

# Effects of Shiitake (*Lentinus edodes*) Extract on Human Neutrophils and the U937 Monocytic Cell Line

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The aqueous extract of the shiitake mushroom was found to decrease IL-1 production and apoptosis in human neutrophils, as measured by ELISA and flow cytometry respectively. It was found to increase IL-1 production and apoptosis in the U937 monocytic cell line. The extract showed no significant effects on the superoxide production of both neutrophils and U937 cells, as measured by chemiluminescence. The extract was further separated into high and low molecular weight components, and it was found that the low molecular weight component retained the activity of the whole extract. This further suggests that the active substance is a novel compound distinct from lentinan, a well-studied high molecular weight anti-tumour agent found in shiitake. Copyright © 1999 John Wiley & Sons, Ltd.

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## INTRODUCTION

The shiitake mushroom, *Lentinus edodes*, is the second most popular edible mushroom in the global market. Ancient Chinese medical theory recommends its consumption for longevity and good health. More recently, research has shown that extracts of *L. edodes* have hypolipidaemic, anti-thrombotic, antibiotic, antiviral and anti-tumour activity. The most well-studied bioactive compound in the shiitake mushroom is the polysaccharide lentinan, a potent anti-tumour agent commercially available for clinical use, which appears to act as a host defence potentiator of T-cell activity (Jong and Birmingham, 1993).

There appears to be little research on the effects of shiitake extract on leukocytes other than T-cells. The aim of our project was to investigate its effects on human neutrophils and the U937 monocytic cell line (Sundstrom and Nilsson, 1976). Three parameters of neutrophil activity were studied, namely superoxide production, interleukin-1 production and apoptosis.

## MATERIALS AND METHODS

**Preparation of shiitake extract.** 100 g of *Lentinus edodes* fruiting bodies purchased at a local supermarket was boiled with 200 mL of distilled water for 1 h, after which the volume was readjusted to 200 mL. To ascertain the concentration, a specific volume was freeze dried, and found to contain 21 mg/mL solid matter. The extract was filtered and adjusted to pH 7.4. The filtrate was divided into 3 mL aliquots and stored below 0°C. This was

designated as extract 1:1, with extract 1:2 being the two-fold dilution of this extract. The extract was further extracted with 60% ethanol. The precipitate was redissolved in the original volume of distilled water and designated the high molecular weight component (HMW). The 60% ethanol extract was blown dry with oxygen-free nitrogen and designated the low molecular weight component (LMW). Both components were stored below 0°C. The HMW and LMW components had 17.6 mg/mL and 3.4 mg/mL solid matter, respectively.

**Preparation of cells.** Neutrophils were isolated from the peripheral heparinized blood of healthy adult volunteers. 5 mL of blood was layered on 3.5 mL of Polymorph-prep™ centrifuged at 1700 rpm for 30 min. The neutrophil band was washed twice with phosphate buffered saline (PBS) at pH 7.4 and diluted to  $1.5 \times 10^6$  cells per mL. Cell viability was above 95% as determined by trypan blue exclusion.

The U937 cell line from American Type Culture Collection was cultured on RPMI 1640 with 2 mM L-glutamine containing 100 U/mL of streptomycin and penicillin in a humidified 5% CO<sub>2</sub> atmosphere at 37°C. The cells were harvested after 2 days, washed once, and diluted to  $1.5 \times 10^6$  cells per mL. Cell viability was above 95% as determined by trypan blue exclusion.

**Superoxide assay by chemiluminescence.** Lucigenin chemiluminescence has been shown to be an accurate measure of the amount of superoxide produced by neutrophils (Gyllenhammar, 1987), and was used to assay superoxide production. 2 mL of 10 mM lucigenin in PBS and 0.25 mL of cells in PBS were preincubated at 4°C in the dark for 2 h. 0.2 mL of PBS, phorbol myristate acetate (PMA, 1 mg in 1 mL DMSO diluted 25-fold with PBS), extract 1:1, extract 1:2, HMW or LMW was then added and the chemiluminescence was measured with a

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Beckman LS6000L scintillation counter operating in single photon mode. The number of photons emitted during three successive 0.3 min intervals at various times were noted. The three readings were averaged to obtain the number of counts per minute.

**Interleukin-1 (IL-1) assay by ELISA.** One mL of cell suspension in RPMI 1640 (with glutamine, 10% fetal calf serum and 100 U/mL penicillin and streptomycin) was incubated with 0.5 mL of PBS and 0.5 mL of PMA, extract 1:1, extract 1:2, HMW or LMW in a humidified 5% CO<sub>2</sub> atmosphere at 37°C for 24 h. The cells were removed by centrifugation and the supernatant was used for IL-1 assay with ELISA kits purchased from Amersham after storage at -20°C. The microplates were read at 450 nm on a Biotek Instruments microplate autoreader.

**Apoptosis assay by flow cytometry.** The cell pellet obtained above (in the IL-1 assay) was washed once with PBS, resuspended in 100 mL PBS and fixed by incubation with 100 mL buffered formalin at room temperature for 30 min. The cells were then washed once with PBS and permeabilized with 100 mL 0.1% Triton X-100 in 0.1% sodium citrate on ice for 2 min. After two washings in PBS, the cells were incubated at 37°C with 50 mL of terminal deoxynucleotide transferase dUTP nick end labelling (TUNEL) mixture (TUNEL apoptosis kit purchased from Boehringer Mannheim) in a humidified atmosphere for 1 h. The cells were then washed twice with PBS and resuspended in 250 mL PBS. The fluorescein isothiocyanate (FITC) fluorescence of the cells was measured by flow cytometry using a Becton-Dickinson FACStar. All measurements in each set of experiment were made with the same instrument settings. The data obtained were analysed with WinMDI 2.13. A high fluorescence value corresponds to more fragmented DNA and indicates more advanced apoptosis. The mean log FITC was taken as a measure of apoptosis.

**Statistics.** Experiments were carried out in sets of four, consisting of PBS (negative control), PMA (positive control) and either extract 1:1 and 1:2, or HMW and LMW. The statistical significance of differences between groups was analysed by Student's (two-tailed) *t*-test for paired samples, comparing each experimental observation with its control. Statistical tests were performed with the software Sigmaplot (Jandel Scientific).

## RESULTS

### Effects of whole extract on human neutrophils

In each set of experiments, PBS served as a negative control while PMA, a known activator of neutrophils, served as a positive control. Neutrophils were collected from three healthy adult males on more than two occasions and the results of each individual were averaged. Tables 1–4 give the grand means for the three individuals. The results for the U937 cell line are means of 3 to 12 experiments.

Neutrophils incubated with shiitake extract showed comparable superoxide production to that incubated with PBS (Table 1). Predictably, neutrophils incubated with

**Table 1. Effects of whole extract (21 mg/mL) on neutrophil superoxide production**

Experimental group	Counts per min $\pm 10^3$	<i>p</i> value
PBS	45.2 $\pm$ 22.0	–
PMA	269.4 $\pm$ 143.0	–
1:1	77.4 $\pm$ 54.2	–
1:2	112.6 $\pm$ 88.5	–

All values are mean  $\pm$  SD., *n* = 3.

**Table 2. Effects of whole extract (21 mg/mL) on neutrophil IL-1 $\alpha$  production**

Experimental group	Wt of IL-1 $\alpha$ produced (pg)	<i>p</i> value
PBS	51.3 $\pm$ 16.0	–
PMA	15.2 $\pm$ 0.8	–
1:1	16.4 $\pm$ 1.8	0.06
1:2	18.9 $\pm$ 1.4	0.08

All values are mean  $\pm$  SD., *n* = 3.

**Table 3. Effects of whole extract (21 mg/mL) on neutrophil IL-1 $\beta$  production**

Experimental group	Wt of IL-1 $\beta$ produced (pg)	<i>p</i> value
PBS	92.6 $\pm$ 18.7	–
PMA	71.9 $\pm$ 31.2	–
1:1	21.4 $\pm$ 2.8	0.02
1:2	30.2 $\pm$ 3.0	0.03

All values are mean  $\pm$  SD., *n* = 3.

**Table 4. Effects of whole extract (21 mg/mL) on neutrophil apoptosis**

Experimental group	FITC log	<i>p</i> value
PBS	4.85 $\pm$ 0.82	–
PMA	4.47 $\pm$ 2.90	–
1:1	2.25 $\pm$ 0.52	0.03
1:2	2.49 $\pm$ 0.77	0.02

All values are mean  $\pm$  SD., *n* = 3.

PMA showed a sharp rise in superoxide production. The extract depressed both IL-1 $\alpha$  (*n* = 3, *p* = 0.06) and IL-1 $\beta$  (*n* = 3, *p* = 0.02) production in a concentration-dependent manner (Tables 2 and 3). Apoptosis of neutrophils was also markedly depressed, as shown by the absence of an apoptotic population in neutrophils incubated with the extract (Fig. 3). The histogram of cell events against FITC log shows two peaks for neutrophils incubated with PBS but only one peak for that incubated with extract 1:1 (Fig. 1). This may indicate selective apoptosis of a subpopulation of neutrophils, which the extract protects. The level of apoptosis was quantified by the mean FITC log, which showed that the suppression of apoptosis was concentration dependent (Table 4). Neutrophils incubated with PMA showed moderate DNA fragmentation, comparable to that of PBS. Visual examination under a light microscope revealed many ruptured cells with diffuse chromatin. This is consistent with previous

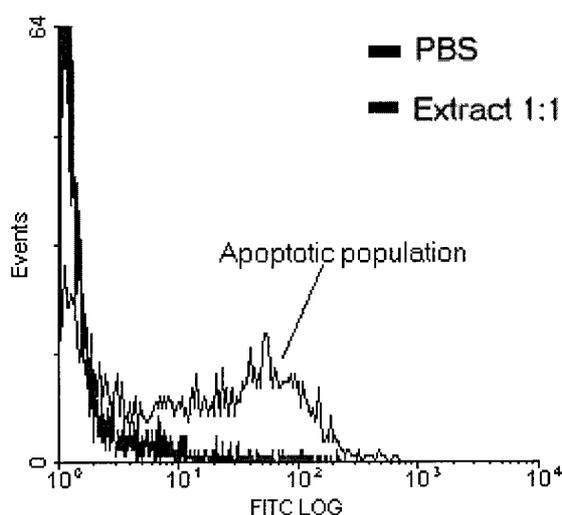


Figure 1. Effect of whole extract on neutrophil apoptosis.

findings that PMA-treated neutrophils undergo a form of cell death distinct from apoptosis and necrosis, characterized by an increase in membrane permeability and a lack of DNA degradation products smaller than 300 kbp (Takei *et al.*, 1996).

#### Effects of whole extract on U937 cells

U937 cells incubated with the extract were comparable to that incubated with PBS (Table 5). The extract increased both IL-1 $\alpha$  ( $n = 7$ ,  $p < 0.01$ ) and IL-1 $\beta$  ( $n = 7$ ,  $p < 0.01$ ) production in a concentration-dependent manner (Tables 6 and 7). The extract also appeared to increase apoptosis ( $n = 6$ ,  $p = 0.05$ ), again in a concentration dependent manner (Table 8).

#### Effects of extract components on U937 cells

Having established the effects of the whole extract on

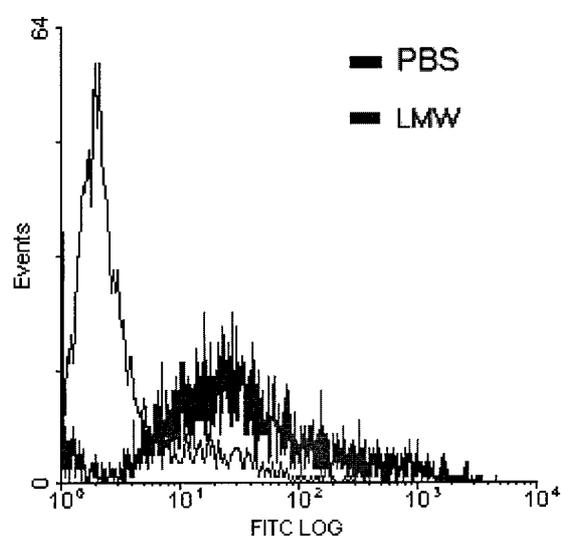


Figure 2. Effect of extract components on U937 apoptosis.

Table 5. Effects of whole extract (21 mg/mL) on U937 superoxide production

Experimental group	Counts per min $\pm$ SD	$p$ value
PBS	13.8 $\pm$ 3.0	–
PMA	53.3 $\pm$ 12.8	–
1:1	17.9 $\pm$ 2.9	–
1:2	15.7 $\pm$ 2.1	–

All values are mean  $\pm$  SD.,  $n = 9$ .

Table 6. Effects of whole extract (21 mg/mL) on U937 IL-1 $\alpha$  production

Experimental group	Wt of IL-1 $\alpha$ produced (pg)	$p$ value
PBS	12.5 $\pm$ 2.6	–
PMA	99.9 $\pm$ 63.3	–
1:1	46.9 $\pm$ 22.8	<0.01
1:2	36.9 $\pm$ 10.9	<0.01

All values are mean  $\pm$  SD.,  $n = 7$ .

Table 7. Effects of whole extract (21 mg/mL) on U937 IL-1 $\beta$  production

Experimental group	Wt of IL-1 $\beta$ produced (pg)	$p$ value
PBS	21.3 $\pm$ 3.2	–
PMA	548.1 $\pm$ 205.4	–
1:1	321.6 $\pm$ 150.6	<0.01
1:2	290.5 $\pm$ 147.9	<0.01

All values are mean  $\pm$  SD.,  $n = 7$ .

Table 8. Effects of whole extract (21 mg/mL) on U937 apoptosis

Experimental group	FITC log	$p$ value
PBS	2.63 $\pm$ 0.42	–
PMA	3.47 $\pm$ 0.81	–
1:1	4.06 $\pm$ 1.79	0.05
1:2	3.17 $\pm$ 1.08	0.10

All values are mean  $\pm$  SD.,  $n = 6$ .

both human neutrophils and the U937 cell line, we then separated the extract into HMW and LMW components and tested for activity with each component. Only U937 cells were used in this phase as the lack of interindividual variation made for more reproducible results. The U937 cells incubated with LMW component were comparable in superoxide production to that incubated with PBS (Table 9). LMW caused an increase in apoptosis ( $n = 6$ ,  $p = 0.02$ ) in U937 cells (Table 12) similar in magnitude to that obtained with the whole extract. The HMW component gave results comparable to that of the control. The histogram of cell events against FITC log showed only one peak for both PBS and LMW (Fig. 2), which indicates that LMW induced apoptosis in the entire cell population. Neither components increased IL-1 production in U937 cells (Tables 10 and 11), despite the fact that the whole extract caused such a response.

**Table 9. Effects of extract components on U937 superoxide production**

Experimental group	Counts per min $\pm 10^3$	<i>p</i> value
PBS	13.6 $\pm$ 2.7	–
PMA	53.3 $\pm$ 12.8	–
HMW (17.6 mg/mL)	12.9 $\pm$ 1.4	–
LMW (3.4 mg/mL)	16.5 $\pm$ 1.4	<0.01

All values are mean  $\pm$  SD., *n* = 12.

**Table 10. Effects of extract components on U937 IL-1 $\alpha$  production**

Experimental group	Wt of IL-1 $\alpha$ produced (pg)	<i>p</i> value
PBS	19.1 $\pm$ 0.3	–
PMA	72.2 $\pm$ 15.2	–
HMW (17.6 mg/mL)	19.9 $\pm$ 4.4	–
LMW (3.4 mg/mL)	36.9 $\pm$ 10.9	–

All values are mean  $\pm$  SD., *n* = 3.

**Table 11. Effects of extract components on U937 IL-1 $\beta$  production**

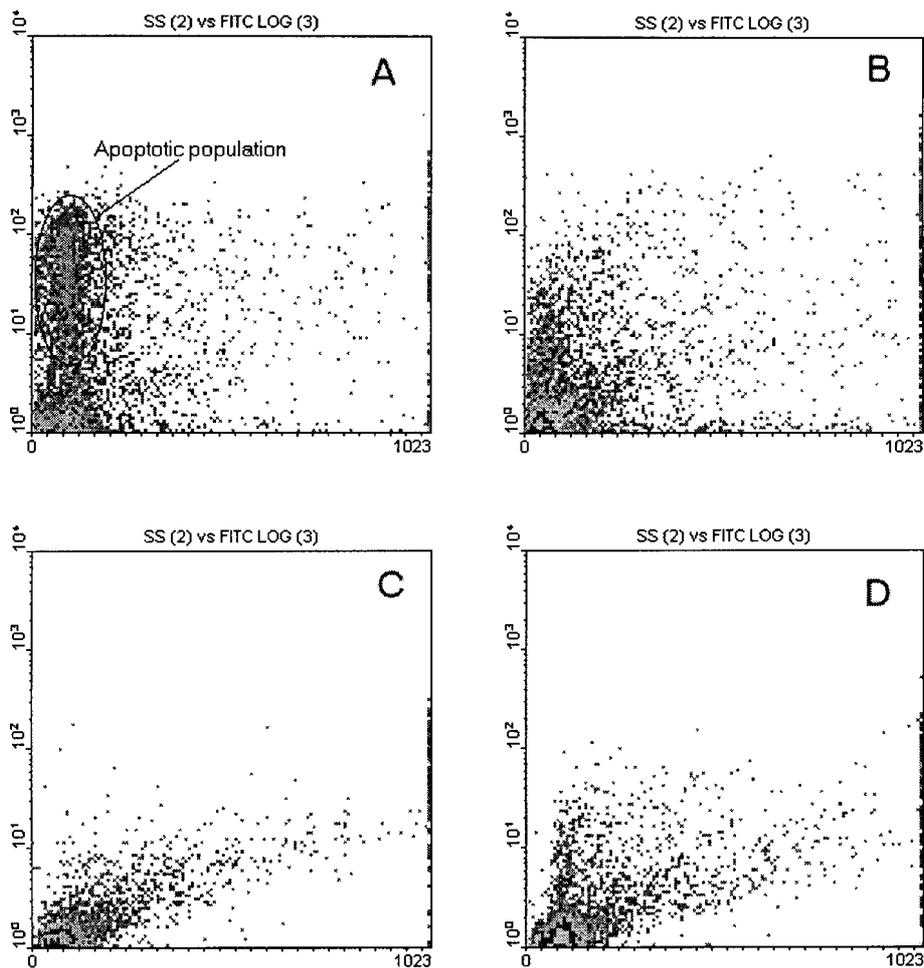
Experimental group	Wt of IL-1 $\beta$ produced (pg)	<i>p</i> value
PBS	23.1 $\pm$ 13.8	–
PMA	681.8 $\pm$ 88.5	–
HMW (17.6 mg/mL)	16.7 $\pm$ 5.6	–
LMW (3.4 mg/mL)	14.5 $\pm$ 3.7	–

All values are mean  $\pm$  SD., *n* = 6.

**Table 12. Effects of extract components on U937 apoptosis**

Experimental group	FITC log	<i>p</i> value
PBS	2.50 $\pm$ 0.90	–
PMA	3.62 $\pm$ 1.57	–
HMW (17.6 mg/mL)	2.76 $\pm$ 1.12	–
LMW (3.4 mg/mL)	3.70 $\pm$ 1.42	0.02

All values are mean  $\pm$  SD., *n* = 6.

**Figure 3.** Dotplots of neutrophils after incubating for 24 h with (A) PBS, (B) PMA, (C) Extract 1:1 and (D) Extract 1:2.

## DISCUSSION

IL-1 is a pro-inflammatory cytokine with a pathogenic role in several diseases, including inflammatory bowel

disease and atherosclerosis (Dinarello and Wolff, 1993). It does not appear to have an essential role in homeostasis, and studies have indicated that blocking its action does not affect host defence in the short term (Dinarello and Wolff, 1993). The large depression of neutrophil IL-1

production by shiitake extract encourages further studies on its potential as a therapeutic agent for IL-1 induced diseases. The extract also decreases apoptosis in neutrophils, which may be helpful in situations when the immune system is compromised. It has been shown by Guichon and Zychlinsky (1996) in time course experiments with macrophages that IL-1 release is a concomitant of apoptosis, so the latter plays a key role in the initiation of inflammation. The observed effects of the shiitake extract are apparently not accompanied by an increase in superoxide production, hence the extract is not likely to aggravate inflammatory conditions such as rheumatoid arthritis and asthma (Smith, 1994). Further work will be directed towards determining the extent to which intestinal absorption of extract components are potentially antiinflammatory in tissues.

The extract appears to have opposite effects on the U937 cell line and human neutrophils. The extract increased IL-1 production and apoptosis in U937 cells, but depressed them in neutrophils. Neutrophils are an end-stage cell present in sites of inflammation, and are an important component of the immune system. The U937 cell line is an immortal monocytic cell line derived from the pleural effusion of a leukaemic patient. The differential effect on cells from the immune system and

on a lymphoma derived cell line is novel and further investigation is required, since the possibility of selective therapeutic targeting is raised.

An increase in apoptosis in U937 cells was seen with the LMW component, suggesting that the active compound in modulating these activities is a peptide or steroid. Lentinan has been shown to increase IL-1 production in peripheral monocytes (Arinaga *et al.*, 1992), so it could be the compound which elicits an increase in IL-1 production in U937 cells. It has also been shown that lentinan is heat stable (Aoki, 1984), but can be denatured by urea and NaOH, with concomitant loss of biological activity, which can be restored by renaturation (Maeda *et al.*, 1988). Lentinan could have been denatured in the ethanol extraction procedure, resulting in a loss of activity. From the present work, it seems more likely that a LMW component is bioactive.

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