Extraction Optimization of Total Phenolic Content and Antioxidant Activity from Teaw (*Cratoxylum Formosum*) by Central Composite Design

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Abstract: The extraction of bioactive compounds from Teaw *(Cratoxylum formosum* (Jack) Dyer) was carried out based on the central composite design and response surface methodology to optimize the three variables: solvent polarity index (5.5-9.0), temperature (50-70°C) and extraction time (35-55 min). The optimization condition was based on yield, total phenolic content (TPC), DPPH radical scavenging (DPPH), ABTS radical scavenging (ABTS) and thiobarbituric acid reactive substances (TBARs) assay. From five variables, only two responses (yield and TPC) were fitted to a quadratic equation with R-square of 0.8479 and 0.8891, respectively. DPPH response was not quite fitted (R^2 = 0.4420) while ABTS and TBARs were insignificantly fitted ($p>0.05$). The optimal extraction condition was obtained with a solvent polarity index of 8.64 at 70°C for 55 min. The software predicted under these optimal conditions that yield was 28.58% whereas the TPC and DPPH, were 289 and 402 mg/g dry sample, respectively. The desirability value was 0.933. The proposed method proves to be simple, cheap and good for natural bioactive extraction from Teaw, being a potential approach for natural antioxidants.

1 INTRODUCTION

Plants has recently been focused as good sources of phytochemicals with strong antioxidant activity. The major responsible compounds are phenolic compounds which are abundantly contained in vegetable, fruits, berries, tea leaves, and herbs (Altermimi *et al*., 2017). A phenolic hydroxy group in the phenolic structure can donate a hydrogen atom to interrupt the propagation of free radical in oxidation processes (Altermimi *et al*., 2017; Kaur and Kapool, 2002). Therefore, the antioxidant can inhibit or delay the oxidation of molecules. From its activity, it can reduce the oxidative damage in foods ultimately increasing the shelf-life and quality of these foods (Lattanzio, 2013). It is a role not only for food preservation, but also for the defense of living systems and environmental stresses such as high light, low temperatures, pathogen infection, herbivores, and nutrient deficiency. These stresses can lead to increase production of free radicals and other oxidative species in plants (Altermimi *et al*., 2017).

Teaw (*Cratoxylum formosum*) is a native plant mostly grown in the North and North-Easts of Thailand and tolerates drought well. This plant is typically consumed as fresh shoots and young leaves. It tastes sour and slightly astringent due to phenolic components (Nakahara *et al*., 2002). It also found that plant have been reported as a medicinal plant. Fresh shoot and young leaves are used as a laxative. Root and leaves help a stomachache and skin disease (Areekul *et al*., 2009). The extract of Teaw (*C.formosum*) leaves showed strongly antioxidant and antimutagenic properties when compared with 108 species of indigenous Thai plants (Nakahara *et al*., 2002).The 80% methanol plant extract effectively scavenged DPPH radicals and contained many total polyphenol and flavonoids (Maisuthisakul *et al*., 2007).

The different extraction conditions including solvent, time and temperature affected on the yield of total phenolic and antioxidant activity due to different properties of each bioactive compound, a biomolecule from plants are chosen based on the polarity of the solute of interest. A solvent of similar polarity to the solute will properly dissolve the solute (Altermimi *et al*., 2017). In this situation, the many

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variables influenced the response. The response surface methodology (RSM) based on the central composite design (CCD) is an effective technique for optimizing the extraction with the various factors and the number of experiments are more useful for modeling (Bas and Boyaci, 2007; Sebswree, 2009).

The aim of this study is to optimize the extraction process from Teaw using central composite design. Three variables were used in this study: solvent polarity index (5.5-9.0), temperature (50-70°C) and extraction time (35-55 min). The optimization condition was based on yield, total phenolic content (TPC), DPPH radical scavenging (DPPH), ABTS radical scavenging (ABTS) and thiobarbituric acid reactive substances (TBARs) assay.

2 MATERIALS AND METHODS

2.1 Materials

Ethyl acetate was purchased from Honeywell (USA).2,-2diphenyl--1picrylhydrazyl(DPPH), Gallic acid ($C_7H_6O_5$), 2,2-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and Linoleic acid were purchased from Sigma-Aldrich (USA).6-hydroxy-2,5,7,8tetramethylchroman-carboxylic acid (Trolox) was purchased from BBL (USA). Folin–Ciocalteu reagent was purchased from VWR- Prolabo. Sodium Carbonate (Na_2CO_3) was purchased from Ajax-Finechem (Australia). Thiobarbituric acid (TBA), 1,1,3,3-Tetraethoxypropane (MDA) and Tween 40 were purchased from Merck (USA), Trichloroacetic acid (TCA) and Ethanol were purchased from RCI-Labscan (Iceland).

Teaw (*C. formosum*) was collected from Ubol Ratchathani province, Thailand. The sample was cleaned and selected fresh leaves and dried at 40°C in hot air oven for 24 hours. The dried Teaw was ground and passed through a sieve with mesh number 40 and frozen at -20°C.

2.2 Extraction Procedure

In this study, three variables, polarity index of solvent (5.5-9.0), temperature (50-70°C) and extraction time (35-55 min) were designed by using the Central Composite Design. The extraction conditions are shown in Table 1. The solvent preparation was calculated by using equation 1 (Poole, 1998).

$$P' = \sum_i P'_i \emptyset_i \quad (1)$$

Where, P'_i is the polarity index of solvent i
$\emptyset_i$ is the quantity of solvent i

The polarity index of ethyl acetate, ethanol and water are 4.4, 5.2 and 10.2, respectively (Katz, 1998; Harris, 2015). These solvents were used for preparing the polarity index as calculated from the formula above. From this, it has five varying solvent including ethyl acetate, 95%ethanol, 60% ethanol, 25% ethanol and water (Table1). For extraction, 200 ml of solvent was placed in the waterbath to obtain the targeted temperature and then mixed with 10 g of plant powder. The sample was set for certain extraction time, cooled with the tap water and filtered through Whatman No. 4. The filtrate was concentrated by rotary evaporator at 35±1 °C, vacuum pressure 50 mbar. After that, the concentrated extract was dehydrated by using freeze-dryer at -80°C for 50 hours and extract powder was kept at -20°C. The moisture content was determined by AOAC method 966.02 (2000).

2.3 Analytics Method

2.3.1 Yield (%)

The percentage ration of total solid of Teaw extract and plant was calculated by using the equation 2.

$$\% \text{ Yield} = \frac{\text{A dry weight of Teaw extract (g)}}{\text{A dry weight of Teaw powder (g)}} \times 100 \quad (2)$$

2.3.2 Total Phenolic Content (TPC)

The total phenolic content was determined by the Folin–Ciocalteu method (Shaghaghi *et al*., 2008). The 40 µl extract was mixed with 100 µl deionized water, 20 µl Folin–Ciocalteau reagent and 40 µl sodium carbonate (10% Na_2CO_3). Then, it was kept at room temperature for 30 minutes in the dark. Absorbance was measured at 765 nm. Results were expressed as mg of gallic acid per gram dry sample.

2.3.3 DPPH Radical Scavenging (DPPH)

The radical-scavenging activity was determined by the DPPH method (Murakami *et al*., 2004). Aliquot of 50 µl of extract was mixed with 150 µl of 0.22 M DPPH (in 95% ethanol) and incubated at room temperature in the dark for 30 minutes. The Absorbance was measured at 517 nm using Trolox as a standard. Results were expressed as mg of Trolox per gram dry sample.

Table 1: Experimental designs using central composite designs (CCD) and results of the response variables studied.

	Independent Variables			Response Variables				
	Polarity:X_1 (Solvent)	Time:X_2 (min)	Temp:X_3 (°C)	Yield (%)	TPC (mg/g)	DPPH (mg/g)	ABTS (mg/g)	TBARs (mg/g)
1	4.4(Ethyl acetate)	45.0	60	13.30	195.34	297.10	123.49	348.29
2	5.45(25% Ethanol)	35.0	50	19.33	207.49	271.46	227.29	272.40
3	5.45(95% Ethanol)	35.0	70	16.23	227.83	262.88	144.73	278.76
4	5.45(95% Ethanol)	55.0	50	19.04	208.41	267.42	141.55	423.49
5	5.45(95% Ethanol)	55.0	70	19.27	275.15	349.12	196.98	302.71
6	7.2(60% Ethanol)	28.2	60	23.96	264.37	379.55	233.10	285.50
7	7.2(60% Ethanol)	45.0	43	24.63	272.71	372.98	276.43	294.57
8	7.2(60% Ethanol)	45.0	60	19.27	275.15	349.12	196.98	302.71
9	7.2(60% Ethanol)	45.0	60	25.22	280.66	388.76	177.44	289.07
10	7.2(60% Ethanol)	45.0	60	27.16	288.23	390.15	172.95	324.42
11	7.2(60% Ethanol)	45.0	77	28.65	273.55	394.32	180.00	311.47
12	7.2(60% Ethanol)	61.8	60	28.90	283.49	372.10	288.91	317.60
13	8.95(25% Ethanol)	35.0	50	25.04	282.49	382.32	203.57	315.74
14	8.95(25% Ethanol)	35.0	70	29.77	297.63	402.40	213.33	296.74
15	8.95(25% Ethanol)	55.0	50	25.70	297.63	406.44	224.88	297.75
16	8.95(25% Ethanol)	55.0	70	27.03	289.98	409.47	203.49	295.89
17	10.2 (water)	45.0	60	21.81	244.72	335.48	177.60	322.17

2.3.4 ABTS Radical Scavenging (ABTS)

The ABTS was determined by ABTS method (Zhou and Yu, 2004). Aliquots of 50 µl extract and 100 µl of 5 mM ABTS solution was mixed and kept in the dark for 5 minutes. The Absorbance was measured at 734 nm and compared with Trolox standard. Results were reported as mg of Trolox per gram dry sample

2.3.5 Thiobarbituric Acid Reactive Substances (TBARs) Assay

The Oxidation resistance was determined by the Ant-TBARs method (McDonald and Hultin, 1987). Briefly, aliquots of 0.2 ml samples and 0.8 ml linolenic acid (1%) was mixed and placed in a water bath at a temperature of 50 ± 1 °C for 18 hours. After that, 2 mL of TCA-TBA-HCl solution was added and boiled in the boiling water for 15 minutes. The sample was cooled with the tap water and centrifuged at a speed of 5,500 rpm for 5 minutes. The supernatant was measured the absorbance at 520 nm and compared to Butylated Hydroxy Toluene (BHT) standard.

2.4 Experimental Designs and Statistical Analysis

A Central composite (CCD) experimental design was used to investigate the effects of three independent variables, namely polarity index of solvent (X_1) extraction time (min; X_2), and temperature (°C; X_3). A total of 17 experimental runs are listed in Table 1 The influence of extraction factors was optimized using Response Surface Methodology (RSM) using by design expert software (Design Expert 7.0 trial).

3 RESULTS AND DISCUSSION

3.1 The Yield, TPC, DPPH, ABTS and TBARs

The response variables of Teaw extract obtained from 17 experiments from extraction factors values were yield (13.30 - 29.77%), TPC (195-297 mg GAE/g dry sample**)**, DPPH (263-409 mg Trolox/g dry sample), ABTS (123-289 mg Trolox/g dry sample**)** and TBARs (272-424 mg BHT/g dry sample**)**. The data of experiment were statistically tested for analysis of variance (ANOVA) for regression model which are shown in Table 2.

Table 2: Analysis of variance (ANOVA) for regression model.

Response	Source of variation	Sum of squares	df	Mean square	*F*-value	*p*- value
Yield	Model	308.45	9	34.27	4.33	0.0331
	Residual	55.35	7	7.91		
	Lack of fit	21.50	5	4.30	0.25	0.9060
	Pure error	33.85	7	7.91		
	Corr total	363.80	16			
R^2 = 0.8479 Adj.R^2 = 0.6522						
TPC	Model	15635.64	9	1737.29	6.24	0.0124
	Residual	1949.85	7	278.55		
	Lack of fit	1863.68	7	287.55		
	Pure error	86.17	2	43.08		
	Corr total	17585.49	16			
R^2 = 0.8891 Adj.R^2 = 0.7466						
DPPH	Model	21209.40	3	7069.80	4.72	0.0193
	Residual	19472.50	13	1497.88		
	Lack of fit	18368.61	11	1671.51	3.08	0.2706
	Pure error	1084.89	2	542.95		
	Corr total	40681.90	16			
R^2 = 0.5213 Adj.R^2 = 0.4109						
ABTS	Model	22651.62	9	2516.85	2.14	0.639
	Residual	8227.19	7	1175.31		
	Lack of fit	7900.75	5	1580.15	9.68	0.0963
	Pure error	326.44	2	163.22		
	Corr total	30878.80	16			
R^2 = 0.7336 Adj.R^2 = 0.3910						
TBARs	Model	13866.30	9	1540.70	2.07	0.1755
	Residual	5220.07	7	745.72		
	Lack of fit	4584.47	5	916.89	2.89	0.2772
	Pure error	635.60	2	317.80		
	Corr total	19086.37	16			
R^2 = 0.7265 Adj.R^2 = 0.3749						

The result showed that 3 of 5 response variables including yield, TPC and DPPH were significant difference ($p \leq 0.05$). This indicated that 3 extraction factors; polarity index of solvent, extraction time and temperature affected on their values. On the other hand, ABTS and TBARs showed insignificant difference ($p > 0.05$).

For extraction yield, response model was fitted to a quadratic equation with correlation coefficient (R^2) of 0.8479 (p=0.0331). In addition, the lack of fit value was insignificantly (p-lack of fit = 0.9060) indicating that model was suitable and no other model could explain this response. The yield regression equation is shown in equation (3).

$$\text{Yield} = 17.528 + 11.968X_1 - 0.343X_2 - 1.337X_3 - 0.0351X_1X_2 + 0.0639X_1X_3 - 0.0000962X_2X_3 - 0.844X_1^2 + 0.00736X_2^2 + 0.00795X_3^2 \quad (3)$$

The correlation coefficient of this equation was 0.8891. The TPC value was good fitted to a quadratic model with R^2 of 0.8891 that mean a model was effective to explain 88.91% of this result. Moreover, the lack of fit value was insignificantly (p-lack of fit > 0.05) representing a suitable model for TPC. The TPC regression equation is presented in equation (4).

$$TPC = -586.204 + 162.768X_1 + 1.892X_2 + 5.042X_3 + 0.291X_1X_2 - 0.568X_1X_3 + 0.0295X_2X_3 - 7.014X_1^2 - 0.0102X_2^2 - 0.0131X_3^2 \quad (4)$$

The DPPH response was not quite fitted to linear equation due to R^2 of 0.5231 which means that linear model was able to explain only 52.31% of this result. Despite, the lack of fit this model was insignificantly (p-lack of fit > 0.05) which means no other models fit. Therefore, this linear model was proper. The TPC regression equation is presented in equation (5).

$$DPPH = 108.461 + 21.546X_1 + 0.738X_2 + 0.961X_3 \quad (5)$$

For the ABTS regression model, the ABTS data is a quadratic model has a R^2 of 0.7336 and lack of fit was insignificantly (p-lack of fit > 0.05). Additionally, the ABTS data was insignificantly (p > 0.05) with any model. This means that the polarity index of solvent, extraction time and temperature had no influence on the ABTS. Similar result was observed in TBARs. The TBARs response was insignificantly fitted in any model (p > 0.05). Although, in a quadratic model the TBARs result performed R^2 of 0.7265 with lack of fit of 0.2772 The ABTS and TBARs regression equation was are shown in Equation 6 and 7 respectively.

$$ABTS = 1303.183 + 66.614X_1 - 30.881X_2 - 22.271X_3 + 0.321X_1X_2 + 0.110X_1X_3 + 0.133X_2X_3 - 5.396X_1^2 + 0.234X_2^2 - 0.1162X_3^2 \quad (6)$$

$$TBARs = -143.818 - 30.549X_1 + 21.749X_2 + 2.568X_3 - 1.384X_1X_2 + 0.668X_1X_3 - 0.137X_2X_3 + 3.309X_1^2 - 0.22X_2^2 - 0.016X_3^2 \quad (7)$$

3.2 Analysis of Response Surface

Equations from mathematical models of three response variables were plotted to a contour plot. The contour plot present two factors between a response and another one factor specifying at central value. Yield, TPC and DPPH were response variables (Y) and a polarity index of solvent (X_1), extraction time (X_2) and temperature (X_3) where independent variables.

Figure 1 shows a contour plot of yield of extraction depending on the solvent polarity, time and heating treatment. It is known as that polarity of solvent is the most important factors under the same extraction time and temperature (Ju *et al*., 2014). Considering, the effect of polarity index of solvent and extraction time in Figure 1a, yield increased by increasing the polarity solvent and time. Yield for 24.81% was the highest yield by increasing 7.5-9 for polarity index of solvent with time in range 35-55 minutes. These results suggested that increasing polarity or water concentration in the solvent raised a yield which may cause of the higher solubility of proteins and carbohydrate in water (10.2 for polarity index) than a low polarity index of solvent (Zielinski and Kozlowska, 2000). That why a yield was increased by increasing polarity solvent. Figure 1b show the effect of polarity index of solvent and temperature. Yield was increased by increasing polarity solvent. The highest value was 26.09% among 7.5-9 for polarity index of solvent with temperature 63-70°C, but it decreased at low temperature (< 63 °C). Thus, yield of Teaw extract affected by temperature. The higher temperature may improve the solubility of antioxidant compounds (Liang *et al*., 2017). The effect of extraction time and temperature with 7.25 for polarity index of solvent was shown in contour plot Figure 1c. Yield of extract was increased by increasing extraction time and temperature. This result confirmed that extraction time and temperature influenced the yield.

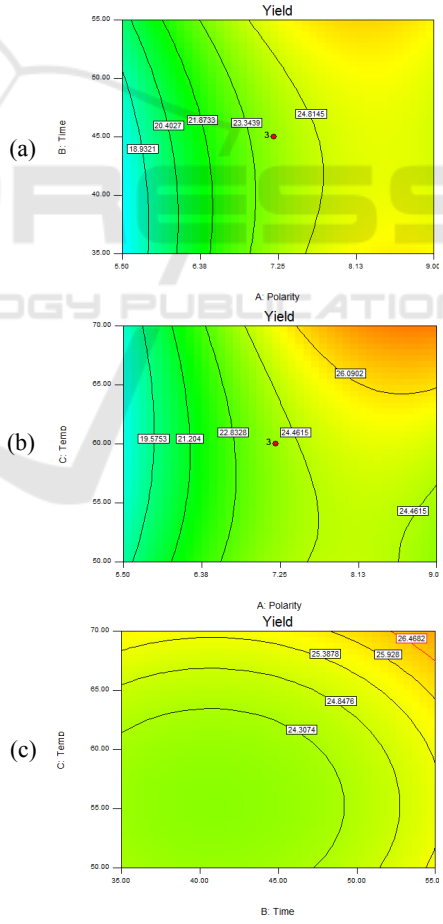

Figure 1: Contour plot for yield (%) as a function of (a) polarity index of solvent and extraction time, (b) polarity index of solvent and temperature, (c) extraction time and temperature of Teaw extract.

Influence of extraction factors on a TPC is presented in contour plot (Figure.2). The TPC value increased by increasing a polarity index of solvent from 7.25 until almost 9 after that slightly decreased (Figure.2a). TPC increased by increasing a polarity index of solvent at higher 7.25 and the highest TPC value was inspected at 8.13 to 9 for polarity index. These results confirmed the polarity of solvent greatly influence on the TPC value. On the other hand, when temperature increasing the TPC value had a slightly decrease as a result of high temperature. The temperature was an important factor affecting of the extract due to thermal degradation or loss of the antioxidant compounds. It suggests molecular change inside the antioxidant compounds structure (Sahin, 2018). In addition, the TPC increased by increasing time and temperature (Figure.2c). High temperature and time might soften the plant tissue and waken the phenol-polysaccharide and phenol-protein interactions in plant therefore, the polyphenol would migrate into solvent (Youseff and Adawi, 2006).

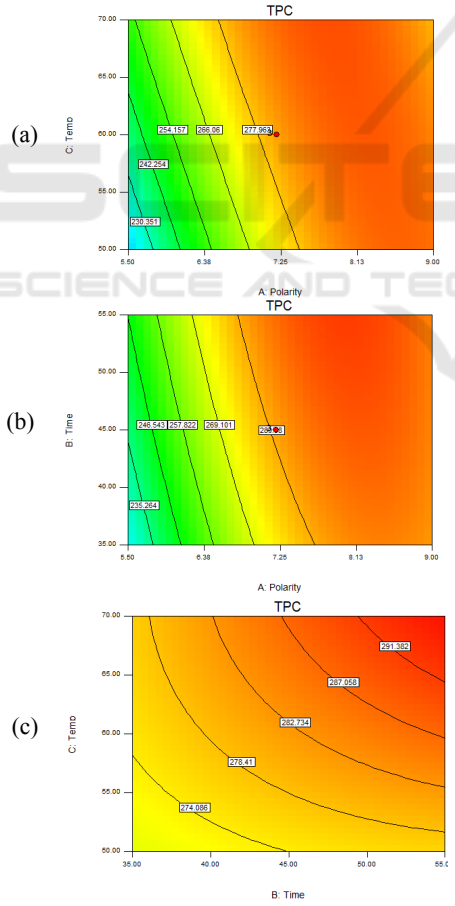

Figure 2: Contour plot for TPC (mgTrolox/g dry sample) as a function of (a) polarity index of solvent and extraction time, (b) polarity index of solvent and temperature, (c) extraction time and temperature of Teaw extract.

Figure 3 shows the contour plot of Teaw extract on DPPH. The DPPH values were fitted to linear model. This meant factors had not interaction term on this result (Equation 5). However, each factor was effective to DPPH value. It increased by increasing the polarity index of solvent, extraction time and temperature. Polarity of solvent was vastly influent factor. When polarity index increased at 8.95, DPPH was highest value. Then, polarity index at 10.2 DPPH was slightly decreased.

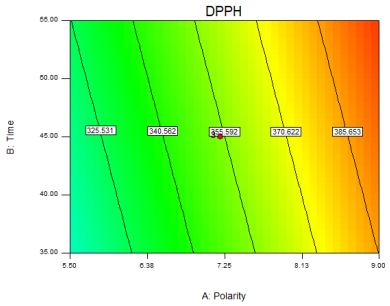

Figure 3: Contour plot for DPPH (mgTrolox/g dry sample) of Teaw extract.

3.3 The Optimum Extraction

Using the design expert software 7.0 trial, the computer program calculated the optimal conditions which was solvent polarity index of 8.64 at 70°C for 55 min. The yield, TPC and DPPH were predicted to be 28.58%, 289 mg/g dry sample and 402 mg/g dry sample, respectively with the desirability value of 0.933. The desirability represents the correlation of independent and response. In this experiment, it was correlation of 93.3% which a “very good” correlation level (Lazic, 2004).

4 CONCLUSIONS

Three extraction factors including polarity index of solvent and extraction condition (temperature and time) significantly pronounced the effect on yield and TPC while the other three variables were insignificant. The central composite design as an experimental design using by design expert program could be used for optimizing the Teaw extraction. The optimum condition was a solvent polarity index of 8.42 at 70°C for 55 min with desirability of 0.933. This technique is simple, cheap and good for natural bioactive extraction from Teaw being a potential approach for natural antioxidants.

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