Original Research Article

AMINO ACIDS ANALYSIS OF THE FOUR MUSHROOM SPECIES, ANTIOXIDANT ACTIVITY AND IMMUNOSTIMULANT EVALUATIONS OF THEIR POLYSACCHARIDES EXTRACTS

Abstract

Mushrooms have been valued as edible and medical resources. This project aimed to evaluate of four mushrooms such as *Pleurotus ostreatus* (Fr.) Kummer, *Pleurotus opuntiae* (Dur.& Lev.) Sacc., *Pleurotus sajor-caju* (Fr.) Sing, and *Ganoderma lucidum* (Curtis) for amino acids, two extraction methods, antioxidant activity, total phenolic content, ^{1}H NMR analysis and *in vitro* immunostimulant activity, compared the cultivation between TISTR and Nakhon Prathom area. The results showed that glutamic acid and aspartic acid were abundant amino acids, while alanine, leucine, lysine, arginine, serine, phenyl alanine and threonine were found varied in those mushrooms. The most common amino acids found in *P. sajor-caju* were found in lower amounts in *G. lucidum.* There amino acids content was contrast with the antioxidant activity and total phenolic content. Meanwhile the ^{1}H NMR chemical shifts illustrated signals of a mixture of polysaccharides resonated at $\boldsymbol{\delta}$ 3.0-4.0 ppm, and fatty acids at $\boldsymbol{\delta}$ 0.70-2.30 ppm of the A extract as well as the two signals of anomeric protons $\boldsymbol{\alpha}$-glucan and $\boldsymbol{\beta}$-glucan of the B extract appeared at δ 5.36 ppm and 4.47 ppm, respectively. The bioassay *in vitro* immunostimulant activity of all mushroom extracts showed good immunostimulant booster at concentration of 0.5 mg/mL. It was found that the A and B extracts of *G. lucidum* were the most potent immunostimulant activity followed by the A extract of *P. sajor-caju*, and they had higher activity than $\boldsymbol{\beta}$-glucan standard with % phagocytic activity of 187.12±2.17, 166.23±0.56, 155.00±2.93, respectively. Future works will focus on their *in vivo* immunostimulant activity, healthy drinks and nutraceutical product development.

Keywords: *Pleurotus ostreatus* (Fr.) Kummer, *Pleurotus opuntiae* (Dur.& Lev.) Sacc., *Pleurotus sajor-caju* (Fr.) Sing, and *Ganoderma lucidum* (Curtis); immunostimulant activity

1. Introduction

Immune response is an important physiological process to identify and destroy foreign harmful substances as well as organisms to protect diseases, in which macrophages play key roles in phagocytosis, cytotoxicity and intracellular killing activities. Macrophages can resist to pathogens either directly through phagocytosis or indirectly by producing related factor such as nitric oxide (NO), interleukins (IL), tumor necrosis factor-α (TNF-α) and reactive oxygen species (ROS). [1] In clinical practice, immunomodulators are classified into three categories including immunosuppressants, immunostimulants and immuno adjuvants. Immune system modulations

are used common as prophylactic medicine for increasing healthy people Where modulations are used common as prophylactic medicine for ncreasing a number of healthy people Whereasimmunomodulators are synthetic or semi-synthetic components therefore,natural immnunomodulators has been in growing interest for overall health- benefiting effects to humans with no or less toxicity [2,3]. Mushrooms,a fungi kingdom member with well-known medicinal properties has drawn significant interest due to their immunomodulatory and antioxidant attributes,anti-inflammatory, antimicrobeial,antidiabetic, cardiovascular protective, hepatoprotective and anticancer potentials [4]. Several active components for example, essential amino acids, proteins, vitamins and mineral, fiber, and polysacchrides in mushrooms fruiting bodies. β-D-glucan polysacchrides and their derivatives extracted from mushrooms showed immunomodulatory responses[5,6]. Therefore, it is essential to extract these active ingredients from mushroom bodies to examine their therapeutic potential for immunostimulant activity in this study.

2. Materials and Methods

2.1 Materials

Methods

Plant materials:

Four mushroom species such as *Pleurotus ostreatus* (Fr.) Kummer, *Pleurotus opuntiae* (Dur.&Lev.) Sacc., *Pleurotus sajor-caju* (Fr.) Sing and *Ganoderma lucidum* (Curtis) were obtained from Dr. Tanapak Inyod, Expert Centre of Innovative Agriculture, Thailand Institute of Scientific and Technological Research, Ministry of Ministry of Higher Education, Science, Research and Innovation, Thailand. All the isolates were collected from the mushroom farm in Ratchaburi Province, Thailand. The mushroom pure cultures were transferred into PDA slants and maintained at room temperature. The three mushrooms species, *Pleurotus opuntiae* (Dur.&Lev.) Sacc., *Pleurotus sajor-caju* (Fr.) Sing and *Ganoderma lucidum* (Curtis) were purchased from the local mushroom farm, Tambon Srapattana Community Enterprise in Nakhon Prathom province, Thailand.

Mushroom production: The upper end of the sample bags were opened. Colonized (by mycelium) substrates were arranged on shelves in a greenhouse (temperature, 25-30°C; relative humidity, (85%). The cultures were constantly wet in order to maintain the required relative humidity (85%). The cultures were irrigated by spraying with water, once or twice a day. Primordia will be initiated after 7 days and mature mushrooms (noted as the point where the pileus started to fold) from each bag were harvested at 10 days after open the bags.

Plant preparation: The mushrooms were individually collected, weighed, washed and torn to pieces by hands, then put each of them separately on aluminium foil sheet to dry in the hot air oven, temp 50^0C for 24 hrs. The dried mushrooms were ground in to powder weighed and kept in zip lock bag at room temperature.

Plant extraction

Extraction A: The dried powder of *Pleurotus ostreatus* (Fr.) Kummer, *Pleurotus opuntiae* (Dur.&Lev.) Sacc., *Pleurotus sajor-caju* (Fr.) Sing and *Ganoderma lucidum* (Curtis) were invidually extracted with DeIonized water, temp 25^0C, stirred for 7 hrs. The supernatant was collected after filtered and then freeze-dried under FreeZone 6, 12 and 18 Liter, Freeze dryer gave DI water extracts.

Extraction B: The dried powder of *Pleurotus Sajor-caju* (Fr.) Sing, *Pleurotus ostreatus* (Fr.) Kummer, *Pleurotus ostreatus* (Jacq. exFr.) Kummer and *Ganoderma lucidum* (Curtis) were individually extracted with with 80% (80:20 ethanol:water) at room temp. for 7 days, then filtered

and dried *in vacuo* using evaporator gave ethanol extracts. mM. Measured their absorbance by using UV-VIS spectrophotometer at 517 nm.

2.2 Total phenolic content analysis : using Folin-ciocaltea colorimetry method; The stock standard solution of gallic acid solution was prepared conc. 5000 mg/mL in ethanol. The standard solution was pipetted to prepared six concentrations(mentioned that concentration value). The sodium carbonate (Na_2CO_3) was added, mixed and adjusted to 10 mL of DI water. The solutions were kept in dark for two hrs. and measured their absorbance by using UV-VIS spectrophotometer at 765 nm.

2.3 Anti-oxidation activity test : Free radical scavenging activity assay; The stock standard solution of 2,2-Diphenyl-1-picrylhydrazyl (DPPH) solutin was prepared conc. 607 µM in methanol. Prepared the DPPH working by pipette DPPH stock solution 1 mL and added methanol 2.1 mL. Pipetted DPPH working solution 200 µL into Well Plate and measured absorbance by Microplate Reader Sunrise. The stock standard solution of trolox was prepared into 10 mM. Measured their absorbance by using UV-VIS spectrophotometer at 517 nm.

2.4 HPLC analysis Sample preparation: **HPLC:** model LC-20A Series, Shimadzu, Japan, column; Shim-pack ISC-07/S 1504 Na. **Mobile phases:** A:0.2N sodium citrate, pH 3.2; B:0.6N sodium citrate+0.2N boric acid, pH10; C:0.2N sodium hydroxide.
Flow rate:0.3 mL/min. **Injection volume**: 10µL. **Post column**: Reaction solution A and B with flow rate 0.3 mL/min.
Detector: Fluorescence detector, RF-515, Ex 348nm, Em 450nm. **Calibration curve and linearity**: Measure of amino acids standard for 5 concentrations by using HPLC-LC-20A, duplicate and read, relative percent different (%RPD)≤10. Peaks concentration (X) and peaks area (Y), read by Regression Coefficient (R^2)≥0.99.
Sample preparation
Weighed mushroom powder into vaccum reaction tube, added 6H HCl 4 mL then mixed by vortex mixer following by Aspirator. The sample solution was digested at 110^0C for 24 hrs., adjusted the volume to 10 mL with 0.1 N HCl after the liquid was at room temperature.
Nuclear Magnetic Resonance (NMR) Spectra: The ^{1}H NMR spectra were measured with a Bruker ASCEND 400 FT-NMR spectrometer, operating at 400 MHz (^{1}H). The chemical shifts (δ_H and δ_C) were recorded in ppm. with reference to residual solvent signal $CDCl_3$ (δ_H 7.24), CD_3OD (δ_H 4.78, 3.31) where appropriated and coupling constants (*J*) were given in Hz.

2.5 Immunostimulant activity
Cytotoxicity: **For MTT assay,** all cell on 96-well plate were washed out. MTT (10 µl, 5 mg/ml) and the medium (190 µl) were added in each well. The cells were then incubated at 370 C 5% CO2 for additional 4 hours. The medium containing MTT was discarded, and MTT formazan that had been produced was extracted with 100 µl of DMSO under 15 minutes agitation. The absorbance was read at 570 nm. The toxicity of extract was indicated by 50% inhibitory concentration (IC_{50}),

comparing the absorbances in control wells which were not exposed to Co, with each of the treated wells.

Amino acid analysis of mushrooms	*Pleurotus ostreatus* (Fr.) Kummer (Nang Rom Tao)	*Pleurotus opuntiae* (Dur.&Lev.) Sacc. (Nang Rom Hungari)	*Pleurotus Sajor-caju* (Fr.) Sing (Nang Fah Phutan Dam)	*Ganoderma lucidum* (Curtis) (Ling Zhi)

Nitrous Oxide (NO) production: For Griess assay, 100 µl of Griess reagent was mixedwith 100 µl of collected medium in a 96-well plate. The mixture was incubated for 15 min at room temperature. The absorbance was determined at 540 nm with the spectrophotometry. The concentration of nitrite was calculated using a calibration standard curve constructed using sodium nitrite dissolved in DMEM.

Phagocytic activity: The RAW264.7 cells were seeded (1×10^5 cells/ml) in a 96-well plate with DMEM medium (10% FBS) and incubated at 37°C in 5% CO_2 for 24 h. The cells were cultured with the eight mushroom extracts KC-MU1 TISTR, KC-MU2 TISTR, KC-MU3 Nakhon Prathom, KC-MU4 TISTR, KC-MU5 Nakhon Prathom, KC-MU6 Nakhon Prathom at 37°C. DMEM and beta-glucan (50 and 100 µg/ml) were used as negative and positive controls, respectively. The stimulated cells were washed twice with phosphate-buffered saline (PBS), and 100 µl of 0.075% neutral red solution was added to each well, followed by incubation for 3 h at 37°C. Following removal of the unphagocytized neutral red by PBS, destain buffer (ethanol:DW:acetic acid, 50:1:49) was added to each well at shaker for 1 h. The optical density (OD) value of each well was measured at 540 nm using a micro-plate reader. Cell phagocytosis (%) was calculated as follows: OD_S / OD_C × 100, where OD_S and OD_C represent the OD values of the stimulated and control wells, respectively. The percentage of phagocytosis in the untreated cells was designated as 100%. All experiments were performed in triplicate.

Statistical Analysis: Significant differences between two normally distributed data were determined with Student's t-test. The values are reported as the means ± SEM. Statistical analysis was performed using SPSS (Version 17.0).

3. Results and Discussion

3.1 Amino acids analysis

The most abundant of amino acids in the four mushrooms included glutamic acid, aspartic acid following by leucine and alanine while phenyl alanine and threonine were found varied in Nang-Rom Hungari (KC-MU2), Nang-Fah-Phuthan-Dam (KC-MU4) and Ling-Zhi (KC-MU6).

Table1 Amino acid analysis of the four mushrooms species, anti-oxidation, total phenolic content and immunostimulant activity of the polysacchraride extracts

Amino acid Mg/100g	KC-MU1 TISTR	KC-MU2 TISTR	KC-MU3 Nakhon Prathom	KC-MU4 TISTR	KC-MU5 Nakhon Prathom	KC-MU6 Nakhon Prathom
Aspartic acid	**1742.90**	**1956.23**	**1708.60**	**1980.42**	**1921.19**	**916.47**
Threonine	**837.36**	**956.79**	**878.69**	**1010.91**	**968.98**	**630.44**
Serine	**929.97**	**1081.02**	**944.46**	**1102.32**	**1024.69**	**573.80**
Glutamic acid	**2897.55**	**3350.86**	**3595.61**	**4541.77**	**3696.89**	**1133.20**
Proline	**681.51**	**793.16**	**707.93**	**846.88**	**832.59**	**419.01**
Glycine	**800.82**	**967.16**	**816.75**	**1009.54**	**906.83**	**493.73**
Alanine	**1110.29**	**1276.92**	**1141.46**	**1456.90**	**1261.88**	**625.97**
Cystine	**86.27**	**101.32**	**109.61**	**105.53**	**131.25**	**88.05**
Valine	**762.79**	**876.80**	**780.86**	**930.84**	**912.08**	**553.19**
Methionine	**231.40**	**254.66**	**256.93**	**246.91**	**303.75**	**106.52**
Isoleucine	**609.93**	**723.51**	**645.41**	**725.92**	**754.06**	**436.95**
Leucine	**1073.77**	**1278.95**	**1113.68**	**1276.90**	**1315.29**	**718.36**
Tyrosine	**429.35**	**458.90**	**438.17**	**466.82**	**523.83**	**260.41**
Phenylalanine	**736.68**	**830.29**	**927.64**	**906.64**	**1442.08**	**650.26**
Histidine	**390.62**	**445.09**	**387.67**	**453.53**	**426.41**	**227.32**
Lysine	**976.52**	**1152.92**	**931.75**	**1134.00**	**1060.24**	**477.29**
Arginine	**875.35**	**1154.38**	**852.41**	**979.40**	**1000.30**	**420.12**
Tryptophan	**234.78**	**273.06**	**214.46**	**268.90**	**224.70**	**107.23**
			Antioxidation			
Extraction A	**56.88±2.46**	**18.30±0.48**	**32.01±0.50**	**71.37±1.61**	**41.01±1.06**	**6.96±0.31**
Extraction B	**58.01±2.10**	**73.93±1.75**	**36.47±4.00**	**82.55±3.35**	**63.56±2.37**	**8.26±0.52**
			Total phenolic content			
Extraction A	**2.01±0.04**	**2.94±0.12**	**1.76 ± 0.09**	**0.99±0.14**	**1.49 ± 0.09**	**3.23±0.01**
Extraction B	**1.25±0.15**	**0.40±0.32**	**0.61 ± 0.41**	**0.37±0.52**	**0.39 ± 0.63**	**2.04±0.02**
			***In vitro* immunostimulant activity**			
Extraction A	**143.95±1.64**	**135.17±4.59**	**-**	**155.00±2.93**	**-**	**187.12±2.17**
Extraction B	**113.67±3.06**	**116.89±3.82**	**-**	**119.44±1.77**	**-**	**166.23±0.56**

3.2 In vitro Immunostimulant activity

The immunostimulant activities of eight mushroom extracts included A and B extracts were performed to evaluate cell viability and nitric oxide (NO) production and % Phagocytic activity, respectively using MTT and Griess assays. The results showed that the cell viability found that Ling-Zhi (KC-MU6) was the highest % viability at concentration 0.5 mg/mL following by Nang-Fah-Phutan-Dam (KC-MU4), Nang-Rom-Tao (KC-MU1) and Nang-Rom-Hungari (KC-MU2) in

extract B, respectively. The viability in A extracts were similarily among the four extracts. Whereas nitric oxide production were varied in the range of 31.06±0.40 to 41.32±1.40 μM, at concentration 0.5 mg/mL in A and B extracts. Meanwhile the % Phagocytic activity of RAW 264.7 cells at concentration 0.5 mg/mL showed that Ling-Zhi (KC-MU6) was the most active

3.3 The ^{1}H NMR spectrum chemical shifts

The ^{1}H NMR spectrum illustrated the A extracts constituting of a mixture of polysaccharidescids, resonated chemical shift at $\boldsymbol{\delta}$ 3.0-4.0 ppm and fatty acids at $\boldsymbol{\delta}$ 0.70-2.30 ppm. While the B extracts showed the signals of $\boldsymbol{\alpha}$-glucan and $\boldsymbol{\beta}$-glucan anomeric protons appeared at δ 5.36 ppm and 4.47 ppm, respectively. The results foundthe extract, containing of polysac Charides possesses quite better immunosti mulant activity than $\boldsymbol{\alpha}$-glucan and $\boldsymbol{\beta}$-glucan composition.

3.4 The antioxidation and total phenolic content

The antioxidation activity of the mushroom in A extracts were increased from KC-MU4, KC-MU1, KC-MU5, KC-MU1 and KC-MU6, respectively. The activity in B extracts were risen from KC-MU4, KC-MU2, KC-MU5, KC-MU1, KC-MU6 and KC-MU3, respectively. The highest activity among all extracts was KC-MU6 (Ling-Zhi) with the extraction A which was related its total phenolic content found the highest amount in both Ling-Zhi Extractions. The lowest antioxidation activity and total phenolic content were KC-MU4.

4. Conclusions

Our results demonstrated that the glutamic acid, aspartic acid and alanine exhibited the most plentiful in the four mushrooms. The content of amino acids was found less effect to the immunostimulant activity for example, the smallest amount of amino acids found in KC-MU6 which exhibited the highest activity compared to the others. On the other hand, the polysaccharides and $\boldsymbol{\alpha}$-glucan as well as $\boldsymbol{\beta}$-glucan composition as a major component in the four mushroom play a key role on the potential to improve and stimulate the phagocytic ability of macrophage RAW 264.7 to produce NO, especially in Ling-Zhi mushroom. The four mushroom with rich polysaccharides than $\boldsymbol{\alpha}$-glucan as well as $\boldsymbol{\beta}$-glucan in the extracts, considering for the greater activity, can be prepared and applied to the development and utilization of future new functional foods and healthy drinks.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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