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Innovative Techniques in Sandalwood Oil Extraction: Optimizing Phenolic and Flavonoid Yields with Subcritical Ethanol

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Abstract: Sandalwood essential oil, known for its rich content of phenolic and flavonoid compounds, holds great promise for applications in perfumery and medicine. However, traditional production methods have raised concerns regarding their environmental impact and sustainability. This study explored subcritical ethanol extraction as a green technique to address these concerns. Under optimized conditions (6.04 MPa, 109 °C, and 2.01 mL/min), maximum oil yield reached 4.04% with substantial total phenolic compounds (TPCs) of 4.11 mg GAE/100 g and total flavonoid compounds (TFCs) of 8.85 mg QE/100 g in the extracted sandalwood oil. Furthermore, the oil displayed notable antioxidant activity AA of 71.68%. The temperature was identified as a significant factor affecting oil yield, TPCs, TFCs, and AA. The fine-tuning of the extraction temperature enhanced the desired characteristics, improving bioactive compound yields and heightening antioxidant potential. This study uses a green extraction technique to contribute to sustainable sandalwood essential oil production.

Keywords: sandalwood essential oil; phenolic; flavonoids; antioxidant activity; subcritical ethanol extraction

Citation: Zhang, W.; Rizkiyah, D.N.; Putra, N.R. Innovative Techniques in Sandalwood Oil Extraction: Optimizing Phenolic and Flavonoid Yields with Subcritical Ethanol. *Separations* **2024**, *11*, 201. https://doi.org/10.3390/separations11070201

Academic Editors: Di Chen and Haobo Zheng

Received: 23 May 2024
Revised: 22 June 2024
Accepted: 26 June 2024
Published: 27 June 2024

1. Introduction

Sandalwood (*Santalum album*) is a highly valued plant species renowned for its distinct aroma and diverse applications in the traditional medicine, perfumery, and cosmetic industries [1–3]. It contains a rich array of bioactive compounds, including phenolic and flavonoid compounds, associated with various health benefits [3,4]. Phenolic compounds, such as phenolic acids and lignans, possess potent antioxidant and anti-inflammatory properties, while flavonoids are known for their antioxidant, antimicrobial, and anticancer activities [1]. Therefore, the extraction and isolation of these compounds from sandalwood hold great significance for their potential therapeutic applications.

Over the years, numerous extraction techniques have been employed to isolate bioactive compounds from plant materials. Among these techniques, subcritical ethanol extraction has emerged as a promising method for obtaining phenolic and flavonoid compounds with high yields and preserving their structural integrity [5,6]. Subcritical ethanol uses ethanol as a solvent in an extraction process conducted at temperatures and pressures below the critical point of ethanol. The critical point of a substance is the specific temperature and pressure at which it transitions from a liquid to a gas (vapor) phase and beyond which it becomes a supercritical fluid. In the case of ethanol, its critical point occurs at approximately 243.3 °C (469.94 °F) and a pressure of around 6.39 MPa (61.8 atm) [7]. In subcritical ethanol extraction, the process occurs at temperatures lower than the critical temperature of ethanol. Typically, subcritical ethanol extraction is performed at temperatures below 120 °C to ensure that the ethanol remains liquid throughout the extraction process.

Secondly, subcritical ethanol extraction operates at lower temperatures, minimizing the degradation of thermally sensitive compounds that may occur during high-temperature extraction [8]. By employing milder conditions, the structural integrity and bioactivity of the extracted compounds are better preserved, ensuring the retention of their beneficial properties. Moreover, subcritical ethanol extraction exhibits high extraction efficiency, facilitating the optimal recovery of target compounds from the plant material. This efficient extraction process maximizes the yield of bioactive compounds, making it a cost-effective and resource-efficient technique [9].

Due to these advantages, subcritical ethanol extraction has gained recognition and application across various industries, including food, pharmaceuticals, and cosmetics. The method enables the effective extraction of bioactive compounds from natural sources, providing a sustainable and environmentally friendly approach to obtaining valuable compounds for product development and formulation. By maintaining the quality and potency of the extracted compounds, subcritical ethanol extraction contributes to producing high-quality ingredients with potential applications in functional foods, nutraceuticals, therapeutic agents, and cosmetic formulations.

Therefore, this study aimed to determine the optimal parameters for subcritical ethanol extraction to obtain a high yield of sandalwood essential oil with high amounts of TPC, TFC, and antioxidant activity.

2. Materials and Methods

2.1. Preparation of Raw Material

The sandalwood used in the extraction process was sourced from Nusa Tenggara Timur, Indonesia, renowned for its high-quality sandalwood. Firstly, the moisture content of the sandalwood was carefully controlled, ensuring it remained below 8%. Next, the dried sandalwood underwent a grinding process, resulting in particles with a desired particle size range of 355 to 425 µm. Following grinding, the prepared sandalwood particles were placed in a freezer at a temperature of −20 °C. Freezing the sandalwood helped preserve the delicate phenolic and flavonoid compounds, which can be degraded under higher temperatures. The sandalwood was optimally prepared for subcritical ethanol extraction through meticulous control of moisture content, particle size reduction, and freezing. This preparation process aimed to ensure maximum extraction efficiency and maintain the quality of the extracted phenolic and flavonoid compounds.

2.2. Chemicals Used

The ethanol used for extraction was procured from Sigma-Aldrich (St. Louis, MI, USA) as an analysis-grade solvent. Na_2CO_3, Al_2NO_3, Folin–Ciocalteu reagent, and CH_3COOK were obtained from Fisher Scientific (Atlanta, GA, USA). Quercetin and gallic acid, used as referent materials for calibration and plotting the regression graphs, were purchased from Sigma-Aldrich (St. Louis, MI, USA). 2,2-Diphenyl-1-picrylhydrazyl was also acquired from Sigma-Aldrich (St. Louis, MI, USA) to analyze antioxidant activity.

2.3. Subcritical Ethanol Extraction

The subcritical ethanol extraction process utilized various equipment, including a 5 mL extraction vessel, a high-pressure pump, a back pressure regulator, a pressure gauge, and an oven. Figure 1 provides a schematic representation of the subcritical ethanol extraction setup. Table 1 outlines the three variables involved in the process: pressure (ranging from 2 to 10 MPa), temperature (ranging from 90 to 110 °C), and flow rate (ranging from 2 to 6 mL/min). The extraction aimed to recover phenolic and flavonoid compounds and achieve a high antioxidant activity in sandalwood oil.

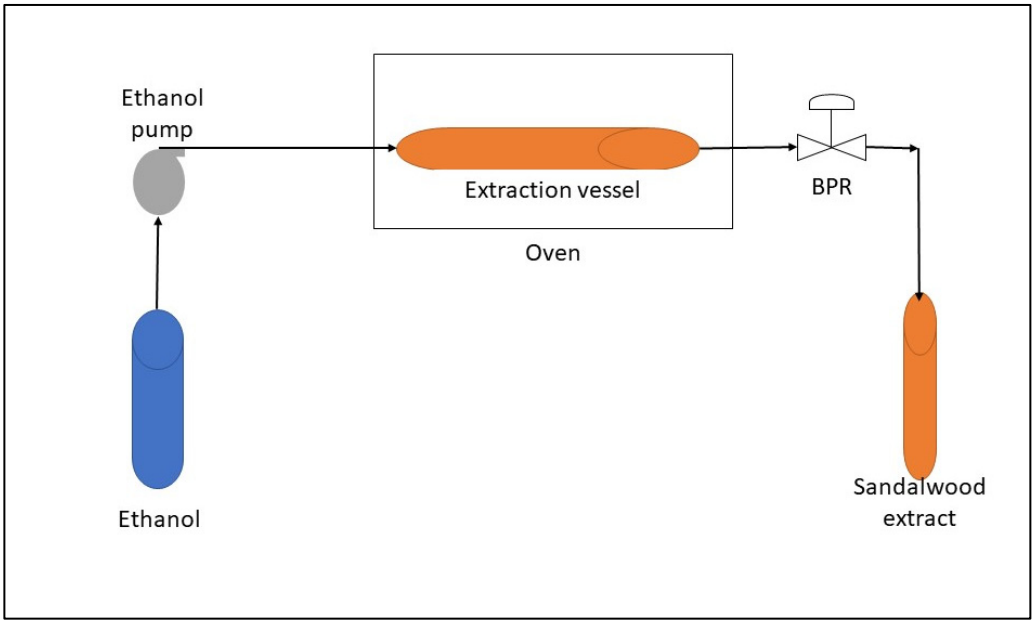

Figure 1. Schematic representation of the subcritical ethanol extraction setup.

Table 1. Parameters and responses of subcritical ethanol extraction for sandalwood.

Run	E_1: Pressure	E_2: Temperature	E_3: Flow rate	S_1: Oil Yield	S_2: Phenolic	S_3: Flavonoid	S_3: AA
	MPa	°C	mL/min	%	Mg GAE/100 g	Mg QE/100 g	%
1	2	100	2	3.84	3.72	8.85	71.68
2	2	100	6	3	2.61	7.3	70.27
3	6	100	4	3.47	3	7.69	70.62
4	6	100	4	3.41	3.11	7.8	70.72
5	10	90	4	2.26	2.26	6.95	69.95
6	6	100	4	3.42	3.42	8.11	71
7	6	90	6	2.82	2.82	7.51	70.46
8	6	100	4	3.38	3.18	7.87	70.79
9	6	110	2	3.84	3.94	8.63	71.48
10	6	90	2	3.59	3.78	8.47	71.33
11	10	110	4	3.51	3.21	7.9	70.81
12	6	110	6	3.58	3.53	8.22	71.11
13	2	90	4	2.56	2.21	6.9	69.92
14	6	100	4	3.42	3.04	7.73	70.66
15	10	100	2	3.86	3.58	8.27	71.15
16	2	110	4	3.78	3.52	8.21	71.1
17	10	100	6	3.46	3.24	7.93	70.84

The chosen parameters for the extraction of sandalwood using subcritical ethanol were pressure, temperature, and flow rate. The pressure range of 2 to 10 MPa was selected to ensure that ethanol remains in its subcritical state, which is crucial for effective penetration and dissolution of a wide range of compounds from the sandalwood matrix [7]. This range optimizes the yield of valuable components while avoiding unnecessary energy consumption and equipment stress that higher pressures might cause. The temperature range of 90 to 110 °C balances the efficiency of extraction with the stability of the extracted compounds. At these temperatures, subcritical ethanol exhibits enhanced solvent properties, improving the solubility of the target compounds [10]. This range prevents the degradation of sensitive compounds that could occur at higher temperatures and ensures sufficient extraction efficiency compared to lower temperatures. A flow rate of 2 to 6 mL/min was chosen to optimize the contact time between ethanol and the sandalwood material [11]. A moderate

flow rate ensures effective interaction for the dissolution of target compounds, avoiding incomplete extraction due to inadequate solvent penetration or inefficient extraction from too rapid solvent flow.

Approximately 1 ± 0.05 g of finely ground sandalwood was placed in the extraction vessel to initiate the extraction. Analysis-grade ethanol was then pumped into the extraction tube containing the powdered sandalwood. The flow rate of subcritical ethanol was adjusted according to the pump's flow rate setting. The extraction process lasted for 20 min. The extraction process was set to 20 min to ensure effective and efficient extraction of sandalwood compounds. This duration is long enough for the ethanol to fully penetrate the sandalwood and dissolve the desired compounds. It also helps prevent the breakdown of sensitive compounds that could occur with longer extraction times [12]. The oven temperature was set based on the specific subcritical ethanol extraction variable being investigated. At the same time, the pressure was controlled through the use of the pressure gauge on the back pressure regulator.

2.4. Method of TPC Analysis

TPC analysis was followed as in Rizkiyah et al. [13]. Firstly, to prepare the Gallic Acid Stock Solution, 0.500 g of dry gallic acid was dissolved in 10 mL of ethanol in a 100 mL volumetric flask. The solution was then diluted with water to the desired volume. Next, for the Na_2CO_3 solution, 200 g of anhydrous Na_2CO_3 was dissolved in 800 mL of water and heated to a boiling point. After cooling, the solution was filtered, and water was added to make a final volume of 1 L. To establish a calibration curve, various volumes (0, 1, 2, 3, 5, and 10 mL) of the Gallic Acid Stock Solution were added to separate 100 mL volumetric flasks. Each flask was diluted with water to reach the desired volume, resulting in concentrations of 0, 50, 100, 150, 250, and 500 mg/L of gallic acid. For each calibration solution, sample, or blank, 20 µL was pipetted into individual cuvettes. To each cuvette, 1.58 mL of water and 100 µL of the Folin–Ciocalteu reagent were added. The contents were mixed thoroughly and allowed to react for a duration of 30 s to 8 min. Following the reaction, 300 µL of the Na_2CO_3 solution was added to each cuvette, and the solutions were shaken to ensure proper mixing. The cuvettes were then left at a temperature of 20 °C for a period of 2 h, and the absorbance of each solution was measured at 765 nm against the blank (the solution with 0 mL of Gallic Acid Stock Solution). Alternatively, the solutions could be incubated at 40 °C for 30 min before measuring the absorbance. It is also worth noting that the spectrophotometer used for these measurements was a Shimadzu model from Japan, ensuring precise and accurate quantification of the extracted compounds. The TPC value was expressed in milligrams of gallic acid equivalents per one hundred grams of the extract (mg GAE/100 g).

2.5. Method of TFC Analysis

TFC analysis was followed as in Idham et al. (2022) [13]. 1 mg of sandalwood oil was diluted with 3 mL of ethanol. Subsequently, 1 mL of the diluted oil was combined with 0.2 mL of 1.0 M CH_3COOK and 0.2 mL of Al_2NO_3 10%. The resulting mixture was allowed to rest at room temperature for 30 min. To establish a calibration curve, various volumes (0, 1, 2, 3, 5, and 10 mL) of the Quercetin Stock Solution were added to separate 100 mL volumetric flasks. Each flask was diluted with water to reach the desired volume, resulting in quercetin concentrations of 0, 50, 100, 150, 250, and 500 mg/L. The absorbance of each solution was measured at 415 nm against the blank (the solution with 0 mL of Quercetin Stock Solution). It is also worth noting that the spectrophotometer used for these measurements was a Shimadzu model from Japan, ensuring precise and accurate quantification of the extracted compounds. The TFC value was expressed in milligrams of quercetin equivalents per one hundred grams of the extract (mg QE/100 g).

2.6. Method of Antioxidant Activity by Radical Scavenging Capacity DPPH

Initially, 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) was diluted using ethanol as the solvent. The concentration of DPPH was set to 0.5 mM. Next, 1 mg of sandalwood oil was diluted with 10 mL of ethanol, resulting in a 0.1 mg/mL concentration. A total of 2 mL of the ethanolic extract (0.1 mg/mL) was mixed with 2 mL of the DPPH solution (0.5 mM). The mixtures were then incubated in darkness at 25 °C for 30 min. The absorbance of the sample was measured using a UV-Vis spectrophotometer (Jasco, Tokyo, Japan) at a wavelength of 517 nm. The DPPH method was measured as shown in Equation (1):

$$\text{AA } (\%) = \left(\frac{D - Sw}{D}\right) \times 100 \quad (1)$$

where AA is the percentage of antioxidant activity, D is the absorbance of the DPPH solution, and Sw refers to the absorbance of the mixture solution between DPPH and sandalwood oil.

2.7. Optimization by Response Surface Methodology (RSM)

A Box–Behnken design (BBD) was formulated by "Design Expert" software (13.0.4, Minneapolis, MN, USA) to analyze the effects of variables. There were 5 repetitions in the middle point of the design experiment. The parameters of temperature (°C), flow rate (mL/min), and pressure (MPa) were studied. The responses of this study were oil yield (%), TPCs (mg/100 g), TFCs (mg/100 g), and AA (%). The second order was applied to correlate the data as follows:

$$S_i = C_0 + \sum_{i=1}^{k} C_i E_i + \sum_i \sum_j C_{ij} E_i E_j + \sum_{i=1}^{k} C_{ii} E_i^2 \quad (2)$$

where S is an investigated response (oil yield (%), TPCs (mg GAE/100 g), TFCs (mg QE/100 g), and AA (%)); C_0 is constant; C_i, C_{ii}, and C_{ij} are coefficients of linear, quadratic, and interaction terms, respectively. E_i and E_j are independent variables of temperature (°C), flow rate (mL/min), and pressure (MPa).

3. Results and Discussion

The extraction of bioactive compounds from natural sources has gained significant attention in recent years due to their potential health benefits and applications in various industries. Sandalwood (*Santalum album*), renowned for its aromatic and medicinal properties, is a valuable source of phenolic and flavonoid compounds. These compounds exhibit a range of bioactivities, including antioxidant, anti-inflammatory, and anticancer properties, making them highly sought after in the pharmaceutical, cosmetic, and food industries [4,14–16].

Traditional extraction methods for these bioactive compounds, such as solvent extraction, often involve the use of toxic chemicals and can be inefficient in terms of yield and selectivity [4,16,17]. In contrast, subcritical ethanol extraction (SEE) presents a promising alternative, offering a more environmentally friendly and efficient method for the recovery of phenolic and flavonoid compounds. Subcritical ethanol, operating at temperatures and pressures below its critical point, provides several advantages, including lower solvent consumption, reduced extraction time, and the ability to extract thermolabile compounds without degradation.

Optimization of the subcritical ethanol extraction process is crucial to maximize the recovery of phenolic and flavonoid compounds from sandalwood. Parameters such as temperature, pressure, and flow rate play a vital role in determining the efficiency and selectivity of the extraction process. This study aims to optimize these parameters to enhance the yield and quality of phenolic and flavonoid compounds extracted from sandalwood. In this research, a systematic approach is employed to investigate the effects of various extraction parameters on the recovery of phenolic and flavonoid compounds. The optimization process involves a series of experiments designed to identify the optimal

conditions for subcritical ethanol extraction. The results of this study will provide valuable insights into the extraction process, contributing to the development of more efficient and sustainable methods for the recovery of bioactive compounds from natural sources.

The following sections present the results and discussion of the optimization experiments, highlighting the impact of different extraction parameters on the yield and quality of phenolic and flavonoid compounds. The findings of this research will not only enhance our understanding of subcritical ethanol extraction but also pave the way for its application in the industrial-scale production of high-value bioactive compounds from sandalwood.

3.1. Parameter-Fixing to Select the Maximum and Minimum Values of Parameters

Parameter-fixing experiments are a valuable methodology employed in research to ascertain the maximum and minimum values of parameters within a specified range or experimental design. This technique involves selecting specific parameters and setting them at extreme values while manipulating others to observe the effect on the system or process under investigation. The parameter-fixing experiments were conducted under specific conditions determined by the apparatus, preliminary study, and theoretical data. The highest pressure of 10 MPa was maintained to ensure the subcritical phase/region of ethanol, as it is crucial for the fluidity of ethanol. Ethanol can transition to a vapor phase without sufficient pressure and high temperature, reducing viscosity and diffusivity [18]. Low viscosity negatively impacts the selectivity of subcritical ethanol and reduces the oil yield [19].

To prevent the degradation of phenolic and flavonoid compounds, the maximum temperature was set at 110 °C. Flavonoids are a class of phytochemicals found in various plants, including sandalwood, known for their beneficial health properties. However, due to their chemical structure and properties, flavonoids can be sensitive to high temperatures [20]. One reason for the sensitivity of flavonoids to high temperatures is their susceptibility to thermal degradation. Flavonoids contain multiple hydroxyl groups and conjugated double-bonds in their structure, which make them susceptible to oxidation and degradation when exposed to high temperatures [21]. The heat can cause the breakage of these chemical bonds, leading to the loss of structural integrity and potential degradation of the flavonoid compounds.

Moreover, flavonoids are often present in plants in a glycosylated form attached to sugar molecules. These glycosidic bonds can also be susceptible to heat-induced hydrolysis, resulting in the separation of the flavonoid from the sugar moiety and subsequent loss of the glycosylated form [22]. Furthermore, flavonoids undergo isomerization reactions under high temperatures, altering their chemical structure and potentially affecting their bioactivity and stability [23]. The specific isomer formed may differ in terms of its biological properties and antioxidant activity.

To avoid solvent channeling effects and ensure sufficient extraction time, the flow rate of solvents was maintained at 6 mL/min. A high flow rate would reduce the residence time of subcritical ethanol in the extraction vessel, which is essential for extracting the desired phenolic and flavonoid compounds. Insufficient recovery of these compounds would subsequently diminish the antioxidant activity of the sandalwood oil [11]. Therefore, an effective extraction procedure requires careful consideration of the flow rate. Additionally, to prevent prolonged exposure to heat during the extraction process, the extraction period was limited to 10 min.

The selection of an optimal particle size range for the extraction of sandalwood using subcritical ethanol was based on various factors. Primarily, the choice aimed to optimize the efficiency of the extraction process by considering the impact of particle size on mass transfer and extraction kinetics [24]. The size of solid particles is known to influence these aspects substantially. Larger particles possess a reduced surface area available for extraction, potentially limiting the contact between the solvent and the target compounds [25,26]. Conversely, smaller particles can exhibit increased resistance to solvent penetration, hindering effective extraction.

By selecting a particle size range of 355 to 425 µm, it was anticipated that an optimal balance would be achieved between the available surface area for extraction and the ease of solvent penetration. This balance would enhance the efficiency of the extraction process,

ensuring effective contact between the subcritical ethanol and the sandalwood particles. Consequently, a higher yield of desired compounds from the sandalwood could be obtained. Furthermore, practical considerations were considered, such as the availability of suitable equipment and techniques for particle size reduction and handling. The chosen particle size range needed to be practically achievable using the available methods and equipment within the extraction process. It ensured that the desired particle size range could be consistently obtained, promoting reproducibility and reliability in the extraction experiments.

3.2. Statistical Analysis and Model Fitting

Phenolic and flavonoid compounds were extracted from sandalwood using subcritical ethanol extraction. Furthermore, the antioxidant activity of sandalwood oil was analyzed using the DPPH method. To maximize phenolic and flavonoid recovery and antioxidant activity, response surface methodology (RSM) was applied. RSM can be applied to determine the most significant factor in the oil yield, phenolic and flavonoid recovery, and antioxidant activity of sandalwood oil. Therefore, the significant factor can be adjusted to enhance the selectivity of subcritical ethanol.

Table 1 presents the factors (temperature, flow rate, and pressure) and corresponding responses (oil yield, TPCs, TFCs, and AA) observed in sandalwood oil's subcritical ethanol extraction process. The adequacy of the model employed to establish relationships between the experimental data was assessed through analysis of variance (ANOVA). Table 2 shows regression of the quadratic model fitted the experimental data for sandalwood essential oil using subcritical ethanol extraction. Additionally, Table 3 provides the ANOVA tables for TPCs, TFCs, and AA. Moreover, Figure 2 compares actual and predicted values for TPCs, TFCs, and AA obtained through subcritical ethanol extraction. The goodness of fit of the quadratic model to the experimental data is supported by Figure 2, where the predicted values align well with the actual values. The R^2 values in Table 3, which exceed 0.8, further validated it.

Table 2. Regression of the quadratic model fitted the experimental data for sandalwood essential oil using subcritical ethanol extraction.

$S_1 = 3.42 - 0.01E_1 + 0.43E_2 - 0.28E_3 + 0.007E_1E_2 + 0.11E_1E_3 + 0.12E_2E_3 - 0.15E_1^2 - 0.23E_2^2 + 0.27E_3^2$
$S_2 = 3.15 + 0.03E_1 + 0.39E_2 - 0.35E_3 - 0.09E_1E_2 + 0.19E_1E_3 + 0.14E_2E_3 - 0.29E_1^2 - 0.06E_2^2 + 0.43E_3^2$
$S_3 = 7.84 - 0.03E_1 + 0.39E_2 - 0.41E_3 - 0.09E_1E_2 + 0.3E_1E_3 + 0.14E_2E_3 - 0.23E_1^2 - 0.11E_2^2 + 0.48E_3^2$
$S_4 = 70.8 - 0.028E_1 + 0.36E_2 - 0.37E_3 - 0.08E_1E_2 + 0.27E_1E_3 + 0.13E_2E_3 - 0.21E_1^2 - 0.1E_2^2 + 0.44E_3^2$

Furthermore, an ANOVA table was employed to evaluate the model's ability to describe the experimental data (Table 3). The selection of the quadratic model for the responses of oil yield, TPCs, TFCs, and AA is justified by the *p*-values in Table 3, which are lower than 0.05. Additionally, the F-value of the model surpasses the critical F-value, indicating a significant fit to the experimental data. Table 3 also demonstrates that the linear coefficients of temperature (E_2) and flow rate (E_3) have a statistically significant effect ($p < 0.05$) and high coefficient value as shown in Table 2 on all the responses. It is further supported by the coefficient values of the linear coefficients of temperature (E_2) and flow rate (E_3), which are higher than the coefficients of other factors, as shown in Table 3.

The analysis of the lack of fit in the model yielded an F-value that suggests its insignificance compared to the pure error, aligning with the expectations set by the mathematical model. Furthermore, the coefficient of variation (CV), which indicates repeatability, for the subcritical ethanol extraction method was below 10% when considering five design trials, including center points. A CV value below 10% is generally regarded as very good, while values between 10% and 20% are considered good, 20% to 30% are acceptable, and values exceeding 30% are deemed unacceptable. Thus, with a CV below 10%, the experiments were conducted with an acceptable level of consistency for all responses.

Table 3. ANOVA table for subcritical ethanol extraction for enhanced phenolic and flavonoid compound recovery from sandalwood.

	S_1= Essential Oil Yield (%)					S_2= TPC (mg GAE/100 g)					S_3= TPC (mg QE/100 g)					S_4= AA (%)				
Source	**Sum of Squares**	**df**	**Mean Square**	**F-Value**	***p*-Value**	**Sum of Squares**	**df**	**Mean Square**	**F-Value**	***p*-Value**	**Sum of Squares**	**df**	**Mean Square**	**F-Value**	***p*-Value**	**Sum of Squares**	**df**	**Mean Square**	**F-Value**	***p*-Value**
Model	2.90	9	0.32	5.45	0.02	3.56	9	0.39	6.56	0.01	4.24	9	0.47	8.30	0.005	3.49	9	0.38	8.44	0.005
E_1	0.001	1	0.001	0.02	0.89	0.01	1	0.01	0.11	0.75	0.01	1	0.01	0.09	0.76	0.01	1	0.01	0.13	0.72
E_2	1.51	1	1.51	25.60	0.0015	1.22	1	1.22	20.28	0.003	1.22	1	1.22	21.58	0.002	1.01	1	1.01	21.92	0.002
E_3	0.64	1	0.64	10.89	0.01	0.99	1	0.99	16.46	0.01	1.33	1	1.33	23.40	0.002	1.10	1	1.10	23.81	0.002
E_1E_2	0.002	1	0.001	0.04	0.95	0.03	1	0.03	0.53	0.48	0.03	1	0.03	0.57	0.47	0.02	1	0.02	0.55	0.47
E_1E_3	0.04	1	0.04	0.81	0.39	0.14	1	0.15	2.46	0.16	0.36	1	0.36	6.45	0.04	0.3	1	0.30	6.58	0.04
E_2E_3	0.06	1	0.06	1.10	0.32	0.08	1	0.07	1.25	0.3	0.08	1	0.07	1.33	0.28	0.06	1	0.06	1.36	0.28
E_1^2	0.10	1	0.10	1.71	0.23	0.35	1	0.35	5.87	0.05	0.23	1	0.23	4.10	0.08	0.18	1	0.18	4.10	0.08
E_2^2	0.23	1	0.23	4.02	0.08	0.02	1	0.01	0.25	0.63	0.06	1	0.01	0.98	0.35	0.04	1	0.04	0.94	0.36
E_3^2	0.31	1	0.31	5.38	0.05	0.76	1	0.76	12.75	0.01	0.98	1	0.98	17.27	0.004	0.81	1	0.81	17.60	0.004
Residual	0.41	7	0.06			0.42	7	0.06			0.39	7	0.05			0.32	7	0.05		
Lack of Fit	0.40	3	0.13	130.09	0.0002	0.31	3	0.10	3.79	0.11	0.28	3	0.09	3.48	0.13	0.23	3	0.07	3.45	0.13
Pure Error	0.004	4	0.001			0.11	4	0.03			0.11	4	0.02			0.08	4	0.02		
Cor Total	3.31	16				3.99	16				4.64	16				3.81	16			
R^2	0.85					0.89					0.92					0.92				
CV	7.23					7.79					3.01					0.3				

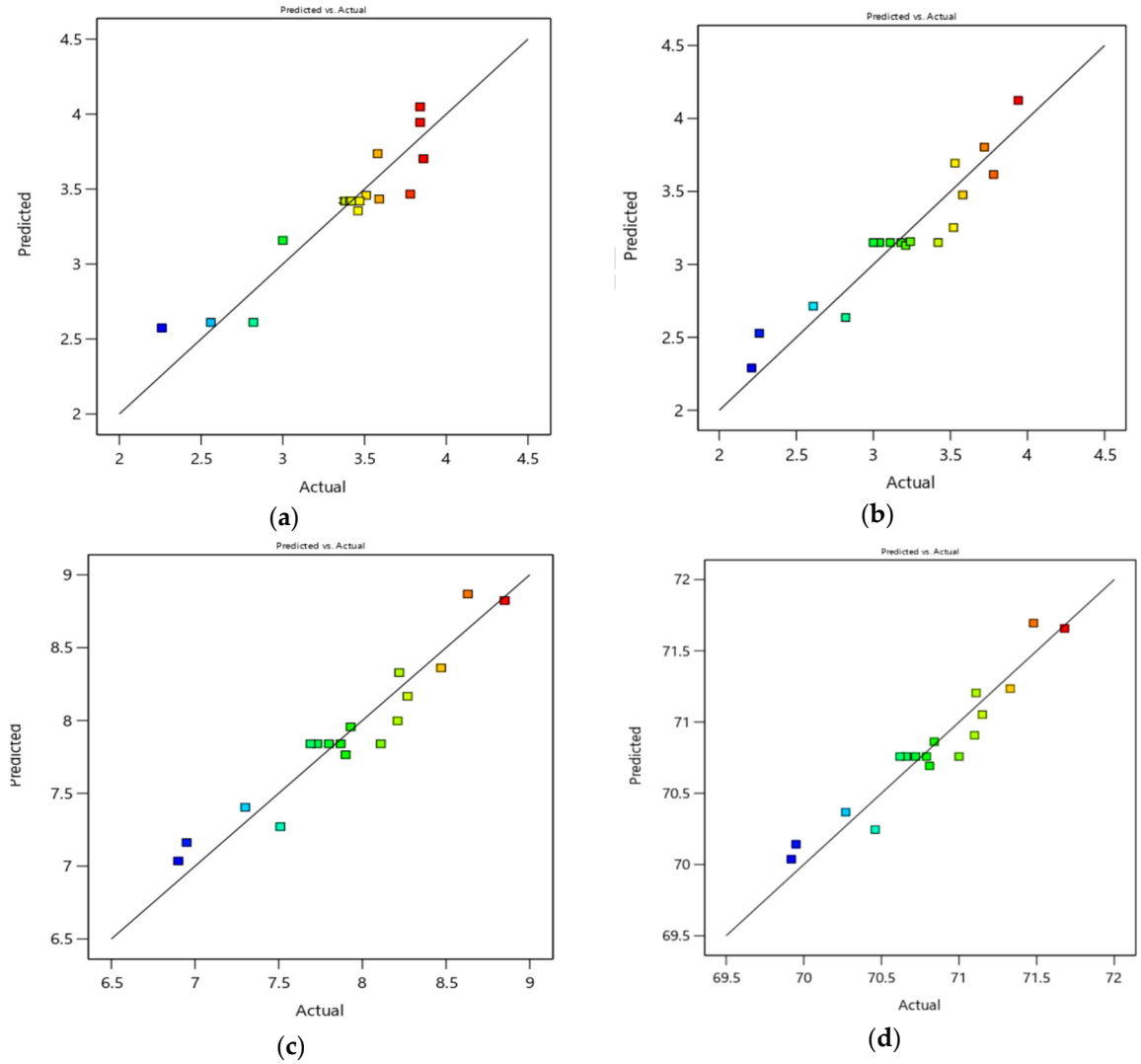

Figure 2. The goodness of fit of the quadratic model to the experimental data of (**a**) Yield, (**b**) TPC (**c**) TFC and (**d**) AA.

3.3. Effect of Parameters on Total Yield Sandalwood Essential Oil

The observed increase in oil yield with an increase in temperature from 90 to 110 °C during subcritical ethanol extraction of sandalwood essential oil can be attributed to various underlying mechanisms. Primarily, the temperature significantly impacts the solubility of the target compounds within the solvent. With higher temperatures, the solubility of desirable components, including essential oils, tends to increase [27–29]. This improved solubility enables more efficient extraction of the desired compounds from the solid sandalwood matrix. This phenomenon is supported by the data presented in Figure 3a,b, where higher temperatures correspond to higher oil yields.

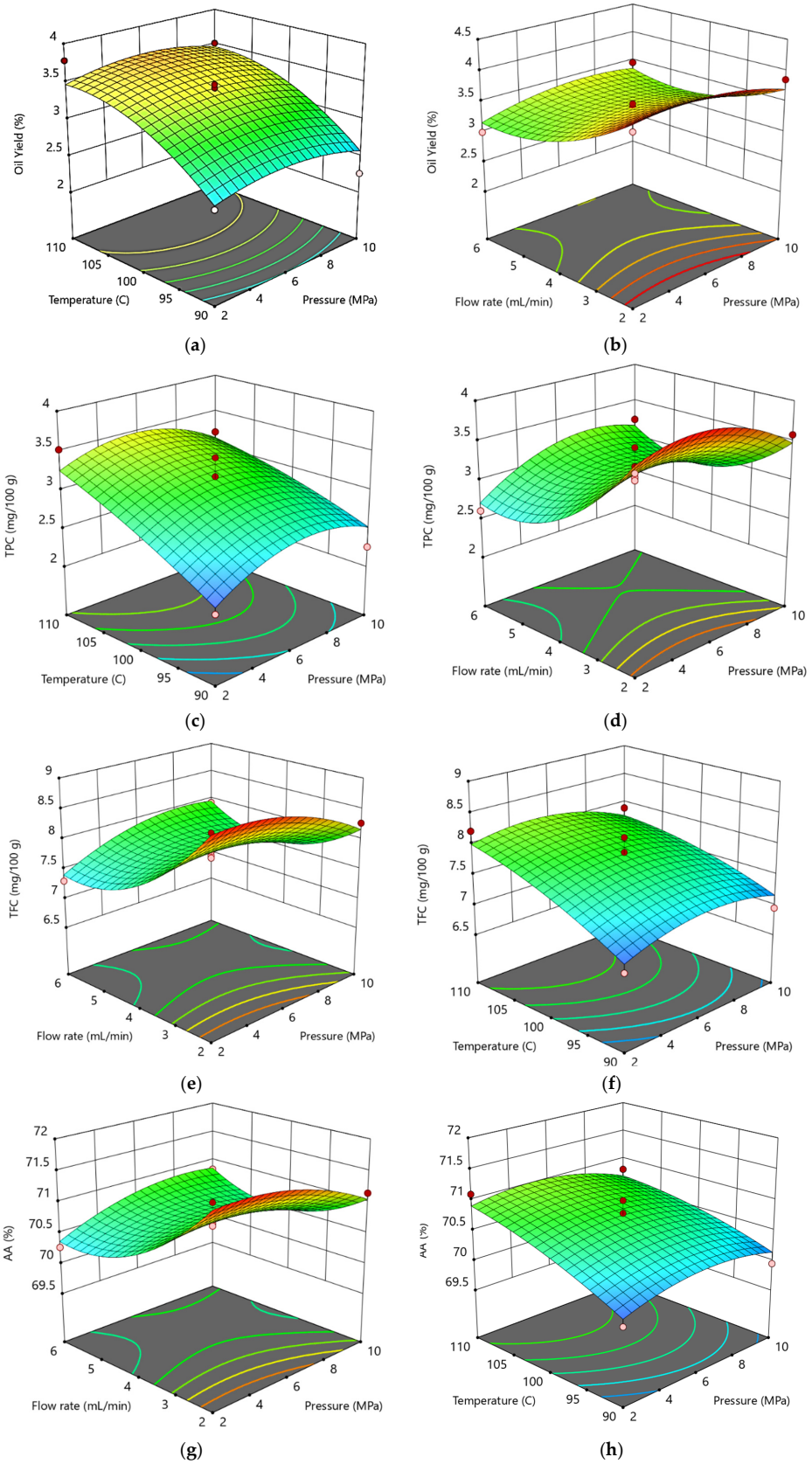

Figure 3. Three-dimensional response of (**a**,**b**) Yield, (**c**,**d**) TPC, (**e**,**f**) TFC and (**g**,**h**) AA of sandalwood essential oil using subcritical ethanol extraction.

Furthermore, temperature influences the rate of diffusion, which affects the release of the target compounds from the solid material. The increased thermal energy at higher temperatures promotes the movement and diffusion of solvent molecules into the sandalwood particles, facilitating the interaction between the solvent and the target compounds [30]. This enhanced penetration contributes to higher extraction yields. In addition, elevated temperatures can induce thermal decomposition or disruption of the cell structure within the sandalwood. This thermal effect leads to the breakdown of cell walls and membranes, facilitating the release of essential oil components. Consequently, oil diffusion from the plant material into the solvent is promoted.

While increasing the temperature can enhance extraction efficiency and oil yield, it is essential to note that there is an optimal temperature range to consider. Beyond this range, the efficiency may start to decline, or undesired changes in the composition of the extracted oil could occur. Therefore, the specific choice of the upper limit, such as 110 °C in this study, may depend on factors such as the stability of the target compounds, the avoidance of thermal degradation, and the desired quality and characteristics of the extracted sandalwood essential oil.

The effect of pressure on oil yield during subcritical ethanol extraction of sandalwood was not statistically significant, as shown in Figure 3a. Several factors may contribute to this observation. Firstly, the pressure range studied needed to be more extensive to induce significant changes in the oil yield. The selected pressure levels might have been within a range where the influence on the extraction process was limited. The pressure conditions explored did not substantially impact the solubility or diffusion of the target compounds, leading to minimal variations in the oil yield.

Additionally, the solubility and diffusion characteristics of the target compounds in the subcritical ethanol system might be more influenced by temperature and other factors rather than pressure [30,31]. The interactions between the solvent and the sandalwood matrix and the release of essential oil components may be primarily driven by temperature rather than pressure-related effects. Therefore, the pressure variations explored in this study may not have significantly influenced these critical factors. Moreover, the sandalwood material and its specific composition may play a role in the observed lack of significant pressure effect on oil yield. Different plant materials can exhibit varying responses to extraction parameters, and the composition and structure of the sandalwood matrix may have inherently low sensitivity to pressure changes.

The decrease in the flow rate of ethanol during subcritical extraction of sandalwood was observed to enhance the oil yield recovery, as depicted in Figure 3b. This phenomenon can be attributed to several underlying factors. Firstly, reducing the flow rate of ethanol allows for a longer residence time of the solvent within the extraction system [32]. This prolonged contact time between the solvent and the sandalwood matrix enhances the extraction efficiency by facilitating a more thorough dissolution and diffusion of the target compounds. As a result, a greater quantity of the desired oil components can be extracted from the solid material.

Furthermore, decreasing the flow rate of ethanol promotes a higher degree of saturation within the extraction system. A slower flow rate provides more time for the solvent to reach its saturation point, thereby increasing the solubility of the oil components in the ethanol. This improved solubility enables more effective oil extraction from the sandalwood matrix, leading to higher oil yield recovery. Additionally, a decreased flow rate of ethanol can mitigate any potential mass transfer limitations during the extraction process. The solvent can better penetrate and interact with the sandalwood particles by reducing the flow rate, ensuring a more comprehensive oil extraction. It aids in overcoming any diffusion barriers and enhances the release of the oil components from the solid matrix.

3.4. Effect of Parameters of Subcritical Ethanol Extraction on TPCs

Increasing the temperature from 90 to 110 °C during subcritical ethanol extraction of sandalwood was observed, as in Figure 3c, to increase the total phenolic compounds

while maintaining a constant pressure of 2 and 10 MPa. This effect can be attributed to several underlying factors. Firstly, temperature plays a crucial role in promoting the solubility of phenolic compounds in the ethanol solvent. As the temperature increases, the solubility of these compounds also increases, leading to higher extraction yields. The elevated temperature provides more thermal energy, which enhances the dissolution of the phenolic compounds from the sandalwood matrix into the solvent.

Furthermore, the increase in temperature can facilitate the breakdown of the cell walls and membranes within the sandalwood material. This breakdown increases the accessibility of the phenolic compounds, allowing for easier extraction. The higher temperatures promote the disruption of the plant's cellular structure, leading to improved release of the phenolic compounds into the solvent. Moreover, the increased temperature may enhance the diffusion rate of the ethanol solvent into the sandalwood particles. The higher thermal energy accelerates the movement of the solvent molecules, enabling them to penetrate the solid matrix more effectively. This improved penetration facilitates the interaction between the solvent and the phenolic compounds, enhancing extraction efficiency.

Reducing the ethanol flow rate from 6 mL/min to 2 mL/min in the subcritical ethanol extraction of sandalwood increased the recovery of total phenolic compounds, as depicted in Figure 3d. The observed phenomenon can be explained by various factors associated with mass transfer and extraction kinetics. Reducing the ethanol flow rate increases the duration of contact between the sandalwood material and the solvent. The prolonged contact duration facilitates the diffusion of phenolic compounds from the solid matrix into the solvent. Reducing the flow rate enhances phenolic compound interaction and extraction, increasing recovery.

Similar to the response of oil yield, the pressure parameter was not statistically significant for the recovery of TPC during the subcritical ethanol extraction of sandalwood. This observation suggests that the pressure range studied did not have a substantial impact on the extraction efficiency of TPC. Furthermore, lowering the flow rate can increase the solute concentration in the solvent during extraction. It reduces the flow rate of ethanol in the extraction vessel, resulting in a higher concentration of phenolic compounds in the solvent. The elevated concentration gradient facilitates the enhanced mass transfer from the solid matrix to the solvent, resulting in heightened retrieval of phenolic compounds. A reduced flow rate can minimize washout. Excessive flow rate can result in insufficient contact time between the solvent and solid matrix, reducing extraction efficiency. Reducing the flow rate enhances the interaction between the solvent and sandalwood material, resulting in the complete extraction of phenolic compounds.

3.5. Effect Parameters of Subcritical Ethanol Extraction on TFC

Elevating the temperature from 90 to 110 °C in subcritical ethanol extraction of sandalwood improved the retrieval of total flavonoid compounds, as shown in Figure 3e. The effect was observed at constant pressure levels of 2 and 10 MPa. Temperature is a significant factor in enhancing the solubility of flavonoids in solvents. With increasing temperature, the solubility of the compounds in the sandalwood matrix increases, leading to improved extraction efficiency. The elevated temperature facilitates the dissolution of flavonoids into the ethanol solvent by providing the required thermal energy [33]. Temperature can affect the diffusion rate of the solvent into the solid material, facilitating the release of flavonoids from sandalwood particles [34]. Increased temperature enhances solvent molecule kinetic energy, facilitating their movement and diffusion into the solid matrix—improved solvent–flavonoid interaction due to enhanced penetration results in higher recovery [35]. Elevated temperatures can lead to the disintegration of cellular structures in sandalwood, which aids in the discharge of flavonoids. Elevated temperature can facilitate the extraction of target compounds into the solvent by disrupting cell walls and membranes.

Elevating the pressure from 2 to 10 MPa in subcritical ethanol extraction of sandalwood at temperatures ranging from 90 to 110 °C was found to enhance the recovery of total flavonoid compounds, as illustrated in Figure 3f. Multiple factors contribute to this

phenomenon. Higher pressures can increase flavonoid solubility in ethanol. Elevated pressure can enhance solvent density, thereby increasing the solubility of the desired compounds [36]. Increased solubility facilitates efficient flavonoid extraction from sandalwood. Higher pressure can improve the solvent's mass transfer and diffusion into the solid material. Increased pressure facilitates solvent penetration into sandalwood particles, promoting the release of flavonoids. Elevated pressure facilitates solvent molecules to penetrate the solid matrix and extract the desired compounds. Increased pressure can alter the physical and structural characteristics of sandalwood [37]. Elevated pressure can alter the porosity and permeability of the matrix, facilitating solvent penetration to flavonoid compounds. Enhanced accessibility improves extraction efficiency and increases recovery rates.

Reducing the flow rate of ethanol from 6 mL/min to 2 mL/min during subcritical ethanol extraction of sandalwood was found to increase the recovery of total flavonoid compounds, as demonstrated in Figure 3f. Reducing the solvent flow rate increases the contact time between sandalwood particles and ethanol. Prolonged contact duration enhances the chance of solvent–solid matrix interaction, thereby promoting the extraction of flavonoids. A slower flow rate enhances the solvent's ability to dissolve and extract flavonoids, increasing recovery rates. Reducing the flow rate can enhance the solvent's penetration and diffusion into the sandalwood particles. A reduced flow rate facilitates improved solvent infiltration into the solid matrix, resulting in a more comprehensive extraction of flavonoids. Enhanced penetration and diffusion improve solvent accessibility to target compounds, increasing recovery.

The decreased flow rate mitigates the "washing effect" when the solvent passes through the solid material too rapidly, diminishing the extraction efficacy. Reducing the flow rate enhances the solvent's ability to dissolve and extract flavonoids efficiently [38]. The slowed solvent flow through sandalwood particles facilitates ample interaction between solvent and flavonoid-rich constituents, leading to increased retrieval of flavonoid compounds. Reducing ethanol flow rate during subcritical ethanol extraction of sandalwood enhances extraction efficiency, resulting in increased recovery of total flavonoid compounds due to longer contact time and improved penetration. The observed response similarity between oil yield and total phenolic compound recovery is consistent.

3.6. Effect Parameters of Subcritical Ethanol Extraction on AA

The decrease in the flow rate of ethanol from 6 mL/min to 2 mL/min during subcritical ethanol extraction of sandalwood was observed to enhance the antioxidant activity of the extract, as shown in Figure 3g. This effect is similar to the responses observed in oil yield, total phenolic compound (TPC) recovery, and total flavonoid compound (TFC) recovery. The slower flow rate allows for a longer contact time between the solvent and the sandalwood particles, facilitating a more thorough extraction of antioxidant compounds [11]. Antioxidants are known to be present in the phenolic and flavonoid compounds, and by extending the contact time, more of these bioactive components can be extracted from the sandalwood, leading to higher antioxidant activity in the extract. Furthermore, reducing the flow rate of the solvent can improve the solubility and diffusion of antioxidant compounds [39]. A slower flow rate provides more time for the solvent to interact with the solid matrix, allowing for better solubility and extraction of the antioxidant compounds. This enhanced solubility ensures that more antioxidants are extracted from the sandalwood, resulting in higher antioxidant activity in the extract.

The increase in temperature from 90 to 110 °C during subcritical ethanol extraction of sandalwood was observed to enhance the antioxidant activity of the extract at constant pressures of 2 and 10 MPa, as shown in Figure 3h. There are several reasons for this effect. Firstly, temperature plays a crucial role in promoting the extraction of bioactive compounds, including antioxidants, from the sandalwood matrix. As the temperature increases, these compounds' solubility and diffusion rates are generally enhanced [31,40,41]. It allows for more efficient extraction of antioxidants from the solid material into the solvent. The

higher temperatures provide greater thermal energy, facilitating the release of antioxidant compounds from the sandalwood and their subsequent dissolution in the ethanol solvent.

Moreover, the increased temperature can also contribute to the breakdown or degradation of certain compounds present in sandalwood, such as polyphenols. Thermal degradation of these compounds can result in the formation of smaller, more bioactive antioxidant molecules. Consequently, the higher temperature promotes the generation of potent antioxidants, increasing the extract's antioxidant activity. Furthermore, the elevated temperature can enhance the solubility and extraction efficiency of antioxidants by improving the fluidity and penetration of the solvent into the sandalwood particles. The thermal energy increases the mobility of the solvent molecules, enabling them to interact more effectively with the target compounds and extract a higher concentration of antioxidants.

3.7. Multiple Optimizations of Subcritical Ethanol Extraction for Enhanced Phenolic and Flavonoid Compound Recovery from Sandalwood

Multiple optimization experiments were conducted to determine the optimum conditions for achieving maximum responses in terms of oil yield, total phenolic compounds (TPCs), total flavonoid compounds (TFCs), and antioxidant activity (AA). The optimized conditions were a pressure of 6.04 MPa, a temperature of 109 °C, and a flow rate of 2.01 mL/min. Under these optimized conditions, the subcritical ethanol extraction of sandalwood yielded a maximum oil yield of 4.04%. Additionally, the extracted sandalwood oil exhibited significant levels of TPCs, with a value of 4.11 mg GAE/100 g and TFCs, 8.85 mg QE/100 g. Furthermore, the extracted oil displayed notable antioxidant activity, as indicated by an AA value of 71.68%.

A comparison was made to validate the optimization results between the predicted values obtained from the optimization process and the actual experimental data. The error between the predicted and actual data was below 10%, indicating satisfactory accuracy in the optimization model. Therefore, these optimized extraction conditions can be applied for the scale-up process, ensuring the production of sandalwood oil with desirable properties. The details of the optimization validation can be found in Table 4.

Table 4. The predicted and observed parameters and responses for sandalwood extraction.

Parameters	Values	Responses	Predicted	Observed	%Error
E_1: Pressure, MPa	6.04	S_1: Oil yield, %	4.05	4.21	3.8%
E_2: Temperature, °C	109.9	S_2: TPC, mg GAE/100 g	4.11	4.43	7.4%
E_3: Flow rate, mL/min	2.01	S_3: TFC, mg QE/100 g	8.86	8.42	4.9%
		S_4: AA, %	71.68	74.5	3/7%

4. Conclusions

In summary, this study investigated the subcritical ethanol extraction of sandalwood to maximize oil yield, TPCs, TFCs, and AA. The optimal conditions were determined through multiple optimizations as 6.04 MPa pressure, 109 °C temperature, and 2.01 mL/min flow rate. Under these conditions, this study achieved a maximum oil yield of 4.04%, significant TPCs of 4.11 mg/100 g, TFCs of 8.85 mg/100 g, and notable antioxidant activity with an AA value of 71.68%. The optimization process was validated, demonstrating an error below 10% between predicted and observed data, ensuring the reliability of the results. It was found that temperature and flow rate significantly affected all responses. These findings provide valuable insights for scaling up sandalwood's subcritical ethanol extraction process, facilitating its practical application.

Author Contributions: Conceptualization, W.Z. and N.R.P.; methodology, W.Z.; software, D.N.R.; validation, D.N.R.; writing—original draft preparation, W.Z., D.N.R., and N.R.P.; writing—review and editing, N.R.P.; visualization, N.R.P. All authors have read and agreed to the published version of the manuscript.

Funding: UTM Postdoctoral Fellowship with grant number Q.J130000.21A2.07E06.

Data Availability Statement: The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

Acknowledgments: The authors would like to acknowledge the Professional Development Research University grant (Q.J130000.21A2.07E06) from Universiti Teknologi Malaysia for supporting this work.

Conflicts of Interest: There are no conflicts of interest.

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