The medicinal benefits of Cordyceps *militaris* are dependent on consistent laboratory growth

Health Works Corporation

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The work consists of the file `hwc-c-militaris-whitepaper.pdf`.

First edition: January 2019
Second edition: September 2019

Health Works Corporation
The medicinal benefits of Cordyceps *militaris* are dependent on consistent laboratory growth. –
1st HWC Press ed.
p. cm.
Includes illustrations and bibliographical references.
1. Medicinal Mushrooms I. Title

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1 An Ancient Medicinal Mushroom

Cordyceps *militaris* is a fungus that has been used as an ancient traditional remedy throughout Asia for millennia. Recently, scientific studies have begun to confirm some of the benefits of C. *militaris* extract and its primary bioactive compound, cordycepin, as having numerous pharmacologically important health effects, such as anti-cancer, anti-diabetic and immuno-protective. The near-extinction of the mushroom and its counterpart, Ophiocordyceps sinensis, in their native ranges, requires laboratory-based production. However, current systems lack quality control and consistency with no product or compound assurances. Growth on a solid culture medium in small vessels under consistent conditions of a particular source strain of C. *militaris* eliminates contamination, increases growth with consistent bioactive concentrations.

2 A Remedy to Many Modern Illnesses

Communicable or infectious diseases were once the highest cause of mortality, however, nowadays most people die from non-communicable or lifestyle diseases (Figure 2). In 2016, roughly 40 million people died from lifestyle illnesses and this number has been steadily increasing throughout the last century. Non-communicable diseases (NCDs) include heart and lung disease, stroke, diabetes, and cancer. As these NCDs rise in society, an interest in traditional herbal medicine has also grown because of its focus on health promotion, prevention

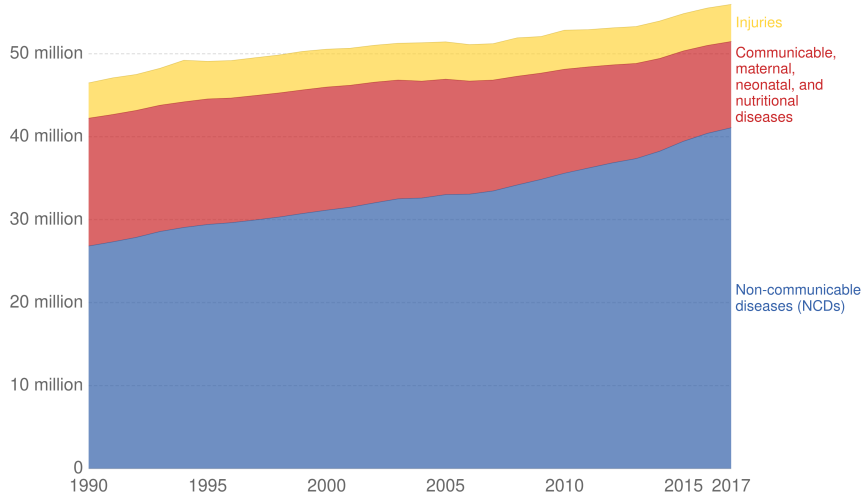

Figure 1: World Total Number of Deaths by Cause.[a]

[a] Non-communicable diseases (NCDs) include cardiovascular diseases, cancers, diabetes and respiratory diseases. Source: Data: Global Burden of Disease Collaborative Network. Image: Our World in Data.

and therapy for chronic disease.

Traditional Chinese medicines originated in ancient China and have evolved over thousands of years. Herbal plants, such as mushrooms, were administered in tonics or teas, for a variety of illnesses or nutritional value. In modern society, herbal medicine use is increasing worldwide with an estimated three-quarters of the world population currently using herbs and other forms of traditional medicine to treat illnesses, highlighting the increasing acceptance of their use in modern medicine [BK02]. The increasing interest and acceptance of traditional and indigenous knowledge of remedies has also been born out of the need to make healthcare affordable. As a result there has been increasing application of scientifically rigorous studies to evaluate the pharmacological activity of historic remedies.

3 The Prized Cordyceps

Cordyceps is a genus of fungi with 400-500 species, many having been used in traditional medicine. Their fruiting bodies grow parasitically on butterflies and moths and their pupae [S+07] [WS07]. They have been classified for many years in the Division Ascomycota. The most popular species in the Cordyceps genus include Ophiocordyceps *sinensis* and C. *militaris* . Both species have been used throughout Asia as an herbal remedy for millennia. In 2007 it was shown by nuclear genetic sampling that it was unrelated to most of the other members of the genus and as a result, a new family was proposed and C. *sinensis* was renamed Ophiocordyceps *sinensis* and classified under Ophiocordycipitaceae family [S+07].

Because of the increased number of diseases and the need for new medicines, the bioactive compounds present in the extracts from C. *militaris* are candidate drugs for many conditions. Apart from the clinical applications, the extracts from these fungi have beneficial impacts on the immune system, offering preventative care with the potential to improve an individual's overall health. Studies have shown that C. *militaris* has many pharmacological functions, such as anti-tumor/anti-cancer/anti-leukemic [P+09] [Y+04a] [LHZ14] [C+11] [LLP04] [MPP12] [LM10], anti-proliferative [MPP12] [LM10], anti- metastatic [Y+04a] [STH07] [JA76] [M+77] [KMYM00] [PRP70], anti-diabetic [Z+06] [C+14], anti-fatigue [JKH04], anti-oxidant/anti-aging [S+14b] [F+11], [DYL14] [X+14] [STH07] [MZ06] [LC08] [Y+07] [L+07] [DYL14], immunomodulatory, pro-sexual, anti-microbial, anti-bacterial [YJ+00], anti-viral [N+01], anti-fungal [STH07] [MZ06], anti-fibrotic [N+01], liver-protective [JKH04] [Y+07] [Y+04b] [WP05], pneumo-protective [Y+07], neuro-protective [J+14], anti-HIV [M+91], and anti-inflammatory [WP05] [S+14a], which make it marketable in Western countries [C+18].

4 Counterfeit Mushrooms

Wild O. *sinensis* is often valued at US$20,000 per kilogram (sometimes upwards of US$100,000), making it the most expensive fungus in the world. The medicinal value and rarity of O. *sinensis* have contributed to its high value. C. *militaris* has been increasingly viewed as a substitute species for O. *sinensis*, sharing similar chemical compounds and medicinal properties [DMSS10] [Z+11]. Compared to O. *sinensis*, C. *militaris* is easily cultured in vitro on a relatively large scale [Z+11]. It has been shown that C. *militaris* has been used to form adulterants or counterfeits found in products sold in the Asian markets [LYT06] [O+18] making customers believe they are consuming wild Cordyceps *sinensis*. This suggests the two fungi could be considered as interchangeable in the health benefits they offer. Products labeled and sold under as O. *sinensis* have shown that the materials are commonly unreliable, uncertain, and unspecified with unknown quality, highlighting the importance of reliable testing and standards in this field.

5 Range and Growth Cycle in Nature

Fungi play an important role in the recycling of organic matter in the terrestrial habitat of the earth [S+16]. Cordyceps species have been found mainly in North America [Mai58], Europe [Win08], Asia [PS11], and in India's subalpine regions of grassy lands and in some villages of Uttarakhand [S+10]. They range in habitat depending on their respective host species [Haj97]. O. *sinensis* is endemic to the Tibetan Plateau, occurring >3,000 m above seal level with a strict host range, growing only on the larvae of the ghost moth (Hepialus *humuli*) [Z+12] [WY11]. Comparatively, C. *militaris* , is a cosmopolitan species, distributed globally between 0 and >2,000 m above seal level [Mai58] [SS05] [SS04]. Although this genus has a worldwide distribution, C. *militaris* is mainly distributed in East Asian countries (i.e. China, Japan and the Korean Peninsula).

C. *militaris* is an annual parasite, growing on the larvae and pupae of butterflies, moths and spiders. The common name for O. *sinensis* meaning "winter worm, summer grass" and C. *militaris* meaning "pupa grass" is an interpretation of the growth cycle of these species as they normally inhabit the host surface in winters, and then form the fruiting body in summers. Spores of the fungus enter the larvae or pupae and once inside, branching tubes called hyphae, collectively known as a mycelium, spread throughout the host [WS07]. The hyphal bodies carry out extra-cellular digestion and the solubilized breakdown products are absorbed as they grow. Club-shaped yellow and orange cylindrical or thin filamentous or filiform fruiting bodies or stromata emerge above the surface of the buried mycosed pupae following death (Figure 2) [WS07] [Z+11]. These stromata grow to a length of around 2 to 8 cm and have a width of about 0.5 cm [Cui14].

One interesting aspect of the life cycle of the mentioned Cordyceps species is

their ability to manipulate host behaviours to maximize reproductive survival in the wild. It is common for pathogenic parasites to manipulate host behaviours through a number of techniques that take control of one or more aspects of the host's body functions. An extreme example of this is the "death grip" of ants caused by species in the Ophiocordyceps genus, whereby the parasite is able to control the muscles in the ant's jaw and cause the ants to bite onto vegetation in an appropriate position prior to being killed [A+09] [HWL11]. When this occurs, ants are attached to the lower side of twigs and others are found attached to leaves [dB+14]. "Summit disease" is another example, where the fungal pathogen causes the infected insect to climb to an uppermost point of the plant they are on to increase dispersal of the fungal spores when the insect dies [E+06]. O. *sinensis*, causes the larvae of the ghost moth to move upward close to the surface of soil to allow the fruiting body to grow prior to the death of the host [SFW15]. There is no such information on the impact of C. *militaris* infection and impact on host behavior.

Figure 2: Fruiting bodies of the fungus C. *militaris* .[b]

[b] Image of Cordyceps *militaris* . Fluff Berger, CC-BY-SA. Via Encyclopedia of Life.

6 Laboratory Cultivation

The popularity of these species has caused rapid declines in natural populations because of over collection and exploitation [LYT06] [WY11], resulting in greater interest in cultivating these fungi in the laboratory. Growth of the complete fungus is important as approximately 80-85% of medicinal mushroom products are extracted from their fruiting bodies and only 15% are derived from the mycelium culture 55 . Small fruiting bodies, slow growth and rareness in nature have meant that these species are threatened with extinction in their native habitat [TSS14]. Therefore, to maintain demand, laboratory cultivation has become an integral part of the production of C. emph*militaris* supplements.

6.1 Methods of Fermentation

In recent years submerged fermentation (SmF) has emerged as a dominant method in large-scale production of fungal byproducts, using antibiotics and enzymes achieving better monitoring, ease of handling and cost savings [dB+14]. However, one of the oldest techniques is the growth of fungi in the absence or near-absence of water, known as solid-state fermentation (SSF). As SSF closely mimics the growth of wild C. *militaris* , the final product is often higher in quality, concentration and activity and additionally materials used for the solid state can be selected and manipulated to increased yields in specific compounds [HL17] [HJWL15] whilst also being sourced from a range of environmentally-conscious and easily accessible substrates [Cui14] [M+11] [HHL04] [RSN01] [VG+03]. Additionally, SSF consumes less energy and water than comparative methods [Pan03] [SPSP09].

Solid culture or SSF mimics the natural growth of C. *militaris* and was used in ancient times [Pan03] [SPSP09]. Considerable differences have been identified in the constituents of wild Cordyceps species when compared with cultured Cordyceps species [C+18] [LLDT01] [L+04] [G+04]. The higher and improved yields of bioactive compounds in fungi grown on laboratory SSF in many fungal species over other methods is partially thought to be because of the resemblance of SSF to its natural growth state [M+11] [SPSP09] [MLMYFL15]. SSF is defined as fermentation involving solids in absence (or near absence) of free water [Pan03]. The fungi are instead grown on moist solid substrates possessing enough moisture to support growth and metabolism of cells [Pan03] [SPSP09]. By comparison and despite its industrial uptake, SmF is characterized as the fermentation in the presence of excess water, and yet the lower water content in SSF cultivation is well adapted to the metabolism of fungi [SPSP09] [DAG+11].

Selection of the appropriate solid substrate is important for the growth of the fungi and production of the compound of interest [M+11]. The solid material acts as both a physical support and source of nutrients and has involved utilization of agricultural crops or inert support [Pan03]. The growth of C. *militaris* has been carried out on a wide variety of solid substrates including germfree silkworm pupae [R+14b], larvae of the domestic silkmoth, Bombyx mori [H+08], germinated soybeans [MPP12], wheat substrates [G+16] and oat substrates [HJWL15]. Comparative studies report benefits of each culture technique, sometimes with a limited ability to clearly predict the preferred or more productive method as one method may provide increases in products not observed in the alternative method and vice versa [G+16]. This was observed by Guo et al. [G+16] in their comparison of SSF using wheat or silkmoth pupae, finding a lower yield of adenosine, polysaccharide, and mannitol on the fruiting bodies from pupae compared with wheat and the yield of cordycepin, carotenoids, and superoxide dismutase was higher in the fruiting bodies on pupae compared with those on wheat. Altogether this highlights the importance of selecting a substrate whereby consistent repeatable quality results can be obtained in the growth of the fungus.

The right selection of solid substrate is important for the efficient and eco-

nomical production of compounds and the production of primary or secondary metabolites can be improved with the suitable choice of substrate or mixture of substrates with the appropriate nutrients [M+11] [NP09]. Hung et al. [HJWL15] observed that the addition of sodium selenite in the solid substrate significantly increased the production of cordycepin, cordycepic acid, Cordyceps polysaccharide, and organic selenium of the fruit body compared with the control group that had no sodium selenite [HJWL15]. One suggestion as to why this might be is the pressure of the C. *militaris* growth environment, which forces C. *militaris* to enter the secondary metabolic phase and begin producing secondary metabolites. Additional ions in the provided medium may serve as enzyme or protein cofactors [HJWL15].

One of the major advantages of SSF is that the substrates are utilized very slowly and steadily, which supports the controlled release of nutrients [SV12]. Research has suggested that antibiotics produced through SSF are more stable and are produced in higher quantities than SmF which has been attributed to lesser production of the intermediate compounds [SV12]. In numerous studies, SSF has been shown to produce far greater quantities of enzymes. For example, in Aspergillus species which is arguably the largest source of fungal enzymes, increases in fungal enzyme production on SSF has been shown to be up to 50-fold greater than with SmF 74 (Figure 3). Bioactive secondary metabolites are also produced in higher concentrations in SSF than SmF and often in faster time frames [M+11]. The steady utilization of the substrate in SSF has a positive impact on enzyme yield and productivity despite the maximal mycelia biomass being lower than that observed in SmF grown fungi [VG+03]. SmF growth displays strong decay during fermentation compared with no apparent decay in SSF, which may contribute to the higher yields and may be a consequence of large cell aggregates spread out in thin layers having a large area to volume ratio (A/V) where SmF cultures produce smaller aggregates with a smaller A/V ratio and these may account for the important physiological difference in relation to gas diffusion [VG+03]. Mycelial growth in SSF expose the fungal hyphae directly to air, this is thought to be one reason productivity is much higher than SmF [RRK03].

Heat and mass transfer in processing are considered to be some of the main difficulties in SSF. Heat is generated in SSF in proportion to the activities of the fungal organism, because the solid materials are often low in thermal conductivity [Pan03]. As such, heat removal occurs slowly, and this may affect growth and production when controlling large scale fermenters [Pan03] [RRK03]. Additionally, novel designs of appropriate bioreactors are being developed with heat removal as a major consideration and the use of evaporative cooling and of small-volume reactors is promising [SPSP09]. Production at a smaller scale in individual vessels overcomes the problem of heat transfer considerably, for example see Figure 4. In normal scale productions, SSF is performed in smaller vessels, and this also has the benefit of a lower water consumption, reduced wastewater treatment costs, and lower energy, and is therefore extremely environmentally friendly. Multiple smaller vessels may assist in the control of bacterial contamination and prevent issues with quality control of the final product [Pan03].

Smaller fermenter sizes also reduce downstream processing, require less stirring, and have lower sterilization costs, thus contamination issues associated with their productions [M+11].

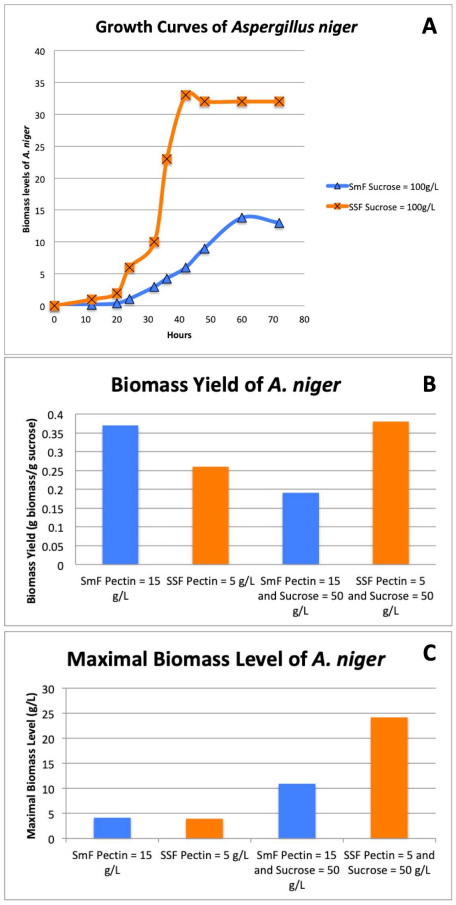

Figure 3: Biomass and yield from Aspergillus *niger* under SmF and SSF growth conditions.[c]

[c] Comparison of the fungus Aspergillus *niger* highlighting the benefits of solid-state fermentation (SSF, orange) over submerged fermentation (SmF, blue) conditions in regards to yield over time (A), biomass yield (B) and biomass level (C). Data adapted from Viniegra-Gonzalez et al.[VG+03].

6.2 Sources of Strains and Stock Cultures

The source of strains varies considerably in published studies. In a small cohort of research papers, there were very few that shared the same source.

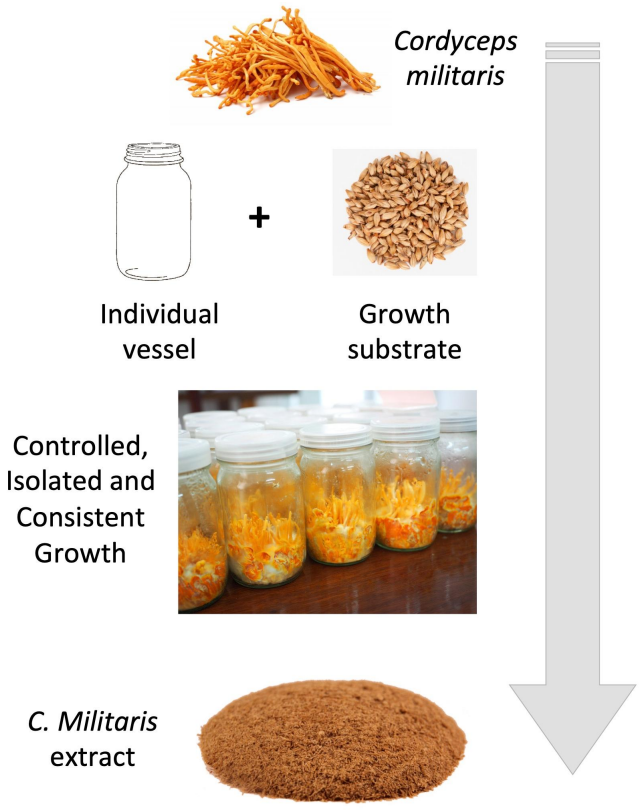

Figure 4: Example cultivation of C. *militaris* using SSF in small, individual vessels.[d]

[d] Solid-state fermentation (SSF) has many benefits and using small individual vessels can improve quality, consistency and reduce environmental waste.

For example, researchers obtained their C. *militaris* from NonGong Mushroom Co. (Kyungbuk, Korea) [KY05]; the Biosource Collection and Research Center (BCRC, Hsinchu, Taiwan) [HJWL15] [Yan12]; the Thailand BIOTEC culture collection (BCC 2816) [R+14a]; a voucher specimen deposited in the Herbarium at the College of Bioscience and Biotechnology, Konkuk University (Seoul, Republic of Korea) [MPP12]; the Honghao Biological Company of Jiangmen, Guangdong, China [T+14]; and collected from wild sources [YXWG17][Y+04a]. Stocks are commonly maintained on potato dextrose agar as either slants or agar plates [L+16b] [JKC08]. Common incubation is 25°C for 7 days 76 and in some cases a 12:12 hour dark/light light cycle was specified [L+16b]. Yin et al.

incubated at 23°C on a shaker at 150 rpm for 7 days in the dark [Y+12]. Alternative mediums include yeast and mold agar at 24°C or Sabouraud dextrose broth [L+16b].

The differences in strains and stock culture techniques will have an impact on the compounds that can be produced. Consistent cultivation methods with an identical starting culture will allow for consistency that will maximize quality, growth and yield of bioactive compounds.

7 Biosynthesis Pathways of Compounds in C. *militaris*

The biosynthetic pathway of cordycepin production by C. *militaris* has not been determined. However, the complete genome has been sequenced [Z+11] and genome-scale metabolic network and gene expression analyses have been constructed [Y+12] [V+17]. Together these have provided insight into the possible cordycepin pathways.

Both adenine and adenosine have been suggested as the precursor to cordycepin production pathway [M+15]. There are many variables known to impact cordycepin production and gene expression in C. *militaris* . Most notable are growth type, temperature, light, humidity, oxygen state, oxygen volume and presence of compounds such as iron, ammonium, and carbon/nitrogen ratio [Cui14] [M+15] [SZZL12] [HHLT17] [WLK14] [J+18] [MUSS06] [SKDP17]. For instance more dissolved oxygen leads to a reduction in cordycepin production [SKDP17] [MJ04] and increases in cordycepin production have been shown when peptone is available as the preferred nitrogen source and adenosine is included as a addition to the culture media [MUSS06].

8 Compounds associated with C. *militaris*

The death of the host and the emergence of the fruiting bodies are associated with the secretion of the compounds cordycepin (3'-deoxyadenosine) and cordycepic acid [d-mannitol], which accumulates in both the mycelia and stromata [KYKA02] [KW+01]. Cordycepin is a derivative of the nucleoside adenosine. It is a bioactive metabolite with numerous therapeutic benefits including anti-viral, anti-cancer, anti-inflammatory, anti-fungal, anti- bacterial and antidepressive [TSSK13] [Pat08] [L+16a]. There are a diversity of compounds that have been routinely identified in C. *militaris* extracts, such as adenosine, ergosterol, polysaccharides, such as beta-glucan, and numerous other bioactive metabolites [S+14a] [Pat08]. Since cordycepin was isolated from cultured C. *militaris* , nucleosides in Cordyceps have become a focus [XQX13]. Nucleosides and bases, including adenosine, guanosine, cytidine, uridine, adenine, uracil, hypoxanthine, 6-acetylpurine [G+04] [GWLY07] [HLG03] [J+07] and homocitrullyl aminoadenosine [G+04] have been isolated from C. *militaris* .

8.1 Recently Identified Compounds

There is much work being conducted to identify the constituents of C. *militaris* and their pharmacological benefits [Pat08]. New metabolites are still being identified, and we are continuing to understand differences in the activation of secondary metabolites from genomic data and through alterations in methods of C. *militaris* cultivation and extraction. C. *militaris* grown on germinated soybean revealed novel isoflavone methyl-glycosides: daidzein, glycitein, genistein and genistein, as well as other phenolics and flavonoids such as, soyasaponin, glycerol, proline, glutamine, pentitol, fructose, inositol, octadecanoic acid, sucrose, pyroglutamic acid, citric acid, histidine, and palmitic acid which were neither found in C. *militaris* extracts or germinated soybeans grown in isolation [PCP12] [C$^+$10].

Novel carotenoids (pigments) from C. *militaris* fruiting bodies have been identified as xanthophylls and named as cordyxanthin-I, cordyxanthin-II, cordyxanthin-III and cordyxanthin-IV [D$^+$13]. Various saccharides and polysaccharides, such as cyclofurans, β-glycans, β-mannans, cross-linkedβ -mannan polymers and complex polysaccharides were isolated from C. *militaris* [HC08] [O$^+$07]. Novel compounds from cell fusion between C. *militaris* and C. *cicadae*, resulted in the isolation of 13 compounds including 2 new ones named 2-(5-(3-oxobutyl) furan-2-yl) acetic acid and cordycepone [Y$^+$18]. Ten-membered macrolides, cepharosporolides C, E and F, cordycepin, pyridine-2,6-dicarboxylic acid and 2- carboxymethyl-4-(30hydroxybutyl) furan were also reported from C. *militaris* [R$^+$14a].

C. *militaris* also contains proteins: a glycoprotein with N-acetylgalactosamine [K$^+$86], a lectin [J$^+$07], haemagglutinin [WWN09], fibrinolytic enzymes [C$^+$07] [K$^+$06], an endochitinase[LM03] and superoxide dismutase [WHLY05]. Peptides have been reported [Y$^+$09] [R$^+$06]. Proteases [H$^+$05], polyamines and all essential amino acids were purified from the dry fruiting body of C. *militaris* . Moreover, cyclic dipeptides [HC08] and sterol-type compounds, including ergosterol, δ-3 ergosterol, ergosterol peroxide, 3-sitosterol, daucosterol and campeasterol [R$^+$14a] [Z$^+$09] [BW10] have been identified. Saturated acids, such as palmitic acid, unsaturated acids like linoleic acid [KW$^+$01] and myriocin; a fungicide, have also been isolated [Pat08] [BW10]. Other compounds that have been purified from the fruiting bodies of Korean C. *militaris* are mannitol [LYT06] and novel carotenoids, which were identified as xanthophylls [D$^+$13]. Besides these compounds, ophiocordin [BHKK94], bioxanthracenes [R$^+$06], pyridine-2, 6-dicarboxylic acid [J$^+$07] and naphthoquinones [U$^+$05] were determined.

For many of these compounds, the benefits, if any, are not fully understood. For the identification of some of these compounds, such as haemagglutinins or lectins which are proteins with carbohydrate-binding specificity [WWN09], it is thought that they have the potential to be exploited due to their health benefits. Below is a summary of some of the most important research experiments highlighting the benefits of C. *militaris* and briefly discussing their mechanisms.

9 Summary

There is further scope for the identification of undiscovered metabolites with the use of experimentation using genetic mutants. Despite the variety of methods and strains yielding different results and a lack of repeatability across the scientific studies, there is much promise in the use of C. *militaris* extracts for a variety of ailments. Below is a non-exhaustive list of some these experiments and Table 1 shows the majority of bioactive constituents of C. *militaris* and their applications.

There are numerous confirmations of the historic traditional Chinese medical benefits of C. *militaris* . Quality and consistent growth of C. *militaris* with a solid-substrate fermentation is likely to provide a higher quality extract with necessary and desired compounds.

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