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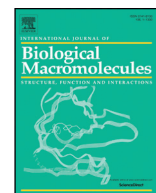
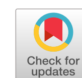


# Protective roles of *Amanita caesarea* polysaccharides against Alzheimer's disease via Nrf2 pathway



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ABSTRACT

This study explores the neuro-protective effects of *Amanita caesarea* polysaccharides (ACPS), obtained by 80% alcohol precipitation of water extract and purified using a DEAE-52 cellulose anion exchange column, related to antioxidant activity. A 3-h pre-treatment of ACPS prior to L glutamic acid (L Glu) co-exposure reversed the decreased cell viability, inhibited apoptosis, suppressed the accumulation of intracellular reactive oxygen species and restored mitochondrial membrane potential in HT22 cells. Compared to L Glu-exposed cells, ACPS enhanced the nuclear levels of NF-E2p45-related factor 2 (Nrf2), reduced the cytoplasmic levels of Nrf2 and cytochrome C, suppressed the expression of Kelch-like ECH-associated protein 1, and enhanced the expression of heme oxygenase 1, superoxide dismutase 1 and cysteine ligase catalytic subunit. In a D galactose and aluminum trichloride Alzheimer's disease (AD) mouse model, 42-day administration of ACPS improved the abnormal behaviors. ACPS suppressed the deposition of β amyloid peptide in the brain and ameliorated oxidative stress via modulating the levels of related enzymes. ACPS improved the functioning of the central cholinergic system, as indicated by an increase in acetylcholine and choline acetyltransferase concentrations, and reduced acetylcholine esterase levels in the serum, hypothalamus and cerebral cortex. Our data suggest that ACPS may be a promising candidate for the treatment of AD.

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## 1. Introduction

Alzheimer's disease (AD) is a severe neurodegenerative disorder that accounts for 60–70% of all dementias [1,2]. The main characteristic of AD is loss of memory and cognitive ability, which ultimately causes disability and dependency [3]. The main cause of AD is a failure to clear β amyloid peptide (Aβ) peptide from brain tissue [4]. The aggregated Aβ located around neurons not only has a direct toxic effect, but also makes the neurons susceptible to free radicals [5]. Oxidative stress, which can damage macromolecules, mitochondria, membrane lipids and nucleic acids and ultimately lead to neuronal death, is known to play an important role in the pathogenesis of neurodegenerative diseases, especially AD [6]. Redox active metal ions catalyze the production of reactive oxygen species (ROS) when bound to Aβ [7,8]. The ROS thus produced, in particular hydroxyl radicals, may contribute to oxidative damage to both the Aβ peptide itself and surrounding molecules [9].

Mitochondria are an important cellular source of ROS. It has been reported that the disruption of mitochondrial homeostasis and subsequent mitochondrial dysfunction caused by the over-accumulation of ROS also play a role in the pathophysiology of neurodegenerative diseases [10]. Hence, the maintenance of mitochondrial integrity through various surveillance mechanisms is critical for neuron survival [11]. The transcription factor NF-E2p45-related factor 2 (Nrf2) is a master regulator of cellular redox homeostasis, which also supports the structural and functional integrity of the mitochondria [12]. The deletion of Nrf2 can increase intracellular levels of APP, Aβ1–41 and Aβ1–40 in AD mice [13].

Fungi are widely appreciated worldwide due to their nutritional properties and pharmacological value as sources of important bioactive compounds [14]. Polysaccharides extracted from fungi are attracting much more attention from researchers [15–17], and numerous studies have investigated the potential of polysaccharides in the treatment of AD [15,18,19]. Our group has demonstrated the protective effect of polysaccharides isolated from *Armillaria mellea* on L glutamic acid (L Glu)-induced HT22 cell injury and mice with AD induced by D galactose (D gal) and aluminum trichloride ($AlCl_3$) [20]. Polysaccharides purified from *Sparassis crispa* protect PC12 cells against L Glu-induced toxicity via regulation of mitochondrial function [21]. *Amanita caesarea*, a type of fungi of the *Amanita* genus, can be found in Yunnan province of China at the elevation of 3800 m [22]. It has been studied for many years; however, most studies have focused on improving the

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growth environment [23], evaluating of heavy metal index [24] or component analysis [25]. In our previous study, the protective effects of *A. caesarea* aqueous extract on AD were successfully confirmed in HT22 cells and AD mice [26]; however, the effects of its polysaccharides have not yet been elaborated.

In this study, polysaccharides obtained from *A. caesarea* aqueous extract were purified and characterized. By using L Glu-induced apoptotic HT22 cells and D gal and $AlCl_3$-developed AD-like mice, an extract of *A. caesarea* polysaccharides (ACPS) was found to show neuroprotective effects against AD-like behavior via regulation of the oxidative stress-mediated Nrf2 pathway.

## 2. Materials and methods

### 2.1. Separation and purification of A. caesarea polysaccharide

*A. caesarea* fruiting bodies (obtained from Yunnan, China) were extracted twice with hot water at 80 °C for 3 h as in our previous study [26]. The extracts were concentrated, deproteinized with Sevag reagent (butanol/chloroform, v/v = 1:4), and then progressively precipitated with 60%, 70%, and 80% alcohol. Based on the results obtained from a cell viability assay, polysaccharides obtained by 80% alcohol precipitation were further purified by DEAE-52 (4 × 60 cm; C8930, Solarbio), eluting with distilled water and 0.2 mol/L NaCl solution successively at a flow rate of 1.0 mL/min. The collected polysaccharides were named ACPS for further experiments (Fig. 1s A). The polysaccharide content was measured using a phenol-sulfuric acid method [27].

### 2.2. Characterization of ACPS

#### 2.2.1. Fourier transform infrared (FT-IR) spectra analysis of ACPS

ACPS (2 mg) was pressed into 1 mm pellets with KBr at ratio of 1:200 for FT-IR measurements at a range of 4000–400 $cm^{-1}$ using an infrared spectrometer (IRPrestige-21, Tosoh, Japan).

#### 2.2.2. Molecular weight distribution of ACPS

A high-performance lipid chromatography (HPLC) system (Agilent 1100, Palo Alto, CA, USA) equipped with a refractive index detector (RID-10A, Shimadzu, Japan) was used to analyze the molecular weight distribution of ACPS. A gel filtration chromatographic column (7.8 × 300 mm, Sepax Technologies, Inc., USA) was used, operated at 35 °C and eluted by triple DD water at a flow rate of 0.45 mL/min with an injection 20 μL of ACPS sample dissolved in DD. water. Dextran (Mw 1000, 5000, 12,000, 25,000, 80,000 Da) (Sigma Chemical Co., St. Louis, MO, USA) was used as standards. The molecular weight of ACPS was calculated according to retention time by reference to the calibration curve.

#### 2.2.3. Monosaccharides analysis of ACPS

20 mg ACPS was hydrolyzed with 5 mol/L $H_2SO_4$ at 105 °C for 4 h, neutralized with $BaCO_3$ and centrifuge at 7000 rpm for 10 min, and then the supernatant was collected. The HPLC systems were used for the detection of monosaccharides in ACPS. The LC–10ATvp HPLC system (Shimadzu, Japan) was equipped with Prevail™ ES carbohydrate analysis column (250 × 4.6 mm) and 2000ES evaporation light scattering detector (Alltech, USA). 80% acetonitrile was chosen as mobile phase and the flow rate was 1.0 mL/min. Results were compared with the following monosaccharide standards: D glucose (Glc), L rhamnose (Rha), D xylose (Xyl), D galactose (Gal), D mannose (Man) and L arabinose (Ara) (Sigma-Aldrich, St. Louis, Missouri, USA).

### 2.3. Cell culture

HT22 cells, a mouse hippocampal neuronal cell line (337709; purchased from BeNa Culture Collection, Beijing, China), were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Invitrogen, USA), 100 U/mL penicillin (Invitrogen, USA) and 1% 100 μg/mL streptomycin in 5% $CO_2$ at 37 °C.

### 2.4. Cell viability assay

HT22 cells were cultured in 96-well plates at a density $5.0 \times 10^3$ cells/well and pretreated with ACPS at doses of 2.5 μg/mL or 5 μg/mL for 3 h. The HT22 cells were then incubated with 25 mM of L Glu (Sigma-Aldrich, USA) for another 24 h. 20 μl of 3 (4,5 dimethylthiazol 2 yl) 2,5 diphenyltetrazolium bromide (MTT) (5 mg/mL) was added to the culture for 4 h. The supernatant was then removed and 100 μL DMSO was added to dissolve the formazan crystals. The optical density (OD) was detected at 570 nm by a Synergy™4 Microplate Reader (BioTek Instruments, Winooski, VT).

### 2.5. Cell apoptosis assay

HT22 cells were cultured into 6-well plates, pre-incubated with 2.5 μg/mL or 5 μg/mL ACPS for 3 h and then co-exposed with 25 mM L Glu for a further 24 h. The cells were then collected and washed twice with phosphate buffered saline (PBS), then resuspended with PBS and incubated with propidium iodide (PI) and Annexin V in the dark at room temperature for 20 min. The fluorescence intensity was measured using a Muse Cell Analyzer (Millipore, Billerica, MA) according to the manufacturer's instructions.

### 2.6. Mitochondrial membrane potential (MMP), intracellular ROS and $Ca^{2+}$ measurement

HT22 cells were cultured into 24-well plates and pre-exposed with ACPS at dose of 2.5 μg/mL or 5 μg/mL for 3 h, then co-incubated with 25 mM L Glu for a further 12 h. To measure MMP, HT22 cells were stained with 2 μM of 5,5′,6,6′ TetrAchloro 1,1′,3,3′ tetraethyl imidacarbocyanine iodide staining (JC-1, Calbiochem, USA) for 15 min at 37 °C in darkness. To analyze the intracellular ROS level, HT22 cells were stained with 10 μM of 2′ 7′ dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma-Aldrich, USA) for 15 min at 37 °C in darkness. To detect the $Ca^{2+}$ concentration, HT22 cells were exposed with 2 μM Fluo-4-AM (Molecular Probes, USA) for 15 min at 37 °C in darkness. All of the cells were washed with PBS three times and the fluorescence intensity of the HT22 cells was determined using fluorescence microscopy (200×; CCD camera, TE2000, Nikon, Japan). Quantitative data analysis was performed using ImageJ software version 1.46 (National Institutes of Health, Bethesda, MD, USA) and data were expressed as the fluorescence intensity.

### 2.7. Western blot

HT22 cells were cultured in 6-well plates and pre-incubated with 2.5 μg/mL and 5 μg/mL ACPS for 3 h and then co-exposed with 25 mM L Glu for a further 24 h. The collected HT22 cells and brain samples of D gal and $AlCl_3$ developed AD mice were analyzed by western blot using 10–12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), as in our previous study [26]. To detect the translation of Nrf2 from the cytoplasm to the nucleus, cytoplasmic and nuclear extracts were prepared using a nuclear and cytoplasmic extraction kit (Bestbio, Shanghai, China) according to the manufacturer's instructions. The antibodies used for western blot analysis were as follows: Bax (ab32503), Bcl-2 (ab7973), cleaved caspase 3 (ab2302), Nrf2 (ab89443), heme oxygenase- (HO-1) 1 (ab137749), beta Actin (ab8229), Kelch-like ECH-associated protein 1 (Keap-1) (ab119403), glyceraldehyde 3 phosphate dehydrogenase (GAPDH, ab181602), cytochrome *c* (ab13575), Lamin B (ab133741), pyruvate dehydrogenase E1-alpha subunit (PDHE1-α; ab168379), dynamin-related protein 1 (DRP1, ab184247) (Abcam, Cambridge, MA, USA), superoxide dismutase 1

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