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Activity-guided isolation and structural identification of immunomodulating substances from *Pleurotus eryngii* byproducts

Yanan Sun[b], Wenxiang Li[a,b,*]

[a] Key Laboratory of Modern Agricultural Quality and Safety Engineering of Qingdao, China
[b] Qingdao Agricultural University, Qingdao 266109, China

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ABSTRACT

A two-step extraction process was used to investigate the bioactive substances from the byproducts of the fungus *Pleurotus eryngii*. Methanol extraction followed by extraction using different polar solvents coupled with silica column chromatography was carried out to separate the bioactive components. The immunomodulating activity of the extracts was investigated using Ana-1 mouse macrophage cells. At 25 μg/mL, the butyl alcohol extract showed the highest enhancement of Ana-1 cell proliferative activity and secretion of cytokines. Using silica-gel column chromatography, the butyl alcohol extract was purified to seven elution fractions. Fr. 2 showed the highest promotion of Ana-1 macrophage cell proliferation and TNF-α, IL-6 secretion. HPLC-QTOF-MS/MS was used to determine the structure of the active substance in Fr. 2. Adenosine was identified as the primary active component in Fr. 2 with a chemical formula of $C_{10}H_{13}N_5O_4$. Adenosine may be useful as a natural immunostimulatory agent and anti-tumor agent.

1. Introduction

Pleurotus eryngii, a tetrapolar basidomycete belonging to *Pleurotus sp.*, commonly called the king oyster mushroom, is a precious commercial edible fungus with almond flavor and abalone texture found in China [1]. *Pleurotus eryngii* is rich in polysaccharides (e.g. hemicellulose, α- and β-glucan), proteins, vitamins, and 18 kinds of amino acids, especially aspartic acid, glutamic acid, and arginine [2–3]. As a popular type of edible mushroom, *Pleurotus eryngii* may have several potential biological effects, as it contains important bioactive molecules such as polysaccharides, lipids, peptides, sterols, and dietary fiber among others [3–4]. Therefore, *Pleurotus eryngii* not only has high nutritional value, but may also be considered a type of health food because it contains numerous bioactive substances [5–6]. In recent years, domestic and foreign research has focused on the biological activity of *Pleurotus eryngii* and its active substances, which include beta glucan [7] with broad spectrum biological activity, and terpenoids [8] and sterol compounds with antibacterial, antiviral and antioxidant activities [9–13]. Its remarkable flavor, nutritional value and biological functions have recently made it popular as an edible mushroom [14], and thus its production has rapidly increased to satisfy consumers in North Africa, Europe, and Asia.

In recent years, *Pleurotus eryngii* has been produced on a large scale with output and consumption increasing year by year in China. In 2013, *Pleurotus eryngii* production reached 1.075 million tons according to the statistics from the Chinese Edible Fungi Association. In the process of *Pleurotus eryngii* cultivation factory management, the fungal byproducts (mushroom bud and stalk) make up about 1/6 of the product, and have the same nutritional value as the commodity mushroom [15]. Generally they are abandoned as waste, resulting in a huge loss of nutrition sources and pollution of the environment. The byproducts of *Pleurotus eryngii* are produced in large quantities at a low cost, are easy to collect, and have a nutrient content similar to the product mushroom [15].

Activation of innate immune signaling pathways through pattern-recognition receptors (PRRs) in the host cell is a crucial step for defense against infection by pathogens [16]. The human defense system is controlled by a series of immune responses mediated through different immune cells which can secrete highly effective cytokines, chemokines, NO and H_2O_2 and multi-functional small polypeptide molecules [17]. In cancer treatment, chemotherapy and radiotherapy are always accompanied by immunosuppression [18–20]. Therefore, it is necessary to identify new antitumor regimens with the potential to stimulate the immune system. It is well known that macrophage pinocytosis plays an important role in cellular defense mechanisms against pathogens, tumors and cancer growth [21–22].

In the present study, a methanol extract of *Pleurotus eryngii* byproducts was used as a raw material and its effect on the immunomodulating activity of mouse macrophage cells was evaluated.

* Corresponding author at: College of Food Science and Engineering, Qingdao Agricultural University, China.
E-mail address: 956551609@qq.com (W. Li).

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Using an activity-guided isolation method, the immunomodulating active substances were screened and purified based on the in vitro assays of immunomodulating activity, and the functional components were characterized by HPLC-QTOF-MS/MS. These data can provide the theoretical basis for the reuse of the byproducts of *Pleurotus eryngii*, the development of separation methods for immune active substances and for functional food applications.

2. Materials and methods

2.1. Plant materials and treatments

2.1.1. Cell lines

The macrophage Ana-1 cells were obtained from the cell bank of the Chinese Academy of Sciences in Shanghai. They were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and 1% Green streptomycin mixture. The cells were maintained in 5% carbon dioxide (CO_2) in a humidified incubator at 37 °C.

2.1.2. Chemicals

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), culture medium RPMI-1640 and trypan blue were purchased from Sigma-Aldrich Co. Fetal bovine serum, Pen Strep, DMEM, dimethyl sulfoxide and phosphate buffered saline (PBS, pH 7.4) were purchased from Thermo Scientific. TNF-α and the IL-6 Elisa kit were purchased from Nanjing Co. The glass chromatographic column (20 mm × 600 mm), silica gel plate GF_{254} and column chromatography silica gel (200–300 mesh) were obtained from Solarbio Co. Acetonitrile, and HPLC grade methanol and acetic acid were obtained from Merck Co (Germany). All other chemicals and reagents used were of analytical grade and were purchased from Qingdao Chemical Reagent Co.

2.1.3. Extract preparation

The *Pleurotus eryngii* byproducts from bud thinning in factory production were dried by vacuum freeze drying. The powder were obtained from the laboratory of Qingdao Agricultural University. The freeze dried powder (1 kg) of the *Pleurotus eryngii* byproducts was reflux extracted three times with 5000 mL of methanol at 55 °C for 12 h each time to yield 98.1 g of methanol extract (PEM) after nitrogen blow drying. The methanol extract from the *Pleurotus eryngii* byproducts was dispersed as a suspension in water, and then sequentially extracted with petroleum ether, ethyl acetate and butyl alcohol. The petroleum ether extract (9.81 g), ethyl acetate extract (17.20 g), butyl alcohol extract (35.38 g) and water extract (27.70 g) were stored at 4 °C until use. The butyl alcohol extract was subjected to gradient elution with a chloroform-methanol solution (chloroform: methanol = 100:0, 95:5, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, 0:100) by silica column chromatography. The components of the collection were concentrated using thin-layer chromatography with the corresponding solvent, merged to the same polarity composition, and the butyl alcohol extract was divided fractionated into seven components: Fr. 1, Fr. 2, Fr. 3, Fr. 4, Fr. 5, Fr. 6, and Fr. 7. The separation step is shown in Fig. 1.

2.2. Determination index

2.2.1. Determination of cell survival rate

The cell survival rate (%) of macrophages was evaluated using the methods (proliferation assay) as described by Maringa et al. [23]. Cells were inoculated into each well (100 μL) of 96-well plates at 10^5 cells/mL and incubated at 37 °C and 5% CO_2 for 24 h. After that, to each well in the experimental group 100 μL of complete medium was added containing different concentrations of extract such that the final concentration of each well was 6.25, 12.5, 25, 50, 100, 200 μg/mL respectively and to each well in the blank control group complete medium was added, six wells with cells were set for each concentrations. After incubation for 24 h, 20 μL MTT solution filtered (5 mg/mL) through a 0.45 μm filter was added for an additional 4 h. The supernatant was discarded and 150 μL of dimethyl sulfoxide (99.9%) was added to solubilize the formed formazan crystals. The absorbance of each well was read at 570 nm using a microplate reader after the plates were shaken for 10 min. The cell survival rate (%) was calculated as: survival rate = $(A_{exp}/A_{control}) \times 100$, where A_{exp} is the absorbance of the experimental group, and $A_{control}$ is the absorbance of the blank control group.

2.2.2. Determination of TNF-α and IL-6 secretion

Cells were inoculated into each well (100 μL) of 96-well plates at 10^5 cells/mL and incubated at 37 °C and 5% CO_2 for 24 h. After that, to each well in experimental group 100 μL of complete medium was added containing different concentrations of extract such that the final concentration of each well was 6.25, 12.5, 25, 50, 100, 200 μg/mL respectively and to each well in the blank control group complete medium was added, six wells with cells were set for each concentrations. The pooled supernatant was collected after 24 h and centrifuged at 1500 × *g* for 10 min, and the TNF-α and IL-6 secretion was determined according to the Elisa kit instructions. According to the Elisa kit directions, three times the immunological assays on Ana-1 cells have been repeated for cytokine determination.

2.2.3. Determination of pinocytic activity

The effect on pinocytic activity of target cells of the extracts of the *Pleurotus eryngii* byproducts were evaluated using the neutral red assay as described by Jacobo-Salcedo [24] with modifications. Cells were inoculated into each well (100 μL) of 96-well plates at 10^5 cells/mL and incubated at 37 °C and 5% CO_2 for 24 h. After that, to each well in experimental group 100 μL of complete medium was added containing different concentrations of extract such that the final concentration of each well was 6.25, 12.5, 25, 50, 100, and 200 μg/mL respectively and to each well in the blank control group complete medium was added, six wells with cells were set for each concentrations. After incubation for 24 h, 200 μL of 0.1% neutral red solution through a 0.45 μm filter was added to each well for an additional 1 h. The supernatant was discarded and 200 μL 0.1 mol/L of the acetic acid/alcohol solution was added to solubilize the cells. The absorbance of each well was read at 490 nm using a microplate reader.

2.2.4. QTOF-LC/MS/MS analysis

2.2.4.1. Liquid chromatography. The analytical column for the liquid chromatography was a Thermo BioBasic C18 column (2.1 mm × 100 mm, 5 μm). Fr. 2 from the butyl alcohol elution dissolved in methanol (0.5 mg/mL) was eluted with a mixture of methanol (A) and 1% acetic acid (B) at a flow rate of 0.2 mL/min at 30 °C. The UV absorbance of the effluent was measured from 200 nm–400 nm. The program for the gradient elution was 0–20 min: 10% A; 20–25 min: 15% A; 25–30 min: 65% A; 30–31 min: 65%–100% A; 31–40 min: 100% A.

2.2.4.2. Mass spectrometry. Ionization methods: electrospray ionization (ESI), negative ion mode, source voltage: 4500 V, debris voltage 130 V, spray pressure: 1.0 bar, drying temperature: 180 °C and dry gas volume flow: 6.0 L/min, Quality scan range: 50–1500 *m/z*.

2.2.5. HPLC analysis

The HPLC analysis of the butyl alcohol extract Fr. 2 was carried out on a Shimadzu LC-2010A HPLC system equipped with a quaternary gradient pump unit, an UV–vis detector (190–700 nm), an autosampler (0.1–100 L) and a column oven (273–333 K). The analytical column was an XTerra Prep RP C18 HPLC column (7.8 × 150 mm, 10 μm). The elution of Fr. 2 was performed with a mixture of acetonitrile (A) and 1% acetic acid (B) at a flow rate of 3 mL/min at 30 °C. The UV absorbance of the effluent was measured at 260 nm. The gradient elution program