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Artificial intelligence-guided optimization of *Suillus lakei* extracts enhancing bioactive profile and functional properties for future food applications

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Abstract

This study aimed to comparatively evaluate the biological properties of *Suillus lakei* (Murrill) A.H. Sm. & Thiers extracts optimized using response surface methodology (RSM) and artificial neural network-genetic algorithm (ANN-GA) approaches. The extracts were systematically analyzed in terms of their antioxidant potential, anticholinesterase capacity, antiproliferative effects, and phenolic composition. The ANN-GA-optimized extract exhibited significantly higher DPPH and FRAP values and significantly lower TOS values than the RSM-optimized extract, whereas the differences in TAS and OSI were not statistically significant. Enzyme inhibition assays further demonstrated that these extracts exhibited stronger inhibitory activity against both acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). In vitro MTT assays showed that the ANN-GA-derived extract produced a greater reduction in MTT absorbance, particularly at higher concentrations, in A549, MCF-7, and DU-145 cancer cell lines. Additionally, phenolic profiling suggested that these extracts were enriched in bioactive compounds, with gallic acid and quercetin occurring at the highest measured concentrations, followed by protocatechuic and caffeic acids. Overall, under the tested experimental conditions, the ANN-GA-optimized extract exhibited a distinct chemical composition and more favorable values for the measured in vitro biological endpoints than the RSM-optimized extract. This work represents one of the early detailed investigations into the bioactivity profile of *S. lakei* and provides preliminary in vitro evidence that warrants further validation before its extracts can be considered for potential functional food-related applications.

Keywords Extraction optimization · Antioxidant activity · Anticholinesterase activity · Antiproliferative effect · Phenolic compounds · Artificial intelligence · Natural products

Introduction

Medicinal mushrooms have attracted considerable interest in natural product research because they contain chemically diverse metabolites, including phenolic compounds, polysaccharides, sterols, terpenoids, proteins, and other secondary metabolites with measurable biological activities[1–3]. The recovery and measured activity of

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these constituents depend strongly on the extraction procedure. Solvent composition, extraction temperature, processing time, solid-to-solvent ratio, and extraction technique can influence compound solubility, diffusion from the fungal matrix, and chemical stability[4–6]. Consequently, extraction conditions should be defined systematically when the objective is to obtain reproducible extracts with specific chemical or biological characteristics. Conventional optimization based on changing one extraction variable at a time does not adequately account for interactions among process factors. Multivariate approaches such as Response Surface Methodology (RSM) allow linear, quadratic, and interaction effects to be evaluated simultaneously and have therefore been widely applied to natural-product extraction. For example, experimental-design-based optimization has been used to investigate the recovery of bioactive constituents from *Pleurotus ostreatus* together with their antioxidant and antimicrobial activities, while accelerated solvent extraction combined with RSM has been applied to optimize crude polysaccharide extraction and antioxidant activity in *Agrocybe aegerita*[7,8]. Artificial neural networks (ANNs) provide an alternative approach for modeling nonlinear relationships that may not be adequately represented by polynomial regression, whereas genetic algorithms (GAs) can search the modeled response space for parameter combinations associated with a predefined optimization target. The combined use of RSM and ANN-GA therefore enables statistical and nonlinear modeling approaches to be compared within the same extraction framework[9].

Suillus lakei (Murrill) A.H. Sm. & Thiers is an ectomycorrhizal basidiomycete belonging to the family Suillaceae. It is native to western North America and is closely associated with Douglas fir, *Pseudotsuga menziesii* (Mirb.) Franco. The species has subsequently been recorded outside its native range in association with introduced Douglas fir populations, including locations in Europe, South America, and New Zealand[10,11]. Morphologically, *S. lakei* is characterized by a dry, dark red to reddish-brown pileus with a fibrillose to scaly surface, dingy yellow to ochre pores that may become reddish brown after bruising, and a firm, solid stipe that is generally equal to slightly clavate and usually bears remnants of a veil[10]. Previous taxonomic and ecological investigations have primarily addressed its distribution, host association, morphology, and ectomycorrhizal characteristics[10–12]. Species-specific information on the chemical composition and biological properties of *S. lakei* remains comparatively limited. In an earlier experimental study, chemical screening of *S. lakei* culture-derived material was positive for alkaloids and tannins, while proteins, carbohydrates, and total phenolic compounds were also quantified[13]. The same study reported antioxidant and immunostimulatory activity and antimicrobial effects for selected *S. lakei* samples, whereas cytotoxicity was not observed in the tested liver-cell model[13]. These findings provide experimental evidence that the species contains chemically and biologically active constituents, but they were obtained using culture-derived samples and different biological assays from those employed in the present study. The available species-specific literature is focused mainly on taxonomy, ecology, and experimental bioactivity, and a clearly documented traditional medicinal use of *S. lakei* has not been established. Accordingly, no traditional therapeutic properties are attributed to this species here.

Previous extraction studies in other mushroom species have shown that optimization strategies can be used to identify combinations of solvent composition, temperature, and extraction time associated with defined chemical or biological responses[7,8]. However, these studies differ in their extraction systems, target responses, and biological endpoints. For *S. lakei*, the available biological study characterized selected activities and chemical groups but did not systematically optimize extraction variables or directly compare conventional statistical modeling with an ANN-GA approach[13]. Thus, it remains unclear whether RSM and ANN-GA identify different extraction conditions when applied to the same *S. lakei* dataset and a common predefined response. It is also unknown whether extracts obtained under the resulting optimum conditions differ in their measured antioxidant parameters, cholinesterase inhibition, effects on cancer-cell viability, or phenolic composition.

The present study was therefore designed to optimize the extraction conditions of *S. lakei* using RSM and ANN-GA and to compare the conditions identified by these two modeling approaches. Total Antioxidant Status (TAS) was defined as the primary and sole response variable used for optimization. Anticholinesterase activity, antiproliferative effects, additional antioxidant parameters, and phenolic composition were not optimization targets; instead, they were subsequently evaluated as secondary outcomes in extracts prepared under the TAS-based

optimum conditions. In this framework, the novelty of the study lies in the comparative application of RSM and ANN-GA to TAS-based extraction optimization of *S. lakei*, followed by chemical and in vitro biological characterization of the resulting extracts under the tested experimental conditions.

Materials and methods

The macrofungus specimens examined in this study were collected during field sampling at the Ataturk Arboretum, next to Belgrad Forest, Istanbul/Türkiye (Fig. 1). The materials obtained after field sampling were transported to the laboratory under appropriate conditions to ensure the preservation of their morphological and taxonomic characteristics. The collected specimens were then delivered to the Ankara University Fungarium for evaluation as reference material and registered in the collection in accordance with the acceptance, registration, and long-term storage procedures of the relevant collection. Thus, the accessibility of the specimens for future taxonomic verification and analytical studies was ensured, and all materials were preserved under standard fungarium storage conditions.

Extraction procedure

To comprehensively assess the combined influence of critical parameters on extraction efficiency, a multi-variable experimental design was employed. Within this framework, temperature, extraction duration, and solvent composition were selected as the principal independent variables governing the process. Each factor was examined at three distinct levels, resulting in a structured design that enabled the evaluation of both individual effects and potential interactions among variables. Accordingly, a total of 27 experimental conditions were generated and implemented. All extraction procedures were conducted using an ultrasonic-assisted system under controlled conditions, with temperature (30–60 °C), time (30–60 min), and ethanol concentration (0-100%) maintained within predefined intervals. Ultrasonic-assisted extraction was performed using an ultrasonic bath device rather than a probe-type sonicator. The extraction procedures were carried out with a WIGGENS UA22MFDN ultrasonic bath device (WIGGENS, Germany) operating at a fixed frequency of 40 kHz and a nominal output power of 400 W. For each extraction, 10 g of dried and powdered *S. lakei* sample was mixed with 100 mL of the appropriate



Fig. 1 *Suillus lakei* (Murrill) A.H. Sm. & Thiers.

ethanol/water solvent system, corresponding to a solid-to-solvent ratio of 1:10 (w/v). The extraction temperature and duration were adjusted according to the experimental design. After extraction, the mixtures were filtered using a non-sterile regenerated cellulose (RC) syringe filter with a pore size of 0.22 µm. The filtrates were concentrated under reduced pressure using a rotary evaporator (Heidolph Hei-VAP Core ML G3, Germany) until the solvent was completely removed. The resulting dried extracts were obtained in powder form and stored at +4 °C until further analyses. For subsequent biological and chemical assays, appropriate amounts of the dried extract were weighed and reconstituted in the assay-specific solvents to obtain the required working concentrations. All extract concentrations were therefore expressed on a dry-extract mass basis. The experimental data were first analyzed using Response Surface Methodology (RSM) to estimate optimal extraction parameters through statistical modeling. In a subsequent step, Artificial Neural Network (ANN) and Genetic Algorithm (GA) techniques were applied based on previously reported artificial intelligence-assisted optimization approaches for mushroom extraction, with modifications according to the experimental variables and optimization target used in the present study[9]. Each extraction condition was independently performed in triplicate using separate sample portions, and each independently prepared extract was subsequently analyzed.

Response surface methodology (RSM)

In this study, RSM was employed to quantitatively investigate the influence of extraction parameters on biological activity and to identify optimal operating conditions. Within this framework, extraction temperature, extraction time, and ethanol/water composition were considered as independent variables, while Total Antioxidant Status (TAS) was defined as the primary and sole response variable used for extraction optimization. The other biological activities and phenolic composition were not included as optimization objectives and were evaluated subsequently as secondary outcomes of the extracts obtained under the TAS-based optimum conditions. The experimental design, data analysis, and optimization procedures were conducted using Design Expert 13 software, which provides advanced tools for statistical modeling and process optimization. Candidate regression models were comparatively evaluated using the model-fit statistics provided by Design-Expert 13, and the quadratic model was selected based on its overall fit to the experimental TAS data. The selected quadratic model was represented by a second-order polynomial equation including linear, quadratic, and two-factor interaction terms. This modeling strategy enabled a comprehensive evaluation of the system by clearly elucidating not only the individual contributions of each parameter but also their interaction effects on the response variable.

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

In the developed regression framework, the dependent variable (Y_k) corresponds to the TAS values of the extracts, whereas the coded predictors (X_i) denote the extraction parameters, namely temperature, extraction time, and solvent composition. The intercept term (β_{k0}) was defined as the coefficient representing the average response at the central point of the experimental design space. The adequacy and robustness of the model were rigorously evaluated through statistical indicators, including the coefficient of determination (R^2), analysis of variance (ANOVA), and associated significance levels (p-values). Optimal extraction conditions were determined by solving the model equations to identify the parameter combinations that maximize the response function. Furthermore, three-dimensional response surface plots were generated to visualize the interactions among the independent variables. These graphical representations facilitated a clearer interpretation of how extraction parameters collectively influence both extraction efficiency and the resulting biological activity.

Artificial neural network-genetic algorithm (ANN-GA)

The ANN-GA modeling framework was implemented based on previously reported artificial intelligence-assisted optimization approaches for mushroom extraction, with modifications according to the input variables, response

variable, and optimization strategy used in the present study[9]. Within this framework, extraction temperature, extraction duration, and ethanol/water proportion were used as input parameters, while TAS was defined as the sole output variable and optimization target of the network. The 27 experimental observations were randomly partitioned into training, validation, and test subsets using an approximately 80:10:10 allocation ([$n=21$], [$n=3$], and [$n=3$], respectively). The Levenberg-Marquardt (LM) algorithm was employed during the training phase to accelerate convergence and minimize the risk of overfitting.

For the optimization of network architecture, the number of neurons in the hidden layer was systematically varied, and multiple configurations ranging from 1 to 20 neurons were evaluated. A feed-forward neural network with a single hidden layer was employed. Network training was terminated when one of the predefined stopping criteria was met: a maximum of 500 epochs, no improvement in validation error for 50 consecutive iterations, or achievement of an error threshold of 1×10^{-5}. Each network configuration was trained 1000 times using randomly initialized weights to enhance robustness, and the resulting models were comparatively assessed. Performance evaluation was carried out using Mean Squared Error (MSE) and Mean Absolute Percentage Error (MAPE), calculated according to the corresponding mathematical formulations provided below.

$$\mathrm{MSE} = \frac{1}{\mathrm{n}} \sum_{\mathrm{i}=1}^{\mathrm{n}} (\mathrm{e_i} - \mathrm{p_i})^2 \tag{1}$$

$$\mathrm{MAPE} = \frac{1}{\mathrm{n}} \sum \left| \frac{\mathrm{e_i} - \mathrm{p_i}}{\mathrm{e_i}} \right| * 100 \tag{2}$$

To achieve a more comprehensive exploration of the solution domain during optimization, a Genetic Algorithm (GA) was incorporated into the modeling framework. The population size, a critical factor influencing algorithm performance, was tested at different levels, and its impact on convergence characteristics and solution consistency was systematically examined. During the selection stage, a fitness-based probabilistic approach, namely the roulette wheel method, was employed to determine individuals for reproduction. To preserve diversity within the population and avoid premature convergence, a single-point crossover strategy was implemented. The search space was restricted to the experimental ranges of 30–60 °C for extraction temperature, 30–60 min for extraction duration, and 0–100% for ethanol concentration. The progression of the optimization process was evaluated through convergence curves obtained across iterations, and the influence of iteration number on solution quality was quantitatively assessed. The GA optimization was terminated when the convergence plot indicated that the objective function had stabilized and no further improvement in the fitness value was observed. To minimize stochastic variability and enhance the robustness of the results, each optimization configuration was executed in at least thirty independent trials. The predictive capability of the ANN model was assessed by comparing experimentally obtained values with the corresponding model outputs. The agreement between experimental and ANN-predicted TAS values was evaluated using MSE, MAPE, and the correlation coefficient (R). In this framework, e_i denotes the experimentally measured TAS values, whereas p_i represents the ANN predicted counterparts. The total number of observations included in the analysis is expressed by n. Based on these parameters, error metrics were calculated and used as primary indicators for quantitatively evaluating the model's prediction accuracy.

Extraction for bioactivity analyses

In order to identify extraction conditions associated with the maximum TAS response, the extraction process was optimized using both RSM and ANN–GA approaches, with TAS serving as the primary optimization target. According to the results obtained from RSM, the optimal extraction parameters were estimated as 42.434 (42.5) °C for temperature, 40.082 (40) minutes for extraction time, and 99.320% (100%) for the ethanol/water ratio. In comparison, the hybrid ANN-GA model suggested slightly different optimal conditions, with temperature, time, and solvent composition predicted as 40.4602 (40.5) °C, 49.0953 (49) minutes, and 88.7691% (90%) ethanol/water ratio, respectively. The optimal values derived from both modeling approaches were considered

collectively, and experimental validation studies were conducted using parameter sets that were both close to these predictions and practically applicable under laboratory conditions. The antioxidant, anticholinesterase, antiproliferative, and phenolic composition analyses subsequently performed on these extracts were considered secondary characterization outcomes and were not used as objective functions during optimization. All extraction experiments were performed using an ultrasonic-assisted system to maintain methodological consistency. The procedures were performed under the ultrasonic extraction conditions described above to ensure methodological consistency. This integrated approach ensured coherence between the theoretical optimization models and the experimental validation phase.

Antioxidant activity tests

The overall redox status of the optimized mushroom extracts was evaluated by determining Total Antioxidant Status (TAS), Total Oxidant Status (TOS), and Oxidative Stress Index (OSI). TAS was measured using a commercially available assay kit according to the manufacturer's instructions and the method described by Erel[14]. The assay is based on the ability of antioxidant compounds present in the extracts to reduce the 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) radical cation. The reduction of the colored ABTS radical cation results in a proportional decrease in absorbance, which was measured spectrophotometrically. TAS values were calculated using Trolox as the calibration standard and expressed as mmol Trolox equivalent/L.

TOS was determined using a commercial assay kit according to the manufacturer's instructions and the method described by Erel[15]. In this assay, oxidant compounds present in the extracts oxidize the ferrous ion–chelator complex to ferric ions. Under acidic conditions, the resulting ferric ions form a colored complex with xylenol orange, and the color intensity is proportional to the total amount of oxidants in the sample. Absorbance was measured spectrophotometrically, and hydrogen peroxide was used as the calibration standard. The results were expressed as µmol H_2O_2 equivalent/L.

OSI was calculated to evaluate the balance between the total oxidant and antioxidant capacities of the extracts. Before calculation, TAS and TOS values were adjusted to compatible units. OSI was calculated using the following equation[16]:

$$\text{OSI}\ (\%)\ =\ \text{TOS}\ (\mu\text{mol}\ \text{H}_2\text{O}_2\ \text{equivalent/L})\ /\ [\text{TAS}\ (\text{mmol Trolox equivalent/L})\ \times\ 10]$$

The radical-scavenging capacity of the optimized extracts was determined using the DPPH assay[17]. The method is based on the reduction of the purple-colored DPPH radical by electron- or hydrogen-donating antioxidant compounds, resulting in a decrease in absorbance. Extract stock solutions were prepared in DMSO at a concentration of 1 mg/mL. For each measurement, 1 mL of the extract solution was mixed with 4 mL of a freshly prepared 0.004% (w/v) DPPH solution in methanol. The reaction mixtures were incubated for 30 min at room temperature under light-protected conditions. Absorbance was subsequently measured at 517 nm. Trolox was used as the calibration standard, and the antioxidant capacity was calculated from the corresponding calibration curve and expressed as mg Trolox equivalent per g of dry extract (mg TE/g dry extract).

The ferric reducing capacity of the optimized extracts was determined using the Ferric Reducing Antioxidant Power (FRAP) assay[17]. The method is based on the reduction of the ferric 2,4,6-tripyridyl-s-triazine (Fe^{3+}–TPTZ) complex to its blue-colored ferrous form (Fe^{2+}–TPTZ) by electron-donating antioxidant compounds. The FRAP working reagent was freshly prepared by combining 300 mM acetate buffer (pH 3.6), 10 mM TPTZ dissolved in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6\ H_2O$ at a volume ratio of 10:1:1, respectively. For each measurement, a 100 µL aliquot of the extract stock solution was added to 2 mL of the freshly prepared FRAP reagent. The reaction mixture was incubated at 37 °C for 4 min, after which the absorbance was recorded at 593 nm. Trolox was used as the calibration standard, and the reducing capacity was determined from the linear regression equation of the Trolox calibration curve and expressed as mg Trolox equivalent/g (mg TE/g).

Anticholinesterase activity tests

The cholinesterase inhibitory potential of the optimized mushroom extracts was determined using a modified Ellman colorimetric assay[18]. Acetylcholinesterase (AChE; Type VI-S, EC 3.1.1.7) and butyrylcholinesterase (BChE; EC 3.1.1.8) were employed as target enzymes, while galantamine hydrobromide served as the standard reference inhibitor. Extract solutions were prepared from the dried extracts at final concentrations ranging from 3.125 to 200 µg/mL, expressed on a dry-extract mass basis. All assays were performed in a microplate system using 0.1 M phosphate buffer (pH 8.0) as the reaction medium. Initially, enzyme and extract solutions were combined in the wells and pre-incubated for 10 min at 25 °C under dark conditions to stabilize the reaction environment. Subsequently, the enzymatic reaction was initiated by the addition of the appropriate substrate, acetylthiocholine iodide (ATCI) for AChE or butyrylthiocholine iodide (BTCI) for BChE, together with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). Hydrolysis of the thiocholine substrates released thiocholine, which reacted with DTNB to produce the yellow 5-thio-2-nitrobenzoate anion; absorbance was measured at 412 nm. Percentage inhibition was calculated relative to the enzyme control without inhibitor. IC_{50} values were determined from concentration–inhibition curves using nonlinear regression analysis in GraphPad Prism software and were expressed as µg/mL.

Antiproliferative activity analyses

The antiproliferative effects of the optimized mushroom extracts were investigated in vitro using three human cancer cell lines. Lung adenocarcinoma (A549), breast adenocarcinoma (MCF-7), and prostate carcinoma (DU-145) cells were employed as tumor models, all obtained from the American Type Culture Collection. Treatment solutions were prepared from the dried extracts at concentrations of 25, 50, 100, and 200 µg/mL on a dry-extract mass basis. Cells were maintained in suitable culture media under standard incubation conditions and were subcultured using Trypsin-EDTA upon reaching approximately 70–80% confluence. Following detachment, cells were seeded into culture plates and allowed to attach for 24 h. After the adhesion period, the culture medium was replaced with fresh medium containing the prepared extract solutions, and the cells were exposed to the treatments for an additional 24 h. Cellular metabolic activity was subsequently assessed using the MTT assay. The formazan crystals produced by metabolically active cells were dissolved in dimethyl sulfoxide (DMSO) prior to absorbance measurement. Absorbance was measured at 570 nm, and the resulting MTT absorbance values were used as an indicator of the cellular response to extract treatment[16].

Phenolic compound analyses

The phenolic composition of the optimized mushroom extracts was characterized using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) approach. In this analysis, a panel of 24 reference standards representing diverse phenolic classes was screened simultaneously to determine both the presence and concentrations of individual compounds. Chromatographic separation was achieved on a reversed-phase C18 column, with the column temperature maintained at 40 °C to ensure analytical stability. The mobile phase system consisted of two solvents: Phase A, comprising ultrapure water with 0.1% formic acid, and Phase B, consisting of LC-MS grade methanol also supplemented with 0.1% formic acid. The analyses were conducted at a constant flow rate of 0.3 mL/min, and each sample was introduced into the system with an injection volume of 2 µL. The selected chromatographic and detection parameters enabled efficient separation and accurate quantification of the phenolic constituents present in the extracts.

Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics software (version 21.0). Data are presented as mean ± standard deviation (SD) from three independently prepared extract replicates ($n = 3$). For comparisons

between two independent groups, an independent-samples t-test was used. Homogeneity of variances was evaluated using Levene's test, and Welch's t-test was applied when the assumption of equal variances was not satisfied. For comparisons involving more than two groups, one-way analysis of variance (ANOVA) was performed, followed by Duncan's multiple range test when a significant overall effect was detected. Statistical significance was accepted at $p < 0.05$.

Results and discussion

Optimization of extraction conditions

In this study, the extraction variables influencing the total antioxidant status (TAS) of the obtained extracts were systematically investigated using a controlled multifactorial experimental design. Within this framework, extraction temperature (30, 45, and 60 °C), extraction time (30, 45, and 60 min), and solvent composition (0%, 50%, and 100% ethanol/water) were selected as the primary factors. Extraction experiments were conducted under various combinations of these factor levels, and the TAS values of the resulting extracts were determined. The collected data were comparatively analyzed to elucidate how different extraction conditions influence antioxidant capacity. A detailed presentation of the obtained results is provided in Table 1.

When the combined influence of extraction temperature, duration, and solvent composition on the TAS of *S. lakei* extracts was examined, it became evident that antioxidant capacity is highly sensitive to both individual factors and their interactions. Among the tested conditions, an extraction time of 45 min consistently produced superior TAS values across different temperatures and solvent systems. The highest antioxidant capacity observed in this study was achieved at 45 °C for 45 min using 100% ethanol, yielding a TAS value of 5.776 ± 0.044 mmol/L. Notably, a comparable extraction performed under the same temperature and duration but with 50% ethanol also resulted in a relatively high TAS value (5.430 ± 0.042 mmol/L), suggesting that moderate temperature combined with an appropriate solvent composition is particularly effective for extracting antioxidant compounds. In general, extractions conducted at 45 °C demonstrated enhanced performance, with both 50% and 100% ethanol systems producing consistently high TAS values, especially at 30- and 45-minute durations. For instance, TAS values of 5.462 ± 0.023 mmol/L and 5.047 ± 0.042 mmol/L were recorded under 45 °C–30 min-100% ethanol and 45 °C–30 min-50% ethanol conditions, respectively. These findings indicate that moderate thermal conditions likely promote efficient diffusion of phenolic compounds into the solvent while preserving their structural integrity. In contrast, prolonging the extraction time to 60 min led to a marked decline in TAS values across most experimental conditions. The lowest antioxidant capacities were observed at 60 °C combined with 60 min of extraction, where TAS values decreased to 2.872 ± 0.028 mmol/L (100% ethanol) and 3.014 ± 0.028 mmol/L (50% ethanol). This pattern suggests that excessive heat exposure and prolonged processing may induce degradation of thermolabile antioxidant constituents. At lower temperature conditions (30 °C), TAS values exhibited a more moderate range. The highest value at this temperature (4.582 ± 0.039 mmol/L) was obtained using 100% ethanol for 45 min, indicating that solvent strength can partially compensate for reduced extraction efficiency at lower temperatures. However, these values remained below those achieved at 45 °C, reinforcing the importance of optimal thermal conditions.

Overall, the experimental data showed that TAS varied markedly across the tested extraction conditions, with the highest observed TAS value occurring at 45 °C, 45 min, and 100% ethanol. These findings support the use of multivariable optimization to identify extraction conditions associated with higher TAS responses.

To further evaluate process efficiency and identify optimal conditions, two modeling strategies were employed: RSM and artificial intelligence-based approaches. Within the RSM framework, candidate regression models were comparatively evaluated using model-fit statistics, and the quadratic model provided the best overall fit to the experimental TAS data. The quadratic model provided an R^2 value of 0.911 and an adjusted R^2 value of 0.864, indicating a good overall fit to the experimental TAS data. The fitted model indicated that the TAS response varied

Table 1 Total Antioxidant Status (TAS) values of *S. lakei* extracts obtained under different extraction conditions.

Experiment number	Ethanol/water ratio (%)	Extraction time (min)	Extraction temperature (°C)	TAS (mmol/L)
1	0	30	30	4.108 ± 0.033^{i}
2	0	45	30	4.425 ± 0.038^{g}
3	0	60	30	3.162 ± 0.033^{o}
4	50	30	30	4.397 ± 0.019^{g}
5	50	45	30	4.573 ± 0.040^{f}
6	50	60	30	3.459 ± 0.038^{m}
7	100	30	30	4.248 ± 0.029^{h}
8	100	45	30	4.582 ± 0.039^{ef}
9	100	60	30	3.327 ± 0.032^{n}
10	0	30	45	4.793 ± 0.037^{d}
11	0	45	45	5.132 ± 0.032^{c}
12	0	60	45	3.662 ± 0.034^{l}
13	50	30	45	5.047 ± 0.042^{c}
14	50	45	45	5.430 ± 0.042^{b}
15	50	60	45	4.231 ± 0.021^{h}
16	100	30	45	5.462 ± 0.023^{b}
17	100	45	45	5.776 ± 0.044^{a}
18	100	60	45	4.253 ± 0.024^{h}
19	0	30	60	4.645 ± 0.032^{e}
20	0	45	60	4.837 ± 0.021^{d}
21	0	60	60	3.722 ± 0.031^{l}
22	50	30	60	3.866 ± 0.023^{k}
23	50	45	60	4.148 ± 0.045^{i}
24	50	60	60	3.014 ± 0.028^{p}
25	100	30	60	3.892 ± 0.029^{k}
26	100	45	60	4.029 ± 0.029^{j}
27	100	60	60	2.872 ± 0.028^{q}

Values are presented as mean ± SD of three independently prepared extract replicates ($n = 3$). Means with different superscript letters in the same column are significantly different according to Duncan's multiple range test ($p < 0.05$).

as a function of the extraction conditions, with the quadratic model providing the best overall fit among the models evaluated. The performance of the quadratic model suggests that it adequately represented the relationships between the extraction variables and TAS within the experimental domain and was therefore used to estimate the optimum extraction conditions. Consequently, this model emerges as the most appropriate statistical approach for defining the optimal extraction conditions required to maximize the antioxidant potential of *S. lakei* extracts

$$TAS = 5.35 - 0.069\,X_1 - 0.486\,X_2 - 0.002\,X_3 + 0.001\,X_1X_2 - 0.239\,X_1X_3 - 0.012\,X_2X_3 - 0.903\,X_1^2 - 0.761\,X_2^2 + 0.033\,X_3^2$$

The quadratic regression model established in this study incorporates three primary variables governing the extraction process as independent factors: extraction temperature (X_1), extraction time (X_2), and solvent composition (X_3; ethanol/water ratio). The influence of these variables on the TAS of *S. lakei* extracts was analyzed using a multivariate framework that accounted for both main effects and interaction terms. This approach enabled the quantitative assessment of the relative contribution of each parameter to the observed antioxidant response. To facilitate interpretation, the results derived from the regression model were visualized through three-dimensional response surface plots (Fig. 2). The response surface plots illustrated how the predicted TAS response changed across combinations of the extraction variables and facilitated visualization of potential interaction patterns within the investigated experimental domain. These plots were also used to support interpretation of the model-derived optimum conditions.

In the advanced phase of the modeling framework, an Artificial Neural Network (ANN) was employed to more effectively capture the nonlinear interactions between extraction variables and the resulting biological response. Among the various network configurations tested, the architecture yielding the best performance was identified as a 3-4-1 structure. In this configuration, the input layer comprises three neurons corresponding to extraction

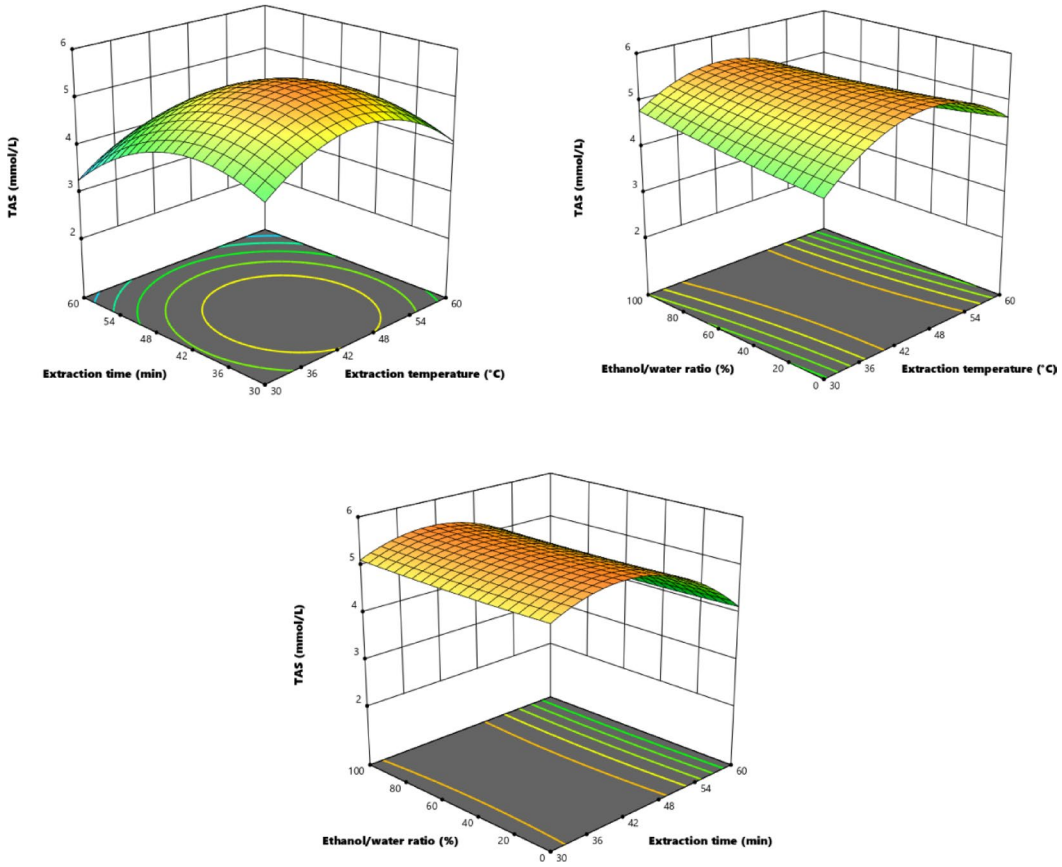


Fig. 2 Response surface plots.

temperature, extraction time, and solvent composition, while the hidden layer includes four neurons. The output layer consists of a single neuron representing the predicted TAS. The predictive performance of the ANN model was evaluated using MSE, MAPE, and the correlation coefficient. For the complete dataset, the selected 3-4-1 network yielded an MSE of 0.005, a MAPE of 1.423%, and an R value of 0.994. These performance metrics indicate close agreement between the experimentally observed and ANN-predicted TAS values within the dataset used for model development and internal assessment. To further refine the optimization process, a Genetic Algorithm (GA) was integrated with the ANN model. During this stage, different population sizes were evaluated, and a population size of 8 was used for the final GA optimization. Each optimization configuration was independently executed 30 times to reduce the influence of stochastic variation and to assess the consistency of the optimization process. The convergence behavior of the GA was monitored using iteration-based convergence curves (Fig. 3), and only minor changes in the best fitness value were observed after approximately the second iteration under the tested settings. This convergence pattern indicates early stabilization of the best fitness value under the tested GA settings but does not by itself demonstrate that the global optimum was reached.

Antioxidant activity

Mushroom-derived natural compounds, particularly phenolics, polysaccharides, and various secondary metabolites, offer significant biological potential in terms of antioxidant activity. The roles of these components in neutralizing free radicals and reducing oxidative stress make mushrooms a valuable resource for both nutritional sciences and pharmaceutical research[19,20]. In this context, the antioxidant properties of optimized extracts obtained from the *S. lakei* species were experimentally determined, and the findings are presented in Table 2.

Fig. 3 Convergence graph.

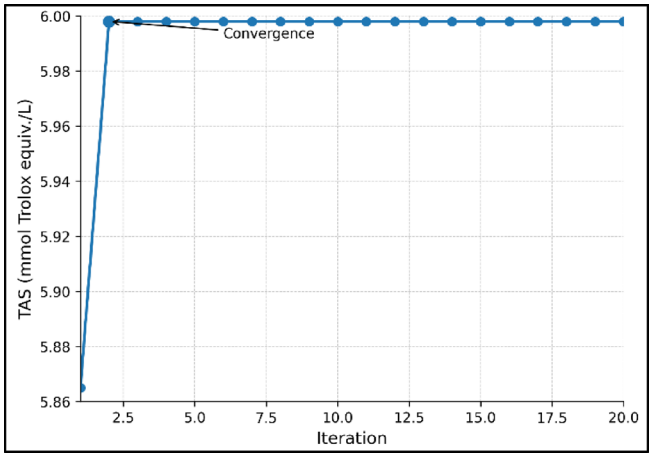


Table 2 Antioxidant parameters of the optimized *S. lakei* extracts.

Parameters	ANN-GA extract values	RSM extract values
TAS (mmol/L)	6.057 ± 0.090^{a}	5.373 ± 0.633^{a}
TOS (µmol/L)	10.692 ± 0.064^{b}	11.878 ± 0.068^{a}
OSI (TOS/(TAS*10))	0.177 ± 0.004^{a}	0.223 ± 0.028^{a}
DPPH (mg TE/g)	155.802 ± 2.724^{a}	127.999 ± 2.761^{b}
FRAP (mg TE/g)	192.685 ± 2.823^{a}	168.458 ± 2.213^{b}

Values are presented as mean ± SD of three independently prepared extract replicates ($n = 3$). Different superscript letters within the same parameter indicate statistically significant differences between the ANN-GA and RSM extracts ($p < 0.05$).

The antioxidant parameters differed between the extracts obtained under the ANN-GA- and RSM-derived conditions. The ANN-GA-optimized extract showed significantly higher DPPH and FRAP values and a significantly lower TOS value than the RSM-optimized extract. Although TAS was numerically higher in the ANN-GA-optimized extract, the difference was not statistically significant. These findings are consistent with reports in the literature indicating that *S. lakei*, especially its water extracts, exhibits high antioxidant activity[13]. The antioxidant activity reported in that study is consistent with the measurable antioxidant properties observed for *S. lakei* in the present investigation. In particular, the significantly higher DPPH and FRAP values obtained for the ANN-GA-optimized extract indicate differences in radical-scavenging and reducing capacity between the two optimized extracts. From this perspective, not only the nature of the extract but also the optimization of extraction parameters stands out as a critical factor in determining antioxidant capacity. No previous findings regarding the TAS, TOS, and OSI values of *S. lakei* have been found in the literature. In studies on different mushrooms, the TAS values of *Ramaria obtusissima (*Peck) Corner, *Hymenopellis radicata* (Relhan) R.H. Petersen, *Morchella importuna* M. Kuo, O'Donnell & T.J. Volk, and *Phylloporia ribis* (Schumach.) Ryvarden were reported as 6.638, 6.308, 5.266, and 6.092 mmol/L, respectively. The TOS values were reported as 10.134, 4.320, 8.144, and 13.389 µmol/L, respectively; OSI values were reported as 0.153, 0.068, 0.155, and 0.220[21–24]. The TAS value is considered an important parameter reflecting the total capacity of antioxidant compounds present in a mushroom[9]. In this context, when the TAS values of *S. lakei* extracts obtained in our study were examined, it was determined that the values obtained with ANN-GA and RSM methods were lower than those of *R. obtusissima*, *H. radicata*, and *P. ribis*, but higher than those of *M. importuna*. These differences reveal that the total antioxidant capacity can vary depending on the interspecies phenolic compound profile and extraction conditions. In particular, it is thought that the parameters optimized with ANN-GA and RSM are decisive in the preservation of antioxidant compounds and extraction efficiency.

In contrast, the evaluation of TOS values, which represent oxidative load, makes it possible to examine the extracts not only in terms of antioxidant but also oxidant components. The TOS value expresses the total level

of oxidant compounds present in the mushroom[9]. According to the results obtained in our study, the TOS values of both ANN-GA and RSM extracts of *S. lakei* were determined to be higher than those of *R. obtusissima*, *H. radicata*, and *M. importuna*, but lower than those of *P. ribis*. This suggests that *S. lakei* extracts may contain not only antioxidant compounds but also secondary metabolites that can exhibit a certain level of pro-oxidant effect. This kind of bidirectional effect can lead to different results in biological systems, especially depending on the concentration. The OSI value, used in the holistic assessment of oxidative stress, is an important indicator revealing the extent to which oxidant compounds are balanced by antioxidant compounds[9]. In our study, the OSI value of the ANN-GA extract of *S. lakei* was determined to be higher than that of *R. obtusissima*, *H. radicata*, and *M. importuna*, but lower than that of *P. ribis*. In contrast, the OSI value of the RSM extract was found to be higher than all compared species. Although the OSI value was numerically lower in the ANN-GA-optimized extract than in the RSM-optimized extract, this difference was not statistically significant. Therefore, the present data do not support a definitive conclusion that the ANN-GA-derived conditions produced a superior antioxidant–oxidant balance. The difference observed between the ANN-GA and RSM methods in our study should be considered not only as a numerical superiority but also as an indicator of how the optimization approach guides the chemical properties of the extract. Each of the variables in the extraction process, such as temperature, time, and concentration, directly affects the solubility, liberation from the matrix, and stability of antioxidant compounds throughout the process. The often nonlinear relationship between these variables makes it difficult to obtain the maximum yield, especially of phenolic and redox-active compounds[25,26]. The different extraction conditions identified by the ANN-GA and RSM approaches may have contributed to the observed differences in the measured antioxidant parameters, particularly the significantly higher DPPH and FRAP values and lower TOS value obtained for the ANN-GA-optimized extract. Overall, the ANN-GA-derived conditions were associated with significantly higher DPPH and FRAP values and a significantly lower TOS value, while the numerical differences in TAS and OSI did not reach statistical significance. While the RSM method yields somewhat effective results, it may be more limited in fine-tuning the determination of optimum conditions, especially in mushroom extracts where biological activity is shaped by the combined contribution of numerous compounds. Therefore, the current findings show that the selection of the optimization model cannot be considered independently in studies aimed at maximizing antioxidant activity, and that AI-assisted approaches are not only a technical tool but also a strategic element determining the functional quality of the extract. Under the tested experimental conditions, the ANN-GA-optimized extract exhibited significantly higher DPPH and FRAP values and a significantly lower TOS value than the RSM-optimized extract, whereas the differences in TAS and OSI were not statistically significant. These findings indicate an association between the ANN-GA-derived extraction conditions and the measured antioxidant profile, but they do not demonstrate a general improvement in biological efficacy beyond the evaluated endpoints.

Anticholinesterase activity

Inhibition of cholinesterase enzymes is considered an important approach in supporting cognitive functions by maintaining synaptic acetylcholine levels. Therefore, cholinesterase inhibitors derived from natural sources are attracting increasing interest, especially in research aimed at the management of neurodegenerative diseases. Mushrooms are considered among the potential natural agents that can exert an inhibitory effect on these enzymes due to their rich bioactive compound content[27]. In this context, the effects of optimized extracts obtained from *S. lakei* on acetylcholinesterase and butyrylcholinesterase enzymes were investigated, and the results obtained are presented in Table 3.

The anticholinesterase results showed significant differences in AChE inhibitory activity among the tested samples. For BChE, the ANN-GA extract yielded a measurable IC_{50} of 153.877 µg/mL, whereas 50% inhibition was not reached for the RSM extract within the tested concentration range. As expected, galantamine, used as the reference inhibitor, exhibited the lowest IC_{50} values for both enzymes. In contrast, the lower IC_{50} values observed for the ANN-GA-optimized extract compared with the RSM-optimized extract indicate greater AChE and BChE inhibitory activity under the tested assay conditions.

Table 3 Anticholinesterase activity of the optimized *S. lakei* extracts.

Sample	AChE µg/mL	BChE µg/mL
Galantamine	7.424 ± 0.392^{c}	15.438 ± 0.376^{c}
ANN-GA extract	98.590 ± 2.162^{b}	153.877 ± 2.454^{b}
RSM extract	123.768 ± 2.164^{a}	>200

Values are presented as mean ± SD ($n = 3$). For AChE, different superscript letters indicate statistically significant differences among the tested samples according to one-way ANOVA followed by Duncan's multiple range test ($p < 0.05$). For BChE, the RSM extract did not reach 50% inhibition within the tested concentration range and was therefore reported as IC_{50} >200 µg/mL and excluded from statistical comparison.

No previous findings regarding the anticholinesterase activity of *S. lakei* have been found in the literature. However, studies on species belonging to the same genus show that this group may have enzyme inhibitory potential. In this context, a study conducted on *Suillus luteus* (L.) Roussel reported that this species exhibited a certain level of anticholinesterase activity[28]. The present findings provide evidence of measurable in vitro cholinesterase inhibitory activity in *S. lakei* extracts, although the specific compounds responsible for this activity were not identified. In our study, the ANN-GA-optimized extract showed lower IC_{50} values for both AChE and BChE than the RSM-optimized extract. However, the present experiments do not establish whether this difference resulted from greater recovery of specific inhibitory compounds or from other compositional differences between the extracts. It is known that mushroom-derived phenolics, flavonoids, and various secondary metabolites interact with cholinesterase enzymes and exhibit inhibitory effects[29,30]. The differences in inhibitory activity may be related to compositional differences arising from the extraction conditions; however, the present experiments did not determine the recovery, stability, or individual contributions of specific cholinesterase-inhibitory compounds. The higher IC_{50} values of extracts obtained using the RSM method suggest that while this method provides a certain level of effectiveness in determining extraction parameters, it is more limited in maximizing the extraction of compounds contributing to enzyme inhibition. Especially in multi-component and complex mushroom extracts, considering that biological activity depends not on a single component but on synergistic interactions[31], the extent to which the optimization model can capture these interactions becomes a critical factor. Overall, the ANN-GA-optimized extract exhibited lower IC_{50} values for both AChE and BChE under the conditions of the present in vitro assays. These findings are restricted to the measured enzyme inhibition endpoints and do not establish enhanced pharmacological efficacy.

Antiproliferative activity

Controlling cell proliferation is one of the fundamental research areas in cancer biology and treatment strategies. In this context, the effects of bioactive compounds obtained from natural sources on tumor cells are attracting increasing attention; fungal metabolites, in particular, stand out due to their potential to modulate cellular growth and proliferation processes[32]. The complex compound profile of fungal extracts can contribute to the emergence of cytotoxic and antiproliferative effects on different cancer cell lines[33]. In this framework, the antiproliferative effects of optimized extracts obtained from *S. lakei* were evaluated on various human cancer cell lines, and the findings are presented in Fig. 4.

In the present study, the MTT assay showed that the cellular response to S. lakei extracts varied according to extract type and concentration. No statistically significant difference in MTT absorbance was observed between the untreated control and DMSO vehicle-control groups in A549, MCF-7, or DU-145 cells, indicating that the DMSO concentration used did not measurably affect the MTT response under the present experimental conditions. Both extracts produced a concentration-dependent reduction in MTT absorbance in the tested cell lines. However, the severity and trend of this decrease differed according to the optimization method used. It is noteworthy that the extracts optimized with ANN-GA exhibited a more pronounced antiproliferative effect, especially at high concentrations (100 and 200 µg/mL), compared to RSM extracts. This observation indicates that the extract obtained under the ANN-GA-derived conditions was associated with a greater reduction in MTT

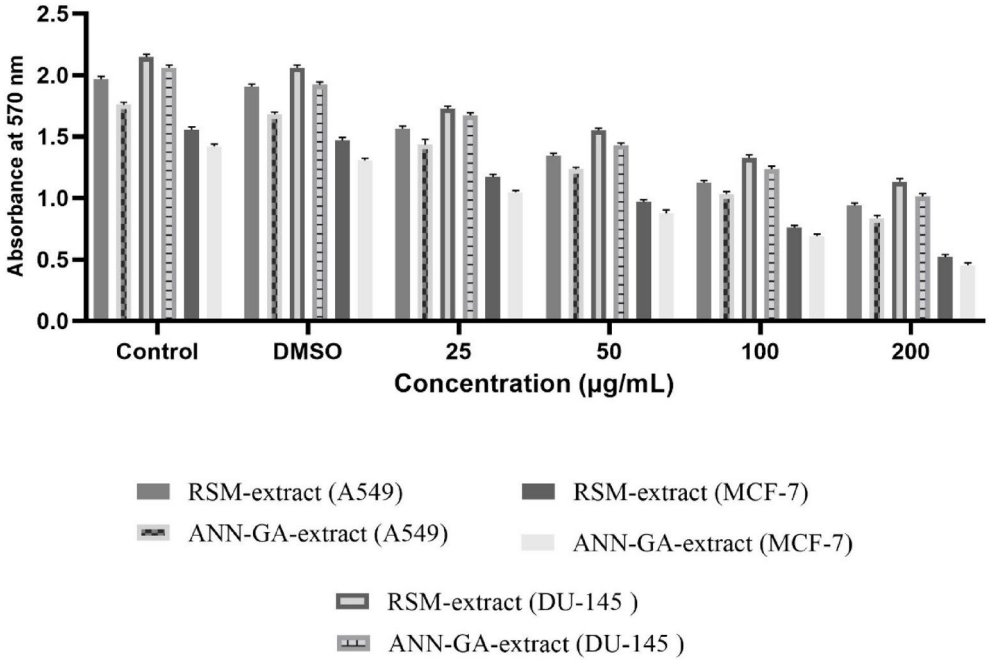


Fig. 4 MTT absorbance responses of A549, MCF-7, and DU-145 cells treated with the optimized *S. lakei* extracts. (In cell culture experiments, the control group was incubated only under standard culture medium conditions without exposure to any chemicals. In the DMSO group, created to reveal the possible effects of the solvent, only a medium containing DMSO was used without the application of the extract. In the experimental groups, dose-dependent biological responses were evaluated by treating the cells with extracts prepared at concentrations of 25, 50, 100, and 200 µg/mL).

absorbance, particularly at the higher tested concentrations, than the RSM-optimized extract ngly, the present results show only that the extract; however, antiproliferative activity was not an optimization target, and the mechanism underlying this difference was not investigated.

No direct evidence regarding the antiproliferative activity of *S. lakei* has been found in the literature. However, studies on species belonging to the same genus show that this group has anticancer potential. In this context, the methanolic extract of *S. luteus* has been reported to be effective against NCI-H460 (lung), AGS (stomach), MCF-7 (breast), and HCT-15 (colon) cancer cell lines[34]. Similarly, suillin, a compound isolated from *Suillus placidus (*Bonord.) Singer, has been reported to show a significant antiproliferative effect against HepG2 human hepatoma cells[35]. Furthermore, it has been determined that the petroleum ether extract of *Suillus flavus* (Quél.) Singer exhibits an inhibitory effect on the H446 small cell lung cancer cell line[36]. When these studies are considered together, it is understood that different species of the *Suillus* genus can be effective on various cancer cell lines, and this effect is largely related to the chemical composition of the extract. In addition, the degree of proliferation reduction observed in A549, MCF-7, and DU-145 cells may be related to the biological characteristics of each cell line and its sensitivity to the extract. In particular, differences in the metabolic activities, membrane permeabilities, and redox balances of cancer cell lines can directly shape the intracellular effects of the extract components[37,38]. Therefore, the fact that ANN-GA extracts show a stronger effect on some cell lines suggests that the phenolic and other secondary metabolites in the composition of these extracts act through cell line-specific mechanisms. The dose-dependent increase in antiproliferative effect indicates that the bioactive compounds in the extract can suppress cellular processes above a certain threshold. However, the limited effect observed at low concentrations may be related to the adaptive response mechanisms of the cells. At high concentrations, this balance is disrupted to the detriment of the cells, and proliferation is significantly inhibited. When considered together with the antioxidant and anticholinesterase results, these findings indicate differences in the measured in vitro biological profiles of the two extracts. Especially in multi-component natural products, considering that

Table 4 Phenolic contents of the optimized *S. lakei* extracts.

Phenolic compounds	LOD	LOQ	ANN-GA extract (µg /kg)	RSM extract (µg/kg)
Catechin hydrate	7.64	25.47	188.13 ± 2.77	ND
Syringic acid	41.83	139.43	790.49 ± 2.50[a]	546.07 ± 2.62[b]
Gallic acid	22.88	76.25	3566.94 ± 3.10[a]	3214.39 ± 3.95[b]
Protocatechuic acid	7.17	23.90	1073.79 ± 2.36[a]	695.57 ± 3.44[b]
4-hydroxybenzoic acid	15.30	51.10	401.88 ± 1.62[a]	116.54 ± 1.30[b]
Caffeic acid	81.80	272.67	938.61 ± 2.56[a]	552.08 ± 2.23[b]
2-hydroxycinnamic acid	7.79	25.98	281.51 ± 2.23[a]	77.16 ± 1.45[b]
Quercetin	68.40	228.10	3178.93 ± 3.09[a]	2794.70 ± 4.61[b]

Values are presented as mean ± SD of three independently prepared extract replicates ($n = 3$). Different superscript letters within the same row indicate statistically significant differences between the ANN-GA and RSM extracts ($p < 0.05$). ND, not detected.

the biological effect depends not on a single component but on synergistic interactions, the extent to which the optimization approach preserves these interactions becomes a critical factor. Accordingly, the present results show only that the extract prepared under the ANN-GA-derived conditions produced a greater reduction in MTT absorbance than the RSM-optimized extract under the specific in vitro conditions tested. No mechanistic experiments were performed to determine the cellular pathways responsible for this difference.

Phenolic contents

Phenolic compounds are among the key components determining the biological activity potential of fungi, and play a critical role, particularly in the manifestation of antioxidant properties[39,40]. In this context, the phenolic component profiles of optimized *S. lakei* extracts were analyzed using LC-MS/MS techniques, and the findings are presented in Table 4.

The results obtained in our study revealed differences in the quantified phenolic profiles of the S. lakei extracts obtained under the ANN-GA- and RSM-derived conditions. For the compounds quantified in both extracts, higher concentrations were measured in the ANN-GA-optimized extract than in the RSM-optimized extract. The increase observed especially in compounds closely related to biological activity, such as gallic acid, quercetin, and caffeic acid, may be directly related to the strengthening of the functional capacity of the extract.

The extraction yield of phenolic compounds is extremely sensitive to extraction conditions, and the interactive effects of parameters such as temperature, time, and solvent concentration play a decisive role in this process. Differences in temperature, extraction time, and ethanol concentration between the ANN-GA- and RSM-derived conditions may have contributed to the observed differences in phenolic composition. However, because individual compound recovery and total extraction yield were not directly determined, the present data do not allow conclusions regarding extraction efficiency or recovery yield. Catechin hydrate was detected and quantified in the ANN-GA-optimized extract but was not detected in the RSM-optimized extract under the applied analytical conditions. This observation indicates a difference in the measurable phenolic profiles of the two extracts. Since the extraction of flavonoid compounds is particularly sensitive to parameters such as solvent polarity and processing temperature[41], it is thought that the optimum conditions determined by ANN-GA support the solubility and stability of such compounds. Considering the relationship between phenolic compounds and biological activity, the obtained results appear to be consistent with findings of antioxidant and antiproliferative activity. Indeed, compounds such as gallic acid, caffeic acid, and quercetin are known for their strong antioxidant and anticancer properties[42–44], and the higher concentrations of these compounds in ANN-GA extracts may explain the higher biological activity of these extracts. This reveals that the biological effect of the extract depends not only on the total phenolic content but also on the diversity of compounds and synergistic interactions. Overall, the ANN-GA-optimized extract contained higher concentrations of the quantified phenolic compounds than the RSM-optimized extract under the analytical conditions used. These findings suggest that AI-assisted optimization may help guide the chemical profile of natural product extracts and support the preliminary selection of extraction

conditions for obtaining candidate bioactive ingredients. However, further validation is required before such extracts can be considered for functional food, nutraceutical, or pharmaceutical applications.

Although differences were observed between the ANN-GA and RSM-optimized extracts in the measured chemical and in vitro biological parameters, the findings should be interpreted within the limitations of the present experimental design. First, the biological and chemical measurements were based on three independent measurements ($n=3$), which limits the extent to which variability and reproducibility can be assessed. Second, the biological evaluations were restricted to in vitro antioxidant, anticholinesterase, and cell viability assays and therefore cannot be directly extrapolated to in vivo efficacy, safety, or physiological relevance. Third, no mechanistic validation studies were performed to determine the molecular or cellular pathways responsible for the observed differences between the extracts. Accordingly, the present results demonstrate differences in the measured outcomes under the tested experimental conditions but do not establish that ANN-GA optimization directly enhances biological or pharmacological efficacy. Further studies with greater experimental replication and appropriate mechanistic and in vivo validation are required, together with evaluations of safety, bioaccessibility, food-matrix compatibility, residual solvent content, and stability during processing and storage.

Conclusion

In this study, RSM and ANN-GA approaches were comparatively applied to optimize the extraction conditions of *S. lakei* using TAS as the primary response variable. The chemical composition and other in vitro biological activities of the extracts obtained under the resulting optimum conditions were subsequently evaluated as secondary outcomes. Compared with the RSM-optimized extract, the ANN-GA-optimized extract exhibited significantly higher DPPH and FRAP values and a significantly lower TOS value, whereas the differences in TAS and OSI were not statistically significant. It also showed lower IC_{50} values against AChE and BChE and produced greater concentration-dependent reductions in MTT absorbance, particularly at higher treatment concentrations. LC-MS/MS analysis further demonstrated that the ANN-GA-optimized extract contained higher concentrations of the quantified phenolic compounds, including gallic acid, quercetin, and caffeic acid. These findings indicate that ANN–GA is not merely an alternative predictive model but a useful decision-support approach for identifying extraction conditions associated with targeted functional characteristics. Its ability to model complex nonlinear interactions among extraction temperature, duration, and solvent composition may offer an advantage in the development and standardization of mushroom-derived extracts intended as potential functional ingredients. The main limitations of the present study include the limited experimental replication ($n=3$), reliance on in vitro assays, absence of mechanistic and in vivo validation, and lack of evaluation of the optimized extracts in an actual food matrix. From a practical perspective, the next stage should first validate the identified optimum conditions using additional independent extraction batches and greater experimental replication to determine their reproducibility. Subsequent studies should evaluate residual solvent levels, chemical stability during processing and storage, bioaccessibility, and safety, together with mechanistic and in vivo investigations of the observed biological responses. For potential food applications, the optimized extracts should then be incorporated into representative food matrices to determine effective incorporation levels, sensory compatibility, interactions with food components, and retention of the measured antioxidant and phenolic characteristics during processing and storage. Finally, pilot-scale extraction studies should assess whether the ANN-GA-derived conditions remain reproducible when the process is transferred beyond the laboratory scale. These sequential validation steps will be necessary to determine whether the present in vitro and ingredient-level findings have relevance for future functional food research. Accordingly, ANN-GA-guided extraction may be useful for identifying extraction conditions associated with selected chemical and in vitro biological characteristics of *S. lakei* extracts, but further safety, formulation, and in vivo validation is required before any functional food application can be considered.

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Data availability Data are available upon request.

Declarations

Competing interests The authors declare no competing interests.

Consent for publication All authors have approved the fnal article and consented to publication.

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References

1. Sevindik, M., Bal, C., Eraslan, E. C., Uysal, I. & Mohammed, F. S. Medicinal mushrooms: a comprehensive study on their antiviral potential. *Prospects Pharm. Sci.* **21**, 42–56 (2023).
2. Cirlincione, F. et al. Antioxidant and aromatic properties of aqueous extracts of pleurotus nebrodensis as potential food ingredients. *Foods* **15**, 296 (2026).
3. Demei, M. & Fansheng, C. Edible fungi: processing, storage preservation, disease control, and potential bioactivities. *Foods* **14**, 3755 (2025).
4. Łysakowska, P., Sobota, A. & Wirkijowska, A. Medicinal mushrooms: Their bioactive components, nutritional value and application in functional food production—A review. *Molecules* **28**, 5393 (2023).
5. Spigno, G., Tramelli, L. & De Faveri, D. M. Effects of extraction time, temperature and solvent on concentration and antioxidant activity of grape marc phenolics. *J. Food Eng.* **81**, 200–208 (2007).
6. Casagrande, M. et al. Influence of time, temperature and solvent on the extraction of bioactive compounds of Baccharis dracunculifolia: In vitro antioxidant activity, antimicrobial potential, and phenolic compound quantification. *Ind. Crops Prod.* **125**, 207–219 (2018).
7. Ianni, F. et al. Extraction optimization by experimental design of bioactives from Pleurotus ostreatus and evaluation of antioxidant and antimicrobial activities. *Processes* **9**, 743 (2021).
8. Du, B. et al. Optimization extraction and antioxidant activity of crude polysaccharide from chestnut mushroom (Agrocybe aegerita) by accelerated solvent extraction combined with response surface methodology (ASE-RSM). *Molecules* **27**, 2380 (2022).
9. Gürgen, A. & Sevindik, M. Application of artificial neural network coupling multiobjective particle swarm optimization algorithm to optimize Pleurotus ostreatus extraction parameters. *J. Food Process. Preserv.* **46**, e16949 (2022).
10. Szczepkowski, A. & Olenderek, T. Suillus lakei (Murrill) AH Sm. and Thiers (Boletales, Basidiomycota) in Poland: new data. *Acta Mycol.* **52** (2017).
11. Pietras, M., Litkowiec, M. & Gołębiewska, J. Current and potential distribution of the ectomycorrhizal fungus Suillus lakei ((Murrill) AH Sm. & Thiers) in its invasion range. *Mycorrhiza* **28**, 467–475 (2018).
12. Durall, D., Todd, A. & Trappe, J. Decomposition of 14 C-labelled substrates by ectomycorrhizal fungi in association with Douglas fir. *New Phytol.* **127**, 725–729 (1994).
13. Barranco, P. G. et al. Evaluation of antioxidant, immunomodulating, cytotoxic and antimicrobial properties of different strains of Basidiomycetes from Northeastern Mexico. *J. Med. Plants Res.* **4**, 1762–1769 (2010).
14. Erel, O. A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. *Clin. Biochem.* **37**, 277–285 (2004).
15. Erel, O. A new automated colorimetric method for measuring total oxidant status. *Clin. Biochem.* **38**, 1103–1111 (2005).

16. Sevindik, M. Evaluation of antioxidant, anticholinesterase and antiproliferative potential of Artemisia herba-alba by artificial intelligence-assisted extraction optimization. *Sci. Rep.* **15**, 37985 (2025).
17. Ünal, O. et al. Optimization of Phellinus hartigii extracts: Biological activities, and phenolic content analysis. *BMC Complement. Med. Ther.* **25**, 113 (2025).
18. Ellman, G. L., Courtney, K. D., Andres Jr, V. & Featherstone, R. M. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* **7**, 88–95 (1961).
19. Aliaño-González, M. J. et al. Ultrasound-assisted extraction of total phenolic compounds and antioxidant activity in mushrooms. *Agronomy* **12**, 1812 (2022).
20. Kumla, J., Lumyong, S., Suwannarach, N. C. Composition bioactive compounds, antioxidant and α-glucosidase inhibitory activities of commercial edible oyster mushrooms at different maturity stages in Northern Thailand. *Foods* **14**, 3511 (2025).
21. Karaltı, İ., Sevindik, M. & Akata, I. Artificial intelligence assisted optimization of *Ramaria obtusissima* extracts and their integrated chemical and biological characterization. *Molecules* **31**, 870 (2026).
22. Gürgen, A. et al. Optimization of Hymenopellis radicata extracts using RSM and ANN-GA and evaluation of biological activities. *Sci. Rep.* (2026).
23. Akata, I. et al. RSM and ANN-GA assisted optimization of Morchella importuna extracts and their effects on antioxidant anticholinesterase and antiproliferative activity. *Sci. Rep.* (2025).
24. Korkmaz, A. F., Gürgen, A., Krupodorova, T., Sevindik, M. & Akata, I. Artificial intelligence-assisted optimization of extraction enhances the biological activity of Phylloporia ribis. *Sci. Rep.* **15**, 41206 (2025).
25. Elshreef, H., Mirghani, M., Sulaiman, S. & Jami, M. *IOP Conference Series: Earth and Environmental Science.* 012109 (IOP Publishing).
26. de Marsiglia, L. Thermal stability of total phenolic compounds and antioxidant activities of jaboticaba peel: Effect of solvents and extraction methods. *J. Indian Chem. Soc.* **100**, 100995 (2023).
27. Jiang, X. et al. Mushroom-derived bioactive components with definite structures in alleviating the pathogenesis of Alzheimer's disease. *Front. Pharmacol.* **15**, 1373660 (2024).
28. Andrade, J. M., Pachar, P., Trujillo, L., Cartuche, L. & Suillin A mixed-type acetylcholinesterase inhibitor from Suillus luteus which is used by Saraguros indigenous, southern Ecuador. *PLoS One*. **17**, e0268292 (2022).
29. Nguyen, T. K., Im, K. H., Choi, J., Shin, P. G. & Lee, T. S. Evaluation of antioxidant, anti-cholinesterase, and anti-inflammatory effects of culinary mushroom Pleurotus pulmonarius. *Mycobiology* **44**, 291–301 (2016).
30. da Silva, D. F. et al. Phytochemical screening, phenolic and flavonoid contents, psilocybin, antioxidant, and acetylcholinesterase inhibition activities of the aqueous extract from the fungi Cyathus striatus, Laternea dringii, and Marasmius haematocephalus. *Brazilian J. Sci.* **3**, 39–50 (2024).
31. Bhambri, A., Srivastava, M., Mahale, V. G., Mahale, S. & Karn, S. K. Mushrooms as potential sources of active metabolites and medicines. *Front. Microbiol.* **13**, 837266 (2022).
32. Xu, J. et al. Current advancements in antitumor properties and mechanisms of medicinal components in edible mushrooms. *Nutrients* **14**, 2622 (2022).
33. Sevindik, M. et al. The role of medicinal mushrooms in cancer treatment: bioactive compounds and therapeutic potential. *Int. J. Med. Mushrooms*. **27**, 1–24 (2025).
34. dos Santos, T. et al. Suillus luteus methanolic extract inhibits cell growth and proliferation of a colon cancer cell line. *Food Res. Int.* **53**, 476–481 (2013).
35. Liu, F. et al. Suillin from the mushroom Suillus placidus as potent apoptosis inducer in human hepatoma HepG2 cells. *Chemico-Biol. Interact.* **181**, 168–174 (2009).
36. Zhao, J. X. et al. Iso-suillin from Suillus flavus induces apoptosis in human small cell lung cancer H446 cell line. *Chin. Med. J.* **129**, 1215–1223 (2016).
37. Montenegro, I. et al. Chemical composition, antioxidant and anticancer activities of Leptocarpha rivularis dc flower extracts. *Molecules* **26**, 67 (2020).
38. Lo Galbo, V. et al. Redox imbalance and mitochondrial release of apoptogenic factors at the forefront of the antitumor action of mango peel extract. *Molecules* **26**, 4328 (2021).
39. Bach, F. et al. Bio compounds of edible mushrooms: In vitro antioxidant and antimicrobial activities. *Lwt* **107**, 214–220 (2019).
40. Chu, M. et al. LC-ESI-QTOF-MS/MS characterization of phenolic compounds in common commercial mushrooms and their potential antioxidant activities. *Processes* **11**, 1711 (2023).
41. Wang, J. et al. Extraction and purification of total flavonoids from Zanthoxylum planispinum Var. Dintanensis leaves and effect of altitude on total flavonoids content. *Sci. Rep.* **15**, 7080 (2025).
42. Mohammed, F. S., Sevindik, M., Uysal, İ. & Sabik, A. E. Quercetin: derivatives, biosynthesis, biological activity, pharmacological and therapeutic effects. *Prospects Pharm. Sci.* **21**, 49–56 (2023).
43. Şabik, A. E., Mohammed, F. S., Sevindik, M., Uysal, I. & Bal, C. Gallic acid: derivatives and biosynthesis, pharmacological and therapeutic effect, biological activity. *Bull. Univ. Agricultural Sci. Veterinary Med. Cluj-Napoca Food Sci. Technol.* **81**, 18–27 (2024).

44. Yazar, M., Sevindik, M., Uysal, I. & Polat, A. O. Effects of caffeic acid on human health: pharmacological and therapeutic effects, biological activity and toxicity. *Pharm. Chem. J.* **59**, 44–55 (2025).

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