

## ORIGINAL ARTICLE

# Optimized Aqueous Extraction Conditions for Maximal Phenolics, Flavonoids and Antioxidant Capacity from *Artocarpus heterophyllus* (Jackfruit) Leaves by Response Surface Methodology (RSM)

Nor Hafiza Sayuti<sup>1</sup>, 'Ammar Akram Kamarudin<sup>1</sup>, Nor Asma Ab. Razak<sup>1</sup>, Norazalina Saad<sup>2</sup>, Mohd Sabri Pak Dek<sup>3</sup>, Norhaizan Mohd Esa<sup>1,4,5</sup>

<sup>1</sup> Laboratory of Molecular Biomedicine, Institute of Bioscience, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

<sup>2</sup> UPM-MAKNA Cancer Research Laboratory, Institute of Bioscience, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia,

<sup>3</sup> Department of Food Science, Faculty of Food Science and Technology, Universiti Putra Malaysia, Serdang, Selangor, 43400, Malaysia

<sup>4</sup> Department of Nutrition and Dietetics, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

<sup>5</sup> Research Centre of Excellent, Nutrition and Non-Communicable Diseases (NNCD), Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

## ABSTRACT

**Introduction:** There are numerous studies on the therapeutic properties of *Artocarpus heterophyllus*. However, studies on the aqueous extraction of *A. heterophyllus* leaves are limited. This present study was conducted to optimize the extraction conditions of *A. heterophyllus* leaves to yield the highest phenolic, flavonoids and antioxidant contents.

**Methods:** Response surface methodology (RSM) was employed to obtain a higher phenolic extraction parameter(s) of *A. heterophyllus* leaves using Central Composite Design (CCD). The antioxidant activity was then determined via ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) and DPPH (2,2-Diphenyl-1-picrylhydrazyl) assay and analysis of the individual phenolics was performed by high performance liquid chromatography (HPLC). **Results:** The optimum extraction conditions with higher phenolics content and antioxidant activity was achieved at 81°C, 100 min and 40 mL/g sample with a good desirability value of 0.87. Under these optimized parameters, total phenolics and flavonoids were  $174.48 \pm 4.05$  mg GAE/g sample and  $21.44 \pm 0.05$  mg RE/g sample, respectively. Meanwhile, antioxidant activity via ABTS and DPPH assays were  $90.88\% \pm 0.09$  and  $87.22\% \pm 0.62$ , respectively. Finally, under optimal extraction conditions revealed 4 compounds identified as chlorogenic acid, quercetin, rutin and kaempferol.

**Conclusion:** The optimisation are promising to improve phenolic yield and antioxidant activity in *A. heterophyllus* leaves. It also proved that *A. heterophyllus* leaves can be used as an alternative natural antioxidant especially in medicinal applications since all identified compound possess significant biological activities for human health.

**Keywords:** Natural antioxidants, Polyphenols, Aqueous extraction, *Artocarpus heterophyllus* leaves, Central composite design

## Corresponding Author:

Norhaizan Mohd Esa, PhD

Email: nnhaizan@upm.edu.my

Tel: +603-9769 2427

## INTRODUCTION

A great deal of attention has been focused on antioxidant agents as they are able to protect the cells from excess free radicals that are produced naturally following cell metabolism in the human body (1). Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are synthetic antioxidant agents that are most commonly used in the food industry as food preservatives despite its reported side effects on human health (2).

Notably, according to Taghvaei and Jafari (3), BHA and BHT are potential carcinogens due to its cytotoxicity in the forestomach of rodents and toxicity effect on rat's liver, kidney and lung, respectively. Hence, natural antioxidant agents such as phenolic compounds are a safer alternative to synthetic antioxidants. Phenolic compounds found abundantly in various plants, herbs and fruits have proven to exhibit strong antioxidant activity *in vitro* and *in vivo* (4).

*Artocarpus heterophyllus* (jackfruit) is a tropical evergreen tree. Different parts of *A. heterophyllus* such as leaves, fruits, roots, peel and bark possess various traditional medicinal properties which depend on the chemical composition of each part of the plant

(5). As shown by Baliga *et al.* (6), certain parts of *A. heterophyllum* including leaves and fruits contain useful compound such as phenolics and flavonoids which are known for its antioxidant properties. In an *in vivo* study reported by Shahin *et al.* (7) reveals that the aqueous extract of *A. heterophyllum* leaves has antidiabetic and antihyperlipidemic agents. These parts of *A. heterophyllum* (leaves, seeds and bark) do not cause any cytotoxic effect as shown from several previous *in vivo* and *in vitro* studies (8–12).

Although there are numerous studies on the therapeutic properties of *A. heterophyllum*, studies on the leaf's aqueous extract are limited. Results from our preliminary study showed that *A. heterophyllum* leaves contain highest phenolics with  $50.71 \pm 3.88$  mg GAE/g sample, as compared to fruit, seed, stem and peel with  $8.53 \pm 1.42$ ,  $21.81 \pm 3.44$ ,  $31.31 \pm 2.25$  and  $19.92 \pm 1.80$  mg GAE/g sample, respectively. So, *A. heterophyllum* leaves were chosen for optimisation of extraction parameter by using response surface methodology (RSM). Extraction process is a very crucial step in order to maximise the extraction of the compounds of interest with therapeutic properties from any samples (13). Various factors can affect the extraction process such as extraction time, extraction temperature and extraction volume.

One of the most effective and widely used technique to optimize the extraction process is the RSM (14). RSM can be used to obtain an optimized response by analysing the combinations of independent factors level. The main advantage of RSM is that it requires a lower number of experimental runs as compared with the full factorials design (15), more economical and user-friendly since it can minimize the total number of experimental runs, thus minimizing the cost and time without compromising the data reliability (16). Hot aqueous extraction is used instead of other extraction approaches such as microwave-assisted and ultrasonication extraction technique due to the relatively low cost and simple instruments needed. Moreover, aqueous is more desirable solvent for the food industry due to its non-toxicity for human consumption, environmentally friendly and inexpensive compared to organic solvents (17). To date, there was no study conducted to improve phenolic extraction from *A. heterophyllum* leaves by aqueous. Therefore, this study aimed to optimize the extraction conditions of *A. heterophyllum* leaves to yield the highest phenolic and antioxidant capacity by using RSM.

## MATERIALS AND METHODS

### Raw material and hot aqueous extraction process

*A. heterophyllum* leaves were purchased from a local farmer in Rawang, Selangor, Malaysia. The collected sample *A. heterophyllum* leaves were washed using tap water and oven-dried at 50°C for a maximum of 48 hours. The dried sample was then ground into a powder

form and sifted using a 35 mm sieve. The extraction procedure was modified from previously reported by Al-Manhel and Niamah (18). Briefly, powdered samples (1g) were extracted with deionized water at different parameters (extraction time 11-448 minutes, extraction temperature 5-100°C, extraction volume 13-47 mL/g sample) by using a water bath. The range of extraction parameters used (Table I) according to experimental design generated by Design Expert software version 6.0.4 (Stat Ease Inc., Minneapolis, USA). The extract was then filtered using Whatman No. 1 filter paper. The filtrate was considered as a sample extract and then allowed to dry in the oven (Memmert, Germany) at 50 °C for a maximum of 72 hours and then dry extract was ready to be used for analysis. The extraction yield obtained was calculated as the ratio between the extract mass in dry basis (x) and the mass of initial dry sample (y). The percentage of total yield was calculated as following equation:

$$\text{Extraction yield \% (w/dw)} = (x / y) * 100 \quad [1]$$

### Experimental design and statistical analysis

Design Expert software version 6.0.4 of RSM was used in this study to optimize the experimental results. The selection of CCD was due to its precision for estimating factors that will affect the response thus provide better interaction between the factors. The experimental runs consist of 8 factorial points, 6 axial points and 6 center points as shown in Table I. Center points runs were

**Table I: The experimental data for the five responses based on Central Composite Design matrix**

Run	Type	Time (min)	Temperature (°C)	Volume (mL)	Yield	TPC	TFC	ABTS	DPPH
1	Fact	360	81	20	18	144.12	36.20	88.59	89.32
2	Center	230	53	30	20	129.87	31.49	89.00	91.32
3	Fact	100	24	20	20	101.07	23.45	89.48	93.40
4	Center	230	53	30	22	154.20	26.74	89.41	91.43
5	Fact	100	81	20	26	183.34	43.16	88.53	90.26
6	Fact	360	24	20	20	157.10	31.64	88.05	91.50
7	Fact	360	24	40	20	126.39	29.28	87.58	91.33
8	Fact	360	81	40	20	163.84	35.61	88.39	91.54
9	Fact	100	81	40	19	177.95	38.84	88.39	90.73
10	Center	230	53	30	21	143.41	28.89	88.32	92.17
11	Fact	100	24	40	23	131.28	29.97	89.55	93.06
12	Axial	230	53	13	22	143.91	29.53	89.00	92.47
13	Axial	11	53	30	17	166.18	27.42	89.21	93.49
14	Axial	230	5	30	18	130.57	26.10	87.24	91.06
15	Axial	230	100	30	19	162.06	23.94	91.04	88.43
16	Center	230	53	30	19	137.74	27.96	89.14	91.73
17	Axial	448	53	30	21	130.22	29.43	88.59	92.16
18	Center	230	53	30	21	130.22	27.86	89.68	91.52
19	Axial	230	53	47	23	131.00	28.99	91.65	95.27
20	Center	230	53	30	21	126.89	25.51	91.85	91.73

Yield = % (w/dw), TPC = total phenolic contents (mg GAE/g dw), TFC = total flavonoid contents (mg RE/g dw), ABTS = 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) radical cation inhibition (%), DPPH = 2,2-diphenyl-1-picrylhydrazyl radical scavenging ability assay (%)

repeated 6 times to reduce the error. The data were statistically analyzed to identify the significant main effects as well as the interaction effects between the variables and responses. Analysis of variance (ANOVA) of the experimental results was carried out to determine individual linear, quadratic and interaction regression coefficient using Design Expert software.

### Phytochemical analysis

#### Total phenolics contents (TPC)

Total phenolic content was determined using the method described by Yusri *et al.* (19) with slight modifications. An aliquot of 100  $\mu$ L of the extract prepared as mention earlier was added with 500  $\mu$ L of Folin-Ciocalteu reagent and mixed using a vortex for 10 seconds. Then, 400  $\mu$ L of 7.5 % sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) solution was added, and the mixture was incubated at 40 °C for 1 hour. After 1 hour, 200  $\mu$ L of reaction mixture was loaded into a 96 wells microplate. The standard curve was constructed using gallic acid and TPC was calculated from the standard curve,  $y = 0.0047x + 0.0483$ ,  $R = 0.992$ . The absorbance was then measured at 765 nm using a microplate reader (BioTek Instruments, United States) and the results were expressed in terms of milligram (mg) gallic acid equivalent (GAE)/g of sample.

#### Total flavonoid contents (TFC)

Total flavonoid of all extract was measured by the colorimetric method as previously reported by Yusri *et al.* (19) with modification. One hundred microliter of each extract mixed with 100  $\mu$ L of 10% aluminium chloride ( $\text{AlCl}_3$ ). The solution then incubated for 10 min at room temperature in the dark. The absorbance was measured using a microplate reader at 435 nm. Quantification of TFC was measured based on rutin standard curve ( $y = 0.0068x - 0.0008$ ,  $R = 0.9998$ ). The concentration of flavonoids was expressed in terms of mg rutin equivalent (RE)/g of sample.

### Antioxidant activity (AA)

#### DPPH radical scavenging ability assay

Radical scavenging ability was determined according to Ramadan *et al.* (20) with some modifications. The 2,2-Diphenyl-1-picrylhydrazyl (DPPH) solution was prepared by dissolving 4.2 mg of DPPH in powder form in 50 mL methanol and were shaken vigorously to let the DPPH dissolved completely. The solution was incubated for 2 hours at room temperature in the darkroom before reacted with the extract. A quantity of 50  $\mu$ L extract mixed with 195  $\mu$ L of DPPH solution in 96 wells plate and the plate was kept in the dark for 1 hour. Then, the absorbance of the solution was measured at 540 nm using the microplate reader. The formula for the percentage of DPPH scavenging activity is as follows:

$$\% \text{ DPPH scavenging activity} = (\text{AB}-\text{AA}) / (\text{AB}) \times 100 \text{ [2]}$$

Where AB is the absorbance of control and AA is the absorbance of the sample.

### ABTS radical cation inhibition antioxidant assay

The free radical scavenging activity of extracts was examined by ABTS radical cation inhibition assay (21) with slight modifications. The ABTS cation radical was prepared by the reaction between 76.8 mg of ABTS in 40 mL of deionized water with 13.24 mg of potassium persulfate ( $\text{K}_2\text{S}_2\text{O}_8$ ). The solution was stored for not more than 16 hours in darkness at room temperature prior to use. The ABTS solution was then diluted with deionized water and was ready to be mixed with the extract. The reaction between 0.1 mL of extract in 0.9 mL of diluted ABTS solution and the mixture left for one hour in dark at room temperature. The absorbance of the mixture was measured after one-hour incubation at 735 nm using microplate reader. All the assays were performed in triplicate in order to obtain an accurate reading of absorbance. Percentage of inhibition was calculated by using the same formula as the percentage of DPPH scavenging activity [2].

### Validation of model

This study was aimed to obtain the optimum extraction parameters that gave the highest yield of phenolics, flavonoids and antioxidant activity. All the responses were analyzed under optimized conditions of the extraction (generated by Design Expert software version 6.0.4). In order to verify the validity of the model, the experimental data were statistically compared with predicted values generated by design expert software based on the coefficient of variation (CV) and independent t-test. The independent t-test were performed using IBM SPSS statistics 21 to determine the validity of the model and to check the significant difference between predicted and actual experimental data.

### Statistical analysis

Design Expert software version 6.0.4 was used to design the experiments and to generate the three-dimensional (3D) graphs for independent and dependent variable. Additionally, Design Expert software was also used to study the regression coefficient and analysis of variance (ANOVA) for experimental data as well as to predict the optimal values and extraction conditions of three independent variables. The accuracy of the fitted model was evaluated based on the coefficient of  $R^2$  value. Differences in means were considered statistically significant at  $p < 0.05$ .

### High performance liquid chromatography (HPLC)

HPLC analysis were conducted according to Rodríguez-Pérez *et al.* (22) with modification using Shimadzu HPLC system model LC-20AT equipped with two pumps and Shimadzu SPD-20 AV UV-Vis detector (ultraviolet-visible (UV-Vis) spectrophotometry detector). The column used for the chromatographic separation was a C18 (1.8  $\mu$ m, 15 cm x 4.6 cm) (Agilent Technologies, Palo Alto, CA, USA). The mobile phases were composed of two, solutions A: 0.5% formic acid in deionized water

(v/v) and solution B: acetonitrile. The gradient was programmed as follows: 0 min, 5% B; 10 min, 35% B; 65 min, 95% B; 67 min, 5% B. The detection was carried out at 280 nm and the flow rate were set at 0.50 mL/min throughout the gradient and injection volume in the HPLC system was 10 µL.

**RESULTS**

**Model fitting**

The regression coefficients of dependent variables (extraction time, temperature and volume) are as shown in Table II. The extraction time showed a significant negative linear effect on yield and DPPH activity. Meanwhile, the extraction temperature was found to be significant for all the response variables. However, extraction volume only showed a significant effect on DPPH activity. Additionally, the quadratic effect of extraction time (A<sup>2</sup>) was found to be significant only on yield and TPC. Likewise, the quadratic effect of extraction temperature (B<sup>2</sup>) produced a significant effect on yield, TPC and DPPH activity whereas extraction volume (C<sup>2</sup>) only give a significant effect on DPPH activity.

On the other hand, the interaction between extraction time and temperature (AB) was highly significant with p < 0.001 on TPC and significant p < 0.05 on DPPH activity. Both interactions between extraction time

**Table II: Fitting of the model based on the regression coefficient (β), coefficient of determination (R<sup>2</sup>) and F-test value for yield, total phenolic and flavonoid contents and antioxidant activities**

	Yield	TPC	TFC	ABTS	DPPH
A-Time	-2.55***	5.42	0.05	-0.66	-0.44**
B-Temperature	0.62***	24.54***	7.98**	-0.92*	-0.87***
C-Volume	-0.43	4.77	-0.12	-0.70	0.50**
A <sup>2</sup>	-0.52*	4.77*	1.45	-0.35	0.24
B <sup>2</sup>	-0.47*	4.12*	0.25	-0.25	-0.85***
C <sup>2</sup>	0.77	0.99	1.75	0.26	0.61**
AB	0.030	-13.06***	-2.21	0.52	0.44*
AC	0.65*	-4.48	-0.64	-0.093	0.24
BC	-0.71*	1.85	ND	0.010	0.40
A <sup>3</sup>	1.32***	-5.70**	ND	0.16	ND
B <sup>3</sup>	-0.13	-5.37*	-3.05**	0.81**	ND
C <sup>3</sup>	0.19	-3.04	ND	0.58*	ND
ABC	1.52**	0.54	ND	ND	ND
R <sup>2</sup>	0.98	0.99	0.81	0.93	0.95
F value (model)	13.95**	20.99**	3.78*	5.95*	15.93***
F value (lack of fit)	0.84	0.87	0.38	0.58	0.21

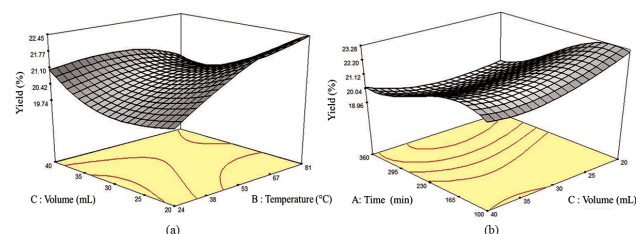
A = Extraction time (min), B = Extraction temperature (C°), C = volume (mL), Yield = % (w/dw), TPC = total phenolic contents (mg GAE/g dw), TFC = total flavonoid contents (mg RE/g dw), ABTS = 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) radical cation inhibition (%), DPPH = 2,2-diphenyl-1-picrylhydrazylradical scavenging ability assay (%), R = Coefficient of determination. ND = not determined, Significance level \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.

and volume (AC) and between extraction temperature and volume (BC) significantly affected the yield of the extract. In contrast, the cubic effect for extraction time (A<sup>3</sup>) only showed a significant effect on yield and TPC, whereas cubic effect for extraction temperature (B<sup>3</sup>) showed a significant effect on TPC, TFC and ABTS activity respectively. Meanwhile, the cubic effect for extraction volume (C<sup>3</sup>) produced no significant effect on all responses.

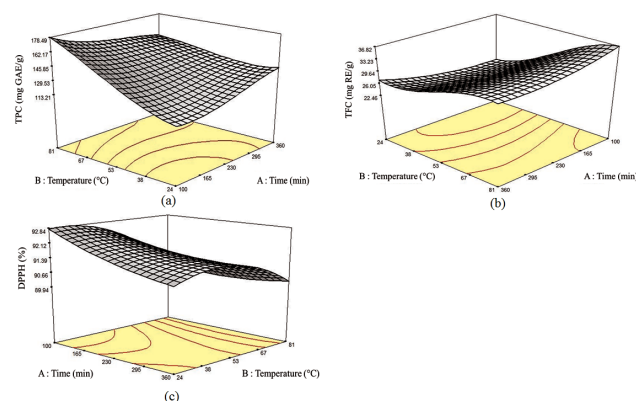
Overall, the lack of fit (F value) for all model term was found statistically non-significant with p > 0.05, indicating that the model fits the response variables well in which the variables have significant effects on responses. The software generates the response surface 3D graph for each response to show the interaction between independent variables on responses (Fig. 1 and Fig. 2).

**Effect of extraction variables on the yield of extract**

The model for the yield of extract showed high significance with the experimental data (p < 0.01) as shown in Table II. In this case A, B, A<sup>2</sup>, B<sup>2</sup>, AC, BC, A<sup>3</sup> and ABC were significant model terms. Using ANOVA, both time and temperature of the extraction fit the linear model, whereas, the extraction time of the first cubic model fits the yield of the extraction with p < 0.001. Meanwhile, the quadratic effects of extraction time and temperature (A<sup>2</sup> and B<sup>2</sup>) were found to be significant yield with p < 0.05. Additionally, the interaction between all variables (ABC) was also statistically significant on the yield of extraction.



**Figure 1. Interaction effect of extraction variables on yield (%) of the aqueous extract**



**Figure 2: Interaction effect of extraction variables on Total Phenolic Contents (TPC)(a), Total Flavonoid Content (TFC)(b) and on antioxidant activity (DPPH)(c) of the aqueous extract.**

Based on the regression coefficient ( $\beta$ ) values, the interaction between all extraction variables (ABC) produced the highest effect on yield, followed by the cubic effect ( $A^3$ ). Interactive effect between extraction time and volume (AC), extraction temperature (B), quadratic effect of time ( $A^2$ ) and temperature ( $B^2$ ), the non-significant variables were eliminated and the equation for coded values in the cubic model fitting the experimental data of yield shown as follows:

$$Y = +20.72 - 2.55A + 0.62B - 0.52A^2 - 0.47B^2 + 0.65AC - 0.71BC + 1.32A^3 + 1.52ABC$$

The non-significant value of lack of fit ( $F = 0.84$ ) indicates that the model is fitted well with a good prediction ( $R^2 = 0.98$ ) as shown in Table II.

#### **Effect of extraction variables on total phenolic contents (TPC)**

The model of TPC showed a high significance ( $p < 0.01$ ) as shown in Table II. Generally, the extraction time and extraction temperature showed a significant effect on total phenolic content (TPC) of the extract. Thus, the non-significant factors associated with the extraction volume for TPC were eliminated, and the coded equation for TPC is as follows:

$$Y = +137.06 + 24.54B + 4.79A^2 + 4.12B^2 - 13.06AB - 5.70A^3 - 5.37B^3$$

The lack of fit ( $F = 0.87$ ) showed that the model fitted the experimental data well with a good prediction of  $R^2 = 0.99$  (Table II). Not only the extraction temperature showed a significant linear positive effect on TPC with  $p < 0.001$ , it also showed significance on quadratic and cubic effects on the TPC.

#### **Effect of extraction variables on total flavonoid contents (TFC)**

The model is significant with  $p < 0.05$ . Putting aside the linear and cubic effects of the extraction temperature which were statistically significant, other factors which were statistically non-significant were removed, and the coded equation for TFC is as shown:

$$Y = +28.02 + 7.98B - 3.05B^3$$

The lack of fit ( $F = 0.38$ ) confirmed that the model was well-fitted with a good prediction ( $R^2 = 0.81$ ) as shown in Table II. The range of TFC in various extraction conditions was 23.45 - 43.16 RE/g of extract (Table I). As mentioned earlier, only the extraction temperature affected the amount of TFC. Specifically, there was a positive significant linear effect and a negative significant cubic effect between the temperature of extraction and TFC, respectively. In this study, the extraction temperature of 81°C yielded the highest value of TFC (Table I). However, at 100°C, the yield of TFC decreased ultimately compared to extraction at 81°C.

#### **Effect of extraction variables on antioxidant activity (AA)**

##### **ABTS radical cation inhibition antioxidant assay**

The antioxidant activity via ABTS assay was expressed as the percentage (%) of inhibition. Overall, the negative linear effect of extraction temperature (B), positive cubic effects of extraction temperature ( $B^3$ ) and extraction volume ( $C^3$ ) on ABTS assay were statistically significant. On the other hand, there was no significant effect observed for interactive and quadratic effects on the ABTS assay. After elimination of the non-significant factors, the coded equation is as shown:

$$Y = +104.30 - 0.92B + 0.8B^3 + 0.58 C^3$$

The lack of fit ( $F = 0.58$ ) showed that the model was well-fitted with a good prediction ( $R^2 = 0.93$ , Table II). As mentioned earlier, the ABTS scavenging activity increased along with the increasing temperature. On the other hand, the extraction time did not show any significant effect on the ABTS assay. Extraction time exhibited a weaker effect whereas the extraction temperature showed a relatively significant effect on ABTS assay. Therefore, we can conclude that the antioxidant activity via ABTS assay increased with increasing temperature.

##### **DPPH radical scavenging ability assay**

In this response, A, B, C,  $B^2$ ,  $C^2$ , and AB were the significant model terms (Table II). Therefore, the coded equation was derived as shown below after removal of the non-significant factors:

$$Y = +91.66 - 0.44A - 0.87B + 0.50C - 0.85B^2 + 0.61C^2 + 0.44AB$$

The model fitted well with good prediction ( $R^2 = 0.95$ ) and non-significant lack of fit ( $F = 0.58$ ). The negative linear effects of extraction temperature were both statistically significant on the antioxidant activity via DPPH assay. Vice versa, there were significantly positive linear and quadratic effects of the extraction volume on the AA. In addition, the extraction temperature gave a significant negative quadratic effect on DPPH with  $p < 0.001$ , followed by a significant interactive effect between the extraction time and temperature with  $p < 0.05$ .

#### **Validation of optimal extraction conditions**

Comparison between the predicted and experimental values closely agreed with the CV ranged from 0.01 to 0.41 % as shown in Table III. The optimal extraction parameters were performed by maximizing the desirability of the responses. The optimal extraction conditions were then used for the extraction process, and the responses were later validated based on the procedure mentioned above for all the responses including TPC, TFC, ABTS and DPPH. The results of the validation suggested that the optimized extraction conditions to obtain the maximum value of the extract

**Table III: Experimental data of the validation of predicted values at optimal extraction conditions: 80°C, 100 min and 40 mL/g sample**

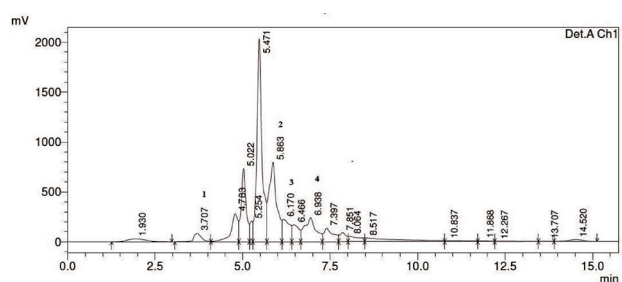
Dependent variables	Predicted value	Experimental value	% Difference (CV)
Yield	19.11	22.78 ± 1.48	0.12
TPC	186.44	174.48 ± 4.05	0.05
TFC	39.06	21.44 ± 0.05	0.41
ABTS	89.10	87.22 ± 0.62	0.02
DPPH	91.50	90.88 ± 0.09	0.01

Yield = % (w/dw), TPC = total phenolic contents (mg GAE/g dw), TFC = total flavonoid contents (mg RE/g dw), ABTS = 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) radical cation inhibition (%), DPPH = 2,2-diphenyl-1-picrylhydrazyl radical scavenging ability assay (%), CV = coefficient of variation, ± standard deviation (n=3)

yield, TPC, TFC and antioxidant activity were at extraction time of 100 min, extraction temperature of 81°C, and extraction volume of 40 mL/g sample with desirability value was at 0.87. Desirability close to one indicates that optimized parameter for extraction seems to achieve favourable results for all responses.

### HPLC analysis of phenolic compounds

Four compounds were quantified and identified as chlorogenic acid, quercetin, rutin and kaempferol from optimized extract of jackfruit leaves (Fig. 3). Peak compound identification in extracts was achieved by comparing HPLC retention time of standards with those found in the chromatograms of prepared extract and quantification was achieved through calibration curve with phenolic standards as shown in Table IV. Among the analyzed compounds, jackfruit leaves extract had the highest content of quercetin (151.04 mg/g), followed by rutin (35.48 mg/g), kaempferol (22.64 mg/g) and chlorogenic acid (11.95 mg/g). Compound at retention time 4.763 and 5.471 (Fig. 3) was not identified even though that compound had a higher peak compared to four identified compounds as the retention time did not match with any of our standard compound.



**Figure 3: HPLC chromatograms of phenolics from jackfruit leaves. (1) chlorogenic acid; (2) quercetin; (3) rutin and (4) kaempferol at 280 nm.**

### DISCUSSION

Phenolics have wide range of complex structures which widely distributed group of plants bioactive molecules. Bioactive compounds are the phytochemicals involved in protection of human health against the chronic degenerative ailments (23). The main classes of phenolic can be classified into phenolic acids, flavonoids, stilbins, phenolic alcohols, and lignans. A plant extract

is an active substance with desirable properties that is removed from plant tissues and made up of various bioactive compounds to the which plants and plant part from which they were extracted (24). The optimization of the extraction condition for *A. heterophyllum* leaves was generated using RSM. Central composite design (CCD) was utilized to analyse the relationship between the response and the independent variables. The matrix of CCD for variables and responses is shown in Table I. The variables including extraction time, temperature and volume adopted in this work were designed based on a preliminary study. The analysis regression coefficient was performed to evaluate the relationship between response and independent variables and the data as shown in Table II. All models showed significant p-value and fit well with the experimental data with R<sup>2</sup> values ranges between 0.81 to 0.99. The non-significant of lack of fit with p > 0.05 for all models indicate that the model can be used to analyse the response. Equations were obtained using a 20-batch run from Design-Expert 6.0.4 software version 6.0.4. It was found that the reduced cubic and quadratic model is the best fit for this study. To generate the models, non-significant terms of p > 0.05 were removed, and p < 0.05 is selected as the variables (25).

Based on the regression analysis of extraction variables on yield, extraction time significantly influenced the yield of extract. In particular, the extraction time produced a statistically significant negative effect (p < 0.001) on the yield. Prolonged extraction time at high temperature causes a significant decrease in yields due to the oxidation and degradation of the desired compound (26). On the other hand, the extraction temperature showed a significantly positive effect (p < 0.001) on yield, but this is highly dependent on the chemical properties of compounds present in the extract. However, the amount of yield and antioxidant activity of the extract reduced significantly at a higher temperature of 100-140°C. This may be due to thermal degradation as a result of overheating. As for volume, there was a significant negative effect in the interaction between the temperature and volume of extraction (AC, Fig. 1(a)), as well as a significant positive effect in the interaction between extraction time and volume (BC) as shown in Fig. 1(b). The mass transfer principle can explain the latter interaction (BC) during extraction in which the increasing solid-to-solvent ratio resulted in a higher diffusion rate (27). However, at a selected range of solvent-to-solid ratio, the solid and solvent will reach an equilibrium state. At this point, the extraction process stopped and thus will not give any significant effect on the yield (28). As for the temperature and volume of extraction (AC), the yield increased rapidly at a higher temperature with decreasing extraction volume. To conclude, the yield of *A. heterophyllum* leaves extract in this study was significantly affected by the extraction time and temperature. Thapa *et al.* (29) reported the highest extraction yield was obtained by

using the aqueous methanol of *A. heterophyllus* leaves compared to methanol and ethyl acetate extract with 10.98%, 0.22% and 4.68% respectively. However, our percentage yield range between 17-23 % and this indicate that the used of aqueous as solvent yielded more dry extract. Omar *et al.* (30) also reported the same pattern of *A. heterophyllus* leaves aqueous fraction that yield 22.8%. The higher amount of extraction yield may attributable by compound other than phenolics such as protein and carbohydrates, that may have been extracted during the extraction process (31).

In this study, the highest temperature of 81°C yielded the highest amount of TPC. This factor showed significant effect as temperature increase a greater contact of plant cell wall with extraction solution as the integrity of plant cell wall is altered, thus releasing more cellular component including phenolic compound (32). Likewise, the time of extraction also showed significant quadratic and cubic effects on the TPC. The interaction between the extraction time and temperature (AB) showed highly significant ( $p < 0.001$ ) effect on TPC (Fig. 2(a), Table II). Hence, in this study, when the extraction temperature was elevated and the time was reduced, this results in a gradual increase in TPC. Azahar *et al.* reported the same pattern as highest phenolic content obtained in Curcuma Zedoaria leaves with high extraction temperature with short periods of extraction time (33). Concerning the time of extraction, conflicting duration of time was found in different reported studies (4,34,35). However, such conflicting time of extraction may depend on the extraction temperature. Indeed, Cheng *et al.* (34) reported that, at a given set of extraction temperatures, longer extraction time caused high degradation of the phenolics due to thermal destruction. Among all the significant factors discussed, the main factors that affected the TPC in *A. heterophyllus* leaves extract were extraction temperature and time. The extraction volume showed no significant effect on TPC. The experimental data of TPC range from 101.07 - 183.34 GAE mg/g (Table I) in aqueous extract of *A. heterophyllus* leaves. In comparison with our data, Awuor Ojwang *et al.* (36) found the total phenolic content in methanolic extract of *A. heterophyllus* leaves to be 35.18 mg/g. Thus, our aqueous extraction of *A. heterophyllus* leaves is more efficient to yield more phenolic compound as compared to methanol extraction. This result is with agreement with Loizzo *et al.* (37) which also reported the high TPC obtained from total aqueous extract of *A. heterophyllus* leaves than ethyl acetate fraction.

The interaction effect between extraction temperature and extraction time on total flavonoid contents (TFC) is shown in Fig. 2(b). As extraction temperature and time increased at a constant volume of 30 mL/g, TFC of the extract gradually increased. The same pattern for aqueous extract of *Coreopsis tinctoria* Nutt. was also reported by Liu *et al.* (38). However, for the cubic effect, extraction temperature gives negative effects on TFC as extraction

temperature causing the TFC to decrease. In contrast, Azahar *et al.* (39) discovered a different pattern where in their study, the extraction temperature gives a weaker effect whereas extraction time show a significant effect on flavonoids yield. Thus, we can conclude that higher yield of TFC can be obtained at certain temperature and when higher temperature is applied, this compound will be degraded. Previous study by Awuor Ojwang *et al.* (36) reported  $5.74 \pm 0.68$  mg/g of total flavonoids in *A. heterophyllus* leaves in methanolic extract. This TFC value was much lower than TFC determined in our aqueous extract of *A. heterophyllus* leaves (23.45 - 43.16 RE mg/g) (Table I).

Meanwhile, for the effect of extraction variables on antioxidant activity (AA), as shown in Fig. 2(c), with decreasing time and temperature of extraction, the AA via DPPH assay increased at a fixed extraction volume (30 mL/g), but no significant interaction effect between extraction variables observed in ABTS assay. Thus, no 3D graph can be generated for ABTS assay. The extraction volume also has significant ( $p < 0.01$ ) effect on the AA via DPPH assay. This was supported by a study of Belwal *et al.* (13) which found that AA was highly influenced by solid to liquid ration. The high antioxidant activity of *A. heterophyllus* leaves extract in both ABTS and DPPH assays may be attributed to the high phenolic content, as evident from the results obtained from the TPC and TFC analysis. As previously reported by Loizzo *et al.* (31), for antioxidant activity of *A. heterophyllus* leaves, total aqueous extract possess highest free radical scavenging activity with lowest values of half maximal inhibitory concentration (IC<sub>50</sub>) 73.5 ug/mL, as compared with aqueous fraction and ethyl acetate fraction which showed IC<sub>50</sub> values 219.9 and 235.8 ug/mL, respectively.

The independent t-test was performed to check the significant differences between predicted and experimental values. The first test performed was the Levene's test to find the homogeneity of variance assumption, and there was a significant difference between two variances for extraction yield, TFC and AA via ABTS assay. This suggested that the variances can be assumed to be not homogenized; thus, the unequal variances t-test value would be used. However, for TPC and AA via DPPH assay, there was no significant difference between two variances, meaning that the variances can be assumed homogenized and hence the equal variances t-test value would be used for these two responses. The second test was the actual t-test which tests for equality of means. In the test, the null hypothesis is that the mean of the predicted value is equal to the mean of experimental data. The t-test for all responses was all significant with  $p < 0.05$  which means that the difference between means was statistically significant. Therefore, it is concluded that all the responses showed significant differences between the predicted experimental results.

Previously, Riyanti *et al.* (40) reported that *A. heterophyllum* leaves contained secondary metabolites that were identified as flavonoids, polyphenols, tannins, saponins, quinones, steroids, triterpenoids, monoterpene and sesquiterpene. Meanwhile, Bhattacharjee and Dutta (9) reported the presence of flavonoids, tannins, saponins and carbohydrates in methanolic and aqueous extracts of *A. heterophyllum* leaves. To date Wang *et al.* (41) isolated two new phenolic compounds which are artocarpilbene B and (E)-3,5-dihydroxy-4-(3-methylbut-1-enyl)benzaldehyde from *A. heterophyllum* leaves. Among all four identified compounds, kaempferol, quercetin, rutin and chlorogenic acid can be naturally found in many types of plants, including *A. heterophyllum* leaves. In general, pharmacological studies have proven that these compounds possessed various biological activities such as antioxidant, anticancer, wound healing and so on. Specifically, the therapeutic potential of kaempferol was well explained against cancer and autoimmune diseases like diabetes, arthritis, and asthma (42). Meanwhile, quercetin has been categorized as a flavonol and is well known for its excellent free radical scavenging activity. This property of quercetin has been associated with its potential in reducing oxidative-stress related chronic diseases such as cancer, heart disease, diabetes, and stroke. In the other hand, rutin is a flavonol and also known as quercetin-3-rutinoside. It has demonstrated a number of pharmacological activities, such as antioxidant, anticancer, neuroprotective and cardioprotective activities (43). Meanwhile, chlorogenic acid (CGA) classed under phenolic acid is an important and biologically active dietary polyphenol. CGA playing several important and therapeutic roles including antioxidant activity, anti-inflammatory properties, antibacterial, hepatoprotective, cardioprotective, anti-inflammatory, neuroprotective and anti-hypertension (44). These health benefits of CGA have been the focus of many epidemiologic studies involving CGA (45). It is possible that the observed antioxidant activity in the present study was due to the presence of these four active compounds. The high antioxidant activity may also be due to the synergism effects between these compounds and other compounds that might present in the extract. The other compounds from other classes of polyphenols such as alkaloids, saponins, glucosides that might present in our extracts but did not detected for this study. Thus, it can be concluded that jackfruit leaves extract has the potential to be utilized therapeutically and pharmacologically due to its high bioactive compounds with high antioxidant activity.

## CONCLUSION

Response surface methodology (RSM) was successfully developed to determine the optimum extraction parameters of *A. heterophyllum* leaves. The best combinations of extraction time, temperature and volume were 100 min with the temperature at 81°C and

extraction volume at 40 mL/g sample. This optimised parameter was promising to improve yield, antioxidant content and antioxidant activity in *A. heterophyllum* leaves, hence maximizing the health benefit of this plant.

## ACKNOWLEDGEMENTS

The authors gratefully acknowledge Universiti Putra Malaysia for the funding for this study (UPM/700-2/1/GPB/2017/9549900) and Laboratory of Molecular Biomedicine, Institute of Bioscience, Universiti Putra Malaysia for the laboratory facilities.

## REFERENCES

1. Miller HE, Rigelhof F, Marquart L, Prakash A, Kanter M. Antioxidant Content of Whole Grain Breakfast Cereals, Fruits and Vegetables. *J Am Coll Nutr.* 2000 Jun;19(sup3):312S-319S.
2. Kumar Y, Yadav DN, Ahmad T, Narsaiah K. Recent Trends in the Use of Natural Antioxidants for Meat and Meat Products. *Compr Rev Food Sci Food Saf.* 2015 Nov 1 ;14(6):796–812.
3. Taghvaei M, Jafari SM. Application and stability of natural antioxidants in edible oils in order to substitute synthetic additives. *J Food Sci Technol.* 2015 Mar ;52(3):1272–82.
4. Salar RK, Purewal SS, Bhatti MS. Optimization of extraction conditions and enhancement of phenolic content and antioxidant activity of pearl millet fermented with *Aspergillus awamori* MTCC-548. *Resour Technol.* 2016 Sep 1;2(3):148–57.
5. Swami SB, Thakor NJ, Haldankar PM, Kalse SB. Jackfruit and Its Many Functional Components as Related to Human Health: A Review. *Compr Rev Food Sci Food Saf.* 2012 Nov 1;11(6):565–76.
6. Baliga MS, Shivashankara AR, Haniadka R, Dsouza J, Bhat HP. Phytochemistry, nutritional and pharmacological properties of *Artocarpus heterophyllum* Lam (jackfruit): A review. *Food Res Int.* 2011 Aug 1;44(7):1800–11.
7. Shahin N, Alam S, Ali M. Pharmacognostical standardisation and antidiabetic activity of *Artocarpus Heterophyllum* leaves Lam. *Int J Drug Dev Res.* 2012 ;4(1):346–52.
8. Bhattacharjee C, Dutta A. Nephro protective activity of bark of *Artocarpus heterophyllum* lam. *Int J Biomed Adv Res.* 2017;8(6):259–63.
9. Bhattacharjee C, Dutta A. Phytochemical and acute toxicity study of leaves of *Artocarpus heterophyllum* Lam. *Int J Allied Mediccal Sci Clin Res.* 2013;1(2):78–81.
10. Burci LM, da Silva CB, Rondon JN, da Silva LM, de Andrade SF, Miguel OG, *et al.* Acute and subacute (28 days) toxicity, hemolytic and cytotoxic effect of *Artocarpus heterophyllum* seed extracts. *Toxicology Reports.* 2018;
11. Patel RM, Patel SK. Cytotoxic activity of methanolic extract of *Artocarpus heterophyllum* against A549,



- Hela and MCF-7 cell lines. *J Appl Pharm Sci*. 2011(07):167–71.
12. Prakash O, Kumar R, Chandra D, Kumar A, Kumar P. Effect of *Artocarpus heterophyllus* Lam. (Jackfruit) on Indomethacin-Induced ulcer model in albino rats. *Sch Res Libr Der Pharm Lett*. 2015;7(1):81–5.
  13. Belwal T, Dhyani P, Bhatt ID, Rawal RS, Pande V. Optimization extraction conditions for improving phenolic content and antioxidant activity in *Berberis asiatica* fruits using response surface methodology (RSM). *Food Chem*. 2016 Sep 15;207:115–24.
  14. Irakli M, Tsifodimou K, Sarrou E, Chatzopoulou P. Optimization infusions conditions for improving phenolic content and antioxidant activity in *Sideritis scardica* tea using response surface methodology. *J Appl Res Med Aromat Plants*. 2018 Mar 1;8:67–74.
  15. Tan C-H, Ghazali HM, Kuntom A, Tan C-P, Ariffin AA. Extraction and physicochemical properties of low free fatty acid crude palm oil. *Food Chem*. 2009 Mar 15;113(2):645–50.
  16. Kuo C-H, Hsiao F-W, Chen J-H, Hsieh C-W, Liu Y-C, Shieh C-J. Kinetic aspects of ultrasound-accelerated lipase catalyzed acetylation and optimal synthesis of 4'-acetoxysesveratrol. *Ultrason Sonochem*. 2013 Jan;20(1):546–52.
  17. Tan S, Stathopoulos C, Parks S, Roach P. An Optimised Aqueous Extract of Phenolic Compounds from Bitter Melon with High Antioxidant Capacity. *Antioxidants*. 2014 Dec 2;3(4):814–29.
  18. Jabbar Al-Manhel A, Kareem Niamah A. Effect of Aqueous and Alcoholic Plant Extracts on Inhibition of Some Types of Microbes and Causing Spoilage of Food. *J Nutr Food Sci*. 2015;5.
  19. Yusri N, Chan K, Iqbal S, Ismail M. Phenolic Content and Antioxidant Activity of *Hibiscus cannabinus* L. Seed Extracts after Sequential Solvent Extraction. *Molecules*. 2012 Oct 25;17(12):12612–21.
  20. Ramadan MF, Kroh LW, Mursel J-T. Radical Scavenging Activity of Black Cumin (*Nigella sativa* L.), Coriander (*Coriandrum sativum* L.), and Niger (*Guizotia abyssinica* Cass.) Crude Seed Oils and Oil Fractions. *J Agric Food Chem*. 2003 Nov 19;51(24):6961–9.
  21. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic Biol Med*. 1999 May 1;26(9–10):1231–7.
  22. Rodríguez-Pérez C, Quirantes-Piñ R, Fernández-Gutiérrez A, Segura-Carretero A. Optimization of extraction method to obtain a phenolic compounds-rich extract from *Moringa oleifera* Lam leaves. *Ind Crops Prod*. 2015 Apr 1;66:246–54.
  23. Abbas M, Saeed F, Anjum M, Afzaal M, Tufail T, Shakeel Bashir M, *et al*. Natural polyphenols: An overview. *Int J Food Prop*. 2017;20(8):1689–99.
  24. Šernaitė Lina. Plant extracts: antimicrobial and antifungal activity and appliance in plant protection (Review). 2018;58–68.
  25. Xujie H, Wei C. Optimization of extraction process of crude polysaccharides from wild edible BaChu mushroom by response surface methodology. *Carbohydr Polym*. 2008 Apr 3;72(1):67–74. 9
  26. Naczki M, Shahidi F. Extraction and analysis of phenolics in food. *J Chromatogr A*. 2004 Oct 29;1054(1–2):95–111.
  27. Wong BY, Tan CP, Ho CW. Effect of solid-to-solvent ratio on phenolic content and antioxidant capacities of “Dukung Anak” (*Phyllanthus niruri*). *Int Food Res J*. 2013;20(1):325–30.
  28. Cacace JE, Mazza G. Mass transfer process during extraction of phenolic compounds from milled berries. *J Food Eng*. 2003 Oct 1;59(4):379–89.
  29. Thapa N, Thapa P, Bhandari J, Niraula P, Shrestha N, Shrestha BG. Study of Phytochemical, Antioxidant and Antimicrobial Activity of *Artocarpus heterophyllus*. *Nepal J Biotechnol*. 2016 Dec 31;4(1):47–53.
  30. Omar HS, El-Beshbishy HA, Moussa Z, Taha KF, Singab ANB. Antioxidant Activity of *Artocarpus heterophyllus* Lam. (Jack Fruit) Leaf Extracts: Remarkable Attenuations of Hyperglycemia and Hyperlipidemia in Streptozotocin-Diabetic Rats. *Sci World J*. 2011 Apr 5 ;11:788–800.
  31. Do QD, Angkawijaya AE, Tran-Nguyen PL, Huynh LH, Soetaredjo FE, Ismadji S, *et al*. Effect of extraction solvent on total phenolic content, total flavonoid content, and antioxidant activity of *Limnophila aromatica*. *J Food Drug Anal*. 2014 Sep 1;22(3):296–302.
  32. Houston K, Tucker MR, Chowdhury J, Shirley N, Little A. The plant cell wall: A complex and dynamic structure as revealed by the responses of genes under stress conditions. Vol. 7, *Frontiers in Plant Science*. Frontiers Media S.A.; 2016.
  33. Azahar NF, Gani SSA, Mohd Mokhtar NF. Optimization of phenolics and flavonoids extraction conditions of *Curcuma Zedoaria* leaves using response surface methodology. *Chem Cent J*. 2017 Dec 2;11(1):96.
  34. Cheng Y, Xu Q, Liu J, Zhao C, Xue F, Zhao Y. Decomposition of Five Phenolic Compounds in High Temperature Water. *J Braz Chem Soc*. 2014;25(11):2102–7.
  35. Upadhyaya V, Pai SR, Hegde H V. Effect of method and time of extraction on total phenolic content in comparison with antioxidant activities in different parts of *Achyranthes aspera*. *J King Saud Univ - Sci*. 2015 Jul 1;27(3):204–8.
  36. Awuor Ojwang R, Muge EK, Mbatia B, Mwanza B, Ogoyi DO, Lee JW, *et al*. Comparative Analysis of Phytochemical Composition and Antioxidant Activities of Methanolic Extracts of Leaves, Roots and Bark of Jackfruit (*Artocarpus heterophyllus*) from Selected Regions in Kenya and Uganda. *J Adv Biol Biotechnol*. 2017;16(1):2394–1081.
  37. Loizzo MR, Tundis R, Chandrika UG, Abeysekera

- AM, Menichini F, Frega NG. Antioxidant and Antibacterial Activities on Foodborne Pathogens of *Artocarpus heterophyllus* Lam. (Moraceae) Leaves Extracts. *J Food Sci.* 2010 Jun;75(5):291–5.
38. Liu XF, Liu L, Wang YG, Leng FF, Wang SW, Li YC. Optimization of Total Flavonoids Extraction from *Coreopsis tinctoria* Nutt. by Response Surface Methodology. *Kem u Ind.* 2014 Nov 4;63(11–12):391–6.
39. Azahar NF, Gani SSA, Mohd Mokhtar NF. Optimization of phenolics and flavonoids extraction conditions of *Curcuma Zedoaria* leaves using response surface methodology. *Chem Cent J.* 2017 Oct 2;11(1):96.
40. Riyanti S, Sunardi C, Falah N. Isolation of Flavonoid from Jackfruit Leaves (*Artocarpus heterophyllus* Lamk.) and its Antioxidant Activity. *Acta Pharm Indones.* 2013;38(2):58–61.
41. Wang X-L, Di X-X, Shen T, Wang S-Q, Wang X-N. New phenolic compounds from the leaves of *Artocarpus heterophyllus*. *Chinese Chem Lett.* 2017 Jan 1;28(1):37–40.
42. Kashyap D, Sharma A, Tuli HS, Sak K, Punia S, Mukherjee TK. Kaempferol – A dietary anticancer molecule with multiple mechanisms of action: Recent trends and advancements. *J Funct Foods.* 2017 Mar;30:203–19.
43. Ganeshpurkar A, Saluja AK. The Pharmacological Potential of Rutin. *Saudi Pharm J SPJ Off Publ Saudi Pharm Soc.* 2017 Feb ;25(2):149–64.
44. Naveed M, Hejazi V, Abbas M, Kamboh AA, Khan GJ, Shumzaid M, *et al.* Chlorogenic acid (CGA): A pharmacological review and call for further research. *Biomed Pharmacother.* 2018 Jan ;97:67–74.
45. Tajik N, Tajik M, Mack I, Enck P. The potential effects of chlorogenic acid, the main phenolic components in coffee, on health: a comprehensive review of the literature. *Eur J Nutr.* 2017 Oct 8; 56(7):2215–44.