Immunomodulating Activities of Cultivated Maitake Medicinal Mushroom *Grifola frondosa* (Dicks.: Fr.) S.F. Gray (Higher Basidiomycetes) on Peripheral Blood Mononuclear Cells

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ABSTRACT: *Grifola frondosa* is a culinary-medicinal mushroom that contains several physiologically active compounds, of which polysaccharides, specifically β-glucans, are known to possess immunomodulating properties. Its extracts are studied for application as adjuncts for chemotherapy, and experiments in animal models support the use of this mushroom for cancer treatment. The effect of extracts obtained from mushrooms cultivated on different substrates and their capacity of inducing the secretion of cytokines from human peripheral blood mononuclear cells were studied. The activity of extracts at concentrations 12.5, 100, and 200 μg/mL on induction of TNF-α, IFN-γ, and IL-12 was screened. Two extracts from substrates fortified with olive oil press cakes showed appreciable activity and induced the secretion of TNF-α, IL-12, and INF-γ. The extracts differed from the others in the amount of sugar, protein, and β-glucans, which can explain their higher activity. Present results show that different substrates and different source materials can reasonably modify the bioactivity of cultivated *G. frondosa.*

KEY WORDS: medicinal mushrooms, *Grifola frondosa*, Maitake mushroom, MD-fraction, immunomodulation, TNF-α, IFN-γ, IL-12

ABBREVIATIONS: BE: biological efficiency; **BFWS:** Biotechnical Faculty Wood Science; **IFN-γ**: interferon gamma; **IL-12:** interleukin 12; **LPS:** lipopolysaccharide; **PBMC:** peripheral blood mononuclear cells; **TNF-α:** tumor necrosis factor alpha

I. INTRODUCTION

Grifola frondosa (Dicks.: Fr.) S.F. Gray (Meripilaceae, Polyporales, higher Basidiomycetes), also know as Maitake and Hen-of-the-Woods, is a recognized culinary and medicinal polypore mushroom with a diverse number of physiologically active compounds.[1] Its edible fruit bodies consist of approximately 86% water and 14% dry matter, of which carbohydrates represent 59%, crude protein 21%, crude fiber 10%, crude fat 3%, and ash 7%.[2] *G. frondosa* fruit bodies often occur on dead or dying deciduous hardwoods (*Quercus*, *Ulmus*, *Acer*, *Nyssa*, *Larix*, and *Fagus*) and sporadically on other tree types. The mushroom's optimal growing conditions are determined by a limited range of humidity, moisture, temperature, and other environmental factors. It mainly occurs in the northern temperate forests of Asia, Europe, and eastern North America.[3]

Since 1981 it is commercially cultivated in Japan and elsewhere.[4] Its primary active compounds are polysaccharides, glycoproteins, and proteins.[5] Medicinal effects of *G. frondosa* are numerous, including its anti-cancer activity,[6,7] immune system stimulation,[8,9] effects on angiogenesis,[10] effects on lipid metabolism, and antidiabetic activity.[11] Active polysaccharides have a typically basic structure of a 1,6-β-branched 1,3-β-D-glucan and heteroglycan, or heteroglycan-protein complex as the major component. Several glucans isolated from *G. frondosa* have been patented in Japan as potential anti-cancer

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TABLE 1. Composition of Substrates for *Grifolas frondosa* Fruiting Body Production

Substrate	Strain	Beech sawdust (%)	Crushed hemp seeds (%)	Olive oil press cake (%)	Crushed corn seeds (%)	$CaCO_3$ (%)	$CaSO_4$ (%)	Water (%)
1	Gf1	0	0	52.5	14.8	1.6	0	31.1
2	Gf1	53.5	0	0	10,7	0	0.1	35.5
3	Gf1	31.6	31.6	0	0	0	1.3	35.5
4	Gf5	0	0	52.5	14.8	1.6	0	31.1
5	Gf5	53.5	0	0	10.7	0	0.1	35.5
6	Gf10	53.5	0	0	10.7	0	0	35.5

and immunomodulating agents. Besides the D and MD-fraction these include GF-1, Grifolan NMF-5N and Grifolan-7N.[12] The MD-fraction is a standardized preparation of *G. frondosa* polysaccharides obtained from fruiting bodies with confirmed *in vitro* and *in vivo* effects.[8]

In this research we studied the response of human peripheral blood mononuclear cells to *G. frondosa* polysaccharides obtained from fruiting bodies that had been cultivated on different solid substrates. The polysaccharides were isolated according to a procedure used for isolation of MD-fraction,[13] which is a specific standardized fraction that is widely studied and has proven immunomodulating activities. The influence of strain and substrate composition on the extract composition (polysaccharides, protein, and glucan content) and immunomodulating effects was investigated.

II. MATERIALS AND METHODS

A. Origin and Cultivation of *Grifola frondosa*

Grifola frondosa strains Gf1 (Slovenian isolate, deposited in Culture Collection of Biotechnical Faculty Wood Science (BFWS), 1996, Ljubljana, Slovenia), Gf5 (Slovenian isolate, deposited BFWS, 2004, Ljubljana, Slovenia), and Gf10 (Slovenian isolate, deposited in Culture Collection of Institute for Natural Sciences Collection, Slovenia, NSC, Gregori 2005, Ljubljana, Slovenia), were maintained on potato dextrose agar (Biolife, Milan, Italy) at 24°C. The cultures were maintained active by regular transfers on fresh agar plates every 14 days. The composition of substrates and strains used for the cultivation of fruiting bodies is presented in Table 1.

Two to four kilograms of substrate was placed in polypropylene bags with microporous breathing filters (Combiness, Belgium) and sterilized at 121°C for 3 hours. After sterilization the substrates were cooled, inoculated (8%–10% w/w), and incubated in the dark at 24°C. When primordia started to form the bags were opened and transferred to a mushroom cultivating facility under the following conditions: T = 17°C, 10-h light cycles, and a relative humidity of 85%–90%. Fruiting bodies were harvested at full maturity.

B. Preparation of *Grifola frondosa* Extracts

The extraction of polysaccharides was done according to Nanba,[13] who described isolation of the MD-fraction. Briefly, 100 grams of homogenized mushrooms were boiled in 150 mL deionized water for 1.5 h and then filtered. Ethanol was added gradually to the filtrate until the ethanol concentration reached 45 vol. %, and left for 24 h at 4°C to allow precipitation. The precipitate was filtered off, and ethanol was added again to 90 vol. %. The suspension was allowed to rest under the same conditions. After the second precipitation the samples were centrifuged at 3000 rpm for 5 min, and the supernatant was decanted. The homogenized mushrooms were extracted twice to ensure an extraction that yielded more than 80% of the extractable material. The extracts were combined and air dried.

C. Preparation of a Commercial *Grifola frondosa* Fruiting Body

For comparison a commercially available lyophilized *G. frondosa* (Edible Fungi Institute, Shanghai, China) was used. The dry fruiting body was rehydrated with 4 volumes of deionized water for 24 h and then subjected to the same extraction procedure as the cultivated fruiting bodies.

D. Analyses

After harvest the fruiting bodies were dried at 105°C for 24 h and the biological efficiency (BE)

TABLE 2. ***Grifola frondosa*** **Yields, BE, and Water Content**

Substrate	Strain	Days to harvest	Weight of fresh fruiting body (g)	BE (%)	Water content (%)
1	Gf1	129	266	9.7	86.3
2	Gf1	104	123	9.5	89.6
3	Gf1	110	222	17.2	80.3
4	Gf5	135	419	15.2	89.2
5	Gf5	95	186	14.4	81.5
6	Gf10	141	389	30.2	87.3

was calculated. BE is defined as the weight of fresh mushrooms divided by the weight of dry substrate.

The isolated extracts were characterized for their content of polysaccharides, proteins, and β-glucan. The polysaccharide content of the extracts was determined by the phenol-sulphuric method,[14] with glucose as the standard. The protein content was determined with the Bradford method[15] and bovine serum albumin as the standard. The content of β-glucan was analyzed with the Beta Glucan (yeast and mushroom) Assay Kit (Megazyme, Bray, Ireland) according to the manufacturer's instructions. Unless noted otherwise, all chemicals where purchased from Sigma (St. Louis, MO, USA).

E. Cytokine Induction in PBMCs

Human peripheral blood mononuclear cells (PBMC) from buffy coat of healthy blood donors were isolated by density gradient centrifugation with Ficoll-Paque (Pharmacia Biotech, Stockholm, Sweden) and washed with phosphate buffered saline. The cells were cultured in RPMI 1640 tissue culture medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and heat-inactivated AB normal human serum. After that, 1 x 10^6 cells (final culture volume: 1.5 mL) were plated in 24-well plates (Nunc, Roskilde, Denmark) with different concentrations of extracts. Final concentration of extracts was 12.5, 100, and 200 μg/mL at 37°C in a 95% humidified (relative humidity) atmosphere with 5% CO_2. Polymixin B (10 μg/mL) was added to samples to rule out a possible contamination with lipopolysaccharide (LPS). Cultures of untreated cells in RPMI 1640 with and without polymixin B, and without the mushroom extracts, were used as negative controls. A control experiment was performed with 10 ng/mL LPS with and without polymixin B addition on untreated cells to check the neutralizing effect of polymixin B. The incubation period for TNF-α determination was 4 h. The incubation period for INF-γ and IL-12 determination was 72 h. Before ELISA analysis the cell suspensions were centrifuged at 3000 rpm for 5 min, and the supernatants were frozen at –70°C. All chemicals used were obtained from Sigma (USA).

F. Analysis of Cytokine Concentrations

Cytokine levels in PBMC culture supernatants were determined by commercially available ELISA kits and in accordance with the manufacturer's instructions. The TNF-α concentration was evaluated with TNF-α Assay Kit from Milenia Biotec (Giessen, Germany), and IFN-γ and IL-12 with respective Assay Kits from Pierce Biotechnology (Rockford, IL, USA). The detection limits for TNF-α were 15 pg/mL, INF-γ 1 pg/mL, and IL-12 1 pg/mL.

III. RESULTS AND DISCUSSION

A. Mushroom Yields, BE, and Water Content

The results of mushroom yields, BE, and water content of the fruiting bodies are shown in Table 2. The shortest cultivation period (95 d) was achieved with strain Gf5 cultivated on the substrate without hemp seeds or olive oil press cakes, while the longest period (141 d) was observed on the same substrate mixture with strain Gf10.

There is scarce information about the cultivation of *G. frondosa* and its isolates. The first commercial production was successfully started in 1980 in Japan. Some authors report growing times on selected substrates in the range of 60 to 70 d with the BE in the range of 22.2% to 48.9%,[16] while several of their substrates did not produce fruiting bodies at all. Other researchers report growth times in the range of 84 to 105 d,[17,18] which

TABLE 3. Extraction Yield, Concentrations of Polysaccharides, and Protein in Extracts of *Grifola frondosa*

Fruiting body*	Mass of extract (g)	Yield (mg/g of dry fruiting body)	Polysaccharide content, expressed as glucose equivalents (%)	Protein content (%)
1	0.69	50.4	70.3	13.2
2	10.9	104.8	42.3	9.5
3	1.25	63.5	39.5	7.2
4	0.71	65.7	75.9	12.4
5	0.87	47	41.9	9.1
6	0.57	44.5	41.8	9.4
7**	0.55	43.8	40.1	8.8

*Obtained from corresponding substrates listed in Table 1; **commercial product.

is comparable to our results with production cycles from 95 to 141 d.

The BE obtained on our substrates ranged from 9.7% to a maximum of 30.2%. Even though the growth period was longer for strain Gf10, the biological efficiency was superior to all other strains or substrate combinations.

The influence of substrate composition had a marked effect on the BE, as is the case in strain Gf1, and the addition of hemp seeds more than doubled the mushroom yield. The water content in the fruiting bodies was uniform and well within expectations.[2]

The addition of olive oil press cake supplements, previously reported as biomass and polysaccharide production stimulants in *G. frondosa* submerged cultures,[19] failed to enhance the BE in our experiments; therefore, further experiments should be performed to elucidate the effect of olive oil (or other oils) addition on enhancement of BE in *G. frondosa* mushroom production.

B. Characterization of *Grifola frondosa* Extracts

The extraction yields (Table 3) were in the range of 43 to 104 mg per gram of dry fruiting body, i.e. 4.3% to 10.4%. Most yields were uniform in the range of 44 to 65 mg/g, while the yield from the fruiting body grown on substrate #2 was almost 105 mg/g. The sugar content ranged from 39.5% to 75.9% and the protein content from 8.8% to 13.2%. The highest sugar and protein content was recorded in extracts obtained from fruiting bodies grown on substrates 1 and 4 (Table 3) that contained only olive oil press cake, crushed corn seeds, calcium carbonate, and water. These two extracts also had the highest biological activity (Figs. 1, 2, and 3) and the highest content of total polysaccharides and β-glucan (Table 4). The values of sugar and protein content do not add up to 100%. The cause of this could be the remaining water in the extracts, as they were only air dried, or the presence of other compounds, such

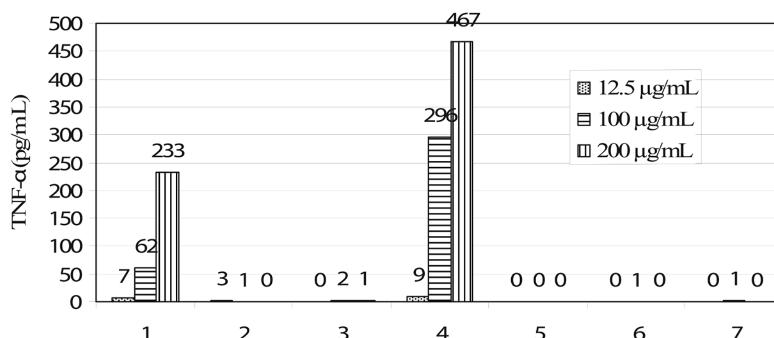

FIGURE 1. TNF-α secretion in PBMC. Human PBMC (1 x 10^6 cells/mL) were treated with 12.5, 100, and 200 μg/mL of *Grifola frondosa* extract. The numbers represent the extracts from corresponding fruiting bodies obtained from different substrates.

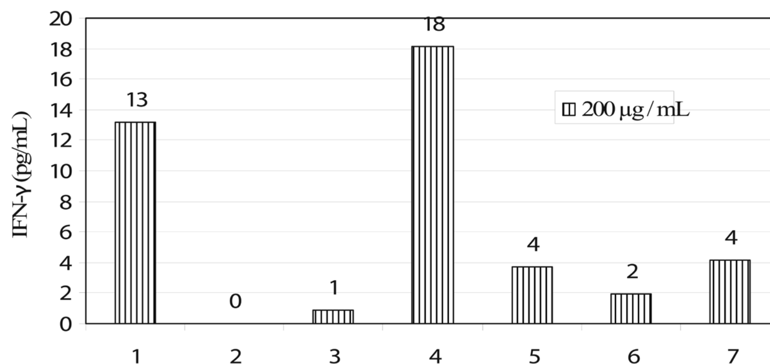

FIGURE 2. IFN-γ secretion in PBMC. Human PBMC (1 x 10^6 cells/mL) were treated with 12.5, 100, and 200 ug/mL of *Grofola frondosa* extract. IFN-γ values for 12.5 and 100 μg/mL treatments are not shown because they were below the detection limit. The numbers represent the extracts from corresponding fruiting bodies obtained from different substrates.

as nucleic acids, fats, or polymerized phenolic compounds.

To our knowledge, there are no other published data on the yields of the polysaccharides obtained from fruiting bodies according to the procedure described for the isolation of MD-fraction other than the original patent, where they claim a yield of merely 6 mg/g.[13] The authors also claim a sugar:protein ratio of 96:4 after purification by gel chromatography and a molecular weight distribution around 10^6. The total glucan, α-glucan, and β-glucan content of the mushroom extracts is presented in Table 4.

The highest content of total and β-glucan was determined in the polysaccharide preparations isolated from the fruiting bodies grown on substrates 1 and 4. The results of total glucan content correlate with the sugar content (Table 3) as expected; the total glucan content analysis is based on the chemical hydrolysis of polysaccharides and the determination of glucose by an enzyme-catalyzed reaction. The α-glucan content is negligible (around 1%) and the β-glucan thus represents the main polysaccharide-containing component in the extract.

The determination of total glucan is based on the chemical hydrolysis of polysaccharides and the determination of the released glucose by glucose-oxidase reaction. The results are slightly lower than the determination of the sugar present in the extracts as assessed by the Dubois method. The difference of around 10% probably lies in the specificity of the glucose-oxidase reaction for glucose in the extract. The determination of β-glucan is based on enzyme hydrolysis with β-glucosidase and is specific for the β-1,3 and β-1,6 linkage.

The extracts obtained from fruiting bodies grown on substrates 1 and 4 (the ones containing olive oil press cakes) had a higher total glucan (and hence also sugar) content as well as higher

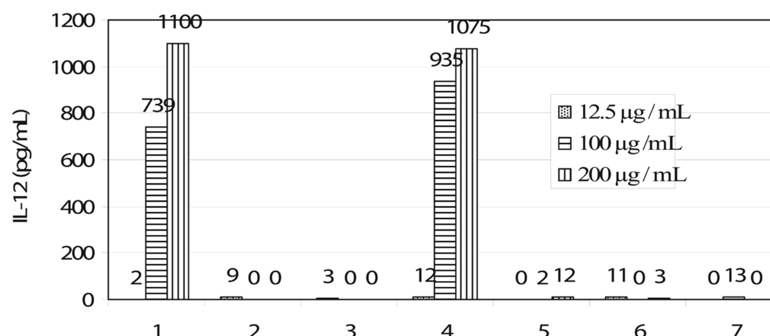

FIGURE 3. IL-12 secretion in PBMC. Human PBMC (1 x 10^6 cells/mL) were treated with 12.5, 100, and 200 μg/mL of *Grifola frondosa* extract. The numbers represent the extracts from corresponding fruiting bodies obtained from different substrates.

TABLE 4. Total Glucan, α-Glucan, and β-Glucan Content of Polysaccharide Extracts of *Grifola frondosa*

Fruiting body*	Total glucan (%)	α-Glucan (%)	β-Glucan (%)
1	65.4	1.1	64.3
2	33.6	1.0	32,6
3	17.2	0.8	16.4
4	69.3	1.2	68.1
5	19.2	0.9	18.3
6	35.6	0.9	34.7
7**	27.8	1.1	26.7

*Obtained from corresponding substrates listed in Table 1; **commercial product.

β-glucan content. Whether the olive oil press cakes were responsible for the higher β-glucan content remains to be answered, but as oils are known to stimulate biomass and polysaccharide production in submerged cultures, this is a feasible assumption.[19]

C. Cytokines Secretion in Human PBMC

1. Secretion of TNF-α

The secretion of TNF-α in PBMC is shown in Fig. 1. The induction of TNF-α in the control (only RPMI medium) was 1 pg/mL; with added polymixin B, 0 pg/mL; with added LPS, 665 pg/mL; and with both LPS and polymixin B, 17 pg/mL. The control experiments demonstrated the effectiveness of polymixin B in suppressing TNF-α production in LPS-contaminated samples. Only the extracts from fruiting bodies 1 and 4 showed a marked increase of TNF-α secretion. In the extract from substrate #4 this amounted to 450 pg/mL TNF-α, which is comparable to the level elicited by 10 ng/mL of LPS. In both cases the TNF-α secretion capacity was in a dose-dependent manner, i.e., more TNF-α was secreted from the PBMC cells when larger doses of the extracts were applied.

Both extracts that showed the highest activity (1 and 4) were grown on substrates containing olive oil press cakes, which seem to modulate activity.

2. Secretion of IFN-γ

The secretion of IFN-γ in PBMC is shown in Fig. 2. The induction of IFN-γ in the control (only RPMI medium) was 0 pg/mL; with added polymixin B, 1.6 pg/mL; with added LPS, 9.7 pg/mL; and with both LPS and polymixin B, 0 pg/mL. Induction of IFN-γ from PBMC was below the detection limit in all cases when the extract concentration was below 200 μg/mL. At the highest concentration the secretion pattern was similar to the TNF-α secretion, with extracts 4 and 1 being the most potent inducers.

3. Secretion of IL-12

The secretion of IL-12 in PBMC is shown in Fig. 3. The induction of IL-12 in the control (only RPMI medium) was 0 pg/mL; with added polymixin B, 11 pg/mL; with added LPS, >1100 pg/mL; and with both LPS and polymixin B, 21 pg/mL. The IL-12 secretion from PBMC followed the pattern of TNF-α and IFN-γ secretion and was best in extracts 4 and 1.

Grifola frondosa extracts exhibit antitumor activity and modulate the expression of various cytokines. These effects can be observed in extracts from fruiting bodies,[20] liquid cultured mycelium,[7,21] and mycelium produced by solid-state fermentation.[22] It has been demonstrated that an extract from *G. frondosa* stimulates the release of TNF-α, IFN-γ, and IL-12 *in vivo* and consequently exhibits anti-tumor effects in C3H/HeN mice.[23] There is evidence that *G. frondosa* polysaccharides induce the differentiation of $CD4^+$ into Th-1 cells in tumor-bearing BALB/c mice by stimulating IL-12 release, which further enhances the Th-1 response.[8]

IV. CONCLUSIONS

Our results show that only two extracts appreciably stimulated the release of TNF-α, while the secretion of IFN-γ and IL-12 was more uniform and modest. The difference between the extract

compositions, specifically the β-glucan content, reflects these biological activities and confirms that β-glucans are the principal components with immunomodulating activity. The differences in the extracts highlight the need for a better characterization and/or documentation of cultivation parameters and extract composition, as well as to correlate these data to immunological and antitumor activities.

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