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OPTIMIZATION OF ANTIOXIDANT EXTRACTION FROM KALUMPIT (*TERMINALIA MICROCARPA* DECNE) FRUITS

Dennis Marvin Santiago[*1], *Claire Zubia*[1], *Shekayna Eunice Pacia*[1], *Sheba Mae M. Duque*[1]

Address(es): Dennis Marvin Santiago, PhD,
[1]University of the Philippines Los Baños, College of Agriculture and Food Science, Institute of Food Science and Technology, 4031 College, Laguna, Philippines, +63495018932.

*Corresponding author: dosantiago1@up.edu.ph

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ABSTRACT

The effects of extraction parameters, including temperature (25 – 80 °C), time (30 – 90 min), solvent to sample (S/S) ratio (10 – 50 mL g^{-1}), initial pH (3 - 8) and ethanol concentration (20 – 100%), on the % 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of *kalumpit* were screened and optimized using 2-level factorial design and Box-Behnken design (BBD) of experiments. Temperature, S/S ratio, and ethanol concentration exhibited significant effects on the % DPPH radical scavenging activity of *kalumpit* extract. Response surface models developed for % DPPH and 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging activities of *kalumpit* fruit extract adequately fit and were used to determine the optimum extraction conditions. A desirability function approach determined the optimum conditions for solvent extraction of antioxidants at 80.0 °C, 10 mL g^{-1} S/S, and 51.66% ethanol concentration. This resulted in a maximum desirability value of 0.977 and predicted % DPPH and ABTS radical scavenging activities of 66.63 and 82.14, respectively. Validation of the adequacy of the predictive models showed no significant difference between experimental data and predicted values ($p > 0.05$), indicating that the models developed were adequate in describing the relationship between factors and responses.

Keywords: Solvent extraction, 2-level factorial design, Box-Behnken design, DPPH scavenging activity, ABTS scavenging activity

INTRODUCTION

The Philippines has a variety of edible and nutritious fruits from indigenous trees that are good sources of ethnobotanical ingredients (**Dela Cruz, 2012**). Traditionally, fruits that contain phytochemicals are being consumed as food and medicine due to the health benefits they bring. However, despite their potential health benefits, the economic significance of these fruits is not yet fully realized; thus, they remain underutilized in terms of food processing. These kinds of fruits are usually seasonal and have a short shelf life. Therefore, it is important to develop processing technologies to fully realize their benefits and extend their availability in the market.

Kalumpit (*Terminalia microcarpa* Decne.) is one of the endemic, underutilized, and widely distributed fruit trees in the Philippines. It can be found in low altitude forests (**Department of Agriculture of the Philippines, 1996; Langenberger *et al*., 2009; Coronel, 2011**). **Ragasa *et al*. (2014)** reported that its dichloromethane leaf extract contains squalene, lutein and fatty alcohols that have preventive and therapeutic effects on tumor promotion and regression, and protective effect against inflammatory damage development. *Kalumpit* fruits can be eaten raw and are used in making wine, jam, and jellies (**Sanchez *et al*., 1976; Ungson, 2001**). It is also rich in ascorbic acid, enzymes, bioflavonoid, chromium, potassium, magnesium, B vitamins and amino acids (**De Leon, 2012**). Moreover, **Garcia *et al*. (2005)** reported that *kalumpit* methanolic fruit extract has antioxidant activity comparable to butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), DL-α tocopherol and bignay (*Antidesma bunius* (L) K. Spreng) fruit in terms of lipid peroxidation inhibition and hydroxyl scavenging activity. It was also verified to have a high total phenolic and flavonoid content that are responsible for its high antioxidant activities (**Santiago *et al*., 2007**).

Antioxidants are compounds that can scavenge free-radicals, which if stabilized and accumulated in the body may cause various pathological conditions such as various types of cancer, atherosclerosis, coronary heart disease, rheumatoid arthritis and other degenerative diseases (**Ramis-Ramos, 2003**). Stabilizing free-radicals is done by means of donating a hydrogen atom to the free-radical to decrease its activation energy, and to prevent it from reacting with other compounds, which in turn produces more free radicals (**Pokorny, 2007; Embuscado, 2015; Rodriguez-Amaya, 2015; Shahidi, 2015**). Polyphenols are compounds with significant antioxidant activity. The bioactive polyphenols are anthocyanin, flavonoid, isoflavone, phenolic acid and lignin, which exhibit high levels of antioxidant activity (**Galili and Hovav, 2014**). The extraction of different compounds associated with antioxidant activity may differ through different sources, methods and factors such as the S/S ratio (**Yuan *et al*., 2015**), solvent concentration (**Chen *et al*., 2015**), extraction temperature (**Belwal *et al*., 2016**), extraction time (**Vaji´c *et al*., 2015**) and initial pH (**Sharmila *et al*., 2016**). The extraction of bioactive compounds was reported to be highly influenced by the processing parameters employed. The efficiency of extraction is a function of the characteristic of both solvent (e.g. concentration and pH) and compound (e.g. particle size, ratio to solvent), and extraction time and temperature (**Gertenbach, 2001**; **Cacace and Mazza, 2003**). The extraction process also depends on the diffusion coefficient or how fast the compound will dissolve and reach equilibrium concentration in the solvent, which is influenced by the concentration of the solute, time and temperature (**Cacace and Mazza, 2003**). Moreover, the denaturation of plant membrane tissues was earlier reported to occur at 55-75 °C and cannot be increased indefinitely (**Schwartzberg and Chao, 1982**).

Research on antioxidant extraction were conducted on mangosteen hull (**Cheok *et al*., 2012**), blueberry pomace wine (**He *et al*., 2016**), pomegranate husk (**Amyrgialakia *et al*., 2014**), and fruits of peach (**Mokrani and Madani, 2016**), apple (**Alberti *et al*., 2014**) and pear (**Guan *et al*., 2015**) to find natural sources to develop food supplements and functional food products (**Silva *et al*., 2007**). This research on *kalumpit* is vital to establish baseline data prior to the utilization of this crop and maximize its potential. Aside from food products that can be developed from *kalumpit*, high value ingredients, such as antioxidants and colorants, can also be extracted for use in various industries.

In order to maximize antioxidant extraction, the optimization of various parameters and conditions that can affect the extraction process should be performed. The traditional way of screening variables is the "one-variable-at-a time' concept that has been shown to work well when applied to process designs, especially in cases where a small number of variables are being studied. However, this may become a problem when applied to an experiment that needs to consider a large number of variables. This traditional method is time consuming, expensive and does not allow the understanding of the interactions between the factors considered (**Weuster-Botz, 2000**). Response surface methodology (RSM) can be a better alternative due to its efficiency and practicality. It utilizes mathematical and statistical techniques in the creation of models for the analysis in which the response of concern can be affected by the numbers of known variables. In a way, those variables are used to attain the goal of optimal results (**Montgomery, 2013**).

This study fundamentally aimed to optimize the extraction protocol for antioxidants from *kalumpit* fruit. Specifically, this study intended to screen factors (temperature, time, S/S ratio, initial pH and ethanol concentration) that significantly affect the antioxidant activity of *kalumpit* extract using 2-level factorial experimental design; optimize levels of significant factors that affect DPPH and ABTS radical scavenging activities using Box-Behnken design of experiment; and validate the optimized extraction parameters generated.

MATERIAL AND METHODS

Sample Preparation

Mature *kalumpit* fruits were obtained from the Fruit Crops Nursery, Institute of Crop Science, College of Agriculture and Food Science, University of the Philippines Los Baños. Fruits were immediately washed, cut to obtain the flesh, dried at 50 °C for 72 hours, ground, and passed through a 40-mesh sieve. Samples were then stored in an amber or foil-covered bottle under freezing temperature until use.

Chemicals

Absolute ACS/USP Grade Ethyl alcohol (PHARMCO-AAPER, Connecticut, USA), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), and 2,2-azino-bis-3-ethylbenzothiazoline 6-sulfonic acid (ABTS) (Sigma Aldrich Pte. Ltd, Singapore, Singapore) were used in this study.

Screening of Factors for Antioxidant Extraction

A randomized 2-level factorial experimental design was generated using Design Expert version 10.0.3 (Stat-Ease, Inc., Minnesota, USA) to screen which among the considered factors significantly affect antioxidant extraction from *kalumpit*. Varying levels of temperature (25, 52.5, 80 °C), extraction time (30, 60, 90 min), S/S ratio (10, 30, 50 mL g^{-1}), pH (3, 5.5, 8) and % ethanol (20, 60, 100%) were considered based on previous studies of **Chew *et al.* (2011), Vaji´c *et al.* (2015) and Belwal *et al.* (2016)**. Table 1 shows the different factors considered, in uncoded levels, consisting of 35 experimental points, including 3 replicates of the center points.

Extraction of antioxidants from *kalumpit* powder was conducted according to **Belwal *et al.* (2016)**, with minor modifications. Approximately 1 g of *kalumpit* powder was weighed into 50-mL centrifuge tubes, added with ethanol (varying amounts and concentration), and adjusted to its corresponding initial pH. The mixture was then thoroughly mixed using a vortex mixer for 2 minutes and incubated in water bath (varying temperature and time). Samples were then filtered through Whatman no. 2 filter paper and the collected extract was analyzed for its DPPH radical scavenging activity.

Optimization and Validation of Significant Factors for Antioxidant Extraction

After identifying the significant factors that influence antioxidant extraction, Box-Behnken Design (BBD) was employed to establish the optimal values of these factors, based on the maximum percentage of DPPH radical scavenging activity of *kalumpit* fruit extract. Design Expert version 10.0.3 was used to generate the model and analyze the results of the experiment. Table 2 shows the different factors, in uncoded levels, to determine the optimal levels of identified significant factors for maximum antioxidant extraction. Evaluation of % DPPH was conducted. At the same time, an additional assessment by ABTS radical scavenging activities was carried out as a complementary test. The desirability function of Design Expert version 10.0.3, which is a unique and powerful multi-response (multivariate) optimization tool, was used to determine the optimum conditions for extraction of *kalumpit* with optimum of % DPPH and ABTS radical scavenging activities. To validate the predictive ability and accuracy of the models developed, three replications at optimum conditions were carried out and compared with the predicted values.

Analysis of Antioxidant Property

The 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay, according to **Zhu *et al.* (2002)**, was used to determine the radical scavenging activity of *kalumpit* extract. Particularly, 1 mM solution of DPPH was prepared in absolute ethanol. A 1 mL aliquot of diluted extract (dilution factor = 30) in 52% ethanol was mixed with 4 mL distilled water and 1 mL of the freshly prepared DPPH ethanolic solution. The mixture was placed in a dark room for 30 minutes. The absorbance was measured at 517 nm against a blank using UV-VIS spectrophotometer Shimadzu UVmini-1240 (Kyoto, Japan). All measurements were carried out in triplicate. The % DPPH radical scavenging activity was calculated using equation 1:

$$\%\text{ DPPH radical scavenging activity} = \frac{A_0 - A_t}{A_0} \times 100\%$$ **Equation 1**

where A_0 represents the absorbance of control reference at t = 0 min while A_t represents the absorbance of the samples.

The ABTS (2,2-azino-bis-3-ethylbenzothiazoline 6-sulfonic acid) free radical scavenging activity of the extract was measured using the method described by **Re *et al.* (1999)**. The ABTS stock solution was prepared by mixing 7 mM ABTS with 2.45 mM potassium persulfate and incubated for 16 hours in the dark at 30 °C. An aliquot of the stock solution was diluted with methanol to adjust the absorbance to 0.70 ± 0.02 at 734 nm. A 20 µL aliquot of diluted extract (dilution factor = 30) was added with 980 µL ABTS reagent. The absorbance of the resulting solution was read at 734 nm using a UV-VIS spectrophotometer Shimadzu UVmini-1240 (Kyoto, Japan) after 1 minute. The antioxidant activity of the extract was calculated against a calibration curve (0, 5, 10, 15, 20, 25, 30, 35, 40 µM) established with Trolox (R^2 = 0.998), and expressed as mg Trolox equivalent per 100 g purée. Results were expressed as % ABTS scavenging activity using equation 2:

$$\%\text{ABTS Scavenging Activity} = \left(1 - \frac{\text{Absorbance}_{sample}}{\text{Absorbance}_{blank}}\right) x\ 100$$

Equation 2

Statistical data analysis

All statistical analyses were performed using Design Expert version 10.0.3 software (Stat-Ease, Inc., Minnesota, USA) to analyze the experimental data and the response surface model for screening and optimization of significant factors affecting % DPPH and ABTS radical scavenging activities. Design Expert version 10.0.3 software was also used to determine the Analysis of Variance (ANOVA) and coefficient of determination (R^2), and to evaluate the goodness of fit of the model. The validity of the model was determined by comparing the observed experimental and predicted values for the response variables with t-test analysis using Minitab version 18.1 (Minitab Pty Ltd, Sydney, Australia).

RESULTS AND DISCUSSION

Screening of Factors for Antioxidant Extraction

A 2-level factorial experimental design, with three-level factor, was employed to screen the extraction factors that significantly affects DPPH radical scavenging activities of *kalumpit* extract. Table 1 shows the DPPH radical scavenging activities of *kalumpit* extracted using different extraction conditions. The model as described in Equation 3, achieved an R^2 value of 0.8671, indicating that 86.71 % of the proportion of the variability in the response can be explained by the model. Moreover, the high value of R^2 (values > 0.70) is indicative that the model is fitting the data very well. A standard deviation value of 3.06 apart from the fitted values, which is only 4.62 % of the mean (66.08%), signifies a precise description of the response. Nevertheless, the model assumption cannot be validated completely through this value, hence the need for further statistical analyses.

DPPH Scavenging Activity = +66.08 +1.96A +0.40B -2.15C -0.11D -1.97E +0.60AC +0.29AD +0.64AE -0.96BC +0.66BD -0.05BE -0.49CD -1.41CE -0.29DE -0.43ACD -0.17ACE +0.75ADE -1.64BCE +1.13BDE -0.58CDE +1.62ACDE **Equation 3**

Where A, B, C, D, and E denote temperature, time, S/S ratio, pH and % ethanol concentration, respectively.

Table 2 shows the analysis of variance (ANOVA), model summary, coefficient estimate and the effects of the selected factorial model for screening of factors that significantly affect the antioxidant activity of the *kalumpit* extract. The F-value of 4.04 implies that the model is significant and there is only a 0.63 % chance that an F-value this large could occur due to noise ($p < 0.05$). The "Lack of Fit F-value" of 0.84 implies the Lack of Fit is not significant relative to the pure error and that there is a 65.93 % chance that a "Lack of Fit F-value" this large could occur due to noise ($p > 0.05$). The non-significant lack of fit indicates that the model fits and the "Adeq Precision" of 8.985 value, which is greater than 4, is suggestive of an adequate signal. Therefore, the model generated can be used to navigate the design space.

A significant effect ($p < 0.05$) on the % DPPH radical scavenging activity of *kalumpit* extract was exhibited by temperature, S/S ratio, % ethanol concentration, two-factorial interaction of S/S ratio and % ethanol concentration,

three-factorial interactions of time, S/S ratio and % ethanol concentration, and four-factorial interaction of temperature, S/S ratio, pH and % ethanol concentration. The effect of individual factors, presented in Table 2, describes that the greater degree of effect is signified by a higher value. Temperature had the highest positive effect value among all the factors and interactions with 13% contribution. The antioxidant activity of *kalumpit* extract increased when the temperature was increased because heat enhances the diffusivity of extraction solvents into plant cells and stimulates higher solubility of phenolic compounds in the extraction solvent **(Chew *et al.*, 2011)**. Similarly, the four-factorial interaction of temperature, S/S ratio, pH and % ethanol concentration had significant positive effect on % DPPH scavenging activities of *kalumpit* extract with 9.19% contribution. In contrast, S/S ratio and % ethanol had a high degree of negative effect with 16.19 and 13.55% contribution, respectively. The observed decrease in the antioxidant activity of *kalumpit* extract, when the S/S ratio was increased, may be due to the limited amount of extractable phenolic compounds **(Rezaei *et al.*, 2013)**. On the other hand, the observed negative effect of ethanol concentration is similar to the result reported by **Li *et al.* (2019)**, wherein, an increase in the ethanol concentration from 20% to 40% increased the antioxidant activity of *Gordonia axillaris* extract and gradually decreased when the concentration was increased further to 80%. Increasing the volume of ethanol in water increases its polarity. Thus, in the case of *kalumpit* extract, it is possible that most of the phenolic compounds present are moderately polar. Furthermore, in the extraction of bioactive components, the use of a binary solvent system is found to be more favorable since water and alcohol can work in synergy. Water can swell the plant matrix, thereby increasing the contact surface area between the solvent and the plant matrix **(Alara *et al.*, 2017)**.
The two-factorial interaction of S/S ratio and % ethanol concentration, three-factorial interaction time, S/S ratio and % ethanol concentration also had significant negative effects with 6.9 and 9.47% contribution, respectively. With this, temperature, S/S ratio and % ethanol concentration were the significant factors considered for the optimization of antioxidant extraction from *kalumpit*. Similarly, **Belwal *et al.* (2016)** reported that the effects of extraction temperature, S/S ratio, and solvent concentration significantly affect the antioxidant activities of *Berberis asiatica* fruits using ABTS, FRAP and DPPH Assays. Moreover, the same variables were optimized by **Karacabey and Mazza (2010)** and **Ilaiyaraja *et al.* (2015)** on the extraction of phenolic compounds with high antioxidant activity from grape cane and wood apple, respectively.

Response surface model of % DPPH radical scavenging activity of *Kalumpit* Extract

Optimization of the extraction process was carried out by applying the Box-Behnken design as shown in the experimental data in Table 3. Table 4 shows the model for % DPPH radical scavenging activity with an R^2 of 0.9147 which indicates that 91.47% of the proportion of variability in the response can be attributed to the model. Design Expert version 10.0.3 suggested a quadratic model represented by Equation 4. The Model F-value of 39.34 implies that the model was significant ($p < 0.05$) with a 0.01% chance that an F-value this large could occur due to noise. The "Lack of Fit F-value" of 0.77 implies that the Lack of Fit was not significant ($p > 0.05$) relative to the pure error and there was a 70.87% chance that a "Lack of Fit F-value" this large could occur due to noise. The model's Adeq precision of 21.031 indicates an adequate signal to noise ratio and that the model can be used to navigate the design space.

$$\% \text{ DPPH Scavenging Activity} = +62.69 + 1.15A - 3.70B - 7.23C + 0.35AB - 0.35AC - 5.32BC + 0.13A^2 - 0.87B^2 - 6.89C^2 \quad \textbf{Equation 4}$$

Where A, B, and C denote temperature, S/S ratio and % ethanol concentration, respectively.

Table 1 2-level factorial experimental design for the screening of factors affecting the antioxidant activity of kalumpit extract

Standard Order	Run Order	Temperature (°C)	Time (min)	Solvent to Sample Ratio (mL·g-1)	pH	Ethanol Concentration (%)	DPPH Radical Scavenging Activity (%)
35	1	52.5	60	30	5.5	60	74.07
28	2	80	90	10	8	100	73.88
21	3	25	30	50	3	100	65.5
10	4	80	30	10	8	20	71.64
27	5	25	90	10	8	100	70.18
32	6	80	90	50	8	100	66.18
19	7	25	90	10	3	100	64.81
18	8	80	30	10	3	100	65.79
8	9	80	90	50	3	20	72.51
15	10	25	90	50	8	20	68.03
2	11	80	30	10	3	20	68.13
1	12	25	30	10	3	20	67.64
3	13	25	90	10	3	20	71.05
20	14	80	90	10	3	100	72.03
22	15	80	30	50	3	100	67.15
13	16	25	30	50	8	20	67.15
9	17	25	30	10	8	20	67.06
14	18	80	30	50	8	20	65.5
11	19	25	90	10	8	20	65.6
16	20	80	90	50	8	20	68.03
33	21	52.5	60	30	5.5	60	67.74
12	22	80	90	10	8	20	69.59
23	23	25	90	50	3	100	56.14
26	24	80	30	10	8	100	66.08
29	25	25	30	50	8	100	55.26
17	26	25	30	10	3	100	63.74
7	27	25	90	50	3	20	63.65
34	28	52.5	60	30	5.5	60	69.4
5	29	25	30	50	3	20	60.43
6	30	80	30	50	3	20	69.98
30	31	80	30	50	8	100	61.7
24	32	80	90	50	3	100	57.7
4	33	80	90	10	3	20	66.37
25	34	25	30	10	8	100	61.7
31	35	25	90	50	8	100	51.56

Table 2 Analysis of variance, model summary, and estimated effects of selected factorial model affecting antioxidant activity of *kalumpit* extract

Source/Factor	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	Coefficient Estimate	Standardized Effect	% Contribution
Model	792.59	21	37.74	4.4	0.0063			
A-Temperature	123.12	1	123.12	13.18	0.0030	1.96	3.92	13.47
B-Time	5.17	1	5.17	0.55	0.4700	0.4	0.80	0.57
C- Solvent to Sample Ratio	147.97	1	147.97	15.84	0.0016	-2.15	-4.30	16.19
D-pH	0.38	1	0.38	0.041	0.8423	-0.11	-0.22	0.042
E- Ethanol Conc.	123.89	1	123.89	13.26	0.0030	-1.97	-3.94	13.55
AC	11.64	1	11.64	1.25	0.2845	0.6	1.21	1.27
AD	2.74	1	2.74	0.29	0.5975	0.29	0.59	0.3
AE	13.9	1	13.9	1.4	0.2577	0.64	1.28	1.43
BC	29.27	1	29.27	3.13	0.1001	-0.96	-1.91	3.2
BD	13.85	1	13.85	1.48	0.2450	0.66	1.32	1.52
BE	0.096	1	0.096	0.01	0.9207	-0.055	-0.11	0.011
CD	7.79	1	7.79	0.83	0.3777	-0.49	-0.99	0.85
CE	63.91	1	63.91	6.84	0.0214	-1.41	-2.83	6.99
DE	2.62	1	2.62	0.28	0.6051	-0.29	-0.57	0.29
ACD	5.99	1	5.99	0.64	0.4378	-0.43	-0.87	0.65
ACE	0.93	1	0.93	0.1	0.7572	-0.17	-0.34	0.1
ADE	17.96	1	17.96	1.92	0.1888	0.75	1.50	1.97
BCE	86.57	1	86.57	9.27	0.0094	-1.64	-3.29	9.47
BDE	40.64	1	40.64	4.35	0.0573	1.13	2.25	4.45
CDE	10.94	1	10.94	1.17	0.2988	-0.58	-1.17	1.2
ACDE	84.02	1	84.02	8.99	0.0103	1.62	3.24	9.19
Residual	121.43	13	9.34					
Lack of Fit	99.84	11	9.8	0.84	0.6593			
Pure Error	21.59	2	10.79					
Cor Total	914.02	34						
Model Summary								
Std. Dev.	3.6			R-Squared		0.8671	Adeq Precision	8.985
Mean	66.08			Adj R-Squared		0.6525	BIC	221.08
C.V. %	4.62			Pred R-Squared		0.4266	AICc	271.2
PRESS	524.13			-2 Log Likelihood		142.87		

Table 4 shows that the linear terms of temperature, S/S ratio, ethanol concentration, interactive effect of S/S ratio, ethanol concentration, and quadratic effect of ethanol concentration were found to significantly affect the % DPPH radical scavenging activity of the *kalumpit* extract ($p < 0.05$). The significant linear effects of extraction temperature and ethanol concentration on the % DPPH radical scavenging activity are consistent with the reports of **Belwal *et al*. (2016)**, **Ilaiyaraja *et al.* (2015)**, **Karacabey and Mazza (2010)**, and **Li *et al*. (2012)** on the antioxidant extraction from *Berberis asiatica* fruits, wood apple, grape, and tomato, respectively. On the other hand, the quadratic effect of ethanol concentration agrees with the report of **Belwal *et al*. (2016)** on the significant quadratic effect of solvent on FRAP activity of *Berberis asiatica* fruit extract.

Positive values of coefficients indicate a positive effect on the response variable while negative coefficients indicate the opposite. Higher coefficient values indicate greater contribution to the response. In this regard, extraction temperature had a significant positive effect on the % DPPH radical scavenging activity of *kalumpit* extract ($p < 0.05$), demonstrated by its coefficient estimate of 1.15 (Table 4) and as shown in Figure 1A. It can be observed that high level of temperature (80 °C) resulted in high % DPPH scavenging activity. This can be attributed to the higher diffusion coefficient of phenolic compounds, resulting in high solubility and extraction rate (**Ju and Howard, 2003; Pompeu *et al.*, 2009; Piovesan *et al*., 2017**). This result agrees with several studies that reported on the positive effect of increasing extraction temperature on antioxidant activity (**Vatai *et al.* 2009; Li *et al*., 2012; Benmeziane *et al*. 2013; Piovesan *et al*., 2017**).

On the other hand, linear effect of S/S ratio was found to have significant negative effect ($p < 0.05$) on % DPPH scavenging activity of *kalumpit* extract as shown in Table 4 and Figure 1B. It can be observed that low level S/S ratio (10 mL/g) resulted in high % DPPH scavenging activity. The significant negative effect of S/S ratio in this study is consistent with the findings of **Kemerli-Kalbaran and Ozdemir (2019)** on the optimization of DPPH scavenging activities of lipophilic and hydrophilic phase, and oil of pine nut.

The linear and quadratic effects of ethanol concentration were observed to have significant negative effects on % DPPH scavenging activity of *kalumpit* extract as shown in Table 4 and Figure 1C. In this regard, ethanol concentration near the midpoint level resulted in high % DPPH scavenging activity. Among interactive effects, only the interaction of S/S ratio and ethanol concentration was found to have significant negative effects as shown in Table 4. Figure 2C shows that high % DPPH scavenging activity can be observed in low-level S/S ratio and midpoint-level of ethanol concentration. The significant negative effect of ethanol concentration, in quadratic term, is consistent with the report of **Lee *et al.* (2016)** on % DPPH scavenging activity of Korean red ginseng. Increasing ethanol concentration up to the midpoint level results in reduced dielectric constant of the solution and energy needed to separate solvent molecules, allowing solutes to easily dissolve in the solvent (**Cacace and Mazza, 2002, 2003; Pompeu *et al.*, 2009**)

The interaction of temperature and S/S ratio had a positive effect while the interaction of S/S ratio and ethanol concentration showed negative effect as shown in Table 4. However, both effects were not significant. Figure 2A shows that high % DPPH scavenging activity can be observed in high-level extraction temperature and low-level S/S ratio. Moreover, Figure 2B shows that a high % DPPH scavenging activity can be observed in high-level extraction temperature and midpoint-level of ethanol concentration.

Table 3 Box-Behnken design for optimization of DPPH and ABTS scavenging activities of *kalumpit* extract

Standard Order	Block	Run Order	Temperature (°C)	Solvent to Sample Ratio (mL·g^{-1})	Ethanol Concentration (%)	DPPH Radical Scavenging Activity (%)	ABTS Scavenging Activity (%)
7	1	1	25	30	100	53.08	2.50
1	1	2	25	10	60	62.31	66.43
6	1	3	80	30	20	65.38	52.86
9	1	4	52.5	10	20	59.62	79.64
13	1	5	52.5	30	60	63.08	76.43
10	1	6	52.5	50	20	62.31	21.79
11	1	7	52.5	10	100	61.54	26.43
5	1	8	25	30	20	62.69	5.36
2	1	9	80	10	60	65.38	78.93
12	1	10	52.5	50	100	38.08	5.71
14	1	11	52.5	30	60	55.00	56.79
15	1	12	52.5	30	60	62.31	34.64
8	1	13	80	30	100	46.54	13.57
4	1	14	80	50	60	60.77	41.43
3	1	15	25	50	60	57.69	2.50
31	2	16	25	10	60	63.46	77.14
44	2	17	52.5	30	60	63.46	76.79
43	2	18	52.5	30	60	62.31	50.71
33	2	19	25	50	60	58.08	8.93
38	2	20	80	30	100	49.23	31.43
41	2	21	52.5	10	100	56.92	30.36
40	2	22	52.5	50	20	60.77	17.14
42	2	23	52.5	50	100	39.23	4.29
35	2	24	25	30	20	62.69	19.64
36	2	25	80	30	20	63.85	46.43
45	2	26	52.5	30	60	63.08	41.79
34	2	27	80	50	60	59.62	23.57
39	2	28	52.5	10	20	61.15	79.64
32	2	29	80	10	60	65.77	79.29
37	2	30	25	30	100	43.85	3.57
21	3	31	80	30	20	67.69	34.64
26	3	32	52.5	10	100	58.46	11.43
18	3	33	25	50	60	57.69	18.93
19	3	34	80	50	60	62.69	30.71
28	3	35	52.5	30	60	67.69	70.71
30	3	36	52.5	30	60	64.23	63.57
29	3	37	52.5	30	60	63.08	37.86
25	3	38	52.5	50	20	61.54	15.36
16	3	39	25	10	60	65.00	51.79
17	3	40	80	10	60	65.00	75.36
23	3	41	80	30	100	49.23	27.14
24	3	42	52.5	10	20	61.15	82.14
20	3	43	25	30	20	63.08	4.29
27	3	44	52.5	50	100	38.46	12.14
22	3	45	25	30	100	43.85	5.00

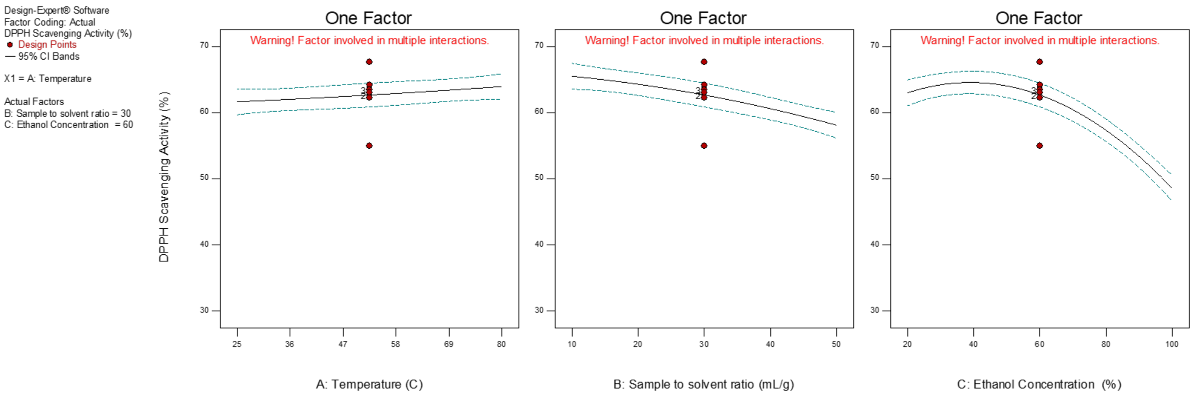

Figure 1 Model graphs for linear effects of extraction temperature (A), S/S ratio (B) and ethanol concentration (C) on % DPPH scavenging activity of *kalumpit* extract

Table 4 Analysis of variance and model summary for response surface quadratic model for % DPPH scavenging activity of *kalumpit* extract

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	Coefficient Estimate
Block	9.18	2	4.59			
Model	2489.7	9	276.63	39.34	< 0.0001	
A-Temperature	31.95	1	31.95	4.54	0.0406	1.15
B-Solvent to Sample Ratio	328.9	1	328.9	46.78	< 0.0001	-3.7
C-Ethanol Concentration	1253.7	1	1253.7	178.3	< 0.0001	-7.23
AB	1.49	1	1.49	0.21	0.6481	0.35
AC	1.49	1	1.49	0.21	0.6481	-0.35
BC	339.69	1	339.69	48.31	< 0.0001	-5.32
A^2	0.18	1	0.18	0.026	0.8731	0.13
B^2	8.3	1	8.3	1.18	0.2853	-0.87
C^2	526	1	526	74.81	< 0.0001	-6.89
Residual	232.03	33	7.3			
Lack of Fit	180.06	27	6.67	0.77	0.7087	
Pure Error	51.97	6	8.66			
Cor Total	2730.91	44				
Model Summary						
Std. Dev.	2.65			R-Squared		0.9147
Mean	58.62			Adj R-Squared		0.8915
C.V. %	4.52			Pred R-Squared		0.8461
PRESS	418.88			Adeq Precision		21.031
-2 Log Likelihood	201.51			BIC		247.19
				AICc		235.26

Table 5 Analysis of variance and model summary for response surface quadratic model for % ABTS scavenging activity of *kalumpit* extract

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	Coefficient Estimate
Block	82.18	2	41.09			
Model	28255.97	9	3139.55	23.23	< 0.0001	
A-Temperature	3021.45	1	3021.45	22.36	< 0.0001	11.22
B- Solvent to sample ratio	11973.86	1	11973.86	88.61	< 0.0001	-22.34
C-Ethanol Concentration	3392.86	1	3392.86	25.11	< 0.0001	-11.89
AB	61.39	1	61.39	0.45	0.505	2.26
AC	158.21	1	158.21	1.17	0.2871	-3.63
BC	1658.43	1	1658.43	12.27	0.0013	11.76
A^2	1337.17	1	1337.17	9.9	0.0035	-10.99
B^2	4.68	1	4.68	3.50E-02	0.8535	0.65
C^2	6958.84	1	6958.84	51.5	< 0.0001	-25.06
Residual	4459.16	33	135.13			
Lack of Fit	2326.34	27	86.16	0.24	0.9954	
Pure Error	2132.82	6	355.47			
Cor Total	32797.32	44				
Model Summary						
Std. Dev.	11.62			R-Squared		0.8637
Mean	37.71			Adj R-Squared		0.8265
C.V. %	30.83			Pred R-Squared		0.7663
PRESS	7646.64			Adeq Precision		13.395
-2 Log Likelihood	334.53			BIC		380.21
				AICc		368.28

Response surface model of % ABTS radical scavenging activity of *Kalumpit* Extract

Table 5 shows the ANOVA table for the special quadratic model for % ABTS radical scavenging activity with an R^2 of 0.8637. The Model F-value of 23.23 implies that the model is significant ($p < 0.05$) and there is only a 0.01% chance that an F-value this large could occur due to noise. The "Lack of Fit F-value" of 0.24 implies that the Lack of Fit is not significant ($p > 0.05$) relative to the pure error and there is a 99.54% chance that a "Lack of Fit F-value" this large could occur due to noise. Moreover, the model's "Adeq precision" of 13.395 indicates an adequate signal to noise ratio and that the model can be used to navigate the design space.

Equation 5 and Table 5 describe the special quadratic model for % ABTS scavenging activity of *kalumpit* extract. The linear effects of temperature, S/S ratio, ethanol concentration, interactive effect of S/S ratio and ethanol concentration, and quadratic effects of extraction temperature and ethanol concentration were found to significantly influence the % ABTS scavenging activity of *kalumpit* extract ($p < 0.05$). Moreover, the linear effect of extraction temperature and interactive effect of S/S ratio and ethanol concentration on % ABTS scavenging activity of *kalumpit* extract were observed to be significantly positive as evidenced by their coefficient estimate values of 11.22 and 11.76, respectively. Figure 3A shows that a high % ABTS scavenging activity of *kalumpit* extract can be observed in midpoint level of extraction temperature. The positive effect of temperature can be attributed to the reduced viscosity and surface tension of the solvent, resulting in higher phenolic solubility, extraction and diffusion rate, and antioxidant activity (**Ju and Howard, 2003; Piovesan *et al.*, 2017**). This was also found to be consistent with **Lee *et al.* (2016)** report on the significant positive effect of extraction temperature on the % DPPH scavenging activity of Korean red ginseng extract. Figure 4C show that a high % ABTS scavenging activity of *kalumpit* extract can be observed in low-level S/S ratio and midpoint-level of ethanol concentration. Although insignificant, the interaction between temperature and S/S ratio also have positive effects as shown in Figure 4A and confirmed by a coefficient estimate of 2.26 (Table 5). A medium level % ABTS scavenging activity of *kalumpit* extract can be observed at midpoint-level of extraction temperature and low-level S/S ratio.

On the other hand, the linear terms of S/S ratio and ethanol concentration, and the quadratic terms of extraction temperature and ethanol concentration, were found to have significant negative effects on the % ABTS scavenging activity of *kalumpit* extract. In this regard, a high % ABTS scavenging activity of *kalumpit* extract can be observed at midpoint level of extraction temperature (Figure 3A), low-level S/S ratio (Figure 3B) and midpoint-level of ethanol concentration (Figure 3C). The linear significant negative effects of S/S ratio and ethanol concentration agrees with **Cheok *et al.* (2012)** report on the negative effects of S/S ratio and methanol concentration on total phenolic content of *Garcinia mangostana* Linn. hull. On the other hand, the significant quadratic effect of the ethanol concentration on % ABTS scavenging activity of *kalumpit* extract is consistent with **Lee *et al.* (2016)** and **Liyana-Pathirana and Shahidi (2005)** report on the significant negative effect of ethanol concentration quadratic term on antioxidant activity of Korean red ginseng and hard wheat, respectively.

Interaction between extraction temperature and ethanol concentration also had an insignificant negative effect on the % ABTS scavenging activity of *kalumpit* extract, as demonstrated by its coefficient estimate of -3.63. An intermediate % ABTS scavenging activity of *kalumpit* extract can be observed at midpoint level of extraction temperature and ethanol concentration as shown in Figure 4B.

% ABTS Scavenging Activity= +56.59 +11.22A -22.34B -11.89C +2.26AB -3.63AC +11.76C -10.99A^2 +0.65B^2 -25.06C^2 **Equation 5**

Where A, B, and C denote temperature, S/S ratio and % ethanol concentration, respectively.

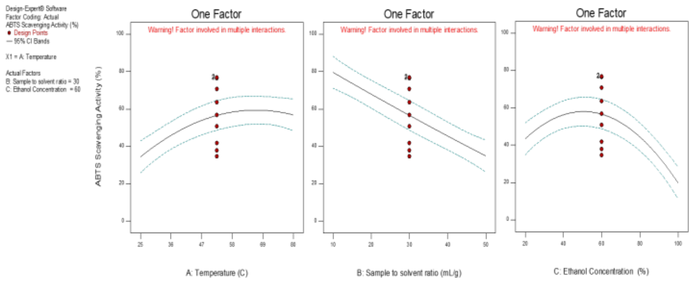

Figure 3 Model graphs for linear effects of extraction temperature (A), solvent to sample ratio (B) and ethanol concentration (C) on % ABTS scavenging activity of *kalumpit* extract

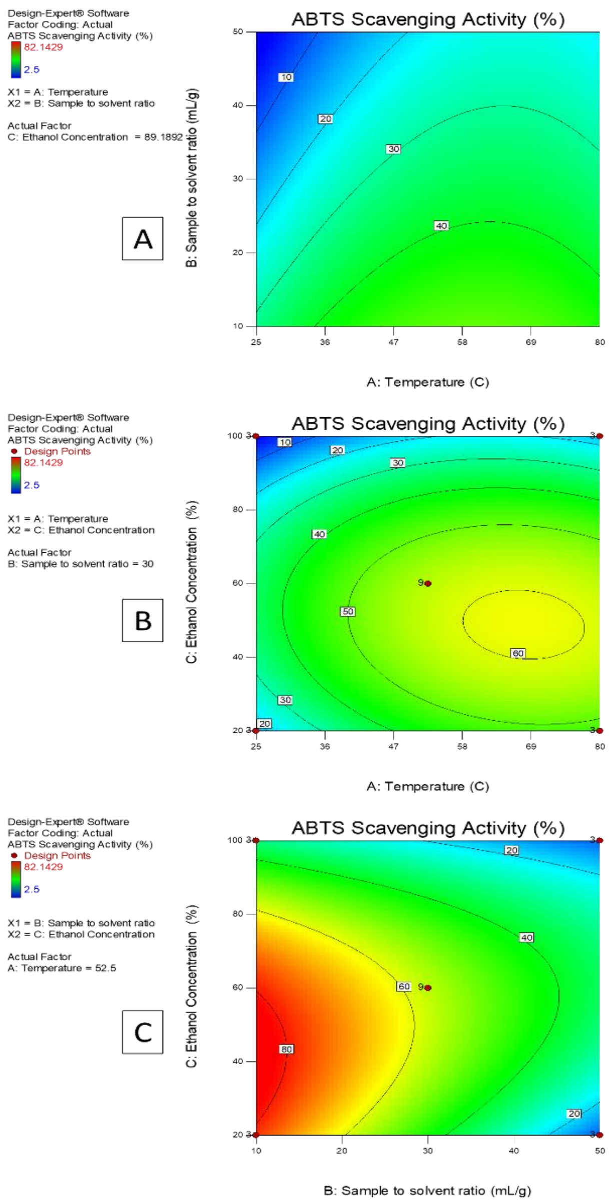

Figure 4 Contour plots for interaction effects of extraction temperature and solvent to sample ratio (A), temperature and ethanol concentration (B), and solvent to sample ratio and ethanol concentration (C) on % ABTS scavenging activity of *kalumpit* extract

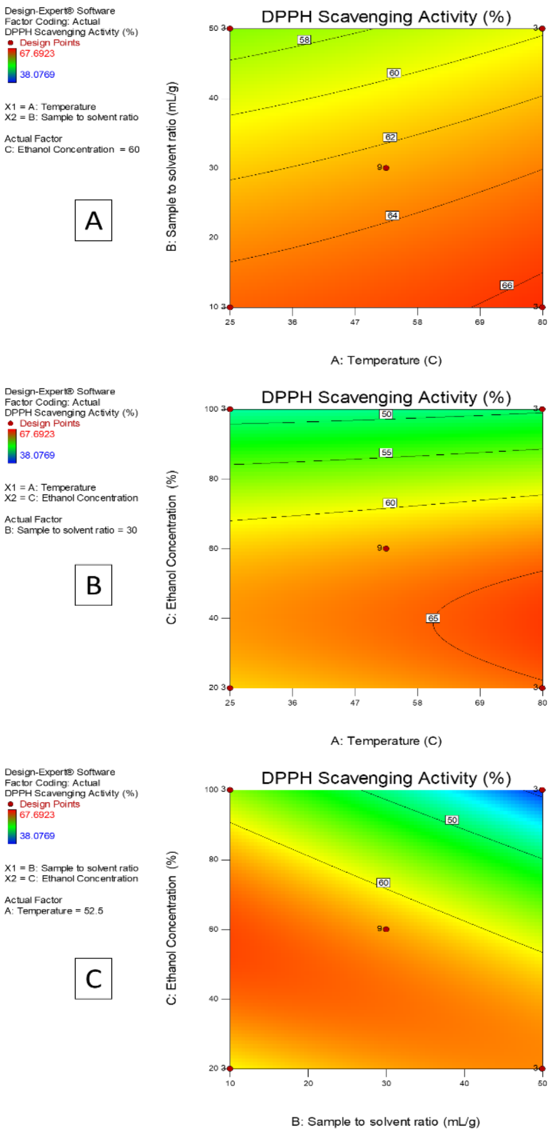

Figure 2 Contour plots for interaction effects of extraction temperature and solvent to sample ratio (A), temperature and ethanol concentration (B), and solvent to sample ratio and ethanol concentration (C) on % DPPH scavenging activity of *kalumpit* extract

Optimization and validation of extraction models developed

Ultimately, the models for % DPPH and ABTS radical scavenging activities of *kalumpit* fruit extract were confirmed to adequately fit the response surface, hence, no evidence supports the claim that the model does not adequately explain the variation in the response. The composite desirability of 0.977 is indicative that the settings for the factors achieved favorable results for all responses as a whole and the goal for optimization was satisfied. A desirability function approach as a numerical optimization technique was used to determine the optimum extraction condition where the highest % DPPH and ABTS radical scavenging activities of *kalumpit* fruit extract were attained. Constraints were defined as the lower and upper limit range of extraction temperature, S/S ratio and ethanol concentration to attain maximum % DPPH and ABTS radical scavenging activities and desirability. Based on the numerical optimization with the desirability function and the constraints given above, the optimum condition of extraction was attained at the extraction temperature of 80.0 °C, S/S ratio of 10 mL g^{-1} min and ethanol concentration of 51.66%, which resulted in the maximum desirability value of 0.977, and predicted % DPPH and ABTS radical scavenging activities of 66.63 and 82.14, respectively.

The adequacy of the predictive models at the optimum condition was validated by performing 3 independent experiments while the experimental data were compared with the predicted values (Table 6). No significant difference was found between experimental data and predicted values ($p > 0.05$) indicating that the models developed were adequate to describe the relationship between the factors and responses. Moreover, less than 5% error was observed for both response parameters.

Table 6 T-test analysis for validation of the optimized conditions for extraction of antioxidants from *kalumpit*.

Response Parameter	Predicted	Experimental	t- Value	p-Value	% Error
% DPPH scavenging activity	66.63±2.65	67.66±2.05	-0.53	0.632	1.54
% ABTS scavenging activity	82.10±11.60	78.98±6.57	0.41	0.709	-3.80

CONCLUSION

The results showed that temperature, S/S ratio and % ethanol are significant factors that affect the extraction of antioxidants from *kalumpit*. In addition, optimum % DPPH and ABTS radical scavenging activities of *kalumpit* extract were generally obtained at high-level extraction temperature, low-level S/S ratio and midpoint-level ethanol concentration. Predictive models adequately described the % DPPH and ABTS radical scavenging activities of *kalumpit* extract as a function of independent factors. The desirability function approach determined the optimum conditions for the extraction of *kalumpit* resulting in maximum % DPPH and ABTS radical scavenging activities. The optimum conditions for the solvent extraction of antioxidants were attained at the extraction temperature of 80.0 °C, S/S ratio of 10 mL g^{-1} min and ethanol concentration of 51.66% resulting in the maximum desirability value of 0.977, and predicted % DPPH and ABTS radical scavenging activities of 66.63 and 82.14, respectively. The baseline data generated by this research is useful in maximizing the potential of *kalumpit*, through the production of high-value food ingredients from *kalumpit* such as health promoting antioxidants and natural colorants.

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