Antioxidant and cytotoxic potential of potentized preparation of Cordyceps sinensis in vitro in cancer cell lines

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Objectives: The objectives of the study were to evaluate the antioxidant and cytotoxicity activity of potentized preparation of Cordyceps sinensis in carcinoma cell-lines.

Methods: In this in vitro study, antioxidant activity was analyzed by the 2,2-diphenyl-1-picryl-hydrazyl-hydrate assay and the dilution with more antioxidant potential was further analyzed for cytotoxicity, using 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide assay on various carcinoma cell lines: Breast cancer cell line (MCF-7), liver cancer cell line (HePG2), lung cancer cell line (A-549), and prostate cancer cell line (PC3). All experiments were carried out in triplicates. Data were analyzed by one-way analysis of variance and the means were compared by Duncan's new multiple range test.

Results: A higher antioxidant potential 377.40 μL/mL was seen in Cordyceps sinensis 30C, which was further analyzed for cytotoxicity in cell lines (MCF-7), (HePG2), (A-549), and (PC3) which it inhibited at concentrations 596.21 ± 3.32 μL/mL, 438.10 ± 2.39 μL/mL, 555.40 ± 3.08 μL/mL, and 656.42 ± 2.68 μL/mL, respectively. When comparing Cordyceps sinensis 30C to other cell lines, its cytotoxic activity against HEPG2 is particularly potent.

Conclusion: This research demonstrates the usefulness of potentized preparation of Cordyceps sinensis, it will take additional studies comparing it to currently used drugs to determine whether or not it is significantly more efficient.

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Antioxidant and cytotoxic potential of potentized preparation of *Cordyceps sinensis* in vitro in cancer cell lines

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Abstract

**Background:** The parasitic fungus *Cordyceps sinensis* (*Ophiocordyceps sinensis*) has been discovered in lepidopteran larvae, having known antitumor effects. Testing potentized *Cordyceps sinensis* in cancer cells could help broaden Homoeopathy cancer therapeutics. **Objectives:** The objectives of the study were to evaluate the antioxidant and cytotoxicity activity of potentized preparation of *Cordyceps sinensis* in carcinoma cell-lines. **Methods:** In this *in vitro* study, antioxidant activity was analyzed by the 2,2-diphenyl-1-picryl-hydrazyl-hydrate assay and the dilution with more antioxidant potential was further analyzed for cytotoxicity, using 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide assay on various carcinoma cell lines: Breast cancer cell line (MCF-7), liver cancer cell line (HePG2), lung cancer cell line (A-549), and prostate cancer cell line (PC3). All experiments were carried out in triplicates. Data were analyzed by one-way analysis of variance and the means were compared by Duncan’s new multiple range test. **Results:** A higher antioxidant potential 377.40 μL/mL was seen in *Cordyceps sinensis* 30C, which was further analyzed for cytotoxicity in cell lines (MCF-7), (HePG2), (A-549), and (PC3) which it inhibited at concentrations 596.21 ± 3.32 μL/mL, 438.10 ± 2.39 μL/mL, 555.40 ± 3.08 μL/mL, and 656.42 ± 2.68 μL/mL, respectively. When comparing *Cordyceps sinensis* 30C to other cell lines, its cytotoxic activity against HePG2 is particularly potent. **Conclusion:** This research demonstrates the usefulness of potentized preparation of *Cordyceps sinensis*, it will take additional studies comparing it to currently used drugs to determine whether or not it is significantly more efficient.

**Keywords:** Anticancer drug, *Cordyceps sinensis*, *in vitro* study, *Ophiocordyceps sinensis*, Homoeopathy.

Introduction

Cancer is a complex set of disease symptoms that advances progressively with a general loss of growth control.[1] According to GLOBOCAN, there were an estimated 19.3 million new cancer cases and nearly 10 million cancer deaths worldwide in 2020. In 2040, there are projected to be 28.4 million cancer cases worldwide, a 47% increase from 2020. With an estimated 2.3 million new cases, female breast cancer has exceeded lung cancer as the most frequently diagnosed cancer, followed by lung, colorectal, prostate, and stomach cancers. With an estimated 1.8 million deaths, lung cancer remains the leading cause of cancer-related mortality, followed by colorectal, liver, stomach, and female breast cancers.[2] This disease is tissue-based, and the tissue variation greatly complicates diagnosis and treatment efficacy.[3] In spite of advanced cancer treatments such as chemotherapy, immunotherapy, radiation therapy, surgery, and hormone therapy people use complementary and alternative therapies in view of their limited risk, natural origin, minimal consequences, and affordability.[4]

According to recent international studies, 29–91% of cancer patients seek complementary and alternative medicine treatments.[5] Patients with metastatic cancers would benefit from additional palliative care that would enhance their quality of life and allow efficient use of available medical resources.[6] To support patients’ lifestyle modifications, to manage both acute and chronic cancer-related symptoms, complementary therapies have been implemented at several oncological centers.[7] Homoeopathy was reported as one of the most popular CIM therapies (40.4% of those used) in a 2015 European survey of 236 facilities providing integrative oncology services as part of the public health system.[8] Several studies show that homoeopathy can help mitigate the toxic effects of oncology treatments, while

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also helping patients experience better health and overall well-being.

_Ophiocordyceps sinensis_ (syn._Cordyceps sinensis_) is an entomopathogenic fungus that grows on insect larvae. More than 450 species linked to cordyceps are now found worldwide based on fungi or insect hosts.[10] Harvesting begins in April and extends till August. This fungus only grows in the high-altitude regions of about 3800 m above sea level, where it can be found in alpine meadows on the Himalayan region.[11] Cordyceps commonly grows on larvae (host) that typically reside 6 inches below the ground’s surface. The fungus advances from the larval stage to maturity, consuming more than 90% of the insect host. The stroma reaches maturity, and it then bulges to form perihelia. A cordyceps weigh about 300–500 mg, which is typical.[11] The reason to select this drug for this study is that its anti-cancer and anti-metastatic effects are established by targeting pathways such as Bcl-2/Bax,[12] caspases,[13] epidermal growth factor receptor,[14] nuclear Factor Kappa B (NF-κB),[15] phosphatidylinositol 3-Kinase/Protein Kinase B (PI3K–Akt),[16] matrix metalloproteinase-2 (MMP-2)/MMP-9,[17] Janus kinase/signal transducers and activators of transcription (JAK/STAT3)[18] Mitogen-activated protein kinase (MAPK), and adenosine Monophosphate-activated protein kinase.[19,20] Carcinoma cell lines of the prevailing cancers such as breast, liver, lung and prostate were taken for the present study.[21] In a recent clinical trial conducted in partnership with Oxford University and NuCana, researchers discovered that NUC-7738, a novel drug for cancer treatment derived from _Ophiocordyceps sinensis_, was 40 times more effective in killing cancer cells than many of its parent compounds.[21] Cordyceps is in extremely limited supply due to its unique environment, and as demand increases, the drug’s value increases, making it an incredibly expensive drug. Homoeopathically prepared _Cordyceps sinensis_ is used in the treatment of mental and physical exhaustion due to overwork; nerve weakness; chronic fatigue; mountain sickness, shortness of breath; and lack of strength and endurance. It increases athletic and general performance, especially in long-distance races. _Cordyceps sinensis_ is used in the treatment of Alzheimer’s disease, backache due to injury, fatigue, stress or simple aging. It is further used in the treatment of various conditions such as lumbar weakness, metrorrhagia, abnormal menstruation increased appetite, tiredness, chronic fatigue syndrome, debility, immune weakness, anaemia, hypercholesterolemia, lymphoma, arrhythmias, weakness of heart, chronic obstructive hepatic diseases. It is therapeutically used for breathlessness, worse exertion, constricted bronchioles, asthma, persistent cough, mountain sickness, emphysema, lung cancer and tuberculosis. Cordyceps sinensis is also used for sexual hypofunction, sexual frigidity, infertility, loss of sexual drive, impotence and seminal emissions with aching of loins and knees. It is used in the preventing liver and spleen atrophy. It also antitodes opium and narcotic withdrawal symptoms.[22] The present study was done to investigate the antioxidant and anti-cancer potential of potentised _Cordyceps sinensis_ using _in vitro_ methods, in all the above-mentioned cell lines.

**MATERIALS AND METHODS**

**Materials**

_Cordyceps sinensis_ 6C and _Cordyceps sinensis_ 30C were procured from Helios Homoeopathy Limited, Tunbridge Wells, The United Kingdom. Phosphate Buffered Saline (PBS), 3-(4,5- dimethyl thiazol–2–yl)–5–diphenyl tetrazolium bromide (MTT), Dulbecco’s Modified Eagle’s medium (DMEM), fetal Bovine serum (FBS), and Trypsin were obtained from Sigma Aldrich Co, St Louis, USA. Ethylenediaminetetraacetic acid (EDTA), dimethyl sulfoxide (DMSO) Propanol from E. Merck Ltd., Mumbai, India, and glucose and antibiotics from Hi-Media Laboratories Ltd., Mumbai.

**Cell lines**

Human breast cancer cell line (MCF-7), human prostate cancer cell line (PC-3), lung cancer cell line (A-549), and liver cancer cell line (HEPG-2) were procured from National Centre for Cell Sciences Pune, India.

**Culture medium**

Cells were grown in stock in DMEM. The medium was supplemented with 10% inactivated FBS, penicillin (100 IU/mL), streptomycin (100 μg/mL), and amphotericin B (5 μg/mL) in a humidified atmosphere of 5% CO₂ at 37°C until confluent. The cells were dissociated with TPVG solution (0.02% EDTA, 0.2% tryspin, and 0.05% glucose in PBS. Experiments were carried out in 96 microtiter plates and the stock cultures were grown in 25 cm² culture flasks (Tarsons India Pvt. Ltd., Kolkata, India).

**Methods**

**Evaluation of the antioxidant property in drug samples**

To test the 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radical scavenging activity, the Molyneux method[23] was employed. In a test tube, 1.0 mL of 100.0 μM DPPH solution in methanol was mixed with an equal volume of the test samples of different-concentration solution in methanol and were incubated in the dark for 30 min. The color change was documented by measuring the light absorbance at 514 nm with a spectrophotometer. Instead of a test sample, only 1 ml of methanol was added to the control tube.

The DPPH radical scavenging activity of the sample was calculated as follows:

\[
\text{% inhibition} = \left( \frac{\text{Absorbance of control} - \text{Absorbance of extract}}{\text{Absorbance of control}} \right) \times 100
\]

The IC50 value was determined using GraphPad Prism 5.0 by using linear regression,

\[ y = mx + c \]
Where,
\( y = \) The dependent variable (i.e., what you measure as the signal)
\( x = \) The independent variable (i.e., what you control, such as, dose, and concentration)
\( m = \) The slope of the fitted line
\( c = \) The intercept of the dependent axis.

**MTT assay**

MTT assay (Denizot and Lang)\(^{23}\) is based on the ability of viable cells with active mitochondria to produce succinate dehydrogenate enzyme which cleave the tetrazolium rings of MTT, where the optical density obtained is proportional to the number of healthy viable cells.

Five samples for each drug were prepared for each cell line. The drug sample which had the highest antioxidant was selected and investigated. For cytotoxicity studies, each measured test drug sample was separately dissolved in distilled DMSO and the volume was made up with DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1 ml/ml concentration and sterilized by filtration. Serially 2-fold dilutions were prepared from this for carrying out cytotoxic studies.

Using a medium containing 10% FBS, the monolayer cell culture was trypsinized, and the cell count was increased to 1.0 \( \times 10^5 \) cells/mL. A total of 0.1 mL of the diluted cell suspension (roughly 10,000 cells) was added to each well of the 96-well microtiter plate. When a partial monolayer had formed after 24 h, the supernatant was removed, the monolayer was washed once with medium, and then test drug concentrations ranging from 50 to 1000 \( \mu \)L/mL, were added on top of the partial monolayer in microtiter plates. The plates were subsequently incubated for 3 days at 37°C in a 5% \( \text{CO}_2 \) atmosphere, during which a microscopic examination was conducted and observations were recorded every 24 h. After 72 h, the drug solutions in the wells were discarded and 50 \( \mu \)L of MTT in PBS was added to each well. The plates were gently shaken and heated to 37°C in a 5% \( \text{CO}_2 \) atmosphere for 3 h. To dissolve the formazan that had formed, 100 \( \mu \)L of propanol was added after the supernatant was drained from the plates. A microplate reader calibrated to measure at 540 nm was used to measure absorbance. The following formula was used to determine the percentage growth inhibition, and the dose-response curves for each cell line were used to determine the concentration of test drug required to inhibit cell growth by 50% (CTC50).

\[
\% \text{Growth inhibition} = \frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}} \times 100
\]

**RESULTS**

**Antioxidant activity**

The homoeopathic drugs, *Cordyceps sinensis* 6C and *Cordyceps sinensis* 30C, were analyzed using the DPPH assay for scavenging activity with 90% v/v of ethanol as control. *Cordyceps sinensis* 6C at concentrations of 50 \( \mu \)L/mL, 250 \( \mu \)L/mL, 500 \( \mu \)L/mL, 750 \( \mu \)L/mL, and 1000 \( \mu \)L/mL showed percentage inhibition of 33.77, 42.76, 48.9, 53.95, and 58.77, respectively. *Cordyceps sinensis* 30C at concentrations 50 \( \mu \)L/mL, 250 \( \mu \)L/mL, 500 \( \mu \)L/mL, 750 \( \mu \)L/mL, and 1000 \( \mu \)L/mL showed percentage inhibition of 43.42, 47.37, 53.95, 59.65, and 64.47, respectively [Table 1].

A standard curve was plotted with the percentage of radical scavenging activity of ethanol, *Cordyceps sinensis* 6C and *Cordyceps sinensis* 30C in Figure 1. The IC50 value was calculated from the slope of the figure using the “\( y = mx + c \)” formula [Table 2]. The values of IC50 of ethanol, *Cordyceps sinensis* 6C, and *Cordyceps sinensis* 30C are 865.24 \( \mu \)L/mL, 603.83 \( \mu \)L/mL, and 377.40 \( \mu \)L/mL, respectively, illustrated in [Figure 2]. The IC50 values obtained were significant \((P < 0.01)\) for free radicals. IC50 for *Cordyceps sinensis* 30C was 377.40 \( \mu \)L/mL and *Cordyceps sinensis* 6C was 603.83 \( \mu \)L/mL, thus *Cordyceps sinensis* 30C showed inhibitory activity at a much lower concentration. Since *Cordyceps sinensis* 30C had higher scavenging activity it was taken to the next stage of analyses for its cytotoxicity in different cancer cell lines.

**Cytotoxic activity**

![Figure 1: Comparison of mean value of % of inhibition of ethanol, Cordyceps sinensis 6C and Cordyceps sinensis 30C](image)

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Concentration (( \mu )L/mL)</th>
<th>Ethanol % of inhibition</th>
<th>Cordyceps sinensis 6C % of inhibition</th>
<th>Cordyceps sinensis 30C % of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50</td>
<td>21.27</td>
<td>33.77</td>
<td>43.42</td>
</tr>
<tr>
<td>2</td>
<td>250</td>
<td>26.31</td>
<td>42.76</td>
<td>47.37</td>
</tr>
<tr>
<td>3</td>
<td>500</td>
<td>35.74</td>
<td>48.9</td>
<td>50.22</td>
</tr>
<tr>
<td>4</td>
<td>750</td>
<td>46.71</td>
<td>53.95</td>
<td>59.65</td>
</tr>
<tr>
<td>5</td>
<td>1000</td>
<td>54.82</td>
<td>58.77</td>
<td>64.47</td>
</tr>
</tbody>
</table>

DPPH: 2,2-diphenyl-1-picryl-hydrazyl-hydrate

![Table 1: Mean value of % of inhibition of ethanol, Cordyceps sinensis 6C and Cordyceps sinensis 30C in different concentrations by DPPH assay](image)
Cordyceps sinensis 30C was tested using MTT assay with different concentrations, that is, 50–1000 μL/mL on MCF-7, HEPG2, and A-549 and PC3. Control tubes were kept which consisted of saline and tumor cells without drug intervention [Figure 3]. Cordyceps sinensis 30C inhibited MCF7 cell line at a concentration of 596.21 ± 3.32 μL/mL, inhibited HEPG2 cell line at a concentration of 438.10 ± 2.39 μL/mL, inhibited A-549 cell line at a concentration of 555.40 ± 3.08 μL/mL, and inhibited PC3 cell line at a concentration of 656.42 ± 2.68 μL/mL [Figure 4].

**Table 2: IC50 values of ethanol, Cordyceps sinensis 6C and Cordyceps sinensis 30C by using linear regression**

<table>
<thead>
<tr>
<th>IC50 Formula</th>
<th>Ethanol</th>
<th>Cordyceps sinensis 6C</th>
<th>Cordyceps sinensis 30C</th>
</tr>
</thead>
<tbody>
<tr>
<td>y=mx+c</td>
<td>y=0.0366x+18.33</td>
<td>y=0.0253x+34.72</td>
<td>y=0.0227x+41.43</td>
</tr>
<tr>
<td>x=(y-c)/m, where, y=50</td>
<td>x=(50–18.33)/0.03</td>
<td>x=(50–34.72)/0.02</td>
<td>x=(50–41.43)/0.02</td>
</tr>
<tr>
<td>IC50</td>
<td>865.24 μL/mL</td>
<td>603.83 μL/mL</td>
<td>377.40 μL/mL</td>
</tr>
</tbody>
</table>

**Discussion**

Numerous previous studies on Cordyceps have demonstrated its wide-ranging medicinal properties, including those that are antimicrobial, fungicidal, larvicidal, inflammatory, diabetic, antioxidant, antitumor, pro-sexual, apoptotic, immunomodulatory, and anti-HIV.[26] Some of the studies demonstrate that it inhibits M1 macrophage polarization mediated by activation of the NF-kB pathway, inhibiting inflammatory cytokines like MMP-9 can reduce tumor growth and metastasis.[27] It can suppress the HEPG2 cell line of liver cancer cells by caspases triggered apoptosis, ergosterol increased lysosomal membrane permeability, by suppressing activated hepatic stellate cells.[28] Cordyceps sinensis and cisplatin treatment significantly reduced cell viability, which subsequently caused a higher number of dead cells. Cordycepin suppressed MAPK and Rb/E2F1 signaling, cell cycle proteins, and fibroblast growth factor (FGF) receptor expression.[29,30] It stimulated Fas, DR5, and caspase-8.[31,32] Cordycepin’s anti-tumor properties resulted in a significant decline of cell viability in a dose-dependent and time-dependent manner. It was salient to understand the potential of potentized homoeopathic Cordyceps sinensis. Hence, this pre-clinical study was done to evaluate the basic properties such as antioxidant and cytotoxicity of homoeopathic Cordyceps sinensis, to test its efficacy to be an anticancer drug.

**Figure 2:** All experiments were carried out in triplicates. Data obtained were analyzed by one-way analysis of variance and means were compared by Duncan’s New Multiple Range test (SPSS 21.0 version). Representation of the results of DPPH assay, where ethanol has 865.24 μL/mL, Cordyceps sinensis 6C has 603.83 μL/mL and Cordyceps sinensis 30C has 377.40 μL/mL.

**Figure 3:** Representative photomicrographs of the cellular morphology and cell viability of (a) breast cancer cell line, (b) liver cancer cell line, (c) lung cancer cell line, (d) prostate cancer cell line at different concentrations.
In this study, it was found that Cordyceps sinensis 30C had higher antioxidant potential as compared to Cordyceps sinensis 6C when evaluated under the DPPH assay. Cordyceps sinensis 30C underwent MTT assay on different cancer cell lines, that is, human lung carcinoma (A549), hepatocellular (HEPG2), breast (MCF7), and prostate (P3). Cordyceps sinensis 6C was not further taken to test cytotoxicity because without enough antioxidant potential it can cause cell damage and oxidative stress which results in organoleptic damage comparing to Cordyceps sinensis 30C. According to the findings, Cordyceps sinensis 30C showed a significant cytotoxic activity in human lung carcinoma (A549), hepatocellular (HEPG2), breast (MCF7), and prostate (P3) cancer cell lines.

**CONCLUSION**

In this study, potentized preparation of Cordyceps sinensis has shown significant effects in all cell lines. Further studies must be done in comparison with prevailing drugs to know its effectiveness. Since significant anticancer properties are exhibited by the cytotoxic activity of Cordyceps sinensis 30C against HEPG2, compared to other cell lines, *in silico* molecular docking studies should be done to know the molecular affinity between HEPG2 and Cordyceps sinensis 30C, to understand the deeper aspects of its cytotoxic properties. Additional *in vivo* studies to assess the toxicological risk will help allay the safety concerns about the drug.

**ACKNOWLEDGMENTS**

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**Financial support and sponsorship**

Nil.

**Conflicts of interest**

None declared.


Antioxidative and cytotoxic potential of the potenziert Zubereitung von Ophiocordyceps sinensis in-vitro in the Brustkrebszelllinie (MCF-7), der Leberkrebszelllinie (HePG2), der Lungenkrebszelllinie (A-549), der Prostatakrebszelllinie (PC3)

Potencial antioxidante y citotóxico de la preparación potenciada de Ophiocordyceps sinensis en-vitro en línea celular de cáncer de mama (MCF-7), línea celular de cáncer de hígado (HePG2), línea celular de cáncer de pulmón (A-549), línea celular de cáncer de próstata (PC3)

Fondo: El hongo parásito Ophiocordyceps sinensis ha sido descubierto en larvas de lepidópteros, teniendo efectos antitumorales conocidos. La prueba de Cordyceps sinensis potenciado en células cancerosas podría ayudar a ampliar la terapéutica del cáncer homeopático. Objetivo: Evaluar la actividad antioxidante y citotóxica de la preparación potenciada de Cordyceps sinensis en líneas celulares de cáncer de mama: línea celular de cáncer de mama (MCF-7), línea celular de cáncer de hígado (HePG2), línea celular de cáncer de pulmón (A-549), línea celular de cáncer de próstata (PC3). Todos los experimentos se llevaron a cabo por triplicado. Los datos se analizaron mediante análisis unidireccional de varianza (ANOVA) y las medias se compararon mediante la prueba de Nueva Rango Múltiple de Duncan. Resultado: Se observó un mayor potencial antioxidante de 377,40 μl/ml en Cordyceps sinensis 30C, que se analizó más a fondo para determinar la citotoxicidad en líneas celulares (MCF-7), (HePG2), (A-549), (PC3), donde se inhibió a concentraciones 596,21 ± 3,32 μl/ml, 438,10 ± 2,39 μl/ml, 555,40 ± 3,08 μl/ml, 656,42 ± 2,68 μl/ml respectivamente. Al comparar Cordyceps sinensis 30C con otras líneas celulares, su actividad citotóxica contra las líneas celulares de cáncer de hígado (HEPG2) es particularmente potente. Conclusión: Esta investigación demuestra la utilidad de la preparación potenciada de Cordyceps sinensis, se necesitarán estudios adicionales que la comparen con los medicamentos utilizados actualmente para determinar si es o no significativamente más eficiente.