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Antioxidant Activity and Infrared Spectroscopy Analysis of Alcoholic Extracts Obtained from *Paecilomyces hepiali* (Ascomycetes)

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ABSTRACT: This research studies the influence of substrate on the antioxidant activity of alcohol extracts of *Paecilomyces hepiali*. We used corn, rice, millet, and peas as substrates. Antioxidant activity was measured with the DPPH radical scavenging method. Concentrations of extracts (6.25, 3.12, 1.56, 0.78, and 0.39 mg/mL) were applied in all evaluations. Overall antioxidant activity was expressed as the concentration of substrate that decreased DPPH radical levels by 50% (IC_{50}^{DPPH}) for 7 methanol and 7 ethanol extracts. A comparison of IC_{50}^{DPPH} allowed us to conclude that the methanol extracts are more active in scavenging stable DPPH radicals than are the ethanol extracts. The substrate with antioxidant properties most suitable for cultivation of *P. hepiali* was rice supplemented with non-defatted soy flour. The extract most effective in scavenging stable radicals was the methanol extract of sample 4 (IC_{50}^{DPPH} = 2.33 mg/mL) cultivated on rice with nondefatted soy flour. The methanol extract of sample 7 cultivated on peas was less effective (IC_{50}^{DPPH} = 11.50 mg/mL). By crystallizing these extracts, we managed to obtain sufficient quantities of 6 samples in a solid state, for which infrared spectra were measured and confirmed the presence of amino acids in the extracts.

KEY WORDS: cultivation, DPPH radical, IR spectroscopy, medicinal mushrooms and fungi, methanol and ethanol extracts, *Paecilomyces hepiali*, scavenging activities

ABBREVIATONS: $\mathbf{IC_{50}^{DPPH}}$, concentration of substrate that decreases DPPH radical levels by 50%; **IR**, infrared; **MYA**, malt extract yeast agar

I. INTRODUCTION

Humans have been using mushrooms for millenia, not only as a source of nutrition but also for other practical uses—starting fires, honing tools, making covers and clothing, and so on. Evidence proves that mushrooms have been used for many purposes, including medicinal uses, since the Neolithic period. Modern science has confirmed this vast amount of old knowledge of mushrooms. Quickly developing interdisciplinary research on medicinal mushrooms, especially from the past 3 decades, confirms the positive effect of biologically active compounds extracted from a plethora of mushroom species. Therefore, even modern practices in Japan, China, Korea, Russia, and many other countries use various mushroom extracts and products.[1–5]

One of the rarest yet traditionally used species is *Ophiocordyceps sinensis* (Berk.) G.H. Sung et al., which has been a staple medicinal mushroom in traditional Chinese medicine since at least the 15th century, when it was described by the Tibetan physician Nyamnyi Dorje.[6] Traditional Chinese medicine uses *O. sinensis* to treat

lung and respiratory system problems, hyposexuality, hypolipidemia kidney, or liver, heart, and immune system conditions. In addition, it has been used to treat various forms of cancer, especially in addition to chemotherapy and radiotherapy. In Tibet it is used to regain strength and life force after various illnesses.[7–9]

Considering the rarity and high price of natural fruiting bodies of *O. sinensis*, research during the second half of the 20th century focused on growing and cultivating this species. One studied strain was named Cs-4, and it was chosen for commercial production. Its medical potential was tested and, on the basis of its potency and safety, it was approved in China in 1988 as a medicine under the name *Jin Shui Bao*.[9–11]

Cs-4 has been studied extensively in China, including in industrial fermentation and its chemical composition, toxicity, and therapeutic functions.[12,13] This isolated strain was believed to be the same species as wild-type *Cordyceps* until 1982 when researchers discovered that Cs-4 in fact belonged to a different genus and species: *Paecilomyces hepiali* Chen (Trichocomaceae, Eurotiales, Ascomycetes).

A study compared the chemical components and pharmacological activity of wild *O. sinensis* and *P. hepiali* and showed that *P. hepiali* has larger amounts of 7 essential amino acids (leucine, lysine, methionine, phenylalanine, tryptophan, tyrosine, and valine) than are found in *O. sinensis*. The total amount of dietary elements analyzed (potassium, magnesium, zinc, copper, manganese, and selenium) was also higher in *P. hepiali*.[10] *P. hepiali* contained more protein, lipid, ash, carbohydrate, ergosterol, and mannitol than *O. sinensis*. A hot water extract of *P. hepiali* was shown to have a number of pharmacological effects: (1) inhibition of human platelet aggregation, (2) inhibition of *IL-8* gene expression, (3) antimutagenic activity, (4) inhibition of melanin production, and (5) improvement of skin texture in humans.[7] Polysaccharides, adenosine, and cordycepin are considered to be the major functional components providing the healthful effects of powdered *P. hepiali* mycelia.[14]

Our current research focuses on testing cultivation of the fungus *P. hepiali* by solid-state fermentation on various plant substrates, and the production of fungus or mycelial biomass containing qualitatively interesting substances. Growth on a solid substrate matches closely growth conditions in the natural environment, and the composition of the substrate can be modified to change the biologically active compounds in the final product and to eliminate the loss of extracellular metabolites into the surrounding medium.

II. MATERIALS AND METHODS

A. Mushroom Material

P. hepiali (Cs-4), provided by Aloha Medicinals Inc. (Carson City, NV), was maintained on potato dextrose agar, malt extract yeast agar (MYA), and malt extract peptone insect agar at 4°C by Mykoforest Company (Velcice, Slovakia; accession no. MFTCCB022/022013). Cultures were cultivated on Petri dishes containing MYA medium (20 g malt extract/L distilled water, 2 g yeast extract/L distilled water, and 20 g agar/L distilled water; Biolife Italiana s.r.l., Milan, Italy) at 22°C in the dark for 14 days.

B. Substrate Preparation

P. hepiali mycelia were grown on 4 different substrates: corn (*Zea mays*), rice (*Oryza glaberrima*), millet (*Panicum miliaceum*), and peas (*Pisum sativum*). Rice (extract 3) was boiled in a nutrient solution consisting of 8 g yeast extract (Biolife Italiana s.r.l.), 1 g KH_2PO_4, 1 g $MgSO_4 \cdot 7H_2O$, 2 g K_2HPO_4, and 8 g glucose (all per liter distilled water; Merck, Bratislava, Slovakia). After centrifugation, the rice was autoclaved at 121°C for 2 hours. The other rice sample (extract 4) was enriched with 12 g non-defatted soy flour after centrifugation. All substrates (except sample 3) were boiled in distilled water, then centrifuged and autoclaved at 121°C for 2 hours. The weight of the substrates after centrifugation is shown in Table 1.

TABLE 1: Yields of the Methanol and Ethanol Extraction for Samples 1–7

Cultivation Substrate[a]	Weight after Centrifugation (g)	Extract	Yield (%)	
			Methanol	Ethanol
Corn	220	1	7.1	5.2
Rice	243	2	2.2	1.7
Rice (nutrient solution)	223	3	8.0	5.5
Rice (non-defatted soy flour)	243	4	4.8	2.3
Millet	220	5	8.3	4.0
Millet (low humidity)	163	6	7.6	**10.0**
Peas	203	7	**9.1**	5.2

The bold values represent the highest yields after extraction into alcohol solvents.
[a]We evaluated 100 g of each substrate.

C. Cultivation

After cooling to room temperature, the substrates were inoculated with 3 mL inoculation solution containing 3 g glucose, 3 g sucrose (Merck), 1 g soy peptone, 2.5 g yeast extract (Biolife Italiana s.r.l.), 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g $CaCl_2 \cdot 2H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.001 $CuSO_4 \cdot 5H_2O$ (Merck), 0.1 g thiamine hydrochloride (Sigma-Aldrich Co., St. Louis, MO), and 3 pieces (4 × 4 mm) of Cs-4 from an MYA culture in a Petri dish. The substrates were shaken (120 rpm) at 22°C for 7 days in 500-mL Erlenmeyer flasks containing 200 mL nutrient solution. The substrates were then incubated at 22°C (12-hour light/12-hour dark cycle) for 30 days. Specimens were dried at 40°C in an APT.line FED Series dryer (Binder GmbH, Tuttlingen, Germany) and ground with an SM-100 cutting mill (Retsch Co., Haan, Germany).

D. Chemicals

We used commercially available chemicals in the state in which they were purchased, without purification (Sigma-Aldrich, Mikro Chem). We used methanol, in its commercially available form (99.9%), to measure the UV/visible spectra.

E. Instruments

We used an IKA Works RV 10 Control Rotary Evaporator (VWR) to prepare the extracts. UV/visible spectra were measured on a Libra S12 spectrophotometer in a 1-cm cell at room temperature. We used a Nicolet 6700 Fourier transform infrared (IR) spectrophotometer (Thermo Scientific) to measure IR spectra. The spectra were measured with the attenuated total reflection method, with the Smart Orbit nozzle.

III. RESULTS AND DISCUSSION

Samples 1–7 were extracted in 2 solvents (99.0% methanol and 96.0% ethanol). Samples 1–7 (10 g of each) were separately mixed with 150 mL methanol and ethanol in a 250-mL round-bottom flask. This mixture was refluxed for 8 hours, then filtered and concentrated to dryness on a rotary evaporator. Table 1 shows the largest and smallest yields. The yields of the methanol extracts from samples 1–7 ranged from 2.2% to

9.1%. It was highest for sample 7 (9.1%), which was grown on peas, and the lowest for sample 2 (2.2%), which was cultured in rice. The yields of the ethanol extracts ranged from 1.7% to 10.0%. Sample 6, which was grown on millet, had the largest yield, and sample 2, grown on rice, had the smallest yield. The largest yield (10.0%) was reported for sample 6, grown on millet with lower humidity. Extracts were white, golden yellow, or brown and had a typical mushroom scent. They were of solid, semisolid, or oily consistency.

We used the DPPH radical method to determine antioxidant activity. We always used freshly prepared stock solution of stable DPPH radicals (0.2 mmol/L) when taking measurements. The DPPH radical solution showed the highest absorption at a wavelength of 517 nm, which was used for further measurements. As a blank we used a solution of 99.9% methanol (2 mL). We measured the absorbance of the DPPH solution, which was prepared by mixing 1 mL 0.2 mmol/L stock solution with 1 mL 99.9% methanol.

The method most commonly used to calculate antioxidant activity is the percentage reduction of the activity of a DPPH radical solution, determined by measuring absorbance at a maximal wavelength of 517 nm. Antioxidant activity was calculated as follows:

$$\text{Inhibition (\%)} = A_{BL} - A_{SMP}/A_{BL} \times 100$$

where inhibition refers to the inhibition of DPPH radicals, A_{BL} represents the absorbance of the blank DPPH solution, and A_{SMP} represents the absorbance of the sample after 30 minutes of incubation with the DPPH solution in the dark at room temperature. The results of the measurements are reported as the concentration of the substrate that caused a 50% loss of activity of DPPH radicals (IC_{50}^{DPPH}), as determined by linear extrapolation of functional dependence, Inhibition (%) = f (*Concentration*), in Microsoft Excel software. All measurements were calculated with concentrations of 6.25, 3.12, 1.56, 0.78, and 0.39 mg/mL.

To the samples was added 1 mL DPPH stock solution (0.2 mmol/L). After addition of the radicals, samples 1–7 were incubated in the dark at 25°C for 30 minutes. The subsequent decrease in absorbance of the samples at a maximal wavelength of 517 nm against the blank is equal to the decrease in free DPPH radicals in the samples. Tables 2 and 3 summarize the results of radical scavenging of the methanol and ethanol extracts of samples 1–7.

On the basis of the measured antioxidant activity of the methanol and ethanol extracts of sample 1, we found that the methanol extract traps radicals more effectively (89.9%) than the ethanol extract (45.3%) at the highest concentration (6.25 mg/mL). When we compared the IC_{50}^{DPPH} we found that the sample that was extracted with methanol, at a concentration of 2.45 mg/mL, has a 2.44-times higher ability to scavenge radicals than the ethanol extract. For sample 2, which was cultured on rice, the methanol extract (6.25 mg/mL) had higher antioxidant activity, at 86.9%. The IC_{50}^{DPPH} indicate a 1.13-times difference in favor of the methanol extract of DPPH. The methanol extract of sample 3, grown on rice with the addition of a nutrient solution, showed the opposite trend. A comparison of IC_{50}^{DPPH} showed that the ethanol extract exhibited only 1.24-times greater antioxidant activity, in contrast to the extracts of samples 1 and 2, in which case the methanol extract had higher antioxidant activity. Antioxidant activity of the ethanol extract was 75.8% at the largest concentration of 6.25 mg/mL. The IC_{50}^{DPPH} of extracts of the sample cultured on rice with the addition of non-defatted soy flour show that the methanol extract has a 1.14-times higher antioxidant activity than the ethanol extract.

The DPPH acceptor of radicals for the highest concentration of the methanol extract of sample 4 was 86.1%. Table 4 provides a comparison of 3 samples that were grown on the same substrate (rice); in the case of sample 3, however, a nutrient solution was added to the substrate, and to sample 4, non-defatted soy flour was added.

The IC_{50}^{DPPH} results presented in Table 4 show that the alcohol extracts of sample 4, obtained by cultivation on rice with the addition of soy flour, have the highest antioxidant activity (2.33 and 2.65 mg/mL).

TABLE 2: Percentage Antioxidant Activity and IC_{50}^{DPPH} of the Methanolic Extracts of *O. sinensis* Samples 1–7[a]

Samples	Absorbance[b]							Antioxidant Activity (%)						
	1	**2**	**3**	**4**	**5**	**6**	**7**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Concentration (mg/mL)														
0.39	0.797	0.675	0.836	0.744	0.837	0.838	0.849	10.1	23.9	5.7	16.2	5.7	5.5	4.3
0.78	0.684	0.756	0.762	0.598	0.773	0.775	0.834	22.8	14.8	14.1	32.5	12.9	12.6	6.0
1.56	0.449	0.506	0.699	0.365	0.671	0.670	0.817	49.4	42.9	21.2	58.9	24.3	24.5	7.9
3.13	0.192	0.262	0.508	0.166	0.433	0.586	0.714	89.0	70.5	42.8	81.3	51.2	34.0	19.5
6.25	0.088	0.116	0.297	0.123	0.209	0.281	0.655	**89.9**	86.9	66.5	86.1	76.5	68.4	26.2
IC_{50}^{DPPH} (mg/mL)[c]	—	—	—	—	—	—	—	2.45	2.78	4.37	2.33	3.74	4.46	11.50

[a]Values were determined with the use of the DPPH radical scavenging method after incubation in the dark at 25°C for 30 minutes.
[b]Values shown are the mean of 3 repeated measurements.
[c]Concentrations inhibiting 50% of DPPH radical activity (IC_{50}^{DPPH}) were determined using linear extrapolation of functional dependence in Microsoft Excel software: I (%) = *f* (*Concentration*).

TABLE 3: Percentage Antioxidant Activity and IC_{50}^{DPPH} of Ethanolic Extracts of *O. sinensis* Samples 1–7[a]

Samples	**Absorbance[b]**							**Antioxidant Activity (%)**						
	1	**2**	**3**	**4**	**5**	**6**	**7**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Concentration (mg/mL)														
0.39	0.848	0.769	0.826	0.801	0.883	0.881	0.970	4.4	13.3	6.9	9.7	0.4	0.7	0.2
0.78	0.844	0.736	0.716	0.625	0.811	0.858	0.910	4.8	17.0	19.3	29.6	8.6	3.2	6.4
1.56	0.833	0.583	0.577	0.456	0.669	0.805	0.823	6.1	34.3	34.9	48.6	24.5	9.2	15.3
3.13	0.487	0.302	0.400	0.229	0.494	0.613	0.613	45.1	65.9	54.9	74.1	44.3	30.9	37.0
6.25	0.485	0.163	0.215	0.121	0.399	0.437	0.382	**45.3**	**81.7**	**75.8**	**86.4**	**55.0**	**50.7**	60.7
IC_{50}^{DPPH} (mg/mL)[c]	—	—	—	—	—	—	—	5.99	3.14	3.52	2.65	4.98	5.96	4.95

[a]Values were determined with the use of the DPPH radical scavenging method after incubation in the dark at 25°C for 30 minutes.
[b]Values shown are the mean of 3 repeated measurements.
[c]Concentrations inhibiting 50% of DPPH radical activity (IC_{50}^{DPPH}) were determined using linear extrapolation of functional dependence in Microsoft Excel software: I (%) = *f* (*Concentration*).

TABLE 4: Comparison of IC_{50}^{DPPH} of Alcoholic Extracts of the Samples

Extracts	Sample 2		Sample 3		Sample 4	
	MeOH	**EtOH**	**MeOH**	**EtOH**	**MeOH**	**EtOH**
IC_{50} (mg/mL)	2.78	3.14	4.37	3.52	**2.33**	2.65

Extracts were obtained by cultivation on rice (sample 2), rice with a nutrient solution (sample 3), or rice with the addition of non-defatted soy flour (sample 4). IC_{50}^{DPPH}, concentration inhibiting 50% of DPPH radical activity.

Extracts of sample 2, grown on pure rice, had the next highest activity (2.78 and 3.14 mg/mL), and the lowest activity was recorded for extracts of sample 3, grown on rice with the addition of a nutrient solution (4.37 and 3.52 mg/mL). In general, the methanol extracts have much higher antioxidant activity than the ethanol extracts, except for sample 3, for which the ethanol extract showed higher activity.

The highest DPPH radical scavenging ability (2.33 mg/mL) was obtained for the methanol extract of sample 4 (cultured on rice with the addition of non-defatted soy flour), which is almost 1.87 times higher than the activity of the methanol extract of sample 3, which was the least active. It is interesting that the extracts of sample 3, obtained by culturing with a nutrient solution, exhibited the lowest activity of all samples, even when compared with the activity of the extracts from sample 2 (grown on pure rice). We believe that the addition of the nutrient solution, which contained a yeast, K_2HPO_4, glucose, and $MgSO_4$, inhibited the formation of substances that are involved in radical scavenging. The opposite trend was observed for sample 4, where the addition of soy flour significantly affected the ability of both alcohol extracts to scavenge DPPH radicals.

As shown in Tables 2 and 3, the methanol extract (at 6.25 mg/mL) of sample 5, grown on millet, has 21.5% higher antioxidant activity than the sample's ethanol extract. This was confirmed by an IC_{50}^{DPPH} that is 1.33 times lower than that of the methanol extract.

Lower humidity was used in the culture of sample 6. As for most of the other samples, the methanol extract was better able to scavenge DPPH radicals. At the highest concentration, this activity was higher by 17.7%, corresponding to an IC_{50}^{DPPH} of 4.46 mg/mL, which has 1.34 times higher antioxidant activity than the IC_{50}^{DPPH} of the ethanol extract. Table 5 shows a comparison of samples 5 (cultivated on millet) and 6 (cultivated on millet with lower humidity). The IC_{50}^{DPPH} shown in Table 5 indicate that the alcohol extracts of sample 5 have on average antioxidant activity that is 1.19 times higher than that of sample 6.

In general, we can say that the methanol extracts of the studied samples have higher antioxidant activity than the ethanol extracts. The methanol extract of sample 5, cultured on millet, had the highest DPPH radical scavenging ability (3.74 mg/mL). The ethanol extract of sample 6, cultured on millet with lower humidity, had the lowest activity. The difference between the most active extract (from sample 5) and the least active extract (from sample 6) is a factor of 1.59.

The ethanol extract of the sample cultivated on peas (sample 7) showed higher activity than the methanol extract at the highest concentration (6.25 mg/mL). The difference was as high as 34.5%. These results were confirmed by the IC_{50}^{DPPH}, which were 3.32 times higher in the case of the ethanol extract.

As shown in Tables 2 and 3, the IC_{50}^{DPPH} values were lower for methanol extracts than for ethanol ones. Extracts of sample 4 (2.33 mg/mL), cultured on rice with the addition of non-defatted soy flour, were the most effective. The least active were the extracts from sample 2, cultivated on pure rice. In all 3 cases the IC_{50}^{DPPH} does not exceed a value of 3. Other methanol extracts are characterized by a lower ability to scavenge radicals. The least active sample (11.50 mg/mL) was cultured on peas. The most active sample (no. 4) was 4.93 times more active than the least active sample.

TABLE 5: Comparison of IC_{50}^{DPPH} of Alcoholic Extracts

Extracts	Sample 5		Sample 6	
	Methanol	Ethanol	Methanol	Ethanol
IC_{50}^{DPPH} (mg/mL)	**3.74**	4.98	4.46	5.96

Extracts were obtained by cultivation on millet (sample 5) and millet with low humidity (sample 6). IC_{50}^{DPPH}, concentration inhibiting 50% of DPPH radical activity.

Analysis of the ethanol extracts (Table 3) showed a trend similar to that observed for the methanol extracts. The most active was the extract of sample 4, grown on rice with the addition of soy flour (2.65 mg/mL), followed by the extracts of sample 2, grown on rice without any additives; sample 3, cultured on rice with a nutrient solution; and sample 7, cultured on peas with lower moisture. The least active was the extract of sample 1, grown on corn. The difference in activity between the least active sample 1 and the most active sample 4 was 2.26-fold.

It is interesting to note that the opposite trend was observed for sample 3 (grown on rice with a nutrient solution) and sample 7 (grown on peas). The ethanol extract of sample 3 was 1.24 times more active than the sample's methanol extract. Antioxidant activity of the ethanol extract of sample 7 was 2.32 times higher than that of the sample's methanol extract. Based on the results shown in Table 2 and 3, we can arrange the antioxidant activity of the samples in the following order: methanol extract of sample 4 (rice and soy flour) > methanol extract of sample 1 (corn) > ethanol extract of sample 4 > methanol extract of sample 2 (rice) > ethanol extract of sample 2 > ethanol extract of sample 3 (rice with nutrient solution) > methanol extract of sample 5 (millet) > methanol extract of sample 3 > methanol extract of sample 6 (millet with lower humidity) > ethanol extract of sample 7 (peas) > ethanol extract of sample 5 > ethanol extract of sample 6 > ethanol extract of sample 1 > ethanol extract of sample 7.

These results show that the substrate significantly affects the amounts of substances with antioxidant activity during the culturing of *P. hepiali*. We assume that sample 4 (cultured on rice with the addition of soy flour), which had the highest activity, contains folate-dihydrofolate,[15,16] vitamin E,[15] and hydroxycinnamates (e.g., 6′-O-(*E*)-feruloylsucrose and 6′-O-(*E*)-*sinapoylsucrose),*[17] which are known antioxidants present in rice.

With the addition of soy flour to the substrate, other compounds in the substrate, such as phenolic substances, tannins (tannic acid), proanthocyanidins[18] (procyanidin A1), and isoflavones[19] (genistein, 2-tocopherols, β-carotenoids, ascorbic acid),[20] might penetrate the mushroom. These compounds could significantly contribute to the antioxidant activities of the given samples, which were higher than that of the sample cultivated only on pure rice (sample 3).

Wang and Daun[21] dealt with the chemical analysis of peas. Their results showed that the chemical compounds contained in peas that may be involved in the scavenging of DPPH radical are mainly ascorbic acid, α-tocopherol, β-tocopherol, and unsaturated fatty acids. Tannins and folic acid were not detected in peas, as opposed to rice and soy. We assume that the deficiency of these substances may have affected the antioxidant activity of sample 7. However, as shown by further studies of the chemical compounds in peas or their antioxidant ability, the decisive criterion is the pea variety. For example, in their work that described the antioxidant ability of peas, Nilsson et al.[22] identified that ascorbic acid was the main agent responsible for this effect. Amarowicz and Troszyńska[23] mention the presence of mainly polyphenols and tannins in peas. Because we did not deal with the aforementioned substances with antioxidant activities in the pea substrate used in our experiments, we can only assume that the pea substrate we used contained minimal amounts of these compounds, as opposed to soy.

All methanol and ethanol extracts, in the form of solutions, were stored in a refrigerator at 8°C. Some extracts began to crystallize during storage. We were able to filter from 6 crystallized extracts an amount necessary to measure IR spectra. Methanol extracts (from 5 samples) crystallized well, but we obtained only one crystallized sample of an ethanol extract. The following extracts crystallized as white powders to pale yellow crystals: the methanol extracts of samples 1, 3, 5, 6, and 7, and the ethanol extract of sample 6. IR spectra were measured with attenuated total reflection on a Thermo Scientific Nicolet 6700 Fourier transform IR spectrophotometer with a Smart Orbit extension. The crude evaporated extract was transferred to the Smart Orbit measuring point and the IR spectra were measured. Individual samples were not modified before being measured.

Carboxylic acids are expressed in the IR spectrum by absorption bands originating from several types of vibrations. Valence vibrations of the carboxylate group appear in the spectra as 2 typical bands that originate from symmetrical and asymmetrical vibrations.[24,25] The asymmetrical vibration of the carboxyl group usually occurs in the region of 1550–1610 cm^{-1}, whereas the band resulting from symmetrical vibration appears in the range 1335–1420 cm^{-1}. For all of our samples, we observed the presence of the aforementioned absorbance bands.

For sample 1, the asymmetrical vibration of the carboxyl group was observed at 1578 cm^{-1}, whereas the symmetrical vibration of the carboxyl group was at 1406 cm^{-1}. In the spectrum of sample 3, bands were present at 1457 and 1371 cm^{-1}. The wavelengths of sample 5 were observed at 1513 and 1405 cm^{-1}. Shifts in wavelength values may be due to the formation of intramolecular hydrogen bonds.

In a similar way, the IR spectrum of the ethanol extract of sample 6 showed bands resulting from asymmetrical vibration of the carboxylate group at 1578 cm^{-1} and from symmetrical vibration at 1406 cm^{-1}. For the methanol extract of sample 6, the given vibrations were observed at 1578 and 1406 cm^{-1}. Sample 7, produced on peas, showed the presence of bands at 1578 and 1406 cm^{-1}. In these cases we also consider that shifts in wavelength values may again be due to the existence of intramolecular hydrogen bonds.

Further evidence of a carboxyl group in all samples is the presence of a wide band in the range of 3300 to 2500 cm^{-1}, associated with valence vibrations of the -OH group. A wide band at the given interval was observed for all the samples.

In the IR spectra of all the samples that crystallized, other distinctive bands were observed for amino group $-NH_2$. In the case of the primary $-NH_2$ group, a bond scissor vibration at 1604 cm^{-1} and a valence band at 3066 cm^{-1} were observed for sample 1; in the range of 650–900 cm^{-1} there was a wide pendulum vibration band characteristic of the primary $-NH_2$ group. A valence vibration occurred around 3380 cm^{-1} for the primary $-NH_2$ group in sample 3; another band appeared at 1637 cm^{-1}, confirming the presence of this functional group, and finally a wide band appeared in the range of 650 to 900 cm^{-1}. For sample 5, bands were found at 3409, 3207, 1577, and 650–900 cm^{-1}, again demonstrating the presence of a primary amino group. In sample 6 we observed bands that were characteristic of the primary amino group; both IR spectra contained similarly intensive bands at about equal wavelengths. For the sample crystallized from methanol we observed vibrations at 3367 and 3062 cm^{-1}, 1604 cm^{-1}, and again a wide band overlaid by others in the range of 650 to 900 cm^{-1}. The IR spectrum of the ethanol extract contained intensive bands at 3357, 3197, and 1605 cm^{-1} and a wide band overlapping with others in the range of 650 to 900 cm^{-1}. For sample 7, the primary amino group was indicated by the presence of bands at 3357, 3195, and 1605 cm^{-1}, and by a broad band in the range of 650–900 cm^{-1}.

These results show that methanol extracts have higher antioxidant activity than ethanol extracts. The most suitable substrate for cultivation was rice with the addition of non-defatted soy flour. *P. hepiali* cultivated this way contained the most substances with antioxidant properties. The most effective extract in scavenging stable DPPH radicals (IC_{50}^{DPPH} = 2.33 mg/mL) was the methanol extract of sample 4, cultivated on rice with the addition of non-defatted soy flour. Less effective was the methanol extract of sample 7,

cultivated on peas (IC_{50}^{DPPH} = 11.50 mg/mL). On the basis of detailed IR spectral analysis and previous experience, we can assume that crystallization resulted in the exclusion of amino acid mixtures from the samples described above. Also, we suppose these are amino acids based on work by Bedlovičová et al.,[26] who isolated from methanol, through the use of crystallization, a mixture of amino acids and confirmed their chemical structure with 1- and 2-dimensional nuclear magnetic resonance spectroscopy. The following amino acids are present in the composition: phenylalanine, valine, alanine, leucine, and isoleucine. The presence of amino acids was demonstrated by 2 spectral methods: an IR method in this work and nuclear magnetic resoance in a previouly published work.[26] Based on recent and continuing research, we conclude that oleic acid[10] and linoleic acid are present as the major antioxidant substances in species in the *Paecilomyces* and *Ophiocordyceps* genera.

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