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Optimal Conditions for Pomegranate Peel Enzymatic Extraction of Phenolics Using Response Surface Methodology and Comparative Analysis With Traditional Soxhlet Extraction

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ABSTRACT

This study introduces a tannase-focused enzyme-assisted extraction (EAE) approach that modifies phenolics in pomegranate peel, significantly boosting antioxidant activity under mild processing conditions. The EAE method yields higher extract yields (≈70%) and antioxidant activity (≈74%) than Soxhlet extraction. Although the Soxhlet method yielded a slightly higher phenolic content (≈196 mg EAE/g), its antioxidant activity was lower (≈67%). The higher TPC extraction parameter, as determined by Soxhlet, does not correlate with biological activity due to the possible thermal degradation of the antioxidants, which reduces the actual biological activity. Scanning electron microscopy (SEM) of the structural changes before and after processing revealed that EAE caused cell wall disruption and surface roughness, enhancing bioactive compound release and polyphenol extraction efficiency. Statistical analyses (RSM and ANOVA) confirmed the adequacy and high predictive accuracy of the optimization model for TPC and AA ($R^2 > 0.96$), which indicates its reliability. This study suggests that EAE, optimized using RSM, represents a greener alternative to conventional techniques, offering both efficiency and sustainability in the valorization of food products.

1 | Introduction

Annual global pomegranate production is around 3 million t, of which 26%–30% consists of peel, the main by-product of processing [1]. Pomegranate peel (PP) represents a promising source of biologically active compounds, yet it is often discarded, contributing to environmental burdens. Valorization of this by-product through efficient extraction strategies can provide an eco-friendly source of functional ingredients while improving resource utilization [2]. Major producers include Turkey, China, Iran, and India, and emerging pomegranate cultivation in Kazakhstan similarly generates underutilized peel with high potential for value-added applications [3, 4].

PP contains high concentrations of bioactive substances: polyphenols, tannins, flavonoids, anthocyanins, vitamins, dietary fiber, and minerals [5, 6]. The content of total phenolic compounds ranges from 18 to 510 mg/g dry matter, depending on the variety and extraction method [7]. PP is also a powerful source of antioxidants, which help reduce oxidative stress, maintain heart and vascular health, and may slow cellular aging [8, 9].

Detailed extraction of compounds from PPs allows for the recovery of valuable resources while ensuring sustainable industrial practices and waste recycling [10].

Extraction is a key technique for isolating specific bioactive compounds or defined chemical constituents from complex plant materials, enabling their subsequent qualitative and quantitative analysis. The methods used to extract phenolic and other bioactive compounds range from traditional to green technologies. Conventional extraction methods, like maceration, percolation, and Soxhlet extraction, are popular because they are simple and can effectively extract many compounds from plant matrices. However, they need large amounts of solvents and labor-intensive manual work, depend on the researcher's skill, and do not guarantee consistent results. Green extraction methods have been developed to overcome the limitations of traditional methods [11, 12].

Table 1 describes the advantages and disadvantages of most extraction methods used in the modern scientific world.

The results indicated that the Soxhlet method had more impact on releasing the bioactive compounds than the ultrasound treatment (UAE) and conventional maceration. It provided the highest yield at 33.5%, while with ethanol, it is about 30.45%. Maceration is recognized as the simplest, but the least effective in terms of extract yield [22].

Ultrasonic extraction is the most effective technique for achieving high yields and efficiency. Ultrasonic waves align cell structures and improve turbulence and depth of a solvent, thereby reducing energy and solvent consumption. Under the conditions (frequency, power, and time), the output amount of phenols is in the range of 81.6–190.9 mg GAE/g dry weight [23].

MAE speeds up the process, increases yield, and requires less solvent. For example, the yield of phenols was up to 18.92 mg GAE/g of extract, surpassing traditional methods. However, overheating can destroy heat-sensitive polyphenols (e.g., anthocyanins) [24].

While Soxhlet and maceration methods are traditional laboratory methods, they are less efficient. Nonconventional extraction methods, including ultrasonic, microwave, and other techniques, achieve 32%–36% higher polyphenol yields than conventional methods while using approximately 17.6 times less energy [25, 26]. For increased efficiency and "greenness," UAE, MAE, SFE, enzyme-assisted extraction (EAE), or their combinations are recommended. Based on the data in Table 1, the EAE method significantly increases the yield of the desired components. The EAE method relies on the action of enzymes (tannases, pectinases, and cellulases) that degrade plant cell walls and release bound phenolic compounds [27].

Although combined enzyme mixtures often increase the overall yield of metabolites [28], some studies report that tannase effectively increases the yield and antioxidant activity (AA) of extracts from polyphenol-rich plant raw materials (e.g., tea, grapes, and pomegranates) without the need for additional enzymes [29–31]. In this study, tannase was employed as a single enzyme to isolate its specific contribution to phenolic extraction, avoiding multienzyme systems commonly used for plant matrices.

Tannase (tannin acyl hydrolase, EC 3.1.1.20) has high specificity for the hydrolysis of complex tannins and the destruction of their complexes with cellular macromolecules. Some authors have also noted the effectiveness of tannase in polyphenol extraction from PP [32, 33].

Although the extraction of phenolics from PP has been widely studied [34–39], the application of tannase in EAE has not received comparable attention.

TABLE 1 | The advantages and disadvantages of most extraction methods.

Method	Advantages	Disadvantages	References
Soxhlet/maceration	Simplicity and applicability to different compounds	Long, a lot of solvent, and low yield	[13]
Ultrasound-assisted extraction (UAE)	High output, fast, and energy-efficient	Need to optimize parameters	[14]
Microwave-assisted extraction (MAE)	Fast, efficient, and less solvent	Risk of overheating and damage to heat-sensitive substances	[15]
Supercritical fluid extraction (SFE-CO_2)	Minimal thermal damage and selective	Expensive specialized equipment	[16]
Pressurized liquid extraction (PLE)	"Green" method and high repeatability	Requires equipment and complexity	[17]
Enzyme-assisted extraction (EAE)	Significantly increases yield	Dependent on enzyme, temp, and pH	[18, 19]
Combined (hybrid)	Increased efficiency and reduced time and solvent	Complex, expensive, and requires optimization	[20]
Water/diluted ethanol	Safe and economical	Less selectivity and extracts less lipophilic substances	[21]

Prior works have applied tannase for punicalin production from PP without comprehensive extraction optimization or bioactivity assessment [32] and tannase treatment of pomegranate juice to modulate phenolic composition and antioxidant properties [40]. However, systematic evaluation of tannase-assisted extraction conditions and functional outcomes in PP remains limited.

Given the importance of environmentally friendly methods, this work compares the efficiency of tannase-based enzymatic extraction with that of traditional Soxhlet extraction for isolating bioactive compounds.

For EAE, reaction temperature and enzyme concentration were varied, while for Soxhlet extraction, reaction temperature and extraction time were considered. This work also assesses the yield, total phenolic content (TPC), AA, and surface morphology of compounds obtained, using scanning electron microscopy (SEM). Extraction conditions (temperature and enzyme concentration) will be optimized using the response surface method (RSM) to maximize the recovery of bioactive compounds from PP.

2 | Materials and Methods

2.1 | Chemicals and Reagents

Methanol, sodium acetate, acetic acid, HCl/NaOH, tannase from *Aspergillus niger*, Folin–Ciocalteu reagent, sodium carbonate, gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, and all other chemicals and reagents used were of analytical grade and commercially available.

2.2 | Sample Preparation

The pomegranate (*Punica granatum* L.) variety "Kazakh pomegranate" peel was collected from a local pomegranate processing plant in Kazakhstan. It was then dried in an infrared dryer at a temperature of 40°C–50°C to a moisture content of 11%–13% and crushed to a size not exceeding 0.5–1 mm, and the powder peels were then stored in a freezer at −18°C. Two different methods were employed for the extraction of PP: EAE and Soxhlet extraction.

2.3 | EAE

A 0.1 M sodium acetate buffer (pH 5.0) was prepared by dissolving 1.961 mL acetic acid and 5/524 g sodium acetate in distilled water, adjusting the pH with 1 M HCl/NaOH, and bringing the final volume to 1 L. One gram of PP was placed in a 50-mL Falcon tube, and tannase from *Aspergillus niger* was added at concentrations of 1, 3, and 5 U/g substrate, which were calculated by Equation (1):

$$\text{Volume of enzyme} = \frac{\text{desiredconcentrationU/g}}{\text{specific activity}} \times W \text{ substrate (g)}, \quad (1)$$

where specific activity of tannase = 150 U/mL and W substrate is mass of substrate = 1 g.

Enzymatic extraction was carried out in 0.1 M acetate buffer under optimal conditions for tannase, using an orbital shaker (150 rpm, dark). The mixtures were incubated for 3 h at 30°C, 40°C, and 50°C, and the reaction was stopped by heating at 80°C for 5 min. The samples were centrifuged in a KUBOTA 2420 centrifuge (Japan) under the conditions of 1020 × g, 10 min, and 4°C, and the supernatants were stored at −20°C after filtration through Whatman No. 41 paper.

2.4 | Soxhlet Extraction

Soxhlet extraction with a closed-cycle system was performed at INBIOSIS (UKM) using 27 g of sample in filter paper and 500 mL of methanol in a 1000-mL round-bottom flask. Extractions were carried out for 4 h at 40°C and 5 h at 50°C to assess the effects of time and temperature on phenolic recovery. The extracts were centrifuged (2612 × g, 10 min), filtered through a 0.22-μm nylon syringe filter, and analyzed for TPC and AA. The difference in weights (1 g for EAE and 27 g for Soxhlet) is due to the high selectivity of enzymatic hydrolysis, which ensures an effective yield of active components from small samples. At the same time, the Soxhlet method requires large volumes to achieve comparable concentrations due to the extensive nature of the process and the partial loss of labile substances.

2.5 | RSM

To optimize the enzymatic extraction of polyphenols from PP, a RSM with a central compositional design (CCD) (Design-Expert software) was used [41]. Enzyme concentration (1–5 U/g) and temperature (30°C–50°C) were varied as independent variables.

The design matrix included 13 experiments, including replicates at the central point (3 U/g, 40°C) to assess statistical error. The extraction time (3 h) was chosen as sufficient based on preliminary studies [42, 43]. The resulting model was generated using the factor levels from Table 2 and used to construct three-dimensional response surface plots and contour plots. This facilitates visualization of factor interactions and the identification of optimal parameter combinations.

2.6 | Extraction Yield

The extraction yield was determined by measuring the amount of extract obtained after the extraction process. The weight of

TABLE 2 | Level of two factors.

Factor	Symbol	Actual levels for each factor		
		−1	0	+1
Enzyme concentration (U/g substrate)	A	1	3	5
Temperature of reaction (°C)	B	30	40	50

the residue after extraction was subtracted from the initial sample weight. The yield was then calculated using the following formula (Equation 2):

$$\text{Extraction yield}\,(\%) = \frac{W\ \text{extract}}{W\ \text{peel}} \times 100, \tag{2}$$

where $W_{extract}$ is the weight of the extracted phenolic compounds in grams and W_{peel} is the weight of the dried PP in grams.

A methanol extract obtained by Soxhlet extraction served as a control, allowing comparison of the EAE effectiveness with the traditional method for polyphenolic compound extraction.

2.7 | TPC

The modified Folin–Ciocalteu method was used to calculate the PP extract's TPC [44]. For the PP extract analysis, 100 mg of extract was weighed and diluted to a final volume of 10 mL with distilled water. To 200 μL of the diluted extract, 2.8 mL of distilled water and 0.5 mL of Folin–Ciocalteu reagent were added and mixed by vortex for 20 s and then incubated for 3 min in the dark at room temperature. Afterward, 2 mL of 20% (w/v) sodium carbonate solution was added and vortexed for 20 s. The mixture was incubated for 60 min in the dark at room temperature, and absorbance was measured at 765 nm using the UV-VIS spectrophotometer (UV-1800; Shimadzu, Kyoto, Japan). TPC of samples was calculated from standard gallic acid solutions under the same conditions, and concentrations were expressed as milligrams of gallic acid equivalent per gram of PP (mg GAE/g). Soxhlet extraction, performed under identical temperature conditions but with a longer extraction time, served as a control, allowing comparison of the effectiveness of enzymatic extraction with the conventional method for the extraction of polyphenolics.

2.8 | AA

Simple and reproducible, the DPPH method is widely used to quantitatively assess the antioxidant potential of plant extracts, facilitating comparisons with the literature. The DPPH free radical scavenging activity of samples was evaluated by the method reported by Padmanabhan and Jangle with a slight modification [45]. For the test, 20 μL of the extract was diluted with distilled water in order to form a total of 10 mL. Next, 5 mL of a 0.1 mM DPPH solution in methanol was added to 3 mL of the diluted extract. The blend was shaken for 20 s to be homogeneous. Following incubation in the dark for 30 min, the absorbance was read at 517 nm on a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). Pure methanol was the blank, while the control was a 0.1 mM DPPH–methanol solution. Inhibition of DPPH radicals or AA (%) was determined by Equation (3):

$$\text{AA}\,(\%) = \frac{A0 - A1}{A0} \times 100\%, \tag{3}$$

where $A0$ and $A1$ represent the control sample's and the extracts' respective absorbencies.

2.9 | Surface Morphological Analysis (SEM)

The surface of PP powder was examined before and after extraction using a SEM (JSM-IT100 In Touch Scope) from Tokyo, Japan. The SEM was operated at a voltage of 10 kV. To make the samples conductive, a thin gold layer (approximately 10–15 nm) was applied to the dried powder, which was attached to the holder for 60 s with a current of 20 mA. The electron beam then scanned the sample surfaces, creating high-resolution images that allowed for detailed observation of the microstructure and assessment of changes after extraction.

3 | Results and Discussion

3.1 | EAE

Previous findings have shown that green and efficient methods, including EAE, are superior to conventional methods in terms of environmental friendliness, lower time and energy consumption, higher yields, and scalability [46, 47].

EAE enhances the extraction of bioactive compounds from plant waste by increasing permeability and disrupting cell walls. Furthermore, there is growing interest in enzymes capable of cleaving specific macromolecular bonds and releasing valuable phytocomponents, expanding the possibilities for producing enriched plant extracts [48].

Tannase was selected for use with PP due to its specific hydrolysis of ellagotannins and complex tannins, enabling effective release of bioactive polyphenols. This approach also supports reproducibility and the potential for scaling the extraction process [49]. Tannase operates at moderate temperatures (30°C–50°C) and loses activity at 60°C, permitting controlled hydrolysis without enzyme denaturation [50].

The obtained data for the analysis of extraction yield, TPC, and AA, performed as a part of the current study, are given in Table 3.

3.2 | Extraction Yield, TPC, and AA of PP Using EAE

Experiments conducted using the RSM design matrix revealed significant effects of enzyme concentration and temperature on the extraction yield, TPC, and AA of PP extracts. A total of 13 experimental runs were conducted with enzyme concentrations of 1, 3, and 5 U/g and temperatures of 30°C–50°C during 3 h (180 min).

From the 13 experimental runs (Table 4), the highest extraction yield (70.00% ± 0.25%) was obtained at 5 U/g enzyme concentration and 40°C, while the lowest (25.00% ± 0.72%) was at 1 U/g and 30°C, confirming the strong influence of these factors. Comparable yields were reported by Cortes-Ferre et al. [51] for habanero chili seeds (73.5%, at 60°C, 150 min), though at a much higher enzyme concentration (2500 UI/L), highlighting the efficiency of the moderate conditions in the present study.

The TPC and AA of PP extracts were strongly influenced by temperature and enzyme concentration, with optimal

TABLE 3 | The experimental design of pomegranate peel extracts using EAE.

Run order	Enzyme concentration (U/g substrate)	Temperature (°C)	Extraction yield (%)	TPC (mg GAE/g)	AA (%)
1	5	50	65.00 ± 0.34	168.65 ± 0.39	69.00 ± 0.61
2	1	50	30.00 ± 1.03	166.11 ± 0.06	60.38 ± 0.18
3	3	50	55.00 ± 0.09	167.71 ± 0.64	58.27 ± 0.33
4	1	40	45.00 ± 0.09	168.17 ± 0.03	55.96 ± 0.13
5	5	40	70.00 ± 0.25	172.62 ± 0.52	64.81 ± 1.35
6	3	40	60.00 ± 0.35	181.96 ± 0.04	72.88 ± 0.69
7	1	30	25.00 ± 0.72	138.35 ± 0.53	67.12 ± 0.67
8	3	30	50.00 ± 0.35	139.04 ± 0.09	68.65 ± 0.47
9	5	30	55.00 ± 0.22	135.98 ± 0.62	44.81 ± 0.21
10	3	40	60.00 ± 0.72	178.43 ± 0.16	72.12 ± 0.08
11	3	40	58.00 ± 0.04	172.62 ± 0.33	73.85 ± 0.04
12	3	40	62.00 ± 0.09	177.35 ± 0.25	72.50 ± 0.35
13	3	40	59.00 ± 1.05	177.86 ± 0.02	71.73 ± 0.19

Note: The experimental results are presented as mean ± standard deviation, reflecting the values obtained from triplicate experiments.

TABLE 4 | The experimental results of Soxhlet extraction.

Run order	Extraction time	Temperature (°C)	Extraction yield (%)	TPC (mg GAE/g)	AA (%)
1	4 h	40	42.50 ± 0.07	183.95 ± 0.03	56.00 ± 0.09
2	5 h	50	49.50 ± 0.03	195.72 ± 0.06	67.00 ± 0.02

Note: The experimental results are presented as mean ± standard deviation, reflecting the values obtained from triplicate experiments.

results at 3 U/g and 40°C. Under these conditions, both TPC (181.96 ± 0.04 mg GAE/g) and AA (72.88% ± 0.69%) reached their peaks, indicating the efficient hydrolysis and high enzyme activity. Lower temperatures (30°C) and excessive enzyme amounts (5 U/g) decreased the values, likely due to reduced enzyme activity, substrate overload, or partial enzyme inactivation. Raising the temperature to 50°C also lowered TPC and AA, confirming temperature sensitivity. Therefore, moderate enzyme load and medium temperature yield the best phenolic and antioxidant release, demonstrating the effectiveness of EAE for PP.

Similar trends were reported by Gómez-García et al. [52], who observed the highest AA (90% ± 0.37%) and TPC (0.40 mg GAE/g) for grape residue treatment at 40°C during 12 h using Novoferm enzyme. This confirms the key role of the temperature–enzymatic regime in increasing phenolic compound yield. Ghandahari Yazdi and Ahmadi Gavligh [53] reported an increased extraction yield of phenolic compounds (up to 51%) from pistachio hulls under conditions similar to ours (tannase concentration 4 U/g and time 3 h).

According to our data, PP contains five times more polyphenols than raspberry pulp (RP), even under optimized conditions in the EAE study for RP (1.2 units of protease/100 g, pH 9, 60°C, 2 h) [54]. The AA of RP was 13.5 g GAE/g, whereas PP provided 72.88% DPPH inhibition, which corresponds to very high activity. This confirms the significantly greater radical-suppressing potential of PP due to its high polyphenol content [55].

In another study, the combined action of pectinase and cellulase (4% vol.) under high pressure (300 MPa, 15 min) was employed, achieving a total extract yield of 41.0% ± 1.9% and TPC of 207.0 ± 2.8 mg GAE/g [56]. Under our milder tannase-based enzymatic extraction conditions, the extract yield was approximately 1.46 times higher, and TPC was slightly lower (1.14 times).

Similar trends have been reported by authors using a preoptimized Viscozyme concentration of 0.6% for 60 min at 40°C and pH 4.5 to extract polyphenols from PP, resulting in a TPC of 165.5 ± 9.8 mg GAE/g [34].

EAE at moderate temperatures and enzyme concentration is an efficient, cost-effective, and environmentally friendly method for extracting bioactive compounds from PP, which can be combined with other extraction technologies and remains a promising modern approach [57].

3.3 | Soxhlet Extraction

Soxhlet extraction is a conventional reference method for isolating phenolic compounds from plant materials, as it ensures exhaustive recovery through continuous solvent reflux and percolation [58]. In this study, the benchmark was applied to evaluate the efficiency of EAE in terms of energy savings, reduced processing time, and preservation of bioactive compounds that can be destroyed by prolonged heating in Soxhlet. Soxhlet conditions (40°C–50°C, 4–5 h) were chosen for a fair comparison with EAE, as they correspond to the moderate enzyme load and mild temperature range of EAE.

Results of extraction yield, TPC, and AA of PP using Soxhlet extraction are shown in Table 4.

3.4 | Extraction Yield, TPC, and AA of PP Using Soxhlet Extraction

According to Table 4, Soxhlet extraction of PP yielded 42.5% (4 h) at 40°C and 49.5% at 50°C (5 h), confirming the influence of temperature and recovery duration. These values are consistent with Kupnik et al. [59], who reported 38.89% yields using methanol, and with Ahmetović et al. [22], who obtained 30.45% with ethanol solvent and 33.5% with methanol at Soxhlet extraction. Overall, the results show that Soxhlet extraction of PP typically yields between 30% and 50%, depending on the solvent and operating conditions.

To assess the efficiency of Soxhlet extraction, two independent experiments were carried out to look at how temperature and extraction time affected the TPC of PPs.

At 40°C for 4 h, the TPC reached 183.95 mg GAE/g, while at 50°C for 5 h, it increased to 195.72 mg GAE/g, highlighting the influence of temperature and extraction time. This experiment only tested these two parameters due to the longer extraction time and the large amount of methanol required. Similar studies report variation depending on plant species, solvent, and conditions: Soxhlet extraction of *C. chrysanthus* with 70% ethanol yielded 127.48 mg GAE/g [60], while date palm seeds gave 125.54 mg GAE/g [61]. The elevated TPC in this study is attributed to optimized temperature and extraction time, which enhance solvent penetration, cell wall disruption, and polyphenol solubilization.

Soxhlet extraction at 40°C for 4 h produced 56% AA, representing the upper range of typical Soxhlet values (Table 5). Increasing the extraction temperature and duration to 50°C for 5 h enhanced activity to 67.00% ± 0.02%, approaching levels usually obtained with ultrasound-assisted extraction. The enhanced radical-scavenging properties of the phenolic compounds were attributed to better solvent penetration and cell wall disruption at elevated temperatures, which resulted in the release of these compounds. Usman and Kumar [62] reported comparable activity (15.0%–56.02%) in PP using Soxhlet with methanol, supporting the present findings. In contrast, Kaur et al. [63] observed higher AA (75.36%) under Soxhlet extraction at 50°C for 5 h, which may be explained by differences in solvent-to-sample ratio, extraction conditions, and the more efficient solubilization of flavonoids, tannins, and anthocyanins. Overall, the results confirm that extraction time and temperature are critical parameters influencing antioxidant potential, with higher values promoting more effective release of bioactive compounds.

3.5 | Surface Morphological Analysis

The surface morphology of PP powder was examined using SEM to evaluate the structural changes before and after EAE.

SEM analysis revealed clear structural differences in PP between the pre- and post-EAE samples. The untreated peel exhibited a dense, smooth, and compact surface, indicating intact cell walls with polyphenols confined within (Figure 1). After EAE, the surface became rough, porous, and fragmented due to tannase activity, confirming effective cell wall disruption and enhanced release of phenolic compounds (Figure 2). The findings align with those of Chiang and Lai [64], who demonstrated that enzymatic processing alters the microstructure of plant materials, rendering their surfaces less dense and more permeable to the extraction of bioactive components. This phenomenon is characteristic of plant materials subjected to enzymatic modification. Similar findings were reported for ginger powder by Chandran and Nangarthody [65], where cellulose-assisted extraction led to cell wall breakage and surface roughness, facilitating the release of bioactive compounds. Overall, SEM images confirm that enzymatic treatment effectively alters the peel's microstructure, thereby improving polyphenol extraction efficiency.

Although SEM analysis could provide valuable visualization of microstructural changes induced by Soxhlet extraction, it was not included in the present study. SEM analysis was focused on EAE due to its pronounced impact on cell structure, whereas Soxhlet extraction is known to induce only limited morphological alterations [66].

TABLE 5 | The data of the fit summary of TPC and AA obtained from RSM.

	Sequential *p* value		Lack-of-fit *p*value		Adjusted R^2		Predicted R^2	
Source	**TPC**	**AA**	**TPC**	**AA**	**TPC**	**AA**	**TPC**	**AA**
Linear	0.0656	0.5221	0.0037	< 0.0001	0.3041	−0.0537	−0.0934	−0.8881
2FI	0.8669	0.14667	0.0027	< 0.0001	0.2294	0.0855	−1.2755	−2.0542
Quadratic	< 0.0001	< 0.0001	0.4968	0.0131	0.9570	0.9473	0.8999	0.7371
Cubic	0.7894	0.0711	0.2096	0.0245	0.9453	0.9744	0.0298	0.0594

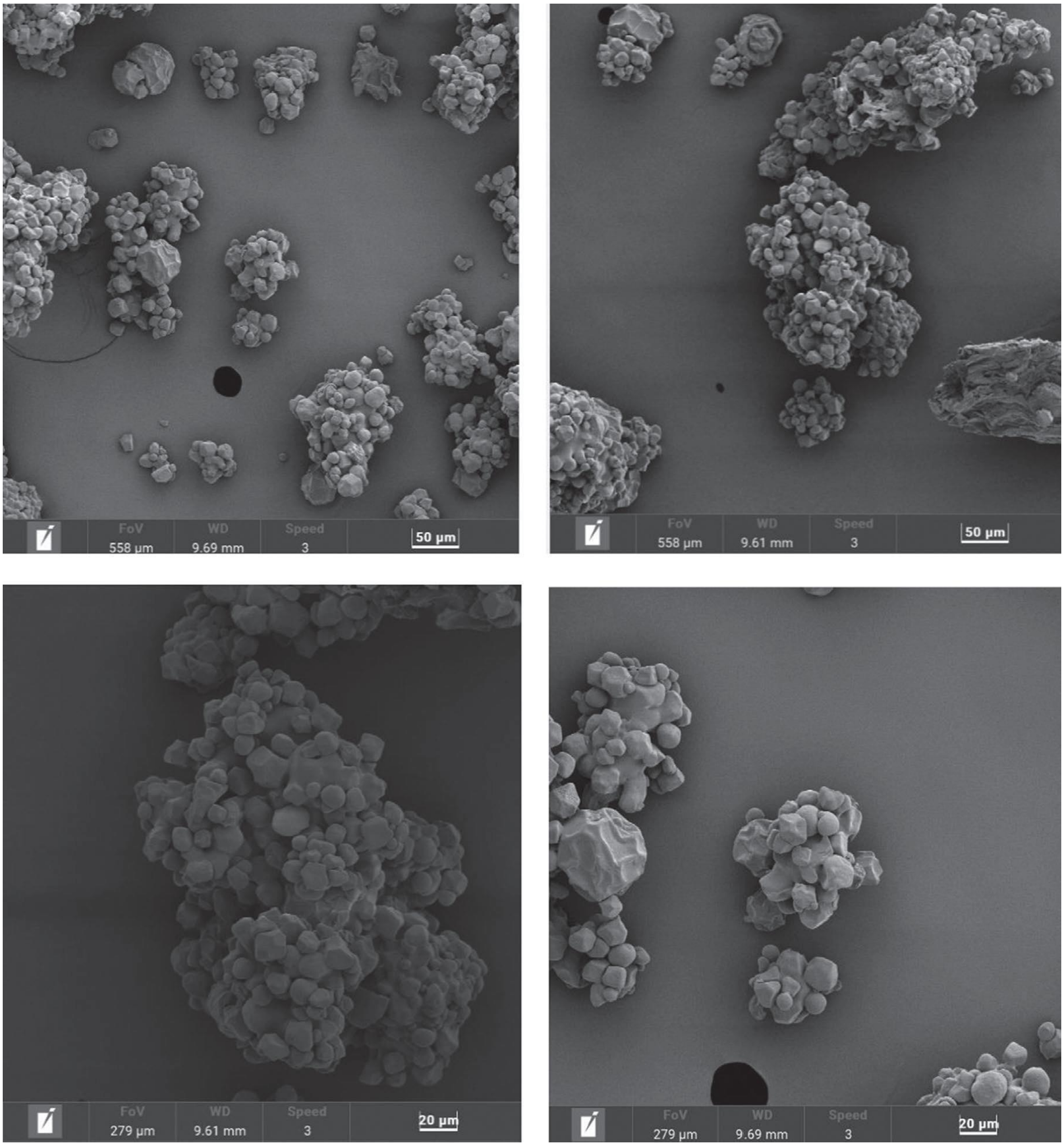


FIGURE 1 | SEM images (MAG 375× and 570×) of PP powder before EAE.

3.6 | Regression Analysis and Model Fitting

3.6.1 | Model

3.6.1.1 | Response 1 (TPC). RSM was employed to evaluate the impact of enzyme concentration (Factor A) and temperature (Factor B) on the yield of TPC from PP. Models constructed using coded and actual factors not only allow response values to be predicted but also enable the significance of individual factors and their interactions to be identified.

The model in coded factors makes it easier to compare coefficients and the relative contribution of variables. In contrast, the equation in actual factors reflects the real conditions of the experiment and is used for practical calculations:

$$\text{TPC (coded factor)} = 176.77 + 0.77A + 14.85B + 1.23AB - 4.27A^2 - 21.29B^2, \quad (4)$$

$$\text{TPC (actual factor)} = -226.58078 + 4.32776A + 18.32901B + 0.061375AB - 1.06629A^2 - 0.212852B^2, \quad (5)$$

where A is the enzyme concentration and B is the temperature.

The equations demonstrate that temperature (B) exerts a more pronounced effect on TPC than enzyme concentration (A). The quadratic terms (A^2 and B^2) indicate the nonlinearity of the effect (i.e., that an increase in the factors will lead to a decrease in polyphenol yield).

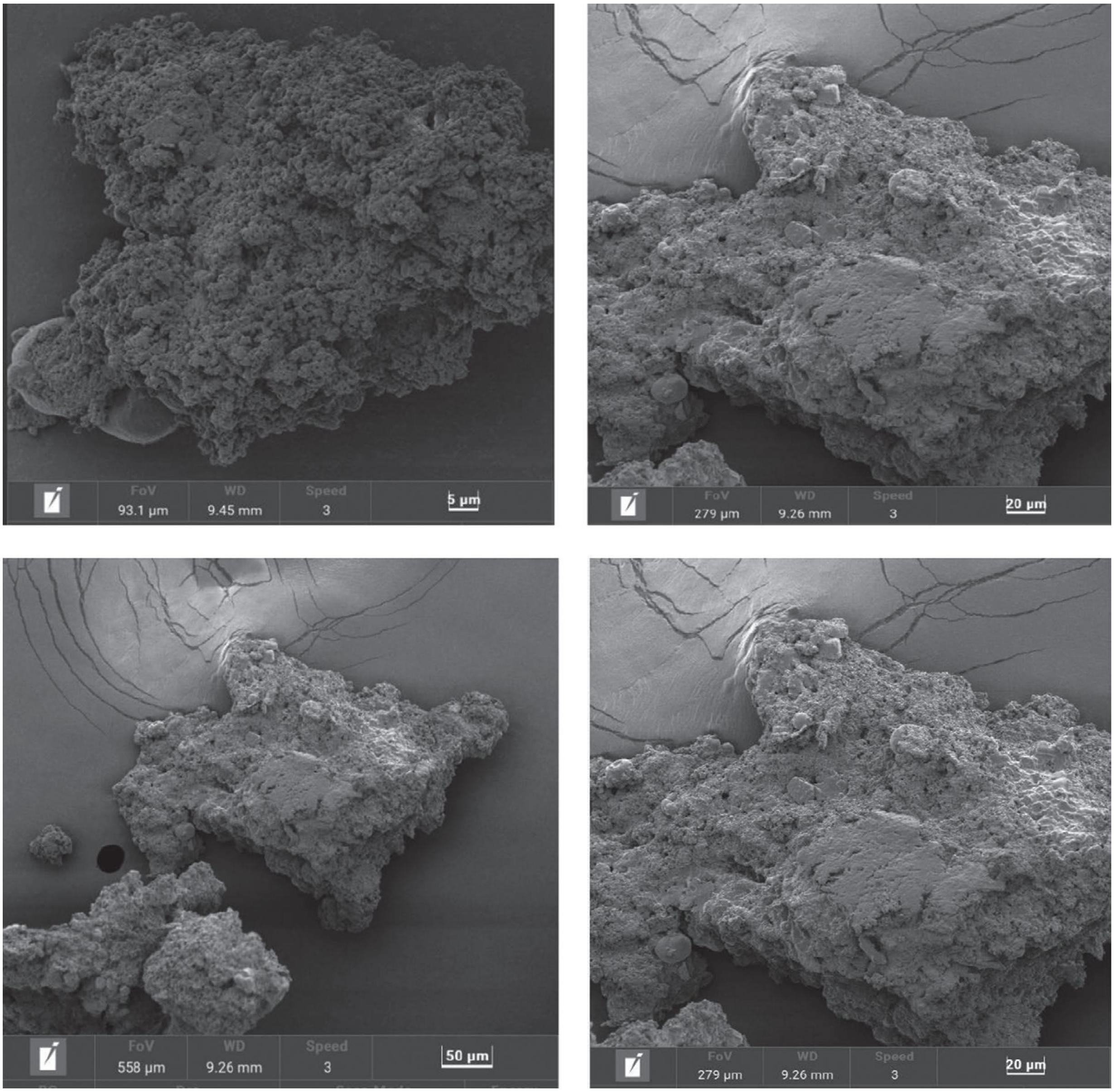


FIGURE 2 | SEM images (MAG 375× and 570×) of pomegranate peel powder residue after EAE.

3.6.1.2 | Response 2 (AA). Furthermore, quadratic models have been developed to define the AA. These factors enable the establishment of relationships and the identification of conditions that allow for the achievement of maximum effect:

$$\text{AA (coded factor)} = 72.14 + 4.26A - 1.54B - 6.97AB - 10.57A^2 - 6.44B^2, \quad (6)$$

$$\text{AA (actual factor)} = -96.76966 + 31.93322A + 6.04470B - 0.348625AB - 2.64276A^2 - 0.064410B^2, \quad (7)$$

where A is the enzyme concentration and B is the temperature.

The models show that an increase in enzyme concentration contributes to an increase in AA, while temperature has a negative effect when the optimal level is exceeded.

The influence of the interaction of factors (AB) and quadratic terms is significant, confirming the need for optimization.

3.6.2 | Interactions Among Factors

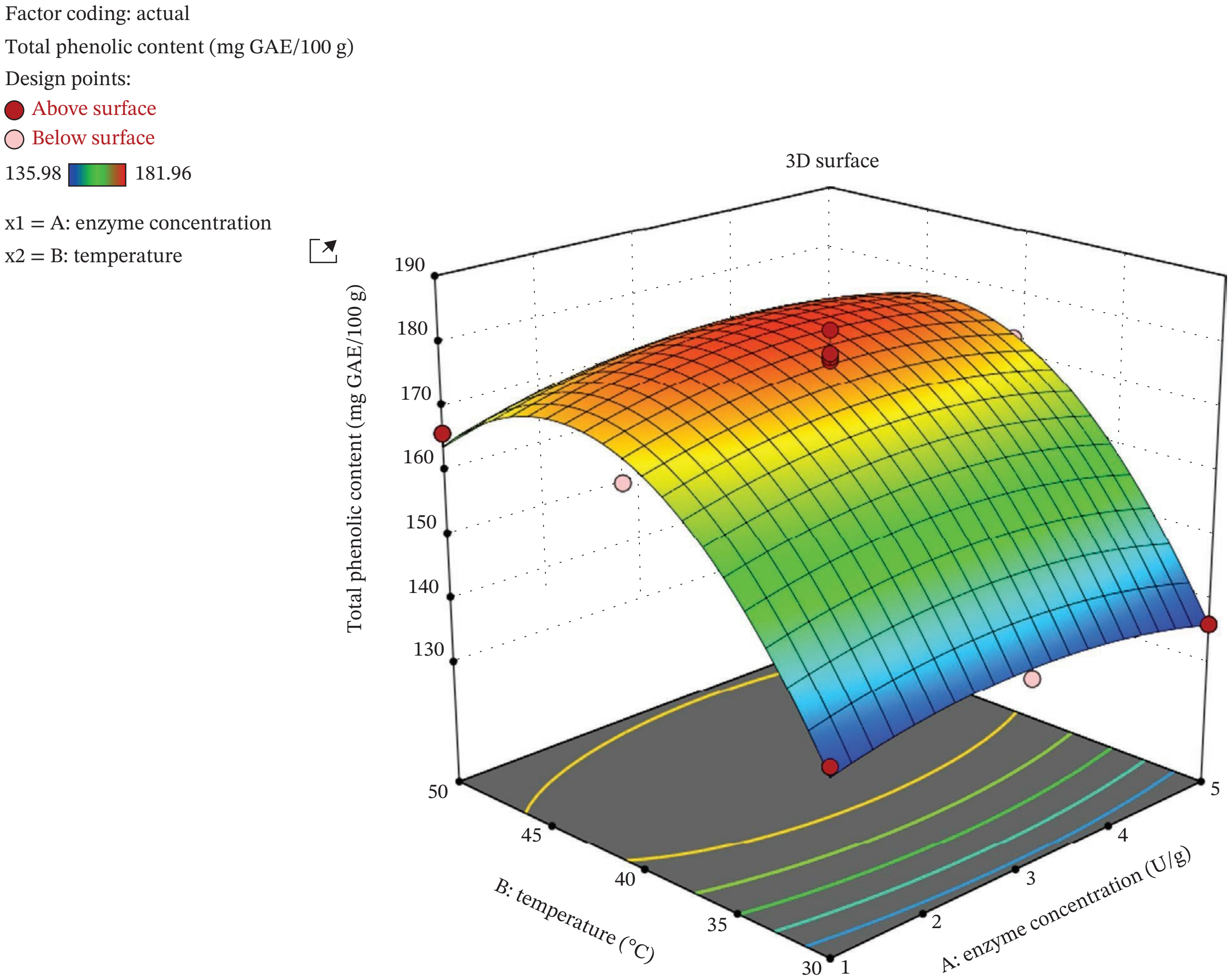
3.6.2.1 | Response 1 (TPC) and Response 2 (AA). Based on the RSM, the fit summary data presented in Table 5 indicates that the quadratic model is the most suitable for predicting TPC and AA. Among the models evaluated, the quadratic model recorded the lowest sequential p value (< 0.0001), signifying that it is statistically significant. Additionally, it achieved the highest adjusted R^2 value for TPC and AA (0.9570 and 0.9473, accordingly), indicating an excellent fit to the data, and a reasonably high predicted R^2 value (0.8999 and 0.7371), reflecting strong predictive capability. The lack-of-fit p value (0.4968 and 0.0131) was also high, suggesting that the model fits the experimental data well without a significant lack of fit. Thus, the quadratic model was the best model to describe the variability of the response as it has a large adjusted R^2 and a high lack-of-fit p value and was statistically significant.

The response surface graphs and contour graph analysis indicating the effects of independent variables on TPC and AA are shown in Figures 3 and 4, respectively. The effects of enzyme concentration (A) and temperature (B) on TPC and AA of PPs were evaluated using RSM. The 3D surface and contour plots demonstrated a significant influence of the interaction of factors (AB). The combined effects of the studied parameters on TPC and AA are essential, and the quadratic model provided a good fit to the experimental data.

3.6.3 | Influence of Enzyme Concentration and Temperature on TPC and AA

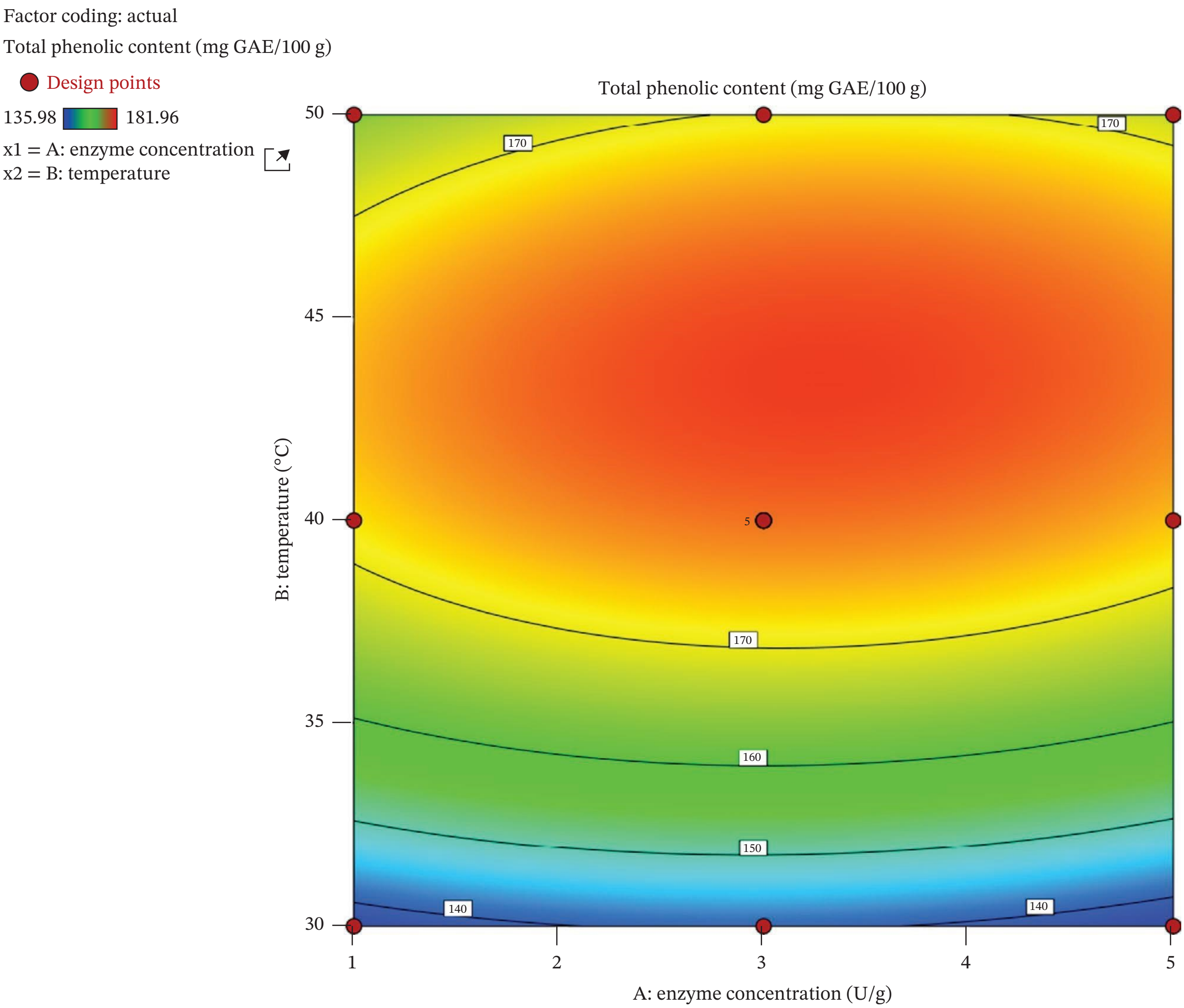
Enzyme concentration (1–5 U/g) and temperature (30°C–50°C) had a significant interaction effect on the yield of phenolic compounds and AA. According to the response surface analysis (Figures 3 and 4), the highest TPC and AA were achieved at 3 U/g enzyme and 40°C (181.96 ± 0.04 mg GAE/g and 73.85% ± 0.04% DPPH inhibition, respectively). This observation is consistent with previous studies on PP [43, 67, 68], which report that mild enzymatic or low-temperature extraction conditions enhance the release of phenolic compounds while preserving their antioxidant functionality.

Furthermore, at the higher enzyme dosage (5 U/g) and lower temperature (30°C), the values decreased, indicating that there was no linear increase in efficiency with increasing enzyme concentration. This is consistent with the results of Ghandahari Yazdi and Ahmadi Gavligh [53] and Stanek-Wandzel et al. [69], which indicate that increasing the dosage above the optimal dosage did not improve the yield due to mass transfer limitations. Similarly, the effect of temperature was quadratic: A moderate increase to 40°C enhanced solvent penetration and enzyme activity. In contrast, a temperature of 50°C resulted in a decrease in TPC and AA, likely due to potential degradation of heat-labile compounds. Similar results were noted by Shiban et al. [44] and other authors, who emphasized the importance of balancing enzyme dosage and temperature to prevent a decrease in yield.



(a) 3D surface analysis on enzyme concentration and temperature of TPC using RSM

FIGURE 3 | Effects of enzymatic concentration and temperature on total phenolic content (TPC) from pomegranate peels using enzyme-assisted extraction.



(b) Contour graph analysis on enzyme concentration and temperature of TPC using RSM

FIGURE 3 | (CONTINUED)

Overall, the highest TPC and AA values were achieved at an enzyme dosage of 3 U/g and a temperature of 40°C. This enzyme concentration provides an optimal balance between catalytic activity and mass transfer limitations, and the moderate temperature facilitates the efficient release of phenolic compounds without their thermal degradation.

3.6.4 | Experimental Versus Optimization of Response Variables

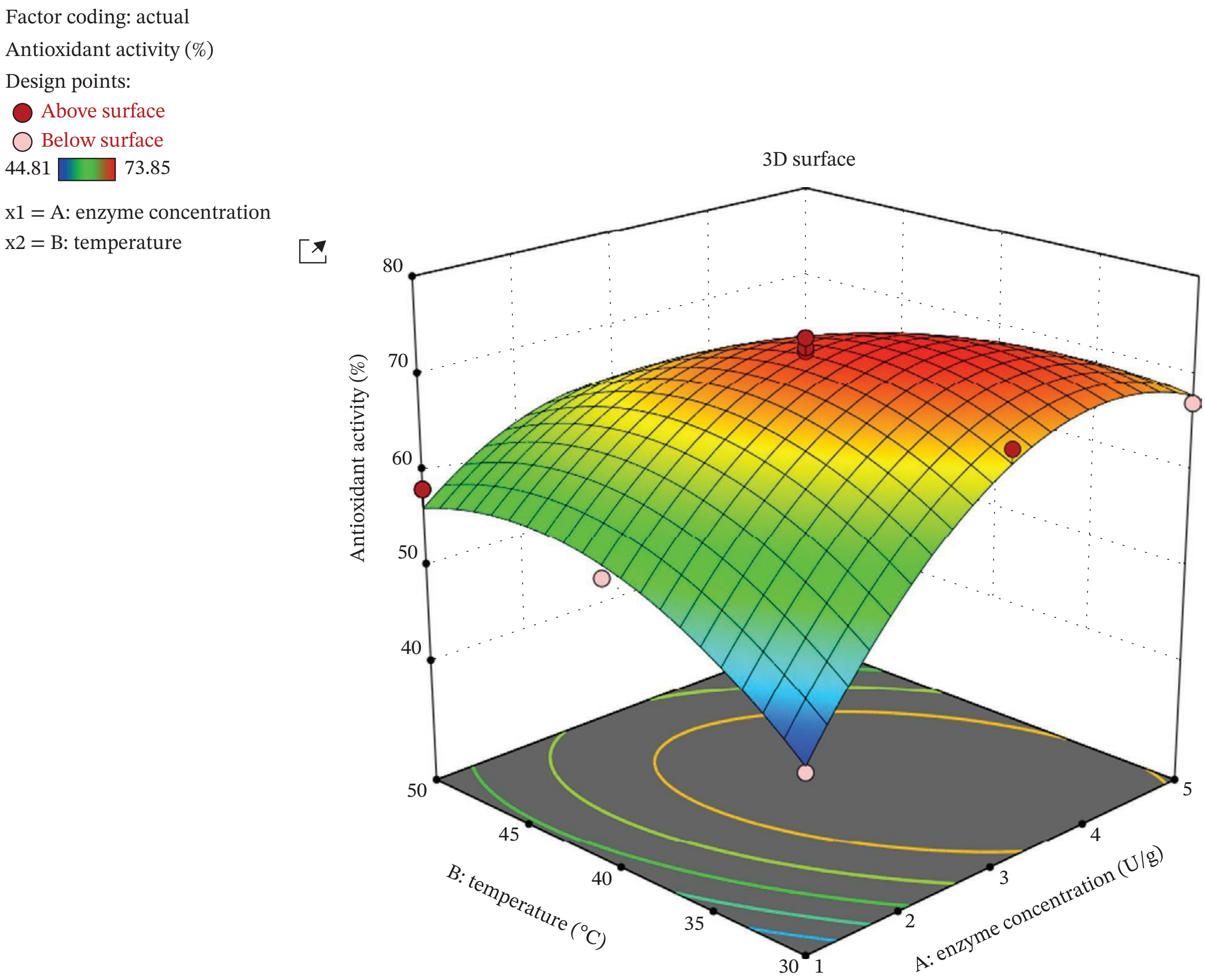
Analysis of variance (ANOVA) was generated by RSM to analyze deeper on the significance of the model (Table 6). It was easy to detect the reliability of the data, optimization, and the analysis by looking at the value from the ANOVA. Not only that, but ANOVA also functions in showing the error in the result produced and suggests the significance of the model.

ANOVA confirmed the significance of the quadratic model for TPC ($F = 54.44$, $p < 0.0001$), with a nonsignificant lack of fit ($p = 0.4968$), indicating good model validity. Temperature (B) had the strongest effect ($F = 116.98$, $p < 0.0001$), while enzyme concentration (A) and interaction AB were not significant. The quadratic term B^2 was highly significant, whereas A^2 had a minor contribution.

For AA, the quadratic model was also significant ($F = 44.18$, $p < 0.0001$). Significant effects were observed for A, AB, A^2, and B^2, while B was not significant. Despite a significant lack of it ($p = 0.0131$), the model retained strong predictive ability.

ANOVA analysis (Table 7) supported the regression coefficient (R^2), confirming the validity of the polynomial model [70]. The high R^2 value (0.9749) indicated excellent model fit and predictive accuracy, while the adjusted R^2 (0.9570) and predicted R^2 (0.8999) demonstrated consistency and strong predictive power. The adequate precision (17.8820) exceeded the threshold of 4, confirming model reliability for optimizing TPC content from PPs.

Similarly, the model for AA showed $R^2 = 0.9693$, explaining 96.63% of the variation, with adjusted R^2 of 0.9473 and predicted R^2 of 0.7371, indicating acceptable agreement. The low



(a) 3D surface analysis on enzyme concentration and temperature of antioxidant activity using RSM

FIGURE 4 | Effects of enzymatic concentration and temperature on antioxidant activity from pomegranate peels using enzyme-assisted extraction.

coefficient of variation (3.28%) and high adequate precision (18.6597) confirmed precision, robustness, and a strong signal-to-noise ratio. Overall, the models were accurate, reliable, and suitable for predicting phenolic content and AA in PP.

3.6.5 | Optimization of the Factors Using RSM

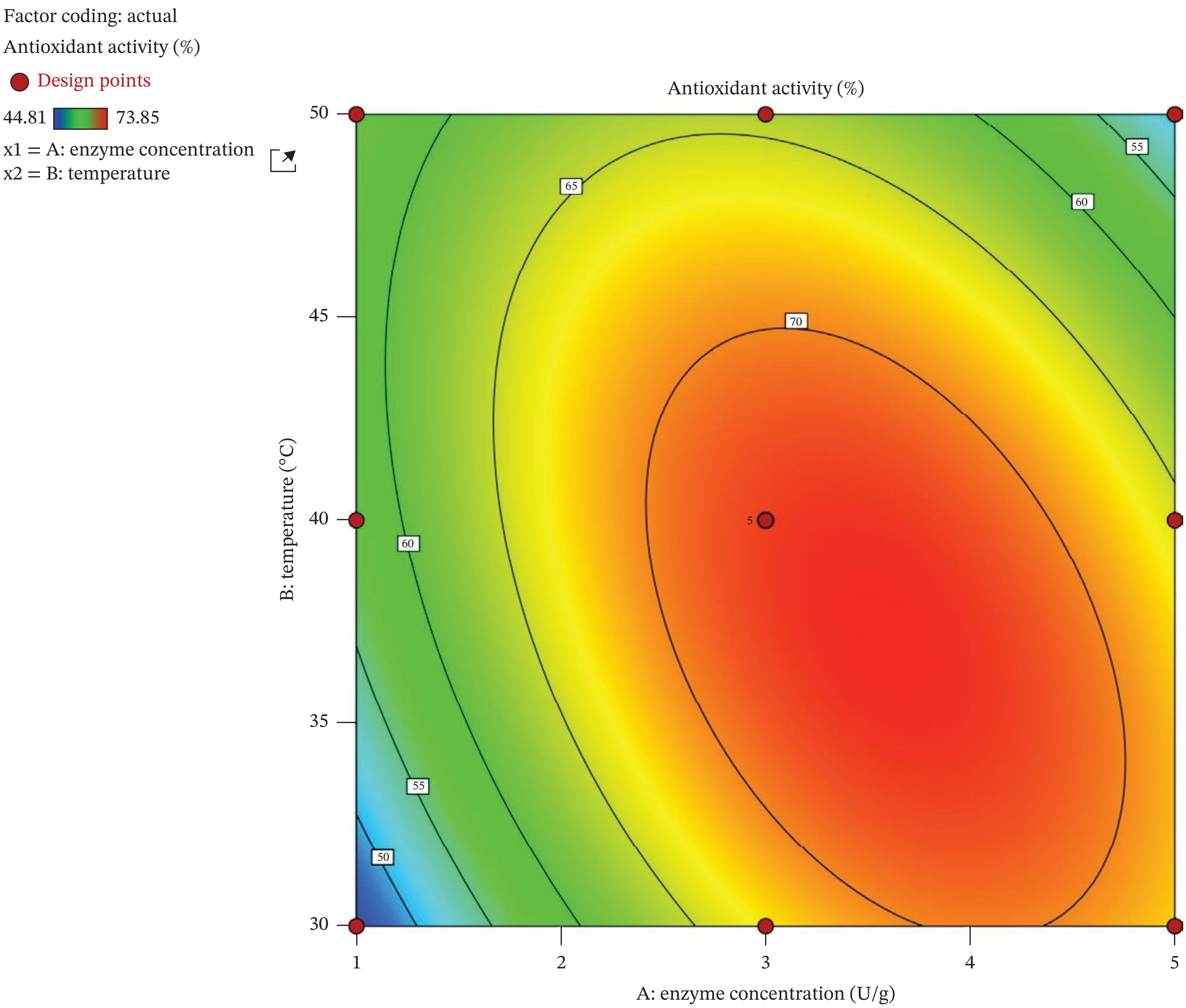
Optimization of TPC from PPs (Table 8) was performed using enzyme concentration (1%–5%) and temperature (30°C–50°C) as key factors of equal importance. The target range was 135.98–181.96 mg GAE/g, with both variables contributing significantly to maximizing phenolic content.

AA optimization considered the same factors—enzyme concentration (1%–5%) and temperature (30°C–50°C) with equal weight. The target range was 44.1%–73.85%, confirming their critical role in enhancing the antioxidant potential of PPs.

Optimization generated two solutions for maximizing TPC from PP. The best result, with a desirability score of 0.945, was obtained at 3.281 U/g enzyme concentration and 43°C, predicting 179.44 mg GAE/g. A second solution showed similar desirability, confirming model consistency and highlighting the role of moderate temperature and specific enzyme levels in phenolic extraction. For AA, the solution with the highest desirability score, 0.970, was selected as the optimal conditions (Table 9).

According to Table 10, the selected formulation for TPC (3.281 U/g enzyme, 43°C) yielded experimental values of 175.40–177.53 mg GAE/g, closely matching the predicted 179.44.mg GAE/g, and confirming model accuracy. For AA, the optimum conditions (3.588 U/g enzyme, 37°C) produced 72.52%–73.15%, in good agreement with the predicted 72.98%. The close match between experimental and predicted values validated the reliability of the optimization model.

Experimental validation confirmed the accuracy and applicability of the models under given conditions, in line with the reliability criteria proposed by Ahmed et al. [71]. For TPC (Table 11), the experimental value was 176.38 ± 1.07 mg GAE/g versus a predicted 179.44 mg GAE/g, with a relative error of 1.706%, within the acceptable < 5% limit. For AA, the experimental



(b) Contour graph analysis on enzyme concentration and temperature of antioxidant activity using RSM

FIGURE 4 | **(CONTINUED)**

value (72.81% ± 0.26%) closely matched the predicted 72.98%, with a relative error of 0.236%.

3.6.6 | Comparison Studies Between EAE and Soxhlet Extraction Methods

In this study, the efficiency of EAE was compared with that of the conventional Soxhlet technique to evaluate its potential for maximizing the recovery of phenolic compounds from PP. The Soxhlet method was chosen as the control/reference method due to its high reproducibility, complete extraction, and compliance with international analytical standards (AOAC and ISO).

3.6.6.1 | Extraction Yield Between EAE and Soxhlet Extraction. The extraction yield reached 70.00% ± 0.25% at EAE, notably higher than the 49.50% ± 0.03% obtained by Soxhlet extraction (Table 12). This indicates the superior efficiency of enzymatic treatment in releasing bioactive compounds, particularly from PP. The action of tannase facilitates the hydrolysis of tannins and degradation of cell wall structures, enhancing the release of phenolics and antioxidants into the solvent. EAE was carried out under milder conditions (40°C, 3 U/g enzyme), resulting in lower energy and solvent consumption than Soxhlet extraction.

By contrast, Soxhlet extraction requires elevated temperature (50°C), longer duration, and larger solvent volumes, which increase energy demand and operational costs. Although Soxhlet is effective in phenolic recovery through continuous solvent reflux, the lack of enzymatic selectivity and potential thermal degradation of sensitive compounds limit its efficiency. Overall, EAE offers higher yield, reduced environmental impact, and greater sustainability, making it a promising alternative for industrial-scale extraction of bioactive compounds.

3.6.6.2 | TPC Between EAE and Soxhlet Extraction. In comparison to Soxhlet extraction, which yielded a maximum TPC of 195.72 ± 0.62 mg GAE/g at 50°C, EAE obtained a slightly lower TPC (181.96 ± 0.04 mg GAE/g) under optimal conditions (3 U/g at 40°C, Table 12). While Soxhlet extraction was more efficient in yield, it required a higher temperature,

TABLE 6 | Analysis of variance using the regression method.

Source	df	Sum of squares		MeanSquare		*F*-value		*p* value		Remarks	
		TPC	AA	TPC	AA	TPC	AA	TPC	AA	TPC	AA
Model	5	3078.69	980.41	615.74	196.08	54.44	5 44.18	< 0.0001	< 0.0001	Significant	Significant
A: Enzyme concentration	1	3.56	109.06	3.56	109.06	0.3145	24.57	0.5924	0.0016		
B: Temperature	1	1323.13	14.23	1323.13	14.23	116.98	3.21	< 0.0001	0.1165		
AB	1	6.03	194.46	6.03	194.46	0.5328	43.81	0.4891	0.0003		
A^2	1	50.24	308.63	50.24	308.63	4.44	69.53	0.0730	< 0.0001		
B^2	1	1251.30	114.58	1251.30	114.58	110.63	25.81	< 0.0001	0.0014		
Residual	7	79.18	31.07	11.31	4.44						
Lack of fit	3	32.93	28.43	10.98	9.48	0.9494	14.38	0.4968	0.0131	Not significant	Significant
Pure error	4	46.25	2.64	11.56	0.6592						
Cor. total	12	3157.87		1011.48							

longer time, and larger solvent volume, making it less sustainable. EAE, by contrast, provided comparable results under milder conditions with reduced solvent use, verifying its potential as a greener and environmentally benign technique. Nguyen et al. [72] reported that EAE using Alcalase recovered more phenolics and polysaccharides from *Padina gymnospora* than conventional ethanol extraction, emphasizing its environmental advantages. Similar findings (a maximum TPC of 432.00 and 522.33 mg GAE/g at Soxhlet extraction and EAE, respectively) were reported by Chandran and Nangarthody [65], who demonstrated that EAE can match or even surpass conventional extractions in TPC recovery. Thus, with further optimization, EAE may provide higher results and improved sustainability.

3.6.6.3 | AA Between EAE and Soxhlet Extraction. EAE showed higher inhibition (73.85% ± 0.04%) under optimal conditions (40°C, 3 U/g) compared to Soxhlet extraction, which reached 67% ± 0.02% at 50°C for 5 h (Table 13). The higher AA of EAE can be attributed to enzymatic degradation of tannins and cell wall components, which facilitates the release of low-molecular phenolic compounds with strong radical-scavenging activity. Similar statements have been reported by several authors [73, 74], who demonstrated that enzymatic treatment affected the phenolic compound profile and antioxidant capacity of extracts from PP.

Furthermore, studies of [75, 76] have shown that the recovery of bioactive phenolics and their AA in PP was influenced by temperature up to a limit above which degradation occurs.

In contrast, Soxhlet extraction, despite providing slightly higher TPC, was less efficient in preserving AA. Prolonged heating and higher temperatures may cause thermal degradation of sensitive compounds, reducing overall activity.

While Soxhlet extraction remains effective for maximizing phenolic yield, the present results indicate that EAE provides extracts with superior AA under milder and more sustainable conditions.

The high TPC value obtained with the Soxhlet extraction does not correlate with biological activity due to the thermal degradation of labile antioxidants during prolonged heating. At the same time, mild EAE conditions ensure better preservation of native compounds and their synergism. Thus, EAE outperforms the Soxhlet method and ensures better preservation of functional properties, despite a slightly lower yield of phenolics.

This highlights its potential as a green technology for industrial applications that target antioxidant-rich materials, where bioactivity and environmental performance are critical.

3.6.6.4 | Assessment of Greenness Between EAE and Soxhlet Extraction. Analytical GREEnness (AGREE) is a metric system for assessing the greenness of analytical methods, based on 12 principles of significance. It is comprehensive and flexible due to the use of scales and is easy to use, as it appears as a colored pictogram that reflects the method's strengths and weaknesses [77].

Sample preparation for EAE is implemented externally using a simplified, three-step procedure (Principle 1) and requires 1.0 g of PP sample (p. 2). The measurement is performed offline (p. 3), and the EAE procedure involves three separate steps: preparation, integrated incubation–extraction, and centrifugation–separation (p. 4). The method is not automated or miniaturized (p. 5). The procedure does not require derivatization, which aligns with green analytics principles and reduces the use of toxic reagents (p. 6). Analytical wastes include bioresidues of the sample (0.3 g) and aqueous buffer solutions (p. 7). Three analytes are determined in a single run; the throughput is ~0.33 samples/h, based on a 3-h time extraction (p. 8). The data in Table 13 confirm that EAE is energy-efficient and does not require extensive heating (p. 9), and the reagents are derived from biological sources (p. 10). The procedure does not require toxic solvents (p. 11) and contains no hazardous components (p. 12).

The external pretreatment for Soxhlet extraction is performed to minimize sample handling and reduce the risk of contamination (p. 1). The PP sample required for the procedure is 27 g (p. 2), and the measurement is performed offline. This method includes five discrete operations (from extraction to

TABLE 7 | Result analysis on ANOVA from RSM, specifically on R^2 values of Response 1 (total phenolic content) and Response 2 (antioxidant activity) from pomegranate peels.

Parameter	Response 1 (total phenolic content)	Response 2 (antioxidant activity)
Std. Dev.	3.36	2.11
Mean	164.98	64.29
CV %	2.04	3.28
R^2	0.9749	0.9693
Adjusted R^2	0.9570	0.9473
Predicted R^2	0.8999	0.7371
Adeq. precision	17.8820	18.6597

TABLE 10 | Predicted and experimental values for TPC and AA for selected corresponding optimum solutions.

Run	Total phenolic content (mg GAE/g)	Antioxidant activity (%)
1	176.213	72.515
2	177.532	73.145
3	175.402	72.750
Average run	176.382	72.810
Predicted value (from RSM)	179.443	72.982

TABLE 11 | Result of statistical analysis for verification of optimization.

Parameter	Experimental (*mean* ± *SD*)	Predicted value (mg GAE/g)	Absolute relative error (%)
Total phenolic content	176.382 ± 1.066	179.443	1.706
Antioxidant activity	72.81 ± 0.26	72.982	0.23

TABLE 8 | Raw data for optimization of Response 1 (TPC) and Response 2 (AA) from pomegranate peels.

Name	Goal	Lower limit		Upper limit		Lower weight		Upper weight		Importance	
A: Enzyme concentration	Within range	1	1	5	5	1	1	1	1	3	3
B: Temperature	Within range	30	30	50	50	1	1	1	1	3	3
Total phenolic content	Maximize	135.98		181.96		1		1		3	
Antioxidant activity	Maximize	44.81		73.85		1		1		3	

TABLE 9 | List of predictions of the Response 1 (total phenolic content) from pomegranate peels by RSM.

Number	Enzyme concentration (U/g)	Temperature (°C)	Total phenolic content	Desirability	Remarks
1	3.281	43	179.443	0.945	Selected
2	3.263	43	179.442	0.945	
Number	**Enzyme concentration (U/g)**	**Temperature (°C)**	**Antioxidant activity**	**Desirability**	**Remarks**
1	3.588	37	72.982	0.970	Selected

TABLE 12 | Comparison of enzyme-assisted extraction (EAE) and Soxhlet extraction.

Parameter	Enzyme-assisted extraction (EAE)	Soxhlet extraction (control)
Condition	Temperature: 40°C	Temperature: 40°C
	Enzyme concentration: 3 U/g	Extraction time: 5 h
	Extraction time: 3 h	
Extraction yield	70.00% ± 0.25%	49.50% ± 0.03%
Total phenolic content	181.96 ± 0.04 mg GAE/g	195.72 ± 0.06 mg GAE/g
Antioxidant activity	73.85% ± 0.04%	67.00% ± 0.02%

TABLE 13 | Energy calculations for EAE and Soxhlet extraction.

Energy calculations for EAE	Energy calculations for Soxhlet extraction
Orbital shaker: 0.15 kWh × 3 h / 13 samples = 0.04 kWh / sample	Hot plate: 0.5 kWh × 5 h / 2 samples = 1.25 kWh / sample
Centrifuge: 0.8 Wh × 0.16 / 13 samples = 0.01 kWh / sample	Centrifuge: 0.8 Wh × 0.16 h / 2 samples = 0.07 kWh / sample
	Cooling water pump aeration: 0.05 kWh × 5 h / 2 samples = 0.13 kWh / sample
Total: 0.05 kWh/sample. Score by AGREE: 1	Total: 1.45 kWh/sample. Score by AGREE: 0

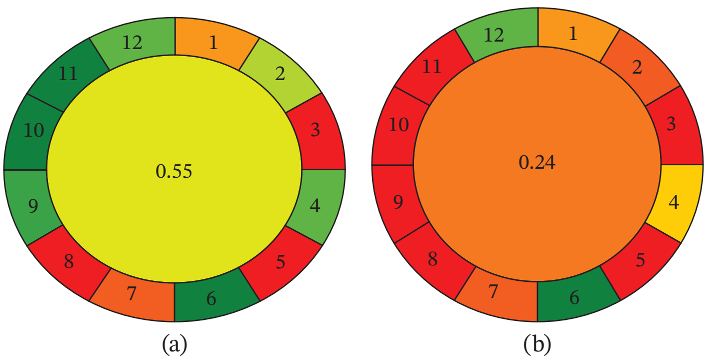


FIGURE 5 | Results of AGREE analysis for (a) EAE and (b) Soxhlet extraction.

evaporation) (p. 4) and is neither automated nor miniaturized (p. 5). Derivatizing agents are not used (p. 6); the amount of waste generated is 13.5 g of the raw material. Most of the solvent is returned to the flask and reused (p. 7). The number of analytes determined is 3, and the analytical throughput is 0.54 samples/h (p. 8). The energy calculations from Table 13 show that the most energy-intensive device is used in this method (p. 9). None of the reagents is of biological origin (p. 10), although 500 mL of methanol is required (p. 11). High flammability cannot be ruled out (p. 12).

In the range from 0 to 1, 0.55 for EAE (Figure 5a) indicates a moderately "green" process, meaning there are positive aspects (low organic solvent consumption and less energy), but there are still areas requiring optimization. Most red and orange segments (Figure 5b) indicate that Soxhlet extraction consumes a significant amount of solvent and energy, generating waste. A low value of 0.24 confirms that the Soxhlet method is not environmentally friendly.

4 | Conclusion

This study validates the effectiveness of EAE for processing the peel of a Kazakh pomegranate variety. A key innovation is the confirmed specificity of tannase to the polyphenolic composition of local raw materials. Good agreement was achieved between the experimental (in the range of 175.400–177.530 mg GAE/g) and predicted (179.443 mg GAE/g) values for total phenolic compounds. For AA, the expected value (72.98%) falls within the experimental range (72.52%–73.15%), confirming the reliability and accuracy of the RSM optimization. SEM analysis revealed significant structural changes in PP after EAE: The smooth, dense surface of untreated raw material became more porous and fragmented. The obtained data and results of the AGREE analysis confirm the potential of this method as a relatively green and alternative to traditional extraction.

Comparative analysis indicates that EAE offers advantages over Soxhlet extraction for processing plant biomass, as it reduces process time, eliminates toxic solvents, and enables selective recovery of bioactive compounds without compromising their activity.

Further research should focus on optimizing combinations of EAE with ultrasonic or microwave-assisted processing to increase the extraction yield to 80% and reduce the process time to 60 min. For industrial applications, further work is necessary to evaluate the enzyme costs, energy consumption, and cost-effectiveness.

Author Contributions

A. A. Saparbekova: conceptualization, funding acquisition, and supervision. G. O. Kantureyeva: conceptualization and writing—original draft, review, and editing. S. M. Mustapa Kamal: formal analysis, data curation, and resources. W. A. Wan Ab Karim Ghani: investigation, methodology, and writing—original draft. Adawiyah Rabiatul: investigation. D. E. Kudasova: methodology. A. Altekey: investigation.

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Conflicts of Interest

The authors declare 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.

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