Research Article

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Optimization of bioactive compounds extraction from *Rosa canina L.* pseudofruit through the action of two hydrolytic enzyme preparations

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Abstract

BACKROUND: The pseudo-fruit of *Rosa canina L.* is a rich source of bioactive compounds with antioxidant, anti-inflammatory, anti-cancer, anti-diabetic, anti-aging, and antimicrobial activities. The aim of the present study is the optimization of a green process based on the action of two hydrolytic enzyme preparations, namely Pectinex® Ultra Color (pectinolytic) and Viscoferm® (hemicellulolytic), for the extraction of bioactive compounds from the pseudo-fruit of *Rosa canina L.* of Greek origin. A Box–Behnken Design (BBD) was employed to optimize extraction conditions by evaluating the effects of enzyme loadings and extraction time on total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity. The optimum extract was evaluated for its biological activities, anti-aging (inhibition of tyrosinase activity), antidiabetic (inhibition of α-glucosidase and α-amylase activity), and for its antibacterial activity against *Escherichia coli* cells.

RESULTS: The optimal conditions were determined using Derringer's desirability function and were found at 0.59% v/v Pectinex® Ultra color, 0.51% v/v Viscoferm®, and approximately 97 min (96.23 min) extraction time. The optimum extract yielded 123.22 ± 2.50 mg GAE/g DW TPC, 49.13 ± 0.92 mg CAE/g DW TFC, and exhibited antioxidant activity with an IC_{50} of 0.98 ± 0.02$\mu L_{extract}$/mL. The biological activities of the extracts revealed strong inhibition of *E. coli* growth, moderate α-glucosidase inhibition, and weak tyrosinase inhibition and α-amylase inhibition.

CONCLUSION: The proposed process offers a rapid approach for the effective extraction of bioactive compounds from the pseudofruit of *Rosa canina L.*, with promising biological activities, thus demonstrating its potential for applications in various industries.
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Keywords: enzyme-assisted extraction (EAE); *Rosa canina L.*; Box–Behnken design (BBD); Pectinex® ultra color; Viscoferm®; biological activities

INTRODUCTION

In recent years, there has been growing scientific interest in plant-derived bioactive compounds due to their health-promoting effects and applications in the food, pharmaceutical, and cosmetic industries.[1] Although these bioactive compounds are not essential for the basic plant survival, they exhibit therapeutic properties. *Rosa canina L.* (dog rose) is a plant of the Rosaceae family, which was traditionally used in herbal medicine across Europe and the Mediterranean.[2] Its pseudo-fruit, commonly known as rosehip, is rich in valuable phytochemicals such as ascorbic acid (vitamin C), phenolic acids, flavonoids, carotenoids, tannins, and minerals.[3,4] These compounds contribute to the plant

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defense mechanisms and are responsible for its strong biological activities such as antioxidant, antimicrobial, anti-inflammatory, antidiabetic, and anti-aging.[5] The beneficial effects of rose hip extracts have been documented in the management of oxidative stress, metabolic syndrome, skin aging, and microbial infections.[5-8] Hence, the increasing interest for *Rosa canina L*. potential in developing nutraceutical, pharmaceutical, and cosmetic products.[3] However, the effective and eco-friendly extraction of the bioactive compounds remains a key challenge, as many of them are trapped within the plant cell wall or cytosol.[9] The plant cell wall is primally composed of complex polysaccharides, including cellulose (a linear chain of glucose units connected by β-1,4-glycosidic bonds), hemicellulose (a non-linear heteropolymer in which main sugars are also linked by β-1,4-glycosidic bonds) and pectin (a linear polymer of D-galacturonic acid connected by α-1,4-glycosidic bonds). In addition to these, the cell wall consists of glycoproteins and lignin (a heterogeneous polymer derived from three lignol precursors chemically linked in various configurations).[10] The complex structure of the plant cell wall hinders the release of the bioactive compounds, hence the need for optimization of innovative extraction methods.[11]

Traditional extraction methods, such as solvent extraction using organic solvents (*i.e*., methanol, ethanol, or acetone) are effective; however, they come with several limitations including high energy consumption, long extraction times, use of toxic solvents, and poor selectivity.[12,13] These limitations have steered research toward the development of green extraction technologies that align with the principles of green chemistry, sustainability, safety, and efficiency.[14] Among these, enzyme-assisted extraction (EAE) has emerged as a promising technique. EAE is based on the inherent ability of enzymes to selectively hydrolyze and degrade the structural components of the plant cell wall, facilitating the release of bound phytochemicals.[15,16] It is regarded as an eco-friendly method due to its minimal use of toxic solvents, mild operating conditions, and ability to achieve high extraction yields.[17] The success of EAE relies on several factors, including enzyme type and load, extraction time, temperature, pH, and the physicochemical characteristics of the plant material.[18,19] Specifically, the combined use of pectinases and hemicellulases has been demonstrated to significantly enhance the release of phenolic compounds from plant tissues.[20-24] Pectinases are a group of enzymes responsible for hydrolyzing the glycosidic bonds in pectic polymers, especially pectin which is one of the complex plant cell wall's polysaccharides. They are primarily categorized intro three main groups based on their mode of action: protopectinases, depolymerases, and esterases.[25,26] Hemicellulases are a diverse group of enzymes that break down the complex, non-linear structure of hemicellulose by targeting specific glycosidic and ester bonds within its various polysaccharide components. This group includes xylanases, mannanases, esterases, arabinanases, and others, each specialized in degrading different structural elements of hemicellulose into simpler sugars or oligosaccharides.[27-29] 29 To date, only our previous study has focused on the EAE of bioactive compounds specifically from *Rosa canina L*. pseudofruit, while none has ever optimized this extraction process using statistical tools.[30] Moreover, the combined action of multiple enzymes on the extraction efficiency and biological activities of rose hip extracts remains underexplored. This knowledge gap limits the development of standardized and sustainable extraction protocols for industrial applications.

In this context, the present study aims to optimize a green extraction process of bioactive compounds from the pseudo-fruit of *Rosa canina L*., based on the combined effect of Pectinex® Ultra Color (a pectinolytic enzyme preparation) and Viscoferm® (a hemicellulolytic enzyme preparation) under mild extraction conditions. A Box–Behnken Design (BBD) was applied to optimize the process by evaluating the interactions of multiple variables. The independent variables considered in the optimization design were the enzyme loading of each enzyme preparation and the extraction time. The extraction performance was assessed based on three key response variables: total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity. The biological potential of the optimum extract was further investigated through standard *in vitro* assays, specifically assessing its anti-aging activity *via* tyrosinase inhibition, antidiabetic activity *via* inhibition of α-glucosidase, and α-amylase and antibacterial activity based on the inhibition growth of *Escherichia coli* (*E. coli*). This study seeks to integrate green chemistry principles, biotechnological and statistical tools to establish a sustainable process for producing high-value extracts. The resulting extracts may serve as multifunctional ingredients supporting the valorization of underutilized plant resources from the Mediterranean biodiversity. Moreover, the findings may contribute to the broader development of eco-innovative strategies in natural product extraction.

MATERIALS AND METHODS

Plant material

The raw material used in the experiments was the dried pseudo-fruit (also referred to as rosehip) of dog rose (*Rosa canina L*.), which was kindly provided by Korres S.A.—Natural Products. It originates from plants cultivated and preserved within the Balkan Botanic Garden of Kroussia, located in northern Greece, near the mountain village of Pontokerasia in the Kilkis Regional Unit (coordinates: 41°05′ N 23°06′ E). The rosehips were collected during the first ten days of October. Dried rosehip shells (hypanthium), manually separated from the seeds, were downsized to an average particle size of <500 μm in a laboratory mill. The milled material was stored in plastic containers at room temperature until further analysis.

Chemical and reagents

The following reagents were used: 2,2-Diphenyl−1-picrylhydrazyl (DPPH); absolute methanol (CH3OH); and Folin–Ciocalteu from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were of the highest purity commercially available and were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Enzyme preparations

Pectinex® Ultra Color and Viscoferm® were a generous gift from Novozymes A/S (Bagsværd, Denmark). Tyrosinase (EC 1.14.1.8.1) from mushroom, α-amylase (EC 3.2.1.1) from porcine pancreas, and α-glucosidase (EC 3.2.1.20) from *Saccharomyces cerevisiae* were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Experimental design

The Box–Behnken Design (BBD) is a statistical experimental design used in response surface methodology (RSM) to optimize processes and model complex relationships between multiple variables. The number of experiments (N) required for the development of BBD is defined as $N = 2\ k(k - 1) + C_0$, (where k is number of factors and C_0 is the number of central points), while the respected number for a central composite design (CCD) is $N = 2^k + 2\ k + C_0$. One of the key advantages of BBD is its efficiency. It requires fewer experimental runs than a CCD for the same number of factors. The design is rotatable or nearly

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rotatable, meaning it provides equal precision of estimation in all directions from the center.[31]

In the present study, a three-factor BBD with three levels (low: −1, medium: 0, and high: +1) was applied to study the combined effect of two commercial enzyme preparations, namely Pectinex® Ultra Color and Viscoferm®, at different loadings and the extraction time on total phenolic content (TPC), and total flavonoid content (TFC) of the extracts as well as on their antioxidant activity. The levels of the independent variables used in the experimental design are shown in Table 1 and were selected based on previous study and preliminary experiments (data not shown).[30] BBD resulted in 15 experimental runs with three replicates at the center point level (Table 2).

Enzyme-assisted extraction

The milled material, at solid to liquid ratio 6% w/v, was mixed with 50 mM phosphate buffer pH 5.5 and a specific amount of the two enzyme preparations was added. Extraction was performed in a thermoshaker (Thermomixer®, Eppendorf, Hamburg, Germany) under constant agitation (1300 rpm) at 50 °C. The specific extraction parameters were determined by BBD. The samples were centrifuged at 10 000 rpm for 10 min at 4 °C and the supernatants were collected and heated at 85 °C for 10 min to inactivate residual enzymes. At this temperature the phenolic and flavonoid compounds remain stable.[32,33] After heat treatment samples were stored at −18 °C until further analysis.

The extraction temperature (50 °C) and pH (5.5) were selected because they fall within the optimal range for the catalytic activity of the pectinolytic and hemicellulolytic enzyme preparations used in this study. The solid-to-liquid ratio was fixed at 6% w/v in order to maintain a balance between extract concentration and efficient enzyme-substrate contact, as also identified as optimal in our previous study.[30] Higher ratios increase viscosity and hinder mass transfer, while lower ratios result in overly dilute extracts.[34] A high agitation rate was selected to ensure homogeneity of the suspension, prevent sedimentation, and enhance mass transfer, thereby maximizing the efficiency of the enzymatic extraction process.[35]

Total phenolic content

The total phenolic content (TPC) was determined according to Folin–Ciocalteu method, as previously described.[30] Briefly, the extract was mixed with 250 µL of Folin–Ciocalteu reagent and 3.95 mL distilled water, then left to stand at room temperature for 1 h. The absorbance was measured at 755 nm and the results were expressed as mg of gallic acid equivalents (GAE) per g of dry material (DM) (mg GAE/g DM) using a gallic acid calibration curve ($C_{GAE} = 0.9377ABS_{755\ nm} + 0.0515$, $R^2 = 0.999$). All measurements were performed in triplicate.

Total flavonoid content

The total flavonoid content (TFC) was measured using the aluminum chloride colorimetric method, following the procedure reported in our previous study.[30] Briefly, the extract was mixed sequentially with $NaNO_2$, $AlCl_3$, NaOH, and distilled water, then left to stand at room temperature for 15 min. The absorbance was measured at 510 nm. The results were expressed as mg Catechin Equivalents (CAE) per g of dry material (DM) (mg CAE/g DM), using a catechin calibration curve ($C_{CAE} = 0.8726ABS_{510\ nm} - 0.021$, $R^2 = 0.998$). All measurements were performed in triplicate.

Antioxidant activity

The antioxidant activity of the extracts was determined using the colorimetric determination of DPPH radical scavenging ability, as previously described.[30] Briefly, different concentrations of the extract were mixed with a DPPH solution (2.5% w/v in methanol) and incubated in the dark at room temperature for 30 min. Absorbance was measured at 515 nm and the DPPH radical scavenging activity of the samples was quantified by the IC_{50} value, which represents the extract concentration that is required to reduce 50% of the initial DPPH absorbance. All measurements were performed in triplicate. IC_{50} values were calculated by the linear plot of extract concentrations *vs*. percentage inhibition of DPPH radicals, and the results were finally expressed as µL of extract per mL of sample ($\mu L_{extract}/mL$).

Anti-aging activity (Tyrosinase inhibition)

Inhibition of tyrosinase (EC 1.14.1.8.1) was determined using the modified dopachrome method with L-DOPA as substrate, according to our previously published protocol.[30] Briefly, in a 96-well plate, 25 µL of sample (diluted in phosphate buffer, 50 mM, pH 6.8), 40 µL of tyrosinase (200 U/mL), 100 µL of the same buffer, and 40 µL of L-DOPA (10 mM) were mixed. Following incubation at 25 °C for 10 min, absorbance was measured at 492 nm. All measurements were performed in triplicate. Kojic acid was used as the positive control (complete inhibition of tyrosinase activity) at a concentration of 1 mg/mL.

Inhibition of tyrosinase activity was calculated as follows:

$$\text{Inhibition of tyrosinase activity } (\%) = \left(1 - \frac{A_{sample} - A_{sample\ control}}{A_{negative\ control} - A_{blank\ control}}\right) \times 100 \quad (1)$$

where A_{sample} is the absorbance of the sample, $A_{sample\ control}$ is the absorbance of the reaction mixture in the absence of substrate, $A_{blank\ control}$ is the absorbance of the reaction mixture in the absence of sample and substrate, and $A_{negative\ control}$ is the absorbance of the reaction mixture in the absence of sample. The anti-aging activity of the samples was quantified by the IC_{50} value, calculated by the linear plot of extract concentrations *vs*.

Table 1. Independent variables and their levels employed in the Box–Behnken Design (BBD)

Coded symbols	Independent variables	Levels		
		−1	0	1
X_1	Extraction time (min)	60	120	180
X_2	Enzyme loading Pectinex® Ultra Color (% v/v)	0.25	0.50	0.75
X_3	Enzyme loading Viscoferm® (% v/v)	0.50	0.75	1.00

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Table 2. The Box–Behnken Design and corresponding response values. The results are expressed as mean values ± SD

A/A	X_1	X_2	X_3	TPC (mg GAE/g DW)	TFC (mg CAE/g DW)	Antioxidant activity ($\mu L_{extract}$/mL)
1	−1 (60)	−1 (0.25)	0 (0.75)	116.6 ± 3.4	42.3 ± 1.5	0.79 ± 0.00
2	+1 (180)	−1 (0.25)	0 (0.75)	120.1 ± 1.6	45.4 ± 2.2	0.76 ± 0.02
3	−1 (60)	+1 (0.75)	0 (0.75)	118.4 ± 1.9	44.2 ± 2.6	0.71 ± 0.00
4	+1 (180)	+1 (0.75)	0 (0.75)	127.0 ± 1.5	47.3 ± 1.6	1.17 ± 0.02
5	−1 (60)	0 (0.5)	−1 (0.5)	119.3 ± 2.0	43.2 ± 1.2	1.11 ± 0.03
6	+1 (180)	0 (0.5)	−1 (0.5)	118.1 ± 2.8	45.9 ± 1.6	1.23 ± 0.01
7	−1 (60)	0 (0.5)	+1 (1)	120.3 ± 1.9	43.9 ± 1.9	1.10 ± 0.01
8	+1 (180)	0 (0.5)	+1 (1)	125.7 ± 4.1	47.6 ± 0.8	1.04 ± 0.02
9	0 (120)	−1 (0.25)	−1 (0.5)	115.2 ± 1.5	44.8 ± 1.6	1.01 ± 0.01
10	0 (120)	+1 (0.75)	−1 (0.5)	127.1 ± 2.4	49.7 ± 2.5	0.99 ± 0.01
11	0 (120)	−1 (0.25)	+1 (1)	116.7 ± 3.2	44.3 ± 1.2	1.03 ± 0.00
12	0 (120)	+1 (0.75)	+1 (1)	117.9 ± 3.2	42.5 ± 1.0	1.10 ± 0.01
13	0 (120)	0 (0.5)	0 (0.75)	119.0 ± 0.3	42.7 ± 1.3	0.73 ± 0.00
14	0 (120)	0 (0.5)	0 (0.75)	122.1 ± 0.6	43.4 ± 1.2	0.80 ± 0.01
15	0 (120)	0 (0.5)	0 (0.75)	118.2 ± 0.6	43.1 ± 0.7	0.75 ± 0.00

percentage inhibition of tyrosinase and the results were finally expressed as mg of extract per mL of sample.

Antidiabetic activity

α-Glucosidase inhibition

Inhibition of α-glucosidase was performed according to our previously described method, based on the use of p-Nitrophenyl-α-D-glucopyranoside as substrate.[30] Briefly, 100 µL of the sample was incubated with 50 µL of α-glucosidase (0.25 U/mL in 0.1 M phosphate buffer, pH 6.8) at 37 °C for 10 min. After adding 50 µL of p-nitrophenyl-α-D-glucopyranoside (1 mM), the mixture was further incubated for 5 min and absorbance was measured at 405 nm against a buffer blank., Absorbance was measured at 405 nm. All measurements were performed in triplicate. Tannic acid is a known inhibitor of α-glucosidase activity and was used as the positive control (complete inhibition of α-glucosidase activity) at a concentration of 1 mg/mL.

Inhibition of α-glucosidase activity was calculated as follows:

$$\text{Inhibition of } \alpha\text{−glucosidase activity } (\%) = \frac{A_{blank} - A_{sample}}{A_{blank}} \times 100 \quad (2)$$

where A_{blank} is the absorbance of the blank and A_{sample} is the absorbance of the sample. The antidiabetic activity of the samples was quantified by the IC_{50} value, calculated by the linear plot of extract concentrations *vs.* percentage inhibition of α-glucosidase and the results were finally expressed as mg of extract per mL of sample.

α-Amylase inhibition

Inhibition of α-amylase activity was performed using the iodine/potassium iodide (IKI) method, as detailed in our earlier work.[30] Briefly, in a 96-well plate, 25 µL of sample was mixed with 50 µL of α-amylase (0.5 U/mL in phosphate buffer, pH 6.9, with 6 mM NaCl) and incubated at 37 °C for 10 min. The reaction was initiated with 50 µL of starch solution (0.025% dissolved in the same buffer) and further incubated for 10 min. Reaction was stopped by adding 25 µL of 1 M HCl, followed by 100 µL of IKI solution, and absorbance was measured at 630 nm. All measurements were performed in triplicate. Tannic acid is a known inhibitor of α-amylase activity and was used as the positive control (complete inhibition of α-amylase activity) at a concentration of 1 mg/mL.

Inhibition of α-amylase activity was calculated as follows:

$$\text{Inhibition of } \alpha\text{−amylase activity } (\%) = \left(1 - \frac{A_{s_s} - A_{s_b}}{A_{s_{c+}} - A_{s_{c-}}}\right) \times 100 \quad (3)$$

where A_{s_s} is the absorbance of the sample, A_{s_b} is the absorbance of the reaction mixture in the absence of enzyme, $A_{s_{c+}}$ is the absorbance of the reaction mixture in the absence of the sample (100% enzyme activity), and $A_{s_{c-}}$ is the absorbance of the mixture without sample and enzyme (0% enzyme activity).

The antidiabetic activity of the samples was quantified by the IC_{50} value, calculated by the linear plot of extract concentrations *vs.* percentage inhibition of α-amylase, and the results were finally expressed as mg of extract per mL of sample.

Antibacterial activity

The antibacterial activity of the extracts was tested against *Escherichia coli* (*E. coli*) using the broth microdilution method, as previously reported.[30] The extracts were first diluted in sterile culture medium. In a 96-well microplate, 50 µL of culture medium was dispensed into each well. Then, 50 µL of each extract was added to the wells of the first column and mixed thoroughly. A serial twofold dilution was performed by transferring 50 µL from the first column into the second column, mixing, and continuing this procedure sequentially down to column 10. After completing the dilutions, 50 µL of bacterial suspension (10^8 CFU/mL) was added to each well. Column 11 was designated as the growth control (containing only bacterial suspension and culture medium), while column 12 served as the sterility control (containing only culture medium). Optical density was recorded at 600 nm after 24 h of incubation. All measurements were performed in triplicate. Antibacterial activity (inhibition of *E. coli* growth) was calculated as follows:

$$\text{Inhibition of growth } (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \quad (4)$$

where $A_{control}$ is the absorbance of the growth control and A_{sample} represents the absorbance of cells containing the extracts.

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Conventional extraction

The milled material at 6% w/v concentration was mixed with ethanol-water mixture (EtOH:H_2O 70:30 v/v). Extraction was performed in a thermoshaker (Thermomixer®, Eppendorf, Hamburg, Germany) under constant agitation (1300 rpm) at 50 °C. The samples were centrifuged at 10 000 rpm for 10 min at 4 °C and the supernatants were collected and stored at −18 °C until further analysis.

Statistical analysis

The experimental design was generated and analyzed using Design-Expert 12.0 software (Stat-Ease Inc., Minneapolis, MN, USA—Trial Version). All data were initially analyzed using Analysis of Variance (ANOVA). The significance of each regression coefficient was evaluated at the 95% confidence level. The results were expressed as mean values with the standard deviation (SD) of three independent measurements ($n = 3$) and significance was assumed at $P < 0.05$. Pareto charts of standardized effects were generated using R programming to visually assess the influence and statistical significance of individual factors and their interactions. To validate the accuracy of the prediction model, a confirmation experiment was carried out under optimal conditions and the experimental results were compared with the model's predicted values.

RESULTS AND DISCUSSION

A three-factor Box–Behnken Design (BBD) with three levels was applied to investigate the combined effect of three independent variables, extraction time, enzyme loading of Pectinex® Ultra Color and enzyme loading of Viscoferm®, on the response variables total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity. The results of the BBD are presented in Table 2.

RSM involves the use of statistical analysis and mathematical techniques for the optimization of experimental designs.[36] The collected data from the experimental design is then subjected to regression analysis to fit mathematical models that describe the relationship between the independent variables and the response. In the present study the data obtained from BBD for each response were fitted to the following quadratic polynomial model (Eq. (5)).

$$y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \sum_{j=i+1}^{n} \beta_{ij} X_i X_j + \sum_{i=1}^{n} \beta_{ii} {X_{ii}}^2 \qquad (5)$$

where Y is the predicted response; β_0 is an offset term; β_i, β_{ii}, and β_{ij} are the linear, quadratic, and interactive coefficients, respectively; n is the number of independent variables; and X_i and X_j are the levels of the independent variables.

TPC of extracts

The TPC data ranged from 115.2 ± 1.5 to 127.1 ± 2.4 mg GAE/g DW (Table 2), indicating a moderate sensitivity to the experimental conditions. In general, increasing the extraction time from 60 to 180 min led to higher TPC yields, as observed in comparisons between runs, such as 1 and 2 or 3 and 4. This trend is consistent with the diffusion-controlled nature of solid–liquid extractions, where prolonged extraction time enhances the release and solubilization of phenolic compounds from plant matrices, until equilibrium is reached.[37] The enzyme loading of Viscoferm® did not have strong impact on TPC release. For instance, in run 9, the enzyme loading of Pectinex® Ultra Color is 3-fold the amount of run 8 and the TPC response is about 10% higher, while at the highest enzyme loading of Viscoferm® (1% v/v) the corresponding responses do not differ significantly from those obtained with lower concentrations. The highest TPC yield was observed in run 10 (127.1 ± 2.4 mg GAE/g DW), which combined medium extraction time (120 min), high enzyme loading of Pectinex® Ultra Color X_2 (0.75% v/v), and low enzyme loading of Viscoferm® (0.5% v/v). Comparable results were obtained in run 4, employing higher enzyme loading of Viscoferm® and longer extraction time, suggesting that increasing either parameter does not significantly enhance TPC yield.

A quadratic model was selected to describe the TPC response. Statistical analysis of the model coefficients was performed at a 95% confidence level. Terms with *P*-values greater than 0.05 and that did not significantly contribute to the model's fit were excluded, except for the main (independent) variables, which were retained to preserve model hierarchy. The ANOVA results of both unmodified and modified models are presented in Table S1. The quadratic term ${X_2}^2$ was excluded from the model as it did not significantly contribute to its fit. The final proposed equations are given in coded (Eq. (6)) and actual values (Eq. (7)). The model F-value of 5.09 indicates that the model is significant, as it is also suggested by the *P*-value of 0.0440 < 0.05. The lack of fit F-value of 0.8574 and *P*-value of 0.5780 imply that the lack of fit is not significant relative to the pure error, confirming the validity of the model. The coefficient of determination R^2 and adjusted R^2 are 0.9015 and 0.7243, respectively, while the predicted R^2 of 0.7131 is in reasonable agreement with the adjusted R^2, as their difference is less than 0.2. The correlation between predicted and actual values for TPC obtained from the BBD indicates adequate model accuracy, as evidenced by the close alignment along the diagonal line (Fig. S1(a)). Therefore, this model can be utilized to make predictions about the response for the studied levels of each factor, within the design space.

In general, the positive sign in front of the terms in a model indicates a synergistic effect, whereas a negative sign indicates an antagonistic effect.[38] According to the regression coefficients of the proposed model (Eq. (7)), all three factors affected positively the response TPC, with Viscoferm® showing the strongest linear effect. The positive interaction between time and Pectinex® Ultra Color is minimal, while a negative interaction between time and Viscoferm® slightly reduces TPC. The strong antagonistic interaction between Pectinex® Ultra Color and Viscoferm® suggests that using both enzymes at high concentrations lowers phenolic release. Overall, moderate extraction time and carefully adjusted enzyme levels enhance TPC yields effectively. The standardized effect of the independent variables and their interaction on the TPC was investigated by preparing a Pareto chart (Fig. S2(a)). The length of each bar in the chart indicates the standardized effect of that factor on the response. The fact that the bar for X_3, X_1X_3, X_1X_2, ${X_1}^2$, and ${X_3}^2$ remained inside the reference line (*P*-value = 0.05), indicated that these terms contributed the least in prediction of TPC of the extracts.

The correlation between the studied factors and the TPC response is further analyzed using the three-dimensional diagrams (3D) surface plots of TPC as a function of two independent variables, while the third remains at a fixed value (Fig. 1), where the previous observations are confirmed. The response surface analysis (RSM) depicts that low enzyme loading of Viscoferm® and high enzyme loading of Pectinex® Ultra Color achieve TPC maximization (red plot area) (Fig. 1(a)). Also, the combination of high enzyme loading of Pectinex® Ultra Color or Viscoferm® with

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longer extraction time yields elevated TPC values (Fig. 1(b), (c)), supporting the observation from comparison between runs 4 and 10. This trend suggests potential synergistic effects between enzymatic cell wall degradation and prolonged solvent contact. These findings indicate that enzyme Pectinex® Ultra Color may be more efficient for phenolic release, possibly due to its substrate specificity. Based on our previous study, the concentration of pectin and hemicellulose in the pseudofruit of *Rosa canina L.* were 11.8 ± 1.9 and 10.9 ± 0.3% w/w dry basis, respectively.[30] In addition to the slightly higher abundance of pectin in the rosehip matrix, the stronger effect of the pectinolytic preparation on phenolic recovery can also be attributed to the specific physicochemical associations between phenolic compounds and plant cell wall polysaccharides. Phenolics in plant tissues are frequently associated with cell wall polysaccharides through both covalent and non-covalent interactions. Covalent ester and ether linkages, particularly involving hydroxycinnamic acids such as ferulic and p-coumaric acid, are commonly formed with pectic and hemicellulosic domains. Non-covalent interactions, such as hydrogen bonding, hydrophobic forces, electrostatic attractions, and π–π stacking, further contribute to the entrapment of phenolics within the cell wall matrix.[39-41] Pectinases hydrolyze α-1,4-glycosidic bonds in galacturonic acid-rich homogalacturonan regions, thereby disrupting the structural integrity of the pectin network and facilitating the release of phenolics that are either chemically bound or physically embedded.[25,26] Although hemicellulase loading did not significantly affect total phenolic content, its positive impact on antioxidant activity suggests the selective release of low-molecular-weight phenolics with high radical scavenging capacity. Hemicellulases target complex heteropolysaccharides such as arabinoxylans and mannans, cleaving β-1,4-glycosidic bonds and side chains, including feruloyl esters.[27-29] This enzymatic action may liberate antioxidant-active compounds or enhance their extractability by increasing cell wall porosity and solvent accessibility, thereby contributing to the observed improvement in antioxidant potential.

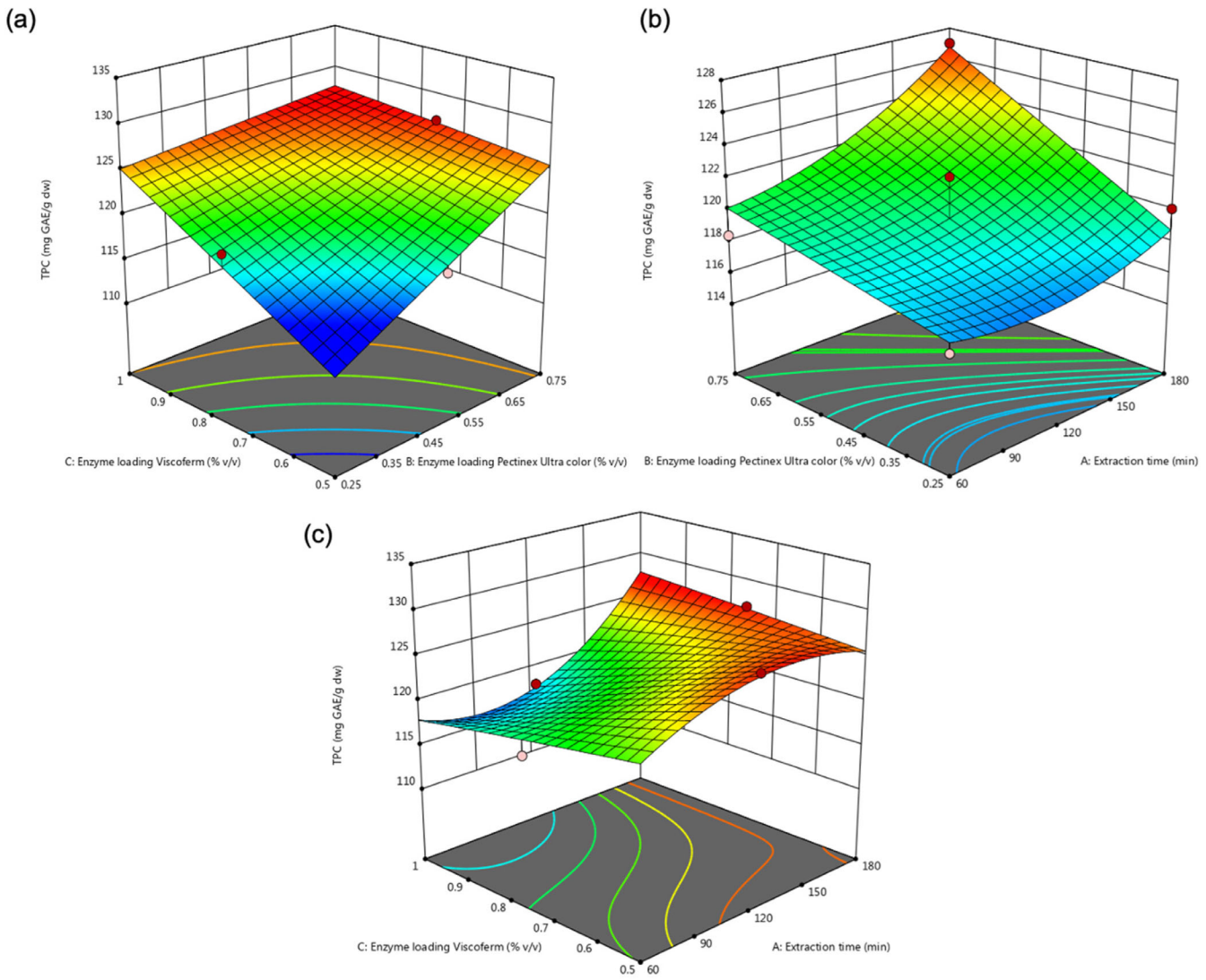

Figure 1. Three-dimensional (3D) surface plot of the total phenolic content (TPC) response, showing the correlation between TPC and the studied factors. (a) Effect of enzyme loading Viscoferm® (% v/v) and enzyme loading Pectinex® Ultra Color (% v/v) at a fixed extraction time of 180 min; (b) effect of enzyme loading Pectinex® Ultra Color (% v/v) and extraction time (min), at a fixed enzyme loading Viscoferm® of 0.75% v/v; and (c) effect of enzyme loading Viscoferm® (% v/v) and extraction time at a fixed enzyme loading Pectinex® Ultra Color 0.75% v/v.

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$$\text{TPC (mg GAE/g DW)} = 119.50 + 2.04X_1 + 2.72X_2 - 1.92X_3 + 1.28X_1X_2 + 1.65X_1X_3 - 2.67X_2X_3 + 1.23{X_1}^2 - 0.08{X_3}^2 \quad (6)$$

$$\text{TPC (mg GAE/g DW)} = 70.02 + 0.64^{*}\,t + 32.80^{*}\,P + 67.5^{*}\,V + 0.09^{*}\,t^{*}\,P - 0.98^{*}\,t^{*}\,V - 42.8^{*}\,P^{*}\,V - 0.003^{*}\,t^2 - 1.20^{*}V^2 \quad (7)$$

where t is the extraction time (min), P is the Pectinex® Ultra Color loading, and V is the Viscoferm® loading.

TFC of extracts

The total flavonoid content (TFC) varied from 42.3 ± 1.5 to 49.7 ± 2.5 mg CAE/g DW (Table 2), following trends similar to those observed for TPC. Longer extraction times resulted in a 10% increase in TFC yields (*i.e.*, runs 1 *vs* 2 or 3 *vs* 4), suggesting that time facilitates the release of flavonoid compounds through matrix breakdown and solvent penetration. The enzyme loading of Pectinex® Ultra Color assist in the release of flavonoids as well; however, the effect of the hemicellulolytic enzyme preparation is less consistent and likely dependent to the interaction with the other factors. For instance, comparing run 10 to central points (run 13–15) indicates that increasing the enzyme loading of Viscoferm® alone does not enhance TFC. The highest TFC value (49.7 ± 2.5 mg CAE/g DW) was obtained in run 10, which featured a medium extraction time (120 min) and high enzyme loading Pectinex® Ultra Color loading (0.75%).

Based on the statistical analysis (ANOVA) (Table S2), a quadratic model was employed to describe the TFC response (coded Eq. (8) and actual Eq. (9)). The interaction term X_1X_2 (combined effect of X_1 and X_2) was eliminated as its *P*-value was over 0.05 and did not contribute to the fitness of the model. The model F-value of 75.46 indicates that the model is significant, as it is also suggested by the *P*-value <0.0001. The lack of fit F-value of 0.6520 and *P*-value of 0.6523 confirm the validity of the model. The coefficient of determination R^2 and adjusted R^2 are 0.9927 and 0.9795, respectively, while the predicted R^2 of 0.8688 is in reasonable agreement with the adjusted R^2. The strong correlation between predicted and actual values for TFC obtained from the BBD is also shown in Fig. S1(b). Therefore, this model can be applied to navigate the design space within the studied ranges of each factor. The standardized effect of the independent variables and their interaction on the TFC is illustrated in a Pareto chart (Fig. S2(b)). The coefficients remained inside the reference line (*P*-value = 0.05) indicating that these terms significantly contributed in prediction of TFC of the extracts.

According to the regression coefficients of the proposed model (Eq. (9)), all three factors have a positive linear effect on TFC. The quadratic terms for both enzymes are also positive, indicating a curvature and suggesting that higher enzyme concentrations enhance TFC up to an optimal point. However, the negative interaction between enzyme loadings suggests a strong antagonistic effect when both enzymes are used together at high levels.

The correlation between the studied factors and the TFC response was analyzed using the 3D surface plots, which illustrate TFC as a function of two independent variables while the third is held at a fixed value (Fig. 2). Notably, TFC minimization (blue plot area) is associated with both low enzyme loadings and short extraction times. As observed in Fig. 1(a) enzyme loading of Viscoferm® appears to have minimal effect on the TFC response, as previously observed, while in enzyme loading of Pectinex® Ultra Color above 0.65% v/v combined with extended extraction times (>120 min) could achieve TFC maximization Fig. 1(b). Overall, the data suggests that flavonoid extraction is primarily driven by higher concentrations of the pectinolytic enzyme, as observed in the case of TPC.

$$\text{TFC (mg CAE/g DW)} = 43.07 + 1.70X_1 + 0.86X_2 - 1.93X_3 + 0.5X_1X_3 - 1.68X_2X_3 + 0.65{X_1}^2 + 1.08{X_2}^2 + 1.18{X_3}^2 \quad (8)$$

$$\text{TFC (mg CAE/g DW)} = 26.91 + 0.41^{*}\,t + 6.28^{*}\,P + 9.80^{*}\,V - 0.57^{*}\,t^{*}\,V - 26.80^{*}\,P^{*}\,V - 0.01^{*}\,t^2 + 17.27^{*}\,P^2 + 18.87^{*}\,V^2 \quad (9)$$

where t is the extraction time (min), P is the Pectinex® Ultra Color loading, and V is the Viscoferm® loading.

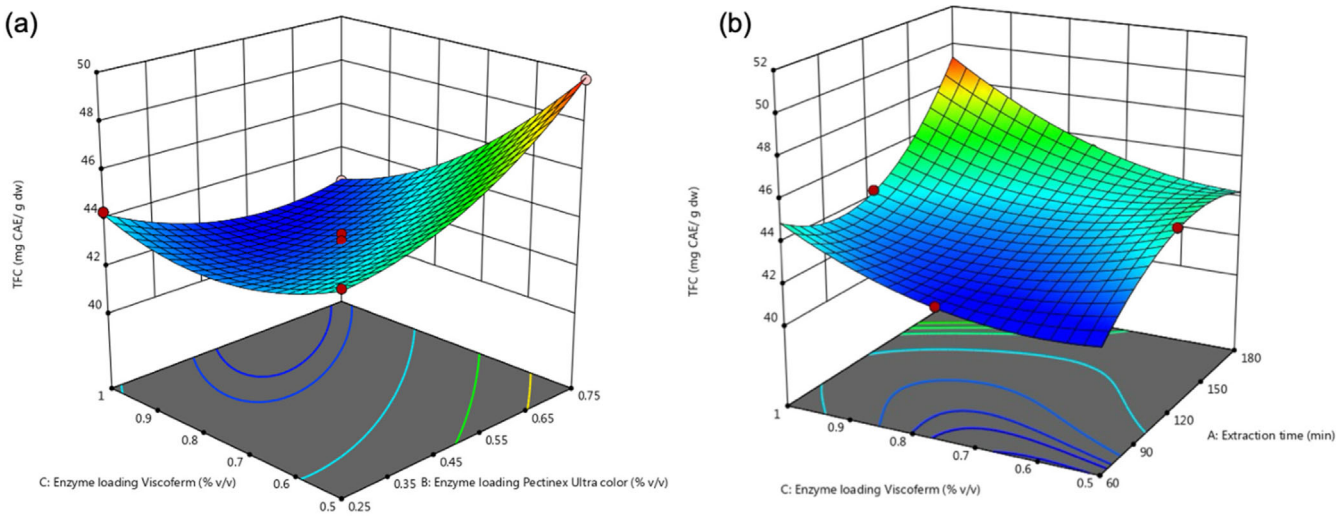

Figure 2. Three-dimensional (3D) surface plot of the TFC response, showing the correlation between TFC and the studied factors. (a) Effect of enzyme loading Viscoferm® (% v/v) and enzyme loading Pectinex® Ultra Color (% v/v) at a fixed extraction time of 120 min and (b) effect of enzyme loading Viscoferm® (% v/v) and extraction time (min), at a fixed enzyme loading Pectinex® Ultra Color of 0.25% v/v.

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Antioxidant activity of extracts

Antioxidant activity, expressed as IC_{50}, ranged from 0.71 ± 0.00 to 1.23 ± 0.01 $\mu L_{extract}$/mL (Table 2), where lower values indicate higher antioxidant activity. In contrast to TPC and TFC, antioxidant activity did not exhibit a linear relationship with extraction time or enzyme loadings. While longer extraction times promoted the release of phenolics and flavonoids, they did not necessarily enhance antioxidant activity. For instance, run 10, which had one the highest TPC and TFC values, exhibited a relatively high IC_{50} value (0.99 ± 0.01 $\mu L_{extract}$/mL), suggesting that not all extracted compounds contribute equally to antioxidant activity. Interestingly, the most potent antioxidant activity was recorded in run 3 (IC_{50} = 0.71 ± 0.00 $\mu L_{extract}$/mL), where the highest amount of the pectinolytic enzyme was applied at the lowest extraction time. These findings suggest that excessive enzymatic treatment or prolonged extraction may lead to the degradation or oxidation of sensitive antioxidant compounds, responsible for the DPPH radical scavenging ability of the studied extracts.[42]

Based on ANOVA results (Table S3), the quadratic model described best the antioxidant activity response (coded Eq. (10) and actual Eq. (11)). The quadratic term X_2^2 was eliminated as it was not statistically significant and did not contribute to the fitness of the model. The model *P*-value and F-value were 0.01 < 0.05 and 7.01, respectively, indicating that the model is significant. The lack of fit F-value of 7.81 and *P*-value of 0.12 confirm the validity of the model. The coefficient of determination R^2 and adjusted R^2 are 0.9033 and 0.7744, respectively, while the predicted R^2 of 0.7388 is in reasonable agreement with the adjusted R^2. The plot of predicted and actual values for TFC obtained from the BBD (Fig. S1(c)) confirmed the strong correlation between them. Therefore, this model can be applied to navigate the design space. The standardized effect of the independent variables and their interaction on the antioxidant activity is illustrated in a Pareto chart (Fig. S2(c)). Only two coefficients (X_3^2 and X_1X_2) remained inside the reference line (*P*-value = 0.05), indicating that these terms significantly contributed in the prediction of antioxidant activity of the extracts.

The regression coefficients of the proposed model (Eq. (11)) suggest that all independent variables have a positive effect on antioxidant activity, with the enzyme loading of Viscoferm® showing the most pronounced impact. Mild interactions between variables suggest some synergistic or antagonistic behaviors, especially between the enzymes.

As illustrated in the 3D surface plots (Fig. 3), the blue plot areas maximize the antioxidant activity, as it is expressed by IC_{50}. Optimal antioxidant activity is achieved under relatively mild conditions, specifically at approximately 0.75% v/v Viscoferm® loading and around 0.25% Pectinex® Ultra Color loading (Fig. 3(a)) and 90 min extraction time (Fig. 3(b), (c)). A notable antagonistic interaction between Pectinex® Ultra Color and Viscoferm® was observed, as their combined use at high levels reduced antioxidant activity. Overall, the model suggests that optimized enzyme concentrations and moderate extraction times favor higher antioxidant activity.

$$\text{Antioxidant activity } (\mu L_{extract}/mL) = 0.76 + 0.06X_1 + 0.05X_2 - 0.01X_3 + 0.12X_1X_2 - 0.04X_1X_3 + 0.02X_2X_3 + 0.09X_1^2 + 0.27X_3^2 \quad (10)$$

$$\text{Antioxidant activity } (\mu L_{extract}/mL) = 3.70 - 0.01^{*}t - 1.06^{*}P - 6.27^{*}V + 0.01^{*}t^{*}P - 0.01^{*}t^{*}V + 0.36P^{*}V + 0.00^{*}t^2 + 4.28^{*}V^2 \quad (11)$$

where t is the extraction time (min), P is the Pectinex® Ultra Color loading, and V is the Viscoferm® loading.

Optimization by Derringer's desirability function and confirmation

The second order polynomial models developed in this study were used to determine the optimal conditions for each individual response. As previously discussed, different optimal conditions were obtained for TPC, TFC, and antioxidant maximization. To identify a single set of conditions that simultaneously optimizes all three responses, Derringer's desirability function method was applied.[43] Derringer's desirability function converts each response into a scaled desirability value (ranging from 0 to 1), determining the optimal conditions that maximize the overall composite desirability.

In this approach, TPC and TFC were set to be maximized, while antioxidant activity was set to be minimized, since lower IC_{50} values indicate higher antioxidant activity. Equal weight and importance were given to all the responses. The optimal levels of the independent variables and the predicted responses are presented in Table 3, the desirability (D) value achieved was 0.70 indicating a satisfactory level of optimization and a reasonable compromise between the targeted responses. Variations in the desirability (D) value provided insight into the effectiveness of the optimization, where values above 0.9 indicate excellent agreement with target criteria, values between 0.7 and 0.9 suggest a good level of optimization with acceptable trade-offs while values below 0.7 reflect increased deviation from the desired response levels and a need for further refinement.[43]

A confirmatory experiment was performed to confirm the validity of the optimal parameters and the predicted responses calculated, therefore the overall accuracy of the predicted models. The extraction time was round down to 96 min. The experimental results (Table 3) were between the 95% low and high prediction intervals, confirming the validity of the model.

Although extraction time of 96 min may not be considered rapid, it is important to emphasize that both conventional and non-conventional extractions of *Rosa canina L.* often require much longer durations, as reported in the literature. For instance, maceration with aqueous ethanol has been reported to last 20 h and up to 72 h, while methanolic extractions can extend to 48 h.[4,44,45] Even among non-conventional approaches, our previous study reported an EAE process requiring 6 h, while another study employing UAE achieved extraction in 1.35 h.[46] In comparison, the present enzyme-assisted process required only 96 min, demonstrating a substantially time-efficient alternative while ensuring high phenolic recovery.

For comparative purposes, a conventional extraction was carried out using an ethanol-water mixture (70:30 v/v) for the optimum extraction time (96 min) depicted by Derringer's desirability function of the BBD. A 70:30 ethanol-water ratio is widely used for the extraction of bioactive compounds from various matrices. It has been employed in studies comparing maceration, microwave and ultrasound assisted extraction of propolis; in traditional percolation of hawthorn (*Crataegus monogyna* JACQ.); and in the extraction of fenugreek (*Trigonella foenum-graecum* L.), where it yielded maximum results.[47-49] This solvent system has also been applied to repeated extraction of olive leaves (*Olea Europea*), as well as in ultrasound assisted extraction of *Rosa canina L.*[50,51] The conventional extraction yielded a TPC of 63.11 ± 0.21 mg GAE/g DM, a TFC of 28.3 ± 0.80 mg CAE/g DM, and an antioxidant activity of 1.54 ± 0.02 $\mu L_{extract}$/mL. It is obvious that the combined action of

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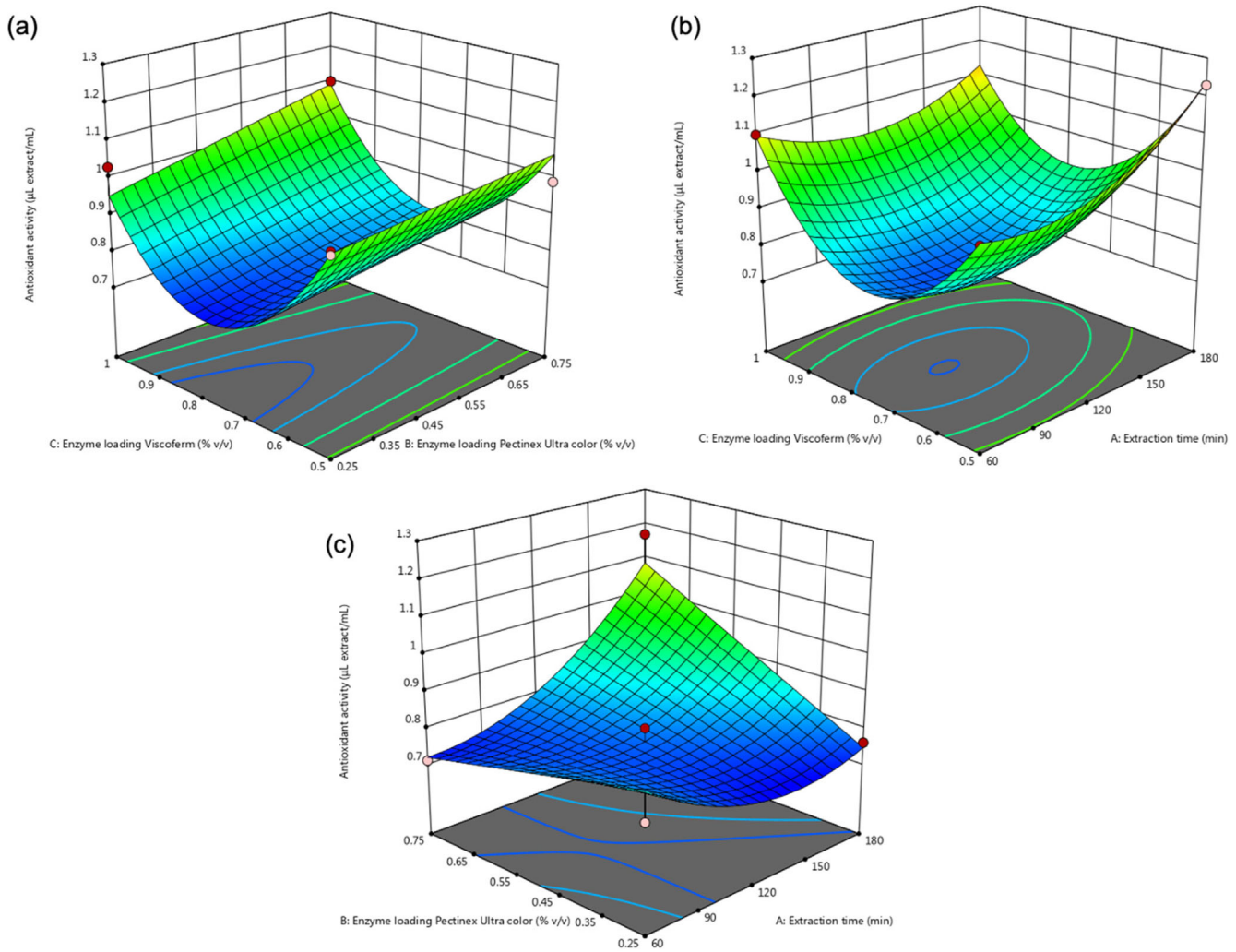

Figure 3. Three-dimensional (3D) surface plot of the antioxidant activity response, showing the correlation between it and the studied factors. (a) Effect of enzyme loading Viscoferm® (% v/v) and enzyme loading Pectinex® Ultra Color (% v/v) at a fixed extraction time of 120 min; (b) effect of enzyme loading Viscoferm® (% v/v) and extraction time (min), at a fixed enzyme loading Pectinex® Ultra Color of 0.50% v/v; and (c) effect of enzyme loading Pectinex® Ultra Color (% v/v) and extraction time (min), at a fixed enzyme loading Viscoferm® of 0.50% v/v.

Table 3. Optimized extraction conditions based on Derringer's desirability function, along with predicted and experimental (confirmation) values for TPC, TFC, and antioxidant activity

Independent variables	
Extraction time (min)	97
Enzyme loading Pectinex® Ultra Color (% v/v)	0.59
Enzyme loading Viscoferm® (% v/v)	0.51

Dependent Variables	Predicted values	Confirmation values
TPC (mg GAE/g DW)	122.42	123.22 ± 2.50
TFC (mg CAE/g DW)	46.34	49.13 ± 0.92
Antioxidant activity ($\mu L_{extract}$/mL)	0.99	0.98 ± 0.02

two enzymes with different catalytic activities yielded substantially higher TPC and TFC yields, and promising antioxidant activity compared to the conventional extraction method. When benchmarked against conventional ethanol–water extraction, the optimum extract of this study yields almost 2-fold higher both TPC and TFC, while about 40% higher antioxidant activity. The method employed in the present study balances both yield and efficiency, resulting in high recovery of bioactive compounds within a significantly reduced extraction time. This contributes not only to improved process economics and environmental sustainability but also minimizes the exposure of phenolic compounds to oxidative stress during extraction.[15,52] Compared to conventional solvent extraction, EAE is generally considered milder and more selective, reducing the risk of oxidative degradation of sensitive bioactives such as polyphenols and flavonoids. Enzymatic methods operate under controlled pH and temperature conditions and avoid harsh solvents, thereby preserving the native structure and functionality of antioxidant compounds.[53] This advantage has been highlighted in comparative studies showing higher retention of antioxidant activity and reduced degradation when enzymatic protocols are employed.[54,55]

Biological activities of the optimum extract

Antidiabetic activity (inhibition of α-glucosidase and α-amylase)

High levels of blood glucose are characteristic of a group of metabolic diseases called diabetes mellitus, which incorporates three

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major types: insulin-dependent diabetes (type 1), non-insulin-dependent diabetes (type 2), and gestational diabetes. Type 1 and 2 diabetes are both chronic conditions that can cause secondary ailments such as neuropathy, retinopathy, angiopathy, and nephropathy, when combined with oxidative stress. Inhibiting carbohydrate-digesting enzymes such as α-amylase (EC 3.2.1.1) and α-glucosidase (EC 3.2.1.20) is an effective therapeutic strategy for managing and treating type 2 diabetes.[56] The inhibitors of α-amylase reduce blood glucose by inhibiting the hydrolysis of the starch, while the inhibitors of α-glucosidase delay the carbohydrate digestion, limiting glucose absorption and maintaining blood sugar levels. Plant extracts exhibit a wide range of α-amylase and α-glucosidase inhibitory effect, largely influenced by their species, plant part, and the extraction method.[56,57]

The optimum *Rosa canina L*. extract exhibited a weak α-amylase inhibition, compared to positive control tannic acid (1 mg/mL, 100% inhibition), with an IC_{50} of 97.95 ± 1.04 mg/mL (Fig. 4(a)). The reported IC_{50} values vary substantially, from highly potent inhibition (*e.g.*, IC_{50} = 0.0108 mg/mL for the methanolic extract of *Englerophytum magalismontanum* leaves) to weak inhibition (*e.g.*, IC_{50} = 13.05 mg/mL for the hexane extract of *Morus nigra* leaves).[56] These variations highlight the critical role of both the botanical source and extraction method in determining bioactivity. Notably, aqueous extracts of *Veronica biloba, Salvia lavandulifolia*, and *Rubus corchorifolius* L. have demonstrated IC_{50} values of 0.11, 0.99, and 4.39 mg/mL, respectively, further illustrating the diverse inhibitory potential across species.[56]

The extract exhibited an IC_{50} of 7.50 ± 1.12 mg/mL against α-glucosidase (Fig. 4(b)). Although its potency is low, there is a significant difference from the α-amylase inhibition. Studies have reported a wide range of α-glucosidase IC_{50} values among plant extracts. For instance, the methanolic extract of *Washingtonia filifera* seeds exhibited strong inhibition with an IC_{50} of 0.53 µg/mL, outperforming the standard drug acarbose (IC_{50} = 90 µg/mL),

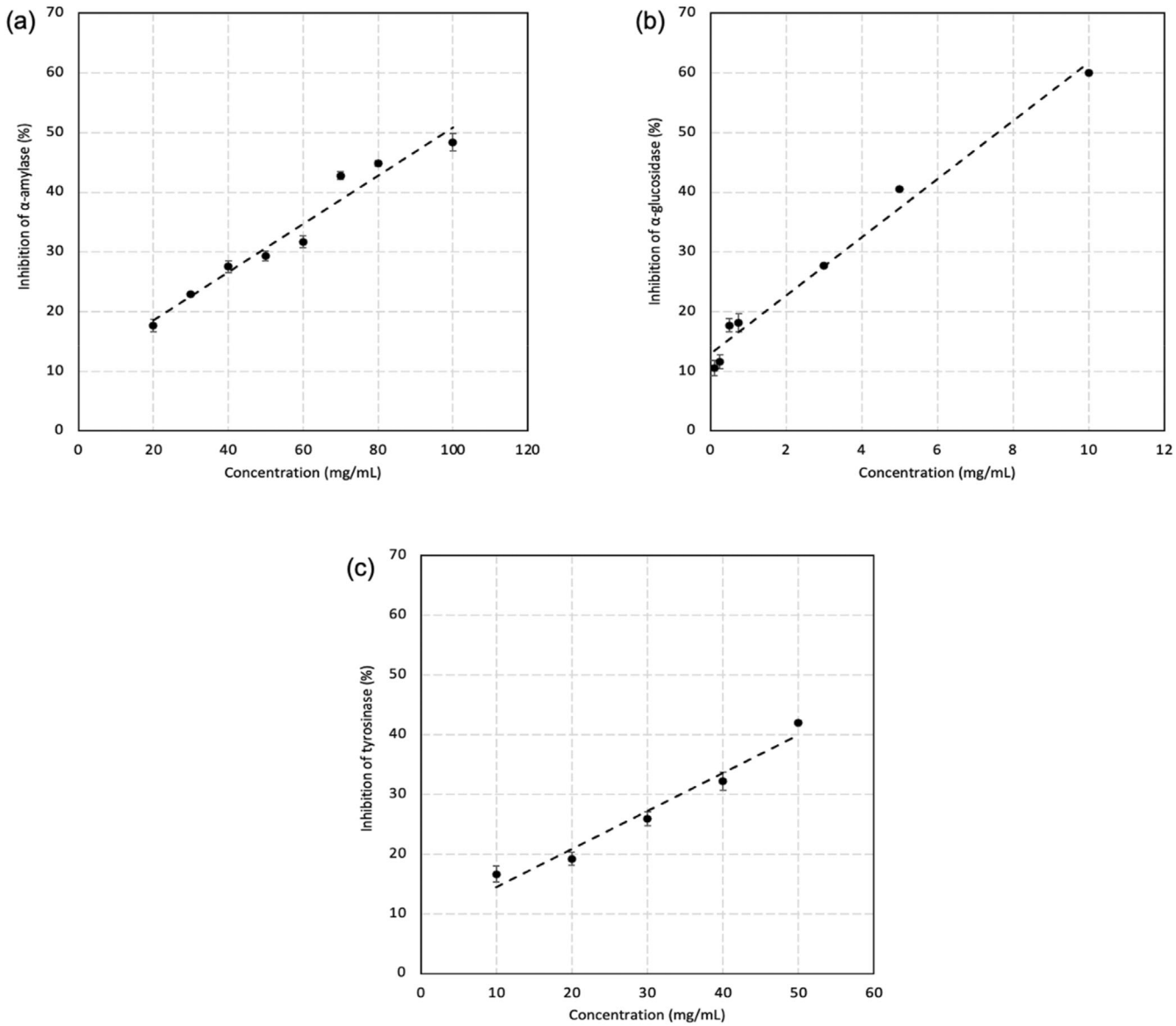

Figure 4. Inhibitory activity of the optimum extract against (a) α-amylase, (b) α-glucosidase, and (c) tyrosinase at various concentrations. Results are expressed as percentage inhibition (%). The dashed line represents the data fitted to linear trendline. The data are expressed as mean values ± SD.

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while the aqueous extract of *Evodiae fructus* have shown 84.6% inhibition of α-glucosidase activity at 4 mg/mL.[58,59]

Anti-aging activity (inhibition of tyrosinase)

Melanin is the pigment responsible for the color of skin, hair, and eyes, and its overproduction can result in hyperpigmentation disorders such as age spots or melanoma. Tyrosinase (EC 1.14.18.1) is a key enzyme in melanin synthesis, catalyzing the conversion of L-tyrosine to L-DOPA and subsequently to dopaquinones, which polymerize to form melanin.[60] Inhibiting tyrosinase can reduce the melanin production and address pigmentation issues. Numerous natural compounds, particularly phenolic compounds like flavonoids and tannins, have been shown to effectively inhibit tyrosinase activity.[61] The optimum extract showed an IC_{50} of 65.77 $\pm$ 1.72 mg/mL toward tyrosinase (Fig. 4(c)), reflecting weak inhibition, compared to kojic acid, which achieved 100% inhibition at a concentration of 1 mg/mL. Plant extracts exhibit a wide range of tyrosinase inhibitory activities. Acetone extracts of *Koelreuteria henryi* (IC_{50} = 289.0 $\pm$ 0.5 µg/mL), *Camellia sinensis* (IC_{50} = 232.5 $\pm$ 3.3 µg/mL), and *Rhodiola rosea* (IC_{50} = 181.8 $\pm$ 11.0 µg/mL) demonstrate moderate inhibition of tyrosinase, while the methanolic extract of *Hypericum laricifolium* Juss. exhibited a higher inhibitory activity with an IC_{50} of 120 µg/mL.[62,63] Aqueous extracts of *Eugenia dysenterica* showed even stronger tyrosinase inhibition, with an IC_{50} of 11.88 µg/mL.[64] Notably, the extracts from *Eugenia dysenterica* displayed tyrosinase inhibitory activities that were better to that of kojic acid, the known tyrosinase inhibitor, which has an IC_{50} value of 13.14 µg/mL.[64]

Although enzyme-assisted extraction (EAE) significantly enhanced the recovery of total phenolic content (TPC) and total flavonoid content (TFC) compared to conventional solvent extraction, the relatively low α-amylase and tyrosinase inhibitory activities observed may be attributed to factors beyond phenolic concentration alone. Structural modifications induced during enzymatic hydrolysis—such as hydroxylation and galloylation of flavonoids like catechins—can enhance α-amylase inhibitory effects, whereas glycosylation, methylation, methoxylation, and hydrogenation of the C2═C3 double bond have been shown to reduce inhibitory potency.[65] Additionally, EAE releases high-molecular-weight polysaccharide fragments, including pectins and hemicelluloses, which may dilute the bioactive fraction or sterically hinder interactions between phenolics and target enzymes. These polysaccharides can also form noncovalent complexes with phenolics, limiting their availability and reducing biological activity.[41] Moreover, TPC measurements do not reflect the specific phenolic subclasses present and the extract may be enriched in compounds with inherently low inhibitory potential against α-amylase and tyrosinase. The structural requirements for enzyme inhibition are highly specific, for example, flavonoids bearing ortho-dihydroxy substitutions on the B-ring exhibit stronger tyrosinase inhibition than their mono-hydroxylated counterparts.[66] In conclusion, the weak enzyme inhibition observed likely results from a combination of altered phenolic structures, matrix effects from co-extracted polysaccharides, and the limited affinity of the phenolic profile for the target enzymes.

Antibacterial activity

Plant-derived bioactive compounds, particularly polyphenols, in plant extracts exhibit antibacterial activity through multiple mechanisms, such as degradation of the cell wall, membrane destabilization, and interference with nucleic acids. Their effectiveness strongly depends on structural features, such as the number and position of hydroxyl groups.[67] Although Gram-negative bacteria like *Escherichia coli (E. coli)* are generally more resistant to antimicrobials.[68] The antibacterial activity of the optimum extract was evaluated by monitoring *E. coli* growth (OD_{600} nm) in the presence and absence of the extract (Fig. 5). After 24 h incubation, the OD_{600} of the untreated control reached 0.43, while the culture supplemented with the extract reached only 0.19, corresponding to an inhibition of 55.1 $\pm$ 1.2% and confirming the moderate antibacterial activity of the extract.

The inhibition of *E. coli* growth is expressed in the literature by different terms such as inhibition percentage or Minimum Inhibitory Concentration (MIC), which represents the lowest concentration required to inhibit the visible growth of a microorganism. Several studies have reported varying levels of inhibition of *E. coli* growth by plant-derived extracts. For instance, the percentage of *E. coli* inhibition by *Lantana camara* leaf extract ranged from weak to strong, specifically, 3% to 100%, with the highest inhibition observed with extract concentrations of 1.5 and 2 g/L.[69] Studies on methanolic and aqueous extracts of *Rosa canina L*. have showed significant antibacterial activity with MIC values as low as 32 µg/mL.[70,71] Other medicinal plant extracts such as *Punica granatum* and *Phyllanthus emblica* have demonstrated MIC values 2.5 mg/mL.[72] According to the literature, MIC values below 100 µg/mL are indicative of strong antibacterial activity, whereas values between 100 and 625 µg/mL suggest moderate activity.[73] Kanarek *et al*. have suggested that the antibacterial activity is strain-dependent and that there is a correlation between the efficacy of the extracts and phytochemicals known to interfere with bacterial cell structures and metabolic pathways namely epicatechin, shikimic acid, quinic acid, gallic acid, and caffeine.[74] Overall, antibacterial activity varies among plant species and is influenced by extract composition, concentration, and bacterial strain.

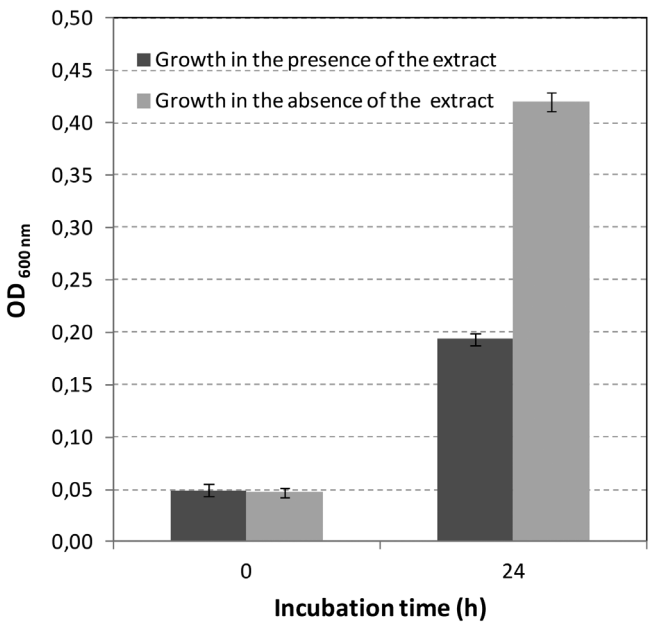

Figure 5. Effect of *Rosa canina* pseudofruit extract on *E. coli* growth. Bacterial growth was monitored by measuring optical density at 600 nm (OD_{600}) in the presence (dark bars) and absence (light bars) of the extract at 0 and 24 h of incubation. Data are expressed as mean $\pm$ standard deviation of three independent replicates.

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CONCLUSIONS

The present study demonstrated that enzyme-assisted extraction (EAE) is an efficient and environmentally sustainable approach for extracting bioactive compounds from the pseudofruit of *Rosa canina* L, especially when the combined action of two hydrolytic enzymes is applied. A Box–Behnken Experimental Design (BBD) was employed to quantify the relationship between the studied variables (extraction time, enzyme loading of Viscoferm® and Pectinex® Ultra Color) and the responses (TPC, TFC, antioxidant activity). The optimal extraction conditions were determined using Derringer's desirability function and validated. Under optimal conditions, the process yielded significantly higher levels of total phenolic content (TPC) and total flavonoid content (TFC), as well as improved antioxidant activity, compared to conventional aqueous-ethanolic extraction. The biological activities of the extracts were evaluated, revealing weak tyrosinase inhibition and α-amylase inhibition, moderate α-glucosidase inhibition and strong inhibition of *E. coli* growth. Future research should focus on tailoring the extraction process to maximize the tested biological activities and performing detailed phytochemical profiling to identify the most active compounds.

AUTHOR CONTRIBUTIONS

Diomi Mamma and Anastasia Detsi – Conceptualization, Methodology, and Supervision. Zafeiria Lemoni –Investigation and Original draft. Styliani Kalantzi, Theopisti Lymperopoulou and Andromachi Tzani- Software and formal analysis. Diomi Mamma, Anastasia Detsi and George Stavropoulos- Resources.

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CONFLICT OF INTEREST STATEMENT

Author G. Stavropoulos is an employee of the of Korres S.A.—Natural Products. None of the authors received research grants from the company, a speaker honorarium from the company, own stocks in the company, are involved as a consultant or an expert witness at the company, or are the inventor of patent. Therefore, the authors have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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