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Optimization Based Evaluation of Biological Activities and Chemical Composition of *Russula vinosa*

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Abstract:

This study aimed to optimize the extraction conditions of *Russula vinosa*, an edible and medicinal mushroom, to enhance its biological activity. The effects of extraction parameters (temperature, time, and ethanol-water ratio) were evaluated using Response Surface Methodology (RSM) and Artificial Neural Network-Genetic Algorithm (ANN-GA) approaches. The results showed that ANN-GA optimization yielded extracts with improved antioxidant properties, reflected by higher FRAP (193.010 mg TE/g), TAS (6.439 mmol/L), and DPPH (159.927 mg TE/g) values, along with lower TOS (11.074 μmol/L) and OSI (0.172) compared to RSM. In addition, ANN-GA extracts exhibited stronger anticholinesterase activity and enhanced antiproliferative effects.

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Chemical profiling revealed that ANN-GA optimization resulted in higher levels of key phenolic compounds, including gallic acid, quercetin, caffeic acid, and fumaric acid, indicating improved extraction efficiency. Overall, these findings demonstrate that ANN-GA provides a more effective optimization strategy than RSM by enhancing both bioactive compound recovery and biological activity. This study highlights the potential of *R. vinosa* as a valuable natural resource for functional food and pharmaceutical applications.

Keywords: Extract optimization; biological activity; artificial neural network; medicinal mushroom; edible mushroom

Introduction

Medicinally valuable and edible mushrooms are important natural resources in both nutrition and health sciences due to their rich bioactive composition. These compounds contribute to immune system support, reduction of oxidative stress, and prevention of metabolic disorders [1,2]. In addition to their bioactive properties, mushrooms are recognized for their high nutritional value, including protein, fiber, vitamins, and minerals, along with low fat content and high digestibility. Furthermore, species traditionally used in folk medicine are increasingly attracting scientific attention for their potential applications in functional foods and natural pharmaceutical products [3,4].

Extraction optimization is a critical process in modern biotechnology aimed at maximizing the biological activity of mushroom-derived extracts. This process involves the systematic control of key variables such as temperature, solvent type, extraction time, and solvent ratio to achieve high yields while preventing the degradation of bioactive compounds. In addition to conventional techniques, advanced methods such as ultrasound- and microwave-assisted extraction have been widely used to enhance extraction efficiency and reduce processing time. In recent years, the integration of statistical modeling tools and artificial intelligence-based algorithms into extraction processes has significantly reduced experimental workload while improving optimization accuracy [5-8]. As a result, the biological potential of medicinal mushrooms can be more effectively evaluated and utilized. Previous studies have also demonstrated that AI-assisted optimization strategies can enhance the biological activity of mushroom extracts[9-12].

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The aim of this study was to determine the optimal extraction conditions that maximize the biological activity of *Russula vinosa* and to evaluate the biological properties of the resulting extracts. *R. vinosa* is a widely consumed wild mushroom species known for its rich nutritional composition and diverse bioactive profile. It contains high levels of protein, fiber, essential minerals (such as phosphorus, potassium, iron, selenium, magnesium, zinc, and manganese), and various metabolites including amino acids, polyphenols, organic acids, terpenoids, fatty acids, sugars, and phospholipids [13,14]. Previous studies have reported that *R. vinosa* exhibits significant antioxidant and hepatoprotective effects, as well as immunostimulatory properties, including increased macrophage activity and cytokine production [15,16]. In addition, certain compounds isolated from this species have shown moderate anti-inflammatory activity without notable toxicity[17]. These characteristics, along with its use in both fresh and processed forms, highlight its potential as a valuable resource for functional food and pharmaceutical applications [18,19].

Despite the growing interest in the biological activities of medicinal mushrooms, studies on *R. vinosa* have mainly focused on its chemical composition and selected biological properties, without applying a systematic optimization strategy or comparing different modeling approaches. In particular, studies integrating statistical methods such as RSM with artificial intelligence-based approaches like ANN-GA are lacking. While RSM is effective, it may be limited in capturing complex nonlinear relationships between extraction parameters. Therefore, the novelty of this study lies in the combined use and comparison of RSM and ANN-GA to optimize extraction conditions and to comprehensively evaluate their effects on phenolic composition and multiple biological activities.

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Materials and Methods

The fungal samples analyzed in this study were collected during fieldwork in the fir forest near Ankara, Turkey. They were then stored at the Ankara University Fungarium and preserved under appropriate conditions.

Extraction Procedure

R. vinosa samples were sliced and dried in a fruit dryer (WMF KITCHENminis, Germany) at 45 °C for 48 hours, after which they were ground into a fine powder using a mechanical grinder. For each extraction, 5 g of mushroom powder was mixed with 100 mL of solvent. Three independent

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variables were selected for optimization: extraction temperature, extraction time, and ethanol-water ratio. These factor levels (30, 45, and 60 °C; 30, 45, and 60 minutes; 0%, 50%, and 100% ethanol) were determined based on published reports on phenolic extraction from edible and medicinal mushrooms as well as preliminary laboratory trials, which indicated that values outside these ranges either resulted in insufficient extraction efficiency or caused thermal degradation of heat-sensitive phenolics. The selection of ethanol-water ratios was further supported by their well-established effectiveness in extracting phenolic compounds with different polarities. Ethanol-water mixtures provide an optimal balance of solvent polarity, enabling efficient extraction of both polar and moderately polar bioactive compounds, while also ensuring low toxicity and compatibility with food and pharmaceutical applications[20]. A total of 27 experimental runs were conducted using a three-factor, three-level Box-Behnken design to evaluate quadratic interactions among the selected variables. This design was selected because it efficiently models nonlinear effects and interaction terms while avoiding extreme experimental conditions, making it particularly suitable for optimization studies involving temperature, extraction time, and solvent composition. In addition, the inclusion of multiple center runs enabled a more reliable estimation of pure experimental error and improved the robustness of the statistical model. All extractions were performed in an ultrasonic bath (WIGGENS UA22MFDN, Germany) operating at 40 kHz and 400 W. Ultrasonic-assisted extraction was selected due to its ability to enhance mass transfer and disrupt cell structures through cavitation effects, thereby improving the release of intracellular bioactive compounds. Compared to conventional extraction techniques, this method offers reduced extraction time, lower solvent consumption, and improved extraction efficiency while minimizing thermal degradation of heat-sensitive compounds[21,22]. After extraction, the samples were filtered through Whatman No. 1 filter paper, and the solvents were evaporated under vacuum at 40 °C. All experiments were carried out in triplicate (n = 3), and the resulting extracts were stored at 4 °C until further analysis. The experimental data obtained from the RSM design were first analyzed to determine the optimal extraction conditions and to model the interactions among variables. Subsequently, the modeling framework was integrated into a hybrid structure combining Artificial Neural Networks and Genetic Algorithms (ANN-GA) to further enhance predictive accuracy and optimize extraction efficiency. To ensure reproducibility, all extraction parameters were strictly controlled, and deviations between replicate experiments were minimized. The selected parameter

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ranges were further validated based on preliminary trials to confirm their suitability for preserving thermolabile compounds.

Response Surface Methodology (RSM)

In our study, Response Surface Methodology (RSM) was used to determine the optimum parameters for the extraction process. Extraction time, solvent ratio (ethanol/water), and extraction temperature were considered as independent variables. Total Antioxidant Status (TAS) of the obtained extracts was used as the response variable.

Optimization procedures were executed utilizing Design Expert 13 software. Analyses were conducted using a statistical framework grounded in a second-order polynomial regression model. The modeling technique was organized according to the subsequent generic mathematical expression:

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

In this model, Y_k represents the TAS value as the dependent variable. The X_i variables represent the extraction time, temperature, and solvent percentage, respectively. The X_i values are independent of each other. The β_{k0} coefficient in the model represents the constant term dependent on the central point. Statistical validity and fit of the model were assessed using basic statistical measures such as analysis of variance (ANOVA), coefficient of determination (R^2), and p-value for significance. Derivatives of the model were calculated to determine the parameter combinations that produced the optimum response values. Three-dimensional response surface plots were constructed to describe the interactions between the factors.

Artificial Neural Network-Genetic Algorithm (ANN-GA)

In this study, an Artificial Neural Network (ANN) model was employed for predictive modeling, using extraction time, temperature, and ethanol–water ratio as input variables and TAS values as the response. The dataset obtained from 27 experimental conditions was divided into training (80%), validation (10%), and testing (10%) subsets to ensure model reliability. The Levenberg–Marquardt (LM) algorithm was used for training.

To determine the optimal network architecture, hidden layer sizes ranging from 1 to 20 neurons were evaluated as part of a hyperparameter optimization process. The final model consisted of a

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single hidden layer with seven neurons, representing a relatively low-complexity structure suitable for the dataset size. Model selection was based on minimizing validation error and ensuring convergence stability, while architectures showing instability or high variance between training and validation were excluded to avoid over-parameterization.

It should be noted that the dataset was not derived from random sampling but from a structured three-factor, three-level Box–Behnken experimental design, which provides a systematic and statistically balanced representation of the experimental domain while minimizing the number of required trials.

Although the dataset size (n = 27) is relatively limited for ANN modeling, such experimental designs are specifically developed for efficient process optimization with a reduced number of runs. To address potential overfitting risks, input variables were normalized prior to training, and the model was trained using the Levenberg–Marquardt backpropagation algorithm with validation-based early stopping. In addition, repeated training procedures (1,000 runs) were applied to improve model robustness and stability. This modeling strategy enabled reliable learning and prediction within the constraints of the experimental design. Nevertheless, the ANN results should be interpreted with caution, as the relatively small dataset limits their generalizability. The importance of hyperparameter control and validation strategies in ANN models trained on small datasets has been emphasized in previous studies [23]. Consistent with these findings, the present approach demonstrates that ANN models can effectively capture nonlinear relationships when combined with structured experimental designs and appropriate validation techniques[24,25].

The predictive effectiveness of the model was determined using Mean Square Error (MSE) and Mean Absolute Percentage Error (MAPE). In addition to these metrics, model reliability was further assessed through the consistency between training, validation, and testing subsets, as well as convergence stability observed across repeated training runs. Residual distributions were also examined to ensure the absence of systematic bias in predictions. These complementary evaluation approaches provide additional support for the robustness of the ANN model under the given experimental conditions. The following mathematical expressions were used to calculate these metrics:

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

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

In the formula, the symbol e_i denotes the values measured empirically. Moreover, p_i denotes the values forecasted by the artificial intelligence model, whereas n signifies the aggregate number of observations. The Genetic Algorithm (GA) was selected as the primary computational method for the optimization process. The overall efficacy of the method was assessed by comparatively analyzing various population sizes and evaluating their impact on solution quality. The roulette wheel selection method, facilitating random selection, was employed to generate new individuals. A single-point crossover method was utilized to maintain genetic variety. Convergence plots illustrating the algorithm's convergence curve were utilized to ascertain the optimal number of iterations. To enhance the likelihood of attaining the global optimum solution, each optimization cycle was executed at least 30 times. In addition, the relatively simple ANN topology (3-7-1) was intentionally selected to maintain an appropriate balance between model flexibility and dataset size, thereby reducing the risk of over-parameterization.

Extraction for Bioactivity

This study identified optimal extraction procedures to enhance the biological activity of extracts derived from mushroom samples. The optimization conducted using RSM identified the optimal settings as a temperature of 40.222 °C, a duration of 38.475 minutes, and an ethanol/water ratio of 72.099%. The optimal settings of the ANN-GA hybrid technique were established as 37.484 °C for temperature, 40.758 minutes for time, and a 41.490% ethanol/water ratio. The extraction procedures were conducted in an ultrasonic bath device (WIGGENS UA22MFDN, Germany) at a frequency of 40 kHz, with 100% power and a capacity of 400 W, by choosing parameters that closely aligned with the values recommended by both approaches. Extracts generated under these conditions were subsequently utilized in investigations of biological activity. After solvent evaporation, all extracts were dried to constant weight in a vacuum oven at 40 °C to ensure complete removal of residual solvent. The optimized extraction conditions obtained from both RSM and ANN-GA approaches were applied to produce extracts, and their biological activities were experimentally evaluated. Although independent validation experiments under newly defined conditions were not performed, this approach provides partial experimental support for the predicted optimal conditions.

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Antioxidant Activity tests

The overall antioxidant status of mushroom extracts obtained under optimized extraction conditions was determined using the Total Antioxidant Status (TAS) kit (Rel Assay Diagnostics, Mega Tıp, Gaziantep, Turkey; RL0017), which is based on the ability of antioxidants present in the sample to suppress the formation of a colored radical cation. The results were expressed as mmol Trolox equivalents per liter according to standard procedures [26]. In addition, the Total Oxidant Status (TOS) was evaluated using the corresponding commercial kit (Rel Assay Diagnostics; RL0024). This method is based on the oxidation of ferrous ion to ferrous ion in the presence of various oxidant molecules, and the values were calculated as μmol hydrogen peroxide equivalents per liter [27]. To provide a more comprehensive indicator of oxidative balance, the Oxidative Stress Index (OSI) was calculated as the ratio of TOS to TAS, and the result was presented as a percentage according to established protocols [28].

The free radical scavenging potential of mushroom extracts obtained under optimized conditions was determined by the DPPH (2,2-diphenyl-1-picrylhydrazyl) method, a widely used spectrophotometric assay. Stock solutions of the extracts were initially prepared at a concentration of 1 mg/mL in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA). A 1 mL aliquot of each stock solution was thoroughly mixed with 4 mL of a solution consisting of 160 μL of 0.267 mM DPPH (Sigma-Aldrich, St. Louis, MO, USA) and 0.004% methanol (Merck, Darmstadt, Germany). The mixtures were then left in the dark at ambient temperature for 30 min to allow the reaction to proceed. After incubation, the absorbance was recorded at 517 nm using a UV-VIS spectrophotometer. Radical scavenging activity was measured and expressed as milligrams of Trolox equivalents per gram of extract (mg TE/g) [29].

The reducing power of the optimized mushroom extracts was examined by Ferric Reducing Antioxidant Power (FRAP) assay, which measures the ability of antioxidants to reduce Fe^{3+} to Fe^{2+}. For analysis, 100 μL of extract stock solution was mixed with 2 mL of freshly prepared FRAP reagent. This reagent was composed of 300 mM acetate buffer (pH 3.6; Merck, Darmstadt, Germany), 20 mM $FeCl_3 \cdot 6H_2O$ (Sigma-Aldrich, St. Louis, MO, USA), 40 mM HCl, and 10 mM TPTZ (2,4,6-tris(2-pyridyl)-s-triazine, Sigma-Aldrich, St. Louis, MO, USA) combined in a 10:1:1 ratio. The reaction mixtures were incubated at 37 °C for 4 min and then the absorbance was measured at 593 nm by UV-VIS spectrophotometer. Antioxidant capacity was calculated

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according to a Trolox calibration curve and expressed as mg Trolox equivalents per gram of extract (mg TE/g) [29].

Anticholinesterase Activity Tests

Anticholinesterase activity of mushroom extracts obtained under optimized extraction conditions was examined using a modified Ellman colorimetric method [30]. Galantamine hydrobromide (Sigma-Aldrich, St. Louis, MO, USA) was used as a standard reference inhibitor. Extracts were prepared in serial dilutions ranging from 200 to 3.125 µg/mL. For each assay, 130 µL of phosphate buffer (0.1 M, pH 8.0; Merck, Darmstadt, Germany) was first added to the wells of a 96-well microplate. Next, 10 µL of the test sample and 20 µL of acetylcholinesterase (AChE, Type VI-S, EC 3.1.1.7) or butyrylcholinesterase (BChE, EC 3.1.1.8) solution (Sigma-Aldrich, St. Louis, MO, USA) were added. The mixtures were incubated for 10 min at 25 °C in the dark to facilitate enzyme-inhibitor interactions. After incubation, 20 µL of DTNB [5,5'-dithiobis-(2-nitrobenzoic acid), Sigma-Aldrich, St. Louis, MO, USA] and 20 µL of the corresponding substrate (acetylcholine iodide for AChE or butyrylcholine iodide for BChE) were added to initiate the enzymatic reaction. Then, based on the formation of the yellow 5-thio-2-nitrobenzoate anion, absorbance at 412 nm was monitored using a microplate reader. Enzyme inhibition was expressed by calculating IC_{50} values (µg/mL) from the dose-response curves.

Antiproliferative Activity Tests

The antiproliferative effects of optimized mushroom extracts were investigated against three human cancer cell lines. These cell lines were A549 (lung adenocarcinoma, ATCC CCL-185), MCF-7 (breast adenocarcinoma, ATCC HTB-22), and DU-145 (prostate carcinoma, ATCC HTB-81). All cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Extract solutions were prepared at concentrations of 25, 50, 100, and 200 µg/mL. When cell confluence reached approximately 70-80%, cultures were detached with 3.0 mL of Trypsin-EDTA solution (Sigma-Aldrich, St. Louis, MO, USA). They were then plated on appropriate culture plates, and cells were allowed to adhere for 24 h under standard incubation conditions. Next, the prepared extract solutions were added to the culture medium, and the cells were incubated for another 24 h, after which the culture medium was removed. It was then replaced with 1 mg/mL MTT solution [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich, St. Louis, MO, USA]. The plates were incubated at 37 °C until insoluble purple

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formazan crystals were formed by metabolically active cells via mitochondrial dehydrogenase activity, and the crystals were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA), with DMSO serving as a negative control. Finally, the absorbance was measured at 570 nm using an Epoch microplate spectrophotometer (BioTek Instruments, Winooski, VT, USA), and cell viability percentages were calculated [31].

Phenolic Analysis

The phenolic composition of the mushroom extracts obtained under optimized extraction conditions was analyzed using a Shimadzu LCMS-8030 triple-quadrupole LC-MS/MS system equipped with an electrospray ionization (ESI) source operating in negative ion mode. The source voltage was set to 4.5 kV, and the capillary temperature was maintained at 320 °C. Chromatographic separation was performed using a SUPELCO Discovery C18 analytical column (50 mm × 2.1 mm, 5 µm) housed in a CTO-10ASvp column oven set at 40 °C. A binary gradient system was used, consisting of mobile phase A (0.1% formic acid with 20% water and 80% methanol) and mobile phase B (0.1% formic acid with 20% methanol and 80% water), delivered at a total flow rate of 0.40 mL/min via LC-20ADXR pumps. The gradient program was applied as follows: 100% B (0-2.5 min), 40% B (2.5-7.0 min), 20% B (7.0-8.0 min), and 100% B (8.0-15.0 min). An autosampler (SIL-20ACXR) was used to inject 2 µL of sample. A total of 24 reference phenolic standards were screened and quantified. Data acquisition was performed in multiple reaction monitoring (MRM) mode to ensure high sensitivity and selectivity for all targeted analytes.

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Statistical Analysis

Statistical analyses were performed using SPSS software (version 21.0; SPSS Inc., Chicago, IL, USA). An independent samples t-test was applied to compare the antioxidant (FRAP, DPPH, TAS, TOS, OSI), anticholinesterase (AChE and BChE), antiproliferative, and phenolic content results between ANN-GA and RSM optimized extracts. One-way analysis of variance (ANOVA) was used to evaluate the effects of different extraction parameter combinations on TAS values obtained during the optimization experiments. Statistical significance was set at $p < 0.05$. When significant differences were detected, Duncan's multiple range test was conducted as a post hoc analysis to identify differences among group means. The results of the post hoc comparisons were used to

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confirm statistically significant differences among treatments; however, for clarity, only the corresponding p-values are reported in the Results section.

Results and Discussion

Extract Optimizations

This study comprehensively investigated the effects of three main extraction parameters on TAS values. The effects of extraction temperature (30, 45, and 60°C), exposure time (30, 45, and 60 minutes), and solvent system (ethanol/water ratio 0%, 50%, and 100%) on TAS values were determined. TAS values of the extracts obtained in different combinations are given in detail in Table 1.

Table 1. TAS values of extracts produced from *Russula vinosa*

Experiment number	Extraction time (min)	Extraction temperature (°C)	Ethanol/water ratio (%)	TAS (mmol/L)
1	30	30	0	5.547 ± 0.017^{i}
2	45	30	0	5.771 ± 0.025^{m}
3	60	30	0	4.521 ± 0.014^{b}
4	30	30	50	5.787 ± 0.031^{m}
5	45	30	50	5.994 ± 0.028^{o}
6	60	30	50	4.758 ± 0.023^{d}
7	30	30	100	5.673 ± 0.023^{k}
8	45	30	100	5.850 ± 0.021^{n}
9	60	30	100	4.636 ± 0.021^{c}
10	30	45	0	5.765 ± 0.021^{m}
11	45	45	0	6.121 ± 0.017^{p}
12	60	45	0	4.842 ± 0.019^{e}
13	30	45	50	5.952 ± 0.039^{n}
14	45	45	50	6.243 ± 0.019^{r}
15	60	45	50	4.955 ± 0.024^{f}
16	30	45	100	5.824 ± 0.018^{m}

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17	45	45	100	6.147 ± 0.026[p]
18	60	45	100	4.818 ± 0.016[e]
19	30	60	0	5.348 ± 0.023[g]
20	45	60	0	5.456 ± 0.031[h]
21	60	60	0	4.434 ± 0.022[a]
22	30	60	50	5.463 ± 0.030[i]
23	45	60	50	5.750 ± 0.027[l]
24	60	60	50	4.560 ± 0.030[b]
25	30	60	100	5.349 ± 0.033[h]
26	45	60	100	5.602 ± 0.031[j]
27	60	60	100	4.455 ±0.030[a]

[a] Different superscript letters within the same column indicate statistically significant differences ($p < 0.05$), as determined by one-way ANOVA followed by Duncan's multiple range test.

TAS values clearly demonstrated the effect of extraction parameters on this biological activity. In particular, the 45°C temperature, 45-minute processing time, and 50% ethanol solvent system provided the highest TAS value of 6.243 ± 0.019 mmol/L, demonstrating that this combination is highly effective in extracting phenolic and similar antioxidant compounds. These conditions represent the critical threshold for achieving maximum solubility before temperature-induced degradation begins. Extending the extraction time to 60 minutes at the same temperature and using 100% ethanol as the solvent resulted in a decrease in the TAS value to 4.818 ± 0.016 mmol/L. This decrease clearly demonstrates the negative effects of changing the extraction time and solvent type. When 60°C was reached, the values ranged between 5.750 ± 0.027 mmol/L (45 min, 50% ethanol) and 4.434 ± 0.022 mmol/L (60 min, 0% ethanol) for all combinations. This result confirms the detrimental effect of thermal conditions on the stability of antioxidant structures. The time factor had a similar suppressive effect on TAS values at all temperature levels. It is suggested that 60-minute applications, in particular, resulted in a thermal interaction that threatened the structural integrity of the compounds. On the other hand, the TAS values measured in extractions performed at 30°C varied within a more limited range (4.521-5.994 mmol/L) and showed less systematic deterioration. These findings indicate that low-temperature conditions contribute to the stability of antioxidant compounds through more controlled and gentle extraction processes. Our study adopted two different optimization approaches based on experimental data. Analyses conducted

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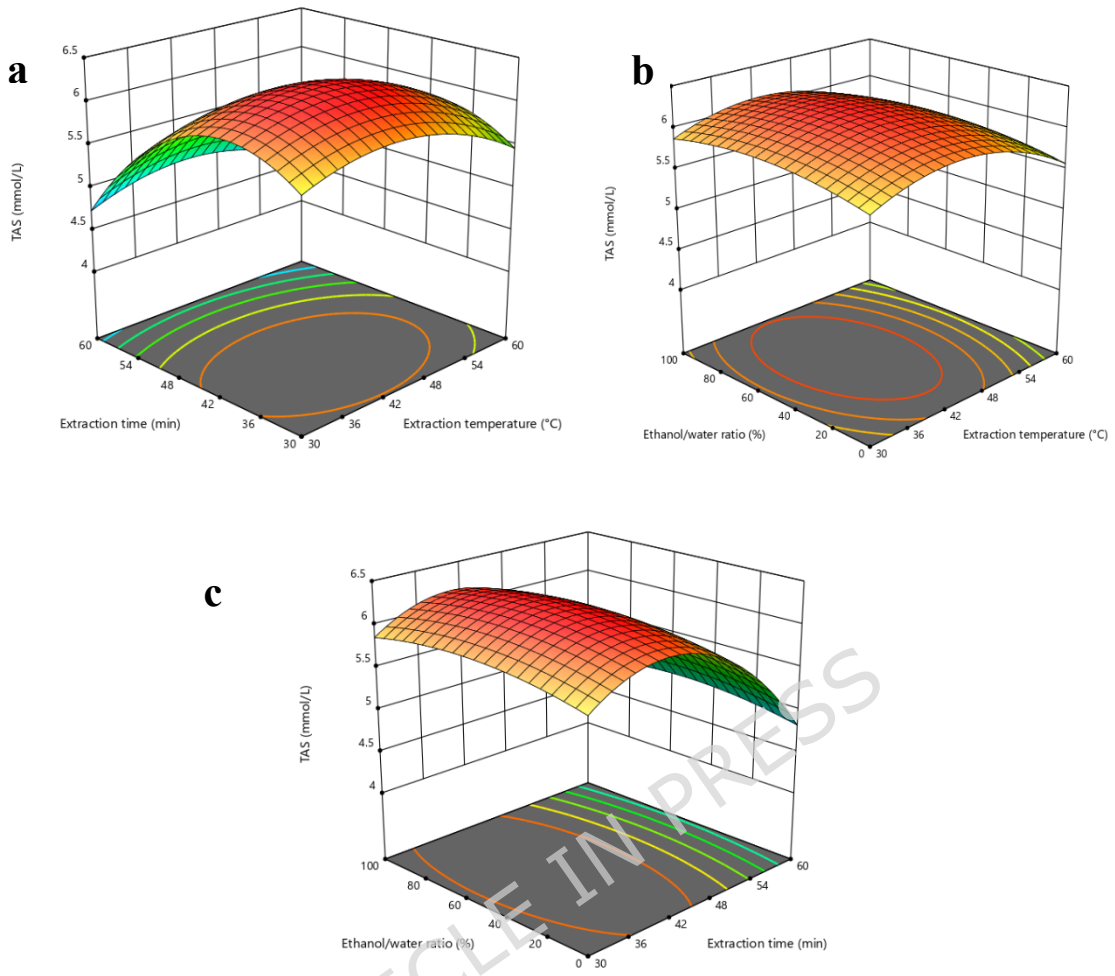
within the RSM framework systematically compared four different regression models: linear, two-factor interaction (2FI), quadratic, and cubic. Statistical evaluations revealed that the quadratic model, with an R^2 value of 0.992, was the most compatible model with the data set. This result demonstrates that 99.2% of the total variance observed in the dependent variable can be explained by the independent variables included in the model, supporting the model's statistical validity.

The high coefficient of determination (R^2) obtained demonstrates that the model accurately reflects the relationship between the variables and reliably represents trends across samples. In this context, it was concluded that the quadratic model provides robust and predictable predictions during the optimization process. The quadratic polynomial regression equation created to estimate the TAS values of *R. vinosa* is presented below:

$$TAS = 6.22 - 0.117\,X_1 - 0.484\,X_2 + 0.030\,X_3 + 0.030\,X_1X_2 - 0.012\,X_1X_3 - 0.006\,X_2X_3 - 0.355\,X_1^2 - 0.733\,X_2^2 - 0.152\,X_3^2$$

In this quadratic regression model, the variable X_1 denotes the extraction temperature, X_2 signifies the extraction time, and X_3 indicates the ethanol/water ratio in the solvent system. Figure 1 (a-c) presents three-dimensional response surface plots demonstrating how extraction temperature, extraction time, and ethanol–water ratio individually and interactively influence the TAS values of *R. vinosa*. These response surface plots indicate a clear interaction between variables, where moderate temperature and solvent ratios maximize antioxidant extraction, while extreme conditions lead to reduced efficiency due to possible compound degradation. The plots clearly show that TAS increases when moderate temperatures (around 40-45 °C) and mid-range extraction times (approximately 40-45 minutes) are combined with a solvent ratio close to 50% ethanol. The curvature observed at higher temperatures reflects the thermal degradation of antioxidant compounds, while the flattened regions at lower temperatures indicate limited mass transfer and reduced extraction efficiency. Overall, the graphical trends emphasize the synergistic effect of temperature, time, and solvent composition in maximizing antioxidant capacity.

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Figure 1. Three-dimensional response surface plots illustrating the interactive effects of (a) extraction temperature and extraction time, (b) extraction temperature and ethanol-water ratio, and (c) extraction time and ethanol-water ratio on the total antioxidant status (TAS) of *Russula vinosa* extracts as determined by the RSM model

ANN-based modeling and GA optimization were performed using the network architecture and training procedure described in the Materials and Methods section. The predictive performance of the ANN-GA model confirmed its suitability for optimization, as detailed in the methodology section. These findings demonstrate that the ANN model is an ideal tool for the optimization process. It is important to clarify that the comparison between RSM and ANN was not conducted solely on the basis of minimizing loss functions. While prediction error metrics (MSE and MAPE) were employed to quantify model performance, the evaluation also considered the fundamental

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structural characteristics of the two approaches. RSM is inherently constrained by predefined polynomial equations and may be limited in capturing complex nonlinear interactions beyond quadratic terms. In contrast, ANN models offer a flexible, data-driven framework capable of approximating nonlinear relationships without requiring an explicit functional form [32,33]. However, it is also acknowledged that the relatively small dataset (n = 27), combined with the high flexibility of ANN architectures, may increase the risk of overfitting. To mitigate this concern, several precautions were implemented. The dataset was generated using a structured Box–Behnken experimental design, which ensures a systematic and uniform distribution of experimental points across the factor space, thereby enhancing the reliability of model training[34,35]. Additionally, early stopping based on validation performance was applied to prevent excessive model fitting. The consistency observed between training, validation, and testing predictions further supports that the model did not rely solely on memorization[36,37]. Nevertheless, given the limited sample size, the ANN results should be interpreted with caution and considered as indicative rather than definitive evidence of superior predictive performance. Therefore, the improved agreement observed in ANN–GA modeling is more appropriately attributed to its ability to capture nonlinear trends within the experimental domain, rather than being viewed as conclusive proof of generalization superiority.

Figure 2 presents the convergence graph of the genetic algorithm (GA), illustrating how the fitness values evolve across successive iterations during the optimization process. The rapid stabilization of the curve suggests that the optimization process efficiently converged to a near-optimal solution without excessive iterations. The curve shows a rapid improvement in solution quality during the early iterations, followed by stabilization around the 12th iteration, indicating that the GA successfully converged toward an optimal solution. This pattern reflects the balance between exploration and exploitation within the GA search space. The GA convergence behavior demonstrated stable optimization performance under the predefined parameters. Overall, the graph demonstrates that the ANN-GA hybrid model reached a stable and reliable optimum extraction condition. This observation is consistent with previous studies reporting that ANN models can perform reliably in structured small datasets.

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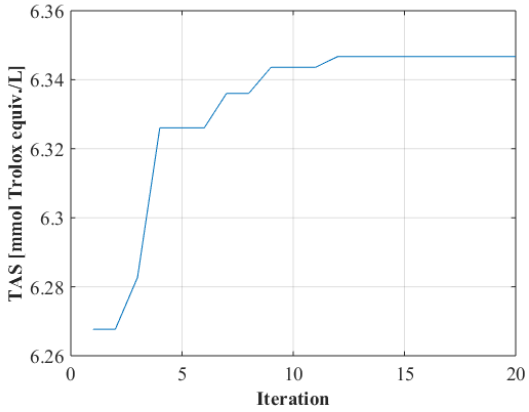


Figure 2. Convergence curve of the genetic algorithm (GA) showing the evolution of fitness values across iterations during ANN-GA-based optimization of extraction conditions for *Russula vinosa*

Antioxidant Activity

Mushrooms significantly mitigate oxidative stress by neutralizing free radicals through their polyphenols, flavonoids, and polysaccharides. These attributes aid in the mitigation of cellular damage and the diminishment of chronic disease risk [38]. This study assessed the antioxidant capabilities of *R. vinosa* extracts generated under ideal circumstances. The findings are displayed in Table 2.

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Table 2. Antioxidant values of *Russula vinosa* optimized extract

Parameters	ANN-GA extract values	RSM extract values
FRAP (mg TE/g)	193.010±1.428[b]	174.927±2.886[a]
TAS (mmol/L)	6.439±0.026[b]	6.317±0.017[a]
DPPH (mg TE/g)	159.927±1.294[b]	144.433±0.885[a]
TOS (µmol/L)	11.074±0.032[b]	12.754±0.038[a]
OSI (TOS/(TAS*10))	0.172±0.001[b]	0.202±0.001[a]

Note: Differences between ANN-GA and RSM extracts were evaluated using an independent samples t-test, and values with different superscript letters indicate statistically significant differences ($p < 0.05$).

In our study, the antioxidant properties of optimized *R. vinosa* extracts were compared using ANN-GA and RSM optimization methods. ANN-GA extracts showed relatively higher activity under the studied conditions in terms of FRAP, TAS, and DPPH values. The observed differences

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between ANN-GA and RSM extracts should be interpreted within the context of the optimized parameter sets, as variations in extraction conditions directly influence the concentration and composition of bioactive compounds. The higher phenolic content observed in ANN-GA optimized extracts can be attributed to the more precise identification of optimal extraction conditions, particularly in terms of solvent polarity, temperature, and extraction time. Phenolic compounds exhibit varying solubility depending on solvent composition, and mixtures of ethanol and water are known to enhance the extraction of both polar and moderately polar compounds. In addition, moderate temperatures improve mass transfer by increasing cell wall permeability without causing thermal degradation of heat-sensitive phenolics[39,40]. The ANN-GA approach, unlike traditional RSM, is capable of capturing complex nonlinear interactions between these parameters, enabling a more accurate identification of conditions that maximize phenolic solubility and stability. As a result, the optimized conditions obtained via ANN-GA likely facilitated more efficient disruption of cellular structures and improved diffusion of bioactive compounds into the solvent phase. In contrast, TOS and OSI values were found to be lower. In parallel with this improved extraction efficiency, ANN-GA extracts showed higher FRAP, TAS, and DPPH values, whereas TOS and OSI values were lower than those of RSM extracts. These findings indicate that ANN-GA optimization enhanced the antioxidant profile of *R. vinosa* by increasing the recovery of redox-active compounds while reducing oxidative burden. The observed antioxidant trends are consistent with previous studies reporting strong radical scavenging and reducing capacity in *R. vinosa*. The literature has reported that *R. vinosa* exhibits significant antioxidant potential in DPPH, ABTS, FRAP, and β-carotene/linoleic acid inhibition assays, that post-fermentation extracts exhibit enhanced antioxidant activity, and that it demonstrates significant activity in DPPH, ABTS, and hydroxyl radical scavenging assays [14,15,41]. In our study, the FRAP, DPPH, and TAS values obtained using ANN-GA optimization support these findings. Furthermore, previous studies have not found any findings regarding the TAS, TOS, or OSI values of *R. vinosa*. Research on various wild mushrooms indicated TAS levels of 4.054, 7.468, 5.511, and 5.426 mmol/L for *Paralepista flaccida, Lactarius deliciosus, Cantharellus cibarius*, and *Hericium erinaceus*, respectively. The TOS values were documented as 10.352, 13.161, 7.289, and 6.621 μmol/L, respectively. OSI values were documented as 0.255, 0.176, 0.132, and 0.122[11,12,42,43]. The TAS number signifies the total concentration of antioxidant chemicals present in the mushroom [8]. The TOS number signifies the total oxidant chemicals contained in the mushroom [8]. The OSI value

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denotes the percentage reduction of oxidant components by antioxidant compounds [8]. In our study, the TAS values of the optimized *Russula vinosa* extracts were determined to be higher than those of *P. flaccida, C. cibarius*, and *H. erinaceus*. However, they were lower than those of *L. deliciosus*. TOS values are relatively low compared to other fungal species reported in the literature. The OSI values of the ANN-GA extract were determined to be lower than those of *P. flaccida* and *L. deliciosus*. However, they were higher than those of *C. cibarius* and *H. erinaceus*. The RSM extract was lower than those of *P. flaccida* but higher than those of *L. deliciosus, C. cibarius*, and *H. erinaceus*. The findings of our study indicated that ANN-GA optimization increased TAS. It was also determined to improve OSI by reducing TOS. Overall, these findings indicate that ANN-GA optimization improves the oxidative stability profile of R. vinosa extracts and that the antioxidant characteristics are consistent with previous literature.

Anticholinesterase Activity

Mushrooms have the potential to exhibit anticholinesterase activity by inhibiting the enzymes acetylcholinesterase and butyrylcholinesterase. Due to these properties, they are considered potential therapeutic agents, particularly in the treatment of Alzheimer's disease and other neurodegenerative diseases [44]. In our study, we evaluated the anticholinesterase activity of optimized *R. vinos*a extracts. The obtained IC_{50} values are presented in Table 3.

Table 3. Anticholinesterase activity of *Russula vinosa* optimized extract

Sample	AChE µg/mL	BChE µg/mL
Galantamine	7.260±0.141^{c}	15.350±0.208^{c}
ANN-GA extract	59.213±0.771^{b}	93.980±0.918^{b}
RSM extract	68.687±0.917^{a}	101.363±1.122^{a}

Our study demonstrates that optimized *R. vinosa* extracts exhibit significant inhibitory effects against AChE and BChE enzymes. While galantamine, used as the reference inhibitor, exhibited the strongest activity, extracts obtained through both ANN-GA and RSM optimizations exhibited significant anticholinesterase activity. In particular, the lower IC_{50} values of ANN-GA extracts compared to RSM extracts suggest a potential advantage of the AI-based optimization approach in preparing more biologically active extracts. Furthermore, the role of phenolic compounds in this

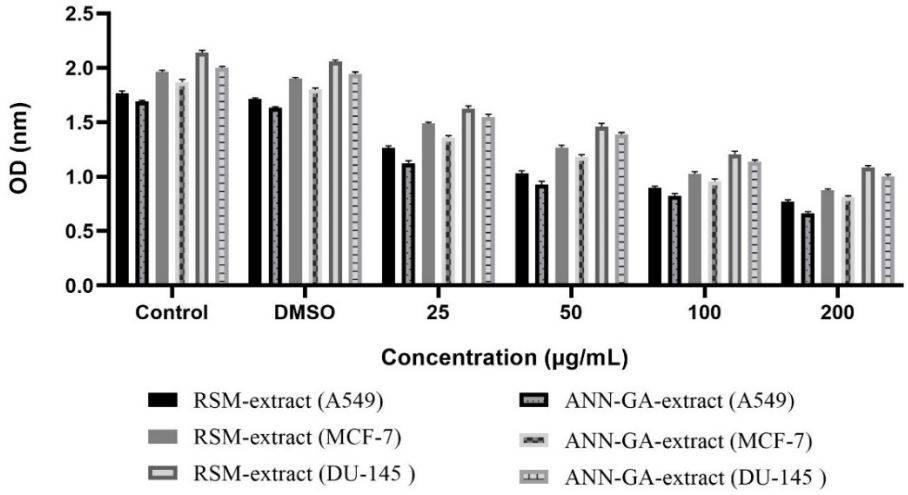
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activity is noteworthy. In our study, extracts optimized using the ANN-GA approach were found to inhibit acetylcholinesterase and butyrylcholinesterase enzymes. Furthermore, considering the high amounts of flavonoids, including quercetin, caffeic acid, and catechin hydrate, detected in ANN-GA extracts, a relationship between phenolic content and anticholinesterase activity is suggested. In the literature, methanol and hot water extracts of *Russula rosacea* have been reported to exhibit moderate inhibitory effects against AChE and BChE [45]. A separate study reported that *Russula aurea* and *R. sanguinea* have low AChE activity and no BChE activity [46]. Furthermore, *Russula ochroleuca* has been reported to exhibit cholinesterase-like enzyme activity capable of hydrolyzing acetylcholinesterase and butyrylcholinesterase substrates [47]. The literature indicates that cholinesterase inhibitor levels vary among different *Russula* species but are generally moderate or low. Therefore, the significant inhibition of AChE and BChE by optimized extracts of *R. vinosa* in our study strongly supports the neuroprotective potential of this species. In conclusion, by confirming the relationship between phenolic content and biological activity, *R. vinosa* appears to be a potential alternative or complementary source of natural cholinesterase inhibitors for the treatment of Alzheimer's and other neurodegenerative disorders.

Antiproliferative activity

Certain edible and medicinal mushroom species may impede cancer progression by modulating the cell cycle and initiating apoptotic processes via their bioactive metabolites[48,49]. These characteristics render them a significant source of natural anticancer compounds. This study assessed the biological activity of optimized *R. vinosa* extract against A549, MCF-7, and DU-145 cell lines. The results are illustrated in Figure 3.

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* Note: (Control group: Cells were cultured only in standard medium and were not exposed to any chemical application. DMSO group: DMSO was added to the cell medium as a solvent, but no extract was applied. Extract application groups: Cells were treated with extract solutions prepared at concentrations of 25, 50, 100 and 200 µg/mL).

Figure 3. Antiproliferative effects of ANN-GA- and RSM-optimized *Russula vinosa* extracts on A549, MCF-7, and DU-145 cancer cell lines at different concentrations

Our results showed that optimized extracts of *R. vinosa* displayed considerable antiproliferative effects in A549, MCF-7, and DU-145 cell lines. The observation that extracts optimized using ANN-GA exhibited reduced cell viability at all concentrations relative to those derived from the RSM technique indicates that AI-based optimization may provide a beneficial effect in augmenting biological activity. This difference, particularly at higher concentrations, suggests that the ANN-GA method more effectively enhances the bioavailability of phenolic compounds, suppresses the cell cycle, and triggers apoptotic mechanisms. This result is consistent with the phenolic profile findings. In our study, the presence of compounds such as gallic acid, caffeic acid, quercetin, and catechin hydrate in the ANN-GA extracts, and the strong antiproliferative activity of these compounds [50-53], explains the chemical basis for the strong antiproliferative effect observed in our study. Compared with previous studies in the literature, the obtained findings indicated that polysaccharide fractions obtained from *R. vinosa* exhibited antiproliferative activity in HeLa and HepG2 cells. In particular, the alkaline extract had a strong effect, the acidic extract had a moderate effect, and the aqueous extract had a weak effect, while all fractions remained at low levels in HepG2 cells [54]. While this study demonstrates that polysaccharides can exert cell-type-selective

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effects, our findings demonstrate that extracts rich in phenolic compounds provide broader-spectrum and more potent antiproliferative effects on various cancer cell lines. Therefore, the biological potential of *R. vinosa* is not dependent on a single compound group but is directly shaped by the extraction conditions and the diversity of metabolites obtained. Consequently, the antiproliferative activity of phenolic-rich extracts obtained through ANN-GA optimization was significantly increased compared to those prepared with RSM. Considered together with the polysaccharide-based selective effects reported in the literature, it appears that *R. vinosa* possesses a versatile anticancer potential through its diverse compound groups, and this potential can be more effectively unlocked with appropriate extraction strategies.

Phenolic Composition

Mushrooms are abundant in phenolic chemicals, which enhance their significant biological activities. The phenolic content greatly enhances both the nutritional value and medicinal potential of mushrooms [55]. This work assessed the phenolic contents of the optimized extract of *R. vinosa* using LC-MS/MS, with results displayed in Table 4.

Table 4. Phenolic contents of optimized extracts of *Russula vinosa*

Phenolic compounds	**ANN-GA extract (mg/kg)**	**RSM extract (mg/kg)**
Fumaric acid	4165.47±3.15[a]	3227.32±1.74[b]
Gallic acid	7166.30±3.53[a]	7068.23±1.90[b]
Protocatechuic acid	164.71±1.11[a]	112.95±0.94[b]
4-hydroxybenzoic acid	1021.50±1.76[a]	1105.95±2.48[b]
Caffeic acid	2701.02±1.46[a]	2562.05±1.17[b]
Quercetin	1647.77±1.03[a]	1295.05±1.84[b]
Catechin hydrate	673.82±1.01[a]	513.87±0.95[b]

Our study demonstrated that optimized *R. vinosa* extracts have significant potential in terms of their phenolic composition and biological activity. Extracts optimized using the ANN-GA approach generally contained more phenolic compounds than those obtained using RSM. This suggests that ANN-GA optimization improved the extraction efficiency of several physiologically relevant phenolic compounds compared with RSM. Gallic acid was the highest detected compound

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in our study. Additionally, phenolics including fumaric acid, caffeic acid, quercetin, and catechin hydrate were acquired in greater quantities using the ANN-GA approach. Compounds including gallic acid, fumaric acid, catechin hydrate, p-coumaric acid, and trans-cinnamic acid have been documented in *R. vinosa* in prior literature[56]. Our research validates the existence of these phenolic compounds. The increased concentration of 4-hydroxybenzoic acid in extracts optimized by RSM indicates that extraction parameters may selectively influence both total phenolic content and phenolic distribution. The biological activities observed in this study are strongly supported by the phenolic composition of the optimized extracts. The ANN-GA extract, which contained higher levels of gallic acid, caffeic acid, quercetin, and catechin hydrate, demonstrated superior antioxidant, anticholinesterase, and antiproliferative performance. Gallic acid and quercetin are well known for their strong reducing capacity and free-radical scavenging activity, which explains the elevated FRAP, TAS, and DPPH values observed in the ANN-GA extract [51,52]. Flavonoids such as quercetin and catechin hydrate, widely reported as inhibitors of acetylcholinesterase and butyrylcholinesterase, provide a mechanistic basis for the lower IC_{50} values in the enzyme inhibition assays [50,51]. Furthermore, phenolic acids including gallic acid and caffeic acid are recognized for their ability to induce apoptosis and regulate cancer-related signaling pathways, supporting the stronger antiproliferative effects recorded in A549, MCF-7, and DU-145 cell lines [52,53]. This relationship can be further explained by the direct influence of extraction parameters on compound solubility and, consequently, biological activity. Extraction temperature, solvent composition, and extraction time play a critical role in determining the solubility and stability of phenolic compounds[57]. For instance, ethanol-water mixtures enhance the extraction of both polar and moderately polar compounds, while moderate temperatures improve mass transfer and facilitate the release of intracellular metabolites without causing thermal degradation. As a result, optimized extraction conditions lead to higher concentrations of bioactive compounds, which in turn contribute to increased antioxidant capacity, enzyme inhibition, and antiproliferative effects. Therefore, the observed improvements in biological activity can be attributed not only to the presence of specific compounds but also to the optimization of physicochemical extraction parameters that govern their availability and functionality. Overall, these results indicate that the enhanced biological activity of ANN-GA extracts is directly linked to their enriched phenolic profile. Consequently, it was determined that *R. vinosa* could be an important natural resource applicable in functional food and medical fields.

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Conclusions

This study provides a comprehensive evaluation of the biological potential of *Russula vinosa* through the integration of Response Surface Methodology (RSM) and Artificial Neural Network-Genetic Algorithm (ANN-GA) optimization strategies. The results indicate that ANN-GA offers a flexible modeling approach for complex extraction systems and showed improved performance under the studied conditions, leading to enhanced extraction efficiency and biological activity. ANN-GA-optimized extracts exhibited a richer phenolic composition, particularly in gallic acid, caffeic acid, quercetin, and catechin hydrate, which was associated with increased antioxidant capacity (FRAP, TAS, DPPH) and improved oxidative balance (lower TOS and OSI values). In addition, the observed anticholinesterase and dose-dependent antiproliferative activities further support the biological relevance of the optimized extracts. These findings highlight the relationship between extraction strategy, chemical composition, and biological activity, and suggest that *R. vinosa* may serve as a promising resource for functional food, nutraceutical, and pharmaceutical applications. Furthermore, the ANN-GA-guided extraction approach offers advantages in terms of reduced solvent use, lower energy consumption, and improved process efficiency, supporting its potential as a sustainable optimization strategy. However, given the relatively small dataset used in this study, the findings should be interpreted cautiously. Future studies should include larger datasets, independent validation experiments, and IC_{50}-based evaluations to further confirm the robustness and general applicability of the proposed approach. In particular, the absence of external validation using independent datasets represents a limitation of the present study. Additionally, the isolation of individual bioactive compounds and in vivo validation studies will be essential to fully elucidate the therapeutic potential of *R. vinosa*.

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Availability of data and materials

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

Ethics declarations

Ethics approval and consent to participate

This research does not involve any ethical issues.

Consent for publication

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Not applicable.

Competing interests

The authors declare no competing interest.

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