

## ORIGINAL ARTICLES

# Medicinal mushrooms *Ophiocordyceps sinensis* and *Cordyceps militaris*

## Liečivé huby *Ophiocordyceps sinensis* a *Cordyceps militaris*

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### Summary

The main component of *Ophiocordyceps sinensis* and *Cordyceps militaris* extracts are polysaccharides. These are natural biopolymers that represent a large class of biologically active components. These contribute to their pharmacological activity and effect on health. They contain monosaccharides that include rhamnose, ribose, arabinose, xylose, mannose, glucose, galactose, mannitol, fructose, and sorbose. The exopolysaccharide fraction has a large number of pharmacological effects, the two most important of which are immunomodulatory and antitumour. Among the contained polysaccharides is also manoglucan, which shows weak cytotoxic activity against the SPC-1<sup>1</sup> cancer cell line. More than ten nucleosides and their related compounds, including adenine, adenosine, inosine, cytidine, cytosine, guanine, uridine, thymidine, uracil, hypoxanthine, and guanosine, have been successively isolated from *Ophiocordyceps sinensis*. It contains many amino acids and polypeptides that are thought to affect the cardiovascular system. They also have a sedative and hypnotic effect, with tryptophan being the most effective component among them.

Polysaccharides were extracted from four samples: sample 1 (grown on the substrate *Oryza sativa indica*, strain *Ophiocordyceps sinensis*), sample 2 (grown on the substrate *Oryza sativa japonica*, strain *Ophiocordyceps sinensis*), sample 3 (grown on the substrate *Oryza sativa indica*, strain *Cordyceps militaris*), sample 4 (grown on *Oryza sativa japonica* substrate, strain *Cordyceps militaris*). Through NMR spectroscopy and subsequent comparison with the literature, the majority of a chemical compound in deproteinized extracts 1 and 4 was found to be a hydrophilic polyglucan referred to as CBHP<sup>2</sup>.

**Key words:** *Ophiocordyceps sinensis* • *Cordyceps militaris* • NMR analysis • CBHP

### Súhrn

Hlavnou zložkou extraktov *Ophiocordyceps sinensis* a *Cordyceps militaris* sú polysacharidy. Ide o prírodné biopolyméry, ktoré predstavujú veľkú triedu biologicky aktívnych zložiek. Tie prispievajú k ich farmakologickej aktivite a pôsobeniu na zdravie. Obsahujú monosacharidy, ktoré zahŕňajú ramnózu, ribózu, arabinózu, xylózu, manózu, glukózu, galaktózu, manitol, fruktózu a sorbózu. Exopolysacharidová frakcia má veľký počet farmakologických účinkov, z ktorých dva najdôležitejšie sú imunomodulačné a protinádorové. Medzi obsahové polysacharidy patrí tiež manoglukán vykazujúci slabú cytotoxickú aktivitu proti rakovinovej bunkovej línii SPC-1<sup>1</sup>. Z *Ophiocordyceps sinensis* sa postupne izolovalo viac ako desať nukleozidov a im príbuzných zlúčenín vrátane adenínu, adenosínu, inozínu, cytidínu, cytozínu, guanínu, uridínu, tymidínu, uracilu, hypoxantínu a guanozínu. Obsahuje mnoho aminokyselín a polypeptidov, u ktorých sa predpokladá, že by mohli mať vplyv na kardiovaskulárny systém. Taktiež vykazujú sedatívny a hypnotický účinok, pričom najúčinnjšou zložkou spomedzi nich je tryptofán.

Polysacharidy sa extrahovali zo štyroch vzoriek: vzorka 1 (pestovaná na substráte *Oryza sativa indica*, kmeň *Ophiocordyceps sinensis*), vzorka 2 (pestovaná na substráte *Oryza sativa japonica*, kmeň *Ophiocordyceps sinensis*), vzorka 3 (pestovaná na substráte *Oryza sativa indica*, kmeň *Cordyceps militaris*), vzorka 4 (pestovaná na substráte *Oryza sativa japonica*, kmeň *Cordyceps militaris*). Prostredníctvom NMR spektroskopie a následným porovnaním s literatúrou sa podarilo zistiť, že majoritná chemická zlúčenina v deproteinizovaných extraktoch 1 a 4 bol hydrofilný polyglukán označovaný ako CBHP<sup>2</sup>.

**Kľúčové slová:** *Ophiocordyceps sinensis* • *Cordyceps militaris* • NMR analýza • CBHP

### Introduction

Species of the genus *Cordyceps*, including *Ophiocordyceps sinensis*, *Cordyceps militaris*, *Cordyceps pruinosus*

and *Cordyceps ophioglossoides*, are valuable traditional medicinal mushrooms<sup>3)</sup> that have been used for medicinal purposes for several centuries, especially in China, Japan, and other Asian countries. Species of the genus *Cordyceps* are a parasitic complex of fungi and caterpillars<sup>4)</sup>. The main collectors of these mushrooms are people in the age group of 15–65 years old, and the price of 1 kg of wild mushrooms in the market in Nepal ranges from 30 000 to 60 000 Nepalese rupees (around 240–480 €), while in India, 1 kg of mushrooms costs around 100 000 rupees (around 800 €)<sup>5)</sup>. Another market survey says that the price varies depending on the quality and location of the *Cordyceps* mushrooms and estimates that the price can go as high as \$ 9000–10 000 per kilogram (around 8300–9200 €) for average quality.

*Cordyceps* mushrooms are found at an elevation of 3600–4200 meters above sea level, mainly in the Nepalese Himalayas, Tibet, Bhutan, Sikkim, Yunnan, and other provinces of China<sup>6)</sup>. In India, they are found in the grassy areas of the Himalayas, where they are known under the name “Keera Ghas”. These mushrooms are also referred to as “Himalayan Viagra” or “Himalayan gold” due to their wide clinical and commercial value<sup>7)</sup>. In recent years, there has been huge exploitation of *Cordyceps*, which has significantly reduced its natural occurrence<sup>8)</sup>. Due to the high price and rare occurrence of these mushrooms, several strains have been isolated from natural mushrooms of the genus *Cordyceps*, which have been successfully cultivated during the

past decades. Products from cultivated *Cordyceps militaris* have shown comparable pharmacological efficacy to natural *Cordyceps militaris*<sup>9)</sup>.

### Experimental methods

Four specimens of *Ophiocordyceps sinensis* (samples 1, 2) and *Cordyceps militaris* (samples 3, 4) cultured on two types of rice substrate, *Oryza sativa indica* (samples 1, 3) and *Oryza sativa japonica* (samples 2, 4), were analysed. The mushroom samples were prepared in cooperation with Assoc. Prof. Ing. Martin Pavlík, PhD., from the Technical University in Zvolen, Faculty of Forestry. Distilled water was used to extract the crude, dried, and milled material from samples 1–4. 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 dimethylsulfoxide DMSO-*d*<sub>6</sub> (Merck, Germany) or deuterated water D<sub>2</sub>O (Acros Organics, New Jersey, USA).

### Sample preparation

Samples 1–4 (9 g) were extracted in 85 ml of distilled water. This mixture was refluxed for 8 hours, then filtered and concentrated to dryness on a rotary evaporator (IKA RV 10 digital, Germany). The yields of the water extracts from samples 1–4 ranged from 0.02% to 0.56% and were highest for samples 2 *Ophiocordyceps sinensis*, which was grown on *Oryza sativa japonica* (0.56%), and 3 *Cordyceps militaris*, which was grown on *Oryza*

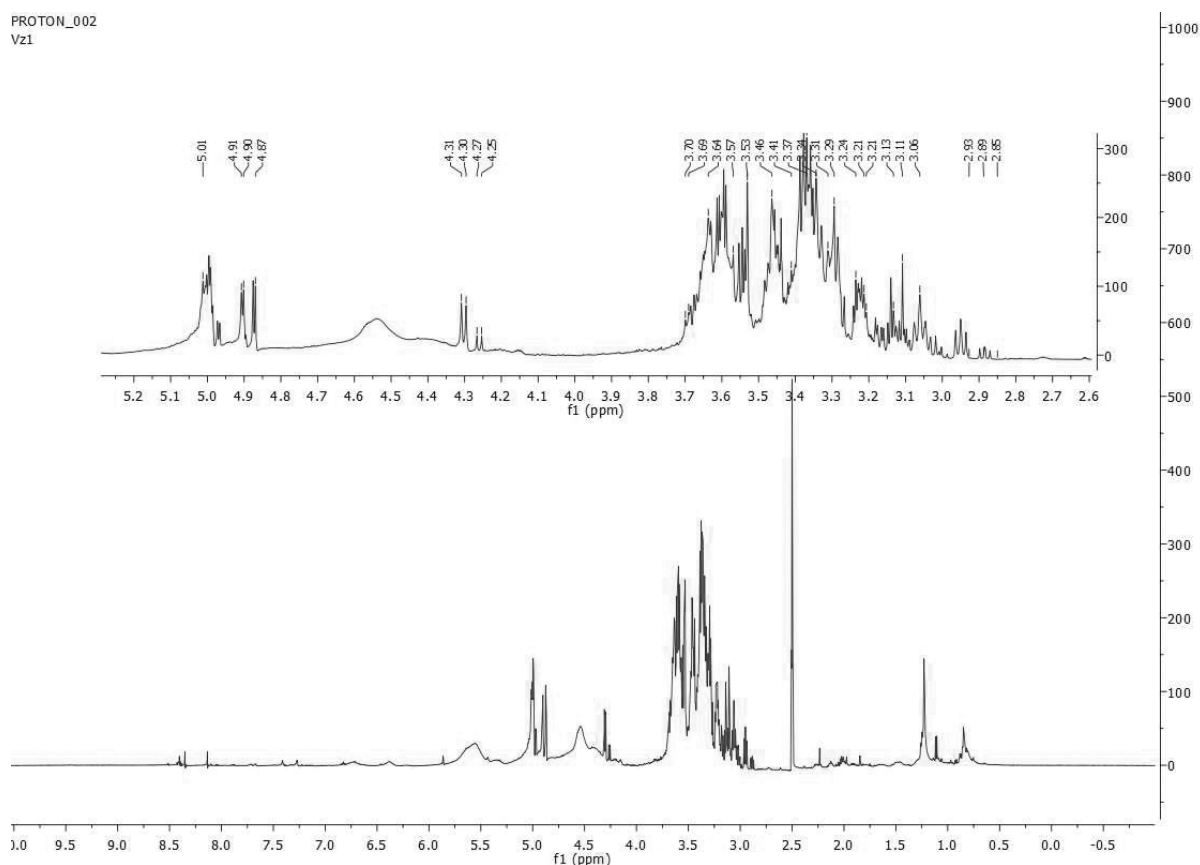


Fig. 1. <sup>1</sup>H NMR spectrum of extract of sample 1 measured in DMSO

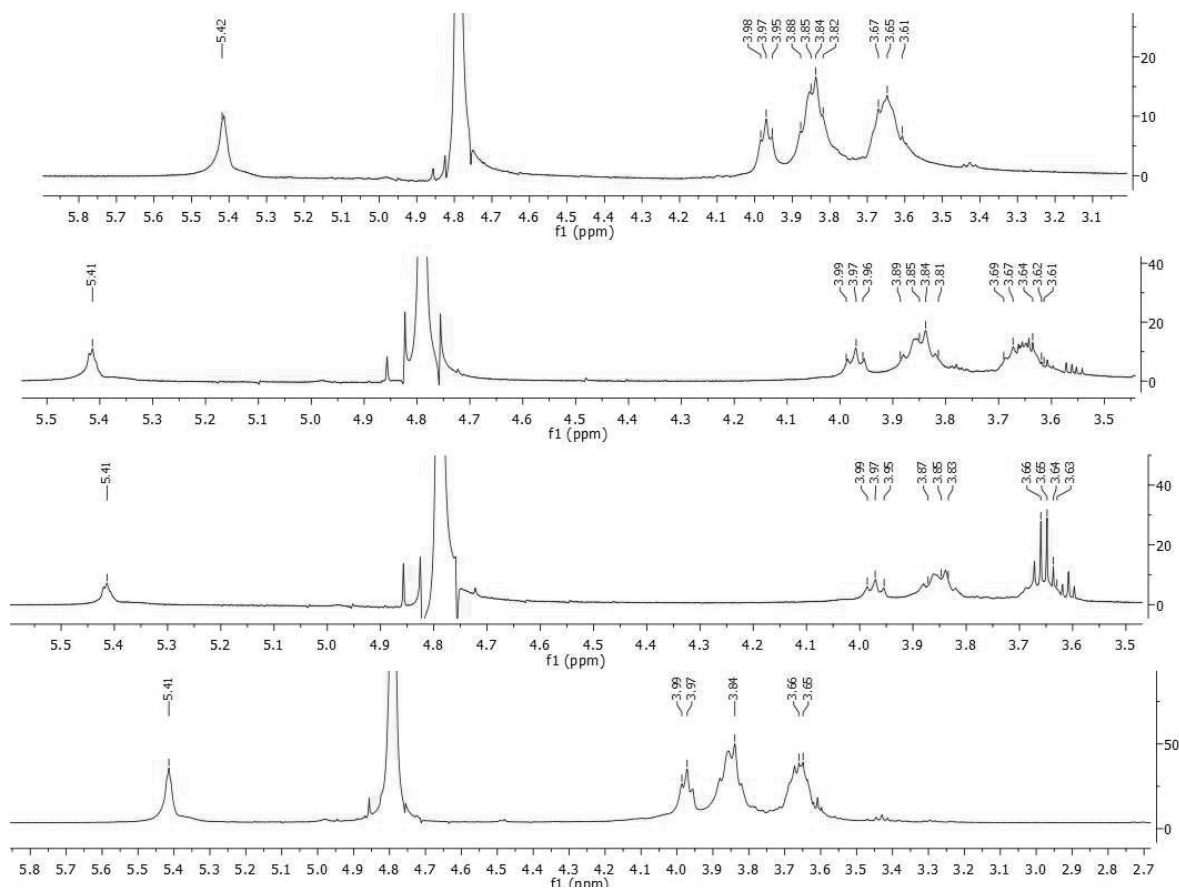


Fig. 2. <sup>1</sup>H NMR spectrum of extracts 1–4

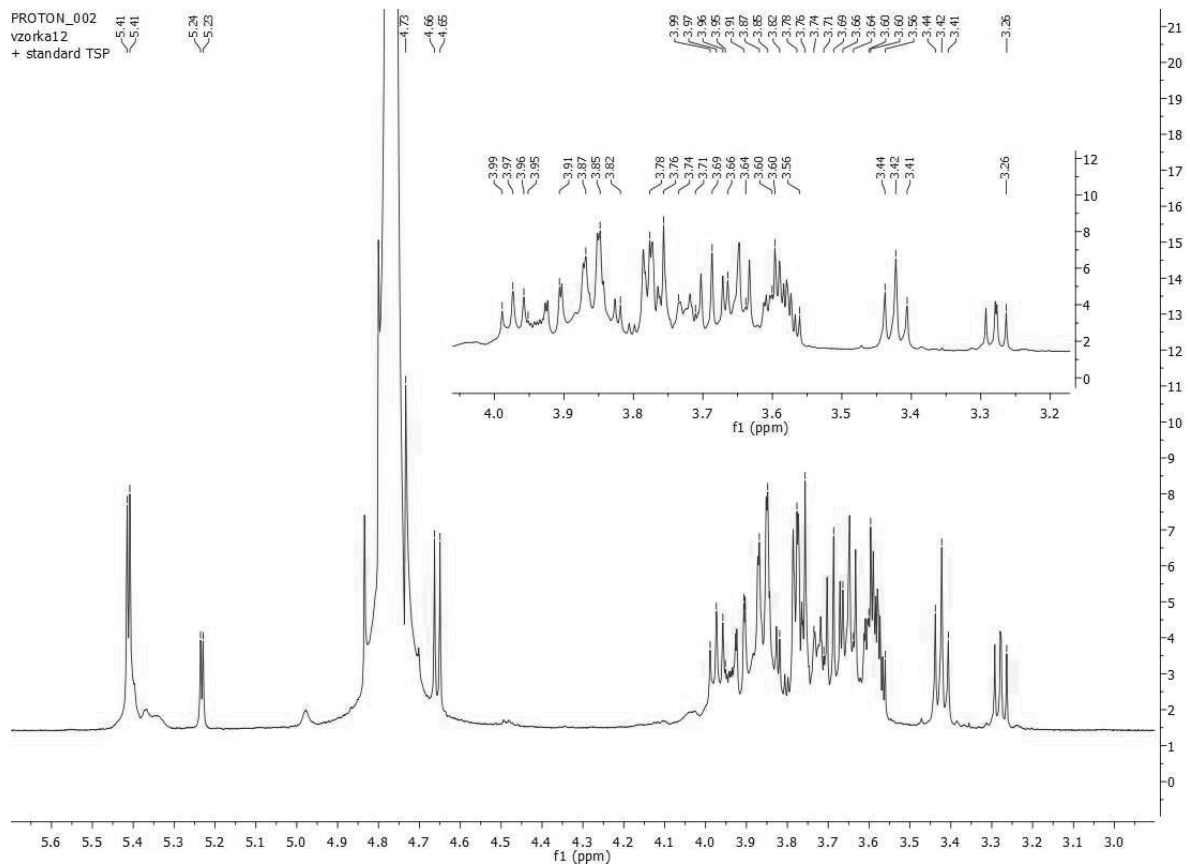


Fig. 3. <sup>1</sup>H NMR spectrum of the CBHP glucan

*sativa indica* (0.56%). Extracts were golden yellow in colour with a typical mushroom scent. They were of solid consistency.

Spectral analysis was performed on a Varian VNMRs 600 MHz spectrometer. Aqueous extracts of deproteinized samples<sup>10</sup>. 1–4 were dissolved in 0.6 ml of deuterated water (D<sub>2</sub>O) or in dimethylsulfoxide (DMSO-*d*<sub>6</sub>) in quartz NMR cuvettes.

The data obtained were as follows: the chemical shift in the  $\delta$ -scale (ppm), signal multiplicity, integrated intensity, and interaction constants *J* in Hz. Determination of the structure of the chemical compounds in the extracts of 1–4 samples required an analysis of 1D (<sup>1</sup>H, <sup>13</sup>C) and 2D (COSY, HSQC, and HMBC) NMR spectra. The spectra were processed using the Mnova-MestReNova software, version 11.0.4-18998, 2017<sup>11</sup>.

## Results and discussion

The chemical composition of deproteinized extracts 1–4 was determined using NMR spectroscopy. Figure 1 contains the proton spectrum of the extract of sample 1. Based on chemical changes, it can be stated that this extract is a mixture of polysaccharides, fatty acids (free or bound in the form of acylglycerols), and amino acids. Hydrophilic polyglucan, known as CBHP, is present in this extract as the major chemical compound<sup>2</sup>. The chemical structure of this polyglucan was also identified by means of 2D NMR spectroscopy in the extract of *Ophiocordyceps sinensis* studied by the Nie<sup>2</sup> working group.

The presence of complex multiplet signals in the region around 2.85–3.75 ppm indicates the presence of protons bound to carbon atoms in the carbohydrate skeleton. By comparing the chemical shifts of anomeric protons with the literature Nie<sup>2</sup> and our measured spectra, we can confirm the chemical structure of the hydrophilic CBHP polyglucan. The doublet signal at 4.87 ppm in the <sup>1</sup>H NMR spectrum is a typical signal of the anomeric terminal hydrogen of  $\alpha$ -D-glucose. The signal at 4.90 ppm belongs to the anomeric proton of  $\alpha$ -D-glucose connected by a 1,4-glycosidic bond. The two weakly intense proton signals at 4.30 ppm and 4.26 ppm belong to the anomeric protons of the 1,2,4,6-connection of  $\alpha$ -D-glucose and 1,2,3,6-connection of  $\alpha$ -D-glucose.

The minor chemical compounds in this extract are unsaturated fatty acids, evidenced by a multiplet signal at 5.01 ppm (signals of the -CH=CH- group), and in the region from 0.80–1.50 ppm long-chain aliphatic hydrogen signals are present.

As can be seen from Figure 2, after purification of the extracted polysaccharides by the Sevag method<sup>10</sup>, the same polysaccharides – polyglucans are found in all four extracts 1–4. The chemical shifts of the anomeric hydrogen atoms at 5.41 ppm are the same in all extracts. This chemical shift indicates that it is a polyglucan –  $\alpha$ -glucan, in which linear glucose units are connected by  $\alpha$ (1→4) glycosidic bonds. Likewise, the presence of the majority of multiplet signals at 3.99–3.96 ppm, 3.89–3.81 ppm, and 3.69–3.61 ppm confirms this involvement of glucose units<sup>12</sup>.

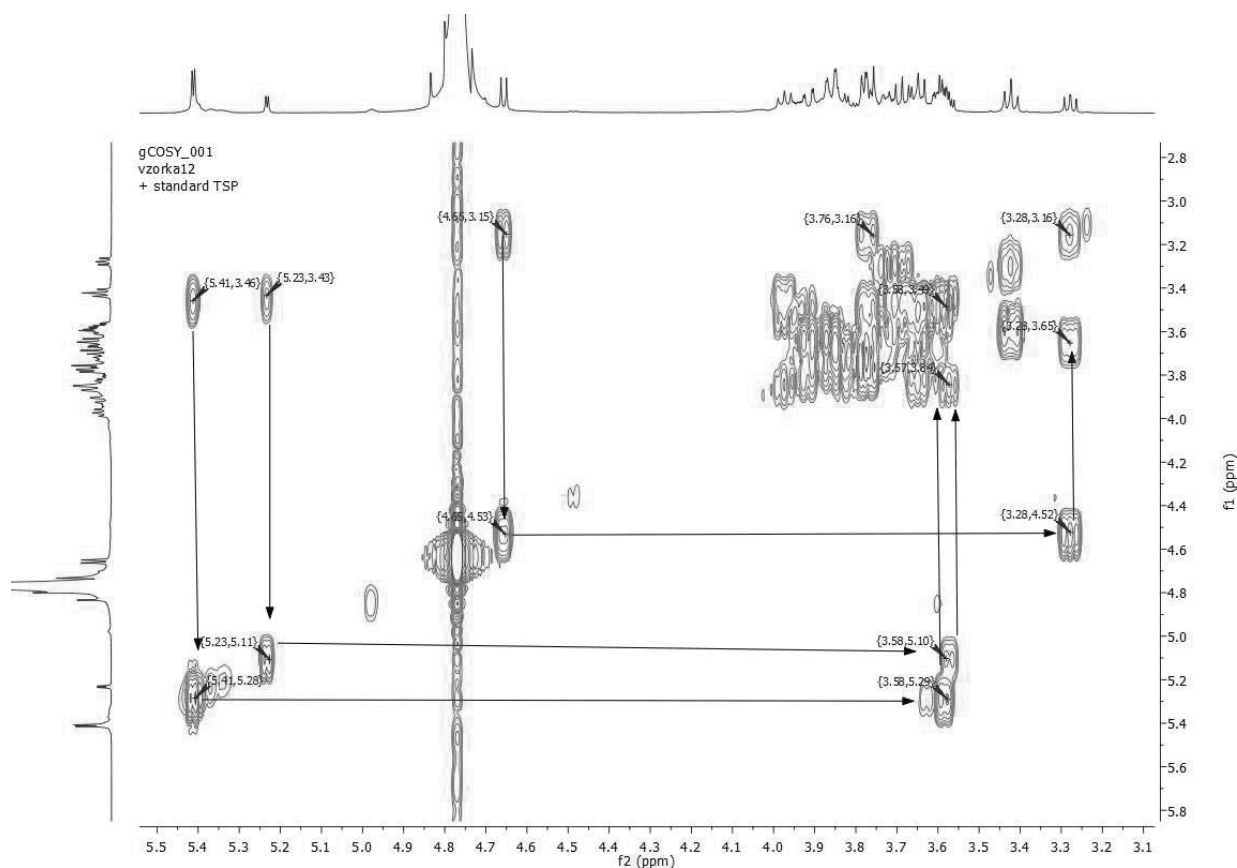


Fig. 4. COSY spectrum of CBHP glucan

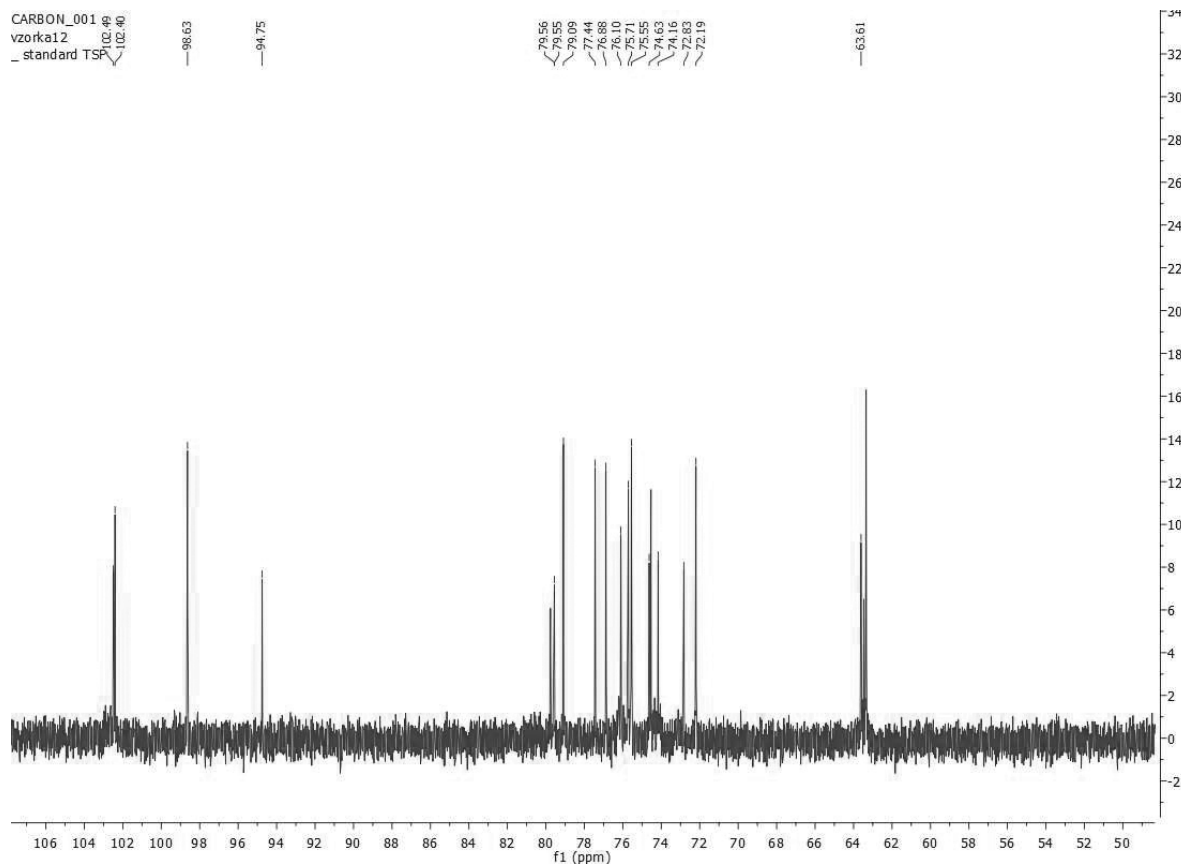


Fig. 5. <sup>13</sup>C NMR spectrum of the CBHP polysaccharide

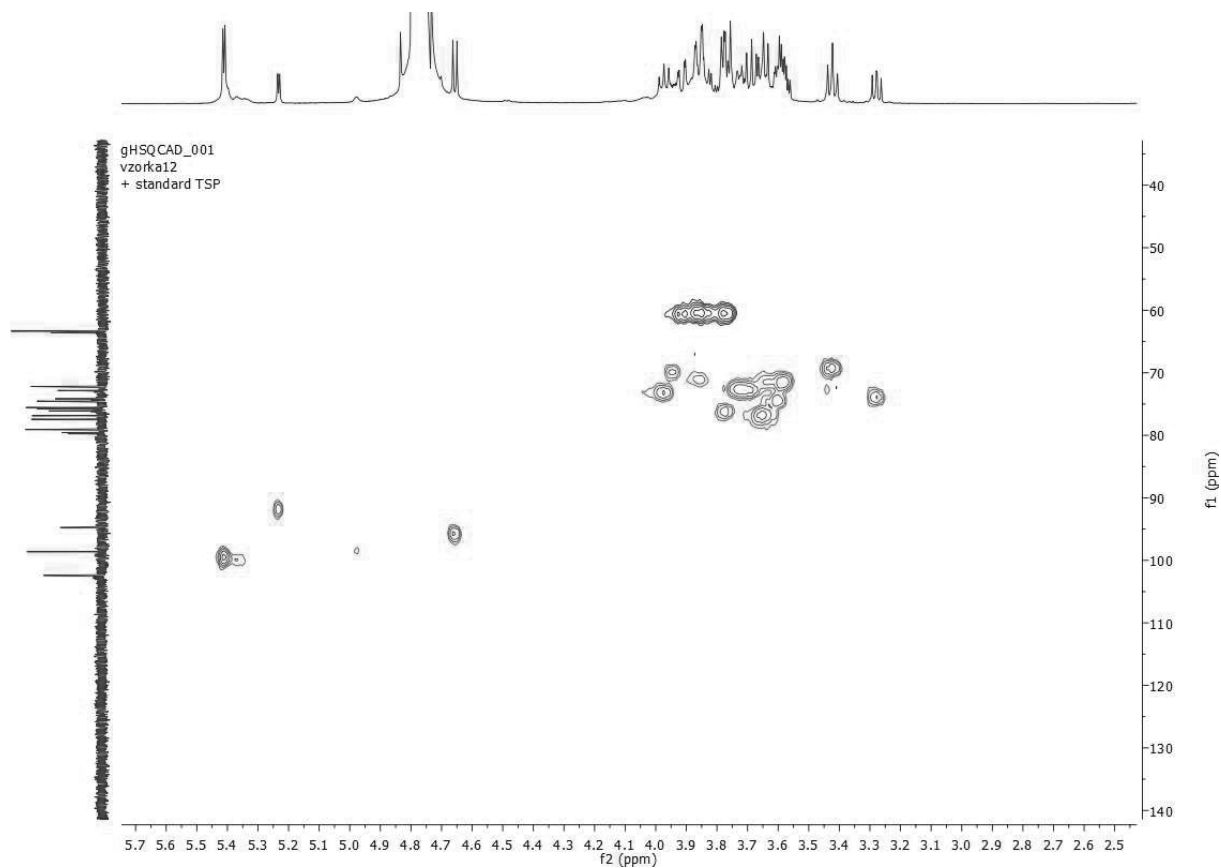


Fig. 6. HSQC spectrum of the CBHP polysaccharide



By placing the 4 extract in D<sub>2</sub>O in the refrigerator for 14 days, new signals began to appear in the <sup>1</sup>H NMR spectrum. Based on a complete 2D analysis (COSY, HSQC, and HMBC), it was found that even in the extract of the 4<sup>th</sup> sample, there is a hydrophilic polyglucan referred to as CBHP<sup>2)</sup>. Figure 3 shows the <sup>1</sup>H NMR spectrum of the CBHP glucan.

According to the literature<sup>2)</sup>, the main component of the CBHP polysaccharide is glucose, which makes up 95.2%. It also contains uronic acid (1.32%). The doublet signal at 5.24 ppm in the <sup>1</sup>H NMR spectrum is the signal of the anomeric terminal hydrogen of  $\alpha$ -D-glucose. The signal at 5.41 ppm belongs to the anomeric proton of  $\alpha$ -D-glucose connected by a 1,4-glycosidic bond. The two proton signals at 4.66 ppm and 4.73 ppm (partially incorporated into the D<sub>2</sub>O signal) belong to the anomeric protons of the 1,2,4,6-connection of  $\alpha$ -D-glucose and the 1,2,3,6-connection of  $\alpha$ -D-glucose. Figure 4 shows the COSY spectrum of the CBHP glucan. In the COSY spectrum, the main correlations between anomeric protons from the  $\alpha$ -terminal D-glucose connected by a 1 $\rightarrow$ 4 glycosidic bond in the chain are marked. Furthermore, there are anomeric protons from the 1,2,4,6-connection- $\alpha$ -D-glucose and 1,2,3,6-connection- $\alpha$ -D-glucose.

Figure 5 contains the carbon spectrum of the polysaccharide extracted from sample 4. In the carbon spectrum are signals of anomeric carbon atoms in the region above 90.0 ppm and carbon atoms belonging to the carbohydrate skeleton. These are located in

the area of 63.0–80.0 ppm. This high chemical shift is caused by the presence of –OH groups bonded directly to carbon atoms. By comparing with the literature<sup>13, 14)</sup>, typical signals for the anomeric carbon atoms belonging to the glucose molecule were found in the <sup>13</sup>C NMR spectrum. The carbon signal with a chemical shift of 102.4 ppm belongs to terminal  $\alpha$ -D-glucose. The signal at 102.5 ppm also belongs to the anomeric carbon atom in  $\alpha$ -D-glucose, which is bound by a 1 $\rightarrow$ 4 glycosidic bond. At the value of 94.7 ppm lies the signal of the anomeric carbon atom from  $\alpha$ -D-glucose, which is connected to the linear chain by a 1,2,4,6 glycosidic bond or a 1,2,3,6 connection (Fig. 5).

Figure 6 is a heteronuclear 2D NMR spectrum – <sup>1</sup>H, <sup>13</sup>C-HSQC. Based on this spectrum, it was possible to identify the signals of anomeric carbon atoms through the transfer of magnetization from the hydrogen atom to the carbon atom through a single bond. In the HSQC spectrum, speckles belonging to the carbon atoms of the –CH<sub>2</sub>–OH group are shown in blue.

Figure 7 shows the HMBC spectrum of the above-mentioned polysaccharide, where, based on the transfer of polarization from H $\rightarrow$ C through three bonds, similar correlations were observed as reported in the literature<sup>2)</sup>.

There is an important peak in the HMBC spectrum, which was formed by the transfer of magnetization from the H-1 hydrogen of  $\alpha$ -(1 $\rightarrow$ 4)-D-glucose to the C-4 carbon of  $\alpha$ -(1 $\rightarrow$ 4)-D-glucose and with the remainder of  $\alpha$ -(1 $\rightarrow$ 2, 4 $\rightarrow$ 6)-D-glucose. The anomeric hydrogen of

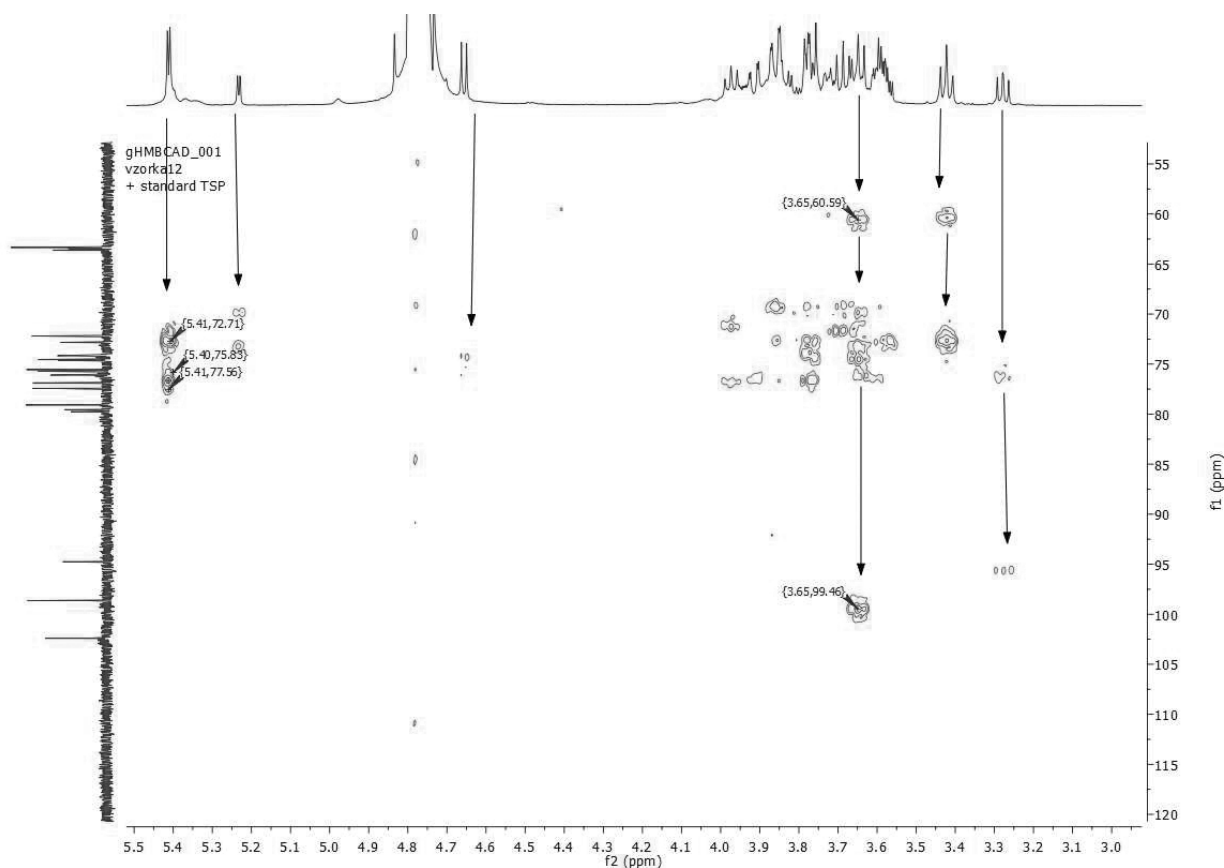


Fig. 7. HMBC spectrum of the CBHP polysaccharide

$\alpha$ -(1 $\rightarrow$ 4)-D-glucose also correlates with the C-3 carbon of  $\alpha$ -(1 $\rightarrow$ 2, 3 $\rightarrow$ 6)-D-glucose. Another important correlation is observed from the H-4 proton of  $\alpha$ -(1 $\rightarrow$ 4)-D-glucose to the C-4 carbon of  $\alpha$ -(1 $\rightarrow$ 4)-D-glucose.

## Conclusions

Based on a complete analysis of 1D and 2D NMR spectra, extract 1 was found to be a mixture of fatty acids, amino acids, and polysaccharides. After comparing with the literature, it was concluded that the hydrophilic polyglucan CBHP was found in this extract as the majority chemical compound. This was also found after the purification of the extracted polysaccharides by the Sevag method. In extracts 1–4, the same polysaccharides – polyglucans were found, which contained linear glucose units connected by an  $\alpha$ (1 $\rightarrow$ 4) glycosidic bond, which was also confirmed by the results of the working group of Shi<sup>12</sup>. After extract 4 in D<sub>2</sub>O was kept in the refrigerator for 14 days, it was found, based on a complete 2D analysis, that CBHP polyglucan was present even in extract 4.

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**Conflict of interest:** none.

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