Full Length Research Paper

Partially purified mushroom exudates induce apoptosis in acute promyelocytic leukemia cell line (HL-60)

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Mycelial exudates of *Lentinula edodes* 1358 strain were observed to be produce profusely among other strains. They were collected, partially purified and evaluated for their antiproliferation activity on acute promyelocytic leukemia cells (HL-60). The partially purified fraction 8 prepared by preparative HPLC using 1% acetic acid and methanol had the strongest inhibition concentration (IC_{50}) values at 21.59 µg/ml (48 h) and 16.89 µg/ml (72 h). Further evaluation using fluorescent dyes and cell cytometry suggested that apoptosis was involved. One sub-fraction of fraction 8 showed substantially low IC_{50} at 1.56 µg/ml (72 h), suggesting it might contain the most potent compound.

**Key words:** Mushroom, exudates, apoptosis, antiproliferation, acute promyelocytic leukemia cells (HL-60).

INTRODUCTION

*Lentinula edodes* (shiitake) is currently the second most commonly cultivated edible mushroom in the world (Chang, 2002). It is the most popular fungus cultivated in China, Japan, and other Asian countries. According to ancient Chinese medicinal theory, consumption of shiitake contributes to longevity and good health (Sia and Candlish, 1999). In China and Japan, shiitake has been used as both a food and a medicinal herb for thousands of years (Jones, 1995; Slaven et al., 2006). Many studies have revealed its strong anti-tumor, immune-modulating and cardiovascular effects. It is the source of several well-studied preparations, with proven pharmacological properties (Mizuno, 1995, 1996; Wasser, 2002; Yap and Ng, 2003), especially the polysaccharide lentinan and shiitake mushroom mycelium (LEM) (Arinaga et al., 1992; Kidd, 2000; Yap and Ng, 2001, 2003; Zhang et al., 2005). Lentinan is a water soluble anti-tumor polysaccharide from the fruiting bodies of shiitake. It induces the lymphocytokines and activates the lymphocytes, resulting in the stimulation of the immune system (Kupfahl et al., 2006). Lentinan is also useful for the treatment of hepatitis B (Mizuno, 1995, 1996; Han, 2001). Leukemia is a form of cancer that involves the bone marrow and blood circulation systems (Bennett, 2000). The cause of leukemia is unknown but it results in the uncontrolled growth of abnormal white blood cells. Acute promyelocytic leukemia is a subtype of acute myelocytic leukemia (AML) which occurs more prevalent in adults than youngsters (ACS, 2010). Cells that normally develop to neutrophils, basophils, eosinophils, and monocytes become cancerous and are rapidly replaced in the bone marrow. So far, investigations involving the anti-cancer activity of shiitake focus mostly on the shiitake fruiting body while the activity in its exudates is rarely reported (Harhaji et al., 2008; Lu et al., 2009). Therefore, this investigation is to report the antiproliferation activity of the partially purified mushroom exudates on the acute promyelocytic leukemia cell line (HL-60), as well as the cause of such activity.

MATERIALS AND METHODS

Cultivation of *Lentinula edodes* mycelia

*L. edodes* strain 1358 was provided by Professor HS Kwan, School of Life Sciences, the Chinese University of Hong Kong. A cuboid (1
Collection and fractionation of exudates

To collect the dark brown exudates which diffused into the PDA, the surface of the PDA was scraped clear of the mycelia, cut to small pieces, and sonicated twice in the milli-Q water for 4.5 h. The aqueous layer was collected, centrifuged and freeze-dried to prepare a dry crude extract which was further purified as shown in Figure 1. Briefly, the extract was redissolved in milli-Q water and precipitated in absolute ethanol at room temperature for 5 h. The mixture was centrifuged for 20 min at 15,000 g. The supernatant was concentrated. Addition of ethanol, followed by centrifugation was repeated until no precipitate was found. The remaining solution was freeze-dried to produce the solid ethanol precipitate.

Based on the separation times of different peaks in the HPLC profile, 12 fractions were collected by the Waters Fraction Collector II (Waters, MA, USA), and their anti-proliferation activity on the HL-60 cells was assessed. The most active HPLC fraction (no. 8) was further separated by preparative Thin layer chromatography (TLC) (Si 60 F254, 20 × 20 cm glass plate, 0.5 mm thickness, Merck, Germany) using a solvent system composed of chloroform, methanol and water at 7:3:1 ratio (v/v). The active fraction and sub-fractions (F08_1 and F08_2) were evaluated for their activities.

Anti-proliferation effect of different fractions on leukemia cells determined by trypan blue exclusion method (Louis and Siegel, 2011)

Briefly, fifty microliters of HL-60 cell suspension (5 × 10^6 cells/ml) were co-incubated in the Roswell Park Memorial Institute medium (RPMI) 1640 culture medium at different concentrations (0.78, 1.56, 3.13, 6.25, 12.50, 25.00, 50.00, and 100.00 μg/ml) of fraction 08 for 48 and 72 h or fraction 08_1 and 08_2 for 72 h in a humidified incubator with humidified atmosphere at 37°C, 5% CO2 and 95% air. One hundred microliters of 0.4% trypan blue in phosphate buffered saline (PBS, w/v, pH at 7.4) was mixed thoroughly with equal volume of cell suspensions of control and treatment for 1 min. The number of unstained (viable) cells and stained cells (dead cells) were counted by a hemocytometer. Both cell viability and inhibition rate were calculated. Inhibition concentration (IC50) was estimated by graphical interpolation (Popioliukiewicz et al., 2005).

Quantification of apoptotic cell death using fluorescent dyes and fluorescence microscopy (Hoores et al., 1996)

HL-60 (5 × 10^4 cells/mL) and exudates fraction were incubated in the RPMI 1640 culture medium in microplate for 12 h (triplicate) in a humidified incubator (37°C, 5% CO2 and 95% relative humidity). HL-60 cells without the addition of partially purified fraction were prepared and used as a control. After incubation, cell mixtures were centrifuged (200 × g, 10 min), pellet was collected, re-suspended and stained by 2 μL of Hoechst 33342 (20 μg/ml in PBS) + propidium iodine (PI) (10 μg/ml in PBS) staining solution. Mixture (10 μL) was placed on slides, covered, and examined with different objectives (40 to 60×) using the epilumination and a filter combination at 340 to 380 nm. Cell number at the following states was recorded: (a) live cells with normal nuclei (LN: blue chromatin with organized structure); (b) live cells with apoptotic nuclei (LA: blue chromatin that is highly condensed or fragmented); (c) dead cells with normal nuclei (DN: pink chromatin with organized structure); and (d) dead cells with apoptotic nuclei (DA: pink chromatin that is highly condensed or fragmented). Apoptotic index was determined using the following formula:

\[
\text{% Apoptotic cells (apoptotic index)} = \frac{\text{LA} + \text{DA}}{\text{LN} + \text{LA} + \text{DN} + \text{DA}} \times 100
\]

Flow cytometric analysis of HL-60 cells in the presence of fractions 08 (Nicoletti et al., 1991; Givan, 1992)

Using a minimum density of 1 × 10^6 HL-60 cells per condition, sample and HL60 were incubated as described before. The pellet of HL-60 cells after centrifugation at 400 × g and 10°C for 5 min was re-suspended in 5 ml of cold PBS. The centrifugation and re-suspension steps were repeated once. Cells were fixed with 3 ml of 70% ethanol, incubated at -20°C for 1 h, centrifuged and finally washed with 5 ml of cold PBS. One milliliter of propidium iodine (PI, 10 μg/ml in PBS) working solution with DNase-free, RNaseA and Triton X-100 was added to the cells and incubated at 37°C in the dark for 30 min. The fixed cells were analyzed by a COULTER Epics XL flow cytometry system (Beckman Coulter, Inc., CA, USA) in sextuplicate and the percentages of sub G1 was recorded.

Gas chromatography-mass spectrum (GC-MS) of sub-fraction F08_01

Four microliter of the sub-fraction F08_01 at 1 g/l was injected into a gas chromatography coupled with mass spectrometry (GCMS-Q5050 system, Shimadzu, Japan) at splitless mode for separation and analysis. An HP-5 column (30 m, 0.25 mm i.d., 0.25 mm film; Agilent, CA, USA) was installed. Helium gas (purity: 99.9999%) was set at a linear flow rate of 30 cm/s. Temperature programme was as follows: initial and final temperatures: 35°C (5 min) and 195°C (179 min); ramp rate: 10°C/min. Mass spectrometry was set at scan mode with mass/charge (m/z) range as: 24 to 600.

RESULTS AND DISCUSSION

Exudates of L. edodes 1358 strain secreted to the solid culture medium (PDA) was extracted and monitored to track the fraction having high antiproliferative activity. Twelve fractions were collected according to the optimal peak separation time period as shown in Table 1. Among them, trypan blue exclusion assay was used to determine the inhibition rate of each fraction at different concentrations and at two different treatment periods. Table 1 shows the calculated IC50 value based on the present findings. Apparently, fraction 8 at both 48 and 72 h treatments had the lowest IC50 at 21.59 and 16.89 μg/ml, respectively; indicative of its high antiproliferation activity towards HL-60 cells. Its yield from the crude extract was
0.316% (w/w) (Figure 1). The fraction was immediately tested for its ability to induce apoptosis in order to explain the cause of antiproliferation.

During apoptosis, the structural integrity of most cytoplasmic organelles is often preserved but morphological changes involving condensation and fragmentation of chromatin into uniformly dense masses occurs dramatically in the nucleus. These changes can be readily detected under a fluorescence microscope with the aid of DNA-binding dyes such as Hoechst dyes (Duke, 2004; Hacker, 2000).

Whether stained cells were living or dead, or with normal or apoptotic nuclei, they were counted and expressed by the apoptotic index. Figure 2A shows the increasing index value as the concentration of fraction 8 increases. This observation suggests that fraction 8 induced apoptosis in the HL-60 leukemia cells.

Further proof of the apoptotic induction by fraction 8 was shown by the results of cell cytometry. Apoptotic cells have deficient DNA content and can be recognized by flow cytometry as compared to that in the G1 cells. On a DNA content histogram, the apoptotic cells form a characteristic sub-G1 peak. Generally, the percentage of cells in sub-G1 peak reflects the extent of apoptosis (Givan, 2001). There were significant differences in the percentage of sub-G1 being detected among the different concentrations of fraction 8 applied.

The highest percentage of sub-G1 found was at the concentration of 100 µg/l. As high percentage of sub-G1 signifies strong apoptosis in HL-60 leukemia cells, Figure 2B suggests that stronger apoptosis generally occurred at concentration higher than 25 µg/l of fraction 8. Typical DNA content histograms of control and experimental groups are shown in Figure 2C and D. In short, both assays show apoptosis in the HL-60 cells occurred when fraction 8 of the exudates was added, and was more effective at higher concentrations. To further our search for the potent component(s), fraction 8 was subjected to preparative TLC separation, and two sub-fractions were established, namely F08_1 and F08_2 (Figure 1). Through the bio-assay, that is trypan blue exclusion method, inhibition rates of the sub-fraction F08_01 were determined and its IC₅₀ was found to be 1.56 µg/l in the 72 h treatment (Figure 3A). Its yield was 0.122% of the crude extract. One major peak was found when F08_1 was separated by the GC-MS having the major m/z fragments as follows: 133, 51, 77, 146, 105, 158, 187 (Figure 3C and D). For sub-fraction F08_02, cells survived well at all concentrations, suggesting its antiproliferation ability was very low (Figure 3B).

There are a number of current reviews on mushrooms (Lindequist et al., 2005; Fan et al., 2006; Moradali et al., 2007). More often, fungal fruiting body was reported to have anticancer property while fungal exudates were rarely investigated and reported (Harhaji et al., 2008; Lu et al., 2009). The property was suggested to be due to the presence of either macromolecules (for example, β-D-gulcoan derivatives, glycopetide complexes, etc.) or small molecules (for example, sesquiterpenes, triterpenes, etc.). Antitumor activity was also reported for the combined treatment of mushroom extract with chemotherapeutic drugs (Wu et al., 2007). Our present investigation showed that the exudates of *L. edodes* 1358 strain also had antiproliferation ability, and is likely to be a small molecule. Yet, further investigation is needed to elucidate the identity of the active component.

**Conclusion**

Exudates were found to possess strong antiproliferation

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<th>S/N</th>
<th>Time (min)</th>
<th>Flow (ml/min)</th>
<th>Solvent A (%)</th>
<th>Solvent B (%)</th>
<th>Fraction collection time (min)</th>
<th>IC₅₀ (µg/ml) at different treatment periods</th>
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A: 0.1% acetic acid; B: absolute methanol.

<p>| Table 1. Separation conditions of low polar fraction of shiitake exudates (1359 strain) by semi-preparative HPLC, and the IC₅₀ values of the fractions on HL-60 cells. |
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A: 0.1% acetic acid; B: absolute methanol.
Crude extract
  → DD water
  → Saturated solution
  → Absolute ethanol
  → Completely mixing
  → Centrifuge
  → Precipitate
  → Supernatant
  → Concentrate
  → Absolute ethanol
  → Completely mixing
  → Centrifuge
  → Precipitate
  → Supernatant
  → Rotaevaporation
  → Concentrate
  → Absolute ethanol
  → Completely mixing
  → Centrifuge
  → Precipitate
  → Supernatant
  → No precipitate
  → Rotaevaporation, Freeze drying
  → Ethanol insoluble fraction
  → Ethanol soluble fraction
  → (22.300% of crude extract)
  → Macroporous resin column separation
  → Low polar fraction
  → (17.990% of ethanol soluble fraction)
  → High polar fraction
  → (4.012% of crude extract)
  → Semi-preparative HPLC
  → F08_01
  → (38.559% of HPLC fraction08)
  → (0.122% of crude extract)
  → F08_02
  → (7.870% of low polar fraction)
  → (0.316% of crude extract)

**Figure 1.** Isolation scheme of F08_01 from the crude extract of *Lentinula edodes* 1358 strain and the recovery in each step.
activity on HL-60 cells which was likely contributed by the apoptotic induction. Further isolation is underway to collect sufficient sample for confirmation of the potent component in the sub-fraction.

ACKNOWLEDGMENT

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Figure 3. Effect of (A) the sub-fraction F08_01 and (B) the sub-fraction F08_02 of the shiitake 1358 strain on the proliferation of HL-60 leukemia cells after 72 h treatment, (C) GC-MS chromatogram of sub-fraction F08_01, (D) mass spectrum of the major peak with retention time at 39.84 min. HL-60 leukemia cells (5 × 10⁴ cells/ml) were incubated with different concentrations of F08_01 (0.78–100 µg/ml) at 37°C for 72 h. Cell proliferation was measured by the trypan blue exclusion method. Each bar in (A) and (B) represents the mean ± SD, n = 6. Different letter means significant difference (ANOVA, p ≤ 0.05).

REFERENCES


