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Acetylcholinesterase inhibition and antioxidant activities of polysaccharide-peptide complexes from edible mushrooms

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Abstract

Edible mushrooms are commonly known to be good source of nutrients and possess biological activities. This study focuses on comparison of anti-acetylcholinesterase and radical scavenging activities of polysaccharide-peptide complexes extracted from three types of edible mushrooms, including white shimeji (*Hypsizygus marmoreus*), brown shimeji (*Hypsizygus marmoreus*), and enokitake (*Flammulina velutipes*). Acetylcholinesterase is an enzyme involved in hydrolysis of acetylcholine, causing a decrease in level of this major neurotransmitter. Over function of acetylcholinesterase and accumulation of free radicals could lead to several neurodegenerative disorders, including Alzheimer's disease. In this work, polysaccharide-peptides from mushrooms were prepared by hot water and following with ethanol precipitation. Total protein and carbohydrate contents were determined. The bioactivities were evaluated by acetylcholinesterase inhibition, DPPH radical scavenging, ABTS radical inhibition, and ferric reducing antioxidant power (FRAP) assays. The results indicated that enokitake extracts showed the highest activities to inhibit acetylcholinesterase (71.39 ± 9.63%), whereas brown shimeji extract exhibited the highest ability to quench DPPH radicals (51.57 ± 10.79%). In the future, the polysaccharide-peptides extracted from mushrooms could be developed as functional food for prevention or treatment of neurodegenerative diseases.

Introduction

Population aging has rapidly become an important transformation of the global society and lead to age-related healthcare challenges. With increasing needs of health awareness, functional food development has been of interest to promote good health and to prevent diseases.[1] Neurodegenerative disorders are considered to be significant problems in aging society, leading to progressive dysfunction of the brains and affecting the patients' quality of life. One of the major causes of dementia diseases is a decrease in the level of the neurotransmitter, acetylcholine, which plays important role in transmission of nerve impulses in the central nervous system and the autonomic nervous system.[2,3] At normal condition, acetylcholine can be broken down to choline and acetic acid by a function of acetylcholinesterase to discontinue the synaptic neurotransmission. However, over function of this enzyme could cause depletion of acetylcholine by hydrolysis reaction, resulting in continuous pathogenesis of the Alzheimer's diseases.[4,5] In addition, another cause of age-related disorders is overrepresentation of free radicals in the cells, which affects biomolecule

destruction and lead to cell death. It has been reported that oxidative damages from free radicals are involved with rapid progression of the neurodegenerative disorders, including Alzheimer's disease and Parkinson's disease.[6] The purpose of this study is to evaluate the use of naturally active compounds from edible mushrooms to maintain level of acetylcholine at the synaptic cleft and to prevent cellular oxidative damage.

Edible mushrooms are known to provide both high nutritional values and medicinal properties. Their bioactivities have been extensively studied, such as antioxidant, anti-inflammatory, anti-acetylcholinesterase, antimicrobial and anticancer activities. These biological properties resulted from a number of active compounds in the mushrooms, including phenolics, flavonoids, alkaloids, terpenoids, carotenoids, tocopherol, proteins, and polysaccharides.[7-12] Polysaccharides in mushrooms are found to be associated with proteins in form of large soluble complexes, called polysaccharide-peptide complexes (PSP). They play crucial roles in biological activities of mushroom extracts.[13] The PSP from several edible and medicinal mushrooms were shown to have biological effects; for examples, PSP extracted from *Ganoderma lucidum* contained antioxidant, hepatoprotective, and anti-proliferative activities[14,15], PSP from *Cordyceps militaris* exhibited acetylcholinesterase inhibition[16], PSP from mycelial culture of *Tricholoma sp*. was shown to promote immune response and inhibit tumor cell proliferation[17], and PSP from fruiting body of abalone mushroom possessed anti-diabetic, antioxidant, and anticancer properties.[18] In this work, comparative studies of acetylcholinesterase inhibition and antioxidant activities of PSP extracted from three types of commonly consumed mushrooms, white shimeji (*Hypsizygus marmoreus*), brown shimeji (*Hypsizygus marmoreus*), and enokitake (*Flammulina velutipes*), were performed. Our work could provide basic information for future development of functional food products from edible mushrooms to alleviate memory loss and to reduce the risk to age-related diseases.

Methodology

Extraction of polysaccharide-peptide complexes from edible mushrooms:

Three types of commercially available mushrooms, *Hypsizygus marmoreus*, *Hypsizygus marmoreus*, and *Flammulina velutipes* were purchased from local supermarket in Chonburi, Thailand, in September, November and December 2017, for triplicate experiments. Each fresh mushroom was cut into small pieces. Fifty grams of each sample was immersed in 200 ml deionized H_2O, followed by grinding to increase the surface area. The mixtures were boiled for 30 min and filtered by No.1 Whatman filter paper. Polysaccharide-peptide complexes were precipitated by incubation with three volumes of ethanol overnight at 4°C, followed by centrifugation at 3500 rpm for 20 min. The pellets were dissolved in 2 ml deionized H_2O for bioactivity evaluations.

Total protein content

Protein concentration was determined by Lowry's protein assay using albumin as a standard protein.[19] Sample and standard protein solutions (200 µl) were mixed with 1 ml Lowry's reagent. After 15 min incubation at room temperature, Folin-ciocalteu's reagent (100 µl) was added to the mixture and incubated for another 30 min at room temperature. Absorbance was recorded at 750 nm using a spectrophotometer (Thermo Fisher Scientific, Madison, WI, USA).

Total carbohydrate content

Total concentration of carbohydrate was determined by anthrone reaction using glucose as a standard sugar. Sample and standard solutions (50 µl) were mixed with 150 µl of 0.1% anthrone solution in H_2SO_4, and incubated at 100°C for 20 min. The reactions were cooled down at room temperature, and absorbance was measured at 620 nm using the VersaMax™ microplate spectrophotometer (Molecular devices, Sunnyvale, CA, USA).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Profile of proteins extracted from three types of mushroom were revealed by SDS-PAGE. Equal volume of each mushroom extract (10 μl) was loaded to 12% polyacrylamide gel and separated by electrophoresis at 100 V for 45 min. Protein bands were visualized by Coomassie blue staining.

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

DPPH radical scavenging assay was modified from Molyneux (2006).[20] Mushroom extracts (5 μl) were diluted with 45 μl H_2O and mixed with 100 μl of 0.2 mM DPPH in methanol in 96 well-plate. The mixtures were incubated at room temperature for 30 min prior to absorbance measurement at 517 nm using the microplate spectrophotometer. The ability of mushroom extracts to scavenge DPPH radical was determined by equation (1). The experiments were performed in triplicate and ascorbic acid was used as positive control.

$$\% \text{ inhibition} = [(A_{control} - A_{blank}) - (A_{sample} - A_{blank\ sample})] / (A_{control} - A_{blank}) \quad \text{equation (1)}$$

2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assay

The assay was modified from Shalaby and Shanab, 2013.[21] ABTS solution was prepared at the concentration of 7 mM in H_2O and oxidized by 2.45 mM $K_2S_2O_8$ for 16 h to form $ABTS^{\bullet+}$ solution before performing an assay. The extracts were diluted 10 times, and 10 μl of each sample solution was mixed with 1 ml $ABTS^{\bullet+}$ solution. The reaction was performed for 6 min at room temperature, and absorbance at 734 nm was recorded. ABTS radical scavenging ability was determined by equation (2). The experiments were performed in triplicate and ascorbic acid was used as positive control.

$$\% \text{ inhibition} = [(A_{control} - A_{blank}) - (A_{sample} - A_{blank\ sample})] / (A_{control} - A_{blank}) \quad \text{equation (2)}$$

Ferric reducing antioxidant power (FRAP) assay

FRAP assay was modified from Benzie and Strain, 1999.[22] FRAP reagent was prepared by combination of 0.28 mM sodium acetate pH 3.6 solution, 10 mM tripyridyltriazine (TPTZ) in 40 mM HCl, and 20 mM $FeCl_3.6H_2O$ at the ratio of 10:1:1. A solution of FRAP reagent (180 μl) was mixed with 10x diluted mushroom extracts (20 μl), and incubated at room temperature for 30 min. Absorbance was recorded at 596 nm to observe the ability of the extracts to reduce FRAP, as compared to $FeSO_4$ solution. Various concentrations of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) were used as a positive control.

Acetylcholinesterase (AChE) inhibition assay

AChE inhibitory assay was modified from Ellman's method.[23] Mushroom extracts (25 μl) were incubated with 0.28 U/ml acetylcholinesterase in 50 mM Tris-HCl buffer containing 0.1% bovine serum albumin, pH 8.0 (25 μl), at room temperature for 5 min. After that, 25 μl of 15 mM acetylthiocholine iodide was added to the mixture as a substrate, followed by addition of 125 μl of 3 mM 5,5'-dithio-bis-(2-nitrobenzoic acid). Absorbance at 405 nm was recorded to measure the ability of AChE to cleave acetylthiocholine iodide, which could be calculated by using an equation (3). Galanthamine hydrobromide was used as a positive control.

$$\% \text{ inhibition} = [(A_{control} - A_{blank}) - (A_{sample} - A_{blank\ sample})] / (A_{control} - A_{blank}) \quad \text{equation (3)}$$

Statistical analysis

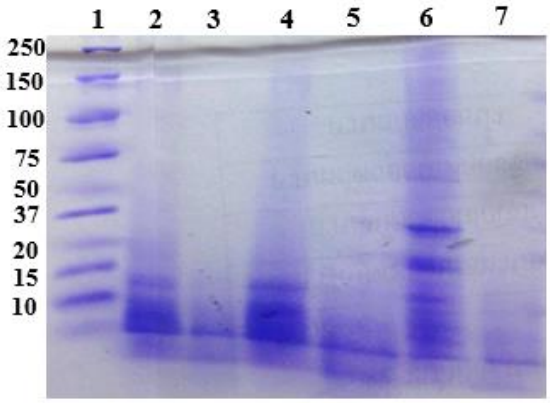
All experiments were performed in triplicate (3 biological replicates and 3 technical replicates). The results were shown as mean ± standard deviation. Statistical analyses were performed by Minitab17 using one-way ANOVA with Tukey's Post-test.

Results and Discussion

Following the methods for mushroom polysaccharide-peptide complex (PSP) extraction with slight modifications[16,18], PSP from white shimeji, brown shimeji, and enokitake mushrooms were obtained, as confirmed by the presence of proteins and carbohydrate in the pellet after ethanol precipitation (Table 1). The highest protein and carbohydrate contents from the precipitates (P) were observed in brown shimeji mushroom extract, followed by enokitake extracts and white shimeji extract, respectively. However, significant amount of proteins and carbohydrates were presented in the supernatant (S) after ethanol precipitation. It is possible that low molecular weight peptides or carbohydrates were able to dissolve in ethanol solution. The results from SDS-PAGE revealed that higher molecular weight proteins were observed in the pellets from all mushrooms, as compared to the supernatant (Figure 1). White shimeji and brown shimeji shared similar patterns of proteins because of similar species, while enokitake's proteins showed different molecular weight pattern.

Table 1. Total protein and carbohydrate concentrations from ethanol precipitates (P) and supernatants after precipitation (S) of extracts from white shimeji, brown shimeji, and enokitake. Data were shown as mean ± standard deviation from triplicate experiments.

Types of mushroom	Total protein concentration (mg/ml)	Total carbohydrate concentration (mg/ml)
White shimeji (P)	0.575 ± 0.242	0.237 ± 0.011
White shimeji (S)	0.510 ± 0.015	0.139 ± 0.020
Brown shimeji (P)	0.791 ± 0.149	0.362 ± 0.024
Brown shimeji (S)	0.706 ± 0.009	0.134 ± 0.012
Enokitake (P)	0.652 ± 0.001	0.305 ± 0.010
Enokitake (S)	0.392 ± 0.016	0.375 ± 0.035



Lane	Samples
1	Molecular weight marker
2	White shimeji (P)
3	White shimeji (S)
4	Brown shimeji (P)
5	Brown shimeji (S)
6	Enokitake (P)
7	Enokitake (S)

Figure 1. SDS-PAGE of proteins extracted from white shimeji, brown shimeji and enokitake mushrooms, presented in ethanol precipitates (P) and supernatants after precipitation (S).

Biological activities of PSP extracted from three types of mushrooms were compared. The ability to reduce the risk of Alzheimer's disease was evaluated by acetylcholinesterase inhibition assay and compared with commercially available medicine for Alzheimer's symptomatic treatment, galantamine hydrobromide.[16] As shown in Figure 2, PSP from enokitake showed the highest activity to inhibit acetylcholinesterase (46.88±5.29%), followed by those from brown shimeji (37.24 ± 6.86 %) and white shimeji (28.47±11.12%), respectively. Acetylcholinesterase inhibition of enokitake PSP was similar to that of 0.05 mg/ml galantamine hydrobromide used as positive control. It was observed that supernatant after precipitation of all mushrooms exhibited more potent activity to reduce acetylcholinesterase function, possibly due to the effects of other types of compounds in the mushrooms, such as β-carboline alkaloids and lanostanoids as presented in previously published reports.[24-26] Also, it was reported that solvent extracted compounds from various mushrooms showed anti-acetylcholinesterase activity[26,27], and these groups of components were likely to be dissolved in ethanol and be presented in the supernatant.

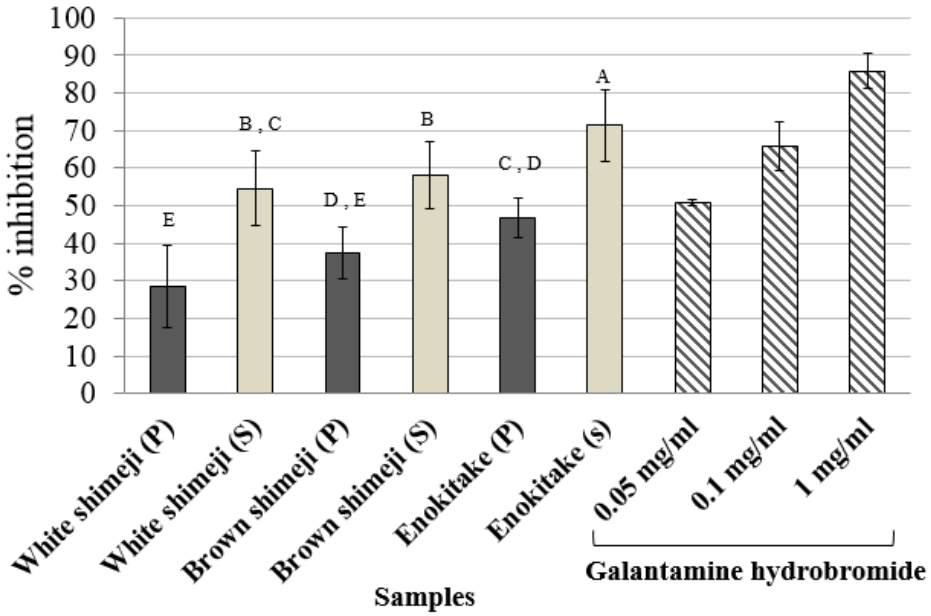


Figure 2. Acetylcholinesterase inhibition activity (mean ± SD) of ethanol precipitates (P) and supernatants after precipitation (S) of the extracts from white shimeji, brown shimeji and enokitake mushrooms. The data points with different letters (A-D) show statistically significant difference (ANOVA and Tukey test, $p<0.05$). The %AChE inhibition values of positive control (galantamine hydrobromide) at different concentrations are shown on the right.

Antioxidant activities of PSP extracts were evaluated using three different assays to observe different mechanisms of antioxidant processes; DPPH radical scavenging assay was used to determine the ability to donate hydrogen atom, ABTS radical scavenging assay was employed to observe electron transfer process, and FRAP assay was carried out to evaluate the ability to reduce other compounds. Figure 3 showed that PSP pellet from brown shimeji had higher ability to scavenge DPPH radicals (26.83 ± 5.63%) than those from white shimeji and enokitake. And ethanol-soluble supernatant from brown shimeji pellet also exhibited the highest activity against DPPH radicals (51.57 ± 10.79%). It was unambiguous that a group of substances dissolved in ethanol solution possessed stronger activity than the PSP precipitates from all three mushrooms (Figure 3). A number of published reports revealed that antioxidant compounds in mushrooms can be identified as phenolic compounds, ergosterol, β-carotene, lycopene, tocopherol, ascorbic acid, and polysaccharide complexes.[8,9,28] It was reasonable that many of these compounds were solubilized in the ethanol supernatants and resulted for higher

DPPH radical scavenging ability, as compared to the macromolecules-containing pellets. For ABTS radicals, PSP from all mushrooms showed similar inhibition ability ranging from 16.66 ± 0.58% to 18.09 ± 2.65%. Although ethanol-soluble supernatant from enokitake and brown shimeji showed slightly higher ability to quench $ABTS^{\bullet+}$ than other samples, the difference in values was not statistically significant (Figure 4). Considering the ability to reduce Fe^{3+}-2,4,6-Tris(2-pyridyl)-s-triazine in the FRAP reagents, PSP from brown shimeji had higher reducing power (102.62 ± 11.33 μM Fe^{2+} equivalent) than those from the other two mushrooms, but it was not considered to be statistically different. The FRAP value of brown shimeji PSP was higher than 10 μg/ml Trolox. Interestingly, both pellet and supernatant displayed comparable reducing ability. It could be implied from the results that both ethanol-soluble and ethanol-insoluble compounds from white shimeji, brown shimeji, and enokitake extracts had similar ability to transfer electron to the quench free radicals and similar reducing capability.

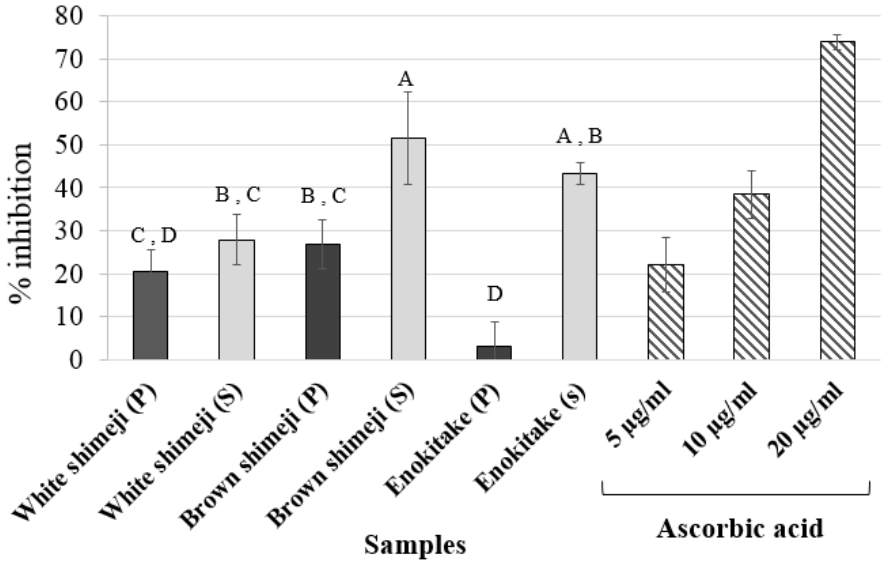


Figure 3. DPPH inhibition activity (mean ± SD) of ethanol precipitates (P) and supernatants after precipitation (S) of the extracts from three mushrooms. The bar charts with different letters (A, B, C, D) indicate significant difference between the samples (ANOVA and Tukey test, $p<0.05$). And %DPPH inhibition of ascorbic acid at different concentrations are also indicated on the right.

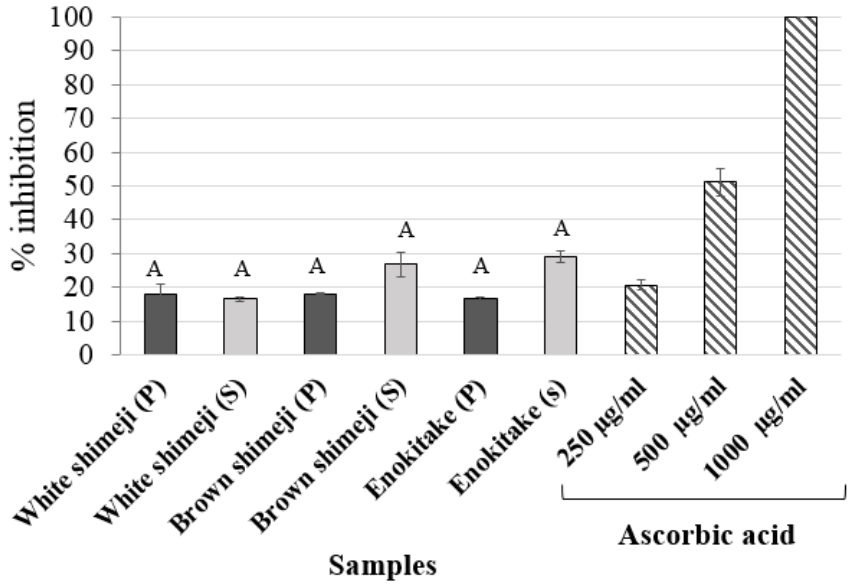


Figure 4. ABTS quenching ability (mean ± SD) of ethanol precipitates (P) and supernatants after precipitation (S) of the extracts from three mushrooms. The data points with the same letter (A) are not significantly different (ANOVA and Tukey test, $p<0.05$). The %ABTS inhibition of ascorbic acid at different concentrations are shown on the right.

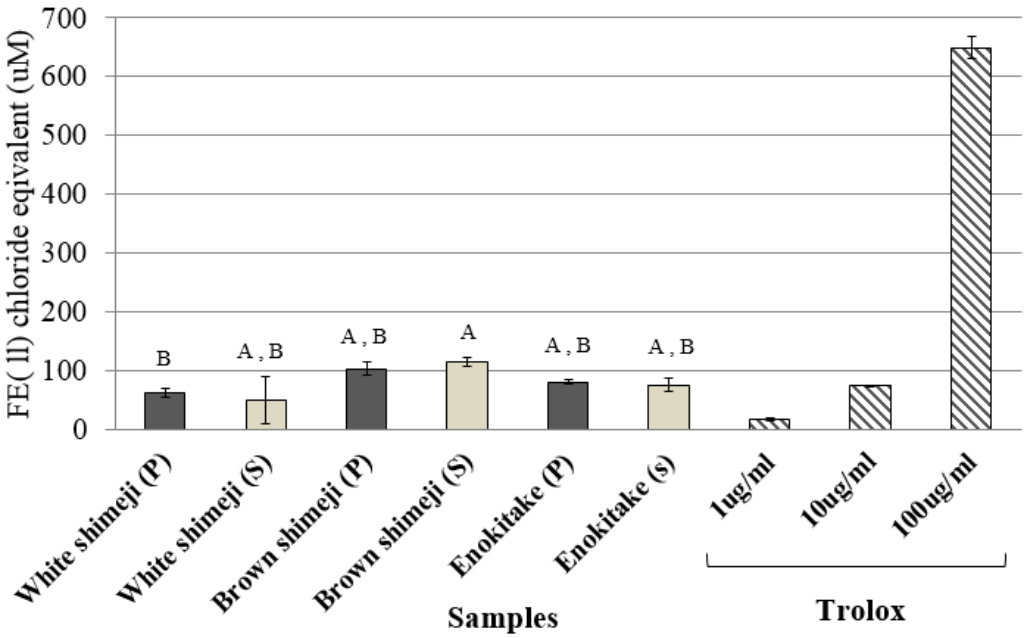


Figure 5. Ferric reducing ability shown as Fe^{2+} equivalent (mean ± SD) of ethanol precipitates (P) and supernatants after precipitation (S) of the extracts from white shimeji, brown shimeji and enokitake mushrooms. The bar charts with the same letters (A-B) indicate no significant difference between the data (ANOVA and Tukey test, $p<0.05$). The reducing power of trolox at different concentrations are shown on the right.

Conclusion

Polysaccharide-peptide complexes could be extracted from white shimeji, brown shimeji, and enokitake mushrooms by hot water extraction followed by ethanol precipitation. PSP from brown shimeji contained higher quantity of both polysaccharides and polypeptides than those from the other two mushrooms. And it also possessed stronger DPPH radical scavenging ability. PSP from enokitake showed distinct SDS-PAGE protein profile, as compared to the other two, and it also displayed the highest ability to inhibit acetylcholinesterase. The results from this preliminary work provide useful information to support the health benefits of mushroom consumption to reduce the risk of age-related diseases, and can be informative for functional food developments from edible mushrooms.

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