Optimization of Phenolic Compound Extraction from Supercritical Fluid Extraction Residues of *Fraxinus mandshurica* Leaf and Evaluation of Anti-inflammatory Activity

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This study investigated the optimization of phenolic compound recovery from *Fraxinus mandshurica* leaves residues generated by supercritical fluid extraction (SFE) to promote the sustainable use of resources. Using Box–Behnken design under response surface methodology, the optimal extraction conditions were determined to be an extraction temperature of 64.3 °C, an extraction time of 12.6 h, and an ethanol concentration of 76.3%. Under these conditions, the experimental yields of total polyphenol content and total flavonoid content were 63.41 ± 3.05 mg GAE/g and 5.12 ± 0.12 mg QE/g, respectively. These results showed high reliability, with relative errors below 10%. Biological analysis using RAW 264.7 cells revealed that the optimized extract exhibited non-toxicity up to a concentration of 125 μg/mL. At a treatment concentration of 62.5 μg/mL, it inhibited the production of nitric oxide and tumor necrosis factor-alpha (TNF-α) 38.0% and 15.8%, respectively, under inflammatory conditions induced by lipopolysaccharide (LPS). These results demonstrate that SFE residues from *F. mandshurica* leaves, which were previously considered waste, can be effectively upcycled into high-value functional materials for use in the pharmaceutical and food industries.

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INTRODUCTION

Fraxinus mandshurica Rupr. (Manchurian ash), a deciduous broad-leaved tree belonging to the Oleaceae family, is widely distributed across East Asia, including China, Japan, and Korea. According to the Chinese Compendium of Materia Medica (Ben Cao Gang Mu), *F. mandshurica* has long been utilized as a traditional medicinal resource.

The leaves of *F. mandshurica* are rich in bioactive phenolic compounds, such as kaempferol, quercetin, and rutin, making them valuable raw materials for the food and pharmaceutical industries (Guo *et al.* 2024). Previous studies have reported various physiological benefits of these extracts; for instance, Jeon and Choi (2007) demonstrated that *F. mandshurica* leaf extract exhibits hepatoprotective effects by reducing acetaminophen-induced hepatotoxicity. Furthermore, phenolic compounds derived from the leaves have shown significant antioxidants (Hidalgo *et al.* 2010) and anti-allergic activities (Kawai *et al.* 2007). Additionally, Sarfraz *et al.* (2017) reported anti-

inflammatory effects mediated by the inhibition of nitric oxide (NO) and tumor necrosis factor-alpha (TNF-α) enzymes.

With the growing consumer preference for natural ingredients, there is increasing interest in sustainable and eco-friendly production methods (Shrivastav *et al.* 2025). Supercritical Fluid Extraction (SFE), which utilizes CO_2 as a solvent, is a representative green technology that minimizes contamination (Luca *et al.* 2022). The SFE process is classified as a "green chemistry" process in the pharmaceutical and food industries, and its application and importance continue to increase (Perrut 2000; Reverchon and De Marco 2006). Consequently, the volume of SFE residues generated following this process also increases significantly each year.

The SFE primarily employs non-polar gaseous CO_2, making it highly effective for extracting non-polar compounds (Uwineza and Waśkiewicz 2020). Indeed, SFE has been extensively used for the extraction of fats, oils, and lipophilic carotenoids (Durante *et al.* 2014; Li *et al.* 2022). These characteristics of the SFE process suggest that a substantial number of phenolic compounds may remain unextracted in the solid residues. This indicates that these residues represent a valuable and underutilized source of phenolic compounds. For example, Thai *et al.* (2024) compared extractions of coffee pulp powder and found a substantial difference in total polyphenol content between SFE (0.11 mg GAE/g) and 80% ethanol (EtOH) extraction (18.00 mg GAE/g). To address this, researchers have incorporated organic co-solvents, such as EtOH, into the SFE process. Cuéllar *et al.* (2024) successfully extracted phenolic compounds (1.69 ± 0.02 g GAE/kg substrate) from *Berberis microphylla* G. Fort using EtOH-modified SFE. However, the efficiency remained moderate compared to Soxhlet extraction. In this context, it is hypothesized that a significant number of phenolic compounds remains in the residues after SFE processing (Tyśkiewicz *et al.* 2018). Paula *et al.* (2013) successfully recovered phenolic compounds from the SFE residues of *Arrabidaea chica* leaves, achieving higher yields than single-solvent extraction methods. Therefore, this study focused on the extraction of residual phenolic compounds from SFE residues using suitable co-solvents such as ethanol, positioning these residues as a promising and largely untapped source of bioactive compounds

Phenolic compounds can be extracted through various processes, with the choice of method aiming to minimize degradation and denaturation. The most common method involves polar organic solvents, such as EtOH and methanol, which are reported to be effective for extracting high quantities of phenolics. Xiang *et al.* (2024) optimized the extraction of total polyphenols from *Cleome polyodonta* residues using 70% methanol. Similarly, Klongdee and Klinkesorn (2022) extracted 333.01 ± 5.84 mg GAE/g of total polyphenols from Rambutan peel using 54% EtOH.

To maximize the recovery of phenolic compounds, it is crucial to optimize process variables such as solvent concentration, extraction temperature, and time (Jung *et al.* 2017; Cheaib *et al.* 2018), as these factors significantly influence extraction efficiency (Bhebhe *et al.* 2016). Response Surface Methodology (RSM) is a widely used statistical technique for optimizing such processes (Weremfo *et al.* 2023). Through ANOVA and second-order polynomial equations, RSM allows for the evaluation of interactions between variables and the determination of optimal conditions (Khuri and Mukhopadhyay 2010). Despite limitations in predicting results outside the experimental range, RSM has been successfully applied to optimize extraction processes for various plant materials (Asoo 2024). However, systematic research on extraction optimization and bioactivity verification for residues following the SFE process remains limited.

The primary objective of this study was to determine the optimal extraction conditions to maximize the efficiency of recovering phenolic compounds from the residues of *F. mandshurica* leaves after SFE processing. EtOH solvent extraction was employed, and the phenolic content of the optimized residues extract was compared with that of the SFE extract. The optimal conditions (extraction time, temperature, and EtOH concentration) were determined using RSM. Finally, the anti-inflammatory efficacy of the extract obtained under optimal conditions was analyzed by measuring NO and TNF-*α* production.

EXPERIMENTAL

Materials

All analytical grade reagents, including Folin-Ciocalteu reagent, sodium carbonate, aluminum chloride, methanol and ethanol, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Standard compounds (gallic acid and quercetin), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent, and lipopolysaccharide (LPS) were also obtained from Sigma-Aldrich. For cell culture and sample preparation, Dulbecco's Modified Eagle Medium (DMEM), penicillin-streptomycin, and phosphate-buffered saline (PBS) were obtained from Gibco (Grand Island, NY, USA), while fetal bovine serum (FBS) was supplied by Sigma-Aldrich. The Griess Reagent System and the mouse TNF-α ELISA kit were purchased from Invitrogen (Thermo Fisher Scientific, Inc., Frederick, MD, USA, and Vienna, Austria, respectively).

SFE Residues Collection

The leaves of *F. mandshurica* (moisture content: 10.57%) were purchased from a local market in Sancheong, Republic of Korea. Without prior grinding, 200 g of the raw leaves were loaded into the extraction vessel and subjected to supercritical fluid extraction using an SFE system (ISA-SCCO-S-1000-500, Ilshin Autoclave, Daejeon, Republic of Korea).The SFE was performed at 40 °C and 250 bar for 120 min, with a CO_2 flow rate of 2 L/min, following a slightly modified protocol of Rosa *et al.* (2019). The resulting SFE residues were pulverized into a fine powder using a commercial grinder and sieved through a 60-mesh screen. The powdered residues were stored at 4 °C until further analysis.

Preparation of Extracts

The SFE residues were pulverized into a fine powder using a commercial grinder and stored at 4 °C. Briefly, 0.5 g of the powdered sample was accurately weighed into a 15 mL conical tube, followed by the addition of 10 mL of ethanol-water solution. The ethanol-water solution was prepared at varying ethanol concentrations of 50, 75, and 99% (v/v), with distilled water constituting the remainder of the solvent composition, in accordance with the experimental design outlined in Table 1.The mixture was vigorously vortexed for 5 s. Extraction was carried out using a water bath (WB-20E, Jeio Tech, Daejeon, Korea) at various temperatures and time intervals according to the experimental design.

After extraction, the mixture was centrifuged at 4,000 rpm for 10 min (Union 32R, Hanil Scientific Inc., Gimpo, Korea). The resulting supernatant was collected and stored at 4 °C, with all analyses completed within one week. For cell-based biological assays, including the cytotoxicity assay (MTT assay), cell viability assay under LPS stimulation, nitric oxide (NO) production assay, and TNF-α quantification assay (ELISA), the extract

obtained under optimized conditions was lyophilized, and the resulting powder was dissolved in PBS at the required concentrations before being applied to the culture medium.

Experimental Design and Statistical Optimization

The optimization of the extraction process was performed using Box-Behnken Design (BBD), a type of RSM, to determine the optimal conditions for maximizing phenolic compound recovery. The experimental design consisted of three independent variables tested at three levels, coded as −1, 0, and +1. The independent variables selected were extraction temperature (A), extraction time (B), and EtOH concentration (C). The effects of these variables on two dependent variables, total polyphenol content (TPC) and total flavonoid content (TFC), were investigated. The experimental data was fitted to a second-order polynomial equation to correlate the relationship between the independent variables and the responses. The coded and actual levels of independent variables are outlined in Table 1.

Table 1. Independent Variables and Their Levels Used in the BBD

Independent Variables	Symbols	Coded Level		
		-1	0	+1
Extraction Temperature (°C)	A	50	60	70
Extraction Time (h)	B	9	12	15
EtOH concentration (%)	C	50	75	99

Determination of Total Polyphenol Content

The total polyphenol content (TPC) of the extract was determined using a modified microplate method described by Attard (2013). Briefly, 20 µL of the extract and 20 µL of Folin-Ciocalteu reagent were added to each well of a microplate and allowed to react at room temperature for 5 min. Subsequently, 200 µL of 7% sodium carbonate (Na_2CO_3) solution and 10 µL of distilled water were added, and the mixture was incubated in the dark at room temperature for 2 h. The absorbance was measured at 750 nm using a microplate reader (SpectraMax 190, Molecular Devices, USA). Prior to measurement, all extracts were diluted 20-fold with distilled water. TPC was expressed as milligrams of gallic acid equivalents per gram of raw sample (mg GAE/g) using a gallic acid calibration curve ($y = 3.668x + 0.1264$, $R^2 = 0.9994$), where y is the absorbance at 750 nm and x is the gallic acid concentration (mg/mL). TPC was calculated as follows Eq (1):

$$\text{TPC (mg GAE/g)} = (C \times V \times 20) / (W) \quad (1)$$

where C is the concentration derived from the calibration curve (mg/mL), V is the total extract volume (mL), 20 is the dilution factor, and W is the dry weight of the sample (g).

Determination of Total Flavonoid Content

The TFC was quantified using a microplate method described by Sari *et al.* (2023). In each well of a microplate, 50 µL of the extract was mixed with 100 µL of methanol and 10% aluminum chloride ($AlCl_3$) reagent. The mixture was allowed to react at room temperature for 3 min. Subsequently, 20 µL of 1 M sodium acetate (CH_3COONa) solution and 60 µL of methanol were added. The mixture was then incubated in the dark at room temperature for 40 min. Absorbance was measured at 430 nm using a microplate reader

(SpectraMax 190, Molecular Devices, USA). TFC was expressed as milligrams of quercetin equivalents per gram of raw sample (mg QE/g) using a quercetin calibration curve ($y = 5.3326x + 0.218$, $R^2 = 0.9955$), where y is the absorbance at 430 nm and x is the quercetin concentration (mg/mL). No dilution was applied prior to measurement. TFC was calculated as follows Eq (2):

$$TFC\ (mg\ QE/g) = (C \times V) / (W) \quad (2)$$

where C is the concentration derived from the calibration curve (mg/mL), V is the total extract volume (mL), and W is the dry weight of the sample (g).

RAW 264.7 Cell Culture

RAW 264.7 cells were obtained from the Korean cell line bank (KCLB No. 40071). Cells were grown in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin and incubated at 37 °C under a 5% CO_2 atmosphere (Kim *et al.* 2010).

Cytotoxicity Assay

The cytotoxicity of the *F. mandshurica* extract on RAW 264.7 cells was evaluated using the MTT colorimetric assay (Gilmore *et al.* 2018). Cells were seeded in a microplate at a density of 5×10^3 cells/well and incubated for 24 h. The medium was then replaced with serum-free medium containing the extract at concentrations of 62.5, 125, 250, and 500 µg/mL, and the cells were incubated for another 24 h. After incubation, the medium was replaced with MTT reagent (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma Chemical Co.) and incubated for 3 h at 35 °C in a 5% CO_2 incubator to allow formazan crystal formation. The medium was removed, and 200 µL of dimethyl sulfoxide (DMSO) was added to dissolve the insoluble formazan crystals. Cell viability was determined by measuring the absorbance at 540 nm using a microplate reader (SpectraMax 190, Molecular Devices, USA).

Evaluation of Cell Viability under LPS Stimulation

To evaluate the effect of the extract on cells under inflammatory stress, cell viability was measured in the presence of lipopolysaccharide (LPS). RAW 264.7 cells were seeded at 5×10^3 cells/well and incubated for 24 h. The cells were treated with the extract (62.5, 125, 250, and 500 µg/mL) in serum-free medium and incubated for 3 h. Subsequently, LPS was added at concentrations of 2.5, 5, and 10 µg/mL, and the cells were incubated for 21 h. After incubation, cell viability was assessed using the MTT assay as described above, with absorbance measured at 540 nm.

Measurement of Nitric Oxide Production

The NO concentration in the culture supernatant was measured as nitrite using the Griess reagent method (Nagano 1999). RAW 264.7 cells were pre-treated with the extract for 3 h, followed by stimulation with 10 µg/mL LPS for 21 h. A mixture of 150 µL of the culture supernatant, 130 µL of distilled water, and 20 µL of Griess reagent was incubated at room temperature in the dark for 30 min. The absorbance was measured at 540 nm using a microplate reader. NO concentration was calculated using a standard curve prepared with sodium nitrite.

Measurement of TNF-α Production

The concentration of TNF-α in the culture supernatant was determined using a Mouse TNF-α ELISA Kit following the manufacturer's instructions. Briefly, RAW 264.7 cells were pre-treated with the extract for 3 h, followed by stimulation with 10 µg/mL LPS for 21 h. After incubation, 50 µL of blanks, standards, and samples were added to the wells of a microplate and incubated at room temperature for 2 h. The wells were then washed six times with phosphate buffer, after which 100 µL of Streptavidin-HRP was added and incubated at room temperature for 1 h. Following six additional washes with phosphate buffer, 100 µL of TMB Substrate Solution was added to each well and incubated at room temperature for 30 min. The reaction was terminated by adding 100 µL of Stop Solution. The absorbance was measured at 450 nm using a microplate reader (SpectraMax 190, Molecular Devices, USA), and the TNF-α concentration was calculated using a standard curve.

Statistical Analysis

All statistical analyses and experimental design optimizations were performed using Design-Expert 13 software (Stat-Ease Inc., Minneapolis, MN, USA). Additionally, GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA) was utilized for statistical comparisons and biological data analysis. All experiments were conducted in triplicate, and the data are presented as the mean ± standard deviation.

RESULTS AND DISCUSSION

Effects of Various Experimental Variables on the Response

The BBD was applied under various conditions of temperature, extraction time, and EtOH concentration, as presented in Table 2. The statistical analysis indicated that these experimental variables exerted a significant impact on the yields of TPC and TFC. The observed values ranged from 12.95 to 66.10 mg GAE/g for TPC and from 0.57 to 5.15 mg QE/g for TFC.

Table 2. Experimental Conditions of the BBD and Corresponding Results Obtained for TPC, TFC

Run	Extraction Temperature (°C)	Extraction Time (h)	EtOH Concentration (%)	TPC (mg GAE /g)	TFC (mg QE /g)
1	70	12	50	66.10	4.19
2	60	12	74.5	65.50	5.15
3	60	15	50	59.99	3.53
4	70	15	74.5	61.31	5.39
5	70	9	74.5	55.51	2.78
6	60	12	74.5	65.50	5.15
7	60	15	99	38.03	3.95
8	50	12	50	12.95	3.15
9	50	15	74.5	25.70	4.39
10	60	9	50	62.25	0.57
11	50	9	74.5	24.14	2.17
12	70	12	99	30.56	4.34
13	60	9	99	25.85	1.20
14	60	12	74.5	65.50	5.15
15	50	12	99	15.40	3.55

Table 3 shows that the quadratic model used for TPC prediction was statistically significant ($p < 0.05$). Specifically, the linear coefficients (A, B, C), interaction effects (AC) and quadratic coefficients (B^2, C^2) were significant, whereas the interaction effects (AB, BC) were not ($p > 0.05$). A regression equation was derived to demonstrate the significance of the model (Eq. 3). Furthermore, the high determination ($R^2 = 0.99$), adjusted R^2 (0.98), coefficient of variation (CV = 4.51), and the lack of fit value (26.98) confirm that the mathematical model in Eq. 3 is suitable for predicting TPC across various experimental conditions.

In practice, Fig. 1(A) demonstrates that mild temperature conditions contribute to the softening of plant tissues and the hydrolysis of bonds between phenolic compounds and cellular components such as phenol–polysaccharide complexes. Solvent diffusion occurs in accordance with Fick's law, driven by the solvent concentration gradient (Yang *et al.* 2015). Therefore, excessively high solvent concentrations may contribute to the saturation of the reaction medium and reduced diffusion rates.

Regarding the TFC the analysis of variance (ANOVA, $p < 0.05$) revealed that the model, linear coefficients (A, B, C) and quadratic coefficients (B^2, C^2) were significant, while the other variables were not (Table 3). Additionally, the estimated model showed a significant correlation coefficient ($R^2 = 0.98$) and a non-significant lack of fit (0.0451), suggesting that the mathematical model in Eq. 4 is suitable for predicting TFC.

Total Polyphenol Content (mg GAE/g) =

$$65.5001 + 16.9109\times A + 2.15916\times B \pm 11.4302\times C + 1.0569\times AB - 9.49685\times AC + 3.60937\times BC - 19.5556\times A^2 - 4.27401\times B^2 - 4.688\times C^2 \quad (3)$$

Total Flavonoids Content (mg QE/g) =

$$5.15295 + 0.429385\times A + 1.31838\times B + 0.20213\times C + 0.098045\times AB - 0.0623254\times AC - 0.0508777\times BC + 0.0155505\times A^2 - 1.47978\times B^2 - 1.3565\times C^2 \quad (4)$$

where A is the extraction temperature (°C), B is the extraction time (h), and C is the EtOH concentration (%).

Table 3. Analysis of Variance of the Quadratic Models for Total TPC and TFC Values

	TPC (mg GAE/g)		TFC (mg QE/g)	
	F-value	*p*-value	F-value	*p*-value
Model	40.21	0.0004	122.27	< 0.0001
A-Extraction Temperature	141.34	< 0.0001	54.52	0.0007
B-Extraction Time	2.30	0.1895	513.99	< 0.0001
C-EtOH Concentration	64.57	0.0005	12.08	0.0177
AB	0.2760	0.6218	1.42	0.2867
AC	22.29	0.0052	0.5743	0.4827
BC	3.22	0.1327	0.3827	0.5632
A²	87.23	0.0002	0.0330	0.8630
B²	4.17	0.0967	298.87	< 0.0001
C²	49.21	0.0009	251.14	< 0.0001
Lack of Fit	26.98 (not significant)		0.0451 (not significant)	
C.V. %	8.95		4.51	
R^2	0.9864		0.9955	
Adjusted R^2	0.9618		0.9873	

Figures 1 and 2 present 3D response surface plots illustrating the effects of extraction conditions (temperature, time, and EtOH concentration) on TPC and TFC. The analysis revealed that TPC and TFC exhibited highly comparable behavior across all independent variable conditions.

While TPC and TFC exhibited broadly comparable trends across most of the range of extraction conditions, a notable discrepancy was observed with respect to extraction time. The effect of extraction time on TFC was highly significant ($p < 0.0001$), but it was non-significant for TPC ($p = 0.1895$). This contrasting behavior may be attributed to the structural and physicochemical differences between the phenolic fractions contributing to each response variable. TPC encompasses a wide range of phenolic compounds, including free phenolic acids and simple polyphenols. These compounds exist predominantly in free or soluble-conjugated forms, and thus, they are readily extractable within a relatively short time (Acosta-Estrada *et al.* 2014). In contrast, flavonoids frequently occur in glycosylated or esterified bound forms within the plant cell matrix, and their complete release is likely governed by the time (dependent hydrolysis of glycosidic bonds and the slower diffusion kinetics of flavonoid) matrix complexes (Shahidi and Yeo 2016; Li *et al.* 2024). These mechanistic differences between freely soluble and matrix-bound phenolic fractions provide a plausible explanation for the higher time dependence observed for TFC compared to TPC.

Regarding the interaction between extraction temperature and time, both response variables initially showed an increasing trend in yield as temperature rose and time elapsed. This phenomenon is attributed to the reduction in solvent viscosity and the increase in diffusion coefficients at higher temperatures within the static environment of the water bath, which facilitated the elution of bioactive compounds from the plant cell matrix (Pompeu *et al.* 2009). However, when conditions exceeded the critical thresholds of 65 °C and 13 h, the contents of both TPC and TFC declined concurrently. This reduction is likely caused by excessive thermal energy, which accelerates the oxidation and thermal degradation of the thermosensitive phenolic and flavonoid skeletons (Deorukhkar and Ananthanarayan 2021).

EtOH concentration also demonstrated a similar optimal range for both groups. TPC and TFC reached their maximum values in the 70 to 80% EtOH concentration range, suggesting that the polarity of the binary solvent system formed at this ratio was most suitable for dissolving the target compounds. Conversely, yields significantly decreased at high EtOH concentrations (> 99%), which is consistent with the findings of Ismail-Suhaimy *et al.* (2021). Similarly, a study on *Rosmarinus tournefortii* extracts reported a maximum TFC (21.38 mg QE/g) at 80% EtOH, with a decreasing trend at higher concentrations (Ziani *et al.* 2023). This decline can be interpreted as the result of the absence of water, which plays a crucial role in inducing sample swelling and reducing mass transfer resistance; consequently, the solvent failed to sufficiently penetrate the plant tissue (Gil-Martín *et al.* 2022). In a comparable study, Hou *et al.* (2016) achieved optimized yields of 88.6 mg GAE/g for TPC and 19.4 mg/g (Rutin equivalents) for TFC from *Melaleuca bracteata* leaves using RSM within the ranges of 40 to 70 °C and 20 to 80% EtOH, which aligns with the experimental range of this study. In conclusion, this study confirmed the existence of optimal extraction conditions capable of simultaneously maximizing the extraction efficiency of both TPC and TFC within the experimental range.

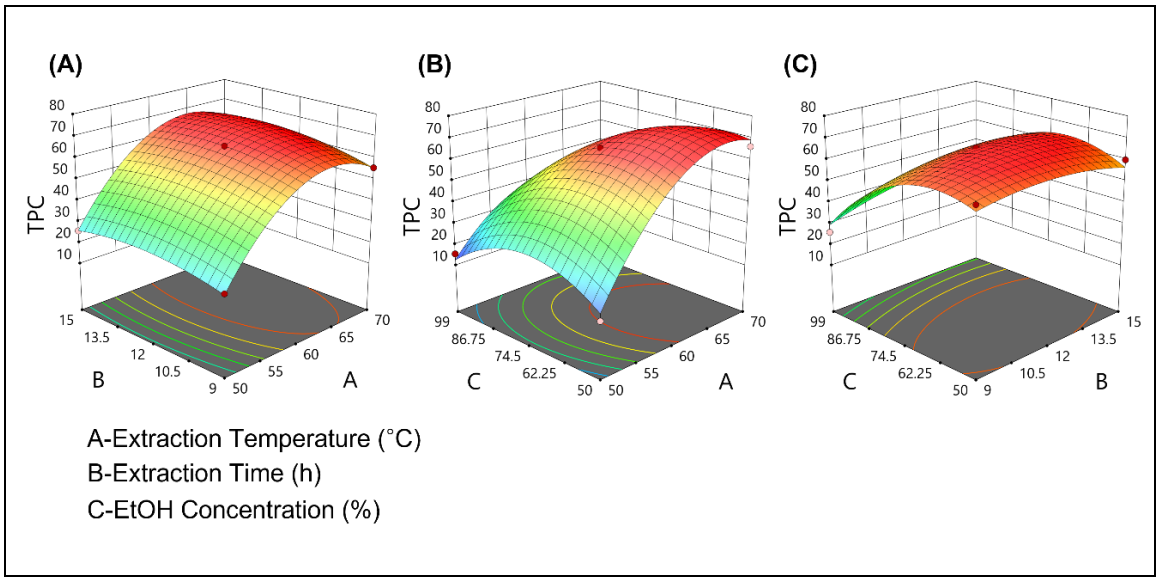

Fig. 1. Response and contour plots illustrate the effect of extraction temperature (A), extraction time (B), and EtOH concentration (C) on TPC

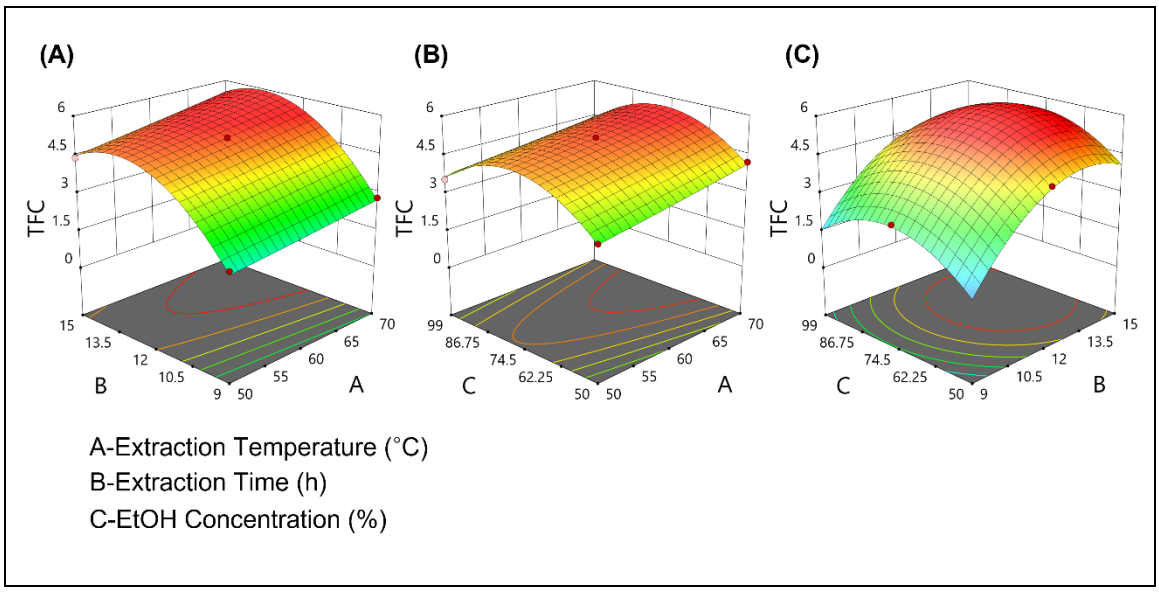

Fig. 2. Response surface plots illustrate the effects of extraction temperature (A), time (B), and EtOH concentration (C) on TFC

Optimal Condition of Extractions Variables

Based on the previously derived quadratic regression models, numerical optimization was performed using a desirability function to simultaneously maximize the yields of TPC and TFC (Fig. 3).

The extraction conditions optimized for simultaneously maximizing the yields of total polyphenol content (TPC) and total flavonoid content (TFC) from the SFE residues of *F. mandshurica* leaves, as derived from the Box-Behnken Design (BBD), were determined to be an extraction temperature of 64.3 °C, a time of 12.6 h, and an EtOH concentration of 76.3%.

Under these conditions, the model predicted response values of 68.36 mg GAE/g for TPC and 5.56 mg QE/g for TFC.

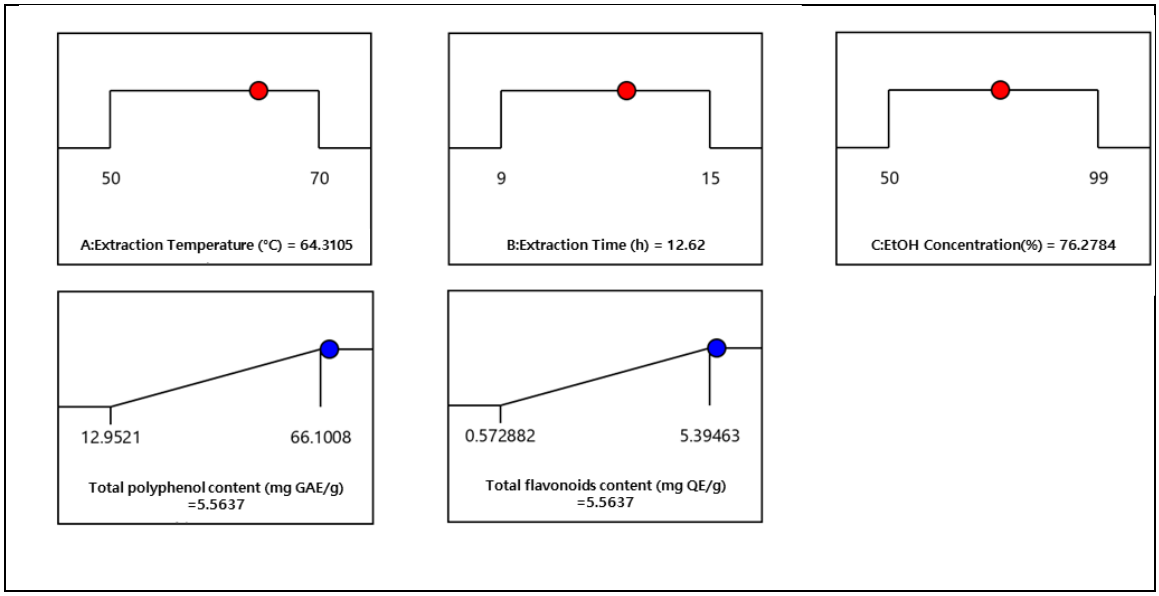

Fig. 3. Optimization ramps showing optimal experimental conditions and predicted response values for maximizing TPC and TFC

To validate the reliability of the optimized extraction conditions derived from the RSM model (64.3 °C, 12.6 h, and 76.3% EtOH), verification experiments were performed in triplicate (Table 4). Under these conditions, the model predicted values of 68.36 mg GAE/g for TPC and 5.56 mg QE/g for TFC. The actual experimental results were measured as 63.41 ± 3.05 mg GAE/g for TPC and 5.12 ± 0.12 mg QE/g for TFC. The relative errors between the predicted and experimental values were calculated to be 7.24% and 7.91%, respectively, which fell within the acceptable range (< 10%). Furthermore, the low coefficient of variation (CV) of the experimental data confirmed the high reproducibility of the process under the optimized conditions. Consequently, the established quadratic regression model was determined to be suitable for optimizing and predicting the extraction process.

Table 4. Experimental and Predicted Values of Each Evaluation Index Under Optimal Conditions

Response Variables	Optimum Extractions Conditions			Maximum Value	
	A	B	C	Predicted Values	Experimental Values
TPC	64.3 °C	12.6 h	76.3%	68.36	63.41 ± 3.05
TFC	64.3 °C	12.6 h	76.3%	5.56	5.12 ± 0.12

As demonstrated in Table 5, the TPC obtained in the present study (63.41 mg GAE/g) was considerably higher than those reported for *Senna alata* leaves (30.61 mg GAE/g; Bao *et al.,* 2025) and *Citrus maxima* albedo (15.79 mg GAE/g; Inthachat *et al.* 2023). These findings suggest that *F. mandshurica* SFE residues are a competitive source

of phenolic compounds. In contrast, the TFC obtained in the present study (5.12 mg QE/g) was comparatively lower than that of S. alata (47.91 mg QE/g), which may reflect the phytochemical characteristics of *F. mandshurica*, a species known to contain a high proportion of non-flavonoid phenolic compounds such as coumarins, secoiridoids, and phenylethanoids rather than flavonoids (Kostova and Iossifova 2007). Collectively, these comparisons indicate that the optimized extraction conditions established in the present study enable efficient recovery of phenolic compounds from *F. mandshurica* SFE residues, with yields that are well within or exceed the range reported for other plant-derived materials.

Table 5. Comparison of TPC and TFC Yields Obtained in the Present Study with Those Reported for Selected Plant Materials Extracted Using Ethanol-based Solvent Extraction Under Optimized Conditions

Material	Part	Solvent	TPC (mg GAE/g)	TFC (mg QE/g)	Reference
F. mandshurica SFE residues	leaves	76.3% EtOH	63.41	5.12	Present study
Senna alata	leaves	75% EtOH	30.61	47.91	Bao *et al.* 2025
Citrus maxima albedo	leaves	50% EtOH	15.79	4.50	Inthachat *et al.* 2023

Cytotoxicity Assessment of Extracts

Prior to evaluating anti-inflammatory activity, the safety range of the optimized *F. mandshurica* SFE residues extract on RAW 264.7 cells were determined, and its effects on LPS-induced stress were investigated (Fig. 4). First, the cytotoxicity of the extract was assessed (Fig. 4A). No significant decrease in cell viability was observed compared to the control group at concentrations up to 125 μg/mL, confirming the absence of toxicity within this range. In contrast, cell viability decreased in a dose-dependent manner at concentrations of 250 μg/mL and above. Therefore, 62.5 and 125 μg/mL were selected as the optimal concentrations for subsequent experiments. Next, to establish an inflammation model, cell viability was examined according to LPS concentration (Fig. 4B).

The selection of 10 μg/mL LPS is consistent with previous studies utilizing RAW 264.7 macrophages, in which concentrations ranging from 1 to 10 μg/mL have been employed to induce inflammatory responses (Moro *et al.* 2012; Kang *et al.* 2015; Suriyaprom *et al.* 2023), and this concentration has been specifically applied in studies evaluating the anti-inflammatory efficacy of plant-derived extracts under stringent inflammatory conditions (Yang *et al.* 2012).
(Min-Jung Kang *et al.* 2015)

Based on these findings, the effect of the extract was analyzed under 10 μg/mL LPS stimulation (Fig. 4C). The LPS-only group (Veh) showed a marked reduction in cell viability compared to the control (CTL); however, co-treatment with the extract showed a tendency to alleviate LPS-induced cytotoxicity. This suggests that the extract, at the selected concentrations, possesses potential cytoprotective effects against inflammatory stress without inducing cell death.

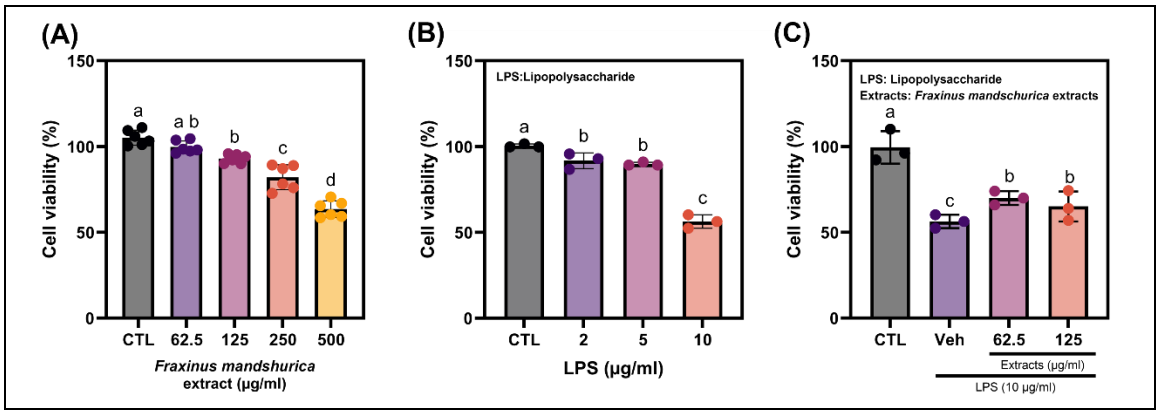

Fig. 4. Cytotoxicity and anti-inflammatory effects of *F. mandshurica* residues extract on RAW 264.7 cells: (A) Effect of extract concentration on cell viability; (B) Effect of LPS concentration on cell viability; (C) Cytoprotective effect of the extract against LPS-induced damage (10 μg/mL); Data are presented as mean ± SD. Different letters (a through d) indicate statistically significant differences ($p < 0.05$). The circular plots on the bar chart represent actual data values.

Anti-inflammatory Evaluation of Extracts

To investigate the anti-inflammatory mechanism of *F. mandshurica* SFE residues extract under LPS-induced inflammatory conditions, quantitative analysis was performed on the production of key inflammatory mediators, namely NO and tumor necrosis factor-alpha (TNF-α) (Fig. 5). Regarding the production of NO, a byproduct of iNOS activation (Fig. 5A), minimal expression was observed in the non-stimulated control (CTL), whereas it increased sharply to approximately 7.1 μM following LPS stimulation (Veh). However, treatment with the extract significantly inhibited this NO production; in particular, at a concentration of 62.5 μg/mL NO levels were reduced to 4.4 μM representing a inhibitory efficiency of approximately 38.0%. At a concentration of 125 μg/mL a level of 5.2 μM was observed, maintaining an inhibitory effect of approximately 26.8%

Furthermore, the secretion of TNF-α, a critical cytokine involved in the initiation of the inflammatory response, was also evaluated (Fig. 5B). The LPS stimulation led to a surge in TNF-α expression to approximately 1,010 pg/mL, but treatment with the extract at 62.5 and 125 ug/mL showed a reduction to 850 pg/mL (15.8% inhibition) and 900 pg/mL (10.9% inhibition), respectively.

Although the inhibitory effect on TNF-α was relatively moderate compared to that on NO, this differential response suggests that the extract may act through a pathway-selective mechanism, preferentially targeting iNOS-mediated NO production rather than exerting broad upstream cytokine suppression. This pattern of selectivity is not uncommon among polyphenol-rich plant extracts. Shih *et al.* (2010) reported that treatment with an ethanol extract of *Euphorbia hirta* L. in LPS-stimulated RAW 264.7 macrophages selectively inhibited iNOS-mediated NO production while exerting comparatively modest effects on TNF-α. Similarly, Peng *et al.* (2015) demonstrated, in the same cell model, that treatment with *Panax notoginseng* flower ethanol extract strongly suppressed NO overproduction and iNOS gene expression without exerting significant influence on TNF-α expression. It is also noted that the TNF-α inhibition did not follow a clear dose-dependent pattern, which may reflect the complexity of cytokine regulation under the experimental conditions employed and warrants further investigation.

Collectively, these results suggest that *F. mandshurica* residues extracted through the optimized SFE process partially modulate inflammatory responses by suppressing the

excessive production of major pro-inflammatory mediators. However, a limitation of this study is that the LPS concentration of 10 µg/mL was relatively high compared to concentrations commonly employed in literature (Moro *et al.* 2012; Kang *et al.* 2015; Suriyaprom *et al.* 2023). This concentration was intentionally selected to establish robust inflammatory stress conditions. Importantly, the co-treatment data in Fig. 4C demonstrated that the extract partially alleviated this LPS-induced viability loss, suggesting that the measured suppression of NO and TNF-α production reflects genuine anti-inflammatory activity rather than a cytotoxicity-driven reduction in cellular inflammatory capacity. Nevertheless, the possibility that the cytotoxic component of LPS at 10 µg/mL partially confounds the anti-inflammatory readout cannot be entirely excluded, and future studies employing lower LPS concentrations would enable a more rigorous mechanistic interpretation.

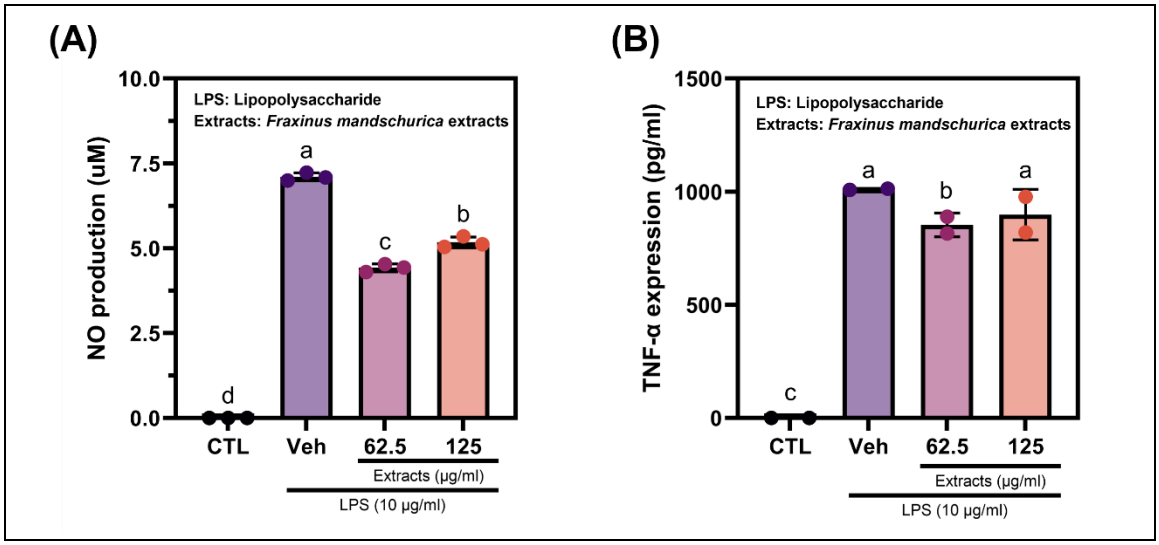

Fig. 5. Anti-inflammatory activity of *F. mandshurica* extract manifested by the inhibition of (A) NO production and (B) TNF-α expression in LPS-induced macrophages. Data are presented as mean ± SD. Different letters (a, b, c) indicate statistically significant differences ($p < 0.05$). The circular plots on the bar chart represent actual data values.

CONCLUSIONS

1. The optimal extraction conditions derived from the Box-Behnken Design (BBD) were determined to be an extraction temperature of 64.3 °C, a time of 12.6 h, and an EtOH concentration of 76.3%. Under these conditions, the experimental yields of total polyphenol content (TPC) and total flavonoid content (TFC) were 63.41 ± 3.05 mg GAE/g and 5.12 ± 0.12 mg QE/g, respectively. The low relative errors (< 10%) between predicted and experimental values confirmed the acceptable reproducibility of the established quadratic model within the experimental range tested.
2. In biological assays using RAW 264.7 macrophages, the optimized extract exhibited no cytotoxicity up to a concentration of 125 µg/mL. Furthermore, the extract demonstrated selective inhibition of inflammatory mediator production, particularly suppressing NO production to approximately 4.4 µM at 62.5 µg/mL, with a comparatively modest reduction in TNF-α secretion in LPS-stimulated cells.

3. This study provides an optimized strategy for recovering residual bioactive compounds from SFE residues. These findings suggest that *F. mandshurica* residues represent a promising candidate for further investigation as a source of bioactive compounds, with potential applicability in the pharmaceutical and food industries pending scalability and application-level validation.

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Data Availability

All datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Use of Generative AI (if applicable)

The authors acknowledge the use of AI assistance during manuscript preparation. This includes English translation, formatting of the Experimental section, formatting of references, and language refinement. All AI-generated content was thoroughly reviewed and verified by the authors to ensure accuracy and adherence to scientific standards.

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