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RESEARCH ARTICLE

Supercritical Fluid Extraction of Phenolics from *Anisophyllea disticha* (Jack) Baill. and Evaluation of their Antioxidant Activities

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Abstract: ***Background*:** *Anisophyllea disticha* (Jack) Baill. (*A. disticha*) is a species of the Anisophylleaceae family that has undergone the least investigation despite being widely used in folk medicine to cure a wide range of illnesses.

***Objective*:** The purpose of this study is to examine the impact of various factors on the supercritical fluid extraction of *A. disticha* in order to maximise recovery of total phenolic content, antioxidant activity, and polyphenol identification.

***Method*:** The total phenolic content (TPC) and antioxidant activities of *A. disticha* were determined using the supercritical fluid extraction (SFE) method and compared with Soxhlet. Box-Behnken design of response surface methodology was performed to examine the effect of independent variables of SFE such as temperature, pressure, and concentration of ethanol as co-solvent on TPC and antioxidant activities of *A. disticha* stem extracts.

***Result*:** At combined effects of different temperatures, pressure, and co-solvent, the total SFE yield were ranged between 0.65 and 4.14%, which was about half of the Soxhlet extract of 8.75 ± 1.54%. The highest concentration (μg/g) of gallic acid (118.83 ± 1.17), p-coumaric (61.60 ± 0.33), ferulic acid (57.93 ± 1.15), and quercetin (24.16 ± 0.41) were obtained at a temperature of 50°C, the pressure of 25 MPa and co-solvent of 20%, while lowest concentration was found 70°C, 30 MPa, and 20% ethanol.

***Conclusion*:** SFE extracts possessed remarkable TPC and concentration of phenolic compounds, indicating superior recovery of compounds. SFE showed more than two-fold higher ferric-reducing antioxidant power compared to Soxhlet with values of 585.32 ± 17.01 mg Fe (II)/g extract and 203.63 ± 16.03 mg Fe (II)/g extract, respectively. SFE demonstrated a potential alternative to the classical solvent extraction methods.

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1. INTRODUCTION

Anisophyllea disticha (Jack) Baill. (*A. disticha*) is a small treelet that is distributed in swamp and lowland forests in Malaysia, Indonesia, Singapore, Brunei, and the Philippines [1]. It belongs to the Anisophylleaceae family that made up of two markedly different sizes of leaf blades arranged along the branches. The leaves and stem are generally used by the folklore to treat diarrhoea, dysentery as well as fever [1]. Quattrocchi [2] stated the ability of leaves in healing jaundice, cuts, and wounds while fruits are useful for poisoned stings by bees and hornets. The stem of *Anisophyllea laurina* is a well-known traditional medication for the remedy of dysentery, malaria, and fungal diseases, contains a high amount of total phenolic content (2382.39 mg GAE/100 g) and total flavonoid concentration (385.79 mg QE/100 g) [3]. The roots of *A. disticha* serve in relieving tiredness and body aches, refresh the body, revitalize the birth canal, delay the aging process as well as treat weakness in men and infertility in women [4, 5].

Conventional extraction methods are generally employed to obtain bioactive compounds from the plant which are time-consuming, involve high consumption of toxic sol-

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vents that require additional evaporation steps, are costly, have low extraction selectivity, and are not suitable for extracting thermo-labile compounds [6]. Drawbacks from conventional methods and increase demand for solvent-free products have encouraged the growing interest in green extraction technology [7]. In this regard, SFE has become a very attractive option as it offers several advantages including high extraction efficiency and selectivity, short extraction time, reduce thermal degradation due to low temperature, and high purity of the extract. Carbon dioxide is the most common solvent for SFE due to its moderate critical conditions (31.1°C and 7.28 MPa), and non-toxic, non-flammable, and cost-effective properties [8]. However, CO_2 is a non-polar solvent; hence adding a small volume of a food-grade modifier like ethanol can significantly enhance the solubility of the extraction [9].

In this study, the Box-Behnken Design (BBD) of a response surface methodology (RSM) approach was employed to maximize the extraction yield by minimizing the pressure, temperature, and concentration of co-solvent. Previous literature reviews are limited to the use of conventional methods in extracting phytoconstituents from the genus *Anisophyllea* namely maceration, percolation, and Soxhlet [3, 10, 11]. To the best of my knowledge, there is no previous research that has been reported on the application of supercritical fluid to extract total phenolic content from *A. disticha*. Therefore, the objective of this research was to investigate the effect of different process variables such as temperature, pressure, and concentration of co-solvent on supercritical fluid extraction of *A. disticha* for maximum recovery of total phenolic content (TPC) and antioxidant activities, and to identify its polyphenols by HPLC.

2. MATERIALS AND METHODS

2.1. Sample Preparation

The stem of *A. disticha* was collected from Kenyir Lake, Terengganu, Malaysia. The sample was authenticated by Dr. Samsul Khamis, a plant taxonomist from Universiti Kebangsaan Malaysia, and a voucher specimen (PIIUM 0003-2) was deposited at Kulliyyah of Pharmacy, International Islamic University Malaysia (IIUM). The sample was cleaned with tap water and dried for a week in the oven set to 40°C in the natural product research laboratory, Kulliyyah of Science, IIUM. The dried stem sample was ground into a fine powder and preserved for further investigation.

The dried stem sample was ground into a fine powder for Soxhlet and SFE extraction. 50 g of powdered stem sample was packed in a thimble and subsequently extracted using Soxhlet (Gerhardt, Germany) apparatus with 250 ml ethanol for 12 hours in the dark. The solvent was dried at 60°C using vacuum rotary evaporators. The yields of Soxhlet extracts were determined in percentage on a dry weight basis.

2.2. Supercritical Fluid Extraction

For each experiment, 20 g of powdered sample was densely packed into a 100 ml extraction vessel (Supercritical Fluid Technologies Inc SFT-150). The extraction method involved two stages namely static and dynamic phase. In the first operating mode, the extraction chamber was occupied with supercritical CO_2 and thermostatic for 15 min to stabilize the temperature of the chamber and to ensure adequate contact of the solvent with the stem powder. Each extraction was performed for 90 minutes at a constant CO_2 mass flow rate of 8 mL/min, with desired temperature, pressure, and concentration of co-solvent. When the scheduled time was accomplished, the extractor column was depressurized, and the extract was detached from the CO_2 and collected in glass vials. The extract was sealed and kept at 4°C prior to analysis to avoid any potential degradation.

For the BBD of RSM, three independent variables with three levels were selected (Table **1**). The selected values of temperature (X_1) range from 50-70°C, pressure (X_2) of 20-30 MPa, and concentration of co-solvent (X3) that varies from 10-20% (Table **1**). The complete design established by BBD consisted of 15 experiments with three replicates for the central point. The experimental order was randomized to lower the effect of unpredicted variability in the responses caused by extraneous factors.

The second-order polynomial regression equation was used to study the correlation between independent variables and responses, hence applied to predict the optimal points. For the three variables, the equation is of the following form:

$$Y = \beta_0 + \sum_{j=1}^{k} \beta_j X_j + \sum_{j=1}^{k} B_{jj} X_j^2 + \sum_i \sum_{<j=2}^{k} \beta_{ij} X_i X_j + e_i$$

where Y is the response, β_o, β_j, $\beta_{jj,}$ and β_{ij} are constant coefficients of intercept, linear, quadratic, and interaction terms, respectively. X_i and X_j are independent variables (temperature, pressure, or concentration of co-solvents), k is the number of independent variables and e_i is the error.

The Stat-Ease Design Expert 10 statistical software was used for multiple regression analysis and the significance of the developed models was verified through analysis of variance (ANOVA). The values of determination coefficient (R^2), sum of squares, and F-value were analyzed to measure

Table 1. Investigated factors and levels in box-behnken design.

Independent Variable	Symbol	Level		
		Low (-1)	Middle (0)	High (+1)
Temperature (°C)	X_1	50	60	70
Pressure (MPa)	X_2	20	25	30
Co-solvent (%)	X_3	10	15	20

the fitness of the regression model. The fitted quadratic polynomial equation was depicted as response surface and contour plots in order to exhibit the association between independent variables and responses as well as to anticipate the optimum conditions.

2.3. Antioxidant Activity of Crude Extracts

2.3.1. Total Phenolic Content Assay

Total phenolic content (TPC) was analysed using the Folin Ciocalteu assay following the method of Zheng & Wang [12] with some alterations. A reaction mixture consisting of 58 µl of diluted extracts, 968 µl of Folin-Ciocalteu reagent (diluted 1:10), and 774 µl of 5% Na_2CO_3 solution was used to neutralize the reaction. The mixture was incubated for 1 hour in a dark place and the absorbance was determined at 760 nm using a multi-detection micro-plate reader. The TPC was calculated and expressed as mg gallic acid equivalents (GAE) per g extract with a calibration curve in the linear range of 0.02-0.20 mg/ml. In our earlier publications [13], we described the sample preparation process in detail for the TPC investigation.

2.3.2. DPPH Radical Scavenging Activity Assay

The free radical scavenging activity of the extracts was evaluated by using a 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay according to Liu *et al*. [14] with some modifications. Initially, a volume of 40 µl of extracts (0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml) was thoroughly mixed with 160 µl of 0.079 mg/ml DPPH in methanol. The blank was prepared using the same procedure; instead, the plant extract was replaced with an equal volume of methanol and added to 160 µl of DPPH solution. The mixture was incubated for 15 mins in a dark place and the absorbance at 517 nm was measured using a microplate reader spectrophotometer. Detail methods were reported in our earlier articles [13].

2.3.3. Ferric Reducing Antioxidant Power Assay (FRAP)

The reducing power was determined according to the procedure described by Benzie & Strain [15] with a slight adaptation. FRAP reagent was prepared from the mixture of 0.031 g 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM hydrochloric acid (HCl), 0.054 g ferric chloride (FeCl3) in deionized water and 300 mM acetate buffer (pH 3.6) in proportions of 1:1:10 (v/v/v). 600 µl of freshly made FRAP reagent was mixed with 80 µl of each appropriate diluted sample (0.2, 0.4, 0.6, 0.8, and 1.0 mg/ml) and 1 ml of deionized water. The mixture was then incubated at 37°C for 30 min. The absorbance was measured at 539 nm using a microplate reader spectrophotometer. Detail methods were reported in our earlier articles (13).

2.4. Determination of Phenolic Compounds

A high-performance liquid chromatography (Perkin Elmer, USA) using a photodiode array (PDA) detector was developed for quantitative estimation of phenolic acids (gallic, *p*-coumaric, and ferulic acids) and flavonoid (quercetin) present in *A. disticha*. The investigated compounds were separated on a reversed-phase C18 column (250 mm x 4.6 mm ID, 5 µm particle size) using isocratic elution. The column temperature was maintained at 25°C. According to the method described by Wang *et al.* [16], after some modifications, the mobile phase adopted for the detection of phenolic acids consisted of methanol (Solvent A) and 1% formic acid in water (Solvent B) (80:20). The detection of flavonoid namely quercetin was done based on the procedure described by Tasioula-Margari & Tsabolatidou [17] with slight adaptations. The mobile phase used contains methanol: acetonitrile (5:50) (Solvent A) and 1% formic acid in water (45) (Solvent B). We provided a detailed explanation for the determination of phenolic compounds in our earlier published papers (13).

2.5. Statistical Analysis

Each experiment was carried out in triplicate, and the findings were presented as mean ± standard deviation. Microsoft Excel and the Statistical Package for Social Science (SPSS) version 20.0 software were used to analyse the experimental data. Analysis of variance (ANOVA) and Duncan's multiple range test were used to determine the significance of differences among the means, and $p < 0.05$ was considered significant.

3. RESULTS AND DISCUSSION

3.1. Model Fitting and Evaluation of the Factors

Table **2** summarizes the experimental runs and their influence on dependent variables. The analysis of variance (ANOVA) in Table **3** showed that the developed model was statistically significant ($p < 0.05$) with the value of the coefficient of determination (R^2) of 0.9004, 0.9410, and 0.9056, respectively, for total phenolic content, DPPH radical scavenging activity and ferric reducing antioxidant power. These values demonstrated that the polynomial regression model is accurate and adequately fits the experimental data. Furthermore, the fitness of experimental data with the model was further justified according to the analysis of lack of fit that showed a non-significant value ($p > 0.05$).

As recorded in Table **3**, it can be inferred that temperature (X_1) and concentration of co-solvent (X_3) contribute as significant parameters for TPC and reducing power of *A. disticha* with $p < 0.05$, while all the three main variables were statistically significant in the model for scavenging activity. The interaction of temperature and concentration of co-solvent (X_1X_3) was significant while the other interactions were insignificant for the extraction of phenolic compounds. Interactions between temperature and concentration of co-solvent (X_1X_3) and between pressure and concentration of co-solvent (X_2X_3) have significant effects on the DPPH radical scavenging activity of *A. disticha*. Interactions of temperature and percentage of modifier (X_1X_3) exhibited a *p*-value smaller than 0.05, suggesting their influences in the reducing power in a significant way for a confidence level of 95%.

Through multiple regression analysis on the experimental data, the following second-order polynomial models were obtained to express the total phenolic content (Y_1), scavenging activity (Y_2), and reducing power (Y_3) as a function of studied independent variables:

Table 2. Box-behnken design matrix with extraction conditions and observed responses.

Run	X_1	X_2	X_3	Total Yield	TPC	DPPH	FRAP
	Temperature (°C)	Pressure (MPa)	Percentage of Modifier (%)	(%)	(mg GAE/g extract)	(% Inhibition)	(mg Fe(II)/g Extract)
1	60	30	20	2.09	20.12 ± 1.10	31.76 ± 2.33	98.29 ± 3.81
2	70	25	20	2.32	22.25 ± 0.25	48.38 ± 4.23	137.49 ± 9.26
3	70	30	15	0.95	11.43 ± 0.21	28.29 ± 1.91	59.52 ± 1.70
4	70	25	10	1.02	14.05 ± 1.19	33.80 ± 6.46	65.77 ± 2.65
5	60	25	15	2.19	28.36 ± 0.39	55.54 ± 1.99	116.61 ± 4.07
6	50	30	15	2.95	40.58 ± 0.64	67.97 ± 1.98	281.01 ± 8.43
7	50	25	20	4.14	84.85 ± 0.35	90.80 ± 0.23	585.32 ± 17.02
8	60	20	20	1.91	52.78 ±1.19	88.94 ± 1.98	412.14 ± 36.61
9	60	25	15	1.89	24.96 ± 0.46	61.12 ± 0.79	159.06 ± 13.65
10	60	25	15	2.00	22.55 ± 0.68	59.13 ± 1.00	128.17 ± 15.48
11	50	20	15	2.53	25.88 ± 0.40	79.26 ± 2.34	218.38 ± 8.18
12	60	30	10	0.65	17.80 ± 0.75	38.86 ± 1.46	117.82 ± 1.99
13	60	20	10	0.32	16.82 ± 0.21	31.06 ± 1.87	130.52 ± 2.63
14	70	20	15	4.00	27.73 ± 0.35	46.97 ± 1.04	193.35 ± 3.36
15	50	25	10	0.87	17.90 ± 1.28	29.32 ± 1.36	84.35 ± 3.02

Table 3. ANOVA analysis for three parameters for SFE.

Source	Total Phenolic Content				DPPH Radical Scavenging Activity				Ferric Reducing Antioxidant Power			
	Sum of Squares	F-value	*p*-value	R^2	Sum of Squares	F-value	*p*-value	R^2	Sum of Squares	F-value	*p*-value	R^2
Model	4444.94	5.02	0.0452	0.9004	6443.26	8.87	0.0135	0.9410	2.610E + 05	5.33	0.0401	0.9056
X_1	1098.63	11.17	0.0205	-	1323.81	16.39	0.0098	-	63533.65	11.67	0.0189	-
X_2	137.61	1.40	0.2901	-	787.05	9.75	0.0262	-	19775.63	3.63	0.1150	-
X_3	1605.46	16.32	0.0099	-	2239.14	27.73	0.0033	-	87107.21	16.00	0.0103	-
$X_1 X_2$	240.25	2.44	0.1789	-	13.65	0.1691	0.6980	-	9649.13	1.77	0.2405	-
$X_1 X_3$	862.89	8.77	0.0315	-	726.30	8.99	0.0301	-	46063.89	8.46	0.0334	-
$X_2 X_3$	284.60	2.89	0.1497	-	1055.60	13.07	0.0153	-	22672.83	4.17	0.0968	-
X_1^2	74.31	0.7555	0.4245	-	3.00	0.0372	0.8546	-	6205.51	1.14	0.3345	-
X_2^2	41.96	0.4266	0.5425	-	15.85	0.1963	0.6762	-	572.85	0.1052	0.7588	-
X_3^2	91.80	0.9333	0.3784	-	290.47	3.60	0.1164	-	6707.99	1.23	0.3175	-
Residual	491.83	-	-	-	403.77	-	-	-	27216.73	-	-	-
Lack of Fit	474.79	18.57	0.0515	-	387.78	16.16	0.0588	-	26253.45	18.17	0.0526	-

Note: X_1 = Temperature, X_2 = Pressure, X_3 = Concentration of co-solvent.

$$Y_1 = 25.29 - 11.72X_1 - 4.15X_2 + 14.17X_3 - 7.75X_1X_2 - 14.69X_1X_3 - 8.44X_2X_3 + 4.49X_1^2 - 3.37X_2^2 + 4.99X_3^2$$

$$Y_2 = 58.60 - 12.86X_1 - 9.92X_2 + 16.73X_3 - 1.85X_1X_2 - 13.47X_1X_3 - 16.25X_2X_3 - 0.9021X_1^2 - 2.07X_2^2 - 8.87X_3^2$$

$$Y_3 = 134.61 - 89.12X_1 - 49.72X_2 + 104.35X_3 - 49.11X_1X_2 - 107.31X_1X_3 - 75.29X_2X_3 + 41.00X_1^2 + 12.46X_2^2 + 42.62X_3^2$$

Fig. (**1a**) was a graphical representation that visualized the effects of combinations of temperature and pressure on the total phenolic content of *A. disticha*. It was noted that when the concentration of ethanol was fixed at 15%, the increase in pressure at the low-temperature range was more effective in extracting phenolic compounds. On the other hand, a reduction in terms of total phenolic content was observed when the temperature was set above 60°C at constant pressure, and no significant effect in the interaction between these two types of variables was observed. As shown in Fig. (**1b**), when pressure was fixed at 25 MPa, the interaction of temperature and concentration of co-solvent was significant. It also depicted that an increase in ethanol concentration from 10 to 20% improves the extraction of total phenolic content. However, further increment of extraction temperature resulted in a decline in the response. The highest phenolic content could be achieved when using an extraction temperature of 50°C and 20% of ethanol. The results from Fig. (**1c**) suggested that the amount of the phenolic compounds extracted could be influenced by ethanol concentration while no significant effect observed when varying the pressure. Furthermore, the interaction between pressure and concentration of co-solvent was not statistically significant ($p > 0.05$). Wozniak *et al*. [18] reported the highest yield of TPC (1.52 g per 100 g of pomace) was acquired at 35° C, 10 MPa, and 80% m/m ethanol addition. An increase in the recovery of phenolic compounds was obtained by maximizing the addition of ethanol and the density of CO_2. An increase in the recovery of phenolic compounds was obtained by minimizing the extraction temperature and maximizing the addition of ethanol and pressure. Three parameters were investigated during the research: temperature (35, 50, and 65 C), pressure (7.5, 10.0, and 12.5 MPa), and the addition of ethanol to the pomace (20%, 50%, and 80% m/m).

The response surface curve in Fig. (**2a**) was developed to demonstrate the main and interactive effects of extraction temperature and pressure on percentage inhibition of DPPH

Figure 1

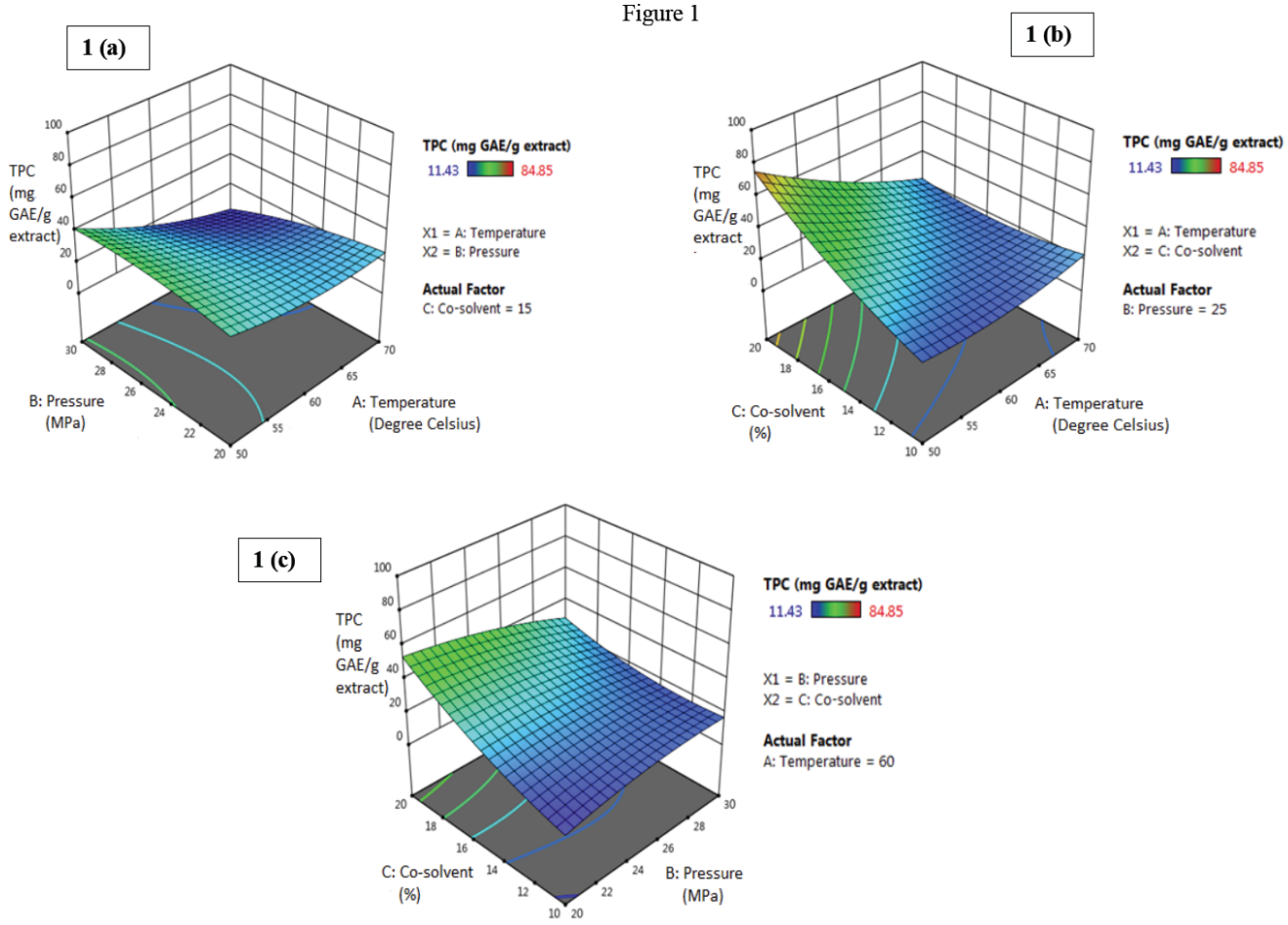

Fig. (1). Response surface and contour plots of total phenolic content showing (**a**) the effect of temperature and pressure at constant 15%, (**b**) the effect of temperature and concentration of co-solvent at constant 25 MPa, (**c**) the effect of pressure and concentration of co-solvent at constant 60°C. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Figure 2

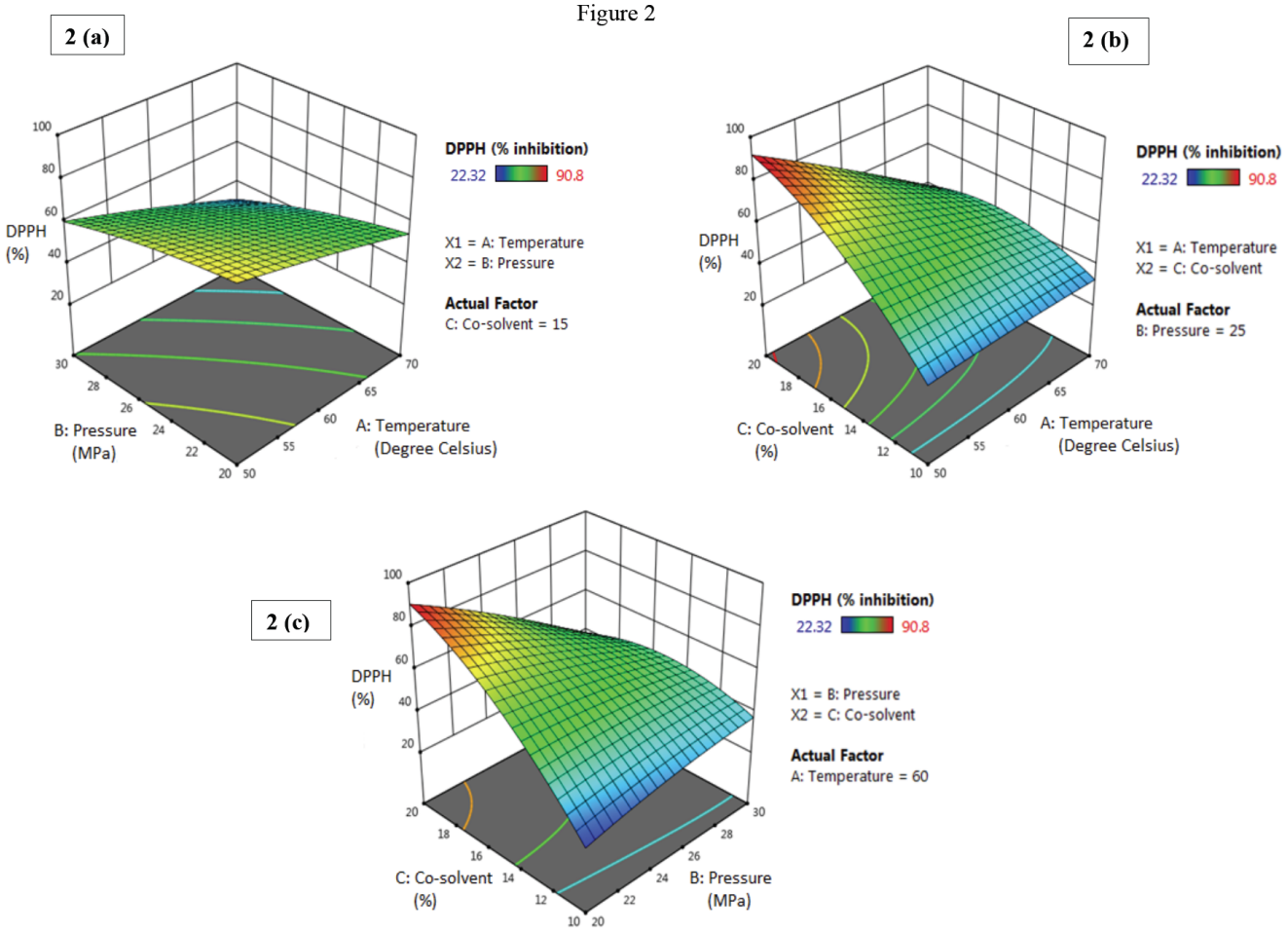

Fig. (2). Response surface and contour plots of DPPH radical scavenging activity showing (**a**) the effect of temperature and pressure at constant 15%, (**b**) the effect of temperature and concentration of co-solvent at constant 25 MPa, (**c**) the effect of pressure and concentration of co-solvent at 60°C. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

radical scavenging activity of stem from *A. disticha*. When the concentration of co-solvent was kept at the middle level, varying the pressure at low temperature was more effective in extracting oxidative compounds, and the highest antioxidant activity was attained at temperature and pressure of 50°C and 20 MPa, respectively. However, the interaction between temperature and pressure was not significant ($p > 0.05$). The effects of temperature and concentration of co-solvent on the level of antioxidant activity was shown in Fig. (**2b**). The potent scavenging effect was attained at the higher range of ethanol concentration and lower temperature of 50°C but slowly decreased when the temperature continued to be extended. Furthermore, temperature and ethanol concentration have a significant interaction for the extraction of compounds responsible for antioxidant activity ($p < 0.05$). In addition, a significantly increasing effect on the percentage inhibition of oxidative DPPH was observed at lower pressure and higher ethanol concentration as shown in Fig. (**2c**). The interaction between both variables was statistically significant for this study.

The response surface and contour plot shown in Fig. (**3a**) described the interaction effects of extraction temperature and pressure on reducing the power of *A. disticha*. In the present study, low temperature showed a positive effect while changing pressure did not show an obvious influence on the response. Further increment of extraction temperature beyond 50°C resulted in the reduction of antioxidant activity. No significant interaction between temperature and pressure was recorded ($p > 0.05$). The effect of temperature and concentration of ethanol on the antioxidant capacity in terms of FRAP was shown in Fig. (**3b**). The result revealed that the reducing power was greatly improved by increasing the concentration of ethanol from 10 to 20% at low extraction temperature. The interaction between temperature and percentage of co-solvent was statistically significant ($p < 0.05$). As shown in Fig. (**3c**), when the temperature was fixed at 60°C, the highest antioxidant activity was obtained at the lowest operating pressure of 20 MPa under a high concentration of ethanol. However, the interaction between pressure and co-solvent concentration was not significant ($p > 0.05$).

3.2. Effect of Process Variables

In the present study, three factors at three levels of BBD were used to analyse the effect of operating conditions namely temperature (50-70°C), pressure (20-30 MPa), and concentration of co-solvent (10-20%) on extraction yield,

Figure 3

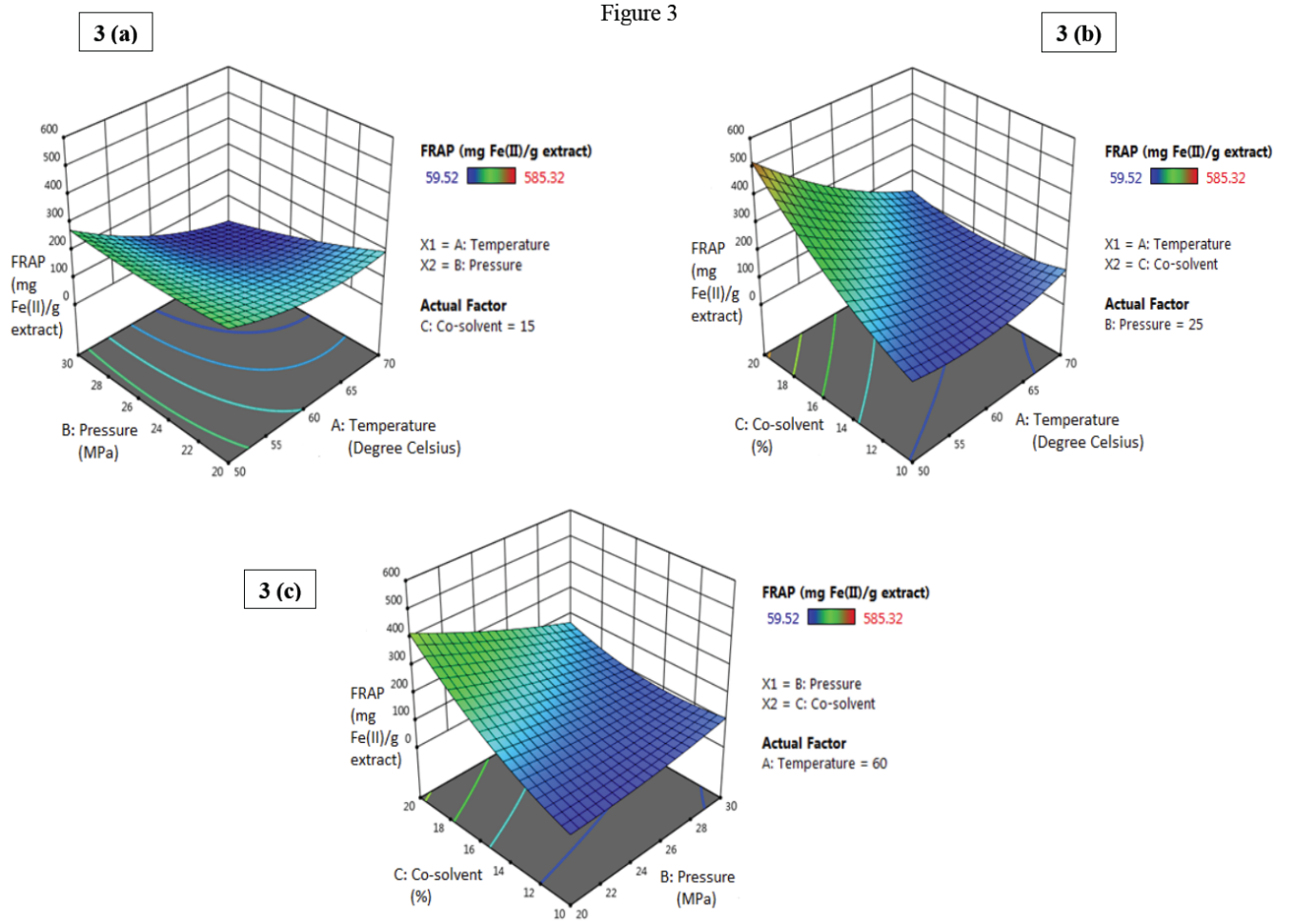

Fig. (3). Response surface and contour plots of ferric reducing antioxidant power showing (**a**) the effect of temperature and pressure at constant 15%, (**b**) the effect of temperature and concentration of co-solvent at constant 25 MPa, (**c**) the effect of pressure and concentration of co-solvent at 60°C. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

total phenolic content, and antioxidant activities of the stem of *A. disticha* obtained by supercritical carbon dioxide extraction.

3.2.1. Effect of Temperature

Basically, temperature is one of the principal factors that significantly affect the physicochemical properties of the solvent and influence the rate and selectivity of SFE. From the results, it can be observed that the highest extraction of phenolic and antioxidative compounds was attained at an extraction temperature of 50°C as shown in Figs. (**1b**, **2a**, and **3a**). The amount of extract decreased significantly as the temperature increased from 50°C to 70°C (Table **2**). This is in correspondence with the findings of other studies [19] which reported the total phenolic compounds from bamboo leaf extracts decreased with increasing temperature from 50°C to 95°C. According to Bimakr *et al*. [20], this enhancement of extraction efficiency is associated with an increase in solute vapor pressure which in turn increases the tendency of phenolic compounds to pass through and dissolve more easily in the supercritical fluid. Furthermore, the solvating power of ethanol-modified CO_2 depends on density, which decreases with temperature and increases with pressure. Higher temperatures also contributed to particle cell wall destruction and acceleration of mass transfer rate, which subsequently increase the compounds available for extraction [21]. However, further temperature increments resulted in the vaporization and decomposition of components, leading to low recovery of bioactive compounds [22]. As to provide an example, the increment of temperature above 60°C at constant pressure resulted in a decline in the extraction of the phenolic compound as shown in Fig. (**1a**), which was in accordance with previously reported literature [23].

3.2.2. Effect of Pressure

The effect of pressure on the amount of phenolic compounds extracted from the stem of *A. disticha* was shown in Table **2**. According to the obtained result, at a constant temperature, the yield of phenolic compounds was increased with increasing operating pressure, whereby the highest TPC recorded was at the pressure of 25 MPa. This result is related to the fact that molecules with a higher molecular weight require higher operating pressure for extraction. Our results are in agreement with other studies [19] which reported the optimal pressure for the extraction was 20 MPa while the operating pressure ranged from 10 to 25 MPa. This perfor-

mance is caused by the enhancement in the fluid density which consequently diminishes the mean distance between molecules as well as promotes stronger interactions between solute and solvent [24]. However, as shown in Figs. (**2c** and **3c**), the opposite finding was observed whereby high pressure resulted in a decrease in extraction yield and antioxidant capacity of *A. disticha*. This might be attributable to the increase in fluid viscosity at an elevated pressure which in turn reduces the ability of the fluid to penetrate the sample matrix and interact with the analyte [25].

3.2.3. Effect of Co-solvent

As a non-polar fluid, supercritical carbon dioxide is generally less effective in extracting highly polar compounds such as polyphenols. Therefore, to overcome this problem, a small amount of suitable polar co-solvent or modifier is introduced into the SFE system. The result from the present study showed that the concentration of co-solvent used significantly influenced the amount of extraction yield. Furthermore, Figs. (**1b** and **c**) showed that the highest total phenolic content was accomplished when using 20% of ethanol at constant pressure and temperature with 84.85 ± 0.35 and 52.78 ± 1.19 mg GAE/g extract, respectively. It was also noteworthy that potent scavenging and reducing effect was achieved when the concentration of co-solvent used was increased from 10-20% (Figs. **2b** and **b**). Our results are in accordance with the previous study [19] which reported total phenolic compound yield increased as the amount of cosolvent increased from 5 to 10% (mol) of ethanol to CO_2. This result shows that highly molecular-weighted phenols could only be extracted using larger concentrations of the cosolvent (ethanol with CO_2) [18]. According to Casas *et al*. [26], co-solvent basically influences extraction in various ways which include enhancing the solubility of compounds in supercritical solvent owing to the compound-modifier bindings. Furthermore, it stimulates structure modification of the cellular matrix *via* osmotic swelling and favours the penetration of the supercritical fluid into the matrix. Maran *et al*. [27] also added that co-solvent is capable of breaking the analyte-matrix complex by competing with the compounds for the active sites in the matrix, which in turn promotes rapid analyte desorption. Due to the small volume of co-solvent required to alter the solvating power of CO_2, its consumption during SFE is still much lower than in conventional extraction techniques. Most of the SFE especially in food and pharmaceutical applications use ethanol as a co-solvent due to its nontoxicity and miscibility in CO_2, but in some cases, other co-solvents such as acetonitrile, dichloromethane, hexane, isopropanol, and methanol have shown to be more efficient [7].

3.3. Total Phenolic Content, Phenolic Compounds and Antioxidant Activities of SFE Extracts

Phenolic compounds of *A. disticha* stem extracts were identified and quantified by HPLC. Out of fifteen experimental runs of SFE, the highest responses of total phenolic content, and antioxidant activities in terms of scavenging and reducing capabilities were found at the temperature of 50°C, the pressure of 25 MPa, and ethanol concentration of 20%, yielded 84.85 ± 0.35 mg GAE/g extract, 90.80 ± 0.23% and 585.32 ± 17.02 mg Fe (II)/g extract (Table **2**). On the other hand, the lowest extracts were recorded at 70°C, 30 MPa, and 15% ethanol, yielding 11.43 ± 0.21 mg GAE/g extract, 28.29 ± 1.91%, and 59.52 ± 1.70 mg Fe (II)/g for TPC, scavenging and reducing capabilities, respectively (Table **2**). Under the described chromatographic conditions, the contents of investigated phenolic compounds are presented in Fig. (**4**).

At lower pressure SFE experimental run 7 showed a significantly higher occurrence of gallic acid, *p*-coumaric, ferulic acid, and quercetin with values of 118.83 ± 1.17, 61.60 ± 0.33, 57.93 ± 1.15, and 24.16 ± 0.41 µg/g extract (Fig. **4**), respectively, compared to SFE run 3 which yielded very minimum amount of total phenolic content (Table **2**) as well as phenolic compounds with values of 45.83 ± 0.47, 10.22 ± 0.12, 20.36 ± 0.08, and 17.34 ± 0.17 µg/g of gallic acid, p-coumaric, ferulic acid, and quercetin, respectively (Fig. **4**). The result agreed with those [28] who demonstrated the increased extraction yield of compounds from grape bagasse (*Vitis vinifera*) using SFE at low pressure of 20 MPa compared to 35 MPa. By analysing the results presented, it can also be concluded that the amount of content of phenolic compounds detected matched well with their antioxidant activities.

3.4. Comparison between Soxhlet and Supercritical Fluid Extraction

Fig. (**4**) illustrated the total phenolic content, antioxidant activities, and concentration of phenolic compounds obtained from Soxhlet and SFE extracts. Based on the best result of antioxidant activities and maximum recoveries of phenolic compounds obtained at experimental run 7 (50°C, 25MPa and 20%) was chosen to represent the SFE method. From the results presented in Fig. (**4**), supercritical carbon dioxide extract was found to have a significantly higher content of phenolic compounds namely p-coumaric (61.60 ± 0.33 µg/g), ferulic acid (57.93 ± 1.15 µg/g) and quercetin (24.16 ± 0.41 µg/g) compared to Soxhlet extracts. However, the opposite finding was observed where superior recovery of gallic acid was achieved by the extract from Soxhlet (319.69 ± 6.69 µg/g) than those recorded by SFE (118.83 ± 1.17 µg/g). In this study, ANOVA analysis showed a significant difference ($p < 0.05$) between TPCs of both extracts from Soxhlet and SFE with values of 27.73 ± 4.10 mg GAE/g extract and 84.85 ± 0.25 mg GAE/g extract, respectively (Fig. **4**). The same trend was found in antioxidant activities where SFE extracts had a considerably high scavenging activity of 90.80 ± 0.23% compared to Soxhlet extract's activity of 66.20 ± 11.75%. SFE showed more than two-fold higher ferric-reducing antioxidant power than Soxhlet with values of 585.32 ± 17.01 mg Fe (II)/g extract and 203.63 ± 16.03 mg Fe(II)/g extract, respectively (Fig. **4**).

The highest yield of Soxhlet extraction and SFE was recorded at 8.75% and 4.14%, respectively, in the present study. Soxhlet extracted yields were double compared to SFE yield. Despite the fact that the total yield of the SFE-produced extracts was not very high, the phenolic concentration was higher than that of the Soxhlet-produced extracts. High extraction yield does not necessarily exhibit the high antioxidant activity of the sample as proved by previous researchers [29]. According to the overall yield and composition of the

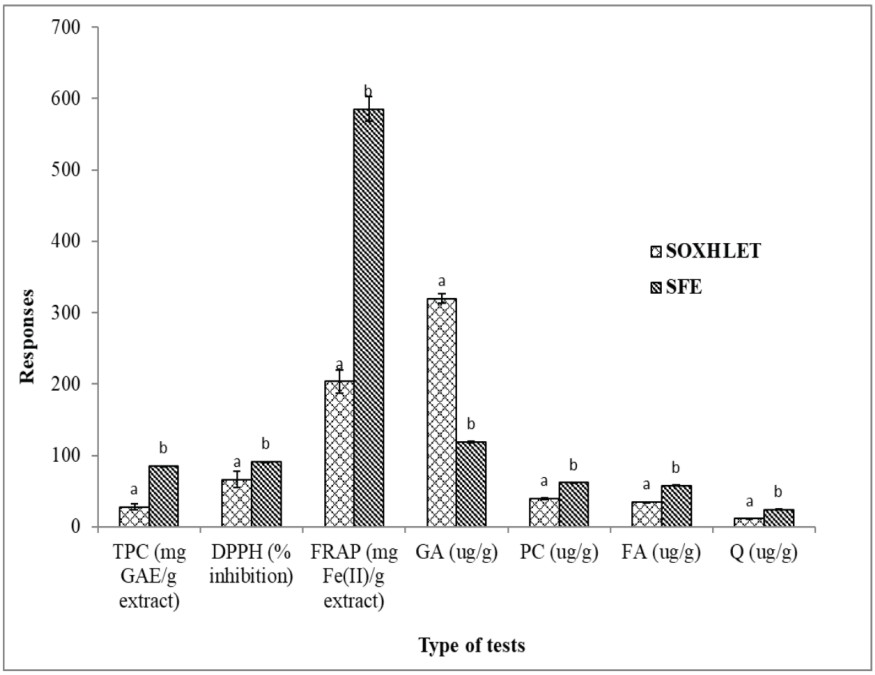

Fig. (4). Comparison of responses between soxhlet and SFE. The values were expressed as mean ± standard deviation (n=3). Different letters indicate significant difference at the level of $p < 0.05$ between types of extraction. **Note:** GA (gallic acid), PC (p-coumaric), FA (ferulic acid), Q(quercetin). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

extracts, it was demonstrated that SFE was more effective at extracting the phenolic compounds from the *A. disticha* than the conventional extraction techniques using ethanol as a solvent. In another study, the recovery of solvent extraction was only 13.5%, while supercritical CO_2 modified with ethanol was able to elute phenolic compounds of about 11.1%-44.6%. The output of solvent extraction can be increased by using a greater volume of solvents, although one of the objectives of the experiment was to reduce the use of these substances [18].

CONCLUSION

Phenolic compounds and their antioxidant capabilities from *A. disticha* stems were successfully determined using SFE and compared with Soxhlet extraction. SFE showed more than threefold higher TPC, and about two-fold higher antioxidant activities in terms of FRAP and DPPH radical scavenging activity than Soxhlet extracts. The results showed that extraction temperature and concentration of co-solvent have a significant effect on TPC and antioxidant activities. There was a strong correlation between phenolic content and antioxidative properties. The highest yield of TPC, the percent inhibition of DPPH, and radical scavenging activity were obtained at a temperature of 50°C, pressure 25 MPa, and 20% ethanol as cosolvent with 80% CO_2. The experimental results indicated that gallic acid was established as the dominating phenolic compound. Although the other phenolic compounds such as p-coumaric, ferulic acid, and quercetin were found in much lower concentrations, they could contribute to a great deal to the antioxidative properties of *A. disticha*.

AUTHORS' CONTRIBUTIONS

Ferdosh S. and Sarker MZI contributed to the project conception/supervision, and funding acquisition. Bari NAA carried out the experiment and drafted the manuscript. All authors reviewed and approved the final manuscript.

LIST OF ABBREVIATIONS

BBD	=	Box-behnken Design
RSM	=	Response Surface Methodology
SFE	=	Supercritical Fluid Extraction
TPC	=	Total Phenolic Content

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Data is available upon request from the authors.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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