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Abstract: Supercritical fluid extraction (SFE) is an innovative green technology for the extraction of phytochemicals from plants. Therefore, this study aimed to evaluate the application of SFE and to optimize the extraction condition of the Thai herbal formula, Kleeb Bua Daeng (KBD). A Box-Behnken design (BBD) with response surface methodology was used to determine the effect of extraction time, temperature, and pressure on response variables including extraction yield, total phenolic content (TPC), total flavonoid content (TFC), total carotenoid content (TCC) and total anthocyanin content (TAC) of KBD formula. The highest percentage extraction yield, TPC, TFC, and TCC of the extracts were 3.81%, 464.56 mg gallic acid equivalents/g extract, 217.19 mg quercetin equivalents/g extract, and 22.26 mg β-carotene equivalents/g extract, respectively. The results indicated that SFE is a suitable method of extraction for green recovery of some phytochemicals from the KBD formula.

Keywords: Supercritical fluid extraction; response surface methodology; Box-Behnken design; Kleeb Bua Daeng formula

1. Introduction

Kleeb Bua Daeng (KBD) is a Thai traditional herbal formula developed by Chao Phraya Abhaibhubejhr hospital in Prachinburi Province, Thailand. Many phytochemicals such as phenolics, flavonoids, and carotenoids have been found in this formula [1]. KBD is used for the treatment of insomnia and to improve memory [2,3]. Safety and efficacy of this formula in mild cognitive impairment symptom were reported [4]. KBD was also found to improve the cognitive impairment in unpredictable chronic mild stress mice model [5]. KBD consists of three herbs: *Nelumbo nucifera* (petals), *Piper nigrum* (fruits) and *Centella asiatica* (aerial part). The petals of *N. nucifera* were found to contain many bioactive compounds with antioxidant, anti-inflammatory, neuroprotective, and anticancer activities [6-8]. In turn, the fruits of *P. nigrum* or black pepper which display anti-inflammatory, antioxidant, anticancer, antidepressant, and analgesic activities [9,10], were found to contain the alkaloid piperine as a major constituent, in addition to polyphenols and flavonoids [11,12], while *C. asiatica*, which has antioxidant, antiulcer, antidepressant and anti-inflammatory activities [13,14], contain asiaticoside, polyphenols, flavonoids and carotenoids [15].

Extraction is the most important step to isolate bioactive compounds from plants and other biological materials. Various traditional extraction techniques such as heat reflux, maceration, and Soxhlet extraction normally require large amount of solvent and long extraction time. As new extraction technologies have emerged, advanced extraction methods, including ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), pulsed electric field-assisted extraction, pressurized liquid extraction, enzyme-assisted extraction, and supercritical fluid extraction, have been recently developed [16]. These modern extraction techniques provide fast and effective extraction with less amount of solvent used. Supercritical fluid extraction (SFE) is a technique which is widely used to extract natural products and usually employs non-toxic solvents such as carbon dioxide (CO_2), water, nitrous oxide and ethane. SFE is conducted at pressures above the critical pressure of the extracting solvent since high pressures increase the solvent density and the diffusion coefficient. Moreover, the extraction selectivity can be altered by small changes of pressure and temperature [17]. SFE allows a separation of the extracting solvent from the extract simply by the expansion of the fluid in the extractor vessel outlet with a pressure drop, causing the fluid to change to a gas phase, and thus separates from the solid that it extracts. CO_2 is the most commonly used extraction solvent in SFE. Supercritical carbon dioxide ($ScCO_2$; critical point: 7.38 MPa, 304 K/31.1 °C and 73.8 bar) is a nonpolar medium with a large quadrupole moment. Its density can be changed as a function of temperature and pressure. At a critical pressure, its compressibility is maximized, and small changes to thermal parameters can lead to large changes in its local density [18]. The advantages of CO_2 are non-flammable, non-toxic, non-explosive, economical and easily removable from the extract. Many parameters that influence the extraction efficiency such as temperature, pressure, particle size, solvent-to-feed-ratio, extraction time, and flow rate of CO_2 [19]. Thus, optimization of these parameters could be performed to increase the percentage yield, the content of active ingredients, and biological activity of the extract.

The Box–Behnken Design (BBD) and response surface methodology (RSM) are the statistical analysis method and simple experimental design tools for the effective optimization of the extraction process. These tools are easy to perform and provide good results. A suitable number of experimental runs from BBD provided higher advantage when compared with other statistical designs [20]. SFE involves many variables, which may affect the efficiency of extraction. Selection of critical variables and their levels is important. RSM has previously been applied for the optimization of SFE for extraction of bioactive compounds from many plants. For example, optimization of SFE with RSM was used for the extraction of essential oils from orange (*Citrus sinensis*) peel [21] as well as the extraction of bioactive compounds from grape (*Vitis labrusca*) peel [22].

Thus, the aim of this study was to evaluate and optimize the effective conditions of the SFE applicable for the extraction of total active compounds from KBD. The effects of various extraction parameters such as extraction time, pressure, and temperature were evaluated to determine the appropriate extraction conditions to achieve the highest percentage extraction yield and active compound contents to improve product development.

2. Results

2.1. Optimization of variable conditions for SFE

The BBD with RSM was used to optimize the SFE conditions. Three extraction variables, i. e. extraction temperature, extraction pressure, and extraction time, and six responses including percentage extraction yield, TFC, TPC, TCC, TAC, and percentage inhibition of DPPH assay were evaluated in this study. $ScCO_2$ was chosen as an extraction solvent because it is safe and non-toxic compared to other organic solvents. From the analysis of variance (ANOVA), the quadratic polynomial model was highly significant with the p-value less than 0.0001 for TCC, 0.0265, 0.0162, and 0.0047 for percentage extraction yield, TPC, and TFC, respectively. The experimental results of 17 runs and their predicted values from the equation model of each response are shown in Table 1.

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Table 1. BBD matrix for percentage yield, TPC, TFC, and TCC from KBD powder by SFE.

Run order	Extraction variables			Response							
	temperature (°C)	pressure (bar)	time (min)	Percentage extraction yield (%)		TPC (mg GAE/g extract)		TFC (mg QE/g extract)		TCC (mg β-CE/g extract)	
				Experimental	Predicted	Experimental	Predicted	Experimental	Predicted	Experimental	Predicted
1	-1 (30)	0 (250)	1 (90)	3.28	3.31	138.78	102.31	206.30	209.48	2.99	2.86
2	0 (45)	0 (250)	0 (60)	3.26	3.49	135.33	155.38	164.97	165.46	9.39	10.74
3	0 (45)	0 (250)	0 (60)	3.51	3.49	130.45	155.38	167.97	165.46	10.95	10.74
4	0 (45)	1 (300)	1 (90)	3.53	3.46	131.45	176.63	175.08	181.82	10.33	10.01
5	-1 (30)	1 (300)	0 (60)	3.57	3.61	134.78	126.07	190.63	180.72	10.5	10.95
6	0 (45)	0 (250)	0 (60)	3.39	3.49	145.36	155.38	159.41	165.46	11.34	10.74
7	-1 (30)	0 (250)	-1 (30)	3.05	3.04	127.22	166.00	189.08	197.53	12.55	12.17
8	-1 (30)	-1 (200)	0 (60)	3.08	3.01	125.22	131.62	170.37	168.66	7.70	7.76
9	0 (45)	-1 (200)	-1 (30)	2.67	2.74	82.33	37.15	148.63	141.89	4.94	5.26
10	1 (60)	-1 (200)	0 (60)	3.48	3.44	237.67	246.38	142.08	151.99	17.13	16.68
11	1 (60)	1 (300)	0 (60)	3.81	3.88	327.22	320.82	183.08	184.79	18.56	18.50
12	1 (60)	0 (250)	-1 (30)	3.50	3.47	115.56	152.03	188.86	185.68	8.72	8.85
13	0 (45)	0 (250)	0 (60)	3.50	3.49	233.44	155.38	171.63	165.46	10.81	10.74
14	1 (60)	0 (250)	1 (90)	3.58	3.59	464.56	425.78	217.19	208.74	22.26	22.64
15	0 (45)	-1 (200)	1 (90)	3.13	3.16	287.78	317.85	152.97	151.51	10.19	10.26
16	0 (45)	1 (300)	-1 (30)	3.52	3.49	277.33	247.26	154.97	156.43	10.59	10.52
17	0 (45)	0 (250)	0 (60)	3.80	3.49	132.33	155.38	163.3	165.46	11.21	10.74

2.2. Effects of SFE conditions on the percentage extraction yield

The quadratic model for percentage extraction yield of KBD powder showed significant linear and quadratic effects as shown by equation (1).

$$Y_{Yield} = +3.49 + 0.1738A + 0.2588B + 0.0975C - 0.0400AB - 0.0375AC - 0.1125BC + 0.0665A^2 - 0.0735B^2 - 0.2060C^2 \quad (1)$$

where Y is the response variable (percentage extraction yield), A is the extraction temperature, B is the extraction pressure, and C is the extraction time. A, B, and C are linear terms of each factor; AB, AC, and BC are interaction terms among factors; A^2, B^2, and C^2 are quadratic terms of factors.

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As shown in Table 2, the model was significant at p-value < 0.05. The model F-value of 4.72 was obtained, implying that the model was significant. The coefficient of determination (R^2) and the adjusted coefficient of determination (Adj.R^2) were 0.8584 and 0.6764, respectively. In this case, A, B, and C^2 were significant model terms due to the high estimated coefficient from the model equation (eq. 1). The experimental results showed the extraction yield was between 2.67-3.81%. No interaction effect to the percentage extraction yield among factors was found, i. e. p-value was more than 0.05 for all interaction terms, AB, AC, and BC. The highest percentage extraction yield (3.81%) was found at 60°C of the extraction temperature, 300 bar of the extraction pressure, and 60 min of the extraction time. Higher temperature and pressure seemed to furnish the higher percentage extraction yield.

Table 2. The analysis of variance (ANOVA) results of response surface model of percentage extraction yield, TPC, TFC, and TCC by SFE of KBD powder.

Source	DF	Percentage extraction yield (%)		TPC (mg GAE/g extract)		TFC (mg QE/g extract)		TCC (mg β-CE/g extract)	
		F-value	*p*-value	F-value	*p*-value	F-value	*p*-value	F-value	*p*-value
Model	9	4.72	0.0265*	5.66	0.0162*	8.67	0.0047*	77.70	< 0.0001*
A	1	9.03	0.0198*	17.16	0.0043*	1.01	0.3474	277.81	< 0.0001*
B	1	20.02	0.0029*	0.8500	0.3872	12.89	0.0089*	25.72	0.0014*
C	1	2.84	0.1357	7.90	0.0261*	7.85	0.0265*	20.61	0.0027*
AB	1	0.2392	0.6397	0.5730	0.4738	1.38	0.2789	0.9617	0.3594
AC	1	0.2102	0.6605	10.20	0.0152*	0.3953	0.5495	273.41	< 0.0001*
BC	1	1.89	0.2114	11.05	0.0127*	0.7964	0.4018	15.56	0.0056
A^2	1	0.6959	0.4317	1.73	0.2304	31.75	0.0008*	61.73	0.0001*
B^2	1	0.8501	0.3872	0.4367	0.5299	17.83	0.0039*	0.0280	0.8718
C^2	1	6.68	0.0363*	0.7516	0.4147	6.10	0.0428*	27.51	0.0012*
R^2		0.8584		0.8792		0.9177		0.9901	
Adj-R^2		0.6764		0.7239		0.8119		0.9773	

*Significant at p-value less than 0.05.

2.3. Effects of SFE conditions on total phenolic content (TPC)

The linear quadratic model for TPC in KBD powder showed significant linear at p-value less than 0.05 and F-value at 5.66. All quadratic effects were shown in equation (2).

$$Y_{TPC} = +155.38 +77.38A + 17.22B + 52.52C + 20.00AB + 84.36AC -87.83BC + 33.82A^2 + 17.02B^2 + 22.32C^2 \quad (2)$$

From the ANOVA analysis, the quadratic model of TPC was well fitted significantly (p-value < 0.05) and revealed the R^2 = 0.8792, and the Adj.R^2 = 0.7239. The high coefficient of a particular factor in the equation indicated a strong impact on the response variable, TPC. In this case, the extraction temperature (A), the extraction time (C), the interaction effect between extraction temperature and time (AC), and the interaction effect between the extraction pressure and time (BC) were significant model terms (p-value < 0.05) and were most significant parameters affecting the TPC.

The results showed that the TPC was between 82.33-464.56 mg gallic acid equivalents/g extract (mg GAE/g extract). The highest TPC (464.56 mg GAE/g extract) was determined at the extraction temperature of 60°C, the extraction pressure of 250 bar, and the extraction time of 90 min. Figure 1 showed the interaction effect between the extraction temperature and the extraction time (AC), and between the extraction pressure and the extraction time (BC) on the TPC.

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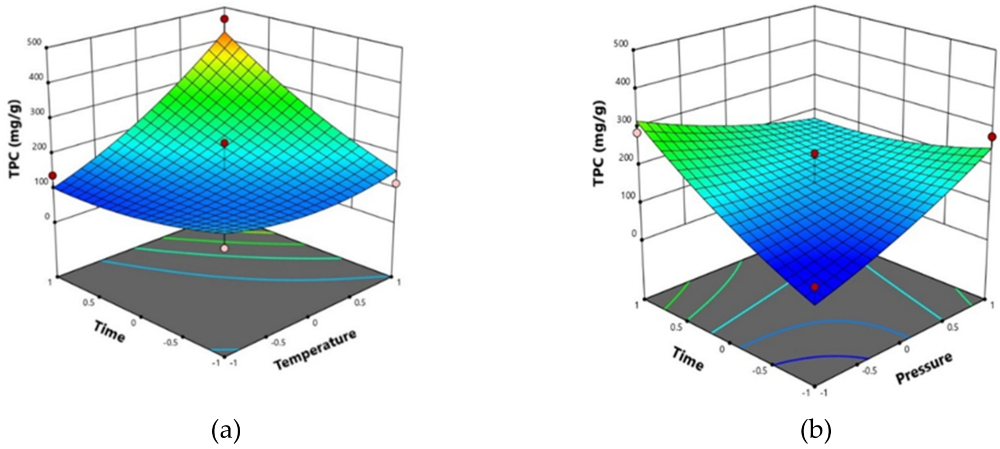

Figure 1. Three-dimensional response surface plots of TPC by Design-Expert software using SFE method against significant interaction factors: (a) between the extraction temperature and the extraction time (AC), and (b) between the extraction pressure and the extraction time (BC).

2.4. Effects of SFE conditions on total flavonoid content (TFC)

The TFC in KBD powder was investigated and the quadratic model showed significant linear and quadratic effects according to equation (3).

$$Y_{TFC} = +165.46 - 3.15A + 11.21B + 8.75C + 5.18AB + 2.78AC + 3.94BC + 24.26A^2 - 18.18B^2 + 10.64C^2 \quad (3)$$

From the ANOVA result in Table 2, the model F-value of 8.67 implied this model is significant and p-value of this quadratic model was 0.0047. The R^2 and Adj.R^2 were 0.9177 and 0.8119, respectively. The extraction pressure and extraction time were the most significant parameter affecting the TFC. The interaction effect between factors was not significant. However, the quadratic terms of this model (A^2, B^2 and C^2) significantly affect the TFC at p-value less than 0.05.

The experimental results showed the TFC between 142.08-217.19 mg quercetin equivalents/g extract (mg QE/g extract). The highest TFC (217.19 mg QE/g extract) was achieved at the extraction temperature of 60°C, the extraction pressure of 250 bar, and the extraction time at 90 min.

2.5. Effects of SFE conditions on total carotenoid content (TCC)

The quadratic model for total carotenoid content (TCC) showed significant linear and quadratic effect according to equation (4).

$$Y_{TCC} = +10.74 + 4.12A + 1.25B + 1.12C - 0.3425AB + 5.78AC - 1.38BC + 2.67A^2 + 0.0570B^2 - 1.79C^2 \quad (4)$$

The model showed p-value < 0.0001 with a high F-value (77.70), implying that the model was significant (Table 2). In this case, the extraction temperature (A) significantly affected the TCC with p-value < 0.0001. Meanwhile, the extraction pressure (B) and the extraction time significantly affected at p-value < 0.005. The interactions between extraction temperature-time (AC) and extraction pressure-time (BC) significantly affected the TCC as shown by the three-dimensional response surface plots (Figure 2). The quadratic term of extraction temperature (A^2) and extraction time (C^2) exhibited a significant effect at p-value < 0.05.

The R^2 and Adj.R^2 were 0.9901 and 0.9773, respectively. Since the Adj.R^2 was close to R^2, the model was highly significant. The results showed that the TCC was between 2.99-22.26 mg β-carotene equivalents/g extract (mg β-CE/g extract). The highest TCC (22.26 mg β-CE/g extract) was found with the extraction temperature at 60°C, the extraction pressure at 250 bar, and the extraction time of 90 min.

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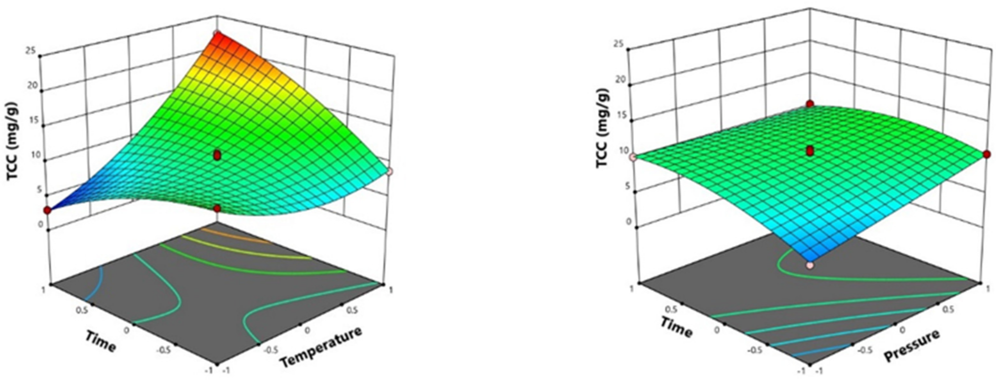

(a) (b)

Figure 2. Three-dimensional response surface plots of TCC by Design-Expert software using SFE method against significant interaction effect: (a) between factors (the extraction temperature and the extraction time (AC) and (b) between the extraction pressure and the extraction time (BC).

2.6. Effects of SFE conditions on total anthocyanin content (TAC)

The results from the experiments to determine the TAC revealed that anthocyanin could not be detected in this study even though the conditions of important factors were varied. That means anthocyanin could not be extracted by this method. The polarity of supercritical CO_2 fluid might not be appropriate for the extraction of less soluble compounds from the KBD powder. Some studies have described the use of a co-solvent such as methanol, ethanol, acetic acid, and formic acid for enhancing the efficiency of extraction [23]. However, ethanol seems to be the preferred co-solvent for consumable products due to its low toxicity. Some polar or nonpolar co-solvents can be used for SFE to improve the solvation of $ScCO_2$ which can enhance the affinity of poorly soluble compounds.

3. Discussion

Different extraction methods, previously described for KBD extraction, including solvent extraction, MAE and UAE were compared [1,24]. Solvent concentration (ethanol in water) was the most influential factor affecting the percentage of extraction yield and total active compounds for both UAE and MAE techniques. Previous results showed that MAE gave higher yields than UAE for the overall percentage yield and for each active compound. Heat generated by microwave radiation through instantaneous heating can cause the remaining moisture in the solid to evaporate and create a high vapor pressure that breaks the cell wall of plant and released the active compounds more easily from a material matrix to the extraction solvent [25]. Several studies have compared UAE, MAE, and SFE for the extraction of natural compounds. Normally, UAE uses longer extraction times and gives lower percentage extraction yields [26]. MAE was more effective than UAE for the extraction of olive leaves [27] and gave higher extraction yields of TPC, TFC and tannins from *Pistacia lentiscus* leaves [28]. Comparative study of UAE, MAE, SFE-CO_2 and classical methods for extraction of alkaloids from *Mitragyna speciosa* leaves showed that MAE gave higher alkaloid yields than other techniques [29]. For UAE, the principal mechanism of extraction is based on the collapse of bubbles which can produce physical, chemical, and mechanical effects and results in the disruption of biological membranes. The different mechanisms among extraction techniques might lead to different extraction efficiency. Previous studies showed that the mechanical disruption created by UAE appeared to be ineffective in breaking the cell wall of KBD powder and the temperature used in the range of 20-30 °C might not be effective enough to release the compounds under extraction [24]. The results of MAE indicated that the variables, especially solvent concentration and material-to-solvent ratio, enhanced the efficiency of the extraction. However, different parameters or extraction conditions in each technique are the main reason that determine the efficiency of extraction.

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In this study, $ScCO_2$ fluid was applied to SFE for the KBD extraction. CO_2 is an excellent solvent since it is chemically inert, economical, easily accessible, and separable from extracts, thus enhancing the penetrative power with high mass transfer rate. CO_2 is also non-toxic and approved food-grade solvent [23]. Thus, CO_2 has found its application for food, feed, and herbal products. Different variables such as extraction temperature, pressure, type of solvent, percentage of co-solvent, and sample particle size can affect SFE, and changing these variables could enhance the extraction yield. It has been reported that the solubility of the desired compounds by SFE depends on temperature and pressure that involve the density of fluid. The solubility of the extractable compounds could be maximized because it is an important factor influencing the quality and effectiveness of the extract. A diffusivity of the supercritical fluid is higher than that of liquid while its viscosity is less than that of liquid. Consequently, the compounds can show better diffusivity in supercritical fluid than in liquid. Low viscosity and high diffusivity of supercritical fluid give better penetration properties than normal liquid which can increase the efficiency and extraction yield. Moreover, high density combined with solvent power can give high solubility and selectivity to supercritical fluid. Thus, new desired compounds can be extracted by supercritical fluid. Temperature and pressure are the primary factors that influence the extraction efficiency. Modification of density by changing the temperature and pressure can affect the solubility [30]. In general, increasing pressure at a specific temperature increases the solvent density and solubility of the desired compounds. Thus, higher pressure and lower solvent volume are needed for the extraction. However, increasing the pressure to a given point can reduce the diffusivity and dissolution which decreases the antioxidant activity in some study [31].

The optimal extraction conditions to achieve high percentage yields of active compounds from KBD were determined with the aid of the Box-Behnken design (BBD) and response surface methodology in this study. The highest percentage yield was obtained at 60 ^{o}C and 300 bar of pressure during 60 min. The highest total active content, including phenolic, flavonoid, and carotenoid, was obtained at 60 ^{o}C and 250 bar during 90 min. Extraction temperature had a significant effect on the percentage extraction yield, TFC and TCC. Meanwhile, extraction pressure showed a significant effect on the percentage extraction yield, TFC, and TCC. Extraction time also showed a significant effect on TPC, TFC and TCC.

All the models of the three variable factors in this study were significant for all the responses (percentage extraction yield, TFC, TFC, TCC). Consequently, these models from three variable factors can be used for the prediction of these responses. High correlation between experimental and predicted values of each response (percentage yield, TPC, TFC and TCC) was also shown in Figure 3. For the extraction of KBD by other different techniques, the percentage extraction yield by SFE was lower than the others [1, 24]. TFC by SFE was similar to that by MAE but higher than those by UAE and conventional solvent extraction. However, TFC and TCC by SFE were the highest when compared to MAE, UAE, and conventional solvent extraction. SFE using $ScCO_2$ as a solvent has a potential for the extraction of flavonoids and carotenoids. Many important and potential flavonoids such as quercetin, kaempferol, rutin and luteolin are found in KBD [32]. Flavonoids and carotenoids exhibited many biological activities. Some studies showed the application of SFE for the extraction of carotenoids (β-carotene and lycopene) in tomato paste waste [33]. However, $ScCO_2$ has some limitations because the extractability of the compounds depends on their chemical structures as well as their polarity and molecular weight [19]. Some compounds have limited solubility in fluid CO_2 because of its non-polar property [34]. Some studies revealed that hydrocarbon and other organic compounds, with low polarity and molecular weight less than 250, can exhibit excellent solubility in $ScCO_2$ which can be carried out at low pressure (75-100 bar). Compounds of moderate polarity with molecular weight between 250-400 are moderately soluble, and higher pressure is needed for extraction. Highly polar compounds or compounds with molecular weight higher than 400 are hardly soluble in $ScCO_2$. For these reasons, TFC and TCC were higher than TFC, especially for anthocyanins which could not be quantified and extracted by SFE in this study. The solubility of the aglycones of anthocyanins in alcohol is higher than their glucosides, however, glycosylated anthocyanins are highly soluble in water. Anthocyanins containing polyphenolic groups show hydrophobic character

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that can make them soluble in some organic solvents such as ethanol and methanol. For example, delphinidin is more soluble in water than malvidin because malvidin is less polar than delphinidin [35]. Thus, the extraction of anthocyanins by water is a preferred method. Therefore, a non-polar $ScCO_2$ might not be appropriate for the extraction of anthocyanins. However, SFE can be used for the extraction of anthocyanins by adding some co-solvents such as ethanol.

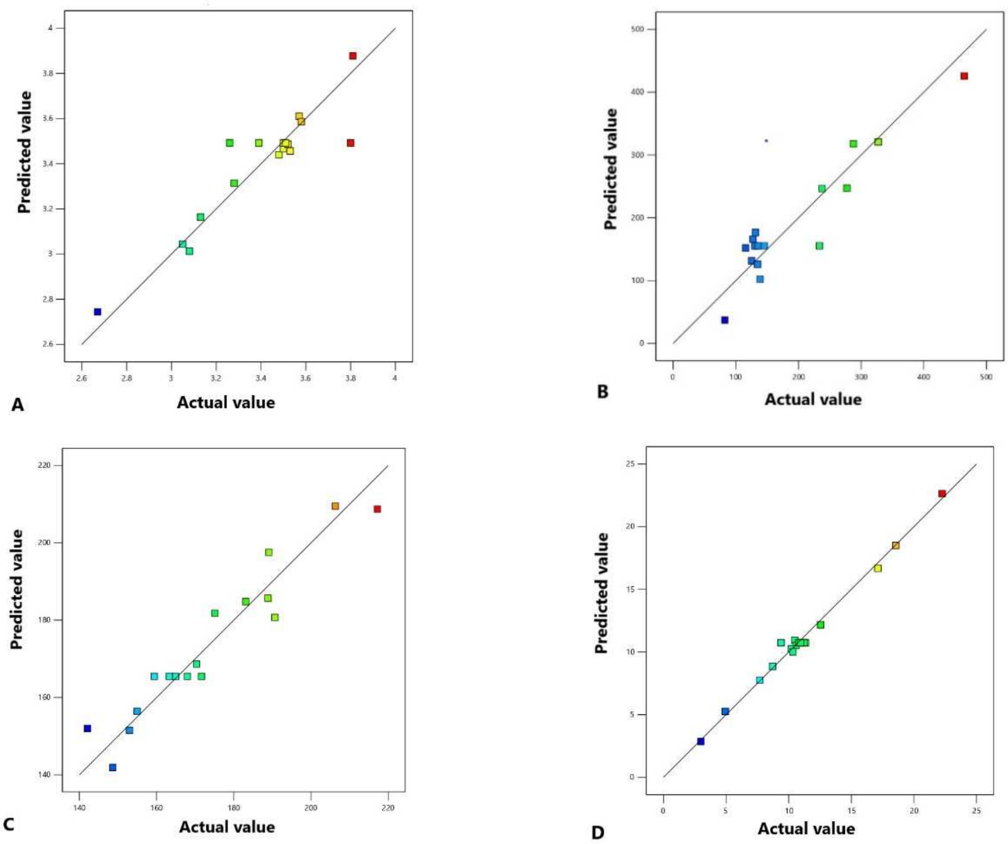

Figure 3. The relationship between the experimental values (actual values) and the predicted values of the percentage extraction yield (A), TPC (B), TFC (C), and TCC (D).

4. Materials and Methods

4.1. Plant materials

KBD used in this study is the Thai traditional herbal formula containing three herbs: *Piper nigrum* Linn. (Voucher specimen ABH18), *Nelumbo nucifera* Garetn. (Voucher specimen ABH15) and *Centella asiatica* Linn. (Voucher specimen ABH17) and their voucher specimens were deposited at Chao Phraya Abhaibhubejhr Hospital Foundation under the Royal Patronage of H.R.H. Princess Bejraratanarajsuda, Prachinburi Province, Thailand.

4.2. Chemicals and reagents

Folin-Ciocalteu phenols reagent, sodium acetate, Trolox, and quercetin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile, methanol and ethanol were purchased from VWR Chemicals BDH (Leicester, England). Gallic acid, phosphoric acid and hydrochloric acid were purchased from Merck (Darmstadt, Germany). Sodium carbonate was bought from loba chemie (Mumbai, India), aluminum chloride from Ajax Finechem (New South Wales, Australia), and potassium chloride from *QRëC* (Auckland, New Zealand).

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4.3. Experimental design

The BBD was used to set up SFE experimental set for response surface optimization. The BBD represents a new paradigm in experimental design. Its fusion of rotatable or nearly rotatable second-order design with a three-level incomplete factorial design creates a powerful framework for conducting experiments. The BBD is easy to perform and interpret compared to other models [36]. The experimental design was performed using Design-Expert software (Version 13, Stat-Ease Inc., Minneapolis, USA). Seventeen experimental runs were used to extract KBD powder by SFE. The variable factors and their levels are presented in Table 3.

Table 3. The variable factors and their levels for optimization of SFE for KBD**.**

Factor	Symbol	Levels		
		-1	0	1
Extraction temperature (°C)	A	30	45	60
Extraction pressure (bar)	B	200	250	300
Extraction time (min)	C	30	60	90

The second-order polynomial model (quadratic model) for the response surface analysis is shown as follows:

$$Y=\alpha_0+\sum \alpha_i X_i + \sum \alpha_{ii} X_i^2 + \sum \alpha_{ij} X_i X_j \quad (5)$$

where Y is the response variable, α_0 is a constant, α_i is the linear effect, αii is the quadratic effect, and αij is the interaction effect, X_i and Xj are the independent variables.

4.4. Supercritical fluid extraction (SFE) for KBD formula

The $ScCO_2$ extraction was performed using supercritical fluid extraction equipment (Taiwan Supercritical Technology Co., LTD, Taiwan) (Figure 4) and the extraction parameters are in accordance with the previously optimized method. The solvent used in the extraction was fluid CO_2. The extraction vessel was a 1000 mL stainless steel vessel. The extractions were conducted at pressure of 200, 250, and 300 bar, temperature of 30, 45, and 60 °C and extraction time of 30, 60, and 90 minutes. KBD powder (30 g) was added into the extraction vessel and extracted with $ScCO_2$ under various conditions according to the experimental design.

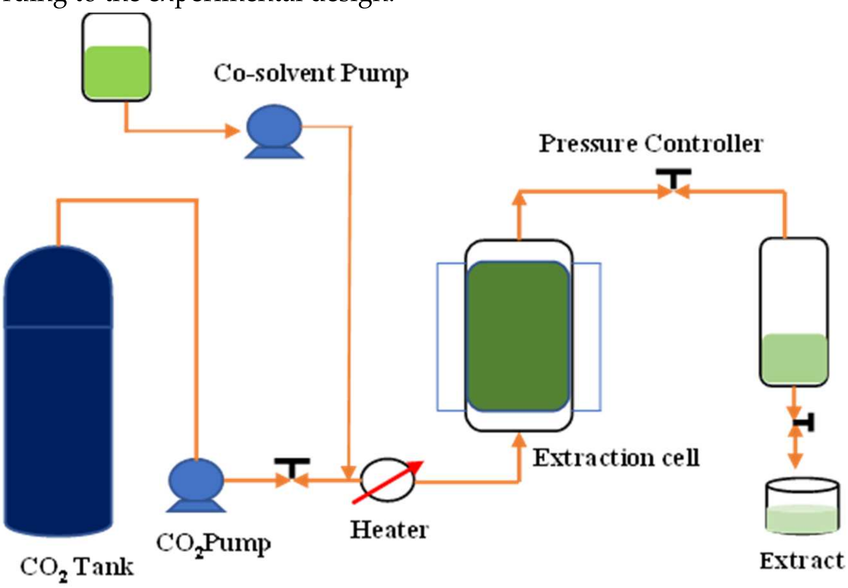

Figure 4. Schematic representation of supercritical fluid extraction instrument.

Table 4 shows all the experimental conditions for each of the extraction runs. After extraction, the extracts were collected, percentage yields of crude extract were calculated for each set of

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experiments. The extracts were stored at -20 °C prior to further analysis. All experiments were performed in triplicate.

Table 4. Experimental conditions from the BBD of each extraction runs for KBD powder by SFE.

Run order	Extraction variables		
	Extraction temperature (°C)	Extraction pressure (bar)	Extraction time (min)
1	30	250	90
2	45	250	60
3	45	250	60
4	45	300	90
5	30	300	60
6	45	250	60
7	30	250	30
8	30	200	60
9	45	200	30
10	60	200	60
11	60	300	60
12	60	250	30
13	45	250	60
14	60	250	90
15	45	200	90
16	45	300	30
17	45	250	60

4.5. Determination of the extraction yield

The extraction yield of KBD by SFE was calculated by the following equation (6):

$$\text{Extraction yield (\%)} = M1/M2 \times 100 \quad (6)$$

where M1 is the weight of KBD crude extract from SFE and M2 is the weight of the KBD dried powder.

4.6. Analysis of phytochemical content in KBD extract

4.6.1. Determination of the total phenolic content (TPC)

The Folin-Ciocalteu method was used for the determination of TPC in the KBD extract as previously described [37]. The extract (20 µL) was mixed with 10% Folin-Ciocalteu reagent (100 µL) and 80 µL of 7 % aqueous solution of Na_2CO_3. Then, the mixture was incubated in the dark at room temperature for 30 min. The microplate reader (PerkinElmer, Inc., Massachusetts, USA) was used for absorbance measurement at 760 nm. The experiments were performed in triplicate. The results are expressed as mg GAE/g extract.

4.6.2. Determination of the total flavonoid content (TFC)

The aluminum chloride colorimetric method was used for the determination of TFC in the KBD extract as previously described [37]. Briefly, 20 µL of the KBD extract was added to $AlCl_3$ solution (15 µL), 10% sodium acetate (20 µL) and distilled water (20 µL). The solution was then incubated in the dark for 15 min and was measured at 430 nm using a microplate reader (PerkinElmer, Inc, Massachusette, USA). The experiments were performed in triplicate and the results are expressed as mg QE/g extract.

4.6.3. Determination of the total carotenoid content (TCC)

The total carotenoid content (TCC) in the KBD extract was determined by UV-visible spectrophotometry as previously described [37]. Briefly, the KBD extract was pretreated with acetone and hexane. Then, 100 μL of the extract solutions were added into 96-well plates and the absorbance was measured at 450 nm using a microplate reader. The results are expressed as mg β-CE/g extract. The experiments were performed in triplicate.

4.6.4. Determination of the total anthocyanin content (TAC)

Determination of TAC in the KBD extract was based on the pH differential method with modification [3]. Briefly, 100 μL of 0.025M potassium chloride solution (pH = 1) were added into 20 μL of the extracts. Then, 20 μL of the extracts were diluted with 0.4M sodium acetate solution (pH = 4.5) in the same dilution factor. The absorbance was measured at 535 and 700 nm using a microplate reader. The experiments were performed in triplicate and the results are expressed as mg cyanidin-3-glucoside equivalents/g extract (mg C3G/g extract).

4.7. Statistical Analysis

The BBD, response surface analysis and ANOVA were performed using Design-Expert software (Version 13, Stat-Ease Inc., Minneapolis, USA) to design the experiments and to evaluate the effectiveness of the variable conditions on the percentage extraction yield and total active content (phenolic, flavonoid, carotenoid, and anthocyanin). The significant results were evaluated at the confidence level of 95% (p-value $\leq$ 0.05).

5. Conclusions

The SFE method was one of the most effective techniques for the extraction of natural products such as flavonoids, phenolic compounds, and carotenoids. $ScCO_2$ used in this technique is proved to be economical, easily accessible, and separable from the extracts. SFE could be the efficient process for extraction of active compounds, especially flavonoids and carotenoids from KBD powder. Optimization of the variables of the extraction technique by the BBD with the aid of response surface method is beneficial for the selection of extraction conditions. The findings from this study could be useful for the development of new KBD formulations from the KBD extract with better yields and higher concentrations of active compounds.

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