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Amino acid composition and biological activity of new powdered vegetable seasonings

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Abstract

To evaluate the amino acid composition and biological activity of new powdered vegetable seasonings included inky cap mushroom (*Coprinopsis radiata*), onion, and radish in three different formulations, namely M1, M2, and M3. The amino acid composition was examined by liquid chromatography with tandem mass spectrometry (LC–MS/MS). Biological activity evaluated antioxidant compounds (total phenolic content [TPC] and total flavonoid content [TFC]), antioxidant activity (2,2-diphenyl-1-picrylhydrazyl [DPPH]), ferric reducing antioxidant power (FRAP)], and cytotoxicity on human liver cancer cells (HepG2) with the sulforhodamine B (SRB) assay. Moreover, the vegetable seasonings powdered enhanced the taste of aspartic and glutamic acids, characteristic of umami, especially in M3, which had the highest umami taste. The ethanolic extract of M3 showed significantly higher concentrations of TPC and TFC ($p< 0.05$) compared to other formula. Furthermore, the antioxidant activity of M3 was similar to antioxidant compounds. Ethanolic extracts of the three formulations induced the death of HepG2 cells, particularly those from M3, in which HepG2 cell death was statistically significant higher ($p< 0.05$) than extracts of M1 and M2. The biological activities of vegetable seasonings powdered were rely on the amount of mushroom powder. These findings suggest that powdered vegetable seasonings could be used as natural seasonings in powder form and may have potential as a source of phytochemicals that possess antioxidant and cytotoxic properties.

Keywords: Powdered vegetable seasonings, Amino acid composition, Antioxidant, Cytotoxicity, Phytochemical

1. Introduction

Epidemiological studies suggest that consuming vegetables reduces the risk of chronic diseases [1]. A previous finding suggested that seasoning vegetables was associated t increasing vegetable intake [2]. In addition, vegetables containing vitamins and phenolic compounds are good sources of phytochemical compounds. In Thailand, vegetables are used as food flavorings. In particular, mushrooms are widely used as functional and healthy food and are typically consumed for their flavor [3]. The potential of medicinal mushrooms to improve health due to their high number of bioactive components can be seen in the number of nutraceutical, nutritional therapy, and pharmaceutical applications [4]. Moreover, plant extracts contain important secondary metabolites, including alkaloids, terpenoids, phenolics, and flavonoids, all promoting health [5]. Phenolic compounds (including flavonoids) of edible plants play a protective role as non-enzymatic antioxidants in cells, protecting them from oxidative damage and, hence, are beneficial to human health [6].

Coprinopsis radiata (C. radiata) is a Basidiomycete fungus, commonly known as an inky cap mushroom, and is a member of the Psathyrellaceae family [7]. In addition, this mushroom is rich in proteins and free amino acids,

the essential compounds in functional foods [8]. It is especially rich in aspartic acid and glutamic acid, which provide the umami taste in many popular dishes, as well as monosodium glutamate (MSG)-like flavor. [9]. Vegetables with an umami taste, such as tomato, seaweed, onion, radish, and mushroom, have been used to develop powdered vegetable seasonings [10,11]. In addition, the glutamate and aspartate contents of onion, radish, and inky cap mushroom were 198, 203, and 65 mg/100g, respectively [12,13].

Interest has increased in the potential use of vegetables as seasonings with increased antioxidant compounds and antioxidant capacity [12]. Our previous study revealed that *C. radiata* is a mushroom with many phytochemical properties that contains antioxidants, as well as some essential amino acids. It has potential as a vegetable seasoning that provides health benefits [13]. In the present study, we developed a new powdered vegetable seasoning, which includes the umami vegetables mushroom, onion, and radish. Although the chemical composition and biological activity of mushroom, onion, and radish have been studied, their combination has not been studied. It has been suggested that consuming novel powdered vegetable seasonings may help reduce the risk of chronic diseases. This research aimed to evaluate the free amino acids in new powdered vegetable seasonings through liquid chromatography with tandem mass spectrometry (LC–MS/MS) analysis and to determine their biological activities, including their antioxidant activity, by determining their antioxidant activity (i.e., 2,2-diphenyl-1-picrylhydrazyl [DPPH] and ferric reducing antioxidant power [FRAP]), antioxidant compounds (total phenolic content [TPC] and total flavonoid content [TFC]), and the cytotoxicity on human liver cancer cells (HepG2).

2. Materials and methods

2.1. Materials

Individual standard amino acids, DPPH, 2,4,6-tripyridyl-S-triazine (TPTZ), Folin-Ciocalteu reagent, gallic acid, and catechin, were obtained from Sigma-Aldrich (MO, USA). All laboratory chemicals and reagents employed in the research were of analytical grade.

2.2. Sample preparation

The powdered vegetable seasonings were prepared using a mixture of inky cap mushrooms, onions, and radish powder modified from Phiangjan [10]. The powdered vegetable seasonings had the following proportions: M1: 50% inky cap mushrooms, 25% onions, and 25% radishes; M2: 60% inky cap mushrooms, 20% onions, and 20% radishes; and M3: 70% inky cap mushrooms, 15% onions, and 15% radishes.

2.3 Free amino acid analysis

Free amino acids in powdered vegetable seasonings were analysed and identified using LC–MS/MS. Free amino acids were extracted by suspending 100 mg of a fine powder with 0.5 mL of 0.05 M aqueous HCl–ethanol (1:1 v/v). The samples were vortexed for 5 min to ensure homogeneity, and then centrifuged at 12,100 x g for 15 min at 4 °C.

The supernatant was analysed by LC–MS/MS. The analysis was carried out using an LCMS-8030 coupled to the power of a triple-quadrupole mass spectrometer (Shimadzu, Kyoto, Japan), operated in ESI mode, and a Shimadzu LC-20AC series HPLC system (Shimadzu, Kyoto, Japan). The sample 10 µL sample was injected and separated conditions: a carrier solvent using a flow rate of 0.2 mL/min and column oven temperature setting at 38 °C. The analysis was carried out in liquid phases consisting of (A) methanol/water (50:50) with 0.1% (v/v) formic acid, and (B) water with 0.1% (v/v) formic acid. The MS/MS equipped with an electrospray ionisation interface (ESI) was used to confirm the amino acid analysis. The mass spectrometer was operated in multiple reaction monitoring (MRM) in the positive ion mode; and ion source temperature at 400 °C.

2.4 Extraction for determination of phytochemical compounds

The powdered form of 25 g dried sample was soaked overnight with 95% ethanol in 250 mL. Next, the ethanolic extracts were filtered using filter paper and removed of solvents from samples by rotary evaporator under reduced pressure and a temperature of 50 °C. The extracts were dried in a freeze-drier and stored at –20 °C for subsequent analysis of antioxidant activity, antioxidant compounds, and anticancer activity.

2.5 Determination of antioxidant compounds of powdered vegetable seasonings included total phenolic content (TPC) and total flavonoid content (TFC)

2.5.1 TPC

The TPC was analysed according to the method described by Yawadio et al. [14]. The absorbance was observed at a wavelength of 765 nm. Gallic acid as a standard for measuring TPC, and the results are expressed in term of gallic acid equivalents (GAE)

2.5.2 TFC

Colorimetric analysis was used to determine the TFC in the extracts using a previously published method [15]. The 0.5 mL of 2% aluminium chloride in ethanol was added to 1 mg/mL of extracts and incubated for 60 min at room temperature. The absorbance was read at a wavelength 420 nm. The TFC is described as mg of catechin equivalents (CA).

2.6 Determination of antioxidant activity

The antioxidant activities of powdered vegetable seasonings were evaluated using several antioxidant assays.

2.6.1 DPPH radical scavenging activity

The radical-scavenging activity used to measure antioxidant activity was based on the DPPH assay described by Brand-Williams et al. [16]. The assay is based on the reduction of DPPH radicals measured by the absorption band at 515 nm. Trolox was used as a positive control, and the assay was carried out in triplicate. The percent of radical-scavenging activity following to the equation: % radical-scavenging activity = $[1- (A_c/A_d)] \times 100$, where A_c is the absorbance of the sample, and A_d is the absorbance of the control.

2.6.2 Determination FRAP assay

The reducing power of extracts from *C. radiata* preparations was determined with the method described by Benzie and Strain [17], with some modifications. The FRAP solution consisted of 300 mM acetate buffer (3.1 g $C_2H_3NaO_2 \cdot 3H_2O$ and 16 mL $C_2H_2O_2$), pH 3.6; and 10 mM TPTZ was mixed with 75 µL of each of the extracts. After 30 min incubation, the absorbance was measured at wavelength 593 nm. The standard curve of Trolox was linear between 20 and100 µg/mL.

2.7 Cytotoxicity assay

The HepG2 used for the cytotoxicity study was purchased from the American Type Culture Collection (ATCC, USA) and maintained according to ATCC's recommendations. To assess the cytotoxicity effect of the extracts on HepG2 cell viability, the sulforhodamine B (SRB) assay was used according to Buranrat et al. [18]. In brief, HepG2 cell line were cultured in a 96-well plate for overnight, and treated with different concentrations of extract were added for 24–72 h, respectively. The cells were subsequently fixed with 10% trichloroacetic acid at 4 °C and incubated for 30 min with 0.4% (SRB). The protein-bound dye solution was prepared with 10 mM Tris base solution, and the absorbance at 540 nm was recorded. Cell cytotoxicity (% cell death) was compared with untreated control groups, and 50% inhibition (IC50) values were calculated.

2.8 Statistical analysis

All statistical analyses were performed using STATA version 13.0 software. The data are presented as mean $\pm$ standard deviation (SD). Statistical significance between groups was analysed by one-way ANOVA followed by the appropriate post-hoc test (Duncan Test), and the statistical difference were considered significant at $p < 0.05$.

3. Results and discussion

Our previous study investigated the biological activity of *C. radiata* or inky cap mushrooms, and the extract displayed high TPC and strong antioxidant activity. Based on the results shown that a high concentration of aspartic acid (8.33 ± 0.93 µg/g dry weight) and glutamic acid (56.67 ± 1.07 µg/g dry weight), TPC (32.27 ± 2.23 mg GAE/g dry weight), TPC (19.06 ± 1.89 mg CA/g dry weight), DPPH assay (65.10 % inhibitions), and FRAP

radicals (620.56 ± 9.51 mmol $FeSO_4$/100 g dry weight) [10]. Thus, we used inky cap mushrooms to develop new powdered vegetable seasonings.

3.1 Free amino acid analysis

The free amino acid profiles of the different powdered vegetable seasoning formulations (M1, M2, and M3) from the LC–MS/MS analysis are shown in Table 1. Fourteen amino acids were identified by searching the standard mass spectral library based on the retention time and mass spectra. The screened samples showed high quantities of aspartic acid and glutamic acid, which provide *the main flavor,* monosodium glutamate. Therefore, we focused on these two amino acids in the powdered vegetable seasonings. The highest glutamic acid concentration was found in M3 (344.13±63.96 μg/g dry weight), as well as the highest aspartic acid concentration (31.07±5.83 μg/g dry weight). Furthermore, arginine was found to be the most abundant free amino acid in all of the powdered vegetable seasonings.

Reyes [19] has analyzed the basic amino acids in the family of *Coprinus* mushrooms and found they were glutamic acid, leucine, and arginine. Onions, radishes, and mushrooms are umami vegetables with high glutamate and aspartate contents [11]. Our results showed that M3 contained the highest percentage of aspartic and glutamic acids, which are components that provide the characteristic umami mushroom taste or the taste of monosodium glutamate [20]. The tastes of aspartic and glutamic acids are reminiscent of umami, which is an important factor in the taste of powdered vegetable seasonings. In addition, M3 contained the lowest amount of arginine, which is the most bitter component of the mixture [21].

Table 1 Free amino acid composition of powdered vegetable seasonings.

Amino acid	Powdered vegetable seasonings (μg/g dry weight)		
	M1	M2	M3
Alanine	56.90 ± 9.20*	75.47 ± 18.77*	124.80 ± 18.92*
Arginine	8161.67 ± 531.31*	7456.10 ± 263.36*	2011.90 ± 116.47*
Aspartic acid	14.83 ± 3.49*	19.93 ± 1.34*	31.07 ± 5.83*
Glutamic acid	161.97 ± 26.85*	225.87 ± 18.38*	344.13 ± 63.96*
Histidine	27.80 ± 1.03*	30.53 ± 1.55*	47.70 ± 6.64*
Isoleucine	115.03 ± 2.46*	95.57 ± 4.89*	84.20 ± 1.97*
Leucine	226.27 ± 33.65*	152.10 ± 11.27*	146.77 ± 7.64*
Lysine	285.17 ± 19.77*	487.50 ± 61.26*	1,049.60 ± 50.49*
Methionine	3.60 ± 1.92*	5.67 ± 0.42*	5.27 ± 0.28*
Phenylalanine	172.00 ± 5.92*	207.67 ± 12.84*	194.63 ± 2.02*
Threonine	24.30 ± 2.89*	37.10 ± 6.46*	49.50 ± 4.42*
Tryptophan	95.43 ± 14.30*	128.27 ± 14.62*	14.37 ± 0.45*
Tyrosine	67.93 ± 11.95*	82.30 ± 14.12*	113.00 ± 1.85*
Valine	46.50 ± 7.02*	67.07 ± 3.45*	86.97 ± 7.92*

*Statistically significant at $p < 0.05$ in the same row to denote a significant difference between powdered vegetable seasonings formula.

3.2 Antioxidant compounds of TPC and TFC

The antioxidant compounds in the ethanolic extracts of the powdered vegetable seasonings, including the TPC and TFC, are shown in Table 2. Ethanolic extracts of powdered vegetable seasonings showed abundant antioxidant compounds, with a TPC comparable to that of standard GAE. The TPCs in powdered vegetable seasonings ranged from 67.24–75.14 mg GAE/100 g. M3 contained the maximum amount of TPC (72.42 mg GAE/100 g), followed by M2 with 70.17 mg GAE/100 g, whereas M1 had the least TPC: (68.84 mg GAE/100 g). With regard to TFC, M3 contained the maximum amount, and M1 contained the minimum. The TPC and TFC were higher in M3 than in M2 and M1. Moreover, the concentrations of ingredients depended on the fluctuations in the phenolic and flavonoid content.

Previous work has indicated that the ethanolic extract of *C. radiata* resulted in a higher phytochemical concentration than that of an extract prepared with a different solvent [13]. The solvent polarity influenced the concentrations of the bioactive substances, including TPC and TFC, contained in the *C. radiata* preparations. Liu [22] has noted that bioactive substances in vegetables include high antioxidant phenolic compounds and flavonoids, which are found in vegetables, including onions, radishes, and mushrooms. The TPCs and TFCs include antioxidant compounds that inhibit oxidative stress and have health-promoting (bioactive) effects [6]. As the total amount of mushrooms in a particular sample increased, the TPC and TFC values increased. The phenolic

compounds exert their antioxidant activity by scavenging free radical species or enhancing endogenous antioxidant activity [23]. Our findings indicate the phytochemical compounds in the powdered vegetable seasonings enhance the antioxidant and bioactive benefits of these formulations proportional to the amount of powdered mushroom.

Table 2 Antioxidant activity and compounds of powdered vegetable seasonings.

Powdered vegetable seasonings	Antioxidant activity		Antioxidant compounds	
	DPPH (% scavenging activity)	FRAP (mmol $FeSO_4$/100 g dry weight)	TPC (mg/100g)	TFC (mg/100g)
M1	60.06 ± 0.77*	780.64 ± 7.88*	68.84 ± 1.60	30.38 ± 5.11*
M2	63.06 ± 1.07*	902.56 ± 4.84*	70.17 ± 2.71	37.14 ± 0.56*
M3	69.10 ± 0.20*	1092.56 ± 13.95*	72.42± 2.72	40.68 ± 2.50*

*significant at $p < 0.05$ for one-way ANOVA comparing the difference between powdered vegetable seasonings formula with values expressed as mean ± SD of the triplicate measurement.

3.3 Antioxidant activity by DPPH and FRAP assays

The antioxidant activity of the ethanol extracts from the powdered vegetable seasonings and the outcome of DPPH radical scavenging assays are presented in Table 2. The inhibition percentages for the DPPH assay were highest for M3 (69.10%), followed by M2 and M1 ($p < 0.05$). Similarly, the M3 formulation possessed the highest antioxidant activity in terms of FRAP radicals (1092.56 ± 13.95 mmol $FeSO_4$/100 g dry weight) ($p < 0.05$), whereas the M1 formulation possessed the lowest antioxidant activity (780.64 ± 7.88 mmol $FeSO_4$/100 g dry weight).

The phenolic and flavonoid compounds might contribute to the antioxidant activity, and it has been shown that the antioxidant activities of powdered vegetable seasonings correlated with their phenolic compound contents [22,23]. The total antioxidant activity values for powdered vegetable seasonings followed a similar trend as that of the antioxidant compounds of the TPC and TFC. The antioxidant activity could act as a radical scavenger through an electron- or hydrogen-donating mechanism. Therefore, different antioxidant assays have been used to measure antioxidant activity [24]. Increasing the amount of mushroom powder in the formulations increased the total antioxidant activity. Such observations show the potential to improve the antioxidant activity of powdered vegetable seasonings by incorporating mushroom powders.

3.4 Cytotoxicity assessments by SRB

The cytotoxicity assessments by SRB assays of HepG2 cells are summarized in Figure 1. HepG2 cells were exposed to various concentrations (125–5000 µg/mL) of ethanol extracts from the powdered vegetable seasonings for 24–72 h. The ethanolic extracts induced a statistically significant ($p< 0.05$) increase in HepG2 cell death, dependent on the product formulation and the concentration. The HepG2 cells exposed to the M3 extracts for >72 h and at higher concentrations experienced increased cell death (%). The increase in cell death determined by SRB assay when exposed to 125, 250, 500, 1000, 2500, and 5000 µg/ mL of M3 at 72 h was 11.53%, 14.81%, 27.83%, 42.91%, 52.75%, and 61.06%, respectively. In addition, M3 was a more effective cytotoxicity agent than other powdered vegetable seasonings formulated with low IC50 values (2,230.90 ± 51.23 µg/mL) (Table 3).

The powdered vegetable seasonings extracts tested in this study increased the death of HepG2 cells in a concentration-dependent manner. Our results are in agreement with previous studies showing the genus *Coprinus* spp. possess cytotoxic activity against human T-cell leukemia cells [25] and liver HepG2 cells [26]. Furthermore, onion and radish showed the strongest inhibition of the HepG2 cell viability [27,28]. The present study suggests that the cytotoxicity of the powdered vegetable seasonings might be due to the free radical scavenging property of the extracts in the presence of antioxidant compounds [26]. In this study, the presence of antioxidant compounds such as TPC and TFC showed cytotoxic activity on HepG2 cells. Fuchs et al. [29] have demonstrated the cytotoxicity of polyphenolic compounds from winery by-products on mitochondrial functions in HepG2 cells. For example, polyphenolic compounds, such as gallic acid and catechin, have been shown to exhibit cytotoxicity after 24 h of incubation on HepG2 cells with cell viability of 12.5 µM and 100 µM, respectively [30,31].

This research supports using vegetables, particularly onions, radishes, and inky cap mushrooms, to develop new powdered vegetable seasonings that show biological activity. The phenolic compounds were the principal components responsible for the biological activity, including the antioxidant and cytotoxic activity of all three powdered vegetable seasonings. In addition, these findings need to be evaluated in further investigations on the isolation and characterization of the active compounds responsible for the antioxidant and cytotoxicity potential of these new powdered vegetable seasonings.

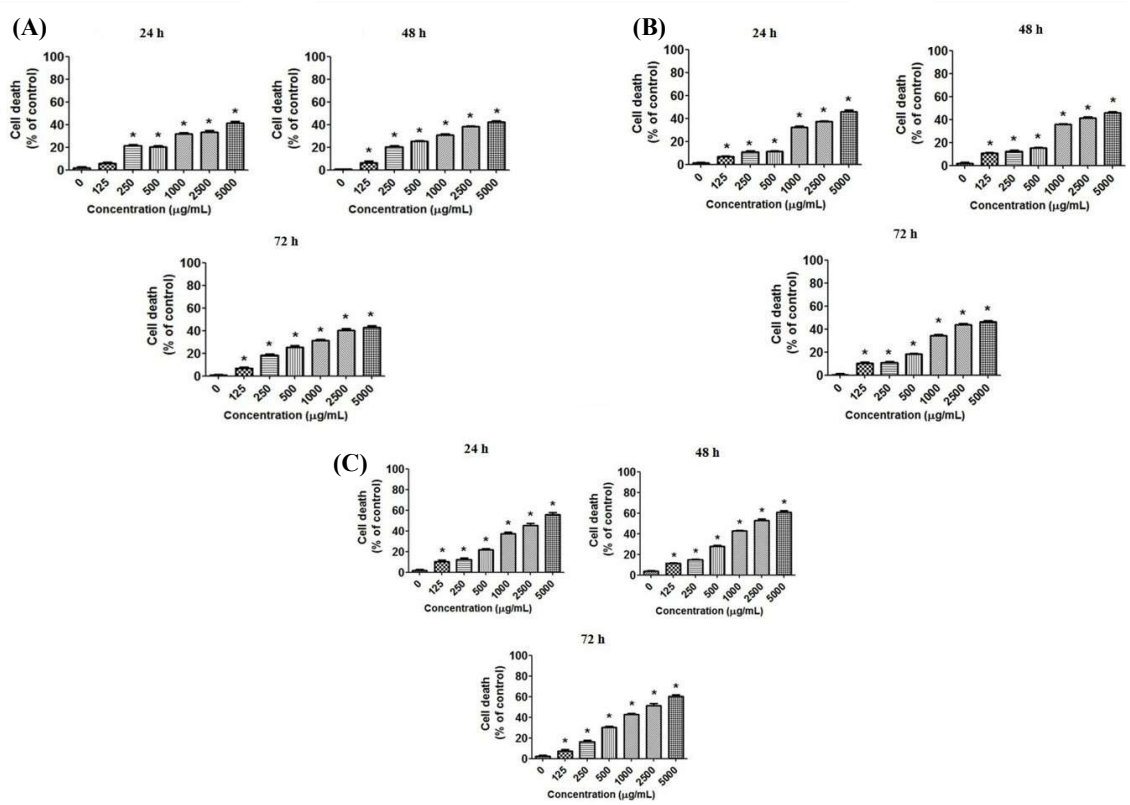

Figure 1 Effect of the extract on HepG2 cell death. The cells were treated with various concentrations of (A) (50% inky cap mushrooms), (B) (50% inky cap mushrooms), and (C) (70% inky cap mushrooms) extract of the powdered vegetable seasonings (0–5000 ug/mL) for 24-72 h.

Table 3 Mean IC50 values for the powdered vegetable seasonings in HepG2 cells following different incubation time periods.

Incubation time	HepG2 Cells		
	M1	M2	M3
24 h	4,995.26 ± 45.00*	4,715.01 + 23.67*	3,723.49 ± 67.19*
48 h	4,701.63 ± 25.27*	4,562.10 + 12.31*	3,253.88 ± 43.16*
72 h	4,487.01 ± 41.79*	4,454.40 + 21.07*	2,230.90 ± 51.23*

*Data having the same superscript are significantly different from each other at $p<0.05$ using one way analysis of variance (ANOVA).

4. Conclusion

The vegetables consumed to promote human health are often included in dietary supplements. Our results show that powdered vegetable seasonings can be used as a new natural seasoning powder, which significantly increases the death of HepG2 cells in a concentration-dependent manner. These encouraging results suggested combining onions, radishes, and inky cap mushrooms into a natural seasoning powder that could contain health-promoting phytochemical compounds. This study also suggests the potential of using culinary powdered vegetable seasonings to increase the phytochemical content and biological activity of food additives.

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