

ORIGINAL ARTICLE

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Dear colleagues, dear friends

It is pleasing to introduce the agenda of the “44th Conference on the Synthesis and Analysis of Drugs” with a wide range of highly interesting issues relating to the development of drugs.

Our Conference is a very important annual gathering of employees of pharmaceutical universities and research institutions it is a great opportunity to renew contacts and discuss topics of mutual interest.

I would like to thank all presenters and other participants who contributed to increasing the awareness of treatment possibilities. I firmly believe that we can meet the upcoming challenges – especially if we continue the tradition of science in the service of public health.

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organizational committee

Determination of biologically active compounds in the fungi of the genus *Cordyceps sinensis* by HPLC and NMR

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Introduction

Cordyceps sinensis is the fungi parasiting larvae, pupae and imagoes of insect as well as fruiting bodies of truffles of the genus *Elaphomyces*¹⁾. The fungi is known in both traditional Chinese medicine and in modern medicinal methods. It is used as a dietary supplement (*CORDYCEPS MRL*®, *ACAI DETOX*®). The fact of *Cordyceps sinensis* consequence is supported by many scientific studies, which have shown its positive effects, for example in anti-tumor therapy²⁾, in the treatment of HIV/AIDS, asthma, liver diseases and it also has a positive effect on female fertility etc.³⁾. Chemical compounds are responsible for these properties, which is currently characterized by the parasite. It was found that the fungi are rich in natural substances such as cordycepin, cordycepic acid, respectively, D-mannitol⁴⁻⁶⁾, polysaccharides^{7, 8)}, nucleotides⁹⁾, proteins and amino acids^{10, 11)}.

This paper is focused on the basic research of studied biologically active compounds (nucleosides, amino acids) identification. To date nucleosides are believed to be the active compounds in *Cordyceps*¹²⁾. Several methods

including thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) and nuclear magnetic resonance spectroscopy (NMR) have been used for the identification of the compounds under study.

Experimental methods

The mushrooms for extraction were obtained dried and grinded from the Technical University of Zvolen, Faculty of Forestry, in co-operation with team of Ing. Martin Paulík, PhD.

For the identification of biologically active compounds occurring in *Cordyceps sinensis* we used methanolic extracts of eight fungi samples (**1–8**). For the separation of the content compounds thin layer chromatography was used (silica gel plates by Kiesselgel 60 F₂₅₄ from Merck) in chloroform. Detection of chromatographs was provided by UV light at 254 nm and by freshly prepared 2% ninhydrine in methanol. For HPLC detection on a UHPLC Ultimate 3000 (ThermoScientific) a DAD detector was used. The measurements were performed at the temperature of 25 °C on the column Polaris 5 C18-A 250 × 4,6 mm (Varian), the injection volumes were 20 µl, flow rate was 1 ml/min. The mobile phase consisted of 2% acetonitrile hypergrade for chromatography (Merck) in water for chromatography (Merck). Standards were purchased: cytidine and uridine (Sigma-Aldrich), guanosine and adenosine (Acros Organics), thymidine (ABCR) and inosine (Calbiochem). NMR spectra were measured on a Varian VNMRs 600 MHz in D₂O (Merck).

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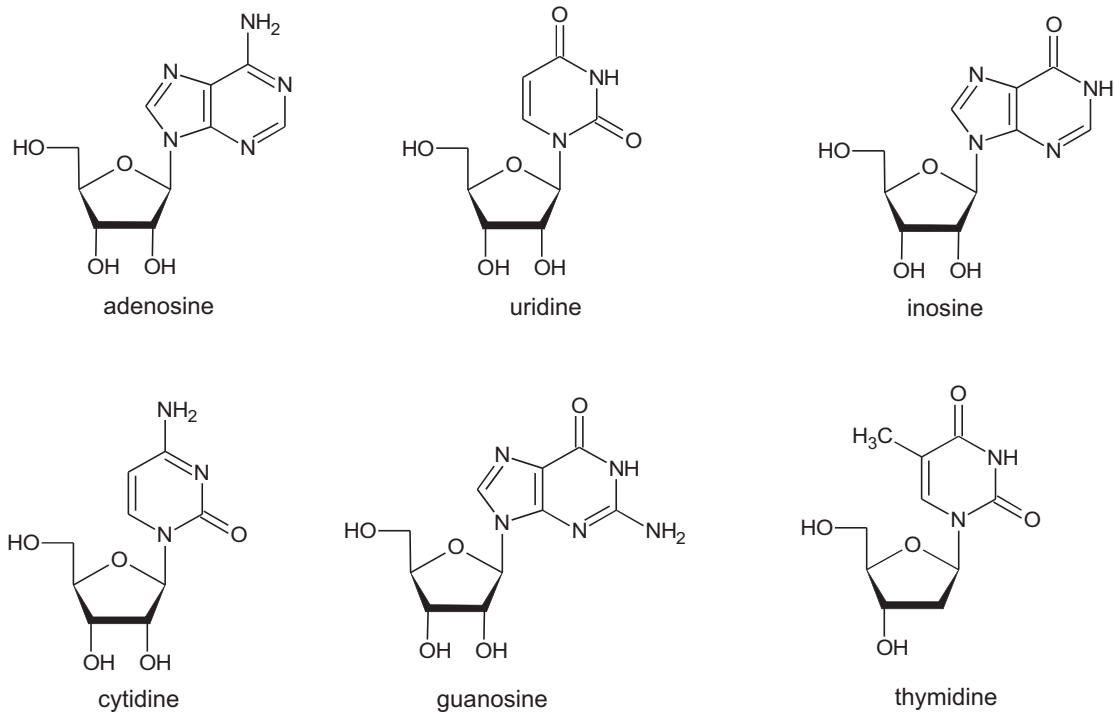


Fig. 1. Structure of studied compounds

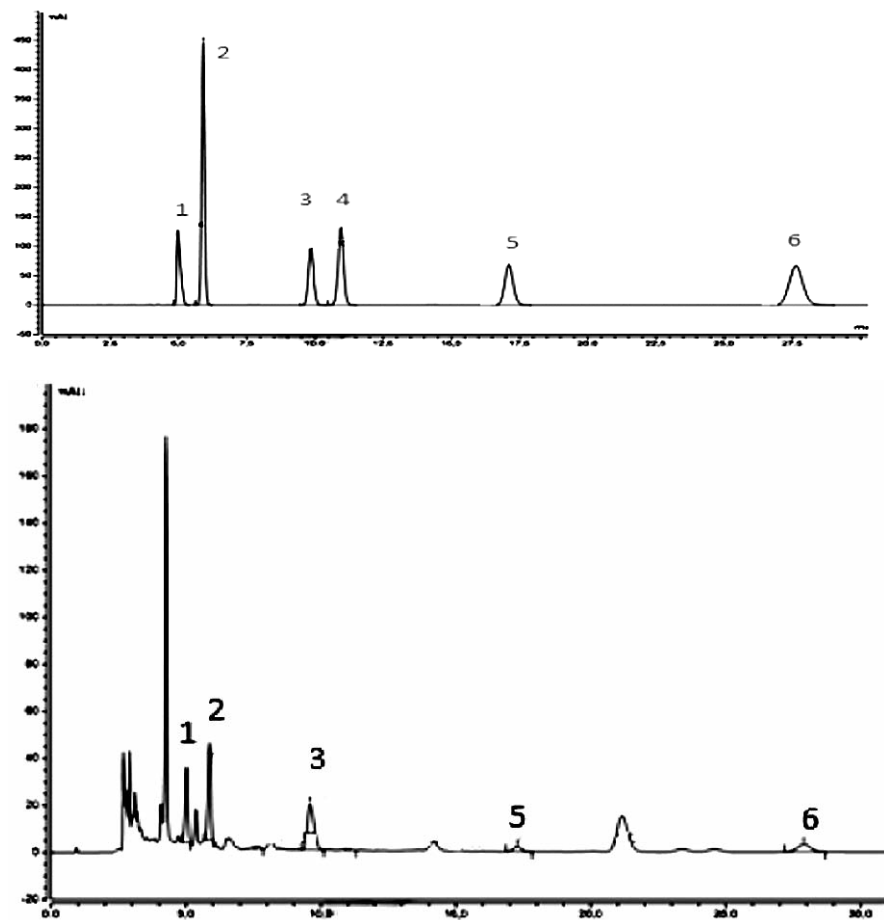


Fig. 2. HPLC-UV/VIS chromatograms of representative samples: above – standards, below – extract 6 (cultivated on millet) of *C. sinensis*
 1 – cytidine, 2 – uridine, 3 – inosine, 4 – guanosine, 5 – thymidine, 6 – adenosine

Table 1. Assignment ^1H and ^{13}C NMR signals for isolated compounds of *Cordyceps sinensis* methanolic extract 7

Compound	Chemical shift (ppm)	^{13}C
	^1H (multiplicity, integral, J value)	
phenylalanine	7.30–7.17 (m, 5H, CH_{Ar}), 3.85 (dd, 1H, $^3J = 8.0, 5.2$ Hz, CH), 3.15 (dd, 1H, $^2J = 14.6$ Hz, $^3J = 5.2$ Hz, CH_2), 2.98 (dd, 1H, $^2J = 14.6$ Hz, $^3J = 8.0$ Hz, CH_2)	173.8 (C = O), 134.9 (C_{r}), 129.3 (C_{o}), 129.0 (C_{m}), 127.6 (C_{p}), 55.8 (C-CH), 36.2 (C- CH_2)
valine	3.46 (d, 1H, $^3J = 4.4$ Hz, CH-NH_2), 2.16–2.10 (m, 1H, CH), 0.90 (d, 3H, $^3J = 7.0$ Hz, CH_3), 0.80 (d, 3H, $^3J = 7.0$ Hz, CH_3)	174.1 (C = O), 60.2 (C- CHNH_2), 29.0 (C- CHCH_3), 17.7 (C- CH_3), 16.5 (C- CH_3)
alanine	3.64 (q, 1H, $^3J = 7.3$ Hz, CH), 1.33 (d, 3H, $^3J = 7.3$ Hz, CH_3)	175.6 (C = O), 50.3 (C-CH), 15.9 (C- CH_3)
leucine	3.59 (m, 1H, CH-NH_2), 1.62–1.50 (m, 3H, CH_2 , CH-CH_3), 0.87 (2 CH_3)	175.4 (C = O), 53.3 (C- CHNH_2), 39.6 (CH_2), 24.1 (C- CHCH_3), 21.8 (C- CH_3), 20.7 (C- CH_3)
isoleucine	3.52 (d, 1H, $^3J = 4.0$ Hz, CH-NH_2), 2.12 (ddd, 1H, $^3J = 14.1$ Hz, 7.0 Hz, 4.0 Hz, CH_2 - CH-CH_3), 1.87–1.81 (m, 2H, CH_2), 0.86 (d, 3H, $^3J = 7.0$ Hz, CH-CH_3), 0.79 (t, 3H, $^3J = 7.4$ Hz, CH_2 - CH_3)	174.0 (C = O), 59.3 (C- CHNH_2), 35.7 (CH_2), 28.9 (C- CH-CH_3), 14.5 (CH_3), 10.9 (CH_3)

Sample preparation

Samples of *Cordyceps sinensis* for analysis were prepared by extraction in methanol. 10 g of crude mushroom was mixed in boiling methanol for 8 h and then filtered. The solvent was evaporated and the extract was dried under vacuum. Extraction yields: 7.1% (**1**), 2.2% (**2**), 8.0% (**3**), 4.8% (**4**), 8.3% (**5**), 7.6% (**6**), 15.1% (**7**), 9.1% (**8**).

Samples were filtered through a 0.22 μm syringe filters before HPLC analysis. Concentrations of samples prepared as water solution were 10 mg/ml.

From the extract of sample **7** we obtained a fraction crystallized from methanol, which we used for NMR experiments (^1H NMR, ^{13}C NMR, DEPT, gCOSY, gNOESY, gHSQC and gHMBC) in amount of 20 mg dissolved in 0.6 ml of D_2O .

Results and discussion

HPLC analysis

In all samples the presence of the nucleosides adenosine, cytidine, uridine, inosine, guanosine and thymidine was studied (Fig. 1).

Figure 2 shows typical chromatograms of nucleoside standards and an example of a *C. sinensis* extract (**6**). The results showed that the components were obviously variant depending on different cultivation conditions of *Cordyceps sinensis*.

In all samples all nucleosides under study were not determined. The qualitative analysis showed that all nucleosides studied are represented only in samples **2**, **3**, **4**, **5**. In other samples cytidine (sample **8**), inosine (samples **1**, **6** and **7**), and guanosine (sample **1**) did not occur. These results demonstrate that qualitative parameters of the

compounds under study are affected by cultivation conditions of fungi.

NMR analysis

With regard to the identification of chemical structures contained in *Cordyceps sinensis*, NMR analysis, which is an invaluable source of new information on the molecular structure, is only little employed.

A NMR study of the extract fraction achieved by crystallisation from methanol (sample **7**) was provided. The TLC and HPLC analyses performed the presence of five compounds in an approximately equal amount. As we could not identify the compounds, we used NMR analysis. On the basis of 1D (^1H NMR, ^{13}C NMR, DEPT) and 2D (gCOSY, gNOESY, gHSQC and gHMBC) NMR experiments, we defined the structure of five amino acids. The chemical shifts (δ , ppm) are described in Table 1. The chemical shifts correlated with the database¹³.

Conclusions

HPLC-UV/VIS and NMR analyses were performed for qualitative determination of nucleosides in *Cordyceps sinensis* fungi. Six nucleosides were determined by HPLC and five unknown compounds were identified by NMR as amino acids.

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Conflicts of interest: none.

References

1. **Hobbs Ch.** Medicinal mushrooms: an exploration of tradition, healing, and culture. Santa Cruz (CA): Botanica Press 1995.
2. **Wang B. J., Won S. J., Yu Z. R., Su Ch. L.** Free radical scavenging and apoptotic effects of *Cordyceps sinensis* fractionated by supercritical carbon dioxide, *Food and Chemical Toxicology* 2005; 43, 543–552.
3. **Gong Y. X., Li S. P., Li P., Liu J. J., Wang Y. T. J.** Simultaneous determination of six main nucleosides and bases in natural and cultured *Cordyceps* by capillary electrophoresis, *Chromatogr. A* 2004; 1055, 215–221.
4. **Huang L. F., Liang Y. Z., Guo F. Q., Zhou Z. F., Cheng B. M.** Simultaneous separation and determination of active components in *Cordyceps sinensis* and *Cordyceps militaris* by LC/ESI-MS, *J. Pharm. Biomed. Anal.* 2003; 33, 1155–1162.
5. **Sprecher M., Sprinson D. B. J.** A reinvestigation of the structure of “*Cordyceps acid*”. *J. Org. Chem.* 1963; 28, 2490–2491.
6. **Cai L. I., et al.** Observation of the structure morphology of *Cordyceps* polysaccharide by atomic force microscope, *J. Chin. Electron. Microsc. Soc.* 1999; 18, 103–105.
7. **Wu Y., et al.** Isolation and characterization of a mannoglucan from edible *Cordyceps sinensis* mycelium, *Carbohydr. Res.* 2007; 342, 870–875.
8. **Li S. P., et al.** The nucleosides content and their variation in natural *Cordyceps sinensis* and cultured *Cordyceps* mycelia, *J. Chin. Pharm. Sci.* 2001; 10, 175–179.
9. **Hsu T. H., et al.** A comparison of the chemical composition and bioactive ingredients of the Chinese medicinal mushroom, *Food. Chem.* 2002; 78, 463–469.
10. **Ji S., et al.** Introduction of the research status in chemical constituents of *Cordyceps*, *J. Fujian Coll. Trad. Chin. Med.* 1999; 9, 46–47.
11. **Fan H., Li S. P., Xiang J. J., Lai C. M., Yang F. Q., Gao J. L., Wang Y. T.** 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 (HPLC-ES-MS/MS), *Analytica Chimica Acta* 2006; 567, 218–228.