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Running Title: Cordyceps sinensis Apoptotic Potential

Comparison of wild and cultivated extracts of *Cordyceps sinensis* apoptotic potential

By

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ABSTRACT

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Cordyceps sinensis is a mushroom which contains the compound cordycepin (3'-deoxyadenosine), an analogue of adenosine. In Traditional Chinese Medicine (TCM), cordycepin has multipurpose pharmacological uses including purported anti-tumor effects. In the present study, cordycepin was extracted from the wild mushroom as well as from various commercially available cultivated extracts. Previous research in this lab has demonstrated that cultivated extracts contain less cordycepin than the wild mushroom. However, it is unclear if the decrease in cordycepin correlates with decreased activity. To measure anti-tumor activity, extracts were used to treat human breast cancer cells (MCF-7 cells). In other labs, cordycepin has been shown to induce apoptosis, or programmed cell death in MCF-7 cells. Activity was evaluated using light microscopy to observe cell morphology and DNA electrophoresis to discern DNA laddering, both of which should be hallmarks of apoptosis. Using these methods the anticipated outcome is to determine if there is a dose and time dependent manner to the cordycepin-induced cell death as well as differential effects across the various cordycepin supplements which may contain other active compounds. These hypotheses were supported by our data with a dose-dependent cell death and marked differential in apoptotic potential among the various types of cordycepin sources.

Introduction

For over 2,500 years Traditional Chinese Medicine (TCM) has been practiced as a study of human physiology and pathology that is based on Chinese philosophy (Wang & Li, 2005). Originally based on Taoism and the idea of holism (the study of the whole person—mind, body, and spirit), its practices include: herbal remedies, acupuncture, moxibustion, cupping, and massage (Wang & Li, 2005; Russell & Patterson, 2008).

While it is often considered as part of complementary and alternative medicine (CAM) in the United States, the popularity of TCM has been on the rise despite the limited scientific evidence of its effectiveness (Russell & Patterson, 2008). It is important to note, however, that this rise in use is as an adjunct to Western medicine and not as the sole treatment.

TCM's holistic approach gives rise to the difficulty found when trying to assess the scientific validity. As it is based on complex principles that exist beyond strict natural science, a unique approach must be taken when analyzing the data. However, in light of new research, TCM treatments can no longer be dismissed as unscientific without thorough experimentation.

Herbal supplements are probably the most popular of all the TCM treatments. They abound in many stores and are not just limited to herbal shops and co-ops. Plus, they lend themselves well to laboratory research. Of the over 13,000 herbal supplements employed in TCM, one of the most sought after herbs comes from the mushroom species *Cordyceps*. This herbal remedy uses cordycepin (3'-deoxyadenosine), an extract from the rare mushroom *Cordyceps sinensis* (CS) found in parts of Asia including Nepal, China, Japan, Korea, Vietnam, and Thailand. CS is a type of ascomycete fungi which

acts in a parasitic manner to insects. It paralyzes the insect victims, grows within their host bodies, and eventually turns into a mature, fruiting fungus. In TCM, the fungus body is boiled and used in chicken soup, duck soup, or herbal tea (Xie & Xie, 2010). Likewise, in experimentation the biochemical cordycepin is extracted from the mycelium of the fungi through a boiling water extraction.

Cordycepin is often considered a “wonder drug” due to its multipurpose pharmacological uses (Russell & Paterson, 2008). It is prescribed for everything from anti-aging, to hypertension, to cancer (He, et al., 2010). As the main active ingredient, cordycepin has been found to have reno-protective activity (Li, He, Yang, & Wang, 2011), anti-inflammation properties (Kim et al., 2011), and anti-tumor activity (He et al., 2010; Kim et al., 2011; Jen et al., 2008; Pan, Lin, & Huang, 2011; Pao, Pan, Leu, Huang, 2012).

One of cordycepin’s most eye-catching claims is its anti-tumor effect, and previous research has shown that this adenosine analogue can induce steroidogenesis in many cell lines including: human colorectal cancer cells (SW480 & SW620), human breast cancer cells (MDA-MB-231 & MCF-7), human neuroblastoma cells (SK-N-BE(2)-C), melanoma cells (SK-MEL-2), human prostate carcinoma cells (PC-3), thyroid carcinoma cells (CGTH W-2), human leukemia cells (U937 & THP-1), oral cancer cells (OEC-M1), and mouse Leydig cells (MA-10). This anti-tumor effect is most likely due to the fact that cordycepin is an analogue of the nucleotide adenosine. The biochemical differs at the 3’ of the ribose moiety where it lacks a hydroxyl group found on adenosine (Fig. 1). This hydroxyl group is necessary for 5’—3’ nucleotide elongation and interfering with this process might be part of the anti-tumor effect (Chen, Stellrecht, & Gandhi, 2008). Also due to the similarity with adenosine, cordycepin has been found to induce

steroidogenesis (steroid hormone synthesis) in many cell lines of the adrenal glands, brain, placenta, testes, and ovaries (Ghayee & Auchus, 2007).

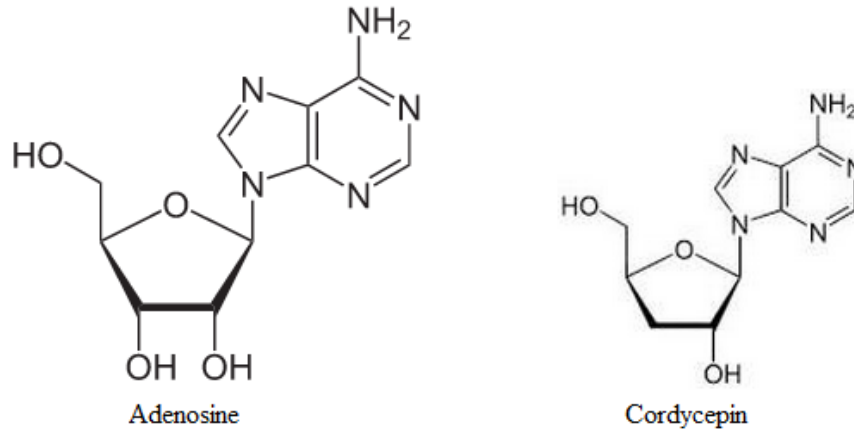


Figure 1. Adenosine and Cordycepin Structures. Adenosine is the purine nucleoside of adenine and the attached ribose sugar molecule. As an analogue of adenosine, cordycepin lacks the 3' hydroxyl on the attached sugar molecule.

Research has found that the anti-tumor role of cordycepin is due in part to its interaction with the adenosine-3 receptor which subsequently activates ERK1/2 (Figure 2). ERK1/2 then interacts with other factors and causes proliferation of the cell and steroidogenesis or apoptosis of the cell and cell death (Pao et al., 2012).

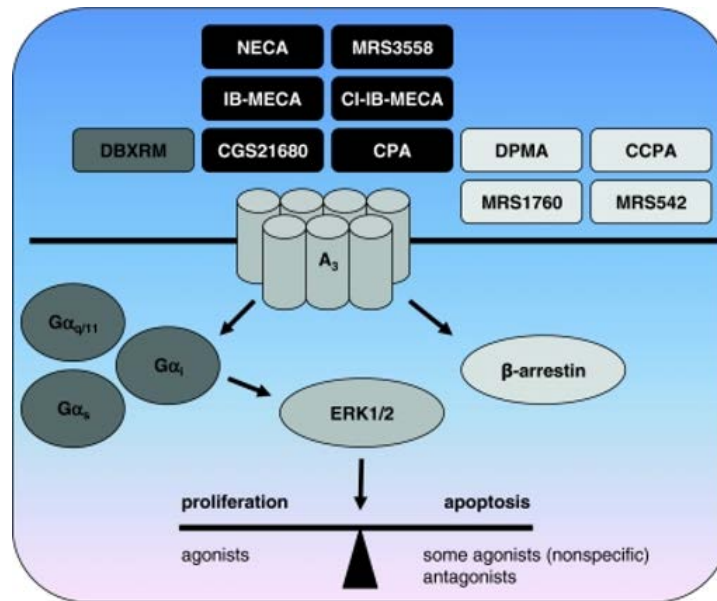


Figure 2. Adenosine-3 Receptor Pathway. A₃ is one type of adenosine receptor that interacts with alpha subunits of the G protein heterotrimer to instigate downstream effects that ultimately activate the ERK1/2 protein and result in proliferation or apoptosis depending on other factors within the cell.

The focus of this thesis is on cordycepin's effects on the MCF-7 cell line, a clonal strain of human breast cancer cells (Choi et al., 2011). Breast cancer is the most common cancer among women in the world. The prevalence of breast cancer has been increasing approximately 2% each year (Jemal et al., 2008). It is most often treated with surgical interventions, chemotherapy, radiotherapy, and endocrine therapy. However, many women also make use of CAM therapies both to lessen the symptoms and as an attempt to cure their cancer (Yong et al., 2004). Therefore, research into cordycepin-induced cell death could provide an alternative to the surgical route and the harsh chemotherapy while also evolving with the widespread integration of CAM into the anti-cancer field.

Given cordycepin's previously described wide range of uses there is a great demand for this extract. However, CS is a rare mushroom that only exists in its wild form in high-altitude areas of the Tibetan Plateau and is quite expensive outside of China. These factors have led to an increase in various cultivation methods globally and in the United States. As this production is generally unregulated the quality and purity of the cultivated species of CS is questionable. It is often listed as having the same bioactive ingredients and properties as wild CS but is sold at a much reduced price. In previous research done by this lab by Lucas First, the cultivated species of CS that is commercially available was found to contain less cordycepin.

This experiment will investigate the differential effects of various types of extracts from cultivated CS, wild CS extracts, and Sigma-purchased pure cordycepin on the viability of MCF-7 cells. At high enough concentrations, these treatments should induce apoptosis in this cell line. Furthermore, even when the amount of cordycepin from each sample type is normalized there might still be differential activity in the cells due to other compounds located within the natural supplements used. In this way the anti-tumor claim of cordycepin can be tested as it is available to the public in its supplemental form as well as in its wild imported form. It will also provide valuable insight into further avenues of cancer treatment and alternatives to chemotherapy.

In order to measure cell apoptosis light microscopy will be employed to determine the cell morphology. DNA electrophoresis will also allow apoptosis to be monitored by the presence or absence of DNA laddering. Using these methods the anticipated outcome is to find a dose and time dependent manner to the cordycepin-induced cell death as well as differential effects across the various cordycepin supplements.

Methods

Materials. Cordycepin (>98% pure) was purchased from Sigma-Aldrich (St. Paul, MN). The MCF-7 cell line was obtained from ATCC. The apoptotic DNA ladder kit was purchased from Roche Applied Science.

Extraction. A boiling water extraction was used for both the wild type and cultivated species. For the wild type, the *Cordyceps* was broken down into a powder sample (half a larvae body or ~ 0.2g) using a mortar and pestle. Next, the powder was combined with 10mL of boiling deionized water in a glass tube with a stopper (Yang et al., 2010). The mixture was then vortexed for approximately 5 minutes and placed into a 25mL round bottom flask. After pre-heating an oil bath, heat reflux extraction (100°C) with a condenser was performed for 30 minutes (the timing began when the solution started to boil). Following extraction, the extract sample was returned to room temperature. The sample was transferred to microfuge tubes and centrifuged for 5 minutes at 12×10^3 rpm (Yang et al., 2010). The supernatant solution was filtered through a 0.20µm filter (Yang et al., 2010).

Table 1. Cultivated Species

Manufacturer:	Amount of <i>Cordyceps</i> (as presented on label):
Now Foods <i>Cordyceps</i>	750mg per capsule
Vitamin Shoppe <i>Cordyceps</i>	520mg per capsule

Jorow Formulations <i>Cordyceps</i>	500mg per capsule
Holistic Herbal Solutions <i>Cordyceps</i> Mushroom Powder	1lb bag of pure powder

For the cultivated species (listed in Table 1), the same water extraction method described above was used for all four cultivated species. The amount of *Cordyceps* used in each extraction was controlled by referencing the manufacturer's published amount contained in each capsule/tablet.

Cell Culture & Treatment. MCF-7 cells were maintained in DMEM medium in an atmosphere of 5% CO₂ at 37°C. The cells were plated in 2 sets of 6-well dishes and allowed to grow for 72 hours. Each well contained approximately 2.5x10⁵ cells and 2.5ml medium. They were treated with 3 doses (100, 150, and 200µM) of four different samples (Sigma-purchased pure cordycepin, wild-type *Cordyceps*, Holistic Herbal Solutions powder *Cordyceps*, and Vitamin Shoppe capsule *Cordyceps*) and incubated at 37°C for 72 hours.

Cell Analysis. The cell morphology was observed and recorded using inverted light microscopy. DNA gel electrophoresis (2% Agarose) was then performed to observe the presence or absence of DNA laddering, a hallmark of apoptosis.

Results

To investigate possible cell growth inhibition, the effect of cordycepin on the MCF-7 human breast cancer cell line was visualized by light microscopy. Through this examination many morphological changes were observed. The first set of wells were treated with pure sigma-purchased cordycepin. In the control well (Figure 3a) the cells appeared normal and attached to the well. In the well treated with 100 μ M (Figure 3b) many cells appeared rounded-up, an indication of cell death. There was quite a bit of cell debris from already dead cells and the remaining viable cells were irregularly shaped. In the well treated with 200 μ M (Figure 3c) there was mostly cell debris and the few cells that remained were rounded up.

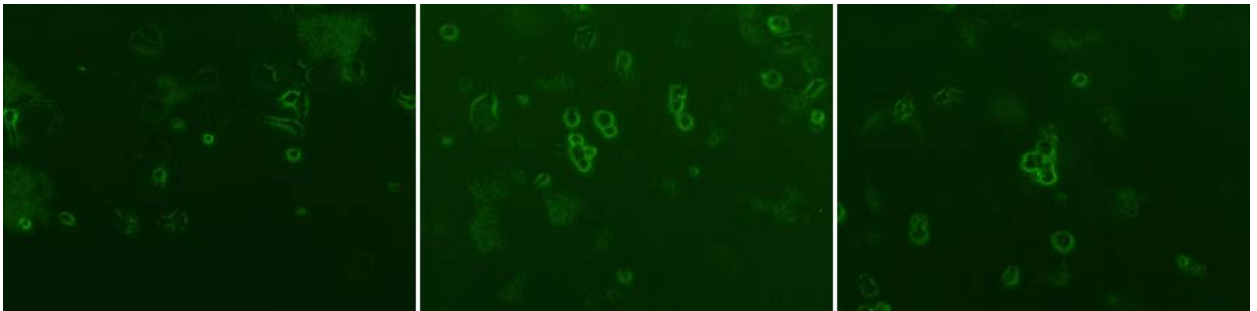


Figure 3. Light microscopy of breast cancer cells treated with pure cordycepin. (a) The control well contained normally-shaped cells. (b) The cells treated with 100 μ M pure cordycepin showed some rounding of cells, cell debris, and irregularly shaped cells. (c) The cells treated with 200 μ M pure cordycepin show mostly rounded up cells.

The second set of wells was treated with the wildtype *Cordyceps* mushroom extract. In the well treated with 100 μ M (Figure 4a) there were once again some viable cells that were irregularly shaped and a few rounded up cells. In the 200 μ M treatment (Figure 4b), there were more rounded up cells and quite a bit of cell debris.

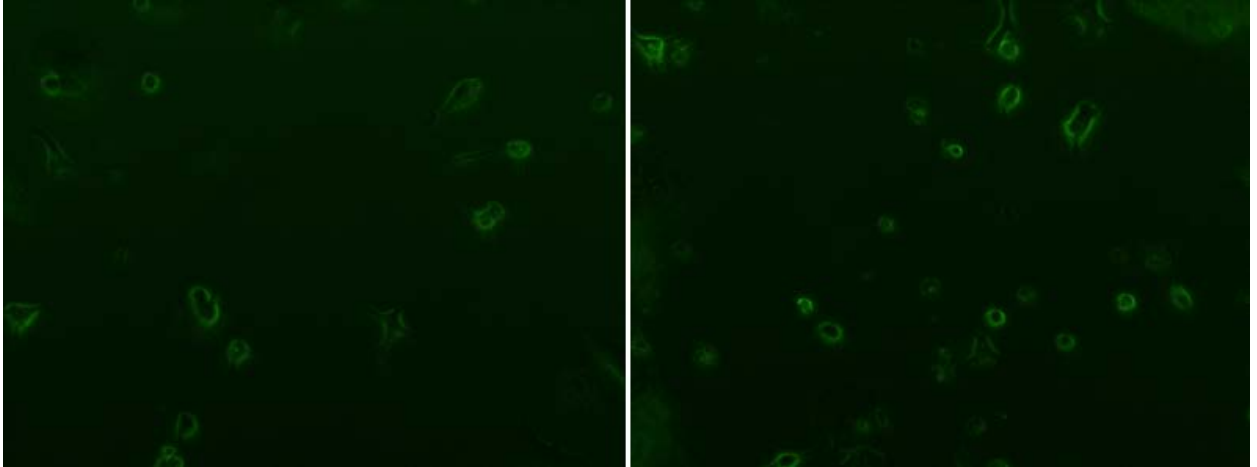


Figure 4. Light microscopy of breast cancer cells treated with wildtype *Cordyceps* extract. (a) The 100 μ M treatment has many irregularly shaped viable cells and some rounded up cells. (b) The 200 μ M treatment has many rounded up cells, a few irregularly shaped viable cells, and visible cell debris.

The third set of wells was treated with the American cultivated Holistic Herbal Solutions *Cordyceps* powder extract. The well treated with 100 μ M (Figure 5a) contains mostly normal cells that are viable and still attached to the well bottom. There are only a few rounded up cells at this treatment concentration and very little cell debris. At 200 μ M (Figure 5b) there is significantly more rounded up cells and very few normal viable cells. There is also a marked increase in cell debris at this concentration.

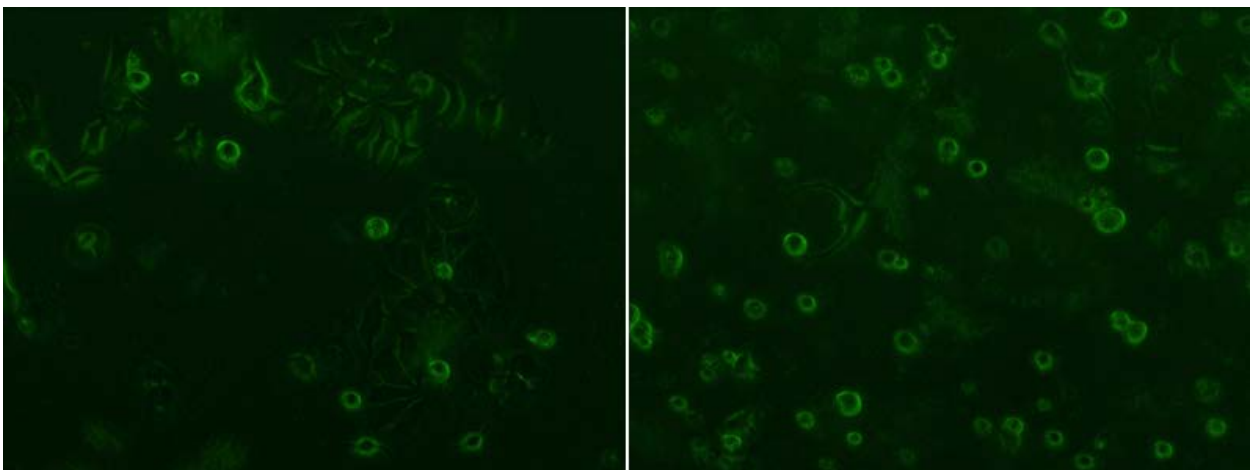


Figure 5. Light microscopy of breast cancer cells treated with cultivated Holistic Herbal Solutions *Cordyceps* powder extract. (a) The 100 μ M treatment resulted in many normal viable cells and a few rounded up cells. (b) The 200 μ M treatment contains many rounded up cells and cell debris.

The last treatment utilized the Vitamin Shoppe Capsule *Cordyceps* extract. In the well treated with 100 μ M (Figure 6a), most cells were normal and attached to the well bottom with only a few rounded up cells. In the 200 μ M treatment (Figure 6b), there were still many normal cells and few rounded up cells.

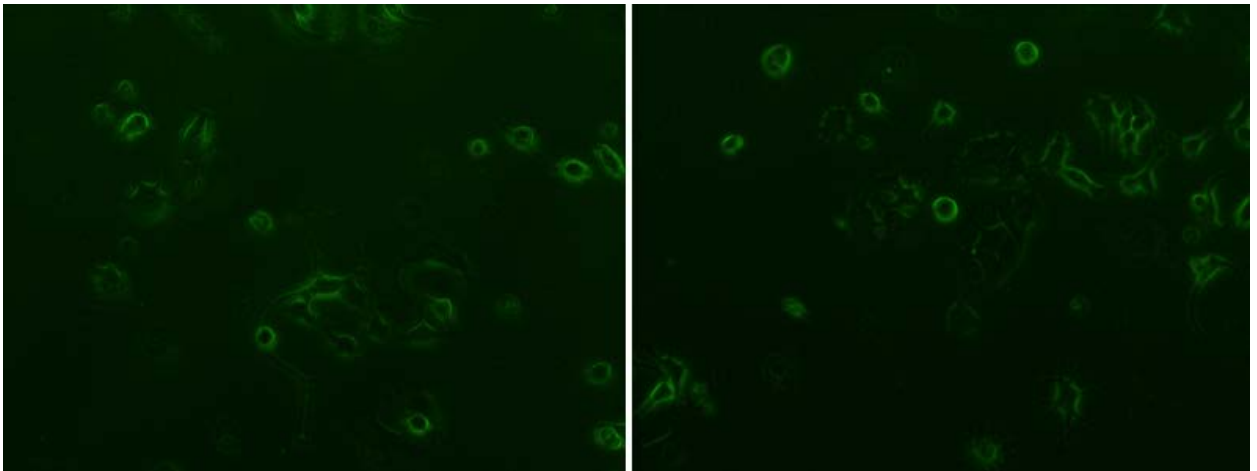


Figure 6. Light microscopy of breast cancer cells treated with Vitamin Shoppe *Cordyceps* extract. (a) The 100 μ M treatment resulted in many normal cells and a few rounded up cells. (b) The 200 μ M treatment also resulted in many normal cells and a few rounded up cells.

The results of the DNA electrophoresis (Figure 7) did not present DNA laddering which would indicate apoptosis. Instead, there was non-specific DNA degradation that was again consistent with the findings of Choi et al., (2011) and Lee et al., (2012) which also worked with this cell line (MCF-7).

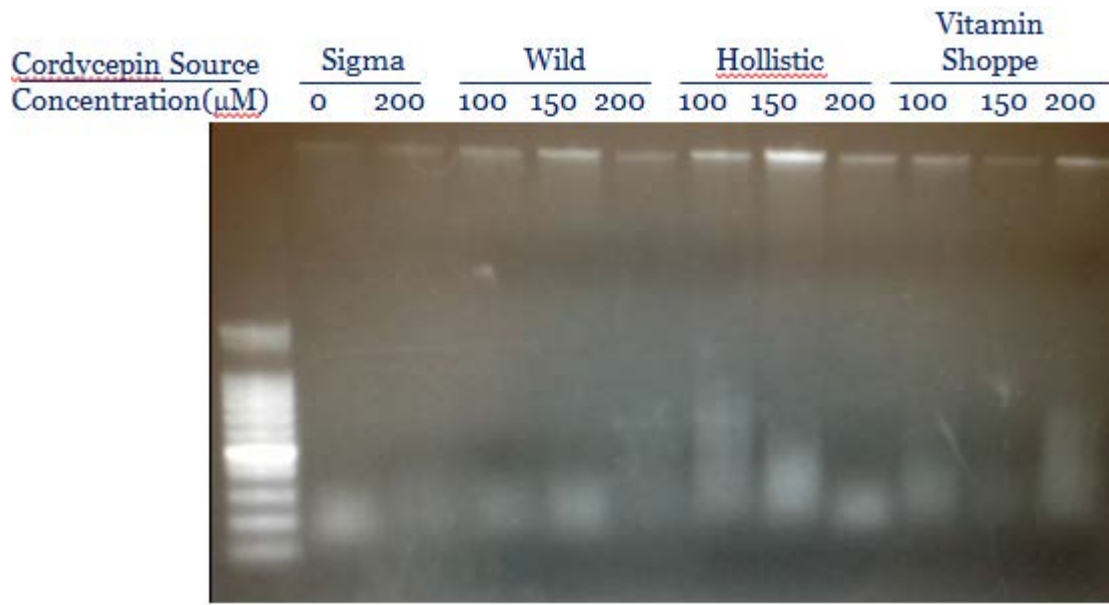


Figure 7. DNA electrophoresis of MCF-7 cells treated with cordycepin. The cells were treated with 4 sources of cordycepin at 3 different concentrations and then analyzed via agarose gel electrophoresis.

Discussion

There were two hypotheses for this study: (1) all forms of *Cordyceps* used would reduce cell survival in the MCF-7 cell line and (2) there will be a differential apoptotic potential across the various types of *Cordyceps* used—specifically that the cultivated form would be less potent than the wildtype which would be less potent than the pure Sigma-purchased cordycepin. To test these hypotheses MCF-7 cells were treated with cultivated CS extracts, wild CS extracts, and their viability was first inferred by observing the cell morphology via light microscopy.

The observations of rounding up of cells, large amounts of cell debris, and irregularly shaped remaining cells in the treatment wells all indicate stages of cell death and support the hypothesis that cell survival in the MCF-7 cell line was reduced due to the treatment of *Cordyceps*. These findings are all consistent with the findings of Choi et al., (2011) and Lee et al., (2012), both of which also did research using this cell line (MCF-7).

Furthermore, while the amount of cordycepin from each sample type was normalized (using previous analysis values found in this lab using HPLC on an Agilent 6400 Series Triple Quad LC/MS) there was still differential activity in the cells and a differential apoptotic potential across the various types used. The pure Sigma-purchased cordycepin treatment and the wildtype CS extract treatment both resulted in large amounts of cell debris, many rounded up cells, and a large percentage of any remaining cells were irregularly-shaped in both the 100 μ M and 200 μ M wells. In the American cultivated Holistic Herbal Solutions *Cordyceps* extract treatment, however, there were only a few rounded up cells at the 100 μ M dosage and there was not significant rounding-up of cells

and cell debris until the 200 μ M treatment. The Vitamin Shoppe capsule *Cordyceps* extract also did not show many rounded-up cells at the 100 μ M dosage nor was there a significant amount at the 200 μ M treatment. These results display the hypothesized differential apoptotic potential across the various *Cordyceps* types as predicted. This effect may have then been due to inherent differences in the wildtype versus cultivated forms of *Cordyceps* such as other active compounds (Table 2) and not based on the actual amount of cordycepin. The method of cultivation is not listed on the packaging for any of the samples used in this study but previous studies have found that different methods produce *Cordyceps* with varying amounts of the bioactive compounds generally found in the wildtype. For example, in the submerged fermentation method there is a loss of extra-cellular compounds which leads to less secondary bio-metabolites (Tuli, Sanhu, & Sharma, 2013). While many studies have been done concerning cordycepin, the remaining bioactive compounds have still not been fully researched to determine their structure-function relationships and effects on the cell.

Table 1. Bioactive compounds extracted from *Cordyceps* (Tuli, Sanhu, & Sharma, 2013).

Number	Bioactive Compound
1	Cordycepin
2	Cordycepic acid
3	<i>N</i> -acetylgalactosamine
4	Adenosine
5	Ergosterol and ergosteryl esters
6	Bioxanthracenes

7	Hypoxanthine
8	Acid deoxyribonuclease
9	Polysaccharide and exopolysaccharide
10	Chitinase
11	Macrolides
12	Cicadapeptins and myriocin
13	Superoxide dismutase
14	Protease
15	Naphthaquinone
16	Cordyheptapeptide
17	Dipicolinic acid
18	Fibrynolytical enzyme
19	Lectin
20	Cordymin

Apoptosis or programmed cell death is normally characterized by chromatin condensation and then endonuclease cleavage of DNA (Cohen, Sun, Snowden, Dinsdale, & Killeter, 1992). This cleavage results in fragments of varying lengths that form a DNA “ladder” when submitted to gel electrophoresis with bands in a ladder pattern corresponding to the increasing fragment weights. This hallmark of apoptosis was, however, not evident and indicated that the cordycepin activated a different pathway or different branch of the normal apoptotic route.

This alternate pathway should not be used as evidence that cordycepin would not be an effective treatment of breast cancer, but instead quite the opposite. In the MDA-MB-231 cell line (another human breast cancer cell line), MA-10 cell line (mouse Leydig tumor cells), and many others, cordycepin treatment did in fact result in DNA laddering and normal apoptosis. However, in the cell line used in this study a different pathway was instead activated but the end goal of cell death was still accomplished. In a study by Choi et al., (2011), a series of tests were ran to determine the exact differences between the apoptosis in the MDA-MB-231 breast cancer cells and the cell death in the MCF-7 breast cancer cells following treatment with cordycepin. This study found that the MCF-7 cells underwent a different type of cell death that mainly consisted of autophagy. There were autophagosome-like structures observed with transmission electron microscopy and many molecules found that indicate autophagy (LC3-II, AVOs, and MDC-positive vacuoles).

Breast cancer cell lines are often referred to as ER-positive, meaning that estrogen promotes tumor growth, or ER-negative, meaning that estrogen decreases tumor growth. However, most breast cancer cell lines actually contain a combination of both. Treatment with cordycepin, which acts irrespective of this ER-response could suppress both types of tumor growth (Choi et al., 2011).

By activating multiple cell-death pathways this form of treatment could be very effective in aggressive multi-drug resistant cancers. Likewise, due to its anti-cancer effects in multiple cell lines across different body parts (testicular, oral, colorectal, neuroblastoma, melanoma, prostate, thyroid, leukemia, etc.) it would also be a good candidate for treatment in metastasized breast cancer.

All of this data concerning cordycepin indicates that it could be used in future therapeutic applications with more research. In fact, much research is being done at this very moment concerning the link between adenosine and cancer. In a review article by Merighi et al., (2003), adenosine was proposed as a potent regulator of tumor cell growth. The study reviewed many others where numerous adenosine receptors were found to play a role in cancer including A₁, A_{2A}, A_{2B}, and A₃ (Figure 8). Current research is also being done on other adenosine analogues similar to cordycepin such as: 3'-ethynyladenosine, pentostatin, and cladribine as well as adenosine antagonists such as caffeine.

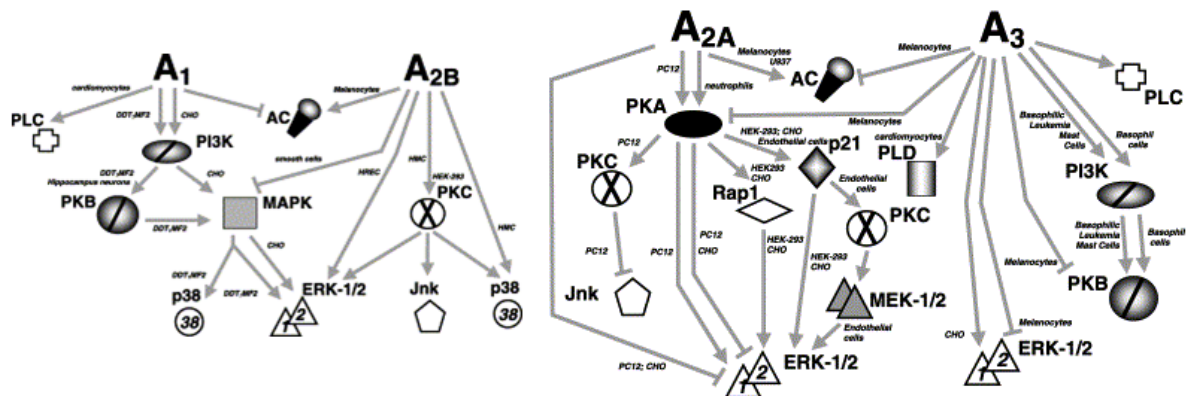


Figure 8. Signaling pathways of A₁, A_{2B}, A_{2A}, and A₃ adenosine receptors. AC: adenylate cyclase; CHO: Chinese hamster ovary cells; ERK1/2: extracellular signal-regulated kinases 1 and 2; HEK-293: human embryonic kidney cells; JNK: c-Jun N-terminal kinase; MEK-1/2: MAP kinase kinases 1 and 2; PC12: pheochromocytoma cell line; PI3K: phosphoinositide 3-kinase; PKA: protein kinase A; PKB: protein kinase B; PKC: protein kinase C; PLC: phospholipase C; PLD: phospholipase D; p21: small G protein, p21(*ras*); rap 1: small G protein, rap 1; U937: monocytic lymphoma cell line.

Cordyceps has been used as a treatment for thousands of years safely and even with present research that have only been a few cases where the treatment caused a negative effect. The most common negative report involves dry mouth, nausea, and diarrhea with very few patients reporting an allergic response. While there are recommended dosages

based on the condition that the *Cordyceps* is supposed to be triggering, there is not a lethal dosage. Studies using mice and rabbits found no fatality even when the dosage was increased dramatically for a long period of time (Tuli, Sandhu, & Sharma, 2013).

As cordycepin is similar to adenosine it is able to cause a wide variety of polypharmacological effects based on the inhibition of RNA synthesis and polyadenylation and should be considered for further research and eventually clinical trials once more of the mechanism is further elucidated. Besides the missing gaps in the mechanism, the effects of all the other bioactive compounds should be clarified and proper cultivation methods should be followed for accurate production of the capsules sold in many vitamin shops and websites. This research would provide a valuable insight into further avenues of cancer treatment and alternatives to chemotherapy.

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