

NOVEL β -GLUCANS ISOLATED FROM HIGHER BASIDIOMYCETES MUSHROOM *GANODERMA TSUGAE* VAR. *JANNIEAE*

FIELD OF THE INVENTION

The present invention relates to polysaccharides isolated from mushrooms and, more particularly, to novel highly soluble in water β -glucan isolated from the higher Basidiomycetes medicinal mushroom variety designated *Ganoderma tsugae* var. *jannieae* and, particularly, from a new and distinct strain thereof designated *Ganoderma tsugae* var. *jannieae* strain Tay-1.

BACKGROUND OF THE INVENTION

Mushrooms or macrofungi represent a major and, as yet, largely untapped source of potent new pharmaceutical products. Of approximately 15000 known species, 2000 are safe for people's health, and about 650 of them possess medicinal properties (Wasser, 2002).

Edible and medicinal mushrooms (macrofungi) not only can convert the huge lignocellulosic biomass waste into human food but, most remarkably, can produce notable mycopharmaceuticals, myconutriceuticals and myco-cosmeceuticals.

The most significant aspect of mushroom cultivation, if managed properly, is to create zero emission of lignocellulosic waste materials. Mushroom biotechnological products have multibeneficial effects to human welfare, e.g., as food, health tonics and medicine, feed and fertilizers, and to protect and regenerate the environment. Pharmaceutical substances with potent and unique health-enhancing properties were isolated recently from medicinal mushrooms and distributed worldwide (Mizuno, 1999; Wasser and Weis, 1999; Borchers et al.,

2004; Lindequist et al., 2005; Sullivan et al., 2006; Moradali et al., 2007). Many of them are pharmaceutical products, while others represent a novel class of dietary supplements (Wasser et al., 2004) or "mushroom nutraceuticals" (Chang and Buswell, 2003). Several antitumor polysaccharides, such as hetero- β -glucans and their protein complexes (e.g., xyloglucans, and acidic β -glucan containing uronic acid) as well as dietary fiber, lectins, and triterpenoids, have been isolated from medicinal mushrooms. The potential of medicinal mushrooms is enormous but mostly untapped. It could and should evolve into a successful biotechnological industry for the benefit of humankind.

Medicinal mushroom mycology has deep and firm roots in fungi's traditional uses in the medicine of the Far East. For centuries, Chinese and other healthcare practitioners employed mushrooms to treat various diseases. Only at the end of the 1960s did Eastern and Western scientists start to investigate the mechanisms of the health effects of mushrooms. The first successful research discovered the antitumor effects of hot water extracts from several mushroom species. The main active components proved to be polysaccharides, specifically β -D-glucans (Chen and Seviour, 2007). Chihara and his co-workers (1969) isolated from the fruiting bodies of shiitake mushroom (*Lentinus edodes*) a water-soluble antitumor polysaccharide, which was named "lentinan" after the generic name of this mushroom and demonstrated powerful antitumor activity, preventing chemical and viral tumor development in mice and experimental models (Mizuno, 1999).

Mushroom Polysaccharides

Polysaccharides belong to a structurally diverse class of macromolecules, in which polymers of monosaccharide residues are joined to each other by glycosidic linkages and have the greatest potential for structural variability. The involvement and importance of polysaccharides in tumor and cancer treatment were first recognized more than 100 years ago when it was found that certain polysaccharides could induce complete remission in patients with cancer (Nauts et al., 1946). Higher Basidiomycetes mushrooms contain a large amount of polysaccharides,

especially β -glucans. The literature (Mizuno, 1999; Wasser, 2002b; Chen and Seviour, 2007; Zhang et al., 2007) suggests β -glucans are effective in treating diseases like cancer, a range of microbial infections, hypercholesterolemia, and diabetes as well as in helping patient recovery from chemotherapy and radiotherapy. β -glucans are also especially beneficial to middle-aged people, people with active and stressful lifestyles, and athletes. The mechanisms of action of β -glucans appear to depend on their capabilities to bind to cell receptors, which are known to include dectin-1, CR3, LacCer, and scavenger receptors. The immune system is stimulated by β -glucans' presence (Brown and Gordon, 2001, 2005). The anti-tumor effects of β -glucans seem to be related to their molecular weight and solubility. Only the low-molecular weight lentinan, for example, had high anti-tumor activity. Unsurprisingly, soluble β -glucans appear to be stronger immunostimulators than insoluble ones (Wasser, 2002b; Zhang et al., 2005; Chen and Seviour, 2007). However, the relationships between the structures of the glucans and their stimulatory activities are still controversial due to a lack of reproducible *in vitro* systems to evaluate the activities of (1 $\rightarrow$ 3)- β -D-glucans (Kataoka et al., 2002).

Polysaccharides are the best known and most potent mushroom-derived substances with antitumor and immunomodulating properties. Polysaccharides from mushrooms do not attack cancer cells directly, but produce their antitumor effects by activating different immune responses in the host.

In Japan, China, Russia, and Korea, several different polysaccharide antitumor drugs have been developed from the fruiting bodies, mycelia, and culture media of various medicinal mushrooms, such as shiitake mushroom (*Lentinus edodes*), ling zhi or reishi (*Ganoderma lucidum*), turkey tail (*Trametes versicolor*), split gill (*Schizophyllum commune*), mulberry yellow polypore (*Phellinus linteus*), and chaga or cinder conk (*Inonotus obliquus*).

As mentioned above, lentinan, a 1,3-beta-glucan polysaccharide fraction extracted from *Lentinus edodes* - shiitake mushroom, was the first polysaccharide extracted from fungi shown to exhibit antitumor activity and was later

commercialized in Japan. Since the discovery of lentinan, several antitumor polysaccharide agents have been developed and commercialized, using the submerged cultured mycelial biomass, including the immunostimulating krestin, polysaccharide-K or PSK, present in a popular Japanese extract made from *Trametes versicolor* (also known as *Coriolus versicolor*) submerged mycelium; the polysaccharide-peptide or PSP, a proteoglycan from *Trametes versicolor*, widely used in China as anticancer and immunomodulatory agent; and schizophyllan (SPG, sonifilan) a polysaccharide known to suppress tumor growth, especially cervical cancer, extracted from medium product of *Schizophyllum commune* cultivated in submerged culture.

These antitumor polysaccharides are regarded as biological response modifiers that activate immunological responses. This basically means that they cause no harm and place no additional stress on the body; they help the body to adapt to various environmental and biological stresses; and they have nonspecific action on the body, supporting some or all of the major systems, including nervous, hormonal, and immune systems, as well as regulatory functions.

Mushroom dietary supplements

Mushrooms represent a valuable source of bioactive agents with potent and unique medicinal properties. Most mushroom-derived preparations and substances find their use not as pharmaceutical products (real medicines), but represent a novel class of functional foods, dietary supplements or “nutriceuticals” (Chang and Buswell, 2003; Wasser et al., 2004).

A mushroom nutraceutical is a refined or partially refined extract or dried biomass from either the mycelium or the fruiting body of the mushroom, which is consumed in the form of capsules or tablets as a dietary supplement (not a conventional food) and which has potential therapeutic applications. Regular intake may enhance the immune responses of the human body, thereby increasing resistance to disease, and in some cases, causing regression of a disease state.

Ganoderma lucidum dietary supplements, for example, are valued for their immunomodulating, anticancer, and antiviral properties and are used during remission of cancer and by hepatitis B patients. They also have anti-hyperlipidemic, hypotensive, and hypoglycemic actions. There are more than 60 brands of *Ganoderma lucidum* products on the market.

Higher Basidiomycetes mushrooms contain a large amount of polysaccharides, proteins, well-balanced essential amino acids, melanins, lipids comprising essential fatty acids, triterpenoids, antioxidant agents, vitamins, and other biological active substances. Also, dietary fibers belonging to glucans, chitin, and heteropolysaccharides including pectinous substances, hemi-celluloses or polyuronides, are abundant in the tissue of all mushrooms, which are capable of absorbing bile acids or hazardous materials in the intestine, and thus can act as carcinostatics and decrease various kinds of poisoning.

The safety of mushroom-based dietary supplements is enhanced through the following controls: (i) the overwhelming majority of mushrooms used for production of dietary supplements are cultivated commercially (and not gathered in the wild), thus guaranteeing proper identification and pure, unadulterated products, meaning in many cases genetic uniformity; (ii) mushrooms are easily propagated vegetatively and thus keep to one clone, the mycelium can be stored for a long time, and the genetic and biochemical consistency may be checked after a considerable period of time; and (iii) many edible and medicinal mushrooms are capable of growing in the form of mycelial biomass in submerged cultures and thus offer a promising future for standardized production of safe mushroom-based dietary supplements. These are advantages of using mushroom-based dietary supplements as opposed to herbal preparations.

Biological activity of medicinal mushroom substances

Fungal substances are known as modulators of NF- κ B activation pathway that plays critical roles in a variety of physiological and pathological processes NF- κ B is related to the promotion of cell proliferation, inhibition of apoptosis, and the

enhancement of tumor metastasis and angiogenesis (Aggarwal, 2004). In addition to its role in activation of immune cells, NF- κ B within the malignant cell is a major modulator of the tumor response to inflammation. Inhibition of NF- κ B in cancer cells leads inflammation-induced tumor growth to tumor regression. Extracts of several fungi were screened for their ability to interfere with the NF- κ B activation pathway. Higher Basidiomycetes mushrooms e.g., *Marasmius oreades*, *Phellinus linteus*, and *Coprinus comatus* as natural sources of low-molecular-weight bioactive substances are able to affect the process of tumorigenesis through the direct blockage of the NF- κ B activation at the I κ B kinase complex level. The review compiles all available data on medicinal mushroom metabolites known to modulate the activity of NF- κ B, thus demonstrating their potential use as novel anti-cancer agents in the rapidly advancing field of molecular therapy, published by Petrova et al. (2008). Twenty-six species including *Ganoderma lucidum* were found to have properties that affected the NF- κ B function (Sliva, 2003; Yuen and Gohel, 2005; Zaidman et al., 2007; Petrova et al., 2008).

1,3:1,6- β -glucans from fungi, yeast and seaweed as well as 1,3:1,4- β -glucans from grasses express immunostimulatory activity through stimulation of granulocytes (neutrophils and eosinophils), monocytes, macrophages, and natural killer (NK) cells. Two membrane β -1,3-glucan receptors that mediate biological responses to β -1,3-glucans have been characterized on a molecular level: CR3, expressed on neutrophils, monocytes, and NK cells, and less present on macrophages, and dectin-1, preferentially expressed on macrophages over granulocytes, while absent on NK cells (Hong et al., 2004; Chen and Seviour, 2007). The grass (1,3:1,4)- β -D-glucan consists of an unbranched, unsubstituted glucose chain with two distinct linkage types that are arranged in a non-repeating, but nonrandom, fashion. The (1,3:1,4)- β -D-glucans are components of dietary fibers that are highly beneficial in the prevention and treatment of serious human health conditions, including colorectal cancer, high serum cholesterol and cardiovascular diseases, obesity, and non-insulin-dependent diabetes (Braaten et al., 1994; Brennan et al., 2005; Chen and Seviour, 2007). (1,3:1,4)- β -D-glucans have

antinutritive effects in monogastric animals such as pig and poultry (Brennan et al., 2005) and are important in many cereal processing applications, including malting and brewing.

Examples of pharmaceutically significant β -glucans exhibiting antitumor activity include grifolan, a β -glucan derived from the fruiting bodies and submerged cultivated mycelia of *Grifola frondosa*, maitake mushroom, a higher Basidiomycetes species (Boh and Berovic, 2007); lentinan, derived from the fruiting bodies of *Lentinus edodes* (Chichara et al., 1969; Sasaki and Takasuka, 1976); schizophyllan or sonifilan, a β -glucan derived from extract from medium product of *Schizophyllum commune* cultivated in submerged culture *Schizophyllum commune* (Kikumoto and Kimura, 1971), used as an immunoadjuvant in the treatment of cancer, especially stomach tumors; and a β -glucan derived from the fruiting bodies of *Auricularia auricula-judae*, an edible mushroom (Misaki et al., 1981; Hobbs, 1995). These β -glucans bear in common a backbone structure of β -1,3-linked D-glucopyranose residues with beta-1,6-linked side chains at some of the C-6 positions, and are used as antitumor drugs.

Mushroom constituents are known to have immunomodulatory properties. The major immunopotential effects of these active substances include mitogenicity, stimulation of hematopoietic stem cells, activation of alternative complement pathway, and activation of immune cells such as T_H cells, T_c cells, B cells, macrophages, dendritic cells and NK cells. Different profiles have been observed in relation to the activated immune cells. Some polysaccharides activate B cells and macrophages, but not T cells, while others stimulate all three types of cells. Lentinan is a stimulator of T cells and macrophages, but not of B cells. Some polysaccharides might promote a T_{H1} response and others a T_{H2} response. In the particular case of β -glucans, despite the structural and functional similarities of some of them, they differ in their ability to elicit various cellular responses, particularly cytokine expression and production, and in their effectiveness against specific tumors.

Mushroom products are known to affect inflammatory processes. Macrophages stimulated by mushroom products release several inflammatory cytokines such as IL-1, IL-6, IL-8, TNF- α , and NO, all of which directly induce tumoricidal activity in macrophages. In other cases mushroom extracts have been shown to assert anti-inflammatory effects by inhibiting the production of NO, PGE₂, IL-1 β , and TNF- α in LPS-stimulated macrophages and LPS administered mice.

Besides their use as dietary supplements, mushroom-derived preparations may also be used as natural and cosmeceutical products (Wasser et al., 2000, 2001; Wasser, 2002a; Chang and Buswell, 2003). For example, preparations containing β -glucans are used in creams to accelerate healing of shallow abrasions and partial thickness burns and as anti-irritant.

Genus *Ganoderma*

Ganoderma P. Karst. (Ganodermataceae, Polyporales, higher Basidiomycetes) is a highly distinctive genus of white-rot polypore fungi that is primarily characterized by the formation of a double-walled, generally echinulate, basidiospores. Most *Ganoderma* species are very variable macromorphologically and lack micromorphological distinctiveness. As a consequence, earlier taxonomic studies in the genus have created many synonymous names and have resulted in largely ambiguous species delimitation and identification systems, making species identification in the genus virtually impossible. Species of *Ganoderma* used in Oriental folk medicine refer to “Ling Zhi,” “Chi Zhi,” “Ling Chi,” “Ling Chih,” “Ling Qi,” “Zi Zhi,” “Reishi,” “10,000 year mushroom,” “Herb of spiritual potency,” and “Mushroom of Immortality” in the Chinese literature and have traditionally been labeled *G. lucidum* in the scientific literature. However, there is now accumulative evidence that most species reported as *G. lucidum* in most studies were wrongly identified (Moncalvo and Ryvarden, 1997; Moncalvo, 2005; Wasser et al., 2006).

In recently published illustrations of Ganodermataceae in China, Wu and Dai (2005) included 77 *Ganoderma* species. Over the years, at least 166 laccate *Ganoderma* species have been described worldwide, of which at least 48 names have been, at some point, considered to be synonyms of others.

In a review on medicinal mushrooms, Wasser (2002b) presented a long list of higher Basidiomycetes mushrooms containing antitumor or immunomodulating polysaccharides, among them 7 *Ganoderma* species described in the literature. The polysaccharides were found in the fruiting bodies, in the submerged mycelium, and in culture medium of the various species of medicinal mushrooms. As to their chemical structure, the polysaccharides identified in *G. lucidum* were β -(1 $\rightarrow$ 3)-glucuronoglucan and mannogalactoglucan and the polysaccharides identified in *G. tsugae* were arabinoglucan and glucogalactan.

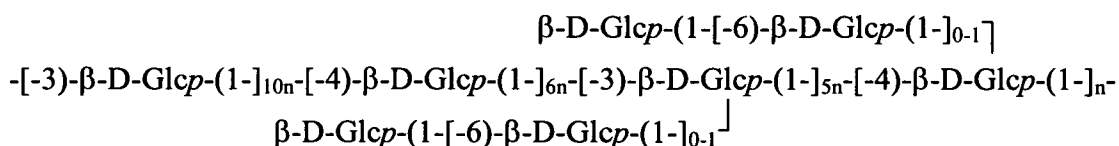
Wasser (2002b) discloses that polysaccharides have been investigated in both the fruiting bodies and mycelia of *Ganoderma tsugae*. Seven glycans with strong antitumor activities were obtained from 14 water-soluble and 15 water-insoluble fractions extracted from *G. tsugae* fruiting bodies (Wang et al., 1993). Water-soluble fractions were protein-containing glucogalactans associated with mannose and fucose, and water-insoluble fractions represented protein-containing β -(1 $\rightarrow$ 3)-glucans with different protein content. Sixteen water-soluble polysaccharides were extracted from *G. tsugae* mycelium and examined for antitumor effects on Sarcoma 180 in mice (Zhang et al., 1994, 1999). The three active polysaccharides obtained were: a glycan-protein complex containing 9.3% protein, with a heteropolysaccharide composed of mannose and xylose; a glucan-protein complex containing 25.8% protein; and a glycan-protein complex with glucose as the main component and associated with arabinose, mannose, xylose and galactose. Comparison of active water-soluble polysaccharides obtained from fruiting body and mycelium showed that those from the fruiting body were glucogalactan-protein complexes, but those of the mycelium were homogalactan-protein complexes or a heteroglycan composed of mannose and xylose (Zhang et al., 1994).

Wasser et al. (2006) recently published a study focused on the examination and comparison of wild growing samples of *Ganoderma* sp. from China, a fungus of the genus *Ganoderma*, which has very important medicinal properties as a producer of hundreds of biologically active substances. Examination and morphological analysis of the wild growing samples of *Ganoderma* sp. and their comparison with specimens of *Ganoderma lucidum* complex, including *G. tsugae* and *G. resinaceum*, showed that it is close to *G. tsugae* but differ in some features from the type of material of *G. tsugae*. It was then identified as a new variety of the latter taxon, namely, *Ganoderma tsugae* var. *jannieae*.

Reference is made to the patent application entitled "New higher Basidiomycetes medicinal mushroom *Ganoderma tsugae* var. *jannieae* strain Tay-1 and biologically active biomass and extracts therefrom", filed on the same date by same applicant and mentioning the same inventors as in the present application.

SUMMARY OF THE INVENTION

The present invention provides a low molecular weight β -glucan composed of a backbone structure of β -1-3- and β -1-4-linked D-glucopyranose residues bearing, at some of the 6-positions, side chains of β -1-6-D-glucopyranose residues, of the structure:



wherein Glcp represents a glucopyranose residue and n is an integer equal to or larger than 1.

The β -glucan of the invention has a molecular weight lower than 10,000 Da, centered at about or lower than 5,000 Da.

The β -glucan may be obtained from *Ganoderma* mushroom, particularly from *Ganoderma tsugae* and varieties thereof, more particularly from dried fruiting bodies or from submerged cultivated mycelium of the new strain *Ganoderma tsugae* var.

jannieae strain Tay-1, deposited as a culture with the Centraalbureau voor Schimmelcultures (CBS), Uppsalalaan 8, P.O. Box 85167, 3508 AD Utrecht, The Netherlands, on October 23, 2006, under the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure, and assigned No. CBS 120394.

The present invention also relates to compositions comprising said β -glucan for use as nutraceuticals or dietary supplements, as an additive to food products or beverages, or for therapy, and to such compositions formulated into food products, beverage products, pet food products, and cosmetic products.

BRIEF DESCRIPTION OF THE FIGURES

Figs. 1A-1B shows a view from the upper side (**Fig. 1A**) and a view from the hymenophore (**Fig. 1B**) of cultivated basidiomata of *Ganoderma tsugae* var. *jannieae*.

Fig. 2 shows basidiomata of various shapes (collection from nature) of *Ganoderma tsugae* var. *jannieae*.

Figs. 3A-3B shows *Ganoderma tsugae* var. *jannieae* culture in a Petri dish with malt agar medium (**Fig. 3A**) and staghorn hyphae of vegetative mycelium under scanning electronic microscope (SEM) (**Fig. 3B**). Scale bar – 10 μ m

Fig. 4 shows general methodology for the production of submerged cultured mycelium of *Ganoderma tsugae* var. *jannieae* using fermentor or bioreactor technology: **a**- preparation of standard agar media for Petri dish; **b**- spores or parts of fruiting body which are used for preparation of culture; **c**- culture on Petri dish; **d**- museum culture on agar slant in tube; **e**- microscopic examination of museum culture; **f**- pre-inoculums culture in 250 mL Erlenmeyer flask; **g**- homogenization of pre-inoculums culture; **h**- cultivation of homogenized mycelial biomass in 2 L Erlenmeyer flasks; **i** - homogenization of mycelial biomass for inoculation in fermentor medium; **j**- growth medium for fermentor; **k**- cultivation of mycelial biomass in fermentor; **l**- harvest of mycelial biomass; **m**- dried biomass formulations for dietary supplements (DS), pharmaceuticals and other products.

Fig. 5 shows a general view of mycelial pellets of *Ganoderma tsugae* var. *janniae* strain Tay-1 CBS 120394 after 5 days cultivation.

Fig. 6 shows high magnification of the vacuolated hyphae with clamp connections of mycelial pellets of biomass of *Ganoderma tsugae* var. *janniae* strain Tay-1 CBS 120394 after 5 days cultivation.

Fig. 7 shows homogenized mycelial pellets of biomass of *Ganoderma tsugae* var. *janniae* strain Tay-1 CBS 120394 in a Petri dish before use as inoculums for culturing in a 2 L Erlenmeyer flask.

Fig. 8 shows the mycelial biomass pellets of *Ganoderma tsugae* var. *janniae* strain Tay-1 CBS 120394 after 7 days of fungus cultivation in a 2 L Erlenmeyer flask.

Fig. 9 shows harvested wet mycelial biomass of *Ganoderma tsugae* var. *janniae* strain Tay-1 CBS 120394

Fig. 10 shows final product dried pulverized biomass of *Ganoderma tsugae* var. *janniae* strain Tay-1 CBS 120394 after fermentation.

Fig. 11 shows vacuolated hyphae with clamp connections of mycelial biomass of *Ganoderma tsugae* var. *janniae* strain Tay-1 CBS 120394 after 7 days of fungus cultivation.

Fig. 12 shows a flow chart describing the procedure for isolation of the β -glucan from *Ganoderma tsugae* var. *janniae* fruiting bodies biomass or mycelium.

Fig. 13 depicts the ^1H NMR spectrum of purified β -glucan from *Ganoderma tsugae* var. *janniae* mushroom (D_2O , 40°) (ppm, parts per million).

Fig. 14 depicts the ^1H NMR spectrum of the mixture of the mannan, galactan, and glucan (void volume fraction of Sephadex G50 size exclusion chromatography).

Fig. 15 depicts a gas liquid chromatography-mass spectroscopy (GLC-MS) chromatogram of the methylated alditol acetates obtained from β -glucan. Labels indicate substitution position of the glucose residues in the polymer. Unlabeled peaks could not be positively identified.

Fig. 16 shows the HSQC (heteronuclear single quantum correlation) and HMBC (heteronuclear multiple bound correlation) spectra of the β -glucan. Signals of 4,6-di-substituted glucose residues are not visible at this intensity (intensity is reduced for noise removal). "Linked" means that this monosaccharide is linked to an indicated position of the other monosaccharide; "substituted" means that this monosaccharide is substituted by another monosaccharide at the indicated position. Labels 1-3, 1-4, and 1-6 indicate HMBC correlations between anomeric protons and corresponding transglycosidic carbon atoms. Star marked signals belong to contaminating α -mannan.

Fig. 17 depicts MALDI mass spectrum of β -glucan. Peaks correspond to sodium $[M+23]^+$ and potassium $[M+39]^+$ adducts. Labels show number of hexose residues in nearest glucose oligomer.

Fig. 18 shows methylation analysis of the β -glucan from *Ganoderma tsugae* var. *janniae* dried fruiting bodies mushroom (lower spectrum) and from submerged cultivated mycelium (upper spectrum). The submerged cultivated mycelium contains less of 4-substituted glucose residues and more of 6- and 3,6-substituted glucose residues. Labels indicate substitution position of the glucose residues in the polymer. T-Glc, terminal glucose.

Fig. 19 depicts ^1H NMR spectra of the purified β -glucan (ppm, parts per million). Upper spectrum: glucan in final fraction isolated from dried fruiting bodies; lower spectrum: glucan in final fraction isolated from submerged cultivated mycelium.

Fig. 20 shows reporter inhibition activity of a mycelial diethyl ether extract of *Ganoderma tsugae* var. *janniae* strain Tay-1 (W01) and of a mycelial ethyl acetate extract of *Ganoderma tsugae* var. *janniae* strain Tay-1 (W02) in MCF7 cancer cell line. Controls: PAR (parthenolide) and MG132, an inhibitor of I κ B α degradation.

Fig. 21 shows the effect of different concentrations of the extract of *Canoderma tsugae* var. *janniae* strain Tay-1 WO2 on MCF7 cancer cell viability.

Fig. 22 shows a Western blot analysis on SDS-PAGE separated cancer line proteins specifically probed with anti-I κ B α , and anti-tubulin antibodies (control) showing the effect of the mushroom extracts of *Ganoderma tsugae* var. *janniae* strain Tay-1 (W02), *Leucoagaricus birnbaumii* (LB12), *Tricholomopsis sulphureoides* (TS03), *Pleurotus ostreatus* (PO01), and *Schizophyllum commune* (SC01) on I κ B α degradation after 10 min TNF- α stimulation. Extracts were applied to cancer cell lines for 10 minutes and the effect was compared to the effect of 10 minute treatment with TNF- α alone as well as to the effects of controls used, PAR and MG132, respectively. Additionally, the effects of all treatments (extracts and controls) are presented as folds to the beta-tubulin expression. Beta-tubulin is a house-keeping protein, which levels do not vary according to the treatments and, thus, can serve as a control protein representing the accuracy of each experiment provided.

Fig. 23 shows a Western blot analysis on SDS-PAGE separated cancer line proteins specifically probed with anti-pI κ B α , antibodies showing the effects of mushroom extracts *Canoderma tsugae* var. *janniae* strain Tay-1 W02 and *Schizophyllum commune* SC01 on I κ B α phosphorylation in comparison with 5 minutes TNF- α stimulation. Extracts effect are compared to the effect of TNF- α alone as well as to the effects of controls used, PAR and MG132, respectively. The level of the house-keeping control protein tubulin is shown in **Fig. 19**.

Fig. 24 shows the effects of mushroom extracts of *Canoderma tsugae* var. *janniae* strain Tay-1 W02 and *Fomes fomentarius* FF04 on cell viability of different cancer cell lines in comparison to the effect of parthenolide (PAR). The concentrations of parthenolide are given in μ M, whereas the extracts concentrations are in μ g/mL.

DETAILED DESCRIPTION OF THE INVENTION

In one aspect, the present invention relates to a β -glucan having the structure as defined hereinabove.

The β -glucan of the invention has a molecular weight of less than 10,000 Da, centered at about or lower than 5,000 Da. In a dried state, the β -glucan is an odorless white powder and it is highly soluble in water producing a brown aqueous solution at high concentrations.

In another aspect, the present invention also relates to a β -glucan having a molecular weight of 5,000-10,000 Da or less, composed of a backbone structure of β -1-3- and β -1-4-linked D-glucopyranose residues bearing, at some of the 6-positions, side chains of β -1-6-D-glucopyranose residues, which in a dried state is a white odorless powder highly soluble in water producing a brown aqueous solution at high concentrations.

As shown herein in the examples, the β -glucan of the invention has terminal 3-, 4-, 3,6- and 4,6-substituted glucose residues in the ratio of 1:0.75:0.25:1.6:0.1:0.6, when subjected to methylation analysis.

The β -glucan of the invention may be obtained, for example, from the higher Basidiomycetes medicinal mushroom variety *Ganoderma tsugae* var. *janniae* (Wasser et al., 2006). In more preferred embodiments, the β -glucan of the invention is obtained from dried fruiting bodies or from submerged cultivated mycelium of *Ganoderma tsugae* var. *janniae* strain Tay-1 CBS 120394.

As described herein, several processes can be used for obtaining the β -glucan from the dried biomass of fruiting bodies of *Ganoderma tsugae* var. *janniae* strain Tay-1 CBS 120394.

According to one embodiment, the β -glucan is obtained from the dried fruiting bodies of *Ganoderma tsugae* var. *janniae* strain Tay-1 CBS 120394 by a process comprising the following steps:

- (i) washing the dried fruiting bodies with ethanol, preferably with boiling 85% ethanol, 3 or more times, and discarding the ethanol extract;
- (ii) extracting the remainder of the fruiting bodies with boiling water, 5 times or more, subjecting the water extract to dialysis, shaking with a solvent mixture of chloroform and isoamyl alcohol (Sevag protocol), concentrating,

centrifuging, treating the water layer with the same solvent mixture, and freeze-drying to give crude β -glucan that is purified.

According to another embodiment, herein called “alkaline extraction”, the β -glucan is obtained from the dried biomass of fruiting bodies of *Ganoderma tsugae* var. *jannieae* strain Tay-1 CBS 120394 by a process depicted in **Fig. 12** comprising the following steps:

(i) washing the dried fruiting bodies with ethanol, preferably with boiling 85% ethanol, 3 or more times, and discarding the ethanol extract; and

(ii) extracting the solid remainder of the fruiting bodies with boiling water, 5 times or more, treating (twice) the insoluble residue from water extraction with boiling 5% NaOH-0.05% NaBH₄, filtering, neutralizing the alkaline extract with HCl to a pH of 4, and removing the precipitate by centrifugation;

(iii) subjecting the neutralized solution to dialysis, concentration, treatment with a solvent mixture of chloroform and isoamyl alcohol (Sevag protocol), ultracentrifugation and freeze-drying, to give β -glucan of dark brown color that is dissolved in water;

(iv) subjecting the brown water solution containing the β -glucan to centrifugation and purification by anion-exchange chromatography, e.g., on a DEAE cellulose column, and eluting with water followed by 0.25 M NaCl; and

(v) recovering the neutral fractions eluted with water and acidic fractions eluted with 0.25 M NaCl, subjecting them to size-exclusion chromatography, e.g., on a Sephadex G-50 column, to give purified β -glucan that is lyophilized (freeze-dried).

According to a further embodiment, herein called “acidic extraction”, the β -glucan is obtained from the dried biomass of fruiting bodies of *Ganoderma tsugae* var. *jannieae* strain Tay-1 CBS 120394 by a process comprising the following steps:

(i) washing the dried fruiting bodies with ethanol, preferably with boiling 85% ethanol, 3 or more times, and discarding the ethanol extract;

(ii) extracting the solid remainder of the fruiting bodies with boiling water, 5 times or more, treating (twice) the insoluble residue from water extraction with boiling 0.01 M H₂SO₄, filtering, and removing the precipitate by centrifugation;

(iii) subjecting the solution to dialysis, concentration, treatment with a solvent mixture of chloroform and isoamyl alcohol (Sevag protocol), ultracentrifugation and freeze-drying, to give β -glucan of light brownish color that is dissolved in water;

(iv) subjecting the brown water solution containing the β -glucan to centrifugation and purification by anion-exchange chromatography, e.g., on a DEAE cellulose column, and eluting with water followed by 0.25 M NaCl; and

(v) recovering the neutral fractions eluted with water and acidic fractions eluted with 0.25 M NaCl, subjecting them to size-exclusion chromatography, e.g., on a Sephadex G-50 column, to give purified β -glucan that is lyophilized (freeze-dried).

According to a further embodiment, the β -glucan is obtained from the submerged cultivated mycelial biomass of *Ganoderma tsugae* var. *jannieae* strain Tay-1 CBS 120394 by alkaline or acidic extraction as described for the fruiting bodies.

In another aspect, the present invention is directed to a composition comprising a β -glucan of the invention.

The composition comprising a β -glucan of the invention may be used as a nutraceutical composition alone or together with other nutrients, for example, agents having antioxidant activity such as carotenoids, flavonoids, tocopherols, ascorbates and mixtures thereof, and/or vitamins and minerals.

The compositions of the invention are preferably formulated in an oral solid dosage form such as, but not limited to, a fine powder, capsules, tablets, caplets, and sachets. These compositions may contain standard excipients and pharmaceutical acceptable carriers such as cellulose, methyl cellulose, carboxymethylcellulose and hydroxypropylmethylcellulose and lubricating agents.

The dosage of the composition to be taken by an individual will depend on his/her health condition. The dosage of β -glucan recommended for healthy adults are 100 to 200 mg daily. In chronic conditions, the daily amount will be higher, usually double or more. In critical conditions, the amount is even higher and may reach from 1000 to 2000 mg, or even more, daily

The compositions of the invention may also be formulated into food and beverage products including bread, cereals, ice creams and fruit drinks. These health products can improve the health conditions of the individual and prevent or treat diseases and disorders as well documented for β -glucans and as described below. The beverage food product may be an energizing beverage product further containing other components such as vitamins, flavors, stabilizers and the like.

The composition comprising the β -glucan of the invention may be also formulated into a pet food products, e.g., dog and cat food products.

In one embodiment, the composition comprising the β -glucan of the invention may be formulated into a cosmetic product such as an aqueous solution, a gel, a cream, a paste, a lotion, a spray, a suspension, a powder, a dispersion, a salve or an ointment.

In another embodiment, the compositions of the invention, preferably in an oral solid dosage form or as a beverage product, are for use as dietary supplements.

In other embodiments of the invention, the compositions comprising the β -glucan of the invention are indicated for prevention or therapy of several diseases, disorders and conditions.

In one embodiment, the compositions comprising the β -glucan of the invention can be used for stimulation of the immune system and are thus useful as adjuvants to chemotherapy or radiotherapy of tumors in the treatment of cancer patients receiving such treatments. These compositions can also increase resistance to microbial agents and are also useful for prevention and treatment of infections, e.g., bacterial, parasitic or viral infections, including human immunodeficiency virus (HIV) infection. In the case of HIV infection, the β -glucan, in addition to

immunostimulation of T-helper (Th)-2 cells, making them resistant to HIV infection, also seems to interfere directly with viral propagation.

In another embodiment, the compositions comprising the β -glucan of the invention can be used for in treatment of an NF- κ B-dependent disease selected from cancer, immunological disorders, septic shock, transplant rejection, radiation damage, reperfusion injuries after ischemia, arteriosclerosis, osteoporosis, and neurodegenerative diseases. In one preferred embodiment, the composition is used in the treatment of a cancer such as breast, lung, bladder, colon, gastric tract, prostate or skin cancer, leukemia or lymphoma, either as sole treatment or together with antitumor drugs such as the monoclonal antibodies Herceptin, Rituxan and Erbitux.

The low molecular weight β -glucan of the invention administered as a composition or incorporated into food products is capable of lowering levels of total cholesterol and LDL ("bad") cholesterol and of enhancing bile acid binding capacity ensuing improved hypocholesterolemic effects. These compositions and food products may thus be used for treatment of hypercholesterolemia and prevention and treatment of cardiovascular diseases and disorders such as arteriosclerosis and coronary artery diseases.

In another embodiment, the composition comprising the β -glucan of the invention is useful for controlling blood sugar levels and reducing glycemic index in Type II diabetes mellitus patients.

The invention will now be illustrated by the following non-limiting Examples.

EXAMPLES

Example 1. Characteristics of the variety *Ganoderma tsugae* var. *jannieae*

The variety *Ganoderma tsugae* var. *jannieae* has the following characteristics as described and depicted in Wasser et al., 2006:

(i) *Basidiomata*: as depicted in **Figs. 1-2**.

(ii) *External habit*. Basidiomata pedicellate orthopleuopodal (**Figs. 1-2**). Pileus dimensions varying between 7–20 cm in diam, the thickness varying between 0.5 and 2 cm. Stipe about 1–2.5 cm in diam. The pileus covered by thin laccate crust, slightly radially wrinkled and regularly concentrically zonate, diamine brown. The pores 5–6 per 1 mm, of normal *G. tsugae* appearance.

(iii) *Section*. Cutis 50–120 μm thick, hard and brittle. Context indistinctly two-layered. Upper layer wood-coloured to ochraceous buff, rather soft. Lower layer is ochraceous buff to coffee brown, penetrated by numerous laccate deposits, rather dense. Tubes one-layered, concolorous with the dense zone.

(iv) *Cutis*. Hymeniderm, consisting of dense layer of pileocystidia 45–100 x 9–11 μm , amyloid (dark brown to almost black), broadly clavate to narrowly clavate, narrowly utriform, thin-walled, few catenulate pileocystidia occur.

(v) *Hyphal system trimitic*. Inflated skeletal not found. Generative hyphae 20–60(>) x 3–5 μm , thin-walled, with short, small clamps not above every septae, slightly branched, seldom anastomosing; skeletal hyphae 40–120 x 3.0–7.5 μm , regularly branched, thick-walled, not regularly septated, clampless; binding hyphae 2.0–5.0 μm in diam, much branched, branches often tapering, thin- or thick-walled. Apart from these types of hyphae lateral and modified skeletal hyphae were observed. Lateral hyphae very thin, 1.6–2.0 μm in diam, branching, seldom septate. Arboriform skeletal hyphae 50–80 x 3.2–5.0 μm , long thick-walled straight with abundant short thin dichotomically branching hyphae (remining of a crown of a tree) at the apex and attenuate at the other end are present. All types of hyphae are inamyloid.

(vi) *Basidiospores*. Spores pale-brown, (8.0)8.8–11.2 x 5.0–6.4 μm , average 9.6 x 6.4 μm ; inamyloid; some spores with vacuoles; ovoid, oblong, germ pore noticeable, truncate or protruding; apiculus lateral, hardly discernable; spore wall two-layered, up to 2.0 μm thick, with rather wide, short, not crowded pillars

between the two layers, endosporium rather thin. SSI ($D \times 100/L$) = 50–80%, average – 66.7%. SI (L/D) = 1.2–2, average – 1.5.

(vii) *Mycelial Characteristics*

Thirty-day-old culture. Colony white, dense, downy (**Fig. 3A**), with faintly outlined concentric zones, some colonies develop well-discernable sectors. Mycelium forming concentric zones and sectors is denser than the mycelium on the rest of the surface of a colony. The outline of the colony are even; mycelium at the margin is raised. Several types of hyphae are observed.

Vegetative mycelium of the center of the colony. *Skeletal hyphae* – 4.8–8.0 μm in diam, thick-walled, cylindrical or slightly attenuate towards the septa, clampless, almost unbranched, not regularly septate. *Generative hyphae* 2.4–3.0 μm in diam, thin-walled, branched, regularly septate, with abundant short small clamps above almost every septa, some sections of generative hyphae are sclerified. *Binding hyphae* thick or thin-walled, strongly branched, 3.2–4.0 μm in diam. *Lateral hyphae* 1.6 μm in diam, thin-walled, dichotomously branching. Furthermore, abundant staghorn hyphae are observed in the center of the colony. *Vegetative mycelium of the growth zone.* Thick-walled unbranched skeletal hyphae, and also generative and binding hyphae similar to those from the center of a colony are observed. Also, thin-walled 35–45 x 28–32 μm cuticular cells and cystidia-like thin-walled utriform terminal cells are present. 24–40 x 18–16 μm cells also occur. H-like anastomoses are abundant in all parts of the colony. Chlamydospores and aleuria not found. All types of hyphae and vegetative mycelial structures are inamyloid.

Seventy-five-day-old culture. Colony white, dense, with concentric zones, reversum unchanged. Aerial mycelium very dense, cottony, felty in the center, subfelty with pronounced hyphal tufts near the edge of the Petri dish, interwoven, outline of margin even, mycelium at the margin is raised.

Vegetative mycelium of the center of the colony. The major types of hyphae (skeletal, generative, binding, and lateral) are observed. However, the number of clamps and staghorn hyphae greatly decreases; they are difficult to find. Chains of

thin or thick-walled not branched hyphae $2.4\text{--}3.2 \times (4.0)6\text{--}10(14) \mu\text{m}$ and a lot of misshaped thick-walled vacuolarized cells $19.2\text{--}28.8 \times 7.2\text{--}9.6 \mu\text{m}$ occurred.

Vegetative mycelium of the growth zone. Same types of hyphae as in the one-month-old culture are observed. Moreover, staghorn hyphae appear and aged vacuolarized cells appear while the number of culticular cells decreases (**Fig. 3B**).

According to the present invention, from the variety *Ganoderma tsugae* var. *jannieae* a new strain was isolated and characterized and was deposited under The Budapest Treaty with the Centraalbureau voor Schimmelcultures (CBS) under Accession No. CBS 120394.

Example 2. Submerged cultivation of mycelial biomass of *Ganoderma tsugae* var. *jannieae* strain Tay-1 in Erlenmeyer flasks and fermentor

Fig. 4 shows the general methodology for the production of submerged cultured mycelium of *Ganoderma tsugae* var. *jannieae* using fermentor or bioreactor technology.

The general scheme of mushroom submerged culture mycelium (SCM) production includes 5 steps of culture growth:

Museum culture (I) —> Intermediate culture (II) —> Pre-inoculums culture (III) —> Inoculums culture (IV) —> Fermentation culture (V).

Three types of culture media are used for SCM production: standard agar medium (steps I and II), liquid standard inoculums medium (steps III and IV), and fermentation medium (step V). Museum cultures are developed on agar slants in tubes; intermediate cultures are developed on agar slants in tubes or Petri dishes. Pre-inoculums and inoculums cultures are developed in Erlenmeyer flasks using a rotary shaker. Fermentation cultures are developed in fermentor Bioflo 2000 (New Brunswick Scientific, USA) that is equipped with instrumentation for the measurement and/or control of agitation, temperature, pH, dissolved oxygen concentration (pO_2), and foam.

For the first pre-inoculums culture, a 250 mL Erlenmeyer flask is inoculated by one to three week old mushroom mycelium from the Petri dish. Five-to-six pieces

(5-7 mm in diameter) from mycelium growing on the edge of the agar plate were transferred into the Erlenmeyer flask and cut on the flask wall into small pieces to increase the number of growth points of mycelia. Mycelium was inoculated in 250-mL Erlenmeyer flasks filled with 100 ml of defined synthetic medium. Fungal inocula were grown on synthetic medium consisting of the following components (g/L of distilled water): glucose, 25.0; peptone, 3.0; KH_2PO_4 , 1.0; K_2HPO_4 , 0.2; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; yeast extract, 3.0. Phosphate salts were sterilized separately (Sigma-Aldrich, St Louis, MO, USA). The cultivation of inoculated flasks is carried out on a rotary shaker at 160 rpm and 27°C for 6-7 days. At the end of cultivation (**Figs. 5-6**), 1 mL of sample is taken from the culture for microscopic observation of culture purity.

For the second inoculumss culture the biomass from the first pre-inoculumss culture (pellets) was homogenized (**Fig. 7**) 2 x 30 seconds using a Waring Laboratory Blender (Waring, USA) and inoculated in a 2 L flask containing 700 mL of the same medium.

After 5-7 days of cultivation, mycelial biomass (pellets) (**Fig. 8**) were homogenized and used as inoculumss culture for growth in a fermentor (Bioflo 2000 10 L, New Brunswick Scientific, USA) with 10 L of working volume on the same synthetic medium mentioned above. Initial parameters of cultivation were as follows: temperature 27°C; pH – 6.1; agitation – 100 rpm, aeration – 0.2 v/v/min. Antifoam used was polypropylene glycol 2000; 4% NaOH and 4% HCl were used to control pH.

Initially, pH of the medium was not controlled. However, when it decreased to 5.2, the pH was kept constant automatically at the level of 6.0 to favor the fungus growth. After 24 h, the speed of agitation was increased to 200 rpm, then after 48 h to 300 rpm. After 48 h, the rate of aeration of the medium was increased to 0.4, then (after 72 h) to 0.5 v/v/min.

The maximal yield of mycelial biomass was 113 g/L of wet biomass (**Fig. 9**) or 11.3 g of dry biomass (**Fig. 10**) achieved on day 7 of fungus cultivation. The conditions of the cultivation are defined in **Table 1**. Vacuolated hyphae with clamp

connections of mycelial biomass of *Ganoderma tsugae* var. *janniae* CBS 120394 after 7 days of fungus cultivation are depicted in Fig. 11.

Table 1. Mycelial biomass production of *Ganoderma tsugae* var. *janniae* strain Tay-1 in submerged culture as a function of time

Time, h	pH	DO, %	Aeration, v/v/min	RPM	Biomass, g/l
0	6.14	100	0.2	100	0.2
24	5.8	28	0.2	100→200	0.8
48	5.8→6.0	19	0.2→0.4	300	3.5
72	6.0	17	0.4→0.5	300	6.0
96	6.0	14	0.5	300	7.9
120	6.0	18	0.5	300	9.5
144	6.0	20	0.5	300	10.5
168	6.0	22	0.5	300	11.3

DO, dissolved oxygen

Example 3. Isolation of β -glucan from dried fruiting bodies biomass of *Ganoderma tsugae* var. *janniae* strain Tay-1

Polysaccharides were isolated from *Ganoderma tsugae* var. *janniae* strain Tay-1 using alkaline or acidic extraction. Dominant β -glucan was most efficiently isolated by boiling diluted sulfuric acid at pH 1.5. Using alkaline extraction, β -glucan, galactan and mannan were isolated, with the two latter products in minor quantity. The β -glucan was characterized by chemical analysis, methylation, acetolysis, and NMR spectroscopy. It was found to have an irregular branched structure with the main chain consisting of β -1-3- and β -1-4-linked glucopyranose residues with side chains consisting of β -1-6-glucopyranose oligomers, attached to O-6 of some monosaccharides of the main chain.

Materials and Methods

3.1 General Methods ^1H and ^{13}C NMR spectra were recorded using a Varian Inova 500 spectrometer (Varian, USA) in D_2O solutions at 40 °C for the polysaccharide with acetone standard (2.225 ppm for ^1H and 31.5 ppm for ^{13}C) using standard pulse sequences COSY (homonuclear correlation spectroscopy),

TOCSY (total correlation spectroscopy; mixing time 120 ms) and NOESY (nuclear Overhauser effect spectroscopy; mixing time 200 ms), HSQC (heteronuclear single quantum correlation), and HMBC (multiple-bond C-H correlation; optimized for a 5 Hz coupling constant).

For monosaccharide analysis, the polysaccharide (0.5 mg) was hydrolyzed (0.2 mL of 3 M trifluoroacetic acid (TFA), 100 °C, 2 h), followed by evaporation to dryness under a stream of air. The residue was dissolved in water (0.5 mL), reduced with NaBH₄ (~5 mg, 1 h), neutralized with acetic acid (0.3 mL), dried, and methanol (1 mL) was added. The mixture was dried twice with the addition of MeOH, and the residue was acetylated with acetic anhydride (Ac₂O) (0.5 mL, 100 °C, 30 min), dried and analyzed by Gas Liquid Chromatography (GLC) on an HP1 capillary column (30 m x 0.25 mm) with a flame ionization detector (Agilent 6850 chromatograph (Agilent, USA) in a temperature gradient of 170 (4 min) to 260 °C at 4 °C/min.

For protein determination 2,2'-bicinchoninate assay kit from Pierce Biotechnology (USA) was used according to the supplier's instructions.

Gel chromatography was carried out on Sephadex G-50 (2.5 m x 95 cm), column (GE Healthcare) using the pyridinium acetate buffer, pH 4.5 (4 mL pyridine and 10 mL AcOH in 1 L water) as the eluent, monitored by a refractive index detector.

Anion-exchange chromatography was performed on a column of DEAE cellulose (15x5 cm), washed with 1 M NaCl and then water. The sample was applied in water, and eluted with water (500 ml), 0.25 M NaCl (500 ml), and then 1 M NaCl for regeneration. 0.25 M NaCl extract was desalted by dialysis.

3.2 Methylation analysis The sample of the polysaccharide (2-3 mg) was dissolved in Me₂SO (1.0 mL), powdered NaOH was added (~50 mg), the mixture was stirred for 30 min, 0.5 ml of MeI was added, then the mixture stirred for 30 min, excess MeI was removed by air stream, water was added (5 ml), methylated polysaccharide was extracted with chloroform, extract washed with water 3 times, and dried. It was then hydrolyzed (3M TFA, 100 °C, 1 h), reduced (NaBD₄),

acetylated, and analyzed by a Varian Saturn 2000 ion-trap GS-MS instrument (Varian, USA).

3.3 Acetolysis. Glucan (80 mg) was dissolved in water (1 ml), diluted with pyridine (10 mL), and acetic anhydride was added (10 mL). The homogeneous mixture was kept 30 min. at 100°, and then evaporated to dryness. Acetylated glucan was dissolved in acetic acid (10 mL), cooled on ice, and Ac₂O (10 mL) and H₂SO₄ (0.5 mL) were added. The mixture was kept three days at room temperature, poured in cold water, after one hour it was extracted with chloroform 3 times. Unexpectedly, organic extract contained only α -glucose pentaacetate; acetylated oligo/polysaccharides were not extracted by chloroform. To extract them, NaCl was dissolved in the aqueous layer to saturation, and products were extracted with chloroform, to give acetylated polysaccharide. It was deacetylated with 0.1 M MeONa in MeOH and analyzed by NMR and methylation (after borohydride reduction to prevent peeling). The hydrolysis of glycosides and polysaccharides to reducing sugars and the concomitant conversion to alditol acetates (borohydride reduction and acetylation) is a standard method to analyse polysaccharides containing aldoses, ketoses, deoxyaldoses, and acetamidohexoses and other related sugars.

3.4 Isolation of β -glucan is carried out by different procedures. The alkaline extraction is schematically depicted in Fig. 12.

Powdered dry fruiting bodies biomass of *Ganoderma tsugae* var. *janniae* (1 kg) were washed with boiling 85% EtOH (5 L, 3h, repeated 3 times) and ethanol extract was discarded.

The remainder was extracted with boiling water (5 L, 100°, 3 h, 5 times), the solution was dialyzed, concentrated by evaporation on the rotary evaporator to ~1 L. This solution was shaken with 200 mL of chloroform-isoamyl alcohol (10:1) for 10 min, centrifuged at 10000 rpm for phase separation, water-layer treated in the same way one more time, and freeze-dried to give crude β -glucan that purified by anion-exchange chromatography, yielded 1.5 g (0.15%) of β -glucan.

Half of the insoluble residue from water extraction was boiled with 5% NaOH - 0.05% NaBH₄ (5 L, overnight, repeated twice). Extract was neutralized with concentrated HCl to a pH of 4, at which point the maximum of the precipitate was formed. Precipitate was removed by centrifugation (10000 rpm, 30 min). Solution was dialyzed against running water, concentrated to 1 L volume, solution was lyophilized, yielding 28 gram (2.8 %). It was dissolved in water (200 mL), centrifuged at 40,000 rpm, and the β -glucan was purified from solution by anion-exchange chromatography. β -glucan was found in water and 0.25 M NaCl eluates. Both neutral and acidic fractions were separated by size-exclusion chromatography on Sephadex G-50 column (2.5x80 cm) to give 3 g (0.6%) of β -glucan, as well as 500 mg of a mixture containing β -glucan, galactan, and mannan.

A quarter of the insoluble residue from water extraction was boiled in 0.01 M H₂SO₄ (5 L, 4h, repeated twice). Extracts were dialyzed and further treated in the same way as alkaline extract, yielding 5 g (2%).

3.5 Results Standard determination of monosaccharide content of the whole mushroom using complete acid hydrolysis and gas chromatography of alditol acetates showed glucose and glucosamine in the respective amount of 6 and 3% by weight. Other monosaccharides were identified in small amounts.

Sawmill dust-like powder prepared by destruction of the dry mushrooms using a high-speed blender was defatted by boiling in three portions of 85% ethanol, and then extracted three times with boiling water. Extract was dialyzed, deproteinized using Sevag protocol (Sevag et al., 1938), concentrated, clarified by ultracentrifugation at 40,000 rpm, and freeze-dried. Monosaccharide analysis (GC of alditol acetates) showed the presence of 56% glucose, 4% galactose, 5% mannose, 1.5% fucose, and 1% xylose. Bicinchoninic acid (BCA) estimation of the protein content showed 32% of protein (possibly overestimated). Anion-exchange and size-exclusion chromatography of the freeze-dried product (dissolved in water) yielded 1.5 g of β -glucan.

The portion of the solid remainder of water extraction was treated essentially as described by Mizuno et al. (1995) with some modifications (particularly, temperature of extraction was increased as it leads to better yield): it was boiled in 5% NaOH-0.05% NaBH₄, filtered, solution neutralized by conc. HCl to pH 4. Massive precipitation occurred; the precipitate was removed by centrifugation. The precipitate was insoluble in water but could be dissolved in alkaline conditions. It gave no signals in NMR spectra. Quantitative monosaccharide analysis showed the presence of a small amount of glucose (it was not analyzed further).

The neutralized solution was dialyzed, concentrated by roto-evaporation, deproteinized using Sevag protocol (Sevag et al., 1938), clarified by ultracentrifugation at 40,000 rpm, and freeze-dried. The product had a dark brown color. Widely used ethanol precipitation was attempted for polysaccharide purification, but it did not give good results: NMR of the EtOH precipitate and solution showed that β -glucan was distributed between both fractions, apparently higher molecular mass glucan dominated in the precipitate, and shorter chains remained in solution. Other polysaccharides were found mostly in the precipitate. Ethanol precipitation was not used for large-scale polysaccharide isolation.

The product was further separated by anion-exchange chromatography on DEAE-cellulose. Most of the brown color was adsorbed on the column packing. Two fractions of glucan were isolated: neutral (eluted with water) and acidic (retained in water and eluted with 0.25 M NaCl). It seems that retention of β -glucan is due to the presence of unidentified acidic group(s), present in very low amounts per one polysaccharide chain. Therefore, glucan was weakly retained on a DEAE sorbent. Each fraction, retained and non-retained, on re-chromatography gave two fractions again, retained and non-retained. Thus retention of the glucan is non-specific, and both products are identical. They had identical NMR spectra (not shown) and contained ~5% of protein. Overall yield of β -glucan from NaOH extraction was 0.6%.

A portion of the remainder after ethanol and water extraction was treated using the procedure described by Yap and Ng (2001). It was frozen in liquid

nitrogen, lyophilized, and extracted again with boiling water. Although there is no description in the original article, it could be assumed that freezing destroys cell walls and thus facilitates extraction of the polysaccharides. However, nothing was extracted in this way, except some proteins.

A portion of the remainder after ethanol and water extraction was extracted using the method described for β -1,3-glucan isolation (Storseth et al., 2005): the material was boiled in 0.01 M H_2SO_4 (pH 1.5), filtered, dialyzed, deproteinized using Sevag protocol (Sevag et al., 1938), concentrated, clarified by ultracentrifugation at 40,000 rpm, and freeze-dried. The product had light brownish color. NMR showed that some protein and/or lipid impurities were still present. The product was mainly β -glucan; the yield was 2%. β -glucan was further purified by anion-exchange chromatography as described above.

3.6 Separation of the β -glucan from other polysaccharides. Size-exclusion chromatography of the mixture of polysaccharides showed a broad molecular mass distribution covering the whole separation range of Sephadex G-50 (500-10000 Da). Three polysaccharides were detected in the mixture – glucan (Glc), galactan (Gal), and mannan (Man). The maximum of molecular weight of the glucan was about 5000 Da. The other two polymers had distinctly higher molecular masses and were eluted close to the void volume of the column. They were not obtained in an individual state, but only as a mixture with two other polysaccharides (not shown). ^1H -NMR spectra of the fraction containing the purified glucan, as depicted in **Fig. 13**, and of the fraction of the void volume containing mannan, galactan, and glucan (**Fig. 14**), show the different compounds present in the two fractions.

3.7 Analysis of the structure of β -glucan.

Monosaccharide analysis of the isolated β -glucan showed the presence of glucose (~90%). Measured protein content (bicinchoninate method) was ~5%. Methylation analysis of the glucan showed the presence of terminal, 3-, 4-, 6-, 4,6-

and 3,6-substituted glucose residues in the ratio of 1:0.64:1.04:0.52:0.12:0.52 (Fig. 15).

Table 2 shows the NMR data for the β -glucan (ppm; D₂O, 40°C). Signals of 4,6-disubstituted glucose were not identified.

Table 2. Chemical shifts of ¹H and ¹³C of glucose

Unit	Nucleus	H/C-1	H/C-2	H/C-3	H/C-4	H/C-5	H/C-6
Glc	H	4.52 or 4.76	3.31	3.51	3.40	3.46	3.72/3.91
	C	104.2	74.7	77.1	71.1	77.4	62.3
-6-Glc	H	4.52 or 4.76	3.32	3.46	3.46	3.62	3.86/4.20
	C	104.2	74.7	77.2	71.2	76.4	70.4
-3-Glc	H	4.52 or 4.76	3.51	3.75	3.51	3.50	3.72/3.91
	C	104.2	74.5	85.9	69.7	77.1	62.3
-4-Glc	H	4.52 or 4.76	3.40	3.66	3.65	3.61	3.80/3.97
	C	104.2	74.7	75.7	80.1	76.3	61.5
-3,6-Glc	H	4.52 or 4.76	3.51	3.75	3.57	3.62	3.86/4.20
	C	104.2	74.5	85.9	69.6	76.4	70.4

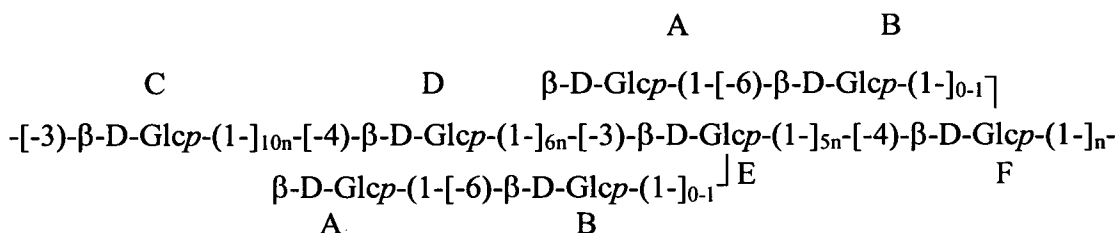
Interpretation of the 2D NMR spectra of the glucan in **Table 2** showed the presence of variably linked β -glucose residues. No clearly defined repeating units were observed. Because of irregular structure, interpretation of ¹H 2D correlation spectra was difficult; assignment of the proton signals – but no sequence information – could be deduced. Substitution types of the monosaccharides were deduced from HMBC spectra (Fig. 15) (Duus et al., 2000)

The signals of all β -glucopyranosides with the substitution types determined by methylation were observed (with the exception of low abundant 4,6-disubstituted glucose residues). It was impossible to determine the arrangement of differently substituted residues in the main chain.

The lengths of the side chains were determined using acetolysis, which is known to break only 1-6-linkages. Treatment of the acetylated glucan with Ac₂O-H₂SO₄ led to isolation of α -glucose tetraacetate, identified by NMR, and of the acetylated main chain of the glucan. This result can be interpreted as a confirmation of the presence of oligomers of 1-6-linked β -glucose in the side chains, whereas the

main chain contained no 1-6-linkages. The number of 6-substituted glucose residues is half of the number of terminal ones according to the methylation data, thus the side chains are composed of single glucose units and β -Glc-(1-6)- β -Glc disaccharide.

The data obtained could be interpreted as belonging to the following structure:



where n is an integer number ≥ 1 .

The different components represented by capital letters in the representation of the β -glucan structure are as follows:

- A - β -D-Glcp-(1-6)
- B - β -D-Glcp-(1-]0-1
- C - -[-3)- β -D-Glcp-(1-]10n
- D - -[-4)- β -D-Glcp-(1-]6n
- E - -[-3)- β -D-Glcp-(1-]5n
- F - -[-4)- β -D-Glcp-(1-]n

The β -glucan is characterized by high solubility in water giving a clear aqueous solution, which becomes brown at high concentrations. The lyophilized dry β -glucan is an odorless white powder.

The signals of all β -glucopyranosides with the substitution types determined by methylation were observed. It was impossible to determine the arrangement of differently substituted residues in the main chain.

The lengths of the side chains were determined using acetolysis. Acetolysis is known to break only 1-6-linkages. Treatment of the acetylated glucan with $\text{Ac}_2\text{O-H}_2\text{SO}_4$ led to isolation of α -glucose tetraacetate, identified by NMR, and of

the acetylated main chain of the glucan. This result can be interpreted as a confirmation of the presence of oligomers of 1-6-linked β -glucose in the side chains.

Various preparations of β -glucan contained different amounts of impurities. Presented spectra contain sharp signals of α -1-3-mannan (marked with stars in **Fig. 16**). Other samples (not shown) contain no such signals; thus mannan is not chemically linked to the glucan structure.

MALDI (Matrix Assisted Laser Desorption Ionization) mass spectra of glucan showed broad mass distribution. Peaks starting from m/z 510 up to 5,000 were observed (**Fig. 17**). This does not reflect real mass distribution, since signal intensity decreases with molecular mass due to lower volatility of the heavy components. Since the spectrum corresponds to molar mass distribution, it also overestimates low-molecular mass components. The spectra showed no variations in peak-to-peak distances, which corresponded to hexose residues (162 amu; peaks are sodium and potassium adducts). Thus, the glucan contained no additional substituents along the chain. However, the overall mass could not be explained as belonging to a polymer built of glucose only. The nearest peaks to that calculated for a molecule containing only hexoses are 16 amu lighter. Thus, the glucan probably has some non-glucose residue at the reducing or non-reducing end. Attempts to determine its nature by NMR and chemical analysis failed.

In summary, β -glucan is present in dried fruiting bodies of *Ganoderma tsugae* var. *jannieae* in an amount not exceeding 6%, total glucose is 6% of the dry weight. It seems that β -glucan is not chemically bound to the other mushroom components, since it can be extracted in neutral, acidic, or basic conditions. The best yield of β -glucan was obtained by acidic extraction. Repeated extractions give additional smaller amounts of β -glucan.

β -glucan has an irregularly branched structure with the main chain consisting of β -1-3- and β -1-4-linked glucopyranose residues with side chains

consisting of short β -1-6-glucopyranose oligomers attached to O-6 of the monosaccharides (mostly 3-substituted) of the main chain.

Molecular mass of the isolated β -glucan is below 10000 Da with broad molecular mass distribution, centered at about 5000 Da.

The glucan contains no additional components except glucose, but seems to have a different reducing end residue. The reason for its retention on anion-exchange column remains unknown.

Two other polysaccharides, mannan and galactan, are co-extracted with the glucan. These polysaccharides are present in low concentration in the mushrooms, but are more easily extracted than β -glucan.

As described in the Background section, β -glucans from fungi possess many biological activities and are beneficial in the prevention and treatment of several human health conditions. The low-molecular mass of *Ganoderma tsugae* var. *janniae* β -glucan may give it an advantage in possibly better internalization using oral administration. Its structure is unique.

Example 4. Isolation and characterization of β -glucan from submerged cultivated mycelium of CBS 120394 *Ganoderma tsugae* var. *janniae* strain Tay-1

Polysaccharides were isolated from submerged cultured mycelium of *Ganoderma tsugae* var. *janniae* strain Tay-1 using water, NaOH or H₂SO₄ extraction. Dominant β -glucan was most efficiently isolated by boiling the mycelium in diluted sulfuric acid at a pH of 1.5. β -glucan and other polysaccharides were characterized by chemical analysis, methylation, and NMR spectroscopy. The β -glucan from submerged cultured mycelium had an irregular branched structure similar to the β -glucan isolated from fruiting bodies, with the main chain consisting of β -1-3- and β -1-4-linked glucopyranose residues with side chains consisting of β -1-6-glucopyranose oligomers, attached to O-6 of some monosaccharides of the main chain.

One can expect that the *Ganoderma tsugae* var. *jannieae* glucan have high biological activity because it combines structural elements of several active polysaccharides: it has alternating β -1,3;1,4-linkages in the main chain as in the glucan from plant cell wall, and branches at O-6 like fungal glucan or lentinan. It also has low molecular weight, which is beneficial for bioavailability.

Results Monosaccharide analysis (acid hydrolysis and gas chromatography of alditol acetates) of the cells of the fruiting bodies and mycelium of *Ganoderma tsugae* var. *jannieae* strain Tay-1 was performed in different hydrolysis conditions: (1) Conc. HCl - 37% HCl, 100°, 15 min., then solution was diluted 1:1 with water and hydrolysis continued for 1 h at 100°; (2) 2M HCl - 100° 4 h; 3M TFA - 120°, 4 h; (3) HF-TFA - anhydrous HF 25°, 1 h, then 3M TFA, 120°, 4 h). The results are shown in **Table 3** below, in which percents are calculated from GC peak integration area relative to internal inositol standard.

The results in **Table 3** demonstrate significant differences in monosaccharide content in the different hydrolysis conditions; glucose (Glc) content varied from 5% to 10%, and glucosamine (GlcN) content varied from 0.5 to 5%. This can be explained by different solubility of the samples in different acids. The highest determined quantity of the monosaccharides still does not reflect the real concentration of the monosaccharides, which can be even higher.

The overall glucose content is lower in the mycelium comparing to dried fruiting bodies biomass of *Ganoderma tsugae* var. *jannieae* strain Tay-1 mushroom, and glucosamine is higher. The results for galactose are unusual in that its concentration in fruiting bodies is very low, or it seems to be absent, whereas the mushrooms contain significant amounts of easily extractable galactan.

Table 3 . Relative amount (%) of monosaccharide of the fruiting bodies and submerged cultivated mycelial biomass of *Ganoderma tsugae* var. *jannieae* strain Tay-1 in different hydrolysis conditions.

Source	Conditions	Xyl	Man	Glc	Gal	GlcN
Fruiting bodies	Conc. HCl	0.8	0.5	28	0	1.8
Mycelium	Conc. HCl		0.7	5	0.7	5
Fruiting bodies	2 M HCl		0.9	18	0	0.2
Mycelium	2 M HCl		1.5	7.5	0.7	0.8
Fruiting bodies	3M TFA		1.2	13.7	0	0
Mycelium	3M TFA	1.2	1.2	5	0.8	0.5
Fruiting bodies	HF-TFA		0.5	50	0	3.2
Mycelium	HF-TFA		1.4	10	0.5	1.2

Xyl, xylose; Man, mannose; Glc, glucose; Gal, galactose; GlcN, glucosamine

4.1 Isolation of polysaccharides. All experiments were as described for the fruiting bodies of *Ganoderma tsugae* var. *jannieae* (Example 2). For isolation of polysaccharides, mycelium of *Ganoderma tsugae* var. *jannieae* strain Tay-1 was washed by boiling 85% ethanol 3 times and then extracted with boiling water for 8 h. The insoluble material was then boiled in diluted H₂SO₄ (~0.01 M, pH adjusted to 1.5) for 8 h, or in 5% NaOH for 12 h. The products were dialyzed and dried. All extractions gave a product containing protein, β -glucan, galactan and other contaminants. The yield of acid extraction was comparable to the yield of alkaline extraction. At the end of all these treatments, the dry insoluble residue of the cells was 30% of the original weight of the starting material. The monosaccharide composition was close to that of the untreated cells, which indicates the presence of tightly bound polysaccharides, probably residing inside the cells.

NaOH extract was cleared by acid precipitation and the precipitate was discarded. All products were then separated by anion-exchange chromatography, which gave a neutral fraction, composed mostly of galactan and mannan with some glucan and other impurities and an acidic fraction of the purified β -glucan. Finally, polysaccharides were purified by size-exclusion chromatography. Overall yield of β -glucan was 3%. The glucan had low molecular weight of <5000 Da, similar to that of the glucan isolated from the fruiting bodies. Some of the glucan was probably lost during dialysis.

4.2 Analysis of the structure of β -glucan from submerged cultivated mycelial biomass of *Ganoderma tsugae* var. *janniae* strain Tay-1. Monosaccharide analysis of the isolated β -glucan from mycelium of *Ganoderma tsugae* var. *janniae* strain Tay-1 showed the presence of glucose (~90%). Measured protein content (by the bicinchoninate method) was ~5%. Methylation analysis of the glucan showed the presence of terminal, 3-, 4-, 6-, 3,6- and 4,6-substituted glucose residues in the ratio of 1:0.75:0.25:1.6:0.1:0.6. The amount of 4-substituted glucose was significantly lower - and the content of 6- and 3,6-substituted glucose was higher - in the glucan extracted from mycelium compared with the glucan extracted from the fruiting bodies (**Fig. 18**).

As with the fruiting bodies in Example 2, two other polysaccharides, galactan and mannan, were co-extracted with the β -glucan. The galactan from the mycelium had the same structure as galactan extracted from fruiting bodies with α -1-6-linked galactopyranose in the main chain and with α -fucose branches at O-2.

NMR spectra of the glucan from submerged cultivated mycelial biomass was similar to the spectra of the glucan from dried fruiting bodies (**Fig. 19**).

Thus, one can conclude that β -glucan from mycelium and from fruiting bodies of *Ganoderma tsugae* var. *janniae* strain Tay-1 has the same overall structure with some quantitative differences, e.g., lower content of 4- and 4,6-substituted glucose and longer side chains of β -1-6-linked glucose. Molecular mass of the isolated β -glucan is below 10000 Da with broad molecular mass distribution centered at ~5000 Da. The glucan contains no additional components except glucose, but seems to have different reducing end residue.

Example 5. *Ganoderma tsugae* var. *janniae* strain Tay-1 extract as a potential modulator of the NF- κ B activation pathway

It is widely accepted in the scientific community that the active component in mushroom extract that have immuno-stimulatory and anti-cancer activity is

comprised largely of β -glucans (Lull et al., 2005, Luhm et al., 2006; Chen and Seviour, 2007). The molecular mechanism by which the β -glucans exert their activity often involves the NF- κ B pathway as already mentioned above (e.g. Kataoka et al., 2002). The activity of the mushroom extract was tested *in vitro* in a human breast cancer cell line.

Material and methods

5.1 Mushroom cultivation and biomass extraction. A pure mycelial culture of *Ganoderma tsugae* var. *jannieae* strain Tay-1 was obtained after being maintained on Suslo-Yeast extract-Agar (SYA) (Conda Laboratories, Spain) medium composed of: 0.5 liter Suslo (an aqueous malt extract boiled with hops), 0.5 liter distilled H₂O, 0.5 g peptone, 0.5 g yeast extract, 17 g agar-agar, 0.5 g MgSO₄ (anhydrous), and 1 g streptomycin; pH – 5.5. Five mycelial disks, with a diameter of 5 mm, were inoculated in 250 mL flasks with 100 ml Yeast Extract (YE) medium containing (g/liter): glucose, 10; yeast extract (Conda Laboratories, Spain), 3; peptone, 2, KH₂PO₄, 0.8; Na₂HPO₄, 0.2 and MgSO₄.7H₂O, 0.25. The inoculums were incubated on a rotary shaker at room temperature. When the grown mycelium filled out the culture medium, the whole flask content was homogenized, divided into 2-liter flasks (containing 1 L YE medium), and put on a rotary shaker (Orbital Shaker Incubator, M.R.C., Ltd., Israel) for 10 days at 27°C and 140 rpm for biomass production. The obtained biomass was washed twice with 1 L of distilled water, dried, ground into a fine powder, and divided into 3 equal portions, each one of which was extracted three times per 30 min with one of the following organic solvents (Frutarom, Israel): ethyl alcohol (EAL), ethyl acetate (EAC), and diethyl ether (DEE) in the ratio of 1 g: 10 mL. Culture liquid (CL) was also extracted with ethyl acetate in the ratio of 1 L (CL): 500 ml ethyl acetate. The total of four crude organic extracts were obtained and named as follows: W01 (mycelial DEE extract); W02 (mycelial EAC extract); W03 (mycelial EAL extract); and W04 (CL extract). All the extracts were diluted with 99.9% DMSO (dimethyl sulfoxide) (Sigma-

Aldrich, St Louis, MO, USA) to a final concentration of 50 mg/mL and kept at -70°C.

5.2 Cell culture. The human breast cancer cell line MCF7 (ATCC, Rockville, MD, USA) was transfected with a NF- κ B responsive reporter plasmid, NF- κ B-LUC (Clontech, USA), connected to a luciferase responsive gene. Transfected cells were maintained in RPMI 1640 medium with L-glutamine, supplemented with 10% fetal calf serum (FCS) and 1% PenStrep (penicillin + streptomycin), and G418 antibiotic (Sigma-Aldrich, St Louis, MO, USA), in the concentration of 1 mg/mL. Baf3/p185 Bcr-Abl (B-lymphocytes, a laboratory model of chronic myelogenous leukemia (CML), 9L (rat glioblastoma), and PC3 and DU145 (human androgen-independent prostate cancer) cell lines, obtained from ATCC (Rockville, MD, USA), were also maintained in RPMI 1640 medium with L-glutamine, supplemented with 10% FCS and 1% PenStrep. Cells were grown in a humidified incubator at 37°C with 5% CO₂ in air and fed twice a week with fresh medium.

5.3 Luciferase reporter gene assay. MCF7 transfected cells (4×10^4) were seeded in 200 μ l of medium, using 96-well plates. After 24 h, mushroom extracts were added in several concentrations: 10, 25, 50, and 100 μ g/ml. Control wells were: solvent-treated wells (1% of DMSO); parthenolide, an inhibitor of I κ B α phosphorylation (Sigma-Aldrich, St Louis, MO, USA); and MG132, an inhibitor of I κ B α degradation (Calbiochem, San Diego, CA, USA). After 24 h, luciferase levels were determined according to the manufacturer's instructions using a microplate luminometer (Anthos Labtec Instruments, Wals, Austria). The extracts' reporter inhibition was expressed as percent relative to DMSO treated cells.

5.4 Cytotoxicity assay. To evaluate the effect of *Ganoderma tsugae* var. *jannieae* strain Tay-1 extracts on cell viability, the trypan blue exclusion method was applied. Cells (2×10^5) were treated with the crude organic extracts in the concentrations of 125, 25, and 5 μ g/mL. After 24 h, cells were collected, stained

with 0.4% trypan blue solution (1:1), and counted using a hemacytometer. Solvent-treated samples were used as a negative control.

5.5 Sodium dodecyl sulphate-polyacrylamide electrophoresis (SDS-PAGE) and Western blot analysis. Transfected MCF7 cells (2×10^5) were seeded and then starved overnight with 0.5% FCS-containing medium. Twenty-four hours later, cells were supplemented with TNF- α (10 ng/mL) for 10 min in the presence of appropriate concentrations of the crude mushroom extracts. A solvent-treated sample was used as a negative control, and positive controls included samples treated with 10 μ M of parthenolide or MG132, respectively. Cells were harvested, washed 3 times with cold PBS, and lysed with 50 μ l of cell lysis buffer/ 1×10^6 cells (Sigma-Aldrich, St Louis, MO, USA), supplemented with 0.33% of PMSF (phenylmethylsulfonyl fluoride), and 1% of both protease and phosphatase inhibitor cocktails (Sigma-Aldrich, St Louis, MO, USA). Protein concentrations of the samples were determined using Bio-Rad DC protein assay (Bio-Rad, Hercules, CA, USA). Equal amounts of proteins (30 μ g) were separated using 12% SDS-PAGE, transferred to a nitrocellulose membrane (Schleicher & Schuell BioScience GmbH, Dassel, Germany) and subjected to Western analysis. Anti-I κ B α or anti-pI κ B α antibodies were used following the manufacturer's instructions (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Target proteins were visualized by an ECL detection kit (Amsterdam, The Netherlands) according to the manufacturer's instructions. The equality of sample loading in each line was confirmed by stripping and reblotting with anti-beta-tubulin antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); however, a portion of blots (mainly for pI κ B α) was confirmed by Coomassie Brilliant Blue gel staining or by Ponceau Red membrane staining following the blotting.

5.6 Cell line sensitivity to parthenolide. A variety of cancer cell lines including MCF7 (breast cancer), Baf3/p185 Bcr-Abl (B-lymphocytes, a laboratory model of CML), 9L (rat glioblastoma), and DU145 and PC3 (human androgen-independent

prostate cancer) were used. Cells (2×10^5) were seeded in 2 ml of medium and maintained for 24 h as previously described. Drugs were then added as follows: 1% DMSO; parthenolide, in the concentrations of 20, 10, 5, and 1 μ M; and *Ganoderma tsugae* var. *jannieae* extracts, in the concentrations of 250, 125, 50, 25, and 5 μ g/ml. Imatinib (Gleevec®, a drug for treatment of CML; Novartis, Switzerland), in 10 μ M and 1 μ M, was used as a positive control for the CML cell line. Cell viability was measured at 48 h post-treatment using the trypan blue exclusion method.

5.7 Statistical analysis. Results from the duplicated luciferase reporter gene assay are presented as an average fold of each extract treatment versus the DMSO values. Data received from the cytotoxicity assay were calculated as sample values versus the solvent-treated value, which was used as a negative control. The analyses of the Western blots for I κ B α and pI κ B α levels in response to the mushroom extracts' effects are presented as folds to only TNF- α treatment for 5 min (for pI κ B α) or 10 min (for I κ B α), respectively.

Results

5.8 *Ganoderma tsugae* var. *jannieae* strain Tay-1 extracts inhibit NF- κ B-mediated reporter activity. The duplicate luciferase-reporter gene assay resulted in two (out of the four tested) *Ganoderma tsugae* var. *jannieae* strain Tay-1 extracts, which demonstrated reporter inhibitory activity by more than 40%. These were the W01 (DEE extract) and W02 (EAC extract), both of which were active only in the higher concentration used, 100 μ g/mL (**Fig. 20**). However, among them, the W02 extract showed higher potential, inhibiting the reporter (luciferase) activity by more than 70%, and was therefore selected for further evaluation.

5.9 Effect of W02 *Ganoderma tsugae* var. *jannieae* strain Tay-1 extract on cell viability. The effect of the W02 extract (EAC extract) on cancer cell viability was evaluated by using three different concentrations, 125, 25, and 5 μ g/mL. As shown

in Fig. 21, the W02 extract, in all tested concentrations, was not toxic for the cells and exerted a growth inhibitory effect lower than 30%. Considering the high reporter inhibition (of more than 70%) and the low cytotoxicity (less than 30%) of W02, it was assumed that the extract's activity is due not to the direct killing of the cancer cells but to the interference in the intracellular surviving mechanisms, which interact with the NF- κ B pathway.

5.10 Effect of W02 *Ganoderma tsugae* var. *janniae* strain Tay-1 extract on I κ B α and p-I κ B α levels. I κ B α is a negative regulator of the NF- κ B pathway and its levels are regulated by its degradation in the proteasomes. TNF- α is known to be the classical activator of the NF- κ B proapoptotic pathway. Therefore, in the present experiment, it was used as a control treatment, according to which the activity of W02 mushroom extract could be estimated.

Our preliminary data have established the kinetics of changes in I κ B α and phospho-I κ B α (pI κ B α) levels in response to TNF- α -stimulation. Results showed that TNF- α treatment (10 ng/mL) for 10 min caused the highest level of I κ B α degradation, and the treatment with 10 μ M of MG132 caused inhibition of I κ B α degradation. Also, a 5 min treatment with TNF- α caused the highest level of I κ B α phosphorylation, and the treatment with 10 μ M of parthenolide significantly inhibited I κ B α phosphorylation. Therefore, in order to check the effect of W02 on I κ B α degradation, it was applied as a 10 min treatment, and for checking its effect on I κ B α phosphorylation, it was applied as a 5 min treatment to the cells. The results, as depicted in Fig. 22, demonstrated that W02 had a strong effect on I κ B α level in the higher concentration used (200 μ g/mL), which could be compared to the effect of parthenolide. Therefore, it was suggested that W02 exerts a mode of action similar to that of parthenolide (PAR), namely, affecting I κ B α phosphorylation. However, W02 appeared to be a stronger inhibitor of I κ B α degradation than the other mushrooms tested: *Leucoagaricus birnbaumii* (LB12 – mycelial ethyl acetate extract), *Tricholomopsis sulphureoides* (TS03- mycelial

ethyl alcohol extract), *Pleurotus ostreatus* (PO01-mycelial diethyl ether extract), and *Schizophyllum commune* (SC01- mycelial diethyl ether extract).

In order to prove the phosphorylation inhibitory activity of W02, it was tested as a modulator of p-I κ B α level. Results showed that W02 acts as a strong inhibitor of I κ B α phosphorylation in both concentrations used and its effect might be considered as similar to the effect of parthenolide (Fig. 23).

5.11 Effect of W02 *Ganoderma tsugae* var. *jannieae* strain Tay-1 extract on cell survival dependency on NF- κ B pathway. Cancer cell lines vary in their dependency on the NF- κ B pathway for their survival. Parthenolide, a NF- κ B inhibitor, was used in order to evaluate the utilization of NF- κ B pathway by various cancer cell lines for their survival.

The effect of *Ganoderma tsugae* var. *jannieae* W02 mushroom extract was compared on the one hand to that of parthenolide (PAR), which was used as a control, and on the other, to that of another active *Fomes fomentarius* mushroom extract, FF04. The latter was obtained after extraction of the culture liquid of *Fomes fomentarius* with ethyl acetate and also demonstrated a strong inhibitory effect on I κ B α phosphorylation in the luciferase reporter gene assay (data not shown).

Five different cancer cell lines, MCF7, Baf3/p185 Bcr-Abl, 9L, DU145, and PC3 were treated with increasing concentrations of parthenolide (1, 5, 10, and 20 μ M) and with the selected mushroom extracts. For each cell line, independently, the IC₅₀ values of parthenolide were determined as well as the IC₅₀s of each extract tested (Fig. 24). Consequently, the effectiveness of the fungal extracts on each cell line was considered relative to the effect of parthenolide on the same cell line. Due to the fact that different cancer cells exert different stability, the IC₅₀ values obtained for parthenolide as well as for the extracts varied. However, in all cell lines used, except MCF7, W02 extract showed stronger effect on cell viability than the FF04.

Results showed that the laboratory model of CML, Baf3/p185 Bcr-Abl, was the most sensitive cell line to parthenolide, with the lowest IC_{50} (3.2 μ M or 0.79 μ g/mL). This cell line appeared to be the most sensitive also to the two extracts tested (**Fig. 24**). Among the other tested cell lines, PC3, androgen-independent cancer cell line, appeared to be the least sensitive to parthenolide, followed by DU145, androgen-independent cancer cell line, 9L, rat glioblastoma cell line, and MCF7, breast cancer cell line. However, considering the extracts' effects, W02 showed a stronger activity in all the cell lines used, except in MCF7 with IC_{50} of 220 μ g/mL. In 9L cell line the two extracts exhibited almost equal effects (IC_{50} of 120 μ g/mL for FF04, and IC_{50} of 117 μ g/mL for W02), while in Baf3/p185 Bcr-Abl, DU145, and PC3, W02 appeared to be much more active with IC_{50} s of 35, 78, and 110 μ g/mL, respectively. These results suggest that W02 might affect the cell viability in a similar way to that of parthenolide, although it appeared to be less effective.

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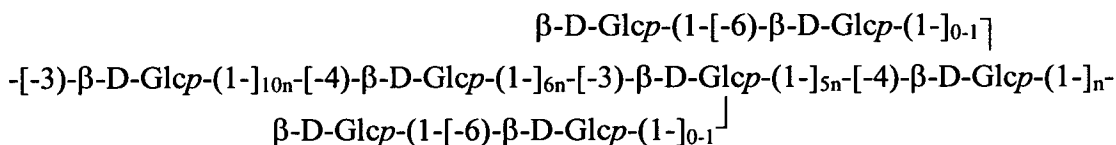
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CLAIMS:

1. A β -glucan composed of a backbone structure of β -1-3- and β -1-4-linked D-glucopyranose residues bearing, at some of the 6-positions, side chains of β -1-6-D-glucopyranose residues, of the structure:



wherein Glcp represents a glucopyranose residue and n is an integer equal to or larger than 1.

2. The β -glucan of claim 1 having a molecular weight of less than 10,000 Da.
3. The β -glucan of claim 2 having a molecular weight of about or less than 5,000 Da.
4. The β -glucan of claim 1, obtained from *Ganoderma tsugae* var. *janniae*.
5. The β -glucan of claim 1, obtained from dried fruiting bodies of *Ganoderma tsugae* var. *janniae* strain Tay-1 CBS 120394.
6. The β -glucan of claim 1, obtained from submerged cultivated mycelium of *Ganoderma tsugae* var. *janniae* strain Tay-1 CBS 120394.
7. A β -glucan having a molecular weight of 5,000-10,000 Da or less, composed of a backbone structure of β -1-3- and β -1-4-linked D-glucopyranose residues bearing, at some of the 6-positions, side chains of β -1-6-D-glucopyranose residues, which in a dried state is a white odorless powder highly soluble in water producing a brown aqueous solution at high concentrations.

8. The β -glucan of claim 7, obtained from dried fruiting bodies or from submerged cultivated mycelium of *Ganoderma tsugae* var. *janniae* strain Tay-1 CBS 120394.
9. A composition comprising a β -glucan as defined in any of claims 1 to 8.
10. The composition of claim 9, for use as a nutraceutical composition.
11. The composition of claim 10, formulated in an oral solid dosage form.
12. The composition of claim 11, wherein said oral solid dosage form is selected from a fine powder, capsules, tablets, caplets, and sachets.
13. The composition of claim 10, formulated into a food products.
14. The composition of claim 10, formulated into a pet food products.
15. The composition of 10, formulated into a beverage products.
16. The composition of 15, formulated into an energizing beverage products.
17. The composition of claim 10, formulated into a cosmetic products.
18. The composition of claim 17, wherein said cosmetic product is selected from an aqueous solution, a gel, a cream, a paste, a lotion, a spray, a suspension, a powder, a dispersion, a salve or an ointment.
19. The composition of any one of claims 9 to 12, for use as a dietary supplement.

20. The composition of claim 10, for stimulation of the immune system.
21. The composition of claim 20, for use as adjuvant to chemotherapy or radiotherapy of tumors.
22. The composition of claim 20, for prevention and treatment of bacterial, parasitic or viral infections.
23. The composition of claim 20, for treatment of human immunodeficiency virus (HIV) infection.
24. The composition of claim 9, for use in treatment of an NF- κ B-dependent diseases.
25. The composition of claim 24, wherein said NF- κ B-dependent disease is selected from cancer, immunological disorders, septic shock, transplant rejection, radiation damage, reperfusion injuries after ischemia, arteriosclerosis, osteoporosis, and neurodegenerative diseases.
26. The composition of claim 10, for lowering levels of total cholesterol and LDL cholesterol.
27. The composition of claim 26, for prevention of cardiovascular diseases.
28. The composition of claim 10, for control of blood sugar levels and reduction of glycemic index in Type II diabetic patients.