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Optimization of Ultrasound-Assisted Extraction for Antioxidant Activity in Relation to Rhoifolin Content of *Fortunella polyandra* using Response Surface Methodology (RSM)

(Pengoptimuman Pengekstrakan Berbantu Ultrabunyi untuk Aktiviti Antioksidan Berkaitan Kandungan Rhoifolin *Fortunella polyandra* menggunakan Kaedah Gerak Balas Permukaan (RSM))

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ABSTRACT

Fortunella polyandra (Ridl) Tanaka or Malayan kumquat, is a family of Rutaceae from Malaysia known for its various active compounds, especially flavonoids. This study aims to verify the optimum condition and correlation of leaves extract in *Fortunella polyandra* by ultrasound-assisted extraction (UAE) for antioxidant activities and apigenin 7-O-neohesperidoside (rhoifolin) contents using the response surface methodology (RSM). Central composite rotatable design (CCD) was performed to investigate the influence of the extraction parameters on maximum antioxidant activity, namely temperatures (X_1), times (X_2), and solvent ratios of ethanol:water (X_3). The optimal extraction conditions for 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity were obtained at extraction time of 5 min, temperature of 30 °C and solvent ratio of 70:30, ethanol:water with IC_{50} value of 0.126±0.004 mg/mL. Rhoifolin content and ferric reducing antioxidant potential (FRAP) assay share the same optimum condition at extraction time of 5 min, temperature of 30 °C, and solvent ratio of 30:70, ethanol:water. Under these conditions, rhoifolin content was measured at 67.83±0.37 ppm, whereas FRAP equivalent value was recorded at 0.22±0.01 mg/mL. All three responses from the model achieved 95% confidence level. Correlation study showed that there is no relationship between the rhoifolin content and the antioxidant activities of *Fortunella polyandra* extract.

Keywords: Antioxidant activity; central composite design (CCD); *Fortunella polyandra*; response surface methodology (RSM); rhoifolin; ultrasound-assisted extraction (UAE)

ABSTRAK

Fortunella poliandra (Ridl) Tanaka atau kumquat Malaya, tergolong dalam famili Rutaceae dari Malaysia yang terkenal dengan pelbagai sebatian aktif terutamanya flavonoid. Walau bagaimanapun, kaedah pengekstrakan tradisi yang digunakan untuk mengekstrak sebatian aktif ini mengambil masa yang lama dan rumit. Secara khususnya, kaedah pengekstrakan berbantukan ultrabunyi (UAE) untuk mengekstrak sebatian aktif telah diketengahkan dan boleh menjadi salah satu kaedah yang berkesan untuk mengekstrak sebatian aktif daripada *Fortunella polyandra*. Kajian ini bertujuan untuk menentusahkan keadaan optimum ekstrak daun *Fortunella poliandra* menggunakan kaedah pengekstrakan berbantu ultrabunyi (UAE) untuk mengkaji aktiviti antioksidan dan korelasi kandungan apigenin 7-O-neohesperidosida (rhoifolin) menggunakan kaedah gerak balas permukaan (RSM). Reka bentuk komposit pusat

(CCD) digunakan untuk mengkaji kesan parameter pengekstrakan ke atas aktiviti optimum antioksidan, iaitu suhu (X_1), masa (X_2) dan nisbah pelarut etanol:air (X_3). Keadaan optimum pengekstrakan untuk aktiviti pemerangkapan 2,2-difenil-1-pikrillhidrazil (DFPH) diperoleh pada masa pengekstrakan 5 minit, suhu 30 °C dan nisbah pelarut 70:30, etanol:air dengan nilai IC_{50} 0.126±0.004 mg/mL. Kandungan rhoifolin dan cerakinan keupayaan antioksidan penurunan ferik (FRAP) menunjukkan keadaan optimum yang sama iaitu pada masa pengekstrakan selama 5 minit, suhu 30 °C dan nisbah pelarut 30:70, etanol:air. Di bawah keadaan optimum ini, kandungan rhoifolin yang diperoleh adalah 67.83±0.37 ppm, manakala nilai setara FRAP direkodkan pada 0.22±0.01 mg/mL. Ketiga-tiga gerak balas daripada model ini mencapai aras keyakinan 95%. Kajian korelasi menunjukkan tiada hubungan antara kandungan rhoifolin dan aktiviti antioksidan ekstrak *Fortunella poliandra*.

Kata kunci: Aktiviti antioksidan; *Fortunella poliandra*; kaedah gerak balas permukaan (RSM); pengekstrakan berbantu ultrabunyi (UAE); reka bentuk komposit pusat (CCD); rhoifolin

Introduction

Fortunella or widely known as kumquat is a vigorous and prolific small bushy tree of the rutaceae family, which gives oval or round-shaped fruits with a smooth, bright orange rind. Kumquat is marketed as confectionery, snack, and can be eaten raw as whole fruit or use as flavor agent in beverages (Çakmakçi et al. 2016). In Taiwan, they are used as traditional folks medicine to manage inflammatory of the respiratory tract which attributed by the present of flavonoid compounds (Lou et al. 2015). A study on the effects of *Fortunella* fruits extracts on metabolic disorders showed that it can prevent body weight gain and control glucose and lipid level in mice (Tan et al. 2014).

Design of Experiments (DOE) is a technique used to guide the choice of the experiments to be performed in an efficient way. Applications of DOE are based on the series of experiments in which the input parameters are varied to identify the reason of the changes in the output response (Cavazzuti 2013). Response surface methodology (RSM) is one of the examples of DOE that often used to optimize an extraction process. Extraction of active ingredient from plant material is one of the important steps in the recovery and purification. The purpose in optimizing an extraction is to give a maximum extract yield obtained from plant and of the highest quality which consist of high concentration of target compound and antioxidant power of extract.

RSM is widely used to design experiments, build model, express the response value, evaluate the multiple factors, and show the optimum condition. Interaction from several factors can be analyzed with minimal data, and intuitive model can be built to get optimal result quickly (Hou et al. 2016). Concentrations of solvent, extraction time and extraction temperature are factors that often used as parameters to optimize the antioxidant extracts from natural resources. Single factor analysis performed by previous researchers demonstrated that there are three factors contributed significantly to the extraction of antioxidant (Jing, Dong & Tong 2015; Lee et al. 2013; Li et al. 2016; Liu 2014; Xu et al. 2016, 2015; Zhao et al. 2014).

Central composite rotatable design (CCD) is one of the tools in RSM that commonly being used to extracts bioactive compound. There are few criteria in CCD that makes it most robust and simple as compared to other tools such as, it contains three types of points: factorial points, a central point, and axial points, the independent variables are studied at five levels ($-\alpha$, -1, 0, $+1$, $+\alpha$), and it consider an extreme point which will give a better overview of the experiment (Weremfo et al. 2022).

The extraction of active compounds from *Fortunella polyandra* (Ridl) Tanaka could be conducted by various technological methods. For examples, there are several extraction processes that can be applied, such as homogenization, maceration and Soxhlet extraction. However, these traditional extraction methods are very time-consuming and require large amount of solvents for extraction. Recently, several studies have been reported on the application of ultrasonic-assisted extraction (UAE) for extracting active compounds (Weremfo et al. 2022). The UAE can reduce extraction time, as well as improves the extraction yield and quality of extracts. Furthermore, UAE is more environmentally friendly and less expensive compared to some other extraction methods. Based on this, UAE could be one of the most efficient method for extraction active compounds from *Fortunella polyandra* (Ridl) Tanaka. In this study, optimum condition and correlation of leaves extract in *Fortunella polyandra* by ultrasound-assisted extraction (UAE) for antioxidant activities and rhoifolin contents was determined using the response surface methodology

(RSM). Three independent factors; concentration of solvent, extraction time and extraction temperature were chosen and further evaluated. The findings provide useful numerical data for the optimization condition of *Fortunella polyandra* extract.

The phytochemical analysis has shown that Kumquat is rich in phenolic antioxidants such as flavonoids and coumarins. The DPPH radical scavenging potency (%/mg/mL) of extracted mature and immature peel Kumquat by hot water was previously reported, the highest scavenging potency was found in immature peel extracts at 46.5 ± 5.3 %/mg/mL (Lou et al. 2015).

The contribution of DGGP as major compound in Kumquat was studied against the flavonoid pool (FP). DPPH bleaching showed that the radical scavenging of DGGP from unripe Kumquat juice was 6.89 µM trolox equivalent and 3.91 µM trolox equivalent for FP and DGGP, respectively. This shows that DGGP responsible of ~57% of the activity of the flavonoids extracts in unripe fruit juice. Ripe juice shows ~40% of DGGP which 2.29 µM TE and 5.73 µM TE for FP (Barreca et al. 2011). DGGP has quite a significant contribution toward the antioxidant activity of Kumquat. 4-hydroxy group in DGGP structure may provide good antioxidant ability (Lou & Ho 2016).

Abd-Elhady El-gizawy and Hussein (2016) tested isolated compounds from *Fortunella* toward the toxicity effect from paracetamol intake. Ferulic acid and coumaric acid showed a significant activity to inhibit the formation of lipid oxidation products. The positive result suggested that these compounds are hepatoprotective agent against paracetamol induced hepatoxicity. Coumaric acid and ferulic acid are known to react with oxidizing radical induced by paracetamol and could serve as free radical inhibitor.

Two new compounds, fortunellone and junosol were isolated together with three other known compounds, limonin, nomilin and deacetylnomilin from an extract of *Fortunella crassifolia* and *Citrus junos*. The isolated compounds were studied for its activity to inhibit the proliferation and cell death to treat human cervical cancer, the result shows that combination of chemotherapy drug with the isolated compound significantly increase the dead cell compared to those single drug treated cell (Kitagawa et al. 2021).

Materials and Methods

Plant Material

The leaves sample of *Fortunella polyandra* was collected at Taman Botani Shah Alam (GPS coordination: N 3°, 5', 45.1", E 101°, 30', 42.6") and stored in -40 °C freezer until further used. The voucher specimen was deposited at UKM Herbarium, Universiti Kebangsaan Malaysia, Bangi, Selangor (herbarium no: SK1507/18). The leaves of *Fortunella polyandra* were dried using freeze dryer (Martin Christ 1-2 LDPLUS), pulverized in an electrical grinder to powder and sieved (kitchen strainer) to give consistent particle size. The samples were then homogenously mixed in a plastic bag by turning the entire sample three times, 23 representative powdered samples of 5 g each was deposited and stored in a screw cap bottles (20 mL) and were extracted under various experimental conditions for optimization purposes.

Chemicals and Reagent

Ethanol and methanol were purchased from Fisher Scientific UK Limited, Glacial acetic acid and hydrochloric acid were supplied by Merck & Co., 2,2-diphenyl-1-picrylhydrazyl (DPPH) was acquired from EMD Millipore Corp., L-ascorbic acid 99%, sodium acetate trihydrate and 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) were obtained from Sigma Aldrich, ferric sulfate solution ($FeSO_4.7H_2O$) and Ferric chloride solution ($FeCl_3.6H_2O$) were supplied by R&M Chemicals. Rhoifolin was purchased from ChromaDex, Inc. All chemicals and reagents used in this study were of analytical reagent grade.

Equipment for UAE and Extraction of *Fortunella polyandra*

The extraction of samples for optimization analysis was carried out by ultrasound-assisted extraction using water bath sonicator (Wisd, WUC-A03H). Dried powdered samples (5 g) were extracted with 300 mL of different ethanol concentrations in water. Samples were extracted at different extraction times using various temperatures (20 different conditions). After the extraction procedure, the extract solutions were filtered with Whatman filter paper (no. 3) and rotavaped until 15-20 mL of concentrated solution remains in the flask. A 1000 µL of the solution above was pippeted out and centrifuged (Christ RVC 2-18) for 10 min at 10,000 rpm, then the supernatant was separated with pipette and speed dried using vacuum concentrator (Christ RVC 2-18) to give 20 powdered samples for evaluations of DPPH assay, FRAP assay, and rhoifolin content.

Central Composite Rotatable Design (CCD) for Extraction Optimization

The 20 different conditions were developed using

response surface methodology based CCD (central composite rotatable design). The experimental design was based on three independent factors (temperature, time, and ethanol concentration) with five-level structure. All the response values obtained from the 20-run-experiment are shown in Table 1.

DPPH RADICAL SCAVENGING MEASUREMENT

The DPPH scavenging capacity protocol was adopted from previous publication (Kassim et al. 2013). In brief, 30 µL of DPPH solution in methanol (300 µM) was mixed with 170 µL of sample in methanol (250 - 31.3 ppm; two-fold dilution) and incubated at room temperature in a dark environment for 30 min. Samples (plus DPPH above) absorbance (As) and methanol (plus DPPH above) absorbance (Am) were measured at 517 nm (microplate reader Biotek Epoch 240292). The DPPH scavenging capacity was calculated by equation (1).

$$\text{DPPH scavenging capacity (\%)} = \frac{A_m - A_s}{A_m} \times 100 \quad (1)$$

FERRIC REDUCING ANTIOXIDANT POWER (FRAP) ASSAY

The method explained by Benzie and Strain (1996) was used in this study for ferric reducing antioxidant potential assay with slight modification. The FRAP reagent was freshly prepared, consist of stock solution of acetate buffer (300 mM, 50 mL), TPTZ (10 mM, 5 mL), $FeCl_3$ (20 mM, 10 mL) and distilled water (6 mL). The FRAP reagent was mixed with sample (5 µL of 0.8 mg/mL in methanol) in 96-well plate and incubated for 10 min at 37 °C in incubator. The absorbance was set at 595 nm and measured using Bio-Rad iMark micro-plate reader after 10 minutes. The experiment was done in triplicates with $FeSO_4.7H_2O$ solutions as standard. A calibration graph of $FeSO_4.7H_2O$ at different concentrations (0.2, 0.4, 0.6, 0.8, 1.0 mM; in water) was plotted. The capability of the extracts to undergo the reduction of ferric ions was calculated as the antioxidant capacity and expressed as mg/mL equivalent to $FeSO_4.7H_2O$.

DETERMINATION OF RHOIFOLIN CONTENT

The samples from optimization extracts (20) were diluted using methanol and put in a water bath sonicator for 10 min. The reduced pressure was used to concentrates the samples to dryness (to give a constant weight) and then diluted to 5000 ppm with HPLC-grade methanol. The sample was analysed with HPLC Perkin Elmer Flexar FX-10 HPLC (PDA) equipped with quaternary pump with column: Luna-5µm C18 (30 mm × 0.25 mm × 4.6 mm). The detection was recorded by photo diode array (PDA) detector. UV spectra of the peaks in the chromatograms were then examine using chromera-PDA. The HPLC analysis was carried out under the following conditions: injection volume: 10 µL; temperature: 40 °C; gradient method: (equilibrium time at 10% MeCN (in 0.1% formic acid in water) for 5 min), 10% MeCN (in 0.1% formic acid in water) for 5 min, 30% MeCN for 3 min, 60% MeCN for 5 min, 50% MeCN for 5 min, and back to 60% MeCN and 30% MeCN for 5 min, each comprises a total run of 23 min; wavelength setpoint: 265 nm. Compound was identified by comparing the UV spectrum and retention time of rhoifolin standard with those of samples.

STATISTICAL ANALYSIS

In this study, a five-level and three-factor CCD was carried out to analyze the linear, cross, and quadratic effects by using RSM of the following parameters on antioxidant anticity and rhoifolin content: X_1: Extraction temperature (°C); X_2: Extraction time (min); X_3: Ethanol concentration (%, v/v). Optimized extraction results were expressed as average values ± standard deviation (n = 3). Determination of the individual linear, quadratic and interaction regression coefficients (β), ANOVA was performed. The fitness of the quadratic model was estimated by employing the coefficient of determination (R^2), and the significance of each coefficient was determined by *p*-values. Specifically, *p*-value ≤ 0.001, 0.001 < *p*-value ≤ 0.01, 0.01< *p*-value ≤ 0.05 and *p*-value > 0.05 indicate that the model terms are remarkably significant, highly significant, significant, and not significant, respectively.

RESULTS AND DISCUSSION

MODEL FITTING

The five-levels and three factors central composite rotatable design (CCD) was carried out to optimize the ultrasound assisted extraction (UAE) of antioxidant activity (DPPH radical scavenging capacity (RSC) and FRAP) and rhoifolin content. The response variables and corresponding fitted model equation of DPPH-RSC (Y_{DPPH}), ferric-reducing antioxidant power (Y_{FRAP}), and rhoifolin content (Y_R) collected from 20 groups of experiments are listed in Table 1. Design-Expert software was used to generate the quadratic regression model and their mathematical expression.

The analysis of variance (ANOVA) indicated that the three models were highly significant (*p*-value<0.001). The values of R^2 were quite close to 1 and the value of adjusted R^2 and predicted R^2 are in the range of 20%, suggesting

that the model is acceptably comply the variability for adequate prediction (Owolabi, Usman & Kehinde 2018; Rai, Mohanty & Bhargava 2015). In addition, the signal-to-noise ratio measured by the adequate precision exceeded four, indicating that the model can be used to navigate the design space. Furthermore, the proposed model showed that the coefficient of variation value was less than 10%, demonstrating the reliability and precision of the experimental run (Zhang et al. 2019). Based on factors mentioned, it can be concluded that the results from Table 2 was appropriate to accommodate and predict the responses.

The 3D response surface analysis was generated to determine the effect of different process parameters by manipulating two variables while keeping the third at a fixed value of zero level. The graph describes the interaction of independent variable for the responses on DPPH-RSC, FRAP, and rhoifolin content, as shown in Figures 1-3.

TABLE 1. Response variables and their fitted model equation

Symbol	Response variable	Fitting the coding equation of the mode
Y_{DPPH}	DPPH radical scavenging capacity (IC_{50}, mg/mL)	$Y_{DPPH} = 0.17 - (3.26 \times 10^{-3})X_1 + (9.14 \times 10^{-3})X_2 - (6.20 \times 10^{-3})X_1X_2 + (6.20 \times 10^{-3})X_2X_3 - (5.20 \times 10^{-3})X_1^2$
Y_{FRAP}	Ferric-reducing antioxidant power (mg/mL equivalent to $FeSO_4.7H_2O$)	$Y_{FRAP} = 0.18 - 0.015X_1 + (5.63 \times 10^{-3})X_2 - (4.28 \times 10^{-3})X_1^2 + (1.03 \times 10^{-3})X_2^2 - (6.04 \times 10^{-3})X_3^2$
Y_R	Rhoifolin content (ppm)	$Y_R = 57.63 + 1.46X_1 - 3.54X_2 - 4.14X_3 + 1.01X_1X_2 - 4.44X_1X_3 + 4.05X_1X_3 + 2.06X_1^2 + 3.05X_2^2 - 2.01X_3^2$

TABLE 2. Analysis of variance results of the predicted quadratic regression model for antioxidant activity and rhoifolin content

Factor	Coefficient (β)		
	DPPH-RSC	FRAP	Rhoifolin Content
Linear			
X_1	0.0035**	<0.0001**	0.0007**
X_2	<0.0001**	0.0079**	<0.0001**
X_3	0.9044	<0.0001**	<0.0001**
Cross product			
X_1X_2	0.0002**	0.2864	0.0273*
X_1X_3	0.3926	1.0000	<0.0001**
X_2X_3	0.0002**	1.0000	<0.0001**
Quadratic			
X_1^2	<0.0001**	0.0272*	<0.0001**
X_2^2	0.1334	0.5479	<0.0001**
X_3^2	0.0501	0.0044**	<0.0001**
R^2	0.9588	0.9848	0.9879
Adjusted R^2	0.9217	0.9710	0.9770
Predicted R^2	0.7552	0.9200	0.9219
Adequate precision	19.239	30.723	41.22
Coefficient of Variance	1.99	3.52	1.86
p-Value (Model)	<0.0001**	<0.0001**	<0.0001**
p-Value (Lack of fit)	0.2638	0.3028	0.0921

(%, v/v). **: indicates highly significant ($p < 0.01$), *: indicates significant ($p < 0.05$)

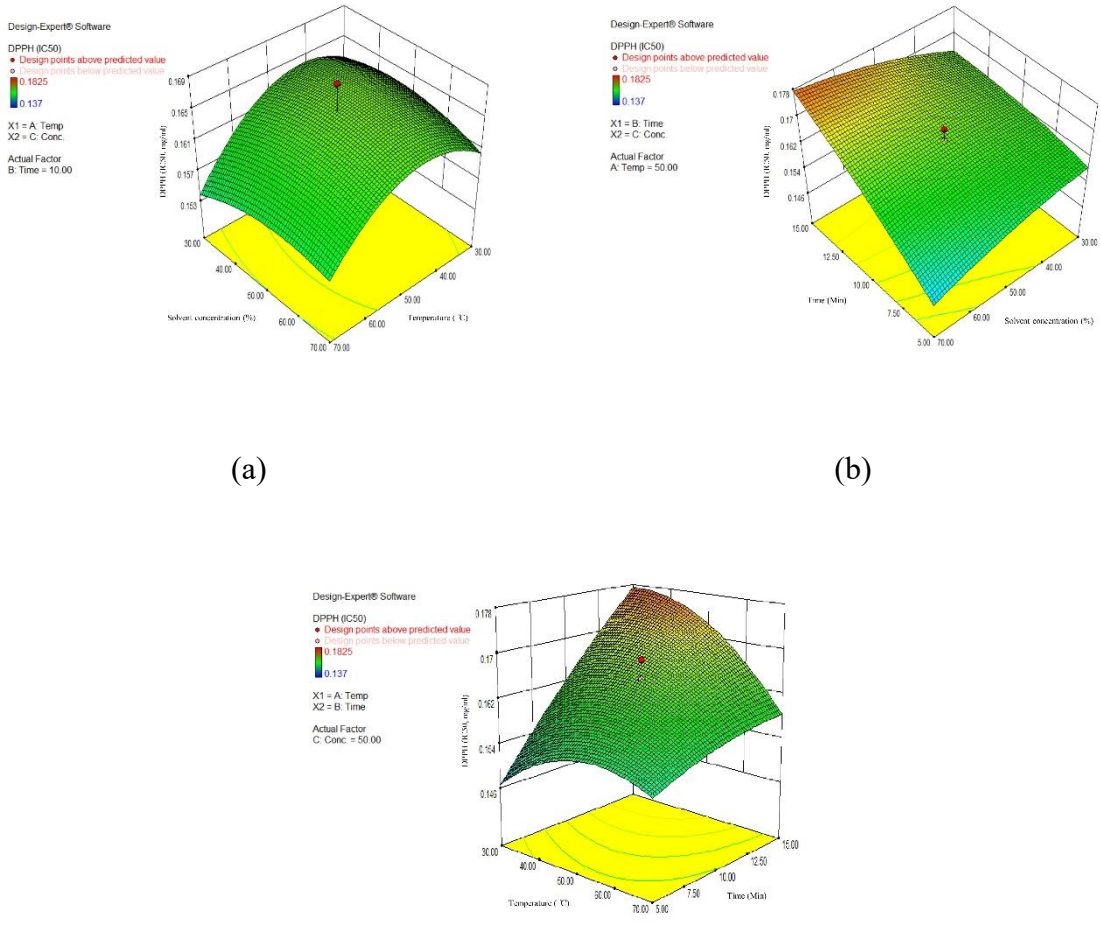
EFFECTS OF THE VARIABLES ON ANTIOXIDANT ACTIVITY

EFFECTS OF VARIABLES ON DPPH ASSAY

Analysis of the regression model for DPPH-RSC was fitted and checked with the determination coefficient value $R^2 = 0.9588$, which demonstrated that only 4.12% of the total variations could be explained by the model. In this study, the R^2 and predicted R^2 of DPPH radical scavenging content are 0.9217 and 0.7552, respectively.

The IC_{50} value of DPPH assay accumulated by the antioxidant extracts ranging from 0.137 to 0.183 mg/mL, with a maximum to minimum ratio of 1.33. Previous study by Lou et al. (2016) reported that the DPPH assay of immature peel from *Citrus japonica* var. *margarita* (kumquat) by hot water extractions at 80 and 90°C were 45.5 and 46.5%/mg/mL, respectively. The comparison of previous study with the existing results demonstrate that the optimized antioxidant extracts show better inhibition at 50% radical scavenging capacity in lower concentration range (0.137 to 0.183 mg/mL) against 45.5 and 46.5% at 1 mg/mL (for the 45.5 and 46.5%/mg/mL above). The normal plot of the residual confirmed that the dataset followed a normal distribution, therefore analysis of variance (ANOVA) was carried out without any power transformation.

Figure 1(a) shows the interaction of temperature and solvent concentration towards the free radical scavenging activity of DPPH. ANOVA from the interaction showed insignificant p-value of 0.3926 ($p > 0.05$). The narrow range of free radical scavenging activity of DPPH from these two factors (0.154 - 0.163 mg/mL)



(a) (b)

(c)

FIGURE 1. The interaction of extraction variables on DPPH-RSC; (a) Solvent concentration (%) vs Temperature (°C); (b) Solvent concentration (%) vs Time (min); Temperature (°C) vs Time (min)

demonstrated that the interaction between these two factors was not significantly affecting the extraction of antioxidant compounds in this experiment.

Figure 1(b) shows the interaction of ethanol concentration and time towards the free radical scavenging activity of DPPH. The lowest IC_{50} value (0.137 mg/mL) can be observed during the highest ethanol concentration with minimum extraction time. Similar trend in Figure 1(c) on the extraction time was also observed on the interaction with temperature which indicate that shorter extraction time gave better antioxidant activity. The contour plots demonstrated a highly significant effect between these two interactions with *p*-value of 0.0002.

Figure 1(c) shows the interaction effect of temperature and time on the antioxidant activity by DPPH assay. As it can be seen in the plots, there is an increase of antioxidant scavenging activity with minimum extraction time in the range of 5.00 to 7.50 min. On the other hand, the effect of temperature showed a high antioxidant scavenging activity at a minimum extraction time with minimum temperature range from 30 to 50 °C. The trend also demonstrated a good extraction of antioxidant properties at a high temperature range from 60 to 70 °C with minimum extraction time. It can be concluded from the contour plot that optimum region for extraction of antioxidant compounds was in the time range of 5.00 to 7.50 min. The interaction of extraction times and temperatures had a highly significant effect towards the antioxidant scavenging activity with *p*-value of 0.0002 from the ANOVA.

EFFECTS OF VARIABLES ON FRAP

Analysis of the regression model for FRAP antioxidant values was fitted and checked with the determination coefficient value $R^2 = 0.9848$, which demonstrated that only 1.52% of the total variations could be explained by the model. In this study, the R^2 and predicted R^2 of FRAP antioxidant assay are 0.9710 and 0.9200, respectively.

Ferric Reducing Antioxidant Potential (FRAP) equivalent values ranged from 0.10 to 0.24 mg/mL with maximum to minimum ratio of 2.4. Previous study conducted by Tan et al. (2016) on different species of *Fortunella* fruits including *Fortunella japonica*, *Fortunella margarita*, and *Fortunella crassifolia* found that the FRAP values ranged from 8.29 to 14.63 μmol TE/g dw. Nonetheless, the normal plot of the residual confirmed that the dataset followed a normal distribution, therefore ANOVA was carried out without any power transformation.

Figure 2(a)-2(c)) shows the interaction effects of temperature and time, temperature and solvent concentration and solvent concentration and time, respectively. ANOVA from these three interactions showed insignificant p-values of 0.2864, 1.000, and 1.000, respectively. The narrow range of FRAP values (0.10 - 0.23 $FeSO_4$ equivalents) demonstrated that the interactions between all factors were not significantly affecting the extraction of antioxidant compounds in this experiment.

EFFECTS OF VARIABLE ON RHOIFOLIN CONTENT

Analysis of the regression model for rhoifolin contents was fitted and checked with the determination coefficient value $R^2 = 0.9879$, demonstrating that only 1.21% of the total variations could be explained by the model. The adjusted R^2 and predicted R^2 value was calculated as 0.9770 and 0.9219, respectively. The obtained values are in acceptable range as defined by Rai, Mohanty and Bhargava (2015).

Rhoifolin contents range from 43.80 to 77.41 ppm, with maximum to minimum ratio of 1.77. The normal plot of the residual confirmed that the dataset followed a normal distribution, therefore ANOVA was carried out without any power transformation.

Figure 3(b) shows the interaction of ethanol concentration and time towards the rhoifolin contents. The highest concentration was achieved at the lower extraction time range from 5 to 7.5 min. Similar trend was also observed on the ethanol concentration that ranged from 30 to 40% and yielded higher rhoifolin contents. In this interaction, it can be concluded that, low extraction time and low ethanol concentration, yielded higher amount of rhoifolin. This result shows that extraction of rhoifolin compound is favourable as the water concentration increase which indicated that the compound is more likely to be extracted in a polar mixture.

Figure 3(c) shows the interaction effect of temperature and time on the extraction of rhoifolin. The ANOVA of these two interactions showed significant effect with *p*-value 0.0273. As it can be seen in the contour plot, the range of rhoifolin content extracted is between the range of 56.64 - 66.65 ppm. The higher rhoifolin content was extracted at shorter extraction time range around 5 to 6 min. Meanwhile, the optimum condition for rhoifolin extraction was achieved regardless of the temperature range with minimum extraction time range from 5 to 6 min. The results concluded that the extraction times (5 - 15 min) and temperatures (30 - 70 °C) played

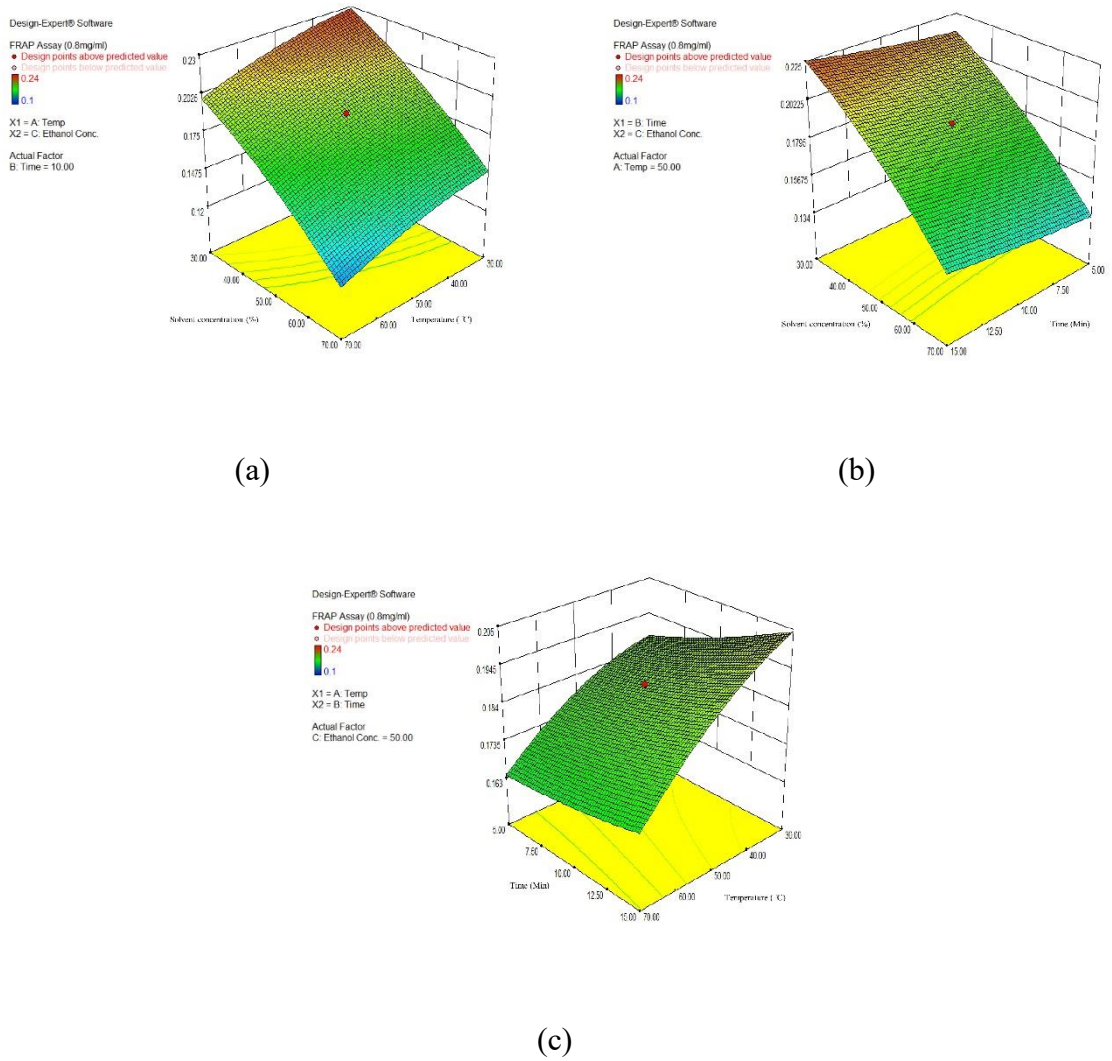


FIGURE 2. The interaction of extraction variables on FRAP; (a) Solvent concentration (%) vs Temperature (°C); (b) Solvent concentration (%) vs Time (min); Temperature (°C) vs Time (min)

a significant effect on the extraction of rhoifolin contents in *Fortunella polyandra*.

OPTIMIZATION EXTRACTION CONDITION AND VERIFICATION OF PREDICTIVE MODEL

The optimal values of the independent variables, temperature, time, and ethanol concentration are as given in Table 3. The optimization criteria were stipulated by minimizing the temperature and time while ethanol concentration was in range to achieve maximum DPPH scavenging capacity by the lowest IC_{50} value of 0.126±0.0039 mg/mL and maximum rhoifolin content 67.83±0.37 ppm.

CORRELATION OF RHOIFOLIN IN BIOACTIVITY

The correlation of rhoifolin contents with DPPH scavenging activity was investigated to determine whether rhoifolin is the key component of the antioxidant activity of the extracts. The two responses were set under the same evaluation to produce one extracts that represent the optimum condition of both responses. The extract was validated and showed 95% confidence interval between experimental and predicted, hence, the extract was used for the correlation. Table 4 shows the experimental and predicted values of each evaluation under the same condition.

Rhoifolin of 65.02 ppm was prepared to match the optimized extract and tested for its DPPH-RSC activity. The results showed low to no activity, suggesting that rhoifolin had small role or may not contribute to the antioxidant activity of *Fortunella polyandra* leaf extract. To further verified the results, an extract with a concentration of 5000 ppm was prepared to match the concentration of rhoifolin (65.02 ppm) and tested for its DPPH-RSC activity. The results showed that the sample recorded a 48.24% of DPPH-RSC in the concentration of 0.151±0.011mg/mL (151 ppm), almost equivalent to that in 5.0 mg/mL (5000 ppm). This results show, higher concentration might contribute to antagonistic interactions that lower the DPPH scavenging capacity.

The FRAP value was calculated using a calibration curve standard of $FeSO_4.7H_2O$ solution with concentration of 0.1 - 0.8 mg/mL. The graph expressed as mg/mL $FeSO_4$ equivalent which were linear over the calibration range with R2 value of 0.9995 (Figure 4). Ascorbic acid was used as standard controls with FRAP equivalent value of 2.59±0.04 mg/mL.

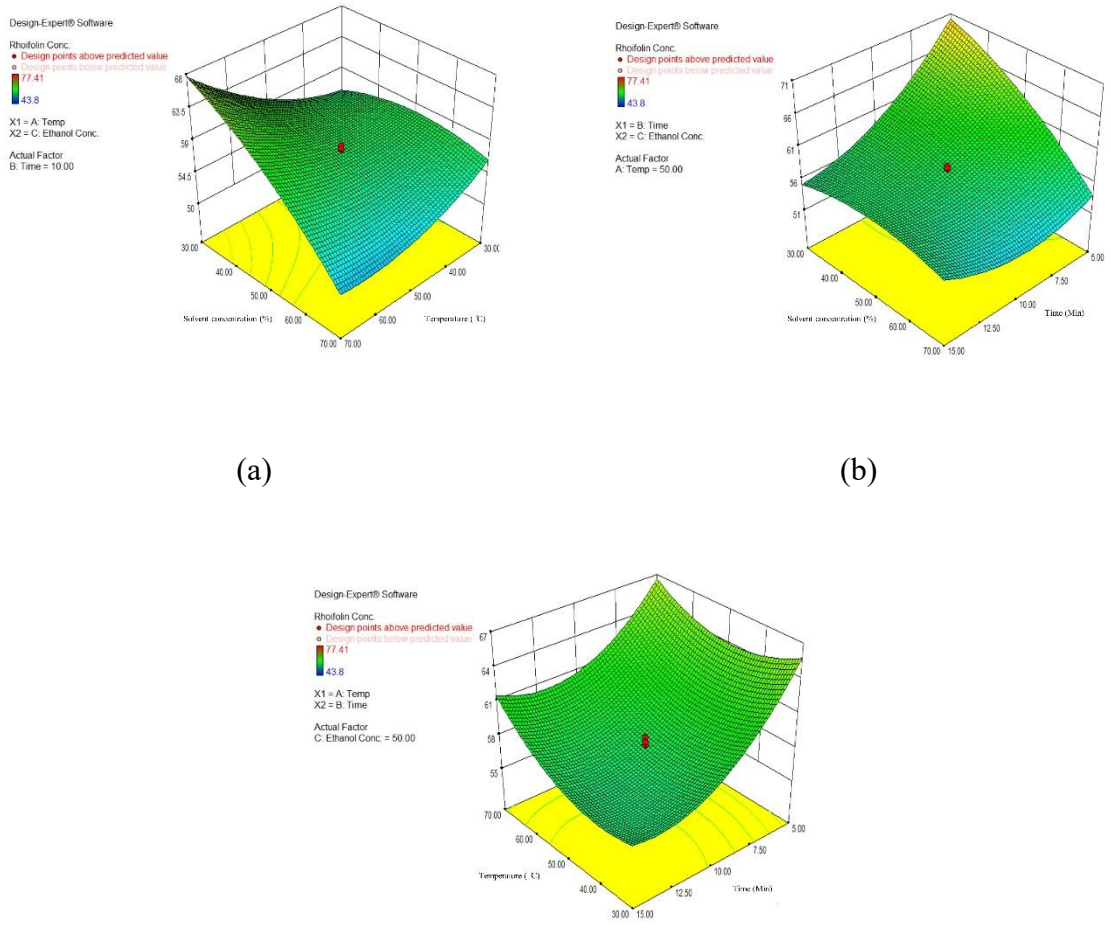


FIGURE 3. The interaction of extraction variables on rhoifolin; (a) Solvent concentration (%) vs Temperature (°C); (b) Solvent concentration (%) vs Time (min); (c) Temperature (°C) vs Time (min)

TABLE 3. Experimental and predicted values of each evaluation index under optimal condition of DPPH scavenging capacity and rhoifolin contents ([a]Responses are the means±SD (n=3))

Responses	Optimum extraction conditions			Optimized value		% Diff. (Approx)
	Temp. (°C)	Time (min)	Ethanol Concentration (%)	Experimental[a]	Predicted	
DPPH scavenging capacity, (mg/mL)	30	5	70	0.126±0.0039	0.137	8.02
Rhoifolin content (ppm)	30	5	30	67.83±0.37	67.58	0.37

TABLE 4. Experimental and predicted values of each evaluation index under the same condition ([a]Responses are the means±SD (n=3)

Optimum condition		DPPH scavenging capacity, IC_{50} (mg/mL)	Rhoifolin content (ppm)
Temperature (30°C) Time (5 min) Ethanol concentration (54%)	Experimental[a]	0.151±0.011	64.04±2.03
	Predicted	0.145	65.02
% Difference (approximately)		4.14	1.51

The optimal values of the independent variables, temperature, time, and ethanol concentration for FRAP value are given in Table 5. The indication of the parameter was specified at a minimum temperature and time, while ethanol concentration was suggested within the range to achieve FRAP equivalent value of 0.22±0.01 mg/mL.

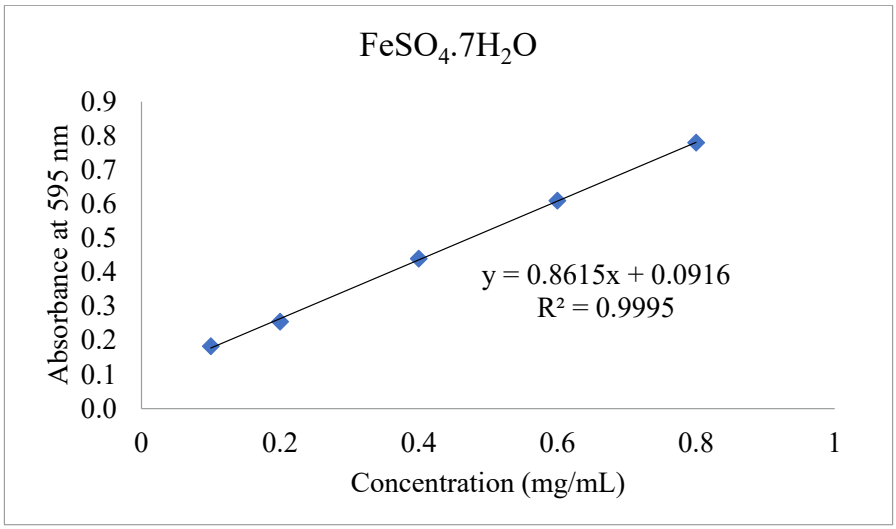


FIGURE 4. Calibration curve of $FeSO_4.7H_2O$

TABLE 5. Experimental and predicted values of each evaluation index under optimal condition of FRAP assay ([a]Responses are the means±SD (n=3))

Optimum condition		FRAP (mg/mL equivalent to $FeSO_4.7H_2O$
Temperature (30°C) Time (5 min) Ethanol Concentration (30%)	Experimental[a]	0.22±0.01
	Predicted	0.22
% Difference (approximately)		0

CONCLUSIONS

This study examines the *Fortunella polyandra* extracts optimization using ultrasound-assisted extraction method by response surface methodology based central composite rotatable design (CCD) approach with three factors and five levels (α, 1, 0, -1, and -α). Extraction temperature (X_1), extraction time (X_2), and solvent concentration (X_3) were chosen as independent factors, while the response analyses performed included free radical scavenging capacity by DPPH assay, ferric reducing antioxidant power assay (FRAP) and quantification of rhoifolin using high performance liquid chromatography (HPLC). The effect between all parameters showed no influence on the interaction for FRAP. Furthermore, the quantification of rhoifolin demonstrated a remarkably significant correlation between X_1X_3 and X_2X_3, while X_1X_2 for DPPH assay. In addition, weak correlation can be observed for X_1X_2 in rhoifolin content. The optimum conditions of independent response were as follows: FRAP and rhoifolin, X_1= 30 °C, X_2= 5 min, X_3= 30% ethanol in water; DPPH assay: X_1= 30 °C, X_2= 5 min, X_3= 70% ethanol in water. Based on the verification experiments, it was found that the predicted values were quite close to the experimental results. Lastly, rhoifolin was quantified to evaluate its contribution and correlation toward the antioxidant activity of the extracts. The results showed that rhoifolin is not the factor for the antioxidant activity. Furthermore, higher concentration of extracts might contribute to antagonistic effect. Further analysis should be carried out to clarify the mechanism of interaction and identify major components that contribute to the antioxidant activity of *F. polyandra*.

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REFERENCES

Abd-Elhady El-gizawy, H. & Hussein, M. 2016. Isolation, structure elucidation of ferulic and coumaric acids from *Fortunella japonica* Swingle leaves and their structure antioxidant activity relationship. *Free Radicals and Antioxidants* 7: 23-30.

Barreca, D., Bellocco, E., Caristi, C., Leuzzi, U. & Gattuso, G. 2011. Kumquat (*Fortunella japonica* Swingle) juice: Flavonoid distribution and antioxidant properties. *Food Research International* 44: 2190-2197.

Benzie, I.F. & Strain, J.J. 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Analytical Biochemistry* 239(1): 70-76.

Çakmakçi, S., Topdaş, E.F., Çakir, Y. & Kalin, P. 2016. Functionality of kumquat (*Fortunella margarita*) in the production of fruity ice cream. *Journal of the Science of Food and Agriculture* 96(5): 1451-1458.

Cavazzuti, M. 2013. *Optimization Methods: From Theory to Design Scientific and Technological Aspects in Mechanics*. 2013th ed. Springer.

Hou, W., Zhang, W., Chen, G. & Luo, Y. 2016. Optimization of extraction conditions for maximal phenolic, flavonoid and antioxidant activity from *Melaleuca bracteata* leaves using the response surface methodology. *PLoS ONE* 11(9): 1-11.

Jing, C.L., Dong, X.F. & Tong, J.M. 2015. Optimization of ultrasonic-assisted extraction of flavonoid compounds and antioxidants from alfalfa using response surface method. *Molecules* 20: 15550-15571.

Kassim, N.K., Rahmani, M., Ismail, A., Sukari, M.A., Ee, G.C., Nasir, N.M. & Awang, K. 2013. Antioxidant activity-guided separation of coumarins and lignan from *Melicope glabra* (Rutaceae). *Food Chemistry* 15(139): 1-4.

Kitagawa, T., Matsumoto, T., Imahori, D., Kobayashi, M., Okayama, M., Ohta, T., Yoshida, T. & Watanabe, T. 2021. Limonoids isolated from the *Fortunella crassifolia* and the *Citrus junos* with their cell death-inducing activity on Adriamycin-treated cancer cell. *Journal of Natural Medicines* 75(4): 998-1004.

Liu, X.F. 2014. Optimization of total flavonoids extraction from *Coreopsis tinctoria Nutt.* by response surface methodology. *Journal of Chemists and Chemical Engineers* 63(11-12): 391-396.

Lee, L.S., Lee, N., Kim, Y.H., Lee, C.H., Pil, H.S., Jeon, Y.W. & Kim, Y.E. 2013. Optimization of ultrasonic extraction of phenolic antioxidants from green tea using response surface methodology. *Molecules* 18: 13530-13545.

Li, H.Z., Zhang, Z.J., Xue, J., Cui, L.X., Hou, T., Li, X.J. & Chen, T. 2016. Optimization of ultrasound-assisted extraction of phenolic compounds, antioxidants and rosmarinic acid from Perilla leaves using response surface methodology. *Food Science and Technology* 36(4): 686-693.

Lou, S.N. & Ho, C. 2016. Phenolic compounds and biological activities of small-size citrus: Kumquat and calamondin. *Journal of Food and Drug Analysis* 25(1): 162-175.

Lou, S.N., Lai, Y.C., Huang, J.D., Ho, C.T., Ferng, L.H.A. & Chang, Y.C. 2015. Drying effect on flavonoid composition and antioxidant activity of immature kumquat. *Food Chemistry* 171: 356-363.

Owolabi, R.U., Usman, M.A. & Kehinde, A.J. 2018. Modelling and optimization of process variables for the solution polymerization of styrene using response surface methodology. *Journal of King Saud University - Engineering Sciences* 30(1): 22-30.

Rai, A., Mohanty, B. & Bhargava, R. 2015. Supercritical extraction of sunflower oil: A central composite design for extraction variables. *Food Chemistry* 192: 647-659.

Tan, S., Li, M., Ding, X., Fan, S., Guo, L., Gu, M., Zhang, Y., Feng, L., Jiang, D., Li, Y., Xi, W., Huang, C. & Zhou, Z. 2014. Effects of *Fortunella margarita* fruit extract on metabolic disorders in high-fat diet-induced obese C57BL/6 mice. *PLoS ONE* 9(4): e93510.

Tan, S., Zhao, X., Yang, Y., Ke, Z. & Zhou, Z. 2016. Chemical profiling using Uplc Q-Tof/Ms and antioxidant activities of fortunella fruits. *Journal of Food Science* 81(7): C1646-C1653.

Weremfo, A., Abassah-Oppong, S., Adulley, F., Dabie, K. & Seidu-Larry, S. 2022. Response surface methodology as a tool to optimize the extraction of bioactive compounds from plant sources. *Journal of the Science of Food and Agriculture* 103(1): 26-36.

Xu, D.P., Zheng, J., Zhou, Y., Li, Y., Li, S. & Li, H.B. 2016. Extraction of natural antioxidants from the *Thelephora ganbajun* mushroom by an ultrasound-assisted extraction technique and evaluation of antiproliferative activity of the extract against human cancer cells. *International Journal of Molecular Sciences* 17(10): 1664.

Xu, D., Zhou, Y., Zheng, J., Li, S., Li, A.N. & Li, H.B. 2015. Optimization of ultrasound-assisted extraction of natural antioxidants from the flower of *Jatropha integerrima* by response surface methodology. *Molecules* 21(1): 18.

Zhang, L., Jiang, Y., Pang, X., Hua, P., Gao, X., Li, Q. & Li, Z. 2019. Simultaneous optimization of ultrasound-assisted extraction for flavonoids and antioxidant activity of *Angelica keiskei* using response surface methodology (RSM). *Molecules* 24(19): 3461.

Zhao, Y., Hou, Y., Tang, G., Cai, E., Liu, S., Yang, H., Zhang, L. & Wang, S. 2014. Optimization of ultrasonic extraction of phenolic compounds from *Epimedium brevicornum* Maxim using response surface methodology and evaluation of its antioxidant activities *in vitro*. *Journal of Analytical Methods in Chemistry* 2014: 1-7.

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