Quantification of Flavonoids and Nucleoside by UPLC-MS in Indian *Cordyceps sinensis* and its In-vitro Cultures

Masood Shah Khan¹,², Washim Khan¹, Manimaran Manickam³, Raj kumar Tulsawani³, Kshipra shipra Misra⁴, Puran Singh Negi⁴ and Sayeed Ahmad¹*

¹Bioactive Natural Product Laboratory, Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Hamdard University, Hamdard Nagar, New Delhi, INDIA.
²Department of Pharmacy, Faculty of Science, Mohammad Ali Jauhar University, Rampur, UP.
³Defence Institute of Physiology and Allied Sciences, Delhi, INDIA.
⁴Defence Institute of Bio-Energy Research, Haldwani, INDIA.

**ABSTRACT**

**Background:** *Cordyceps sinensis*, a comestible mushroom growing in Himalayan regions, is extensively familiar in traditional system of medicine. The paper presents the results of quantification of some of the flavonoids and nucleosides (kaempferol, isorhamnetin and cordycepin) in the Indian variety of *Cordyceps sinensis*. Since, the plant is known for its high therapeutic potential and not easily available, hence it was also tissue cultured in the laboratory as an alternative source of medicine. **Methods:** Ultra Performance Liquid Chromatography (UPLC) along with Q-TOF–MS detector was used for the characterization and quantification of active components in the aqueous extracts of both the samples of *Cordyceps sinensis* (natural as well as in vitro cultured). **Results:** Results indicate that both the extracts have almost similar concentration of the kaempferol, isorhamnetin and cordycepin which supports use of in vitro biomass as an alternative source of medicine.

**Key words:** Cordycepin, *Cordyceps sinensis*, Isorhamnetin, Kaempferol, UPLC-MS/MS.

**INTRODUCTION**

*Cordyceps sinensis* is a medicinal herb that grows at an altitude more than 3800 m above sea level in cold, grassy, alpine meadows of Himalayan Mountains and is used for various ailments by the local people. *Cordyceps sinensis* is a parasitic mushroom, a type of gascomycetes fungus and has found its use in traditional Chinese medicine for many centuries. Various studies have shown that *C. sinensis* modulates immune response,¹ inhibits tumor cell proliferation,² improves liver function,³ regulates insulin sensitivity,⁴ decreases plasma cholesterol levels,⁵ has hypotensive and vasodilatory activity,⁶ and increases physical performance.⁷ It has also shown protection against cerebral ischemia reperfusion injury, oxidative damage,⁸ and radiation induces bone marrow and intestinal injury.⁹ The various medicinal properties of *C. sinensis* have been reviewed in detail by Zhu *et al*.¹⁰ Historically, plants and medicinal fungus provide the basic building blocks for a number of highly effective drugs and they remain an attractive option for discovery of the new molecular entities, due to their largely tapped chemical diversity.¹¹ Flavonoids are polyphenolic compounds found in plants and are known to possess antioxidant, cyto protective and anti-inflammatory activities.¹² - ¹⁴ The nucleosides, which are some of the bioactive compounds present in *C. sinensis* are found responsible to increase mitochondrial activity and play major role in the production of ATP.¹⁵ The flavonoids like kaempferol (Figure 1c) and isorhamnetin (Figure 1b) have been reported in *C. sinensis*.¹⁶ Cordycepin (Figure 1a)
1a) one of the major nucleoside and important active component of *C. sinensis* considered as a known marker to evaluate its quality. Few analytical methods have been reported on *C. sinensis* for cordycepin, isorhamnetin and kaempferol using HPLC, LCMS etc. However, no one has reported till date its production using *in vitro* condition followed by quantification of its metabolites to check its completeness in having all the enzyme and co-enzyme system. This can be used as an alternative source of medicine, since the drug is only growing at Himalayan region.

UPLC uses small columns packed with 2 µm particles and has emerged as a powerful method in recent years. The UPLC separation is 5–10 times faster and has much better resolution than the normal analytical HPLC. Combining UPLC with Q TOF-MS detector fulfills key requirements in terms of rapidity, sensitivity, selectivity, peak-assignment and quantification of the analytes at low concentrations in complex matrices. Therefore, the present study was carried out to compare the potential *in vitro* produced culture of *C. sinensis* of Indian variety with natural drug in terms of the concentrations of kaempferol, isorhamnetin and cordycepin as determined by UPLC-QTOF-MS.

**MATERIALS AND METHODS**

**Chemicals and Reagents**

Cordycepin (C<sub>10</sub>H<sub>13</sub>N<sub>5</sub>O<sub>3</sub>; assigned purity > 99.5%), Isorhamnetin (C<sub>16</sub>H<sub>12</sub>O<sub>7</sub>; assigned purity > 99.5%), Kaempferol (C<sub>15</sub>H<sub>10</sub>O<sub>6</sub>; assigned purity > 99.5%), was purchased from Sigma-Aldrich, Germany. LC-MS grade acetonitrile (Assigned purity 99.9%; Lot No: 90525) was purchased from Sigma-Aldrich, Germany. MS grade ammonium acetate and ammonium formate were obtained from Fluka analytical, Sigma- Aldrich, Germany. Formic acid (assigned purity > 98%; Lot No: 1439632) was commercially obtained from Fluka analytical, Sigma-Aldrich, Germany. Water used in the entire analysis was of LC-MS grade. Other chemicals used were of analytical grade from commercial sources. The solvent for sample preparations were of analytical grade obtained from Merck (India). The *C. sinensis* (Natural) and *C. sinensis* (Cultured) were obtained from Defence Institute of Bio-Energy Research, Haldwani.
Plant Material

*Cordyceps sinensis* (natural) was collected during the months of May–April 2011, from hilly regions of Himalayas, India, where the plant grows widely under natural conditions. It was characterized by the Ethno-Botany Division of DIBER, Haldwani, where the voucher specimen of the plant is preserved in the herbarium. *C. sinensis* (cultured) was also obtained from of DIBER, Haldwani where it was tissue cultured in the laboratory and both the varieties were processed for the preparation of extracts.

The live specimens of *C. sinensis* were collected carefully from their natural habitat in the high altitude region (above 12000 fts) of Central Himalayan hills. The specimens were washed with the tap water to remove the adhering dust particles on it. Further, it was washed cleanly thrice with double distilled water followed by sterilization using mercuric chloride or sodium hypochlorite or propanol or ethanol. Finally the sterilized specimens were washed several times with sterile distilled water. In order to propagate the mycelium *in vitro*, tissues were excised from the different parts of the fungus body like spores, stalk tissue and tissue from stroma region. These tissues were excised from the fungus body with the help of a sterilized blade inside a laminar flow and cultured on to the various culture media. Eight different types of the culture media were used to grow the pure culture of the fungus.

The culture media utilized for the pure mycelium culture are potato dextrose agar (PDA), casein hydrolysate dextrose agar (CHDA), beef extract dextrose agar (BEDA), soybean seed extract dextrose agar (SEDa), rice extract dextrose agar (REDA), mushroom extract dextrose agar (MEDa), soyabari extract dextrose agar (SBEDA) and black soya seed extract dextrose agar (BSEDa). pH of these media varied from 5.5 to 6.5. The cultures were incubated at the various range of temperature (5 to 15) inside an incubator. Maximum growth of mycelium was observed on potato dextrose agar (PDA) which was used for growth of *In vitro* cultures. Twelve months old culture was used for analysis.

Preparation of Extracts

Both the mycelium were cleaned and washed thoroughly with water and dried under shade, in a clean dust free environment and crushed using a laboratory blender. Aqueous extracts were prepared using Accelerated Solvent Extraction system (ASE) equipped with a solvent controller unit (ASE 350, Dionex Corporation, Sunnyvale, CA, USA). Extractions were carried out using distilled water at 25°C for 15 min. in 33 mL stainless steel extraction cells containing 2 g of sample and equal volume of diatomaceous earth in three cycles. The extraction procedure followed was as per the method described previously by Misra *et al.*\(^16\). The crude yield of the lyophilized extract was determined gravimetrically by weighing the dried residues obtained from the known volumes of extract. The percent-yields of both the extracts (C. sinensis natural and cultured) ranged between 25-30 % w/w.

Preparation of Sample Solutions

Weighed, 50 mg of the sample extract was dissolved in 5 mL of water in a volumetric flask, sonicated for 30 minutes. Filtered the mixture using 0.2 μm syringe filter and collected the filtrate sample, which was used for UPLC analysis.

Preparation of calibration standards and quality control (QC) samples

The standard stock solution containing 1000 μg mL\(^{-1}\) of cordycepin, isorhamnetin and kaempferol were prepared by dissolving requisite amount in LC-MS grade Methanol:Water 50:50 v/v (Sonicated: 44 kHz, 250 W at 25°C for 20 min). The stock solutions were appropriately diluted to prepare a series of standard working solutions and then stored at 4°C. The solutions were brought to room temperature and filtered through 0.22 μm membrane filter before UPLC/Q-TOF-MS analysis. Calibration curve of standards consisting of a set of ten non-zero concentrations (A-J) were prepared by making different dilutions of above stock solution for cordycepin, isorhamnetin and kaempferol in methanol. However, QC samples were prepared at three levels; 800 ng mL\(^{-1}\) (HQC, high quality control), 400 ng mL\(^{-1}\) (MQC, middle quality control) and 10 ng mL\(^{-1}\) (LQC, low quality control). All the solutions were stored at 2 - 8°C until use.

Ultra Performance Liquid Chromatography Conditions

UPLC was performed on a Water’s ACQUITY UPLC™ system (Serial No# F09 UPB 920M; Model Code# UPB; Waters Corp., MA, USA) equipped with a binary solvent delivery system, an auto-sampler, column manager and a tunable MS detector (Serial No# JAA 272; Synapt; Waters, Manchester, UK). Chromatographic separation was performed on a Water’s ACQUITY UPLC™ BEH C18 (100.0 mm x 2.1 mm; 1.7 μm) column at 40 ± 5°C. The mobile phase used for UPLC analysis consisted of acetonitrile–20 mM ammonium acetate (90:10; v/v), which was degassed before analysis. For isocratic elution, the flow rate of the mobile phase was kept at 0.20 mL min\(^{-1}\) and 10 μL of sample solution was injected in each run. The total chromatographic run time was 3.50 min. The column and auto-sampler were maintained at 40 ± 5 and 25 ± 5°C, respectively and the pressure of the system was set to 15000 psi.
RESULTS AND DISCUSSION

Q-TOF-MS conditions for the calibration of cordycepin, isorhamnetin and kaempferol

The mass spectrometry was performed on a quadrupole orthogonal acceleration time of flight tandem mass spectrometer (Waters Q-TOF PremierTM). The nebulizer gas was set to 500 L h⁻¹, the cone gas was set to 50 L h⁻¹ and the source temperature was set to 100°C. The capillary voltages were set to 3.0 KV and sample cone voltages were set to 40 V, respectively. Argon was employed as the collision gas at a pressure of 5.3 x 10⁻⁵ Torr. The optimum values for compound-dependent parameters like trap collision energy (Trap CE). The Q-TOF Premier TM was operated in V mode with resolution over 8500 mass with 1.0 min scan time and 0.02 s inter-scan delay. The accurate mass and composition for the precursor ions and for the fragment ions were calculated using the Mass Lynx V 4.1 software incorporated in the instrument.

Cordycepin quantitation was performed using selective reaction monitoring (SRM) of the transitions of m/z 252.08→136.04 for cordycepin with a scan time of 1.0 and 0.02 s inter scan per transition and transfer collision
energy (Tran CE) were set to 16.0 and 1.0 V, respectively for fragmentation information.

Isorhamnetin quantitation was performed using selective reaction monitoring (SRM) of the transitions of m/z 317.06→152.99 for isorhamnetin with a scan time of 1.0 and 0.02 s inter-scan per transition. The optimum values for compound-dependent parameters like trap collision energy (Trap CE) and transfer collision energy (Tran CE) were set to 34.0 and 2.0 V, respectively for fragmentation information.

Kaempferol quantitation was performed using selective reaction monitoring (SRM) of the transitions of m/z 285.24→115.05 for kaempferol with a scan time of 1.0 and 0.02 s inter-scan per transition. The optimum values for compound-dependent parameters like trap collision energy (Trap CE) and transfer collision energy (Tran CE) were set to 37.0 and 4.0 V, respectively for fragmentation information. The MS scan and MS-MS spectra of cordycepin, isorhamnetin and kaempferol are presented in the (Figure 2–6).

**Calibration and Linearity**

Calibration standards of nine concentrations of Cordycepin, Isorhamnetin and Kaempferol ranged 1–1000 ng mL\(^{-1}\), were analyzed. The calibration graphs were plotted.
using peak areas versus drug concentrations. For assessing the linearity, the least square regression equation and correlation of coefficient were determined. The proposed method was validated as per the ICH guidelines for linearity, accuracy, precision, LOD & LOQ similar to other method reported by laboratory for salannin, silybin, glabridin gallic, ellagic and ascorbic acid.

**Linearity**

Linearity was assessed with the aid of serially diluted calibration solutions as mentioned above. Calibration graphs were plotted on the basis of triplicate analysis of each calibration solutions by using peak area against concentration. The proposed method was found to be linear over a wide range of concentration 1-1000 ng mL$^{-1}$ for cordycepin, isorhamnetin and kaempferol with good regression coefficient of 0.998, 0.997 and 0.996 respectively. The slope and intercept was found 0.702 ± 0.001, 0.007 ± 0.0003, 0.107 ± 0.005 and 1.529 ± 0.04, 0.023 ± 0.0005, 2.319 ± 0.02 respectively, for cordycepin, isorhamnetin and kaempferol. The results of the experiment are incorporated in (Table 1).

**Accuracy**

The accuracy of the method was evaluated as recovery by standard addition method. The pre analyzed samples were spiked with standard at three different concentration levels i.e. 50, 100 and 150% and the mixtures were re-analyzed by the proposed method. The results of the experiment are incorporated in (Table 2,3).

**Precision**

The precision of the methods were carried out by doing intermediate precision. In intermediate precision, intra-day and inter-day precisions were carried out. Intra-day and inter-day precisions were done by preparing and applying three different concentrations of

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Range</th>
<th>Cordycepin</th>
<th>Isorhamnetin</th>
<th>Kaempferol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity</td>
<td>1-1000ng mL$^{-1}$</td>
<td>1-1000ng mL$^{-1}$</td>
<td>1-1000ng mL$^{-1}$</td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>1.529</td>
<td>0.023</td>
<td>2.319</td>
<td></td>
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<tr>
<td>Slope</td>
<td>0.702</td>
<td>0.007</td>
<td>0.107</td>
<td></td>
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<tr>
<td>Regression equation</td>
<td>$Y=0.702x + 1.529$</td>
<td>$Y=0.007x + 0.023$</td>
<td>$Y=0.107x + 2.319$</td>
<td></td>
</tr>
<tr>
<td>Correlation Coefficient (rr)</td>
<td>0.998</td>
<td>0.997</td>
<td>0.996</td>
<td></td>
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<tr>
<td>LOD</td>
<td>310 pg</td>
<td>305 pg</td>
<td>317 pg</td>
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</tr>
<tr>
<td>LOQ</td>
<td>929 pg</td>
<td>913 pg</td>
<td>950 pg</td>
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</table>

*pg = pico gram
standard in triplicate six times a day and similarly on six different days, respectively. Assay for each analysis was calculated and % RSD was determined (Table 4).

**Lower Limit of Detection (LLD) and Quantification (LLQ)**

The lower limit of detection for each compound was stated to be the smallest concentration detectable by mass spectrometer with signal-to-noise ratio of 5:1. The limit of quantification was defined as the lowest concentration on the calibration curve which could be measured with an intra-assay precision and accuracy $< 20\%$.\textsuperscript{18}

**Sample analysis**

The analysis of aqueous extract of natural and cultured varieties of *C. sinensis* using proposed UPLC/MSMS method showed presence of cordycepin, isorhamnetin and kaempferol\textsuperscript{0} (Table 5). The content of these metabolites in *in vitro* cultures were showing no signifi-
<table>
<thead>
<tr>
<th>Conc (ng mL⁻¹)</th>
<th>Intra-day precision</th>
<th>Inter-day precision</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean peak area ± S.D</td>
<td>%RSD</td>
</tr>
<tr>
<td>CORDYCEPIN</td>
<td></td>
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</tr>
<tr>
<td>100</td>
<td>74.58 ± 1.34</td>
<td>1.8</td>
</tr>
<tr>
<td>400</td>
<td>272.81 ± 7.66</td>
<td>2.8</td>
</tr>
<tr>
<td>800</td>
<td>577.67 ± 8.56</td>
<td>1.4</td>
</tr>
<tr>
<td>ISORHAMNETIN</td>
<td></td>
<td></td>
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<tr>
<td>100</td>
<td>0.70 ± 0.3</td>
<td>1.6</td>
</tr>
<tr>
<td>400</td>
<td>2.94 ± 0.01</td>
<td>0.39</td>
</tr>
<tr>
<td>800</td>
<td>5.34 ± 0.01</td>
<td>0.56</td>
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<tr>
<td>KAEMPFEROL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>15.86 ± 0.26</td>
<td>1.68</td>
</tr>
<tr>
<td>400</td>
<td>48.76 ± 1.0</td>
<td>2.05</td>
</tr>
<tr>
<td>800</td>
<td>88.16 ± 1.7</td>
<td>1.94</td>
</tr>
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</table>

a) Standard deviation; b) Relative standard deviation.

Table 5: Results of analysis

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cordycepin % ww⁻¹</th>
<th>Isorhamnetin % ww⁻¹</th>
<th>Kaempferol % ww⁻¹</th>
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</thead>
<tbody>
<tr>
<td>Cordyceps sinensis (Natural)</td>
<td>0.000114</td>
<td>0.003433</td>
<td>0.000124</td>
</tr>
<tr>
<td>Cordyceps sinensis (Cultured)</td>
<td>0.000124</td>
<td>0.004556</td>
<td>0.000079</td>
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</tbody>
</table>

CONCLUSION

Based on the results of the study, it can be concluded that the tissue culture variety of Indian *Cordyceps sinensis* is at par with the natural variety in terms of the quantity of active principles as determined by UPLC-MS. The highly sensitive newly developed and validated UPLC MS method can be used for quality control of *C. sinensis*, its formulations available in market as well as other plant materials/formulations containing them as an ingredient.

ACKNOWLEDGMENT

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Highlights of Paper

- *Cordyceps sinensis* is an edible parasitic mushroom.
- It is an important source of bioactive flavonoids and nucleosides.
- Kaempferol, isorhamnetin and cordycepin were quantified using UPLC/MS method.
- In vitro cultures of *Cordyceps* were produced as an alternative source of medicine.
- Validated analytical method can be used for quality control.
Author Profile

- **Dr Ahmadhas** been associated in teaching and research since 2005 after completion of his Doctorate in Pharmacognosy and Phytochemistry. He has been honoured with the University Gold Medal, DST fast track young scientist award (2007), CST-UP young scientist award (2008-09), DST BOYSCAST, AICTE Career award (2009-10), PD Sethi award 2013 and Al Ameen College of Pharmacy award (2014). He did his postdoc from Albert Einstein College of Medicine New York (2011) and has to his credit >150 international publications and author of text book “Introduction to Pharmacognosy”. He has been associated with more than sixteen Govt. of India sponsored research projects and supervised 20 PhDs till date.

REFERENCES