MEDICINAL MUSHROOMS *OPHIOCORDYCEPS SINENSIS* AND *PAECILOMYCES HEPIALI*

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ABSTRACT

The fungi of the genus *Ophiocordyceps sinensis* (Berk.) belong to entomopathogenic fungi. *Ophiocordyceps sinensis* has been used as a tonic and medicinal product in China for more than 2,000 years. A number of scientific papers have described the clinical use of this parasitic fungus with subsequent biological and pharmacological effects. The various chemical compounds identified in these fungi are responsible for a wide range of biological activity: cordycepin, cordycepic acid, D-mannitol, polysaccharides, nucleotides, proteins, amino acids, and unsaturated fatty acids. Our research focused on the determinations of the biologically active chemical compounds in extracts from cultivated *Ophiocordyceps* fungi using UV/VIS (Ultraviolet/Visible) spectrophotometry and NMR (Nuclear Magnetic Resonance) spectroscopy. The testing of antimicrobial activity of extracts against the collection strains of *Escherichia coli* and *Staphylococcus aureus* demonstrated that the percentage of RIZD (relative inhibition zone diameter) ranged from 83 % to 166 %. The increased antimicrobial

activity against *E. coli* was observed in comparison with that against *S. aureus*.

Key words: cultivation; *Ophiocordyceps sinensis*; *Paecilomyces hepialid*; UV/VIS and NMR spectroscopy

INTRODUCTION

Cordyceps sinensis (Fr.) Link (CS), after the new *Ophiocordyceps sinensis* (OS) (Fig. 1.) from the family *Ophiocordycipitaceae* [11], is one of the entomopathogenic fungi, i.e. fungi parasitizing insects. It occurs naturally in Asia at altitudes above 3500 meters in the Tibetan Plateau, especially in the provinces of Yunnan, Qinghai, Sichuan and Gansu, as well as in the north of Nepal, Bhutan, and India [10].

The life cycle of OS consists of a sexual (teleomorph) and asexual stage (anamorph). Host larvae are infected between July and late August [14]. In the later autumn period, chemicals from the surface of the larvae interact with the spores of the fungus released from the head of the

Fig. 1. *Ophiocordyceps sinensis* [13]

stroma. A fungal mycelium is formed, which then infects a larva surviving the Tibetan winter buried underground. The larva, under the influence of the fungus begins to travel closer to the earth's surface. The OS gradually begins to grow at the expense of the inner tissue of the larva, initially only killing non-vital tissue. By spring, the contents of the larva are completely replaced by fungal tissue, and the mycelium have formed by the fibrous hyphae. The larval exoskeleton remains intact [13]. At the beginning of summer, it is possible to observe a fruiting body (stroma) growing from the head of the larva. This, then produces spores that are released into the environment, which can infect other larvae, and the cycle is repeated [15]. The natural form of OS is harvested from April to August. It is also relatively rare, which, along with its medical and culinary status, has made Chinese consumers willing to pay astronomical prices for it. The harvest of this mushroom is the main source of income for the Tibetan countryside [13]. The demand for OS has increased significantly since about 1950, and the scientific community has therefore attempted to cultivate it artificially, or to cultivate other species of the genus *Cordyceps* with a similar positive effect on the human body [11]. One of the first isolated strains of OS used for cultivation was a strain called CS-4, *Paecilomyces hepialid* (HP). The fermented mycelium of this strain contains pharmacologically active substances similar to those of native OS. In total, more than 20 bioactive substances have been detected in OS [4]. The two most important active substances are cordycepin and D-mannitol (cordycepic acid) [3, 13]. In addition, others chemical compounds have been discovered, i.e., nucleosides, cyclic peptides, sterols, polyketides, alkaloids, and polysaccharides [15]. These chemical com-

pounds are responsible for a wide variety of biological activities, such as, hyposexuality, hyperlipidemia, and weakening of the immune system, respiratory problems, kidney, liver, and heart problems [5]. It is also used as a complement to modern cancer treatments (chemotherapy, irradiation and surgery).

The aim of our study was to determine the biologically active chemical compounds in cultivated *Ophiocordyceps sinensis* using UV/VIS spectrophotometry and NMR spectroscopy, and to examine the isolated extracts for their antioxidant and antibacterial activities.

MATERIALS AND METHODS

Samples of OS and PH were provided by the Technical University in Zvolen, Slovakia. The mushroom samples were cultured and prepared into a final powder form [1]. Six samples were tested: four samples of OS and two samples of PH. Mushroom samples were cultivated on two different rice substrates, *Oryza sativa* var. *japonica* and *Oryza sativa* var. *indica*: Sample 1—OS, MFTCCB026/0216, *Oryza sativa* var. *japonica*; Sample 2—OS, MFTCCB026/0216, *Oryza sativa* var. *indica*; Sample 3—OS, MFTCCB025/0216, *Oryza sativa* var. *indica*; Sample 4—OS, MFTCCB025/0216, *Oryza sativa* var. *japonica*; Sample 5—PH, MFTCCB023/0216, *Oryza sativa* var. *japonica*; Sample 6—PH, MFTCCB023/0216, *Oryza sativa* var. *indica*.

The extracts were prepared by two methods, reflux and ultrasound (Bandelin, Sonorex Digitec, Germany), in order to develop the most effective extraction method. All extractions were performed in a 1:1 water-methanol (methanol, Sigma Aldrich, USA) solvent. The solvent was evaporated to dryness by a digital vacuum rotary evaporator (IKA RV 10, Germany).

A Libra S12 UV/VIS spectrophotometer (Biochrom Ltd., USA) was used to test the antioxidant activity employing the DPPH method (2,2-diphenyl-1-picrylhydrazyl radical, Merck Millipore, USA). A Varian VNMRs 600 MHz spectrometer (Agilent Technologies, Palo Alto, USA) was used to analyse the content of the sample extracts.

NMR spectra of the samples were measured in deuterated water (D_2O , d_2 , Organic Acros, USA) and deuterated dimethyl sulfoxide (DMSO, d_6 , Merck Millipore, Germany). These spectra were measured at the University of Pavol Jozef Šafárik in Košice, Slovakia.

The antimicrobial activities of the extracts were tested by the agar diffusion method according to Rojás et al. [8]. The test bacteria *Staphylococcus aureus* and *Escherichia coli* were obtained from the Czech Collection of Microorganisms, Brno, CR (*S. aureus* CCM 4223, ATCC 29213, *E. coli* CCM 3988, ATCC 10536). The bacteria were cultured in BHI broth (Brain Heart Infusion broth, Oxoid) at 37 °C for 20 hours. Gentamicin sulphate (50 µg.ml⁻¹) was used as a positive and dimethyl sulfoxide (Sigma Aldrich, USA) as a negative control.

After culturing the bacteria, they were diluted in phosphate saline to a concentration of 0.5–1.0 McFarland turbidity. This scale indicated the number of colony forming units (CFU) per 1 ml. At a concentration of 0.5 to 1.0, there were 1.5–3 × 10⁸ bacteria in 1 ml of suspension. One ml of this suspension was inoculated into 100 ml of tempered liquid agar (Standard plate count agar, Oxoid). Twenty (20) ml of this agar were transferred to Petri dishes with a diameter of 9 cm. After solidification, wells with a diameter of 0.5 cm were cut into the agar and 50 µL of a sample were applied to the wells. An aqueous solution of 50 µg.ml⁻¹ gentamicin sulphate was used as a positive control and DMSO as a negative control. The plates were incubated at 37 °C for 24 hours.

RESULTS

The series of samples of *Ophiocordyceps* fungi consisted of six samples (species *Ophiocordyceps sinensis* and *Paecilomyces hepiali*) that were cultivated on two types of rice: *Oryza sativa* var. *indica* and *Oryza sativa* var. *japonica*. Of the two extraction methods used the most effective was the extraction by reflux (the highest yield was 9.86 % in the extract of sample 4, OS, *Oryza sativa* var. *japonica*). The extracts obtained by refluxing had the lowest value of the half-maximal inhibitory concentration IC₅₀ = 3.03 ± 0.05 mg.ml⁻¹ (sample 5, PH, *Oryza sativa* var. *japonica*). Ultrasound extraction was the least effective method. The antioxidant activity of the extracts prepared by this method was 2.5-times lower (the lowest value of IC₅₀ was 6.67 ± 0.01 mg.ml⁻¹, sample 3, OS, *Oryza sativa* var. *indica*) in the uptake of DPPH radical compared to the extracts obtained by reflux.

To determine the content of individual chemicals in the extracts, 1D, 2D NMR was used. By comparing the signals in the measured proton NMR spectra with the NMR database of chemical compounds [9] and the available literature [6], we were able to identify the chemical structure of the respective compounds. The results showed that the ex-

Fig. 2. 1H NMR spectrum of the chemical structure of D-mannitol in sample 5, PH extract

Tab. 1. Antimicrobial activity of extracts 1—4 and 6 tested on *S. aureus* and *E. coli* strains

Sample	Strain, substrate	Extraction method	IZD (mm)		RIZD (%)	
			<i>S. aureus</i>	<i>E. coli</i>	<i>S. aureus</i>	<i>E. coli</i>
1	<i>O. sinensis</i> MFTCCB026/0216	reflux	10	9	166	150
	<i>Oryza sativa</i> var. <i>japonica</i>	ultrasound	5	5	83	83
2	<i>O. sinensis</i> MFTCCB026/0216	reflux	9	9	150	150
	<i>Oryza sativa</i> var. <i>indica</i>	ultrasound	0	8	0	133
3	<i>O. sinensis</i> MFTCCB025/0216	reflux	5	10	83	166
	<i>Oryza sativa</i> var. <i>indica</i>	ultrasound	7	10	116	166
4	<i>O. siensis</i> MFTCCB025/0216	ultrasound	7	8	116	133
6	<i>P. hepiali</i> MFTCCB023/0216	reflux	6	10	100	166
	<i>Oryza sativa</i> var. <i>indica</i>	ultrasound	0	5	0	83

IZD—inhibition zone diameter [mm]; RIZD—relative inhibition zone diameter
concentration of gentamicin = 50 µg.ml⁻¹; IZDDMSO = 0 mm; IZDGENTAMICIN = 6 mm

Fig. 3. Antimicrobial activity of the most active extracts of OS against *E. coli* (EC, left) and *S. aureus* (SA, right) collection strains

traction into 1:1 water-methanol gives: oleic acid, isomers of linoleic acid, acetic acid, 1,4-butanedioic acid, malonic acid; polysaccharides were present in some samples; D-mannitol, tyrosine, uracil and some amino acids.

Figure 2 shows the ¹H NMR spectrum of the chemical structure of D-mannitol present in sample 5, PH, MFTCCB023/0216, *Oryza sativa* var. *japonica*.

The antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* strains, expressed as a percentage of relative inhibition zone diameter RIZD, varied from 83 %

to 166 % for the active samples. The following formula was used to calculate the % of RIZD:

$$\% \text{ RIZD} = (\text{IZD}_{\text{SAMPLE}} - \text{IZD}_{\text{NEGATIVE CONTROL}}) / \text{IZD}_{\text{GENTAMICIN}} \times 100$$

where IZD is the inhibition zone diameter (mm).

The values of the antimicrobial activity of extracts are presented in Table 1.

Table 1 shows an average increase in antimicrobial activity of extracts against *E. coli* in comparison with *S. aureus*. It is interesting that the extracts prepared from sample 5, PH strain MFTCCB023/0216, *Oryza sativa* var. *japonica*, did not show any antimicrobial activity.

Figure 3 illustrates the results of the agar diffusion test of the antimicrobial activity of extracts against *E. coli* and *S. aureus* (IZD).

DISCUSSION

The aim of this study was to isolate the substances contained in fungi of the species *Cordyceps*. The prepared extracts were examined for antioxidant and antimicrobial activities and the content of chemical compounds in the extracts were determined.

Extracts of substances were prepared by two different extraction methods, reflux and ultrasound extraction. Reflux is one of the most widely used methods for isolating substances from fungi. According to our previous experience, water-methanol mixture was used as a suitable solvent. The yield of the extracts ranged from 1.93 to 9.86 %. The values of the given yields were comparable with the yields reported in the literature [12]. Yields ranging from 1.7 % to 10 % have been reported.

The antioxidant activities of the prepared solutions were determined by the DPPH method. It is one of the most frequently used methods for determining the antioxidant activity *in vitro*. From the prepared extracts, the highest antioxidant activity ($IC_{50} = 3.03 \pm 0.05 \text{ mg.ml}^{-1}$) was shown by the extract from sample number 5, which represented the PH strain MFTCCB023/0216 cultured on *Oryza Sativa* var. *japonica* substrate. In the case of ultrasonic extraction, the highest IC_{50} value was shown by sample 3 (IC_{50} was $6.67 \pm 0.01 \text{ mg.ml}^{-1}$, OS, *Oryza sativa* var. *indica*).

Dong et al. [2] compared the antioxidant activities of natural and artificially cultured mycelia by the DPPH method. They found that the aqueous extracts from natural mycelium had an IC_{50} of 0.93 mg.ml^{-1} and for artificially cultured mycelium 1.03 mg.ml^{-1} . The difference in these values may be due to the different *Cordyceps* strain used, the different culture conditions and the substrate, or different procedure. An interesting observation was that the

naturally growing *Cordyceps* showed better antioxidant activity in the uptake of DPPH radical than the artificially cultured one [10]. However, the common result was that *Cordyceps* fungi are a functional antioxidant.

Comparison of the signals in the measured proton NMR spectra with the NMR database of chemical compounds and the available literature [6] showed that the extracts contained oleic acid, isomers of linoleic acid, acetic acid, 1,4-butanedioic acid and malonic acid. Some samples contained also polysaccharides D-mannitol, tyrosine, uracil and some amino acids.

D-mannitol belongs to the category of compounds that show a significant therapeutic potential [7]. When evaluating the NMR spectra, D-mannitol was captured only in sample 5. In the other spectra, the above compounds were detected as the majority. We assume that the bands of minor compounds, including D-mannitol, overlap with the bands belonging to the major compounds.

Testing of the antimicrobial activity on *Escherichia coli* and *Staphylococcus aureus* strains showed that the % RIZD varied from 83 % to 166 % for active samples. Compounds belonging to the group of peptides, glycoproteins and, to a large extent, cordycepin itself are responsible for this antimicrobial activity.

CONCLUSIONS

Our research focused on the determination of antioxidant and antimicrobial activities of OS and PH methanol extracts. The extract of sample 5, PH, *Oryza sativa* var. *japonica* (sample 5) had the best ability to scavenge the DPPH radical ($IC_{50} = 3.03 \pm 0.05 \text{ mg.ml}^{-1}$). The analysis of NMR spectra confirmed the presence of the following compounds in extracts 1–6 in various ratios: oleic acid, isomers of linoleic acid, acetic acid, 1,4-butanedioic acid, malonic acid, polysaccharides, D-mannitol, tyrosine, uracil, and some amino acids. The testing of extracts for their antimicrobial activities against collection bacterial strains *Escherichia coli* and *Staphylococcus aureus* showed that the % of RIZD ranged from 83 % to 166 %. An increased antimicrobial activity against *E. coli* was observed in comparison with that against *S. aureus*.

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