

NEW *GANODERMA TSUGAE* VAR. *JANNIEAE* STRAIN TAY-1 AND
BIOLOGICALLY ACTIVE BIOMASS AND EXTRACTS THEREFROM

FIELD OF THE INVENTION

The present invention relates to medicinal mushrooms, more particularly to *Ganoderma* mushrooms and to a new and distinct strain of higher Basidiomycetes designated *Ganoderma tsugae* var. *jannieae* Tay-1. The invention further relates to fruiting bodies, submerged cultivated mycelial biomass and extracts from *Ganoderma tsugae* var. *jannieae* Tay-1 comprising various biologically active compounds and their use as dietary supplements, cosmeceuticals, and in the therapy of several diseases and conditions.

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

Higher Basidiomycetes mushrooms contain a large amount of polysaccharides, especially β -glucans. The literature (Chen and Seviour, 2007) suggests β -glucans are affective 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. 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, 2002; Zhang et al., 2005; Chen and Seviour, 2007).

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 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.

10 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” or “nutraceuticals”.

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.

5 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 and also benefit to conservation of
10 biodiversity; (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
15 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

20 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
25 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
30 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).

Some mushrooms have been found to possess an antioxidant activity due to the presence of antioxidant agents such as flavonoids, carotenoids and phenolic compounds. They can thus be used as antioxidant supplements in the human diet for preventing or reducing oxidative damage caused by oxidative stress reactions.

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, Tc cells, B cells, macrophages, dendritic cells, and natural killer (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, and 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, 2002; Chang and Buswell, 2003). For example, preparations containing melanin may be used for skin protection against UV solar radiation,

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 *Ganoderma 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 (2002) 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 various species of the 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 (2002) 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 comprised protein-containing glucogalactans associated with mannose and fucose, and water-insoluble fractions comprised 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 and 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 homoglycan-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 material of *G. tsugae*. It was then identified as a new variety of the latter taxon and designated *Ganoderma tsugae* var. *jannieae*.

Reference is made to the patent application entitled "Novel β -glucan isolated from *Ganoderma tsugae* var. *jannieae*", filed on the same date by same applicant and mentioning the same inventors as in the present application.

SUMMARY OF THE INVENTION

In one aspect, the present invention relates to a new and distinct strain of *Ganoderma* mushroom, *Ganoderma tsugae* var. *jannieae* strain Tay-1, herein identified as the strain Tay-1, that has been deposited as 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 was assigned the No. CBS 120394.

In another aspect, the present invention relates to a biomass of the *Ganoderma tsugae* var. *jannieae* strain Tay-1 rich in nutraceutical agents and biologically active substances including proteins rich in essential amino acids and carbohydrates and further comprising vitamins, lipids rich in essential fatty acids, minerals, melanin, antioxidants and other biologically active agents. The biomass can be obtained from the submerged cultivated mycelium or the fruiting bodies of *Ganoderma tsugae* var. *jannieae* strain Tay-1.

In a further aspect, the present invention relates to extracts from the *Ganoderma tsugae* var. *jannieae* strain Tay-1 rich in nutraceutical agents and biologically active substances. The extracts can be obtained from the mycelium or the fruiting bodies of *Ganoderma tsugae* var. *jannieae* strain Tay-1.

In still another aspect, the invention relates to compositions comprising the biomass or a freeze dried extract from the *Ganoderma tsugae* var. *jannieae* strain Tay-1 for use as nutraceuticals or dietary supplements, for skin protection 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 µm

Fig. 4 shows general methodology for the production of submerged cultured mycelium of *Ganoderma tsugae* var. *jannieae* in fermentor or in 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 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. *jannieae* strain Tay-1 CBS 120394 after 5 days cultivation. These pellets are homogenized and used as inoculums for culturing in a 2 L Erlenmeyer flask.

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

Fig. 7 shows homogenized mycelial pellets of biomass of *Ganoderma tsugae* var. *jannieae* 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 depicts the ^1H NMR spectrum of purified β -glucan from *Ganoderma tsugae* var. *janniae* strain Tay-1 mushroom (D_2O , 40°) (ppm, parts per million).

Fig. 13 depicts the ^1H NMR spectrum of the mixture of the mannan, galactan, and glucan (void volume fraction of Sephadex G50 size exclusion chromatography).

Fig. 14 shows UV absorption spectrum of 0.001% melanin of *Ganoderma tsugae* var. *janniae* strain Tay-1.

Fig. 15 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 transfected MCF7 breast cancer cell line. Controls: PAR (parthenolide), an inhibitor of $\text{I}\kappa\text{B}\alpha$ phosphorylation, and MG132, an inhibitor of $\text{I}\kappa\text{B}\alpha$ degradation.

Fig. 16 shows the effect of different concentrations of the extract *Ganoderma tsugae* var. *janniae* strain Tay-1 W02 on MCF7 cancer cell viability.

Fig. 17 shows a Western blot analysis of SDS-PAGE separated cancer line proteins specifically probed with anti- $\text{I}\kappa\text{B}\alpha$, anti-p $\text{I}\kappa\text{B}\alpha$, 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 $\text{I}\kappa\text{B}\alpha$ degradation after 10 min $\text{TNF-}\alpha$ stimulation. Extracts were applied

to cancer cell lines for 10 min and the effect was compared to the effect of 10 min 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. 18 shows a Western blot analysis of SDS-PAGE separated cancer line proteins specifically probed with anti-pI κ B α antibodies, showing the effects of mushroom extracts *Ganoderma tsugae* var. *janniae* strain Tay-1 W02 and *Schizophyllum commune* SC01 on I κ B α phosphorylation in comparison with 5 min TNF- α stimulation. Effect of the extracts is 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. 17**.

Fig. 19 shows the effects of mushroom extracts *Ganoderma 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

The present invention is directed to a new strain of medicinal mushroom *Ganoderma tsugae* var. *janniae*, namely *Ganoderma tsugae* var. *janniae* strain Tay-1 deposited under the Budapest Treaty with Centraalbureau voor Schimmelcultures (CBS) as Accession No. CBS 120394.

In one aspect, the present invention relates to a biomass of the *Ganoderma tsugae* var. *janniae* strain Tay-1 of claim 1 rich nutraceutical agents and biologically active compounds including proteins rich in essential amino acids and carbohydrates and further comprising vitamins, lipids rich in essential fatty acids, antioxidant agents, minerals, and melanin. The biomass also contains triterpenoids.

In one embodiment, the biomass is obtained from the mycelium of the *Ganoderma tsugae* var. *jannieae* strain Tay-1. The mycelial biomass may be obtained by cultivation of the strain in submerged culture on nutrient media.

In another embodiment, the biomass is obtained from the fruiting bodies of the *Ganoderma tsugae* var. *jannieae* strain Tay-1.

The mycelial biomass has about 50% carbohydrates and about 37% proteins of the dry weight of mycelium, and the fruiting bodies biomass has about 80% carbohydrates and about 16% proteins of the dry weight.

The carbohydrates in the biomass include both polysaccharides and monosaccharides.

Examples of polysaccharides present in the biomass include β -glucan, galactans, mannan, and chitin. As mentioned before, β -glucans have antitumor and immunomodulating, particularly immunostimulatory activities, and the presence of the polysaccharides makes the biomass of the new strain subject of the present application promising as a herbal medicine to prevent and treat diseases and conditions in which strengthening of the immune system is important, such as to prevent and treat cancer, viral diseases such as AIDS, diabetes, heart diseases, blood pressure, and as hypocholesterolemic agents to treat high cholesterol conditions. Chitin, also present in the biomass, is an important constituent of dietary fibers.

The monosaccharides found in the mycelial biomass include glucose, arabinose, xylose, mannose, galactose and glucosamine. The fruiting bodies monosaccharides apparently lack galactose, but further include rhamnose. All these monosaccharides are important for the health. In addition, mannose has been shown to prevent the adhesion of bacteria to tissues of the urinary tract and bladder, and glucosamine is known for treatment of osteoarthritis and to rebuild cartilage.

The mycelial biomass proteins are rich in alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, leucine, isoleucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine and valine. The fruiting-body biomass proteins consist of the same set of amino acids but lacks methionine. Thus, the proteins of mycelium contain 8, and those of fruiting body contain 7, out of the

10 essential amino acids; threonine, valine, isoleucine, leucine, histidine, lysine, and arginine. The biomass of the present invention thus constitutes an important dietary supplement due to the presence of the proteins rich in essential amino acids.

Vitamins found in the mycelial biomass include the vitamins B₁, B₂, B₃, B₅,
5 B₉, B₁₂, C, Q₉, pro-A- β -carotene, and pro-D-ergosterol. The fruiting-body biomass vitamins comprise B₁, B₂, B₅, B₉, C, and carotenoids. Deficiency in vitamins in the ordinary diet may cause a large variety of symptoms that may be treated with food additives containing these vitamins. Since *Ganoderma tsugae* var. *janniae* biomass and extracts contain high levels of important vitamins they may be used as
10 nutraceuticals and/or may be added to food and beverage products as dietary supplements and serve as an excellent source of vitamins.

The mycelial biomass comprises lipids including the fatty acids palmitic acid, oleic acid, linoleic acid, stearic acid and palmitoleic acid. The fruiting-body biomass comprises lipids including the fatty acids palmitic acid, oleic acid, linoleic
15 acid, stearic acid and elaidic acid. The fatty acids are found in the mushroom in the form of their esters with glycerol. A high nutritional quality of the mushroom is made evident by the presence of the essential unsaturated fatty acids palmitoleic acid, elaidic acid and linoleic acid. The latter gives rise to the omega-6 series of polyunsaturated fatty acids, which incorporation into phospholipids affect cell
20 membrane properties such as fluidity, flexibility, permeability and the activity of membrane bound enzymes.

The mycelial biomass comprises minerals, both macroelements and microelements, including aluminum, copper, iron, potassium, magnesium, manganese, phosphorus, silicon, sodium, titanium and zinc. The fruiting-body
25 biomass comprises similar minerals but is richer in calcium and lead. Iron deficiency (or "sideropenia") is the most common known form of nutritional deficiency and may cause anemia with symptoms including fatigue, pallor, irritability and weakness. A daily dose of *Ganoderma tsugae* var. *janniae* biomass endows an excellent source of iron and other minerals.

As shown herein in the examples, the mycelial and fruiting-body biomasses of the strain of the invention comprise melanin, phenolic compounds, anti-oxidant agents and free-radical scavenging agents. Melanin confers protection against photo-aging of the skin, particularly protects the skin from solar UV radiation, and also protects against damage to internal organs caused by ionizing radiation, and may serve to sequester potentially toxic metal ions through its carboxylate and phenolic hydroxyl groups. Phenolic compounds are known to possess anti-oxidant activity.

In another aspect, the present invention is directed to a process for producing a biomass rich in vitamins, polysaccharides, monosaccharides, proteins, essential amino acids, essential fatty acids, minerals and microelements from higher Basidiomycetes mushroom *Ganoderma tsugae* var. *jannieae* strain Tay-1 (CBS 120394), said process comprising: cultivating the mushroom *Ganoderma tsugae* var. *jannieae* strain Tay-1 (CBS 120394) in submerged culture on nutrient media, isolating the resulting biomass of medicinal mushroom from the culture broth, and drying and grinding said biomass into fine powder. In one preferred embodiment, the nutrient media used for cultivating the mushroom is of the following composition (g/L of distilled water): glucose, 15.0-25.0; peptone, 3.0; yeast extract, 3.0; KH_2PO_4 , 1.0; K_2HPO_4 , 0.2; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5.

The invention further relates to a pure submerged mycelial culture of *Ganoderma tsugae* var. *jannieae* strain Tay-1.

In yet another aspect, the instant invention is directed to a composition comprising a biomass rich in nutraceutical agents and biologically active substances obtained from the mycelium or from the fruiting bodies of *Ganoderma tsugae* var. *jannieae* strain Tay-1 CBS 120394.

In one embodiment, the composition is formulated in an oral solid dosage such as, but not limited to, a fine powder, capsules, tablets, caplets, and sachets.

The composition for oral administration may be used as a nutraceutical composition as a dietary supplement, vitamin supplement, dietary fiber supplement, protein supplement, amino acid supplement, fatty acids supplement, and mineral

and microelement supplement. Due to the presence of the nutraceutical and biologically active polysaccharides, these compositions have other activities including immunostimulatory and hypocholesterolemic effects.

In another embodiment, the composition or the biomass may be incorporated/formulated into a food product for human consumption, e.g. cereals, ice cream and the like or into a pet food products, e.g. dog and cat food products.

A composition comprising the biomass may also be formulated into a cosmetic product such as an aqueous solution, gel, cream, paste, lotion, spray, suspension, powder, dispersion, salve or ointment. The presence of melanin makes the cosmetic product beneficial for protecting the skin from solar UV radiation and photoaging.

In another aspect, the instant invention is directed to a *Ganoderma* extract
5 having biological activity comprising an extract of *Ganoderma tsugae* var. *jannieae* strain Tay-1 Accession No. CBS 120394.

In one embodiment, the extract is obtained from the fruiting bodies of *Ganoderma tsugae* var. *jannieae* strain Tay-1, for example by extraction with an aqueous solvent, preferably hot water.

10 In another embodiment, the extract is obtained from the mycelium of *Ganoderma tsugae* var. *jannieae* strain Tay-1, preferably from a pure submerged mycelium culture, which is concentrated, dried, and ground, the resulting fine powder is subjected to organic or aqueous solvent extraction and the solvent is removed by freeze-drying. About 10 g dried biomass is obtained from 100 g wet
15 biomass, which results in about 7g freeze-dried extract. The organic solvent may be ethyl alcohol, ethyl acetate and diethyl ether, or a mixture thereof. In preferred embodiments, the organic solvent is ethyl alcohol or ethyl acetate.

To obtain an extract rich in nutraceutical agents the biomass is preferably extracted with 40% ethyl alcohol or hot water. The extracts resulting from
20 extraction of the biomass with ethyl alcohol or water differ in their nutraceutical agent composition. These extracts may be used separately or in combination to obtain different compositions of nutrients and biologically active agents.

To obtain an extract having high anti-oxidant and free radical scavenging activity the biomass is preferably extracted with culture liquid (water).

According to another aspect, the present invention relates to a composition having biological activity comprising an extract of *Ganoderma tsugae* var. *jannieae* strain Tay-1 Accession No. CBS 120394.

The composition may contain an organic solvent extract, an aqueous extract, or both. When a mixture of both organic and aqueous extracts is used, it may comprise equal or unequal parts of the different extracts.

As defined above for the biomass, the composition comprising the freeze-dried extract may be used alone or the composition or freeze-dried extract may be incorporated into human food products, pet food products and cosmetic products.

The freeze-dried extract is completely water-soluble and may be dissolved in an aqueous solution providing nutrient-loaded energy drinks that can be packaged in bottles or cans. The inventions thus further provide beverages, particularly energizing beverages, comprising an extract of *Ganoderma tsugae* var. *jannieae* strain Tay-1 Accession No. CBS 120394, and possibly other ingredients and additives.

As shown herein in the examples, the biological activity of *Ganoderma tsugae* var. *jannieae* strain Tay-1, particularly of an extract thereof, include a nuclear factor κ B (NF- κ B) pathway modulating activity and/or anti-oxidant activity and/or free radical scavenging activity and/or anti-radiation activity and/or metal ion scavenging activity. Thus, it may be used for treatment of an NF- κ B-dependent disease, such as cancer (e.g., breast cancer, lung cancer, chronic myelogenous leukemia, prostate cancer and glioblastoma), immunological disorders, septic shock, transplant rejection, radiation damage, reperfusion injuries after ischemia, arteriosclerosis and neurodegenerative diseases.

The invention further relates to a process for producing an *Ganoderma* extract comprising an extract of *Ganoderma tsugae* var. *jannieae* having biological activity, wherein the biological activity is nuclear factor κ B (NF- κ B) pathway modulating activity and/or anti-oxidant activity and/or free radical scavenging

activity and/or anti-radiation activity and/or metal ion scavenging activity, and the process comprises: cultivating mycelium of *Ganoderma tsugae* var. *jannieae* strain Tay-1 (CBS 120394) in submerged culture on nutrient media, isolating the resulting biomass of mushroom from the culture broth by drying and grinding said biomass into fine powder which is subjected to solvent extraction and freeze drying.

All *Ganoderma* mushrooms contain triterpenoids that are known to possess the bioactivities of antioxidation, hepatoprotection, cholesterol stasis, anti-hypertension and inhibiting platelet aggregation due to the inhibition of enzymes such as β -galactosidase, cholesterol synthase, angiotension-converting enzyme, etc.

Recently, triterpenoids isolated from *Ganoderma* spp. were reported to exhibit cytotoxic activity against tumor cells (e.g. Lin et al., 2003). Although triterpene content in *Ganoderma tsugae* var. *jannieae* strain Tay-1 was not measured in the instant application, there is no reason to assume that this strain does not contain significantly different levels of triterpenoids compared to other *Ganoderma* mushrooms described in the prior art.

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 orthopleuropodal (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 μ m 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 skeletals 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 (reminding 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 2.4–3.2 x (4.0)6–10(14) μm and a lot of misshaped thick-walled vacuolarized cells 19.2–28.8 x 7.2–9.6 μm 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 cuticular 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. *janniae* 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. *janniae* 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 (pO₂), and foam.

For the first pre-inoculums culture, 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; KH₂PO₄, 1.0; K₂HPO₄, 0.2; MgSO₄·7H₂O, 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 inoculums culture the biomass from the first pre-inoculums 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 inoculums 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. *jannieae* CBS 120394 after 7 days of fungus cultivation are depicted in Fig. 11.

Table 1. Mycelial biomass production of *Ganoderma tsugae* var. *jannieae* 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

Time, h	pH	DO, %	Aeration, v/v/min	RPM	Biomass, g/l
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 of *Ganoderma tsugae* var. *janniae* strain Tay-1

5 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, 10 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.

15 **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), 20 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 25 (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 NaBH_4 (~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 (Ac_2O) (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 Me_2SO (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 (NaBD_4), 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 Ac_2O (10 mL) and H_2SO_4 (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. Powdered dry fruiting bodies of *Ganoderma tsugae* var. *jannieae* (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%).

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.

10 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
15 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.

20 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
25 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
30 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 β -glucan had low molecular-weight centered at 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. 12**, and of the fraction of the void volume containing mannan, galactan, and glucan (**Fig. 13**), show the different compounds present in the two fractions.

Example 4. Isolation 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 H_2SO_4 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 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. However, the amount of 4-substituted glucose was significantly lower in the glucan extracted from mycelium, and the content of 6-substituted glucose was higher. Other polymers included α -1-6-galactan and mannan.

Results Monosaccharide analysis (acid hydrolysis and gas chromatography of alditol acetates) of the cells of the fruiting bodies and mycelium of *Ganoderma tsugae* var. *janniae* strain Tay-1 was performed in different hydrolysis conditions:

5 (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.

10 The results in **Table 2** 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

15 concentration of the monosaccharides, which can be even higher.

The overall glucose content is lower in the mycelium comparing to fruiting bodies of *Ganoderma tsugae* var. *janniae* strain Tay-1, but glucosamine contents is higher. The results for galactose are unusual in that its concentration in fruiting bodies is very low, or it seems to be absent.

20

Table 2 . Relative amount (%) of monosaccharide of the fruiting bodies and mycelium of *Ganoderma tsugae* var. *janniae* 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

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 the 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 centered at 5000 Da, similar to that of the β -glucan isolated from the fruiting bodies. Some of the β -glucan was probably lost during dialysis.

As with the fruiting body in Example 3, 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, with α -fucose branches at O-2.

Example 5. General chemical composition of *Ganoderma tsugae* var. *jannieae* strain Tay-1

The chemical composition of *Ganoderma tsugae* var. *jannieae* strain Tay-1 was determined in the mushroom fruit bodies and mycelium. The following contents were examined: lipids, vitamins, polysaccharides, monosaccharides, amino acids, fatty acids and minerals.

Our results demonstrate that mushroom fruit bodies and mycelium have significantly different biochemical compositions. The results also show that the mushroom is rich in most of the nutritionally important components of a well-balanced diet.

5 *Ganoderma tsugae* var. *jannieae* strain Tay-1 is rich in important nutraceutical agents and the biomass or extract from the mushroom may be formulated in oral dosage forms for administration as dietary supplements for a well-balanced diet or for clinical purposes. Due to FDA and FTC regulations, nutraceutical compositions cannot be labeled for clinical or medical purposes, but
10 they can indicate the contents of the composition regarding vitamins, minerals, protein, dietary fiber, and potassium. For example, according to the FDA's Food Labeling Guide--Appendix B, "High", "Rich In", or "Excellent Source Of" is defined as products that contain 20% or more of the Daily Value (DV) to describe protein, vitamins, minerals, dietary fiber, or potassium per reference amount. "Good
15 Source of", "Contains" or "Provides" is defined as products that contains 10%-19% of the DV per reference amount. "More", "Added", "Extra", or "Plus" is defined as products that contains 10% or more of the DV per reference amount.

The daily intake of medicinal mushrooms routinely used in North America, Europe and the Far East is in the range of 3-9 g dry biomass which is equivalent to
20 30-90 g wet biomass and approximately 2.1-6.3 g mushroom extract. Thus, the results below show that a similar dosage of *Ganoderma tsugae* var. *jannieae* strain Tay-1 fruiting bodies or mycelial biomass or extract would bestow the consumer with high levels of a range of important nutrients.

25 **Material and Methods** For each of fruit bodies and mycelia, three samples were used for the determination of every quality attribute. The experimental data were subjected to an analysis of variance for a completely random design to determine the least significant difference among means at the level of $\alpha=0.05$.

The proximate compositions of fruit bodies and mycelia of *Ganoderma tsugae*
30 var. *jannieae*, were determined according to the methods of AOAC (1990) (James,

1995). The biomass of the fruiting bodies and mycelium was prepared by concentrating, drying, and grinding the mushroom tissues.

The protein was determined by the method of Lowry et al. (1951). Carbohydrates - was determined by the method of Dubois et al. (1956) and
5 Varbanets et al. (2006).

5.1 Vitamins Deficiency in vitamins in the ordinary diet may cause a large variety of symptoms that may be treated with food additives containing these vitamins. *Ganoderma tsugae* var. *janniae* biomass and extracts contain high levels of some
10 vitamins of importance for the individual's health, such as vitamins B₁ (thiamine), B₂ (riboflavin), B₃ (niacin), B⁵ (pantothenic acid), B⁹ (folic acid), B₁₂ (cobalamin), C (ascorbic acid), Pro-A (beta-carotene), Pro-D (ergosterol) and Q₉ (ubiquinone).

The contents of vitamins in the fruit bodies and in the mycelium are shown in **Tables 3**. Vitamins are more actively accumulated in mycelium, while the fruit
15 bodies contain considerably less vitamins than mycelium (except for B₂). The amount of B₅ is 39.6 times less in fruit bodies, vitamin C is 12 times less, and B₁ is 2.9 times less than in mycelium. Vitamin B₁₂ is relatively abundant in mycelium (0.2 mg/100 g) and almost absent from fruit bodies (only trace amounts). Beta-carotene (a precursor of vitamin A) also dominates in mycelium: found in amounts
20 greater than 2.6 times that of fruit bodies. The only exception from this tendency is vitamin B₂, which is present in fruit bodies at 2.7 times higher levels than in mycelium. Nicotinic acid (niacin) was not found in the mushroom fruiting body.

25 **Table 3. Vitamins content of submerged mycelia biomass and fruiting bodies**

Vitamins		Mycelium	Fruiting bodies
		Content, mg/100 g dry weight	
Vitamin B ₁	Thiamine	5.22 ± 0.06	1.79±0.12
Vitamin B ₂	Riboflavin	2.22 ± 0.35	5.98±0.19
Vitamin B ₃	Niacin	34.17 ± 1.67	21.15±1.17
Vitamin Q ₉	Ubiquinone	4.75 ± 0.20	2.65±0.15
Vitamin B ₁₂	Cyancobalamin	0.2 ± 0.001	Trace amounts
Vitamin B ₅	Pantothenic acid	198.0 ± 10	5.0±0.1
Vitamin B ₉	Folic acid	0.082 ± 0.01	0.049±0.002
Vitamin Pro-A	Beta-carotene	0.56 ± 0.02	Trace amounts
Vitamin C	Ascorbic acid	68.7 ± 1.37	5.71±0.14
Vitamin Pro-D	Ergosterol	5.91 ± 0.24	4.18±0.17
Carotenoids			0.21±0.01

In summary, the fruiting bodies and mycelium of *Ganoderma tsugae* var. *jannieae* strain Tay-1 contain high amounts of important vitamins, and thus, a daily dose of biomass or extracts of this mushroom could provide an essential contribution to the daily requirement of these vitamins.

5.2 Carbohydrates and total protein With regard to the total polysaccharide and protein contents, it is shown in Table 4 that mycelium accumulates 50% of carbohydrates, while fruit bodies contain much more, namely 80% of their dry weight. On the contrary, proteins make up 37% of dry weight of mycelium, and only 16% of fruiting bodies (2.3 times less).

Besides β -glucan, galactan and mannan (see Examples 3 and 4 above), another polysaccharide, chitin, is present in fruit bodies in considerably greater amounts than in mycelium. We found 18.4 % dry weight of chitin in the fruiting bodies and 8.9% chitin dry weight in cultivated mycelium (not shown).

Table 4. Total polysaccharides and protein content

	Mycelium	Fruiting bodies
	Content, % of dry weight	
Carbohydrates	50.0	80.0
Protein	37.0	16.0

With regard to monosaccharides present in *Ganoderma tsugae* var. *janniae* strain Tay-1, three have high nutritional value: glucose, mannose and glucosamine.

Monosaccharide composition, both of neutral and charged monosaccharides, is not very different in fruiting bodies (Table 5) and mycelium (Table 6). The dominating monosaccharide in both is glucose (16.25% in fruit bodies and 16.61% in mycelium). Arabinose, xylose, and mannose are present in both preparations, but in greater amounts in fruit bodies than in mycelium. Fruiting bodies also contain rhamnose and some unidentified monosaccharide.

Table 5. Monosaccharide composition of fruiting bodies

Monosaccharide	Exposure time	% quantity in a peak area of chromatogram	% of dry weight
Neutral monosaccharides			
Rhamnose	6.28	0.9	0.2
Arabinose	7.55	1.8	0.35
Xylose	9.07	4.8	0.9
Mannose	16.02	4.4	0.85
Glucose	19.02	84.7	16.25
Unidentified monosaccharide	15.47	3.4	0.65
Charged monosaccharide			
Glucosamine	8.66		3.25

Glucosamine is present in considerable amounts in both preparations. However, its quantity was measured by different methods, and it is hard to compare the data for mycelium and fruit bodies.

Table 6. Monosaccharide composition of submerged mycelia biomass

Monosaccharide	Exposure time	% quantity in a peak area of chromatogram	% of dry weight
Neutral monosaccharides			
Rhamnose	-	-	-
Arabinose	7.53	6.72	0.85
Xylose	9.03		0.79
Mannose	15.94	9.68	2.8
Galactose	17.34	0.85	0.85
Glucose	18.8	61.77	16.61
Unidentified monosaccharide	-	-	-
Charged monosaccharide			
Glucosamine	8.71	8.66	6.46

5.3 Amino acid composition Analysis of amino acids shows that 17 amino acids are found in mycelium and 15 in fruit bodies (**Table 7**). The fruit bodies lack cysteine and methionine. In each preparation certain amino acids clearly dominated. The biomass of fruit bodies is rich in tyrosine (38.8% from the whole protein content, which makes 6.21 grams in 100 grams of biomass). The four most abundant amino acids in the mycelium biomass are glycine (12%), glutamic acid (11.3%), aspartic acid (9.4%), and alanine (8.9%). All amino acids, except for tyrosine, are accumulated in mycelium in greater quantities than in fruit bodies.

Essential amino acids that cannot be synthesized de novo by the organism and therefore must be supplied in the diet include the following ten amino acids: histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, arginine, and valine. In addition, the amino acids cysteine, glycine and tyrosine are considered conditionally essential, meaning they are not normally required in the diet, but must be supplied exogenously to specific populations that do not synthesize it in adequate amounts.

The biological value of the analyzed mushroom proteins is extremely high; The proteins of mycelium contain 8 out of 10 essential amino acids: threonine, valine, isoleucine, leucine, methionine, histidine, lysine, and arginine. The protein

of the mushroom fruiting body, lacking methionine, contains seven essential amino acids, all of them in lower quantities than in mycelium.

Table 7. Amino acid composition of submerged mycelia biomass and fruiting bodies

Amino acid	Mycelium		Fruiting body	
	% of all proteins	% of dry weight	% of all proteins	% of dry weight
Alanine	8.9	3.29	6.5	1.04
Arginine	5.2	1.92	1.2	0.19
Aspartic acid	9.4	3.48	6.8	1.09
Cysteine	0.2	0.7	-	-
Glutamic acid	11.3	4.18	4.8	0.77
Glycine	12.0	4.44	7.8	1.25
Histidine	5.7	2.11	6.4	1.02
Isoleucine	4.5	1.67	1.8	0.29
Leucine	7.0	2.59	4.8	0.77
Lysine	7.2	2.67	3.2	0.51
Methionine	0.1	0.44	-	-
Phenylalanine	4.8	1.78	2.9	0.46
Proline	2.9	1.07	1.2	0.19
Serine	6.4	2.37	6.7	1.07
Threonine	5.2	1.92	4.6	0.74
Tyrosine	2.6	0.96	38.8	6.21
Valine	6.6	2.44	2.5	0.40
Total	100	37	100	16

5.4 Lipid and fatty acid composition Fatty acids and, particularly, essential fatty acids such as the omega-6 fatty acids, play important roles in the organism. Palmitic acid is known to possess antioxidant properties and oleic acid is known to decrease the development of atherosclerosis and lower serum cholesterol by diminishing oxidative stress and inflammatory mediators while promoting antioxidant defenses. The fatty acids are usually present in the mushroom in the form of esters with glycerol (triglycerides).

Mycelium is richer in lipids: 10.2% versus 4.4±0.20% (w/w) in mushroom fruit bodies.

The fatty acids in the analyzed fruit bodies and mycelium preparations are both saturated (C_{16:0} palmitic acid and C_{18:0} stearic acid) and monounsaturated (C_{16:1} palmitoleic acid and C_{18:1} oleic acid) or diunsaturated (C_{18:2} oleic acid) fatty acids (as shown in Table 8).

5

Table 8. Content of fatty acids in submerged mycelia biomass and fruiting bodies of *Ganoderma tsugae* var. *janniae* strain Tay-1.

Fatty Acid	Mycelia	Fruiting body
	Content, % total methyl esters	
C14:0 Myristic	—	—
C16:0 Hexadecanoic acid (Palmitic acid)	16.87	11.4
C16:1 cis-9- Hexadecenoic acid (Palmitoleic acid)	4.23	—
C18:0 Octadecanoic acid (Stearic acid)	3.03	1.02
C18:1 cis-9- Octadecenoic acid (Oleic acid)	13.87	43.0
C18:1 trans-9- Octadecenoic acid (Elaidic acid)	—	28.13
C18:2 cis-9, cis-12- Octadienoic acid (Linoleic acid)	62.0	9.75
Unidentified	—	6.7
Total	100	100

Content derived from % quantity in a peak area of the chromatogram

The investigated mushroom synthesizes most usual fatty acids in the form of their triglycerides: C_{16:0} palmitine, C_{18:0} stearine, and C_{18:2}, linoleine. It also produces less abundant fatty acids such as C_{16:1}, palmitoleine and C_{18:1} elaidine. A high nutritional quality of the mushroom is made evident by the presence of unsaturated C_{18:2} linoleic acid, which belongs to the indispensable (essential) fatty acids.

As shown in Table 8, both quality and quantity of fatty acids differ between mushroom fruit bodies and mycelium. The fatty acids of fruit bodies are dominated by unsaturated C_{18:1} fatty acids, namely, cis-9-octadecenoic acid (oleic acid) (43.0%) and trans-9-octadecenoic acid (elaidic acid) (28.13%). Elaidic acid is specific to the fatty acid composition of fruit bodies.

Mycelium and fruit bodies differ in fatty acids also by the presence of C_{16:1} palmitoleic acid in mycelium (4.23%) and its absence in fruit bodies.

5.5 Mineral composition. The mineral nutrients are defined as all the inorganic elements or inorganic molecules that are required for life. As far as human nutrition is concerned, the inorganic nutrients include water, sodium, potassium, chloride, calcium, phosphate, sulfate, magnesium, iron, copper, zinc, manganese, iodine, selenium, and molybdenum. *Ganoderma tsugae* var. *janniae* strain Tay-1 fruiting body and mycelium are rich in most of these nutrients, and possess therefore a very high nutritional value.

Abnormally low mineral concentration may lead to an impairment in a function dependent on the mineral. Iron deficiency, for example, may cause iron deficiency anemia.

The investigated mushroom was analyzed for macroelements in oxides: silicon, titanium, aluminum, iron, manganese, magnesium, calcium, sodium, potassium, and phosphorus; other macroelements: sulfur and chlorine; and microelements nickel, copper, zinc, bromine, rubidium, lead, gallium, germanium, arsenium, selenium, strontium, yttrium, thorium, silver, mercury, cadmium, tin, indium, iodine, lanthanum, cerium, praseodymium, and neodymium.

The mineral composition is also different in the fruiting bodies and in the mycelium (Tables 9 and 10) preparations. In fruit bodies, silicon, magnesium, aluminum, iron, and titanium dominate.

Table 9. Mineral composition of submerged mycelia biomass and fruiting bodies of *Ganoderma tsugae* var. *janniae*

Mineral elements in oxides	Quantity, mg/100 g dry weight	
	Mycelium	Fruiting bodies
Al	11.6	177.8
Ca	0.7	7.9
Fe	160.2	254.6
K	406.0	57.3
Mg	1521.8	631.5
Mn	8.5	24.8
Na	53.4	24.5
P	15.3	1.83

Si	182.0	1020.4
Ti	79.7	165.4

Mycelium accumulates more magnesium, potassium, and sodium than the fruiting body. Manganese, calcium, phosphorus (Table 9), sulfur, chlorine, nickel, and copper (Table 10) are present in both preparations in lesser amounts. Some elements were found only in trace amounts or were below the sensitivity limit of the measurements.

Table 10. Microelements in submerged mycelia biomass and fruiting bodies of *Ganoderma tsugae* var. *janniae*

Microelements	Quantity, mg/100 g dry weight	
	Mycelium	Fruiting bodies
Ag	≥ 0.5 *	**
As	≥ 0.1 *	**
Cd	≥ 0.5 *	**
Ce	≥ 0.5 *	**
Cl	0.058	0.011
Cu	1.5	2.7
Ga	≥ 0.2 *	**
Ge	≥ 0.2 *	**
Hg	≥ 0.2 *	**
I	≥ 0.5 *	**
In	≥ 0.1 *	**
La	≥ 0.5 *	**
Nd	≥ 0.5 *	**
Ni	0.17	0.1
Pb	0.24	2.4
Pr	≥ 0.5 *	**
Rb	0.33	0.05
S	0.638	0.232
Se	≥ 0.1 *	**
Sn	≥ 0.5 *	**
Sr	≥ 0.1 *	0.78
Th	≥ 0.3 *	**
Y	≥ 0.1 *	**
Zn	5.6	5.5

* elements found only in trace amounts; ** elements outside the scope of measurements

Gallium, germanium, arsenic, selenium, strontium, yttrium, thorium, silver, mercury, cadmium, tin, indium, iodine, lanthanum, cerium, praseodymium, and neodymium were present as trace elements in the mycelium but were not detectable

in fruiting body (except strontium that accumulated more in the fruiting body).

Example 6. Antioxidant and free-radical scavenging activity of submerged cultured mycelium extract of CBS 120394 *Ganoderma tsugae* var. *jannieae* strain Tay-1

Ganoderma tsugae var. *jannieae* was grown in submerged conditions and screened for antioxidant (AA) and free-radical scavenging capacity. Three different solvents were used for extraction: ethanol, water (culture liquid) and ethyl acetate. The yield of extracts received from mushroom biomasses significantly depended on the solvent used.

Materials and Methods

6.1 Organisms and cultivation conditions. The inocula were prepared by growing fungi on a rotary shaker at 150 rpm in 250 ml flasks containing 100 mL of synthetic medium (g/L): glucose, 15.0; peptone, 3.0 g; KH_2PO_4 , 0.8; K_2HPO_4 , 0.2; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; and yeast extract 5.0. Initial pH of the media was 5.8-6.0. After 5-7 days of cultivation, mycelial pellets were harvested and homogenized with a Waring laboratory blender.

Submerged cultivation was carried out at 25-27°C on a rotary shaker (150 rpm) in 2L Erlenmeyer flasks containing 1L of the synthetic medium. The medium consisted of (g/L) glucose, 20.0; peptone, 5.0; KH_2PO_4 , 0.8; K_2HPO_4 , 0.2; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; yeast extract 5.0.

6.2 Biomass dry weight determination. After 8 days of submerged cultivation, mycelial biomass was harvested with filtration and dried at 50°C to a constant weight. The dried mycelia were milled to a powder form and freeze-dried as were the culture liquids.

6.3 Extraction. Three different solvents (culture liquids, ethanol, and ethyl acetate) were used to extract the antioxidant compounds from the mushroom mycelia in ascending polarity. At the first stage, the mycelium was extracted 3 h with culture liquid (1 g/10 mL) at 80°C (on a water bath). After extraction, insoluble compounds were separated by centrifugation at 6000 g, 15 min and the supernatant was filtrated through Whatman filter paper N 4. Filtrate were evaporated.

The residues after centrifugation were extracted during 3 h on a rotary shaker at 150 rpm at 27°C with ethanol (80%) and subsequently with ethyl acetate. After extraction, the solutions were centrifuged, filtrated, and the organic solvents in the extracts were evaporated.

6.4 Antioxidant Activity Assay. The antioxidant activity of the mushroom extracts was determined according to the β -carotene bleaching method described by Velioglu et al. (1998). A reagent mixture containing 1 mL of β -carotene (Sigma) solution (0.2 mg/ml in chloroform), 0.02 ml of linoleic acid (Sigma) and 0.2 mL of Tween 80 (Sigma), was evaporated to dryness under a nitrogen stream and 50 mL of oxygenated distilled water was added to the mixture. To determine antioxidant activity, 4.8 mL of reagent mixture and 0.2 ml of mushroom crude extracts with different concentrations (2-8 mg/mL) were added. Pure methanol or water (0.2 mL) was used as a control, whereas the blank contained all mentioned chemicals except β -carotene. All these mixtures were then incubated at 50°C for 2 h to form liposome solutions. The absorbance of an aliquot (1 mL) of these solutions at 470 nm was monitored by a spectrophotometer at 20 min time intervals. Butylated hydroxyanisole (BHA) (Sigma) (2 mg/ml in methanol) was used as the standard. The bleaching rate (R) of β -carotene was used as the standard and calculated according to Equation (1):

$$R = \ln (a/b)/t \quad \text{Equation (1)}$$

where: ln - natural log, a - absorbance at time 0, b - absorbance at time t, and t – incubation interval 20, 40, 60, 80, 100, or 120 min.

The antioxidant activity (AA) was calculated in terms of percent inhibition relative to the control, using Equation (2):

$$AA = [(R_{\text{control}} - R_{\text{sample}}) / R_{\text{control}}] \times 100 \quad \text{Equation (2)}$$

5 6.5 Scavenging activity on 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radicals.

The scavenging activity of the ethanol and water extracts from the mushroom on DPPH radicals was measured as follow: An aliquot of 0.5 ml of 0.1 mM DPPH radical (Sigma) in methanol was added to a test tube with 1 mL of mushroom methanol or water extract of different concentrations (1.5 to 9 mg/mL). Methanol or
10 water was used instead of the mushroom sample as a control. The reaction mixture was vortex mixed at room temperature and the absorbance (Abs) was determined immediately after mixing by measuring at 520 nm with a spectrophotometer. Inhibition of free radical by DPPH in percent (I %) was calculated in following way:

$$15 \quad I\% = (A_{\text{blank}} - A_{\text{sample}} / A_{\text{blank}}) \times 100 \quad (\text{Burits and Bucar, 2000})$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. The values of inhibition were calculated for the various concentrations extract.

20 Results

6.6 Extraction results

The extraction of antioxidant compounds from dried and milled submerged mushroom mycelia of *Ganoderma tsugae* var. *janniae* was performed with water (culture liquid), ethanol, and ethyl acetate.

25 Culture liquid (water) appeared to be the most appropriate solvent for the extraction. It solubilized 65.8% of the material from submerged mycelium of *Ganoderma tsugae* var. *janniae*. Extraction with ethanol was less effective than culture liquid and it showed 16.5% yield of extract. Finally, the extraction of residual biomass with ethyl acetate showed just 4.1%.

30

6.7 Antioxidant properties of *Ganoderma tsugae* var. *jannieae* extracts

In water extracts from *Ganoderma tsugae* var. *jannieae* we observed 40.7, 47.0, and 94.0% of antioxidant activity at concentration 2.0, 4.0, and 8.0 mg/mL, respectively. When the ethanol extracts from the *Ganoderma tsugae* var. *jannieae* biomasses were tested, 42.1, 50.8, and 59.3% antioxidant activity was observed at concentrations of 2.0, 4.0, and 8.0 mg/mL, respectively.

The comparison of the antioxidant potential of extracts obtained from mushroom biomasses with two different solvents shows that the water extract has the highest antioxidant activity at concentration 8.0 mg/mL.

The EC₅₀ values for *Ganoderma tsugae* var. *jannieae* was 4.3 and 3.7 mg extract/ml for water and ethanol extracts, respectively.

6.8 Free radical scavenging properties of *Ganoderma tsugae* var. *jannieae* extracts

Free radical scavenging is one of the known mechanisms by which antioxidants inhibit lipid oxidation. DPPH, a stable free radical with a characteristic absorption at 520 nm, was used to study the radical-scavenging effects of *Ganoderma tsugae* var. *jannieae* extracts. The water extract showed 66.3% of scavenging activity at the concentration 3 mg/ml. At low concentrations of water extract, scavenging activity increased with increasing concentrations and at 9 mg/ml it decreased (38.6%). When the ethanol extract from mushroom mycelia was evaluated, the activity increased from 21.9, 39.9, 50.0, to 65.9% at the 0.5, 1.5, 3.0, 9.0 mg/mL, respectively, and highest activity was shown at 9.0 mg/mL (65.9%).

This example shows that submerged mycelium of *Ganoderma tsugae* var. *jannieae* possesses high antioxidant activity and free radical scavenging potentials and indicates that it may serve as a good source for safe natural antioxidants.

Example 7. Analysis of biochemical composition of melanin and phenolic compounds of CBS 120394 *Ganoderma tsugae* var. *jannieae* strain Tay-1

Materials and Methods

7.1 Extraction of melanin. Melanin was extracted from mycelial biomass by hydrolysis in 2% NaOH solution for 2 hours in a boiling water-bath. After cooling the extract, pH was adjusted to 2 with concentrated HCl. The coagulated pigment was separated from the liquid by centrifugation for 15 minutes at 6000g. The precipitate was diluted in 2% NaOH and used for further melanin detection. The quantity of melanin was determined by measuring the adsorption at 490 nm in a spectrophotometer using a calibration plot. The absorption of the UV- and visible light of the 0.001 – 0,1% solutions of pigment was estimated using a spectrophotometer “Specord M-40” (Germany). IR-spectra of melanins in form of tablet with KBr was estimated using a spectrophotometer “Specord M-80” (Germany). The quantity of paramagnetic centers (PMC) was estimated using a radiospectrometer “Varion E-112” (USA) with Mn^{++} in powder MnO as standard.

7.2 Molecular weight was estimated by gel filtration on a sorbent Toyopearl HW (Japan) in 0.25% LiOH, at a flow speed of 0.5 mL/min. Photometrical detection was made at 490 nm and the molecular weight was rated using a calibration plot. A calibration curve was built according to dextrans – 40, 70, 500 and 2000 (Blue dextran) kDa (Fluka). To estimate the molecular mass of melanins the gel-filtration method was used. To determine the zero volume blue dextran (2000 kDa) was applied to the columns, and to estimate elution volumes corresponding to defined masses of molecules, dextrans with known molecular mass (40, 70, 500 kDa) were applied to the columns. Then melanins were applied to the columns and the elution volume was estimated for them. The molecular mass was determined against the calibrating plot.

7.3 Melanin pigments were identified by a qualitative reaction with $KMnO_4$, H_2O_2 , and $FeCl_3$.

7.4 Phenolic compounds were detected in the extracts, obtained by the following method. Submerged mycelium was finely ground, repeatedly frozen in liquid nitrogen and extracted exhaustibly by 70% ethanol for 30 minutes in a boiling water-bath. The extract was cooled until receiving a negative test for phenolic compounds, and then centrifuged for 15 minutes at 8000 rpm. Mono- and polyphenol yields were detected using the Folin-Denis chemical reagent (10% Na₂WO₄ (w/v), 2% phosphomolybdenic acid (w/v), 4.25% H₃PO₄ (w/w))

7.5 All spectral measurements (the absorption of the UV- and visible light) were estimated on a spectrophotometer (Specord M-40, Germany).

7.6 For polysaccharides detection, 100 mg of ground dry mycelium was placed into a 20-mL tube, 5 ml of 1 M NaOH was added, and the tube was tightly closed. Extraction was performed in a thermostat at 60°C for an hour, the mycelium was mixed several times during the incubation by inverting the tube. The extract was centrifuged for 20 minutes at 8000 rpm. The phenol-sulfurous method was used to detect and evaluate the polysaccharides obtained as follows: One ml of supernatant was placed into a 20-mL tube, 1 mL of 5% soluble phenol was added, and the supernatant was thoroughly mixed. Five ml of concentrated sulfuric acid was added quickly to the supernatant with constant, but slight, shaking. The solution was incubated for 10 min, mixed, and placed in a water-bath at 25°–30°C for 10–20 min. Measurements were taken using a spectrophotometer (wavelength of 490 nm) using 5 mm cuvettes. As a control, a mixture of 1 mL distilled water, 1 mL of 5% soluble phenol, and 5 ml of concentrated sulfuric acid was used.

Results

7.7 Physicochemical characteristics of the melanin pigment of CBS 120394 *Ganoderma tsugae* var. *jannieae* Strain Tay-1

In order to check if the pigments extracted from CBS 120394 *Ganoderma tsugae* var. *jannieae* strain Tay-1 belong to the melanin group, pigments were

subjected to several tests based on the physicochemical characteristics of melanin, as follows: solubility in alkali, and in concentrated H_2SO_4 , and HNO_3 ; color loss under the influence of H_2O_2 , $\text{Na}_2\text{S}_2\text{O}_4$, KMnO_4 and bromine water; and interaction with FeCl_3 .

5 The results showed that the pigment extracted from CBS 120394 could be dissolved in 0.1N NaOH. When potassium hypermanganate was added, the mixture changed color from brown to light brown, then to green, and finally became colorless and precipitated. The minimum quantity of KMnO_4 , required to make the mixture colorless, was 13.0 mmol/g melanin.

10 When 0.5 mg/ml FeCl_3 and 0.1N NaOH were added to the 0.01% alkali solution of the pigment, a flake-like precipitation was observed. After the addition of a saturating amount of FeCl_3 , the precipitate disappeared.

 Melanin content in the biomass was determined at 1.46% of dry weight. The molecular weight of the pigment complexes is in the range of 40 – 45 kDa, as
15 estimated by gel filtration,

7.8 Spectral characteristic of melanin pigment of CBS 120394.

 The UV spectrum of absorption of 0.001% melanin of *Ganoderma tsugae* var. *jannieae* Strain Tay-1 is depicted in Fig. 14. It has the shape of an inclined line,
20 which is typical for melanins of fungal origin.

 Thus, the study of *Ganoderma tsugae* var. *jannieae* strain Tay-1 pigment's behavior as described above allowed us to identify it as a true melanin.

7.9 Phenolic compound content in CBS 120394.

25 The biomass of CBS 120394 contained 2702–2903 mg phenolic compounds per 100 g absolutely dry biomass.

Example 8. *Ganoderma tsugae* var. *jannieae* extract as a potential modulator of the NF- κ B activation pathway

 The activity of the mushroom extract was tested *in vitro* in a human breast
30 cancer cell line.

Material and methods

8.1 Mushroom cultivation and biomass extraction. A pure mycelial culture of *Ganoderma tsugae* var. *janniae* 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 inoculumss 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.

8.2 Cell culture. The human breast cancer cell line MCF7 (ATCC, Rockville, MD, USA) was transfected with a NF-κB responsive reporter plasmid, NF-κB-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) a gift from Dr. J. Duyster, Germany), 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.

8.3 Luciferase reporter gene assay. Transfected MCF7 cells (4×10^4) were seeded in 200 µL of medium, using 96-well plates. After 24 h, mushroom extracts were added in several concentrations: 10, 25, 50, and 100 µg/mL. Control wells were: solvent-treated wells (1% of DMSO); parthenolide, an inhibitor of IκBα phosphorylation (Sigma-Aldrich, St Louis, MO, USA); and MG132, an inhibitor of IκBα 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 that of DMSO treated cells.

8.4 Cytotoxicity assay. To evaluate the effect of *Ganoderma tsugae* var. *janniae* 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 µg/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.

8.5 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
5 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
10 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
15 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
20 confirmed by Coomassie Brilliant Blue gel staining or by Ponceau Red membrane staining following the blotting.

8.6 Cell line sensitivity to parthenolide. A variety of cancer cell lines including MCF7 (breast cancer), Baf3/p185 Bcr-Abl (B-lymphocytes, a laboratory
25 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. *janniae* extracts, in the concentrations of 250, 125, 50, 25,
30 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 **8.7 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
10 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

15 **8.8 *Ganoderma tsugae* var. *jannieae* strain Tay-1 extracts inhibit NF- κ B-mediated reporter activity.**

 The extracts were evaluated for their ability to inhibit the reporter activity in the transfected MCF7 breast cancer cell line. The duplicate luciferase-reporter gene assay resulted in two (out of the four tested) *Ganoderma tsugae* var. *jannieae* strain
20 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. 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. The results are
25 depicted in **Fig. 15**.

8.9 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
30 evaluated by using three different concentrations, 125, 25, and 5 μ g/mL. As shown

in Fig. 16, the W02 extract, in all tested concentrations, was not toxic for the MCF7 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.

8.10 Effect of W02 *Ganoderma tsugae* var. *jannieae* 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. 17, 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).

5 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. 18).

10 8.11 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.

15 The effect of 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).

20 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. 19). Consequently, the effectiveness of the fungal extracts on each cell
25 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 IC₅₀ (3.2 µM or 0.79 µg/mL). This cell line appeared to be the most sensitive also to the two extracts tested (Fig. 19). 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 IC₅₀ of 220 µg/ml. In 9L cell line the two extracts exhibited almost equal effects (IC₅₀ of 120 µg/mL for FF04, and IC₅₀ of 117 µg/mL for W02), while in Baf3/p185 Bcr-Abl, DU145, and PC3, W02 appeared to be much more active with IC_{50s} of 35, 78, and 110 µg/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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CLAIMS

1. A new and distinct variety of higher Basidiomycetes medicinal mushroom *Ganoderma tsugae* var. *jannieae* strain Tay-1 deposited under The Budapest Treaty with the Centralbureau voor Schimmelcultures (CBS) under Accession No. CBS 120394.
2. A biomass of the *Ganoderma tsugae* var. *jannieae* strain Tay-1 of claim 1 rich in nutraceutical agents and biologically active compounds including carbohydrates and proteins rich in essential amino acids.
3. The biomass of claim 2, further comprising vitamins, lipids rich in essential fatty acids, antioxidant agents, minerals, and melanin.
4. The biomass of claim 2 or 3, obtained from the mycelium of the *Ganoderma tsugae* var. *jannieae* strain Tay-1.
5. The mycelial biomass of claim 4, obtained by cultivation of the *Ganoderma tsugae* var. *jannieae* strain Tay-1 in submerged culture on nutrient media.
6. The mycelial biomass of claim 4, wherein said biomass has about 50% carbohydrates and about 37% proteins of the dry weight of mycelium.
7. The mycelial biomass of claim 6, wherein said carbohydrates include polysaccharides and monosaccharides.
8. The mycelial biomass of claim 7, wherein said polysaccharides include soluble in water, low molecular-weight β -glucan, galactans, mannan, and chitin.
9. The mycelial biomass of claim 7, wherein said monosaccharides include glucose, arabinose, xylose, mannose, galactose and glucosamine.
10. The mycelial biomass of claim 6, wherein said proteins are rich in alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, leucine, isoleucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine and valine.
11. The mycelial biomass of claim 4, further comprising the vitamins B₁, B₂, B₃, B₅, B₉, B₁₂, C, Q₉, Pro-A- β -carotene, and pro-D-ergosterol.

12. The mycelial biomass of claim 4, further comprising lipids including the fatty acids: palmitic acid, oleic acid, linoleic acid, stearic acid and palmitoleic acid.

13. The mycelial biomass of claim 4, further comprising minerals
5 including aluminum, copper, iron, potassium, magnesium, manganese, phosphorus, silicon, sodium, titanium and zinc.

14. The mycelial biomass of claim 4, further comprising melanin.

15. The mycelial biomass of claim 4, further comprising phenolic compounds, anti-oxidant agents and free-radical scavenging agents.

10 16. The mycelial biomass of any one of claims 4 to 15, for use in nutraceutical compositions.

17. The biomass of claim 2 or 3, obtained from the fruiting body of the *Ganoderma tsugae* var. *janniae* strain Tay-1.

18. The fruiting body biomass of claim 17, wherein said biomass has
15 about 80% carbohydrates and about 16% proteins of the dry weight of the fruiting body.

19. The fruiting body biomass of claim 17, wherein said carbohydrates include polysaccharides and monosaccharides.

20. The fruiting body biomass of claim 19, wherein said polysaccharides
20 include soluble in water, low molecular-weight β -glucan, galactans, mannan, and chitin.

21. The fruiting body biomass of claim 19, wherein said monosaccharides include glucose, rhamnose, arabinose, xylose, mannose, and glucosamine.

25 22. The fruiting body biomass of claim 18, wherein said proteins are rich in alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, leucine, isoleucine, lysine, phenylalanine, proline, serine, threonine, tyrosine and valine.

23. The fruiting body biomass of claim 17, further comprising the vitamins B₁, B₂, B₅, B₉, C, and carotenoids.

24. The fruiting body biomass of claim 17, further comprising lipids including the fatty acids palmitic acid, oleic acid, linoleic acid, stearic acid and elaidic acid.

25. The fruiting body biomass of claim 17, further comprising minerals including aluminum, calcium, copper, iron, lead, magnesium, manganese, phosphorus, potassium, silicon, sodium, titanium and zinc.

26. The fruiting body biomass of claim 17, further comprising melanin.

27. The fruiting body biomass of claim 17, further comprising phenolic compounds, anti-oxidant agents and free-radical scavenging agents.

28. A composition comprising a biomass rich in nutraceutical agents and biologically active substances obtained from the mycelium or from the fruiting body of *Ganoderma tsugae* var. *jannieae* strain Tay-1 Accession No. CBS 120394.

29. A composition of claim 28, for use as a nutraceutical compositions.

30. The composition of claim 29, for use as a dietary supplement.

31. The composition of claim 30, for use as vitamin supplement, dietary fiber supplement, protein supplement, amino acid supplement, fatty acids supplement, and mineral and microelement supplements.

32. The composition of claim 28, wherein the nutraceutical agents or biologically active agents are polysaccharides including beta-glucan, galactans, mannan, and chitin.

33. The composition of claim 28, wherein the biologically active agents have immunostimulatory or hypocholesterolemic activity.

34. The composition of claim 28, rich in melanin.

35. The composition of claim 34, for protecting the skin from solar UV radiation or internal organs from ionizing radiation damage.

36. The composition of any of claims 28-35, formulated in an oral solid dosage form.

37. The composition of claim 36, wherein said oral solid dosage form is selected from a fine powder, capsules, tablets, caplets, and sachets.

38. The composition of claim 36, formulated into a food products.

39. The composition of claim 36, formulated into a pet food products.

40. The composition of claim 28, formulated into cosmetic products.

41. The composition of claim 40, 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.

42. A *Ganoderma* extract having nutraceutical and biological activity comprising an extract of *Ganoderma tsugae* var. *jannieae* strain Tay-1 Accession
5 No. CBS 120394.

43. The extract of claim 42, obtained from the fruiting body of *Ganoderma tsugae* var. *jannieae* strain Tay-1.

44. The extract of claim 43, obtained from the mycelium of *Ganoderma tsugae* var. *jannieae* strain Tay-1.

10 45. The extract of claim 44, obtained from a pure submerged mycelium culture.

46. The extract of claim 45 as a freeze-dried extract obtained by the following steps: (i) concentration of the submerged mycelium culture; (ii) drying the concentrate; (iii) grinding the resulting fine powder; (iv) extracting the ground
15 powder with an organic or aqueous solvent; and (v) removing the solvent by freeze-drying.

47. The freeze-dried extract of claim 46, wherein said organic solvent is selected from ethyl alcohol, ethyl acetate or diethyl ether.

20 48. The freeze-dried extract of claim 47, wherein said solvent is ethyl acetate.

49. The freeze-dried extract of claim 47, wherein said solvent is ethyl alcohol.

50. The freeze-dried extract of claim 46, wherein said aqueous solvent is water.

25 51. A composition comprising a freeze-dried extract as defined in any one of claims 47 to 50.

52. The composition of claim 51, formulated into a food product.

53. The composition of claim 51, formulated into a beverage product.
58. 54. The composition of claim 53, wherein said beverage product is an energizing beverage product.
55. The composition of claim 51, formulated into a pet food product.
- 5 56. The composition of claim 52, formulated into a cosmetic product.
57. The composition of claim 56, 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.
58. The *Ganoderma tsugae* var. *janniae* extract of any one of claims 42
10 to 50, wherein said biological activity is nuclear factor κ B (NF- κ B) pathway modulating activity and/or anti-oxidant activity and/or free radical scavenging activity and/or anti-radiation activity and/or metal ion scavenging activity.
59. The extract of claim 58, wherein said biological activity is NF- κ B pathway modulating activity.
- 15 60. The extract of claim 59, for use in treatment of an NF- κ B-dependent diseases.
61. The extract of claim 60, wherein said NF- κ B-dependent diseases are selected from cancer, immunological disorders, septic shock, transplant rejection, radiation damage, and reperfusion injuries after ischemia, arteriosclerosis and
20 neurodegenerative diseases.
62. A process for producing a *Ganoderma* extract having biological activity, wherein the biological activity is NF- κ B pathway modulating activity and/or anti-oxidant activity and/or free radical scavenging activity and/or anti-radiation activity and/or metal ion scavenging activity, said process comprising:
25 cultivating fruiting bodies or the mycelial biomass of mushroom *Ganoderma tsugae* var. *janniae* strain Tay-1 (CBS 120394) in submerged culture in nutrient media, isolating the resulting biomass of mushroom from the culture broth, drying and grinding said biomass into fine powder which is subjected to solvent extraction and freeze drying.

63. A process for producing a biomass rich in polysaccharides, monosaccharides, proteins, essential amino acids, vitamins, essential fatty acids, minerals and microelements from higher Basidiomycetes medicinal mushroom *Ganoderma tsugae* var. *jannieae* strain Tay-1 (CBS 120394), said process
5 comprising: cultivating fruiting bodies or the mycelial biomass of the mushroom *Ganoderma tsugae* var. *jannieae* strain Tay-1 (CBS 120394) in submerged culture on nutrient media, isolating the resulting biomass of edible fungi from the culture broth, and drying and grinding said biomass into fine powder.

64. The process of claim 62 or 63, wherein the nutrient media for
10 submerged cultivation of mycelium is of the following composition (g/L of distilled water): glucose, 15.0-25.0; peptone, 3.0; yeast extract, 3.0; KH_2PO_4 , 1.0; K_2HPO_4 , 0.2; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5.

65. A pure submerged mycelial culture of *Ganoderma tsugae* var. *jannieae* strain Tay-1 (CBS 120394).