

OPTIMIZATION OF ENZYME-ASSISTED EXTRACTION OF PHENOLICS AND SAPONINS FROM PARAMIGNYA TRIMERA (OLIV.) BURKILL ROOT AND EVALUATION OF ULTRASOUND-ENZYME COMBINED TECHNIQUES

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Abstract

Xao tam phan (XTP) root (*Paramignya trimera*), a well-known Vietnamese medicinal plant, is rich in bioactive phenolics and saponins. This work optimized an enzyme assisted ultrasound extraction to maximize total phenolic content (TPC) and total saponin content (TSC) from the root. Screening with a Plackett–Burman design identified the most influential variables, and Response Surface Methodology (RSM) then refined the process. The optimal enzyme pre-extraction conditions were 0.91% pectinase–cellulase, 47°C, and 105 minutes. Under these conditions, TPC and TSC increased by 43.5% and 52.9%, respectively, compared to untreated control samples. The study also evaluated different ways of combining ultrasound with enzymatic treatment. Ultrasound treatment before enzyme assisted extraction (U EAE) produced the highest yields, delivering 12.63 mg GAE/g d.b for TPC and 274.46 mg AE/g d.b for TSC. These values correspond to 1.77fold and 3.2fold improvements over the controls, respectively. The findings indicate that U EAE is a promising, efficient approach for recovering valuable phytochemicals from *P. trimera* root and could support future development of functional extracts and related products. In addition, the optimized protocol may reduce solvent use and processing time, improving sustainability for laboratory and industrial applications. Further studies on compound profiles, bioactivity, and scale up will help translate these extraction gains into standardized preparations.

Keywords: enzyme, *Paramignya trimera*, total phenolics, total saponins, Ultrasound, XTP.

1. Introduction

Xao tam phan (*Paramignya trimera* (Oliv.) Guillauum) is a woody shrub species belonging to the Rutaceae family (Son, 2018). This plant is widely distributed in Southeast Asia, including countries such as Southern Vietnam, Southern Philippines, Thailand, Malaysia, Java-Indonesia, Australia, and Sri Lanka (Son, 2018). In Vietnam, seven *Paramignya* species have been found, including *P. trimera* (Son, 2018). *P. trimera*, also known as xao

tam phan (XTP) in Vietnamese, is a plant species endemic to Vietnam, particularly in the South (Dang et al., 2017).

According to traditional medicine, XTP roots have been used by Vietnamese people as a folk remedy to treat hepatitis and diabetes (Nguyen et al., 2025). However, there were few records of its use in medicine before 2012 (Quan et al., 2021). Phytochemical research on XTP, particularly in the roots, has revealed the presence of many bioactive compounds. The main compound groups include coumarins and coumarin glycosides, acridone alkaloids, flavonoids, and saponins (Son, 2018; Nguyen et al., 2025). Notably, acridones isolated from XTP roots have shown alpha-glucosidase inhibitory activity more potent than acarbose, a blood glucose control medication (Dang et al., 2017). Research results indicate that XTP roots have potential in diabetes treatment.

Studies have also focused on quantitative analysis of chemical components in *P. trimera* roots, including total phenolic content (TPC), total flavonoid content (TFC), and saponin content (TSC) (Nguyen et al., 2016; Nguyen et al., 2017). HPLC analysis has identified and quantified numerous phenolic compounds in the methanol extract of the roots, such as kaempferol, chlorogenic acid, gallic acid, and rutin (Nguyen et al., 2015). Other studies have investigated the effects of different solvents and extraction methods (e.g., conventional extraction, ultrasound-assisted, and microwave-assisted) on the extraction efficiency and antioxidant capacity of compounds from the roots (Nguyen et al., 2015; Nguyen et al., 2023). Methanol and microwave-assisted extraction showed the highest extraction efficiency for bioactive compounds from XTP roots (Nguyen et al., 2015). These studies demonstrate the potential for developing new pharmaceutical materials from XTP roots.

Green extraction methods (which avoid chemical solvents) remain understudied for XTP roots. Among these techniques, enzyme-assisted extraction with ultrasound support has shown promise in producing bioactive compounds at an industrial scale. The growing demand for environmentally friendly technologies has increased interest in enzyme-assisted extraction (Marathe et al., 2019). Ultrasound-assisted extraction also offers notable benefits: shorter processing times, lower energy consumption, and reduced solvent requirements. This study focuses on optimizing the extraction of phenolics and saponins from XTP roots using enzyme-based aqueous extraction and comparing combined enzyme-ultrasound methods.

2. Literature review

The application of EAE, UAE, and their hyphenated configurations has been widely documented in medicinal plants, particularly for the targeted recovery of polyphenols and saponins embedded in complex plant matrices.

In *Paramignya trimera*, roots accumulate substantially higher levels of phenolics and saponins than leaves or stems (Nguyen et al., 2023). These compounds underpin the pharmacological activities of Xao tam phan, particularly its strong antioxidant capacity and cytotoxicity against cancer cell lines (Nguyen et al., 2025). Although *Paramignya trimera* roots have attracted considerable attention as a rich source of these bioactive compounds, most studies rely on chemical solvent extraction or single-assisted techniques, such as standalone microwave or ultrasound extraction (Nguyen et al., 2015; Nguyen et al., 2023). Such work typically depends on empirical, single-variable-at-a-time optimization, leaving multivariable kinetic interactions and the potential for enzymatic integration unexamined.

A rigorous analysis of the literature reveals three critical gaps in scientific knowledge regarding the processing and utilization of *Paramignya trimera*:

Absence of a Green, Purely Aqueous Framework: Most pharmacological studies confirming the anti-cancer and anti-diabetic traits of XTP roots have used crude organic fractions (methanol or chloroform). Data are lacking on whether equivalent or superior yields can be achieved using a 100% green, water-based solvent system. This limits the direct integration of XTP extracts into functional foods, beverages, or clean-label nutraceuticals due to regulatory limits on residual solvent toxicity.

Unexplored Multi-Enzyme Synergies on Lignified Root Matrices: While standard EAE has been mapped for soft tissues (leaves and fruits), its efficacy on the dense, fibrous subterranean root tissues of *Paramignya trimera* remains unknown. The specific combinations of cellulolytic and pectinolytic enzymes needed to break down this highly cross-linked woody matrix have not been systematically evaluated.

Lack of Comparative Kinetic Mapping for Coupled Processing Methods: To date, no study has investigated the operational sequence for combining ultrasound and enzymes for this genus. The literature lacks comparative analyses determining whether ultrasound first (mechanical dismantling), enzyme first (biochemical softening), or simultaneous treatment (co-current physical-biochemical degradation) is most efficient. The thermodynamic and kinetic thresholds governing enzyme stability under specific cavitation fields within this matrix have yet to be established.

Therefore, this study aimed to: (i) identify key factors affecting EAE using a Plackett–Burman design; (ii) optimize extraction conditions via response surface methodology; and (iii) evaluate different ultrasound–enzyme combinations to determine the most effective strategy for enhancing bioactive compound recovery.

3. Materials and Methods

3.1. Materials

Four-year-old xao tam phan roots were obtained from Phu Yen province, Vietnam. The roots underwent careful preparation: washing and draining, followed by drying under controlled conditions ($40 \pm 2^\circ\text{C}$) until reaching approximately 10% moisture content. The dried material was ground into powder, sieved through a 0.3 mm mesh, sealed in polyethylene bags, and stored at -10°C for future analysis. The enzymes used for this assay were pectinase (EC 232-885-6) and cellulase (EC 232-734-4) from Novozymes, Denmark. Analytical-grade chemicals used in this study included Folin-Ciocalteu reagent, gallic acid (GA), vanillin, and aescin, all obtained from Sigma-Aldrich Co., USA.

3.2. Experimental methods

3.2.1. Identifying critical parameters in enzymatic processing

To determine the factors influencing extraction efficiency, five parameters were investigated: pectinase–cellulase enzyme mixture concentration (% v/w, ratio 1:1), treatment time (min), treatment temperature ($^\circ\text{C}$), water/material ratio (v/w), and pH. The low (–1) and high (+1) levels of these factors are shown in Table 1. Using the Plackett–Burman design matrix, 12 experiments were conducted (Table 2) to identify the key factors affecting the yield of phenolics (mg GAE/g dried basis) and saponins (mg AE/g dried basis).

All treatments were conducted in triplicate using a thermostatic water bath (Mettmert, Germany), with a control sample without enzymatic treatment included. After treatment, samples were heated to 90°C for 10 min to deactivate enzymes. The mixture was then filtered through cloth, and the filtrate was centrifuged at 5000 rpm for 15 min to obtain the supernatant for TPC and TSC analysis.

Table 1. Variables examined in the Plackett-Burman experimental design

Coded factors	Variables	Factor values	
		Low level (-1)	High level (+1)
A	Concentration of enzyme mixture (% v/w)	0.2	1.0
B	Treatment time (min)	60	180
C	Treatment temperature (°C)	30	70
D	Water/material ratio (v/w)	10:1	80:1
E	pH	3.0	5.0

Table 2. Variables and levels used in the Plackett-Burman design

Pattern	Variables				
	A	B	C	D	E
0a0	0.2	60	30	80	3
---	0.2	60	70	10	3
--+	0.2	60	70	10	5
+--	0.2	180	30	10	5
+--+	0.2	180	30	80	5
a00	0.2	180	70	80	3
00a	1.0	60	30	10	5
000	1.0	60	30	80	3
000	1.0	60	70	80	5
000	1.0	180	30	10	3
00A	1.0	180	70	10	3
A00	1.0	180	70	80	5

3.2.2. Parameter optimization for enzymatic extraction of phenolics and saponins from XTP roots

Based on the Plackett-Burman design (PBD) results, optimization of enzymatic treatment parameters was conducted using central composite design (CCD) combined with response surface methodology (RSM). As shown in Table 3, this optimization established ranges that maximize the extraction yields of phenolics (mg GAE/g dried basis) and saponins (mg AE/g dried basis).

Table 3. Experimental design for optimizing enzyme extraction parameters

Survey parameters	Range of variation
Concentration of enzyme mixture (% v/w)	0.8 – 1.0
Treatment time (min)	75 - 105
Treatment temperature (°C)	45 - 55
Fixed parameters	Values
Water/material ratio (v/w)	40: 1
pH	4.0

Quadratic models were developed through regression analysis (Equation 1) (Wang et al., 2014). The accuracy of the optimization was verified by comparing experimental results with predicted values.

$$Y_n = \beta_0 + \sum_{i=1}^k \beta_i X_i + \sum_{i=1}^k \beta_{ii} X_i^2 + \sum_{i=1}^k \sum_{j=i+1}^{k-1} \beta_{ij} X_i X_j \quad (1)$$

In Equation 1, Y_n represents the response variables, with β_0 as the intercept term. The linear effects are represented by β_i , while β_{ij} and β_{ii} indicate interaction and quadratic effects, respectively. The variables X_i and X_j are input parameters, where k equals 3 (total number of variables). ANOVA helps determine regression coefficients for linear, interaction, and quadratic components. The relative importance of each factor is shown by the magnitude of standardized coefficients - larger values indicate greater influence. To help understand variable interactions and their impact on responses, 3-D surface plots complement the quadratic model visualization.

3.2.3. Investigation of combined enzyme-ultrasonic extraction techniques

Optimized parameters for ultrasonic treatment of roots in an aqueous medium (220W power, 52°C temperature, 8-minute duration) (Nguyen et al., 2024), were utilized, and three extraction methods combining enzyme treatment with ultrasonic techniques were evaluated: ultrasound treatment followed by enzyme incubation, simultaneous enzyme incubation with ultrasound treatment, and enzyme incubation followed by ultrasound treatment. Through these approaches, the extraction efficacy of TPC and TSC was assessed.

3.3. Analytical methods

Total phenolic content (TPC) and total saponin content (TSC) were quantified using spectrophotometric methods, as outlined by Nguyen et al.(2023).

3.3.1. Quantification of total phenolics

Total phenolic content (TPC) in XTP root extracts was measured using the Folin-Ciocalteu assay. The analysis began by combining 0.5 mL of extract (diluted with distilled water) with 2.5 mL of 10% (v/v) Folin-Ciocalteu reagent and leaving it for 6 mins to stabilize. Next, 2 mL of 7.5% (w/v) Na_2CO_3 solution was added followed by 2 mins of vortexing. After incubating the mixture at room temperature in dark conditions for 60 mins, absorbance readings were taken at 765 nm with an Agilent 8453 Spectrophotometer. The TPC values were calculated using a gallic acid calibration curve (Equation 2) and reported as mg gallic acid equivalent (GAE) per gram dry basis (d.b.).

$$TPC = \frac{A \times V_m \times d}{m \times (100 - W_m) \times 1000} \quad A = \frac{(D_m - D_0) - b}{a} \quad (2)$$

Where:

TPC: total polyphenol content (mg GAE/g d.b.)

A: gallic acid concentration from the standard equation

V_m : volume of extracted liquid (mL)

d: dilution factor

m: mass of the sample (g, dry matter)

W_m : moisture content of the sample (%)

D_m : OD of sample

D_0 : OD at the intercept point between the standard curve and the y-axis

a, b: are values of the equation $y = ax + b$ using gallic acid as a standard.

3.3.2. Quantification of triterpenoid saponins

Total saponin content (TSC) in XTP root extracts was quantified through a colorimetric assay. The procedure involved combining 0.5 mL of XTP sample with 0.5 mL of 8% (w/v) vanillin solution in a flask. Following this, 5 mL of 72% (v/v) H_2SO_4 solution was incorporated. After 2 mins of vortexing, the mixture underwent incubation at 70°C for 30 mins, followed by immediate cooling to room temperature in an ice water bath. The solution's absorbance was then evaluated at 560 nm using a UV-vis spectrophotometer. Using aescin as a reference standard, TSC was determined through Equation 3 and reported as mg of aescin equivalents (AE) per g of dried basis (d.b.).

$$TSC = \frac{C_x \times V_m \times d}{m \times (100 - W_m) \times 1000} \quad (3)$$

Where:

TSC: Triterpenoid saponin content (mg AE/g d.b.)

C_x : aescin concentration from the standard equation

d: dilution factor

V_m : volume of extracted liquid (mL)

W_m : moisture content of the sample (%) m: mass of the sample (g)

3.4. Statistical analysis

All experiments and measurements were performed three times. The data were analyzed statistically using JMP software (version 13 Pro), with results expressed as mean $\pm$ standard deviation. Significant differences between means were evaluated through analysis of variance (ANOVA) and least significant difference (LSD) tests, with statistical significance set at $p < 0.05$. Data visualization, including means and error bars, was accomplished using Microsoft Excel 2016.

4. Results and Discussion

4.1. Key parameters affecting enzymatic extraction efficiency

The study investigated five key variables that could affect extraction efficiency: pectinase-cellulase enzyme concentration (A), treatment duration (B), temperature (°C), water/material ratio (D), and pH (E). These variables were tested at two levels – low (-1) and high (+1), as shown in Table 4. The investigation employed a Plackett-Burman experimental design consisting of 12 trials testing 5 factors (Table 5). The primary objective was to identify which factors had the most significant impact on TPC (mg GAE/g d.b.), and TSC (mg AE/g d.b.).

Table 4. Influence coefficients of factors in the Plackett-Burman experiment

Coded factors	Variables	Low level (-1)	High level (+1)	Total phenolics (TPC)		Total saponins (TSC)	
				Effect coefficient	p-value (Prob>F)	Effect coefficient	p-value (Prob>F)
A	Pectinase-cellulase enzyme concentration (% v/w)	0.2	1.0	9.53*	<0.0001	2.56*	0.0431
B	Treatment duration (min)	60	180	2.84*	0.0295	5.19*	0.0020
C	Temperature (°C)	30	70	-6.26*	0.0008	-6.40*	0.0007
D	Water/material ratio (v/w)	10:1	80:1	-2.00	0.0924	-0.70	0.5097
E	pH	3.0	5.0	1.95	0.0994	0.87	0.4170

Significant at confidence level $\alpha = 0.05$

Table 5. Results of the Plackett-Burman experiment

Pattern	A	B	C	D	E	TPC (mg GAE/g d.b.)		TSC (mg AE/g d.b.)	
						Experimental value	Predicted value	Experimental value	Predicted value
0a0	0.2	60	30	80	3	7.42	7.56	83.6	83.9
---	0.2	60	70	10	3	7.34	7.29	82.4	81.3
--+	0.2	60	70	10	5	7.46	7.42	80.6	81.7
+--	0.2	180	30	10	5	7.95	7.99	87.6	87.1
+--	0.2	180	30	80	5	7.88	7.87	87.2	86.8
a00	0.2	180	70	80	3	7.42	7.35	82.7	83.4
00a	1.0	60	30	10	5	8.41	8.42	85.5	85.9
000	1.0	60	30	80	3	8.23	8.17	85.2	85.1
000	1.0	60	70	80	5	7.88	7.89	83.1	82.5
000	1.0	180	30	10	3	8.58	8.47	87.5	87.9
00A	1.0	180	70	10	3	7.92	8.08	85.0	84.9
A00	1.0	180	70	80	5	8.07	8.07	84.9	85.0

Results from Table 4 demonstrate that the experimental factors significantly influence TPC and TSC. A positive effect coefficient indicates that a factor has greater influence at its high level, while a negative coefficient shows greater influence at its low level. Analysis of the effect coefficients (based on their absolute values) identified three factors with the strongest impact at 95% confidence ($p < 0.05$): pectinase-cellulase enzyme concentration (A), treatment time (B), and temperature (°C). These three factors significantly affect TPC and TSC extraction efficiency and were therefore selected for the subsequent optimization. The water/material ratio (D) and pH (E) showed no significant effects. The relationships for TPC (Y_1) and TSC (Y_2) can be expressed as follows:

$$Y_1 \text{ (mg GAE/g d.b.)} = 7.578 + 0.754A + 0.002B - 0.010C - 0.002D + 0.062E \quad (4)$$

$$Y_2 \text{ (mg EE/g d.b.)} = 84.421 + 1.498A + 0.020B - 0.075C - 0.005D + 0.204E \quad (5)$$

4.2. Determining optimal enzyme-based extraction conditions for XTP roots

Three key factors identified through PBD analysis – pectinase-cellulase enzyme concentration (X_1), treatment temperature (X_2), and treatment time (X_3) – underwent further optimization using RSM-CCD. The study examined each variable at five coded levels ($-\alpha$, -1 , 0 , 1 , α), with all variables centered at a coded value of zero. The matrix for CCD, along with experimental and predicted results, is presented in Table 6.

Table 6. Experimental design matrix with coded variables

Variables	Coded factors	Encoding level				
		$-\alpha$	-1	0	1	α
Pectinase-cellulase enzyme concentration (% v/w)	X1	0.732	0.8	0.9	1.0	1.068
Treatment temperature ($^{\circ}\text{C}$)	X2	36.6	40	45	50	53.4
Treatment time (min)	X3	79.8	90	105	120	130.2

Table 7 shows the optimized extraction parameters obtained from the roots

Table 7. Impact of enzyme treatment parameters on TPC and TSC in root extracts

Run order	Pattern	X_1	X_2	X_3	TPC (mg GAE/g d.b.)		TSC (mg AE/g d.b.)	
					Experimental value	Predicted value	Experimental value	Predicted value
1	a00	0.732	45	105	10.59	10.55	101.6	100.7
2	---	0.8	40	90	10.13	10.16	117.2	114.4
3	--+	0.8	40	120	10.38	10.44	111.2	111.3
4	-+-	0.8	50	90	10.41	10.47	114.6	115.0
5	+++	0.8	50	120	10.36	10.34	112.3	115.7
6	0a0	0.9	36.6	105	10.24	10.16	124.3	128.6
7	00a	0.9	45	79.8	10.14	10.06	119.6	123.6
8	000	0.9	45	105	10.58	10.59	129.3	130.4
9	000	0.9	45	105	10.54	10.59	130.1	130.4
10	000	0.9	45	105	10.62	10.59	131.9	130.4
11	00A	0.9	45	130.2	10.21	10.18	126.7	123.7
12	0A0	0.9	53.4	105	10.46	10.43	133.6	130.2
13	+--	1	40	90	9.77	9.87	133.6	129.6
14	++-	1	40	120	10.11	10.13	130.1	129.0
15	++-	1	50	90	10.27	10.29	127.8	127.1
16	+++	1	50	120	10.09	10.15	128.3	130.4
17	A00	1.068	45	105	10.22	10.14	123.8	125.7

Results in Table 7 show that TPC in the extracts ranged from 9.77 - 10.62 mg GAE/g d.b. and TSC from 101.6 - 133.6 mg AE/g d.b. The highest TPC achieved was 10.62 mg GAE/g d.b. under conditions of 45°C temperature, 105 min duration, and 0.9% enzyme concentration. For TSC, the highest value reached was 133.6 mg AE/g d.b. with 105 min duration, 0.9%

enzyme concentration at 53°C. The influence of each factor: pectinase-cellulase enzyme concentration (X_1), temperature (X_2), and extraction time (X_3), as well as the interactions between these factors, were determined based on Table 8.

Table 8. Statistical Analysis of the RSM-CCD Model

Regression coefficients and statistical values	TPC			TSC		
	Regression coefficients	F-values	<i>p</i> -values	Regression coefficients	F-values	<i>p</i> -values
Constant						
X_0	-17.343		<0.001*	-539.447		<0.0001*
Linear						
X_1	11.955	29.1232	0.0010*	1193.995	51.1234	0.0002*
X_2	0.486	12.9873	0.0087*	1.354	0.2111	0.6598
X_3	0.222	2.4061	0.1648	1.261	0.0021	0.9646
Quadratic						
X_{11}	-8.499	11.6876	0.0112*	-608.962	28.0958	0.0011*
X_{22}	-0.004	17.6618	0.0040*	-0.014	0.0899	0.7730
X_{33}	-0.001	44.8466	0.0003*	-0.010	4.3108	0.0765
Interaction						
X_{12}	0.055	0.8708	0.1648	-1.525	0.3135	0.5930
X_{13}	-0.003	0.0288	0.3818	0.432	0.2260	0.6490
X_{23}	-0.001	12.0974	0.8701	0.013	0.5047	0.5004
R^2	0.9395			0.9221		
<i>p</i> -values	0.0017*			0.0039*		

*Statistically significant with $p < 0.05$.

Analysis of variance results in Table 8 indicates that the regression model is statistically significant ($p < 0.05$), with correlation coefficients R^2 of 0.9395 for TPC and 0.9221 for TSC. This demonstrates strong compatibility between the experimental and predicted models. The quadratic polynomial equations predicting TPC (Y_1) and TSC (Y_2) are:

$$Y_1 = -17.343 + 11.955X_1 + 0.486X_2 - 8.499X_1^2 - 0.004X_2^2 - 0.001X_3^2 \quad (6)$$

$$Y_2 = -539.447 + 1193.995X_1 - 608.962X_1^2 \quad (7)$$

Where Y_1 and Y_2 represent TPC (mg GAE/g d.b.) and TSC (mg AE/g d.b.), respectively; X_1 is the pectinase-cellulase enzyme concentration (%v/w); X_2 is the treatment temperature (°C); and X_3 is the time (min).

The significance of each coefficient is determined by its p -value – lower p -values indicate higher significance. The results reveal that first-order terms for enzyme concentration (X_1) and temperature (X_2) significantly affect TPC ($p < 0.05$), while extraction time (X_3) shows no significant effect. For TSC, only the enzyme concentration among first-order terms shows significance ($p = 0.0002$). The quadratic terms of all three factors significantly influence TPC ($p < 0.0001$), with the enzyme concentration showing the strongest effect. For TSC extraction efficiency, only the quadratic term of enzyme concentration shows a significant impact ($p = 0.0011$, regression coefficient -608.962). These results indicate that extraction outcomes are significantly influenced by factor combinations, with enzyme concentration being the primary driver for both TPC and TSC extraction efficiency. Temperature and time only influence TPC extraction. Notably, no significant interaction effects were observed for extraction yield in XTP root parts.

The three-dimensional response surface plots shown in Figure 1 demonstrate the relationships between variables and their effects on the extraction efficiency from XTP roots.

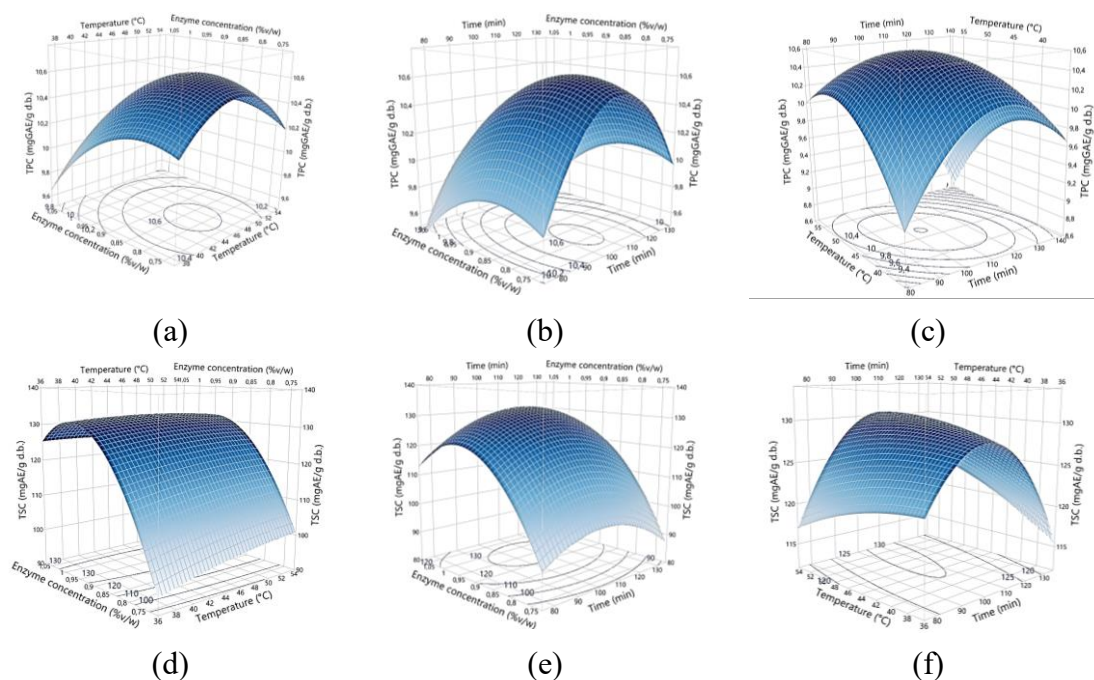


Figure 1. Response surface plots of (a-c) TPC and (d-f) TSC of extracts from XTP roots

The response surface plots in Figure 1 illustrate the effects of enzyme concentration, temperature, and extraction time on TPC and TSC. As shown in Figure 1 (a–c), TPC is strongly influenced by enzyme concentration (F-value of 29.1232) and temperature ($F = 12.9873$), with maximum values observed at approximately 0.8–0.9% enzyme concentration, 44–48°C, and 100–115 mins. In contrast, the response surfaces for TSC (Figure 1 (d–f)) indicate that enzyme concentration is the dominant factor ($F = 51.1234$), while temperature and time have relatively weaker effects. These extraction conditions facilitated cell wall disruption in the presence of enzymes, enhancing solvent penetration into cells and releasing bioactive compounds, which increased extraction yield (Meyer et al., 1998).

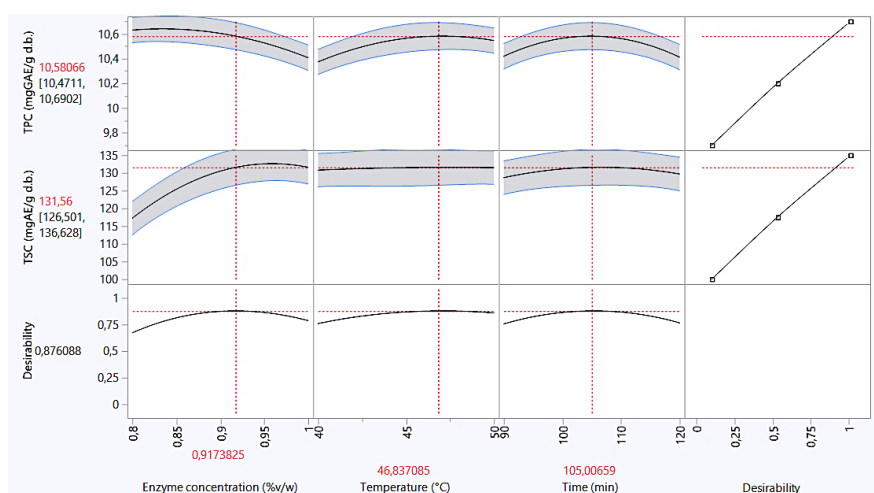


Figure 2. Optimization of factors influencing the root extraction of TPC and TSC

The optimization results (Figure 2) indicate a compromise optimum at 47°C, which differs slightly from the temperature associated with the maximum TSC observed in Table 7. Although the highest TSC was recorded at 53°C, this increase is not statistically significant and is not accompanied by a corresponding improvement in TPC. In contrast, TPC exhibits a clear optimum at lower temperatures (approximately 45°C). Therefore, the final optimal condition was determined based on simultaneous optimization of both responses, where a moderate temperature (47°C) ensures near-maximal values for both TPC and TSC. This difference reflects the distinct sensitivity of the two responses to temperature, with TPC being more temperature-dependent than TSC. The TPC and TSC obtained were 10.58 ± 0.11 mg GAE/g d.b. and 131.56 ± 5.07 mg AE/g d.b., respectively, with optimal enzyme concentration, temperature, and extraction time of 0.91%, 47°C, and 105 mins.

To confirm the model's accuracy, three independent replications were performed to measure TPC and TSC under optimal conditions. The actual TPC and TSC obtained in the extract under real conditions were 10.53 ± 0.18 mg GAE/g d.b. and 132.93 ± 1.78 mg AE/g d.b. Therefore, the actual experimental values were similar to the predicted values from the equation. When comparing extraction yields, enzyme-treated samples showed a 43.5% increase in TPC and 52.9% increase in TSC compared to untreated samples (TPC 7.34 ± 0.33 mg GAE/g d.b., TSC 86.96 ± 5.42 mg AE/g d.b., respectively).

4.3. Evaluating water-based extraction using enzyme-ultrasound combination methods

The optimized parameters for enzyme extraction from XTP roots (0.91% pectinase-cellulase enzyme concentration (1:1), 47°C treatment temperature, 105-min treatment time), and ultrasound-assisted extraction (220W power, 50°C temperature, 8-minute duration) (Nguyen et al., 2024), were selected to extract TPC and TSC using different methods: enzyme treatment (EAE), ultrasound (UAE), ultrasound before enzyme (U-EAE), simultaneous ultrasound and enzyme (UES-AE), enzyme before ultrasound (E-UAE) and no treatment (CONTROL). The TPC and TSC of the extracts from XTP roots are shown in Figure 3.

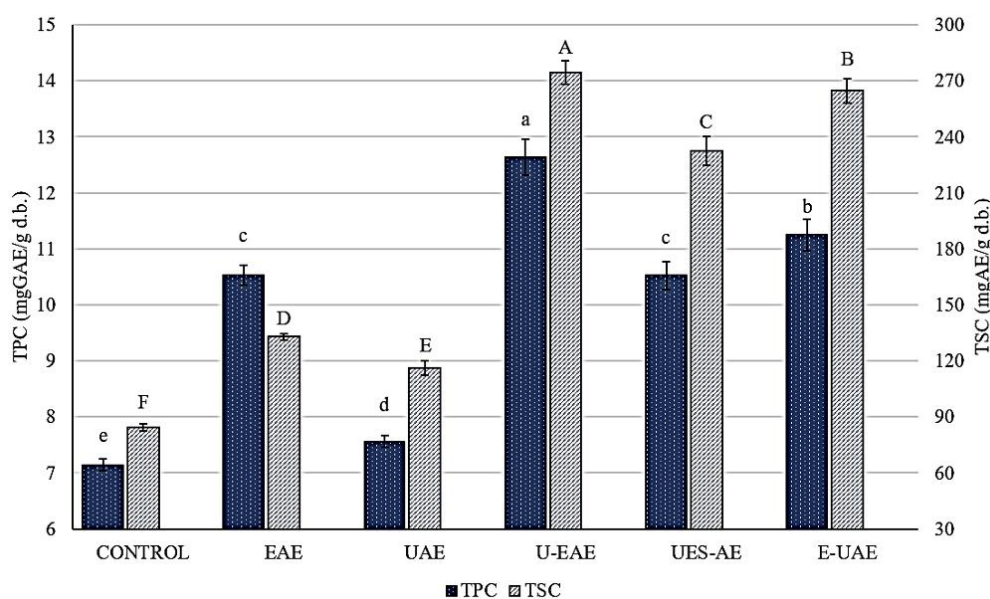


Figure 3. Comparison of TPC and TSC from XTP roots using various extraction techniques

(The letters on the columns respectively indicate statistically significant differences at $p \leq 0.05$ of the control sample and the treated sample, $n = 3$)

Figure 3 demonstrates that XTP root extracts using enzyme treatment, ultrasound, or combined enzyme-ultrasound methods all yielded significantly higher TPC and TSC compared to the untreated sample ($p < 0.05$).

The enhanced extraction efficiency observed for the combined ultrasound–enzyme treatments can be attributed to the complementary mechanisms of enzymatic hydrolysis and ultrasonic cavitation. The root matrix of *P. trimera* is rich in structural polysaccharides, including cellulose and pectin, which form a rigid cell-wall network that limits the release of bound phenolics and saponins. The use of cellulase and pectinase facilitates the hydrolysis of these polysaccharide components, thereby loosening the cell-wall structure and promoting the release of intracellular and cell wall–associated compounds.

Ultrasound treatment further enhances this process through cavitation-induced microstreaming, shear forces, and localized cell disruption, which improve solvent penetration and increase enzyme–substrate contact. This synergistic effect is particularly evident in the U–EAE sequence, where preliminary ultrasound treatment may increase substrate accessibility, allowing subsequent enzymatic hydrolysis to proceed more efficiently.

When enzyme and ultrasound treatments were combined for XTP root, the resulting TPC and TSC exceeded those achieved by either method alone. The U-EAE method (TPC 12.63 ± 0.32 mg GAE/g d.b.; TSC 274.46 ± 6.22 mg AE/g d.b.) and E-UAE method (TPC 11.25 ± 0.29 mg GAE/g d.b.; TSC 264.56 ± 6.52 mg AE/g d.b.) both outperformed individual UAE (TPC 7.56 ± 0.35 mg GAE/g d.b.; TSC 116.24 ± 4.41 mg AE/g d.b.) or EAE treatments (TPC 10.53 ± 0.18 mg GAE/g d.b.; TSC 132.93 ± 1.78 mg AE/g d.b.). Similarly, Van Thanh et al. (2023) found that combined ultrasound–enzyme methods (U-EAE or E-UAE) extracted significantly more phenolics, flavonoids, and catechins from cashew nut shells than single methods (EAE or UAE).

In contrast, the relatively lower performance observed for the simultaneous ultrasound–enzyme treatment (UES-AE) (TPC 10.52 ± 0.25 mg GAE/g d.b.; TSC 232.50 ± 7.67 mg AE/g d.b.) may be related to the potential influence of ultrasonic energy on enzyme activity. However, given the absence of direct measurements of enzyme stability under the applied conditions, this effect should be interpreted with caution. It is also possible that competing physical and biochemical processes during simultaneous treatment reduce the overall efficiency compared to sequential approaches.

Results from combined enzyme–ultrasound extraction methods revealed that U-EAE treatment yielded the highest TPC and TSC extraction efficiency, showing 1.77 and 3.25-fold increases, respectively, compared to untreated samples (CONTROL). Gao et al. (2022) demonstrated that ultrasound-assisted enzymatic extraction (U-EAE) effectively extracted TPC from *Empetrum nigrum*, with TPC yields 1.62 and 1.73 times higher than using ultrasound-assisted extraction (UAE) or enzyme-assisted extraction (EAE) alone. Similarly, Zhao et al. (2023) showed that extraction using cellulase combined with ultrasonic waves was the most effective. Ultrasound enhanced the binding between cellulase and substrate, increasing their affinity and promoting compound dissolution – improving efficiency compared to traditional and individual methods. This enhancement likely stems from the synergistic effect of ultrasound and enzymes: ultrasonic waves

create cavitation, turbulence, and compound fragmentation, breaking particles into smaller sizes that enable more effective enzyme catalysis with substrates during extraction.

All figures and tables in this article are the results of the authors' own research.

5. Conclusion

This study successfully optimized enzyme-assisted extraction (EAE) conditions for recovering phenolics and saponins from *Paramignya trimera* roots using response surface methodology. The optimized parameters significantly improved extraction efficiency, and the integration of ultrasound, particularly in the U–EAE sequence, further enhanced the recovery of bioactive compounds through a synergistic effect.

From a practical perspective, the optimized aqueous EAE combined with ultrasound offers a promising green extraction strategy with reduced solvent use and improved efficiency, which may be applicable for the development of functional ingredients from medicinal plant materials.

However, several limitations should be noted. The study focused on total phenolic and saponin contents, and further work is needed to characterize individual compounds and their bioactivities. In addition, validation at pilot or industrial scale, as well as economic feasibility and enzyme cost considerations, should be addressed before practical implementation.

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