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Free-Radical Scavenging Activities of Cultured Mycelia of *Paecilomyces hepiali* (Ascomycetes) Extracts and Structural Characterization of Bioactive Components by Nuclear Magnetic Resonance Spectroscopy

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ABSTRACT: Current research is focused on testing the cultivation of *Paecilomyces hepiali* mycelia on various plant substrates and producing fungus or mycelial biomass with qualitatively interesting substances. *P. hepiali* mycelia was cultivated using solid-state fermentation of different substrates. Mycelial biomass was then analyzed, and antioxidant activity was evaluated using the DPPH radical scavenging method for different ethanolic extracts based on a millet substrate (extract 1) or a chickpea substrate (extract 2). Extract 1 corresponds to a half-maximal DPPH radical inhibitory concentration of 1.73 mg/mL; the inhibitory concentration of ethanol extract 2 was almost 4.5 times higher at 7.92 mg/mL. Extracts 1 and 2 were separated into fractions by column chromatography and the chemical structures were determined for the substances that formed the most effective fraction of sample 1. The chemical structures of all compounds in the most active fraction of sample 1 were analyzed by ^{1}H, ^{13}C, distortionless enhancement by polarization transfer, correlation spectroscopy, heteronuclear single-quantum correlation spectroscopy, and heteronuclear multiple-bond correlation spectra.

KEY WORDS: 1- and 2-dimensional NMR spectroscopy, cultivation, free radical scavenging activity, DPPH radical, medicinal mushroom and fungi, *Paecilomyces hepiali*

ABBREVIATIONS: COSY, correlation spectroscopy; **DPPH**, 1,1-diphenyl-2-picrylhydrazyl; **HMBC**, heteronuclear multiple-bond correlation; **HSQC**, heteronuclear single-quantum correlation spectroscopy; $\mathbf{IC_{50}}^{\mathbf{DPPH}}$, concentration of substrate that decreases DPPH radical levels by 50%; **MYA**, malt extract yeast agar; **NMR**, nuclear magnetic resonance; R_f, retention factor

I. INTRODUCTION

Recent scientific research has focused on the study of natural materials such as plants and mushrooms. This is interesting from a chemical point of view because natural substances contain various biologically active compounds such as polysaccharides, which are strong antioxidant and antitumor agents and also positively regulate the immune system. Highly prized is the Chinese caterpillar mushroom, *Ophiocordyceps sinensis* (Berk.) G. H. Sung et al. (=*Cordyceps sinensis*), which is known in traditional Chinese medicine.[1–3]

Because of its rarity and medicinal benefits, the fruiting bodies are in demand not only in China. Uncontrolled, excessive, and aggressive mushroom picking on the extensive Tibetan Plateau, in Nepal, and in Bhutan caused devastation and the breakdown of the ecological equilibrium of that region. Since then, intensive research has focused on cultivating *Cordyceps*. *Cordyceps* was first successfully cultivated in 1972. The first 3 strains, named Cs-1, Cs-2,

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and Cs-3, did not have the fast growth characteristics that make commercial cultivation practical. It was the fourth culture, named Cs-4, that was hardy and grew quickly. Therefore this Cs-4 strain was chosen for commercial production. It was tested for its medical potential and, based on its potency and safety, was approved in China in 1988 as a medicine under the name *Jin Shui Bao*.[3,4]

Cs-4 has been extensively studied in China, including industrial fermentation, chemical composition, toxicity, and therapeutic functions.[5,6] 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 known as *Paecilomyces hepiali* Q. T. Chen & R. Q. Dai (Trichocomaceae, Eurotiales, Ascomycetes). *P. hepiali* was described based on the Cs-4 strain isolated from natural Chinese *Cordyceps*.[7,8]

The results of previous studies compared the chemical components and pharmacological activity of wild *O. sinensis* and *P. hepialid*, showing that *P. hepiali* has a larger total amount of 7 essential amino acids (leucine, lysine, methionine, phenylalanine, tryptophan, tyrosine, and valine) than *O. sinensis*. The total amount of dietary elements analyzed (potassium, magnesium, zinc, copper, manganese, and selenium) was also larger in *P. hepiali*. The total amount of 4 vitamins (B_1, B_2, B_6, and E) was almost equal in both fungi. *P. hepiali* contained more protein, lipid, ash, carbohydrate, ergosterol, and mannitol than *O. sinensis*. The hot water extract of *P. hepiali* has been shown to have a number of pharmacological effects, including (1) inhibition of aggregation of human platelets, (2) inhibition of *IL-8* gene expression, (3) antimutagenic activity, (4) inhibition of the production of melanin, and (5) improvement of the texture of human skin.[9]

The mycelial powder of *P. hepiali* has been intensively studied and developed into a functional food in China. Polysaccharides, adenosine, and cordycepin in the mycelial powder of *P. hepiali* are considered to be the major functional components having health effects.[10] Studies have shown that *P. hepiali* can inhibit tumor proliferation, invasion, metastasis, and neovascularization; induce apoptosis; reverse drug resistance; enhance immunity; and protect hepatic function.[6]

Although there is not a long tradition of *P. hepiali* cultivation, many new methods of cultivation have been developed since the isolation of the first production strain 40 years ago. Within the framework of our project, fungal biomass has been produced using solid-state fermentation under precisely defined conditions without free water. The use of a solid substrate is closer to the growth conditions in the natural environment; it also allows the composition of the substrate to be modified in order to change the amounts of biologically active compounds in the final product and eliminate the loss of extracellular metabolites into the surrounding medium.

We report here the ethanolic extraction and chromatographic separation of biologically interesting organic compounds from cultured *P. hepiali* mycelia, the DPPH radical scavenging activity of total and fractionated extracts, and the elucidation of the chemical structure of isolated compounds by 1- and 2-dimensional nuclear magnetic resonance (NMR) spectroscopy.

II. MATERIALS AND METHODS

A. Mushroom Material

P. hepiali (Cs-4), provided by Aloha Medicinals Inc. (Carson City, NV), was maintained at 4°C on potato dextrose agar, malt extract yeast agar (MYA), and malt extract peptone insect agar by Mykoforest Company (Velčice, Slovakia) under accession number MFTCCB022/022013. Cultures were cultivated on Petri dishes of MYA medium, containing (per liter of distilled water) 20 g malt extract, 2 g yeast extract, and 20 g agar (Biolife Italiana s.r.l., Milan, Italy) at 22°C for 14 days in the dark.

B. Substrate Preparation

Two different substrates, millet (*Panicum miliaceum*) and chickpea (*Cicer arietinum*), were used to grow mycelia of *P. hepiali*. Millet (100 g dry weight) was boiled in a nutrient solution consisting of (per liter of distilled water) 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 (Merck, Bratislava, Slovakia) for 30 minutes. After centrifuging, the millet (208 g) was autoclaved at 121°C for 2 hours. Dry chickpeas (100 g) were boiled in distilled water for 30 minutes, then centrifuged and autoclaved at 121°C for 2 hours.

C. Cultivation

After cooling to room temperature, the substrates were inoculated with 3 mL of 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 g $CuSO_4 \cdot 5H_2O$ (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. Cultivation was carried out at 22°C for 7 days in shaken (120 rpm) 500-mL Erlenmeyer flasks containing 200 mL of nutrient solution. The substrate was then placed inside the incubator for 30 days at 22°C (12-hour light/12-hour dark cycle). Specimens were dried at 40°C using an APT.Line FED Series dryer (Binder GmbH, Tuttlingen, Germany) and ground using an SM-100 cutting mill (Retsch Co., Haan, Germany).

D. Chemicals

Chemicals and solvents for measuring ultraviolet/visible spectra were from commercial sources (Sigma-Aldrich, MikroChem) and used without further purification. Compounds in extracts were monitored using thin-layer chromatography with an aluminum TLC Silica gel 60 system (EMD Millipore) with F254 fluorescent indicator, and spots were visualized with iodine vapor. Silica gel 60 (0.040–0.063 mm; CAS no. 7631-86-9, EC no. 231-545-4; Merck) was used for column chromatography (230–400 mesh ASTM).

E. Instruments

^1H and ^{13}C NMR spectra (parts per million) were measured on a Varian NMR Mercury Plus system (400 MHz) at room temperature in deuterated dimethyl sulfoxide using tetramethylsilane as the internal standard (δ = 0.00 ppm). The following data obtained were obtained: the chemical shift in the δ-scale, multiplicity of signals, integrated intensity, and coupling constant J (Hertz). Proton and carbon assignments were based on analysis of ^1H, ^{13}C, correlation spectroscopy (COSY), heteronuclear single-quantum correlation spectroscopy (HSQC), heteronuclear multiple-bond correlation (HMBC), and distortionless enhancement by polarization transfer spectra. Ultraviolet/visible spectra were measured using 1-cm cells at room temperature in methanol (99.9%) with a Libra S12 spectrophotometer.

III. RESULTS AND DISCUSSION

Ten grams each of samples 1 (cultured on millet) and 2 (cultured on chickpea) were suspended separately in 150 mL ethanol in 250-mL round-bottom flasks and refluxed for 3 hours. After filtration, filtrates were evaporated to dryness in a rotary evaporator. Yields were 8.66% (sample 1) and 14.95% (sample 2). Although visually distinguishable from each other, both extracts were oily, with a golden yellow color, and had a distinctive mushroom smell. Antioxidant activity was measured by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method at 517 nm using a freshly prepared 0.2 mmol/L solution of stable DPPH radicals. The blank solution was 99.9% methanol (2 mL). In contrast to this solution, absorbance of the DPPH radical solution, prepared in a cuvette by mixing 1 mL of 0.2 mmol/L stock solution with 1 mL of 99.9% methanol, was measured. Antioxidant activity was calculated from the percentage reduction in the absorbance at λ_{max} 517 nm using the equation:

$$I\,(\%) = (A_{BL} - A_{SMP}/A_{BL}) \times 100,$$

where I represents the percentage inhibition of DPPH radicals, A_{BL} is the marked absorbance of the blank DPPH radical solution, and A_{SMP} is the absorbance of the sample measured after 30 minutes' incubation in the dark at room temperature after adding the DPPH radical solution. For easier comparison, measurements are denoted as IC_{50}^{DPPH}

TABLE 1: Antioxidant Activity of *Paecilomyces hepiali* Ethanolic Extracts (Samples 1 and 2), Determined by the DPPH Radical Scavenging Method after 30 Minutes' Incubation in the Dark at 25°C

Concentration (mg/mL)	Absorbance*		Antioxidant Activity (%)	
	Sample 1	Sample 2	Sample 1	Sample 2
12.50	**0.330**	**0.272**	73.8	**76.0**
6.25	**0.228**	**0.700**	**81.9**	38.3
3.13	0.260	0.812	79.3	28.5
1.56	0.587	0.980	53.3	13.7
0.78	0.904	1.027	28.1	9.5
0.39	1.013	1.099	19.5	3.2
0.19	1.150	1.117	**8.6**	**1.6**
0.10	1.145	**1.120**	8.9	**1.3**
IC_{50}^{DPPH} (mg/mL)†			**1.73**	7.92

The bold value 81.9% represents the highest antioxidant activity at a concentration of 6.25 mg/mL for sample 1. The bold value 76.0% represents the highest antioxidant activity at a concentration of 12.50 mg/mL for sample 2. The bold value 1.73 represents the fact that sample 1 is more effective than sample 2.

*Average absorbance is based on 3 replicate measurements.

†Determined using measurement data of the linear extrapolation of functional dependence $I(\%) = f(\text{concentration})$ in Microsoft Excel software (Figs. 3 and 4).

values (concentration of substrate that decreases DPPH radical levels by 50%). Antioxidant activity was determined using total extracts and separated fractions. All measurements (total ethanol extracts of samples 1 and 2, and their fractions) were carried out over a concentration range of 0.10–12.50 mg/mL. Prepared stock solution (1 mL) with a DPPH radical concentration of 0.2 mmol/L was gradually added to the diluted sample. After adding the radicals, samples were incubated in the dark at room temperature (25°C) for 30 minutes. The decrease in absorbance at 517 nm against the blank is equal to the percentage decrease in free DPPH radicals in the samples. The uptake of stable radicals in total extracts of samples 1 and 2 is summarized in Table 1.

Data in Table 1 show that sample 1, at a concentration of 6.25 mg/mL, exhibited the highest antioxidant activity (81.9%), although less activity was recorded at higher concentrations (12.5 mg/mL). This deviation from the observed trend may be due to the poor solubility of sample 1 at higher concentrations. After comparison of IC_{50}^{DPPH} values, samples cultured on millet were 4.5 times more active (IC_{50}^{DPPH} = 1.73 mg/mL) than those cultured on chickpea.

To determine which chemical compounds were most involved in the antioxidant activity, total ethanol extracts of samples 1 and 2 were fractionated by column chromatography on silica gel using chloroform as the mobile phase. In both cases, highly polar substances were eluted when the column was washed with methanol. Sample 1 was divided into 4 fractions (each faction was a mixture of about 2–3 substances with different retention factor [R_f] values). Total extract of sample 2 was separated into 3 fractions (in this case, each fraction consisted of a mixture of about 1–3 compounds with different R_f values; Table 2).

Figure 1 shows the antioxidant activity of the individual fractions of sample 1 using the same method adopted for the total ethanolic extracts. Fraction I exhibited virtually no radical scavenging activity, whereas fraction II had only moderate antioxidant activity at the highest concentrations. Fraction III exhibited relatively high antioxidant

TABLE 2: Fractionation of the Total Extract Samples 1 and 2 by Column Chromatography Based on Retention Factor Values Using Chloroform as the Mobile Phase

Fraction	Retention Factors	
	Sample 1	Sample 2
I	**0.431, 0.275**	**0.532, 0.539, 0.851**
II	0.137, 0.113	**0.279**
III	**0.121, 0.151, 0.094**	0*
IV	0*	—

*Fractions IV (sample 1) and III (sample 2) were obtained from the beginning of column washing with methanol.

activity, but fraction IV, obtained by initially washing the column with methanol, was the most effective (70.4% at a 12.5 mg/mL concentration). IC_{50}^{DPPH} values of fractions III and IV, determined from the linear extrapolation function of I (%) = f (concentration) of the trend line using Microsoft Excel software (Fig. 2), were 22.19 and 7.72 mg/mL, respectively. IC_{50}^{DPPH} values of fractions I and II were 509.94 and 102.73 mg/mL, respectively. Comparison of the measured and calculated inhibitory values revealed that the total sample 1 had higher antioxidant activity than the individual fractions (1.73 mg/mL; Table 1).

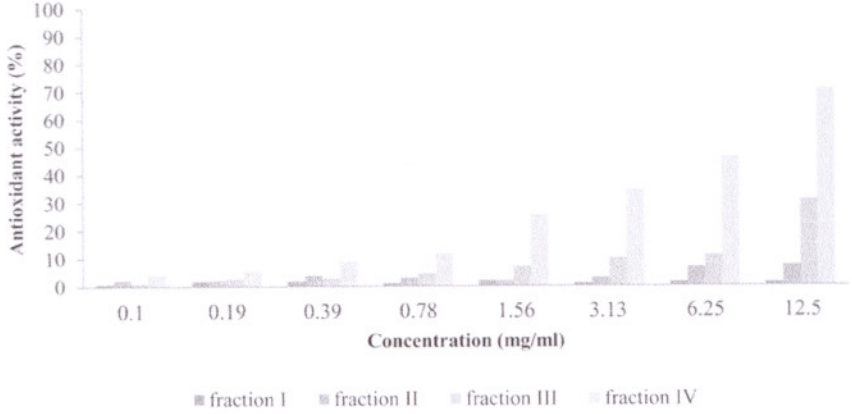

FIG. 1: Antioxidant activity of individual fractions of *Paecilomyces hepiali* sample 1.

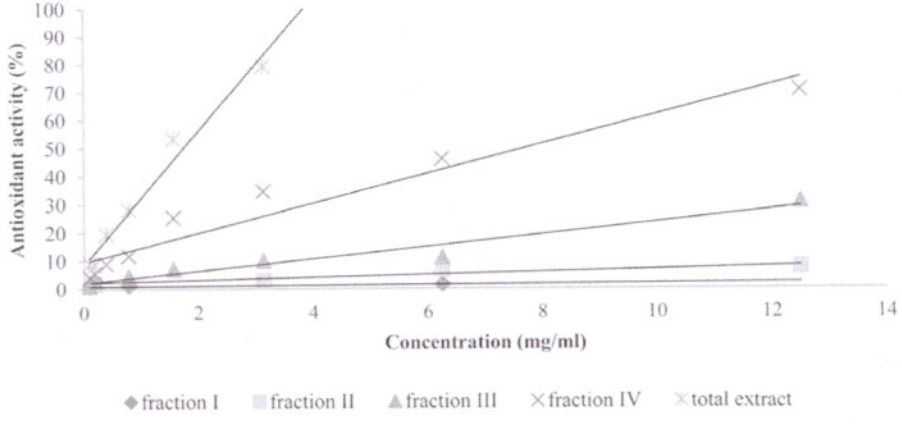

FIG. 2: Comparison of the antioxidant activity of the total extract of sample 1 with the 4 fractions obtained by column chromatography. The half-maximal DPPH radical inhibitory concentrations for the total extract and all fractions were determined from a linear extrapolation function I (%) = f (concentration) of the trend line using Microsoft Excel software.

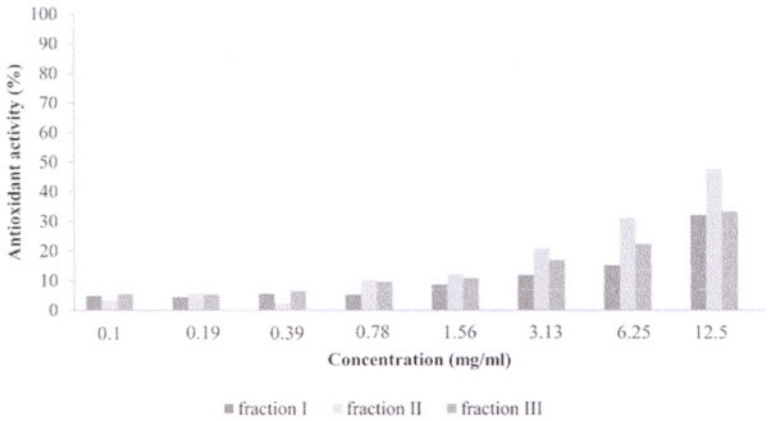

FIG. 3: Antioxidant activity of individual fractions of *Paecilomyces hepiali* sample 2.

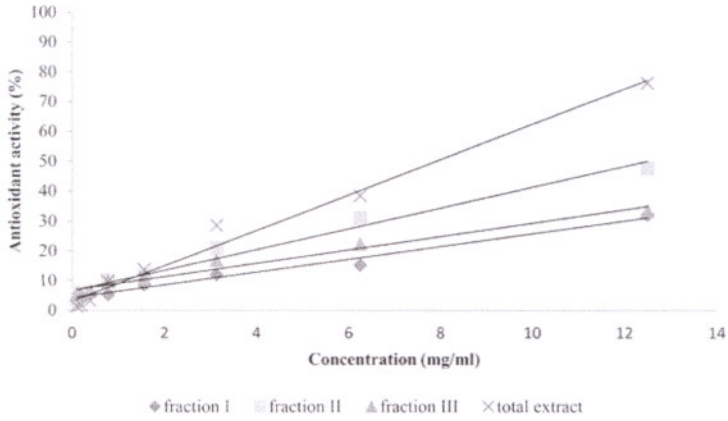

FIG. 4: Comparison of the antioxidant activity of the total extract of sample 2 with 3 fractions obtained by column chromatography. The half-maximal DPPH radical inhibitory concentrations for the total extract and all fractions were determined from a linear extrapolation function I (%) = f (concentration) of the trend line using Microsoft Excel software.

A similar pattern was observed for fractions derived from sample 2, grown on chickpea (Fig. 3). Here, the highest antioxidant activity was recorded for fraction II (47.5% at 12.5 mg/mL; IC_{50}^{DPPH} = 12.55 mg/mL); fraction III was less effective and fraction I the least effective of the 3. After comparison of the results, it is obvious that the concentrations inhibit radicals at 50%. Figure 4 shows the antioxidant activity of the individual fractions of sample 2; 3 fractions obtained by column chromatography. As in the case of sample 1, the total sample 2 exhibited higher antioxidant activity than the individual fractions.

Comparison of the fractionated extracts of samples 1 and 2 shows that the sample produced on millet and its most polar fraction (fraction IV) exhibited the highest antioxidant activity (70.4% at 12.5 mg/mL) compared with the most effective fraction of sample 2 (fraction II; 47.5% at 12.5 mg/mL), containing only one chemical substance with a different R_f value and having a significantly lower polarity (fraction II of sample 2 was obtained as the chloroform fraction). Interestingly, in the case of sample 2, the IC_{50}^{DPPH} values of fractions I–III fell within the range of 12.55–21.41 mg/mL. For fractions derived from sample 1, the

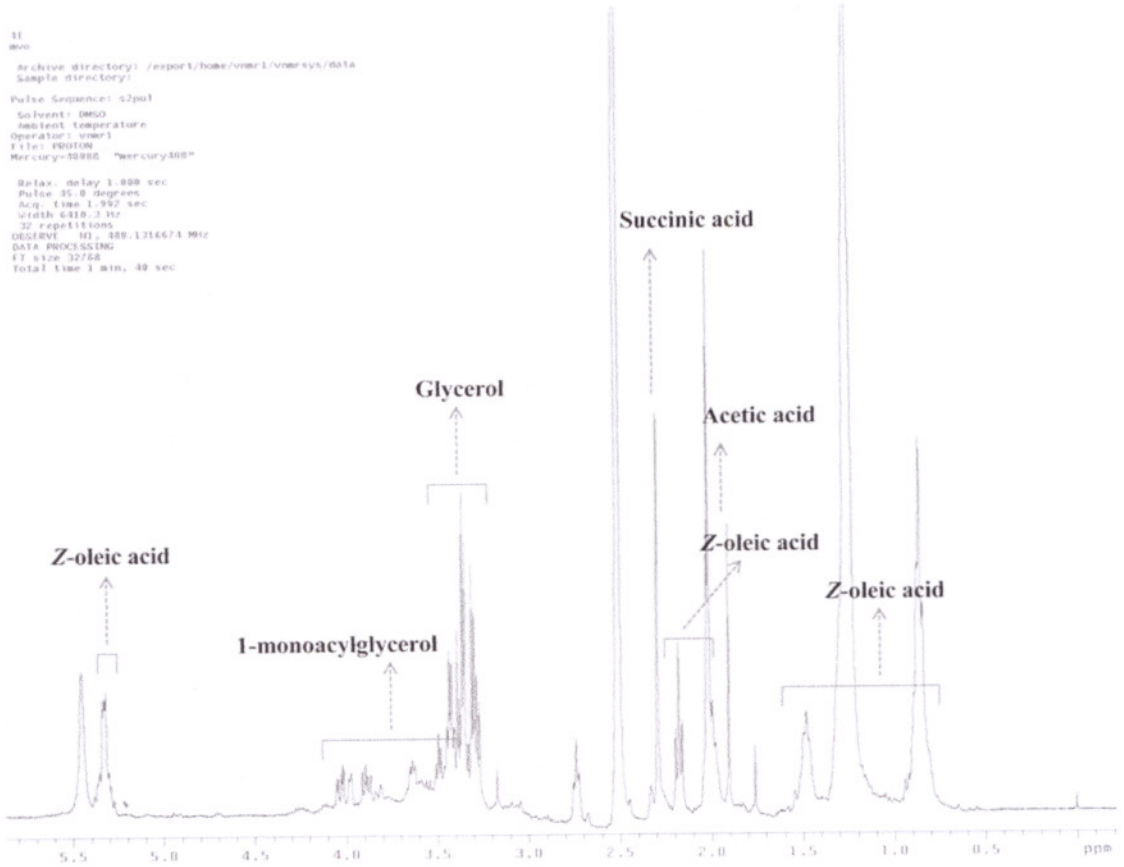

FIG. 5: ^{1}H nuclear magnetic resonance spectrum (400 MHz) of *Paecilomyces hepiali* ethanolic extract fraction IV from sample 1 measured in deuterated dimethyl sulfoxide.

IC_{50}^{DPPH} value of fraction I indicated that this fraction was practically inactive (509.94 mg/mL), and the maximum concentration (12.5 mg/mL) of fraction II also exhibited minimal antioxidant activity (7.3%). This observation shows that samples 1 and 2 are significantly different and contain chemical substances with different polarities, each of which contributes to the free radical scavenging ability to varying degrees. A common feature of the 2 samples is that the antioxidant activity of a total extract is higher than the highest activity of any fraction. This may be caused by the synergistic effect of all the components in unfractionated ethanol extracts.

The structures of the components of the most effective fraction of sample 1 (fraction IV) were determined by NMR spectroscopy (1-dimensional ^{1}H and ^{13}C NMR spectroscopy; 2-dimensional COSY, HSQC, and HMBC). Figure 5 shows the proton spectrum of fraction IV of sample 1.

COSY spectra were analyzed in detail to determine the individual spin systems of all 5 chemical compounds, followed by HSQC spectrum analysis, where the relevant signals of protons are assigned to carbon atoms on the basis of the transfer of proton magnetization to carbons atoms through a single bond. The signals of the protons bearing carbon atoms were assigned from the ^{13}C NMR spectra. Finally, HMBC spectra were analyzed; in this case the transfer of the magnetization of the protons to the carbon atoms through 3 bonds were assigned

TABLE 3: Assignment of Proton Signals in the ^{1}H Nuclear Magnetic Resonance Spectrum of the Fraction IV of Sample 1

Compound	Chemical Shift, parts per million (Multiplicity, Number of Protons, *J*)
Glycerol	3.43 (qi, 1H, H-2, $^3J_{H2\text{-}H1A} = {}^3J_{H2\text{-}H1B} = {}^3J_{H2\text{-}H3A} = {}^3J_{H2\text{-}H3B}$ = 3.6 Hz) 3.36 (dd, 2H, H-1_A,3_A, $^2J_{H1A\text{-}H1B}$ = 7.2 Hz, $^3J_{H1A\text{-}H2}$ = 3.6 Hz, $^2J_{H3A\text{-}H3B}$ = 7.2 Hz, $^3J_{H3A\text{-}H2}$ = 3.6 Hz) 3.29 (dd, 2H, H-1_B,3_B, $^2J_{H1B\text{-}H1A}$ = 7.2 Hz, $^3J_{H1B\text{-}H2}$ = 3.6 Hz, $^2J_{H3B\text{-}H3A}$ = 7.2 Hz, $^3J_{H3B\text{-}H2}$ = 3.6 Hz)
Z-oleic acid	5.32 (m, 1H, H-10) 5.31 (m, 1H, H-9) 2.17 (t, 2H, H-2,$^3J_{H2\text{-}H3}$ = 4.8 Hz) 2.00–1.98 (m, 4H, H-8,11) 1.46 (qi, 2H, H-3, $^3J_{H3\text{-}H2} = {}^3J_{H3\text{-}H4}$ = 4.8 Hz) 1.33–1.24 (m, 20H, H-4,5,6,7,12,13,14,15,16,17) 0.85 (t, 3H, H-18, $^3J_{H18\text{-}H17}$ = 2.8 Hz)
1-Monoacyl-glycerol	4.03 (dd, 1H, H-1_A,$^2J_{H1A\text{-}H1B}$ = 7.2 Hz, $^3J_{H1A\text{-}H2}$ = 2.4 Hz) 3.88 (dd, 1H, H-1_B,$^2J_{H1B\text{-}H1A}$ = 7.2 Hz, $^3J_{H1B\text{-}H2}$ = 4.4 Hz) 3.64 (m, 1H, H-2) 3.42 (dd, 1H, H-3_A, $^2J_{H3A\text{-}H3B}$ = 7.2 Hz, $^3J_{H3A\text{-}H2}$ = 3.6 Hz) 3.36 (dd, 1H, H-3_B, $^2J_{H3B\text{-}H3A}$ = 7.2 Hz, $^3J_{H3B\text{-}H2}$ = 3.6 Hz) 2.02 (s, 3H, CH_3)
Succinic acid	2.30 (s, 4H, CH_2)
Acetic acid	1.90 (s, 3H, CH_3)

dd, doublet of doublets; *J*, interaction constant; m, multiplet; qi, quintet; s, singlet; t, triplet.

a quaternary carbon atom in acetic, succinic, and *Z*-oleic acids. This also reaffirmed the correct assignment of proton and carbon signals in each of the identified substances (Table 3).

Based on the integrated intensity of the protons signals at δ/parts per million, which were sufficiently distinct, the percentage of each substance in the measured mixture was determined. In the case of glycerol, the integrated signal was a signal of protons H-1,3 at 3.36 ppm; for *Z*-oleic acid it was a triplet signal at 2.17 ppm; a doublet of doublets at 4.03 ppm for 1-monoacylglycerol; a singlet signal at 2.30 ppm for succinic acid, and the same singlet signal at 1.90 ppm for acetic acid. The identified chemical compounds were glycerol (38.8%), *Z*-oleic acid (22.4%), 1-monoacylglycerol (18.6%), succinic acid (10.8%), and acetic acid (9.4%).

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