



Extraction Process of O-Coumaric Acid from *Gliricidia Sepium* Leaves: Optimization and Kinetic Studies

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ARTICLE INFO	ABSTRACT
Article history: Received 20 October 2024 Received in revised form 19 December 2024 Accepted 25 January 2025 Available online 28 February 2025 Keywords: Ultrasound-assisted extraction; o-coumaric acid; <i>Gliricidia sepium</i>; optimization	Extraction and product recovery are among the most significant processes for assessing valuable active chemicals from distinct plant sections. This study explores the potential of ultrasound-assisted extraction (UAE) method to optimize the o-coumaric acid extraction from <i>Gliricidia sepium</i> leaves, with the goal of shifting conventional extraction method. Response surface methodology was applied to observe interaction of three independent variables (ratio of solvent to solid, extraction temperature and extraction time) on the dependent variable (yield of o-coumaric acid) for optimal extraction conditions. This research reveals that the optimal conditions for the extraction of o-coumaric acid were established at 80% (v/v) methanol with optimum level of extraction temperature (45°C), extraction time (46.60 min) and ratio of solvent to solid (10:1 mL g⁻¹) resulted to o-coumaric acid yield of 36.14 ± 0.015 mg L⁻¹. The reduction in particle size distribution through UAE led to a notable increase in the surface area of solid materials, consequently enhancing mass transfer and extraction yield. This approach yielded a remarkable 62% higher o-coumaric acid yield (1148.2 mg g⁻¹) compared to conventional Soxhlet extraction (435.02 mg g⁻¹). Additionally, o-coumaric compound was confirmed through HPLC at a retention time of 2.78 minutes and successfully analyzed via FTIR. This study not only establishes optimal conditions for o-coumaric acid extraction but also highlights UAE as a transformative technique for sustainable and high-yield recovery of bioactive components from plants, paving the way for advancements in bioresource utilization and eco-friendly extraction methodologies.

1. Introduction

Plants are valuable sources of various natural bioactive compounds, including secondary metabolites and antioxidants [1,2]. As stated by Golawska *et al.*, [3], natural bioactive components such as flavonoids, carotenoids, and phenolic acids are crucial for the production of functional foods

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and medicinal products, which also hold industrial significance. These bioactive components are often stored in plant leaves, and many, particularly phenolic acids and flavonoids, are commercially significant [4]. Hydroxycinnamic acids, a subgroup of phenolic phytochemicals, are natural compounds commonly found in fruits, vegetables, and coffee. Among hydroxycinnamic acids, caffeic, ferulic, and p-coumaric acids derived from plants have garnered significant attention for their biological activities [5]. However, research on the biological activities of o-coumaric acid remains limited.

Gliricidia sepium, often known as the mother of cocoa, is a shade tree that is widely used in plantation agriculture [6]. The plants were initially introduced into Sri Lanka from the West Indies and later to India. In Malaysia, it is known as 'Pokok Bunga Jepun' due to its beautiful flowers and it contains various medicinal values. According to Wafaey *et al.*, [7] *G. sepium* exhibits a wide range of pharmacological activities, including cytotoxic, antimicrobial, antibacterial, anti-inflammatory, antioxidant, thrombolytic, antisickling, wound healing, larvicidal, and anthelmintic activities. These findings highlight its notable importance in the medical field. In fact, another significant application of *G. sepium* is the extraction of o-coumaric acid from its leaves using a solid-liquid extraction process. This compound holds promise as a potential precursor for the bioconversion into dicoumarol, which is valued for its natural properties as both a rodenticide and anticoagulant [8]. Therefore, the extraction process of *G. sepium* leaves with optimal conditions is highly required and vital to get the highest yield of o-coumaric acid as the desired anticoagulant compound to be used in further bioconversion process.

In recent years, there has been a significant surge in interest in the study of bioactive substances, particularly phenolic compounds derived from plants [9-11]. Phenolic compounds comprise lignin, tannin, lignan, simple phenol, flavonoid, phenolic acid and coumarin. All these compounds can either being obtain through conventional or the modern extraction technique. The choice of extraction technique depends on factors such as the yield, expenses, and effectiveness of the process. Solid-liquid extraction stands out as a prevalent approach for transferring a soluble component (solute) from a solid material into a liquid solution [12]. In solid-liquid extraction, solute molecules move from the solid substrate to the surrounding solvent by processes such as dissolution and diffusion, which are influenced by the concentration gradient between the solid and liquid phases. This technique exhibits fundamental ideas similar to those used for liquid-liquid extraction regardless the solute initially resides in a solid.

From an engineering point of view, solid liquid extraction is a multiphase process involving unsteady state of mass transfer operation. Mass transfer is defined as migration of a system component from phase to phase or in the same phase due to concentration gradient between two points. The mass transfer is carried out by molecular flow due to molecular diffusion. In diffusion, the concentration gradient is the cause of flow and heat gradient is the driving force. The diffusion phenomenon occurs in all mass transfer operation which involve at least one phase and usually in both [13]. Traditionally, the extraction of most phenolic compounds was accomplished by conventional method- Soxhlet extraction [14]. However, this method has been associated with a high consumption of organic solvents, high temperature, high energy cost, slow in operation and might lead to degradation of extracted compounds [15,16].

Ultrasound-assisted extraction (UAE) has emerged as a modern method for recovering various plant extracts, offering several advantages over traditional solvent extraction methods [17]. It provides a cost-effective solution with simple equipment, significantly reducing solvent consumption, extraction time, and operating temperatures. UAE stands out as an environmentally friendly alternative, minimizing pollutant production compared to conventional approaches [18]. Furthermore, the UAE appears to have a significant ability to increase the output of targeted

bioactive constituents, rendering it an appropriate approach across various sectors has presented novel research opportunities in chemistry, biology, pharmaceuticals, food science, and related fields [19]. Nonetheless, there still remains a need for further research into the variables influencing the extraction process. Among the numerous factors influencing solid-liquid extraction efficiency, ratio of solid to solvent, extraction time and reaction temperature are particularly critical. Temperature affects solubility and diffusion rates, optimizing extraction efficiency up to a certain point before potential degradation occurs. Longer extraction times enhance thoroughness but may reach diminishing returns and risk compound degradation [20,21].

Apart from that, the solvent-to-solid ratio may influence extraction efficiency by affecting concentration gradients and compound accessibility, with higher ratios generally yielding better results [22,23]. Thus, this present research endeavors to investigate the optimal conditions of extraction process on *G. sepium* leaves to get the highest yield of o-coumaric acid as the desired compound for further use in bioconversion process. The study will also assess physical and chemical analysis included morphology, particle size distribution, thermal behaviour and other assessment to fully compare the efficacy of ultrasound assisted extraction to the traditional Soxhlet extraction method.

2. Methodology

2.1 Sample Collection

The leaves of *Grilicidia sepium* (Jacq) Steud. were collected along some local residential areas in Nilai, Negeri Sembilan, Malaysia (latitude 2° 50' 21.49"N, longitude 101° 47' 41.50"E), in March 2016. The samples were preserved in wax-quoted paper bags and deposited in Biodiversity Unit, Institute Bioscience, UPM for authentication and registration of voucher specimen. The voucher specimen was authenticated and registered with no SK 3133/17.

2.2 Plant Drying and Grinding

About 10 g of *G. sepium* fresh leaves sample were placed in the oven for 18 hr at 50°C for the drying process and the weight loss were measured at an interval of 10 minutes. The process for drying was considered complete when constant weight was observed. The sample weight loss was measured at an interval of 10 minutes and the final constant weight was recorded as 2.711 g. The temperature of ambient air was between 26 to 28°C while the relative humidity of air was between 80 to 88%. The dried leaves were grounded into powder, sieved and the particle sizes were recorded.

2.3 Soxhlet Extraction

About 5g of *G. sepium* leaf samples in dried powder were placed into an extraction thimble, which was then transferred into a Soxhlet apparatus (Bionics, Model BST/SX-1A, India). Approximately 150 mL of 80% methanol was added to each flask connected to the extractor. Each extraction was conducted in triplicate for a duration of 6 hours.

2.4 Ultrasonic-Assisted Extraction

About 5g of *G. sepium* leaf samples in dried powder were used for the extraction of o coumaric acid using methanol as the solvent with ultrasound-assisted extraction method. The extraction method involved immersing the ultrasound probe in the mixture containing 80% of methanol and *G.*

sepium leaf samples with various solvent-to-solid ratios (5:1, 10:1, 15:1). The mixture was then heated in a water bath at different temperatures (50°C, 55°C, and 60°C) for varying durations (40, 45, and 50 minutes). The ultrasound probe was equipped with a constant power of 130 W and a frequency of 20 kHz (refer to Figure 1). Subsequently, the leaves extract containing o-coumaric acid was filtered with Whatman No.1 filter paper and the filtrate was evaporated to obtain a dry extract using air evaporation at room temperature under hood for 24 h. The extracts were stored at -20 °C in a deep freezer for further analysis.

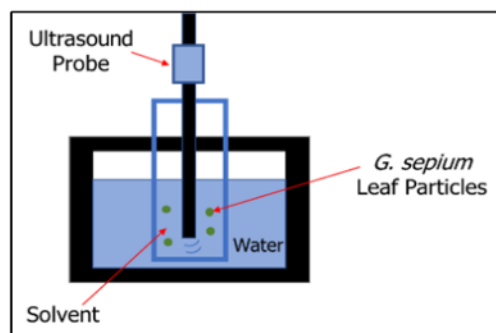


Fig. 1. A schematic diagram of ultrasonic assisted extraction [24]

2.5 Single Factor Experiments of Extraction

For o-coumaric acid extraction, five types of solvents were primarily used in the experiments including n-hexane (Hex), ethyl acetate (EA), chloroform (Chloro), methanol (MetOH) and pure water (H₂O) which were selected based on their polarity ranges from most non-polar, less non polar, less polar, medium polar to the most polar. Each solvent extract was dissolved in respective solvents (1 mg/mL) for further studies. 1 mg/mL sample solution was prepared in HPLC grade methanol and filtered through a 0.22 µm filter membrane for HPLC analysis under operating conditions in Table 1. All the experiments were conducted in triplicates.

Table 1

HPLC operating condition for extraction of o-coumaric acid

HPLC Parameters	Operating Conditions
Mobile phase	Methanol + 0.4% acetic acid (80:20 v/v)
Column type	C-18 RP column
Column temp	25°C
Detector type	UV-Vis detector
Injection volume	5µL
Flow rate	5µL
Detection rate	290 nm

2.6 Extraction Kinetic Models

In general, some important parameters in solid liquid extraction (extraction rate, rate constant and activation energy) can be determined through a kinetic model by experiments. Solid liquid extraction is found to be most appropriate fitted by a Pseudo-second-order [25,26]. The model is more suitable for kinetic study as it enables the determination of the most important kinetic parameters.

A second order process typically takes place in two subsequent stages. The first stage is the rapid extraction of the major parts of the solutes due to the scrubbing and dissolution caused by the solvent driving force. The second stage is the slower extraction process due to external diffusion of the remainder solute into the solution. The second order kinetic rate equation which represent the dependence of the extraction rate on the solute dissolved in the solution is expressed as Eq. (1):

$$\frac{dC_t}{dt} = k(C_s - C_t)^2 \quad (1)$$

where k is the second order extraction rate constant (L/mg min), C_s is the extraction capacity (concentration of solute at saturation in mg/L) and C_t is the concentration of solute in the solution at any time t (min). By considering the boundary condition $t=0$ to t and $C_t=0$ to C_t , the integrated rate law for a second-order extraction is obtained as Eq. (2) [27].

$$C_t = \frac{C_s^2 kt}{1 + C_s kt} \quad (2)$$

The linearized above equation would be written as Eq. (3). The extraction rate can be further written as Eq. (4). Then, as t approaches 0, the initial extraction rate, h as C_t/t can be expressed as Eq. (5).

$$\frac{t}{C_t} = \frac{1}{kC_s^2} + \frac{t}{C_s} \quad (3)$$

$$\frac{C_t}{t} = \frac{1}{\left(\frac{1}{kC_s^2}\right) + \left(\frac{t}{C_s}\right)} \quad (4)$$

$$h = kC_s^2 \quad (5)$$

Experimentally, to determine the initial extraction rate (h), the extraction capacity (C_s) and the second-order extraction rate constant (k) from the slope and intercept, one can plot t/C_t versus t [28]. The Arrhenius law describes the dependence of the extraction rate constant on the temperature. The activation energy of the process can be calculated using the Arrhenius equation in Eq. (6) [29].

$$D_e = A \exp\left(-\frac{E_a}{RT}\right) \quad (6)$$

where:

A = pre-exponential factor of Arrhenius equation (m^2s^{-1})

E_a = energy of activation (J/mol)

R = universal gas constant (8.314 J/molK)

T = temperature (K)

2.7 Optimization of O-Coumaric Acid Extraction Using Respond Surface Methodology

Respond Surface Methodology was applied to optimize the process parameter of *G. sepium* leaves extraction using UAE. Central composite design (CCD) was selected to fit the model using the least squares technique. This method is suitable for fitting a quadratic surface and it helps to optimize the effective parameters with a minimum number of experiments, and to analyse the

interaction between the parameters [30]. Analysis of variance (ANOVA), residual plots, and contour plots were used to determine the optimum Design Expert version 13.0 (Stat-Ease, Minneapolis, MN, USA) was used to design the study. Three parameters (ratio of solid to solvent, temperature and extraction time) were studied while the response was the concentration of o-coumaric acid. In the RSM analysis, yield was set as the response variable (Y) and was a function of coded independent variables including temperature (X_1) and ratio of solvent to solid (X_2). The independent variables were converted to 5 coded levels of assessment, ranging from $-\alpha$, -1, 0, +1 and $+\alpha$.

20 experiments were performed and randomized to assess the potential interaction of process variables and their impact on the yield and concentration of o-coumaric acid using analysis of variance (ANOVA). A p-value of 0.05 was chosen, establishing a 95% significance level. The adequacy of the CCD for ultrasound-assisted extraction of o-coumaric acid concentration was evaluated using the correlation coefficient, R^2 to predict the experiments. The regression coefficient model was established by fitting the experimental data to a second-order polynomial model. The response variable and independent variables were defined using the second-order polynomial model as in Eq. (7).

$$Y = b_0 + b_1 \times X_1 + b_2 \times X_2 + b_3 \times X_3 + b_{11} \times (X_1 \times X_1) + b_{22} \times (X_2 \times X_2) + b_{33} \times (X_3 \times X_3) + b_{12} \times (X_1 \times X_2) + b_{13} \times (X_1 \times X_3) + b_{23} \times (X_2 \times X_3) \quad (7)$$

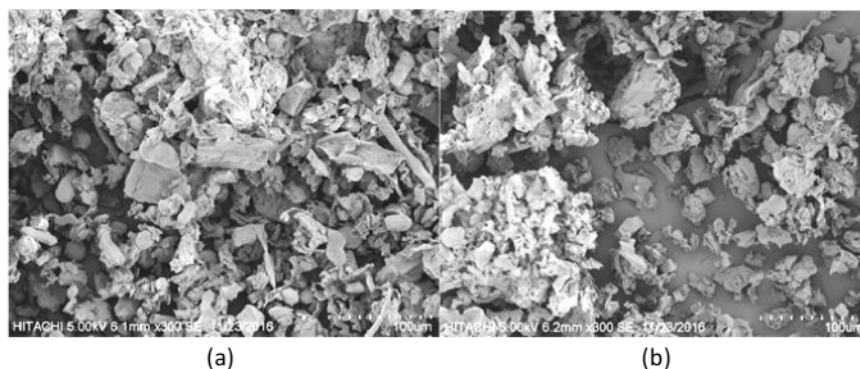
where Y represents the measured response variables; b_0 is a constant; b_1 , b_2 , and b_3 are the linear, quadratic, and interactive coefficients of the model, respectively; and X_1 , X_2 and X_3 are independent variables.

3. Results

3.1 Physical and Chemical Analysis of Dried *G. Sepium* Leaves

3.1.1 Scanning electron micrograph

The scanning electron micrograph of *G. sepium* leaves is shown in Figure 2. The untreated leaf appears filled and intact (Figure 2(a)). The cell structures of Soxhlet extracted leaves were intact but emptied due to transfer of cell content within the solvent. A gradual degradation of cell walls was obtained after UAE which were affected at various degrees and duration of times. During UAE (Figure 2(c)), there was slight fragmentation of *G. sepium* leaves in the early of sonication and continued to show significant effect within 30 minutes allowing solvent diffusion into the plant matrix cells. It was comparable when the leaves did not seem impacted during conventional extraction performed by SE (Figure 2(b)). The cells structures of UAE were destructed to undefined shapes. This finding could be assumed that such cell destruction fragmentation favored accessibility to the solvent reaction.



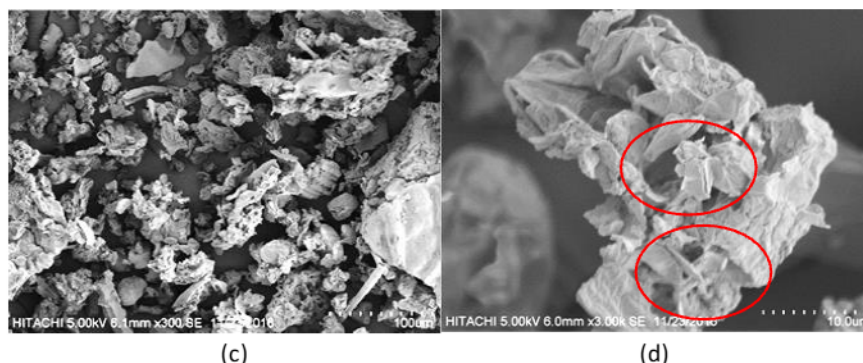


Fig. 2. Effect of ultrasound power on *G. sepium* leaves. SEM microscopic observation of *G. sepium* leaves at room temperature (a) Untreated raw material (b) SE (c) UAE (US probe, 20kHz, 15min)

3.1.2 Particle size distribution

The *G. sepium* residues were collected after filtration to measure the particle size distribution. The distribution of particle size is an initial key in understanding both physical and chemical properties. In Figure 3, it shows that the particle size distributions were below 1100 μm for both residues from UAE and SE. The particle size distribution was calculated by volume-based. The result indicated that 9.6% of the size distribution was in the category of 181.97 to 239.88 μm , which means the total volume of all particles with diameters in this range represents 9.6% of the total volume of all particles. Comparatively, it was 1.8% lower in size distribution in the category of 208.93 to 239.88 μm for SE.

Ultrasound cavitation has led to a fragmentation of friable solids. The cells fragmentation could be due to inter-collision between particles and from shockwaves created from collapsing cavitation bubbles in the solvent [31-33]. The reduction in particle size distribution by ultrasound action can lead to direct consequence of increase surface area of the solid materials which resulted in higher mass transfer and increase extraction rate and yield. The average particle size of *G. sepium* residue after UAE was lower than the conventional SE extraction. The specific surface area of UAE and SE were $0.1852 \text{ m}^2 \text{ g}^{-1}$ and $0.0782 \text{ m}^2 \text{ g}^{-1}$ respectively. By creating finer particles in UAE as compared to SE, it increases the surface area which reduces the physical barriers to dissolution, thus making the quicker extraction process. This concluded that UAE technique reduced the inner size particles and external mass transfer limitation and hence increased the extraction yield.

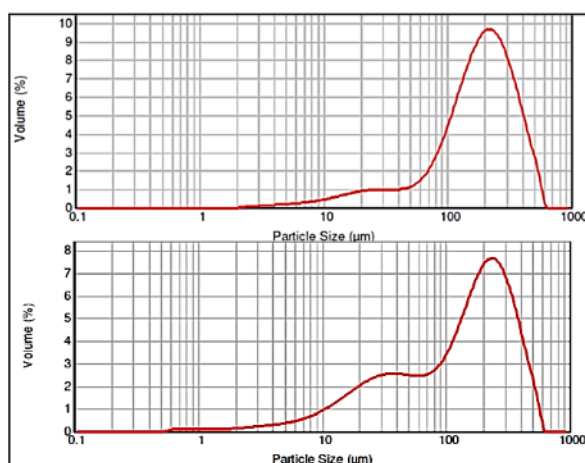


Fig. 3. The average particle size of leaf by (a) UAE (b) SE at room temperature

3.2 Single-Factor Experiments of Leaf Extraction

Methanol has a polarity index of 5.1. Methanol is mostly used for extraction various polar compounds [34,35] but certain group of non-polar and unready soluble compounds are fairly soluble in methanol. Moreover, methanol among all other solvents has low boiling point of just 65°C. Thus, extraction and recovery of o-coumaric acid was easy accomplished by both extraction systems. O-coumaric acid was semi-standard non polar compound and it was slightly soluble in water but well dissolved in methanol as polar solvent.

Another point to the finding was the molecule of methanol which consists in a single atom of a tetrahedral carbon, linked to 3 hydrogens and a -OH group. The -OH group is the polar group and the three hydrogens, the water-insoluble hydrocarbon chain. This contributes to the ability of methanol to dissolve polar molecules and also non-polar ones. Figures 4 and 5 show the comparison of HPLC chromatograms between UAE and SE respectively.

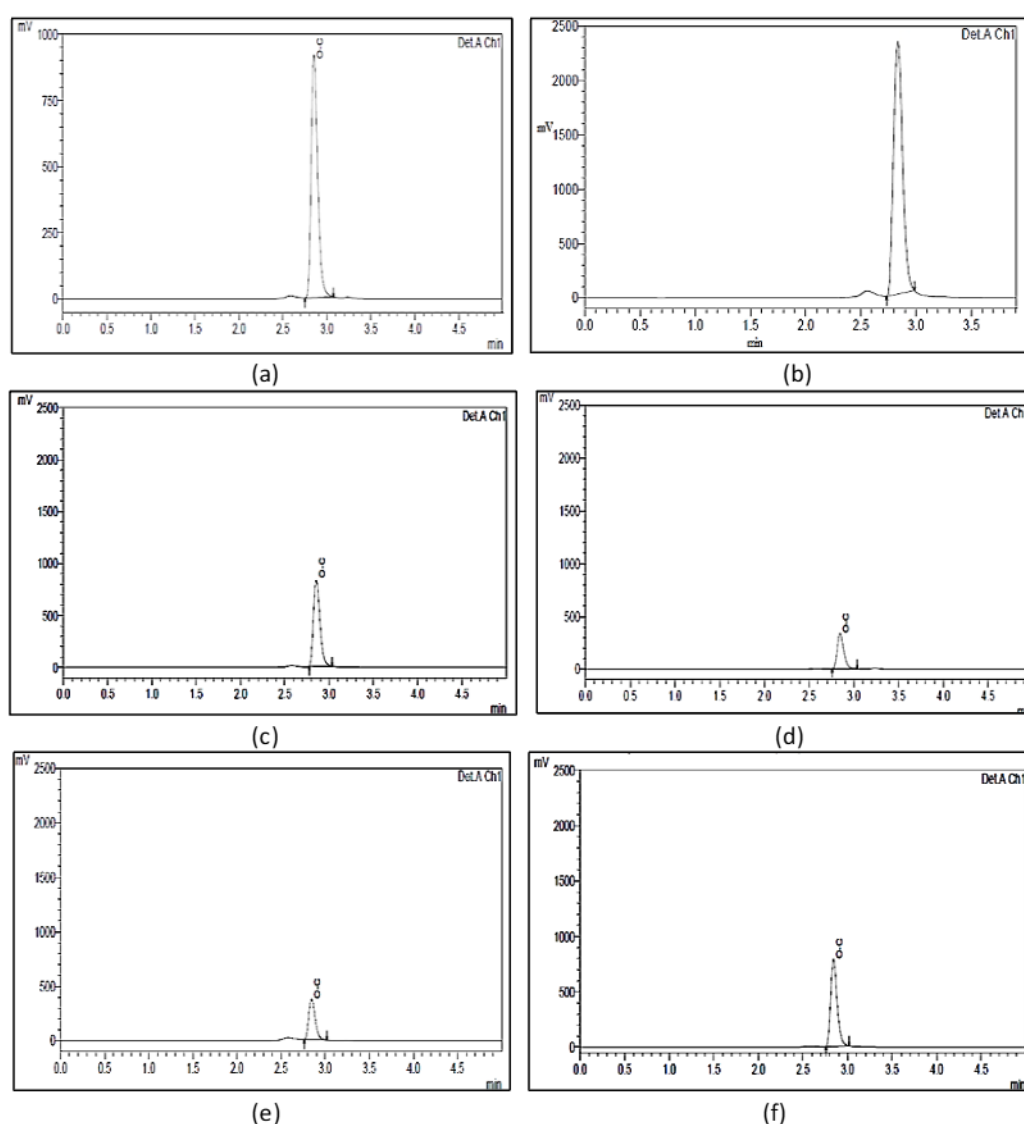


Fig. 4. HPLC Chromatograms of o-coumaric acid solute separated at specific retention time from UAE (a) Standard (10 µL) (b) MetOH extract (c) Chloro extract (d) EA extract (e) H₂O extract (f) Hex extract

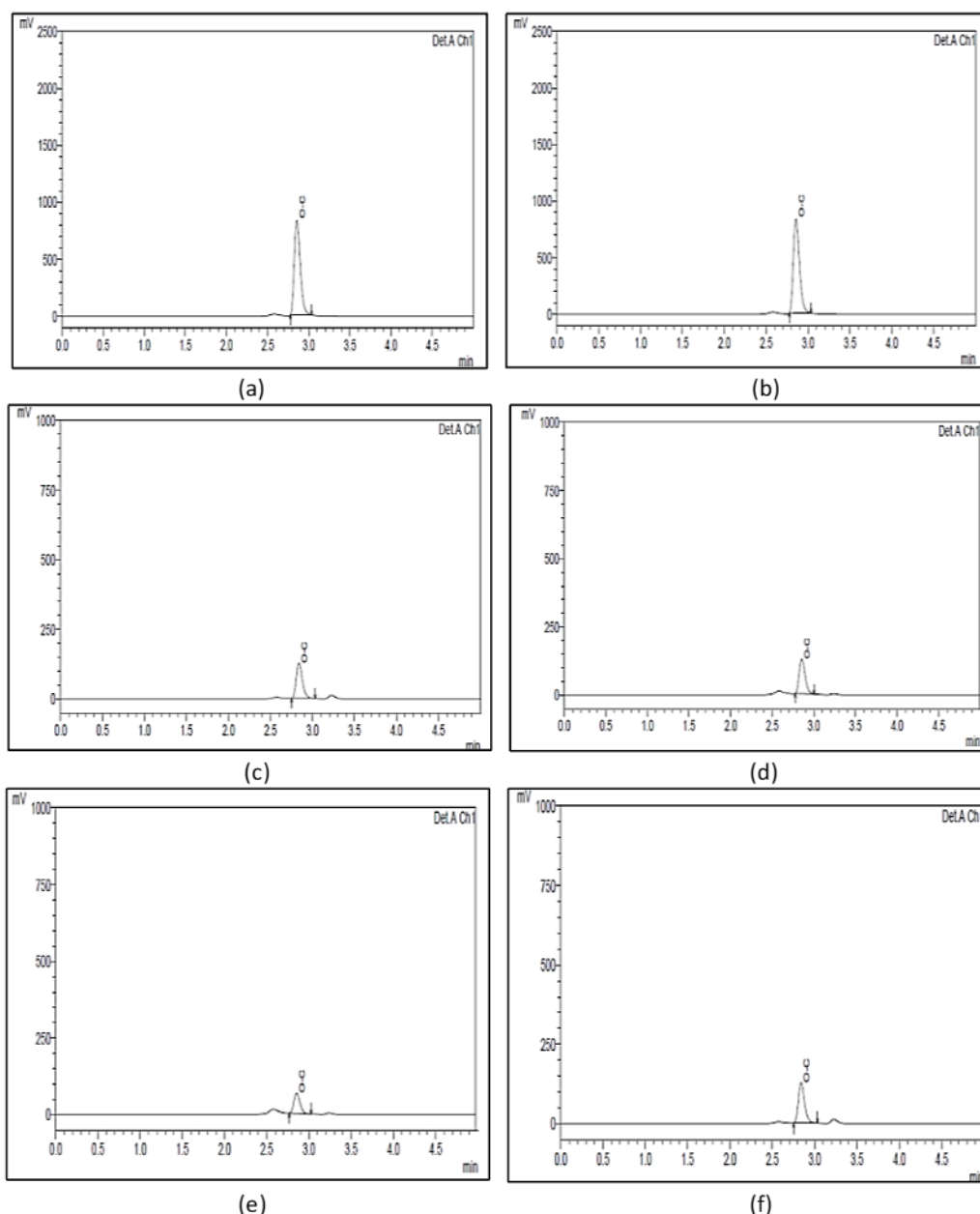


Fig. 5. HPLC Chromatograms of o-coumaric acid solute separated at specific retention time from SE (a) Standard (10 μ L) (b) MetOH extract (c) Chloro extract (d) EA extract (e) H₂O extract (f) Hex extract

The SEM analysis (refer Figure 2) revealed that UAE promotes better cell ruptures within the *G. sepium* leaves, facilitating better solvent penetration. In terms of solvent consumption, conventional extraction utilized about 100-200 ml, while the UAE method required only 50 ml of solvent, marking a fourfold decrease in solvent consumption (refer Figure 6). As a result, it is apparent that UAE performs more efficiently than SE. Figure 7 illustrates the concentration of o-coumaric acid in response to varying levels of methanol concentration. It indicated that methanol alone could suppress the concentration of o-coumaric acid to be extracted. The 80:20 (v/v) of methanol to water was the ideal as solvent extraction for o-coumaric acid compound from *G. sepium* leaves. The reason for this range could be due to the o-coumaric acid bioactive compound belongs to large chemical class, so solvent system for extraction is required, which can dissolve the large quantity of medium polar compound. This also parallel to the HPLC parameters used in this study.

The presence of higher concentration of methanol in the extraction solution creates a larger concentration gradient. This acts as a driving force for higher diffusion of solvent into the plant cells, thereby, improving the overall mass transfer of the system [36-38]. Moreover, increased amount of methanol enhances the contact area between the solvent and the solute (due to the relative polarity and the increase in effective swelling of the plant by water), thus improving the solubility of the phenolic compounds from within the plant cells [38,39]. Moreover, this study discovered that when the as methanol concentration increased to pure methanol, the concentration of o-coumaric acid phenolic compounds extraction yield decreased. This phenomenon can be attributed to the high concentrations of methanol can cause molecule congestion, which hinders the mass transfer and subsequently reducing the extraction efficiency of phenolic compounds [40,41].

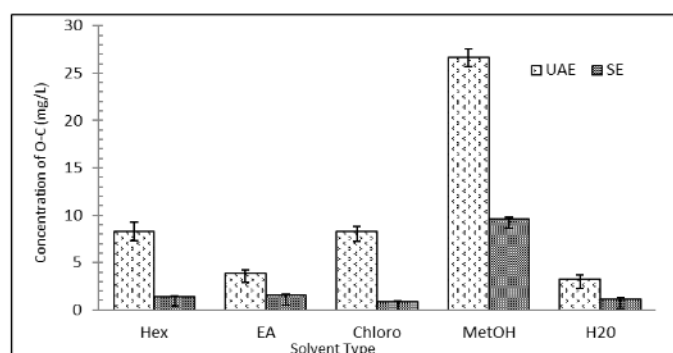


Fig. 6. Comparison of yield concentration from different solvent types and extraction systems

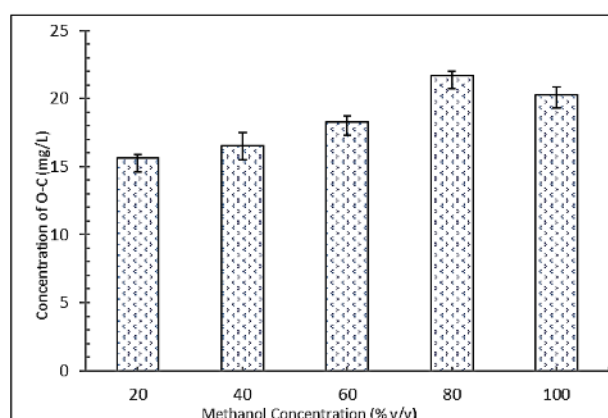


Fig. 7. Yield concentration from different methanol concentration

3.3 UAE Parameter Optimization with CCD

A total of 20 runs were conducted to study the effect of different variables on the amount of o-coumaric acid extracted from *G. sepium*. The experimental runs were randomized to minimize errors due to machining set-up. CCD was performed for three factors at five levels ($-\alpha$, -1, 0, +1, $+\alpha$) as in Table 2. In the Response Surface Methodology (RSM) analysis, the yield was set as the response variable (Y) and was a function of the coded independent variables: temperature (A), time (B), and the ratio of solvent to solid (C). The complete design matrix of the experiments, along with the results obtained, are displayed in Table 3.

Table 2
The employed variables in CCD experiment

Level	Independent parameters		
	Temperature (°C)	Time (min)	Solvent to solid (mL/g)
$-\alpha$	46.6	36.6	1.6
-1	50	40	5
0	55	45	10
+1	60	50	15
$+\alpha$	63.4	53.4	18.4

Table 3
Experimental design matrix and results

Run	Independent variables			Amount of o-coumaric acid, (mg L ⁻¹)	
	A: Temperature (°C)	B: Extraction time (min)	C: Solvent-Solid ratio (mL g ⁻¹)	Experimental	Predicted
1	50.01	40.00	5	26.84	26.92
2	50.00	40.01	15	16.37	16.46
3	46.60	45.00	10	36.14	35.92
4	50.01	50.00	5	34.03	34.12
5	50.00	50.00	5	29.16	30.05
6	50.00	50.00	10	10.04	9.920
7	50.01	50.00	15	18.73	18.91
8	55.00	45.00	18.41	21.02	21.46
9	55.00	45.00	1.59	15.20	15.37
10	55.00	50.00	5	22.18	22.31
11	55.00	53.41	10	10.09	9.97
12	55.00	36.59	10	10.09	9.97
13	55.00	53.41	10	10.09	9.97
14	55.00	50.00	15	13.92	14.74
15	60.00	40.00	5	10.47	10.37
16	60.00	40.00	5	10.47	10.37
17	60.00	40.00	15	9.27	9.27
18	63.41	45.00	10	9.43	9.53
19	60.00	50.00	5	9.93	9.93
20	60.00	50.00	5	9.93	9.93

3.3.1 Fitting the response surface models

The influence of extraction parameters on the yield of o-coumaric acid can be described using ANOVA to observe the adequacy and fitness of the developed model. Model terms are considered significant if their p-value is less than 0.05; they are not significant if their p-value is more than 0.1. The independent parameters of temperature (A), extraction time (B) and ratio of solvent to solid (C), along with the interactive parameters of AB, AC, BC and the quadratic parameters of A², B² and C², were identified as significant model terms in this study. The results from the single factor test, response surface analysis, and experimental values were considered. Additionally, the data from linear regression and binomial fitting analysis were summarized in Table 4.

Each parameter's impact on the experimental response is shown by the regression coefficient, where the value denotes an increase or decrease and whose magnitude is correlated with the weight of the effect [42]. The regression coefficient for A and C were negative and positive for B. The negative regression coefficient means that an increase in A and C would lead to a decrease in the yield of o-coumaric acid. The positive regression coefficient for C indicated that its increase would lead to an increase in the yield of o-coumaric. The regression coefficient of AB was negative and that of A² and C² were positive. The value stated for the regression coefficients indicated the size of the effect on the dependent variable. For parameter A, an increase by 1 unit would lead to a 7.84-fold decrease in the yield of o-coumaric acid, while an increase by 1 unit of B would lead to a 0.50-fold increase in the yield of o-coumaric acid.

The empirical model for the parameters on the yield of o-coumaric acid was given as in Eq. (8).

$$Y = 15.37 - 7.84A + 0.50B - 4.08C - 1.91 AB + 2.34 AC - 1.18 BC + 2.60A^2 - 2.21 B^2 + 0.5C^2 \quad (8)$$

Table 4
ANOVA for the model of the yield of o-coumaric acid

Source	Regression coefficient	df	Mean square	F-value	p-value prob > p	Remarks
Model	15.37	9	119.62	2252.07	< 0.0001	significant
A-Temp	-7.84	1	683.47	14582.09	< 0.0001	significant
B-Time	0.50	1	1.84	39.32	0.0004	significant
C-Rss	-4.08	1	120.48	2570.45	< 0.0001	significant
AB	-1.91	1	20.93	446.66	< 0.0001	significant
AC	2.34	1	31.51	672.36	< 0.0001	significant
BC	-1.18	1	8.05	171.76	< 0.0001	significant
A ²	2.60	1	85.89	1832.41	< 0.0001	significant
B ²	-2.21	1	36.38	776.26	< 0.0001	significant
C ²	0.50	1	1.84	39.29	0.0004	significant
Residual	0.33	7	0.047			significant
Lack of fit	0.15	2	0.077	2.23	0.2029	not significant
Pure error	0.17	5	0.035			
Cor total	1076.88	16				

The model gave a satisfactory fit with the experimental data because R^2 showed a value of 0.9997. This means that the model could explain about 99% of the total variability with the studied range. Figure 8 showed the comparison between the predicted versus actual data of yields. The plot indicated high level of agreement between the experimental and predicted data. Thus, this model can be used to calculate the yield of extraction with the studied range.

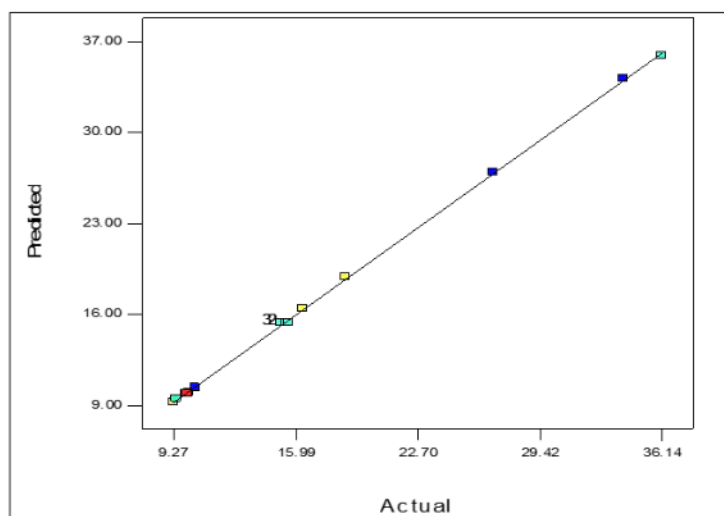


Fig. 8. Relationship between the experimental and predicted data

The ANOVA results and regression coefficients indicated that the contribution of the quadratic model was significant ($p < 0.05$). The Model F-value of 2552.07 implied the model was significant. The values of Prob > F which less than 0.0500 indicated the model terms were significant. Thus, in this study A, B, C, AB, AC, BC, A², B², C² were significant model terms. The fitness of the model was also investigated using the lack-of-fit test ($p > 0.05$), indicating the suitability of models to predict variations accurately [43]. In this study, the Lack of Fit F-value of 2.23 implied the Lack of Fit was not significant relative to the pure error. There was a 20.29% chance that the Lack of Fit F-value this large could occur due to noise. The non-significant lack of fit was good as the model was well fit.

3.4 Validation of Optimization Parameter

Optimal conditions for ultrasound-assisted extraction (UAE) to achieve the ideal *o*-coumaric acid content in the extract were determined. All factors and responses, along with their respective high-limit and low-limit experimental regions, were considered as listed in Table 3 to meet the criteria defined for optimal operations. The predictive values aligned closely with experimental values obtained using the optimum extraction parameters, as confirmed by a high regression coefficient ($R^2 = 0.9997$). Response surface and contour plots illustrating the effect of independent variables on *o*-coumaric acid extraction yield are shown in Figure 9.

Based on the fitted model, response surface plots have been generated and are presented in Figure 9. Two of the variables were put inside the experimental range, and one was set at a medium level when the plots were generated. Figure 9(a) shows the plot for variables of temperature and time with a medium level of time. As stated in Table 3, temperature was found to be highly significant ($p < 0.0001$), while time also showed statistical significance ($p < 0.0005$). An increase in temperature to 60°C would increase the yield of *o*-coumaric acid. The increase in temperature would lead to a greater number of cavitation bubbles, expanding the solid-solvent contact area, thereby enhancing solvent diffusivity and promoting the desorption of the desired compound. However, as the temperature approaches the boiling point of the solvent at 65°C, this effect diminishes. This can be explained by the increased vapor pressure of the solvent at high temperatures, which fills the cavitation bubbles formed during the rarefaction cycle. Consequently, the pressure gradient between the inside and outside of the bubble decreases. While there may be a higher number of cavitation bubbles at higher temperatures, they implode with less intensity, causing less cellular damage and resulting in decreased yield [44].

Figure 9(b) depicts the correlation between temperature and solvent-to-solid ratio at a moderate temperature level. In accordance with the findings presented in Table 3, factor time was shown to be statistically significant ($p < 0.0005$) in the study, and it was observed that a reduction in time led to an increase in *o*-coumaric acid concentration. This unusual occurrence could be explained by certain compounds degrading over time when exposed to ultrasonic radiation. Prolonged ultrasonic exposure may cause free radicals to form in the solvent, which may cause cell rupture [45]. In light of this, the UAE method offers a more efficient alternative to traditional SE method owing to the shorter extraction time.

Lastly, Figure 9(c) shows the plot for solvent-to-solid ratio and time with a medium level of solid-to-solvent ratio, where both solvent-to-solid ratio and time were significant. An increase in solvent-to-solid ratio and a decrease in time are associated with an increase in *o*-coumaric acid yield. This is due to a higher solvent-to-solid ratio increases the concentration gradient between the solid and liquid phases, which increases the driving force of mass transfer and facilitates the diffusion of phenolic chemicals from plant cells. However, further increases in this ratio can lead to a slight decrease in extraction yield, as seen in Figure 9(c). As stated Yancheshmeh *et al.*, [46], higher solvent-to-solid ratios intensify the cavitation effect, potentially causing the degradation of desirable solutes and hindering extraction efficiency. Despite this, when compared to variations in temperature and time, changes in solvent-to-solid ratio have minimal impact on *o*-coumaric acid yield.

Based on statistical analysis, the developed model was found to be significant at F-value of 2552.07. The high values of R^2 (0.9997) and adjusted R^2 (0.9941) indicate a strong correlation (see Table 4). The predicted correlation coefficient (0.9993) also confirms its high significance. Adeq Precision, which measures the signal-to-noise ratio, was greater than 4, indicating desirable precision. The obtained ratio of 160.499 suggests an adequate signal in this study. Moreover, the coefficient of variation (CV = 1.30%) and adequate precision values further support the model's ability

to delineate the relationship between extraction variables and the response of *o*-coumaric acid.

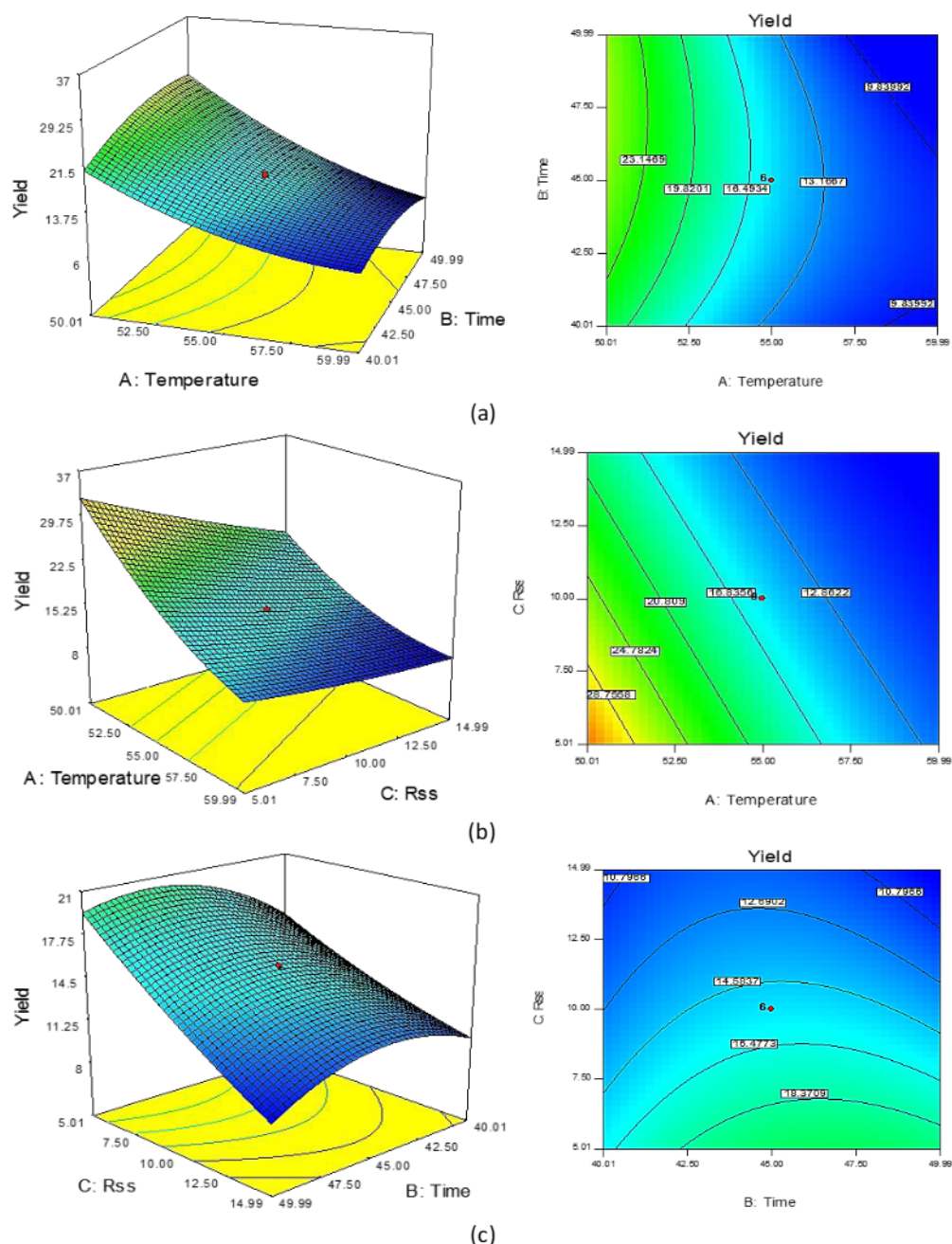


Fig. 9. Response surface and contour plots for the effect of independent variables on *o*-coumaric acid extraction yield (a) A: Interaction between temperature (°C) and time (min) (b) B: Temperature (°C) and Rss (mLg⁻¹) (c) C: Rss (mLg⁻¹) and time (min)

Table 4

Significance test table of regression coefficient

Std. Dev	0.22	R ²	0.9997
Mean	16.68	Adj R ²	0.9993
C.V %	1.30	Pred R ²	0.9941
PRESS	6.39	Adequate precision	160.449

Applying the methodology of desired function, the optimum level of various parameters was obtained and it indicated that extraction temperature of 45°C, extraction time of 46.60 min and ratio

of solvent to solid of 10:1 mg L⁻¹ resulted to *o*-coumaric acid content of 34.12 ± 0.015 mg L⁻¹. These optimize conditions could be considered as optimum as well as feasible conditions.

3.5 Characterisation of *O*-Coumaric Acid in *G. Sepium* Leaf Extract

3.5.1 High performance liquid chromatography

The extraction procedure was optimized for extracting *o*-coumaric acid from *G. sepium* leaves. Complete extraction of *o*-coumaric acid was achieved through successive solvent extraction with methanol. An accurate isocratic RP-HPLC method was developed and validated using optimized chromatographic conditions as presented in Table 3. The obtained chromatograms displayed a peak corresponding to *o*-coumaric acid at a retention time of 2.78 minutes. The regression coefficient obtained from the linearity plot for *o*-coumaric acid was 0.979, indicating good linearity of the method, as depicted in Figure 10. The chromatogram in Figure 11(a) shows the similarity peak detected in *G. sepium* leave extract as comparable to the standard at retention time of 2.78 min, indicating the compound was identified as *o*-coumaric acid as shown in Figure 11(b).

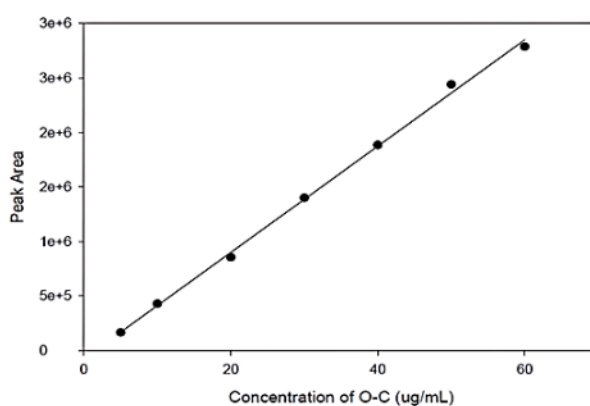


Fig. 10. Linearity plot of *o*-coumaric acid

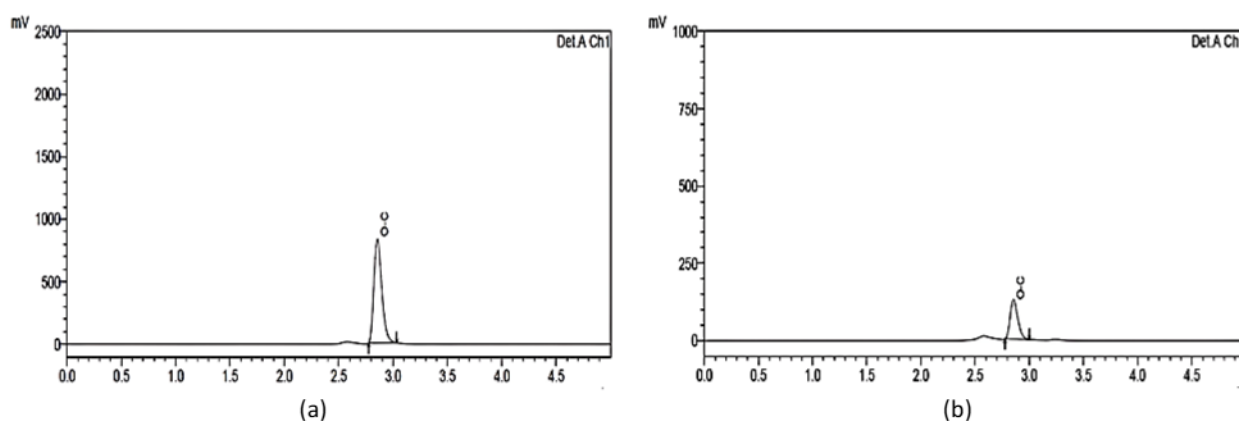


Fig. 11. HPLC chromatograms of *o*-coumaric acid (a) Standard (b) Dried extract

3.5.2 Fourier-transform infrared spectroscopy of *o*-coumaric acid

This study utilized infrared (IR) spectroscopy to identify the functional group of active components based on the peak value in the infrared region. The determination and identification of the functional groups in the extract from *G. sepium* leaves were confirmed through infrared spectroscopy by comparing its spectra with that of standard *o*-coumaric acid (refer to Figure 12(a)).

As shown in the Figure 12(b), the FTIR spectrum of the extract from *G. sepium* 's leaves, obtained through the UAE, contains peaks occurring at wavenumbers similar to those in the spectrum of standard o-coumaric acid, indicating the formation of o-coumaric acid on the surface of the extract derived from *G. sepium* leaves.

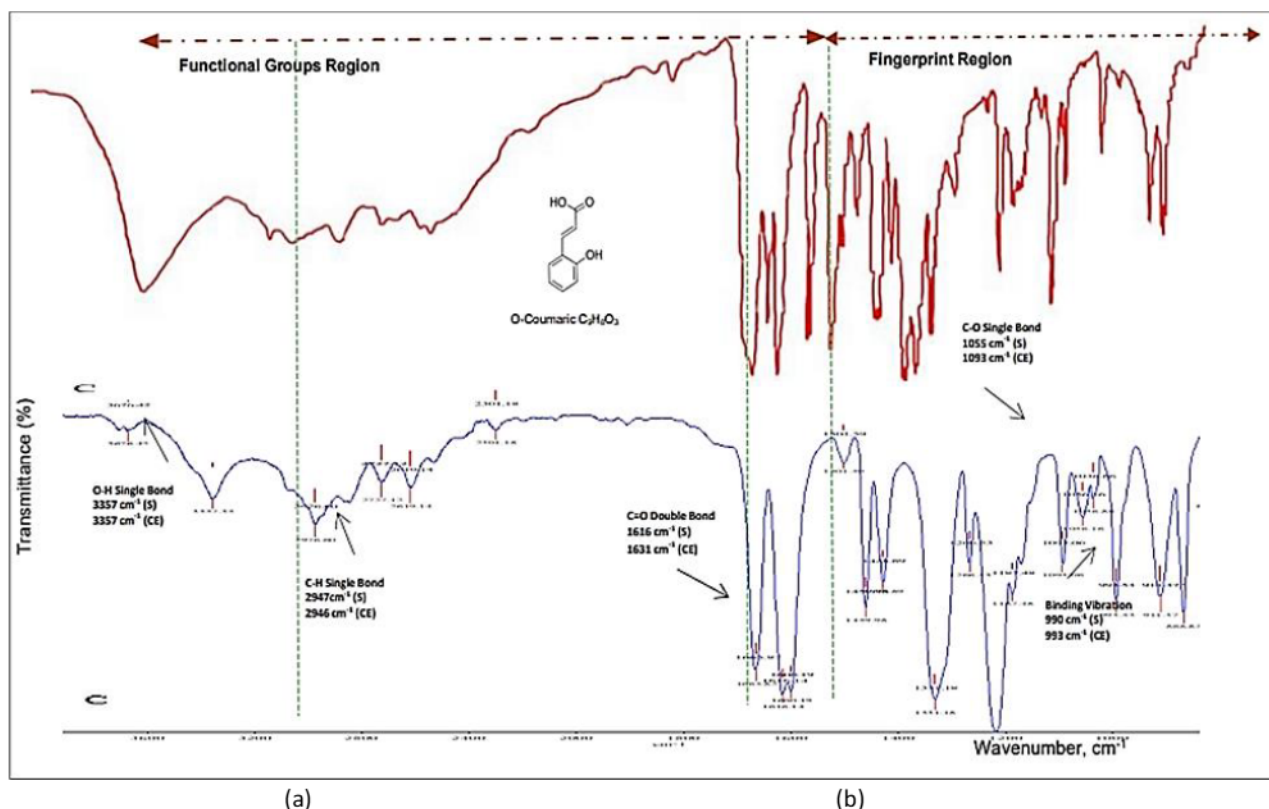


Fig. 12. FT-IR spectra of o-coumaric acid for (a) Standard (b) Extract from *G. sepium* 's leaves

When comparing the spectra of the extract with standard o-coumaric acid, a disappearance of bands characteristic of stretching vibrations of the carboxyl group can be observed. The 1700 cm^{-1} band (carboxylic acid) and the 1631 cm^{-1} band (C-O group) were absent due to the presence of hydrogen bonds, as indicated by the presence of a 1266 cm^{-1} band between hydroxyls (1616 cm^{-1}) and carboxylic acid groups (1200 cm^{-1}). Apart from that, the very strong bands assigned to the stretching vibrations of the carboxylate group are located in the range $1543\text{--}1380 \text{ cm}^{-1}$, including peaks at 1459 cm^{-1} and 1501 cm^{-1} , which are caused by the absorption of bending deformation $\delta(\text{C-H})$ vibration [47]. This is in agreement with the study from [48], which claims that the disappearance of bands characteristic of stretching vibrations of the carboxyl group likely occurs in the structure of the obtained o-coumaric acid.

In the present study, the extract derived from *G. sepium* 's leaves through the UAE contained phenolic and alcohol compounds that were responsible for antibacterial activity. Based on the standard IR spectra of the o-coumaric acid, the slightly broad and intense -OH single bond stretching frequencies at 3354 cm^{-1} shifted to 3357 cm^{-1} in the *G. sepium* extraction. The intensity at 990 cm^{-1} (vinyl group) was partially attributed to the C-O (phenol) stretch from alkyl ester, ether, methoxy and alcohol groups [49,50]. Additionally, strong peaks at 1428 cm^{-1} indicate asymmetric and symmetric stretching vibrations, which are related to carboxylate anions (COO^-) [51]. All these identified organic compounds exhibited good antimicrobial activity [52].

Additionally, in the region of C-H stretching frequencies, two or three bands of weak to medium intensity originally observed at 2945 cm^{-1} shifted to 2976 cm^{-1} . Furthermore, the bands located in

the range of 2045-2945 cm^{-1} also experienced a shift to 2301-2936 cm^{-1} in the extract, attributable to the C-H stretching modes of the aromatic ring and the double bond between C₈ and C₉ atoms. The results indicate variations in the molecular structure (shifts or disappearances of certain peaks, as well as the emergence and of new peaks) in the obtained extract. All these changes are likely due to the destructive effects of ultrasonic cavitation induced by UAE process. Overall, the application of the novel UAE extraction method on *G.sepium* provides valuable insights into the structural characteristics of *o*-coumaric acid, shedding light on its properties and potential applications.

3.6 Kinetic Study of *O*-Coumaric Acid Extraction

As shown in the Figures 13, around 20% of *o*-coumaric acid was obtained in the first hour of extraction. The amount of *o*-coumaric acid extracted after one hour was 22.85, 25.9, 28.65, 29.55 and 31.9 mg g^{-1} for the extraction temperatures of 45, 50, 60 and 65°C respectively. For all studied temperatures, the extraction rate was highly fast at the beginning of the operation followed by the slowing rate for the remaining of extraction period. As any raw materials were dipped into a solvent, the bioactive compounds inside the material were solubilized and get extracted quickly inducing a fast increase in the extraction rate. As the *o*-coumaric acid concentration was low in the solvent at the beginning of the extraction process, the compound diffused rapidly from the leaf surface to the liquid phase.

As the time passing by, the extraction rate becomes slower. The trend was due to the external diffusion phenomenon, which means diffusion took place through stagnant liquid film around the solid particles. It was also due to the increase of *o*-coumaric acid concentration in the solvent, the diffusion rate decreased and the extraction rate was slower consequently. When the maximum amount of *o*-coumaric acid was obtained, the yield level remained invariable even by extending the reaction time.

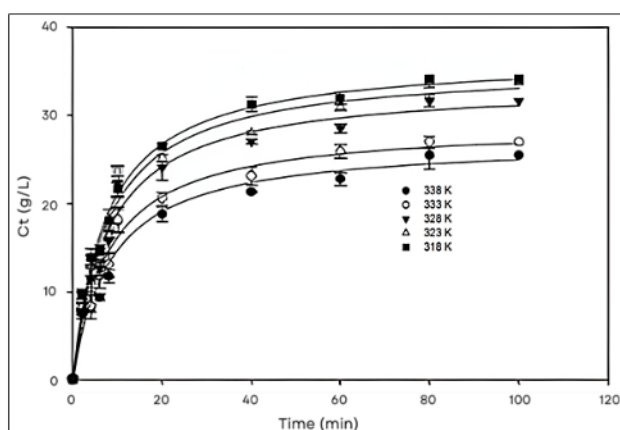


Fig. 13. Concentration of extracted *o*-coumaric acid versus time at different temperatures in ultrasound assisted extraction of *G. sepium* leaves for 80 min

3.7 Extraction Predictive Model

The experimental results were analyzed using a second order model by plotting t/C_t versus time. Figure 14 implied a good agreement between the second order model and the experimental results with high coefficient of determination. This agreement confirmed the assumptions that ultrasound assisted extraction of *G. sepium* leaves took place in two subsequent stages of fast and slow. From Table 5, all the saturated extraction capacity, C_s , extraction rate constant, k , and initial extraction

rate, h , were linearly proportional to the extraction temperature. The C_s increased from 25.64 g/L to 34.72 g L⁻¹ by increasing the temperature from 318 to 338 K. For extraction constant rate, k , it increased from 4.75×10^{-3} to 7.67×10^{-3} L g⁻¹ min and the initial extraction rate, h , increased from 3.12 to 9.24 g L⁻¹ min K with the increasing temperature from 318 to 338, respectively.

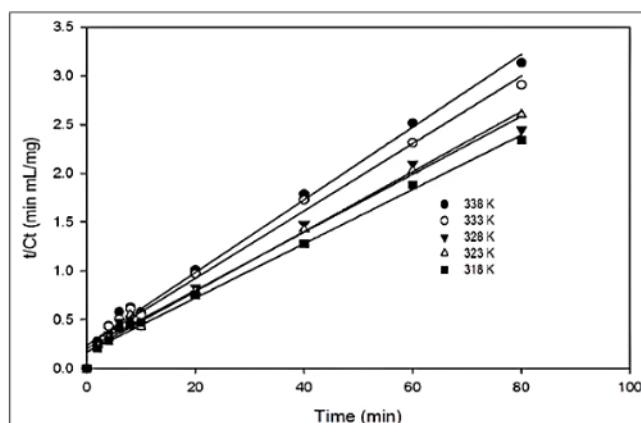


Fig. 14. Second order extraction kinetics of *G. sepium* leaves at different temperatures using methanol for 80 min

Table 5

Linearization of kinetic model of second order ultrasound assisted extraction of *G. sepium* leaf under different temperatures

T (K)	Linear equation	C_s (g L ⁻¹)	K (L g ⁻¹ min)	H (g L ⁻¹ min)	R^2
318	$y = 0.039x + 0.3204$	25.64	4.75×10^{-3}	3.12	0.9901
323	$y = 0.0373x + 0.2661$	26.81	5.23×10^{-3}	3.75	0.9945
328	$y = 0.0331x + 0.1801$	30.15	6.11×10^{-3}	5.55	0.9906
333	$y = 0.0308x + 0.136$	32.46	6.95×10^{-3}	7.32	0.9912
338	$y = 0.0288x + 0.1081$	34.72	7.27×10^{-3}	9.24	0.9921

3.8 Activation Energy

The second order extraction rate constants increased with the increasing temperatures. The phenomena can be described by the Arrhenius equation. Temperature independent factor (A) and activation energy of extraction (E) was further calculated by plotting $\ln(k)$ against $1/T$ as in Figure 15.

$$k = 0.00757 \text{ Exp} \left[\frac{-7309.67}{8.314T} \right] \quad (9)$$

From Figure 15, there was a linear relationship between the second order extraction rate constant and the inverse temperature with linear equation of $y = -2671.5x + 3.0406$ and high R^2 value of 0.9942.

$$h = 0.00757 \text{ Exp} \left[\frac{-7309.67}{8.314T} \right] \times (0.0033T^2 - 1.6792T + 227.09) \quad (10)$$

The temperature independent factor (A) and activation energy (E) were found to be 0.00757 L g⁻¹ min and 7309.67 J mol⁻¹ respectively from the slope and intercept. Since the activation energy is positive, thus the extraction of *G. sepium* leaf was considered to undergo endothermic reaction.

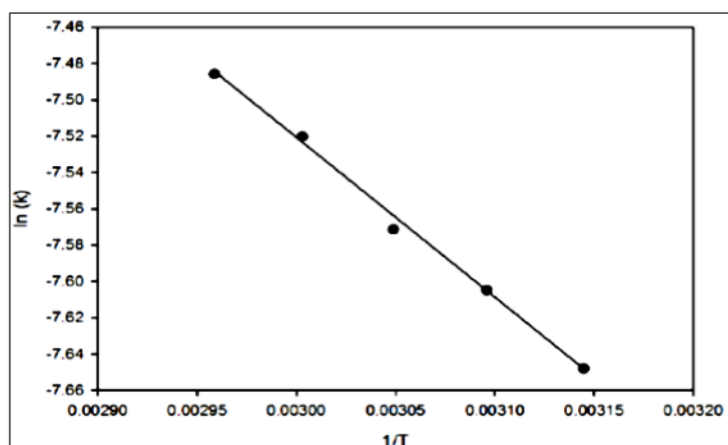


Fig. 15. Linear relationship between second order extraction constant, (ln k) and temperature for solid liquid extraction of *G. sepium* leaves

3.9 Modelling of Extraction Process

A relationship between the saturated extraction capacity and temperature was further established by plotting C_s versus T as shown in Figure 16. Polynomial equation was derived from the graph as shown in Eq. (11).

$$Y = 0.033 X^2 - 1.679 X + 227.09 \quad (11)$$

Therefore, C_s can be furthered correlated by the following empirical equation:

$$C_s = 0.0033T^2 - 1.6792T + 227.09 \quad (12)$$

Based on the relation between curve plot and by using experimental data, it showed that there was a positive correlation between saturated extraction capacity, C_s and temperature. C_s values are non-linearly proportional with extraction temperature. The values of h could also be obtained through the following Eq. (13). The evolution of C_t versus time and temperature for ultrasound assisted extraction can be further described as.

$$C_t = \frac{t}{\frac{1}{h} + \left[\frac{t}{0.0033 T^2 - 1.6792 T + 227.09} \right]} \quad (13)$$

Eq. (14) represented the predictive model for ultrasound assisted extraction of *G. sepium* leaf using methanol for specific range of extraction time and temperatures. However, this equation was valid for only to predict the extracted amount of *o*-coumaric acid at any given extraction time up to 40 minutes and temperature in range of 318 to 338 K. This finding indicated that more *o*-coumaric acid could be extracted at longer extraction temperature up to methanol boiling point. By using the predictive model, the amount of *o*-coumaric acid (C_t) in different combination of time (t) and temperature (T) can be calculated. This feature was useful for economic assessment in industrial application. The kinetics of extraction was developed based on the assumption of a second order extraction mechanism with regression coefficient value of $R^2 > 0.98$. The *o*-coumaric acid concentration obtained (C_t) was compared with the experimental results for the second order model validation at the lowest and highest temperature range limits (313 ± 338K). The good fitting between

experimental and calculated data was obtained for the model, showing the validity of the relationship.

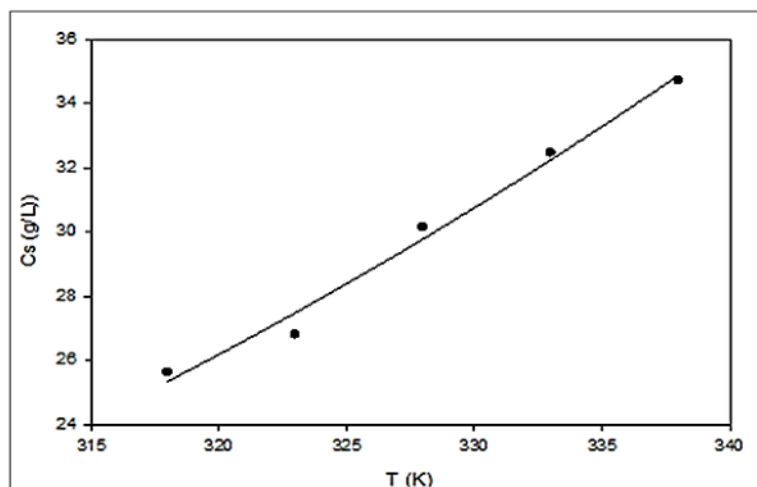


Fig. 16. Relationship between saturated extraction capacity, C and temperature for UAE of *G. sepium* leaves

4. Conclusions

In conclusion, this work indicates that the ultrasound-assisted extraction (UAE) can efficiently replace the traditional Soxhlet extraction technique for extracting *o*-coumaric acid from *G. sepium* leaves. Through RSM analysis, the optimization conditions for *o*-coumaric acid concentration were determined: extraction temperature of 46.60°C, extraction time of 45 minutes, and a solvent-to-solid ratio of 10:1 mg L⁻¹, resulting in an *o*-coumaric acid content of 35.92 ± 0.015 mg L⁻¹. Notably, UAE achieved higher concentrations of *o*-coumaric acid at lower extraction temperatures, indicating its superior efficiency over SE methods. Additionally, ultrasound cavitation increases the surface area of solid materials, enhancing mass transfer and extraction rates, which improves the yield of *o*-coumaric acid from *G. sepium* leaves. The extract derived from UAE has also induced the presence of phenolic and alcohol compounds, which contribute to its remarkable antibacterial action.

Apart from that, the kinetic extraction study clearly showed that the predictive model showed good fit between experimental and calculated data, enabling the calculation of *o*-coumaric acid amounts under different time and temperature combinations, which is valuable for economic assessment in industrial applications. This research not only discloses ideal conditions for *o*-coumaric acid extraction, but it also establishes UAE as a transformative approach in achieving sustainable and high-yield recovery of bioactive components from plants.

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