

## ORIGINAL ARTICLE

## NMR and IR analysis of natural substances isolated from *Cordyceps* medicinal mushrooms

### NMR a IR analýza prírodných látok izolovaných z liečivých húb *Cordyceps*

Jarmila Harvanová • Lucia Ungvarská Maľučká • Anna Uhrinová • Mária Vilková • Martin Vavra • Martin Pavlík • Martin Rajtar • Anna Furmaníková

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#### Summary

There exist about 750 species of *Cordyceps* at present. A high price of natural *Cordyceps* and its lack in nature caused that the attention has been focused to its cultivation in laboratory conditions. The demand for this “fungus-parasite” is still quite high nowadays, as shown by the amount of commercial nutritional supplements. Phytochemical diversity has ensured that *Cordyceps* is used as an immunomodulatory and an antioxidant; it has anti-cancer, anti-inflammatory, anti-diabetic, antibacterial, anti-HIV effects. In the present study we focused on NMR and IR analyses of natural substances isolated from two species of *Cordyceps*: *Cordyceps sinensis* MFTCCB025/0216, MFTCCB026/0216 and *Paecilomyces hepiali* MFTCCB023/0216. Two types of rice substrates (*Oryza sativa Indica* and *Oryza sativa Japonica*) were used for cultivation. A total of five methanol extracts obtained by a reflux method of the ground mushroom were analysed. To determine the quality and quantity of the major chemical compounds, 1D and 2D NMR analysis has been used with  $^1\text{H}$ ,  $^{13}\text{C}$ , COSY, NOESY, HSQC, HMBC and DEPT spectra. IR

spectroscopy was chosen as a complementary analysis to determine functional groups. Linoleic acid, oleic acid and mannitol were identified as major compounds of the methanol extracts. Tyrosine, alanine, urea and the others biologically interesting substances were found as minor components.

**Key words:** *Cordyceps sinensis* • NMR and IR analysis • oleic acid • D-mannitol

#### Súhrn

V súčasnej dobe existuje 750 druhov húb rodu *Cordyceps*. Vysoká cena prírodného *Cordycepsu* a jej nedostatok v prírode spôsobil, že sa pozornosť upriamila na jej pestovanie, kultiváciu v laboratórnych podmienkach. Dopyt po tejto „hube-parazitovi“ je aj v dnešnej dobe pomerne vysoký, čo dokazuje aj množstvo komerčných výživových doplnkov. Fytochemická diverzita zabezpečila, že *Cordyceps* sa používa ako imunomodulátor, antioxidant; má protirakovinové, protizápalové, antidiabetické, antibakteriálne a anti-HIV účinky. Práca sa zaoberá NMR a IR analýzou prírodných látok izolovaných z dvoch druhov húb rodu *Cordyceps*: *Cordyceps sinensis* MFTCCB025/0216, MFTCCB026/0216 a *Paecilomyces hepiali* MFTCCB023/0216. Tie boli umelo kultivované na dvoch substrátoch ryže (*Oryza sativa Indica* a *Oryza sativa Japonica*). Celkovo sa analyzovalo päť metanolových extraktov, ktoré boli pripravené refluxovaním pomletého materiálu húb. Na stanovenie kvality a kvantity majoritných chemických zlúčenín sa využila 1D a 2D NMR analýza, ktorej riešením  $^1\text{H}$ ,  $^{13}\text{C}$ , COSY, NOESY, HSQC, HMBC a DEPT spektier sa priradili protóny a uhlíky jednotlivým organickým zlúčeninám. Ako doplnková analýza na stanovenie funkčných skupín sa zvolila IR spektroskopia. V extraktoch boli identifikované ako majoritné nasledujúce chemické zlúčeniny: kyselina linolová, kyselina olejová, manitol; ako minoritné tyrozín, alanín, močovina a iné biologicky zaujímavé látky.

**Kľúčové slová:** *Cordyceps sinensis* • NMR a IR analýza • kyselina olejová • D-manitol

J. Harvanová • RNDr. Lucia Ungvarská Maľučká, PhD. (✉) • A. Uhrinová • A. Furmaníková  
University of Veterinary Medicine and Pharmacy  
Komenského 73, 041 81 Košice, Slovak Republic  
e-mail: lucia.ungvarska.malucka@uvlf.sk

M. Vilková • M. Vavra  
University of Pavol Jozef Šafárik  
Faculty of Science, Institute of Chemistry, Košice, Slovak Republic

M. Pavlík  
Technical University, Faculty of Forestry, Department of Forest Protection and Game Management, Zvolen, Slovak Republic

M. Rajtar  
Mykoforest, Veľčice, Slovak Republic

## Introduction

Species of the genus *Cordyceps* (Fr.) Link are fungi that live on larvae, hoods and images of insects or on the breeds of *Elaphomyces*<sup>1)</sup>. Western civilization has been interested in these mushrooms only for the past 30 years, and more and more scientific evidence of the possibilities of their use in medicine has been seen, although in traditional Chinese medicine it has been used for centuries<sup>2)</sup>. As a part of traditional Chinese medicine, *Cordyceps* has been used in respiratory problems, kidney, liver and heart problems, hyposexuality, hyperlipidemia, and to combat weakening of the immune system. It has been also used as a complement to modern cancer treatments (chemotherapy, radiation and surgery)<sup>3)</sup>. Modern science has confirmed the effectiveness of *Cordyceps* fungus treatment in most commonly used traditional medicine practices<sup>4)</sup>. Modern pharmaceutical studies show that *Cordyceps* has a really beneficial effect on several biological systems, immune, cardiovascular, respiratory, and glandular<sup>5)</sup>. A number of scientific works have described the clinical use of this parasitic fungus<sup>3, 6)</sup> with subsequent biological and pharmacological effects<sup>7)</sup>. Various chemical compounds identified in these fungi, such as cordycepin, cordycepic acid, D-mannitol<sup>8–10)</sup>, polysaccharides<sup>11, 12)</sup>, nucleotides<sup>13)</sup>, proteins and amino acids<sup>14)</sup>, and unsaturated fatty acids<sup>15)</sup> are responsible for a wide range of their biological activity.

This study deals with the determination of the chemical structure of the compounds with biological activity in methanol extracts of *Cordyceps* and *Paecilomyces hepiali*, cultivated on two types of rice (*Oryza sativa Japonica* and *Indica*). Modern spectroscopic methods, such as nuclear magnetic resonance (NMR) and infrared spectroscopy (IR), were used to analyse the extracts.

## Experimental part

Five specimens of *Cordyceps* mushrooms cultured on two types of rice substrate were analysed: *Cordyceps sinensis* MFTCCB026/0216 (*Oryza sativa Japonica*) specimen 1, *C. sinensis* MFTCCB026/0216 (*O. sativa Indica*) specimen 2, *C. sinensis* MFTCCB025/0216 (*O. sativa Indica*) specimen 3, *Paecilomyces hepiali* MFTCCB023/0216 (*O. sativa Japonica*) specimen 4, and *P. hepiali* MFTCCB023/0216 (*O. sativa Indica*) 5. Mushroom samples were prepared in cooperation with Ing. Martin Pavlík, Ph.D. from the Technical University in Zvolen, Faculty of Forestry. Methanol (p.a., Sigma-Aldrich) was used to extract the crude, dried and milled material from samples 1–5. A rotary vacuum evaporator IKA, VWR, RV10 control was used to evaporate and thicken the extracts. NMR spectra were measured on a Varian VNMRs 600 MHz spectrometer in deuterated dimethyl sulfoxide DMSO-*d*<sub>6</sub> (Merck). Infrared spectra were measured on a Thermo Scientific spectrophotometer, a NICOLET 6700 FT-IR, an ATR method on a Smart Orbit extension.

## Sample preparation

The production strains came from the collections of Aloha Medicinals (USA) and Mykoforest Velčice (Slovakia). Mushroom cultures were maintained on agar and used to inoculate rice-based substrates (*O. sativa Indica* and *O. sativa Japonica*) which were sterilized in an autoclave at 121 °C at 100 kPa for 2 hours after cooking in nutrient solutions. After cooling down to room temperature, the substrates were inoculated with 3 ml of the inoculum solution and with three pieces of this 4 × 4 mm fungi from a Petri dish. Cultivation was carried out in a shaker (120 beats per minute) for 7 days. 200 mL of the nutrient solution was placed in the incubator for 30 days at 22 °C and the light/dark cycle for 12/12 hours. Afterwards, the substrate was dried in a drier at 40 °C and milled with a laboratory mill.

In total, five samples were labelled as 1–5. The samples 1–5 (10 g) were macerated in 50 ml of 99.9% methanol for six hours in the dark. After maceration, an additional 100 mL of methanol was added and the samples were refluxed for 4 hours, filtered and evaporated to dryness on a rotary vacuum evaporator. The yield of methanol extracts was 6.38 % for sample 1; 2.77% for sample 2; 8.10% for sample 3; 7.00% for sample 4; and 8.61% for sample 5, respectively. 30 mg of all extracts were dissolved in DMSO-*d*<sub>6</sub> (0.6 mL) and were analysed spectroscopically. <sup>1</sup>H, <sup>13</sup>C NMR spectra were measured on a 600 MHz NMR spectrometer at room temperature. The data obtained are as follows: chemical shift in  $\delta$ -scale (ppm), signal multiplicity, integrated intensity and interaction constants *J* in Hz. Proton and carbon assignment was performed on the basis of <sup>1</sup>H, <sup>13</sup>C, COSY, HSQC, HMBC and DEPT spectra. Infrared spectra were measured by the ATR method on the Smart Orbit extension. The crude, evaporated extract was transferred to the Smart Orbit measuring point. The analysed extracts were not modified before the measurement.

## Results and discussion

### NMR analysis

Based on the detailed analysis of 1D and 2D NMR spectra, unsaturated fatty acids (*Z*-oleic acid and linoleic acid) were found in all five samples. The chemical structures of *Z*-oleic acid, linoleic acid and D-mannitol were determined by a detailed and demanding analysis of the COSY spectra (Fig. 1), where the spin systems of all three chemical compounds were determined. The HSQC spectra analysis followed, whereby the carbon atoms signals were assigned to the respective proton of the hydrogen, based on the transfer of the magnetization from the protons to the carbon atoms via one bond. At <sup>13</sup>C NMR spectra were assigned carbon signals that carry protons. Then HMBC spectra were analysed, in which the quaternary carbon atoms in *Z*-oleic and linoleic acid were assigned, based on the transfer of the magnetization from the protons to the carbon atoms via three bonds. Also, this experiment reaffirmed the correct assignment

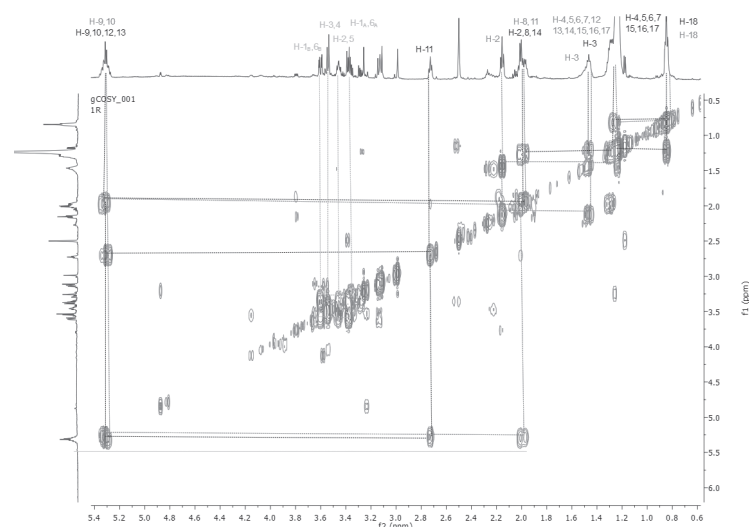


Fig. 1. Assignment of spin systems of Z-oleic acid, linoleic and D-mannitol based on COSY spectra sample number

of proton and carbon signals in each of the identified substances.

To determine the quantity (%) and the ratio of Z-oleic acid, linoleic acid and D-mannitol, proton spectra were used to compare the integrated intensity of the distinct proton signals of the individual chemical compounds (Table 1). All samples had almost double the content of Z-oleic acid compared to linoleic acid, except sample number 4, *P. hepiali* cultivated on *O. sativa Japonica*. Sample No. 1 *C. sinensis* cultivated on *O. sativa Japonica* and sample No. 2 cultivated on *O. sativa Indica* also contained D-mannitol in contrast to samples 3, 4 and 5.

As shown in Table 1, sample extract nos. 3, 4 and 5 did not contain D-mannitol, probably due to strain variation between the individual specimens. In the case of *C. sinensis* 1 and 2 extracts only slight differences in the ratio of Z-oleic acid, linoleic acid and D-mannitol were observed. A more significant difference has been seen in the *P. hepiali* strain, where the substrate effect on the unsaturated fatty acids was more evident. Extract number 4 of *P. hepiali* cultivated on *O. sativa Japonica* contained 1.2-times more linoleic acid, whereas extract 5 cultivated

on *O. sativa Indica* contained 2-times more Z-oleic acid. We assume that different substrates and their different lipids contents (*O. sativa Indica* vs. *O. sativa Japonica*) are responsible for different amounts of lipids which fungi can consume and metabolize to unsaturated fatty acids during their growth. As the literature shows, fatty acid composition of the rice substrate is dependent on the growing season and the ecogeographical varieties<sup>16</sup>. In addition, the difference between the individual strains (*C. sinensis* vs. *P. hepiali*) also affects the content of the chemical compounds.

These initial results indicate that not only the substrate but also the mushroom strain contains Z-oleic acid, linoleic acid and D-mannitol. We will continue to study this issue with a larger number of extracts from several strains of *C. sinensis*, *C. militaris* and *P. hepiali*, the results of which will give us a more comprehensive view of the problem and the relationship between the substrate, strain and chemical composition.

Table 2 shows the complete assignment of hydrogen and carbon at the mixture of substances: Z-oleic acid, linoleic acid and D-mannitol.

Table 1. Integrated intensity and percentage of Z-oleic acid, linoleic acid and D-mannitol in samples of extracts 1–5

| Samples | Integrated intensity                             |                                  |  | Percentage ratio %                           |
|---------|--|----------------------------------|--|--|
|         | Chemical shift/multiplicity/integrated intensity |                                  |  |  |
|         | Z-oleic acid<br>2.15/t/2H – H2                   | Linoleic acid<br>2.73/t/2H – H11 | D-mannitol<br>3.61/dd/2H – H <sub>1B</sub> , H <sub>6B</sub> | Z-oleic acid : Linoleic acid :<br>D-mannitol |
| 1       | 1.99   | 1.0                              | 1.16   | 48.0 : 24.1 : 27.9                           |
| 2       | 1.92   | 1.0                              | 0.85   | 51.0 : 26.5 : 22.5                           |
| 3       | 1.54   | 1.0                              | 0  | 60.6 : 39.4 : 0                              |
| 4       | 0.82   | 1.0                              | 0  | 45.0 : 55.0 : 0                              |
| 5       | 2.03   | 1.0                              | 0  | 67.0 : 33.0 : 0                              |

**IR analysis**

In order to confirm the functional groups in the *Z*-oleic acid, linoleic acid and D-mannitol molecule, IR spectroscopy was used. Infrared spectra were measured by the ATR method on the Smart Orbit extension. The analysed extracts were not modified before the measurement. Table 3 shows vibrations for characteristic functional groups: hydroxyl, carbonyl, SP<sup>3</sup> carbon methylene or SP<sup>2</sup> carbon methylene groups. This method

also confirmed the presence of the aforementioned chemical compounds identified by NMR analysis. The IR spectra of extracts 3, 4 and 5 were identical, D-mannitol was absent in these samples. In all spectra, strong bands in the 1707–1650 cm<sup>-1</sup> region were present for the -C=O group of the carboxyl group in *Z*-oleic acid and linoleic acid. All samples had bands for a -OH group in the range of 3369–3208 cm<sup>-1</sup> in the D-mannitol or in the -COOH group.

Table 2. <sup>1</sup>H a <sup>13</sup>C NMR chemical shift in DMSO\*

| Chemical compounds   | Chemical shift <sup>1</sup> H (multiplicity, integrated intensity, sign of proton, <i>J</i> ); chemical shift <sup>13</sup> C (sign of carbon, number of carbons) ppm  |
|--|--|
| <i>Z</i> -oleic acid;<br>(9 <i>Z</i> )-octadec-9-enoic acid                                    | δ <sup>1</sup> H: 5.32 (m, 1H, H-10); 5.30 (m, 1H, H-9); 2.15 (t, 2H, H-2, <sup>3</sup> <i>J</i> <sub>H2-H3</sub> = 6.6 Hz); 2.03–1.96 (m, 4H, H-8,11); 1.46 (qi, 2H, H-3, <sup>3</sup> <i>J</i> <sub>H3-H2</sub> = <sup>3</sup> <i>J</i> <sub>H3-H4</sub> = 6.6 Hz); 1.32–1.23 (m, 20H, H-4,5,6,7,12,13,14,15,16,17); 0.85 (t, 3H, H-18, <sup>3</sup> <i>J</i> <sub>H18-H17</sub> = 6.6 Hz); δ <sup>13</sup> C: 174.6 (C-1); 129.7 (C-10); 127.7 (C-9); 33.9 (C-2); 26.6 (C-8,11); 24.6 (C-3); 22.0–29.1 (C-4,5,6,7,12,13,14,15,16,17); 13.9 (C-18).  |
| Linoleic acid; ( <i>cis,cis</i> )-<br>-octadeca-9,12-dienoic acid                              | δ <sup>1</sup> H: 5.35–5.27 (m, 4H, H-9,10,12,13); 2.73 (t, 2H, H-11, <sup>3</sup> <i>J</i> <sub>H11-H12</sub> = <sup>3</sup> <i>J</i> <sub>H11-H10</sub> = 6.6 Hz); 2.01 (t, 2H, H-2, <sup>3</sup> <i>J</i> <sub>H2-H3</sub> = 6.6 Hz); 2.00–1.96 (m, 4H, H-8,14); 1.46 (qi, 2H, H-3, <sup>3</sup> <i>J</i> <sub>H3-H2</sub> = <sup>3</sup> <i>J</i> <sub>H3-H4</sub> = 6.6 Hz); 1.32–1.23 (m, 14H, H-4,5,6,7,15,16,17); 0.84 (t, 3H, H-18, <sup>3</sup> <i>J</i> <sub>H18-H17</sub> = 6.6 Hz); δ <sup>13</sup> C: 176.9 (C-1); 129.7 (C-9,10); 127.7 (C-12,13); 34.0 (C-2); 29.0 (C-14); 27.0 (C-8); 25.3 (C-11); 25.2 (C-3); 22.0–29.1 (C-4,5,6,7,15,16,17); 13.9 (C-18).   |
| D-mannitol;<br>(2 <i>R</i> ,3 <i>R</i> ,4 <i>R</i> ,5 <i>R</i> )-hexane-<br>-1,2,3,4,5,6-hexol | δ <sup>1</sup> H: 3.61 (dd, 2H, H-1 <sub>B</sub> , 6 <sub>B</sub> , <sup>2</sup> <i>J</i> <sub>H1B-H1A</sub> = 10.8 Hz, <sup>3</sup> <i>J</i> <sub>H1B-H2</sub> = 3.6 Hz, <sup>2</sup> <i>J</i> <sub>H6B-H6A</sub> = 10.8 Hz, <sup>3</sup> <i>J</i> <sub>H6B-H5</sub> = 3.6 Hz); 3.55 (d, 2H, H-3,4, <sup>3</sup> <i>J</i> <sub>H3-H2</sub> = <sup>3</sup> <i>J</i> <sub>H4-H5</sub> = 7.8 Hz); 3.46 (m, 2H, H-2,5); 3.38 (dd, 2H, H-1 <sub>A</sub> , 6 <sub>A</sub> , <sup>2</sup> <i>J</i> <sub>H1A-H1B</sub> = 10.8 Hz, <sup>3</sup> <i>J</i> <sub>H1A-H2</sub> = 6.0 Hz, <sup>2</sup> <i>J</i> <sub>H6A-H6B</sub> = 10.8 Hz, <sup>3</sup> <i>J</i> <sub>H6A-H5</sub> = 6.0 Hz); δ <sup>13</sup> C: 71.3 (C-2,5); 69.7 (C-3,4); 63.9 (C-1,6). |

\*The chemical shifts of the compounds are compared with the NMR database<sup>17)</sup>

*d* – doublet, *t* – triplet, *qi* – quintet, *m* – multiplet

Table 3. Characteristic bands observed in IR spectra \* of extracts 1–5

| Sample/Vibration (cm <sup>-1</sup> ) | 1                  | 2                  | 3, 4, 5                    |
|--------------------------------------|--------------------|--------------------|----------------------------|
| <b>v(O–H)</b>                        | 3369 br<br>3236 br | 3361 br<br>3217 br | 3364 br<br>3208 br         |
| <b>v<sub>as</sub>(C–H)</b>           | 2922 s<br>2852 s   | 2925 m<br>2854 m   | 2954 s<br>2924 s<br>2854 s |
| <b>v(C=O)</b>                        | 1709 s             | 1650 s             | 1707 sh, m                 |
| <b>v(C=C)</b>                        | 1655 m             | 1604 s             | 1577 s<br>1516 m           |
| <b>δ(–CH<sub>2</sub>–)</b>           | 1456 m             | 1394 m             | 1404 m                     |
|                                      | 1201 m             | 1226 m             | 1188 w                     |
| <b>v(C–C)</b>                        | 1082 m<br>1059 m   | 1076 m<br>1039 m   | 1088 w<br>1041 w           |
| <b>ρ(–CH<sub>2</sub>–)</b>           | 972 w              | 930 w              | 845 w                      |
| <b>ρ(–CH=CH–)</b>                    | 723 m              | 509 m              | 669 m<br>534 m             |

\* *v* – stretching vibration, *δ* – scissoring deformation vibration, *ρ* – rocking deformation vibration

*br* – broad, *w* – weak, *m* – middle, *s* – strong, *sh* – sharp

## Conclusions

To determine the chemical structure of the majorities of substances in methanol extracts prepared from the fungi *C. sinensis* and *P. hepiali*, which were cultured on two rows of rice, NMR and IR spectroscopy were used. In all 1–5 samples, unsaturated acids were present, *Z*-oleic acid was almost twice as high as linoleic acid. Samples 1 and 2 contained, among others, D-mannitol, which was not confirmed in other extracts. The D-mannitol content was 1.4-times higher in the sample of *Cordyceps* cultivated on *O. sativa Japonica* compared with sample No. 2, which was cultivated on *O. sativa Indica*.

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**Conflict of interest:** none.

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