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Optimization of *Phellinus hartigii* extracts: Biological activities, and phenolic content analysis

Orhan Ünal[1], Ayşenur Gürgen[2], Tetiana Krupodorova[3*], Mustafa Sevindik[4], Şanlı Kabaktepe[5] and Ilgaz Akata[6]

Abstract

Background Medicinal mushrooms are sources of natural substances with diverse biological functions. The study evaluated the biological activity of *Phellinus hartigii* (Allesch. & Schnabl) Pat. and optimized extraction conditions to the maximize its bioactive potential.

Methods Extraction was performed using a Soxhlet apparatus under varying conditions: temperatures (30, 50, and 70 °C), durations (1, 5.5, and 10 h), and ethanol/water ratios (0%, 50%, and 100%). Total antioxidant status (TAS) was analyzed across 17 experiments, and the optimal conditions were identified using response surface methodology (RSM). Extracts from optimal conditions were further analyzed for antioxidant capacity (Rel assay kits, DPPH, FRAP), anticholinesterase activity (acetyl- and butyrylcholinesterase inhibition), antiproliferative activity (A549 lung cancer cell line), total phenolic content (Folin-Ciocalteu method), and phenolic compound profile (LC–MS/MS).

Results Optimal extraction conditions were determined to be 48.22 ˚C, 9.04 h, and an ethanol/water ratio of 52.22%. The extract exhibited significant antiproliferative effects against the A549 lung cancer cells, with activity increasing in a concentration-dependent manner. The inhibition values (IC_{50}) of acetylcholinesterase and butyrylcholinesterase were 21.29 ± 0.41 and 35.51 ± 0.53 µg/mL, respectively. The TPC (total phenolic content) value of the optimized extract was determined as 88.21 ± 1.50 mg/g, FRAP value as 137.81 ± 1.72 mg/g, DPPH value as 106.07 ± 2.44 mg/g, TOS (total oxidant status) value as 9.27 ± 0.06 µmol/L, TAS value as 4.98 ± 0.03 mmol/L and OSI (oxidative stress index) value as 0.19 ± 0.002. LC–MS/MS analysis identified nine phenolic compounds, with gallic acid and catechin hydrate as the most abundant.

Conclusions The extract of *P. hartigii* obtained under optimal conditions demonstrated substantial antioxidant, anticholinesterase, and antiproliferative activities, highlighting its therapeutic potential.

Keywords Antialzheimer, Anticancer, Antioxidant, *Phellinus hartigii*, Phenolic, Response surface method

*Correspondence:
Tetiana Krupodorova
krupodorova@gmail.com
Full list of author information is available at the end of the article

Introduction

People have used different natural products in the natural ecosystem for different purposes such as nutrition, heating, shelter, self-defense or fighting diseases [1]. Among these natural products, mushrooms are one of the most commonly used. They encompass various nutritional components, including vitamins, minerals, and proteins [2]. These features render them essential components of human dietary lists. Mushrooms possess significant medicinal properties in addition to their nutritional benefits. Numerous studies indicate that mushrooms exhibit various activities, including antioxidant, anticancer, antimicrobial, anti-inflammatory, hepatoprotective, antiaging, antiallergic, and DNA protective properties [3–7]. In this context, evaluation of the biological activities of mushrooms is important for their potential applications. In this context, in our study, biological activities such as antioxidant, anticholinesterase and antiproliferative of optimized extracts of *Phellinus hartigii* (Allesch. & Schnabl) Pat. were determined. Also, the A549 lung cancer cell line was selected for the antiproliferative activity study because this cell line represents a human lung adenocarcinoma model and is a widely used in vitro system in lung cancer research [8]. The A549 cell line is considered a powerful tool for studying the molecular biology of lung cancer and this study aimed to evaluate potential biological activities against lung cancer.

The genus *Phellinus* (Hymenochaetaceae) causes white rot in woody plants. The fruiting bodies found on the wood are erect, stemless and perennial. Fruiting bodies are hard, woody and brown in color. *Phellinus* species produce many natural chemicals such as hispidin and phelligridin. The fruiting bodies of *Phellinus* species are burned and the smoke released is inhaled by those suffering from sore throat. Scrapings taken from slightly charred fruit bodies have been reported to be drunk with water to treat cough, sore throat, fever, and diarrhea [9, 10]. Based on current knowledge, the pharmacological significance and medicinally important species of *Phellinus* have been comprehensively summarized in several reviews [11–13]. Among the most widely used species with health-promoting effects are *Phellinus igniarius, P. baumii, P. lonicerinus, P. gilvus, P. ribis, P. pini, P. tremulae, P. linteus, P. tuberculosus, P. rhabarbarinus, P. merrillii, P. pomaceus,* and *P. vaninii* [11, 14–17]. In contrast, the current understanding of other *Phellinus* species, such as *P. badius, P. fastuosus, P. grenadensis* [18], *P. torulosus* [19, 20], and *P. hartigii* [21, 22] remains episodic and fragmentary. Comprehensive studies of mushrooms, including the search for novel biologically active compounds, remain highly relevant. Such efforts could significantly contribute to the discovery of new natural drugs for modern diseases and to alleviating the course of existing conditions. Therefore, the aim of this study was to investigate the antioxidant, antiproliferative and anticholinesterase activities of *P. hartigii*.

Materials and methods

Samples of *P. hartigii* utilized in this study were procured from Antalya, Turkey (Fig. 1). Ethanol and water extracts were obtained from dry samples of the mushroom using the Gerhardt SOX-414 device (Königswinter, Germany). The identification of the mushroom samples was made by Dr. Ilgaz Akata and Dr. Mustafa Sevindik. The fungarium samples of the mushroom are kept in Ankara University Fungarium. Sample numbers are ANK-FA Akata & Sevindik 32. Permissions for the collection of mushroom samples were obtained from the Ministry of Agriculture and Forestry of the Republic of Turkey.

Experimental design

The Box-Behnken Design was used in planning the experiments. Within the scope of the experimental design, experiments were conducted at three levels for each factor as low (−1), medium (0), and high (+1). The number of experiments was determined as 17 in accordance with the requirements of the design.

First, experiments were conducted in the Soxhlet apparatus under extraction conditions of 30, 50 and 70˚C extraction temperature, 1-, 5.5- and 10-h extraction time and 0, 50 and 100% ethanol/water ratio. Each experiment was repeated 3 times, and the response variable was measured. The obtained data was optimized by the response surface method (RSM) [23].

Fig. 1 Phellinus hartigii (Allesch. & Schnabl) Pat (Photo: Dr. Ilgaz AKATA)

Optimization

In this study, RSM was used for optimization. Extraction temperature, extraction time and ethanol/water ratio were selected as the independent variables of the study. The response variable was determined as the total antioxidant activity (TAS) value of the obtained extract.

The optimization process was carried out using Design Expert 13 software with a second-order polynomial response as:

$$Y_k = \beta_{k0} + \sum_{i=1}^{n} \beta_{ki} x_i + \sum_{i=1}^{n} \beta_{kii} x_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{kij} x_i x_j$$

where Y_k was response variable (Y_i was TAS value of extract); x_i was coded process variables (x_1 was extraction temperature, x_2 was extraction time, and x_3 was ethanol/water ratio) and β_{k0} is the value of fitted response at the design center point, respectively.

The suitability of the model was tested using coefficient of determination (R^2), ANOVA analysis and p-values. Critical points were calculated from the derivatives of the model to optimize the response variable. In addition, three-dimensional surface plots were prepared to visualize the effects of independent variables. These plots were used to better understand the effects of variables on the response.

Extraction processes for biological activities

A study on optimization was performed to identify the extraction parameters that enhance the biological activities of the tested mushroom extract. The optimization analysis identified the ideal extraction parameters as a temperature of 48.22 ˚C, a duration of 9.04 h, and an ethanol/water ratio of 52.22%. Following the establishment of these settings, extracts were procured under parameters nearest to the optimal extraction conditions utilizing the Gerhardt SOX-414 device, which is adjustable via a computer interface. Following the extraction process, the solvent content of the produced extract was evaporated with the Buchi R100 Rotary Evaporator (New Castle, United States), resulting in crude extracts.

Antiproliferative activity

The antiproliferative efficacy of the mushroom extract was tested against the A549 lung cancer cell line using the MTT assay [24]. Stock solutions were made from the extracts at concentrations of 25, 50, 100, and 200 µg/mL. Subsequently, the cells were permitted to reach 70–80% confluence. The separation process was conducted utilizing 3.0 mL of Trypsin–EDTA solution (Sigma-Aldrich, MO, USA). Subsequent to this procedure, the plates (NIST, China) were inoculated. Subsequent to the seeding operation, a 24-h incubation period was conducted. Subsequently, the stock solutions that had been prepared before were uploaded individually. Subsequent to this procedure, it was incubated for 24 h. The supernatants were subsequently dissolved in the growth medium, replaced with 1 mg/mL MTT, and incubated at 37 °C until a purple precipitate developed. Subsequently, dimethyl sulfoxide (DMSO) (Sigma-Aldrich, MO, USA) was included into MTT. The measurement was conducted at 570 nm with an Epoch spectrophotometer (BioTek Instruments, Winooski, USA).

Anticholinesterase activity

The anticholinesterase activity of the optimized mushroom extracts the Ellman method [25]. Galantamine served as the benchmark in the study The extracts were diluted to amounts ranging from 200 to 3.125 µg/mL. Subsequently, 130 µL of 0.1 M phosphate buffer at pH 8, 10 µL of stock solution, and 20 µL of enzyme solution (AChE or BChE) were introduced to the microplate (Abcam/Cambridge, United Kingdom). Subsequent to this procedure, it was incubated at 25 °C in darkness for 10 min. Subsequent to the incubation period, 20 µL of DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)) solution and 20 µL of substrate (acetylcholine iodide or butyrylcholine iodide) were introduced. Subsequent to these treatments, measurements were conducted at 412 nm using a microplate spectrophotometer ($PowerWave_X$, BioTek Instrument, Winooski, Vermont, USA). Activities are expressed as IC_{50} values in µg/mL.

Total phenolic content

1 mL stock solutions were formulated from the mushroom extract.1 mL of Folin-Ciocalteu reagent (1:9, v/v) was incorporated into the prepared solutions and thoroughly stirred. Subsequently, 0.75 mL of 1% Na_2CO_3 was incorporated into the mixture and allowed to incubate for 2 h at room temperature. Ultimately, measurements were conducted at 760 nm using a spectrophotometer (Thermo Fisher Scientific Evolution, Waltham, MA, USA). The total phenolic content was quantified as mg/g based on the calibration curve of the gallic acid standard solution [26].

Antioxidant activity

Total antioxidant and oxidant analysis

The antioxidant capacities of the mushroom extract generated under optimal conditions were assessed using Rel Assay TAS and TOS kits (Rel Assay Diagnostics, Megatıp, Gaziantep, Turkey). Total antioxidant status (TAS) and total oxidant status (TOS) were assessed following the manufacturer's methodology. TAS readings were quantified as mmol of trolox equivalent per liter. TOS levels

were quantified as μmol hydrogen peroxide equivalent per liter [27, 28]. The oxidative stress index (OSI) was calculated by dividing the total oxidative status (TOS) data, after standardizing the units to match the total antioxidant status (TAS) values, and expressing the result as a percentage [29].

DPPH Free radical scavenging activity

Dilution procedures were conducted with DMSO derived from the mushroom extract generated under optimal circumstances. In this context, stock solutions of 1 mg/mL were produced from the crude extract. 1 mL of the solution was combined with 160 μL of DPPH solution (0.267 mM in 4 mL, 0.004% methanol) and incubated for 30 min in a dark atmosphere at room temperature. The absorbance was measured at 517 nm using a spectrophotometer (Shimadzu UV-1601, Kyoto, Japan). Values were reported as mg of Trolox Equivalent per gram of extract [30].

Ferric reducing antioxidant power assay

100 μL of stock solution was formulated from mushroom extract generated under optimal circumstances. Subsequently, 2 mL of FRAP reagent was included into this solution. Subsequently, a solution comprising 300 mM acetate buffer (pH 3.6), 40 mM HCl, 20 mM $FeCl_3 \cdot 6H_2O$, and 10 mM 2,4,6-tris(2-pyridyl)-*S*-triazine was introduced. The solution was combined with the FRAP solution in a ratio of 10:1:1. The sample was incubated at 37 °C for 4 min and subsequently measured at 593 nm using a spectrophotometer (Shimadzu UV-1601, Kyoto, Japan). Values were presented as mg of Trolox Equivalent per gram of extract [30].

Phenolic content analysis

24 standard compounds were screened using LC–MS/MS device in mushroom extract produced under optimum conditions. Instrument used: Shimadzu LCMSMS-8030, Column: Inertsil ODS 4; 2um, 2.1X 50 mm, Mode: Binary gradient, Pump A: LC-20ADXR, Pump B: LC-20ADXR, Total Flow: 0.4000 mL/min, Mobile Phase A: 0.1% Formic acid + water, Mobile Phase B: 0.1% Formic acid + Methanol, B Conc.: 5.0%, B Curve: 0, PressMax: 660 bar, Autosampler Model: SIL-20ACXR, Oven (Column Oven) Model: CTO-10ASvp, Oven (Column Oven)Temperature: 40 °C, Maximum Temperature: 85 °C.

Statistical analysis

The statistical analysis for all evaluations performed in this study utilized the 'SPSS 21.0 for Windows' software. A Basic Variance Analysis (BVA) was conducted to ascertain the differences among the groups in the analyzed tests; the Duncan test was utilized at a confidence level ($\alpha = 0.05$) to evaluate the differences between the groups. IC_{50} values were determined through nonlinear regression analysis using GraphPad Prism version 6.0 (GraphPad Software Inc., USA).

Results and Discussions

Optimization of extraction conditions

The extraction conditions of the tested mushroom were optimized using the response surface methodology. Consequently, the extraction parameters were refined to enhance the overall antioxidant capacity. The TAS values derived from the experimental study are presented in Table 1.

An examination of Table 1 reveals that the extraction condition yielding the lowest TAS value (3.02 ± 0.05) is 70 °C, 5.5 h, and a 0% ethanol/water ratio (100% water). It was observed that the extraction condition with 50°C, 5.5 h and 50% ethanol/water ratio gave the highest TAS value of 4.98 ± 0.04 among the studied limits. Homogeneity groups are given in Table 1. It was determined that the difference between the studied groups was statistically significantly different ($p < 0.05$).

Optimization

Linear, cubic and two-factor interaction models were examined in the Box-Behnken design, which was applied for experimental studies in which the TAS values of *P. hartigii* was determined. As a result of the examination, it was determined that the model that best explains the extraction

Table 1 TAS values of the extracts obtained in the study

Extraction temperature (°C)	Extraction time (h)	Ethanol/water ratio (%)	TAS (mmol/L)	Homogeneity groups
50	5.5	50	4.978 ± 0.039	J*
50	5.5	50	4.976 ± 0.014	J
50	5.5	50	4.953 ± 0.024	J
70	5.5	100	3.169 ± 0.037	B
50	5.5	50	4.962 ± 0.022	J
50	10	100	4.465 ± 0.042	I
70	1	50	3.373 ± 0.104	D
50	1	0	3.723 ± 0.028	F
30	1	50	3.078 ± 0.061	AB
50	1	100	3.868 ± 0.047	G
30	55	0	3.269 ± 0.017	C
50	5.5	50	4.987 ± 0.042	J
70	10	50	3.772 ± 0.047	FG
70	5.5	0	3.020 ± 0.045	A
30	5.5	100	3.571 ± 0.060	E
30	10	50	3.855 ± 0.150	G
50	10	0	4.279 ± 0.034	H

* Means followed by different letter(s) differ significantly at $p < 0.05$ (Duncan's multiple range test)

conditions is the quadratic model. Fit summary was presented at Table 2.

The quadratic polynomial equation created as a result of the multiple regression analysis to determine the TAS values of *P. hartigii* is shown below.

$$TAS = 4.97 - 0.055X_1 + 0.291X_2 + 0.097X_3 - 0.094X_1X_2 - 0.038X_1X_3 + 0.010X_2X_3 - 1.14X_1^2 - 0.312X_2^2 - 0.574X_3^2$$

In the equation, X_1, X_2 and X_3 represent the extraction temperature, extraction time and ethanol/water ratio, respectively. Response surface plots of TAS of *P. hartigii* were shown at Fig. 2.

R^2 (coefficient of determination) shows the capacity of the model to explain the response of the independent variables. A high R^2 (e.g. ≥ 0.90) indicates that the model is suitable. In this study, the R^2 of the model was found to be 0.989.

Table 2 Fit summary

Source	Sequential *p*-value	Lack of Fit *p*-value	Adjusted R^2	Predicted R^2	
Linear	0.7406	< 0.0001	−0.1216	−0.4085	
2FI	0.9966	< 0.0001	−0.4505	−15.827	
Quadratic	< 0.0001	0.0001	0.9751	0.8272	**Suggested**
Cubic	0.0001		0.9997		Aliased

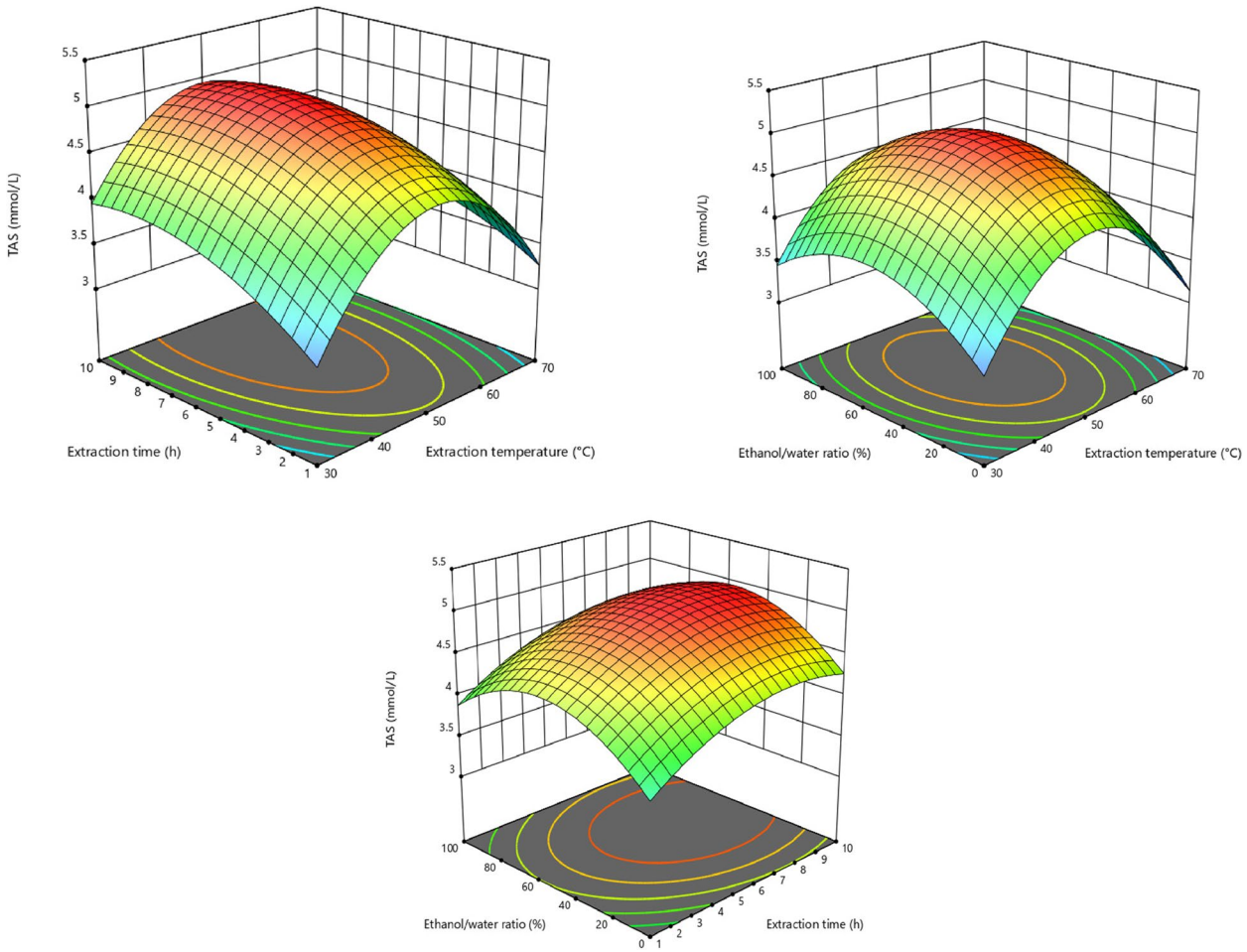

Fig. 2 Response surface plots of TAS of *P. hartigii*

As can be seen from Fig. 2, the TAS value of *P. hartigii* extracts is mostly affected by the extraction time and ethanol/water ratio among the extraction conditions studied (statistically $p < 0.05$), and less affected by the extraction temperature. The optimum extraction conditions were found to be 48.22 °C temperature, 9.04 h and % 52.22 ethanol/water ratio, and the model estimated the TAS value of the extract studied under these conditions as 5.04 mmol/L.

Antioxidant activity

Antioxidants significantly mitigate the impact of free radicals. Although modest concentrations of these chemicals may be mitigated, significant cellular damage becomes evident as their concentrations rise [31]. Oxidative stress arises from an imbalance between antioxidants and oxidant molecules. Severe illnesses, including cancer, diabetes, obesity, cardiovascular diseases, multiple sclerosis, and neurological disorders, can manifest due to oxidative stress. Supplemental antioxidants may mitigate the consequences of oxidative stress [32, 33]. We assessed the antioxidant potential of the extract from *P. hartigii* cultivated under optimal conditions that yield maximum biological activity. The results are presented in Table 3.

The TAS, TOS, and OSI values of *P. hartigii* remain unreported in the literature. Our investigation established this for the first time. Values for TAS, TOS, and OSI of various *Phellinus* species have been documented. The TAS values for *P. igniarius* and *P. rimosus* were recorded as 5.364 and 3.772 mmol/L, respectively, while the TOS values were 20.854 and 24.323 µmol/L, and the OSI values were 0.389 and 0.645, respectively [34]. In comparison to this investigation, the TAS value of the extract exhibiting the highest biological activity of *P. hartigii* was found to be lower than that of *P. igniarius* and greater than that of *P. rimosus*. The TOS and OSI values of *P. hartigii* were found to be inferior to those of *P. igniarius* and *P. rimosus*. Our investigation identified the extraction parameters that yielded the highest biological activity of *P. hartigii*, revealing that the extracts obtained under these circumstances exhibited significant antioxidant activity. The literature contains TAS, TOS, and OSI research on many wild mushrooms. The TAS values recorded for *Ramaria stricta, Hebeloma sinapizans, Morchella esculenta*, *Laccaria laccata*, and *Hericium erinaceus* were 4.223, 4.540, 4.580, 3.857, and 5.426 mmol/L, respectively. The TOS values were recorded as 8.201, 10.303, 13.549, 9.526, and 6.621 µmol/L, respectively. OSI values were documented as 0.194, 0.227, 0.296, 0.247, and 0.122, respectively [35–39]. In comparison to these investigations, the TAS value of *Phellinus hartigii* in our research was shown to be superior than that of *R. stricta*, *H. sinapizans*, *M. esculenta*, and *L. laccata*, although inferior to that of *H. erinaceus*. The TAS value signifies the totality of antioxidant molecules generated in natural products [40]. Our analysis revealed that the *P. hartigii* extract, generated under optimal conditions, exhibited significant antioxidant capacity. The TOS value signifies the aggregate of oxidant chemicals generated in natural products [40]. The TOS value of *P. hartigii* in our study was found to be greater than that of *R. stricta* and *H. erinaceus*, however lower than *H. sinapizans, L. laccata*, and *M. esculenta*. In this regard, it was noted that the oxidant compound levels of *P. hartigii* extract generated at optimal circumstances, which yield the highest biological activity, were within normal ranges. The OSI value is determined by contrasting the TOS values with the TAS values. The OSI score indicates the extent to which antioxidant molecules inhibit oxidant compounds [40]. The OSI value of *P. hartigii* in our study was found to be superior to that of *H. erinaceus*, although inferior to *R. stricta, H. sinapizans, M. esculenta*, and *L. laccata*. It was established that the *P. hartigii* extract, generated under optimal conditions for maximum biological activity, exhibits substantial antioxidant potential and effectively inhibits oxidant chemicals. The antioxidant capability of the *P. hartigii* extract, generated under optimal conditions for maximum biological activity, was assessed using DPPH and FRAP assays. The literature documents the antioxidant capabilities of various *Phellinus* species. The DPPH test results for ethanol extracts of *P. contiguus, P. gilvus, P. hippophaecola, P. igniarius, P. linteus, P. pini, P. torulosus, P. tremulea, P. trivalis,* and *P. tuberculosus* ranged from 15.31 to 154.50 µg/mL, while the results for water extracts ranged from 79.59 to 332.85 µg/mL [17]. The DPPH test result for the extract of *P. hartigii*, generated under optimal conditions yielding the maximum biological activity, was found to be 106.07 ± 2.44 mg/g. It was concluded that *P. hartigii* possesses antioxidant potential. In a different study, the antioxidant activity of ethanol and water extracts of *Phellinus nigricans, Phellinus rimosus,* and *Phellinus wahlbergii* was reported as 0.06–0.33 mmol/mg using the FRAP test [41]. In our study, the FRAP value of the extract of *P. hartigii* produced under optimum conditions that gave the highest

Table 3 Antioxidant parameters of *Phellinus hartigii*

Parameter	Values*
TPC (mg/g)	88.21 ± 1.50
FRAP (mg Trolox Equi/g)	137.81 ± 1.72
DPPH (mg Trolox Equi/g)	106.07 ± 2.44
TOS (µmol/L)	9.27 ± 0.06
TAS (mmol/L)	4.98 ± 0.03
OSI (TOS/(TAS*10))	0.19 ± 0.002

* Values are given as mean ± standard deviation. ($n = 3$)

biological activity was determined as 137.81 ± 1.72 mg Trolox Equi/g. In this context, it was determined that *P. hartigii* used in our study, in addition to different *Phellinus* species reported in the literature, also has significant antioxidant potential.

Antiproliferative activity

Contemporary therapy modalities for various cancer patients are evolving. Furthermore, numerous natural compounds are utilized to enhance the therapeutic procedure [42]. The study examined the impact of *P. hartigii* extract, generated under optimal conditions, on the A549 cancer cell line. The results are illustrated in Fig. 3.

There is no evidence in the literature regarding the anticancer activity of *P. hartigii* against the a549 lung cancer cell line. Various *Phellinus* species have been reported to be effective against the A549 human lung cancer cell line. *P. baumii* has been reported to be effective against the A549 human lung cancer cell line [43]. A separate investigation indicated that *P. linteus* and *P. baumii* exhibited significant activity against (5.48 µg/mL) the A549 human lung cancer cell line [44]. Our study assessed the biological activity of the extract from *P. hartigii*, produced under optimal conditions, against the A549 human lung cancer cell line. The analyses indicated that the effects intensified with increasing concentration (25, 50, 100 and 200 µg/mL). Furthermore, it was established that the maximum activity occurred at an extract concentration of 200 µg/mL. It was determined that *P. hartigii* extract produced under optimum conditions exhibited significant biological activity and had remarkable anticancer potential. These findings indicate that the extract can be further investigated as a natural anticancer agent.

Anticholinesterase activity

The prevalence of Alzheimer's disease, particularly among individuals over 65 years of age, is rising [45]. It is anticipated that the number of these instances would surpass 80 million in the imminent future. No effective treatment for Alzheimer's disease is currently known. Nonetheless, various therapeutic methodologies exist. The suppression of cholinesterase enzymes is one such instance [45, 46]. In this study, we assessed the anticholinesterase activity of the extract from *P. hartigii* generated under optimal conditions. Table 4 presents the IC_{50} values of the results.

No prior research exists in the literature regarding the anticholinesterase activity of *P. hartigii*. Research on various *Phellinus* species indicates that *P. gilvus* exhibits elevated anti-acetylcholinesterase and anti-butyrylcholinesterase activity [47]. Various components of *P. igniarius* exhibit acetylcholinesterase activity such as fruiting body, mycelia and culture filtrate [48]. In other study, it was found that *P. xeranticus* exhibits acetylcholinesterase inhibitory activity [49]. In our study, acetyl and butyrylcholinesterase inhibitory activities of optimized *P. hartigii* extract were evaluated. The analyses revealed that it had reduced activity in comparison to galantamine, which served as the reference. Identifying the enzymes responsible for illness etiology and inhibiting these enzymes is crucial for managing disorders [50]. In this

Table 4 Anticholinesterase activity of *Phellinus hartigii* extract

Sample	AChE (IC_{50}: (µg/mL))	BChE (IC_{50}: (µg/mL))
Optimized Extract	21.29 ± 0.41[b]	35.51 ± 0.53[b]
Galantamine	9.45 ± 0.23[a]	16.95 ± 0.52[a]

* Means followed by different letter(s) differ significantly at $p < 0.05$ (t- test)

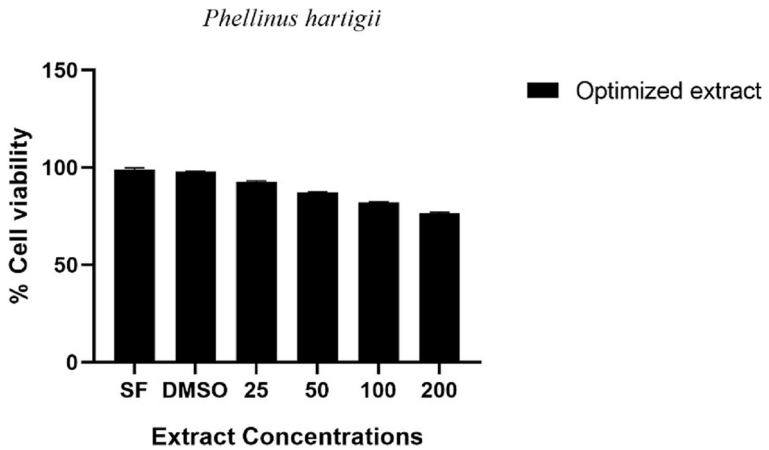

Fig. 3 Antiproliferative activity of *P. hartigii* optimized extract. * DMSO: dimethyl sulfoxide; SF: serum-free medium

context, it is thought that *P. hartigii* used in our study may be effective in the treatment of neurological diseases by inhibiting cholinesterase enzymes.

Phenolic profile

Total phenolic contents

The results of the total phenolic content (TPC) of the *P. hartigii* extract cultivated under optimal conditions was presented in Table 5. To the best of our knowledge, the literature contains no findings about the TPC of *P. hartigii*. The TPC of methanol extracts from various *Phellinus* species, specifically *P. rimosus, P. gilvus*, and *P. badius*, were documented to range from 44.76 to 49.31 mg GAE/g extract [51]. A separate investigation indicated that the total phenolic concentrations of methanol extracts from *P. fastuosus, P. grenadensis*, and *P. badius*, ranged from 96.96 to 209.76 mg GAE/g extract [16]. The total phenolic content of the extract obtained under optimal conditions, which yielded the maximum biological activity of *P. hartigii*, was quantified as 88.21 ± 1.50 mg GAE/g extract. In this context, *P. hartigii* was identified as a significant source of total phenolic content.

Table 5 LC–MS/MS analysis of selected phenolic and non-phenolic compounds in analyzed *P. hartigii* extract

Compounds	Ret. Time	Values (ppb)
Acetohydroxamic acid	0.460	3494.28 ± 14.26
Fumaric acid	0.859	137.51 ± 1.71
Gallic acid	1.483	15,544.27 ± 22.11
Protocatechuic acid	2.060	732.46 ± 5.20
Catechin hydrate	2.482	12,571.29 ± 16.60
4-hydroxybenzoic acid	2.550	1020.72 ± 5.91
Caffeic acid	2.850	344.05 ± 1.75
Vanillic acid	2.853	n.d.*
Syringic acid	2.973	n.d
Thymoquinone	3.338	n.d
Salicylic acid	3.480	n.d
Phloridzindyhrate	3.523	n.d
2-hydoxycinamic acid	3.553	872.68 ± 8.41
Oleuropein	3.587	n.d
Resveratrol	3.632	n.d
2-hyroxy-1,4-naphthaquinone	3.654	n.d
Myricetin	3.725	n.d
Naringenin	3.960	n.d
Silymarin	4.000	n.d
Quercetin	4.000	3170.32 ± 16.27
Luteolin	4.122	n.d
Kaempferol	4.222	n.d
Alizarin	4.552	n.d
Curmin	4.605	n.d

* **n.d: Not detected**

LC–MS/MS profile of phenolic and non-phenolic compounds

Secondary metabolites are non-nutritive molecules that hold significance for medicinal applications. Mushrooms possess the capability to synthesize several secondary metabolites inside their structures. These secondary metabolites are accountable for many biological actions [52]. In this study, 24 phenolic and non-phenolic compounds in the extract of *P. hartigii*, generated under optimal conditions, were analyzed using the LC–MS/MS method. The results acquired are presented in Table 5. Additionally, the chromatogram of the standards is shown in Fig. 4.

Our study identified the presence of acetohydroxamic acid, fumaric acid, gallic acid, protocatechuic acid, quercetin, caffeic acid, 2-hydroxycinnamic acid, catechin hydrate, and 4-hydroxybenzoic acid in the extract of *P. hartigii* produced under optimal conditions that yielded the highest biological activity. The literature contains no findings about phenolic compounds in *P. hartigii*. Research on various *Phellinus* species identified the occurrence of protocatechualdehyde, protocatechuic acid, caffeic acid, hispidin, davalliallactone, ellagic acid, hypholomine B, inoscavin A, interfungins A, and methyldavallialactone in *P. linteus* [53]. A separate investigation identified the presence of ferulic acid, *p*-coumaric acid, caffeic acid, epicatechin, catechin, 1,3-dicaffeoylquinic acid, gallic acid, rutin, and chlorogenic acid in *P. badius, P. grenadensis*, and *P. fastuosus* [16]. In our study, the presence of 24 traditional compounds in *P. hartigii* was screened and 9 compounds were identified as a result of the analysis. This shows that the chemical content of the mushroom may vary depending on environmental factors, growing conditions and extraction method. In particular, the optimal extraction conditions used may have highlighted the compounds that maximize the biological activity of the mushroom. In addition, the natural composition and metabolic profile of *P. hartigii* may have contributed to the higher presence of certain compounds.

Conclusion

In conclusion, in this study, the biological activities of the extract of *P. hartigii* prepared under optimum conditions were evaluated and the obtained results revealed that this extract exhibited strong antioxidant, anticholinerase and antiproliferative activities. In particular, it was determined that the optimized extract was effective in eliminating free radicals, inhibited cholinesterase enzymes associated with neurodegenerative diseases and showed cytotoxic effects against cancer cells. In the analyses, it was determined that the extract was rich in phenolic and non-phenolic compounds and it is thought that these compounds make a significant

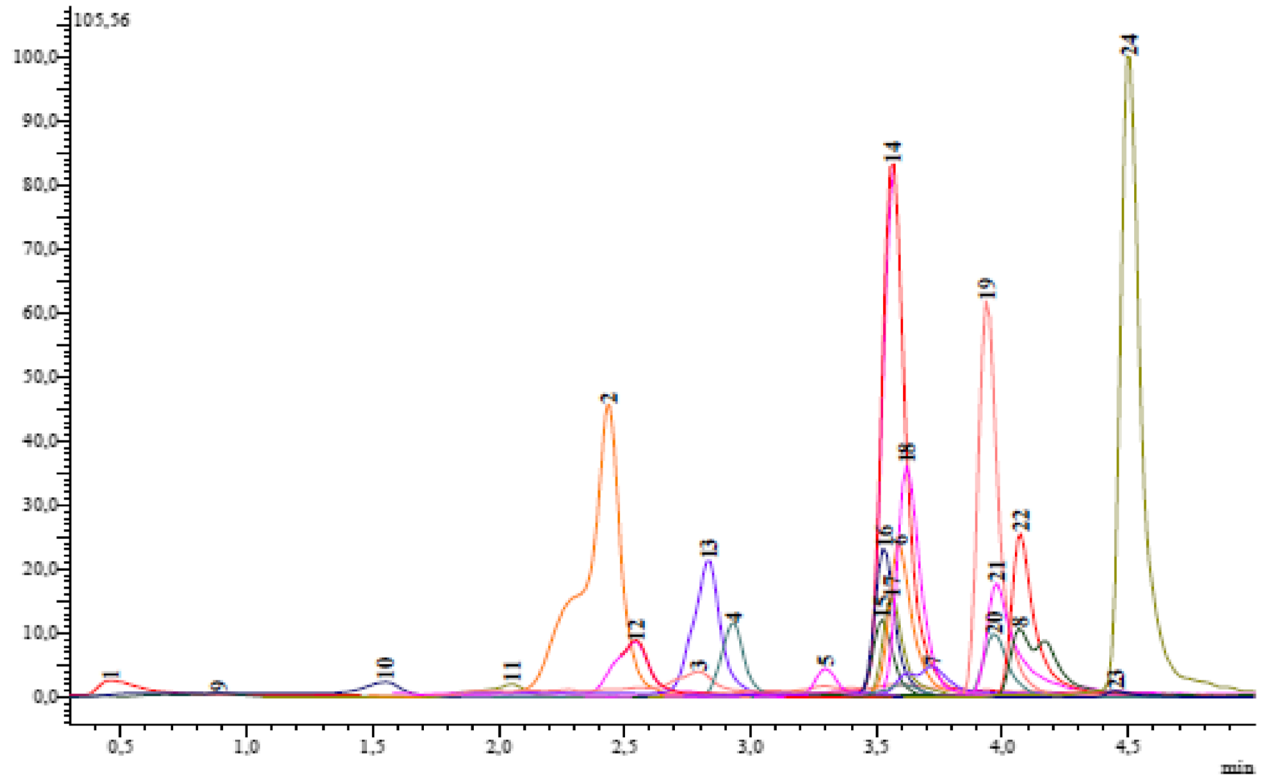

Fig. 4 Standard chromatogram

contribution to biological activities. These findings suggest that *P. hartigii* is a valuable natural source of bioactive compounds and has therapeutic potential. The obtained data emphasize that the pharmacological properties of this fungus should be investigated in more detail and provide a scientific basis for its evaluation in future drug development processes. Although the study showed promising biological activities, further in vivo studies are required to confirm the efficacy and safety of *P. hartigii* extracts in living organisms.

Abbreviations

AChE	Acetylcholinesterase
BChE	Butyrylcholinesterase
DMSO	Dimethyl Sulfoxide
DPPH	1,1-Diphenyl-2-Picrylhydrazil
FRAP	Ferric Reducing Antioxidant Power
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
RSM	Response Surface Methodology
TAS	Total Antioxidant Status
TOS	Total Oxidant Status
TPC	Total Phenolic content

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Authors' contributions
MS and OU supervised the research. AG and MS performed the experiments, analyzed the data, and wrote the manuscript. AG and TK designed the experiments. IA and ŞK provided assistance during the experiments. AG, MS, TK, IA, ŞK and OU revised the manuscript. All authors read and approved the final manuscript.

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Data availability
The datasets generated analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
This research does not involve any ethical issues.

Consent for publication
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Competing interests
The authors declare no competing interests.

Author details
[1]Faculty of Science, Department of Biology, Akdeniz University, Antalya 07058, Turkey. [2]Faculty of Engineering and Natural Sciences, Department of Industrial Engineering, Osmaniye Korkut Ata University, Osmaniye 80000, Turkey. [3]Department of Plant Food Products and Biofortification, Institute of Food Biotechnology and Genomics, National Academy of Sciences of Ukraine, Kiev 04123, Ukraine. [4]Faculty of Engineering and Natural Sciences, Department of Biology, Osmaniye Korkut Ata University, Osmaniye 80000, Turkey. [5]Battalgazi Vocational School of Higher Education, Malatya Turgut Ozal University, Malatya 44210, Turkey. [6]Faculty of Science, Department of Biology, Ankara University, Ankara 06100, Turkey.

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