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OPTIMIZING EXTRACTION CONDITIONS TO ENRICH POLYPHENOL AND FLAVONOID FROM *Ehretia asperula* LEAF EXTRACT AND EVALUATING ITS *IN VIVO* HEPATOPROTECTIVE PROPERTIES

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ABSTRACT

Ehretia asperula is a medicinal plant rich in polyphenols and flavonoids, with potential bioactive properties, and is used in traditional medicines. This study aimed to extract and optimize the antioxidant activity of the extract and determine its hepatoprotective effects. The extraction conditions were optimized using the Box-Behnken method at different temperatures, ethanol concentrations, and solid-liquid ratios. Antioxidant capacity was assessed using $ABTS^{\bullet+}$, DPPH, reducing power (RP), and total antioxidant capacity (TAC) assays, and hepatoprotective efficacy was evaluated in a CCl_4-induced liver injury mouse model. The results showed that the optimal conditions for antioxidant extraction were 58.97°C, 59.08% ethanol, and a 1:26.16 w/v liquid ratio. The maximum polyphenol and flavonoid contents were 182.44 mg GAE/g and 336.77 mg QE/g, respectively. The antioxidant activities under optimal conditions were 1.21–2.17 fold higher than those of the initial extracts. CCl_4-injured mice fed the extract showed a lower liver/body ratio and higher ALT, AST, and MDA levels, whereas GSH levels

remained unchanged. The liver tissue of mice fed with the extract was similar to that of healthy mice. The optimized *E. asperula* extract exhibited strong antioxidant and hepatoprotective properties, making it a promising natural therapeutic agent for liver disorders associated with oxidative stress.

KEYWORDS: Antioxidant, Box-Behnken design, *Ehretia asperula*, flavonoids, hepatoprotective, polyphenols

1. INTRODUCTION

Oxidative stress has been implicated in various clinical conditions, including liver damage (Ramos-Tovar & Muriel, 2020; Vona, Pallotta, Cappelletti, Severi, & Matarrese, 2021). It induces hepatic injury by altering the structure and function of biomolecules and disrupting molecular signaling pathways that regulate normal liver functions (Allameh, Niayesh-Mehr, Aliarab, Sebastiani, & Pantopoulos, 2023). Consequently, reducing oxidative stress through antioxidant administration has emerged as a promising therapeutic approach to prevent or treat oxidative stress-induced liver damage (Ponnampalam et al., 2022).

In some Asian countries, several plants are rich in antioxidants and are known as herbs (Nguyen, Nguyen, & Kim, 2021). Dietary phytochemicals are known for their potent antioxidant properties (Lee, Park, Kang, & Paik, 2023; Tuan & Men, 2024). Flavonoids are a class of phytochemicals found in plants, fruits, vegetables, and leaves and have garnered attention for their potential medicinal benefits. Flavonoids exhibit diverse pharmacological activities including anticancer, antioxidant, anti-inflammatory, and antiviral effects. In addition, they exhibit neuroprotective and cardioprotective properties. These biological actions depend on the type of polyphenol and flavonoid, their mechanisms of action, and their bioavailability. Flavonoids have demonstrated significant potential as cost-effective pharmaceutical agents for treating various diseases including liver disorders (Damavandi, Shojaei, & Esfahani, 2023).

Ehretia asperula, a member of the Boraginaceae family, grows widely across provinces in Vietnam including Ha Nam, Ninh Binh, Hoa Binh, and Tay Ninh.

Traditionally, it has been used to treat ulcers, tumors, liver detoxification, and inflammation. Ethanol extracts from the fruits and flowers of *E. asperula* have shown anticancer activity against colon and liver cancer and inhibitory effects on HIV replication in H9 lymphocytes cells.(Tram, Suong, & Tien, 2021; Tram, Suong, & Tien, 2022) Additionally, Tam et al.(Tam et al., 2021) reported that rosmarinic acid, lithospermic acid B, astragalin, and kaempferol-3-rutinoside from *E. asperula* leaf extract effectively prevented oxidative stress-induced retinal cell death. The leaf extract also exhibited anti-inflammatory activity against lipopolysaccharide-stimulated RAW264.7 macrophages (Le, Hong, & Yang, 2024).

Previous study (Duc et al., 2024) identified four flavonoid compounds, kaempferol, astragalin, nicotiflorin, and rutin, in the ethyl acetate fraction of *E. asperula*. These compounds demonstrated strong antioxidant activity in TAC, RP, FRAP, DPPH, and $ABTS^{\bullet+}$ assays. However, the hepatoprotective properties and *in vivo* antioxidant activities of *E. asperula* remain poorly understood.

Recent research has explored advanced methodologies and animal models to isolate flavonoids, synthesize analogs, and evaluate their health effects. Thousands of flavonoids have been identified, with this list continuously expanding (Ullah et al., 2020). To optimize the extraction of polyphenols and flavonoids, Response Surface Methodology (RSM), particularly the Box-Behnken model, has been widely employed in experimental design and mathematical analysis (Elgudayem et al., 2023).

To harness the health benefits of *E. asperula* extract, optimizing polyphenol and flavonoid extraction is essential. This study aimed to optimize the extraction conditions for polyphenol- and flavonoid-enriched *E. asperula* extracts and evaluate their in vitro and in vivo efficacies. Key parameters, such as solvent type, temperature, time, and solid-to-solvent ratio, were investigated. Furthermore, the hepatoprotective potential of the extracts was assessed in a CCl_4-induced liver injury mouse model.

2. MATERIALS AND METHODS

2.1. Plant material and extraction

Ehretia asperula was collected from Tay Ninh Province, Vietnam, and was botanically identified. A voucher specimen (SGN) was deposited at the Southern Institute of Ecology in Vietnam. The leaves were cut into small pieces, air-dried, and ground into a fine powder using a high-speed universal grinder (DFY-600, Linda Machinery Co., Ltd, China). The powdered sample was extracted using 99.5% ethanol (w/v) at a solid-to-liquid ratio of 1:10 (w/v) at 30°C for 24 h. Subsequently, the solvent was removed from the extract using a rotary vacuum evaporator. The resulting extract was stored at -20°C for further analysis and biological testing.

2.2. Quantitative analysis of the phytochemical in E. asperula leaf extract

The total phenolic content (TPC) and total flavonoid content (TFC) of the *E. asperula* leaf extract (EALE) were quantified using a modified colorimetric method (Dai, Chau, Truong, Tran, & Nguyen, 2024). TPC and TFC were expressed as milligrams of gallic acid equivalents (mg GAE) and milligrams of quercetin equivalents (mg QE) per gram dried extract, respectively. All measurements were performed in triplicates to ensure accuracy and reproducibility.

2.3. Determination of antioxidant activities

The antioxidant activities of the non-optimized and optimized *E. asperula* leaf extracts (EALE) were evaluated using 2,2-Azino-bis-3-ethylbenzothiazoline-6-sulphonic acid ($ABTS^{\bullet+}$), 2,2-diphenyl-1-picryhydrazyl (DPPH) radical scavenging, reducing power (RP), and total antioxidant capacity (TAC) assays. All spectrophotometric assays were conducted following established protocols as described in previous studies, without modifications (Anh et al., 2021). Antioxidant activity results are expressed as EC_{50} values.

2.4. Optimization of conditions using a one-factor-at-a-time experimental design

The primary factors influencing the total phenolic content (TPC) and total flavonoid content (TFC) include ethanol concentration, extraction temperature, extraction time, and–d the liquid ratio. Based on the extraction parameters reported in previous studies, the ranges for the critical factors in this study were determined as follows: ethanol concentrations of 40%, 50%, 60%, 70%, 80%, 90%, and 99.5%; extraction temperatures of 30°C, 40°C, 50°C, 60°C, 70°C, 80°C, and 90°C; extraction durations of 6, 12, 18, 24, 30, 36, and 42 h; and solid-liquid ratios (w/v) of 1:10, 1:15, 1:20, 1:25, 1:30, 1:35, and 1:40.

2.5. Experimental design and equation analyses

A Box-Behnken experimental design, as outlined by Elgudayem et al. (2023) (Elgudayem et al., 2023), was utilized to develop a predictive model and analyze response patterns influenced by three independent variables: temperature (X_1), ethanol concentration (X_2), and liquid-to-solid ratio (X_3). The experimental design involved three levels for each factor: low (-1), medium (0), and high (+1), to systematically explore their effects. Seventeen experimental runs were conducted to estimate the experimental error and ensure robustness including five replicates at the center point.

A second-order polynomial equation (1) was employed to investigate the individual and interactive effects of the independent variables as well as their optimal levels and impact on the responses:

$$Y_{1,2} = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_{i^2} + \sum_{i=1, j=2, i \neq j} \beta_{ij} X_i X_j \quad (1)$$

Where, Y_1: the response of the phenolic compounds content, Y_2: the response of the flavonoid content, β_0: the regression coefficient of the model (intercept

coefficient), X_i and X_j were the independent factors, β_{ij}, β_{ii} and β_i were the coefficient of the interaction, squared and linear coefficient, respectively.

Analysis of variance (ANOVA) was conducted to assess the significance of the factors and their interactions on the target responses. Key statistical metrics, including the coefficient of determination (R^2), adjusted R^2, lack-of-fit significance, and regression coefficients (β), were evaluated to determine the quality and accuracy of the model. R^2 was specifically used to assess the predictive strength of the model, while the lack of fit evaluated its overall suitability. The F-value was calculated to compare the experimental and predicted values, further validating the reliability and performance of the model.

2.6. Experimental animals

The animals used in this study were bred and maintained at the animal facility of the Pasteur Institute in Nha Trang, and subsequently transported to the Department of Biology, College of Natural Sciences. A total of 42 healthy adult male and female, aged 6-12 weeks and weighing 25-30 g, were selected for experimentation (Tuan & Men, 2024). The mice were randomly grouped and housed in individual glass cages, each lined with clean paddy-husk bedding. Prior to experimentation, the animals were acclimatized to laboratory conditions for one week to minimize nonspecific stress (Girish et al., 2009). Male and female mice were housed in separate cages throughout the study, to ensure controlled experimental conditions.

2.7. Induction liver damage in mice

The hepatoprotective effects of EALE were evaluated using a mouse model of hepatic injury induced by carbon tetrachloride (CCl_4) (Kang & Koppula, 2014). Liver damage was initiated by the oral administration of CCl_4 dissolved in olive oil. One hour after CCl_4 administration, mice were treated with EALE at different

concentrations to assess their protective potential. Silymarin served as a positive control, providing a benchmark for the hepatoprotective effects of EALE.

2.8. Experimental design

EALE was prepared by dissolving it in 1% DMSO solution and stored under appropriate conditions. Carbon tetrachloride (CCl_4) was dissolved in olive oil at a ratio of 1:4 and administered orally at a dose of 2.5 mL/kg body weight (b.w) per day for four consecutive weeks(Mclean & Mclean, 1966). This dose was below the LD_{50} of CCl_4, which ranged between 12,000 and 14,000 mg/kg BW, as reported in previous studies. Furthermore, CCl_4 at a level of 1,200 mg/kg BW has been shown to induce chronic hepatotoxicity over 90 days without causing mortality in experimental animals(Hayes, Condie, & Borzelleca, 1986).

All solutions were administered orally using a syringe with a curved needle to deliver 200 µL directly into the stomachs of mice. The physiological conditions of the animals were monitored twice daily, in the morning and evening. In the event of death of a mouse, necropsy was conducted to examine the internal organs. In this study, mice were randomly assigned to six groups, each consisting of six animals.

Group I (Control): Mice received only food and water.

Group II: Mice received 1% DMSO one hour after olive oil administration.

Group III (CCl_4-intoxicated control): Mice were given CCl_4.

Group IV (positive control): Mice received silymarin at a dose of 16 mg/kg b.w one hour after CCl_4 administration.

Groups V, VI, VII (EALE-treated groups): Mice were treated with EALE at doses of 100, 200, and 400 mg/kg b.w, respectively, one hour after CCl_4 administration.

2.9. Biochemical analysis and histopathological examination

Following four weeks of treatment, the animals were anesthetized using diethyl ether and subsequently sacrificed. Body weight (b.w) and liver weight were recorded, body weight gain was calculated as the difference between the initial and final body weights, and relative liver weight was determined as the ratio of liver weight to body weight. Blood samples were immediately collected and liver tissues were dissected. Each liver sample was divided into two portions: one was washed with 0.9% ice-cold saline and stored at -20°C for biochemical analysis, while the other was fixed in 10% formalin for histopathological examination.

Plasma was isolated from blood samples by centrifugation at 3,000 rpm for 10 min at room temperature. The levels of alanine transaminase (ALT) and aspartate transaminase (AST) were measured using a semi-automated Erba Chem-7 analyzer (Erba, Germany).

To assess the effects of EALE on endogenous oxidants and antioxidants in the liver, malondialdehyde (MDA) and glutathione (GSH) concentrations were analyzed, representing lipid peroxidation and antioxidant status, respectively. MDA and GSH concentrations (nM/g tissue) were calculated using linear regression equations derived from their respective standard curves (Moron, Depierre, & Mannervik, 1979; Ohkawa, Ohishi, & Yagi, 1979).

For histopathological analysis, a small portion of the liver was preserved in 4% formaldehyde for at least 24 hours. The tissues were then gradually dehydrated with ethanol, cleared with xylene, and embedded in paraffin wax (Russell, 1951). Sections of 5 µm thickness were prepared using a microtome (Leica, Germany), stained with hematoxylin and eosin, and examined under a microscope to evaluate histopathological alterations.

2.10. Ethical Approval and Animal Welfare

This study adhered to the ethical guidelines outlined in the US National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996). Approval for the study was granted by the Animal Ethics Committee of Can Tho University (CTU-AEC) under permission code CTU-AEC24012. Measures were taken to minimize the number of animals used and to reduce pain and distress throughout the experimental procedures. Humane euthanasia was performed at the conclusion of the experiment to ensure the animals did not experience unnecessary suffering.

2.11. Statistical Analysis

All experiments were conducted with a minimum of three replicates. Experimental data are expressed as the mean ± standard deviation. Statistical analyses were performed using Minitab 16 software, employing ANOVA followed by Tukey's post-hoc test for multiple comparisons. Correlation analyses were conducted using the Pearson's correlation method. Statistical significance was set at $p < 0.05$. Graphical representations of the results were created in Microsoft Excel 2016 and GraphPAD Prism 9.5.0.

3. RESULTS

3.1. Single-factor effects on the extraction of flavonoids and polyphenols

Figure 1A illustrates the effects of varying ethanol concentrations on the polyphenol and flavonoid contents of *E. asperula* leaf (EAL) powder. Polyphenol content increased from 62.13 mg GAE/g extract at 40% ethanol to 74.65 mg GAE/g extract at 60% ethanol. Similarly, flavonoid content rose from 207.85 mg QE/g extract at 40% ethanol to 282.06 mg QE/g extract at 60% ethanol. However, both polyphenol (60.95 mg GAE/g extract) and flavonoid (246.21 mg QE/g extract) contents

significantly decreased at 70% ethanol concentration. Based on these results, 60% ethanol was selected as the center point for the RMS model.

Temperature is another critical factor that affects the extraction of polyphenols and flavonoids. Total polyphenol content (TPC) increased proportionally from 39.29 mg GAE/g extract at 30°C to 71.44 mg GAE/g extract at 60°C. A similar trend was observed for total flavonoid content (TFC), which increased from 192.59 mg QE/g extract at 30°C to 255.17 mg QE/g extract at 60°C (Figure 1B). However, further increase in temperature resulted in a marked decline in both TPC and TFC. Consequently, 60°C was chosen as the central temperature for the RMS.

The solid-to-liquid ratio also significantly influences the extraction process. Various ingredient-to-solvent ratios (w/v), ranging from 1:10 to 1:40, were tested. TPC and TFC increased substantially as the ratio increased from 1:10 to 1:25. Beyond this ratio, a decline in both TPC and TFC was observed. Accordingly, 1:25 was fixed as the central ratio for the RMS.

The effect of the extraction time on the TPC and TFC yields is shown in Figure 1D. The polyphenol and flavonoid contents steadily increased with longer extraction times from 6 to 42 h. TPC values ranged from 29.29 mg GAE/g extract at 6 hours to 48.85 mg GAE/g extract at 42 hours. TFC values similarly rose from 192.59 mg QE/g extract at 6 hours to 242.28 mg QE/g extract at 42 hours. However, the increase in TPC and TFC over time was gradual, and 6 h was deemed sufficient for the EAL extraction procedure based on its practical efficiency.

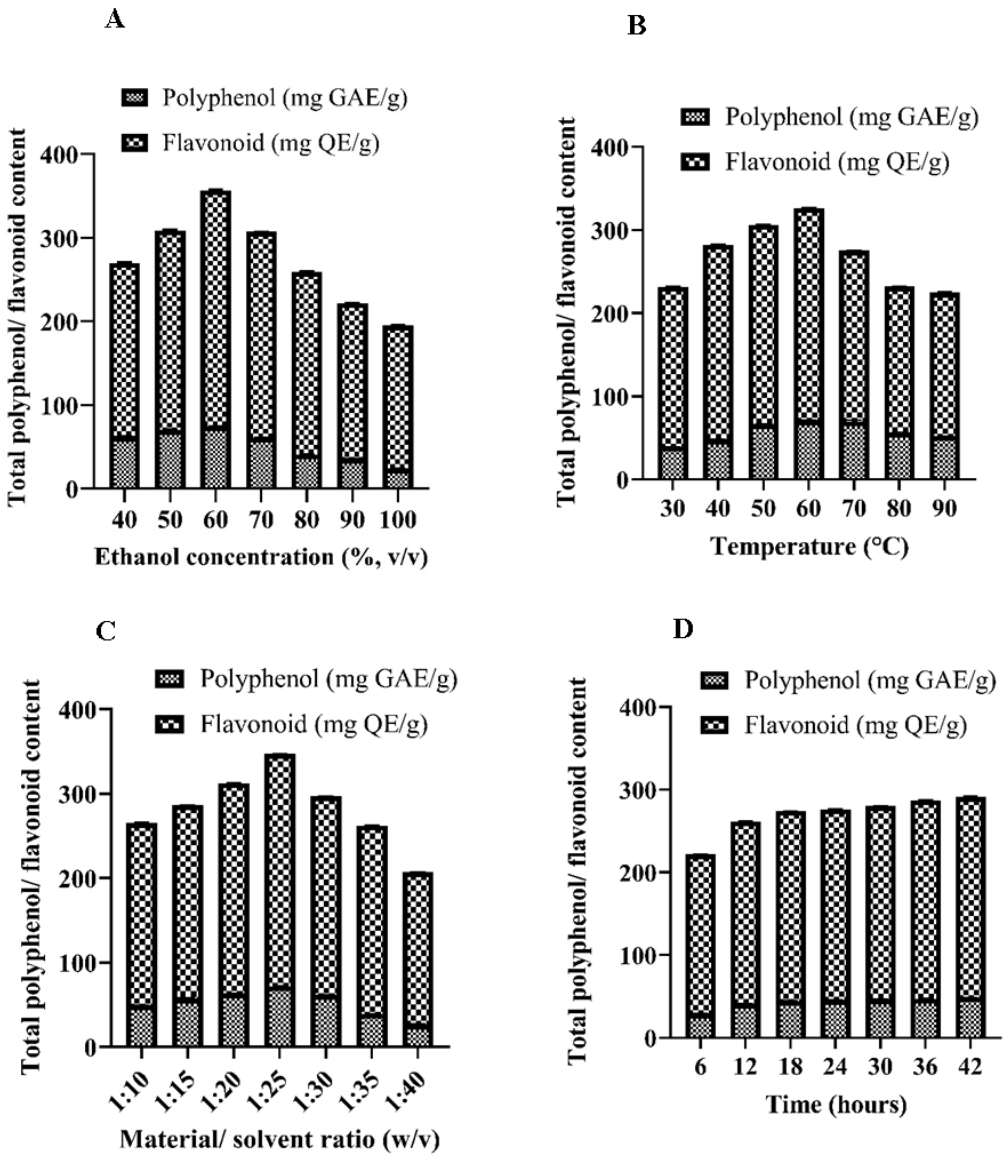

Figure 1. Effect of ethanol concentration (A), temperature (B), material/ solvent ratios (C), and extraction time (D) on TPC and TFC. TPC and TFC were expressed as mg GAE/g and mg QE/g extract, respectively. Data are presented as the mean ± SD of triplicate experiments.

3.2. Optimal conditions for polyphenol and flavonoid-enriched extract

Temperature, ethanol concentration, and solid-to-liquid ratio were identified as critical factors that significantly influenced the polyphenol and flavonoid contents of the extract, as indicated by single-factor analyses. However, the extraction duration did not have a significant impact. Consequently, the Box-Behnken model was developed using the temperature, ethanol concentration, and solid-to-liquid ratio as independent variables.

The central treatments (13-17) demonstrated superior total polyphenol content (TPC) and total flavonoid content (TFC) compared to other treatments. Treatment 16 produced the highest TPC and TFC values, with 181.81 mg GAE/g extract and 335.6 mg QE/g extract, respectively. The TPC and TFC values obtained across all 17 treatments closely matched the model predictions, as shown in Table 1.

Table 1. Rotable central composite design setting in the original and code forms of the independent variables (A, B, C) and experimental results of TPC (Y_1) and TFC (Y_2).

Run				TPC (mg GAE/mL)		TFC (mg QE/mL)	
	A	B	C	Observed	Predicted	Observed	Predicted
1	50	50	1/25	100.95^{g}±0.14	100.73	185.49^{g}±0.51	185.66
2	70	50	1/25	105.02^{f}±0.57	104.58	193.87^{f}±0.46	193.41
3	50	70	1/25	61.71^{i}±0.76	62.16	113.92^{i}±0.77	114.39
4	70	70	1/25	52.44^{j}±0.60	52.66	96.80^{k}±0.51	96.64
5	50	60	1/20	126.47^{c}±0.91	126.39	233.45^{c}±0.85	233.31
6	70	60	1/30	120.71^{d}±0.75^{d}	120.86	222.83^{d}±0.70	223.31
7	50	60	1/30	133.73^{b}±0.20	133.59	246.85^{b}±0.56	246.38
8	70	60	1/30	133.38^{b}±0.25	133.47	246.21^{b}±0.57	246.37
9	60	50	1/20	104.70^{f}±0.89	105.08	193.27^{f}±0.97	193.26
10	60	70	1/20	60.201^{i}±0.75	59.84	111.13^{j}±0.75	110.83
11	60	50	1/30	114.629^{e}±0.56	114.99	212.60^{e}±0.52	212.91
12	60	70	1/30	69.948^{h}±0.83	69.65	127.27^{h}±0.51	127.30
13	60	60	1/25	180.82^{a}±0.57	181.27	333.79^{a}±0.60	334.62
14	60	60	1/25	181.23^{a}±0.98	181.27	334.53^{a}±0.72	334.62
15	60	60	1/25	181.76^{a}±0.60	181.27	335.51^{a}±0.76	334.62
16	60	60	1/25	181.81^{a}±0.56	181.27	335.60^{a}±0.98	334.62
17	60	60	1/25	180.74^{a}±0.39	181.27	334.63^{a}±0.73	334.62

A: temperature (°C); B: ethanol concentration; C: ratio solid-liquid

Regression analysis and ANOVA were used to fit the model and evaluate the statistical significance of the terms. Table 2 presents the ANOVA results, which revealed a coefficient of determination (R^2) of 0.9999 for both TPC and TFC. The lack-of-fit test was not statistically significant ($p>0.05$), indicating that the model reliably predicted the responses. Additionally, the 3D response surface graphs (Supplementary Figures 1 and 2) depict the interactive effects of the independent variables on the responses.

Table 2. ANOVA for the effect of temperature (A), ethanol concentration (B), ratio solid-liquid (C) on TPC and TFC of the *E. asperula* leaf extract using a response surface model.

Source	df	Sum of Squares		Mean Square		F-value		*p*-value	
		TPC	TFC	TPC	TFC	TPC	TFC	TPC	TFC
Model	9	33924.17	1.160E+05	3796.35	12888.95	13288.23	19525.83	<0.0001	<0.0001
A	1	15.95	50.02	15.95	50.02	56.22	75.78	0.0001	<0.0001
B	1	4095.03	14121.05	4095.03	14121.05	14436.37	21392.37	<0.0001	<0.0001
C	1	196.07	652.73	196.07	652.73	691.23	988.84	<0.0001	<0.0001
AB	1	44.56	162.44	44.56	162.44	157.09	246.09	<0.0001	<0.0001
AC	1	7.31	24.91	7.31	24.91	25.77	37.74	0.0014	0.0005
BC	1	0.0080	2.52	0.0080	2.52	0.0281	3.82	0.8717	0.0916
A^2	1	3794.27	12928.69	3794.27	12928.69	13376.07	19586.03	<0.0001	<0.0001
B^2	1	21358.04	73010.38	21358.04	73010.38	75294.26	1.106E+05	<0.0001	<0.0001
C^2	1	2165.63	7379.24	2165.63	7379.24	7634.57	11179.02	<0.0001	<0.0001
Residual	7	1.99	4.62	0.2837	0.6601				
Lack of Fit	3	0.9770	1.18	0.3257	0.3946	1.29	0.4592	0.3919	0.7256
Pure Error	4	1.01	3.44	0.2522	0.8592				
Cor Total	16	33926.16	1.160E+05						
R^2		0.9999	0.9999						
$R^2_{Adjusted}$		0.9999	0.9999						
$R^2_{Predicted}$		0.9995	0.9998						
CV%		0.43	0.35						
N		17	17						

Among the variables, the quadratic effects of temperature (X_1, coded A), ethanol concentration (X_2, coded B), and solid-to-liquid ratio (X_3, coded C) significantly influenced TPC and TFC ($p < 0.05$), followed by the interactive effects AB and AC. However, the BC interaction was not significant ($p>0.05$). The

relationships between the variables and responses were described by the following second-order polynomial equations:

$$Y_{TPC} = -3991,84 + 37,21A + 85,23B + 44,78C - 0,03AB + 0,03AC - 0,0009BC - 0,30A^2 - 0,71B^2 - 0,91C^2 \quad (2)$$

$$Y_{TPC} = -3991,84 + 37,21A + 85,23B + 44,78C - 0,03AB + 0,03AC - 0,0009BC - 0,30A^2 - 0,71B^2 - 0,91C^2 \quad (3)$$

Equations (2) and (3) provide accurate predictions of experimental observations. TPC and TFC values peaked under both experimental and predicted conditions, as shown in Supplementary Figures 1 and 2, and Figure 2.

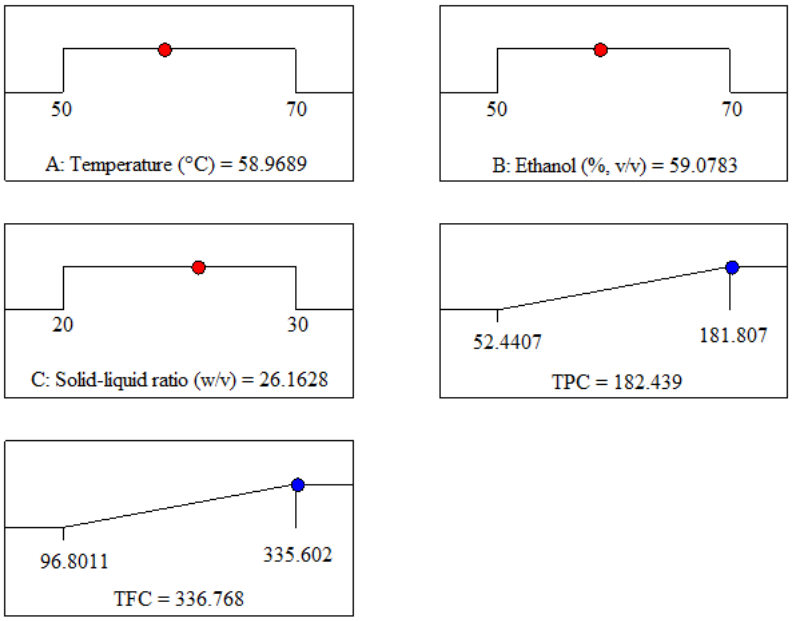

Figure 2. Optimization of extraction conditions for polyphenol and flavonoid. Total polyphenol content (TPC, mg GAE/g) and total flavonoid content (TFC, mg QE/g) derived from the response surface methodology. The graphs illustrate the optimal levels of temperature (°C), ethanol concentration (% v/v), and solid-to-liquid ratio (w/v) corresponding to maximum TPC and TFC values. Predicted optimal TPC and TFC values are shown as 182.439 mg GAE/g and 336.768 mg QE/g, respectively.

The optimal conditions for maximizing TPC and TFC were determined to be a temperature of 58.97°C, an ethanol concentration of 59.08%, and a solid-to-liquid ratio of 1:26.16 (w/v). Under these conditions, the *E. asperula* leaf extract (EALE) achieved TPC and TFC concentrations of 182.44 mg GAE/g and 336.77 mg QE/g, respectively (Figure 2). In the actual experiments, the observed TPC and TFC values were 186.22 mg GAE/g and 335.09 mg QE/g, respectively, which closely aligned with the model's predictions.

3.3. Antioxidant properties of optimized EAL extract

As previously reported, *E. asperula* leaf (EAL) extract obtained using a non-optimized extraction method exhibited antioxidant properties. This method involved extraction with 99.5% ethanol (w/v), solid-to-liquid ratio of 1:10 (w/v), extraction temperature of 30°C, and duration of 24 h. Key parameters, including total polyphenol content (TPC), total flavonoid content (TFC), ABTS radical scavenging activity, DPPH radical scavenging activity, reducing power (RP), and total antioxidant capacity (TAC), were evaluated for both non-optimized and optimized extracts to assess their impact on antioxidant properties. The results are summarized in Table 3.

Table 3. Total phenolic contents (TPC), total flavonoid contents (TFC), antioxidant activities (ABTS, DPPH, RP, TAC) of EAL extract obtained from non-optimized and optimized extraction

	Non-optimized	**Optimized**	**Fold changes** (Optimized/Non- optimized)
TPC (mg GAE/g extract)	49.78 ± 0.98	186.22 ± 0.76	3.74
TFC (mg QE/g extract)	169.63 ± 2.88	335.09 ± 2.05	1.98
$ABTS^{\bullet+}$ (EC_{50}, µg/mL)	143.40 ± 4.68	66.22 ± 0.53	2.17
DPPH (EC_{50}, µg/mL)	93.98 ± 3.64	49.75 ± 4.38	1.89
RP (EC_{50}, µg/mL)	16.67 ± 0.70	13.80 ± 1.34	1.21
TAC (EC_{50}, µg/mL)	147.25 ± 5.46	115.94 ± 3.86	1.27

The findings revealed that all measured parameters were significantly enhanced in the optimized extraction method compared to the non-optimized method. This demonstrates that the optimization of the extraction process using the Box-Behnken

Design (BBD) and Response Surface Methodology (RSM) effectively enhanced the antioxidant activity of the EAL extract. Thus, the optimized EAL extract represents a superior formulation for harnessing the antioxidant properties of plants.

3.4. Hepatoprotective assay of optimized E. asperula leaf extract by against carbon tetrachloride-induced liver toxicity in mice

3.4.1. Effects of EALE on body weight and liver weight

Table 4 summarizes the effects of *E. asperula* leaf extract (EALE) on body weight (b.w.), liver weight, body weight gain, and relative liver weight in mice subjected to CCl_4-induced hepatotoxicity. In the hepatotoxic group (Group III), CCl_4 treatment significantly reduced body weight and increased liver weight, resulting in lower body weight gain (1.93 ± 0.73 g) and elevated relative liver weight (8.71 ± 0.95%) compared to the control group (1.59 ± 0.12 g and 6.09 ± 0.48%, respectively).

Administration of silymarin (16 mg/kg b.wt) in Group IV significantly improved body weight gain (2.70 ± 0.61 g) and reduced liver weight (1.63 ± 0.07 g), leading to a lower relative liver weight (6.04 ± 0.27%), which was comparable to that of the control group (Group I) and significantly better than that of Group III.

In contrast, Group V, treated with the lowest dose of EALE, did not exhibit a significant improvement in body weight gain or relative liver weight compared to Group III. However, Groups VI and VII, treated with higher doses of EALE (200 and 400 mg/kg b.wt, respectively), demonstrated significant improvements. Body weight gain in Groups VI (3.80 ± 0.49 g) and VII (4.47 ± 0.31 g) was significantly higher than that in Group II (negative control), although not significantly different from that in Group I (healthy control). Additionally, the relative liver weights in Groups VI (6.82 ± 0.59%) and VII (5.63 ± 0.46%) were significantly reduced compared to Group III and closely aligned with the control group values.

These findings indicate that higher doses of EALE effectively mitigated the adverse effects of CCl_4 on body weight and liver weight, demonstrating its potential hepatoprotective efficacy.

Table 4. Effect on EALE on final body weight, liver weight and relative liver weight against CCl_4 induced liver damage in mice

	Groups	Body weight (g)	Liver weight (g)	Body weight gain (g)	Relative liver weight (%)
I	Normal control	26.20±1.42	1.59±0.12	4.28^{ab}±0.63	6.09^{cd}±0.48
II	Olive, DMSO 1%	27.13±1.12	1.52±0.12	4.67^{a}±0.63	5.64^{d}±0.69
III	CCl_4, DMSO 1%	23.48±0.12	2.03±0.12	1.93^{d}±0.73	8.71^{a}±0.95
IV	CCl_4, silymarin 16	26.95±0.68	1.63±0.07	2.70^{c}±0.61	6.04^{cd}±0.27
V	CCl_4, EALE 100	25.46±1.15	1.95±0.05	2.08^{d}±0.24	7.91^{ab}±0.40
VI	CCl_4, EALE 200	26.28±1.04	1.79±0.09	3.80^{abc}±0.49	6.82^{bc}±0.59
VII	CCl_4, EALE 400	27.62±1.55	1.55±0.46	4.47^{ab}±0.31	5.63^{d}±0.46

3.4.2. Effects of EALE on serum biomarkers AST and ALT

Mice intoxicated with CCl_4 exhibited significantly elevated levels of aspartate transaminase (AST) and alanine transaminase (ALT), which are key indicators of liver injury, compared with the control group. AST levels increased to 1660.0 ± 89.6 U/L and ALT levels to 986.3 ± 29.9 U/L in the CCl_4-intoxicated group, compared to the control group's values of 86.0 ± 3.8 U/L and 65.0 ± 2.8 U/L, respectively (Table 5).

Treatment with EALE demonstrated significant hepatoprotective effects by reducing the biomarker levels in a dose-dependent manner. AST levels were reduced by 133.3, ± 20.7, 74.2, ± 8.0, and 55.0 ± 5.5 U/L at EALE doses of 100, 200, and 400 mg/kg body weight (b.w.), respectively. Similarly, ALT levels decreased by 65.8 ± 10.2, 38.3 ± 6.8, and 23.3 ± 5.2 U/L at the same respective doses when compared to the CCl_4-intoxicated group.

Notably, EALE treatment at doses of 200 and 400 mg/kg b.w. resulted in AST and ALT levels comparable to those in the control and silymarin-treated groups, with no significant differences observed between them (Table 5).

These findings highlight the potent hepatoprotective efficacy of EALE, particularly at high doses, in mitigating CCl_4-induced hepatic damage. These results suggest that EALE effectively reduces liver injury by decreasing serum AST and ALT levels, indicating its therapeutic potential in protecting against hepatotoxicity.

Table 5. Effect on EALE on serum AST, ALT, MDA and GSH against CCl_4 induced liver damage in mice

	Groups	AST (U/L)	AST (U/L)	MDA (nmole MDA/ g tissue)	GSH (nmole GSH/ g tissue)
I	Normal control	86.0^{b}±3.8	65.0^{b}±2.8	8.91^{e}±0.54	246.25^{d}±6.05
II	Olive, DMSO 1%	76.5^{b}±18.2	62.2^{b}±11.9	8.88^{e}±0.46	247.40^{d}±6.52
III	CCl_4, DMSO 1%	1660.0^{a}±89.6	986.3^{a}±29.9	89.10^{a}±3.50	57.18^{f}±4.72
IV	CCl_4, silymarin 16	83.2^{b}±15.4	55.2^{b}±10.4	19.66^{d}±0.51	657.10^{b}±23.17
V	CCl_4, EALE 100	133.3^{b}±20.7	65.8^{b}±10.2	56.53^{b}±2.61	153.32^{e}±4.53
VI	CCl_4, EALE 200	74.2^{b}±8.0	38.3^{bc}±6.8	33.34^{c}±0.63	598.88^{c}±14.98
VII	CCl_4, EALE 400	55.0^{b}±5,5	23.3^{c}±5.2	7.55^{e}±0.87	720.92^{a}±15.67

3.4.3. In vivo antioxidant activity of EALE in CCl_4-intoxicated mice

A significant reduction ($p < 0.05$) in hepatic glutathione (GSH) content and a marked increase ($p < 0.05$) in malondialdehyde (MDA) levels were observed in CCl_4-intoxicated mice compared to the normal control group (Table 3). Treatment with EALE at doses of 100, 200, and 400 mg/kg body weight (b.w.) significantly increased hepatic GSH levels to 153.32 ± 4.53, 598.88 ± 14.98, and 720.92 ± 15.67 nmol GSH/g tissue, respectively, compared to the reduced levels in CCl_4-intoxicated mice (57.18 ± 4.72 nmol GSH/g tissue). Notably, hepatic GSH levels in the 400 mg/kg b.w. EALE-treated group surpassed those in the normal control group.

In addition, EALE treatment at all tested doses significantly mitigated CCl-induced elevation in hepatic MDA levels. In particular, at a dose of 400 mg/kg b.w., MDA levels in EALE-treated mice were comparable to those in the normal control

group and were significantly lower than those in CCl_4-intoxicated mice treated with silymarin.

These findings underscore the potent *in vivo* antioxidant activity of EALE, as demonstrated by its ability to restore hepatic GSH levels and reduce MDA accumulation, thereby offering robust protection against CCl_4-induced oxidative stress and liver damage.

3.4.4. Histopathological findings

Histopathological analysis of liver sections from the normal control group (Group I) revealed a typical hepatic architecture, including a normal central vein, well-organized hepatocytes arranged in radiating plates, and polygonal cells with central, spherical, and vesicular nuclei. Additionally, intact hepatic sinusoids surrounded by endothelial cells were observed (Figure 3A).

In the DMSO-treated group (Group II), liver sections exhibited architecture that remained largely intact. Key features include a central vein with normal morphology, radiating hepatic cords, polygonal hepatocytes with clear nuclei, and well-defined sinusoidal spaces. No significant pathological changes were observed, indicating that DMSO treatment did not adversely affect hepatic structure (Figure 3B).

In contrast, liver sections from the CCl_4-intoxicated group (Group III) exhibited severe histopathological alterations, including extensive necrosis, ballooning degeneration, lymphocyte infiltration, and loss of cellular boundaries, indicative of significant hepatotoxic damage (Figure 3C).

Silymarin treatment (16 mg/kg b.w.) in Group IV mitigated the deleterious effects of CCl_4, resulting in improved hepatic architecture. While patches of necrotic hepatocytes were still present, the overall cellular organization was markedly improved compared to the CCl_4-intoxicated group (Figure 3D).

Treatment with EALE at doses of 100 mg/kg (Figure 3E), 200 mg/kg (Figure 3F), and 400 mg/kg (Figure 3G) progressively alleviated the hepatic damage. The liver sections of these groups displayed a relatively normal lobular pattern, with reduced necrosis and moderate lymphocyte infiltration. Notably, the 400 mg/kg b.w. EALE-treated group showed the most substantial improvement, closely resembling normal hepatic architecture.

These histopathological findings highlight the hepatoprotective potential of EALE, particularly at high doses, in mitigating CCl_4-induced liver damage and restoring hepatic integrity.

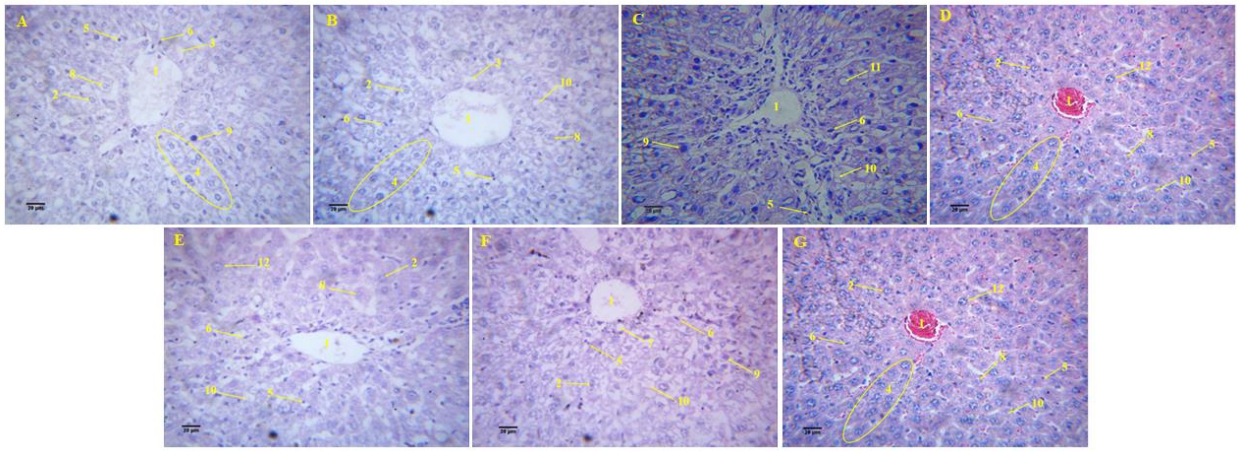

Figure 3. Histopathological examination of liver tissues.

Histopathological analysis of liver sections from mice treated with Ehretia asperula leaf extract (EALE) under varying experimental conditions. (A) Normal control group showing typical hepatic architecture with well-organized hepatocytes and intact sinusoids. (B) DMSO-treated group with no significant histopathological changes. (C) CCl_4-intoxicated group showing severe necrosis, lymphocyte infiltration, and loss of cellular structure. (D) Silymarin-treated group exhibiting partial restoration of hepatic architecture. (E–G) EALE-treated groups at doses of 100, 200, and 400 mg/kg body weight, respectively, demonstrating progressive restoration of hepatic

structure, with the highest dose showing near-complete recovery. Hematoxylin and eosin staining, scale bar = 20 µm. Central vein (1), radiating hepatic cords (2), polygonal hepatocytes with centrally located nuclei (3), intact sinusoidal spaces surrounded by endothelial cells (4), (5), hepatic sinusoids monocyte (6), bile ducts (7), (8) lymphocyte infiltration and mild necrosis, (9) lymphocyte infiltration, mild necrosis (10).

4. DISSCUSSION

In previous study, flavonoid compounds isolated from *E. asperula* leaf extract (EALE) demonstrated notable antioxidant activity (Duc et al., 2024). However, the extraction was performed under non-optimized conditions, which can reduce the extraction efficiency, prolong the processing time, and increase the use of plant material. In this study, extraction conditions were optimized using the Box-Behnken design (BBD) and Response Surface Methodology (RSM) to enhance the antioxidant potential of the extract. The optimized EALE was subsequently evaluated for its antioxidant activity *in vitro* and hepatoprotective effects in a CCl_4-induced liver injury model.

The analysis of variance (ANOVA) results (Table 2) demonstrated the reliability of the optimization process, with a high coefficient of determination (R^2>99%) and low probability values ($p < 0.05$), indicating a significant relationship between the independent variables and extraction outcomes. The non-significant lack of fit (p>0.05) further confirmed the predictive accuracy (Elgudayem et al., 2023). The coefficients of variation (CV) for total polyphenol content (TPC) and total flavonoid content (TFC) were 0.43% and 0.35%, respectively, reflecting the precision of the extraction process (Koraqi et al., 2023; Lubis, Sumaiyah, Lubis, Fitri, & Astyka, 2024). Optimized extraction conditions resulted in significantly higher TPC, TFC, and antioxidant activities ($ABTS^{\bullet+}$, DPPH, RP, and TAC) than non-optimized conditions (Table 3).

CCl_4 is a potent hepatotoxin widely used to induce liver injury in experimental models, mimicking human hepatotoxicity and serving as a platform for screening hepatoprotective agents (Frank et al., 2020). Upon activation by cytochrome P450 enzymes, CCl_4 generates trichloromethyl (CCl_3) and trichloromethyl peroxy (CCl_3O_2) radicals, which propagate lipid peroxidation, oxidative stress, DNA damage, and hepatocyte necrosis (Ritesh, Suganya, Dileepkumar, Rajashekar, & Shivanandappa, 2015). This cascade depletes antioxidant defenses such as glutathione (GSH) and stimulates reactive oxygen species (ROS) production, exacerbating hepatic inflammation (Mooli, Mukhi, & Ramakrishnan, 2022; Sharma et al., 2023).

In this study, CCl_4 administration caused severe hepatotoxicity, as evidenced by elevated serum ALT and AST levels, significant histopathological damage, increased hepatic malondialdehyde (MDA) levels, and reduced GSH levels. These findings align with those of previous studies on CCl_4-induced oxidative stress (Ayala, Muñoz, & Argüelles, 2014; Ritesh et al., 2015).

Treatment with optimized EALE at doses of 100, 200, and 400 mg/kg b. w. effectively mitigated these effects. EALE significantly reduced ALT and AST levels and normalized hepatic MDA levels. At higher doses, EALE's effects of EALE were comparable to those of silymarin, which is a standard hepatoprotective agent. Furthermore, EALE treatment restored GSH levels, suggesting improved antioxidant defenses. Histopathological analysis confirmed these biochemical findings, showing reduced necrosis and preserved hepatic architecture in the EALE-treated group.

The antioxidant activity of EALE, demonstrated through $ABTS^{\bullet+}$, DPPH, reducing power, and total antioxidant capacity assays, is primarily attributed to its flavonoid and polyphenol content. Flavonoids such as kaempferol, astragalin, nicotiflorin, and rutin, previously isolated from EALE (Duc et al., 2024), exhibit strong radical scavenging activity and contribute to the hepatoprotective effects observed in this study (Liao, Lv, Quan, Wang, & Li, 2024; Sahu, Goswami, Narahari Sastry, & Rawal, 2023).

This study is the first to demonstrate the antioxidant and hepatoprotective properties of the optimized EALE *in vitro* and *in vivo*, highlighting its potential as a natural therapeutic agent for liver diseases caused by oxidative stress.

5. CONCLUSIONS

This study recommends the use of the Box-Behnken design to optimize the extraction of polyphenols and flavonoids from *E. asperula* leaves. The optimized EALE exhibited strong antioxidant potential and hepatoprotective efficacy against CCl_4-induced liver injury, which was attributed to its high antioxidant capacity and free radical scavenging ability. Furthermore, optimized EALE enhan ced the endogenous antioxidant defense system disrupted by CCl_4, likely due to the abundance of bioactive polyphenol and flavonoid compounds. These findings suggest that EALE may serve as a promising natural remedy for oxidative stress-related liver disorders.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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