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Effects of ultrasound-assisted extraction on antioxidant activity and bidirectional immunomodulatory activity of *Flammulina velutipes* polysaccharide

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ABSTRACT

In order to investigate the impacts of ultrasound-assisted extraction (UAE) on *Flammulina velutipes* polysaccharides (FVPs), the differences between FVPs extracted by ultrasound-assisted extraction (FVPU) and FVPs extracted by hot water extraction (FVPH) were compared in terms of yield, primary compositions, surface microstructure, helix-coil transition structure, molecular weight distribution, antioxidant activity, and bidirectional immunomodulatory activity. Results indicated that UAE changed the above properties of FVPs. Compared with FVPH, higher yield, protein content, and uronic acid content but lower polysaccharide and polyphenol contents were observed in FVPU. UAE changed the surface microstructure, destroyed the triple helix structure, and increased the proportion of low molecular weight polysaccharide components of FVPU. Compared with FVPH, FVPU showed a stronger reducing power and scavenging activities on DPPH radical, hydroxyl radical, and superoxide anion radical. FVPU was a better inhibitor of inflammation compared with FVPH. However, FVPH had a better immunity enhancing effect compared with FVPU. These results were attributed to the cavitation effect of ultrasonic waves on the structure of polysaccharides during the extraction process of UAE. These findings suggested that UAE was an efficient and environmentally friendly method to produce new polysaccharides from *F. velutipes* for the development of functional foods or nutraceuticals.

1. Introduction

Flammulina velutipes (F. velutipes), also named "enokitake" in Japan or "golden needle mushroom" in China, is a tasty fungus cultivated extensively with high medicinal therapeutic value [1,2]. In recent years, large-scale artificial cultivation of *F. velutipes* has been established in Asia due to the increasing consumption demand. As a delicious ingredient, *F. velutipes* could be processed to various foods, such as noodles, jellies, and drinks [3–5]. Polysaccharide has been known as an important active ingredient in *F. velutipes* [2]. Various bioactivities of *F. velutipes* polysaccharides (FVPs) such as antitumor, hepatocyte protection, antioxidant effect, immunomodulatory activity, intestinal flora regulation, and memory improvement activity in mice have been reported [6–9]. There are considerable potentials for FVPs to be investigated as additives of functional foods [10]. In recent years, ultrasound-assisted extraction (UAE) has been applied in the extraction of polysaccharides due to its many advantages including time-saving, yield-improvement, and low environment-polluting risks [11]. UAE works through the cavitation process which makes mechanical erosion of solid particles and small bubbles in liquids. The cavitation could cause a local increase in pressure and temperature which promotes the diffusivity, solubility, and transport of solute molecules [12–16]. However, previous studies on the UAE of FVPs mainly focused on the optimization of parameters during the extraction process to enhance higher yield [17,18]. The effects of UAE on the structure and bioactivity of FVPs have not been explored. Therefore, the objective of this study was to investigate the effects of UAE on FVPs in terms of primary characterizations, antioxidant activity, and bidirectional immunomodulatory activity. This study provides references for the efficient and environmentally friendly extraction of FVPs with high bioactivity.

2. Materials and methods

2.1. Materials

Fresh *F. velutipes* (FV088) were provided by Jiangnan Biological Technology Co., Ltd. (Zhenjiang, China). Expressed on dry weight basis

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(*w*/w), the contents of polysaccharide, crude protein, crude fat, and ash were 13.93 ± 3.92%, 21.03 ± 0.49%, 0.44 ± 0.08%, and 5.55 ± 0.11%, respectively. RAW264.7 cells were obtained from CBCAS (Cell Bank of the Chinese Academy of Sciences, Shanghai, China). Dexamethasone was purchased from Saiguo Biotech Co., Ltd. (Guangzhou, China). Vitamin C, polymyxin B, penicillin-streptomycin Liquid (100×) and fetal bovine serum were obtained from Solarbio Technology Co., Ltd. (Beijing, China). Lipopolysaccharide (LPS) was obtained from Yuanye Biological Technology Co., Ltd. (Shanghai, China). Enzyme-linked immunosorbent assay (ELSA) kits for detecting nitric oxide (NO), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and type II interferon (IFN-γ) secretions were provided from Jiancheng Biological Technology Co., Ltd. (Nanjing, China). Dulbecco's modified Eagle's medium (DMEM) culture (high glucose) was purchased from GE Healthcare Life Sciences Hyclone Laboratories (Utah, United States). All other chemicals were of analytical grade unless specified and purchased from local suppliers.

2.2. Preparation of FVPs

2.2.1. Hot water extraction

Fresh *F. velutipes* were freeze-dried (FreeZone 2.5, Labconco Corporation, Missouri, United States) and powdered *via* superfine comminution technique (300 mesh, WFJ-20, Wenzhou Dingli Medical Equipment Co., Ltd. Zhejiang, China). The pretreated powder (10 g) was extracted using hot ultrapure water at 80 °C for 2 h with a material/liquid ratio of 1:25(*w*/*v*). Subsequently, the extracted solution was centrifuged (5000 rpm for 5 min) and the supernatant was collected. The supernatant was then concentrated by a rotary evaporator (N-1300D-WB, EYELA Co., Ltd.) at reduced pressure. Thereafter, the supernatant was well-mixed with 4 times volume Sevage reagent and centrifuged (5000 rpm for 30 min). This operation was repeated 6 times to remove the proteins impurities of FVPs. Finally, the concentrated extract was precipitated by adding 4 volumes of ethanol and stored at 4 °C overnight. The precipitates were collected and freeze-dried to obtain FVPs. Taking D-glucose as the standard, the phenol-sulfuric acid method was used to determine the contents of polysaccharides.

2.2.2. Ultrasound-assisted extraction

FVPU was extracted according to procedures established by our laboratory with some modifications [18,19]. Specifically, *F. velutipes* powders were weighed accurately (10 g) and extracted in ultrapure water (1:25 *w*/*v*) with a JOYN-3000A ultrasonic extractor (Shanghai Qiao Yue Electronics Co. Ltd., China) under optimal conditions (620 W, 45 °C, and 20 min) [18]. The ultrasonic probe was applied into 250 mL of each sample solution through a horn of 10 mm diameter at 20 kHz, in pulsed mode (1 s on/2 s off). After centrifuging (5000 rpm for 5 min), the supernatant was concentrated, deproteinized, and lyophilized following the same procedures in Section 2.2.1.

2.3. Characterization of FVPs

2.3.1. Chemical components of FVPs

Polysaccharides content of crude FVPs was measured by the phenol-sulfuric acid method [20]. Protein content was determined with a bicinchoninic acid protein assay kit (Nanjing Built Biotechnology Research Institute Co., Ltd.). Polyphenol content was tested following the method of He et al. (2017) [21]. Uronic acid content was analyzed according to the reported method [22] with the glucuronic acid used as standard.

2.3.2. Scanning electron microscopy assay

A scanning electronic microscope (HITACHI TM-3000, Hitachi High-Technologies Co., Ltd., Tokyo, Japan) was applied to investigate the microstructure of FVPU, FVPH, and corresponding *F. velutipes* powder residues (UAER and HWER). Samples were coated with gold on the surface and examined subsequently under a high vacuum. The voltage was 15.0 kV and images were photographed at 200× and 500× magnifications [23].

2.3.3. UV–vis spectroscopy assay

Ultraviolet-visible (UV) spectra were applied according to the reported method with minor modifications [24,25]. Briefly, FVPs solution at a concentration of 4 mg/mL was scanned by a UV–vis spectrophotometer (TU1900, Beijing Persee General Instrument Co., Ltd., China) from 200 nm to 800 nm. The absorptions at 260 nm and 280 nm represent the absorption peaks of nucleic acids and proteins, respectively.

2.3.4. Helix-coil transition assay

The structure of helix-coil transition in FVPs was determined according to Gongo red staining test described by Kang et al. (2019) [26] with some modifications. Precisely, FVPs (4 mg) were dissolved in ultrapure water (2 mL) and then the solutions were mixed with 80 μmol/L Congo red solution (2 mL). Finally, different volumes of NaOH solution (1 mol/L) were added to change the final concentration of NaOH in the mixed solution from 0 mol/L to 0.5 mol/L. Visible spectra of the mixture at various concentrations of NaOH were scanned on a UV–vis spectrophotometer from 400 nm to 800 nm and the maximum absorption wavelength was determined. Ultrapure water, treated in the same way as the polysaccharide solution, was tested as the negative control.

2.3.5. Molecular weight distribution

Molecular weight distribution of FVPH and FVPU were measured by HPLC system (Agilent 1200, Agilent Technologies, USA) equipped with TSK G4000PW$_{XL}$ column (7.8 mm × 300 mm, Tosoh Crop., Tokyo, Japan) and evaporative light-scattering detector (ELSD) [27]. T-series dextran standards (T-2000, T-70, T-40, and T10) were used to calibrate the standard curve. The mobile phase was ultrapure water (35 °C, 0.5 mL/min).

2.4. Antioxidant activity of FVPs in vitro

2.4.1. DPPH radical scavenging activity

Scavenging activity on 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical was measured according to the previously reported method with some modifications [26]. Briefly, The DPPH ethanol solution (2 mmol/L, 2 mL) was added to FVPs samples (1–5 mg/mL, 2 mL). The mixtures were kept in the dark and incubated for 30 min at 25 °C. Vitamin C was regarded as the positive control, the absorbance was measured at 517 nm with a spectrophotometer. The scavenging percentage was calculated according to the following Eq. (1):

$$\text{Scavenging activity}\ (\%) = \left(A_0 - \frac{A_1 - A_2}{A_0}\right) \times 100\% \quad (1)$$

where A_0, A_1, and A_2 represented the absorbance of DPPH solution, sample mixed with the DPPH solution, and the sample without DPPH solution, respectively.

Table 1
Comparison of the yield and basic physicochemical characteristics of *F. velutipes* polysaccharides extracted by UAE (FVPU) and HWE (FVPH).

Method	FVPU	FVPH
Extraction temperature (°C)	45	80
Extraction power (W)	620	–
Extraction time (min)	20	120
Ratio of water to raw material (mL/g)	25	25
Yield (%)	5.59 ± 0.01^a	4.45 ± 0.05^b
Polysaccharide content (%)	80.65 ± 0.73^b	84.86 ± 2.50^a
Crude protein (%)	8.18 ± 0.77^a	6.04 ± 0.79^b
Polyphenol (%)	0.61 ± 0.03^b	0.81 ± 0.03^a
Uronic acid (%)	25.24 ± 0.69^a	20.01 ± 1.03^b

Note: Values are expressed as the mean ± SD (n = 3). Different letters within a row represent a significant difference at P < .05.

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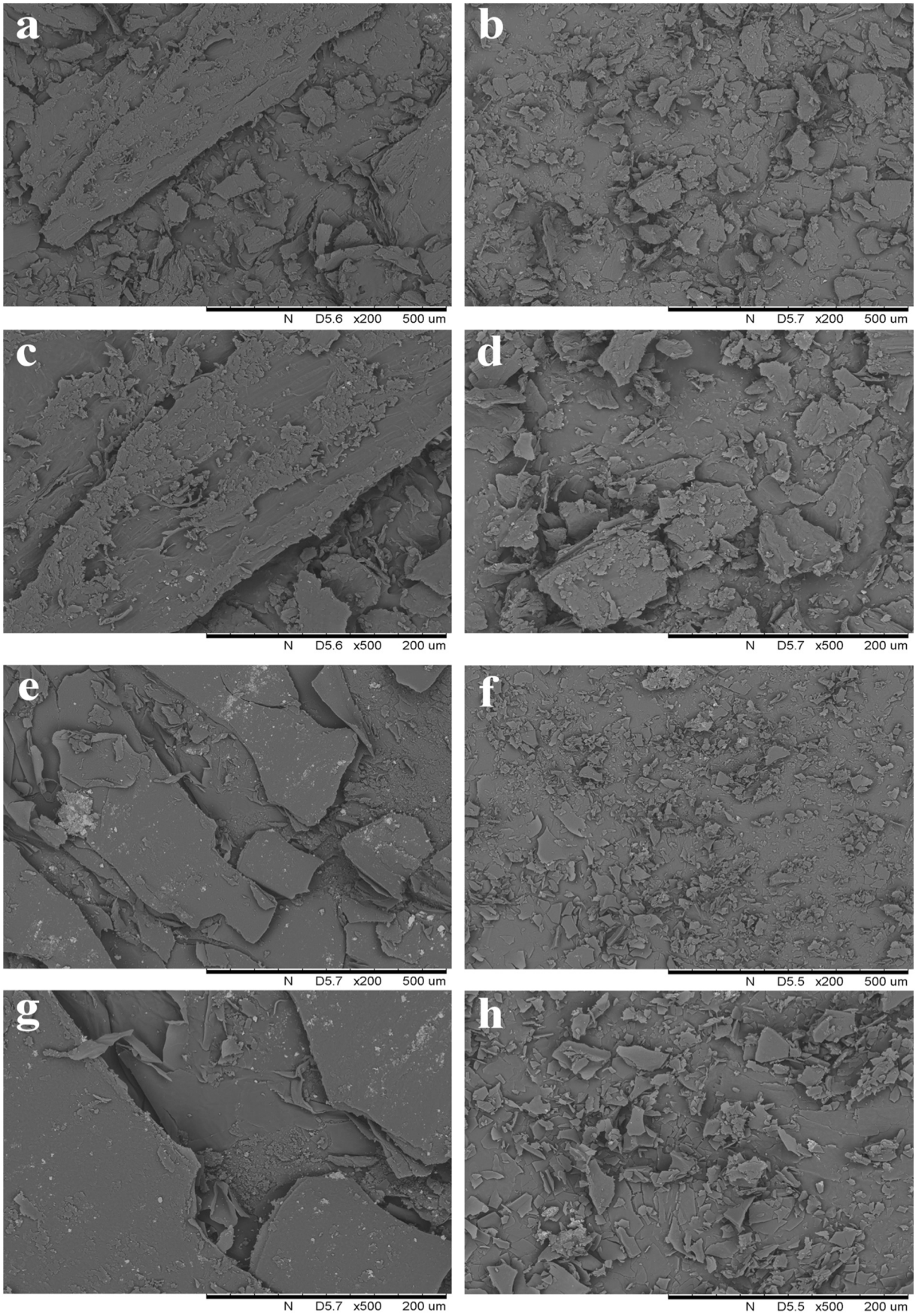

Fig. 1. Scanning electron microscope images of hot water extracted residues (HWER) at 200× (a), 500× (c) magnification; ultrasound-assisted extracted residues (UAER) at 200× (b), 500× (d) magnification; hot water extracted polysaccharides (FVPH) at 200× (e) and 500× (g) magnification; ultrasound-assisted extracted polysaccharides (FVPU) at 200× (f) and 500× (h) magnification.

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2.4.2. Superoxide anion radical scavenging activity

Superoxide anion scavenging activity of FVPs (1–5 mg/mL) was tested according to the reported method with some modifications [28]. Tris-HCl buffer (pH 8.2, 0.05 mol/L, 4.5 mL) was kept at 25 °C for 20 min. Subsequently, tested samples solution (1 mL) and 1,2,3-phentriol (25 mmol/L, 0.4 mL,) were added and incubated at 25 °C for 5 min. Finally, HCl solution (8 mmol/L, 1 mL) was added quickly. The absorbance (299 nm) was determined and the scavenging activity was calculated from the following Eq. (2):

$$\text{Scavenging activity}\ (\%) = \left(1-\frac{A_1-A_2}{A_0}\right)\times 100\% \quad (2)$$

where A_0, A_1, and A_2 represented the absorbance of the control, the sample, and the blank, respectively.

2.4.3. Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was measured according to the method reported by Zhou et al. (2019) [29]. The absorbance was measured at 510 nm and vitamin C was used as the positive control.

2.4.4. Reducing power

Reducing power was estimated using the Prussian blue method according to the report of Yin et al. (2018) [23]. Taking ultrapure water as the blank and vitamin C as a positive control, the absorbance was measured at 700 nm.

2.5. Bidirectional immunomodulatory activity of FVPs in vitro

2.5.1. Cell culture

RAW264.7 macrophages were cultured with DMEM medium in a cell incubator (37 °C, 5% CO_2). These macrophages were seeded in a 96-well plate (200 μL/well) at a density of 2×10^5 cells/mL and incubated for 24 h before further tests. The outer wells of the plate were filled with phosphate buffer (200 μL) as there was much more variation compared with the inner wells [30]. FVPs were dissolved in DMEM, sterilized (aperture: 0.22 μm) and kept at 4 °C.

2.5.2. Design of experimental models

FVPs were prepared and added to the medium of RAW 264.7 macrophages in the absence or presence of LPS under two experimental models to assess the differences of bidirectional immunomodulatory activity between FVPU and FVPH [31].

For the Model A, RAW264.7 macrophages were cultured with FVPs (5 μg/mL, 10 μg/mL and 50 μg/mL) for 24 h to evaluate the effects of FVPs on RAW264.7 Cells. LPS (0.1 μg/mL) was used to substitute the polysaccharides as the positive control group. Polymyxin B (4 μg/mL) was added to the medium to eliminate LPS contamination in FVPs in half of the treatment groups.

For Model B, RAW264.7 macrophages were cultured with FVPs (5 μg/mL, 10 μg/mL and 50 μg/mL) for 2 h firstly. Dexamethasone (50 μg/mL) was used to substitute the polysaccharides as a positive control group. Subsequently, the supernatants containing FVPU and FVPH were removed and the cells carefully washed twice with PBS (100 μL/well) Then, a new medium that contained LPS (2.5 μg/mL) was added and incubation continued for another 24 h.

2.5.3. Assessment of cell viability

Cell viability was determined by MTT assay [32]. The absorbance was determined at 570 nm with a plate reader (MQX200, BioTek Instruments, Inc., Vermont, USA).

2.5.4. Phagocytosis of macrophages assay

The RAW264.7 macrophages were cultured for 24 h as described in Model A. The phagocytosis of RAW264.7 cells was determined with the Neutral Red Test reported by Chen et al. (2018) [33].

2.5.5. Nitric oxide and IFN-γ, TNF-α, IL-6 analysis

The RAW264.7 macrophages were cultured according to Models A and B for 24 h. Subsequently, the supernatant of RAW264.7 cells was collected to determine the concentrations of NO, IFN-γ, TNF-α, and IL-6 with corresponding enzyme-linked immunosorbent assay kit according to the manufacturer's instructions.

2.6. Statistical analysis

All experiments were performed in triplicate and data were expressed as means ± standard deviation (SD). Statistical analysis of data was performed using SPSS software (Version 22; SPSS Inc., Chicago, IL, USA). Significant differences were identified by one-way analysis of variance (ANOVA).

3. Results and discussion

3.1. Analysis of physicochemical property

Table 1 shows the efficiencies of FVPU and FVPH extraction. Compared with FVPH (4.45 ± 0.05%), the extraction yield of FVPU (5.59 ± 0.01%) was significantly higher ($P < .05$). In addition, the extraction time and temperature of UAE were shorter and lower than HWE. This phenomenon was due to the acoustic cavitation of ultrasound, which could promote the dissolution of active ingredients in solution [34]. The wall and membrane of cells were broken down and the transferred mass was strengthened by ultrasonic waves, which made the solvent molecules penetrate the tissue cells [35]. This is consistent with reported results which have shown that ultrasound-assisted extraction is a high-efficient technical method to extract polysaccharides [17,18]. Protein and uronic acid contents of FVPU were 2.14% and 5.23% higher ($P < .05$) than those of FVPH, respectively. However, polysaccharide and polyphenol contents of FVPU were 4.21% and 0.20% lower ($P < .05$) than those of FVPH, respectively. This phenomenon indicated that the composition of FVPs was changed by ultrasound with the strong cavitation in the extraction process which could promote the dissolution of polysaccharides and gain a higher yield of extraction. Simultaneously, other substances such as proteins in the *F. velutipes* powders were dissolved which caused the higher ($P < .05$) protein content and lower polysaccharide content of FVPU. The lower ($P < .05$) polyphenol content of FVPU was due to the reason that polyphenol was degraded by the ultrasonic cavitation [36,37].

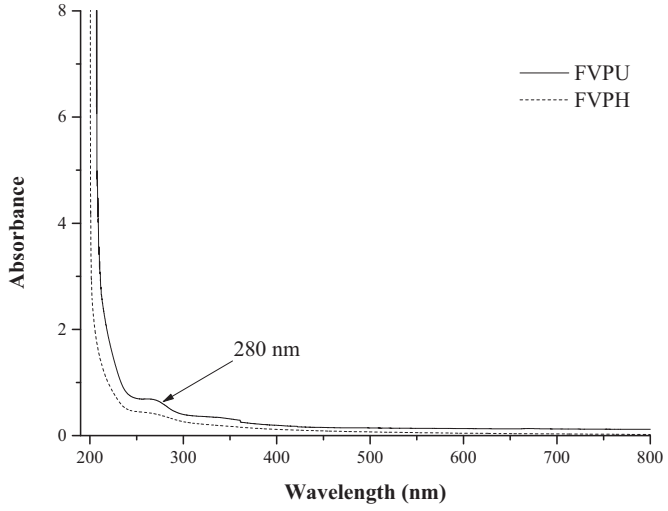

Fig. 2. UV–Vis spectroscopy of FVPU and FVPH.

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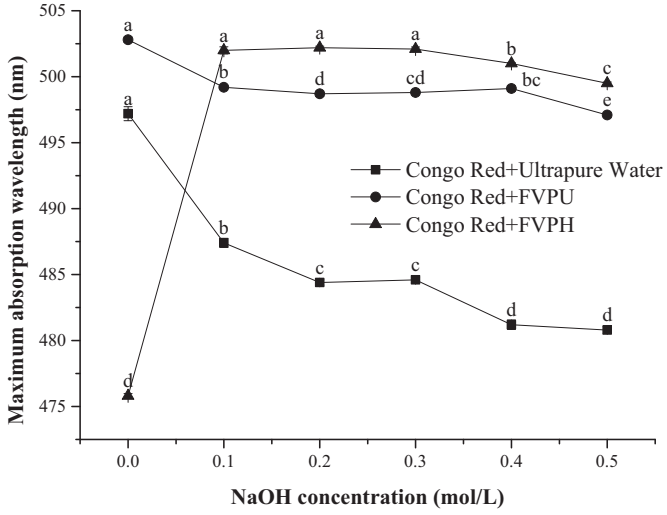

Fig. 3. The maximum absorbance value of (FVPU + Congo red), (FVPH + Congo red) and (Ultrapure Water + Congo red) at different concentrations of NaOH. Note: Values are expressed as the mean ± SD ($n = 3$). Different letters within a legend represent a significant difference at $P < .05$.

3.2. Analysis of microstructure

In order to clarify the mechanism of UAE and the microstructure of FVPs, the micrographs of FVPs and corresponding residues of *F. velutipes* after extraction were scanned with scanning electronic microscope (Fig. 1).

For magnifications of 200× and 500×, the hot water-extracted residue (HWER) showed an overlapping adhesion sheet structure (Fig. 1a & c), whereas the structure of ultrasound assisted-extracted residue (UAER) was small independent sheet (Fig. 1b & d). Compared with HWER, a higher degree of breaking was observed on the micrographs of UAER. This phenomenon could be ascribed to the cavitation and the mechanical effects of ultrasonic treatment [38,39]. The surface of *F. velutipes* powders was subsequently changed by the ultrasound treatment which promoted the diffusion process and made osmotic powder rupture or crumble more readily. A strong force of crush was produced at the contact point when cavitation bubbles burst due to ultrasound. This finding was congruent with a previous study by Hou et al. (2016) [39]. They observed that ultrasound-assisted-extracted residue of chestnut powder displays a higher broken degree. Compared with HWE, UAE was a more efficient ($P < .05$) method to extract FVPs because of the strong disruption of mushroom fruiting body's structure which could explain above-observed surface microstructure differences between HWER and UAER. What's more, this result was consistent with the data in Table 1.

FVPH showed a sheet-like smooth structure (Fig. 1 e & g) whereas particles of FVPU were much smaller (Fig 1. f & h). The differences observed in the morphological characteristics always contribute to physical and chemical properties. The instantaneous high pressure or shear force, produced by the cavitation of ultrasound, could break down polysaccharide chains. Polysaccharides with high molecular weight were degraded to small ones and the macromolecular structure was as well destroyed by the cavitation effect of ultrasonic waves. These results were consistent with previous studies which proved that the structure, shape and surface topology of polysaccharides could be affected by the method of extraction [40].

3.3. Analysis of UV–vis spectroscopy

The absorption peaks of UV scanning spectra will be observed if there were nucleic acids (260 nm) or proteins (280 nm) in tested samples [24,29,41,42]. Fig. 2 shows the UV spectra of FVPU and FVPH. A downward trend, regarded as the general character of polysaccharide, was observed both from the UV scanning spectra of FVPU and FVPH in the range of 200–800 nm. As shown in the spectra, there were no absorption peaks at 260 nm on the spectrograms of FVPU and FVPH.

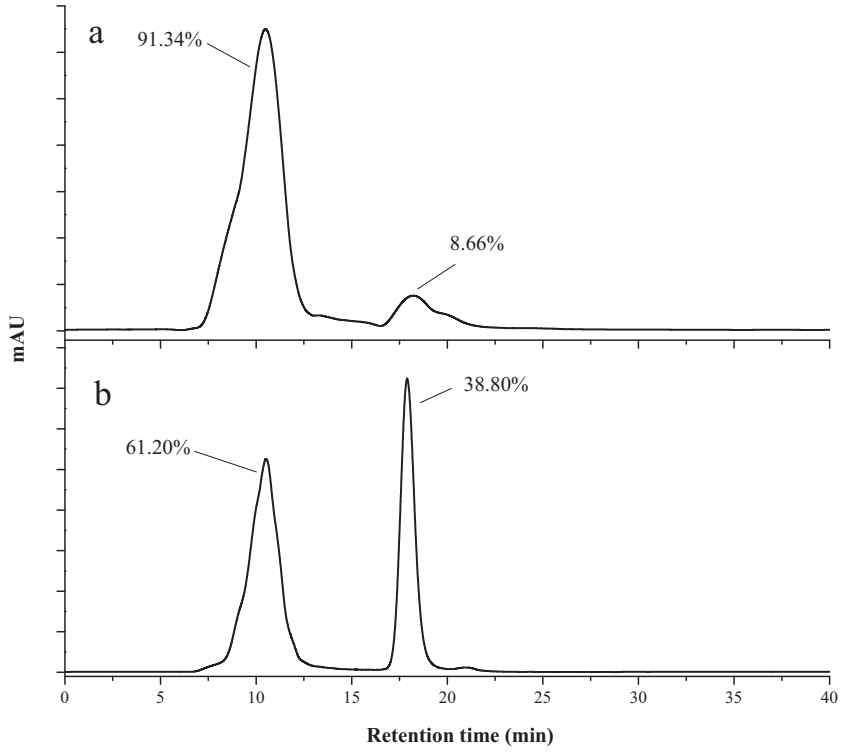

Fig. 4. High-performance gel permeation chromatogram of FVPH (a) and FVPU (b).

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However, FVPU and FVPH both showed weak peaks at 280 nm with a weaker peak for FVPH [43]. This phenomenon indicated that all crude FVPs contained protein, which could be validated by chemical components assay of crude polysaccharides. As exhibited in Table 1, the protein content of FVPU (8.18 ± 0.77%) was higher ($P < .05$) than that of FVPH (6.04 ± 0.79%), resulting in the differences of the UV spectrograms between FVPU and FVPH.

3.4. Analysis of helix-coil transition

The shift of maximal absorption wavelength (λ max) of the polysaccharide solution containing Congo red was measured when NaOH was added at different concentrations (0–0.5 mol/L). It could be concluded that the triple helical structure exists when the maximum absorption wavelength of the mixture increased initially and then decreased with the NaOH concentration increasing [26,44]. Fig. 3 shows changes in the maximal absorption wavelength of the mixture of Congo red and FVPs at various concentrations of NaOH. The maximal absorption wavelength of FVPH increased with the NaOH concentration increasing from 0 mol/L to 0.1 mol/L. As the NaOH concentration increased from 0.1 mol/L to 0.5 mol/L, the maximal absorption wavelength of FVPH (Congo Red + FVPH) gradually declined. Similar to the negative control group (Congo Red + Ultrapure Water), the maximal absorption wavelength of FVPU (Congo Red + FVPU) decreased with the concentration of NaOH increasing. These results signified that the triple helical structure existed in FVPH. However, the triple helical structure was destroyed by ultrasound effects including cavitation effect and mechanical effect during the UAE process. Therefore, it could be concluded that the helix-coil transition structure of FVPs was affected by the method of extraction with UAE destroying the triple helical structure.

3.5. Molecular weight distributions of FVPU and FVPH

Fig. 4 shows the molecular weight distributions of FVPU and FVPH. The elution peaks of FVPH (Fig. 4 a) and FVPU (Fig. 4 b) were both multiple. The equation of the standard curve was: Log Mw = $-0.235\ T_R + 8.6189$ with a correlation coefficient of 0.99. Mw represented the molecular weight and T_R represented retention time. There were two peaks in the elution profiles of FVPU and FVPH at 10.55 and 17.93 min, while the corresponding molecular weights were calculated to be 1379 kDa and 25 kDa, respectively. This result indicated that both FVPU and FVPH were composed of two components which have different

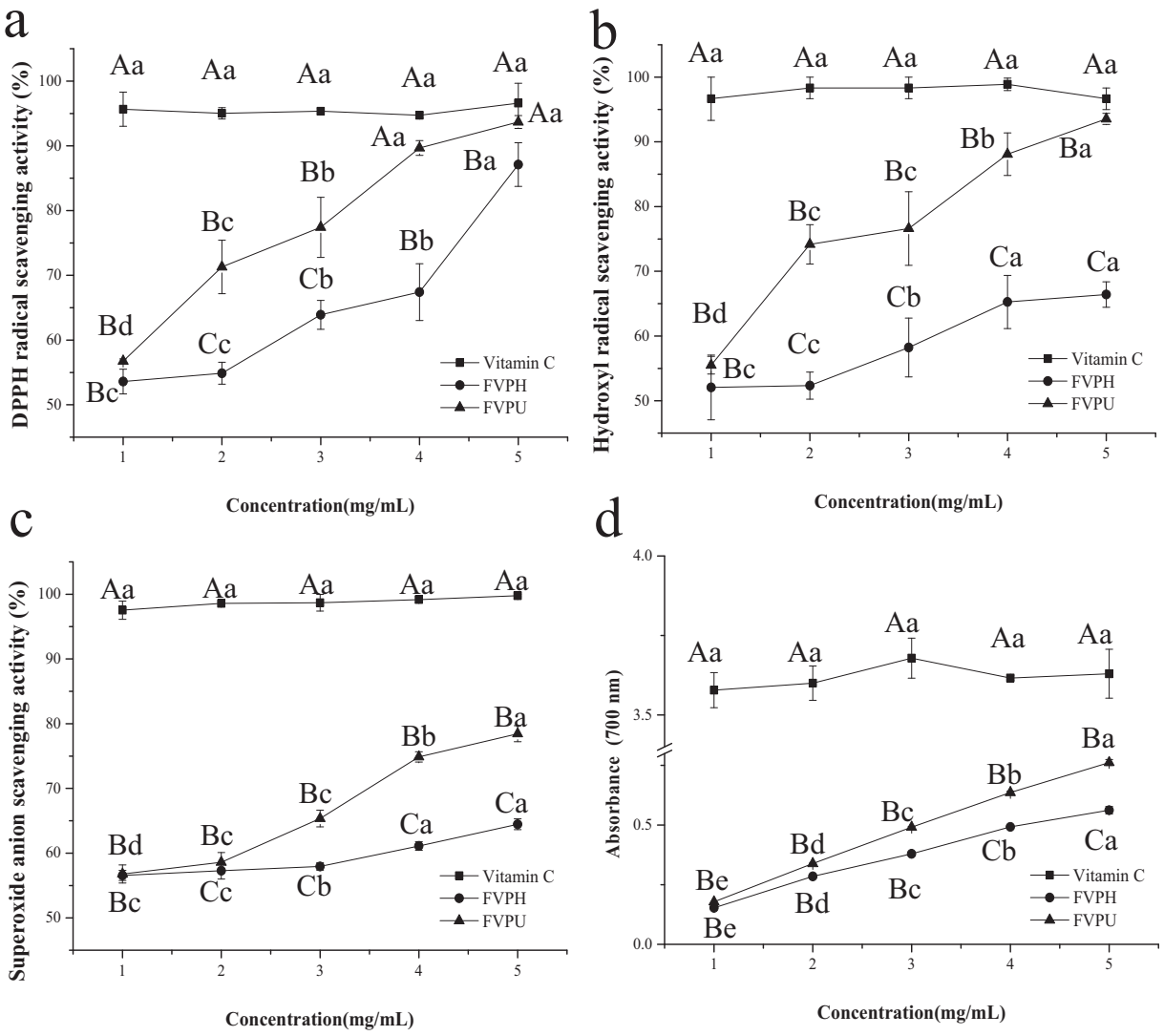

Fig. 5. Antioxidant activities of FVPH, FVPU and Vitamin C. a: DPPH radical scavenging activity; b: Hydroxyl radical scavenging activity; c: Superoxide radical scavenging activity; d: Reducing power. Note: Values are expressed as the mean ± SD ($n = 3$). Different capital letters within same concentration represent a significant difference at $P < .05$. Different lower case letters within same legend represent a significant difference at $P < .05$.

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molecular weight. Furthermore, the composition ratios of the two components were different in FVPU and FVPH. The proportion of lower molecular weight polysaccharides component in FVPU (38.80%) was higher than that in FVPH (8.66%), which made the lower average molecular weight of FVPU (853.65 kDa) compared with FVPH (1261.74 kDa). This phenomenon might be due to the acoustic cavitation of UAE, which made high molecular weight components reduce to smaller ones. The result was in agreement with a previous study which proved that the starch extracted by ultrasound-extraction has a lower average molecular weight compared with the conventional method of extraction [26].

3.6. Antioxidant activity of FVPs in vitro

3.6.1. Analysis of DPPH radical scavenging activity

The DPPH radical scavenging model is universally used in the antioxidant investigation of natural compounds [26]. In comparison with vitamin C, the DPPH scavenging effects of FVPU and FVPH were measured. As shown in Fig. 5 a, the DPPH scavenging effects of FVPs depicts a concentration-dependent rate. There were no significant differences ($P > .05$) between FVPU and FVPH on DPPH scavenging effects when the concentration of FVPs was 1 mg/mL. However, with the FVPs concentration further increasing (2–5 mg/mL), the scavenging effects of FVPH were significantly lower ($P < .05$) than that of FVPU at the same concentration. When the concentration of FVPs increased from 1 mg/mL to 3 mg/mL, FVPs showed a significant lower ($P < .05$) DPPH radical scavenging activity than vitamin C. Nevertheless, when the concentration of FVPU increased to 4 mg/mL, the DPPH scavenging effect of FVPU was close to that of vitamin C ($P > 0.05$). At the highest tested concentration of 5 mg/mL, compared with FVPH, the scavenging activity on DPPH radical of FVPU was significantly higher ($P < .05$). These results indicated that the extraction method did influence the DPPH radical scavenging activity of FVPs. FVPU had a stronger DPPH radical scavenging activity compared with FVPH. This result was consistent with the findings of Zhang et al. (2013) [34]. The higher DPPH radical scavenging activity of FVPU may be related to the degradation of polysaccharides caused by ultrasonic treatment. The chemical groups in the degraded sample could have a wider range of probability of contact with the radical because of the larger surface area and better solubility in water. FVPU may have a stronger hydrogen-donating ability which results in the stronger DPPH radical scavenging activity of FVPU compared with that of FVPH [34,45].

3.6.2. Analysis of hydroxyl radical scavenging activity

The hydroxyl radical, produced by superoxide radical, can trigger radical chain reactions and destroy macromolecules in living cells [32]. Fig. 5b shows hydroxyl radical scavenging effects of FVPU and FVPH. The hydroxyl radical scavenging activities of FVPs portrayed concentration-dependent reaction ability. When the concentration of polysaccharides was 1 mg/mL, the hydroxyl radical scavenging activity of FVPU was close to that of FVPH. However, the scavenging activity of FVPU on the hydroxyl radical was significantly higher ($P < .05$) than that of FVPH when the concentration of polysaccharides further increased from 2 mg/mL to 5 mg/mL. At the highest tested concentration of 5 mg/mL, compared with FVPH, the scavenging activities on hydroxyl radicals of FVPU was significantly higher ($P < .05$). These results proved that FVPU had a better activity on scavenging hydroxyl radical compared with FVPH. Polysaccharides act as electron or hydrogen donors in the scavenging reaction of hydroxyl radical. The lower molecular weight and special structural characteristics of FVPU, caused by ultrasonic effects, made FVPU a better donor of electron or hydrogen, which may lead to its higher hydroxyl radical scavenging activity [26].

3.6.3. Analysis of superoxide anion radical scavenging activity

Superoxide anions radical, created by the mitochondrial electron transport system, can cause tissue damage and various diseases [28,46,47]. Fig. 5c shows superoxide anion radical scavenging activities of FVPU and FVPH. Both FVPU and FVPH had an increasing trend in superoxide anion radical scavenging capacity with the increase in concentration. When the concentration of the polysaccharides was 1 mg/mL, the superoxide anion radical scavenging activity of FVPU was close to FVPH ($P > 0.05$). However, the superoxide anion radical scavenging activity of FVPU was significantly higher than that of FVPH when the concentration of polysaccharides further increased from 2 mg/mL to 5 mg/mL. At the highest tested concentration of 5 mg/mL, compared with FVPH, the scavenging activities on superoxide anion radical of FVPU improved significantly ($P < .05$). Furthermore, superoxide could initiate lipid peroxidation indirectly, resulting in the formation of H_2O_2, and create precursors of hydroxyl radicals [48]. These results suggested that the higher hydroxyl radical scavenging activity of FVPU is also related to the better superoxide radical scavenging ability. But the mechanism of scavenging superoxide anion free radical for polysaccharides is still not clear. The lower molecular weight and special structure of FVPU may result in the higher superoxide anion radical scavenging activity.

3.6.4. Analysis of reducing power

Reducing power is a significant indicator of antioxidant activities [23]. Fig. 5d shows the reducing power of FVPU, FVPH and vitamin C, in which a higher absorbance value corresponds to a stronger reducing power. Generally, the reducing power of all tested FVPs was lower than that of vitamin C at the same concentration. Both FVPU and FVPH had an increasing trend in reducing power with increasing test concentration.

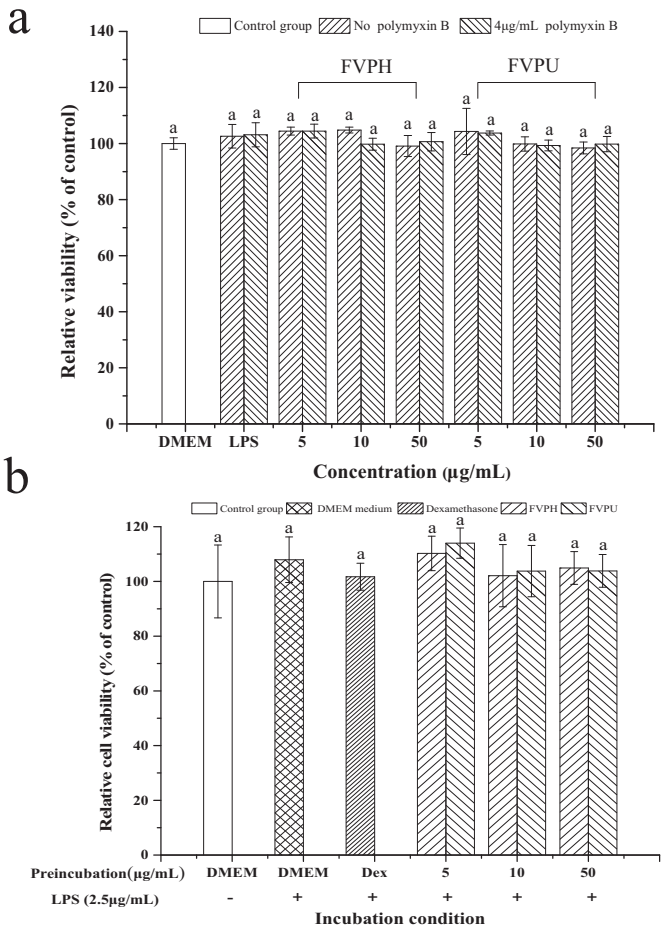

Fig. 6. The viability of RAW264.7 macrophages cultured under the conditions of Model A (a) and Model B(b). Note: Values are expressed as the mean ± SD ($n = 3$). Different letters represent a significant difference at $P < .05$.

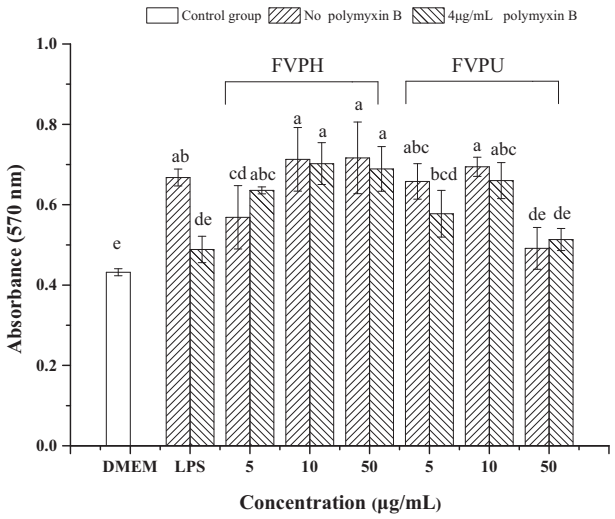

Fig. 7. Pinocytic activity of RAW264.7 macrophages in Model A. Note: Values are expressed as the mean ± SD ($n = 3$). Different letters represent a significant difference at $P < .05$.

When the concentration of polysaccharides increased from 1 mg/mL to 3 mg/mL, there was no significant difference ($P > 0.05$) between FVPU and FVPH. However, when the concentration of polysaccharides increased from 4 mg/mL to 5 mg/mL, the reducing power of FVPU was significantly higher than that of FVPH. Particularly, compared with FVPH, the total reducing power of FVPU improved significantly at the highest tested concentration of 5 mg/mL. This result was in accordance with a previous study which indicated that UAE improved the reducing power of *Lentinus edodes* polysaccharides compared with HWE [49]. It has been reported that polysaccharides with lower molecular weight have stronger reducing power [23].Similarly, the stronger reducing power of FVPU might be ascribed to the lower average molecular weight.

3.7. Analysis of bidirectional immunomodulatory activity

3.7.1. Cell viability

Fig. 6 shows the viability of RAW264.7 macrophages cultured under Model A and Model B conditions. There were no significant viability differences ($P > .05$) between the control group and tested group in each model. These results indicated that these experimental conditions (5–50 μg/mL) could be used in subsequent experiments both in Model A (Fig. 6a) and Model B (Fig. 6b).

3.7.2. Effects of FVPU and FVPH on pinocytic activity

As the first and pivotal step in immune response, phagocytosis is an important indicator to test the activation status of macrophages [50]. Therefore, pinocytic activity can reflect the immune function of macrophages indirectly. Fig. 7 shows the pinocytic activity of RAW264.7 macrophages in Model A. Significantly higher pinocytic activity ($P < .05$) was observed in FVPH-group than FVPU-group when the concentration of polysaccharides was 50 μg/mL. As a positive control, LPS significantly increased the phagocytic activity of RAW264.7 macrophages. However, due to the specific combination of polymyxin B and LPS, this enhancement is eliminated ($P < .05$) by the addition of polymyxin B [51]. Simultaneously, the addition of polymyxin B didn't affect the phagocytic activity significantly in FVPH-group and FVPU-group, which suggested that there was no contamination of LPS in FVPs. These results indicated that both FVPU and FVPH could increase the phagocytic activity of RAW264.7macrophages in Model A but the activation effect of FVPH was better than FVPU at the highest tested concentration of 50 μg/mL. This result might be attributed to the ultrasonic effects on polysaccharides structure during the extraction process of UAE.

3.7.3. Effects of FVPU and FVPH treated alone on nitric oxide and cytokine secretion levels at different concentrations in RAW264.7 macrophage cultured with LPS as a positive control (Model A)

To examine the differences of immunoregulatory activity between FVPU and FVPH, the two polysaccharides were added into DMEM medium at different concentrations for 24 h. And LPS was selected as the positive control (Model A). As shown in Table 2, the concentrations of nitric oxide (NO) and cytokines (IFN-γ, TNF-α, IL-6) in the positive control group increased significantly ($P < .05$) compared to the control group. Additionally, the addition of polymyxin B didn't affect the secretion of NO and cytokines in FVPs-groups significantly. This phenomenon suggested that there was no contamination of LPS in FVPs. Significantly higher ($P < .05$) concentrations of NO and IFN-γ were observed in FVPH-group compared with groups treated with FVPU when the concentration of polysaccharides was 50 μg/mL. This result indicated that FVPH exhibited a higher NO and IFN-γ production stimulating the activity of RAW264.7 cells in Model A. FVPH might be a better inhibitor of bacteria and tumor cells compared with FVPU. This result was consistent with the result of pinocytic activity assay. Multiple cytokines and NO are secreted by activated macrophages to modulate the immune system [52,53]. A modest increase in these indicators indicates the activation of macrophages. Some substances with immune-enhancing functions can promote the secretion of nitric oxide and pro-inflammatory cytokines. These results indicated that FVPH was a better immunity enhancing factor of immunity compared with FVPU. This might be attributed to

Table 2
The contents of NO, TNF-α, INF-γ, and IL-6 in supernatant of RAW264.7 macrophages in Model A.

Incubation condition	Polysaccharide concentration (μg/mL)	NO (μmol/L)	TNF-α (pg/mL)	IL-6 (pg/mL)	IFN-γ (pg/mL)
DMEM		3.34 ± 1.92^{f}	139.54 ± 9.85^{d}	14.10 ± 1.89^{d}	33.33 ± 3.54^{d}
LPS		53.54 ± 2.99^{a}	404.81 ± 10.04^{a}	62.58 ± 3.52^{abc}	117.00 ± 6.43^{abc}
LPS + PB		7.15 ± 2.36^{e}	123.80 ± 14.59^{d}	22.53 ± 8.08^{d}	49.49 ± 8.13^{d}
HFVP	5	22.99 ± 2.56^{bcd}	367.78 ± 20.03^{abc}	69.84 ± 8.08^{a}	115.99 ± 4.41^{abc}
	10	26.47 ± 1.46^{b}	379.81 ± 21.03^{ab}	63.03 ± 2.85^{abc}	122.90 ± 12.47^{abc}
	50	25.94 ± 1.68^{b}	350.19 ± 13.20^{bc}	61.51 ± 4.22^{abc}	132.49 ± 10.32^{a}
HFVP+PB	5	20.44 ± 1.17^{d}	346.94 ± 21.83^{bc}	66.97 ± 2.75^{ab}	123.91 ± 12.52^{abc}
	10	26.00 ± 1.54^{b}	365.46 ± 17.37^{abc}	60.43 ± 3.10^{abc}	121.89 ± 7.08^{abc}
	50	24.38 ± 0.59^{bc}	326.11 ± 21.38^{c}	59.80 ± 4.04^{abc}	130.13 ± 2.59^{ab}
UFVP	5	23.60 ± 0.50^{bcd}	365.00 ± 32.66^{abc}	67.33 ± 6.77^{ab}	118.52 ± 3.04^{abc}
	10	20.39 ± 3.25^{d}	359.44 ± 14.43^{bc}	64.64 ± 4.88^{abc}	106.73 ± 11.71^{c}
	50	21.93 ± 1.46^{cd}	352.50 ± 44.38^{bc}	57.47 ± 7.68^{bc}	108.25 ± 21.84^{c}
UFVP+PB	5	22.86 ± 2.08^{bcd}	335.37 ± 30.24^{bc}	62.22 ± 5.70^{abc}	117.17 ± 13.16^{abc}
	10	19.94 ± 0.88^{d}	336.30 ± 16.74^{bc}	61.77 ± 2.10^{abc}	104.38 ± 8.33^{c}
	50	21.05 ± 3.03^{cd}	328.89 ± 32.30^{c}	54.96 ± 8.03^{c}	110.27 ± 15.24^{bc}

Note: Values are expressed as the mean ± SD ($n = 3$). Different letters in the same column represent a significant difference at $P < .05$.

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Table 3
The contents of NO, TNF-α, INF-γ, and IL-6 in supernatant of RAW264.7 macrophages in Model B.

Incubation condition	Polysaccharide concentration (μg/mL)	NO (μmol/L)	TNF-α(pg/mL)	IL-6 (pg/mL)	IFN-γ (pg/mL)
DMEM+DMEM		2.29 ± 1.09^{e}	117.31 ± 15.49^{g}	11.51 ± 4.76^{d}	31.48 ± 3.04^{g}
DMEM+LPS		59.65 ± 1.28^{a}	412.22 ± 19.70^{a}	72.61 ± 7.90^{a}	263.97 ± 8.23^{a}
DEX + LPS		28.04 ± 2.92^{b}	316.85 ± 13.49^{ef}	62.31 ± 3.43^{bc}	79.29 ± 2.81^{f}
HFVP+LPS	5	26.82 ± 2.48^{bc}	377.96 ± 5.61^{b}	68.41 ± 1.02^{ab}	131.48 ± 8.70^{c}
	10	23.92 ± 1.12^{c}	366.85 ± 16.45^{bc}	67.51 ± 2.95^{ab}	123.74 ± 7.94^{cd}
	50	23.58 ± 0.51^{c}	336.30 ± 13.20^{cde}	67.15 ± 3.05^{ab}	113.97 ± 4.80^{d}
UFVP+LPS	5	26.71 ± 1.04^{bc}	363.61 ± 16.90^{bcd}	72.08 ± 5.80^{a}	143.27 ± 4.11^{b}
	10	24.35 ± 2.31^{c}	329.81 ± 29.18^{def}	65.90 ± 1.01^{ab}	127.61 ± 6.27^{c}
	50	19.83 ± 1.89^{d}	297.41 ± 33.37^{f}	55.05 ± 7.60^{c}	101.52 ± 6.83^{e}

Note: Values are expressed as the mean ± SD ($n = 3$). Different letters in the same column represent a significant difference at $P < .05$.

the ultrasonic effects on polysaccharides structure during the extraction process of UAE.

3.7.4. Curative effects of FVPs on LPS-stimulated nitric oxide and cytokine secretion levels in RAW264.7 macrophages cultures under a specified preventive experiment (Model B)

To identify the different preventive effects of FVPU and FVPH on LPS-induced inflammation, FVPs was added into DMEM medium at the noncytotoxic concentrations before stimulation of LPS (Model B). As shown in Table 3, excessive secretions of NO, IFN-γ, TNF-α, and IL-6 caused by the stimulation of LPS were inhibited significantly ($P < .05$) by FVPU and FVPH. Compared with the blank control group, significant increases of NO and cytokines (IFN-γ, TNF-α, IL-6) in the supernatant were observed by the stimulation of LPS. Meanwhile, the excessive activation was inhibited significantly with the preincubation of dexamethasone (DEX). Similar inhibitions were observed in the FVPU and FVH groups. The concentrations of NO and cytokines (IFN-γ, TNF-α, IL-6) in the supernatant of macrophages that were pre-cultured with FVPU were significantly lower ($P < .05$) compared those pre-incubated with FVPH when the concentration of polysaccharides was 50 μg/mL. This result indicated that for the macrophages stimulated into inflammation by LPS in Model B, the preincubation of FVPs inhibited the increase of NO and cytokines (IFN-γ, TNF-α, IL-6) induced by LPS and the inhibitory effect of FVPU was better than FVPH.

For LPS-induced inflammatory cells in Model B, the levels of NO and pro-inflammatory cytokines (IFN-γ, TNF-α, IL-6) in the supernatant were elevated by the stimulation of LPS. The secretion of nitric oxide and pro-inflammatory cytokines increased in LPS-induced inflammatory cells. Excessive secretion of these cytokines is a hallmark of inflammation. In this case, some substances with anti-inflammatory activity can reduce the secretion of nitric oxide and pro-inflammatory cytokines [53–56]. Our results indicated that FVPU was a better inhibitor of inflammation compared with FVPH. This phenomenon might be attributed to the ultrasonic effects on polysaccharides structure during the extraction process of UAE.

4. Conclusions

Compared with HWE, the extraction yield of FVPs improved significantly with the application of UAE. Significantly higher uronic acid content, better ($P < .05$) antioxidant activity as well as stronger ($P < .05$) ability to suppress overactivity in LPS-induced macrophages were observed in FVPU compared with FVPH. However, UAE also decreased macrophages activation activity of FVPU. The above differences between FVPU and FVPH may be due to different chemical composition, molecular weight distribution and helix-coil transition structure triggered by cavitation of ultrasonic waves. However, the structure-activity relationship and mechanism of ultrasonic effects resulting in structural changes of FVPs still need further investigation. These findings strongly indicated that UAE changed the antioxidant activity and bidirectional immunomodulatory activity of FVPs. The UAE was an efficient and environmentally friendly method to produce value-added polysaccharides with special functional activity from *F. velutipes* for the development of functional foods or nutraceuticals.

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