

**NEW *CORDYCEPS MILITARIS* STRAIN CBS 132098 AND BIOLOGICALLY
ACTIVE FRUITING BODIES, MYCELIAL BIOMASS AND EXTRACTS
THEREFROM**

5

FIELD OF THE INVENTION

The present invention relates to medicinal mushrooms, more particularly to *Cordyceps* fungi and to a new and distinct strain of caterpillar fungus designated *Cordyceps militaris* CBS 132098. The invention further relates to fruiting bodies, submerged cultivated mycelial biomass and extracts from *Cordyceps militaris* comprising various biologically active compounds and their use as dietary supplements and in the therapy of several diseases and conditions.

BACKGROUND OF THE INVENTION

The study of medicinal mushrooms includes the study of biology, chemistry, as well as practical applications in the field of pharmacology, in order to prepare new drugs by biotechnological means. This field is currently becoming more important and has been widely investigated due to practical benefits. Synthetic drugs are available in the commercial market and are being used in daily life, but they are not safe enough as they have some side effects and could be harmful to human health (Das et al. 2010).

Edible and medicinal mushrooms represent a valuable source of useful natural products having diverse biological activities, which may provide more efficacious and safer drugs having no side effects. Of approximately 15,000 known species, 2,000 are safe for human health, and about 700 of them possess medicinal properties (Wasser 2002, 2011). Most of traditional knowledge about medicinal properties of mushrooms comes from the Far East (China, Japan, Korea, and Russia), where such mushrooms as Chinese caterpillar fungi (*Cordyceps sinensis* and *C. militaris*), reishi (*Ganoderma lucidum*), shiitake (*Lentinus edodes*) and others were collected, cultivated and used for thousands of years. The western countries have become more interested in mushroom diversity and their great potential in medicinal therapies. In the second half of the 20th century, mushroom-producing technologies have grown enormously. It is estimated that more than 24 million metric tons of edible and medicinal mushrooms were produced in 2009 in various

countries (Royse 2005; Chang and Wasser 2012). Numerous bioactive polysaccharides or polysaccharide-protein complexes from medicinal mushrooms are described that appear to enhance innate and cell-mediated immune responses, and exhibit antitumor activities in animals and humans. Stimulation of host-immune defense systems by bioactive polymers
5 from medicinal mushrooms has significant effects on the maturation, differentiation, and proliferation of many kinds of immune cells in the host. Many of these mushroom polymers were reported previously to have immunotherapeutic properties by facilitating growth inhibition and the destruction of tumor cells. Whilst the mechanism of their antitumor actions is still not completely understood, stimulation and modulation of key
10 host-immune responses by these mushroom polymers appears central. Particularly, and most importantly for modern medicine, are numerous bioactive polysaccharides or polysaccharide-protein complexes from medicinal mushrooms, which appear to enhance innate and cell-mediated immune responses, and exhibit antitumor activities in animals and humans. Stimulation of host-immune defense systems by bioactive polymers from
15 medicinal mushrooms has significant effects on the maturation, differentiation, and proliferation of many kinds of immune cells in the host. Several of the mushroom polysaccharide compounds have proceeded through Phase I, II, and III clinical trials and are used extensively and successfully in Asia to treat various cancers and other diseases. Mushroom polysaccharides prevent oncogenesis, show direct antitumor activity against
20 various syngeneic tumors, and prevent tumor metastasis. Their activity is especially beneficial in clinics when used in conjunction with chemotherapy (Chang and Wasser 2012; Petrova 2012).

A total of 126 medicinal functions are thought to be produced by medicinal mushrooms and fungi including antitumor, immunomodulating, antioxidant, radical
25 scavenging, cardiovascular, anti-hypercholesterolemia, antiviral, antibacterial, anti-parasitic, antifungal, detoxification, hepato-protective, anti-diabetic, and many other effects.

Mushrooms produce beneficial effects not only as drugs but also as dietary supplements (DS) or nutraceuticals. These are not strictly pharmaceutical products, but
30 produce their healthy effects through everyday use as part of a healthy diet. The market of DS from mushrooms is quickly growing and comprises a value of more than \$15 billion USD today (representing 10% of the general market of dietary supplements, approximately \$150 billion USD). Every year we accumulate new evidence for the beneficial effects from

DS made from mushrooms. One significant problem with DS is their safety. They do not go through the same strict phases of laboratory and clinical evaluations as do pharmaceutical drugs. Furthermore, a major problem associated with mushroom-based DS is their wide variability and the current lack of standards for production and testing protocols necessary to guarantee product quality. The active ingredient components of the majority of present commercial mushroom products have not been identified (Chang and Wasser 2012).

The development of medicines and dietary supplements from mushroom polysaccharides is hampered by the fact that high-molecular-weight compounds are used. These compounds cannot be synthesized artificially and their production, therefore, is restricted to the extraction from fruit bodies or cultured mycelium. Such an approach imposes high-market prices. Today, science should concentrate on the beneficial medicinal effects of low-weight-molecular compounds produced by mushrooms, i.e., low-molecular-weight secondary metabolites targeting processes such as apoptosis, angiogenesis, metastasis, cell cycle regulation, and signal transduction cascades.

The use of herbal supplements in Europe and the United States continues to grow and questions have been raised concerning their safety, efficacy, and effects on patient care. However, the use of natural products including medicinal mushrooms has increased in recent years and the increased growth of the medicinal mushroom product industry has led to increased concerns regarding their safety (Wasser and Akavia 2008).

Genus *Cordyceps*

Cordyceps Fr. (Cordycipitaceae, Ascomycota) is the most diverse genus in the family Clavicipitaceae. There are currently more than 680 documented species of *Cordyceps*, found on six continents and in many climatic zones and habitats, and occurring parasitically or commensally with a range of hosts (Holliday et al. 2010). *Cordyceps* is characterized and distinguished from other genera of the family Cordycipitaceae by its production of superficial to completely immersed perithecia on stipitate and often clavate to capitate stromata and its ecology as a pathogen of arthropods and the fungal genus *Elaphomyces* (Kobayasi 1941; Mains 1957, 1958; Kobayasi and Shimizu 1960; Rogerson 1970; Sung et al. 2007). *Cordyceps* has a long history as a rare and exotic medicinal fungus. The first record about medicinal properties of *Cordyceps* mushrooms comes from China, in the year AD 620 at the time of the Tang Dynasty. Tibetan scholars

wrote about *Cordyceps* through the 15th to 18th centuries, and in 1757, the earliest objective and scientifically reliable depiction of the *Cordyceps* mushroom was written by the author Wu-Yuluo in the Ben Cao Congxin ("New Compilation of Materia Medica") during Qing Dynasty (Holliday et al. 2010).

5 The medicinal mushroom *Cordyceps militaris* (L.) Link, known to the Chinese as "Bei Dong Chong Xia Cao" or "Bei Chong Cao", is an entomopathogenic fungus (belonging to the family Cordycipitaceae in the phylum Ascomycota), which infects and grows parasitically on *Lepidoptera* larvae or pupae in temperate countries (Isaka et al. 2005). It is characterized by the production of orange to orange-red colored stromata that
10 erupt from the carcass of the dead host (Mains 1958; Kobayasi 1982). *Cordyceps militaris* is one of the most important and well known medicinal mushrooms frequently collected from all major continents except Antarctica. The fruiting bodies of *Cordyceps militaris*, which produce the active ingredients, have a rich and interesting history.

Cordyceps militaris contains many kinds of active components such as cordycepin
15 (3'-deoxyadenosine), ergosterol, mannitol, and polysaccharides and due to its various physiological activities is now used for multiple medicinal purposes (Mizuno 1996; Song et al. 1998). To date, more than 10 nucleosides and their related components, including adenine, adenosine, cytidine, cytosine, guanine, guanosine, uracil, uridine, hypoxanthine, inosine, thymine, thymidine, 2'-deoxyuridine, 2'-deoxyadenosine, cordycepin, N6-
20 methyladenosine, and 6-hydroxyethyl-adenosine, have been isolated and/or identified in *Cordyceps* (Feng et al. 2008).

 The main active constituent of *Cordyceps militaris* is cordycepin, which was first extracted from *Cordyceps militaris* and then found to be present in *Cordyceps sinensis* (Cunningham et al. 1950) and *Cordyceps kyushuensis* (Ling et al. 2002). The cordycepin
25 (3'-deoxyadenosine, C₁₀H₁₃N₅O₃, m. pt. 225°C, [α]_D-47°C, molecular weight is 251), a nucleotide analogue (Cunningham et al. 1950; Ahn et al. 2000), is considered as a nucleic acid antibiotic that might inhibit canceration of cells and thus contribute to the normalization of cancer cells as one of the constituents of gene DNA (Cunningham et al. 1950; Das et al. 2010).

30 Masuda et al. (2006) reported that the cordycepin is intracellularly converted into its 5'-mono-, di- and triphosphates that inhibit the activity of several enzymes in the purine biosynthetic pathway, and affects a multitude of pharmacological actions, i.e., inhibition of human glomerular mesangial cell proliferation, anti-fibrotic, anti-angiogenetic,

improvement of insulin resistance and insulin secretion, anti-inflammatory and growth inhibition of Leukemia cells.

Moreover, cordycepin itself acts as an antitumor (Penman et al. 1970; Johns and Adamson 1976; Mueller et al. 1977; Liu et al. 1997; Kodama et al. 2000), anti-proliferative
5 (Liu et al. 1997), anti-metastatic (Liu et al. 1997; Shih et al. 2007), hypoglycemic (Yu et al. 2004; Shen and Chen 2001), anti-diabetic (Choi et al. 2004), anti-HIV (Mueller et al. 1991), insecticidal (Mao and Zhong 2006; Kim et al. 2002), and anti-bacterial (Ahn et al. 2000) compounds. *Cordyceps militaris* also can be used to treat conditions such as night sweating, night enuresis, asthenia after severe illness, respiratory disease, renal
10 dysfunction, renal failure arrhythmias, and other heart disease (Mizuno 1999).

Cordycepic acid, an isomer of quinic acid, is one of the main active medicinal components. Cordycepic acid was identified as D-mannitol. Mannitol is a major bio-product with important biological activity. The chemical formula of manitol is $C_6H_{14}O_6$; its molecular weight is 182. Mannitol is a functional polyol with notable properties. The
15 content of mannitol varies with the original habitat. In general, there are about 25-80 mg/g in *Cordyceps* species fruiting bodies (more in mycelia than in fruiting bodies) (Cunningham et al. 1950; Chatterjee et al. 1957; Liu et al. 1989).

Furthermore, a number of polysaccharides and other sugars have been identified from *Cordyceps militaris* extracts and their pharmacological activity has been reported.
20 Research has shown these polysaccharides are affective in regulating blood sugar as well as having anti-metastatic and anti-cancer effects, hepatoprotective and antihypertensive effects (Kim et al. 2002; Yu et al. 2004). Nucleotides (including adenosine, uridin, and guanosin) are effective components in *Cordyceps militaris* (Kredich and Guarino 1961; Guarino and Kredich 1963). Ergosterol is a sterol unique to fungi and is an important
25 precursor of Vitamin D₂, which has important medicinal value (Peterson 2008). The content of crude protein in *Cordyceps militaris* is 28-33%. Fatty acids of *Cordyceps militaris* are composed of carbon, hydrogen, and oxygen and are major components of lipids, phospholipids, and glycolipids. They are saturated and unsaturated fatty acids. Generally, the contents of unsaturated fatty acids in *Cordyceps militaris* are higher than
30 that of saturated fatty acids. The content of linoleic and oleic acids are higher than other fatty acids. Many investigations show that *Cordyceps militaris* contains many micro- and macroelements, including K, P, Ca, Al, Mo, Na, Zn, Mg, Mn, etc.

The novel components already obtained from *Cordyceps militaris* already have more than 21 clinically approved beneficial effects for human health (Mizuno 1999).

Mushroom dietary supplements

5 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 rather represent a novel class of functional foods, dietary supplements or “nutriceuticals” or “nutraceuticals”.

10 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 conventional food) and also 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.

15 *Cordyceps* dietary supplements is believed to have many beneficial properties such as increased exercise performance, improved fatigue and stress resistance, improved immune function, cholesterol lowering properties, anti-tumor properties as well as anti-ageing and antioxidant properties. One of the most useful things that *Cordyceps* appears to do is the simple act of oxygenation. The fungus dilates the airways in the lungs, resulting
20 in more oxygen to the blood. Thus more oxygen reaches every cell of the body, resulting in better cell and greater energy levels. Today there are several brands of *Cordyceps* supplements available on the market.

25 Edible and medicinal mushrooms contain a large amounts 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 a carcinostatic and decrease various kinds of poisoning.

30 The safety of mushroom-based dietary supplements is enhanced through the following controls: (i) the overwhelming amount of mushrooms used for production of dietary supplements are cultivated commercially (and not gathered in the wild), thus guaranteeing identification and pure, unadulterated products, which would result, in many

cases, with genetic uniformity and would also benefit the conservation of biodiversity; (ii) mushroom 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
5 capable of growing in the form of mycelial biomass in submerged cultures and thus offer a promising future for standardized production of safe mushroom-based dietary supplements. These are advantages of using mushroom-based dietary supplements as opposed to herbal preparations.

10 SUMMARY OF THE INVENTION

In one aspect, the present invention relates to a new strain of caterpillar fungus, *Cordyceps militaris* strain Phytopharma, which has been deposited as culture with the Centraalbureau voor Schimmelcultures (CBS), Uppsalalaan 8, P.O. Box 85167, 3508 AD Utrecht, the Netherlands, on March 2, 2012, under the Budapest Treaty on the International
15 Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure, and was assigned the No. CBS 132098.

In a further aspect the present invention relates to a pure submerged mycelial culture of *Cordyceps militaris* strain CBS 132098 and *Ganoderma tsugae* var. *janniae* Tay-1 (CBS 120394).

20 In another aspect, the present invention relates to a biomass of the *Cordyceps militaris* strain CBS 132098 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, nucleosides, mannitol and other biologically active agents. The biomass can be obtained from the submerged cultivated
25 mycelium or the cultivated fruiting bodies of *Cordyceps militaris* strain CBS 132098.

In a further aspect, the present invention relates to extracts from the *Cordyceps militaris* strain CBS 132098 rich in nutraceutical agents and biologically active substances. The extracts can be obtained from the fruiting bodies or the submerged cultivated mycelium of *Cordyceps militaris* strain CBS 132098.

30 In further aspects, the present invention provides compositions comprising the biomass and extracts of the invention, and processes for producing these biomasses and extracts.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 shows macro- and microstructure of vegetative mycelium of *Cordyceps militaris* strain CBS 132098 on potato dextrose agar media. A – mycelial colony with orange tint. B - anamorph stage (*Lecanicillium* sp.-like conidiophores, with phialides and globose conidia).

Fig. 2 shows weight of dry mycelial biomass of *Cordyceps militaris* strain CBS 132098 at three cultivation temperatures after 14 days of cultivation.

Fig. 3 shows HPTLC chromatogram of the standard cordycepin (Track 1) and HPTLC chromatogram of the fruiting bodies biomass of *Cordyceps militaris* strain CBS 132098 (Track 2).

Fig. 4 shows HPTLC chromatogram of the standard cordycepin (Track 1) and HPTLC chromatogram of the mycelial biomass of *Cordyceps militaris* strain CBS 132098 (Track 2).

Fig. 5 shows the effect of different *Cordyceps militaris* strain CBS 132098 fruiting body and culture liquid crude extracts on the viability of HPAF-II cells by XTT assay. Cells were seeded in a 96-well plate (1×10^4 cells/mL) and were treated with different extracts 24 hours later at different concentrations and incubated for 24, 48, and 72 hours. At the end of treatment, cell viability was measured using XTT. All results expressed as percentages of control (non-treated cells).

Fig. 6 shows the effect of different *Cordyceps militaris* strain CBS 132098 fruiting body and culture liquid crude extracts on the viability of FaDu cells by XTT assay. Cells were seeded in a 96-well plate (1×10^4 cells/mL) and were treated with different extracts 24 hours later at different concentrations and incubated for 24, 48, and 72 hours. At the end of treatment, cell viability was measured using XTT. All results expressed as percentages of control (non-treated cells).

Fig. 7 shows the effect of different *Cordyceps militaris* strain CBS 132098 fruiting body and culture liquid crude extracts on the viability of HCT116 cells by XTT assay. Cells were seeded in a 96-well plate (1×10^4 cells/mL) and were treated with different extracts 24 hours later at different concentrations and incubated for 24, 48, and 72 hours. At the end of treatment, cell viability was measured using XTT. All results expressed as percentages of control (non-treated cells).

Fig. 8 shows the effect of different *Cordyceps militaris* strain CBS 132098 fruiting body and culture liquid crude extracts on the viability of T47D cells by XTT assay. Cells were seeded in a 96-well plate (1×10^4 cells/mL) and were treated with different extracts 24 hours later at different concentrations and incubated for 24, 48, and 72 hours. At the end of treatment, cell viability was measured using XTT. All results expressed as percentages of control (non-treated cells).

Fig. 9 shows the effect of different *Cordyceps militaris* strain CBS 132098 fruiting body and culture liquid crude extracts on the viability of PC3 cells by XTT assay. Cells were seeded in a 96-well plate (1×10^4 cells/mL) and were treated with different extracts 24 hours later at different concentrations and incubated for 24, 48, and 72 hours. At the end of treatment, cell viability was measured using XTT. All results expressed as percentages of control (non-treated cells).

Fig. 10 shows the effect of different *Cordyceps militaris* strain CBS 132098 fruiting body and culture liquid crude extracts on the viability of all cell lines by XTT assay. Cells were seeded in a 96-well plate (1×10^4 cells/mL) and were treated with different extracts 24 hours later at different concentrations and incubated for 24, 48, and 72 hours. At the end of treatment, cell viability was measured using XTT. All results expressed as percentages of control (non-treated cells).

Fig. 11 shows the effect of DMSO on the viability of all cell lines by XTT assay. (0 – control untreated cells, DMSO – 1% DMSO treated cells). Cells were seeded in a 96-well plate (1×10^4 cells/mL) and were treated with different extracts 24 hours later at different concentrations and incubated for 24, 48, and 72 hours. At the end of treatment, cell viability was measured using XTT. All results expressed as percentages of control (non-treated cells).

Fig. 12 shows the effect of different *Cordyceps militaris* strain CBS 132098 and *Ganoderma tsugae* var. *jannieae* strain Tay-1 (CBS 120394) combination (1:1 v/v) crude extracts on the viability of HPAF-II cells by XTT assay. Cells were seeded in a 96-well plate (1×10^4 cells/mL) and were treated with different extracts 24 hours later at different concentrations and incubated for 24, 48, and 72 hours. At the end of treatment, cell viability was measured using XTT. All results expressed as percentages of control (non-treated cells).

Fig. 13 shows the effect of different *Cordyceps militaris* strain CBS 132098 and *Ganoderma tsugae* var. *jannieae* strain Tay-1 (CBS 120394) combination crude extracts on

the viability of FaDu cells by XTT assay. Cells were seeded in a 96-well plate (1×10^4 cells/mL) and were treated with different extracts 24 hours later at different concentrations and incubated for 24, 48, and 72 hours. At the end of treatment, cell viability was measured using XTT. All results expressed as percentages of control (non-treated cells).

5 Fig. 14 shows the effect of different *Cordyceps militaris* strain CBS 132098 and *Ganoderma tsugae* var. *jannieae* strain Tay-1 (CBS 120394) combination (1:1 v/v) crude extracts on the viability of HCT116 cells by XTT assay. Cells were seeded in a 96-well plate (1×10^4 cells/mL) and were treated with different extracts 24 hours later at different concentrations and incubated for 24, 48, and 72 hours. At the end of treatment, cell
10 viability was measured using XTT. All results expressed as percentages of control (non-treated cells).

Fig. 15 shows the effect of different *Cordyceps militaris* strain CBS 132098 and *Ganoderma tsugae* var. *jannieae* strain Tay-1 (CBS 120394) combination (1:1 v/v) crude extracts on the viability of T47D cells by XTT assay. Cells were seeded in a 96-well plate
15 (1×10^4 cells/mL) and were treated with different extracts 24 hours later at different concentrations and incubated for 24, 48, and 72 hours. At the end of treatment, cell viability was measured using XTT. All results expressed as percentages of control (non-treated cells).

Fig. 16 shows the effect of different *Cordyceps militaris* CBS 132098 and *Ganoderma tsugae* var. *jannieae* strain Tay-1 (CBS 120394) combination crude extracts on the viability of PC3 cells by XTT assay. Cells were seeded in a 96-well plate (1×10^4 cells/mL) and were treated with different extracts 24 hours later at different concentrations and incubated for 24, 48, and 72 hours. At the end of treatment, cell viability was measured using XTT. All results expressed as percentages of control (non-treated cells).

25 Fig. 17 shows the effect of different *Cordyceps militaris* strain CBS 132098 and *Ganoderma tsugae* var. *jannieae* strain Tay-1 (CBS 120394) combination crude extracts and *Cordyceps militaris* strain CBS 132098 crude extracts on the viability of HPAF-II cells by XTT assay. Cells were seeded in a 96-well plate (1×10^4 cells/mL) and were treated with different extracts 24 hours later at different concentrations and incubated for 24, 48, and 72
30 hours. At the end of treatment, cell viability was measured using XTT. All results expressed as percentages of control (non-treated cells).

Fig. 18 shows the effect of different *Cordyceps militaris* strain CBS 132098 and *Ganoderma tsugae* var. *jannieae* strain Tay-1 (CBS 120394) combination crude extracts

and *Cordyceps militaris* strain CBS 132098 crude extracts on the viability of FaDu cells by XTT assay. Cells were seeded in a 96-well plate (1×10^4 cells/mL) and were treated with different extracts 24 hours later at different concentrations and incubated for 24, 48, and 72 hours. At the end of treatment, cell viability was measured using XTT. All results expressed as percentages of control (non-treated cells).

Fig. 19 shows the effect of different *Cordyceps militaris* strain CBS 132098 and *Ganoderma tsugae* var. *janniae* strain Tay-1 (CBS 120394) combination crude extracts and *Cordyceps militaris* CBS 132098 crude extracts on the viability of HCT116 cells by XTT assay. Cells were seeded in a 96-well plate (1×10^4 cells/mL) and were treated with different extracts 24 hours later at different concentrations and incubated for 24, 48, and 72 hours. At the end of treatment, cell viability was measured using XTT. All results expressed as percentages of control (non-treated cells).

Fig. 20 shows the effect of different *Cordyceps militaris* strain CBS 132098 and *Ganoderma tsugae* var. *janniae* strain Tay-1 CBS 120394 combination crude extracts and *Cordyceps militaris* strain CBS 132098 crude extracts on the viability of PC3 cells by XTT assay. Cells were seeded in a 96-well plate (1×10^4 cells/mL) and were treated with different extracts 24 hours later at different concentrations and incubated for 24, 48, and 72 hours. At the end of treatment, cell viability was measured using XTT. All results expressed as percentages of control (non-treated cells).

Fig. 21 shows the effect of different *Cordyceps militaris* strain CBS 132098 and *Ganoderma tsugae* var. *janniae* strain Tay-1 CBS 120394 combination crude extracts and *Cordyceps militaris* strain CBS 132098 crude extracts on the viability of T47D cells by XTT assay. Cells were seeded in a 96-well plate (1×10^4 cells/mL) and were treated with different extracts 24 hours later at different concentrations and incubated for 24, 48, and 72 hours. At the end of treatment, cell viability was measured using XTT. All results expressed as percentages of control (non-treated cells).

Fig. 22 shows the effect of *Cordyceps militaris* strain CBS 132098 Eth extract and *Cordyceps militaris* strain CBS 132098 and *Ganoderma tsugae* var. *janniae* strain Tay-1 (CBS 120394) combination EA extract on the viability of T47D cells by XTT assay. Cells were seeded in a 96-well plate (1×10^4 cells/mL). Cells were treated 24 hours later with different extract concentrations and incubated for 24, 48, and 72 hours. At the end of treatment, cell viability was measured using the XTT assay (Biological Industries, Kibbutz Beit Haemek, Israel). All results presented are an average of at least three independent

experiments: four repeats each (mean $\pm$ SEM) and expressed as percentages of the respective vehicle treated control. Statistical significance was determined by one way ANOVA $P < 0.001$ or two-tailed Kruskal-Wallis test $P < 0.001$. Each letter above the bars represents relations to fellow concentrations in the same treatment period.

5 Fig. 23 shows the effect of *Cordyceps militaris* strain CBS 132098 and *Ganoderma tsugae* var. *jannieae* strain Tay-1 (CBS 120394) combination Eth extract and *Cordyceps militaris* strain CBS 132098 EA extract on the viability of PC3 cells by XTT assay. Cells were seeded in a 96-well plate (1×10^4 cells/mL). Cells were treated 24 hours later with different extract concentrations and incubated for 24, 48, and 72 hours. At the end of
10 treatment, cell viability was measured using the XTT assay (Biological Industries, Kibbutz Beit Haemek, Israel). All results presented are an average of at least three independent experiments: four repeats each (mean $\pm$ SEM) and expressed as percentages of the respective vehicle treated control. Statistical significance was determined by one way ANOVA $P < 0.001$ or two-tailed Kruskal-Wallis test $P < 0.001$. Each letter above the bars
15 represents relations to fellow concentrations in the same treatment period.

 Fig. 24 shows the effect of *Cordyceps militaris* strain CBS 132098 and *Ganoderma tsugae* var. *jannieae* strain Tay-1 (CBS 120394) EA extract and *Cordyceps militaris* strain CBS 132098 Eth extract on the cytotoxicity of T47D cells by LDH leakage. Cells were seeded in a 96-well plate (1×10^4 cells/mL). Cells were treated 24 hours later with different
20 extract concentrations and incubated for 24 hours. At the end of treatment, LDH leakage was measured using LDH Cytotoxicity Detection Kit (Roche Diagnostics, Mannheim, Germany). All results presented are an average of at least three independent experiments: four repeats each (mean $\pm$ SEM) and expressed as percentages of the respective vehicle treated control. Statistical significance was determined by one way ANOVA $P < 0.001$.
25 Each letter above the bars represents relations to fellow concentrations in the same treatment period.

 Fig. 25 shows the effect of *Cordyceps militaris* strain CBS 132098 and *Ganoderma tsugae* var. *jannieae* strain Tay-1 (CBS 120394) combination EA extract and *Cordyceps militaris* strain CBS 132098 Eth extract on the cytotoxicity of PC3 cells by LDH leakage.
30 Cells were seeded in a 96-well plate (1×10^4 cells/mL). Cells were treated 24 hours later with different extract concentrations and incubated for 24 hours. At the end of treatment, LDH leakage was measured using LDH Cytotoxicity Detection Kit (Roche Diagnostics, Mannheim, Germany). All results presented are an average of at least three independent

experiments: four repeats each (mean $\pm$ SEM) and expressed as percentages of the respective vehicle treated control. Statistical significance was determined by one way ANOVA $P < 0.001$. Each letter above the bars represents relations to fellow concentrations in the same treatment period.

- 5 **Fig. 26** shows the effect of *Cordyceps militaris* strain CBS 132098 Eth extract on Sub-G1 accumulation of T47D cells by FACS flow cytometry analysis. *Cordyceps militaris* strain CBS 132098 Eth extract induced cell accumulation in SubG1-phase of the cell cycle in T47D cells. Exponentially growing cells were exposed to either medium (control) or *Cordyceps militaris* strain CBS 132098 Eth extract (25 $\mu\text{g/mL}$) for 24, 48, and 72 hours.
- 10 Cells were then harvested, washed in PBS, and fixed in 70% ethanol. DNA content was evaluated with propidium iodide staining, and fluorescence was measured and analyzed as described in Materials and Methods. All results presented are an average of at least three independent experiments (mean $\pm$ SEM). Statistical significance was determined by Student's *t*-test. Asterisks denote the level of significance as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

- 15 **Fig. 27** shows the effect of *Cordyceps militaris* strain CBS 132098 and *Ganoderma tsugae* var. *janniae* strain Tay-1 (CBS 120394) Eth extract on Sub-G1 accumulation of PC3 cells by FACS flow cytometry analysis. *Cordyceps militaris* strain CBS 132098 Eth extract induced cell accumulation in SubG1-phase of the cell cycle in PC3 cells.
- 20 Exponentially growing cells were exposed to either medium (control) or *Cordyceps militaris* strain CBS 132098 Eth extract (25 $\mu\text{g/mL}$) for 24, 48, and 72 hours. Cells were then harvested, washed in PBS, and fixed in 70% ethanol. DNA content was evaluated with propidium iodide staining, and fluorescence was measured and analyzed as described in Materials and Methods. All results presented are an average of at least three independent
- 25 experiments (mean $\pm$ SEM). Statistical significance was determined by Student's *t*-test. Asterisks denote the level of significance as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

- 30 **Fig. 28** shows the effect of *Cordyceps militaris* strain CBS 132098 Eth extract on apoptosis induction of T47D cells by Annexin V-FITC and propidium iodide staining. Exponentially growing cells were exposed to either medium (control) or *Cordyceps militaris* strain CBS 132098 Eth extract (25 $\mu\text{g/mL}$) for 24, 48, and 72 hours. Cells were then harvested, washed in PBS, and 2×10^5 cells were counted and stained with annexin and PI for the flow cytometry analysis. Early apoptotic cells (Annexin V-FITC positive) and

late apoptotic cells (Annexin V-FITC and propidium iodide positive) together are presented as percentage of total cells observed. All results presented are an average of at least three independent experiments (mean $\pm$ SEM). Statistical significance was determined by Student's *t*-test. Asterisks denote the level of significance as follows: *, p < 0.05; **, p < 0.01; ***, p < 0.001.

Fig. 29 shows the effect of *Cordyceps militaris* strain CBS 132098 and *Ganoderma tsugae* var. *janniae* strain Tay-1 (CBS 120394) Eth extract on apoptosis induction of PC3 cells by Annexin V-FITC and propidium iodide staining. Exponentially growing cells were exposed to either medium (control) or *Cordyceps militaris* strain CBS 132098 Eth extract (25 μ g/mL) for 24, 48, and 72 hours. Cells were then harvested, washed in PBS, and 2×10^5 cells were counted and stained with annexin and PI for the flow cytometry analysis. Early apoptotic cells (Annexin V-FITC positive) and late apoptotic cells (Annexin V-FITC and propidium iodide positive) together are presented as percentage of total cells observed. All results presented are an average of at least three independent experiments (mean $\pm$ SEM). Statistical significance was determined by Student's *t*-test. Asterisks denote the level of significance as follows: *, p < 0.05; **, p < 0.01; ***, p < 0.001.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed, in one aspect, to a new strain of medicinal mushroom *Cordyceps militaris*, namely *Cordyceps militaris* strain Phytopharma deposited under the Budapest Treaty with Centraalbureau voor Schimmelcultures (CBS) as Accession No. CBS 132098.

The invention further relates to a pure submerged mycelial culture of *Cordyceps militaris* strain CBS 123098.

In another aspect, the present invention relates to a biomass of the *Cordyceps militaris* strain CBS 132098, or a biomass of a pure submerged mycelial culture of *Cordyceps militaris* strain CBS 123098, rich in 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, minerals, nucleosides, and mannitol. 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 *Cordyceps militaris* strain CBS 132098. In certain embodiments, the mycelial biomass is

for use in nutraceutical compositions, in a dietary supplement, vitamin supplement, dietary fiber supplement protein supplement, amino acid supplement, fatty acids supplement and mineral and microelement supplement. The biomass may also be incorporated/formulated into a food product for human consumption, e.g. cereals, ice cream and the like or into pet food products, e.g., for dogs and cats.

The fruiting bodies have about 29.1% carbohydrates, about 59.8% proteins and about 8.8% fats of the dry weight, and the mycelial biomass has about 39.6% carbohydrates, about 39.5% proteins and about 2.2% fats of the dry weight of mycelium.

The fruiting-body biomass proteins are rich in alanine, arginine, aspartic acid, cysteic acid, isoleucine, glutamic acid, glycine, histidine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, and valine. The mycelial biomass is rich in aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, lysine, histidine, and arginine. The biomass of the present invention thus constitutes an important dietary supplement due to the presents of the proteins rich in essential amino acids.

Vitamins found in the fruiting-body biomass include the vitamins A, B₂, B₃, C, E, and D-mannitol. The mycelial biomass vitamins comprise A, B₂, B₃, and E. 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 *Cordyceps militaris* biomass and extracts contain important vitamins they may be used as nutraceuticals and/or may be added to food and beverage products as dietary supplements and serving as an excellent source of vitamins.

The fruiting-body biomass comprises lipids including fatty acids: palmitic, palmitoleic, stearic, oleic, linoleic, and linolenic. The mycelial biomass comprises lipids including the fatty acids palmitic, palmitoleic, stearic, trans-vaccenic, oleic, linoleic, linolenic and arachidic acids. 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 (oleic, trans-vaccenic, linoleic, and linolenic acids) and saturated (palmitic, stearic, and arachidic acids) fatty acids. The latter gives rise to the omega-6 and omega-9 series of polyunsaturated fatty acids, which incorporation into phospholipids affect cell membrane properties such as fluidity, flexibility, permeability and the activity of membrane bound enzymes.

The fruiting-body biomass comprises minerals, both microelements and macroelements, including aluminum, boron, calcium, copper, iron, potassium, magnesium, manganese, sodium, phosphorus, and sulfur. The mycelial biomass comprises macroelements: aluminum, calcium, chromium, iron, potassium, magnesium, manganese, sodium
5 and phosphorus; and microelements: silver, arsenic, boron, barium, beryllium, cadmium, cobalt, copper, mercury, lithium, molybdenum, nickel, lead, sulfur, selenium, tin, strontium, titanium, vanadium, and zinc. Thus, the mycelial biomass comprises similar minerals but with low levels of calcium, high phosphorus content, and the presence of such important microelements, such as zinc. Iron deficiency (or "sideropenia") is the most
10 common known form of nutritional deficiency and weakness. A daily dose of *Cordyceps militaris* biomass endows an excellent source of iron and other minerals.

As shown herein in the examples, the mycelial and fruiting-body biomasses of strain of the invention comprise cordycepin. Cordycepin, or 3'-deoxyadenosine, is a known polyadenylation inhibitor with a large spectrum of biological activities including anti-
15 proliferative, pro-apoptotic and anti-inflammatory effects.

The mycelial biomass of *Cordyceps militaris*, as shown herein in the examples of the invention, comprise ergothioneine. Ergothioneine is a water-soluble, naturally occurring thiol compound and has been recognised an antioxidant substance *in vivo* and a protection factor that helps cellular to against the oxidative stress.

20 Many studies indicates that ergothioneine shows effective intrinsic anti-hydroxyl, anti- peroxyl and anti-peroxynitrite radical antioxidant activity, as compared to classic molecules with antioxidant capacity as reduced glutathione, uric acid and trolox. Recently, ergothioneine has attracted attention because it is identified as the biogenic key substrate of the organic cation transporter OCTN1 (gene symbol: SLC22A4). OCTN1 seems to have a
25 pivotal protective role in monocytes, which has been associated as a susceptibility factor in the etiopathology of autoimmune disorders such as rheumatoid arthritis and Crohn's disease.

In certain embodiments, the biomass is a mixture of the biomass of *Cordyceps militaris* and the biomass of medicinal mushroom *Ganoderma tsugae* var. *janniae* strain
30 Tay-1 Accession No. CBS 120394 (Lee and Wasser 2009, Patent WO/2009/017462). In particular, the ratio between the two biomasses is 1:1 (v/v).

In certain embodiments, the mixed biomass is rich in nutraceutical agents and biologically active compounds including carbohydrates and proteins rich in essential amino

acids, and comprises vitamins, lipids in essential fatty acids, macro- and microelements, and cordycepin. The mixed biomass has about 20.0% carbohydrates, 51.3 % proteins, and about 5.9% fats of the dry weight of mycelium. The mixed biomass is also rich amino acids, vitamins, fatty acids, macro-elements, microelements and ergothioneine as described
5 below for each of the separate biomasses.

The mixed biomass may be obtained from the fruiting body of the *Cordyceps militaris* strain CBS 132098 and mycelial biomass of the *Ganoderma tsugae* var. *jannieae* Tay-1 (CBS 120394).

The present invention further provides *Cordyceps* extracts having nutraceutical and
10 biological activity comprising an extract of *Cordyceps militaris* strain Phytopharma, Accession No. CBS 132098, and *Cordyceps* and *Ganoderma* mixed extracts having nutraceutical and biological activity comprising of *Cordyceps militaris* strain Phytopharma Accession No. CBS 132098 and *Ganoderma tsugae* var. *jannieae* strain Tay-1 Accession No. CBS 120394 mixture. In one embodiment, the ratio of *Cordyceps militaris* extract to
15 *Ganoderma tsugae* extract is 1:1 (v/v). The *Cordyceps militaris* extract may be obtained from the fruiting body and the culture liquid of submerged mycelium culture of the mushroom, and the *Cordyceps militaris* and *Ganoderma tsugae* mixed extract may be obtained from the mixture of fruiting body of *Cordyceps militaris* and mycelial biomass of the *Ganoderma tsugae*.

20 In certain embodiments, the extracts may be obtained from the organic or aqueous extraction solvent. In particular, the organic extract solvent is selected from ethyl alcohol, ethyl acetate and chloroform.

It has been shown in accordance with the present invention that the extracts (a) inhibits growth of cancer cells; (b) arrests cancer cell cycle; and (c) induces apoptosis in
25 cancer cells. Some of the more important biological activities of the extract 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 (Lee and Wasser (2009), Patent WO/2009/017462). The extracts, or pharmaceutical compositions containing them, may be used for treating NF- κ B-dependent
30 associated diseases selected from cancer, immunological disorders, septic shock, transplant rejection, radiation damage, and reperfusion injuries after ischemia, arteriosclerosis, and neurodegenerative diseases (Lee and Wasser (2009), Patent WO/2009/017462).

In yet an additional aspect, the instant invention is directed to a composition comprising a biomass rich in nutraceutical agents and biologically active substances obtained from the mycelium and/or from the fruiting bodies of *Cordyceps militaris* strain CBS 132098 or a composition comprising a biomass rich in nutraceutical agents and
5 biologically active substances obtained from the fruiting body of *Cordyceps militaris* strain Phytopharma Accession No. CBS 132098 and mycelial biomass of *Ganoderma tsugae* var. *janniae* strain Tay-1 Accession No. CBS 120394.

The composition may alternatively comprise *Cordyceps militaris* and *Ganoderma tsugae* mixed extracts as described herein above.

10 In certain embodiments, the composition comprises a biomass or extract rich in nutraceutical agents and biologically active substances obtained from the fruiting body of *Cordyceps militaris* strain Phytopharma Accession No. CBS 132098 and mycelial biomass of *Ganoderma tsugae* var. *janniae* strain Tay-1 Accession No. CBS 120394.

As shown herein in the examples, the biological activities of *Cordyceps militaris*
15 strain CBS 132098 separately and mixed with *Ganoderma tsugae* var. *janniae* strain Tay-1, CBS 120394 (1:1 v/v), particularly of extracts thereof, include immunostimulatory or antitumor activity.

Apoptosis, or programmed cell death, is a central regulator of normal tissue homeostasis. The physiological "cell suicide" program is essential for the elimination of
20 redundant, damaged, and infected cells. Disturbed apoptosis is involved in the pathogenesis of multiple diseases, especially cancer. The acquisition of mechanisms to evade apoptosis is a hallmark of cancer, with both the loss-of-function of pro-apoptotic signals and gain-of-function of anti-apoptotic mechanisms contributing to tumorigenesis and the cancer phenotype.

25 Most chemotherapies act by the induction of apoptosis. Therefore, the evasion of apoptosis is mainly responsible for the current insufficiency therapies. Tumor cells use multiple pathways to escape apoptosis. Defective apoptotic mechanisms allow genetically unstable cancer cells to avoid elimination and confer resistance to chemotherapy.

As such, modulating the apoptotic pathways likely represents a propitious strategy
30 for inducing tumor-cell death and increasing responses to chemotherapy, radiotherapy, and even targeted therapies.

A most beneficial feature of the extract, as shown in example 6, is that it is capable of inducing apoptosis in prostate and breast cancer cells. Since a multitude of active agents

are comprised in the extract of the present invention that may induce apoptosis by different pathways, it is likely that the cells in a cancer tumor exposed to the extract would not have enough time to develop mutated proteins that would render all these pathways resistant to the active agents in the extract.

5 Thus, in view of the above, in one aspect, the present invention is directed to a pharmaceutical composition comprising a pharmacologically acceptable carrier and the biomasses and/or extracts defined herein above. In particular, the pharmaceutical composition comprises the extracts of the present invention.

10 In some embodiments, the pharmaceutical compositions are useful for treating diseases associated with NF- κ B-dependent defects, such as cancer, immunological disorders, septic shock, transplant rejection, radiation damage, and reperfusion injuries after ischemia, arteriosclerosis, and neurodegenerative diseases (Lee and Wasser (2009), Patent WO/2009/017462). Thus, the pharmaceutical compositions of the present invention may be used for treatment of for example pancreatic cancer, colon cancer, prostate cancer,
15 breast cancer, head and neck cancer, etc.

The term "treating cancer" as used herein refers to the inhibition of the growth of cancer cells. Preferably such treatment also leads to the regression of tumor growth, i.e. to the decrease in size or complete regression of the tumor.

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

Some of the more important nutraceutical agents or biologically active agents in the composition are cordycepin and cordycepic acid. Due to the presence of the nutraceutical and biologically active nucleoside – cordycepin, these compositions have certain activities including immunistimulatory and anti-cancer effects.

25 In addition to being useful in anti-cancer treatment, 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.

30 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 pet food products, e.g., for dogs and cats.

In another aspect, the present invention is directed to a process for producing a biomass rich in vitamins, carbohydrates, proteins, essential amino acids, essential fatty

acids, minerals and microelements from the caterpillar fungus *Cordyceps militaris* strain CBS 132098, said process comprising: cultivating the mushroom *Cordyceps militaris* strain CBS 123098 in submerged culture on nutrient media, isolating the resulting biomass into fine powder. In certain embodiments, the nutrient media used for cultivating the mushroom is of the following composition (g/L of distilled water) glucose, 40.0; peptone 20.0; yeast extract, 5.0; KH_2PO_4 , 1.0; MgSO_4 , 0.5; pH, 6.0.

The invention is further directed to a process for producing a biomass rich in carbohydrates, proteins, essential amino acids, vitamins, essential fatty acids, minerals and microelements from caterpillar medicinal fungus *Cordyceps militaris* strain CBS 132098 and higher Basidiomycetes medicinal mushroom *Ganoderma tsugae* var. *janniae* Tay-1 (CBS 120394), wherein in addition to the biomass of *Cordyceps militaris*, biomass of *Ganoderma tsugae* is produced by cultivating fruiting bodies or mycelial biomass of the medicinal mushroom *Ganoderma tsugae* var. *janniae* 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.

In yet another aspect, the present invention provides a process for producing *Cordyceps* extract having biological activity, wherein the biological activity inhibits growth of cancer cells, arrest cancer cell cycle, and induces apoptosis in cancer cells, said process comprising: cultivating fruiting bodies or mycelial biomass of caterpillar fungus *Cordyceps militaris* strain Phytopharma Accession No. CBS 132098 in submerged culture in nutrient media, isolating the resulting biomass of mushroom from the culture broth, drying and graining said biomass into fine powder which is subjected to solvent extraction, thereby producing *Cordyceps* extract.

In still another aspect, the present invention provides a process for producing the *Cordyceps* and *Ganoderma* mixed extracts having biological activity, wherein the biological activity inhibits growth of cancer cells, arrest cancer cell cycle, and induces apoptosis in cancer cells, said process comprising: cultivating fruiting bodies or mycelial biomass of caterpillar fungus *Cordyceps militaris* strain Phytopharma Accession No. CBS 132098 and *Ganoderma tsugae* var. *janniae* strain Tay-1 Accession No. CBS 120394 in submerged culture in nutrient media, isolating the resulting biomass of mushroom from the culture broth, drying and graining said biomass into fine powder which is subjected to solvent extraction and mixing the *Cordyceps* extract with the *Ganoderma* extract, thereby producing *Cordyceps* and *Ganoderma* mixed extract.

The *Cordyceps* extract or the mixed extract is subjected to organic or aqueous solvent extraction and the solvent is removed by drying (evaporation). The organic solvent may be, but is not limited to, ethyl alcohol, ethyl acetate, chloroform, or a mixture. In certain embodiments, the organic solvent is ethyl alcohol, or ethyl acetate.

- 5 In certain embodiments, the nutrient media for submerged cultivation of mycelium is of the following composition (g/L of distilled water): glucose, 40.0; peptone 20.0; yeast extract, 5.0; KH_2PO_4 , 1.0; MgSO_4 , 0.5; pH, 6.0 (*Cordyceps militaris* strain CBS 132098; and mycelial biomass of *Ganoderma tsugae* var. *jannieae* Tay-1 (CBS 120394) was obtained according to Lee and Wasser (2009), Patent WO/2009/017462. For the fruiting
10 bodies' cultivation of *Cordyceps militaris* strain CBS 132098, the following nutrient media was used: 200 mL potato residue; 2.0g KH_2PO_4 ; 1.0g MgSO_4 ; 1.0 g $(\text{NH}_4)_2\text{C}_6\text{H}_6\text{O}_7$; 30.0 $\text{C}_6\text{H}_{12}\text{O}_6$; 3.0g bacteriological peptone; 50.0 mg Vitamin B₁; 800 mL distilled water, and rice as a solid substrate.

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

15

EXAMPLES

Example 1. Characteristics of the *Cordyceps militaris* strain CBS 132098

Cordyceps militaris has the following characteristics:

- Cordyceps militaris* (L.) Link, Handbuck zur Erkennung der Nutzbarsten und am
20 Häufigsten Vorkommenden Gewächse 3: 347 (1833).
= *Clavaria militaris* L., Sp. pl. 2: 1182 (1753).
= *Clavaria granulosa* Bull., Hist. Champ. France 10: 1999 (1971).
= *Hypoxyton militare* (L.) Mérat, Nouv. Fl. Environs Paris: 137 (1821).
= *Corynesphaera militaris* (L.) Dumort.: 1-10 (1822).
25 = *Xylaria militaris* (L.) Gray, Nat. Arr. Brit. Pl. 1: 510 (1821).
= *Torrubia militaris* (L.) Tul. & C. Tul., Selecta Fungorum Carpologia: Nectrici-Phacidiei-Pezizei 3: 6 (1865).

- Fruit body 15-30 × 2-3 mm, clavate, indistinctly separated into head and stalk.
Head fertile, irregularly clavate to cylindrical, orange-yellow when fresh, rouge and
30 punctuate with projecting peritecia. Stalk sterile, pale orange to ochre, sometimes mottled with orange, smooth distinctly set off from the head. Arising slightly to clustered from stromatized pupal tissue. Hyphae with simple septa, 3-3.8 μm in diam. Asci eight-spored,

breaking apart into many individual ascospores up to $250 \times 5 \mu\text{m}$. Ascospores thin cylindric, hyaline, thin-walled, smooth, $5-6 \times 1.5-2 \mu\text{m}$, lined up on each other, inside the ascus like a chain. Paraphyses absent.

Habitat and general distribution: Mostly on lepidopteran pupae with rare reports on lepidopteran and coleopteran larvae. **Asia:** China, Japan, Korea, Nepal, Taiwan **Europe:** Belgium, Czech Republic, Denmark, France, Germany, Italy, the Netherlands, Russia, Sweden, Switzerland, Ukraine, United Kingdom. **Central America:** Mexico. **North America:** Canada, USA.

Vegetative mycelium in pure culture.

10 The mycelial colony white to cream, dense, not transparent, with well developed aerial mycelium and numerous developed hyphal strands, hyphal formations, and concentric zones.

A novel strain was isolated and deposited at Phyto Pharma Pte Ltd. (Singapore) under the name *Cordyceps militaris* strain Phytopharma. It was also deposited under The Budapest Treaty with the Centraalbureau voor Schimmelcultures (CBS) under Accession No. CBS 132098 (hereinafter *Cordyceps militaris* CBS 132098). The novel strain was isolated as follows:

Fruiting bodies of *Cordyceps militaris* were collected from their natural habitat in China. The mushroom submerged culture productions include 4 steps of culture growth: 20 Museum culture (I) → Intermediate culture (II) → Inoculums culture (III) → Fermentation culture (IV).

Morphological characteristics of *Cordyceps militaris* strain CBS 132098: colony was at first white to cream, with orange tint, dense, downy, mycelium well-developed, slightly appressed to agar; in the center of the colony the mycelium forms dense knots, on 25 the edges of the colony mycelium with powdery (farinose) texture and clearly visible accumulations of conidial sporulation, margin even. Reverse of the colony cracked on the center, achromatic or slightly yellowish (Fig. 1).

Hyphal characteristics: Vegetative hyphae septate, hyaline, branched, thin- and smooth-walled, hyphae $2.3-4.6 \mu\text{m}$ wide. Anamorph stage *Lecanicillium* sp.-like, 30 conidiophores little differentiated from conidiogenous hyphae, commonly arising from aerial hyphae, initially erect with one or two whorls phialides, becoming prostrate and bearing large numbers of phialide whorls or single phialides. Conidia hyaline, single-

celled, from globose to ellipsoid, varying in size from 1.98-2.3 × 1.98-2.3 μm to 4.6-6.92 μm, produced in slimy heads.

Example 2. Submerged cultivation of mycelial biomass of *Cordyceps militaris* strain

5 CBS 132098 in Erlenmeyer flasks

The general scheme of mushroom submerged culture mycelium (SCM) production includes 4 steps of culture growth:

Museum culture (I) → Intermediate culture (II) → Inoculums culture (III) → Fermentation culture (IV).

10 Three types of culture media are used for SCM production: standard agar medium (steps I and II), liquid standard inoculums medium (steps III), and fermentation medium (step IV). Museum cultures are developed on agar slants in tubes; intermediate cultures are developed on agar slants in tube or Petri dishes. Pre-inoculums and inoculums cultures are developed in 250-mL Erlenmeyer flasks using rotary shaker. Fermentation cultures are
15 developed in 2L Erlenmeyer flasks using a rotary shaker.

For the first pre-inoculums culture, 250-mL Erlenmeyer flask is inoculated with three-four week 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
20 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, 40.0; peptone 10.0; yeast extract, 3.0; KH₂PO₄, 1.0; MgSO₄, 0.5. The cultivation of inoculated flasks is carried out on a rotary shaker at 130 rpm and 24 ± 1 °C for 7-14 days. At the end
25 of cultivation, 1 mL of sample is taken from the culture for microscopic observation of culture purity.

After 7-14 days of cultivation, mycelial biomass (pellets) was homogenized 2 × 15 seconds using the Waring Laboratory Blender (Waring, USA) and used as inoculum culture for growth in 2L-Erlenmeyer flasks with 1L of working volume on the synthetic
30 medium consisting of the following components (g/L of distilled water): glucose, 40.0; peptone 20.0; yeast extract, 5.0; KH₂PO₄, 1.0; MgSO₄, 0.5; pH, 6.0. The cultivation of inoculated flasks is carried out on a rotary shaker at 130 rpm and under three different

temperature conditions 15°C, 20°C, and 25°C for 14 days. At the end of cultivation, 1 mL of sample is taken from the culture for microscopic observation of culture purity.

Cultivation was carried out using three different temperature conditions with intervals of 5 degrees: 15°C, 20°C, and 25°C. Results showed that dry mycelia yield varies from an average of 12.8 to 14.78 g/L (Fig. 2). The highest yield was 14.78 mg/L at 20 °C, which in general, corresponds to the natural growth conditions of *Cordyceps militaris*. The highest dry mycelium weight (14.78 g/L) and cordycepin yield (0.182%) were obtained at a temperature of 20°C and pH 6.0.

Mycelial biomass of *Ganoderma tsugae* var. *jannieae* Tay-1 was obtained according to Lee and Wasser (2009), Patent WO/2009/017462.

Example 3. Determination of the cordycepin content in dried fruiting bodies of *Cordyceps militaris* strain CBS 132098

To determine cordycepin content in dried fruiting bodies of *Cordyceps militaris* strain CBS1320 the standard cordycepin (Sigma-Aldrich Inc., USA), Soxhlet extraction, and high performance thin layer chromatography (HPTLC) were used.

Materials and Methods

3.1. Reagents. Cordycepin standard was from Sigma-Aldrich Inc. USA. Chloroform, methanol and water were AR grade. A stock solution (1 mg/mL) of cordycepin was prepared by dissolving 25 mg cordycepin standard, accurately weighed, in 10 mL water in a 25-mL volumetric flask and diluted to volume with water. Standard solutions containing 200, 250, 300, 350, 400, 450, and 500 µg/mL were prepared by diluting 2, 2.5, 3.0, 3.5, 4.0, 4.5, and 5.0 mL of the stock solution with 10 mL separately.

3.2. Soxhlet extraction. The powder (5 g) of fruiting bodies was extracted with chloroform: methanol (2:1 v/v) using Soxhlet extraction for 4 hours. The liquid extracts were membrane filtered and concentrated under reduced pressure. The residue was dissolved in 5 mL methanol and used for quantification of cordycepin.

3.3. HPTLC analysis. HPTLC analysis was carried out on a CAMAG HPTLC system. Chromatography was performed on silica gel 60 F₂₅₄ TLC pre-coated aluminum plates (20 × 10 cm, layer thickness 0.2 mm, E. Merck) were used as a stationary phase. The standard (1.5 - 4.5 µL) and samples (10 - 15 µL) were applied to the plates as 6 mm bands

(slit dimension 5.00×0.45 mm, micro), application position 8 mm, starting 15 mm from the edge of the plate, by means of a Linomat 5 applicator equipped with 100 μ L syringe. The plates were developed to a distance of 80 mm in a twin trough chamber previously saturated with mobile phase chloroform: methanol (3:1 v/v) for 20 minutes. After removal
5 from the chamber, the plates were completely dried in air at ambient temperature, scanned and quantified at 263 nm in absorbance/reflectance mode the CAMAG TLC scanner 3 using winCATS 3.2.1 software incorporating track optimizing option. The standard plot of cordycepin was established by plotting the peak area Vs concentration (ng/ μ L) corresponding to each spot. The analysis was performed in triplicate, and the results were
10 averaged.

Results

The mobile phase of chloroform: methanol (3:1 v/v) for fruiting bodies afforded a
15 good resolutions with R_F 0.49. The spectrum of cordycepin exhibited maxima at 263 nm.

The identity of cordycepin in *Cordyceps militaris* strain CBS 132098 extract was confirmed by an overlaying absorption spectra acquired for standard cordycepin. The purity of the cordycepin bands in sample extracts was also confirmed by comparing absorption spectra acquired at the start, middle, and end of the bands. HPTLC analysis of
20 the sample was carried out using the procedure described by Varshney et al. (2011), and cordycepin content (% and mg/g) was determined. HPTLC analysis showed the presence of 0.110% (~1.101 mg/g; Fig. 3) cordycepin in fruiting bodies' biomass of *Cordyceps militaris* strain CBS 132098.

25 Example 4. Determination of the cordycepin content in mycelial biomass of *Cordyceps militaris* strain CBS 132098

To determine the cordycepin content in mycelial biomass of *Cordyceps militaris* strain CBS 132098 the same equipment and methods as for fruiting bodies biomass of *Cordyceps militaris* CBS 132098 were used. Cordycepin standard was from Sigma-Aldrich
30 Inc. USA. A stock solution (1 mg/mL) of cordycepin was prepared by dissolving 25 mg cordycepin standard, accurately weighed, in 10 mL water in a 25-mL volumetric flask and diluting to volume with water. Standard solutions containing 200, 250, 300, 350, 400, 450, and 500 μ g/mL were prepared by diluted 2, 2.5, 3.0, 3.5, 4.0, 4.5, and 5.0 mL of the stock

solution with 10 mL separately. The powder (10.8 g) of mycelial biomass was extracted with chloroform: methanol (2:1 v/v) using Soxhlet extraction for 4 hours. The liquid extracts were membrane filtered and concentrated under reduced pressure. The residue was dissolved in 5 mL methanol and used for quantification of cordycepin.

5 HPTLC analysis was carried out on a CAMAG HPTLC system. Chromatography was performed on silica gel 60 F₂₅₄ TLC pre-coated aluminum plates (20 × 10 cm, layer thickness 0.2 mm, E. Merck) were used as a stationary phase. The standard (1.0 – 4.5 µL) and samples (4-5 µL) were applied to the plates as 6 mm bands (slit dimension 5.00 × 0.45 mm, micro), application position 8 mm, starting 15 mm from the edge of the plate, by
10 means of a Linomat 5 applicator equipped with 100 µL syringe. The plates were developed to a distance of 80 mm in a twin trough chamber previously saturated with mobile phase chloroform: methanol (6:1 v/v) for 20 minutes. After removal from the chamber, the plates were completely dried in air at ambient temperature, scanned and quantified at 263 nm in absorbance/ reflectance mode the CAMAG TLC scanner 3 using winCATS 3.2.1 software
15 incorporating track optimizing option. The standard plot of cordycepin was established by plotting the peak area Vs concentration (ng/µL) corresponding to each spot.

Results. The mobile phase of chloroform: methanol (6:1 v/v) for mycelial biomass afforded a good resolutions with R_F 0.21. The spectrum of cordycepin exhibited maxima at 263 nm.

20 The identity of cordycepin in *Cordyceps militaris* strain CBS 132098 extract was confirmed by overlaying absorption spectra acquired for standard cordycepin. The purity of the cordycepin bands in sample extracts was also confirmed by comparing absorption spectra acquired at the start, middle, and end of the bands. HPTLC analysis of the sample was carried out using the procedure described by Varshney et al. (2011), and cordycepin
25 content (% and mg/g) was determined. HPTLC analysis showed the presence of 0.182% (~1.827 mg/g; Fig. 4) cordycepin in mycelial biomass of *Cordyceps militaris* strain CBS 132098.

The cordycepin content is lower in the fruiting body biomass compared to the mycelial biomass of *Cordyceps militaris* strain CBS 132098.

30

Example 5. Determination of the ergothioneine content in mycelial biomass of *Cordyceps militaris* strain CBS 132098

The ergothioneine content was analyzed following the method of Dubost et al. (2006) with some modification.

Materials and Methods

- 5 Dried mushroom powder (1 g) was added to 20 mL extracting solution (10 mM 1,4-Dithiothreitol, 100 μ M betaine, 100 μ M 2-mercapto-1-methylimidazole in 70% ethanol) and vortexed for 90 s. After 4 mL of 1% sodium dodecyl sulphate solution was added and the mixture was centrifuged at 25°C and 3000g for 10 min. The supernatant was then rotary evaporated at 40°C to 5 mL and filtered through a 0.45 μ m CA non-sterile filter.
- 10 The HPLC system consisted of a Hitachi L-2130 pump, a Hitachi L-2455 Diode Array Detector, and a Luna 5 μ PFP(2) 100A column (4.6 $\times$ 250 mm, Phenomenex Inc., Ca, USA). The mobile phase was 500 mM sodium phosphate in water with 3% acetonitrile and 0.1% triethylamine adjusted to a pH of 7.3 with at a flow rate of 1 mL/min and UV detection at 254 nm. Ergothioneine was quantified by the calibration curve of the authentic
- 15 standard (Sigma).

Results. According to our research the content of ergothioneine in the mycelial biomass of *Cordyceps militaris* is 130.65 mg/kg, which correspond to fourth levels Chen et al's gradation (Chang et al. 2012).

- 20 The concentration of ergothioneine had been found to be 1–2 mM in human and mammalian tissues, suggesting that ergothioneine might serve as a non-toxic antioxidant *in vivo*. Cells lacking ergothioneine were readily susceptible to oxidative stress, and thereby resulting in increased mitochondrial DNA damage, protein oxidation and lipid peroxidation. Since mushrooms are an abundant source of antioxidants, including ergothioneine, it is another reason to incorporate mushrooms into the human diet.

25

Example 6. General chemical composition of *Cordyceps militaris* strain CBS 132098 separately and with *Ganoderma tsugae* var. *janniae* Tay-1, CBS 120394 (1:1 v/v) combination

- 30 The chemical composition of *Cordyceps militaris* strain CBS 132098 was determined in the mushroom fruit bodies and mycelium. Additionally, the chemical composition of mixture (1:1 v/v) of the fruit bodies of *Cordyceps militaris* strain CBS 132098 and mycelium of *Ganoderma tsugae* var. *janniae* strain Tay-1 (CBS 120394) were determined in order to make a comparison of two biologically active medicinal

mushrooms. The following contents were examined: moisture, ash, lipids, carbohydrates, amino acids, fatty acids, vitamins, and minerals.

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

Cordyceps militaris strain CBS 132098 separately and mixed with *Ganoderma tsugae* var. *janniae* strain Tay-1, CBS 120394 (1:1 v/v) 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 medicinal purposes, but they can indicate the contents of the composition regarding vitamins, minerals, protein, 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, or potassium per reference amount. "Good 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 contain 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 30-90 g wet biomass and approximately 2.1-3.6 g mushroom extract. Thus, the results below show that a similar dosage of *Cordyceps militaris* strain CBS 132098 fruiting bodies or mycelial biomass, or extracts or its mixture with *Ganoderma tsugae* var. *janniae* Tay-1 (CBS 120394) would bestow the consumer high levels of a range of important nutrients.

Materials and methods

Proximate analysis. The proximate composition the fruiting body and mycelia of *Cordyceps militaris* strain CBS 132098 separately and mixed with *Ganoderma tsugae* var. *janniae* Tay-1, CBS 120394 (1:1 v/v) were determined according to the methods of Association of Official Analytical Chemists (AOAC) (1990). The total carbohydrate content was calculated by subtracting the contents of ash, fat, and protein from 100, and expressed as % of dry mass. Total reducing sugars (RS) were determined using the 3,5-

dinitrosalicylic acid (DNS) method as described by James (1995). The energy provided by 100 grams of dry mushrooms is calculated as follows:

$$\text{Energy (kcal/100g)} = \text{RS} \times 4 + \text{Fat} \times 9 + \text{Protein} \times 4$$

5 *Macro- and microelements analysis.* Powder of dried fungi samples were analyzed by inductively coupled plasma optical emission spectroscopy (ICP-OES) (Thermo Electron Corporation iCAP 600 series).

10 *Amino acid analysis.* The amino acid composition was determined by hydrolyzing powder of dry biomass with 6N HCl for 24 h at 105°C, then deriving the amino acids in a Water PICO-TAG™ Work Station. The derivative amino acids were analyzed by liquid chromatography consisting of Waters 515 pumps, Water 486 detector, and Reodyne injector, equipped with Water PICO-TAG™ column (3.9 ×150 mm). Amino acids were identified by comparing retention times and areas to an authentic standard mixture (Hur 2008).

15 *Fatty acid analysis.* Fatty acids were extracted from dried samples using the method of Hamilton and Hamilton (1992). Fatty acids were determined as fatty acid methyl esters by gas chromatography using Hewlett-Packard, Model 5890A – 5971A gas chromatography (Germany) equipped with 100 M x 0.25 mm and 0.20 µm film thickness Fused Silica Capillary Column (SP-2560, Supelco Ltd.). The sample was injected into a gas chromatograph using Hewlett Packard HP 7673 GC/SFC Autoinjector Model 18593B
20 (Agilent Co.). The temperature program was 140°C for 5 min, increased to 240°C at 4°C/min, and maintained at 240°C for 15 min. Helium was used as the carrier gas and was maintained at a flow rate of 20 cm/s. The injection port and the flame ionization detector oven temperatures were 260°C. Fatty acid methyl esters were identified by comparing retention times to an authentic standard mixture (Supelco® 37 Component FAME Mix,
25 Sigma).

Vitamin analyses. The HPLC system (Hewlett-Packard CA, USA) consisted of an HP 1050 system controller pump, an HP 1050 Series degassing device, an HP 1100 autosampler with 20 µl fixed loop injector, and an HP 1050 Series UV detector. Data acquisition was accomplished by a Chemstation system HP 3365-II. The separation was
30 performed on a Tracer Spherisorb ODS 2 C₁₈ column 250 × 4.6 mm, 5 µm (Teknokroma, Barcelona, Spain), with a matching guard cartridge. Analyses were carried out isocratically at room temperature at a flow rate of 1 mL/min. The total run time required was less than 55 min.

6.1. **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. *Cordyceps militaris* strain CBS 132098 biomass contain high levels of some vitamins of importance for the individual's health, such as vitamin B₂ (riboflavin), B₃ (niacin), A (retinol), C (ascorbic acid), E (tocopherol), and D-mannitol (cordycepic acid). In the combination of *Cordyceps militaris* strain CBS 132098 and the *Ganoderma tsugae* var. *janniae* strain Tay-1, CBS 120394 (1:1 v/v), content of vitamins different comparing with *Cordyceps militaris* strain CBS 132098 alone, due to the mixture of two mushrooms with high medicinal value. The mixture of *Cordyceps militaris* strain CBS 132098 and the *Ganoderma tsugae* var. *janniae* strain Tay-1, CBS 120394 (1:1 v/v) contain high levels of some vitamins of importance the individual's health, such as vitamin B₁ (thiamine), B₂ (riboflavin), B₃ (niacin), Q₉ (ubiquinone), B₁₂ (cyanocobalamin), B₅ (pantothenic acid), B₉ (folic acid), Pro-D (ergosterol), A (retinol), C (ascorbic acid), E (tocopherol), and D-mannitol (cordycepic acid).

The contents of vitamins in the fruit body, mycelium of *Cordyceps militaris* strain CBS 132098 separately and in the mixture of *Cordyceps militaris* strain CBS 132098 and the *Ganoderma tsugae* var. *janniae* strain Tay-1, CBS 120394 (1:1 v/v) are shown in Table 1. Vitamins are more actively accumulated in mycelia, while the fruit bodies contain considerably less vitamins than mycelium (except for E and C). The amount of B₃ is 3 times less in fruiting bodies; B₂ is 2 times less than in mycelium. D-mannitol is relatively abundant in mycelium than in fruiting body. According to our results, a mixture of *Cordyceps militaris* and *Ganoderma tsugae* var. *janniae* is richer in vitamins than *Cordyceps militaris* only.

In summary, the fruiting bodies and mycelia of *Cordyceps militaris* CBS 132098 separately and mixed with *Ganoderma tsugae* var. *janniae* Tay-1, CBS 120394 (1:1 v/v) 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.

Table 1. Vitamin content of submerged mycelial biomass and fruiting bodies of *Cordyceps militaris* strain CBS 132098 separately and in the mixture of *Cordyceps militaris* strain CBS 132098 and the *Ganoderma tsugae* var. *janniae* strain Tay-1, CBS 120394 (1:1 v/v)

5

Content	Units	<i>Cordyceps militaris</i>		<i>Cordyceps militaris</i> and <i>Ganoderma tsugae</i> var. <i>janniae</i> mixture (1:1 v/v)
		Fruiting body	Mycelium	
Vitamin B ₁ (Thiamine)	mg/100g	--	--	2.61
Vitamin B ₂ (Riboflavin)	mg/100g	0.16	0.32	1.19
Vitamin B ₃ (Niacin)	mg/100g	4.9	15.2	19.5
Vitamin Q ₉ (Ubiquinone)	mg/100g	--	--	2.37
Vitamin B ₁₂ (Cyanocobalamin)	mg/100g	--	--	0.1
Vitamin B ₅ (Pantothenic acid)	mg/100g	--	--	99.0
Vitamin B ₉ (Folic acid)	mg/100g	--	--	0.041
Vitamin Pro-D (Ergosterol)	mg/100g	--	--	2.95
Vitamin A (Retinol)	mg/100g	96.0	100	48
Vitamin C (Ascorbic acid)	mg/100g	<2.0	--	68.7
Vitamin E (α - Tocopherol)	mg/100g	3.6	1.3	1.8
D-Mannitol (Cordycepic acid)	g/100g	4.7	5.2	2.35

6.2. Proximate composition. Due to the proximate composition of fruiting body and mycelium of *Cordyceps militaris* CBS 132098 and the mixture with *Ganoderma tsugae* var. *janniae* Tay-1, CBS 120394 (1:1 v/v), data are shown in Table 2. The moisture content in fruiting body of *Cordyceps militaris* strain CBS 132098 separately and in the mixture with *Ganoderma tsugae* var. *janniae* strain Tay-1 (CBS 120394) were in the range 5.7-8.1%. For the mycelial biomass of the *Cordyceps militaris* strain CBS 132098 the moisture content was 13.1%, which is dependent on drying conditions.

Based on dry weight, the mycelium of *Cordyceps militaris* strain CBS 132098 accumulate 39.5% of protein, while fruit bodies contain much more, namely, 59.8% of their dry weight. On the contrary, carbohydrates make up 39.6% of dry weight of mycelium, and only 29.1% of fruiting bodies (1.3 times less). The ash content in mycelium was a little bit higher (5.7%) than in fruiting bodies (5.1%). We found 8.8% dry weight of crude fat in the fruiting bodies and 2.2% crude fat dry weight in cultivated mycelium (4 times less). No significant differences in proximate composition were found between fruiting bodies of

Cordyceps militaris strain CBS 132098 separately and in a mixture with *Ganoderma tsugae* var. *jannieae* Tay-1, CBS 120394 (1:1 v/v).

Table 2. Proximate composition submerged mycelial biomass and fruiting bodies of *Cordyceps militaris* strain CBS 132098 separately and in the mixture of *Cordyceps militaris* strain CBS 132098 and the *Ganoderma tsugae* var. *jannieae* strain Tay-1, CBS 120394 (1:1 v/v)

Component	Content (% of dry weight)		
	<i>Cordyceps militaris</i>		<i>Cordyceps militaris</i> and <i>Ganoderma tsugae</i> var. <i>jannieae</i> mixture (1:1 v/v)
	Fruiting body	Mycelium	
Protein*	59.8	39.5	51.3
Moisture	5.7	13.1	8.1
Ash	5.1	5.7	6.2
Fat & Oils	8.8	2.2	5.9
Carbohydrate**	29.1	39.6	20.0
Energy***	401	336	372

* - Protein was calculated as N $\times$ 6.25.

10 ** - Carbohydrate was calculated from the moisture, ash, protein, fat & oils in the sample.

*** - Energy was calculated from the content of protein, carbohydrate and fat & oils in the sample, and expressed as Kcal/100g.

15 According to the energy values of fruiting bodies of *Cordyceps militaris* strain CBS 132098 (401 Kcal/100g) separately and in a mixture with *Ganoderma tsugae* var. *jannieae* strain Tay-1, CBS 120394 (1:1 v/v) (372 Kcal/100g) is higher than mycelium of *Cordyceps militaris* strain CBS 132098 (336 Kcal/100g).

20 6.3. Amino acid composition. Analysis of amino acids shows that 17 amino acids are found in fruiting bodies and 15 in mycelial biomass of *Cordyceps militaris* strain CBS 132098 (Table 3). The mycelium lack cysteic acid and methionine sulfon. The biomass of fruit bodies is rich in glutamic acid 5.65% from the whole protein content. The four most abundant amino acids in the mycelium biomass are alanine (3.61%), glycine (3.12%), arginine (2.76%), and aspartic acid (1.92%). The concentration of all amino acids, except
25 for alanine, are much higher in fruiting bodies than in mycelial biomass. In a mixture of the

Cordyceps militaris strain CBS 132098 with *Ganoderma tsugae* var. *janniae* strain Tay-1, CBS 120394 (1:1 v/v), amino acids present at concentrations of more than 3% were glutamic acid (5.40%), valine (4.78%), aspartic acid (4.58%), lysine (4.17%), glycine (3.73%), threonine (3.18%), and arginine (3.13%).

5

Table 3. Amino acid composition in fruiting bodies and submerged mycelial biomass of *Cordyceps militaris* strain CBS 132098 separately and in the mixture of *Cordyceps militaris* strain CBS 132098 and the *Ganoderma tsugae* var. *janniae* strain Tay-1, CBS 120394 (1:1 v/v)

Amino acid	Content (% of dry weight)		
	<i>Cordyceps militaris</i>		<i>Cordyceps militaris</i> and <i>Ganoderma tsugae</i> var. <i>janniae</i> mixture (1:1 v/v)
	Fruiting body	Mycelium	
Cysteic acid	1.00	--	0.88
Aspartic acid	5.49	1.92	4.58
Methionine sulfon	1.14	--	0.86
Threonine	3.87	1.13	3.18
Serine	2.43	1.37	2.13
Glutamic acid	5.65	1.82	5.40
Proline	2.59	1.87	2.53
Glycine	4.21	3.12	3.73
Alanine	2.48	3.61	2.48
Valine	5.45	1.36	4.78
Isoleucine	1.98	0.46	1.74
Leucine	3.13	1.85	2.63
Tyrosine	4.51	0.45	2.90
Phenylalanine	2.66	1.68	2.05
Lysine	5.53	1.29	4.17
Histidine	1.54	0.29	1.35
Arginine	3.73	2.76	3.13
Total	57.39	24.98	48.52

10

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 high. The proteins of the fruiting body contain 9 out of 10 essential amino acids: methionine, threonine, valine, isoleucine, leucine, phenylalanine, lysine, histidine and arginine. The protein of the mycelium, lacking methionine, contains eight essential amino acids, all of them in lower quantities than in the fruiting body. The protein of the mixture of *Cordyceps militaris* strain CBS 132098 with *Ganoderma tsugae* var. *janniae* strain Tay-1 (CBS 120394) contain also 9 out of 10 essential amino acids but in lower quantities than in fruiting bodies of *Cordyceps militaris* strain CBS 132098 only.

6.4. Lipid and fatty acid composition. Fatty acids, 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).

The fruiting bodies are richer in lipids: 8.8% versus 2.2% in mushroom mycelium.

The fatty acids in the analyzed fruiting bodies and mycelia of *Cordyceps militaris* strain CBS 132098 and in the mixture with *Ganoderma tsugae* var. *janniae* strain Tay-1 (CBS 120394) preparations are both saturated ($C_{16:0}$ palmitic acid, $C_{18:0}$ stearic acid, and $C_{20:1}$ arachidic acid) and monounsaturated ($C_{16:1}$ palmitoleic acid, $C_{18:1n7}$ trans-vaccenic acid, and $C_{18:1n9c}$ oleic acid) or polyunsaturated ($C_{18:2}$ linoleic acid and $C_{18:3}$ linolenic acid) fatty acids (as shown in Table 4).

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 as $C_{16:1}$ palmitoleine and $C_{18:1n7}$ trans-vaccenic acid. A high nutritional quality of the mushroom is made evident by the presence of unsaturated $C_{18:2}$ linoleic acid and $C_{18:3}$ linolenic acid, which belong to the indispensable (essential) fatty acids.

As shown in Table 4, both quality and quantity of fatty acids differ between mushroom fruit body and mycelium. The fatty acids of fruit bodies are dominated by

monounsaturated C_{18:1n9c} oleic acid (32.0%) and polyunsaturated C_{18:3} linolenic acid (44.2%). The fatty acids of mycelium are dominated by saturated C_{16:0} palmitic acid

Table 4. Content of fatty acids in fruiting bodies and submerged mycelial biomass and of *Cordyceps militaris* strain CBS 132098 separately and in the mixture of *Cordyceps militaris* strain CBS 132098 and the *Ganoderma tsugae* var. *janniae* strain Tay-1, CBS 120394 (1:1 v/v)

Fatty acid	Content *, (% of total fatty acids)		
	<i>Cordyceps militaris</i>		<i>Cordyceps militaris</i> and <i>Ganoderma tsugae</i> var. <i>janniae</i> mixture (1:1 v/v)
	Fruiting body	Mycelium	
C16:0 Hexadecanoic acid (Palmitic acid)	8.8	23.5	8.6
C16:1 cis-9-Hexadecanoic acid (Palmitoleic acid)	2.5	2.1	2.3
C18:0 Octadecanoic acid (Stearic acid)	1.1	5.5	1.5
C18:1n7 11-trans-Octadecanoic acid (Trans-Vaccenic acid)	--	2.2	--
C18:1n9c cis-9-Octadecanoic acid (Oleic acid)	32.0	24.9	30.6
C18:2 cis-9, cis-12-Octadecanoic acid (Linoleic acid)	11.4	40.7	16.2
C18:3 (n-3) cis-9, cis-12, cis-15-Octadecanoic acid (Linolenic acid)	44.2	0.9	40.9
C20:0 Eicosanoic acid (Arachidic acid)	--	0.2	--
Total	100	100	100

*- Relative percentage from the oil

- 10 (23.5%) and polyunsaturated C_{18:2} linoleic acid (40.7%). Arachidic acid is specific to the fatty acid composition of mycelium.

There is no significant difference between fatty acids composition of *Cordyceps militaris* strain CBS 132098 separately and the mixture with *Ganoderma tsugae* var. *janniae* strain Tay-1, CBS 120394 (1:1 v/v).

15

6.5. Macro- and microelements composition. The mineral nutrients are defined as all of the inorganic elements or organic molecules that are required for life. As far as human nutrition is concerned, the inorganic nutrients include water, sodium, potassium, calcium, phosphorus, sulfur, magnesium, iron, copper, zinc, manganese, selenium, and

molybdenum. *Cordyceps militaris* strain CBS 132098 fruiting body and mycelium are rich in most of these nutrients and therefore possess a very high nutritional value.

Abnormally low mineral concentration may lead to impairment in a function dependent on the mineral. Iron deficiency (or "sideropenia") is the most common
5 nutritional deficiency and may cause anemia with symptoms including fatigue, pallor, irritability, and weakness.

The investigated mushroom was analyzed for macro-elements: aluminum, calcium, chromium, iron, potassium, magnesium, manganese, sodium and phosphorus; and
10 microelements: silver, arsenic, boron, barium, beryllium, cadmium, cobalt, copper, mercury, lithium, molybdenum, nickel, lead, sulfur, selenium, tin, strontium, titanium, vanadium, and zinc.

The macro- and microelements composition is also different in the fruiting bodies and in the mycelium (Table 5) preparations. In fruiting bodies, boron, calcium, copper, iron, potassium, magnesium, manganese, and sulfur dominate.

15

Table 5. Macro- and microelements composition in fruiting bodies and submerged mycelial biomass of *Cordyceps militaris* strain CBS 132098 alone and in the mixture of *Cordyceps militaris* strain CBS 132098 and *Ganoderma tsugae* var. *janniae* strain Tay-1, CBS 120394 (1:1 v/v)

Macro- and microelements*	Content (mg/kg)		
	<i>Cordyceps militaris</i>		<i>Cordyceps militaris</i> and <i>Ganoderma tsugae</i> var. <i>janniae</i> mixture (1:1 v/v)
	Fruiting body	Mycelium	
Silver (Ag)	<0.2	<0.2	<0.2
Aluminum (Al)	1.0	1.0	2.0
Arsenic (As)	0.4	<0.2	0.3
Boron (B)	5.0	1.0	3.0
Barium (Ba)	0.3	<0.1	1.0
Beryllium (Be)	<0.2	<0.1	<0.2
Calcium (Ca)	797.0	11	654.0
Cadmium (Cd)	0.2	<0.1	0.1
Cobalt (Co)	<0.2	<0.2	<0.2
Chromium (Cr)	0.3	0.6	0.4
Copper (Cu)	17.0	0.7	12.0
Iron (Fe)	31.0	9.0	32.0
Mercury (Hg)	<0.5	<0.5	<0.5
Potassium (K)	15,938	12,183	9,999
Lithium (Li)	0.2	<0.2	<0.2
Magnesium (Mg)	4,227	3,414	9,722
Manganese (Mn)	7.0	0.3	6.0
Molybdenum (Mo)	0.2	0.4	≤0.2
Sodium (Na)	171.0	1,567	605.0
Nickel (Ni)	0.2	0.3	0.5
Phosphorus (P)	7,196	14,293	9,865
Lead (Pb)	<0.2	<0.2	≤0.2
Sulfur (S)	5,088	2,558	4,559
Selenium (Se)	0.4	<0.5	0.4
Tin (Sn)	0.6	0.5	0.5
Strontium (Sr)	1	<0.2	1
Titanium (Ti)	<0.2	0.2	0.2
Vanadium (V)	<0.2	0.3	<0.2
Zinc (Zn)	--	10.0	--

5 *ICP scan – results including "<" = below detection limits

10 Mycelium accumulates more chromium, molybdenum, sodium, and phosphorus than the fruiting body. Zinc is present only in mycelium; it is not detected in fruiting bodies of *Cordyceps militaris* strain CBS 132098 or in the mixture with *Ganoderma tsugae* var. *janniae* strain Tay-1, CBS 120394 (1:1 v/v). The mixture of *Cordyceps militaris* strain CBS 132098 with *Ganoderma tsugae* var. *janniae* strain Tay-1, CBS 120394 (1:1

v/v) contains higher amounts of aluminum, barium, iron, and magnesium (Table 5) than the fruiting bodies and mycelium of *Cordyceps militaris* strain CBS 132098. Some elements were found only in trace amounts or were below the sensitivity limit of the measurements.

5

Example 7. Anticancer activity of extracts from fruiting bodies of *Cordyceps militaris* strain CBS 132098 and extracts from mixture of *Cordyceps militaris* strain CBS 132098 and the mycelial biomass of *Ganoderma tsugae* var. *janniae* strain Tay-1, CBS 120394 (1:1 v/v)

10

For the screening of anti-cancer activity of fruiting bodies of *Cordyceps militaris* strain CBS 132098 and in a mixture with *Ganoderma tsugae* var. *janniae* Tay-1, CBS 120394, four different solvents were used for extraction: water, ethyl acetate, ethanol, and chloroform. The yields of extracts received from mushroom biomasses significantly depended on the solvent used.

15

Materials and methods

7.1 Organisms and cultivation conditions. Fruiting bodies of *Cordyceps militaris* strain CBS 132098 were cultivated on white rice. Artificial cultivation of fruiting bodies of *Cordyceps militaris* can be divided into the following stages: inocula preparation → solid-state substrate preparation → inoculation of solid-state substrate → cultivation → grass → harvest.

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For the fruiting bodies' cultivation, the inocula were prepared by growing fungi on a rotary shaker at 130 rpm in 500-mL Erlenmeyer flasks containing 300 mL of synthetic medium. For liquid cultivation, nutrient media was prepared using the following composition: 200 mL potato residue; 2.0g KH₂PO₄; 1.0g MgSO₄; 1.0 g (NH₄)₂C₆H₆O₇; 30.0 C₆H₁₂O₆; 3.0g bacteriological peptone; 50.0 mg Vitamin B₁; 800 mL distilled water. Cultivation was carried out at 25±1°C on a rotary shaker for 7 days. For solid-state cultivation, 30.0 g white rice were put into the 350-mL glass jar and 35 mL nutrient media were added, which was used for inocula preparation in the first stage. Jars were sealed with a polypropylene plastic film and were sterilized at 121°C for 30 minutes. About 5 mL of inocula were transferred into the each jar. Inoculated jars were transferred into the growth chamber at temperatures 15°-18°C and 60% humidity for 5 days. After 5 days the

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temperature was raised to 20-23°C for about 15 days incubation. About 7-10 days, day/night conditions prevailed: day with light illumination to 1000 lux, duration 14 hours, and 20°C temperature and night condition: darkness, duration 10 hours and temperature 10°C. Day-by-day day lighting increased slowly, illumination to 1000 lux, and in this stage mycelium gradually changed color from white to orange and mycelium primordium formation was stimulate. After growth of granular primordia, the *Cordyceps militaris* fruiting bodies appeared, ventilation with fresh air was used, room temperature at 18 C - 22 C was maintained, relative humidity was kept at 80%-85%. After 40-60 days of cultivation, mature fruiting bodies grew up to 7 - 8 cm, generally growth no longer occurred and therefore the fruiting bodies were harvested.

For submerged cultivation, the inocula were prepared by growing fungi on a rotary shaker at 130 rpm in 250-mL Erlenmeyer flasks containing 100 mL of synthetic medium (g/L of distilled water): glucose, 40.0; peptone 10.0; yeast extract, 3.0; KH_2PO_4 , 1.0; MgSO_4 , 0.5; pH, 6. After 5-7 days of cultivation, mycelial pellets were harvested and homogenized with a Warning laboratory blender.

Submerged cultivation was carried out at $20 \pm 1^\circ\text{C}$ on a rotary shaker (130 rpm) in 2L of the synthetic medium. The medium consisted of (g/L of distilled water): glucose, 40.0; peptone 20.0; yeast extract, 5.0; KH_2PO_4 , 1.0; MgSO_4 , 0.5; pH, 6.0.

Mycelial biomass of *Ganoderma tsugae* var. *janniae* Tay-1 (CBS 120394) was obtained according to Lee and Wasser (2009), Patent WO/2009/017462.

7.2. Biomass dry weight determination. After 14 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.

7.3. Preparation of mushroom extracts.

Dried fruiting body or mycelium biomass was homogenized to a fine powder. In order to receive low-molecular-weight fungal metabolites with different chemical strictures and activities, the powder was extracted three times with the different organic solvents (50 mL/g dry weight): chloroform (Chl), ethanol (Eth), ethyl acetate (EA), and water (Wtr):

Water extract – powder of dry fruiting body or mycelia biomass extracted for 3 h with distilled H_2O (1 g/10mL) at 80°C (using a water bath). After extraction, insoluble compounds were separated by centrifugation at 6000 rpm at 4°C for 15 min and filtrated through the Whatman® filter paper N 1. Filtrates (supernatant) were evaporated.

Ethanol extract - powder of dry fruiting body or mycelia biomass was extracted on the rotary shaker at 150 rpm with ethanol (80%) (1 g/10mL) at 27°C for 3 h. After extraction, insoluble compounds were separated by centrifugation at 6000 rpm for 15 min at 4°C and filtrated through the Whatman® filter paper N 1, and the organic solvents were evaporated from the extracts.

Chloroform and ethyl acetate extracts - powder of dry fruiting body or mycelia biomass was extracted on the rotary shaker at 150 rpm with chloroform or ethyl acetate (1 g/10mL) at 27°C for 3 h. After extraction, insoluble compounds were separated by centrifugation at 6000 rpm for 15 min at 4°C and filtrated through the Whatman® filter paper N 1, and the organic solvents were evaporated from the extracts.

The crude extracts were collected in previously weighed glass tubes, then weighed again in order to measure the exact weight of the obtained fungal extracts (Yassin et al. 2003). Stock solutions (50 mg/mL) were prepared in 99.9% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) and kept at -20°C.

Culture liquid extraction - After biomass filtration, pH of the culture growth media was decreased to 2.0 using 96% sulphuric acid (H₂SO₄). One liter of culture liquid was separated 3 times with 500 mL of ethyl acetate, and the extract-solvent mixture was washed once with 0.5 L distilled water using a glass chemical separator. The extract-solvent mixtures were left in a chemical hood for solvent evaporation till a resin (or powder) was formed, which represented the actual crude fungal extract, collected in previously weighed 4-mL plastic tubes.

The crude culture liquid (CL) extracts were collected in previously weighed glass tubes, then weighed again in order to measure the exact weight of the obtained fungal extracts. Stock solutions (50 mg/mL) were prepared in 99.9% DMSO (Sigma-Aldrich, St. Louis, MO, USA) and kept at -20°C.

7.4. Cell cultures

Five cell lines were used for the evaluation of the extracts effects on cell viability: HPAF-II, HCT116, PC3, T47D, and FaDu. All human cancer cell lines (ATCC, Rockville, MD, USA) will be maintained in a suitable media, supplemented with 1% L-glutamine, 10% fetal calf serum (FCS), and 1% PenStrep (penicillin + streptomycin) (Biological Industries, Kibbutz Beit-Haemek, Israel). Cells will be grown in a humidified incubator at

37°C with 5% CO₂ in air and will be fed twice a week with fresh media. Table 6 presents cell lines with their descriptions, which were used in this study.

Table 6. Human cancer cell lines used for this research

Designations	Disease	Source	Differentiation	Propagation
HPAF-II	Pancreatic cancer	Adenocarcinoma	Well differentiated	Temperature: 37°C; Atmosphere: air – 95% carbon dioxide (CO ₂) – 5%
HCT116	Colon cancer	Adenocarcinoma	Well differentiated	Temperature: 37°C; Atmosphere: air – 95% carbon dioxide (CO ₂) – 5%
PC3	Prostate cancer	Adenocarcinoma	Poorly differentiated	Temperature: 37°C; Atmosphere: air – 95% carbon dioxide (CO ₂) – 5%
T47D	Breast cancer	Ductal carcinoma	Moderately differentiated	Temperature: 37°C; Atmosphere: air – 95% carbon dioxide (CO ₂) – 5%
FaDu	Head and neck cancer	Squamous cell carcinoma	Well differentiated	Temperature: 37°C; Atmosphere: air – 95% carbon dioxide (CO ₂) – 5%

5 7.5. Cytotoxicity assays

XTT assay

Evaluations of fungal extracts' effect on cell line viability were performed by XTT ((sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolinum]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate)) assay (Biological Industries, Kibbutz Beit Haemek, Israel). Cells (1×10⁴ cells) were seeded in 100 µL of medium, using 96-well plates. After 24 hours, fungal extract was added in several concentrations: 25, 50, 100, 250, and 500 µg/mL for

24, 48, and 72 hours. Control wells were medium-treated wells and 1% DMSO-treated wells. After 24, 48, and 72 hours, viability levels were determined according to the manufacturer's instructions using an Elisa reader at 450 nm waves and subtracted from the reference absorbance at 620 nm (PowerWave Microplate Spectrophotometer, BioTEK, USA). Experiments were repeated 3-5 times in at least three replicates. Data were presented as the average proliferation percentage of the respective vehicle-treated control.

Lactate dehydrogenase (LDH) leakage assay

LDH is a cytoplasmic enzyme that catalyzes the oxidation of L-lactate to pyruvate with NAD^+ as a hydrogen acceptor, the final step in the metabolic chain of anaerobic glycolysis. The extracellular appearance of LDH serves as a marker for tissue lysis since cell damage, such as necrosis, causes a rise of LDH in the cells' medium (Moran and Schnellmann 1996). Detection of LDH activity released from damaged cells were performed in order to exclude the possibility for cytotoxic effects of the extracts on the cell lines. LDH leakage into the medium was measured in aliquots of the extracellular fluid of each sample by the LDH Cytotoxicity Detection Kit (Roche Diagnostics, Mannheim, Germany). Cells (1×10^4 cells) were seeded in 100 μL of medium, using 96-well plates. After 24 hours, fungal extract was added in several concentrations: 25, 50, 100, 250, and 500 $\mu\text{g/mL}$ for 24 hours. After 24 hours, viability levels were determined according to the manufacturer's instructions using an Elisa reader at 450 nm wave and subtracted from the reference absorbance at 620 nm (PowerWave Microplate Spectrophotometer, BioTEK, USA). Experiments were conducted three times in at least 3 replicates. Data presented as the average LDH level percentage of the respective vehicle-treated control.

7.6. Cell cycle and apoptosis assays

Cell cycle analysis

7×10^5 T47D and 5×10^5 PC3 cells were seeded in T-25 flasks. On the next day, T47D cells were treated with 25 $\mu\text{g/mL}$ *Cordyceps militaris* strain CBS 132098 Eth extract and PC3 cells were treated with 100 $\mu\text{g/mL}$ *Cordyceps militaris* CBS 132098 and *Ganoderma tsugae* var. *janniae* Tay-1 (CBS 120394) combination extract for 24, 48 and 72 hours. At the end of that time, cells was trypsinized and collected with the growth medium, centrifuged, washed with PBS and fixed with 70% ethanol for one hour. This was then followed by the incubation with 0.1% NP-40 for 5 minutes in 4°C and then incubation on ice with 100 $\mu\text{g/mL}$ RNase for 30 minutes. Finally, 50 $\mu\text{g/mL}$ PI was added for 20 minutes. Cell cycle phase distributions were determined by Fluorescence Activated Cell

Sorter (FACS) flow cytometry (Becton Dickinson); 10,000 cells were counted for each control and treatment group.

Annexin V-FITC staining

Cell apoptosis was measured using Annexin V-FITC Apoptosis Detection Kit (MBL, Woburn, MA, USA). T47D and PC3 cells were seeded in T-25 flasks. On the next day, T47D cells were treated with 25 µg/mL *Cordyceps militaris* CBS 132098 Eth extract and PC3 cells were treated with 100 µg/mL *Cordyceps militaris* CBS 132098 and *Ganoderma tsugae* var. *janniae* Tay-1 (CBS 120394) combination extract for 24, 48 and 72 hours. At the end of that time, cells were trypsinized and collected with the growth medium, centrifuged, washed with PBS and 2×10^5 cells/mL were counted and collected for the analysis. Cells were resuspended in 85 µL of 1X binding buffer, 10 µL of Annexin V-FITC and 5 µL of propidium iodide were added. Cells were incubated for 15 minutes at room temperature in the dark. Cell apoptosis was determined using a BD FACS Calibur flow cytometer.

7.7. Statistics

All results are expressed as mean $\pm$ SEM. ONE WAY ANOVA was used for the evaluation of the differences between treatment groups and control groups. $P < 0.05$ was considered significant, and SPSS software was used for calculation of differences. All experiments were performed at least three times and with three replicates.

Results

Cytotoxicity assays

XTT assay

Effect of *Cordyceps militaris* strain CBS 132098 fruiting body extracts on human cancer cell lines' (HPAF-II, HCT116, PC3, T47D and FaDu) viability

A total of four crude fungal extracts, from fruiting bodies and from culture liquid of *Cordyceps militaris* strain 132098, were evaluated for their ability to inhibit the growth of several human cancer cell lines: HPAF-II, FaDu, HCT116, T47D, and PC3 at different concentrations following exposure of 24, 48, and 72 hours.

For the cell line HPAF-II, Eth extract exhibited a 50% decrease in cell viability after exposure of cells to a concentration of 25 µg/mL of the extract for 72 h (Fig. 5). Higher concentrations (100 and 500 µg/mL) of the Eth extract induced a 70% decrease in cell

viability after 72 h. EA extract also exhibited a 60% decrease in cell viability after treatment with a concentration of 500 $\mu\text{g/mL}$ for 48 h.

Extracts effect were also examined using FaDu cells (head and neck cancer) several of which showed good results. Eth extract caused a 60% decrease in cell viability after treatment with 25 $\mu\text{g/mL}$ for 72 h and an 85% decrease after treatment with 100 $\mu\text{g/mL}$ for 72 h. A period of 48 h of treatment with the Eth extract caused a 70% decrease of viability at a concentration of 250 $\mu\text{g/mL}$ of the extract. Other extracts were effective also; Chl extract exhibited a 60% decrease in cell viability after a treatment period of 72 h with a 250 $\mu\text{g/mL}$ concentration. EA extract caused a 60% decrease in cell viability after exposure of 72 h at a 250 $\mu\text{g/mL}$ concentration and a 75, 87, and 92% decrease was seen in cell viability after exposure to a 500 $\mu\text{g/mL}$ for 24, 48, and 72 h, respectively (Fig. 6).

Examination of HCT116 cells, human colon cancer, 25 $\mu\text{g/mL}$ of Eth extract, for 72 h caused a 45% decrease in cell viability. After 48 h 50 $\mu\text{g/mL}$ induced a 50% decline and a 60% decline after 72 h. After 72 h treatment with 250 and 500 $\mu\text{g/mL}$ of Eth extract a 75% and 82% decrease in cell viability was induced, respectively (Fig. 7). Chl extract at a concentration of 500 $\mu\text{g/mL}$ caused a 60% decrease after 72 h treatment and EA extract at a concentration of 500 $\mu\text{g/mL}$ decreased cell viability by 60 and 75% after 24h and 48 h, respectively.

The human breast cancer cell line, T47D, exhibited a 40% decrease in cell viability after treatment with 25 $\mu\text{g/mL}$ EA extract for 72 h and a 60% decrease after treatment with Eth extract for the same treatment period and at the same dose. A 73% and 80% decline in cell viability was seen after 72 h treatment with 50 and 100 $\mu\text{g/mL}$ Eth extract, respectively.

A concentration of 100 $\mu\text{g/mL}$ EA extract caused a 53 and 57% decrease, after treatment of the cells for 48 and 72 h, respectively. The same extract at a dose of 250 $\mu\text{g/mL}$ produced a 61% decline in cell viability after treatment for 72 h.

The highest concentration used 500 $\mu\text{g/mL}$, which caused a decline in cell viability for all extracts used except CL extract. Chl extract gave rise to a 55% and 62% decrease after 48 and 72 h of treatment, respectively; cells treated with EA extract showed a 55%, 77%, and 82% decrease after 24, 48, and 72 h. Eth extract exhibited an 82% decrease in the viability of cells following 72 h of treatment (Fig. 8).

For human prostate cancer cell line, PC3, with concentrations of 25 and 50 $\mu\text{g/mL}$, from any of the extracts examined, did not induce a decrease in the viability of cells. Chl

extract at a concentration of 100 µg/mL for 48 and 72 h decreased the viability of cells by 40% and 55%, respectively. Following treatment with 250 µg/mL with the same extract, the decrease in cell viability ascended to 55% and 74% for 48 and 72 h of treatment. CL extract at the same concentration gave rise to a 42% decrease following a 24 h treatment
5 that did not increase when treatment periods were longer (48 and 72 h). A 70% and 90% decrease was seen following treatment of 250 µg/mL EA extract for 48 and 72 h, respectively.

For this cell line a dose of 500 µg/mL induced impressive results for both the Chl extract and the EA extract. A decrease of 75%, 92%, and 95%, following treatment for 24,
10 48, and 72 h, respectively, was seen after treatment with the Chl extract. As for the EA extract, for all periods of treatment that were examined, more than a 95% decrease in cell viability was seen. CL extract also gave rise to a 40% and 75% decrease in cell viability after treatment for 48 and 72 h, respectively (Fig. 9).

Since, all extracts were diluted with DMSO, the experiments also included DMSO
15 treated wells that contained 1% DMSO (as the highest DMSO levels that the 500 µg/mL concentrations contained) which had shown no significant change in cell viability except for the Eth extract (500 µg/mL) (Fig. 11).

Amongst all extracts examined in this screening phase, the most active extract was the Eth extract, showing the most profound decrease in cell viability. The effect seen
20 following a 25 µg/mL treatment for 72 h of the Eth extract (for cell lines; FaDu, HPAF-II and T47D) is the most interesting of all and requires further investigation.

At higher concentrations the HCT116 human colon cancer cell line had also shown the ability to decrease cell viability. Unfortunately, the effect of *Cordyceps militaris* strain CBS 132098 Eth extract had not been examined for PC3, the human prostate cancer cell
25 line. Since this cell line had shown sensitivity to other *Cordyceps militaris* strain CBS 132098 extracts, i.e., Chl, CL, and EA, it would be interesting to examine the effect of the Eth extract.

Amongst all cell lines examined, the FaDu, human head and neck cancer was the most sensitive cell line to the Eth extract although T47D, human breast cancer cell line and
30 HPAF-II, human pancreatic cancer cell line, had shown good results also (Fig. 10).

Effect of mixture of *Cordyceps militaris* strain CBS 132098 and *Ganoderma tsugae* var. *janniae* strain Tay-1, CBS 120394 (1:1 v/v) extracts on human cancer cell lines (HPAF-II, HCT116, PC3, T47D and FaDu) viability

A total of 4 crude fungal extracts (Wtr, EA, Eth, and Chl) from the combination of
 5 *Cordyceps militaris* strain CBS 132098 and *Ganoderma tsugae* var. *janniae* strain Tay-1 (CBS 120394) mushrooms were evaluated for their ability to inhibit the growth of several human cancer cell lines; HPAF-II, FaDu, HCT116, T47D, and PC3, at different concentrations, following exposure of 24, 48, and 72 hours.

All of the extracts exhibited some degree of effectiveness; for the cell line HPAF-
 10 II, Eth extract exhibited a 70% decrease in cell viability after exposure of cells to a concentration of 100 µg/mL of the extract for 72 h (Fig. 12). Higher concentrations (250 and 500 µg/mL) of the Eth extract induced an 80% decrease in cell viability after 72 h. EA extract also exhibited a 90% decrease in cell viability after treatment with a concentration of 500 µg/mL for 72 h.

15 Extracts effect were also examined using FaDu cells (head and neck cancer) and some of them showed significant results. Eth extract caused more than a 60% decrease in cell viability after treatment with 100 µg/mL for 72 h. Treatments with the highest concentration used (500 µg/mL) exhibited more than a 50% decrease in cell viability for the Chl extract (treatment periods of 48 and 72h). The EA extract exhibited a 90% decrease
 20 in cell viability following treatment with 500 µg/mL of the extract. Wtr extract also showed good results following treatment with 500 µg/mL of the extract for 48 and 72 h, a 65% and more than an 80% decrease was seen in cell viability (Fig. 13).

Examination of the HCT116 cells, human colon cancer, showed that 250 µg/mL of the Eth extract for 72 h caused a 60% decrease in cell viability. Treatment of 500 µg/mL of
 25 the same extract induced more than 70% decline after 72 h (Fig. 14). Chl extract at a concentration of 500 µg/mL caused a 55% decrease after 48 h.

The human breast cancer cell line, T47D, exhibited promising results. A 53% and 56% decrease was seen after treatment with a dose of 25 µg/mL EA extract for 48 h and 72 h, respectively. As the concentration of the extract increased, the decrease in cell viability
 30 increased. At a concentration of 50 µg/mL, cell viability declined in 50, 60, and 63% following treatment for 24, 48, and 72 h with the EA extract. A treatment of the cells with Eth and Chl extract at a concentration of 50 µg/mL caused a decrease of 52 and 62%, respectively, in cell viability following a 72 h treatment. As for higher concentrations used,

100, 250, and 500 µg/mL, the effect seen was found dose- and time-dependent for the Chl and EA extracts. Viability levels were decreased to 67, 50, and 35% after treatment with Chl extract at a concentration of 100 µg/mL, and at a concentration of 250 µg/mL viability levels continued to decrease; 62, 44, and 33% cell viability was seen after a period of 48 h. After 72 h of treatment, viability levels were the lowest: 35, 33, and 14% viable cells were left. Cells treated with the EA extract for the same treatment periods and at the same doses exhibited the highest decrease in the levels of viability. Treatment at a dose of 100 µg/mL for a period of 24, 48, and 72 h caused a 55, 67, and 71% decline, respectively, in cell viability. A dose of 250 µg/mL decreased cell viability in 59, 65, and 78% after 24, 48, and 72 h, respectively. A 65, 84, and 89% decline was seen after treatment at a dose of 500 µg/mL for 24, 48, and 72 h, respectively (Fig. 15).

The human prostate cancer cell, PC3, also exhibited remarkable effects. At a concentration of 50 µg/mL, of the Eth extract, a 55% decline was seen following a treatment period of 48 h. A dose of 100 µg/mL with the same extract for the same period of time, resulted in a 65% decrease in cell viability, and a period of 72 h of treatment resulted in 78% decrease in cell viability. EA extract also exhibited a 78% decrease in cell viability following treatment at the same dose and the same period of time. At higher concentrations, almost all extracts caused more than a 50% decrease in cell viability (Fig. 16).

Eight different crude fruiting body fungal extracts were examined. Chl, Eth, CL, and EA extract from the mushroom *Cordyceps militaris* strain CBS 132098 and Chl, Eth, EA, and Wtr from a combination of *Cordyceps militaris* strain CBS 132098 and *Ganoderma tsugae* var. *jannieae* strain Tay-1, CBS 120394 (1:1 v/v). Amongst all extracts examined in this screening phase, the most active extract was the Eth extract from the *Cordyceps militaris* strain CBS 132098 and *Ganoderma tsugae* var. *jannieae* strain Tay-1 (CBS 132098) combination and *Cordyceps militaris* strain (CBS 132098) extract separately as well, showing the most profound decrease in cell viability and exhibiting anti-proliferative activity against all cell lines used for this research. The most impressive effect was seen on PC3 cells and T47D cells.

Eth extract combination from *Cordyceps militaris* strain CBS 132098 and *Ganoderma tsugae* var. *jannieae* strain Tay-1 (CBS 120394) caused a 55% decline in PC3 cell viability following treatment of 50 µg/mL for a period of 48 h and 65% decline following 100 µg/mL treatment for a period of 48 h (Fig. 21).

The EA extract combination from the mushroom *Cordyceps militaris* strain CBS 132098 and *Ganoderma tsugae* var. *jannieae* Tay-1 (CBS 120394) also exhibited a valuable effect on PC3 cell viability; a 78% decrease was seen after treatment with 100 µg/mL for a period of 72 h (Fig. 20).

5 As for the *Cordyceps militaris* strain CBS 132098 extract separately, the most promising effect was seen on the human breast cancer cells, T47D. A treatment with 25 µg/mL from the Eth induced a 60% decrease in cell viability following a period of 72 h (Fig. 21). The effect of this extract was found to be dose dependent, as the concentrations used increased (50, 100, 250, and 500 µg/mL), the decrease in cell viability increased 72.5,
10 78, 81, and 83%, respectively. EA extract from the combination of *Cordyceps militaris* strain CBS 132098 and *Ganoderma tsugae* var. *jannieae* Tay-1 (CBS 120394) also exhibited interesting results; a concentration of 50 µg/mL for 48 h of treatment caused a 55% decrease in cell viability (Fig. 21).

The other cell line used for this research: human head and neck cancer, FaDu;
15 human colon cancer, HCT116, and human pancreatic cancer, HPAF-II, showed less promising results (Figs. 17, 18 and 19).

Based on the results of the experiments concerning the influence of the extracts of *Cordyceps militaris* strain CBS 132098 separately and its mixture with *Ganoderma tsugae* var. *jannieae* strain Tay-1 (1:1 v/v) on the human cancer cell lines (HPAF-II, FaDu,
20 HCT116, T47D, and PC3), we have chosen two extracts for each fungal sample: the combination of *Cordyceps militaris* strain CBS 132098 and *Ganoderma tsugae* var. *jannieae* strain Tay-1, CBS 120394 (EA and Eth) and *Cordyceps militaris* strain CBS 132094 separately (EA and Eth) for the evaluation of their ability to inhibit the growth of two human cancer cell lines, T47D and PC3, at different concentrations, following
25 exposure of 24, 48, and 72 hours.

All of the extracts exhibited some degree of effectiveness. The most discernable effect seen in the lower concentration used for T47D cells was the result of *Cordyceps militaris* strain CBS 132098 - Eth extract treatment; exhibiting a 60% decrease in cells viability after exposure of the cells to a concentration of 25 µg/mL of the extract for 72 h
30 (Fig. 22). Higher concentrations (250 and 500 µg/mL) of the extract induced 70% and 80% decrease in the cell's viability after 72h. EA extract also exhibited a 90% decrease in the cell's viability after treatment with a concentration of 500 µg/mL for 72 h. The combination extract of *Cordyceps militaris* strain CBS 132098 and *Ganoderma tsugae* var.

janniae strain Tay-1 (CBS 120394) with EA exhibited a lower anti-proliferative effect; only the highest concentrations (250 and 500 µg/ml) attained more than 50% inhibition of living cells following the extract treatment at all periods of time examined (Fig. 22).

5 PC3 cells treated with either *Cordyceps militaris* strain CBS 132098 and *Ganoderma tsugae* var. *janniae* strain Tay-1 (CBS 120394) combination Eth extract or *Cordyceps militaris* strain CBS 132098 EA extract exhibited a dose- and time-dependent effect of both extracts, however, the most profound effect seen at the lower dose used, was a result of *Cordyceps militaris* strain CBS 132098 and *Ganoderma tsugae* var. *janniae* Tay-1 (CBS 120394) Eth extract following a 72 hour treatment with 100 µg/ml (Fig. 23).

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LDH leakage

LDH leakage is a cytoplasmic enzyme constantly expressed in most mammalian cells. It is well accepted to use the amount of LDH in extracellular space to assess plasma membrane integrity (Korzeniewski and Callewaert 1983). The capability of LDH assay to
15 detect cytotoxic effects is limited to the agents causing direct damage on cellular membranes.

In order to exclude a possible necrotic effect of the extracts on the cells, an LDH cytotoxicity test was performed for all the extracts examined by the XTT assay for both the T47D cell line and the PC3 cell line.

20 The results obtained for the examination made for the T47D cell line treated with *Cordyceps militaris* strain CBS 132098 and *Ganoderma tsugae* var. *janniae* strain Tay-1 (CBS 120394) combination EA extract showed that the 25, 50, 100, and 250 µg/ml doses did not differ significantly from the LDH levels found in control non treated cells (Fig. 24). *Cordyceps militaris* strain CBS 132098 Eth extract treatment also showed relatively
25 no differences between all doses examined except 500 µg/ml that exhibited higher levels of LDH than in control non-treated cells (125%) (Fig. 24).

LDH levels measured following treatment with *Cordyceps militaris* and *Ganoderma tsugae* var. *janniae* combination EA extract and *Cordyceps militaris* Eth extract of the PC3 cell line, was also found as in the control non treated cells, with the
30 exception of the 500 µg/ml dose of *Cordyceps militaris* Eth extract, that exhibited higher levels of LDH in comparison to control non-treated cells (140%) (Fig. 25).

According to the result obtained by the XTT assay and the LDH assay, the lowest effective dose of one extract per cell line was chosen for further evaluation of the effect on

cells. The *Cordyceps militaris* Eth extract in a dose of 25 µg/ml was chosen for the T47D cell line, and the *Cordyceps militaris* and *Ganoderma tsugae* var. *janniae* Eth extract in a dose of 100 µg/ml was chosen for the PC3 cell line.

Apoptosis and cell cycle analysis

5 *Cell cycle analysis*

Cell cycle analysis was used for the evaluation of the chosen extract on the cell cycle and more specifically, on the accumulation of cells in the Sub-G1 phase of the cell cycle as a marker for the occurrence of the apoptosis process, following the treatment with the extracts. Figures 26 and 27 demonstrate the effect of the *Cordyceps militaris* Eth
10 extract on the Sub-G1 phase of T47D cells following a dose of 25 µg/ml for 24, 48, and 72 hours. The most significant effect occurred after 72 hours of treatment with the extract-40% of the cells was found in the Sub-G1 phase of the cell cycle (Fig. 26).

PC3 cells were treated with 100 µg/ml of *Cordyceps militaris* and *Ganoderma*
15 *tsugae* var. *janniae* combination Eth extract for 24, 48, and 72 hours as well. Again, the most significant increase in the Sub-G1 phase was found following 72 hours of treatment with the extract (46%) (Fig. 27).

Following the results obtained by cell cycle analysis, we examined whether the accumulation of cells in the sub-G1 phase of the cell cycle are the result of apoptosis. Thus
20 T47D and PC3 cells was stained with FITC labeled annexin and PI and analyzed by flow cytometry. For both cell lines, only the treatment for 72 hours yields significant results. 25 µg/ml of the *Cordyceps militaris* Eth extract for 72 hours induced apoptosis in 28.1% of the cells (15.25% was found positive in the control non-treated cells). A concentration of 100 µg/ml of the *Cordyceps militaris* and *Ganoderma tsugae* var. *janniae* combination
25 Eth extract for 72 hours induced apoptosis in 21.2% of the cells (3.5% was found positive in the control non-treated cells) (Figs. 28, 29).

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CLAIMS

1. A pure submerged mycelial culture of *Cordyceps militaris* strain CBS 132098 and *Ganoderma tsugae* var. *janniae* Tay-1 (CBS 120394).
2. A biomass of the pure submerged mycelial culture of claim 1, rich in nutraceutical agents and biologically active compounds including carbohydrates and proteins rich in essential amino acids.
3. The mycelial biomass of claim 2, obtained by cultivation of the *Cordyceps militaris* strain CBS 132098 in submerged culture on nutrient media.
4. The biomass of claim 2, obtained from fruiting bodies of the *Cordyceps militaris* strain CBS 132098.
5. The mycelial biomass of claim 2 or 3, for use in nutraceutical compositions.
6. A biomass mixture comprising the biomass of claim 2 or 3 mixed with a biomass of medicinal mushroom *Ganoderma tsugae* var. *janniae* strain Tay-1 Accession No. CBS 120394 (Lee and Wasser 2009, Patent WO/2009/017462).
7. The biomass of claim 6, obtained from the fruiting body of the *Cordyceps militaris* strain CBS 132098 and mycelial biomass of the *Ganoderma tsugae* var. *janniae* Tay-1 (CBS 120394).
8. A *Cordyceps* and *Ganoderma* mixed extract having nutraceutical and biological activity comprising of *Cordyceps militaris* strain Phytopharma Accession No. CBS 132098 and *Ganoderma tsugae* var. *janniae* strain Tay-1 Accession No. CBS 120394 mixture.
9. The extract of claim 8, obtained from the mixture of fruiting body of *Cordyceps militaris* strain Phytopharma Accession No. CBS 132098 and mycelial biomass of the *Ganoderma tsugae* var. *janniae* strain Tay-1 Accession No. CBS 120394.
10. The extract according to any one of claims 8 to 9, obtained from the organic or aqueous extraction solvent.

11. The extract according to claim 10, wherein said organic extract solvent is selected from ethyl alcohol, ethyl acetate and chloroform.
12. A composition comprising a biomass of any one of claims 2 to 7 or an extract of any one of claims 8 to 11 further comprising the biomass or extract rich in nutraceutical agents and biologically active substances obtained from the fruiting body of *Cordyceps militaris* strain Phytopharma Accession No. CBS 132098 and mycelial biomass or extract of *Ganoderma tsugae* var. *janniae* strain Tay-1 Accession No. CBS 120394.
13. The composition of claim 12, wherein the biologically active agents have immunostimulatory or antitumor activity.
14. A pharmaceutical composition of claim 12, for use in treating cancer.
15. The pharmaceutical composition of claim 14, wherein said cancer is selected from pancreatic cancer, colon cancer, prostate cancer, breast cancer and head and neck cancer.
16. A composition of claim 12, for use as nutraceutical compositions.
17. The composition of any of claims 12 to 16, formulated in an oral solid dosage form.
18. The composition of claim 17, wherein said oral solid dosage form is selected from a fine powder, capsules, tablets, caplets, and sachets.
19. The composition of claim 18, formulated into a food products.
20. The compositions of claim 18, for use as a dietary supplement.
21. The compositions of claim 18, for use as vitamin supplement, dietary fiber supplement, protein supplement, amino acid supplement, fatty acids supplement, and mineral and microelements supplement.

22. A process for producing a biomass rich in carbohydrates, proteins, essential amino acids, vitamins, essential fatty acids, minerals and microelements from caterpillar medicinal fungus *Cordyceps militaris* strain CBS 132098 and higher Basidiomycetes medicinal mushroom *Ganoderma tsugae* var. *janniae* Tay-1 (CBS 120394), said process comprising: cultivating fruiting bodies or mycelial biomass of the caterpillar fungus *Cordyceps militaris* strain CBS 132098 and medicinal mushroom *Ganoderma tsugae* var. *janniae* 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, thereby producing *Cordyceps* and *Ganoderma* biomass.

23. A process for producing a *Cordyceps* and *Ganoderma* mixed extract having biological activity, wherein the biological activity inhibits growth of cancer cells, arrest cancer cell cycle, and induces apoptosis in cancer cells, said process comprising: cultivating fruiting bodies or mycelial biomass of caterpillar fungus *Cordyceps militaris* strain Phytopharma Accession No. CBS 132098 and *Ganoderma tsugae* var. *janniae* strain Tay-1 Accession No. CBS 120394 in submerged culture in nutrient media, isolating the resulting biomass of mushroom from the culture broth, drying and graining said biomass into fine powder which is subjected to solvent extraction and mixing the *Cordyceps* extract with the *Ganoderma* extract, thereby producing *Cordyceps* and *Ganoderma* mixed extract.

FIGURES

Fig. 1

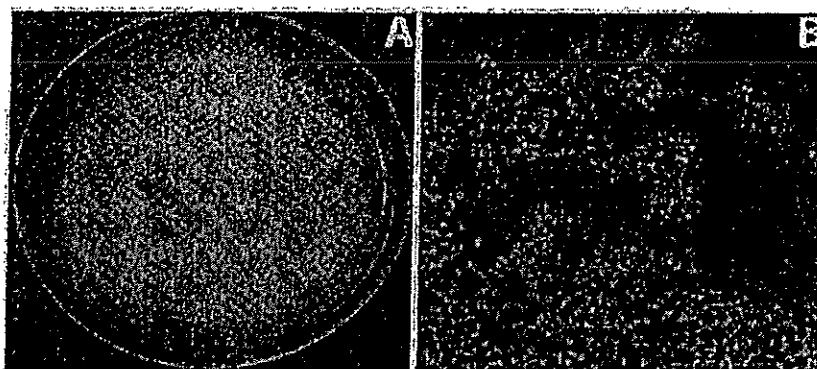
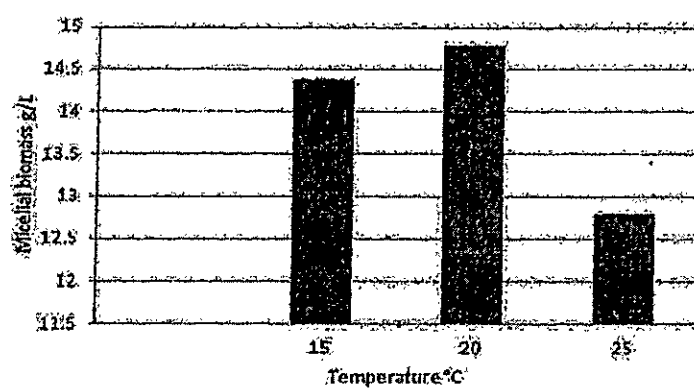


Fig. 2



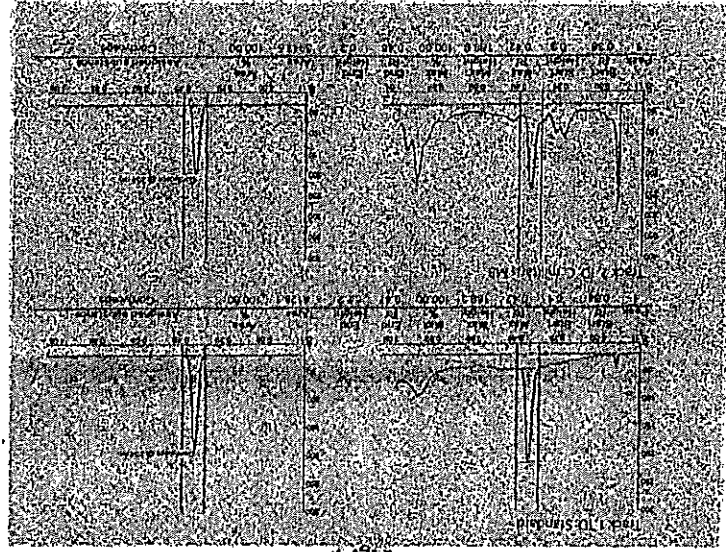


Fig. 4

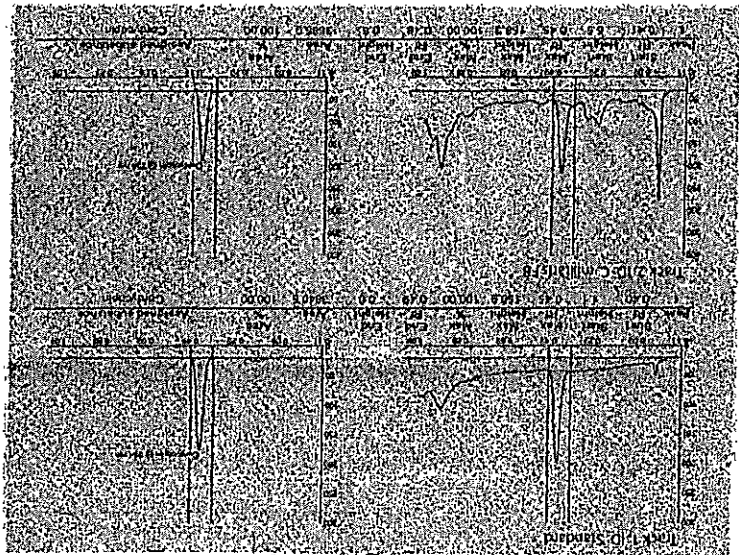


Fig. 3

Fig. 5

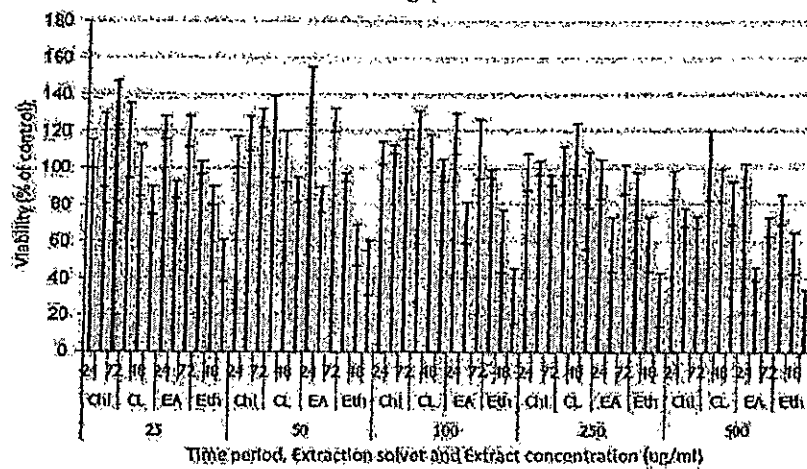


Fig. 6

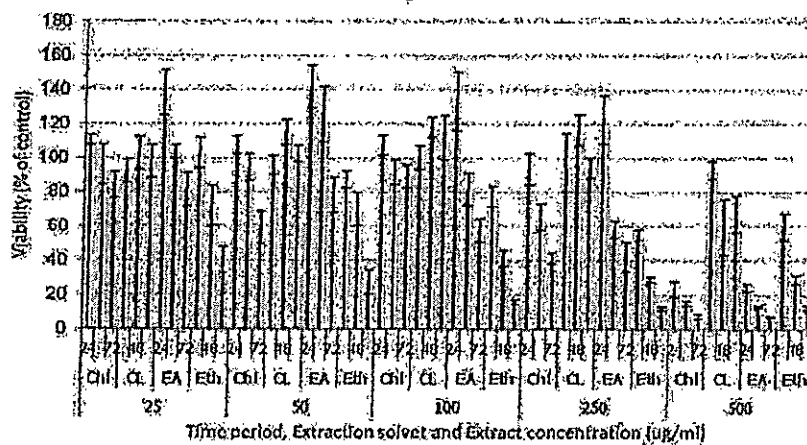


Fig. 7

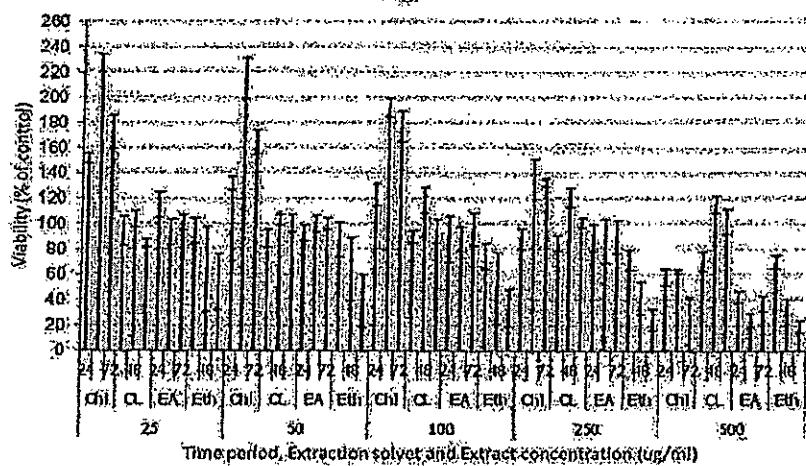


Fig. 8

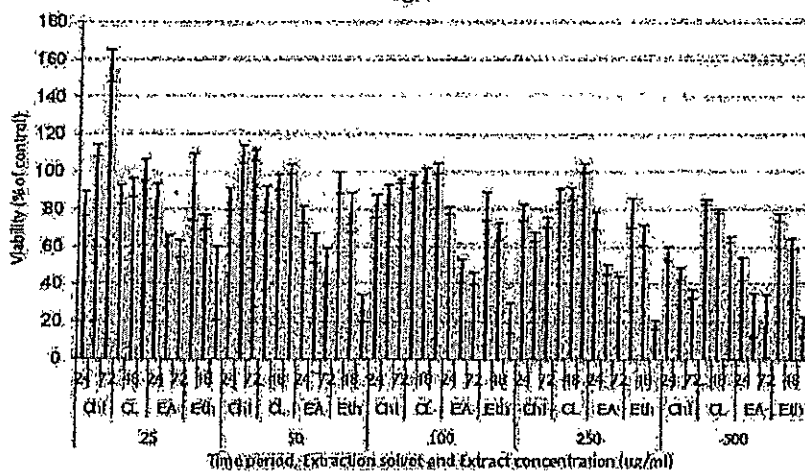


Fig. 9

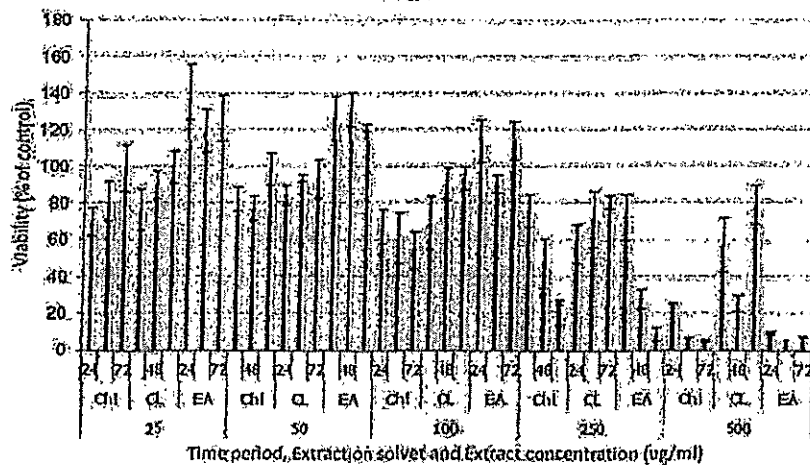


Fig. 10

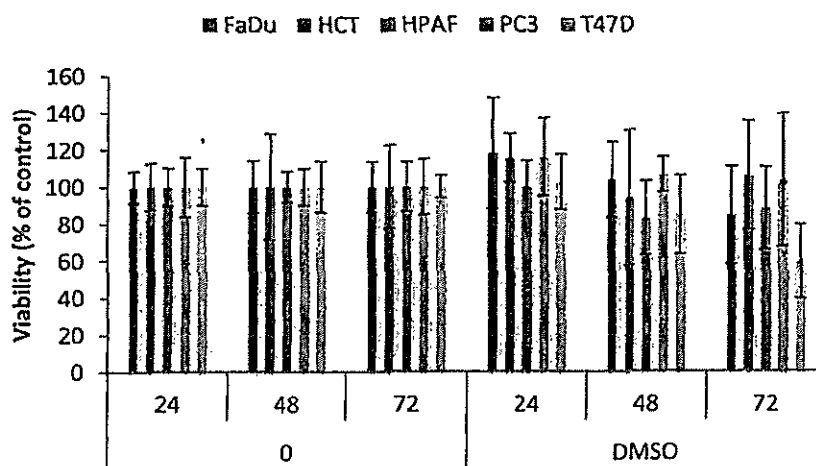


Fig. 11

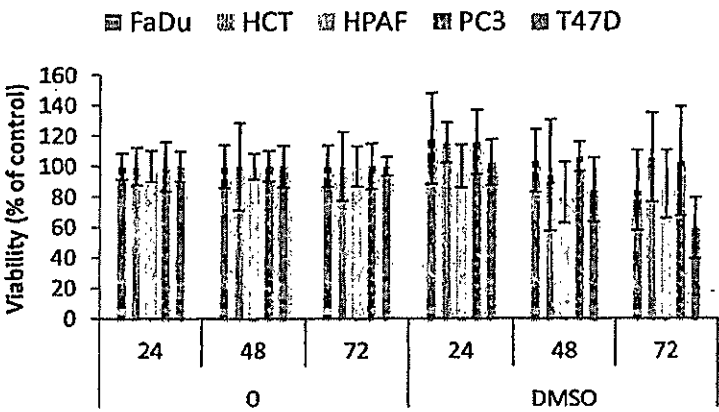


Fig. 12

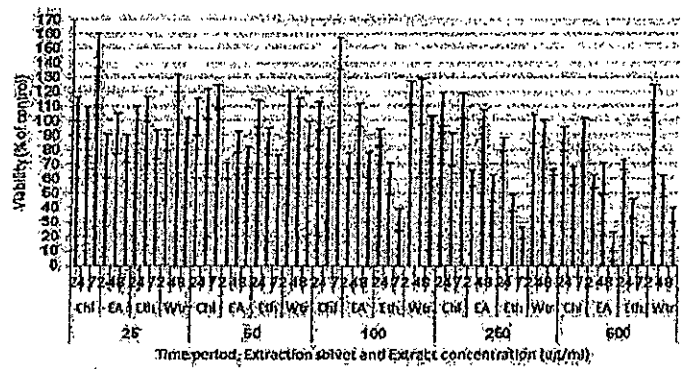


Fig. 13

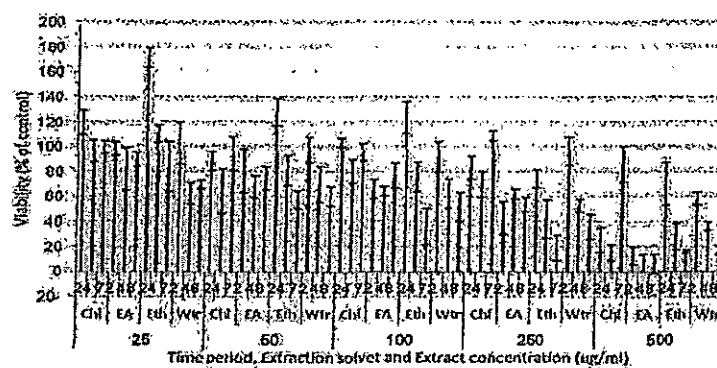


Fig. 14

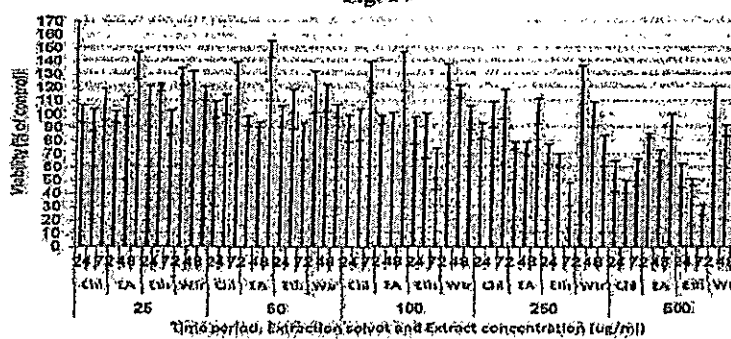


Fig. 15

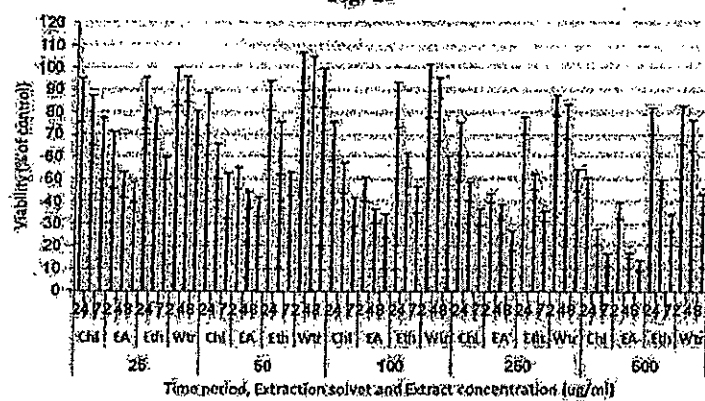


Fig. 16

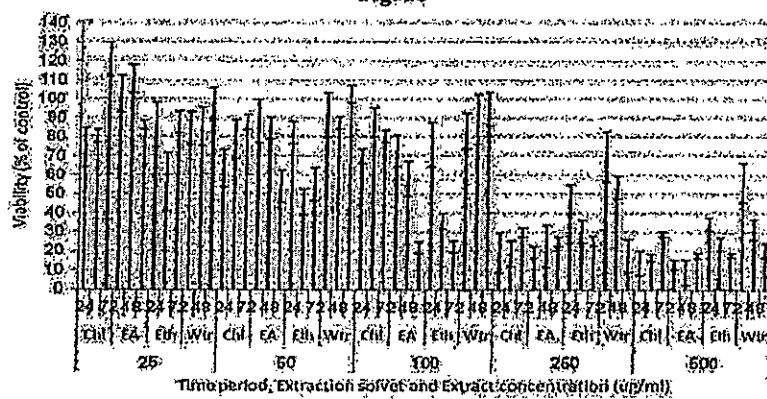


Fig. 17.

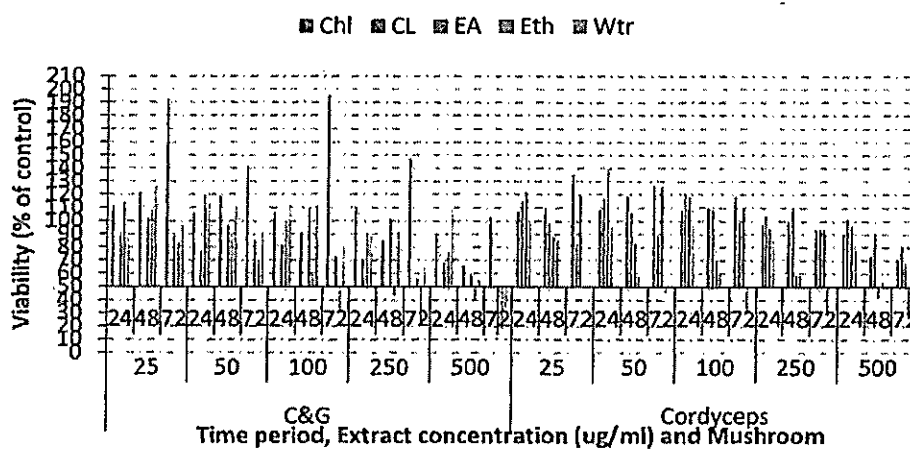


Fig. 18

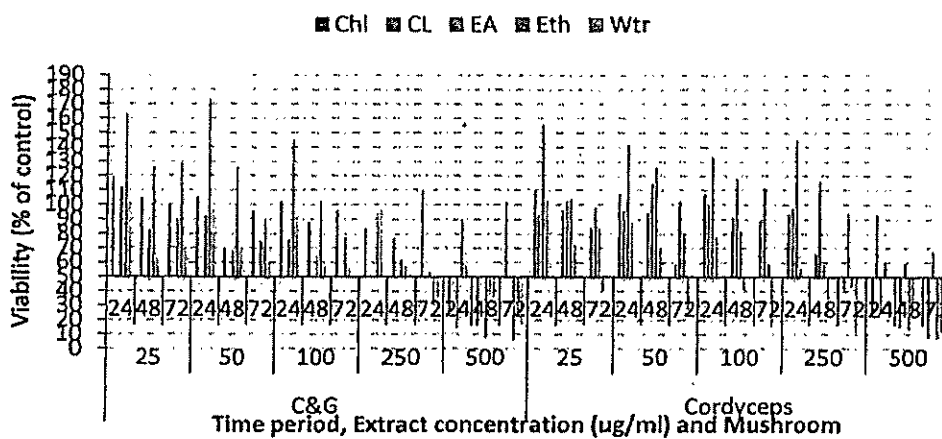


Fig. 19

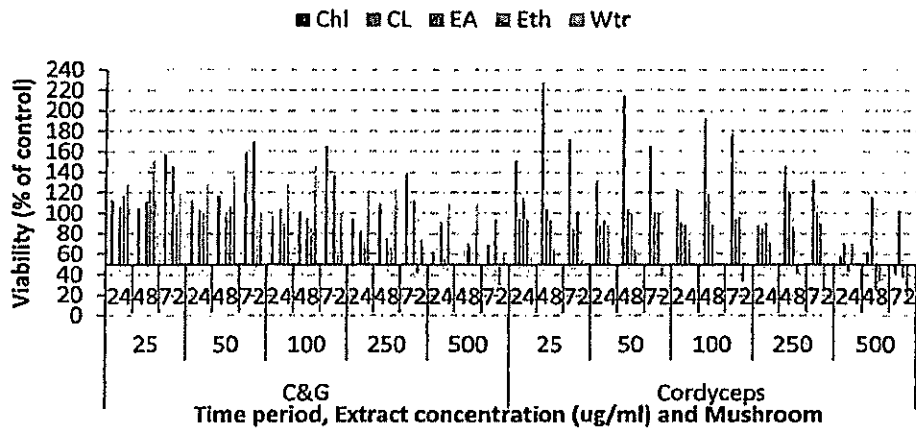


Fig. 20

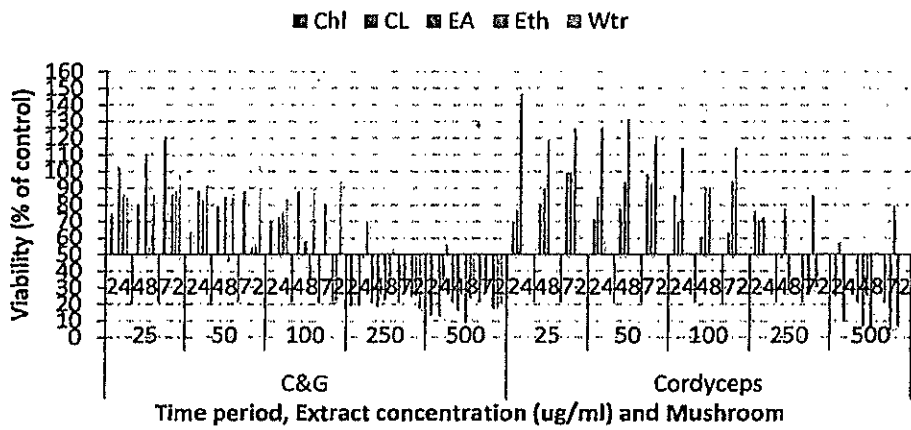


Fig. 21

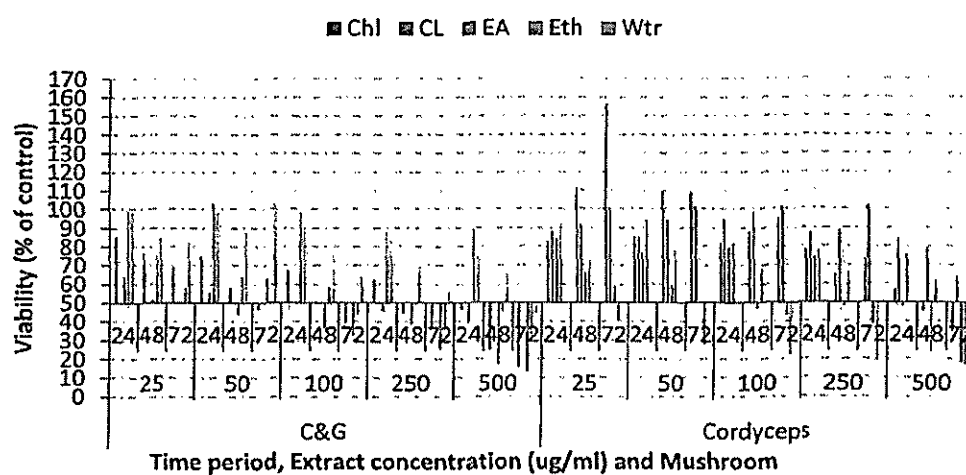
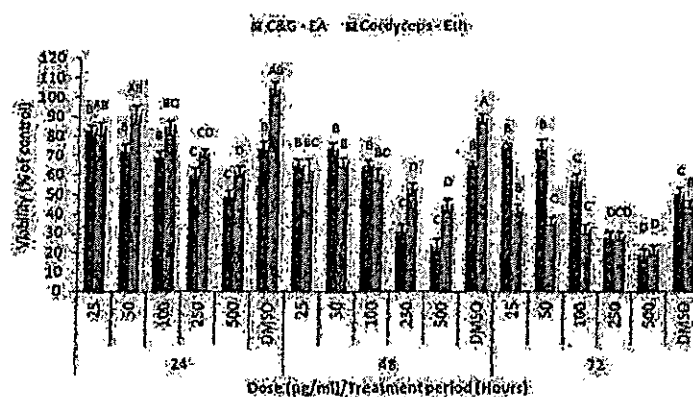


Fig. 22



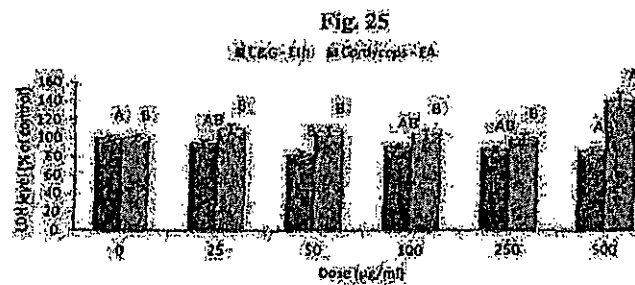
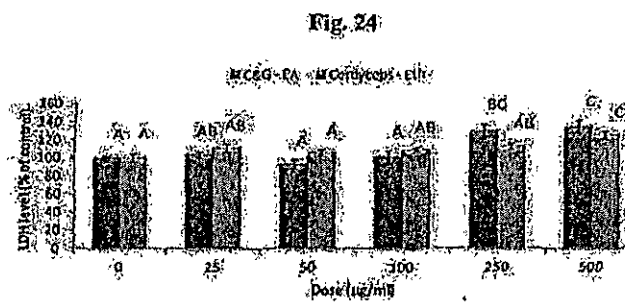
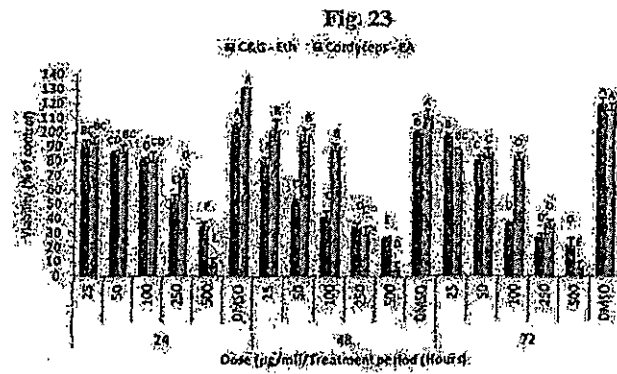


Fig. 26

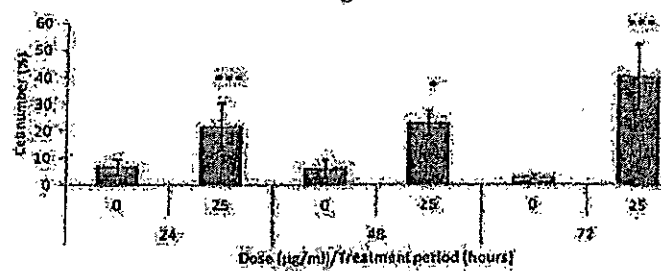


Fig. 27

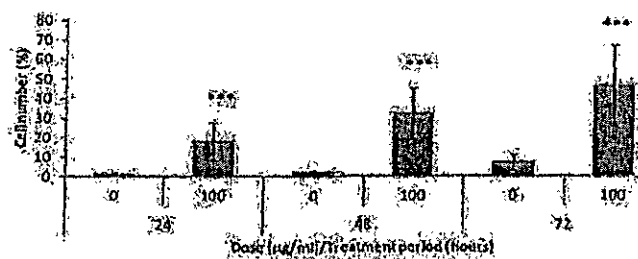


Fig. 28

