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Properties of *Cordyceps Sinensis*: A review

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ABSTRACT

A great mystique and aura surrounds *Cordyceps sinensis* (syn.: *Cephalosporium sinensis*), an endoparasitic fungus which has claims of anti-cancer and anti-aging properties. Much research has been conducted over the years on crude extracts and its bioactivity. More research is now focused on culturing *C. sinensis* and on isolating and identifying pure compounds novel to *C. sinensis* in an attempt to alleviate strain on demand for the natural fungi. Several polysaccharides, nucleosides and sterols all have had reports of promoting health both *in vitro* and *in vivo*. Specific and novel compounds which are characteristic to *C. sinensis* are emerging with reports of two new epipolythiodioxopiperazines, gliocladicillins A and B capable of inhibiting growth of HeLa, HepG2 and MCF-7 tumor cells. Exclusive to natural *C. sinensis*, five constituents of cordysinin (A–E) has also been reported for the first time and has been linked to anti-inflammatory properties. Although it may still be premature to believe these results should translate into pharmaceutical use, there is sufficient evidence to warrant further research.

Contents

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1. Introduction

A parasitic fungus known as *Cordyceps* has long been coveted as an exotic traditional Chinese medicine (TCM) with an overwhelming list of pharmacological properties. *Cordyceps* are the most diverse genus of the *Clavicipitaceae* family in the Order Hypocreales. Over 400 species have been reported (Sung et al., 2007) making classification a rather tedious task. Each individual species is specific to a singular host species, insect caterpillars. The caterpillar fungus known as *Cordyceps sinensis* (Berk) Sacc. stands out as the most documented species of *Cordyceps* with reports from Tibetan doctors dating as far back as the late 1400 (Winkler, 2008a; Winkler, 2008b).

The natural production of *Cordyceps* undoubtedly summoned the interest of first discoverers. The fungal spores infect and take over the host organism causing its eventual demise. The fungus continues to grow and emerges from the corpse of the host organism. Adding to the mystique is the location in which the relationship between the fungus and the larva of the ghost moth occurs. The caterpillar fungus lives on the Tibetan Plateau including parts of India and western China, otherwise known as the "Roof of the World". During the 1993 National Games in Beijing, China where three female runners set 5 world records for the 1500, 3000 and 10,000 meter races. After testing negative for anabolic steroids and other banned substances, it was later revealed by the coach that the runners had taken *C. sinensis* extracts. Anecdotal evidence of the potential performance enhancing properties of *C. sinensis* ignited consumer interest. In fact, the larva-fungi complex has long been used in TCM to "invigorate the lung and nourish the kidney" in China for hundreds of years (Dong & Yao, 2008).

Demand for the fungi was further accentuated by the numerous scientific reports stating specific pro-health-related claims. *C. sinensis* tonic can be purchased over the counter and is believed to provide the immune system with a boost especially after serious illness (Bai, Ren, & Yu, 1997; Chen, Zhang, Shen, & Wang, 2010; Cheung et al., 2009). An ever growing list of symptoms remedied using *C. sinensis* include respiratory, renal, liver, nervous system, cardiovascular diseases, cancerous tumors, decreased libido and even stress, fatigue and aging (Belo, Marchbank, Fitzgerald, Ghosh, & Playford, 2006; Benowitz, Goldberg, & Irwin, 2002; Chen et al., 2005; Dong & Yao, 2008; Ji et al., 2009; Koh, Kim, Chang, & Suh, 2003b; Koh, Kim, Kim, Song, & Suh, 2003c; Koh, Suh, & Ahn, 2003a; Liu, Li, Zhao, Tang, & Guo, 2010; Song, Ming, Peng, & Xia, 2010; Woo Bok, Lermer, Chilton, Klingeman, & Towers, 1999; Yamaguchi, Kagota, Nakamura, Shinozuka, & Kunitomo, 2000). In fact, long before any of these studies had been published, *C. sinensis* has officially been classified as a drug in the Chinese Pharmacopoeia since 1964 (Committee of Pharmacopoeia, Chinese Ministry of Health, 1964, 2005).

The value of the fungi received a sharp increase following the severe acute respiratory syndrome (SARS) outbreak in China in 2003. With the inflated value of the fungi comes increased demand leading to severe price hikes. According to 2008 prices, the price of natural *C. sinensis* ranged from 3000 to over 18,000 USD per kg depending on size of the larvae (Winkler, 2008a). In a decade (1998–2008), the price of *C. sinensis* has increased 900% (Winkler, 2008b). This creates a strong socioeconomic strain in the Tibetan Plateau where many villagers have fought over the desecration of grasslands containing the sought-after fungi. The availability of the fungi is limited by its confined geographic location. With the added effects of large-scale harvests, the fungi has been classified as an endangered species by CITES (*Convention on International Trade in Endangered Species*) Management Authority of China and China Customers. The scarcity of natural *C. sinensis* sparked novel approaches such as artificial cultivation of the pure mycelium in liquid culture or on grains.

Many subsequent studies were conducted using cultured *C. sinensis* and have yielded positive results suggesting the cultivated fungi might possess the same health-promoting functions as the natural counterpart (Chen et al., 2010; Cheung et al., 2009; Dong & Yao, 2008). On the backs of these reports, the USA passed the Dietary Supplement Health and Education Act in 1994 giving way to a vast market consisting of websites selling capsules and tonics made from independently grown *Cordyceps*. Fueled by an increasing wave of health-conscious consumers and countless supporting scientific literature to boot, demand for *Cordyceps* products are at an all-time high. Yet with all this, no major pharmaceutical

company has developed a supplement to capitalize on the market. The lack of support from big name pharmaceuticals puts into questions the validity of some reports and if the findings are substantial or merely overblown. Dong and Yao (2008) called out a study on *C. sinensis* and its protective effects on PC12 cells from hydrogen-peroxide induced neuronal toxicity (Li et al., 2003). Although the claim involved *Cordyceps*, the actual strain used was named *Cephalosporium sinensis* Chen sp. nov. supplied by Wan Fong Pharmaceutical Factory (Zhejiang, China). It would appear as though the lack of regulation and quality control has affected factories and their distribution of *C. sinensis*. Yamaguchi et al. (2000) purchased their artificially cultivated fruiting-bodies of *C. sinensis* from the Xinhui Xinhan Artificial *Cordyceps* Factory (Guangdong, China) and successfully radical scavenging power of both the water and ethanol extracts. However, both Dong and Yao (2008) and Paterson (2008) believe the fungal material to be unauthentic claiming that the manufacturer is actually selling *C. militaris*. These findings can cause damage to the reputation of the overall body of work involving *C. sinensis* and its therapeutic properties. Asian countries have a marked advantage in the *Cordyceps* market due to its inherent oriental origin; these nations are also responsible for delivering the vast majority of literature regarding the beneficial properties of *Cordyceps*.

The following review focuses on the different methods used to extract and identify compounds derived from *C. sinensis* and how they have been reported to possess pharmaceutical properties.

2. Extraction

An overwhelming body of literature involving *C. sinensis* has been conducted using solvent extracts of the fungus. Extracting solvents vary based mainly on polarity and ease of use. The large volume of research related to *C. sinensis* extracts (CSE) has whittled down the choice of solvents to a critical few that have been shown to be effective in extracting the desired compounds. In order of increasing polarity, ethyl acetate, ethanol, methanol and water figure amongst the most commonly used solvents for extraction although combination and sequential extractions are also common practice.

2.1. *Water extracts*

Water, a polar solvent, has long been used in the field of food chemistry in part due to its nontoxicity and invasiveness (Cheung et al., 2009). Its molecular structure contains dipoles and can solvate ions by orienting itself according to electrostatic attraction between the ion and the water molecule. A hydration shell is formed and stability is achieved. Yamaguchi et al. (2000) performed a simple hot water extraction using 10 g of dried fruiting bodies of cultured *C. sinensis* blanched in 200 mL of hot water at 70 °C for 5 min. This produced a brown-colored extract which was then filtered and lyophilized into a gray powder which was administered to rats to determine its effects on cholesterol levels (Yamaguchi et al., 2000). Ji et al. (2009) seemingly followed the same extraction protocol however, at a higher temperature and longer extraction time in order to prove antiaging effects of *C. sinensis* extract. In brief summary, *C. sinensis* powder was added to water at a 1:20 ratio (w/v) and heated by autoclaving for 20 min to a temperature of 120 °C. The extracted powder was then filtered and lyophilized and a yield of 30% was ascertained. This simple extraction technique was also employed by Song et al. (2010) to test reparative effects of the extract on kidney function and its potential as a therapeutic drug for kidney diseases (Song et al., 2010). Slight modifications included the 1:5 (w/v) powder to water ratio and extraction temperature and time set to 90 °C and 2 h, respectively. Koh et al. (2003c) performed a series experiments involving hot-water extract from mycelia of *C. sinensis* in 2003 and effectively demonstrated physiological function as antibiotic growth promoter (Koh et al., 2003a), antistress and antifatigue effect (Koh et al., 2003c) as well as hypocholesterolemic effect (Koh et al., 2003b) of the hot water extract. Extract preparation protocol for the antibiotic growth promoting followed that of Ji et al. (2009) and Yamaguchi et al. (2000) however, at 100 °C (Koh et al., 2003a). A more thorough and elaborate extraction process was employed for the subsequent experiments involving antistress, antifatigue and hypocholesterolemic effects of *C. sinensis* extract whereby 50 g of freeze dried *C. sinensis* powder was boiled quickly at 100 °C for 5 min, homogenized and centrifuged. A sequential fractionation of the resulting residue ensued using solvents of increasing polarity beginning with ethylacetate, methanol and hot-water. The final hot-water extract was centrifuged and the supernatant freeze-dried. This more thorough hot-water extraction process did not provide a greater yield (29.3%) compared to that of Ji et al. (2009). Liu et al. (2010) prepared separate hot water and ethanol extract, which were later combined for their experiments involving the therapeutic effects of the extract on brain damage (Liu et al., 2010). Once again, a straight forward extraction procedure was followed with temperature set to 100 °C and extraction time of 3 h. An aliquot was removed and lyophilized and yield of 29% was determined prior to the subsequent ethanol extraction. It would appear that based on the above mentioned extract preparation protocols, time of extraction appears to be of minimal value seeing as a 5-min extraction proved just as effective as a 3 h extraction. Temperature may be a more critical parameter.

Fractionating *C. sinensis* extract with various solvents is a relatively novel approach and certainly critical for thorough investigation of active components directly responsible for the many physiological roles associated with *C. sinensis*. One such study involved structural analysis of polysaccharides in cultured mycelia of *C. sinensis* responsible for monocyte activation and improved innate immunity (Akaki et al., 2009). Cultured mycelia were defatted by incubating for 6 h in ethanol three times. The extraction was then performed on the residue using distilled water at 100 °C for another 6 h. Following centrifugation, the supernatant was recovered and concentrated down to half its initial volume and ethanol was added to make up the difference. The resulting precipitate was freeze-dried and represented the crude polysaccharide fraction with a yield of 4.2%. A portion of this crude extract was dissolved in water and dialysis was performed

to remove low-molecular-weight constituents. After 3 days of dialysis, the liquid within the membrane was removed and centrifuged resulting in a water soluble polysaccharide fraction (2.6%) and a water insoluble polysaccharides fraction (0.2%). All three fractions were then subjected to further analysis after suspension in distilled water at a concentration of 1 mg/mL. Sugar content, cytokine production, Fourier transform infrared spectroscopy (FTIR), nuclear magnetic resonance (NMR) and methylation analyses were performed on all three fractions in order to pinpoint which fraction was responsible for the immunostimulation and subsequently invest efforts into one particular fraction.

2.2. Ethanol extraction

In 2011, a group of researchers set out to explore the anti-inflammatory properties of extracts of *C. sinensis* mycelia (Yang, Kuo, Hwang, & Wu, 2011). The intensive extraction protocol was based on a crude ethanol extract subsequently partitioned into *n*-hexane-soluble and water-soluble fractions. The *n*-hexane fraction was further separated into *n*-hexane/*n*-hexane and *n*-hexane/MeOH–H_2O fractions. The water-soluble fraction was further partitioned with ethyl acetate to give ethyl acetate and water-soluble fractions. The crude and the partitioned fractions were tested for inhibition of superoxide anion generation and elastase activity with the *n*-hexane/*n*-hexane, hexane/MeOH–H_2O and ethyl acetate fractions containing the most inhibition and elastase activity. Further purification of these fractions led to the discovery of five novel compounds and 45 known compounds. NMR and mass spectroscopy was utilized to analyze structures. The five novel compounds comprise cordysinins and its various constituents simply defined as compounds 1 through 5 (Fig. 1).

2.3. Methanol extraction

In an attempt to elucidate specific bioactive compounds from mycelia of *C. sinensis* with antitumor activity, an 'activity guided chemical fractionation' of the methanol extract of *C. sinensis* was performed and led to the isolation of two antitumor compounds (Woo Bok et al., 1999; González-Sarrías, Li, & Seeram, 2012). A crude methanol extract was prepared using 150 g of dried mycelium and 500 mL of MeOH. The process was repeated three times and the supernatants combined and the solvent was evaporated under pressure. The crude extract was used in an antiproliferation assay using a K562 erythroleukemia cell line and was shown to have the ability to inhibit proliferation by 36%. No further testing was performed on the crude extract and instead extraction focused on the remaining residue from the crude MeOH extraction. The residue was redissolved in a 1:1 (v/v) mixture of MeOH/H_2O and partitioned using hexanes. The aqueous layer was then extracted with ethyl acetate. Ethyl acetate extracts were concentrated under pressure to remove the solvent and a brown oil remained as a result. Silica gel-flash column chromatography elution was performed using $CHCl_3$ with stepwise increase of the solvent polarity. In all, 14 fractions were collected and tested for antitumor activity. Fractions that tested positive were further chromatographed. This led to two fraction FB1 and FB2. FB1 was reported as a white solid and was recrystallized into a white powder and subsequently labelled compound 2. Further separation of FB2 using silica gel chromatography resulted in the isolation of compound 1a and a fraction FC1 which was injected into HPLC and gave compounds 3 and 4. The structure of these compounds were resolved using NMR and in sum, a steroidal glycoside 5α,8α-epi-dioxy-24(R)-methylcholesta-6,22-dien-3β-D-glucopyra-noside (compound 1a) and 5α,6α-epoxy-24(R)-methylcholesta-7,22-dien-3β-ol (compound 2) were isolated and identified for the first time as anti-tumor compounds. Ergosteryl-3-O-β-D-glucopyranoside (compound 3) and 22,23-dihydroergosteryl-3-O-β-D-glucopyranoside (compound 4) were also isolated following this extraction protocol but have previously been isolated (Shiao, Lin, Lien, Tzean, & Lee, 1989).

2.4. Ethyl acetate extraction

The vast majority of research conducted on the extract of *C. sinensis* utilized ethanol, methanol or hot water as extraction solvents mainly due to common practice and their ability to extract polar components (Wu, Zhang, & Leung, 2007). Ethyl acetate, although the least polar of the three aforementioned solvents, has garneshed more attention of late and has gained in popularity. Studies conducted by Zhang, Wu, Hu, and Li (2004) and Zhang and Wu, (2007) have effectively shown that ethyl acetate extracted *C. sinensis* mycelium to be a potent inhibitor of proliferation of cancer cells (Wu et al., 2007; Zhang et al., 2004). Preparation of the extracts followed a sequential order starting from non-polar to polar solvents. The dried mycelium powder was first extracted using petroleum ether, ethyl acetate, ethanol and water at a 1:10 (w/v) for each solvent. The extraction process involved the use of an orbital shaker at room temperature for 24 h except in the case of the hot water extraction where a hot plate and stir bar were used for a period of 3 h. After each round of extraction, the extract was filtered and the solvent was rotovapped to dryness to afford the crude extract.

In a more recent study aimed at identifying novel anti-cancer agents from *C. sinensis*, Chen et al. (2009) extensively extracted the fungi with ethyl acetate six times over. After vacuum evaporation of the solvent, 40 g of the crude extract was obtained and further fractioned by silica gel chromatography using a CH_2Cl_2–MeOH elution gradient. At a ratio of 99: 1 (v/v) CH_2Cl_2–MeOH, a 1 g active fraction was collected and separated once more on a smaller silica gel column using 99.5: 0.5 (v/v) CH_2Cl_2–MeOH as eluents. Purification of a 150 mg active fraction using reversed-phase high-performance liquid chromatography (RP HPLC) yielded 30 mg of glicladicillin A. Purification of a 120 mg active fraction using RP HPLC resulted in 25 mg of glicladicillin B. A third compound was isolated from a 100 mg active fraction again using RP HPLC and was identified as 11,11′-dideoxyverticillin (18 mg).

2.5. Pressurized liquid extraction (PLE)

PLE promotes the high extraction properties of the solvent used by sustaining high temperature and pressure within the extraction cartridge or cell. The high temperature enables high solubility and diffusion while high pressure ensures the

solvent is below boiling point. Both parameters combined, this allows the solvent to penetrate the sample with much more efficiency resulting in fast extraction time using less solvent (Luthria 2012). Li et al. (2004) performed PLE on powdered *C. sinensis* in a study investigating the presence of ergosterols and nucleosides by HPLC. The extraction process involved mixing the *Cordyceps* powder with diatomaceous earth in a 1:1 proportion and subsequently placed in a stainless steel extraction cell. Optimization trials scrutinized parameters including type of solvent, temperature, static extraction time and static cycles. Temperature and extraction time proved to be most influential to extraction efficiency. Taking everything into account, the proposed optimal PLE conditions were: solvent, methanol; temperature, 160 °C; static extraction time, 5 min; pressure, 1500 psi; static cycle, 1 (Li et al., 2004). This extraction technique successfully recovered a plethora of compounds most notably cordycepin which had previously not been found in *C. sinensis* following HPLC detection (Shiao, Wang, Lin, Lien, & Wang, 1994). Cordycepin is largely prevalent in *C. militaris* and rarely detected in *C. sinensis* although this study using PLE confirmed its presence in both cultured and natural *C. sinensis*.

3. Nucleoside determination

Nucleosides are considered the main bioactive component in *C. sinensis* (Li, Yang, & Tsim, 2006). Various physiological processes are mediated via nucleoside receptors including nucleic acid synthesis, stimulating immune response (Cheung et al., 2009), influence fatty acid metabolism (Koh et al., 2003a), assisting in iron absorption in the gut and contribute reparative properties in damaged gastrointestinal tract (Marchbank, Ojobo, Playford, & Playford, 2011; Yang, Li, Feng, Hu, & Li, 2010). Soluble nucleosides are considered by some the main bioactive components in *C. sinensis* (Xie, Huang, Hu, He, & Wong, 2010). To date, more than 10 nucleosides, nucleobases and related compounds have been isolated and identified in *Cordyceps* (Fan et al., 2006). These include adenine, adenosine, cytosine, cytidine, uridine, guanine, guanosine, hypoxanthine, inosine, thymine, thymidine, 2′-deoxyuridine and cordycepin (Yang, Ge, Yong, Tan, & Li, 2009). Adenosine has been shown to have regulatory function on the central nervous system (CNS) and has anticonvulsant activity as evidenced *in vivo* in rats (Ballarin, Herrera-Marschitz, Casas, & Ungerstedt, 1987). Oxidative deamination by the enzyme adenosine deaminase substitutes the amino group on adenosine with a hydroxyl group resulting in the related nucleoside inosine which has proven to mediate axonal growth and could help improve the condition of patients suffering from CNS injury (Benowitz et al., 2002).

Cordycepin (3′-deoxyadenosine) is pure compound certainly worthy of attention. The presence of cordycepin has been firmly established in *C. militaris* using a highspeed counter-current chromatography technique yielding cordycepin with purity of 98.9 and 91.7% recovery (Zhu, Liang, Lao, Zhang, & Ito, 2011). However, controversy lingers regarding its availability in *C. sinensis* (Fan et al., 2006). Cordycepin has been classified as an anti-cancer compound stemming from its ability to substitute adenosine thereby terminating synthesis of RNA molecules (Paterson, 2008). Extraction and detection of nucleosides enables differentiation between various strains of *Cordyceps* as well as to ensure identification of counterfeits that have become more wide spread in recent years due to high value driven by scarcity of the natural product.

Several detection methods are commonly available such as thin layer chromatography (TLC) (Ma & Wang, 2008), HPLC (Song, Liu, Li, & Jin, 2008) and capillary electrophoresis (Li, Feng, Ni, & Zhang, 2008) however, limitations are encountered in the way of sensitivity, selectivity and suitability. Ion-exchange chromatography (IEC) is yet another method for separation of nucleosides however, the K^+ and Na^+ present in the mobile phase prevents further analysis and detection via mass spectrometry (MS). Ion-pairing reversed-phase liquid chromatography (IP-RP-LC-MS) has been commonly used in other fields for the separation of nucleotides (Yang et al., 2010). Reagents used here are critical for optimal mass spectrometry results. Another effective method of separation and detection involves liquid chromatography separation coupled with electrospray ionization-mass spectrometry (LC/ESI-MS) (Xie et al., 2010).

3.1. *High performance liquid chromatography (HPLC)*

One of the first attempts at generating a profile of nucleosides and nitrogen bases for *C. sinensis* was performed by Shiao et al. (1994) and employed the use of reversed-phase HPLC. Gradient elution using two-solvent system consisting of 2.5% MeOH and 20% MeOH in 0.01 M ammonium phosphate revealed the presence of major nucleosides and nitrogen bases, uracil, guanine, uridine, guanosine and adenosine. Shiao et al. (1994) were successful in producing RP-HPLC profiles of various *Cordyceps* species and *Paecilomyces* species providing the first step in distinguishing between the various species and the use of metabolites as markers for quality control. The use of a phosphate buffer improves chromatographic performance however can result in ion suppression for mass spectrometry detection and poor separation (Klawitter, Schmitz, Klawitter, Leibfritz, & Christians, 2007).

RP HPLC was used by Chen et al. (2009) to identify three anti-cancer compounds from active fractions of a crude ethyl acetate extracted *C. sinensis*. Mobile phase parameters employed in detecting these compounds varied and therefore three separate runs were performed. In the isolation of gliocladicillin A, 50–55% acetonitrile in water ran for 5 min and the concentration of acetonitrile was held constant at 55% for the remaining 25 min with peak retention time observed at 14.3 min. Gliocladicillin B was observed at a retention time of 19.5 min using 60–85% MeOH as mobile phase for 40 min. The last compound 11,11′-dideoxyverticillin was eluted at 18.0 min using 60% acetonitrile in water for 5 min followed by 60–70% acetonitrile for 25 min.

3.2. *Capillary electrophoresis (CE)–mass-spectroscopy (MS)*

CE provides an attractive method for the identification of nucleosides and nucleobases due to its simplicity of use, high

separation efficiency, small sample volume and low organic solvent consumption (Yang et al., 2009). Optimized CE–MS conditions were used involving 100 mM formic acid containing 10% (v/v) methanol as CE electrolyte. The sheath liquid assists the transfer of the analyte from liquid phase to gas phase. Along with the CE electrolyte, this ensures stable electrospray. Optimal sheath liquid conditions were 75% (v/v) methanol containing 0.3% formic acid. Following optimal conditions, twelve nucleosides and nucleobases were detected. This includes cytosine, adenine, guanine, cytidine, cordycepin, adenosine, hypoxanthine, guanosine, inosine, 2′-deoxyuridine, uridine and thymidine in both natural and cultured *C. sinensis* (Yang et al., 2009). Quantitative results indicate total content of nucleosides is much higher in the cultured fungi with the exception hypoxanthine and inosine. Cordycepin, which is mostly found in *C. militaris*, was only detected in minute amounts in natural *C. sinensis* and was not detected in cultured samples.

3.3. *Liquid Chromatography/electron spray ionization-MS (LC/ESI-MS)*

Selection of the mobile phase must take into consideration of the separation of the nucleosides but also the ESI component whereby the analyte must be in a volatile state. For example, previous HPLC methods (Guo, Zhu, Zhang, & Zhang, 1998) used gradient elution consisting of a KH_2PO_4 buffer and methanol however the low volatility and high salt content rendered this system incompatible for LC/ESI-MS. An optimized method was developed and validated. Xie et al. (2010) suggests the use of ammonium acetate and methanol for the chromatographic component. Optimization of ESI-MS involved testing in positive and negative ion mode and scanning between *m/z* 50–350 per second. Positive ion mode enabled the detection of thymine, adenine, adenosine, cordycepin and 2-chloroadenosine (internal standard). Selective ion monitoring (SIM) mode was used to detect as well as to quantify the four main nucleosides (thymine, adenine, adenosine and cordycepin). Xie et al. (2010) used $[M+H]^+$ at *m/z* 127, 136, 268, 252 and 302 for monitoring. The ionization temperature was set to 400 °C. The *Cordyceps* samples from different sources were extracted using distilled water and ultrasonicated. The vacuum dried filtrate was dissolved in methanol prior to chromatographic separation and subsequent ESI-MS detection. Following this protocol, the four main nucleosides were detected with 98.47–99.32% recovery rates.

The limit of detection (LOD) is the lowest quantity of a detectable substance distinguishable from the blank sample while the limit of quantification (LOQ) encompasses not only the lowest concentration of detection but also the limit at which one can reasonably distinguish differences between two different values by acknowledging a predefined level for bias and imprecision (Armbruster & Terry, 2008). LOQ and LOD for thymine, adenine, adenosine and cordycepin were respectively 1.0 and 0.2, 1.8 and 0.6, 0.6 and 0.1 and 0.5 and 0.1 μg/mL (Xie et al., 2010). Concentrations of thymine, adenine, adenosine and cordycepin were 138.5–174.2, 20.4–38.1, 79.6–186. and 31.3–91.2 μg/g (Xie et al., 2010). Fan et al. (2006) claim to have developed a method combining pressurized liquid extraction (PLE) and HPLC–ESI-MS that "significantly increases the selectivity, sensitivity and repeatability of assay". Similar to that of Xie et al. (2010), ammonium acetate and methanol were used as the mobile phase with UV detection occurring at 254 nm. Positive ion mode was used however; negative ion mode was used for uridine detection. The addition of PLE prior to LC/ESI-MS resulted in the discovery of 43 compounds and the identification of 16 of those compounds. The identified compounds include cytosine, cytidine, uracil, uridine, thymine, thymidine, adenine, adenosine, 2′-deoxyadenosine, cordycepin, hypoxanthine, inosine, guanine, guanosine, 3′-amino-3′deoxy adenosine and 6-hydroxyethyladenosine (Fan et al., 2006). With the exclusion of 2′-deoxyadenosine, 3′-amino-3′deoxy adenosine and 6-hydroxyethyladenosine, the remaining 13 compounds were further investigated for linear range and detection limits. For comparison sake, LOQ and LOD for thymine, adenine, adenosine and cordycepin were respectively 45.21 and 11.32, 24.39 and 6.11, 3.44 and 1.72 and 4.64 and 1.16 ng/mL (Fan et al., 2006). A much lower detection limit was observed in relation to Xie et al. (2010). In terms of concentration of these four nucleosides, comparison between the two studies is rather cumbersome since Xie et al. did not specify the nature (culture or natural) of the *Cordyceps* used in the study leading one to make assumptions based on the source of the *Cordyceps*. Thymine and cordycepin were detected in much greater concentrations according to Xie et al. (2010) while concentrations of adenine and adenosine measured by Fan et al. (2006) overwhelmingly trumped that of Xie et al. (2010). The recovery rate was more than 93.6% on average (Fan et al., 2006). The 13 compounds were further quantified and compared amongst natural and cultured *C. sinensis* from various regions in China along with data from culture *C. militaris*. Briefly, uridine, adenosine and guanosine were dominant in cultured over natural *C. sinensis*. However, inosine figured more prominently in natural *C. sinensis*. Cordycepin was detected in small amounts in natural *C. sinensis* and essentially not present in the cultured fungus. The small amount of cordycepin in natural *C. sinensis* pales in comparison to that found in *C. militaris*.

3.4. *Ion-pairing-reverse phase liquid chromatography–MS (IP-RP-LC–MS)*

Ion-pairing chromatography requires an additional reagent in the mobile phase charged for interaction with the incoming analyte. The reagent used here can influence the quality of the ensuing MS by clouding and polluting the resulting spectra. The volatility of the reagent is therefore tremendously important to increase the sensitivity of the MS for the sampled analytes. Some commonly used reagents include N,N-dimethylhexylamine (DMHA) (Vinas et al., 2010), 1,5-dimethylhexylamine (1,5-DMHA) (Yang et al., 2010) and hexylamine (HMA) (Fromentin, Gavegnano, Obikhod, & Schinazi, 2010). The amine interacts with the positively charged nucleotide phosphates. Although capable of analysis nucleotides, these solvent systems required high concentrations (5–20 mM) of the ion-pairing reagent often resulting background signals and decreased sensitivity. Pentadecafluorooctanoic acid (PDFOA), a perfluorinated carboxylic acid, had previously been used to detect 10 underivatized amino acids (Asp, Asn, Ser, Gly, Gln, Cys, Glu, Thr, Ala, Pro) which were less common

using previous detection methods (Petritis, Chaimbault, Elfakir, & Dreux, 1999). Compared with other perfluorinated carboxylic acids, the long *n*-alkyl chain PDFOA proved best at separation and was employed for the detection nucleotides and nucleobases along with derivative compounds in *Cordyceps* (Yang et al., 2010). Yang et al. (2010) performed separation of the extract using a gradient mobile phase consisting of 0.25 mM PDFOA in solution and acetonitrile with peaks detecting at 260 nM. Validation of this solvent system comes via results indicating low limits of detection. The overall LOD and LOQ for all 16 investigated compounds were less than 0.16 and 0.41 μg/mL, respectively (Yang et al., 2010). The values are not staggering and are in fact comparable to previously conducted MS (Fan et al., 2006; Xie et al., 2010). The advantage to using PDFOA in ion-pairing chromatography is attributed to highly resolved peaks due to decreased peak tailing which occurs when compounds are strongly interacting with the stationary phase as well as the stain-less steel hardware. This is effectively reduced with the addition of an ion-pairing reagent in the mobile phase facilitating identification of peaks.

4. Polysaccharide determination

Polysaccharides are water soluble and are obtained by ethanol precipitation. Polysaccharides comprise a major chemical constituent found in *C. sinensis* and they are responsible for many of its pharmacological properties. Based on a 2008 review, polysaccharides have been shown both *in vitro* and *in vivo* to possess anti-inflammatory, antioxidant, anti-tumor, anti-metastic, immunomodulatory, hypoglycemic, steroidogenic and hypolipidaemic (Paterson, 2008).

4.1. *Isolation and purification*

Ethanol has previously been used to precipitate polysaccharides from water (Chen et al., 2010; Wang et al., 2011). Chen et al. (2010) examined effects of the extract while Wang et al. (2011) focused primarily on structural characterization of the extract. Preparation of the polysaccharide fraction differed in approaches. Chen et al. (2010) isolated the mycelia from the liquid medium by centrifugation prior to boiling with distilled water and then precipitating with ethanol. After washing with ethanol to get rid of unwanted polar compounds, the precipitate was dried and redissolved in water. DEAE-32 cellulose column was used to elute the neutral and acid polysaccharide fractions. Water and a gradient NaCl solution (0–2 M) was used for elution with monitoring of the eluate via phenol–sulfuric monitoring. The supernatant was freeze-dried after dialysis thus completing the extraction process. DEAE elution profile showed two peaks; first peak was that of neutral polysaccharides eluted with water while the second peak is that of the acid polysaccharides eluted from the NaCl gradient. A more thorough investigation of the acidic polysaccharides fraction was performed by Wang et al. (2011). In their extraction protocol, the boiling of the mycelia step was bypassed and instead used centrifugation to collect the liquid supernatant from the liquid culture medium used to ferment the fungus. Ethanol precipitation was then used to collect the exopolysaccharide which was subsequently freeze-dried and reported as a 'whitish and fibrous solid' (Wang et al., 2011).

To get rid of protein content, the crude exopolysaccharide extract was treated with Sevag reagent (1-butanol/chloroform 1:4, v/v). Dialysis and freeze-drying completed the exopolysaccharide preparation step. Further purification was required in order to obtain acidic polysaccharides. The dried exopolysaccharide was dissolved in a Tris–HCl solution overnight and the supernatant collected and loaded onto a DEAE-52 cellulose column for ion-exchange chromatography. Somewhat similarly to Chen et al. (2010), a NaCl gradient (0.2 and 0.4 M in Tris–HCl) was used for elution with monitoring for the presence of polysaccharides by the anthrone-sulfuric acid method. Elution profile showed a total of four distinct peaks. The first peak was eluted with the buffer solution at 0 M NaCl and the three later peaks were eluted at 0.2 M NaCl. The combined eluates representing the three latter peaks were collection and further purified by gel-permeation chromatography on a Sephadex G-75 column with each fraction eluted with 0.2 M NaCl. The collected eluates from this step were dialysed against water and freeze-dried to produce the acidic polysaccharide fraction representing 52% of the original crude exopolysaccharide (Wang et al., 2011). Agarose gel electrophoresis stained with the positively charged toluidine blue displayed a single red dot upon being subjected to the acidic polysaccharide fraction. This change in color indicates the presence of polyanions within the fraction thereby confirming the acidic property as well as its homogeneity as evidenced by the presence of a singular point. A single peak was also observed when subjected to high performance gel permeation chromatography (HPGPC). A retention time of 25 min would suggest a molecular weight of 36.3 kDa.

In a study focused on polysaccharide from *C. sinensis* and its ability to combat renal failure, a crude polysaccharide extract (21 g; 7% yield) was obtained and subjected to a DEAE-52 cellulose anion-exchange column eluting with 0.05 M NaCl solution (Wang et al., 2010). Gel-permeation purification was performed on a Sephadex G-100 column and eluted with distilled water. The collected eluate was lyophilized resulting in a white powder (1.6 g) representing 7.6% of the crude polysaccharide. Purity of the final fraction (CPS2) was verified with the Bradford assay displaying negative results indicating the lack of proteins. Spectrophotometer analysis at 260 and 280 nm saw no absorption indicating the absence of protein and nucleic acid. HPGPC analysis using 0.1 M $NaNO_3$ displayed a singular sharp peak at roughly 16 min thus suggests fraction homogeneity. A dextran standard was used to determine a molecular weight of 43.9 kDa. Wang et al. (2009) extracted a polysaccharide fraction called CPS1 from fruiting bodies of cultured *C. sinensis*, which was shown to possess antioxidant activity and was subjected to further characterization. The impressive health claims promised by both fractions were intriguing and prompted the researchers to dig deeper and attempt to characterize their respective structures.

4.2. *Structural analysis*

A variety of analysis methods are currently available to researchers. In almost all cases, multiple methods are used

to provide a thorough and more conclusive structure determination.

4.2.1. PMP pre-column derivation (monosaccharide determination)

Monosaccharide composition of a polysaccharide fraction can be determined using a 1-phenyl-3-methyl-5-pyrazolone (PMP) pre-column derivation (Wang et al., 2009; Wang et al., 2010). Wang et al. (2009), Wang et al. (2010) have been demonstrating the positive health effects of water-soluble polysaccharides isolated from *C. sinensis* following hot-water extraction and ethanol precipitation, as discussed previously. These health claims included protection against kidney damage (Wang et al., 2010) and antioxidant activity *in vitro* (Wang et al., 2009). Monosaccharide composition of both polysaccharide fractions were investigated using PMP labelling and was determined that CPS1 was composed of glucose, mannose and galactose at a molar ratio of 2.8:2.9:1, respectively. CPS2 was composed of mannose, glucose and galactose at a ratio of 4:11:1, respectively. The same protocol was followed in both cases. Briefly, 2 M trifluoroacetic acid (TFA) hydrolyzed the polysaccharide with the hydrolysate labeled with PMP by incubating with 0.6 M NaOH and 0.4 M PMP. Aqueous phase from a chloroform extraction was then analyzed via HPLC with all standards labeled following the same procedures. CPS1 alone was subjected to a partial acid hydrolysis experiment involving 0.05, 0.2, 0.5 M TFA and following dialysis with a membrane size cut-off at 1000 Da, four fractions (f1, f2, f3, and f4) were obtained and underwent HPLC analysis. The fraction with the largest molecular weight was collected in f1 with fraction resulting from 0.5, 0.2 and 0.05 M TFA hydrolysis ensuing in decreasing order of molecular weight. The significant presence of mannose and glucose in f1 is indicative of the backbone structure composition of CPS1. Monosaccharides of f2, f3 and f4 showed an abundance of glucose and galactose suggesting their presence in the branch structure of CPS1.

4.2.2. Periodate-oxidation and Smith degradation

Periodate oxidation aims at deducing structural information relating to branching and linkage of a polysaccharide based on the uptake of periodate (Stoddart, 1984). Smith degradation is a useful extension of periodate oxidation whereby the previously oxidized product is reduced to a polyalcohol with borohydride and subsequently hydrolysed with a mild acid. Wang et al. (2009), Wang et al. (2010) performed this oxidation – degradation procedure with the components of the polyalcohol determined using HPLC. The polysaccharide of CPS1 and CPS2 showed large periodate consumption indicating the existence of a 1,2-glycosidic linkage or 1,4-glycosidic linkage or both. Trace amounts of formic acid was also detected implying the existence of a 1,6-glycosidic linkage. HPLC detected the presence of mannose, glucose, galactose, glycerol and erithritol in the hydrolyzed product following periodate-oxidation and Smith degradation of CPS1. Mannose, glycerol and erithritol were detected in the hydrolyzed product of CPS2. The presence of mannose, galactose and glucose indicates a 1,3-, 1,2,3-, 1,2,4-, 1,3,4-, 1,3,6- or 1,2,3,4-linkage which was not oxidized by periodate. Glycerol stems from 1,2-glycosidic linkage or 1-linked glycosidic linkage and erithritol from 1,4 or 1,4,6-linked glycosidic linkage. Predictions were made in regards to the backbone structure of CPS1 and was hypothesized to have $1 \rightarrow 2$ and $1 \rightarrow 4$-linkage of mannose, $1 \rightarrow 3,6$ and $1 \rightarrow$ -linked glucose and $1 \rightarrow 3$-linked galactose (Wang et al., 2009). NMR spectroscopy was used later on to complete the structural characterization of CPS2.

4.2.3. Gas chromatography coupled mass spectrometry (GC/MS)

GC is a commonly used analytical tool for separating and analyzing vaporized compounds. As the name would suggest, the compound must be first converted into a volatile derivative. Several derivatization procedures exists such as trimethylsilyl ether, deuterioalditol acetate, peracetylation and aldononitrile acetate (Price, 2004). Derivatization to aldononitrile acetate allows for short sample preparation time and produces well resolved peaks and stable compounds for quantitative and qualitative analysis (Guan, Yang, & Li, 2010). Cultured and natural *C. sinensis* extracts were prepared using a pressurized liquid extraction (PLE) technique and analyzed using GC/MS in a recent study conducted by Guan et al. (2010). Under optimized conditions, polysaccharides from a water extract were hydrolyzed at 100 °C for 2 h using 2 M of TFA in order to release saccharides. The hydrolyzed product was then derivatized. Derivatization involves firstly, conversion of the analyte into an oxime by reacting with hydroxylamine hydrochloride followed by an acylation reaction involving acetic anhydride. Optimal derivatization conditions were used based on knowledge from a previous study suggesting amount of reagent, temperature and duration of oximation and acylation can influence derivatization (Li, Wu, & Zhang, 1981). Based on their own preliminary optimization experiments, Guan et al. (2010) found that oximation and acylation reactions performed best at a temperature of 90 °C and a reaction time of 30 min. The ratio of reagent to analyte was optimal at 0.5–2.0 (mg/mg). Column separation was performed using 5% phenyl methyl siloxane at a temperature of 175 °C with gradual increase to 185 °C and finally to 230 °C. MC parameters includes ionization energy of 70 eV and a scan range of 40–550 amu. From these experimental parameters, ten monosaccharides (rhamnose, ribose, arabinose, xylose, mannose, glucose, galactose, mannitol, fructose and sorbose) were detected in both cultured and natural *C. sinensis* samples. Contrast between the carbohydrate profile of cultured and natural *C. sinensis* was established and showed that natural *C. sinensis* contained more than 7.99% free mannitol compared to that from the cultured samples.

4.2.4. Nuclear magnetic resonance (NMR) spectroscopy

NMR spectroscopy is a popular method used for structure determination generating highly informative spectra all the while providing high-resolution and quantitative results. This non-destructive method, preserves the ingredient of the original sample. One-dimensional (1D) carbon or proton NMR exploits the magnetic properties of NMR active nuclei (^{1}H or ^{13}C) and can be analyzed further to determine physical and chemical characteristics of the atom and, in a larger sense, the molecule itself. Although 1D NMR can provide the user with

adequate information pertaining to the structure of simple unknown compounds, large complex compounds will usually generate crowded spectra resulting in the overwhelming of small NMR peaks and heavy signal overlap. This problem is alleviated by incorporating an additional parameter or dimension. Multidimensional spectra is considered as a major achievement in NMR spectroscopy capable of resolving the structures of large proteins and nucleic acids. For bioactive compounds determination within *C. sinensis* extracts, 1D and 2D NMR spectroscopy are most frequently employed. Homo- and heteronuclear NMR experiments such as correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), heteronuclear single quantum correlation (HSQC), heteronuclear multiple quantum correlation (HMQC) and heteronuclear multiple-bond correlation (HMBC) can be used for structure elucidation.

Polysaccharides extracted from cultured and natural *C. sinensis* have drawn conflicting conclusions as to the structure of the active compounds. Recently, a hydrophilic polysaccharide fraction isolated from cultured *C. sinensis*, was examined after receiving positive results suggesting antifibrotic effect in renal fibrosis (Zhang, Liu, Al-Assaf, Phillips, & Phillips, in press). Using this bioactive fraction, monosaccharide composition and structural analysis was studied by way of methylation analysis and 1D and 2D NMR spectroscopy (Nie et al., 2011). Methylation analysis produced partially methylated alditol acetates (PMAA) which were identified via mass spectrometry. This type of analysis determines the mode of linkage and based on this study, would suggest that the hydrophilic polysaccharide fraction is composed mainly of a glucopyranosyl backbone $1 \rightarrow 4$ linked (65.7%). Non-reducing terminal-D-glucopyranosyl residue had a peak area percentage of 20.7% (Nie et al., 2011). At this juncture, discrepancies in the assigned linkage have already emerged. Akaki et al. (2009) reported $1 \rightarrow 3$ linkage with supporting evidence by Bai et al. (1997). Discrepancies in literature can be attributed to many factors however most likely due to difference in species and extraction methods. Akaki et al. (2009) however, failed to perform further structural investigation using NMR spectroscopy in order to confirm their methylation analysis conclusions. Nie et al. (2011) further elucidated structural characteristics of the polysaccharide fraction. Based on methylation analysis, ^{1}H and ^{13}C NMR, HMQC, HMBC, COSY, and TOCSY, a conclusion was made as to the hypothesized structure of the bioactive fraction comprising a glucopyranosyl α-linked $1 \rightarrow 4$ backbone with $1 \rightarrow 3$ linked branching at the O-2 or O-6 glucopyranosyl and an α-terminal-D-glucopyranosyl in the side chain.

FTIR analysis complemented with ^{13}C NMR analysis determined the main compound in CS-Pp was 1,3-β-D-glucan (Akaki et al., 2009). Alditol acetates obtained from hydrolysis of methylated CS-Pp was investigated using GC and GC/MS. Three peaks emerged corresponding to 2,3,4,6-tetra-O-methylglucitol, 2,4,6-tri-O-methylglucitol and 2,4-di-O-methylglucitol. These conclusions were enough for the authors to deem the active compound found in CS-Pp is a 1,3-β-D-glucan with 1,6-branched chains. Similar immunostimulating results have previously been determined and credited to the insoluble 1,6-branched, 1,3-β-D-glucan, Letinan (Bai et al., 1997). However, the compound Akaki et al. (2009) isolated, had a much smaller particle size and could stimulate an immune response following oral administration as previously demonstrated (Koh et al., 2003a).

5. Biological functions of extract

As summarized in Table 1 and Table 2, the following section described major pharmaceutical compounds in *Cordyceps* extracts and their therapeutic properties (i.e. antiaging, reparative properties, anti-cancer/anti-tumor, immuno-stimulation and antioxidant) (Akaki et al., 2009; Chen et al., 2009; Dong & Yao, 2008; Ji et al., 2009; Koh et al., 2003c; Woo Bok et al., 1999; Yang et al., 2011).

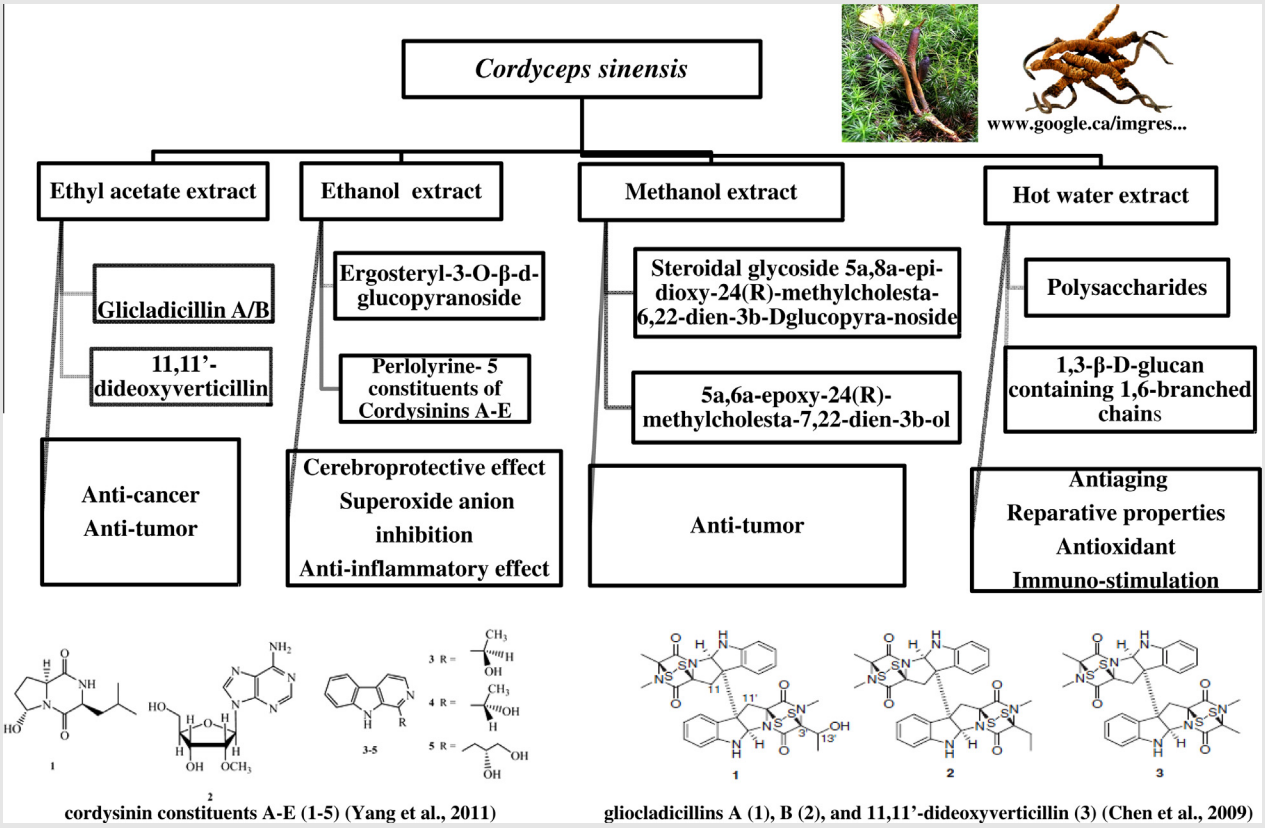
5.1. *Antiaging*

Solvation properties of water have been exploited and used to determine various health benefits of *C. sinensis*. Antiaging effect of *C. sinensis* is of particular interest (Ji et al., 2009). Hot water extract was prepared and administered to male and female rats. Under D-galactose induced aging, accumulation of metabolites occurs in nerve cells leading to symptoms associated with aging (Song, Bao, Li, & Li, 1999). This aging model was effectively employed by Ji et al. (2009) to demonstrate common aging symptoms such as memory dysfunction, decline in sexual function and decreased activity of antioxidant enzymes. Water extract of *C. sinensis* (WECS) fed rats showed improved resistance to the symptoms of induced aging in a dose-dependent manner. Although pure compounds within WECS were not identified nor isolated, a thorough investigation of the effects of the extract was conducted. The rats were subjected to learning and memory tests and sexual function test. The ultrastructure of the hippocampus was observed with an electron microscope and visually demonstrated the preservative effects of WECS on the cellular structure of the hippocampus. The brains and livers were subsequently removed in order to perform age-related enzyme assays, which once again demonstrated WECS ability to improve the activity of antioxidative enzymes such as superoxide dismutase, catalase, GSH-Px all the while lowering the activity of lipid peroxidation and the activity of monoamine oxidase B, both indicators of aging.

5.2. *Reparative properties*

More recently, the reparative properties of *C. sinensis* extracts were investigated using HT29 cells, a human colon cancer cell line (Marchbank et al., 2011). Effect of *C. sinensis* extracts on gastric damage was explored *in vivo* on rats that had been administered indomethacin, a non-steroidal anti-inflammatory drug. *C. sinensis* grown on rice and potato medium along with a powdered dried extract from mycelia grown in liquid culture were further extracted using hot-water. Whole and finely ground up fruiting bodies of *C. sinensis* grown on rice and potato medium were extracted for comparison purposes and concluded that the supplemental step of grinding the fruiting bodies only improved pro-proliferative activity. Ethanol-soluble and ethanol-insoluble sub-fractions were also collected from ground-rice and potato hot water extract in order to fur-

Table 1 – Major bioactive compounds in Cordyceps extracts and their reported properties.



References: Chen et al. (2009),Yang et al. (2011), Woo Bok et al. (1999), Akaki et al. (2009), Ji et al. (2009), Koh et al. (2003c), and Dong and Yao (2008).

ther examine possible active components. Wound healing involves the migration, invasion and proliferation of cells. On average, *C. sinensis* extract stimulated cell proliferation threefold, cell migration increased by 69% and invasion by 17%. In comparison, the liquid-phase-grown product was less effective however yielded similar pro-proliferative effects. Of interest, 93% of the pro-proliferative activity was found in the ethanol-soluble sub-fraction and pro-migratory activity was split 61:39 between ethanol-soluble and ethanol-insoluble sub-fractions.

Protective effects of *C. sinensis* extracts were also seen *in vivo* on rats with gastric damages induced by indomethacin. Based on *in vitro* studies, *C. sinensis* extracts displayed no anti-apoptosis activity as it showed no influence on caspase-3 activity thereby suggesting its gastroprotective effects may stem from its ability to stimulate cell migration and proliferation. Previous research by the same group showed that pure adenosine compounds had pro-proliferative, pro-migratory and gastric injury protective effects therefore relevance can be made in regards to the ethanol soluble sub-fraction which contains nucleotides and nucleosides (Belo et al., 2006).

C. sinensis extracts role as a protective agent stretches beyond the stomach and into the kidneys, this according to recent studies on extracellular accumulation of glomerular sclerosis by Song et al. (2010). A simple hot water extraction was performed using *C. sinensis* powder and the filtrate was administered to one of four groups of male rats. Adriamycin was administered to three of four test groups and induced kidney damage. Urine protein measurement as well as biochemistry index examination were conducted to quantify the damage induced by adriamycin and the protective effects of the extract. Hematoxylin and eosin staining, and immunological histochemistry examination were used to visually detect changes in the renal organ. In non-treated adriamycin rats, otherwise referred to as the model group, urine protein level saw consistent increase over the 12-week period compared to the sham group subjected to isotonic sodium chloride injections. This definitively shows adriamycin effectiveness at inducing detectable kidney damage. *C. sinensis* extracts role as a protective agent was tested on rats fed *C. sinensis* extract on a daily basis (5 mL/kg). Another group of rats were fed with 7.5 mg/kg of fosinopril sodium on a daily basis as the positive control group. Fosinopril sodium, or commonly known as Monopril, is a angiotensin converting enzyme inhibitor and used for the prevention of kidney failures among other conditions. Urine protein levels in rats treated with either *C. sinensis* extract or fosinopril sodium were much lower in comparison to the model group. Furthermore, blood urine nitrogen (BUN) and serum creatinine (SCr) also showed decreased expression in rats treated with either *C. sinensis* extract or fosinopril sodium with the former demonstrating more effectiveness than its counterpart. BUN is an

Table 2 – Compilation of C. sinensis extraction method and results from a selection of current and pertinent research.

Extraction	Extraction methods	Bioactive compounds or fractions	*In vitro/in vivo* evaluations	Observations	References
– Sequential water/ethanol	– Water extraction at 100 °C for 3 h – Absolute ethanol extraction – Combination of aqueous and ethanol extracts	– CSE	– In vivo	– Cerebroprotective effect of CSE on ischemic neuronal damage – CSE reduced lactate dehydrogenase activity; indicator of low oxidative stress – Reduced glutathione levels increased in treated groups relative to ischemic group; indicating heightened antioxidant defense system	Liu et al. (2010)
– Ethanol extraction with partitioning	– Ethanol extraction under reflux for 8 h – Partitioned into n-hexane/n-hexane fraction (CSEHH), an n-hexane/MeOH–H_2O fraction (CSEHM), an ethyl acetate fraction (CSEE), and a water-soluble fraction (CSEW)	– Identification of 45 known compounds (i.e. ergosteryl-3-O-β-D-glucopyranoside and perlolyrine) – 5 Constituents of Cordysinins A–E	– In vitro	– CSEHH, CSEHM and CSEE fractions displayed significant inhibition of superoxide anion generation as well as inhibiting elastase release	Yang et al. (2011)
– 80% Medicinal alcohol (ethanol) – Aqueous extraction – Trypsin extraction	– Soaked in 80% ethanol and vacuum concentrated – Mixed in boiling water for 2 h and vacuum concentrated and heated – Trypsin treated and subsequently inactivated – The three extracts combined, homogenized and sterilized at 60	– CSE	– In vivo (Inbred male BN (Brown-Norway) and Lewis rats)	– CSE attenuates ICAM-1 and TNF-α expression levels in transplanted aortas in addition to reducing serum levels of both ICAM-1 and TNF-α – CSE inhibited the proliferative activity of vascular smooth muscle cells by decreasing PCNA expression, and effectively reduced the development of transplant arteriosclerosis, in addition to conferring protective effects on allograft vasculopathy	Zhang et al. (2011a)
– Water-based ethanol extraction	– Extracted in water via decoction – Filtered and precipitated in 95% ethanol – Dissolved CSE was sterilized and diluted in Dulbecco modified eagle medium (DMEM)	– CSE	– In vitro	– CSE was found to suppress hypoxia-induced proliferation of pulmonary artery smooth muscle cells (PASMCs) – The expression of PCDNA was observed as it is an indicator for cell proliferation; CSE inhibited the expression of PCDNA – CSE inhibits c-jun and c-fos oncogenes – Cell proliferation measured by MTT assay	Gao et al. (2010)

– Methanol extraction – Ethyl acetate extraction	– Dry mycelia were extracted with MeOH three times, combined and concentrated under reduced pressure – Residue redissolved in 1:1 MeOH/H_2O and aqueous layer extracted with EtOAc and extracts concentrated under reduced pressure – Extract chromatographed on silica gel-flash column with increasing solvent polarity	– Steroidal glycoside 5a,8a-epi-dioxy-24(R)-methylcholesta-6,22-dien-3b-D-glucopyranoside – 5a,6a-Epoxy-24(R)-methylcholesta-7,22-dien-3b-ol – Ergosteryl-3-O-b-D-glucopyranoside – 22,23-Dihydroergos-teryl-3-O-b-D-glucopyranoside	– In vitro (erythroleukemia K562, T-lymphoblastic Jurkat, promyeloctic leukemia HL-60, malignant melanoma WM1341, and multiple myeloma RPMI 8226)	– Antitumor activity tested on K562 (erythroleukemia), Jurkat (T-lymphoblastic), HL-60 (promyelocytic leukemia), WM1341 (malignant melanoma) and RPMI 8226 (multiple myeloma) malignant cell lines – The glycosylated form of ergosterol peroxide was found to be a greater inhibitor to the proliferation of K562, Jurkat, WM-1341, HL-60 and RPMI-8226 tumor cell lines by 10–40% than its previously identified aglycone, 5a,8a-epidioxy-24(R)-methylcholesta-6,22-dien-3b-ol	Woo Bok et al. (1999)
– Hot water extraction	– Extraction performed at 120 °C for 20 min – Filtered and freeze-dried	– CSE	– In vitro and in vivo (male and female ICR mice and Sprague–Dawley rats)	– Chronic injections of D-galactose subcutaneously into mice induced changes that resembled accelerated aging – CSE increased learning and memory significantly in a dose-dependent manner – Brain morphology showed that CSE ameliorated the ultrastructure of the hippocampus – Results showed that CSE has a mildly beneficial effect on sexual function – CSE appeared to increase antioxidant enzyme function and slow the overall aging progress	Ji et al. (2009)
– Hot water extraction	– Whole fruiting bodies boiled in distilled water for 30 min (common preparation) – Dried fruiting bodies ground to fie powder and heated in distilled water at 90 °C for 2 h (max. water soluble material) – Repeated for liquid medium-grown fruiting bodies	– Extract 1 (whole rice- and potato-grown hot water extract) – Extract 2 (ground rice- and potato-grown extract) – Extract 3 (powdered liquid medium-grown hot water extract) – Extract 4A (ground rice- and potato-grown ethanol insoluble extract) – Extract 4B (round rice- and potato-grown ethanol soluble extract)	– In vitro (human colonic HT29 cells) and in vivo	– Rice- and potato-grown and liquid-phase-grown C. sinensis possess pro-migratory, invasive and proliferative activity – Ethanol-soluble fraction showed pro-proliferative activity – Ethanol-soluble and insoluble fractions showed pro-migratory activity – In vivo study displayed protective effect of C. sinensis extract on rat gastric damage induced by indomethacin	Marchbank et al. (2011)

(continued on next page)

Table 2 (continued)

Extraction	Extraction methods	Bioactive compounds or fractions	*In vitro/in vivo* evaluations	Observations	References
– Water extraction	– Defatted with ethanol and residue suspended in water and treated with hot water at 100 °C – Hot water extract concentrated and added 1 vol. of EtOH – Precipitate used as the crude polysaccharide (CS-P) – CS-P redissolved in water and dialysis performed against distilled water to remove low molecular weight constituents – Liquid within dialysis membrane fractionated into soluble and insoluble polysaccharide (CS-Ps and CS-Pp)	– Polysaccharides – CS-Pp is a 1,3-β-D-glucan containing 1,6-branched chains with mean particle diameter of 1.5 μm	– In vitro (mouse splenocyte cell line C3H/HeJ and macrophage cell line RAW264.7)	– CS-Pp induced the most TNF-α production – FTIR and 13C-NMR analysis revealed the immunostimulating polysaccharide of CS-Pp was 1,3-β-D-glucan – GC and GC/MS analysis revealed CS-Pp contained a 1,6-branched chain sugar – Particle size of CS-Pp has a mean diameter of 1.5 μm contributing to its effectiveness when administered orally	Akaki et al. (2009)
– Hot water extraction	– Sequential fractionating with ethylacetate, methanol and hot water; in increasing order of polarity	– CSE	– In vivo (male ICR mice and Sprague–Dawley rats)	– Swimming endurance capacity of mice administered orally with CSE was prolonged from 75 to 90 min indicating a lessening of fatigue symptoms. – In rats fed CSE, stress symptoms were suppressed by observing weight changes of the adrenal gland, spleen, thymus and thyroid	Koh et al. (2003a, 2003b, 2003c)
– Hot water extraction	– Natural and cultured C. sinensis extracted using hot water for 2 hr – Vacuum filtered – Rotary evaporated and lyophilized	– CSE	– In vitro (inhibition of linoleic acid peroxidation; scavenging abilities on DPPH, hydroxyl and superoxide anion radicals; the reducing power and the chelating ability on ferrous ions)	– Extracts showed inhibition of linoleic acid peroxidation, scavenging activities on superoxide anion and hydroxyl radicals and DPPH scavenging activities – Moderate reducing power and ferrous ion chelating activity was also observed	Dong and Yao (2008)

– Hot water extraction	– C. sinensis powder heated in 90 °C for 2 h and filtered	– CSE	– In vivo (male Wistar rats)	– CSE markedly decrease urine protein, BUN and SCr levels in Adriamycin damaged kidneys – CSE attenuated the pathological alteration in rat glomerular sclerosis – Immunohistochemical results show decreased expressions of fibronectin (FN), collagen-IV (Col-IV), connective tissue growth factor (CTGF) and plasminogen activator inhibitor-1 (PAI-1), and increasing the expression of matrix metalloproteinase-2 (MMP-2) in rats treated with CSE	Song et al. (2010)
– Sequential three-solvent extraction (ethyl acetate, methanol, and 50% aqueous methanol)	– Ethyl acetate extraction with filtrate redissolved in acetone – Residue extraction using 100% methanol followed by 50% methanol with filtrate redissolved in respective extraction solvents	– Ethyl acetate extract – Methanol extract – Aqueous methanol extract	– In vitro	– Extracts from the most polar solvent and least polar solvents contained the lowest antioxidant activity – Not much remains post aqueous methanol extraction – Very hydrophilic solvents are less effective at extracting phenolic compounds which represents a direct correlation with antioxidant activity – Based on ORAC values from 55 medicinal herbs, 15.7%, 39.1%, and 45.2% of the total antioxidant activity measured were attributable to the ethyl acetate, methanol, and aqueous methanol extracts, respectively	Wojcikowski, Stevenson, Leach, Wohlmuth, and Gobe, (2007)
– Ethyl acetate extraction	– Crude ethyl acetate extract further fractioned by silica gel chromatography	– Two new epipolythiodioxopiperazines, named gliocladicillins A and B	– In vitro (breast cancer MCF-7, hepatocellular carcinoma HepG2 and cervical cancer HeLa tumor cell lines – In vivo (C57BL/6J mice)	– Inhibited growth of HeLa, HepG2 and MCF-7 tumor cells – Arrested cell cycle at G2/M phase and induced apoptosis – In vivo studies showed inhibitory effect on cell population growth of melanoma B16 cells in immunodeficient mice	Chen et al. (2009)

end product of protein metabolism and excreted by kidneys and is an indicator of acute or chronic renal failure. SCr is an amino acid derived from muscle tissue excreted by glomerular filtration therefore decreased filtration rate caused by renal damage increases SCr levels. These observations were all further concretized via visual inspections. Based on this study, *C. sinensis* extract can be considered an effective therapeutic agent in reducing proteinuria, improve renal function and inhibit glomerular sclerosis adding yet another benefit to the long list of health benefits associated with water extracted *C. sinensis*.

5.3. *Anti-cancer/anti-tumor*

Ethyl acetate extracted *C. sinensis* has been previously shown to exhibit potent inhibitory effects on various cancer cell lines and also *in vivo* anti-tumor activity has been detected in B16-induced melanoma C57BL/6J mice (Wu et al., 2007). Zhang et al. first released a study on the effects of various *C. sinensis* extracts against the proliferation of human premyelocytic leukemia cell HL-60 in 2004 (Zhang et al., 2004). Petroleum ether, ethyl acetate, ethanol and water extracts were prepared and subjected to MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay to measure cell proliferation as well as to determine cytotoxicity potential of the various extracts on HL-60 cells. Caspase-3 activity as well as cleavage of poly ADP-ribose polymerase (PARP) were also analyzed; both are characteristic of cell apoptosis. During apoptosis, activation of caspase-3 enables the cleavage of the nuclear enzyme, PARP, and subsequently prevents it from repairing DNA. Although all four extracts exhibited significant inhibition of cell growth, the ethyl acetate extract proved the overwhelming favorite with an ED_{50} (median effective dose; a dose required for quantal effect in 50% of the population) of $\leqslant$25 μg/mL and became the focus for the remainder of the study. The ethyl acetate extract continued to impress by activating caspase-3 in a dose- and time-dependent manner. This had a cascading effect of PARP as evidenced by the cleavage of the 113 kD protein into 89 kD fragments. These results confirms ethyl acetate extracted mycelium's ability to induce apoptosis in HL-60 cells via caspase-3 mediated PARP cleavage. Of significant interest is the low ED_{50} value obtained in this study which warrants further screening in an attempt to identify the active compound involved and its use as potential therapeutic drug. The authors did suggest the extract contained ergosterols, glycosides and polysaccharides.

Sterols have previously been proven to have antitumor activity against Jurkat cells and K562 cells. Results from this study concluded that fraction FB1 had 61% antitumor activity against Jurkat immortalized T lymphocyte cells and 36.3% activity against K562 cells at 100 μg/mL. Fraction FB2 was found to have 96% activity against Jurkat cells and 77% activity against K562 cancer cells at 100 μg/mL (Woo Bok et al., 1999). Sterols are therefore the primary suspect involved in growth inhibition and apoptosis induction. Based on this study, Zhang et al. (2005) continued their research using four cancer cell lines (breast cancer MCF-7, mouse melanoma B16, human premyelocytic HL-60 and human hepatocellular carcinoma HepG2). The same extraction protocol was employed as in the 2004 study and four extracts were subjected to testing. HPLC analysis was also incorporated in the latter study to determine the major chemical constituents in each extract. *In vivo* anti-tumor activity of the ethyl acetate extract was tested on B16-induced melanoma in C57BL/6 mice. Initial cytotoxicity tests revealed nothing new to the researchers as yet again ethyl acetate extract proved to be the most potent inhibitor in all the cancer cell lines based on IC_{50} (concentration required for 50% inhibition) values. In fact, the potency of the ethyl acetate is comparable to harringtonine, a positive control drug. Due to the overwhelming cytotoxic nature of the ethyl acetate extract, its potential damaging effects on normal cells was put into question. Normal mouse bone marrow cells witnessed no more than 30% growth inhibition when treated with the extract; much lower than that against cancer cells at the same dose. The extract was tested *in vivo* on B16-induced mice and was shown to reduce the weight and volume of the tumor by 48% and 62% in just 27 days. Cytoxan was used as a positive control drug and although it was able to suppress tumor growth almost completely, damage to the spleen and strong *in vivo* toxicity was observed; none of which was observed in the group treated with the extract. HPLC analysis provided insight into possible chemical constituents involved in inhibiting cancer cell proliferation. Polysaccharide and cordycepin were the least prevalent constituents in all four extracts except in water where water-soluble sugars contributed to the high concentration of carbohydrates in the water extract. Ethyl acetate extract had the highest ergosterol content compared to the other extracts. Pure compounds found in *C. sinenesis* were assayed and ergosterol and β-sitosterol displayed significant inhibitory effect on cancer cell proliferation. Although one may jump to conclusion and proclaim sterols and its relatives as the main active anti-tumor components in *C. sinensis* and its ethyl acetate extract, synergy between various complexes is most likely *in vivo* and involving various mechanisms.

A library consisting of 200 crude ethyl acetate extract of *C. sinensis* was screened using a MTT assay on HeLa cervical cancer cells with one strain (*Gliocladium* sp.) emerging as the most potent inhibitor of proliferation with a GI_{50} (dose required for 50% growth inhibition) of 15 μg/mL. Further separation of the extract produced an even more potent fraction with a GI_{50} value of 2.0 μg/mL. The stepwise 'bioassay-directed' purification resulted in the isolation of three active compounds with a respective GI_{50} value of 0.50, 0.10 and 0.25 μg/mL. The structures of compounds 1, 2 and 3 were determined using MS and NMR. Compound 3 was first identified as 11,11′-dideoxyverticillin and data from NMR and MS revealed clues pertaining to the structure of compound 1 and comparisons compound 3 provided enough information to elucidate a structure for compound 1 and subsequently compound 2. Compound 3 (11,11′-dideoxyverticillin) had already been defined as a growth factor receptor tyrosine kinase with anti-tumor activity with two papers firmly establishing this conclusion (Chen et al., 2009; Zhang et al., 2005).The former two compounds are novel epipolythiodioxopiperazines and accordingly named gliocladicillin A and B (Fig. 2). Further evaluations revolved around these two novel compounds. Anti-proliferative effects were substantiated using three human cancer cell lines; HeLa, HepG2 and MCF-7. The mechanism by which proliferation is inhibited was analysed using

HeLa cells and the percentage of cells in each phase of the cell cycle was determined using flow cytometry. When treated with compound 1, an increase in the percentage of cells in the G2/M phase from 22.7% to 41.8% was detected suggesting G2/M phase arrest was responsible for inhibiting cell proliferation. This apoptotic mechanism was further confirmed by Annexin V staining and DNA fragmentation analysis. Both time and dose were critical factors and directly proportional to the inhibition effect. The effects of compound 1 and 2 on the expression of the proteins p21 and p53 were examined by Western blotting and showed significant increase in expression following exposure to compounds 1 and 2 leading to induction of cells to arrest in the G2/M phase. Mitotic exit requires the degradation of the cyclin B subunit composing the protein kinase dimer CDK2/cyclin B. Western blots showed increased accumulation of cylcin B suggesting compounds 1 and 2 effectively prevents the degradation of cyclin B. The caspase pathway was also investigated via immunoblots with results indicating activation of caspase-8, caspase-9, executioner caspase-3 and its substrate PARP-1 by cleavage. The ratio of Bax/Bcl-Kl protein levels were examined and shown to modulate the activity of caspase-3.The effects of these two novel compounds in inhibiting cancerous cell growth are firmly evidenced *in vitro* however, how would results translate *in vivo*? B16-induced C57BL/6J mice were injected with controlled doses of compound 1 (0.25 and 0.50 mg/kg) or 2 (0.10 and 0.40 mg/kg) and examined after 21 days of treatment. When compared to tumors retrieved from the control group, compound 1 was 69.8–87.2% inhibition while compound 2 was a similar 56.7–82.5% growth inhibition.

5.4. *Immuno-stimulation*

A study from Japan in 2009 identified a potential polysaccharide that may be responsible in inducing monocyte activation (Akaki et al., 2009). The cultured *C. sinensis* was defatted using ethanol prior to extraction of the residue with hot water. The supernatant layer was further concentrated and ethanol was added to precipitate the crude polysaccharide (CS-P). A portion of the crude polysaccharide was further fractionated to yield soluble (CS-Ps) and insoluble polysaccharides (CS-Pp). Cytokine production assay measured tumor necrosis factor alpha (TNF-α) production of mouse spleen cells C3H/HeJ as determined by ELISA. CS-Pp seemingly induced an overwhelming production of cytokine compared to the other fractions. A mouse macrophage cell line RAW264.7 was used to determine the effect of CS-Pp, which was time and concentration-dependent.

A more recent study focuses on the effect of polysaccharide from the fruiting bodies of cultured *C. sinensis* on splenic lymphocytes, macrophages, delayed-type hypersensitivity (DTH), anti-oxidant activity and cytokine expression in BALB/c mice exposed to ^{60}Co (Zhang et al., 2011a; Zhang et al., 2011b). An immunosuppresion model was used in this study by subjecting the mice to the ionizing radiation of ^{60}Co thereby stimulating inflammation and causing suppression of the immune system by way of repressing lymphocyte proliferation. Results from the lymphocyte proliferation assay would suggest the polysaccharide fed mice were more apt in stimulating splenic lymphocyte proliferation and at 100 mg/kg dose, irradiated mice displayed enhanced immunomodulatory activity when compared to normal non-irradiated mice. The bioactive polysaccharide may also affect cytokine regulation and assist in immuno-stimulation in this manner. Cytokines play a major role in all aspect of immunity and oxidation. Cytokines act in a synergistic manner with each cytokine amplifying the effects of each other. A bead-based multiplexed method assay was used to quantify IL-1α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-17, IFN-γ, TNF-α and GM-CSF. This method allows for the quantification of several cytokines simultaneously thereby only requiring a small volume of sample. This method also offers high precision and overall more rapid and cost-effective. Five cytokines (IL-4, IL-5, IL-6, IL-10, IL-17) emerged above detection limits. When compared to the non-treated irradiated control group, IL-4 was significantly decreased as a result of treatment with the polysaccharide fraction. A 25.0% and 34.1% decrease was observed in the 50 and 100 mg/kg body weight dose groups, respectively. Meanwhile, IL-5 levels were significantly increased by 38.6% and 49.1% in the 50 and 100 mg/kg body weight dose groups, respectively. IL-17 was the only other cytokine to have exhibited noteworthy results. In the 50 and 100 mg/kg body weight dose groups, levels of IL-17 decreased by 14.5% and 11.1%, respectively. IL-4 and IL-17 both display a variety of proinflammatory and immune modulatory activities. The authors therefore assumed the low levels of IL-4 and IL-17 may contribute to the inhibition of reactive oxygen species (ROS) production and may protect the organism against inflammation and subsequently protecting the immune system. IL-5 is secreted by T cells with murine IL-5 capable of inducing the proliferation and differentiation of pre-activated B-cells. This enables the secretion of natural antibodies such as IgM and IgA by mature B-cells. The observed increase in IL-5, along with the decrease in IL-4 and IL-17 levels, can therefore be suggested as a possible mechanisms induced by the immunostimulating polysaccharide.

5.5. *Antioxidant*

There are increasing evidence suggesting correlations between the accumulation of ROS and associated degenerative diseases including senescence and cancer. Oxidative damage can affect DNA, proteins and other macromolecules (Dong & Yao, 2008). The role of antioxidants is to scavenge these free radicals and prevent diseases caused by ROS. Several previous reports successfully demonstrate hydroxyl and superoxide anion radical scavenging activities of natural and cultured *C. sinensis* extracts *in vitro* (Cai, Chen, Yin, & Zhang, 2004; Li, Su, Dong, & Tsim, 2002; Zhang, Pu, Yin, & Zhong, 2003). Metal chelating and reducing power of *C. sinensis* extracts had yet to be observed. *In vitro*, hot water extracts from natural and cultured *C. sinensis* were subjected to determinations of antioxidant activities including inhibition of linoleic acid peroxidation, scavenging abilities on DPPH, hydroxyl and superoxide anion radicals and the reducing power and the chelating ability on ferrous ions (Dong & Yao, 2008). Reports from this study would suggest that the natural and cultured mycelia of the fungus contains potent antioxidant activities with the cultured mycelia outperforming the natural mycelia

in all *in vitro* assays except for the linoleic acid peroxidation assay. In terms of reducing power and metal chelating abilities, the hot-water extracts proved to have a moderate affect. From this study, it is interesting to note the strong performance of the cultured mycelia prompting its use over the depleted natural fungus.

In vivo antioxidant activities in the serum of ^{60}Co irradiated BALB/c mice were investigated using two assays involving malondialdehyde (MDA) and superoxide dismutase (SOD) detection (Zhang et al., 2011a; Zhang et al., 2011b). MDA is a lipid peroxidation indicator while SOD protects the cell from oxidative damage by converting superoxide anions into hydrogen peroxide. In mice administered low and intermediate doses of *C. sinensis* extract, MDA in serum was decreased by 17% and 20% compared to the irradiated untreated control group. SOD levels was enhanced by 11%, 2% and 15% in the 50, 100 and 200 mg/kg groups respectively. The authors claim the enhanced immunity function of a polysaccharide extracted from cultured *C. sinensis* may be attributed to the reduction of oxidative stress and the stimulating of antioxidant enzymes activity (Zhang et al., 2011a; Zhang et al., 2011b).

6. Toxicity and side effects

Although *Cordyceps* is recognized as a dietary supplement by the U.S. FDA in many nations, *Cordyceps* is still regarded as an unknown substance. This has shielded *Cordyceps* from the full scrutiny of modern science. Little information is available regarding detailed side effects and toxicity. In rare cases, dry mouth, nausea, or diarrhea has been reported. An even rarer occurrence is that of a systemic allergic drug allergy seen in a patient taking Cs-4. Reports of toxicity towards human have not been proven. In animal trials, mice having received intraperitoneal injections of a *Cordyceps* extract suffered no fatalities after 7 days at doses of up to 80 g/kg daily. When orally administered to rabbits for a period of 3 months at a dose of 10 g/kg daily, no significant changes in kidney or liver function and blood tests revealed any signs of toxicity. To put things into perspective, clinical trials involving *C. sinensis* normally require 3–4.5 g of *Cordyceps* daily and up to 3–5 daily, when treating severe liver disease (Holliday, Cleaver, & Wasser, 2005). Anecdotal use of *Cordyceps* in the treatment of cancer patients has produced promising results at 3–5 g of *Cordyceps* daily with zero reports of *Cordyceps* related toxicity.

Ethyl acetate extract of *C. sinensis* have been shown to have strong anti-tumor activity both *in vitro* and in *vivo* (Wu et al., 2007). It have been reported anti-proliferative effect of the ethyl acetate extract possess towards four cancer cells, including human breast cancer *MCF-7* cells, mouse melanoma B16 *cells*, Human promyelocytic leukemia HL-60 cells, and Human hepatocellular liver carcinoma cell HepG2 cells, At a dose of 200 μg/mL, 50% growth of above four cells were inhibit after 2 day treatment, and near complete inhibition by day four. Interestedly, ethyl acetate extract of *C. sinensis* was less toxic to normal mouse bone marrow cells. Such selective toxicity of ethyl acetate *Cordyceps* extract permits the potential of *C. sinensis* as a promising chemotherapy drug for treatments certain type of cancers. In melanoma-induced mice, ethyl acetate extract treatment at a dose of 0.05 g/kg/day for 27 days displayed a significant overall decrease in tumor weight and volume all the while causing no harm to the spleen, a major immunity organ. In contrast, the positive control drug Cytoxan, showed strong *in vivo* toxicity as witnessed by the lowered spleen index (Wu et al., 2007).

Immunoglobulin A nephropathy (IgAN) is a chronic disease whereby resting mesangial cells in the kidney are stimulated promoting release of cytokines and growth factors. The combined action of cytokines and growth factors enables a vicious cycle of mesangial proliferation which can lead to glomerular injury and sclerosis. Cultured human mesangial cells (HMC) stimulated with interleukin-1 (IL-1) and IL-6 caused an increase in mesangial cell proliferation, increased production of mediators and superoxide anion (Lin et al., 1999). A fraction from the methanolic *C. sinensis* extract was successful at inhibiting HMC activation by IL-1 and IL-6 *in vitro*. This active fraction was fed to mice as part of their normal diet at a ratio of 2%. Acute toxicity test revealed no changes in body weight and liver function as a result of the 2% *Cordyceps* diet. Liver to body weight ratio however did increase and was speculated to be the result of an increase in liver cytochrome P450 content. The authors of the study point out that this increase in cytochrome P450 does not cause any adverse effects and instead may even have beneficial effects in IgAN (Lin et al., 1999).

Although there is a lack of evidence suggesting that *Cordyceps* may be toxic at high doses, more certainly doesn't always equal better. In a study focused on the protective effects of *C. sinensis* extract on ischemia-induced brain infarction, basal grip strength, serum lactate dehydrogenase (LDH) levels, hippocampus reduced glutathione (GSH), cerebral cortex GSH, glutathione peroxidase (GPx) activity, glutathione reductase (GR) activity and glutathione S transferase (GST) activity were measured in a rat model at three dosages (4, 8, 10 mg/kg/day) (Liu et al., 2010). Data from these indexes showed *Cordyceps* extract having a restorative property when compared to the positive and negative controls. At a dose of 8 mg/kg/day, greatest improvements were witnessed. At an increased dose of 10 mg/g/kg, a decrease in health restoring properties was observed suggesting that too much *Cordyceps* may not always provide optimal results.

Of real concern to all consumers of traditional Chinese medicine is the possibility of lead contamination and adulterations of the materials. Lead wire or solder is often inserted into the fungi to increase the weight of the product. This practice is widespread and the use of fillers is often times unavoidable. Lead wire is now the filler of choice due to its heavier mass compared to the less harmful twigs. Lead poisonings caused by *Cordyceps* have been reported with one patient having a blood lead levels of 130 μg/dl while another patient was asymptomatic with a blood lead level of 46 μg/dl (Wu et al., 1996).

7. Traditional uses and edibility

Other well-known fungi including mushrooms, truffles and morels are considered rare and exotic delicacies and in some cases have reported immunomodulating and anti-tumor

effects (Roupas, Keogh, Noakes, & Taylor, 2012). Although *Cordyceps* are rare and expensive, they are by no means considered a delicacy due mainly to their tough texture. Instead, *Cordyceps* are believed to possess medicinal properties much like the Penicillium fungi from which the antibiotic penicillin is synthesized. Capsules are now available typically containing 600–750 mg of *Cordyceps*. Traditionally, *Cordyceps* are cooked as part of "medicinal dishes" with various types of meats depending upon the targeted illness (Zhu, Halpern, & Jones, 1998). Combinations of *Cordyceps* with certain foods have been reported to possess certain functionalities. Representative examples of traditional medicinal dishes include (1) a mixture of *Cordyceps* and old duck meat could be used for the treatment of cancer, asthenia or recovery after sever illness; (2) a mixture of *Cordyceps* and hen yield hyposexuality; (3) a mixture of *Cordyceps* and black-bone hen could teat asthenia; (4) a mixture of *Cordyceps* and lean pork could release the symptoms of fatigue, male impotence and kidney asthenia (5) a mixture of *Cordyceps* and sparrow could use for antiaging/senescence; (6) a mixture of *Cordyceps* and quail could treat fatigue, poor appetite, kidney asthenia and tuberculosis; (7) a mixture of *Cordyceps* and steamed turtle used for male/female hyposexuality, and finally a mixture of *Cordyceps* and abalone could use for treat chronic bronchitis, COPD, tuberculosis, arteriosclerosis, cataracts, and for healthy overall individuals (Zhu, Halpern, & Jones, 1998).

8. Conclusion

Health-promoting properties stemming from *Cordyceps* was once considered mere Chinese folklore but has since been accepted within the scientific community. However, shortcomings in obtaining authentic fungi and loose taxonomic regulations are key detriments to the legitimacy of most papers. The papers that were discussed in this review provide a small sampling of the plethora of studies praising the health contributions of *C. sinensis*. The substantial nature of literature suggesting positive outcomes derived from *C. sinensis* use is not met with an equal amount of literature discussing the biochemistry behind the transformation of the living insect into fungi. Furthermore, as evidenced in Table 1, a limited variety of solvents are employed in the extraction of *C. sinensis*. Non-polar solvents deserve more attention as they are especially effective for fungal compounds (Paterson, 2008). Nevertheless, the current stock of scientific literature, both mainstream and alternative, contributes to the ever-growing wealth of knowledge revolving *Cordyceps* and can provide leads for future research.

REFERENCES

Akaki, J., Matsui, Y., Kojima, H., Nakajima, S., Kamei, K., & Tamesada, M. (2009). Structural analysis of monocyte activation constituents in cultured mycelia of *Cordyceps sinensis*. *Fitoterapia, 80*, 182–187.

Armbruster, D. A., & Terry, P. (2008). Limit of blank, limit of detection and limit of quantitation. *Clinical Biochemical Review, 29*, S49–S52.

Bai, R., Ren, Y., & Yu, H. (1997). Elements determination and their relation to immunity. *Gansu Chinese Medical College Bulletin, 14*, 25–27 (in Chinese).

Ballarin, M., Herrera-Marschitz, M., Casas, M., & Ungerstedt, U. (1987). Striatal adenosine levels measured 'in vivo' by microdialysis in rats with unilateral dopamine denervation. *Neuroscience Letter, 3*, 338–344.

Belo, A., Marchbank, T., Fitzgerald, A., Ghosh, S., & Playford, R. (2006). Gastroprotective effects of oral nucleotide administration. *Journal of Gastroenterology and Hepatology, 55*, 165–171.

Benowitz, L., Goldberg, D., & Irwin, N. (2002). Inosine stimulates axon growth in vitro and in the adult CNS. *Progress in Brain Research, 137*, 389–399.

Cai, Z., Chen, S., Yin, D., & Zhang, Y. (2004). Scavenging effect of *Cordyceps* growing in different environment on hydroxyl radical. *Chinese Traditional and Herbal Drugs, 35*, 57–59.

Chen, Y., Guo, H., Du, Z., Liu, X.-Z., Che, Y., & Ye, X. (2009). Ecology-based screen identifies new metabolites from a cordyceps-colonizing fungus as cancer cell proliferation inhibitors and apoptosis inducers. *Cell Proliferation, 42*, 838–847.

Chen, Y., Zhang, Y., Li, M., Zhao, W., Shi, Y., Miao, Z., Zhang, X. W., Lin, L. P., & Ding, J. (2005). Antiangiogenic activity of 11,11′-dideoxy-verticillin, a natural product isolated from the fungus *Shiraia bambusicola*. *Biochemical and Biophysical Research Communications, 329*, 1334–1342.

Chen, W., Zhang, W., Shen, W., & Wang, K. (2010). Effects of the acid polysaccharide fraction isolated from a cultivated *Cordyceps sinensis* on macrophage in vitro. *Journal of Cellular Immunology, 262*, 69–74.

Cheung, J., Li, J., Cheung, A., Zhu, Y., Zheng, K., Bi, C., Duan, R., Choi, R., Lau, D., Dong, T., Lau, B., & Tsim, K. (2009). Cordysinocan, a polysaccharide isolated from cultured *Cordyceps*, activates immune responses in cultured T-lymphocytes and macrophages: Signaling cascade and induction of cytokines. *Journal of Ethnopharmacology, 124*, 61–68.

Dong, C., & Yao, Y. (2008). In vitro evaluation of antioxidant activities of aqueous extracts from natural and cultured mycelia of *Cordyceps sinensis*. *Swiss society of Food Science and Technology, 41*, 669–677.

Fan, H., Li, S., Xiang, J., Lai, C., Gao, J., & Wang, Y. (2006). Qualitative and quantitative determination of nucleosides, bases and their analogues in natural and cultured *Cordyceps* by pressurized liquid extraction and high performance liquid chromatography–electrospray ionization tandem mass spectrometry. *Analytica Chimica Acta, 567*, 218–228.

Fromentin, E., Gavegnano, C., Obikhod, A., & Schinazi, R. (2010). Simultaneous quantification of intracellular natural and antiretroviral nucleosides and nucleotides by liquid chromatography–tandem mass spectrometry. *Analytical Chemistry, 82*, 1982–1989.

Gao, B., Yang, J., Huang, J., Cui, X., Chen, S., Den, H., & Xiang, G. (2010). *Cordyceps sinensis* extract suppresses hypoxia-induced proliferation of rat pulmonary artery smooth muscle cells. *Saudi Medical Journal, 31*, 974–979.

González-Sarrías, A., Li, L., & Seeram, N. P. (2012). Anticancer effects of maple syrup phenolics and extracts on proliferation, apoptosis and cell cycle arrest of human colon cells. *Journal of Functional Foods, 4*, 185–196.

Guan, J., Yang, F.-Q., & Li, S.-P. (2010). Evaluation of carbohydrates in natural and cultured *Cordyceps* by pressurized liquid extraction and gas chromatography coupled with mass spectrometry. *Molecules, 15*, 4227–4241.

Guo, C., Zhu, J., Zhang, C., & Zhang, L. (1998). Determination of adenosine and 3-deoxuadenosine in *Cordyceps militaris* (L.) link by HPLC. *Zhongguo zhong yao za zhi, 23*, 236–237 (in Chinese).

Holliday, J., Cleaver, M., & Wasser, S. P. (2005). Cordyceps. In *Encyclopedia of dietary supplements*. Taylor & Francis Publishing.

Ji, D.-B., Ye, J., Li, C.-L., Wang, Y.-H., Zhao, J., & Cai, S.-Q. (2009). Antiaging effect of *Cordyceps sinensis* extract. *Phytotherapy Research, 23*, 116–122.

Klawitter, J., Schmitz, V., Klawitter, J., Leibfritz, D., & Christians, U. (2007). Development and validation of an assay for the quantification of 11 nucleotides using LC/LC–electrospray ionization-MS. *Analytical Biochemistry, 365*, 230–239.

Koh, J.-H., Kim, J.-M., Chang, U.-J., & Suh, H.-J. (2003b). Hypocholesterolemic effect of hot water extract from mycelia of *Cordyceps sinensis*. *Biological and Pharmaceutical Bulletin, 26*, 84–87.

Koh, J.-H., Kim, K.-M., Kim, J.-M., Song, J.-C., & Suh, H.-J. (2003c). Antifatique and antistress effect of the hot-water fraction from mycelia of *Cordyceps sinensis*. *Biological and Pharmaceutical Bulletin, 26*, 691–694.

Koh, J., Suh, H., & Ahn, T. (2003a). Hot-water extract from mycelia of *Cordyceps sinensis* as a substitute for antibiotic growth promoters. *Biotechnology Letter, 25*, 585–590.

Li, J., Feng, C., Ni, X., & Zhang, W. (2008). Determination of nucleosides of natural *Cordyceps sinensis* in Qinghai province by capillary electrophoresis. *Journal of Chinese Pharmaceutical Sciences, 43*, 1105–1107.

Li, S., Li, P., Lai, C., Gong, Y., Kan, K., Dong, T., Tsim, K. W., & Wang, Y. (2004). Simultaneous determination of ergosterol, nucleosides and their bases from natural and cultured *Cordyceps* by pressurised liquid extraction and high-performance liquid chromatography. *Journal of Chromatography A, 1036*, 239–243.

Li, S., Su, Z., Dong, T., & Tsim, K. (2002). The fruiting body and its caterpillar host of *Cordyceps sinensis* show close resemblance in main constituents and anti-oxidation activity. *Phytomedicine, 9*, 319–324.

Li, T., Wu, C., & Zhang, Y. (1981). Studies of gas chromatographic analysis of saccharides and alditols II. Some improvements in the analysis of acetylated aldononitriles by gas chromatography. *Chinese Journal of Analytical Chemistry, 10*, 272–276.

Li, S., Yang, F., & Tsim, K. (2006). Quality control of *Cordyceps sinensis*, a valued traditional Chinese medicine. *Journal of Pharmaceutical and Biomedical Analysis, 41*, 1571–1584.

Li, S., Zhao, K., Ji, Z., Song, Z., Dong, T., Lo, C., Cheung, J., Zhu, S., & Tsim, K. (2003). A polysaccharide isolated from *Cordyceps sinensis*, a traditional Chinese medicine, protects PC12 cells against hydrogen peroxide-induced injury. *Life Sciences, 73*, 2503–2513.

Lin, C. Y., Ku, F. M., Kuo, Y. C., Chen, C. F., Chen, W. P., Chen, A., & Shiao, M. S. (1999). Inhibition of activated human mesangial cell proliferation by the natural product of Cordyceps sinensis (H1-A): An implication for treatment of IgA mesangial nephropathy. *Journal of Laboratory and Clinical Medicine, 133*, 55–63.

Liu, Z., Li, P., Zhao, D., Tang, H., & Guo, J. (2010). Protective effect of extract of *Cordyceps sinensis* in middle cerebral artery occlusion-induced focal cerebral ischemia in rats. *Behavioral and Brain Functions, 6*, 61–68.

Ma, Y., & Wang, Y. (2008). Determination of nucleosides in *Cordyceps sinensis* preparation by dual-wavelength TLC-scanning. *China Pharmacy, 20*, 2375–2377.

Marchbank, T., Ojobo, E., Playford, C., & Playford, R. (2011). Reparative properties of the traditional Chinese medicine *Cordyceps sinensis* (Chinese caterpillar mushroom) using HT29 cell culture and rat gastric damage models of injury. *British Journal of Nutrition, 105*, 1303–1310.

Nie, S.-P., Cui, S., Phillips, A., Xie, M.-Y., Phillips, G., Al-Assaf, S., & Zhang, X.-L. (2011). Elucidation of the structure of a bioactive hydrophilic polysaccharide from *Cordyceps sinensis* by methylation analysis and NMR spectroscopy. *Carbohydrate Polymers, 84*, 894–899.

Paterson, R. R. (2008). Cordyceps – A traditional chinese medicinie and another fungal therapeutic biofactory? *Phytochemistry, 69*, 1469–1495.

Petritis, K., Chaimbault, P., Elfakir, C., & Dreux, M. (1999). Ion-pair reversed-phase liquid chromatography for determination of polar underivatized amino acids using perfluorinated carboxylic acids as ion pairing agent. *Journal of Chromatography A, 833*, 147–155.

Price, N. P. (2004). Acylic sugar derivatives for GC/MS analysis of ^{13}C-enrichment during carbohydrate metabolism. *Analytical Chemistry, 76*, 6566–6574.

Roupas, P., Keogh, J., Noakes, C. M., & Taylor, P. (2012). The role of edible mushrooms in health: Evaluation of the evidence. *Journal of Functional Foods, 4*, 687–709.

Shiao, M., Lin, L., Lien, C., Tzean, S., & Lee, K. (1989). Natural products in *Cordyceps*. *Proceedings of the National Science Council of the Republic of China, 3*, 382–387.

Shiao, M.-S., Wang, Z.-N., Lin, L.-J., Lien, J.-Y., & Wang, J.-J. (1994). Profiles of nucleosides and nitrogen bases in Chinese medicinal fungus *Cordyceps sinensis* and related species. *Botanical Bulletin of Academia Sinica, 35*, 261–267.

Song, X., Bao, M., Li, D., & Li, Y. (1999). Advanced glycation in D-galactose induced mouse aging model. *Mechanism of Ageing and Development, 108*, 239–251.

Song, J., Liu, C., Li, D., & Jin, B. (2008). Determination of cordycepin from cultured *Cordyceps sinensis* by HPLC-DAD. *Food Science, 40*, 352–354.

Song, L.-Q., Ming, Y.-S., Peng, M.-X., & Xia, J.-L. (2010). The protective effects of *Cordyceps sinensis* extract on extracellular matrix accumulation of glomerular sclerosis in rats. *African Journal of Pharmacy and Pharmacology, 4*, 471–478.

Stoddart, R. (1984). *The biosynthesis of polysaccharides*. Minnesota: Macmillan Pub. Co..

Sung, G.-H., Hywel-Jones, N., Sung, J.-M., Luangsa-ard, J., Shrestha, B., & Spatafora, J. (2007). Phylogenetic classification of *Cordyceps* and the *Clavicipitaceous* fungi. *Studies in Mycology, 57*, 5–59.

Vinas, P., Campillo, N., Melgarejo, G., Vasallo, M., Lopez-Garcia, I., & Hernandez-Coroba, M. (2010). Ion-pair high-performance liquid chromatography with diode array detection coupled to dual electrospray atmospheric pressure chemical ionization time-of-flight mass spectrometry for the determination of nucleotides in baby foods. *Journal of Chromatography A, 32*, 5197–51203.

Wang, Z.-m., Peng, X., Lee, K.-L., Tang, J.-o., Cheung, P.-K., & Wu, J.-Y. (2011). Structural characterisation and immunomodulatory property of an acidic polysaccharide from mycelial culture of *Cordyceps sinensis* fungus Cs-HK1. *Food Chemistry, 125*, 637–643.

Wang, Y., Wang, M., Ling, Y., Fan, W., Wang, Y., & Yin, H. (2009). Structural determination and antioxidant activity of a polysaccharide from the fruiting bodies of cultured *Cordyceps sinensis*. *American Journal of Chinese Medicine, 37*, 977–989.

Wang, Y., Yin, H., Lv, X., Wang, Y., Gao, H., & Wang, M. (2010). Protection of chronic renal failure by a polysaccharide from *Cordyceps sinensis*. *Journal of Fitoterapia, 81*, 397–402.

Winkler, D. (2008a). The mushrooming fungi market in Tibet exemplified by *Cordyceps sinensis* and *Tricholoma matsutake*. *Journal of the International Association of Tibetan Studies, 4*, 1–47.

Winkler, D. (2008b). Yartsa gunbu (*Cordyceps sinensis*) and the fungal commodification of Tibet's rural economy. *Economic Botany, 62*, 291–305.

Wojcikowski, J., Stevenson, L. M., Leach, D. N., Wohlmuth, H., & Gobe, G. (2007). Antioxidant capacity of 55 medicinal herbs traditionally used to treat the urinary system: a comparison using a sequential three solvent extraction process. *The Journal of Alternative and Complementary Medicine, 13*, 103–109.

Woo Bok, J., Lermer, L., Chilton, J., Klingeman, H. G., & Towers, G. N. (1999). Antitumor sterols from the mycelia of *Cordyceps sinensis*. *Phytochemistry, 51*, 891–898.

Wu, T. N., Yang, K. C., Wang, C. M., Lai, J. S., Ko, K. N., Chang, P. Y., & Liou, S. H. (1996). Lead poisoning caused by contaminated Cordyceps, a Chinese herbal medicine: two case reports. *Science of the Total Environment, 182*, 193–195.

Wu, J., Zhang, Q., & Leung, P. (2007). Inhibitory effects of ethyl acetate extracts of *Cordyceps sinensis* mycelium on various cancer cells in culture and B16 melanoma in C57BL/6 mice. *Journal of Phytomedicine, 1*, 43–49.

Xie, J.-W., Huang, L.-F., Hu, W., He, Y.-B., & Wong, K. (2010). Analysis of the main nucleosides in *Cordyceps sinensis* by LC/ESI-MS. *Molecules, 15*, 305–314.

Yamaguchi, Y., Kagota, S., Nakamura, K., Shinozuka, K., & Kunitomo, M. (2000). Inhibitory effects of water extracts from fruiting bodies of cultured *Cordyceps sinensis* on raised serum lipid peroxide levels and aortic cholesterol deposition in atherosclerotic mice. *Phytotherapy Research, 14*, 650–652.

Yang, F.-Q., Ge, L., Yong, J., Tan, S., & Li, S.-P. (2009). Determination of nucelosides and nucleobases in different species of *Cordyceps* by capillary electrophoresis–mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis, 50*, 307–314.

Yang, M.-L., Kuo, P.-C., Hwang, T.-L., & Wu, T.-S. (2011). Anti-inflammatory principles from *Cordyceps sinensis*. *Journal of Natural Products, 74*, 1996–2000.

Yang, F., Li, D., Feng, K., Hu, D., & Li, S. (2010). Determination of nucleotides, nucleosides and their transformation products in *Cordyceps* by ion-pairing reversed-phase liquid chromatography–mass spectrometry. *Journal of Chromatography A, 34*, 5501–5510.

Zhang, Y., Chen, Y., Guo, X., Zhang, X., Zhao, W., Zhong, L., Zhou, J., Xi, Y., Lin, L. P., & Ding, J. (2005). 11,11′-Dideoxy-verticillin: A natural compound possessing growth factor receptor tyrosine kinase-inhibitory effect with anti-tumor activity. *Anti-Cancer Drugs, 16*, 515–524.

Zhang, X., Liu, B., Al-Assaf, S., Phillips, G., & Phillips, A. (in press). Cordyceps sinensis decreases TGF-B1 dependent epithelial to mesenchymal transdifferentiation and attenuates renal fibrosis. *Nephrology Dialysis and Transplantation*.

Zhang, X., Pu, Y., Yin, L., & Zhong, W. (2003). Studies on the scavenging effect on superoxide anion free radical and hydroxyl free radical of *Cordyceps sinensis*. *Chinese Journal of Gerontology, 23*, 773–775.

Zhang, Q. X., & Wu, J. Y. (2007). Cordyceps sinensis mycellium extract induces human premyelocytic leukemia cell apoptosis through mitochondrion pathway. *Experimental Biology and Medicine, 232*, 52–57.

Zhang, Q., Wu, J., Hu, Z., & Li, D. (2004). Induction of HL-60 apoptosis by ethyl acetate extract of *Cordyceps sinensis* fungal mycelium. *Life Sciences, 75*, 2911–2919.

Zhang, Y., Yang, M., Gong, S., Yang, Y., Chen, B., Cai, Y., Zheng, S., Yang, Y., & Xia, P. (2011a). *Cordyceps sinensis* extracts attenuate aortic transplant arteriosclerosis in rats. *Journal of Surgical Research, 175*, 1–8.

Zhang, J., Yu, Y., Zhang, Z., Ding, Y., Dai, X., & Li, Y. (2011b). Effect of polysaccharide from cultured *Cordyceps sinensis* on immune function and anti-oxidation activity of mice exposed to 60Co. *International Immunopharmacology, 11*, 2251–2257.

Zhu, J. S., Halpern, G. M., & Jones, K. (1998). The scientific rediscovery of a previous ancient Chinese herbal regimen: Cordceps sinensis: part II. *Journal of Alternative Complementary Medicine, 4*, 429–457.

Zhu, L., Liang, Y., Lao, D., Zhang, T., & Ito, Y. (2011). Preparative separation of high-purity cordycepin from *Cordyceps militaris* (L.) link by high-speed countercurrent chromatography. *Journal of Liquid Chromatography Related Technology, 37*, 491–499.