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Research article

# Investigating the cellular antioxidant and anti-inflammatory effects of the novel peptides in lingzhi mushrooms

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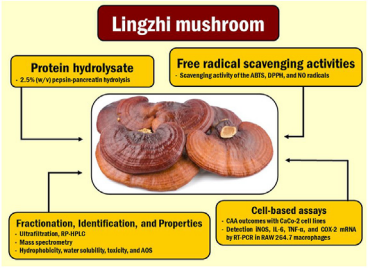
## GRAPHICAL ABSTRACT

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## ABSTRACT

The lingzhi mushroom (*Ganoderma lucidum*) is well known for its medicinal properties and has long played a role in traditional oriental medicine due to its health-giving benefits and potential to extend life expectancy. The mushroom contains a number of highly bioactive compounds and can also act as an excellent source of protein. This research investigated the peptides obtained from the protein hydrolysates of lingzhi mushrooms to assess their free radical scavenging abilities. These peptides were acquired via different proteases (Alcalase, Neutrase, papain, and pepsin-pancreatin) and were tested at a range of different concentrations (1.0%, 2.5%, and 5.0% w/v). The highest levels of 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and nitric oxide (NO) radical scavenging activities were presented by lingzhi mushroom hydrolysate using 2.5% (w/v) pepsin-pancreatin after 6 h of digestion. The hydrolysate was then fractionated using 10, 5, 3, and 0.65 kDa molecular weight cut-off membranes. The results showed that the MW 0.65 kDa fraction had the highest level of free radical scavenging activity. Further analysis of this MW 0.65 kDa fraction began with another RP-HPLC fractionation technique to obtain three further sub-fractions. *De novo* peptide sequencing using electrospray ionization quadrupole time-of-flight mass spectrometry (ESI-Q-TOF-MS/MS) was chosen as the optimum method for studying the $F_3$ sub-fraction. DRVSIYGWG and ALLSISSF were discovered as new peptides with different antioxidant properties. Adenocarcinoma colon (Caco-2) cells showed the antioxidant action of these synthesized peptides. This activity was linked to peptide concentration. The peptides and their pure synthetic

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counterparts were found to reduce NO generation by RAW 264.7 macrophages without causing cytotoxicity. The results of gene expression reveal that the DRVSIYGWG and ALLSISSF peptides were able to cut the expression of the proinflammatory cytokine genes iNOS, IL-6, TNF-α, and COX-2 in the context of RAW 264.7 macrophages.

## 1. Introduction

Humans are frequently exposed to hazardous substances or physiological imbalances that can disrupt the body's normal activities and lead to sickness. The ensuing chronic or acute health issues can be addressed by physiological homeostasis or by utilizing health-promoting drugs. The threat posed by human disease is growing, with some of it being caused by free radicals whose activity can contribute to the oxidation process [1, 2]. Free radicals are ions, atoms, or molecules that have an electron that is not part of a pair. They can be created both exogenously and endogenously. Exogenous sources of free radicals include tobacco, microbial toxins, air pollution, grilled meals, pesticides, heavy or transition metals, and some medicines [3, 4]. Endogenous free radicals are often produced as a result of inflammation, emotional stress, excessive exercise, or immune cell activation. When free radicals are discovered in excess in the human body, they cause oxidative stress, which damages the proteins, lipids, and DNA inside cells, limiting their normal functions. Free radicals have also been linked to the onset of a variety of health problems, including diabetes, stroke, cardiovascular disease, myocardial infarction, and cancer. However, organisms with antioxidant capacities can limit and control these free radicals. It is well understood that both nutritional and non-nutritional food components might increase the risk of acquiring disease or worsening an existing health condition. As a result, functional foods and nutraceutical products have been utilized as alternative or adjuvant treatments to control disease and enhance good health, and this is a study subject that is gaining traction as the food industry strives to attract health-conscious consumers [5, 6, 7].

An important macronutrient, protein provides both needed amino acids and energy. The bioactive peptides (BPs) encoded within some proteins can also enhance health. It is well known that protein hydrolysates or bioactive peptides can be used to create value-added ingredients for food items. BPs are short amino acid sequences that provide physiological effects when consumed *in vivo*. Proteolytic hydrolysis utilizing commercial enzymes or proteolytic microorganisms and fermentation processes can release them from the parent proteins. Using dietary peptide proteins to treat chronic diseases and improve overall health is also gaining popularity [8, 9, 10]. An enzyme is used to hydrolyze dietary proteins to liberate peptide sequences, which are then used to make these peptides. The BPs must then be separated from other molecules in the mixture by further processing. Peptides are created by proteolysis of native dietary proteins and are not found naturally. A variety of human physiological processes can be supported by peptides of low molecular weight [11]. These peptides will be more bioactive than the proteins they come from. Enzymatic hydrolysates with antioxidant activity have been described recently from plant and animal protein sources [12]. Casein [13], peanut [14], fish [15] and soybean proteins [16] are among examples. In food products claiming to be natural antioxidants, these hydrolysates are often employed as food additives. Although mushrooms have long been used as a source of natural medicines and functional foods, little is known about their hydrolysates.

The usage of mushrooms has been thoroughly researched in nations like China, Japan, and Korea, confirming traditional benefits and revealing new prospective uses. Steroids, terpenoids, phenols, nucleotides and their derivatives, polysaccharides, and glycoproteins are common bioactive compounds found in mushrooms [17, 18, 19]. Mushrooms' low-fat content and high polyunsaturated fatty acid to total fatty acid ratio also contribute to their health benefits. The lingzhi mushroom (*Ganoderma lucidum*) is a popular medicinal mushroom believed to extend life and improve general health [20, 21]. There are many commercially available mushroom products, such as teas, powders, and diet supplements [22]. This mushroom has been the subject of a lot of attention because it could be a source of bioactive substances and pharmaceuticals. A lot of chemical compounds that have a pharmacological effect have been found in the mycelium, or the parts of the mushroom that produce fruit, such as triterpenoids, polysaccharides, proteins, and amino acids, were isolated. Lingzhi's bioactive components and their pharmacological properties were covered in a larger collection of papers that looked at previous studies. These compounds can help with diseases like anti-angiogenic, anti-allergic, inflammation, anti-arthritic, anti-osteoporotic, anti-viral, anti-fungal, antibacterial, and anti-androgenic. They can also help with diseases like cardioprotective and carcinostatic [23, 24]. These advantages are not often offered by proteins, and little research has been conducted to investigate the protein content of mushrooms or the bioactivities of their peptides. To maximize the potential of these mushrooms, particularly in the field of natural non-toxic antioxidants, this study aims to synthesize BPs from lingzhi mushroom proteins and then assess their antioxidant capacity in terms of ABTS, DPPH, and NO radical scavenging.

Lingzhi mushroom proteins were hydrolyzed using several protease enzymes in this study, and the antioxidant peptides generated from the lingzhi mushroom protein hydrolysate were then tested (LPH). Ultrafiltration and reversed-phase high-performance liquid chromatography were used to purify the antioxidant peptide (RP-HPLC). The fractions were collected and analyzed for radical scavenging activity by measuring the quantity of any radical scavenging. The peptide sequence was then determined using a quadrupole time-of-flight (Q-TOF) mass spectrometer (LC-MS/MS), and tests were performed to assess the capacity of these peptides to decrease oxidative stress in human colon adenocarcinoma (Caco-2) cells. The anti-inflammatory activity on macrophage RAW 264.7 cells was then evaluated in vitro. The results of this study can be utilized to further develop peptides as viable replacements for chemotherapeutic medicines that have significant side effects.

## 2. Materials and methods

### 2.1. Biological material

The lingzhi mushrooms (*G. lucidum*) were supplied by AU Farm, Khlong Toei District, Bangkok, Thailand. The powder from the lingzhi mushrooms was produced using a slightly modified version of the technique proposed by Ketprayoon et al. [25] Initially, the mushrooms were dried at 60 °C in a hot air oven. A grinder was then used to make a fine powder which was then sieved using a 90 μm mesh. The surface of the mushroom powder then acts to support the hydrolyzing protein. This powder was then stored in a vacuum-sealed polypropylene bag in a desiccator at room temperature until required for further use.

American Type Culture Collection (ATCC, Manassas, VA, USA) provided the human intestinal epithelial cell line, Caco-2 cells (ATCC® HTB-37™) which then underwent culturing in Eagle's Minimum Essential Medium (EMEM) which was supplemented with L-glutamine and 10% fetal bovine serum supplied by Gibco (Rockville, MD, USA). American Type Culture Collection (ATCC, Manassas, VA, USA) also supplied the RAW 264.7 cell lines (ATCC® TIB-71™) which were then grown in Dulbecco's Modified Eagle's Medium (DMEM) and supplemented using high glucose 4.5 g/L with sodium pyruvate, L-glutamine, and 3.7 g/L NaHCO3 (PAN Biotech, Aidenbach, Bavaria, Germany). The cells were stored until use in a humidified chamber under 5% carbon dioxide at a temperature of 37 °C.

### 2.2. Chemicals

L-ascorbic acid, 2,2′-Azobis (2-methylpropionamidine) dihydrochloride (ABAP), 2, 2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

(ABTS), bovine serum albumin (BSA), Coomassie Brilliant Blue G, curcumin from *Curcuma longa* (turmeric), budesonide, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), 2′-7′ dichlorodihydrofluorescein diacetate (DCFH-DA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Dimethyl sulfoxide (DMSO), ferrous sulfate ($FeSO_4$), monosodium dihydrogen orthophosphate ($NaH_2PO_4$), disodium hydrogen phosphate ($Na_2HPO_4$), L-glutamine, hydrogen peroxide ($H_2O_2$), lipopolysaccharides (LPS) from *Escherichia coli*, naphthylethylenediamine dichloride (NED; $C_{12}H_{16}Cl_2N_2$), pancreatin from porcine pancreas (8 × USP specifications), pepsin from porcine gastric mucosa (≥250 U/mg), papain from papaya latex (≥10 U/mg), potassium persulphate ($K_2O_8S2$), protease from *Bacillus amyloliquefaciens* (Neutrase; ≥0.8 U/g), proteinase from *B. licheniformis* (Alcalase; ≥2.4 U/g), quercetin, sulphanilamide ($C_6H_8N_2O_2S$), and sodium nitroprusside (SNP; $C_5FeN_6Na_2O$) were obtained from Sigma-Aldrich, Merck (St. Louis, MO, USA). The chromatographic grade ethanol, acetonitrile (ACN), formic acid, and trifluoroacetic acid (TFA) were provided by Thermo Fisher Scientific (San Jose, CA, USA). All of the substances utilized in this study were of analytical grade.

### 2.3. Amino acid content determination

Standard AOAC techniques were also used to measure the amino acid content of the lingzhi mushrooms, with results of 994.12 [26] and 988.15 [27] via the acid hydrolysis method. This approach requires dissolving 25 mg of lingzhi mushroom powder in 5 mL of 6 M HCl before placing the test tube containing the solution in a heating block for a period of 24 h at a temperature of 110 °C for the purposes of releasing the amino acids. Addition of the internal standard (10 μL of 2.5 mM L-amino-n-butyric acid in 0.1 M HCl) took place prior to diluting the solution with deionized water to 250 μL. The newly diluted solution was then positioned in a heating block for 10 min at a temperature of 55 °C. RP-HPLC was then performed. A Hypersil GOLD column C18 (4.6 mm × 150 mm, 3 μM) was employed for elution using a sodium acetate buffer (pH 4.90) and 60% (v/v) ACN at 0.3 mL/min. The amino acid content was determined with the assistance of ALS Laboratory Group (Thailand) Co., Ltd. (Suan Luang, Bangkok, Thailand).

### 2.4. Preparation of the protein hydrolysates from lingzhi mushrooms

A total of 0.5 g of lingzhi mushroom powder was mixed with 10 mL of 20 mM phosphate buffer with a pH of 7.2. The mixture was then agitated overnight with 150 mM NaCl while the temperature was kept at 4 °C. This was followed by hydrolysis with varied amounts of Alcalase, Neutrase, papain, or pepsin-pancreatin (1.0, 2.5, and 5.0 (w/v)) [28]. The control sample was just a sample with no additive. The samples were activated using the procedure for the enzyme conditions shown in Table 1. Following activation, the temperature was quickly elevated to 80 °C in order to stop the enzyme activity. The resultant hydrolysates were then centrifuged for 30 min at 15,900 g while the temperature was kept at 4 °C. The supernatants were then collected and stored at -20 °C until needed.

### 2.5. Determination of the protein content

The protein hydrolysates obtained was investigated by applying the Bradford procedure to determine the concentration of protein, which was then compared to the BSA standard [29]. The absorbance at 595 nm ($A_{595}$) was then measured using a spectrophotometer (Multiskan GO; Thermo Fisher Scientific, Waltham, MA, USA).

**Table 1.** Enzymatic hydrolysis conditions using lingzhi mushrooms.

| Conditions | Alcalase | Neutrase | Papain | Pepsin-Pancreatin |
|---|---|---|---|---|
| pH | 8.0 | 7.0 | 7.0 | 2.5/7.5 |
| Temperature (°C) | 50 | 50 | 60 | 37 |
| Time (h) | 4 | 4 | 6 | 6 |

### 2.6. Free radical scavenging assay

The ABTS and DPPH assays were used to assess free radical scavenging activity in accordance with the approach proposed by Saisavoey et al. [30]. The radical scavenging activity of nitric oxide (NO) was investigated using a technique followed by Suttisuwan et al. [31].

### 2.7. Calculation of the percentage inhibition

The radical scavenging percentage was calculated using Eq. (1) as shown below:

$$\frac{[(\text{Abs control} - \text{Abs blank}) - (\text{Abs sample} - \text{Abs background})]}{(\text{Abs control} - \text{Abs blank})} \times 100, \quad (1)$$

where Abs control showed the control absorbance (which had no sample content), Abs blank showed the absorbance of deionized water, Abs sample showed the absorbance of the protein hydrolysates from the lingzhi mushrooms, while Abs background showed the color absorbance of the samples. The $IC_{50}$ value for the protein hydrolysate concentration achieving 50% inhibition of antioxidant activity was established using GraphPad Prism v. 6.01 for Windows (GraphPad Software Inc., San Diego, CA, USA).

### 2.8. Isolation and enrichment of bioactive peptides

#### 2.8.1. Ultrafiltration

Ultrafiltration was used to separate the hydrolyzed proteins of the lingzhi mushrooms with MW cut-off membranes as 0.65, 3, 5, and 10 kDa. The resulting fractions were then collected and tested for the antioxidant capabilities.

#### 2.8.2. RP-HPLC (reverse phase high performance liquid chromatography)

The fraction demonstrating the greatest free radical scavenging activity was selected for further purification via reverse phase HPLC (RP-HPLC) using a Luna 5U 250 × 4.6 mm column (Phenomenex, California, USA) and a linear gradient for mobile phase A of 0.1% TFA (v/v), and for mobile phase B of 70% ACN (v/v) in 0.05% TFA (v/v) when the flow rate was 0.7 mL/min. The process was carried out at 25 °C with an injection volume of 20 μL, while the protein concentration of the injected sample was 1.50–2.00 mg protein/mL. The chromatogram was assessed for UV absorbance at 280 nm, and the purified peak was collected and repeated more than 20 times under identical conditions. Centrifugal evaporation was then employed to concentrate the pooling fractions at 5,000 rpm and a temperature of 4 °*C prior* to determining the amino acid sequences and the free radical scavenging activity.

### 2.9. Electrospray (ESI)-Q-TOF-MS/MS and de novo peptide sequencing

An LC-MS/MS system consisting of a liquid chromatography system (Dionex Ultimate 3000, Thermo Scientific, USA) equipped with an electrospray ionization (ESI)/quadrupole ion trap mass spectrometer (Model Amazon SL, Bruker, Germany) was used for the LC-MS/MS experiments. The compounds were separated using a Hypersil Gold reversed-phase column (50 × 0.5 mm, 5 μm), which was protected with a Hypersil Gold guard column (30 × 0.5 mm, 5 μm). The separation was performed at a flow rate of 100 μl/min under the following gradient conditions: 5–80% eluent B from 0-50 min. The solvent systems consisted of solvent A (water with 0.1% formic acid) and solvent B (100% acetonitrile). The initial application of *de novo* sequencing and mascot had the purpose of examining the collected ESI-Q-TOF-MS/MS data in order to then compare the sequences with those found in the NCBI database through the use of BLASTp.

### 2.10. Synthesis of peptides

The peptide which was chosen from the RP-HPLC fraction on the basis of its antioxidant qualities was then used in the process of chemical synthesis via fluorenylmethoxycarbonyl (Fmoc)-strategy using a simultaneous multiple peptide synthesizer (model Applied Biosystems Model 433A) by Applied Biosystems (Foster City, CA, USA) as described by Chen et al. [32]. The purity of the peptides was measured via analytical mass spectrometry with a quadrupole ion trap Thermo Finnigan™ LXQ™ LC-ESI-MS (San Jose, CA, USA) linked to a Surveyor HPLC (Thermo Fisher Scientific, San Jose, CA, USA) on a Phenomenex Luna C18 (10 × 250 mm, 10 mm particle size) column, eluted at a flow-rate of 5 mL/min with a linear gradient of 0–60% acetonitrile (ACN; 0.07% trifluoroacetic acid; TFA) in 0.1% TFA/$H_2O$ (1% ACN/min). This resulted in synthetic peptide purity of no less than 98% according to the HPLC analysis. The identified peptide sequences were DRVSIYGWG and ALLSISSF, with the respective molecular weights of 1053.02 Da and 1052.52 Da. Measurements were also taken to determine the $IC_{50}$ values for these synthetic peptides.

### 2.11. Bioinformatics search in silico for antioxidant peptides derived from LPH

In order to predict the possible antioxidant activity of the DRVSIYGWG and ALLSISSF sequences obtained from the lingzhi mushroom hydrolysates, this study made use of the BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep). To evaluate toxicity, ToxinPred (http://crdd.osdd.net/raghava/toxinpred/) was used, involving a virtual scanning method (VSM) Swiss-Prot based with the SVM (support vector machine) threshold of 0.0. The SVM prediction method was used with the threshold value of 0.0 in order to classify peptides in terms of the toxicity or non-toxicity, while the hydrophobicity was assessed with Peptide 2.0 (https://www.peptide2.com/main_about.php). Peptide solubility was investigated via the online Innovagen server (www.innovagen.com/proteomics-tools).

Antioxidant scores were used to identify lingzhi mushroom protein-derived peptides which might potentially serve as strong antioxidants. The score was based on the peptide sequences which were analyzed for the existence and location of particular amino acid residues. The technique proposed by Conway et al. [33] was used to determine the antioxidant score: Trp (W) within the amino acid sequence, 5 points; Tyr (Y) within the amino acid sequence, 2 points; short sequence length (2–10 residues), 2 points; His (H), Lys (K), Pro (P), Phe (F), Val (V), or Ile (I) within the sequence, 1 point; Tyr (Y), Trp (W), Val (V), or Leu (L) residues at N-terminus, 0.5 points; and Trp (W), Tyr (Y), and Met (M) residues at C-terminus, 0.5 points.

### 2.12. Cell-based antioxidant activity of the synthetic peptides toward Caco-2 cells

#### 2.12.1. Cell viability

An *in vitro* assessment of the synthetic peptides DRVSIYGWG, and ALLSISSF for cytotoxic activity affecting the Caco-2 was carried out, whereby the prepared cell suspensions in EMEM (described in Section 2.1) underwent dilution before plating in 200 μL/wells in 96-well plates to reach the $A_{540}$ value of 1.0 (with density of $5 \times 10^3$ cells/well). This was followed by incubation with 5% carbon dioxide (v/v) for 72 h at a temperature of 37 °C. Fresh EMEM including different concentrations of the two synthetic peptides then replaced the original cell culture medium for further incubation for 72 h. A quantity of 10 μL of 5 mg/mL MTT in normal saline solution was then introduced to each of the wells before mixing and incubation for 4 h. After the subsequent removal of the media, 150 μL of dimethyl sulfoxide was introduced to each of the wells in order to dissolve the insoluble purple formazan crystals. Measurements were finally taken at $A_{540}$ with a microplate reader spectrophotometer. This value was considered to be in direct proportion to the number of viable cells, enabling Eq. (2) to be used to determine the relative percentage cell viability:

$$\text{Cell survival (\%)} = (\text{A540-sample} \times 100)/(\text{A540-control}), \quad (2)$$

in which the control, containing no sample, was indicated to offer cell survival of 100%. Version 6.01 of the GraphPad Prism software was used to calculate the $IC_{50}$ value from data obtained from the assays which were carried out in triplicate.

#### 2.12.2. Cellular antioxidant activity (CAA)

In Caco-2 cells, the CAA of the synthetic peptides DRVSIYGWG and ALLSISSF was determined using the Wolfe and Liu [34] technique. Caco-2 cells seeded in 96-well plate at density of $1.5 \times 10^5$/mL were cultured for 24 h, as described previously with minor modifications [35]. The cells were washed with sterile 20 mM phosphate buffer saline (PBS; pH 7.4) to remove dead or non-adherent cells. Each well was then given 100 μL of various synthetic peptide concentrations as well as 50 μL of 50 μM DCFH-DA probe solution. After 1 h of incubation at 37 °C, the cells were withdrawn from the treatment solutions and washed three times with 100 μL of PBS. With the exception of the blank and negative control wells, each well received 100 μL of 600 M ABAP solution in the last stage. A microplate reader was used to measure the emitted fluorescence at 528 nm after illumination at 485 nm at 5-minute intervals. This was done for 90 min at 37 °C. The CAA unit (% reduction) was calculated using Eq. (3):

$$\text{CAA Unit} = \%\text{Reduction} = (1 - \text{AUCsample/AUCcontrol}) \times 100 \quad (3)$$

where $AUC_{sample}$ indicates the area integrated beneath the curve showing sample fluorescence against time, and $AUC_{control}$ indicates the area integrated from the quercetin control curve obtained after the triplicate findings from four independent trials which were expresses in the form of mean ± standard error.

### 2.13. Synthetic peptide anti-inflammatory activity in the RAW 264.7 cell model

#### 2.13.1. Macrophage RAW 264.7 cells pre-treatment

The macrophage RAW 264.7 cells were placed in 96-well plates at a density of $10^4$ cells per well in 100 μL DMEM, as explained in Section 2.1 before overnight incubation under a humid 5% carbon dioxide atmosphere at a temperature of 37 °C. As a negative control, the medium was replaced by DMEM, while the experiment saw DMEM replaced by various concentrations of the DRVSIYGWG or ALLSISSF peptides, or with budesonide (2.5 μg/mL) to serve as the positive control. Incubation then followed for a period of 1 h. The production of NO was promoted by adding 100 ng/mL of LPS followed by a 12-hour incubation period.

#### 2.13.2. MTT assay for cell viability and proliferation

The cytotoxicity of the DRVSIYGWG and ALLSISSF peptides was performed using a modified version of the technique proposed by Inkanuwat et al. [36] whereby the RAW 264.7 macrophages were added to 96-well plates at a density of $10^4$ cells/well, prior to incubation under 5% carbon dioxide for 24 h at a temperature of 37 °C. Handling of the cells then proceeded with separate peptide test samples and LPS levels. The wells then received supplementation with 100 μL of a 5 mg/mL MTT solution (in PBS) before further incubation for 4 h under humidified 5% carbon dioxide at 37 °C. The medium was then removed, and DMSO added for the purpose of solubilizing the formazan crystals which formed at 100 μL/well. Finally, the measurements at $A_{540}$ nm were performed so that the relative percentage cell viability could be determined via Eq. (2).

#### 2.13.3. Analysis of NO production using RAW 264.7 macrophages

NO production was evaluated via the Griess reaction. In the first step, the RAW 264.7 macrophages were incubated at different concentrations along with the peptides under examination (control 0 mM). Subsequently, the Griess reagent was added in the amount of 50 μL–50 μL of

supernatant culture and then placed in a 96-well plate at room temperature for further incubation for 10 min. An additional 50 μL of 12.5 mM of NED solution was then introduced, followed by a further 10-minute period of incubation. Measurements were then taken at $A_{540}$ nm using a microplate reader.

#### 2.13.4. Determination of iNOS, interleukin 6 (IL-6), tumor necrosis factor α (TNF-α), and cyclooxygenase-2 (COX-2) mRNA levels via real-time quantitative PCR (qRT-PCR) analysis

RAW 264.7 macrophages underwent pre-treatment for 1 h in which the negative control was solvent, and the positive control was synthesized peptide or 2.5 μg/mL budesonide. The pre-treated cells were stimulated by adding 100 ng/mL LPS and incubating for 12 h. Total RNA subsequently underwent extraction using a MasterPure™ Complete DNA and RNA Purification Kit (Epicentre; Lucigen, under LGC, Biosearch Technologies, Middleton, WI) in accordance with the procedures recommended by the manufacturer. RNA concentration measurements were gathered at 260 nm using a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific, Inc.). The reverse transcription process for the total RNA (1 μg) made use of oligo-dT primers and a Precision nanoScript II Reverse Transcription Kit (PrimerDesign, Camberley, UK) in line with the manufacturer's instructions.

To carry out the qRT-PCR analysis, it is necessary to prepare the PCR mixture of 1 μL of cDNA, 1 μL of each primer (10 mM), 10 μL of 2× qPCRBIO SyGreen Mix (PCR Biosystems Ltd., London, UK), and 7 μL of ultrapure water. The qRT-PCR reactions then took place separately to ensure no interference if the different genes were to undergo amplification by MyGo Pro® Real time PCR apparatus (IT-IS International Ltd., Stokesley, UK). This was followed by 2 min of thermal treatment at 95 °C before 40 cycles of 10 s at 95 °C, 20 s at 68 °C (or a cooler 60 °C for TNF-α and IL-6), and 30 s at 72 °C. The final step involved 1 min of melting at 55–95 °C. The RT-PCR analysis was performed using selective primers for mouse β-actin (5′-GATCAAGATCATTGCTCCTCCTG-3′ and 5′-CGCAGCTCAGTAACAGTCCG-3′), TNF-α (5′-GGGCAGGTCTACTTTGGAGTCA-3′ and 5′-ACAGACTGGGGGCTCTGAGG-3′), iNOS (5′-CGGCAAACATGACTTCAGGC-3′ and 5′-TAGGTCGATGCACAACTGGG-3′), IL-6 (5′-CTCTCTGCAAGAGACTTCCATCC-3′ and 5′-ACAGGTCTGTTGGGAGTGGTATC-3′), and COX-2 (5′-CTGACCCCCAAGGCTCAAAT-3′ and 5′-AAGTCCACTCCATGGCCCAG-3′). The internal reference gene used was β-Actin. The Ct value, denoting the threshold cycle, was applied to quantify the relative level of gene expression level using the equation which holds that relative gene expression $= 2^{-\Delta\Delta Ct}$, where ΔΔCt indicates the increase in the gene threshold cycle. While a score equal to 1 represents no change, a score above 1 indicates increased gene expression, while scores below 1 show reduced gene expression.

## 3. Results and discussion

### 3.1. Lingzhi mushroom amino acid composition

The typical kind of amino acid composition found in lingzhi mushroom is shown in Table 2. Edible mushrooms have a relatively high protein content, making them an appealing source for producing protein hydrolysates for food applications, especially in undeveloped countries where complete proteins are expensive. People in these countries typically eat cereal-based diets, which would benefit from the addition of mushrooms [18, 19, 37, 38]. Aps was found to be most abundant in Lingzhi mushroom, whereas Leu and Gln and were found to be the second and the third most abundant amino acids, respectively (Table 2). This was in agreement with the reports of Wang et al. [39], Sanodiya et al. [23], and Zhu et al. [40]. Aps and Gln, which are non-essential amino acids, were the prominent amino acids in all mushrooms. The fact that different mushrooms vary in their amino acid content may be explained by various factors including the species, the developmental conditions, especially the availability of nitrogen during the growth phase, and the part of the mushroom which is sampled. Additional considerations include the substrate used for growth, the pileus size, and the time of harvesting, all of which can affect the nutritional content of the mushroom, as well as the available amino acid. According to Guo et al. [41], Asp was the most abundant endogenous amino acid in the pink oyster mushroom (*Pleurotus djamor*), accounting for 19% of the total. It is well known that Asp and Gln can result in foods with an umami flavor, and they are commonly thought to be monosodium glutamate-like (MSG-like) amino acids. As a result, the high amounts of Asp and Gln content can be attributed to the pleasant taste of these mushrooms [42]. *Volvariella volvacea* may be used in replacement of sodium aspartate monohydrate and sodium glutamate monohydrate in foods to enhance their umami flavor [43].

**Table 2.** Amino acid profiles of the lingzhi mushroom powder (mg/100 mg).

| Amino acid type | Amino acid | mg/100 g |
|---|---|---|
| Hydrophilic | Arginine (Arg) | 436 ± 0.022 |
| | Aspartic acid (Asp) | 973 ± 0.014 |
| | Glutamic acid (Gln) | 868 ± 0.071 |
| | Histidine (His) | 522 ± 0.006 |
| | Lysine (Lys) | 556 ± 0.011 |
| | Serine (Ser) | 709 ± 0.031 |
| | Threonine (Thr) | 766 ± 0.048 |
| Hydrophobic | Alanine (Ala) | 844 ± 0.077 |
| | Cysteine (Cys) | 29.6 ± 0.045 |
| | Glycine (Gly) | 676 ± 0.002 |
| | Isoleucine (Ile) | 444 ± 0.007 |
| | Leucine (Leu) | 935 ± 0.018 |
| | Methionine (Met) | 42.3 ± 0.086 |
| | Phenylalanine (Phe) | 417 ± 0.091 |
| | Proline (Pro) | 640 ± 0.074 |
| | Tryptophan (Trp) | 38.1 ± 0.031 |
| | Tyrosine (Tyr) | 229 ± 0.054 |
| | Valine (Val) | 670 ± 0.008 |
| Total | | 9,795 ± 0.051 |

*All values take the form of mean ± standard deviation and trials were carried out in triplicate.

Mushrooms also contain amino acids, which have antioxidant effects, though these vary greatly depending on the type of amino acid. Hydrophobic amino acids (HAA) have been linked to antioxidant action [44, 45, 46]. According to Table 2, the various species have significant amounts of HAA content, accounting for more than half of all amino acids. Hence, mushrooms with a higher content of HAA will perform better in terms of free radical scavenging activity [47]. A variety of factors influence mushroom composition, including species, harvesting period, and other environmental factors. Sun et al. (2017) discovered that the overall amino acid composition ranged from 14.62 to 131.06 mg/g in the context of 13 examined species of edible mushrooms growing wild in China [48].

### 3.2. Enrichment of peptides obtained from LPH treated with various proteases

Finding bioactive peptides from source proteins might be challenging. Enzymatic hydrolysis (using digestive enzymes like pepsin-pancreatin, or enzymes found in plants and other microbes) and microbial fermentation are illustrations. Proteases like Alcalase, Flavourzyme, Neutrase, Protex 30L, Protease G6, Protamex, and Protease GN are notable examples of industrial proteolytic enzymes obtained from microbes. Plants are the second major source of cysteine proteases, including which comes from pineapple (*Ananas comosus*) stems, and papain which comes from papaya (*Carica papaya*). Following hydrolysis, more active amino acid R groups are exposed, increasing antioxidant activity. Additionally, it has been shown that hydrolysis might result in the creation of hydrophobicity when

proteolysis unfolds the protein chains [10, 49]. However, the breaking of peptide bonds results in an increase in the amount of free amino and carboxyl groups, which results in increased solubility. Hydrolysis can thus vary hydrophobicity based on the source protein's characteristics and the peptides' molecular weight [49, 50]. The enzymes tested in this study were Alcalase, Neutrase, papain, and pepsin-pancreatin.

ABTS, DPPH, and NO radical scavenging assays were used to determine the free radical scavenging capabilities of the LPH. Table 3 presents the findings in the form of $IC_{50}$ values. Antioxidant activity was demonstrated by all of the LPH which had been obtained using a range of proteases, but the best performance was associated with the treatment using 2.5% (w/v) pepsin-pancreatin. In this case, the $IC_{50}$ values for the ABTS, DPPH and NO radical scavenging assays were 30.63 ± 1.11, 76.64 ± 5.79, and 84.54 ± 0.64 μg/mL respectively. It is also important, however, to look at enzyme specificity when hydrolyzing proteins due to the fact that each different enzyme requires its own particular substrate. It is understood that peptide bonds in the protein structure, and also in the aromatic amino acids such as Phe, Tyr, and Tryp, can be broken by pepsin to create highly active polypeptide fragments [51]. Furthermore, the pepsin which occurs in pancreas, a digestive enzyme, is able to hydrolyze the peptide bonds found on the carboxyl side of the Arg and Lys amino acids to produce oligopeptides, which have superior biological attributes [52]. It can therefore be postulated that antioxidant peptides found in rice bran protein hydrolysates which are obtained via the process of pepsin-pancreatin digestion could contain amino acid sequences which have strong hydrophobic residues, leading to greater antioxidant activity in such peptides. In the work of Saisavoey et al. [53] pepsin-pancreatin hydrolysates which were obtained from rice bran (*Oryza sativa* L.) were reported to be capable of scavenging free radicals. Meanwhile, the research of Petsantad et al. [54] revealed that pepsin-pancreatin hydrolysates from the spotted babylon snail (*Babylonia areolata*) demonstrated excellent performance in ABTS, DPPH, and NO assays to determine the radical scavenging activity. In this research, pepsin and pancreatin were used in sequence as the treatment offering the greatest radical scavenging activities, and were thus chosen for the ultrafiltration stage.

**Table 3.** $IC_{50}$ values for LPH generated by several enzymes in the ABTS, DPPH, and NO radical scavenging assays.

| Enzyme | Free radical scavenging activity ($IC_{50}$) (μg/mL) | | |
|---|---|---|---|
| | 1 wt.% | 2.5 wt.% | 5 wt.% |
| **ABTS assay** | | | |
| Alcalase | $82.11 \pm 2.95^{I}$ | $69.55 \pm 2.64^{G}$ | $63.72 \pm 0.94^{F}$ |
| Neutrase | $44.09 \pm 1.62^{C}$ | $50.79 \pm 1.60^{D}$ | $37.27 \pm 1.03^{B}$ |
| Papain | $75.12 \pm 1.87^{H}$ | $99.74 \pm 0.96^{J}$ | $125.30 \pm 1.47^{K}$ |
| Pepsin-Pancreatin | $59.27 \pm 4.05^{E}$ | $30.63 \pm 1.11^{A}$ | $25.49 \pm 2.05^{A}$ |
| **DPPH assay** | | | |
| Alcalase | $12.92 \pm 0.05^{a}$ | $12.86 \pm 0.08^{a}$ | $9.97 \pm 0.31^{a}$ |
| Neutrase | $19.07 \pm 2.19^{bb}$ | $14.88 \pm 0.33^{aabb}$ | $10.16 \pm 0.19^{aa}$ |
| Papain | $18.97 \pm 1.16^{B}$ | $13.60 \pm 0.06^{AB}$ | $9.34 \pm 0.08^{A}$ |
| Pepsin-Pancreatin | $119.77 \pm 4.43^{g}$ | $76.64 \pm 5.79^{a}$ | $82.52 \pm 2.75^{ab}$ |
| **NO assay** | | | |
| Alcalase | $97.65 \pm 0.26^{cc}$ | $111.97 \pm 0.90^{eeff}$ | $88.35 \pm 0.59^{aabb}$ |
| Neutrase | $106.23 \pm 3.91^{ddee}$ | $110.33 \pm 7.17^{ddee}$ | $118.80 \pm 1.15^{ff}$ |
| Papain | $95.49 \pm 2.12^{bbcc}$ | $106.93 \pm 2.57^{ddee}$ | $131.27 \pm 8.91^{gg}$ |
| Pepsin-Pancreatin | $102.80 \pm 1.41^{ccdd}$ | $84.54 \pm 0.64^{aa}$ | $85.81 \pm 2.57^{aa}$ |

[a–i, A–K, aa–gg] values are shown in the form of mean ± standard deviation from tests carried out in triplicate and separation within the column using Duncan's new multiple range test at $p < 0.05$. Means indicated using different letters show the significance between different groups of $p < 0.05$. The positive control used was ascorbic acid in the DPPH and ABTS radical scavenging assays, and the respective recorded $IC_{50}$ values were 18.82 ± 0.34 μg/mL and 123.15 ± 0.27 μg/mL. The positive control in the NO radical scavenging assay was curcumin, with an $IC_{50}$ value of 67.23 ± 5.66 μg/mL.

### 3.3. Free radical scavenging after the fractionation of LPH using ultrafiltration

It is normally the case that when protein hydrolysates are obtained via processes involving biological treatments, they will contain both active and inactive peptides of different sizes and with varying sequences of amino acids, and as a consequence will be able to perform multifunctional roles. Suitable peptides offering the required biological functions are normally present in low concentrations. For the food and pharmaceutical industries, it is therefore necessary to examine the fractionation as well as the concentration of the bioactive peptides in protein hydrolysates. Ultrafiltration (UF) is both rapid and uncomplicated as a means to fractionate mixtures of peptides which involve similar molecular weights. It can be performed in a laboratory, or scaled up for industrial purposes. The technique usually applies different molecular weight cut-off (MWCO) membranes, usually at 100, 20, 10, 5, 3, 1, and 0.65 kDa in order to segregate biopeptides on the basis of their molecular size [8, 10, 55]. The LPH obtained using 2.5% pepsin-pancreatin underwent ultrafiltration using MWCO membranes of 10, 5, 3, and 0.65 kDa. The findings are presented in Table 4, which reveals that the highest activity levels in the ABTS, DPPH and NO radical scavenging assays were achieved by the fraction of MW < 0.65 kDa, for which the respective $IC_{50}$ values were recorded as 0.71 ± 0.06, 3.53 ± 0.08, and 4.20 ± 0.09 μg/mL. It was found that only this smallest fraction produced a lower value for $IC_{50}$ ($p < 0.05$) than the LPH overall or any of the other fractions, and as a consequence, this fraction was selected for ongoing examination. Low molecular weight peptides offer higher antioxidative characteristics, according to the literature, due to reduced steric hindrance and the active core of short chains, which includes hydrophilic and hydrophobic amino acid residues that may easily react with free radicals [8, 55]. These findings support previous study that found peptides obtained by ultrafiltration from the protein hydrolysates of other mushroom species, *Schizophyllum commune* (split gill mushrooms) [45] and *Hericium erinaceus* (lion's mane mushrooms) [46], showed the highest antioxidant performance in the case of the fraction with the lowest molecular weight (MW < 0.65 kDa) when fractions of similar concentration were compared. This heightened efficacy in the case of lower molecular weight is a consequence of the greater absorption of the small peptides since they are more readily capable of crossing the intestinal barrier to then exert their influence within the body.

### 3.4. Fractionation of MW < 0.65 kDa via RP-HPLC and the radical scavenging activity assessment

RP-HPLC often plays a role in separating and purifying proteins and peptides. The method is based upon the fact that proteins may exhibit varying levels of surface hydrophobicity, and can thus be separated as a result of the reversible interactions which take place between the hydrophobic patches found on the surface of the protein, and the hydrophobic groups which form covalent bonds with the relevant matrix. As a

**Table 4.** The $IC_{50}$ values of ABTS, DPPH, and NO radical scavenging activities of 2.5% pepsin-pancreatin hydrolysate derived from lingzhi mushroom protein and its ultrafiltration membrane fractions.

| Molecular weight Cut off (kDa) | Radical scavenging activity ($IC_{50}$) (μg/mL) | | |
|---|---|---|---|
| | ABTS | DPPH | NO |
| >10 | $33.10 \pm 0.67^{D}$ | $38.75 \pm 2.64^{d}$ | $67.34 \pm 5.00^{dd}$ |
| 10–5 | $16.92 \pm 1.14^{C}$ | $39.88 \pm 0.28^{d}$ | $50.67 \pm 1.25^{cc}$ |
| 5–3 | $5.08 \pm 0.67^{B}$ | $11.70 \pm 0.42^{c}$ | $13.10 \pm 0.65^{bb}$ |
| 3–0.65 | $1.83 \pm 0.12^{A}$ | $7.20 \pm 0.16^{b}$ | $7.36 \pm 0.13^{aa}$ |
| <0.65 | $0.72 \pm 0.06^{A}$ | $3.53 \pm 0.08^{a}$ | $4.20 \pm 0.09^{aa}$ |

[a–d, A–D, aa–dd] indicate the difference in mean value with the standard deviation of triplicate determination and separation within the column by Duncan's new multiple range test at $p < 0.05$.

consequence of the characteristics of reversed-phase matrices (in the stationary phase), the bonds typically show great strength, requiring organic solvents including acetonitrile, and ion-pairing agents including TFA. The mobile phases A and B contained 0.1% TFA, which served to enhance and separate the hydrolysate peaks. It was suggested that the concentration of TFA should not be increased, however, since higher levels would cause damage to the packing materials of the column, and in particular to the hydroxyl functional groups. The use of RP-HPLC can take both analytical and preparative forms. The aim of analytical HPLC is to determine the properties and quantitative aspects of the compound under investigation, while preparative HPLC aims to isolate and purify a product for further application [56, 57]. This study further analyzed the MW < 0.65 kDa fraction which had been obtained via ultrafiltration from LPH and which offered the greatest level of antioxidant activity. RP-HPLC was employed to analyze the sample in which there were differences in the molecular polarity. In Figure 1., information about the RP-HPLC elution of the MW < 0.65 kDa fraction is presented. This fraction leads to the production of the four further fractions, $F_{1-4}$. Among these, $F_3$, with the greatest antioxidant activity, exhibited an elution period of 25:06 min, while the respective ABTS, DPPH, and NO assay results shown in Table 5 were 7.11 ± 1.34%, 18.94 ± 1.55%, and 6.47 ± 0.52% ($p < 0.05$). The peptide concentrations for the peptides from these four fractions were about 6.5–7.0 μg/mL Table 6 presents the recorded yield from each of the purification processes. Antioxidative properties of peptides can be determined using the hydrophobic chromatography column for which the timing is particularly important. If the elution takes a long time, this is indicative of greater hydrophobicity, also associated with antioxidative activity [55, 58]. In peptide sequences, the antioxidative activity is related to the time required for peptides in LPH, and the findings can be given further support by the assay for free radical scavenging activities. Earlier research involving the protein hydrolysates of split gill mushrooms [45] and lion's mane mushrooms [46] revealed that RP-HPLC fractionation was followed by enhanced ability in free radical scavenging despite the lack of change in the peptide concentrations. The $F_3$ amino acid sequences could then be identified with confirmation coming from LC-QTOF-MS/MS and UniProt databases with the process of *de novo* sequencing.

### 3.5. Peptide sequence identification and free radical scavenging activities of synthetic peptides

To identify the peptides from the HPLC fractions which demonstrated the greatest antioxidant activity, liquid chromatography quadrupole time of flight mass spectrometry (LC-QTOF-MS/MS) was employed. In order to lower the chance of finding molecules which were not peptides, fragmentation was carried out on several charge ion peaks. The peptide sequences were then determined via examination of the MS/MS peak lists through UniProt databases via the process of *de novo* sequencing. In Figure 2(A, B), the mass spectra (MS) for the antioxidant peptides obtained from the $F_3$ sub-fraction are displayed. Mass spectral analysis revealed the following peptides: The $F_3$ sub-fraction was confirmed to be Asp-Arg-Val-Ser-Ile-Tyr-Gly-Trp-Gly (DRVSIYGWG; $m/z = 1{,}053$ Da) and Ala-Leu-Leu-Ser-Ile-Ser-Ser-Phe (ALLSISSF; $m/z = 838$ Da). Earlier work has shown that most peptides with antioxidant properties obtained from food proteins exhibit molecular weights in the range of 500 Da to 1,800 Da, and typically have from 2-20 amino acids [8]. The DRVSIYGWG and ALLSISSF peptides were similar, respectively, to the hypothetical protein GSI_14420 (7/9), and the hypothetical protein GSI_06145 (6/8), while the identity of the amino acid sequence stood at 75% from *G. sinense* ZZ0214-1 (Accession numbers PIL23111.1 and PIL31444.1). It may therefore be the case that this protein could play a role in transport or signaling. The Innovagen server data confirm that the water solubility of DRVSIYGWG and ALLSISSF is poor, as shown in Table 7. In contrast, however, our research reported that the water solubility of the DRVSIYGWG and ALLSISSF peptides was not poor at concentration below 5.0 mg/mL. As the concentration rose above 5.0 mg/mL, the solubility showed a gradual decline. ToxinPred was used to predict the toxicity based on a hybrid model of motif scanning and dipeptide composition which allows the toxic regions within a protein to be identified. No antioxidant proteins obtained from the lingzhi mushrooms were shown to be potentially toxic according to the findings shown in Table 7. They are therefore suitable candidates for application in the food or pharmaceutical sectors.

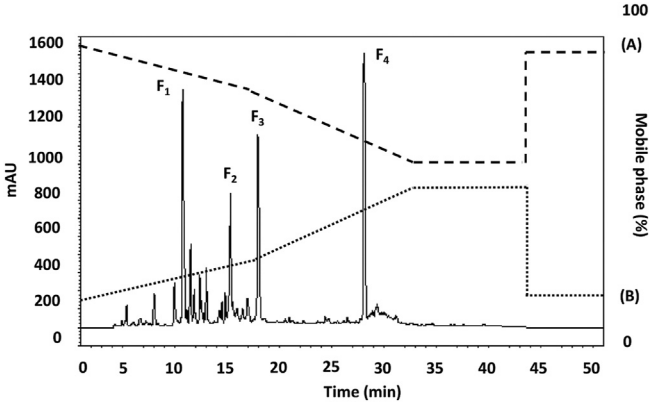


**Figure 1.** RP-HPLC profile for the active fraction (<0.65 kDa) obtained from LPH. Mobile phase A of 0.1% TFA (v/v), and for mobile phase B of 70% ACN (v/v) in 0.05% TFA (v/v).

**Table 5.** Percentage inhibition (%) for the DPPH, ABTS, and NO radical scavenging activity of the fractions obtained via RP-HPLC ($F_{1-4}$).

| Fractions | Percentage inhibition (%) | | |
|---|---|---|---|
| | ABTS | DPPH | NO |
| $F_1$ | $4.05 \pm 0.90^{B}$ | $11.47 \pm 2.69^{b}$ | $5.11 \pm 0.24^{aa}$ |
| $F_2$ | $2.43 \pm 0.26^{B}$ | $12.66 \pm 4.36^{b}$ | $2.81 \pm 1.51^{bb}$ |
| $F_3$ | $7.11 \pm 1.34^{A}$ | $18.94 \pm 1.55^{a}$ | $6.47 \pm 0.52^{aa}$ |
| $F_4$ | $3.66 \pm 1.55^{B}$ | $13.02 \pm 0.14^{b}$ | $3.24 \pm 0.49^{bb}$ |

*All values take the form of mean ± standard deviation and trials were carried out in triplicate (with significant differences considered at $p \leq 0.05$). The positive control used was ascorbic acid (1 μg/mL) in the DPPH and ABTS radical scavenging assays, and the respective recorded inhibition percentages were 95.63 ± 0.46% and 99.82 ± 0.42%. The positive control in the NO radical scavenging assay was curcumin (1 μg/mL), with an inhibition percentage of 85.21 ± 0.61%.

**Table 6.** Yields from each of the purification processes.

| Fractions | Yield (mg protein) | Yield (%) |
|---|---|---|
| Crude extract | 982.75 ± 1.67 | 100 |
| Crude protein hydrolysate | 817.22 ± 7.46 | 83.16 |
| Ultrafiltration | | |
| MW > 10 kDa fraction | 970.95 ± 10.44 | 98.80 |
| MW 5–10 kDa fraction | 427.14 ± 8.42 | 43.46 |
| MW 3–5 kDa fraction | 99.83 ± 8.74 | 10.16 |
| MW 0.65–3 kDa fraction | 37.65 ± 0.60 | 3.89 |
| MW < 0.65 kDa fraction | 15.90 ± 2.82 | 1.62 |
| RP-HPLC | | |
| $F_1$ sub-fraction | 6.53 ± 1.52 | 0.66 |
| $F_2$ sub-fraction | 6.67 ± 3.90 | 0.68 |
| $F_3$ sub-fraction | 6.87 ± 1.96 | 0.70 |
| $F_4$ sub-fraction | 7.02 ± 5.46 | 0.71 |

All values take the form of mean ± standard deviation and trials were carried out in triplicate.

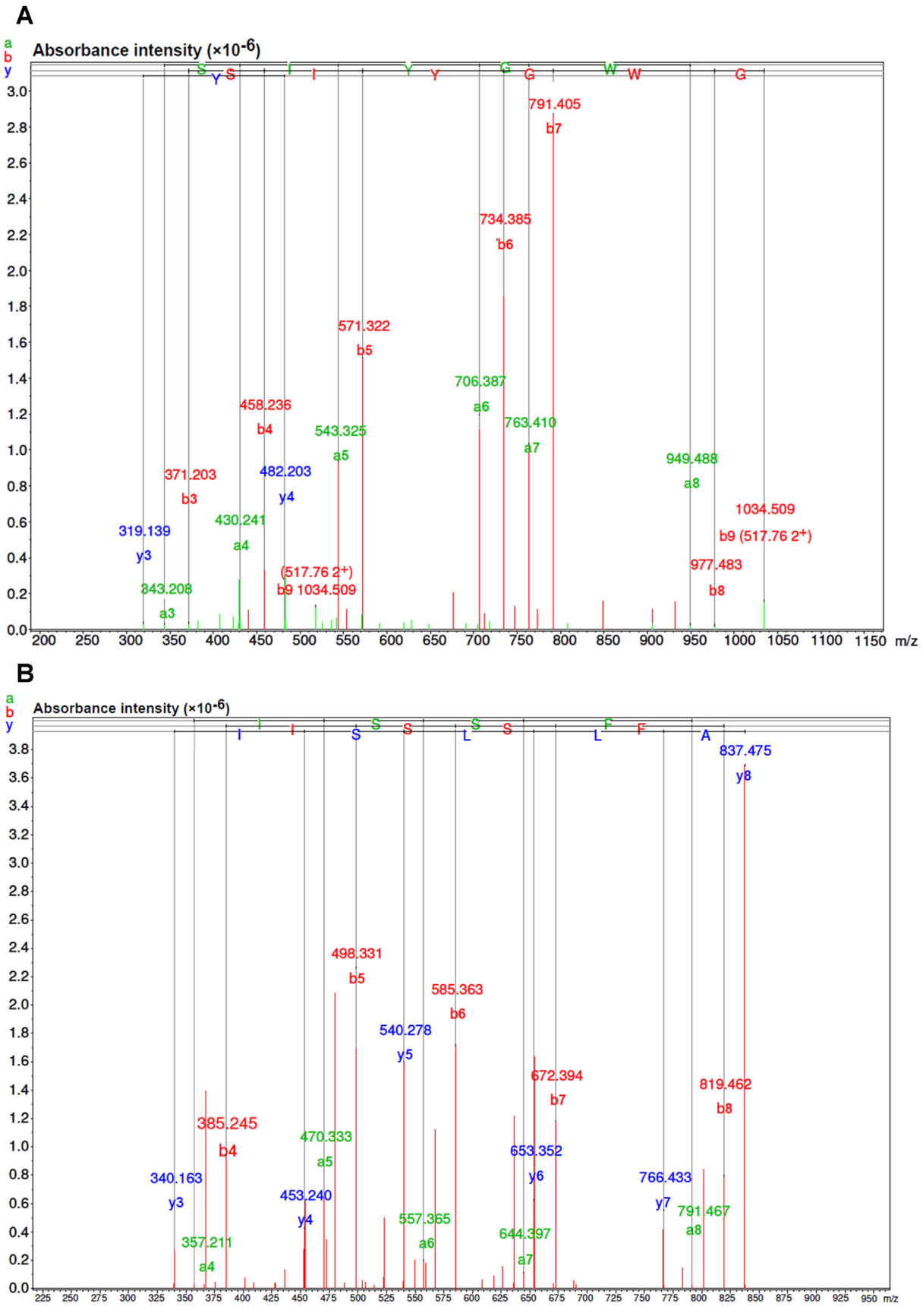


**Figure 2.** The mass fragmentation spectrum for the $F_3$ sub-fraction obtained via RP-HPLC (see Figure 1). (A) DRVSIYGWG, and (B) ALLSISSF.

**Table 7.** The free radical scavenging activity for the synthesized peptides of the $F_3$ sub-fraction.

| Synthesized peptides | Percentage inhibition (%) (1 mg/mL) | | | Antioxidative sequences* | Hydrophobicity (%)** | Water Solubility† | Toxicity# | AOS |
|---|---|---|---|---|---|---|---|---|
| | ABTS | DPPH | NO | | | | | |
| DRVSIYGWG | $2.37 \pm 1.00^{A}$ | $8.01 \pm 0.87^{a}$ | $3.16 \pm 2.17^{aa}$ | IY, WG | 33.33 | Poor | non-toxic | 10 |
| ALLSISSF | $1.72 \pm 0.54^{A}$ | $7.53 \pm 0.68^{a}$ | $1.69 \pm 0.88^{aa}$ | - | 62.50 | Poor | non-toxic | 3 |

[a,A]those means which have the same superscript letter within a row or column show statistical non-significance ($p > 0.05$).
[*] Data are obtained from the BIOPEP database.
[**] Calculations were performed using the peptide property calculator (www.peptide2.com).
[†] The Innovagen server was used for peptide solubility data (www.innovagen.com/proteomics-tools).
[#] Peptide toxicity analysis was performed using the ToxinPred server (http://crdd.osdd.net/raghava/toxinpred/).

The two peptides which had been identified underwent synthesis prior to assessing the antioxidant capabilities, which were clearly demonstrated by both peptides, although for DRVSIYGWG, the ABTS, DPPH, and NO radical scavenging was superior. The $F_3$ peptides, DRVSIYGWG and ALLSISSF, had antioxidant properties which were significantly influenced by the respective hydrophobic amino acid residue content of 33.33% and 62.50%. It was found that the hydrophobic amino acid levels in ALLSISSF exceeded those in DRVSIYGWG, but the composition of amino acids in ALLSISSF differed from DRVSIYGWG due to a greater proportion of aromatic amino acids including Trp and Tyr. The antioxidant properties of peptides can be enhanced by hydrophobic amino acids, since they make hydrophobic polyunsaturated fatty acid chains inside the biological membranes which then serve to restrict oxidative harm. In any biological system this can be very important since there is a tendency for unsaturated fatty acids within cell membranes to sustain oxidative damage caused by free radicals and oxygen species [8, 9, 16]. Furthermore, it was apparent that the Asp amino acid occurred at the N-terminus location of DRVSIYGWG. Studies seeking to examine the antioxidant properties of fish protein hydrolysates showed that Asp is able to donate electrons while also playing a role in the DPPH scavenging, ferric reduction, and hydrogen peroxide scavenging capabilities of some types of food protein hydrolysates.

The peptide AOS could be approximated by considering the presence and location of specific amino acids along with the peptide lengths. Under this scoring approach, those peptides allocated a score exceeding 5 were determined to offer potential antioxidant activities [33]. In this context, the AOS for DRVSIYGWG was greater than for ALLSISSF, as shown in Table 7. Meanwhile, the most suitable residues to promote antioxidant activity were determined to come from the aromatic amino acids, Trp and Tyr. This could be attributed to hydrogen-donating indole and phenolic groups. Furthermore, even greater effectiveness has been suggested in the case of the amino acids being positioned at the N- and C-terminus locations [8].

### 3.6. Caco-2 cell-based antioxidant activity assay

In order to study the extent to which bioactive components can permeate the intestines, *in vitro* studies can be performed using human adenocarcinoma colon (Caco-2) cell monolayers since they are very similar to the intestinal endothelium cells [34, 35]. The tight junctions created by these cells provide them with the necessary functional and morphological attributes of enterocytes, while it is possible that the fully intact absorbance of smaller peptides may be absorbed intact across the brush border membrane. Caco-2 cellular models have long been used for assessments of the potential for new drugs under the conditions of human intestinal absorption, so in this case the antioxidant activity assay makes use of Caco-2 cells to determine how the initial intestinal absorption will later affect the influence of the antioxidants within the body [59]. Given that the normal path for antioxidant food compounds to enter the body is via the mouth, the antioxidant functions are most significantly affected by the absorption properties and the ability to cross the intestinal barrier. Therefore, it may be the case that the Caco-2 cellular model might be the ideal approach for screening phytochemicals and food products to assess any antioxidant potential [60]. Through the process of chemical assays performed *in vitro*, DRVSIYGWG and ALLSISSF demonstrated antioxidant capabilities, but it is important to note the absence of biological relevance as well as the matrix environment of bioavailability. There was no involvement of the action and metabolism mechanisms which would take place *in vivo*, so further studies within a biological cell system would be necessary to establish the *in vivo* antioxidant properties of DRVSIYGWG and ALLSISSF.

To establish the most suitable peptide treatments for cells involved in the *in vivo* assay, Caco-2 cell viability was evaluated via CAA. Figure 3(A) shows that there were no changes in cell viability as a consequence of the activity of the DRVSIYGWG and ALLSISSF peptides, suggesting that the concentration range in the study (0.24–120 mM) had no adverse effects during incubation upon cell integrity. All samples with concentrations selected for this study were non-cytotoxic on Caco-2 cells. The cell viability was shown to be marginally greater when hydrolysates or their fractions were present, possibly due to the improved nutrient balance required for cell survival. In a study of the spotted babylon snail, Petsantad et al. [54] found that the synthetic peptides which were obtained caused no difference in the cell viability for Caco-2 cells at the tested concentrations (1.88–120 mM). It was noted, however, that cell metabolism might require the amino acids, short chain proteins, or peptides which are produced through hydrolysis. Meanwhile, research into split gill mushroom protein hydrolysates by Wongaem et al. [45] revealed that the MW < 0.65 kDa fraction was not toxic towards the human intestinal cell line (HT-29) at concentrations in the range of 0.00781–0.45 μg/mL.

It was clear that the involvement of the DRVSIYGWG and ALLSISSF peptides in the pre-treatment could lead to a reduction in the intensity of DCF fluorescence. The results in Figure 3(B) showed similarity among all concentrations up to 60 mM for DCF fluorescent intensity ($p > 0.05$) in the case of the DRVSIYGWG and ALLSISSF peptides, allowing the conclusion that a cellular radical scavenging effect took place. These findings indicate that cells can be protected from ROS oxidative harm by both of the peptides, which may therefore be suitable biomolecules for further development to prevent the formation of ROS. This is significant since ROS generation is cytotoxic and has been connected to a variety of degenerative disorders, and elevated ROS concentrations can also increase the probability of acquiring intestinal illnesses. As a result, there is a growing interest in determining the ability of dietary antioxidants to protect intestinal epithelial cells from oxidative stress. Similarly, peptides produced by the split gill mushroom [45] and the spotted babylon snail [54] protected ABAP-stimulated HT-29 and Caco-2 cells. These peptides have multifunctional attributes which have been demonstrated *in vitro* to be able to hold ROS concentrations to a very low level. The compounds are believed to be both safe and healthy, and are characterized by a low molecular weight, good ease of absorption, and a high level of activity. These peptides can also act alongside other non-peptide antioxidants, thus improving the overall protection they can offer.

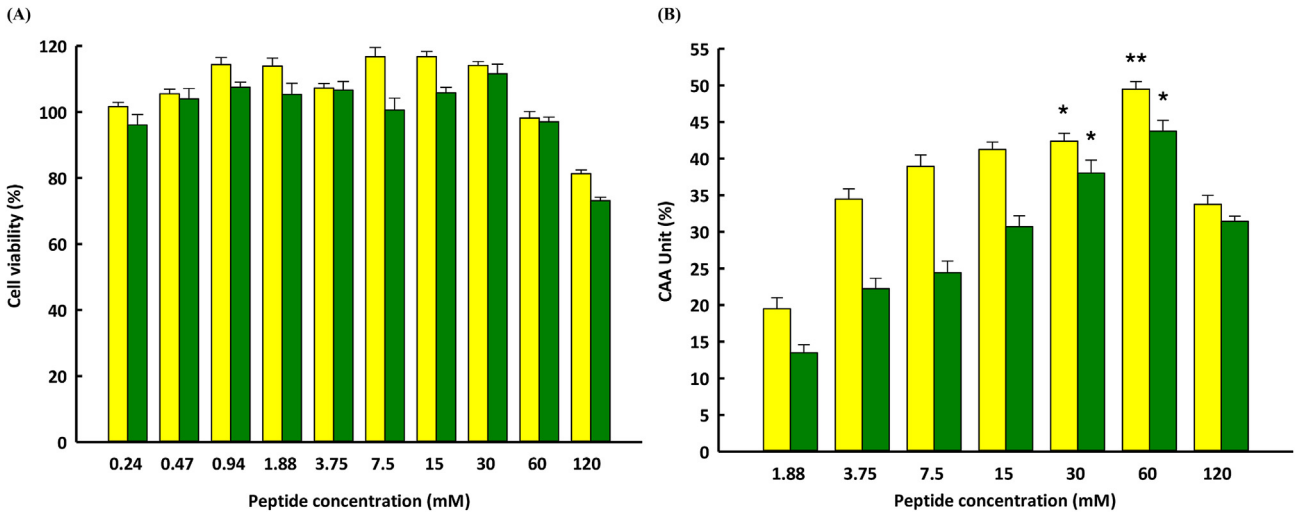


**Figure 3.** (A) Caco-2 cell viability after treatment at different concentrations of (●) DRVSIYGWG, and (●) ALLSISSF for 72 h, and (B) cellular antioxidant activity of (●) DRVSIYGWG, and (●) ALLSISSF. The standard deviation (n = 3) is indicated by the bars.

Numerous researchers have suggested that peptide mixtures will exhibit bioactivity levels which depend upon their molecular sizes, with stronger antioxidant activity occurring in fractions of low molecular weight. This can be explained by the fact that hydrophobic amino acids can be found in peptides of low molecular weight. It has been shown, for instance, that whey hydrolysate peptides of low molecular weight (0.1–2.8 kDa) offer strong antioxidative activity and are able to prevent lung fibroblast MRC-5 cells from becoming damaged when exposed to hydrogen peroxide [61]. One earlier study revealed that peptides of low molecular weight (941.43 Da) derived from the by-products of duck skin are effective free radical scavengers and also prevent oxidative damage to the liver [62]. In the earlier works, it was found that the 0–3 kDa of monkfish muscle protein hydrolysate (MPTH-I) [63], blood plasma hydrolysate (BPH4) [64], and peptides obtained from biodegraded chicken feathers (PBCF) [65] achieved greater levels of antioxidant activity when compared to other molecular weights, offering support to our finding that low molecular weight produce the greatest antioxidant activity.

### 3.7. Anti-inflammatory effects in RAW264.7 macrophage cells induced by LPS

Macrophages act as the primary mechanism to react to infections, and have a key role to play in the inflammatory response to counter any invasion of pathogens. In this research, the LPS-stimulated macrophage cell line RAW 264.7 was the model employed to assess the anti-inflammatory qualities of the DRVSIYGWG and ALLSISSF peptides. This particular cell line often serves in this capacity for studies of anti-inflammatory activity as well as the molecular mechanisms which underpin the activity of various agent types. It is helpful that the mouse macrophages generate similar inflammatory mediators to those found in humans. Furthermore, any compound which acts to counter inflammation in the mouse macrophage cell line is likely to work well in the human context. The MTT assay was employed to assess the cytotoxicity of DRVSIYGWG and ALLSISSF towards RAW 264.7 cells. The results are shown in Figure 4(A)., and clearly indicate no cytotoxicity from the DRVSIYGWG and ALLSISSF peptides in the context of RAW264.7 cells for the range of concentrations tested

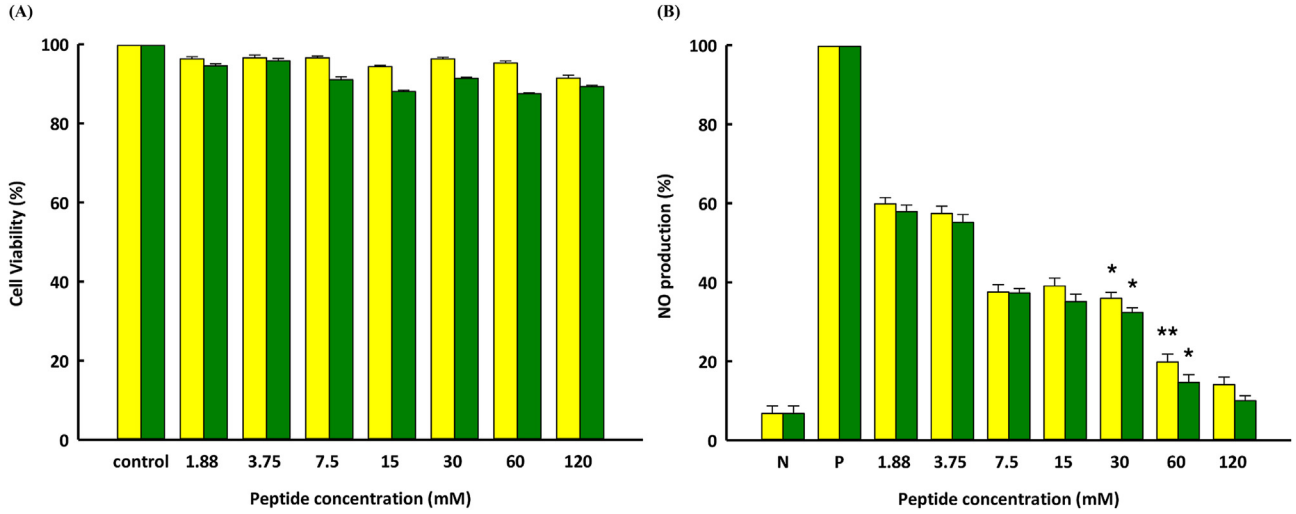


**Figure 4.** The influence of various synthesized peptide concentrations for (●) DRVSIYGWG, and (●) ALLSISSF on: (A) viability of, and (B) NO production by, *E. coli* lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. The negative controls are represented by N and P (only cells) while the positive controls involve cells + LPS. Mean ± SD, $n = 3$, and $^{*}P < 0.05$ and $^{**}P < 0.001$.

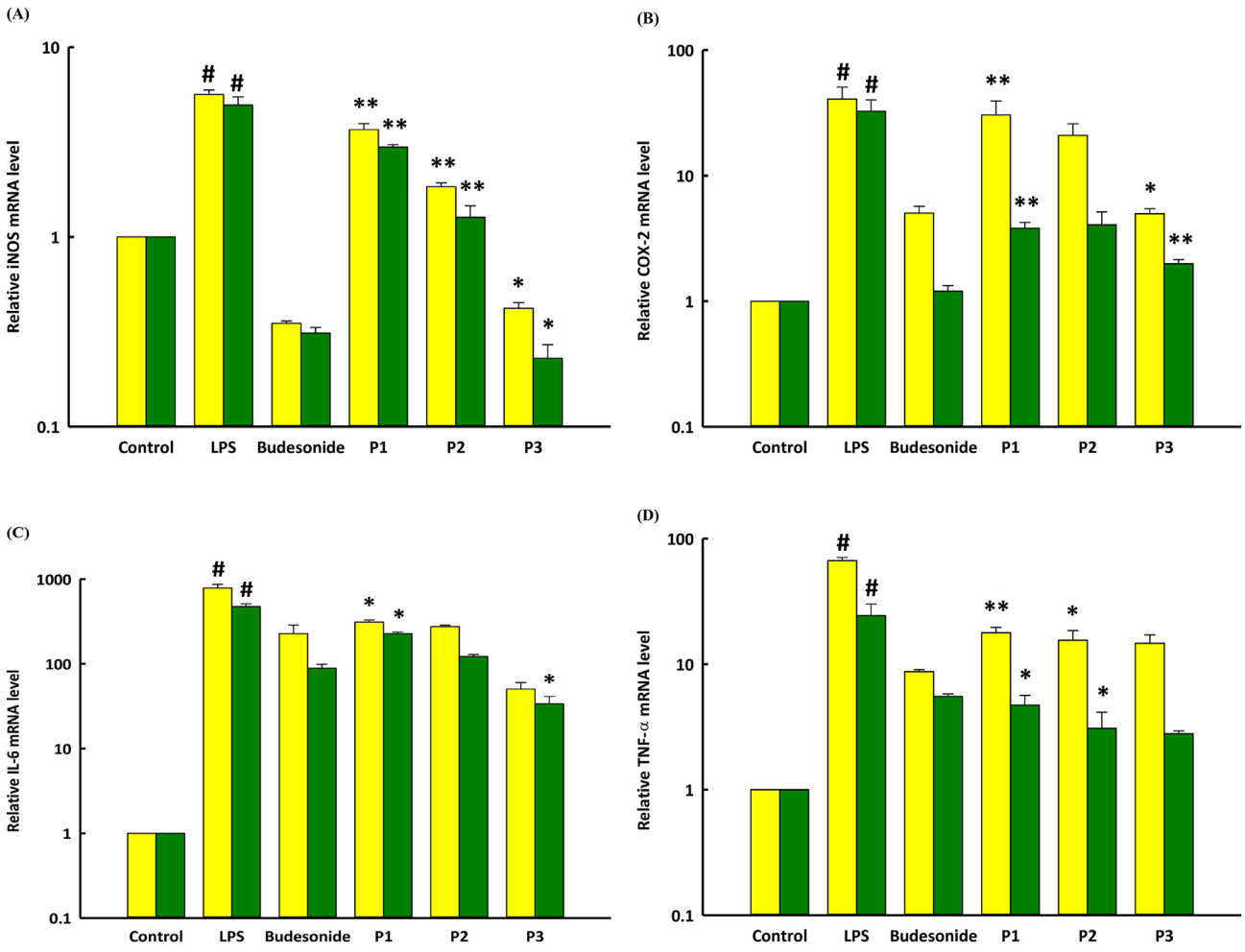


**Figure 5.** The influence of the synthesized peptides (●) DRVSIYGWG, and (●) ALLSISSF upon the levels of transcript expression for: (A) inducible nitric oxide synthase (iNOS); (B) cyclooxygenase-2 (COX-2); (C) interleukin 6 (IL-6); and (D) tumor necrosis factor (TNF-α) in LPS-stimulated RAW264.7 cells evaluated using two-stage qRT-PCR analysis. The cells underwent incubation in the presence of the peptide [30 ($P_1$), 60 ($P_2$) or 120 ($P_3$) mM] for 12 h with 100 ng/mL LPS, or with 2.5 μg/mL budesonide which served as the positive control. All values are presented in the form of mean ± standard deviation from trials performed in triplicate. The levels of statistical significance are set to $^{*}P < 0.05$ and $^{**}P < 0.001$ for comparison with LPS, and to $^{\#}P < 0.01$ for comparisons with the negative control.

(1.88–120 mM). When oxidative stress arises in the cells, NO production is the natural inflammatory response. Excessive NO production is implicated in cases of diabetes mellitus, neurodegenerative problems, and health conditions related to inflammation. Oxidative signaling is known to be inhibited by peptides with antioxidant capabilities, and this will mitigate the inflammatory response [66]. The production of NO in LPS-stimulated macrophage cells was significantly greater than in the case of untreated cells. NO production was shown to be significantly increased as a consequence of the stimulation of RAW264.7 cells using LPS (100 ng/mL), when tested against an unstimulated sample. The inhibition of NO production on the basis of dosage was reported with $IC_{50}$ values of 76.76 ± 0.18 and 65.86 ± 0.05 mM for DRVSIYGWG and ALLSISSF respectively, as shown in Figure 4(B). Earlier research showed that soy protein hydrolyzed with Alcalase could restrict NO production in the range of 18–35% in macrophage cells induced by LPS. Various mechanisms might explain the way proteins and peptides can inhibit the effects on macrophage cell lines induced by LPS. These include the potential for binding to the lipid A moiety of LPS, or interfering with the LPS-CD14 interaction through the effect upon the LPS-binding protein. The amino acid sequence has also been suggested as a possible factor contributing to the inhibitory effect due to the ability to alter the cell internalization of the peptides [67].

In this study, the researchers also examined the role of DRVSIYGWG and ALLSISSF by conducting qRT-PCR analysis of iNOS and COX-2 to determine the gene and protein expressions. Figure 5(A) and (B) present the results of treatment solely with LPS, in which the gene expression iNOS and COX-2 showed a notable increase, while in contrast, the effect of DRVSIYGWG and ALLSISSF was inhibition of iNOS and COX-2 gene expression. Three NO synthases (NOS) capable of forming NO include NOS-I (neural NOS, nNOS), NOS-II (inducible NOS, iNOS), and NOS-III (endothelial NOS, eNOS). While iNOS is not usually found in living cells, its induction is possible via stimulation from cytokines, LPS, bacterial toxins, or the activity of NF-κB. The outcome is substantial NO production over long periods of time. The role of NO is reacted with superoxides in the body, thus supporting the inflammatory reaction by triggering macrophages among other inflammatory substances. However, this can cause tissue damage due to the strength of the inflammatory response. There are two kinds of cyclooxygenases (COX): COX-1 and COX-2. COX-1 occurs widely and plays a part in normal physiology, whereas the expression of COX-2 is rather unlikely in normal circumstances. Its induction can be rapidly stimulated by cytokines and LPS and it contributes to the inflammatory response [68]. The findings indicate that a reduction in the gene expression of iNOS and COX-2 serves to confirm the anti-inflammatory qualities of DRVSIYGWG and ALLSISSF.

By examining the extent of the gene expression of IL-6 and TNF-α arising in RAW 264.7 cells it is possible to establish the extent to which DRVSIYGWG and ALLSISSF treatment can reduce the pro-inflammatory cytokines. It can be observed in Figure 5(C) that the use of

DRVSIYGWG and ALLSISSF at different concentrations could lower the secretion of IL-6 on the basis of dosage. Indeed, as the concentration of DRVSIYGWG and ALLSISSF rises there is usually a drop in IL-6 gene expression; if the concentration of DRVSIYGWG and ALLSISSF exceeds 60 mM, IL-6 gene expression drops rapidly. The production of TNF-α falls in line with the applied dosage in the presence of DRVSIYGWG and ALLSISSF at 30–120 mM, as shown in Figure 5(D). As shown by the treatment of cells with DRVSIYGWG and ALLSISSF, TNF-α expression in RAW 264.7 macrophages was reduced by both peptides in a dose-dependent manner. Inflammatory reactions are mediated by inflammatory cytokines, including IL-6 and TNF-α in the case of the initial responses. In cases of cancer, and some other inflammatory diseases, these mediators tend to be produced in excessive quantities. Once very important inflammatory cytokine is TNF-α, since it plays a major role in physiological immunity. However, it can also be responsible for the onset of chronic inflammation and associated diseases after LPS stimulation. IL-6 is known to be a central inflammatory cytokine, so where the production of these cytokines can be managed, it is possible to prevent inflammatory diseases from developing and progressing [69, 70]. In this context, DRVSIYGWG and ALLSISSF are able to provide inflammatory response regulation through the influence exerted upon IL-6 and TNFα secretion, with a high probability of also affecting NF-κB and MAPK signaling.

For instance, the work of Inkanuwat et al. [36] showed that another anti-inflammatory peptide, SNPSVAGVR, could significantly upregulate the expression of iNOS and TNF-α at 120 mM, while IL-6 expression was downregulated. Meanwhile, it was reported by Sangtanoo et al. [71] that the synthesized peptides LSPLLAAH and TVNLAYY obtained from the protein hydrolysates of Peanut worm (Sipunculus nudus Linn.) could boost the level of NO while simultaneously reducing the levels of iNOS, IL-6, TNF- α, and COX-2 in RAW 264.7 macrophages. This suppression effect was also seen in the bioactive chemical Artepillin C, which inhibited IL-1β and TNF-α, while having no effect upon IL-6 or the anti-inflammatory cytokine IL-10 [72]. It will, however, be necessary to carry out further *in vivo* work on the anti-inflammatory and antioxidant qualities of these particular peptide fractions.

## 4. Conclusion

During the course of this research, it was confirmed that it was possible to obtain peptides offering free radical scavenging capabilities demonstrated in ABTS, DPPH, and NO assays, from LPH hydrolysates which underwent preparation with 2.5% (w/v) pepsin-pancreatin. Fractions obtained using various membranes through ultrafiltration were examined to determine their activity levels, revealing that peptides of low molecular weight (MW $<$ 0.65 kDa) exhibited significant antioxidant activity. Further fractionation using RP-HPLC resulted in the creation of the $F_{1\text{-}4}$ fractions, with $F_3$ demonstrating the greatest activity levels in the free radical scavenging assay. Q-TOF-LC-MS/MS amino acid sequencing therefore followed, and the sequences of two peptides were revealed: DRVSIYGWG and ALLSISSF. These identified peptides underwent synthesis and an investigation of their antioxidant properties was carried out, with both demonstrating clear antioxidant activity, albeit with the superior radical scavenging exhibited by DRVSIYGWG. The two synthesized peptides had cytoprotective qualities in Caco-2 cells during CAA, but this varied depending on the peptide concentration, was non-cytotoxic, and had the ability to inhibit the pro-inflammatory mediators iNOS, COX-2, IL-6, and TNF-, on a selective basis at the transcription level in RAW264.7 macrophage cells following LPS stimulation. This study holds the distinction of being the first to confirm the ability of peptides derived from lingzhi mushroom proteins to reduce oxidative stress and inflammation. In future, it would be beneficial to examine the mechanisms by which this can be achieved, as well as the peptide bioavailability which should be studied both *in vitro* and *in vivo*.

## Declarations

### *Author contribution statement*

Thanyaporn Aursuwanna: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Sajee Noitang, Tanatorn Saisavoey, Songchan Puthong: Performed the experiments.

Papassara Sangtanoo, Piroonporn Srimongkol: Performed the experiments; Analyzed and interpreted the data.

Onrapak Reamtong: Analyzed and interpreted the data.

Aphichart Karnchanatat: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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### *Data availability statement*

Data included in article/supplementary material/referenced in article.

### *Declaration of interest's statement*

The authors declare no conflict of interest.

### *Additional information*

No additional information is available for this paper.

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