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In vitro cytostatic and immunomodulatory properties of the medicinal mushroom *Lentinula edodes*

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Abstract

Lentinula edodes, known as "shiitake" is one of the widely used medicinal mushrooms in the Orient. Antitumour activity of extracts of this mushroom has been widely demonstrated in animals and humans. However, this activity was shown to be host mediated and not by direct cytotoxic activity to cancer cells. This study demonstrates cytotoxic and cell growth inhibitory (cytostatic) effect of aqueous extracts of the mushroom on MCF-7 human breast adenocarcinoma cell line using an MTT cytotoxicity assay. Such effect was demonstrated with fruit body and mycelial extracts, the difference being that there was no significant suppression on normal cells with the latter. Furthermore mycelial extracts did not induce any cytostatic effect in both cancer and normal cell lines based on a DNA synthesis assay. The significant suppression of the proliferation of cancer cells was reflected by the comparatively low IC_{50} values and the simultaneous higher respective values on normal fibroblast cells. The immunostimulatory activity of both fruit body and mycelial extracts was tested by the lymphocyte transformation test (LTT), which is based on the capacity of active immunomodulators to augment the proliferative response of rat thymocytes to T mitogens *in vitro*. Both fruit body and mycelial preparations were able to enhance the proliferation of rat thymocytes directly and act as co-stimulators in the presence of the T-mitogen PHA. Interestingly both extracts, similarly to zymosan showed SI_{comit}/SI_{mit} ratios of about 2, indicating adjuvant properties. Overall *L. edodes* aqueous extracts have demonstrated direct inhibition of the proliferation of human breast cancer cells *in vitro* and immunostimulatory properties in terms of mitogenic and co-mitogenic activity *in vitro*.
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Keywords: *Lentinula edodes*; Cancer; Cytotoxic; Cytostatic; Antitumour; Mitogenic and comitogenic activity

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Introduction

Medicinal mushroom extracts have been considered as important remedies for the prevention and treatment of many diseases for thousands of years especially in the Orient (Israilides and Philippoussis, 2003; Kidd, 2000; Wasser and Weis, 1999). A plethora of medicinal effects has been demonstrated for many traditionally used mushrooms including antibacterial, antiviral, antifungal, antitumour and immuno-potentiating activities (Hobbs, 2003; Ooio and Liu, 1999). Among the various bioactive components which have been demonstrated to be most effective as antitumor and immunomodulatory agents are polysaccharides and polysaccharopeptides.

Lentinula edodes is the source of many therapeutic polysaccharide macromolecules among which the ones with proven pharmacological effects are lentinan, LEM and KS-2. Lentinan is a high molecular weight (about one million) homopolysaccharide in a triple helix structure, with linear chains consisting of $(1\rightarrow3)$-β-D-glucopyranosyl (Gl*cp)* residues with two β-$(1\rightarrow6)$-linked Gl*cp* branchings for every five β-$(1\rightarrow3)$-Gl*cp* residues (Aoki, 1984). LEM is a mycelial extract preparation of *L. edodes* harvested before the cap and stem grow. It is a heteroglycan–protein conjugate containing 24.6% protein and 44% sugars, comprising mostly pentoses as well as glucose and smaller amounts of galactose, mannose and fructose (Iizuka, 1986; Sugano et al., 1982). It also contains nucleic acid derivatives, B complex vitamins, ergosterol, eritadenine (an anticholesteremic amino acid), and water-soluble lignins (Sugano et al., 1985). KS-2 is a peptide–polysaccharide complex. The comparison of fruit body and mycelial extracts was carried out for the following reasons:

1. The production of fruit bodies and mycelium in *L. edodes* as well as in many other medicinal mushrooms, comprise the two main production methods (Wasser and Weis, 1999). The production of fruit bodies does not always guarantee a consistent product while the mycelial growth in fermenters under vigorously controlled conditions gives improved product purity and reproducibility.
2. The main antitumor polysaccharide in *L. edodes* fruit bodies is a single compound, lentinan. On the other hand there are many different active compounds in mycelia which have been demonstrated to have "antitumor" properties. This provides the opportunity for enhanced activity from crude extracts of fruit bodies or mycelium. The mechanism of antitumor activity of either lentinan, which is the main biologically active compound in *L. edodes* fruit bodies, or the mycelial extract has not been fully elucidated, but it has been reported as host mediated by activating the host's immune responses and not attacking cancer cells directly (Aoki, 1984; Meiqin et al., 1998). Therefore there is a need for comparison of the two kinds of extracts in an effort to investigate and differentiate tumor selective cytotoxicity.

Since many of the compounds, which are found in *L. edodes*, have been shown to act synergistically (Yamasaki et al., 1989), it is worth testing the cytotoxic and/or cell growth inhibitory effects of the whole mushroom and mycelium extract rather than its individual components. This principle (synergy) is compatible with similar natural biological products like the essential oils, which allow the achievement of strong effects when used as whole products, while quenching or nullifying potential unwanted side-effects by the presence of individual components.

The objectives of this project were to investigate the cytotoxic and cell growth inhibitory effect on normal and cancer cell lines of active *Lentinula edodes* extracts produced from both the mushroom and mycelia as well as their immunostimulatory activity with the '*in vitro*' comitogenic rat thymocytes test (lymphocyte transformation test, LTT).

Materials and methods

The strain of *L. edodes* (Berk.) Pegler used in this study, was originated from China and registered in the fungal culture collection of the Edible Fungi Laboratory of NAGREF with the code number AMRL 118. It was selected for its phenotypic characteristics concerning productivity and quality. The culture substrate preparation and growing procedure for sporophore production has been previously described (Philippoussis et al., 2007). The culture was maintained on a 2% potato dextrose agar (PDA, Merk) for routine culture and storage purposes.

Mycelia were grown in a submerged liquid fermentation in a Bioengineering L1523, 7 liter bench fermentor. The initial pH was 5.50, temperature 28 °C, and the aeration was 10 liter/min. The substrate composition was (w/v): malt extract 0.3%, yeast extract 0.3%, peptone 0.3% and glucose 1.0%. The inoculum, 500 ml, was grown in the same medium and the duration of fermentation was 3 days. The fruiting bodies and mycelia were dried by lyophilization and powdered. All extracts were stored at −40 °C.

Methanol and distilled water extracts from mushrooms and mycelia of *L. edodes* were prepared to an initial concentration of 100 mg/ml. The extracts were incubated for 2 h at room temperature under continuous shaking. They were centrifuged for 30 min at 1500*g* and the supernatant was passed through a 0.2 μm filter. Samples were further diluted with plain culture medium

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(Dulbecco's minimal essential medium (DMEM)) to the defined concentrations as indicated.

Cell cultures

Human breast adenocarcinoma cell line MCF-7 and human normal fibroblasts from a 30 year-old healthy volunteer were cultured in DMEM supplemented with antibiotics and 10% fetal bovine serum (FBS) and they were subcultured using trypsin-citrate (0.25–0.3%, respectively) solution. In the incubation chamber the gas mixture consisted of 5% CO_2 and 95% air. Furthermore, the humidity was adjusted to 85%, so as to diminish evaporation of the culture medium and the consequent changes to its osmolarity that could have been detrimental to the cultured cells. Cells were tested periodically and found to be mycoplasma-free. All cell culture media were from Gibco–BRL.

For the assessment of the cytotoxic and cytostatic activities of *L. edodes* extracts cells were seeded in 96-well flat-bottomed microplates at a density of approximately 5000 cells/well, in DMEM 10% FBS. After 18 h to ensure cell attachment, serial dilutions of the extracts in culture medium were added and incubated for 24 or 48 h. Then, cytotoxicity and DNA synthesis rate were determined using the MTT assay and tritiated thymidine incorporation, respectively.

In testing the cytotoxic or cytostatic effects of different substances on cancer cells the ideal control is always an issue. Such control ideally could be normal epithelial cells originating from a neighboring area with healthy tissue of the same patient. However, this is not always feasible, especially regarding commercially available cancer cell lines. On the other hand, tumors *in vivo* are surrounded by stroma, thus understanding the effect of the studied substances on normal fibroblasts is equally important. In this study we have chosen to use a commercially available human cancer cell line, MCF-7, which is one of the most popular cell lines in the literature, because this would facilitate replication as well as comparisons with similar work. As a control we have used normal human stroma fibroblasts. Furthermore, MCF-7 cells and fibroblasts grow in the same medium, while normal epithelial cells require special serum-free, chemically-defined media for their culture, which would introduce further unequal parameters in the experiments.

Cytotoxicity assay

The assay detects the reduction of MTT [3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, Sigma] by mitochondrial dehydrogenase to blue formazan product, which reflects the cell viability, as well as the actual cell number of the culture. Following a 48-h-incubation of the cells with the extracts, the culture medium was replaced with MTT dissolved at a final concentration of 1 mg/ml in serum-free, phenol-red-free DMEM (Biochrom KG), for a further 4-h-incubation. Then, the MTT-formazan was solubilized in isopropanol and the optical density was measured at a wavelength of 550 nm and a reference wavelength of 690 nm. The results were assessed based on IC_{50}, the concentration that reduced by 50% the optical density of treated cells with respect to untreated controls.

DNA synthesis assay

In this assay, the rate of novel DNA synthesis in the cell nuclei is monitored, based on the incorporation of radiolabelled thymidine. Following a 24-h-incubation of the cells with the extracts, fresh culture medium was added along with [^{3}H]-thymidine (0.15 μCi/ml, 25 Ci/mmol) (Amersham, Buckinghamshire, UK). After incubation for further 14 h, the radioactivity incorporated in DNA was counted, by fixing the cells with trichloroacetic acid (10% w/v), washing copiously under running tap water and air-drying. Then DNA was solubilised by addition of 0.3 N NaOH–1% SDS and the lysates were subjected to scintillation counting.

Lymphocyte transformation test (LTT)

For the immunostimulatory activity testing (LTT) test, samples were dissolved in physiological solution (8.5 g NaCl/1000 ml d H_2O) to a 2% (w/v) concentration and mixed by a magnetic stirrer, until the suspension was homogeneous. Then it was centrifuged at 3000 rpm and the supernatant was sterilized (at 120 °C for 20 min) and was used for the test.

LTT was performed according to a slightly modified method elaborated for muramyl glycopeptides (Iribe and Koba, 1984), which do not stimulate thymocyte proliferation markedly. On the other hand, several polysaccharides were reported to be directly mitogenic for rat thymocytes (Ebringerová et al., 2002; Hromádková et al., 2003). Rat thymocytes (strain Wistar, males weighing about 200 g) in RPMI-1640 medium, HEPES modification (Sigma) supplemented with 5% fetal calf serum (Sigma) were cultivated at 1.5×10^6 cells in 0.2 ml per well either without or with 25 μg/ml phytohaemagglutinin (PHA). Test compounds were added at final concentrations ranging from 3–2000 μg/ml. After 72 h cultivation, thymocyte proliferation was measured by incorporation of [^{3}H]-thymidine expressed in counts per minute (cpm). In each of at least two experiments, means of the counts per minute (cpm) for each set of 4 replica experiments were used to calculate the stimulation indices (SI). The direct mitogenic effect of the compounds tested was expressed as: SI_{mit} = mean cpm

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for test compound/mean cpm for control. The comitogenic effect was expressed as: SI_{comit} = (mean cpm for PHA + test compound)/mean cpm for PHA. The eventual contamination with endotoxin was checked by cultivation of the thymocytes in presence of polymyxin B, which inhibits stoichiometrically its biological effect including the mitogenic activity. As positive control the commercial immunomodulator zymosan – a particulate β-glucan from baker's yeasts (Likospol Ltd., Bratislava, Slovakia) was used. Zymosan gave a fine suspension of non-sedimenting particles. Polymyxin B sulfate was from Wellcome (UK) and PHA from Murex Biotech Ltd. (UK).

Chemical and FTIR analyses

These were performed on lyophilised samples prepared from the fruit body and mycelium of *L. edodes* as described in a previous paper (Israilides and Philippoussis, 2003).

Statistical analysis

Multiple extracts from both fruiting bodies and mycelia were prepared and analysed on multiple occasions. The results for the cytotoxicity assay are presented as the mean of three independent experiments performed in quadruplicate wells. Differences from control cultures were considered significant when $p \leqslant 0.01$ (Student's *t*-test). In Figs. 2 and 3 asterisks above data points indicate significant differences from the control.

In the LTT test, the means of SI in repeated testing of the extracts were evaluated by analysis of variance (ANOVA), and calculations were done by the EP16 programme.

Results and discussion

Chemical and FTIR analyses

The analytical characteristics of the extracts isolated from the fruit body and mycelium of *L. edodes* are listed in Table 1. The glucose content, indicating the presence of glucan-type polysaccharides, is higher in the case of the mycelium, whereas, the mannoglycan content was twice as high in the fruit body. Mannose, galactose, glucose as well as the minor sugars are components of mannoglycans and mannoproteins of fungal cell walls (Kim et al., 2003; Peng et al., 2003).

The FT-IR spectra (Fig. 1) of lentinan samples showed the presence of considerable amount of proteins indicated by the absorption bands **c** at 1660 cm^{-1} ($\nu C=O$, amide I) and **d** at 1550 cm^{-1} (δ_{NH}, amide II).

Table 1. Analytical data of *L. edodes* mushroom and mycelium extracts

Sample	Fruit body	Mycelium
Nitrogen (wt%)	6.81	4.19
Klason lignin (wt%)	12.3	3.1
Ash (wt%)	5.14	4.15
Neutral sugar composition (rel. wt%)		
Fucose	0	0.6
Ribose	2.3	0.6
Arabinose	1.5	8.3
Xylose	1.5	1.1
Mannose	28.6	15.0
Glucose	55.9	70.8
Galactose	10.1	3.5

The bands **a** at 2850–3000 cm^{-1} (νCH_2 and νCH_3) and **b** at 1745 cm^{-1} and 1702 cm^{-1} (νCO), related to vibrations of alkyl chain, ester and free carboxyl groups, respectively, are indicative of lipids, and are particularly intense in the fruit body. The absorption bands in the mid-infrared region 1200–800 cm^{-1} are useful for the identification of polysaccharides with different structure and composition (Kačuráková et al., 2000). In contrast to the fruit body, the spectral pattern of the mycelium in this region showed some similarity to that of the β-glucan from *S. cerevisiae* (Hromádková et al., 2003).

Cytostatic/cytotoxic activity

L. edodes fruit body water extracts at 10 to 800 μg/ml exhibited significant dose-dependent inhibitory effects on the proliferation of MCF-7 cells (Fig. 2) with more than 90% suppression, and an average IC_{50} of 73 ± 14 μg/ml. In normal cells under the same range of extract concentration, there was a similar inhibitory trend. However the number of normal cells always remained higher at the same extract concentration compared to cancer cells at all concentrations tested, with an average IC_{50} of 140 ± 30 μg/ml.

Similar inhibitory effects were found with *L. edodes* after incubation with mycelial extract (Fig. 3). The difference was that MCF -7 proliferation was much less suppressed in the case of mycelia, showing a much higher average IC_{50} value ($11{,}236 \pm 4856$ μg/ml). On the other hand, the mycelia extract seem to induce a suppressive effect on the proliferation of normal fibroblast cells only at high doses (over 10 mg/ml) with an IC_{50} of $15{,}490 \pm 2310$ μg/ml). The data in Figs. 2 and 3 appear to suggest a biphasic effect. However these differences were not statistically significant.

The same effect was also verified with a DNA synthesis assay, which proved that the cytostatic effect of this fruit body extract was much more potent on cancer cells, compared to normal cells (IC_{50} 119 ± 23 vs

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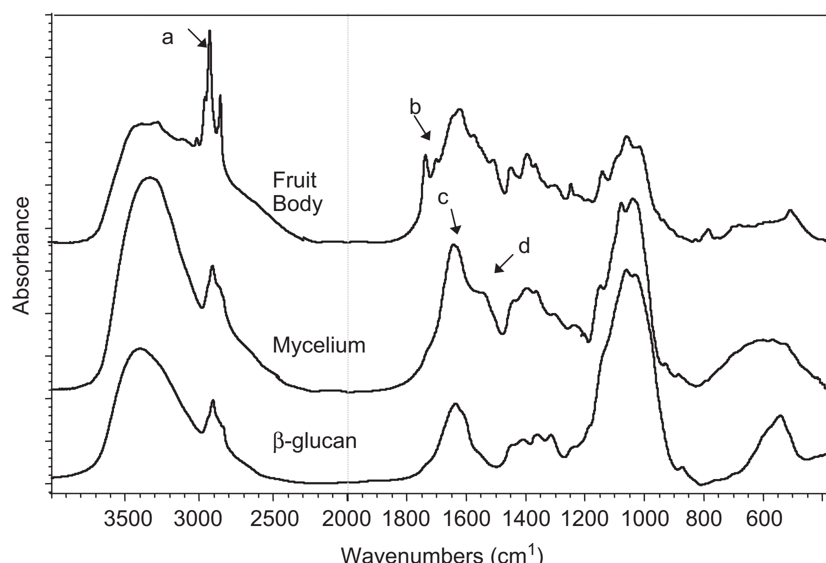

Fig. 1. FT-IR spectra (in KBr) of lentinan samples and the immunologically active β-glucan from *Saccharomyces cerevisiae*. The arrows indicate absorption bands typical of lipids (a and b), and proteins (c and d).

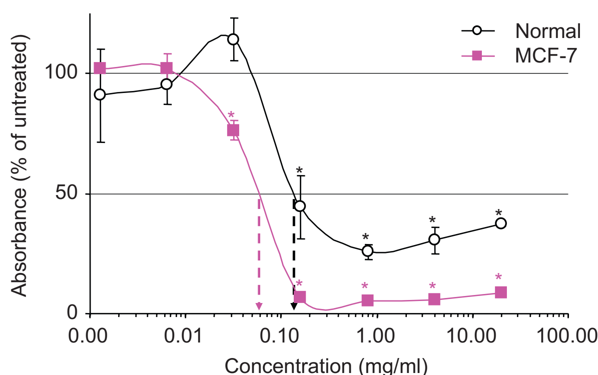

Fig. 2. Effects of *L. edodes* fruit body water extracts on the proliferation of MCF-7 and normal cell lines (one representative experiment). *Statistically significant.

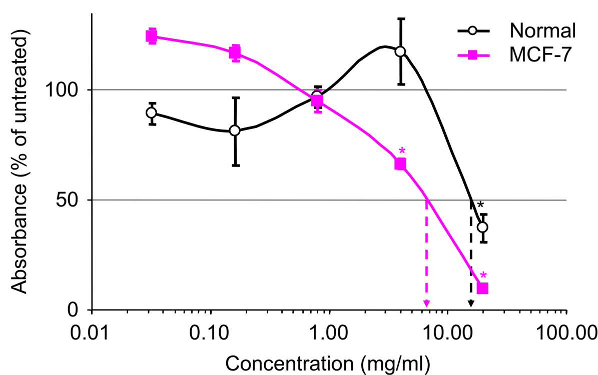

Fig. 3. Effects of *L. edodes* mycelia extracts on the proliferation of MCF-7 and normal cell lines (one representative experiment). *Statistically significant.

Table 2. Concentration producing 50% cytostatic effect of DNA synthesis (IC_{50})[a] of *Lentinus edodes* aqueous extracts on MCF-7 and fibroblast cell lines

L. edodes extract	IC_{50} (μg/ml)
Mushroom (MCF-7)	119±23
Mushroom (fibroblasts)	251±74
Mycelium (MCF-7)	>1000
Mycelium (fibroblasts)	>1000

[a] IC_{50} values were expressed as the mean±SD, determined from the results of DNA synthesis assay in triplicate experiments.

251±74, respectively). However, this was not shown with mycelial extracts where there was an absence of any significant cytostatic effect, reflected by the high IC_{50} values (>1000 μg/ml) in both cancer and normal cell lines (Table 2). Zhang et al., (2005) who used lentinan in an MTT test to study cell proliferation in 5–180 sarcoma tumor cells showed less than 50% inhibition even with concentrations up to 500 μg/ml. Although our test results are expressed in a different way if appears that the crude extracts of the fungus have higher activity.

The IC_{50} in either mushroom or mycelia extracts in absolute methanol was >2500 in all cases, indicating that methanolic extracts contrary to aqueous ones did not have any inhibitory (cytotoxic) effect on MCF-7 cancer cell line.

Mitogenic response of T-cells

The mitogenic and comitogenic activities of fruit body and mycelium determined by the '*in vitro*' rat thymocyte

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test are shown in Table 3. Both samples, containing glucan as the main polysaccharide component, showed dose-dependent stimulatory activities, similar to the immunomodulator zymosan. The mean values of cpm for control cultures without any stimulant was 1084 (1024–1143) and for the PHA-stimulated cultures 1320 (1289–1350).

Both the fruit body and mycelium showed dose-dependent stimulatory activities. The lowest concentration, at which the mitogenic and comitogenic responses showed a significant increase in comparison to the controls, appeared with fruit body and mycelium at 200 and 600 μg/m, respectively. Maximum stimulation indices were achieved at 1500 μg/ml. At higher doses, the decrease of the responses indicated an inhibitory effect. This has been observed also for zymosan (Kardošová et al., 2003) and other polysaccharides (Ebringerová et al., 2002; Ebringerová et al., 2003) The biological responses might also be affected by contaminants present in the tested preparations. The presence of polymyxin B in the cultivation medium excluded the influence of potential endotoxin contamination. The results suggest that both samples were able to enhance the proliferation of rat thymocytes directly and act as co-stimulators in the presence of the T-mitogen, PHA. The SI_{comit}/SI_{mit} ratio was ~2. In accord with the interpretation of the comitogenic test the ratio indicated adjuvant properties of the tested extracts similar to zymosan (Iribe and Koba, 1984; Rovenskỳ et al., 1990).

There are not significant differences in the immuno-enhancing effect of both extracts. Interestingly, the polysaccharides isolated from the mycelium of *Ganoderma tsugae* have been reported to possess the same antitumor activity as those from the *L. edodes* fruiting bodies (Zhang et al., 1994; Wang et al., 1993). More pronounced immunostimulatory effect in the comitogenic rat thymocyte test was reported with the particulate β-glucan from *S. cerevisiae* (Hromádková et al., 2003), which is structurally related to the β-glucan lentinan (Aoki, 1984; Surenjav et al., 2006; Zheng et al., 2005) but is only sparsely branched. As the β-1,3-glucan (lentinan) has been suggested to be the dominating antitumor and immunostimulatory component in *L. edodes*, it could be suggested that the low immunostimulatory activity of the fruit body and mycelium is due to the low glucose content, which comprises only about 60 and 70% (on dry weight basis), respectively, of the sugar components. However, the mannoglycans and protein-containing mannans have been reported to posses biological activities as well (Križková et al., 2001; Tizard et al., 1989), but their biological response in the comitogenic rat thymocyte test have not been reported.

In using *L. edodes* extracts in comparison to lentinan one has to consider the fact that *L. edodes* extracts are given as dietary supplements and lentinan as a drug. Although lentinan is considered fairly safe in doses used by i.v. administration (0.001–30 mg/kg) for 5–6 weeks, the long term clinical use of lentinan is not recommended and there have been reports of its toxicity (Aoki, 1984; Mori, et al., 1977). Also lentinan has very little oral activity and may cause gastrointestinal disturbances and allergic reactions if taken orally.

On the other hand mycelial extracts of *L. edodes* like LEM have the advantage to be used *per os* for long term as dietary supplements, without reported side effects even in massive doses (over 50 mg/day for a week). In the future the *L. edodes* extracts have the potential of being used more widely than lentinan.

Conclusion

Aqueous extracts of *Lentinula edodes* can significantly suppress the proliferation of cancer cell line MCF-7 *in vitro*. This is reflected by the comparative low IC_{50} values and the simultaneous higher IC_{50} values on normal cells.

L. edodes mushroom water extracts are more cytotoxic than mycelial aqueous extracts. Methanolic extracts of either mushroom or mycelia of *L. edodes*

Table 3. Mitogenic and comitogenic activities of the lentinan samples

Sample	20	60	200	600	1500	2000
SI_{mit} dose (μg/ml)						
Fruit body	0.63±0.08	0.90±0.17	1.45±0.16[a]	4.48±0.5	7.50	1.56
Mycelium	0.61±0.08	0.72±0.01	1.37±0.23	2.61±0.20[b]	7.01	5.45
SI_{comit} dose (μg/ml)						
Fruit body	0.87±0.20	1.51±0.30	3.46±0.12[c]	11.58±0.55	13.44	2.90
Mycelium	0.71±0.04	1.11±0.15	1.44±0.17	5.66±0.55[d]	11.88	10.72

[a,b]The first lowest concentration which gives significant increase of SI mit (p-values 0.802, 0.075) in comparison to the control: SINE 1084 (1024–1143).

[c,d]The first lowest concentration which gives significant increase of SI comit (p-values 0.011, 0.017) in comparison to the control: PHA 1320 (1289–1350).

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do not exhibit any inhibitory (cytostatic) effect on MCF-7 cancer cell line.

Both fruit body and mycelial extracts are able to enhance the proliferation of rat thymocytes directly and act as co-stimulators in the presence of the T-mitogen, PHA. The SI_{comit}/SI_{mit} ratio about 2, indicated adjuvant properties of the tested extracts.

This paper supports the direct cytostatic/cytotoxic action of the *L. edodes* extracts on cancer cells, which is in parallel action with its host-mediated antitumour activity.

Furthermore it was demonstrated that *L. edodes* can act as an immunomodulator to augment the proliferative response of rat thymocytes to T mitogens *in vitro*, indicating another mechanism for immunostimulatory activity. Overall there seems to be a therapeutic advantage in using *L. edodes* extracts orally administered instead of a single substance like lentinan given i.v.

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