standard curves and the final results were expressed as µmol/mL.

Table 1. The pre-set flow gradient conditions in HPLC method for amino acids analysis.

Time (min)	Mobile phase B (%)	Mobile phase A (%)
0	2	98
0.84	2	98
58.4	57	43
58.5	100	0
64.3	100	0
64.4	2	98
65	End	End

2.3 Antioxidant analysis

Sample solutions with concentration of 10 mg/mL were used in antioxidant tests. The snail slime solution was prepared by directly dissolving the dried slime powder in the distilled water. However, for EBN, they were heated in distilled water at 100°C for around 2 h using dry block heater (Grant, United Kingdom) in order to be dissolved, then subjected to the non-refrigerated Centrifuge 5430 (Eppendorf, Germany) with 6400 rpm for 10 min and the supernatant was collected [18].

2.3.1 DPPH free radical scavenging assay

The scavenging activity of DPPH free radical was determined using the procedures mentioned by Matusiewicz and colleagues [19] with some modifications. Firstly, about 400 µL of the 0.25 mM DPPH radical solution was added to 100 µL of sample in microcentrifuge tube and mixed well. A control was also prepared by replacing the sample solution above with distilled water and mixed it with DPPH working solution [20]. The mixtures were incubated at 37°C for 30 min in dark environment with the use of Elite Dry Bath Incubator (Major Science, USA). After incubation, 200 µL of 80% methanol was added to remove the protein suspension and the mixtures were then centrifuged at 10 rpm for 5 min using Sorvall Legend Micro 21R Microcentrifuge (Thermo Scientific, USA). Afterwards, 200 µL of supernatants were transferred to the wells of 96-well microtiter plate and 100 µL of distilled water was added. The solutions were mixed well and their absorbances were measured at 517 nm against the blanks, employing FLUOstar Omega microplate reader (BMG Labtech, Germany). The DPPH radical scavenging activity was expressed in percentage and they were calculated based on the formula stated by Prabu and Natarajan [21].

$$\text{Scavenging activity (\%)} = [(Ac - Ab) - (Ae)] / (Ac - Ab) \times 100 \quad (1)$$

Where Ac represents the absorbance of DPPH• solution with methanol as negative control, Ab represents the absorbance of methanol as blank control, Ae represents the absorbance of DPPH• solution with methanol extract of sample.

2.4 Mineral test

The mineral test was determined using the procedures mentioned by Saengkrajang, Matan and Matan [22] with some modifications. First, about 50 mg of sample was weighed in boiling tube and a mixture made up of 2 mL of HNO_3, 1 mL of H_2O_2 and 3 mL of deionized water was then added to digest it. The boiling tube containing sample was subjected to heat treatment by the dry block heater (Grant, United Kingdom) for 45 min at 220°C. A transparent solution was formed after the digestion and it was allowed to cool down for approximately 20 min. The solution was topped up to 10 mL with deionized water to make a 5 mg/mL sample solution. Afterwards, it was filtered through a PES syringe filter, and the filtrate was transferred to the centrifuge tube to be kept inside the fridge prior to instrumental analysis. The elemental composition of samples was analyzed by Optima 7000 DV ICP-OES (Perkin Elmer, USA). A blank (deionized water) and several concentrations (2, 4, 6, 8 and 10 ppm) of different standards (Na, Ca, K, Mg, Ba, Zn, Cu and Fe) were prepared and analysed in order to plot the calibration curves. The final results regarding the mineral content were expressed as mg/L.

2.5 Statistical analysis

The data collected from experiments were analyzed with statistical software IBM SPSS Statistics, with Version 25 (SPSS Inc, USA). All the experimental results acquired were reported as mean ± standard deviation with replication, n = 3. Independent-samples t test was performed for each test to evaluate whether significance difference existed among two types of samples at significance level, $\alpha = 0.05$.

3 Results and discussion

3.1 Amino acid analysis

The most abundant amino acid in snail slimes was reported as Asp (3.80 ± 0.69 μmol/mL), then followed by Glu (2.87 ± 0.27 μmol/mL), Ala (2.23 ± 0.26 μmol/mL) and the fourth major amino acid, Ser (1.80 ± 0.21 μmol/mL) as shown in Table 2. There are aspartic acid and glutamic acid found in the snail slime as reported by Dolashka [23] and Matusiewicz [19] as compared to this study. Different species of snails and different environmental conditions being set up in snails' habitat will exert a great impact on the composition of snail slimes [24]. The species of the snail studied were belong to *Achatina fulica* while the researchers [19, 23] utilized *Helix aspersa* snails as their testing subjects. Moreover, the snails of this experiment were said to live in a habitat different with those of previous research because they grew in the natural environment but the latter were commercially bred and even raised in a ventilated plastic box for a period of time after purchasing by researchers. All of these have contributed to the dissimilarities of the results.

The four major amino acids in EBN were noticed to be Pro (5.31 ± 1.3 μmol/mL), Ser (5.27 ± 1.23 μmol/mL), Asp (4.78 ± 1.16 μmol/mL) and Thr (3.97 ± 0.89 μmol/mL). This findings have slight differences in terms of amino acid composition compared to those stated by [18, 25], in which only serine and aspartic acid are matched with this results. Amino acid composition of EBN is actually affected by the breeding sites, climate and also the food intake of swiftlets [26]. In fact, the EBNs of this test were collected from Sitiawan, Perak while the EBNs in previous works were bred and obtained from other different places including Terengganu, Pahang, Penang, Selangor, Johor, Sabah and Sarawak. All the EBNs in this test were also secreted by the swiftlets that inhabit in man-made bird houses, however

the EBN studied by [18] was a mixture of cave-harvested EBN and house-farmed EBN. The swiftlets that live in cave may have diets different with those live in the man-made swiftlet farms and cause the amino acid content to be different.

Some similarities can be noted between the amino acid profiles of snail slimes and EBN, for instance, containing serine and aspartic acid as two of the major amino acids, and do not possess methionine and lysine. The serine and aspartic acid are members of these twenty amino acids and they are classified as non-essential amino acids, which mean that they can be synthesized by the body and thus not necessary must be taken from the diets [27]. Both of them are responsible for different functions, for example, serine can assist in converting the folate to tetrahydrofolate in the one-carbon metabolism and generating the choline and carnitine, while aspartic acid is vital for synthesis of nucleotides and D-Asp [28]. In general, the EBNs were detected to be significantly ($p < 0.05$) higher in total amino acid concentration (42.63 ± 11.15 μmol/mL) compared to the snail slimes (23.59 ± 3.93 μmol/mL), and the significant difference ($p < 0.05$) was only present in serine, histidine, threonine, valine and proline levels of these two samples.

Table 2. Amino acids analysis of snail slime and edible bird's nest.

Amino acids	**Amino acid concentration (μmol/mL)**	
	Snail slimes	**Edible bird's nests**
Aspartic acid (Asp)	3.80 ± 0.69[a]	4.78 ± 1.16[a]
Glutamic acid (Glu)	2.87 ± 0.27[a]	3.10 ± 0.72[a]
Serine (Ser)	1.80 ± 0.21[b]	5.27 ± 1.23[a]
Histidine (His)	0.37 ± 0.12[b]	1.18 ± 0.36[a]
Glycine (Gly)	0.94 ± 0.12[a]	1.20 ± 0.38[a]
Threonine (Thr)	1.63 ± 0.21[b]	3.97 ± 0.89[a]
Arginine (Arg)	1.56 ± 0.13[a]	2.50 ± 0.71[a]
Alanine (Ala)	2.23 ± 0.26[a]	3.15 ± 0.83[a]
Tyrosine (Tyr)	0.76 ± 0.21[a]	1.53 ± 0.61[a]
Cystine (Cys)	1.37 ± 0.86[a]	0.67 ± 0.48[a]
Valine (Val)	1.33 ± 0.21[b]	3.87 ± 1.06[a]
Methionine (Met)	-	-
Phenylalanine (Phe)	1.07 ± 0.21[a]	1.89 ± 0.67[a]
Isoleucine (Ile)	0.88 ± 0.20[a]	0.94 ± 0.41[a]
Leucine (Leu)	1.78 ± 0.25[a]	3.26 ± 0.92[a]
Lysine (Lys)	-	-

Proline (Pro)	1.20 ± 0.48^{b}	5.31 ± 1.37^{a}
Total amino acids	**23.59 ± 3.93^{b}**	**42.63 ± 11.15^{a}**

Note: These data are expressed as mean ± SD with n = 3. The difference in superscript lowercase letters within same row indicates significant differences at $p < 0.05$.

3.2 Antioxidant analysis

Based on Fig. 1, the mucus secreted by *A. fulica* possessed 16.02 ± 0.98% of DPPH free radical scavenging activities. This result has higher antioxidant activity compared to the value reported by [29]. The differences of antioxidant activity obtained could be due to the difference of environmental conditions experienced by the snails, as *A. fulica* used in the previous study were collected from Beijing, China (country with four distinct seasons throughout a year) while snails used in this study were collected from Perak, Malaysia (a place which do not have four seasons). Snail slime is an extremely complex matrix which its quality or composition is highly affected by the environmental factors [24]. Next, the EBN was identified to have 25.12 ± 1.48% of DPPH free radical scavenging rate and this is also very similar to the value revealed by [30].

Free radicals are molecules which are not stable in nature and will cause cell damage in human body and further lead to those degenerative and chronic diseases, while the antioxidants are molecules which able to fight off these harmful free radicals [31]. The higher antioxidant activities of EBN detected in DPPH assay test indicated that the EBN is more effective in slowing down the aging process of human body and lowering the risk of getting diseases such as heart diseases and cancers, as compared to the snail slimes.

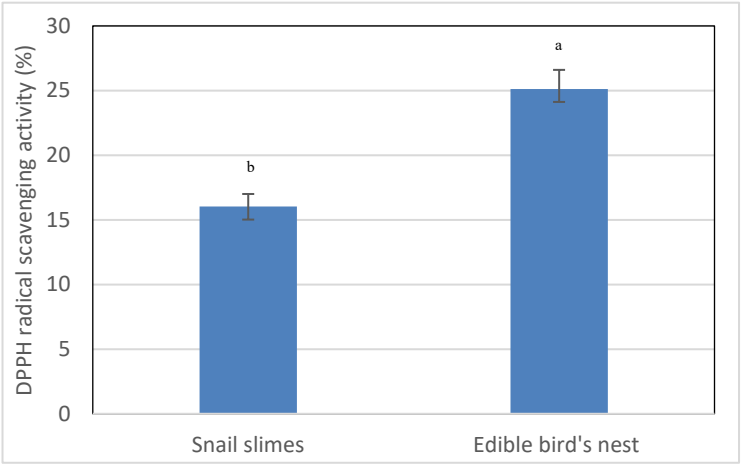

Fig. 1. DPPH free radical scavenging activity of *A. fulica* snail slime and edible bird's nest. Data are presented as mean ± standard deviation in triplicate (n=3). Data are presented as mean ± SD of triplicate groups. Significant differences ($p < 0.05$) are indicated by different superscript lowercase letters on the bar.

3.3 Mineral test

Based on Table 3, the order of mineral inside snail slimes can be arranged as Na > Ca > K > Mg > Ba > Zn > Cu > Fe (absent). This order is not in accordance with the results reported by [32]. This may happen because the *A. fulica* snails in this study were collected from the location (Kampar, Perak) that is different with the place (Columbia) that mentioned by previous research. The mineral content of snail is greatly influenced by locations, seasons, feeding habits and biological cycles [1].

Next, in this project, the mineral content detected inside edible bird's nests was arranged in descending order of Ca > Na > K > Mg > Zn, while Ba, Cu and Fe were absent. This order is also not in agreement with the previous studies done by [18, 33]. The difference in mineral profile of EBN is due to the difference in environmental factor and the diets of swiftlet, which are almost the same main reasons of differences found between different EBN in terms of amino acid composition [8]. Apart from that, as the EBN of this study were obtained from Sitiawan, Perak, which is a region that directly accesses to Malacca Straits, it is explained that the sodium from marine aerosols accumulates via atmospheric deposition towards the edible bird's nests, leading to sodium has becoming the second highest mineral content[8]. Besides, the copper and iron contents detected were said to be complied with the maximum regulatory limits of Cu (1.0 mg/L) and Fe (0.3 mg/L) stated by SIRIM [34].

Table 3. The comparison of mineral content between snail slime and edible bird's nest.

Parameters	Snail slimes	Edible bird's nests
Sodium (Na) , mg/L	331.07 ± 43.22^{a}	53.97 ± 6.96^{b}
Calcium (Ca), mg/L	265.28 ± 32.52^{a}	61.20 ± 4.75^{b}
Potassium (K), mg/L	215.93 ± 47.61^{a}	11.63 ± 0.10^{b}
Magnesium (Mg), mg/L	106.65 ± 7.60^{a}	7.43 ± 0.40^{b}
Barium (Ba), mg/L	12.97 ± 3.57^{a}	-
Zinc (Zn), mg/L	2.28 ± 0.79^{a}	0.85 ± 0.04^{b}
Copper (Cu), mg/L	0.39 ± 0.16	-
Iron (Fe), mg/L	-	-
Total mineral content (mg/L)	934.57 ± 39.03^{a}	135.08 ± 2.38^{b}

Note: The data above were stated in mean ± standard deviation in triplicate (n=3). The different superscript lowercase letters within same row indicates significant differences at $p < 0.05$.

Furthermore, the results of this research showed that snail slimes and EBN shared some similarities such as lacking of iron and having sodium, calcium, potassium and magnesium as their major minerals. The latter is in accordance with the results obtained by [18, 32, 33]. These top four minerals are actually macrominerals which are required in amount of more than 100 mg per day by our bodies, while the others such as copper, iron, zinc and barium are classified as microminerals which our bodies only need them in quantity of lower than 100 mg per day [35]. In overall, snail slimes were discovered to possess greater amount of minerals (934.57 ± 39.03 mg/L) compared to EBN (135.08 ± 2.38 mg/L) regardless of what

type of mineral being tested, and there was a significant difference ($p < 0.05$) in Na, Ca, K, Mg, Ba and Zn levels of these two types of samples. These indicate that snail slimes are literally better source of minerals than the EBN.

4 Conclusion

In conclusion, amino acid analysis showed that the snail slimes contained aspartic acid, glutamic acid, alanine and serine as their four major amino acids, while for EBN, the four predominant amino acids were proline, serine, aspartic acid and threonine. EBN was a more significant source of amino acids than the snail slimes owing to its significantly ($p < 0.05$) higher in total amino acid content. Next, EBN contained higher antioxidant activity compared to snail slimes. In addition, the four predominant mineral elements inside these two types of samples were sodium, calcium, potassium and magnesium, regardless of the order.

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