Effects of Extraction Parameters on Total Flavonoid Content and Antioxidant Activity of *Cyperus rotundus*

Zainol N.*, Aziz N.H. and Mohd Nazaruddin M.H.
Faculty of Chemical and Process Engineering Technology, Universiti Malaysia Pahang Al-Sultan Abdullah, Lebuh Persiaran Tun Khalil Yaakob, 26300, Kuantan, Pahang, MALAYSIA
*azwina@umpsa.edu.my

Abstract

This study aims to evaluate the factors affecting total flavonoid content (TFC) and antioxidant activity (AA) extracted from Cyperus rotundus. The factors under investigation include extraction time (2 and 5 hours), solvent percent (70 and 80%), solvent type (methanol and ethanol), temperature (50 and 80°C) and agitation (0 and 100 rpm). The factors were analyzed using Design-Expert software through a two-level factorial analysis. The results show that temperature and agitation contributed the most to TFC and AA concentration respectively. The best conditions for the phytochemical analysis were obtained at 5 hours extraction time, 85% solvent, methanol as solvent, 80°C temperature and with agitation, with the concentrations of TFC at 0.171 mg/ml and AA at 6.331 nmol/µl.

The findings concerning the most influential factors in phytochemical extractions are beneficial for scaling up yields. Process parameters can be tailored during extraction to obtain maximum yields as desired. The results suggest that the design of experiments is helpful in improving the TFC and AA production by considering all the interactions of variables involved.

Keywords: Antioxidant activity, *Cyperus rotundus*, Factorial analysis, Phytochemical, Total flavonoid content.

Introduction

Humans have relied on medicinal plants as sources of phytochemical compounds since ancient times[16]. Approximately 20% of plant species recorded in biological nomenclature have been commercially employed by pharmaceutical lines, affecting the healthcare system positively[3]. Phytochemicals, synthesized as secondary metabolites by plants, serve as natural antioxidants beneficial in treating or reducing the risk of major chronic diseases and exhibit biological activity[16,21,31]. Phytochemicals are known for their capabilities to combat inflammation, viruses, cancer and microbes[1,8,17].

Natural resources, particularly animals, plants and bacteria have been used in medicines by humans for at least 60,000 years to prevent and cure illnesses[51]. Despite being classified as non-essential components for the human body's functioning, phytochemical compounds are advantageous to human health and can facilitate disease recovery[32]. To date, various plant-derived phytochemical compounds have been documented, including phenolics, flavonoids, anthocyanins, alkaloids, carotenoids, terpenoids, phytosterols and isoflavonoids[1,3,26,43,44]. These non-nutrient elements depend on their antioxidant properties and ability to scavenge free radicals to uphold the enzymatic activity responsible for detoxification[41].

Many plant species have been demonstrated to be rich in antioxidants. *Cyperus rotundus*, for example, is a plant species with a wide range of phytochemical compounds. *C. rotundus* belongs to Cyperaceae, with the genus *Cyperus* having more than 700 identified plant species. *C. rotundus* is dubbed the worst weed; however, the rhizomes of *C. rotundus* have been useful in treating many ailments since ancient times[1]. *C. rotundus* has often been used as medicine since most medicinal herbs have been known as a source of safety, low toxicity, cheap cost and effective natural medications since ancient times.

The study on rhizome oil demonstrated the antifungal and antimicrobial properties of the plant species[13]. The phytochemical analysis of the leaves and rhizomes shows that the plant consists of flavonoids, alkaloids, polyphenols, tannins, glycosides and saponins[33]. Phytochemical compounds possess antioxidants, antibacterial, anti-inflammatory, antidiarrheal, analgesic, antidiabetic, antisaturative, appetizer, digestant, thirst-quenching and tranquillizing properties[1].

Researchers are actively researching plant phytochemicals due to their significance in the pharmacological line. Nevertheless, extraction of these compounds renders various encounters; thus, effective extraction methods are essential to obtain extracts with concentrated phytochemical contents. Experts have developed various extraction methods to safeguard the efficacy and potency of crude medications used to treat illnesses. Several factors have been recognized to affect the extracted phytochemicals including the extraction method, solvents and the plant part used[16]. Each extraction method affects both the quantity and types of phytochemicals produced. Factors such as solvent properties (polarity and concentration), extraction time and temperature also play crucial roles[3,31].

Plants may contain many naturally active chemical compounds that can be extracted by a solvent-based separation[21]. Therefore, the choice of solvent utilized for phytochemical extraction is important in determining the efficacy of the process[44]. Several researchers have investigated the effect of different solvents on

phytochemical extraction including water, ethanol, methanol, acetone, chloroform, dichloromethane, ether and hexane[3,44]. This exploration is based on the reason that the choice of solvent significantly influences the efficiency of phytochemical extraction which subsequently affects the phytochemical content.

Solvents used for extraction are selected based on their polarity, which should match the solute of interest to ensure proper dissolution. It is essential to employ solvents with varying polarities to extract different phenolic compounds accurately. Highly polar solvents, particularly methanol, exhibit strong antioxidant properties. For instance, Koffi et al[27] reported that methanol was particularly efficient in extracting more phenolic compounds than ethanol from walnuts. Conversely, the extraction of ivorian plants using ethanol produced higher phenolic concentrations than water, acetone and methanol.

Additionally, factors such as plant components, development stage, soil pH, fertilization, as well as weather conditions with light intensity and water availability, for instance, have been shown to influence the phytochemical content of a plant significantly[6]. The design of experiments (DOE) is an approach used to determine the correlation between various factors that affect a process and its outcomes[22]. The DOE includes factorial analysis, which reduces many variables to a few understandable basic factors, resulting in simple-to-comprehend conclusions[4]. The two-level factorial analysis (TLFA) provides a broader inductive basis, encompassing a larger range to support inferences about the process[30].

However, the need for a powerful theoretical basis in the correlation between dependent and independent variables represents its flaw. These are attributed to the potential loss of information if the original variables are reduced to fewer factors[40]. Nevertheless, factorial analysis implements a process to allow the inclusiveness of the information in the correlated variables in the regression by extracting a few linearly independent factors[40].

This study aims to identify and to characterize phytochemical compounds derived from *C. rotundus* and to determine the factors affecting their phytochemical compound extraction through a two-level factorial analysis of the Design-Expert software in order to maximize extraction yields.

Material and Methods

Collection and preparation of *C. rotundus*: The *C. rotundus* sample used in this study was collected from the house compound, backyard and roadside area in Pahang. The collected sample was oven-dried at 40°C for 3 days. The dried sample was then ground to obtain a powdered sample using a grinder.

Extraction procedure: The procedure for phytochemical extraction was performed following the conventional extraction method using different solvents (methanol and ethanol). Briefly, 5 g of powdered *C. rotundus* was mixed with 100 ml of solvent at room temperature. The ratio between the powdered plant sample and the solvent volume was kept constant. The solvent-powdered plant mixture was then placed in an incubator shaker at selected temperatures and times based on the constructed design table (Table 1). The extracts were filtered and the solvents were then evaporated before being kept in the freezer at 4°C until further use.

Table 1
Experimental outputs of TFC and AA analysis

Std	Factors					TFC (mg/ml)	AA
	A	B	C	D	E		
1	2.00	70.00	Methanol	50.00	Yes	0.134	6.465
2	5.00	70.00	Methanol	50.00	No	0.066	4.430
3	2.00	85.00	Methanol	50.00	No	0.084	4.720
4	5.00	85.00	Methanol	50.00	Yes	0.154	6.576
5	2.00	70.00	Ethanol	50.00	No	0.100	6.090
6	5.00	70.00	Ethanol	50.00	Yes	0.112	5.661
7	2.00	85.00	Ethanol	50.00	Yes	0.151	5.843
8	5.00	85.00	Ethanol	50.00	No	0.091	5.059
9	2.00	70.00	Methanol	80.00	No	0.175	5.997
10	5.00	70.00	Methanol	80.00	Yes	0.168	6.519
11	2.00	85.00	Methanol	80.00	Yes	0.131	5.697
12	5.00	85.00	Methanol	80.00	No	0.291	5.442
13	2.00	70.00	Ethanol	80.00	Yes	0.213	5.983
14	5.00	70.00	Ethanol	80.00	No	0.105	5.261
15	2.00	85.00	Ethanol	80.00	No	0.162	6.360
16	5.00	85.00	Ethanol	80.00	Yes	0.160	6.479

A: Extraction Time (hour), B: Solvent Percent (%), C: Solvent Type, D: Temperature (˚C), E: Agitation

The experiment was designed with the aid of Design-Expert software with randomized factors. Five factors affecting phytochemical extraction were selected in this study. These include extraction time (2 and 5 hours), solvent percent (70 and 85%), solvent type (methanol and ethanol), temperature (50 and 80˚C) and agitation (yes and no). Experiments were performed according to the setup as portrayed in table 1.

Experimental design: The significant independent factors influencing the extraction process were identified using Two-Level Factorial Analysis (TLFA) from the Design-Expert software[5,23,28]. The dependent factors were investigated based on phytochemical production and antioxidant activities. The regression model presenting the relationship between the independent and dependent factors is shown in eq. (1):

$$y_i = \beta_0 + \sum_{i=1}^{n} \beta_i X_i \quad (1)$$

where y_i represents the response value, β_0 is the constant coefficient, β_i represents the linear parameters coefficient, n is the number of variables and X_i represents the interaction parameters.

Sample analysis

(a) Total flavonoid content: The determination of TFC followed the Dowd method, as described by Shirin and Prakash[39]. Precisely, 1 ml of plant extract was mixed with 0.3 ml of 2% aluminium trichloride and allowed to rest for 5 minutes. Then, 2 ml of sodium hydroxide was added to the mixture, followed by an additional 6-minute incubation period. Deionized water was then added to each sample until a total volume of 8 ml was reached. The samples were quantified and analyzed through a UV-Vis spectrophotometer at 450 nm, in which quercetin served as the standard solution for generating the calibration curve.

(b) Antioxidant activity: The plant extract was evaluated using the FRAP assay (ferric ion reducing antioxidant power), according to Nile and Park[34]. 10 µl of sample extract was mixed with a reaction solution consisting of 152µl FRAP assay buffer, 19µl $FeCl_3$ (0.01%) and 19µl FRAP probe in a microplate well before being incubated in an incubator shaker for 1 hour at 300 rpm. The sample was monitored by a spectrophotometer at 366 nm using glutathione as a standard solution for the calibration curve. The step was repeated using different plant extracts.

(c) Data analysis: The collected data were input into the Design-Expert software as organized in table 1. Analysis of Variance (ANOVA) was conducted on the data, adhering to a significance level of $p < 0.5$[11,20,38] to identify the most significant factors influencing the phytochemical analysis.

Results and Discussion

Experimental outputs: Table 1 displays the 16 experimental runs and the phytochemical analysis of TFC and AA. The experimental outputs were analyzed through ANOVA based on a 95% confidence level. The value of TFC ranges from 0.066 to 0.291 mg/ml while AA ranges from 4.430 to 6.576 nmol/µl. The result shows that the maximum amount of TFC obtained in this experiment is at an experimental condition of 5 hours extraction time with 85% methanol concentration at 80°C temperatures and without agitation (Std 12), with 0.291 mg/ml.

Meanwhile, the lowest TFC content was obtained in an experimental condition of 5 hours of extraction time, with 70% methanol concentration at 50°C and without agitation (Std 2). The highest AA value is obtained at five hours of extraction times with 85% methanol and 50°C with agitation (Std 4), with 6.576 nmol/µl.

Meanwhile, the lowest percentage of AA was seen in 5 hours of extraction time, 70% methanol, 50°C temperatures and without agitation (Std 2). Interestingly, the lowest TFC and AA yields were obtained from similar experimental conditions (Std 2). It is also worth noting that both TFC and AA were maximized at the same extraction times and methanol concentration.

Analysis of total flavonoid compound (TFC)

(a) Analysis of variance (ANOVA) for TFC: The percent contribution of the factors to total flavonoid content is shown in table 2. As shown in the table, temperature contributed the most to the TFC, with 36.14% whereas extraction time contributed the least. According to Ioku et al[19], total flavonoids improved after being heated at a specific temperature and time, in which the heating at 150°C for 3 hours lowered total flavonoid content. The degradation of flavonoids might cause a reduction in total flavonoids at higher temperatures.

In addition, TFC is also influenced by the structure of the individual flavonoids. Most vegetables and fruits contain flavonoids with C-glycoside bonds, existing as oligomers and dimers. During industrial processing, such as heating or boiling, the C-glycoside bonds are hydrolyzed, forming monomers[29]. The ANOVA for TFC is summarized in table 3. ANOVA is utilized to calculate the coefficient of the model, to validate the significance of process factors and to identify the interaction level among these factors. The R^2 and adjusted R^2 (Adj-R^2) values are 0.9850 and 0.9248 respectively indicating a good fit for a bioprocess model, as determined by Olmez[35]. This model could, therefore, be seen as a representation of the process.

(b) Main and interaction effect of factors on TFC: The Pareto chart in figure 1 illustrates the effect of main and interaction factors on TFC. The chart shows that temperature is the only significant factor (as shown in the ANOVA table) contributing to TFC.

Meanwhile, the effect of agitation, extraction time, solvent type and solvent percent was insignificant on the total flavonoid content as the bar lays under the t-value limit.

Table 2
Percent contribution of each factor on total flavonoid content and antioxidant activity

Factor	% Contribution	
	TFC	AA
A: Extraction Time	1.75×10^{-3}	2.85
B: Solvent Percent	3.10	0.05
C: Solvent type	1.60	0.76
D: Température	36.14	8.01
E: Agitation	3.04	32.89

Table 3
ANOVA table for TFC

Source	Sum of squares	Df	Mean squares	F value	P-value
Model	0.045	12	3.771E-003	16.37	0.0207
A	8.042E-007	1	8.042E-007	3.490E-003	0.9566
B	1.424E-003	1	1.424E-003	6.18	0.0888
C	7.337E-004	1	7.337E-004	3.18	0.1723
D	0.017	1	0.017	72.07	0.0034
E	1.398E-003	1	1.398E-003	6.07	0.0906
AB	7.190E-003	1	7.190E-003	31.21	0.0113
AC	6.056E-003	1	6.056E-003	26.29	0.0144
AD	4.909E-004	1	4.909E-004	2.13	0.2405
BE	2.870E-003	1	2.870E-003	12.46	0.0387
CD	1.225E-003	1	1.225E-003	5.32	0.1044
CE	2.661E-003	1	2.661E-003	11.55	0.0425
DE	4.599E-003	1	4.599E-003	19.96	0.0209
R^2	0.9850				
Adj. R^2	0.9248				

A: Extraction Time (hour), B: Solvent Percent (%), C: Solvent Type, D: Temperature (˚C), E: Agitation

The temperature presented a positive effect on the total flavonoid content, as portrayed by the figure. The concentration of total flavonoid content increases as temperature rises (Figure 2). The positive effect occurs when the factor corresponds directly to the response value. Tzani et al[45] obtained an optimum operating temperature and time of 70°C and 60 minutes for *Zingiber officinale* extraction. At higher temperature settings, some volatile chemicals may have evaporated and some other compounds may have broken down, generating less extract. Based on figure 1, the interactions of AC (extraction time and solvent percent), DE (temperature and agitation) and BE (solvent percent and agitation) present negative effects. Meanwhile, the interaction of AB (extraction time and solvent percent) and CE (solvent type and agitation) contributed to the total flavonoid content with positive effects.

Figure 2 presents the effect of the independent factor on TFC. TFC concentration was increased with temperature increment. This is due to the enhanced extraction efficiency as temperature rises. Higher temperatures naturally increased the solubility of TFC in the solvent, improving mass transfer kinetics, thereby enhancing the extraction efficiency of TFC from the plant matrix. Also, the solubility of TFC is generally increased along with temperature increment, allowing for greater TFC extraction from plant materials. The disruption of cell walls as temperature increases, can lead to the increased accessibility of TFC to the extraction solvent (methanol or ethanol), which facilitates the release of TFC from the plant matrix. Comparable findings were also observed by Tagliazucchi et al[42]. The increase in temperature during the extraction of grape skin enhanced the extraction process through the increased diffusion coefficient and solubility of polyphenols.

The interaction effect between factors on TFC is shown in figure 3. As shown in figure 3(a), at lower extraction times i.e. 2 hours, TFC concentration was higher in 70% solvent percentage than that of 85%. However, as the extraction times increased to 5 hours, more solvent concentration significantly increased the TFC concentration. This is attributed to the concentration-dependent of the solvent in extracting TFC. Higher solvent concentrations can dissolve more flavonoid compounds from plant materials. TFC exhibits greater solubility in higher solvent percentages and temperatures.

Additionally, longer extraction times allow more solvents to penetrate deeper into the plant material, reaching more TFC-rich regions within the tissue. The increase in extraction times and solvent percentage enables more contact between the solvent and plant material, enhancing the dissolution and extraction of TFC. Also, longer extraction times allow for more periods for the mass transfer process. It is interesting

to notice the insignificant differences in TFC concentration with increased extraction times at lower solvent percentages. This is due to the limited solvent's ability to penetrate the plant material deeply. As a result, extending the extraction time did not significantly enhance the extraction efficiency due to the reduced capability of the solvent to access TFC-rich regions in the plant tissue.

An equilibrium may be reached in which the rate of TFC release from the plant equals the rate of TFC dissolution in the extraction solvent where additional extraction time did not increase TFC. The interaction between extraction times and solvent type is displayed in figure 3(b). In general, it is observed that at a longer extraction time, methanol produced more flavonoid compounds compared to ethanol. Compared to ethanol, methanol is a more polar solvent[3]. Flavonoids contain a hydroxyl group, making them a polar compound. The higher polarity of methanol compared to ethanol allows it to form stronger hydrogen bonds with flavonoids, thereby facilitating the dissolution and extraction from the plant material.

Methanol generally has a higher solubility for flavonoids than ethanol[15], which is attributed to the stronger interactions between methanol and flavonoids. As mentioned by Koffi et al[27], methanol is the most effective than ethanol in extracting many phenolic compounds from walnuts. Meanwhile, no significant variation is observed in the amount of TFC extracted through ethanol at both shorter and longer times. The most possible reason is that the equilibrium has been reached in the system, where the rate of flavonoid extraction equals the rate of flavonoid degradation. Prolonging the extraction times after the equilibrium point does not increase the flavonoid extraction as maximum extraction capacity has been reached.

Some TFC might be evaporated or degraded during prolonged extraction times, leading to reduced TFC in the extract. However, TFC is higher in ethanolic extraction than methanolic at shorter extraction times. The possible reason might be due to the difference in the affinity of ethanol and methanol for specific flavonoids[7]. Certain flavonoids may have better solubility in ethanol or stronger interactions with ethanol molecules, resulting in higher extraction efficiency compared to methanol at shorter extraction times. Also, the extraction kinetics of ethanol might be faster than that of methanol for some kinds of flavonoids, which allows for rapid extraction within a shorter time.

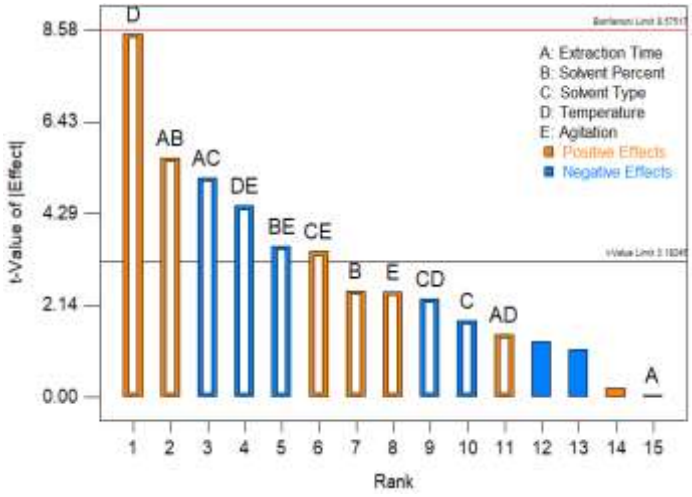

Figure 1: Pareto chart of total flavonoid content analysis

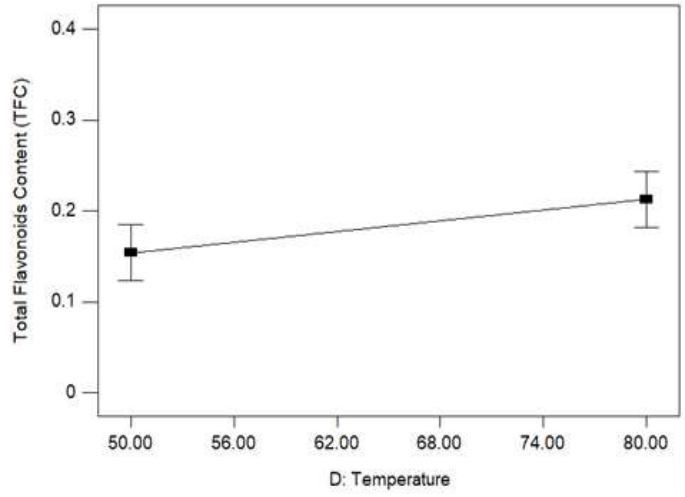

Figure 2: Effect of temperature on total flavonoid content

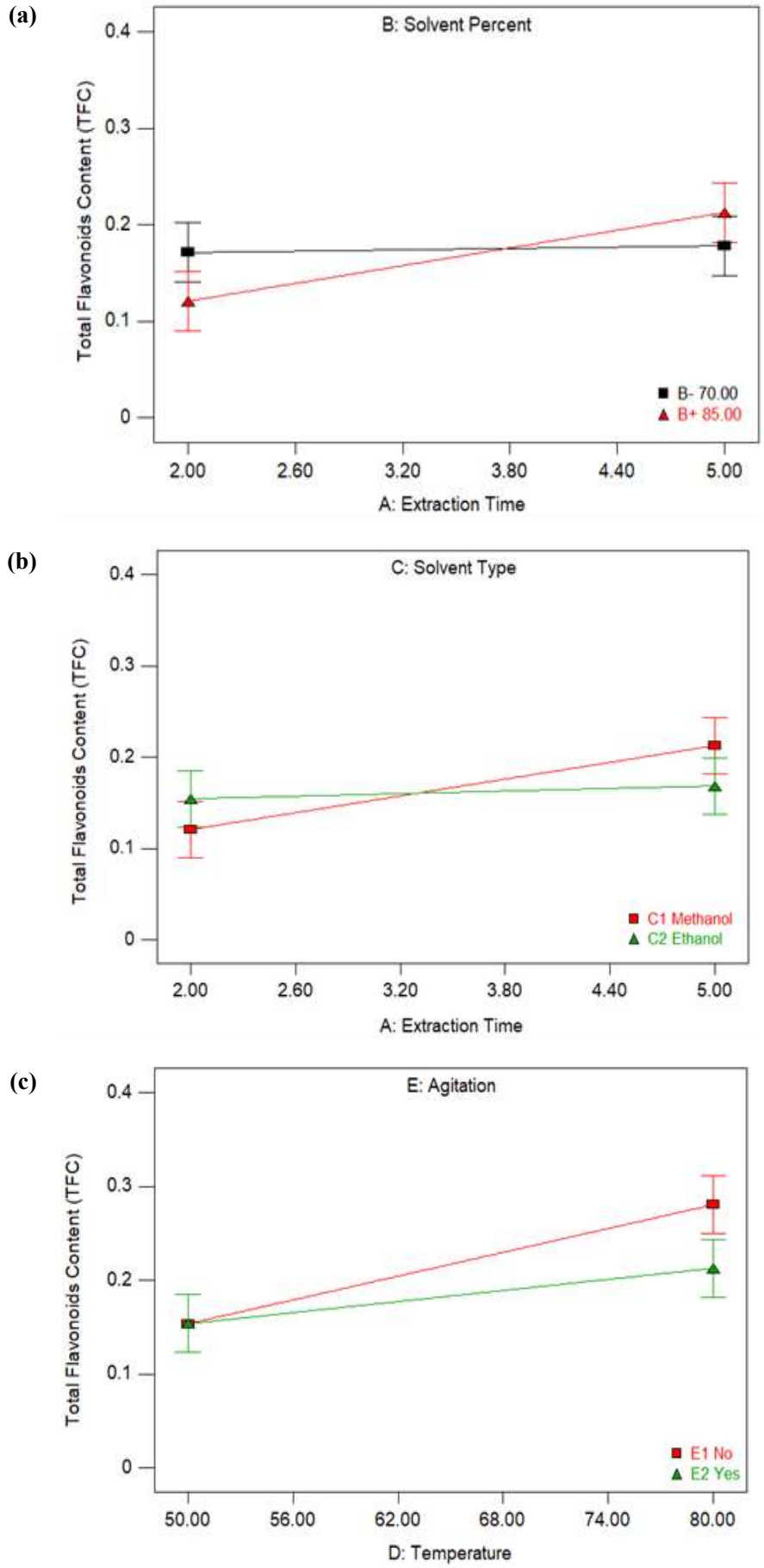

Figure 3: Effect of interaction between the main factors of (a) extraction time and solvent percent; (b) extraction time and solvent types; (c) temperature and agitation on TFC

Temporarily, figure 3(c) presents the interaction effects between temperature and agitation. It is observed from the figure that the flavonoid concentration increased with temperature increments and without agitation. Ideally, plant tissue is being softened under high temperatures, affecting the cell membranes, resulting in flavonoids being easily extracted from the plant tissue into the solvent. The finding is comparable to the theory of mass transfer, in which convective mass transfer occurs when a fluid is at the outer surface of the solid[42]. Higher temperatures accelerate mass transfer processes, including the diffusion of flavonoids from the plant materials into the solvent. The mobility of flavonoids within the plant matrix increases as thermal energy increases, which consequently promotes the release of flavonoids into the solvent.

The reduced viscosity of the solvent at high temperatures facilitates the diffusion of flavonoids, in which lower solvent viscosity enhances mass transfer and promotes effective extraction. Comparable outcomes relating to the increased solubility of polyphenols with rising temperatures are also described elsewhere[46-48,52]. At higher temperatures, the solubility of flavonoids increases due to the increasing kinetic energy of the solvent molecules, which promotes stronger interactions with flavonoids.

As the figure shows, the TFC is higher in the extraction condition without agitation (0 rpm) at higher temperatures. This explains the insignificant contribution of agitation to TFC at a high temperature. Increasing the temperature is sufficient to increase the TFC without agitation. Agitation can cause mechanical disruption of plant material, resulting in the discharge of undesirable substances that might interfere with and compete with flavonoids during extraction.

Also, agitation can lead to the increased exposure of flavonoids to oxygen, which results in oxidation or degradation. The absence of agitation can reduce the possibility of flavonoids being oxidized or degraded, increasing the extraction yields.

At a lower temperature setting, the TFC amount is comparable between both conditions with and without agitation. To conclude, despite being insignificant for TFC concentration i.e. extraction time, solvent type, solvent percent and agitation (based on ANOVA table), all main factors show a sign of significance when interacting with each other. Therefore, it is crucial to understand the behaviour of each factor for better extraction yields. From the figures, one can conclude that methanolic extraction is much more desirable in terms of high temperature settings and longer extraction times to obtain high TFC content.

Analysis for antioxidant activity (AA)

(a) Analysis of variance (ANOVA) for AA: The percentage of contribution for each factor on AA is portrayed in table 2. Agitation is seen to contribute the most, while solvent percentage demonstrated the least contribution to antioxidant activity. Table 4 presents the ANOVA for AA, with an R^2 value of 0.9955 and an adjusted R^2 of 0.9774, showing the highly significant value and, therefore, could represent the process.

(b) Main and interaction effect of factors on AA: Figure 4 depicts the effects of the main and interaction factors on AA. From the Pareto chart, agitation, temperature and extraction time exhibit significance in the AA. Temporarily, solvent type and solvent percent demonstrated an insignificant contribution, with the bar positioned under the t-value limit line. Agitation is the most contributing factor to AA, as presented in table 2, with 32.89% contributions. The interaction effects of CE (solvent type and agitation), DE (temperature and agitation) and AC (extraction time and solvent type) displayed negative effects. Meanwhile, the interactions of AB (extraction time and solvent percent), AE (extraction time and agitation) and BC (solvent percent and solvent type) contributed to AA with positive effects.

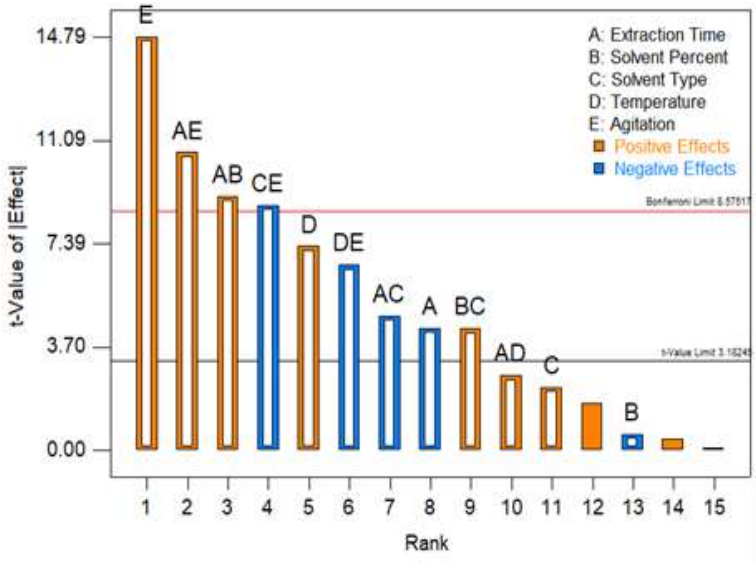

Figure 4: Pareto chart of antioxidant activity using FRAP analysis

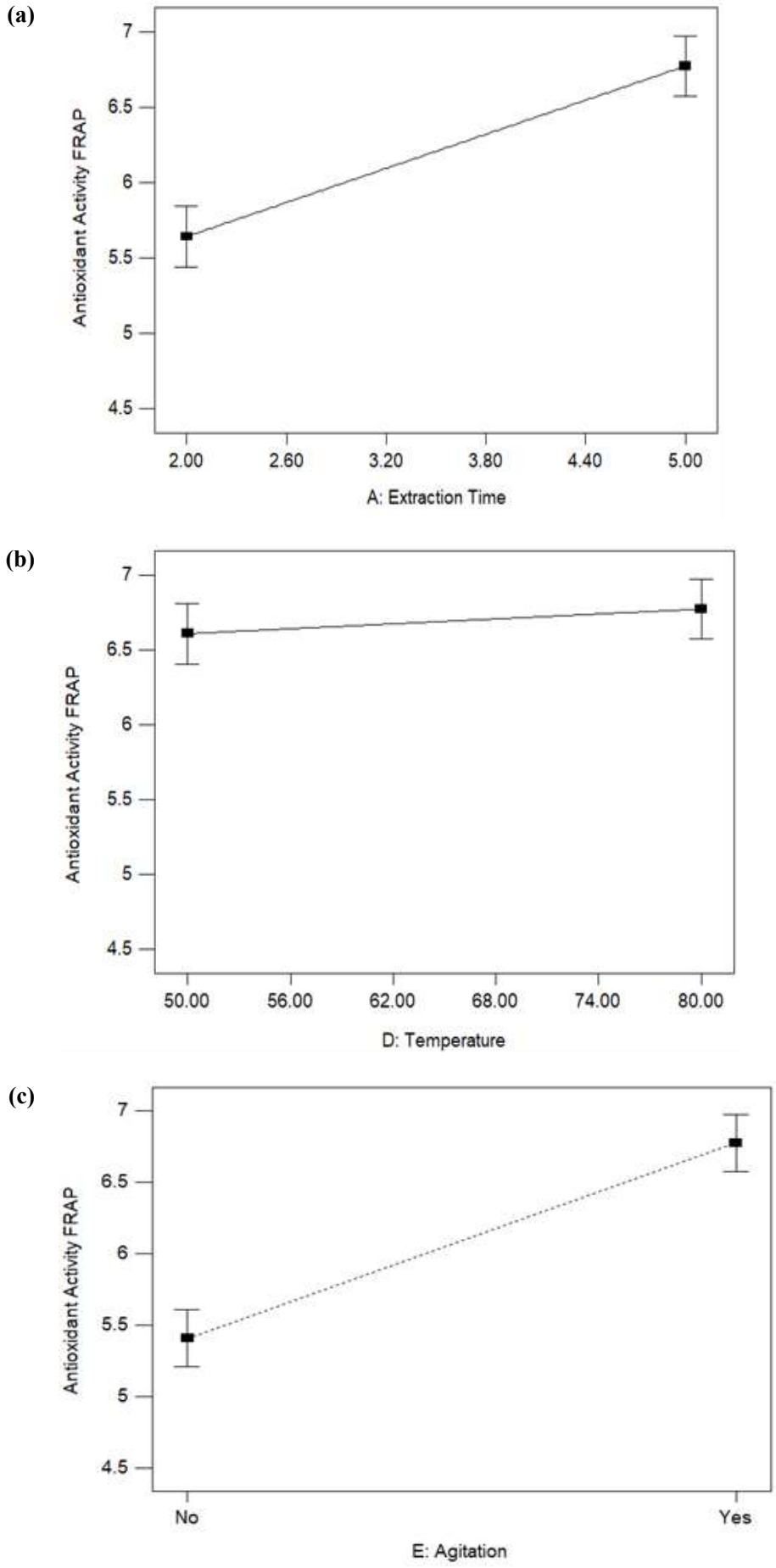

Figure 5: Effect of (a) extraction time, (b) temperature and (c) agitation on antioxidant activity

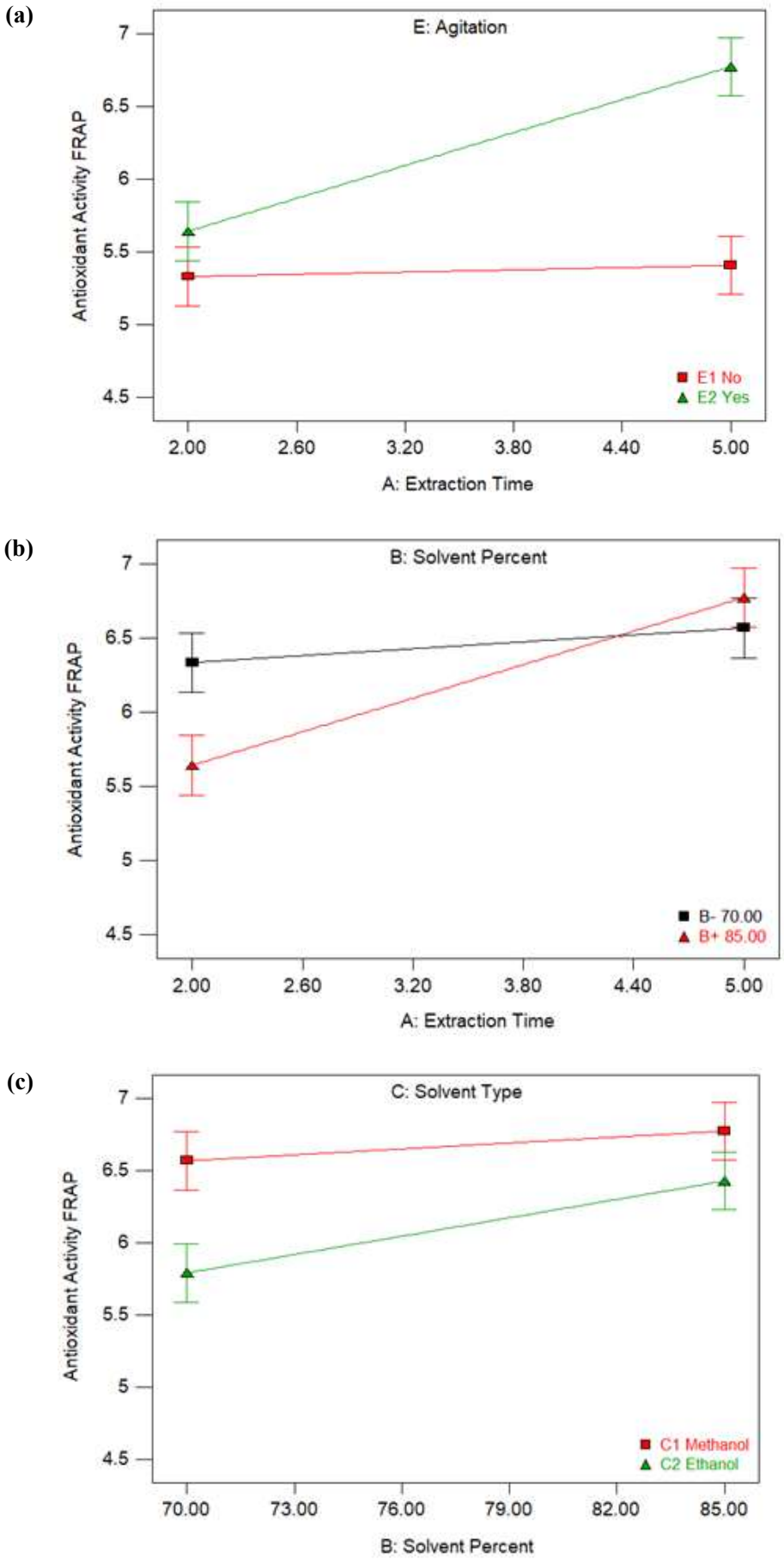

Figure 6: Effect of interaction between the main factors of (a) extraction time and agitation, (b) extraction time and solvent percentage, (c) solvent percentage and solvent type on AA

Table 4
ANOVA table for antioxidant activity

Source	Sum of squares	Df	Mean squares	F value	P-value
Model	6.50	12	0.54	55.15	0.0035
A	0.19	1	0.19	18.97	0.0224
B	3.272E-003	1	3.272E-003	0.33	0.6043
C	0.050	1	0.050	5.05	0.1103
D	0.52	1	0.52	53.22	0.0053
E	2.15	1	2.15	218.64	0.0007
AB	0.81	1	0.81	82.43	0.0028
AC	0.23	1	0.23	23.10	0.0172
AD	0.070	1	0.070	7.09	0.0762
AE	1.11	1	1.11	113.49	0.0018
BC	0.19	1	0.19	18.89	0.0225
CE	0.75	1	0.75	76.73	0.0031
DE	0.43	1	0.43	43.92	0.0070
R^2	0.9955				
Adjusted R^2	0.9774				

A: Extraction Time (hour), B: Solvent Percent (%), C: Solvent Type, D: Temperature (˚C), E: Agitation

Table 5
The best extraction condition for TFC and AA obtained from the Design-Expert software

Factor	Value
Extraction time (hr)	5
Solvent percent (%)	85
Solvent type	Methanol
Temperature (°C)	80
Agitation	Presence (100 rpm)

Figure 5 displays the effect of the independent factor on AA. It was observed in figure 5(a) that AA increases over time. Prolonged extraction times allow for more comprehensive extraction of antioxidant compounds from plant tissue, providing more opportunities for antioxidant compounds to be released[25,50]. A comparable observation was also reported by Che Sulaiman et al[9], in which the longer extraction time from 80 to 120 minutes significantly increases the phenolic compounds in *Clinacanthus nutans* leaves. The solubility of some antioxidants plays a crucial role during extraction. This is especially true since some antioxidants might have limited solubility in the solvent and thus require more time to dissolve fully. Longer extraction times permit the gradual dissolution of antioxidants into the solvent, resulting in greater antioxidant activity when measured.

The effect of temperature on AA is presented in figure 5(b). The small increment in AA as the temperature rises, presents the factor as being less critical to AA. However, higher temperature maximizes AA. Heating accelerates the initiation of reactions, leading to a reduction in the activity of existing or added antioxidants[37]. Nevertheless, temperature variations can alter the action mechanism of particular antioxidants or can affect them by different means[49]. Also, elevated temperatures can result in the oxidation and degradation of phytochemical compounds. In this case, degradation of antioxidants might occur at elevated temperatures, thus losing their antioxidant activity. Higher temperatures can also accelerate degradation reactions, thereby reducing overall antioxidant activity. Also, a temperature threshold might have been reached and a further increase in temperature did not significantly enhance antioxidant activity.

Figure 5(c) shows the effect of agitation on AA. It is observed that agitation significantly affects AA, in which the presence of agitation remarkably increases the AA. Agitation can promote the movement of solvent molecules and can increase their contact with plant tissue, facilitating the diffusion of antioxidants into the solvent. The increase in mass transfer results in higher antioxidant concentration, hence increasing the antioxidant activity. Agitation assists in the penetration of solvent into plant material, leading to increased antioxidant activity. The mechanical disruption might also occur with increased agitation speed, causing the breakdown of cell walls and releasing intracellular components. This exposes more surface area to the solvent, enhancing the accessibility of antioxidants.

Chan et al[8] reported similar findings in which the increase in agitation speed causes the dissolution and disruption rate of active compounds. The interaction effect between the main factors is shown in figure 6. As shown in figure 6(a), longer extraction time did not significantly increase the AA in non-agitated conditions. The absence of agitation causes no shears in plant tissues, in which further increase in extraction

time did not necessarily increase the AA. On the other hand, in the presence of agitation at 100 rpm, AA increased remarkably with longer extraction times. Prolonged contact of the plant tissue in the solvent permitted sufficient time for the desired compounds to migrate into the solvent.

The interaction between extraction time and the solvent percentage is portrayed in figure 6(b). A significant difference in AA is observed when 85% solvent percentage is used with the increase in extraction time. A small increment of AA exhibited in the extraction conditions of a lower solvent percentage (75%) might refer to the lower polar molecule available in the system with a lower solvent concentration, thus affecting the amount of antioxidants being solubilized. A higher solvent percentage serves as a more suitable condition for the solubilization of antioxidants as more solvent molecules are available to come into contact with and dissolve antioxidant molecules.

The effect of the interaction between solvent percentage and solvent type is exhibited in figure 6(c). It is well-portrayed that the antioxidant activity is much higher in methanolic than in ethanolic extracts at both solvent concentrations. The higher antioxidant activity observed in methanolic extraction compared to ethanolic extraction is attributed to solvent polarity. Methanol is a more polar solvent due to the presence of a shorter hydrocarbon chain compared to ethanol. The hydrocarbon chain dilutes the effect of the polar hydroxyl group, resulting in the less polar attribute. The increase in antioxidant compounds with methanol aligns with existing reports[10,24].

Solvent polarity is essential during the extraction, as it increases the solubility of antioxidants[2]. Onyebuchi and Kavaz[36] also reported the highest number of bioactive compounds in methanolic extracts compared to ethanol and water extracts. Therefore, in spite of being insignificant as a main factor, both solvent type and solvent percentage play a crucial role when interacting with one another.

The best extraction condition for TFC and AA: As proposed by the Design-Expert software, the best extraction conditions are shown in table 5, where TFC and AA were at the highest concentration. At this condition, TFC and AA concentrations were up to 0.171 mg/ml and 6.331 nmol/µl, respectively.

Conclusion

The most significant factors contributing to the phytochemical extraction of *C. rotundus* were determined in this study. The effect of independent and interaction factors on TFC and AA was investigated using two-level factorial analysis with randomized factors. The extraction time, solvent percent, solvent type, temperature and agitation were the contributing factors for all responses. The result shows that the temperature was the most contributing factor to TFC. Meanwhile, extraction time, temperature and agitation contributed most to AA. An additional study on phytochemical analysis should be carried out to enhance the extraction yield.

Acknowledgement

The authors are grateful for the financial aid supported by the Ministry of Higher Education under the Fundamental Research Grant Scheme (FRGS) No. FRGS/1/2023/TK05/UMP/02/7 (University reference RDU230130) and Universiti Malaysia Pahang Al-Sultan Abdullah for laboratory facilities.

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(Received 04th October 2024, accepted 07th December 2024)