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Optimization of *Stropharia rugosoannulata* Extracts Using Artificial Intelligence and Response Surface Methodology and Comparative Evaluation of Their Biological Activities

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Abstract

Stropharia rugosoannulata Farl. ex Murrill is an edible mushroom species recognized for its nutritional value and potential functional properties. In this study, extraction conditions were optimized to maximize the biological potential of its extracts. Ultrasonically assisted extraction parameters, including temperature, extraction time, and ethanol/water ratio, were optimized using two advanced modeling approaches: Response Surface Methodology (RSM) and Artificial Neural Network–Genetic Algorithm (ANN-GA). The biological activities of the optimized extracts were evaluated through antioxidant assays (TAS, TOS, OSI, DPPH, and FRAP), cholinesterase inhibition tests (AChE and BChE), and antiproliferative activity against A549, MCF-7, and DU-145 cancer cell lines. Chemical characterization of the extracts was performed by LC-MS/MS for phenolic compounds and by GC-MS for volatile and semi-volatile constituents. The results indicated that the RSM optimized extract exhibited stronger biological performance than the ANN-GA extract, including higher antioxidant capacity, more pronounced cholinesterase inhibition, and greater antiproliferative activity. LC-MS/MS analysis revealed higher concentrations of major phenolic compounds such as gallic acid, caffeic acid, protocatechuic acid, and quercetin in the RSM extract, while GC-MS analysis showed differences in the distribution of terpenoid and fatty

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acid-derived compounds between the extracts. These findings demonstrate that extraction parameters significantly influence both the chemical composition and biological activity of mushroom extracts and highlight *S. rugosoannulata* as a promising natural source of bioactive compounds for functional food and natural product development.

Keywords: Ultrasonic-assisted extraction; bioactive compounds; cholinesterase inhibition; cytotoxicity; phenolic profiling; oxidative stress; LC-MS/MS; GC-MS

Introduction

Medicinal mushrooms are currently considered not only for their ethnomycological uses but also as natural resources with high biological value in the fields of nutritional science, pharmaceutical chemistry, and biotechnology. These organisms offer a matrix rich in highly bioavailable proteins, structural and functional polysaccharides, dietary fiber, B vitamins, and essential micronutrients such as potassium, selenium, and zinc, while their low lipid and energy content provides advantages in terms of healthy nutrition [1,2]. However, the therapeutic potential of medicinal mushrooms is primarily determined by secondary metabolites such as β-glucans, phenolic compounds, flavonoids, triterpenoids, and sterols. The multifaceted biological effects of these components, such as immune response modulation, suppression of oxidative stress, microbial inhibition, and limitation of cell proliferation, have been extensively reported in the literature [3]. However, the extent to which these effects occur largely depends on the optimization of the methods and parameters applied during the extraction process [4].

Stropharia rugosoannulata Farl. ex Murrill (wine-cap) is among the edible mushroom species recommended by the FAO, especially for developing countries, due to its high biomass yield, ease of cultivation, and low production costs. Studies have shown that this species is not only a nutritionally rich food source but also plays important ecological roles in soil improvement and the recovery of organic waste. *S. rugosoannulata* is rich in protein and dietary fiber, as well as B vitamins, and is described as a mineral-balanced and low-fat food, particularly due to its high potassium and low sodium content [5-8]. Furthermore, its rich chemical profile in terms of polyphenols, flavonoids, sterols, triterpenoids, and aromatic compounds enhances both the nutritional and functional value of this species [6,9-11]. It has been reported to be hallucinogenic and a safe edible mushroom due to the absence of biosynthetic genes responsible for psilocybin

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production [6-8]. When grown using agricultural residues, pesticide and heavy metal content has been reported to remain below regulatory limits [8,9]. Beyond its nutritional properties, polysaccharides and other extract fractions derived from *S. rugosoannulata* are reported to exhibit significant biological activities. These extracts have been shown to have antioxidant, antitumor, antihyperglycemic, and immunomodulatory effects, particularly enhancing macrophage activation and cytokine release via TLR4/NF-κB signaling pathways [5,6,9,12]. These findings suggest that *S. rugosoannulata* is a strong candidate for functional foods and fungal-derived bioactive products.

The main objective of this study is to determine the extraction conditions using advanced optimization approaches to ensure the efficient recovery of bioactive components obtained from *S. rugosoannulata*, which has high nutritional, functional, and ecological potential. In this context, optimization of extraction parameters was performed using response surface methodology (RSM) and artificial neural network-genetic algorithm (ANN-GA) models; the performance of both models on bioactive component yield and biological activity was evaluated comparatively. The antioxidant, anticholinesterase, and antiproliferative activities, as well as the phenolic content of the optimized extracts, were determined, revealing the decisive role of extraction strategies on biological activity. Thus, the study aims to scientifically evaluate the potential of *S. rugosoannulata* for the development of functional foods and mushroom-derived bioactive products by considering statistical and artificial intelligence-based optimization approaches within a holistic framework. The novelty of this study is that, unlike previous work focusing mainly on the protein fraction of *S. rugosoannulata*[13], it comparatively optimizes whole mushroom extracts using RSM and ANN-GA and evaluates their antioxidant, anticholinesterase, antiproliferative, LC-MS/MS, and GC-MS profiles together.

Materials and Methods

The sampling was conducted in non-protected, publicly accessible areas in Antalya province, Türkiye, where no specific collection permit is required under current national regulations. Therefore, no special permission was necessary for sample collection. Following the collection process, the samples were preserved under appropriate conditions to ensure the preservation of taxonomic characteristics and transferred to the laboratory. Fresh mature basidiomata of *S. rugosoannulata* were used as the fungal material in this study. Species identification was carried out by Prof. Dr. Ilgaz Akata and Prof. Dr. Mustafa Sevindik based on detailed macro and

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micromorphological examination. Diagnostic macromorphological characters included the reddish-brown convex to plane pileus, crowded adnate lamellae becoming dark purplish-brown at maturity, and a long whitish stipe with a persistent rugose annulus. Micromorphological examination revealed ellipsoid, smooth, thick-walled basidiospores with a distinct germ pore and a dark purplish-brown to purplish-black spore print, consistent with the diagnostic characteristics of the species. Identification was further confirmed by comparison with the taxonomic key and detailed species description of *S. rugosoannulata* provided by Cortez and Silveira[14], with the examined specimen fully matching the diagnostic macro- and micromorphological characters reported for the species. The voucher specimen was recorded under collector number MS-IA-378. The obtained materials were delivered to the Ankara University fungarium to establish a reference collection and were included in the collection in accordance with the acceptance, registration, and long-term storage procedures of the relevant collection. The fungal material used in this study was obtained from multiple mature basidiomata of *S. rugosoannulata* collected from the same locality during the same sampling period. After taxonomic identification, the fruiting bodies were cleaned, combined, and homogenized to obtain a representative bulk sample for extraction and subsequent analyses. Therefore, the experimental material represents a pooled sample prepared from multiple individual basidiomata rather than a single fruiting body. The optimized extraction procedures were carried out independently under the conditions predicted by the RSM and ANN-GA models. For each optimized extract, analytical measurements were performed in triplicate. Accordingly, the reported n = 3 values refer to technical/analytical replicates performed for each extract, unless otherwise stated, and the results are presented as mean ± standard deviation. All samples were stored under standard fungarium conditions for further identification and analytical studies.

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Chemicals, Reagents, Culture Media, and Reference Standards

All chemicals, reagents, culture media, and reference standards used in the study were of analytical grade. TAS and TOS assay kits were obtained from Rel Assay Diagnostics (Gaziantep, Türkiye). DPPH, TPTZ, DTNB, acetylcholinesterase, butyrylcholinesterase, acetylcholine iodide, butyrylcholine iodide, galantamine hydrobromide, Trolox, and phenolic reference standards used for LC-MS/MS analysis were commercially supplied by Sigma-Aldrich/Merck (Darmstadt, Germany) or equivalent certified suppliers. Cell culture media, fetal bovine serum, antibiotics, trypsin-EDTA, MTT reagent, and DMSO used in antiproliferative assays were obtained from

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standard commercial cell culture suppliers. All solvents used for chromatographic analyses were LC-MS or GC-MS grade.

Extraction Procedure

In order to simultaneously evaluate the critical parameters determining extraction performance, the study was designed within a multivariate experimental framework. In this context, temperature, processing time, and solvent ratio were selected as independent factors defining the extraction process, and a three-stage level structure was created for each factor. The experimental design was arranged to allow the quantitative demonstration of both individual factor effects and inter-factor interactions; accordingly, 27 different experimental conditions were applied. Extraction processes were performed using an ultrasonically assisted system, with temperature (30-60 °C), time (30-60 min), and ethanol content (0-100%) controlled within predetermined ranges. For each extraction run, 10 g of dried and homogenized mushroom material was used as the initial sample weight. The extraction yield was not calculated in the present study, since the optimization process was primarily based on TAS response rather than extract mass recovery. TAS was selected as the primary response variable because it represents the overall antioxidant status of the extract and reflects the cumulative contribution of different antioxidant constituents. Since oxidative balance is closely associated with several biological activities of mushroom extracts, TAS was considered a suitable screening criterion for determining the initial optimum extraction conditions. Other biological activities, including DPPH, FRAP, TOS/OSI, anticholinesterase, and antiproliferative effects, were subsequently evaluated to compare and validate the biological performance of the optimized extracts. However, multi-response optimization may provide a broader optimization strategy and should be considered in future studies. Experimental outputs were first analyzed using Response Surface Methodology (RSM) to statistically predict optimum conditions, then, Artificial Neural Network and Genetic Algorithm (ANN-GA) based optimization approaches were applied to improve modeling accuracy and predictive power. This integrated approach provided a comprehensive optimization framework that accurately describes the complex and nonlinear effects of extraction parameters. The overall experimental workflow and comparative optimization strategy applied in this study are summarized schematically in Figure 1.

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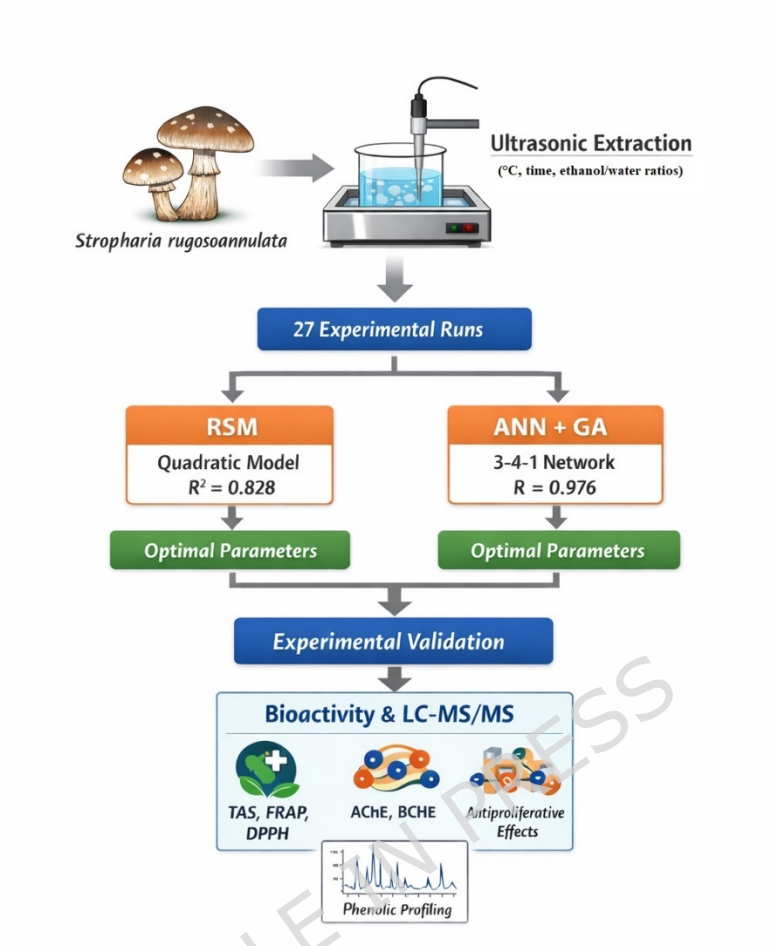


Figure 1. Schematic representation of the experimental workflow and comparative optimization strategy applied for ultrasonic extraction of *Stropharia rugosoannulata*. The workflow includes sample preparation, ultrasonic-assisted extraction, optimization of extraction parameters using RSM and ANN-GA, production of optimized extracts, and subsequent biological and chemical analyses.

Response Surface Methodology (RSM)

To quantitatively determine the effects of extraction parameters on biological response and to identify optimal processing conditions, the RSM approach was applied in this study. In this context, extraction temperature, processing time, and ethanol/water ratio were defined as control variables of the experimental system; the effects of these parameters on the Total Antioxidant Status (TAS) values of the extracts were examined based on the response variable. All experimental design, data analysis, and optimization processes were performed using Design Expert 13 software, which provides a statistical modeling infrastructure. Experimental results were

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evaluated by adapting them to a second-order polynomial model to define both linear and curvilinear components of factor-response relationships.

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

In the constructed regression model, the response variable (Y_k) represents the TAS values of the extracts, while the coded independent variables (X_i) represent temperature, time, and solvent composition. The model constant (β_{k0}) is considered as the coefficient defining the response level at the center point of the experimental area. The model's fit and statistical reliability were comprehensively tested through the coefficient of determination (R^2), analysis of variance (ANOVA) results, and relevant p-values. Optimum extraction conditions were calculated by mathematically solving the model equations to determine the points where the response function is maximized. In addition, three-dimensional response surface graphs were created to visually evaluate the interactions between the independent variables, and the obtained trends were interpreted within a scientific framework.

Artificial Neural Network-Genetic Algorithm (ANN-GA)

To model the complex relationship between extraction parameters and the resulting biological response, a data-driven, AI-powered prediction approach was applied in this study. Accordingly, an Artificial Neural Network (ANN) model was constructed, with extraction temperature, processing time, and ethanol/water ratio defined as input variables, TAS was considered as the model's output. To ensure consistent performance across different datasets, the experimental dataset was randomly distributed, with 80% used for training, 10% for validation, and 10% for testing. To prevent overlearning and increase convergence speed, the network was trained using the Levenberg-Marquardt (LM) algorithm. To determine the optimal ANN architecture, the number of neurons in the hidden layer was gradually varied, and between 1 and 20 different network topologies were tested. In all trials, the learning rate and momentum parameters were kept constant, allowing the network behavior to be evaluated solely based on architectural variables. The training process was limited to a maximum of 500 iterations, and an early stop criterion was applied if no error reduction was observed after 50 iterations during the validation phase. The error tolerance was defined as 1×10^{-5}. Each network configuration was independently trained 1000 times with randomly assigned initial weights, and the results were analyzed comparatively. Model performance was quantitatively evaluated using Mean Squared Error (MSE) and Mean Absolute

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Percentage Error (MAPE) values; these indicators were calculated using the mathematical expressions given below.

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

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

To efficiently scan the solution space during the optimization phase, the Genetic Algorithm (GA) approach was applied. Population size, one of the key parameters affecting the algorithm's performance, was defined at different levels, and its effects on solution stability and convergence success were evaluated comparatively. In the individual selection process, the roulette wheel selection method, a probabilistic mechanism based on fitness values, was preferred, a single-point crossover operation was applied to preserve genetic diversity in successive generations. The progress of the optimization process was monitored through the convergence curves obtained throughout the iterations, and the effect of the number of iterations on solution quality was quantitatively analyzed. To increase the repeatability and reliability of the results, each optimization scenario was run with at least thirty independent runs. In evaluating the prediction accuracy of the ANN model, the difference between experimental measurements and model outputs was taken as the basis. In this context, ei represents the actual TAS values obtained experimentally, p_i represents their equivalents generated by the ANN, and the total number of data points used in the analyses is expressed by the parameter n. Error metrics calculated in accordance with these definitions have been used as the basis for quantitatively evaluating model performance.

Extraction for Bioactivity

To determine the process parameters that can maximize the biological activity of mushroom extracts, the extraction process was evaluated using statistical and artificial intelligence-based multivariate optimization approaches. As a result of optimization analyses conducted with RSM, the optimum value for the extraction temperature was calculated as 55.894 (55.9) °C, the optimum for the processing time as 41.603 (41.6) minutes, and the optimum solvent composition as 98.880% (98.9%) ethanol/water ratio. In contrast, the ANN-GA predicted the most suitable extraction conditions as 59.8268 (59.8) °C temperature, 41.1635 (41.2) minutes extraction time, and 97.8305% (97.8%) ethanol/water ratio. Theoretical optimum parameters obtained from both

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models were evaluated together, and in the experimental validation phase, extraction processes were carried out based on parameters closest to the calculated values and applicable under laboratory conditions. All extractions were performed using an ultrasonic-assisted system to ensure methodological consistency; the processes were carried out under standardized conditions at 40 kHz frequency, 100% power level, and 400 W output power.

Antioxidant Activity Tests

The antioxidant properties of the optimized mushroom extracts were evaluated using multiple complementary methods to reveal both the total redox capacity and the free radical scavenging ability. Total antioxidant status (TAS) measurement was performed using a commercial analysis kit to determine the total antioxidant capacity. The analysis principle is based on the reduction of the 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) radical cation by antioxidant molecules present in the extracts. The decrease in absorbance associated with radical reduction was monitored spectrophotometrically, and the results were expressed as mmol Trolox equivalent/L [15]. Total Oxidant Status (TOS) analysis was applied to determine the oxidant potential of the extracts. In this method, it is based on the conversion of ferrous ions to ferric form by the oxidant compounds present in the samples, and the formation of a colored complex of the resulting ferric ions with xylenol orange. The change in absorbance due to complex formation was measured, and the results were reported in μmol hydrogen peroxide equivalent/L [16]. The Oxidative Stress Index (OSI) was calculated using TOS and TAS values together; OSI was expressed as a percentage (%) based on the TOS/TAS ratio. This parameter was used to numerically define the balance between the antioxidant capacity and oxidant load of the extracts [17].

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) method was used to evaluate free radical scavenging activity. Before analysis, extracts were dissolved in dimethyl sulfoxide to prepare stock solutions at a concentration of 1 mg/mL. Predetermined volumes were taken from these stocks, reacted with the DPPH solution, and a solvent mixture containing low-concentration methanol was added to ensure homogeneity of the medium. Considering the photosensitivity of the DPPH radical, the mixtures were incubated at room temperature and in the dark. At the end of incubation, absorbance measurements were performed at a wavelength of 517 nm, and the results were calculated in mg Trolox equivalent/g extract (mg TE/g) [18].

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The Ferric Reducing Antioxidant Power (FRAP) test was applied to determine the reducing power of the extracts. In this context, specific volumes of the extract solutions were mixed with the FRAP reagent, freshly prepared before analysis. The reagent; The reaction mixtures were prepared by combining acetate buffer (pH 3.6), ferric chloride solution, and 2,4,6-tripyridyl-s-triazine (TPTZ) in appropriate proportions. The reaction mixtures were subjected to short-term incubation at 37 °C, and the color change resulting from the reduction of ferric ions to ferrous form was measured at a wavelength of 593 nm. Using the obtained absorbance values, the antioxidant capacity was calculated in mg Trolox equivalent/g extract (mg TE/g) [18].

Anticholinesterase Activity Tests

The inhibitory effects of optimized mushroom extracts on cholinesterase enzymes were investigated using a modified version of the Ellman method based on colorimetric measurement [19]. Acetylcholinesterase (AChE; Type VI-S, EC 3.1.1.7) and butyrylcholinesterase (BChE; EC 3.1.1.8) were selected as target enzymes, and galantamine hydrobromide was used as the reference inhibitor. Extract solutions were prepared in the concentration range of 3.125-200 µg/mL (final concentrations) to reveal the concentration-dependent change in inhibition. Experiments were carried out in a microplate setup, and the reaction medium was provided with 0.1 M phosphate buffer (pH 8.0). After adding the extracts and enzyme solutions to the wells, the mixtures were pre-incubated for 10 minutes at 25 °C under light-free conditions. The reaction was initiated by the addition of 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) and a suitable substrate (acetylcholine iodide or butyrylcholine iodide). The absorbance of the chromogenic product formed as a result of enzymatic hydrolysis was measured at a wavelength of 412 nm; percentage inhibitions were calculated from the obtained values, and the anticholinesterase activities of the extracts were expressed as IC_{50} (µg/mL) values. IC_{50} values were estimated from concentration-dependent inhibition data obtained over the tested concentration range of 3.125-200 µg/mL. When 50% inhibition was not reached within the tested range, the IC_{50} value was reported as greater than the highest tested concentration. Since nonlinear fitting curves and confidence intervals were not generated in the original analysis, this point has been acknowledged as a methodological limitation.

Antiproliferative Tests

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The effects of optimized fungal extracts on cell proliferation were investigated in vitro using three different human cancer cell lines. These included lung adenocarcinoma (A549), breast adenocarcinoma (MCF-7), and prostate carcinoma (DU-145) cell lines, all obtained from the American Type Culture Collection. Extracts were prepared at concentrations of 25, 50, 100, and 200 µg/mL to determine dose-dependent efficacy. Cells were cultured in appropriate media under standard culture conditions; when 70–80% confluence was reached, they were separated with Trypsin-EDTA and added to culture plates. After a 24-hour pre-incubation period to allow cell adhesion to the surface, extract solutions were added to the culture medium, and the cells were treated for 24 hours. At the end of this period, cell viability was evaluated using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) test. Formazan crystals formed in metabolically active cells were dissolved with dimethyl sulfoxide (DMSO). DMSO was considered as a negative control. Absorbance measurements were performed at a wavelength of 570 nm, and the obtained data were used to determine the antiproliferative effects of the extracts on A549, MCF-7, and DU-145 cell lines [17]. In the antiproliferative assays, serum-free medium (SF) was used as the control medium and DMSO as the solvent control. However, no standard anticancer drug was included as a positive control; therefore, the results should be interpreted as a preliminary comparative evaluation of the optimized extracts.

Phenolic Analysis

The phenolic compound composition of the optimized mushroom extracts was determined using a targeted liquid chromatography-tandem mass spectrometry (LC-MS/MS) method based on reference standards [18]. In the analyses, the presence and amounts of a total of 24 standard compounds representing different phenolic classes were simultaneously evaluated in the extracts. LC-MS/MS analysis was performed using a Shimadzu LCMSMS-8030 system equipped with LC-20ADXR binary pumps, a SIL-20ACXR autosampler, and a CTO-10ASvp column oven. Chromatographic separation was achieved on a SUPELCO Discovery C18 column (50 mm × 2.1 mm, 5 µm), and the column temperature was maintained at 40 °C throughout the analysis. A binary gradient elution program was applied at a total flow rate of 0.400 mL/min. Mobile phase A consisted of 0.1% formic acid, 20% water, and 80% methanol, while mobile phase B consisted of 0.1% formic acid, 20% methanol, and 80% water. The gradient program was as follows: 100% B at 2.50 min, 40% B from 4.00 to 7.00 min, 20% B from 7.01 to 8.00 min, and return to 100% B at 9.00 min, with a total run time of 15 min. The maximum system pressure was set at 400 bar, and

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2 µL of sample was injected into the system for each analysis. The determined chromatographic and detection conditions enabled reliable separation and quantitative determination of phenolic compounds. Since the LC-MS/MS analysis was performed as a targeted phenolic profiling approach using reference standards, the quantified compounds were presented in tabular form. The available analytical documentation is provided in the Supplementary Information. For LC-MS/MS analysis, compound assignment was based on comparison with reference standards, including retention behavior and target ion transitions used for the selected phenolic compounds. Since the analysis was designed as targeted phenolic profiling rather than structural elucidation of unknown metabolites, detailed fragmentation pattern tables were not included. A representative LC-MS/MS chromatogram of the 24-component phenolic reference standard mixture is provided as Supplementary Figure S1. The corresponding LC-MS/MS acquisition parameters and analytical validation data for all 24 reference standards are summarized in Supplementary Table S1.

Chemical Composition Analysis

The chemical composition of the optimized mushroom extracts was analyzed using gas chromatography-mass spectrometry (GC-MS) in order to identify volatile and semi-volatile constituents[20]. All analyses were performed in triplicate to ensure reproducibility. Chromatographic separation was carried out on an HP-5MS capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness). Helium was used as the carrier gas at a constant flow rate of 3 mL/min. Sample injections were performed in split mode, and the injector temperature was maintained at 280 °C. The oven temperature program was set as follows: the initial temperature was held at 50 °C for 2 min, then increased to 120 °C at a rate of 15 °C/min and held for 2 min. Subsequently, the temperature was increased to 300 °C at a rate of 5 °C/min and maintained for 16 min. The mass spectrometer detector temperature was set to 280 °C. Compound identification was performed by comparing the obtained mass spectra with those available in the NIST and Wiley spectral libraries, together with literature data. The relative abundance of the detected compounds was calculated using the percentage peak areas obtained from the total ion chromatogram (TIC). As authentic reference standards were not used, the identified compounds should be considered tentative. Detailed experimental fragmentation patterns for each tentatively identified compound were not presented, because the GC-MS analysis was intended for comparative chemical profiling and library-based tentative identification rather than full structural elucidation of unknown metabolites. Therefore, the identified compounds were summarized in tabular form based on

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retention time, relative peak area, and library-based spectral matching, and the raw chromatographic outputs are available from the corresponding author upon reasonable request.

Statistical Analysis

Statistical evaluation of experimental data was performed using IBM SPSS Statistics software (v21.0). For pairwise comparisons, differences between group means were analyzed using the independent samples t-test. For comparisons involving three or more groups, one-way analysis of variance (ANOVA) was applied to examine group effects. In all statistical tests, the significance level was accepted as $p < 0.05$, based on a 95% confidence level. In cases where a significant difference was found as a result of ANOVA, Duncan's multiple comparison test was used to reveal the source of the differences between the groups.

Results and Discussions

Optimization of Extraction Conditions

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The process parameters affecting the TAS of the *S. rugosoannulata* extracts were investigated within the framework of a controlled and multi-level experimental approach. For this purpose, the extraction temperature was set at 30, 45, and 60 °C; the application time at 30, 45, and 60 minutes and the solvent system was set at 0%, 50%, and 100% ethanol/water ratios. The TAS values obtained as a result of the extraction processes carried out under different combinations of the determined factor levels were measured and evaluated comparatively, and the quantitative results obtained are presented in detail in Table 1.

Table 1. Total antioxidant status (TAS) values of *Stropharia rugosoannulata* extracts obtained under different ultrasonic-assisted extraction conditions

Experiment number	Extraction time (min)	Extraction temperature (°C)	Ethanol/water ratio (%)	TAS (mmol/L)
1	30	30	0	3.389 ± 0.026^{j}
2	45	30	0	3.751 ± 0.039^{f}
3	60	30	0	2.491 ± 0.024^{o}
4	30	30	50	3.627 ± 0.039^{h}

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5	45	30	50	4.054 ± 0.048^{c}
6	60	30	50	2.850 ± 0.028^{n}
7	30	30	100	3.468 ± 0.050^{i}
8	45	30	100	3.863 ± 0.036^{e}
9	60	30	100	2.752 ± 0.034^{no}
10	30	45	0	3.674 ± 0.046^{h}
11	45	45	0	4.072 ± 0.031^{c}
12	60	45	0	2.850 ± 0.033^{n}
13	30	45	50	3.727 ± 0.039^{f}
14	45	45	50	4.216 ± 0.032^{b}
15	60	45	50	3.011 ± 0.035^{m}
16	30	45	100	3.824 ± 0.032^{e}
17	45	45	100	4.166 ± 0.025^{b}
18	60	45	100	2.918 ± 0.027^{n}
19	30	60	0	3.212 ± 0.026^{l}
20	45	60	0	3.482 ± 0.027^{i}
21	60	60	0	2.359 ± 0.030^{p}
22	30	60	50	3.383 ± 0.027^{j}
23	45	60	50	3.548 ± 0.029^{h}
24	60	60	50	3.433 ± 0.037^{i}
25	30	60	100	4.247 ± 0.040^{b}
26	45	60	100	4.414 ± 0.034^{a}
27	60	60	100	3.352 ± 0.048^{j}

Note: Values are presented as mean ± standard deviation (SD). Means with different superscript letters in the same column are statistically significant according to Duncan's multiple comparison test ($p < 0.05$).

In this study, when the effects of different extraction temperatures, durations, and solvent ratios on the TAS values of *S. rugosoannulata* were evaluated together, it was observed that the antioxidant capacity changed in a sensitive manner to both the individual and combined effects of these parameters. In general, the 45-minute extraction time stood out as the time in which the highest TAS values were obtained, considering all temperatures and solvent ratios. In particular, the TAS value obtained as a result of 45 minutes of extraction at 60 °C and 100% ethanol/water

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ratio (4.414 ± 0.034) represents the highest antioxidant capacity determined in the study. This shows that increasing the solvent ratio under appropriate duration conditions significantly increases the extraction yield of antioxidant compounds.

Similarly, in extractions carried out at 45 °C, it was observed that the 45-minute duration and solvent systems containing 50%-100% ethanol stood out with high TAS values. The fact that the TAS values obtained under these conditions (4.216 ± 0.032 and 4.166 ± 0.025) are statistically in the upper groups suggests that medium temperature conditions are favorable for the preservation and transfer of antioxidant compounds to the solvent. In contrast, extending the extraction time to 60 minutes at the same temperature levels led to a significant decrease in TAS values, regardless of the solvent ratio.

It was clearly observed that the TAS values were in a lower range in all experimental conditions where the extraction time was 60 minutes. In particular, the low TAS values obtained in the 60 °C-60 minute combinations (2.359 ± 0.030-3.352 ± 0.048) suggest that long duration and high temperature may negatively affect the structural stability of antioxidant compounds and trigger possible degradation processes. This trend reveals that extending the duration under high temperature conditions is a limiting factor on antioxidant capacity.

The TAS values obtained under low temperature conditions (30 °C) showed a more balanced distribution, but the maximum values lagged behind those obtained under medium and high temperature conditions. At this temperature, the highest TAS value was determined at 45 minutes and with 50% ethanol (4.054 ± 0.048), while significant decreases occurred at all solvent ratios when the extraction time was increased to 60 minutes. Overall, it is understood that reaching extreme values in extraction time and temperature negatively affects TAS, while medium-high temperature conditions supported by a medium time (45 min) and appropriate solvent ratios are more favorable for maximizing the antioxidant capacity of Stropharia rugosoannulata extracts. In this study, statistically based RSM and artificial intelligence-supported approaches were examined in a comparative framework to model the extraction process and determine the optimal conditions. Within the scope of the RSM application, four different regression structures were created: linear, two-factor interaction (2FI), quadratic, and cubic. The performance of the developed models was evaluated considering the coefficient of determination (R^2), analysis of variance (ANOVA) outputs, and significance levels (p-values). As a result of model comparisons, it was determined

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that the quadratic regression model, with an R^2 value of 0.828, represented the changes in TAS values with higher accuracy compared to the other models. This result shows that the model offers a statistically reliable structure in predicting the TAS levels of *S. rugosoannulata* extracts. In addition, the analyses showed that extraction temperature, application time, and solvent composition had significant and determining effects on TAS. Accordingly, the quadratic model emerged as the most suitable approach for statistically improving extraction processes in terms of both predictive power and optimization potential.

$$TAS = 4.12 + 0.065\,X_1 - 0.363\,X_2 + 0.206\,X_3 + 0.057\,X_1X_2 + 0.209\,X_1X_3 + 0.004\,X_2X_3 - 0.180\,X_1^2 - 0.698\,X_2^2 - 0.078\,X_3^2$$

The second-order regression approach developed in this study is structured around three main variables that define the extraction process. The independent parameters included in the model are extraction temperature (X_1), application time (X_2), and solvent system composition (X_3; ethanol/water ratio), and the effects of these variables on the TAS values of *S. rugosoannulata* extracts were comprehensively investigated. The analyses were conducted considering not only the contribution of each parameter individually but also the interactive effects arising from the combination of variables. To make the obtained regression outputs more understandable and interpretable, the model results were transferred to three-dimensional response surface graphs (Figure 2). These visual presentations, by going beyond quantitative values, clearly revealed the trending effects of changes in extraction conditions on TAS, showing the parameter-response relationships. In addition, these graphs, by allowing the evaluation of the direction and intensity of interactions between variables, were used as a complementary analytical tool in identifying optimum extraction regions.

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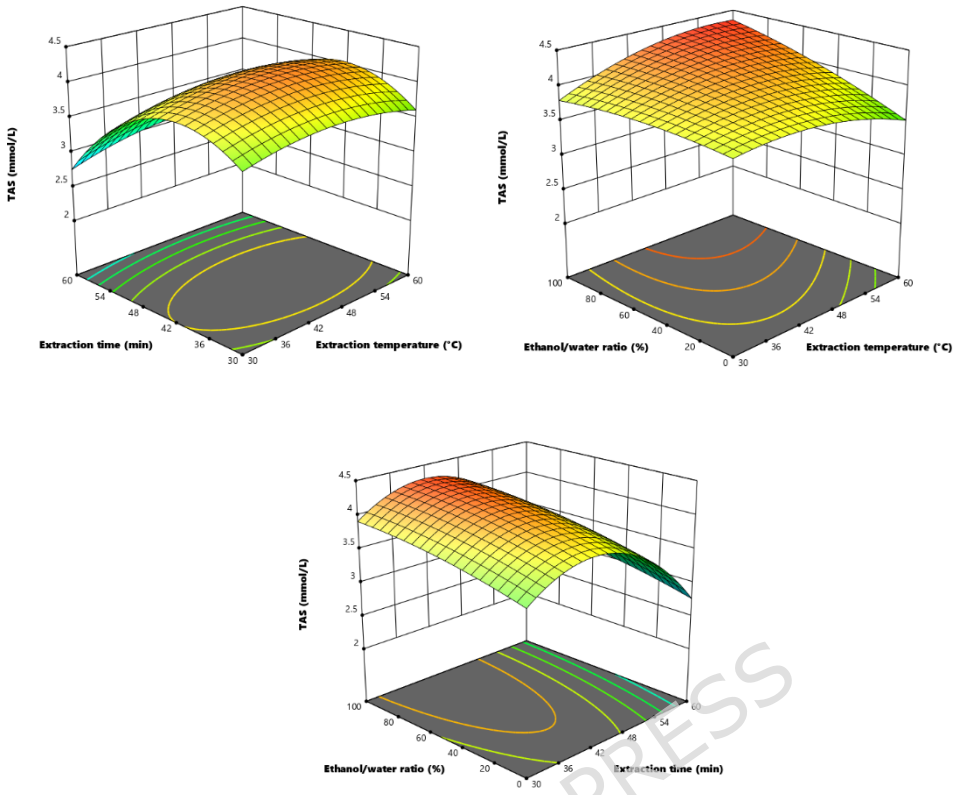


Figure 2. Three-dimensional response surface plots illustrating the interactive effects of extraction temperature, extraction time, and ethanol/water ratio on TAS values of *Stropharia rugosoannulata* extracts. The plots show how changes in the selected extraction parameters influence the total antioxidant status and help visualize the optimum extraction region.

In the advanced stage of the modeling process, an ANN based approach was applied to more accurately predict the response variable. Different network configurations were tested, and comparisons revealed that the 3-4-1 architecture, consisting of three input variables, four hidden layer neurons, and one output neuron, provided the highest prediction performance. This structure was able to effectively represent the nonlinear relationships between the extraction parameters and the response variable. The performance of the established ANN model was evaluated using three key statistical indicators: MSE, MAPE, and correlation coefficient (R). The calculated MSE value for the model was 0.013, the MAPE value was 2.803%, and the R coefficient was 0.976. These indicators show that the model exhibits high fit with a low error level and demonstrates a strong correlation with experimental data. Based on the prediction outputs generated by ANN, GA

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assisted optimization process was conducted to determine the optimum extraction conditions. Different population sizes were tested in the GA application, and their effects on the algorithm's stability and solution quality were compared. As a result of these evaluations, it was determined that a population structure containing 10 individuals provided the most consistent and effective results. The iterative progress of the optimization process was monitored through convergence curves; it was observed that after approximately 15 iterations, the solution stabilized and reached values very close to the global optimum (Figure 3).

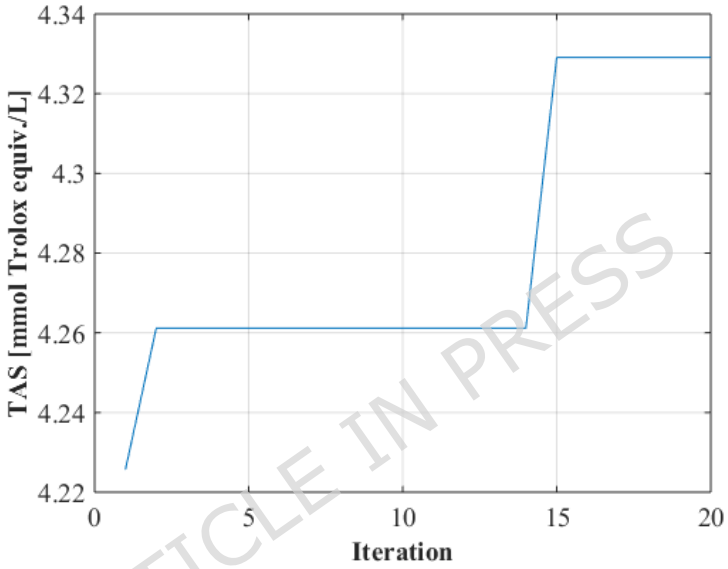


Figure 3. Convergence behavior of the genetic algorithm during the ANN-GA optimization process. The curve represents the progression of the optimization process across iterations and indicates the stabilization of the predicted optimum solution.

Antioxidant Activity

Mushrooms exhibiting antioxidant properties are considered effective biological resources in limiting oxidative stress due to their rich content of phenolic compounds, polysaccharides, and various secondary metabolites. These qualities make these mushrooms noteworthy in terms of functional food development and pharmaceutical applications [21,22]. In this context, the antioxidant properties of optimized extracts obtained from *S. rugosoannulata* were experimentally evaluated, and the findings are presented in Table 2.

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Table 2. Antioxidant activity parameters of optimized *Stropharia rugosoannulata* extracts obtained by RSM and ANN-GA

Parameters	RSM extract values	ANN-GA extract values
FRAP (mg Trolox Equi/g)	178.130 ± 4.590[a]	163.710 ± 2.490[b]
TAS (mmol/L)	4.449 ± 0.038[a]	4.171 ± 0.053[b]
DPPH (mg Trolox Equi/g)	113.320 ± 3.360[a]	102.940 ± 2.940[b]
TOS (μmol/L)	7.272 ± 0.081[b]	7.745 ± 0.047[a]
OSI (TOS/(TAS*10))	0.163 ± 0.001[b]	0.186 ± 0.003[a]

Note: Means followed by different superscript letters within the same parameter indicate significant differences between RSM and ANN groups ($p < 0.05$).

Directing ultrasonic-assisted extraction with two different optimization methods (RSM and ANN-GA) resulted in a significant difference in the antioxidant capacity and redox stability of *S. rugosoannulata* extracts. The significantly higher FRAP, TAS, and DPPH values in the RSM-optimized extract ($p < 0.05$) indicate that this approach achieves a more favorable combination of parameters for the extraction of compounds with high reducing power and radical scavenging potential [23]. In contrast, the increase in TOS and related OSI values in the ANN-GA group suggests that in a scenario where antioxidant gain is relatively weak, the oxidative load increases and the redox stability of the extract may become more fragile [24]. Although the optimum extraction conditions predicted by RSM and ANN-GA were relatively close to each other, small differences in temperature, extraction time, and ethanol/water ratio may have altered the selective recovery and stability of bioactive compounds. The superior biological performance of the RSM extract may be associated with its higher phenolic content, particularly gallic acid, caffeic acid, protocatechuic acid, quercetin, and catechin hydrate, as well as its lower TOS and OSI values. Therefore, even minor changes in extraction parameters can influence the chemical composition of the extract and result in measurable differences in biological activity.

The literature strongly supports the antioxidant potential of *S. rugosoannulata* at both *in vivo* and *in vitro* levels. The fact that selenium-enriched mycelia increased GSH-Px and SOD activities and decreased MDA levels in vivo demonstrates that the species can exhibit a profile capable of suppressing oxidative damage in biological systems [25]. *In vitro* studies show that acidic polysaccharide fractions (SRP-2) exhibit higher activity in $ABTS^{+}$, DPPH, and hydroxyl radical

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scavenging capacity [26], indicating that antioxidant performance is sensitive to fraction composition. Furthermore, it has been reported that the drying method and growing medium significantly affect antioxidant capacity; fractions dried with hot air and samples grown under bamboo forest show stronger activity [5,27,28]. Despite the limited activity of natural EPS, the sulfated derivative exhibits a higher radical scavenging capacity, suggesting that structural modifications can enhance biological efficacy [29].

The cavitation-based effect of ultrasonic extraction weakens cell wall integrity, accelerating mass transfer and facilitating the passage of target compounds into the solvent phase. However, high energy input and local temperature increases can trigger some oxidative side reactions, which can be reflected in the TOS and OSI values of the extract [30,31]. In this context, the fact that the extract optimized with RSM is characterized by higher TOS and lower OSI shows that the oxidative load can be limited while preserving the antioxidant capacity.

Furthermore, no previous findings regarding the TAS, TOS, and OSI values of *S. rugosoannulata* have been found in the literature. Studies on different fungi have reported the TAS value of *Paralepista flaccida* as 4.054 mmol/L, the TOS value as 10.352 μmol/L, and the OSI value as 0.255 [32]. In *Hericium erinaceus*, the TAS value was determined as 5.426 mmol/L, the TOS value as 6.621 μmol/L, and the OSI value as 0.122 [33]. In *Phylloporia ribis*, the TAS value was reported as 6.092 mmol/L, the TOS value as 13.389 μmol/L, and the OSI value as 0.220 [34]. TAS value is an indicator of the total antioxidant compounds detected in a fungus [35]. In this context, the fact that the TAS values obtained in our study are higher than those of *P. flaccida* and lower than those of *H. erinaceus* and *P. ribis* shows that *S. rugosoannulata* has a medium-to-high level of total antioxidant capacity in interspecies comparison. TOS value is an indicator of the oxidizing compounds present in the fungus [35]. In this context, the fact that the TOS values obtained are lower than those of *P. ribis* and *P. flaccida*, but higher than those of *H. erinaceus*, suggests that the species does not exhibit a balanced but completely suppressed profile in terms of oxidative load. The OSI value indicates the percentage suppression of oxidant compounds by antioxidant compounds [35]. Accordingly, the fact that the OSI values are lower than those of *P. ribis* and *P. flaccida*, but higher than those of *H. erinaceus*, shows that the redox balance of *S. rugosoannulata* is more advantageous than that of species with high oxidative stress tendencies, although it is not as dominant as that of strongly antioxidant species. Overall, the antioxidant performance of *S.*

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rugosoannulata appears to be consistent with the strong biological effects reported in the literature; however, the optimization approach plays a decisive role in the redox balance of the extract. In particular, the lower OSI and higher TAS values obtained with RSM demonstrate that accurate modeling of ultrasonic extraction parameters shapes not only the yield but also the quality of biological activity.

Anticholinesterase Activity

Fungi exhibiting anticholinesterase activity are natural biological resources that can contribute to the maintenance of acetylcholine levels by inhibiting AChE and BChE enzymes. This effect is generally associated with phenolic and terpenoid compounds and has supportive potential for neurodegenerative diseases [36]. In this context, the anticholinesterase activities of optimized extracts of *S. rugosoannulata* were experimentally evaluated, and the calculated IC_{50} values are presented in Table 3.

Table 3. Anticholinesterase activity of optimized *Stropharia rugosoannulata* extracts expressed as IC_{50} values

Sample	AChE IC_{50} (µg/mL)	BChE IC_{50} (µg/mL)
Galantamine	6.713 ± 0.360^{c}	17.610 ± 0.240^{c}
ANN-GA extract	168.487 ± 2.098^{a}	$>200^{a}$
RSM extract	151.567 ± 2.476^{b}	188.430 ± 2.760^{b}

Note: Values are expressed as mean ± SD (n = 3). Different superscript letters within the same column indicate statistically significant differences among samples ($p < 0.05$). Lower IC_{50} values indicate stronger enzyme inhibitory activity.

As a result, it was determined that the extracts obtained from both optimization methods of *S. rugosoannulata* showed different effects. The fact that the IC_{50} values for AChE and BChE enzymes were found to be significantly lower in the RSM extract compared to the ANN-GA group ($p < 0.05$) suggests that the extraction conditions determined by RSM support the recovery of compounds with higher cholinesterase inhibitor potential. Acoustic cavitation generated during the ultrasonic extraction process disrupts cell wall integrity, facilitating the passage of phenolics, terpenoids, and low molecular weight secondary metabolites into the solvent phase [37,38].

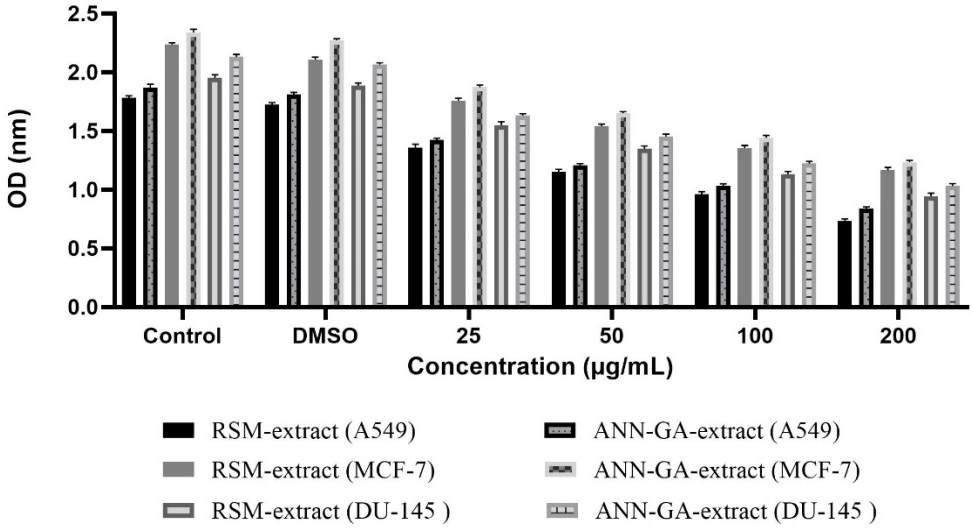
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Optimizing the parameters applied in this process can enable the selective enrichment of fractions with high enzyme inhibition capacity [39]. The RSM method's ability to determine the optimum condition by considering inter-parameter interactions may have contributed to the lower IC_{50} values of AChE and BChE inhibition. The fact that both extracts have higher IC_{50} values compared to the reference inhibitor galantamine indicates lower inhibitory power compared to pure pharmaceutical agents. However, the multi-component nature of natural extracts necessitates evaluation not only through a single enzyme target but also in conjunction with simultaneous antioxidant and neuroprotective effects. No studies directly reporting the anticholinesterase activity of *S. rugosoannulata* have been found in the literature. However, mushroom-derived bioactive compounds have been reported to show neuroprotective potential through mechanisms associated with Alzheimer's disease, including cholinesterase dysfunction, oxidative stress, neuroinflammation, amyloid aggregation, tau pathology, neuronal apoptosis, and mitochondrial impairment. Researchers have reported that polysaccharides, terpenoids, phenolics, sterols, and other secondary metabolites derived from different mushrooms may contribute to the alleviation of pathological processes associated with Alzheimer's disease through multiple target mechanisms[40]. In this context, the AChE and BChE inhibitory effects observed in the present study suggest that S. rugosoannulata may also be considered a promising mushroom species for future studies on natural cholinesterase inhibitors. The results obtained in our study showed that AChE inhibition in both optimized extracts occurred at lower IC_{50} values than BChE inhibition. Overall, ultrasonic extraction conditions optimized with RSM more effectively revealed the anticholinesterase potential of *S. rugosoannulata*. These findings indicate that the optimization strategy affects not only the extraction process but also the enzyme inhibition capacity of the resulting extract.

Antiproliferative activity

Fungi exhibiting antiproliferative activity are considered natural bioactive resources that can suppress tumor cell proliferation in cancer cells through mechanisms such as cell cycle arrest, inducing apoptosis, and regulating oxidative stress [41,42]. Accordingly, the biological effects of optimized extracts of *S. rugosoannulata* on A549, MCF-7, and DU-145 cell lines were investigated under in vitro conditions, and the results obtained are shown in Figure 4.

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(In cell culture experiments, control group cells were incubated only in standard culture medium and were not treated with any chemical agent. In the DMSO group, DMSO was added to the cell medium to serve as a solvent control, without applying the extract. In the treatment groups, cells were treated with extract solutions prepared at concentrations of 25, 50, 100, and 200 µg/mL, and dose-dependent effects were evaluated).

Figure 4. Antiproliferative activity of RSM- and ANN-GA-optimized *Stropharia rugosoannulata* extracts against A549, MCF-7, and DU-145 cancer cell lines.

The antiproliferative effects of optimized *S. rugosoannulata* extracts were evaluated. Both optimization approaches resulted in a significant suppression of proliferation in A549, MCF-7, and DU-145 cell lines with increasing concentration. The similarity of OD values in the control and DMSO groups indicates that the decrease in cell viability is due to the direct application of the extract, not the solvent effect. The dose-dependent decrease observed in the 25-200 µg/mL range in the application groups reveals that the extracts can suppress growth by reducing cellular metabolic activity. The fact that the RSM optimized extract produced lower OD values compared to the ANN-GA extract, especially at high concentrations such as 100 and 200 µg/mL, suggests that RSM optimization may provide a parameter combination that supports the extraction of components with higher antiproliferative activity. In ultrasonic extraction, cavitation accelerates mass transfer by causing microdestruction in the cell wall and matrix structure. This can increase

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the transfer of phenolics, terpenoids, and biologically active polysaccharide fractions to the solvent phase [31,43]. Therefore, the optimization strategy can be considered a critical factor shaping not only the overall yield but also the distribution of compounds that determine antiproliferative activity. The presence of significant differences in sensitivity among cell lines suggests that the effect of the extract may be related to cell type-specific biological processes. In particular, a more pronounced suppression of proliferation was observed with increasing concentration in the A549 cell line, while DU-145 cells exhibited moderate sensitivity. In contrast, the higher OD values in the MCF-7 cell line compared to other cell lines suggest that this cell type may be relatively more resistant to the extract. This differentiation appears consistent with the multi-component nature of the extract and the variability of cell type-specific signaling pathways. The literature reports that the antiproliferative potential of *S. rugosoannulata* is supported particularly through its polysaccharide and protein/lectin components. The significant inhibition of proliferation by the SR-1 polysaccharide isolated from the species in CT26.WT (colon) and MFC (gastric) cancer cell lines [44] suggests that the dose-dependent suppression observed in our study may be related to polysaccharide-weighted fractions. Similarly, the dose- and time-dependent antiproliferative effect of homogeneous polysaccharide fractions purified by alkaline extraction and DEAE-52 column chromatography in the HT-29 cell line [45] reveals that the antiproliferative response in this species is sensitive to fraction composition. Furthermore, the significant antiproliferative activity of the lectin (SRL) isolated from the species in HepG2 and L1210 cell lines [46] demonstrates that *S. rugosoannulata* can suppress tumor cell growth not only through polysaccharides but also through protein-based bioactives. In this context, the cavitation-based effect of ultrasonic-assisted extraction, which leads to more efficient release of different classes of molecules from the cell matrix, can be considered a possible mechanism that strengthens the antiproliferative profile of optimized extracts. In particular, the fact that the optimum conditions determined by RSM create stronger proliferation pressure compared to ANN-GA may be related to the enrichment of active fractions or the protection of sensitive compounds from degradation. Therefore, the obtained findings are consistent with the antiproliferative component potential of *S. rugosoannulata* reported in the literature [44-46], and show that the optimization approach is a key variable determining the level of biological activity. Overall, the cell line-dependent antiproliferative activity observed in this study may be mainly attributed to the combined effects of phenolic

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compounds, terpenoid/aromatic constituents, fatty acid derivatives, and polysaccharide- or lectin-related bioactives rather than to a single compound class.

Phenolic Contents

Mushrooms are rich natural sources of phenolic compounds, which play an important role in the formation of antioxidant, antimicrobial, and antiproliferative activities [47]. Phenolic structures are among the key components determining the biological activity of mushroom extracts due to their capacity to neutralize free radicals and reduce cellular oxidative stress. In addition, extraction conditions and methods used can directly affect the quantitative and qualitative distribution of the phenolic profile [48]. In this context, the phenolic component profiles of optimized *S. rugosoannulata* extracts were analyzed using LC-MS/MS technique, and the findings are presented in Table 4.

Table 4. Phenolic compounds quantified in optimized *Stropharia rugosoannulata* extracts by LC-MS/MS and expressed as mg/kg extract

Phenolic compounds	RSM extract (mg/kg)	ANN-GA extract (mg/kg)
Gallic acid	4871.400 ± 3.841[a]	2946.937 ± 4.078[b]
Protocatechuic acid	1287.493 ± 6.491[a]	264.997 ± 2.844[b]
Caffeic acid	2365.583 ± 3.605[a]	1002.793 ± 3.480[b]
Kaempferol	861.937 ± 4.075[a]	565.317 ± 2.823[b]
Quercetin	1684.880 ± 4.263[a]	1124.703 ± 3.052[b]
Vanillic acid	756.070 ± 4.373[a]	511.770 ± 1.769[b]
Luteolin	395.733 ± 3.578[b]	445.917 ± 5.671[a]
Catechin hydrate	1791.070 ± 2.057[a]	1570.110 ± 3.057[b]

Note: Values are expressed as mean ± SD (n = 3). Different superscript letters within the same row indicate statistically significant differences between RSM and ANN groups ($p < 0.05$). Results are expressed as mg/kg extract after correction for sample preparation and dilution factors.

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When the phenolic profile of the optimized *S. rugosoannulata* extracts in our study was examined, significant compositional differences were observed between the two optimization methods. The significantly higher levels of gallic acid, protocatechin acid, caffeic acid, kaempferol, quercetin, vanillic acid, and catechin hydrate in the RSM extract compared to the ANN-GA group ($p < 0.05$) indicate that the ultrasonic extraction conditions determined by RSM are more effective in terms of phenolic compound recovery. In contrast, the higher amount of luteolin in the ANN-GA extract reveals that the optimization strategy can lead to selective enrichment on a compound basis. In ultrasonic extraction, the microjet streams and cell wall damage generated by acoustic cavitation facilitate the release of phenolic compounds from the cell matrix. However, the delicate balance of applied energy density and time parameters determines both the yield and stability of phenolic compounds [49,50]. The RSM approach's mathematical modeling of inter-parameter interactions may have enabled the optimal extraction of phenolic acids and flavonoids in particular. The significantly higher levels of compounds with strong reducing capacities, such as gallic acid, caffeic acid, and protocatechic acid [51-53], in the RSM group suggest that this extract offers a chemical basis consistent with the previously determined high FRAP and TAS values.

Literature reports that *S. rugosoannulata* contains phenolic acids such as caffeic acid, ferulic acid, and vanillic acid, as well as flavonoids such as quercetin, catechin, and flavonols. It is also reported to be rich in coumarins and tannins [12]. These compounds are suggested to contribute to antioxidant and biological activity. In our study, the detection of particularly high levels of caffeic acid, vanillic acid, quercetin, and catechin hydrate supports the phenolic diversity reported in the literature and confirms the chemical basis of the species' biological potential. The higher amount of luteolin found in the ANN-GA extract suggests that the optimization algorithm may have focused on a parameter combination that enriches specific flavonoid fractions. This indicates that AI-based modeling can shape the chemical profile in a different compositional direction. In conclusion, it was observed that ultrasonic extraction conditions optimized with RSM resulted in higher recovery for the vast majority of phenolic compounds, while the ANN-GA approach produced selective increases in some specific compounds. These findings reveal that the optimization strategy directly shapes not only the extract yield but also the chemical composition and, consequently, biological activities such as antioxidant and antiproliferative properties.

GC-MS Based Profile of Volatile Compounds in Extracts

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The biological efficacy of mushroom-derived extracts is closely associated with the diversity and distribution of their chemical constituents. These constituents may include fatty acids, aromatic compounds, terpenoids, esters, and other classes of secondary metabolites. Such compounds are known to play a fundamental role in determining the biological properties of mushroom extracts, including their antioxidant capacity, cholinesterase inhibitory activity, and antiproliferative potential [54-56]. Accordingly, the chemical profiles of optimized extracts obtained from *S. rugosoannulata* were characterized using gas chromatography-mass spectrometry (GC–MS) analysis, and the identified components are presented in Table 5.

Table 5. Chemical composition of extracts obtained from *Stropharia rugosoannulata*

Compound name	Chemical class	R.Time (min)	Area (%) – RSM	Area (%) – ANN-GA
α-Pinene	Monoterpene	3.647	1.69	–
β-Pinene	Monoterpene	4.740	2.69	–
Sabinene	Monoterpene	4.931	1.37	–
1,8-Cineole (Eucalyptol)	Oxygenated monoterpene	6.169 / 5.971	5.02	0.79
Benzene, methyl(1-methylethyl)-	Aromatic hydrocarbon	7.090	2.60	–
Caryophyllene	Sesquiterpene	11.772	3.73	–
α-Terpinenyl acetate	Terpene ester	13.027 / 12.988	7.11	1.46
Benzene, 1-methoxy-4-(1-propenyl)-	Aromatic compound	15.176	4.57	–

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trans-Anethole	Phenylpropanoid	15.150	–	11.97
Caryophyllene oxide	Oxygenated sesquiterpene	18.547	3.68	–
Eugenol	Phenolic compound	22.867	5.84	–
Hexadecanoic acid, methyl ester	Fatty acid methyl ester	24.732 / 24.716	5.84	9.27
Methyl stearate	Fatty acid methyl ester	30.375	–	3.04
9-Octadecenoic acid, methyl ester	Fatty acid methyl ester	30.886 / 30.857	17.69	7.30
9,12-Octadecadienoic acid, methyl ester	Fatty acid methyl ester	32.123 / 32.095	4.18	16.61
Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester	Fatty acid ester	41.257	–	1.84
n-Hexadecanoic acid	Fatty acid	42.660 / 42.621	26.09	14.03
Octadec-9-enoic acid	Fatty acid	49.225	–	15.81
Squalene	Triterpene	47.165	7.90	–

Note: Compounds were tentatively identified by comparison of mass spectra with library databases (e.g., NIST), and relative abundances were calculated based on the total ion chromatogram (TIC) peak areas.

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In the present study, the chemical profiles of RSM and ANN-GA optimized extracts obtained from *S. rugosoannulata* were characterized by GC-MS analysis. The results demonstrated that both extracts exhibit a complex multi-component chemical composition; however, the diversity and relative distribution of the identified compounds differed markedly depending on the optimization strategy employed. Among the detected constituents, n-hexadecanoic acid (palmitic acid) was identified as one of the dominant compounds in both extracts and represented a major proportion of the total chromatographic area. Palmitic acid and related fatty acids are widely reported as common lipid components in fungal extracts and have been associated with antioxidant properties and regulatory roles in cellular metabolic processes[57,58]. Therefore, the high abundance of this compound may partially contribute to the biological activities observed in the extracts. The RSM-optimized extract exhibited a relatively richer profile in terms of terpenoid and aromatic compounds, including α-pinene, β-pinene, sabinene, caryophyllene, caryophyllene oxide, eugenol, and squalene. Such compounds are widely recognized for their antioxidant and bioactive potential[59-62]. For instance, sesquiterpenes such as caryophyllene and its oxide derivative have been associated with anti-inflammatory and antioxidant activities, while phenolic compounds such as eugenol are known to exhibit strong radical scavenging and enzyme inhibitory properties [59,61]. The detection of these compounds suggests that the RSM-based optimization strategy may favor the extraction of volatile and semi-volatile terpenoid constituents.

In contrast, the ANN-GA optimized extract was characterized by a higher proportion of long-chain fatty acids and their methyl ester derivatives, particularly 9-octadecenoic acid (oleic acid), 9,12-octadecadienoic acid (linoleic acid) methyl ester, and hexadecanoic acid methyl ester. Polyunsaturated fatty acid derivatives such as linoleic acid have been reported to play important roles in antioxidant defense mechanisms and the regulation of cellular proliferation [63,64]. Therefore, the higher abundance of these lipid-derived compounds in the ANN-GA extract provides a chemical explanation consistent with the stronger antioxidant and antiproliferative activities observed in the biological assays. Additionally, the presence of aromatic compounds such as trans-anethole further contributes to the chemical diversity of the ANN-GA extract and may enhance its biological potential.

Furthermore, the accumulation of fatty acids, terpenoid derivatives, and aromatic compounds in the extracts may also be related to the ecological and physiological characteristics of *S.*

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rugosoannulata. This species is a saprotrophic fungus, obtaining nutrients through the decomposition of lignocellulosic plant residues and other organic materials in its natural habitat. During the degradation of complex substrates such as cellulose, hemicellulose, and lignin, saprophytic fungi activate diverse metabolic pathways associated with lipid metabolism and secondary metabolite biosynthesis[65,66]. These metabolic processes often lead to the production and accumulation of long-chain fatty acids, phenolic derivatives, and terpenoid-related compounds, which may function in membrane stability, oxidative stress regulation, and ecological interactions with surrounding microorganisms. In addition, the decomposition of plant-derived materials can supply precursor molecules that contribute to the biosynthesis of aromatic and phenolic structures within fungal cells [65,67]. Therefore, the presence of compounds such as fatty acid derivatives, terpenoid constituents, and phenolic molecules in the optimized extracts may reflect not only the extraction conditions but also the intrinsic metabolic capacity of this saprotrophic mushroom to transform and accumulate bioactive metabolites from its lignocellulosic environment.

Overall, the GC-MS findings clearly indicate that the optimization strategy strongly influences the qualitative and quantitative distribution of bioactive compounds in the extracts. While RSM optimization tends to enrich terpenoid and aromatic constituents, ANN-GA optimization appears to favor the extraction of lipid-derived compounds such as fatty acids and their esters. These differences in chemical composition provide a plausible explanation for the variations observed in antioxidant capacity, enzyme inhibition potential, and antiproliferative activity between the extracts. Consequently, the biological activities of *S. rugosoannulata* extracts can be attributed to a synergistic multi-component chemical matrix, whose composition is significantly shaped by the selected extraction optimization approach. However, it should be emphasized that the GC-MS-based compound assignments should be interpreted as tentative because authentic reference standards were not used for structural confirmation. Therefore, the GC-MS results mainly provide a comparative chemical profile of the optimized extracts rather than definitive identification of all volatile and semi-volatile metabolites. Therefore, the possible relationships between specific compounds and biological activities should be interpreted cautiously. The associations discussed in this study are based on the known bioactive potential of the detected compound classes and the comparative chemical profiles of the extracts, rather than direct correlation analysis or compound-specific mechanistic validation.

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Conclusion

The biological potential of extracts obtained from *S. rugosoannulata* was comprehensively evaluated using two different optimization strategies. Both Response Surface Methodology (RSM) and Artificial Neural Network–Genetic Algorithm (ANN-GA) models demonstrated high predictive performance in determining the optimal extraction parameters. However, the RSM-optimized extract exhibited more pronounced biological activity, including higher total antioxidant capacity, lower oxidative stress index values, and stronger cholinesterase inhibitory effects. In addition, a significant and dose-dependent antiproliferative activity was observed at higher extract concentrations. Chemical characterization revealed that the optimization strategy directly influenced the chemical composition of the extracts. In particular, variations in phenolic compounds as well as the distribution of fatty acids and terpenoid-related constituents were found to be consistent with the differences observed in biological activities. These findings indicate that the selection of an appropriate optimization approach not only affects extraction efficiency but also plays a decisive role in shaping the bioactive profile of the resulting extract. Overall, the results highlight *S. rugosoannulata* as a promising natural source of bioactive compounds with potential applications in functional foods, natural antioxidant formulations, and neuroprotective product development. Moreover, the study demonstrates that integrating advanced optimization models with chemical and biological analyses provides an effective strategy for maximizing the therapeutic potential of mushroom-derived extracts.

Like any experimental study, this work has several limitations. The original LC-MS/MS sample chromatograms and integration reports were unavailable, preventing independent verification of sample-level peak integration. Other limitations include the lack of molecular authentication, independent biological replicates, extract yield calculations, detailed IC_{50} fitting curves, metabolite fragmentation data, and a standard anticancer drug control. Future studies should address these limitations to improve reproducibility and facilitate independent validation of the findings.

Data availability

Data are available upon request.

Ethics declarations

Ethics approval and consent to participate

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This research does not involve any ethical issues.

Consent for publication

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

Conflict of interest

The authors declare that they have no conficts of interest in the manuscript.

Research involving animals

None

Informed consent

This study did not involve human participants.

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Supplementary Figure S1. Representative LC-MS/MS chromatogram of the 24-component phenolic reference standard mixture at 100 ppb. The chromatogram includes the reference standards used for the targeted phenolic profiling of the optimized *Stropharia rugosoannulata* extracts.

Supplementary Table S1. LC-ESI-MS/MS acquisition parameters and analytical validation data for the 24 phenolic reference standards used in the targeted analysis of *Stropharia rugosoannulata* extracts.

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